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Effects of glyphosate on nodulation and nitrogen fixation of transgenic glyphosate-tolerant soybean

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Abstract: Background: The use of glyphosate on glyphosate-tolerant soybean crops led to improved control of a wide range of weeds, which resulted in reduced costs with the no-till system. The emergence of the first herbicide-resistant weeds have driven an increase in glyphosate applications, and even though those soybean materials have a low sensitivity to glyphosate, the rhizobial symbionts could be affected by the herbicide, and plants might be indirectly injured.

Objective: This study aimed to determine the effect of multiple glyphosate applications throughout the soybean crop cycle on plant growth, nodulation and biological nitrogen fixation (BNF).

Methods: The effects of one, two and three treatments of a recommended dose of glyphosate on BNF and growth of glyphosate-tolerant soybean plants were evaluated in greenhouse and field experiments.

Results: Two or more applications of glyphosate inhibited the BNF and growth of soybean plants. Under controlled conditions, at least one glyphosate application at V1 affected the number and mass of nodules per plant, and successive applications in advanced phonological stages resulted in the inhibition of nodule growth. With two and three sprayings of glyphosate, the proportion of N derived from the air in plants was reduced by 41% compared with the treatment without glyphosate. In field experiments, detrimental effects of three sequential applications of glyphosate on number of nodules per plant (-25%), biomass production (-21%) and grain yield (-36%) were detected.

Conclusions: Multiple glyphosate applications of glyphosate inhibited the BNF and growth of soybean plants and it could be as damaging as +weed interference.

Nomenclature: Glyphosate; soybean, *Glycine max* (L.) Merril

Keywords: Biological Nitrogen Fixation; N derived from the air; RR Soybean

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1. Introduction

Soybean (*Glycine max* (L.) Merril) is the most economically important legume in the world. Soybean grains are used as a source of protein for the human diet and livestock and as a raw material for cooking oil and biofuel (Hartman et al., 2011). Before the 1960s, Asian farmers were responsible for producing the majority of the world's soybeans. The expansion of the crop led to the annual world production of 28.6 million metric tons between 1961 and 1965. However, this production has increased markedly since the introduction of genetically modified soybeans and reached 348.7 million metric tons in 2018 (James, 2010; Food and Agriculture Organization, 2022). Brazil is the largest producer, generating 37% of that volume; the United States is the second producer with 31% of world production, and Argentina is the third-largest producer harvesting 13% of the global production (US Department of Agriaulture, 2022). The three countries have approved genetically modified soybean varieties and at least 75% of the world's total area planted with soybean is genetically modified, where the main transgenic properties confer glyphosate resistance (Brookes, Barfoot, 2018). The use of glyphosate on soybean crops leads to improved control of a wide range of weeds, which has resulted in reduced costs with the no-till system (Reddy, 2001; James, 2010; Brookes, Barfoot, 2018).

Glyphosate is the most used herbicide worldwide with about 600 to 750 thousand tonnes applied annually, and, far from decreasing, an expected consumption of 740 to 920 thousand tonnes has been projected by 2025 (Maggi et al., 2019). This herbicide inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs; EC. 2.5.1.19) and interrupts the synthesis of aromatic amino acids in susceptible plants (Franz et al., 1997). Thus, glyphosate affects protein synthesis, inhibiting plant growth (Duke, Powles, 2008).

Although photosynthesis is not a primary inhibitory target of glyphosate, it has been reported to be affected by the herbicide, triggering a rapid inhibition of photosynthetic CO_2 assimilation and a reduction of photoassimilate translocation (Vivancos et al., 2011; Olesen, Cedergreen, 2010; Yanniccari et al., 2012; Zobiole et al., 2010). Glyphosate-tolerant soybeans treated with the herbicide have shown inhibition of net photosynthesis, transpiration rate, stomatal conductance, and nodulation process (Zobiole et al., 2010; Zobiole et al., 2012; Krenchinski et al., 2017). However, these effects should not affect the soybean yield (Elmore et al., 2001).

