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 Lee[1], Robert E Clark[1], Sayanta Bera[2,3], Clare L Casteel[2], and David

5 W. Crowder[1]

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

Introduction

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

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

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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 lineatu*s 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, 21-24ºC during light cycles, and 16-18ºC during dark cycles.

104

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 111ºC 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

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 N_2 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

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149   for 5 d at 37ºC 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 N_2, and kept

162   on dry ice before storing in -80 °C. Samples were ground using a mortar and pestle in liquid N_2,

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 Psβ-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* (*PR1*) and *Isochorismate synthase1* (*ICS1)* are associated with the salicylic acid

180 (SA) pathway, with *ICS1* involved upstream of SA biosynthesis and *PR1* 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 *1-*

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 S*uper Oxide Dismutases* (*FeSOD, CuZnSOD, MnSOD*), *Catalase* and *Glutathione*

195   *reductase 1(GR1)*, 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, ICS1, OPR3, LOX2, AO3, ACS2, β-1,3 Glucanase, Calcium-regulated/ATP-*

210   *independent ferisome protein gene, CuZnSOD, FeSOD, MnSOD, Catalase, GR1 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

11

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 though 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

Author Contributions

322   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.

323   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

324   data; all authors contributed critically to the drafts and gave final approval for publication.

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575 **FIGURE LEGENDS**

