Glyphosate effects on photosynthesis, nutrient accumulation, and nodulation in glyphosate-resistant soybean

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Abstract

Previous greenhouse studies have demonstrated that photosynthesis in some cultivars of first- (GR1) and second-generation (GR2) glyphosate-resistant soybean was reduced by glyphosate. The reduction in photosynthesis that resulted from glyphosate might affect nutrient uptake and lead to lower plant biomass production and ultimately reduced grain yield. Therefore, a field study was conducted to determine if glyphosate-induced damage to soybean (*Glycine max* L. Merr. cv. Asgrow AG3539) plants observed under controlled greenhouse conditions might occur in the field environment. The present study evaluated photosynthetic rate, nutrient accumulation, nodulation, and biomass production of GR2 soybean receiving different rates of glyphosate (0, 800, 1200, 2400 g a.e. ha^{-1}) applied at V2, V4, and V6 growth stages. In general, plant damage observed in the field study was similar to that in previous greenhouse studies. Increasing glyphosate rates and applications at later growth stages decreased nutrient accumulation, nodulation, leaf area, and shoot biomass production. Thus, to reduce potential undesirable effects of glyphosate on plant growth, application of the lowest glyphosate rate for weed-control efficacy at early growth stages (V2 to V4) is suggested as an advantageous practice within current weed control in GR soybean for optimal crop productivity.

Key words: *Glycine max* / herbicide impacts / photosynthetic rate / root nodulation

Accepted December 15, 2011

1 Introduction

Soybean is one of the major world crops with most of the global production devoted to glyphosate [N-(phosphonomethyl)-glycine]-resistant (GR) soybean cultivars. The area cultivated with conventional, non-GR soybeans has decreased mainly due to the efficacy of glyphosate for weed control in current crop-management systems. Glyphosate-resistant soybean was developed by insertion of a transgene (cp4) from an *Agrobacterium* species that encodes for an insensitive version of EPSPS (*Franz* et al., 1997). In 1996, the "first generation—GR1" was commercially available for production in the U.S. (*Duke*, 2005). Subsequent development of GR soybean designated as "second generation—GR2" was based on a new technique of *Agrobacterium*-mediated gene delivery to the soybean meristem, which directly induced cells to differentiate into transgenic plants (*Martinell* et al., 2002). In 2008, GR2 cultivars developed by this procedure with reportedly higher-yielding traits than GR1 cultivars became commercially available for farmers.

Although GR crops are resistant to glyphosate, recent reports suggest that glyphosate or a metabolite of degradation, aminomethylphosphonic acid (AMPA), may decrease photosynthesis (*Zobiole* et al., 2010a), water absorption (*Zobiole* et al., 2010a), symbiotic N_2 fixation (*Zobiole* et al., 2010b), and shoot mineral concentrations in leaf tissue and seed (*Zobiole* et al., 2010c, d) in GR1 soybean cultivars. Also, visual plant injury in some GR1 soybean varieties after glyphosate application is often reported (*Zablotowicz* and *Reddy*, 2007). The typical symptom, known as "yellow flashing", is attributed to the accumulation of the primary phytotoxic metabolite (AMPA; *Reddy* et al., 2004) or to immobilization of divalent cations, including Fe (*Bellaloui* et al., 2009) and Mn (*Johal* and *Huber*, 2009), due the formation of insoluble glyphosate–metal complexes (*Jaworski*, 1972; *Kabachnik* et al., 1974; *Coutinho* and *Mazo*, 2005), possibly leading to nutrient immobilization and interference with uptake and translocation (*Cakmak* et al., 2009).

Several reports suggest that glyphosate may interfere with the symbiotic N_2 fixation in GR soybean. Glyphosate may restrict Ni availability to symbiotic rhizobia by chelation effects, which could reduce efficiency of biological N_2 fixation in GR soybeans (*Zobiole* et al., 2010b). Decreased nodulation of GR soybeans may be due directly to toxicity of glyphosate or its metabolites translocated to roots and contacting bradyrhizobia within root and nodular tissue. *Bradyrhizobium japonicum* possesses a glyphosate-sensitive EPSP synthase and accumulates shikimic, hydroxybenzoic, and protocatechuic acids (PCA) upon exposure to glyphosate, potentially leading to growth inhibition and death at high concentrations (*Moorman* et al., 1992). Although decreased nodulation may vary among soybean cultivars, previous research demon-

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strated that nodulation of 45% of the most widely planted GR soybean cultivars grown in Brazil were affected by glyphosate (*Oliveira* et al., 2008).

An initial greenhouse experiment conducted with GR1 and GR2 cultivars receiving different glyphosate rates (800, 1200, and 2400 g a.e. ha^{-1}) applied at various growth stages (V2, V4, and V6; *Pederson,* 2009) demonstrated that photosynthesis, leaf area, and shoot biomass in both soybean cultivars were decreased by glyphosate, especially at high rates when applied at growth stage V6 (*Zobiole* et al., 2010e). We examined effects of glyphosate at various soybean growth stages to simulate the multiple field applications often necessary for complete weed control. In contrast, low rates and early applications caused less damage in both GR soybean generations. Very few reports of glyphosate effects on GR soybean physiology are available, especially those related to photosynthesis, mineral nutrition, and biological N_2 fixation, and even less information is currently available about the performance of GR2 soybean beyond commercial and farmer testimonials. Therefore, the aim of this research was to evaluate the nutrient accumulation and nodulation in GR2 soybean under field conditions treated with different rates of glyphosate applied at various growth stages.

2 Material and methods

2.1 Field location and seed establishment

Field experiments were carried out in 2009 at two sites on Sanborn Field (38°56″ N, 92°28′ W) at the University of Missouri, Columbia, MO. The soil was a Mexico silt loam (fine, smectitic, mesic Aeric Vertic Epiaqualfs) with chemical properties of C_{org}, 31 g kg^{-1}; P, 15 mg kg^{-1}; K, 55 mg kg^{-1}; Ca, 1523 mg kg^{-1}; Mg, 112 mg kg^{-1}; Fe, 90 mg kg^{-1}; Mn, 27 mg kg^{-1}; B, 3.2 mg kg^{-1}; Cu, 1.1 mg kg^{-1}; Zn, 3.1 mg kg^{-1}; pH_{CaCl2}, 5.7, as determined based on methods in *Brown* (1998). The soil had an indigenous population of *Bradyrhizobium japonicum* of approximately 10^6 cells (g dry soil)$^{-1}$ determined by the most-probable-number technique (*Woomer*, 1994).

