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Foliar nutrients shape fungal endophyte communities in Western white pine (*Pinus monticola*) with implications for white-tailed deer herbivory

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ABSTRACT

Asymptomatic fungal endophytes colonize tissues of woody plants worldwide, with largely unknown ecological effects. Using culture-based methods and ITS1-5.8S-ITS2 rDNA sequence analysis, we investigated differences between foliar endophyte communities in disease-resistant hybrid and wild-type *Pinus monticola* (Western white pine) trees with observed variation in tree growth, vigor, and browsing damage by white-tailed deer (*Odocoileus virginianus*). We isolated 69 phylotypes of endophytic fungi in at least 39 genera, including 26 that have not previously been reported in *P. monticola*. Principal components analysis revealed that endophyte communities differed between browsed seedlings, unbrowsed seedlings, and unbrowsed adult trees. Sulfur, nitrate and calcium concentrations correlated with endophyte community differences among tree groups based on a distance-based redundancy analysis. Our results indicate that foliar nutrient variation influences endophyte community assembly and deer herbivory in *P. monticola* on a small landscape scale (80 hectares).

Introduction

Asymptomatic foliar fungal endophytes in Ascomycota are ubiquitous in vascular and nonvascular plants from the tropics to sub-polar regions (Arnold 2007; Higgins *et al.* 2007; Rosa *et al.* 2010). Endophyte function in plant hosts has been described along a continuum from latent pathogen to mutualist (Saikkonen *et al.* 1998; Moricca & Ragazzi 2008). Endophytes that inhabit cool season (C3) grasses provide the most extensive support of mutualistic interactions (Clay & Schardl 2002). These fungi, in the family *Clavicipitaceae*, increase plant growth (Marks & Clay 1996), reduce damage by fungal pathogens (Clarke *et al.* 2006), cause intestinal distress or death in grazing mammals (Wolfe *et al.* 1998), and reduce insect herbivory in their grass hosts (Hardy *et al.* 1986; Clay & Cheplik 1989). Endophyte-grass mutualisms alter plant succession in grasslands (Clay & Holah 1999) and forests (Rudgers *et al.* 2007; Rudgers & Orr 2009), and shift C:N ratios through a reduction in soil microbial activity (Franzluebbers & Hill 2005).

Endophyte symbioses in dicotyledonous forbs, shrubs, and trees have received less attention than grass endophyte systems (Carroll 1995; Saikkonen *et al.* 1998; Sieber 2007). Woody species endophytes display high diversity in host plants, disperse horizontally, and are thought to be commensal opportunists rather than mutualists (Rodriguez 2009). They may occur in alternate hosts if succession or disturbance reduces the abundance of their preferred host (the host jumping hypothesis, Carroll 1999; Rogers 2000). Some may also be latent saprotrophs (Deckert *et al.* 2002), or have evolved from pathogens that adopt an asymptomatic lifestyle as

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a self-preservation strategy (Ganley et al. 2004). Despite the challenges to characterizing mutualisms between horizontally dispersed endophytes and woody plants (Moricca & Ragazzi 2008), and the lack of empirical support in some cases (Faeth & Hammon 1997), evidence of endophyte and woody plant mutualisms has accumulated. In a greenhouse trial, endophytes limited damage caused by a *Phytophthora* sp. pathogen in *Cacao theobroma* (Arnold et al. 2003). Endophytic fungi also produced an insect toxin that reduced the growth rate of spruce budworm in *Picea glauca* (white spruce) *in vitro*, and could reduce budworm herbivory in field conditions (Miller et al. 2002). In the interior northwest of the United States, mutualistic endophytes improved the survival of *Pinus monticola* (Western white pine) seedlings inoculated with the nonnative pathogen *Cronartium ribicola*, causal agent of blister rust (Ganley et al. 2008).

Although the additive effects of proper silviculture (Ostry et al. 2010), selective breeding programs (McDonald et al. 2004; King et al. 2010), and now potentially endophyte inoculations (Ganley et al. 2008) contribute to an encouraging outlook for *P. monticola* recovery, barriers to reforestation still exist. In restoration plots, white-tailed deer (*Odocoileus virginianus*) frequently browse the apical meristems of planted *P. monticola* seedlings, diminishing future growth potential. Individual tree exclosures reduce browsing damage, but add cost to restoration activities. These initial costs and anticipated mortality associated with *P. monticola* restoration often stymie recovery efforts (Harvey et al. 2008).

