

The effect of Rhizobium-field pea symbiosis on host plant quality and subsequent herbivory by the pea leaf weevil, *Sitona lineatus* (Coleoptera: Curculionidae)

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Abstract

Legumes develop symbiotic relationships with rhizobia bacteria, enabling biological nitrogen fixation and providing nitrogen for plant growth and development. This nitrogen availability influences plant nutrition and chemical defense that affect herbivores. The pea leaf weevil (PLW), *Sitona lineatus* L. is an herbivore of legumes that depends on nutritious foliage and root nodules for food in different parts of its lifecycle. Adult PLW overwinter and emerge in spring to seek hosts and mates. Following host colonization, reproductively active weevils mate and oviposit in the soil. Larval development occurs in root nodules followed by pupation in the soil. Adults that emerge in the fall prepare to overwinter in a reproductively inactive state. We tested the hypothesis that *Rhizobium*-field pea interactions influence plant traits and subsequent herbivory by adult PLW in different physiological states. Field pea plants were inoculated with different *Rhizobium leguminosarum* strains that either fix nitrogen (wild-type; *nif+*) or do not fix nitrogen (mutant-type; *nif-*) and were compared to nitrogen and water-treated plants as positive and negative controls. The *nif+* positively affected field pea height, nitrogen and chlorophyll content compared to the *nif-* inoculated plants. *Rhizobium*-mediated plant traits only affected adult PLW in the fall when weevils were preparing to overwinter. The fall weevils preferred the *nif+* over the *nif-*, nitrogen or water-treated plants. Fall weevil preference for *nif+* treated plants suggests that host nutritional quality, rather than plant defense, drives weevil feeding behaviour before winter. Understanding the effect of rhizobia-mediated plant-herbivore interactions will help develop sustainable management strategies for pests of legumes.

Introduction

Mutualistic relationships between soil microbiota and plants contribute to plant diversity and ecosystem productivity (Carney and Matson 2005; van der Heijden et al. 2008). Legumes in the family Fabaceae exhibit mutualistic relationships with nitrogen-fixing rhizobia bacteria (Biddle 2017) that support plant growth through biological nitrogen fixation (Gopalakrishnan et al 2015; Jaiswal et al 2021). The beneficial symbiosis promotes plant tolerance to biotic and abiotic stress as well (Van Oosten et al. 2008; Kempel et al. 2009). Nitrogen fixation by rhizobia also affects the carbon-nitrogen (C:N) ratio of plant tissue (Lou and Baldwin 2004; Scheible et al. 2004), and subsequent herbivory (Fischer and Fiedler 2000; Coley et al. 2006). Understanding mechanisms that drive plant-mediated indirect interactions between soil microbes and aboveground herbivores is a major development in food web ecology (Basu et al. 2022).

Inoculation of legumes with *Rhizobium* bacteria induces the formation of root nodules in which the bacteria make nitrogen available for plant use and receive carbon nutrients in exchange (Ferguson and Mathesius 2014). Plant nitrogen content can affect both the nutritional quality and the defensive capacity of herbivore host plants (Awmack and Leather 2002). The C:N ratio of the plant affects herbivore nutrition directly or indirectly through secondary plant metabolites (Ballhorn et al. 2013). The carbon-nutrient hypothesis postulates that the carbon-nitrogen balance determines plant allocation of resources to carbon and nitrogen-based defense compounds (Bazzaz et al. 1987; Bryant et al. 1983).

Rhizobia inoculation benefits legumes by regulating plant defenses (Pieterse and Dicke 2007; Pieterse et al. 2009) through systemically priming for defense response to biotic and abiotic stress (Conrath et al. 2006).

Nitrogen fixed by rhizobia in the root nodules of legumes contributes to the production of nitrogen-based defense compounds (Thamer et al. 2011; Basu et al. 2021). The legume-rhizobia symbiosis mediates interactions with below and aboveground herbivores by causing changes to the nutritional and chemical defense profile of the plant (Kempel et al. 2009; Dean et al. 2014; Basu et al. 2021, 2022). Consumption of plants inoculated with nitrogen-fixing rhizobia can alter the life history and fitness of insect herbivores (Awmack and Leather 2002; Kempel et al. 2009; Leather 2018; Cowie et al. 2019; Simard et al. 2022). Legumes grown without rhizobia can be more susceptible to attack by insect herbivores, compared to rhizobia-treated plants (Millet et al. 2010; Rashid and Chung, 2017). The mutualism can alternatively make plants more susceptible to insects through increased plant growth and nutritive content (Van Der Heijden et al. 2008; Pinēda et al. 2010). According to the Plant Vigor Hypothesis, insect herbivores should benefit from feeding on vigorous (nutritious) plants (Price 1994).

The effects of rhizobia-legume symbioses on plant defense and subsequent host plant use by herbivores is inconsistent and can impact insect herbivores either positively or negatively (Wijerathna et al. 2025). Rhizobia-inoculated lima bean (*Phaseolus lunatus*) is less preferred by *Epilachna varivestis* (Coleoptera: Coccinellidae) than the rhizobia-free lima bean (Ballhorn et al. 2013; Thamer et al. 2011). Inoculation of cyanogenic strains of white clover, *Trifolium repens*, with *Rhizobium leguminosarum* bv. *trifolii* inhibits growth of the *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Kempel et al. 2009). Rhizobia-inoculated plants have more hydrogen cyanide (Kempel et al. 2009; Thamer et al. 2011) and induced production of indole, a nitrogen containing defensive compound, that negatively affect *Spodoptera littoralis* (Ballhorn et al. 2013). Inoculation of soybean (*Glycine max*) with *Bradyrhizobium japonicum* reduces *Helicoverpa zea* (Lepidoptera: Noctuidae) development (Dean et al. 2014). In contrast, the generalist herbivore *Spodoptera exigua* (Lepidoptera: Noctuidae) survives and develops best on alfalfa (*Medicago truncatula*) and alsike clover (*T. hybridum*) inoculated with nitrogen-fixing rhizobia (Wargin et al. 2026). Plant nutrition can drive the choice of some insect herbivores and override deterrent effects of induced defensive compounds (Wargin et al. 2026). Although legume-rhizobia symbioses affect herbivore infestation, limited research exists on the effect of rhizobia symbiosis on herbivory by the oligophagous, pea leaf weevil (PLW), *Sitona lineatus*.

