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Assessment of two nondestructive assays for detecting glyphosate resistance in horseweed (*Conyza canadensis*)

Clifford H. Koger
Corresponding author. U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS), Crop Genetics and Production Research Unit and Southern Weed Science Research Unit, P.O. Box 350, Stoneville, MS 38776; ckoger@ars.usda.gov

Dale L. Shaner
USDA-ARS, Water Management Research Unit, 2150 Centre Avenue, Building D, Suite 320, Fort Collins, CO 80526

W. Brien Henry
USDA-ARS, Central Plains Research Station, 40335 County Road GG, Akron, CO 80720

Talia Nadler-Hassar
Colorado State University, Biological Science and Pest Management, Weed Science Lab, Fort Collins, CO 80527

Walter E. Thomas
Crop Science Department, North Carolina State University, Raleigh, NC 27695-7620

John W. Wilcut
Crop Science Department, North Carolina State University, Raleigh, NC 27695-7620

Two rapid, nondestructive assays were developed and tested for their potential in differentiating glyphosate-resistant from glyphosate-susceptible biotypes of horseweed. In one assay, leaves of glyphosate-resistant and -susceptible corn, cotton, and soybean plants, as well as glyphosate-resistant and -susceptible horseweed plants, were dipped in solutions of 0, 300, 600, and 1,200 mg ae L^{-1} glyphosate for 3 d, and subsequent injury was evaluated. In the second assay, plant sensitivity to glyphosate was evaluated in vivo by incubating excised leaf disc tissue from the same plants used in the first assay in 0.7, 1.3, 2.6, 5.3, 10.6, 21.1, 42.3, and 84.5 mg ae L^{-1} glyphosate solutions for 16 h and measuring shikimate levels with a spectrophotometer. The leaf dip assay differentiated between glyphosate-resistant and -susceptible crops and horseweed biotypes. The 600 mg L^{-1} rate of glyphosate was more consistent in differentiating resistant and susceptible plants compared with the 300 and 1,200 mg L^{-1} rates. The in vivo assay detected significant differences between susceptible and glyphosate-resistant plants of all species. Shikimate accumulated in a glyphosate dose–dependent manner in leaf discs from susceptible crops, but shikimate did not accumulate in leaf discs from resistant crops, and levels were similar to nontreated leaf discs. Shikimate accumulated at high ($\geq$ 21.1 mg ae L^{-1}) concentrations of glyphosate in leaf discs from all horseweed biotypes. Shikimate accumulated at low glyphosate concentrations ($\leq$ 10.6 mg L^{-1}) in leaf discs from susceptible horseweed biotypes but not in resistant biotypes. Both assays were able to differentiate resistant from susceptible biotypes of horseweed and could have utility for screening other weed populations for resistance to glyphosate.

Nomenclature: Glyphosate; horseweed, *Conyza canadensis* (L.) Cronq. ERICA; corn, *Zea mays* L. 'Dekalb 687RR', 'Pioneer 31B13'; cotton, *Gossypium hirsutum* L. 'Delta and Pine Land 444RR', 'Suregrow 747'; soybean, *Glycine max* (L.) Merr. 'Delta and Pine Land 4748', 'Asgrow 4702RR'.

Key words: Absorbance, bioassay, glyphosate resistance, herbicide resistance, leaf dip, leaf disc, shikimate.

Glyphosate is a systemic, nonselective herbicide that has been used for > 20 yr to control most annual and perennial monocot and dicot weeds (Blackshaw and Harker 2002; Faircloth et al. 2001; Gower et al. 2003; Koger et al. 2005; Scott et al. 2002; Shaw and Arnold 2002; Wilcut and Askew 1999). Crops resistant to glyphosate have been widely adopted by growers in the United States and provide a simple, broad-spectrum weed control option in corn, cotton, and soybean production systems (Reddy and Koger 2005). More than 80 and 60% of the soybean and cotton hectareage, respectively, in the United States were planted to glyphosate-resistant (GR) varieties in 2004 (USDA-NASS 2004).

Repeated use of glyphosate over years in GR crops as well as noncrop areas has resulted in the selection of weeds resistant to glyphosate. Resistance to glyphosate has been found in horseweed biotypes from Delaware (VanGessel 2001); Tennessee (Mueller et al. 2003); Mississippi (Koger et al. 2004a); and Kentucky, Indiana, Maryland, New Jersey, Ohio, Arkansas, and North Carolina (Heap 2004). GR horseweed biotypes infest over 100,000 ha in the United States (Heap 2004). GR biotypes of goosegrass [*Eleusine indica* (L.) Gaertn.] from Malaysia; Italian ryegrass (*Lolium multiflorum* Lam.) from Brazil and Chile; rigid ryegrass (*Lolium rigidum* Gaud.) from Australia, South Africa, and the United States; buckhorn plantain (*Plantago lanceolata* L.) from South Africa; and hairy fleabane [*Conyza bonariensis* (L.) Crong.] from South Africa have also been documented (Heap 2004; Lee and Ngim 2000; Perez and Kogon 2003; Powles et al. 1998; Pratley et al. 1999).