The experimental area was naturally infested with giant foxtail (*Setaria faberi* Herrm.), morningglory species (*Ipomoea* spp.), *Amaranthus* spp., and yellow nutsedge (*Cyperus esculentus* L.). Field preparation consisted of moldboard plowing in the fall followed by disking/harrowing in the spring; management was consistent with practices common to soybean production in Missouri (*Wiebold* and *De Felice*, 1993). Nitrogen-free fertilizer was applied at disking/harrowing to provide 22 kg P ha^{-1} and 42 kg K ha^{-1}. Previous crops at this site had been grass and legume forages, which had not received herbicides within the preceding 5 years.

Glyphosate-resistant soybean (*Glycine max* L. Merr. cv. Asgrow AG3539) was sown on August 5 with seeds inoculated with a commercial peat-based inoculant (> 10^9 viable cells g^{-1}) of *Bradyrhizobium japonicum* (SEMIA 5079) and *B. elkanii* (SEMIA 587) at a rate of 500 g inoculant per 20 kg seeds. Soybean seeds were sown 1.5 cm deep using a precision seeder at a rate of 400 000 seeds ha^{-1}. Plot size was 3.8 m × 5.5 m, and row spacing was 76 cm, providing 4 rows per plot. Weather conditions were nearly normal during the experiment (Fig. 1).

2.2 Glyphosate application

The applications were accomplished using a backpack sprayer equipped with SF110.02 nozzles calibrated to deliver a spray volume of 190 L ha^{-1} at a pressure of 2 kg cm^{-2}. Environmental conditions during the applications included air temperature between 25°C and 27°C, relative humidity between 80% and 89%, wind speed between 5 and 10 km h^{-1}, and open sky with no clouds.

Plants at different growth stages (*Pederson*, 2009) identified during the season (Fig. 1), including V2 (10 DAS—days after sowing), V4 (20 DAS), and V6 (34 DAS), were sprayed at 0700h using the commercially formulated potassium salts of glyphosate 540 g a.e. L^{-1} (Roundup Weather Max®, Monsanto Company) at different rates (0, 800, 1200, and 2400 g a.e. ha^{-1}). The labeled rate for single glyphosate application at V4 growth stage in GR soybeans, varies from 600 to 1200 g a.e. ha^{-1} (*Gazziero* et al., 2008). The rate 2400 g a.e. ha^{-1}

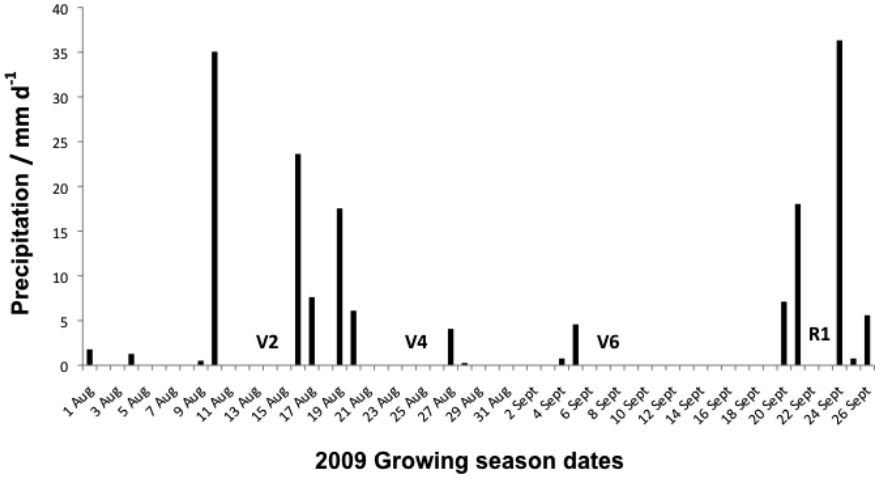

Figure 1: Rainfall during the experimental period and occurrence of different developmental growth stages for glyphosate-resistant soybean AG3539.

was selected to represent the "worst-case scenario" to promote soybean injury. Independent of the treatment applied, all plots were hand-weeded manually once every 7 d. The single application label rate for the crop production region of the study site was 800 g a.e. ha^{-1}.

To prevent contamination of neighboring plots, care was taken during application of the different glyphosate rates by covering perimeter field plots with 1 m high, framed cages covered with plastic sheeting (*Fagliari* et al., 2005). The cages remained on the plots for at least 5 min after glyphosate application to assure cessation of drift, and then removed.

2.3 Photosynthetic rate and SPAD readings

At R1 growth stage (48 DAS), the last fully expanded trifoliate (diagnostic leaf) was randomly selected from four plants in the middle of the two center rows, which represented 38, 28, and 12 d after glyphosate application at V2, V4, and V6 growth stages, respectively. The photosynthetic rate (*A*) was recorded using an infrared gas analyzer (IRGA; Li-Cor, LI 6400XT, Lincoln, NE, USA) and calculated using the equations of *von Caemmerer* and *Farquhar* (1981). Two evaluations were carried out between 1100h and 1200h under different weather conditions. The first assessment was on a cloudy day, with the air temperature between 24°C and 26°C, relative humidity between 86% and 88%, and photosynthetic photon-flux density (PPFD) between 100 and 140 μmol m^{-2} s^{-1} at the top of the leaf canopy. In contrast, the second evaluation was on a sunny day, no clouds, with the air temperature ranging between 29°C and 32°C, relative humidity between 78% and 82%, and PPFD between 1700 and 1900 μmol m^{-2} s^{-1} at the top of the leaf canopy.

The SPAD (Minolta SPAD-502 meter) evaluations were taken on these same diagnostic leaves, whereby the meter was placed randomly on leaf mesophyll tissue avoiding the veins. Three SPAD readings were taken per leaflet of the terminal leaflet of the diagnostic leaf (*Richardson* et al., 2002) and averaged to provide a single SPAD unit.

