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Rhizobia–Bean Symbiosis Increases Root Herbivore Attraction and Growth via Volatile Signals and Enhanced Nutrition

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ABSTRACT

The symbiosis between nitrogen-fixing rhizobia and plants is considered mutually beneficial, yet its indirect effects on other organisms remain understudied. We examined how rhizobia symbiosis in *Phaseolus vulgaris* influences the behaviour and performance of *Diabrotica balteata* larvae. Specifically, we tested larval preference for nodulated (R^+) vs. non-nodulated (R^-) roots and assessed the impact on larval growth. We also analysed root nutrient content and volatile organic compounds (VOCs) to identify potential chemical cues driving feeding preferences. Larvae strongly preferred R^+ roots, where they exhibited enhanced growth and higher survival post-metamorphosis. Nutritional analysis revealed that R^+ roots had greater nutrient content, supporting improved larval performance. VOC profiles differed significantly between treatments, and olfactometer assays confirmed that larval attraction was mediated by VOCs, likely signalling enhanced nutritional benefits from rhizobia symbiosis. Our results demonstrate that rhizobia-induced metabolic changes in bean roots make them more attractive and nutritious to herbivorous larvae. This highlights a complex belowground interaction between nitrogen-fixing bacteria, host plants and herbivores, with potential implications for ecological theory and sustainable agriculture. Understanding these interactions could inform pest management strategies and improve legume cultivation by balancing plant–microbe mutualisms with herbivore dynamics.

1 | Introduction

Plants interact with a diverse array of organisms throughout their life cycle, both above- and belowground, shaping their development, survival and ecological roles. Belowground interactions, in particular, involve complex relationships with plant roots, microorganisms and other soil-dwelling organisms, which are critical for nutrient cycling, plant productivity and ecosystem functioning (Johnson and Rasmann 2015; Pineda et al. 2013; Van Dam 2009). One of the most well-known belowground interactions is the symbiosis between legumes and rhizobia nitrogen-fixing bacteria, such as rhizobia, which convert atmospheric nitrogen into a form that plants can readily use (Kawaka 2022). This relationship provides legumes with a clear nutrient advantage, particularly in nitrogen deficient soils, while supplying the bacteria with carbon from plant photosynthesis (Lee and Hirsch 2006; Keller and Lau 2018).

While the benefits of rhizobia-legume symbiosis for plant nutrition and productivity are well established, its ecological consequences

extend beyond nutrient acquisition. Rhizobia can influence aboveground interactions, including those with herbivores, often enhancing herbivore performance (Kempel et al. 2009). However, several studies have found that rhizobia can also provide better protection against herbivores. For example, Endo et al. (2007) demonstrated that soybean plants inoculated with rhizobia exhibited enhanced resistance to herbivory by the common cutworm (*Spodoptera litura*), potentially due to increased production of defensive secondary metabolites. Similarly, Pandharikar et al. (2020) found that rhizobia-inoculated *Medicago truncatula* plants exhibited reduced feeding damage by the pea aphid (*Acyrthosiphon pisum*), likely as a result of changes in plant nutrient content and induced defences. These contrasting results suggest that the effects of rhizobia on herbivore performance are context-dependent, varying with plant species, herbivore identity and environmental factors, which highlights the need to explore how these interactions shape ecological outcomes in this study. These effects can extend to higher trophic levels, influencing the natural enemies of herbivores (Bustos-Segura et al. 2024; Godschalx et al. 2023). However, the impact of rhizobia on belowground herbivores remains poorly understood, despite their critical roles in root health, plant fitness and soil ecology.

Belowground herbivores, such as root-feeding insects, are directly influenced by the nutritional quality and defensive compounds of their host plant's roots. The symbiotic relationship with rhizobia enhances nutrient uptake in plants, leading to increased root biomass and changes in exudate composition, which may influence herbivore behaviour and performance (Lagunas et al. 2023). In some cases, enhanced nutrient acquisition through rhizobia symbiosis allows plants to allocate more resources towards defence, such as the production of secondary metabolites that deter herbivores (Agrawal and Fishbein 2006; Basu et al. 2022; Bustos-Segura et al. 2024; Godschalx et al. 2023). However, the influence of which rhizobia-induced changes in plant nutrition and root chemistry on the attraction or deterrence of belowground herbivores remains largely unexplored.

This study examines the impact of rhizobia–bean symbiosis on belowground herbivores, specifically its effects on the attraction and performance of root-feeding larvae of *Diabrotica balteata*. We hypothesised that nodulated (R^+) bean roots attract larvae and enhance their performance due to improved nutritional quality and altered volatile organic VOC emissions. To test this, we conducted behavioural assays to assess larval preference for R^+ versus non-nodulated (R^-) roots and evaluated the effects of rhizobia symbiosis on larval growth and development. Additionally, we analysed root VOCs and nutritional changes to understand their roles in mediating these interactions. Our findings provide novel insights into how rhizobia symbiosis influences belowground plant–herbivore interactions, with broader ecological and agricultural implications.

2 | Materials and Methods

2.1 | Plants and Insects

Native to the Americas, *Phaseolus vulgaris* (common bean) was domesticated around 8000 years ago in Central Mexico, then in both Mesoamerican and Andean regions (Bitocchi et al. 2012). As a staple food worldwide, it is prized for its high protein content and its role in sustainable agriculture, as the bean–rhizobia association reduces dependence on synthetic fertilisers (Castro-Guerrero et al. 2016). Legume-based cropping systems also enhance soil health by reducing carbon and nitrogen losses (Drinkwater et al. 1998). Seeds of *P. vulgaris* L. var. Coco Noir Starazagorski were obtained from the Sainte Marthe Farm (Angers, France).

The banded cucumber beetle *D. balteata* is a chrysomelid beetle native to the tropical Americas but has expanded to temperate regions (Eben 2022). Its larvae feeds on roots, while adult beetles consume leaves. This generalist herbivore feeds on various crops including cucurbits, maize and beans (Capinera 2001). Eggs of *D. balteata* were supplied weekly by Syngenta (AG, Switzerland) and maintained in a nylon mesh-covered box ($21 \times 21 \times 6$ cm) under quarantine conditions at the University of Neuchatel (25 ± 2°C, 60% Relative Humidity, 16:8 L/D photoperiod). Larvae were transferred to plastic boxes (20×20 cm) with organic soil (RICOTER Erdaufbereitung AG, Switzerland) and reared on 3- to 4-day-old maize seedling roots (DSP, Delley, Switzerland) grown in vermiculite. The roots were buried in the soil and sprayed with water to maintain humidity.

