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Isoflavone, Glyphosate, and Aminomethylphosphonic Acid Levels in Seeds of Glyphosate-Treated, Glyphosate-Resistant Soybean

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The estrogenic isoflavones of soybeans and their glycosides are products of the shikimate pathway, the target pathway of glyphosate. This study tested the hypothesis that nonphytotoxic levels of glyphosate and other herbicides known to affect phenolic compound biosynthesis might influence levels of these nutraceutical compounds in glyphosate-resistant soybeans. The effects of glyphosate and other herbicides were determined on estrogenic isoflavones and shikimate in glyphosate-resistant soybeans from identical experiments conducted on different cultivars in Mississippi and Missouri. Four commonly used herbicide treatments were compared to a hand-weeded control. The herbicide treatments were (1) glyphosate at 1260 g/ha at 3 weeks after planting (WAP), followed by glyphosate at 840 g/ha at 6 WAP; (2) sulfentrazone at 168 g/ha plus chlorimuron at 34 g/ha applied preemergence (PRE), followed by glyphosate at 1260 g/ha at 6 WAP; (3) sulfentrazone at 168 g/ha plus chlorimuron at 34 g/ha applied PRE, followed by glyphosate at 1260 g/ha at full bloom; and (4) sulfentrazone at 168 g/ha plus chlorimuron at 34 g/ha applied PRE, followed by acifluorfen at 280 g/ha plus bentazon at 560 g/ha plus clethodim at 140 g/ha at 6 WAP. Soybeans were harvested at maturity, and seeds were analyzed for daidzein, daidzin, genistein, genistin, glycitin, glycitein, shikimate, glyphosate, and the glyphosate degradation product, aminomethylphosphonic acid (AMPA). There were no remarkable effects of any treatment on the contents of any of the biosynthetic compounds in soybean seed from either test site, indicating that early and later season applications of glyphosate have no effects on phytoestrogen levels in glyphosate-resistant soybeans. Glyphosate and AMPA residues were higher in seeds from treatment 3 than from the other two treatments in which glyphosate was used earlier. Intermediate levels were found in treatments 1 and 2. Low levels of glyphosate and AMPA were found in treatment 4 and a hand-weeded control, apparently due to herbicide drift.

KEYWORDS: Aminomethylphosphonic acid; glyphosate; herbicide-resistant crop; isoflavone; shikimic acid; transgenic crop

INTRODUCTION

The isoflavones of soybeans have a number of nutraceutical properties, including estrogenic and hypocholesterolemic activities (*1*, *2*), as well as reportedly being able to reduce the risk of cancer (*3*). They may have adverse health effects on certain animals fed soybean meal (*4*). Thus, the levels of these compounds in soybeans are of great interest to both human and animal nutritionists.

The most successful transgenic crop in the world has been glyphosate-resistant (GR) soybeans (*5*). Its use has steadily increased since it was introduced in 1995, until approximately 75% of all U.S. soybeans planted in 2002 were GR (*6*). Before and since transgenic crops were introduced, questions have been posed regarding potential subtle, pleiotrophic effects of the transgenes on food quality (*7*) and similar effects that might be due to positional effects of the transgene in the genome (*8*). One study indicates that GR soybean lines contain lower levels of estrogenic isoflavones than non-GR soybean lines (*9*). A more thorough study has shown that there are no effects of the CP4 5-enolpyruvylshikimatae-3-phosphate synthase (CP4 EPSPS) gene, which confers glyphosate resistance to all GR cultivars sold, on isoflavone content of soybeans (*10*).

There is the possibility of sublethal levels of the herbicide to which the crop has been made resistant, if resistance is not complete. Isoflavones are products of the shikimate pathway,

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the target pathway of glyphosate (*11*). Glyphosate has been shown to reduce the levels of related compounds in nontransgenic soybeans (see, e.g., refs *12* and *13*) and of the phytoestrogenic compound genistein in *Lupinus luteus* L. (*14*). Reddy et al. (*15*) found that GR soybeans are not completely resistant, with significant inhibition of growth occurring at application rates as low as 2.24 kg/ha under certain growing conditions. Therefore, it is possible that glyphosate affects the levels of estrogenic isoflavones in seed produced from GR soybeans. Taylor et al. (*16*) addressed this question and found no effects. However, their study did not examine all estrogenic isoflavones and their glycosides. Furthermore, the glyphosate application rates used were not as high or applied as late in plant development as those commonly used by many farmers.

