JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simulated Glyphosate Drift Influences Nitrate Assimilation and Nitrogen Fixation in Non-glyphosate-Resistant Soybean

NACER BELLALOUI,*,† KRISHNA N. REDDY,‡ ROBERT M. ZABLOTOWICZ,‡ AND ALEMU MENGISTU§

Crop Genetics and Production Research Unit and Southern Weed Science Research Unit, Agricultural Research Service, U.S. Department of Agriculture, 141 Experiment Station Road, Stoneville, Mississippi 38776; and Crop Genetics and Production Research Unit, Agricultural Research Service, U.S. Department of Agriculture, 605 Airway Boulevard, Jackson, Tennessee 38301

Nontarget injury from glyphosate drift is a concern among growers using non-glyphosate-resistant (non-GR) cultivars. The effects of glyphosate drift on nitrate assimilation and nitrogen fixation potential, nodule mass, and yield of non-GR soybean were assessed in a field trial at Stoneville, MS. A non-GR soybean cultivar 'Delta Pine 4748S' was treated with glyphosate at 12.5% of use rate of 0.84 kg of active ingredient/ha at 3 (V2), 6 (V7), and 8 (R2, full bloom) weeks after planting (WAP) soybean to simulate glyphosate drift. Untreated soybean was used as a control. Soybeans were sampled weekly for 2 weeks after each glyphosate treatment to assess nitrate assimilation and N_2 fixation potential. Nitrate assimilation was assessed using in vivo nitrate reductase assay in leaves, stems, roots, and nodules. Nitrogen fixation potential was assessed by measuring nitrogenase activity using the acetylene reduction assay (ARA). Nitrogen content of leaves, shoots, and seed and soybean yield were also determined. In the first sampling date (4 WAP), glyphosate drift caused a significant decrease in NRA in leaves (60%), stems (77%), and nodules (50%), with no decrease in roots. At later growth stages, NRA in leaves was more sensitive to glyphosate drift than stems and roots. Nitrogenase activity was reduced 36−58% by glyphosate treatment at 3 or 6 WAP. However, glyphosate treatment at 8 WAP had no effect on nitrogenase activity. Nitrogen content was affected by glyphosate application only in shoots after the first application. No yield, seed nitrogen, protein, or oil concentration differences were detected. These results suggest that nitrate assimilation and nitrogen fixation potential were significantly reduced by glyphosate drift, with the greatest sensitivity early in vegetative growth. Soybean has the ability to recover from the physiological stress caused by glyphosate drift.

KEYWORDS: Acetylene reduction assay; glyphosate; nitrate assimilation; nitrate reductase activity; nitrogen fixation; nitrogenase activity

INTRODUCTION

In 2005, 87% of soybean hectareage was planted to glyphosate-resistant cultivars in the United States (*1*). Ten years after the introduction of glyphosate-resistant soybean cultivars, ~13% of soybean area is still planted to conventional (non-glyphosate-resistant, non-GR) cultivars. Spray drift is common when herbicides are applied under windy conditions and environmental conditions that favor volatilization and redisposition (*2*). Wet fields can delay timely glyphosate application, and aerial applications under these conditions can increase potential damage to off-target crops by glyphosate drift. Furthermore, the frequency of glyphosate application has increased with the adoption of glyphosate-resistant cotton and glyphosate-resistant corn.

Off-target movement of herbicides during application can range from 0.01 to 10% of the applied rate (*3*, *4*). Although these drift rates appear to be sublethal, the injury can be severe in susceptible crops, depending on the growth stage. Previous research has shown that simulated drift of 0.8−12.5% of the usage rate of 1.12 kg of active ingredient (ai)/ha glyphosate has injured soybean; however, yields were not affected (*5*).

Glyphosate application has decreased chlorophyll content, nodule biomass, and leghemoglobin content of soybean (*6*) and nitrogen fixation, accumulation, and nodulation (*6*, *7*). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme in the shikimate pathway, and thus blocks aromatic amino acid biosynthesis (*8*). The soybean nitrogen fixing symbiont, *Bradyrhizobium japonicum*, possesses

* Corresponding author [telephone (662) 686-2531; fax (662) 686-5218; e-mail nbellaloui@msa-stoneville.ars.usda.gov].
† Crop Genetics and Production Research Unit, Stoneville, MS.
‡ Southern Weed Science Research Unit.
§ Crop Genetics and Production Research Unit, Jackson, TN.

10.1021/jf053198l
Published on Web 04/01/2006

a glyphosate-sensitive EPSPS enzyme, and exposure to glyphosate may interfere with N_2 fixation. Soybean having a symbiotic association with nitrogen fixing *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 (*9*). For the nitrate to be used by plants, it has to be reduced to nitrite by nitrate reductase, the key enzyme that catalyzes the first step in nitrate assimilation, leading to nitrite production. The enzyme is substrate inducible (*10*) and requires NADH or NADPH reductant (*11*). Nitrate reductase enzyme is found in both roots and shoots of plants, but the proportion of nitrate taken up that is subsequently reduced to its nitrite form varies between these plant parts (*12*, *13*). On the other hand, atmospheric N_2 is fixed by the enzyme nitrogenase in the bacteroids of nodules (*14*), and both nitrate reductase and nitrogenase enzymes coexist in nodules competing for reductant (*15*).

Because there is a lack of information on the effect of glyphosate drift on physiological disturbances on non-GR soybean, the objective of this study was to investigate the effect of glyphosate drift on nitrate assimilation and nitrogen fixation at different developmental stages of non-GR soybean. Nitrate assimilation was assessed by measuring nitrate reductase activity (NRA) in leaves, stems, roots, total plant, and nodules. Nitrogen fixation was investigated using the acetylene reduction assay (ARA). The effect of glyphosate on growth was evaluated by determining root, shoot, and nodule biomass and root respiration. Nitrogen content in leaves and shoots and yield and seed composition of non-GR soybean were assessed under weed-free conditions.

