Colonization by nitrogen-fixing Frankia *bacteria causes short-term increases in herbivore susceptibility in red alder (*Alnus rubra*) seedlings*

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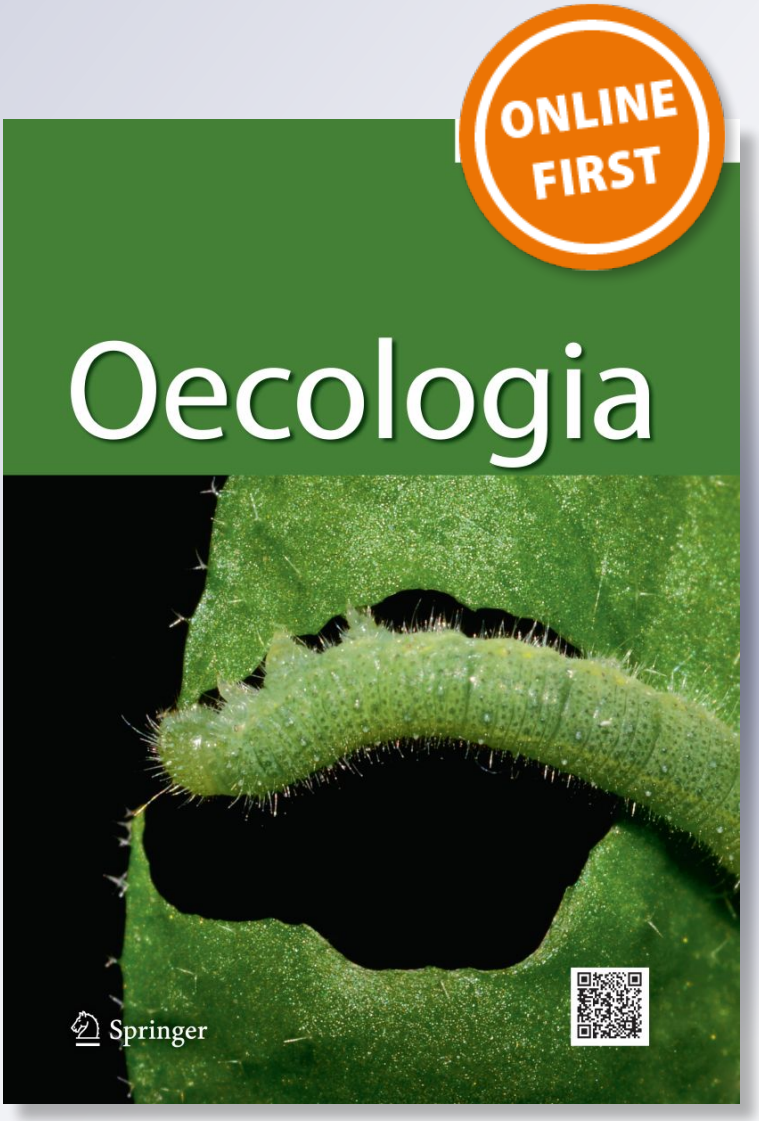

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PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Colonization by nitrogen-fixing *Frankia* bacteria causes short-term increases in herbivore susceptibility in red alder (*Alnus rubra*) seedlings

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Abstract Carbon allocation demands from root-nodulating nitrogen-fixing bacteria (NFB) can modulate the host plant's chemical phenotype, with strong bottom–up effects on herbivores. In contrast to well-studied rhizobia, the effects of other important NFB on plant chemistry and herbivory are much less understood. Here, combining field surveys in the Oregon Coast Range, USA with laboratory experiments, we analyzed how N_2-fixing *Frankia* bacteria influenced plant growth, chemistry, and herbivory on *Alnus rubra* (red alder) seedlings. In the field, we quantified *Frankia* nodulation, herbivore damage, and plant size. In the laboratory, we grew seedlings with *Frankia* (F+), *Frankia*-free but nitrogen-fertilized (N+), or both uncolonized and unfertilized (F−N−) and assessed growth and leaf chemistry. We further conducted choice trials with black slugs, *Arion rufus*, a natural red alder herbivore. In the field, *Frankia* nodulation was significantly positively correlated with herbivory and negatively with seedling height. In contrast, in the lab, F+ as well as N+ seedlings were significantly taller than the F−N− controls. Seedlings from both treatments also had significantly increased leaf protein concentration compared to controls, whereas carbon-based nutritive compounds (carbohydrates) as well as leaf palatability-decreasing condensed tannins, lignin, and fiber were decreased in F+ but not in N+ treatments. In the choice assays, slugs preferred leaf material from F+ seedlings, but the effects were only significant in young leaves. Our study indicates that colonization by *Frankia* causes short-term ecological costs in terms of susceptibility to herbivory. However, the ubiquity of this symbiosis in natural settings suggests that these costs are outweighed by benefits beyond the seedling stage.

