GENOTYPIC VARIATION IN BELOW-TO ABOVEGROUND SYSTEMIC INDUCTION OF GLUCOSINOLATES MEDIATES PLANT FITNESS CONSEQUENCES UNDER HERBIVORE ATTACK

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Running title: Fitness impact of induced systemic resistance

30   **Abstract-** Plants defend themselves against herbivore attack by constitutively producing toxic

31   secondary metabolites, as well as by inducing them during herbivore feeding. Induction of secondary

32   metabolites can cross plant tissue boundaries, such as from root to shoot. However, whether the

33   potential for plants to systemically induce secondary metabolites from roots to shoots shows genetic

34   variability, and thus, potentially, is under selection conferring fitness benefits to the plants is an open

35   question. To address this question, we induced 26 maternal plant families of the wild species

36   *Cardamine hirsuta* belowground (BG) using the wound-mimicking phytohormone jasmonic acid (JA).

37   We measured resistance against a generalist (*Spodoptera littoralis*) and a specialist (*Pieris brassicae*)

38   herbivore species, as well as the production of glucosinolates (GSLs) in plants. We showed that BG

39   induction increased AG resistance against the generalist but not against the specialist, and found

40   substantial plant family-level variation for resistance and GSL induction. We further found that the

41   systemic induction of several GSLs tempered the negative effects of herbivory on total seed set

42   production. Using a widespread natural system, we thus confirm that BG to AG induction has a strong

43   genetic component, and it can be under positive selection by increasing plant fitness. We suggest that

44   natural variation in systemic induction is in part dictated by allocation trade-offs between constitutive

45   and inducible GSL production, as well as natural variation in AG and BG herbivore attack in nature.

46

47   **Key Words -** Brassicaceae, Fitness impact of herbivory, Generalist herbivore, Glucosinolates,

48   Induced systemic resistance, Plant-herbivore interaction.

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INTRODUCTION

52   The selective pressure of insect herbivores on plants has led to the evolution of a wide variety of

53   secondary metabolites that can intoxicate or inhibit digestion capacities of the herbivores during

54   feeding (Futuyma and Agrawal 2009; Schoonhoven et al. 2005). While secondary metabolites can be

55   constitutively stored in plant tissues prior to herbivore attack, herbivore feeding on one organ of a

56   plant can induce the *de novo* production, or increase accumulation of the toxins locally, on the same

57   organ, or systemically, on other organs of a plant (Kessler and Baldwin 2002). Within-plant induction

58   of toxic chemicals often reduces the performance of current or subsequent herbivores (Karban and

59   Baldwin 1997; Poelman et al. 2008), and therefore, locally- or systemically-induced chemical defenses

60   should be linked to plant fitness (Agrawal 1998; Agrawal 2000). Moreover, the induction of defenses

61   can cross widely-separated plant organs, such as travelling from roots to shoots (Bezemer et al. 2003).

62   Indeed, a growing body of literature is showing that a range of belowground (BG) organisms can

63   induce defense responses in aboveground (AG) tissues and *vice versa* (Bezemer et al. 2003; Erb et al.

64   2009; Rasmann and Agrawal 2008; Soler et al. 2005; Staley et al. 2007; van Dam et al. 2005).

65   Reviews on the topic suggest that the magnitude and direction of chemically-mediated AG-BG

66   interactions in plants largely depend on plant genotypic variation as well as the attacking species'

67   identity (Kabouw et al. 2011; van Geem et al. 2013; Vandegehuchte et al. 2011). Therefore,

68   potentially, selection is acting on plants' natural genetic variation to optimize their ability to induce

69   defenses systemically. While significant levels of genetic variation, as well as a heritable genetic basis

70   for both constitutive and inducible defense traits expression has been shown in several systems

71   (Agrawal et al. 2002; Havill and Raffa 1999; Humphrey et al. 2018; Stevens and Lindroth 2005;

72   Underwood et al. 2000; Wagner and Mitchell-Olds 2018), to date, we have practically no information

73   on whether BG-AG defense induction is under positive selection for plants harboring such trait

74   variation in nature. . Measuring BG to AG root induction is also ecologically-relevant since in nature,

75   plants can be potentially in contact with a wide range of root herbivores, that phenologically, can

76   induce the plants before the leaf herbivores arrive (Erb et al. 2008; Huang et al. 2017; Rasmann and

77   Agrawal 2008).

78   For a trait to be under selection, it needs to display a significant degree of genetically based variation

79   in nature. Whereas most of such variation is generated by random mutation, and evolutionary and

80   genetic mechanisms (Caliskan 2012), the maintenance of genetic variability can also be affected by

81   energetic costs. Optimal defense theory suggests that inducible defenses have evolved as a cost-saving

82   strategy, and the relative allocation of constitutive and inducible defenses in plant organs, individuals

83   or populations depends on predictability of attack from herbivores, the susceptibility of plants to

84   attacks, and the context dependency of the interaction (e.g. environmental variation) (Agrawal et al.

85   2002; McKey 1974; Zangerl and Bazzaz 1992; Zangerl and Rutledge 1996). In other words, the

86   simultaneous expression of constitutive and induced defense is thought to be costly (Brody and

87   Karban 1992; Rasmann and Agrawal 2009; Strauss et al. 2002; Zangerl and Bazzaz 1992) and should

88   result in negative genetic correlations (trade-offs) between individual traits and between defense

89   deployment strategies (Agrawal et al. 2010). Therefore, high constitutive expression of a defense trait

90   is predicted to be associated with lower induction abilities. While trade-offs between constitutive and

91   induced defenses on the same organs have been shown in several systems (Heil et al. 2004; Rasmann

92   et al. 2015; Rasmann et al. 2011; Thaler et al. 1999; Thaler and Karban 1997), we still lack evidence

93   for whether AG inducibility of defenses after BG induction is trading-off with constitutive defenses.

94

95   In brassicaceaous plants, glucosinolates (GSLs), Sulphur- and nitrogen-containing plant secondary

96   metabolites, are the main defensive compounds conferring plant resistance against insect herbivores

97   (Howe and Jander 2008). The defensive function of GSLs breakdown products, when either expressed

98   constitutively or when induced, against both specialist and generalist insect herbivores has been amply

99   documented (Agrawal 1998; Agrawal 2000; Agrawal et al. 2002; Baldwin 1998; Karban and Baldwin

100 1997; van Dam and Oomen 2008; van Dam and Raaijmakers 2005; van Dam et al. 2005). Several

101 individual GSLs show strong inducibility following herbivory and generally, the plant hormone

102 jasmonic acid (JA) is a key player in the regulation of induced plant responses against chewing

103 herbivores such as caterpillars (Farmer et al. 2003; Howe and Jander 2008). Emerging patterns from

104 studies on *Brassica* spp. indicate that BG insect herbivory, or JA application to roots increase total

105 GSLs levels in shoots (Griffiths et al. 1994; Pierre et al. 2012; Soler et al. 2005; van Dam et al. 2004).

106 For instance, previous work with *Cardamine hirsuta* demonstrated that the overall abundance and

107 identity of GSLs in the leaves is affected by JA induction in the roots (Bakhtiari et al. 2018).

108 Therefore, if genetic variation for root-to-shoot induction exists in nature, it should correlate to plant

109 fitness, particularly, when plants are under herbivore attack.