On a 80 hectares private conservation property in Montana's Swan Valley, we manage a remnant population of local genotype white pines and their regeneration (about 60 trees). In 2005, approximately 500 blister rust resistant, F_2 generation "Bingham" hybrid *P. monticola* seedlings were planted on the property during restoration efforts. By 2008, about half of the seedlings had endured severe, continuous browsing by deer while the other half showed no signs of browsing damage. White-tailed deer will habitually browse preferred plants (Welch et al. 1992), but what features of white pine seedlings drive their original choice? Nitrogen applications encouraged deer browsing in *Pseudotsuga menziesii* (Douglas fir, Crouch & Radwan 1981) and deciduous shrubs in New Mexico (Anderson et al. 1974). Deer also browse more heavily on plants that grow in nutrient-rich soils (Gill 1992). Since endophyte deterrence of insect herbivores and antagonism of mammalian herbivores have been documented, we speculated that asymptomatic foliar endophytes in *P. monticola* also affect deer preference for particular seedlings. In this study, we assessed the diversity of asymptomatic foliar endophytes and foliar nutrient variation in Swan Valley white pine needles to investigate differences in endophyte communities among three tree groups (local genotype trees, browsed, and unbrowsed seedlings), and determine the relationship between endophytes, foliar nutrients and deer browsing of white pines.

Methods

Study site and tree group designation

The study site sits at the foot of the Swan range on the east side of the Swan Valley in Western Montana (centered at 47°31′20″N 113°40′15″W). The 80 hectares property receives 610–710 mm of precipitation per year which encourages dense forest growth, but a century of management for timber production has shifted dominant upland conifer species from *Larix occidentalis* (Western larch) and *Pseudotsuga menzeisii* (Douglas fir) to *Pinus contorta* (lodgepole pine). *Pinus ponderosa* (Ponderosa pine) and *P. monticola* grow where canopy gaps provide light for seedlings. Recruitment of these shade-intolerant species is limited due to browsing pressure, introduced exotic grasses, and soil compaction. *C. ribicola* (white pine blister rust) and logging activities have further limited reestablishment of Western white pine on the property. In riparian areas, deciduous trees and shrubs that established during past flood and fire disturbances gave way to thick *Picea engelmannii* (Engelmann spruce) and occasional *Thuja plicata* (Western red cedar) emergence.

For the present study, 18 members of the browsed seedling group (IF2br) were chosen on the basis of significant damage by deer (several branches or growth leaders browsed and evidence of repeated browsing by deer). The unbrowsed seedling group (IF2ubr) included 19 seedlings that showed little evidence of deer herbivory and no apical meristem damage. Both seedling groups contain Idaho "Bingham" F_2 hybrid blister rust resistant stock grown at the Center for Forestry Nursery and Seedling Research at the University of Idaho in Moscow (USA). We also selected 21 local regeneration, Swan Valley genotype trees (SVG) from the approximately 60 that grow on site based on evidence of past browsing. Some evidence of deer browsing on lower tree branches in the SVG group did occur, but growth characteristics in these older and taller trees precluded severe leader damage in the past. For the purposes of this study, SVG trees were considered unbrowsed. SVG trees included in the study ranged in age from 10 to 72 yr old; seedlings were all 5 yr old (2 yr in the nursery and about 3 in the ground) in late 2008 when sampling occurred.

Field data collection

Needle collections occurred on 20 Dec. 2008 and needle sampling from individual trees followed Ganley & Newcombe (2006). From the Swan Valley genotype trees, we collected at least 80 needles from each of four branches spaced evenly about the circumference of the tree. Many SVG trees had lower branches removed as part of past management practices on the property; as a result, differences in sampling height between seedlings and taller SVG trees were unavoidable. All needles included in the study were accessible from standing height at ground level. Mid-branch needles were chosen to avoid bias towards young or old needles, and needles that displayed obvious blemishes, senescence, or evidence of fungal pathogens were discarded. Many seedlings displayed sufficient growth for us to follow this procedure for needle collection exactly, but those seedlings with extensive browsing often had fewer than four branches. In that case, about 80 mid-branch fascicles were collected from available foliage. Needles from each tree were placed in plastic zipper bags, labeled, and stored on ice in a cooler for transfer to the lab, where they were stored at −20 °C until processing. Tree height, age and increment (average annual growth), were recorded at the time of needle collection. Because Western

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white pines grow a discernable whorl of branches every year, average increment and tree age can be determined visually in the field.

Endophyte isolation and morphotyping

Culture-based methods were employed to screen white pine needles for fungal endophytes using established protocol (Ganley & Newcombe 2006; Arnold *et al.* 2007; Marquez *et al.* 2007). We randomly chose 30 unblemished needles among those sampled from each tree for plating. Needles were surface sterilized in 70 % ethanol, 6 % sodium hypochlorite, and 70 % ethanol for 1 min, 5 min, and 1 min, respectively. After surface sterilization, needles were allowed to dry on flamed microscope slides and sectioned into three or four even pieces depending on overall needle length. Two sectioned needles were placed on each 90 mm plate with either 2 % malt extract agar (MEA) or potato dextrose agar (PDA), both amended with 5 mg ml^{-1} of the antibiotics chloramphenicol and streptomycin. Of the thirty needles from each tree, 15 were plated on MEA and 15 on PDA, for a total of 1 740 needles in culture. Because the SVG trees frequently had longer needles than the seedlings, needle length was measured to avoid overestimating total endophyte colonization based solely on the plating of a larger amount of tissue. Culture plates were incubated at room temperature immediately after plating and were inspected daily from the time the first fungal growth appeared (within 1 week) until new fungal emergence ceased about 2 months later. Fresh fungal outgrowths were collected for isolation on 60 mm plates. Each individual endophyte isolate was subcultured on both MEA and PDA treated with antibiotics. Sterile procedures were observed during all fungal isolations.

