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Library Copy -- 2007 -- #452

Weed Science 2007 55:1–5

Shikimate Accumulation in Sunflower, Wheat, and Proso Millet after Glyphosate Application

W. Brien Henry, Dale L. Shaner, and Mark S. West*

Experiments were conducted to examine the utility of a spectrophometric leaf disc assay for detecting shikimate accumulation after glyphosate application in sunflower, proso millet, and wheat. The assay was conducted on both greenhouse- and field-grown plants. Glyphosate was applied at five rates ranging from 840 to 53 g ae ha^{-1}. Shikimate accumulation data were generated at 1, 4, 7, and 14 d after application (DAA). Sunflower accumulated shikimate more rapidly and at lower glyphosate rates than the other two species. At 14 DAA, glyphosate at the two highest rates remained detectable in all three species. Plants receiving lower glyphosate doses (210, 105, and 53 g ae ha^{-1}) had begun to grow out of the injury, or at least the shikimate levels in the plants were no longer significantly different than that present in the untreated controls. This spectrophotometric assay is both rapid and simple, with respect to other means of detecting shikimate, and it can be used to detect glyphosate drift. For it to be used by crop managers, samples from potentially drift-affected crops should be taken as soon as possible after the suspected drift event or immediately after the appearance of glyphosate injury.

Nomenclature: Glyphosate; proso millet, *Panicum miliaceum* L. 'Sunup'; sunflower, *Helianthus annus* L. 'Triumph 765C'; wheat, *Triticum aestivum,* L. 'Stak-Tite N25550'.

Key words: Drift, shikimate, glyphosate, EPSPS, leaf disc, spectrophotometer.

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 2004; Shaner 2000). Glyphosate is foliar-applied and nonselective and is used for both burndown and POST applications in GR crops (James and Krattiger 1996). Inhibition of growth occurs almost immediately, followed by chlorosis at the newest growing points and necrosis throughout the entire plant within 1 to 2 wk. Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase in the shikimate pathway (Amrhein et al. 1980). In 2004, GR-cotton (*Gossypium hirsutum* L.) and GR-soybean [*Glycine max* (L.) Merr.] were planted in the United States on more than 60 and 80% of planted hectares, respectively (USDA-NASS 2004). As glyphosate use increases, so does the possibility of misapplication and drift.

Spray drift, as defined by the U.S. Environmental Protection Agency, is "The physical movement of a pesticide through air at the time of application or soon thereafter, to any site other than that intended for application (often referred to as off-target)" (Anonymous 1999). This definition of drift does not include injury caused by volatilization, erosion, or windblown soil particles to which herbicides are attached. Drift is influenced by a variety of variables such as environmental conditions (wind, temperature, humidity), herbicide formulation, pressure, nozzle type, droplet size, cultivar, growth stage, and distance that the herbicide is released from the target (Al-Kahatib and Peterson 1999; Auch and Arnold 1978; Cranmer and Linscott 1990; Miller 1993; Nordby and Skuterud 1975).

A rapid and accurate method is needed to detect glyphosate drift because, with the increase in GR crops, as well as a rise in chemical fallow and conservation tillage, glyphosate applications have increased with a concomitant increase in glyphosate drift incidents (Drapala 2001). In 1998, drift accounted for 21% of the insurance claims in Iowa, and by 1999, > 80% of the complaints investigated by the Iowa Department of Agriculture and Land Stewardship dealt with drift (Pringnitz 1999). Glyphosate drift became quite a problem in the Mississippi Delta in 2000 and 2001, with 145 cases of drift reported to the Bureau of Plant Industries in 2001 (T. McDaniel, personal communication). Because the frequency of drift events was increasing, aerial application of glyphosate in certain Mississippi Delta counties was banned March 15 through April 30 (T. McDaniel, personal communication).

Glyphosate drift can be a problem with a wide variety of crops. Early in the growing season (April–May) is a particularly windy time of the year in the Central Great Plains of the United States, thereby increasing the likelihood of herbicide drift. In Colorado, the number of hectares planted to GR-corn (*Zea mays* L.) on both dryland and irrigated fields is increasing rapidly (J. Tichota, personal communication) because of the declining price of glyphosate and the efficacy of this weed control package. In addition, glyphosate is widely used to control weeds in fallow. However, many other crops, such as winter wheat, sunflower, and proso millet are planted in close proximity to GR-corn and fallow fields. At times during the early to mid-June portion of the growing season in the Central Great Plains, both burndown and early POST applications of glyphosate to GR-corn overlap with emergence of proso millet and sunflower. The risk of glyphosate injury from drift is particularly likely during this time, especially given the region's reduction in tillage and increased reliance on glyphosate for burndown and in-crop usage. Fallow management with glyphosate can also coincide with emerging wheat during the fall.