2.2 | Soil and Seed Sterilisation Procedures

Experiments were conducted using bean plants with (R^+) and without (R^-) root nodules. To create these treatments, half of the plants were inoculated with rhizobia, while the other half received only water as a control. To prevent rhizobia contamination, the soil used for both treatments was sterilised before inoculation as followed: The soil was autoclaved using the dry setting, applied three times over three consecutive days. Gloves were worn throughout the sterilisation process. Dry heat sterilisation is a widely used method to effectively eliminate pathogens, minimise soil contamination risks and preserve nutrient integrity (Wood 2024). Seeds were surface sterilised by rinsing them in an approximately 5% sodium hypochlorite solution for 2 min, followed by five rinses with distilled water. The sterilised seeds were then germinated in an incubator for 48 h.

2.3 | Rhizobia Inoculant Preparation

The rhizobia inoculant was prepared following Godschalx et al. (2015). Nodules were collected from 1-month-old bean plants, crushed using a pestle and mortar and diluted in 1 L of distilled water. A total of 150 nodules was used for the inoculant solution which was stored at 4°C until use.

2.4 | Bean Cultivation and Inoculation

Seeds were germinated in sterilised Petri dishes lined with moistened cotton. The dishes used were disinfected with 70% ethanol, wrapped in cellophane and aluminium foil and placed in an incubator for 48 h under controlled conditions (day/night: 30/28°, 60%–80% Relative Humidity, 12:12 h L/D photoperiod). Pots (4 cm diameter, 11 cm height) and trays were thoroughly

cleaned with soap, rinsed with water and autoclaved for one cycle before use. After germination, seedlings were transplanted into the sterilised pots filled with sterile organic soil and placed in a greenhouse (24 ± 5°C, 60% Relative Humidity, 16:8 h L/D photoperiod). To prevent cross-contamination, plants from the control (R^-) and treatment (R^+) groups were placed on separate trays, arranged randomly to account for potential light and temperature variability within the greenhouse. Inoculation was performed immediately after potting and repeated once a week for 4 weeks. For R^+, 10 mL of the rhizobia inoculant was applied directly to the soil, while R^- plants, received an equivalent volume of sterile water. Thereafter, a clean glass bottle, disinfected with a 5% sodium hypochlorite solution and rinsed thoroughly with water, was used to water plants.

After 7–14 days, R^+ plants develop symbiosis with bacteria, resulting into nodules formation (Ferguson et al. 2010), and were ready for experimental use.

2.5 | Fertilisation Protocol

When plants showed signs of nutrient deficiency (e.g. yellowing leaves), a liquid fertiliser (CAPITO Flüssigunger universal, Dotzigen, Switzerland) was applied every 2 days during the vegetating and budding stages until the start of the experiment. This fertiliser contained: 7% total nitrogen, 1.75% nitric nitrogen (NS), 1.75% ammoniacal nitrogen (NA), 3.75% ureic nitrogen (NU), 3% soluble phosphate (P_2O_5) and 6% soluble potassium (K_2O). Micronutrients: 0.01% soluble boron (B), 0.002% soluble copper (Cu, EDTA-chelated), 0.02% soluble iron (Fe, EDTA-chelated), 0.01% soluble manganese (Mn, EDTA-chelated), 0.001% soluble molybdenum (Mo) and 0.002% soluble zinc (Zn, EDTA-chelated). The fertiliser was applied uniformly to all plants in both R^+ and R^- treatments to ensure consistent nutrient availability while avoiding interference with the rhizobia symbiosis.

2.6 | Larval Choice for Roots of R^+ or R^- Plants: Bioassay

We conducted a choice test to determine whether larvae of *D. balteata* were more attracted to R^+ or R^- roots. In a square Petri dish (12 × 12 cm), two virtual lines were set to divide the dish into three equal zones. The experiment was conducted in a controlled environment (L16:D8, $T = 24$°C). To maintain hydration and prevent desiccation, a moistened filter paper was placed at the bottom of the dish. On one side of the dish, 0.55 mg of rinsed R^+ roots were placed, while an equal biomass of rinsed R^- roots was positioned on the opposite side. The roots were weighed with a MX5 micro-balance (Mettler Toledo, Greifensee, Switzerland). A hole was made in the lid to release the larvae at an equidistant point between the root treatments. Five second-instar larvae were then placed in the centre of each dish. The dishes were sealed with parafilm to prevent escape and kept in the dark, as root-feeding larvae feed and develop belowground. Larval choice was recorded every 10 min during the first hour, then once per hour for the next 4 h, with a final observation 24 h after the start of the experiment. A total of 36 plants were used, resulting in 18 replicates for larval choice between roots of R^+ or R^- plants.

To investigate whether the larval choice was influenced directly by the presence of nodules, or by the quality of the roots containing the nodules, we conducted a follow-up experiment using the same setup. Square Petri dishes were again divided into three equal sections. In this experiment, we compared larval preference for detached nodules (N) vs. R^+ roots from which the nodules had been removed (R^+wn). Nodules were detached from the roots using a pair of fine forceps. Both roots and nodule samples were standardised to approximately 0.55 mg. Detached nodules were placed on one side of the Petri dish, and rinsed R^+wn roots were placed on the other side. Five second-instar larvae were then released at the centre of the dish. Larval choices were recorded following the same procedure as in the previous experiment. A total of 24 plants were used, resulting in 12 replicates for larval choice between nodules and R^+wn roots.