2.4 Nutrient accumulation

Immediately after collecting the SPAD and IRGA measurements at R1 growth stage, the upper three leaves consisting of the diagnostic trifoliate and the leaves from the nodes above and below the diagnostic leaf (including petioles) were collected from each of the four sampled plants. Leaves were washed in deionized water, packed in paper bags, and dried in an oven at 60°C–70°C and weighed after 48 h when a constant dry weight was achieved. The mineral composition (P, K, Ca, Mg, S, Zn, Mn, Fe, Cu) of the leaves was determined by complete perchloric nitric digestion (6 : 1), and B concentration was obtained after dry digestion (*Embrapa*, 1997). All elements, except N, were measured using an AES Perkin Elmer ICP (inductively coupled plasma) spectrophotometer. Nitrogen was determined using the Kjeldahl method (*Baker* and *Thompson*, 1992), after complete sulfuric acid digestion. The data of nutrient accumulation per plant were calculated based on total shoot (leaves, stems, and petioles) dry weight and nutrient concentrations in the leaves of the upper plant parts. Leaves and petioles comprise approximately 90% of the dry biomass of soybean through the V6 growth stage (*Pederson*, 2009), and because leaves are the dominant nutrient sinks, nutrient contents were expressed on an aboveground plant basis.

2.5 Leaf area, nodulation, and biomass

The leaf area was measured using a leaf-area meter (Delta T. Devices) per entire four plants per plot and then averaged to obtain a total area (cm^2 $plant^{-1}$). After these assessments, shoots were clipped at the soil surface and roots were carefully removed from soil, washed under running water and nodules were removed and counted. Immediately after nodule counting, the roots, shoots, and nodules were then placed in separate paper bags and transferred to an oven at 60°C–70°C for 48 h after which dry weights were determined.

2.6 Experimental design and statistical analysis

The two separate experiments were organized in a randomized complete block design, using a factorial scheme 3 × 3 + 1 replicated four times. The first factor was represented by glyphosate rate (800, 1200, and 2400 g a.e. ha^{-1}), and the second was the application timing at different growth stages (V2, V4, and V6). An additional treatment was a no-glyphosate-application (control) treatment.

The data errors passed the normality test of *Shapiro* and *Wilk* (1965), and because there was homogeneity of error variances, the data for the two repeated experiments were combined. No transformations were necessary. Data were subjected to ANOVA by SAS statistical program (*SAS*, 2006), and when F values were significant ($p < 1\%$), regression analyses were conducted and equations were adjusted using the polynomial model $\hat{y} = a + bx + cx^{0.5}$ by SigmaPlot 10.0 statistical package (*SPSS*, 2000).

3 Results

3.1 Photosynthetic rate and SPAD readings

In both experiments, the photosynthetic rate (*A*) at the R1 growth stage was severely depressed by glyphosate application (Fig. 2A, Tab.1). In addition, the same decreased *A* caused by glyphosate was noticed on either sunny or cloudy days. However, as expected, the *A* was lower during the cloudy than sunny day. The value of *A* for the treatment control (no applied glyphosate) was approximately 34 μmol m^{-2} s^{-1} on a sunny day while under cloudy conditions *A* was decreased to around 20 μmol m^{-2} s^{-1}. The SPAD readings also decreased as glyphosate rate increased (Fig. 2B, Tab.1). According to *Richardson* et al. (2002) the SPAD meter can estimate chlorophyll levels and thus, the chlorotic symptoms noticed at R1 growth stage might reflect decreased chlorophyll concentration.

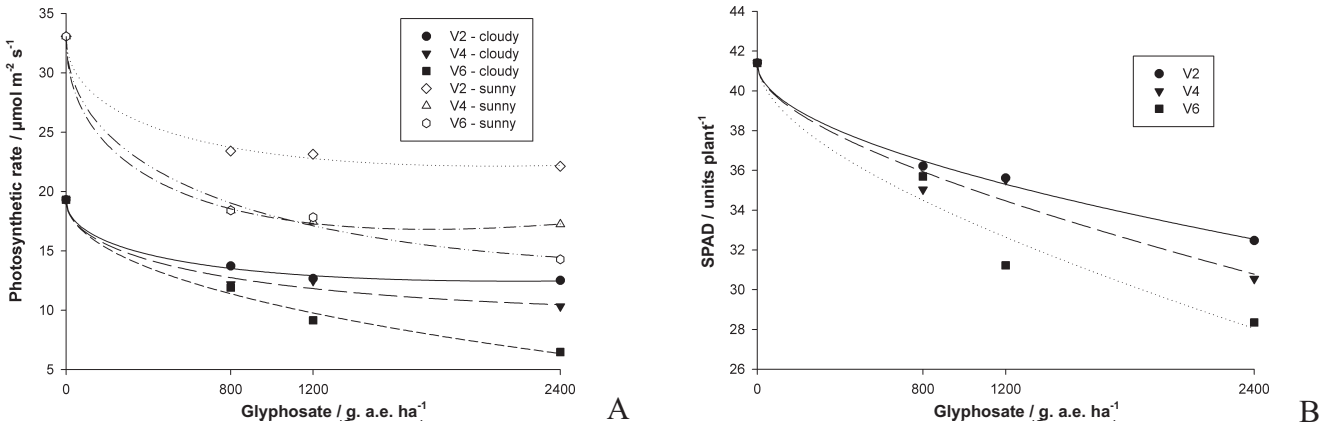

Figure 2: Photosynthetic rate (A) and SPAD measurements (B) at R1 growth stage of GR2 soybean across increasing rates of glyphosate application at different growth stages, V2, V4, and V6 (n = 8, p < 1%). See Tab. 1 for fitted regression equations.

3.2 Nutrient accumulation, nodulation, and biomass

Non-treated soybeans exhibited higher nutrient accumulation than those treated with glyphosate (Figs. 3 and 4, Tab. 1). In general, the macro- and micronutrient accumulations were proportionally reduced as glyphosate rates increased and applications were delayed. However, nutrient concentrations did not respond to glyphosate effects to the magnitude of nutrient accumulation responses (Tab. 2). Macronutrient and all micronutrient concentrations except Cu were within the nutrient-sufficiency ranges for soybean (*Mills* and *Jones*, 1996). Concentrations of Ca, Mg, S, and Cu were significantly (p < 5%) lower in glyphosate-treated soybean yet all values were within the sufficiency ranges for those nutrient concentrations to provide acceptable soybean growth. Concentrations of P and Fe appeared to be increased by glyphosate.

