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Growth response of Douglas-fir seedlings to nitrogen fertilization: importance of Rubisco activation state and respiration rates

DANIEL K. MANTER,[1–3] KATHLEEN L. KAVANAGH[4] and CATHY L. ROSE[5]

[1] *USDA Forest Service, PNW Research Station, 3200 SW Jefferson Way, Corvallis, OR, USA*

[2] *Present address: USDA-ARS, Soil–Plant–Nutrient Research, 2150 Centre Avenue, Fort Collins, CO 80526, USA*

[3] *Corresponding author (daniel.manter@ars.usda.gov)*

[4] *Department of Forest Resources, University of Idaho, Moscow, ID, USA*

[5] *Department of Forest Science, Oregon State University, Corvallis, OR, USA*

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Summary High foliar nitrogen concentration ([N]) is associated with high rates of photosynthesis and thus high tree productivity; however, at excessive [N], tree productivity is reduced. Reports of excessive [N] in the Douglas-fir forests of the Oregon Coast Range prompted this investigation of growth and needle physiological responses to increasing foliar N concentrations in 1-year-old Douglas-fir seedlings. After 1 year of N fertilization, total seedling biomass increased with each successive increase in N fertilizer concentration, except in the highest N fertilization treatment. Of the many physiological responses that were analyzed, only photosynthetic capacity (i.e., V_{cmax}), respiration rates and leaf specific conductance (K_L) differed significantly between N treatments. Photosynthetic capacity showed a curvilinear relationship with foliar [N], reaching an apparent maximum rate when needle N concentrations exceeded about 12 mg g^{-1}. In vitro measurements of ribulose-1,5-bisphosphate carboxylase (Rubisco) activity suggested that photosynthetic capacity was best related to activated, not total, Rubisco content. Rubisco activation state declined as foliar [N] increased, and based on its significant correlation ($r^2 = 0.63$) with foliar Mn:Mg ratios, it may be related to Mn inactivation of Rubisco. Respiration rates increased linearly as foliar N concentration increased ($r^2 = 0.84$). The value of K_L also increased as foliar [N] increased, reaching a maximum when foliar [N] exceeded about 10 mg g^{-1}. Changes in K_L were unrelated to changes in leaf area or sapwood area because leaf area to sapwood area ratios remained constant. Cumulative effects of the observed physiological responses to N fertilization were analyzed by modeling annual net CO_2 assimilation (A_{net}) based on treatment specific values of V_{cmax}, dark respiration (R_{dark}) and K_L. Estimates of A_{net} were highly correlated with measured total seedling biomass ($r^2 = 0.992$), suggesting that long-term, cumulative effects of maximum Rubisco carboxylation, R_{dark} and K_L responses to N fertilization may limit seedling production when foliar N exceeds about 13 mg g^{-1} or is reduced to less than about 11 mg g^{-1}.

Keywords: biomass, foliar nitrogen, photosynthesis, V_{cmax}.

Introduction

Nitrogen (N) is often a limiting resource in forest ecosystems and N fertilization frequently results in increased photosynthesis and enhanced growth; however, excessive amounts of N can have negative effects on physiology and growth of trees (e.g., Schulze 1989). High foliar N concentrations are usually associated with high rates of atmospheric N deposition, although amounts of foliar N can also be excessive in areas with low N deposition rates. In the Douglas-fir forests of the Oregon Coast Range, where N deposition rates are less than 1.6 kg ha^{-1} $year^{-1}$ (Fenn et al. 2003), foliar [N] can reach 14 mg g^{-1} (Rothe et al. 2002). In addition to high foliar [N], these forests are commonly infected by a foliar pathogen that causes Swiss needle cast disease (SNC) (Hansen et al. 2000).

Although studies have shown that SNC directly affects host physiology, thus reducing growth rates (Manter et al. 2000, 2003, Manter and Kavanagh 2003), the additional consequences of high foliar [N] on physiology and growth have not been studied in these forests. Preliminary evidence suggests that the periodic annual increment (PAI) of infected trees declines more abruptly and independently of the severity of SNC, when foliar [N] is above about 11 mg g^{-1} (D.A. Maguire, Oregon State University, personal communication). Recent surveys indicate that foliar [N] in coastal Oregon Douglas-fir often exceed 11 mg g^{-1} and may be as high as 20 mg g^{-1} (authors' unpublished data).

