

1 **Genomic and chemical evidence for local adaptation in resistance to different herbivores in**  
2 *Datura stramonium*

3

4 **Running title:** Local adaptation of plant resistance to herbivory

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24 **Author contributions**

25 Conceived and designed the experiments: JNF, IMDC. Performed the experiments: IMDC, JNF,  
26 PLV. Analyzed the data: IMDC, JM, JNF. Contributed reagents/materials/analysis tools: IMDC,  
27 JNF, CMFO, PLV, JM. Wrote the paper: IMDC, JNF, JM.

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46

47 **Data availability**

48 Scripts, commands and the entire workflow to obtain the Identity by Descent (IBD) can be  
49 consulted in <https://github.com/icruz1989/IBDcalculation>. ddRad-seq data can be found in  
50 DDBJ/ENA/GenBank under the BioProject PRJNA663170. Phenotype data can be found in  
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52

53 **Ethics approval and consent to participate**

54 All authors approved the manuscript

55

56 **Competing interests**

57 The authors declare that they have no competing interests

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71 **Abstract**

72  
73 Since most species are collections of genetically variable populations distributed to habitats  
74 differing in their abiotic/biotic environmental factors and community composition, the pattern  
75 and strength of natural selection imposed by species on each others' traits are also expected to be  
76 highly spatially variable. Here, we used genomic and quantitative genetic approaches to  
77 understand how spatially variable selection operates on the genetic basis of plant defenses to  
78 herbivores. To this end, an F<sub>2</sub> progeny was generated by crossing *Datura stramonium*  
79 (Solanaceae) parents from two populations differing in their level of chemical defense. This F<sub>2</sub>  
80 progeny was reciprocally transplanted into the parental plants' habitats and by measuring the  
81 Identity by Descent (IBD) relationship of each F<sub>2</sub> plant to each parent, we were able to elucidate  
82 how spatially variable selection imposed by herbivores operated on the genetic background  
83 (IBD) of resistance to herbivory, promoting local adaptation. The results highlight that plants  
84 possessing the highest total alkaloid concentrations (sum of all alkaloid classes) were not the  
85 most well-defended or fit. Instead, specific alkaloids and their linked loci/alleles were favored by  
86 selection imposed by different herbivores. This has led to population differentiation in plant  
87 defenses and thus, to local adaptation driven by plant-herbivore interactions.

88  
89 **Key words.** *Datura stramonium*, identity by descent, local adaptation, plant-herbivore  
90 interactions, phenotypic selection, resistance.

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## Introduction

95  
96 Coevolution between plants and insects that feed on them is thought to be fueled by reciprocal  
97 selection imposed by traits (or trait states) that mediate the interaction, potentially given rise to  
98 arms races (Ehrlich and Raven 1964; Dawkins and Krebs 1979; Thompson 2005; Janz 2011). At  
99 the microevolutionary scale, spatial environmental variation may result in a selection mosaic that  
100 favors different traits (or trait states), hence promoting phenotypic and genetic/genomic  
101 divergence among populations, and thereby local adaptation (Gomulkiewicz *et al.* 2002;  
102 Thompson and Cunningham 2002; Thompson 2005; Briscoe Runquist *et al.* 2020). For instance,  
103 plant populations are likely to encounter different communities of herbivores both in space and  
104 time (Stam *et al.* 2014), making it highly improbable that selection pressures on plant defense  
105 traits (*e. g.*, chemical secondary compounds) would be homogenous across populations  
106 (Berenbaum *et al.* 1986; Charlesworth 1998; Züst *et al.* 2012). Thus, it is expected that natural  
107 selection on plant-herbivore interactions between environments can lead to population  
108 differentiation of plant defense traits and ultimately to local adaptation. However, evidence of  
109 how varying herbivore communities impose selection of phenotypic defense variation, and their  
110 role in shaping the genomic constitution of populations is still scarce (Briscoe Runquist *et al.*  
111 2020).

112       Local adaptation of plant defense against insect herbivores has been primarily studied  
113 using traditional quantitative genetic approaches such as common garden and reciprocal  
114 transplant experiments (Kawecki and Ebert 2004; de Villemereuil *et al.* 2016). These traditional  
115 approaches along with recent advances in genomics and mass spectrometry have made it  
116 possible to conduct detailed analyses of the genetic basis of chemical-based plant defense  
117 (Savolainen *et al.* 2013). For example, Identity by Descent analyses (IBD), genome-wide

118 association analyses (GWAS), quantitative trait loci mapping (QTL), or  $F_{ST}$  vs.  $Q_{ST}$  comparisons,  
119 provide methodologies to conduct in-depth studies on how plant chemical defense have evolved  
120 in response to spatial variation in plant-insect interactions (Browning and Browning 2012;  
121 Savolainen *et al.* 2013; Anderson *et al.* 2014; Flood and Hancock 2017).

122 In particular, IBD analysis estimates to what extent two or more individuals inherit a  
123 similar nucleotide sequence from a common ancestor (Thompson 2013) and describes the degree  
124 of genetic/familial similarity among a group of individuals (*e. g.*, parents-offspring; Albrechtsen  
125 *et al.* 2010; Thompson 2013). Thus, IBD can be used to evaluate whether the genetic background  
126 of a plant is associated with its ability to face its herbivores. Furthermore, it also allows to detect  
127 patterns of very recent or ongoing selection in the genome (Albrechtsen *et al.* 2010). For  
128 instance, if insect herbivores are reducing the fitness of individual plants, one might suppose that  
129 more resistant plants to herbivory will produce more progeny than less resistant plants (Núñez-  
130 Farfán *et al.* 2007). If so, then ongoing natural selection will increase, across generations, the  
131 amount of IBD sharing in a population in the region surrounding the allele(s) that confer(s)  
132 resistance to herbivory (Browning and Browning 2012). The reasoning behind this is that as a  
133 positively selected allele increases in frequency, the region containing the resistance allele will  
134 increase in homozygosity and experience less intra-allelic recombination at the population level  
135 (Albrechtsen *et al.* 2010). While IBD analysis has been used to identify how recent or ongoing  
136 selection operates on human diseases caused by pathogens (Albrechtsen *et al.* 2009; 2010;  
137 Daniels *et al.* 2015; Wong *et al.* 2017; Henden *et al.* 2018), to best of our knowledge, no studies  
138 have used this approach to evaluate how the genetic background of plant resistance to herbivory  
139 is driven by natural selection.

140           The main aim of this study was to assess the extent to which the evolution of plant  
141 defenses to insect herbivores has been driven by natural selection. To this end, we generated an  
142 F<sub>2</sub> progeny derived from the cross between two populations of the annual herb *Datura*  
143 *stramonium* (Asteridae; Solanaceae), known to differ in their level of chemical defense and  
144 herbivore community (De-la-Cruz *et al.* 2020). The F<sub>2</sub> plants were reciprocally transplanted to  
145 the natural environments (populations) of the grandparents. In this way, we were able to 1)  
146 determine the level of infestation and damage exerted by different herbivores on plants sowed in  
147 each locality, 2) to determine whether the seven most abundant constitutive alkaloids of *D.*  
148 *stramonium* are linked to the level of herbivore infestation. 3) by estimating the Identity by  
149 Descent (IBD) relationship of each F<sub>2</sub> plant to each grandparent, we were able to evaluate  
150 whether genomic similarity to either of the grandparents predicts survival/fitness and resistance  
151 to herbivores in each experimental site. Finally, 4) by quantifying the strength of natural  
152 selection on plant defense traits in the two experimental sites, we assessed whether natural  
153 selection favors an increase in plant resistance against herbivores in each of the two study sites.

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155

## Materials and Methods

156

### The study sites

157

The two study sites, Teotihuacán (State of Mexico, 19°41'6.96"N, 98°52'19.63"W) and Ticumán  
158 (State of Morelos, 18°45'39.90"N, 99° 7'13.86"W), were selected for four main reasons. First,  
159 the two populations occur in different habitats with distinct climatic characteristics (xerophytic  
160 shrub and tropical dry forest, respectively; Valverde *et al.* 2001). Second, species of herbivores  
161 that infest upon *D. stramonium* differ between the sites (see also Results); in Ticuman, *D.*  
162 *stramonium* is attacked mainly by the specialist flea beetle *Epitrix* sp. (Valverde *et al.* 2001;

163 Fornoni *et al.* 2004), whereas in Teotihuacán it is consumed mainly by the specialists beetles  
164 *Lema daturaphila*, *Epitrix parvula* and the specialist seed predator *Trichobaris soror* (Bello-  
165 Bedoy and Núñez-Farfán 2010; Miranda-Pérez *et al.* 2016). In fact, *L. daturaphila* is absent in  
166 the Ticumán population. Third, low degree of genetic differentiation at neutral loci has been  
167 detected between the two populations ( $F_{ST} = 0.06$ ; De-la-Cruz *et al.* 2020). Finally, the two  
168 populations are highly differentiated in their level of tropane alkaloid concentrations (De-la-Cruz  
169 *et al.* 2020). Previous evidence indicates that tropane alkaloids are central for resistance against  
170 herbivores in this species (Shonle and Bergelson 2000; Miranda-Pérez *et al.* 2016). Indeed, at  
171 least the tropane alkaloid scopolamine has been implicated as a defense against herbivores in one  
172 population (Castillo *et al.* 2014).

173

#### 174 **Experimental design**

175 To produce the F<sub>2</sub> generation progeny for this study, we randomly collected fruits from 45 and  
176 47 different plants from the Teotihuacán and Ticumán sites, respectively. Ten seeds from each of  
177 the 92 plants were soaked in water containers and maintained in an environmental chamber at a  
178 photoperiod of 12:12 L:D, and at a temperature of 30°C during the day and 25°C at night, at  
179 constant humidity of 85%. Seeds were scarified to facilitate germination (Fornoni *et al.* 2000).  
180 Germinated seeds were transferred to plastic pots (237 ml) and randomly allocated to positions  
181 on benches in the greenhouse. When the first true leaves developed, each plant was transplanted  
182 into 10 L plastic pots filled with a 1:1 mix of sand and vermiculite, and again, the pots were  
183 placed randomly on the benches. Each plant received the same daily quantity of water (500 ml)  
184 during the entire experiment. When the plants reached the flowering stage, flowers were hand  
185 pollinated. Plants from Teotihuacán were used as pollen receptors and plants from Ticumán were



186 used as pollen donators. Prior to manual pollination, flowers from Teotihuacán were emasculated  
187 before dehiscence and covered with bags to avoid pollen contamination from other plants.

