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Research

Nitrogen and carbon dynamics of a foliar biotrophic fungal parasite in fertilized Douglas-fir

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Summary

• We investigated the nutritional dynamics of *Phaeocryptopus gaeumannii* and the impact of nitrogen (N) fertilization of Douglas-fir (*Pseudotsuga menziesii*) on the production of *P. gaeumannii* fungal fruiting bodies. Emergence of *P. gaeumannii* fungal fruiting bodies (pseudothecia) in Douglas-fir stomata has been directly linked to premature needle loss, a symptom of Swiss needle cast disease.

• Douglas-fir trees (10-yr-old) naturally infected with *P. gaeumannii* were treated with soil applications of N fertilizer isotopically enriched with ^{15}N to increase foliar N and track the movement of N from the host to the fungus. Foliar N, free amino acids, percent of stomata occluded by pseudothecia, N isotope and carbon (C) isotope levels were assessed on treated and control trees.

• Higher foliar N resulted in increased %N and %C in *P. gaeumannii*, as well as increased fungal fruiting and thus disease severity. Comparisons of $\delta^{15}N$ levels between *P. gaeumannii* pseudothecia and associated needles indicated an increase in $\delta^{15}N$ of needles and a simultaneous decline in $\delta^{15}N$ of pseudothecia coupled with increased levels of foliar and fungal percentage N.

• These findings confirm that *P. gaeumannii* responds to host nutrient status and that increased N availability inside Douglas-fir needles is linked to increased severity of Swiss needle cast disease.

Key words: *Pseudotsuga menziesii*, *Phaeocryptopus gaeumannii*, pathogenic fungi, stable isotope, fertilization, needle age, endophyte.

Introduction

Phaeocryptopus gaeumannii (Rohde) Petr., the causal agent of Swiss needle cast (SNC) disease on Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is a common ascomycetous endophyte in the natural range of Douglas-fir (Chastagner & Byther, 1984; Tainter & Baker, 1996; Hood, 1997). Recent SNC intensification in western Oregon has substantially reduced Douglas-fir growth (Maguire *et al.*, 2002) on over 119 500 ha in the Oregon Coast Range, USA (Hansen *et al.*, 2000). The processes influencing this host-pathogen relationship are poorly understood, limiting our ability to predict or prevent future increases in the incidence and severity of this disease. To understand and potentially ameliorate the impact of cultural practices and environmental conditions on disease intensification, the underlying mechanisms controlling disease progression must be better understood.

Previous studies have described the mode of fungal dispersal, infection processes, and symptom development in Douglas-fir. The fungus disperses via windborne ascospores released during rainfall at budbreak, from May through July in coastal areas of Oregon and Washington (Hood, 1982). Fungal hyphae enter through the stomata of newly emerging needles following germination on needle surfaces (Capitano, 1999). Infection in fully expanded needles is rare (Hood, 1997). Within needles, *P. gaeumannii* hyphae colonize intercellular spaces without disrupting cellular integrity (Capitano, 1999). In addition to occupying intercellular spaces, *P. gaeumannii* hyphae colonize the lower outer surfaces of infected needles (Capitano, 1999). Following colonization, fruiting structures known as pseudothecia, emerge from stomata. The timing and abundance of pseudothecia emerging from Douglas-fir needles is highly variable; initial

emergence can occur in needles that range from 3 months to 7 yr in age.

It is important to understand processes controlling the timing and number of pseudothecia emerging from needles because symptom development and thus disease severity associated with SNC disease is tightly coupled with emergence of pseudothecia (Hansen *et al.*, 2000 Manter *et al.*, 2000 Tainter & Baker, 1996). Emergence of pseudothecia from stomata is linked to a sharp reduction in stomatal conductance and CO_2 assimilation rates (Manter *et al.*, 2000). Models of Douglas-fir needle productivity indicate that individual needles become carbon sinks following the emergence of pseudothecia in *c.* 25% of the stomata (Manter *et al.*, 2003). Pseudothecia abundance increases with needle age and the percentage of stomata occluded by pseudothecia is coupled with increasing rates of defoliation (Hansen *et al.*, 2000). Thus, high levels of infection result in premature senescence of infected needles in younger needle age classes, causing a marked reduction in Douglas-fir productivity (Maguire *et al.*, 2002). The relationship between pseudothecia density and foliar retention was demonstrated by the application of fungicides, which decreased fungal density and increased needle retention relative to the controls (Hansen *et al.*, 2000).

The processes by which *P. gaeumannii* obtains nutrients and the role of nutrient availability in pseudothecia production are not well understood. Since *P. gaeumannii* lacks haustoria (specialized structures for host cell penetration and nutrient absorption) to physically penetrate cells (Capitano, 1999), it is inferred that the fungus acquires nitrogen (N) and carbon (C) from available nutrients in intercellular (i.e. apoplastic) spaces. Therefore, a logical assertion is that increases in apoplastic nutrient levels may improve nutrition, growth, and fruiting of this fungus.

