Weed Science, 53:769–774. 2005

A rapid in vivo shikimate accumulation assay with excised leaf discs

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An in vivo shikimate accumulation assay with excised leaf tissue was developed to provide a fast and reliable method for identifying glyphosate-resistant plants. The assay is based on glyphosate-induced accumulation of shikimate. There was a linear accumulation of shikimate in excised leaf discs of soybean and canola treated with 250 μM glyphosate for 48 h. The IC_{50} for the accumulation of shikimate in soybean and corn leaf discs was 34 and 87 μM, respectively. Leaf discs excised from glyphosate-resistant corn or soybean did not accumulate shikimate when treated with 500 μM glyphosate. Leaf discs taken from a number of field-grown plants accumulated shikimate in a glyphosate dose–dependent manner. The accumulation of shikimate was dependent on light and the age of the leaf from which the disc was taken. The assay worked either in 96-well microtiter plates or in vials, and it clearly differentiated between glyphosate-resistant and -susceptible crops in which the resistance is due to an alteration of the target site for glyphosate. The assay was simple and robust and has the potential to be used as a high throughput assay to detect glyphosate resistance in weeds.

Nomenclature: Glyphosate; canola, *Brassica napus* L. 'Hayola 420'; corn, *Zea mays* L. 'Pioneer 37M34', 'Dekalb DK493RR/BTY'; soybean, *Glycine max* (L.) Merr., 'Asgrow A2869', 'Asgrow AG3003'.

Key words: Glyphosate resistance, resistance detection.

Glyphosate is a versatile herbicide that controls many annual and perennial weeds. The introduction of glyphosate-resistant (GR) crops in the late 1990s provided farmers with a simple, broad-spectrum weed control option (Reddy and Koger 2005; Shaner 2000). In 2004, GR-cotton (*Gossypium hirsutum* L.) and GR-soybean were planted in the United States on more than 60% and 80% of planted hectares, respectively (USDA-NASS 2004).

The increased use of glyphosate for weed control in GR crops has led to the selection of resistant weed biotypes. The first GR-horseweed [*Conyza canadensis* (L.) Cronq.] biotypes were discovered in Delaware in 2000 (VanGessel 2001) and are now found in 10 U.S. states and are reported to infest over 100,000 ha (Heap 2004). GR-weed biotypes have also been found throughout the world in five other species, including rigid ryegrass (*Lolium rigidum* Gaudin), Italian ryegrass (*Lolium multiflorum* Lam.), goosegrass [*Eleusine indica* (L.) Gaertn.], hairy fleabane [*Conyza bonariensis* (L.) Cronq.], and buckhorn plantain (*Plantago lanceolata* L.) (Heap 2004).

The rapid appearance of glyphosate-resistant weed populations in GR-crops has alarmed many farmers and crop advisors (Brunoehler 2004). However, there are several reasons in addition to resistance why glyphosate may provide inadequate and inconsistent weed control. These include misapplication, water or heat stress, spraying weeds that are too large, or using inadequate surfactants. It is important to rapidly determine if poor control by glyphosate is due to resistance so that the existing weed management practices can be altered before the resistant population spreads. Thus, there is a need for a rapid and accurate method to detect glyphosate resistance.

There are multiple methods to screen for resistance (Beckie et al. 2000). Field and greenhouse whole-plant screens are probably the most definitive, but such assays are slow and expensive. Petri plate assays testing seed germination and root elongation in the presence of herbicides have been used to detect resistance to acetyl coenzyme A carboxylase (ACCase) and acetolactate synthase (ALS) inhibitors and to glyphosate (Beckie et al. 2000; Escorial et al. 2001; Perez and Kogan, 2003; Tal et al. 2000). These assays are relatively rapid but require seeds, which often limits their utility for making decisions early in the growing season.

A different screening approach developed for grasses is to take cuttings with roots from plants that have escaped herbicide treatment, transplanting them in the greenhouse where they are treated again with the herbicide (Boutsalis 2001). However, this assay could be expensive, requires extensive manipulation of the material, and may not be suitable for all species.