Even though tolerant soybean materials have a low sensitivity to glyphosate, the rhizobial symbionts could be affected by the herbicide, and plants might be indirectly injured (Reddy et al., 2000; King et al., 2001; Heatherly et al., 2003). The nitrogen requirement of soybean plants is supplied by the N from the soil and provided by the biological nitrogen fixation (BNF) resulting from the rhizobia-soybean symbiosis (Thilakarathna, Raizada, 2017). Collino et al. (2015) estimated that 46–71% of the N requirements of soybean crops are supplied by BNF in Argentinian agroecosystems. The amount of N fixed depends on the growth of the plant, as there is a close association among growth, yield and N uptake (van Kessel, Hartley, 2000). However, many factors impact the rhizobia-soybean interaction, such as the physical-chemical properties of the soil, composition of the community of soil microorganisms, climate conditions and the interactions among these factors (Hungria, Vargas, 2000; Thilakarathna, Raizada, 2017; Valentine et al., 2018).

It is known that agrichemicals and environmental contaminants can impact the rhizobial population and inhibit the symbiotic process (Fox et al., 2007; Drouin et al., 2010). Among xenobiotic compounds, glyphosate is the main pesticide used for soybean crop protection, and it has been pointed out as an inhibitor of rhizosphere microorganisms, altering the microbial communities involved in nutrient transformations and changing the balance of beneficial and pathogenic microorganisms (Kremer, Means, 2009). In contrast, Nakatani et al. (2014) found that microbial communities of different edaphic environments were not directly affected by the use of glyphosate in terms of biomass.

Although the greatest negative impact on soybean grain yield is due to weed interference from the V4 to R1 stage (Eyherabide, Cendoya, 2002), given the initial slow growth of soybeans, it is advisable to control weeds as soon as possible. In this context, late weed emergences allow escape from chemical treatment (Scursoni et al., 2007). Added to this, shifts in weed communities favouring species less susceptible to glyphosate and the emergence of first glyphosate-resistant weed populations has led to an increase in the use of herbicide mixtures and the number of applications of herbicides to control surviving weeds (Powles, 2008; Benbrook, 2016; Cruz et al., 2020). However, multiple glyphosate treatments could affect soybean-rhizobial symbiosis. Under in vitro conditions, Moorman et al. (1992) demonstrated that the growth of *Bradyrhizobium japonicum* is inhibited by glyphosate at concentrations above 0.5 mM, suggesting that nodulation and BNF could be affected by repeated applications of glyphosate to glyphosate-tolerant soybeans. In this context, the current study tested the hypothesis that the number of glyphosate applications performed during the soybean crop cycle inhibits the BNF. The aim was to determine the effect of multiple glyphosate applications throughout the soybean crop cycle on plant growth, nodulation and BNF.

2. Materials and Methods

2.1 Greenhouse Experiment

Soybean seeds ('AW3806 Intacta RR2Pro' resistant to glyphosate and Lepidoptera) were sown in pots filled with 2 dm^3 of soil from an agricultural field (lat. 38° 19' S and long. 60° 14' W). This soil is classified as Petrocalcic Argiudoll with chemical properties of SOM 2.67%, pH 6.3, P 32.4 mg kg^{-1} and N-NO_3 (0-40 cm) 27.3 kg ha^{-1}.

Previously, seeds were inoculated with a commercial inoculant (> 10^9 viable cells g^{-1}) of *Bradyrhizobium japonicum* at a rate of 3 mL per kg of seeds. Emerged seedlings were thinned to three per pot. The plants were grown in a greenhouse and pots were irrigated daily to field capacity.

The assay was conducted twice with a completely randomised design balanced with four replicates, where a pot was the experimental unit. Four treatments were applied according to the number of glyphosate applications performed during the crop cycle: (a) control without glyphosate applications, (b) one application of glyphosate at V1, (c) two applications of glyphosate at V1 and V3 and (d) three applications of glyphosate at V1, V3 and V4.

