

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

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4 Saumik Basu^{1*}, Benjamin W Lee¹, Robert E Clark¹, Sayanta Bera^{2,3}, Clare L Casteel², and David
5 W. Crowder¹

6 ¹ Department of Entomology, Washington State University, Pullman, WA, USA, 99164

7 ² School of Integrative Plant Science, Plant Pathology and Plant-Microbe Biology Section, Cornell
8 University, Ithaca, NY, USA, 14850

9 ³ Present address: Department of Cell Biology and Molecular Genetics, University of Maryland
10 College Park, College Park, MD 20742, USA; sbera@umd.edu

11 * Corresponding author

12 saumik.basu@wsu.edu

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14

15 **Abstract**

16 Soil bacteria that form mutualisms with plants, such as rhizobia, affects susceptibility of plants to
17 herbivores and pathogens. Soil rhizobia also promote nitrogen fixation, which mediates host
18 nutrient levels and defenses. However, whether aboveground herbivores affect the function of soil
19 rhizobia remains poorly understood. We assessed reciprocal interactions between *Sitona lineatus*,
20 a chewing herbivore, and pea (*Pisum sativum*) plants grown with or without rhizobia (*Rhizobium*
21 *leguminosarum* biovar *viciae*). We also examined the underlying plant-defense and nutritional
22 mechanisms of these interactions. In our experiments, soil rhizobia influenced feeding and
23 herbivory by chewing herbivores. Leaf defoliation by *S. lineatus* was lower on plants treated with
24 rhizobia, but these insects had similar amino acid levels compared to those on un-inoculated plants.
25 Plants grown with soil rhizobia had increased expression of gene transcripts associated with
26 phytohormone-mediated defense, which may explain decreased susceptibility to *S. lineatus*.
27 Rhizobia also induced expression of gene transcripts associated with physical and antioxidant-
28 related defense pathways in *P. sativum*. Conversely, *S. lineatus* feeding reduced the number of
29 root nodules and nodule biomass, suggesting a disruption of the symbiosis between plants and
30 rhizobia. Our study shows that aboveground herbivores can engage in mutually antagonistic
31 interactions with soil microbes mediated through a multitude of plant-mediated pathways.

32

33 **Keywords:** pea leaf weevil, defense genes, phytohormones, physical defense, plant nutrients

34

35 **Introduction**

36 Soil harbors abundant and diverse microbe communities that affect ecosystem functions like
37 biomass production, carbon sequestration, pollution mitigation, and nutrient cycling (A’Bear,
38 Johnson & Jones, 2014; Bardgett & van der Putten, 2014). Plant-root associated soil bacteria such
39 as rhizobia can also affect plant susceptibility to herbivores and pathogens by altering plant
40 nutrient levels or physical and chemical defenses (Dean, Mescher & De Moraes, 2014; Rashid &
41 Chung, 2017; Heinen, Biere, Harvey & Bezemer, 2018; Blundell et al., 2020). By affecting plant
42 traits, soil microbes often may indirectly alter interactions between plants, herbivores, and plant
43 pathogens, and integrating aboveground and belowground interactions is a key priority in food
44 web ecology (Hooper et al., 2005; Pangesti, Pineda, Pieterse, Dicke & Van Loon, 2013; van Geem
45 et al., 2013; de Vries & Wallenstein, 2017; Ramirez et al., 2018).

46 Soil microbes have cascading bottom-up impacts on aboveground organisms by altering
47 plant traits (Pineda, Soler, Pozo, Rasmann & Turlings, 2015; Tao, Hunter & de Roode, 2017;
48 Valencia et al., 2018). For example, legume plants grown in soil inoculated with rhizobia have
49 greater biomass than plants grown without rhizobia as well as greater systemic resistance against
50 herbivores and pathogens (Gopalakrishnan et al., 2015). Soil rhizobia may also indirectly affect
51 herbivores and pathogens by altering plant defense signaling, release of volatile organic
52 compounds, and plant nutrients (Rasmann, Bennett, Biere, Karley & Guerrieri, 2017; Tao et al.,
53 2017; Heinen et al., 2018). Similarly, aboveground pathogens and herbivores may often disrupt
54 plant-microbe mutualisms, resulting in reduced biological nitrogen fixation and weakened plant
55 defense (Heath & Lau, 2011; Ballhorn, Younginger & Kautz, 2014; Simonsen & Stinchcombe,
56 2014). These studies suggest reciprocal interactions between microbes, herbivores, and pathogens
57 may often be mediated *via* plant-mediated interactions. Yet, few studies have extensively

58 characterized the mechanistic chemical, physical, and nutritional properties of plants and how they
59 may mediate interactions between aboveground and belowground organisms.

60 Direct herbivore-soil microbe interactions may also occur when herbivores spend part of
61 their life belowground. For example, *Sitona lineatus* (pea leaf weevil) larvae consume nodules of
62 legume roots that harbor rhizobia. However, the majority of interactions between soil microbes
63 and aboveground organisms are likely to be indirect and plant-mediated. For example, rhizobia-
64 inoculated legumes are often less susceptible to herbivory, as physical defenses such as greater
65 callose deposition and induction of antioxidants are promoted in these rhizobia-inoculated plants
66 (Millet et al., 2010; Cawoy et al., 2014; Rashid, Khan, Hossain & Chung, 2017). On the other
67 hand, herbivory may interfere with legume-rhizobia symbiosis, reducing the number and size of
68 root nodules, if aboveground herbivores decrease photosynthesis and plant vigor (Simonsen &
69 Stinchcombe, 2014; Heath & Lau, 2011). By limiting nodule growth and rhizobia function,
70 herbivores might benefit by interfering with anti-herbivore defense signaling induced by rhizobia
71 (Pineda, Zheng, van Loon, Pieterse & Dicke, 2010; Shikano, Rosa, Tan & Felton, 2017; Heinen et
72 al., 2018).

