Research Article

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Lack of transgene and glyphosate effects on yield, and mineral and amino acid content of glyphosate-resistant soybean

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Abstract

BACKGROUND: There has been controversy as to whether the glyphosate resistance gene and/or glyphosate applied to glyphosate-resistant (GR) soybean affect the content of cationic minerals (especially Mg, Mn and Fe), yield and amino acid content of GR soybean. A two-year field study (2013 and 2014) examined these questions at sites in Mississippi, USA.

RESULTS: There were no effects of glyphosate, the GR transgene or field crop history (for a field with both no history of glyphosate use versus one with a long history of glyphosate use) on grain yield. Furthermore, these factors had no consistent effects on measured mineral (Al, As, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe, Ga, K, Li, Mg, Mn, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn) content of leaves or harvested seed. Effects on minerals were small and inconsistent between years, treatments and mineral, and appeared to be random false positives. No notable effects on free or protein amino acids of the seed were measured, although glyphosate and its degradation product, aminomethylphosphonic acid (AMPA), were found in the seed in concentrations consistent with previous studies.

CONCLUSIONS: Neither glyphosate nor the GR transgene affect the content of the minerals measured in leaves and seed, harvested seed amino acid composition, or yield of GR soybean. Furthermore, soils with a legacy of GR crops have no effects on these parameters in soybean.

Supporting information may be found in the online version of this article.

Keywords: amino acid; glyphosate; glyphosate-resistant soybean; metal; mineral; soybean yield; transgene

1 INTRODUCTION

Transgenic, glyphosate-resistant (GR) crops have helped to make glyphosate [*N*-(phosphonomethyl) glycine] the most heavily used herbicide worldwide.[1] The *CP4* gene from *Agrobacterium tumefaciens* strain CP4 that encodes a GR version of the glyphosate target site, 5 – enolpyruvylshikimate-3-phosphate synthase (EPSPS), has been used as the GR transgene for most GR crops.[1] In the USA, following the introduction of GR canola and soybean in 1996, cotton in 1997, maize in 1998, alfalfa in 2005 and sugar beet in 2008, adoption of the these crops was very rapid, reaching >90% in most of these crops. Similar adoption rates have occurred in other countries such as Argentina and Brazil after these crops were approved for production. Although yields of cotton, maize and soybean in the USA have continued upward since these GR crops were introduced,[2,3] some have claimed that glyphosate causes deficiencies in cationic minerals in GR crops, due to either the chelation of glyphosate with divalent metal cations and/or toxic effects on rhizosphere microbes involved in the assimilation of these nutrients from soil.[4−15] Others have found no effects of glyphosate on mineral content in GR crops.[16−27] In a review of most of this literature,[2] the authors concluded that the strongest available evidence in the literature supported the view that glyphosate does not significantly affect mineral nutrition in GR crops. However, the authors stated that there might

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be effects with particular soil types, crop cultivars, glyphosate formulations and/or environmental conditions. Additionally, despite publications finding no substantial difference in the harvested seed composition of GR crops,[28,29] some have claimed that GR crops do not have the same amino acid content of conventional crops.[30]

The studies reported here are intended to address whether the GR transgene, glyphosate use on GR soybean and glyphosate use history on a field affect: (1) plant tissue content of minerals that can chelate glyphosate as cations, (2) seed amino acid composition (free and protein) and (3) yield of soybean. Furthermore, we determined the glyphosate and aminomethylphosphonic acid (AMPA; the metabolic degradation product of glyphosate in soybean)[31] content of leaves and seeds of glyphosate-treated plants. Our results provide further support for the hypothesis that the GR transgene, glyphosate use on GR plants and glyphosate use over multiple years have no significant effect on the concentration of a range of mineral elements, amino acid content or yield of soybean.

2 MATERIALS AND METHODS

2.1 Field experiments

Field experiments were conducted on Dundee silt loam soil (fine-silty, mixed, active, thermic Typic Endoqualf) in 2013 and 2014 at the Crop Production Systems Research farm, near Stoneville, Mississippi, USA. The experiment was conducted at two adjacent sites, one with a legacy of glyphosate use and another where glyphosate had not been used. The field with a history of glyphosate use had either GR soybean or GR cotton grown on it for the last 15 years. The no-glyphosate history field had cogongrass [*Imperata cylindrica* (L.) Beauv.] (maintained for weed biology studies) with no herbicides applied for the past 12 years. In 2012, cogongrass was killed with repeated tillage, and non-GR soybean and non-GR corn were then planted in alternate rows to prepare the land for this study. Corn and soybean were grown until maturity and flail mowed. Fields were prepared by disking and bedding in the fall of 2012 and 2013. Each year, at planting, soil samples were collected from the surface 0–15 cm, using a 7.5-cm diameter core sampler, from all plots. Soil samples consisted of a composite of four subsamples collected randomly from the center two rows of the plot. Soil samples were analyzed by the Agricultural Analytical Services Laboratory, Pennsylvania State University. Ca, Mg, K and P are Mehlich 3 extractable, and all other metals are total sorbed using the EPA 3050 method. The treatments were a non-GR cultivar with no glyphosate, a GR cultivar with no glyphosate and a GR cultivar with glyphosate applied at 0.87 kg ae ha^{-1} twice at 5 and 7 weeks after planting. The experimental design was a randomized complete block with four replications. The plots were four rows (spaced at 102 cm) wide and 15.2 m long. Soybean cultivars that are near isolines, USG Allen (GR) and USG 5601 T (non-GR)[32,33] were planted using a planter at a seeding rate of 350 000 seeds ha^{-1} on 30 April 2013 and 24 April 2014, and grown using standard production practices under irrigation. After planting, *S*-metolachlor at 1.12 kg ae ha^{-1} plus pendimethalin at 1.12 kg ai ha^{-1}, and paraquat at 1.12 kg ai ha^{-1} were applied using a tractor-mounted sprayer to the entire experimental area to ensure early-season weed control. For glyphosate treatment, a potassium salt formulation of glyphosate (Roundup WeatherMax, Monsanto Agricultural Co., St. Louis, MO, USA) was used. All plots were hand-hoed periodically throughout the season to keep them weed-free.

