Differential response of *Amaranthus tuberculatus* (Moq ex DC) JD Sauer to glyphosate†

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Abstract: Midwest USA farmers have reported inconsistent control of *Amaranthus tuberculatus* (= *rudis*) (Moq ex DC) JD Sauer by glyphosate in glyphosate-resistant crops. The potential of selection for decreased *A tuberculatus* sensitivity to glyphosate was therefore investigated in a reportedly resistant Everly, IA population (P0–EV). Evaluation of six *A tuberculatus* populations from the Midwest USA estimated a seedling baseline sensitivity of 2.15 mM glyphosate. Based on these results, three generations of divergent recurrent selection were implemented on P0–EV to isolate resistant and susceptible populations. A seedling assay was developed to screen large amounts of seeds and thus expedite the selection process. Whole-plant and seedling rate responses of P0–EV and a known pristine *A tuberculatus* population from Paint Creek, OH (P0–WT) identified no significant difference in response to glyphosate; however, greater phenotypic variance was ostensibly evident in P0–EV. The first recurrent generation selected for resistance at 3.2 mM glyphosate (RS1–R) had a 5.9- and 1.7-fold resistance increase at the seedling and whole-plant levels, respectively, compared with the susceptible generation selected at 32 µM glyphosate. After three cycles of recurrent selection, 14.6-fold difference in resistance at the seedling level and 3.1-fold difference at the whole-plant level were observed when comparing the populations selected for resistance (RS3–R) and susceptibility (RS3–S). Overall, recurrent selection increased the frequency of resistant individuals and decreased the variability to glyphosate at the population level. Nevertheless, variability for glyphosate resistance was still evident in RS3–R. Results herein suggested that *A tuberculatus* is inherently variable to glyphosate and that selection decreased the sensitivity to glyphosate. We purport that evolved glyphosate resistance in *A tuberculatus* may require multiple cycles of selection under field conditions. Historic estimated use of glyphosate alludes to the evolution of tolerant weed populations.

Keywords: asexual propagation; baseline sensitivity monitoring; non-destructive assay; recurrent selection; shikimic acid; herbicide resistance

1 INTRODUCTION

Since their commercialization in 1996, N-(phosphonomethyl)glycine (glyphosate)-resistant crops have provided an excellent control strategy for broad-spectrum weed suppression, thus possibly simplifying farm management, presumably lowering production costs, and permitting the management of weed biotypes resistant to herbicides other than glyphosate.1,2 These elements have propelled the broad adoption of this technology in such crops as cotton (*Gossypium hirsutum* L), soybean (*Glycine max* L Merr), and canola (*Brassica napus* L). In 2001, glyphosate-resistant soybean comprised more than 60% of the cultivated soybean area in the USA, and nearly 100% in Argentina.3 Producers can now spray high glyphosate rates without injury concerns to the crop, and, given the lack of residual activity, to meet with weed control expectations, crops typically receive multiple glyphosate applications per growing season. This represented an increase from the average total of 340 Mg acid equivalents (AE) of glyphosate sprayed per state within the Midwest USA in 1995 (prior to glyphosate-resistant crops) to 2060 Mg AE glyphosate per state by 2002.3,4 In the past few years, much debate has centered on the potential implications of such unparalleled change of farming operations on the environment, the world economy, and human health. Evidence put forward suggests that the large-scale adoption of herbicide-resistant crops may be accompanied by significant biological implications; chiefly reduction in biodiversity,5,6 weed population shift,7,8 and vertical and horizontal transgenic flow to adjacent plants.9–12

Enough evidence exists to assert that the herbicide selection pressure in the production of herbicide-resistant crops increases the fitness of weeds in that agroecosystem. In the case of glyphosate-resistant

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crops, cited weed responses include variable or differential response, tolerance and resistance. The first response encompasses the variable efficacy within a weed species to a single, otherwise lethal glyphosate rate; examples include *Convolvulus arvensis* L., *Abutilon theophrasti* Medicus, and *Amaranthus tuberculatus* (= *rudis*) (Moq ex DC) JD Sauer.13–15 Tolerance is the inherent ability of some weed species to survive glyphosate rates that effectively control other species in the weed community; species in this category include *Commelina communis* L., *Chenopodium album* L and *Dicliptera chimensis* (L.) Juss.3 Mecha...nisms by which tolerant species compensate for the damaging glyphosate effects include reduced herbicide absorption and translocation, and higher activity of the target enzyme, 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS; EC 2.5.1.19).16,17 Presumably, similar mechanisms exist in species that demonstrate a differential response to glyphosate. Resistance is the evolved ability of some species, through selection pressure or genetic engineering, to survive glyphosate rates that have effectively controlled the species in the past and that are lethal to the wild-type species. Glyphosate resistance has been confirmed in seven species worldwide; resistance mechanisms include target site modification and differential glyphosate cellular transport.18–21 All three, resistance, tolerance and differential response, have been implicated in weed succession changes within glyphosate-resistant agroecosystems.3,8,22 The cosmopolite pigweed complex is represented by at least eight weedy species in the Great Plains of the USA.23 Control with post-emergence herbicides is variable, depending on the pigweed species and the application timing.15,24 Furthermore, interspecific hybridization and multiple resistance to triazine, acetolactate synthase (ALS; EC 2.2.1.6) and protoporphyrinogen oxidase (PPO; EC 1.3.3.4) inhibiting herbicides has complicated *A. tuberculatus* management, even with alternating herbicide programs.25–27 In recent years, however, Iowa farmers have reported inconsistent *A. tuberculatus* control with glyphosate in glyphosate-resistant crops. Most field reports of inconsistent glyphosate control of *A. tuberculatus* are attributable to an ineffective herbicide application, sub-lethal herbicide doses, or delayed glyphosate application with respect to the weed’s phenological stage. Nevertheless, some reports of inconsistent glyphosate control do not fit this profile.

In 1998, a producer from Everly, IA asserted that application of glyphosate-isopropylammonium at 0.63 kg AE ha⁻¹ on 10-cm tall plants, followed by 0.83 and 1.25 kg AE ha⁻¹ one and two weeks after the initial application, failed to effectively control *A. tuberculatus*. This incident occurred during the producer’s first year adoption of glyphosate-resistant soybean, yet different control levels were observed between adjacent *A. tuberculatus* plants within soybean rows. Field assessment ruled out delayed or other application problems as the probable cause for the inconsistent *A. tuberculatus* control. In 1999, another similar incident was reported in Badger, IA where 12-cm tall *A. tuberculatus* plants reportedly survived two sequential applications of 1.25 kg glyphosate AE ha⁻¹. Since plants were treated at the labeled height and glyphosate dose, and no application problems were detected, the inconsistent control at Badger, IA was attributed to a differential response in the *A. tuberculatus* population. Endogenous shikimic acid accumulation estimates from putative susceptible and resistant plants from Badger, IA, and from untreated plants in adjacent areas, confirmed the observations of variable whole-plant response chronicled in the field.28 Similar cases of inconsistent *A. tuberculatus* control with glyphosate have been reported in other Midwest states.29 Preliminary greenhouse assessments suggested that, compared with unselected *A. tuberculatus* populations, plants that originated from the Everly, IA population demonstrated an enhanced fitness to glyphosate. Subsequently, an investigation was undertaken to estimate the baseline sensitivity of *A. tuberculatus* populations to glyphosate, and to assess the potential for selection of glyphosate resistance in the Everly, IA population.