2.7 | Larval Choice for R^+ and R^- Roots: Olfactometer Assay

This experiment investigated whether volatiles emitted by R^+ and R^- roots influence larval attraction, using a soil olfactometer. Seeds were germinated as described previously and transplanted into cylindrical pots (11 × 4 cm) filled with vermiculite to minimise interference from other odour sources. Plants were inoculated with rhizobia or water (R^+ and R^- treatments, respectively), following the established protocol. After 3–4 weeks, plants were placed in the olfactometer system. The olfactometer, as outlined by Arce et al. (2021), consisted of two static L-shaped glass vases (5 × 11 cm) connected to glass tubes (24–29 mm × 8 cm), which were further linked to specialised funnels to prevent direct contact between larvae and plants. The glass tubes containing the 3–4-week-old plants were wrapped in aluminium foil, while dark plastic enclosed the connecting tubes to maintain darkness in the larval environment. Larvae were introduced to the system and their initial choice was recorded after 1 h, followed by observations every hour for 8 h, and then finally after 24 h. At each observation point, the glass tubes were briefly uncovered to record larval location. This experiment included 50 plants, resulting in 25 replicates for larval choice between R+ and R^- roots in the olfactometer.

2.8 | Volatile Collection and Analysis

For each treatment (R+ and R^-), 20 fresh roots were collected and washed in clean water. A 6 cm and an average of 0.480 g section of each root was cut, dried and weighted before being individually introduced into a 20 mL glass vial closed with a metallic cap with a silicon/PTFE septum. A solid phase microextraction (SPME) Arrow fibre (PDMS phase, 100 µm thickness, 1.1 mm diameter, 20 mm length, PAL System, CTC Analytics AG, Switzerland) was inserted into the vial to trap the volatiles released in the headspace. The sample was incubated for 3 min at 35°C. All the volatiles were analysed using an Agilent 8890 gas chromatograph (GC) coupled to an Agilent 5977B mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA). The fibre was thermally desorbed for 3 min in the GC front injector set at 250°C in pulsed splitless mode (total flow 64 mL min^{-1}, injection pulsed pressure 15 psi, helium

carrier gas). The volatile compounds were separated on a non-polar column (HP5-ms Ultra Inert; 30 m length, 0.25 mm internal diameter, 0.25 μm film thickness; J & W Scientific, Agilent Technologies). After injection, the column temperature was kept at 40°C for 1 min, then ramped up to 100°C at 6°C min^{-1} and then to 250°C at 3°C min^{-1} (hold 1 min) followed by a 2 min post run at 270°C. Helium at a constant flow mode of 1.1 mL min^{-1} was used as a carrier gas.

In all the analyses, the MSD transfer line temperature was set at 280°C and the ion source and quadrupole temperatures were set at 230°C and 150°C respectively. Electron impact (EI, ionisation potential 70 eV) mode was used with a scanning over the mass range of 33–205 *m*/*z*.

Compounds were tentatively identified by comparing their mass spectra with the NIST mass spectra library (NIST MS Search program version 2.3). For quantification, 1 μL of a 20 ng/μL (nanograms) of naphthalene (CAS# 91-20-3, Sigma-Aldrich) in dichloromethane (CAS# 75-09-2, Honeywell GmBH) was added to each sample as an internal standard before extraction. Volatile compound quantities were normalised to the internal standard and the fresh weight of root samples, expressed in ng/g (nanograms per gram) of fresh weight. For this experiment, we had 20 root samples for R^+ and 20 for R^-.

2.9 | Performance of *D. balteata* Larvae on R^+ and R^- Roots

This experiment evaluated the performance of *D. balteata* larvae when fed on either R^+ or R^- plant roots. Before the experiment, larvae were starved for 24 h by keeping them in rearing boxes without food. Five second instars (~ 10 days old) were then placed into 18 red-coloured Petri dishes, designed to simulate the dark soil environment (Jaccard et al. 2021). Each Petri dish contained approximately 0.55 g of plant material weighed using a MX5 micro-balance (Mettler Toledo, Greifensee, Switzerland), along with filtered soil (pore size = 1.5 mm, RICOTER Erdaufbereitung AG, Switzerland). The dishes were sealed with parafilm to prevent larvae from escaping and kept in the dark at room temperature for 48 h. The experiment compared larvae fed on 3–4-week-old roots from R^+ and R^- plants. To evaluate larval performance, the Relative Growth Rate (RGR) was calculated using the formula: $RGR = (\ln(W_2) - \ln(W_1))/t_2 - t_1$; where $W1$ and $W2$ are the initial and final larval masses (mg), and t_1 and t_2 are the start and end times of the experiment. For this experiment, we had 36 plants, consisting of 18 root samples for R^+ and 18 for R^-.

2.10 | Performance of *D. balteata* Larvae on Nodulated Root, Non-Nodulated Roots and Isolated Nodules

A second experiment investigated whether larvae performed differently on nodulated roots, roots without nodules or isolated nodules. Five-second instars were placed into round plastic boxes (250 mL, Ø: 11.7 cm, *h*: 4.3 cm) with small holes in the lids for ventilation. The bottoms of the boxes were filled with soil, and the larvae were left to feed for 48 h. Their masses were recorded at the start and end of the experiment to calculate RGR. The three following treatments were tested: Nodules only (N), roots from R^+ plants with nodules removed (R^+wn), and roots from R^- plants (R^-). Each box contained approximately 0.400 g of plant tissue, with 18 replicates per treatment. The plants used were 3–4 weeks old. For this experiment, we had 32 plants, consisting of 16 root samples for R^+ and 16 for R^-, with the third treatment consisting solely of nodules from the R^+ treatment.

2.11 | Adult Emergence From Larvae Fed on R^+ and R^- Roots

To evaluate adult emergence when larvae fed either on R^+ or R^- roots, a follow-up experiment was conducted using a similar set-up. Forty plastic boxes were filled with 3 cm of soil to provide a suitable environment for pupation. Twenty late second instars (14 days old) were introduced into each box. Every other day, larvae were fed with 1 g of rinsed R^+ roots with nodules or 1 g of rinsed R^- roots. The boxes were monitored daily for adult emergence, and the experiment continued until no more adults emerged (approximately 25 days after the start of the experiment). The total number of adults emerging from each treatment was recorded. For this experiment, there were 20 replicates for each treatment.