188 Cross-pollination was achieved by rubbing anthers of pollen donors onto the stigma of a  
189 flower. Mating pairs were set at random. After pollination, flowers were tagged and bagged.  
190 Because a plant can produce several flowers, each flower could be pollinated by different pollen  
191 donors. Thus, we produced *ca.* 200 crosses. Fruits of each cross (F<sub>1</sub> generation progeny) were  
192 tagged and collected in paper bags and stored at room temperature. When plants reached the  
193 flowering stage and second bifurcation (~30 days after planting in pots), 6-8 leaves from each  
194 plant were harvested to quantify the diversity and concentration of tropane alkaloids. There is  
195 evidence that the highest concentration of tropane alkaloids in *D. stramonium* occurs at the  
196 flowering stage, which is related to the timing of infestation by the main herbivores of *D.*  
197 *stramonium* (Kariñho-Betancourt *et al.* 2015). A total of 21 tropane alkaloids were identified and  
198 analyzed for all parental plants using methods described in De-la-Cruz *et al.* (2020).

199 Once the total tropane alkaloid concentration of the parental plants was completed, we  
200 selected the individual plant with the lowest (Teotihuacan) and the highest (Ticumán)  
201 concentration of tropane alkaloids (grandparents Teotihuacán 1 and Ticumán 23) (S1). These  
202 plants differed 58-fold in their total alkaloid concentration (1,013 vs. 59,000 ng/g of leaf,  
203 respectively) (S1). F<sub>1</sub> seeds derived from the cross between these two parental plants were  
204 sowed, following the procedure described above, to produce the F<sub>2</sub> progeny (single family: S1).  
205 To this end, we used seeds from three fruits of the same crossing. From the germinated F<sub>1</sub> plants  
206 (n = 8), we randomly chose one plant whose flowers were bagged to avoid pollen contamination  
207 from other plants (although plants were grown in a glasshouse; S1). We allowed this F<sub>1</sub>  
208 individual to self-pollinate to produce the F<sub>2</sub> generation progeny (S1).

209

## 210 **Transplant experiment in the two sites**

211 *Experiment.* F<sub>2</sub> seeds, taken randomly, were germinated and grown in the greenhouse as  
212 described above. When the two true leaves appeared, F<sub>2</sub> seedlings (n = 430) were transplanted to  
213 experimental plots in Teotihuacán (n = 230) and Ticumán (n = 200) in order to expose the F<sub>2</sub>  
214 plants to the local herbivores and natural environmental conditions of their grandparents (S1).  
215 During the first days after transplanting, high seedling mortality occurred at the tropical site  
216 (Ticumán), reducing sample size to 103 plants. In each site, seedlings were planted in the  
217 experimental plot according to a complete randomized design. Plants were spaced 1 m apart in a  
218 regular grid. Experimental plots were regularly weeded to prevent interference and competition  
219 by other species.

220 *Damage by herbivores.* Leaf damage to plants by herbivores was measured with the mobile  
221 application BioLeaf (Machado *et al.* 2016) on four sampling periods (15, 30, 45, 60 days after  
222 planting). On each sampling date, we took photographs of eight randomly chosen full expanded  
223 leaves per plant using a mobile phone (Samsung Galaxy S6 edge). The app automatically  
224 calculates the injured leaf regions caused by insect herbivory and then estimates the damage (in  
225 percentage) relative to the total leaf area (Machado *et al.* 2016). Thus, we were able to quantify  
226 the damage inflicted by herbivores to the plants during the experiment. Likewise, the average  
227 proportion of leaf damage by herbivores per plant was obtained. However, it is important to  
228 highlight that in the Teotihuacán site, most leaf tissue was completely eaten by herbivores in  
229 many plants. In these cases, we assigned 100% of the damage to these plants.

230 *Herbivore infestation.* At the Teotihuacán site, three species of herbivores were recorded during  
231 three sampling periods (15, 30, 45 days after planting). In each plant, we counted the number of

232 1) adults *Epitrix parvula*, 2) adults *Lema daturaphila*, 3) larvae of *Lema daturaphila*, and 4)  
233 adults *Trichobaris soror*. Since larval development and pupation of *E. parvula* occur in the soil,  
234 we were unable to record these stages. Therefore, only the number of adults on plants was  
235 obtained for this insect species, as well as for *T. soror*. To minimize bias in insect counting, only  
236 one person counted the herbivores on each plant in all the sampling periods. In the Ticumán site,  
237 we recorded the infestation accounted only by *Epitrix* sp., since *L. daturaphila* is absent and *T.*  
238 *soror* is very rare (only 3 individuals registered at this site). At the end of the experiments, we  
239 had a measurement of total infestation that each plant experienced by each herbivore in both  
240 sites.

241 *Leaf tissue sampling*. In order to determine alkaloid concentration, we collected one leaf (10 cm  
242 in length) per plant when plants reached their second bifurcation and were flowering (~25 days  
243 after sowing). The leaf sampled was packed in aluminum foil, labeled and immediately frozen in  
244 liquid nitrogen. All samples were transported and stored in a freezer at -80°C. In order to obtain  
245 DNA from each F<sub>2</sub> plant for genetic analyses, one additional leaf was collected, frozen and  
246 stored as described above.

247 *Plant survival and reproduction*. In the Teotihuacán site, plant mortality was caused by heavy  
248 damage exerted by insects (n = 66). We recorded plant survival as a nominal variable  
249 (dead/alive). At the Ticumán site, however, there was no record of single plant mortality due to  
250 damage exerted by herbivores.

251 At the end of the experiment (two months after sowing), we collected all fruits produced  
252 by each plant in each experimental site. Fruits were bagged individually and labelled. In the lab,  
253 seed set per fruit was counted and total number of seeds per plant was used as an estimator of

254 maternal plant fitness (*see* statistical analyses section; Motten and Antonovics 1992; Núñez-  
255 Farfán *et al.* 1996; Mauricio and Rausher 1997).

256

### 257 **Alkaloid extraction of F<sub>2</sub> plants**

258 In order to extract tropane alkaloids from each plant, frozen leaf tissue was first transferred to 2  
259 mL Eppendorf tubes, grinding it with a plastic pestle while keeping it frozen by adding liquid  
260 nitrogen. Second, we weighted the pulverized frozen leaf tissue in Eppendorf tubes. Third, we  
261 added two steel balls to each Eppendorf tube along with 1.5 mL of extraction buffer (80%  
262 methanol; MeOH and 1% formic acid); the tubes were then shaken for 60 s at 30 Hz in a  
263 TissueLyser II (QIAGEN). Finally, the samples were centrifuged for 20 min at 14,000 rpm; 700  
264  $\mu$ L of supernatant was collected and stored in glass vials (1.5 mL) and maintained at -4°C until  
265 quantified in the Liquid Chromatography/Time-of-Flight/Mass Spectra (HPLC-TOF-MS).

266

### 267 **Liquid Chromatography/Time-of-Flight/Mass Spectra**

268 Before analysis, 300  $\mu$ L of MeOH was added to each sample (stored in a glass vial; *see* above)  
269 and then injected into an Agilent 1260 Infinity, coupled to an Accurate-Mass Time-of-Flight  
270 (TOF) LC/MS-6230, with an auto-sampler Agilent Technology 1200 Infinity. The  
271 chromatographic separations were performed in a HPLC Agilent ZORBAX column. Before  
272 samples were injected into the column, it was cleaned with 15 mL of MeOH. For this, a gradient  
273 of mobile phase A (1% (v/v formic acid in water) and mobile phase B (1% (v/v formic acid in  
274 methanol) were used. The gradient profile was set to 0.00 min 90% A eluent, 10 min 10% A  
275 eluent, 17 min 90% A eluent, 17.10 min 90% A eluent. Conditions of this last step were  
276 maintained for 5 min to balance the column. The flow rate was 0.200  $\mu$ L 1 min<sup>-1</sup> each 5 min, so

277 each sample was analyzed for 23 min, and the column temperature was 50°C. The injection  
278 volume was 1  $\mu$ l for all samples. The electrospray source (ESI) was operated in the positive  
279 mode, and the interface conditions were as follows: the fragmentor of 200 V; Skimmer 65 V; oct  
280 1 RF Vpp 750 V; gas temperature of 350°C; drying gas flow rate of 6 L min<sup>-1</sup>; the nebulizer  
281 worked at 50 psig. The ions of the compounds and their retention times are given in S2.

282 To standardize the method and optimize the detection of alkaloids in the HPLC-TOF-MS  
283 system, we prepared standard solutions (1:1000; mg/ml) of Atropine and Scopolamine (Sigma-  
284 Aldrich, St. Louis, MO, USA) of MeOH and injected these at volumes of 2, 4 and 8  $\mu$ l. Since  
285 atropine showed a better calibration curve, we used this curve to calculate the concentration for  
286 each identified alkaloid per plant.

287

### 288 **Identification and quantification of alkaloids in *D. stramonium* leaves**

289 First, we identified the seven most abundant constitutive alkaloids in *D. stramonium*: four  
290 tropane alkaloids (atropine, scopolamine, 3-hydroxy-6-tigloyloxytropine and anisodamine; De-  
291 la-Cruz *et al.* 2020), one alkaloid derived from the phenylalanine biosynthesis  
292 (phenylacetaldehyde), one pyrrolizidine alkaloid (pyrroline), and one triterpenoid of unknown  
293 name but of similar structure and molecular weight to azadirone triterpenoid (Álvarez-Caballero  
294 and Coy-Barrera 2019) (S2). Each alkaloid was searched and integrated (peak integration)  
295 individually in each chromatogram of each plant. The MassHunter Workstation software (v. B.  
296 06.00; Agilent Technologies) was used to identify the alkaloids using data of mass spectra,  
297 retention time, and molecular formula obtained in the chromatograms (S2). The total  
298 concentration for each alkaloid per plant was obtained using the slope and the intersect from the  
299 regression equation of the calibration curve (curve from atropine standard):

300 
$$\left( \left( \frac{(a + bX) \times 1000}{d} \right) \times 1000 \right)$$

301 where  $a$ , is the intercept obtained from the regression of the calibration curve;  $b$ , is the slope  
302 obtained from the regression of the calibration curve,  $X$  is the concentration of given alkaloid in  
303 each plant and  $d$  is the dry weight of the sample. Alkaloid concentration was expressed in  $\mu\text{g/g}$   
304 units of leaf weight. Total alkaloid concentration was obtained as the sum of the seven alkaloids  
305 per plant (Kariñho-Betancourt *et al.* 2015).