Tree growth is related to long-term estimates of net carbon dioxide (CO_2) assimilation (A_{net}) (Ledig 1969, Manter et al. 2003), and foliar [N] may have impacts on both photosynthetic and respiration rates. Increases in respiration rates with increasing foliar [N] can be related to increased rates of protein turnover and repair as metabolic contents increase (Lambers et al. 1983, Ryan 1991, 1995, Lusk and Reich 2000).

Photosynthesis also has a complex relationship with foliar N that includes changes in both photosynthetic capacity and duration (i.e., diel patterns). When examined over a wide range of foliar N concentrations, photosynthetic capacity typically

shows a curvilinear relationship with foliar N (Evans 1983, DeJong and Doyle 1985, Terashima and Evans 1988, Sinclair and Horie 1989, Cheng and Fuchigami 2000). It has been hypothesized that the curvature in the relationship between photosynthetic capacity and foliar N is associated with: (1) a CO_2 transfer resistance between intercellular spaces and the chloroplast (Evans 1983, Terashima and Evans 1988); (2) a decline in total Rubisco content, because gene activity and protein synthesis are influenced by high N:P ratios, low P contents and high carbohydrate contents (Stitt 1996, Nakaji et al. 2001); or (3) a decline in activated Rubisco content (Cheng and Fuchigami 2000) associated with concomitant nutrient limitations. For example, Rubisco activation may decline as the Mn:Mg ratio increases (Jordan and Orgren 1981, Houtz et al. 1988, Nakaji et al. 2001) because Mn may replace the Mg^{2+} cofactor, which is necessary for Rubisco activation (Portis 1992).

In our study, SNC-free Douglas-fir seedlings were fertilized with five rates of N fertilizer to explore the impact of foliar N concentration on seedling growth, and the underlying limitations on A_{net} that may arise. We used a modeling approach to determine if the quantified needle physiology and estimates of annual A_{net} were related to the observed pattern of growth in the various N fertilizer treatments.

Materials and methods

Plant material

In January 2001, 1-year-old bare-root Douglas-fir seedlings (open-pollinated north-western Oregon *Pseudotsuga menziesii* var. *menziesii* supplied by Starker Forests, Corvallis, OR) were potted in 7.6-l pots containing a 1:1 (v/v) mix of loam, peat, pumice and fine Douglas-fir bark plus micronutrients (15 g, Scotts STEP Hi Mag Formula, Teufel Nursery, Portland, OR). The 60 seedlings were randomly assigned to one of five N fertilizer treatments. Initially, all seedlings were grown in ambient conditions in a cold frame (Oregon State University Botany Farm, Corvallis, OR) and irrigated twice per week. Five fertilizer solutions were formulated based on the recommendations of Walker and Gessel (1991). The fertilizer formulations consisted of increasing N concentrations (N1 = 0.2, N2 = 0.4, N3 = 0.8, N4 = 1.6 and N5 = 3.2 g NH_4NO_3 l^{-1}), in combination with macronutrients (P = 1.10 g NaH_2PO_4 l^{-1}, K = 1.19 g KCl l^{-1}, Ca = 1.18 g $CaCl_2$ l^{-1} and S = 0.17 g Na_2SO_4 l^{-1}). Fertilizer solutions (125 ml per application) were applied weekly from January to May (20 applications) and every other week from June to December (15 applications). Fertilizer application rates were equivalent to applying 0.95, 1.9, 3.8, 7.6 and 15.2 kg N ha^{-1} $year^{-1}$, respectively, assuming 1087 seedlings per hectare.

Physiology measurements

In February 2002, CO_2 response curves (A/C_i), chlorophyll fluorescence and in vitro Rubisco activity measurements were conducted on five seedlings per treatment. Seedlings were acclimated to laboratory conditions (photosynthetic photon flux (PPF) = 150 ± 20 μmol m^{-2} s^{-1}, air temperature = 22 ± 1 °C) for 3 days before analysis. Each set of measurements was conducted over the course of a week with one seedling per treatment measured each day in random order.

