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RESEARCH PAPER

Legume plant defenses and nutrients mediate indirect interactions between soil rhizobia and chewing herbivores

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Abstract

Soil bacteria that associate with plant roots promote host vigor. Legume plants form mutualisms with rhizobial bacteria, and legumes grown with rhizobia have more nutrients and defenses than those grown without rhizobia. However, few studies have tested how stressors such as herbivores affect soil rhizobia, and the mechanisms mediating these interactions. Here we tested reciprocal interactions between a chewing herbivore, *Sitona lineatus* (pea leaf weevil), and *Pisum sativum* (pea) plants grown with or without rhizobia (*Rhizobium leguminosarum* biovar. *viciae)*, and the plant-defense and nutritional mechanisms mediating these interactions. We hypothesized that plants grown with rhizobia would have less feeding from *S. lineatus* due to greater expression of phytohormones or physical defenses. We also predicted that herbivory might impede the mutualism between *P. sativum* and rhizobia. Our experiments showed that leaf defoliation by *S. lineatus* was indeed lowest on plants with rhizobia. Plants grown with rhizobia had increased gene transcript expression associated with hormone-related defense (jasmonic acid, ethylene, abscisic acid) as well as physical and antioxidant-related defense, which may explain reduced feeding by *S. lineatus*. Conversely, *S. lineatus* feeding reduced the number of root nodules and nodule fresh weight, suggesting a disruption of the symbiosis between plants and rhizobia. Our study shows that aboveground herbivores can engage in mutually antagonistic interactions with soil microbes that are mediated through multiple plant-mediated pathways.

Keywords: Pea leaf weevil; Defense genes; Phytohormones; Physical defense; Plant nutrients

Introduction

Soil harbors abundant and diverse microbe communities that affect ecosystem services like biomass production, nutrient cycling, and carbon sequestration (A'Bear, Johnson & Jones, 2014; Bardgett & van der Putten, 2014). Plant-root associated bacteria such as rhizobia can also affect plant susceptibility to herbivores and pathogens by altering plant nutrients or defense (Blundell et al., 2020; Rashid & Chung, 2017). Similarly, herbivores and pathogens that attack plant hosts aboveground can disrupt plant-microbe mutualisms in the soil, reducing nitrogen fixation and weakening plant defense (Ballhorn, Younginger & Kautz, 2014; Simonsen &

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Stinchcombe, 2014). A key emerging priority in food web ecology is thus to better understand the mechanisms that mediate plant-mediated indirect interactions between soil microbes and aboveground biotic stressors such as herbivores and pathogens (A'Bear et al., 2014; de Vries & Wallenstein, 2017).

The symbiosis between legumes and soil rhizobia is a highly specialized mutualism in the Fabaceae family (Gopalakrishnan et al., 2015; Wang, Yang, Tang & Zhu, 2011). Soil rhizobial bacteria form nodules on roots that aid legume growth and nitrogen fixation (Gopalakrishnan et al., 2015; Jaiswal, Mohammed, Ibny & Dakora, 2021). Legumes grown in soil with rhizobia are often less susceptible to herbivores, as physical defenses like callose and antioxidants are induced, compared to plants without rhizobia (Millet et al., 2010; Rashid & Chung, 2017). Inoculation of rhizobia also promotes synthesis and release of phytohormones like gibberellins, jasmonic acid, ethylene, and brassinosteroids, as well as volatile organic compounds, that directly or indirectly enhance plant defense (Jaiswal et al., 2021; Rasmann, Bennett, Biere, Karley & Guerrieri, 2017; Tao, Hunter & de Roode, 2017). Yet, there is a considerable need to better understand how biotic stressors such as herbivores and pathogens attacking legume plant shoots and leaves may affect symbiosis between plants and rhizobial bacteria in the soil.

Herbivores affect soil microbes directly if they feed on them, such as when larvae of the pea leaf weevil (*Sitona lineatus*) consume root nodules containing rhizobia (Carcamo, Herle & Lupwaya, 2015). However, most interactions between soil microbes and herbivores feeding on plants are indirect. As plant-rhizobia symbiosis often promotes plant defense against stressors, herbivores and pathogens may benefit from an interruption of plant-rhizobia symbiosis (Pineda, Zheng, van Loon, Pieterse & Dicke, 2010; Shikano, Rosa, Tan & Felton, 2017). For example, viruses transmitted by aphids can be highly antagonistic to plant-rhizobia symbiosis, as infected plants have limited nodule formation; pathogens also impede signaling pathways that directly affect nodule formation (Base et al., 2021b). However, few studies have assessed reciprocal interactions between herbivores or pathogens aboveground and rhizobia in the soil.

Here we addressed mechanisms mediating indirect interactions between a legume host (*P. sativum*), soil rhizobia (*Rhizobium leguminosarum),* and a chewing herbivore (*S. lineatus).* In the Palouse region of northern Idaho and eastern Washington, USA, these organisms commonly co-occur in natural and managed ecosystems (Basu, Clark, Bera, Casteel & Crowder, 2021a; Chisholm, Eigenbrode, Clark, Basu & Crowder, 2019). However, it is largely unknown if *S. lineatus* are affected by soil rhizobia, or whether herbivory from *S. lineatus* affects rhizobia. We predicted that plants grown in soil with rhizobia would have greater defense induction and nutrients, which may decrease defoliation by *S. lineatus*. In contrast, we predicted feeding by *S. lineatus* would inhibit symbiosis between *P. sativum* and rhizobia. We combined greenhouse experiments with molecular assays to test these hypotheses and assess the mechanisms underlying interactions between *S. lineatus*, soil rhizobia, and *P. sativum* hosts. Our results reveal complex mechanisms by which aboveground biotic stressors can indirectly interact with soil microbes belowground.