After 2 months, we obtained 479 isolates and organized them into morphotaxa based on colony form, elevation, depth of agar penetration, margin, surface texture, opacity and surface color (Arnold 2002). Qualitative observations (form, margin, surface texture, opacity, surface color) were assigned sequential numeric codes, and these codes were included with quantitative measurements (elevation and depth of agar penetration) for analysis. A principal components analysis (PCA, Canoco, ter Braak & Smilauer 2006) was used to produce an ordination plot and identify isolates that clustered based on shared morphology. Visual assessment of clusters helped us place isolates that were indeterminate based on the PCA. We then randomly chose at least 10 % of plates from each morphotaxon and all unassignable singletons for DNA extraction and sequencing.

DNA extraction, amplification and sequencing

DNA extraction methods were modified from a protocol used for melanized fungi found in Douglas fir needles (Lori Winton and George Carroll, personal communication). Tissue samples from each isolate were suspended in a CTAB + 2-mercaptoethanol buffer, homogenized in a Geno/Grinder 2000 (BT&C Incorporated, Lebanon, NJ, USA) for 5 min, frozen in liquid nitrogen, thawed briefly and separated with 24:1 chloroform:isoamyl alcohol in a centrifuge at 10 000×*g*. The supernatant was drawn off and precipitated with cold isopropanol. Pellets obtained after centrifugation were washed with 70 % ethanol, suspended in 0.5 M Tris–EDTA buffer, and stored at −20 °C. Ribosomal DNA (the 5.8S rDNA gene and surrounding ITS regions 1 and 2) was amplified with universal fungal primers ITS1F (CTTGGTCATTTAGAGGAAGTA) and ITS4 (TCCTCCGCTTATTGATATGC, White *et al.* 1990). Each isolate was amplified in a 25 μl PCR reaction mixture containing 1 μl of undiluted template, 10 μl of 2.5 × 5 Prime HotMasterMix (1.0 U HotMaster *Taq* DNA polymerase, 45 mM KCl, 2.5 mM Mg^{2+}, and 200 μM each dNTP, 5 Prime, Gaithersburg, MD, USA), 10 μl deionized water, 0.5 μl of each primer, and 3 μl betaine (Henke *et al.* 1997) and the following thermocycling parameters; 3 min at 94 °C, followed by 37 cycles at 94 °C for 30 s, 52 °C for 40 s, and 65 °C for 40 s, and a final extension phase of 68 °C for 7 min. Amplicon samples were electrophoresed in 1 % agarose and evaluated against a standard ladder for single bands of appropriate size (400–800 bp). Suitable PCR products were diluted 50× for shipment to a sequencing facility (High Throughput Genomics Unit, Seattle, Washington) where they were cleaned with Exo-SAP-IT (USB, Cleveland, OH, USA) and sequenced. Obtained sequences were edited with DNA Baser v2 (Heracle Biosoft, Pitesti, Romania), uploaded to the NCBI/GenBank database and assigned accession numbers (Supplementary Table 1).

All sequences were compared to the NCBI/GenBank database using BLAST, and putative species designations were based on the closest match whenever species (or genera) matches were available. Phylotype groups were assembled around putative species based on a conservative pairwise nucleotide difference of <3 % (Marquez *et al.* 2007). CLUSTALW alignment of selected ITS sequences and confirmation of percentage nucleotide difference between sequenced isolates in phylotype groups were completed with MEGA v4 (Tamura *et al.* 2007).

Analysis of microscopic reproductive structures was conducted on each isolate that produced spores or conidia to support the gene-based identification. Unsequenced isolates from morphotaxa were added to phylotype groups based on micromorphological analysis, and additional extractions were performed and sequences obtained to support isolate inclusion in phylotype groups with variable or indeterminate micromorphology. Isolate sequences that returned insufficiently identified matches through BLAST searches were identified to the most specific taxa possible using BLAST search results and micromorphological evidence. The five remaining unidentifiable fungi were treated as missing data.

Needle nutrient analyses

Uncultured *P. monticola* needles were immediately dried in an incubator at 65 °C for 24 hr and sent to Ward Laboratories (Kearney, NE, USA) for analysis of NO^{3-}, total N, P, K, S, Mg, Ca, Mn, Cu, Fe and Zn. Percentage nutrient concentrations were arcsin transformed prior to statistical analysis. Foliar nutrient differences among tree groups were tested for significance with an ANOVA (PAST v2.04, Hammer *et al.* 2001).

Community data analysis

To test the extent of our sampling effort, phylotype collection curves were constructed using EstimateS (Colwell 2009) with

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each tree as the sampling unit. Diversity indices were also produced with EstimateS. To compare endophyte abundance and phylotype richness among tree groups, we first corrected the data for variations in needle length sampled by dividing the number of endophytes or phylotypes detected in a particular tree by the total needle length (in meters) measured in the tree. As a result, abundance and richness calculations presented are in the units of endophytes per needle-meter or phylotypes per needle-meter, respectively.