Herbicide treatments were performed using a laboratory sprayer calibrated to deliver 200 L ha^{-1} at a dose of glyphosate (potassium salt, 50.6% acid equivalent, Sulfosate touchdown®) of 1,012 g ae ha^{-1}.

At the end of the experiment (at the R1 stage), the highest leaf of each of the plants was harvested. In addition, the aerial parts of the plants were dried at 65 °C for 72 hours before the determination of dry aerial biomass.

2.1.1 *Soil Plant Analysis Development (SPAD) Readings:*

At the R1 stage, a SPAD sensor (Minolta SPAD-502 meter) was used on the youngest fully developed trifoliate leaf of two branches of the three plants of each experimental unit. Readings were taken on leaf blades (avoiding the midrib) of the terminal leaflet of the diagnostic leaves (Richardson et al., 2002). The values obtained per plant and per pot were averaged to provide a single SPAD value per experimental unit.

2.1.2 *Biological N-fixation*

It was determined using the natural ^{15}N abundance method (Shearer, Kohl, 1986). Corn seeds were planted in four pots filled with 2 kg of soil, following the same methodology and conditions described above. These plants were grown together to the pots with soybean plants and were used as a reference for the estimation of BNF. At the R1 stage, 4 g of dry aerial biomass were collected from three plants of each experimental unit and reference plants. Samples were dried in a forced-air oven at 65 °C for 72 hours and then ground in a Wiley mill. All plant samples were analysed for total N content using a semi-micro Kjeldahl method (Nelson, Sommers, 1973).

Sub-samples containing approximately 35 mg of total N were used for determining ^{15}N abundance. For this purpose, an automated continuous-flow isotope-ratio mass spectrometer (Thermo Scientific DELTA V Advantage spectrometer coupled to a ConFlo IV interface to a Flash 2000 Elemental Analyzer) was employed.

The proportion of N derived from the air (%Ndfa) was calculated according to:

$$\%Ndfa = 100\ \frac{\delta^{15}N_{Ref} - \delta^{15}N_{Soy}}{\delta^{15}N_{Ref} - (-1.032)}$$

where $\delta^{15}N_{Ref}$ and $\delta^{15}N_{Ref}$ are the natural ^{15}N abundances of the reference and soybean plants, respectively, and –1.032 is the estimated value (‰) of the ^{15}N natural abundance of N in soybean that relies only on BNF (Collino et al., 2015).

The total BNF-derived N per plant (BNF, grams per plant) was calculated considering the soybean biomass, total N content and the percentage Ndfa.

2.2 Field Experiment

Two experiments (A and B) were carried out in fields of the south of Buenos Aires Province (Argentina) (lat. 37° 50' S and long. 60° 17' W; elevation 215 m) during 2018-2019. In both experimental sites, the soils are classified as Petrocalcic Argiudoll, where the chemical properties were SOM 3.4 and 3.8%, pH 6.4 and 6.1, P-Bray 9.2 and 12.1 mg kg^{-1} and N-NO_3^- (0–40 cm)—106 and 127 kg ha^{-1}, respectively for A and B sites. In both cases a no-till system for at least the last three years, following soybean-oat-wheat and wheat-soybean-wheat sequences, respectively.

Four weeks before planting, areas of 500 m^2 were treated with glyphosate (1012 g ae ha^{-1}; potassium salt of glyphosate, 50.6% acid equivalent, Sulfosate touchdown®) to control established weeds. In November, pre-inoculated soybean seeds were sown (45 seeds m^{-2}) using a seed drill at a 0.35 m row spacing. The soybean varieties were 'AW3806 Intacta RR2 Pro'®, which stacks resistance to glyphosate and lepidopteran species, and 'Bioceres 4.11' and with glyphosate resistance (Roundup Ready®, RR) in A and B experiments, respectively.

