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Ectomycorrhizal community structure and function in interior spruce forests of British Columbia under long term fertilization

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ABSTRACT

Long-term fertilization is being considered as a mechanism to increase growth of pine and spruce trees in British Columbia following a major outbreak of mountain pine beetle. It is important, however, that long-term fertilization not disrupt colonization by ectomycorrhizal fungi or impede their ability to release nutrients from soil organic matter. In order to determine the effect of long-term (up to 14 years) fertilization on the structure and function of ectomycorrhizal (ECM) fungal communities on roots of interior spruce, we characterized the ECM fungal community and, on separate samples collected at a different time of year, assayed for extracellular enzymes in the mycorrhizoplane. The study was conducted at three interior spruce (naturally occurring hybrids of *Picea glauca* [Moench] Voss and *Picea engelmannii* Parry) plantations in the interior of south-central British Columbia. Three 0.164 ha plots per site received no (controls), periodic (200 kg ha−1 N, plus P, K, S, Mg, and B every 6 years), or annual (same nutrients adjusted to maintain foliar N concentration at 1.3% and other nutrients in balance; 50 to 75 kg ha^{-1} of N, depending on site and year) fertilization. There was no overall effect of fertilization on the composition of the ECM fungal community (OTUs) on spruce roots (permutational MANOVA P = 0.4) nor on alpha diversity per plot. At single sites, there was reduced relative abundance of *Cortinarius* spp. mycorrhizas under periodic fertilization, and increased relative abundance of *Lactarius* spp. or *Tylospora* spp. mycorrhizas under annual fertilization. None of the dominant genera increased or decreased significantly across sites. The ratio of carbon-acquiring to mineral nutrient-acquiring enzyme activities in the mycorrhizoplanes increased with frequency of fertilizer application. Increased litterfall may explain the increase in plant cell wall-degrading enzyme activity in fertilized plots. A decrease in activities involved in the release of sulphur was as expected in fertilized plots because the fertilizer contained inorganic nitrogen, phosphorus and sulphur. Although activities of two enzymes involved in nutrient cycling (sulfatase and phosphomonoesterase) tended to decrease, there did not appear to be an overall loss of organic matter-degrading potential from the ECM fungal community on spruce roots under long-term fertilization.

1. Introduction

The use of fertilization to overcome nutrient constraints to aboveground productivity has become standard practice for increasing tree yields in many forest ecosystems (Tamm, 1991; Bergh et al., 1999; Elser et al., 2007; Egnell and Björheden, 2013). While single applications of fertilizer yield only temporary increases in stand and tree growth, frequent applications produce large increases in harvest volume and sustained growth in boreal forests (Tamm, 1991; Bergh et al., 1999; Tamm et al., 1999). In order to assess the effects of fertilization in both lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) and interior spruce (*Picea glauca* [Moench] Voss and *Picea engelmannii* Parry, or naturally occurring hybrids of these species) stands, the former British Columbia Ministry of Forests and Range began to establish "Maximum Productivity" field installations in 1992 (Brockley and Simpson, 2004). Regular balanced fertilization in the Maximum Productivity Study was effective in accelerating tree and stand development in these immature stands (Fisher and Binkley, 2000; Brockley, 2006, 2010) and, hence, could be used to justify long-term fertilization as part of a strategy of intensive forest management.

Many tree species rely upon a symbiotic relationship formed with ectomycorrhizal (ECM) fungi to enhance nutrient acquisition

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(Smith and Read, 2008). As part of their nutrient acquisition strategy, ECM fungi, like other fungi, excrete hydrolytic and oxidative enzymes (Bending and Read, 1995; Luis et al., 2005; van Aarle and Plassard, 2010). These enzymes break down plant cell walls during litter degradation, and then release nitrogen, phosphorus and sulphur from organic molecules (Perez-Moreno and Read, 2003). Given their considerable biomass in forest soils (Högberg and Högberg, 2002), ECM fungi are likely to contribute significantly to nutrient (Talbot et al., 2008; Phillips et al., 2014) and carbon (Godbold et al., 2006; Hobbie, 2006; Clemmensen et al., 2013) fluxes in forest soils. Consequently, the implications of forest fertilization on the structure and nutrient cycling capacity of ECM fungal communities should be considered by forest managers.

Nitrogen or phosphorus enrichment of forest soils generally reduces the abundance of ectomycorrhizas (Treseder, 2008). Studies of mixed fertilizer application are fewer and tend to be of short duration, but report lower ECM fungal species richness, percent root length colonized, mycelium production or total sporocarp biomass, in response to fertilization (Baum and Makeschin, 2000; Nilsson and Wallander, 2003; Berch et al., 2006). Shifts in ECM fungal community structure have also been observed, including a reduction or elimination of some dominant ECM fungal taxa (Berch et al., 2006; Wright et al., 2009). These shifts may impact the nutrient cycling capacity of forest soils, as nitrogen fertilizers select against ECM fungi with enhanced abilities to attack N-containing biomolecules (Lilleskov et al., 2011 and references therein). Nutrient additions may also directly impact enzymatic activity. For example, the addition of phosphorus alone suppresses phosphatase activities in the ectomycorrhizosphere (Ali et al., 2009). Conversely, the annual application of balanced fertilizer to pine sites within the Maximum Productivity Study resulted in higher ligno-cellulolytic enzyme activities (Jones et al., 2012).

