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Aminomethylphosphonic Acid, a Metabolite of Glyphosate, Causes Injury in Glyphosate-Treated, Glyphosate-Resistant Soybean

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Glyphosate-resistant (GR) soybean [*Glycine max* (L.) Merr.] was developed by stable integration of a foreign gene that codes insensitive enzyme 5-enolpyruvylshikimate-3-phosphate synthase, an enzyme in the shikimate pathway, the target pathway of glyphosate. Application of glyphosate to GR soybean results in injury under certain conditions. It was hypothesized that if GR soybean is completely resistant to the glyphosate, injury could be caused by a metabolite of glyphosate, aminomethylphosphonic acid (AMPA), a known phytotoxin. Glyphosate and AMPA effects on one- to two-trifoliolate leaf stage (16−18-days old) GR and non-GR soybean were examined in the greenhouse. In GR soybean, a single application of glyphosate−isopropylammonium (1.12−13.44 kg/ha) with 0.5% Tween 20 did not significantly reduce the chlorophyll content of the second trifoliolate leaf at 7 days after treatment (DAT) or the shoot dry weight at 14 DAT compared with Tween 20 alone. A single application of AMPA (0.12−8.0 kg/ha) with 0.5% Tween 20 reduced the chlorophyll content of the second trifoliolate leaf by 0−52% at 4 DAT and reduced shoot fresh weight by 0−42% at 14 DAT in both GR and non-GR soybeans compared with Tween 20 alone. AMPA at 0.12 and 0.50 kg/ha produced injury in GR and non-GR soybean, respectively, similar to that caused by glyphosate−isopropylammonium at 13.44 kg/ha in GR soybean. AMPA levels found in AMPA-treated soybean of both types and in glyphosate-treated GR soybean correlated similarly with phytotoxicity. These results suggest that soybean injury to GR soybean from glyphosate is due to AMPA formed from glyphosate degradation.

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

INTRODUCTION

Glyphosate inhibits the biosynthesis of aromatic amino acids (phenylalanine, tryptophan, and tyrosine), which leads to several metabolic disturbances, including the arrest of protein production and prevention of secondary product formation (*1*) and the deregulation of the shikimate pathway, leading to general metabolic disruption (*2*, *3*). Glyphosate-resistant (GR) soybean was created by stable integration of a transgene from *Agrobacterium* species that codes insensitive enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimate pathway (*4*). Expression of the glyphosate-resistant EPSPS enzyme helps to maintain normal aromatic amino acid levels in GR soybean treated with glyphosate. Several crops resistant to glyphosate have been commercialized since the mid-1990s (*5*−*8*). The most successful transgenic crop in the world has been GR soybean (*5*, *8*). Its use has steadily increased from 2% of the U.S. soybean acreage in 1996 to 81% in 2003 (*9*).

Although transgenic soybean is resistant to glyphosate, application of glyphosate to GR soybean may result in injury under certain conditions and with certain formulations. Glyphosate can decrease chlorophyll content, plant growth, nodule biomass and leghemoglobin content, and nitrogen fixation and accumulation in GR soybean (*10*−*15*). The visible injury symptoms following glyphosate treatment include foliar speckling, necrosis, and chlorosis (*10*, *14*). These injury symptoms develop within 1−2 h or days after glyphosate treatment, and GR soybeans usually recover from injury over time. Speckling and necrosis may have been due to the salts, surfactants, and other ingredients of the formulations of glyphosate. For example, 1-aminomethanamide dihydrogen tetraoxosulfate salt of glyphosate (Engame, Entek Corp., Elkridge, MD) caused burning and necrosis in GR soybean within hours after glyphosate treatment (*14*). During the past 7 years of field research with GR soybean, the senior author (K.N.R.) has observed chlorosis

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following isopropylamine salt of glyphosate (Roundup Ultra, Monsanto Co., St. Louis, MO) application in two of seven years. However, the exact cause of chlorosis in glyphosate-treated GR soybean is unknown.