306

### 307 **DNA extraction, library preparation for ddRad- sequencing**

308 Genomic DNA (gDNA) was extracted from 163 individuals planted in Teotihuacán and 51  
309 individuals planted in Ticumán. Since we had high mortality of seedlings at the beginning of the  
310 experiment in Ticumán, we extracted DNA from more individuals sowed in Teotihuacán. gDNA  
311 was isolated from fresh leaves with a modified CTAB mini-prep protocol for ddRad-seq (Doyle  
312 and Doyle 1987). The total amount of gDNA was measured using Qubit dsDNA HS Assay Kit  
313 (Invitrogen, Thermo Fisher Scientific, Waltham, USA). A total of 200 ng of gDNA was used for  
314 library preparation. The qualified DNA samples were digested with EcoRI and Hin1II (NlaIII)  
315 restriction enzymes (Takara, Osaka, Japan) and subjected to adapter ligation. The digestion and  
316 ligation were performed at 37°C for 16 hrs. The ligation products barcoded with unique P1  
317 adapter were pooled and purified by size selection using E-Gel SizeSelect 2% agarose (Life  
318 Technologies, Carlsbad, CA, USA). Approximately 400-600 bp fragments were retrieved. The  
319 selected size and adaptor-ligated DNA was subsequently amplified by PCR. The PCR products  
320 were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA). The purified library  
321 was sequenced using Illumina Hiseq X Ten platform (Illumina, San Diego, CA, USA). Library  
322 preparation and sequencing were carried out by CD Genomics company (Shirley, NY, USA). For

323 the two grandparents, gDNA was isolated and measured as above. However, whole genome  
324 sequencing was carried out for both, rather than ddRad-seq. Libraries were sheared on the  
325 Covaris and then prepped for 150PE (paired-end) Illumina HiSeq 4000 sequencing using the  
326 Kapa Hyper prep Illumina library prep kits. Final libraries were visualized on the Agilent  
327 Fragment Analyzer, then quantified and pooled at equimolar amounts with Kapa qPCR Illumina  
328 library quant Universal Kits. The sequencing and library preparations for the grandparents were  
329 carried out in the QB3 Functional Genomics and Vincent J. Coates Sequencing Laboratories at  
330 the University of California, Berkeley.

331

### 332 **Identity by Descent (IBD)**

333 Two haplotypes are identical by descent (IBD) if they share the same alleles inherited from a  
334 common ancestor (Thompson 2013). Thus, closely related individuals have a high proportion of  
335 IBD (Thompson 2013). We estimated IBD between each individual F<sub>2</sub> plant and each of the two  
336 grandparents (214 F<sub>2</sub> plants vs. grandparent from Teotihuacán/grandparent from Ticumán). This  
337 information was used to evaluate whether F<sub>2</sub> plants more related to a given grandparent (*i. e.*,  
338 grandparent from Ticumán or grandparent from Teotihuacán) were more or less resistant to  
339 herbivory or had higher or lower fitness/survival in the experimental sites (*see* below).

340 For IBD estimation, demultiplexing was performed with the Illumina bcl2fastq v2.19  
341 software, which returned sequence data in fastq format for each individual. Barcodes and indexes  
342 had been removed previously by CD Genomics and QB3 services. Illumina reads were trimmed  
343 using a Phred quality score > 20 in TRIMMOMATIC v0.32 (Bolger *et al.* 2014). We visually  
344 verified the quality of the grandparents and some individuals (~80) before and after trimming  
345 with FastQC (Andrews 2010). This allowed us to keep only high-quality reads for IBD analyses.

346 Sequences of each individual were aligned to the *Datura stramonium* reference genome (De-la-  
347 Cruz *et al.* under review; available in NCBI, BioProject PRJNA622882, biosample  
348 SAMN14531593, accession JAAWWY000000000) using the BWA v0.7.17 software (Li and  
349 Durbin 2009) with default parameters. SAM files from BWA were converted to BAM format  
350 and these BAM files were sorted using SAMtools v1.10 (Li *et al.* 2009).

351 The calculation of IBD values between each individual in relation to the grandparents  
352 was based on the genotype posterior probabilities (GPP) of each individual (Li *et al.* 2009;  
353 Rastas 2017). These GPPs were calculated using SAMtools mpileup (Li *et al.* 2009) and custom  
354 scripts provided in the tutorial of the Lep-MAP3 program (Rastas 2017;  
355 <https://sourceforge.net/p/lep-map3/wiki/LM3%20Home/>). These scripts also account for the  
356 alignment quality and filtering. The Lep-MAP3 program (Rastas 2017) was then used to  
357 calculate the IBD values between each individual and their grandparents.

358

### 359 **Statistical analysis**

360 All statistical analyses were performed using the JMP statistical package (v. 15.0; SAS Institute).  
361 Plotting was made using ggplot2 (Wickham 2016) in RStudio version 1.1.463 (R Core Team  
362 2020).

363

### 364 **Damage, herbivore infestation and concentration of alkaloids**

365 Estimates of herbivore damage, herbivore infestation and alkaloid concentration were natural log  
366 transformed ( $\log n + 1$ ) to meet normality assumptions. An ANOVA was performed to test for  
367 differences in the levels of infestation by different herbivore species. A repeated measures  
368 ANOVA was used to test for differences in the level of damage between sampling dates in each



369 experimental site. To assess the severity of damage as a function of the total infestation rate by  
370 each species of herbivore in each of the two populations, a Pearson correlation analysis (Zar  
371 1999) was performed between the average damage and the total infestation by each herbivore in  
372 each population.

373

#### 374 **IBD and survival in Teotihuacán**

375 To analyze if genomic similarity among the F<sub>2</sub> individuals to their Ticumán and Teotihuacán  
376 grandparent was associated with their survival probability (alive/dead), two-tailed *t*-tests were  
377 used to compare mean IBD of individuals that survived or died. This analysis was only carried  
378 out for plants grown at the Teotihuacan site, because there was not plant mortality due to damage  
379 exerted by herbivores at the Ticumán site (*see above*).

380

#### 381 **Relationships between resistance, IBD and herbivory**

382 Prior to analyses, all variables were standardized to a mean of zero and a standard deviation of  
383 one ( $\bar{x} = 0$ ,  $SD = 1$ ). Generalized linear models (GLMs) were employed to evaluate the  
384 relationships between resistance and IBD and herbivory. The GLMs described hereafter, were  
385 selected based on the statistical significance of the model and on the lowest corrected AIC  
386 values, *i. e.*, models that best explained the relationship between the variables (Akaike 1974).

387 First, general plant resistance ( $R_i$ ) of the plant  $i$  was defined as  $R_i = 100 - def$ , where  $def$  is  
388 the average proportion of leaf damage experienced by each plant (Núñez-Farfán and Dirzo  
389 1994). To evaluate the relationship between resistance and IBD, two GLMs (link = identity,  
390 distribution = normal) were constructed; one using the IBD values between F<sub>2</sub> plants and the  
391 Teotihuacán grandparent, and the other using the IBD values between F<sub>2</sub> plants and the Ticumán

392 grandparent. In these models, the response variable was resistance, whereas IBD, the  
393 experimental site, and their interaction were used as predictors. Adding the interaction between  
394 experimental site and the covariate in the models allowed us to assess whether the effect of the  
395 IBD to each one of the grandparents (Teotihuacán or Ticumán) differed depending on the site of  
396 testing (*cf.* Zar 1999).

397         Since herbivore species differed between the sites, we independently assessed resistance  
398 as a function of herbivore species by GLMs (link = identity, distribution = normal). In  
399 Teotihuacán, we used the abundance of adults and/or larvae of *L. daturaphila*, *T. soror* and *E.*  
400 *parvula* as covariates, whereas in Ticumán, only the abundance of *Epitrix* sp. (the only herbivore  
401 detected in this site) was used as a predictor.

402

#### 403 **Relationship between herbivore infestation and alkaloid concentration**

404 To assess the effect of the alkaloids on herbivore infestation, we also carried out GLMs (link =  
405 identity, distribution = normal) in which the response variables were *E. parvula*, *Epitrix* sp.  
406 adults or larvae of *L. daturaphila* or *T. soror* abundances on plants. The predictors in these  
407 models were the concentrations of the seven alkaloids. In addition, we performed stepwise  
408 GLMs (link = identity, distribution = normal) following a backward selection, which starts with  
409 all predictors in the model (seven alkaloids), and iteratively removes the least contributive  
410 predictors (Sokal and Rohlf 1994; Zar 1999). This allowed us to detect which alkaloid  
411 configuration had a greater positive or negative effect (or both) on the infestation of each  
412 herbivore species. The best GLMs were selected based on the statistical significance of the  
413 model and on the lowest corrected AIC values (Akaike 1974). An additional GLM (link =  
414 identity, distribution = normal) with the total alkaloid concentration as a predictor was carried

415 out to see the impact of total alkaloid concentration on herbivores. Tests for the interaction  
416 between experimental site and one particular herbivore were not possible because different  
417 species were present in the two sites.

418

#### 419 **Natural selection on alkaloids, resistance, herbivore infestation and IBD**

420 To quantify the magnitude and direction of natural selection acting on the seven alkaloids, we  
421 used the number of seeds produced by each plant as a fitness proxy to perform phenotypic-  
422 selection analyses (Lande 1979; Lande and Arnold 1983). For this purpose, standardized  
423 individual fitness (relative fitness) was calculated as  $w_i = x_i/\bar{x}$ , where  $x_i$  is the total number of  
424 seeds produced per plant, and  $\bar{x}$  is the average number of seeds per plant in the population in  
425 each site. In all analyses,  $w_i$  was used as a response variable. Thus, one GLM (link = identity,  
426 distribution = normal) was constructed using the concentrations of seven alkaloids, the  
427 experimental site, and their interactions. An additional two separate GLMs were constructed  
428 using resistance and total alkaloid concentration as predictors, as well as experimental site as a  
429 factor. Interactions between site and predictors allowed us to test if the effects of predictors on  
430 fitness differed between the two sites.

431 Two separate models, one for each experimental site, were carried out to assess selection  
432 on the infestation by each herbivore (independent variables). As pointed out earlier, we could not  
433 evaluate the effect of the experimental site and its interaction with predictors, since different  
434 species of herbivores were present in the two populations.

435 Finally, to evaluate the effects of identity by descent (IBD) on fitness (seed production),  
436 two GLMs were constructed (one using the IBD values between F<sub>2</sub> plants and the Teotihuacán  
437 grandparent, and the other using the IBD values between F<sub>2</sub> plants and the Ticumán

438 grandparent). In these models, the response variable was relative fitness, whereas IBD,  
439 experimental site and its interaction were predictors.

440 The generalized linear coefficients (*i. e.*, the selection gradients;  $\beta_i$ , Lande and Arnold  
441 1983) obtained from the selection analyses represent the strength and direction of selection  
442 acting directly on each alkaloid, resistance, infestation by each herbivore and IBD in comparable  
443 units (standard deviations; Wise and Rausher 2013).

444

445

## Results

### 446 **Damage, herbivore infestation and alkaloid concentrations in the two experimental sites**

447 Damage by herbivores varied between sampling dates in each site (Teotihuacán:  $F_{710} = 110.98$ ,  
448  $R^2 = 0.41$ ,  $p = 0.0001$ ; Ticumán:  $F_{262} = 27.16$ ,  $R^2 = 0.27$ ,  $p = 0.0001$ ; S3 a, b, S4). There were  
449 clear differences in level of infestation by the different species of herbivores ( $F_{545} = 215.32$ ,  $R^2 =$   
450  $0.61$ ,  $p = 0.0001$ ; S3 c, S4). Correlation analyses indicated that plant damage in Teotihuacán site  
451 was mainly imposed by larvae of *L. daturaphila*, whereas that in the Ticumán site mainly by  
452 *Epitrix* sp. (S5).

