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Shikimic Acid Accumulation in Field-Grown Corn (*Zea mays*) Following Simulated Glyphosate Drift

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Field studies were conducted in 2001 through 2003 to determine if shikimic acid accumulation could be used to accurately predict yield reductions in field corn exposed to sublethal rates of glyphosate. Glyphosate (0−0.32 kg ae/ha) was applied to corn at the V6 to V8 growth stage. Corn whorls were randomly collected up to 14 days after application (DAA), and shikimic acid accumulation in the whorls was determined using HPLC-UV. Maximum shikimic acid accumulation occurred 3−7 DAA in corn receiving 0.16 and 0.32 kg/ha. Shikimic acid accumulation 3, 5, and 7 DAA did correlate ($r = 0.80-0.86$) to yield losses from a sublethal application of glyphosate. Shikimic acid accumulation 3, 5, and 7 DAA was better correlated to visual injury at 14 DAA than to yield reductions. Visual injury ratings 14 DAA were a slightly better indicator of potential yield losses ($r = 0.93$) than shikimic acid accumulation in field-grown corn whorls ($r = 0.8-0.86$).

KEYWORDS: ***Zea mays*; glyphosate; herbicide drift; shikimic acid**

INTRODUCTION

In the past few years, problems with glyphosate drift to susceptible crops have increased. This has indirectly been due to the increase in acreage in glyphosate-resistant crops. After a glyphosate drift event occurs, producers are often confronted with the difficult decision to either terminate the crop and replant or keep the crop in production with maximum or minimal agronomic inputs (depending on yield potential). Many methods have been examined to predict potential yield losses from sublethal rates of glyphosate. The most common method involves using visual injury to predict yield losses. Rowland (*1*) found that visual injury estimates from sublethal rates of glyphosate do not strongly correlate with yield losses. Rowland (*1*) also concluded that percent plant height reductions do not strongly correlate to yield losses.

Glyphosate inhibits 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthase in the shikimate pathway (*2*, *3*). EPSP is produced from shikimate-3-phosphate and phosphoenolpyruvate. This reaction is necessary for the production of the aromatic amino acids tryptophan, phenylalanine, and tyrosine (*4*). Because of glyphosate inhibition of the shikimate pathway, the metabolic precursor of shikimate 3-phosphate, shikimic acid, cannot be converted into EPSP, which results in the accumulation of high levels of shikimic acid (*2*−*4*). Even though shikimate 3-phosphate is the precursor to EPSP, it does not accumulate at high levels due to the cleavage by a phosphatase enzyme in the tonoplast or vacuole, which then yields shikimic acid (*7*). Herbicides that affect AHAS and ACCase enzymes often result in visual injury symptoms similar to glyphosate. However, AHAS- and ACCase-inhibiting herbicides do not affect the shikimate pathway as with glyphosate (*8*). Therefore, shikimic acid accumulation could be used to differentiate between glyphosate drift from other herbicides that results in similar visual injury symptoms.

Previous research has reported that shikimic acid accumulation began within 1 day after the sublethal application of glyphosate to field-grown corn and generally peaked between 4 and 7 days after the application (*9*). Koger et al. (*10*) also reported that shikimic acid accumulation following a sublethal application of glyphosate to rice (*Oryza sativa* L.) peaked at 7 days after the application. This research also concluded that shikimic acid accumulation was highly correlated to potential yield reductions. Becerril et al. (*11*) found that shikimic acid accumulated in velvetleaf (*Abutilon theophrasti* Medic.) 6 days after treatment with sublethal rates of glyphosate. Singh and Shaner (*8*) found that shikimic acid accumulated within 24 h of a sublethal glyphosate application to soybean [*Glycine max* (L.) Merr.]; by 96 h, shikimic acid levels were lower than at 24 h but still detectable (*8*). Also, Harring et al. (*12*) reported that shikimic acid accumulation was proportional to glyphosate rate. The ED_{50} values based on visual plant death ratings 14 DAA were the same as those observed with shikimic acid accumulation.