Glyphosate is labeled for use in GR soybeans from emergence to flowering. Two applications of glyphosate alone or one application of glyphosate following preemergence herbicide applications are commonly used to achieve effective weed control. Use of preemergence herbicides at planting provides the flexibility for late-season glyphosate application (*17*). In this paper, we reinvestigate this question, comparing several commonly used herbicide-based weed management regimes, including herbicide programs that include other herbicides that have been reported to affect the synthesis of compounds related to isoflavones. Environmental conditions are known to influence the level of resistance of glyphosate-resistant crops (see, e.g., ref *18*), so we conducted these experiments with GR soybeans at two locations with different environmental conditions.

MATERIALS AND METHODS

Field Experimental Conditions. *Mississippi Experiment.* This experiment was conducted in 2000 at the USDA-ARS Southern Weed Science Research farm, Stoneville, MS (33° N latitude). The soil was a Dundee silt loam (fine-silty, mixed, thermic Aeric Ochraqualf) with pH 6.4, 1.6% organic matter, and soil textural fractions of 19% sand, 57% silt, and 24% clay. The experimental area was tilled in the fall of 1999 and the following spring with a disk harrow, followed by a field cultivator before planting. The experimental area was naturally infested with weeds. Predominant weed species in the experimental area included barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.], browntop millet [*Brachiaria ramosa* (L.) Stapf], hemp sesbania [*Sesbania exaltata* (Raf.) Rydb. *ex* A.W. Hill], hyssop spurge (*Euphorbia hyssopifolia* L.), pitted morningglory (*Ipomoea lacunosa* L.), prickly sida (*Sida spinosa* L.), sicklepod [*Senna obtusifolia* (L.) Irwin and Barneby], and yellow nutsedge (*Cyperus esculentus* L.).

Glyphosate-resistant soybean cultivar DP 5806 RR (determinant, late V maturity group) was planted at a population of 359000 seeds/ha on May 12, 2000, in four-row plots with rows 100 cm apart and 24.3 m long. Preemergence (PRE) and postemergence (POST) herbicide treatments were applied as described below and summarized in **Table 1**. All herbicides were applied with a tractor-mounted sprayer with TeeJet 8004 (Spraying Systems Co., Wheaton, IL) standard flat spray tips delivering 187 L/ha water at 179 kPa at a ground speed of 6.8 km/h.

The experiment was conducted in a randomized complete block design with four replications. Rainfall during May and June was normal, but the months of July and August were extremely hot and dry. Soybeans were irrigated five times during July and August. At maturity, soybeans were harvested from two center rows of each plot using a combine.

Missouri Experiment. This experiment was conducted in 2000 at the University of Missouri Bradford Research Farm near Columbia (39° N latitude). The soil was a Mexico silt loam (fine, montmorillonitic, Mesic, Aeric Vertic Epiaqualf) with pH 6.0, 2.3% organic matter, and soil textural fractions of 6% sand, 73% silt, and 21% clay. The experimental area was tilled in the fall 1999 with a disk harrow and the seedbed prepared in spring 2000 with a field cultivator. Predominant weeds in the experimental area included common waterhemp (*Amaranthus rudis* Sauer), giant foxtail (*Setaria faberi* Herrm.), ivyleaf morningglory [*I. hederacea* (L.) Jacq.], pitted morningglory, and prickly sida.

Table 1. Herbicide Treatments Used in Glyphosate-Resistant Soybean Experiments in Mississippi and Missouri in 2000

treatment[a]	rate[b] (g/ha)	application method[c]	application timing
1. glyphosate	1260	POST	3 weeks after planting
	840	POST	6 weeks after planting
2. sulfentrazone +	168	PRE	at planting
chlorimuron fb	34	PRE	at planting
glyphosate	1260	POST	6 weeks after planting
3. sulfentrazone +	168	PRE	at planting
chlorimuron fb	34	PRE	at planting
glyphosate	1260	POST	at full bloom (8 weeks after planting)
4. sulfentrazone +	168	PRE	at planting
chlorimuron fb	34	PRE	at planting
acifluorfen +	280	POST	6 weeks after planting
bentazon +	560	POST	6 weeks after planting
clethodim	140	POST	6 weeks after planting
5. hand-weeded	manual hoeing as needed to remove weeds		

[a] fb, followed by. [b] Rate refers to acid equivalent for glyphosate and active ingredient for all other herbicides. [c] Pre- (PRE) or post- (POST)emergence.