Keywords Trophic interactions · Ecological costs · Nitrogen fixation · Condensed tannins · Pacific Northwest · Plant defense · Plant–herbivore interactions

Introduction

Mutualistic interactions between plants and soil microbiota are ubiquitous in terrestrial ecosystems and strongly influence plant diversity and ecosystem productivity (Carney and Matson 2005; Van der Heijden et al. 2008). In particular, plant-associated nitrogen-fixing bacteria (NFB) are widely recognized for their key function in terrestrial ecosystems. Although gaseous nitrogen (N_2) is abundant in Earth's atmosphere (~78%), N is commonly the most limiting resource for plant growth. Thus, NFB that convert atmospheric nitrogen into forms available to plant hosts are considered important plant mutualists. However, symbiotic nitrogen fixation also is costly for plants, as maintaining the microbial partner requires significantly more energy stored in photoassimilates than direct uptake of ammonium or nitrate from the soil. Chapin et al. (1987) estimate that 25–40% of plant net photosynthetic carbon gain is

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allocated to microbial symbionts. Although the benefits of microbial nitrogen fixation for plants have been studied in great depth, associated costs for hosts and effects on other plant-associated organisms are not well understood. This seems particularly true in terms of the effects; microbial nitrogen-fixing symbionts have on the expression of plant defenses and thus on higher trophic levels. Plant defenses can contribute to greater fitness in the face of herbivory; however, the costs of maintaining defenses vs. growth and reproduction can result in resource allocation constraints (Coley et al. 1985; Herms and Mattson 1992).

Mounting evidence suggests that soil microbial mutualists can significantly affect above-ground food webs (Goverde et al. 2000; Gehring 2003; Gange et al. 2003; Bezemer and van Dam 2005; Pozo and Azcón-Aguilar 2007; Hempel et al. 2009). Depending on the mode of feeding and degree of specialization in above-ground herbivores, colonization of plant roots with mycorrhizal fungi has been demonstrated to have negative (generalists and leaf-chewing herbivores) or positive effects (specialists and sap-feeders) on the plant consumers (Koricheva et al. 2009). Similarly, existing studies focusing on NFB indicate significant but inconsistent bottom–up effects on herbivores. For example, when comparing rhizobia-free and rhizobia-colonized plants, Kempel et al. (2009) observed increased performance of the generalist leaf-chewing herbivore *Spodoptera littoralis* (Lepidoptera) on some *Trifolium repens* (white clover) strains but not on others. In the legume host *Phaseolus lunatus* (lima bean), Thamer et al. (2011) found distinct effects of rhizobia on cyanogenesis, an N-based chemical plant defense. Rhizobia-colonized plants showed higher levels of cyanogenesis and were better defended against an oligophagous insect herbivore, the Mexican bean beetle (*Epilachna varivestes*). Working in the same study system, Ballhorn et al. (2013) showed that rhizobial colonization also altered the quantity and composition of herbivore-induced volatile organic compounds (HI-VOCs) in *P. lunatus*, which resulted in a significant herbivore deterrence. In contrast, extensive colonization of *P. lunatus* plants by rhizobia decreased the quantity of extrafloral nectar, which resulted in lower attractiveness of plants for mutualistic ants, thereby reducing indirect defense against herbivores (Godschalx et al. 2015). These mixed effects on herbivory of plants hosting rhizobia highlight the complexity of carbon–nitrogen balance dynamics, wherein differences in defense (N-based or C-based; induced or constitutive) can drive differential outcomes in trophic relationships.

Despite the increasing awareness that NFB alter the outcome of above-ground food webs, specific bottom–up effects of non-rhizobial NFB are still little understood (Van der Putten et al. 2001; Sprent 2001; Van der Heijden et al. 2008). This is surprising, since NFB play a key role for global and local nitrogen cycles (Sprent and Sprent 1990) and are widely distributed in the plant kingdom. A second major group of NFB in soils is composed of actinobacteria of the genus *Frankia*, which fix atmospheric nitrogen in root nodules of a diverse array of woody plant hosts (Benson and Dawson 2007). Like other plant-associated NFB, *Frankia* generally have a positive effect on the growth of their hosts and can contribute to significant gains in biomass relative to uncolonized plants, though the strength and direction of this effect can be variable depending on host-*Frankia* strain combinations (Monaco et al. 1982; Teissier du Cros et al. 1984; Hendrickson et al. 1993). As with rhizobia in legumes, the presence of *Frankia* in actinorhizal plants has also been observed to influence host plant–herbivore interactions. In one study, Hendrickson et al. (1991) found that two of three *Frankia*-inoculated *Alnus* species were more susceptible to herbivory by a leaf-mining sawfly than uninoculated plants. Similarly, Hendrickson et al. (1993) also observed higher levels of aphid infestation in *Alnus* species that had been inoculated with *Frankia* relative to uninoculated individuals.