For each seedling, R_{dark} (respiration in the absence of light), A/C_i curves and chlorophyll fluorescence of attached current-year needles were measured with an LI-6400 (Li-Cor, Lincoln, NE) with the Model 6400-40 cuvette set to maintain a leaf temperature of 25 °C, a water vapor concentration ≥ 10 mmol mol air^{-1} and a PPF of 1500 μmol m^{-2} s^{-1}. Ambient CO_2 concentration (C_a) in the cuvette was controlled with a CO_2 mixer at values of 30, 20, 10, 40, 50, 60, 80, 100, 160 and 200 Pa, and measurements were recorded after equilibration to a steady state (coefficient of variation ≤ 2%). Leakage of CO_2 into and out of the empty cuvette was determined at each reference C_a value and used to correct measured leaf fluxes with the equations provided in the Li-Cor operator's manual (see also, Bernacchi et al. 2002). Following measurements, one-sided projected leaf area was estimated by placing needles between glass plates and digitally estimating leaf area (Agimage, Decagon Devices, Pullman, WA). The A/C_i curves were used to estimate V_{cmax} (maximum Rubisco activity), J_{max} (maximum electron transport capacity) and R_{day} (respiration in the presence of light), based on the equations of Farquhar et al. (1980) and recommendations in Manter and Kerrigan (2004).

On completion of all A/C_i curves, foliage samples were collected and stored in liquid N until analyzed spectrophotometrically for Rubisco activity as follows. Foliage was homogenized in 3 ml of extraction buffer (100 mM Bicene, 5 mM EDTA, 0.75% (w/w) polyethylene glycol, 14 mM 2-mercaptoethanol and 1% (v/v) Tween 80, adjusted to pH 7.8 with 2 N KOH). The extract was centrifuged at 13,000 *g* for 40 s, and 50 μl of the supernatant was added to each of two samples of 900 μl of analysis buffer (100 mM Bicene (pH 8.0 at 25 °C), 25 mM $KHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 80 nkat glyderaldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase, 80 nkat creatine phosphokinase and 0.25 mM NADH). To measure initial Rubisco activity, 50 μl RuBP was added to one sample of the analysis buffer immediately (total preparation time was < 2 min) and changes in A_{340} were measured 15 s later when a steady slope was observed. To assay for total (fully activated) Rubisco activity, RuBP was added after 15 min of activation.

Needle area (A_N; cm^2 needle^{-1}) and specific leaf area (SLA; cm^2 g^{-1}) were measured on an adjacent set of 10 current-year needles and used to express measurements on a leaf area (gas exchange and Rubisco measurements) or dry mass basis (chlorophyll). All nutrient analyses were conducted on dry-ashed needles (about 2 g) by plasma optical emission spectrometry (Perkin Elmer Optima 3000DV) at the Central Analytical Laboratory (Department of Crop and Soil Science, Oregon State University, Corvallis, OR) and values are reported on a dry mass basis (mg g_{DM}^{-1}).

The following year (January 2003), seedling biomass and leaf-specific conductance (K_L; mmol m^{-2} s^{-1} MPa^{-1}) were measured on an additional subset of seedlings (n = 5) as follows. Seedling biomass (g_{DM}) was determined for root, shoot

and leaf area tissues after drying for 72 h at 70 °C. We measured K_L as described by Kavanagh et al. (1999). Briefly, four consecutive 60-s flux measurements were made on each main stem segment (about 5 cm) by connecting one end of the stem segment to tubing containing degassed deionized H_2O pressured to 12.5 kPa.

Gas exchange modeling

Total seedling A_{net} was estimated for each treatment group for the period January to December 2002 using the gas exchange model described by Manter et al. (2003). Briefly, this model assumes that stomata regulate leaf water potential above a critical threshold and then estimates carbon assimilation using the biochemical model of Farquhar et al. (1980). Group-specific estimates of gas exchange were determined based on the observed values of K_L, V_{cmax} and R_{dark} for each N fertilization treatment. Climate data needed to parameterize the model were collected at the OSU Botany Farm with Watchdog data loggers (Spectrum Technologies, Plainfield, IL).

