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Horseweed with Reduced Susceptibility to Glyphosate Found in the Czech Republic

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The physiological and molecular basis of apparent resistance to glyphosate in horseweed (*Conyza canadensis* L. Cronq.) plants that had survived being sprayed with the herbicide at Prague-Bubny railway station in the Czech Republic was investigated. For the sake of comparison, plants expected to be susceptible were collected in areas where no herbicides had been used. Plants of both sets were treated, at the rosette stage (10−25 leaves, diameter of 3−5 cm), with herbicide at the rate recommended for use in the Czech Republic to control horseweed (960 g of glyphosate-IPA/ha; Roundup Klasik, Monsanto, 480 g of glyphosate-IPA ae L^{-1}). Phytotoxic symptoms of the treated plants varied substantially, both between and within these sets of plants. Leaves of susceptible (S) plants wilted and turned yellow, and the plants subsequently died; leaves of plants with reduced susceptibility (RS) remained green, or new leaves were created in the center of their rosettes a few weeks after glyphosate application. There were no significant differences in the accumulation of shikimate between S and RS plants 3 days after treatment (DAT). However, the time course of changes in shikimic acid contents differed between the two biotypes; from 3 to 10 DAT, they decreased more than 4-fold in RS plants, while in S plants, they increased (3-fold, on average) from 3 to 7 DAT. A conserved region of the *epsps* gene, in which mutations are known to confer resistance in several plant species, was amplified from samples of both S and RS plants and sequenced, but no changes in the encoded amino acid sequence were found, indicating that mutations at another *epsps* site were responsible for the observed resistance, or that the mechanism may be at least partially non-target-based. Our results suggest that the reduced susceptibility to glyphosate may be due to impaired herbicide translocation, as previously found in studies of horseweed in the United States.

KEYWORDS: *Conyza canadensis*; 5-enolpyruvylshikimate-3-phosphate synthase; *epsps* gene; glyphosate-IPA; susceptibility; shikimate variability

INTRODUCTION

In plants, the shikimic acid pathway is an important biochemical pathway that is responsible for the biosynthesis of aromatic amino acids and several secondary compounds derived from these amino acids. Glyphosate, one of the most widely used nonselective herbicides, inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in this pathway, by binding to a site on the enzyme and preventing binding of the substrate phosphoenolpyruvate (*1*). The widespread use of glyphosate has exerted selection pressure on various species of weeds, and many have evolved resistance, including horseweed. Resistance to glyphosate in horseweed was first described by VanGessel in the United States in 2000 (*2*), and resistant biotypes have been discovered in 16 states of the United States, Brazil, China, and Spain to date (*3*). The widescale occurrence of horseweed in glyphosate-resistant crops after repeated long-term application of herbicides containing glyphosate has become a serious problem.

Horseweed occurs widely across the Czech Republic from the lowlands to piedmont areas and is a common component of the weed flora along Czech railways (*4*). Herbicides containing the active ingredient glyphosate-IPA were applied at railway sites in the Czech Republic between 2000 and 2003 at doses of 3840 g/ha (Roundup Klasik, Monsanto) and since 2004 at doses of 3642−4856 g/ha (Roundup Rapid, Monsanto) in volumes equivalent to 192 L/ha, twice a year. Individual horseweed plants, suspected of having lower susceptibility or even resistance to glyphosate, have been observed since 2005 at railway stations at Prague-Bubny and Prague-Libeň, both of which are long-term monitoring sites. We have also detected triazine-resistant horseweed plants at Czech railway sites, after almost application of herbicides containing the active ingredient simazine for 20 years (*5*).

Glyphosate resistance in goosegrass [*Eleusine indica* (L.) Gaertn.] (*6*), Italian ryegrass (*Lolium multiflorum* Lam.) (*7*), and

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annual ryegrass (*Lolium rigidum* Gaudin) (*8*) has been shown to be due to a target site mechanism; resistant plants carry a mutation of the *epsps* gene at amino acid position 106. Important factors in the survival of resistant horseweed biotypes include increases in EPSPS mRNA levels, a reduced level of translocation of glyphosate to crown and root tissues (*9*, *10*), and the production of new branches after glyphosate treatment (*11*).Glyphosate resistance is also strictly dependent on the growth stage of the plants and may depend on the characteristics of the biotype (*9*, *10*, *12*−*15*).