MATERIALS AND METHODS

Growth Conditions. A field study was conducted in 2005 at the USDA-ARS Southern Weed Science Research Unit farm, Stoneville, MS. The soil was a Dundee silt loam (fine-silty, mixed, thermic Aeric Ochraqualf) with pH 6.8, 1.2% organic matter, a cation exchange capacity of 21 cmol/kg, and soil textural fractions of 23% sand, 51% silt, and 26% 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 2004. The experimental area was under glyphosate-resistant soybean production in 2004. A non-GR soybean cultivar ('Delta Pine 4748S') was planted at a rate of 355 000 seeds/ha on April 18, 2005. Corn was grown on all four sides of the experimental area as border to minimize spray drift from periodic glyphosate applications in neighboring fields. Corn was planted on March 31, 2005, and was harvested on August 22, 2005. A single application of glyphosate at 12.5% of use rate of 0.84 kg of ai/ha was applied at 3 (V2, first trifoliate), 6 (V7, sixth trifoliate), and 8 (R2, flower at node immediately below the uppermost node with completely unrolled leaf) (*32*) weeks after planting (WAP) soybean to simulate glyphosate drift. T1, T2, and T3 refer to glyphosate treatments at 3, 6, and 8 WAP, respectively. For comparison purposes, a no-glyphosate control was included. Therefore, there are four different treatments (T1, T2, T3, and control, respectively, refer to glyphosate application at 3, 6, and 8 WAP and no glyphosate). At any given sampling date, plant material was taken from each treatment. For example, at the 10 WAP sampling date, we took samples from T1, T2, T3 (10 WAP), and the no-glyphosate control. Metolachlor at 2.30 kg of ai/ha plus flumetsulam at 0.07 kg of ai/ha plus paraquat at 1.12 kg of ai/ha were applied to entire experimental area immediately after planting. Paraquat was applied to kill existing vegetation, and metolachlor and flumetsulam were applied to provide residual weed control. Herbicide treatments were applied with a tractor-mounted sprayer with TeeJet 8004 standard flat spray tips delivering 187 L of water/ha at 179 kPa. The commercial formulation of glyphosate was used with no additional adjuvant (Roundup Weathermax, Monsanto Agricultural Co., St. Louis, MO).

Soybean was grown nonirrigated for at least 5 WAP and was irrigated thereafter as needed because of late-season dry weather. Each treatment consisted of four soybean rows spaced 102 cm apart and 12.2 m long. All plots including glyphosate-treated were hand weeded periodically throughout the season to keep weed-free. Soybean was harvested from each plot using a combine on September 6, 2005, and grain yield was adjusted to 13% moisture.

Nitrate Reductase Assay. Four to six soybean plants were randomly sampled from the middle two rows of each plot approximately 4, 5, 7, 8, 9, and 10 WAP. These sampling dates correspond to about 1 and 2 weeks following 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 (*16*). 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. The concentration of nitrite was calculated from a standard calibration curve.

NRA was measured in leaves, stems, and roots. The youngest fully expanded leaf was used to measure NRA in leaves. To measure NRA in roots, only non-noduled 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. To determine potential NRA (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 and Root Respiration. Soybean plants (10−15) were randomly sampled from the middle two rows of each plot approximately 4, 5, 7, 8, 9, and 10 WAP. These sampling dates correspond to about 1 and 2 weeks following 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 as described elsewhere (*17*, *18*). Roots with nodules intact were excised and incubated in 60 mL plastic syringes (4 and 5 WAP, three replicates per block) or 1 L Mason jars (7, 8, 9, and 10 WAP, two replicates per block). Two roots were placed in the syringes and six roots in the Mason jars and sealed. A 10% volume of air was then removed and replaced with an equal volume of acetylene. After 1 h of incubation at room temperature, duplicate 1.0 mL gas samples were removed and analyzed by gas chromatography for ethylene formation and carbon dioxide evolution. An Agilent HP6960 (Agilent Technologies, Wilmington, DE) gas chromatograph was equipped with manual injector, injector loop, and sample splitter. A flame ionization detector (FID) and a thermal conductivity detector (TCD) were used. Using the sample loop and splitter, 0.25 mL of gas was directed into a 30 m length × 0.53 mm i.d. alumina megabore column (115-3532) connected to the FID, and 0.25 mL of sample was injected into a HP-PLOT D column (30 m length × 0.53 mm i.d. megabore with 40 μm film; 1905D-Q04) connected to the TCD using helium as a carrier gas. Chromatographs were integrated using Chem Station software. Standard curves for ethylene and carbon dioxide were developed for each day of analysis and used to determine ethylene and carbon dioxide evolved. Samples having <9% acetylene were not used in the analysis. 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 4−5 days.

Total Nitrogen. At harvest, ~200 soybean pods were randomly hand sampled from the middle two rows for seed nitrogen determination. For the first two samples at 4 and 5 WAP, nitrogen content was determined on total shoot biomass. At 7, 8, 9, and 10 WAP, nitrogen analysis was conducted on the youngest fully expanded trifoliate leaf (composite of 24 leaves per plot). The shoots, leaf, and seed samples were oven-dried (60 °C) and then finely ground twice in a Wiley mill

Table 1. Glyphosate Simulated Drift Effects on Root and Shoot Biomass Accumulation in Non-glyphosate-Resistant Soybean[a]

treatment	when applied (WAP)[b]	root dry wt (g/plant)						shoot dry wt (g/plant)					
		4 WAP	5 WAP	7 WAP	8 WAP	9 WAP	10 WAP	4 WAP	5 WAP	7 WAP	8 WAP	9 WAP	10 WAP
no glyphosate		0.15 a	0.29 a	0.67 a	1.13 a	1.58 a	2.96 a	0.66 a	1.38 a	4.90 a	8.01 a	19.9 a	24.8 a
glyphosate	3	0.17 a	0.24 b	0.55 a	0.94 ab	1.21 b	2.03 b	0.51 a	1.37 a	4.68 a	7.41 a	18.6 a	17.3 b
glyphosate	6			0.59 a	0.89 b	1.36 ab	1.84 b			4.87 a	7.10 a	18.6 a	16.2 b
glyphosate	8					1.18 b	1.70 b					17.2 a	15.6 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. [b] WAP, weeks after planting soybean.