The nutritional requirements of *P. gaeumannii* are poorly understood, yet a variety of closely related ascomycetes achieve rapid growth via direct assimilation of free amino acids (FAA) (Cochrane, 1963:; Hancock & Huisman, 1981 Solomon & Oliver, 2001, 2002). The nutritional status of plant tissues can alter FAA accessibility with implications for fungal nutrition (Nordin *et al.*, 1998; Schaberg *et al.*, 2002). Increases in foliar FAA are associated with conditions that lead to a surplus plant N relative to demand such as chronic N inputs (Näsholm & Ericsson, 1990; Ericsson *et al.*, 1993), decreasing light levels to the foliage (Shainsky & Rose, 1995), needle senescence (Chapin & Kedrowski, 1983) and budbreak (van den Driessche & Webber, 1977). In addition, an increase in foliar N has been associated with increased cell wall permeability (McLaughlin & Wimmer, 1999; Schaberg *et al.*, 2002), thereby potentially influencing apoplastic nutrient availability. Environmental effects on apoplastic nutrient availability and resulting host-pathogen nutrition may explain the observed variability in the number of *P. gaeumannii* pseudothecia emerging from infected Douglas-fir needles.

Stable isotope analysis is a useful tool for analyzing host-pathogen nutrient movement. Isotopic signatures of fungi associated with plant tissues are influenced by internal metabolism of the fungi as well as by the isotopic ratios of their nutrient source (Gebauer & Taylor, 1999; Henn & Chapela, 2001). Mycorrhizal fungi have a higher $\delta^{15}N$ value compared with associated host tissues due to isotopic fractionation that favors incorporation of ^{15}N into fungal biomass, resulting in a subsequent transfer of N depleted in ^{15}N (lower $\delta^{15}N$) to the plant (Hobbie *et al.*, 1999; Emmerton *et al.*, 2001). Saprophytic fungi have a $\delta^{15}N$ value similar to or higher than that of their nutrient sources (Gebauer & Taylor, 1999; Henn & Chapela, 2001). Both mycorrhizae and saprophytes have a higher $\delta^{13}C$ relative to host tissues (Hobbie *et al.*, 1999; Henn & Chapela, 2001). Thus, stable isotopes may be used to track nutrient flow from tree host to fungal pathogen however, compared with soil-dwelling fungi, stable isotope abundance in plant-parasitic fungi has been poorly studied.

Analyzing host-pathogen nutrient dynamics of *P. gaeumannii* will assist in determining a possible nutritional basis for the recent severe SNC outbreaks. The objectives of this study are to examine the relationship of foliar percentage N and foliar FAA to the number of stomata occluded by fungi (disease severity), the nutrition of the fungus in Douglas-fir foliage, and to document nutrient flow from host to pathogen using enriched N and natural abundance C isotopic signals. We hypothesize that first increased N and FAA levels in Douglas-fir needles will be positively correlated with the number of pseudothecia emerging from stomata, that fungal values for %N and %C will be correlated with levels in the needles, and isotopic values of the fungus will mirror values in fertilized and unfertilized Douglas-fir needles. Increased N amounts in the host tissue and subsequently in the fungus would indicate that fungal nutrition may play a role in the intensification of SNC disease and thus give us clues for disease management.

Materials and Methods

Study site

The study was conducted during 2001 and 2002 at the USDA-Forest Service Priest River Experimental Forest (PREF) in Priest River, ID, USA (latitude: 48°22′-N, longitude: 116°92′-W, altitude: 700 m). The area receives an average annual precipitation of 801 mm and a yearly average maximum temperature of 13°C and average minimum temperature of 0.7°C (WRCC, NOAA). Experiments were conducted on 10-yr-old Douglas-fir from a progeny-test of low elevation open-pollinated seed sources at PREF. All trees in the experiment were naturally infected with *P. gaeumannii* with an average severity of 0, 0.33 (0.1), 2.3 (0.1) and 3 (0.1) for needle age classes current, 1, 2 and 3-yr-old trees, respectively, using a 0–4 scale, where 0 = no pseudothecia, 1 = light infection, 2 = moderate infection, 3 =

severe infection. The average needle retention was 2.7 (0.06) yr. (Van Aelst-Bouma, unpublished data, SE in parenthesis).

Fertilizer treatments

In June 2001 *c.* 2 wk post budbreak; two levels of granular urea (46% N, $\delta^{15}N$ = 0.28‰) were incorporated into the soil around randomly selected trees. Five trees received a high N treatment (HU) of 490 g urea per tree, while five other trees received a low N treatment (LU) of 40 g urea per tree, for a total of 10 trees. In addition, five randomly chosen control trees received no N amendments. In October 2001, preliminary samples of current-year needle and pseudothecia were collected from fertilized and control trees, and analyzed for percent N (%N), $\delta^{15}N$, and FAA. The single application of urea was deemed inadequate since there were no changes in %N or $\delta^{15}N$ of needles and associated pseudothecia, or in FAA concentrations of needles (data not shown). Consequently, new amendments of 1000 g of ^{15}N-enriched urea ($\delta^{15}N$ = 3.69‰, Isotec Inc., Champaign, IL) were applied before budbreak in April 2002 to the 10 previously fertilized trees. A ^{15}N tracer was added to the 2002 urea applications to augment the isotopic signature, thereby making it easier to track foliar and fungal uptake. A total of 1040 g and 1490 g of urea were added per tree to the LU and HU treatments, respectively.

Sample collection

In August 2002, foliar samples from all treated and control trees were collected for FAA quantification and in October 2002, additional samples were collected for %N, stable isotope analysis and evaluation of disease severity. Several branches containing all foliar age class (current-year growth to 3-yr-old needles) were randomly sampled from each tree at 1.2 m from the soil. Needle age class was determined by the presence of bud scars on the stem. Samples were placed in plastic bags and transported in a cooler to the laboratory at the University of Idaho (Moscow, ID, USA) and kept frozen at −20°C until processed.

