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Effects of Glyphosate on the Mineral Content of Glyphosate-Resistant Soybeans (*Glycine max*)

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ABSTRACT: There are conflicting claims as to whether treatment with glyphosate adversely affects mineral nutrition of glyphosate-resistant (GR) crops. Those who have made claims of adverse effects have argued links between reduced Mn and diseases in these crops. This article describes experiments designed to determine the effects of a recommended rate (0.86 kg ha^{-1}) of glyphosate applied once or twice on the mineral content of young and mature leaves, as well as in seeds produced by GR soybeans (*Glycine max*) in both the greenhouse and field using inductively coupled plasma mass spectrometry (ICP-MS). In the greenhouse, there were no effects of either one application (at 3 weeks after planting, WAP) or two applications (at 3 and 6 WAP) of glyphosate on Ca, Mg, Mn, Zn, Fe, Cu, Sr, Ba, Al, Cd, Cr, Co, or Ni content of young or old leaves sampled at 6, 9, and 12 WAP and in harvested seed. Se concentrations were too low for accurate detection in leaves, but there was also no effect of glyphosate applications on Se in the seeds. In the field study, there were no effects of two applications (at 3 and 6 WAP) of glyphosate on Ca, Mg, Mn, Zn, Fe, Cu, Sr, Ba, Al, Cd, Cr, Co, or Ni content of young or old leaves at either 9 or 12 WAP. There was also no effect on Se in the seeds. There was no difference in yield between control and glyphosate-treated GR soybeans in the field. The results indicate that glyphosate does not influence mineral nutrition of GR soybean at recommended rates for weed management in the field. Furthermore, the field studies confirm the results of greenhouse studies.

KEYWORDS: *glyphosate, minerals, soybean, Glycine max, glyphosate resistance, herbicide, ICP-MS*

■ INTRODUCTION

Glyphosate (*N*-(phosphonomethyl)glycine) is the most widely used herbicide in the world, due mainly to its extensive use with glyphosate-resistant (GR) crops.[1,2] Glyphosate is a divalent metal cation chelator,[3,4] although not a strong one compared to certain synthetic and natural metal ion chelators.[5−7] In glyphosate-sensitive plants, glyphosate does affect mineral nutrition of the plant. For example, Eker et al.[8] found that glyphosate reduced uptake and translocation of Mn and Fe in non-GR sunflower. Whether such effects are due to chelation effects or are due to secondary effects from the phytotoxicity is unknown. However, the almost 50-fold level of resistance of GR crops[9] indicates that if there is a significant effect on mineral nutrition on non-GR plants, it is a secondary effect of glyphosate's phytotoxicity.

Nevertheless, published data on the effects of glyphosate on mineral nutrition of GR crops are contradictory. Three groups have claimed adverse effects on mineral nutrition in GR crops in peer-reviewed journals: Zobiole et al.,[10−16] Bellaloui et al.,[17] and Bott et al.[18] All but one[16] of the Zobiole et al. studies and the Bott et al. study were conducted in a greenhouse or growth chamber. Eight other research groups have found no effect of glyphosate on mineral nutrition of GR crops, mostly in the field.[19−27] Published data on the mineral content of GR crops do not address the question of whether glyphosate has an effect, as these papers do not compare glyphosate-sprayed plants with a no glyphosate control.[28−34] However, the published mineral contents are within the normal ranges for these crops. Others have tried to connect the reported effects of glyphosate on the mineral content of GR crops to the greater susceptibility of these crops to plant disease.[35−37]

The objective of the experiments described in this paper was to determine whether glyphosate applied at field rates has an effect on the mineral content of young and mature leaves, as well as in seeds produced by GR soybeans in both the greenhouse and field. This is the first study on this topic to use high resolution inductively coupled plasma mass spectrometry (ICP-MS) to examine glyphosate effects on the content of almost all metals in a GR crop. Other analytical techniques commonly employed to measure elemental content of plant tissues include atomic absorption spectrometry (AAS) and inductively coupled plasma-atomic emission spectrometry (ICP-AES). Unlike the former, ICP-MS is considered a multielement technique which dramatically increases sample throughput. ICP-MS also generally has lower detection limits than both AAS and ICP-AES, and is capable of measuring isotope ratios. There are two types of mass analyzers commonly employed in ICP-MS: the quadrupole, by far the most common, and the sector field. Whereas each has its advantages and limitations, sector field instruments allow for high resolution measurements, which were used in this study for select elements to eliminate certain isobaric interferences. Moreover, sector field instruments generally have lower backgrounds and higher sensitivity and thus lower limits of

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detection. All mineral elements of interest were measured in our study. We found no effects on any of the metal contents in leaves or harvested seed.

