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Effect of glyphosate on symbiotic N_2 fixation and nickel concentration in glyphosate-resistant soybeans

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ABSTRACT

Decreased biological nitrogen fixation in glyphosate-resistant (GR) soybeans has been attributed directly to toxicity of glyphosate or its metabolites, to N_2-fixing microorganisms. As a strong metal chelator, glyphosate could influence symbiotic N_2 fixation by lowering the concentration of nickel (Ni) that is essential for the symbiotic microorganisms. Evaluation of different cultivars grown on different soil types at the State University of Maringá, PR, Brazil during the summer 2008 revealed, significant decreases in photosynthetic parameters (chlorophyll, photosynthetic rate, transpiration and stomatal conductance) and nickel content with glyphosate use (single or sequential application). This work demonstrated that glyphosate can influence the symbiotic N_2 fixation by lowering nickel content available to the symbiotic microorganisms.

1. Introduction

Glyphosate is a non-selective, broad-spectrum metal chelating herbicide that inhibits the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS), which is necessary for the synthesis of aromatic amino acids (Jaworski, 1972). In some circumstances plant pathogens contribute to infection of roots of glyphosate-susceptible plants by soil-borne microorganisms due to decreased production of plant protection compounds known as phytoalexins (Kremer et al., 2005).

Glyphosate-resistant (GR) soybeans are developed biotechnologically by introducing of the *cp4* gene coding for resistant forms of EPSP synthase (Duke et al., 1991). The introduction of this type of glyphosate resistance may have unforeseen consequences for symbiotic microorganisms associated with soybeans due to the translocation of glyphosate to important metabolic sinks such as root nodules (Reddy and Zablotowicz, 2003) and the exudation of relatively large quantities of glyphosate into the rhizosphere of GR soybeans (Duke, 1996; Kremer et al., 2005). The soybean nitrogen fixing symbiont, *Bradyrhizobium japonicum*, possesses a glyphosate-sensitive EPSP synthase and accumulates shikimic, hydroxybenzoic and protocatechuic acids (PCA) upon exposure to glyphosate which inhibits growth and induces death at high concentrations (Moorman et al., 1992; De Maria et al., 2006). The toxic effect of glyphosate to *B. japonicum* also has been attributed to the inability of the organism to synthesize aromatic amino acids. The loss of energy and fixed N_2 provided by *B. japonicum* may be significant factors responsible for reduced growth and yield in GR soybean (Moorman et al., 1992; Hernandez et al., 1999).

Herbicides can influence nitrogen metabolism through direct effects on the rhizobial symbiont (Zobiole et al., 2007) or indirectly by effecting the physiology of the host plant (Moorman, 1989). In addition, glyphosate affects the balance of IAA in GR soybeans, which leads to lower root nodulation by *B. japonicum* (Kremer and Means, 2009). Several metabolites or degradation products of glyphosate have been identified or postulated (Rueppel et al., 1977; Sprankle et al., 1978). Among these compounds are aminomethylphosphonic acid (AMPA), sarcosine and glycine (Hoagland, 1980). Chlorotic symptoms in GR soybean following glyphosate application have been attributed to the accumulation of AMPA (Reddy et al., 2004).

Nickel (Ni) is directly related to N_2 fixation, and increases hydrogenase activity in bacteroids isolated from nodules (Klucas et al., 1983). Urease is the only known Ni-containing enzyme in higher plants, although N_2-fixing microorganisms require Ni for

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the hydrogen uptake hydrogenase that processes hydrogen gas generated during N_2 fixation (Evans and Sorger, 1966; Taiz and Zeiger, 1998) and low levels of Ni in agricultural soils can limit the symbiotic hydrogenase activity of *Rhizobium leguminosarum* (Ureta et al., 2005).

The roles of Ni in plant metabolism remain mostly unknown; however effects attributable to Ni deficiency suggest that it may be involved in the transport of nutrients to the seed or grain and movement of Fe into plant cells (Brown et al., 1987). In nature, substantial amounts of H^+ are reduced to H_2 gas that can compete with the reduction of N_2 by nitrogenase electrons. In *Rhizobium*, 30–60% of the energy supplied to nitrogenase can be lost as H_2, decreasing the efficiency of N_2 fixation. *Rhizobia* that have the hydrogenase enzyme to cleave H_2 formed during fixation can generate electrons for N_2 reduction and increase the efficiency of N_2 fixation (Marschner, 1995).

