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Nitrogen Metabolism and Seed Composition As Influenced by Glyphosate Application in Glyphosate-Resistant Soybean

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Previous research has demonstrated that glyphosate can affect nitrogen fixation or nitrogen assimilation in soybean. This 2-year field study investigated the effects of glyphosate application of 1.12 and 3.36 kg of ae ha^{-1} on nitrogen metabolism and seed composition in glyphosate-resistant (GR) soybean. There was no effect of glyphosate application on nitrogen fixation as measured by acetylene reduction assay, soybean yield, or seed nitrogen content. However, there were significant effects of glyphosate application on nitrogen assimilation, as measured by in vivo nitrate reductase activity (NRA) in leaves, roots, and nodules, especially at high rate. Transiently lower leaf nitrogen or ^{15}N natural abundance in high glyphosate application soybean supports the inhibition of NRA. With the higher glyphosate application level protein was significantly higher (10.3%) in treated soybean compared to untreated soybean. Inversely, total oil and linolenic acid were lowest at the high glyphosate application rate, but oleic acid was greatest (22%) in treated soybean. These results suggest that nitrate assimilation in GR soybean was more affected than nitrogen fixation by glyphosate application and that glyphosate application may alter nitrogen and carbon metabolism.

KEYWORDS: Transgenic crops; nitrogen assimilation; nitrate reductase; seed composition

INTRODUCTION

Glyphosate-resistant (GR) soybean [(*Glycine max* (L.) Merr.)] is a result of the insertion of an insensitive 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), EC 2.5.1.19, gene from *Agrobacterium* strain CP4 allowing expression of a functional shikimic acid pathway (*1*). Although GR soybean has an insensitive EPSPS gene, the nitrogen-fixing symbiont in the soybean root nodules, *Bradyrhizobium japonicum*, possesses a glyphosate-sensitive enzyme. Nitrogen nutrition in soybean is ensured by dinitrogen fixation and nitrogen assimilation, and these nitrogen sources can be complementary or antagonistic, depending on environmental stress factors or developmental stages of the plant (*2*). The highest nitrogen fixation rate occurs at the end of flowering (R2) and during seed fill (R5−R6). However, the highest rate of nitrogen assimilation occurs at an earlier stage of ontogeny (R1−R2) (*3, 4*). Therefore, improving nitrogen fixation can facilitate productivity (*5*) and high seed protein content (*6*), indicating that nitrogen fixation is a decisive physiological parameter in productivity and seed quality (*7*). Soybean nodulated with *B. japonicum* has the ability to use both inorganic soil nitrogen and atmospheric N_2 to meet the crop's optimum yield and protein requirements (*3*). Exposure of *B. japonicum* to glyphosate may interfere with N_2 fixation, leading to alteration of nitrogen metabolism (*8*) and carbon metabolism (*9*) alteration.

For nitrate to be used by plants, it has to be reduced to nitrite by nitrate reductase (NR). The enzyme is substrate inducible (*10*) and requires NADH or NADPH reductant (*11*). NR enzyme is found in plant leaves, stems, and roots. The relative proportion of nitrate taken up and reduced by NR varies between these plant parts (*4, 12, 13*). On the other hand, atmospheric N_2 is reduced to ammonia by the enzyme nitrogenase in the bacteroids of nodules (*14*). Both NR and nitrogenase coexist in nodules (*4, 15, 16*), competing for reductant (*15*).

Herbicides are known to influence nitrogen metabolism, on which seed protein production and yield depend. This influence can occur through direct effects on the rhizobial symbiont or indirect effects on the physiology of the host plant (*17*). Therefore, understanding the impacts of herbicides on the crop and the symbiont is essential (*18*). Reddy et al. (*18*) and King et al. (*19*) studied the effects of glyphosate on the nodulation of GR soybean. The results showed a significant reduction in nodulation by glyphosate at label use rate. However, there was

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no consistency in the results mentioned in the literature (*19, 20*). King et al. (*19*) observed a significant reduction in acetylene reduction activity (ARA) (12–20%) in three of four studies at 21 days after emergence in GR soybeans (TV5866RR). These data suggested that both nodulation and nitrogen fixation activity were more sensitive in the early stages of soybean development. It was reported that the effect of glyphosate on symbiotic N_2 fixation was due to inhibition of photosynthesis and carbon substrate availability (*21*), in that ARA in bacteroids isolated from treated conventional soybeans was 10−30% lower than that of nontreated plants. Also, it was reported that the level of ARA inhibition corresponded to the sensitivity of the *B. japonicum* strain to glyphosate under in vitro conditions. In glyphosate-sensitive soybean glyphosate drift significantly reduced the rate of nitrogen fixation and nitrate assimilation in an early stage of soybean (*16*), but there were no differences in yield, protein, or total oil between treated and nontreated plants when soybean was treated with glyphosate at 12.5% of use rate of 0.84 kg of ae ha^{-1} at several growth stages.