Previous research has indicated that shikimic acid accumulation could be potentially used as an assay method for glyphosate spray drift (*8*). However, correlations between percent yield reductions and accumulated shikimic acid concentrations have not been investigated in field-grown corn. Therefore, the objectives of this research were to (i) measure shikimic acid concentrations over time following a sublethal application of glyphosate to non-glyphosate-resistant field-grown corn and (ii)

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Table 1. Recovery of Endogenous[a] Shikimic Acid from Corn Tissue Using Different Extraction Methods

extraction medium[b]	tissue processing method[c]	shikimic acid (ppmw)
1 N HCl	liquid N_2	651
	food processor	607
0.01 M H_2SO_4	liquid N_2	538
	food processor	534
LSD (0.05)		6

[a] Endogenous levels of accumulated shikimic acid in greenhouse-grown corn 3 DAT with 0.64 kg ae/ha of glyphosate applied at the V2 growth stage. [b] 20 g of tissue was added to 100 mL of 1 N HCl or 100 mL of 0.01 M H_2SO_4 and extracted for 72 h. [c] Tissue either finely ground with mortal and pestle in liquid N_2 or roughly chopped with a food processor.

Table 2. Recovery of Freshly Fortified and Endogenous Shikimic Acid from Corn Tissue Using 1.0 N HCL, as a Function of Time[a]

shikimic acid treatment	extraction time (h)	average recovery
200 μg/g of freshly fortified shikimic acid[b]	24	84.2 ± 7.1%
	48	85.2 ± 2.5%
	72	74.6 ± 1.2%
2000 μg/g of freshly fortified shikimic acid[b]	24	89.9 ± 2.3%
	48	84.5 ± 1.3%
	72	82.6 ± 5.3%
endogenous shikimic acid[c]	24	972 ± 21.2 μg/g
	48	926 ± 22.2 μg/g
	72	987 ± 97.1 μg/g

[a] Extraction time on orbital shaker at 1500 rpm using 5 mL of 1 N HCl per gram of tissue. [b] Applied to 5 g of untreated, field-grown tissue roughly chopped with a food processor; recovery results are corrected for background shikimic acid concentrations, which ranged from 505 to 672 ppmw. [c] Endogenous levels of accumulated shikimic acid in corn 3 DAT with 0.32 kg ae/ha of glyphosate.

determine if visual injury and yield losses correlate with shikimic acid levels in affected corn plants.

MATERIALS AND METHODS

Field Methods. Experiments were conducted at the R.R. Foil Plant Science Research Center near Starkville, MS in 2001, 2002, and 2003. The soil was a Leeper silty clay loam (fine, montmorillonitic, nonacid, thermic Chromudertic Haplaquept) with 1.6% organic matter and a pH of 6.1. Corn "Pioneer 3167" was planted in four 96.5 cm × 12.2 m rows in 2001, and eight 96.5 cm × 12.2 m rows in 2002 and 2003 at a rate of 85 000 seed ha^{-1}. The experimental area was treated with 0.96 kg ai ha^{-1} of atrazine and 1.16 kg ai ha^{-1} of metolachlor (preemergence) and kept weed-free the entire growing season. Corn was grown without supplemental irrigation. Standard agronomic practices were implemented to optimize yield.

Treatments were arranged in a randomized complete block design with four replications. Glyphosate was applied at 0.32, 0.16, 0.04, 0.01, and 0.0 kg ae ha^{-1}. These application rates represent 50, 25, 6, and 1.5% of the labeled rate. The glyphosate formulation, Roundup D-PAK, was used since it contained no surfactant. A nonionic surfactant was added to all glyphosate treatments at 1% (v/v) to ensure a uniform surfactant concentration among glyphosate treatments. An untreated check was included to monitor background levels of shikimic acid and visual injury. All treatments were applied at the V6 to V8 growth stage with a CO_2−-pressurized sprayer delivering a volume of 48 L ha^{-1}. This application timing was chosen because corn appears most susceptible to glyphosate at this growth stage (*1*).

Corn whorls were collected 0, 1, 3, 5, 7, and 14 DAA. Corn whorls were cut approximately 5 cm below the first collared leaf from the top portion of the plant. Preliminary research indicated that shikimic acid accumulated most in the middle to upper portion of corn plants following a sublethal application of glyphosate (data not shown). Corn whorls were placed in a freezer within 1 h of collection and stored at −20 °C until processed. In 2001, six corn whorls were collected and analyzed as a composite from each plot. In 2002 and 2003, two corn whorls were collected and analyzed individually from each plot. Visual injury ratings were also taken at these collection times in 2002 and 2003. These rating were based on chlorosis, necrosis, and stunting, using a 0−100 scale (0 = no visible injury and 100 = total death). Corn yield data were collected at the end of the growing season.