Statistical analyses

Differences in the response variables between the fertilizer treatments were analyzed by the MIXED procedure in SAS Version 8 (The SAS Institute, Cary, NC), assuming a completely randomized design with seedling as the experimental unit ($n \leq 5$). Because we were interested only in differences among fertilizer treatments, the experimental structure was assumed to be qualitative and mean comparisons were analyzed by Fisher's LSD. All reported values are least-square means and pooled standard errors.

Results

The N fertilizer treatments resulted in significantly different values of foliar N per unit mass and area (Table 1), increasing about 2.7-fold over the range of N fertilizer treatments. Changes in the other foliar macronutrients with respect to increasing N fertilization were variable: Ca and Mn concentrations increased, Mg and K concentrations remained unchanged, and P and S concentrations decreased. Other significant changes in seedling anatomy and physiology were observed over the range of N fertilizer treatments. For example, chlorophyll concentration, A_N and K_L all increased (Table 2). Total seedling biomass increased with each successive N fertilization treatment, except the highest (N5), which did not significantly increase seedling biomass above that of seedlings in the N4 treatment (Figure 1). The N fertilizer treatments had no effects on SLA or the leaf area to sapwood area ratio (A_L:A_S), except at the lowest N treatment (N1; Table 2).

The maximum rate of carboxylation (V_{cmax}), which is dependent on the amount, kinetics and activity of Rubisco, showed a curvilinear response to foliar [N] (Figure 2). Based on in vitro Rubisco activity measurements, this curvilinear response was unrelated to total Rubisco activity, which showed a linear relationship with foliar N (Figure 3b), but was similar to the curvilinear relationship observed between initial Rubisco activity and foliar N (Figure 3a). Initial in vitro Rubisco activity estimates were lower than estimated in vivo (V_{cmax}) activity, although the relative rates remained constant across all N treatments. Because of the different amounts of initial and total Rubisco activities, we observed a decline in Rubisco activation as foliar [N] increased (Figure 3c), which was linearly

Table 1. Effects of nitrogen (N) treatments on current-year foliar nutrient concentrations (mg g^{-1}) of Douglas-fir seedlings sampled in December 2002. Means with different letters are significantly different ($P < 0.05$). Abbreviations: P = phosphorus; K = potassium; Ca = calcium; Mg = magnesium; Mn = manganese; and S = sulfur.

Treatment	N	P	K	Ca	Mg	Mn	S
N1	6.32 a	3.10 b	10.0 a	2.1 a	1.7 a	0.17 a	1.10 a
N2	8.02 ab	3.80 bc	10.4 a	2.3 ab	1.6 a	0.23 ab	0.96 b
N3	9.53 b	4.30 c	10.1 a	2.3 ab	1.8 a	0.27 bc	0.81 c
N4	12.72 c	3.10 b	10.2 a	2.8 bc	1.7 a	0.32 cd	0.69 d
N5	17.28 d	1.50 a	10.6 a	3.3 c	1.8 a	0.37 d	0.65 d

Table 2. Effects of nitrogen treatments on Douglas-fir current-year chlorophyll concentration (Chl), needle leaf area (A_N), specific leaf area (SLA), root biomass:shoot biomass ratio (M_R:M_S), leaf area:sapwood area ratio (A_L:A_S) and leaf-specific conductance (K_L) of Douglas-fir seedlings sampled in December 2002. Means with different letters are significantly different ($P < 0.05$).

