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Soil moisture, N, P, and forest cover effects on N fixation in alders in the southern boreal forest

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Abstract. Outside of tropical and arid regions, plant productivity is limited by the availability of soil N. Ironically, these are the regions where N-fixing plants are restricted to early successional habitats. The energy cost of N fixation has been used to explain the paucity of N-fixing plants in late-successional ecosystems, and models predict reduced light and increased soil N availability will reduce N-fixing plant success. The lack of success of N-fixing plants has also been explained by their greater need for some elements, especially phosphorus. We examined the effect of N and P fertilizer over four years on N fixation in *Alnus alnobetula* spp. *crispa* in a mature *Pinus banksiana* forest in the southern boreal forest, and an adjacent site where wildfire had removed the tree canopy. We also monitored N fixation throughout two growing seasons examined the effect of artificial shading on photosynthesis. The addition of N or P fertilizer resulted in a 60% decrease in plant nodule nitrogenase activity. N fertilizer also reduced the proportion of cells in the root nodules containing N-fixing vesicles in the forest site. Shrubs in the forest had the highest photosynthetic rate when measured on a leaf mass basis, indicating an increased carbon capture efficiency compared with shrubs in the open. Over the course of two growing seasons, soil temperature had a positive effect on specific nitrogenase activity, and soil in the open site was warmer than soil in the forest in the spring. However, as soil moisture decreased, temperature was a weaker predictor of nitrogenase activity. Although all shrubs consistently acquired most of their N from fixation, shrubs in the forest derived more than shrubs in the open, which were more water limited. Overall, these results show that N-fixing plants may benefit from being in the understory for improved water balance in this region of the boreal forest. As such, light availability may not be the primary factor limiting the abundance of N-fixing shrubs.

Key words: Alnus; boreal forest; canopy cover; green alder; N fixation; nutrient amendment.

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INTRODUCTION

In regions of the globe where N limits terrestrial ecosystem productivity, symbiotic N-fixing plants tend to be rare in late-successional communities (Houlton et al. 2008, Menge et al. 2014). This is especially true in the boreal forests of North America, where there are no N-fixing trees, and the native N-fixing actinorhizal shrubs and herbaceous legumes are mostly restricted to early successional communities. Unlike other plants, N fixers tend to reabsorb only a small proportion of their N from senescing leaves (Stewart et al. 2008). Therefore, their leaf litter provides a large input of N into the soil. While decomposition and N mineralization are dependent on favorable climatic conditions (Hobbie 1996, Moore et al. 1999), N-fixing plants can increase overall soil decomposition rates and increase soil fertility (Chapin et al. 1994, Binkley 2003, Kohls et al. 2003). We have well-documented cases of how N-fixing plants

facilitate succession by increasing soil fertility (Chapin et al. 1994, Kohls et al. 2003). What is less clear are the reasons for the lack of success of N-fixing plants in late-successional communities. Understanding limitations to N fixation are therefore crucial to understanding limitations to ecosystem productivity and carbon storage.

Several hypotheses have been put forward to explain why N-fixing plants are not dominant in N-limited ecosystems. N fixation is more energetically costly than absorbing inorganic N from the soil (Tjepkema and Winship 1980). Decreased access to light and/or increased access to inorganic N is therefore predicted to reduce the advantages of N fixation and, consequently, lower the abundance of N fixers (Vitousek et al. 2002). Models based on this reasoning predict that N fixers will be confined to early successional communities because of competitive exclusion by non-N-fixing plants (Rastetter et al. 2001, Menge and Crews 2016). It has also been suggested that N fixers are less shade tolerant than non-N-fixing plants, even though they belong to clades with shade-tolerant species (Menge et al. 2010). This implies that there is an ecophysiological constraint on symbiotic N-fixing plants in shaded conditions. N fixation is also more sensitive to temperature than is carbon acquisition, and a model with this assumption predicts the decrease in abundance of N-fixing plants at higher latitudes (Houlton et al. 2008).

There are also two crucial interactions between N fixation and P nutrition in plants. N-fixing plants tend to have a higher P requirement than non-N fixers (Ingestad 1980). Consequently, some P addition experiments have shown increases in N-fixing nodule formation, rates of fixation, and plant growth (Uliassi et al. 2000, Valverde et al. 2002, Ruess et al. 2013). On the contrary, phosphatase enzymes used for P acquisition have a high N content (Treseder and Vitousek 2001). N fixation can therefore supply the N needed to generate phosphatases (Maseko and Dakora 2019), and N-fixing legumes tend to have a higher phosphatase activity than non-N-fixing plants (Olde Venterink 2011). This increased phosphatase production has been used to explain the success of N-fixing plants in P-limited tropical environments (Wang et al. 2007). A corollary to the temperature effect on N fixation is that in tropical climates, some N fixers become dominant because the temperature is high enough to allow them to generate the fixed N needed to produce phosphatases but are unable to do so at higher latitudes (Houlton et al. 2008). It has also been shown that N fixers in high latitude forests also do not have increased phosphatase production compared with non-N fixers (Ruess et al. 2019). Recently, the ability of N fixation to increase plant P supply has been brought into question. Studies suggest that both legumes and actinorhizal plants have a broad range of nutrient acquisition strategies, and their higher phosphatase activity is not related to N fixation (Png et al. 2017, Batterman et al. 2018, Soper et al. 2018, Guilbeault-Mayers et al. 2020).

There are several ways plants can acclimate to abiotic and biotic conditions that could complicate our understanding of N-fixing plants lack of success in late-successional habitats. First, N fixers can have a facultative N fixation strategy, that is, they can downregulate both nodule formation and activity when N fixation is no longer advantageous. The depressive effect of N fertilizer on the N-fixing symbiosis in both legumes and actinorhizal plants has been well documented in laboratory studies (Markham and Zekveld 2007, Hiltbrunner et al. 2014) and to some extent in the field (Ruess et al. 2013). However, N isotope ratio data suggest that the higher latitude N fixers (mostly actinorhizal plants) tend not to downregulate N fixation, indicating an obligate N fixation strategy under natural field conditions (Andrews et al. 2011, Menge et al. 2014). Second, N fixation depends on the identity of the symbiont partner, with some symbionts providing little fixed N (Markham 2008*b*). The influence of light and nutrient availability on this interaction has not been studied. Still, nutrient additions can cause a shift in the identity of N-fixing symbionts in actinorhizal plants (Ruess et al. 2013) and different plant/symbiont combinations can result in substantial variation in N fixation in different habitats (Markham and Chanway 1999). Finally, many species can show substantial adjustment in their leaf morphology under shaded conditions, allowing them to compensate for the reduced available energy for photosynthesis (Givnish 1988, Mathur et al. 2018). How this affects N fixation has received little attention.