In previous studies, application of glyphosate to a glyphosate-sensitive soybean generally decreased NRA activity with different patterns of inhibition in specific soybean parts (*16*). The reduction in NRA due to herbicide application is not unique in that other studies have demonstrated that herbicides such as imazethapyr (*22*) and 2,4-D (*23*) decreased NRA, whereas metobromuron increased NRA (*24*). Thus, herbicides with a wide range of mode of actions can alter nitrogen assimilation in legumes. Recently, Zablotowicz and Reddy (*20*) indicated that glyphosate may alter nitrogen assimilation/fixation in GR soybean.

Our objective in this study was to elucidate which component of nitrogen nutrition (N_2 fixation or N assimilation) could be most affected and if glyphosate use could also affect seed composition.

MATERIALS AND METHODS

Growth Conditions. A field study was conducted in 2006 and 2007 at the USDA-ARS Southern Weed Science Research Unit farm, Stoneville, MS. The soil was a Dundee silt loam (fine-silty, mixed, active, thermic Typic Endoqualf) with pH 6.3, 1.1% organic matter, a cation exchange capacity of 15 cmol/kg, and soil textural fractions of 26% sand, 56% silt, and 18% clay, and it contained an abundant native population of *B. japonicum*. The experimental area was tilled with a disk harrow followed by a field cultivator in the fall of 2005 and 2006. The experimental area was under glyphosate-resistant soybean production in previous years. GR soybean cultivars AG4503RR and AG4604RR were planted on May 4, 2006, and May 3, 2007, respectively, at a seed rate of 355,000 seeds ha^{-1}. *s*-Metolachlor at 1.68 kg of ai ha^{-1} plus paraquat at 1.12 kg of ai ha^{-1} were applied to the experimental area immediately after planting. Paraquat was used to kill existing weeds at planting, and *s*-metolachlor was used to provide early-season weed control. A different experimental site with similar soil conditions was used in each year.

Glyphosate treatments consisted of two rates (1.12 and 3.36 kg of ae ha^{-1}), and each rate was applied postemergence twice in a sequence at 4 and 6 weeks after planting (WAP). Four and six WAP soybean corresponded to 28 and 42 days after planting. At 4 and 6 WAP, soybean was at V4 and V7 (*25*) growth stages. Glyphosate at 3.36 kg of ae ha^{-1} followed by 3.36 kg of ae ha^{-1} was higher than the suggested label use rate for single and total in-crop application of glyphosate. This rate was selected to represent the "worst case scenario" to promote soybean injury. A hand-weeded control with no glyphosate was included for a comparison. Herbicide treatments were applied with a tractor-mounted sprayer with TeeJet 8004 standard flat spray tips delivering 187 L of water ha^{-1} at 179 kPa. The commercial formulation of glyphosate (Roundup Weathermax, Monsanto Agricultural Co., St. Louis, MO) was used with no additional adjuvant.

Soybean was irrigated as needed to minimize crop stress especially during late-season dry weather conditions. All plots including glyphosate-treated were hand weeded periodically throughout the season to keep them weed-free. Soybean was harvested from each plot using a combine on September 5, 2006, and September 17, 2007, and grain yield was adjusted to 13% moisture. Each treatment consisted of four soybean rows spaced 102 cm apart and 16.8 m long. Treatments were arranged in a randomized complete block design with four replications. The data for each variable were analyzed by analysis of variance (*26*). Treatment means were separated at the 5% level of significance using Fisher's LSD test.

Nitrate Reductase Assay (NRA). Four to six soybean plants were randomly sampled from the middle two rows of each plot at 1 week after each glyphosate application. Plants were excavated with roots and shoot intact, immediately transported to the laboratory, and assayed for NRA. NRA was measured on the basis of the method of Klepper and Hageman (*27*). Approximately 0.3 g of tissue was placed in 10 mL of potassium phosphate buffer at a concentration of 100 mM, pH 7.5, containing 1% (v/v) 1-propanol, in the flask. The incubation solution was vacuum filtered for 1 min, and the flask and contents were flashed with nitrogen gas for 30 s and then incubated at 30 °C. Samples of 0.5 mL were taken at regular intervals (0, 60, 120, 180, and 300 min) for nitrite determination. Samples were extracted with 5 mL of deionized water and reacted with 1.0 mL of 1% (w/v) sulfanilamide in 10% v/v HCl and 1.0 mL of *N*-naphthyl-1-ethylenediamine dihydrochloride (0.1%). After 30 min, the samples were read at 540 nm using a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA). The concentration of nitrite was calculated from a calibration curve made of potassium nitrite (KNO_2). NRA was measured in leaves, stems, roots, and nodules as described previously (*16*). Briefly, the youngest fully expanded leaf was used to measure NRA in leaves. To measure NRA in roots, only non-nodulated root segments were used for the analysis. Nodule NRA was measured after nodules had been gently removed from the roots and placed in the above buffer solution and assayed as described above. NRA represents the actual nitrate reducatse activity (ANRA) to distinguish it from the potential nitrate reductase activity (PNRA). To determine PNRA under conditions when nitrate concentration could not be a limiting factor, exogenous nitrate was added to the incubation solution at a concentration of 10 mM.