FAA quantification

Randomly selected needles from all age classes were used to quantify total FAA following Moore and Stein's ninhydrin reagent method (Moore & Stein, 1954). One-half gram of fresh needles were homogenized in 80% ethanol using a polytron homogenizer (Brinkmann, NY). Phenolic compounds were precipitated from the homogenized solution using insoluble polyvinylpolypyrrolidone (PVPP) followed by centrifugation (3250 ***g***). The supernatant was then reacted with the ninhydrin reagent and FAA were quantified colorimetrically using a spectrophotometer (570 nm). Results were reported as μmoles of leucine–equivalents per gram dry weight of foliage.

Foliar and fungal analysis

Quantification of %N, $\delta^{15}N$, percent carbon (%C) and $\delta^{13}C$ in needles and fungal pseudothecia from needles sampled in October 2002 was performed using a continuous-flow elemental analyzer (CE Instruments, Italy) coupled to an isotope ratio mass spectrometer (Finnigan, Bremen, Germany). Pseudothecia were dislodged from stomata of 2- and 3-yr-old needles by suction using a glass pipette connected to a side arm flask and vacuum line. Pseudothecia quantity was inadequate for analysis in current- and 1-yr-old needles, whereas early needle senescence limited availability of 4-yr-old needles. Because of the small size of the individual pseudothecia (*c.* a minimum of 50 needles were vacuumed to obtain 1 mg of fungal sample), detached pseudothecia were collected in deionized water and freeze dried (Labconco/Freezone 4.5, Kansas City, MO). The vacuumed 2- and 3-yr-old needles and randomly sampled needles from the current and 1-yr-old age classes were oven dried at 65°C and ground to a fine homogeneous powder for isotope analysis. Needles and associated pseudothecia were pooled by needle age class across all sample branches within individual trees for analysis.

SNC severity

To quantify the effect of N on the severity of Swiss needle cast, pseudothecia density was estimated in accordance with Hansen *et al.* (2000). One hundred adjacent stomata per needle were examined by needle age class using ×50 magnification. Numbers of pseudothecia were quantified on 10 randomly chosen needles per needle age class per tree.

Statistical analysis

Experiments were conducted in a split-plot design, where N fertilization served as the plot, individual trees served as blocks and needles were observational units. Initial analysis of the data could not be performed in an analysis of variance (ANOVA) framework because of the violation of the assumption of independence of variables, that is needle age. Needles are infected soon after emergence; therefore, needles acquire the *P. gaeumannii* inoculum at age zero (budbreak). Two approaches that accommodate for the lack of independence among needle ages in such repetitive measures experiments were employed. The two models used to investigate the effects of fertilization on %N, %C, $\delta^{15}N$, and $\delta^{13}C$ levels (foliar and fungal), as well as foliar FAA levels and infection consisted of a comparison of residuals and an order-one autoregressive structure. All statistical analyses were performed in SAS release 8.2 (SAS Institute, Cary, NC) using PROC Mixed. The approach that yielded the smallest Akaike's information criterion corrected (AICC) was selected, with the condition of congruence of the results generated by the two methods. Least squares mean separations were performed in the best-fitted model, using Tukey's

Table 1 F ratios for analysis of fertilizer and needle age effects on percent foliar nitrogen (%N), nitrogen isotope ratio ($\delta^{15}N$), foliar free amino acids (FAA) and percent of stomata occluded by fungi (severity) for needles and associated emerging pseudothecia from Douglas-fir (*Pseudotsuga menziesii*) in N. Idaho. Significance of ratios is footnoted

	Treatment response variables					
	Needles				Pseudothecia	
Predictor variables	%N (% g^{-1} d. wt)	$\delta^{15}N$ [‰]	FAA μmol g^{-1} d. wt	Severity (%)	%N (% g^{-1} d. wt)	$\delta^{15}N$ [‰]
Treatment	6.98**	32.85**	12.35**	15.69**	260.89**	189.25**
Age	6.03**	9.65**	16.87**	82.27**	16.26**	340.69**
Treatment × Age	2.16	17.19**	3.93**	7.67**	39.38**	42.52**

*, $P < 0.05$; **, $P < 0.01$; $n = 5$.

adjustment among significant main effect factors, that is fertilizer treatments and needle age classes. Where interactions between factors were significant, an ANOVA within each needle age class was performed to assess differences in response to N attributable to needle age. Where ANOVAs within age classes were significant, a least squares mean separation using Tukey's adjustment was performed, to compare the impact of individual N levels.

The relationship between percentage N and $\delta^{15}N$ in pseudothecia collected from 2- and 3-yr-old needles on fertilized trees and controls was investigated using a comparison of the slopes of the trend line for each individual tree in the experiment. Slopes were compared with each other in an ANOVA analysis followed by a least squares mean separation using Tukey's analysis in SAS. In this particular case, using trees as variables, as opposed to needles per tree, avoided violating the assumption of independence of variables faced in previous analyses.

Results

Needle nitrogen and carbon

Nitrogen fertilization increased foliar %N in HU treatments relative to controls in all needle age classes. The LU treatment was significantly higher in percentage N relative to the control in all needle ages except the current year (needle age = 0) (Table 1, Fig. 1a). In the HU treatment, uptake of N fertilizer enriched in ^{15}N is indicated by a 2–4‰ increase in $\delta^{15}N$ in all needle ages relative to the CK treatments (Table 1, Fig. 1b). LU and CK treatments were not significantly different from each other in foliar $\delta^{15}N$. There was some indication of increasing foliar $\delta^{15}N$ with increasing needle age in the LU and CK treatments (Fig. 1b) although the differences were not significant.