Glyphosate injury can decrease growth, reduce yield, or kill the susceptible crop if the drift dosage exceeds the target tolerance level. Determining the type and degree of injury is important to a producer. To use shikimate accumulation to

DOI: 10.1614/WS-06-065.1

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identify potential herbicide injury to crops, the manner in which glyphosate affects crops must be considered.

It is a challenge to detect herbicide injury caused by drift because crops often do not exhibit extensive injury symptoms; however, yield can still suffer. Rowland (2000) determined that low rates of glyphosate could reduce the yield of corn and that stand height was one of the best parameters for estimating the degree of damage. If a crop is injured to the degree that height is limited and yield is decreased, perhaps shikimate accumulation could be used to quantify glyphosate-related injury and potentially predict yield loss.

Shikimate accumulation has been used to assess the exposure of plants to glyphosate. Glyphosate inhibits EPSPS (Steinrucken and Amrhein 1980), resulting in the accumulation of shikimate, the dephosphorylated substrate of the enzyme (Amrhein et al. 1980). Methods for extracting and measuring shikimate levels in plant tissue are relatively simple (Cromartie and Polge 2000; Singh and Shaner 1998). Shikimate accumulation after glyphosate treatment has been used to determine whether susceptible crops have been exposed to glyphosate drift (Henry et al. 2005; Koger et al. 2005; Pline-Srnic 2005). Shikimate accumulation in rice (*Oryza sativa* L.) appears to be a better predictor of yield reduction than either height reduction or visual injury (Koger et al. 2005).

The use of a shikimate assay to determine the extent and location of herbicide injury would provide a producer with proof that the crop was injured by glyphosate and an opportunity to seek out the guilty party whose herbicide drifted onto the grower's property. Determining the herbicide that drifted onto the crop is the first step before seeking out the guilty applicator. Second, the status of the crop must be determined. A producer needs to know how much acreage was affected and to what extent. With this information, the producer could make an informed crop management and mitigation decision.

The objective of this research was to use a spectrophotometric-based leaf disc assay to (1) measure shikimate accumulation in sunflower, wheat, and proso millet after glyphosate application; (2) determine what rates of glyphosate cause significant shikimate accumulation in these crops; and (3) determine how long after treatment shikimate levels remain elevated.

Materials and Methods

Greenhouse and field experiments were conducted at the USDA-ARS Central Great Plains research station in Akron, CO, during the summer and fall of 2005. Both experiments were repeated, and all spectrophotometric data were generated in Ft. Collins, CO.

Plant Material

Greenhouse-Grown Plants

Seeds of sunflower 'Triumph 765C', wheat 'Stak-Tite N25550', and proso millet 'Sunup' were planted in pots (6.3 by 6.3 by 8.5 cm deep) containing Green Formula[1] 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 16 to 18/22 to 30 C night/day temperatures. Plants were watered daily and fertilized once a week with half-strength Hoagland's solution beginning 1 wk after emergence (Hoagland and Arnon 1950). Glyphosate was applied with a trac-sprayer at 840, 420, 210, 105, and 53 g ae ha^{-1} in 140 L ha^{-1} to all three crops in the four- to six-leaf stage for both the first and second experiments. Surfactant[2] was added to all treatments at 0.25% (v/v). Twelve leaf discs, each 4 mm diam, were excised from the youngest two leaves of proso millet and wheat and the youngest three leaves of sunflower, for a total leaf area per sample of 150 mm^2. Each sample was generated from an individual plant and was stored in a glass scintillation vial. One milliliter of 0.25 N HCl was pipetted into each vial, and then the vials were placed in a freezer and kept frozen until assayed for shikimic acid. Sample weight (12 discs) varied slightly, ranging between 0.03 and 0.04 g across species and samples. Proso millet and wheat sample weights were typically near the bottom of this range, whereas sunflower, with thicker leaves, was closer to the top. The plants receiving the 840 and 420 g ae ha^{-1} rates began to exhibit necrosis by 7 to 14 d after application (DAA), so these samples were often slightly lighter (0.02–0.03 g) than samples collected before the onset of injury symptoms.

