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Original Research Article

Glyphosate influence on the soil microorganism sensibility, physiological parameters of the plant, isoflavones and residues in the seeds and soil

ABSTRACT

The use of Glyphosate herbicide is the best way to control weed growing when cultivating genetically modified soybean [Glycine max (L.) Merr.] resistant to glyphosate (N-phosphono-methyl-glycine) (GMSRG). However, there have been doubts about the safety of this herbicide use concerning its effects on the plant, quality of grains and on the soil cultivated. For this purpose, two soybean genotypes, one GMSRG BRS 243 R and a conventional (isogenic genotype) were grown under different weed control methods: hand weeding and glyphosate herbicide application. The study was performed in Rio Grande do Sul State with evaluations of soil microbial biomass and respiration, chlorophyll, nitrogen, ureides, nitrates, carotenoid, isoflavone content in seeds, and glyphosate and aminomethylphosphonic acid (AMPA) residues in seeds and soil. The use of glyphosate positively affected the microbiologica biomass, basal respiration and seeds yield. On the other hand, the chlorophyll, nitrogen, ureides, nitrates, carotenoid, and isoflavone contents in seeds were unaffected by the treatment. Even though we used the recommended application doses, the glyphosate residues in the seeds were above the levels permitted by the Brazilian law. Also, AMPA residues were detected in the soil and the seeds.

Keywords: residues, isoflavones, chlorophyll, aminomethylphosphonic acid, transgenic soybean.

1. INTRODUCTION

Brazil is the second largest global producer of glyphosate-resistant soybean [Glycine max (L.) Merr.] (GMSRG), already occupied over 20 Mha at 2012 [1], approximately 85% of the Brazilian soybean crop area was planted with GR varieties that was accompanied by heavy use of glyphosate [N-(phosphono-methyl) glycine] [2]. Glyphosate works by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; E.C. 2.5.1.19, CP4 EPSPS), which participates in the biosynthesis of aromatic amino acids, having as substrates shikimate-3-phosphate and phosphoenolpyruvate [3]. The inhibition of shikimate pathway by glyphosate results in the accumulation of shikimic acid and/or hydroxybenzoic acids as well as gallic acid in sensitive plant species [4, 5]. Thus, both primary and secondary metabolism are affected with accumulation of intermediate compounds in different plant organs [6, 7]. In addition, glyphosate is an ionic molecule that easily forms complexes with metals and other soil components, and can be desorbed and/or degraded to aminomethylphosphonic acid (AMPA) [8]. The glyphosate used at recommended doses is not toxic to plants in the soil. In contrast, the AMPA moves to ground water in some soils [9]. Glyphosate resistance in soybean was produced with a transgene encoding GR EPSPS from a microbe that allows normal functioning of the shikimate pathway when the plant´s EPSPS is inhibited by glyphosate [10].

Possible impacts of this genetic modification or glyphosate application were reviewed by Cerdeira et al. [11], Cerdeira and Duke [12], Bohm and Rombaldi [13]. According to them

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there is little evidence that GR soybean affects either soil microorganisms or physical and chemical plant composition (protein, lipid, carbohydrate and mineral contents) including isoflavone levels in soybean seed. Zablotowicz and Reddy [14] showed little sign of inhibition of nodulation or nitrogenase activity by glyphosate application, even when the plants were treated with twice the recommended rate of herbicide on two occasions during growth. Cerdeira et al. [11] reported that early applications of glyphosate can decrease the resistance of soybean to glyphosate GR, causing chlorosis, incidence of infection by Fusarium solani, reductions in biological N2 fixation (BNF), losses in productivity and biomass reduction. According to Correa et al. [15], glyphosate has broad spectrum of against weeds, however, the exclusive use of this herbicide in areas of GR soybeans, may have limitations. For adequate control some species may require higher rates of glyphosate, applied sequentially or even the addition of another herbicide. For adequate control, some species may require higher doses of glyphosate, applied sequentially or combination with another herbicide.

The ureides, allantoin and allantoic acid, are synthesized in nodules of soybeans exported via xylem main products of BNF, reaching about 80% of the nitrogen transported, being used for synthesis of nitrogenous compounds and proteins, essential for plant growth [16]. The content of ureides, or xylem of leaf area is a reliable indicator of the efficiency of BNF, since high concentrations of ureides in soybeans and other legumes are usually associated with effective nodulation and high rates of BNF [17].

Negative effects in plant nutrition or high residue levels in harvested grains are unlikely to occur if the recommendations for glyphosate use are followed [18, 19, 20]. However, in studies carried out in Brazil [2, 21], high residual levels of glyphosate in soybean seeds were detected after applying the recommended rate for the crop. Residue levels were above the limits permitted by Brazilian legislation (10 mg kg-1), which is half of that allowed by most countries consuming this commodity [22]. Even though GR soybean has been widely adopted, very few field studies have been carried out to investigate for possible negative impacts of glyphosate on the plant itself and on the environment and harvested seed. Hence, the present study aimed at evaluating the effects of weed management two soybean cultivars (GR and its conventional isogenic genotype) with the use of different doses of glyphosate on soil processes and microorganisms, physiology and metabolism of the plant along with possible contamination of soil and seed by herbicide residues.

2. MATERIAL AND METHODS

The experiment was performed in the experimental area of the Centro Agropecuário da Palma, Pelotas Federal University, located at Capão do Leão, in the state of Rio Grande do Sul, Brazil (27°57'21" S, 51°48'32"W, at 643 m above sea level.). The soil is classified as Dystrophic Red Yellow Podzolic (U.S. soil taxonomy) with, pH 5.6, 1.4% organic matter and 16% clay.