2 MATERIALS AND METHODS

2.1 Plant materials

Ninety-seven plants (53 ♂, 44 ♀) with a resistant phenotype were collected from the Everly, IA field where glyphosate reportedly failed to control *A. tuberculatus* and the rescued plants were grown in controlled greenhouse conditions (Section 2.2). At anthesis, plants were allowed to cross-pollinate without restrictions. The female inflorescences were then covered with a PQ218 DelNet® bag (Speciality Nets & Profiles, 601 Industrial Drive, Middletown, DE 19709-1083) and the seed permitted to develop on the mother plant. Seeds from individual female plants (henceforth maternal lines) were then collected, cleaned in an air-column separator (Seedburo Equipment Company, 1022 W Jackson Boulevard, Chicago, IL 60607–2914), and stored at 5 °C and darkness until utilized; the combined (53) maternal lines comprised the parental Everly, IA population (P₀–EV). Collected at Paint Creek Dam, OH, the wild-type *A. tuberculatus* population (P₀–WT) was acquired through the USDA–ARS National Plant Germplasm System (NPGS; PI 603888). The P₀–WT population evolved in a wild environment without exposure to glyphosate (D Pratt, pers comm). After collection, P₀–WT was maintained at the North Central Regional NPGS station in Ames, IA and propagated without selection to any herbicide (D Brenner, pers comm). Two *A. tuberculatus* populations from Iowa agroecosystems were extracted from the Iowa State University weed seed collection; records indicated that the seed was collected at Curtiss Farm, Ames, IA in 1996 (P₀–AM) and Ogden, IA in 1997 (P₀–OG). The
homogenous cross-resistant \emph{A. tuberculatus} population (P\(_0\)–CR) was isolated in the greenhouse through two cycles of recurrent selection at 2.8 kg imazethapyr AE ha\(^{-1}\) + 1.6 kg primisulfuron-methyl ha\(^{-1}\) from a Davis City, IA population previously described.\(^{30}\) The multiple-resistant \emph{A. tuberculatus} was selected from a triazine-resistant population from Fillmore County, NE.\(^{31}\) Two cycles of recurrent selection at 2.8 kg imazethapyr AE ha\(^{-1}\) + 4.8 kg atrazine ha\(^{-1}\) isolated the homogenous multiple-resistant \emph{A. tuberculatus} population (P\(_0\)–MR). The grain amaranth cultivar ‘Plainsman’ (\emph{A. hypochondriacus} L \(\times\) \emph{A. hybridus} L cv K343) (P\(_0\)–GA) was acquired from a local dealer (Calvin Oliverius, PO Box 25, Albin, WY, 82050–9776).

### 2.2 Seed conditioning and growth environment

Dormancy was overcome by preconditioning seeds in filter paper moistened (20 ml) with a 1 mM GA\(_3\) [90% (+)-gibberellic acid] solution in plastic petri dishes and stored for one month in darkness at 5°C. Seeds were then planted in a 30 \(\times\) 90 cm flat containing a peat:perlite:loam (1:2:1) soil mix and seedlings were grown in a greenhouse set at 25–30°C and 40–60% relative humidity (RH) diurnal and 20–25°C and 50% RH nocturnal conditions. One week post-emergence, seedlings were transplanted to 12-cm diameter pots, irrigated as needed, and fertilized (Miracle Gro Excel, Scott–Sierra Co. 14111 Scottslawn Road. Marysville, OH 43041–0001) twice during the reproductive growth cycle. Natural light was supplemented with artificial illumination delivering 600–1000 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) photosynthetic photon flux density (PPFD) to create a 16-h photoperiod.

### 2.3 Glyphosate efficacy assessment

#### 2.3.1 Seedling dose response

All \emph{Amaranthus} populations were evaluated for response to glyphosate utilizing a novel seedling assay that permitted large-scale screening of individuals. This approach was relevant as \emph{A. tuberculatus} produced >80,000 seeds under the aforementioned greenhouse conditions. Twenty seeds per well in a 24-well cell culture cluster plate (Corning Incorporated. One Riverfront Plaza Cornng, NY 14831–0001) were placed on four 3-mm diameter 3 MM chromatography paper discs (Whatman Inc 100 Ames Pond Drive, Tewksbury, MA 01876-1240), previously moistened (500 \(\mu\)l) with distilled deionized water \((dH_2O)\) control or doses of 0.032, 0.1, 0.32, 1, 3.2, 10 or 32 mM glyphosate. Doses were prepared by solubilizing the salt of glyphosate (Sigma-Aldrich Corporation. 3050 Spruce Street, St Louis, MO 63103-2530) in \(dH_2O\) by adjusting the solution to pH 7.0 with 0.5 M potassium hydroxide. Therefore, each 24-cell culture plate allocated for three replications of the control and the seven glyphosate doses; evaporation of the treatment solution was prevented by enveloping plates with plastic wrap film. Plates were also established with potassium hydroxide alone to assess the effect of the base on the germination and development of \emph{A. tuberculatus} seedlings. Finally, plates were placed in a germinator (Model SG30, Hoffman Manufacturing, Inc 353 29th Avenue SW, Albany, OR 97322-3899) set at 30°C and 14 h diurnal and 20°C and 10 h nocturnal conditions at 500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PPFD. Seed germination, and seedling hypocotyl and radicle length were recorded two weeks after plate establishment. Germination was defined by radicle protrusion from the seed, while dimensional measurements were recorded from the hypocotyl base to the distal apex or root end.

#### 2.3.2 Whole-plant rate response

Three seedlings per 12-cm diameter pot were equidistantly transplanted and permitted to grow in the aforementioned greenhouse conditions (Section 2.2). The manufacturer’s recommended glyphosate rate for 15-cm tall \emph{A. tuberculatus} is 0.85 kg AE ha\(^{-1}\) (Roundup UltraMAX\(^{\text{TM}}\), Monsanto Co, 700 Chesterfield Parkway North, St Louis, MO 63198–0001), thus rate responses were conducted at 0.0 \((dH_2O)\) control, 0.21, 0.54, 0.85, 1.27, 1.69 and 2.5 kg glyphosate AE ha\(^{-1}\). Treatments were sprayed at 30 cm from the canopy of the plants through an even flat flow nozzle delivering 187 liter ha\(^{-1}\) at 2.8 kg cm\(^{-2}\) (275 kPa) in a CO\(_2\)-powered spray chamber (Model SB5–66, DeVries Manufacturing. Route 1 Box 184, Hollandale, MN 56045). Visual glyphosate injury was assessed 14 days after application by estimating the percentage foliage damage of treated plants compared with the \(dH_2O\)-treated controls; dead plants received a 100% score while asymptomatic plants received 0%. Typical \emph{A. tuberculatus} injury symptoms to glyphosate included plant stunting, leaf chlorosis and meristem necrosis. At evaluation, plant height was also measured by recording the distance from the soil surface to the base of the plant apex. Furthermore, biomass was assessed by cutting plants at the soil surface, drying the tissue at 70°C for 48 h, and determining the weight of individual plant samples. Endogenous shikimic acid quantification was performed on the dry plant apices (Section 2.3.3).

#### 2.3.3 Endogenous shikimic acid determination

Dry apex samples (0.2 g) were ground in liquid nitrogen with a mortar and pestle and were then moistened with 0.25 M hydrochloric acid at a 1:20 (w:v) proportion. Cells were disrupted by sonication (2210 Ultrasonicator. Branson Ultrasonic Corporation. 9999 Markham Road, PO Box 150, Markham, ONT. Canada L3P 3J6) at 25°C for 30 min and the solution and plant debris were separated by centrifugation at 20,000 \(\times\) g for 15 min. The supernatant (5–10 \(\mu\)l) was then used directly for shikimic acid determination, by mixing with \(dH_2O\) (240 \(\mu\)l) and 250 \(\mu\)l of an aqueous solution of periodic acid (5 g liter\(^{-1}\)) and sodium metaperiodate (5 g liter\(^{-1}\)). The mixture was then incubated at 37°C for 30–45 min, quenched with a mixture of aqueous sodium hydroxide (1.0 M; 300 \(\mu\)l) and aqueous sodium...
sulfite (0.056 M; 200 µl), and absorbance detected at 382 nm in a Lambda 18 UV/Vis Spectrometer (Perkin–Elmer, 710 Bridgeport Avenue, Shelton, CT 06484–4750). Apex samples were assayed in duplicate and standard curves constructed with shikimic acid (Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103–2530) at a range of 1–60 µmol ml⁻¹. The shikimic acid chromophore had a molar absorptivity (ε382) of 4.76 × 10⁴ liter mol⁻¹ cm⁻¹ at pH 11–12.