A more diagnostic screening method involves extracting EPSPS (5-enolpyruvoylshikimate-3-phosphate synthase), which is the site of action of glyphosate (Steinrucken and Amrhein 1980) and testing its sensitivity to glyphosate in vitro. The in vitro assay was used to determine that a GR goosegrass population has a less sensitive target site (Baerson et al. 2002) and that a GR ryegrass population did not (Baerson et al. 2000). The major limitation of this assay is the availability of shikimate-3-phosphate (S3P), which is one of the substrates for EPSPS. Producing S3P requires access to sophisticated biochemistry lab equipment that is often unavailable to many researchers.

An alternative method to determining whether a suspected GR weed has a resistant EPSPS is to monitor the effect of glyphosate on shikimate levels. When glyphosate inhibits EPSPS, shikimate, the dephosphorylated substrate of the en-

zyme, accumulates (Amrhein et al. 1980). There are relatively simple methods for extracting and measuring shikimate levels in plant tissue (Cromartie and Polge 2000; Singh and Shaner 1998). Shikimate accumulation after glyphosate treatment has been used to identify GR-soybean and GR-cotton with a resistant EPSPS (Pline et al. 2002; Singh and Shaner 1998). Similar methods were used to detect resistance in GR-weeds. Shikimate did not accumulate in a GR–rigid ryegrass population treated with glyphosate (Simarmata et al. 2003) or in a GR-horseweed population treated with a sublethal rate of glyphosate, although the same rate did cause shikimate accumulation in a susceptible horseweed population (Feng et al. 2004).

Leaf discs assays have been used to detect resistance to Photosystem II inhibitors (Hensley 1981) and ALS inhibitors (Gerwick et al. 1993, Uchino et al. 1999). If a similar assay could be developed to detect glyphosate resistance, then this assay could be used to test weed populations in the field. Shikimate has been shown to accumulate in excised buckwheat (*Fagopyrum esculentum* Moench.) hypocotyls and cotyledons (Amrhein et al. 1980, Tokhver and Pal'm 1986) treated with glyphosate. It may be possible to measure changes in shikimate accumulation in leaf discs incubated in a solution containing glyphosate as a simple method for screening populations for resistance to glyphosate.

The objectives of this research were (1) to develop an excised leaf assay to measure the inhibition of EPSPS by glyphosate based on shikimate accumulation, (2) to determine what factors influence the accumulation of shikimate in excised leaf tissue, and (3) to determine if this assay could differentiate between glyphosate-susceptible and -resistant plants.

Materials and Methods

Plant Material

Greenhouse-Grown Plants

Seeds of corn ('Pioneer 37M34'; 'Dekalb DK493RR/BTY'), soybean ('Asgrow A2869'; 'Asgrow AG3003'), canola ('Hayola 420') sunflower () ('Cargill SF 187'), dry beans () ('Pinto beans Bill Z'), sugar beet () ('Monohikori') and wheat (*Triticum aestivum* L.) ('Avalanche') were planted in 26- by 26- by 6-cm-deep trays containing Metro Mix® 200[1] potting soil supplemented with 14–14–14 Osmocote®[2] pellets. Spotted knapweed (*Centaurea maculosa* Lam.) seed was sown in 4.8-L pots containing Metro Mix® 200 potting soil and grown for 3 mo. Plants were watered daily and fertilized every 14 d. Horseweed seed was sown in 10-L pots containing Metro Mix® 350[3] potting soil. Pots were subirrigated until seed had germinated and then thinned to 1 plant per pot. All plants were grown in the greenhouse at 21–27/14–18 C (day/night) with supplemental metal halide lamps to provide a 14-h photoperiod. All plants were watered with an automatic sprinkler system twice a day and sprayed weekly with an insecticide to control insects. Leaf discs were collected from crop plants in the three- to seven-leaf growth stage and from spotted knapweed and horseweed plants that were 26 cm tall and in the 25- to 30-leaf growth stage.

Field-Grown Plants

Leaf discs were collected in September and October 2004 from prickly lettuce (*Lactuca sativa* L.), dandelion (*Taraxacum officinale* Weber in Wiggers), field bindweed (*Convolvulus arvensis* L.), common lambsquarters (*Chenopodium album* L.), blue mustard [*Chorispora tenella* (Pallas) DC], hairy nightshade (*Solanum sarrachoides* Sendtner), and common mallow (*Malva neglecta* Wallr) that were growing near Akron and Fort Collins, CO. Prickly lettuce, dandelion, blue mustard, and common mallow plants were in the rosette stage with 10 to 40 leaves. Field bindweed, common lambsquarters, and hairy nightshade were flowering when the plants were sampled.