Glyphosate-resistant soybeans (Asgrow 3701 RR; determinant, mid III maturity group) were planted on May 8 at a population of 432000 seeds/ha in four-row plots with rows 76 cm apart and 13.7 m long. The PRE and POST herbicide treatments were applied as described below (**Table 1**). All herbicides were applied with a CO_2-pressurized backpack sprayer at a carrier volume of 187 L/ha and a spray pressure of 207 kPa using flat fan TeeJet XR8003 nozzle tips at a ground speed of 4.8 km/h. Rainfall was adequate throughout the growing season, negating the use of irrigation.

The experiment was conducted in a randomized complete block design with four replications. At maturity, the two center rows of each plot were harvested with a combine.

Herbicide Treatments. Herbicide treatments at both locations included glyphosate at 1260 g of ae/ha at 3 weeks after planting (WAP) followed by (fb) glyphosate at 840 g ae/ha at 6 WAP; sulfentrazone at 168 g of ai/ha plus chlorimuron at 34 g of ai/ha applied PRE fb glyphosate at 1260 g ae/ha at 6 WAP; sulfentrazone at 168 g of ai/ha plus chlorimuron at 34 g of ai/ha applied PRE fb glyphosate at 1260 g of ae/ha at full bloom (8 WAP); sulfentrazone at 168 g of ai/ha plus chlorimuron at 34 g of ai/ha applied PRE fb acifluorfen at 280 g of ai/ha plus bentazon at 560 g of ai/ha plus clethodim at 140 g of ai/ha at 6 WAP; and a hand-weeded control (**Table 1**). PRE herbicides were applied broadcast immediately after planting. POST herbicides were applied at several stages of soybean growth. A nonionic surfactant (paraffinic petroleum oil concentrate) was added to all POST treatments except glyphosate as suggested by the manufacturer.

Soybean Isoflavone Analysis. *Extraction.* Ten-gram samples of oven-dried (100 °C, 15−16 h), ground (Cyclotech 1093 sample mill, Foss Tecator, Höganäs, Sweden) soybeans were extracted with 80% methanol/20% water using a Dionex 200 ASE extractor (Dionex Corp., Sunnyvale, CA), programmed to four cycles to ensure complete extraction of the isoflavones. Extracts were dried in a vacuum.

Analysis. Samples were analyzed for their content of daidzin, genistein, genistin, glycitin, formononetin, and biochanin A by HPLC (Hewlett-Packard 1050, Agilent Technologies Inc., Palo Alto, CA) using a reversed-phase C18 column, Zorbax SB-Aq, 5 μm, 4.6 × 150 mm i.d. × length (Agilent Technologies Inc.) maintained at 26 °C. The mobile phase and solvent elution were as follows (solvent A is 0.05% acetic acid in water; solvent B is 0.05% acetic acid in acetonitrile): 0−2 min, 20% B; 2−18 min, 20−40% B; 18−23 min, 40−100% B; 23−26 min, 100% B; 26−27 min, 100−20% B; 27−34 min, 20% B. The mobile phase flow rate was 0.6 mL/min. Sample volume injection was 10 μL. The isoflavones were detected using a photodiode array detector while on-line monitoring was done at 260 nm.

Daidzein, glycitein, and shikimic acid were analyzed by GC-MS on an Agilent 6890 gas chromatograph coupled to a JEOL GC Mate II mass spectrometer (JEOL Corp., Peabody, MA). The capillary column used was a DB-17HT (15 m length × 0.25 mm i.d. × 0.15 μm film; J&W Scientific, Folsom, CA). The carrier gas was helium (flow rate = 1.0 mL/min). The injection port was maintained at 250 °C. The volume of injection was 1 μL, splitless injection. The GC temperature was programmed as follows: initial temperature, 120 °C; held for 2 min; then increased to 250 °C at a rate of 25 °C/min; held at this temperature for 2 min; then increased to 340 °C at a rate of 60 °C/min; and held at this temperature for 3 min. The GC interface and MS ionization chamber were kept at 250 and 200 °C, respectively. For the quantitation of the isoflavones, standard curves were prepared for each isoflavone using chalcone as an internal standard.