453

### 454 **Effect of the Identity by Descent (IBD) on fitness/survival and on resistance**

455 The GLM between fitness and the IBD to the Ticumán grandparent as measured by genome wide  
456 IBD was significant, revealing a positive effect of increasing IBD on fitness ( $L-R$  chi-square<sub>3</sub> =  
457  $12.91$ ,  $AICc = 273.68$ ,  $p = 0.0048$ , Table 1, Fig. 1 a, b). The model between fitness and the IBD  
458 with the Teotihuacán grandparent was not significant ( $L-R$  chi-square<sub>3</sub> =  $2.68$ ,  $AICc = 283.90$ ,  $p$   
459 =  $0.4424$ , Table 1, Fig. 1 c, d). Our results also showed that plants more related to the Ticumán  
460 grandparent had higher survival than F<sub>2</sub> plants less related to the Ticumán grandparent in the

461 Teotihuacán site ( $F_{129} = 17.52$ ,  $R^2 = 0.12$ ,  $p = 0.0001$ ; Fig. 1 e). In contrast, plant survival was not  
462 significantly associated with IBD to the Teotihuacán grandparent in Teotihuacán ( $F_{129} = 1.92$ ,  $R^2$   
463  $= 0.014$ ,  $p = 0.1682$ ; Fig. 1 f). The effect of IBD on plant survival in the Ticumán site was not  
464 evaluated because there was not plant mortality due to damage exerted by herbivores in this site  
465 (see above). The mean  $F_2$  full-sibs relatedness (IBD) was 0.47 (range 0.006-0.803, standard error  
466  $= 0.0007$ ). Identity by descent between the  $F_2$  plants to each grandparent range between 0.006-  
467 0.5 (Teotihuacán grandparent) and 0.031-0.5 (Ticumán grandparent). Relatedness between the  
468 two grandparents was zero (Fig. 1 g).

469 The GLM between resistance and the IBD with the Ticumán grandparent was significant  
470 ( $L-R$  chi-square<sub>3</sub> = 54.21,  $AICc = 441.47$ ,  $p = 0.0001$ ). Significant effects included population,  
471 and the interaction between population and IBD to the Ticumán grandparent (positive  
472 relationship in Teotihuacán site, while an opposite effect was observed in the Ticumán site;  
473 Table 2, Fig. 2 a, b). The GLM between resistance and the IBD with the Teotihuacán  
474 grandparent was significant ( $L-R$  chi-square<sub>3</sub> = 32.50,  $AICc = 463.18$ ,  $p = 0.0001$ ). However,  
475 only the population effect was significant (Table 2, Fig. 2 c, d).

476

### 477 **Resistance against herbivore infestation levels in the two experimental sites**

478 Resistance to herbivory was significantly related to herbivore infestation levels in Teotihuacán  
479 ( $L-R$  chi-square<sub>4</sub> = 111.09,  $AICc = 426.37$ ,  $p = 0.0001$ ). Resistance was only positively related to  
480 levels of *E. parvula* infestation and negatively related to infestation by larvae of *L. daturaphila*  
481 in Teotihuacán (Table 2, Fig. 2 e, f). However, resistance and *Epitrix* sp. infestation levels were  
482 negatively related in Ticumán ( $L-R$  chi-square<sub>1</sub> = 14.96,  $AICc = 223.89$ ,  $p = 0.0001$ ; Table 2, Fig.  
483 2 g).

484

### 485 **Effect of the alkaloid concentration on herbivore infestation levels**

486 The effect of the seven alkaloid concentrations on *E. parvula* infestation level was significant (*L-*  
487 *R* chi-square<sub>7</sub> = 15.67, *AICc* = 444.78; *p* = 0.0282). However, the effects of individual alkaloids  
488 were different: triterpenoid had a positive significant effect, whereas negative significant effects  
489 were detected in case of phenylacetaldehyde and pyrroline (S6). The effect of the total alkaloid  
490 concentration on *E. parvula* infestation was negative and significant (*L-R* chi-square<sub>1</sub> = 3.91,  
491 *AICc* = 462.23; *p* = 0.0479; S6).

492 The effect of alkaloid concentrations on infestation levels by larvae of *L. daturaphila* was  
493 significant (*L-R* chi-square<sub>5</sub> = 12.53, *AICc* = 460.49, *p* = 0.0281). Significant effects included  
494 phenylacetaldehyde (positively) and the triterpenoid (negative effect; S6). The GLM of  
495 infestation levels by larvae of *L. daturaphila* against the total alkaloid concentration was not  
496 significant (*L-R* chi-square<sub>1</sub> = 1.45, *AICc* = 473.76; *p* = 0.2272; S6).

497 The effect of alkaloid concentration on infestation levels by adults of *L. daturaphila* was  
498 significant (*L-R* chi-square<sub>3</sub> = 9.49, *AICc* = 453.93; *p* = 0.023). In this model we observed that 3-  
499 hydroxy-6-tigloyloxytropine was significantly and negatively related with *L. daturaphila* adults,  
500 whereas atropine showed a significant positive relationship (S6). The GLM of infestation levels  
501 by adults of *L. daturaphila* against the total alkaloid concentration was also not significant (*L-R*  
502 chi-square<sub>1</sub> = 0.49, *AICc* = 469.65; *p* = 0.4797; S6).

503 The GLM testing for the effects of alkaloid concentration on *T. soror* infestation rate was  
504 significant (*L-R* chi-square<sub>4</sub> = 10.95, *AICc* = 467.18, *p* = 0.027). In this model, *T. soror*  
505 infestation level was negatively related to scopolamine concentration, but positively related to  
506 the concentration level of the triterpenoid (S6). The effect of the total alkaloid concentration on

507 *T. soror* infestation was also not significant ( $L$ - $R$  chi-square<sub>1</sub> = 0.11,  $AICc$  = 478.92;  $p$  = 0.7367;  
508 S6).

509 The GLM between *Epitrix* sp. infestation levels and the alkaloid concentrations in  
510 Ticumán was significant ( $L$ - $R$  chi-square<sub>7</sub> = 17.89,  $AICc$  = 134.49,  $p$  = 0.0125; S6). Significant  
511 effects included 3-hydroxy-6-tigloyloxytropine (negative effect) and pyrroline (positive effect;  
512 S6). The effect of the total alkaloid concentration on *Epitrix* sp. infestation was not significant  
513 ( $L$ - $R$  chi-square<sub>1</sub> = 0.70,  $AICc$  = 141.09;  $p$  = 0.4000; S6).

514

### 515 **Natural selection on resistance, alkaloids and herbivore infestation in the two experimental** 516 **sites**

517 The GLM of relative fitness against resistance to herbivory was significant ( $L$ - $R$  chi-square<sub>3</sub> =  
518 20.77,  $AICc$  = 487.04;  $p$  = 0.0001). While there was no main effect of the population, the  
519 population  $\times$  resistance interaction was significant, revealing that the relative fitness was  
520 positively related to resistance in Teotihuacán, but negatively in Ticumán (Table 1, S7 a, b).

521 The GLM of relative fitness as a function of the concentration of seven alkaloids was  
522 significant ( $L$ - $R$  chi-square<sub>15</sub> = 26.14,  $AICc$  = 400.98;  $p$  = 0.036). There was a significant  
523 positive main effect of the pyrroline concentration on fitness (Table 1), but the experimental site  
524  $\times$  pyrroline interaction was also significant, revealing a negative trend between fitness and  
525 pyrroline concentration in Teotihuacán, whereas the opposite was true in Ticumán (Table 1, S8).  
526 Similarly, the significant experimental site  $\times$  triterpenoid interaction revealed that fitness was  
527 positively related to triterpenoid concentration in Teotihuacán, but with a negative trend in  
528 Ticumán (Table 1, S8). The GLM of relative fitness against total alkaloid concentration was not

529 significant in either of the populations ( $L-R$  chi-square<sub>3</sub> = 2.86,  $AICc$  = 418.28;  $p$  = 0.41; Table  
530 1).

531 The GLM of relative fitness against level of herbivore infestation (larvae and adults of  
532 *Lema*, *E. parvula* and *T. soror*) was significant in Teotihuacán ( $L-R$  chi-square<sub>4</sub> = 17.29,  $AICc$  =  
533 342.00;  $p$  = 0.002) (Table 1). Nevertheless, only the negative effect of *L. daturaphila* larvae on  
534 fitness was significant (Table 1). Likewise, a significant positive effect of *Epitrix* sp. infestation  
535 level on fitness was detected in Ticumán ( $L-R$  chi-square<sub>1</sub> = 13.06,  $AICc$  = 130.18,  $p$  = 0.001;  
536 Table 1).

537

538

### Discussion

539 Our results revealed differentiation in plant-herbivore interactions among the study sites. First,  
540 different herbivore species are present in each population, and the infestation levels and the  
541 amount of foliar damage exerted by each herbivore on plants differed within and between  
542 populations. Second, different chemical compounds were related to infestation by each specific  
543 herbivore. Third, variable spatial selection was detected on identity by descent (IBD), resistance,  
544 chemical defensive traits and herbivore infestation levels.

545 A number of studies have also documented geographic variation in the level of herbivory  
546 and chemical defenses (Castells *et al.* 2005; Muola *et al.* 2010; Agrawal *et al.* 2012; Züst *et al.*  
547 2012; Castillo *et al.* 2014; Verçosa *et al.* 2019; Hanh *et al.* 2019). However, there has been no  
548 previous attempts to determine how the plants' genetic background (IBD) is driven by ongoing  
549 natural selection-imposed by herbivores. Furthermore, the results provide strong evidence of  
550 local adaptation in plant-herbivore interactions in both populations of *D. stramonium*.



551 In the locality of Teotihuacán, F<sub>2</sub> plants more related to the local grandparent (selected as  
552 a parent due to its low alkaloid concentration) were less resistant and had higher mortality due to  
553 herbivory than F<sub>2</sub> plants more related to the Ticumán grandparent. Furthermore, we did not  
554 detect any relationship between fitness and IBD to the Teotihuacán grandparent in this site. This  
555 result was anticipated since it is not expected that natural selection would favor poorly defended  
556 plants in a habitat where damage by herbivores can be lethal (*e. g.*, plant deaths due to herbivory  
557 caused by larvae of *Lema daturaphila*) (Valverde *et al.* 2001; 2003; Fornoni *et al.* 2004). Hence,  
558 it is plausible that F<sub>2</sub> plants more related to the Teotihuacán grandparent inherited the loci/alleles  
559 that do not confer resistance (Albrechtsen *et al.* 2010; Browning and Browning 2012). Likewise,  
560 alkaloid concentration in F<sub>2</sub> plants more related to the Teotihuacán grandparent remained at low  
561 levels after damage by different herbivores in Teotihuacán. This result indicates that the  
562 chemical defenses studied here are not induced and have a genetic basis, since a positive  
563 significant relationship between plant resistance and IBD to the Teotihuacán grandparent would  
564 be expected if plant defenses were induced after herbivore damage (Baldwin 1998; Karban and  
565 Baldwin 2007).