LU treatment significantly increased %C of Douglas-fir needles in some needle age classes (Table 2, Fig. 1c); however, foliar ^{13}C levels remained unaffected by soil N treatments (Table 2, Fig. 1d).

Table 2 F ratios for analysis of fertilizer and needle age effects on percent foliar carbon (%C), carbon isotope ratio ($\delta^{13}C$) for needles and associated emerging pseudothecia from Douglas-fir (*Pseudotsuga menziesii*) in N. Idaho. Significance of ratios is footnoted

	Treatment response variables			
	Needles		Pseudothecia	
Predictor variables	%C (% g^{-1} d. wt)	$\delta^{13}C$ [‰]	%C (% g^{-1} d. wt)	$\delta^{13}C$ [‰]
Treatment	4.45*	0.01	98.03**	1095.35**
Age	4.40**	5.77**	20.77**	11.76**
Treatment × Age	0.49	1.46	2.51	7.59**

*, $P < 0.05$; **, $P < 0.01$; $n = 5$.

Foliar FAA

Foliar FAA concentration increased with N fertilization in current-year-needles only (Table 1, Fig. 2a). FAA concentration in current year HU-treated needles was *c.* 14% higher in FAA concentrations compared with the LU treatment. Foliar FAA content of needles of fertilized trees leveled-off in older needle age classes with a trend toward slightly higher foliar FAA concentrations in the HU treatment.

Disease severity

Disease severity in needle ages two and three increased with N fertilization relative to controls (Table 1, Fig. 2b). In 2-yr-old needles, infection was 2.2 and 3.6 fold higher in HU and LU, respectively, than in controls (a mean of 13 and 21% of stomata occluded vs 6%). In 3-yr-old needles, infection was 2.3 and 2.4 fold higher in HU and LU, respectively, than in controls (a mean of 18% of stomata occluded vs 8%) (Fig. 2b). No relationship was found between infection severity and foliar FAA levels in Aug ($r^2 = 0.06$).

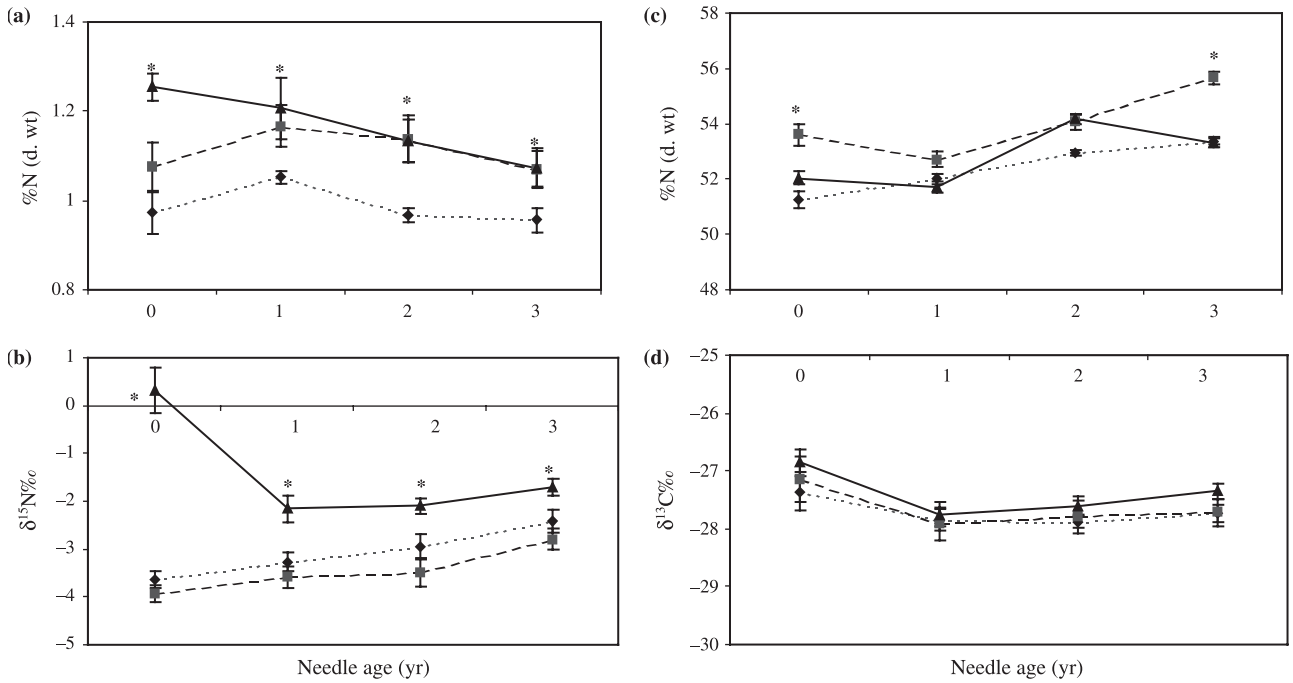

Fig. 1 Current, 1-, 2-, and 3-yr-old Douglas-fir (*Pseudotsuga menziesii*) needles sampled in Oct 2002 following different levels of soil N applications control (CK, diamond), low urea (LU, square), and high urea (HU, triangle). (a) Percent N (%N), (b) nitrogen stable isotope ratio ($\delta^{15}N$), (c) percent carbon (%C), and (d) carbon stable isotope ratio ($\delta^{13}C$). *indicate significantly different values within an age class. Bars represent SE, $n = 5$.