Seeds were inoculated with a commercial inoculant (> 10^9 viable cells g^{-1}) of *Bradyrhizobium japonicum* at a rate of 3 mL g^{-1} of seeds. Plots were 2.10 by 7 m in size (experimental unit) and arranged in a randomised complete block design with four repetitions. The following treatments were applied according to the weed management: (a) without weed control, (b) hand-pulling weed control every 15 days, (c) one application of glyphosate at V1, (d) two applications of glyphosate at V1 and V4 and (e) three applications of glyphosate at V1, V4 and R2. The dose of glyphosate (potassium salt, 50.6% acid equivalent, Sulfosate touchdown®) was 1,012 g ae ha^{-1} and included methylated seed oil (0.25% v/v). A CO_2 constant pressure backpack sprayer equipped with four flat-fan nozzles (Teejet 11002) was used, delivering 150 L ha^{-1}.

At the R5 stage, five plants were harvested at random from each experimental unit. These samples were taken using a shovel to preserve the roots. The number of nodules by plant was determined and aboveground biomass dry weight was determined by drying the samples at 60 °C for 72 hours.

At harvest maturity, all plants of two central rows of each plot were cut at the base. The grains of every sample were separated from the stubble with a static threshing machine and were dried to constant weight at 60 °C. Finally, grain yield was expressed in kg ha^{-1} after adjusting the values to a constant moisture basis (13.5%).

Botanical identity and density of weed species recorded on experimental units were determined at R1 in plots without hand-pulling control (b).

2.3 Statistical Analysis

The data obtained in greenhouse experiments were subjected to analysis of variance using the R-Commander package of the R 3.6.1 program (Fox, 2005). The data from field experiments were subjected to analysis of combined experiments according to (Mclntosh, 1983). Residual plots indicated that the variances were normally distributed, and the Levene test was used to determine variance homogeneity. Means were compared by the least significant difference (LSD; $P<0.05$) test. The experiments were replicated twice.

3. Results and Discussion

Nodulation was significantly affected by the application of glyphosate in number ($P=0.015$) and weight of nodules ($P=0.001$) in greenhouse conditions. The herbicide applications reduced the number of nodules per plant by 31.6% (117 nodules per plant) compared to the control treatment without herbicide (171 nodules per plant) (Figure 1). In addition, the nodule mass per plant decreased with the glyphosate treatment. With one application of herbicide, the nodule weight per plant was reduced by 26.3% compared with the treatment without glyphosate and after two and three applications, the reductions were 35 and 44%, respectively, but no significant differences were detected among the effects of the number of applications (Figure 2). Meanwhile, BNF was also affected by glyphosate applications ($P=0.002$). With two and three sprayings of glyphosate, Ndfa was reduced by 41% compared with the treatment without glyphosate (107.2 vs 180.8 mg per plant, respectively). Two and three applications of glyphosate were associated with 56.1% and 54.4% of Ndfa, respectively, while plants without herbicide showed 70.3% of Ndfa (Figure 3).

The total nitrogen per plant was significantly affected by the glyphosate applications, which was reduced by 21% compared to the treatment without glyphosate (257.5 mg N per plant). At R1, SPAD values decreased significantly with two and three applications of glyphosate, decreasing on average by 15%, compared to the treatment without glyphosate (Figure 4). Aerial dry biomass was affected by glyphosate applications (P=0.014), decreasing on average by 17% compared to the control without herbicide (Figure 5).

Under field conditions, the number of nodules per plant was significantly different in both experiments (P=0.007). The plants from the experiment A showed 16.2 ±1.1 (SE) nodules per plant and 9.8 ±1.1 (SE) nodules per plant were recorded in the experiment B (Figure 6). Although no interaction between experiments and treatments was detected (P=0.98), the number of applications of