In order to better understand how ECM fungi and roots in interior spruce forests respond to repeated fertilization, we sampled roots from three spruce sites within the Maximum Productivity Study. We characterized the ECM fungal community colonizing the roots, and, on separate samples collected at a different time of year, assayed for a suite of extracellular enzymes in the ectomycorrhizoplane. We knew that increased frequency of fertilization at these sites was associated with increased soil and foliar C and N (Harrison, 2011) and increased litter volumes (up to 3 × higher in fertilized than unfertilized stands [L.A. Phillips, unpublished]). Although previous research at one of the spruce sites (Berch et al., 2009) had detected minimal effects on ectomycorrhizas, earlier work on the pine sites found a reduction in the relative abundance of *Suillus* and *Cortinarius* mycorrhizas (Jones et al., 2012). Based on this information and the review of Lilleskov et al. (2011), we predicted that with fertilizer additions (i) ECM fungal diversity on colonized roots would decrease; (ii) the relative abundance and frequency of some of the dominant ECM fungal genera, especially *Cortinarius* and *Piloderma*, would decrease; and (iii) activities of plant cell wall-degrading enzymes would increase, while those associated with the release of soluble nitrogen, phosphorus and sulphur would decrease.

2. Methods

2.1. Study description

Forests in British Columbia are classified into biogeoclimatic zones based on macroclimate and into subzones based on local moisture and temperature regimes (Pojar et al., 1987). This research was conducted at three interior spruce (*Picea glauca* × *engelmannii*) sites in central interior British Columbia, which are thoroughly described in Brockley and Simpson (2004). The Crow Creek site is located at 54°20′N, 126°17′W, within the moist cold subzone of the Sub-Boreal Spruce Biogeoclimatic Zone (SBSmc2; www.for.gov.bc.ca/hre/becweb/). The site was clear-cut harvested in 1985, broadcast burned, and replanted in the spring of 1986. The Hand Lake site is located at 54°N, 122°53′W within the Mossvale variant of the moist cool subzone of the Sub-Boreal Spruce Biogeoclimatic Zone (SBSmk1). In 1985 the previous mature stand was clear-cut harvested and, in 1986, the cutblock was broadcast burned and replanted. The Lodi Lake site is located at 53°22′N, 122°06′W, within the wet cool subzone of the Sub-Boreal Spruce Biogeoclimatic Zone (SBSwk1). In 1985, the previous stand was clear-cut harvested and broadcast burned. The stand was replanted in 1987.

2.2. Experimental design

Stands were thinned to 1100 stems per hectare and fertilization commenced in 1995 at Crow Creek, 1996 at Lodi Lake, and 2000 at Hand Lake. Two fertilizer treatments and an unfertilized control were replicated three times for a total of nine 0.164 ha treatment plots at each site. Adjacent plots were separated by at least 5 m. For the periodic treatments, granular fertilizer was applied every 6 years at a rate of 200 kg ha^{-1} of N, 100 kg ha^{-1} of P, 100 kg ha^{-1} of K, 50 kg ha^{-1} of S, 25 kg ha^{-1} of Mg, 1.5 kg ha^{-1} of B (see Brockley and Simpson, 2004 for formulation). The most recent application prior to sampling (late summer 2008 and spring 2009) was in 2007 for Crow Creek, 2005 for Hand Lake, and 2008 for Lodi Lake (Table A.1). The annual treatments were fertilized at rates formulated to maintain foliar N concentrations at 1.3%, with other nutrients in balance with N. The rate of N applied in the annual treatment ranged from 50 to 75 kg ha^{-1}, depending on the year (Table A.1). No fertilization was applied to the control treatment. Fertilization was performed by hand soon after spring snowmelt.

2.3. Soil sampling and processing

From August 30 to September 4, 2008, seven randomly-located 10 cm × 10 cm × 10 cm soil samples, including both forest floor and mineral soil, were collected from the three replicate plots for each fertilization regime treatment from each site (3 sites × 3 fertilizer treatments × 3 replicate plots × 7 samples = 189). Samples were then divided into four sub-samples by quartering. Each sub-sample was used for a different measurement: fine root length, ectomycorrhizas, soil chemistry, and archaeal and bacterial assessment. In this paper, we present data on ectomycorrhizas. Subsamples for ECM characterization were weighed and then frozen at −20 °C until processing was initiated in January 2009.

2.4. Characterization of ectomycorrhizas

For characterization of ectomycorrhizas, soil samples were thawed, soaked in tap water, gently shaken, and then washed over a 2 mm sieve. Mineral and forest floor subsamples were combined. Forty independent ECM root tips were randomly selected per soil sample. In cases where fewer than 40 root tips were available, all root tips were characterized. Each tip or system was examined in detail under dissecting and compound microscopes and described according to Agerer (1987–2002) and Goodman et al. (1996). Two of the most turgid and light-coloured tips from each morphotype were frozen at −80 °C (Lee et al., 2007) for molecular analyses.

2.5. Molecular identification of ectomycorrhizal fungi from fall samples

DNA was extracted from 449 tips, each representing one morphotype per soil sample, using the Extract-N-Amp Plant PCR Kit,

(Sigma, St. Louis, Missouri, USA). The manufacturer's protocol was followed with the exception that only 25 μl of the extraction and dilution solutions was added to each tip rather than 100 μl and GoTaq® Green Master Mix (Promega, Madison, WI) was used during amplification. The internal transcribed spacer (ITS) region of the fungal ribosomal RNA gene was used for identification of fungal operational taxonomic units (OTUs) (Goodman et al., 1996–2002; Martin and Rygiewicz, 2005). Thermocycler conditions for PCR amplification of the entire ITS using primers ITS1F and ITS4 (Gardes and Bruns, 1993; White et al., 1990) were as follows: 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C and 10 min incubation at 72 °C. Success of amplification was assessed using a 1% agarose gel, stained with SYBRsafe (Invitrogen, Carlsbad, CA). Reactions displaying clear single bands were cleaned of excess primers and nucleotides using Mag-Bind™ E-Z Pure (Omega Bio-Tek, Norcross, GA). When no or multiple bands were observed, PCR was repeated on genomic DNA diluted by 1/10. When multiple or no PCR products were observed after dilution, the second tip from that morphotype and sample was extracted.