### 2.4 Asexual *Amaranthus tuberculatus* propagation

An asexual propagation method (cloning) was developed in an attempt to perpetuate specific *A. tuberculatus* genotypes, permit non-destructive assessment of glyphosate efficacy (Section 2.5), and facilitate seed increase for recurrent selection purposes (Section 2.6). The method comprised defoliating *A. tuberculatus* branches in active vegetative growth, cutting branches at the base insertion with the stem, and treating the abaxial end of branches with Rootone® (TechPac, LLC. PO Box 24830. Lexington, KY 40504). The branch propagules were then planted in vermiculite and allowed to develop in a growth cabinet (Model CMP–3244, Conviron, 222 South 5th Street, Pembina, ND 58271) set at 16h of 1100 µmol m⁻² s⁻¹ PPFD and approximately 95% RH. Adventitious roots developed from propagules within one to four weeks after planting. Propagules were then transplanted to an autoclaved soil-mix and fertilized weekly for one month. Note that the long photoperiod and frequent fertilization instigated the re-differentiation of floral *A. tuberculatus* meristems back to vegetative reproduction.

### 2.5 Non-destructive plant assay

Branches from asexually propagated *A. tuberculatus* plants (clones) were cut at the base insertion with the stems and placed in 13 × 100 mm culture tubes with Hydro-grow® hydronic solution (Hummert International. 4500 Earth City Expressway, Earth City, MO 63045–1329). The branch cuttings were then sprayed with 0.85% glyphosate AE ha⁻¹ and placed under a 73% PAK shade fabric (Hummert International) with natural light supplemented to 16 h of 600 µmol m⁻² s⁻¹ PPFD for one week; the Hydro-grow® solution was replenished as needed. Glyphosate efficacy was estimated visually (Section 2.3.2) and by measuring the accumulation of shikimic acid in the leaves of treated branches (Section 2.3.3). Susceptible phenotypes accumulated at least twice the amount of shikimic acid of resistant phenotypes. The non-destructive plant assay therefore permitted assessment of glyphosate efficacy in selected phenotypes, without compromising the survival of cloned plants.

### 2.6 Selection of P₀ –EV

#### 2.6.1 Baseline sensitivity monitoring

The natural variation to glyphosate between samples of the same population was estimated by assessing glyphosate efficacy in the 53 P₀ –EV maternal lines with the seedling dose response (Section 2.3.1). Glyphosate resistance was defined as the reduction in susceptibility beyond that estimated in the natural variation of the baseline sensitivity for the population. Maternal lines were therefore considered resistant if the glyphosate dose required to reduce *A. tuberculatus* biomass accumulation by 50% (GR₅₀) lay outside the 95% confidence intervals (CI) for the baseline sensitivity across all six *A. tuberculatus* populations evaluated (Sections 2.1; 2.8).

#### 2.6.2 Divergent recurrent selection

Using the P₀ –EV baseline sensitivity data as reference, maternal lines were selected for three generations of recurrent selection in a divergent strategy, thus isolating resistant (R) and susceptible (S) populations (Table 1). Recurrent selection was performed with a modified seedling assay (Section 2.3.1). Six well cell culture clusters were used and approximately 500 seeds per well on two 3.5-cm diameter chromatography paper disks were moistened (1 ml) with the screening glyphosate dose (Table 1). The amount of seed used was estimated by a previous determination that 100 P₀ –EV seeds weighed 20.8 mg. Plates

### Table 1. Summary of the divergent recurrent selection program to isolate glyphosate resistant (R) and susceptible (S) plants within the Everly, IA *Amaranthus tuberculatus* population (P₀ –EV). Identification of R and S plants was performed at the seedling stage (Section 2.6.2)

<table>
<thead>
<tr>
<th>Source material</th>
<th>Selected lines</th>
<th>Estimated seeds selected</th>
<th>Screening dose</th>
<th>Rescued seedlings</th>
<th>Relative frequency</th>
<th>♀♂ ratio at maturity</th>
<th>Relative frequency</th>
<th>z (P &gt; z)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₀ –EV</td>
<td>24</td>
<td>3.54 × 10⁵</td>
<td>32 µM</td>
<td>376</td>
<td>1.06 × 10⁻³</td>
<td>24:29</td>
<td>1.50 × 10⁻⁴</td>
<td>0.69 (0.49)</td>
</tr>
<tr>
<td>P₀ –EV</td>
<td>29</td>
<td>3.84 × 10⁵</td>
<td>3.2 mm</td>
<td>159</td>
<td>4.14 × 10⁻³</td>
<td>7:6</td>
<td>3.38 × 10⁻⁵</td>
<td>0.28 (0.78)</td>
</tr>
<tr>
<td>RS₁ –S</td>
<td>24</td>
<td>2.51 × 10⁵</td>
<td>10 µM</td>
<td>320</td>
<td>1.27 × 10⁻³</td>
<td>16:21</td>
<td>1.47 × 10⁻⁴</td>
<td>0.82 (0.41)</td>
</tr>
<tr>
<td>RS₁ –R</td>
<td>7</td>
<td>1.38 × 10⁵</td>
<td>10 mm</td>
<td>113</td>
<td>8.19 × 10⁻⁴</td>
<td>0:12</td>
<td>8.70 × 10⁻⁵</td>
<td>3.46 (&lt;0.01)</td>
</tr>
<tr>
<td>BC₂₀</td>
<td>7</td>
<td>2.01 × 10⁵</td>
<td>8 mm</td>
<td>121</td>
<td>6.02 × 10⁻⁴</td>
<td>7:11</td>
<td>8.96 × 10⁻⁵</td>
<td>0.94 (0.35)</td>
</tr>
<tr>
<td>RS₂ –S</td>
<td>16</td>
<td>1.73 × 10⁵</td>
<td>10 µM</td>
<td>242</td>
<td>1.40 × 10⁻³</td>
<td>12:15</td>
<td>1.56 × 10⁻⁴</td>
<td>0.58 (0.56)</td>
</tr>
<tr>
<td>RS₂ –R</td>
<td>7</td>
<td>1.37 × 10⁵</td>
<td>8 mm</td>
<td>209</td>
<td>1.53 × 10⁻³</td>
<td>5:18</td>
<td>1.68 × 10⁻⁴</td>
<td>2.71 (&lt;0.01)</td>
</tr>
</tbody>
</table>


ᵇ Based on a mean A *tuberculatus* seed weight of 20.8 mg per 100 seeds.

ᶜ Proportion of confirmed R or S individuals relative to the estimated seed selected.

ᵈ The one-tailed sign test assessed whether the observed sex ratios in selected lines were equally proportioned (1 ♀ : 1 ♂).
were treated as described in Section 2.3.1 and evaluated two weeks after establishment. The R phenotype was classified as *A. tuberculatus* seeds that germinated and the resulting seedlings grew above the baseline glyphosate dose; conversely, the S phenotype comprised those seeds that germinated in the medium, but seedlings developed glyphosate toxicity symptoms below the baseline dose (Table 1). Individuals that met the R or S classifications were rescued from the selection medium, rinsed of superfluous glyphosate with deionized H$_2$O$_2$, and roots treated with Rootone. Seedlings were then transplanted into an autoclaved soil mix in 12-cm diameter pots and grown in a growth cabinet (Section 2.4) at 500 μmol m$^{-2}$ s$^{-1}$ and approximately 80–90% RH; pots were covered with plastic bags to maintain the high RH. Plants that survived these conditions were transferred to the aforementioned greenhouse condition (Section 2.2). At anthesis, R or S plants were intercrossed in clear plastic tents where pollen contamination was less than 0.01%. Finally, seeds from maternal lines were collected, cleaned and conditioned as previously indicated (Sections 2.1; 2.2). At least 12 *A. tuberculatus* plants per recurrent generation were crossed to mitigate the potential for genetic drift and inbreeding depression and sustain enough genetic variability for recurrent selection.