Field-Grown Plants

The same varieties of crops that were used in the greenhouse study were also planted in the field study. Sunflower was 35 cm tall in the 10- to 15-leaf stage at the time of application for the first experiment and 50 cm tall and in the 15- to 18-leaf stage at the time of application for the second experiment. Proso millet was 30 cm tall in the four- to six-leaf stage at the time of application for the first experiment and 35 cm tall in the five- to seven-leaf stage at the time of application for the second experiment. Wheat was grown at two field locations: one receiving an initial irrigation event and the other grown without supplemental irrigation. Sufficient fall rainfall provided enough soil moisture for wheat at the second site to be actively growing at the time of glyphosate application. Wheat was 10 to 15 cm tall and at the three- to four-leaf stage at the time of glyphosate application. Glyphosate was applied with a CO_2 backpack sprayer at the same rates and volumes as the greenhouse experiment. The same number of leaf discs were collected for assaying shikimate as in the greenhouse experiment.

Shikimate Assay

Vials were placed in a –20 C freezer until the 0.25 N HCl solution froze and then were later thawed at room temperature or at 60 C for 30 min. Shikimate was determined spectrophotometrically following the procedure of Cromartie and Polge (2000). Two aliquots of 25 µl were transferred from each vial to a microtiter plate to which 100 µl of 0.25% (wt/v) periodic acid[3]/0.25% (wt/v) *m*-periodate[4] was added to each well. Plates were incubated at room temperature (25 C) for 90 min, 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 with a microtiter plate spectrophotometer.[5] Background optical density was determined from the wells containing the extract from discs of untreated plants and was subtracted from each of the glyphosate treatments. A shikimate standard curve was developed by adding known amounts of shikimate[6] to wells containing extract from leaf discs not exposed to glyphosate so that shikimate levels could be reported as micrograms of shikimate per milliliter of HCl extraction solution. The standard curve for shikimate ranged from 0.01 to 1 µg ml^{-1} of HCl extraction solution.