Globally, PLW is a major pest of field peas (*Lathyrus oleraceus*, Lamb (syn. *Pisum sativum*, Linnaeus)), and faba beans, *Vicia faba* L. (Fabales: Fabaceae) (Vankosky et al. 2009; Evenden 2018; Kiju et al. 2024). Pea leaf weevil is an invasive, univoltine pest in North America (Fisher and O'Keefe 1979). After overwintering, adult weevils emerge and feed on legumes (Schotzko and O'Keefe 1988) before dispersing to primary hosts, pea or faba bean, for reproduction in the spring. Adults feed on the leaves of the primary hosts by causing characteristic notches on the foliage (Jackson 1920). Adult PLW feeding reduces plant nitrogen content (Cárcamo and Vankosky 2011; Cárcamo et al. 2015) and contributes to yield loss in field pea (Corre-Hellou and Crozat 2005; Vankosky et al. 2011) and faba bean (Wijerathna et

al. 2021). Reproductive PLWs mate on or near primary hosts, and females lay eggs in the soil around the primary hosts. Upon hatching, larvae develop and feed on root nodule tissues of primary host plants containing *Rhizobium* bacteria (Johnson and O'Keefe 1981, Hamon et al. 1987). Larval feeding decreases plant growth, fitness, and nitrogen content (Johnson and Rasmann 2015; Cárcamo et al. 2015), and decreases crop yield (Corre-Hellou and Crozat 2005). In the summer, pupae develop in the soil and the non-reproductive fall adults emerge and disperse to feed on legumes prior to overwintering (Vankosky et al. 2009). Little is known about the effect of the *Rhizobium*-field pea interaction on host plant quality, and subsequent feeding behaviour of PLWs in different physiological states throughout the year. *Rhizobium*-faba bean symbiosis had no effect on spring-feeding PLWs in a laboratory assay (Wijerathna et al. 2025), but symbiosis resulted in less PLW herbivory on *Rhizobium*-inoculated field peas in a greenhouse assay (Basu et al. 2022). Reduced herbivory on inoculated plants could be due to induction of plant defences or enhancement of the defensive response with access to fixed nitrogen (Katayama et al. 2011; Basu et al. 2022).

Preference for plants of differing nutritional quality and defensive capacity can vary with insect physiological state (Anton and Cortesero 2022). Insect herbivore response to plant cues should be greatest when the insect is in the physiological state to use the host for feeding or oviposition (Lemmen-Lechelt et al. 2018). Host preference by adult PLWs is state-dependent, as weevils prefer faba bean over field pea in the spring when they search for primary hosts to support oviposition and larval development, but not in the fall when they feed on many legumes in preparation for overwintering (Wijerantha et al. 2026). Pea leaf weevil is a good system to study state-dependent plasticity in response to plant host cues, but it has yet to be tested on rhizobia-inoculated vs. uninoculated plants that vary in nutritional quality and defensive capability.

In this study, we tested the effect of inoculation of different strains of *Rhizobium leguminosarum* biovar *viciae* on host plant traits and subsequent PLW herbivory when adult weevils are in different physiological states. We hypothesized that the rhizobia-field pea symbiosis affects adult weevil feeding preferences differently depending on weevil physiological state because weevils in different physiological states require different host plants (Wijerathna et al. 2026). We predicted that PLW will prefer field pea without rhizobia because inoculated plants have higher levels of direct and indirect plant defences against herbivores (Basu et al. 2022; Godschalx et al. 2023, Bustos-Segura et al. 2024; Wijerathna et al. 2025). Feeding preference may vary with weevil physiological state depending on the nutritional needs of the insect throughout the adult life stage. A trade-off between nutritional food quality and chemical defense in field pea may also dictate host preference by PLW, due to improved host nutritional quality because of nitrogen fixation by rhizobia. Since nitrogen is essential for chlorophyll synthesis for photosynthesis (Bassi et al. 2018; Chai et al. 2024), abundant nitrogen will promote host plant growth and condition. We predicted that the host plant quality of field pea for PLW, as measured by chlorophyll content and C:N ratio, will improve with rhizobia inoculation (Jaiswal and Dakora, 2025) and affect subsequent herbivory.

Methodology

Study system and treatments

Field pea seeds (Smirnoff AAC, Lacombe) were planted 3 cm deep in 500 ml plastic pots containing a plant growing mixture (Sun Gro Horticultural Canada Ltd.) and sand (Quikrete, Home Depot, Canada), in a 3:1 ratio (v:v). We manipulated nitrogen availability to plants with four treatments in all experiments: 1) wild-type *Rhizobium leguminosarum* biovar *viciae* 3841 (*nif*⁺), that induces nodulation and nitrogen fixation (Thilakarathna and Raizada 2018); 2) a near isogenic mutant-type *Rhizobium leguminosarum* biovar *viciae* 3940 (*nif*⁻), that induces nodulation but does not fix nitrogen (Thilakarathna and Raizada 2018); 3) nitrogen fertilizer, as a positive control; and 4) water, as a negative control. In treatments 1 and 2, we inoculated field pea seeds with 1 ml of the *Rhizobium leguminosarum* biovar *viciae* wild-type and mutant-type inoculum respectively, with a rhizobial density adjusted to OD₆₀₀ = 0.1 (Thilakarathna and Raizada 2018). Seeds for the water-treatment control plants (treatment 4) were treated similarly but received 1 ml of water prior to planting. The fertilizer treatment (treatment 3) received quarter-strength nitrogen-included Hoagland's nutrient solution (HOP03-50LT; Caisson Labs), whereas both rhizobia inoculated and negative control plants received quarter-strength of nitrogen-free Hoagland's nutrient solution (HOP03-50LT; Caisson Labs) (Thilakarathna and Reizada 2019). Individual plant received 50 ml of the corresponding fertilizer solution per week, from planting to the end of the experiment. We maintained plants in each experiment in a growth room at 22°C and 16 : 8 h (Light : Dark) cycle with supplemental lighting in the photophase (1224 lm/m²). We re-inoculated rhizobia-treated plants 1 week after planting to ensure proper nodulation (1 ml/plant, OD₆₀₀ = 0.1).

Pea leaf weevil collection (*Sitona lineatus*)

Adult PLWs for use in experiments were collected from three field sites (Table 1). Weevils were sampled from alfalfa (spring) or faba bean plants (fall) using sweep nets (39 cm diameter). Weevils in the overwintered physiological state were collected before winter (October 2022) and held under controlled conditions in the laboratory to simulate winter (Table 1). One hundred male and female weevils were introduced into plastic 5.1 L containers with a surface layer of oven dried leaf litter (35 g) on top of oven-dried soil (376 g), water was added to maintain 35% moisture content. Six containers with overwintering PLWs were held at 5°C for 4 months in dark conditions. Weevils in the spring reproductively active and fall reproductively inactive physiological states were collected directly from the field in May and August 2023, respectively (Table 1). Prior to use in experiments, weevils in all physiological groups were fed alfalfa in a meshed dome cages (60 cm X 60 cm X 60 cm) at 22°C and 16: 8 h (Light: Dark) cycle for at least 2 days and males and females were separated by sex (Jackson 1920). Humidity was maintained by misting cages as needed.