To compare average endophyte community distances among tree groups, a between-tree Bray–Curtis similarity matrix was calculated from phylotype abundance data using PrCoord v1.0 (included with CANOCO v4.55, ter Braak & Smilauer 2006). The software removed singleton species and corrected negative eigenvalues before analysis (Legendre & Anderson 1999). The resultant principle coordinate ordination (PCO) solution file provided coordinates for each sample (individual tree) and percentage phylotype variation in the species matrix explained by each axis. Using methods adapted from Feris *et al.* (2003), sample coordinates thus obtained for each tree were averaged along PC1 and 2 for all the trees in each group. Axis distributions between groups were tested for significance with an ANOVA in PAST v2.04 (Hammer *et al.* 2001).

We tested the interactions of host environmental variables (11 foliar nutrients, tree height, age, and average annual growth) and tree group on endophyte community assemblage with a distance-based redundancy analysis (db-RDA, Legendre & Anderson 1999; Mummey *et al.* 2009). A Bray–Curtis similarity matrix was produced as previously described and analyzed with environmental variables and covariables in CANOCO v4.55 (ter Braak & Smilauer 2006). Monte Carlo permutation tests (1 000 iterations) were performed in forward selection to assess the significance of environmental variables, and in a global test to assess the significance of canonical axes produced by the db-RDA.

We also investigated the contributions of specific phylotypes to the species distance matrix and correlated phylotype abundance to varying nutrient levels among trees and tree groups with PAST v2.04 (Hammer *et al.* 2001). To assess the significance of individual phylotype contributions to the species distance matrix, we used a *post-hoc* component loadings test included with the PCA in PAST. The loadings test produces a correlation coefficient and constructs bootstrapped 95 % confidence intervals for each phylotype included in the analysis. Phylotypes were identified as significant based on a correlation coefficient >0.50 and a non-overlapping confidence interval. Linear regressions were used to determine the extent to which significant phylotype abundances varied with foliar nutrient levels.

Results

Endophyte diversity in tree groups

Of the 479 isolates obtained, 24 were removed from analysis due to external plate contamination. In the remaining 455 isolates, 69 phylotypes in at least 39 genera were found (Supplementary Table 1), including 26 that have not previously been described in *P. monticola* (see Ganley & Newcombe 2006). The majority of these fungi belong in the *Ascomycota*, representing at least eight orders. Rhytismataceous fungi comprised the largest group of *P. monticola* endophytes (50 %), and 93 % of the endophytes in this family belong in the *Lophodermium s. lat.* Three members of the *Basidiomycota* in three orders were also detected. The isolate collection included 34 singleton phylotypes.

Endophyte communities in tree groups

In the entire isolate collection, endophyte phylotype detections continued to accumulate with increased sampling effort. A collection curve produced with all trees pooled into one group indicated that the sampling effort was not sufficient to characterize all endophytes colonizing white pines in this study: 97 phylotypes were expected based on a bootstrap estimate (Supplementary Fig 1). In tree groups, phylotypes accumulated at similar rates (Fig 1), and 95 % confidence intervals overlapped (not shown). This suggests no substantial difference in endophyte richness among tree groups. Similarly, corrected endophyte abundance (endophytes/meter of needle tissue sampled per tree) did not differ significantly ($F = 2.858$, $P = 0.07$), and overlapping 95 % confidence intervals suggest that corrected phylotype richness (phylotypes/meter of needle sampled per tree), and diversity indices also did not differ among tree groups (Supplementary Table 2).

Of the 69 phylotypes detected, 11 occurred in all three tree groups surveyed. The majority of these shared endophytes belong in *Lophodermium s. lat.* or *Cladosporium s. lat.*, genera often isolated from *Pinus* spp. (Carroll & Carroll 1978; Ganley & Newcombe 2006). One phylotype in *Xylaria* also occurred in all tree groups; endophytes in this genus are common in the tropics but less so in temperate forests (Arnold 2007). Six phylotypes occurred in seedlings only; these included less numerous *Lophodermium s. lat.* and *Cladosporium s. lat* phylotypes, but also a potential coprophilous fungus in the genus

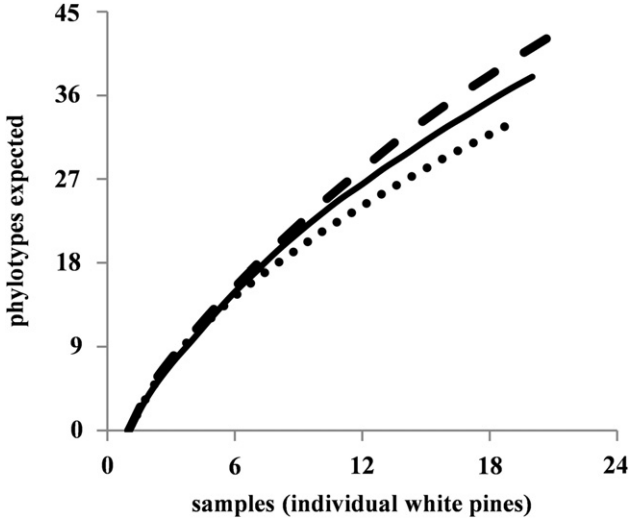

Fig 1 – Comparison of expected endophyte phylotype accumulation in "Bingham" seedlings that were browsed by deer (dotted line), unbrowsed "Bingham" seedlings (solid line), and Swan Valley genotype white pines (dashed line). 95 % confidence intervals for each tree group overlapped (not shown).

