

# EFFECT OF GLYPHOSATE ON NODULE FORMATION AND ITS INTERRELATION WITH NICKEL CONTENT IN GLYPHOSATE-RESISTANT SOYBEANS

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**Abstract** Recent research has demonstrated that glyphosate can decrease biological nitrogen fixation in glyphosate-resistant (GR) soybeans, however this reduction has been attributed directly to the effect of glyphosate on the microorganisms or indirectly through its intermediates that are also toxic to these microorganisms. This work aims to demonstrate that glyphosate can influence the symbiotic N<sub>2</sub> fixation by lowering nickel content available to the microorganisms. The experiment was conducted in the greenhouse at the State University of Maringá, PR, Brazil during the summer cropping season, 2008. The experimental design was a completely randomized blocks in a factorial scheme 4 x 3 x 2 where the first factor was: (T1 - single application of glyphosate (900 g a.e. ha<sup>-1</sup>) at the four-leaf stage; T2 - sequential application (450 + 450 g a.e. ha<sup>-1</sup>) at the four-leaf and five-leaf stage; T3 - without glyphosate; T4 - non-GR parental line]; the second factor, maturity group (early, medium and late;) and third factor soil type. Under glyphosate use, GR soybeans showed reduced photosynthetic parameters, nickel contents, and nodule, shoot and root dry biomass.

**Keywords** GR soybean; glyphosate; nitrogen fixation; nickel

## Introduction

The toxic effect of glyphosate to symbiotic microorganisms has been attributed to the inability of the organism to synthesize aromatic amino acids; the toxicity of accumulated intermediates of the shikimic acid pathway also represents a loss of energy and may be a significant factor responsible for reduced growth and yield in GR soybean (Zablotowicz and Reddy, 2004).

Herbicides influence nitrogen metabolism and this influence can occur through direct effects on the rhizobial symbiont (Zobiole et al., 2007) or indirect effects on the physiology of host plant (Moorman, 1989). In addition, glyphosate applied to GR soybeans, affects the balance of IAA in GR soybeans, which leads to lower root colonization by *B. japonicum* (Kremer and Means, 2006).

Nickel is directly related to N<sub>2</sub> fixation, since it increases the hydrogenase activity in bacteroids isolated from nodules (Klucas et al., 1983). Urease is the only known nickel-containing enzyme in higher plants, although nitrogen-fixing microorganisms require nickel for the enzyme that processes some of hydrogen gas generated during fixation (hydrogen uptake hydrogenase) (Evans and Sorger, 1966; Taiz and Zeiger, 1998). Recently Ureta et al. (2005) demonstrated that low level of Ni in agricultural soils can limit the symbiotic hydrogenase activity of *Rhizobium leguminosarum*.

Since glyphosate is considered a phosphonic acid (Franz et al., 1997) and that these acids are known as “chelators” of metallic cations (Jaworski, 1972; Bromilow et al., 1993; Coutinho and Mazo, 2005), it may be hypothesized that if glyphosate can chelate cations, the nickel could also be affected and this could explain the direct effect of glyphosate in low N<sub>2</sub> fixation by symbiotic microorganisms. The objective of the present study was therefore to investigate the effect of glyphosate on nodule formation and its interrelation with nickel content in GR soybean plants.

## Materials and Methods

### *Soil and growing conditions*

The experiment was conducted in the greenhouse at the State University of Maringá, PR, between October 14<sup>th</sup>, 2007 and February 15<sup>th</sup>, 2008 (location: 23° 25' S, 51° 57' W). The experimental units were 5.0 dm<sup>3</sup> polyethylene pots, filled with two different soils: Typic Hapludox (75% clay, 16% sand, pH CaCl<sub>2</sub>: 5.40; Al: 0.0; Ca: 8.22; Mg: 3.03; K: 0.47 cmolc.dm<sup>-3</sup>; P: 10.90; S: 5.47; Fe: 88.02; Zn: 11.98; Cu: 32.38; Mn: 95.04 mg.dm<sup>-3</sup> and C<sub>org</sub>: 7.82 g.dm<sup>-3</sup>) and Rhodic Ferralsol (21% clay, 71% sand, pH CaCl<sub>2</sub>: 5.10; Al: 0.0; Ca: 1.85; Mg: 1.24; K: 0.26 cmolc.dm<sup>-3</sup>; P: 18.10; S: 27.06; Fe: 264.30; Zn: 1.73; Cu: 3.08; Mn: 32.82 mg.dm<sup>-3</sup> and C<sub>org</sub>: 7.82 g.dm<sup>-3</sup>). The soil were collected from the A horizon and sieved (10 mesh).