This is the first report of the occurrence of horseweed plants with reduced susceptibility to glyphosate in the Czech Republic. The plants were collected along the railway tracks and other sites near the Prague-Bubny railway station building after repeated application of glyphosate. This study was undertaken to characterize the reduced susceptibility of these horseweed plants to glyphosate, focusing on the accumulation of shikimic acid and the sequence of the *epsps* gene in G_1 offspring of glyphosate-treated, collected plants.

MATERIALS AND METHODS

Seed Sources. In total, 20 horseweed plants at various developmental stages suspected of having reduced, i.e., lower than usual, susceptibility to glyphosate (designated RS plants) were collected 8 weeks after treatment with glyphosate-IPA (4856 g/ha; Roundup Rapid, Monsanto, 607 g ai L^{-1}) from sites along the railway tracks and other sites within 20−40 m of the Prague-Bubny railway station building in the Czech Republic (50°6'7.904"N, 14°26'20.132"E) in 2006 (generation G_0). The plants were transferred to the greenhouse, and 2 weeks after being established in pots, they were treated with 960 g of glyphosate-IPA/ha (Roundup Klasik, 480 g ai L^{-1}) in 200 L of water/ha. Survivors (generation G_0) were grown to maturity, and their inflorescences were bagged before flowering to avoid cross pollination. The collected seeds were stored at −18 °C and showed very good germinability (90 ± 5%). Seeds of plants that were expected to be susceptible, and provided controls (designated S plants), were collected in fields of the Crop Research Institute in Prague-Ruzyně (50°5'12.137"N, 14°18'11.053"E) where no herbicides had been used.

Cultivation of Plants and Glyphosate Treatment. Seventy seeds obtained from the G_0 plants following the glyphosate treatment mentioned above were sown in plastic pots. The resulting plants (G_1 generation) were transplanted individually, at the 2−4-leaf stage, into plastic pots (7 cm×7 cm) containing commercial soil substrate at pH 6.8. Plants were grown under controlled conditions (12 h−12 h day−night regime, light intensity of 130 μmol m^{-2} s^{-1}, temperature of 20−24 °C, relative humidity of 65−80%) and watered daily. At the rosette stage (10−25 leaves, diameter of 3−5 cm), 50 of the plants were sprayed with 960 g of glyphosate-IPA ai ha^{-1} (Roundup Klasik, Monsanto, 480 g ai L^{-1}) using an AVIKO 5 chamber sprayer (prototype fy AVICO s.r.o., Na Hutmance 2, 158 00 Praha 5, Czech Republic) at 200 L/ha, which enabled complete foliar coverage. Most of the plants died. The others wilted and turned moderately yellow. After a few weeks, the survivors produced new leaves in the center of the rosette or grew a short branched stem and were considered to be plants with reduced susceptibility to glyphosate (RS). After recovery, these plants were used for the shikimic acid determinations and EPSP synthase gene sequencing analysis described below. Each pot containing a single horseweed plant was considered to be an individual experimental unit, as described by Mueller et al. (*12*).

Susceptible (S) plants, originating from seeds collected from the fields that had not been sprayed with glyphosate, were also cultivated and treated with glyphosate as described above. Leaves were harvested from each of eight S and eight RS plants prior to spraying (day 0) and 3, 7, and 10 days after treatment (DAT) with glyphosate, for the determination of dry weight (after drying to a constant weight at 75 °C), determination of shikimic acid content, and *epsps* gene sequence analysis (see below). Leaf samples were stored at −80 °C before analysis. In addition, the total plant decline was visually evaluated 14 DAT.

Extraction of Shikimic Acid. An extraction procedure similar to one previously reported by Mueller et al. (*12*) was used for the shikimate analysis. Briefly, 20−25 leaves from each sampled plant were ground in liquid nitrogen using a mortar and pestle; 5 mL of 1 M HCl was added per gram of tissue, and the samples were agitated on an orbital shaker at 1500 rpm for 24 h. The extracts were filtered through Whatman No. 1 filter paper, and their pH was adjusted to 3.0−3.2 using saturated NaOH and/or 0.01 M NaOH, as required. The volumes of the extracts were then reduced in a rotary evaporator under reduced pressure at 40 °C; they were centrifuged at 10000*g* for 5 min, and the volumes of the supernatants were measured and adjusted to 2 mL using 0.001 M HCl. Finally, the samples were filtered through nylon syringe filters (13 mm, 0.2 μm; Varian, Palo Alto, CA), and the level of shikimic acid was measured in 5 or 10 μL samples by high-performance liquid chromatography (HPLC), as described below.