Table 1

Weevil collection sites, hosts and time for specimens used in experiments to examine the effect of *Rhizobium*-field pea symbiosis on host preference by weevils in different physiological states

Site	Latitude: Longitude	Host Plant at Collection	Collection Date (month, year)	Weevil Physiological State at Collection	Weevil Physiological State in Experiments
Lacombe, Alberta	52.42° N, 113.75° W	Faba bean	Oct, 2022	Fall	Overwintered
Edmonton, Alberta	53.50° N, 113.53° W	Alfalfa	May, 2023	Spring	Spring
Lacombe, Alberta	52.42° N, 113.75° W	Faba bean	Aug, 2023	Fall	Fall

Effect of rhizobia on herbivory by adult pea leaf weevils in different physiological states

We conducted three choice experiments to test the hypothesis that the rhizobia-field pea symbiosis and nitrogen fixation affects feeding preference of adult PLWs in varying physiological states. Pea leaf weevil adults were tested in three different physiological states: i) overwintered weevils that were tested immediately post overwintering prior to dispersal to reproductive hosts; ii) spring weevils that were reproductively active after dispersal to reproductive hosts; and iii) new generation fall weevils that were reproductively inactive prior to overwintering (Wijerathna et al. 2026). Weevils in each physiological state were tested for feeding preference using both 3- and 5-week-old field pea plants (before and after nodule initiation) in three separate experiments (Table 1). In each preference experiment, one plant of each of the four treatments at either the 3- or 5-week post planting stage ($n = 10$ per treatment and plant developmental stage) were positioned in cages (80 cm x 39 cm x 39 cm). Plants were physically connected with a square (13 cm x 13 cm) piece of white polypropylene sheeting, coroplast (BioQuip, CA, USA) (Wijerathna et al. 2025) so that adult weevils could move between plants. Four male and four female PLWs were introduced into cages and allowed to feed for 8 days, after which leaf notching on each plant was counted (Cárcamo and Vankosky 2011; Vankosky et al. 2011; Basu et al. 2022; Wijerathna et al. 2025). After each experiment, roots of individual plants were washed with tap water, and the root nodules were counted (Basu et al. 2022). Experiments on weevils in different physiological states were necessarily conducted at different times of year (Table 1).

Effects of rhizobia on nitrogen, carbon and chlorophyll content of field pea (dup: abstract ?)

The C:N ratio of plant tissues was measured for the variously manipulated field pea plants at both 3 and 5 weeks old ($n = 10$ per treatment). Plants were seeded, treated and grown as described above. At 3 or 5

weeks post planting, three young, unfolded leaves per plant were randomly selected for chlorophyll content measurement using a photosynq system (ROHS, MultispeQ V2.0, USA) that provided Soil Plant Analysis Development (SPAD) values (Kashiwagi et al. 2010, Roger et al. 2013). Following leaf chlorophyll content measurement, the roots of each plant were washed, and the root nodules were counted (Basu et al. 2022). Five randomly selected plants in each treatment were dried in an oven at 65°C for 3 d and pulverized into powder with a TissueLyser II grinder (Quiagen, Retsch, Richmond Scientific, Great Britain). The CNH/O dry combustion method with a Thermo FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Bremen, Germany 2016) was used to analyze the carbon and nitrogen content of the treated plants.

Statistical analyses

R version 4.4.0 (R Core Development Team 2024) was used to analyze all the collected data. Tidyverse was used for data manipulation, and the figures were obtained with ggplot2 (MacDonald et al. 2026) from the model outputs. In the three choice experiments, the number of notches per plant, plant height and the number of nodules per plant were treated as dependent variables in separate mixed models for each experiment using glmmTMB library models (Zuur et al. 2009; Bates et al. 2015). Plant manipulation treatments and plant developmental stages and the interaction were the independent variables in all models, and cages was treated as a random variable in mixed effect models. Models with different count (Poisson, negative binomial, compois, genpois) and continuous (gaussian and gamma) error distribution families were compared using AIC to identify the model with best fit (McDonald et al. 2026) (Online Resource 1). As there was no interaction effect between treatments and the plant developmental stages ($P > 0.05$) in all models, the interaction term was removed, leaving the treatments and plant developmental stages as predictors. The residuals of the best model were checked for uniformity, goodness of fit, homogeneity of variance, over-dispersion, and zero-inflation using one sample Kolmogorov–Smirnov tests (KS test) in DHARMA package (Hartig 2022).

To statistically test for the effects of plant manipulation treatments on plant traits, plant tissue nitrogen, carbon and chlorophyll content, the C:N ratio, SPAD value, and nodule number per plant were assigned as the dependent variable in separate models. Count or continuous data were modeled using the glmmTMB library model (Zuur et al. 2009; Bates et al. 2015) with the poisson, negative binomial, compois, genpois, gaussian and gamma error families specified in glmmTMB. The row in which each plant was grown was treated as a random factor in linear mixed effect models to analyze the SPAD values, plant height and nodule number. The C:N ratios were analyzed with a generalized linear model with specified gaussian and gamma error families in the glmmTMB. All models were compared with AIC to identify the model with best fit (McDonald et al. 2026) (Online Resource 1). ANOVA hypothesis testing ($P < 0.05$) was used to compare the means of the four treatments derived from the best models. A TukeyHSD post-hoc test was used if the model was significant to determine treatment-specific differences (Tukey 1977).

Results

Effect of rhizobia on herbivory by adult Pea leaf weevils in different physiological states

Although not statistically compared, the amount of feeding done by weevils varied with physiological state. Fall weevils preparing to overwinter fed the most and reproductive weevils in spring fed the least, while overwintered weevils produced an intermediate number of notches (Fig. 1). Overwintered weevils showed no host preference for the differently manipulated plants as the mean number of feeding notches was statistically similar ($\chi^2 = 6.2595$, $df = 3$, $p = 0.09964$) among the *nif+* *Rhizobium*, *nif-* *Rhizobium*, nitrogen and water treatments of pea plants at both phenological stages. Similarly, the mean number of feeding notches caused by the spring weevils did not differ by treatment at the two plant stages ($\chi^2 = 3.9960$, $df = 3$, $P = 0.2619$) (Fig. 1b). No interaction effect was detected between the treatments and plant age across the three weevil physiological stages. The mean number of feeding notches generated by the reproductively inactive fall weevils differed with plant treatment at both plant stages ($\chi^2 = 14.7506$, $df = 3$, $P = 0.0020$) (Fig. 1c). Plant stage did not directly influence the mean number of notches caused by the weevils in the Fall ($\chi^2 = 2.8634$, $df = 1$, $P = 0.0906$). Fall weevils fed more on plants treated with *nif+* *Rhizobium* than on the *nif-* mutant-type, nitrogen and water-treated plants in the 3- and 5-week old plants (Fig. 1c). Feeding by fall weevils on 3- and 5-week old plants treated with the *nif-* *Rhizobium* had a similar number of notches as nitrogen and water-treated plants (Fig. 1c).

As would be expected, 5-week-old plants were significantly taller than the 3-week-old plants, in all treatment categories (Overwintered: $\chi^2 = 612.3932$, $df = 1$, $P < 0.0001$; Spring: $\chi^2 = 535.7359$, $df = 1$, $P < 0.0001$; Fall: $\chi^2 = 588.3116$, $df = 1$, $P < 0.0001$; Fig. 2). In all experiments, the *nif+* *Rhizobium* treated plants grew taller than the other treatments at 5 weeks, however, the mean plant height of the *nif+* plants and the nitrogen treated plants were statistically similar in the experiment conducted on Spring weevils (Fig. 2b). There was no effect of treatments on plant height for 3-week-old plants in any of the experiments (Fig. 2).