Acetylene Reduction Assay (ARA) and Root Respiration. Ten to fifteen soybean plants were randomly sampled from the middle two rows of each plot one week after each glyphosate application. Plants were excavated with roots and shoot, immediately transported to the laboratory, and assayed within 30 min of collection. Nitrogenase activity was assayed using the acetylene reduction assay to measure ARA as described elsewhere (*16, 20*). Roots with nodules intact were excised and incubated in 1 L Mason jars (two jars per plot). Six roots were placed in the Mason jars and sealed, and a 10% volume of acetylene was added. After 1 h of incubation at room temperature, gas samples were removed and analyzed by gas chromatography using a flame ionization detector (FID) and a thermal conductivity detector (TCD) for determination of ethylene and carbon dioxide, respectively. Following the incubation, roots were washed, the nodules were removed from the roots, and the dry weight of nodules and roots was determined following oven-drying at 60 °C for 4 days.

Total Nitrogen. Leaf material, ∼50 fully expanded, trifoliolate leaves, was sampled per plot at the V6, V9, and R4 stages of growth. At harvest (R8), ∼200 soybean pods were randomly hand sampled from the middle two rows for seed nitrogen determination. Samples were oven-dried (60 °C) and then finely ground twice in a Wiley mill (0.42 mm sieve). Samples were redried the night before nitrogen analysis to remove any moisture that may have been absorbed prior to analysis. Total nitrogen was determined from triplicate samples (10–15 mg) using a Flash EA 112 elemental analyzer (CE Elantech, Lakewood, NJ). Nitrogen was expressed as percent of leaf and seed dry weight.

Delta ^{15}N ($^{15}N/^{14}N$ Ratio Using Natural Abundance Method). Delta ^{15}N abundance was evaluated from nitrogen isotope $^{15}N/^{14}N$ ratio (*28*) on 0.9 mg of tissue samples as described above. A Thermo Finnigan Delta Plus Advantage Mass Spectrometer with a Finnigan ConFlo III and Isomass Elemetal Analyzer (Bremen, Germany) was

Table 1. Analysis of Variance with *F* and *P* Values[a] of Glyphosate Application (Treatment), Year, and Their Interactions for Nitrate Reductase Activity in Plant Parts (Leaves, Stems, Roots, and Nodules) and Seed Composition Constituents

Nitrate Reductase Activity (Micromoles of Nitrite per Gram of Fresh Weight per Hour)								
	leaves		stems		roots		nodules	
source	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
treatment (T)	59.00	<0.0001	1.88	0.17	4.12	0.031	37.68	<0.001
year (Y)	18.65	<0.0001	13.08	0.0008	5.90	0.019	6.94	0.012
T × Y	0.34	0.71	0.15	0.86	0.27	0.77	0.67	0.52

Seed Composition (Percent)								
	protein		oil		oleic acid		linolenic acid	
source	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
treatment (T)	60.38	<0.0001	28.6	<0.0001	92.60	<0.0001	38.48	<0.0001
year (Y)	12.08	0.0016	21.2	<0.0001	15.11	0.0005	2.79	0.11
T × Y	2.40	0.11	0.23	0.79	1.04	0.37	0.88	0.42

[a] Level of significance was $p \leq 0.05$.

Table 2. Yield of Glyphosate-Resistant Soybean in 2006 and 2007 As Influenced by Two Glyphosate Application Rates (1.12 and 3.36 kg of ae ha^{-1}) at 4 and 6 Weeks after Planting Soybean[a]

		soybean yield (kg ha^{-1})	
herbicide	rate (kg of ae ha^{-1})	2006	2007
hand-weeded		4636	4713
glyphosate	1.12	4706	4917
glyphosate	3.36	4767	4779
F test		NS[b]	NS

[a] Values are means of six replicates. Samples were taken 1 week after each application. [b] Not significant at 5% level.

used for isotopic analysis. Delta values were obtained using Isodat software version 2.38. The elemental combustion system was Costech ECS 4010 with an autosampler (Bremen, Germany).

Protein, Oil, and Fatty Acids Analysis. Seeds from each replicate were analyzed for protein, oil, and fatty acids by near-infrared (NIR) reflectance (*29*) using a diode array feed analyzer AD 7200 (Spring Field, IL). Calibrations were developed by the University of Minnesota, using Perten's Thermo Galactic Grams PLS IQ software (Spring Field, IL). To improve accuracy, the calibration curve was updated as needed for oil, fatty acids, and protein, using high performance liquid chromatography (HPLC). The analysis was performed on the basis of percent dry matter (*16*).