566 In marked contrast, we detected strong positive selection on IBD to the Ticumán  
567 grandparent in the locality of Teotihuacán. Also, plant resistance to herbivores and IBD to the  
568 Ticumán grandparent were positively related in this site. We suggest that F<sub>2</sub> plants more related  
569 to the Ticumán grandparent - selected as a parent on the basis of its high alkaloid concentration -  
570 had higher survival in Teotihuacán as they inherited the loci/alleles that confer resistance to  
571 herbivores; positive selection of these loci/alleles would be associated with different defensive  
572 chemical compounds which are produced in high concentration (Albrechtsen *et al.* 2010; Lowry  
573 *et al.* 2019). In fact, our findings indicate that the higher resistance of F<sub>2</sub> plants more related to

574 the Ticumán grandparent in the Teotihuacán site was provided by specific alkaloids that are  
575 produced in very high concentration to face different herbivore species. Total alkaloid  
576 concentration (sum of the concentration of all classes of alkaloids; Moore *et al.* 2014) only seems  
577 to affect negatively the infestation levels of *E. parvula*. Since alkaloid concentrations vary in  
578 wild Teotihuacán plants (Castillo *et al.* 2014; Miranda-Pérez *et al.* 2016; De-la-Cruz *et al.* 2020),  
579 we think that wild plants from Teotihuacán that produce specific alkaloids in very high  
580 concentrations (*i. e.*, plants more related to the Ticumán grandparent) have strong chemical  
581 defense against the herbivores in this site. For instance, we observed strong positive selection to  
582 increase the concentration of the triterpenoid compound in Teotihuacán, which seems to affect  
583 negatively the infestation levels of the most harmful herbivore of *D. stramonium*, the larvae of *L.*  
584 *daturaphila*.

585         The defensive role of specific alkaloids in the Teotihuacán site revealed unexpected  
586 results, namely, changing the sign of their relationship with the infestation by different  
587 herbivores. For instance, while the triterpenoid compound appears to reduce the infestation of  
588 *Lema* larvae (the most dangerous herbivore of *D. stramonium*), it was also positively associated  
589 with infestation levels by *E. parvula* and *T. soror*. Triterpenoids are structurally similar to insect  
590 hormones known as ecdysones (Oliveira *et al.* 2019) known to control metamorphosis as insects  
591 pass from larva to pupa to adult (Yamanaka *et al.* 2013). It has been reported that many  
592 triterpenoids function as ecdysone blockers (*e. g.*, azadirone; Ujváry 2010; Oliveira *et al.* 2019).  
593 Therefore, the most parsimonious explanation for our observations is that this triterpenoid of *D.*  
594 *stramonium* is acting mainly on larvae of *Lema* (Miller *et al.* 1989; Ujváry 2010), and since this  
595 compound is structurally similar to insect hormones (Ujváry 2010), it may be used by *E. parvula*  
596 and *T. soror* adults to trace *D. stramonium* plants (and potential mates on them). Complex

597 interactions where one compound is toxic to insects at one developmental stage (*e. g.*, larvae) or  
598 to a particular herbivore species, but functioning as an attractant at other stage (adults) or to other  
599 herbivore species have been reported, for instance, in *Nicotiana attenuata* (Zhou *et al.* 2017).

600         Local adaptation of plant defenses to herbivores depends on (1) the strength of selection  
601 as a result of the interaction, and (2) the level of specificity on the interaction (*e. g.*, folivores,  
602 seed predators, stem-borers) (Thompson *et al.* 2005; Cogni and Futuyma 2009; Agrawal *et al.*  
603 2012). In the Teotihuacán site, our results suggest that the strong selection pressure exerted by  
604 one herbivore (the folivore *L. daturaphila*) on *D. stramonium* plants may affect the interaction  
605 between plants and other insects, leading to local adaptation of plant defenses to different  
606 herbivore species (Wise 2009, 2010).

607         On the other hand, in the Ticumán site, we detected strong positive selection on pyrroline  
608 alkaloid. It has been reported that pyrroline is a defensive compound against many insect species  
609 and pathogens (bacteria, virus, fungi) (Qamar *et al.* 2015; Martins *et al.* 2015; Tamariz *et al.*  
610 2018). Pyrroline has also been related to different physiological processes such as plant growth  
611 (Chen *et al.* 2018; Tamariz *et al.* 2018). It is worth mentioning that polyamine oxidase, an  
612 enzyme involved in the biosynthesis of the pyrroline, is a growth-regulating enzyme (Chen *et al.*  
613 2018). Nevertheless, an unexpected finding is that we observed a positive association between  
614 pyrroline concentration and infestation level by *Epitrix* in Ticumán. It has reported that some  
615 herbivore insects can tolerate pyrrolizidine alkaloids and use them for defense against their  
616 predators or as precursors of insect hormones (Martins *et al.* 2015). Thus, our most parsimonious  
617 explanation is that *Epitrix* sp. is surpassing the defensive role of the pyrroline alkaloid in  
618 Ticumán. This could explain why the F<sub>2</sub> plants more genetically related to the Ticumán  
619 grandparent (with higher concentration of pyrroline) had lower resistance towards *Epitrix* sp.

620 infestation. Furthermore, since pyrroline could be positively related to plant growth (Chen *et al.*  
621 2018; Tamariz *et al.* 2018), it is also possible that *Epitrix* sp. searches for more vigorous plants,  
622 which have more biomass to feed (Agrawal 2005; Wise and Rausher 2013). On the other hand,  
623 we observed that 3-hydroxy-6-tigloyloxytropine negatively affected the infestation levels of  
624 *Epitrix* sp. in Ticumán. Then, it seems that the latter alkaloid is providing resistance against this  
625 herbivore in this site.

626         Interestingly, pyrroline affected negatively the infestation levels of *E. parvula* in  
627 Teotihuacán. However, negative selection on this compound was detected also in Teotihuacán.  
628 Thus, while *Epitrix* sp. appears to be adapted to this compound in Ticumán, the production of  
629 this compound in high concentrations in Teotihuacán may involve physiological costs, as plants  
630 also have to allocate resources for production of other compounds (*e. g.*, triterpenoid) to tackle  
631 their most harmful herbivore (*Lema* larvae). Indeed, as we mentioned above, it seems that total  
632 alkaloid concentration should be the option to face with *E. parvula* infestation in Teotihuacán.

633         De-la-Cruz *et al.* (2020) found that plants from Ticumán have on average higher alkaloid  
634 concentration than those in Teotihuacán. Why we did not observe strong selection to increase the  
635 IBD to the Ticumán grandparent (higher alkaloid concentration) in Ticumán? Our most  
636 parsimonious explanations are, first, as mentioned above, that *Epitrix* sp. (the main herbivore in  
637 this site) seems locally adapted to plant chemical defenses (pyrroline) in Ticumán, and that other  
638 alkaloids could now be providing defense against this herbivore. Second, since these compounds  
639 are expressed constitutively, it is possible that all these powerful chemical weapons are being  
640 used to face other natural enemies (virus, bacteria, nematodes, fungi, oomycete, other herbivore  
641 species) that we did not detect or that were not present during our experiment. Third, it is also  
642 possible that these compounds have other functions in this habitat (*e. g.*, growth, plant-plant

643 communication). For instance, recent genomic evidence from *D. stramonium* indicates that  
644 tropane alkaloids such as atropine and scopolamine also act as defenses against pathogens and  
645 viruses (De-la-Cruz *et al.* under review).

646 Finally, the lack of association between the IBD to the Teotihuacán grandparent with  
647 resistance or fitness in Ticumán suggests that the chemical defenses studied here are not induced  
648 (see above; Karban and Baldwin 2007).

649

650

### Conclusions

651 The methodology used in this study allowed us to get insights on how natural selection imposed  
652 by herbivores drives the genetic underpinnings of plant resistance traits. The lack of association  
653 between plant fitness and IBD to the Teotihuacán grandparent (low resistance) in both  
654 populations, as well as different magnitude and direction of selection on the IBD to the Ticumán  
655 grandparent (high resistance) across populations, provides evidence of how ongoing natural  
656 selection operates on plant resistance and promotes local adaptation. Likewise, the results of this  
657 study shed some new light on how plants defend themselves against the attack from different  
658 herbivores. It seems that in populations where plants are suffering frequent or heavy damage by  
659 different herbivores, plants are able to produce and “use” different chemical defensive  
660 compounds to face each insect species that feed on them (Wittstock and Gershenzon 2002). The  
661 same alkaloids were produced by plants in both populations, but plants possessing the highest  
662 total alkaloid concentrations were not the most well-defended or fit in either of the populations.  
663 Instead, different specific alkaloids appear to be favored by natural selection imposed by  
664 herbivores in the two study populations.

665 Our results also revealed that the strong negative selection imposed by one herbivore  
666 species (*e. g.*, larvae of *L. daturaphila*) on plants likely affects interactions with other insects.  
667 Most importantly, the results provide evidence for local adaptation by showing that selection  
668 favors different loci/alleles related to plant resistance to herbivores in the two populations  
669 (Briscoe Runquist *et al.* 2020). Hence, intraspecific diversity in secondary metabolites of *D.*  
670 *stramonium* seems to be maintained and selected to cope with varying local conditions among  
671 populations (Moore *et al.* 2014), giving rise to a geographic coevolutionary mosaic (Thompson  
672 2005).

673

674

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**Table 1.** Analyses of natural selection testing the effects of (a) Identity by Descent to Ticumán grandparent, (b) Identity by Descent to Teotihuacán grandparent, (c) resistance, (d) the seven alkaloids, (e) total alkaloid concentration, (f) herbivores at Teotihuacán and (g) *Epitrix* sp. infestation at Ticumán. N = number of individuals, d.f. = degrees of freedom,  $\beta_i$  = selection gradients (generalized linear coefficients), se = standard error,  $t$  = t-ratio. Significant  $p$ -values ( $p$ ) are in bold. Pop = effect of the experimental site.