In Vivo Shikimate Accumulation Assay

Microtiter Plate Assay

Leaf discs (4-mm diam) were excised from leaves with a modified cork borer equipped with a spring-loaded plunger. One disc was placed in each well of a 96-well microtiter plate.[4] Control wells contained 10 mM ammonium phosphate plus 0.1% (v/v) Tween 80 surfactant. The glyphosate treatment wells contained 100 μl of 10 mM ammonium phosphate (pH 4.4), 0.1% (v/v) Tween 80 surfactant and various concentrations of glyphosate[5] ranging from 4 μM to 500 μM. Plates were covered with a lid, sealed with a strip of Parafilm,®[6] and wrapped in plastic wrap to minimize evaporation. Plates were then incubated under fluorescent lights (150 μM m^{-2} s^{-1}) at 26 C for 16 to 23 h, except for the time course study. After incubation, plates were placed in a −20 C freezer until the solution froze and then thawed at room temperature or at 60 C for 30 min. Twenty-five microliters of 1.25 N HCl were pipetted into each well, giving a final concentration of 0.25 N HCl per well. The plates were incubated at 60 C for 15 min. At the end of this incubation, the discs had turned a uniform gray–green, indicating complete penetration of the tissue by the acid. Shikimate was determined spectrophotometrically following the procedure of Cromartie and Polge (2000). Aliquots of 25 μl were transferred from each well to another microtiter plate to which 100 μl of 0.25% (w/v) periodic acid[7]/0.25% (w/v) *m*-periodate[8] was added to each well. This plate was incubated at room temperature (25 C) for 90 min and then 100 μl of 0.6 N sodium hydroxide/0.22 M sodium sulfite was added to each well. The optical density at 380 nm was measured within 30 min using a microtiter plate spectrophotometer[9]. Background optical density was determined from the wells containing the control discs and was subtracted from each of the glyphosate treatments. A shikimate standard curve was developed by adding known amounts of shikimate[10] to wells containing leaf discs not exposed to glyphosate so that shikimate levels could be reported as micrograms shikimate per milliliter HCl solution.

Vial Assay

Ten to 20 leaf discs (4-mm diam) were collected from 3 to 6 plants and placed in a 20-ml vial containing 1 ml of the assay solution. Control vials contained 10 mM ammonium phosphate (pH 4.4) plus 0.1% (v/v) Tween 80. Glyphosate treatment vials contained 10 mM ammonium phosphate (pH 4.4), 0.1% (v/v) Tween 80, plus varying concentrations of glyphosate (1000, 500, 125, 32, or 8 μM glyphosate). Vials were capped and transferred to the lab where they were incubated under light (150 μM m^{-2} s^{-1}) for 16

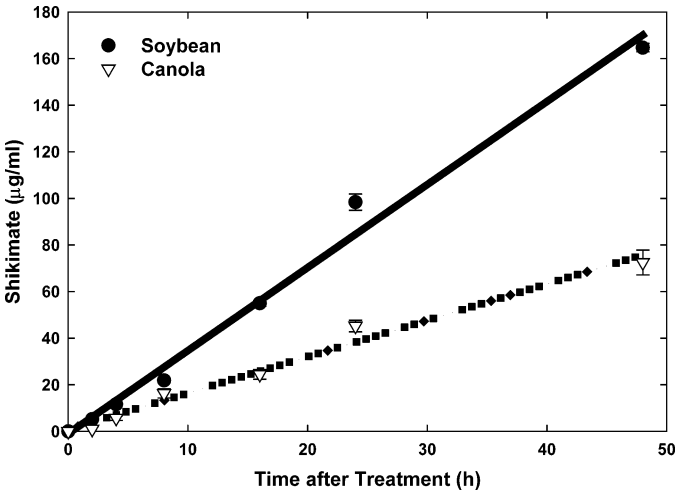

FIGURE 1. Accumulation of shikimate in soybean and canola leaf discs. Discs (4-mm diam) were excised from the third true leaf of soybean and canola and incubated in 250 µM glyphosate. Data indicate the average amount of shikimate accumulated in eight discs that were harvested at different times after initiation of the treatment. Bars indicate ± 1 standard error.

