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Shikimate Accumulates in Both Glyphosate-Sensitive and Glyphosate-Resistant Horseweed (*Conyza canadensis* L. Cronq.)

THOMAS C. MUELLER,*,† JOSEPH H. MASSEY,‡ ROBERT M. HAYES,† CHRIS L. MAIN,† AND C. NEAL STEWART, JR.†

Department of Plant Sciences and Landscape Systems, The University of Tennessee, 2431 Center Drive, Knoxville, Tennessee 37996, and Department of Plant and Soil Sciences, Mississippi State University, Mississippi State, Mississippi 39762

Horseweed (*Conyza canadensis*) is a cosmopolitan weed that commonly grows throughout North America. Horseweed that is not completely controlled by normal applications of glyphosate has been reported in western Tennessee. This research had three objectives: (1) to develop and validate an analytical procedure for the quantitative determination of shikimate, an important indicator of glyphosate activity in plants; (2) to confirm resistance to glyphosate in a horseweed population; and (3) to examine the accumulation of shikimate in both glyphosate-resistant and glyphosate-susceptible horseweed plants. The analytical procedure to determine shikimate used extraction with 1 M HCl for 24 h, followed by liquid chromatography using photodiode array detection, and shikimate recoveries were ≥82%. Glyphosate applications of both 0.84 kg ae/ha (the standard application rate) and 3.8 kg ae/ha to susceptible plants caused complete plant death. The same glyphosate applications to putative resistant populations caused less than 15% growth reduction as determined by visual evaluations, and fresh weights of these resistant plants 17 days after glyphosate treatment (DAT) were reduced an average of 45% in one population and were not affected in a different population. This direct comparison conclusively confirms that horseweed plants collected in western Tennessee in 2002 are resistant to 4 times the normal application dosage of glyphosate. The glyphosate-resistant horseweed biotypes still exhibited some herbicidal effects from the glyphosate, such as yellowing in the most actively growing, apical shoot meristems. The yellowing in the shoot apexes was transitory, and the plants recovered from this damage. Shikimate concentrations in all untreated horseweed plants were less than 100 μg/g, which was significantly less than that in all plants which had been treated with 0.84 kg ae/ha of glyphosate. Unexpectedly, shikimate accumulated (>1000 μg/g) in both resistant populations and the susceptible population. However, there were differences in shikimate accumulation patterns between resistant and susceptible horseweed biotypes. Shikimate concentrations in resistant populations declined about 40% from 2 to 4 DAT, while shikimate concentrations in the susceptible horseweed plants increased about 35% from 2 to 4 DAT. The confirmed resistance of a widespread weed implies that alternative control strategies for glyphosate-resistant horseweed will be needed in those no-tillage production systems where it commonly occurs.

KEYWORDS: Shikimate; HPLC; weed-resistance; glyphosate; EPSPS; herbicide resistance

INTRODUCTION

A common perspective in the late 1990s was that weed resistance to the herbicide glyphosate was not probable (*1*). This was believed because the complex manipulations of the target EPSPS enzyme required for the development of glyphosate-resistant crops were not expected to be duplicated in nature to evolve glyphosate-resistant weeds. This assessment is no longer true.

Horseweed (*Conyza canadensis* L. Cronq.) (also referred to as Canada fleabane or mare's-tail) is an annual plant, native to North America (*2*). Horseweed is a substantial problem in conservation tillage production systems in cotton in Alabama (*3*), in grain sorghum in Georgia (*4*), in corn in Wisconsin (*5*), in soybean and corn in Iowa and Minnesota (*6*), in fallow periods in the southern Great Plains (*7*), and in the production of container-grown ornamentals (*8*). Horseweed is present

* To whom correspondence should be addressed. Phone: (865) 974-8805. Fax: (865) 974-7997. E-mail: tmueller@utk.edu.
† The University of Tennessee.
‡ Mississippi State University.

