

1 Title: Fitness costs of herbicide resistance across natural populations of the common  
2 morning glory, *Ipomoea purpurea*

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12 Running head: Fitness cost of herbicide resistance

13 Keywords: cost of herbicide resistance, fitness cost, glyphosate, *Ipomoea purpurea*,  
14 germination, early life history, seed quality, trade-offs

15 Data is archived on Dryad doi:xxx

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17

## 18 **Abstract**

19 Although fitness costs associated with plant defensive traits are widely expected, they are  
20 not universally detected, calling into question their generality. Here we examine the  
21 potential for life history trade-offs associated with herbicide resistance by examining seed  
22 germination, root growth, and above-ground growth across 43 naturally occurring  
23 populations of *Ipomoea purpurea* that vary in their resistance to RoundUp®, the most  
24 commonly used herbicide worldwide. We find evidence for life history trade-offs  
25 associated with all three traits; highly resistant populations had lower germination rates,  
26 shorter roots and smaller above-ground size. A visual exploration of the data indicated  
27 that the type of trade-off may differ among populations. Our results demonstrate that  
28 costs of adaptation may be present at stages other than simply the production of progeny  
29 in this agricultural weed. Additionally, the cumulative effect of costs at multiple life  
30 cycle stages can result in severe consequences to fitness when adapting to novel  
31 environments.

32

## 33 **Introduction**

34 Plant defense is generally hypothesized to involve a cost. This expectation stems  
35 from the surprising observation of genetic variation underlying plant defense traits in  
36 many natural systems, whether the elicitor of damage is an herbivore, a pathogen, or an  
37 herbicide (Simms and Rausher 1987, 1989; Stahl et al. 1999; Baucom and Mauricio  
38 2004; Bakker et al. 2006; Menchari et al. 2006; Délye et al. 2010; Kuester et al. 2015). If  
39 there were no costs associated with defense, traits conferring either resistance or  
40 tolerance to damage should increase to fixation rendering all individuals in the population

41 highly defended (Rausher and Simms 1989). Despite our expectations of a trade-off  
42 between fitness and defense, however, reviews of the literature consistently show that  
43 costs are not ubiquitous regardless of the elicitor of selection or the study organism at  
44 hand (Bergelson and Purrington 1996; Coustau and Chevillon 2000).

45 Three main ideas have been proposed to explain the absence of such costs. First,  
46 there are a diverse number of potential mechanisms responsible for adaptation to a  
47 damaging agent, only some of which may incur a cost (Powles and Yu 2010; Vogwill et  
48 al. 2012). A single gene nucleotide substitution that leads to herbicide resistance, for  
49 example, may not alter the efficiency of translated proteins and therefore not incur a cost  
50 (e.g. Yu et al. 2007; Yu et al. 2010). On the other hand, a mechanism that provides  
51 resistance to a range of different herbicides through changes in growth may be more  
52 likely to impose fitness costs. Second, costs may not be detected if the genetic  
53 background is not properly controlled (Bergelson and Purrington 1996; Vila-Aiub et al.  
54 2009b; Vila-Aiub et al. 2011). Control of the genetic background, either by performing  
55 crosses (Baucom and Mauricio 2004; Menchari et al. 2008; Giacomini et al. 2014) or  
56 ensuring replication across multiple genetic backgrounds (Cousens et al. 1997; Strauss et  
57 al. 2002) increases the likelihood that a cost will be detected (Bergelson and Purrington  
58 1996). Third, researchers often examine only a portion of the life cycle (i.e., seed  
59 production or fecundity) and may do so in artificial and/or non-competitive conditions  
60 (Vila-Aiub et al. 2009b; Vila-Aiub et al. 2011). Studies that examine a range of traits are  
61 more likely to identify potential growth and/or fitness differences associated with plant  
62 defense compared to those that focus solely on measures of fecundity (Vila-Aiub et al.  
63 2009b).

64           The phenomenon of herbicide resistance in plant weeds provides a particularly  
65 useful system to investigate the nature and types of costs associated with plant defense,  
66 since we know when selection by the herbicide began, the strength of selection, and often  
67 the frequency of herbicide use. However, as in other systems examining the evolution of  
68 plant defense, fitness costs of herbicide resistance are often not detected (Bergelson and  
69 Purrington 1996; Gemmill and Read 1998; Vila-Aiub et al. 2009b). Despite  
70 recommendations to control/increase the number of genetic backgrounds (Bergelson and  
71 Purrington 1996), and to examine multiple life history stages when determining if  
72 resistance incurs a cost (Primack and Kang 1989; Vila-Aiub et al. 2009b), only 25% of  
73 herbicide resistance studies control for background effects; further, only 7-10% of cost  
74 studies examine multiple stages of the life cycle (Vila-Aiub et al. 2009b). Fewer still  
75 examine the potential for fitness costs using a large number of naturally occurring  
76 populations sampled from a species' range, an approach suggested almost 20 years ago  
77 (Cousens et al. 1997; Strauss et al. 2002). Just as the mechanism of resistance can vary  
78 among species, populations of the same weed have been shown to harbor different  
79 mechanisms of resistance to the same herbicide (Christopher et al. 1991; Christopher et  
80 al. 1992; Christopher et al. 1994; Preston and Powles 1998; Yu et al. 2008; Délye et al.  
81 2010), thus increasing the likelihood that costs may likewise vary among populations. It  
82 is also possible, though rarely tested, that fitness costs have been ameliorated in some  
83 herbicide resistant populations relative to other populations due to the evolution of  
84 modifier loci (i.e. compensatory evolution, Darmency et al. 2015). The above hypotheses  
85 for the lack of costs are all interrelated: because resistance could be due to a variety of  
86 mechanisms (Délye et al. 2013a), costs may be apparent at only certain life history

87 stages, expressed in particular environments (Vila-Aiub et al. 2009b), or apparent in  
88 some populations but not others. Thus, there remain crucial gaps in our understanding of  
89 where in the life history of a plant tradeoffs between fitness-enhancing traits and  
90 resistance might be apparent, and further, how ubiquitous such trade-offs may be across a  
91 species' range (Vila-Aiub et al. 2011; Neve et al. 2014).