2.12 | Protein Content in R^+ and R^- Roots

To evaluate the nutritional quality of R^+ and R^- roots, the protein content of roots was analysed with 15 replicates per treatment. Root samples were ground into a fine powder using liquid nitrogen, and protein extraction was performed using the Pierce BCA protein assay kit (Nguyen et al. 2012), a high-precision, detergent-compatible method for determining protein concentration. Protein concentrations were measured using an Ultrospec 3100 Pro spectrophotometer. Results were expressed as protein concentrations per gram of fresh root weight. For this experiment, there were 15 replicates for each treatment.

2.13 | Statistical Analyses

All the statistical analyses were conducted using RStudio (version 2024.04.2+764). For the bioassay data assessing larval choice between R^+ and R^- roots, we employed a generalised linear model (GLM) with a binomial error distribution using the *glm* function in the stats package (R Core Team 2022), with time as a fixed effect. For the soil olfactometer experiment, a binomial GLM was also applied, with time and time as fixed effect. To analyse the volatile collection data, we used redundancy analysis (RDA). The proportion matrix, based on the proportion of each volatile relative to the total volatiles emitted was transformed with centred log-ratio (CLR) before the analysis. The RDA was performed with the *rda* function in the vegan package (Oksanen et al. 2005). Univariate comparisons of individual VOCs between treatments were performed using the Wilcoxon signed-rank test (*wilcox. test* function in the stats

package; R Core Team 2022). Total VOC emissions were calculated by summing the concentrations of all VOCs per plant (ng/g fresh plant). Since the data for the olfactometer assay was not normally distributed a GLM with a Gamma distribution and log link was applied, with treatment as the explanatory variable. Model significance, for the olfactometer assay, was tested using a Chi-squared test, then we performed post hoc test using Tukey's pairwise comparisons. To assess larval performance across different treatments, we used a linear model (LM). For adult emergence, a GLM with a binomial error distribution (using the *glm* function in stats; R Core Team 2024) was applied. The response variable was a two-column matrix comprising the number of emerged adults and the number of non-emerged adults. Treatment was included as the explanatory variable. A Cox Proportional Hazards regression model (*coxme* function in coxme; Therneau 2020) with frailty for box was applied to assess the impact of treatment on the timing of emergence, accounting for random variability between boxes. The model was fit using the coxme package to include both fixed effects for treatment and random effects for box. The significance of treatment on emergence timing was evaluated using the likelihood ratio test. Finally, protein content in roots was analysed with an independent samples *t*-test (*t*-test function in stats; R Core Team 2022).

3 | Results

3.1 | Choice of *D. balteata* Larvae Between Roots From R^+ or R^- Plants

D. balteata larvae showed a significant preference for R^+ roots compared to R^- roots ($\chi^2(1) = 76.21$, $p < 0.0001$). There was a significant treatment effect on larval attraction in 8 out of the 11-time measurements Figure 1A (Table S1). In contrast, larvae displayed a preference for R^+ roots without nodules over the nodules themselves ($\chi^2(1) = 59.84$ $p < 0.0001$). There was a significant effect of treatment on larval attraction in 9 out of the 11-time measurements Figure 1B (Table S2).

3.2 | Attraction of *D. balteata* Larvae to Volatiles Emitted by Roots of R^+ and R^- Plants

Seventeen volatile compounds were detected in the headspace of isolated bean roots with (R^+) and without (R^-) nodules. The total VOC emissions were significantly influenced by treatment ($\chi^2(1) = 6.29$, $p = 0.013$; Gamma GLM with log link). Roots from R^+ plants emitted significantly more VOCs than roots from R^- plants. The estimated difference in log-transformed VOC emissions between treatments was 1.15 (SE = 0.42, $z = 2.75$, $p = 0.013$). Enhanced VOC identifications (Table S4) were then carried out using the laboratory in-house library dedicated to plant volatiles (Table S3). Redundancy analysis revealed that volatile blend emitted by roots with nodules (R^+) is significantly different from that emitted by R^- roots. The overall model showed a significant difference in volatile blends emitted between R^+ and R^- roots ($F(1, 18) = 2.699$, $p = 0.006$). The total variance of the data set was 16.27, with the treatment effect accounting for 13.04% of the variation in volatile emissions. These findings indicate that the treatment significantly influences the composition of the emitted volatile blends (Figure 2A). The univariate analyses for each compound (Table S3) showed that (E)-2-hexenal (compound 2, $p = 0.0079$) was more abundant in the R^+ treatment. Other compounds such as hexanal (compound 1, $p = 0.096$), linalool (compound 5, $p = 0.0665$), and nonanal (compound 6, $p = 0.0956$) were marginally also showing a trend but however not significant (Table S3). Results from the olfactometer bioassay showed that *D. balteata* larvae were significantly more attracted to R^+ roots compared to R^- roots, even without visual stimuli ($\chi^2(1) = 447.3$, $p < 0.001$.). There was a significant effect of treatment on larval attraction all of the-time measurements (Figure 2B and Table S4).

3.3 | Performance of *D. balteata* Larvae on R^+Roots With and Without Nodules and on Nodules Alone

Larval relative growth rate (RGR) was significantly greater on R^+ roots compared to R^- roots ($F_{(2, 47)} = 34.23$, $p < 0.0001$, Figure 3A). When comparing larval growth on nodules vs. roots, larvae showed significantly higher RGR when fed only on nodules than on any other of the other three treatments ($F_{(3, 73)} = 34.9$, $p < 0.0001$, Figure 3B). In addition, protein content analysis of the roots showed a significant difference between R^+ and R^- roots. R^+ roots had a significantly higher protein content per unit of fresh root tissue (μg/mg) compared to R^- roots ($t_{(14)} = 11$, $p < 0.0001$) (Figure 3C).

A total of 230 adults emerged from the R^+ treatment roots, compared to 129 adults from R^- treatment roots. The analysis revealed a significant effect of treatment on adult emergence ($\chi 2(1) = 52.14$, $p < 0.0001$), with larvae fed on R^+ roots exhibiting a significantly higher emergence proportion (estimate = 1.045, $p < 0.0001$). However, treatment did not significantly influence the time to emergence when comparing larvae fed on R^+ and R^- roots ($z = -0.48$, $p = 0.634$), even after accounting for random variability among experimental boxes.