### *Cultivation practices and glyphosate application*

Six seeds were sown per pot at 3 cm depth. At one-leaf stage only three plants per pot were left. Spraying was accomplished with pots placed outside the greenhouse, using a backpack sprayer with SF110.02 nozzles, under constant pressure 2 kgf cm<sup>-2</sup> of CO<sub>2</sub>, and sprayed at 190 L ha<sup>-1</sup>. The environmental conditions during the applications were air temperature between 25 and 29°C, relative humidity between 80 and 89%, wet soil, wind speed between 5 and 10 km h<sup>-1</sup> and open sky without cloudiness. After herbicide applications, pots were returned to greenhouse and irrigated only the following day, thereby ensuring absorption of leaf's herbicide. Herbicide spraying ceased before causing run-off from leaves. The pots were irrigated daily in order to keep the soil moist, and kept free of weeds by hand weeding.

### *Measurement of response variables*

When plants reached the R1 stage, the last fully expanded trifolium (diagnostic leaf) was collected from three plants in each pot to determine the nickel content. After dry digestion, Ni concentration was measured using AES Perkin Elmer ICP (inductively coupled plasma spectrometry). R1 stage was slightly different for cultivars: BRS 242 GR (46 days after sowing – DAS); BRS 245 (54 DAS); BRS 247 (65 DAS). Before these leaves were collected, the photosynthetic rates (A) were evaluated using an infrared gas analyzer (IRGA) and the chlorophyll index (CI) was measured using a Minolta SPAD-502. After these assessments all harvested materials (aerial part, root and nodules) were then packed in paper bags and dried in a circulating air oven at 65 – 70 °C until a constant weight was achieved. Biomass was determined by weighing plant parts.

### *Data analysis*

Main effects and two factor interactions were the accounted with 96 experimental units, which were distributed in a completely randomized block experimental design. Treatments were combined in a factorial scheme: 4 x 3 x 2 with four replicates. The first factor was represented by four herbicide treatments, using the commercially formulated isopropylamine salts of glyphosate (360 g a.e. L-1) for the treatments to GR soybean [T1 - single application of glyphosate (900 g a.e. ha<sup>-1</sup>) at the four-leaf stage; T2 - sequential application (450 + 450 g a.e. ha<sup>-1</sup>) at the four-leaf and five-leaf stage; T3 - without glyphosate; T4 - non-GR parental line]. The no-GR parental line was considered the treatment control for each cultivar, and did not receive any glyphosate. The second factor was the cultivar maturity groups (BRS 242 GR – early maturity group, BRS 245 GR – medium maturity group and BRS 247 GR – late maturity group), and their respective non-GR parental lines Embrapa 58, BRS 133 and BRS 134, respectively, and the last factor was soil type. All data were subjected to analysis of variance and then tested by Scott Knott groupment test to 5% probability by SISVAR variance analysis software.

## **Results and Discussion**

### *Nickel content and biomass production*

Results obtained for both soils (Table 1) showed that, nodule number, shoot, root and nodule dry weight were lower in GR soybeans under glyphosate treatment (T1 and T2) compared with treatments without glyphosate both in GR and non-GR parental lines (T3 and T4). The only difference between soils was that in the sequential glyphosate application (T2) the nodule dry weight in Rhodic Ferralsol soil was more affected than in the single application. In addition, total nickel content in plants was also lowered by glyphosate use in treatments T1 and T2, compared to T3 and T4 (Table 1). In general, for both soils studied and for all cultivars evaluated, in treatments where glyphosate was applied (T1 and T2), parameters correlated with biological N<sub>2</sub> fixation were reduced (Table 1). This could be related to the products of glyphosate metabolism that can inhibit biochemical processes related to symbiosis between plants and micro-organisms (González et al. 1996).

Analyzing the effects of treatments on different cultivar maturity groups (Table 2), every group was affected by glyphosate application. A strong reduction in nodule number, shoot, root and nodule dry weight occurred under glyphosate use (T1 and T2). However there was no difference between T3 and T4, with the exception of the non-GR parental line Embrapa 58 (T4).

The sufficient tissue concentration on dry matter basis for nickel that may be required by plants is 0.1 mg kg<sup>-1</sup> (Epstein and Bloom, 2005). Although contents shown in Tables 1 and 2 vary from 0.03 to 0.14 mg kg<sup>-1</sup>, the use of glyphosate reduced Ni contents from 25 to 40%, compared with GR cultivars without glyphosate. This lower content may have occurred due to glyphosate's ability to chelate cations (Jaworski, 1972; Glass 1984; Bromilow et al., 1993; Coutinho and Mazo, 2005), leading to low Ni availability to symbiotic microorganisms and consequently to low nodule number and nodule dry weight (Table 1 and 2).

Ions such as Ca, Fe, and Ni are transported within the xylem as organic complexes while Mn and Zn appear to have complexed forms within the phloem (Cataldo et al., 1978); therefore if glyphosate can chelate these elements, one of these being nickel, this lower biological fixation may result and, consequently, lower shoot and root yield.