At the R2 (flower at node immediately below the uppermost node with a completely unrolled leaf) soybean growth stage (∼ 3 weeks after glyphosate application or 10 weeks after planting), uppermost fully expanded leaflets without petiole were sampled. At harvest, 20 soybean plants were sampled at random from the center two rows and seed collected. Soybean was harvested using a combine and grain yield was adjusted to 13% moisture. Leaf and seed samples were stored at 4 °C and room temperature, respectively.

2.2 Sample preparation for mineral analyses

Prior to digestion, leaves were dried for 24 h at 60 °C to obtain constant weight. Soybean seeds were digested without drying. However, the moisture content of the seeds was negligible ($0.16 \pm 0.03\%$), as determined by drying for 8 h at 75 °C in an oven. Between 0.15 and 0.25 g of each sample was digested with 5 ml of HNO_3, 1 ml of H_2O_2 and 100 μl of HF in acid-washed Teflon PFA vessels using a Milestone Ethos microwave digestion system. Reagents were either trace metal grade (HNO_3) or optima grade (H_2O_2 and HF) from Fisher Scientific. Microwave operation was at 1200 W with a 30-min ramp to 120 °C, followed by a 60-min ramp to 180 °C, ending with 20 min at 180 °C. The digests were then diluted to 50 ml with 18.2 MΩ deionized water. Before analysis, 3 ml of each sample and standard were diluted with 7 ml of an internal standard solution containing 2 ppb Rh and 10 ppb Y in 2% HNO_3.

2.3 ICP-MS analysis

Concentrations of 24 elements (Al, As, Ba, Be, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, K, Li, Mg, Mn, Ni, Pb, Se, Sr, Tl, U, V, Zn) were determined by sector field inductively coupled plasma mass spectrometry (SF-ICPMS) using a Thermo Fisher Element-XR. Data acquisition and instrumental parameters are given in Table S2. The instrument utilizes reverse Nier-Johnson geometry and features resolving power ($m\ \Delta m^{-1}$) settings of low (∼ 300), medium (∼ 3000) and high (10 000) resolving power. The sample introduction system comprised a glass concentric nebulizer with a glass cyclonic spray chamber. The instrument was tuned prior to analysis for sensitivity, stability and oxide levels, yielding ∼1 million counts per second for 1 ng g^{-1} of In; < 3% relative standard deviation (RSD; short-term); and <8% uranium oxide.

For quantitation, a six-point calibration curve ranging from ∼0.1 to 20 ppb was used. Standards were prepared in 2% HNO_3 using a multi-element standard solution (Spex Certiprep). Linearity (r^2 value) for the calibration plots for all isotopes was >0.99. Rh was used as an internal standard for elements being run in low resolution and Y for elements in medium and high resolution. Data are reported on a dry-weight basis for leaves and fresh-weight basis for seeds. Recoveries for the reference material (NIST SRM 1547, Peach Leaves) generally ranged from 80% to 120%. The RSD between samples run in triplicate was generally <10%.

2.4 Seed analysis for free and hydrolyzed amino acids

Harvested soybean seed samples were analyzed for both free and hydrolyzed amino acids as described by Hacham *et al.*[34] Free amino acids were determined on a 150 mg seed sample. Hydrolyzed amino acids were determined on a 3 mg dry ground seed sample. Seed samples were analyzed by Donald Danforth Plant Science Center, Proteomics and Mass Spectrometry Facility (St. Louis, MO, USA).

2.5 Analysis of glyphosate and aminomethylphosphonic acid

2.5.1 *Sample preparation*

Dried soybean seeds were ground on a Foss Cyclotec 1093 (Höganäs, Sweden) sample mill, and extracted following published procedures,[35] with minor modifications. One gram of ground seeds was extracted with 15 ml of H_2O, sonicated for 20 min, then centrifuged at 2000 ***g*** for 10 min. Four milliliters of supernatant was transferred to a 20 ml vial. Extraction was repeated by adding 5 ml of H_2O to the sample; the vial was shaken and sonicated for 20 min, then centrifuged at 2000 ***g*** for 10 min. Two milliliters of supernatant was taken and combined with the 4 ml obtained from the first extraction. Concentrated HCl (30 μl) was added to this combined supernatant and shaken. A 4-ml portion was pipetted into a 20-ml vial, and 2 ml of CH_2Cl_2 was added, shaken, and centrifuged at 2000 ***g*** for 10 min. A portion (1.8 ml) of the H_2O layer was mixed with 200 μl of acidic modifier (16 g KH_2PO_4, 160 ml H_2O, 40 ml MeOH, and 13.4 ml HCl). One milliliter of this was transferred to a cation-exchange resin column (2-ml packed volume; AG 50 W-X8, H+; Bio-Rad Laboratories, Hercules, CA, USA). Conditions for column elution and sample derivatization were the same as in Duke *et al.*[35]

2.5.2 *Analysis of glyphosate and aminomethylphosphonic acid by gas chromatography–mass spectrometry*

Gas chromatography–mass spectrometry (GC–MS) analysis was performed according to Duke *et al.*,[35] with modifications. The GC temperature program was as follows: initial, 80 °C; held for 2.5 min; raised to 120 °C at 30 °C min^{-1}; raised to 200 °C at 17 °C min^{-1}; raised to 300 °C at 45 °C min^{-1}; and finally held at this temperature for 1 min. The injection port, GC interface and ionization chamber were maintained at 250, 250 and 150 °C, respectively. The MS spectra were acquired in the positive, low-resolution, selected ion monitoring mode. AMPA was observed at 6.57 min (*m/z* 571, 446, 372), and glyphosate was observed at 7.59 min (*m/z* 611, 584, 338).

2.6 Statistical analyses

The data were subjected to analysis of variance using SAS PROC GLM (SAS Institute, Cary, NC, USA). Data from glyphosate legacy and no legacy sites were analyzed separately. Treatment means were separated at the 5% level of significance using Fisher's protected least significant difference test. Data were averaged across years as treatment by year interactions were not significant.

