

# Multiple and contrasting pressures determine intraspecific phytochemical variation in a tropical shrub

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## Research Article

**Keywords:** intraspecific variation, plant chemistry, trophic interactions, resource availability, soil heterogeneity

**Posted Date:** March 3rd, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1347564/v1>

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**Multiple and contrasting pressures determine intraspecific phytochemical variation in a tropical shrub**

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Running Head: Intraspecific phytochemical variation

**Author Contribution**

A.E.G. wrote the first draft of the manuscript. W.P.C., L.A.D., A.M.S., and L.A.R. contributed substantially to revisions. A.E.G., L.A.D., A.M.S, C.S.J., and G.L.H. generated hypotheses and designed experiments. L.A.D., A.M.S., and C.S.J. funded experiments. A.E.G., A.M.S., and G.L.H. collected the field data. A.E.G., C.S.J., C.D.D., and C.S.P. conducted chemical analyses. A.E.G., L.A.D., and L.A.R. conducted statistical analyses. G.L.H. identified *Piper imperiale* plants and *Eois apyraria* caterpillars in the field.

23 **Abstract:** Intraspecific phytochemical variation across a landscape can cascade up trophic  
24 levels, potentially mediating the composition of entire insect communities. Surprisingly, we  
25 have little understanding of the processes that regulate and maintain phytochemical variation  
26 likely because these processes are complex and operate simultaneously both temporally and  
27 spatially. To assess how phytochemistry varies within species, we tested the degree to which  
28 resource availability, contrasting soil type, and herbivory generate intraspecific chemical  
29 variation in growth and defense of the tropical shrub, *Piper imperiale* (Piperaceae). We  
30 quantified changes in both growth (*e.g.*, nutritional protein, above- and below-ground biomass)  
31 and defense (*e.g.*, imide chemicals) of individual plants using a well-replicated fully factorial  
32 shade-house experiment in Costa Rica. We found that plants grown in low light and nutrient-  
33 poor residual soil had *reduced* foliar protein, while those grown in high light and richer old  
34 alluvial soil had *increased* protein. Thus, investment into growth was determined by resource  
35 availability and soil composition. Surprisingly, we found that chemical defenses *decreased* in  
36 response to herbivory. We also found that changes in plant protein were more plastic compared  
37 to plant defense, indicating that constitutive defenses may be relatively fixed, and thus an  
38 adaptation to chronic herbivory that is common in tropical forests. We demonstrate that  
39 intraspecific phytochemical variation of *P. imperiale* is shaped by resource availability from  
40 light and soil type. Because environmental heterogeneity occurs over small spatial scales (tens of  
41 meters), herbivores may be faced with a complex phytochemical landscape that may regulate  
42 how much damage any individual plant sustains.

43

44 **Keywords:** intraspecific variation, plant chemistry, trophic interactions, resource availability,  
45 soil heterogeneity

## 46 **Introduction**

47 Phenotypic plasticity determines the degree of intraspecific variation in key traits, which in  
48 turn modifies species interactions across food webs (Turcotte and Levine 2016). Parasites can  
49 cause changes in the chemical profiles of their hosts, and this occurs in a broad array of  
50 phylogenetically distinct taxa and in nearly all ecosystems. For example, herbivory, changes in  
51 resource availability, or both can induce chemical defenses in algae (Van Alstyne 1988, Cronin  
52 and Hay 1996), freshwater macrophytes (Morrison and Hay 2011), endophytes (Saikkonen et al.  
53 2013), and herbs, shrubs, and trees (Fowler and Lawton 1985, Kessler and Kalske 2018, Moore  
54 et al. 2014, Glassmire et al. 2016, Dyer et al. 2018). Most studies on intraspecific variation in  
55 phytochemical defenses have either focused on specific mechanisms in isolation, such as induced  
56 defenses, or have only examined interspecific differences among species (Feeny 1976, Rhoades  
57 and Cates 1976, Herms and Matson 1992, Hay 2002, Fink 2007, Smilanich et al. 2016). This  
58 metabolic variation that occurs within species is a key dimension of diversity, but surprisingly,  
59 the putative ecological processes that determine intraspecific variation in plant defense are rarely  
60 compared experimentally and are thus poorly understood (Morrison and Hay 2011, Hahn and  
61 Maron 2016, Hunter 2016). Phytochemical defenses are hypothesized to shape herbivore host  
62 plant use and may underlie patterns of diversification in phytophagous insects (Endara et al.  
63 2017). Across most taxa and ecosystems, the processes that cause intraspecific variation in  
64 phytochemical landscapes are mostly unresolved, even for abundant species (Hunter & Price  
65 1992, Hunter 2016). Here, we directly compare different sources of intraspecific variation in  
66 chemical defense by experimentally quantifying the degree to which key limiting resources (light

67 and nutrients), enemies, and soil type, mediate intraspecific variation in nutritional and defensive  
68 secondary metabolites.

69         Intraspecific phytochemical variation is shaped by top-down and bottom-up pressures,  
70 and it is likely that simultaneous effects of resource availability and herbivory impact plant  
71 fitness across the landscape (Hunter & Price 1992, Massad et al. 2012, Hahn and Maron 2016,  
72 Hunter 2016). Variation in resource availability acts as a bottom-up pressure that regulates  
73 growth and defense (Coley et al. 1985, Loomis 1932, Herms and Mattson 1992, Stamp 2004).  
74 Similarly, changes in availability of both light and nutrients are hypothesized to cause tradeoffs  
75 between plant growth and defense. For example, when plants obtain nutrients and light beyond  
76 their physiological demands, then resources can be diverted from growth toward defense. In  
77 contrast, herbivory is a top-down pressure that can alter plant metabolism; the focus of these  
78 effects has been on changes in plant defensive compounds and relies on the assumptions that  
79 certain compounds are metabolically expensive and that they are produced to mitigate additional  
80 damage from parasites (Myers 1988, Agrawal and Karban 1999, Dicke and Hilker 2003). While  
81 we have clear predictions of individual responses to variation in both resource availability and  
82 herbivory, we do not have clear predictions of how these top- and bottom-up processes interact.  
83 Thus, we do not have a realistic assessment of what underlies intraspecific phytochemical  
84 variation. Even more, some of these factors may have contrasting effects that likely negate the  
85 effects of any single process or factor. Overall, there is a compelling need to evaluate the  
86 individual and interactive effects of abiotic and biotic processes on intraspecific phytochemical  
87 variation.