Statistical and Experimental Design. Treatments were arranged in a randomized complete block design with six replications. Only four replicates were used for NRA measurements. The data were subjected to analysis of variance using Proc GLM (*26*). Means were separated by Fisher's least significant difference (LSD) test at the 5% level of probability.

RESULTS AND DISCUSSION

Analysis of variance showed that there were significant effects of glyphosate application on NRA in leaves, roots, nodules, and seed composition (**Table 1**). There was no significant effect of glyphosate application on NRA in stems, yield (**Table 2**), ARA, total respiration, nodule mass, and nodule number (**Table 3**). Because there was no interaction between glyphosate application (treatment) and year for NRA in leaves, roots, nodules, and seed composition constituents (**Table 1**), results are presented as the mean of two years. Weather data (*30*) showed that year 2006 was warmer than 2007, and rainfall was more uniform in 2006. The experiments were irrigated, and rain should not be a source for differences in our measurable variables. Because statistical analysis showed that there were no glyphosate application × year interactions (**Table 1**), the ranking of measured variables did not change. Because temperature is an important factor in our measurable variables, especially in seed composition, temperature effect cannot be entirely excluded as a source of variability of year effect.

Table 3. Acetylene Reduction Activity (ARA), Respiration, Nodule Number, and Nodule Mass As Influenced by Two Glyphosate Application Rates (1.12 and 3.36 kg of ae ha^{-1}) at 4 and 6 Weeks after Planting (WAP) Soybean[a]

application timing (WAP)	glyphosate rate (kg of ae ha^{-1})	ARA (μmol of C_2H_4 $plant^{-1}$ h^{-1})	total respiration (mmol of CO_2 $plant^{-1}$)	nodules (number $plant^{-1}$)	nodule mass (g $plant^{-1}$)
4	0	12.2	1081	15.0	0.031
	1.12	11.0	1080	15.0	0.030
	3.36	12.3	1067	17.0	0.039
	F test	NS	NS	NS	NS
6	0	15.5	1106	21.8	0.029
	1.12	15.0	1157	25.0	0.034
	3.36	15.5	1115	23.0	0.039
	F test	NS	NS	NS	NS

[a] Samples were taken 1 week after each application. Values are means of four replicates for NRA and means of six. NS, not significant at 5%.

Yield, Nitrogen Fixation, and Nodulation Components. There was no significant effect of glyphosate application on yield (**Table 2**) or nitrogen fixation parameters (**Table 3**). Yield results are in agreement with those found by others (*8, 31, 32*) on glyphosate-resistant soybean or non-glyphosate-resistant soybean (*16*). Under high-yield environments (2000–4000 kg ha^{-1} seed yield), there was generally no effect of glyphosate on soybean yield compared with untreated plants under weed-free conditions (*31*). In our study, in both years chlorosis was observed in newly expanded leaves in soybean treated with the highest glyphosate application rate as previously reported (*32*). The chlorosis under our experiment conditions was apparent for several weeks after the second glyphosate application.

King et al. (*19*) concluded that it is unlikely that glyphosate has any long-term effects on N_2 fixation or processes that are critical for yield under their experimental conditions. In addition, Elmore et al. (*33*) showed that GR sister lines yielded 5% less than the non-GR sisters, but it was suggested that the decrease in yield was associated with the gene and its insertion rather than glyphosate. The conflicting results indicate that yield decrease due to glyphosate application has not been consistently demonstrated, although changes in nitrogen content were observed (*20*).

The analysis of variance showed that there was no effect of glyphosate treatment on parameters of nitrogen fixation such as ARA, nodule number or mass, or total root respiration (**Table 3**). In glyphosate-sensitive soybean, ARA, nodule number, and nodule mass were reduced under simulated glyphosate drift at a rate of 12.5% of use rate of 0.84 kg of ae ha^{-1} at V2 and V7, although a recovery of ARA and NRA took place at a later stage of soybean development (*16*). A small reduction in N_2 fixation potential may have long-term effects on sustainable soil nitrogen pools, considering the widespread adoption of the GR soybean system. It should be noted that the nodule mass observed in the current study is about 50% lower than that of other field studies at this location (*16, 20*). As there was no effect of glyphosate application on root respiration there was most likely no reduction in photosynthate translocation to the roots. In addition, ARA determined at 6 and 8 WAP is also lower than previously reported (*16, 20*). Reddy et al. (*34*)

