JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Glyphosate-Resistant and -Susceptible Soybean (*Glycine max*) and Canola (*Brassica napus*) Dose Response and Metabolism Relationships with Glyphosate

VIJAY K. NANDULA,*,† KRISHNA N. REDDY,‡ AGNES M. RIMANDO,§ STEPHEN O. DUKE,§ AND DANIEL H. POSTON†

Delta Research and Extension Center, Mississippi State University, P.O. Box 197, Stoneville, Mississippi 38776, Southern Weed Science Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 350, Stoneville, Mississippi 38776, and Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 8048, University, Mississippi 38677

Experiments were conducted to determine (1) dose response of glyphosate-resistant (GR) and -susceptible (non-GR) soybean [*Glycine max* (L.) Merr.] and canola (*Brassica napus* L.) to glyphosate, (2) if differential metabolism of glyphosate to aminomethyl phosphonic acid (AMPA) is the underlying mechanism for differential resistance to glyphosate among GR soybean varieties, and (3) the extent of metabolism of glyphosate to AMPA in GR canola and to correlate metabolism to injury from AMPA. GR_{50} (glyphosate dose required to cause a 50% reduction in plant dry weight) values for GR (Asgrow 4603RR) and non-GR (HBKC 5025) soybean were 22.8 kg ae ha^{-1} and 0.47 kg ha^{-1}, respectively, with GR soybean exhibiting a 49-fold level of resistance to glyphosate as compared to non-GR soybean. Differential reduction in chlorophyll by glyphosate was observed between GR soybean varieties, but there were no differences in shoot fresh weight reduction. No significant differences were found between GR varieties in metabolism of glyphosate to AMPA, and in shikimate levels. These results indicate that GR soybean varieties were able to outgrow the initial injury from glyphosate, which was previously caused at least in part by AMPA. GR_{50} values for GR (Hyola 514RR) and non-GR (Hyola 440) canola were 14.1 and 0.30 kg ha^{-1}, respectively, with GR canola exhibiting a 47-fold level of resistance to glyphosate when compared to non-GR canola. Glyphosate did not cause reduction in chlorophyll content and shoot fresh weight in GR canola, unlike GR soybean. Less glyphosate (per unit leaf weight) was recovered in glyphosate-treated GR canola as compared to glyphosate-treated GR soybean. External application of AMPA caused similar injury in both GR and non-GR canola. The presence of a bacterial glyphosate oxidoreductase gene in GR canola contributes to breakdown of glyphosate to AMPA. However, the AMPA from glyphosate breakdown could have been metabolized to nonphytotoxic metabolites before causing injury to GR canola. Injury in GR and non-GR canola from exogenous application of AMPA was similar.

KEYWORDS: Aminomethylphosphonic acid; AMPA; *Brassica napus*; canola; dose response; *Glycine max*; glyphosate; glyphosate resistance; GOX; shikimic acid; soybean

1. 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 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. It apparently also prevents the metabolic disruption caused by inhibition of the shikimate pathway (*2*). Several crops resistant to glyphosate have been commercialized since the mid-1990s (*5*−*8*). Although transgenic soybean is resistant to glyphosate, application of glyphosate to GR soybean may result in injury under certain conditions and with certain formulations (*9*−*11*). The visible

* Author to whom correspondence should be addressed [telephone (662) 686-3271; fax (662) 686-7336; e-mail vkn8@msstate.edu].
† Mississippi State University.
‡ USDA-ARS Southern Weed Science Research Unit.
§ USDA-ARS Natural Products Utilization Research Unit.

10.1021/jf063568l CCC: $37.00
Published on Web 04/07/2007

injury symptoms following glyphosate treatment include foliar speckling, necrosis, and chlorosis (*10*, *11*). These injury symptoms develop within 1−2 h or days after glyphosate treatment, and GR soybeans usually recover from injury (*11*).