Amplicons were sequenced using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) on an ABI3130xl capillary sequencer at the Fragment Analysis and DNA Sequence Service at University of British Columbia's Okanagan campus. Alignment and correction of sequences were performed in Sequencher 4.2 (GeneCodes, Ann Arbor, MI, USA). Each sequence was then BLASTed (Basic Alignment Search Tool; Altschul et al., 1990) against National Centre for Biotechnology Information (NCBI) and User Friendly Nordic ITS Ectomycorrhizas (UNITE) databases. The species name of the accessioned sequence was applied to the sample sequence if at least 97% of base pairs (bp) were identical over a sequence length of at least 450 bp. Next, all sample sequences BLASTed to the same genus or family were aligned using ClustalX (Thomspon et al., 1997), pairwise sequence similarities calculated, and phylogenetic trees constructed by the neighbour-joining method using PHYLIP 3.69 (Felsenstein, 1989). Sequences that matched database sequences at only 85–96% or that matched sequences over less than 450 bp (but greater than 250 bp) were not given species names. Instead, they were named at genus, family or higher levels, based on the taxonomic affiliations of the closest BLAST hits in the databases, and with the morphological descriptions. Sequences from this study are listed in the NCBI database under accession numbers KF53579-753617 (Table A.2). If amplification was not successful from either saved tip, that morphotype from that sample was named at the genus level based on morphological data and sequence data from similar morphotypes from other samples.

2.6. Assays of mycorrhizoplane enzymes from spring samples

Samples for enzyme activity were collected from the same plots 9–10 months later, after soils had warmed in the growing season of 2009 (Crow Creek, June 7; Hand Lake, June 21; Lodi Lake July 5). Ten 5 × 5 cm by 10 cm deep soil subsamples were collected from each plot and stored on ice in coolers for transport to the laboratory. Samples were then stored at 4 °C for no more than 4 days. Subsamples were combined to create one single composite sample for each plot. From these composite samples, seven active tips for each of the four most common morphotypes found within each sample were chosen (28 tips per plot). The tips were cleaned of all debris using forceps while rinsing in water, and then each tip was placed in an individual micro-sieve (Pritsch et al., 2004) within 96-well microplates. Each well was then filled with 200 μL 75 mM Tris–maleic acid buffer (pH 4.5) and the tips were allowed to equilibrate for at least 5 min prior to beginning the plate-based enzyme assays. This microplate enzyme method is based on previously published methods (Courty et al., 2005; Pritsch et al., 2004), and our lab-specific protocols are described in detail in Jones et al. (2012). The enzyme assays were performed in the following order: β-xylosidase, β-cellobiohydrolase, β-glucosidase, β-*N*-acetyl-glucosaminidase, phosphomonoesterase, sulfatase, leucine-aminopeptidase, and laccase. Between each assay, tip-containing sieves were placed into a rinse plate that contained Tris-maleic buffer at a pH appropriate for the next assay, and tips were again allowed to equilibrate for at least 5 minutes. All assays except laccase were fluorogenic, using methyl-umbelliferyl (MU) or 7-amino-4methyl coumarin (AMC) linked substrates; laccase assays were colorimetric using 2,2′-Azino-bis(3-ethylbenzothiazoline-6 sulfonic acid diammonium salt (ABTS). All enzyme substrates were sourced from Sigma–Aldrich (St. Louis, MO, USA).

2.7. Data analysis

Non-metric multidimensional scaling (NMS) ordinations with Bray Curtis dissimilarity distances were used to visualize the total ECM fungal communities, based on relative abundances of all OTUs, using the metaMDS function of the vegan package in R 3.0.2. Relative abundances per plot were used to examine communities at each site separately, and across all sites. The overall significance of both treatment and site on ECM community structure was determined by non-parametric permutational MANOVA (Permanova; Anderson, 2001), using Bray Curtis similarity resemblance matrices with 9999 permutations of residuals under a reduced model (Permanova + software package v1.0.6; PRIMER-E Ltd, Ivybridge, UK). Abundance data for each fungal OTU in each sample were square root transformed prior to analysis, with samples nested in plots and treatment plots nested within site.