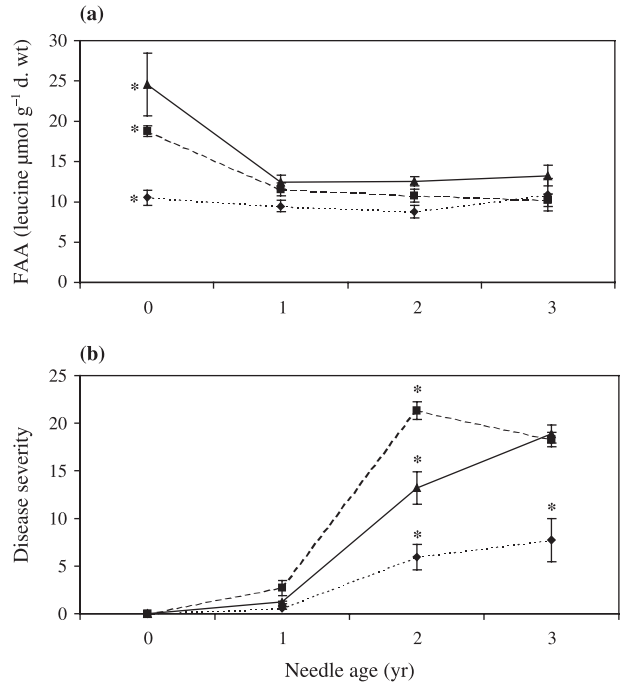

Fig. 2 Current, 1-, 2-, and 3-yr-old Douglas-fir (*Pseudotsuga menziesii*) needles sampled for foliar free amino acids and disease severity in Aug and Oct 2002, respectively, following different levels of soil N applications: control (CK, diamond), low urea (LU, square), and high urea (HU, triangle). (a) Foliar free amino acids (FAA), (b) disease severity (percent of stomata clogged with pseudothecia). *indicate significantly different values within an age class. Bars represent SE, $n = 5$.

Pseudothecia nitrogen and carbon

The percentage *N*-values were higher in pseudothecia than in needles for all treatments (Figs 3a and 1a). Nitrogen applications substantially increased %N in pseudothecia compared with controls (Table 1, Fig. 3a). Fertilization with HU had a larger effect on %N in pseudothecia emerging from 2-yr-old relative to 3-yr-old needles. Pseudothecia had significantly lower $\delta^{15}N$ values relative to the needles in all treatments (Figs 3b and 1b). Pseudothecia $\delta^{15}N$ values declined even further in fertilized needles compared with control (Fig. 3b). Pseudothecia $\delta^{15}N$ also declined noticeably with needle age, except in the HU treatment where uptake of fertilizer enriched in ^{15}N masked such trends.

The percentage *C*-values were lower in pseudothecia than in needles for all treatments (Figs 3c and 1c). Nitrogen applications increased %C in pseudothecia by 40–50% in the HU treatment, and to a lesser degree in 3-yr-old needles in the LU treatment relative to the controls (Table 1, Fig. 3a). Pseudothecia had significantly higher $\delta^{13}C$ value relative to the needles in the control and HU treatments (Figs 3d and 1d). Pseudothecia emerging from needles receiving the LU treatment had significantly reduced $\delta^{13}C$ values compared with control pseudothecia, whereas the same was true only in 3-yr-old pseudothecia in the HU treatment (Fig. 3d).

The percent N in needles and pseudothecia was positively correlated when averaged across all treatments ($r^2 = 0.60$, Fig. 4). However, as indicated by the error bars, the percentage N in the