The mean number of nodules per plant differed among treatments in all 5-week-old plant experiments (Overwintered; $\chi^2 = 37.512$, $df = 3$, $P < 0.0001$, Spring; $\chi^2 = 115.41$, $df = 3$, $P < 0.0001$, Fall; $\chi^2 = 144.43$, $df = 3$, $P < 0.0001$) (Table 2). Even though the *nif-* *Rhizobium* symbiosis does not fix nitrogen, plants inoculated with *nif-* *Rhizobium* produced significantly more inactive nodules (Fall; 797.06 ± 101.97 SE; Spring; 845.14 ± 91.36 SE) than all other treatments in plants grown in the spring and fall (Table 2). Plants treated with the *nif-* *Rhizobium* produced a similar number of nodules to plants treated with *nif+* *Rhizobium* and more than nitrogen and water treated plants in the experiment targeting overwintered weevils (Table 2). A statistically similar low number of nodules was produced by plants treated with nitrogen (Fall; 24.39 ± 10.15 SE; Spring; 83.71 ± 22.83 SE) or water (Fall; 6.68 ± 3.87 ; Spring; 112.24 ± 26.43 SE) (Table 2). The *nif+* *Rhizobium* treated plants produced more nodules (Fall; 110.92 ± 26.84 SE; Spring; 286.38 ± 47.30 SE) than water- and nitrogen-treated plants in the Fall and Spring. The mean number of nodules produced by the *nif+* inoculated plants (137.28 ± 43.75 SE) did not differ from *nif-* inoculated (161.62 ± 47.78 SE) plants or water (55.83 ± 20.82 SE) treated plants in the experiment on

overwintered weevils. Both *nif* + and *nif*- plants produced more nodules than the nitrogen (10.21 ± 5.35 SE) and water (55.83 ± 20.82 SE) treatments in the experiment on overwintered weevils.

Table 2

Significant Linear Mixed effect model results for choice experiment to test the *Rhizobium*-field pea symbiosis on pea leaf weevil (PLW) adult host preference when insects are in different physiological states. Separate mixed effect models analyzed the number of nodules per 5-week-old plant in all treatments at each adult weevil physiological stage. There were ten replicates per treatment: wild-type 3841 *Rhizobium* inoculant (*nif*+), mutant-type 3940 *Rhizobium* inoculant (*nif*-), nitrogen fertilizer, and a water control. Values in bold format are significantly different at $P < 0.05$. Pairwise comparisons of the treatments were conducted using Tukey's HSD post hoc test. Adjusted P-values are reported.

Response variable	Plant age	Weevil stage	Post-hoc test	P-value
Number of nodules/plant	5-week-old	Overwintered	wild-type vs mutant-type	0.9446
			wild-type vs Nitrogen	< 0.0010
			wild-type vs Water	0.0644
			mutant-type vs Nitrogen	< 0.0010
			mutant-type vs Water	0.0302
			Nitrogen vs Water	0.0086
Mean number of nodules/plant	5-week-old	Spring	wild-type vs mutant-type	< 0.0001
			wild-type vs Nitrogen	0.0002
			wild-type vs Water	0.0022
			mutant-type vs Nitrogen	< 0.0001
			mutant-type vs Water	< 0.0001
			Nitrogen vs Water	0.8032
Mean number of nodules/plant	5-week-old	Fall	wild-type vs mutant-type	< 0.0010
			wild-type vs Nitrogen	0.0012
			wild-type vs Water	< 0.0010
			mutant-type vs Nitrogen	< 0.0010
			mutant-type vs Water	< 0.0010
			Nitrogen vs Water	0.1290

Effects of rhizobia on nitrogen, carbon and chlorophyll content of field pea

In the 3-week-old treated plants, the mean C:N ratio of the *nif+* *Rhizobium*, *nif-* *Rhizobium*, and water treatments were similar (Fig. 3). However, positive control plants with the synthetic nitrogen treatment had a lower C:N ratio that differed from the water treatment, but not the *nif+* *Rhizobium* and *nif-* *Rhizobium* treatments ($\chi^2 = 9.0151$, $df = 3$, $P = 0.0290$) (Fig. 3). The *nif+* *Rhizobium*-treated plants had the lowest C:N ratio in the 5-week-old treated plants, that differed from all other treatments ($\chi^2 = 451.43$, $df = 3$, $P < 0.0001$). Nitrogen-treated plants had an intermediate C:N ratio and the *nif-* *Rhizobium* and the water-treated plants had similar high C:N ratios, indicating low levels of nitrogen (Fig. 3).

The SPAD values that represent chlorophyll content did not differ by plant treatment in 3-week-old plants ($\chi^2 = 4.2738$, $df = 3$, $P = 0.2334$) (Fig. 4). In the 5-week-old plants when root nodules have formed, the chlorophyll content of the *nif+* *Rhizobium* treated plants was higher than that of all other treatments ($\chi^2 = 398.46$, $df = 3$, $P < 0.0001$) (Fig. 4). Chlorophyll content of plants was intermediate in nitrogen-treated plants and lowest in plants treated with the *nif-* *Rhizobium* and water.

As in the previous experiment when plant height was measured after herbivory (Fig. 2), plant height did not vary with treatment on 3-week old plants that were not fed on by weevils ($\chi^2 = 2.35$, $df = 3$, $P = 0.5030$) (Fig. 5). Plant treatment significantly affected plant height of non-herbivorized 5-week old plants ($\chi^2 = 83.73$, $df = 3$, $P < 0.0001$) in ways that differed from the previous experiment (Fig. 2). The *nif+* -treated 5-week-old plants were significantly taller than plants in all other treatments. Plants with the synthetic nitrogen fertilizer treatment were significantly taller than the *nif-* *Rhizobium* and water treatments, which did not differ (Fig. 5).

The number of nodules on 5-week old plants varied by treatment in plants that did not experience herbivory ($\chi^2 = 201.80$, $df = 3$, $P < 0.0001$). The *nif-* *Rhizobium* (919.49 ± 54.47 SE) treated plant had significantly more nodules than the *nif+* *Rhizobium* (157.47 ± 21.32 SE), water (68.40 ± 27.25 SE), and nitrogen (6.94 ± 5.95 SE) treatments ($P < 0.0010$). The *nif+* *Rhizobium* treated plants produced an intermediate number of nodules that was significantly greater than plants in the nitrogen treatment ($P < 0.0010$) but not the water treatment ($P = 0.1528$). Nodule production did not vary between nitrogen and water treatments ($P = 0.0562$).

Discussion

The symbiosis between plants and associated nitrogen-fixing rhizobia affects the quality and defensive strategy of host plants against insect herbivores (Katayama et al. 2010; Thamer et al. 2011; Pinēda et al. 2010; Dean et al. 2014; Pangesti et al. 2015). This interaction may contribute to plant growth and defense trade-offs (Hahn and Maron 2016). Our results highlight that nutrient acquisition through symbiosis benefits field pea plants but also makes them a more nutritious host for the PLW. Previous work suggests that *Rhizobium*-inoculated pea plants produce defensive compounds that negatively

affect PLW herbivory (Basu et al. 2022). In contrast, our work shows that inoculation of field pea with the *nif+* *Rhizobium* strain promotes adult herbivory but only in weevils that are preparing to overwinter in the fall.