Nodulation was affected by glyphosate application as reflected by significantly decreased nodule number and dry weight (Fig. 5A and 5B, Tab. 1). In contrast with other results, a tendency was noted for reduced effects at late applications compared with early applications (Fig. 5A and 5B, Tab. 1). Similar findings were noted for root dry weight, which was more severely depressed with glyphosate applied at V2 growth stage compared with V6 growth stage (Fig. 5C, Tab. 1). However, leaf area and shoot dry weight were more strongly decreased at the late growth stage than at the early stage (Fig. 5D and 5E, Tab. 1).

4 Discussion

Photosynthetic rates (A) in control plants (Fig. 2) were comparable with those reported by *Kumudini* et al. (2008) who found A values around 26 µmol m^{-2} s^{-1} at R3 growth stage for cv. Asgrow 3905 with PPFD of 1500 µmol m^{-2} s^{-1} at the top of the leaf canopy at ambient temperature and CO_2. Although there was no difference in A values among the glyphosate rates applied at the V2 growth stage, reduced A values in GR2 soybean were more pronounced with increased glyphosate rate and later applications (V6). In contrast, low rates and early applications resulted in lower damage in GR2 soybean plants (Fig. 2A, Tab. 1). These findings agree with previous greenhouse results in which different glyphosate rates applied at different growth stages of the first and second generation of GR soybeans significantly decreased all photosynthetic parameters (*Zobiole* et al., 2010e). Previous studies also demonstrated that photosynthetic parameters (A, E, g_s) were severely affected by glyphosate in GR cultivars of different maturity groups growing in different soils. However, there were no differences between the nontreated GR soybeans and their respective near-isogenic non-GR parental lines (*Zobiole* et al., 2010c). Reduced SPAD readings (Fig. 2B) were proportional to increasing glyphosate rates and were pronounced further with later applications (V6). Nontreated soybean showed higher SPAD values (Fig. 2B, Tab. 1) compared with those treated with glyphosate. The chlorotic symptoms associated with low SPAD in GR1 soybeans frequently occur in field reports of visual plant injury in some GR1 soybean varieties after glyphosate application (*Zablotowicz* and *Reddy*, 2007).

Although yellow flashing tends to disappear within the first 2 weeks after herbicide application (*Reddy* and *Zablotowicz*, 2003), the injury noted in Fig. 2 occurred at the R1 growth stage, several weeks after herbicide application, suggesting that either glyphosate or AMPA exerts long-term effects on the physiology of the plant. This decrease could be due to direct damage of chloroplasts by glyphosate (*Campbell* et al., 1976; *Nilsson*, 1985), or to the accumulation of AMPA, which is phytotoxic (*Reddy* et al., 2004). Nevertheless, because glyphosate is a known strong chelator (*Kabachnik* et al., 1974; *Coutinho* and *Mazo,* 2005) of cations, especially Fe (*Bellaloui* et al., 2009), Mg (*Zobiole* et al., 2010c), and Mn (*Johal* and *Huber*, 2009), the immobilization of these essential nutrients affects chlorophyll formation and function (*Beale*, 1978; *Taiz* and *Zeiger*, 1998) thereby compromising the photosynthetic apparatus. *Zobiole* et al. (2010c) also noted that GR1 soybean from different maturity groups exposed to a single or sequential application of glyphosate frequently had chlorophyll concentrations lower than plants not exposed to the herbicide. According to *Cakmak* et al. (2009), the period for the observed "yellow flashing" is most likely dependent on the ability of the plants to recover through adequate root uptake of the specific elements that are immobilized by glyphosate in plant tissues.

Table 1: Regression analyses and correlations for the variables analyzed with increasing rates of single glyphosate application at different growth stages of soybean, V2, V4, and V6 presented in Figs. 2, 3, 4, and 5.

	Estimation of model parameters adjusted			
Variable and soybean growth stage	**a**	**b**	**c**	R^2
Photosynthetic rate (Fig. 2A):				
V2—cloudy	19.31	0.0031	–0.2932	0.99*
V4—cloudy	19.25	0.0025	–0.2999	0.99*
V6—cloudy	19.34	0.0008	–0.3035	0.99*
V2—sunny	33.05	0.0052	–0.4778	0.99*
V4—sunny	33.06	0.0092	–0.7745	0.99*
V6—sunny	33.02	0.0056	–0.6532	0.99*
SPAD (Fig. 2B):				
V2	41.38	–0.0004	–0.1632	0.99*
V4	41.33	–0.0012	–0.1571	0.98*
V6	41.49	–0.0013	–0.2103	0.98*
N accumulation (Fig. 3A):				
V2	890.08	–0.0082	–4.8098	0.98*
V4	891.69	0.0195	–6.4851	0.95*
V6	893.98	0.0097	–8.7145	0.94*
P accumulation (Fig. 3B):				
V2	59.34	0.0015	–0.2650	0.99*
V4	59.41	0.0018	–0.3214	0.96*
V6	59.46	0.0021	–0.3586	0.94*
K accumulation (Fig. 3C):				
V2	289.25	–0.0261	–0.5116	0.97*
V4	289.24	–0.0077	–1.6364	0.98*
V6	289.95	0.0109	–2.5760	0.94*
Ca accumulation (Fig. 3D):				
V2	197.12	0.0187	–2.6058	0.99*
V4	197.71	0.0217	–2.7739	0.98*
V6	197.89	0.0121	–2.8706	0.98*
Mg accumulation (Fig. 3E):				
V2	58.32	0.0002	–0.4045	0.99*
V4	58.47	0.0031	–0.5455	0.96*
V6	58.56	–0.0013	–0.5192	0.96*
S accumulation (Fig. 3F):				
V2	48.29	–0.0006	–0.2659	0.97*
V4	48.36	0.0006	–0.3749	0.95*
V6	48.36	–0.0014	–0.3646	0.97*
Zn accumulation (Fig. 4[A]):				
V2	0.77	0.000056	–0.0086	0.99*
V4	0.77	0.000078	–0.0100	0.97*
V6	0.77	0.000079	–0.0109	0.95*
Mn accumulation (Fig. 4B):				
V2	0.89	0.000017	–0.0073	0.99*
V4	0.89	0.0001	–0.0125	0.98*
V6	0.89	0.000064	–0.0114	0.97*

Table 1: continued.