Table 2. Glyphosate Simulated Drift Effects on Nodule Biomass in Non-glyphosate-Resistant Soybean[a]

treatment	when applied (WAP)[b]	soybean nodule dry wt (mg/plant)					
		4 WAP	5 WAP	7 WAP	8 WAP	9 WAP	10 WAP
no glyphosate		14.4 a	70 a	68 a	90 a	111 a	51 c
glyphosate	3	11.4 b	78 a	69 a	58 b	105 a	93 bc
glyphosate	6			63 a	54 b	90 a	108 ab
glyphosate	8					92 a	158 a

[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. [b] WAP, weeks after planting soybean.

(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 or seed weight. Total seed nitrogen (kilograms) per hectare was calculated as the product of yield and nitrogen content.

Oil and Protein Analysis. Seeds from each replicate were analyzed for oil and protein, using near-infrared (NIR) reflectance (diode array feed analyzer, Perten). Calibrations were developed by Perten using Thermo Galactic Grams PLS IQ. The calibration curve has been updated for unique samples, using HPLC. The analysis was performed on the basis of percent dry matter.

Statistical and Experimental Design. Treatments were arranged in a randomized complete block design with four replications. The data were subjected to analysis of variance using Proc GLM (*19*). Means were separated by Fisher's least significant difference test at the 5% level of probability.

RESULTS AND DISCUSSION

Soybean Injury. Soybean was injured from glyphosate applied at all growth stages. The visible injury symptoms (e.g., chlorosis, necrosis) were observed usually on young leaves following glyphosate application (data not shown). Soybean injury decreased over time, and soybean completely recovered from injury within 14 days after treatment, similar to that reported by Reddy and Zablotowicz (*20*).

Root, Shoot, and Nodule Mass. The effects of simulated glyphosate drift on soybean root and shoot biomass accumulation are shown in **Table 1**, and nodule biomass accumulation is presented in **Table 2**. Temporal and inconsistent effects of glyphosate on all components of soybean biomass were observed in this study. At early stages of plant ontogeny no effect of glyphosate was observed on shoot or root biomass. After 8 WAP, significant reductions in root biomass were observed in response to several glyphosate treatments. However, a significant reduction in shoot biomass was observed in all glyphosate-treated plants only at 10 WAP. Although the effect of glyphosate on growth is still a matter of debate, our study showed that both root and shoot biomass in control plants showed a higher biomass accumulation compared to the all-glyphosate-treated soybean at 10 WAP. Significant reduction of nodule dry weight was noticed 4 and 8 WAP. At 4 WAP sampling, the nodule mass of glyphosate-treated plants was reduced by 20% compared with the nodule mass of untreated plants. At 8 WAP sampling, glyphosate treatments at both 3 or 6 WAP had reduced nodule mass by 35−40% compared to untreated soybean. At 10 WAP sampling, the untreated control had the lowest nodule mass compared to the three glyphosate treatments. Control plants sampled at 10 WAP were lacking crown nodulation; at this stage of ontogeny some of the early-formed nodules would have been senesced and likely sloughed off during excavation. Nodule mass in treated plants recovered by 10 WAP, indicating that nodule growth is more sensitive to glyphosate drift at early stages.

Nitrate Reductase Activity. Nitrate reductase activity was significantly lower in leaves, stems, roots, and the whole plant in glyphosate-treated soybean compared to non-glyphosate-treated soybean. The influence of glyphosate was severe at early developmental stages of soybean plants. NRA recovered gradually toward later stages of plant development. The NRA (micromoles of nitrite per gram per hour) in leaves and stems of the treated plants was significantly lower than those of the nontreated plants at 3.4 and 4 WAP (**Figure 1A**,**B**). The decreases in NRA of leaves and stems were 68 and 42%, respectively, at 3.4 WAP, and 60 and 77%, respectively, at 4 WAP. No NRA decrease was noticed in roots. This indicates that, although leaves were the major site for nitrate reduction, leaves were more sensitive to glyphosate drift than roots. It appears that, under glyphosate drift conditions, soybean roots at early stages of ontogeny were more important for nitrate reduction than leaves and stems. This may reflect the ability of roots to compensate for the decrease in nitrate reduction that occurred in leaves and stem and caused by exposure to glyphosate. The same trend was followed by NRA in plant parts (micromoles of nitrite per part per hour) (**Figure 2A**,**B**) and NRA in the whole plant (micromoles of nitrite per plant per hour). For example, at 3.4 WAP, NRA (micromoles of nitrite per plant per hour) was 8.4 in untreated plants compared to 6.6 in treated plants. The same trend was observed at 5 WAP, when NRA in untreated plants was 27.3 compared to the 22.6 that was recorded in treated plants. Nitrate reductase activity (micromoles of nitrite per plant per hour) recovered toward later stages of plant development (data not shown). There is