Glyphosate was reported to be metabolized to aminomethylphosphonic acid (AMPA) in soybean, and AMPA residues were detected in leaves and seeds of glyphosate-treated, field-grown GR soybean (*12*, *13*). Injury from glyphosate in GR soybean was hypothesized to be caused by AMPA formed from glyphosate degradation, and evidence was provided to validate this theory (*9*). AMPA at 0.12 kg ha^{-1} produced chlorosis in both GR and conventional soybean similar to that caused by glyphosate-isopropylammonium (IPA) at 13.44 kg ha^{-1} in GR soybean. AMPA levels found in AMPA-treated soybeans of both types and in glyphosate-treated GR soybean correlated similarly with phytotoxicity. The extent of injury from AMPA depends on GR soybean variety and environmental conditions (*9*). Several farmers have observed severe chlorosis in GR soybean varieties Delta King 4868RR (field observation by Daniel Poston) and HBK 4820RR (Alan Blaine, personal communication) following glyphosate application. We hypothesized that severe chlorosis in these two varieties was due to greater degradation of glyphosate to AMPA in Delta King 4868RR and HBK 4820RR than in other GR varieties. In this study, we attempted to compare AMPA formation in Delta King 4868RR and HBK 4820RR with another predominantly used GR variety in the region (Asgrow 4603RR).

Detection of AMPA following glyphosate treatment suggests that a plant glyphosate oxidoreductase (GOX) was responsible for glyphosate conversion to AMPA. GR canola (oilseed rape) has a glyphosate-insensitive EPSPS as well as a bacterial GOX (*14*). The GOX enzyme in GR canola prevents accumulation of glyphosate in canola oil seed cake. Apparently, AMPA does not cause a problem in canola; however, nothing is known about the susceptibility of canola to AMPA, or the efficacy of bacterial GOX in canola in converting glyphosate to AMPA in vivo. Therefore, the objectives of this research were to determine (1) the dose response of GR and non-GR soybean and canola to glyphosate, (2) if differential metabolism of glyphosate to AMPA is the underlying mechanism for differential resistance to glyphosate among three GR soybean varieties, and (3) the extent of metabolism of glyphosate to AMPA in GR canola and to correlate metabolism to injury from AMPA.

2. MATERIALS AND METHODS

2.1. General Experimental Conditions. Greenhouse experiments were conducted from March 2005 to July 2006 at the Southern Weed Science Research Unit, U.S. Department of Agriculture, Stoneville, MS. Four soybean varieties [Asgrow 4603RR, Delta King 4868RR, HBK 4820RR, all GR varieties; HBKC 5025, non-GR variety] and two canola varieties (Hyola 514RR, GR variety; Hyola 440, non-GR variety) were used in the study. Soybean and canola 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 and canola to one plant per pot. The greenhouse was maintained at 25/20 °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 and fertilized as needed. Soybean plants at one- to two-trifoliate leaf (22 days old, 45 cm tall) growth stage and canola plants at four- to five-leaf (29 days old, 14 cm tall) 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^{-1} at 140 kPa using 8002E flat-fan nozzles.

2.2. Glyphosate-Potassium (K) Dose−Response in GR and Non-GR Soybean. Glyphosate-K was applied at 0.87, 1.73, 3.47, 6.93, 13.86, 27.72, 55.44, and 110.88 kg ae ha^{-1} to Asgrow 4603RR GR soybean and at 0.007, 0.015, 0.03, 0.06, 0.11, 0.22, 0.44, and 0.87 kg ha^{-1} to HBKC 5025 non-GR soybean. Spray solutions were prepared using a commercial potassium salt formulation of glyphosate. We recognize that injury to GR soybean could be in good part from inert ingredients of the formulation at the higher rates. At 21 days after treatment (DAT), plants were excised at the soil surface and oven-dried at 60 °C for 48 h, and dry weights were recorded. There were nine replications per treatment.