Materials and methods

Study system and experimental conditions

Many legumes such as *P. sativum* are found in the Palouse, where they are attacked by pathogens and chewing herbivores like *S. lineatus* (Basu et al., 2021a; Chisholm et al., 2019). *Sitona lineatus* adults overwinter in weedy hosts and migrate into legume crop fields in the late spring to lay eggs; after eggs hatch, larvae burrow into soil to feed and pupate before adults re-emerge (Chisholm et al., 2019). As *S. lineatus* larvae feed on legume roots belowground, directly affecting rhizobia abundance, *S. lineatus* adults feed aboveground and indirectly interfere with legume-rhizobia symbiosis. Given the relative ease of working with adults compared to larvae, and our focus on plant-mediated mechanisms, we assessed effects of adults rather than larvae.

Adult *S. lineatus* were collected from pea fields one week prior to experiments, and field-collected soil was taken from the Palouse Conservation Farm (Pullman, WA, USA). Experiments were conducted in greenhouses at Washington State University (Pullman, WA, USA) with a 16:8 h light: dark cycle, with 21−24 °C during light cycles and 16−18 °C during dark cycles.

Effects of rhizobia on *S. lineatus* feeding

We assessed effects of rhizobia on *S. lineatus* with three treatments: (i) no treatment of soil or seeds; (ii) soil autoclaved to remove microbes and seeds untreated; and (iii) soil autoclaved and seeds inoculated with rhizobia. Untreated soil was soil taken directly from the field site. For autoclaving soil, field-collected soil was placed in 61 × 91 cm bags in a steam autoclave at 7 psi and 111°C overnight. All soil was standardized to 75% moisture after treatment.

To establish rhizobia treatments, pea seeds were treated with an inoculation of pea-specific rhizobia (*Rhizobium leguminosarum* biovar. *viciae)* by mixing N-Dure^{R}, a peat-based inoculant with *P. sativum* seeds using the manufacturer's protocol (Verdasian Life Sciences, Cary, NC). The inoculum concentration was 2×10^8 colony forming units per g of seed. Seeds grown with no rhizobia were mixed in water. After seeds had been treated, individual seeds were grown in sheet pots (50 cm^2) in potting mix (Sunshine® LC1) for 2 weeks to allow them to form a root complex before they were transplanted to field soil; our prior work shows this promotes establishment.

After 2 weeks, plants were transplanted to 1 L pots with field-collected soil with the proper treatment and placed in mesh cages (0.6 × 0.3 × 0.3 m) for 2 weeks before *S. lineatus* treatments. There were two *S. lineatus* treatments: (i) none and (ii) two adult *S. lineatus* feeding for 48 h, after which they were removed. The experiment was a 3 × 2 factorial, with 3 soil/seed and 2 *S. lineatus* treatments; each was replicated 10 times per block with two temporal blocks. There was a total of 120 experimental units (2 blocks × 3 soil/seed × 2 *S. lineatus* × 10 replicates). In each replicate, the total leaf notches were counted by visual observation of the above-ground portion of plants. Leaf notches are a reliable indicator of the amount of *S. lineatus* feeding (Chisholm, Sertsuvalkul, Casteel, & Crowder, 2018). Following the experiment, plants were uprooted from the soil after 7 d, soil was washed off roots with tap water, and the root nodules were counted.

Analyses of amino acids

We measured amino acid content of *S. lineatus* adults from different treatments to assess herbivore nutrient acquisition using methods priorly used for piercing-sucking insects (Basu et al., 2021a; Guo et al., 2019). This analysis was designed to assess whether short-term feeding on different plants affected adult amino acid levels. Two adult *S. lineatus* were collected from each replicate of the feeding experiment (4 replicates per treatment) into liquid N_2 and lyophilized. After lyophilization, *S. lineatus* tissue was weighed and extracted with 20 mM of HCL (Patton, Bak, Sayre, Heck, & Casteel, 2020). Amino acids were derivatized using AccQ-Fluor kits (Waters, Milford, MA), with L-Norleucine as a standard. 10 μl from each sample were injected into an Agilent 1260 Infinity HPLC (Agilent, Santa Clara, CA) with a Nova-Pak C18 column.

Amino acid derivatives were detected with excitation and emission wavelengths of 250 nm and 395 nm, respectively. Peak areas were compared to a standard curve made from a serial dilution of amino acid standards (Sigma-Aldrich, St. Louis, MO). Solvent A, AccQ−Tag Eluent A, was premixed from water; Solvent B was acetonitrile:water (60:40). The gradient used was 0−0.01 min, 100% A; 0.01−0.5 min, linear gradient to 3% B; 0.5−12 min, linear gradient to 5% B; 12−15 min, linear gradient to 8% B; 15−45 min, 35% B; 45−49 min, linear gradient to 35% B; 50−60 min, 100% B. The flow rate was 1.0 ml min^{-1}. Amino acid derivatives and peak areas were measured with an Agilent fluorescence detector and ChemStation software. To calculate concentrations, standard curves were created for each amino acid using dilutions of standards.

Effects of *S. lineatus* on soil rhizobia

We next assessed how *S. lineatus* feeding affected nodulation, which indicates the function of rhizobia. There were two *S. lineatus* (present or absent) and two rhizobia (present or absent) treatments. *Pisum sativum* plants were treated with rhizobia by mixing them with an inoculation of pea-specific rhizobia as described previously or were left untreated. Treated or untreated seeds were grown in an autoclaved soil mix consisting of equal volume of Sunshine Mix LC1 potting soil and sand (1:1) to facilitate nodule development. For autoclaving, the mix was placed in 61 × 91 cm bags in a steam autoclave at 7 psi and 111 °C overnight to eliminate microbial load. These treatments were crossed with the *S. lineatus* treatments (present or absent). For treatments with *S. lineatus*, we released two adults for 48 h on 2 wk old plants, after which they were removed. Following treatments, plants were uprooted after 7 days, and soil was washed off roots with tap water. Nodules were counted from each plant and then excised. Nodule fresh weights were taken and then dried for 5 d at 37 °C before dry weight measurements were taken. Plants failed to develop any root nodules in the autoclaved soil when seeds were not inoculated with rhizobia.