Treatment	Chl (nmol g^{-1})	A_N (cm^2 needle^{-1})	SLA (cm^2 g^{-1})	M_R:M_S (g g^{-1})	A_L:A_S (m^2 m^{-2})	K_L (mmol MPa^{-1} m^{-2} s^{-1})
N1	649 a	0.218 a	75.0 a	0.34 a	1166 a	273 a
N2	793 ab	0.227 a	79.9 b	0.40 ab	1408 b	319 b
N3	939 b	0.256 b	80.3 b	0.45 b	1507 b	343 c
N4	1212 c	0.272 bc	80.8 b	0.46 b	1446 b	404 d
N5	1648 d	0.290 c	80.1 b	0.45 b	1452 b	416 d

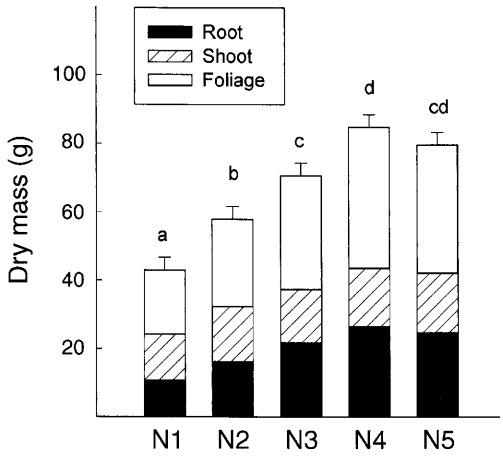

Figure 1. Biomass of Douglas-fir seedlings fertilized with increasing concentrations of nitrogen (N1–N5). Measurements were made in January 2003. Means with different letters are significantly different ($P < 0.05$). Error bars are the pooled standard errors for total biomass (root + shoot + foliage).

related to foliar Mn:Mg ratios (Figure 4). In addition to changes in photosynthetic capacity, we observed significant increases in R_{dark} as foliar [N] increased (Figure 5).

A significant linear relationship between estimated A_{net} and total seedling biomass was observed (Figure 6). Therefore, like total seedling biomass, carbon assimilation was maximized at the foliar [N] found in the N4 treatment (about 12.7 mg g^{-1}).

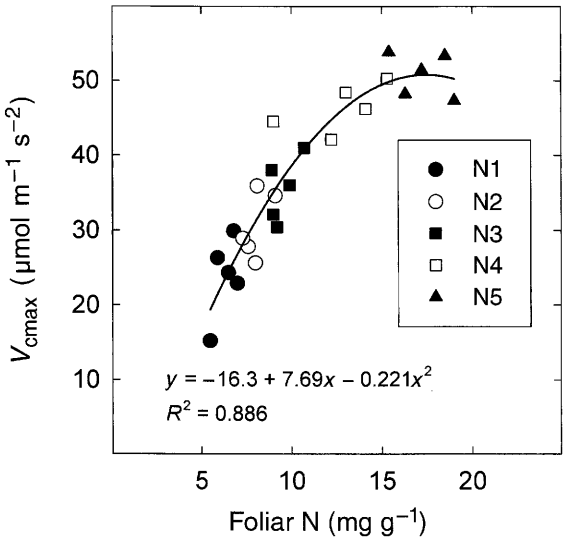

Figure 2. Relationship between foliar nitrogen (N) concentration and maximum Rubisco carboxylation (V_{cmax}) for Douglas-fir seedlings fertilized with increasing concentrations of N. Measurements were made in December 2002.