2.3. Chlorophyll and Shoot Fresh Weight in Glyphosate-Isopropylammonium (IPA)-Treated GR Soybean. Three GR soybean varieties (Asgrow 4603RR, Delta King 4868RR, and HBK 4820RR) were treated with glyphosate at 0.87 kg ha^{-1}. Spray solution was prepared using technical grade glyphosate-isopropylammonium (>95% purity, Chem Service, West Chester, PA) with Tween 20 (0.5%, v/v). Technical grade glyphosate-IPA 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 DAT, distal leaflets of the second trifoliolate leaf from two plants/pot in each treatment were sampled for chlorophyll determination. Chlorophyll was extracted with 10 mL of dimethyl sulfoxide, and chlorophyll concentrations were determined spectrophotometrically (*15*). At 14 DAT, soybean plants (two plants per pot) were excised at the soil surface, and fresh weights were recorded. Chlorophyll content and shoot fresh weight were expressed as percent of nontreated control. There were eight replications per treatment.

2.4. Glyphosate, Shikimate, and AMPA Accumulation in Glyphosate-IPA-Treated GR Soybean. Three GR soybean varieties were treated with glyphosate-IPA at 0.87 kg ha^{-1} as described above. At 14 DAT, soybean plants were harvested by clipping at the base, washed with running water, rinsed with distilled water to remove glyphosate-IPA remaining on the leaf surface, and blotted dry with paper towels. All of the leaves (without petioles) were air-dried, ground, and analyzed for glyphosate, shikimate, and AMPA. There were eight replications per treatment.

2.5. Glyphosate-K Dose−Response in GR and Non-GR Canola. Glyphosate-K was applied at 0.63, 1.26, 2.52, 5.04, 10.08, 20.16, 40.32, and 80.64 kg ha^{-1} to GR canola and at 0.005, 0.01, 0.02, 0.039, 0.079, 0.158, 0.315, and 0.63 kg ha^{-1} to non-GR canola. Spray solutions were prepared using commercial glyphosate formulation as described in section 2.2. At 21 DAT, plants were excised at the soil surface and oven-dried at 60 °C for 48 h, and dry weights were recorded. There were nine replications per treatment.

2.6. Chlorophyll and Shoot Fresh Weight in Glyphosate-IPA-Treated GR Canola. GR canola plants were treated with technical grade glyphosate-IPA at 0.63 kg ha^{-1}. Tween 20 (0.5%, v/v) was added to the spray solution. At 7 DAT, distal 2 cm portion of the fourth leaf was sampled for chlorophyll determination as described in section 2.3. At 14 DAT, canola plants were excised at the soil surface, and fresh weights were recorded. Chlorophyll content and shoot fresh weight were expressed as percent of nontreated control. There were 10 replications per treatment.

2.7. Glyphosate, Shikimate, and AMPA Accumulation in Glyphosate-IPA-Treated GR Canola. GR canola plants were treated with technical grade glyphosate-IPA at 0.63 kg ha^{-1}. At 14 DAT, canola plants were harvested by clipping at the base, washed with running water, rinsed with distilled water to remove glyphosate-IPA remaining on the leaf surface, and blotted dry with paper towels. All of the leaves (without petioles) were air-dried, ground, and analyzed for glyphosate, shikimate, and AMPA. There were eight replications per treatment with five plants per replication.

2.8. AMPA Dose−Response and AMPA Concentrations in GR Canola and Non-GR Canola. AMPA at 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 kg ha^{-1} was applied to GR and non-GR canola. 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 at 4 DAT as described in section 2.3 with amendments as described in section 2.6. At 14 DAT, canola plants were excised at the soil surface, and fresh weights were recorded.

After weights were recorded, 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 AMPA. There were five replications per treatment.

2.9. Extraction of Soybean and Canola Leaves and Derivatization of Extracts. Soybean leaf samples were analyzed for glyphosate, shikimic acid, and AMPA, and canola leaf samples were analyzed for glyphosate and AMPA. For glyphosate and AMPA analysis, extraction and derivatization were performed according to a published procedure (*16*), 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 mL 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 mL 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 up to the top surface 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-mL aliquot was taken and added to 640 mL 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 mL of ethyl acetate containing 0.2% citral and analyzed by GC−MS.