### 2.7 Estimation of the number of expressed EPSPS homologs in plants

The National Center for Biotechnology Information (NCBI) database was used to identify the ten plant species with the largest number of available expressed sequence tags (dbEST). The keyword ‘EPSP synthase’ was used to acquire annotated tentative consensus (TC) sequences from these species from the Eukaryotic Gene Orthologs (EGO) database available at the Institute for Genomic Research (TIGR). To verify that the TC sequences encoded different genes, pairwise nucleotide–nucleotide comparisons of EPSPS orthologous genes within species were conducted with the BLASTN algorithm in the Basic Local Alignment Search Tool (BLAST). Moreover, the BLASTX algorithm was utilized to compare TC sequences with previously characterized EPSPS genes in the GenBank non-redundant database and to identify the highest homologous species.

### 2.8 Statistical analysis

Analyses were performed with the Statistical Analysis Software. Experiments replicated in time were tested for patterns of covariance matrices that satisfied the Huynh–Feldt condition (option PRINTTE), if accepted, $F$–statistics tested within the time effects and related interactions. Dose and rate response experiments repeated once in time were arranged in a randomized complete block (RCB) design with four replications and analyzed by ANOVA (PROC GLM); Fisher’s least significant difference (LSD; $\alpha = 0.05$) test was used to separate means when ANOVA identified significant population or maternal line effects. In addition, biomass data were used to estimate gamma ($\gamma$) and test whether sub-lethal glyphosate concentrations had a stimulatory effect on *A. tuberculatus* development (hormesis). A parameterized Brain–Cousens model was adopted to estimate GR$_{50}$ values if a positive hormetic response was obtained; otherwise, data were analyzed by PROC NLIN following the log–logistic model. For the difference between two estimated GR$_{50}$ values ($\lambda_{50}$), were calculated by reparameterization of the Brain–Cousens model. Visual injury data were transformed to a binomial format utilizing the classifications for R ($\leq 30\%$) and S ($>30\%$) phenotypes and the glyphosate dose that inflicted 50% mortality in the *A. tuberculatus* population (LD$_{50}$) estimated by a modified Newton–Raphson algorithm (PROC PROBIT). The overall data fit to the models was assessed graphically by the distribution of residuals, and statistically by a lack-of-fit (LOF) tests and by pseudo coefficients of determination. Confidence intervals (CI) for means and GR$_{50}$ estimates were calculated with PROC SURVEYMEANS, while the magnitude in variability to glyphosate was calculated by the sensitivity index (SI):

$$SI = \frac{\text{GR}_{50} \text{ of most tolerant maternal line}}{\text{GR}_{50} \text{ of most sensitive maternal line}}$$

Total phenotypic variances ($\sigma^2$) for the components of the ANOVA were estimated with PROC VARCHART using the restricted maximum likelihood (REML) method and a one-tailed sign test was used to assess whether the observed sex ratios in selected populations were equally proportioned 1:1 (♂:♀). The relationship strength between endogenous shikimic acid and the measured plant response parameters was estimated by Spearman’s correlation analysis.

### 3 RESULTS AND DISCUSSION

#### 3.1 Differential *Amaranthus tuberculatus* response to glyphosate

**3.1.1 Natural variation of *Amaranthus tuberculatus* species to glyphosate**

The grain amaranth (P$_0$–GA) was the most susceptible and least variable population to glyphosate (Fig 1). Both *A. tuberculatus* populations from agroecosystems (P$_0$–AM and P$_0$–OG) had a similar median GR$_{50}$ to glyphosate compared with the wild-type *A. tuberculatus* (P$_0$–WT); however, the agroecosystem populations had a greater magnitude (at least 18-fold) in variance to glyphosate as indicated by the SI index (Fig 1). In addition, the herbicide-resistant *A. tuberculatus* populations (P$_0$–CR and P$_0$–MR) had a higher SI index to glyphosate than P$_0$–WT. P$_0$–CR particularly demonstrated a broad native variability to glyphosate (Fig 1). This is relevant as most *A. tuberculatus* populations in the Midwest are

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suspected to be resistant to ALS-inhibiting herbicides\(^3\) and further selection pressure by glyphosate may select for multiple-resistant populations. Combined, these data suggest that *A. tuberculatus* populations from agroecosystems are more variable to glyphosate than pristine populations. Notably, the *A. tuberculatus* population collected from Everly, IA (P\(_0\)–EV) demonstrated the most variability to glyphosate and had a higher SI index (32-fold) than the other evaluated *A. tuberculatus* populations (Fig 1). The median GR\(_{50}\) value for P\(_0\)–EV was 1.85 mM glyphosate, compared with 1.11 mM glyphosate of the P\(_0\)–WT population. The data therefore confirmed that P\(_0\)–EV was inherently variable to glyphosate.

### 3.1.2 Baseline sensitivity monitoring of P\(_0\)–EV

Baseline sensitivity monitoring provided an estimate of the natural variation of P\(_0\)–EV to glyphosate and permitted identification of resistant maternal lines for recurrent selection purposes. The range of sensitivity across P\(_0\)–EV maternal lines was 0.33–10.66 mM glyphosate, with a mean (\(n = 103\)) baseline GR\(_{50}\) of 2.15 mM glyphosate (95% CI = 1.75–2.54) across all six *A. tuberculatus* populations evaluated. Considering our definition of resistance (Section 2.6.1), eleven maternal lines 1, 3, 11, 19, 20, 23, 26, 27, 28, 44 and 47 were identified as resistant to glyphosate (Fig 1). Other researchers have suggested that herbicide-resistant populations comprise those with a 3× difference in sensitivity to the baseline.\(^48\) Under these parameters, only three maternal lines, 3, 23 and 47, were considered resistant to glyphosate (Fig 1).

Characterization of insect natural variation via baseline monitoring has been proposed as an important tool for insect resistance management\(^49\) and similar strategies could be applied to plant systems. Resistance risk assessment through controlled studies, including baseline sensitivity monitoring, is pivotal in developing effective pest resistance management strategies.\(^50\)

### 3.2 *Amaranthus tuberculatus* from Everly, IA is inherently variable to glyphosate

To minimize possible environmental, rate and phenological factors that affect herbicide efficacy in the field, the response of P\(_0\)–EV to glyphosate was assessed at the seedling and whole-plant levels under controlled environments. The pristine P\(_0\)–WT was used as a control since records indicated that the population evolved in a non–agricultural environment without previous exposure to glyphosate (Section 2.1). Most studies that evaluate plant performance to herbicides model the rate response to the log–logistic equation, but this model is insensitive to discontinuous increasing or decreasing trends that may result at sub-lethal herbicide rates.\(^14\) The Brain–Cousens model was proposed as an alternative to the accepted log–logistic model, specifically when discontinuous response trends are observed that may distort proper estimation of the response parameters.\(^45\) Therefore, the response of

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**Figure 1.** Main plot: response of *Amaranthus tuberculatus* from Everly, IA (P\(_0\)–EV) to glyphosate estimated by the dose that reduced plant growth by 50% (GR\(_{50}\)). The 1× and 3× P\(_0\)–EV baseline sensitivity to glyphosate is indicated by the dashed line; 95% upper and lower confidence intervals (CI) of the 1× baseline are depicted by the dotted lines. Insets: natural variability (box plot) and variability magnitude (bar plot) of *Amaranthus* species to glyphosate as estimated by GR\(_{50}\) and sensitivity index (SI; eqn (1)) values. Boxes denote 5% and 95% percentiles; the line represents the median. *A. hypochondriacus × A. hybridus*: P\(_0\)–GA (grain amaranth). *A. tuberculatus*: P\(_0\)–WT (wild-type Paint Creek, OH), P\(_0\)–AM (Ames, IA), P\(_0\)–OG (Ogden, IA), P\(_0\)–CR (cross-resistant Davis City, IA), P\(_0\)–MR (multiple-resistant Fillmore County, NE), P\(_0\)–EV (Everly, IA), RS\(_1\)–EV (P\(_0\)–EV first recurrent), RS\(_2\)–EV (P\(_0\)–EV second recurrent). Numbers in parentheses designate the numbers of observations (\(n\)) in each box estimate. For all graphs, extensions on boxes or bars designate the 95% upper and lower CI associated with the median or estimated GR\(_{50}\) value, respectively.
A tuberculatus at the seedling and whole-plant levels was tested for stimulatory glyphosate effects at sub-lethal doses (hormesis). The response of P0–EV maternal lines to glyphosate was variable (Fig 1). Resistant lines had seedlings survive the 3.2 mM and 10 mM glyphosate doses, while seedlings in susceptible lines demonstrated pronounced injury symptoms at the 0.1 mM glyphosate concentration (data not shown).