Little is known of the degradation of glyphosate to aminomethylphosphonic acid (AMPA) in plants. It has been conjectured that glyphosate can be metabolized by plants via two pathways similar to those present in microorganisms (*1*). One involves oxidative cleavage of the C−N bond to yield AMPA and the other, breaking of the C−P bond by a C−P lyase to generate sarcosine. The metabolite, AMPA, is phytotoxic to plant species, although it is considerably less active than glyphosate (*1*, *16*). Although glyphosate is normally metabolized very little by plants (*3*), AMPA is found as a major metabolite in seeds of GR soybean treated with glyphosate (*17*). AMPA residues were also detected in leaves and seeds of field-grown GR soybean treated with glyphosate at label use rates, indicating metabolism of glyphosate in GR soybean (*18*).

We have previously reported that application of glyphosate at label use rates to GR soybean can result in chlorosis and reduced plant growth (*13*, *14*). If GR soybean is completely resistant to glyphosate, then injury could be caused by its degradation product, AMPA. In this paper, we investigated glyphosate and AMPA effects on GR soybean under greenhouse conditions. We present evidence that GR soybean injury following glyphosate treatment is caused by AMPA formed from glyphosate degradation.

MATERIALS AND METHODS

General Experimental Conditions. Greenhouse experiments were conducted during September 2002−March 2003 at the Southern Weed Science Research Unit, U.S. Department of Agriculture, Stoneville, MS. Soybean varieties (Asgrow 4702RR, GR variety; HBK 4891, non-GR variety) used were determinant, highly adaptive to the Mississippi Delta region, and belonged to the late IV maturity group. Five soybean seeds were planted in a 30-cm diameter plastic pot containing a 1:1 (v/v) mixture of Bosket sandy loam soil and Jiffy mix (Jiffy Products of America Inc., Batavia, IL). After emergence, soybean plants were thinned to two uniform plants per pot. The greenhouse was maintained at 28/22 °C (±3 °C) day/night temperature with natural light supplemented by sodium vapor lamps to provide a 13-h photoperiod. Plants were subirrigated with water as needed to maintain adequate soil moisture. Soybean plants at one- to two-trifoliolate leaf (16−18 days old) growth stage were used for treatment. Spray solutions were applied using an indoor spray chamber equipped with an air-pressurized system at a volume of 190 L/ha at 140 kPa using 8002E flat-fan nozzles.

Experiment 1. Glyphosate-Isopropylammonium Dose−Response in GR Soybean. Glyphosate-isopropylammonium at 1.12, 2.24, 3.36, 4.48, 6.72, and 13.44 kg/ha was applied to GR soybean. Spray solutions were prepared using technical grade glyphosate-isopropylammonium (>95% purity, Chem Service, West Chester, PA) with Tween 20 (0.5%, v/v). Technical grade glyphosate-isopropylammonium was used to minimize interference associated with unknown ingredients in the commercial formulations of glyphosate. Nontreated plants and Tween 20 treated plants were included as appropriate controls. At 7 days after treatment (DAT), distal leaflets of the second trifoliolate leaf from two plants/pot in a given treatment were sampled for chlorophyll determination. Chlorophyll was extracted with 10 mL of dimethyl sulfoxide, and chlorophyll concentrations were determined spectrophotometrically (*19*). At 14 DAT, soybean plants (two plants/pot) were excised at the soil surface and oven-dried, and dry weights were recorded. Chlorophyll content and shoot dry weight were expressed as percent of nontreated control. Treatments were arranged in a randomized complete block design with eight replications. Data were subjected to analysis of variance, and means were separated using Fisher's protected least significant difference (LSD) test at $P = 0.05$ (*20*).