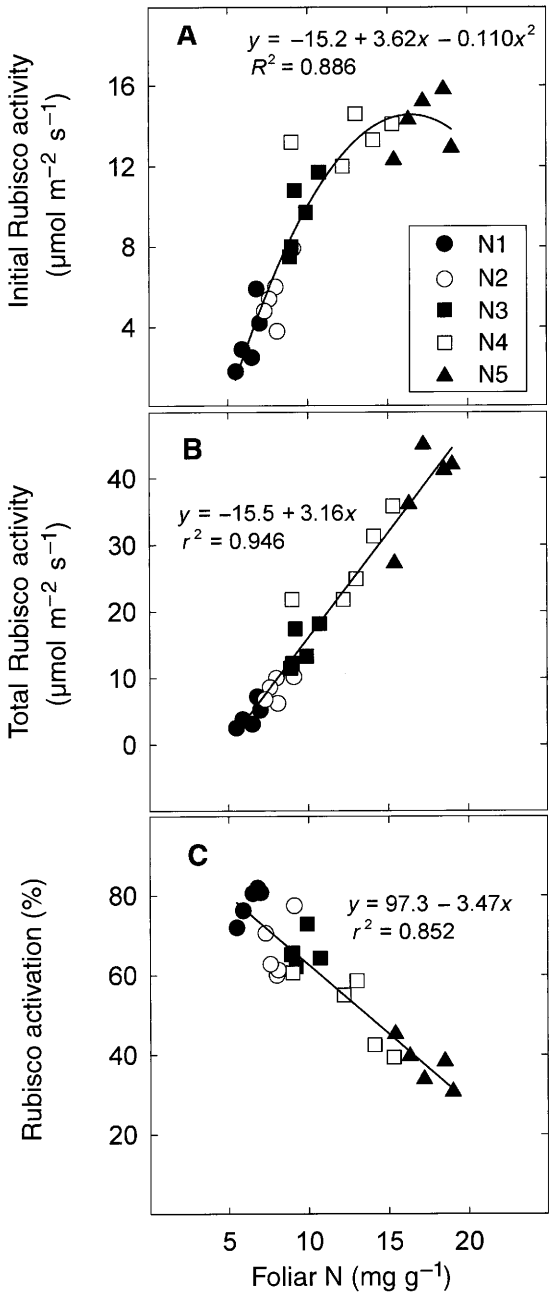

Figure 3. Relationship between foliar nitrogen (N) concentration and in vitro Rubisco measurements: initial Rubisco activity (A), total Rubisco activity (B) and percent of Rubisco activated (C) in Douglas-fir seedlings fertilized with increasing concentrations of N. Measurements were made in December 2002.

Discussion

Although a general correlation between net photosynthetic rates and foliar [N] exists (Evans 1983, 1989, DeJong and Doyle 1985, Field and Mooney 1986, Sinclair and Horie 1989), photosynthetic capacity or V_{cmax} typically reaches a maximum because of a limitation elsewhere in the photosynthetic system. As foliar [N] increased, both in vivo and in vitro estimates of Rubisco activity showed a linear increase up

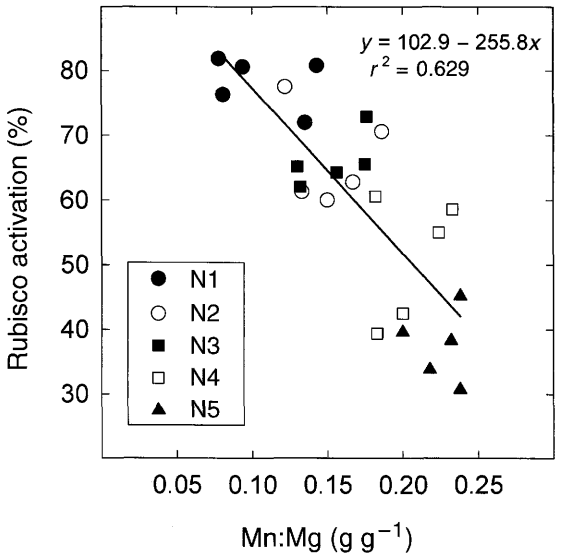

Figure 4. Relationship between the Mn:Mg ratio and the percent of Rubisco activated for Douglas-fir seedlings fertilized with increasing concentrations of nitrogen (N1–N5). Measurements were made in December 2002.

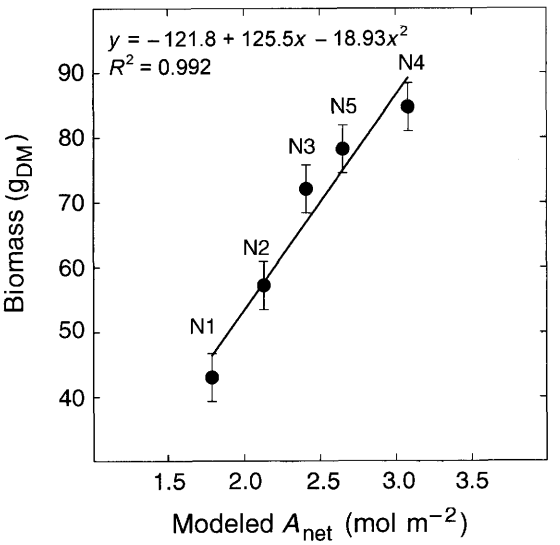

Figure 6. Relationship between total modeled net CO_2 assimilation (A_{net}) for January–December 2002 and total seedling biomass measured in January 2003. Estimates of A_{net} were calculated based on the mean values of leaf-specific conductance, maximum rate of carboxylation and dark respirations for each treatment, and hourly observations of meteorological data collected at the OSU Botany farm using the gas exchange model described by Manter et al. (2003).