3 RESULTS

3.1 Soil analyses

Results of soil mineral analyses are provided in Table S1. There were no significant differences in content of any of the elements between any of the treatment plots.

3.2 Soybean yield

There was no effect of the GR transgene (non-GR vs GR near isogenic cultivar) and glyphosate (GR no glyphosate vs GR with glyphosate) on soybean, yield regardless of glyphosate legacy site in both years (Table 1).

Table 1. Glyphosate and transgene effects on yield in glyphosate-resistant soybean near isogenic cultivars grown on glyphosate legacy and non-legacy soils at Stoneville, MS, 2013 and 2014

	Glyphosate legacy site		No glyphosate legacy site	
	2013	2014	2013	2014
Cultivar/glyphosate	Soybean yield, kg/ha			
Non-GR, no glyphosate	4572 a	4377 a	4659 a	4428 a
GR, no glyphosate	4543 a	4424 a	4580 a	4303 a
GR with glyphosate	4406 a	4473 a	4594 a	4481 a

Non-GR, non-glyphosate resistant; GR, glyphosate resistant.
Means within a column followed by same letter are not significantly different at the 5% level as determined by Fisher's least significant difference test.

3.3 Mineral composition

For leaf tissue sampled 3–4 weeks after glyphosate application, all minerals were measured in both 2013 and 2014 (Table 2), except for As which was measured only in 2013, and Li, Tl and Ca measured only in 2014 (Table 3). There were no effects of glyphosate, the GR transgene or glyphosate legacy soil on any mineral content, except for a significantly lower content in Sr in the non-legacy soil in the glyphosate-treated GR plants and a significantly lower content of Mg in the non-legacy soil in non-GR plants that had not been treated with glyphosate. This may be due to slightly lower levels of Sr and Mg in non-glyphosate legacy soils (Table S1) and/or the likelihood of a few false positives (see discussion).

For soybean seed, all minerals were measured in both 2013 and 2014 (Table 4), except for Pb and Cr which were measured only in 2013, and Li, Be, Rb, Ag, Cs, Tl and Ga measured only in 2014 (Table 5). Cd and Mg (Table 4) were slightly lower in GR seed whether from glyphosate-treated plants or not from the glyphosate legacy plots, whereas Ba and Sr were slightly lower in GR seed whether treated with glyphosate or not in the non-glyphosate legacy plots (Table 4). Ag and Li were slightly lower in seed of GR plants not treated with glyphosate in glyphosate legacy plots (Table 5). Be and Tl were lower in GR seed from plants not treated with glyphosate in glyphosate legacy plots and GR seed from glyphosate-treated plants in the non-glyphosate legacy plots (Table 5). Ga was lower in GR seed from non-glyphosate legacy plots whether treated with glyphosate or not (Table 5). In general, there were no dramatic effects on any mineral content measured, nor was there a consistent effect of the GR gene, the type of glyphosate legacy or whether the plant was treated with glyphosate on the elements measured. We did not measure the essential elements P, S, Cl, B and Mo, but there have been no claims that any of these elements are affected by glyphosate in GR crops.

3.4 Amino acid composition

There were no effects of the GR transgene, soil legacy or glyphosate application on the seed free amino acids (Table 6) or protein amino acids (Table 7), other than slightly less free isoleucine in GR seed from plants grown on glyphosate legacy soil, whether from plants treated with glyphosate or not.

3.5 Glyphosate and aminomethylphosphonic acid content

Glyphosate and AMPA were found only in samples from glyphosate-treated plants. In 2013, the leaves of the no history soil had 780 ± 90 ng g^{-1} glyphosate and no detectable AMPA,

Table 2. Soybean leaf mineral content 3 weeks after glyphosate application in glyphosate-resistant soybean near isogenic cultivars grown on glyphosate legacy and no legacy sites at Stoneville, MS in 2013 and 2014

	Glyphosate legacy site			No glyphosate legacy site		
Leaf mineral content	Non-GR, no Gly	GR, no Gly	GR + Gly	Non-GR, no Gly	GR, no Gly	GR + Gly
Al	377 a	347 a	335 a	585 a	522 a	492 a
Ba	33.3 a	43.9 a	31.3 a	32.2 a	30.1 a	28.9 a
Cd	0.04 a	0.05 a	0.05 a	0.06 a	0.05 a	0.06 a
Co	0.21 a	0.19 a	0.20 a	0.29 a	0.25 a	0.25 a
Cr	12.6 a	11.4 a	12.7 a	16.3 a	14.5 a	13.6 a
Cs	0.11 a	0.09 a	0.08 a	0.07 a	0.07 a	0.06 a
Cu	10.6 a	11.0 a	11.4 a	11.8 a	11.4 a	11.2 a
Fe	264 a	248 a	252 a	359 a	304 a	300 a
Ga	0.07 a	0.06 a	0.06 a	0.11 a	0.10 a	0.09 a
K*	19.3 a	20.3 a	20.9 a	23.8 a	23.0 a	23.3 a
Mg	4593 a	4546 a	4711 a	3487 b	3773 a	3784 a
Mn	34.1 a	33.1 a	36.5 a	39.6 a	39.1 a	40.8 a
Ni	10.0 a	10.2 a	10.9 a	19.8 a	14.9 a	14.2 a
Pb	0.15 a	0.20 a	0.12 a	0.20 a	0.17 a	0.15 a
Rb	28.1 a	32.7 a	30.0 a	33.2 a	36.6 a	36.1 a
Se	8.12 a	6.48 a	6.88 a	6.45 a	7.64 a	8.53 a
Sr	33.6 a	34.6 a	33.6 a	29.3 a	26.7 ab	25.3 b
U	0.03 a	0.03 a	0.03 a	0.04 a	0.04 a	0.04 a
V	0.52 a	0.47 a	0.47 a	0.89 a	0.73 a	0.70 a
Zn	38.5 a	45.4 a	45.3 a	41.8 a	41.0 a	39.6 a

GR, glyphosate-resistant; non-GR, non-glyphosate-resistant; Gly, glyphosate.
Leaf mineral content in μg g^{-1} unless indicated otherwise; *mg g^{-1}. Data represent an average of two years. Means within a row for each site followed by the same letter are not significantly different at the 5% level as determined by Fisher's least significant difference test.