Experiment 2. Glyphosate, Shikimate, and AMPA Accumulation in Glyphosate-Isopropylammonium-Treated GR Soybean. Glyphosate-isopropylammonium (technical grade, >95% purity) at 6.72 kg/ha was applied to GR soybean. This is 3.8 and 2.6 times higher rate than the suggested label use rate for single and total in-crop application of glyphosate, respectively, in GR soybean. We selected this rate to represent the "worst case scenario" to promote soybean injury and to enable detection of AMPA residues in leaf tissue. Preparation of spray solutions and application was as described for experiment 1. Two sets of Tween 20 treated plants were included in the study. Soybean plants were harvested at 1, 3, 5, 7, 14, and 22 DAT. Plants were clipped at the base, washed with running water, rinsed with distilled water to remove glyphosate-isopropylammonium remaining on the leaf surface, and blotted dry with paper towels. Leaves without petioles were partitioned into leaves treated with glyphosate-isopropylammonium and new leaves produced after spraying. Treated leaf samples consisted of a pair of primary leaves, and first, second, and third trifoliolate leaves. At treatment, plants had tiny third trifoliolate leaves that were exposed to glyphosate spray. New leaf samples consisted of fourth trifoliolate leaves and above. New leaves were collected only from 7, 14, and 22 DAT. Leaves were sampled from one set of Tween 20 treated plants for 1 DAT (treated leaves) and another set for 22 DAT (new leaves). All leaf samples were air-dried, ground, and analyzed for glyphosate, shikimate, and AMPA. Treatments were arranged in a randomized complete block design with five replications. Data were subjected to analysis of variance and means separation test as previously described.

Experiment 3. AMPA Dose−Response and AMPA Concentrations in GR Soybean and Non-GR Soybean. AMPA at 0.12, 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 kg/ha was applied to GR and non-GR soybean. Spray solutions were prepared using technical grade AMPA (99% purity, Sigma-Aldrich, St. Louis, MO) with Tween 20 (0.5%, v/v). Nontreated plants and Tween 20 treated plants were included as appropriate controls. Chlorophyll content was determined as described in the previous study at 4 DAT. At 14 DAT, soybean plants (two plants/pot) were excised at the soil surface and fresh weights recorded. After recording weights, plants were washed with water to remove AMPA residue remaining on the leaf surface and blotted dry with paper towels. All leaves were sampled without petioles, air-dried, ground, and analyzed for shikimate and AMPA. Treatments were arranged in a randomized complete block design with eight replications; however, shikimate and AMPA were analyzed from only five replications. Data were subjected to analysis of variance and means separation test as previously described.

Extraction of Soybean Leaves and Derivatization of Extracts. Soybean leaf samples were analyzed for glyphosate, shikimic acid, and AMPA at the Natural Products Utilization Research Unit, U.S. Department of Agriculture, University, MS. For glyphosate and AMPA analysis, extraction and derivatization were performed according to a published procedure (*21*), with modifications. One gram of ground leaves was extracted with 15 mL of water in a 20-mL vial, shaken, placed in a sonicating bath for 20 min, and then centrifuged (Sorvall RC 5C Plus; Kendro Laboratory Products, Asheville, NC) at 2000*g* and 20 °C, for 20 min. Four milliliters of supernatant was taken and filtered. The tissue sample pellet was extracted a second time by adding 5 mL of water, and procedures were performed as in the first extraction. Two milliliters of supernatant was taken, filtered, and combined with the 4 mL from the first extraction; then 30 μL of concentrated HCl was added and shaken. Four milliliters was transferred to a 20-mL scintillation vial provided with a Teflon-lined cap, shaken with 4 mL of methylene chloride, and centrifuged (Savant speed vac model SVC 200, Savant Instruments, Inc., Holbrook, NY) for 10 min. 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, 13.4 mL of HCl) was added. One milliliter was loaded to a cation-exchange resin column (AG 50W-X8, H^+; Bio-Rad Laboratories, Hercules, CA) previously equilibrated with two 5-mL portions of water. The sample was eluted until the level of the column bed. CAX mobile phase [160 mL of H_2O, 40 mL of MeOH, 2.7 mL of HCl (0.7 mL)] was added, eluted, and discarded. Twelve milliliters of CAX mobile phase was again added to the column to elute the analytes. The eluate was collected in a 20-mL vial and evaporated to dryness using a Savant speed vac (model SVC 200, Savant Instruments, Inc.). To the dried sample was added 1.5 mL of CAX mobile phase, and then the vial was placed in a

sonicating bath for 30 min. 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 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. This method afforded 90 and 86% recoveries of glyphosate and AMPA, respectively, based on duplicate extraction experiments in which samples were fortified with 100 ng standards per gram of sample.