Glyphosate resistance in biotypes of horseweed in the United States and rigid ryegrass in Australia is due to limited translocation of glyphosate to the plant's growing points (Feng et al. 2004; Koger and Reddy 2005; Lorraine-Colwill et al. 2003), whereas glyphosate resistance in a rigid ryegrass biotype in California and a goosegrass biotype in Malaysia is due to an alteration of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Baerson et al. 2002; Simarmata and Penner 2004).

The selection of weed biotypes resistant to glyphosate alarms many growers, researchers, and crop consultants (Brunoehler 2004). However, in some cases, insufficient control of weeds with glyphosate might not be because of resistance, but other factors such as sublethal rate (Koger et al. 2004b) or large plant size at time of application (Chachalis et al. 2001). It is critical to quickly determine whether poor control by glyphosate is because of resistance or some other factor so that the resistant population can be contained with alternative weed management strategies. A rapid and easy assay that can determine whether glyphosate resis-

tance exists in a population would be a useful tool to growers and researchers alike.

There are multiple methods for screening herbicide resistance (Beckie et al. 2000). Field and greenhouse whole-plant screens are the most widely used and definitive. Drawbacks to these types of assays are the time required to complete the tests and expense. A potential alternative to whole-plant screening is to initially screen a population with a simpler test with the use of seeds, pollen, or excised plant tissue. Any population that appears to be resistant by the simpler test can be further examined with more definitive assays. However, an early answer would be valuable because most weed management decisions must be made early in the growing season before plants mature and develop seeds.

Various assays for screening glyphosate resistance have been proposed. One assay is to germinate seeds in petri plates containing a range of glyphosate concentrations and measure root or shoot length 4 to 8 d after treatment (Escorial et al. 2001; Perez and Kogan 2003). Another assay is to soak seeds in glyphosate solutions and then plant the seeds in soil. Main et al. (2004) found that susceptible soybean seeds did not germinate after soaking for 4 h in 1, 2, and 5% solutions of glyphosate, whereas seeds containing a GR gene germinated at all concentrations. One major drawback of both the petri plate and pot assay is the need for seeds. To screen weed populations from the field, seeds have to be collected and dormancy broken before running the assay. The time required to obtain seeds that will germinate results in more time needed to determine whether a population is resistant or susceptible and the possibility that seeds from resistant plants have already dehisced or migrated to noninfested areas.

Boutsalis (2001) used a quick test to screen for herbicide resistance in several grass species. Shoot cuttings were harvested from rigid ryegrass that had survived herbicide treatment in the field and were treated 7 d after cuttings were cultured in the greenhouse. Resistance to both acetyl-CoA carboxylase and acetolactate synthase inhibitors were confirmed by this method. The assay was relatively quick (3 to 4 wk) and could be used to sample plants taken from the field. The assay requires very little equipment and could be used by growers to test for resistance. However, this assay requires extensive manipulation of plant material and would only be practical on species in which cuttings are easy to culture.

An easier alternative to treating transplanted cuttings is to incubate leaves in herbicide solution immediately after excision and monitor subsequent injury. If the mechanism of glyphosate resistance is from limited subcellular translocation or an altered target site mutation, it is possible that leaves of resistant plants, compared with leaves of susceptible plants, are not injured with sublethal rates of glyphosate.

An in vivo measurement of shikimate accumulation in glyphosate-treated tissue could be another alternative for screening suspected glyphosate-resistant populations. Competitive inhibition of EPSPS by glyphosate results in uncontrolled flow of carbon and subsequent accumulation of shikimate in affected plant tissue (Bresnahan et al. 2003). Shaner et al. (2005) recently described an in vivo assay as a method to detect glyphosate resistance. In this assay, leaf discs from plants are incubated in different concentrations of glyphosate for 16 to 23 h, and the amount of shikimate accumulated in the discs is measured with a spectrophotometer. The assay can be used to differentiate between susceptible and glyphosate-resistant crops expressing an insensitive EPSPS. However, the assay has not been used to detect glyphosate resistance because of reduced translocation of glyphosate to the growing points of plants.

The objectives of this research were (1) to test two assays, a leaf dip assay measuring injury at the leaf level and an in vivo assay measuring shikimate accumulation in excised leaf discs, for their potential in differentiating known resistant and susceptible crop varieties and horseweed biotypes and (2) to test the ability of both assays to detect resistance in suspected glyphosate-resistant populations of field-grown horseweed.

Materials and Methods

General Information

Seeds of GR corn 'Dekalb 687RR', cotton 'DP 444RR', and soybean 'Asgrow 4702RR', as well as non-GR corn 'Pioneer 31B13', cotton 'Suregrow 747', and soybean 'Delta and Pine Land 4748,' were planted 2-cm-deep in individual 11-cm-diam pots containing a mixture of soil (Bosket sandy loam, fine-loamy, mixed thermic Molic Hapludalfs) and Jiffy Mix potting soil[1] (1:1, v/v). Pots were subirrigated with distilled water (DW) as needed for the first 10 d after planting, after which, pots were subirrigated with DW every third day and a 1% Hoagland solution (Hoagland and Arnon 1950) every 10th day. After emergence, plants were thinned to three plants per pot. Plants were grown in a growth chamber maintained at 25/22 C day/night temperature with a 12-h photoperiod (200 μmol m^{-2} s^{-1}) and 75% relative humidity. Plants were in the three-leaf growth stage and were 30 cm tall (cotton and soybean) to 40 cm tall (corn) when leaves were harvested for use in leaf dip and leaf disc assays. Plants of GR corn, cotton, and soybean varieties will be referred to hereafter as RRCN, RRCT, and RRSY, respectively, and plants of conventional non-GR corn, cotton, and soybean varieties will be referred to hereafter as CVCN, CVCT, and CVSY, respectively.