Response variable	Effects	N	d.f.	$\beta_i$	se	$t$	$p$
(a) $w_i$ fitness	IBD-Ticumán grandparent	135	3	<b>2.19</b>	0.99	2.20	<b>0.0302</b>
	Pop	135	3	-0.13	0.10	-1.22	0.2262
	Pop $\times$ IBD	135	3	1.39	0.99	1.40	0.1646
(b) $w_i$ fitness	IBD-Teotihuacán grandparent	135	3	0.41	0.89	0.46	0.6484
	Pop	135	3	-0.17	0.11	-1.49	0.1390
	Pop $\times$ IBD	135	3	-0.71	0.89	-0.80	0.4252
(c) $w_i$ fitness	Resistance	177	1	0.11	0.27	0.43	0.6624
	Pop	177	1	-0.15	0.31	-0.50	0.6143
	Pop $\times$ Resistance	177	1	<b>0.64</b>	0.27	2.36	<b>0.0179</b>
(d) $w_i$ fitness	3-hydroxy-6-tigloyloxytropene	136	1	-0.36	0.21	-1.71	0.0899
	Anisodamine	136	1	-0.07	0.28	-0.25	0.7961
	Atropine	136	1	-0.07	0.12	-0.56	0.5719
	Triterpenoid	136	1	0.26	0.18	1.46	0.1459
	Scopolamine	136	1	-0.24	0.19	-1.24	0.2151
	Phenylacetaldehyde	136	1	-0.21	0.21	-0.98	0.3254
	Pyrroline	136	1	<b>0.32</b>	0.14	2.19	<b>0.0304</b>
	Pop	136	1	-0.12	0.09	-1.29	0.1997
	Pop $\times$ 3-hydroxy-6-tigloyloxytropene	136	1	0.13	0.21	0.62	0.5362
	Pop $\times$ Anisodamine	136	1	-0.06	0.28	-0.24	0.8104
	Pop $\times$ Atropine	136	1	0.19	0.12	1.55	0.1224
	Pop $\times$ Triterpenoid	136	1	<b>0.38</b>	0.18	2.10	<b>0.0378</b>
	Pop $\times$ Scopolamine	136	1	-0.04	0.19	-0.20	0.8375
	Pop $\times$ Phenylacetaldehyde	136	1	-0.14	0.21	-0.67	0.5015
Pop $\times$ Pyrroline	136	1	<b>-0.43</b>	0.14	-2.93	<b>0.0040</b>	
(e) $w_i$ fitness	Total alkaloid concentration	144	1	-0.05	0.08	-0.58	0.5657
	Pop	144	1	-0.12	0.09	-1.34	0.1838
	Pop $\times$ Total alkaloid concentration	144	1	0.09	0.08	1.04	0.2988
(f) $w_i$ fitness	Adults of <i>Lema daturaphila</i>	113	1	0.08	0.09	0.90	0.3692
	Adults of <i>Epitrix parvula</i>	113	1	0.09	0.09	0.97	0.3324
	Adults of <i>Trichobaris soror</i>	113	1	0.09	0.08	1.03	0.3070
	Larvae of <i>Lema daturaphila</i>	113	1	<b>-0.27</b>	0.08	-3.22	<b>0.0017</b>
(g) $w_i$ fitness	Adults of <i>Epitrix</i> sp.	63	1	<b>0.30</b>	0.081	3.75	<b>0.0004</b>

**Table 2.** General linear models testing the effect of (a) Identity by Descent to the Ticumán grandparent, (b) Identity by Descent to the Teotihuacán grandparent, (c) herbivore infestation at Teotihuacán and (d) *Epitrix* sp. infestation at Ticumán, on whole plant resistance. N = number of individuals, d.f. = degrees of freedom, Estimate = generalized linear coefficients, se = standard error, *t* = t-ratio. Significant *p*-values (*p*) are in bold. Pop = effect of the experimental site.

Response variable	Effects	N	d.f.	Estimate	se	<i>t</i> ratio	<i>p</i>
(a) Resistance	IBD-Ticumán grandparent	182	3	0.98	0.64	1.52	0.1256
	Pop	182	3	<b>-0.40</b>	0.06	-5.95	<b>0.0001</b>
	Pop × IBD	182	3	<b>2.06</b>	0.64	3.18	<b>0.0015</b>
(b) Resistance	IBD-Teotihuacán grandparent	182	3	0.31	0.48	0.64	0.5182
	Pop	182	3	<b>-0.40</b>	0.07	-5.69	<b>0.0001</b>
	Pop × IBD	182	3	0.51	0.48	1.05	0.2896
(c) Resistance	Adults of <i>Lema daturaphila</i>	185	1	0.02	0.05	0.38	0.7009
	<i>Epitrix parvula</i>	185	1	<b>0.27</b>	0.06	4.51	<b>0.0001</b>
	<i>Trichobaris soror</i>	185	1	0.03	0.05	0.53	0.5944
	Larvae of <i>Lema daturaphila</i>	185	1	<b>-0.52</b>	0.05	-9.00	<b>0.0001</b>
(d) Resistance	<i>Epitrix</i> sp.	82	1	<b>-0.40</b>	0.10	-4.00	<b>0.0001</b>

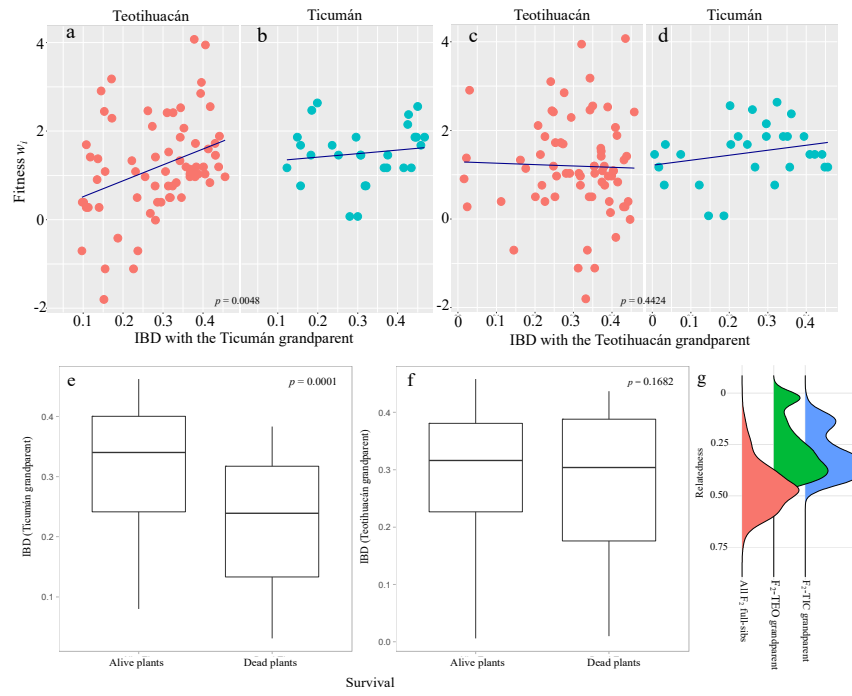
## Figure legends

**Fig. 1.** Relationships between plant fitness (log scale) and identity by descent (IBD) relationship to the Ticumán grandparent for (a) plants grown in the Teotihuacán population and (b) plants grown in the Ticumán population. Relationships between plant fitness and IBD relationship to the Teotihuacán grandparent for (c) plants grown in the Teotihuacán population and (d) plants grown in the Ticumán population. (e) Box plot of IBD to the Ticumán grandparent for plants that survived and died. (f) Box plot of IBD to the Teotihuacán grandparent for plants that survived and died. (g) Distribution of the relatedness between all F<sub>2</sub> full-sibs, and relatedness of F<sub>2</sub> plants to each grandparent. A relatedness of ~0.5 is the mean expected value between all F<sub>2</sub> full-sibs. A relatedness of ~0.5 is the maximum expected value between the F<sub>2</sub> progeny and each one of the grandparents (Falconer and Mackay 1996). *p*-values of full GLMs are shown in each plot (a-f). Each dot depicts observation for an individual. See also Table 1.

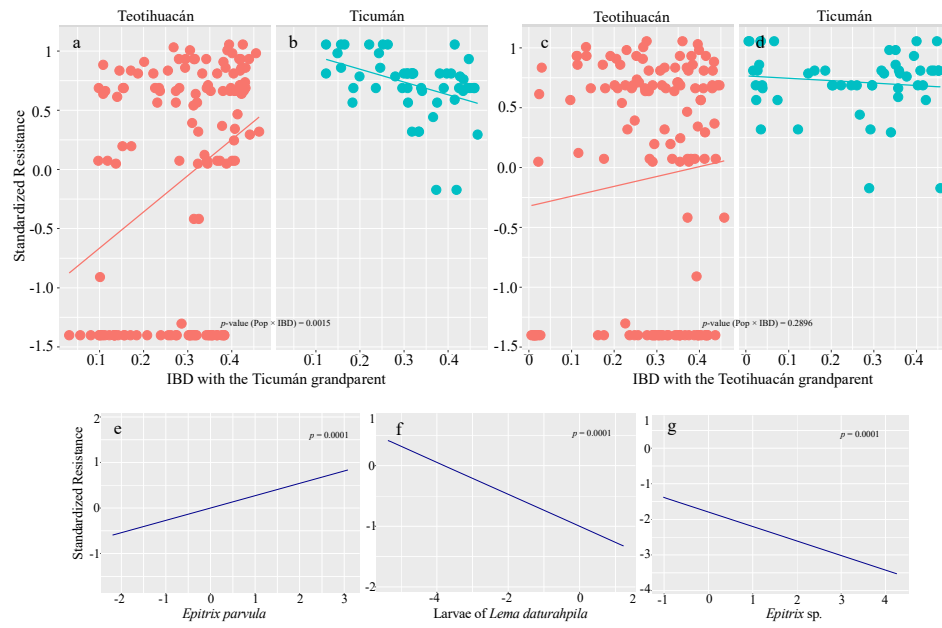
**Fig. 2.** Relationships between resistance to herbivory and identity by descent (IBD) relationship to the Ticumán grandparent for (a) plants grown in the Teotihuacán population and (b) plants grown in the Ticumán population. Relationships between resistance to herbivory and IBD relationship to the Teotihuacán grandparent for (c) plants grown in the Teotihuacán population and (d) plants grown in the Ticumán population. Relationship between resistance and (e) adults of *Epitrix parvula* in Teotihuacán, (f) larvae of *Lema daturaphila* in Teotihuacán, (g) adults of *Epitrix* sp. in Ticumán. *p*-values of GLMs are shown in each plot (a-g). Each dot depicts observation for an individual. Pop = population. See also Table 2.



**Fig. 1**



**Fig. 2**



**Supplementary Information (Figures and Tables) of the manuscript**  
**“Genomic and chemical evidence for local adaptation in resistance**  
**to different herbivores in *Datura stramonium*”**

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**Evolution**

S1. Depiction of the experimental design used to produce the F<sub>2</sub> generation progeny used in the study (see Methods for details).