Twenty plots 4-m wide by 5-m long were set up in the area where five treatments were employed with 4 replicates. The treatments were a combination of the GR soybean cultivar BRS 243RR, or the isogenic conventional cultivar, with different weed-control methods, as follows: (i)T1- BRS 243RR; hand weeding at 28 days after planting (dap); (ii) T2- BRS isogenic; hand weeding at 28 dap; (iii) T3 - BRS 243 RR; one application of glyphosate at 960 g ai ha-1 28 dap; (iv) T4 - BRS 243 RR; one applications of glyphosate at 1920 g ai ha-1 at 28 dap; (v) T5 - BRS 243 RR; one application glyphosate at 3840 g ai ha-1 at 28 dap;

The experiment was repeated in the crop seasons of 2008 to 2009, 2009 to 2010, 2010 to 2011 and 2011 to 2012, always in a different area of the same soil. Each crop season was considered as a block, even though the statistical analysis did not show a block effect.

The soybean cultivars were supplied by the Wheat Centre of Embrapa (Passo Fundo, Rio Grande do Sul State). Soybean seeds were inoculated with Bradyrhizobium japonicum strain SEMIA 5079 and Bradyrhizobium elkanii SEMIA 5878. A fertilizer rate of 250 kg ha-1 of a 0-20-20 NPK was applied in the seedbed just before seeding.

Yield, chlorophyll a and b, carotenoid, total nitrogen, ureides, nitrates and soil microbial biomass and activity were analyzed in samples taken from the experiment along with residues of glyphosate and aminomethylphosphonic acid (AMPA) in soil and seeds and isoflavone content in seeds.

Thirteen days after glyphosate application four plant samples were taken from each plot. The contents of chlorophyll 'a' and 'b' and carotenoids were determined by the method adopted

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by Zaicovski et al. [23]. Samples of 1 g of leaf disks taken from young leaves disregarding the central ribbed leaflets were macerated with 5 mL of 80% acetone (v/v). The material was centrifuged at 14000 g for 10 minutes and the supernatant was retained. This operation was repeated three times. The final volume was adjusted to 25 mL with v. The absorbance of the solution was read in a spectrophotometer Ultraspec 2000 ®, the 647 and 663 nm. The contents of chlorophyll and carotenoids of the samples were determined based on the relationships described by Cardoso et al [24]: Chla = [(12,25*Abs663) – (2,79*Abs647)]*[V/(1000*MF)]; Chlb = [(21,50*Abs647) – (5,10*Abs663)]*[V/(1000*MF)]; Car = [((1 0 0 0 * A b s 4 8 0) – (1 , 8 2 * C h l a) –(85,02*Chlb))/198]*[V/(1000*MF)].
Where: Chla, Car Chlb and are, respectively, the contents of chlorophyll 'a', 'b' and carotenoids; Abs 663 Abs 647 Abs 480 and the absorbance in the length of 663 nm, 645 nm and 480 nm; V is the volume used (ml), and MF fresh weight (g) .The results were expressed in mg g-1.
The quantification of total ureides, allantoin and allantoic acid, the dry tissue of the plant canopy was performed according to the method adopted by Tajima et al. [25] estimated that the content of ureides taking as a basis a standard curve of allantoin in amounts ranging between 12.5 and 125.0 nmol and absorbance readings at 535 nm and results were expressed in nmol N-ureides/g dry mass.
The method described by Ramos et al. [26] was used for determine the total nitrogen. Samples of 0.3 g of shoot dry along with 2 g of catalyst mixture (K2SO4 and CuSO4), 3 glass beads and 5 mL of H2SO4 were slowly digested in digestion block until 380ºC to obtain a colorless extract, used for the determination of nitrogen total. In the distiller 20 mL of 50% NaOH was reacted with colorless extract allowing the collection of 80 mL in 50 mL conical flask containing 4% of boric acid diluted to 10 drops of the mixed indicator mixture. This was titrated with 0.1N HCl solution.
Determination of nitrate (NO3) was performed according to the method adopted by Beninni et al. [27]. The extract dried tissue of the plant in a solution of 5 mL of 0.1 M phosphate buffer, pH 7.0, and 2.5 mL of ethanol. Estimates using as a basis a standard curve of nitrate in amounts ranging from 50 to 500.0 nmol and absorbance readings at 410 nm.
Ninety days after soybean planting (R5 growth stage), four soil samples were taken from each plot at a depth of 0 to 20 cm within plant rows. Carbon in the microbial biomass (MBC) was determined by the method described by Bohm et al [28]. According to this method, soil microorganisms are killed by irradiation at 2450 Mhz for four minutes instead of fumigation with chloroform. The MBC was determined by the difference between the irradiated and non-irradiated soil sample after K2SO4 extraction and C determination by dichromate oxidation and titration with ferrous ammonium sulphate. The value for MBC was calculated by the following formula: MBC = (Ci - Cni)/Kc, where Ci and Cni are the C content of the irradiated and non-irradiated samples, respectively; and Kc is the correction factor with a value of 0.33 (Bohm et al. 2011). The results were expressed in µg CO2 g-1 soil.
Basal respiration (BR), which consists in measuring microbial activity derived from organic carbon decomposition and the quantification of CO2 released, was determined according to the method described by Bohm et al [28]. The quantity of CO2 released from soil of each treatment was trapped in alkali during seven days of incubation and subsequently titrated against HCl. For the calculation of CO2 efflux the formula BR = (B-S) x M x 4 was used, where B is the volume of HCl to titrate the blank flask; S is the volume of HCl to titrate the remaining NaOH from the soil sample; M is the HCl concentration; and 4 (standard value) is the equivalent gram of respired carbon by soil microorganisms. The results were expressed in mg C-CO2 100 g-1.
Soybean yield was estimated by sampling a central area of 1 m2 from each plot at harvest and seed was dried to 12% moisture. Seed was separated and weighed. Also, soil samples from the layer of 0-20 cm of each plot were taken for the analyses glyphosate and AMPA. The method described by Veiga et al. [29] was used for detection and quantification of glyphosate and AMPA residues in soil and seed. Soil samples were dried at 40°C and sieved to pass a 2 mm mesh. Glyphosate and AMPA were extracted by a potassium dihydrogenphosphate solution. A 5 g sample was shaken during 15 min in 25 mL 0.1 M KH2PO4 then centrifuged for 10 min at 2500 g and passed through a Whatman N° 2 filter. The extraction was repeated twice until 70 to 75 mL liquid extract were obtained from each sample. The extracts were concentrated under low temperatures (freeze-dried) and dissolved in Milli-Q® water until a final volume of 10 ml was reached. These extracts were poured through a Millipore® 0.45 µm membrane and kept at a temperature of -20°C until