■ MATERIALS AND METHODS

Greenhouse Experiment. A greenhouse experiment was conducted during November, 2010–January, 2011 at the USDA-ARS Crop Production Systems Research Unit, Stoneville, MS. Glyphosate-resistant soybean (*Glycine max*) cultivar (Asgrow 4605RR/S) was grown in 20-cm diameter plastic pots containing Bosket sandy loam soil (fine-loamy, mixed, thermic Mollic Hapludalfs; pH 8.2, 0.5% organic matter, cation exchange capacity = 16.7 meq 100 g^{-1}, 51.3% sand, 37.1% silt, and 11.6% clay). The greenhouse was maintained at 28/22 ± 3 °C day/night temperature with natural light supplemented by sodium vapor lamps to provide a 13-h photoperiod. Soybeans were seeded and thinned to one uniform plant per pot after emergence and subirrigated with distilled water as needed. Plants were supplied with nitrogen (urea, 46% N, 2.6 g/L, 100 mL/pot) at 4, 6, and 8 weeks after planting (WAP). Soybean plants at the two-trifoliolate leaf (3 WAP) stage were used for glyphosate treatment. Treatments were (1) glyphosate at 0.86 kg ae/ha applied at 3 WAP; (2) glyphosate at 0.86 and 0.86 kg ae/ha applied at 3 and 6 WAP; and (3) no glyphosate control. Treatments were replicated eight times. Spray solutions, prepared using a commercial formulation of the potassium salt of glyphosate (Roundup WeatherMax, Monsanto Agricultural Co., St. Louis, MO), were applied using an indoor spray chamber equipped with 8002E flat-fan nozzles and pressurized at 140 kPa to deliver 190 L/ha. Young and old leaflets were sampled at 6 (prior to second application of glyphosate), 9, and 12 WAP. At 6 WAP, the young leaves were leaf 6 at node 7, and old leaves were trifoliate leaf 2 at node 3; at 9 WAP, young leaves were trifoliate leaves 6 and 7 at nodes 7 and 8, and old leaves were trifoliate leaves 2 and three at nodes three and four; at 12 WAP, new leaves were trifoliate leaves 10 and 11 at nodes 11 and 12, and old leaves were trifoliate leaves 3 and 4 at nodes 4 an 5. At 12 WAP, soybean seeds (physiological maturity) were also collected. Leaf and seed samples were stored in sealed plastic bags and stored at 4 °C and room temperature, respectively.

Field Experiment. A field study was conducted in 2011 at the USDA-ARS Crop Production Systems Research farm, Stoneville, Mississippi, under an irrigated environment. The soil was a Dundee silt loam (fine-silty, mixed, active, thermic Typic Endoqualf) with pH 6.7, 1.1% organic carbon, a cation exchange capacity of 15 meq 100 g^{-1} with soil textural fractions of 26% sand, 55% silt, and 19% clay. At planting, soil samples from the top 15-cm depth were collected by taking four random cores (7.5-cm diameter) in both no glyphosate and glyphosate plots. The samples were analyzed by the private soil testing laboratory, Waters Agricultural Laboratories, Inc. Camilla, Georgia. The Mehlich 1 double acid extraction method,[38] followed by inductively coupled argon plasma emission spectrophotometry was used for mineral determinations.

The experimental area was under glufosinate-resistant soybean production for two years prior to this study. Seedbed preparation consisted of disking, subsoiling, disking, and bedding in the fall of the previous year. Prior to planting, the raised beds were smoothed as needed. GR soybean (Asgrow 4605RR/S) was planted in 102-cm wide rows using a MaxEmerge 2 planter (Deere and Co., Moline, IL) at 350,000 seeds/ha on May 6, 2011. *S*-Metolachlor at 1.12 kg ai/ha plus pendimethalin at 1.12 kg ai/ha plus paraquat at 1.12 kg ai/ha were applied to the entire experimental area immediately after planting. Paraquat was applied to kill existing weeds at planting, and *S*-metolachlor and pendimethalin were used to provide early season weed control. Treatments were (1) glyphosate at 0.86 and 0.86 kg ae/ha applied at 3 and 6 WAP and (2) no glyphosate control. The commercial formulation of potassium salt of glyphosate (Roundup WeatherMax, Monsanto Agricultural Co., St. Louis, MO) was used. Herbicides were applied with a tractor-mounted sprayer with TeeJet 8004 standard flat spray nozzles (TeeJet Spraying Systems Co., Wheaton, IL), delivering 187 L/ha water at 179 kPa. All plots including glyphosate-treated ones were hand weeded periodically throughout the season to keep them weed-free. No fertilizer nitrogen was applied, and the crop was irrigated on an as-needed basis. The experiment was conducted in a randomized complete block design with eight replications. Each treatment plot consisted of four 15.2-m long rows spaced 102-cm apart.