Variable and soybean growth stage	Estimation of model parameters adjusted			
	a	b	c	R^2
Fe accumulation (Fig. 4C):				
V2	2.79	–0.00005	–0.0117	0.99*
V4	2.80	0.0001	–0.0299	0.99*
V6	2.79	0.0001	–0.0226	0.99*
Cu accumulation (Fig. 4D):				
V2	0.1166	0.000047	–0.0009	0.99*
V4	0.1167	–0.000017	–0.0008	0.98*
V6	0.1168	–0.000037	–0.0008	0.97*
B accumulation (Fig. 4E):				
V2	0.65	0.00008	–0.0087	0.99*
V4	0.65	0.00006	–0.0089	0.99*
V6	0.65	0.00008	–0.0110	0.99*
Nodule number (Fig. 5A):				
V2	2333.68	0.5015	–42.2575	0.99*
V4	2344.09	0.4135	–42.4206	0.98*
V6	2343.18	0.5912	–51.9479	0.98*
Nodule dry weight (Fig. 5B):				
V2	158.56	0.0179	–1.5763	0.98*
V4	158.88	0.0105	–1.0301	0.99*
V6	158.80	0.0174	–1.3960	0.99*
Root dry biomass (Fig. 5C):				
V2	0.5634	–1.39E5	–0.0043	0.99*
V4	0.5664	–4.30E5	–0.0031	0.98*
V6	0.5664	3.68E6	–0.0038	0.96*
Leaf area (Fig. 5D)				
V2	2.94	0.0003	–0.0455	0.99*
V4	2.95	0.0005	–0.0469	0.99*
V6	2.94	0.0005	–0.0484	0.99*
Shoot dry biomass (Fig. 5E):				
V2	14.80	0.0008	–0.1509	0.99*
V4	14.88	0.0016	–0.1980	0.97*
V6	14.87	0.0027	–0.2579	0.98*

*(n = 8, p < 1%)

In general, all macro- and micronutrient contents were significantly decreased by glyphosate at increased rates (Figs. 3 and 4), suggesting that GR2 soybean was rendered less efficient in nutrient uptake and translocation as well suffering from potential chelating effects of glyphosate. Concentrations of Ca, Mg, S, and Cu in leaves were depressed by glyphosate application. However, there was no direct evidence of induced nutrient deficiencies in leaves treated with glyphosate based on the measured concentrations (*Mills* and *Jones*, 1996). After glyphosate is absorbed by plants, uptake and transport of nutrients may be inhibited by the formation of poorly soluble glyphosate–metal complexes within plant tissues (*Eker* et al., 2006). It is possible that higher levels of nutrients may actually be required in leaves to achieve physiological sufficiency in GR2 soybean after treatment with glyphosate. Other factors in addition to glyphosate complexation may contribute to reduced nutrient uptake and accumulation within soybean include root growth inhibition, as suggested by *Bott* et al. (2008) and the root-growth data reported in the present study. Further, when nutrient analyses were expressed as concentrations in plant tissue biomass, the impact of glyphosate on nutrient relationships was seemingly