Analyses of transcripts related to defense signaling

We conducted a separate experiment to assess mechanisms mediating interactions between soil rhizobia and weevils. This experiment was set up similarly to the experiment testing effects of weevils on rhizobia, but had two end points (3 and 7 d) to allow for analysis of changing plant signals. There were two *S. lineatus* treatments (present or absent) and two rhizobia treatments (present or absent). *Pisum sativum* plants were either treated with rhizobia by mixing with an inoculation of pea-specific rhizobia as described previously or untreated. Treated or untreated seeds were grown in an autoclaved potting mix with an equal volume (1:1) of potting soil and sand. For autoclaving, the mix was placed in 61 × 91 cm bags in a steam autoclave at 7 psi and 111 °C overnight. These treatments were crossed with the two *S. lineatus* treatments (present or absent). For treatments with *S. lineatus*, we released two adults for 48 h on plants, after which they were removed. Plant tissue was harvested 3 and 7 d after *S. lineatus* were removed. The experiment included four randomly assigned replicates of each treatment for two temporal blocks in a 2 × 2 × 2 factorial (2 soils × 2 *S. lineatus* × 2 times × 4 replicates = 32 experimental units).

Aboveground plant tissue was wrapped in aluminum foil, frozen in liquid N_2, and kept on dry ice before storing in −80 °C. Samples were ground using a mortar and pestle in liquid N_2, and 50 to 100 mg of tissue was used for total RNA extraction using Promega SV total RNA isolation kits (Promega, Madison, WI) and cDNA from 1 μg of total RNA using Bio-Rad iScript cDNA kits. These gene-specific primers (Appendix A: Table A1) were used in qRT-PCR reactions (10 μl) containing 3 μl of ddH2O, 5 μl of iTaq Univer SYBR Green Supermix, 1 μl of primer mix (forward and reverse), and 1 μl of diluted (1:25) cDNA template. The

qRT-PCR program had an initial denaturation for 3 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. For melting curve analysis, a dissociation step cycle was used (55 °C for 10 s, and then 0.5 °C for 10 s until 95 °C). The relative expression of genes was calculated using the delta-delta Ct method, ($2^{-\Delta\Delta Ct}$) with Psβ-tubulin as a housekeeping gene (Kozera & Rapacz, 2013; Livak & Schmittgen, 2001).

Harvested plant tissue was assessed for expression of 14 gene transcripts associated with hormone signaling, physical, or antioxidant defense (Fondevilla, Küster, Krajinski, Cubero & Rubiales, 2011; Kimura & Kawano, 2015; Tran, You & Barbetti, 2018). Gene sequences were obtained using accession numbers of genes in the pea marker database (Kulaeva et al., 2017) and searching the pea genome (Kreplak et al., 2019). We assessed 7 genes related to phytohormones across multiple signaling pathways, as feeding from *S. lineatus* on *P. sativum* has been shown to affect gene transcripts associated with salicylic acid, jasmonic acid, abscisic acid, and gibberellic acid as well as genes associated with antimicrobial peptides and lectin Basu et al. (2021a). The genes *Pathogenesis-related protein 1* (*PR1*) and *Isochorismate synthase1* (*ICS1*) are associated with salicylic acid, with *ICS1* acting upstream of salicylic acid biosynthesis and *PR1* triggering downstream systemic acquired defenses (Fondevilla et al., 2011; Zhang et al., 2010). Two genes, *Lipoxygenase 2* (*LOX2*) and *12-oxophytodienoate reductases 3* (*OPR3*) function upstream and downstream of jasmonic acid synthesis, respectively (Fondevilla et al., 2011; He, Fukushige, Hildebrand & Gan, 2002; Wasternack & Hause, 2013). Other genes were *1-aminocyclopropane-1-carboxylic acid synthases 2* (*ACS2*), which is associated with ethylene biosynthesis, and *Aldehyde oxidase* 3 (*AO3*), which catalyzes abscisic acid biosynthesis. Beside abscisic acid biosynthesis, *AO3* also affects production of reactive oxygen species (Yergaliyev et al., 2016).

We also assessed transcript expression of two genes related to physical defense: (i) viz. *β−1,3 Glucanase*, an enzyme that regulates callose and (ii) *calcium-regulated/ATP-independent ferisome protein gene*, which affects P protein plugs that seal phloem (Srivastava, Tuteja & Tuteja, 2015; Zavaliev, Ueki, Epel & Citovsky, 2011). Six additional genes for antioxidant related defense were assessed: 3 S*uper Oxide Dismutases* (*FeSOD, CuZnSOD, MnSOD*), *Catalase* and *Glutathione reductase 1(GR1*), and *Peroxidase* (*PsPOX11*) (Fondevilla et al., 2011; Tran et al., 2018). Induction of these defenses can catalyze superoxides (reactive oxygen species) in plants (Kimura & Kawano, 2015) and affect induction of salicylic acid in peas (Kawahara et al., 2006).