Horseweed seeds were collected at maturity from field-grown plants in Arkansas (AR), Delaware (DE), and Mississippi (MS). One resistant and one susceptible biotype were collected from each state. Seeds of resistant biotypes from MS (Tunica County) and AR (Lawrence County) were collected from plants that survived at least two applications of 0.84 kg ae ha^{-1} glyphosate in GR cotton in 2003. GR cotton was grown in all three fields for at least 3 consecutive yr. Seeds of a resistant biotype from Sussex County, DE, were collected from plants that survived at least two in-season 0.84 kg ae ha^{-1} applications of glyphosate in a no-till field that had been planted to GR soybean for 5 consecutive yr. Resistant biotypes used in these studies were confirmed as resistant to glyphosate because of limited translocation of ^{14}C-glyphosate from site of application as reported in Koger and Reddy (2005). Seeds from plants of susceptible biotypes were collected from noncrop areas in the same counties as their respective resistant biotypes. Seeds were stored in separate screw-cap plastic bottles in the dark at 4 C until further use.

Seeds of each horseweed biotype were planted in the greenhouse (32/25 C day/night temperature) in 15 by 15

by 6 cm trays containing a mixture of soil (Bosket sandy loam, fine-loamy, mixed thermic Molic Hapludalfs) and Jiffy Mix potting soil[1] (1:1, v/v). Seeds were spread on top of the soil and subirrigated with DW. After emergence, seedlings in the cotyledon growth stage were transplanted to 11-cm-diam pots containing potting soil. Plants were transferred to the growth chamber and maintained at 25/22 C day/night temperature with a 12-h photoperiod (200 μmol m^{-2} s^{-1}) and 75% relative humidity. Plants were subirrigated with DW as needed. Youngest fully expanded leaves of plants in the 23- to 32-leaf growth stage (24–33-cm-diam rosette) were harvested for use in both assays. Plants of these resistant and susceptible horseweed biotypes will be referred to hereafter as ARR and ARS for the two AR biotypes, DER and DES for the two DE biotypes, and MSR and MSS for the two MS biotypes, respectively.

Leaves were collected from suspected GR, field-grown horseweed plants growing in a GR soybean field near Benoit, MS (Bolivar County), and three GR cotton fields near Walls, MS (DeSoto County). For the soybean field, GR soybean had been grown in the field each year since 2001. One GR cotton field was planted to GR soybean in 2000 and GR cotton in 2001 through 2004. The two remaining cotton fields have been planted to GR cotton each year since 2001. Leaves were collected from plants that had survived at least two applications of 0.84 kg ha^{-1} glyphosate. Leaves from suspected glyphosate-susceptible horseweed plants were also collected from plants growing in a noncropped field of the Mississippi State University Delta Branch Experiment Station near Stoneville, MS (Washington County), and three noncrop roadside sites near Tunica, MS (Tunica County); Clarksdale, MS (Coahoma County); and Cleveland, MS (Bolivar County).

Plants of the suspected GR biotypes (Bio) will be referred to hereafter as Bio1 (Bolivar County) and Bio2, Bio3, and Bio4 (DeSoto County), respectively. Plants of the suspected glyphosate-susceptible biotypes will be referred to hereafter as Bio5 (Washington County), Bio6 (Tunica County), Bio7 (Coahoma County), and Bio8 (Boilvar County), respectively. Plants of Bio1 and Bio5 were 140 to 180 cm tall and flowering when leaves were harvested for use in the leaf dip and leaf disc assays. Plants of all other biotypes were 45 to 90 cm tall and in the vegetative growth stage when leaves were harvested.

Leaf Dip Assay

The top (leaf tip) 10-cm segment of the youngest leaf of corn plants, one fully expanded leaf (~ 5 cm long) from the youngest whorl of leaves of horseweed plants, and the youngest fully expanded leaf of cotton and soybean plants, including the petiole, were excised from each plant with a scalpel. The bottom 2.54 cm of each corn leaf segment and the petiole, along with bottom one-fourth of each horseweed, cotton, and soybean leaf, was submerged in 6.8 ml of glyphosate solution contained in a 7-ml plastic vial.[2] Each leaf was placed in an individual vial. A commercial formulation[3] of the isopropylamine salt of glyphosate was diluted in a 10 mM ammonium phosphate[4] solution to give concentrations of 0, 300, 600, and 1,200 mg ae L^{-1} of glyphosate. Vials were placed in a growth chamber maintained at 25/22 C day/night temperature with a 12-h photoperiod (200 μmol m^{-2} s^{-1}) and 75% relative humidity for 72 h. Additional solution was added as needed to account for evaporation losses.