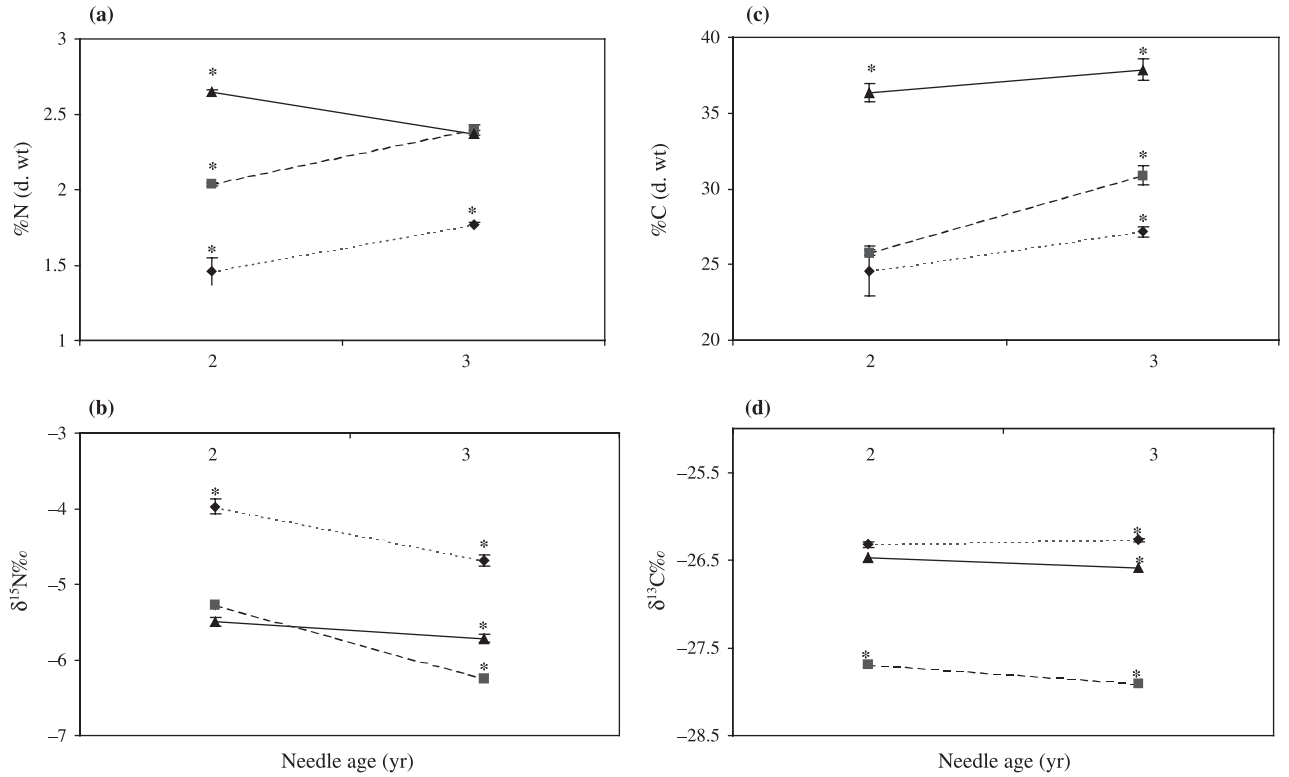

Fig. 3 Pseudothecia associated with 2- and 3-yr-old Douglas-fir (*Pseudotsuga menziesii*) needles sampled in Oct 2002 following different levels of soil N applications: control (CK, diamond), low urea (LU square), and high urea (HU triangle). (a) Percent N (%N), (b) nitrogen stable isotope ratio (δ^{15}N), (c) percent carbon (%C), and (d) carbon stable isotope ratio (δ^{13}C). *indicate significantly different values within age classes. Bars represent SE, some error bars are smaller than the symbols and are, therefore, not visible. $n = 5$.

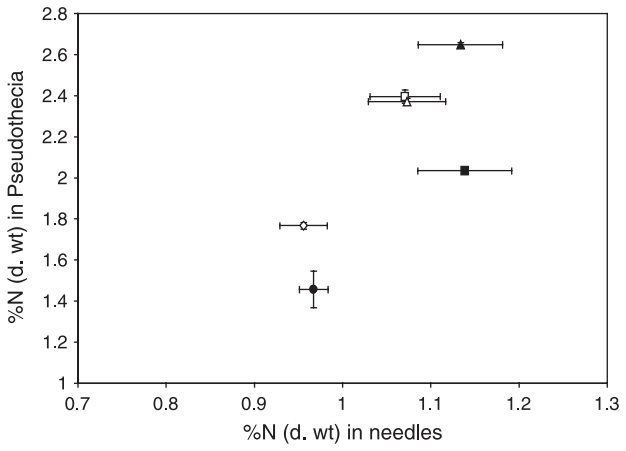

Fig. 4 Percent nitrogen in 2- and 3-yr-old Douglas-fir (*Pseudotsuga menziesii*) needles and associated pseudothecia sampled in Oct 2002 following different levels of soil fertilizer applications. Solid triangle, High urea for 2-yr-old needles; open triangle, high urea for 3-yr-old needles; solid square, low urea for 2-yr-old needles; open square, low urea for 3-yr-old needles; solid diamond, control for 2-yr-old needles; and open diamond, control for 3-yr-old needles. Points represent means. Bars represent SE. $P = 0.001$. $n = 5$. $y = 4.3705x - 2.5046$, $r^2 = 0.6026$.

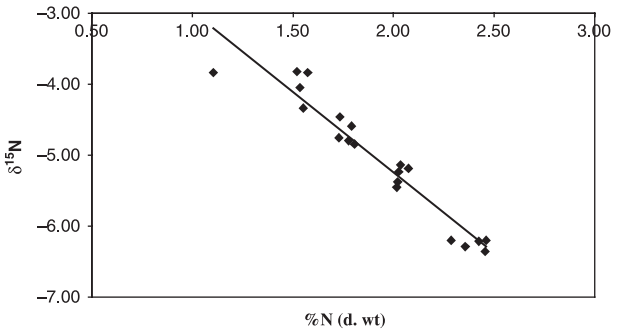

Fig. 5 Nitrogen isotope ratio (δ^{15}N) versus percent nitrogen (%N) in pseudothecia emerging from 2- and 3-yr-old Douglas-fir (*Pseudotsuga menziesii*) foliage that did not incorporate enriched N from fertilizer (i.e. low urea (LU) and control (CK)). $y = 2.3x - 0.72$, $r^2 = 0.92$.

foliage was much more variable relative to percentage N in the pseudothecia.