92         The common morning glory, *Ipomoea purpurea*, a noxious weed of US  
93 agriculture (Webster and MacDonald 2001), provides an excellent system to examine the  
94 strength and type of potential costs that may be present in natural populations. This  
95 species exhibits variability in resistance to glyphosate (Baucom and Mauricio 2008;  
96 Kuester et al. 2015), which is the main ingredient in the herbicide RoundUp®.  
97 RoundUp® is currently the most widely used herbicide in agriculture (Fernandez-  
98 Cornejo et al. 2014), and of the approximately 30 resistant weeds that have been  
99 examined (Heap 2015), only a third are reported to express fitness costs (Ismail et al.  
100 2002; Pedersen et al. 2007; Brabham et al. 2011; Giacomini et al. 2014; Shrestha et al.  
101 2014; Vila-Aiub et al. 2014; Glettner and Stoltenberg 2015; Goh et al. 2015). *I. purpurea*  
102 has long been considered to exhibit low-level resistance to glyphosate (Culpepper  
103 (2006)), and previously we have shown that this low-level resistance (estimated as  
104 proportion leaf damage) has an additive genetic basis and is under positive selection in  
105 the presence of the herbicide (Baucom and Mauricio 2008). Further, a recent replicated  
106 dose-response experiment of 43 populations sampled from the southeastern and Midwest  
107 US showed that some populations of *I. purpurea* exhibit ~100% survival after application  
108 of the field dose of RoundUp® (*i.e.*, resistance), whereas other populations exhibit high  
109 susceptibility (Kuester et al. 2015). Although we find variability in resistance across

110 natural populations, it is unclear if this defense trait involves a cost. We investigated this  
111 question within one population using artificial selection for increased/decreased  
112 resistance and discovered that the seed production of individuals from the increased  
113 resistance lines was not significantly lower than that of susceptible lines in the absence of  
114 the herbicide, suggesting that there may not be a fecundity cost associated with resistance  
115 in this species. However, there was some indication that progeny quality may be lower in  
116 resistant individuals – resistant lines exhibited a trend for reduced seed viability  
117 compared to susceptible lines (Debban et al. 2015). This finding suggests that trade-offs  
118 between fitness enhancing traits (e.g., germination and resistance) may be present within  
119 this species, which could manifest as a cost by reducing the overall fitness of resistant  
120 compared to susceptible lineages in the absence of herbicide.

121 Here we determine if there are trade-offs associated with resistance by examining  
122 germination, early root growth and above-ground growth across 43 populations of *I.*  
123 *purpurea*. We specifically ask the following: (1) are there potential trade-offs associated  
124 with resistance across this species' range in the US, manifest in the form of (i) lower  
125 germination and/or (ii) smaller size at early life history stages (i.e., early germinant,  
126 young plant)?, and (2) do resistant populations exhibit the same type of potential trade-  
127 off, which may indicate the nature and expression of fitness costs may vary across  
128 populations?

129

130

## 131 **Materials and Methods**

132 *Seed collection and control of maternal/environmental effects*

133 Multiple fruits were collected from up to 79 individuals separated by at least 2 m from 43  
134 populations located across the Midwest and Southeastern US (Table S1; Fig S1). These  
135 seeds (hereafter field-collected seeds) were used in several experiments to determine  
136 resistance, germination and early growth characteristics. To homogenize the effects of  
137 maternal environment on seed quality, we chose a subset of the populations (N=18), grew  
138 them in a common greenhouse for one generation and collected the autonomously self-  
139 pollinated seeds from a similar growing and mating system environment (hereafter once-  
140 selfed seeds).

141

#### 142 *Estimate of herbicide resistance*

143 To determine glyphosate resistance across populations, a dose-response experiment was  
144 conducted by planting a single field-collected seed from 10 randomly chosen maternal  
145 lines from each population in six glyphosate treatments (including a non-herbicide  
146 control treatment) in each of two greenhouse rooms. Full details of the dose-response  
147 experiment are presented in Kuester *et al.* (2015) - for simplicity, we present resistance as  
148 the percent survival per population at 1.70 kg a.i./ha of glyphosate, a rate which is  
149 slightly higher than the suggested field rate of 1.54 kg a.i./ha. Individual seeds were  
150 scarified, planted, allowed to grow for three weeks, and then treated with the herbicide  
151 (PowerMax Roundup; Monsanto, St. Louis, Missouri) using a hand-held CO<sub>2</sub> pressurized  
152 sprayer (Spraying Systems Co., Wheaton, IL). Survival was scored three weeks after  
153 treatment application, and the population estimate of resistance was determined as the  
154 proportion of individuals that survived glyphosate.

155

156 *Germination*

157 We performed three germination experiments to determine if resistance influenced seed  
158 traits. First, we examined germination using field-collected seeds in a petri-dish assay in  
159 the laboratory; second, we examined germination of the field-collected seeds in the soil in  
160 the greenhouse; and third we performed a petri-dish assay in the lab using seeds  
161 generated via selfing in the greenhouse (once-selfed seeds) to examine the potential for  
162 maternal field environmental effects. For the first experiment using field-collected seeds,  
163 we measured seed weight and germination characteristics using field-collected seeds  
164 from each population (N=43). Up to five (ave 4.6) seeds from 8-79 maternal lines per  
165 population (ave 38, total 1621, see Table S1 for exact sample sizes per population) were  
166 randomly chosen for the germination test. From this pool of seeds we randomly chose a  
167 subset of families per population (8-49 maternal lines per population; Table S1) for  
168 which the selected seeds were weighed (as a group) to determine the average seed  
169 weight. All of the selected seeds were placed in a small petri dish (one dish per family),  
170 submerged in filtered water and allowed to germinate in the lab under ambient light and  
171 temperature. Water was added as necessary every three days to prevent drying out. Petri  
172 dishes were completely randomized across lab benches. Germination was scored  
173 periodically until no further germination was recorded. Final pre-scarification  
174 germination was scored after 16 days, with successful germination considered the  
175 emergence of a normal radicle. At this time, seeds that had not imbibed water (by visual  
176 determination) were scarified and germination was again scored after 1 week. We  
177 recorded the final number of seeds exhibiting normal germination, the number of seeds  
178 needing scarification, the number of scarified seeds that germinated, and the number that



179 had abnormal germination. For the second germination assay, we examined germination  
180 data from one replicate (housed in a single greenhouse room) of the dose-response  
181 experiment mentioned earlier in which seeds were scarified and planted in containers  
182 with 1 seed per pot (10 maternal families per population). Germination was scored after  
183 three weeks and used to calculate the percentage of seeds that germinated.

184 For the third and final germination assay, we used seeds from maternal lines that  
185 were selfed once in the greenhouse. Two sets of five seeds for up to 8 maternal lines  
186 (randomly selected) for each of 18 populations were placed in petri dishes with water.  
187 Germination was scored after 11 days. If seeds had not imbibed water they were scarified  
188 and scored again in one week. We calculated the percentage of seeds with normal  
189 germination prior to scarification, the percentage of seeds that germinated after  
190 scarification, and the percentage that had abnormal germination.

191

### 192 *Early root and above-ground growth*

193 To examine early root growth, we again used the once-selfed seeds and measured root  
194 length four days after the germination assay began. We chose to first scarify the seeds in  
195 this assay to standardize water absorption among individuals. Two sets of five seeds for  
196 up to 8 maternal lines (randomly selected) for each of 18 populations were scarified and  
197 placed in petri dishes with water. Germination was scored after 1, 4 and 7 days. On day 4  
198 petri dishes were scanned and the root length was measured using Image J (Abramoff et  
199 al. 2004) for each germinated seed.