88         A second key gap in resolving the putative ecological processes that determine intraspecific  
89 variation in plant defense is understanding the role of soil heterogeneity on phytochemical

90 variation (Fine et al. 2006, Cacho and Strauss 2014, Glassmire et al. 2017). Soils are dynamic  
91 and heterogeneous across the landscape because of changes in chemical and physical properties  
92 due to climate, parent material, topography, the resident plant and microbial community, and  
93 many other factors (Clark et al. 1998, Laliberte et al. 2013, van der Putten et al. 2013).  
94 Interactions between plants and their soil microbes create positive and negative soil feedbacks  
95 (e.g., Bever 2003, Pendergast et al. 2013) to such an extent that they can alter growth, leaf  
96 chemistry, and allelopathic potential among co-occurring plant species (Miener et al. 2017).  
97 Contrasting soil type likely underlies a considerable proportion of intraspecific variation in plant  
98 defensive chemistry and growth, although it is rarely considered in studies of intraspecific  
99 phytochemical variation (Massad et al. 2012, Hunter 2016). Resolving this gap requires  
100 conducting controlled experiments that manipulates soil type, in addition to resource availability  
101 and herbivory to obtain a more rigorous understanding of how ecological processes mediate  
102 intraspecific phytochemical variation.

103         We tested the degree to which resource availability, contrasting soil type, and herbivory  
104 generate intraspecific chemical variation by manipulating these putative sources of intraspecific  
105 phytochemical variation for a common understory shrub, *Piper imperiale*, (Piperaceae) in a  
106 lowland tropical rainforest in Costa Rica. We examined whether there were tradeoffs between  
107 plant growth and defense using a full factorial design where we manipulated soil nutrients, light  
108 availability, soil type, and the presence or absence of herbivory. We addressed the following  
109 questions: (1) What are the relative effects of soil nutrients, light availability, soil type, and  
110 herbivory on plant defensive chemistry? (2) To what degree do these factors mediate plant  
111 growth? (3) To what degree do these factors interact and contribute to intraspecific variation in  
112 growth and defense? Currently, there is a considerable knowledge gap about tradeoffs between

113 growth and defense among conspecifics and whether these tradeoffs occur when there is  
114 variation in resource availability herbivory, and the soil substrate. Each of these processes vary  
115 across small spatial scales beneath forest understories and can change phytochemical variation  
116 on the scale of meters thus affecting entire communities. Overall, the degree that each of these  
117 process shapes intraspecific phytochemical variation is poorly understood.

## 118 **Materials and Methods**

119 *Plant Study System* – We conducted this study in Costa Rica from June – December  
120 2014 at the La Selva Biological Station (10.4306° N, 84.0070° W), a lowland rainforest that  
121 receives ~4000 mm of precipitation annually (Clark 2013). *Piper imperiale* C.DC. (Piperaceae)  
122 is an abundant and shade tolerant shrub that is one of 54 co-occurring *Piper* species at La Selva  
123 and is a model plant system to test hypotheses based on chemical defense (Dyer and Palmer  
124 2004; Gentry 1990). Its chemical defenses are well described and include two biosynthetically  
125 related nitrogen containing imides, sintenpyridone and piplaroxide, (where sintenpyridone is the  
126 precursor of piplaroxide, Figure 1), and at least five different sesquiterpenes, all of which likely  
127 contribute synergistically to plant defense (Fincher et al. 2008). *Piper imperiale* invests in  
128 relatively lower concentrations of more highly toxic defensive compounds compared to other  
129 *Piper* species (1.7% dry weight, Fincher et al. 2008, Richards et al. 2010).

130 *Experimental Design* – We manipulated soil type, nutrient additions, and herbivory using  
131 a factorial design that was nested within low and high light levels. Our treatments included: 1)  
132 nutrient-poor old alluvial versus a richer residual soil type, 2) low versus high nutrient additions,  
133 and 3) presence or absence of herbivores, totaling 16 treatment combinations. Each treatment  
134 combination was replicated 10 times for a total of 160 plant individuals that were randomly

135 assigned to each treatment combination (Figure S1). Individual vegetative cuttings of *P.*  
136 *imperiale* were collected throughout La Selva from 12 different plant populations.

137 *Soil type, nutrient additions, and herbivore manipulations* – Both light and soil nutrient  
138 availability are limiting and highly patchy within understories of closed canopy tropical forests  
139 (*for light* Montgomery and Chazdon 2002, *for nutrients* see Wright et al. 2011, Pasquini et al.  
140 2015). Light availability also influences plant phytochemistry; higher light levels are associated  
141 with 17% increase in total imide content in *Piper cenocladum* shrubs (e.g. sintenpyridone, 4'-  
142 desmethylpiplartine, and cenocladimide, Dyer et al. 2004). We used reflective shade cloth  
143 (AgFabric, Corona, CA) to create two levels of light availability (within an 8 X 3 X 3 m  
144 enclosure), an ambient level that commonly occur in forest understories, and a deep shade  
145 treatment (see below). We measured photosynthetically active radiation throughout the day  
146 beneath our two light treatments using H21-002 HOBO micro station data logger with two S-  
147 LIA-M003 sensors (MicroDAQ, Ltd., Contoocook, NH, USA). Our low light treatment reduced  
148 light to 2% full sun and ranged from a minimum of 1.2  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to a maximum of 106.2  
149  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Our high light treatment reduced light to 30% full sun and ranged from a minimum  
150 of 1.2  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to a maximum of 363.7  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (t-test,  $P < 0.001$ ). The low light  
151 treatment mimicked deeply shaded understory patches that occur beneath the shrub and herb  
152 layer, and a high light treatment that occurs in the understory above this layer (Montgomery and  
153 Chazdon 2002). All individual plants were rotated within the light treatment block every two  
154 weeks throughout the duration of the experiment to ensure homogeneity in light and  
155 environmental conditions of the shade-house. We watered plants daily during the first two weeks  
156 of establishment and then twice weekly for weeks three and four. Thereafter, plants received  
157 ample ambient precipitation via perforations in the shade cloth.

158 We manipulated soil type by collecting the two main soils (alluvial and residual) that  
159 occur throughout La Selva (Sancho and Mata 1987). Old alluvial soils are clay soils with  
160 intermediate nutrient content, high water holding capacity, and low drainage, whereas residual  
161 soils are derived from highly weathered lava flows and are nutrient poor with high drainage  
162 (Sancho & Mata 1987, Clark et al. 1998). Contrasting plant communities occur on these two soil  
163 types (*for extensive details see* Sancho & Mata 1987, Clark et al. 1998). We collected soil from  
164 four different areas for each soil type by excavating soil down to 15 cm. We bulked and  
165 thoroughly mixed soil within a soil type before use. Plant individuals from each population were  
166 randomly assigned to one of the two soil types and grown for one month in plastic pots (3.79 L)  
167 prior to applying the nutrient and herbivory treatments.

