

Defence against the herbicide RoundUp® predates its widespread use

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ABSTRACT

Background: The herbicide RoundUp® became widely used in agriculture in the mid-1990s. Current populations of the crop weed *Ipomoea purpurea* exhibit both resistance and tolerance to RoundUp, which are two types of defence that have previously been shown to have a genetic basis in this weed species.

Questions: Did the genetic basis of RoundUp defence antedate the introduction of RoundUp? If so, how does defence today compare with defence then?

Organism: A preserved collection of *Ipomoea purpurea* seeds (the common morning glory) was germinated from 10 accessions sampled from the early 1980s. A contemporary population of *I. purpurea* derived from a crossing scheme was used for comparison.

Methods: Replicate seeds from both the historical and contemporary populations were planted into a field experiment located at the University of Georgia's Plant Sciences Farm. Individuals were sprayed with the field rate of glyphosate, the active ingredient in the herbicide RoundUp. Tolerance, or the fitness response following herbicide application, and resistance, a measure of the amount of damage a plant exhibits after herbicide application, were determined for each line of both the historical and contemporary populations.

Results: Genetic variation for both types of defence was already present in this weed species prior to the herbicide's introduction in the mid-1990s. Furthermore, the level of tolerance to glyphosate has not increased over time. In contrast, our results suggest that resistance is higher in the contemporary population than in the historical accessions.

Keywords: adaptation, agro-ecosystem, evolution, *Ipomoea purpurea*, resistance, tolerance.

INTRODUCTION

The evolution of herbicide defence traits in weedy plant species is perhaps one of the best examples of rapid adaptation to a changing environment (Cousens and Mortimer, 1995; Yuan *et al.*, 2006). It is also a phenomenon that is of immense economic importance, with the worldwide cost of herbicide resistance estimated to be as high as US\$8 billion per year (D. Pimentel, personal communication). Currently, there are more than 300 herbicide-resistant weed biotypes, with new cases of herbicide resistance reported annually (Heap, 2008).

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Although there are many weeds that exhibit defence to herbicide, investigations of its historical origin are rare. Herbicide resistance is usually ‘diagnosed’ when a population with a history of herbicide treatment exhibits a high level of resistance. To document evolution by herbicide-imposed selection, one must ascertain resistance levels of naive weed populations, or those that have not previously been exposed to the herbicide. Only rarely are samples of weed populations collected and stored before herbicide exposure. This is unfortunate, as some weed species can remain dormant in the seed stage for many years (Baskin and Baskin, 1998), and seed dormancy and storage can provide an avenue by which the historical state of defence can be ascertained. For example, researchers have reconstructed ancestral plant populations to study adaptation to other human-mediated selective agents, such as climate change (Franks *et al.*, 2007).

Present-day populations of the common morning glory, *Ipomoea purpurea*, exhibit both tolerance and resistance to applications of glyphosate (Baucom and Mauricio, 2008), the active ingredient in Roundup®, a herbicide of global use (Duke and Powles, 2008). Tolerance is defined as the ability to re-grow and produce progeny after being damaged (Stowe *et al.*, 2000), whereas resistance refers to the ability of the plant to prevent damage (Simms and Rausher, 1989). Tolerance and resistance have been central to studies of plant–herbivore interactions (reviewed in Nunez-Farfan *et al.*, 2007), but they have also been studied in terms of defence against abiotic stresses, such as frost (Agrawal *et al.*, 2004). Here, as in previous work (Baucom and Mauricio, 2004, 2008), we borrow the definitions of resistance and tolerance from the evolutionary ecology literature and apply it to the study of the evolution of herbicide defence in a crop weed.

We have previously considered the evolutionary dynamic of both tolerance and resistance to glyphosate in a contemporary population of *I. purpurea*, but herbicide defence traits have not been considered in an historical population, or one collected before the widespread use of the herbicide. Such an assessment would inform us if the defence characters are a consequence of mutations arising after the introduction of the herbicide, or if they predate widespread use of the herbicide and were selected from genetic variation that already existed in the population. In the latter case, these mechanisms of defence would be exaptations, originally evolving for some function other than defence against glyphosate.