These hydrogenases play a specific role in maintaining the energy efficiency of symbiotic nitrogen fixation. The synthesis of hydrogenases is dependent on a supply of Ni and a mechanism to "sense" that the substrate H_2 is available (Maier and Triplett, 1996). Glyphosate is a phosphonic acid (Franz et al., 1997) chelator of metallic cations (Jaworski, 1972; Kabachnik et al., 1974; Bromilow et al., 1993; Coutinho and Mazo, 2005) that could affect the availability of Ni and may explain the direct effect of glyphosate on N_2 fixation by symbiotic microorganisms. The objective of the present study was to investigate the effect of glyphosate on nodule formation and its interrelation with Ni in GR soybean plants.

2. Material and methods

2.1. Soil and growing conditions

The experiment was conducted in the greenhouse equipped with an evaporative cooling system (25–35: 20–22 °C day/night) under natural daylight conditions at the State University of Maringá, Paraná State, Brazil, between October 14, 2007 and February 15, 2008 (location: 23°25′S, 51°57′W). 5 dm^{-3} polyethylene pots were, filled with either a Typic Hapludox (75% clay, 16% sand, pH $CaCl_2$: 5.40, Al: 0.0, Ca: 8.22, Mg: 3.03, K: 0.47 cmolc dm^{-3}, P: 10.90, S: 5.47, Fe: 88.02, Zn: 11.98, Cu: 32.38, Mn: 95.04 mg dm^{-3} and C_{org}: 7.82 g dm^{-3}) or a Rhodic Ferralsol (21% clay, 71% sand, pH $CaCl_2$: 5.10; Al: 0.0, Ca: 1.85, Mg: 1.24, K: 0.26 cmolc dm^{-3}, P: 18.10, S: 27.06, Fe: 264.30, Zn: 1.73, Cu: 3.08, Mn: 32.82 mg dm^{-3} and C_{org}: 7.82 g dm^{-3}) soil. Characteristics of the soils, organic matter (C_{org}) and pH in $CaCl_2$ were determined as described by Embrapa (1997). The soils were collected from the A horizon and sieved to pass a (10 mesh screen). Independent of chemical analyses, samples of 10 kg the Typic Hapludox soil were fertilized with 100 mg K_2O and 250 mg P_2O_5 per kg of soil and samples of 10 kg the Rhodic Ferralsol soil was amended with 80 mg K_2O, 80 mg P_2O_5 and 1 mg $ZnSO_4$ per kg of soil.

2.2. Seed and glyphosate treatments

Seeds of near-isogenic normal and GR soybean varieties of early (BRS 242 GR and Embrapa 58), medium (BRS 245 GR and BRS 133), and late (BRS 247 GR and BRS 134) maturity groups were treated with 40 g carboxim + 40 g thiram L^{-1} fungicides and 13.5 g Co + 135.0 g Mo L^{-1} 100 kg^{-1} seeds. Seeds were then inoculated with a double commercial rate at 300 mL 100 kg^{-1} of seeds of a culture of *Bradyrhizobium elkanii*, strains SEMIA 587 and SEMIA 5019 at a concentration of 5×10^9 *Rhizobia* per gram. Six seeds per pot were sown at 3 cm depth and thinned to three plants per pot at the one-leaf stage.

The commercially formulated isopropylamine salts of glyphosate (480 g a.e. L^{-1}) was applied to GR soybean: T1—single application of glyphosate (1200 g a.e. ha^{-1}) at the four-leaf stage (25 days after sowing, DAS); T2—sequential application (600 + 600 g a.e. ha^{-1}) at the four-leaf and five-leaf stage (25 DAS and 35 DAS); T3—without glyphosate; and T4—non-GR parental line. The non-GR parental line was considered the treatment control for each cultivar, and did not receive any glyphosate.

Plants were spraying outside the greenhouse, using a backpack sprayer with SF110.02 nozzles, under 2 kgf cm^{-2} of CO_2 at 190 L ha^{-1} to prevent runoff. Air temperature was between 25 and 29 °C, relative humidity was between 80% and 89%, wet soil and wind speed between 5 and 10 km h^{-1} under open sky without cloudiness during glyphosate treatment. After glyphosate application, plans were returned to the greenhouse and irrigated the following day to keep the soil moist, to ensure absorption of the herbicide. The pots were irrigated daily to keep the soil moist, and hand weeded for weed control.