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 per 23.3 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 Puradisc 25 AS disposable filter device (Whatman, Inc., Clifton, NJ), and the filtrate was lyophilized. One milligram of the lyophilized extract was treated with 100 mL of bis(trimethylsilyl)trifluoroacetamide/dimethylformamide (1:1 mixture) and heated at 70 °C for 30 min. This was used for GC−MS analysis.

2.10. GC−MS Analysis of Glyphosate, AMPA, and Shikimic Acid in Soybean and Canola 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 m 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 230 °C, respectively. The carrier gas was ultrahigh-purity helium at a 1 mL min^{-1} 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^{-1} rate, raised to 270 °C at 70 °C/min rate, raised to 310 °C at 35 °C min^{-1} 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^{-1} rate, raised to 270 °C at 40 °C min^{-1} rate, raised to 310 °C at 45 °C min^{-1} 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.

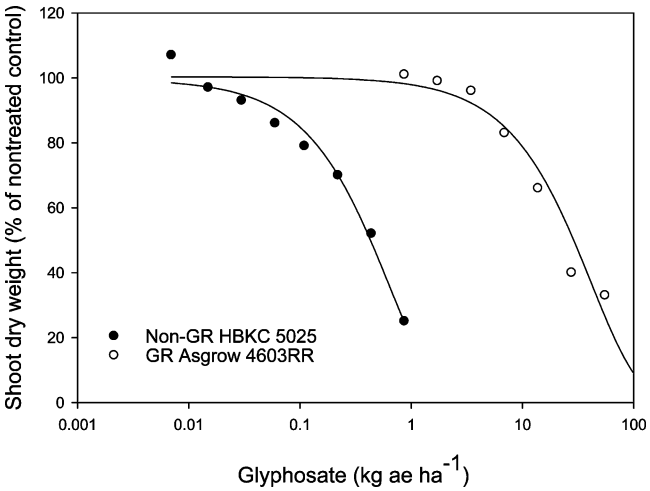

Figure 1. Response of glyphosate-resistant (GR) Asgrow 4603RR and non-GR HBKC 5025 soybean in the one- to two-trifoliolate leaf (22 days old, 45 cm tall) growth stage to glyphosate-potassium 3 wk after treatment. Mean values of nine replications are plotted.

2.11. Statistical Analyses. Treatments were arranged in a completely randomized design. Data were subjected to analysis of variance, and means were separated using Fisher's protected least significant difference (LSD) test at $P = 0.05$ (*17*). GR_{50} (glyphosate dose required to cause a 50% reduction in plant dry wt accumulation) values for GR and non-GR soybean and canola were calculated fitting nonlinear regression equations [soybean, $y = ae^{-bx}$, where a is an asymptote, x is GR_{50}, and b is slope, Sigma Plot 9.0, Systat Software Inc., Point Richmond, CA; and canola, $f = c + (d - c/(1 + \exp(b(\log(x) - \log(e)))))$, where c and d are lower and upper response limits, e is GR_{50}, and b is slope, *R* (*18*)] to raw data.

3. RESULTS AND DISCUSSION

GR_{50} values for the GR Asgrow 4603RR and non-GR HBKC 5025 soybean varieties were 22.8 and 0.47 kg ae ha^{-1}, respectively, indicating that GR Asgrow 4603RR variety is 49-fold more resistant to glyphosate as compared to the non-GR HBKC 5025 variety (**Figure 1**, **Table 1**). This is the first report of the GR_{50} ratio of GR soybean and non-GR soybean. Asgrow 4603RR was highly resistant to glyphosate under conditions of this study. Chlorophyll content at 7 DAT by a single application of glyphosate at 0.87 kg ha^{-1} in GR Delta King 4868RR (69% of control) and GR HBK 4820RR (68% of control) was significantly lower when compared to GR Asgrow 4603RR (85% of control), but there were no differences among the three GR varieties in shoot fresh weight reduction (95−97% of control) at 14 DAT (**Table 2**). This indicates that the GR soybean varieties were able to overcome glyphosate-induced chlorotic injury, and growth was not adversely affected. Reduction of chlorophyll (% of Tween-20-treated control) by glyphosate at 0.87 kg ha^{-1} ranged from 15% to 32%, and shoot fresh weight reduction ranged from 3% to 5% in the GR soybean varieties. In a previous study, another GR soybean variety Asgrow 4702RR treated with glyphosate-IPA at 1.12−13.44 kg ha^{-1} rates exhibited 12% reduction in chlorophyll content at 7 DAT and 8% reduction in shoot dry weight at 14 DAT (*9*). Other instances of GR soybean injury (speckling, necrosis,