In the present study, we focus on red alder (*Alnus rubra* Bong.), a common deciduous tree widely distributed throughout the Douglas-fir region of the Pacific Northwest, USA (González-Hernández et al. 2000). Seasonally, red alder represents an important food source for cervids, in particular, black-tailed deer (*Odocoileus hemionius columbianus*) and Roosevelt elk (*Cervus elaphus roosevelti*) (Crouch 1976; Jenkins and Starkey 1991; Radwan et al. 1978). Furthermore, it is a food source for various oligophagous to specialist insect herbivores such as tent caterpillars (*Malacosoma disstria*, *M. californicum*), red alder flea beetles (*Altica ambiens*), red alder woolly sawflies (*Eriocampa ovata*), striped red alder sawflies (*Hemichroa crocea*), and leaf beetles (*Pyrrhalta punctipennis*) (Gara and Jaeck 1978; Resch 1980). While both mammalian and insect herbivores seem to feed predominantly on red alder plants ranging from 1 m to tree size, generalist invasive black slugs (*Arion rufus*) can also be important herbivores of smaller-sized plants in Pacific Northwest forests (Lauren and Whitlow 2012).

To better understand the bottom–up trophic effects of non-rhizobial NFB, we first made field observations relating *Frankia* colonization in seedlings with both height growth and herbivore leaf damage. We then grew *Frankia*-colonized and *Frankia*-free red alder seedlings as well as *Frankia*-free but fertilized seedlings in a laboratory experiment. We measured various nutritive and defensive chemical leaf traits in leaves of different developmental stages (young, intermediate, and mature) in each treatment to identify effects of the belowground microbial mutualists on above-ground plant traits. Finally, we conducted a feeding choice assay with black slugs using the leaf material from the laboratory-grown plants. Based on results from

studies examining rhizobial NFB, we hypothesized that there would be *Frankia*-mediated changes in host plant leaf traits with subsequent effects on herbivores. In particular, we predicted an increase in nitrogen-based leaf traits in extensively *Frankia*-colonized seedlings due to microbial nitrogen provisioning, whereas carbon-based traits were expected to decrease as a result of the microbial carbon demand.

Materials and methods

Field seedling survey

In June 2013, 110 red alder seedlings (10–15 cm) were collected in a 10 × 40 m area at a forest clearing near the Nehalem River (Clatsop County, OR, USA). Seedlings were dug out carefully and soil attached to roots was washed off on site. Plants were individually stored in Ziploc bags and transported to the lab at Portland State University. In the lab, plants were washed again to remove remaining soil particles, after which all *Frankia* root nodules were removed with forceps. Nodules were placed in pre-weighed 1.5 mL microcentrifuge tubes and dried in an oven at 60 °C for 7 days. Subsequently, tubes were closed and reweighed to determine dry mass (New Classic MF, Mettler Toledo, Zurich, Switzerland). To quantify removed leaf area on each seedling, leaves were digitally photographed (Canon 5D) on a scale using the analySIS software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Laboratory seedling experiment

Red alder seeds collected from Hoquiam, WA, USA were obtained from Silvaseed Company (Roy, WA). To facilitate seed germination, the seeds were rehydrated by soaking for 24 h and then stratified at 8 °C for 14 days. Soil was collected from a natural red alder stand in Portland, Oregon, USA (Riverview Natural Area; 45°27′12.47″N, 122°40′4.16″W), sieved (2 mm mesh size) to remove large roots and rocks, and then autoclaved for 60 min three separate times (with 24 h in between). Eighty mL of soil was placed into individual surface-sterilized cone-tainers (Steuwe and Sons, Corvallis, OR, USA). A small wad of plastic pillow filling was placed at the bottom of the containers to prevent soil leakage (while still allowing for water drainage). The cone-tainers were arrayed in 30 × 60 cm racks, with trays below to catch water that dripped through after watering. After stratification, multiple seeds were placed in the center of each cone-tainer and watered with 10 mL deionized water. Racks were placed in a growth chamber with 14/10 h light dark cycle, 23/15 °C temp cycle. Cone-tainers were moistened every 2–3 days, and once secondary leaves developed on seedlings (~1 month), they were weeded (via scissors) to one seedling per cone-tainer.

Frankia nodules were collected from the same alder stand as the soils. Roots were gently excised from the soil and *Frankia* nodules (3–4 nodules per root section, about 2 cm in diameter each) were removed and placed in a Ziploc bag. In the lab, nodules were rinsed thoroughly to remove any remaining soil (without breaking the individual nodes off) and then weighed. A bulk inoculum was prepared by transferring the nodules into a clean mortar and thoroughly crushing them in 40 ml of sterile deionized water. The mixture was poured into a 50 mL cone-bottomed tube and additional deionized water was added to reach 50 mL. Before inoculation, the concentration of *Frankia* slurry (in mg/mL) was calculated to ensure that each container received 100 mg of *Frankia* nodule homogenate. The *Frankia* solution was briefly vortexed before inoculation, and the corresponding volumes were pipetted next to the seedlings in the *Frankia* inoculation (F+) treatment into small indentations in the soil made with a sterile pipette tip. At the same time, plants in the N-fertilized treatment (N+) began to be watered with 10 ml of a 2.5 mM NH_4NO_3 (Sigma-Aldrich) solution every 3–4 days. In a preliminary experiment, this fertilizer concentration was identified as generating similar development (i.e., seedling height, leaf size, and leaf color) between fertilized and *Frankia*-inoculated plants (P. Kennedy, unpublished data). The *Frankia*-inoculated plants and plants in the non-fertilized and non-inoculated treatment (F−N−) were watered with 10 ml of sterile deionized water every 3–4 days. To avoid position effects in the chamber, racks were rotated once a week and individual cone-tainers were also shuffled. After 3 months in the growth chamber, plants were transferred to a greenhouse with a light regime of 16 h light:8 h dark cycle and a photon flux density of 550–600 μmol photons m^{-2} s^{-1} at table height. Additional light was provided by a combination (1:1) of HQI-BT 400 W (Osram) and RNP-T/LR 400 W (Radium) lamps. Temperature in the greenhouse was set at 23:15 °C (i.e., 23 °C in the light period and 15 °C in the dark period) and air humidity was maintained at 65–75%.