2.3. Data collection

The last fully expanded trifolium (diagnostic leaf) was collected from three plants in each pot to at R1 growth stage to determine the nickel concentration. After dry digestion, Ni was measured by ICP (inductively coupled plasma spectrometry) spectrometry (AES PerkinElmer). The R1 growth stage was slightly different for the cultivars maturity pairs: BRS 242 GR (46 DAS); BRS 245 (54 DAS) and BRS 247 (65 DAS). Prior to collecting leaves, photosynthetic rates (A) of the diagnostic leaf of three different plants in each pot were determined between 7:00 and 11:00 am by infrared gas analysis (IRGA, ADC Model LC*pro+*, Analytical Development Co. Ltd, Hoddesdon, UK).

The chlorophyll index (CI) was measured with a Minolta SPAD-502 meter to measure absorption at 650 and 940 nm wavelengths to estimate chlorophyll concentration (Singh et al., 2002; Richardson et al., 2002; Pinkard et al., 2006). SPAD readings were taken randomly on leaf mesophyll tissue only (with veins avoided) of the terminal leaflet of the diagnostic leaf. Two leaves were chosen per plant in each pot and measurements were averaged to provide a single CI reading per pot. After CI and SPAD assessments, the shoots were clipped close to the ground and roots were carefully removed from soil, washed under running water, packed in paper bags to dry in an air circulation oven at 65–70 °C weighed after a constant dry weight was achieved. Nodules were counted from three plants in each pot immediately after the roots were washed and then placed in the air circulation oven to determine the nodule dry biomass.

2.4. Statistical analyses and experimental design

Main effects and two factor interactions accounted for 96 experimental units distributed in a completely randomized block experimental design. Treatments were combined in a $4 \times 3 \times 2$ factorial scheme with four replicates. The first factor was represented by four herbicide treatments (T1, T2, T3 and T4), the second factor was the cultivar maturity groups and the last factor was soil type. The three near-isogenic pairs of soybean cultivars consisting of the glyphosate-resistant and normal parent of each were selected from early, medium, and late maturity groups commonly grown in Brazil. Embrapa 58 and BRS 242 GR are early maturity cultivars, BRS 133 and BRS 245 GR are medium maturity cultivars, and BRS 134 and BRS 247 GR are late maturity cultivars.

The data errors passed the normality test of Shapiro and Wilk (1965). All data were subjected to analysis of variance and the

Table 1
Nodule number; shoot, root and nodule dry biomass; Ni leaf concentration; and photosynthetic parameters in GR soybeans and their respective non-GR parental lines cultivated in two different soils.

Soil type	Glyphosate treatment/cultivar type	Treatment	Nodules ($plant^{-1}$)	Shoot ($g\,plant^{-1}$)	Root ($g\,plant^{-1}$)	Nodules ($g\,plant^{-1}$)	Ni ($mg\,kg^{-1}$)	Chlorophyll, CI (SPAD units)	Photosynthetic rate (micro mol $CO_2\,m^{-2}\,s^{-1}$)
Typic Hapludox	Single (1200 g a.e. ha^{-1})/GR	T1	223.53 b[a]	8.93 b	4.31 b	1.14 b	0.09 b	28.7 b	12.88 b
	Sequential (600/600 g a.e. ha^{-1})/GR	T2	187.47 b	7.53 b	3.89 b	0.99 b	0.09 b	24.5 b	12.08 b
	Without glyphosate/GR	T3	354.14 a	11.42 a	5.80 a	1.66 a	0.12 a	34.4 a	15.11 a
	Without glyphosate/non-GR	T4	235.61 a	11.19 a	5.19 a	1.51 a	0.18 a	32.7 a	14.42 a
Rhodic Ferralsol	Single (1200 g a.e. ha^{-1})/GR	T1	94.08 b	8.95 b	4.99 b	1.11 b	0.03 b	24.4 b	13.00 b
	Sequential (600/600 g a.e. ha^{-1})/GR	T2	76.81 b	8.00 b	4.40 b	0.87 c	0.03 b	23.9 b	12.72 b
	Without glyphosate/GR	T3	150.42 a	12.29 a	7.11 a	1.43 a	0.05 a	35.7 a	16.09 a
	Without glyphosate/non-GR	T5	121.06 a	12.17 a	6.84 a	1.27 a	0.06 a	36.2 a	18.18 a
CV (%)			28.80	20.49	24.91	21.69	40.15	23.68	24.26

[a] Data represents the average of three maturity group cultivars with four independent replicates. For each column, within each soil type, statistically significant differences at $P < 0.05$ according to the Scott–Knott test, are indicated by different characters.

groupment test of Scott–Knott at 5% probability by SISVAR (Ferreira, 1999).