4 | Discussion

The symbiotic relationship between legumes and nitrogen-fixing bacteria strongly influences plant metabolism and ecological interactions shaping herbivore behaviour and performance. This study demonstrates that rhizobia can significantly impact herbivore–plant interactions by modifying the nutritional profile and volatile emissions of plant roots. Our findings reveal that *D. balteata* larvae prefer rhizobia-inoculated roots (R^+) over non-inoculated roots (R^-), and this preference is coupled with enhanced larval growth and performance on R^+ roots as shown in Figure 4.

The increased nutritional value of R^+ roots, as suggested by their higher protein content, may partially explain the improved larval performance observed in this study. However, it is important to note that high protein content does not necessarily equate to better nutrition, as some proteins may serve defensive

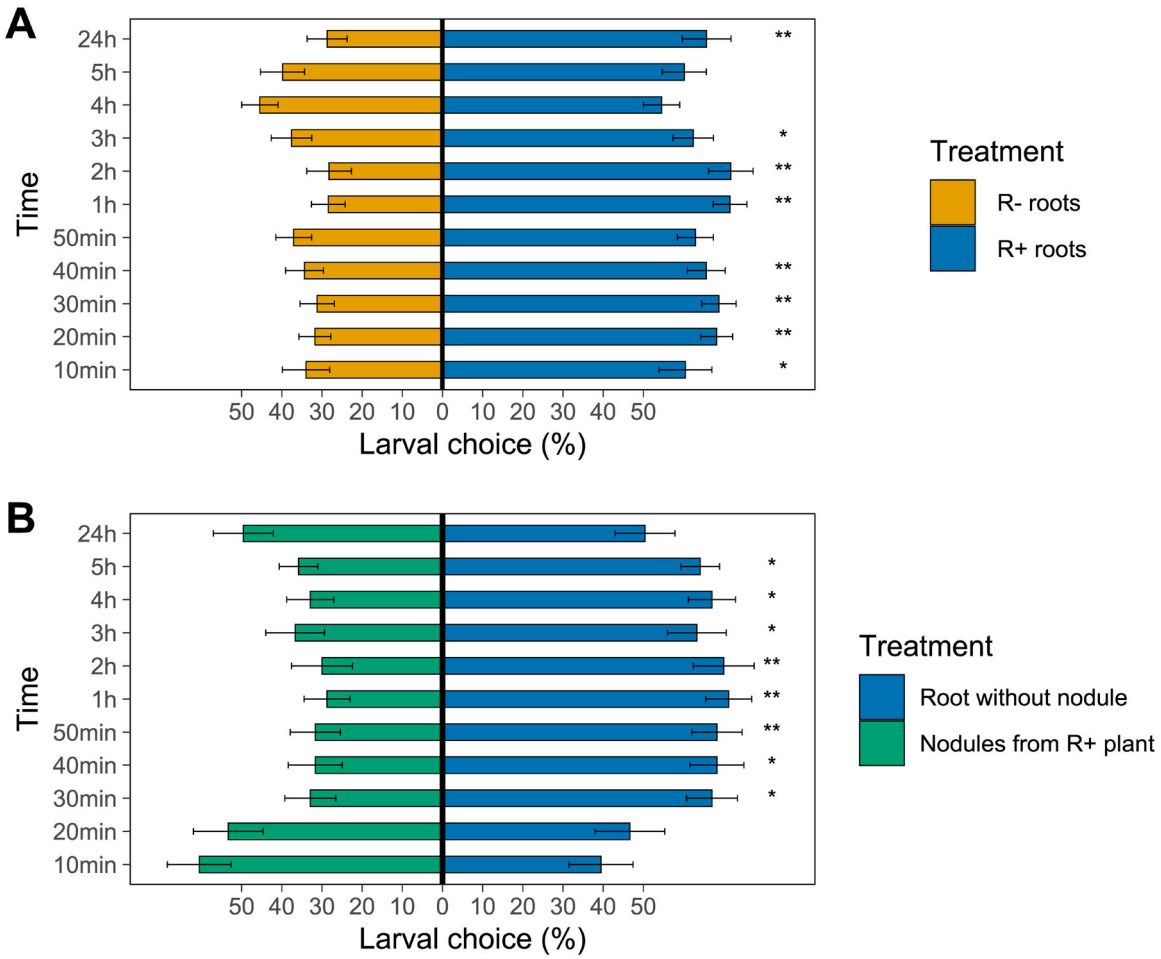

FIGURE 1 | Effect of rhizobia on the choice of *D. balteata* larvae. Shown are the mean percentages of larval choice based on the generalised linear model (GLM) with a binomial error distribution. (A) represents the choice test between R^+ ($n = 18$) and R^- ($n = 18$) roots, the percentage of non-choosing insects was 14.12% not showing a preference (B) represents the choice test between nodules and R^+ ($n = 12$) roots with the nodules removed ($n = 12$), where 4.55% of insects did not show a preference. Bars represent mean percentages of larval choice (± SE) at each time point (*y* axis). Asterisks indicate statistically significant differences between groups, with significance levels denoted as: $^*p < 0.05$, $^{**}p < 0.01$.

roles or may not be easily digestible for herbivores. Nitrogen fixation by rhizobia enriches plant tissues with nitrogen, which can facilitate larval growth. These findings align with previous studies showing that nitrogen-fixing bacteria can enhance herbivore performance by improving the nutritional quality of plant tissues (Bustos-Segura et al. 2024; Dean et al. 2014; Kempel et al. 2009). While the association between nitrogen-fixing rhizobia and plants enhances herbivore performance by improving plant nutritional quality, the broader ecological impact of symbiotic nitrogen fixation on trophic interactions remains complex. The dual role of rhizobia in promoting both plant growth and herbivore attraction highlights the potential trade-offs between mutualism and herbivory in legume systems.