Alnus alnobetula subsp. *crispa* (Aiton Raus) (hereafter referred to as *A. crispa*) is one of the

most common and widely distributed N-fixing shrubs in the North American boreal forest. *Alnus* species are currently expanding their range in the boreal forest, subarctic, and montane regions. Since their N-fixing capability can profoundly alter ecosystem function, understanding their ecology is becoming increasingly important (DeMarco et al. 2014, Hiltbrunner et al. 2014). Like other *Alnus* species, *A. crispa* is always found to possess N-fixing nodules in the field. However, laboratory studies have shown N fixation rates per mass of nodules to be highly variable (Markham 2008*b*). Also, like other actinorhizal plants, laboratory assays show that nodule formation and N fixation are negatively affected by soil N availability, that is, they can be facultative N fixers to some degree (Markham and Zekveld 2007). Therefore, it is possible that under conditions that favor absorption of inorganic N from the soil over N fixation (lower light and P, and higher soil N), plants growing in the field would be expected to reduce the amount of N they fix.

We compared the performance of *A. crispa* in the understory of a mature *Pinus banksiana* forest and an adjacent open area where the trees, but not the understory vegetation, had been killed by fire. While this allowed us to compare plants growing in forested vs. a non-forested habitat after a natural disturbance, this approach necessarily limits the implications of our study. It cannot address the broad range of conditions that occur in forest understories where *A. crispa* is found. We compared rates of photosynthesis, leaf morphology, and N reabsorption prior to leaf senescence of plants in the two habitats. Over four years, we monitored shrubs receiving annual additions of N and P fertilizers, and in two years, we monitored N fixation over the growing seasons. Additionally, seasonal changes in soil temperature and moisture were monitored to investigate their effects on N fixation.

Materials and Methods

Study site

The study was conducted in southeastern Manitoba, Canada, near the transition between the boreal forest and the central grasslands of North America (latitude 49.36° N, longitude 96.19° W). The average growing season (May–August) total precipitation is 336 mm, and the mean temperature is 16°C (http://climate.weather.gc.ca). The landscape consists of a series of sandy beach ridges formed from Lake Agassiz during the Pleistocene. The two study sites were originally part of a single *P. banksiana* stand. In 2008, a wildfire removed the overstory trees from half the site, but almost all the understory plants resprouted the following year (Markham and Essery 2015). Trees were 97 yr old at the start of the study with a density of 400 tree/ha, a diameter at breast height of 25 ± 6 cm (mean ± standard deviation [SD]), and a canopy openness of 33%, measured from hemispherical images of the tree canopy (Frazer et al. 1999). The understory consists of an ericaceous shrub/feathermoss type of community (community type CNVC00207 in Chapman et al. 2020), which is considered nutrient poor. Soils are low in inorganic N and phosphate (see *Results*). Summer droughts are common in the region.

Shade experiment

In 2012, 20 *A. crispa* shrubs in the open site and 10 shrubs in the forest were selected at random over an area of ~3 ha. To separate the effects of overstory shading from other differences between the forest and open site, 10 of the shrubs in the open site had shade structures set up over them just prior to their buds opening. The structures were made of a wooden A-frame with the ridge pole running in an east–west direction ~1 m past the edge of the perimeter of the shrubs and 50 cm above their tops. Two layers of green nylon monofilament shade cloth (Lee Valley Ltd., Ottawa, Ontario, Canada) were erected on the south side of the frames from ~10 cm above the ground to the ridge pole and secured with guy lines to prevent the cloth from touching the shrubs. Mid-day light levels on the top of the shrubs were measured on July 15 using a LiCor PAR light sensor (LiCor, Lincoln, Nebraska, USA). Hemispherical images were also taken over the shrubs without shade structures in the open and forest sites (this could not be done over the artificially shaded shrubs). These images were then used to calculate the mean daily amount of light reaching the shrubs using GLA version 2.0 (Frazer et al. 1999). Photosynthetic response to light was measured on a fully expanded leaf on each shrub between 5th August

and 23rd August using a LiCor LI-6400 (LiCor) portable photosynthesis system with an LED light source. The leaf chamber CO_2 level was set at 400 ppm, and carbon dioxide uptake was measured consecutively at light levels of 1500, 1000, 500, 200, 100, 50, 20, and 2 μmol PAR·m^{-2}·s^{-1}. The leaf temperature during measurements was 21.5° ± 1.6°C. Leaf photosynthetic rates were expressed on a leaf area and a leaf dry mass basis (Givnish 1988). The relationship of CO_2 uptake vs. light level was fitted using the exponential model of Chalker (1981), adding a parameter for respiration rate (R). Least-squares fits of the data were used to estimate the maximum photosynthetic rate (P_{max}), the light level at which the initial slope of the photosynthesis response curve intersects with P_{max} (Ik), and R. Parameter estimation was performed using the nonlinear fit function in JMP Pro 14.0 (SAS Institute 2018). The coefficient of determinations (r^2) for the least-squares fits ranged from 0.90 to 0.99.

Nutrient addition experiment

In 2012, 40 *A. crispa* shrubs in both open and forest sites were selected at random. Each shrub was separated by at least 10 m, and in the forest, they were separated by overstory trees. We therefore considered individual shrubs to be independent replicates of the shading effect of the trees but recognize that the degree of shade differs from stand to stand. So, our results are may not be applicable across a range of stand types. Twenty shrubs in each site were controls and 10 each received either N or P additions. The three treatments were assigned in groups of four shrubs near one another, with the groups used to account for variation within the sites. Nutrients were added each spring from 2012 to 2015 just as the *A. crispa* buds started to open. N was added as $CaNO_3$ at a rate of 50 kg N/ha over a 2 m radius around each shrub. P was added as triple superphosphate at 13 kg P/ha applied over the same area. Soil samples were collected around the base of each shrub in 2012, 2013, and 2015 by taking three 2.5 cm diameter × 15 cm deep cores of the mineral soil and bulking them. The soil was air-dried before being analyzed for total inorganic N using the microdiffusion technique (Kahn et al. 2000) and phosphate using Bray extractions (Kalra and Maynard 1991). In 2014, a decomposition assay was conducted by placing oven-dried wood blocks of aspen under the litter layer of each shrub for one year and determining their mass loss.