Here we provide experimental evidence that glyphosate defence predated the use of the herbicide in the crop weed *I. purpurea*. We reconstructed a historical plant population from seed accessions that were collected more than 20 years ago, well before common use of glyphosate. Fortunately, species of the genus *Ipomoea* are known to remain viable as dormant seed for up to ~20 years (Baskin and Baskin, 1998), and we had access to seed accessions of *I. purpurea* collected and stored well before the widescale, increased use of glyphosate in 1996, a result of the adoption of Roundup Ready® technology (Powles, 2008).

Our specific aims were to determine if glyphosate defence traits were present in these historically collected seed accessions, and if the accessions exhibited variation in tolerance and resistance. Furthermore, because replicates of these accessions were randomly planted alongside a contemporary population of *I. purpurea*, we were able to determine if the levels of both resistance and tolerance had increased over time.

MATERIALS AND METHODS

The common morning glory, *Ipomoea purpurea* (L.) Roth. (Convolvulaceae), is a weedy annual vine found throughout the southeastern United States. This species is a predominant weed in cotton, soy, and corn crops, and is often found growing in areas of recent

disturbance (personal observation). We have previously shown that a population of *I. purpurea* collected from an agricultural field in Oconee County, Georgia is tolerant of glyphosate, the main ingredient in the herbicide RoundUp, and that there is genetic variation for this tolerance (Baucom and Mauricio 2004).

Glyphosate is a non-specific, post-emergence herbicide (Grossbard and Atkinson, 1985) that enters the plant by diffusion and is mobile throughout the phloem (Caseley and Coupland, 1985). Glyphosate accumulates in the apical meristems and other sites of sugar utilization (Franz *et al.*, 1997), and causes plant death by inhibiting a key enzyme in the shikimate pathway (Amrhein *et al.*, 1980; Steinrucken and Amrhein, 1980). Although the herbicide was first commercialized in the USA in the late 1970s, its widespread use began in the late 1990s with the introduction of Roundup-Ready soybeans (Fig. 1). USDA data for glyphosate use begins in 1990; before this date, agriculturists relied on other herbicides for weed control (Baucom and Mauricio, 2004) (see Fig. 1).

The Plant Genetic Resources Conservation Unit of the USDA Agricultural Research Service (Griffin, GA) provided us with *Ipomoea purpurea* accessions originally collected between 1978 and 1981, well before the widespread use of glyphosate (Fig. 1; Table 1). Seeds were maintained at a constant temperature until germination trials in August 2003. We were able to germinate seeds from two replicate lines of five historically collected accessions (Table 1). Replicates of these accessions were randomly planted in 12-inch pots and fertilized every week with a 10-30-20 fertilizer (Peter's Blossom Booster, J.R. Peters, Inc., Allentown, PA). Individuals were allowed to self-pollinate to produce sufficient numbers for the field experiment. Seeds from each replicate were bulked according to accession.

The contemporary population was originally collected from two agricultural fields located in Oconee County, Georgia. To generate replicate seed of the contemporary