Table 3. Soybean leaf mineral content 3 weeks after glyphosate application in glyphosate-resistant soybean near isogenic cultivars grown on glyphosate legacy and no legacy sites at Stoneville, MS in 2013 and 2014

		Glyphosate legacy site			No glyphosate legacy site		
Year	Leaf mineral content	Non-GR, no Gly	GR, no Gly	GR + Gly	Non-GR, no Gly	GR, no Gly	GR + Gly
2013	As	0.14 a	0.13 a	0.12 a	0.26 a	0.19 a	0.25 a
2014	Ca	3940 a	3976 a	4161 a	3921 a	3724 a	3551 a
	Li	0.08 a	0.09 a	0.06 a	0.07 a	0.07 a	0.04 a
	Tl*	1.6 a	1.3 a	1.7 a	1.4 a	1.3 a	1.4 a

GR, glyphosate-resistant; non-GR, non-glyphosate-resistant; Gly, glyphosate.
Leaf mineral content in μg g^{-1} unless indicated otherwise; *ng g^{-1}. Data are for one year as indicated. Means within a row for each site followed by same letter are not significantly different at the 5% level as determined by Fisher's least significant difference test.

whereas the leaves of plants treated with glyphosate in history soil had 7790 ± 440 ng g^{-1} glyphosate and 94 ± 17 ng g^{-1} AMPA. In 2013, no AMPA was found in any seeds, and 101 ± 29 and 456 ± 49 ng g^{-1} glyphosate was found in only glyphosate-treated GR seeds from plants grown in soils with no history and a history of glyphosate use, respectively. These results were surprising because of previous findings,[35] so the seed samples were analyzed twice with the same result. The seed data were qualitatively similar to the leaf data.

In 2014, glyphosate and AMPA were found in leaves and seed of glyphosate-treated plants grown both in glyphosate history and no history soils (Fig. 1), although the AMPA levels in leaves were very low (5.4 ± 0.4 and 194 ± 36 ng g^{-1} in no history and history soils, respectively). Both glyphosate and AMPA levels were higher than in 2013. As in 2013, the glyphosate history soil sample had both higher levels of glyphosate and AMPA.

4 DISCUSSION

Overall, our results discount previous claims of glyphosate effects on mineral nutrition of GR crops.[4–15] These previous papers have claimed effects on Mn, Mg and Fe in particular, and have related purported effects of glyphosate on these minerals to claims of increased susceptibility to crop disease,[4,8,9] chlorosis,[7,10] and decreases in growth and/or yield.[7,15] Although we found some statistically significant differences for some of the minerals, there were no consistent effects between years, treatments and plant tissues. Furthermore, in some cases, there were statistically significant increases in the mineral content of glyphosate-treated plants for minerals that others have claimed to be decreased by glyphosate treatment (e.g. lower Mg in non-GR soybean not treated with glyphosate than in GR soybean with or without glyphosate). Our results are similar to results of an earlier study,[16] in which we concluded that there were false positives at a frequency

Table 4. Soybean seed mineral content in glyphosate-resistant soybean near isogenic cultivars grown on glyphosate legacy and no legacy sites at Stoneville, MS in 2013 and 2014

	Glyphosate legacy site			No glyphosate legacy site		
Seed mineral content	Non-GR, no Gly	GR, no Gly	GR + Gly	Non-GR, no Gly	GR, no Gly	GR + Gly
Al	24.4 a	15.9 a	30.5 a	23.3 a	18.0 a	33.9 a
Ba	15.4 a	14.1 a	16.1 a	21.5 a	19.6 b	18.8 b
Ca	2692 a	2511 a	2560 a	2668 a	2602 a	2548 a
Cd	0.14 a	0.12 b	0.13 b	0.16 a	0.15 a	0.14 a
Co	0.11 a	0.29 a	0.09 a	0.14 a	0.12 a	0.09 a
Cu	17.2 a	17.3 a	16.4 a	18.2 a	18.9 a	18.5 a
Fe	87.2 a	86.6 a	87.8 a	86.4 a	80.7 a	88.5 a
K*	27.7 a	26.9 a	26.2 a	27.4 a	26.6 a	27.4 a
Mg	3747 a	3583 b	3524 b	3433 a	3521 a	3486 a
Mn	29.5 a	27.9 a	29.2 a	34.0 a	33.3 a	32.7 a
Ni	14.8 a	14.5 a	8.1 a	17.4 a	13.1 a	10.9 a
Sr	14.7 a	13.5 a	14.4 a	17.3 a	15.8 b	15.1 b
Zn	40.9 a	40.8 a	40.1 a	43.5 a	44.7 a	43.5 a

GR, glyphosate-resistant; non-GR, non-glyphosate-resistant; Gly, glyphosate.
Seed mineral content is ng g^{-1} unless indicated otherwise; *μg g^{-1}. Data represent an average of two years. Means within a row for each site followed by same letter are not significantly different at the 5% level as determined by Fisher's least significant difference test.