Data analysis

Analyses were done in R 4.0.5 (R Core Team, 2021). We used a generalized linear model (GLM) with a negative binomial distribution to assess how soil treatments affected *S. lineatus* feeding notches; negative controls without *S. lineatus* were excluded. We also used a GLM with a negative binomial distribution to assess if soil treatments affected the number of soil nodules; treatments with *S. lineatus* were excluded. We used GLMs with a negative binomial distribution to assess if *S. lineatus* affected the number of nodules, and GLMs with a gaussian distribution to assess effects of *S. lineatus* on nodule weight. Negative binomial distributions were used in GLMs to account for overdispersion. We tested effects of soil rhizobia and *S. lineatus* treatments, and their interaction, on gene expression using MANOVA (multiple analysis of variance) on delta CT values ($2^{-\Delta\Delta CT}$ values) for relative transcript abundance for 14 different genes: *PR1, ICS1, OPR3, LOX2, AO3, ACS2, β−1,3 Glucanase, Calcium-regulated/ATP-independent ferisome protein gene, CuZnSOD, FeSOD, MnSOD, Catalase, GR1*, and *PsPOX11*. MANOVA was used because gene responses were measured from the same plants and may have a correlated response to treatment. Parameter estimates and subsequent calculations for delta-delta CT ($2^{-\Delta\Delta Ct}$) were plotted on the log 10 scale. Finally, average amino acid content for 13 amino acids was fit to a linear mixed model (lme4 package, Bates, Maechler, Bolker & Walker, 2015), with soil treatment as a fixed effect and amino acid as a random effect. To test effects on specific amino acids, we also fit concentrations of individual amino acids to separate linear models with soil treatment as a fixed effect. For both analyses, amino acid concentrations were log-transformed. Estimated marginal means and all post-hoc tests were assessed using the emmeans package (Lenth, 2016), with significance tests via analysis of deviance tables generated using the car package (Fox & Weisberg, 2011).

Results

Effects of soil rhizobia on *S. lineatus* feeding and amino acid uptake

Soil and seed treatments affected the number of plant root nodules ($Z = 20.78$; $P < 0.001$; Appendix A: Fig. A1). Seeds not treated with rhizobia produced zero nodules in autoclaved soil, and relatively few nodules in untreated field soil (mean = 3.65 ± 0.69), but seeds inoculated with rhizobia and grown in autoclaved soil produced over 50 nodules per plant (mean = 53.4 ± 2.3). Rhizobia inoculation also altered *S. lineatus* feeding ($\chi 2 = 39.4$, $P < 0.001$). Pea plants that were untreated and grown in autoclaved soil had the most feeding notches, while pea plants inoculated with rhizobia and grown in autoclaved soil had the least (Fig. 1A). Plants that were untreated and grown in untreated field-collected soil had an intermediate number of feeding notches. Soil and seed treatment, however, did not affect total amino acid concentrations in *S. lineatus* ($\chi^2 = 1.66$, df = 2, $P = 0.44$, Fig. 1B, Appendix A: Fig. A2) nor concentrations of amino acids excluding

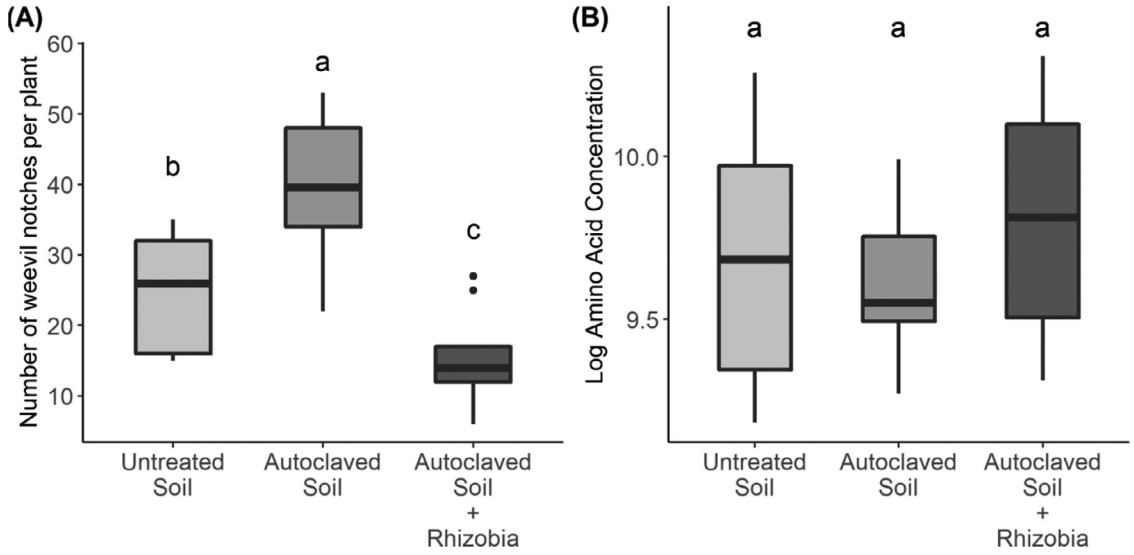

Fig. 1. Effects of soil and seed treatments on (A) the number of feeding notches on plants and (B) log-transformed concentrations (nmol/mg dry weight) of amino acids in *S. lineatus*. Box plots show the mean and quartiles for each metric, and boxes not connected with the same letter were significantly different in the GLMs ($P < 0.05$, Tukey HSD).

Proline, which was highest in weevils from plants that were inoculated with rhizobia and grown in autoclaved soil ($t_{27} = 3.22$, $P = 0.003$, Appendix A: Table A2).

Effects of *S. lineatus* herbivory on soil rhizobia

Herbivory from *S. lineatus* had a negative effect on symbiosis between rhizobia and plant hosts (Fig. 2, Appendix A: Fig. A3). Plants that were fed on by *S. lineatus* had slightly fewer plant root nodules ($\chi 2 = 3.25$, $P = 0.071$, Fig. 2A), and lower nodule fresh weight ($\chi 2 = 9.41$, $P = 0.002$, Fig. 2B) than plants that did not experience any herbivory. However, treatments with *S. lineatus* did not significantly affect plant root nodule dry weight ($\chi 2 = 2.46$, $P = 0.12$, Fig. 2C).