110 We here sought for natural genetic variability in BG-to-AG systemic induction in nature and

111 specifically asked the following questions: 1) Does the exogenous application of JA in roots increase

112 resistance against specialist and generalist insect leaf-chewing herbivores? 2) Is there genetically-

113 based variation in resistance against insects and BG-to-AG induction of GSLs? 3) Is there a trade-off

114 between the constitutive and inducible production of shoot GSLs following root induction? and 4)

115 What is the impact of systemic induction of different GSLs on plant fitness? We answered these

116 questions by inducing the roots of 26 maternal half-sib families of *Cardamine hirsuta* (Brassicaceae),

117 measured GSL production in the leaves, and measured the growth of a specialist herbivore, the large

118 cabbage butterfly *Pieris brassicae,* and a generalist noctuid butterfly, *Spodoptera littorali*s, to assess

119 the potential impact of GSLs on adapted and non-adapted herbivores, respectively Our work builds

120 toward a better understating of the ecological and evolutionary drivers of plant chemical defense

121 variation in nature.

122

123 <div align="center">METHODS AND MATERIALS</div>

124 *Plants and Insects* - The hairy bittercress, *Cardamine hirsuta* (Brassicaceae) is a common weed

125 growing in a variety of habitats in Europe but mainly at low elevations (Pellissier et al. 2016). Seeds

126 from 26 half-sib families were collected from three different natural populations separated by at least

127 10 Km (pop A = 9 pop B = 10, and pop C = 7 families) at the foothills of the Swiss Jura mountains.

128 After an overwintering period of four months at 4 °C, seeds were germinated in Petri dishes lined with

129 humid filter paper, and one week after germination, 15 seedlings per family (total of 390 plants) were

130 transplanted independently into plastic potting pots (13 cm width × 10 cm height) filled with 500 ml of

131 sieved soil (1 cm mesh size) mixed with sand in a 3:1 ratio. The soil/sand mixture was sterilized by

132 autoclave at 120 °C for four hrs. Plants were immediately transferred to climate-controlled chamber

133   and kept at 16h/22°C - 8h/16°C day-night, and 50% relative humidity conditions. Plants were

134   fertilized (universal liquid fertilizer containing N: P: K ratio of 7:3:6% per liter) twice a week until the

135   beginning of experiment. Although our common garden experiment was specifically designed to

136   measure genetic variation across different maternal lines, we would like to acknowledge that the

137   potentially observed genetic differences among families cannot be fully isolated from maternal

138   environmental effects, but because *C. hirsuta* practically completely relies on autogamous selfing for

139   reproduction (Hay et al. 2014), such maternal effects should be minimized in this system. We used the

140   large cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae) and the African cotton leaf worm

141   *Spodoptera littoralis* (Lepidoptera: Noctuidae) as specialist and generalist herbivore insects,

142   respectively. *P. brassicae* is a specialist herbivore that feeds exclusively on plants producing GSLs,

143   especially on species of the Brassicaceae (Chew 1988). The caterpillars used in this experiment were

144   obtained from a culture maintained on *Brassica rapae* ssp. *chinensis* (L) plants. *S. littoralis* is a

145   generalist herbivore known to feed on species belonging to more than forty families of plants (Brown

146   and Dewhurst 1975). However, it does not occur in Switzerland, therefore, it functioned as a

147   generalist, non-adapted, herbivore in our study. Eggs were obtained from Syngenta, Stein AG,

148   Switzerland, and newly hatched *S. littoralis* larvae to be used in the bioassays were reared on corn-

149   based artificial diet until the beginning of the experiment.

150

151   *Experimental Design and Insect Bioassay* - After three weeks of growth, we randomly assigned the

152   plants to three treatment groups. Six plants per family were randomly assigned to the JA treatment,

153   another six group plants to the no-induction treatment, and the rest (three plants per family) to the no-

154   herbivory control treatment. Each plant in the JA treatment were inoculated with 20 ml of JA solution

155   in the roots by spiking the solution into the soil, 0.5 cm below the surface. The JA solution consisted

156   of 2.4 µmoles (500 µg) of JA (± - jasmonic acid, Sigma, St Louis, IL, USA) per plant in 10 ml

157   demineralized water and 0.5% EtOH (van Dam and Oomen 2008; van Dam et al. 2004). The no-

158   induction group of plants received 20 ml of 0.5% EtOH in acid water (pH 3.7 with HCl). We chose to

159   induce roots with JA instead of using a root herbivore (e.g. cabbage root maggots), in order to

160   standardize the induction event across all plant families. Moreover, by applying JA, we intentionally

161 avoided the effect of tissue removal per se on plant fitness. In other words, we were able to measure

162 the fitness impact of defense induction independently from herbivore damage.

163 To measure the effect of BG induction on leaf chemistry, four days after JA root application, we

164 collected two fully-expanded new leaves per plant in the JA and the no-induction treatments, and

165 immediately froze and stored them at -80°C for further chemical analyses. Since the leaves from both

166 treatments were collected prior to AG herbivory, the plant materials collected from no-induction

167 treatment served for measuring constitutive secondary metabolites expression. Immediately after leaf

168 removal, we infested half of the plants in the herbivory treatments (three plants per family per

169 induction treatment = six plants/family) with two 7-days old *S. littoralis* larvae, and the other half,

170 with one 6-days old *P. brassicae* larvae. We next covered all plants with gauze bags to prevent escape

171 or cross-movement of insects between plants. After one week of herbivory, bags were removed, the

172 insects were retrieved from individual plants, and their combined weight per plant was measured and

173 recorded to obtain the average insect weight per plant. We used the formula $\ln(final\ weight -$

174 $initial\ weight)$ to determine the insects' weight gain and plant resistance (i.e. lower growth rate

175 indicate that plants are more resistant). After the herbivore bioassay, we allowed the plants to complete

176 their life cycle and produce seeds. To estimate the total seed production on each plant, we first

177 randomly selected one seedpod per plant from 50 plants, measured each pod's length, and counted the

178 number of seeds per pod. Using these data, we fitted a linear regression of the seed number as the

179 function of seedpod length in order to obtain the seed set of each plants based on the seedpod length

180 (equation: $14.92 \times total\ pods\ length + 1.65$). At the end of the experiment, when all seedpods

181 were mature, AG plant parts were separated from roots, oven-dried at 40°C for 48h and weighted to

182 determine their dry biomass, which served as covariate in the statistical analyses (see below).

183

184 *Glucosinolate Analyses* - We assessed the concentration of individual GSLs in leaf tissues in no-

185 induction and root-JA-induction plants prior to the AG herbivore application. This allowed measuring

186 the chemical content of the leaves to which the herbivores were immediately exposed across different

187 treatments, and to measure the direct effect of the root induction treatment on plant chemistry without

188 the confounding effect of additional herbivore feeding. To this end, we ground the fresh leaves to

7

189   powder using mortars and pestles in liquid nitrogen. A 100-mg aliquot of fresh leaf powder was then

190   added with 1.0 ml Methanol: H2O: formic acid (80:19.5:0.5, v/v) and 5 glass beads in Eppendorf

191   tubes, shaken in a tissuelyser (Mixer Mill MM 400, Retsch GmbH, Haan, Germany) for 4 min at 30

192   Hertz, and centrifuged them at 12800 g for 3 min. The supernatant was then transferred to HPLC vials

193   for liquid chromatography analysis. Glucosinolate identification and quantification was performed

194   using an Acquity UPLC from Waters (Milford, MA, USA) interfaced to a Synapt G2 QTOF from

195   Waters with electrospray ionization, using the separation and identification method as described in

196   Glauser et al. (2012).

197

198   *Statistical Analyses*-All statistical analyses were carried out with R software (R Development Core

199   Team 2017).

200   *1) Does the exogenous application of JA in roots increase resistance against specialist and generalist*

201   *insect leaf-chewing herbivores?* To answer this question we performed two ANOVAs on the larval

202   weight gain, for generalist and specialist respectively, with the JA treatment (two levels) as fixed

203   factor.