Our findings demonstrate that rhizobia influence root volatile emissions, potentially serving as chemical cues for herbivores. As highlighted by Soto et al. (2021), rhizobia, best known for their role as nitrogen-fixing bacteria in leguminous plants, can alter VOC emissions, playing a significant role in interkingdom signalling, affecting nodulation, plant growth, and defence, with potential implications for herbivore attraction. In our study, R^+ roots emitted a distinct blend of VOCs compared to R^- roots, with overall higher emissions and differences in specific compounds, such as (E)-2-hexenal, which has been implicated in attracting Chrysomelid beetles to leguminous plants (Chen et al. 2023). These changes in VOC profiles may explain the observed larval preference for R^+ roots, aligning with previous studies showing that microbial symbionts can alter VOC profiles in ways that positively influence herbivore behaviour positively (Ballhorn et al. 2013; Bustos-Segura et al. 2024). However, the potential confounding effect of root biomass in the olfactometer experiments warrants further investigation. Future research should investigate the broader role of VOCs in root larval attraction using controlled biomass conditions or synthetic volatile blends to isolate specific effects.

A novel finding of this study is that *D. balteata* larvae directly feeding on nodules grow better than in any other diet. Nodules are highly nutritious structures, likely accounting for their exceptional suitability as a food source (Çakir et al. 2019; Legocki and Verma 1980; Samal et al. 2023). Similar interactions have been documented for other root-feeding insects, such as the weevil beetle *Sitona lepidus*, which feeds on nodules of leguminous crops and impact the plant's performance and the

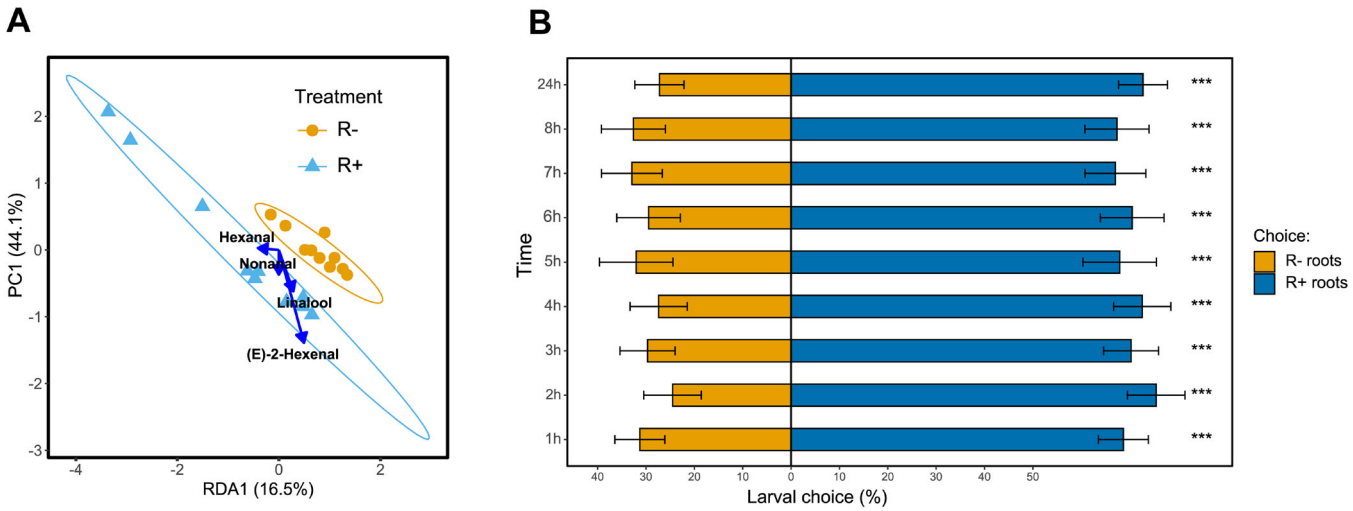

FIGURE 2 | Effects of rhizobia on VOCs emission and larval attraction in *D. balteata*. (A) Redundancy analysis (RDA) comparing the volatile composition emitted by roots of plants inoculated with rhizobia (R^+, blue ellipse and triangles, $n = 20$) versus non-inoculated plants (R^-, orange ellipse and circles, $n = 20$). Ellipses represent 95% confidence intervals for the point clouds. (B) Based on the binomial GLM bars represent the mean percentage of larval choice (± SEM) between R^+ ($n = 25$) and R^- ($n = 25$) over time, with 33.58% of insects not showing a preference. Asterisks indicate statistically significant differences, with significance levels denoted as: ***$p < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

symbiotic relationship with rhizobia (Johnson et al. 2004). Despite the apparent nutritional value advantage of nodules, larvae in our study still preferred R^+ roots without nodules. This suggests that the increased VOC emissions and nutritional value of R^+ roots drive larval preference. However, several factors may have influenced these outcomes in the olfactometer assay. For instance, the greater root mass in R^+ plants may have provided a larger surface area, and the lack of volatile emissions from the nodules compared to roots could have contributed to the observed effects.

Similar interactions with symbiotic organisms have been observed in other plant structures, such as mycorrhizas (Willsey et al. 2017). For instance, Zhang et al. (2022), found that arbuscular mycorrhizal fungi in *Elymus nutans* increased the production of defence enzymes and enhanced the release of VOCs that deterred *Locusta migratoria*. Rhizobia symbiosis can also influence plant chemistry, as seen in *Trifolium repens* (Kempel et al. 2009) and *Phaseolus lunatus* (Bustos-Segura et al. 2024). In plants with rhizobial symbionts, herbivores like *Spodoptera littoralis* benefit from improved tissue nutrition due to nitrogen fixation (Kempel et al. 2009). However, these benefits can be offset by plant defences. For example, nitrogen-based compounds, like cyanogenic compounds in lima bean and some strains of *Trifolium repens*, can reduce the advantage for herbivores (Kempel et al. 2009). In our study, the nitrogen enrichment from rhizobia not only enhanced plant tissue quality but also improved larval development, highlighting the important role of bacterial symbioses in herbivore–plant interactions.