Ectomycorrhizal fungal diversity (Fisher's Alpha, Shannon's, and inverse Simpson's), as well as extrapolated (Colwell et al., 2012) and rarefied estimated (Abundance Coverage-based, Chao 2) richness were determined per plot with EstimateS 9.1.0 (Colwell, 2013), using data from each soil sample as replicates, and based on all ECM fungal OTUs. Relative abundance of the six frequently encountered genera was calculated per plot as the number of root tips colonized by that fungus, divided by the total number of ECM root tips identified in all samples from that plot. Treatment effects on diversity indices, richness estimators, and relative abundance of the six dominant genera were assessed for each site separately with fertilizer treatment as a fixed categorical effect, or across sites, with site and block added as random effects, using the lmer function (lme-4 package in R 3.1.3, R Core Team, 2015), with plots as replicates. In addition, because the sites varied from 9 to 14 years under fertilization, we also tested for relationships between total N + P + K applied (kg as shown in Table A.1) and the response variables listed above. By using lmer with Site/Block as random factors, we could account for slightly different experimental designs at the three sites (i.e., completely randomized design at Crow Creek and Hand Lake = 1 block per site, and a randomized block design at Lodi Lake = 3 blocks; Brockley and Simpson, 2004). A t value of <-2 or >2 was considered to indicate a significant relationship with fertilizer treatment. Assumptions of normality and homogeneity of variance were evaluated using exploratory data analysis. Residuals were examined to ensure compatibility with assumed distributions. In particular, relative abundance data was analyzed first assuming a binomial distribution (family = binomial), but both examination of the residuals and exploratory data analysis supported analysis assuming a normal distribution. When assumptions of normality and homogeneity of variance could not be met, square root transformations or log transformations were used.

To quantify mycorrhizoplane enzyme activities, the concentration of fluorescent product was calculated based on calibration

curves generated with 4-methylumbelliferone (MU) or 7-amino-4-methyl coumarin (AMC) standards (Sigma–Aldrich, St. Louis, MO, USA) and expressed as pmol MU or AMC min^{-1} mm^{-2} surface area of the root tip. Laccase concentrations were determined using the Beer-Lambert law A = εbc, where A = absorbance, ε = wavelength dependent molar absorptivity for ABTS under the conditions of this assay (3.6×10^4 M^{-1} cm^{-1}), b = path length (0.32 cm), and c = sample concentration. Enzyme activities were then averaged across the five to seven tips per analysis group, and then again among all analysis groups in a plot. To facilitate ordination, these data were then standardized across sites by dividing each plot mean by the average activity for each enzyme for that site, then averaged by plot. Effect of fertilizer treatment was examined for each enzyme individually using the lmer function, as described above, on log-transformed data converted to positive values. Principal components analysis (PCA; Borcard et al., 2011) was used to visualize, and non-parametric permutational MANOVA (adonis from package vegan, R 2.12.2 Oksanen et al., 2012) was used to test for, treatment effects on the suite of enzyme activities across sites.

Indices of potential nutrient acquisition based on the relative activities of carbohydrate-hydrolyzing, P-releasing, and N-releasing extracellular enzyme activities were calculated as per Sinsabaugh et al. (2008). The index of potential C:N acquisition activities from soil was calculated using the ratio of β-glucosidase to β-N-acetylglucosaminidase plus leucine amino peptidase activities, potential C:P acquisition activities was calculated using the ratio of β-glucosidase to phosphomonoesterase activities, and potential C:S acquisition activities was calculated using the ratio of β-glucosidase to sulfatase activities. Individual activities were averaged per plot prior to calculation of the ratios.

3. Results

3.1. Ectomycorrhizal fungi from colonized root tips in fall samples

Overall, 38 fungal OTUs were identified in this study, with considerable amount of overlap in the taxa of ECM fungi detected among sites and among treatments at each site. At Crow Creek, 23 ECM fungal OTUs were observed; 12 of these were found in plots of all three treatments. At Hand Lake, 23 ECM fungal OTUs were observed; 16 of these were observed across all three treatments. At Lodi Lake 18 ECM fungal OTUs were detected, with 7 of these found in all three treatments. A total of 15 OTUs were common to all three sites. As is common in studies of ECM roots, sample-based rarefaction curves of ECM fungal OTUs for each treatment at each site did not reach asymptotes (Fig. A.1), indicating that sampling did not detect all fungal OTUs present at each treatment for each site. Some rare taxa would have been missed during sampling.

There was an indication that the ECM fungal communities found on spruce roots differed among sites ($P = 0.055$, PERMANOVA), but were not affected by long-term fertilization. No fertilization effects were detected across sites ($P = 0.4$, PERMANOVA with treatments nested within site), nor for each site considered separately ($P > 0.05$; Fig. A.2). Because some soil samples contained fewer than 40 root tips and some from Lodi Lake did not contain any root tips, we calculated both extrapolated and rarefied richness estimators of ECM fungal OTUs per plot. Richness estimators did not differ among treatments at individual sites or across sites (Table A.3). Likewise, fungal diversity was not affected by fertilization (Table A.3). Between 99% and 100% of root tips were ectomycorrhizal at all sites (data not shown).

Six ECM fungal genera dominated (i.e., >5% of ectomycorrhizas identified per plot) samples collected from the spruce stands: *Amphinema*, *Cenococcum*, *Cortinarius*, *Lactarius*, *Piloderma* and *Tylospora*. No significant effect of fertilization was detected on the relative abundance of any of the dominant types of ectomycorrhizas across sites (Fig. 1). Furthermore, no relationship was apparent between the total amount of N + P + K applied per plot and the relative abundance of any of the taxa across sites. When sites were considered separately, *Lactarius* spp. mycorrhizas increased in relative abundance under annual fertilization and *Cortinarius* mycorrhizas decreased under periodic fertilization at Lodi Lake (Fig. 1). At Hand Lake, *Tylospora* spp. mycorrhizas, increased in relative abundance with annual fertilization, and there was a marginal negative effect on *Cenococcum* with periodic fertilization.