N fixation, reabsorption, and carbon isotope discrimination assays

Root nodule activity was measured using acetylene reduction assays. These assays give a short-term (approximately one hour) rate of N fixation activity. Shrubs in the nutrient amendment experiment were assayed only once each year in 2012 and 2013, in late July, to minimize disturbance to the shrubs. To assess seasonal variation in nodule activity, additional non-nutrient amended shrubs were also assayed from mid-May to the end of October in 2013 and 2017. Measurements were taken weekly or bi-weekly. To avoid any effects previous digging and nodule removal might have on N-fixation activity, different shrubs were sampled on each date. Nodules were selected by excavating a randomly selected area, ~20 × 20 cm under the base of a shrub until a nodule was encountered. Nodules were excised from the roots and placed in a sealed 50-mL syringe, and 10% acetylene was added to the air. The syringe was placed next to the root it was excised from and covered with soil and incubated for one hour. Soil temperature and moisture (measured using a theta probe; Delta-T Devices Ltd, Cambridge, UK) were measured at the time of the assay next to the syringe. Gas samples were then taken from the syringe by displacing water from a sealed vial, thereby maintaining the gas at atmospheric pressure. Gas samples were extracted from the vial by water displacement and analyzed for ethylene and acetylene using a FID on gas chromatograph fitted with a HaySep T column. Control samples consisted of two types: samples of 10% acetylene without nodules and samples with nodules incubated with no acetylene. These were periodically included in the sampling as controls for ethylene in the acetylene supply and non-nitrogenase production of ethylene by the nodules. The quantity of acetylene in the headspace sample was used to test for any sample leakage. On each measurement date, 4–15 shrubs in each site and in each treatment were measured (during the field sampling a minimum of six shrubs were sampled, but on some dates, samples were lost because of leakage). Acetylene reduction was expressed as

specific nodule activity (SNA), that is, the amount of ethylene produced per nodule dry mass per unit time. In 2013, after the assays were complete a single lobe of each nodule was sectioned longitudinally and stained with Fabil's stain. The length and width of the region of cells containing N-fixing vesicles were measured and expressed as a percentage of the total nodule lobe length and width (Appendix S1: Fig. S1).

From 2012 to 2015, N fixation over the growing season was estimated from the ratio of stable nitrogen isotopes in leaves of non-nutrient amended and P-amended plants. (The N fertilizer was enriched in ^{15}N and non-fixing plants had depleted levels of ^{15}N compared with plants getting all their N from fixation. It was therefore not possible to estimate levels of N derived from fixation for plants receiving N fertilizer.) The leaves used in the assay were the third to fifth leaf from the terminal end of branches in the upper part of the shrubs, collected in late August each year. They were dried at 65°C and ground in a ball mill before being sent for analysis at the UC Davis Stable Isotope Facility. Leaves of two other non-N-fixing species (*Amelanchier alnifolia* and *Prunus virginiana*) were used as reference species. The proportion of nitrogen derived from fixation (Ndfa) was calculated according to Boddey et al. (2000). We used −1.1 as the $\delta^{15}N$ value of plants deriving all their N from fixation, obtained by growing *A. crispa* plants on acid-washed Turface (Profile Products, Buffalo Grove, Illinois, USA), fertilized with N-free Rorrison's nutrient solution. In 2013, N reabsorption in senescing leaves was estimated. The N content of leaves collected in midsummer was compared with leaves senescing in September. Senesced leaves were obtained by gently shaking branches and collecting the leaves that detached. The N content of leaves was estimated from the stable isotope analyses. Carbon discrimination was calculated using the same samples used for the N isotope analysis, according to Farquhar et al. (1989).

Statistical analysis

The experiments were analyzed using restricted maximum likelihood mixed-effects models. The open, forest, and artificial shade environments were considered fixed effects. However, since we were taking advantage of a nature disturbance, the open and forest environment is not replicated for each shrub. This limits the applicability of our results, and we caution that the patterns we report can be the result of the particular conditions of the site history. For the shade experiment, the sampling day was included as a random variable for the photosynthesis data. For the nutrient addition experiment, the nutrient amendment treatment was crossed with site. For variables measured in multiple years (Ndfa, carbon isotope discrimination, soil N, and soil phosphate), the year and individual shrub were added to the model as random effects. Because there was a large variation in growing season precipitation among years, we compared the relationship between annual growing season precipitation and Ndfa for plants in the forest and the open using an exponential model of the form:

$$y = a + b^{cx}.$$

We compared this three-parameter model with a two-parameter model (b and c) and a one-parameter model (c only) using Akaike's information criterion (AIC). Differences in parameters among plants in the different environments were compared using an analysis of means approach (SAS Institute 2018). Precipitation data were obtained from the St. Labre weather station, ~10 km away. To examine variability in SNA over the growing season, least-squares models were used to compare soil temperature and moisture in the open and forest over different intervals throughout the growing seasons. Least-square fits were also used to examine the effect of soil temperature and soil moisture, site and year, as linear effects on non-nutrient amended shrubs. To log transform the data and include samples with a no acetylene reduction, 0.1 was added to all values. The best model fit was based on AIC. All analyses were performed using JMP Pro 14.0 (SAS Institute 2018).

Results

Shade experiment

The mid-day light levels above shrubs were significantly higher in the open (941 ± 17 $\mu mol \cdot m^{-2} \cdot s^{-1}$, mean ± SD) than above the shrubs under the artificial shade treatment (346 ±52 $\mu mol \cdot m^{-2} \cdot s^{-1}$), which were significantly higher than the light levels over the shrubs

in the forest (202 ± 45 μmol·m^{-2}·s^{-1}; $F = 84.65$, $P < 0.0001$). The growing season average daily amount of light reaching shrubs was also significantly higher in the open (40.53 ± 0.27 mol·m^{-2}·d^{-1}) than in the forest (27.05 ± 1.12 mol·m^{-2}·d^{-1}; $F = 185.9$, $P < 0.0001$). On a leaf area basis, shrubs in the open had significantly ($F = 3.510$, $P = 0.028$) higher maximum photosynthetic rates than shrubs in the forest (Table 1). Maximum photosynthetic rates in shrubs under artificial shade did not differ from those in either the open or the forest. Leaves from the shrubs in the open also had significantly higher Ik values ($F = 7.388$, $P = 0.0019$), respiration ($F = 10.76$, $P = 0.0002$), and light compensation points ($F = 16.87$, $P < 0.0001$) than leaves from the under the artificial shade treatment or the forest site. However, leaves from the forest shrubs had a specific leaf area 1.7 times greater than leaves from plants in open and 1.3 times greater than leaves from plants in the shade ($F = 102.9$, $P < 0.0001$). This difference resulted in plants from the forest having the greatest maximum photosynthetic capacity on a leaf mass basis ($F = 4.064$, $P = 0.0322$) and higher rates of carbon capture per gram of leaf tissue at all light levels (Appendix S1: Fig. S2). Combining the light response estimates and light availability data for each shrub, the potential CO_2 uptake in the different light environments did not differ statistically: 147 ± 37, 148 ± 22, and 143 ± 15 nmol CO_2·g^{-1}·s^{-1} for plants in the open, artificial shade and forest, respectively.