168 We manipulated nutrient resources by adding both macro and micronutrients to the soil.  
169 Recent research demonstrates that soil nutrients limit plant performance even in deeply shaded  
170 forest understories (*reviewed by* Wright et al. 2011, Griffin et al. 2017). Higher nutrient  
171 additions are associated with 3% increase in total imide content in *Piper cenocladum* shrubs (e.g.  
172 sintenpyridone, 4'-desmethylopiplartine, and cenocladimide, Dyer et al. 2004). We had high (10  
173 grams of macro- and micronutrients per m<sup>2</sup>) and low (2 grams of macro- and micronutrients per  
174 m<sup>2</sup>) nutrient addition treatments; macronutrients included NPK 12-8-16 and micronutrients  
175 included magnesium, calcium, copper, sulfur, iron, boron, and zinc (Blaukorn classic, Kyiv,  
176 Ukraine). Nitrogen was added in two forms, nitrate and ammonium.

177 Finally, we manipulated herbivory using the specialist native herbivore, *Eois apyraria*,  
178 which commonly causes extensive damage on *P. imperiale* (Fincher et al. 2008, Hansen et al.  
179 2017). *Eois apyraria* is a leaf chewer and bioassays have shown conclusively that the defensive  
180 sintenpyridone and piplaroxide chemicals reduce the survival and performance of *E. apyraria*

181 (Fincher et al. 2008, Richards et al. 2010, Hansen et al. 2017). We placed two caterpillars on  
182 plants that were assigned herbivory. We added new caterpillars to plants assigned herbivory  
183 whenever old ones pupated, died, or disappeared for the 6-month duration of the experiment. We  
184 quantified the absolute amount of herbivore damage per plant, which ranged from 0 - 30 % leaf  
185 area reflecting natural levels of damage on *P. imperiale* (see Fincher et al. 2008).

186 *Harvest* - Plants were harvested in December 2014, six months after commencing the  
187 experiment. Leaf Surface Area (LSA) of freshly harvested leaves was measured using LI-3100  
188 Leaf Area (LI-COR, Inc., Lincoln, NE, USA). Total herbivore damage was assessed for all  
189 leaves on each plant by quantifying the Leaf Area Removed (LAR) due to herbivory using LI-  
190 3100 Leaf Area (LI-COR, Inc., Lincoln, NE, USA). Plant stems, roots, and leaves were  
191 separated, dried at 28 °C for three days, weighed, and stored in paper bags at -28 °C until  
192 chemical analyses were conducted.

193 *Foliar defense chemical and protein analyses* – Samples of dried leaves from individuals  
194 from each of the sixteen shade-house treatments were extracted for quantification using seven-  
195 point calibrations. Defensive imide chemical response units are reported as percent dry weight.  
196 We modified a chemical extraction protocol specific to *Piper imperiale* (Dodson et al. 2000).  
197 Briefly, 1.00 g samples aliquot from each plant were extracted with 5 mL of 1:1 aqueous  
198 methanol and 5 mL of chloroform. This mixture was sonicated for 20 minutes and centrifuged to  
199 allow layers to separate. The organic layer was retained before repeating this liquid-liquid  
200 extraction two more times by adding 5 mL of chloroform to the aqueous layer. Organic extracts  
201 were pooled and 1.00 mL aliquots of samples for each plant were combined with commercially  
202 available piperine (MilliporeSigma; St. Louis) as an internal standard (final concentration: 80.0  
203 ug/L) and analyzed using a Gas Chromatography–Flame Ionization Detector or GC-FID (Agilent

204 7890A; Santa Clara, CA). Seven-point calibrations (11.72, 23.44, 46.88, 93.75, 187.5, 375.0, and  
205 750.0 ug/L) were prepared using imides **1** ( $R^2=0.999$ ) and **2** ( $R^2=0.999$ ). External standards for  
206 analysis were provided by the Joe Richards lab (Colorado Mesa University, Grand Junction, CO;  
207 Dyer et al. 2004). The sintenpyridone imides are unstable in solution, so all reported  
208 concentrations are estimates (Dodson et al. 2000). For a detailed description of GC methods see  
209 Dodson et al. (2000) and Dyer et al. (2001).

210 Total foliar protein concentration for each plant was quantified using the Pierce BCA  
211 Protein Assay protocol (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, two mg of  
212 dried leaf material was extracted from each plant with 500  $\mu$ L of buffer (100 mM Tris pH 7.5,  
213 150.00 mM NaCl, 10.00 mM  $MgCl_2$ , and 1% of SDS). The extracted protein was quantified  
214 using a six-point calibration curve of albumin (0.0, 4.0, 10.0, 20.0, 40.0 and 60.0  $\mu$ g/mL). Foliar  
215 protein extractions were replicated for each plant three times to ensure accuracy. The samples  
216 and protein standard were analyzed using a SpectraMax M Series Multi-Mode Microplate  
217 Reader (Molecular Devices, LLC., Sunnyvale, CA, USA) measuring absorbance at 560 nm. The  
218 units of total protein concentration for each sample are reported as the protein mass fraction  
219 relative to plant dry weight.

220 *Statistical Analyses* – All analyses were conducted in program R version 3.6.2 (R Core  
221 Team 2018). Analyses were run separately for individuals exposed to high versus low light  
222 conditions, due to the availability of one shade-house per light level. We conducted 3-way  
223 ANOVAs for each response variable. All analyses included interactions between predictor  
224 variables. For response variables that were significant and intercorrelated (*i.e.* as in biomass or  
225 chemical compounds), multivariate analysis of variance (MANOVA) was used to examine  
226 responses of plant chemical traits to soil type, nutrient additions, and presence or absence of

227 herbivores. The response variables for plant biomass (g) were above-ground based on total mass  
228 of leaves and stems and below ground based on total mass of roots. The response variables for  
229 plant chemistry were protein and the known defense compounds sintonpyridone (1), and  
230 piplaroxide (2) calculated as percent dry weight. Profile analysis followed all MANOVAs, which  
231 compares effects of predictors for each response variable (Tabachnick & Fidel 1989; Tabachnick  
232 et al. 2001). Residuals of all models were approximately normally distributed. We also report  
233 standardized Cohen's d and raw effect sizes to help interpret biological effects of manipulations  
234 (Tables S1-S5, Cohen 1994; Torchiano2020). We used the 'effsize' package to calculate Cohen's  
235 d (Torchiano 2020).