200 We next examined early growth traits of greenhouse-grown individuals to  
201 determine if there was a relationship between resistance and plant size (i.e., are plants

202 from resistant populations smaller?). To do so we used measurements from plants from  
203 the dose-response experiment prior to herbicide application. Three weeks after planting  
204 (and prior to spraying) we measured the height of the stem, the number of leaves and  
205 length of the largest leaf on each individual planted per treatment per population (total  
206 N=2908, Table S1 for exact sample sizes per population).

207

### 208 *Statistical analysis*

209 *Field-collected seeds*—We assessed the relationship between resistance and progeny  
210 quality using mixed model analyses of variance. We used a generalized linear mixed-  
211 effect model to examine final germination, germination before scarification, abnormal  
212 germination, seeds needing scarification, and germination after scarification with  
213 resistance and population (random) as predictors using the glmer function in the R  
214 package lme4 with a binomial distribution. All of the binary measures were coded as 1 or  
215 0. Seed weight (g) was modeled using a mixed model with resistance and population  
216 (random) as predictors using the lmer function in the R package lme4. Additionally,  
217 previous studies have indicated a geographic pattern of resistance in this species (Kuester  
218 et al. 2015). To ensure that the above results were not an artifact of geography, we added  
219 latitude and longitude (scaled) of the population in the above models. For the experiment  
220 examining germination in soil, we modeled germination with resistance level and  
221 population (random) as predictors using a binomial distribution.

222 *Once-selfed seeds*—Similar to the field-collected seeds, we used mixed-model binomial  
223 regressions to assess the effect of resistance on germination characteristics of the once-  
224 selfed, greenhouse generated seeds. We modeled germination before scarification and

225 germination after scarification with resistance and population (random) as predictors  
226 using a binomial model. To determine if the maternal environment in which the seeds  
227 developed influenced germination, in a separate model we compared germination  
228 between maternal environments (i.e., field collected seeds versus seeds propagated in the  
229 greenhouse) by including maternal environment as a treatment effect in the model. To do  
230 so, we modeled final germination using treatment, resistance, population (random) and  
231 treatment\*resistance as predictors using a binomial distribution. An interaction between  
232 treatment and resistance would indicate that the maternal environment differently  
233 influenced germination.

234 *Early growth and size*—We next used mixed model analyses of variance to determine if  
235 more resistant populations exhibited early growth life-history trade-offs. We separately  
236 considered root length of the early germinant and plant size. We examined root length  
237 using the once-selfed seeds in two different models. The first and more basic model  
238 examined the influence of resistance and population (random) on log-transformed root  
239 length (cm) 4 days post germination. A difference in root length, however, could be due  
240 to differences in either growth rate of the radicle or differences due to the timing of  
241 germination, *i.e.*, when growth began following germination. To distinguish between  
242 these two potential explanations, we calculated an estimate of germination speed, the  
243 time to 50% germination – a shorter time would suggest that seeds began growing sooner  
244 after water was added. We used the germination data from days 1, 4, and 7 to obtain a  
245 population level estimate of the time to 50% germination using a germination Hill  
246 function (El-Kassaby et al. 2008). This function decomposes germination into 4  
247 parameters: a, the germination capacity; b, the steepness of the curve; c, the time to 50%

248 germination; and  $y_0$ , the lag time before germination. We used the nonlinear least squares  
249 (nls) function in R to estimate the b and c parameters. We chose to pool the data on a  
250 population level to increase the accuracy of the estimation. The time to 50% germination  
251 (c) was then used as a covariate in the more complex model of root length that included  
252 resistance, population (random) and time to 50% germination.

253 We next examined height (cm), leaf number and leaf size (cm) of plants grown  
254 from field-collected seeds (~3 weeks growth in greenhouse) to determine if resistance  
255 incurs early growth life-history trade-offs. We used each trait in separate mixed models  
256 with replicate, rack within replicate (random), resistance and population (random) as  
257 predictors. Residuals of leaf size were not normal so a box-cox transformation ( $\lambda = 2.0$ )  
258 was used to achieve better fit.

259 Finally, we performed a Principle Components Analysis (PCA) using the  
260 population averages of several traits from the field-collected seeds, which included seed  
261 weight, germination percentage, percentage of abnormally germinating seeds, percentage  
262 of successfully germinating scarified seeds, early plant height, leaf number and leaf size  
263 to visually examine the data and determine how populations differed along the two axes  
264 retained. This analysis was performed using PROC FACTOR in SAS with a varimax  
265 rotation to obtain more easily interpretable axes. Loadings and the proportion variance  
266 explained for each factor with an eigenvalue  $>1$  can be found in Table S3.

267

## 268 **Results**

### 269 *Germination*

270 We found a strong and significant negative relationship between resistance and  
271 the percentage of field-collected seeds that germinated (Fig. 1a). This is true both of  
272 seeds that germinated before scarification ( $\beta = -4.93$ ,  $\chi^2_1 = 24.66$ ,  $P < 0.0001$ ) and the total  
273 number that germinated (including those that germinated after being scarified;  $\beta = -5.20$ ,  
274  $\chi^2_1 = 24.80$ ,  $P < 0.0001$ ; Fig. 1a). In addition to a decline in germination, several other  
275 measures of seed quality also declined with increasing resistance. We found a higher  
276 percentage of abnormally germinating seeds ( $\beta = 4.24$ ,  $\chi^2_1 = 33.20$ ,  $P < 0.0001$ ) in that,  
277 instead of exhibiting normal germination, a non-viable embryo would be ejected from the  
278 seed coat with no further growth. Furthermore, some seeds simply did not imbibe water;  
279 we scarified these seeds to determine if they were viable but potentially dormant.  
280 Populations with greater resistance had more seeds that needed scarification ( $\beta = 1.52$ ,  $\chi^2_1$   
281  $= 5.00$ ,  $P = 0.03$ ), of which fewer seeds that subsequently germinated ( $\beta = -5.50$ ,  $\chi^2_1 =$   
282  $8.81$ ,  $P = 0.003$ ). We also found that populations with higher resistance produced lighter  
283 seeds ( $\beta = -0.005$ ,  $\chi^2_1 = 4.69$ ,  $P = 0.03$ ), indicating that resistance influenced multiple  
284 measures of seed quality for seeds collected from the field. All of these relationships  
285 remain significant after accounting for longitude and latitude of the populations except  
286 for the percentage needing scarification (Table S2), suggesting that the patterns we find  
287 are not due to a simple geographic pattern. We similarly uncovered a negative  
288 relationship between germination and resistance when seeds from these populations were  
289 planted in soil in the greenhouse ( $\beta = -0.79$ ,  $\chi^2_1 = 16.09$ ,  $P < 0.0001$ ; Fig. 1b).