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throughout the North American continent. Large numbers of small, wind-dispersed seeds are produced in late summer (*2*). It serves as a wild host of the tarnished plant bug and of aster yellows, a mycoplasma disease transmitted by the aster leaf hopper.

The first reported occurrence of glyphosate-resistant horseweed in North America was in Delaware in 2000 (*9*). No-till corn and soybean production has been widely adopted in the mid-Atlantic region, which has favored the establishment of horseweed. Within three years of using only glyphosate for weed control in continuous cropping of glyphosate-resistant soybeans, glyphosate failed to control horseweed in some fields. Seedlings originating from seed of one horseweed population in Delaware were grown in a greenhouse and exhibited greater than 10-fold resistance to glyphosate compared with a susceptible population. There were no reported differences in tolerance between different salts of glyphosate. Historically, glyphosate provided essentially complete control of horseweed (*10*−*12*), so this decreased control is markedly different. This weed resistance phenomenon differs from a herbicide-induced weed shift, where species that were never controlled or were poorly controlled by glyphosate increase in relative abundance in that environmental setting. The glyphosate resistance present in these horseweed populations represents a change at the physiological level with agronomic implications.

Glyphosate is a potent herbicide (*13*). It works by competitive inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which catalyzes an essential step in the aromatic amino acid biosynthetic pathway. The measurement of shikimic acid accumulation in response to glyphosate inhibition of EPSPS is a rapid and accurate assay to quantify glyphosate-induced damage in sensitive plants. Pline et al. (*14*) examined the accumulation of shikimic acid in cotton varieties that were either resistant or susceptible to glyphosate. All tissues of susceptible cotton plants accumulated shikimic acid in response to glyphosate treatment, while glyphosate-resistant plants accumulated much less shikimic acid. The active site of the enzyme EPSPS has been probed using site-directed mutagenesis and inhibitor binding techniques (*15*). The studies suggest a high degree of structural conservation from bacteria compared to plant EPSPS enzymes.

Previous research has indicated the propensity of horseweed to develop resistance to herbicides. Populations of horseweed resistant to the herbicide paraquat were found in Ontario, Canada (*16*). These paraquat-resistant populations required doses >25 times higher than susceptible populations for equivalent control. Horseweed resistant to paraquat (*17*) and triazines (*18*) was also documented from collections in Hungary.

MATERIALS AND METHODS

Greenhouse Study. Horseweed plants were collected from two suspected resistant populations in western Tennessee located in Lauderdale County (100 km northeast of Memphis, latitude 89.5°, longitude 35.7°) and from a nonresistant, susceptible horseweed population in Knoxville, TN (latitude 84°, longitude 36°). Plants contained within an intact soil core were carefully removed from their native location and transferred to pots (15 cm diameter by 12 cm height) for study in the greenhouse. Each pot contained a single horseweed plant and was used as an individual experimental unit. The collections were from three distinct populations and were different sizes at the time of collection. The two suspected resistant populations are hereafter denoted as Resistant-East and Resistant-West in the manuscript. Approximate heights at the time of herbicide application were 20 cm for Resistant-East, 10 cm for Resistant-West, and 15 cm for Susceptible. Plants size and plant heights were uniform within a given population. Each data point for the greenhouse trials presented in the tables is the numerical mean of five individual experimental units, each consisting of one plant. The study design was constrained by the limited number of glyphosate-resistant plants that were available. The plants had not been sprayed with glyphosate prior to collection.

Plants were allowed to acclimate to greenhouse conditions for 2 weeks and were watered the evening prior to initiation of the study. Watering was resumed 24 h after glyphosate application. On May 6, plants were randomly distributed for two studies and sprayed. The first study was to confirm that these horseweed populations were in fact resistant to glyphosate. The second study was for shikimate analysis after glyphosate application.

Glyphosate was applied using an enclosed spray booth to prevent movement to nontarget plants. Application was made in a water carrier at 190 L/ha, applied in two passes (95 L/ha per pass) to provide more complete foliar coverage.