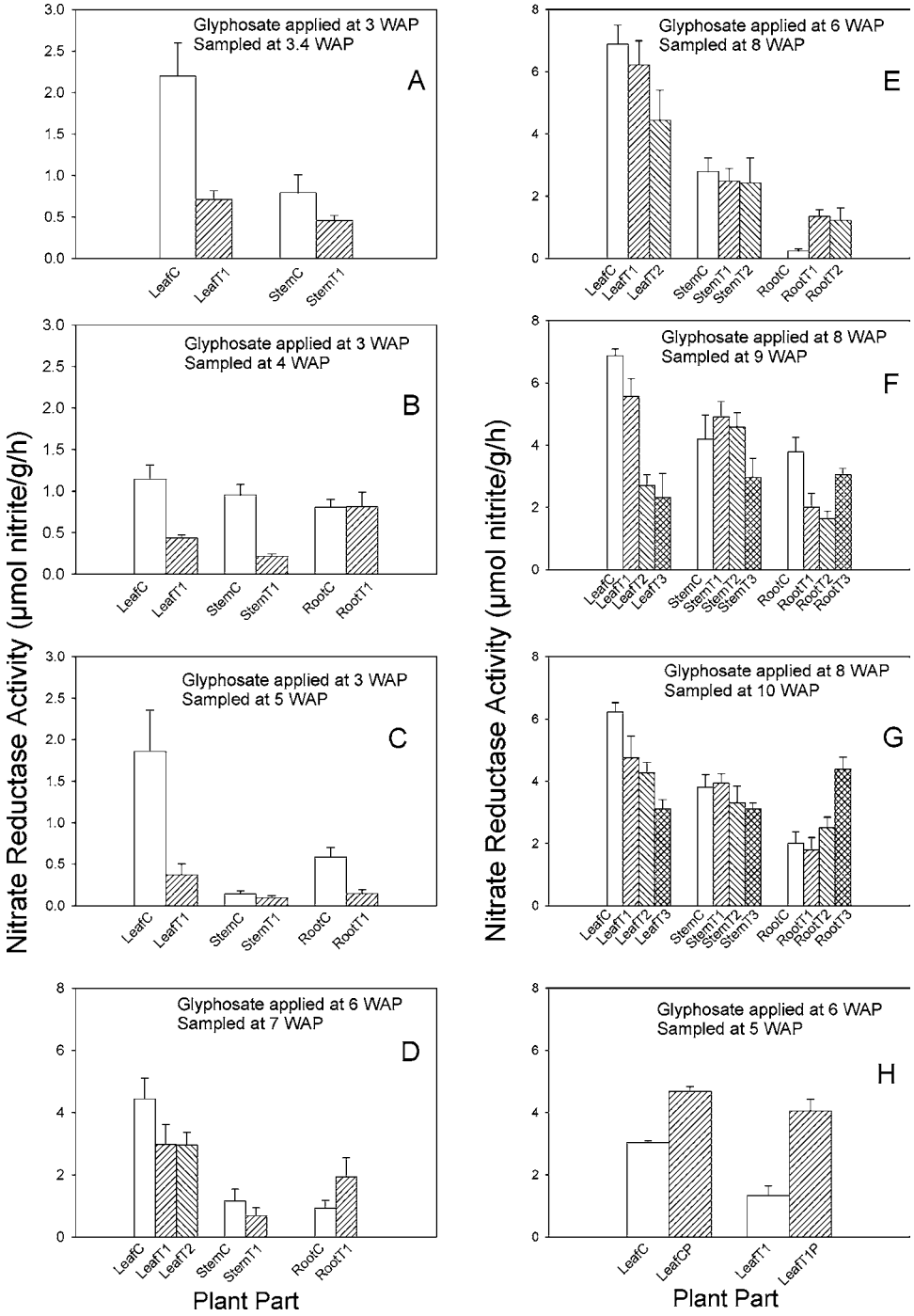

Figure 1. Effect of simulated glyphosate drift on nitrate reductase activity (micromoles of nitrite per gram per hour) in the youngest fully expanded leaf, stems, and roots in the control (LeafC, StemC, and RootC) and treatments (LeafT1, LeafT2, LeafT3,StemT1, StemT2, StemT3, RootT1, RootsT2, and RootsT3). Sampling dates were 3.4 WAP (**A**), 4 WAP (**B**), 5 WAP (**C**), 7 WAP (**D**), 8 WAP (**E**), 9 WAP (**F**), and 10 WAP (**G**). NRA (LeafC and LeafT1) and potential NRA (LeafCP and LeafT1P) were measured at 5 WAP in the youngest fully expanded leaf (**H**). Bars represent mean ± SE.

consistency in the effect of glyphosate on nitrate reduction at early plant stage. A decrease in NRA in roots of treated plants occurred only at 5 WAP (**Figure 1C**), and this is consistent with the previous observation that roots are less sensitive to glyphosate drift compared to leaves and stems. However, NRA in roots can be affected negatively by glyphosate drift at early stage as well. The same general pattern was observed when NRA was measured in the individual plant part (**Figure 2**) or in the whole plant, as mentioned above. Application of glyphosate at 6 WAP caused a decrease in NRA in leaves only, but not in stems and roots; NRA in roots of the treated soybean was significantly higher than that in roots of the untreated control plants (**Figure 1D**). This suggests that NRA in roots of glyphosate-treated plants recovered quickly and exceeded that of NRA in roots of untreated plants, especially at a later stage. This may be attributed to the ability of roots to compensate for the NRA decrease in leaves and stems caused previously by glyphosate treatment.

Both roots and stems played a significant role in nitrate reduction under glyphosate drift. This is may be attributed to the ability of roots and stems to play a significant role in supplying assimilates for growth and development when the plants under physiological and biochemical stresses resulted from glyphosate drift that took place in leaves. At 9 and 10