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analysis by high efficiency liquid chromatography (HPLC). For derivation, 0.1 mL of extract was mixed to 0.9 mL borate buffer 0.025 M (pH 9.0), 0.9 mL of acetone and 0.1 ml of 9-fluorenylmethyl chloroformate. The sample was shaken for 5 min and left for 20 min, then washed three times with ethyl ether. Glyphosate and AMPA were separated by HPLC composed by 50 µl loop Rheodyne® injector, an anionic exchange column C18 of 150 x 2.0 mm with 5 µm particles (Supelco, Bellefonte, PA, USA) and spectrofluormetric detector (Shimadzu®). The mobile phase consisted of standard water for liquid chromatography (LC) at pH 2.5 (adjusted with formic acid) in phase 1 and an aqueous mixture of 5 mM ammonia acetate (pH 4.8), standard water LC and acetonitrile in phase 2.

Glyphosate and AMPA residue analysis in soybean seeds were similar to those used for soil, except that the sample consisted of 1 g of ground soybean seeds, which was extracted with 15 mL of Milli-Q® water, shaking for 30 min, sonicated for 20 min, then centrifuged at 2,000 g at 20°C for 20 min. After that, 4 mL of the supernatant was filtered in Millipore® 0.45 µm membrane. A quantity of 5 mL of water was added to the mixture and the sonication, centrifugation and filtration were repeated. Hence, the same procedures of derivation and quantification were followed as described before. The limits for quantification were 0.04 µg kg-1 for glyphosate and 0.60 µg kg-1 for AMPA, with 90 to 95% recuperation for both molecules.

For the analysis of isoflavone content in seeds, samples were ground in a Janke and Kunkel A-10 (Wilmington, U.S.A.) mill with 0.25 mm sieve. Isoflavones were extracted under mechanical shaker agitation during 2 hours at 4 oC in a 1:20 (m/v) proportion using 80% aqueous methanol [30]. The extracts were then filtered through a Whatman Nº 6 filter paper and evaporated under vacuum (Rotavapor-RE 120 – Büchi, Flawil, Switzerland) to a 2 mL final volume. This solution was then completed to 5 mL with methanol (HPLC degree) and filtered with polyethylene filters with PTFE membrane (Millipore Ltd., Bedford, U.S.A.) of 0.22 µm pores for HPLC analysis. The extractions were done in triplicate. Isoflavones were separated in C18 Nova-pak 4 µm column (Waters, Milford, U.S.A.) according to the method proposed by Song et al [31]. The liquid chromatograph used was a Hewlett Packard (Palo Alto, U.S.A.) model 1100 equipped with a diode detector (DAD) and the ChemStation software. Samples were injected twice. Identification was made based on the spectra and retention time in comparison to known standards, and quantitation was based on external calibration. The 12 isoflavone standards were from LC Laboratories (Woburn, USA). Calibration was performed by injecting the standards three times at five different concentrations ($R2 \geq 0.999$). Total isoflavone contents were expressed as mg of isoflavone aglycone per 100 g of sample (FW.) after normalization of individual isoflavones to account for differences in molecular weight between glycoside derivatives. The mass of each isoflavone form (β-glucosides, malonylglucosides and acetylglucosides) was multiplied by the ratio of its aglycone molecular weight to the molecular weight of the individual form before summing [31].

All the analysis was performed in triplicate and results expressed as mean ± standard deviation. Differences between means were first analyzed by ANOVA test and then Tukey test ($p < 0.05$).

3. RESULTS

At R5 stage of soybean plant development, the amount of microbial biomass C (MBC) did not vary among treatments (Table 1). On the other hand, the basal respiration (BR) was greater where glyphosate applications were performed (Figure 1). The highest metabolic quotients (qCO2) were obtained for treatments with glyphosate application. This result is a consequence of greater microbial activity, with greater release of CO2 per unit of MBC, caused by the presence of easily assimilable substrate for the development of microbial activity. The qCO2 has been used as a biological indicator of the balance of the soil, since as microbial biomass becomes more efficient, less carbon is released as CO2 and respiration by a higher proportion of carbon is incorporated into microbial biomass.