to a foliar [N] of about 12 mg g^{-1}, reaching a maximum of about 45 and 14 μmol m^{-2} s^{-1}, respectively. The reduced rate of in vitro activity relative to in vivo activity suggests incomplete extraction of Rubisco prior to activation; however, the qualitative consistency between the two values indicates the treatment effects are valid (Rogers et al. 2001).

The curvilinear relationship between V_{cmax} and foliar [N] (Figure 2) is best explained by a decline in Rubisco activation as foliar [N] increases. Previous studies suggest that the curvilinear relationship between V_{cmax} and foliar N is associated with the amount of total (e.g., Nakaji et al. 2001) or activated Rubisco (Cheng and Fuchigami 2000). In our study, the latter best explains the curvilinear relationship between photosynthetic capacity and foliar N, because we observed increasing amounts of inactivated Rubisco as foliar N increased (Figure 3c). Activation of Rubisco requires the presence of CO_2 and Mg^{2+} (Portis 1992), and Mn competes with Mg^{2+} for binding to Rubisco, which results in an inactivated form of Rubisco (Houtz et al. 1988, Nakaji et al. 2001). This may be the mechanism underlying the decline in Rubisco activation as foliar [N] increased, because we observed a linear relationship between Rubisco activation and Mn:Mg ratio (Figure 4). However, because all nutrient concentrations (relative to N) declined as foliar [N] increased, and were also significantly correlated with Rubisco activation ($R \geq 0.521$), we cannot rule out the possibility that other nutrient limitations, such as P, also influenced the amount of activated Rubisco (e.g., Brooks 1986, Lauer et al. 1989, Nakaji et al. 2001, Warren and Adams 2002).

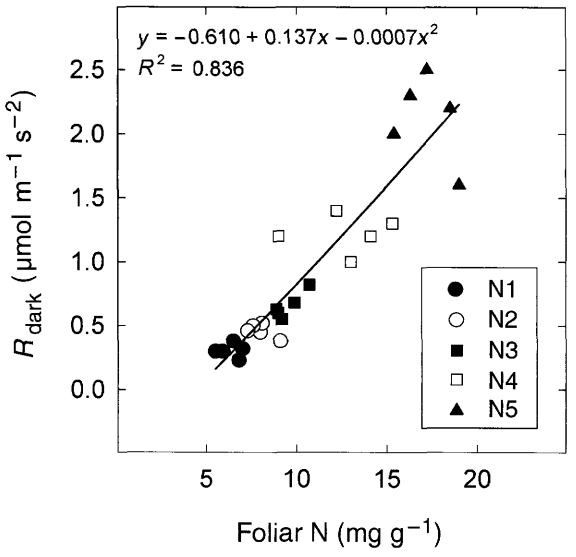

Figure 5. Relationship between foliar N concentration and dark respiration (R_{dark}) for Douglas-fir seedlings fertilized with increasing concentrations of N. Measurements were made in December 2002.

In another study with Douglas-fir seedlings, Ripullone et al. (2003) observed a linear relationship between V_{cmax} and foliar N. Although they reported foliar N on an area basis, the relatively constant SLA observed here suggests that the difference between the two studies, linear versus curvilinear response, is not associated with area versus mass-based measurements. Furthermore, assuming similar SLA between the two studies, the range of foliar N concentrations was similar between the two studies (about 5 to 20 mg g^{-1}). We propose that the difference between the two studies is related to a non-N nutrient limitation. For example, other macronutrient contents in the Ripullone et al. (2003) fertilizer treatments were adjusted

based on the amount of supplied N, whereas our formulations were not. As a result, changes in nutrient ratios, such as the Mn:Mg ratio observed here, may not have developed in the Ripullone study, decreasing the likelihood of Mg-limited Rubisco activation. Unfortunately, this cannot be verified because neither Mn:Mg ratios nor in vitro Rubisco activity measurements were reported.