Modeling P0–EV and P0–WT radicle length, plant height and biomass data to the parameterized Brain–Cousens equation estimated values that disregarded a putative hormetic response. Therefore, response parameters were calculated based on the log–logistic model (Table 2). The rate response of P0–EV hypocotyl length data, however, resulted in a value (38.47) that did not overlap zero at the 95% CI and thus confirmed hormesis. Estimates of the model fit to the data resulted in LOF tests and $R^2_{(pseudo)}$ coefficients that confirmed adequacy of the model in describing the response of P0–EV and P0–W to glyphosate. Overall, comparisons at the seedling and whole-plant levels provided non-significant $|\lambda_{50}|$ values suggesting that the P0–EV and P0–W populations responded similarly to glyphosate (Table 2). While the P0–EV population tended to germinate and survive higher glyphosate rates than P0–W, the differences were statistically not significant (Fig 2). Nevertheless, P0–EV demonstrated a greater overall $\sigma^2$ for hypocotyl (5.88) and radicle length (2.17) in response to glyphosate compared with either parameter in P0–W ($\sigma^2 < 0.02$). Greater $\sigma^2$ in the response of P0–EV to glyphosate was also observed for biomass and plant height measurements. These results indicated that, while P0–EV and P0–WT responded similarly to glyphosate, individuals within the P0–EV population were more variable to the effects of the herbicide. In addition, results reaffirmed seedling assay (Section 3.1.1) and visual estimates of variability to glyphosate assessed in the Everly, IA field.

Variable responses to glyphosate within Amaranthaceae have been documented, and A tuberculatus and A hybridus were found to be the most tolerant species within the family.22 Reports of incomplete A tuberculatus control with glyphosate in Illinois and Missouri suggested that 10–15% of the individuals within these populations survived 0.84 kg glyphosate AE ha$^{-1}$, compared with less than 2% in a known glyphosate-susceptible population.29 Furthermore, evaluations at 0.21 kg glyphosate AE ha$^{-1}$ identified broad injury ranges of 10–100% within 59 A tuberculatus populations in Illinois.15 Collectively these reports confirm that A tuberculatus in the USA Midwest is inherently variable to glyphosate.

### Table 2. Dose (hypocotyl; radicle) or rate (biomass; height) response summary of the pristine Paint Creek, OH (P0–WT), the parental Everly, IA (P0–EV), and first (RS1) and third (RS3) recurrent Amaranthus tuberculatus populations selected for susceptibility (S) or resistance (R) to glyphosate

| Parameter   | Material | Model          | $GR_{50}$ | CI | LOF | $R^2_{(pseudo)}$ | $|\lambda_{50}|$ | $P$ value |
|-------------|----------|----------------|-----------|----|-----|------------------|----------------|-----------|
| Hypocotyl   | P0–EV    | Brain–Cousens  | 1.56      | 1.12–2.00 | 0.78 | 0.78 | 0.004 | 0.32 |
|             | P0–WT    | Log–logistic   | 1.57      | 1.14–1.99 | 0.34 | 0.84 | 3.918 | 0.33 |
|             | RS1–S    | Log–logistic   | 0.79      | 0.57–1.01 | 0.21 | 0.84 | 8.061 | 0.02 |
|             | RS1–R    | Log–logistic   | 4.71      | 3.19–6.22 | 0.16 | 0.81 | 1.919 | 0.07 |
|             | RS3–S    | Log–logistic   | 0.59      | 0.35–0.84 | 0.76 | 0.88 | 1.664 | 0.28 |
|             | RS3–R    | Log–logistic   | 8.65      | 6.62–10.68 | 0.89 | 0.88 | 1.919 | 0.07 |
| Radicle     | P0–EV    | Log–logistic   | 0.07      | 0.05–0.10 | 0.11 | 0.88 | 1.919 | 0.07 |
|             | P0–WT    | Log–logistic   | 0.27      | 0.19–0.34 | 0.91 | 0.87 | 0.59 | 0.69 |
|             | RS1–S    | Log–logistic   | 0.39      | 0.25–0.54 | 0.11 | 0.88 | 1.919 | 0.07 |
|             | RS1–R    | Brain–Cousens  | 2.31      | 1.81–2.82 | 0.17 | 0.88 | 1.664 | 0.28 |
|             | RS3–S    | Log–logistic   | 0.11      | 0.08–0.15 | 0.52 | 0.88 | 1.664 | 0.28 |
|             | RS3–R    | Log–logistic   | 1.78      | 1.19–2.37 | 0.52 | 0.88 | 1.664 | 0.28 |
| Biomass     | P0–EV    | Log–logistic   | 0.62      | 0.52–0.72 | 0.44 | 0.69 | 0.381 | 0.25 |
|             | P0–WT    | Log–logistic   | 0.24      | 0.15–0.32 | <0.01 | 0.89 | 0.354 | <0.01 |
|             | RS1–S    | Log–logistic   | 0.53      | 0.48–0.59 | 0.75 | 0.44 | 0.624 | <0.01 |
|             | RS1–R    | Log–logistic   | 0.89      | 0.83–0.94 | 0.75 | 0.73 | 0.104 | 0.37 |
|             | RS3–S    | Log–logistic   | 0.29      | 0.14–0.43 | 0.41 | 0.86 | 0.483 | 0.13 |
|             | RS3–R    | Log–logistic   | 0.87      | 0.80–0.94 | 0.82 | 0.76 | 0.321 | 0.54 |
| Height      | P0–EV    | Log–logistic   | 0.47      | 0.39–0.55 | 0.82 | 0.76 | 0.321 | 0.54 |
|             | P0–WT    | Log–logistic   | 0.37      | 0.29–0.44 | 0.82 | 0.76 | 0.321 | 0.54 |
|             | RS1–S    | Log–logistic   | 0.39      | 0.33–0.45 | 0.82 | 0.76 | 0.321 | 0.54 |
|             | RS1–R    | Log–logistic   | 0.87      | 0.80–0.94 | 0.82 | 0.76 | 0.321 | 0.54 |
|             | RS3–S    | Log–logistic   | 0.27      | 0.22–0.32 | 0.82 | 0.76 | 0.321 | 0.54 |
|             | RS3–R    | Log–logistic   | 0.59      | 0.50–0.69 | 0.82 | 0.76 | 0.321 | 0.54 |

| Parameter | Material | Model          | $GR_{50}$ | CI | LOF | $R^2_{(pseudo)}$ | $|\lambda_{50}|$ | $P$ value |
|-----------|----------|----------------|-----------|----|-----|------------------|----------------|-----------|
| $\sigma^2$ |          |                |           |    |     |                  |                |           |
More important is that survival of \textit{Amaranthus tuberculatus} plants with a pre-existing fitness to glyphosate may increase the proportion of resistant individuals within the population and result in reduced herbicide efficacy in the field due to tolerance or evolved resistance.

### 3.3 \(P_0\)-EV seedling recurrent selection

#### 3.3.1 The seedling assay permitted screening of a large \textit{Amaranthus tuberculatus} population

Considering that a variable response to glyphosate was confirmed in \(P_0\)-EV, divergent recurrent selection was implemented to ascertain whether isolation and mating of \textit{Amaranthus tuberculatus} individuals with confirmed resistant or susceptible phenotypes changed the overall response to glyphosate at the population level. Greenhouse estimates suggested that an individual \textit{Amaranthus tuberculatus} plant produced more than 80,000 seeds under ideal conditions. Thus, a seedling assay was developed to cope with the prolific nature of \textit{Amaranthus tuberculatus} and segregate individuals that demonstrated relatively high or low responses to glyphosate. The seedling assay effectively identified individuals at \(10^{-4}\) frequencies within the population which would have otherwise required significant greenhouse resources if selection were conducted at the whole-plant level (Table 1). Since no difference was observed between germinating \textit{Amaranthus tuberculatus} seeds in \(\text{HH}_2\text{O}\) or potassium hydroxide solution (data not shown), survival of plants in the seedling assay was attributed to the effect of glyphosate and not to the potassium hydroxide used to solubilize the herbicide (Section 2.3.1).