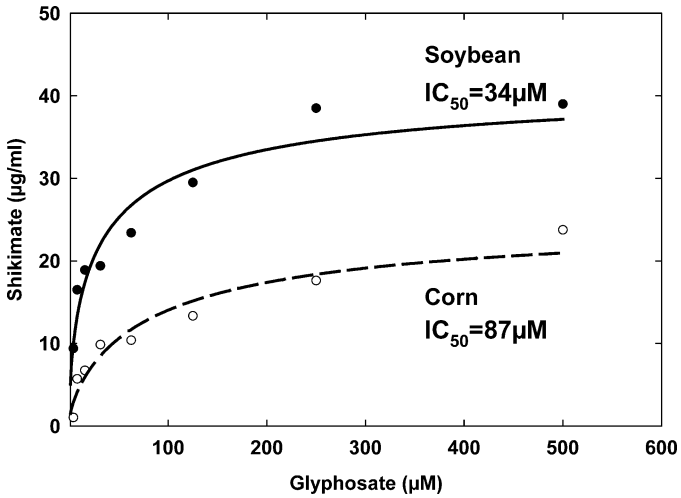

FIGURE 2. Dose response of corn and soybean leaf discs to glyphosate. Discs were taken from the third leaf of soybeans and corn. Equation for corn: $y = 0.97 + 25/1 + [x/88]^{-0.8}$); $R^2 = 0.98$. Equation for soybean: $y = 2.6 + 40/1 + [x/33]^{-0.68}$); $R^2 = 0.98$.

h. After incubation, 0.25 ml of 1.25 N HCl was pipetted into each vial and then the vials were placed in a freezer and kept frozen until assayed for shikimic acid. Shikimate levels were determined by transferring 25 µl solution from each vial to a microtiter plate and continuing as described previously. Each treatment was replicated three to eight times and incubation was in a completely random design. All experiments were repeated at least once.

Effect of Light on Shikimate Accumulation

The in vivo shikimate accumulation assay was conducted as described for the microtiter plate assay. Soybeans were transferred from the greenhouse to a growth chamber[11] (26/20 C [day/night] with a 14-h photoperiod (200 µM m^{-2} s^{-1}) and allowed to grow for 3 d. Discs were removed from the youngest, rapidly expanding leaf of plants that had been exposed to 0, 4, or 8 h of light. The assay solutions were as described previously. Each treatment was replicated eight times and the experiment repeated three times.

Statistical Analysis

Treatments were arranged in a completely randomized design within each experiment. Means and standard errors were calculated for each treatment. Leaf-disc data from corn and soybean and weeds collected in the field were best fit to a sigmoidal logistic regression Equation 1 using SigmaPlot 9.0[12] software:

$$Y = y0 + \frac{a}{1 + (X/X_0)^b} \quad [1]$$

In this equation, a is the difference of the upper and lower response limits (asymptotes); X_0 is the glyphosate rate that results in a 50% reduction in shikimate levels (IC_{50}). $y0$ is the y intercept 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. 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.

Data for measuring the effect of light pretreatment on shikimate accumulation was analyzed with a mixed model using the SAS PROC MIXED procedure[13] and means were separated at the P = 0.05 level using Tukey's multiple comparison procedure.

Results and Discussion

Time Course and Dose Response for Shikimate Accumulation

Shikimate accumulation was linear for 48 h after treating soybean and canola leaf discs with 250 µM glyphosate and accumulation was greater in soybean than in canola (Figure 1). Based on the approximate leaf disc fresh weight (FW) of 2.5 mg $disc^{-1}$, the concentrations of shikimate were 8,200 µg g^{-1} FW in soybean and 3,600 µg g^{-1} FW in canola after 48 h. These levels were similar to those found in soybean (Singh and Shaner 1998) and canola (Harring et al. 1998) leaves 48 h after treatment with glyphosate. The similar response of intact and excised leaf tissue to glyphosate suggests that the inhibition of EPSPS by glyphosate is the same in both systems and that leaf discs might be used to predict the response of whole plants to the herbicide.