For the analysis of shikimic acid, ground leaves were dried in an oven at 80 °C overnight. The leaves were extracted with water (1 g/25 mL) in a sonicating bath for 1 h and then centrifuged at 2000*g* and 20 °C, for 20 min. The supernatant was filtered using a Puradics 25 AS disposable filter device (Whatman, Inc., Clifton, NJ) and the filtrate lyophilized. One milligram of the lyophilized extract was treated with 100 μL of bis(trimethylsilyl)trifluoroacetamide/dimethylformamide (1:1 mixture) and heated at 70 °C for 30 min. This was used for GC-MS analysis.

GC-MS Analysis of Glyphosate, AMPA, and Shikimic Acid in Soybean Leaves. Analysis of glyphosate, AMPA, and shikimic acid by GC-MS (Agilent 6890 series GC coupled to a JEOL GCMateII mass spectrometer) was performed using a DB-5 capillary column (J&W Scientific, Inc., Folsom, CA), 30 m length × 0.25 mm i.d. × 0.25 μm film. The MS detector was a magnetic sector; spectra were acquired in the positive, low-resolution, selected-ion monitoring mode. The injection port, GC interface, and ionization chamber were maintained at 260, 230, 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. Glyphosate, AMPA, and shikimic acid in the samples were quantitated from a calibration curve of respective derivatized standards.

For the analysis of glyphosate and AMPA, the temperature program was as follows: 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. 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). The limit of detection (LOD) and limit of quantitation (LOQ) for glyphosate were 0.250 and 0.834 ppb, respectively. The LOD and LOQ for AMPA were 0.052 and 0.172 ppb, respectively. For shikimic acid analysis, the temperature program was as follows: initial, 80 °C, held for 2.5 min, raised to 160 °C at 30 °C/min rate, raised to 270 °C at 40 °C/min rate, raised to 310 °C at 45 °C/min rate, and finally held at this temperature for 3 min. Shikimic acid derivative was observed at 6.38 min (*m*/*z* 462, 447, 357, 204).

Glyphosate, shikimic acid, and AMPA were determined in duplicate samples. Treatments were arranged in a randomized complete block design, and data were subjected to analysis of variance and means separation test as previously described.

RESULTS AND DISCUSSION

Experiment 1. Glyphosate-Isopropylammonium Dose−Response in GR Soybean. Statistically, application of glyphosate-isopropylammonium at as high as 13.44 kg/ha had no effect on chlorophyll content and shoot dry weight of GR soybean. However, numerically, a single application of glyphosate-isopropylammonium at 1.12−13.44 kg/ha reduced the chlorophyll content of the second trifoliolate leaf by 10% at 7 DAT and reduced the shoot dry weight by 8% at 14 DAT compared with Tween 20 alone in GR soybean (**Table 1**). Evidently, the GR soybean variety used was highly resistant to glyphosate under conditions of this study. Soybean injury (speckling, necrosis, and chlorosis) following glyphosate treatment in GR soybean has been documented (*10*, *13*, *14*). Reddy et al. (*13*) have observed that treatment of GR soybean with glyphosate at 0.84 kg of ae/ha had little effect or no effect on chlorophyll content and dry weights of shoots and roots in five of five trials. However, treatment of glyphosate at 1.68 kg of ae/ha reduced these parameters in three of five trials. Speckling and necrosis may have been due to the ingredients of the formulations of glyphosate. The senior author (K.N.R.) has observed chlorosis following glyphosate (Roundup Ultra, Monsanto Co., St. Louis, MO) application in two of the past seven years of field research with GR soybean. However, the exact cause of chlorosis in glyphosate-treated GR soybean is not known. If GR soybean is completely resistant to glyphosate, then injury could be caused by a plant-derived metabolite of glyphosate, AMPA, a known phytotoxin. To test this hypothesis, we measured glyphosate, shikimate, and AMPA levels in leaves of GR soybean following glyphosate treatment.