Young and old leaflets were sampled at 9 and 12 WAP. At 9 WAP, young leaves were trifoliate leaves 6 and 7 at nodes 7 and 8, and old leaves were trifoliate leaves 2 and 3 at nodes 3 and 4; at 12 WAP, new leaves were trifoliate leaves 10 and 11 at nodes 11 and 12, and old leaves were trifoliate leaves 3 and 4 at nodes 4 and 5. At harvest, about 200 soybean pods were randomly sampled from the middle two rows for seed chemical analysis. Soybean from all four rows in each plot was harvested using a combine, and grain yield was adjusted to 13% moisture. Samples were stored as described above.

Sample Preparation for Mineral Analyses. Leaves were removed from storage and dried at 60 °C for ~24 h to constant weight prior to digestion. The mean moisture content of the leaves before drying was 73.1% ± 3.3% (1 SD). Soybeans were digested without drying. The mean moisture content of the beans, determined on separate portions, was 7.1% ± 0.9% (1 SD). Between 0.1 and 0.3 g of each sample was digested with 5 mL of HNO_3, 1 mL of H_2O_2, and 50 μL of HF using a microwave digestion system (Ethos; Milestone Inc.) equipped with a multiprep rotor (42 vessels). All reagents were high purity grade from SeaStar Chemicals Inc. As noted earlier, there were eight replicates (plants) per treatment. From each of these plants, three leaves were collected and analyzed together as a single sample. Beans from each plant were analyzed in duplicate, using 3 or 4 seeds per digest, and the average of the two analyses was used for each plant. The relative percent difference between the beans from a single plant was generally less than 10%. In addition, each digestion batch included three method blanks to monitor contamination, and three samples of NIST SRM 1547 (peach leaves) reference material to monitor accuracy. The microwave was operated at 1200 W, and the temperature program consisted of a 30 min ramp to 120 °C, followed by a 60 min ramp to 180 °C, after which the temperature was held for an additional 20 min. The resulting clear digests were diluted to 50 mL with deionized (DI) water (≥18.2 MΩ). Before analysis, 3 mL of each sample was further diluted to 10 mL with DI water, yielding a final solution of approximately 3% HNO_3 and 0.03% HF.

ICP-MS Analysis. ICP-MS measurements were made using a sector field mass spectrometer (Element-XR; Thermo-Fisher). The Element XR allows for three resolution settings: $m/\Delta m \approx 400$ (low resolution), $m/\Delta m \approx 4000$ (medium resolution) and $m/\Delta m \approx 10000$ (high resolution). Medium and high resolutions are used to separate certain polyatomic interferences with the elements of interest. The sample introduction system consisted of a glass concentric nebulizer outfitted with a glass cyclonic spray chamber. Before the samples were analyzed, the system was optimized for sensitivity, stability, and oxide levels. The following was achieved for 1 ng g^{-1} ^{115}In in low resolution mode: ~1 million counts per second; <2% RSD (short-term); and <5% oxides. For those isotopes measured under medium and high resolutions, mass offset was determined prior to the analysis in order to center the peak in the mass window. Instrumental and data acquisition parameters are given in Table 1.

For quantitation of the leaves, external calibration was used with a reagent blank and five standards ranging from 0.1 ng g^{-1} to 20 ng g^{-1}. For seeds, we employed the method of standard additions because we expected a somewhat more complex matrix. Spikes ranged from 0.1 ng g^{-1} to 50 ng g^{-1}. Standards were prepared in 3% HNO_3 using a multielement standard solution (Spex Certiprep). Linearity (r^2 value) for the calibration plots for all isotopes was >0.999. Internal standardization was performed online using a 2 ng g^{-1} solution of Y and Sc. Y was used for elements in low and high resolutions and Sc for elements in medium resolution. Recoveries for the reference material for the leaf analyses generally ranged from 80 to 120%, except for Ca and K where the recovery was low (56% and 42%, respectively). However, the low recovery of Ca and K was consistent so that the relative values are valid, although the absolute values are low. Concentrations of elements were above their corresponding method