Shikimate accumulation in glyphosate-susceptible (GS) soybean and corn leaf discs reached a maximum at 250 µM glyphosate (Figure 2), and the IC_{50} values calculated for soybeans and corn were approximately 34 and 87 µM, respectively. There was no accumulation of shikimate in GR corn or soybean leaf discs incubated in 500 µM glyphosate (data not shown). The data show that the in vivo shikimate accumulation assay can differentiate between GS and GR plants when the resistance is due to a resistant target site. Singh and Shaner (1998) and Pline et al. (2002) also found no accumulation of shikimate in GR soybean or cotton leaves after spraying with glyphosate.

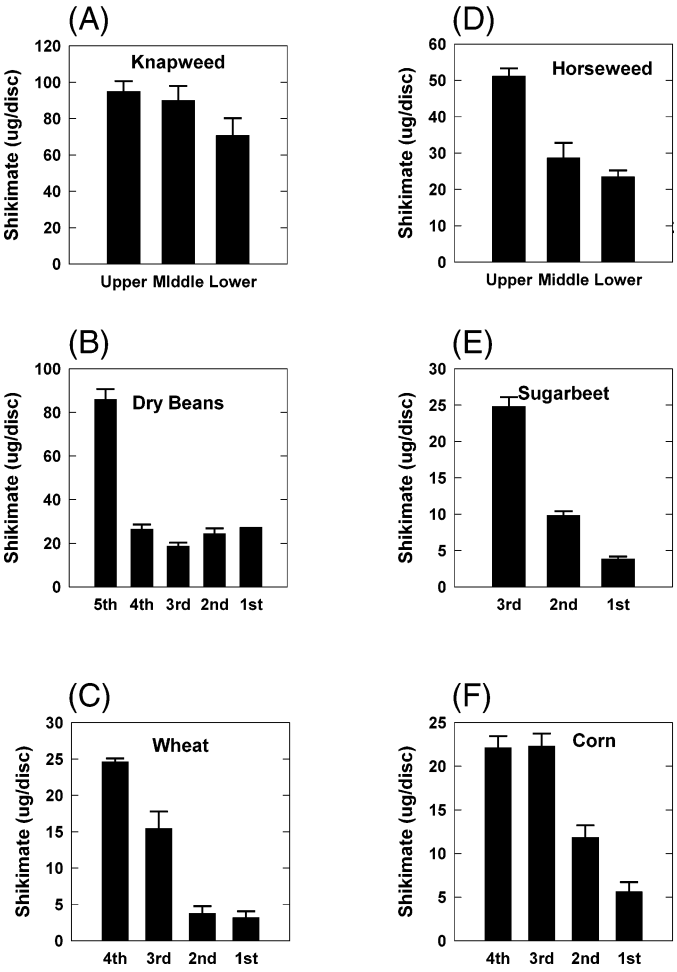

FIGURE 3. Effect of leaf age on shikimate accumulation in leaf discs taken from different leaves of spotted knapweed (A), dry bean (B), wheat (C), horseweed (D), sugar beet (E), and corn plants (F). All discs were treated with 250 μM glyphosate and incubated for 16 h under continuous light. Data indicate the average amount of shikimate accumulated in eight discs taken from different plants. (A) Spotted knapweed: plants had 20 to 30 leaves; upper were young leaves and lower were oldest leaves. (B) Dry bean: fifth was youngest leaf and first was the oldest leaf. (C) Wheat: plants in three-leaf stage; third is youngest emerging leaf and first is oldest leaf. (D) Horseweed: plants had 20 to 40 leaves; upper were young leaves and lower were oldest leaves. (E) Sugar beet: plants had three leaves; third was youngest leaf and first was oldest leaf. (F) Corn: plants had four leaves; fourth was the youngest leaf and first was oldest leaf. Bars indicate ± 1 standard error.

Effect of Leaf Age

The age of the leaf from which a disc was taken affected the rate of shikimate accumulation across a number of species. Shikimate accumulated in excised leaf discs from all species tested. In most cases the highest shikimate accumulation was detected in leaf discs taken from the youngest leaves (Figure 3). When leaf discs were removed from different positions along the youngest leaf of wheat or corn, the highest shikimate accumulation was found in the youngest tissue toward the base of the leaf (Figure 4). These results agree with reports that young leaves of soybean and pigweed plants sprayed with glyphosate accumulated four- to eightfold more shikimate than mature leaves of the same plants (Lydon and Duke 1988). In vitro measurements show that EPSPS activity is the highest in wheat stem meristems (Arnaud et al. 1994) and crown tissue in goosegrass (Baerson et al. 2002). In a similar manner, in vivo ALS assays detect the highest activity in the youngest leaves (Gerwick et al. 1993; Shim et al. 2003). EPSPS and ALS are enzymes in amino acid biosynthesis pathways that are most active in young, rapidly dividing plant tissue (Schmid and Amrhein 1999; Singh 1999). To maximize the accumulation of shikimate in the in vivo shikimate accumulation assay it is important to sample young, rapidly growing leaves.