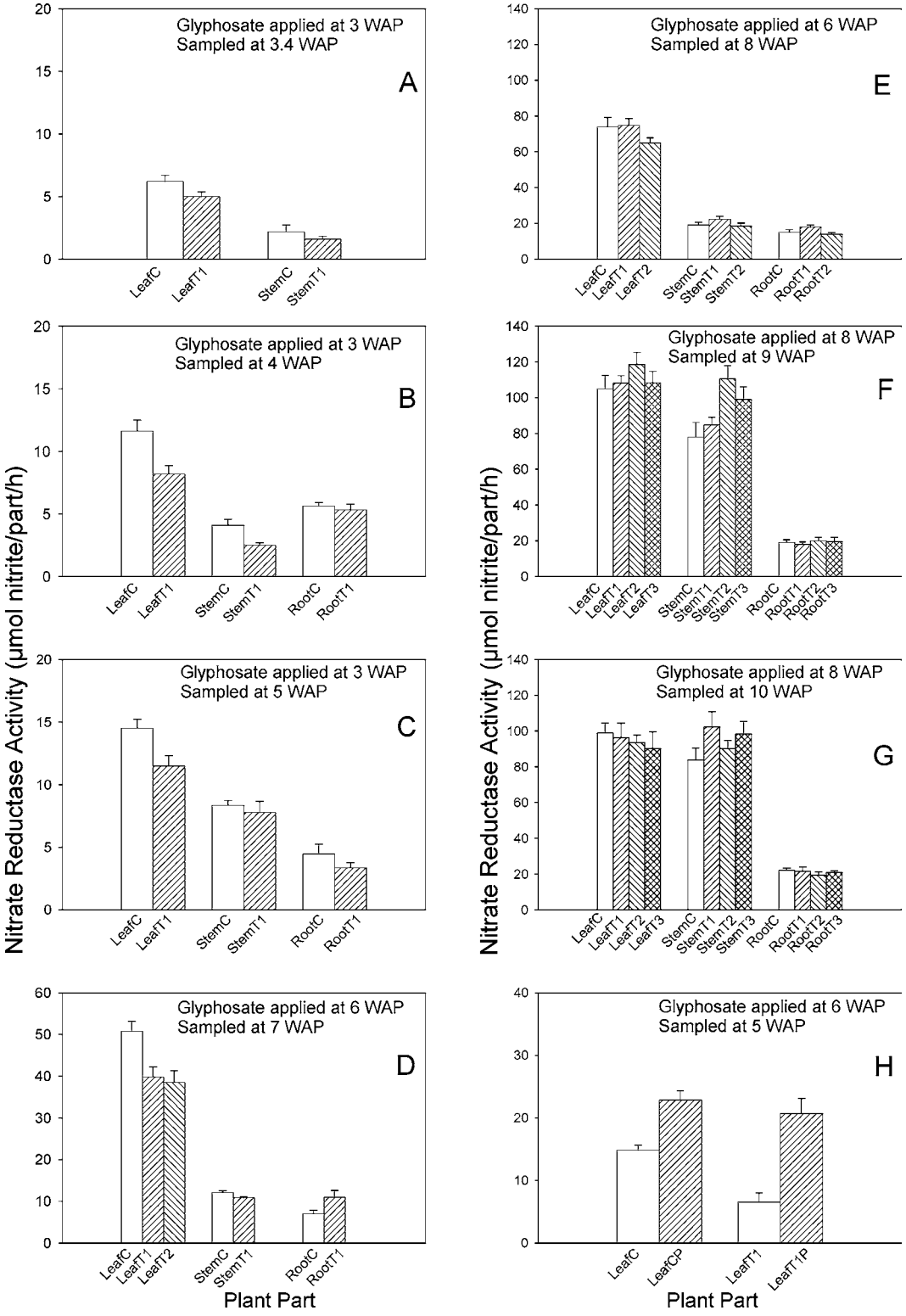

Figure 2. Effect of simulated glyphosate drift on nitrate reductase activity (micromoles of nitrite per part per hour) in the youngest fully expanded leaf, stems, and roots in the control (LeafC, StemC, and RootC) and treatments (LeafT1, LeafT2, Leaf T3, StemT1, StemT2, StemT3, RootT1, RootT2, and RootsT3). Sampling dates were 3.4 WAP (**A**), 4 WAP (**B**), 5 WAP (**C**), 7 WA (**D**), 8 WAP (**E**), 9 WAP (**F**), and 10 WAP (**G**). NRA (LeafC and LeafT1) and potential NRA (LeafCP and LeafT1P) were measured at 5 WAP in the youngest fully expanded leaf (**H**). Bars represent mean ± SE.

WAP, NRA in leaves of treated soybean had lower rates compared to roots and stems of untreated soybean, although leaves of the untreated soybeans were always higher (**Figure 1F**,**G**). It is interesting to note that, at 10 WAP, roots of treated soybean showed a greater NRA than the untreated soybean (**Figure 1F**,**G**). Nitrate reductase activity in leaves of treated soybean started recovering 4 weeks after glyphosate application (**Figure 1D**). This indicates that NRA in leaves is more sensitive at the early stage of growth and development and less sensitive as the plants reach maturity. For example, at 4 WAP, NRA in treated plants decreased 60% in leaves and 78% in stems compared to the NRA in untreated soybeam, and there was no decrease in roots. At 10 WAP, NRA was only 23 and 3% lower in leaves and stems, respectively, and 120% higher in roots (4.4