236 We paired traditional MANOVA analyses with structural equation models (SEM using  
237 the 'lavaan' and 'blavaan' packages, Rosseel 2012) and hierarchical Bayesian models (using  
238 'rjags' package, Plummer 2021) to test specific *a priori* hypotheses, to examine specific direct  
239 and indirect effects, and to estimate relationships among endogenous variables. The hierarchical  
240 Bayesian models treated the two light experiments as the upper level of the hierarchy, with all  
241 treatment level combinations nested in the lower level; these analyses were used to support  
242 inferences about the different results found in the two different light levels. The Bayesian  
243 approaches were also utilized to minimize Type II error - providing probabilities of nulls for  
244 parameter estimates that were close to zero and that had high alpha values in the MANOVAs or  
245 SEMs.

## 246 **Results**

247 The results for high versus low light levels are described separately for ease of interpretation and  
248 because soil type, nutrients, and herbivore treatment combinations were nested within each light  
249 treatment. However, complex differences in the high and low light experiments were clearly

250 supported by the hierarchical Bayesian models, 95% credibility intervals did not cross zero  
251 (Figure S2), with substantial overall decreases in biomass, protein, and chemical defense for  
252 plants in low light versus highlight experiments (Figure S1). Inferences of "nonsignificant"  
253 effects were supported by the Bayesian models, with 50% credibility intervals of parameter  
254 estimates for nonsignificant effects crossing zero (Figures S3-S6). For all statistical models, plant  
255 biomass and protein were a proxy for growth, while the defensive imide compounds were a  
256 proxy for defense.

### 257 ***High Light Conditions***

258 *Plant biomass* – Nutrient enrichment (low versus high) significantly increased above-  
259 ground biomass by 41% (low nutrients  $\bar{x} = 16 \text{ g} \pm 1.14 \text{ SE}$  compared to high nutrients  $\bar{x} = 22.5$   
260  $\text{g} \pm 1.54 \text{ SE}$ ; Figure S7) and below-ground biomass by 33% (low nutrients  $\bar{x} = 5.4 \text{ g} \pm 0.38 \text{ SE}$   
261 compared to high nutrients  $\bar{x} = 7.1 \text{ g} \pm 0.61 \text{ SE}$ ; Figure 2, Figure S7). Our results demonstrate  
262 that soil nutrients increase plant biomass additively (above biomass *MANOVA*:  $F_{1,71} = 10.89$ ,  $P <$   
263  $0.01$ ; below biomass *MANOVA*:  $F_{1,71} = 5.65$ ,  $P = 0.02$ ; Figure 2, Table S1).

264 *Foliar protein and defensive chemistry* – A three-way interaction among herbivory, soil  
265 type, and nutrient enrichment increased the amount of foliar protein (*significant interaction*  
266 *MANOVA*:  $F_{1,52} = 3.31$ ,  $P = 0.07$ ; Figure 3, Table S4). High levels of herbivory on plants grown in  
267 alluvial soils with high nutrient enrichment caused a 55% increase in foliar protein, however, if  
268 these plants were also grown at low nutrient levels, then there was only a 2% increase in foliar  
269 protein (Figure 3, Figure S8). The path analysis confirmed the finding that the damage caused  
270 by herbivores increased foliar protein by 13% (*path analysis*:  $\text{spc} = 0.13$ ; Figure 4). Furthermore,  
271 because the effect sizes were relatively large across the combination of herbivory, soils, and

272 nutrients, it is clear that a combination of these factors together caused increases in foliar protein  
273 (*refer to tables for effect sizes and summary stats*, Table S3, Figs. S8-S10).

274 For defensive imide compounds, the path analysis highlighted the strong, positive effect  
275 of sintenpyridone on piplaroxide, which is the biosynthetic precursor to the formation of  
276 piplaroxide, and thus regulates how much piplaroxide is produced (*path analysis: spc = 0.65*;  
277 Figure 4). We also found that nutrient enrichment caused contrasting effects on the levels of  
278 imide compounds in residual soils. Specifically, plants grown in residual soil, suffering high  
279 levels of herbivory, and low levels of nutrient additions caused *reductions* in piplaroxide by 99%  
280 and sintenpyridone by 58%, whereas high nutrient additions caused an increase in piplaroxide by  
281 67% (*significant interaction for piplaroxide MANOVA:  $F_{1,52} = 3.21, P=0.07$* ; Figure 5). Plants  
282 grown in old alluvial soil, having high levels of herbivory, and low levels of nutrient additions  
283 caused reductions in piplaroxide by 33% and sintenpyridone by 2%, whereas high nutrient  
284 additions further reduced piplaroxide by 75% (*significant interaction for piplaroxide MANOVA:*  
285  *$F_{1,52} = 3.21, P=0.07$* ; Figure 5). Counter to our predictions, herbivory alone caused a decline in  
286 sintenpyridone by 21% (*path analysis: spc = -0.21*; Figure 4, Figures S11-S13). Overall, the  
287 production of the defensive piplaroxide compound was mediated by an interaction between soil  
288 type, herbivory, and nutrient enrichment and these factors typically vary over fairly small spatial  
289 scale throughout forest understories (*refer to tables for effect sizes and summary stats*, Table S3,  
290 Table S5).

### 291 ***Low Light Conditions***

292 *Plant biomass* – Nutrient enrichment (low versus high) caused above-ground biomass to  
293 increase by 31% (low  $\bar{x} = 14.7 \pm 1.05$  g vs. high  $\bar{x} = 19.3 \pm 1.35$  g), even under the low light  
294 conditions that are characteristic of deeply shaded understories (Tables S1-S2, Figure S7).

295 Nutrient enrichment did not significantly change below-ground biomass (low  $\bar{x} = 3.3 \pm 0.25$  g  
296 vs. high  $\bar{x} = 4.1 \pm 0.46$  g; Tables S1-S2, Figures S7). Plants established in old alluvial soils  
297 having high levels of nutrient enrichment and herbivory caused above-ground plant biomass to  
298 decrease by 91%, whereas low levels of nutrient enrichment increased above-ground biomass by  
299 96% (*significant interaction MANOVA:  $F_{1,71} = 3.75, P=0.05$* ; Figure 2, Tables S1-S2).