Glyphosate and Aminomethylphosphonic Acid (AMPA) Determination. *Extraction and Derivatization.* Soybeans were dried in the oven at 100 °C for 15−16 h and milled (Cyclotec 1093 sample mill, Högantäs, Sweden). Soybeans were extracted and derivatized following a published procedure (*19*), with minor modifications. One gram of ground soybeans was extracted with 5 mL of water in a sonicating bath for 20 min and then centrifuged at 200*g* for 10 min. Two milliliters of supernatant was taken and transferred to a 20-mL vial. Extraction was repeated by adding 5 mL of water to the sample; the vial was shaken and sonicated for 20 min and then centrifuged at 200*g* for 10 min. One milliliter of supernatant was taken and combined with the 2 mL obtained from the first extraction. Concentrated HCl (15 μL) was added to this combined supernatant and shaken. A 2.5-mL portion was pipeted into a 20-mL vial, and 2.5 mL of CH_2Cl_2 was added, shaken, and centrifuged for 10 min at 200*g*. A portion (1.8 mL) of the water layer was taken, and 200 μL of acidic modifier (16 g of KH_2PO_4, 160 mL of H_2O, 40 mL of MeOH, and 13.4 mL HCl) was added. One milliliter was transferred to a cation-exchange resin column (2-mL packed volume; AG 50W-X8, H^+; Bio-Rad Laboratories, Hercules, CA) that had been previously washed with two 5-mL portions of water. The sample was drained to the top of the column bed, and to the column was added 0.7 mL of CAX mobile phase (160 mL of H_2O, 40 mL of MeOH, and 2.7 mL of HCl), eluted, and discarded. Twelve milliliters of CAX mobile phase was again added to the column to elute the analyses. The eluate was collected in a 20-mL vial and evaporated to dryness using a Savant Speed Vac (model SVC 200, Savant Instruments, Inc., Holbrook, NY). The dried sample was dissolved in 1.5 mL of CAX mobile phase. A 20-μL aliquot was taken and added to 640 μL of a solution of 2,2,3,3,4,4,4-heptafluoro-1-butanol and trifluoroacetic anhydride (1:2) in a chilled 4-mL vial. The mixture was allowed to equilibrate at room temperature for 10−15 min. The vial was transferred to a heating block maintained at 90 °C for 1 h and then allowed to cool to room temperature. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in 80 μL of ethyl acetate containing 0.2% citral; 50 μL was transferred to a GC vial and analyzed by GC-MS.

Analysis. GC-MS (Agilent 6890 Series GC coupled to a JEOL GCMateII mass spectrometer) analysis was done using a DB-5 capillary column (J&W Scientific, Inc.), 30 m length × 0.25 mm i.d. × 0.25 μm film, run under the following GC temperature program: initial, 70 °C; held for 3.5 min; raised to 160 °C at 30 °C/min rate; raised to 270 °C at 70 °C/min rate; raised to 310 °C at 35 °C/min rate; and finally held at this temperature for 3 min. The injection port, GC interface, and ionization chamber were maintained at 260, 200, and 120 °C, respectively. The carrier gas was ultrahigh-purity helium at a 1 mL/min flow rate. The sample injection volume was 1 μL. The MS detector was a magnetic sector; spectra were acquired in the positive, low-resolution, selected-ion monitoring mode. AMPA derivative was observed at 7:23 min (*m*/*z* 571, 502, 446, 372), and glyphosate derivative was observed at 7:59 min (*m*/*z* 611, 584, 460). Glyphosate and AMPA in the samples were quantified from a calibration curve of derivatized standards of glyphosate and AMPA.

Statistical Analysis. Values from HPLC and GC-MS quantification were statistically analyzed through analysis of variance (ANOVA) using the GLM procedure of SAS software (SAS Institute Inc., Cary, NC). Treatment means were separated using the least significant difference (LSD) test in the GLM procedure. Fisher's protected LSD (*20*) was implemented in that the LSD was interpreted only if the ANOVA *F* test for treatment effect was significant ($P = 0.05$).

Table 2. Effects of Different Herbicide Treatments on Shikimate, Isoflavones and Their Glycosides, Glyphosate, and AMPA in Soybean Seed[a]

seed constituent	μg/g for herbicide treatment				
	1	2	3	4	5
			Stoneville		
shikimate	52	45	55	42	26
daidzein	1023a	634b	883ab	625b	612b
daidzin	1102	773	973	1049	888
genistein	258	150	147	107	113
genistin	1136	962	1105	1202	1041
glycitein	973	656	806	636	676
glycitin	383	441	394	477	459
glyphosate	0.181b	0.480b	2.18a	0.166c	0.103c
AMPA	0.602b	0.729b	7.27a	0.269b	0.263b
			Columbia		
shikimate	29	24	60	41	57
daidzein	805	856	967	1013	1002
daidzin	1367	1562	1704	1398	1696
genistein	250	311	389	382	294
genistin	1403	1413	1347	1385	1451
glycitein	631	562	940	810	674
glycitin	583	555	556	502	502
glyphosate	0.234b	0.552b	3.08a	0.086c	0.126c
AMPA	0.862b	0.492b	25.00a	0.158b	0.126b

[a] Means in the same row with different letters are significantly different ($P = 0.05$) based on Fisher's protected LSD. There were no significant differences between means in rows without letters.