73 Here we addressed the mechanisms driving trait-mediated indirect interactions between a
74 legume host (*Pisum sativum*, pea), soil rhizobia (*Rhizobium leguminosarum* biovar. *viciae*), and a
75 chewing herbivore (*S. lineatus*). In the Palouse region of northern Idaho and eastern Washington,
76 USA, these organisms commonly co-occur in natural and managed ecosystems. However, it is
77 largely unknown if *S. lineatus* herbivores are affected by the presence of rhizobia in soil, or
78 whether herbivory from *S. lineatus* affects symbioses between rhizobia and pea plants. We used
79 greenhouse experiments to assess whether *S. lineatus* affected soil rhizobia and how soil rhizobia
80 affected plant susceptibility to *S. lineatus*. These experiments were complemented with molecular

81 assays that examined chemical, physical, and antioxidant defense signaling and nutritional
82 properties of *P. sativum* hosts exposed to *S. lineatus* and inoculated with *Rhizobium*.

83

84 **Materials and Methods**

85 *Study system and experimental conditions*

86 Many native and cultivated legumes, including *P. sativum*, are found in Palouse region of
87 eastern Washington and northern Idaho, USA (Clement, Husebye & Eigenbrode, 2010; Chisholm,
88 Eigenbrode, Clark, Basu & Crowder, 2019). These plants are attacked by insect vectors, pathogens,
89 and chewing herbivores such as *S. lineatus* (Chisholm et al., 2019; Basu, Clark, Bera, Casteel &
90 Crowder, 2021b). *Sitona lineatus* adults overwinter outside of *P. sativum* fields and migrate into
91 fields in the late spring to lay eggs (Carcamo et al., 2018). After eggs hatch, larvae burrow into the
92 soil to feed and pupate before adults re-emerge in the summer (Carcamo et al., 2018). Thus, *S.*
93 *lineatus* populations attack *P. sativum* hosts above- and belowground for several months. While *S.*
94 *lineatus* larvae feed on legume roots belowground, directly affecting the abundance of rhizobia,
95 we focused on adults feeding aboveground to isolate plant-mediated mechanisms by which
96 rhizobia affected this herbivore (Mutch & Yang, 2004).

97 Adult *S. lineatus* were collected from *P. sativum* fields one wk before experiments, and soil
98 was collected from the Palouse Conservation Farm (Pullman, WA, USA) before being exposed to
99 treatments. For rhizobia treatments, soil was inoculated with pea-specific rhizobia (*Rhizobium*
100 *leguminosarum* biovar. *viciae*) by mixing N-Dure^R, a peat-based inoculant with *P. sativum* seeds
101 using the manufacturer's protocol (Verdasian Life Sciences, Cary, NC, USA). All experiments
102 were conducted in greenhouses at Washington State University (Pullman, WA, USA) with a 16:8
103 h light:dark cycle, 21-24°C during light cycles, and 16-18°C during dark cycles.

104

105 ***Effects of rhizobia on S. lineatus feeding***

106 We assessed effects of rhizobia on *S. lineatus* feeding with three soil treatments: (i) control
107 (no treatment); (ii) autoclaved to remove microbes; and (iii) autoclaved with rhizobia added. In
108 autoclaved treatments, field-collected soil was placed in 61 × 91 cm bags in a steam autoclave at
109 7 psi and 111°C overnight. As autoclaving soil affects soil moisture, all soil treatments were
110 standardized to 75% moisture before plants were added.

111 Plants were grown in potting mix (Sunshine® LC1) before transplantation into treated soil
112 at 2 wk old. Plants were then individually placed in 1 L pots with soil exposed to one treatment,
113 which were placed in bucket cages (0.6 × 0.3 × 0.3m) for an additional 2 wk before *S. lineatus*
114 treatments were applied. There were two *S. lineatus* treatments: (i) none (control) and (ii) two adult
115 *S. lineatus* feeding on plants for 48 h. After 48 h, both adult *S. lineatus* were removed from each
116 plant to prevent further feeding. The experiment was a 3 × 2 factorial design, with 3 soil treatments
117 and 2 *S. lineatus* treatments; each treatment was replicated 10 times per block, and two temporal
118 blocks were performed. There were a total of 120 experimental units (2 blocks × 3 soil treatments
119 × 2 *S. lineatus* treatments × 10 replicates). In each replicate, the total numbers of leaf notches were
120 counted by visually observing the aboveground portion of all the plants. Leaf notches is a reliable
121 indicator of the amount of *S. lineatus* feeding (Chisholm, Sertsuvalkul, Casteel & Crowder, 2018).

122

123 ***Analyses of amino acids***

124 We measured amino acid content of *S. lineatus* adults from the different treatments to assess
125 herbivore nutrient acquisition. Two adult *S. lineatus* were collected from each replicate of the
126 feeding experiment (4 replicates per each soil treatment) into liquid N₂ and lyophilized. After

127 lyophilization, *S. lineatus* tissue was weighed and extracted with 20mM of HCL (Patton, Bak,
128 Sayre, Heck & Casteel, 2019). Amino acids were derivatized using AccQ-Fluor reagent kits
129 (Waters, Milford, MA, USA), with L-Norleucine as an internal standard. 10 µl from each sample
130 were injected into a Agilent 1260 Infinity HPLC (Agilent, Santa Clara, CA, USA) with a Nova-
131 Pak C18 column (c).