Leaf chemical analyses

At time of leaf chemical analyses (and the feeding choice assay described below), red alder seedlings were approximately 20 cm tall and had developed 6–7 leaves. Leaves of three different developmental stages (young: first unfolded leaf from the top of the plant; medium: leaf inserting two positions below young leaves; and mature: leaves inserting two positions below medium leaves) were selected to account for ontogenetic variation of chemical traits

depending on leaf age (Ballhorn et al. 2008). Leaves were removed with a razor blade between 8:00 and 9:00 a.m. and subjected to analyses or feeding trials immediately. All leaves were analyzed for the following chemical traits: crude protein, total available carbohydrate, tissue water content, acid-detergent fiber, acid-detergent lignin, and condensed tannin. These traits are broadly associated with either nutritive (total available carbohydrate, crude protein, and tissue water content) or defensive (acid-detergent fiber, acid-detergent lignin, and condensed tannin) strategies (Barbehenn and Constabel 2011; War et al. 2012).

Crude protein was determined by the micro-Kjeldahl technique (Concon and Soltess 1973). Briefly, 2.3 g of a K_2SO_4–TiO_2 mixture (190:4, w/w) and 50 mg of dry pulverized leaf samples were mixed in a 30 ml Kjeldahl flask (Merck, NY, USA). Concentrated sulfuric acid (2.3 ml) was added under shaking followed by 0.05 ml of warm (40 °C) 50% lauric acid in ethanol and 3 ml of H_2O_2. The mixture was heated rapidly for 15–30 s without letting developing foam escape from the neck of the flask, and then, while still hot, 1 ml of 30% H_2O_2 was slowly added. The sample was heated again for an additional 5 min after the digest had cleared and the concentration of ammonia was measured against a blank through back titration. Total available carbohydrates were extracted and hydrolyzed with 0.2 N H_2SO_4 (Smith et al. 1964), with the resulting sugars determined as glucose by the ceric sulfate method (Hassid 1937). To assess tissue water content, fresh leaf pieces were weighed to the nearest 0.001 g (New Classic MF, Mettler Toledo, Zurich, Switzerland), dried in an oven (IncuMax CV250 Convection Oven, Amerex Instruments, Inc., Lafayette, CA) for 5 days at 70° C, and weighed again. Acid-detergent fiber and lignin were determined according to the method described by Van Soest (1963), with adjustments for the small quantities of samples. Condensed tannins were analyzed following Tikkanen and Julkunen-Tiitto (2003). Leaf homogenates were extracted three times for 15 min in 5 mL of acetone diluted 60:40 (v/v) with distilled water. After each extraction, samples were incubated in an ultrasonic bath (3 min) and then centrifuged for 10 min at 5000×*g*. The supernatant was transferred to 2 mL of concentrated acetic acid (Merck, NY, USA). Acetone was removed under vacuum (60 mbar) at 40° C, and the residue was quantitatively transferred using distilled water. Samples were diluted with 2.5% acetic acid, and 1 mL of this solution was mixed with 0.5 mL Folin–Ciocalteus phenol reagent (Merck, NY, USA). After adding 2 mL 20% Na_2CO_3, the solution was increased to 10 mL with distilled water. Finally, samples were incubated at 70 °C, and after cooling, spectrophotometrically quantified at 730 nm against a blank containing water instead of sample. Different concentrations of Epicathechin (Sigma, Deisenhofen, Germany) served as the standard.

Feeding choice assay

Juvenile black slug individuals of a similar size (~2 cm in length; $N = 30$) were collected at the same study site, where seedlings were harvested. The slugs were collected no longer than 4 days before the assay experiments and maintained individually in transparent 250 ml plastic cups (water supplied on cotton) at a temperature regime of 18 °C (day, 14 h) and 15 °C (night, 10 h) to resemble natural outdoor conditions (climatic chamber; Conviron BDW 160-R walk-in CE chamber; Conviron, Winnipeg, Canada). Slugs were provided with ad libitum food (organic spinach) and then deprived of food for 24 h prior to feeding choice assays to ensure a similar physiological state. Petri dishes were used for feeding assay arenas (14 cm in diameter) and experiments were conducted in the dark (15 °C) to account for the fact that slugs are mostly nocturnal. To guarantee for high air humidity in the feeding arenas, Petri dishes were placed in a large plastic box containing wet cotton tissue. For the feeding assays, individual slugs were simultaneously offered three leaf discs (2.0 cm in diameter), each derived from plants of the different inoculation and fertilizer treatments [*Frankia* (F+), N fertilizer (N+), none (F−N−)]. In a given feeding trial, only leaves of a similar developmental stage (young, intermediate, and mature) were compared, respectively. This allowed for the detection of effects of changing leaf traits in response to the different plant treatments separately from potential effects of leaf age on the herbivores. After 6 h, leaf discs were removed and digitally photographed (Camedia C-4000 Zoom, Olympus, Hamburg, Germany) on a scale to calculate leaf area removed using the analySIS software.