Preussia and all of the undetermined endophytes isolated. The 19 phylotypes that occurred only in SVG trees represented 18 genera and included 16 singletons. Forty-one phylotypes occurred in only one tree group. Among these were the more common *Lophodermium* s. lat. and *Cladosporium* s. lat. as before, but each of the phylotypes in the *Basidiomycota* appeared to segregate within a particular tree group, as did phylotypes in the pathogen-containing genera *Mycosphaerella* and *Herpotrichia*. Two phylotypes that appeared in only one tree group belonged to genera with known fungal antagonists: an endophyte in *Chaetomium* (Park *et al.* 2005) was isolated from a SVG needle, and an *Epicoccum* endophyte (Madrigal *et al.* 1991) occurred in IF2br foliage (Supplementary Table 1).

Per tree Bray–Curtis similarity matrix scores, averaged in tree groups, revealed that endophyte communities were more similar within than between tree groups (Fig 2). PCO coordinate means were significantly different between all tree groups along axis 1 ($F = 26.08$, $P < 0.00001$) and between the SVG and both seedling groups along axis 2 ($F = 6.89$, $P < 0.005$). PCO axes 1 and 2 explained 16.3 % and 9.0 % of the total phylotype community variation, respectively.

Component loadings analysis revealed that the varying abundances of two phylotypes explained the majority of endophyte community distance between tree groups. The disproportionate representation of L33 in the SVG trees relative to seedling groups was significant in determining endophyte community distance along PC1 (coefficient 0.96). Do78 abundance was higher in seedlings, and determined community distance along PC2 to a large extent (coefficient 0.96, loadings plot not shown). Significant loadings attributed to these phylotypes partially reflect the dominance of L33 and Do78 in the overall endophyte community (33.8 % and 21.8 % of total abundance, respectively, Supplementary Table 1).

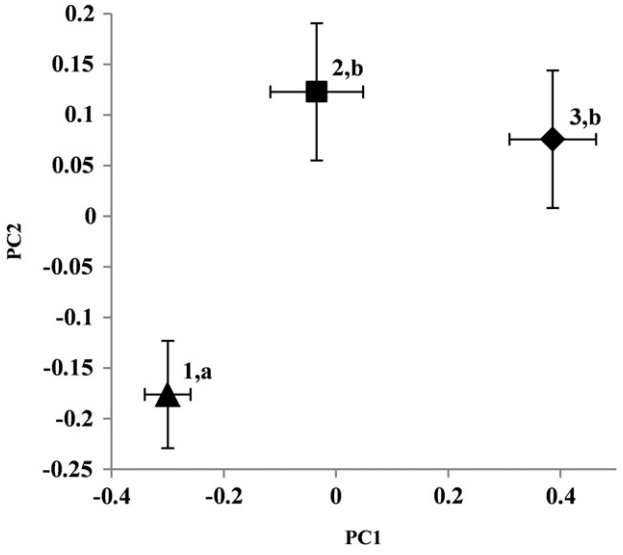

Fig 2 – Distance between tree groups based on a db-RDA of endophyte community similarity averaged for all trees in each group. Horizontal and vertical bars indicate standard error. Axis 1 explained 16.3 % of the total phylotype community variation; numeric annotations show significant differences along axis 1 between Swan Valley genotype trees (triangle), unbrowsed (square), and browsed (diamond) "Bingham" seedlings based on a Tukey's pairwise comparison at $P < 0.05$. Axis 2 explained 9.0 % of the total phylotype community variation; alphabetic annotations indicate significant pairwise differences along axis 2 between the SVG and seedling groups at $P < 0.05$.

Endophyte host environment differences – foliar nutrients

Foliar nutrient profiles differed among tree groups (Fig 3). The macronutrients P, K and S were elevated in seedlings relative to SVG trees based on an ANOVA (phosphorus, $F = 10.66$, $P < 0.01$; potassium, $F = 30.54$, $P < 0.00001$; sulfur, $F = 25.48$, $P < 0.00001$). Total N and NO^{3-} were higher in the browsed seedlings (total nitrogen, $F = 10.66$, $P < 0.001$; nitrate, $F = 35.48$, $P < 0.00001$). SVG needles contained elevated calcium relative to seedling groups ($F = 5.342$, $P < 0.01$).