3.2. Activities of extracellular enzymes from spring samples

Across sites, the combination of activities of the eight mycorrhizoplane enzymes was affected by fertilizer application (permutational MANOVA, $P = 0.049$). The enzyme profiles of annually fertilized plots tended to be located on the negative side of Axis PC1 of the Principal Components Analysis, which explained 46% of the variation, and at the positive end of Axis PC2, which explained 28% of the variation (Fig. 2). Three enzymes were negatively correlated with Axis 1 and positively correlated with Axis 2: xylosidase, cellobiohydrolase and β-glucosidase. These three activities increased significantly under annual fertilization (Fig. 3). By contrast, activities of the remaining enzymes were negatively correlated with both axes (Fig. 2), with significant inhibition of sulfatase activities under both rates of fertilization. Laccase, β-N-acetylglucosaminidase and leucine aminopeptidase activities were not significantly affected by fertilization across sites, whereas phosphomonoesterase was marginally reduced under periodic fertilization (Fig. 3). Consequently, indices of potential C:N, C:P, and C:S acquisition activities from soil were higher in fertilized plots (Table 1).

4. Discussion

Application of a balanced fertilizer every six years or annually, had no detectable effect on overall ECM fungal community structure at three interior spruce forests. A few dominant genera decreased or increased in relative abundance with fertilization at single sites, but no consistent positive or negative effect on a genus was observed across sites. Furthermore, our prediction of reduced fungal richness and diversity was not seen. By contrast, fertilization affected mycorrhiza-associated exoenzymes as expected: a general increase in plant cell-wall degrading enzymes and a decrease in some of the enzymes that release soluble mineral nutrients.

4.1. Diversity and richness of spruce ectomycorrhizas

There were no obvious effects of fertilization on ECM richness or diversity indices at any of the sites individually or across sites. The responses of ectomycorrhizas to nitrogen fertilization are known to be variable. Some studies report no changes to ECM fungal species richness, evenness or diversity indices in coniferous forests (Fransson et al., 2000; Peter et al., 2001; Berch et al., 2009; Wright et al., 2009), while others report significant reductions in diversity indices (Frey et al., 2004) or richness (Lilleskov et al., 2002; Berch et al., 2006). At the pine sites within the Maximum Productivity Study, 10–12 years of annual fertilization decreased ECM roots by 40% (albeit at a higher rate of application than tested here), and ECM fungal richness also decreased (Jones et al., 2012). It is possible that richness of ECM fungal communities in pine forests was more affected by balanced fertilization because of high