3. Results

3.1. Effect of soil on glyphosate response

Nodule number, Ni concentration, and shoot, root and nodule dry weight were lower in GR soybeans treated with glyphosate (T1 and T2) in both soils compared with treatments without glyphosate in GR and their non-GR parental lines (T3 and T4). The only difference between soils was lower nodule dry weight in Rhodic Ferralsol soil with the single application (T1) than with the sequential glyphosate application (T2) (Table 1).

3.2. Effect of maturity group on glyphosate response

Varieties from every cultivar maturity groups were affected by glyphosate application (Table 2). Nodule number and shoot, root and nodule dry weight were severely reduced by glyphosate (T1 and T2); however, presence of the GR gene(s) had no effect (T3 and T4) except that the non-GR parental line Embrapa 58 (T4), had lower shoot and nodule dry weight than its near-isogenic variety BRS 242 GR without glyphosate (T3). The more limited root growth of Embrapa 58, may have reduced the nutrient supply to the nodules to cause their dry weight.

Nickel concentration was reduced by glyphosate in two of the three cultivars in this study. All GR plants from the late maturity group treatments had lower Ni than the non-GR parental isoline (BRS 134).

3.3. Effect of glyphosate on chlorophyll and photosynthesis

Chlorophyll (SPAD units) was lower in glyphosate treated plants (T1 and T2) compared with plants without glyphosate (T3 and T4) (Table 1). These observations may be due to direct damage by AMPA to leaf chlorophyll content, produced in soybean treated with glyphosate at rates as low as 1.12 kg ha^{-1} or chelation of the Mg components of chlorophyll or Mn involved in electron transfer during photosynthesis.

Glyphosate treated plants (T1 and T2) exhibited chlorotic symptoms (yellow) compared with plants without glyphosate (T3 and T4) to reflect possible damage to chlorophyll leading to a decreased photosynthetic rate. Thus, the photosynthetic rate was affected by glyphosate through the R1 growth stage. The GR soybeans had less chlorophyll (SPAD units) than their near-isogenic non-GR parental lines (Table 2) and chlorophyll was even lower in GR soybeans treated with glyphosate (T1 and T2)

Table 2
Nodule number; shoot, root and nodule dry biomass; Ni leaf concentration and photosynthetic parameters in GR soybeans and their respective non-GR parental lines in three cultivars of different maturity groups.

Cultivar and maturity group	Glyphosate treatment	Treatment	Nodules ($plant^{-1}$)	Shoot ($g\,plant^{-1}$)	Root ($g\,plant^{-1}$)	Nodules ($g\,plant^{-1}$)	Ni ($mg\,kg^{-1}$)	Chlorophyll, CI (SPAD units)	Photosynthetic rate (micro mol $CO_2\,m^{-2}\,s^{-1}$)
BRS 242—early GR	Single (1200 g a.e. ha^{-1})	T1	148.33 b[a]	9.62 b	5.08 b	1.13 b	0.07 b	31.5 a	14.42 b
BRS 242—early GR	Sequential (600/600 g a.e. ha^{-1})	T2	132.33 b	7.92 b	4.35 b	0.93 b	0.07 b	25.2 b	12.02 b
BRS 242—early GR	Without glyphosate	T3	239.87 a	12.62 a	7.24 a	1.72 a	0.10 a	37.3 a	16.49 a
Embrapa 58—early non-GR	Without glyphosate	T4	156.92 a	13.54 a	4.48 b	0.92 b	0.12 a	35.7 a	20.97 a
BRS 245—medium GR	Single (1200 g a.e. ha^{-1})	T1	167.08 b	8.17 b	4.54 b	1.14 b	0.05 b	25.9 c	11.81 b
BRS 245—medium GR	Sequential (600/600 g a.e. ha^{-1})	T2	140.71 b	7.15 b	3.72 b	0.88 b	0.05 b	21.7 c	12.10 b
BRS 245—medium GR	Without glyphosate	T3	291.75 a	11.20 a	6.66 a	1.45 a	0.08 a	30.3 b	15.79 a
BRS 133—medium non-GR	Without glyphosate	T4	198.83 a	9.33 a	6.94 a	1.60 a	0.14 a	35.5 a	15.37 a
BRS 247—late GR	Single (1200 g a.e. ha^{-1})	T1	161.00 b	9.04 b	4.33 b	1.11 b	0.06 b	25.2 c	12.58 a
BRS 247—late GR	Sequential (600/600 g a.e. ha^{-1})	T2	123.37 b	8.24 b	4.36 b	0.93 b	0.07 b	24.9 c	13.06 a
BRS 247—late GR	Without glyphosate	T3	225.21 a	11.76 a	5.47 a	1.47 a	0.07 b	31.8 b	14.52 a
BRS 134—late non-GR	Without glyphosate	T4	179.25 a	12.17 a	6.63 a	1.64 a	0.10 a	36.9 a	12.52 a
CV (%)			28.80	20.49	24.91	21.69	40.15	23.68	24.26