204

205   *2) Is there genetically-based variation in insect resistance and BG-to-AG GSLs induction?* First, we

206   assessed the effect of JA treatment (two levels) and maternal families (26 families) on the abundance

207   and composition of all GSLs simultaneously using a permutational multivariate analysis of variance

208   (PERMANOVA) with the *adonis* function in the package *vegan* (Oksanen et al. 2017). We included

209   plant biomass as covariate to control for potential direct effect of plant size (Züst et al. 2015) on GSL

210   production, and populations as strata in the model. The results were visualized using a non-metric

211   multidimensional scaling (NMDS) ordination. The Bray–Curtis metric was used to calculate a

212   dissimilarity matrix of all compounds among samples for both the PERMANOVA and the NMDS.

213   Second, to address the effect of root JA-addition and family variation on all i) individual GSLs

214   production, ii) the total amount of GSLs, iii) the AG resistance against *P. brassicae* and *S. littoralis*

215   (insect weight gain), iv) and the seed production, we ran linear mixed-effect models with JA treatment

216   as fixed factor, plant families nested within populations as random factor, and plant biomass as

217 covariate, using the function *lme* in the package *nlme* (Pinheiro et al. 2017). Because families were

218 included as random factor in the initial model, we estimated their effect by running a second model

219 without the nested family factor. Differences between the first and the second models (AIC scores)

220 would inform on potential maternal family variation, which were assessed using log-likelihood ratios

221 and Chi-Square tests (function *Chisquare* in R). In addition, to test for family-level genetic variation in

222 inducibility (G x E) on all individual GSLs and total amount of GSLs per plant, we ran ANCOVAs

223 with JA treatment, plant families nested within populations and their interactions as fixed factors, and

224 plant biomass as covariate, using the function *lm* in R.

225

226 3) *Is there a trade-off between the constitutive and inducible production of shoot GSLs following root*

227 *induction?* To test for trade-offs between the constitutive production and the inducibility of total GSLs

228 among the 26 plant families, we employed a Monte Carlo simulation procedure proposed by Morris et

229 al. (2006) using MATLAB (Version 7.5.0.342 –R2007b, MathWorks Inc., USA). This statistical

230 approach accounts for several issues that have apparently confounded previous attempts to assess a

231 trade-off between constitutive and induced defenses (Morris et al. 2006). Specifically, this approach

232 uses the difference in mean GSL production between JA-treated and control plants for measuring

233 induced production of GSLs, and uses a modified Monte Carlo procedure that takes into account

234 sampling variation due to limited sample size, measurement error from environmental and genetic

235 differences.

236

237 4) *What is the impact of systemically inducing different GSLs on plant fitness?* First, we tested for the

238 effect of treatment (3 levels in this case: root JA induction, no-induction, no-herbivory control

239 treatment) on lifetime seed production using mixed effect models with JA treatment as fixed factor

240 and families nested in populations as random factor, including biomass as covariate (*lme* function),

241 followed by pairwise comparisons using *Tukey HSD* post-hoc tests (*lsmeans* function in the package

242 *lsmeans* (Lenth 2016). Second, to estimate the lifetime fitness effect of root JA induction across all

243 different GSLs, we ran mixed effect ANCOVA models with seed production per plant as response

244 variable, individual and total GSLs in interaction with JA induction treatment as continuous and

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245   categorical fixed factors in the model, respectively. Plant families nested within population were

246   included as random factor using the function *lme* in the package *nlme*. The aim here was to detect a

247   significant interaction between JA induction and GSLs on seed set (as a proxy for plant fitness). If this

248   were the case, it would indicate that the effect of JA treatment on a particular GSL compound would

249   affect plant fitness, positively or negatively.

250

251   RESULTS

252   *Effect of JA Treatment on Insect Resistance* - We found that *S. littoralis* larvae on JA-treated plants

253   grew 47% less compared to control plants (Fig. 1, Table 1), and maternal families responded

254   differently in resistance against this generalist herbivore (Fig. 2a, Table 1). In contrast, *P. brassicae*

255   larval weight gain did not differ between treatments and there was no family effect on larval weight

256   gain (Table 1).

257

258   *Effect of JA Treatment and Family Level Variations on GSL Production* - The GSL profile of the *C.*

259   *hirsuta* leaves consisted of 28 GSL compounds: 15 aliphatic-GSLs, 8 aromatic-GSLs, 3 indole-GSLs,

260   and 2 unknown GSLs (Supplementary materials Table S1; Fig. 3a). We found that the maternal family

261   background, but not the JA application, affected the multivariate GSL matrix in *C. hirsuta* leaves

262   (Table 2, Fig. 3b). Specifically, maternal families explained 35% of the variance in the

263   PERMANOVA, and such variation was also marginally explained by plant biomass (Table 2). We

264   also found a maternal family effect for 16 out of the 28 GSLs (Table 1), a JA effect for five GSLs

265   (GSL9: glucohirsutin, GSL12: 8-methylthiooctyl gsl, GSL14: hydroxymethylbutyl gsl, GSL17:

266   veratryl gsl, GSL26: neoglucobrassicin; Table 1), and a biomass effect for four GSLs (GSL11:

267   glucoberteroin, GSL24: glucobrassicin, GSL25: methoxyglucobrassicin, GSL26: neoglucobrassicin;

268   Table 1). JA treatment significantly decreased the production of four out of those five compounds,

269   except neoglucobrassicin, which increased its production by 25%. The production of GSL

270   neoglucobrassicin was also significantly affected by plant biomass and the maternal families

271   treatment, which explained 11% of the total variances (Table 1). We found no effect of JA treatment

and maternal family on total levels of GSLs (Table 1). In addition, we found a significant interactive effect of family × JA (maternal family effect for induced production) for five GSLs (GSL1: glucoraphanin, GSL9: glucohirsutin, GSL10: glucoerucin, GSL13: gluconapoleiferin, GSL20: 5-benzoyloxypentyl) (Table S2).

Effect of herbivory on Seed Set - Across all families, lifetime seed production in the control (no-herbivory) treatment was significantly higher compared to plants in induced and no-induction treatment that experienced herbivory (Fig. 4, $F_{1,144}$ = 54.70, p <.0001). While *P. brassicae* and *S. littoralis* herbivory generally decreased seed set by 68% and 40%, respectively, we found strong genetic effect on seed set production after *S. littoralis* herbivory (Table 1, Fig. 2b). Finally, we found no significant JA treatment effect on seed set (Table 1, Fig. 4)

Trade-offs Analyses - We detected a significant negative correlation (trade-off), between the constitutive production and the inducibility of total GSLs across all maternal families of *C. hirsuta* (r = - 0.82, p = 0.01, Fig. S1).