290 The negative relationship between resistance and germination was supported by  
291 the results from the once-selfed seeds grown in a common environment for a generation  
292 (Fig. 1c). Prior to scarification, very few of the greenhouse-grown seeds imbibed water

293 and germinated (2.0%) and there was no effect of resistance ( $\beta = 184.2$ ,  $\chi^2_1 = 0.81$ ,  $P =$   
294 0.37). After scarification, however, there was a significant negative relationship between  
295 germination and resistance ( $\beta = -1.18$ ,  $\chi^2_1 = 6.42$ ,  $P = 0.01$ , Fig. 1c). This effect remained  
296 significant after accounting for latitude and longitude ( $\beta = -1.13$ ,  $\chi^2_1 = 5.49$ ,  $P = 0.02$ ).  
297 Interestingly, the decrease in germination of once-selfed, greenhouse-generated seeds  
298 was significantly less than the field collected seeds (treatment \* resistance:  $\beta = 2.14$ ,  $\chi^2_1 =$   
299 27.8,  $P < 0.0001$ ) suggesting that maternal environmental conditions influence the quality  
300 of seeds produced. In addition, we found a much lower rate of abnormal germination in  
301 the once-selfed seeds (~10%) compared to the field collected seeds, and the level of  
302 abnormal germination showed no relationship with resistance ( $\beta = 0.31$ ,  $\chi^2_1 = 0.23$ ,  $P =$   
303 0.63). These results suggest that, while germination costs are consistently detected  
304 between experiments in which the maternal environment differed, field environmental  
305 conditions exacerbate the strength of the germination cost.

306

### 307 *Early root and aboveground growth*

308 To test whether growth differed according to resistance status, we scarified and  
309 germinated the once-selfed seeds then measured root growth after 4 days. There was a  
310 much higher germination rate of these seeds (86%) compared to the previous experiment  
311 (2% pre-scarification) and the majority occurred before day 4. While there was a nearly  
312 significant negative relationship between root length and resistance ( $\beta = -0.38$ ,  $\chi^2_1 = 3.19$ ,  
313  $P = 0.07$ ; Fig. 2a), including the time to 50% germination in the model removed this  
314 effect (resistance:  $\beta = -0.07$ ,  $\chi^2_1 = 0.09$ ,  $P = 0.76$ ; time to 50% germination:  $\beta = -0.37$ ,  $\chi^2_1$   
315  $= 4.85$ ,  $P = 0.03$ ), suggesting that the difference in root length was due to the timing of

316 germination rather than a difference in growth rate. A difference in plant size was also  
317 found in the 3-4 week old plants grown in soil from the field-collected seeds - plants from  
318 more resistant populations had smaller above-ground structures on average than plants  
319 from less resistant populations (height:  $\beta = -7.42$ ,  $\chi^2_1 = 7.20$ ,  $P = 0.007$ ; leaf number:  $\beta =$   
320  $-1.11$ ,  $\chi^2_1 = 15.86$ ,  $P < 0.0001$ ; largest leaf width:  $\beta = -4.97$ ,  $\chi^2_1 = 17.32$ ,  $P < 0.0001$ ; Fig.  
321 2b-d).

322

### 323 *Visualization of cost-related traits*

324 We next examined germination and early growth traits from the original field-collected  
325 seeds using a principle components analysis (PCA) to determine if there was variation  
326 among populations in the expression of cost-related traits (full results Table S3). The first  
327 3 principle components of the PCA explained 77% of the variance, with the first principle  
328 component (PC) loading with the early growth traits while the second loaded with seed  
329 traits and the third with the proportion of seeds that required scarification to successfully  
330 germinate. Populations with higher resistance scored lower on PC1 ( $b = -2.22$ ,  $r^2 = 0.28$ ,  
331  $t_{41} = -4.03$ ,  $P = 0.0002$ ) and PC2 ( $b = -2.12$ ,  $r^2 = 0.26$ ,  $t_{41} = -3.79$ ,  $P = 0.0005$ ), but not on  
332 PC3 ( $b = -1.02$ ,  $r^2 = 0.06$ ,  $t_{41} = -1.61$ ,  $P = 0.12$ ). Using the first two PCs to plot the results,  
333 resistant populations occur mostly in the lower left quadrant (smaller plants, lighter seeds,  
334 lower germination and more abnormally germinating seeds) and have a wider spread than  
335 less resistant populations (Fig. 3). Furthermore, while resistant populations scored lower  
336 on PC1 and PC2 in comparison to susceptible populations, some resistant populations  
337 exhibited early growth traits that were similar to susceptible populations and yet scored  
338 very low on germination traits (e.g., pop num 5) whereas other resistant populations

339 exhibited similar germination traits compared to the susceptible populations, but were  
340 smaller in stature than susceptible populations (e.g., pop num 51). Thus, it appears that  
341 the type of cost may vary among populations sampled from North America.

342

### 343 **Discussion**

344 Here we show that glyphosate resistant populations of the common morning glory  
345 exhibit life-history trade-offs associated with resistance, and, that these trade-offs may  
346 vary among populations. Our series of experiments uncovered three notable findings:  
347 First, we found a negative linear relationship between germination and resistance  
348 indicating that resistant populations have a lower germination rate than susceptible  
349 populations. This negative relationship persisted when using seeds generated from a  
350 common greenhouse environment showing that this result is not due solely to field  
351 environmental and/or maternal effects. Second, we found that individuals from resistant  
352 populations were smaller than individuals from susceptible populations, indicating that  
353 resistance influences early plant growth. Interestingly, we also found evidence that the  
354 two types of trade-off may differ among populations—using PCA, we show that some  
355 resistant populations produce normally sized plants, but score low on germination traits,  
356 and *vice versa*. Below, we detail how these results add further strength to the suggestion  
357 that a variety of life stages and populations sampled across the species' range should be  
358 assessed when testing the hypothesis that resistance incurs a fitness cost (Délye et al.  
359 2013a).

360

361 *Fitness costs: seed germination and early plant size*



362           It is difficult to determine how common germination differences associated with  
363 resistance may be among weeds since many studies focus on seed quantity rather than  
364 seed quality. There is some indication that germination may be affected in other  
365 glyphosate resistant species. Dinelli et al. (2013) found reduced germination of  
366 glyphosate resistant *Ambrosia trifida* populations, while Ismail et al. (2002) found greater  
367 germination of resistant biotypes of goosegrass (*Eleusine indica*). More broadly, life  
368 history trade-offs may be specific to the herbicide and/or species in question or the type  
369 of mutation conferring resistance (O'Donovan et al. 1999; Vila-Aiub et al. 2005; Délye et  
370 al. 2013b). For example, only one of two different resistance mutations in ACCase  
371 resistant *Lolium rigidum* had more stringent germination requirements (seeds germinated  
372 poorly in the dark and required fluctuating temperatures to break dormancy) than the  
373 susceptible genotype (Vila-Aiub et al. 2005). Similarly, Délye *et al.* (2013b) found  
374 differential effects on germination among resistance mutations to ACCase in *Alopecurus*  
375 *myosuroides*. Both of these studies report that the resistance mutation led to delayed  
376 germination. Such a delay in germination may affect fitness, especially in agricultural  
377 settings where germinating too early can lead to removal by pre-sowing practices and  
378 germinating too late can lead to intensified competition with already established plants  
379 (Weaver and Cavers 1979; Barrett 1983; Mortimer 1997; Forcella et al. 2000; Owen et al.  
380 2014). Our analysis of root growth suggests that differences in plant size in *I. purpurea*  
381 may be due to a similar delay in germination in resistant populations.