Table 1. Effect of Glyphosate-isopropylammonium on Chlorophyll Content and Shoot Dry Weight of Glyphosate-Resistant Soybean[a]

glyphosate-isopropylammonium rate,[b] kg/ha	% of control	
	chlorophyll, 7 DAT	shoot dry wt, 14 DAT[c]
untreated control	100 a	100 a
Tween 20	96 a	92 ab
1.12	96 a	93 ab
2.24	88 a	88 b
3.36	87 a	89 b
4.48	88 a	87 b
6.72	84 a	86 b
13.44	86 a	84 b

[a] Means within a column followed by the same letter are not significantly different at the 5% level as determined by Fisher's protected LSD test. [b] Tween 20 at 0.5% (v/v) was added to spray solutions in all treatments except untreated control. [c] DAT, days after treatment.

Experiment 2. Glyphosate, Shikimate, and AMPA Accumulation in Glyphosate-isopropylammonium-Treated GR Soybean. In treated leaves (exposed to glyphosate at spray) of GR soybean, glyphosate concentration was highest at 1 DAT (527 μg/g of tissue) and gradually decreased to 37 μg/g of tissue at 22 DAT (**Table 2**). This decrease was mainly due to glyphosate translocation to other plant parts and degradation. Shikimate levels were not affected by glyphosate-isopropylammonium at 6.72 kg/ha, regardless of time after treatment. AMPA (42 μg/g of tissue) was found at highest level within 1 DAT (**Table 2**). Similar to glyphosate levels, AMPA levels gradually decreased from 42 μg/g of tissue at 1 DAT to 1 μg/g of tissue at 22 DAT. Detection of AMPA following glyphosate treatment suggests that a plant glyphosate oxidoreductase (GOX) was responsible for this conversion. Reductions of AMPA after accumulation could be due to either translocation or further metabolic alterations.

In new leaves (developed after glyphosate spray) of GR soybean, glyphosate concentration was highest at 7 DAT (239 μg/g of tissue) and lowest at 22 DAT (3 μg/g of tissue) (**Table 2**). Shikimate levels in new leaves were unaffected by glyphosate, similar to that of treated leaves. AMPA was also detected in the new leaves, and the concentration ranged from 42 μg/g of tissue at 7 DAT to 1 μg/g of tissue at 22 DAT. Little is known about AMPA translocation within the soybean plant. AMPA in new leaves may have been derived from the degradation of glyphosate in situ, translocation of AMPA formed in the treated leaves, or both.

Experiment 3. AMPA Dose−Response and AMPA Concentrations in GR Soybean and Non-GR Soybean. A single application of AMPA at 0.12−8.00 kg/ha reduced chlorophyll content of the second trifoliolate leaf by 0−52% at 4 DAT and reduced shoot fresh weight by 0−42% at 14 DAT compared with Tween 20 alone in both GR and non-GR soybean (**Table 3**). Evidently, AMPA as low as 0.50 kg/ha produces injury in both GR and non-GR soybean, similar to that of glyphosate-