#### 3.3.2 Isolation of the first recurrent (RS1) generation

Dose responses were conducted on seeds of the 53 \(P_0\)-EV maternal lines to characterize the inherent glyphosate response within each line (Fig 1). Based on the baseline sensitivity data (Section 3.1.2), 24 maternal lines were identified susceptible to glyphosate and the corresponding 3.54 \(\times\) \(10^5\) seeds were selected for susceptibility at 32 \(\mu\)M glyphosate. Conversely, the remaining 29 maternal lines were classified resistant to glyphosate and an approximate \(3.84 \times 10^5\) seeds were selected for resistance at 3.2 mM glyphosate (Table 1). Seedlings selected at 32 \(\mu\)M glyphosate demonstrated chlorosis; nonetheless, the development of stems and roots was visually not affected by glyphosate. In contrast, seedlings surviving 3.2 mM glyphosate had green healthy tissues but with severely atrophied roots. Treatment of these stunted roots with Rootone\textsuperscript{®} promoted the development of adventitious roots from stems and facilitated the rescue of seedlings selected for resistance to glyphosate. The divergent glyphosate selection of \(P_0\)-EV resulted in the isolation of 376 susceptible and 159 resistant \textit{Amaranthus tuberculatus} seedlings, which comprised the first recurrent selection generation (RS1). RS1 seedling mortality post isolation from the selection medium was 86% and 92% for the susceptible and resistant populations, respectively. The 53 susceptible (RS1-S) and 13 resistant (RS1-R) plants eventually transferred to the greenhouse had sex ratios at anthesis that did not differ statistically from the 1:1 \(\bigodot:\bigotimes\) proportion (Table 1).

#### 3.3.3 Isolation of the second recurrent (RS2) generation

The second recurrent generation (RS2) was selected from 2.51 \(\times\) \(10^5\) seeds for susceptibility at 10 \(\mu\)M glyphosate, while 1.38 \(\times\) \(10^5\) seeds were selected for resistance at 10 mM glyphosate (Table 1). The 113 asymptomatic \textit{Amaranthus tuberculatus} seedlings rescued after 10 days in 10 mM glyphosate developed injuries when transplanted to the growth chamber and 89% eventually perished. Similarly, 88% mortality was estimated in the 320 seedlings selected for susceptibility to glyphosate. At maturity the susceptible (RS2-S) and resistant (RS2-R) populations were comprised of 16:21 (\(\bigodot:\bigotimes\)) and 0:12 (\(\bigotimes:\bigodot\)) plants, respectively.
is interesting that all 12 individuals selected for resistance differentiated into male plants.

These preliminary results suggested that glyphosate may differentially affect the germination of male and female *A. tuberculatus* seeds. Alternatively, it is possible that resistance to glyphosate is inherited concomitantly with maleness in *A. tuberculatus*. Limited information exists regarding the inheritance of sex in *A. tuberculatus*. Crosses between dioecious and monoecious Amaranthaceae provided evidence that dioeciousness was epistatic over monoeciousness. When a monoecious female parent was crossed to a male dioecious plant, the progeny exhibited a 1:1 male : female sex ratio; in the reciprocal cross however, with a dioecious female as the parent, the progeny resulted in 100% female hybrids. This suggested that males may be heterogametic (XY) while females are monogametic (XX), and that sex has a single locus with two alleles, an X and Y loci, that determined plant sex in *A. tuberculatus*. Under this supposition, the gene(s) associated with glyphosate resistance in *A. tuberculatus* may be located in the Y loci in the chromosome.

### 3.3.4 Assessment of the association between maleness and glyphosate resistance in RS2

Ascertaining whether glyphosate instigated differential germination of *A. tuberculatus* was difficult since seeds cannot ordinarily be sexed. Therefore, the putative linkage of maleness and glyphosate resistance was tested by backcrossing RS2–R individuals to RS1–R females, selecting the progeny at 8 mM glyphosate, and inter-crossing the selected plants to generate a new resistant lineage (BCR). Differential sex germination would have resulted in less germination of female seeds in the 8 mM glyphosate media and reduced the overall germination in BCR. Since RS2–S demonstrated a normal 1:1 male : female sex ratio (Table 1) and the combined germination across rates (82%) was not statistically different (LSD0.05 = 6%) from BCR (81%), differential sex germination in the 8 mM glyphosate media was disregarded. This selection resulted in the isolation of 7:11 BCR:BCR plants which demonstrated statistically equal male and female proportions (Table 1). Hence, the putative sex-linkage to glyphosate resistance observed in the RS2–R was attributed to the small sample size in the selected material (n = 12).

### 3.3.5 Isolation of the third recurrent (RS3) generation

Since the offspring of RS1–R resulted in exclusively male plants and thus no progeny were produced, the third recurrent resistant population (RS3–R) was selected at 8 mM glyphosate from the 1.37 × 10^5 seed produced by BCR (Table 1). The third recurrent susceptible population was selected at 10 μM glyphosate from the 1.73 × 10^3 RS2–S seeds. Concurrently, 242 RS3–S and 209 RS1–R seedlings were isolated. Mortality in these selected population ranged from 85 to 90%, thus at maturity RS3–S and RS3–R were comprised of 12:15 and 5:18 plants, respectively (Table 1). Noteworthy, RS3–R had sex ratios that statistically departed from the 1:1 ratio. Therefore, we concluded that glyphosate resistance may be associated with maleness in *A. tuberculatus*. Further investigation is needed to identify the genes and understand the process of sex differentiation in *A. tuberculatus*.

Few reports exist that cite departure from the 1:1 distribution in natural *A. tuberculatus* populations. In isolated native Ohio *A. tuberculatus* populations, female plants can comprise up to 90% of the individuals in a population. Conversely, a tendency for more *A. tuberculatus* males was reported in response to composted swine manure. Spatial segregation of sexes across environmental gradients is well documented in dioecious plant species, such that increased competition and stressful conditions may favor the prevalence of males in a particular time and space. In *A. cannabinus* males may be located in the Y loci in the chromosome.

### 3.4 The fitness of *Amaranthus tuberculatus* to glyphosate increased with selection

Evaluation of RS1–S seeds confirmed that the selected material did not develop well above 1 mM glyphosate, while radicles and hypocotyls of RS1–R seedlings grew in 3.2 mM glyphosate (Fig 3). Furthermore, less than 0.2% of RS1–R seedlings demonstrated minor or no toxicity symptoms at 10 mM glyphosate. Stimulatory responses at sub-lethal glyphosate concentrations were non-significant except for the radicle response of RS1–R (γ = 20.59) (Table 2). Evaluations at the seedling and whole-plant levels based on values indicated that the response of RS1–S and RS1–R differed only when comparing biomass as a function of increasing glyphosate rates (Table 2). This difference accounted for an approximately 1.7-fold increase in resistance to glyphosate (Fig 3). At the population level, 0.55 kg glyphosate AE ha^-1 was required to inflict 50% mortality in the RS1–S compared with 0.78 kg glyphosate AE ha^-1 in the RS1–R (Table 3).

Concurrently, shikimic acid accumulation in the apex tissue of treated RS1 plants correlated positively with the visual herbicide ratings and negatively with plant height or biomass measurements (Table 3). Phenotypic variance comparisons between the parental and RS1 *A. tuberculatus* populations suggested that the recurrent selection process reduced the overall population variability to glyphosate. For example at the
Differential response of *Amaranthus tuberculatus* to glyphosate

Seeding level, the $\sigma^2$ of RS$_1$–R ($<0.27$) and RS$_3$–S ($<1.84$) was less than the hypocotyl (5.88) and radicle length (2.17) response in the P$_0$–EV.