300 *Foliar protein and defensive chemistry under low light* – High nutrient additions caused a  
301 reduction of foliar protein by 20 % compared to low nutrient additions (*additive effect MANOVA:*  
302  *$F_{1,59} = 2.94, P=0.09$* ; Figure S8, Table S4). Plants grown in residual soils with high levels of  
303 herbivory and nutrient additions reduced protein by 167%, although this was not significant  
304 (*three-way interaction MANOVA:  $F_{1,59} = 0.13, P > 0.7$* ; Figure 3, Table S4). For chemical  
305 defense, sintenpyridone significantly increased piplaxoxide, although the strength of this effect  
306 decreased under low light compared to high light (*path analysis:  $\text{spc} = 0.46$* ; Figure 4).  
307 Herbivory decreased the amount of sintenpyridone (*path analysis:  $\text{spc} = -0.18$* ; Figure 4; Figures  
308 S11-S13). There were no significant interactions among nutrients, herbivory, and soil type with  
309 regard to leaf protein and the defensive compounds (*MANOVA summary statistics in Table S3*  
310 *and Table S5*).

## 311 Discussion

312 The cause of intraspecific phytochemical variation is poorly understood even though we  
313 know that the consequences of intraspecific phytochemical variation can cascade up to higher  
314 trophic levels. We examined how ecological processes due to resource availability, herbivory,  
315 and contrasting soil type influenced patterns of resource allocation between growth or defense  
316 for a common and geographically widespread understory shrub, *Piper imperiale*. We found that  
317 high light availability increased plant allocation toward protein production and high levels of

318 nutrient additions increased plant allocation toward biomass, which is an important indicator of  
319 growth. Pervasive interactions among nutrient availability, herbivory, and contrasting soils  
320 determined the degree to which plants allocated resources to chemical defense. Surprisingly,  
321 herbivory decreased sinentpyridone by 31% and piplaroxide by 36%. Moreover, we found  
322 piplaroxide decreased as much as 99% in response to herbivory, but only for plants grown in  
323 residual soils, and under low nutrient additions. Conversely, high levels of nutrient additions on  
324 plants grown in residual soils under high light conditions caused piplaroxide to increase by 67%,  
325 but plants grown in old alluvial soils under the same conditions caused piplaroxide to decrease  
326 by 75%. These results provide compelling evidence that the effect of herbivory on growth and  
327 defense is mediated by soil type and nutrient availability. We also found the imide  
328 phytochemicals followed the classic metabolic patterns of constitutive defenses and did not  
329 increase in response to herbivory. These results demonstrate unequivocally that the degree to  
330 which herbivory changes plant defensive chemistry is determined by variation in resource  
331 availability (light and soil nutrients) and soil type. An important consequence of these results is  
332 that small-scale variation in key factors such as light availability will likely create mosaics of  
333 phytochemical variation among conspecifics. Herbivores must then “sample” this chemical  
334 variation when searching for and “evaluating” the suitability of host plants; such variation  
335 hinders herbivore acclimation to chemistry and may well lower overall herbivore loads on a plant  
336 species across its range (Adler and Karban 1994, Underwood 2014, Barbosa et al. 2009, Hunter  
337 2016, Glassmire et al. 2019). In sum, these results demonstrate that to evaluate our hypotheses  
338 and understand intraspecific variation in phytochemistry we must simultaneously consider the  
339 availability of key limiting resources, soil mosaics, as well as the presence and absence of  
340 herbivores. While this adds complexity to our understanding of phytochemistry and plant

341 defense, it means we cannot ignore abiotic factors that are well known to structure forest  
342 understories (Clark et al. 1998, Wright et al. 2011). It also means that herbivores are almost  
343 certainly encountering a highly variable phytochemical landscape on relatively small spatial  
344 scales that are relevant to these herbivores finding suitable hosts.

345

346 *Light initiated a reduction in plant growth*

347 Decreasing light availability caused a reduction in plant biomass and foliar protein, an indicator  
348 for plant growth. This, however, was not observed for the defensive compounds. Both the  
349 quantity and quality of light availability changes substantially throughout tropical forest  
350 understories (e.g., Chazdon & Fetcher 1984), and can cause major changes in intraspecific  
351 chemical variation in Ecuadorian *Piper* shrubs across small spatial scales (Glassmire et al. 2019).  
352 While it is well known that light is a critical limiting resource for understory plants, particularly  
353 in tropical forests, oddly, it is rarely measured in studies focused on tradeoffs between growth  
354 and defense among conspecifics or heterospecifics (Hahn and Maron 2016, Hahn et al. 2019).  
355 Effect sizes for plant growth documented here were substantive; high light levels increased  
356 proteins related to plant growth by 55% (Figure 3, Table S3), whereas low light levels decreased  
357 the production of proteins by -167% (Figure 3, Table S3). Leaves are costly for plants to produce  
358 under low light conditions (Bryant et al. 1983, Lerda & Coley 2008), and we found that plants  
359 invested in foliar protein in high light environments but not in low light environments.

360

361 *Complex relationships determined the production of defensive compounds*

362 The concentrations of secondary metabolites depended substantially on conditions and  
363 factors known to vary widely across tropical forest landscapes, specifically resource availability,

364 soil type, and herbivory. It is worth noting the substantial effect sizes of manipulations on  
365 chemistry - the percent dry weight of piperoxide increased by 67% when favorable light and soil  
366 resources were applied, while this defensive compound decreased by 75% when plants were  
367 exposed to high levels of herbivory (Figure 5). Growing plants in different soil types, exposing  
368 them to different levels of herbivore damage, and changing resource availability interacted to  
369 modify the production of key defensive compounds. There is little doubt that variation in these  
370 conditions and processes will create a very complex chemical landscape in tropical forests,  
371 whereby herbivores will encounter host plants that vary substantially in host plant quality. We  
372 speculate that this variation is likely the norm for most plant species and future research should  
373 explore, not only this possibility, but the consequences of this variation for herbivore  
374 assemblages and community processes, such as trophic cascades. What we do know is that even  
375 subtle changes (0.2 % dry weight) in *Piper* imide levels can seriously disrupt the immune  
376 response of specialist herbivores (Hansen et al. 2017). Moreover, Richards et al. (2010)  
377 demonstrated that synergistic effects of three *Piper imperiale* defensive compounds, including  
378 piperoxide, even at very low amounts (< 0.1% dry weight), can decrease survival in herbivores  
379 by as much as 25%. Prior to this research, it would be difficult to make the case that soil type  
380 would be the template upon which there were numerous interactions with herbivory and resource  
381 availability, which together determined chemical changes in *P. imperiale*. These findings show  
382 that soil type can be key to determining community interactions (Clark et al. 1998, Fine et al.  
383 2006, Cacho and Strauss 2014) and must be considered in future studies (Hahn et al. 2016). The  
384 striking differences in forest communities found at La Selva between our two soil types (Clark et  
385 al. 1998) may be at least partly due to their impacts on plant chemical defenses, which may  
386 cascade up and down trophic webs to mediate forest plant species composition and diversity.