Table 1. ICP-MS Instrumental Settings

plasma	
cool gas flow	16 L min^{-1}
aux. gas flow	0.9 L min^{-1}
sample gas flow	1.19 L min^{-1}
RF power	1280 W
data acquisition	
resolution	isotopes
low (LR)	^{88}Sr, ^{111}Cd, ^{137}Ba, ^{208}Pb, ^{238}U
medium (MR)	^{24} Mg, ^{27}Al, ^{44}Ca, ^{51}V, ^{53}Cr, ^{55} Mn, ^{57}Fe, ^{59}Co, ^{62}Ni, ^{65}Cu, ^{66}Zn
high (HR)	^{39}K, ^{78}Se,
mass window	20% for LR; 150% for MR; 200% for HR
points per peak	50 for LR; 20 for MR and HR
scan type	E-scan
integration time	10 ms
passes and runs	3 and 2

detection limit except for Se in leaves. Because Se levels in the leaves were near or below the MDL (~ 0.7 μg/g), those data are not reported. The Se results for the seeds are reported because the levels exceeded the MDL, which was found to be lower for seeds (~0.04 μg/g). Future work will investigate ways of lowering the MDL for leaf analysis to allow examination of the behavior of trace levels of Se in GR soybean leaves.

Sample data are reported on a dry-weight basis (for leaves) and wet-weight (fresh) basis for the seeds; mean moisture content for both are reported above. The data reported in the figures represent the averages with standard deviations of eight measurements (representing eight plants) for the leaves. For seeds, the data are averages with standard errors of eight means (representing eight plants) of two samples of three or four beans each per plant.

Statistical Analyses. Data from the greenhouse study were subjected to analysis of variance using SAS PROC GLM (SAS Institute, Cary, North Carolina), and treatment means were separated at the 5% level of significance using Fisher's Protected LSD test. Data from the field study were subjected to Student's *t*-test using Microsoft Excel (Microsoft), and means were separated at the 5% level of significance.

RESULTS

Soil Analyses. No significant differences, except for As, were found in mineral content or other characteristics of the soil samples that were used for glyphosate treatments versus control plots (Table 2).

Greenhouse Studies. Statistically, there were no effects of either one or two applications of glyphosate on Ca, Mg, Mn, Zn, Fe, Cu, or Ni content on young or old leaves sampled at 6, 9, and 12 WAP (Figure 1), except for a reduction of Ni in young leaves 12 WAP with one treatment and an increase in Cu in young leaves at 9 WAP with two treatments. Not shown are no effect results on Sr, Ba, Al, Cd, Cr, and Co, minerals less associated with requirements for plant biochemical processes. No effects of either treatment were seen on Mg, Ca, Sr, Ba, Mn, Fe, Ni, Cu, Zn, Al, Cd, Cr, Co, or Se in harvested seeds of greenhouse-grown plants, except for a decrease in Sr and an increase in Ni with two treatments (Figure 2).

Field Studies. There were no effects of two applications (at 3 and 6 WAP) of a recommended rate (0.86 kg ha^{-1}) of glyphosate on Ca, Mg, Mn, Zn, Fe, Cu, or Ni content of young or old leaves at either 9 or 12 WAP, except for a decrease in Mn in young leaves at 12 WAP and an increase in Zn in old leaves at 9 WAP (Figure 3). The content of some metals changed with leaf age. For example, Ni was higher in younger than older leaves, especially at 9 WAP. Conversely, Fe was higher in older leaves. Not shown are no effect results on Sr, Ba, Al, Cd, Cr, and Co, minerals less associated with requirements for plant biochemical processes. Furthermore, there were no effects on Mg, Ca, K, Sr, Ba, Mn, Fe, Ni, Cu, Zn, Cd, Cr, Co, or Se content of harvested seed of glyphosate-treated, field-grown plants (Figure 4). There was no difference in yield between control and glyphosate-treated GR soybean (Table 3).

Table 2. Physical and Chemical Characteristics of Soil (0−15 cm Depth) from the Field Experiment at Stoneville, MS, in 2011

soil characteristics	no glyphosate	glyphosate	*t* test, *P* ≥
pH (water)	6.54	6.57	0.70
organic matter, %	0.79	0.83	0.11
cation exchange capacity, meq/100 g	9.5	9.3	0.43
P (kg/ha)	200.2	212.1	0.14
K (kg/ha)	319.7	321.4	0.93
Mg (kg/ha)	555.4	551.3	0.83
Ca (kg/ha)	2587	2567	0.74
S (kg/ha)	57.4	56.1	0.39
B (kg/ha)	1.47	1.38	0.58
Zn (kg/ha)	3.5	3.6	0.73
Mn (kg/ha)	41.7	43.7	0.56
Fe (kg/ha)	48.9	51.8	0.28
Cu (kg/ha)	2.25	2.46	0.09
As (ppm)	3.48	3.77	0.05
Al (ppm)	8095	8415	0.48
Ba (ppm)	1.05	1.07	0.61
Cd (ppm)	3.69	3.51	0.24
Co (ppm)	7.22	7.17	0.87
Cr (ppm)	13.11	13.38	0.65
Ni (ppm)	17.7	17.4	0.77
Pb (ppm)	33.5	39.8	0.16
Se (ppm)	0.30	0.28	0.67
Sr (ppm)	0.19	0.21	0.24
V (ppm)	0.0011	0.0011	1