Table 5. Soybean seed mineral content in glyphosate-resistant soybean near isogenic cultivars grown on glyphosate legacy and no legacy sites at Stoneville, MS in 2013 and 2014

		Glyphosate legacy site			No glyphosate legacy site		
Year	Seed mineral content	Non-GR, no Gly	GR, no Gly	GR + Gly	Non-GR, no Gly	GR, no Gly	GR + Gly
2013	Pb	0.12 a	0.12 a	0.06 a	0.13 a	0.12 a	0.10 a
	Cr	0.22 a	0.56 a	0.20 a	0.20 a	0.35 a	0.09 a
2014	Ag	0.10 a	0.08 b	0.10 a	0.40 a	0.10 a	0.08 a
	Be	0.50 a	0.39 b	0.47 a	0.52 a	0.48 ab	0.42 b
	Cs	0.39 a	0.33 a	0.36 a	0.38 a	0.35 a	0.34 a
	Ga	17.8 a	15.9 a	17.6 a	25.1 a	21.5 b	19.6 b
	Li	0.67 a	0.53 b	0.64 a	0.70 a	0.64 a	0.58 a
	Rb	41.6 a	42.6 a	36.6 a	40.5 a	45.1 a	48.3 a
	Tl	0.15 a	0.11 b	0.14 a	0.15 a	0.14 ab	0.12 b

GR, glyphosate-resistant; non-GR, non-glyphosate-resistant; Gly, glyphosate.
Seed mineral content is ng g^{-1}. Data are for one year as indicated. Means within a row for each site followed by same letter are not significantly different at the 5% level as determined by Fisher's least significant difference test.

of ~5%, which one can expect at the 95% level of confidence. In fact, 5.4% of the means in the present study were significantly different from means with which they were compared. The lack of effect of glyphosate or the resistance gene on yield indicates that even if the small number of apparently random mineral content effects were real, the effects were biologically irrelevant to the plants. However, we think that the small number of seemingly random significant means in the present study were probably false positives or negatives, depending on the point of view. Taken as a whole, our results indicate no effect of glyphosate, the GR transgene or soil history on content of the elements measured in GR soybean.

As shown previously,[31,35–38] GR soybeans treated with glyphosate contain glyphosate and AMPA, although we found none in seed from the no glyphosate history plot in 2013. The levels of glyphosate in leaves were at least 10-fold higher than in seeds in plants from the same soils, although AMPA levels were comparable (history soil) or higher (no history soil) in seeds (Fig. 1). A higher AMPA to glyphosate ratio in seeds can be explained by the much later time of sampling of seeds than of leaves, giving the plant longer to metabolize glyphosate. Although sprayed before flowering, glyphosate accumulation in seeds is to be expected, because glyphosate translocates preferentially to metabolic sinks such as developing seeds.[39] In previous work,[31] the glyphosate and AMPA levels found in GR soybean seed of plants treated with glyphosate at 3 and 6 weeks after planting with 1260 and 840 g ae ha^{-1} were similar to the values in the present study, except the ratio of glyphosate to AMPA was reversed. In this earlier study, 1260 g ae ha^{-1} at full bloom (8 WAP) greatly increased (10–20-fold) the amount of both glyphosate and AMPA in the seed. Bohm *et al.*[37] reported higher levels of glyphosate and AMPA (~ 20 μg g^{-1} of both) in seeds of field-grown, glyphosate-treated GR soybean in Brazil. Sampling of glyphosate-treated GR soybean seed from different batches sampled from different fields in Iowa (USA) found a range of glyphosate and AMPA concentrations (glyphosate and AMPA combined were found at 1–15 μg g^{-1}),

Table 6. Transgene and glyphosate effects on free amino acids in glyphosate-resistant soybean near isogenic cultivars grown on soil with legacy and no-legacy of glyphosate at Stoneville, MS, 2013 and 2014

	Glyphosate legacy site			No glyphosate legacy site		
Amino acid	Non-GR, no Gly	GR, no Gly	GR + Gly	Non-GR, no Gly	GR, no Gly	GR + Gly
His	5.9 a	7.5 a	7..2 a	5.9 a	6.7 a	8.4 a
Asn	48.0 a	30.5 a	29.2 a	43.0 a	28.6 a	29.0 a
Ser	2.8 a	3.1 a	2.8 a	3.3 a	2.8 a	3.4 a
Arg	51.9 a	54.1 a	42.3 a	58.4 a	48.8 a	56.0 a
Gly	8.5 a	8.9 a	7.7 a	8.9 a	7.3 a	9.3 a
Asp	45.8 a	39.5 a	41.8 a	43.4 a	39.3 a	31.6 a
Glu	50.3 a	52.3 a	48.0 a	47.0 a	49.6 a	49.3 a
Thr	2.5 a	2.3 a	2.1 a	2.2 a	2.1 a	2.2 a
Ala	34.1 a	35.5 a	31.2 a	35.6 a	29.5 a	36.8 a
Pro	8.8 a	7.9 a	8.0 a	9.6 a	7.3 a	8.9 a
Lys	6.4 a	2.3 a	2.1 a	4.3 a	3.1 a	2.2 a
Tyr	8.3 a	5.3 a	4.6 a	5.8 a	4.5 a	5.0 a
Met	5.1 a	5.1 a	4.4 a	4.9 a	4.5 a	5.0 a
Val	6.0 a	5.9 a	5.3 a	5.9 a	5.8 a	6.4 a
Ile	3.5 a	2.5 b	2.3 b	3.9 a	2.9 a	3.1 a
Leu	5.5 a	4.7 a	4.1 a	5.0 a	4.5 a	5.0 a
Phe	8.5 a	8.0 a	7.2 a	9.1 a	8.0 a	8.4 a
Trp	10.2 a	11.7 a	9.9 a	9.6 a	12.8 a	16.2 a

GR, glyphosate-resistant; non-GR, non-glyphosate-resistant; Gly, glyphosate.
Amino acids are given in nmol mg^{-1}. Soybean seed samples were analyzed by Proteomics and Mass Spectrometry Facility, Danforth Plant Science Center, St. Louis, MO, USA. Data represent an average of two years. Means within a row for each site followed by same letter are not significantly different at the 5% level as determined by Fisher's least significant difference test.