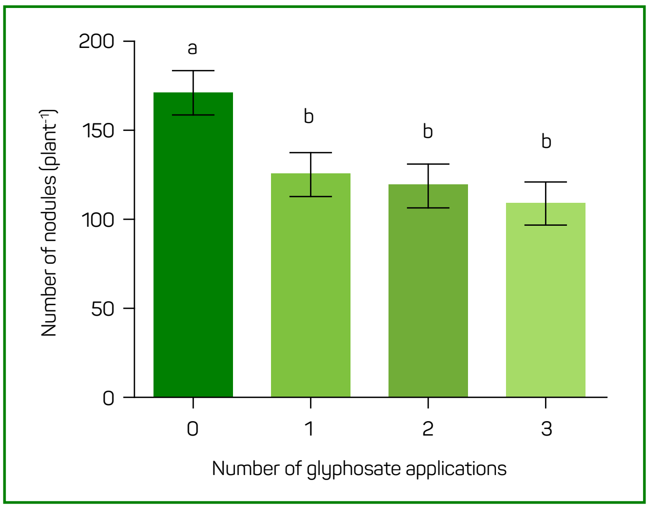

Figure 1 - Effects of the number of glyphosate applications on the number of nodules per plant in greenhouse experiments. Vertical bars represent ± 1 standard error. Letters above the bars indicate statistical significance (P<0.05)

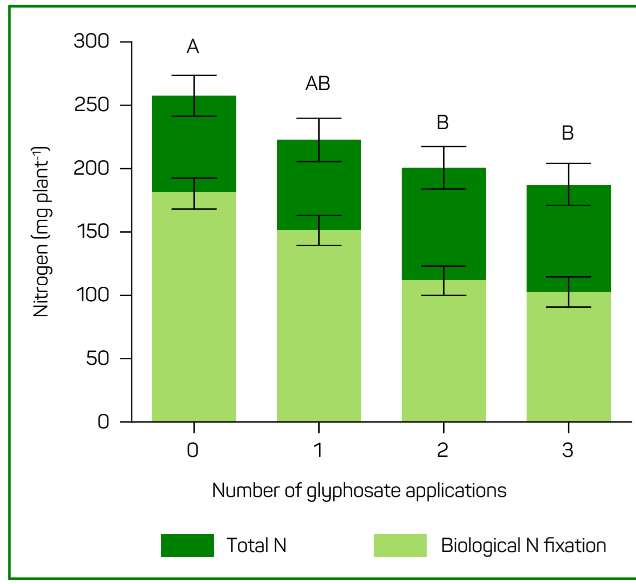

Figure 3 - Total nitrogen accumulation and estimation of N derived from N_2 fixation in response to the number of glyphosate applications in greenhouse experiments. Vertical bars represent ± 1 standard error. Capital letters above the bars and small letters indicate statistical significance (P<0.05)

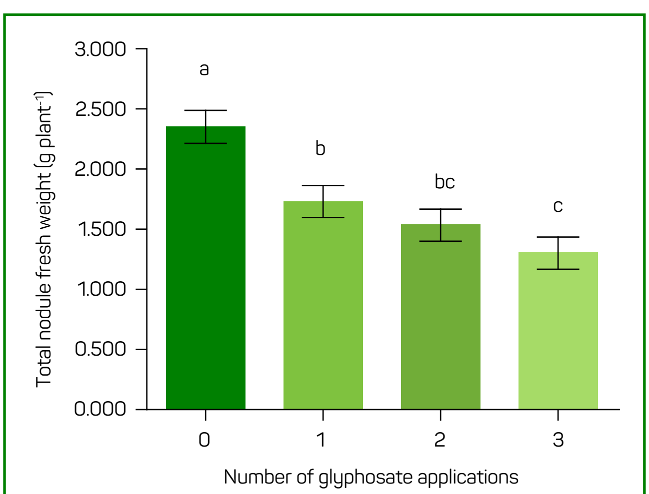

Figure 2 - Effects of the number of glyphosate applications on total nodule fresh weight per plant in greenhouse experiments. Vertical bars represent ± 1 standard error. Letters above the bars indicate statistical significance (P<0.05)