Data analyses

To analyze the relationships between *Frankia* nodule biomass and herbivore leaf damage as well as seedling height for the field-collected alder seedlings, we applied linear regression models. Inspection of residual plots revealed variance assumptions that were met. For the laboratory-grown seedlings, we compared differences in seedling height across the three inoculation and fertilization treatments (F+, N+, and F−N−) using a one-way analysis of variance (ANOVA). Prior to running the ANOVA, the among-group variances were confirmed to be homogenous using Cochran's C test. To determine differences in leaf chemistry across the three inoculation and fertilization treatments, we used a series of two-way factorial ANOVAs, with treatment and leaf age as the predictor variables. Differences among means were determined using Tukey's post hoc tests. All six response variables met the assumptions of variance homogeneity, so no transformations were applied. To assess the difference in leaf removal by treatment in feeding choice assay, we used a two-way factorial ANOVA, with treatment and leaf age

as the predictor variables. For this analysis, we log $(x + 1)$ transformed the data to meet variance assumptions. Differences among means were determined using Tukey's post hoc tests. All analyses were run in R v. 3.0.2 (R Development Core Team 2008) and considered significant at $P < 0.05$.

Results

In the field survey, there was a significant positive correlation between *Frankia* nodule biomass and leaf area consumed by mollusks ($F_{1,108} = 464.3$, $P < 0.001$; Fig. 1a). In contrast, seedling height was significantly negatively correlated with *Frankia* nodule biomass ($F_{1,108} = 77.7$, $P < 0.001$; Fig. 1b).

The height of the laboratory-grown seedlings differed significantly among treatments. *Frankia*-colonized (F+) and fertilized (N+) seedlings were both significantly taller, being, on average, 35% taller than control (F−N−) seedlings ($F_{2,57} = 112.9$, $P < 0.001$; Fig. 2). The leaf traits of the laboratory-grown seedlings varied by treatment and leaf age for five of the six traits assessed (Table 1; Fig. 3). Results for the three nutrient-related traits (protein, carbohydrate, and water) were nutrient dependent. Leaves of *Frankia*-colonized and fertilized seedlings had higher amounts of leaf protein compared to control seedlings (Fig. 3a), while leaves from the fertilized and control seedlings had higher amounts of leaf carbohydrate than the *Frankia*-colonized plants (Fig. 3b). In both cases, there were differences by leaf age as well; young and medium leaves had significantly higher leaf protein than mature leaves (Tukey HSD test, $P < 0.05$), while mature leaves had significantly higher leaf carbohydrate than the medium leaves, which were both significantly higher than the young leaves. Leaf water content was not significantly affected by either treatment or leaf age (Fig. 3c). For the three defense-related traits (fiber, lignin, and tannin), leaves from *Frankia*-colonized seedlings were consistently lower in these traits compared to leaves from the fertilized or control seedlings (Fig. 3d–f). There were, however, significant treatment × leaf age interactions for both leaf fiber and tannin (Table 1). For the former, leaf age had no significant effect in the control treatment, but fiber increased

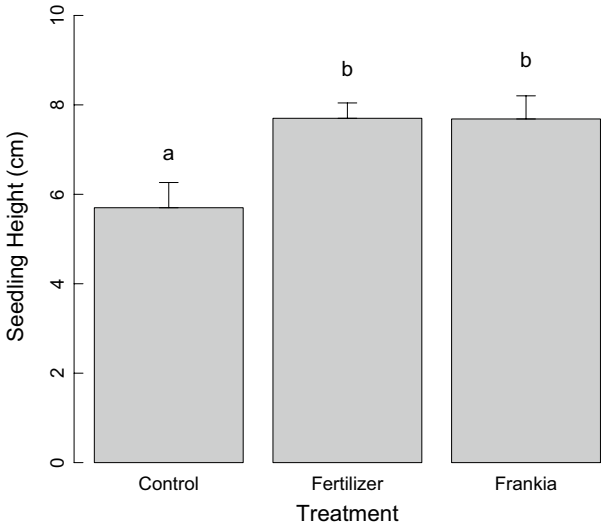

Fig. 2 Differences in laboratory-grown red alder seedling height by treatment. *Bars* represent mean ± S.D. Significant differences among treatments are indicated by *different lower case letters*