387           When there are sharp differences among soil types, selection can lead to the evolution of  
388 distinct plant traits adapted to different soils, even when plant species are in fairly close  
389 proximity (Fine et al. 2004). For example, Misiewicz and Fine (2014) found evidence of local  
390 adaptation to soils, with significant morphological variation, higher levels of genetic  
391 differentiation, and lower migration rates among populations of the tropical tree *Protium*  
392 *subserratum* growing parapatrically on white-sand, brown-sand, and clay soils in Peruvian  
393 Amazonia. Similarly, Cacho and Strauss (2014) found the shift for plants to serpentine soils from  
394 bare, sparsely vegetated ground was extremely important for plant diversification in the  
395 Streptanthoid complex (Brassicaceae). Plants have a long history of being edaphic specialists and  
396 soils have unique micro-climates and biotic factors that plants must adapt to.

397

398 *Constitutive defenses may be a better defense against herbivores in tropical plant systems—*

399           We found little support for herbivory being solely responsible for up- or down-regulating  
400 plant chemical defenses. Herbivore damage caused *P. imperiale* to reduce the amount of  
401 defensive compounds *except* when plants are exposed to high levels of herbivory and when they  
402 are also grown on residual soils with nutrient additions and high light (Figs. 4-5). It is important  
403 to note that low light conditions and year-round pest pressure are the norm in deeply shaded  
404 tropical forest understories (Dyer and Letourneau 1999). Thus, induced defenses caused by  
405 herbivory may not apply to certain phytochemicals that are constitutive defenses and remain at  
406 high concentrations even when herbivores are absent (Bixenmann et al. 2016) and depend more  
407 on plant ontogenetic development (Fuchs and Bowers 2004, Quintero and Bowers 2011, Barton  
408 2013). We found, however, that nutrients and soil type were more consistent predictors of  
409 defensive state. Another experiment with three *Piper* species at La Selva found no effect of

410 herbivory on chemical defenses (Fincher et al. 2008). This suggests that some *Piper* plants in  
411 tropical forests produce and maintain a constant level of constitutive chemical defenses that are  
412 not inducible based exclusively on herbivory. Our results are in contrast to plant communities  
413 that experience seasonal pulses in pest pressure where plants rely more heavily on induced  
414 defenses for protection against herbivores (Williams & Whitham 1986, Karban et al. 1997,  
415 Agrawal 2001). Chemical defenses expressed by the plant are not a unified trait that can be  
416 defined simply as herbivore-induced or not, and our results show that limiting resources are a  
417 major component to plant defense strategies.

#### 418 **Conclusion**

419 Plants simultaneously experience multiple and sometimes contrasting pressures and growing  
420 conditions. Growth and defensive phytochemistry of *P. imperiale* exhibited tradeoffs depending  
421 on light availability and interactions among nutrient availability, contrasting soil types and  
422 presence or absence of herbivores. Our results demonstrate that light and soil heterogeneity,  
423 which vary from very small to larger spatial scales, can have dramatic effects on the  
424 phytochemical profile of plants, leading to intraspecific variation in defense and palatability.  
425 Whether or not this intraspecific variation found in *P. imperiale* leads to variation in plant fitness  
426 has yet to be tested, but implications from our data show that there most likely is not one  
427 phenotype that will be universally fit. Furthermore, plant defense theory and experimental  
428 designs focused on intraspecific chemical variation should consider multiple drivers of plasticity  
429 simultaneously rather than in isolation. These results are relevant to terrestrial and aquatic  
430 ecosystems alike, and a renewed focus on issues such as elemental stoichiometry and allocation  
431 to defense in terrestrial and aquatic systems (e.g., Sardans et al. 2012) will yield a clearer picture  
432 of how and why anti-parasite defense varies intraspecifically across the landscape.

433 **Acknowledgments:** We would like to thank Diego Salazar Amoretti and Bernal Matarrita  
434 Carranza for help establishing *Piper imperiale* individuals in the shade-house at La Selva.  
435 Furthermore, Earthwatch volunteers were a huge help with harvesting plants. The authors have  
436 no conflict of interest to report.

#### 437 **Declarations**

438 **Funding:** This research was funded by the Hitchcock Center for Chemical Ecology and  
439 supported by the Garden Club of America Award in Tropical Botany and by the National  
440 Science Foundation grant numbers DEB 1502059 and DEB 1442103.

441 **Conflicts of interest:** The authors have no conflicts of interest to declare.

442 **Ethics approval:** Not applicable.

443 **Consent to participate:** Not applicable.

444 **Consent for publication:** Not applicable.

445 **Availability of data and material:** This data has not been published elsewhere. Data is  
446 available in Dryad repository (DOI #) and on an open access website maintained by Lee A. Dyer  
447 <<http://caterpillars.org/>>.

448 **Code availability:** Not applicable.

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## Figure captions

620 **Figure 1.** Testing the degree to which resource availability, contrasting soil type, and herbivory  
621 generate intraspecific chemical variation via estimating tradeoffs between growth and defensive  
622 compounds. The top panel depicts the chemical structures of the two nitrogen-based defensive  
623 compounds that were quantified in this experiment. The imide compounds, (1) sintenpyridone  
624 and (2) piplaroxide, are biosynthetically related, where (1) is a precursor to (2). The bottom panel  
625 depicts the *Piper imperiale* individual plants used in this experiment with treatment level  
626 combinations. We used a factorial design and exposed established plant fragments to random  
627 treatment level combinations of either (a) low or high levels of light, (b) alluvial (light brown in  
628 image) or residual soils (black in image), and (c) herbivory (*Eois* herbivore shown feeding on  
629 leaf). (d) Above- and below-ground plant biomass were harvested and weighed for each plant.