Effect of JA Root Induction on Plant Fitness after Herbivore Attack - Mixed effect ANCOVA analyses showed that five GSLs (GSL4: glucoalyssin, GSL8: glucobrassicanapin, GSL10: glucoerucin, GSL11: glucoberteroin, GSL18: glucotropeolin), as well as the total GSLs production interacted with JA treatment for explaining seed production (Fig. 5, Table S3). In other words, JA induction changed the slope of the relationship between the GSLs and seed production from negative to neutral or even positive (Fig. 5). We also found marginally significant effect of JA×GSL for GSL16: sinalbin and GSL13: gluconapoleiferin.

DISCUSSION

We found that the systemic induction, from below- to aboveground, of *C. hirsuta* plants significantly decreased the weight gain of a generalist leaf chewing herbivore, but such effect was highly variable

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299   across plant maternal families. Analyses of plants' secondary metabolites showed that JA root

300   induction affected the production of several GSLs aboveground, and significantly ameliorated plant

301   fitness after leaf chewing herbivore attack. Below, we discuss the implications of these findings for the

302   ecology and evolution of plant defense against herbivores in natural brassicaceaous systems.

303

304   *Effect of Root JA Treatment on Insect Resistance and Aboveground Glucosinolate Production.*

305   One of the principal results of our study is that the JA root application increased resistance against the

306   generalist herbivore (*S. littoralis*), while JA application had no effect on the specialist herbivore (*P.*

307   *brassicae*). These results are in line with several previous studies (Bodenhausen and Reymond 2007;

308   Giamoustaris and Mithen 1995; Lankau 2007; Raybould and Moyes 2001). For instance, root JA

309   application to *Brassica oleracea* roots resulted in reduced weight gain of generalist herbivore

310   *Mamestra brassicae*, whereas the specialist *P. rapae* was unaffected (van Dam and Oomen 2008), and

311   root induction resulted in more infestation by AG specialists in field-grown *B. oleracea* plants (Pierre

312   et al. 2013). Indeed, specialist herbivores of the Brassicaceae not only tolerate GSLs, they also utilize

313   these compounds in host recognition (Raybould and Moyes 2001). On the other hand, the negative

314   effect of GSLs on generalist herbivore performance has also been confirmed in previous studies

315   (Rasmann et al. 2015; Schlaeppi et al. 2008; Schweiger et al. 2014; Schweizer et al. 2017), which

316   confirms strong context-dependency in plant-herbivore interaction.

317   Contrary to general expectations, we did not detect differences in the production of total GSLs

318   between control and JA-treated plants, and found that the GSL production in leaves was related by a

319   large extend to plant biomass, a common phenomenon when studying secondary metabolite

320   production in plants (Glynn et al. 2003; Traw 2002; Züst et al. 2015). Although previous works with

321   Brassicaceae plants showed that BG herbivory, or root induction by JA, increases total levels of GSL

322   in shoots (Griffiths et al. 1994; Pierre et al. 2012; van Dam and Oomen 2008; van Dam et al. 2004),

323   similarly to this one, other studies failed to detect such changes in production of total GSLs (Agrawal

324   2000; Pierre et al. 2013). These results indicate that the systemic induced responses in plants from BG

325   to AG can be species (as well as genotypic, such as in this study) specific, and that uniquely measuring

326   the total amount can often be misleading in plant-herbivore interaction studies. .

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327   Changes in phytochemical diversity in response to induction is, on the other hand, likely a more

328   important component of plant defense against herbivory (Agrawal 2000; Berenbaum and Zangerl

329   1996; Lindig-Cisneros et al. 1997). Accordingly, the results of our multivariate analysis showed that

330   among the five families that are distinctive with respect to their GSL profiles (Fig. 1b.), two families

331   exhibited greater resistance against *S. littoralis* (family 5 & 7). In fact, family 7, which showed the

332   most distinctive GSL composition in the NMDS, was the most responsive family to JA treatment in

333   terms of inducibility of overall GSLs and the most-resistant family against herbivory by *S. littoralis*. In

334   addition, in contrast to total GSL levels, we observed that an indolic GSL, neoglucobrassicin, is the

335   only compound that was both significantly induced by JA (Table 1) and also negatively correlated

336   with *S. littoralis* weight gain (results not shown, but results from linear mixed model for testing the

337   interactive effect of the JA treatment and neoglucobrassicin production on *S. littoralis* weight gain: JA

338   effect; $F_{x,y} = 18.34$, $p < 0.001$; neoglucobrassicin effect; $F_{x,y} = 5.34$, $p = 0.02$; and their interaction: $F_{x,y}$

339     $= 3.46$, $P = 0.07$). Indole GSLs have been shown to be induced by herbivory and to affect the growth

340   and development of insect herbivores in other systems (Irwin et al. 2003; Rostás et al. 2002). Selective

341   induction of indolic GSLs have been reported in *B. napus*, *B. rapae* and *B. juncea* in response to

342   herbivory by flea beetles (Bodnaryk 1992). For instance, the concentration of neoglucobrassicin was

343   increased considerably in leaves of *B. napus* as a result of topical application of methyl JA to aerial

344   part of the plant (Doughty et al. 1995), as well as in *B. rapae* and *B. napus* plants treated with

345   specialist herbivores (Koritsas et al. 1991; Rostás et al. 2002). The same pattern of induction of

346   neoglucobrassicin was observed in the roots of *B. napus* that were damaged by *Delia floralis* root

347   maggots (Hopkins et al. 1998). In another study, the only compound that was shown to affect the

348   performance of *P. rapae* feeding on *B. oleracea* plants was neoglucobrassicin (Harvey et al. 2007)..

349   Together, these results suggest that the total amount of GSLs in Brassicaceaous plants can often be

350   misleading when predicting plant resistance, while, on the other hand, individual GSLs bear

351   differential toxicities might be better predictors of plant resistance.

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353   *Does Below-to-Aboveground Systemic Induction of GSLs Affect Plant Fitness?*

Demonstrating the effect of induced response on plant fitness is crucial for documenting that they truly serve as a defensive response (Erb 2018). We found that herbivory, overall, decreased plant fitness (seed production) by more than 50%, clearly confirming the well-documented negative consequence of herbivory on plant fitness (Agrawal 1998; Agrawal 1999; Kessler and Baldwin 2004; Maron 1998; Mothershead and Marquis 2000). If herbivory decreases plant fitness and plants possess genetic variation for traits affecting herbivory and enhancing fitness, then herbivores may act as strong selective agents for more resistant plants by promoting inducibility of specific toxic molecules. Accordingly, in *C. hirsuta*, we showed that root JA-mediated induced systemic production of seven GSL compounds in shoots increases seed production in plants exposed to shoot herbivory, compared to plants that did not received JA treatment. This fitness impact has important implications. First, inducible systemic resistance may be an example of adaptive plasticity in plants. Adaptive plasticity is defined by the higher fitness of individuals expressing different phenotypes in a particular environment (Vijendravarma et al. 2015). Thus, the induction of GSLs compounds after root damage can be seen as an adaptive plastic response for *C. hirsuta* plants (Agrawal 1999; Agrawal 2000). Nonetheless, to be fully convincing, arguments about adaptive plastic responses should also be linked to ecological setting. In this case, we could speculate that *C. hirsuta* plants are likely damaged in their roots, by e.g. root fly maggots, every spring before pierids or other generalist butterflies start feeding on these plants. To date, due to obvious methodological limitations of measuring rates of root herbivory in the field, we only have anecdotal information on the timing and amount of root damage in natural systems (Johnson and Rasmann 2015). For now we can only speculate that the observed genetic variation in inducibility from below to aboveground is shaped by the natural variation in root and shoot herbivory.