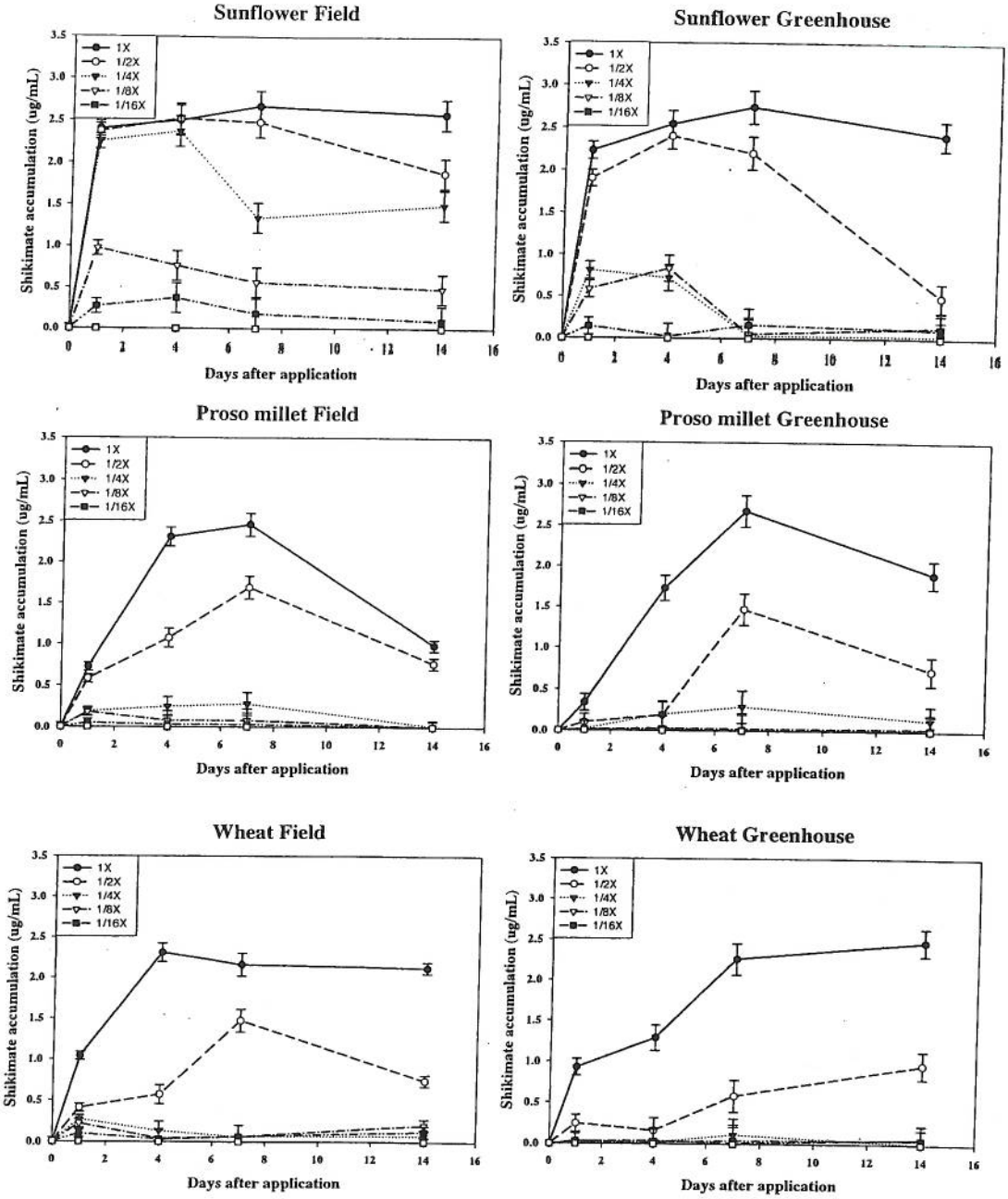

Figure 1. Shikimate accumulation in sunflower, proso millet and wheat with respect to greenhouse or field grown plants, days after application, and glyphosate rate.

Statistical Analyses

Field and greenhouse treatments were arranged in a completely random design. All data, both from the field and greenhouse studies, were fit to a linear mixed model with repeated measures. Experiment and replication within experiment were treated as random effects. SAS[7] PROC MIXED was used to assess the effects of days after application, application rate, species, and all possible interactions.

Results and Discussion

Shikimate accumulated in leaf tissue in all three species after glyphosate treatment. Significant shikimate accumulation was detectable several days before the onset of visual injury symptoms. The spectrophotometric shikimate assay only detects increases in shikimate over background levels. The minimum amount of increased shikimate levels that could be detected was 0.25 $\mu g\ g^{-1}$ fresh tissue weight. Shikimate in the leaf tissue reached maximum levels between 4 and 7 DAA at the 840 g ae ha^{-1} application rate in all three species (Figure 1), which is similar to corn and soybean (Henry et al. 2005). Although not statistically the same, the shikimate accumulation trends were similar regardless of where the plants were grown and at what growth stage they were sampled. The second proso millet field experiment was conducted midway though the proso millet growing season.

Table 1. Shikimate accumulation (presented as averages of both field and greenhouse data) in treated plants beyond endogenous levels in untreated plants with respect to time and glyphosate application rate.

Rate	Shikimate accumulation[a,b]			
	1 DAA	4 DAA	7 DAA	14 DAA
(g ha^{-1})	(μg ml^{-1})			
Sunflower				
840	2.29***	2.53***	2.72***	2.46***
420	2.06***	2.43***	2.29***	0.95**
210	1.78***	1.98***	1.08***	0.52
105	0.86***	0.73***	0.20	0.25
53	0.18	0.13	0.17	0.08
Proso millet				
840	0.54***	1.99***	2.43***	1.38***
420	0.27	0.31	1.34***	0.52***
210	0.12	0.27	0.24	0.16
105	0.12	0.04	0.03	0.06
53	0.02	0.02	0.02	0.08
Wheat				
840	0.93***	1.81***	2.33***	2.19***
420	0.34*	0.41*	1.16**	0.91***
210	0.14	0.10	0.12	0.02
105	0.12	0.06	0.05	0.11
53	0.06	0.02	0.07	0.08

[a] DAA, sampling date, days after application.
[b] Shikimate accumulation was significantly different from untreated controls at * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$.

Although shikimate accumulation was somewhat reduced in proso millet in the second field experiment (approximately 25%), possibly because the plants were entering their reproductive phase, shikimate was still easily detectable above background levels at the two highest glyphosate rates, and the trend of not being able to detect the lower three rates as seen in the greenhouse was also repeated in the field.