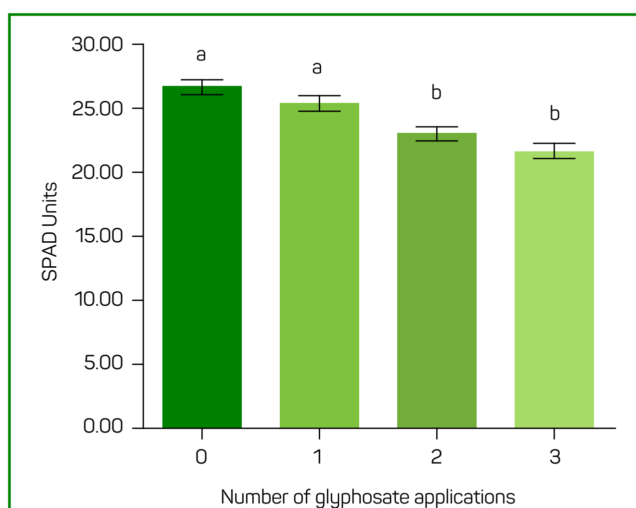

Figure 4 - Effects of the number of glyphosate applications on SPAD values of leaves of soybean in greenhouse experiments. Vertical bars represent ± 1 standard error. Differing letters above the bars indicate statistical significance (P<0.05)

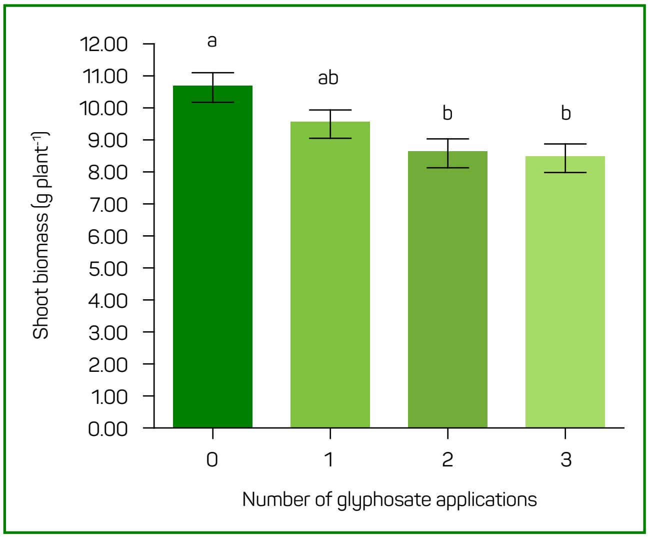

Figure 5 - Effects of the number of glyphosate applications on total shoot biomass produced per plant in greenhouse experiments. Vertical bars represent ± 1 standard error. Differing letters above the bars indicate statistical significance (P<0.05)

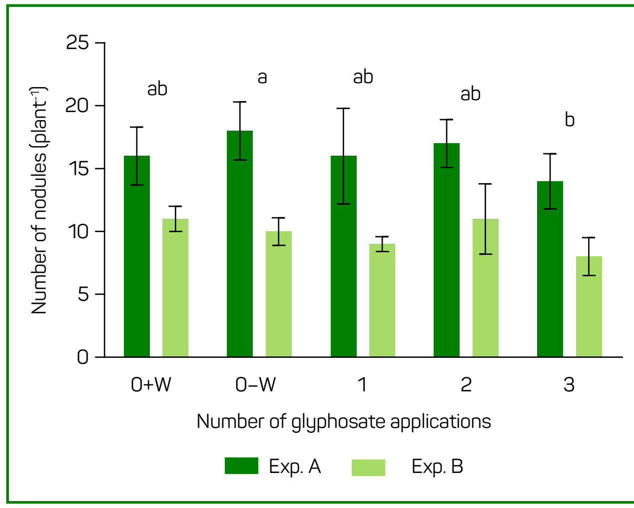

Figure 6 - Number of nodules per plant growing with and without weeds (0 + W and 0 - W, respectively) and after one, two or three glyphosate applications in two field experiments (A and B). Vertical bars represent ± 1 standard error. The same letters accompanying each treatment indicate that the differences are not significant (P>0.05)

glyphosate affected the number of nodules formed per plant (Figure 6). Three glyphosate applications provoked a reduction of nodules by 25% compared to the control treatment without herbicide and free of weeds. One and two glyphosate applications did not significantly affect the number of nodules compared with the treatment free of weeds (Figure 6).