Vials were removed from the growth chamber after 72 h and percent leaf injury was assessed by overlaying a 1.25-cm^2 (crops) or 0.635-cm^2 (horseweed) grid quadrat onto leaves and subtracting the number of squares containing necrosis from the total number of squares for the entire leaf and multiplying that value by 100. Treatments were arranged in a completely randomized design for crop varieties. Treatments were arranged for horseweed biotypes so that each replicate was from individual plants. Each treatment was replicated four times, and each experiment was repeated.

In Vivo Assay

The following solutions, made up in double-distilled deionized water, were used in all leaf disc assay experiments: 10 mM $NH_4H_2PO_4$ (pH 4.4) plus 0.1% Tween 20 surfactant[5] solution (solution A), solution A plus 84.5 mg ae L^{-1} glyphosate[6] solution (solution B), 1.25 N hydrochloric acid[7] (HCl) solution, 0.25% periodic acid[8] with 0.25% metaperiodate[9] solution, and 0.6 M sodium hydroxide[10] with 0.22 M sodium sulfite[11] solution.

Crop Species and Greenhouse Horseweed Biotypes

For crop varieties and the ARR, ARS, DER, DES, MSR, and MSS horseweed biotypes, 100 μl of solution A was added to each well of rows 2 through 8 of eight-row, 96-well microtiter plates.[12] In row 1 of columns 1, 4, 7, and 10 of the 96-well plate, 100 μl of solution A was added to each well. In rows 1 and 2 of columns 2, 3, 5, 6, 8, 9, 11, and 12, 100 μl of solution B was added to each well. Beginning with row 2 of each column, 1:2 dilutions were performed down the columns so that 84.5, 42.3, 21.1, 10.6, 5.3, 2.6, 1.3, and 0.7 mg ae L^{-1} glyphosate could be evaluated. After dilutions, 100 μl of solution was removed from the bottom row of each column and discarded so that each well contained 100 μl of solution. Columns 1, 4, 7, and 10 contained solution A only and served as a nontreated control.

Leaf discs (4 mm diam) were excised from the youngest fully expanded leaves of the crops or the ARR, ARS, DER, DES, MSR, and MSS horseweed plants with a cork borer. Discs were harvested parallel to the midrib of corn leaves (~ 25 cm in length) approximately halfway between the leaf tip and collar. Discs were harvested intervenously from cotton and horseweed leaves and soybean leaflets. One disc was placed in each well of the 96-well microtiter plate.[12] One microtiter plate was used for each crop variety or horseweed biotype. The microtiter plates were covered with transparent plastic wrap and sealed with a rubber band to reduce evaporation, then incubated in a growth chamber at 25 C under continuous light (200 μmol m^{-2} s^{-1}) for 16 h at 75% relative humidity. After incubation, plates were placed in a freezer (−20 C) until solutions were frozen. Plates were thawed at 60 C for 15 min in a forced-air oven. The concentration of shikimate in each well was measured according to the procedure of Cromartie and Polge (2000). Shikimate was extracted from the freeze-thawed leaf discs by adding 25 μl of 1.25 N HCl solution to each well and incubating the plates at 60 C for 15 min in a forced-air oven. A 25-μl aliquot of solution from each well was placed in the

corresponding well of another microtiter plate that contained 100 µl of 0.25% periodic acid with 0.25% metaperiodate solution. Plates were incubated at 22 C for 60 min. A 100-µl aliquot of 0.6 M sodium hydroxide with 0.22 M sodium sulfite solution was then added to each well of each plate, and the optical density at 380 nm was measured with a Synergy HT microplate reader[13] equipped with KC4 software.

A shikimate[14] standard curve was developed by adding known amounts of shikimate to wells containing leaf discs not exposed to glyphosate to report shikimate levels (µg shikimate ml^{-1} HCl solution).

Plates were set up so that leaf discs placed in each column were from a single plant. Each glyphosate concentration including the nontreated control was repeated four times for each crop variety and ARR, ARS, DER, DES, MSR, and MSS horseweed biotype. Each crop and horseweed biotype experiment was conducted in a randomized complete block with individual plants serving as a block. Each experiment was repeated.

Field Horseweed Biotypes

For the Bio1, Bio2, Bio3, Bio4, Bio5, Bio6, Bio7, and Bio8 horseweed biotypes, five 4-mm-diam leaf discs were harvested intervenously from the youngest fully expanded leaves (~ 5 cm length) of each biotype and placed in glass 20-ml vials containing 1 ml of 42.3, 10.6, 2.6, or 1.3 mg ae glyphosate L^{-1} solution. Solution B was diluted with solution A accordingly to develop necessary glyphosate concentration solutions. Vials were capped and transported back to the lab. Caps were removed and vials were placed in a growth chamber maintained at 25 C under continuous light (200 µmol m^{-2} s^{-1}) for 16 h at 75% relative humidity. After incubation, vials were placed in a freezer (−20 C) until solutions were frozen. Vials were thawed at 60 C for 15 min in a forced-air oven. A 0.5-ml aliquot of 1.25 N HCl was added to each vial, and vials were incubated at 60 C for 15 min. A 25-µl aliquot from each vial was placed in an individual well of a 96-well microtiter plate, and the concentration of shikimate was determined as described above.