132 Amino acid derivatives were detected with excitation and emission wavelengths of 250 nm
133 and 395 nm, respectively. Peak areas were compared to a standard curve made from a serial
134 dilution of amino acid standards (Sigma-Aldrich, St. Louis, MO). Solvent A, AccQ-Tag Eluent A,
135 was premixed from water; Solvent B was acetonitrile:water (60:40). The gradient used was 0–
136 0.01 min, 100% A; 0.01–0.5 min, linear gradient to 3% B; 0.5–12 min, linear gradient to 5% B;
137 12–15 min, linear gradient to 8% B; 15–45 min, 35% B; 45–49 min, linear gradient to 35% B; 50–
138 60 min, 100% B. The flow rate was 1.0 ml min⁻¹. Amino acid derivatives and peak areas were
139 measured with an Agilent fluorescence detector and ChemStation software. To calculate
140 concentrations, standard curves were created for each amino acid using dilutions of standards.

141

142 ***Effects of S. lineatus on soil rhizobia***

143 We next assessed how *S. lineatus* feeding affected nodulation and nodule biomass, two key
144 metrics of rhizobia function, with two treatments: (i) control - no *S. lineatus* and (ii) *S. lineatus*
145 feeding. In *S. lineatus* treatments, we released two adults for 48 h on 2 wk old *P. sativum* plants,
146 after which the adults were removed. Following treatments, plants were uprooted from the soil
147 after 7 d and soil was washed off roots with tap water. Nodules were counted from the root of each
148 plant and then excised. Nodule fresh weights were taken immediately after collection, then dried

149 for 5 d at 37°C before dry weight measurements were taken. Plants that failed to develop any root
150 nodules served as a control for this experiment (no rhizobia inoculation).

151

152 *Analyses of transcripts related to defense signaling*

153 We next conducted an experiment with two *S. lineatus* treatments: (i) control, no *S. lineatus*
154 and (ii) two adult *S. lineatus* feeding for 48 h. These treatments were crossed with two rhizobia
155 treatments: (i) control, no rhizobia inoculum and (ii) seeds treated with rhizobia. For preparation
156 of potting mix, soil and sand were mixed in equal volume (1:1) to facilitate nodule development;
157 plants were in treated soil for 2 wk before *S. lineatus* treatments. Plant tissue samples were
158 harvested 3 d and 7 d after *S. lineatus* addition. In total, the experiment included four randomly
159 assigned replicates of each treatment for two temporal blocks in a $2 \times 2 \times 2$ factorial design (2 soil
160 treatments \times 2 *S. lineatus* treatments \times 2 time points \times 4 replicates = 32 total experimental units).
161 Aboveground harvested plant tissue was wrapped in aluminum foil, frozen in liquid N₂, and kept
162 on dry ice before storing in -80 °C. Samples were ground using a mortar and pestle in liquid N₂,
163 and 50 to 100 mg of tissue was used for total RNA extraction using Promega SV total RNA
164 isolation kits (Promega, Madison, WI) and cDNA from 1 µg of total RNA using Bio-Rad iScript
165 cDNA synthesis kits. Gene-specific primers (Table S1) were used in qRT-PCR reactions (10 µl)
166 containing 3 µl of ddH₂O, 5 µl of iTaq Univer SYBR Green Supermix, 1 µl of primer mix (forward
167 and reverse), and 1 µl of diluted (1:25) cDNA template. The qRT-PCR program had an initial
168 denaturation for 3 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, annealing
169 for 30 s at 60 °C, and extension for 30 s at 72 °C. For melting curve analysis, a dissociation step
170 cycle was used (55 °C for 10 s, and then 0.5 °C for 10 s until 95 °C). The relative expression of

171 genes were calculated using the delta-delta Ct method, ($2^{-\Delta\Delta Ct}$) with Ps β -tubulin as a housekeeping
172 gene (Livak & Schmittgen, 2001; Kozera & Rapacz, 2013).

173 Harvested plant tissue was assessed for expression of 14 gene transcripts associated with
174 hormone signaling, physical, or antioxidant-related defense pathways (Fondevilla, Küster,
175 Krajinski, Cubero & Rubiales, 2011; Tran, You & Barbetti, 2018; Kimura & Kawano, 2015). Gene
176 sequences were obtained using accession numbers of available pea genes or by using the pea
177 marker database (Kulaeva et al., 2017) and blast searching the reference pea genome (Kreplak et
178 al., 2019). We assessed expression of 7 gene transcripts related to phytohormones. *Pathogenesis-*
179 *related protein 1 (PRI)* and *Isochorismate synthase1 (ICS1)* are associated with the salicylic acid
180 (SA) pathway, with *ICS1* involved upstream of SA biosynthesis and *PRI* triggering downstream
181 systemic acquired defenses (Zhang et al., 2010; Fondevilla et al., 2011; Seguel et al., 2018). Two
182 genes, *Lipoxygenase 2 (LOX2)* and *12-oxophytodienoate reductases 3 (OPR3)* are associated
183 upstream and downstream, respectively, of jasmonic acid biosynthesis (He, Fukushige, Hildebrand
184 & Gan, 2002; Fondevilla et al., 2011; Wasternack & Hause, 2013). Other genes included were *l-*
185 *aminocyclopropane-1-carboxylic acid synthases 2 (ACS2)*, which is associated with ethylene
186 biosynthesis, and *Aldehyde oxidase 3 (AO3)*, which catalyzes abscisic acid biosynthesis. Beside
187 abscisic acid biosynthesis, *AO3* also affects production of reactive oxygen species (Yergaliyev et
188 al., 2016).