Adult physiological state influences herbivory and response to the pea-*Rhizobium* symbiosis. Spring PLWs that were collected after dispersal to reproductive hosts consumed the least amount of plant tissue and may prioritize mating and oviposition over feeding. After dispersal to reproductive hosts in the spring, weevils feed, mate and lay eggs (Vankosky et al. 2009). Overwintered PLWs prior to dispersal may feed more than spring weevils to fuel the energetically expensive dispersal to reproductive hosts. Adult weevils produced the most notches in the fall when they were not reproductively active and were preparing for overwintering. Fall adult weevils probably feed more to obtain energy in preparation for winter, although previous work showed that Spring PLWs feed more on faba bean than Fall weevils (Wijerathna et al. 2026). Fall PLWs in our experiment were selective in their feeding choices and preferred the nitrogen fixing *nif+* treated plants possibly to obtain more nutrition in preparation for overwintering or for future fitness gains (Bernstein and Jervis 2008). Host nutritional quality can determine insect foraging behaviour based on insect reproductive state (Damien et al. 2019; Le Gall et al. 2021). Organisms make foraging decisions to maximize fitness at a low energy cost based on Optimal Foraging Theory (Pyke et al. 1984).

The mutualistic rhizobia have positive effects on plant growth (Kempel et al. 2009; Thamer et al. 2011; Heath and Lau 2011), but not necessarily on associated herbivores (Kempel et al. 2009; Ballhorn et al. 2013; Basu et al. 2022; Wijerathna et al. 2025). We predicted that PLWs would prefer field pea without nitrogen fixing rhizobia because rhizobia inoculation improves direct and indirect defence against herbivores (Thamer et al. 2011; Ballhorn et al. 2013; Godschalx et al. 2015; Basu et al. 2022; Wijerathna et al. 2025). In contrast to what we predicted, weevils tested after overwintering or when reproductively active in the spring showed no feeding preference for the variously treated field pea plants. Spring PLWs subjected to *Rhizobium*-treated or untreated faba bean plants in a laboratory choice assay also had no preference based on feeding notch counts but oriented to *Rhizobium*-treated faba bean plots in the field (Wijerathna et al. 2025). In a greenhouse study that tested the effect of *Rhizobium* inoculation on PLW feeding in the spring, less feeding occurred on *Rhizobium*-inoculated plants due to upregulation of defensive compounds in treated plants (Basu et al. 2022). These contradictory results may be due to differences in the *Rhizobium* strains (Wijerathna et al. 2025, Wargin et al. 2026). Basu et al. (2022) and Wijerathna et al. (2025) used commercially available *Rhizobium leguminosarum* biovar *viciae* strains, and we used *Rhizobium leguminosarum* biovar *viciae* 3841 and the near isogenic non-nitrogen fixing mutant-type 3940 strains (Thilakarathna and Raizada 2018). *Spodoptera exigua* fed alsike clover (*Trifolium hybridum*) inoculated with a highly mutualistic strain of *Rhizobium leguminosarum* has better survival, development time, pupal weight and adult wing area than when fed plants inoculated with a less mutualistic *Rhizobium leguminosarum* strain (Wargin et al. 2026). Local environmental conditions, plant genotypes or soil types that rhizobia-plant interactions depend on (Taylor et al. 1991; Streeter 1994; Denton et al. 2002; Thrall et al. 2007) also differ among the published PLW experiments (Basu et al. 2022; Wijerathna et al. 2025) and could account for the variable results.

Fall weevils in our study, that are reproductively inactive and preparing for overwintering showed a preference for pea plants treated with *nif+* *Rhizobium*. The amount of feeding on *nif+* treated plants in the fall was significantly more than the nitrogen or water treated three- and five-week-old plants, respectively. Rhizobia-treated plants increase the performance of other chewing herbivores, including *S. littoralis* (Kempel et al. 2009) and the soybean podworm (*Helicoverpa zea*) (Dean et al. 2014). Similarly, velvet armyworm caterpillars, *Spodoptera latifascia*, prefer and grow better on rhizobia-treated lima bean (*Phaseolus lunatus*), despite increased defensive compounds in the *Rhizobium*-treated plants (Bustos-Segura et al. 2024). The positive effects of *Rhizobium* inoculation on herbivores results from the high nutritional plant quality due to nitrogen fixation regardless of plant chemical defenses. Since nitrogen is imperative for insect herbivore feeding and fitness (Mattson 1980; Schädler et al. 2007), more notching on the wild-type *Rhizobium* treated plants by the fall weevils probably reflects improved host plant quality as a result of nitrogen fixation (Kempel et al. 2009). As the primary driver of the development and survival of insect herbivores is the nutritional content of plant diets (Mattson 1980; Scriber and Slansky 1981; Audusseau et al. 2015; Pöyry et al. 2017), our findings support the “Plant Vigor Hypothesis” that states insect herbivores should benefit from feeding on vigorous (nutritious) plants (Price 1994).

Plant developmental stages can affect herbivore feeding behaviour due to changes in plant nutritional quality, tissue toughness, and defensive chemistry throughout plant ontogeny (Boege and Marquis 2005). Young plants are consistently preferred to older plants by invertebrate herbivores, and this preference alters herbivore behaviour, performance and density (Boege and Marquis 2005). For instance, young banana plants (*Musa species*) attract more banana weevils (*Cosmopolites sordidus*) than older host plants for oviposition (Masanza et al. 2009). This is associated with an altered defense system in older plants compared to younger plants (Boege and Marquis 2005). In legumes, biological nitrogen-fixing rhizobia inoculation may modify age-dependent changes in defense, because the rhizobia can simultaneously alter plant nutrient status and defense signaling pathways in nodulating and non-nodulating leguminous plants (Dean et al. 2014). Adult PLW host colonization occurs before the sixth node stage in early spring (Cárcamo et al. 2018) when inoculated plants have not developed nodules. Basu et al. (2022) tested the impact of the mutualism on PLW feeding four weeks after inoculation with a 48 h bioassay. In comparison, we examined the mutualism at 3 weeks post inoculation before pea plants nodulate and at 5 weeks post inoculation after nodulation and found no difference across the two plant growth stages. The lack of a plant age effect on feeding could be due to weevil adaptation to plant defenses (Boege and Marquis 2005) across plant developmental stages. Plant metabolic changes as a result of inoculation with *Rhizobium* bacteria occur prior to nodule formation (Zhang et al. 2012; Liu et al. 2026) and may be detectable by weevils. The volatile organic compounds released from pea vary with plant development in the vegetative, flowering and pod developmental stages (Ceballos et al. 2015). Both plant age categories tested in our study were in the vegetative state but the 5-week old plants were larger and had root nodules. Future research should examine the volatile profile of plants before and after the nodule formation.

