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Development of RR soybean in function of glyphosate doses and *Bradyrhizobium* inoculation

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herbicide

ABSTRACT
Soybean has traditionally been produced in systems that include the use of herbicides, often in higher than recommended doses. The process of symbiotic nitrogen fixation in legumes can be hampered by these herbicides, both by direct effects on rhizobia and indirect effects on the host plant. An outdoor experiment was performed to evaluate the effects of different doses of a glyphosate herbicide on *Bradyrhizobium* strains and biological nitrogen fixation in soybean BMX Potência RR plants. Soybean seeds were inoculated with *Bradyrhizobium elkanii* (SEMIA 5019) and *Bradyrhizobium japonicum* (SEMIA 5079) strains in a commercial liquid inoculant. The treatments consisted of the absence and presence of *Bradyrhizobium* genotypes inoculated via seed and four doses of the herbicide glyphosate applied on the leaves (0, 1.0, 2.0, and 4.0 L ha^{-1} of the commercial product) at the V_3 stage. The leaf chlorophyll index of inoculated RR soybean plants did not change on the application of glyphosate and, regardless of inoculation, plants had the capacity to recover from the effects of glyphosate application, without impaired development.

Palavras-chave:
Bradyrhizobium sp.
Glycine max (L.) Merril
herbicida

Desenvolvimento da soja RR em função de doses de glifosato e da inoculação com *Bradyrhizobium*

RESUMO
A soja tem sido produzida, tradicionalmente, em sistemas que incluem a utilização de herbicidas, muitas vezes em doses acima das recomendadas. O processo de fixação simbiótica de nitrogênio em leguminosas pode ser prejudicado por esses herbicidas, tanto por efeitos diretos sobre o rizóbio, como indiretos, sobre a planta hospedeira. Com o objetivo de avaliar o efeito de doses do herbicida glifosato sobre estirpes de *Bradyrhizobium* e a fixação biológica de nitrogênio em plantas de soja BMX Potência RR, foi realizado um experimento em vasos, conduzido a céu aberto. As sementes de soja foram inoculadas com as estirpes de *Bradyrhizobium elkanii* (SEMIA 5019) e *Bradyrhizobium japonicum* (SEMIA 5079). Foi utilizado um inoculante líquido comercial. Os tratamentos constituíram-se na ausência e presença das bactérias do gênero *Bradyrhizobium* inoculadas via semente e quatro doses do herbicida glifosato via foliar (0; 1,0; 2,0 e 4,0 L ha^{-1} do produto comercial) e aplicadas no estádio fenológico V_3. A soja RR inoculada não altera o índice de clorofila foliar pela aplicação de glifosato e independentemente da inoculação, possui a capacidade de se recuperar da aplicação do glifosato, não prejudicando seu desenvolvimento.

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Introduction

Genetically modified soybean (*Glycine max* (L.) Merril) Roundup Ready (RR), glyphosate-resistant, is one of the most economically important biotech products developed in the last 20 years (Bervald et al., 2010). Very little of the area under cultivation today is conventional (CONAB, 2017). Soybeans have traditionally been produced in systems that include the use of herbicides, often at doses above the recommended levels, owing to the ability of some weeds to acquire herbicide tolerance (Bervald et al., 2010).

The expansion of the no-tillage system and recent biotechnological advances that have resulted in glyphosate-tolerant plants, have led to an increase in the sales and use of this herbicide in agroecosystems, thereby increasing the presence of this molecule in the environment, especially in the soil (Böhm & Rombaldi, 2010). Concomitantly with the increase in herbicide use, greater tolerance has developed in weedy species, leading to the greater and more frequent use of herbicides than recommended by the manufacturers (Vilvert et al., 2014). Biological nitrogen fixation (BNF) may be impaired by glyphosate, by effects both directly on the rhizobia and indirectly on the host plant, which may reduce its efficacy (Chagas Junior et al., 2013).

According to Bervald et al. (2010), herbicide resistance in transgenic soybean was achieved by the insertion of the AroA gene from the genome of the *Agrobacterium* sp., strain CP4, which encodes a variant of the enzyme 5 enolpyruvylchikimate-3-phosphate synthase (EPSPs). CP4 EPSPs is especially resistant to inhibition by glyphosate. Resistant plants are not affected by glyphosate, as they are insensitive to the action of this enzyme (Serra et al., 2011). However, there are questions about the effects on symbiotic bacteria, which do not have this same enzyme (Gonçalves et al., 2014).