189 We assessed relative transcript accumulation of two additional genes related to physical
190 defense: (i) viz. *β -1,3 Glucanase*, an enzyme that regulates callose production and (ii) *calcium-*
191 *regulated/ATP-independent ferisome protein gene*, which is associated with P protein plugs that
192 seal phloem pathways (Zavaliev, Ueki, Epel & Citovsky, 2011; Srivastava, Tuteja & Tuteja, 2015;
193 Moravčíková et al., 2016). Six additional genes for antioxidant related defense pathways were

194 assessed: 3 *Super Oxide Dismutases* (*FeSOD*, *CuZnSOD*, *MnSOD*), *Catalase* and *Glutathione*
195 *reductase 1(GRI)*, and *Peroxidase (PsPOX11)* (Fondevilla et al., 2011; Tran et al., 2018).
196 Induction of these defense pathways can catalyze superoxides (reactive oxygen species, ROS) in
197 plants (Kimura & Kawano, 2015) and affect induction of salicylic acid in peas (Kawahara et al.,
198 2006).

199

200 ***Data analysis***

201 Analyses were conducted in R 4.0.5 (R Core Team 2021). We used a generalized linear
202 model (GLM) with a Poisson distribution to assess whether soil treatments (control, autoclaved,
203 rhizobia) affected the number of *S. lineatus* feeding notches; negative controls without *S. lineatus*
204 never had feeding damage, and these treatments were not included. We also used GLMs with a
205 Poisson distribution to assess if *S. lineatus* (present or absent) affected plant nodule weight and
206 nodule biomass. We analyzed effects of soil rhizobia and *S. lineatus* treatments, and their
207 interaction, on fold change gene expression using MANOVA (multiple analysis of variance) on
208 delta CT values ($2^{-\Delta\Delta CT}$ values before transformation) for relative transcript abundance for 14
209 different genes, *PR1*, *ICSI*, *OPR3*, *LOX2*, *AO3*, *ACS2*, β -1,3 *Glucanase*, *Calcium-regulated/ATP-*
210 *independent ferisome protein gene*, *CuZnSOD*, *FeSOD*, *MnSOD*, *Catalase*, *GRI* and *PsOX11*.
211 MANOVA was used as we assumed responses of multiple gene transcripts came from the same
212 plants and therefore have a partially correlated response to treatment. Parameter estimates and
213 subsequent calculations for delta-delta CT ($2^{-\Delta\Delta Ct}$) were plotted on the log 10 scale. Finally,
214 average amino acid content for 13 amino acids was fitted to a linear mixed model (LMM, lme4
215 package, Bates, Maechler, Bolker & Walker, 2015), with soil treatment as a fixed effect and amino
216 acid and replicate as random effects; amino acid concentration was log-transformed to meet

217 normality assumptions. Estimated marginal means and all post-hoc tests were assessed using the
218 emmeans package (Lenth, 2016), with significance tests via analysis of deviance tables generated
219 using the car package (Fox & Weisberg, 2011).

220

221 **Results**

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

223 Rhizobia inoculation altered the amount of herbivory from *S. lineatus* on *P. sativum* hosts
224 ($\chi^2 = 106.0$, $P < 0.001$, Fig. 1). Plants grown in autoclaved soil had the most feeding, while plants
225 grown in autoclaved soil with rhizobia had the least, with plants grown in control soil having
226 intermediate levels (Figs. 1, S1). Soil treatment, however, did not significantly affect the
227 concentration of amino acids in *S. lineatus* from plants ($\chi^2 = 1.66$, $df = 2$, $P = 0.44$, Fig. 1B).

228

229 **Effects of *S. lineatus* herbivory on soil rhizobia**

230 Herbivory from *S. lineatus* had a negative effect on symbiosis between rhizobia and plant
231 hosts (Figs. 2, S2). Plants that were fed on by *S. lineatus* had fewer root nodules ($\chi^2 = 6.49$, $P =$
232 0.010 , Fig. 2A) and lower total nodule biomass ($\chi^2 = 9.41$, $P = 0.002$, Fig. 2B) than plants that did
233 not experience herbivory. However, treatments with *S. lineatus* did not significantly affect nodule
234 dry mass ($\chi^2 = 2.46$, $P = 0.12$, Fig. 2C).

235

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

237 Plants grown with rhizobia had higher expression of the ethylene biosynthetic gene, *ACS2*,
238 compared to plants grown in control soil with or without *S. lineatus* (Fig. 3A). The presence of *S.*
239 *lineatus* affected expression of other gene transcripts when plants were grown with rhizobia. Plants

240 grown with rhizobia and no herbivory had greater expression of two gene transcripts associated
241 with jasmonic acid, *OPR3* and *LOX2*, and one associated with the final step of abscisic acid
242 biosynthesis, *AO3*, compared to plants grown with rhizobia but no herbivory (Figs. 3B, D, E).
243 *Sitona lineatus* increased levels of the salicylic acid-associated gene transcript, *ICS1*, on control
244 plants compared to plants grown with rhizobia (Fig. 3C). Soil rhizobia also strongly induced β -1,3
245 *glucanase*, which is associated with callose-mediated defense (Fig. 4A, S3).

246 For the six gene transcripts related to antioxidant-mediated defense, three genes associated
247 with the superoxidase disumaste (*FeSOD*, *CuZnSOD*, *MnSOD*) had greater expression in plants
248 grown in rhizobia-inoculated soil compared to control soil (Figs. 5B, C, E, S3). However, other
249 gene transcripts were not impacted by rhizobia. We found that *S. lineatus* induced the antioxidant
250 gene transcript, *Catalase*, on plants grown in soil without rhizobia (Figs 5A, S3), and the gene
251 transcript peroxidase (*PsPOX11*) on plants grown in soil with rhizobia (Figs. 5F, S3).