### 3.5 Evident variability to glyphosate even after three cycles of recurrent selection

Consistent with the RS$_1$ response, RS$_3$–S seedlings did not grow above 1 mM glyphosate (Fig 4). Conversely, more radicle and hypocotyl growth was observed at 3.2 mM glyphosate in the RS$_1$–R compared with the RS$_3$–R (Fig 4; Fig 4). Estimates of hormesis were non–significant for the radicle, hypocotyl, biomass and plant height data ($\gamma > 0.23$), therefore the log–logistic model was used to estimate fitness to glyphosate. Comparisons based on GR$_{50}$ values estimated 3-fold and 14-fold resistance increases between RS$_3$–R and RS$_3$–S for the biomass and hypocotyl length responses, respectively (Table 2). Concomitantly, an approximate 2.6-fold resistance increase was estimated when comparing mortality as a function of glyphosate concentration at the population level and endogenous shikimic acid correlated with the expected *A. tuberculatus* response (Table 3). The fact that RS$_1$ demonstrated an enhanced fitness to glyphosate compared with RS$_1$ and in turn RS$_3$ was better fit to glyphosate than P$_0$–EV, suggested that the response of RS$_3$ to glyphosate was at least in part genetic and heritable. RS$_1$ and RS$_3$ also had less $\sigma^2$ in response to glyphosate compared with P$_0$–EV, which suggested that the recurrent selection procedure reduced the inherent variability to glyphosate observed in P$_0$–EV. However, fewer differences in $\sigma^2$ were observed when comparing the RS$_1$ and RS$_3$ populations. For instance, the $\sigma^2$ of the hypocotyl length response to glyphosate was 0.27 for RS$_1$–R and 1.84 for RS$_3$–S, which was similar to that of 0.27 for RS$_3$–R and 1.91 RS$_3$–S. Biomass response to glyphosate had $\sigma^2$ of 0.01, 0.02, 0.50 and 0.24 for RS$_1$–R, RS$_3$–S, RS$_1$–R and RS$_3$–S, respectively.

Recently, experiments to ascertain whether recurrent selection reduced *A. tuberculatus* sensitivity to glyphosate were initiated with several Midwest populations, including P$_0$–EV.\textsuperscript{56} Conversely to our approach

### Table 3. Association strength between shikimic acid concentration, plant height, biomass and visual injury, and estimate of glyphosate lethality to *Amaranthus tuberculatus*. Numbers in parentheses indicate the probability of the calculated Spearman’s correlation coefficient ($r^2$)

<table>
<thead>
<tr>
<th>Material\textsuperscript{a}</th>
<th>Plant height</th>
<th>Biomass</th>
<th>Visual injury</th>
<th>LD$_{50}$\textsuperscript{c}</th>
<th>FL\textsuperscript{d}</th>
<th>R/S\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>P$_0$–EV</td>
<td>DNP</td>
<td>DNP</td>
<td>DNP</td>
<td>0.57</td>
<td>0.43–0.69</td>
<td>2.7</td>
</tr>
<tr>
<td>P$_0$–WT</td>
<td>–0.65 (&lt;0.01)</td>
<td>–0.74 (&lt;0.01)</td>
<td>0.74 (&lt;0.01)</td>
<td>0.21</td>
<td>0.11–0.29</td>
<td></td>
</tr>
<tr>
<td>RS$_1$–S</td>
<td>–0.79 (&lt;0.01)</td>
<td>–0.83 (&lt;0.01)</td>
<td>0.85 (&lt;0.01)</td>
<td>0.55</td>
<td>0.38–0.68</td>
<td>1.4</td>
</tr>
<tr>
<td>RS$_1$–R</td>
<td>–0.85 (&lt;0.01)</td>
<td>–0.79 (&lt;0.01)</td>
<td>0.91 (&lt;0.01)</td>
<td>0.78</td>
<td>0.61–0.96</td>
<td></td>
</tr>
<tr>
<td>RS$_3$–S</td>
<td>–0.60 (&lt;0.01)</td>
<td>–0.62 (&lt;0.01)</td>
<td>0.70 (&lt;0.01)</td>
<td>0.32</td>
<td>0.22–0.43</td>
<td>2.6</td>
</tr>
<tr>
<td>RS$_3$–R</td>
<td>–0.43 (&lt;0.01)</td>
<td>–0.90 (&lt;0.01)</td>
<td>0.61 (&lt;0.01)</td>
<td>0.83</td>
<td>0.87–0.99</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}P$_0$–EV, parental Everly, IA; P$_0$–WT, pristine Paint Creek, OH; RS$_1$–S, first susceptible recurrent generation; RS$_1$–R, first resistant recurrent generation; RS$_3$–S, third susceptible recurrent generation; RS$_3$–R, third resistant recurrent generation.

\textsuperscript{b}Determination not performed.

\textsuperscript{c}Glyphosate rate in kg AE ha$^{-1}$ that inflicted 50% mortality in the *A. tuberculatus* population.

\textsuperscript{d}95% lower and upper fiducial limits (FL) of the calculated LD$_{50}$ estimated by PROC PROBIT.

\textsuperscript{e}Calculated by dividing the LD$_{50}$ of the resistant material by that of the susceptible material.

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\textbf{Figure 3.} Upper: *Amaranthus tuberculatus* seedling dose responses of the first recurrent material selected at (RS$_1$–R; gray bars; RS$_1$–S; white bars; O) 3.2 mM glyphosate and (RS$_3$–S; white bars; O) 32 $\mu$m glyphosate. Inset: *A. tuberculatus* radicle length (circles) and germination (bars) as affected by glyphosate. Individual points or bars in the graph represent the mean of four separate experiments, each containing three replications ($n = 12$). Lower: *Amaranthus tuberculatus* whole-plant rate response of (gray bars; RS$_1$–R and (white bars; O) RS$_1$–S. Inset: *A. tuberculatus* height (circles) and mortality (bars) associated with glyphosate rates. Data points represent the mean of two experiments with four replications ($n = 8$). Least significant difference at the $P < 0.05$ level (LSD = 0.05) was estimated by Fisher’s test. Extensions on circles or bars designate the standard error associated with individual means (ns).
of selecting for resistance at the seedling level, recurrent selection in this investigation was performed on whole-plants by treating 1000 individuals with 0.22–0.33 kg glyphosate AE ha\(^{-1}\) and allowing 100 surviving plants to intercross. Preliminary data suggested a reduction in glyphosate sensitivity after three cycles of selection and that quantitative genetics may be implicated in the response to glyphosate. In addition, the authors purported an association between plant architecture and the observed differential response of *A. tuberculatus* to glyphosate.\(^{56}\)

While the variability to glyphosate was reduced in RS\(_1\) and RS\(_3\), segregation for glyphosate resistance was still evident in the S\(_1\)–R and S\(_1\)–S populations. This observed persistent variability to glyphosate could be attributed to the several factors, including the reproductive nature of *A. tuberculatus* and the genetics of glyphosate resistance. Genetic diversity is an inherent characteristic among species of Amaranthaceae.\(^{57,58}\)

Recently, *A. radis* JD Sauer and *A. tuberculatus* were grouped in a single-polymorphic species, where populations with more genetic diversity occur in Ohio.\(^{52}\) Because *A. tuberculatus* is an obligate-allogamous species, genetic recombination during mitosis could explain the phenotypic variability observed in RS\(_1\) and RS\(_3\). Alternatively, glyphosate resistance in *A. tuberculatus* could be determined by a polygenic event. Under this hypothesis, alleles associated with resistance could recombine during mitosis, produce different allele combinations, and generate a progeny with different levels of resistance as observed in the segregant RS\(_3\). While glyphosate resistance in *Lolium rigidum* Gaudin and *Conyza canadensis* (L) Cronq is monofactorial,\(^{59,60}\) at least two resistance mechanisms have been reported in *Eleusine indica* (L) Gaertner with potentially independent alleles.\(^{61,62}\) Glyphosate resistance in *Ceratopteris richardii* (L) Brongn is governed by two independent loci that are inherited in an incompletely dominant and recessive fashion.\(^{63}\) Moreover, quantitative genetics of glyphosate tolerance has been reported in other plant species.\(^{64,65}\) These cases exemplify that enhanced fitness to glyphosate can be mediated by monogenic and polygenic events in plants.