Table 7. Transgene and glyphosate effects on hydrolyzed protein amino acids in glyphosate-resistant soybean near isogenic cultivars grown on soil with legacy and no-legacy of glyphosate at Stoneville, MS, 2013 and 2014

	Glyphosate legacy site			No glyphosate legacy site		
Amino acid	Non-GR, no Gly	GR, no Gly	GR + Gly	Non-GR, no Gly	GR, no Gly	GR + Gly
His	13.6 a	18.9 a	19.5 a	13.2 a	16.3 a	16.7 a
Ser	19.7 a	25.7 a	24.9 a	16.8 a	21.9 a	21.3 a
Arg	36.8 a	47.8 a	44.3 a	34.6 a	40.0 a	43.9 a
Gly	50.0 a	64.5 a	58.7 a	46.6 a	57.7 a	59.1 a
Asp	14.4 a	28.0 a	22.9 a	20.1 a	26.6 a	22.6 a
Glu	17.1 a	33.4 a	26.1 a	22.0 a	34.1 a	25.3 a
Thr	19.2 a	24.0 a	23.2 a	16.3 a	20.7 a	21.7 a
Ala	26.6 a	33.6 a	30.7 a	25.2 a	31.1 a	34.2 a
Pro	35.6 a	43.5 a	39.5 a	32.2 a	40.0 a	42.1 a
Cys	0.3 a	0.5 a	0.4 a	0.3 a	0.4 a	0.4 a
Lys	26.0 a	34.6 a	30.9 a	25.3 a	28.6 a	35.1 a
Tyr	22.0 a	27.5 a	26.8 a	19.7 a	25.6 a	23.1 a
Met	6.9 a	8.4 a	8.2 a	6.2 a	8.0 a	7.5 a
Val	31.7 a	39.1 a	36.1 a	28.6 a	37.9a	38.7 a
Ile	29.6 a	35.1 a	33.2 a	26.4 a	36.5 a	34.5 a
Leu	42.5 a	51.2 a	47.8 a	38.0 a	54.1 a	50.1 a
Phe	34.7 a	43.7 a	43.4 a	32.2 a	43.6 a	37.0 a

GR, glyphosate-resistant; non-GR, non-glyphosate-resistant; Gly, glyphosate.
Amino acids are given in nmol mg^{-1}. Soybean seed samples were analyzed by Proteomics and Mass Spectrometry Facility, Danforth Plant Science Center, St. Louis, MO, USA. Data represent an average of two years. Means within a row for each site followed by same letter are not significantly different at the 5% level as determined by Fisher's least significant difference test.

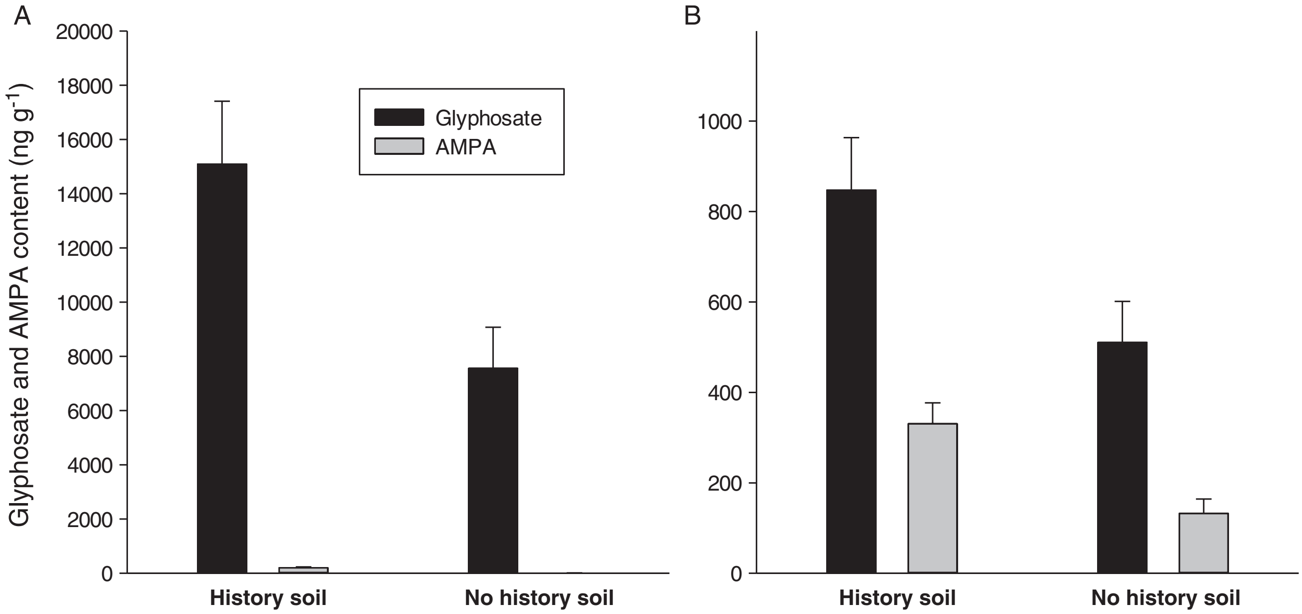

Figure 1. Glyphosate and AMPA content of leaves (A) and harvested soybean seed (B) from soil with a history of glyphosate use versus soil without a history of glyphosate use in 2014. Error bars are 1 SE of the mean.

with AMPA concentrations usually higher than glyphosate.[38] Our 2013 results were similar to the lower levels found by Bøhn *et al.*[38] in some seed samples. Results of the present study compared with our previous findings[35,36] and those of Bøhn *et al.*[38] suggest that the level of glyphosate and AMPA in glyphosate-treated GR soybean seed can vary considerably. Parameters such as glyphosate dose, time of treatment before pod filling, physiological state of the plant, and others should affect the seed glyphosate and AMPA content.

Our amino acid results indicate that the GR transgene, glyphosate use on GR soybean, and soils with a history of glyphosate use have no effect on either free or protein amino acid content of soybean seed (Tables 6 and 7). This is consistent with previous studies,[28,29,40] although some have claimed that glyphosate negatively affects the amino acid composition of GR soybean.[30]

As mentioned earlier, some have claimed that glyphosate reduces growth and yield in GR soybean.[7,15] We found no effects of any of the variables studied on yield. This is in line with previous studies which found no negative effect of glyphosate or the GR transgene on yield of GR soybean.[16,27,37,41–46] The most extensive set of studies on the effects of glyphosate on yield of GR soybean was published in 1995, the year before this GR crop was first grown commercially by farmers.[43] This study evaluated the effects at 17–23 locations over 3 years and found no effect on yield. Kandel *et al.*[27] reported at some locations glyphosate-treated GR soybean had significantly greater yields than GR soybean in which herbicides other than glyphosate were used for weed management. Similarly, Williams *et al.*[46] found glyphosate-treated GR maize to have higher yields than the same cultivar with weeds managed by means other than with glyphosate. A possible explanation of the yield enhancement is a glyphosate hormesis (a stimulatory effect of a toxin at a low, non-toxic dose) effect. Glyphosate hormesis is common with low glyphosate doses used on glyphosate-sensitive plants,[47] so recommended rates on GR crops might sometimes function like a low, hormetic dose on conventional crops.