Percent N and δ^{15}N in pseudothecia from LU and CK treatments were negatively correlated ($r^2 = 0.92$, Fig. 5). Only the LU and CK treatments were considered in this analysis because foliar δ^{15}N indicated no incorporation of ^{15}N from the fertilizer. A significantly different slope for the HU

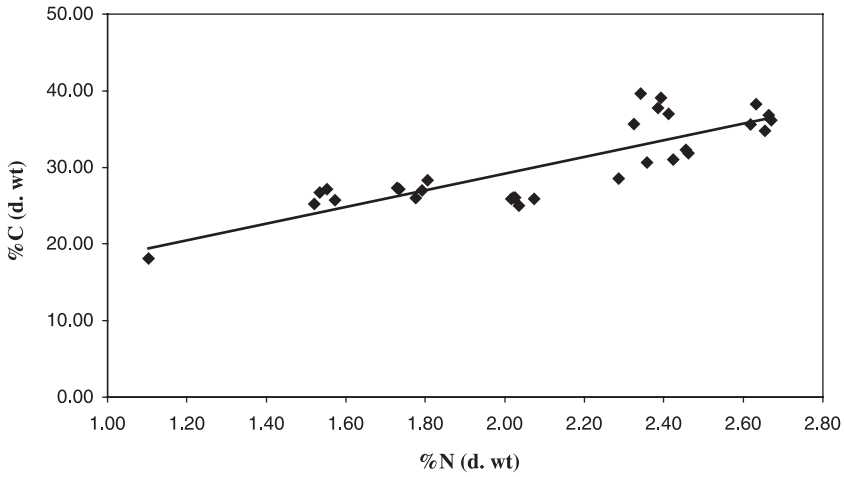

Fig. 6 Percent carbon versus percent nitrogen in pseudothecia, associated with 2- and 3-yr-old Douglas-fir (*Pseudotsuga menziesii*) foliage sampled in Oct 2002, 7 months following application of fertilizer. $y = 10.9x + 7.5$, $r^2 = 0.70$

treatment was consistent with uptake of fertilizer N enriched in ^{15}N (data not shown).

Percent N and C in pseudothecia were positively correlated across all treatments ($r^2 = 0.7$, Fig. 6). The C : N ratio of *c.* 11 is highly conserved across a range of %N and %C values in pseudothecia.

Discussion

The need for two applications of fertilizer to manipulate conifer foliar N levels *in situ* is an indication of the complexity of nutrient cycles in forested ecosystems. A delayed response to fertilization in conifer forests has commonly been attributed to N immobilization by soil microorganisms and soil particles (Kaye & Hart, 1997; Magill *et al.*, 1997). However, it was surprising that the higher N application before budbreak in 2002 did not elicit a response in foliar %N in current-year-needles in the LU treatments (total application of 1040 g urea per tree). This finding could be explained by either low N uptake or dilution of fertilizer N in new biomass. In this instance, low $\delta^{15}N$ values in current-year-needles confirm minimal uptake of fertilizer N in the LU treatment. Millard & Proe (1992) observed a similar lag in fertilizer uptake in *Picea sitchensis* in the first year of fertilization due to N immobilization. This forest ecosystem evidently had a considerable capacity to immobilize N below ground.

Foliar N isotope results indicated that N accumulation in older needles was due to uptake of fertilizer N in the HU treatment, but not in the LU treatment. The increased foliar N levels in LU older needles is most likely due to a reduction in N retranslocation from older to younger needles This is consistent with the findings that N fertilization reduces rates of retranslocation from older to new foliage in Douglas-fir (van den Driessche, 1985).

The increase in foliar percentage N within a needle age class was associated with increasing numbers of pseudothecia emerging from stomata relative to the control. Although we have no direct estimates of actual pseudothecia biomass within the needle, a strong positive correlation exists between pseudothecia density and fungal biomass (Winton *et al.*, 2003). The greatest increase in pseudothecia density occurred in the LU needles between age class one and two; then values level off in year 3. By contrast, in the HU pseudothecia, the density peak is in year 3 (Fig. 2b). None of the needles sampled from any treatment had a pseudothecia density greater than 37% indicating needles were abscised once they reached a point where a negative carbon balance occurred. Thus, the lack of an increasing trend in pseudothecia density in 3-yr-old needles of the LU treatment may be due a negative needle carbon balance and thus premature needle abscission once pseudothecia density exceeds *c.* 25% (Manter *et al.*, 2003). In the HU treatment, reduced pseudothecia density in 2-yr-old needles relative to the LU treatment may have resulted from loss of the most infected needles before we sampled. However, needle abscission rates were not assessed in this study.

An increase in foliar FAA in conjunction with higher %N is well established (Ericsson *et al.*, 1993; Nordin *et al.*, 1998). The incorporation of N into mobile FAA's is a plant mechanism to avoid ammonium toxicity, and an efficient means of storing surplus N (Näsholm *et al.*, 1993). In Douglas-fir, needles exhibit similar seasonal patterns in FAA regardless of age: FAA levels are elevated at budbreak and taper off as needles mature over a 4–6-wk period (van den Driessche & Webber, 1977). We did not measure foliar bulk FAA seasonally, however, near the end of the growing season in Aug, the FAA levels in current year foliage remained elevated relative to controls indicating a surplus of foliar N.

We did not detect a positive correlation between bulk needle FAA and pseudothecia density. In fact, as bulk FAA levels in the needles declined with age, the abundance of pseudothecia increased. The lack of a significant positive correlation between FAA levels and disease severity within a needle age class contrasts with one of the few reports reporting on host and fungal nutrition Nordin *et al.* (1998) found that increased foliar FAA

was positively correlated with incidence of *Podosphaera* and *Valdensia*, on *Vaccinium myrtillus* leaves.