Shikimic Acid Analysis. HPLC analysis was performed using a Dionex chromatographic system equipped with a P680 HPLC pump, an ASI-100 automated sample injector, a TCC-100 thermostated column compartment, a PDA-100 photodiode array detector, a Chromeleon software data system for collecting, integrating, and analyzing the chromatographic data (Dionex Corp., Sunnyvale, CA), and a Waters Spherisorb ODS-2 (250 mm × 4.6 mm, 5 μm particles) column (Supelco, Ballefonte, PA). The mobile phase used in the shikimic acid determinations was 0.01 M sulfuric acid at a flow rate of 0.5 mL/min at 45 °C. Portions (5 or 10 μL) of the samples were injected; the eluate was monitored at 210 nm, and the column was washed with 80% methanol for 10 min and then allowed to equilibrate in 0.01 M H_2SO_4 for 20 min between samples. Shikimic acid was identified in the eluates by comparing its retention time and UV spectrum with those of a pure (99%) standard (Sigma-Aldrich, St. Louis, MO).

DNA Extraction. DNA was extracted from leaf tissues of single plants using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR). In both bacteria and plants, resistance to glyphosate can be conferred by a mutation in the conserved region of the gene encoding EPSP synthase (*6*, *16*, *17*). Primers were designed to amplify this conserved region based on EPSP synthase sequences of *Conyza canadensis* in GenBank (accession numbers ay545666, ay545667, and ay545668) and used in 25 μL PCR reaction mixtures containing 10 ng of DNA, each primer at 0.5 μM, each dNTP at 0.2 mM, 1× thermophilic DNA polymerase buffer (Promega, Madison, WI), and 1 unit of Taq DNA polymerase (Promega). Amplification was conducted using an automated DNA thermal cycler (PTC-200, MJ Research, Waltham, MA) and 30 cycles consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 61 °C, and elongation for 2 min at 72 °C, with a 5 min final extension at 72 °C. PCR amplification products were electrophoretically separated in a 1.7% agarose gel, stained with ethidium bromide, and visualized under UV light. The band of interest was excised from the gel and purified using a QIAquick gel extraction kit (QIAGEN) following the manufacturer's instructions. PCR products were sequenced (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic) to obtain sequences of both S and RS plants.

Statistical Analysis. The significance of differences in shikimic acid content between individual plants and between S and RS plants and the significance of the effects of the level of sensitivity and day of evaluation on their shikimate contents were tested by analysis of variance (ANOVA/MANOVA) with subsequent post hoc comparisons (Tukey HSD test). Statistica 7.0 CZ was used for all of these analyses.

RESULTS

Visual Evaluation of Glyphosate-Treated Plants. There were significant visual differences between LS and S plants following glyphosate treatment; 14 DAT, LS plants of the G_1 generation were wilted and had turned moderately yellow, but most of the S plants were already dead by 6−10 DAT. Marked differences in the responses of plants to glyphosate application were also observed among individuals of each population.

Accumulation of Shikimic Acid in Glyphosate-Treated Plants. Shikimic acid levels were determined in the leaves of individual horseweed plants of both populations from day 0, just prior to the glyphosate application, until 10 DAT. The average background