RESULTS AND DISCUSSION

Qualitatively, the results at the Stoneville, MS, and Columbia, MO, sites were similar (**Table 2**). Shikimate levels were low and not significantly affected by any treatment. In healthy plants of most species, including nontransgenic soybean, shikimate levels are low. By blocking EPSPS, glyphosate causes manyfold increases in shikimate levels in glyphosate-treated soybean plants (*21*, *22*). The effects on shikimate are much more dramatic than glyphosate-induced decreases in levels of compounds derived from shikimate such as anthocyanin (*12*, *13*). In fact, elevated shikimate levels are used as an early and highly sensitive indicator of glyphosate effects on plant tissues (*22*). In transgenic, glyphosate-resistant cotton, shikimate levels rise when the plants are treated with enough glyphosate to cause sublethal effects on reproductive tissues (*23*). Thus, the absence of an effect or only slight increases in shikimate observed in this study indicated that the CP4 EPSPS was either not inhibited or minimally inhibited and that the CP4 EPSPS utilized all or most of the shikimate that would have accumulated from inhibition of the native EPSPS. If so, one would expect no effects of glyphosate on shikimate products, such as isoflavones and their glycosides. There are no reports of inhibitors of acetolactate synthase (chlorimuron), protoporphyrinogen oxidase (acifluorfen and sulfentrazone), or photosystem II (bentazon) on shikimate levels.

There was no effect of glyphosate or any other herbicide treatment on isoflavone levels at the Columbia site (**Table 2**). At the Stoneville location, glyphosate used alone (treatment 1) elevated daidzein levels. At the 5% level of confidence, at least one significant difference would be expected in this number of treatments, even if there were no effects. Only trace amounts of formononetin and biochanin A were found in all samples from both sites (data not shown). Acetolactate synthase inhibitors, such as chlorimuron, and protoporphyrinogen oxidase inhibitors, such as sulfentrazone and acifluorfen, can cause elevated levels of products of the shikimate pathway, such as isoflavones (see, e.g., refs *24* and *25*). There was no evidence of such an effect in this study.

Glyphosate and its metabolite, AMPA, were found at the highest levels when treated at the latest date with glyphosate at both locations (treatment 3). The highest level of glyphosate found (treatment 3, Columbia; 3.08 μg/g) was below the EPA tolerance level of 5 μg/g (*26*). The high levels of AMPA in treatment 3 (7 and 25 μg/g in Stoneville and Columbia, respectively) were a surprise, because glyphosate has not been reported to be readily degraded in soybean plants. There are no EPA tolerance levels for AMPA in soybean. We were also surprised to find low levels of glyphosate and AMPA in treatments 4 and 5, which had not included glyphosate. These findings were not due to contaminated chromatograpy columns, as we found similar values when using fresh columns that had not had a glyphosate treatment sample passed through it. The most likely explanation is that there was herbicide drift at both locations, both from the glyphosate treatments in this study and, perhaps, from surrounding fields. With widespread adoption of GR soybean and cotton, it is difficult to find an experimental site free from glyphosate drift from neighboring fields. Others have explained glyphosate contamination of seeds of untreated wheat and canola by herbicide drift (*27*, *28*). In these previous papers, unsuccessful efforts were made to shield untreated plants from glyphosate drift. Drift of glyphosate to nontarget crops and areas has been a significant problem in glyphosate-resistant crops (*29*).

Little is known of the degradation of glyphosate to AMPA in plants. No plant-derived enzyme has been shown to make this conversion. Soybean cell cultures degrade glyphosate to AMPA more efficiently that those of wheat or maize (*30*). AMPA is mildly phytotoxic to soybean, and its mode of action is apparently different from that of glyphosate (*31*). AMPA can cause anthocyanin levels to increase in soybean seedlings (*31*). AMPA levels were apparently insufficient to affect isoflavone levels in soybean seed in our study (**Table 2**).

In a recent study (*32*) with another legume (field pea, *Pisum sativum* L.), much higher levels of glyphosate and much lower levels of AMPA were found in the seed of plants treated with 1.7 kg of glyphosate at an early seed maturation stage of development. In this case, the crop was nontransgenic, so the lack of metabolism of glyphosate to AMPA could have been due to the high degree of toxicity of the glyphosate treatment.

In summary, even at higher application rates and with later applications than used by Taylor et al. (*16*), glyphosate had little or no effect on shikimate or isoflavones in GR soybeans. These results confirm that there should be no concern about effects of glyphosate on this aspect of the nutritional and nutraceutical properties of GR soybeans when used at the times and rates used in our study.

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