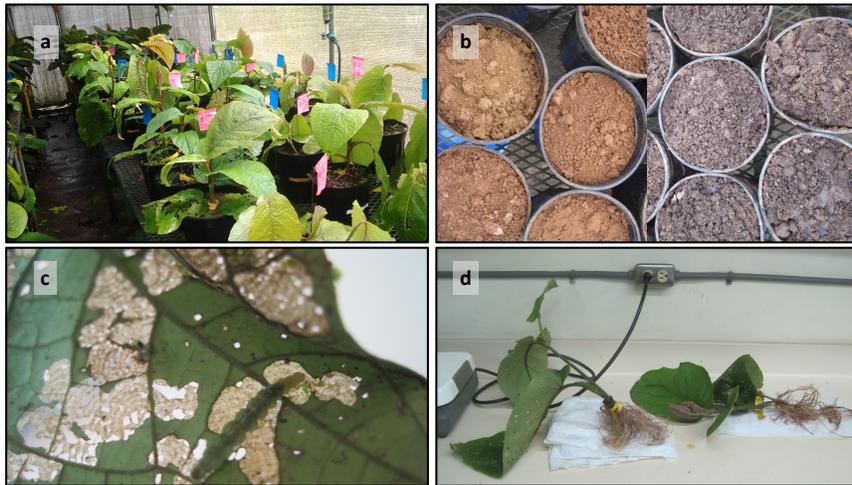
630 **Figure 2.** Comparisons of above- and below- ground plant biomass along an herbivory gradient  
631 from 0-30% are shown here. Low light is represented by the gray panels and high light is  
632 represented by the yellow panels. The green line represents aboveground biomass and the purple  
633 line represents belowground biomass. Herbivory generally decreased above-ground plant  
634 biomass and residual soil facilitated reductions in above- and below-ground plant biomass.

635 **Figure 3.** Protein expressed as percentage dry weight in leaf tissue along an herbivory gradient  
636 from 0-30%. Low light is represented by the gray panels and high light is represented by the  
637 yellow panels. In low light conditions, herbivory caused the greatest reductions of protein  
638 content, when plants were established in residual soils and with high nutrient additions.  
639 Conversely, in high light conditions, herbivory caused large increases in protein content,  
640 especially when plants were established in old alluvial soils and with enhanced nutrient  
641 additions.

642 **Figure 4.** Path analysis examining the direct and indirect effects of herbivory, contrasting soil  
643 type, and nutrient enrichments on plant growth and chemistry. The grey panel represents the  
644 plants established under low light levels ( $\chi^2 = 0.09$ ;  $df = 1$ ;  $P > 0.77$ ), while the yellow panel  
645 represents the plants established under high light levels ( $\chi^2 = 3.36$ ;  $df = 1$ ;  $P = 0.07$ ). Positive  
646 relationships have arrow heads and negative relationships have blunt ends.

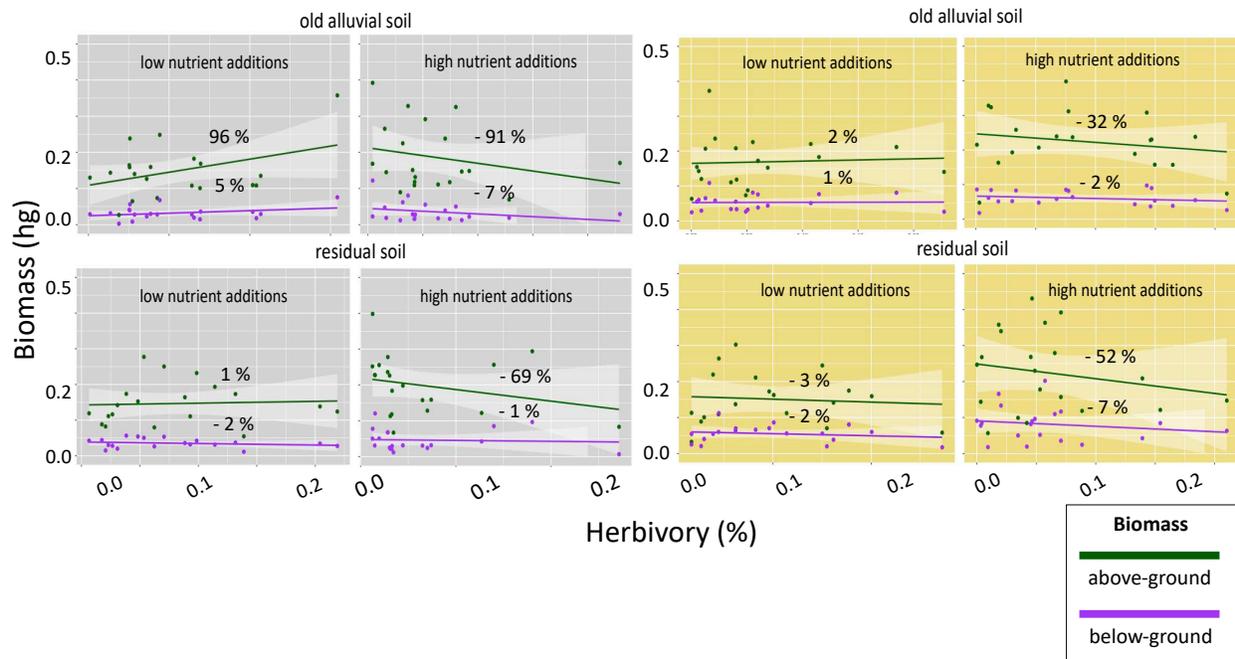
647 **Figure 5.** Defensive compounds expressed as percent dry weight in leaf tissue along an  
648 herbivory gradient from 0-30%. Low light is represented by the gray panels and high light is  
649 represented by the yellow panels. The purple line represents piplaroxide and the green line  
650 represents sintenpyridone. Sintenpyridone generally had stable levels no matter nutrient  
651 additions, herbivory, and contrasting soil type. Piplaroxide, however, fluctuated more and  
652 depended on the treatment combination. Herbivory caused piplaroxide to decrease in plants  
653 established in residual soils and with low nutrient additions under both low and high light levels.