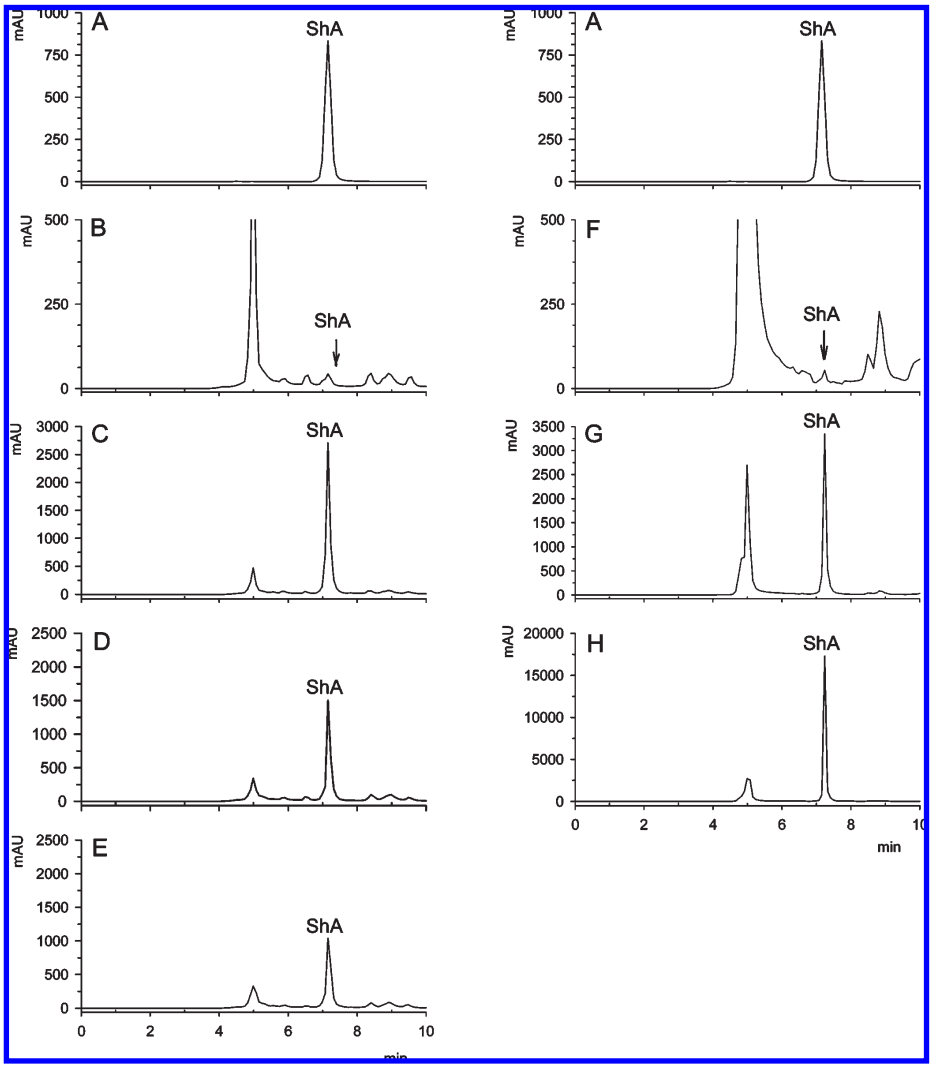

Figure 1. High-performance liquid chromatograms of shikimic acid standard solution (**A**), shikimic acid extracted from leaves of RS horseweed plants 0 (**B**), 3 (**C**), 7 (**D**), and 10 DAT (**E**), and shikimic acid extracted from leaves of S horseweed plants 0 (**F**), 3 (**G**), and 7 DAT (**H**). Each profile represents an equivalent amount of extract, normalized per 5 mg of tissue. ShA denotes shikimic acid. Please note the different scales of the *y*-axes.

level of shikimate on day 0 observed in untreated RS plants was slightly higher than that of untreated S horseweed plants. Three DAT, there were no significant differences in measured shikimate levels between RS and S plants. However, the time courses of subsequent changes in shikimic acid concentrations differed substantially between the two horseweed biotypes: in RS plants, it decreased more than 4-fold from 3 to 10 DAT, but it increased 3-fold on average in S plants from 3 to 7 DAT (**Figure 1** and **Table 1**). Seven DAT, the level of shikimate was more than 10 times higher in S plants than in RS plants. Marked alterations in the level of shikimic acid were also observed among the individual plants of both populations after glyphosate treatment (**Figure 2**). In one S plant (S1), the shikimate concentration increased more than 4-fold from 3 to 7 DAT, from 1846 to 8107 μg/g of fresh weight, while another S plant (S2) that had accumulated extremely high levels of shikimate 3 DAT (3870 μg/g of fresh weight) died 6 DAT (**Figure 2**). However, despite the variability between individual plants, there were consistent differences in time trends of shikimate concentrations between the two biotypes. **Table 1** presents mean values of shikimic acid contents detected in both horseweed biotypes obtained in three independent experiments (means ± standard errors, obtained from two to three measurements of extracts from eight plants of each biotype). These data differ from those in **Figure 2**, where mean values (±standard error) obtained from three measurements of extracts from two selected plants of each biotype are presented to illustrate the great variability in accumulation of shikimic acid between plants. A 286 bp fragment of the *epsps* gene containing codon 106 of each of seven apparently glyphosate-resistant and four susceptible horseweed plants was sequenced. Comparison of their sequences showed no polymorphisms among RS and S plants (**Figure 3**), indicating that mutations at another *epsps* site were responsible for the observed resistance in horseweed and/or that the mechanism may be at least partially non-target-based.