DISCUSSION

In the greenhouse, there were few effects on any minerals in leaves with either treatment, and few effects were seen on any minerals of harvested seeds. Similarly, few effects of two applications of glyphosate at recommended doses were measured on the mineral content of young or old leaves at two time points after the last treatment in the field. Two glyphosate treatments had no effect on the content of any minerals of harvested seeds or on yield. The statistically significant effects appeared random, with on only one instance of an effect for any one metal and both increases and decreases in these six metals (Mn, Zn, Ni, Sr, Cu, and Ca). There were 120 treatment means analyzed in this study at the 95% confidence level, so one might expect a 5% false positive rate (six). Exactly 6 of 120 treatment means were found to be statistically significant. The randomness (six minerals, increases and decreases, and different tisues) of these six "significant" means suggests that they are false negatives and positives.

Our results are in general agreement with the eight groups who have found no effect of glyphosate treatment on mineral content of GR crops[19−27] and in disagreement with the three groups who have reported deficiencies caused by glyphosate in one or more minerals in similar experiments.[10−18] Only two of

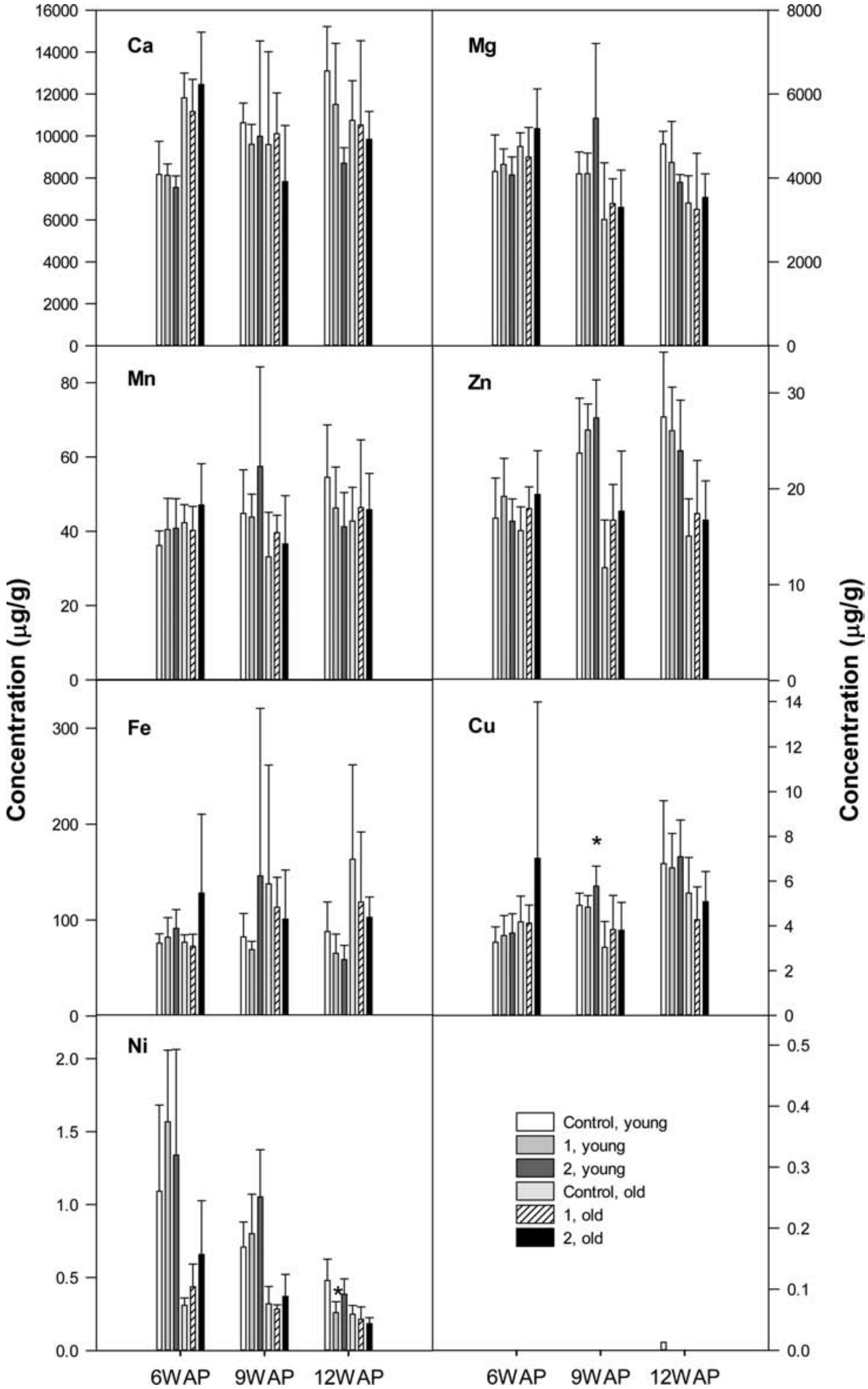