Table 4. Actual Nitrate Reductase Activity (ANRA) and Potential Nitrate Reductase Activity (PNRA) in Leaves, Stems, and Roots As Influenced by Two Glyphosate Application Rates at 4 and 6 Weeks after Planting (WAP) Soybean[a]

application timing (WAP)	glyphosate rate (kg of ae ha^{-1})	plant part	ANRA (μmol of nitrite g^{-1} of FWT h^{-1})	PNRA (μmol of nitrite g^{-1} of FWT h^{-1})	NRA (μmol of nitrite $part^{-1}$ of FWT h^{-1})
4	0	leaf C	5.93 a	7.19 a	224 a
	1.12	leaf T1	4.3 b	6.14 b	166 b
	3.36	leaf T2	3.19 c	5.45 c	111 c
	0	stem C	3.8 a	4.39 a	48 a
	1.12	stem T1	3.69 ab	4.58 a	46 a
	3.36	stem T2	3.55 b	4.16 b	43 a
	0	root C	3.93 a	4.59 a	45 a
	1.12	root T1	2.64 b	4.46 a	30 b
	3.36	root T2	2.33 c	3.90 b	27 b
	0	nodule C	3.7 a	4.4 b	2.00 a
	1.12	nodule T1	2.0 b	4.2 a	1.35 b
	3.36	nodule T2	1.1 c	3.0 c	1.65 b
6	0	leaf C	5.79 a	6.53 a	216 a
	1.12	leaf T1	4.80 b	5.65 b	186 b
	3.36	leaf T2	3.26 c	6.36 a	126 c
	0	stem C	4.26 a	5.09 a	50 a
	1.12	stem T1	3.76 b	4.15 b	44 a
	3.36	stem T2	3.76 b	4.00 b	44 a
	0	root C	3.36 c	4.60 b	38 b
	1.12	root T1	3.85 b	4.40 b	42 b
	3.36	root T2	5.66 a	5.61 a	63 a
	0	nodule C	3.7 a	4.6 b	1.90 a
	1.12	nodule T1	1.5 c	2.9 a	0.80 b
	3.36	nodule T2	2.7 b	3.6 c	0.75 b

[a] Samples were taken after 1 week after each application. Means within a column for each plant part and for each application separately followed by the same letter are not significantly different at the 5% level as determined by Fisher's LSD test. Values are means of four replicates. C, control; T1, 1.12 kg of ae ha^{-1}; T2, 3.36 kg of ae ha^{-1} .

showed that a single foliar application of a sublethal treatment (0.21 kg of ae ha^{-1} glyphosate) to glyphosate-sensitive soybean reduced nodule number by 32%, and nodule mass accumulation by 75%, 2 weeks after treatment. In GR soybean Reddy et al. (*34*) found that early glyphosate application of 2.24 kg of ae ha^{-1} reduced nodule number by 30%, nodule mass by 39%, and total nitrogen content of shoots by 14%.

Previous research by Moorman et al. (*35*) found that there was a growth inhibition of *B. japonicum* in a culture supplied with a glyphosate concentration that is likely a concentration found in the roots and nodules of glyphosate-treated plants (*36*). Other experiments showed that the effect of glyphosate application on biomass and nitrogen content depends on the availability of soil nitrogen. It was shown that early application to soybean of glyphosate decreased biomass and N content by 20−47% by 19 days after emergence when soybean was grown in the presence of soil N. When soil nitrogen was absent, glyphosate did not decrease the biomass or N content at 19 days after emergence (*19*).

NRA in Leaves, Stems, Roots, and Nodules. One week after the first glyphosate application with 1.12 kg of ae ha^{-1} a significant reduction of ANRA was observed in leaves, roots, and nodules compared with the nontreated plants (**Table 4**). The same observation was recorded on a plant part basis (μmol of nitrite $part^{-1}$ h^{-1}) and whole plant basis (μmol of nitrite $plant^{-1}$ h^{-1}). NRA in stems was relatively constant between treatments. Adding exogenous nitrate into the buffer solution to measure potential nitrate reductase activity (PNRA) in leaves, stems, roots, and nodules resulted in a significant increase in the enzyme activity in all plant parts of treated and nontreated plants. The increase in PNRA ranged from 12 to 95% in leaves, from 6 to 24% in stems, and from 14 to 68% in roots and was over 100% in nodules. These ranges depend on glyphosate application rate and stage of application. At a glyphosate application rate of 3.36 kg of ae ha^{-1} and 1 week after the glyphosate application, ANRA showed a significant decrease in all plant parts compared with untreated plants. Adding exogenous nitrate to the buffer solution increased PNRA in leaves, stems, roots, and nodules, indicating that the NR did not reach its full potential activity in the cell.