382           The reduced growth of resistant compared to susceptible populations that we  
383 uncovered could lead to decreased competitive ability and subsequent lower fitness in the  
384 presence of competition if, as has been found in other herbicide resistant weeds, the

385 difference in growth persists to adult plants (Weaver and Warwick 1982; Ahrens and  
386 Stoller 1983; Holt 1988; Alcocer-Ruthling et al. 1992; Williams et al. 1995; Vila-Aiub et  
387 al. 2005; Tardif et al. 2006; Vila-Aiub et al. 2009a). This type of life-history trade-off,  
388 which ultimately may manifest as a fitness cost, is also likely to be species, mutation and  
389 environment specific. For example, *Lolium rigidum* has evolved herbicide resistance via  
390 a variety of mutations ranging from target site (Christopher et al. 1992; Yu et al. 2008) to  
391 non-target site (Christopher et al. 1991; Christopher et al. 1994; Preston and Powles  
392 1998). Target site mutations in the acetohydroxyacid synthase gene result in little cost in  
393 growth (Yu et al. 2010). On the other hand, herbicide resistance mediated by the  
394 cytochrome P450 complex resulted in reduced biomass and decreased competitive ability  
395 (Vila-Aiub et al. 2009a).

396         An alternative explanation for the decline in germination and growth we identify  
397 using the field-collected seeds is that some other co-varying population characteristic  
398 such as soil fertility, spraying regime, herbivore levels, or the many other biotic and  
399 abiotic factors that can influence seed development differed among resistant and  
400 susceptible populations (Roach and Wulff 1987; Fenner 1991; Schmitt et al. 1992;  
401 Platenkamp and Shaw 1993; Galloway 2001). These differences may explain the stronger  
402 decline in germination in the field-collected seeds compared to the once-selfed seeds.  
403 Several lines of evidence, however, suggest the relationship between resistance and  
404 germination that we uncovered represents a true fitness cost rather than simply an effect  
405 of the environment (e.g., driven by maternal effects, latitude, spray environment). First,  
406 the relationship between resistance and germination appears approximately linear, which  
407 is expected as the frequency of resistant individuals increases. If the trade-offs identified

408 herein were due simply to glyphosate exposure, with resistant populations exhibiting  
409 abnormal seed development after surviving glyphosate application, we would expect to  
410 see a binary distribution of seed quality of populations that had been sprayed and those  
411 that had not, rather than a linear trend with resistance. Second, the negative relationship  
412 between resistance and germination is maintained after a generation in a common  
413 greenhouse environment—an effect should disappear if the decrease in fitness was due to  
414 glyphosate exposure in the field. Finally, our results parallel those from a recent  
415 experiment that specifically controlled for genetic background and environmental effects  
416 using *I. purpurea* plants from a single population (Debban et al. 2015). Individuals from  
417 this population were artificially selected for increased or decreased resistance for three  
418 generations under controlled greenhouse conditions, and, similar to results presented  
419 here, the increased resistance lines had a larger percentage of “bad” seeds that ejected the  
420 embryo. That the results from one population utilizing a controlled genetic background  
421 are mirrored across many populations collected from the landscape provides strong  
422 evidence that lower germination represents a fitness cost of glyphosate resistance in this  
423 weed species.

424         While we detected lower germination in herbicide resistant populations across  
425 multiple experiments suggesting a true trade-off, we also found differences between  
426 experiments in the strength of the relationship. This suggests both an underlying genetic  
427 basis as well as an environmental component influence the expression of the trade-off.  
428 Compared to the field-collected, unscarified seeds, the once-selfed, unscarified seeds  
429 (Fig. 1) had a low germination rate suggesting that the seed coat was perhaps more  
430 pristine in seeds generated in the greenhouse. However, if scarified, we found that the

431 once-selfed seeds exhibited high germination and the relationship between resistance and  
432 germination remained negative indicating a fitness decline associated with resistance.  
433 The physical seed coat is the primary mechanism of dormancy in this species (Brechu-  
434 Franco et al. 2000); thus, environmentally-induced physical differences in the seed coat  
435 (e.g. thickness or waxiness) or its degree of degradation (e.g. mechanical disruption or  
436 seed storage differences) likely influences germination timing. Although it is clear that  
437 the environment influences seed germination in this species, that we consistently  
438 observed a decline in germination with resistance across multiple experiments suggests  
439 that there is an underlying genetic basis to the cost of resistance.

440         Another striking difference between experiments was in the frequency of  
441 abnormal seeds produced. The once-selfed seeds had almost no abnormal germination  
442 (i.e., no dead embryos that were ejected from the seed coat) while some of the field-  
443 collected populations had a high level of abnormal germination. In fact, the strong  
444 decline in germination for field-collected seeds was due primarily to this abnormal  
445 germination. Abnormal germination could be due to a variety of environmental causes  
446 (e.g. herbicide application, nutrient availability, competition, etc) or be a cost of  
447 resistance that is only induced under field conditions. Our results suggest that in a benign  
448 environment, such as the greenhouse, the seeds in general are of high quality (high  
449 germination, fewer abnormal germinants) but there is a cost of resistance that increases  
450 the time it takes to germinate (based on root growth experiment), possibly leading to  
451 smaller plants at any given point. On the other hand, under field conditions, populations  
452 with higher resistance produce more abnormal seeds (due to either environmental  
453 differences or an environmentally induced cost of resistance) and the normal seeds may

454 still have an increase in the time it takes to germinate leading to smaller plants at any  
455 given point.

456           Interestingly, by visually examining the germination and growth traits in a PCA,  
457 we find variation in the type of potential cost among resistant populations. While some  
458 resistant populations fell into the “poor germination” axis, other resistant populations fell  
459 into the “poor growth” axis compared to susceptible populations. There are at least three  
460 possible reasons for this difference: different resistance genes, different compensatory  
461 mutations or different genetic backgrounds. First, the gene(s) involved in resistance may  
462 vary among populations leading to different costs. Independent origins of resistance to  
463 herbicide have been found in other species (Délye et al. 2010) and these different  
464 mutations often incur different fitness costs (Vila-Aiub et al. 2005; Délye et al. 2013b).  
465 Second, the resistance gene(s) may be the same amongst populations but each population  
466 may have different compensatory mutations that lead to different costs (Darmency et al.  
467 2015). Third, the resistance gene(s) may behave differently in different genetic  
468 backgrounds (Paris et al. 2008). These distinctions are important because they would  
469 differentially affect the evolutionary trajectory of herbicide resistance. For example, if  
470 populations differ in the gene(s) involved, each population may have a very different set  
471 of costs, benefits and evolutionary trajectories, which would need to be incorporated in  
472 models.