Effects of *S. lineatus* and soil rhizobia on expression of defense gene transcripts

Sitona lineatus or rhizobia did not strongly affect one gene transcript related to salicylic acid, *PR1*, but did affect the other, *ICS1* ($Z = 2.14$, $P = 0.033$) (Fig. 3A, B; Appendix A: Table A3). Specifically, plants attacked by *S. lineatus* had higher *ICS1* levels when they were grown without rhizobia compared to those grown with rhizobia (Fig. 3B). Plants grown with rhizobia but not attacked by *S. lineatus* had higher expression of transcripts related to jasmonic acid, *LOX2* and *OP3*, compared to plants grown with rhizobia that were attacked by *S. lineatus* ($Z = 2.00$, $P = 0.045$, Fig. 3C; $Z = 2.69$, $P = 0.009$, Fig. 3D; Appendix A: Table A3). Plants grown with rhizobia had higher expression of the gene transcript associated with ethylene, *ACS2*, compared to plants without rhizobia, with or without *S. lineatus* ($Z = 3.95$, $P < 0.001$; $Z = 4.11$, $P < 0.001$; Fig. 3E; Appendix A: Table A3). The gene transcript associated with abscisic acid, *AO3*, had a complex response to rhizobia and weevils ($Z = 2.85$, $P = 0.004$, Fig. 3F; Appendix A: Table A3). *AO3* levels were highest when only rhizobia or *S. lineatus* were present, and lowest when neither or both were present (Fig. 3F). Soil rhizobia also seemed to induce *β−1,3 glucanase,* associated with callose, but only when *S. lineatus* was not present ($Z = 2.95$, $P = 0.003$, Fig. 4A, Appendix: Fig. A5, Table A4), while expression of the Ca-regulated ATP independent ferisome protein gene was unresponsive to any treatment (Fig. 4B, Appendix: Fig. A5, Table A4).

Sitona lineatus feeding induced the antioxidant-related gene transcript, *Catalase,* on plants grown with no rhizobia compared to untreated plants ($Z = 2.18$, $P = 0.029$, Fig. 5A; Appendix A: Fig. A4, Table A5). Another antioxidant-related gene transcript, *GR1*, was not affected by *S. lineatus* or rhizobia (Fig. 5B, Appendix A: Fig. A4, Table A5), but three gene transcripts associated with the superoxidase disumaste (*CuZnSOD, MnSOD, FeSOD*) had greater expression in plants grown with rhizobia compared to plants grown without rhizobia ($Z = 2.16$, $P = 0.031$, Fig. 5C; $Z = 2.77$, $P = 0.006$, Fig. 5D; $Z = 3.44$, $P < 0.001$, Fig. 5E, respectively; Appendix A: Fig. A4, Table A5). *Sitona lineatus* feeding also induced the gene transcript peroxidase (*PsPOX11*) on plants grown with rhizobia compared to plants grown with rhizobia but no *S. lineatus* feeding ($Z = 2.02$, $P = 0.044$, Fig. 5F, Appendix A: Fig. A4, Table A5).

Discussion

Herbivores and soil microbes interact both directly and indirectly, and our study highlights mechanisms that