The second glyphosate application of 1.12 kg of ae ha^{-1} caused a significant decrease in ANRA in leaves, roots, and nodules, but the decrease was not as great as found at the first harvest (**Table 4**). This may be because the plants became less sensitive to glyphosate because of previous exposure to glyphosate from the first application or exposure of plants to glyphosate occurred at a later stage of development when there was more biomass for dilution of intercepted glyphosate. All parts showed a higher PNRA, indicating NR was present in active form, but the substrate nitrate was probably a limiting factor for NRA. Glyphosate application at 3.36 kg of ae ha^{-1} resulted in a significant decrease in NRA in leaves and nodules, but a significant increase in NRA in root (**Table 4**). Zabalza et al. (*22*) showed that when imazethapyr (a branched amino acid inhibitor) was applied to soybean, nitrate uptake by roots was drastically reduced. This reduction in nitrate uptake was associated with a decrease in nitrate translocation to the shoots and actual NRA of shoots and roots. Omokaro and Ajakaiye (*24*), working on cowpea, found that the application of metobromuron herbicide at 0.125 kg ha^{-1} increased nitrate concen-

tration in leaves and decreased NRA, but increased protein by 29% compared to the control in 60D cultivar. However, 0.625 kg of metobromuron ha^{-1} resulted in a 52.5% increase in NRA throughout the growth period. Our results support those reported by Zabalza et al. (*22*) in that NRA inhibition in leaves could be due to reduced or limited nitrate uptake by roots and nitrate translocation to shoot.

Adding exogenous nitrate resulted in an increased NRA in leaves and nodules, but there was no response of NRA in roots. This may be explained by the fact that root NRA increased at full potential under higher glyphosate application to compensate for the previous NRA reduction in roots and leaves that was caused by the previous exposure of the plants to glyphosate at the first glyphosate application. In addition, root tissue would more likely be associated with a higher nitrate concentration, whereas any nitrate would be diluted due to the amount of biomass in other tissue. The increase of root NRA to full potential under high rate of glyphosate application may be a response to the reduction of NRA in leaves and nodules that occurred at first glyphosate application. Although the first exposure of roots to glyphosate application decreased root NRA, glyphosate may have given the root adaptability against a higher glyphosate application. Because there was a need for reduced nitrogen for leaves reflected by noticeable decrease of leaves NRA, root NR used all available NR molecules for nitrate reduction and worked at full rate to satisfy growth and development needs for the rest of the plant parts, especially leaves. Also, it is interesting to note that at later stages, glyphosate application inhibited the actual NRA but did not affect the de novo synthesis of NR because all parts showed a higher PNRA. Recently, Bellaloui et al. (*16*) showed that exposure of nonresistant soybean to glyphosate drift caused a significant reduction in both NRA and ARA at early stages of soybean development. It appears that ARA in GR soybean is less sensitive to glyphosate application than ARA in sensitive glyphosate soybean. Glyphosate application at higher rates severely inhibited NRA, especially in leaves, roots, and nodules. Previous work showed that although N_2 fixation in soybean is more sensitive to water deficit than other processes such as gas exchange (*37*), transpiration (*38*), and uptake and assimilation of inorganic soil N (*39*), nitrate assimilation could be more sensitive than N_2 fixation under higher rates of glyphosate applications.

King et al. (*19*) found that early applications of glyphosate delayed N_2 fixation and decreased biomass and N accumulation in the cultivar Terral TV5866RR harvested at 19 days after emergence. However, plants had recovered by 40 days after emergence. It was suggested that dinitrogen fixation may not be the primary physiological process that is affected by glyphosate application (*19*). It was reported that a decrease in flux of carbon to the nodule causes a decline in nitrogen fixation (*40*). De Maria et al. (*9*) reported that glyphosate may cause the alteration of nodular metabolism by limitation of carbohydrates from shoots as substrates for bacteroids. Under carbohydrate limitation, bacteroid respiration and incorporation of fixed nitrogen will be inhibited. In lupin nodules (*9*) glyphosate treatments decreased nitrogenase activity in 24 h after application at sublethal dose (1.25 mM). Increasing glyphosate concentrations (5 mM) and time after exposure (5 days) decreased nodule starch content and sucrose synthase activity but increased sucrose content within the nodule. These effects were accompanied by a great inhibition of the activity of phosphoenolpyruvate carboxylase. It was shown that the inhibition effect of glyphosate on 5-enolpyruvylshikimic-3-phosphate synthase enzyme (EPSPS; EC 2.5.1.19) would divert most phosphoenolpyruvate into the shikimate pathway, depriving bacteroids of energy substrates to maintain nitrogen fixation.