The levels of chlorophyll a and b did not vary among treatments (Table 2). In average, the chlorophyll content varied from 1.275 to 1.663 mg/gMF for chlorophyll a and from 1.294 to 1.988 mg/gMF for chlorophyll b. At R5 stage of plant development, higher levels of carotenoids were obtained by treatments with glyphosate, with no differences among the tested doses (Table 2). For example, untreated plants had 0.419 µmol g-1 and glyphosate

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treated soybean had 0.460 µmol g-1. The total nitrogen did not vary among treatments, reaching averages from 4.575 to 6.025 g/m2 (Table 2). Concerning ureides, the results were inconsistent, i.e., there was not a clear influence of genotype or glyphosate in this parameter. For example, BRS isogenic hand-weed showed higher ureide level, while BRS 243 RR hand-weed or treated with higher glyphosate dose showed the lower level. The highest levels of ureides were obtained by isogenic cultivar hand-weed (7.329 nmol/gms), but did not vary between the other treatments (Table 2).

The yield were obtained by treatments with glyphosate (Table 2). For example, treatment were we used 960 g a.i. ha-1, the yield was 2057.81 kg ha-1, contrasting to 1394.21 kg ha-1 in the treatment BRS 243 RR hand-weed. It indicates that glyphosate contributed to increasing in 47,6 % the productivity.

Total isoflavone contents ranged from 63 to 78 mg/100 g FW for isogenic BRS and BRS 243RR receiving or not glyphosate applications (Table 3), and no significant difference was found among them. These contents were similar to those previously reported for organically grown soybeans (58 to 77 mg/100 g), meanwhile more than twice these values were found in conventionally grown BRS 258 soybeans, from 161 to 193 mg/100 g [32]. There was also a significant difference in the content of daidzein conjugates (almost 50% in isogenic vs 30% in RR) and genistein conjugates (45% in isogenic vs almost 60% in RR). The percentage of glycitein conjugates was half that found in RR (Table 4).

Both glyphosate and AMPA were found in soil and GR soybean seed in the tree glyphosate treatments. Applications of glyphosate at 3840 g a.i. ha-1 brought about a residue of 5.66 mg kg-1 and AMPA residues of 13.8 mg kg-1 (Table 5). Glyphosate concentrations mean of 13.21 mg kg-1 were detected in the treatments with glyphosate applications (Table 5). AMPA concentration was similar to that of glyphosate, reaching 18.23, 28.57 and 32.25 mg kg-1 seed for 960, 1920 and 3840 g a.i. ha-1 applications of glyphosate, respectively.

Table 1. Microbial biomass C (MBC) and basal respiration (BR) of soil samples taken from the areas under different soybean cultivars and weed control methods.

Soybean cultivar; weed control method	MBC µg g^{-1}	BR µg C-CO_2 g^{-1} h^{-1}	qCO_2 (10^{-4})
BRS 243 RR hand-weed[1]	372.23	0.16 [c2]	4.58[c]
BRS isogenic hand-weed	369.93	0.18[c]	5.12[c]
BRS 243RR 960 g ai ha^{-1} glyphosate	410.35	0.24 [bc]	6.91[bc]
BRS 243RR 1920 g ai ha^{-1} glyphosate	383.93	0.32 [ab]	9.44[ab]
BRS 243RR 3840 g ai ha^{-1} glyphosate	352.98	0.41 [a]	11.79[a]
Mean	376.59	0.26	7.57
CV (%)	14.13	18.48	19.03

[1] *At 28 days after planting (dap)*
[2] *Means followed by the same letter are not statistically different according to the Tukey test at a probability < 0.05.*

Table 2 - Chlorophyl, carotenoid, total nitrogen, ureides, nitrates and yield in samples plants soybean taken from the areas with different soybean cultivars and weed control methods.

Soybean cultivar; weed control method	chlorophyll a (mg/gMF)	chlorophyll b (mg/gFW)	Carotenoid µmol g^{-1}	Nitrogen g/m^2	Ureides (nmol /gms)	Nitrates µmol g^{-1}	Yield kg ha^{-1}

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BRS 243 RR hand-weed	1.663 [a]	1.694 [a]	0.419 [ab]	6.019 [a]	4.737 [b]	90.941[a]	1394.21[c]
BRS isogenic hand-weed	1.498[a]	1.793[a]	0.388[b]	6.025 [a]	7.329 [a]	67.062[b]	1654.52[bc]
BRS 243RR 960 g ai ha^{-1} glyphosate	1.275 [a]	1.294 [a]	0.460 [a]	4.799 [a]	5.232 [ab]	96.753[a]	2057.81[a]
BRS 243RR 1920 g ai ha^{-1} glyphosate	1.488 [a]	1.849 [a]	0.465 [a]	4.813 [a]	5.204 [ab]	93.942[a]	2177.60[a]
BRS 243RR 3840 g ai ha^{-1} glyphosate	1.489 [a]	1.988 [a]	0.436[a]	4.575 [a]	4.773 [b]	90.935[a]	2194.41[a]
mean	1.476	1.815	0.434	5.246	5.456	87.434	1895.67
CV (%)	11.42	11.62	7.56	9.40	19.43	10.89	17.41

[*] *Médias com letras idênticas na coluna não diferiram entre si pelo Teste de Tukey (p≤0,05).*

Table 3. Total content (mg/100 g) and profile of isoflavones (%) in seeds of GR soybean BRS 243RR and isogenic BRS receiving applications of glyphosate.