In study one (glyphosate-resistance confirmation), the plants were allowed to grow for 17 d after application of either 0, 0.84, or 3.8 kg ae/ha of glyphosate (commercial formulation of RoundupUltraMax was used). A visual evaluation of total plant decline was conducted at 14 DAT. This visual evaluation utilized a 0−100 scale, with 0 being no visible effects and 100 being plant death. Shoot fresh weights were obtained by excising each plant at the soil level and weighing on a top-loading balance.

In study two (determination of shikimate accumulation), plants were sprayed as previously described with glyphosate at 0 or 0.84 kg ae/ha. Shoot tissue (top 10 cm of each plant) was harvested 2 and 4 DAT. These sampling periods were chosen to bracket the anticipated time of maximum shikimate accumulation, based upon accumulation times reported for soybean (*19*), tomato (*20*), and oil seed rape (*21*). Each plant tissue sample was collected and weighed prior to analysis. Immediately after the plant tissue fresh weight was recorded, each sample was stored on dry ice and transported to the processing facility. The sample size was five plants per population per treatment.

Laboratory Methods. Upon receipt of the plant samples (less than 12 h), the tissue samples were placed into freezer storage (−20 °C) until processed and analyzed. All shikimate analyses were completed within 54 days of sample collection. Shikimate is stable for up to 90 d in corn tissue stored at −20 °C (Massey, unpublished data). On the basis of these findings, it is anticipated that shikimate will be stable in horseweed tissue when stored at −20 °C for this period of time.

An extraction procedure similar to one previously reported for corn and soybean (19) was used to analyze the horseweed tissue for shikimate. Frozen horseweed tissue was finely ground in liquid nitrogen using a mortar and pestle. After grinding, the tissue was weighed into 50-mL screw-cap polypropylene centrifuge tubes, and 1 M HCl was added at a ratio of 5 mL of HCl solution per 1 g of tissue. The tissue sample sizes ranged from 0.95 to 7.85 g (fresh weight). The samples were placed on an orbital shaker at 1500 rpm for 24 h. For each set of 10 samples, a minimum of two untreated blanks and two fresh fortifications (50 and 500 ppmw shikimate) were prepared. The shikimate (Sigma-Aldrich, St. Louis, MO; 99% purity) fortification solution was prepared in acetonitrile containing 5% water (v/v); the solvents were allowed to evaporate thoroughly in a fume hood before the addition of extraction solution.

Pilot studies indicated that shikimate recovery from horseweed tissue that had been finely ground in liquid N_2 and extracted for 24 h in 1 M HCl were acceptable (**Table 1**). Recovery of shikimate from horseweed fortified to 50, 500, and 2000 μg shikimate/g and shaken for 24 h averaged 109 ± 21.5%, 95.0 ± 1.5%, and 82.5 ± 9.6%, respectively. Moreover, recovery of endogenous shikimate did not change significantly after 24 h of shaking (**Table 1**). Taken together, these results indicated that 24 h of shaking with 1 M HCl was a satisfactory means of extracting shikimate from horseweed. After extraction, each HCl extract was filtered through Whatman No. 1 filter paper into a graduated cylinder, and the volume of the filtered extract was recorded. Next, the pH of the filtered extract was adjusted to 3.0−3.3 using saturated NaOH and/or 0.01 N NaOH, as needed. The final volume of the pH-adjusted extract was recorded and returned to the initial extract volume using 0.001 M HCl. A 2-mL portion of the extract was diluted with 1.0 mL of acetonitrile and passed through a 0.45-μm nylon syringe

Table 1. Recovery of Freshly Fortified and Endogenous Shikimate from Horseweed Tissue Using 1 M HCl, as a Function of Extraction Time[a]

shikimate treatment	extraction time (h)	average recovery	*n*
50 μg/g[b] freshly fortified shikimate	24	108.7 ± 21.5%	2
	48	98.9 ± 14.7%	2
	72	86.6 ± 15.5%	2
500 μg/g[b] freshly fortified shikimate	24	95.0 ± 1.5%	3
	48	94.9 ± 3.0%	3
	72	84.1 ± 5.4%	3
2000 μg/g[b] freshly fortified shikimate	24	82.5 ± 9.6%	3
	48	71.5 ± 3.1%	3
	72	71.2 ± 2.5%	3
endogenous[c] shikimate	24	5807 ± 129 μg/g	3
	48	5964 ± 348 μg/g	3
	72	5854 ± 562 μg/g	3