The second implication of our fitness-related results concerns the evolution of the systemic response from root to shoots. In order for such a trait to evolve by natural selection, there must be heritable variation that affects fitness. We detected genetic variation in induced production of five GSL compounds (significant interactive family ×JA effect). Within these five GSLs, two compounds (GSL10 and 13) were among the seven individual compounds found to be positively affecting seed set

381   when induced by JA. In other words, plant families possessing the ability for increased production of

382   these seven compounds in the induced state could hinder the negative fitness effect of herbivory.

383   Finally, genetic variation in inducibility could also have been maintained by physiological trade-offs.

384   Accordingly, we showed that the inducibility of total GSLs and neoglucobrassicin negatively

385   correlated with constitutive investment in both traits. It is generally assumed that constitutive and

386   induced defenses should trade off, as the anti-herbivore defenses are costly for plants (Karban and

387   Baldwin 1997; Karban and Myers 1989; Zangerl and Bazzaz 1992). Thus, most *C. hirsuta* families

388   employ economy in direct chemical defense production, by favoring either a constitutive or an

389   inducible strategy. Altogether, ecological and physiological trade-offs may contribute in maintaining

390   the necessary genetic variation in inducibility of specific GSLs, ultimately generating the raw material

391   for selection to act upon.

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409 **Author contributions**

410 MB and SR conceived and designed the experiments. MB conducted experiments and chemical

411 analyses. MB and SR analysed the data and wrote the manuscript. The authors declare no conflicts of

412 interest.

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Tables

Table 1. Mixed effect model table for testing the effect of JA induction treatment in the roots of *Cardamine hirsuta* plants, maternal families, and their biomass on individual and total glucosinolates (GSL*), as well as seed production and *Spodoptera littoralis* larval growth. *C. hirsuta* plant families nested within populations was the random factor. Family effect was calculated from the log-likelihood difference (LLR) between the full model and the model missing the random effect. 16 GSL out of 28 showed a significant Family effect. GSL 9, 12, 14, 17, 26 showed significant JA effect. GSL 11, 24, 25, 26 showed significant biomass effect

GSL †	Factor	Value	Df	t-value/LLR	p-value	
GSL1	JA	0.11	91	1.154	0.252	
	Plant biomass	0	91	-0.896	0.373	
	Family		4	1.735	0.187	
GSL2	JA	-0.062	91	-1.19	0.237	
	Plant biomass	0	91	0.055	0.956	
	Family		4	0	1	
GSL3	JA	0.063	91	0.88	0.381	
	Plant biomass	0	91	0.769	0.444	
	Family		4	12.671	0	***
GSL4	JA	0.047	91	0.433	0.666	
	Plant biomass	0	91	1.362	0.177	
	Family		4	51.395	0	***
GSL5	JA	0.036	91	0.394	0.695	
	Plant biomass	0	91	0.673	0.502	
	Family		4	6.591	0.01	**
GSL6	JA	0.025	91	0.15	0.881	
	Plant biomass	0	91	-0.104	0.918	
	Family		4	0.099	0.753	
GSL7	JA	0	91	-0.005	0.996	
	Plant biomass	0	91	0.118	0.907	
	Family		4	7.301	0.006	**
GSL8	JA	0.047	91	0.284	0.777	
	Plant biomass	0.001	91	1.491	0.139	
	Family		4	18.37	0	***
GSL9	JA	-0.086	91	-2.846	0.006	**
	Plant biomass	0	91	-0.299	0.766	
	Family		4	27.569	0	***
GSL10	JA	-0.02	91	-0.297	0.768	
	Plant biomass	0	91	0.33	0.742	
	Family		4	3.254	0.071	°
GSL11	JA	0.094	91	1.178	0.242	
	Plant biomass	0	91	2.168	0.033	**
	Family		4	19.56	0	***
GSL12	JA	-0.095	91	-1.769	0.08	°
	Plant biomass	0	91	0.808	0.421	
	Family		4	3.021	0.082	°

GSL13	JA	0.036	91	0.605	0.547	
	Plant biomass	0	91	1.476	0.143	
	Family		4	21.435	0	***
GSL14	JA	-0.112	91	-2.165	0.033	**
	Plant biomass	0	91	0.046	0.963	
	Family		4	3.043	0.081	°
GSL15	JA	-0.081	91	-1.019	0.311	
	Plant biomass	0	91	-0.517	0.606	
	Family		4	0.031	0.859	
GSL16	JA	0.062	91	0.884	0.379	
	Plant biomass	0	91	0.172	0.864	
	Family		4	1.783	0.181	
GSL17	JA	-0.094	91	-1.74	0.085	°
	Plant biomass	0	91	0.754	0.453	
	Family		4	2.751	0.097	
GSL18	JA	0.021	91	0.126	0.9	
	Plant biomass	0	91	0.44	0.661	
	Family		4	0	1	
GSL19	JA	-0.092	91	-1.71	0.091	
	Plant biomass	0	91	0.778	0.439	
	Family		4	2.579	0.108	
GSL20	JA	-0.057	91	-1.535	0.128	
	Plant biomass	0	91	-0.393	0.696	
	Family		4	0.857	0.005	**
GSL21	JA	-0.091	91	-1.768	0.081	
	Plant biomass	0	91	-0.052	0.959	
	Family		4	2.735	0.098	°
GSL22	JA	0.113	91	0.804	0.424	
	Plant biomass	0	91	-0.228	0.821	
	Family		4	14.837	0	***
GSL23	JA	-0.091	91	-1.768	0.081	°
	Plant biomass	0	91	-0.052	0.959	
	Family		4	2.735	0.098	°
GSL24	JA	0.069	91	0.634	0.528	
	Plant biomass	-0.001	91	-4.379	0	***
	Family		4	0.812	0.367	
GSL25	JA	-0.009	91	-0.081	0.935	
	Plant biomass	-0.002	91	-5.913	0	***
	Family		4	3.536	0.06	°
GSL26	JA	0.04	91	1.888	0.062	°
	Plant biomass	0	91	-3.367	0.001	**
	Family		4	9.61	0.002	***
GSL27	JA	-0.089	91	-1.649	0.103	
	Plant biomass	0	91	0.199	0.843	
	Family		4	1.311	0.252	

GSL28	JA	-0.109	91	-2.082	0.04	
	Plant biomass	0	91	-0.027	0.978	
	Family		4	1.966	0.161	
GSL Total	JA	0.029	91	0.194	0.847	
	Plant biomass	0	91	0.489	0.626	
	Family		4	0	1	
Seed production	JA	-80.991	116	-1.697	0.092	°
	Plant biomass	0.171	116	1.206	0.23	
	Family		4	19.551	0	***
S. littoralis	JA	-0.835	92	-4.736505	0	***
	Plant biomass	0	92	-0.087	0.93	
	Family		4	13.989	0	***
P. brassicae	JA		92		0.2	
	Plant biomass		92		0.4	
	Family		4		0.32	

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644 Signif. codes: *** < 0.001,** <0.01, * <0.05, ° <0.1

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646 † GSL1 = Glucoraphanin; GSL2 = Hydroxypropyl gsl; GSL3 = Progoitrin; GSL4 = Glucoalyssin;
647 GSL5 = Glucoputranjivin; GSL6 = Gluconapin; GSL7 = Butyl gsl; GSL8 = Glucobrassicanapin;
648 GSL9 = Glucohirsutin; GSL10 = Glucoerucin; GSL11 = Glucoberteroin; GSL12 = 8-Methylthiooctyl
649 gsl ; GSL13 = Gluconapoleiferin; GSL14 = Hydroxymethylbutyl gsl ; GSL15 = 2-Methylbutyl gsl;
650 GSL16 = Sinalbin; GSL17 = Veratryl gsl; GSL18 = Glucotropeolin; GSL19 = Trimethoxy gsl; GSL20
651 = 5-Benzoyloxypentyl ; GSL21 = 2-Hydroxy-2-phenylethyl gsl; GSL22 = Gluconasturtiin; GSL23 =
652 Hydroxybenzyl-methylether gsl; GSL24 = Glucobrassicin; GSL25 = Methoxyglucobrassicin; GSL26
653 = Neoglucobrassicin; GSL27 = Unknown.C16H23NO10S2; GSL28 = Unknown.C19H28N3O12S3.