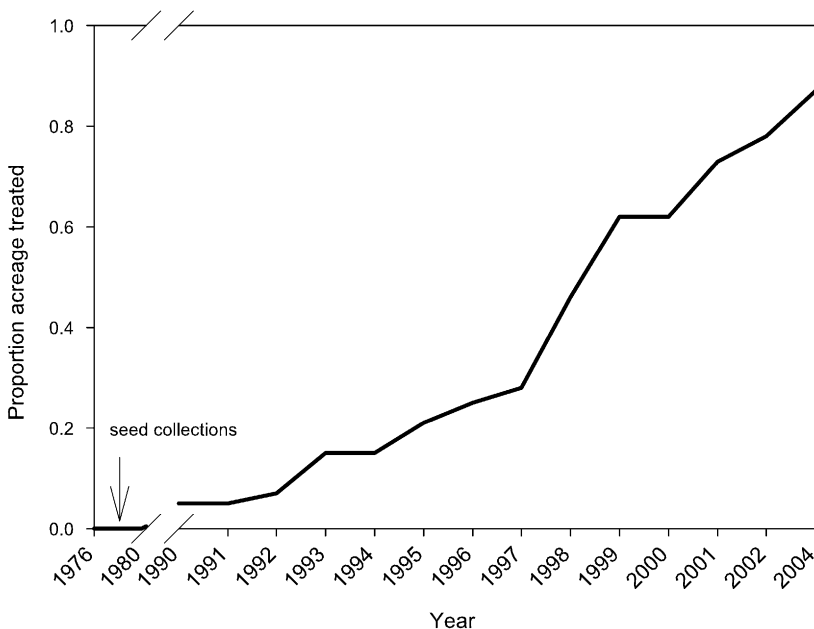


Fig. 1. The proportion acreage of soybeans treated with Roundup per year in the USA. Data from the National Agricultural Statistics Service, USDA (NASS-USDA, 2003).

Table 1. Origin and year of collection of the historically collected accessions

Accession	Origin	Year of collection
1	Virginia, USA	1980
2	Alabama, USA	1978
3	Argentina	1981
4	Turkey	1981
5	Unknown origin	1981

population, 12 field-collected maternal lines were randomly planted in the greenhouse and used as parents in a full-sibling breeding design. Six individuals from lines determined to be ‘least susceptible’ to the effects of glyphosate were reciprocally crossed among themselves, as were six lines considered ‘most susceptible’. This crossing scheme generated replicates of 12 paternal half-sibling families for a larger experiment assessing the evolutionary dynamic of glyphosate defence traits, and is further outlined in Baucom and Mauricio (2008).

We planted replicates of each of the five historically collected and 12 contemporary *I. purpurea* lines in a field experiment on 9 July 2004 in an agricultural field at the University of Georgia’s Plant Sciences Farm in Oconee County, Georgia. We utilized a randomized split-plot design with treatment plots (glyphosate/no glyphosate) nested within two spatial blocks. We planted 14 replicate seeds of the historically collected accessions in each treatment/block combination, and 35 replicate seeds of each paternal half-sibling family of the contemporary population for a total of 1960 randomly planted individuals. Treatment with herbicide was the whole-plot factor with genetic line as the sub-plot factor. Within each plot we planted seeds in a grid with 1 m² separating experimental individuals, and marked planted seeds with plastic straws to facilitate finding experimental plants. Seeds were scarified with a razor blade to ensure equal timing of germination among individuals. We removed the vegetation within 0.3 m around experimental individuals once over the course of the experiment to deter herbivory from the cotton rat, *Sigmodon hispidus*, but otherwise let competitive weeds grow undeterred. Each plant was allowed to grow up a 1-m tall bamboo stake that mimics *I. purpurea* growth in agricultural fields and allows for easy identification of experimental plants. Seedlings began emerging within one week of being planted, and plants began flowering by 31 August in the control plots. Fruits began maturing by 18 September. We applied glyphosate at a rate of 1.121 kg a.e. ha⁻¹ with a pressurized CO₂ plot sprayer, which keeps droplet size and spray intensity constant (R&D Sprayers, Opelousas, LA), on 15 August 2004 to experimental plants within the glyphosate treatment plots of each block.

On 9 September, approximately 3 weeks after the glyphosate application, we counted the total number of leaves that remained on each sprayed plant as well as the number of leaves exhibiting symptoms of glyphosate damage for an estimate of the proportion of plant that was damaged. After fruits began maturing, we collected seeds during four rounds of collection until all plants were killed by a frost on 4 December. Viable seeds were counted, and the number of seeds produced by each plant was used as an estimate of fitness. Non-viable morning glory seeds are shrivelled in appearance and are easily distinguished from viable seeds. Only individuals that survived to glyphosate application were included in

the analysis; those that survived to the application of glyphosate but died as a result of its application or did not produce seed were given a fitness score of '0'.