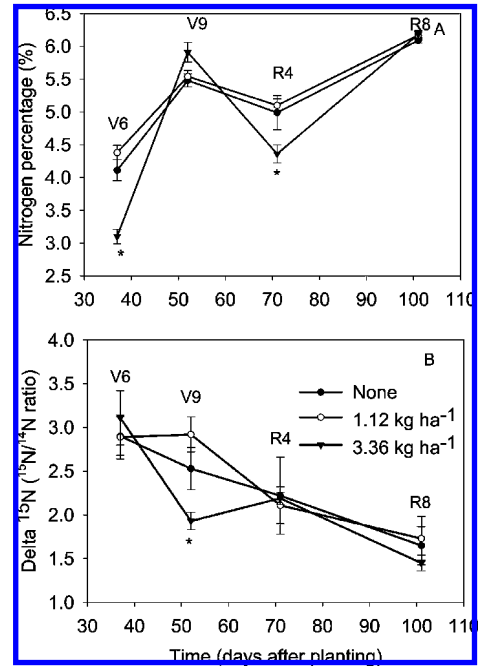

Figure 1. Effect of glyphosate application on nitrogen content (percent) (**A**) and delta ^{15}N ($^{15}N/^{14}N$ ratio) (**B**) of soybean at four stages of ontogeny, in 2006. Nitrogen percent and delta ^{15}N were measured in leaf at 37 (V6), 52 (V9), and 71 (R4) days after planting and in seed at 101 (R8) days after planting. Glyphosate treatments were 0, 1.12, and 3.36 kg of ae ha^{-1} applied at 28 and 42 days after planting. Samples were taken 1 week after each application. $*$ indicates that there was a significant difference between treatments at the time of sampling.

Nitrogen Content and Nitrogen Fixation Using ^{15}N Natural Abundance Method. ARA is an instantaneous estimation of N_2 fixation that allows the comparison of different treatments at a given time, but any extrapolation to the estimate of fixation over a growing season is delicate because environmental conditions differ within and between days (*41*). Therefore, nitrogen fixation using ^{15}N natural abundance method remains accurate and appropriate for estimating nitrogen fixation on the basis of isotopic discrimination of the nitrogenase enzyme (*28*). The natural abundance method would be more applicable to characterizing fixation versus uptake, especially during a full growing season and under field conditions (*42*). This method is based on the discrimination of the fertilizer nitrogen and atmospheric nitrogen, and we used this method to test if there is any change in N assimilation versus fixation based on the ratio between $^{15}N/^{14}N$ under glyphosate treatment.

Analysis of leaf material sampled indicated that soybean treated with the highest glyphosate rate had ~24% reduction in N at V6 and 12% reduction in N at R4 stage of development compared to the nontreated soybean (**Figure 1A**). At the V9 stage there was ~8% greater N in the higher glyphosate rate compared to the nontreated control. At maturity (R8), there was no difference among treatments in seed N. Symptoms of chlorosis in soybean have been described following glyphosate application (*43*). It has been demonstrated that the chlorosis is caused by aminomethylphosphonic acid, a metabolite of glyphosate (*43*). Data from the current study suggest that altered patterns of nitrogen assimilation, for example, inhibition of nitrate reductase and lower leaf N content, may also have caused

Table 5. Percentage of Protein, Oil, and Oleic and Linolenic Acids (as Percentage of Total Oil) As Influenced by Two Glyphosate Application Rates at 4 and 6 Weeks after Planting Soybean[a]

glyphosate rate (kg of ae ha^{-1})	protein (%)	oil (%)	oleic (%)	linolenic (%)
0	40.8 b	23.2 a	25.5 c	8.7 a
1.12	41.0 b	23.0 a	25.9 b	8.6 a
3.36	45.1 a	20.8 b	31.1 a	6.4 b

[a] Means within a column followed by the same letter are not significantly different at the 5% level as determined by Fisher's LSD test. Values are means of six replicates.

chlorosis. Whether aminomethylphosphonic acid affects nitrogen assimilation, especially nitrate reductase, needs further investigation.

On the basis of delta ^{15}N results, similar values were observed for all treatments, in three of the four harvests (V6, R4, and R8). However, at the second vegetative harvest a lower delta ^{15}N was observed for the highest glyphosate rate, indicating an alteration for increased N_2 fixation (**Figure 1B**). These data suggest that temporal changes in nitrogen nutrition were observed in response to the highest glyphosate application, with lower net N availability at two stages of development. Patterns of delta ^{15}N indicate a continuous decrease with time, indicating that N_2 fixation played a greater role in nitrogen nutrition as the soybean matured, as reported by others (*44*). The lower nitrogen content of leaves during the early vegetative harvest (V6) and early reproductive growth (R4) in the high glyphosate application treatment is in agreement with a lower nitrate assimilation due to reduced NRA. The significantly lower delta ^{15}N observed at the V9 does indicate that perhaps N_2 fixation was a greater contributor to total nitrogen assimilated compared to either the control or 1× glyphosate application treatment.