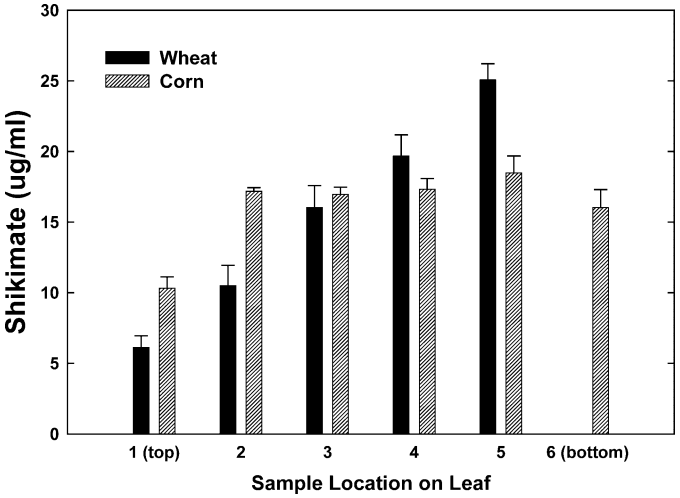

FIGURE 4. Effect of leaf age on shikimate accumulation in leaf discs taken at different positions from the tip (position 1) to the base of the leaf (position 5 or 6) of the third leaf of corn and wheat. All discs were treated with 250 μM glyphosate and incubated for 16 h under continuous light. Data indicate the average amount of shikimate accumulated in eight discs taken from different plants. Bars indicate ± 1 standard error.

Effect of Light

The most consistent accumulation of shikimate occurred when the discs were incubated under continuous light (Table 1). A similar response was noted in excised buckwheat hypocotyls and cotyledons in which shikimate accumulation was greatly increased when the tissues were incubated with glyphosate in the light vs. the dark (Amrhein et al. 1980; Tokhver and Pal'm 1986). In addition, in our assay the time of day in which a disc was taken had a major impact on whether or not shikimate accumulated in the dark. Discs taken from plants that had not been exposed to light did not accumulate shikimate in the dark, whereas discs taken from plants that had been exposed to 8 h of light accu-

TABLE 1. Effect of light pretreatment and incubation conditions on shikimate accumulation in soybean leaf discs.[a,b,c]

Light pretreatment	Incubation	
	Light	Dark
h	μg shikimate ml^{-1}	
0	48.7a	3.7b
4	57.3a	20.1b
8	46.4a	46.2a

[a] Discs were incubated for 23 h under continuous light (150 μM m^{-2} sec^{-1}) or in darkness in 250 μM glyphosate.

[b] Means followed by the same letter within a row are not different (P = 0.05).

[c] Discs were excised from plants that were exposed to 10 h of dark followed by 0, 4, or 8 h of light (200 μM m^{-2} sec^{-1}).

TABLE 2. Accumulation of shikimate in leaf discs from field-grown plants.[a,b,c]

Variable	Prickly lettuce	Dandelion	Field bindweed	Common lambsquarters	Blue mustard	Hairy nightshade	Common mallow
Glyphosate concentration μM	μg shikimate ml^{-1}						
1,000	50.3	49.2	44.9	23.1	65.4	11.6	16.8
250	35.0	43.9	55.0	9.5	43.4	12.3	13.4
63	31.4	26.6	41.9	6.1	8.5	4.4	10.9
16	1.6	1.5	24.1	4.4	1.9	0.0	2.0
4	0.3	1.6	6.2	3.6	0.2	0.0	0.7
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Logistic regression parameters							
a	51	49.1	44.8	23.8	67.1	12.3	16.8
b	−2	−2	−1.7	−2	−2	−2	−2
IC_{50}	53	59	15	363	193	104	50
R^2	0.98	0.99	0.99	0.89	0.99	0.98	0.99

[a] Discs were collected from plants growing near Akron and Fort Collins, CO. Discs were taken from the youngest leaves of populations of plants and placed in vials containing the assay solution of 10 mM ammonium phosphate (pH 4.4) and 0.1% (v/v) Tween 80 plus various concentrations of glyphosate. See Materials and Methods for growth stages of weeds.