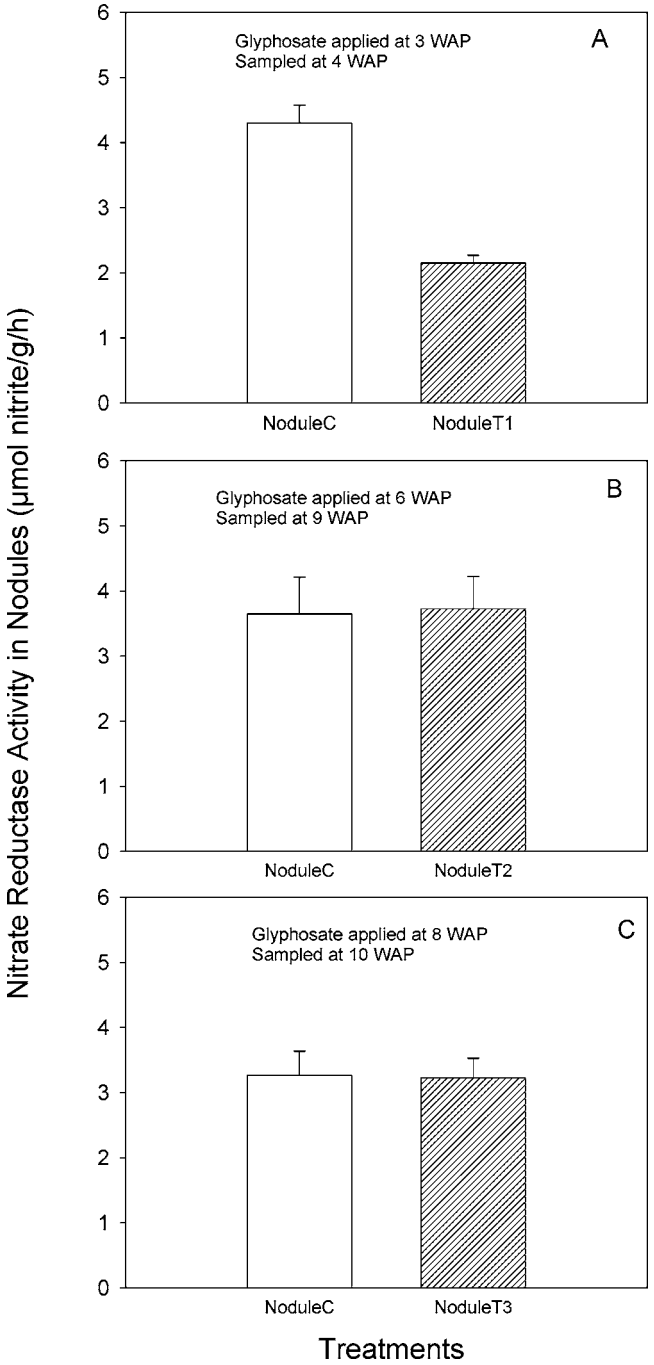

Figure 3. Effect of simulated glyphosate drift on nitrate reductase activity (micromoles of nitrite per gram per hour) in nodules in the control (NoduleC) and treatments (NoduleT1, NoduleT2, and NoduleT3). Sampling dates were 4 WAP (**A**), 9 WAP (**B**), and 10 WAP (**C**).

μmol of nitrite/g/h in RootT3) compared to the untreated (2.0 μmol of nitrite/g/ h in RootC) soybean. This indicates that, although NRA per whole plant decreased, the NRA in the roots is an increased proportion of whole plant NRA so that the assimilation of nitrate in the roots helps minimize the stress effect of glyphosate on plant growth.

Nitrate Reductase in Nodules. NRA in nodules of treated plants showed a significant decrease compared to NRA in nodules of the untreated soybean only when exposed to glyphosate at early stages of development. At 4 WAP, glyphosate caused 50% decrease in NRA of nodules (4.3 vs 2.15 μmol of nitrite/g/h in untreated and glyphosate-treated plants, respectively) (**Figure 3A**). However, at 9 and 10 WAP, glyphosate had no significant effect on nodule NRA (**Figure 3B**,**C**), indicating that nodule NRA is more sensitive at early stages, and treated nodules may be a factor in the sensitivity of nodule NRA.

Potential Nitrate Reductase Activity. To assess whether the glyphosate-induced decrease of NRA in leaves was due to lower nitrate supply to leaves, exogenous nitrate was supplied to leaves in the buffer medium at a concentration of 10 mM. PNRA in leaves of both treated and untreated soybean was much higher than leaf NRA (**Figures 1H** and **2H**). This indicates that either NR enzyme was already present in a potentially active form or de novo synthesis of the enzyme occurred. This observation indicates that nitrate availability in the cell was a limiting factor for NRA and not the enzyme.

Nitrogenase Activity (Acetylene Reduction) and Root Respiration. Nitrogenase activity per plant was significantly reduced by early glyphosate exposure at 3 or 6 WAP. For example, nitrogenase activity at 4 WAP sampling was 12 μmol of ethylene formed/plant/h in control plants versus 4 μmol of ethylene formed/plant/h in treated plants. A similar trend was observed at 5, 7, 8, and 9 WAP sampling (data not shown). Nitrogenase activity when the mass of nodules was taken into consideration (nitrogen fixation potential) was reduced 41−65% by glyphosate treatment at 3 or 6 WAP (**Table 3**). The significant decrease in nitrogenase activity reflects the negative effects of glyphosate on nitrogen fixation. There was no effect of glyphosate on root respiratory activity at initial samplings (**Table 3**). However, at the 9 and 10 WAP samplings, the control had a lower respiratory activity compared to most glyphosate-treated plants. There was a lower root biomass in treated plants, and total respiration per root was significantly greater in control plants (data not shown). In addition, stress induced by glyphosate exposure may have enhanced the respiratory activity per gram of tissue in treated plants. Studies reported on the effect of glyphosate on nodule inhibition, nitrogen fixation, and yield are still inconsistent (*21*), and the mechanism of this effect is still unknown. Glyphosate accumulates in nodules of field-grown GR soybean, but its effects on nitrogenase activity were inconsistent in field studies. Nitrogenase activity in GR soybean was transiently decreased in early stages following glyphosate application under greenhouse conditions. Although GR soybean has an insensitive EPSPS gene, the effect of reduced nitrogen fixation in early stages, observed in GR soybean, on yield was not demonstrated (*21*). On the other hand, the soybean nitrogen fixing symbiont, *B. japonicum*, possesses a glyphostae-sensitive enzyme, and upon exposure to glyphosate it accumulates shikamic acid and hydroxybenzoic acids such as protocatechuic acid. This exposure is accompanied with *B. japonicum* growth inhibition and death at high concentrations (*22*−*25*). The toxic effect of glyphosate on nodules may be attributed to inability of the organism to synthesize aromatic amino acids; energy drain on the organism resulting from ATP and PEP spent in the accumulation of shikimate, 3-deoxy-D-arabino-heptulose-7-phosphate, and hydroxybenzoic acid; and toxicity of accumulated intermediates of shikimic acid pathway (*26*).