Nutrient amendment experiment

On both sites, adding $CaNO_3$ significantly increased inorganic soil N, quadrupling the amount in the final year of the study, compared with soil under control shrubs, but there were no differences in the first two years of the study (Table 2). There were no differences in soil inorganic N in the forest and open sites under non-nutrient amended shrubs over the three sampled years. Buried wooden blocks in the open site had significantly higher mass loss (33.2% ± 21.4%) than blocks placed under shrubs in the forest (21.8% + 11.8%; $F = 6.793$, $P = 0.0112$). Nutrient additions had no effect on mass loss from wooden blocks placed under shrubs ($F = 0.2890$, $P = 0.7499$). Adding phosphate fertilizer resulted in no detectable change in the available phosphate in the soil ($F = 1.234$, $P = 0.2944$), and there were no significant interactions with site or year. There were significant differences in soil phosphate under the non-fertilized shrubs among years, with soil in 2012 having significantly higher phosphate levels (15.8 ± 6.2 mg P/kg) than in 2013 (10.3 ± 3.6 mg P/kg) or 2015 (7.8 ± 5.9 mg P/kg).

Table 1. Effect of light treatment on light response curve parameters (maximum photosynthesis [P_{max}], irradiance saturation point [Ik], and respiration [R]), expressed on a leaf area and a leaf mass basis, light compensation point, specific leaf area (SLA), leaf stable carbon isotope discriminations (‰), and nitrogen derived from fixation (Ndfa).

Parameter	Open	Shade	Forest
Leaf area basis			
P_{max} (μmol·m^{-2}·s^{-1})	15.3 ± 3.0a	13.3 ± 2.8ab	11.7 ± 3.0b
Ik (μmol m^{-2}·s^{-1}/μmol·m^{-2}·s^{-1})	256 ± 51a	189 ± 38b	179 ± 76b
R (μmol CO_2·m^{-2}·s^{-1})	1.4 ± 0.3a	1.0 ± 0.3b	0.8 ± 0.3b
Leaf mass basis			
P_{max} (nmol·g^{-1}·s^{-1})	166 ± 4.8b	179.2 ± 6.1ab	215.0 ± 5.6a
Ik(nmol·g^{-1}·s^{-1}/μmol·m^{-2}·s^{-1})	232 ± 23a	181 ± 20ab	156 ± 13b
R (nmol·g^{-1}·s^{-1})	11.4 ± 3.7	7.3 ± 4.4	12.8 ± 6.1
Light compensation point (μmol·m^{-2}·s^{-1})	23.4 ± 5.2a	15.4 ± 5.0b	12.3 ± 5.7b
SLA (cm^2/g)	110 ± 12c	151 ± 16b	192 ± 21a
Carbon discrimination (‰)	19.7 ± 1.0a	21.3 ± 0.6b	22.1 ± 0.3b
Ndfa (%)	80.4 ± 7.4	78.0 ± 10.1	84.3 ± 3.3

Notes: Values are means ± standard deviation. For each parameter, values with same letter are not significantly different according to a Tukey's honestly significant difference test ($n = 10$).

Table 2. Soil in inorganic nitrogen levels (mg/kg) under shrubs amended with N or P fertilizer.

Year	Control	Nitrogen	Phosphorus
2012	7.5 ± 2.7bc	13.1 ± 5.7b	7.4 ± 2.7bc
2013	4.9 ± 2.2c	6.4 ± 3.2bc	5.6 ± 2.3bc
2015	9.2 ± 11.2bc	29.1 ± 24.7a	7.3 ± 10.2bc

Notes: Values averaged over the open and forest site. There was a significant treatment by year interaction ($F = 9.2026$, $P < 0.0001$), with no differences between the sites ($F = 2.637$, $P = 0.1083$) or site by fertilizer treatment interaction ($F = 0.2533$, $P = 0.7766$).

On both sites, SNA in midsummer was significantly reduced in plots receiving N or P fertilizer by 64% and 60%, respectively, over the two years that it was measured ($F = 4.7767$, $P = 0.0112$ for the nutrient addition effect on log-transformed data, Fig. 1). Shrubs in the forest had 43% higher SNA than shrubs in the open ($F = 15.78$, $P = 0.0002$) with no effect of sampling year or interactions among sampling year, nutrient addition, or site. The area of root nodules containing N-fixing vesicles was three times lower in N-amended shrubs in the forest compared with control shrubs ($F = 3.402$, $P = 0.0396$, Fig. 2) but there were no differences in the open site, and there was no effect of P addition on the size of the vesicle infected zone. In 2013, shrubs growing in the open reabsorbed 35.1% ± 3.0% of their N from leaves whereas those growing in forest absorbed 10.5% ± 3.7% ($F = 11.10$, $P = 0.0221$). Nutrient amendments had no effect on N reabsorption ($F = 1.226$, $P = 0.3063$).

Annual N derived from fixation

Stable isotope analysis showed all plants acquired the majority of their N from fixation (Table 3). Each year shrubs in the forest got more of their N from fixation than those growing in the open, with the greatest difference between the sites in the first year of the study, that is, the year in which plants had the lowest level of N derived from fixation. Amending shrubs with phosphate did not alter the proportion of N derived from fixation ($F = 2.646$, $P = 0.1093$ for the nutrient addition effect). Individual shrubs accounted for only 7% of the variation in the amount of N derived from fixation. Growing season precipitation was highly variable over the four years. In each year, shrubs in the forest had greater carbon isotope discrimination, suggesting these shrubs were less water stressed, and in drier years, the differences between the sites were greater ($F = 26.58$, $P < 0.0001$ for the site by year effect). The best model describing the relation between nitrogen derived from fixation and growing season precipitation was a three-parameter asymptotic model (Fig. 3). Analysis of means of the parameters showed that shrubs from the forest had significantly higher asymptotes for the amount of nitrogen derived from fixation with increasing precipitation ($P = 0.005$) but there were no differences in the other two parameters between shrubs growing in the forest and the open.

Seasonal variation in specific nodule activity

Over the two sampling years, SNA of the non-nutrient amended plants remained low until the second week in June, that is, Julian date 160, about a week after the buds had burst (Fig. 4). Acetylene reduction peaked in late July and then declined. Nodules had some acetylene reduction activity until the end of October, while shrubs typically started senescing leaves by mid-September. The seasonal variation in nodule activity could partly be explained by variation in soil conditions.