[a] Extraction time on orbital shaker at 1500 rpm using 5 mL of 1 M HCl per gram of tissue. [b] Applied to 2 g of untreated, field-grown tissue finely ground in liquid N_2; recovery results are corrected for background shikimate concentrations, which ranged from 21 to 85 ppmw. [c] Endogenous levels of accumulated shikimate in horseweed 3 DAT with 1.9 kg ae/ha glyphosate applied as Roundup UltraMax herbicide.

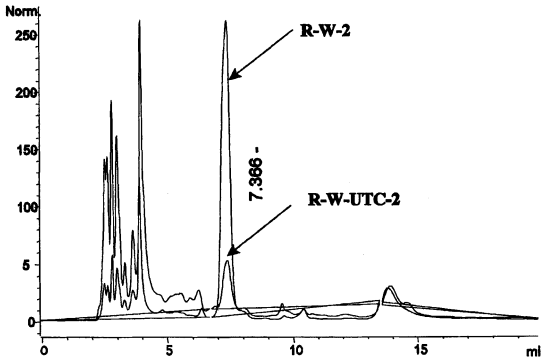

Figure 1. Representative chromatograms for shikimate accumulation and quantification in glyphosate-resistant horseweed in the Resistant-West population 2 d after glyphosate treatment (R-W-2) and corresponding untreated control (UTC).

filter into a chromatography vial. The extract was refrigerated at 4 °C until analysis using HPLC.

Analytical Method for Shikimate. The concentration of shikimate in horseweed tissue was determined by HPLC (*19*) using an Agilent (Wilmington, DE) series 1100 chromatograph equipped with Chemstation software, autoinjector, and photodiode array detector using a detection wavelength of 215 nm. A Phenomenex (Torrance, CA) Luna NH_2 100A column (250 mm × 4.0 mm; 5 μm particle size) was used with an injection volume of 10 μL. The isocratic system used 90:9:1 acetonitrile/deionized water/phosphoric acid and a flow rate of 1.0 mL/min. The total run time was 20 min, with shikimate retention time at 7.4 min. A six-point calibration curve with shikimate concentrations ranging from 3.65 to 52.3 ppm was used to externally quantify shikimate levels in the tissue extracts. The method detection limit for shikimate was approximately 20 ppmw. Representative chromatograms showing extract concentrations of shikimate in horseweed before and after glyphosate treatment are shown in **Figure 1**. The shikimate data were analyzed as a completely randomized design using SAS Proc GLM procedure (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Greenhouse Study. This research conclusively confirmed that the suspected glyphosate-resistant horseweed is resistant to glyphosate (**Table 2**). Visual evaluations 14 DAT indicated less than 15% control in resistant populations, while the susceptible plants showed 99% control. However, all resistant plants showed slight phytotoxicity from glyphosate application. The glyphosate-resistant horseweed plant shoot apexes turned light green to yellow; the plants were slightly stunted and then resumed normal growth after 5−10 days (variable with plants). There were some differences between the two resistant populations. Resistant-East plants were larger at the time of glyphosate application, and they had approximately 45% growth reduction on fresh weight basis compared with the untreated plants. Resistant-West plants increased in size about 20% at the low glyphosate rate or stayed the same size when treated with the higher glyphosate application rate. The resistant populations were contrasted by the susceptible populations that had greater than 80% decline in plant fresh weight. This small amount of fresh weight plant material was essentially a dead stem that remained from the original plant. These results are in agreement with those of VanGessel, who first reported glyphosate-resistant horseweed in Delaware (*9*).