252

253 Discussion

254 Herbivores and soil microbes interact through many direct and indirect, trait-mediated,
255 pathways. Our study highlights plant-mediated mechanisms that may underlie these interactions.
256 Root-associated bacteria can alter transcript levels of important anti-herbivore defensive genes in
257 host plants, which can impact the nutritional quality of plants and nutrient uptake by herbivores.
258 In turn, these changes led to reduced leaf herbivory on plants. We observed that *P. sativum* host
259 plants grown in rhizobia-inoculated soil had reduced leaf defoliation from *S. lineatus* compared to
260 plants grown without rhizobia (either control or autoclaved). However, *S. lineatus* individuals
261 obtained similar levels of amino acids on plants grown in rhizobia-inoculated and control plants.
262 This shows weevils obtained more nutrients per unit of leaf area on plants grown in rhizobia-

263 inoculated soil, which may be due to improved nutritional quality of the host plants (Kempel,
264 Schädler, Chrobock, Fischer & van Kleunen, 2011). Our results raise the intriguing possibility that
265 mutualistic soil microbes promote plant health by increasing plant nutrients, which in turn reduces
266 total feeding by herbivores.

267 Our results are in line with studies showing rhizobia and arbuscular mycorrhizal fungi are
268 keystone microbes that decrease plant susceptibility to insects and pathogens (Jaber & Vidal, 2009;
269 Pineda et al., 2010; Santos et al., 2014; Yang et al., 2014; Gopalakrishnan et al. 2015; Mabrouk et
270 al., 2018). Our study provides evidence that several mechanisms may underlie these results. Soil
271 rhizobia can modify plant nutrients in legumes as well as various defense related signaling
272 pathways against insects, which can alter insect feeding responses and performance (Dean et al.,
273 2014). For example, soil rhizobia increase tolerance of soybean plants (*Glycine max*) to soybean
274 aphid (*Aphis glycine*), although different rhizobia strains vary in their effects (Dean et al., 2014).
275 Similarly, growth and performance of cotton leaf worm (*Spodoptera littoralis*) is limited by
276 rhizobia on clover (*Trifolium repens*), as rhizobia increased production of nitrogen-based defense
277 compounds in hosts (Kempel, Brandl & Schädler, 2009). However, these effects were not observed
278 on clover plants that were naturally cyanogenic, suggesting benefits of rhizobia may only occur
279 on plants that are not naturally well defended (Kempel et al., 2009).

280 Rhizobia-induced changes in phytochemical and nutritional traits of plants have increasing
281 been recognized as important drivers of ecosystem function in multi-trophic food webs (Qchieno
282 et al., 2021). For example, beneficial root colonizing soil rhizobia often elicit induced systemic
283 resistance against insects and pathogens through activation of JA and ET signaling (Romera et al.,
284 2019). Symbiotic association of legume roots by soil rhizobia has been associated with enhanced
285 resistance against aboveground consumers including beetles (Soundararajan, Chitra, & Geetha,

286 2013; Thamer, Schädler, Bonte & Ballhorn, 2011; Godschalx, Tran, & Ballhorn, 2017).
287 Conversely, aboveground feeders damage leaves through defoliation and interfere with
288 photosynthesis by consuming sugars and other nutrients that are required for root nodulation
289 (Katayama et al., 2014). We observed plants attacked by *S. lineatus* had fewer nodules and lower
290 nodule biomass than controls, suggesting antagonistic effects of *S. lineatus* on legume-rhizobia
291 symbiosis. Previous studies have also shown that outbreaks of *S. lineatus* can promote the spread
292 of aphid-borne viruses that also impede the function of rhizobia (Chisholm et al., 2019; Basu et
293 al., 2021a); thus, we have shown that *S. lineatus* may negatively affect plant-rhizobia symbiosis
294 through multiple indirect pathways.

295 Our analysis of phytohormone transcripts suggests interactions between rhizobia and *S.*
296 *lineatus* were mediated by phytohormones. Rhizobia induced plant defense against *S. lineatus* by
297 activating jasmonic acid, ethylene, and abscisic acid signaling. Jasmonic acid and ethylene are two
298 key systemic defense pathways induced in plants against chewing herbivores (Pangesti et al., 2015;
299 2016; Rashid & Chung, 2017; Zhu et al., 2018). Similarly, beneficial microbes often also stimulate
300 biosynthesis of abscisic acid (Sgroy et al., 2009; Jha & Subramanian, 2013), even though abscisic
301 acid signaling can have negative effects on nodulation (Tominaga et al., 2010; Roy Choudhury,
302 Johns & Pandey, 2019). Our study provides further evidence that rhizobia can affect herbivores by
303 altering physical defenses such as callose (Ballhorn et al., 2014; Gaudioso-Pedraza et al., 2018)
304 and antioxidants (Walz, Juenger, Schad & Kehr, 2002, Dumanović, Nepovimova, Natić, Kuča, &
305 Jaćević, 2021). Antioxidant mediated defenses are found in cellular organelles such as
306 chloroplasts, mitochondria, and peroxisomes, and we found evidence that these defenses (*MnSOD*,
307 *FeSOD* and *CuZnSOD*) were impacted positively by rhizobia. These results suggest broad

308 induction of both chemical and physical defense by rhizobia can affect herbivores through trait-
309 mediated indirect pathways.

310 Overall, our study shows soil rhizobia improve plant health by inducing broad-spectrum
311 systemic resistance against herbivores while also improving plant quality. On the other hand,
312 herbivores can interfere strongly with legume-rhizobia symbiosis by inhibiting root nodule
313 development. Thus, assessing reciprocal interactions between soil rhizobia and herbivores is
314 crucial for understanding broader dynamics of agricultural and natural food webs. As legumes are
315 commonly included in rotations with cereals (corn, rice, wheat, barley) in agroecosystems around
316 the world, understanding how soil microbes (e.g. rhizobia) can be affected by chewing herbivores
317 could lead to more effective management of biological nitrogen fixation and crop sustainability.
318 Manipulation of soil microbes, for example, may also provide a novel tactic to manage devastating
319 herbivores while improving crop yield and nitrogen fixation.