Sunflower accumulated more shikimate than either proso millet or wheat. Although we attempted to control as many variables as possible, such as growth stage, herbicide, surfactant concentration, spray volume, which leaf, area of tissue sampled, weight of tissue sampled, time of sampling, and plant vigor, other factors need to be considered before concluding that sunflower is more sensitive to glyphosate than these other species. One potential factor is the difference in leaf surface area among the three species. A sunflower seedling in the three- to four-leaf stage has considerably more leaf area than a similarly aged proso millet or wheat seedling; therefore, a sunflower plant would receive more glyphosate from a given herbicide application than either of the other two species. A possible solution would be to collect leaf discs from untreated plants and submerge them in a glyphosate solution so that all species are exposed to an equal concentration of herbicide per unit leaf area (Shaner et al. 2005). A drift event would be far more similar to the experimental conditions of this experiment as opposed to a submersible leaf disc assay.

Sunflower accumulated shikimate more rapidly and at lower glyphosate application rates than the other two species (Figure 1), perhaps because of its greater leaf surface area and subsequently enhanced glyphosate capture. The three higher application rates resulted in 1.78 to 2.29 μg shikimate ml^{-1} of solution at 1 DAA compared with levels of 0.54 and 0.93 μg shikimate ml^{-1} at the highest application rate for proso millet and wheat, respectively (Table 1). Shikimate was detected at the 105 g ae ha^{-1} application rate at both 1 and 4 DAA in sunflower. This shikimate accumulation trend is different than for proso millet. Proso millet exhibited significant shikimate accumulation at a discriminating rate of 420 g ae ha^{-1}, but only at 7 and 14 DAA. Wheat behaved similarly to proso millet. The discriminating rate was also 420 g ae ha^{-1} at 1, 4, 7, and 14 DAA, but the highly significant ($P < 0.001$) levels were only present at 7 and 14 DAA.

At 14 DAA, the two highest glyphosate rates remained detectable in all three species ($P < 0.001$). Plants receiving lower rates (210, 105, and 53 g ae ha^{-1}) of glyphosate had begun to grow out of the injury, or at least the excess shikimate remaining in the plants was not greater than that present in untreated controls. Although the 105 g ae ha^{-1} rate caused significant elevation in shikimate concentrations in sunflower at 1 and 4 DAA, by 7 DAA it was no longer detectable at this rate, and by 14 DAA, neither the 105 nor the 210 g ae ha^{-1} rates were distinguishable from the untreated controls.

We were able to use a spectrophotometric leaf disc assay to detect elevated levels of shikimate after glyphosate application in wheat, proso millet, and sunflower. Sunflower appears to be more susceptible to glyphosate and also appears to accumulate higher levels of shikimate more quickly than either proso millet or wheat. In sunflower, we were able to detect the 105 and 210 g ae ha^{-1} rates at 1 and 4 DAA; however, shikimate levels in these plants began to decline and, by 14 DAA, were no longer detectable. Only the highest two rates were detectable at 14 DAA across all three species. Typically, by 14 DAA, the plants exposed to the highest two rates were seriously injured or stunted. If producers or crop managers were to use the leaf disc assay to detect shikimate, they would want to sample the most severely injured plants along the edge of the potential drift event (Henry et al. 2005).

Sources of Materials

[1] Green Formula Potting Soil, Lamber Peat Moss Inc., 106 Lambert Road, Riviere-Oulle, Quebec GOL 2CO, Canada.
[2] Activator 90 Surfactant, Loveland Industries Inc., P.O. Box 1289, Loveland, CO 80632.
[3] Periodic acid, Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201.
[4] *m*-Periodate, Aldrich Chemical Co., P.O. Box 355, Milwaukee, WI 53201.
[5] Kinetic microplate reader model UVmax, Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA 94089.
[6] Shikimic acid, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.
[7] SAS Software, Version 6.12, Statistical Analysis Systems Inc., Cary, NC 27513

Acknowledgments

The authors thank ARS research technician Paul Campbell and ARS summer student Kristin Kraich for their efforts in collection and preparation of these data. The authors also thank Jeff Tichota of Monsanto for providing glyphosate for this and other research projects.

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Received March 29, 2006, and approved July 5, 2006.

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