We used two different methodologies to determine if the historically collected accessions varied in tolerance to glyphosate. First, we used the MIXED procedure of the SAS statistical software package (v. 9.1) to model the fitness response of individuals following glyphosate application. In this analysis, relative fitness was the response variable, with accession, block, block \times treatment, and accession \times treatment as the independent, random variables. Treatment environment was considered fixed. The term of interest with regard to tolerance is the interaction between accession and treatment – a significant interaction indicates that glyphosate did not affect the fitness of all accessions equally and is evidence that the level of tolerance is variable. However, a significant interaction can arise because of a difference in the variance of fitness between treatments, rather than simply the response of the accessions to the treatment. To address this, we estimated 'pseudo-tolerance' for each historical accession and tested for variation among accessions, again using the MIXED procedure of SAS. We performed this test since many experimental plants in the spray environment did not produce seed, and we wanted to determine if a significant accession \times treatment effect in the first analysis of variance (ANOVA) was caused by unequal variance in fitness among treatments, rather than variation in tolerance *per se*. To do this, we randomly matched replicates of each historically collected accession from the glyphosate present and absent treatments, and for each pair estimated tolerance as relative fitness in the glyphosate environment minus relative fitness in the control environment. Thus, for each block we produced 14 tolerance estimates per accession. We then used these tolerance estimates in the mixed-model ANOVA as the dependent variable, with accession and block as the independent, random variables. The tolerance estimates were not transformed before analysis, as examination of the residuals suggested that the data did not display heteroscedasticity and did not deviate substantially from normality.

We similarly tested for variation in the level of resistance among accessions by assessing if the proportion of the plant damaged from glyphosate differed among accessions, again using the MIXED procedure of SAS. The proportion of plant damage was estimated by dividing the number of leaves exhibiting yellowing or necrosis following glyphosate application by the total number of leaves remaining on the plant. For this trait, we utilized only the plants treated with glyphosate. In the ANOVA, the arcsine square-root proportion damage was the response variable, with accession and block as independent, random variables. We have demonstrated elsewhere that resistance and tolerance are variable among the paternal half-sibling families of the contemporary population (Baucom and Mauricio, 2008), and for brevity this information will not be presented here.

We operationally estimated both tolerance and resistance for each of the five historically collected accessions and the 12 paternal half-sibling families of the contemporary population. We defined resistance as the average of $(1 - p)$ for each line, where p is the proportion vegetative damage a plant displayed following glyphosate application (Simms and Triplett, 1994). Tolerance to glyphosate application was estimated by subtracting the mean relative fitness of individuals that were not treated with glyphosate from the mean relative fitness of individuals from the same line, but sprayed with glyphosate (Baucom and Mauricio, 2004). The NPAR1WAY procedure of SAS was used to perform a Wilcoxon two-sample test to determine if lines from the contemporary population exhibited higher tolerance and/or resistance compared with the lines from the historically collected population. A retrospective power analysis was performed for each trait using G*Power to determine

if the comparisons between the historically collected accessions and the contemporary population lacked power or if the null hypothesis of no difference should be accepted.

RESULTS

The historically collected *I. purpurea* accessions exhibit both tolerance and resistance to glyphosate (Fig. 2), and survival after the application of glyphosate was high. While 97% of