Table 1. GR_{50} Values and Glyphosate Dose Response Model Parameter Estimates for Glyphosate-Resistant (GR) Asgrow 4603 and Non-GR HBKC 5025 Soybean and GR Hyola 514RR and Non-GR Hyola 440 Canola[a]

		model parameters					
crop	variety	GR_{50}, kg ae ha^{-1}	GR_{50} ratio	*a*	*b*	*c*	*d*
soybean	GR Asgrow 4603RR	22.8	49	99.6	1.6		
soybean	non-GR HBKC 5025	0.47		100.3	0.02		
canola	GR Hyola 514RR	14.1	47		1.23	30.3	107.6
canola	non-GR Hyola 440	0.3			0.85	31.2	111.2

[a] Model parameters are described in section 2.11.

Table 2. Effect of Glyphosate-isopropylammonium (Glyphosate-IPA) Treatment at 0.87 kg ae ha^{-1} on Chlorophyll Content 7 days after Treatment (DAT), Shoot Fresh Weight 14 DAT, and Glyphosate, Aminomethylphosphonic Acid (AMPA), and Shikimate Concentration (in Leaves) 14 DAT in Glyphosate-IPA-Treated Glyphosate-Resistant (GR) Soybean[a]

variety	chlorophyll, % of control	shoot fresh wt, % of control	glyphosate, μg g of tissue^{-1}	AMPA, μg g of tissue^{-1}	shikimate, ng g of tissue^{-1}
GR Asgrow 4603	85 ± 8	96 ± 5	87 ± 8	6 ± 2	120 ± 30
GR Delta King 4868	69 ± 6	97 ± 9	63 ± 18	4 ± 1	113 ± 20
GR HBK 4820	68 ± 8	95 ± 16	59 ± 13	4 ± 2	151 ± 35
LSD (0.05)	9	ns	ns	ns	ns

[a] Values represent mean of eight replications and corresponding standard deviations.

and chlorosis) following glyphosate treatment have been documented (*10*, *11*). Reddy et al. (*9*) determined that injury to GR soybean from glyphosate was due to AMPA formed from glyphosate degradation in plants. Following this premise, we hypothesized that anecdotal reports and our own confirmation of differential tolerance of GR soybean varieties to glyphosate, based on chlorotic injury, could be due to differential metabolism of glyphosate to AMPA among GR varieties. To verify this hypothesis, we measured glyphosate, AMPA, and shikimate levels in leaves of GR soybean following glyphosate treatment.

No significant differences in the levels of glyphosate (59−87 μg g of tissue^{-1}), AMPA (4−6 μg g of tissue^{-1}), and shikimate (113−151 μg g of tissue^{-1}) were found among the three GR varieties (**Table 2**), which indicates that differential chlorotic injury in GR soybean varieties from glyphosate application is not related to metabolism of glyphosate to AMPA. However, it is conceivable that variable rates of detoxification of AMPA to other metabolites among the GR varieties may have a role or that AMPA levels at earlier time points might compare better with the physiological effect. The levels of AMPA in glyphosate-treated leaves at 14 DAT in this study, 4−6 μg g of tissue^{-1}, are comparable to those reported earlier, 3 μg g of tissue^{-1} (*9*). Detection of AMPA following glyphosate treatment suggests that a plant glyphosate oxidoreductase (GOX) was responsible for this conversion (*9*).