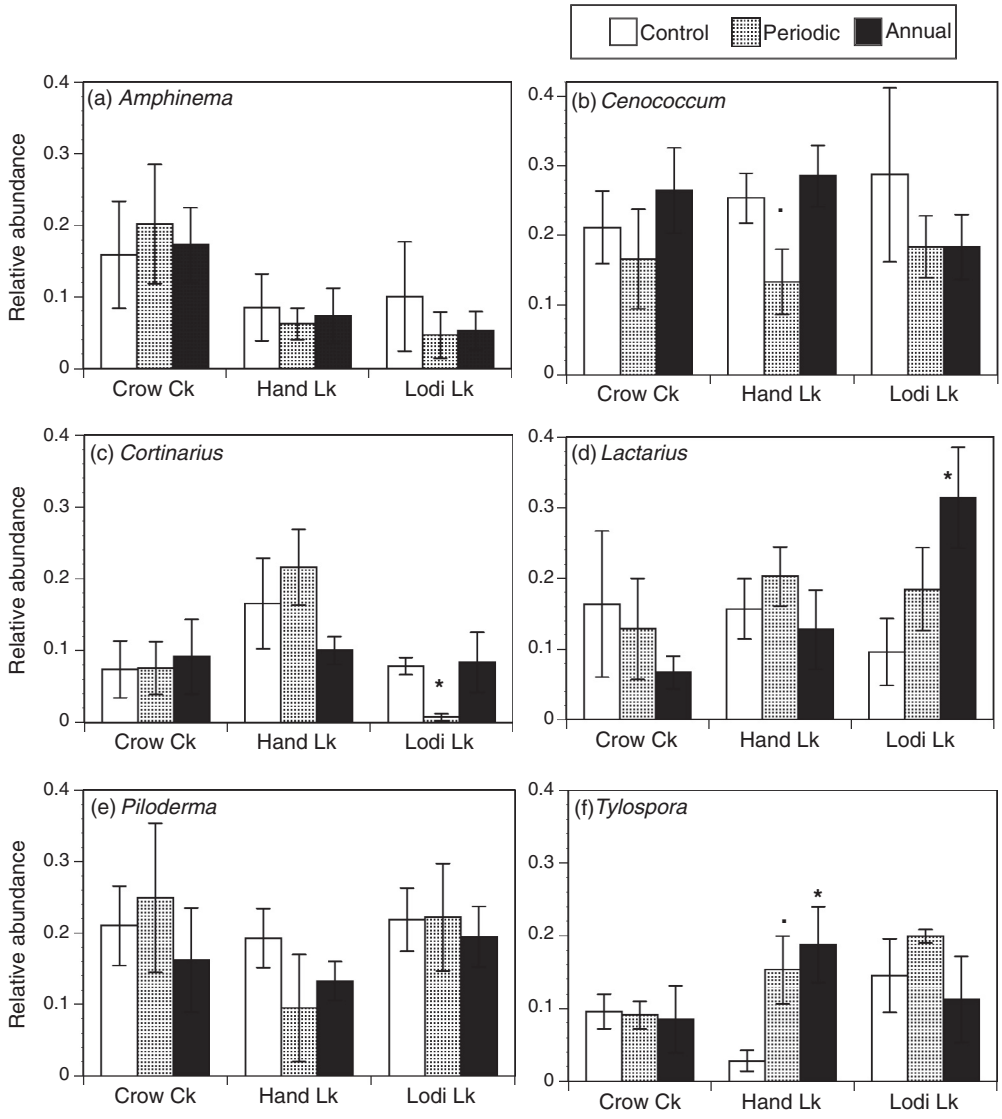

Fig. 1. Mean relative abundance (the proportion of spruce mycorrhizas examined) per plot. (a) *Amphinema* spp. (b) *Cenococcum* spp. (c) *Cortinarius* spp. (d) *Lactarius* spp. (e) *Piloderma* spp. and (f) *Tylospora* spp. mycorrhizas in the control (white bars), periodic (gray bars) and annual (black bars) fertilization treatments ($n = 3$ plots per site). Error bars represent 1 SEM. *indicates a significant difference from control plots ($t < -2$ or >2) and · indicates a marginal effect (<-1.8 or >1.8) according to general linear models (lmer, lme-4 package in R), with block as a random effect at Lodi Lake.

sensitivity to nutrient addition by *Suillus* spp. (Jones et al., 2012). *Suillus* mycorrhizas were abundant at the pine sites, but not at the spruce sites.

4.2. Effects of fertilization on overall ectomycorrhizal community composition

A shift in the species composition of ECM fungal communities is a very common response to forest fertilization in a range of forest types (Kårén and Nylund, 1997; Baxter et al., 1999; Frey et al., 2004; Berch et al., 2006; Lilleskov et al., 2008). In particular, a relationship between the quality and availability of N and ECM community structure has been firmly established (Lilleskov and Parrent, 2007; Treseder et al., 2007; Kranabetter et al., 2009). Although repeated fertilization increased available N in the soil solution at the spruce sites (Harrison, 2011), the overall ECM fungal community appeared remarkably resistant to change. Likewise, Berch et al. (2009) found no detectable changes to the ECM community at Crow Creek after 10 years of fertilization. It is possible that, after such a long period of treatment, the system had returned to a stable equilibrium state, which differed primarily in the size of the nutrient pools. Furthermore, species shifts with N fertilization are often attributed to induced P limitation (e.g., Vitousek et al., 2010), but the balanced fertilizer applied here would be unlikely to produce such limitations.

We predicted that we would see a reduction in *Cortinarius* or *Piloderma* mycorrhizas, because both are considered sensitive to N (Lilleskov et al., 2011) and a reduction in *Cortinarius* mycorrhizas was observed at the pine sites of the Maximum Productivity Study (Jones et al., 2012). While we did observe a significant reduction in *Cortinarius* mycorrhizas at the periodically fertilized plots at Lodi Lake, no relationship with fertilizer application was observed for *Piloderma*. The periodic plots at Lodi Lake were fertilized in the spring of 2008, just 3 months before mycorrhizal samples were collected. Because periodic plots receive at least two times as much N as annually fertilized plots in the year of application, this may have inhibited a sensitive genus such as *Cortinarius* from forming mycorrhizas in the year of application. This appears to be only a short-term response to high nutrient inputs, from which *Cortinarius* recovers, because the relative abundance of

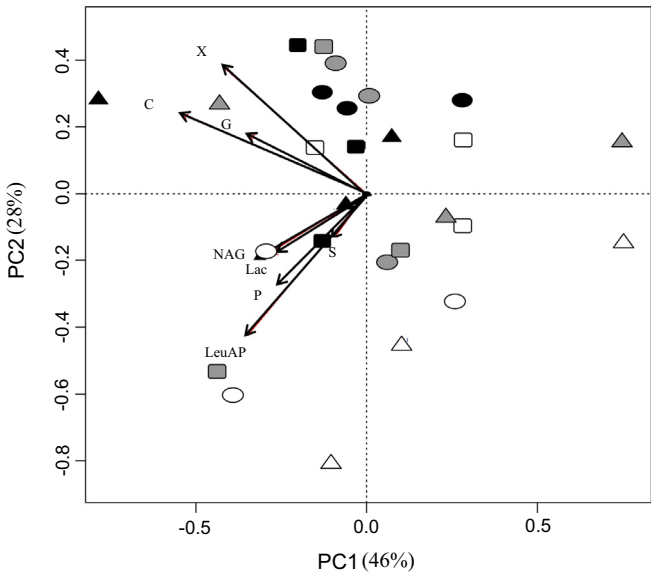

Fig. 2. Principal components analysis of extracellular mycorrhizoplane enzyme activities from root tips harvested from the forest floor, averaged by plot (three plots per treatment) for three interior spruce stands (ovals – Crow Creek, rectangles – Hand Lake, triangles-Lodi Lake), fertilized annually (black symbols), every six years (gray symbols) or left unfertilized (open symbols). Enzymes measured included xylosidase (X), cellobiohydrolase (C), β-glucosidase (G), β-N-acetylglucosaminidase (NAG), laccase (Lac), phosphomonoesterase (P), leucine aminopeptidase (LeuAP) and sulfatase (S).

Cortinarius mycorrhizas was not affected by periodic fertilization at the other two sites, which had been fertilized in 2006 or 2007, nor by annual fertilization at any of the sites.

Cenococcum geophilum is considered to be weakly sensitive to N enrichment (Lilleskov et al., 2011); however, Kranabetter et al., (2009) found the highest percent colonization by *Cenococcum geophilum* along a natural productivity gradient at sites with 11 and 13 N g kg^{-1} foliar N. Foliar N concentrations in this range were found in spruce undergoing annual fertilization; hence, it is surprising that an increase in *Cenococcum geophilum* mycorrhizas was not observed for that treatment, but rather, a tendency to decrease in relative abundance in the periodically fertilized plots at Hand Lake. We saw no reduction in abundance in *Cenococcum* mycorrhizas with fertilization of the pine sites of the Maximum Productivity Study either (Jones et al., 2012).