In summary, our results over 2 years on soils with and without a history of glyphosate use indicate that there is no effect of the GR transgene, history of glyphosate use or recommended glyphosate use rates in GR soybean on content of minerals measured, seed amino acid composition or yield of soybean.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Duke SO, Biotechnology: Herbicide-resistant crops, in *Encyclopedia of Agriculture and Food Systems, Vol. 2*, ed. by Van Alfen N. Elsevier, San Diego, USA, pp. 94–116 (2014).
2 Duke SO, Lydon J, Koskinen WC, Moorman TB, Chaney RL and Hammerschmidt R, Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. *J Agric Food Chem* **60**:10375–10397 (2012).
3 Kowitt B, Can Monsanto save the planet? *Fortune* **173**:95. http://fortune.com/monsanto-fortune-500-gmo-foods/ (2016).
4 Yamada T, Kremer RJ, Kremer RJ, de Camargo e Castro PB and Wood BW, Glyphosate interactions with physiology, nutrition, and diseases of plants: threat to agricultural sustainability. *Eur J Agron* **31**:111–113 (2009).
5 Zobiole LHS, Oliveira RS, Kremer RJ, Muniz AS and Oliviera Jr A, Nutrient accumulation and photosynthesis in glyphosate-resistant soybeans is reduced under glyphosate use. *J Plant Nutr* **33**:1860–1873 (2010).

6 Zobiole LHS, Oliviera RS, Visentainer JV, Kremer RJ, Bellaloui N and Yamada T, Glyphosate affects seed composition in glyphosate-resistant soybean. *J Agric Food Chem* **58**:4517–4522 (2010).

7 Zobiole LHS, Oliveira RS, Kremer RJ, Constantin J, Yamada T, Castro C *et al.*, Effect of glyphosate on symbiotic N_2 fixation and nickel concentration in glyphosate-resistant soybeans. *Appl Soil Ecol* **44**:176–180 (2010).

8 Zobiole LHS, Oliveira RS, Huber DM, Constantin J, Castro C, Oliveira FA *et al.*, Glyphosate reduces shoot concentrations of mineral nutrients in glyphosate-resistant soybeans. *Plant Soil* **328**:57–69 (2010).

9 Zobiole LHS, Kremer RJ, Oliveira RS and Constantin J, Glyphosate affects micro-organisms in rhizospheres of glyphosate-resistant soybeans. *J Appl Microbiol* **110**:118–127 (2011).

10 Zobiole LHS, Kremer RJ, Oliveira RS and Constantin J, Glyphosate affects chlorophyll, nodulation and nutrient accumulation of 'second generation' glyphosate-resistant soybean (*Glycine max* L.). *Pestic Biochem Physiol* **99**:53–60 (2011).

11 Zobiole LHS, Oliveira RS, Constantin J, Kremer RJ and Biffe DF, Amino acid application can be an alternative to prevent glyphosate injury in glyphosate-resistant soybeans. *J Plant Nutr* **35**:268–287 (2012).

12 Zobiole LHS, Kremer RJ, Oliveira RS and Constantin J, Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean. *J. Plant Nutr Soil Sci* **175**:319–330 (2012).

13 Moreira A, Moraes LA, Furlan T and Heinrichs R, Effect of glyphosate and zinc application on yield, soil fertility, yield components, and nutritional status of soybean. *Commun Soil Plant Anal* **47**:1033–1047 (2016).

14 Bellaloui N, Reddy KN, Zablotowicz RM, Abbas HK and Abel CA, Effects of glyphosate application on seed iron and root ferric (III) reductase in soybean cultivars. *J Agric Food Chem* **57**:9569–9574 (2009).

15 Bott S, Tesfamariam T, Candan H, Cakmak I, Römheld V and Newmann G, Glyphosate-induced impairment of plant growth and micronutrient status in glyphosate-resistant soybean (*Glycine max* L.). *Plant Soil* **68**:185–194 (2008).

16 Duke SO, Reddy KN, Bu K, and Cizdziel JV, Effects of glyphosate on the mineral content of glyphosate-resistant soybeans (*Glycine max*). *J Agric Food Chem* **60**:6764–6771 (2012).

17 Andrade GJM and Rosolem CA, Uptake of manganese in RR soybean under glifosate application. *Rev Bras Cienc Solo*, **35**:961–968 (2011).

18 Henry RS, Wise KA and Johnson WG, Glyphosate's effect upon mineral accumulation in soybean. *Plant Manag Network Crop Manag*, https://doi.org/10.1994/CM-2011-1024-01-RS (2011).

19 Bailey WA, Poston DH, Wilson HP and Hines TE, Glyphosate interactions with manganese. *Weed Technol* **1**:792–799 (2002)

20 Ebelhar SA, Varsa EC and Hart CD, Soil pH and manganese effects on yield of Roundup Ready^ soybeans. *Illinois Fert Conf Proc* 54–65 (2006).

21 Rosolem CA, Andrade GJM, Lisboa IP and Zoca SM, Manganese uptake and redistribution in soybeans as affected by glyphosate. *P Bras Ci Solo* **34**:1915–1922 (2010).

22 Loecker JL, Nelson NO, Gordon WB, Maddux LD, Janssen KA and Schapaugh WT, Manganese response in conventional and glyphosate resistant soybean. *Agron J* **102**:606–611 (2010).