The direct consumption of nodules has significant importance for plant performance, defence mechanisms, and the overall rhizobia symbiosis. Nodule damage may reduce nitrogen fixation efficiency, thereby affecting plant growth (Lindström and Mousavi 2020). In response to nodule herbivory, plants can activate defence mechanisms comparable to those triggered by foliar herbivores. For instance, Santamaria et al. (2013) demonstrated that plants activate highly specific inhibitors and oxidative stress responses to counter herbivore attacks, reflecting the interaction between direct and indirect defences. Although induced defences specific to nodules remain poorly understood, it is plausible that plants produce secondary metabolites or structural barriers to deter further feeding as shown by Xiao et al. (2019). In *Triadica sebifera*, herbivory triggers differential allocation of secondary metabolites and extrafloral nectar depending on the type of herbivory and whether the attack is below or aboveground. Additionally, nodule damage could elicit compensatory responses, such as increased nodule formation or enhanced rhizobia infection rates, to maintain nitrogen acquisition (Patra and Mandal 2022). Patra and Mandal (2022) showed that legumes can form nodules even without the typical Nod-factor signaling, suggesting alternative pathways for rhizobia infection and nodule development. This flexibility implies that plants may compensate for nodule damage by increasing nodulation or rhizobia infection. Exploring further these compensatory mechanisms could provide valuable insights into the resilience of legume-rhizobia symbiosis under herbivore pressure.

Our findings highlight the dual role of rhizobia in enhancing root nutritional quality while influencing plant defences. Nitrogen fixation enriches root tissues, supporting herbivore growth. While rhizobia-colonised plants are generally expected to enhance herbivore performance due to improved plant nutrition, they may also produce higher levels of defensive compounds, including VOCs, which can deter herbivores or attract natural enemies (Agrawal and Fishbein 2006; Basu et al. 2022). Interestingly, our results challenge with the traditional trade-off notion, where increased plant defence is thought to come at the expense of growth (Coley and Kursor 1996; Giolai and Laine 2024). Instead, we found that rhizobia-induced benefits to plants were associated with increased benefits to the belowground herbivore. While

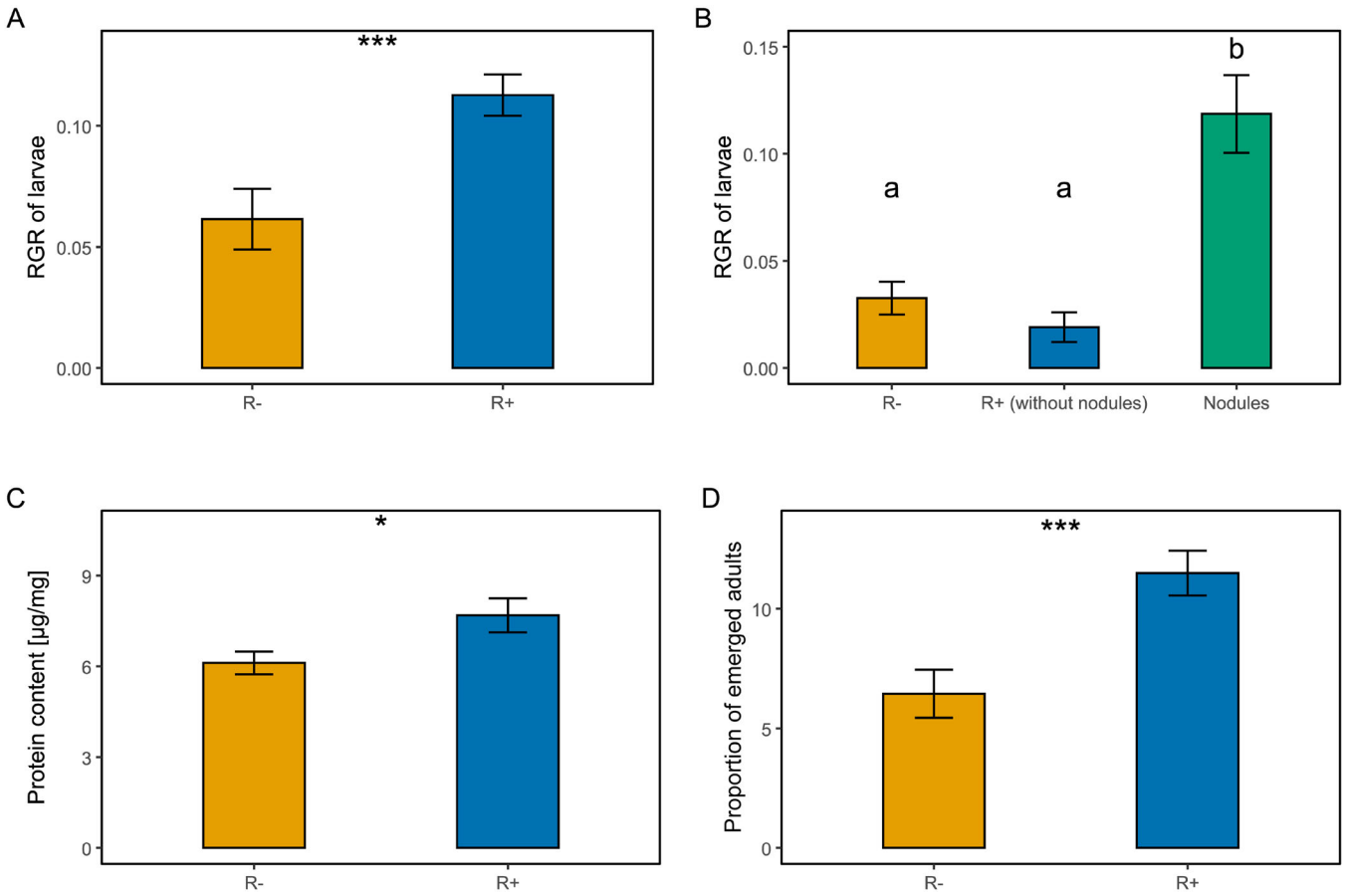

FIGURE 3 | Effects of rhizobia on the performance of *D. balteata* larvae. (A) Linear model (LM) depicts the mean relative growth rate (RGR) of *D. balteata* larvae on two diet treatments: R^- (without nodules, $n = 18$) and R^+ (roots with nodules, $n = 18$). Bars represent the mean RGR (± SE) after four days of feeding. (B) Linear model depicts the mean relative growth rate of larvae on three diet treatments: R^- (without nodules, $n = 16$), R^+ with nodules removed ($n = 16$) and N (nodules, $n = 16$). Bars represent the mean RGR (± SE) of larvae after four days of feeding. (C) Independent samples *t*-test depicts the mean protein content (µg/mg) (± SE) in roots from R^+ ($n = 15$) and R^- ($n = 15$) plants, (D) Generalised linear model (GLM) with binomial error distribution depicts the mean number of *D. balteata* adults emerged from larvae fed on R^+ roots and R^- roots. Asterisks indicate statistically significant differences. Asterisks indicate level of significance: $^*p < 0.05$ and $^{***}p < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

previous studies suggest rhizobia may enhance aboveground defences (van der Heijden et al. 1998; Kempel et al. 2009), our study does not find evidence for this enhanced defence in roots. Instead, the rhizobia–legume symbiosis appeared to optimise both nutrient enrichment and VOC emissions. However, the extent to which these traits outweigh herbivore attraction remains to be investigated.