320

321 **Author Contributions**

322 S.B¹. and D.W.C. conceived the ideas and methodology; S.B.¹, B.W.L., R.E.C., S.B². and C.L.C.
323 collected the data; B.W.L., S.B.¹, R.E.C., S.B²., C.L.C and D.W.C. analyzed and interpreted the
324 data; all authors contributed critically to the drafts and gave final approval for publication.

325

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330

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573

574

575 **FIGURE LEGENDS**

576 **Figure 1.** Soil rhizobia confer resistance against *S. lineatus* feeding. (A) Reduced number of *S.*
577 *lineatus* induced feeding notches were observed in pea leaves in presence of soil rhizobia (N = 10).
578 Average number of feeding notches in response to soil treatments (Poisson GLM). Rhizobia
579 addition reduced feeding notches, with intermediate levels of herbivory in control soils ($p < .05$,
580 Tukey HSD). (B) Effect of soil treatments on uptake of amino acids by *S. lineatus*. Log-
581 transformed mean concentrations (nmol/mg DW) among 13 amino acids in weevils feeding on pea
582 plants undergoing various soil treatments.

583 **Figure 2.** Effect of *S. lineatus* herbivory on nodulation: Fig 2A, B & C. Nodule count based on
584 Poisson-fit GLM. Nodule wet and dry mass based on Gaussian-fit GLM. *S. lineatus* feeding
585 negative affects nodule number and wet weight but not dry mass ($p < 0.05$, Tukey HSD).

586 **Figure 3.** Relative transcript accumulation of SA responsive genes: *ICS1* (A), *PR1* (B); ABA
587 responsive gene: *AO3* (C); JA responsive genes: *OPR3* (D), *LOX2* (E); Ethylene responsive genes:
588 *ACS2* (F) in *Pisum sativum* at 7dpi. Bars not connected with same number are significantly
589 different.

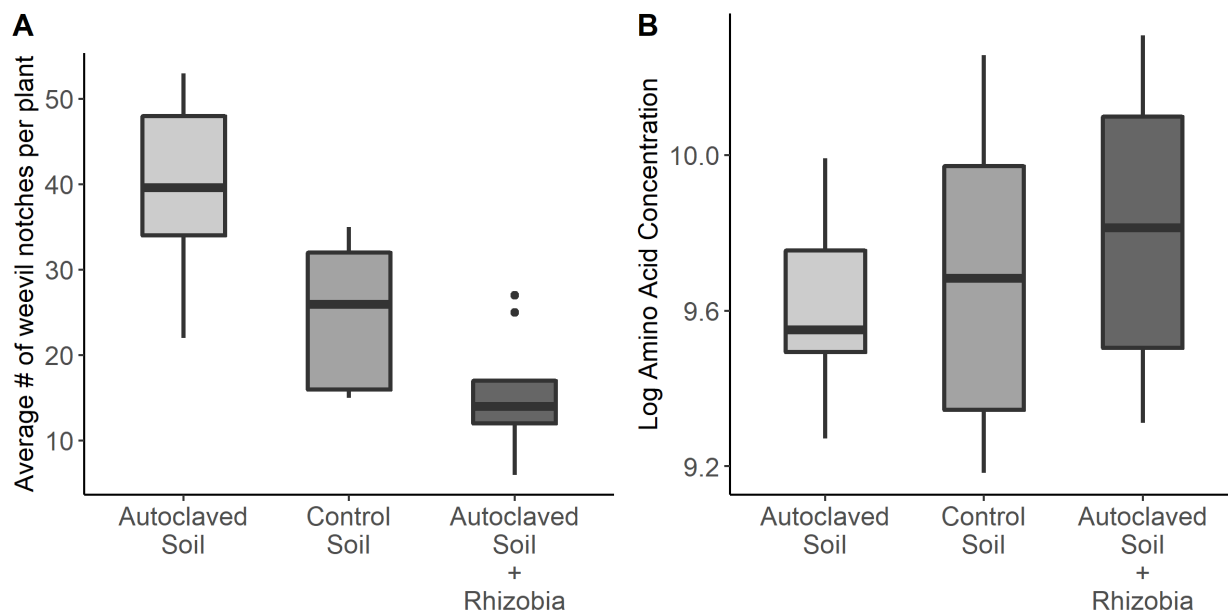
590 **Figure 4.** Relative transcript accumulation of callose mediated defense genes: *Beta-1, 3 glucanase*
591 (A), *PR1* (B) and *Calcium-regulated/ATP-independent ferisome protein gene* in *Pisum sativum*
592 7dpi. Bars not connected with same number are significantly different.

593 **Figure 5.** Relative transcript accumulation of antioxidant related defense genes: *CuZnSOD* (A),
594 *FeSOD* (B), *MnSOD*(C), *Catalase* (D), *GRI* (E) and Peroxidase, *PsOX11* (F) in *Pisum sativum* at
595 7 dpi Bars not connected with same number are significantly different.