DISCUSSION

Glyphosate blocks the biosynthesis of proteins and synthesis of all cinnamate derivatives (flavonoid substances, lignin, etc.) through the inhibition of EPSP synthase, generally leading to the accumulation of high levels of shikimate, benzoic acids, and benzoic acid derivatives (*18*−*21*), inhibition of plant growth, and ultimately death (*22*−*24*). A considerable decrease in the quantity of cinnamic acid derivatives and the presence of a broader spectrum of hydroxybenzoic acids in glyphosate-treated alfalfa cell lines, versus untreated ones, suggest the activation of an alternative pathway not regulated by phenylalanine ammonia lyase (*25*).

Table 1. Shikimate Acid Contents of Leaves from Glyphosate-Susceptible Horseweed Plants (S) and Counterparts with Reduced Susceptibility to Glyphosate (RS) Determined 0, 3, 7, and 10 DAT[a]

	shikimate content (μg/g of dry weight)			
biotype	0 DAT	3 DAT	7 DAT	10 DAT[b]
S	24.1 ± 6.9 a	2531.8 ± 1047.8 b	6901.7 ± 4359.7 c	dead
RS	35.1 ± 14.9 a	1111.5 ± 425.9 ab	592.4 ± 190.5 ab[ab]	325.6 ± 195.1

[a] Means ± standard errors obtained from two to three measurements of extracts from eight horseweed plants of each biotype. The differences between values with no common letter are statistically significant at $p \leq 0.05$. [b] Data for the S plants that were dead 10 DAT were not statistically analyzed.

In horseweed plants, the degree of EPSP synthase inhibition depends on the dose of glyphosate used. In a study of susceptible and resistant horseweed biotypes collected from several states in the United States, shikimate accumulated in leaf discs of both biotypes treated with high concentrations of glyphosate ($\leq$21.1 mg ae L^{-1}), but only in discs from susceptible plants when they were treated with low concentrations ($\leq$10.6 mg ae L^{-1}) (*13*). In our study, shikimate concentrations in untreated plants (the S and RS controls) were always low (20−35 μg/g of fresh weight). Unexpectedly, they were slightly higher in RS plants prior to treatment (0 DAT), probably due to repeated glyphosate treatment of the plants before the experiment. Glyphosate application resulted in increased levels of shikimic acid in both horseweed biotypes, and there were no significant differences in shikimate levels between the biotypes 3 DAT (**Table 1**). However, the time courses of subsequent changes in shikimate contents in RS plants and S plants following treatment were different. In the S biotype, the shikimate content increased with time, ~3-fold from 3 to 7 DAT, while in RS plants, levels 7 and 10 DAT were 50 and 70% lower, respectively, than those recorded 3 DAT (**Table 1** and **Figure 1**). Similar changes in shikimate levels with time have been detected in glyphosate-sensitive and glyphosate-resistant horseweed plants by Mueller et al. (*12*).

Marked differences in the responses of plants to application of glyphosate were observed among individual plants of both biotypes (**Figure 2**). Similarly, significant differences in glyphosate responses have been observed under controlled conditions among hairy fleabane (*C. bonariensis*) plants, sampled from perennial crop fields by Urbano et al. (*14*). However, although the degree of shikimate accumulation in RS plants differed (as illustrated by the data for RS1 and RS2 presented in **Figure 2**), the trends following glyphosate application in them were very similar. The extremely high susceptibility of plant S2, which died 6 DAT, was reflected in its very high shikimate concentration (3870 μg/g of fresh weight) recorded 3 DAT. (**Figure 2**). Strong correlations between visual phytotoxic effects and shikimic acid contents (peaking 3−7 DAT) have been previously observed in field-grown corn (*26*), but no apparent relationship between the speed of the appearance of herbicidal symptoms and shikimate accumulation following glyphosate application was detected in nine weedy species examined by Mueller et al. (*27*). Three mechanisms of glyphosate resistance have been identified: (i) reduced level of translocation of glyphosate to meristematic tissues, (ii) increased levels of EPSP synthase transcription, and (iii) a target site mechanism, involving mutation at amino acid position 106 of the *epsps* gene (*9*, *11*). Comparison of sequences of 286 bp fragments of the *epsps* gene containing codon 106 of seven glyphosate-tolerant and four susceptible horseweed plants showed no polymorphisms between tolerant and susceptible plants (**Figure 3**), implying either that there is at least one site at which mutations confer resistance that is not in this conserved region in horseweed or that the mechanism could be at least partly