For the Bio1, Bio2, Bio3, Bio4, Bio5, Bio6, Bio7, and Bio8 horseweed biotypes, vials and plates were arranged in a randomized complete block design with individual plants of each biotype serving as a block for all five glyphosate concentration treatments. Each treatment was replicated four times for each biotype, and each biotype experiment was repeated.

Statistical Analysis

Data represent the average of the two experiments because no experiment by treatment interaction occurred for any study. Data for leaf dip assay experiments and the Bio1, Bio2, Bio3, Bio4, Bio5, Bio6, Bio7, and Bio8 horseweed biotype experiments were subjected to an analysis of variance with the use of the general linear models procedure in SAS (1998). Means were separated by Fisher's Protected LSD test at P = 0.05. Data for glyphosate treatments of leaf disc experiments were presented as amount of shikimate minus amount in nontreated check for each replication within each experiment. Leaf disc data for the crop variety and ARR, ARS, DER, DES, MSR, and MSS horseweed biotype experiments were best fit to sigmoidal logistic regression equations (three-parameter) with SigmaPlot 2000[15] software. A sigmoidal logistic model (Equation 1) (Seber and Wild 1989) was used to relate shikimate levels (Y) to glyphosate concentration (X).

$$Y = a/[1 + (X/X_0)^b] \quad [1]$$

In this equation, a is the difference between the upper and lower response limits (asymptotes), X_0 is the glyphosate rate that results in a 50% reduction in shikimate levels (I_{50}), and b is the slope of the curve around X_0. Pseudo R^2 values were calculated to assess the goodness of fit for the appropriate equation. The R^2 value was obtained by subtracting the ratio of the residual sum of squares to the corrected total sum of squares from 1.0. The residual sum of squares was attributed to that variation not explained by the fitted line. The R^2 and residual mean squares were used to determine the goodness of fit to the regression model.

TABLE 1. Effect of glyphosate concentration on percent necrosis 3 d after treatment of whole leaves from greenhouse-grown plants of conventional and glyphosate-resistant corn, cotton, and soybean.[a–c]

Glyphosate rate[d]	Corn		Cotton		Soybean	
	GR	CV	GR	CV	GR	CV
mg ae L^{-1}	%					
0	0	0	0	0	0	0
300	0	4	2	14	8	22
600	0	8	4	24	10	46
1,200	0	10	5	29	15	56
LSD (0.05)	1.9		3.2		6.2	

[a] Abbreviations: GR, glyphosate-resistant; CV, conventional.

[b] Entire petiole and bottom one-third of next to youngest leaf (second true leaf) of three-leaf cotton and soybean plants and top 10-cm segment of youngest leaf of three-leaf corn plants were dipped in glyphosate/double distilled water solutions for 3 d at 26/21 C (± 3 C) day/night temperature and 12-h photoperiod in a growth chamber.

[c] Percentage of entire leaf area containing necrosis was determined by overlaying a 1.25-cm^2 grid quadrat on the leaf and subtracting the number of squares containing necrosis from the total number of squares for the entire leaf and multiplying that value by 100.

[d] Solutions were developed using double-distilled water and the isopropylamine salt of glyphosate in the formulation of Roundup Custom (contains no surfactant).

Results and Discussion

Leaf Dip Assay

Crop Species

More necrosis was observed on soybean leaves than on cotton and corn leaves at all glyphosate rates (Table 1). Leaves from glyphosate-susceptible plants had more necrotic spots compared with glyphosate-resistant plants of all three species at all glyphosate rates. However, the 600 and 1,200 mg L^{-1} rates of glyphosate were more favorable for differentiating glyphosate-resistant plants from susceptible plants (Table 1). Necrosis was often located around the leaf tip and leaf perimeter of corn leaves, whereas necrosis was distributed uniformly over cotton and soybean leaves. The leaf dip assay clearly differentiated between susceptible and glyphosate resistant crops.

TABLE 2. Effect of glyphosate concentration on percent necrosis 3 d after treatment of whole leaves of greenhouse-grown glyphosate-resistant and -susceptible horseweed plants from Mississippi, Arkansas, and Delaware biotypes and whole leaves of field-grown plants from four glyphosate-resistant and four glyphosate-susceptible horseweed biotypes from Mississippi.[a–c]

Glyphosate rate[d]	Greenhouse-grown plants[e]		Field-grown plants[f]	
	Resistant	Susceptible	Resistant	Susceptible
mg ae L^{-1}	%			
0	0	0	0	0
300	14	38	3	25
600	35	54	8	45
1,200	59	70	24	78
LSD (0.05)	8		7	

[a] Data averaged across resistant or susceptible biotypes for greenhouse-grown plants and resistant or susceptible biotypes for field-grown plants.

[b] Entire petiole and bottom one-third of youngest fully expanded leaves of greenhouse-grown plants (10 cm diam, 32 ± 8 leaves) and field-grown plants (1.5–2 m in height) were dipped in glyphosate/double distilled water solutions for 3 d at 25/22 C (± 3 C) day/night temperatures and 12-h photoperiod in a growth chamber.