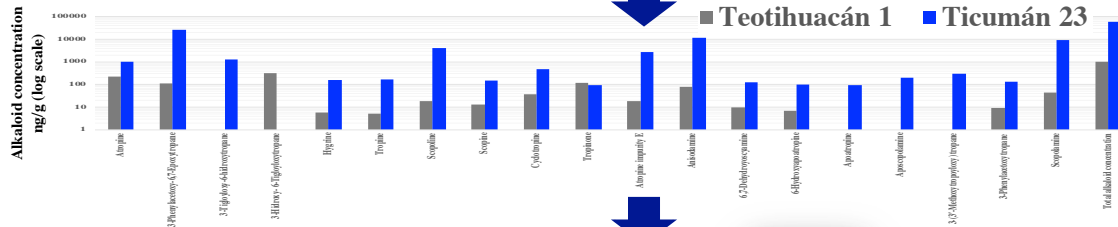
**Experiment design to select the parents and to produce the F<sub>2</sub> generation progeny**



Random crosses (ca. 200) were carried out with plants from **Ticumán** and **Teotihuacán**. Blue labels pots belong to Teotihuacán plants, while orange belong to Ticumán plants.

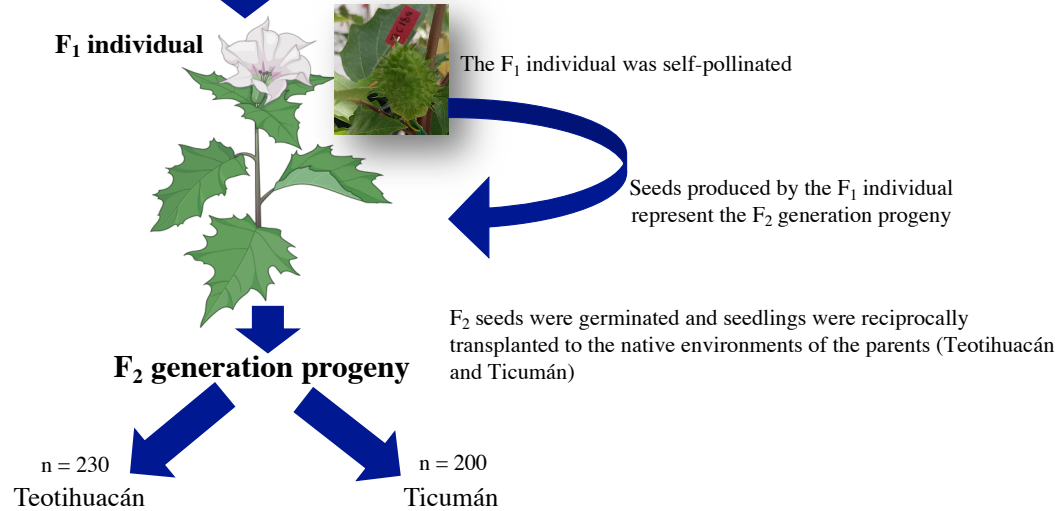
We screened the concentration of 21 tropane alkaloids for each plant. From all the crosses, we selected the couple with the most differentiated individuals in their concentration of tropane alkaloids.

Ticumán 23  
59051.20527 ng/g  
vs.  
Teotihuacán 1  
1013.064785 ng/g



Plant couple with the most differentiated individuals in their concentration of tropane alkaloids

Seeds (F<sub>1</sub> generation progeny) from these plant couple were germinated, and we only took one individual.

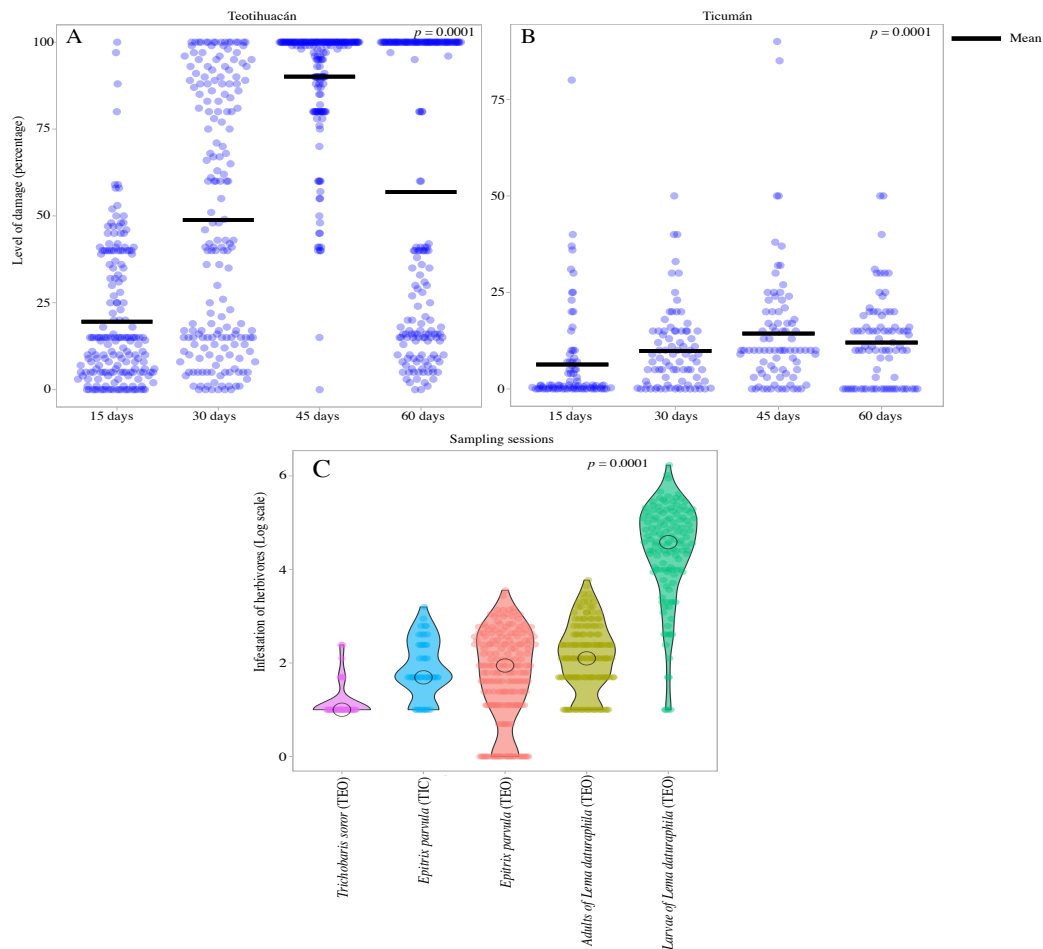


**S2.** Alkaloids identified in leaves of *Datura stramonium*. RT, the retention time of each alkaloid;  $m/z$  = mass/charge,

MS = mass spectrometry reference.

<b>Alkaloid</b>	<b>Formula</b>	<b>RT (min)</b>	<b><math>m/z</math></b>	<b>MS Ref.</b>
3-Tigloyloxy-6-Hydroxytropane	C <sub>13</sub> H <sub>21</sub> NO <sub>3</sub>	10.1	240.1594	Witte 1987
6-Hydroxyhyoscyamine (Anisodamine)	C <sub>17</sub> H <sub>23</sub> NO <sub>4</sub>	13.2	306.1699	Ionkova <i>et al.</i> 1994
Atropine (Hyoscyamine)	C <sub>17</sub> H <sub>23</sub> NO <sub>3</sub>	8.7	290.1751	Witte 1987
Scopolamine	C <sub>17</sub> H <sub>21</sub> NO <sub>4</sub>	15	304.1543	Witte 1987
Pyrroline	C <sub>4</sub> H <sub>7</sub> N	5.5	70.0655	Pedrol and Tiburcio 2001
Phenylacetaldehyde	C <sub>8</sub> H <sub>8</sub> O	9.4	121.0641	Cantelo and Jacobson 1979
Triterpenoid	C <sub>28</sub> H <sub>36</sub> O <sub>4</sub>	15.6	437.2721	González-Coloma <i>et al.</i> 2011

**S3.** Plant damage (in percentage) experienced by each F<sub>2</sub> plant during four sampling dates at (A) Teotihuacán and (B) Ticumán. Plants experienced the most severe damage at 45 days after transplanting in the two localities, and damage levels were higher in Teotihuacán than in Ticumán. (C) Violin plot showing total infestation accounted by different herbivore (log scale). The circle inside each violin depicts the mean value. Overall, *Lema daturaphila* larvae was the most abundant insect herbivore. A quasi-random jittering was used to reduce datapoint overlap. TEO = Teotihuacán, TIC = Ticumán. *p*-values of ANOVAs are showed in each plot. For figures (A) and (B): black line = mean.



**S4.** Mean differentiation in the level of damage (expressed as percentage) between sampling sessions in (a)

Teotihuacán and (b) Ticumán. (c) Mean differentiation in the level of herbivore infestation (Log transformed data).

N = number of individuals, se = standard error, d.f. = degree of freedoms, SS = Sum of Squares, MS = Mean Square,

*F* = Fisher-statistic, *p* = *p*-values of ANOVAs.

ANOVA	N	Mean	se	Source	d.f.	SS	MS	<i>F</i>	<i>p</i>
(a) Damage at Teotihuacán									
15 days	164	19.55	0.07	Model	3	309.61	103.20	99.04	<b>0.0001</b>
30 days	179	48.84	0.07	Error	706	735.63	1.04		
45 days	184	90.08	0.07	Total	709	1045.24			
60 days	183	56.86	0.07						
(b) Damage at Ticumán									
15 days	83	6.40	0.12	Model	3	211.30	70.43	50.33	<b>0.0001</b>
30 days	83	9.96	0.12	Error	317	443.61	1.39		
45 days	73	14.51	0.13	Total	320	654.91			
60 days	82	12.15	0.13						
(c) Herbivore									
<i>E. parvula</i> (TEO)	185	1.74	0.06	Model	4	666.49	166.62	215.32	<b>0.0001</b>
<i>Epitrix</i> sp. (TIC)	47	1.87	0.12	Error	541	418.65	0.77		
Adults of <i>L. daturaphila</i> (TEO)	143	2.15	0.07	Total	545	1085.14			
Larvae of <i>L. daturaphila</i> (TEO)	131	4.36	0.07						
<i>T. soror</i>	40	1.16	0.13						

**S5.** Correlations between infestation by each herbivore and plant leaf average damage. a = Teotihuacán and b = Ticumán. ALd = adults of *Lema daturaphila*, Ep = *Epitrix parvula*, Ts = *Trichobaris soror*, LLd = larvae of *Lema daturaphila*.