[a] Data represents the average over two soil types and four independent replicates. For each column, within each cultivar maturity group, statistically significant differences at $P < 0.05$ according to the Scott–Knott test are indicated by different characters.

compared with the non-treated GR control (T3). The early maturity group GR cultivar (BRS 242 GR) had less chlorophyll at R1 growth stage only with the sequential application of glyphosate (T2). The photosynthetic rate (A) was lower in glyphosate treated (T1 and T2) than in non-glyphosate treated (T3 and T4) early and medium maturity group cultivars (BRS 242 GR and BRS 245 GR, respectively) but not for the late maturity group cultivar (BRS 247 GR) (Table 2).

4. Discussion

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 (Harper, 1974). Exposure of *B. japonicum* to glyphosate may interfere with N_2 fixation, leading to an alteration in nitrogen metabolism (Zablotowicz and Reddy, 2004) and a subsequent alteration of carbon metabolism (De Maria et al., 2006).

Nodule dry weight is one of the minimum set parameters to quantify the efficiency of biological nitrogen fixation in soybean (Souza et al., 2008a,b), and an acceptable coefficient of variation (CV) should be <33%. In this study, the CV for nodule dry weight was 21.69% (Tables 1 and 2). The nodule number CV of 28.80% is classified as a medium CV since this variable is considered a parameter of high variability (Souza et al., 2008a); therefore the CV's of this study are considered in an optimum range. It general glyphosate (T1 and T2) reduced parameters correlated with biological N_2 fixation (Table 1) independent of soil type and cultivar. This could be a direct effect of the glyphosate or related to products of glyphosate metabolism that can inhibit biochemical processes related to symbiosis between plants and microorganisms (Moorman et al., 1992).

King et al. (2001) noticed that glyphosate (1.26 kg a.e. ha^{-1}) applied as two applications 5 and 12 days after emergence (DAE) to GR soybeans, significantly reduced nodule biomass accumulation by 33% compared with untreated GR soybean plants at 19 DAE. This was attributed to the lack of resistant EPSPS in *B. japonicum*, in GR soybean nodules.

Reddy and Zablotowicz (2003) studied the effects of a single (two-leaf stage) or two applications (two-leaf and four-leaf stage) of four formulations of glyphosate on the nodulation of GR soybeans under field conditions. They found that nodule number was unaffected 28 DAE by the single glyphosate application at two-leaf stage, however, two applications (two-leaf and four-leaf stage) of all formulations significantly reduced nodule dry weight 28% compared with the untreated control. In addition, glyphosate visibly injured GR soybean, yellow, specking and necrosis ranging from 8% to 38% two days after treatment. Soybean completely recovered from injury over time, and chlorophyll content and dry weight of shoots and roots were unaffected by glyphosate 14 days after treatment. In the research presented here, except for CI in the early GR cultivar, there was no difference between the single and sequential applications of glyphosate in relation to nodulation, when plants were at the R1 growth stage (Tables 1 and 2).

Chlorophyll content did not recover after glyphosate treatment, at the R1 growth stage since they still exhibited chlorotic symptoms (Tables 1 and 2). This research was conducted under greenhouse conditions where temperatures can be higher than the field, and this could have contributed to the low chlorophyll content. Pline et al. (1999) reported that the chlorophyll loss in glyphosate treated GR soybean was rate and temperature dependent, which greater loss at higher glyphosate rates and higher temperatures.