Laboratory Methods and Analytical Procedures. The extraction procedure used in these studies was similar to ones reported for corn, soybean, and horseweed [*Conyza canadensis* (L.) Cronq.] (*8*, *13*). The corn whorls were homogenized with a food processor until roughly chopped. A preliminary study indicated that roughly chopping corn tissue and extracting with 1 N HCl resulted in acceptable shikimic acid recovery (**Table 1**). In 2001, approximately 20 g of the homogenized plant tissue was added to a 125 mL flask. In 2002 and 2003, approximately 5 g of plant tissue was added to a 50 mL screw cap polypropylene centrifuge tube. In all years, 1 N HCl was added at a ratio of 5 mL of HCl per 1 g of leaf tissue. The samples in 2001 were extracted for 72 h and occasionally stirred. In 2002 and 2003, samples were extracted for 24 h on an orbital shaker at 1500 rpm. The extraction time in 2002 and 2003 was slightly modified from 2001 due to laboratory experiments that indicated that a 24 h extraction period was equivalent to a 72 h extraction period (**Table 2**). For each set of samples extracted (15−20 samples per set), an untreated control and two fortified samples (200 and 2000 ppmw shikimate) were prepared. The carrier solvent, acetonitrile, was allowed to evaporate prior to addition of the 1 N HCl extraction solvent. The fortified samples were corrected by subtracting background shikimic acid levels to determine shikimic acid recovery. New tissue samples were extracted and reanalyzed whenever the extraction recovery for a sample set fell outside of a 70−120% recovery range. Long-term shikimate recovery from fortified samples averaged 95 ± 4% (n = 37) and 85 ± 4% (n = 37) at the 200 and 2000 ppmw levels, respectively.

After the extraction period, the extract was filtered through Whatman No. 1 filter paper into a graduated cylinder, and the filtered extract volume was recorded. The pH of the filtered extract was adjusted to 3.0−3.3 with saturated NaOH and/or 0.01 N NaOH, as needed. The final volume of the pH-adjusted extract was adjusted to its initial volume with 0.001 N HCL and recorded. A 2 mL sample of extract was diluted with 1.0 mL of acetonitrile and passed through a 0.45 μm nylon syringe filter into a glass chromatography vial. The extract was refrigerated at 4 °C until analysis using HPLC, according to the method of Mueller et al. (*13*).

The concentration of shikimic acid in corn tissue was determined using an Agilent (Wilmington, DE) series 1100 HPLC chromatograph equipped with Chemstation software, autoinjector, and photodiode array detector using a detection wavelength of 215 nm. A Phenomenex (Torrance, CA) Luna NH_2 column (250 mm × 4.0 mm; 5 μm particle size) was used with an injection volume of 10 μL. The isocratic system used 95:4:1 actonitrile/deionized water/phosphoric acid and a flow rate of 1.0 mL/min. The total run time was 20 min, with shikimic acid retention time at ca. 7.4 min. A six-point calibration curve with shikimate concentrations ranging from 3.65 to 52.3 ppm was used to externally quantify shikimic acid levels in tissue extracts. The method of detection limit for shikimic acid was approximately 20 ppmw above background levels in untreated corn tissue. A 5 g subsample of each corn tissue sample was dried at 105 °C for 24 h to allow the shikimic acid concentrations to be reported on an oven-dry weight basis. A chromatogram showing shikimic acid levels in field-grown corn before and after a sublethal application of glyphosate is given in **Figure 1**.

A pilot study was conducted to determine the storage stability of shikimic acid in corn tissue stored under study conditions. Three replications of corn tissue (20 g) were fortified with 27 or 270 ppm shikimic acid and stored for up to 94 days at −20 ± 2 °C. The acetonitrile carrier solvent was allowed to thoroughly evaporate before the samples were placed in the freezer. The tissue samples were extracted and analyzed over time as previously described. Results indicate that shikimic acid is stable in corn tissue stored at −20 °C for up to 94 days (**Figure 2**). All tissue samples collected from the field were extracted within 90 days.