Further studies were conducted with GR canola, which unlike GR soybean has two transgenes, an *epsps* gene as in GR soybean and a *gox* gene encoding a modified form of GOX enzyme. GR_{50} values for the GR Hyola 514RR and non-GR Hyola 440 canola varieties were 14.1 and 0.3 kg ae ha^{-1}, respectively, indicating that the GR Hyola 514RR variety is 47-fold more resistant to glyphosate as compared to the non-GR Hyola 440 variety (**Figure 2**, **Table 1**). This is the first report of the GR_{50} ratio of GR canola and non-GR canola. A single application of glyphosate at 0.63 kg ha^{-1} did not reduce chlorophyll content at 7 DAT and shoot fresh weight reduction at 14 DAT in GR Hyola 514RR, indicating that glyphosate did not cause injury to GR canola unlike in GR soybean.

GR canola had less glyphosate per unit leaf weight (1 μg g of tissue^{-1}) as compared to GR soybean (59−87 μg g of tissue^{-1}). This suggests that the rapid breakdown of glyphosate

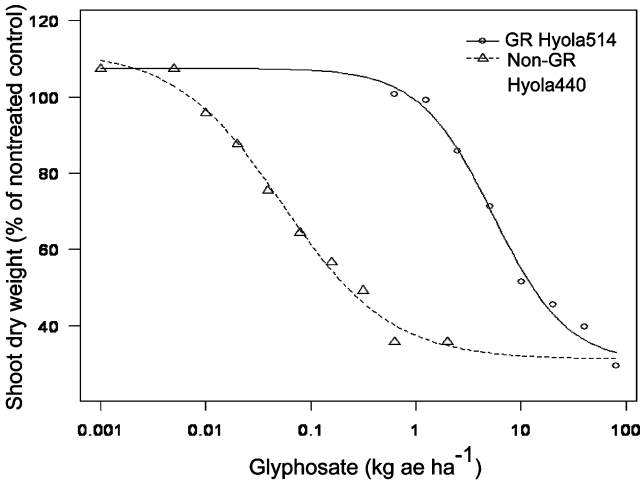

Figure 2. Response of glyphosate-resistant (GR) Hyola 514RR and non-GR Hyola 440 canola in the four- to five-leaf (29 days old, 14 cm tall) growth stage to glyphosate-potassium 3 wk after treatment. Mean values of nine replications are plotted.

in GR canola was due in part to the activity of transgenic, bacterial GOX. If that is the case, then GR canola must have accumulated higher levels of AMPA per unit leaf weight as compared to GR soybean with a greater potential to cause chlorotic injury in GR canola than was seen with GR soybean. The level of AMPA in GR canola was 7 μg g of tissue^{-1}, which was comparable (not statistically analyzed) to AMPA levels of 4−6 μg g of tissue^{-1} in GR soybean. It is conceivable that differences in absorption and translocation of glyphosate between GR soybean and GR canola had a major role in obtaining comparable levels of AMPA despite soybean and canola not treated with equal levels of glyphosate. Also, we have not measured initial levels of glyphosate on the treated plants. There was no chlorotic injury from glyphosate or AMPA from glyphosate in GR canola. AMPA from glyphosate breakdown could have been further metabolized to nonphytotoxic secondary metabolites before causing injury to GR canola.

Chlorophyll levels and shoot fresh weights were reduced by application of AMPA to GR Hyola 514RR and non-GR Hyola

Table 3. Effect of Aminomethylphosphonic Acid (AMPA) Treatment on Chlorophyll Content 4 days after Treatment (DAT), Shoot Fresh Weight 14 DAT, and AMPA Concentration (in Leaves) 14 DAT in Glyphosate-Resistant (GR) Hyola 514RR and Non-GR Hyola 440 Canola[a]

	chlorophyll, % control		shoot fresh weight, % control		AMPA, μg g of tissue^{-1}	
AMPA, kg ha^{-1}	GR Hyola 514RR	non-GR Hyola 440	GR Hyola 514RR	non-GR Hyola 440	GR Hyola 514RR	non-GR Hyola 440
Tween 20	95 ± 3	94 ± 4	97 ± 8	95 ± 2	0	0
0.25	85 ± 4	87 ± 2	96 ± 6	92 ± 3	9 ± 2	10 ± 3
0.5	70 ± 5	74 ± 7	92 ± 6	89 ± 8	11 ± 4	9 ± 3
1	64 ± 4	69 ± 2	93 ± 4	87 ± 7	21 ± 5	18 ± 2
2	51 ± 6	55 ± 9	90 ± 7	84 ± 4	34 ± 8	31 ± 4
4	40 ± 7	46 ± 3	80 ± 4	78 ± 1	183 ± 15	126 ± 11
8	29 ± 6	30 ± 5	74 ± 2	67 ± 4	193 ± 12	145 ± 9
LSD (0.05)	11		9		31	