Soil temperatures where the nodules were sampled ranged from 9°C to 21°C and soil moisture ranged from 2.5% to 36%. From mid-June to late July, the soil temperature in the open site was significantly higher than soil in the forest site by up to 3 degrees. There was little difference in soil moisture between the sites. On 71% of the sampling days, there was no difference in soil moisture between the open and forest site; on 12% of the days, soil moisture was higher in the forest, and on 17% of the days, it was higher in the open site. Below a soil temperature of 12°C, acetylene reduction was always less than 2 μmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$. The best model explained 27.1% of the variation in the rate of nodule N fixation. The model included significant effects of temperature ($P < 0.0001$), the interaction between temperature and soil moisture ($P = 0.0148$), and sampling year ($P < 0.0001$, and Appendix S1: Table S1). However, site was not a significant effect ($P = 0.1114$), suggesting that these other factors can account for differences between the sites. To examine the interaction between soil moisture and temperature, samples

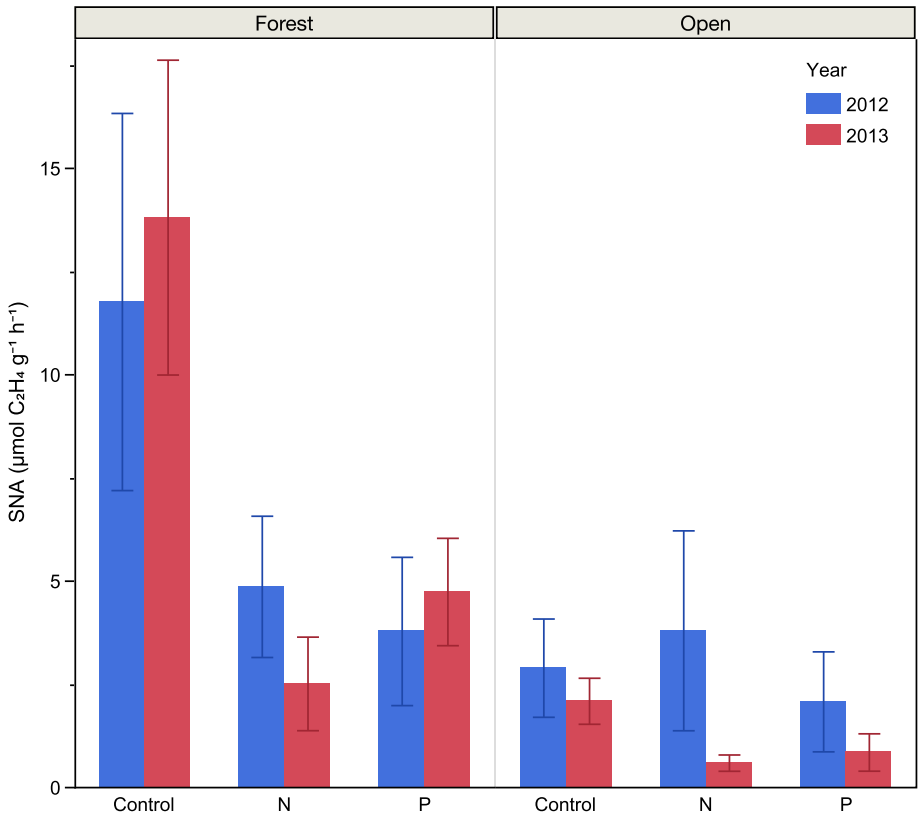


Fig. 1. Specific nodule activity in midsummer for nutrient amended shrubs. Bars are means with standard errors.

were divided into soil moisture classes. The relation between acetylene reduction and soil temperature was then examined with least-squares models. This showed that as soil moisture increases, temperature had an increasingly significant effect on the rate of acetylene reduction (Fig. 5).

If only the periods of peak N fixation are compared (July–August), shrubs in the forest had a significantly higher rate of acetylene reduction (7.5 + 1.5 μmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$) compared with shrubs in the open site (5.4 + 1.4 μmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$, $F = 4.01$, $P = 0.0475$ for the site effect). The mid-summer peak in SNA in these non-nutrient amended shrubs was associated with increases in the proportion of nodule lobe cross-sectional area occupied by N-fixing vesicles (Appendix S1: Fig. S3). A least-squares model with Julian date as a quadratic effect ($F = 11.82$, $P = 0.0007$) showed a significant site by date effect ($F = 4.239$, $P = 0.049$) with the peak in N-fixing vesicles occurring on Julian day 217 in open and day 232 in the forest.

Discussion

These results show that N fixation by actinorhizal shrubs is not inhibited by shade from the tree canopy in a mature boreal pine stand. Although *A. crispa* in both habitats were able to obtain most of their N from fixation, plants growing in the forest obtained more and had higher rates of N fixation per nodule mass. Plants growing in the forest also reabsorbed less N from senescing leaves, indicating less of a need for the plants to reuse N. Theory predicts that the high energy cost of N fixation becomes disadvantageous in shaded conditions (Rastetter et al. 2001). However, *A. crispa* made thinner leaves when growing in the shade. This made leaves more efficient in carbon uptake at all light intensities. Accounting for the reduced light

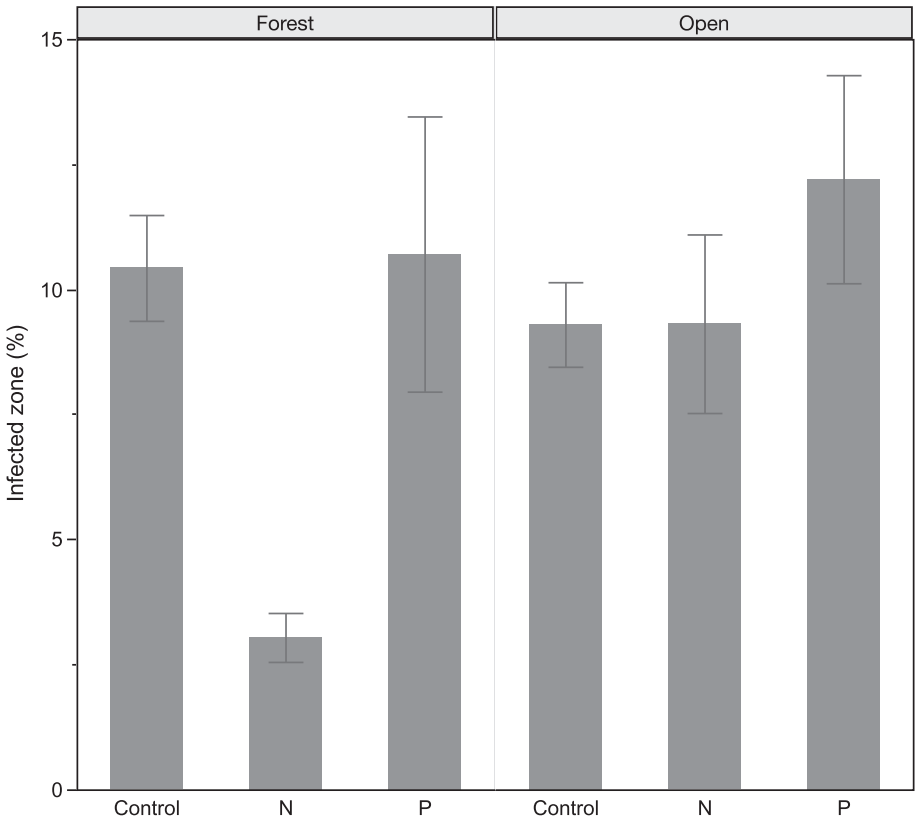


Fig. 2. Proportion of root nodules occupied by nitrogen-fixing nodule vesicles after two years of nutrient amendments.