Statistical Analysis. The relationship between shikimic acid accumulation and (a) yield reduction and (b) visual injury were evaluated

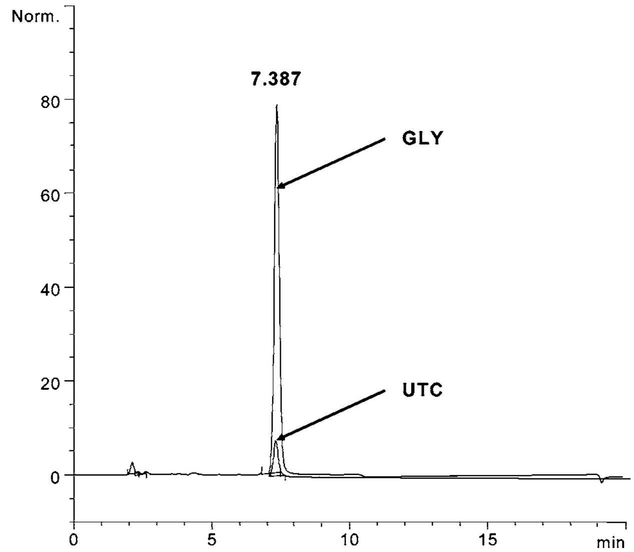

Figure 1. HPLC chromatogram showing shikimic acid concentrations in field corn 5 days after treatment with 0.32 kg ae/ha of glyphosate (GLY) and corresponding untreated control (UTC).

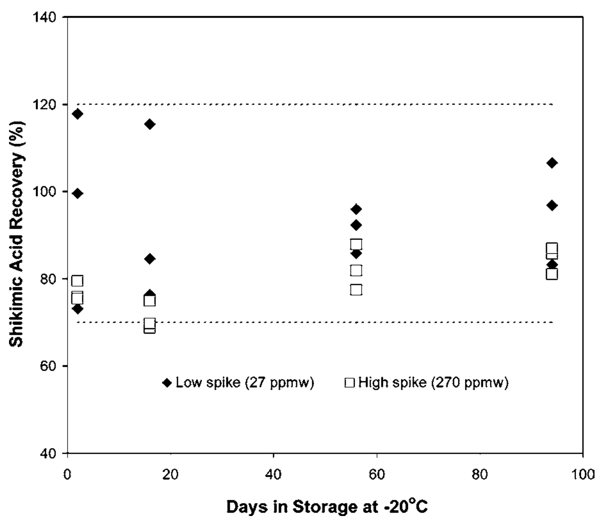

Figure 2. Storage stability of shikimic acid in corn tissue stored from 0 to 94 days at –20 ± 2 °C.

using a nonlinear, segmented quadratic regression model. This analysis was conducted using a PROC NLIN procedure in Statistical Analytical Systems version 9.1 (SAS Institute, Inc., Cary, NC). For these analyses, shikimic acid accumulation (independent variable) was used to predict corn yield reduction or visual injury (dependent variable) following the glyphosate treatment. The regression model used is as follows:

$$y = a + bx + cx^2, \text{ if } x < x_0 \tag{1}$$

$$y = p, \text{ if } x > x_0 \tag{2}$$

where y is the corn yield reduction or visual injury; x is the shikimic acid increase in corn tissue; and a, b, and c are regression coefficients. x_0 is the value on the x-axis where the yield reduction plateaus and is equivalent to $-b/2c$, while p is the value on the y-axis where the yield reduction plateaus and is equivalent to $a - (b2/4c)$. When values of x are less than x_0, the equation relating y and x is quadratic (a parabola), and for values of x greater than x_0, the equation is constant (p).