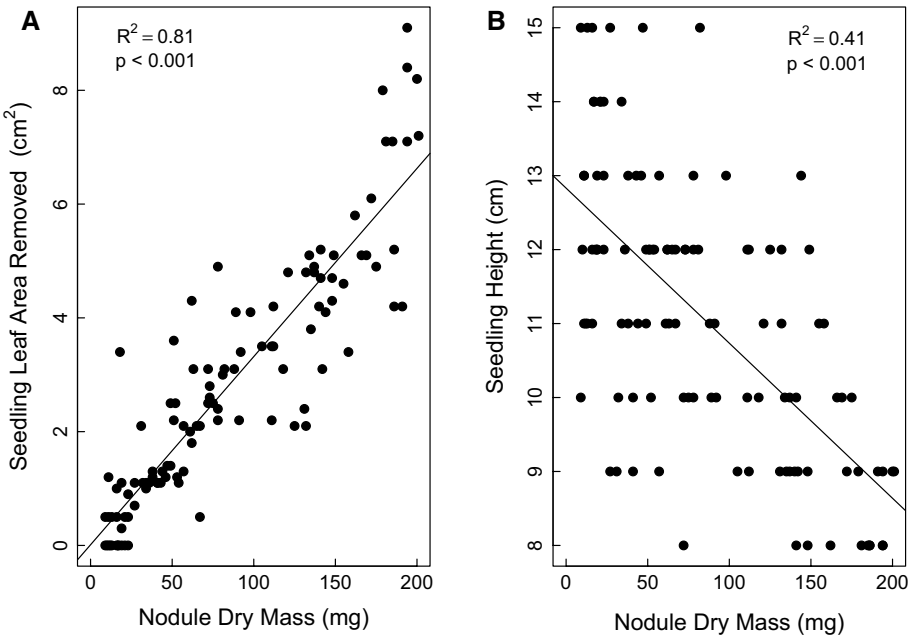

Fig. 1 Correlation between *Frankia* nodule dry mass and **a** leaf area removal by herbivory and **b** seedling height in red alder in the field

Table 1 Lead nutritive and defensive traits of red alder leaves from three developmental stages

Leaf trait	ANOVA	Treatment (*T*)	Leaf age (LA)	*T* * LA
Protein	*F*	26.2	23.7	0.7
	P	<0.001	<0.001	0.569
	Sig.	***	***	NS
Carbohydrate	*F*	22.8	61.6	0.2
	P	<0.001	<0.001	0.919
	Sig.	***	***	NS
Water	*F*	<0.1	2.8	<0.1
	P	0.984	0.061	0.995
	Sig.	NS	NS	NS
Fiber	*F*	71	27.1	3.5
	P	<0.001	<0.001	0.009
	Sig.	***	***	**
Lignin	*F*	115.1	16.2	0.8
	P	<0.001	<0.001	0.511
	Sig.	***	***	NS
Tannin	*F*	318.7	350.2	35.9
	P	<0.001	<0.001	<0.001
	Sig.	***	***	***
	df	2171	2171	4171

Significant differences are defined as *** if $P < 0.001$ and ** if $P < 0.01$ after Tukey post hoc analyses

with age in leaves from both the fertilized and *Frankia*-colonized seedlings (Fig. 3d). Although leaf tannin decreased with leaf age in all three treatments, the decrease was significantly lower in leaves from *Frankia*-colonized seedlings compared to fertilized and control seedlings (Fig. 3f).

In the laboratory feeding choice assay, slugs demonstrated significant differences in their consumption preferences across the three treatments (Fig. 4). Overall, slugs consumed significantly greater quantities of leaf tissue from *Frankia*-colonized seedlings than tissue from either fertilized or control seedlings ($F_{2,171} = 48.9$, $P < 0.001$). While leaf age alone did not have a significant effect on consumption ($F_{2,171} = 0.9$, $P = 0.446$), there was a significant treatment × leaf age interaction ($F_{4,171} = 3.3$, $P = 0.012$). Specifically, while the amount of leaf tissue consumed from *Frankia* seedlings was nearly two times higher for young and medium leaves compared to the other two treatments, there was no significant difference in the amount of tissue consumed among the three treatments for mature leaves.

Discussion

Despite the well documented the effects of nitrogen-fixing bacteria (NFB) on plant growth, the effects of NFB on the chemical phenotype of their hosts and potentially associated costs, particularly for non-rhizobial NFB, have received much less study. In our lab experiment, we observed a significant increase in leaf protein in *Frankia*-inoculated alder seedlings compared to *Frankia*-free control seedlings. Similar levels of soluble leaf protein were also observed in alder seedlings supplemented with nitrogen fertilizer. Thus, increases in leaf protein can be explained by enhanced nitrogen access in both *Frankia*-colonized and fertilized seedlings. In contrast to elevated levels of leaf protein which were observed in both *Frankia*-inoculated and fertilized alder seedling, only in seedlings colonized by *Frankia* did we observe a significant decrease in multiple carbon-based leaf traits (Fig. 2). Although this effect was present at all leaf developmental stages, young leaves in particular showed the greatest decrease in carbon-based traits. We speculate that the observed decreases are likely due to the demand for photoassimilates by *Frankia* NFB. Increased sink strength due to the presence of a rhizospheric symbiont has been shown to influence host carbon allocation in various plant systems (Kaschuk et al. 2009; Millar and Ballhorn 2013; Wright et al. 1998) and NFB can consume up to 40% of the hosts' soluble carbohydrates (Finke et al. 1982; Chapin et al. 1987; Kaschuk et al. 2009). Whereas plants associated with arbuscular mycorrhizal fungi and rhizobia have been reported to increase photosynthesis beyond the carbon costs of the symbiont via sink stimulation (Kaschuk et al. 2009), competition for photoassimilates between growth, defense, and symbiotic demand may result in conditional resource allocation trade-offs depending on plant developmental stage. We believe that our data are consistent with the latter scenario, as decreases in the differences between carbon-based compounds with leaf age strongly suggest carbon allocation competition in young plants among leaf growth, defense, and root-associated NFB.