Significant effects of the treatments and experiments on the aerial biomass per plant were detected (P<0.05), however no interaction between both factors was evidenced. Three glyphosate applications conduced to a reduction of 21% in aerial biomass of soybean compared to the treatment free of weeds (Figure 7). The spontaneous species recorded were *Digitaria sanguinalis* (L.) Scop. (1.5-2.3 plants m^{-2}), *Setaria viridis* (L.) Beauv. (1.7-4.2 plants m^{-2}), *Conyza sumatrensis* (Retz.) E. Walker (0.8-1.8 plants m^{-2}) and *Euphorbia serpens* H. B. K. (1.8-2.2 plants m^{-2}); but, no significant effects of the weeds on soybean biomass production were detected compared with the parcels treated with hand-pulling weed control (Figure 7). The highest shoot biomass was obtained in response to one glyphosate application and this treatment did not differ significantly from two glyphosate applications and hand weeding treatment (Figure 7). The experiment B produced in average 16% more shoot biomass than the plants from the experiment A (Figure 7).

The number of glyphosate applications significantly affected the soybean grain yield (P<0.001). The highest yield of grain soybean was reached when one glyphosate applications were performed. However, the application of three glyphosate treatments conduced to a reduction of yield about 36% compared to the maximum yield recorded (Figure 8). One or two glyphosate applications did not

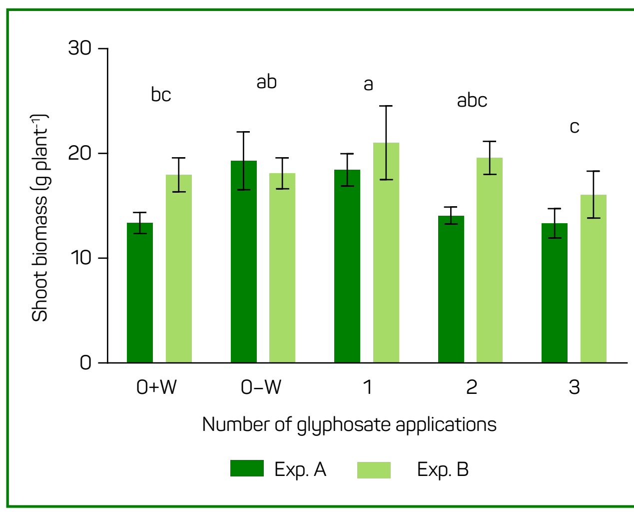

Figure 7 - Shoot biomass per plant growing with and without weeds (0 + W and 0 - W, respectively) or after one, two or three glyphosate applications in two field experiments (A and B). Vertical bars represent ± 1 standard error. The same letters accompanying each treatment indicate that the differences are not significant (P>0.05)

significantly affect the grain yield respect to the hand-pulling control (Figure 8).

According to the results, two or more applications of glyphosate inhibited the BNF and growth of soybean plants. Under controlled conditions, at least one glyphosate application at V1 affected the number and mass of nodules per plant (Figure 1) and successive applications in advanced phenological stages (V3 and V4) highlighted the inhibition of nodule growth (Figure 2). According to that, Reddy et al. (2000) reported an inhibitory effect of