Glyphosate may be partly metabolized to AMPA in soybean, which has been detected in metabolic sinks including seeds and leaves (Arregui et al., 2003; Duke et al., 2003). Shoot and root dry weight, in general, were reduced by glyphosate (T1 and T2). Glyphosate at 1.68 kg a.e. ha^{-1} (Reddy et al., 2000) and 6.3 kg a.e. ha^{-1} (King et al., 2001) have reduced shoot and root dry weights of GR soybean under greenhouse conditions. The potential for GR soybean injury from glyphosate has been reported previously (Reddy et al., 2004). Part of the injury in GR soybean may be caused by AMPA formed from glyphosate degradation with the extent of injury largely dependent on levels of AMPA formed within the plant (Reddy et al., 2004; Zablotowicz and Reddy, 2007). This metabolite is a known phytotoxin and is phytotoxic to GR soybean resulting in reduced chlorophyll and shoot fresh weight (Reddy et al., 2004).

Besides direct toxicity to nodule formation by glyphosate and its intermediates (Moorman et al., 1992; Hernandez et al., 1999; De Maria et al., 2006), another explanation for low nodulation could be low Ni, since Ni is an essential element for microbial nitrogen fixation (Evans and Sorger, 1966; Klucas et al., 1983; Brown et al., 1987; Marschner, 1995; Maier and Triplett, 1996; Taiz and Zeiger, 1998; Ureta et al., 2005).

The sufficient tissue concentration of Ni on a dry matter basis required by plants is reported as 0.1 mg kg^{-1} (Epstein and Bloom, 2005). Although Ni concentrations in Tables 1 and 2 vary from 0.03 to 0.14 mg kg^{-1}, glyphosate reduced Ni 25–40% compared with GR cultivars without glyphosate. This lower Ni content may have occurred due to the ability of glyphosate to chelate cations (Jaworski, 1972; Kabachnik et al., 1974; Lundager Madsen et al., 1978; Glass, 1984; Bromilow et al., 1993; Coutinho and Mazo, 2005; Eker et al., 2006), leading to low Ni availability to symbiotic microorganisms and consequently to fewer nodules and lower nodule dry mass (Tables 1 and 2).

Symptoms of nickel deficiency include chlorosis due to lower absorption of iron, reduced growth of roots and shoots, deformation of various parts of the plant and necrotic spotting of leaves (Mishra and Kar, 1974). The reduced nickel by glyphosate (Tables 1 and 2) is closely correlated with reduced chlorophyll, where reduced chlorophyll was directly related to reduced photosynthetic rate. Either index could explain the visual symptom of chlorosis, observed on GR soybean cultivars treated with glyphosate in this study at the R1 growth stage. Although the photosynthetic rate in the early and medium maturity group cultivars (BRS 242 GR and BRS 245 GR, respectively) were affected by glyphosate, there was no difference with the late maturity group (BRS 247 GR) cultivar. Perhaps the longer growth period of the late maturity group cultivar contributed to its recovery; nevertheless, all cultivars had lower SPAD units that correlate with low chlorophyll content.

Several ions are chelated by glyphosate and it appears that Ca, Fe, and Ni complexes are transported within the xylem while Mn and Zn appear as complexed forms within the phloem (Cataldo et al., 1978); therefore glyphosate chelation of these elements (Glass, 1984; Lundager Madsen et al., 1978), especially Ni, would reduce biological N_2 fixation and result in reduced shoot and root yield.

5. Conclusions

Glyphosate strongly affected the symbiotic N_2 fixation of GR soybeans. Such effects were reduced by decreases on physiological activity (photosynthesis and respiration) and functional chlorophyll and nickel content, which significantly reflected decreases on number of nodules per plant and dry biomass of nodule, shoot and root.

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References

Arregui, M.C., Lenardón, A., Sanchez, D., Maitre, M., Scotta, R., Enrique, S., 2003. Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. Pest. Manage. Sci. 60, 163–166.

Bromilow, R.H., Chamberlain, K., Tench, A.J., Williams, R.H., 1993. Phloem translocation of strong acids: glyphosate, substituted phosphonic, and sulfonic acids in *Ricinus communis* L. Pestic. Sci. 37, 39–47.

Brown, P.H., Welch, R.M., Cary, E.E., 1987. Nickel: a micronutrient essential for higher plants. Plant Physiol. 85, 801–803.

Cataldo, D.A., Garland, T.R., Wildung, R.E., Drucker, H., 1978. Nickel in plants: distribution and chemical form in soybean plants. Plant Physiol. 62, 566–570.