Table 2. Growth Reduction and Control of Horseweed Biotypes Treated with Glyphosate

horseweed biotype	glyphosate dosage (kg ae/ha)	control at 14 d (%)	fresh weight at 17 d (g)	fresh weight of untreated (%)
Resistant-East	0	0	14.91	100
Resistant-East	0.84	4	9.10	61
Resistant-East	3.8	6	7.91	53
Resistant-West	0	0	5.79	100
Resistant-West	0.84	6	7.03	120
Resistant-West	3.8	14	5.54	96
Susceptible	0	0	11.9	100
Susceptible	0.84	99	1.94	16
Susceptible	3.8	99	1.53	13
LSD (0.05)		4.4	2.1	

Laboratory Study. Shikimate recovery from the freshly fortified control samples was corrected using the appropriate untreated control concentrations. The average background level of shikimate in untreated horseweed was 69 ± 55 ppmw (n = 16) for all untreated horseweed populations and study times. The average recoveries of shikimate from freshly fortified horseweed tissue were 99 ± 20% (n = 5) at the 50 ppmw level and 86 ± 5% (n = 5) at the 500 ppmw level of fortification.

Shikimate Accumulation in Glyphosate-Resistant and Glyphosate-Susceptible Horseweed. Shikimate accumulated in concentrations significantly greater than background levels after glyphosate treatment in all horseweed populations (**Figure 2**). There were no significant differences (α = 0.05) in shikimate levels among the glyphosate-resistant (i.e., East and West) and glyphosate-susceptible populations 2 and 4 DAT (**Figure 2**). The two horseweed biotypes differed in the trend over time in shikimate concentration: it decreased from 2 to 4 DAT in the resistant plants but increased from 2 to 4 DAT in the susceptible plants. One would expect resistant biotypes to have lower pools of shikimate compared to susceptible plants upon herbicide treatment, supposedly at levels close to those exhibited by plants not exposed to glyphosate. Blockage of the EPSPS enzyme is the mechanism of glyphosate activity in plants, so less plant effect would imply less shikimate. On the basis of prior studies with glyphosate-tolerant crops (*14*), the accumulation of shikimate in a resistance population was unexpected.

Taken together with the whole plant bioassays, the shikimate accumulation data indicate that the mechanism of glyphosate resistance in horseweed is not due solely to a single, glyphosate-insensitive EPSPS. If a glyphosate-resistant EPSPS were present, we would not expect to see significant increases in shikimate.

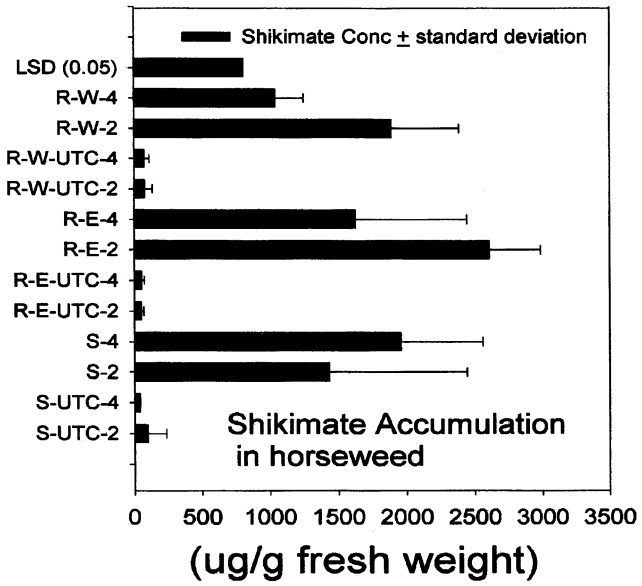

Figure 2. Accumulation of endogenous shikimate in two (denoted East and West) glyphosate-resistant (R) and glyphosate-susceptible (S) horseweed populations determined at 2 and 4 d after glyphosate treatment. Data from untreated control (UTC) plants are also shown.