#### *Photosynthetic parameters*

At R1 stage, changes in chlorophyll content were observed with glyphosate use. For both soils evaluated, the chlorophyll content (SPAD units) was lower in glyphosate treatments (T1 and T2) than in treatments without glyphosate (T3 and T4) (Table 1). These observations may be due to direct damage by glyphosate to leaf chlorophyll content (Reddy et al. 2004). There was a relationship between chlorophyll and photosynthetic rate, which in glyphosate treatments (T1 and T2) this herbicide caused chlorosis symptoms (yellow) in plants different than in treatments without glyphosate (T3 and T4) where there were not this injury, reflecting possible damage to chlorophyll leading to decreases in photosynthetic rate. Thus, the photosynthetic rate was affected by glyphosate on GR soybean growth through the R1 stage.

The photosynthetic rate (A) was lower in glyphosate presence (T1 and T2) than in glyphosate absence (T3 and T4) for early and medium maturity group cultivar (BRS 242 GR and BRS 245 GR, respectively). However, for the late maturity group cultivar (BRS 247 GR) there was no difference among the treatments (Table 2), perhaps due its longer growth that may contribute to its recovery, nevertheless both showed low SPAD units that correlate with low chlorophyll content.

#### **Conclusions**

GR soybeans under glyphosate use showed reduced of all photosynthetic parameters, nickel content, nodule, shoot and root dry biomass.

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**Table 1 Nodule number, shoot, root and nodules dry biomass per pot, nickel leaf contents and photosynthetic parameters at R1 stage in GR soybeans and their respective non-GR parental lines cultivated in two different soils**

Soil type	Herbicide treatment / Cultivar	Treatment	Nodules	Shoot	Root	Nodules	Ni	Chlorophyll (CI)	Photosynthetic Rate
			plant <sup>-1</sup>	-----g plant <sup>-1</sup> -----	-----mg kg <sup>-1</sup> -----	SPAD units	micro mol CO <sub>2</sub> .m <sup>-2</sup> .s <sup>-1</sup>		
Typic Hapludox	Single (900 g a.e. ha <sup>-1</sup> ) / GR	T1	223.53 b*	8.93 b	4.31 b	1.14 b	0.09 b	28.7 b	12.88 b
	Sequential (450 / 450 g a.e. ha <sup>-1</sup> ) / 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 (900 g a.e. ha <sup>-1</sup> ) / GR	T1	94.08 b	8.95 b	4.99 b	1.11 b	0.03 b	24.4 b	13.00 b
	Sequential (450 / 450 g a.e. ha <sup>-1</sup> ) / 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
<b>CV (%)</b>			28.80	20.49	24.91	21.69	40.15	23.68	24.26

\*Data represents average over three cultivar maturity groups per pot and four independent replicates. For each column, within each soil type, statistically significant differences at P<0.05 according to Scott-Knott test, are indicated by different characters.

**Table 2 Nodule number, shoot, root, nodules dry biomass, nickel leaf contents and photosynthetic parameters per pot, at R1 stage in GR soybeans and their respective non-GR parental lines in three cultivars of different maturity groups**

Cultivar and maturity group	Herbicide Treatment	Treatment	Nodules	Shoot	Root	Nodules	Ni	Chlorophyll (CI)	Photosynthetic Rate
			plant <sup>-1</sup>	-----g plant <sup>-1</sup> -----	-----g plant <sup>-1</sup> -----	--mg kg <sup>-1</sup> --	SPAD units	micro mol CO <sub>2</sub> .m <sup>-2</sup> .s <sup>-1</sup>	
Early GR	Single (900 g a.e. ha <sup>-1</sup> )	T1	148.33 b*	9.62 b	5.08 b	1.13 b	0.07 b	31.5 a	14.42 b
Early GR	Sequential (450 / 450 g a.e. ha <sup>-1</sup> )	T2	132.33 b	7.92 b	4.35 b	0.93 b	0.07 b	25.2 b	12.02 b
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
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
Medium GR	Single (900 g a.e. ha <sup>-1</sup> )	T1	167.08 b	8.17 b	4.54 b	1.14 b	0.05 b	25.9 c	11.81 b
Medium GR	Sequential (450 / 450 g a.e. ha <sup>-1</sup> )	T2	140.71 b	7.15 b	3.72 b	0.88 b	0.05 b	21.7 c	12.10 b
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
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
Late GR	Single (900 g a.e. ha <sup>-1</sup> )	T1	161.00 b	9.04 b	4.33 b	1.11 b	0.06 b	25.2 c	12.58 a
Late GR	Sequential (450 / 450 g a.e. ha <sup>-1</sup> )	T2	123.37 b	8.24 b	4.36 b	0.93 b	0.07 b	24.9 c	13.06 a
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
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
<b>CV (%)</b>			28.80	20.49	24.91	21.69	40.15	23.68	24.26

\*Data represents 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 Scott-Knott test are indicated by different characters.