473           It is currently unknown if the trait trade-offs identified here are pleiotropic or due  
474 to linkage to the resistance gene. The most restrictive definition of a cost requires that the  
475 decrease in fitness is due to the resistance allele itself - either the actual allele or through  
476 it acting pleiotropically (Bergelson and Purrington 1996). Given that we do not know the

477 identity of the loci involved in either resistance or the abnormal germination and reduced  
478 growth, we cannot entirely rule out physical linkage between resistance genes and cost  
479 genes, in which case the “cost” could quickly become unlinked over generations  
480 (Lewontin 1974; Hartl and Clark 1989). For some species, easily identifiable mutations in  
481 the enzyme targeted by the herbicide can be linked to resistance, i.e. target site resistance  
482 (TSR). However, preliminary work suggests that glyphosate resistance in *I. purpurea* is  
483 due to non-target site mechanism (NTSR; Leslie and Baucom, *unpublished data*), and as  
484 such elucidating the genetic basis of both resistance and the cost will be a non-trivial  
485 endeavor. Furthermore, it is rare that genes underlying costs are identified; most  
486 documented cases of the genes involved in the cost of resistance is when TSR mutations  
487 lead to poor performance of the enzyme on its natural substrate (Vila-Aiub et al. 2009b).  
488 As far as we are aware, no study has identified the genes involved in the cost of  
489 resistance when the mechanism of resistance is NTSR. One intriguing possibility for this  
490 species stems from a previous study that compared transcript expression levels of  
491 artificially selected lines of resistant and susceptible *I. purpurea* plants following  
492 herbicide application (Leslie and Baucom 2014). One of the differences between the  
493 replicated resistant and susceptible lines was a lower expression of pectin methylesterase  
494 (PME) in the resistant plants. This enzyme has been shown to play a role in breaking seed  
495 dormancy (Ren and Kermode 2000) and stem elongation (Pilling et al. 2000). Thus, the  
496 decreased expression of PME in resistant plants may explain both the reduced  
497 germination and growth in populations with higher resistance.  
498

499 *How might life history trade-offs influence the evolutionary trajectory of resistance in*  
500 *this species?*

501       Recent work in this system has shown that populations of the common morning  
502 glory sampled from 2012 exhibit higher levels of resistance compared to the same  
503 populations sampled in 2003 (Kuester et al, *In Review*). Interestingly, however, the  
504 difference in resistance between sampling years was only slight, i.e., 62% survival at 1.7  
505 kg ai/ha in 2012 vs 57% survival in 2003 samples. It is possible that the life-history  
506 differences that we identified here are responsible, at least in part, for maintaining  
507 resistance between sampling years. For example, the lower germination of resistant types  
508 would manifest as a fitness cost if resistant and susceptible types produce approximately  
509 the same number of total seeds (or if  $R < S$ ); if, however, resistant types produce enough  
510 viable seed to offset the lowered germination, then overall fitness would not be impacted  
511 and resistant types would not be at a relative disadvantage. While we have not examined  
512 seed production across all 43 populations examined herein, a common garden study of  
513 glyphosate susceptible and resistant families of this species found there was no difference  
514 in total seed production of resistant compared to susceptible lines (Debban et al 2015),  
515 indicating there is no cost of resistance in terms of seed quantity. That we similarly find  
516 poor germination between these experiments and those using genetic lines developed  
517 from a single population strongly supports the finding that germination quality is a true  
518 fitness cost of glyphosate resistance in this species. Further, the differences in growth that  
519 we have detected between resistant and susceptible populations could potentially  
520 manifest as a fitness cost if in competition, an effect which remains to be tested in this  
521 system.

522 In summary, we found reductions in seed quality across replicated herbicide  
523 resistant populations of the common morning glory. Although most studies use seed  
524 quantity as a proxy for fitness, our results highlight that reductions in progeny quality are  
525 an equally, if not more, important cost of adaptation in *I. purpurea*. Given that fitness  
526 costs are thought to arise from a variety of mechanisms (allocation of resources,  
527 ecological costs, etc.), our results suggest that a high priority should be placed on the  
528 examination of multiple stages of the life cycle when assessing potential costs and not  
529 just seed quantity. Furthermore, because the strength of this cost could maintain the  
530 efficacy of a globally important herbicide, this work illustrates the utility and importance  
531 of integrating evolutionary principles into management scenarios (Gould 1995).

532

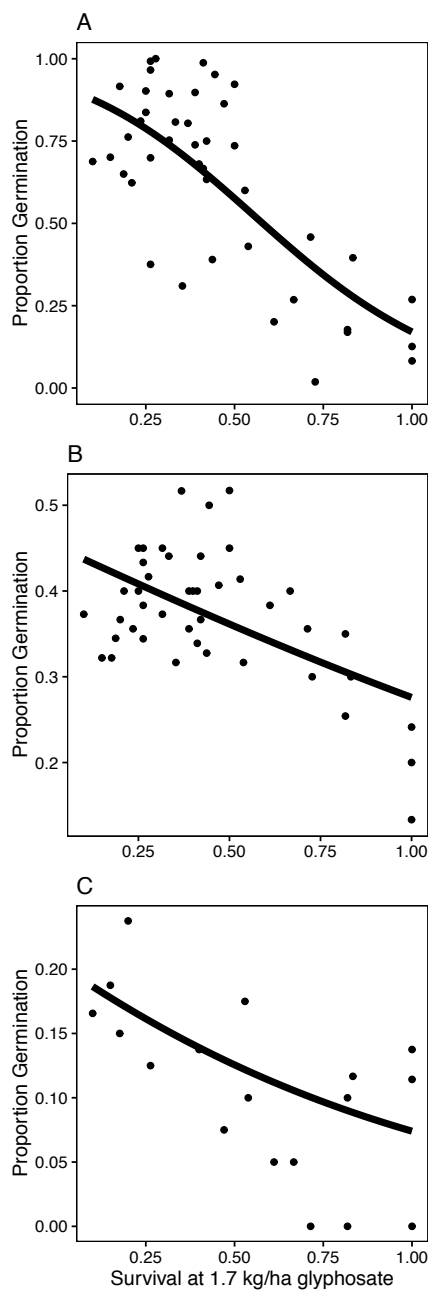
533 **Acknowledgements:** We thank E. Fall, A Wilson, A Jankowiak, D York, N Gabry, S  
534 Sanchez for assistance and J Vandermeer, MA Duffy, and PJ Tranel for comments on  
535 earlier drafts of the manuscript. This work was funded by USDA NIFA grants 04180 and  
536 07191 to RSB and SMC.