Coutinho, C.F.B., Mazo, L.H., 2005. Complexos metálicos com o herbicida glyphosate: Revisão. Química Nova 28, 1038–1045.

De Maria, N., Becerril, L.M., Garcia-Plazaola, J.I., Hernandez, A., De Felipe, M.R., Fernandez-Pascual, M., 2006. New insights on glyphosate mode of action in nodulant metabolism: role of shikimate accumulation. J. Agric. Food Chem. 54, 2621–2628.

Duke, S.O., Christy, A.L., Hess, F.D., Holt, J.S., 1991. Herbicide-Resistant Crops. Comments CAST (Council for Agricultural Science and Technology) , pp. 1–24.

Duke, S.O., 1996. Herbicide Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. CRC Press, Boca Raton, FL.

Duke, S.O., Rimando, A.M., Pace, P.F., Reddy, K.N., Smeda, R.J., 2003. Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. J. Agric. Food Chem. 51, 340–344.

Eker, S., Ozturk, L., Yazici, A., Erenoglu, B., Romheld, V., Cakmak, I., 2006. Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus* L.) plants. J. Agric. Food Chem. 54, 10019–10025.

Embrapa, 1997. Manual de métodos de análises do solo, 2nd ed. Centro Nacional de Pesquisa de Solos Embrapa, Rio de Janeiro, RJ, Brazil, p. 212.

Epstein, E., Bloom, A.J., 2005. Mineral nutrition of plants: principles and perspectives. Sinauer Associates, Sunderland, 400 pp.

Evans, H.J., Sorger, G.J., 1966. Role of mineral elements with emphasis on the univalent cations. Annu. Rev. Plant Physiol. 17, 47–76.

Ferreira, D.F., 1999. Sistema de análise de variância (Sisvar). versão 4.6. Universidade Federal de Lavras, Lavras.

Franz, J.E., Mao, M.K., Sikorski, J.A., 1997. Glyphosate: A Unique Global Herbicide, ACS Monograph 189. American Chemical Society, Washington, DC, 653 pp.

Glass, R.L., 1984. Metal complex formation by glyphosate. J. Agric. Food Chem. 32, 1249–1253.

Harper, J.E., 1974. Soil and symbiotic nitrogen requirements for optimum soybean production. Crop Sci. 14, 255–260.

Hernandez, A., Garcia-Plazaola, J.I., Bacerril, J.M., 1999. Glyphosate effects on phenolic metabolism of nodulated soybean (*Glycine max* L. Merril). J. Agric. Food Chem. 47, 2920–2925.

Hoagland, R.E., 1980. Effects of glyphosate on metabolism of phenolic compounds. VI. Effects of glyphosine and glyphosate metabolites on phenylalanine ammonia-lyase activity, growth, and protein, chlorophyll, and anthocyanin levels in soybean (*Glycine max*) seedlings. Weed Sci. 28, 393–400.

Jaworski, E.G., 1972. Mode of action of N-phosphonomethyl-glycine: inhibition of aromatic amino acid biosynthesis. J. Agric. Food Chem. 20, 1195–1198.

Kabachnik, M.I., Medved, T.Y., Dyatolva, N.M., Rudomino, M.V., 1974. Organophosphorus complexones. Russ. Chem. Rev. 43, 733–744.

King, A.C., Purcell, L.C., Vories, E.D., 2001. Plant growth and nitrogenase activity of glyphosate-tolerant soybean in response to glyphosate applications. Agron. J. 93, 179–186.

Klucas, R.V., Hanus, F.J., Russel, S.A., Evans, H.J., 1983. Nickel: a micronutrient element for hydrogen-dependent growth of *Rhizobium japonicum* and for expression of urease activity in soybean leaves. Proc. Natl. Acad. Sci. U.S.A. 80, 2253–2257.

Kremer, R.J., Means, N.E., Kim, K.S., 2005. Glyphosate affects soybean root exudation and rhizosphere microorganisms. Int. J. Environ. Anal. Chem. 85, 1165–1174.

Kremer, R.J., Means, N.E., 2009. Glyphosate and glyphosate-resistant crop interactions with rhizosphere microorganisms. Eur. J. Agron. 31, 153–161.

Lundager Madsen, H.E., Christensen, H.H., Gottlieb-Petersen, C., 1978. Stability constants of copper (II), zinc, manganese (II), calcium, and magnesium complexes of *N*-(phosphonomethyl)glycine (glyphosate). Acta Chem. Scand. 32, 79–83.