Endophyte community – host environment interactions

Db-RDA tests showed correlations between endophyte community assemblage and foliar nutrient differences among tree groups. All axes produced were significant according to a Monte Carlo permutation test (trace = 0.253, $F = 1.414$, $P < 0.001$). Under forward selection for significance of environmental variables, axis 1 explained 7.6 % of species variation and 54.7 % of species-environment variation; axis 2 explained 2.6 % of species variation and 19.0 % of species-environment variation. Sulfur ($F = 3.12$, $P < 0.001$), calcium ($F = 1.7$, $P < 0.02$) and nitrate ($F = 1.74$, $P < 0.02$) segregated with tree group clusters (Fig 4) and explained 11.0 % of the total species-environment variation. The abundance of phylotype L33 declined as foliar sulfur and nitrate increased, but increased with calcium (Fig 5).

Discussion

Endophyte diversity in Swan Valley P. monticola

Using culture-based methods, we isolated fungi belonging to at least 39 genera from asymptomatic *P. monticola* needles. Twenty-six genera are previously undescribed in *P. monticola* (Supplementary Table 1); the endophytic species richness discovered here partially reflects a greater sampling effort than previously employed (Ganley & Newcombe 2006). The non-asymptotic phylotype collection curve produced in this study (Supplementary Fig 1) suggests that additional fungal diversity exists in this tree species. Based on our work, much of this additional fungal richness includes species with relatively low abundance: singletons comprised 49 % of the phylotype richness described here.

Previous work found an almost complete dominance of rhytismataceous fungi in *Pinus* spp. and *P. monticola* (Carroll & Carroll 1978; Deckert & Peterson 2000; Guo *et al.* 2003; Ganley & Newcombe 2006), but here the singletons and co-dominance of heavily melanized fungi in the *Dothidiomycetes* lowered the relative prevalence of rhytismataceous fungi. Although dramatic variation in endophyte community structure within a tree species that inhabits a wide geographic range is

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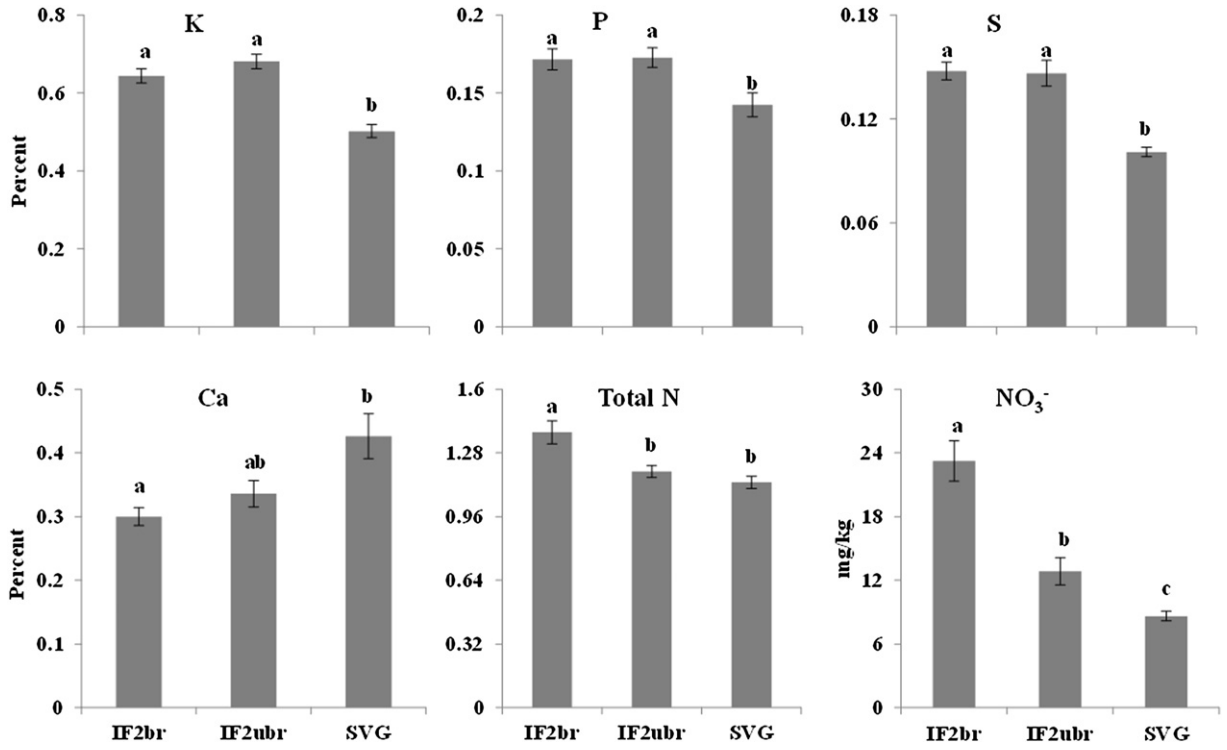

Fig 3 – Foliar nutrient comparison among tree groups. Untransformed data shown for clarity; arcsin transformed data were used for statistical comparisons. Vertical bars indicate standard error; alphabetic annotations show significant differences based on a *post-hoc* Tukey's pairwise comparison at $P < 0.05$.

expected (Hoffman & Arnold 2008), our findings also suggest that continued efforts to exhaustively sample endophyte diversity will further shape our understanding of endophytic community structure in *P. monticola*. Future sampling should include environmental PCR methods in order to characterize the unculturable endophyte diversity that inhabits white pine foliage (Arnold *et al.* 2007).