537



538 **Figure Legends**

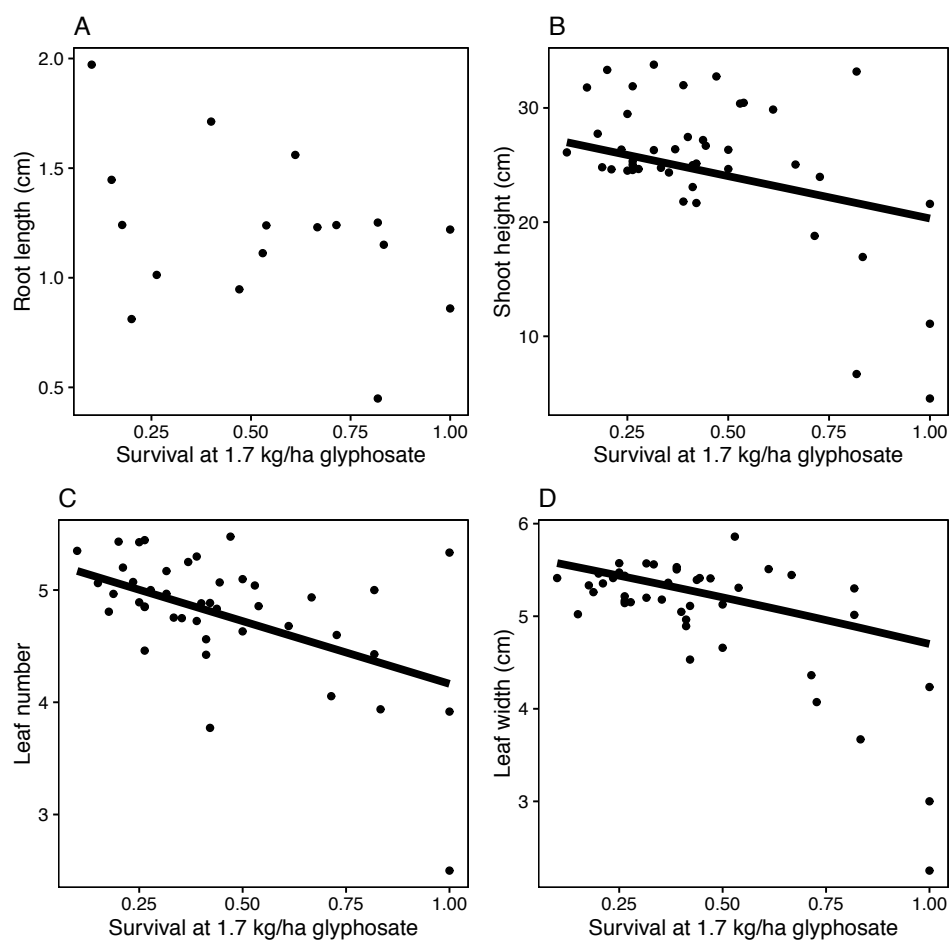
539 Fig 1. Relationship between herbicide resistance and proportion germination for (a) field-  
540 collected seeds in petri dishes, (b) field-collected seeds in soil, and (c) once-selfed seeds  
541 (note the differences in the y-axis scale). Points are the mean per population, lines are the  
542 average marginal predicted probabilities from the appropriate model.



543

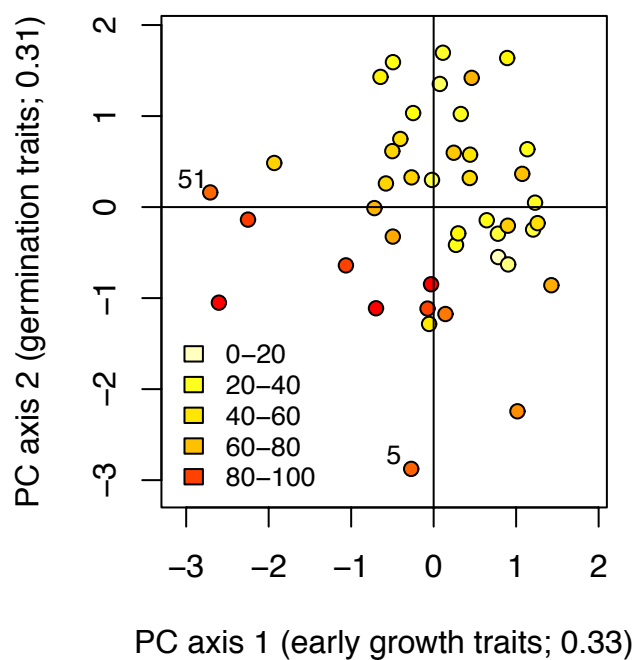
544

545 Fig 2. Relationship between herbicide resistance and (a) root growth after 4 days, (b)  
546 shoot height (c) leaf number and (d) width of the largest leaf after 3 weeks. Points are the  
547 mean per population and lines are the average marginal predicted probabilities from the  
548 appropriate model.



549

550 Fig 3. Scatter plot of PCA results showing average principle components axis 1 (higher  
551 values indicate larger plants) and principle components axis 2 (higher values indicate  
552 heavier seeds, higher germination, and fewer abnormally germinating seeds) values for  
553 field-collected seeds with circle color indicating survival at 1.7 kg/ha glyphosate. The  
554 proportion variation explained by each axis is noted in axis labels.



555

556 **Supporting information**

557 Table S1. Population characteristics including population number, state, crop type,  
 558 latitude and longitude and sample sizes for experiments with field-collected seeds  
 559 ( $N_{fam\ germ}$  = number of families in the germination assay for field collected seeds;  
 560  $N_{seeds\ germ}$  = number of seeds in the germination assay for field collected seeds;  $N$   
 561 early growth = number of seeds in the early growth experiment,  $N_{fam\ once\ selfed}$  =  
 562 number of families in the germination assays for the once-selfed seeds).  
 563

| Population number | State | Crop    | Latitude  | Longitude  | $N_{fam\ germ}$ | $N_{seeds\ germ}$ | $N$ early growth | $N_{fam\ once\ selfed}$ |
|-------------------|-------|---------|-----------|------------|-----------------|-------------------|------------------|-------------------------|
| 1                 | TN    | Corn    | 35.775237 | -85.903419 | 8               | 31                | 10               | 4                       |
| 2                 | NC    | Corn    | 34.595714 | -77.927484 | 79              | 387               | 60               |                         |
| 4                 | NC    | Corn    | 34.556672 | -79.125602 | 39              | 178               | 79               |                         |
| 5                 | SC    | Soy     | 33.859875 | -79.909072 | 34              | 146               | 27               |                         |
| 8                 | SC    | Corn    | 34.297195 | -79.991259 | 40              | 180               | 73               | 8                       |
| 9                 | NC    | Soy     | 34.924044 | -77.796171 | 40              | 189               | 46               | 8                       |
| 10                | NC    | Soy     | 34.983161 | -78.039309 | 28              | 123               | 24               | 8                       |
| 11                | NC    | Corn    | 34.527135 | -78.756704 | 38              | 178               | 81               | 8                       |
| 12                | SC    | Cotton  | 34.145812 | -79.865313 | 42              | 202               | 79               | 8                       |
| 14                | NC    | Soy     | 35.424763 | -77.917121 | 40              | 192               | 76               | 8                       |
| 15                | SC    | Soy     | 34.104209 | -79.073735 | 41              | 193               | 81               | 8                       |
| 16                | SC    | Alfalfa | 34.10535  | -79.183234 | 41              | 203               | 78               |                         |
| 17                | SC    | Soy     | 34.159155 | -79.272908 | 27              | 135               | 82               |                         |
| 18                | SC    | Corn    | 34.156593 | -79.27027  | 39              | 193               | 79               |                         |
| 19                | NC    | Corn    | 34.508193 | -78.70899  | 35              | 138               | 70               | 8                       |
| 20                | TN    | Corn    | 35.830692 | -85.777871 | 38              | 169               | 13               | 2                       |