Nitrogen fixation affects the carbon-nitrogen ratio of plant tissue (Lou and Baldwin 2004; Scheible et al. 2004), and *nif+* rhizobia-treated plants in our study had a significantly lower C:N ratio than other

treatments at 5 weeks post inoculation. The non-nitrogen fixing *nif*⁻ and water treatments had high C:N ratio due to the inability of the host plant to fix nitrogen. The C:N ratio is similar across all treatments in 3-week old plants except for the nitrogen and water treatment that differed from each other. Thus, the initial effect of the mutualists on plant quality of legumes before and after the production of root nodules needs further investigation.

Plant shape, texture, reflectance, colour or size can mediate host location by insect herbivores (Grunseich et al. 2019). Bustos-Segura et al. (2024) suggest that the dark leaves of rhizobia-treated lima beans attract *S. latifascia* caterpillars to nutritious plant hosts. In our study, the chlorophyll content of the *nif*⁺ *Rhizobium*-treated plants was significantly higher than in the other treatments in the 5-week-old plants. This indicates that nitrogen-fixing rhizobia do not only increase the plant nutritional quality but also improve chlorophyll biosynthesis. Plants treated with the *nif*⁺ *-Rhizobium* strain were taller than the other treatments. Plant height could be a cue that Fall PLWs used to assess hosts for selection. Egyptian cotton leafworms larvae prefer the largest (tallest) plants regardless of *Rhizobium* inoculation (Roger et al. 2013). Beneficial microbes that modify plant growth and biomass can indirectly influence the orientation of insect herbivores (Roger et al. 2013). In the Fall 3-week-old plants, the chlorophyll content and plant height were similar across all treatments. The host preference of the Fall Weevils for the *nif*⁺ inoculated 3-week-old plants could indicate that variation in plant size (Roger et al. 2013), and colour is less important than concentrations of defensive compounds and leaf nutritional quality for orientation (Yasui et al. 2006; Thamer et al. 2011).

In conclusion, our study reveals that *Rhizobium*-plant symbiosis differentially influences plant health and insect herbivory, but the effect varies with weevil physiological state. This is the first study to test the effect of symbiosis on PLW feeding preference at different weevil physiological stages. The effect of the rhizobia-field pea symbiosis on PLW feeding is likely determined by host plant nutritional quality (Bustos-Segura et al. 2024), and to a lesser extent, by chemical defence compounds (Basu et al. 2022). In addition, we show that nitrogen fertilizer treatment does not completely replace the benefits of rhizobia to host plant quality. Here we assess the effect of the rhizobia-field pea mutualism on adult PLW feeding and it will be important to test the effect of the symbiosis on larvae in the future. Pea leaf weevil larvae that feed on root nodules and *Rhizobium* bacteria directly (Johnson and O'Keefe 1981, Hamon et al. 1987) are more damaging to crop production and yield (Corre-Hellou and Crozat 2005). Rhizobia directly improve plant nutritional quality that appears to benefit adult weevils despite the potential for more defensive compounds in inoculated plants (Basu et al. 2022). Further research needs to identify and quantify the volatile organic compounds released from the different *Rhizobium*-treated plants since adult PLWs orient to host plant volatiles (Blight and Wadhams 1987; Blight et al. 1991; Landon 1997; Evenden et al. 2016; St Onge et al. 2018) and orientation varies with weevil physiological state (Landon 1997). Globally, legumes are important crops in crop rotation systems with cereals, understanding the effect of soil rhizobia-plant symbiosis on herbivore behaviour will help to develop a sustainable management strategy for oligophagous pest of legumes while improving plant health and crop yield through nitrogen fixation.

Declarations

Declaration of Competing Interest

The authors declare that there is no competing interests that could have seemed to affect the work reported in this paper.

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Author Contribution

ME created the study concept, and all authors contributed to the experimental design of this study. OF performed the experimental set-up, data collection, analysis and manuscript preparation. ME and MT reviewed and edited the prepared manuscript and approved the final manuscript for submission.

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Data Availability

All data supporting this research are available on request.

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Figures

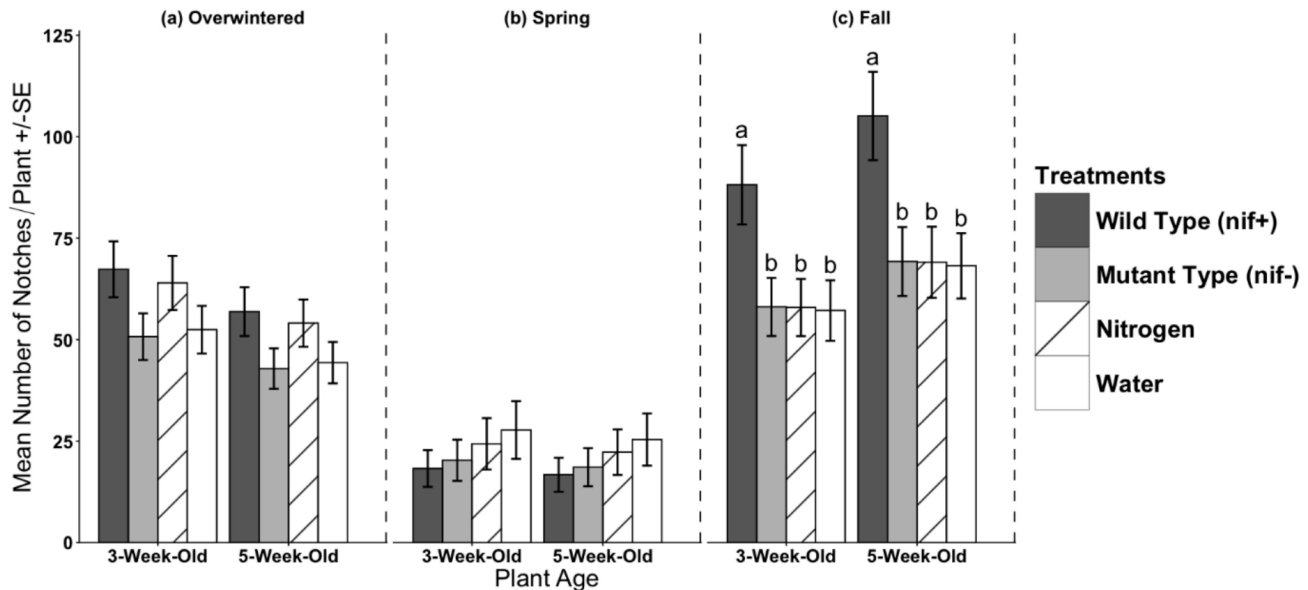


Figure 1

Mean number of notches produced by Pea leaf weevil (PLW) adults in different the physiological stages: (a) Overwintered, (b) Spring, and (c) Fall on variously treated field pea plants presented to weevils in a choice bioassay at two growth stages (3- and 5- week-old). Plants were treated with: 1) wild-type 3841 *Rhizobium* inoculant (*nif*⁺); 2) mutant-type 3940 *Rhizobium* inoculant (*nif*⁻); 3) nitrogen fertilizer; and 4) a water control. Error bars represent the standard error (SE). Treatments with different letters within each weevil physiological state and plant age category are significantly different from each other (Tukey's HSD, $\alpha = 0.05$).