		%			
Soybean cultivar; weed control method	Total isoflavones	β-glucosides	Malonylglucosides	Acetylglucosides	Aglycones
BR 243RR; hand-weed[‡]	63.1 ± 11.7a	56.32[a]	41.08[b]	n.d.	2.59[b]
BR isogenic; hand-weed	73.6 ± 12.4a	49.20[b]	46.92[a]	n.d.	3.88[a]
BR 243RR; 960 g ai ha^{-1} glyphosate [¶]	77.7 ± 6.1a	55.88[a]	41.93[b]	n.d.	2.19[b]
BR 243RR; 1920 g ai ha^{-1} glyphosate [¶]	70.3 ± 10.8a	55.21[a]	41.96[b]	n.d.	2.83[b]
BR 243RR; 3840 g ai ha^{-1} glyphosate [#]	72.3 ± 5.8a	55.57[a]	41.96[b]	n.d.	2.46[b]
Mean	71.4	54.44	42.77	-	2.79
CV (%)	7.5	1.40	1.55	-	16.87

Means followed by the same letter in the same column are not statistically different according to the Tukey test at a probability < 0.05.

Table 4. Contents of main types of isoflavone in seeds of GR soybean BRS 243RR and isogenic BRS receiving applications of glyphosate.

Soybean cultivar; weed control method	Fractions (%)[†]

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	Daidzein	Glycitein	Genistein
		-%	
BR 243RR; hand-weed‡	30.32[b]	10.74[a]	58.92[a]
BR isogenic; hand-weed	49.37[a]	5.09[b]	45.52[b]
BR 243RR; 960 g ai ha^{-1} glyphosate	30.59[b]	10.51[a]	58.90[a]
BR 243RR; 1920 g ai ha^{-1} glyphosate	30.22[b]	11.30[a]	58.48[a]
BR 243RR; 3840 g ai ha^{-1} glyphosate	29.51[b]	10.56[a]	59.92[a]
Mean	34.00	9.64	56.38
CV (%)	5.19	17.96	3.57

† The total percentage of each aglicone represents the sum of free and conjugated isoflavones expressed as aglicones.
‡ At 28 d after planting (dap).
§ Within columns, means followed by the same letter are not statistically different according to the TUKEY test at a probability < 0.05.

Table 5. Concentration of glyphosate and aminometylphosphonic acid (AMPA) in soil and seed of GR soybean BRS 243RR receiving applications of glyphosate.

Soybean cultivar; weed control method	glyphosate (mg.kg^{-1})	AMPA (mg.kg^{-1})	glyphosate (mg.kg^{-1})	AMPA (mg.kg^{-1})
	solo		grãos	
BRS 243RR 960 g ai ha^{-1} glyphosate	3.28[c]	10.30[b]	15,37[b]	18,23[b]
BRS 243RR 1920 g ai ha^{-1} glyphosate	4.36[b]	12.11[ab]	18,07[a]	28,57[ab]
BRS 243RR 3840 g ai ha^{-1}glyphosate	5.66[a]	13.80[a]	19,63[a]	32,25[a]
CV	14.74	15.01	13.21	19.50
P	0.001	0.020	0.006	0.040

n.d. not detected. Means followed by the same letter are not statistically different according to the Tukey test at a probability < 0.05.

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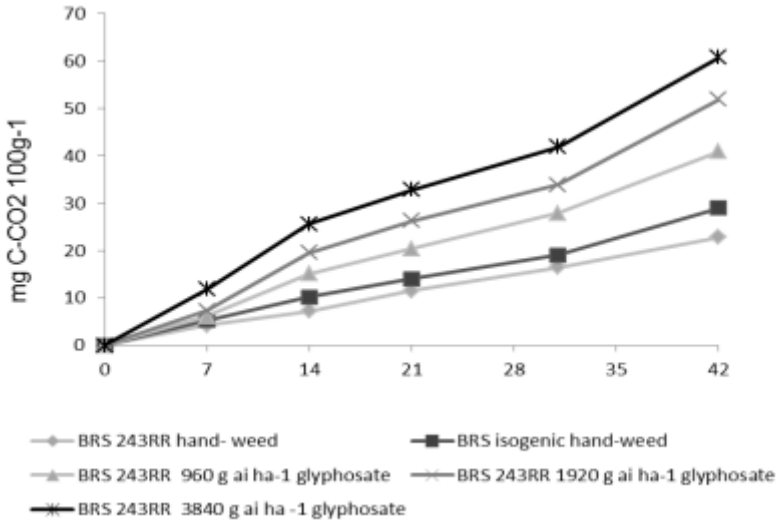

Figure 1. Basal respiration of soil samples, incubated for 42 days, taken from the areas under different soybean cultivars and weed control methods, the results were expressed in mg C-CO_2 100 g^{-1}.

4. DISCUSSION

The putative responses of glyphosate in the GR soybean cultivation on the soil microorganisms activity, grains yield and quality remains barely answered. For example, Haney, Senseman and Hons [33] detected a higher MBC in soils in which there was application of glyphosate herbicide. On the other hand Gomez et al [34] detected lower levels of CBM in soils with application of glyphosate. Here, in our experiment (four seasons), not changes were observed between treatments. The exact causes of these differences have not been studied, but may be associated with differences in soil and microbial activity of studied environments. Concerning the basal respiration (BR) and metabolic quotients (qCO2), higher levels were detected in soil were the glyphosate was applied. This change in respiration may be related to the increase in concentration of the easily degradable glyphosate molecule in soil that could stimulate soil N mineralization [33] and decrease the plant reliance on BNF [35]. However, per the authors literature review [7, 11, 12, 20, 29, 33] glyphosate has generally not been documented to have negative effects on soil.