[c] Percentage of entire leaf area containing necrosis. Percentage determined by overlaying a 0.635 cm^2 grid quadrat on the leaf and subtracting number of squares containing necrosis from total number of squares for entire leaf and multiplying that value by 100.

[d] Solutions were developed using double-distilled water and the isopropylamine salt of glyphosate in the formulation of Roundup Custom (contains no surfactant).

[e] Resistant biotypes collected from glyphosate-resistant cotton fields in Arkansas and Mississippi and glyphosate-resistant soybean in Delaware. Susceptible biotypes collected from noncropland areas within each state.

[f] Resistant biotypes were collected from three glyphosate-resistant cotton fields near Walls, MS (DeSoto county), and one glyphosate-resistant soybean field near Greenville, MS (Bolivar county). Susceptible biotypes were collected from roadside areas in Coahoma, Bolivar, and Washington counties.

Greenhouse Horseweed Biotypes

The interaction among horseweed biotypes within glyphosate-resistant and -susceptible horseweed types was not significant ($P > 0.05$); thus, data were averaged across horseweed biotypes within resistant and susceptible horseweed types. Leaves of glyphosate-susceptible plants had more necrosis compared with leaves of glyphosate-resistant plants across all glyphosate rates (Table 2). Unlike the crops, the 300 mg L^{-1} rate of glyphosate differentiated between the susceptible and resistant biotypes better than the 600 and 1,200 mg ae L^{-1} rates. At 600 and 1,200 mg L^{-1}, the glyphosate-resistant biotypes showed increasing necrosis and the differences between resistant and susceptible biotypes decreased. Glyphosate-resistant horseweed biotypes do not have the same level of resistance as glyphosate-resistant crops. The result of this assay is not surprising because glyphosate-resistant horseweed biotypes can be controlled with high rates of glyphosate (Mueller et al. 2003; VanGessel 2001). Injury to leaves of glyphosate-resistant and -susceptible plants was necrotic spots on the intervenous portions of the leaf and necrosis around the leaf perimeter, although there was more necrosis to the susceptible leaves compared with resistant leaves.

Field Horseweed Biotypes

The interaction among horseweed biotype within suspected glyphosate-resistant and -susceptible horseweed types was not significant ($P > 0.05$); thus, data were averaged across horseweed biotypes within the four resistant and four susceptible horseweed types. More necrosis was observed on leaves of glyphosate-susceptible horseweed compared with leaves of glyphosate-resistant plants at all glyphosate rates (Table 2). Percent necrosis increased with each successive increase in glyphosate rate. Leaves of resistant plants had only minimal necrosis (3 and 8%) at 300 and 600 mg L^{-1} glyphosate. Necrosis on leaves of resistant plants increased to 24% at 1,200 mg L^{-1} glyphosate. In contrast to greenhouse-grown plants, differences in necrosis between leaves of resistant and susceptible plants were greatest at 600 and 1,200 mg L^{-1} glyphosate. Differences in necrosis between greenhouse- and field-grown horseweed could be attributed to increased hardiness of leaves from field-grown plants. Necrosis of suspected glyphosate-resistant and -susceptible biotypes sampled from grower's fields were similar to known resistant and susceptible horseweed biotypes cultured in the greenhouse described previously.

The leaf dip assay was successful in differentiating suspected glyphosate-resistant and -susceptible horseweed biotypes. The assay has potential for screening field-grown populations of horseweed and confirming their resistance or susceptibility to glyphosate. The 600 mg L^{-1} rate of glyphosate was more consistent in differentiating resistant and susceptible plants compared with the 300 and 1,200 mg L^{-1} rates, particularly for field-grown plants. Similar findings were reported by Koger and Reddy (2005) with a similar rate of glyphosate in a nondestructive leaf dip assay to differentiate leaves of suspected glyphosate-resistant and -susceptible horseweed plants to be used in a subsequent ^{14}C-glyphosate absorption and translocation study.

In Vivo Assay

Crop Species

The in vivo assay clearly differentiated between conventional and GR crops. Leaf discs of conventional corn, cotton, and soybean accumulated shikimate in a dose-dependent response to glyphosate treatment, whereas there was no accumulation of shikimate in GR varieties regardless of glyphosate concentration tested compared with the nontreated check (Table 3). The sensitivity of each glyphosate-susceptible crop to glyphosate was reflected by I_{50} values of 3.6 to 12.4 mg ae L^{-1}. These results support previous work that examined shikimate accumulation in intact plants of conventional corn, soybean, and cotton, but not in GR varieties of these same crops (Henry et al. 2004; Pline et al. 2002; Shaner et al. 2005; Singh and Shaner 1998).

The differences in accumulation of shikimate among the three crops reflect the necrosis levels observed in the leaf dip assay. Corn accumulated the least amount of shikimate and also had the least amount of necrosis. Soybean accumulated the most shikimate, particularly at lower concentrations of glyphosate, and had the greatest amount of necrosis. Although it is difficult to directly compare the two assays because the glyphosate concentrations used in the in vivo assay were much lower than those used in the leaf dip assay, the results of the two assays were complementary.