|    |    |      |           |            |    |     |    |   |
|----|----|------|-----------|------------|----|-----|----|---|
| 21 | TN | Soy  | 35.369816 | -77.877314 | 40 | 200 | 70 |   |
| 22 | NC | Corn | 36.1436   | -78.053422 | 17 | 78  | 69 |   |
| 23 | TN | Corn | 35.067905 | -86.62955  | 40 | 195 | 77 |   |
| 26 | TN | Soy  | 35.533413 | -85.951902 | 50 | 250 | 86 |   |
| 28 | SC | Corn | 34.097917 | -80.377715 | 35 | 172 | 80 |   |
| 29 | NC | Corn | 34.705135 | -78.738897 | 30 | 122 | 75 | 7 |
| 30 | TN | Corn | 35.31105  | -85.945003 | 42 | 206 | 82 |   |
| 31 | TN | Corn | 35.608482 | -85.846379 | 31 | 141 | 69 | 8 |
| 32 | TN | Corn | 35.099356 | -86.225509 | 15 | 56  | 33 | 5 |
| 33 | OH | Corn | 39.858763 | -83.669821 | 52 | 260 | 79 |   |
| 34 | OH | Corn | 39.44316  | -83.910189 | 48 | 238 | 82 |   |
| 35 | IN | Corn | 39.853945 | -85.770156 | 28 | 120 | 80 |   |
| 36 | IN | Corn | 40.565608 | -85.503826 | 33 | 146 | 85 |   |
| 37 | OH | Corn | 39.583755 | -83.758264 | 49 | 243 | 79 |   |
| 38 | OH | Soy  | 39.515447 | -83.407431 | 46 | 230 | 88 |   |
| 39 | IN | Corn | 39.988984 | -85.742262 | 38 | 188 | 75 |   |
| 40 | OH | Soy  | 41.284684 | -83.847252 | 50 | 237 | 84 |   |
| 41 | VA | Corn | 38.636343 | -78.472921 | 41 | 200 | 78 |   |
| 42 | VA | Corn | 38.373523 | -78.662516 | 49 | 230 | 82 | 8 |
| 43 | VA | Soy  | 36.886448 | -78.553156 | 24 | 89  | 28 | 2 |
| 44 | VA | Soy  | 38.285415 | -78.797088 | 20 | 98  | 75 |   |
| 45 | VA | Soy  | 36.847945 | -78.595042 | 42 | 209 | 76 |   |
| 46 | TN | Soy  | 35.536019 | -86.17985  | 34 | 100 | 17 | 2 |
| 47 | SC | Soy  | 34.282132 | -79.746597 | 41 | 201 | 83 |   |
| 48 | TN | Corn | 35.31653  | -87.35373  | 31 | 151 | 74 | 8 |
| 51 | TN | Soy  | 35.533413 | -85.951902 | 24 | 118 | 56 | 8 |
| 52 | OH | Corn | 41.284684 | -83.847252 | 62 | 310 | 88 |   |

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564 Table S2. Regression results between resistance and seed quality traits, accounting for geography (latitude and longitude) for field-  
 565 collected seeds.

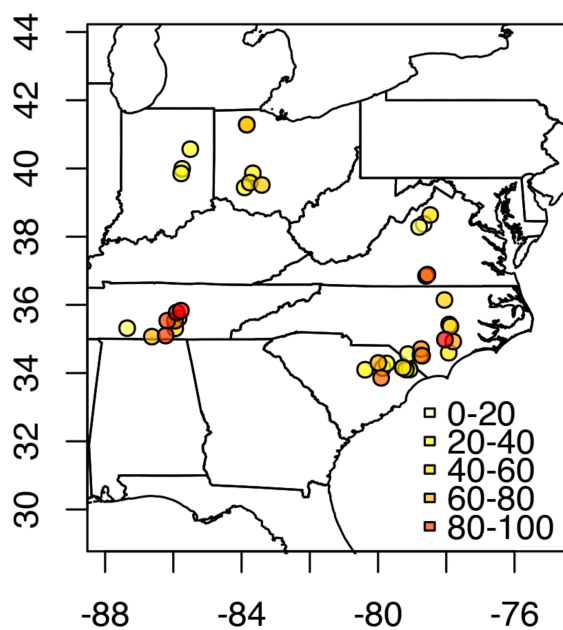
| Trait                                      | Population number | Survival at 1.7 kg a.i.<br>/ha |            | Latitude (scaled) |            | Longitude (scaled) |            |
|--|-------------------|--------------------------------|------------|-------------------|------------|--------------------|------------|
|  |                   | <i>b</i>                       | $\chi^2_1$ | <i>b</i>          | $\chi^2_1$ | <i>b</i>           | $\chi^2_1$ |
| Seed weight (g)                            | 43                | -0.0047                        | 4.63*      | 0.0016            | 7.33**     | -0.0010            | 3.91*      |
| Final germination                          | 43                | -4.89                          | 28.62***   | 0.6298            | 8.80**     | -0.3784            | 4.13*      |
| Abnormally germinating seeds               | 42                | 4.24                           | 32.52***   | -0.1774           | 1.24       | 0.1826             | 1.58       |
| Seeds needing scarification                | 43                | 0.83                           | 1.77       | -0.4636           | 7.19**     | -0.3488            | 5.22*      |
| Scarified seeds that germinated            | 40                | -5.87                          | 8.85**     | 0.0061            | 0.0002     | -0.3342            | 0.65       |
| Height (cm)                                | 43                | -7.39                          | 7.26**     | -0.3336           | 0.38       | 1.2210             | 4.76*      |
| Leaf number                                | 43                | -1.08                          | 17.57***   | -0.0070           | 0.02       | 0.1589             | 9.17**     |
| Largest leaf size (Box cox transformed cm) | 43                | -4.40                          | 14.04***   | 0.4080            | 2.96       | 0.4642             | 3.81       |

567 Table S3. PCA loadings and variance explained.

| Trait                                   | Factor1  | Factor2  | Factor3  |
|---|----------|----------|----------|
| Seed weight                             | 0.09506  | 0.8414   | -0.16627 |
| Percent germination                     | 0.29276  | 0.82272  | 0.37541  |
| Percent viable dormant seeds            | 0.0339   | 0.12801  | 0.92794  |
| Percent abnormally<br>germinating seeds | -0.15651 | -0.81953 | -0.42013 |
| Height                                  | 0.94253  | 0.04143  | 0.05526  |
| Leaf number                             | 0.86728  | 0.13535  | 0.06511  |
| Largest leaf size                       | 0.74853  | 0.28353  | 0.00642  |
| Prop. Variance explained                | 0.3316   | 0.3105   | 0.1734   |

568

569 Fig S1. Map of the Eastern US showing the population locations and their survival at  
570 1.7 kg a.i./ha (color of circle).



571



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