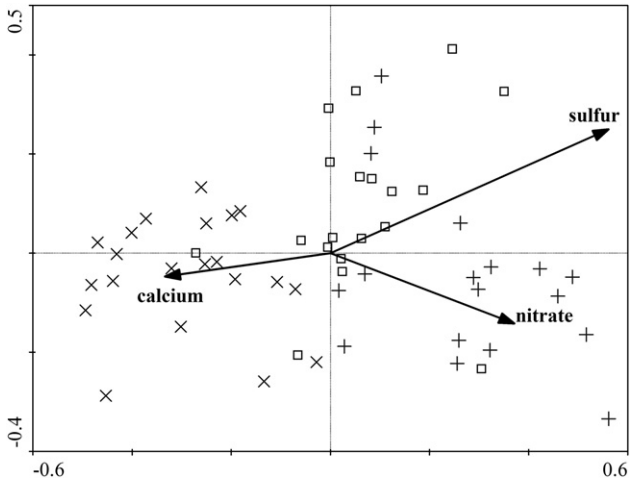

Fig 4 – Distance between Swan Valley genotype trees (×), unbrowsed (square), and browsed (+) "Bingham" seedlings based on a db-RDA of Bray–Curtis endophyte community similarity and foliar nutrient levels. Those nutrients that produced significant between-tree differences along axes 1 and 2 are shown; the arrow indicates both the magnitude and direction of variation in the nutrient.

Tree age and genotype

Endophyte communities differed between all tree groups on PC1 (Fig 2). Along PC2, seedling endophyte communities segregated together, leaving the SVG community as an outgroup. Since endophyte community distance is greater between SVG and both seedling groups than among all groups, tree age is a potential confounding factor in endophyte community assemblage. SVG trees averaged 23.4 yr (range 10–72 yr) but all seedlings were 5 yr at the time of needle collection. Although needle age was likely similar among tree groups (mid-branch needles are less than 5 yr old), young seedlings could have "missed" local endophyte sporulation and dispersal events that predated their planting on the property. Past logging activities in and around the study area altered conditions several times over the last five decades with unknown consequences for local endophyte sporulation and dispersal. For example, latent saprobes and needle cast pathogens in *Lophodermium* s. *lat.* sporulate in large number when drought stress and stand overstocking reduce the ability of individual trees to mount a defensive reaction (Sinclair & Lyon 2005). The relatively greater colonization of L33 in the older SVG trees could be the result of past conditions that favored dispersal of this endophyte. Further, since diversity index measures are similar in tree groups (Supplementary Table 2), the evidence would seem to support the hypothesis that endophyte communities form stochastically from a pool of

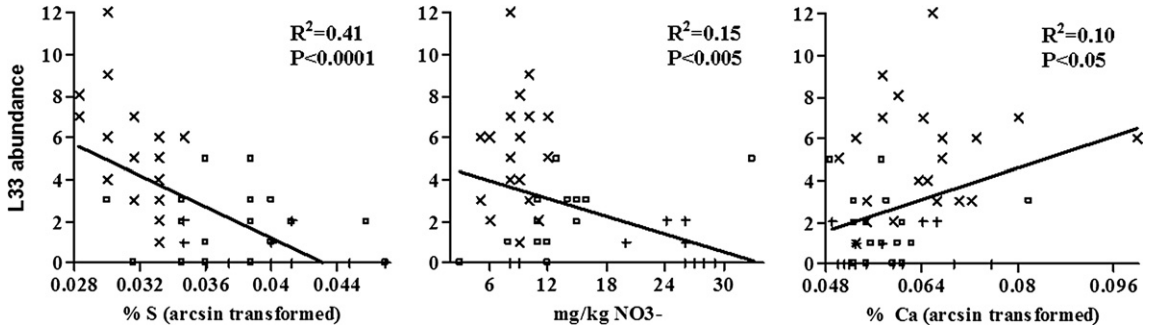

Fig 5 – Phylotype L33 abundance was less in trees with higher S and NO^{3-}, but increased with Ca levels. Percentage nutrient data were arcsin transformed and endophyte abundances in Swan Valley genotype trees (×), unbrowsed (square) and browsed (+) "Bingham" seedlings were pooled prior to linear regression analysis.

available colonizers and exclude new colonizing species once they reach a "colonization threshold" that begins to impair needle function and decreases the quality of endophyte habitat (reviewed in Sieber 2007). In this case, host age as an environmental variable should partially explain endophyte community assemblage, but db-RDA forward selection of environmental variables identified age as non-significant ($F = 0.71$, $P = 0.834$), and canonical axes remained significant with tree age treated as a covariable (trace = 0.220, $F = 1.225$, $P < 0.02$). More investigations are needed to discover the relationship that large differences in tree age have on endophyte community assemblage.