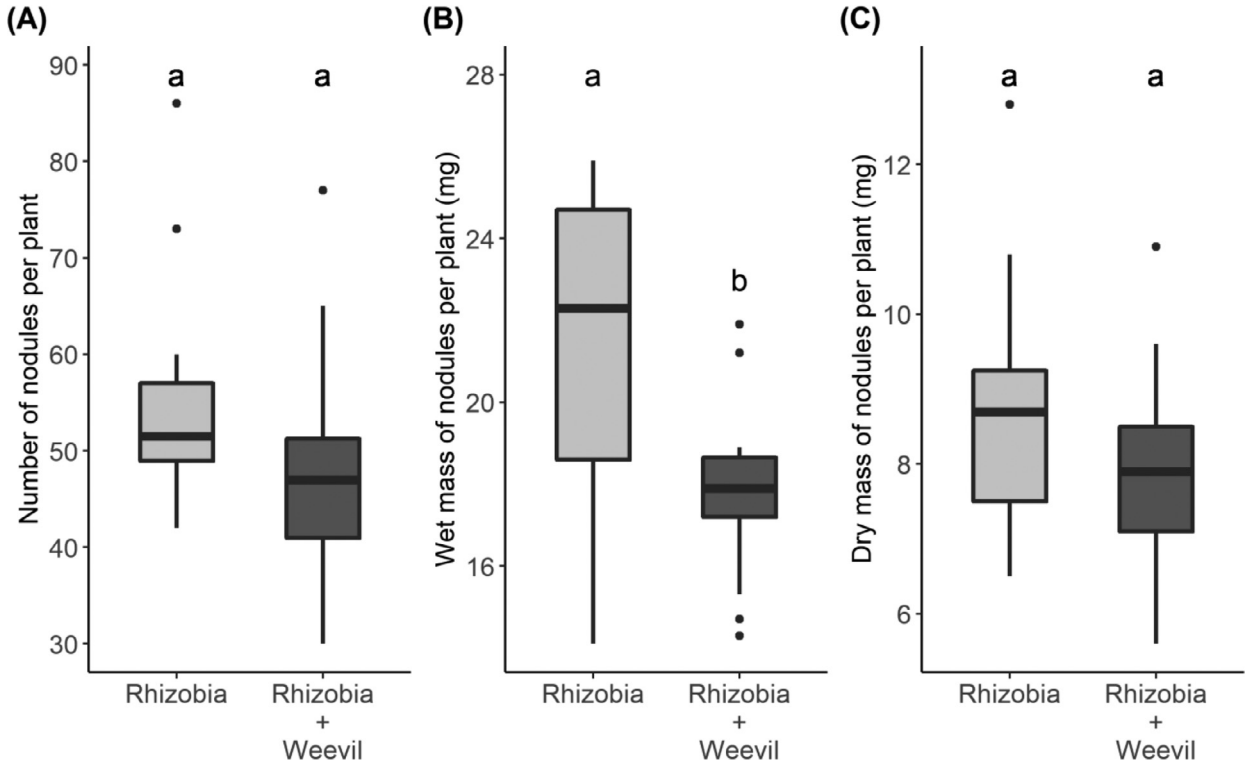

Fig. 2. Effects of *S. lineatus* on (A) the number of root nodules, (B) root nodule wet mass, and (C) root nodule dry mass. Box plots show the mean and quartiles for each metric, and boxes not connected with the same letter were significantly different in the GLMs ($P < 0.05$, Tukey HSD).

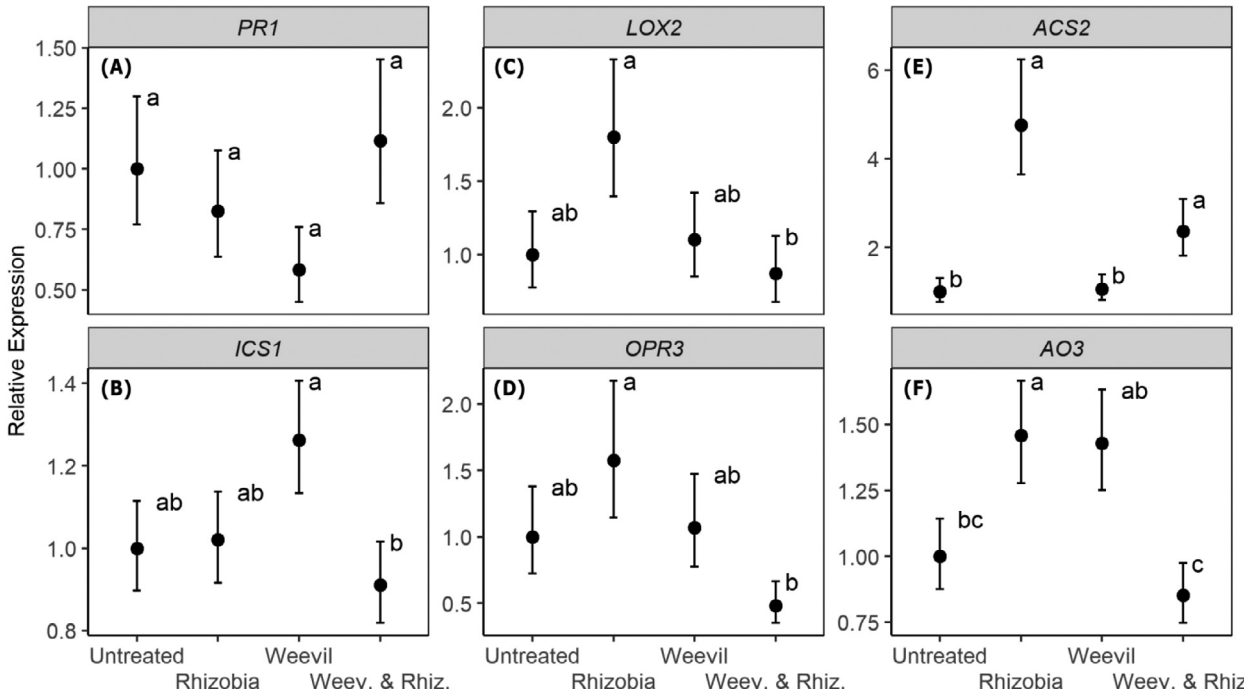

Fig. 3. Effects of rhizobia and *S. lineatus* on gene transcript accumulation of (A, B) salicylic acid responsive genes *PR1* and *ICS1*, (C, D) jasmonic acid responsive genes *LOX2* and *OPR3*, (E) ethylene responsive gene *ACS2*, and (F) abscisic acid responsive gene *AO3* in *P. sativum* at 7 days post infection. Shown are the mean and 95% confidence interval, and points not connected with the same letter were significantly different in GLMs ($P < 0.05$, Tukey HSD).