**Figures**



654

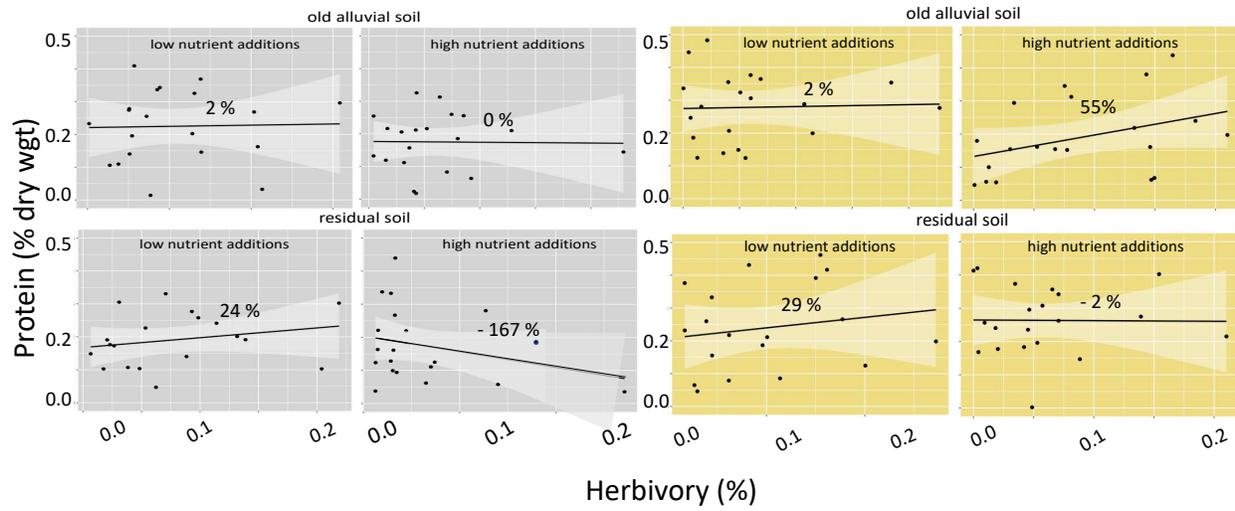
655 **Figure 1.**



656

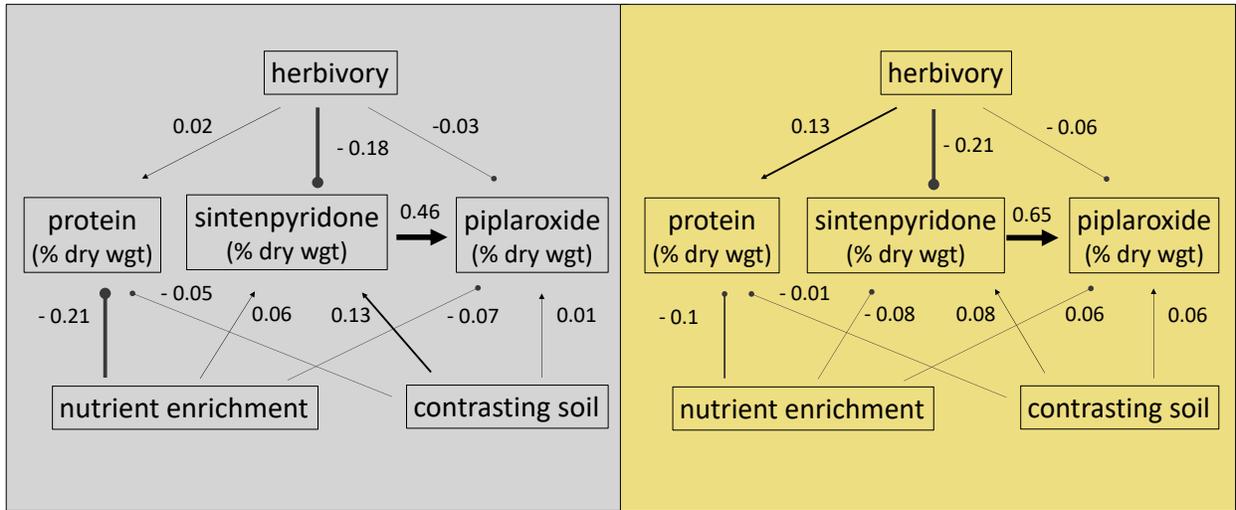
657 **Figure 2.**

658



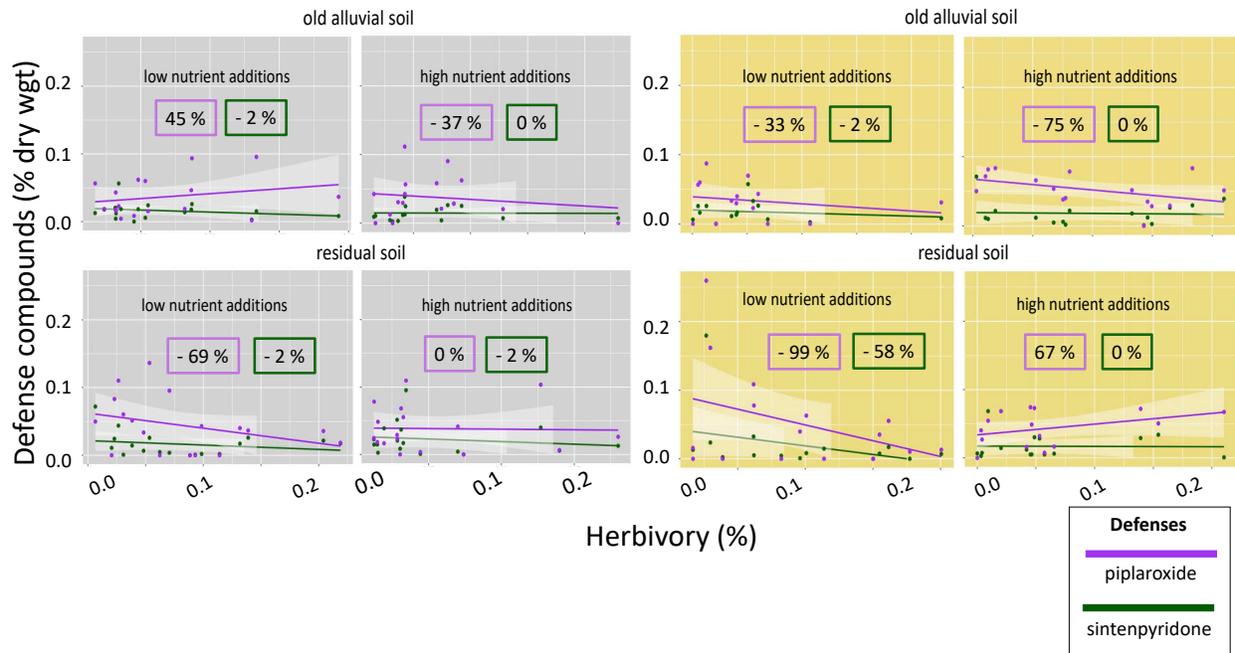
659

660 **Figure 3.**



661

662 **Figure 4.**



663

664 **Figure 5.**

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