[a] Values represent mean of five replications and corresponding standard deviations.

440 canola (**Table 3**). The effects corresponded with AMPA dose. A single application of AMPA at 0.25−8.00 kg ha^{-1} reduced chlorophyll content by 13−71% at 4 DAT and reduced shoot fresh weight by 8−33% at 14 DAT as compared to nontreated control in both GR and non-GR canola. Others have reported similar results in GR soybean (*9*). Evidently, AMPA as low as 0.25 kg ha^{-1} causes injury in both GR and non-GR canola, greater than glyphosate-IPA at 0.63 kg ha^{-1} at which concentration there was no effect on GR canola. There was similar sensitivity to AMPA in GR and non-GR canola, but we do not know if this would be true for all canola varieties. AMPA levels in the leaves increased with increased rate of AMPA application in both GR and non-GR canola (**Table 3**). AMPA levels were significantly higher in GR canola as compared to non-GR canola at 4 and 8 kg ha^{-1} application rates. AMPA levels of 9−11 μg g of tissue^{-1} apparently caused reduction in chlorophyll content in both GR and non-GR canola, but AMPA formed from glyphosate breakdown (7 μg g of tissue^{-1}) did not injure canola. These results suggest that AMPA originating from metabolism of glyphosate causes injury to GR soybean, but not to GR canola. Differential compartmentalization could account for this. Furthermore, AMPA formed from degradation of glyphosate in GR canola seems to be further metabolized to a nonphytotoxic compound(s).

Shikimate levels in glyphosate-treated GR canola were similar to those in nontreated GR canola leaves (data not shown). By blocking EPSPS, glyphosate causes pronounced increases in shikimate levels in glyphosate-treated non-GR canola plants (*19*). In other research, Duke et al. (*12*) also observed that shikimate levels in GR soybean seed were unaffected by commonly used glyphosate treatments in soybean production. Thus, the absence of an effect on shikimate observed in GR canola 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.

In summary, these results have demonstrated that differences exist between GR soybean varieties in extent of chlorosis caused by glyphosate. However, GR soybean plants were able to outgrow the initial injury, resulting in no significant reduction (95−97% of control) in fresh weight. Although earlier work indicated that injury to GR soybean from glyphosate is due to AMPA formed from glyphosate metabolism (*9*), in the present "work" we found no differential metabolism of glyphosate to AMPA among GR soybean varieties to explain differential chlorotic injury. There tended to be less glyphosate (per unit leaf weight) in GR canola as compared to GR soybean. The presence of a bacterial GOX gene in GR canola has apparently resulted in substantial breakdown of glyphosate to AMPA. However, AMPA from glyphosate metabolism did not accumulate to the extent expected, considering the low glyphosate levels and the presence of the GOX transgene, indicating that AMPA was metabolized to nonphytotoxic secondary metabolites before causing injury to GR canola. Taken together, these data suggest that AMPA from glyphosate degradation is apparently subjected to different secondary reactions in GR soybean and GR canola. This needs additional research. The effect of insertion of the bacterial GOX gene in GR soybean, as suggested before (*9*), warrants further investigation.

ACKNOWLEDGMENT

We thank Albert Tidwell, Efren Ford, Gloria Hervey, Robert Johnson, and Dr. Frank Dayan for their excellent technical assistance.

LITERATURE CITED

(1) Franz, J. E.; Mao, M. K.; Sikorski, J. A. *Glyphosate: A Unique Global Herbicide*; ACS Monograph 189; American Chemical Society: Washington, DC, 1997; 653 pp.