Is cited [14] that Nitrogen content could be affected by glypohosate application. According Reddy et al. [6] and King et al. [36] the glyphosate applied at the dosage recommended by the manufacturer reduces the number and mass of nodules, the leg-hemoglobin content and the nitrogen content in the plant. In contrast, Bohm et al. [35] in a field experiment with application of glyphosate at 1920 g ha-1 did not find effect in the nitrogen contents of the plant, although the treatments with glyphosate in GM soybean reverberated less dependent on plant by FBN (below 70%) when compared to GM soybean under weeding, whose dependence by BNF was estimated at 80%. Here, we observed that Nitrogen did not vary among treatments.

Previous study [14] carried out in a field experiment, showed that glyphosate application had little effect at ureides and total amino acids when applied in the recommended doses. However, when glyphosate was applied at a dose of 3360 g ha-1 decreases in BNF were observed, as well as the levels of foliar nitrogen and total ureides. The reduction of BNF by glyphosate application was also observed by Bohm et al. [35]. Here, we observed that the ureides contents changes did not showed a co-variation response in function of genotype or glyphosate application.

Also, is cited that glyphosate could affect plant chlorophyll stability, if applied at high doses (at 2240 g a.i. ha-1) [6]. However, they observed that, in general, glyphosate application at a dose of 1120 g a.i. ha-1 had little or no effect on the chlorophyll content in

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GR soybean. Here, in this study, doses from 960 to 3840 g a.i. ha-¹ glyphosate were tested in four seasons and none effect was observed in the leaves chlorophyll content. The main differences between these works are related to model of cultivation: Redddy et al. [6] cultivated the soybean in the greenhouse and we cultivated in the field. In addition, the cultivars were not the same. The carotenoids, that are important photossynthetic protectors [37], increased in the in the soybean treated with glyphosate. It´s possible that the increasing of carotenoid content had a protective effect of chlorophylls, despite of high glyphosate doses. This hypothesis is based in works that shows increasing of carotenoids synthesis in plants submitted to the moderate abiotic stress [38].

As expected the better grain yield was obtained from treatment were weeds were controlled by glyphosate. Cerdeira et al. [11] mentioned that increasing productivity occurs because the application of glyphosate provides excellent weed control favoring less competition, better photosynthesis and nutrient utilization. In contrast, yields of GR soybean crops have been reported to be unaffected by glyphosate application [39, 40].

In order to monitoring grains quality, the isoflavone contents was also quantified. The weeds control methods did not affected the accumulation of theses specialized metabolites. Soybean contains three types of isoflavones, in four chemical forms: the aglycones daidzein, genistein, and glycitein; the β-glycosides daidzin, genistin, and glycitin; the acetyl-β-glycosides 6''-O-acetyl-β-daidzin, 6''-O-acetyl-β-genistin, 6''-O-acetyl-β-glycitin; and the malonyl-β-glycosides 6''-O-malonyl-β-daidzin, 6''-O-malonyl-β-genistin, and 6''-O-malonyl-β-glycitin [41]. Aglycones and acetylglycosides are not normally present in seeds and are formed as a result of processing such as drying, defatting, and storage. No significant differences were found among BRS 243 RR receiving or not glyphosate applications in relation to the profile and distribution of isoflavones. However, isogenic BRS presented a higher content of malonylglycosides and a lower content of β-glucosides than 243 RR. There was also a significant difference in the content of daidzein conjugates and genistein conjugates. It is known [41] that malonylglucosides are highly unstable and easily converted to β-glucosides. The sum of malonylglucosides plus β-glucosides is among the expected values, for all the samples. However, regarding the distribution of isoflavones (the total percentage of daidzein and its conjugates, glycitein and its conjugates, and genistein and its conjugates), important differences were detected, with daidzein conjugates being the prevalent forms only in isogenic BRS. Similarly to BRS RR, ten BRS Brazilian varieties previously analyzed presented prevalence of genistein conjugates [41]. This alteration is probably related to an up or down-regulation of their biosynthesis. Daidzein is known to be the precursor of the phytoalexin glyceollin, and the accumulation of glyceollin correlates with resistance to various fungal pathogens [42].

Both glyphosate and AMPA were found in soil and GR soybean seed in the tree glyphosate treatments. The fact that concentration of AMPA in soil was higher than of glyphosate is explained by Ginsing et al. [43], who reported that glyphosate biodegradation happens faster than that of AMPA. This is confirmed in the study of Araujo et al. [44], who found proportionally higher AMPA accumulation in soils with successive glyphosate applications. Even though glyphosate and AMPA levels detected in soil are a matter of concern there are no official parameters establishing a threshold. The glyphosate content in seeds were above the limits permitted by Brazilian Agency for Sanitary Vigilance is 10 mg kg-1 [45], with is half of that allowed by USA (20 mg kg-1) and almost all countries of the European community (20 mg kg-1). The reason(s) for the high levels was not explained in this work. However, it is known that the response to treatments, and therefore the prevalence of residues is dependent on edaphoclimatic conditions. The experiment was carried out in an area known to have a significant incidence of abiotic stresses (water stress, temperature changes and intense solar radiation), which are not common (at least concomitantly) in most areas of soybean production.

5. CONCLUSION

The use of glyphosate positively affected the microbiological biomass, basal respiration and seeds yield. On the other hand, the chlorophyll, nitrogen, ureides, nitrates, carotenoid, and isoflavone contents in seeds were unaffected by the treatment. However, even though we used the recommended application doses, the glyphosate residues in the seeds were above the levels permitted by the Brazilian law. Also, AMPA residues were detected in the soil and the seeds.