Table 2. Effect of Glyphosate-isopropylammonium (Glyphosate-Ipa) Treatment at 6.72 kg/ha on Glyphosate, Shikimate, and Aminomethylphosphonic Acid (AMPA) Concentration in Treated and New Leaves of Glyphosate-Resistant Soybean over Time[a]

		treated leaves[b]			new leaves[c]		
treatment[d]	time after treatment, days	glyphosate, μg/g of tissue	shikimate, ng/g of tissue	AMPA, μg/g of tissue	glyphosate, μg/g of tissue	shikimate, ng/g of tissue	AMPA, μg/g of tissue
Tween 20 only	1	0 f	139 a	0 e			
glyphosate-ipa	1	527 a	131 a	42 a			
glyphosate-ipa	3	336 b	146 a	19 b			
glyphosate-ipa	5	167 c	135 a	10 c			
glyphosate-ipa	7	149 c	141 a	8 cd	239 a	126 a	42 a
glyphosate-ipa	14	99 d	167 a	3 de	121 b	147 a	21 b
glyphosate-ipa	22	37 e	147 a	1 e	3 c	148 a	1 c
Tween 20 only	22				0 c	121 a	0 c

[b] Means within a column followed by the same letter are not significantly different at the 5% level as determined by Fisher's protected LSD test. [b] Treated leaves included first pair, and first, second, and third trifoliolate leaves. [c] New leaves included fourth trifoliolate leaf and above. [d] Tween 20 at 0.5% (v/v) was added to all treatment solutions.

Table 3. Effect of Aminomethylphosphonic Acid (AMPA) Treatment on Chlorophyll Content 4 Days after Treatment and Shoot Fresh Weight 14 Days after Treatment of Glyphosate-Resistant (GR) and Non-GR Soybean[a]

	chlorophyll, % of control		shoot fresh wt, % of control	
AMPA rate,[b] kg/ha	GR soybean	non-GR soybean	GR soybean	non-GR soybean
untreated control	100 a	100 a	100 a	100 a
Tween 20	86 b	83 ab	98 a	93 ab
0.12	72 c	84 ab	96 ab	93 ab
0.25	58 d	82 bc	91 bc	90 bc
0.50	59 d	66 c	90 cd	91 bc
1.00	50 de	41 d	88 cd	85 c
2.00	40 ef	36 d	86 d	84 c
4.00	40 ef	41 d	74 e	66 d
8.00	34 f	31 d	61 f	51 e

[a] Means within a column followed by the same letter are not significantly different at the 5% level as determined by Fisher's protected LSD test. [b] Tween 20 at 0.5% (v/v) was added to spray solutions in all treatments except untreated control.

Table 4. Effect of Aminomethylphosphonic Acid (AMPA) Treatment on Shikimate and AMPA Concentration at 14 Days after Treatment in Leaves of Glyphosate-Resistant (GR) and Non-GR Soybean[a]

	shikimate, ng/g of tissue		AMPA, μg/g of tissue	
AMPA rate,[b] kg/ha	GR soybean	non-GR soybean	GR soybean	non-GR soybean
Tween 20	161 a	171 a	0 d	0 e
0.12	178 a	185 a	2 d	1 e
0.25	156 a	159 a	2 d	3 e
0.50	170 a	153 a	7 d	4 e
1.00	173 a	180 a	30 c	23 d
2.00	177 a	169 a	41 c	33 c
4.00	183 a	178 a	64 b	65 b
8.00	180 a	175 a	112 a	117 a

[a] Means within a column followed by the same letter are not significantly different at the 5% level as determined by Fisher's protected LSD test. [b] Tween 20 at 0.5% (v/v) was added to spray solutions.

isopropylammonium at 13.44 kg/ha observed in GR soybean (**Table 1**). AMPA at 0.12 kg/ha reduced leaf chlorophyll content in GR soybean, whereas a 4 times greater rate was required to reduce chlorophyll content in non-GR soybean, indicating differential sensitivity to AMPA. Differential sensitivity to AMPA in GR and non-GR soybean might be attributed to differences in soybean varieties. However, it should be stressed that GR and non-GR varieties used in the studies were from different seed companies and their chance of being sister lines is low. AMPA concentrations in leaves increased with increased rate of AMPA application in both GR and non-GR soybeans (**Table 4**). AMPA levels of 4−7 μg/g of tissue apparently caused significant reduction in chlorophyll content of both GR and non-GR soybean. Shikimate levels were not affected by AMPA treatment in both GR and non-GR soybean (**Table 4**), indicating that it does not act like glyphosate. AMPA is phytotoxic to soybean as evidenced from the reduction in chlorophyll content and shoot fresh weight (**Table 3**), by an unknown mechanism. The stronger dose−response relationship when GR soybean was treated with AMPA (**Table 3**) compared with the dose−response relationship when GR soybean was treated with glyphosate (**Table 1**) suggests that GR soybean injury from glyphosate is primarily dependent on the rate of degradation of glyphosate to AMPA.