Horseweed

The in vivo assay detected major differences between susceptible and GR horseweed populations grown in the green-

TABLE 3. Effect of glyphosate concentration on shikimate levels in leaf discs from greenhouse-grown conventional and glyphosate-resistant corn, cotton, and soybean.[a–d]

Glyphosate concentration	Corn		Cotton		Soybean	
	GR	CV	GR	CV	GR	CV
mg ae L^{-1}	μg shikimate ml^{-1}					
84.5	0	2.7	0	33.1	0	39.0
42.3	0	2.1	0	28.6	0	38.5
21.1	0	2.0	0	24.3	0	29.5
10.6	0	1.5	0	19.6	0	23.4
5.3	0	1.1	0	12.6	0	19.4
2.6	0	0.9	0	8.3	0	18.9
1.3	0	0.6	0	1.3	0	16.5
0.7	0	0.1	0	0.8	0	9.4
LSD (0.05)	0.8		5.8		8.7	
Logistic regression parameters[e]						
a	—	3.2	—	34.3	—	40
b	—	−0.7	—	−1.1	—	−0.7
X_0	—	12.4	—	8.8	—	3.6
R^2	—	0.97	—	0.99	—	0.98

[a] Abbreviations: GR, glyphosate-resistant; CV, conventional.
[b] Plants were grown in the greenhouse at 26/21 C (± 3 C) temperature with a 12-h photoperiod. Natural light was supplemented with sodium halide lights (400 μmol m^{-2} s^{-1}).
[c] Leaf discs were incubated in ammonium phosphate plus glyphosate solutions for 16 h at 25 C under continuous light (300 μmol m^{-2} s^{-1}).
[d] Amount of shikimate in nontreated check (not presented) was subtracted from shikimate level in glyphosate-treated leaf discs of each variety.
[e] Sigmoidal logistic regression equation: $Y = a/[1 + (X/X_0)^b]$. X_0 is the glyphosate rate that results in a 50% reduction in shikimate levels (I_{50}).

house and the field. Shikimate accumulated to similar levels in leaf discs from both biotypes grown in the greenhouse at 21 to 84 mg L^{-1} glyphosate concentrations (Table 4). However, there was much less accumulation of shikimate in leaf discs from resistant biotypes compared with susceptible biotypes at glyphosate levels < 21.1 mg L^{-1} (Table 4). I_{50} values were calculated for the GR biotypes and ranged from 4.8 to 21.5 mg ae L^{-1} (Table 4). However, meaningful I_{50} values for the susceptible biotypes could not be calculated because of little difference in shikimate levels across the glyphosate concentrations tested.

TABLE 5. Effect of glyphosate concentration on shikimate levels in leaf discs from field-grown glyphosate-resistant and -susceptible horseweed biotypes from Mississippi.[a–d]

Glyphosate concentration	Resistant				Susceptible			
	Bio1	Bio2	Bio3	Bio4	Bio5	Bio6	Bio7	Bio8
mg ae L^{-1}	μg shikimate ml^{-1}							
42.3	9.9	35.9	62.3	26.3	11.7	23.6	22.9	22.2
10.6	4.4	28.9	41.1	17.1	11.9	20.2	18.6	19.3
2.6	0.0	9.6	6.1	1.5	8.8	16.9	18.1	19.2
1.3	0.0	3.1	0.5	0.5	6.3	15.8	16.1	17.6
LSD (0.05)	7.2							

[a] Abbreviation: Bio, biotype.
[b] Leaf discs were obtained from the youngest fully expanded leaves and incubated in ammonium phosphate plus glyphosate solutions for 16 h at 25 C under continuous light (300 μmol m^{-2} s^{-1}).
[c] Plants of resistant biotypes (1.2–2 m in height) were collected from three glyphosate-resistant cotton fields near Walls, MS (DeSoto county), and one glyphosate-resistant soybean field near Greenville, MS (Bolivar county). Plants of susceptible biotypes (1.8–2 m in height) were collected from roadside areas in Coahoma, Bolivar, and Washington counties.
[d] Amount of shikimate in nontreated check (not presented) was subtracted from shikimate level in glyphosate-treated leaf discs of each biotype.

The distinctions between susceptible and GR field-grown horseweed biotypes were also demonstrated by the in vivo EPSPS assay. All biotypes accumulated similar amounts of shikimate at glyphosate concentrations of 10.6 to 42.3 mg L^{-1}, but accumulation of shikimate in the resistant biotypes at 1.3 to 2.6 mg L^{-1} glyphosate was much less than in the susceptible biotypes (Table 5). As with the greenhouse-

TABLE 4. Effect of glyphosate concentration on shikimate levels in leaf discs from greenhouse-grown glyphosate-resistant and -susceptible horseweed biotypes from Mississippi, Arkansas, and Delaware.[a–c]