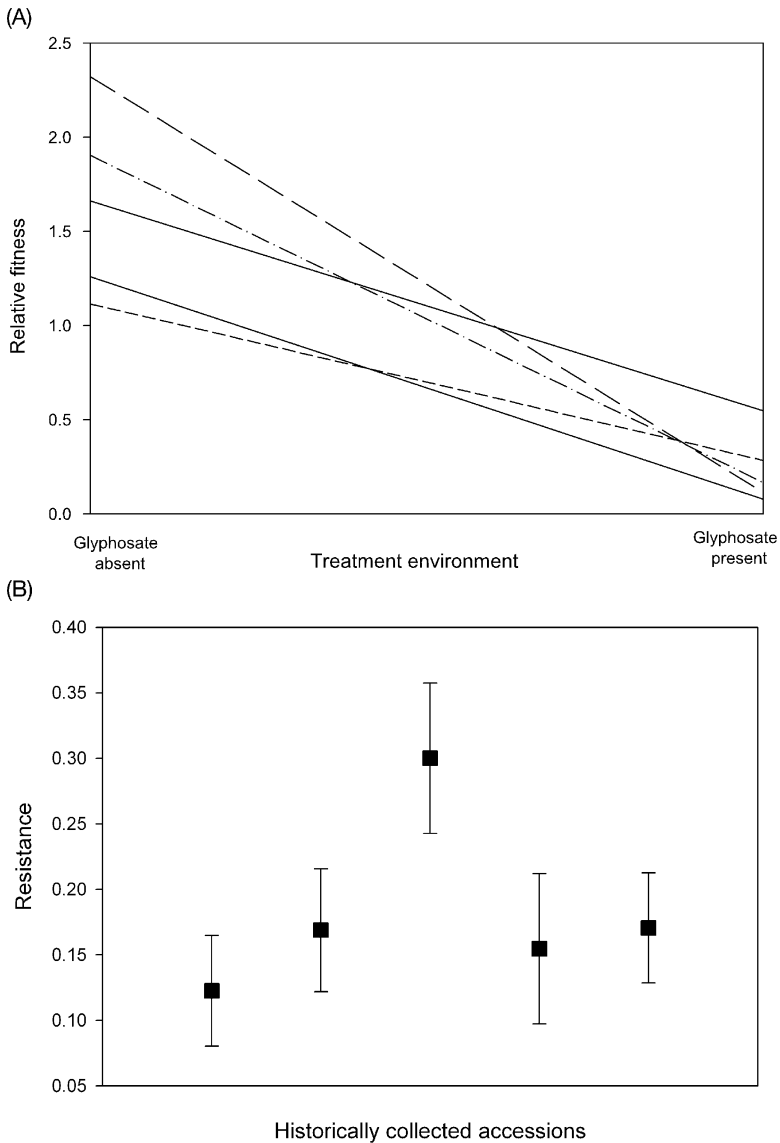


Fig. 2. The level of defence in the historically collected accessions. (A) The relationship between relative fitness and treatment environment. The slope of the line represents the level of tolerance for each of the five historically collected accessions. (B) The level of resistance present in each accession.

the planted individuals germinated, only 20% (28 individuals) died as a result of glyphosate application. The herbicide did impact reproduction, however, with only 30% (42 individuals) of those that survived subsequently setting seed. The contemporary population exhibited similar effects of glyphosate, with fewer than 20% of experimental plants dying as a result of being sprayed (153 of 804), and 44% of those that survived producing seed.

Although 70% (98 individuals) of the replicates of the historical accessions did not reproduce, variation for tolerance to glyphosate was present among the accessions, as evident by the significant Accession \times Treatment interaction in the mixed-model ANOVA (Table 2). We similarly uncovered variation among accessions using our ‘pseudo-tolerance’ estimates (Accession, $\chi^2_1 = 4.2$, $P = 0.04$), indicating that the variation was caused by differences among accessions in the level of tolerance rather than heterogeneity of variance in fitness among treatment environments. Variation for resistance was also present among the historically collected accessions (Table 3); however, average values of resistance were

Table 2. Mixed-model analysis of variance for tolerance to glyphosate application

Source	Covariance estimate \pm s.e.	χ^2	P
Random effects			
Block	0		
Block \times Treatment	0		
Accession	0.009 \pm 0.075	0	1
Accession \times Treatment	0.103 \pm 0.100	5.3	0.021
Residual	1.006 \pm 0.088		
Source	d.f.	F	P
Fixed effects			
Treatment	1, 4	46.25	0.002

Note: A significant interaction between Accession and Treatment indicates the presence of variation for tolerance to glyphosate among accessions. For random effects we present the χ^2 value from a likelihood ratio test, and for fixed effects we provide the F -statistic.