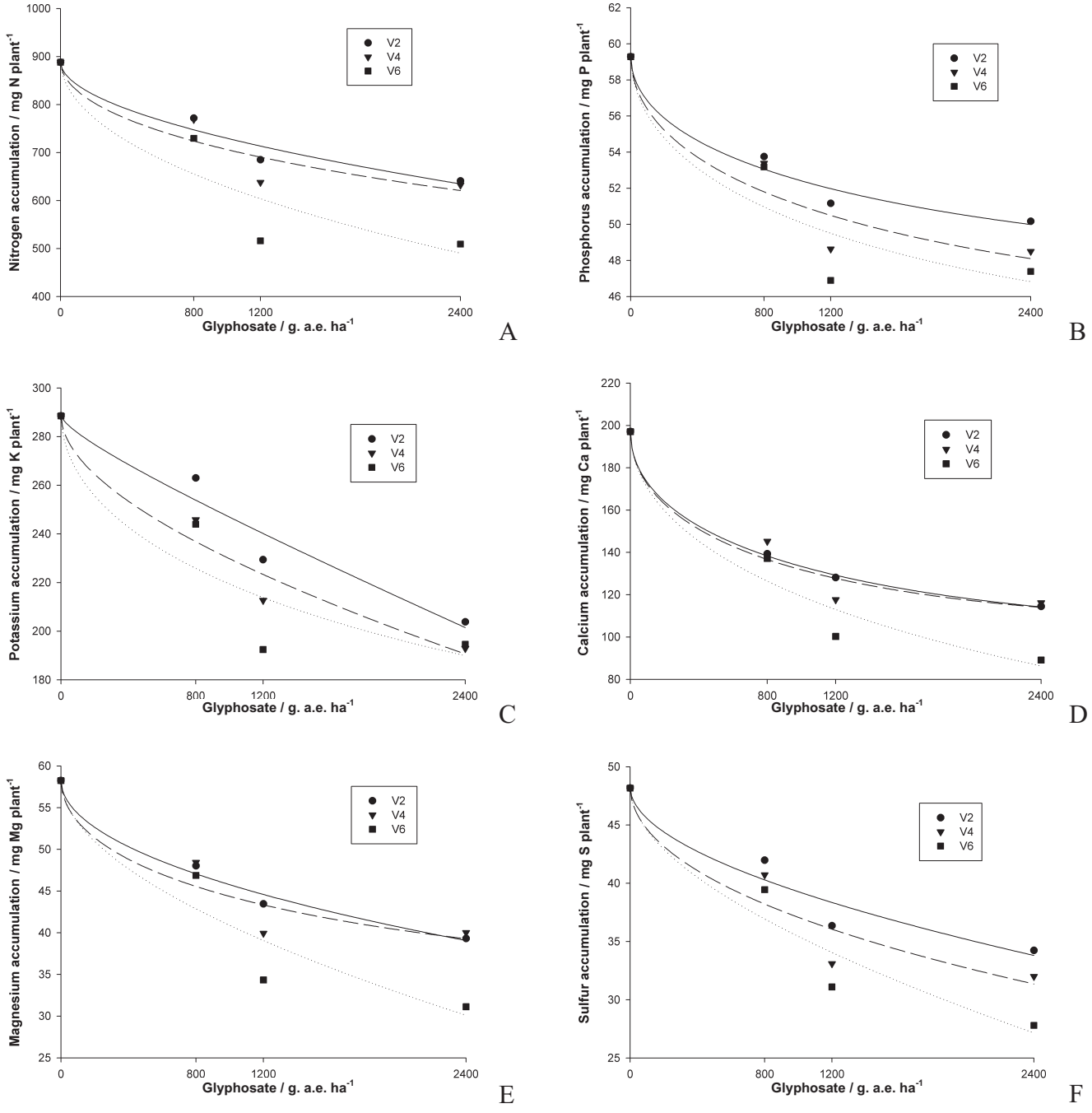

Figure 3: Macronutrient accumulation for nitrogen (A), phosphorus (B), potassium (C), calcium (D), magnesium (E), and sulfur (F) at R1 growth stage of GR2 soybean across rates of glyphosate application at different growth stages, V2, V4, and V6 ($n = 8$, $p < 1\%$). Values are based on leaf and petiole nutrient concentrations in upper shoots, which are then expressed on a whole-plant basis excluding the roots. See Tab. 1 for fitted regression equations.

less than when considered as accumulation (Tab. 2). This may be due to a short-term effect of glyphosate resulting in a temporary nutrient deficiency (*Bell*, 2000). The depression in leaf Ca appeared to be temporary as glyphosate at V2 stage no longer depressed leaf Ca at R1 stage, while more recent applications at V4 and V6 did. Leaf sampling closer to the time of glyphosate application may be necessary to detect the short-term depression in nutrient concentrations. For example, the leaf Mn concentrations appeared to be depressed by glyphosate but effects were not significant. Alternatively, if a nutrient deficiency is sustained in glyphosate-treated GR2 soybean, it may be less dependent on temporarily reduced nutrient accumulation than on the interactions of glyphosate with the nutrients within the plant (*Cakmak* et al., 2009), which may lead to insoluble glyphosate–nutrient complexes, as previously demonstrated for Fe and Mn in sunflower (*Helianthus annuus*; *Eker* et al., 2006). In the present study, leaf Fe concentrations increased

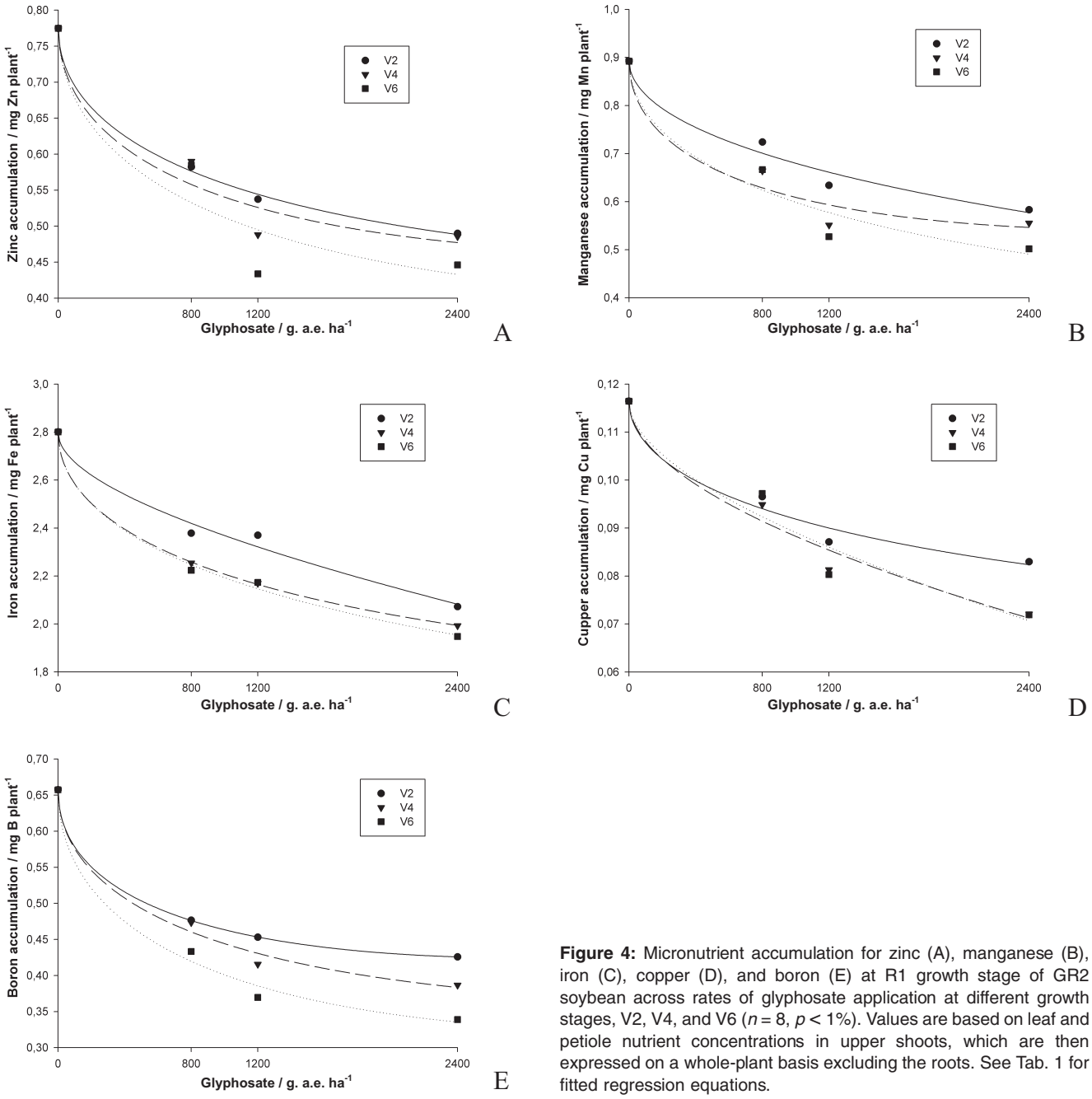

Figure 4: Micronutrient accumulation for zinc (A), manganese (B), iron (C), copper (D), and boron (E) at R1 growth stage of GR2 soybean across rates of glyphosate application at different growth stages, V2, V4, and V6 ($n = 8$, $p < 1\%$). Values are based on leaf and petiole nutrient concentrations in upper shoots, which are then expressed on a whole-plant basis excluding the roots. See Tab. 1 for fitted regression equations.