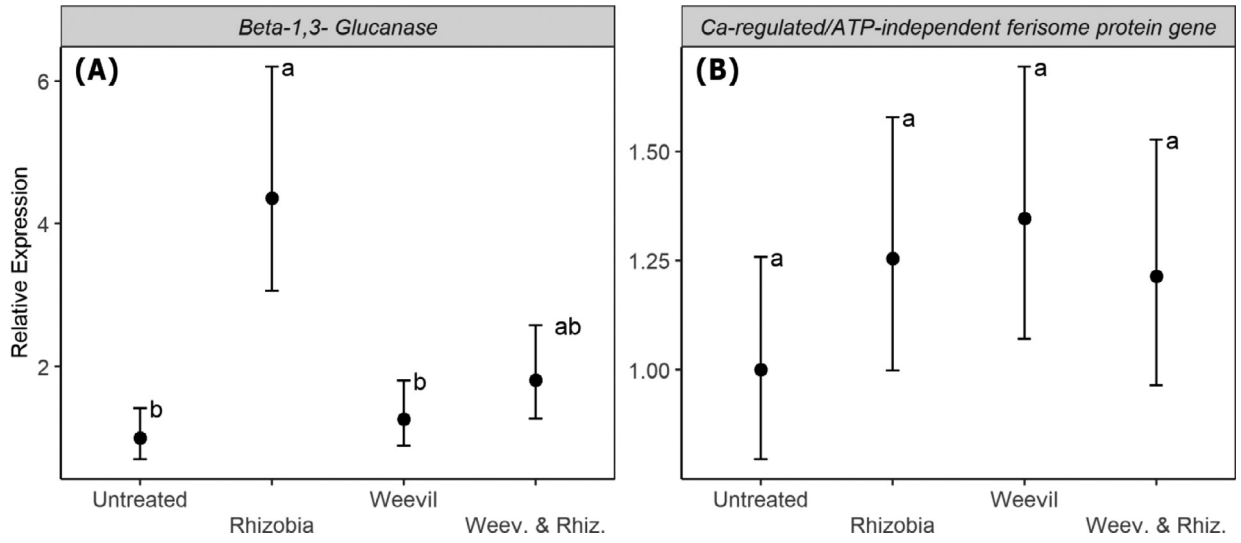

Fig. 4. Effects of rhizobia and *S. lineatus* on gene transcript accumulation of callose-mediated genes (A) *Beta-1, 3 glucanase* and (B) *Calcium-regulated/ATP-independent ferisome protein gene* in *P. sativum* 7 days post infection. Shown are the mean and 95% confidence intervals, and points with different letters were significantly different in GLMs ($P < 0.05$, Tukey HSD).

mediate such interactions. Our results validate studies showing rhizobia and arbuscular mycorrhizal fungi are keystone microbes that can decrease plant susceptibility to herbivores (Gopalakrishnan et al., 2015; Santos et al., 2014; Yang et al., 2014). We show plants grown with rhizobia inoculation had considerably less feeding than plants grown in untreated field-collected soil. This suggests a sufficient density of rhizobia in soil may be necessary to produce a change in plant traits that leads to a reduction in herbivore feeding. We also showed that *S. lineatus* disrupted symbiosis between *P. sativum* and rhizobia. *Sitona lineatus* reduced the number and size of nodules despite causing the fewest feeding notches on plants with rhizobia, suggesting *S. lineatus* feeding disrupted rhizobia-plant symbiosis without lowering plant biomass aboveground. Our results provide further evidence that herbivores may

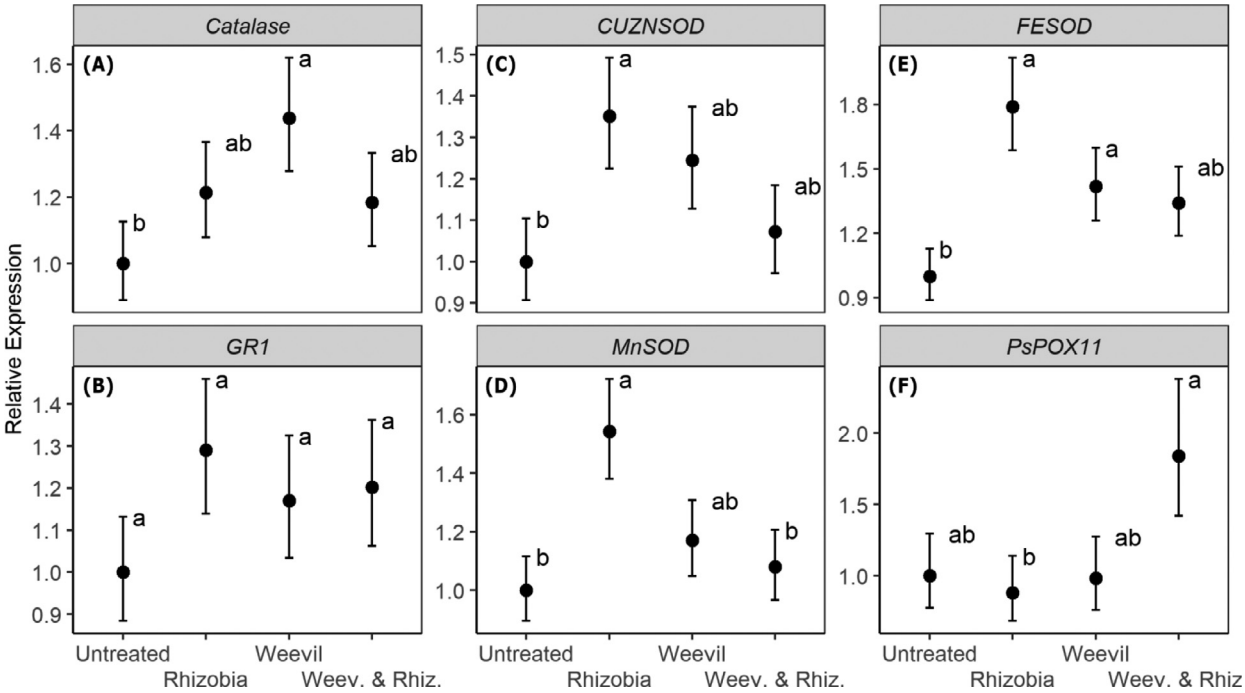

Fig. 5. Effects of rhizobia and *S. lineatus* on gene transcript accumulation of antioxidant-related genes (A) *Catalase*, (B) *GR1*, (C) *CuZnSOD*, (D) *MnSOD,* (E) *FeSOD*, and (F) *PsPOX11* in *P. sativum* at 7 days post infection. Shown are the mean and 95% confidence intervals, and points with different letters were significantly different in GLMs ($P < 0.05$, Tukey HSD).

benefit by interrupting plant-rhizobia symbiosis (Pineda et al., 2010; Basu et al., 2021b).