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598 **FIGURE 1**



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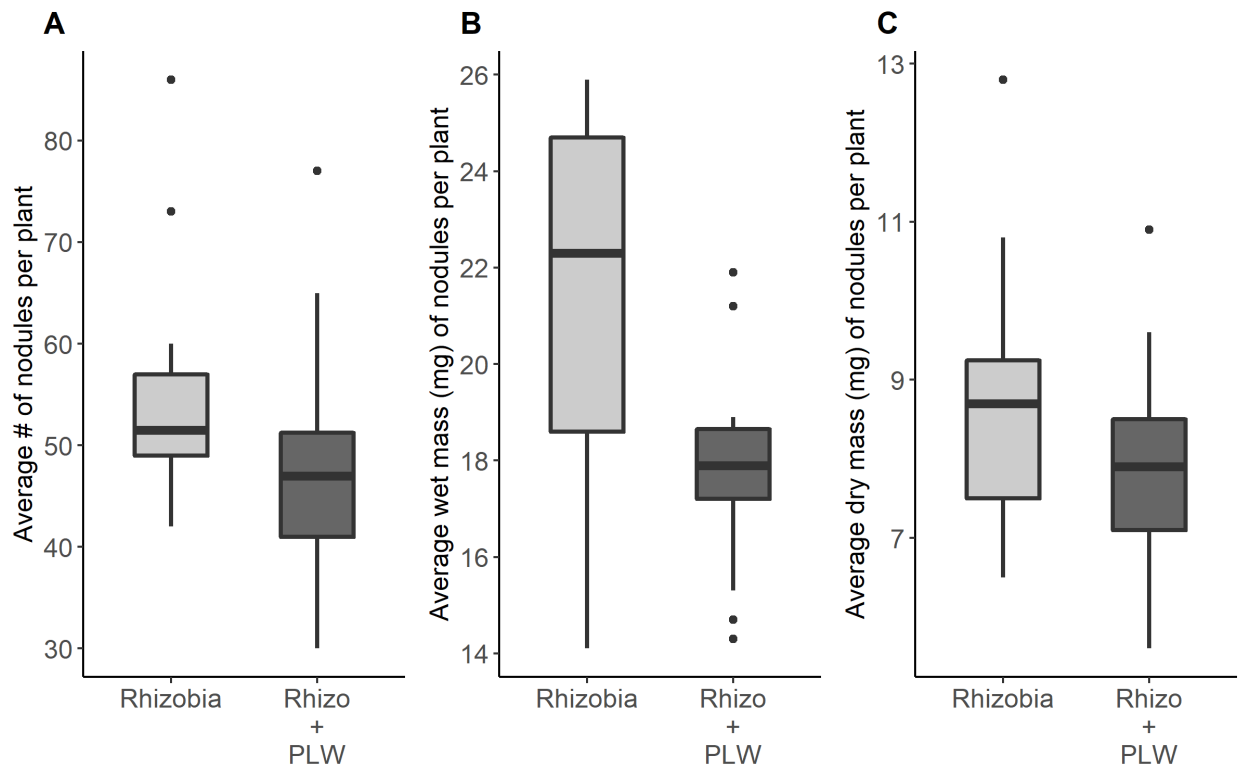
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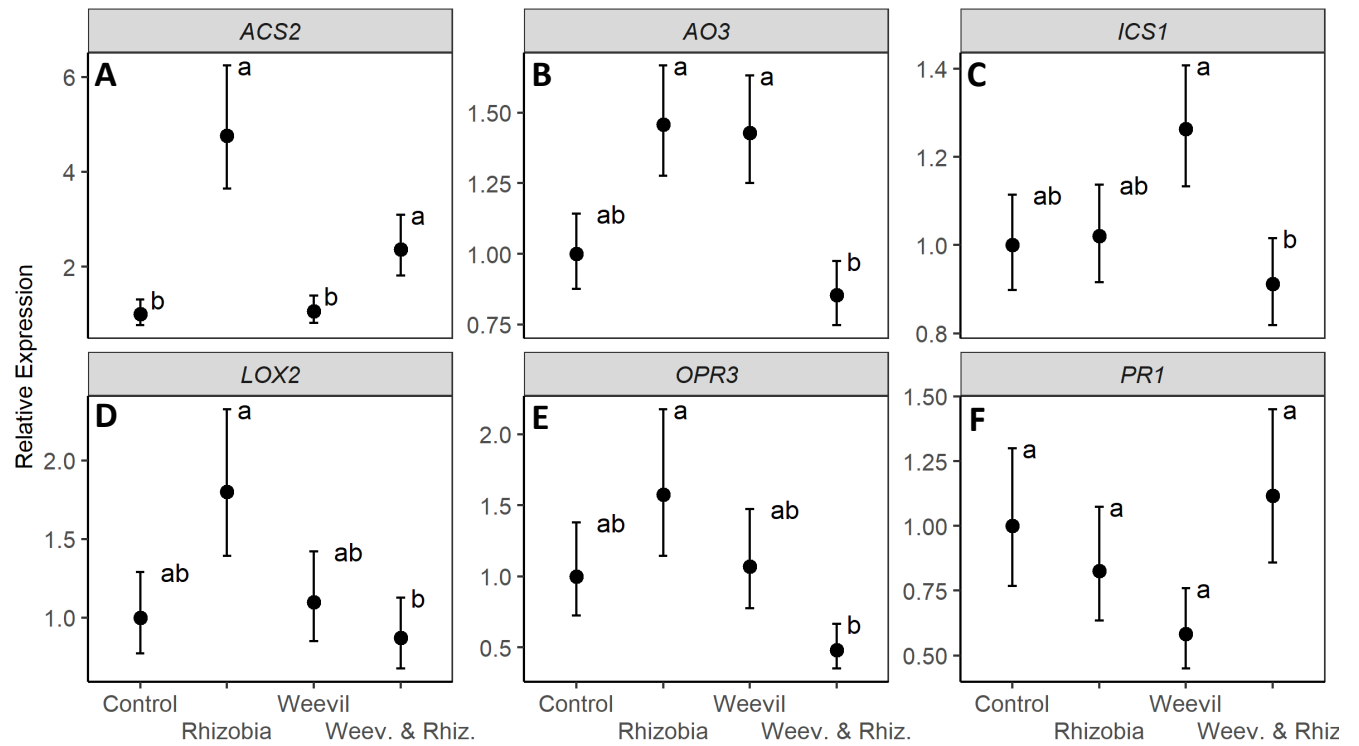
614 **FIGURE 2**