Table 3. Mixed-model analysis of variance for resistance to leaf damage

Source	Covariance estimate \pm s.e.	χ^2	P
Random effects			
Accession	0.025 \pm 0.022	8.8	0.003
Block	0.013 \pm 0.022	3.2	0.074
Residual	0.151 \pm 0.020		

Note: The dependent variable, proportion of leaf damage, was arcsine square-root transformed before analysis.

low, with line means of the proportion of the plant damaged ranging from 0.56 to 0.89. Thus, while individuals survived the application of glyphosate, and lines exhibited variation for both tolerance and resistance to the herbicide, its application led to high levels of vegetative damage and significantly lowered fitness among both the historically collected accessions and the contemporary population.

Furthermore, lines of the contemporary population exhibit a significantly higher level of resistance than the historically collected accessions (Wilcoxon two-sample test, $S = 22.0$, $P = 0.0068$; Fig. 3A). In comparison, the average level of tolerance in the contemporary population appears to be slightly higher than that in the historical lines (Fig. 3B), but this difference was not significant (Wilcoxon two-sample test, $S = 46.0$, $P = 0.4796$). However, a *post-hoc* power analysis found the ability to accept the null hypothesis of no difference in tolerance between collection dates to be low ($1 - \beta = 0.379$), whereas the power of the comparison for resistance was adequate ($1 - \beta = 0.888$).

DISCUSSION

All of the historically collected lines in our experiment expressed tolerance and resistance. Thus, glyphosate defence traits were present in the common morning glory before the widespread use of the herbicide, which supports the idea that tolerance and resistance to glyphosate are exaptations – they likely originally evolved for some function other than defence to glyphosate.

Not only does this small historical collection of seeds exhibit tolerance and resistance to glyphosate, we found variation among the accessions for both defensive traits. We did not have enough replication within our design to determine how much of the variation in tolerance could be explained by additive genetic variation, rather than other potential sources, such as population or collection locality effects. In addition, these accessions were derived from widely separated geographic locations, precluding the possibility of gene flow among them. For this reason, we cannot make predictions regarding the evolution of glyphosate defence traits using these data, since the apparent variation could be a consequence of spatial differences, maternal effects, slight temporal differences or even variability in the ability to remain viable while dormant. However, what we can determine from the data is that (1) glyphosate defence traits were present before the introduction of the herbicide, and (2) to the extent that variation among populations is genetic, the apparent variability among accessions could be caused by an underlying genetic basis. We have consistently uncovered the existence of genetic variation for tolerance and resistance to glyphosate in contemporary populations of morning glories (Baucom and Mauricio, 2004, 2008), so this assertion is plausible.

Furthermore, we find that the contemporary population exhibits higher resistance than the historical accessions. While the average level of tolerance was slightly higher in the contemporary population, this difference was not significant. This suggests that the level of tolerance has been maintained over time but that the level of resistance to glyphosate has increased. Tolerance has previously been shown to incur a significant fitness cost (Baucom and Mauricio, 2004, 2008), and this is one such mechanism that could explain maintenance in the level of tolerance (Simms and Rausher, 1987). However, our power to detect a difference between the contemporary and historical accessions in their respective level of tolerance was low and likely driven by the high variance in tolerance among historical accessions. Thus, we cannot say with a high degree of certainty that tolerance evolution has not occurred.