Figure 1. Effects of different glyphosate treatments (one treatment at 3 WAP or two treatments at 3 and 6 WAP; all treatments were 0.86 kg ai h^{-1}) on the metal content of young and old leaves of greenhouse-grown GR soybean plants at three different times after planting. Bars respresent 1 SD. Differences between any treatment and the paired control mean value at the 95% confidence level, using Fisher's Protected LSD test, are designated with an asterisk.

the papers reporting glyphosate-caused mineral reductions were not conducted in a greenhouse or growth chamber.[16,17] In general, we found less of all minerals in greenhouse-grown plants compared to those from the field, suggesting mineral availability was limited in greenhouse-grown plants.

If there are glyphosate effects on plant mineral content under some circumstances, understanding the mechanism of the effect could be useful in understanding how there could be an effect under some conditions and not others. We are aware of three potential mechanisms of glyphosate effects on mineral uptake and translocation in plants. The first is through phytotoxicity. If

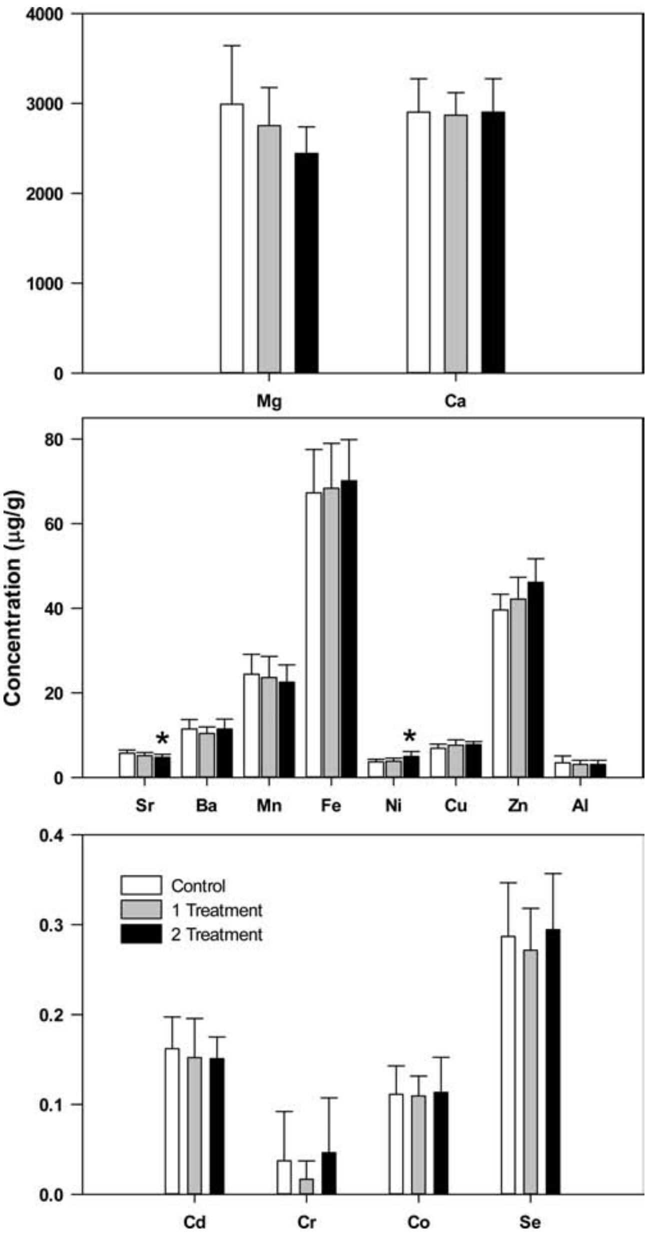

Figure 2. Effects of different glyphosate treatments (one treatment at 3 WAP or two treatments at 3 and 6 WAP; all treatments were 0.86 kg ai h^{-1}) on the metal content of mature seeds from greenhouse-grown GR soybean plants. Bars respresent 1 SE. Differences between any treatment and the paired control mean value at the 95% confidence level, using Fisher's Protected LSD test, are designated with an asterisk.