Unlike plants associated with rhizobial NFB, which show various nitrogen-based defense traits such as alkaloids, toxic amino acids, or cyanogenic glucosides (Thamer et al. 2011), members of the genus *Alnus* and others within the family Betulaceae do not produce nitrogen-based defense compounds (Julkunen-Tiitto et al. 1996). Considering this fact, along with the absence of detectable differences in physical leaf properties such as water content [which is a proxy for leaf toughness and may critically affect herbivore behavior (Lucas et al. 2000; Loney et al. 2006; Raupp 1985; Malishev and Sanson 2015)], it seems likely that a quantitative shift in carbon- versus nitrogen-based traits in response to *Frankia* colonization is the functional basis for the increased mollusk herbivory in the *Frankia*-colonized seedlings that we observed in the laboratory as well as for the greater leaf

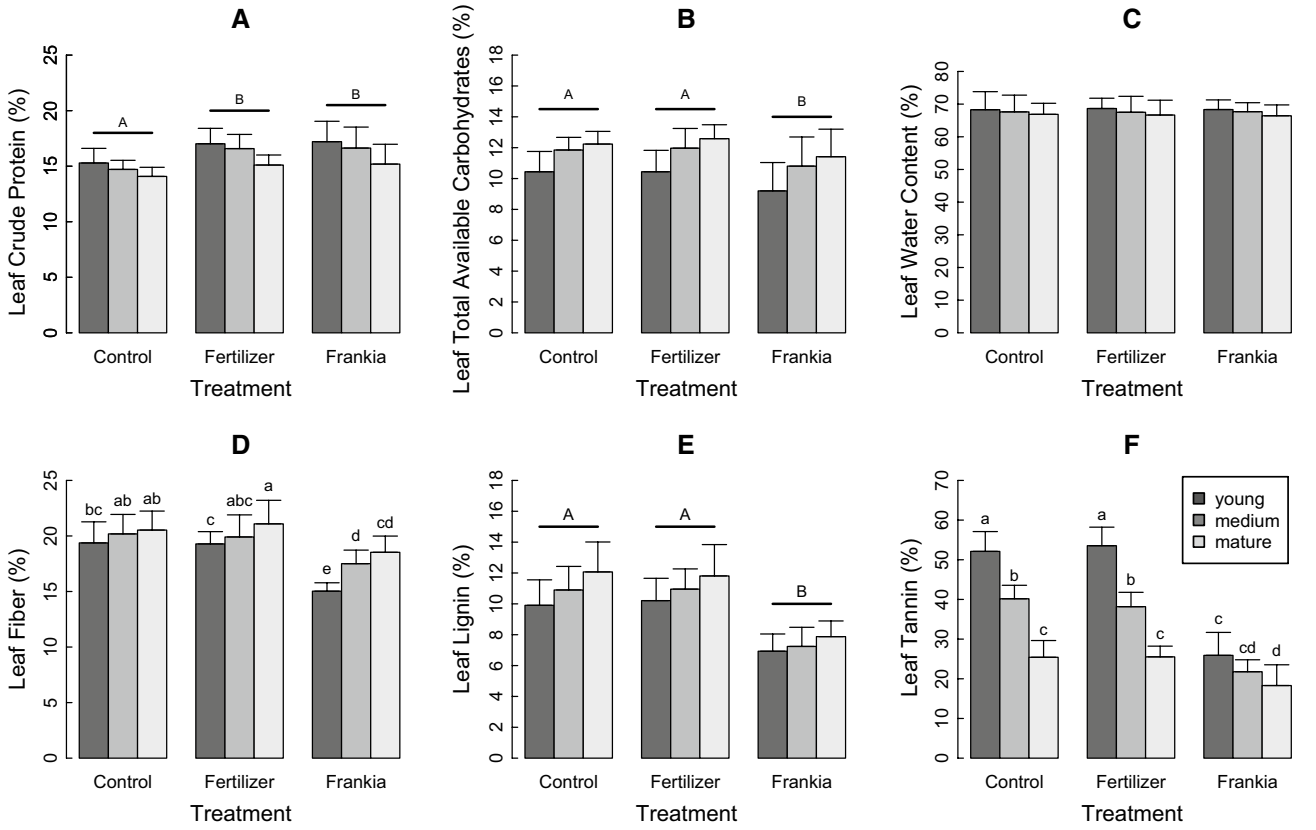

Fig. 3 Chemical leaf traits from three ontogenetic stages of laboratory-grown red alder seedlings in response to treatment. The *upper panel* (**a–c**) includes nutritive traits, and the *lower panel* (**d–f**) is defense-associated leaf traits. *Bars* represent mean ± SD. Significant differences are indicated above *bars* by *different upper case letters* for treatment effects and by *lower case letters* for interactions between treatment and developmental stage