Soil bacteria can alter insect feeding (Basu et al., 2021a; Dean, Mescher & De Moraes, 2014; Kempel, Brandl & Schädler, 2009). For example, rhizobia increase legume tolerance to insects by promoting nitrogen-based defenses (Dean et al., 2014; Kempel et al., 2009). However, such effects are not observed on cyanogenic legumes, suggesting benefits of rhizobia may occur only on poorly defended plants (Kempel et al., 2009). Conversely, by damaging leaves, herbivores can lower levels of plant sugars and nutrients required for nodulation (Katayama et al., 2014). We show that *P. sativum* plants grown in soil inoculated with rhizobia had fewer feeding notches than plants grown in autoclaved soil without rhizobia or in field-collected soil. Plants grown in field-collected soil without rhizobia inoculation had less than 5 nodules on average (with many having 0), while all seeds grown in inoculated soil grew nodules (mean of over 50 per plant). These results suggest that while our untreated field soil treatment did have low natural levels of rhizobia, the rhizobia present did not form strong associations with plants (based on a lack of nodulation), and in turn plant traits were not altered sufficiently to affect herbivore feeding. More broadly, our study shows rhizobia function as a keystone soil microbe that alter plant function and plant-insect interactions aboveground, but perhaps only when a sufficient density of rhizobia are present in the soil (Blundell et al., 2020; Vannette & Hunter, 2011).

Reduced leaf defoliation by *S. lineatus* on plants inoculated with rhizobia could be linked with several plant-response pathways. For example, *S. lineatus* individuals had similar amino acid levels on plants of all treatments despite consuming less leaf area on plants grown with rhizobia. This may have occurred if weevils obtained more nutrients per unit leaf area on plants with rhizobia (Kempel, Schädler, Chrobock, Fischer & van Kleunen, 2011). However, as we only exposed *S. lineatus* individuals to treatments for 48 h, a more likely explanation is that reduced herbivory on plants with rhizobia resulted from alteration of non-nutritive traits. Rhizobia often alter chemical pathways in plants that mediate interactions in multi-trophic food webs (Ochieno et al., 2021), and our analysis of gene transcripts suggests that rhizobia induced jasmonic acid and ethylene in *P. sativum*, two key systemic pathways involved in anti-herbivore defense (Pangesti et al., 2015, 2016; Romera et al., 2019; Thamer, Schädler, Bonte & Ballhorn, 2011). Similarly, rhizobia induced abscisic acid synthesis, even though abscisic acid can negatively affect root nodulation (Choudhury, Johns & Pandey, 2019; Jha & Subramanian, 2013; Tominaga et al., 2010). Our study also provides further evidence that rhizobia affect herbivores by altering physical defenses such as callose (Gaudioso-Pedraza et al., 2018) and antioxidants (Dumanović, Nepovimova, Natić, Kuča & Jaćević, 2021). Yet, our results also show the complexity of plant-mediated pathways that ultimately mediate interactions between soil microbes and herbivores aboveground.

Plants attacked by *S. lineatus* had less nodules and lower nodule fresh weight than plants without herbivory, suggesting antagonistic effects of *S. lineatus* on the association between *P. sativum* and soil rhizobia. However, *S. lineatus* feeding did not affect nodule dry weight, perhaps because the feeding time was too short (48 h) compared to the overall life of plants (2 to 4 wk). Although *S. lineatus* impeded legume-rhizobia synthesis, prior work shows *S. lineatus* herbivory to indirectly promote aphid-borne viruses by altering plant chemical and physical defenses in ways that promote aphid fitness and plant attractiveness (Basu et al., 2021a; Chisholm et al., 2019). Similarly, virus-infected *P. sativum* are preferred by *S. lineatus* compared to uninfected plants. Overall, it appeared that *S. lineatus* feeding had stronger effects on gene transcripts associated with hormone signaling (Fig. 3) compared to transcripts associated with physical defenses (Figs. 4,5). While our study only included adult weevils, it is likely that inclusion of larvae that feed on soil roots and nodules directly might further (and perhaps more significantly) affect plant signals and growth traits. Given that mutualisms between soil microbes and plants are often mediated by both phytohormone signaling and polysaccharides like callose (Gaudioso-Pedraza et al., 2018; Tominaga et al., 2010) our results suggest that aboveground herbivory may often impact soil microbes indirectly by altering plant chemical and physical defense signaling.

Overall, our study shows soil rhizobia improve plant health by inducing systemic resistance against herbivores. In contrast, herbivores interfered strongly with legume-rhizobia symbiosis by inhibiting nodule development, even though *S. lineatus* had fewer feeding notches on plants with rhizobia. This shows *S. lineatus* had strong indirect effects on rhizobia not primarily mediated by the amount of defoliation. Most prior studies involving soil microbes and herbivores assess either bottom-up effects of microbes on herbivores, or top-down effects of herbivores on microbes, but not both. Our study, in contrast, shows assessing reciprocal interactions between rhizobia and herbivores allows for a broader understanding of how aboveground stressors and belowground microbes interact through plant-mediated pathways. While there is a need to better understand direct effects of *S. lineatus* larvae feeding on nodules, our study shows that *S. lineatus* may alter the ecology and evolution of plant-rhizobia symbiosis solely through indirect and plant-mediated mechanisms. As legumes are included in crop rotations with cereals worldwide, understanding how soil rhizobia affect herbivores could promote management of biological nitrogen fixation and crop sustainability. Manipulation of soil microbes, for example, may also provide a novel tactic to manage devastating herbivores while improving crop yield and nitrogen fixation.

Author contributions

S.B[1]. and D.W.C. conceived the ideas and methodology; S.B.[1], B.W.L., R.E.C., S.B[2]. and C.L.C. collected the data;

B.W.L., S.B.[1], R.E.C., S.B[2]., C.L.C. and D.W.C. analyzed and interpreted the data; all authors contributed critically to the drafts and gave final approval for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.baae.2022.08.005.

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