Finally, variation to glyphosate could be explained by the differential expression of EPSPS homologs at various *A. tuberculatus* phenological stages. The EGO database was used to identify EPSPS orthologs across plant species (Section 2.7). Individual TC numbers comprise the compiled annotated consensus sequence of individual expressed sequence tags (ESTs) and thus potentially represent a unique gene. Therefore, comparison of EPSPS TCs within a species allowed for estimation of the minimum number of expressed EPSPS homologs in the species (Table 4). Homologs in *Pinus* spp and *S. bicolor* failed to bear any similarity, yet the TC sequences demonstrated homology to previously characterized EPSPS genes, thus suggesting that the TCs possibly represented opposite ends of the same gene. Overall, the analysis confirmed that plant genomes contained 1–3 copies of EPSPS; nonetheless, additional EPSPS homologs may be identified as plant genome projects are further sequenced. The data also suggested that most EPSPS homologs were tissue specific. For example, *M. truncatula* TC77645 was composed of nine ESTs, six of which were differentially expressed in roots. Conversely, eight of the 13 ESTs comprising TC77644 were expressed in above-ground organs. It has been suggested that the expression of EPSPS isozymes could represent an additional level of regulation of the shikimic acid pathway.\(^{66,67}\)

### 3.6 Several generations of selection may be required for resistance to evolve in the field

Collectively the data herein presented confirmed that variability in response to glyphosate exists naturally in
Differential response of *Amaranthus tuberculatus* to glyphosate
growing season

**Figure 5.** Glyphosate use in glyphosate-resistant soybean within USA Midwest states (IL, IN, IA, KS, MI, MN, MO, NE, OH, WI). Gray bars: producer average number of applications per year; dark gray bars: average kg AE glyphosate per application per ha. Inset: Total kg AE glyphosate applied per ha per year among Midwest states; the solid line represents the expected total use as estimated by the four-parameter sigmoidal model. Averages were estimated from the number of states as indicated in parenthesis above bars in the main plot. Extensions on circles and bars designate the 95% confidence interval (CI) associated with the average. Data were compiled from the US Department of Agriculture (USDA) National Agricultural Statistics Service (NASS) database.4

**Table 4.** Frequency of 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS; EC 2.5.1.19) homologs in ten plant species with the largest number of expressed sequence tags (dbEST)

<table>
<thead>
<tr>
<th>Species</th>
<th>Putative EPSPS homologs</th>
<th>TC numbers</th>
<th>BLASTN 2 sequences (%)</th>
<th>E value</th>
<th>Species</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>2</td>
<td>TC253016</td>
<td>94</td>
<td>0.0</td>
<td><em>Arabidopsis thaliana</em></td>
<td>NM_130093</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>2</td>
<td>TC272122</td>
<td>93</td>
<td>0.0</td>
<td><em>Sarracenia purpurea</em></td>
<td>AY545663</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>1</td>
<td>TC140189</td>
<td>—</td>
<td>8e−83</td>
<td><em>Vitis vinifera</em></td>
<td>AY150520</td>
</tr>
<tr>
<td><em>Medicago truncatula</em></td>
<td>2</td>
<td>TC77646</td>
<td>91</td>
<td>0.0</td>
<td><em>Medicago truncatula</em></td>
<td>AC119419</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>1</td>
<td>TC250561</td>
<td>—</td>
<td>0.0</td>
<td><em>Oryza sativa</em></td>
<td>AB016765</td>
</tr>
<tr>
<td><em>Pinus spp.</em></td>
<td>1–2</td>
<td>TC53076</td>
<td>NSSF(^a)</td>
<td>0.0</td>
<td><em>Petunia hybrida</em></td>
<td>M21084</td>
</tr>
<tr>
<td><em>Saccharum officinarum</em></td>
<td>2</td>
<td>TC66045</td>
<td>95</td>
<td>3e−12</td>
<td><em>Abutilon theophrasti</em></td>
<td>BX15732</td>
</tr>
<tr>
<td><em>Sorghum bicolor</em></td>
<td>1–3</td>
<td>TC53450</td>
<td>NSSF(^a)</td>
<td>0.0</td>
<td><em>Sorghum bicolor</em></td>
<td>X63683</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>1</td>
<td>TC192224</td>
<td>—</td>
<td>0.0</td>
<td><em>Triticum aestivum</em></td>
<td>AJ417034</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>1</td>
<td>TC270442</td>
<td>—</td>
<td>0.0</td>
<td><em>Triticum aestivum</em></td>
<td>AY106729</td>
</tr>
</tbody>
</table>

\(^a\) Additional EPSPS homologs may be elucidated as plant genome projects are further sequenced.
\(^b\) Annotated tentative consensus sequence (TC); available at TIGR database (http://www.tigr.org/).
\(^c\) Used to determine if multiple homologs identified from the same species truly represented different EPSPS genes.
\(^d\) Homology of TC sequences to sequences in the GenBank non–redundant database was estimated with the BLASTX algorithm.
\(^e\) No significant similarity was found.

*A. tuberculatus* populations, and that the potential for the evolution of glyphosate resistance is significant. The frequency of resistant individuals within P0–EV was extremely low (<4.0 × 10^-4) and thus required high selection pressure to isolate resistant individuals (Table 1). However, selection pressure under field...
conditions may be less stringent, since survival of *A. tuberculatus* plants depends on several biotic and abiotic factors. Therefore, several generations of glyphosate selection may be necessary to isolate *A. tuberculatus* genotypes with a stable trait for resistance in the field. At least 8–15 years of glyphosate selection pressure in the field was necessary for resistance to evolve in other weed species.3 This suggested that (1) the frequency of resistant individuals within the population is extremely low, (2) several genes are involved in the trait and recombination plus selection may be required for the accumulation of resistant alleles, or that (3) the trait for resistance imposed a physiological penalty on plants. Evidence for the later hypothesis was reported in petunia (*Petunia* sp), where a single point mutation of glycyl101 to alanyl encoded for an EPSPS with increased apparent dissociation constant (*K*ₐ) for glyphosate and apparent Michaelis–Menten constant (*Kₘ*) for PEP.68 Models predicted that the low frequency of evolved glyphosate resistance in the field was associated with the application timing of the herbicide, the frequency of resistant individuals within the population, the dominance of the resistance allele(s) and the fitness of the resistant weed biotype.69

At present, glyphosate resistance has only been confirmed in *Ambrosia artemisiifolia* L and *C. canadensis* in Midwest cropping systems.18 However, increases in the area planted with glyphosate-resistant crops may enhance the selection pressure and lead to the evolution of additional resistant biotypes.3 Historic estimates of pesticide use allude to the fact that weed populations have increased fitness to glyphosate since the introduction of glyphosate-resistant crops in 1996. Midwest farmers on average applied 0.64 kg glyphosate AE ha⁻¹ on a single application to manage weeds in 1995 (Fig 5). By 2002, the dose and frequency had increased to an average 0.91 kg glyphosate AE ha⁻¹ and 1.3 applications year⁻¹, respectively. This translated to an average increase from 0.65 to 1.2 kg glyphosate AE ha⁻¹ year⁻¹. This change is in part due to the different glyphosate formulations and recommendations available to farmers at the time. In 1996, the commercial formulation (Roundup Ultra®) recommended rates of 0.62 kg AE ha⁻¹ for control of most annual weeds, including 15-cm tall *A. tuberculatus*. Both commercial formulations (Roundup UltraMAX® and WeatherMAX®) available to farmers at present recommend 0.84–0.87 kg AE ha⁻¹ for 15-cm tall *A. tuberculatus*. Regardless, anecdotal accounts of increased tolerance in *C. communis, A. theophrasti, C. album* and other species have been reported by USA farmers in the past few years.3 More research is needed to understand the impact of continuous glyphosate use on the dynamics of weed populations and the evolution of resistance. This information may be relevant in developing effective weed-control strategies and maintaining the productivity of current USA cropping systems.

**ACKNOWLEDGMENTS**

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