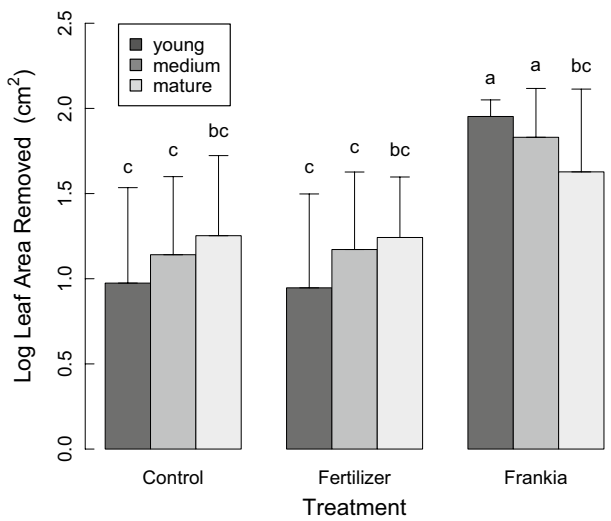

Fig. 4 Slug herbivory of leaf discs from laboratory-grown *red* alder seedlings in the three experimental treatments. *Bars* represent mean ± SD. Significant differences are indicated by *different lower case letters* above *bars*

area removal in extensively nodulated red alder seedlings in the field. Support for the importance of altered ratios of C- and N-based leaf traits for mollusk herbivory was shown by Mølgaard (1986), who found that slugs and snails significantly avoid plant species with extensive carbon-based defenses such as phenolics and tannins. Likewise, Fritz et al. (2001) demonstrated notable slug avoidance of tannins in *Salix* species.

Like other research showing positive effects of *Frankia* colonization on alder performance (e.g., Schwintzer and Tjepkema 1990), we found that *N* provision by *Frankia* bacteria promoted seedling growth. However, the significant preference of slugs for leaves from *Frankia*-inoculated plants in laboratory feeding choice assay, together with the pronounced herbivore damage on extensively *Frankia*-colonized plants in the field, indicates a potential ecological cost of this symbiosis. Given the rapid and ubiquitous colonization of red alder seedlings by *Frankia* NFB in nature, one may wonder how this symbiosis is evolutionarily stable. One factor could be the relatively short temporal window upon which herbivores must attack leaves of alder seedlings, while defenses are low. Red alder leaves

typically mature in a period of 10–14 days of bud break (D.J. Ballhorn, pers. obs.), so there is relatively narrow time period during which *Frankia*-colonized alder leaves are more poorly defended compared to leaves of uncolonized individuals. Given the large benefits to growth shown in *Frankia*-colonized versus uncolonized plants (Arnone and Gordon 1990; Huss-Danell 1997; Hendrickson et al. 1993), this may represent a favorable evolutionary trade-off for red alder seedlings, which typically need to grow quickly due to relatively low shade tolerance ability and high intraspecific competition (Koo 1989).

As a colonizer of disturbed forest locations with strong intraspecific competition, it seems likely that red alder contributes greater resources toward growth than defense, especially in early development. If the development of alder stands precedes the recruitment of herbivores, then the temporal role of *Frankia*—directly enhancing growth in early development and later enhancing defense—may render its host a particularly capable colonizer. This raises a larger question: are pioneer species (especially those associated with NFB) more apt to compensate for intense defoliation pressure than other species? Giertych et al. (2006) found different strategies of leaf defense among mature trees with similar successional status when comparing black alder and European white birch (the latter, although closely phylogenetically related to alder, does not host *Frankia*). *Frankia*-colonized black alder exhibited higher leaf turnover and greater induced defense when confronted with herbivory relative to white birch. This increased compensatory response of alder may be attributed to enhanced nutrient provisions made by *Frankia*. Given the specialized ability to thrive under nutrient-poor conditions, NFB-associated hosts may not face as much simultaneous pressure from herbivory and competition and, therefore, favor regrowth as their preferred ecological strategy.

Conclusion

Overall, our data on leaf chemistry support the hypothesis that carbon are costs involved in maintaining nitrogen providing *Frankia* and that NFB can influence seedling tissue C:N ratios in ways that have consequences for interactions with other trophic levels. Although the down-regulation of carbon-based defenses caused by *Frankia* colonization may temporarily increase rates of herbivory, this effect appears to be largely confined to very early leaf and seedling stages. Given the well-established benefits of the symbiosis in terms of growth, photosynthetic performance, and inducible defense, it is likely that this short-term elevation in susceptibility to herbivory is not sufficiently detrimental to disrupt a net positive outcome between alder trees and *Frankia* NFB in natural settings.

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Author contribution statement DJB and PGK conceived and designed the experiments. DJB, PGK, and RFF performed the experiments. DJB, PGK, and JDE analyzed the data. DJB, PGK, JDE, and MAB co-wrote the manuscript.

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