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REFERENCES

1.ISAAA- International Service for the Acquisition of Agri-biotech Applications - Global Status of Commercialized Biotech/GM Crops: 2006. Online. Disponível na Internet em: <http://www.isaaa.org/Resources/Publications/briefs/35/executivesummary/default.html >. Acesso em 15 ago 2015.

2. Bohm GMB, Rombaldi CV, Genovese MI, Castilhos D, Alves BJR, Rumjanek NG (2014) Glyphosate Effects on Yield, Nitrogen Fixation, and Seed Quality in Glyphosate-Resistant Soybean. Crop Science 54(4):1737-1743.

3. Fisher RS, Berry A, Gaines CG, Jensen RA (1986) Comparative action of glyphosate as a trigger of energy drain in Eubacteria. Journal Bacteriology 168:1147-1154.

4. Duke SO (1988) Glyphosate. In Herbicides: Chemistry, Degradation, and Mode of Action; Kearney PC, Kaufman D D, Eds.; Dekker: New York 3 :1-70.

5. Nandula VK, Reddy KN, Rimando AM, Duke SO, Poston DH (2007) Glyphosate- resistant and susceptible soybean (Glycine max) and canola (Brassica napus) dose response and metabolism relationships with glyphosate. J. Agric. Food Chem. 55(9):3540-3545.

6. Reddy KN, Hoagland RE, Zablotowicz RM (2000) Effect of glyphosate on growth, chlorophyll, and nodulation in glyphosate-resistant and susceptible soybean (Glycine max) cultivars. Journal of New Seeds 2:37–52.

7. Moldes CA, Camiña JM, Medici LO, Tsai SM, Azevedo RA (2012) Physiological effects of glyphosate over amino acid profile in conventional and transgenic soybean (Glycine max). Pesticide Biochemistry and Physiology 102(2):134-141.

8. Reddy KN, Rimando AM, Duke SO (2004) Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. Journal of Agricultural and Food Chemistry 52:5139-5143.

9. Landry D, Dousset S, Fournier JC, Andreux F (2005) Leaching of glyphosate and AMPA under two soil management practices in Burgundy vineyards (Vosne-Romanée, 21-France). Environmental Pollution 138(2):191-200.

10. Green JM (2009) Evolution of glyphosate-resistant crop technology. Weed science 57:108-117.

11. Cerdeira AL, Gazziero DPL, Duke SO, Matallo MB, Spadotto CA (2007) Review of potential environmental impacts of transgenic glyphosate-resistant soybean in Brazil. Journal of Environmental Science and Health (Part B) 42:539-549.

12. Cerdeira AL, Duke SO (2007) Environmental impacts of transgenic herbicide-resistant crops. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2:44-53.

13. Bohm GMB, Rombaldi CV (2010) Effect of genetic transformation and application of glyphosate in soil microbiota, biological nitrogen fixation, quality and safety of genetically modified soybeans. science Rural 40: 213-221.

14. Zablotowicz RM, Reddy KN (2007) Nitrogenase activity, nitrogen content, and yield responses to glyphosate in glyphosate-resistant soybean. Crop Protection 26:370–376.

15. Correia NM, Durigan JC, Spanish M (2011) Weed management in GM soybeans tolerant to glyphosate. Agricultural Research, 41(2):242-247. Doi: 10.5216/pat.v41i2.8807

16. Ohyama T, Ohtake N, Sueyoshi K, Tewari K, Takahashi Y, Ito S, Nishiwaki T, Nagumo Y, Ishii S, Sato A (2008) Nitrogen fixation and metabolism in soybean plants. In Nitrogen fixation research progress, Editor by Guilhem N. Couto. Nova Science Publishers, Inc. New York, 1-131.

17. Herridge DF, Peoples MB (1990) Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by 15N methods. Plant Physiology 93: 495-503.

18. Arregui MC, Lenardón A, Sanchez D, Maitre MI, Scotta R, Enrique S (2003) Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. Pest Management Science 60: 163-166.

19. Duke SO, Rimando AM, Pace PF, Reddy KN, Smeda RJ (2003) Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. Journal of Agricultural and Food Chemistry 51:340-344.

20. Duke SO, Reddy KN, Bu K, Cizdziel JV (2012) Effects of glyphosate on the mineral content of glyphosate-resistant soybeans (glycine max). Journal of Agricultural and Food Chemistry 60(27):6764-6771.

21. Bohm GMB, Genovese MI, Pigosso G, Trichez D, Rombaldi CV (2008) glyphosate residues and aminomethylphosphonic acid, and isoflavones content in soybean BRS 244