Our observation of an increase of *Tylospora* mycorrhizas under annual fertilization at one site is consistent with several other studies. In response to atmospheric N deposition over 27 years, *Tylospora fibrillosa* was not observed in the low N sites, but was a dominant taxon in the high N sites in a white spruce (*Picea glauca*) forest (Lilleskov et al., 2002). Similarly, in response to 4 years of 100 kg ha^{-1} per year of ammonium sulphate in a 30-year-old Norway spruce (*Picea abies*) stand, the relative frequency of *Tylospora fibrillosa* increased (Karen and Nylund 1997). In response to 150 kg ha^{-1} yr^{-1} of ammonium nitrate additions in a 35-year-old subalpine spruce forest, Peter et al. (2001) observed an increase in relative frequency for only the first two years. In reviewing these studies, Lilleskov et al. (2011) concluded that, as a genus, *Tylospora* was tolerant to N additions. Our study extends this to application of balanced fertilizer; *Tylospora* increased in relative abundance at one site and was not affected at the other two. *Lactarius* mycorrhizas were the other dominant type that increased with fertilization at one site, and was unaffected at the other two. This was not surprising given that *Lactarius* spp. tend to respond positively to N additions more frequently than negatively (Lilleskov et al., 2011 and references therein). *Amphinema byssoides* has been labelled as highly nitrophillic by Kranabetter et al. (2009) based on its distribution across natural productivity gradients in BC. As such, we would expect it to become more abundant in fertilized plots, but this was not observed.

None of the dominant genera were significantly affected by more than one of the two fertilizer treatments, or at more than one of three sites, supporting our conclusion that any effect of repeated application of a balanced fertilizer had only minor effects on the ECM fungal community. The apparent insensitivity of these ECM fungi could be an artefact of analyzing the relative abundance of the fungus at the genus level; however, we feel confident in our conclusions because Lilleskov et al. (2011) found quite consistent patterns in response to nitrogen addition at the genus level. Furthermore, when we evaluated the communities as a whole, we used OTUs, not genera, so any major shift in species composition would have been detected. The fact that no overall change in community was detected indicates that we did not miss any major change by comparing relative abundances at the genus level.

4.3. Enzyme activities in the mycorrhizoplane

The extracellular enzyme activity of mycorrhizas can indicate the potential of the ECM fungal community and their associated bacteria to cycle nutrients (Koide et al., 2007; Courty et al., 2010). The eight enzymes measured in our study fell into two groups in terms of their response to fertilization: the activities of cellulose- and hemicellulose-degrading enzymes increased with fertilization, whereas two enzymes associated with the release of mineral nutrients decreased in activity. Because of increased rates of aboveground litter deposition with annual fertilization (Phillips et al., 2014), we expected xylosidase, cellobiohydrolase, β–glucosidase and laccase activities to increase with fertilization, as observed on the pine sites (Jones et al., 2012) and by others (Hobbie et al., 2012). While we observed significant increases in xylosidase, cellobiohydrolase and β-glucosidase activities in response to fertilization in our spruce sites, laccase activity did not change. It is possible that laccase activities were not affected because lignin contents of litter can decrease with N addition (Hobbie et al., 2012). Furthermore, our results are consistent with a recent meta-analysis by Whittinghill et al. (2012) that indicated N enrichment of forest soils increases cellulose decomposition, but decreases lignin decomposition. This may be because fertilization can decrease soil pH, and oxidase activities tend to increase linearly with pH (Sinsabaugh et al., 2008).

Soil enzymes are often inhibited by high concentrations of their products (Geisseler et al., 2010) and, hence, we expected a decrease in enzymes that release soluble nitrogen (leucine aminopeptidase and β-*N*-acetyl-glucosaminidase), phosphorus (phosphatase) or sulphur (sulfatase). Although both phosphatase and sulfatase showed the predicted response, β-*N*-acetyl-glucosaminidase and peptidase were not affected, perhaps because the stimulatory effect of increased soil organic matter (Sinsabaugh et al., 2008; Harrison, 2011) counteracted the inhibitory effects of increased N, P and S. Certainly the ratio of C-acquiring to N-, P-, or S-acquiring enzymes increased with fertilization. In addition, enrichment of mineral nutrients can stimulate microbial activity, including excretion of extracellular enzymes, especially if the stoichiometry of C:N:P in the soil differs from that in fungal or bacterial tissues (Burke et al., 2012; Mooshammer et al., 2012). In contrast to the spruce stands, lodgepole pine stands exposed to the same treatments showed no decrease in activities of phosphatase or sulfatase with fertilization (Jones et al., 2012). This may be because, at the pine site, we measured activities on only *Cenococcum*, and *Piloderma* mycorrhizas, whereas sampling was more representative at the spruce sites.