Table 3. Growing season percent nitrogen derived from fixation (Ndfa) and carbon isotope discrimination for non-nutrient amended shrubs, and growing season precipitation.

	Ndfa (%)		Carbon discrimination (‰)		
Year	Forest	Open	Forest	Open	Precipitation (mm)
2012	84.3 ± 4.8d	76.0 ± 6.0e	21.7 ± 0.51a	18.6 ± 1.0d	278
2013	96.4 ± 2.2ab	92.9 ± 2.9c	20.5 ± 0.80b	19.4 ± 0.76c	393
2014	96.2 ± 1.8ab	93.0 ± 3.0c	20.9 ± 0.66b	19.4 ± 0.75c	309
2015	99.3 ± 1.8a	94.7 ± 2.7bc	20.8 ± 1.0b	19.2 ± 0.87cd	368

Notes: Values are means ± standard deviations. A repeated measures model indicated a significant site by year interaction on Ndfa ($F = 7.009$, $P = 0.0002$) and carbon discrimination levels ($F = 17.56$, $P < 0.0001$) in leaves. Letters indicate significant differences between sites and years according to Tukey's honestly significant difference tests on a repeated measure analysis.

availability in the forest, plants were able to acquire the same amount of carbon per mass of leaf tissue as plants in the open. We therefore find no support for the idea that plants in a mature conifer stand are less able to support the energy cost of N fixation. Since this study sampled only one mature stand, it is possible that under more shaded conditions (that are seen in younger stands), that N fixation would be inhibited. Given the higher efficiency of the leaves from the forest at all light levels, it is unlikely that the change in *A. crispa* leaf morphology we found is driven by acclimation to shade, as seen in many shade-tolerant species (Givnish 1988, Meir et al. 2002). Rather, the dry conditions in the region likely result in thicker leaves in the

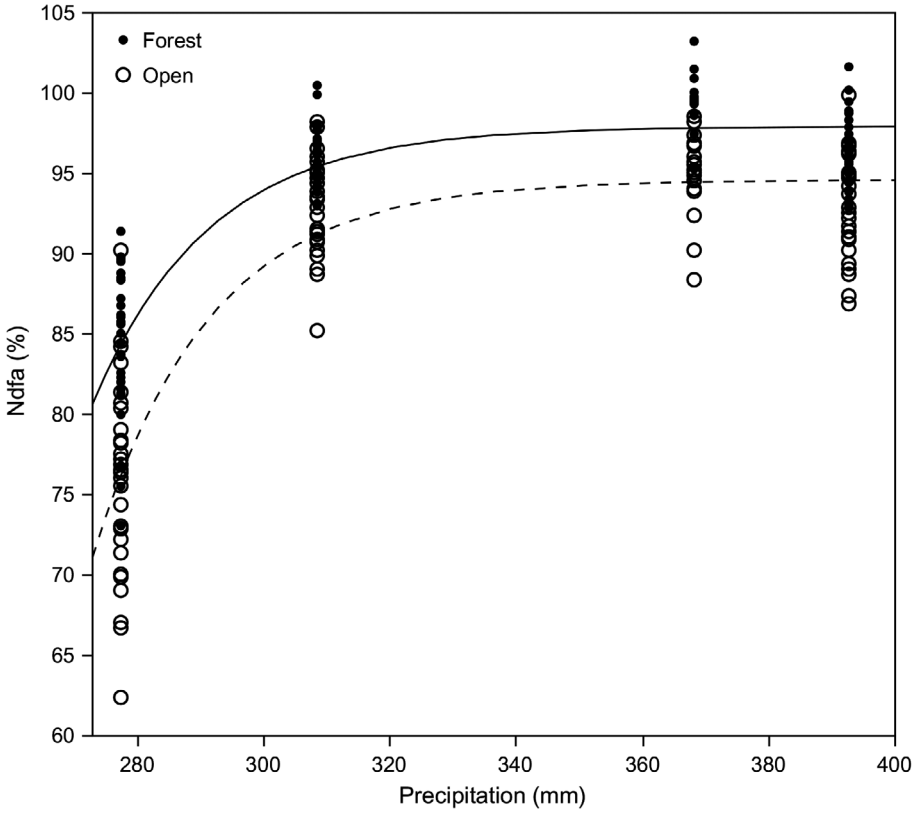


Fig. 3. Relationship between percent nitrogen derived from fixation (Ndfa) and growing season (May–August) precipitation for shrubs in the forest (closed symbols, solid line) and open (open symbols, dashed lines) sites. Each point is an individual shrub, sampled each year from 2012 to 2015. Lines are the exponential model fits: $y = 97.3 - 48{,}000{,}000^{(-0.0544 \times x)}$ for the forest and $y = 94.6 - 66{,}000{,}000^{(-0.0544 \times x)}$ for the open site.

open as a way of reducing water loss (Marron et al. 2003). *Alnus* species, like all N-fixing plants, are generally considered to be shade intolerant (Menge et al. 2010). We confirmed this by comparing shade tolerance in the 51 N-fixing, to the 637 non-N-fixing, angiosperms from across the Northern Hemisphere in the dataset from Niinemets and Valladares (2006). Shade tolerance was based on species occurrence in different light environments. The non-N-fixing plants were more shade tolerant (2.5 ± 0.9, on a standardized five-point scale) than the N-fixing plants (1.8 ± 0.7; $F = 29.16$, $P < 0.0001$ for a one-way ANOVA). Typically, canopy openness in young jack pine stands in the area of the boreal forest studied here tends to less than 15% (J. Markham, *unpublished data*) with understory shrubs, including non-N fixers, typically being absent after canopy closure. This absence suggests all boreal shrub species cannot adjust their leaf morphology to compensate for the reduced light availability that occurs during the early stages of canopy closure. In other boreal stand types, *Alnus* shrub growth has also been shown to decrease with forest stand development, with N fixation activity of nodules being reduced (Uliassi et al. 2000) or unaffected (Mitchell and Ruess 2009, Ruess et al. 2013). As stands mature, canopy openness increases to levels similar to our forest site, allowing for an increase in abundance of understory species. Therefore, while this study cannot address how deeper shade restricts N fixation during canopy closure, N-fixing shrubs do not seem to be at a disadvantage in mature stands.