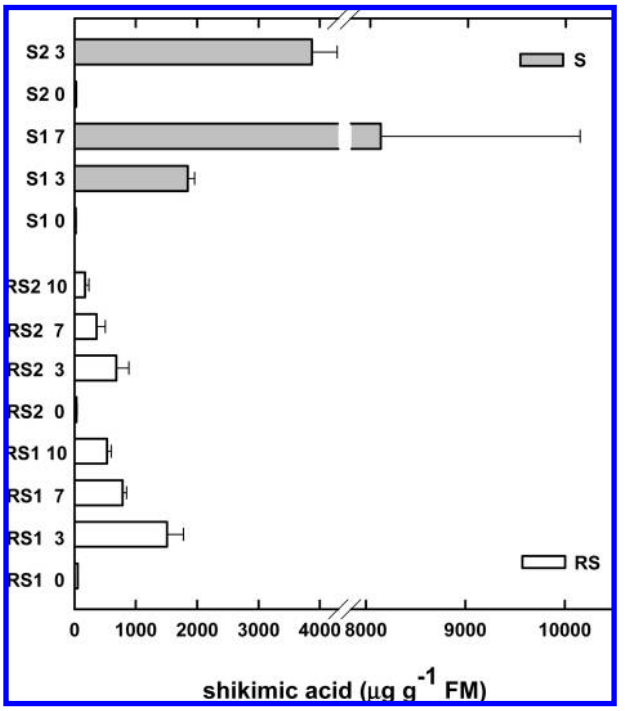

Figure 2. Accumulation of shikimic acid in two glyphosate-susceptible (S1 and S2) and two reduced-susceptibility-to-glyphosate (RS1 and RS2) horseweed plants, determined 0, 3, 7, and 10 DAT. Means ± standard errors obtained from three measurements of extracts of leaves from single horseweed plants.

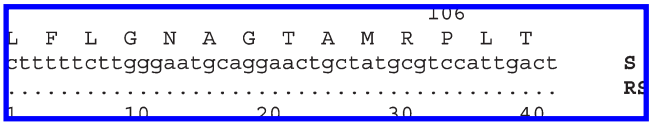

Figure 3. Alignment of the nucleotide sequences of the conserved region of the glyphosate-susceptible plants (S) and horseweed plants with reduced susceptibility to glyphosate (RS). Dots indicate the same nucleotides as in the reference sequence.

non-target-based. However, since leaves from both S and RS biotypes showed elevated levels of shikimate (suggesting that EPSPS remained sensitive to glyphosate), our results suggest that the RS plants we examined have a non-target site-based mechanism of glyphosate resistance. Nontarget site resistance has been documented in glyphosate-resistant populations of horseweed from the United States and Spain, in which impaired translocation of the herbicide and increased EPSPS transcript levels have been confirmed (*9*, *11*, *14*, *28*). Differences in levels of accumulated shikimate from 3 to 7 DAT between the biotypes suggest that the RS plants we examined were able to metabolize accumulated shikimate.

Gaines et al. (*29*) reported that nuclear basis of glyphosate resistance in *Amaranthus palmeri* was likely due to an increased level of production of EPSPS due to gene amplification. This is the first documented occurrence of *epsps* gene amplification in a weed population under glyphosate selection pressure. There is the possibility that one or a few genomic copies have stronger expression due to promoter changes or have a target site mutation that has not been detected. The mechanism of glyphosate resistance in horseweed is not fully known, but Mueller et al. (*12*) presented one possible hypothesis; the species may carry multiple *epsps* genes encoding various EPSPS isoforms, and in plants under glyphosate-stress conditions, the biosynthesis of an altered form of the EPSPS

enzyme may be induced. Our results, showing declining concentrations of shikimate in populations with reduced susceptibility to glyphosate, are consistent with this hypothesis. Further, the plants recovered, a few weeks after being sprayed with glyphosate, by producing new leaves from the center of the rosette, strongly suggesting that the level of translocation of glyphosate toward the meristematic tissues was reduced in them. Such reductions in the level of translocation of glyphosate have been shown to play a major role in glyphosate resistance in resistant biotypes of horseweed examined by several other authors (*9*, *10*).

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