with glyphosate application suggesting that glyphosate may affect Fe complexation. Sensitive analytical techniques for detecting these complexes, such as electron paramagnetic resonance spectroscopy, were used to demonstrate very limited translocation of glyphosate complexed with Fe in velvetleaf (*Abutilon theophrasti*) leaves treated with glyphosate–-fertilizer mixtures (*Bernards* et al., 2005). Such techniques could be adapted to verify whether glyphosate complexation with nutrient elements does occur in GR soybean.

Reduction in nutrient accumulation can affect important physiological processes within the plant including symbiotic N_2 fixation. *Zobiole* et al. (2010b) reported that nickel was immobilized by glyphosate and could thereby compromise symbiotic N_2 fixation by interfering with hydrogenase activity. Four mechanisms for inhibition of bacterial cell growth by glyphosate have been hypothesized: (1) inability to synthesize aromatic amino acids (*Zablotowicz* and *Reddy*, 2004); (2) an energy drain resulting from formation of terminal metabolites, *e.g.,* 3-deoxy-D-arabino-heptulose-7-phosphate (*Fisher* et al., 1986); (3) toxicity of accumulated intermediates (*Moorman* et al., 1992); and (4) an imbalance of IAA in GR soybeans leading to reduced root colonization and infection by *B. japonicum* (*Kremer* and *Means*, 2009). Decreased nodulation likely occurs by one of these four mechanisms, which, in addition to nutrient immobilization, affects the symbiotic N_2 fixation by reducing available metal cofactors necessary for activity of the many enzymes involved in the process. How-

Table 2: Soybean nutrient concentrations in plant tissue.

Growth stage and glyphosate rate	**Macronutrients** / g (kg plant DW)$^{-1}$						**Micronutrients** / mg (kg plant DW)$^{-1}$				
	N	P	K	Ca	Mg	S	Zn	Mn	Fe	Cu	B
Stage V2											
0	60.1	3.6	17.6	13.3	5.8	4.8	52.4	60.2	155.6	10.3	44.4
800	70.0	4.8	22.4	12.6	4.8	4.0	52.9	60.4	204.9	8.8	42.3
1200	69.2	5.4	23.7	13.0	4.8	4.2	57.6	42.6	214.3	8.9	48.0
2400	76.1	5.8	26.2	14.2	4.6	3.9	60.9	43.4	330.6	9.1	44.7
Stage V4											
0	62.1	3.5	17.0	13.5	5.8	4.8	52.8	65.2	154.4	10.1	53.5
800	59.6	4.4	18.5	11.4	4.3	3.6	46.7	55.1	214.4	7.6	39.8
1200	66.0	5.0	20.9	12.8	3.9	3.1	53.0	59.9	214.3	7.8	42.0
2400	54.9	4.6	20.5	10.6	3.4	2.8	46.1	53.4	247.2	7.6	36.6
Stage V6											
0	61.0	3.6	17.4	13.5	5.8	4.8	53.0	64.7	158.6	10.2	48.0
800	61.0	4.8	19.4	10.9	3.9	3.4	46.6	55.5	189.8	7.9	40.6
1200	77.8	6.4	27.0	8.5	4.1	3.5	37.4	43.5	225.2	8.0	42.0
2400	58.8	5.2	21.6	9.9	3.1	2.8	49.6	48.5	216.4	8.4	42.0
LSD (0.05)	10.1	1.0	9.0	1.0	0.5	1.0	NS	NS	90.0	0.8	10.0

ever, nontreated GR soybean exhibited high nodulation relative to GR soybean receiving glyphosate treatments.

Leaf area and shoot dry weight were depressed by glyphosate, more so at the late growth stage than at the early stage (Fig. 5D and 5E, Tab.1), suggesting that with early applications, plants may have more time to recover from effects of glyphosate or its metabolites. *Huber* et al. (2004) also found that late application of glyphosate generally resulted in lower yields because of increased weed competition and changes in rhizosphere microflora that favored more potential phytopathogens that could lead to plant damage such as root and crown rots. Nontreated soybean showed higher leaf area and biomass production compared with soybean receiving glyphosate treatments.

In general, the parameters A, SPAD, leaf area, and shoot biomass production (Figs. 2A, 2B, 5D, 5E; Tab. 1) correlated significantly and negatively with higher and later glyphosate applications. *Thompson* et al. (1996) also noticed strong correlations of chlorophyll concentration with SPAD readings and leaf area in soybean and reported that SPAD meter readings could be used to distinguish high- and low-leaf-area genotypes in experimental lines selected for differences in these traits.

From an economic point of view, the production of grain is more important than total dry biomass, however, the grain yield of corn and soybean crops is closely linked to the accumulation of dry biomass (*Daughtry* et al., 1992). Although we did not evaluate soybean grain yield (due the weather conditions at R8 growth stage), we can infer that the non-glyphosate control in GR2 soybean likely produced higher yields relative to glyphosate application, because treatments without glyphosate always had high A, SPAD, nodulation, leaf area, shoot and root dry biomass. Further studies should be conducted to verify effects of glyphosate applications on grain yield in GR2 soybeans.