23 Cavalieri SD, Velini ED, Silva FML, São José AR and Andrade GJM, Nutrient and shoot dry matter accumulation of two GR soybean cultivars under the effect of glyphosate formulations. *Planta Daninha* **30**:349–358 (2012).

24 Serra AP, Marchetti ME, da Silva Candido AC, Ribiero Dias AC and Christoffoleti PJ, Glyphosate influence on nitrogen, manganese, iron, copper and zinc nutritional efficiency in glyphosate resistant soybean. *Ciênc Rural, Santa Maria* **41**:77–84 (2011).

25 Lundry DR, Alba RM, Culler AH and Bleek MS, Mineral levels in mature soybean seed are not altered by glyphosate treatment or the glyphosate tolerance trait. *Joint 46th and 39th Great Lakes Regional Meeting of the American Chemical Society*, **MWGL-46**, 19–22 October (2011).

26 Stefanello FF, Marchetti ME, da Silva EF, Stefanello J, Doreto RBS and Novelino JO, Effect of glyphosate and manganese on nutrition and yield of transgenic glyphosate-resistant soybean (in Portuguese). *Ciênc Agrár* **32**:1007–1014 (2011).

27 Kandel YR, Bradley CA, Wise KA, Chilvers MI, Tenuta AU, Davis V *et al.*, Effect of glyphosate application on sudden death syndrome of glyphosate-resistant soybean under field conditions. *Plant Dis* **99**:347–354 (2015).

28 Lundry DR, Ridley WP, Meyer JJ, Riordan SG, Nemeth MA, Trujillo WA *et al.*, Composition of grain, forage, and processed fractions from second-generation glyphosate-tolerant soybean, MON 89788, is equivalent to that of conventional soybean (*Glycine max* L.). *J Agric Food Chem* **56**:4611–4622 (2008).

29 Taylor NB, Fuchs RL, MacDonald J, Shariff AR and Padgette SR, Compositional analysis of glyphosate-tolerant soybeans treated with glyphosate. *J Agric Food Chem* **47**:4469–4473 (1999).

30 Zobiole LHS, Bonini EA, Silvero de Oliveira R, Kremer RJ and Ferrarese-Filho O, Glyphosate affects lignin content and amino acid production in glyphosate-resistant soybean. *Acta Physiol Plant* **32**:831–837 (2010).

31 Duke SO, Glyphosate degradation in glyphosate-resistant and -susceptible crops and weeds. *J Agric Food Chem* **59**:5835–5841 (2011).

32 Pantalone VR, Allen FL and Landau-Ellis D, Registration of '5601T' soybean. *Crop Sci* **43**:1123–1124 (2003).

33 Pantalone VR, Allen FL and Landau-Ellis D, Soybean varieties. US Patent 7 777 102 B2, 17 August (2010) 74 pp.

34 Hacham Y, Avraham T and Amir R, The N-terminal region of *Arabidopsis* cystathionine γ-synthase plays an important regulatory role in methionine metabolism. *Plant Physiol* **128**:454–462 (2002).

35 Duke SO, Rimando AM, Pace PF, Reddy KN and Smeda RJ, Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. *J Agric Food Chem* **51**:340–344 (2003).

36 Nandula VK, Reddy KN, Rimando AM, Duke SO and Poston DH, Glyphosate-resistant and -susceptible soybean (*Glycine max*) and canola (*Brassica napus*) dose response and metabolism relationships with glyphosate. *J Agric Food Chem* **55**:3540–3545 (2007).

37 Bohm GMB, Rombaldi CV, Genovese MI, Castilhos D, Alves BJR and Rumjanek NG, Glyphosate effects on yield, nitrogen fixation, and seed quality in glyphosate-resistant soybean. *Crop Sci* **54**:1737–1743 (2014).

38 Bøhn T, Cuhra M, Traavik T, Sanden M, Fagan J and Primicerio R, Compositional differences in soybeans on the market: glyphosate accumulates in Roundy Ready GM soybeans. *Food Chem* **153**:207–215 (2014).

39 Duke SO, Baerson SR and Rimando AM, Herbicides: glyphosate, in *Encyclopedia of Agrochemicals*, ed. by Plimmer JR, Gammon DW, and Ragsdale NN. Wiley, New York (2003). Available: http://www.mrw.interscience.wiley.com/eoa/articles/agr119/frame.html.

40 Lepping M, Herman RA and Potts BL, Compositional equivalence of DAS-44406-06 (AAD-12 + 2mEPSPS + PAT) herbicide-tolerant soybean and nontransgenic soybean. *J Agric Food Chem* **61**:11180–11190 (2013).

41 Zablotowicz RM and Reddy KN Nitrogenase activity, nitrogen content, and yield responses to glyphosate in glyphosate-resistant soybean. *Crop Protect* **26**:370–376 (2007).

42 Bellaloui N, Zablotowicz RM, Reddy KN and Abel CA, Nitrogen metabolism and seed composition as influenced by glyphosate application in glyphosate-resistant soybean. *J Agric Food Chem* **56**:2765–2772 (2008).

43 Delannay X, Baumann TT, Beighley DH, Buettner MJ, Coble HD, DeFelice MS *et al.*, Yield evaluation of glyphosate-tolerant soybean line after treatment with glyphosate. *Crop Sci* **35**:1461–1467 (1995).

44 Lee CD, Penner D and Hammerschmidt R, Influence of formulated glyphosate and activator adjuvants on *Sclerotinia sclerotiorum* in glyphosate-resistant and -susceptible *Glycine max*. *Weed Sci* **48**:710–715 (2003).

45 Sanogo S, Yang XB and Lundeen P, Field response of glyphosate-tolerant soybean to herbicides and sudden death syndrome. *Plant Dis* **85**:773–770 (2001).

46 Williams MM, Bradley CA, Duke SO, Maul JE and Reddy KN, Goss's wilt incidence in sweet corn is independent of transgenic traits and glyphosate. *Hort Sci* **50**:1791–1794 (2015).

47 Belz RG and Duke SO, Herbicides and plant hormesis. *Pest Manag Sci* **70**:698–707 (2014).