Although endophyte communities differed between the seedling groups (Fig 2), which would tend to suggest that host genotype alone is insufficient to determine endophyte community assemblage, tree genotype provides another potentially confounding explanation for endophyte community assemblage in this study. The F2 hybrid "Bingham" seedlings house a different, robust genotype compared to SVG trees. Intraspecies genotypic variation in plants affects fungal pathogen colonization (Morrison 1996; Meyer *et al.* 2010). Since the seedlings were bred for multi-gene blister rust resistance, they may also repel opportunistic or latent saprobes or weak pathogens masquerading as endophytes. This could explain the declining abundance of phylotype L33 in "Bingham" seedlings surveyed here. Phylotype L33 belongs with *Lophodermium* s. *str.*, and BLAST searches revealed 97–100 % similarity between the 52 sequences obtained and the known white pine endophyte *Lophodermium nitens* (Ganley & Newcombe 2006). Declining abundance of L33 in seedlings further supports a defensive reaction in IF2 seedlings because of its close relationship to *L. nitens*: *L. nitens* endophytes are suspected latent saprobes in *Pinus* spp. (Deckert *et al.* 2002) that can become biotrophic in weakened trees (Sinclair & Lyon 2005). The possibility that disease resistant genotype plants could influence endophyte community assemblage is intriguing as it could have unintended functional consequences.

Endophyte communities, deer herbivory and foliar nutrients

Although endophyte communities differed among browsed and unbrowsed trees (Fig 2), the dominance of these communities by a few shared phylotypes and the presence of many singleton phylotypes suggests that endophyte communities have little influence on deer browsing preference. Rather, our evidence indicates that nutrient variation among white pines in the study area influenced deer browsing and endophyte community assemblage (Fig 4). Since mammalian herbivory induces chemical defense in woody plants (Bryant *et al.* 1991, *but see* Danell *et al.* 1985), and damage to seedlings breaches protective structures (bark, leaf cuticles), deer browsing could alter fungal community structure in seedlings irrespective of foliar nutrient status. This implies a potential interaction between deer browsing and foliar nutrients that influences endophyte community assemblage.

Seedlings contained higher P, K and S levels relative to SVG trees, and N levels tended to be elevated in seedlings (Fig 3); this is probably due in part to a latent nursery effect. *A priori* nutrient levels in seedlings were likely exacerbated by management practices like uneven fertilizer applications at planting and past timber clearing events; the latter can shift nitrogen species availability in soils from NH^{4+} to NO^{3-} (Vitousek *et al.* 1979; Vitousek *et al.* 1989; Lavoie *et al.* 1992) with deleterious consequences to some conifer species (Kronzucker *et al.* 1997). Based on our results, disturbance or management action that alters plant nutrient status may have consequences for endophyte community assemblage, with unknown functional effects on ecosystems. Research in progress will investigate the effect of controlled nutrient differences on white pine browsing and endophyte community assemblage.

Foliar nutrients and L33 abundance

In the case where endophytes are latent saprobes (*sensu* Deckert *et al.* 2002) or weak pathogens that hasten tissue senescence (Sieber 2007), it would be reasonable to predict that they trigger a defensive reaction in the host, however mild or localized that reaction might be. Phylotype L33 abundance varies with foliar sulfur and calcium in a manner consistent with this prediction. Elemental sulfur functions in the induced defensive response to fungal pathogens in four agricultural plant families (Cooper & Williams 2004), and sulfur derived compounds contribute to the synthesis of antifungal compounds and reduction of reactive oxygen species (Hell 1997). Plant annexins form passive Ca^{2+} channels in simulated high reactive oxygen species environments,

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indicating that Ca^{2+} mediates plant response to stress signaling (Laohavisit *et al.* 2010).

In seedlings, higher foliar sulfur could result from constitutive defense upregulation. Young foliage in many tropical tree species contains constitutive antifungal compounds that decrease in concentration as the leaves mature (Coley & Barone 1996; Lee & Collins 2001). That the seedlings have a history of nursery care and a rust-resistant genotype bears repetition in this context, but these confounding elements fail to explain all of the relationship between L33 abundance and foliar sulfur. In the SVG tree group alone, L33 abundance declined with increasing sulfur ($R^2 = 0.29$, $P < 0.02$), mirroring the inverse relationship between sulfur and L33 abundance found in the analysis of all trees (Fig 5). The species–environment interaction was also significant in the SVG trees, with sulfur alone explaining 10 % of species–environment relation in a db-RDA ($F = 1.4$, $P < 0.03$). The question of why some trees would have higher sulfur levels than others and what implications this has for pathogen defense or endophyte colonization needs further study.

The results of this study support the intriguing possibility that foliar nutrient variation among white pines influences endophyte community assemblage. Tree age, genotype and deer browsing produce unknown, but probably additive effects on these findings. Future work will attempt to test these observations through manipulation of initial endophyte communities and foliar nutrients in paired tests of trees with varying age and genotype. We further suggest that researchers investigating endophyte diversity or ecology consider sampling plant tissues that contain a wide range of nutrient concentrations in order to more accurately describe endophyte communities.

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Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.funeco.2011.11.002.

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