Our findings have significant ecological and agricultural implications. The preference of *D. baleata* larvae for nodulated roots illustrates a potential trade-off in agricultural systems: while rhizobia inoculation enhances plant nutrition and productivity, it may also increase susceptibility to herbivory. Nevertheless, rhizobia's ability to modulate plant defences provides opportunities for developing integrated pest management strategies. Selecting rhizobial strains that optimise both plant nutrition and defence could mitigate herbivore damage while maintaining high crop yields (Ballhorn et al. 2013; Pineda et al. 2013). This approach aligns with sustainable agricultural practices that prioritise reduced reliance on synthetic fertilisers and improved soil health (Vessey 2003; Youseif et al. 2017).

Further research should explore the mechanisms driving the changes in plant–herbivore interactions changes mediated by rhizobia. Investigating how rhizobia interact with other belowground symbionts, like mycorrhizal fungi, could reveal potential synergies that enhance both plant growth and defence (Johnson and Rasmann 2015). Comparative studies across various legume species and herbivore types would help to determine whether these findings hold true across broader ecological contexts (Tao et al. 2017). Furthermore, molecular studies could shed light into the genetic and biochemical processes that shape these interactions (Ma et al. 2003).

In summary, this study highlights the intricate interactions between rhizobia–legume symbiosis and plant–herbivore dynamics. While rhizobia inoculation affects root nutrition and volatile emissions, which attract and improve the growth of *D. balteata* larvae, it also exposes a trade-off between enhanced plant growth and herbivore susceptibility. Understanding how rhizobia help plant defend themselves, can guide future research and farming practices that enhance crop growth and resilience, promoting sustainable agriculture.

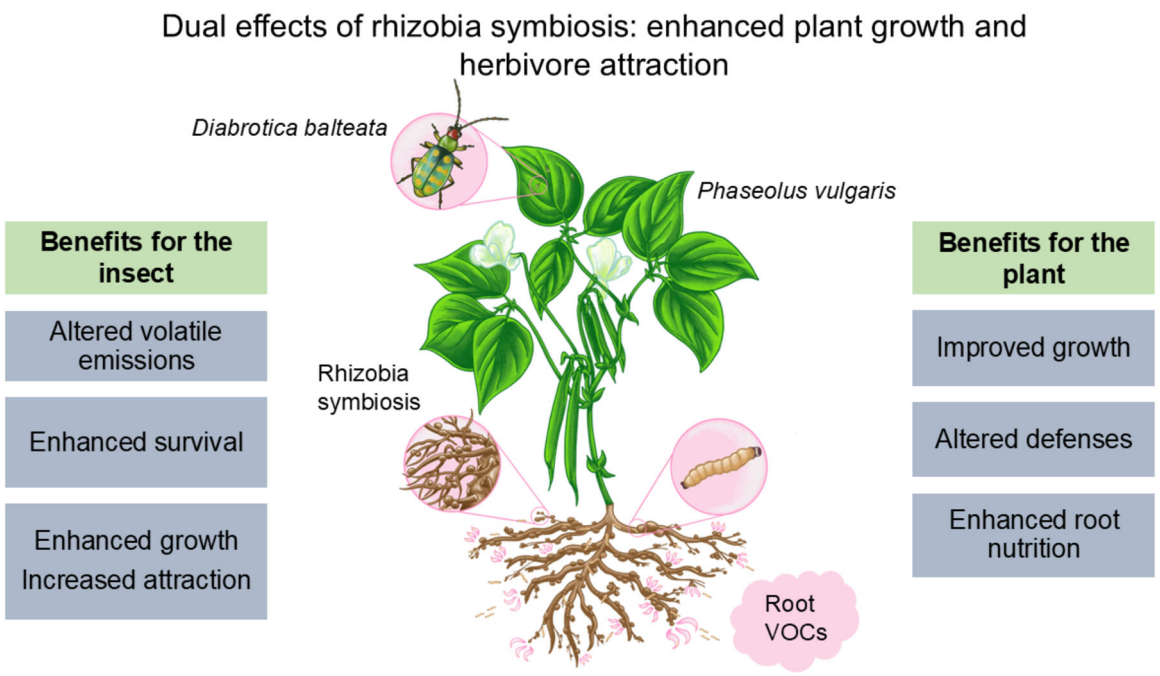

FIGURE 4 | Schematic representation of the effects of rhizobia symbiosis in *Phaseolus vulgaris* on both plant and herbivore outcomes. Rhizobia improve plant growth by enhancing root nutrition and altering plant defences, including volatile organic compound (VOC) emissions. However, these changes also benefit the herbivore *Diabrotica balteata*, as larvae exhibit increased attraction and growth, and adults show enhanced survival. This highlights a trade-off where improved plant nutrition and altered defences increase susceptibility to herbivory. [Color figure can be viewed at wileyonlinelibrary.com]

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/records/14987803?preview=1&token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjJjYTQ2YmQ3LTQzMmUtNDM5MS04ZDU4LWYxYmY1ZDdkZmRlZiIsImRhdGEiOnt9LCJyYW5kb20iOiI5NDk5N2RiN2ExNDhjYjM2Nzc3MmEzZDNmZmJmYzcxNiJ9.6sHGEOhRfSdh6LN_PeHOp2_7l93L_8fm0VU1EZWhV9AUJ5rPl-uRZR6xmStju5nRaM4GpL_6RR4ccrfqgYtWkA.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.