UNDER PEER REVIEW

448 RR and BRS 154 grown in Planosol. Journal of Food Science and Technology 28 (supl.):
449 192-197.
450 22. AGROFIT (2015) Sistema de Agrotóxicos Fitossanitário. Available at
451 <http://www.agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Accessed in
452 february 25th, 2015.
453 23. Zaicovski CB, Pegoraro C, Ferrarezze JP, Dal Cero J, Lund DG, Rombaldi CV (2008)
454 Effect of mechanical damage, temperature reduction and 1-MCP in Legacy broccoli
455 postharvest metabolism. Science and Technology Food 28 (4):840-845.
456 24. SN Cardoso Oliveira LM, LG Fernandez, Pelacani CR, Souza CLM, ARMF Oliveira
457 (2012) priming on seed germination, early growth and content Myracrodruon urundeuva fr
458 pigments. Allemão. Journal of Biosciences 10(4):457-461.
459 25. Tajima S, Nomura M, Kouchi H (2004) Ureide biosynthesis in legume nodules. Frontiers
460 on Bioscience 1 (9):1374-81.
461 26. Ramos MG, Villatoro MAA, Urquiaga S, Alves BJR, Boddey RM (2001) Quantification of
462 the contribution of biological nitrogen fixation to tropical green manure crops and the residual
463 benefit to a subsequent maize crop using 15N-isotope techniques. Journal of Biotechnology
464 91:105–115.
465 27. Beninni ERY, Takahashi HW, Neves CSVJ, Fonseca ICB (2002) nitrate content in
466 lettuce grown in hydroponic and conventional systems. Brazilian horticulture 20: 183-186.
467 28. Bohm GMB, Schneider L, D Castilhos, Rombaldi CV (2011) Weed control, microbial
468 biomass and soil metabolism due to the application of glyphosate and imazethapyr in
469 soybeans. Semina: Agricultural Sciences, 32(3):919-930.
470 29. Veiga F, Zapata JM, Fernandez-Marcos ML, Alvarez E (2001) Dynamics of glyphosate
471 and aminomethylphosphonic acid in a forest soil in Galicia, north-west Spain. The Science of
472 the Total Environment 271:135-144.
473 30. Genovese MI, Lajolo FM (2001) DETERMINAÇÃO em isoflavones derived from
474 soybeans. Food Science and Technology 21:86-93. Doi.org/10.1590/S0101-
475 20612006000400032.
476 31.Song T, Barua K, Buseman G, Murphy PA (1998) Soy isoflavone analysis: quality control
477 and a new internal standard. American Journal of Clinical Nutrition 68:1474-1479.
478 32. Balisteiro DM, Rombaldi CV, Genovese MI (2013) Protein, isoflavones, trypsin inhibitory
479 and in vitro antioxidant capacities: Comparison among conventionally and organically grown
480 soybeans. Food Research International 51: 8-14.
481 33. Haney RL, Senseman SA, Hons FM (2002) Bioremediation and biodegradation: effect of
482 roundup ultra on microbial activity and biomass from selected soils. Journal Environmental
483 Quality 31:730-735.
484 34. Gomez E, Ferreras L, Lavitti L, Fernandez E, 2009. Impact of glyphosate application on
485 microbial biomass and metabolic activity in a vertic argiudoll from Argentina. European
486 Jornal of Soil Biology 45(2):163-167.
487 35. Bohm GMB, Alves BJR, Urguiaga S, Boddey R M, Xavier GR, Hax F, Rombaldi CV
488 (2009) Glyphosate and imazethapyr-induced effects on yield, nodule mass and biological
489 nitrogen fixation in field-grown glyphosate-resistant soybean. Soil Biology & Biochemistry
490 41(2): 420-422.
491 36. King CA, Purcell LC, Vories E (2001) Plant growth and nitrogenase activity of
492 glyphosate-tolerant soybean in response to foliar glyphosate applications. Agronomy Jornal
493 93:179–186.
494 37. Azmat R (2013) An important role of carotenoids in protection of photosynthetic
495 apparatus under VAM inoculation on Momordica charantia. Curr Pharm Biotechnol
496 14(9):829-34.
497 38. Gururani MA, Venkatesh J, Ganesan M, Strasser RJ, Han Y, Kim J-I, Song P-S. (2015).
498 In Vivo Assessment of Cold Tolerance through Chlorophyll-a Fluorescence in Transgenic
499 Zoysiagrass Expressing Mutant Phytochrome A. PLoS ONE, 10(5), e0127200.
500 Doi:10.1371/journal.pone.0127200.
501 39. Elmore RW, Roeth FW, Nelson LA, Shapiro A, Klein RN, Knezevic SZ, Martin A (2001)
502 Glyphosate-resistant soybean cultivar yields compared with sister lines. Agronomy Journal
503 93(2):408-412. Doi:10.2134/agronj2001.932408x

UNDER PEER REVIEW

504 40. Norsworthy JK (2004) Broadleaved weed control in wide-row soybean (Glycine max)
505 using conventional and glyphosate herbicide programmers. Crop Protection 23(12):1229-
506 1235.
507 41. Genovese MI, Hassimotto NMA, Lajolo FM (2005) Isoflavone profile and antioxidant
508 activity of Brazilian soybeans varieties. Food Science and Technology International
509 11(3):205-211.
510 42. Lozovaya VV, Lygin AV, Zernova OV, Ulanov AV, Li SX, Hartman GL, Widholm JM
511 (2007) Modification of phenolic metabolism in soybean hairy roots through down regulation
512 of chalcone synthase or isoflavone synthase. Planta 225(3):665-679.
513 43. Gimsing AL, Borggaord OK, Jacobsen OS, Aamand J, Sorensen J (2004) Chemical and
514 microbiological soil characteristics controlling glyphosate mineralization in Danish surface
515 soils. Applied Soil Ecology 27:233–242.
516 44. Araújo ASF, Monteiro RTR, Abarkeli RB, Souza LS (2003) Biodegradation of glyphosate
517 in two Brazilian soils. Pesticides: Journal of Ecotoxicology and Environmental 13: 157-164.
518 45. ANVISA (2007) National Health Surveillance Agency. Available at
519 <http://www.anvisa.gov.br>. Accessed in july 25th, 2014.
520
521