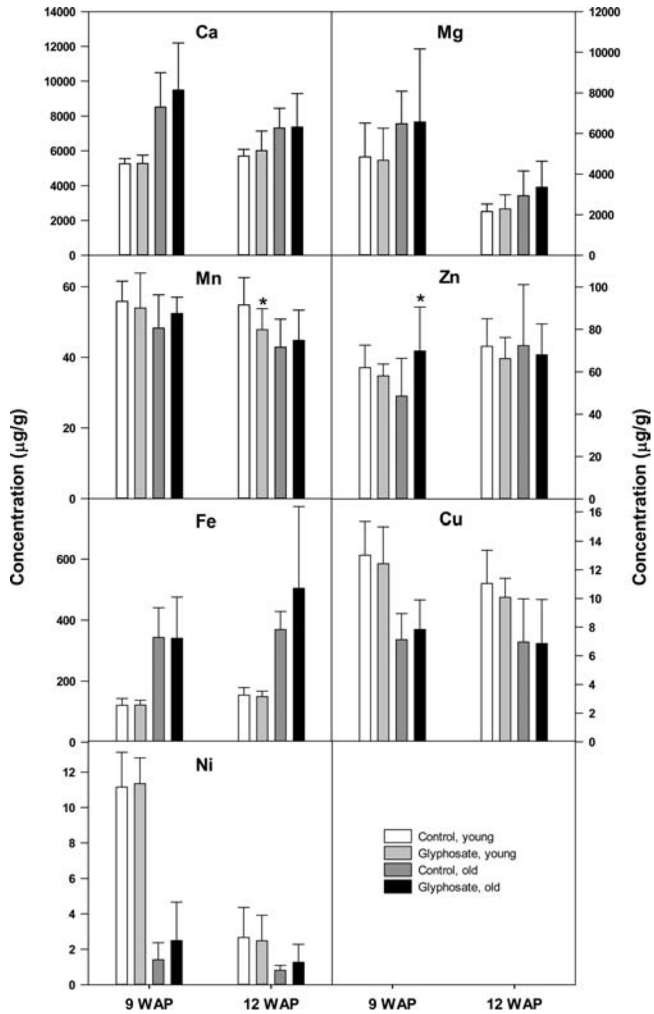

Figure 3. Effects of two different glyphosate treatments (0.86 kg ai h^{-1} at both 3 and 6 WAP) on the metal contents of young and old leaves of field-grown GR soybean plants at two different times after planting. Bars respresent 1 SD. Differences between any of the paired mean values at the 95% confidence level, using Student's *t*-test, are designated with an asterisk.

a compound is herbicidal to a plant, it will eventually affect most all physiological processes, i.e., secondary effects, including mineral uptake and translocation. This is exactly what happens in glyphosate-sensitive plants when treated with glyphosate,[8,39−41] but, because GR crops are about 50-fold less sensitive to glyphosate than non GR crops,[9] any effects on mineral nutrition in GR plants treated with recommended levels of glyphosate should be trivial.

The second is through the chelation of metal cations by glyphosate.[3−7] Glyphosate is a relatively weak chelator,[5−7] although metal ions present in tank mixes of glyphosate can reduce the uptake of glyphosate by weeds due to the poor uptake of chelated glyphosate.[42] There are natural products in plants (e.g., citrate and some amino acids) that are strong metal ion chelators. Furthermore, strong metal ion chelators like EDTA are commonly used to enhance the uptake of metal ions such as Fe and Zn. In general, none of the research on chelating agent effects on metal uptake would indicate that a weak chelator such as glyphosate would reduce the uptake of micronutrient cations from soil, even though glyphosate is certainly chelating mineral ions both *in vitro* and *in vivo*.