Glyphosate concentration	Arkansas		Delaware		Mississippi	
	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
mg ae L^{-1}	μg shikimate ml^{-1}					
84.5	35.6	37.4	29.2	27.7	36.6	23.7
42.3	34.7	36.2	27.9	26.6	25.1	22.6
21.1	21.6	35.1	27.3	26.4	23.2	21.2
10.6	11.7	34.1	26.6	24.8	11.6	20.6
5.3	4.6	33.2	17.4	24.2	4.8	19.2
2.6	0	32.2	5.1	22.9	1.5	18.6
1.3	0	31.3	0	22.5	1.2	17.8
0.7	0	29.8	0	21.9	0	17.2
LSD (0.05)	6.8		5.2		3.3	
Logistic regression parameters[d]						
a	38.3	—	28.3	—	40.8	—
b	−1.9	—	−2.9	—	−1.3	—
X_0	16.9	—	4.4	—	21.5	—
R^2	0.99	—	0.99	—	0.97	—

[a] Plants were grown in the greenhouse at 26/21 C (± 3 C) temperature with a 12-h photoperiod. Natural light was supplemented with sodium halide lights (400 μmol m^{-2} s^{-1}).
[b] Leaf discs were incubated in ammonium phosphate plus glyphosate solutions for 16 h at 25 C under continuous light (300 μmol m^{-2} s^{-1}).
[d] Amount of shikimate in nontreated check (not presented) was subtracted from shikimate level in glyphosate-treated leaf discs of each biotype.
[e] Sigmoidal logistic regression equation: $Y = a/[1 + (X/X_0)^b]$. Accurate I_{50} values for the susceptible biotypes could not be calculated because of little difference in shikimate levels across all glyphosate concentrations tested.

grown horseweed, we could not calculate reasonable I_{50} values for the susceptible biotypes.

The responses of horseweed biotypes to glyphosate are consistent with previous research. Shikimate accumulated in susceptible and GR horseweed biotypes sprayed with 840 g ha^{-1} glyphosate (Mueller et al. 2003). Shikimate did not accumulate in GR horseweed plants treated with a sublethal dose (8.9 μg $plant^{-1}$) of glyphosate (Feng et al. 2004; Koger and Reddy 2005). This same glyphosate concentration of 8.9 μg $plant^{-1}$ caused shikimate accumulation in a susceptible horseweed biotype. Similar distinctions between susceptible and GR biotypes were observed with the in vivo assay. Shikimate accumulated at high concentrations of glyphosate in all biotypes, but there was much less accumulation in resistant biotypes compared with susceptible biotypes at low glyphosate concentrations.

These results suggest the in vivo assay can detect not only target site–based resistance but also resistance from limited absorption and translocation of glyphosate. Shikimate did not accumulate in GR crops that contained an insensitive EPSPS. The in vivo assay results were consistent with a reduced absorption and translocation mechanism of resistance. At high glyphosate concentrations, shikimate accumulated in GR horseweed leaf discs, suggesting that EPSPS was still sensitive to the herbicide. However at low glyphosate concentrations, little or no shikimate accumulated in the GR horseweed because of reduced movement of herbicide to the active site. The mechanism of glyphosate resistance in horseweed is reduced glyphosate uptake by the chloroplast and reduced translocation to the growing points, not an altered EPSPS (Feng et al. 2004).

The leaf dip assay and in vivo assay are complementary and should be used in tandem. An artifact of the leaf dip assay could be injury that is unrelated to glyphosate, such as surfactant, excess salts, etc. However, if the injury rating is related to increased shikimate levels, there is greater certainty that differences among biotypes are due to glyphosate tolerance. These two assays should have utility in screening other weed populations for resistance to glyphosate.

Sources of Materials

[1] Jiffy mix, Jiffy Products of America Inc., 951 Swanson Drive, Batavia, IL 60510.

[2] High-density polyethylene vial, Fisher Scientific, Liberty Lane, Hampton, NH 03842.

[3] Roundup CUSTOM®, isopropylamine salt of glyphosate, Monsanto Company, 800 North Linbergh Boulevard, St. Louis, MO 63167.

[4] Ammonium phosphate monobasic, A.C.S. grade, Mallinckrodt Chemical Works, 2nd and Mallinckrodt Streets, St. Louis, MO 63160.

[5] Tween 20, Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201.

[6] Roundup Weathermax®, potassium salt of glyphosate, Monsanto Company, 800 North Linbergh Boulevard, St. Louis, MO 63167.

[7] Hydrochloric acid 37%, A.C.S. grade, Mallinckrodt Chemical Works, 2nd and Mallinckrodt Streets, St. Louis, MO 63160.

[8] Periodic acid, A.C.S. grade, Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201.

[9] Potassium meta-periodate, A.C.S. grade, J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, NJ 08865.

[10] Sodium hydroxide, A.C.S. grade, Fisher Scientific Co., 1 Reagent Lane, Fair Lawn, NJ 07410.

[11] Sodium sulfite anhydrous, A.C.S. grade, Mallinckrodt Chemical Works, 2nd and Mallinckrodt Streets, St. Louis, MO 63160.

[12] Fisher Scientific, Fisherbrand flat-bottom 96-well plate, Liberty Lane, Hampton, NH 03842.

[13] Microplate reader, Synergy HT, BIO-TEK Instruments Inc., Highland Park, P.O. Box 998, Winooski, VT 05404.

[14] Shikimic acid, technical grade, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.

[15] SigmaPlot, 2000 for Windows version 6.10. SPSS Inc., 233 South Wacker Drive, Chicago, IL 60606.

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