Nitrogenase activity in nodules (micromoles of ethylene formed per gram per hour) exhibited a greater sensitivity to simulated glyphosate drift than either NRA in roots or NRA in nodules as the pattern of NRA inhibition was not as consistent as nitrogenase activity at 4, 5, 7, and 8 WAP. This was not the case in NRA in roots or nodules, where NRA in roots showed a decrease at only 5 and 9 WAP sampling dates after glyphosate application. The relationship between nitrogenase activity and NRA is not well understood and needs further studies. Nitrate

Table 3. Glyphosate Simulated Drift Effects on Nitrogenase (Acetylene Reduction) Activity and Soybean Root Respiratory Activity in Non-glyphosate-Resistant Soybean[a]

treatment	when applied (WAP)[b]	nitrogenase activity (μmol of ethylene formed/g of nodule/h)						soybean root respiration (mmol of CO_2 evolved/g of root/h)					
		4 WAP	5 WAP	7 WAP	8 WAP	9 WAP	10 WAP	4 WAP	5 WAP	7 WAP	8 WAP	9 WAP	10 WAP
no glyphosate		913 a	384 a	442 a	271 a	382 a	480 a	3.5 a	4.1 a	7.3 a	6.5 a	6.7 b	4.3 b
glyphosate	3	387 b	225 b	229 b	230 ab	410 a	352 a	4.4 a	4.8 a	8.1 a	6.9 a	9.4 a	5.5 ab
glyphosate	6			151 b	173 b	300 a	435 a			7.1 a	7.1 a	7.5 ab	6.1 a
glyphosate	8					303 a	429 a					9.1 a	5.0 ab

[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. [b] WAP, weeks after planting soybean.

Table 4. Effect of Simulated Glyphosate Drift on Nitrogen Content of Soybean Shoots or Leaf Tissue[a]

treatment	when applied (WAP)[c]	nitrogen content[b] (%)					
		4 WAP	5 WAP	7 WAP	8 WAP	9 WAP	10 WAP
no glyphosate		3.61 a	2.76 a	5.97 a	5.60 a	5.60 a	5.48 a
glyphosate	3	3.56 a	2.19 b	5.97 a	5.65 a	5.60 a	5.56 a
glyphosate	6			5.81 a	5.81 a	5.30 a	5.55 a
glyphosate	8					5.69 a	5.56 a

[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. [b] Nitrogen content at 4 and 5 WAP represents total nitrogen content of shoots (leaves and stems), and 7–10 WAP, nitrogen content in the youngest fully expanded leaves. [c] WAP, weeks after planting soybean.

Table 5. Glyphosate Simulated Drift Effects on Yield, Seed Nitrogen Content, Protein, and Oil Concentration in Non-glyphosate-Resistant Soybean[a]

treatment	when applied (WAP)[b]	soybean yield (kg/ha)	seed nitrogen (%)	total seed nitrogen (kg/ha)	oil (%)	protein (%)
no glyphosate		3900 a	5.97 b	233 a	21.1 a	39.5 a
glyphosate	3	3880 a	6.25 a	242 a	21.1 a	38.7 a
glyphosate	6	3780 a	6.08 ab	230 a	21.0 a	39.1 a
glyphosate	8	3920 a	6.02 ab	236 a	20.8 a	39.0 a

[a] Means within a column followed by the same letter are not significantly different at the 5% level of significance. [b] WAP, weeks after planting soybean.

concentration in the shoot was considered to be a major factor for nitrate inhibition of nitrogen fixation (*27*). NRA in nodules was reported in legumes (*28*) and present in *B. japonicum* (*29*, *30*). The contribution of root NRA and nodule NRA to the total NRA and its physiological significance is not fully understood. Maximizing nitrate reduction in roots and nodules and nitrogen fixation may increase the efficiency in using atmospheric and soil or fertilizer nitrogen to maximize yield (*31*).

Yield, Seed Quality, and Nitrogen Content. The effects of glyphosate on soybean nitrogen content were minimal, and significant effect on shoot nitrogen content (26% decrease) was only observed 2 weeks after the first application (**Table 4**). As plant biomass increased the most recent fully expanded trifoliate was used as an indicator of crop nitrogen status. Despite the chlorosis observed, no significant effect on leaf nitrogen content was observed in relation to glyphosate application.

Yield, seed composition (oil and protein), and nitrogen content in leaves and shoots are shown in **Table 5**. No yield difference was detected between untreated and treated soybean. This is consistent with other results. For example, Ellis and Griffin (*5*) reported that there was no significant yield reduction in soybean at simulated glyphosate drift of 0.8−12.5%. Nitrogen content in seed increased by application of glyphosate at 3 WAP compared to the control, whereas the other glyphosate treatments were not different from the control (**Table 5**). Protein and oil in soybean seed did not show any glyphosate treatment effect. This does not exclude the possibility that there may be a significant effect of glyphosate drift on fatty acids and amino acids, especially aromatic amino acids, as indicated above. Further research is needed to investigate the effect of glyphosate drift on fatty acids and amino acids, especially aromatic amino acids.

ABBREVIATIONS USED

ARA, acetylene reduction assay; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GR, glyphosate-resistant; NRA, nitrate reductase activity; PNRA, potential nitrate reductase activity; WAP, weeks after planting.

ACKNOWLEDGMENT

We thank Sandy Mosley, Albert Tidwell, Earl Gordon, and Annie Johnson for field and laboratory technical assistance.

LITERATURE CITED

(1) U.S. Department of Agriculture. National Agricultural Statistics Service; Web page http://usda.mannlib.cornell.edu/reports/nass/field/pcp-bba/acrg0605.pdf, accessed July 28, 2005.

(2) Hanks, J. E. Effect of drift retardant adjuvants on spray droplet size of water paraffinic oil applied at ultra volume. *Weed Technol*. **1995**, *9*, 380−384.

(3) Al-Khatib, K.; Peterson, D. Soybean (*Glycine max*) response to simulated drift from selected sulfonylurea herbicides, dicamba, glyphosate, and glufosinate. *Weed Technol*. **1999**, *13*, 264−270.

(4) Snipes, C. E.; Street, J. E.; Mueller, T. C. Cotton (*Gossypium hirsutum*) injury from simulated quinclorac drift. *Weed Sci*. **1992**, *40*, 106−109.

(5) Ellis, J. M.; Griffin, J. L. Soybean (*Glycine max*) and cotton (*Gossypium hirsutum*) response to simulated drift of glyphosate and glufosinate. *Weed Technol*. **2002**, *16*, 580−586.