In this region of the boreal forest, bordering on the central grasslands of North America, where water availability limits forest establishment, the

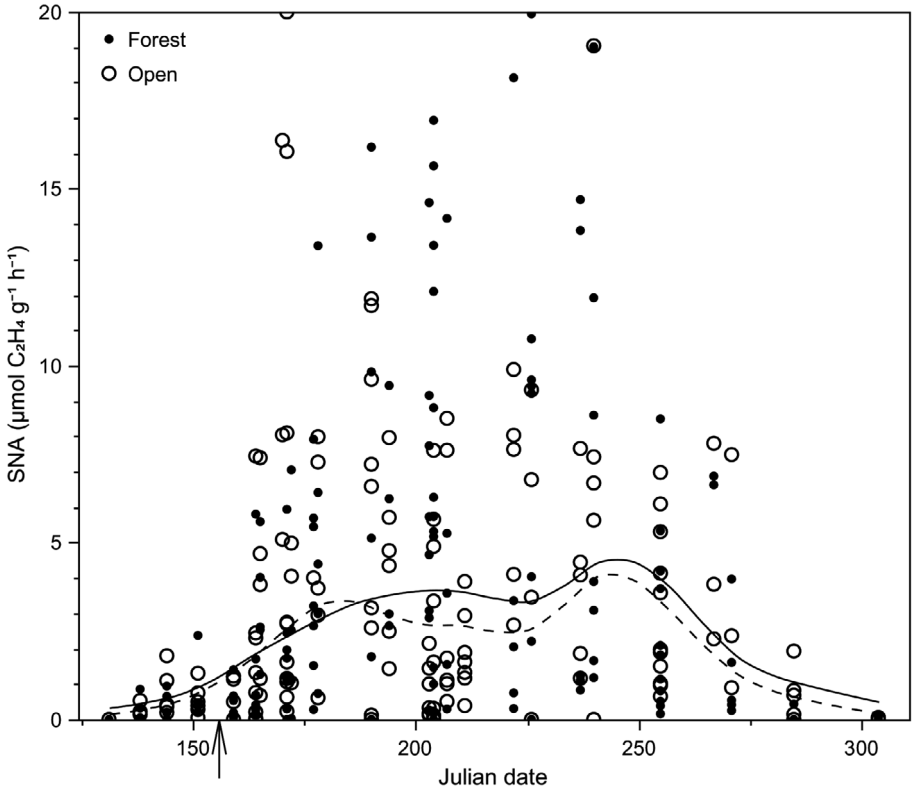


Fig. 4. Specific nodule activity (SNA) measured as the amount of ethylene reduced per nodule dry mass per unit time over the growing season for non-nutrient amended shrubs in 2013 and 2017. Lines are back transformed fits from least the squares model of log-transformed SNA with soil temperature, soil moisture, and their interaction for shrubs in the forest (solid) and open (dashed) sites. The arrow indicated the average date of budburst.

understory environment provides shrubs with protection from desiccation. Shrubs growing in the open have their stomates closed more often because of drier conditions, as indicated by the lower leaf carbon isotope discrimination (Ehleringer et al. 2002). This results in less energy capture to drive N fixation. This may explain the lower proportion of N derived from fixation in the shrubs growing in the open. It is also possible that the higher rate of decomposition in the open may allow plants to access more soil N, accounting for the reduced proportion of fixed N. The lack of trees in the open could also make more soil N available for the shrubs. The lower levels of N derived from fixation in the driest growing season also supports the idea that dry conditions limit N fixation in *A. crispa* in this region. Reduced N fixation under dry growing season conditions may apply to other boreal N-fixing species. However, in temperate and tropical arid regions many N-fixing species have drought tolerance traits, especially the legumes (Liao et al. 2017, Tedersoo et al. 2018). Using the angiosperm database of Niinemets and Valladares (2006) described above, we found that the 30 legume species in the dataset had a significantly higher drought tolerance (3.9 ± 0.8, measured on a five-point scale) than the non-N-fixing angiosperms (2.9 ± 1.0), but the 21 actinorhizal species had a significantly lower drought tolerance (2.2 ± 0.9; $F = 20.75$, $P < 0.0001$). This would suggest that where actinorhizal plants are more abundant (i.e., at higher latitudes), N fixation will be more sensitive to drought.

While the tree cover provides the understory shrubs with better water balance, the forest soil was cooler in the spring, resulting in delayed development of vesicles within nodules. This