Given the life history of *P. gaeumannii*, it may not be the bulk nutrient content of the needle that is important, but nutrient availability in the apoplast where the fungus resides and the ability of fungus to influence apoplastic nutrient content. The apoplastic concentration of most amino acids and total N increased in *Lycopersicon esculentum* (tomato) infected with *Cladosporium fulvum*; an intercellular biotrophic pathogen with a life history similar to *P. gaeumannii* (Solomon & Oliver, 2001). Additionally, increasing soil N supply and thus foliar N can cause leaf cell membrane leakage (Mclaughlin & Wimmer, 1999; Schaberg *et al.*, 2002), resulting in increased availability of nitrogenous compounds such as FAA for fungal uptake in intercellular spaces. Despite uncertainties in the mechanism, our data demonstrate a clear relationship between increased needle %N and infection severity in needles 2 yr of age and older.

The response of the fungi to increased foliar N was consistent with a N-limited organism. Increased N levels in the needle resulted in improved fungal N assimilation and productivity. The addition of N enriched in ^{15}N also allowed us to track the N from the soil application into the needle and into fungal fruiting bodies, thus helping us to distinguish cause and effect. The isotopic enrichment of N in HU treated needles indicates that it is the fertilizer causing increased needle N, not the fungi. In addition, percentage N and C in pseudothecia were positively correlated ($r^2 = 0.70$, Fig. 6), signifying that the processes of assimilating both elements by fungal hyphae are tightly coupled.

To our knowledge, this is the first report describing the N and C isotopic relationship between an endophytic fungus and its host. Previous fungi-host studies involved mycorrhizae (Hobbie *et al.*, 1999; Emmerton *et al.*, 2001) and saprophytes (Gebauer & Taylor, 1999; Henn & Chapela, 2001). These studies concluded that fungi could be differentiated based on their nutritional substrates rather than their ecophysiological roles (Gebauer & Taylor, 1999; Henn & Chapela, 2001). Both mycorrhizal and saprophytic fungi were higher in ^{15}N and ^{13}C relative to their host tissues. By contrast, the nutritional substrate of endophytic *P. gaeumannii* resulted in an *c.* 1‰ increase in $\delta^{13}C$ (except for the LU treatment) and a 2–4‰ decrease in $\delta^{15}N$ of pseudothecia relative to Douglas-fir needles, with an inverse relationship between $\delta^{15}N$ and N uptake (Fig. 5). The inverse nitrogen isotope fractionation of *P. gauemannii* relative to the needle (host) N underscores a different nutritional relationship of this fungus to its host compared with other fungi.

The isotopic shift in N and C between host and fungi can be the result of several processes. The decline in $\delta^{15}N$ values may indicate uptake of amino acids since biosynthetic processes in foliage lead to the production of amino acids that are isotopically depleted relative to source N (Mack *et al.*, 1986; Hobbie *et al.*, 1999). Thus, as %N in pseudothecia followed the increase in foliar %N (Fig. 3b), *P. gaeumannii* took up and utilized FAA causing a further decline in $\delta^{15}N$ relative to bulk host N (Fig. 5). In addition to fungal uptake of nutrients low in ^{15}N, fractionation during nutrient uptake, or biosynthetic processes within the fungus can cause a similar reduction in $\delta^{15}N$. Mycorrhizal fungi fractionate during N assimilation, however, the discrimination against ^{15}N or ^{14}N is dependent on the species of fungi and N form of the substrate (Emmerton *et al.*, 2001) Since the $\delta^{15}N$ value of the pseudothecia become more negative as increasing amounts of N are taken up it is likely the declining $\delta^{15}N$ values are caused by a combination of utilizing depleted N compounds and fractionation upon uptake. Fractionation of N due to biosynthetic processes is not as likely since compounds enriched in ^{15}N would have to be excreted from the fungus. Compound-specific ^{15}N analysis of N compounds in the needle apoplast and the fungus are needed to more precisely identify the class(es) of N compounds released in the apoplast and utilized by the fungus.

By contrast to the N, the fungal $\delta^{13}C$ levels were generally more enriched relative to the bulk C in the needle and there was no correlation between %C and the fungi and $\delta^{13}C$. The 1‰ increase in $\delta^{13}C$ could be linked to utilization of sucrose or starch since both compounds have $\delta^{13}C$ values that are higher than bulk leaf C (Gleixner *et al.*, 1998; Scott *et al.*, 1999). The lack of shift in $\delta^{13}C$ with increasing C uptake indicates that fractionation does not occur with fungal assimilation of C.

P. gaeumannii has long been considered a relatively inconsequential foliar biotrophic endophytic fungal parasite that coexists with Douglas-fir in the natural range of this taxon. The declining productivity of Douglas-fir stands affected by *P. gaeumannii* throughout the PNW is characterized by the development of fungal pseudothecia in younger needles and a subsequent loss of host productivity associated with carbon depletion and premature needle abscission (Manter *et al.*, 2000). This study indicates that foliar mineral nutrition may increase the susceptibility of trees to *P. gaeumannii*, however, other factors may also play an important role in disease severity such as climate conditions and host availability (Hansen *et al.*, 2000). The results of this study have far-reaching implications for disease dynamics between plant pathogenic fungi and their host plants especially given changes in foliar biochemistry associated with nutritional imbalances in forests worldwide.

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