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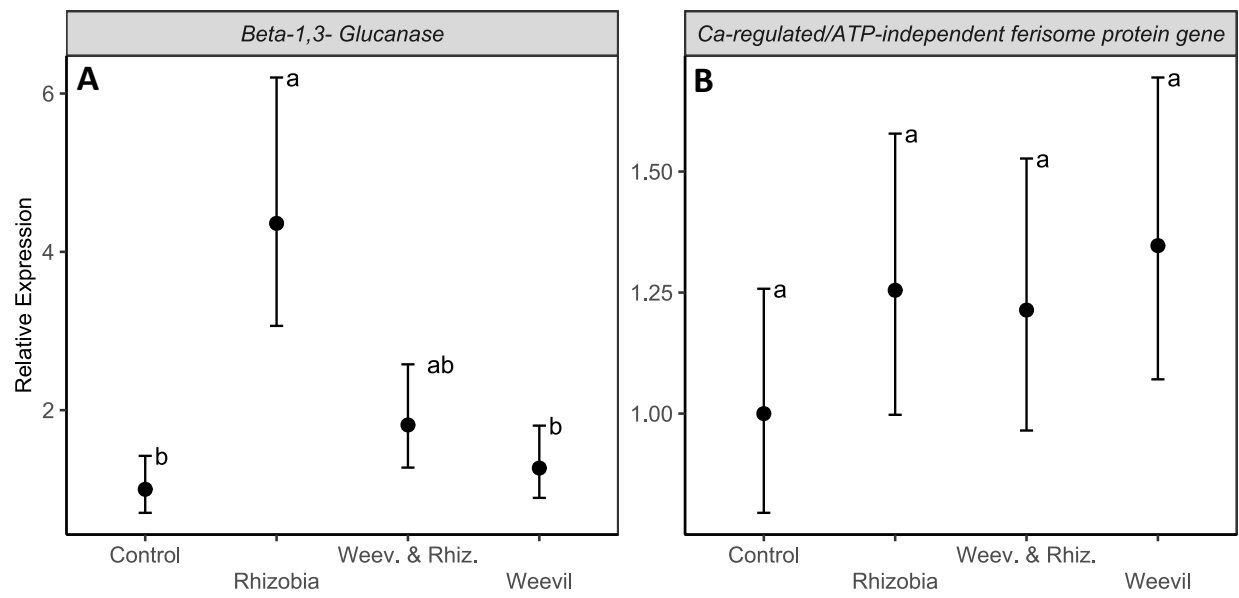
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617 **FIGURE 3**



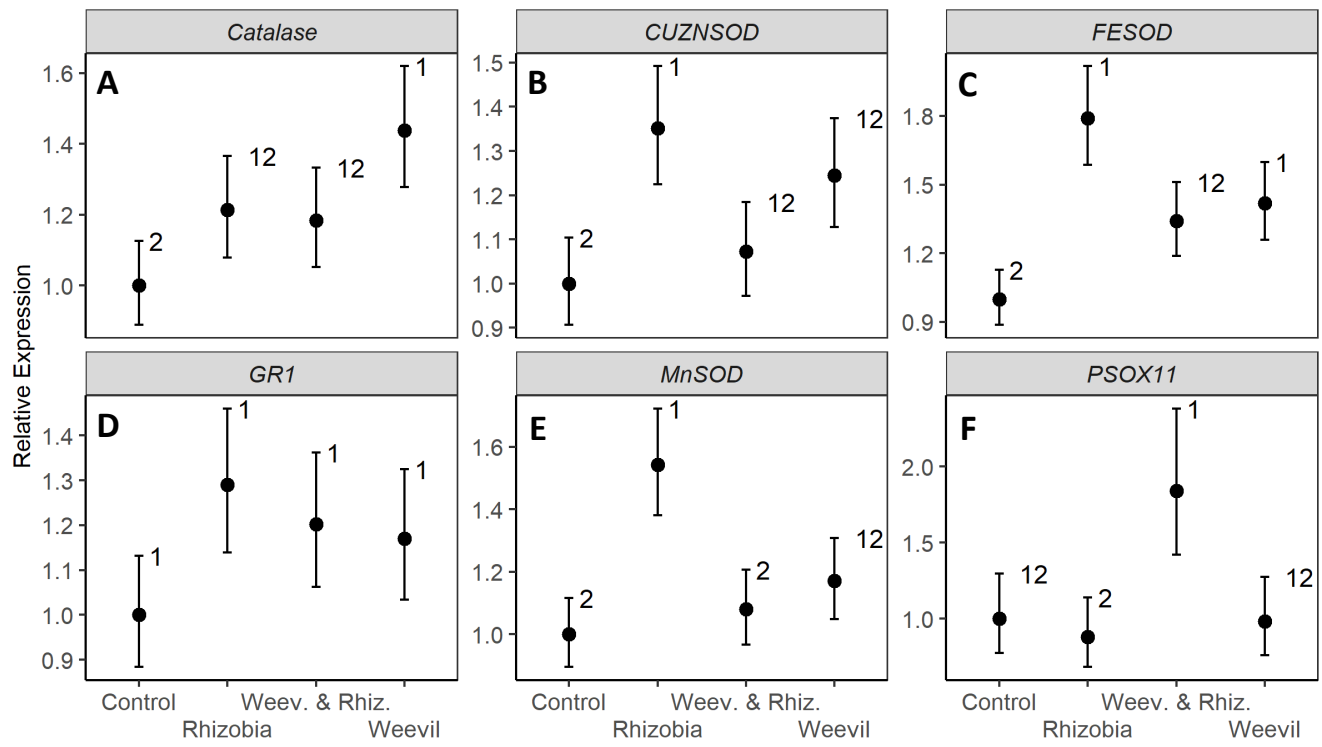
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619 **FIGURE 4.**



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621 **FIGURE 5**



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