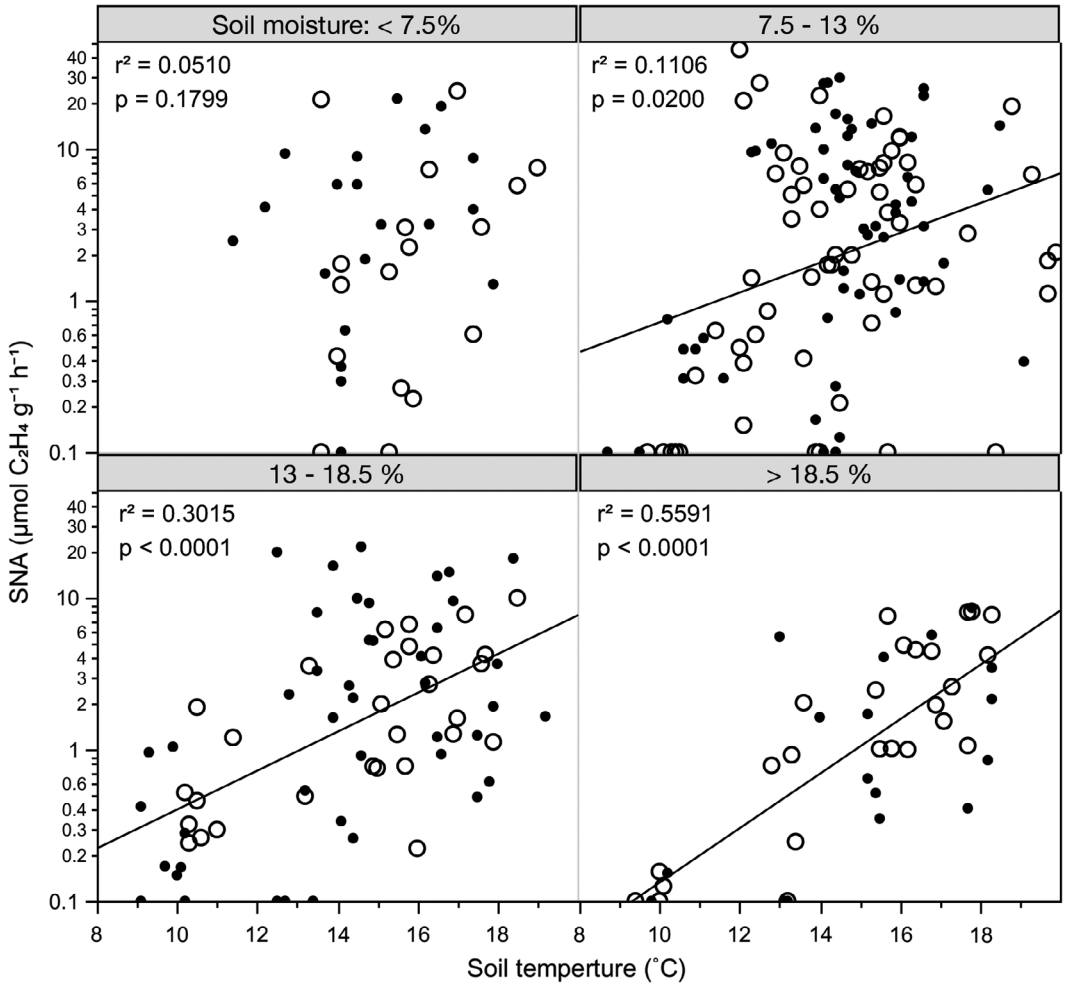


Fig. 5. The effect of soil temperature on nodule acetylene reduction activity (on a log scale) over the growing season, in different classes of soil moisture. The dataset was divided into four categories of soil moisture: two above, and two below, the mean of all soil moisture value. Lines are least-squares fits with their r^2 and P values for each soil moisture class, when the relationship was significant.

shows that besides a limitation on nitrogenase activity, low soil temperatures can limit the N-fixing symbiosis in the boreal forest. The temperature optimum for N fixation is considered to be around 25°C (Houlton et al. 2008), well above the temperatures where we found nodules. Other studies in boreal forests have also found a positive relationship between soil temperature and nodule N-fixation activity. For example, Mitchell and Ruess (2009) found a positive relationship between nitrogenase activity and soil temperatures in the range of 6.5–11.5°C in *Alnus viridis* subsp. *fruticosa*. The negative effect of delayed soil warming in the spring on N fixation has not been considered in theories used to explain the distribution of N-fixing plants. However, it likely contributes to both reduced levels of N fixation and plant growth.

This study confirms an earlier field study and laboratory assays that increased inorganic soil N has a negative effect on N fixation in actinorhizal plants (Huss-Danell et al. 1982, Markham and Zekveld 2007, Ruess et al. 2013). It has been suggested that actinorhizal plants have an obligate N-fixing strategy (Menge et al. 2014). *Alnus* species, in particular, are always found to have

nodules in the field, although in other actinorhizal species nodules are not always found (Markham 2008*a*). *Alnus rubra* has also been shown to maintain its N-fixation activity even when there is sufficient soil N to have a high rate of N leaching (Binkley et al. 1992). Our results show a limited ability of actinorhizal plants to downregulate N fixation, but not to the degree that has been found in tropical legumes, where the proportion of N derived from fixation can be as low as 42% (Andrews et al. 2011). It is possible that soils in our sites would take many years of N addition to have a stronger effect on plants. Boreal forests are typically N limited. Consequently, added N might be immobilized in soil, limiting any effects on plant growth (Houle and Moore 2008). The amount of N used to induce a reduction in N fixation both in laboratory and field studies may be higher than the amount of N naturally available in most natural field settings. In the boreal forest, inorganic N levels in the soil are typically less than 10 mmol/L (Jerabkova et al. 2006) and N nutrition is complicated by the fact that organic soil N may be accessed by plants (Högberg et al. 2017). It is also likely that the short growing season and cool climate in high latitude forests may limit the ability of plants to switch between fixing N and taking up inorganic soil N since nodules typically take several weeks to develop and become functional. It would be insightful to examine the effect of anthropogenic N pollution on rates of N fixation in temperate actinorhizal plants, as has been done for tropical legumes and free-living N-fixing organisms (Sullivan et al. 2014, Zheng et al. 2019).

The mechanism for reduced N fixation in response to increased soil N has not been identified. Presumably, it involves feedback similar to the ability of other N-fixing species to reduce the growth nodules when they are ineffective (Wall et al. 2003, Kiers and Denison 2008). This is the first field study to show a reduction in nitrogen-fixing vesicles in nodules in response to environmental conditions. In a greenhouse experiment, we have also shown a similar reduction in vesicles in response to salt stress (Shao et al. 2019). The reduction in the proportion of N-fixing vesicles in the N-fertilized shrubs in the forest, but not in the open or in response to P fertilizer, suggests that under forest conditions, N fertilization makes devoting resources to nodules activity particularly disadvantageous.

The reduced nodule activity associated with P addition suggests a reduced need for fixed N to produce phosphatases. Using a growth chamber experiment, we have recently shown that nodulated *A. crispa* plants have a higher phosphatase activity in their roots than non-nodulated plants (Chen et al. 2020). Our results are also consistent with earlier studies on non-N-fixing plants showing reduced phosphatase activity with the additions of P fertilizer (Treseder and Vitousek 2001). Although it has been suggested that N fixation increases with P addition since N-fixing plants have a greater requirement for P (Binkley et al. 2003), field studies have shown varying effects of P on N fixation. The metanalysis of Zheng et al. (2019) showed that N fixation decreased with added P in temperate forests, no response in boreal forests, and increased in all other biomes (savanna, grasslands, and tundra). In a P addition study on *Alnus tenuifolia*, Uliassi and Ruess (2002) found that P addition affected nodule mass but not N fixation rate, and only when *A. tenuifolia* was the dominant plant in the community. When *Populus balsamifera* trees formed the overstory, there was no effect of added P on nodule mass or N fixation. In another study in the same region, Ruess et al. (2013) found P addition increased fixation, possibly due to an increased rate of turnover in nodules, with younger nodules have a greater rate of fixation.

Contrary to previous explanations of the distributions of N-fixing species, we found that in the area of the boreal forest we studied, shading from a mature conifer stand provided a benefit to N-fixing shrubs, allowing them to obtain more of their N from fixation. This result depended on the shrubs in the open having to create less-efficient leaves for photosynthesis. However, shaded conditions may be a drawback in the spring, as the canopy can delay the warming of the soil. This study was also able to demonstrate that *Alnus* can downregulate their N fixation activity to some extent. Because most terrestrial boreal ecosystems are N limited and N-fixing plants can survive in forest understories, where they can acquire a majority of their N from fixation, we still do not have an adequate explanation as to why they are not more abundant in these ecosystems.

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Data Availability

Data are available from Dryad: https://doi.org/10.5061/dryad.4qrfj6q99.

Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3708/full