Although we observed a significant increase in respiration rates with increasing foliar N concentrations (Figure 5), the mechanistic-basis for this relationship is unknown and may not represent a direct linkage. Similar foliar N–R_{dark} relationships have been observed in a variety of other woody plant species (Ryan 1991, 1995, Reich et al. 1998*a*, 1998*b*, Lusk and Reich 2000). However, studies vary as to the presence and degree of the relationship between foliar [N] and R_{dark} (e.g., Ryan 1991, 1995, Lusk and Reich 2000, Schaberg et al. 1997). The foliar N–R_{dark} relationship, when present, presumably reflects the cost of protein turnover and maintenance (Lambers et al. 1983, Penning de Vries 1975). However, in some cases, it has been shown that, as foliar [N] increases, protein contents may reach a maximum or even decline, as a result of other nutrient limitations such as P, K or S (e.g., Brooks 1986, Lauer et al. 1989, Nakaji et al. 2001, Warren and Adams 2002). Nevertheless, higher rates of respiration will increase the consumption and depletion of carbon reserves in foliage tissues, which alone could result in a decline in growth and vigor of coniferous species (e.g., McLaughlin et al. 1990, 1991). In addition, higher respiration rates could affect cellular defense and repair mechanisms, causing other stresses that are dependent on repair and maintenance respiration to have a greater and more persistent detrimental impact on host physiology. For example, SNC affected trees, which may experience photooxidative damage at high irradiances (Manter 2002), may be unable to repair damaged proteins as quickly or as efficiently as healthy trees.

The third physiological change that we observed in N-fertilized seedlings, which would increase stomatal conductance and thus long-term carbon assimilation and growth, was a trend for K_L to increase as foliar [N] increased, reaching maximum values as foliar [N] reached about 10 mg g^{-1} (Table 2). Increases in K_L were unrelated to changes in A_L or A_S, because A_L:A_S ratios remained constant. Instead, changes in K_L were most likely related to growth differences and increased sapwood permeability in the fastest growing seedlings. This result is consistent with the results of Manter and Kavanagh (2003) who showed that disease-induced declines in stem growth limit sapwood permeability and stomatal conductance.

We observed the greatest seedling biomass production at a foliar [N] of about 12 mg g^{-1} (Figure 1), which is close to the value of 11 mg g^{-1} at which the plant area index of SNC-infected Douglas-fir trees decline, independent of the severity of SNC. In addition, given that recent surveys have shown that foliar N in coastal Oregon Douglas-fir often exceeds 11 mg g^{-1} and may be as high as 20 mg g^{-1} (authors' unpublished data), it is possible that the declining tree productivity associated with SNC is related not only to the disease but also to concurrent shifts in physiology caused by excess foliar N.

Annual estimates of A_{net}, determined by a modeling approach based on treatment means of V_{cmax}, R_{dark} and K_L support the observation that A_{net} is maximized at a foliar N concentration of about 12 mg g^{-1}. The physiological response to changes in foliar [N] is complex, influencing all aspects of seedling carbon budgets—assimilation capacity (V_{cmax}), duration (K_L) and consumption (R_{dark}). The nature of the observed relationships resulted in a limitation on A_{net} and growth rates, such that further increases in foliar N concentration no longer have a beneficial effect. This threshold will differ depending on the species studied and concomitant changes in other macronutrients.

In conclusion, foliar N concentrations affected several physiological processes that ultimately impact tree growth. Once foliar N concentrations exceeded about 13 mg g^{-1}, seedling productivity declined. The significance of these results is apparent from the common occurrence of Douglas-fir foliar N concentrations in excess of 13 mg g^{-1} in the Oregon Coast Range (Rothe et al. 2002, authors' unpublished data).

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