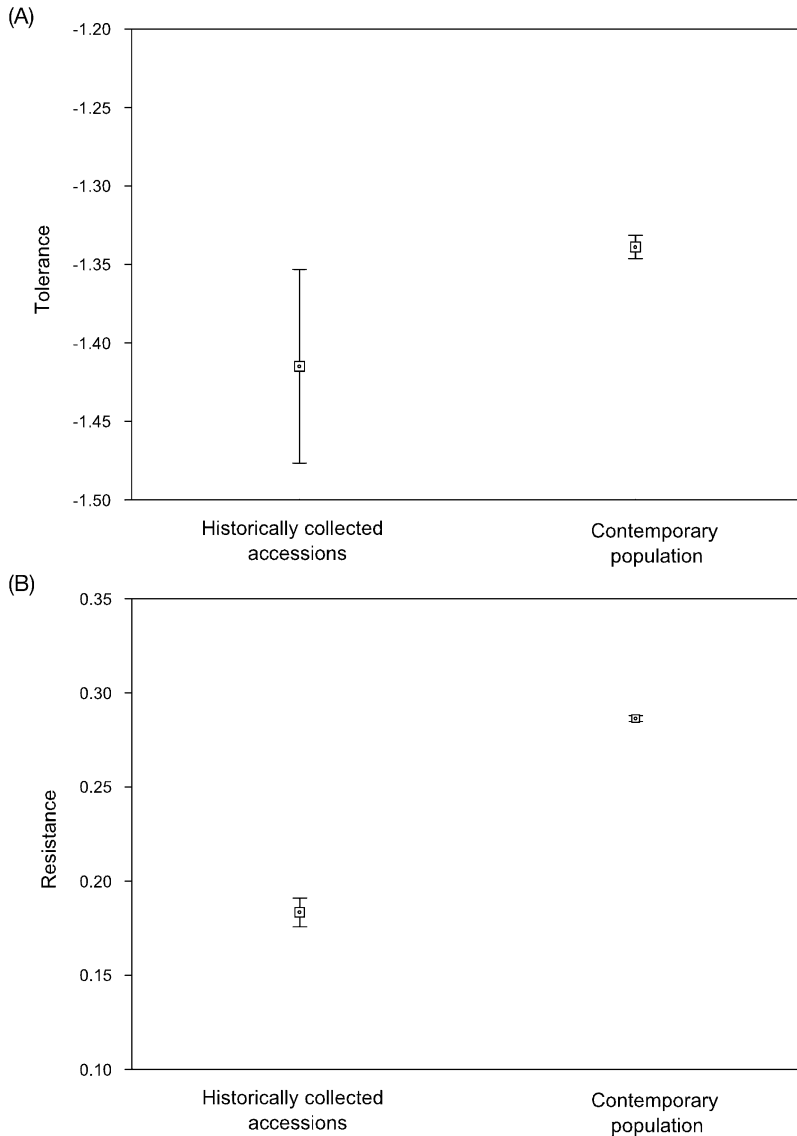


Fig. 3. The average level of defence in the historically collected accessions compared with the contemporary population. (A) The level of tolerance and (B) level of resistance were averaged among the five historical accessions and 12 paternal half-sibling families of the contemporary population. Standard errors were estimated by jackknifing tolerance or resistance estimates across either the accessions or paternal half-sibling families.

In comparison, the power to detect a difference in resistance among the two groups was adequate.

Although these data provide evidence of resistance evolution, there are further caveats to this conclusion. First, the contemporary population was derived from two fields located relatively close to one another, whereas the historical accessions were collected from widely

separated geographic localities – thus, this comparison confounds both spatial and temporal differences. In addition, the historical accessions that remained viable throughout storage as seed could be exhibiting lower resistance because of physiological effects of such dormancy, rather than a reduced level of resistance *per se*. Thus, we can only state that the difference observed is *suggestive* of resistance evolution in the contemporary population. We have previously shown that resistance is a variable trait within the contemporary population, and that glyphosate exerts positive selection for increased resistance (Baucom and Mauricio, 2008); as such, the potential that resistance evolution is occurring and/or has occurred is substantial.

In conclusion, the presence of variation among the historically collected accessions suggests glyphosate defence traits likely arose from selection on standing genetic variation rather than following a very recent mutation(s), or one arising immediately before the contemporary population's collection date. Adaptation via standing genetic variation is thought to occur more rapidly than from a recent mutation, given that advantageous alleles start at a higher frequency (Innan and Kim, 2004), and alleles present as standing genetic variation are thought to have been either 'pre-selected' in previous environments or introduced through gene flow from another population within the species range (Rieseberg *et al.*, 2003). We currently do not have any data to suggest what aspect of the pre-herbicide environment selected on herbicide defence traits, and we do not know what function either defence trait would have served before the introduction of glyphosate.

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