[b] Leaf discs were incubated in ammonium phosphate plus glyphosate solutions for 16 h at 25 C under light (300 μM m^{-2} s^{-1}).

[c] Logistic regression equation: $Y = y0 + a/(1 + [x/IC_{50}])$.

mulated similar shikimate levels in the dark or the light (Table 1). The in vivo ALS assay also requires light to get maximum accumulation of acetolactate (Uchino et al. 1999).

In Vivo Shikimate Accumulation Assay with Field-Grown Plants

Discs were taken from the youngest leaves of various weed species growing in the field. This assay used vials instead of microtiter plates and discs were taken from a number of plants within a population. Because the sampling was done late in the season, some of the plants were growing vigorously and others were senescing. Leaf discs taken from senescing plants did not accumulate shikimate at 1,000 μM glyphosate (data not shown). Discs taken from plants that were actively growing at the time of sampling accumulated shikimate (Table 2). There were differences among the species in both the amount of shikimate accumulated and in the calculated IC_{50} values. The IC_{50} varied from a high of 363 μM for common lambsquarters to a low of 15 μM for field bindweed. Common lambsquarters is relatively tolerant to glyphosate (Shaner 2000), and this high IC_{50} may reflect this natural tolerance. However, field bindweed is also tolerant to glyphosate (Westwood and Weller 1997) but had the lowest IC_{50}, so it is difficult to draw any conclusions from these data without sampling more populations of both species at multiple growth stages.

Utility of In Vivo Shikimate Accumulation Assay

These data show that the in vivo shikimate accumulation assay can be used on field-grown plants and that it works across a number of crop and weed species. However, inaccurate findings may result if plant tissue is not collected from actively growing plants. The in vivo shikimate accumulation assay could differentiate between susceptible and GR crops in which the resistance is due to an alteration of EPSPS. The assay might be used to detect GR weed populations when plants are small, early in the growing season, and would provide a quick turnaround time for determining if GR is present due to an alteration in EPSPS. It has yet to be shown if this same assay can detect resistance due to an alteration in uptake or translocation of glyphosate. The assay is rapid and has high throughput because shikimate is extracted by freeze-thawing and shikimate is detected by an improved spectrophotometric method. Other methods for detecting shikimate are more cumbersome and time- and instrument-intensive (Lydon and Duke 1988; Pline et al. 2002). However, this assay has limitations in that it requires sophisticated equipment, which would restrict its use by producers.

Sources of Materials

[1] Metro Mix® 200 soil, Scott-Sierra Horticultural Products Co., 14111 Scottslawn Road, Marysville, OH 43041.

[2] Osmocote®, Scott-Sierra Horticultural Products Co., 14111 Scottslawn Road, Marysville, OH 43041.

[3] Metro Mix® 350 soil, Scott-Sierra Horticultural Products Co., 14111 Scottslawn Road, Marysville, OH 43041.

[4] Nunc Microwell 96-well plate, VWR International Inc., 17750 East 32nd Place, Suite 10, Aurora, CO 80011.

[5] Roundup Ultramax®, isopropylamine salt of glyphosate, Monsanto Co., 800 North Linbergh Boulevard, St. Louis, MO 63167.

[6] Parafilm®, Fisher Scientific, 4500 Turnberry Drive, Hanover Park, IL 60103.

[7] Periodic acid, Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201.

[8] *m*-periodate, Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201.

[9] Kinetic microplate reader model UVmax, Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA 94089.

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

[11] Conviron model E15, Controlled Environments Inc. (CONVIRON), 222 South 5th Street, Pembina, ND 58271.

[12] SigmaPlot 9.0, Systat Software, Inc., 501 Canal Boulevard, Suite C, Point Richmonds, CA 94804.

[13] SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513.

Acknowledgment

This research was partially supported by Vaadia-BARD Postdoctoral Fellowship Award No. FI-348-2003 from BARD, the United States-Israel Binational Agricultural Research and Development Fund.

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Received February 3, 2005, and approved June 8, 2005.