(6) Reddy, K. N.; Hoagland, R. E.; Zablotowicz, R. M. Effect of glyphosate on growth, chlorophyll, and nodulation in glyphosate-resistant and susceptible soybean (*Glycine max*) varieties. *J. New Seeds* **2000**, *2*, 37−52.

(7) King, C. A.; Purcell, L. C.; Vories, E. D. Plant growth and nitrogenase activity of glyphosate-tolerant soybean in response to foliar glyphosate applications. *Agron. J*. **2001**, *93*, 179−186.

(8) Amrhein, J.; Schab, J.; Steinrücken, H. J. The mode of action of the herbicide glyphosate. *Naturwissenschaften* **1980**, *67*, 356−357.
(9) Harper, J. E. Soil and symbiotic nitrogen requirements for optimum soybean production. *Crop Sci.* **1974**, *14*, 255−260.
(10) Beevers, L.; Schrader, L. E.; Flesher, D.; Hageman, R. H. The role of light and nitrite in the induction of nitrate reductase in radish cotyledons and maize seedlings. *Plant Physiol*. **1965**, *40*, 691−698.
(11) Tischner, R. Nitrate uptake and reduction in higher and lower plants. *Plant, Cell Environ*. **2000**, *23*, 1005−1024.
(12) Bellaloui, N.; Pilbeam, D. J. Effect of nitrate withdrawal and re-supply on nitrate assimilation of nitrate in leaves in tomato. *J. Exp. Bot*. **1991**, *42*, 81−88.
(13) Bellaloui, N.; Pilbeam, D. J. Reduction of nitrate in leaves of tomato during the vegetative growth. *J. Plant Nutr*. **1990**, *13*, 39−55.
(14) Kanayama, Y.; Kimura, K.; Nakamura, Y.; Ike, T. Purification and characterization of nitrate reductase from nodule cytosol of soybean plants. *Physiol. Plant*. **1999**, *105*, 396−401.
(15) Caba, J. M.; Lluch, C.; Ligero, F. Distribution of nitrate reductase activity in *Vicia faba*: effect of nitrate and plant genotype. *Physiol. Plant*. **1995**, *93*, 667−672.
(16) Klepper, L.; Hageman, R. H. The occurrence of nitrate reductase in apple leaves. *Plant Physiol*. **1969**, *44*, 110−114.
(17) Hardy, R. W. F.; Holsten, D.; Jackson, E. K.; Burns, R. C. The acetylene−ethylene assay for nitrogen fixation: laboratory and field evaluation. *Plant Physiol*. **1968**, *43*, 1185−1205.
(18) Zablotowicz, R. M.; Focht, D. D.; Cannell, G. H. Nodulation and N fixation of field grown California cowpeas as influenced by irrigated and drought conditions. *Agron. J*. **1981**, *73*, 9−12.
(19) SAS. *SAS 9.1 TS Level 1M3, Windows version 5.1.2600*; SAS Institute: Cary, NC, 2001.
(20) Reddy, K. N.; Zablotowicz, R. M. Glyphosate-resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. *Weed Sci*. **2003**, *51*, 496−502.
(21) Zablotowicz, R. M.; Reddy, K. N. Impact of glyphosate on the *Bradyrhizobium japonicum* symbiosis with glyphosate-resistant transgenic soybean: a minireview. *J. Environ. Qual*. **2004**, *33*, 825−831.
(22) Lydon, J.; Duke, S. O. Glyphosate induction of elevated levels of hydroxybenzoic acids in higher plants. *J. Agric. Food Chem*. **1988**, *36*, 813−818.
(23) Becerril, J. M.; Duke, S. O.; Lydon, J. Glyphosate effects on shikimate pathway products in leaves and flowers of velvetleaf. *Phytochemistry* **1989**, *28*, 695−699.
(24) Moorman, T. B.; Becerril, J. M.; Lydon, J. M.; Duke, S. O. Production of hydroxybenzoic acids by Bradyrhizobium japonicum strains after treatment with glyphosate. *J. Agric. Food Chem*. **1992**, *40*, 289−293.
(25) Hernandez, A.; Garcia-Plazzola, J. I.; Becerril, J. M. Glyphosate effects on phenolic metabolism of nodulated soybean (*Glycine max* L. Merr.). *J. Agric. Food Chem*. **1999**, *47*, 2920−2925.
(26) Fisher, R. S.; Berry, A.; Gaines, C. G.; Jensen, R. A. Comparative action of glyphosate as a trigger of energy drain in *Eubacteria*. *J. Bacteriol*. **1986**, *168*, 1147−1154.
(27) Walsh, K. B.; Carroll, B. J. A reappraisal of nitrate inhibition of nitrogenase in A317, a nitrate reductase-X deficient mutant of pea (*Pisum sativum*). *Physiol. Plant*. **1992**, *84*, 283.
(28) Caba, J. M.; Lluch, C.; Hervás, A.; Ligero, F. Nitrate metabolism in roots and nodules of *Vicia faba* in response to exogenous nitrate. *Physiol. Plant*. **1990**, *79*, 531.
(29) Zablotowicz, R. M.; Eskew, D. L.; Focht, D. D. Denitrification in *Rhizobium*. *Can. J. Microbiol*. **1978**, *24*, 757−760.
(30) Zablotowicz, R. M.; Focht, D. D. Denitrification and anaerobic, nitrate-dependent acetylene reduction in cowpea *Rhizobium*. *J. Gen. Microbiol*. **1979**, *111*, 445−448.
(31) Heckmann, M. O.; Drevon, J. J.; Saglio, P.; Salsac, L. Effect of oxygen and malate on NO_3^- inhibition of nitrogenase in soybean nodules. *Plant Physiol*. **1989**, *90*, 224−229.
(32) Fehr, W. R.; Caviness, C. E.; Burmood, D. T.; Pennington, J. S. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. *Crop Sci*. **1971**, *11*, 929−931.

Received for review December 21, 2005. Revised manuscript received March 6, 2006. Accepted March 8, 2006. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF053198L