Total biomass production by soybean fundamentally depends on energy supplied by photosynthesis (*Shibles* and *Weber*, 1965). Thus, a decreased photosynthetic rate due to glyphosate (Fig. 2A, Tab. 1) may affect carbon production and contribute to decreased leaf area and biomass production. Decreased shoot and root biomass due to glyphosate likely occurred because of additive effects of decreased photosynthesis and lower nutrient accumulation. Previous reports also demonstrated reduced shoot and root dry weight in GR1 soybean receiving glyphosate at various rates: 600 and 900 g a.e. ha^{-1} (*Zobiole* et al., 2010a), 1200 g a.e. ha^{-1} (*Zobiole* et al., 2010b, c, d), 1680 g a.e. ha^{-1} (*Reddy* et al., 2000), 1800 and 2400 g a.e. ha^{-1} (*Zobiole* et al., 2010a), and 6300 g a.e. ha^{-1} (*King* et al., 2001). These findings agree with those of *Bott* et al. (2008), who noted that glyphosate at recommended label rates applied to a GR soybean cultivar significantly inhibited root biomass and root elongation.

5 Conclusion

Based on the results reported here, late glyphosate applications can be more damaging to GR2 soybean than early applications and contribute to the “yellow flashing” symptom noticed after glyphosate field applications. Thus, an approach to reduce undesirable effects of glyphosate in GR2 soybean

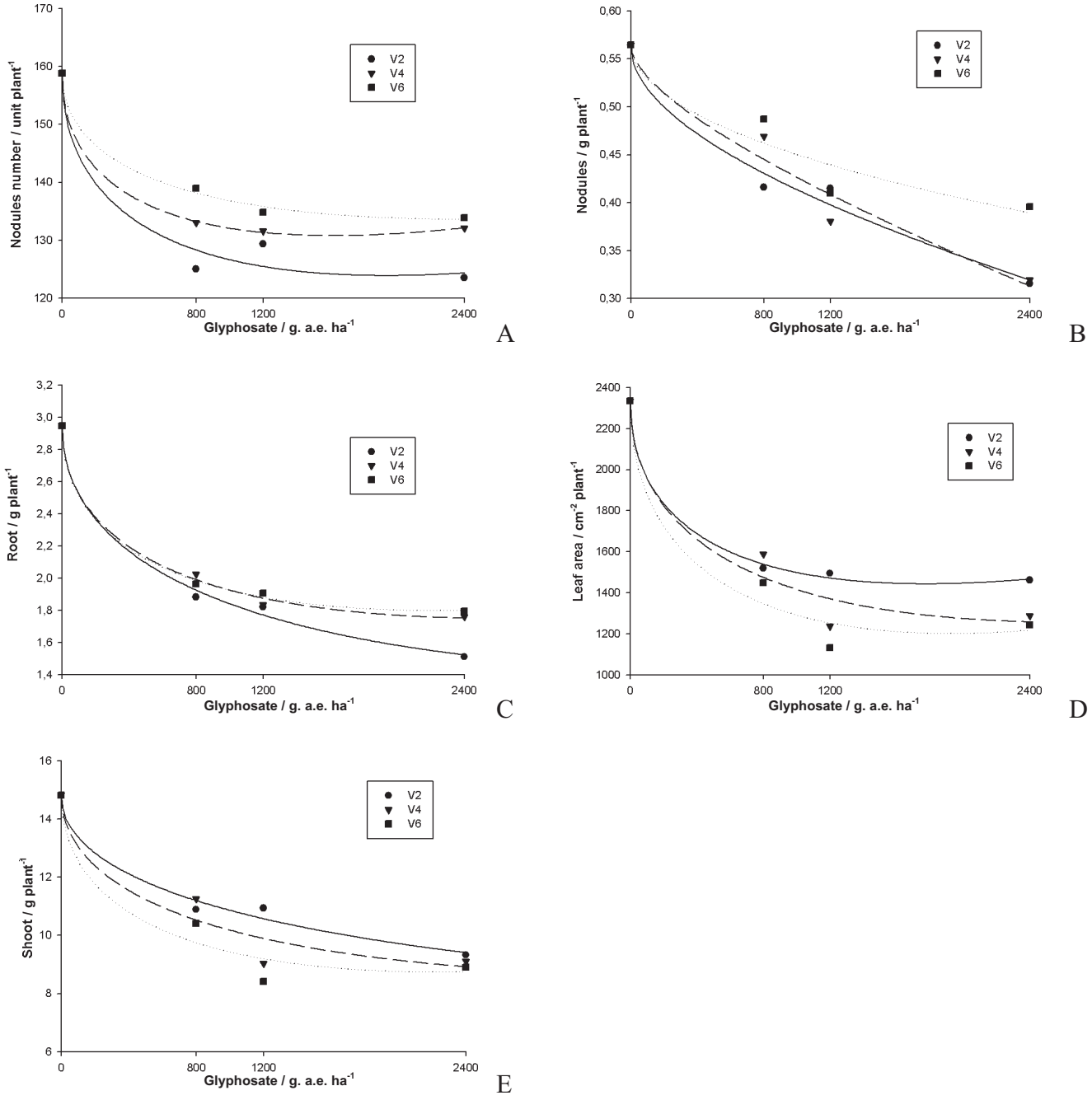

Figure 5: Nodule number (A), nodule dry weight (B), root biomass (C), leaf area (D), and shoot biomass (E) at R1 growth stage of GR2 soybean across rates of glyphosate application at different growth stages, V2, V4, and V6 (n = 8, p < 1%). See Tab. 1 for fitted regression equations.

could be the use of pre-emergence herbicides to prevent early weed interference, and in some cases, glyphosate applied to weeds at an early stage of development. This would likely increase the action of glyphosate, as suggested previously where timing appeared to be more important than rate of herbicide application for adequate weed control (*Ateh* and *Harvey,* 1999). However, the ability to reduce glyphosate rates and implement earlier applications may require deployment of more intensive weed-control strategies by farmers.

Acknowledgments

We thank the *National Council for Scientific and Technology Development (CNPq-Brasilia, DF, Brazil)* for the scholarship and financial support for this research. We also thank Dr. *Randy Miles* for providing plot space on Sanborn Field and *John Gardner* for excellent technical assistance. Trade names are used for clarity and do not represent endorsement by USDA-ARS, the University of Missouri, or the State University of Maringá.

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