Maier, R.J., Triplett, E.W., 1996. Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. Crit. Rev. Plant Sci. 15, 191–234.

Marschner, H., 1995. Mineral Nutrition of Higher Plants, 2nd ed. Academic Press, London, 889 pp.

Mishra, D., Kar, M., 1974. Nickel in plant growth and metabolism. Bot. Rev. 40, 395–452.

Moorman, T.B., 1989. A review of pesticides on microorganisms and soil fertility. J. Prod. Agric. 2, 14–22.

Moorman, T.B., Becerril, J.M., Lydon, J., Duke, S.O., 1992. Production of hydroxybenzoic acids by *Bradyrhizobium japonicum* strains after treatment with glyphosate. J. Agric. Food Chem. 40, 289–293.

Pinkard, E.A., Patel, V., Mohammed, C., 2006. Chlorophyll and nitrogen determination for plantation-grown *Eucaliptus nitens* and *E. globulus* using a non-destructive meter. Forest Ecol. Manage. 223, 211–217.

Pline, W.A., Wu, J., Hatzios, K.K., 1999. Effects of temperature and chemical additives on the response of transgenic herbicide-resistant soybeans to glufosinate and glyphosate applications. Pestic. Biochem. Physiol. 65, 119–131.

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

Reddy, K.N., Rimando, A.M., Duke, S.O., 2004. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. J. Agric. Food Chem. 52, 5139–5143.

Reddy, K.N., Zablotowicz, R.M., 2003. Glyphosate-resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. Weed Sci. 51, 496–502.

Richardson, A.D., Duigan, S.P., Berlyn, G.P., 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytol. 153, 185–194.

Rueppel, M., Brightwell, B.B., Schaefer, J., Marvel, J.T., 1977. Metabolism and degradation of glyphosate in soil and water. J. Agric. Food Chem. 25, 517–528.

Shapiro, S.S., Wilk, M.B., 1965. An analysis of variance test for normality. Biometrika 52, 591–611.

Singh, B., Singh, Y., Ladha, J.K., Bronson, K.F., Balasubramanian, V., Singh, J., Khind, C.S., 2002. Chlorophyll meter- and leaf color chart-based nitrogen management for rice and wheat in Northwestern India. Agron. J. 94, 821–829.

Souza, R.A., Hungria, M., Franchini, J.C., Chueire, L.M.O., Barcellos, F.G., Campo, R.J., 2008. Avaliação qualitativa e quantitativa da microbiota do solo e da fixação biológica do nitrogênio pela soja. Pesq. Agrop. Bras. 43, 71–82.

Souza, R.A., Hungria, M., Franchini, J.C., Maciel, C.D., Campo, R.J., Zaia, D.A.M., 2008. Conjunto mínimo de parâmetros para avaliação da microbiota do solo e da fixação biológica do nitrogênio pela soja. Pesq. Agrop. Bras. 43, 83–91.

Sprankle, P., Sandberg, C.L., Meggit, W.F., Penner, D., 1978. Separation of glyphosate and possible metabolites by thin-layer chromatography. Weed Sci. 26, 673–674.

Taiz, L., Zeiger, E., 1998. Plant Physiology. Sinauer Associates, Sunderland, 719 pp.

Ureta, A.C., Imperial, J., Ruiz-Argueso, T., Palacios, J.M., 2005. *Rhizobium leguminosarum* biovar viciae symbiotic hydrogenase activity and processing are limited by the level of nickel in agricultural soils. Appl. Environ. Microbiol. 71, 7603–7606.

Zablotowicz, R.M., Reddy, K.N., 2004. Impact of glyphosate on the *Bradyrhizobium japonicum* symbiosis with glyphosate-resistant transgenic soybean. J. Environ. Qual. 33, 825–831.

Zablotowicz, R.M., Reddy, K.N., 2007. Nitrogenase activity, nitrogen content, and yield responses to glyphosate in glyphosate-resistant soybean. Crop Prot. 26, 370–376.

Zobiole, L.H.S., Oliveira Jr., R.S., Tormena, C., Constantin, J., Cavalieri, S.D., Alonso, D.G., Brighenti, A.M., Castro, C., 2007. Efeito da compactação do solo e do sulfentrazone sobre a cultura da soja em duas condições de água no solo. Planta Daninha 25, 537–545.