While the mechanism of glyphosate resistance in horseweed is not known, we have several possible hypotheses. First, multiple EPSPS genes encoding various EPSPS isoforms may be present that are responsible for varying levels of inhibition by glyphosate herbicide. Second, this glyphosate-resistant horseweed may possess a glyphosate oxidase reductase (GOX)-like enzyme. This scenario is unique in plant science, since no wild, nontransformed plants have been documented to have native GOX genes. The GOX gene was originally derived from nonplant sources and inserted into several plants to increase the selectivity level of those crops to glyphosate (22). The GOX enzyme accelerates the normal degradation of glyphosate into aminomethylphosphonic acid and glyoxylate. Differences in accumulated shikimate levels between 2 and 4 DAT for the resistant and susceptible populations suggest that the resistant plants were able to metabolize accumulated shikimate. This metabolism would support the hypothesis that the biosynthesis of an altered, secondary form of EPSPS enzyme may be induced when the resistant plants are placed under stress by treatment with glyphosate. Specifically, the phenotypic characterizations of glyphosate-resistant horseweed plants postapplication and the dynamics of shikimate accumulation, in which shikimate quickly builds up, indicate that glyphosate is initially inhibiting EPSPS, but later the shikimate concentration decreases in glyphosate-resistant plants at 4 DAT and they survive and grow. This is in contrast to the continual increase in shikimate in susceptible plants, which are subsequently killed. An example of this scenario is the case of some herbicide safeners, which act using a chemical induction mechanism to involve enzymes in herbicide metabolism (*23*).

A third possible hypothesis deals with the presence of an altered EPSPS. In this scenario, glyphosate competitively binds to EPSPS in the cytosol as well as in the chloroplast. Since natural isoforms of the EPSPS are not overexpressed through genetic manipulation, it is possible that a small pool of altered EPSPS functions normally deplete the large pool of shikimate that builds up after glyphosate binds to and inhibits the susceptible form of EPSPS. As glyphosate binds to the susceptible form of EPSPS, resistant plants would display altered growth due to a lack of aromatic amino acids. Altered EPSPS would then slowly restore the pathway leading to a depletion of the shikimate pool and continued vegetative development.

Glyphosate-resistant horseweed from Delaware has previously been examined to elucidate the resistance mechanism (*24*). Initial indications are that glyphosate uptake into the plant and subsequent translocation to the active site were not responsible for the observed resistance. However, enhanced glyphosate metabolism was also not implicated in this preliminary report. A hypothesis of that research group (*24*) was that an altered form(s) of the EPSPS enzyme was present in glyphosate-resistant horseweed, although the plants retained some susceptible isoforms of the same enzyme. Our results, showing a recovery of growth and declining shikimate concentration, are consistent with this hypothesis.

The present study confirms glyphosate resistance in horseweed populations different than those previously reported in Delaware (*9*). The full extent of the occurrence of glyphosate-resistant horseweed in the mid-southern United States is not known, although a preliminary estimate for western Tennessee is 200 000 hectares (unpublished data). To date, there are few confirmed locations of the occurrence, but others are suspected.

Our research also presents a series of novel findings, such as the occurrence of shikimate accumulation in both glyphosate-resistant and glyphosate-susceptible horseweed plants. It appears as if the horseweed may either contain a secondary glyphosate-insensitive EPSPS enzyme or contain additional enzymes capable of slowly detoxifying herbicides such as glyphosate. Future research efforts include further studies to determine the molecular mechanism for the observed glyphosate resistance. A molecular analysis for both resistant and other horseweed populations with varying glyphosate susceptibility, focusing on sequence analysis of genes encoding EPSPS and GOX-like proteins, will be conducted. These results will be useful in studying the population genetics of the observed resistance, and in generating potential solutions and recommendation for glyphosate resistance management. Additional research conducted under field conditions is currently underway to determine the best management practices to control glyphosate-resistant horseweed while maintaining no-tillage production practices.

ABBREVIATIONS USED

EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; DAT, days after treatment with glyphosate; GOX, glyphosate oxidase.

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