(2) Duke, S. O.; Baerson, S. R.; Rimando, A. M. Herbicides: Glyphosate. In *Encyclopedia of Agrochemicals*; Plimmer, J. R., Gammon, D. W., Ragsdale, N. N., Eds.; Wiley: New York, 2003; http://www.mrw.interscience.wiley.com/eoa/articles/agr119/frame.html.

(3) Duke, S. O. Glyphosate. In *Herbicides: Chemistry, Degradation, and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1988; Vol. 3, pp 1−70.

(4) Padgette, S. R.; Re, D. B.; Barry, G. F.; Eichholtz, D. E.; Delannay, X.; Fuchs, R. L.; Kishore, G. M.; Fraley, R. T. New weed control opportunities: Development of soybeans with a Roundup Ready gene. In *Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects*; Duke, S. O., Ed.; CRC Press: Boca Raton, FL, 1996; pp 53−84.

(5) Duke, S. O.; Scheffler, B. E.; Dayan, F. E.; Dyer, W. E. Genetic engineering crops for improved weed management traits. *Am. Chem. Soc. Symp. Ser.* **2002**, *No. 829*, 52−66.

(6) Anonymous. U.S. GM crops edge higher to set new record. *Agrow World Crop Prot. News* **2002**, *No. 404 (July 12)*, 15.

(7) Thayer, A. M. Ag biotech. *Chem. Eng. News* **2000**, *78*, no. 40 (October 2), 21−29.

(8) Reddy, K. N.; Koger, C. H. Herbicide-resistant crops and weed management. In *Handbook on Sustainable Weed Management*; Singh, H. P., Batish, D. R., Kohli, R. K., Eds.; Haworth Press: Binghamton, NY, 2006; pp 549−580.

(9) Reddy, K. N.; Rimando, A. M.; Duke, S. O. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. *J. Agric. Food Chem.* **2004**, *52*, 5139−5143.

(10) Krausz, R. F.; Young, B. G. Response of glyphosate-resistant soybean (*Glycine max*) to trimethylsulfonium and isopropylamine salts of glyphosate. *Weed Technol.* **2001**, *15*, 745−749.
(11) Reddy, K. N.; Zablotowicz, R. M. Glyphosate-resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. *Weed Sci.* **2003**, *51*, 496−502.
(12) Duke, S. O.; Rimando, A. M.; Pace, P. F.; Reddy, K. N.; Smeda, R. J. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. *J. Agric. Food Chem.* **2003**, *51*, 340−344.
(13) Arregui, M. C.; Lenardón, A.; Sanchez, D.; Maitre, M. I.; Scotta, R.; Enrique, S. Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. *Pest Manage. Sci.* **2003**, *60*, 163−166.
(14) Felsot, A. *Herbicide Tolerant Genes, Part 1: Squaring up Roundup Ready Crops*; Web page www.biotech-info.net/felsot1.html; accessed May 25, 2005.
(15) Hiscox, J. D.; Israelstam, G. F. A method for the extraction of chlorophyll from leaf tissues without maceration. *Can. J. Bot.* **1979**, *57*, 1332−1334.
(16) Alferness, P. L.; Wiebe, L. A. Determination of glyphosate and aminomethylphosphonic acid in crops by capillary gas chromatography with mass-selective detection: Collaborative study. *J. AOAC Int.* **2001**, *84*, 823−846.
(17) SAS. *Proprietary software release 9.2*; SAS Institute, Inc.; Cary, NC, 2003.
(18) Ritz, C.; Streibig, J. C. Bioassay Analysis using R. *J. Statist. Software* **2005**, 12; http://www.bioassay.dk.
(19) Harring, T.; Streibig, J. C.; Husted, S. Accumulation of shikimic acid: A technique for screening glyphosate efficiency. *J. Agric. Food Chem.* **1998**, *46*, 4406−4412.

Received for review December 10, 2006. Revised manuscript received March 6, 2007. Accepted March 8, 2007.

JF063568L