The relationship between visual injury ratings and reductions in corn yield was evaluated using a quadratic regression model as shown next

$$y = a + bx + cx^2 \tag{3}$$

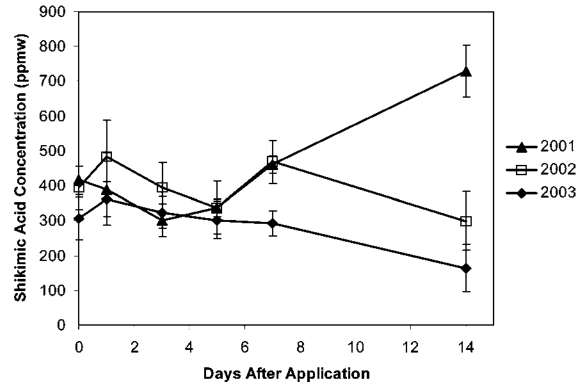

Figure 3. Shikimic acid concentration (ppmw) in untreated field-grown untreated corn whorls (V6 to V8).

Table 3. Relative Shikimic Acid Increase in Field Corn 0–14 Days after Glyphosate Application at the V6 to V8 Growth Stage

glyphosate rate[a] (kg ae/ha)	days after application					
	0 (%[b])	1 (%[b])	3 (%[b])	5 (%[b])	7 (%[b])	14 (%[b])
0.01	–8	–8	–1	8	1	2
0.04	–8	2	14	40	12	11
0.16	12	52	195	191	120	–13
0.32	25	157	355	545	237	54
LSD (0.05)			80			

[a] Nonionic surfactant was added at 1% (v/v) for all glyphosate treatments. [b] Percent increase in shikimic acid concentration relative to the untreated control.

where y is the corn yield reduction; x is the visual injury rating; and a, b and c are regression coefficients.

RESULTS AND DISCUSSION

Shikimic Acid Accumulation in Corn. Shikimic acid concentrations in untreated tissue samples varied over harvest times (**Figure 3**). Plant stress from mechanical wounding or fungal elicitation can induce DAHP synthase mRNA accumulation, which increases carbon flow into the shikimate pathway (*14*−*17*). Sunlight intensity may also indirectly impact shikimic acid levels in plants as the shikimate pathway provides intermediates used to synthesize flavinoids and other pigments used to protect against UV−B irradiation (*18*, *19*). Thus, biotic and abiotic stresses can influence background levels of shikimic acid in plants. Because of differences in shikimic acid concentration in untreated tissue over the collection times, the data are presented as a percent increase in shikimic acid over background levels at corresponding harvest intervals.

Elevated levels of shikimic acid accumulated within 1 day after application (DAA) of glyphosate at 0.32 kg/ha (**Table 3**). The highest shikimic acid accumulation (545% of control) was observed 5 DAA with glyphosate at 0.32 kg/ha. Shikimic acid accumulation was also high (355 and 237% of control) at 3 and 7 DAA with glyphosate applied at 0.32 kg/ha. It has been reported that shikimic acid concentrations in corn, soybean, rice, and wheat (*Triticum aesitivum* L.) tissue generally accumulate at the highest level between 4 and 7 days after a sublethal application of glyphosate (*9*, *10*, *20*). There was no difference in the amount of shikimic acid that accumulated in field-grown corn from the 0.16 kg/ha rate at 3, 5, and 7 DAA. By 14 DAA, shikimic acid concentrations that were once induced by the glyphosate application decreased to background levels of the untreated control. Other researchers have found that increased