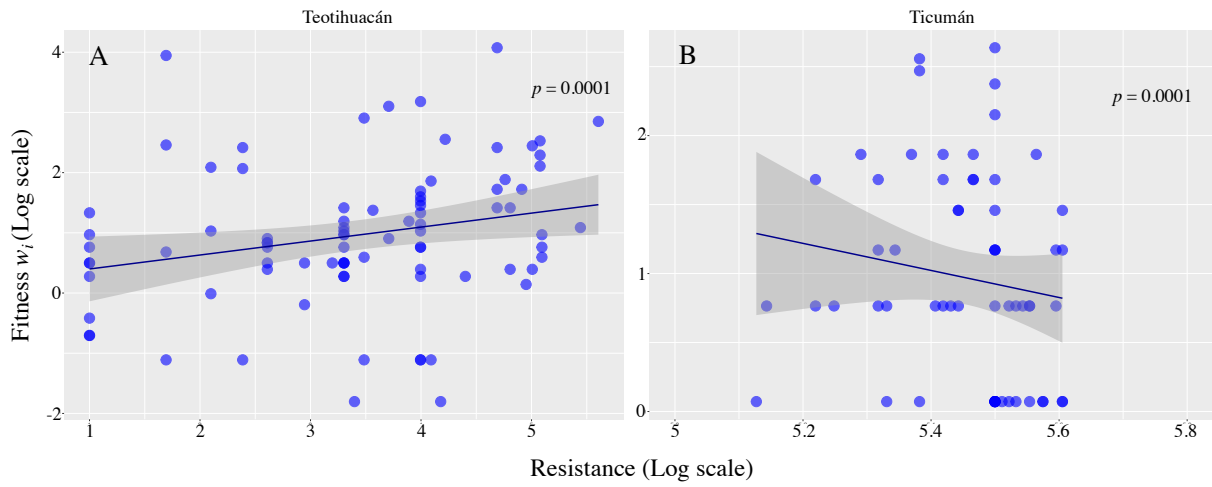
<b>Variable</b>	<b>by Variable</b>	<b>Correlation</b>	<b>Lower 95%</b>	<b>Upper 95%</b>	<b>p-value</b>
(a) Teotihuacán					
Damage	LLd	0.615779	0.517482	0.698035	<b>1.079E-20</b>
Ts	Ep	0.241138	0.100361	0.37245	<b>9.446E-04</b>
Ep	ALd	0.168386	0.024718	0.305239	<b>2.195E-02</b>
Ts	ALd	0.137232	-0.00718	0.276036	<b>6.250E-02</b>
LLd	ALd	-0.03421	-0.1776	0.110605	<b>6.439E-01</b>
Damage	Ts	-0.123	-0.26261	0.021649	<b>9.531E-02</b>
Damage	ALd	-0.13037	-0.26956	0.014169	<b>7.694E-02</b>
LLd	Ts	-0.15396	-0.29175	-0.00991	<b>3.640E-02</b>
LLd	Ep	-0.31356	-0.43802	-0.17731	<b>1.386E-05</b>
Damage	Ep	-0.32261	-0.44611	-0.18704	<b>7.516E-06</b>
(b) Ticumán					
Damage	<i>Epitrix</i> sp.	0.387549	0.187536	0.556704	<b>2.949E-04</b>



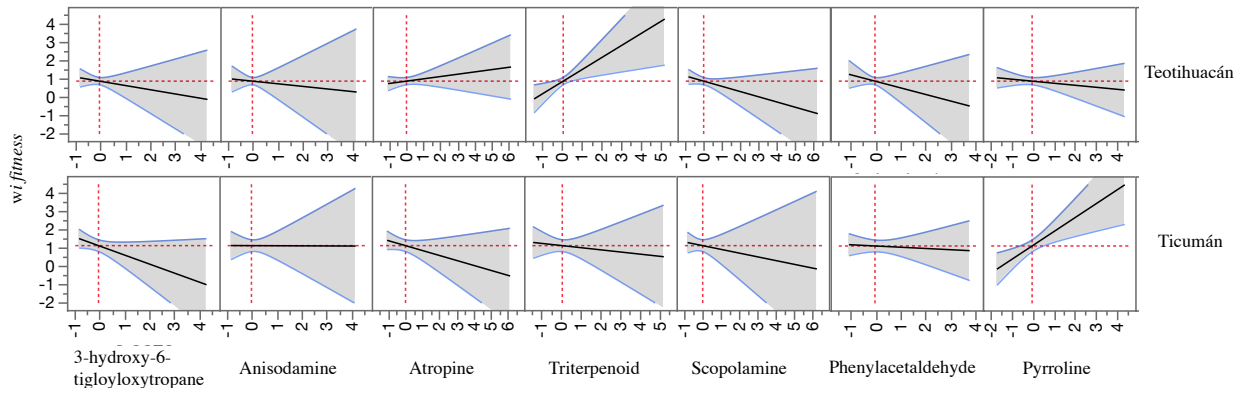
**S6.** Generalized linear models (GLMs) between herbivore infestation levels and the seven alkaloids and total alkaloid concentration: (a) infestation by adults of *E. pavula*, (b) stepwise GLM between larvae of *Lema* and alkaloids. (c) stepwise GLM between adults of *Lema* and alkaloids, (d) stepwise GLM between adults of *T. soror* and alkaloids. (e) GLM between *Epitrix* sp. and alkaloids in Ticumán. N = number of individuals, d.f. = degree of freedoms, Estimate = generalized linear coefficients, se = standard error,  $t$  =  $t$ -ratio, Significant  $p$ -values are in bold. Notice that for the total alkaloid concentration was performed a different model for each herbivore (*i. e.*, the effect of this variable was not included in the GLMs between each herbivore and the seven alkaloids (see methods).

Teotihuacán							
Response variable	Effects	N	d.f.	Estimate	se	$t$	$p$
(a) # of adult of <i>Epitrix parvula</i>	3-hydroxy-6-tigloyloxytropone	153	1	-0.29	0.17	3.09	0.0808
	Phenylacetaldehyde	153	1	<b>-0.32</b>	0.14	5.24	<b>0.0234</b>
	Pyrroline	153	1	<b>-0.26</b>	0.12	4.41	<b>0.0375</b>
	Triterpenoid	153	1	<b>0.46</b>	0.18	6.12	<b>0.0145</b>
	Scopolamine	153	1	-0.22	0.16	1.78	0.1831
	Anisodamine	153	1	0.27	0.21	1.65	0.1997
	Atropine	153	1	0.01	0.11	0.00	0.9272
	Total alkaloid concentration	161	1	<b>-0.15</b>	0.079	-1.98	<b>0.0496</b>
(b) # of larvae of <i>Lema daturaphila</i>	Phenylacetaldehyde	156	1	<b>0.31</b>	0.13	2.38	<b>0.0188</b>
	Pyrroline	156	1	0.10	0.12	0.88	0.3820
	Triterpenoid	156	1	<b>-0.56</b>	0.17	-3.24	<b>0.0015</b>
	Scopolamine	156	1	0.08	0.16	0.48	0.6326
	Anisodamine	156	1	0.06	0.12	0.55	0.5837
	Total alkaloid concentration	161	1	-0.09	0.08	-1.20	0.2308
	(c) # adult of <i>Lema daturaphila</i>	3-hydroxy-6-tigloyloxytropone	156	1	<b>-0.24</b>	0.09	-2.65
Phenylacetaldehyde		156	1	-0.08	0.09	-0.84	0.4031
Atropine		156	1	<b>0.25</b>	0.10	2.45	<b>0.0152</b>
Total alkaloid concentration		161	1	0.05	0.08	0.70	0.4832
(d) # adult of <i>Trichobaris soror</i>	Phenylacetaldehyde	158	1	-0.17	0.13	-1.29	0.1978
	Triterpenoid	158	1	<b>0.33</b>	0.16	2.05	<b>0.0419</b>
	Scopolamine	158	1	<b>-0.37</b>	0.14	-2.65	<b>0.0088</b>
	Atropine	158	1	0.13	0.11	1.16	0.1978
	Total alkaloid concentration	161	1	-0.02	0.08	-0.33	0.7387
Ticumán							
(e) # of adult of <i>Epitrix</i> sp.	3-hydroxy-6-tigloyloxytropone	48	1	<b>-0.43</b>	0.20	4.56	<b>0.0388</b>
	Phenylacetaldehyde	48	1	0.044	0.17	0.06	0.7982
	Pyrroline	48	1	<b>0.54</b>	0.18	8.52	<b>0.0057</b>
	Triterpenoid	48	1	-0.19	0.20	0.90	0.3463
	Scopolamine	48	1	-0.21	0.18	1.32	0.2566
	Anisodamine	48	1	0.14	0.24	0.34	0.5596
	Atropine	48	1	-0.00	0.16	0.00	0.9807
	Total alkaloid concentration	50	1	0.11	0.15	0.83	0.4412

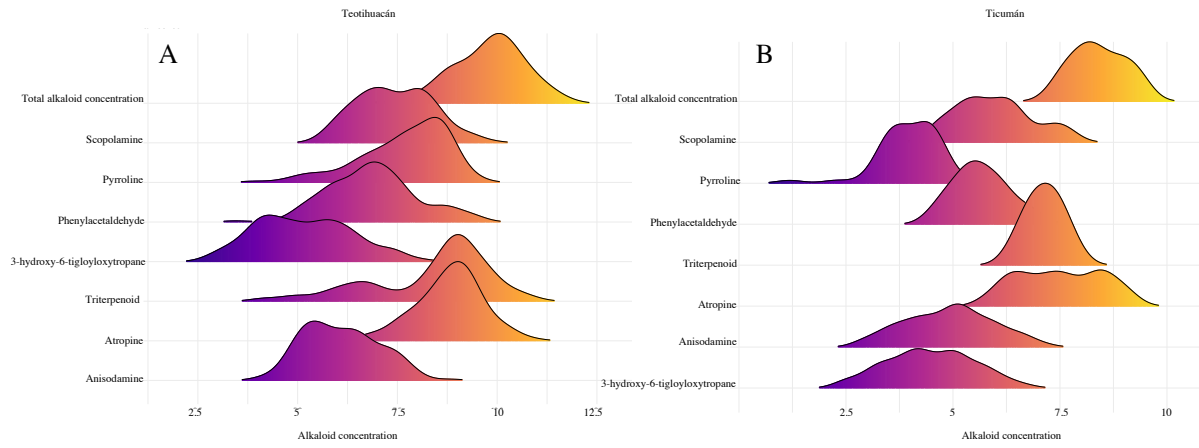
**S7.** Relationships between fitness and resistance in (A)  $F_2$  plants grown in Teotihuacán and (B)  $F_2$  plants grown in Ticumán. Positive selection to resistance was observed in Teotihuacán while a negative trend was detected in Ticumán. Each dot depicts observation for an individual.  $p$ -value of full model is shown in the plots. See Table 1.



**S8.** Prediction profilers from the GLM between fitness ( $w_i$ ) and concentrations of the seven alkaloids. Response effects are shown separately for the two experimental sites. Envelops = confidence intervals (95% CI). See table 1.



**S9.** Ridgeline plot showing the distribution of the concentration of the seven alkaloids and total alkaloid concentration in (A) F<sub>2</sub> plants grown in Teotihuacán and (B) F<sub>2</sub> plants grown in Ticumán. Data was log-transformed.



## References

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