654   **Table 2.** Permutational multivariate analysis of variance (PERMANOVA) table for testing the effect

655   of JA treatment and family on the structure of the glucosinolate (GSLs) matrix.

Factor	Df	MSQ	F value	R^2	P value
JA treatment	1	0.136213	2.04795	0.01494	0.118
Family	25	0.126121	1.89621	0.34592	**0.005****
Plant biomass	1	0.192319	2.89149	0.0211	**0.054**·
JA * Family	25	0.063047	0.94791	0.17292	0.571
Residuals	61	0.066512		0.44512	

656   Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

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662   **Figure legends**

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664   **Fig. 1**. Root to shoot induction of resistance against generalist and specialist herbivores. Boxplots

665   show average weight gain of (a) *Spodoptera littoralis*, and (b) *Pieris brassicae* caterpillars feeding on

666   plants that received jasmonic acid (JA) in the roots 4 days prior herbivory (JA, grey boxes), or

667   received no JA in the roots (Control, open boxes)). Weight gain was calculated as the natural

668   logarithm of the difference between final and initial fresh weight. Asterisks show significant

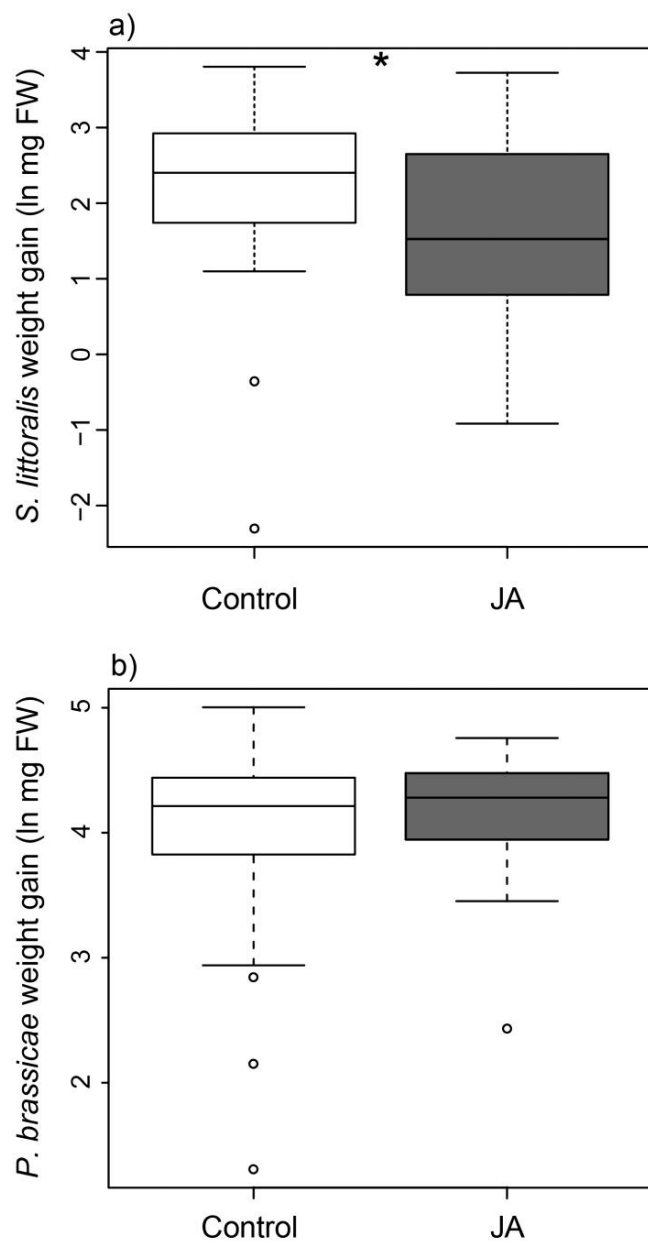669   differences across the two treatments ($p < 0.05$).

670

671   **Fig. 2.** Genetic variation in resistance and fitness related traits. Shown are reaction norm plots for the

672   effects of JA treatment in roots on a) the weight gain of *S. littoralis* caterpillars, and b) total seed

673   production across 26 maternal half-sib families of *C. hirsuta* plants subjected to herbivory by *S.*

674   *littoralis*. Grey lines represent maternal families' averages in the constitutive (Control) state and in the

675   induced state after JA addition the roots (JA). Black lines and dots represent overall averages across

676   families.

677   **Fig. 3.** Glucosinolate (GSL) production across *Cardamine hirsuta* half-sib families. **a)** Barplot

678   representation of the concentration of the individual GSLs in leaves of *C. hirsuta* plants that either

679   received JA in the roots 4 days prior to the start of herbivory (JA, grey bars), or did not receive JA

680   treatment in the roots (Control, open bars). Asterisks indicate a significant effect of JA treatment in

681   production of GSLs. GSL1 = Glucoraphanin; GSL2 = Hydroxypropyl gsl; GSL3 = Progoitrin; GSL4 =

682   Glucoalyssin; GSL5 = Glucoputranjivin; GSL6 = Gluconapin; GSL7 = Butyl gsl; GSL8 =

683   Glucobrassicanapin; GSL9 = Glucohirsutin; GSL10 = Glucoerucin; GSL11 = Glucoberteroin; GSL12

684   = 8-Methylthiooctyl gsl ; GSL13 = Gluconapoleiferin; GSL14 = Hydroxymethylbutyl gsl ; GSL15 =

685   2-Methylbutyl gsl; GSL16 = Sinalbin; GSL17 = Veratryl gsl; GSL18 = Glucotropeolin; GSL19 =

686   Trimethoxy gsl; GSL20 = 5-Benzoyloxypentyl ; GSL21 = 2-Hydroxy-2-phenylethyl gsl; GSL22 =

687   Gluconasturtiin; GSL23 = Hydroxybenzyl-methylether gsl; GSL24 = Glucobrassicin; GSL25 =

688   Methoxyglucobrassicin; GSL26 = Neoglucobrassicin; GSL27 = Unknown.C16H23NO10S2; GSL28

689   = Unknown.C19H28N3O12S. **b)** Non-multidimensional scaling (nMDS) ordination of the individual

690   glucosinolates found in *C. hirsuta* leaves across 26 plant families at the constitutive state (open dots),