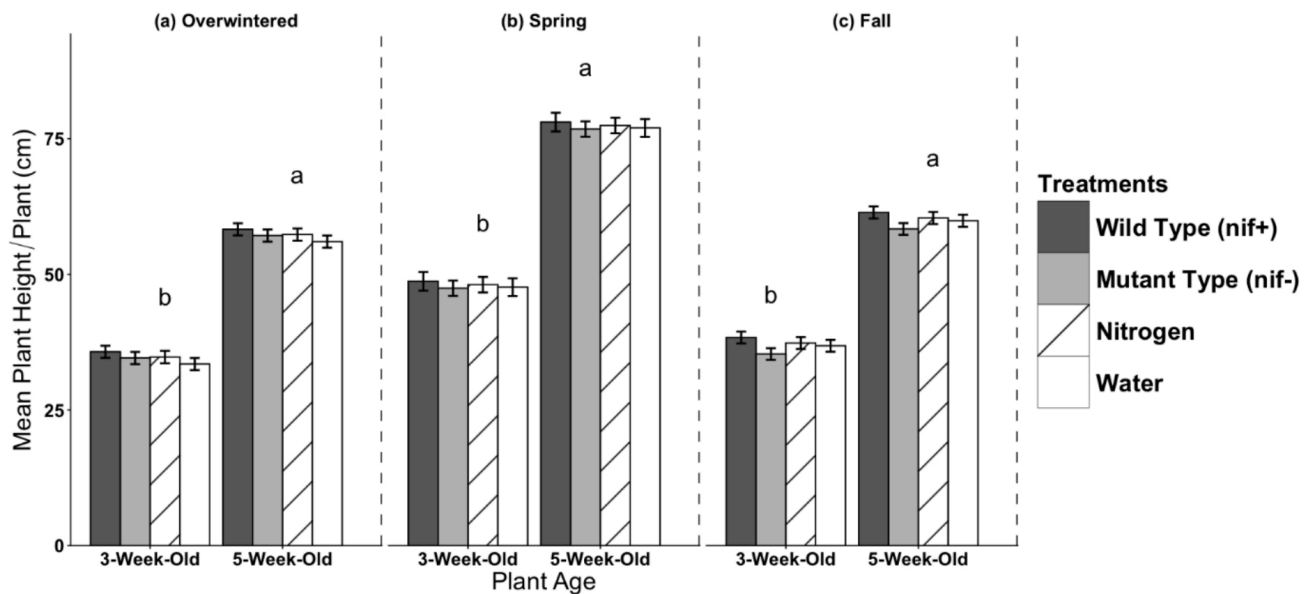


Figure 2

Mean plant height of variously treated field pea plants in two growth stages (3- and 5-week-old) presented to weevils in different physiological stages; (a) Overwintered, (b) Spring, and (c) Fall in a choice bioassay. Plants were treated with: 1) wild-type 3841 *Rhizobium* inoculant (*nif*+); 2) mutant-type 3940 *Rhizobium* inoculant (*nif*-); 3) nitrogen fertilizer; and 4) a water control. Error bars represent the standard error (SE). Plant ages marked with different letters within each weevil physiological state are significantly different from each other (Tukey's HSD, $\alpha = 0.05$).

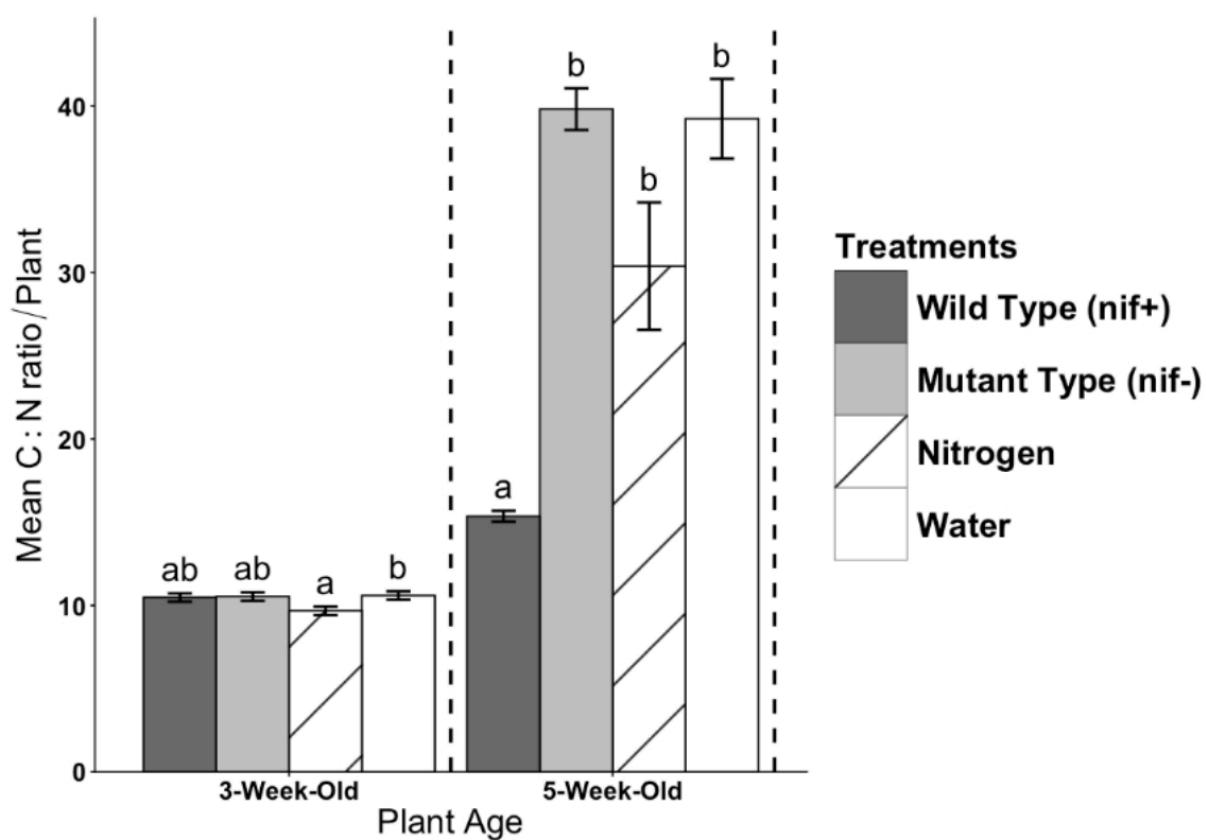


Figure 3

Mean C:N ratio of the variously treated field pea plants: 1) wild-type 3841 *Rhizobium* inoculant (*nif*+); 2) mutant-type 3940 *Rhizobium* inoculant (*nif*-); 3) nitrogen fertilizer; and 4) a water control in each of two growth stages (3- and 5-week old) (n=5 per treatment). Error bars represent the standard error (SE). Treatments with different letters within a plant age category are significantly different from each other (Tukey's HSD, $\alpha = 0.05$).