576 **Figure 1.** Soil rhizobia confer resistance against *S. lineatuus* 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), *GR1* (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**
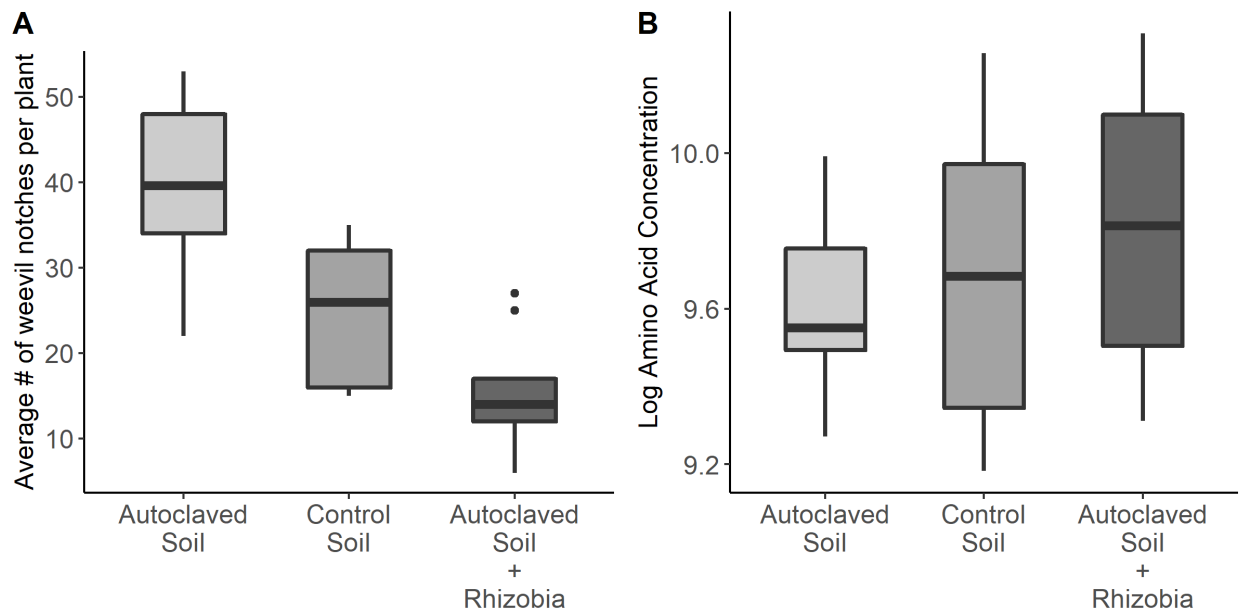
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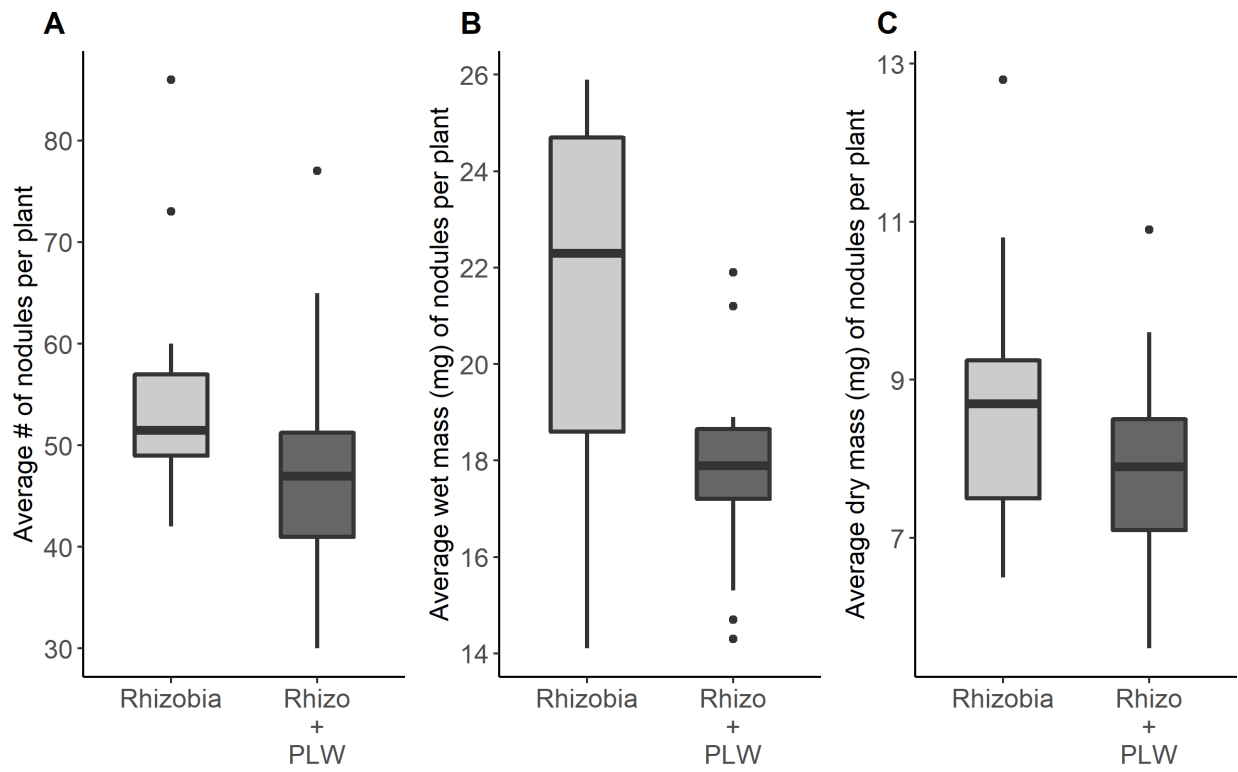
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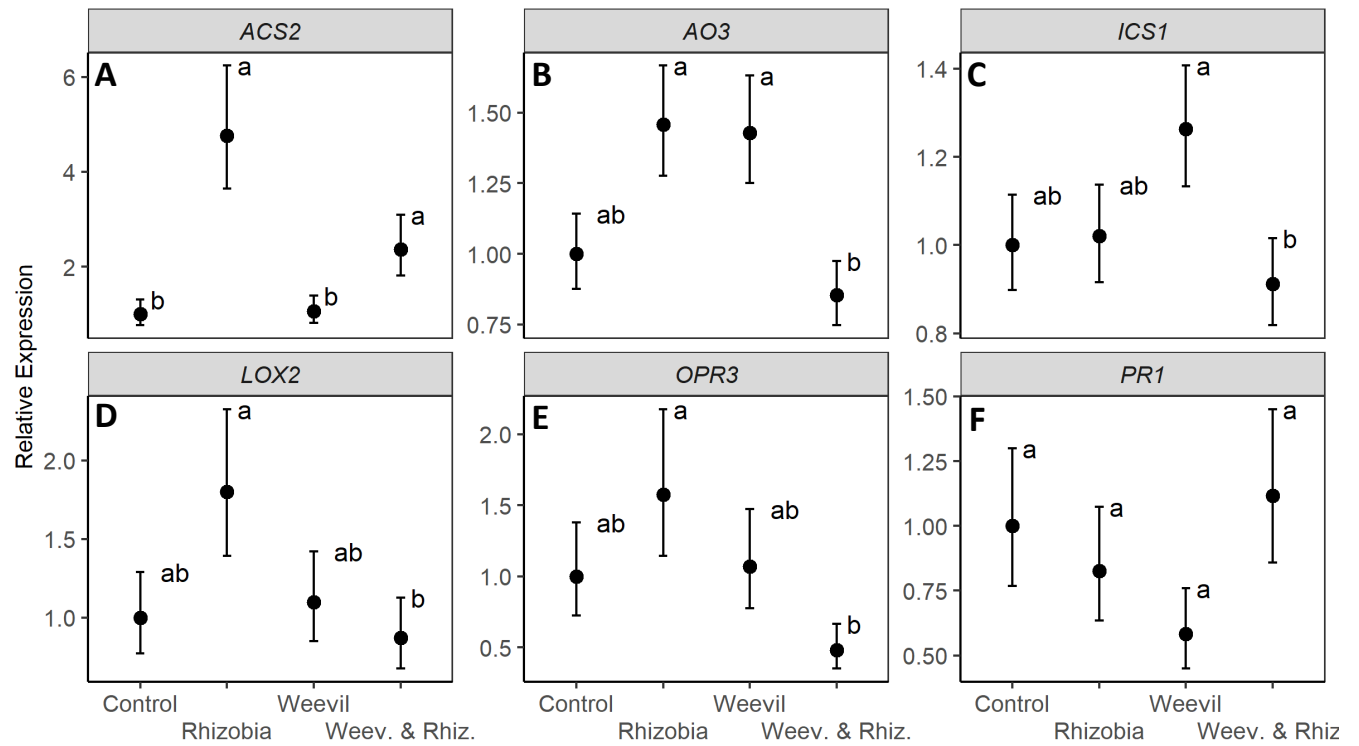
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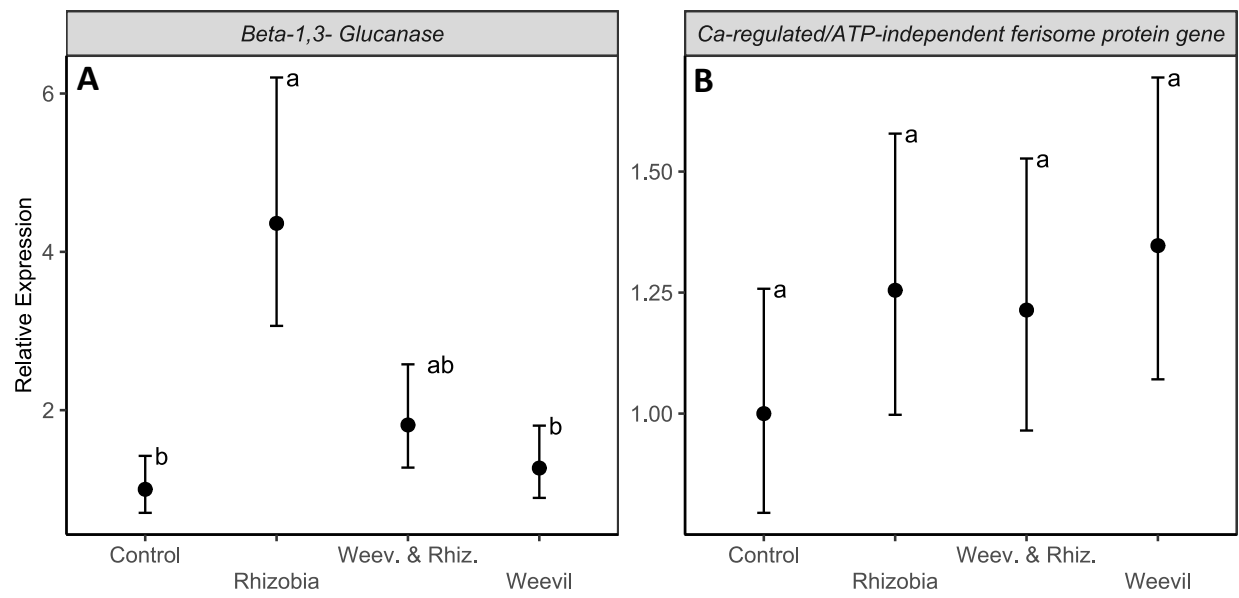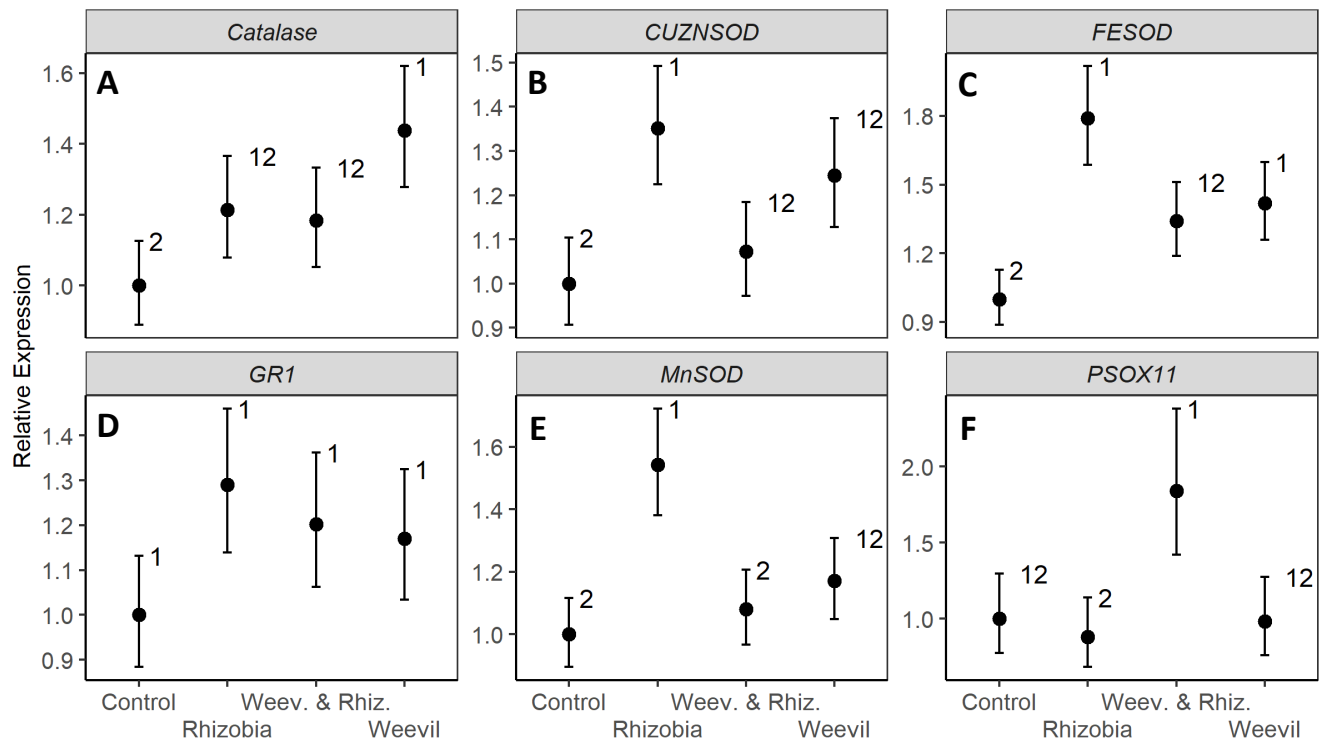614   **FIGURE 2**

615

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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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