In GR soybeans, shikimate levels were not significantly affected by glyphosate treatment compared with Tween 20 control (**Table 2**). By blocking EPSPS, glyphosate causes manyfold increases in shikimate levels in glyphosate-treated, non-GR soybean (*22*) and oilseed rape (*Brassica napus* L. cv. Iris) (*23*) plants. In other research, Duke et al. (*17*) also observed that shikimate levels in GR soybean seed were unaffected by commonly used glyphosate treatments in soybean production. Elevated shikimate levels are used as an early and highly sensitive indicator of glyphosate effects on glyphosate-sensitive plant tissues (*23*). In transgenic, glyphosate-resistant cotton, shikimate levels rise when the plants are treated with enough glyphosate to cause sublethal effects on reproductive tissues (*24*). Thus, the absence of an effect on shikimate observed in this study indicated either that the insensitive EPSPS was not inhibited or that the insensitive EPSPS utilized all of the shikimate that would have accumulated from inhibition of the native EPSPS.

Glyphosate and its metabolite, AMPA, were found at the highest levels within 1 day after glyphosate treatment in GR soybean. Evidently, a plant GOX makes this conversion, although little is known of the degradation of glyphosate to AMPA in plants. AMPA is phytotoxic to soybean, and its mode of action is apparently different from that of glyphosate (*16*). External application of AMPA as low as 0.50 kg/ha injured both GR and non-GR soybeans, similar to glyphosate-isopropylammonium at 13.44 kg/ha observed in GR soybean (**Table 3**). Similar AMPA levels in glyphosate-treated GR soybean and in AMPA-treated GR soybean or conventional soybean were associated with similar phytotoxicity symptoms (**Tables 1** and **3**), indicating that mild phytotoxicity of glyphosate in GR soybean is due to AMPA formed.

The rapid conversion of glyphosate to AMPA in soybean found in this paper does not support the view that the AMPA formation is due to conversion of glyphosate to AMPA by leaf

surface microflora. AMPA residues were found in leaves, stems, and seeds of GR soybean following glyphosate applications (*17*, *18*). Furthermore, over time, there is more AMPA in some plant tissues than glyphosate. We found much more AMPA than glyphosate in seeds of glyphosate-treated, glyphosate-resistant soybean (*17*). Previous studies have shown axenic cell cultures of soybean to degrade glyphosate much better than cells of other crops (*25*). Taken together, these data suggest that soybean has a plant GOX. However, no plant-derived GOX has been described in the literature, making this an area ripe for further investigation.

In summary, the potential for GR soybean injury from glyphosate treatments exists. Our results indicate that injury is caused by AMPA formed from glyphosate degradation. If so, the extent of injury in glyphosate-treated GR soybean is largely dependent on levels of AMPA formed within the plant. Under field conditions, the extent of AMPA formation may depend on glyphosate rate, genotype, and environmental conditions. This uncertainty in AMPA formation within plant explains why some soybean farmers confront glyphosate injury in GR soybean and others do not, and why the same farmer often observes injury in one year and not in other years. Our results predict that insertion of the bacterial GOX into GR soybean, as has been used in GR canola (*Brassica napus* L.) (*26*), would exacerbate the phytotoxicity problem. Why the bacterial GOX gene has been commercially used in only one crop is not known. Apparently, it does not cause a problem in canola, but we do not know the susceptibility of canola to AMPA, nor do we know the efficacy of bacterial GOX in canola in converting glyphosate to AMPA in vivo.

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