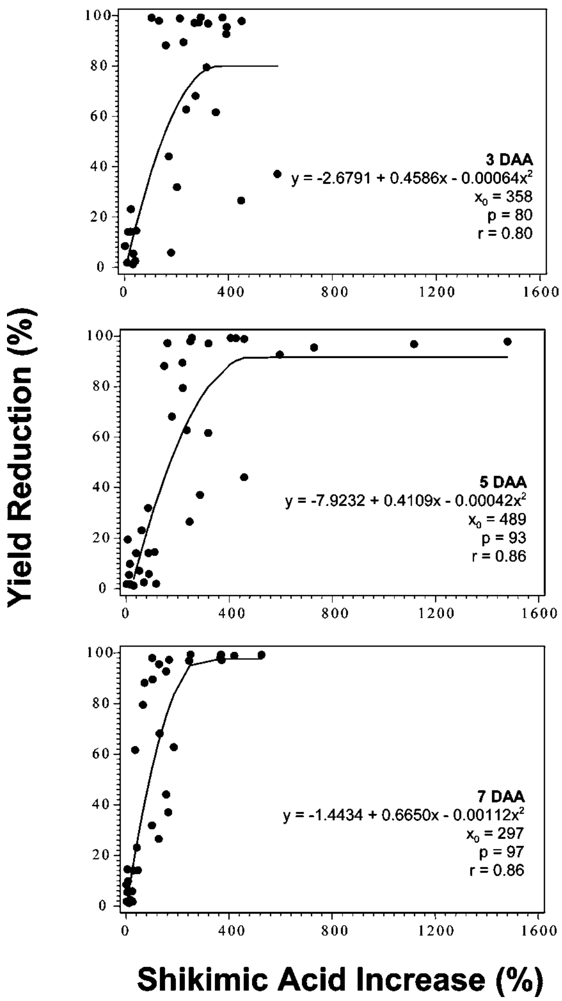

Figure 4. Percent yield reduction in field corn as a function of shikimic acid accumulation 3, 5, and 7 days after application.

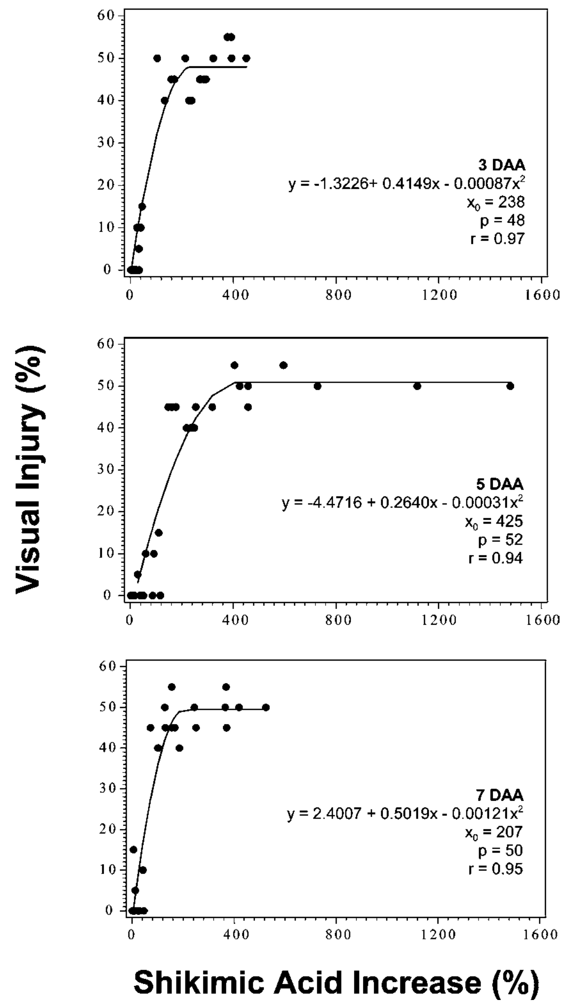

Figure 5. Percent visual injury 14 days after glyphosate application in field corn as a function of shikimic acid accumulation 3, 5, and 7 days after application.

Table 4. Percent Visual Injury of Field Corn 0–14 Days after Glyphosate Application at the V6 to V8 Growth Stage

	days after application					
glyphosate rate[a] (kg ae/ha)	0 (%[b])	1 (%[b])	3 (%[b])	5 (%[b])	7 (%[b])	14 (%[b])
0.01	0	0	0	0	0	0
0.04	0	0	1	6	9	5
0.16	0	0	11	17	31	43
0.32	0	0	15	21	38	51
LSD (0.05)			4			

[a] Nonionic surfactant was added at 1% (v/v) for all glyphosate treatments. [b] Percent relative to untreated control.

shikimic acid levels resulting from sublethal glyphosate applications do not remain at elevated levels (*8*−*11*). This and other research indicates that as the plant recovers from a sublethal application of glyphosate, accumulated shikimic acid is metabolized by the plant and/or diluted with resumed plant growth. Therefore, when a glyphosate drift event occurs in field corn, plant tissue samples would need to be collected within 3−7 days after the drift event to assay for shikimic acid accumulation. Also, tissue samples would need to be collected from unaffected plants to determine background concentrations. Glyphosate at 0.01 and 0.04 kg/ha did not affect shikimic acid levels at any of the collection periods, which was similar to the findings of Henry et al. (*9*).