691   or after roots induction with JA (black dots). Numbers besides dots correspond to plant families.
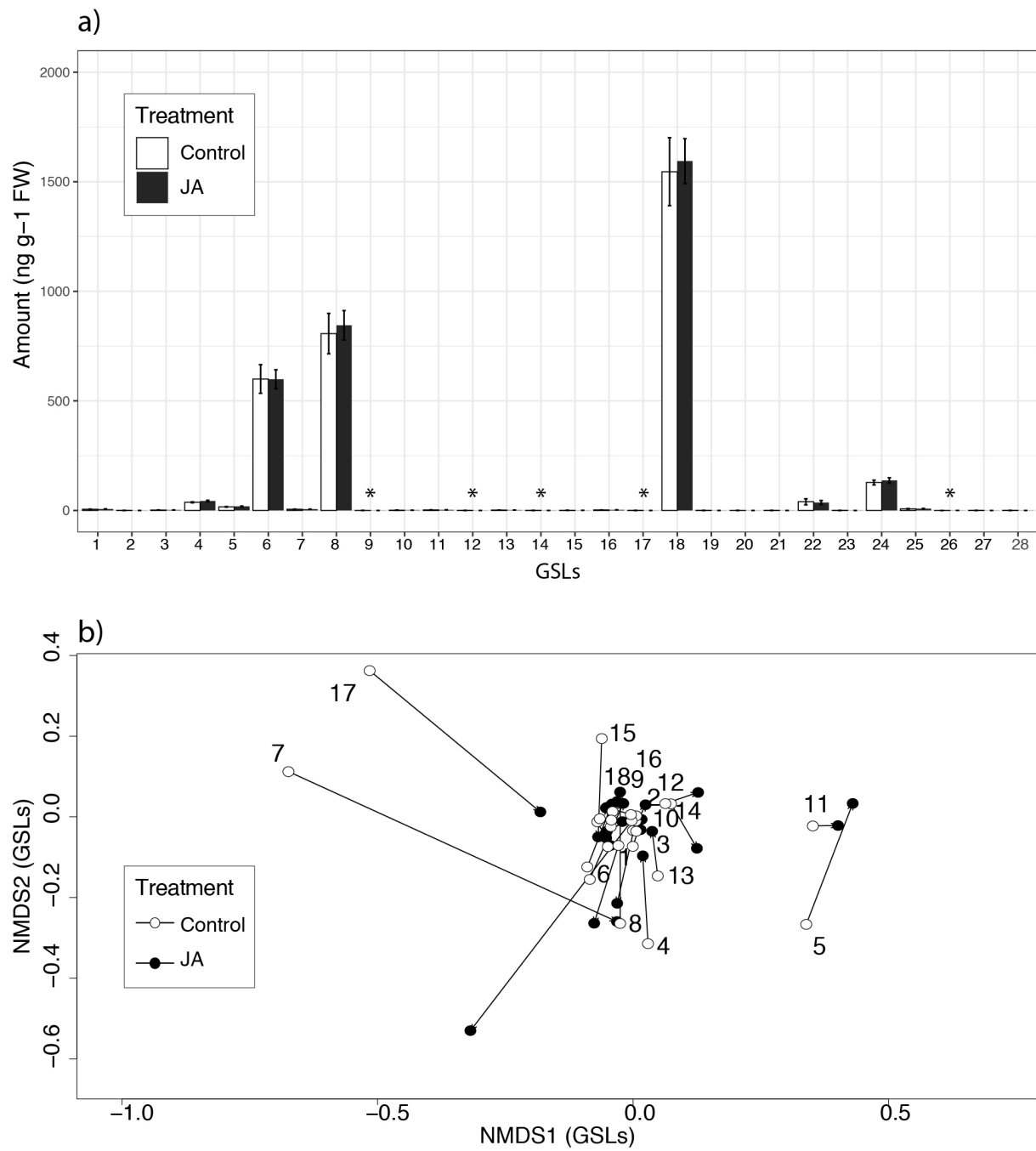
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694 **Fig. 4.** Herbivore impact on plant fitness. Boxplots show the total seed number produced by 26
695 maternal half-sib families of *C. hirsuta* plants in plants that did not receive JA in the roots nor treated
696 aboveground herbivory (No herbivory), plants that received jasmonic acid (JA) in the roots 4 days
697 prior to the start of herbivory by *Spodoptera littoralis* or *Pieris brassicae* (*P. brassicae* / JA, *S.*
698 *littoralis* / JA), and plants subjected to herbivory either by *S. littoralis* or *P. brassicae* but that that
699 were not treated by JA in the roots (*P. brassicae* / C, *S. littoralis* / C).

700

701 **Fig. 5.** Fitness benefits of JA root induction during herbivory. Shown are interaction plots indicating
702 the relationship between the total seed production and total glucosinolates, as well as seven individual
703 GSLs that showed interaction with JA across all plants subjected to herbivory by *S. littoralis* in control
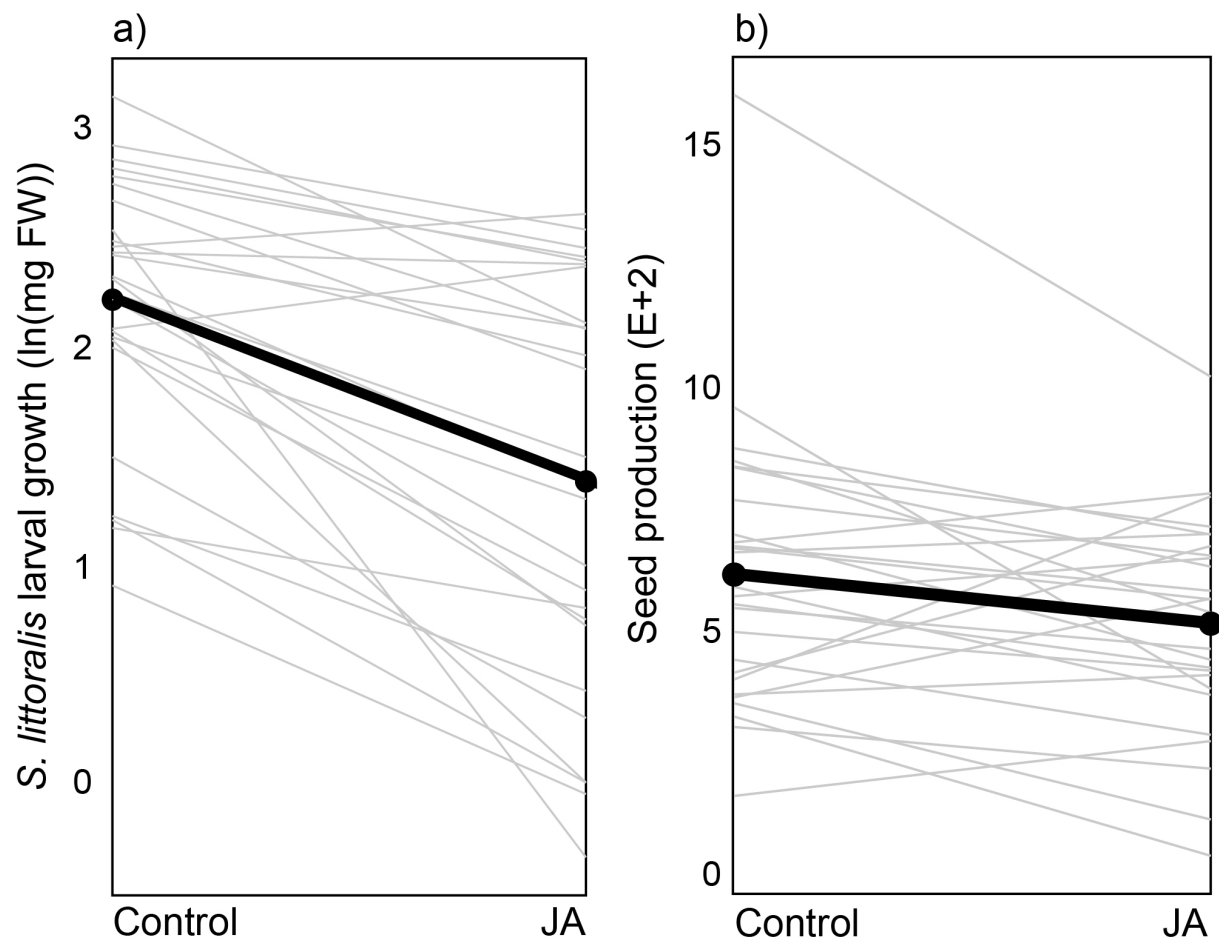704 and JA treatment (see Table S3).