Application of either glyphosate rate did not significantly affect the delta ^{15}N values of soybean seed, and the delta ^{15}N values (~1.5) were low regardless of treatment, indicating that nitrogen reduced by nitrogenase was the main source of nitrogen (**Figure 1B**). In another experiment (data not shown) it was found that growing soybean under water stress resulted in a lower ^{15}N/^{14}N ratio, suggesting more ^{14}N was used in nitrogen fixation and less nitrate from soil compared with soybean grown under irrigation (*45*). The interrelationships between ^{15}N and ^{14}N under environmental or chemical stresses and how these stresses affect the ^{15}N/^{14}N ratio are not yet understood. Understanding these changes under environmental stresses will provide soybean breeders with information for germplasm development for higher nitrogen metabolism efficiency. Although reductions in NRA and available nitrate were observed during the first 8 weeks after planting, the soybean more than likely recovered in its ability to assimilate nitrogen and produce a substantial soybean yield. Studies by Zablotowicz and Reddy (*20*) have shown that soybean treated with high glyphosate application rates used in these studies reduced nitrogen content in two of the three years studied; however, a different soybean genotype AG4702RR was used in the present study. The reduction in seed nitrogen was hypothesized to be due to glyphosate application effects on nitrogen assimilation.

Protein, Oil, and Fatty Acids. Analysis of variance showed that glyphosate application had the main effect on seed composition constituents (**Table 1**). Application of glyphosate at a rate of 1.12 kg of ae ha^{-1} did not change protein or oil percentages (**Table 5**). However, glyphosate application at rate of 3.36 kg of ae ha^{-1} resulted in a higher protein percentage and lower oil percentage compared with the nontreated soybean. There was a 10% increase in protein content despite a similar nitrogen content among treatments. Saturated fatty acids, palmitic and stearic acids, were stable under both rates of glyphosate (data not shown). Unsaturated fatty acids, oleic and linolenic acids, showed significant change, an increase in oleic acid percentage and a decrease in linolenic acid percentage (**Table 5**). Our previous work showed that application of 0.11 kg of ae ha^{-1} to non-glyphosate-resistant soybean did not show significant differences in oil and protein (*16*). The increase of protein under higher glyphosate application may be due to altered patterns of nitrogen repartitioning for seed fill in this treatments. Although total seed nitrogen did not show differences between treated and nontreated plants, altered nitrate translocation and assimilation due to glyphosate may have resulted in protein differences between treated and nontreated plants. Not all nitrogen in seed is associated with protein, as N is also a component of other constituents, for example, nucleic acids, and certain secondary metabolites. Intrinsic differences between species in the proportion of nitrate supply and nitrate assimilated in roots and translocated to the shoots exist, as nitrate is reduced in both leaves and roots of all plants (*16*). Because the main site of nitrogen assimilation in soybean is leaves (*4, 16*), translocation of nitrate from root to stem and leaves may significantly influence protein production. It is well established that nitrogen assimilation can be affected by the rate of translocation not only between shoot and root but also by the translocation of nitrate from tonoplast (nitrate cell vacuole) to accessible/available cytoplasmic nitrate where nitrogen assimilation takes place. This situation can exist under stress factors such as exposure to glyphosate and other herbicides. In cowpea, application of pendimethalin increased leaf nitrate concentration and leaf protein (*24*), but did not influence seed protein. However, the herbicide metobromuron increased leaf nitrate concentration, decreased NRA, but increased seed protein by 29.6% at 0.125 kg ha^{-1} in 60D cultivar (*24*). Metolachlor and prometryne herbicides were most inhibitory to seed protein development (*24*). The impairment of the translocation of vegetative protein to developing seed was indicated by the negative correlation between leaf crude protein and seed protein. It can be concluded from the above discussion that the increase in protein in seed may not always be necessarily associated with higher nitrate concentration in leaf tissues, but it is related to reduction of nitrogen and translocation of vegetative protein to seed protein.

The increase in oleic acid and decrease in linolenic acids could be due to the stress response of soybean to glyphosate application or the indirect physiological disturbances that affected oleic and linolenic acid pathways. This may suggest a possible alteration to carbon metabolism caused by glyphosate application. Previous studies showed that glyphosate application decreased chlorophyll content and inhibited photosynthesis and carbon substrate availability in glyphosate-sensitive soybean (*21*). Other research has shown an alteration in phosphoenolpyruvate (*9*). The reduction in photosynthesis and carbon substrate may have affected the production of oleic and linolenic acids.

This study showed that glyphosate application affects nitrogen assimilation but not nitrogen fixation, suggesting nitrogen assimilation may be more sensitive to glyphosate application at the vegetative and early reproductive stages where nitrate assimilation is highest (*44*). Also, a high rate of glyphosate application caused temporal changes in nitrogen dynamics, and patterns of delta ^{15}N indicated a continuous decrease with time, suggesting that N_2 fixation played a greater role in nitrogen nutrition. Glyphosate application did not affect yield, but resulted

in significant differences in seed composition, especially protein and unsaturated fatty acids, suggesting carbon metabolism alteration.

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