Correlation between Shikimic Acid Accumulation and Yield Reduction. Because shikimic acid accumulation was greater at 3, 5, and 7 DAA than any of the other tissue collection times, these data were used to predict potential yield losses from a sublethal application of glyphosate. Correlation coefficients (r) for corn yield and shikimic acid accumulation ranged from 0.80 to 0.86 (**Figure 4**). The r values were slightly lower at 3 than 5 and 7 DAA, but there was no difference between 5 and 7 DAA. These data suggest that glyphosate exposure resulting in a shikimic acid accumulation of 297% (7 DAA) to 489% (5 DAA) above background levels reduced corn yields by at least 80%. Although the correlation coefficients were moderately high, the results indicated that shikimic acid accumulation was not as strong of an indicator for potential yield reductions as found in rice (*10*).

Correlation between Shikimic Acid Accumulation and Visual Injury. Foliar chlorosis is commonly observed in susceptible plants exposed to glyphosate (*4*). Chlorosis became evident by 3 DAA with glyphosate at 0.16 and 0.32 kg/ha and increased over time (**Table 4**), which was similar to the results found by other researchers (*9*, *10*). These results indicate that even though visual injury was observed up to 14 DAA, the field corn was already metabolically recovering from the sublethal glyphosate application indicated by the return of shikimic acid concentrations to background levels. Minimal visual injury was

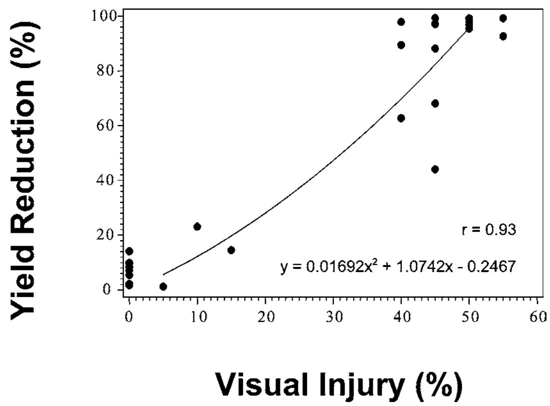

Figure 6. Percent corn yield reduction as a function of visual injury 14 days after glyphosate application.

observed with the 0.04 kg/ha rate, and no injury was observed with the 0.01 kg/ha rate.

The nonlinear, segmented model described in eqs 1 and 2 was also used to compare visual injury ratings 14 DAA to shikimic acid accumulation 3, 5, and 7 DAA (**Figure 5**). These time periods were selected on the basis that visual injury was most evident at 14 DAA but shikimic acid accumulation was highest 3−7 DAA. A high correlation ($r = 0.95$) was observed between visual injury at 14 DAA and the percent increase in shikimic acid at 3, 5, and 7 DAA. Harring et al. (*12*) also reported a high correlation between percent plant death at 14 DAA and shikimic acid accumulation 48 h after glyphosate treatment.

To determine if visual injury ratings were a better indicator of potential corn yield reductions than shikimic acid accumulation, visual injury ratings 14 DAA were used to predict yield reductions using eq 3. Visual injury ratings at 14 DAA were used in this analysis because visual injury was most evident at this time. As indicated in **Figure 6**, visual injury was slightly better ($r = 0.93$) at indicating potential corn yield reductions than shikimic acid accumulation.

In summary, the purpose of this study was to determine if shikimate accumulation in field corn resulting from simulated glyphosate spray drift is a good indicator of potential yield loss. If shikimic acid accumulation and corn yield reduction were moderately correlated (r values ranged from 0.8 to 0.86), shikimate accumulation could be useful as a potential indicator of yield loss. A limitation of this assay would be the narrow time frame in which plant tissue collection would need to occur for shikimic acid determinations: plant tissue samples would need to be collected at the onset of visual injury symptoms, which typically occurs within 7 DAA. Also, plant tissue samples would need to be collected from unaffected portions of the field to determine background shikimic acid levels. If these procedures are done in a timely manner, shikimic acid accumulation could be a valuable method in detecting and assessing glyphosate drift. Visual injury at 14 DAA would also appear to be a reliable predictor ($r = 0.95$) of potential yield loss in field corn exposed to glyphosate spray drift.

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