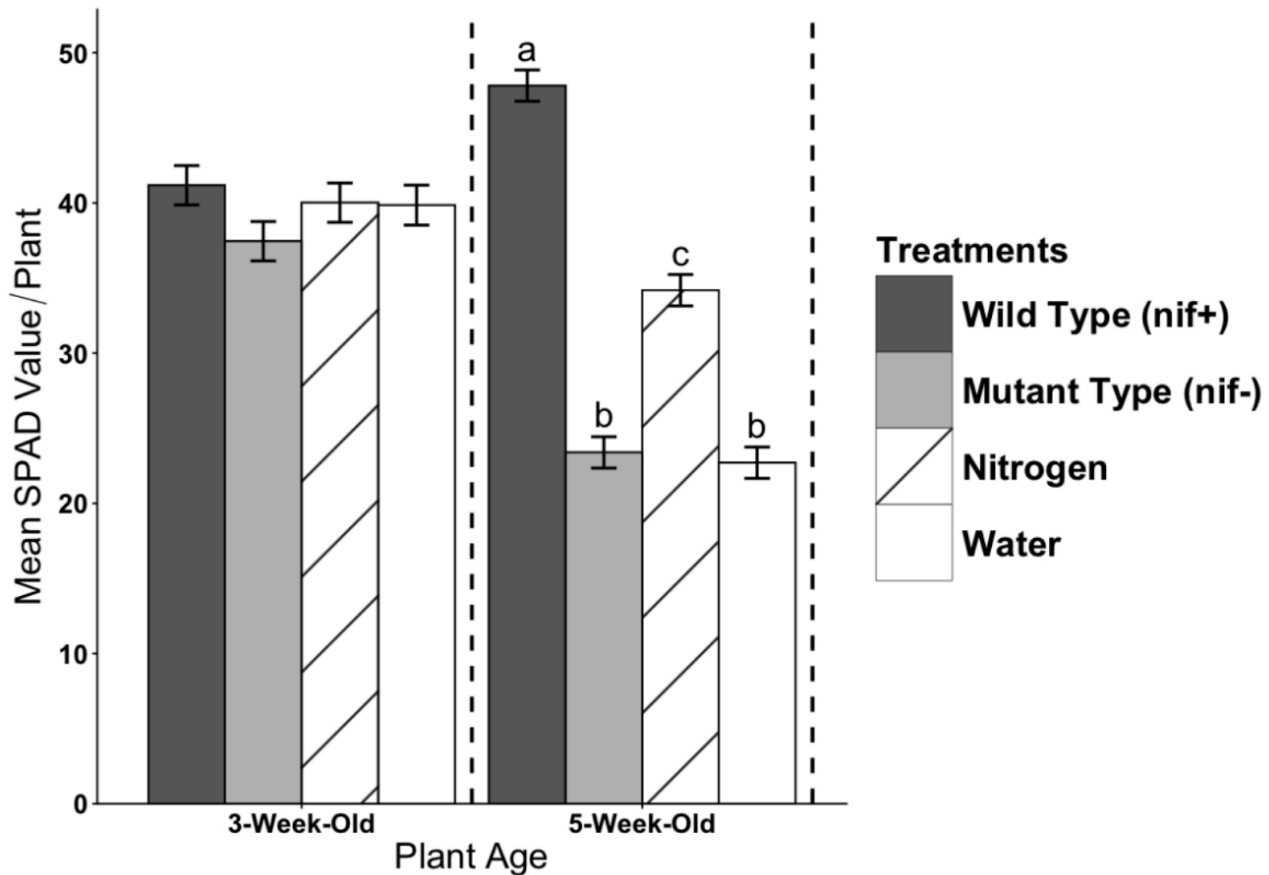


Figure 4

Mean Soil Plant Analysis Development (SPAD) value measuring chlorophyll content of the variously treated field pea plants: 1) wild-type 3841 *Rhizobium* inoculant (*nif*⁺); 2) mutant-type 3940 *Rhizobium* inoculant (*nif*⁻); 3) nitrogen fertilizer; and 4) a water control in each of two growth stages (3- and 5-week-old) ($n=30$ per treatment per plant age). Error bars represent the standard error (SE). Treatments with different letters within a plant age category are significantly different from each other (Tukey's HSD, $\alpha = 0.05$).

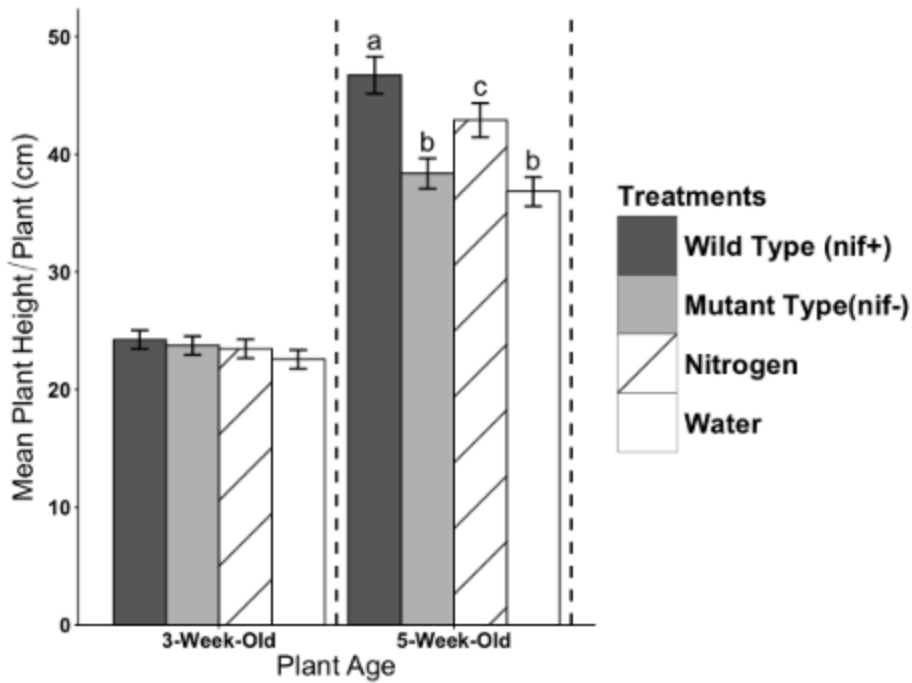


Figure 5

Mean plant height of variously treated field pea plants in two growth stages (3- and 5-week-old). Plants were treated with: 1) wild-type 3841 *Rhizobium* inoculant (*nif*⁺); 2) mutant-type 3940 *Rhizobium* inoculant (*nif*⁻); 3) nitrogen fertilizer; and 4) a water control. Error bars represent the standard error (SE). Treatments with different letters within a plant age category are significantly different from each other (Tukey's HSD, $\alpha = 0.05$).

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