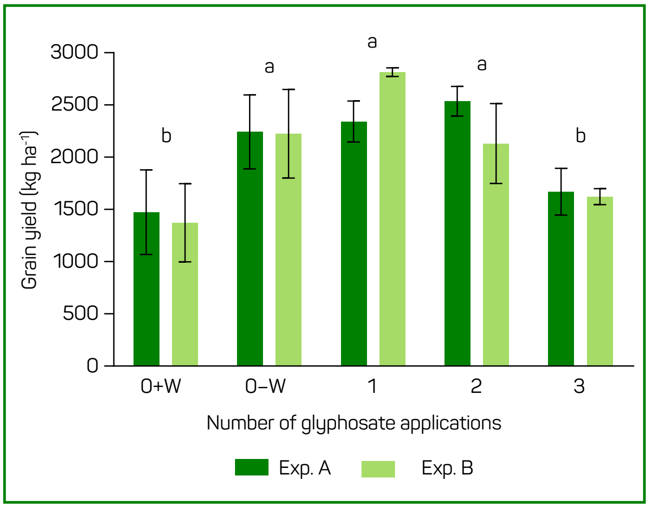

Figure 8 - Grain yield produced on plots with and without weeds (0 + W and 0 - W, respectively) or treated with one, two or three glyphosate applications in two field experiments (A and B). Vertical bars represent ± 1 standard error. The same letters accompanying each treatment indicate that the differences are not significant (P>0.05)

glyphosate on nodule number and weight after two weeks of the treatment with the herbicide (2,240 g ae ha^{-1}) at the vegetative stage of soybean. In consistence with the disruption of normal nodule development, the BNF was reduced around 41%. Comparing the effects of one and two glyphosate applications, both treatments affected the nodulation without significant difference between them (Figures 1 and 2); however, significant inhibition of BNF was only detected as a response to two glyphosate applications (Figure 3). These results suggest an effect of the herbicide on the BNF process regardless of nodulation inhibition. In that sense, a direct effect of glyphosate on nitrogenase activity has been reported (Hernandez et al., 1999; King et al., 2001) and a reduction (6 to 18%) in leghemoglobin content of nodules has been detected in response to a glyphosate treatment (Reddy, 2001).

The total N per plant was affected by glyphosate and no significant differences were detected among the number of applications (Figure 3). Therefore, after two and three glyphosate treatments, the level of N would be partially compensated at the expense of soil N. Nonetheless, the effect of two and three applications of the herbicide has resulted in lower SPAD values compared to control plants according to previous works (Zobiole et al., 2010; Zobiole et al., 2012). Consistently, reductions of 14 and 36% in the percentage of nitrogen have been associated with the effects of glyphosate applications in soybean (King et al., 2001; Reddy et al., 2000). Zablotowicz and Reddy (2007) showed that the largest and most consistent reductions in nitrogen uptake were observed when glyphosate was applied at higher doses respective to the recommended rate. The current results indicate that multiple glyphosate applications at recommended doses can inhibit the nitrogen status of soybean plants, affecting the chlorophyll content (Figure 4). In response to that, the shoot biomass accumulation and grain production would be inhibited according to previous reports (King et al., 2001; Reddy et al., 2000; Zobiole et al., 2012).

In field experiments, negative effects of one or two sequential applications of recommended doses of glyphosate on BNF or biomass production of soybean were no detected according to previous reports (Bellaloui et al., 2008; Bohm et al., 2014; Duke et al., 2018). The results obtained from plants grown under field conditions were consistent with those found at controlled conditions when three glyphosate applications were performed in the field. Only this treatment affected the nodulation, production of aerial biomass and grain yield of the soybean crop. These negative impacts were similar or higher to those of weed interference (Figure 7 and Figure 8).

The current evidence obtained in greenhouse and field experiments show the negative effects of sequential applications of glyphosate on nodulation, BFN, biomass production and grain yield of soybean. It is well known that the effects of weeds are associated with allelopathy and competition for water, light and nutrients affecting the yield production of soybean. However, an increase in the number of glyphosate applications for control weeds could be as damaging as weed interference.

4. Authors' contributions

RAC, and MY: conceptualization of the manuscript and development of the methodology. RAC, GG, and MY: data collection and curation. RAC, and MY: data analysis. RAC, GG, and MY: data interpretation. MY: funding acquisition and resources. RAC, and GG: writing the original draft of the manuscript. MY: writing, review and editing. All authors read and agreed to the published version of the manuscript.

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