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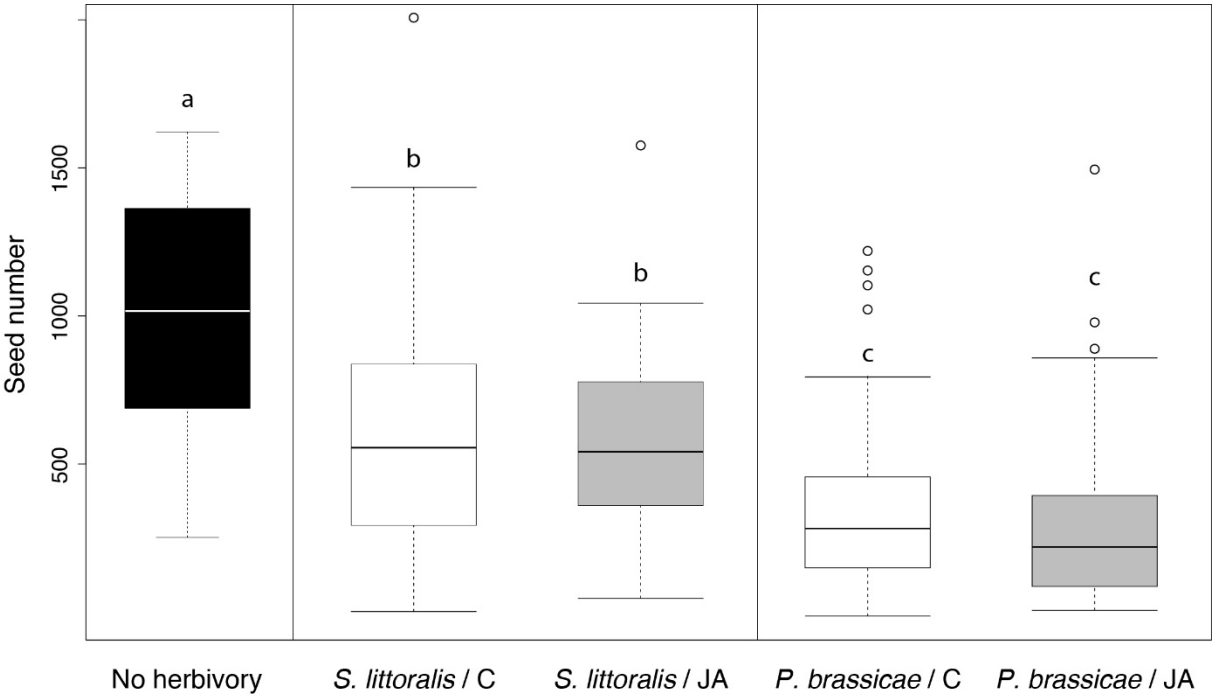706

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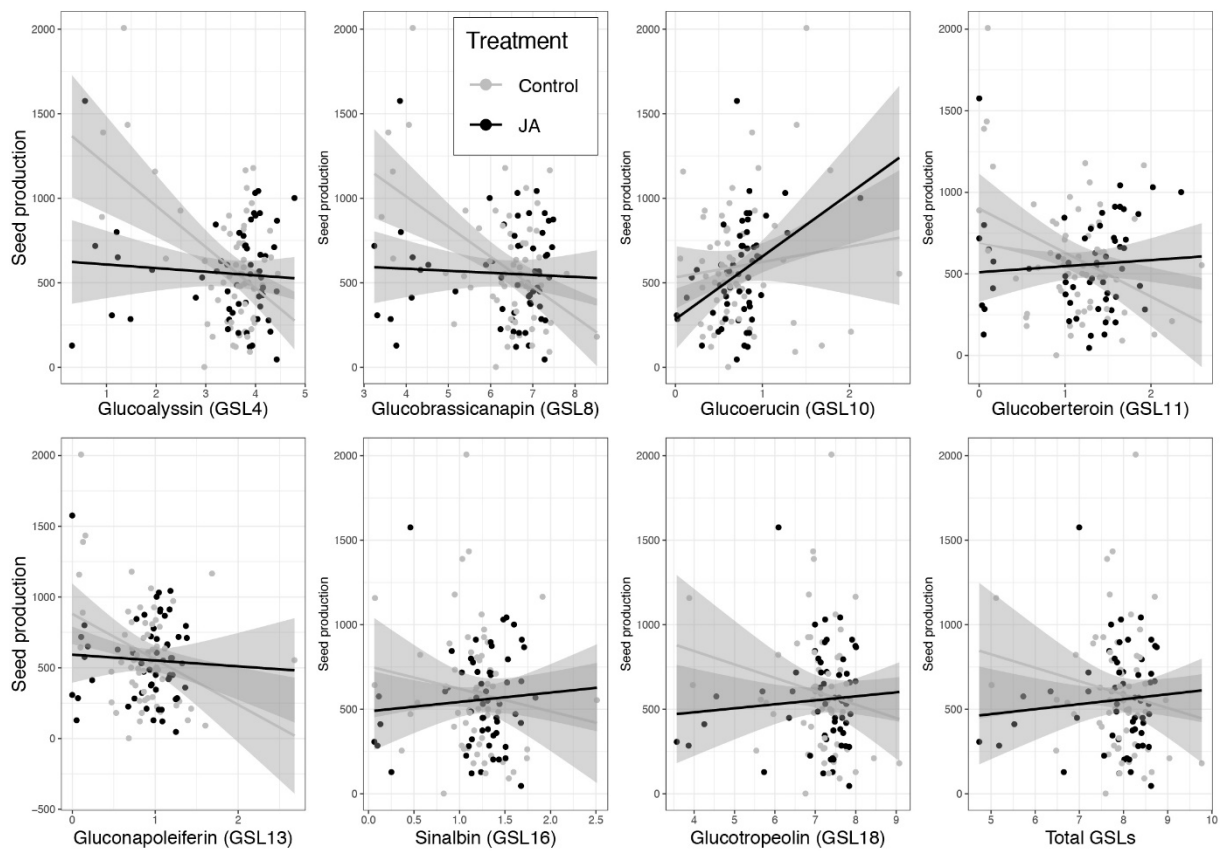
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725 **Figures**

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731 **Fig. 1**
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738 **Fig. 2**

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745   **Fig. 3**

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771 **Fig. 5**

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