Even if glyphosate were a very strong metal ion chelator, examination of glyphosate levels in glyphosate-treated GS soybean seeds at maturity[43] and mineral levels in soybean seed[44] show that on a molar basis the ratio can be from almost 10,000 times more Mn to about 100,000 times more minerals such as Mg or Ca. Comparing glyphosate content of leaves of glyphosate-treated GR soybean[9] with the mineral contents of GR soybean leaves in this article, the ratios are smaller (ca. 300 for Ca, 30 for Fe, 20 for Mn, and only 2 for Cu), but the ratio of total metal atoms to glyphosate molecules is close to 1000. Even if a substantial fraction of the minerals in the plant tissue were unavailable to glyphosate due to chelation with other compounds, sequestration, or other means, the ratio of mineral

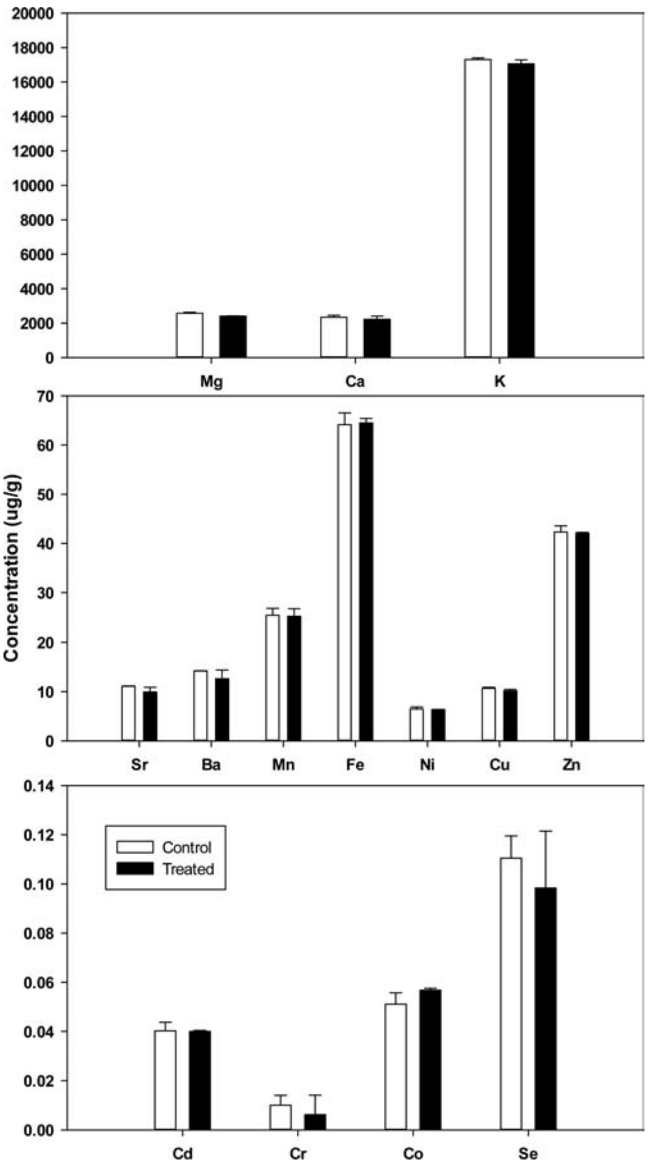

Figure 4. Effects of different two glyphosate treatments (0.86 kg ai h^{-1} at both 3 and 6 WAP) on the metal contents of mature seeds of field-grown GR soybean plants. Bars respresent 1 SE. There were no differences among any of the paired mean values at the 95% confidence level.

Table 3. Glyphosate Effect on Soybean Yield in the Field Experiment at Stoneville, MS, 2011

treatment	soybean yield (kg/ha)
no glyphosate	4327
glyphosate at 0.86 followed by 0.86 kg/ha	4394
t test, $P \geq$	0.70

cations to glyphosate anions would still be large. These sizable ratios do not support the view that the chelator properties of glyphosate would interfere substantially with plant mineral nutrition.

Some rhizosphere microbes, particularly arbuscular mycorrhizal fungi, assist plants in taking up minerals.[45,46] Many microbes, particularly fungi, are sensitive to glyphosate.[47] Some of the glyphosate from foliar applications translocates to roots, where a portion of it is exuded into the rhizosphere.[48−53] This glyphosate exuded into the rhizosphere could adversely affect microbes involved in mineral nutrition. However, several studies have found no effect of glyphosate on mycorrhizae,[54−56] although other studies have reported effects on other rhizosphere microbes.[57] Thus, there are good rationales for why none of these mechanisms would operate to reduce mineral uptake and/or translocation in GR crops.

In summary, no effects of recommended doses of glyphosate on 14 metals were found in leaves or seeds of greenhouse- or field-grown GR soybean plants. Our results support the findings of others that recommended rates of glyphosate do not affect the mineral nutrition of GR soybeans.

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Notes

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