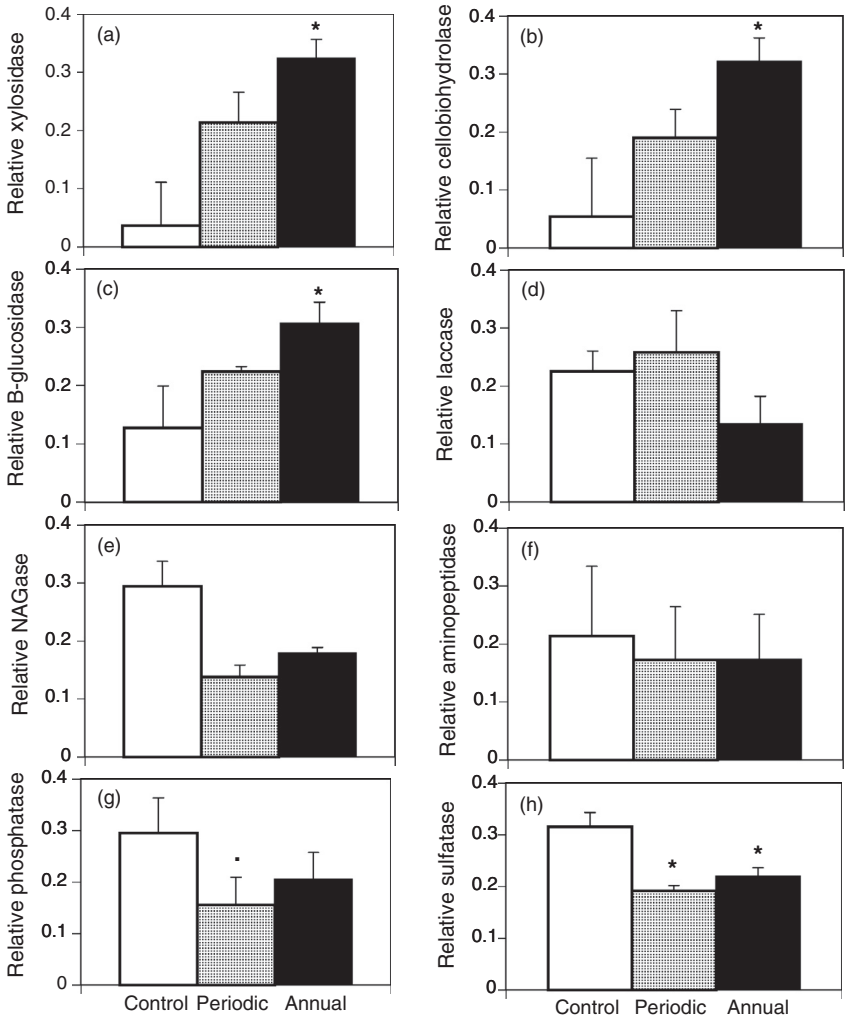

Fig. 3. Extracellular mycorrhizoplane activities of (a) xylosidase, (b) cellobiohydrolase, (c) β-glucosidase, (d) laccase, (e) β-N-acetylglucosaminidase, (f) leucine aminopeptidase, (g) phosphomonoesterase, and (h) sulfatase from root tips harvested from the forest floor of three interior spruce sites, standardized by dividing the activities per mm^2 per tip by the average for each enzyme for each site, and then averaged by plot, log-transformed, and scaled to be plotted on a the positive axis. Error bars represent 1 SEM ($n = 3$ sites). *indicates a significant difference from control plots ($t < -2$ or >2) and · indicates a marginal effect (<-1.8 or >1.8) according to general linear models (lmer, lme4 package in R), with site/block as a random effect.

Table 1
Indices of potential acquisition efficiencies for nitrogen, phosphorus and sulphur in the mycorrhizoplane of spruce ectomycorrhizas from long-term fertilization plots, modified from Sinsabaugh et al. (2008).

Fertilizer treatment[b]	Index of potential nutrient acquisition efficiency[a]		
	C:N	C:P	C:S
Control	0.59 (0.06)	0.45 (0.06)	1.61 (0.19)
Periodic	0.74 (0.02)	0.57 (0.01)	2.26 (0.06)
Annual	0.80 (0.02)	0.60 (0.02)	2.39 (0.17)

[a] C:N index = ln(BG + 1)/ln((NAG + 1) + (LAP + 1)), C:P index = ln(BG + 1)/ln(AP + 1), and C:S index = ln(BG + 1)/ln(S + 1) where BG = activity of β-glucosidase, NAG = activity of β-N-acetylglucosaminidase, LAP = activity of leucine aminopeptidase, AP = activity of phosphomonoesterase, and S = activity of sulfatase, all expressed per mm^2 surface area per root tip, with means per site shown (SEM; $n = 3$ sites).

[b] A balanced fertilizer was added every six years to periodic treatments and every year to annual treatments. Control plots were not fertilized. All three ratios were significantly higher in periodic and annual plots than control plots according to a general linear model with site and block as random factors.

5. Conclusions

The findings reported in this study and those by Berch et al. (2009) are suggestive of an ECM fungal community on interior spruce that is reasonably resistant to perturbation from long-term application of balanced fertilizer. This is an important finding for developers of policy on broad-scale forest fertilization. A change in the decomposer community can result in altered soil organic matter properties (Wickings et al., 2012) or indicate a loss in potential to break down some litter components (Burke et al., 2012). Instead, we found evidence of increased cellulose and hemi-cellulose degrading activities with fertilization. Given the important contribution of ECM fungi to overall enzyme activities in soil (Talbot et al., 2013; Phillips et al., 2014), we are confident that nutrient-cycling capacities of these soils have been maintained even after 9–14 years of annual fertilization. Our observations on enzyme activities are robust, given that effects occurred across three widely-dispersed spruce sites. It is noteworthy, however, that several enzymes that were unaffected or increased in activity with fertilization in pine stands (Jones et al., 2012) were decreased or were unaffected in spruce stands, so it is important not to generalize across forests comprised of different tree species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2015.04.023.

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