The objective of this study was to evaluate the effects of different doses of glyphosate herbicide on the development of RR soybean, with or without inoculation with *Bradyrhizobium* strains.

Material and Methods

The experiment was conducted in open air vases in an experimental area belonging to the State University of São Paulo (UNESP), Faculty of Engineering, Ilha Solteira-SP. Before setting up the experiment, the soil used to fill the vases was sampled and chemical analysis performed according to the methodology described by Raij (2011). The results were as follows: pH in $CaCl_2$: 6.0, M.O.: 9.0 g dm^{-3}, P (resin): 1.0 mg dm^{-3}, K: 0.1 mmol$_c$ dm^{-3}, Ca: 8.0 mmol$_c$ dm^{-3}, Mg: 6.0 mmol$_c$ dm^{-3}, S: 3.0 mg dm^{-3}, Al: 0 mmol$_c$ dm^{-3}, H+Al: 13.0 mmol$_c$ dm^{-3}, CTC: 28.0 mmol$_c$ dm^{-3}, V%: 52.03, Cu: 0.7 mg dm^{-3}, Zn: 0.1 mg dm^{-3}, Fe: 2.0 mg dm^{-3}, Mn: 1.1 mg dm^{-3}, and B: 0.07 mg dm^{-3}. The soil used was sampled from a site with no crop cultivation, thus minimizing the chances of nodule formation by the existing colonies of symbiotic bacteria. Each vase received approximately 4 g of limestone to supply Ca and Mg. The limestone used contained 42% CaO and 14% MgO and a PRNT of 89% and was applied two weeks before sowing of the crop. Prior to sowing, each vase also received 100 mg of N, using urea as a source; 450 mg of P_2O_5 as 'supersimples;' 500 mg of K_2O as KCl; 10 mg of B as H_3BO_3; 15 mg of Mn as $MnSO_4$; and 45 mg of Zn as $ZnSO_4$. Since Fe was at a low soil concentration, it was also supplied as Fe-EDTA 5 ppm at the dose of 5 mL vase^{-1} week^{-1}.

The experimental design was completely randomized, with a double factorial scheme (2 × 4) with four replications. The treatments consisted of the absence and presence of *Bradyrhizobium* inoculated via sowing furrows and four doses of the herbicide glyphosate applied on leaves (0, 1.0, 2.0, and 4.0 L ha^{-1} of a commercial product). Eight soybean seeds of the cultivar BMX Potência RR were sown in each vase with 10 dm^3 capacity, and plant density in each vase was uniformly thinned to two 10 days after seedling emergence.

The sowing occurred on February 5, 2013. Soybean seeds were inoculated with the SEMIA 5019 (*Bradyrhizobium elkanii*) and SEMIA 5079 (*Bradyrhizobium japonicum*) strains shortly after sowing in the vases, via sprays of a solution of water + liquid inoculant at a ratio of 2:1. The volume of inoculant used per vase was 15 mL. After application, the seeds were covered by a soil layer of approximately 3 cm and the vases were irrigated, all receiving the same amount of water. The vases of the treatments without inoculation were set up in the same way; however, they received only irrigation water. The application of glyphosate at different doses was performed 31 days after sowing, when the soybean plants had the second fully developed trifoliate leaf (V_3 stage according to the scale by Fehr et al. (1971)), simulating a normal period of application of this herbicide in commercial cultivations. Application used a costal sprayer with a volume calculated to 150 L ha^{-1}, equipped with a full cone tip. The herbicide was applied at 5 p.m., under conditions of 29 °C temperature, 75% relative humidity of the air (RH), wet soil, and 6.7 km h^{-1} wind speed. After application of glyphosate, chlorophyll content was measured using the Falker digital chlorophyllometer at several phenological stages [V_3 - 0 day (day of application); V_4 - 5 days after application (DAA); V_5 - 10 DAA; V_6 - 15 DAA; R_2 - 25 DAA], taking three readings in each leaflet of the third trifoliate leaf, between 9 and 10 a.m.

The leaf N content in the crop was also evaluated, using the third trifoliate leaf, collected at the reproductive stage R_1. Other variables were analyzed at the end of the R_2 stage, when the plants were harvested from the vases by a basal cut of the stem. The plant height, number of main stem nodes, number of branches, number of leaves, and aerial part dry mass obtained after oven drying at 65 °C for 72 h were evaluated.

The results were submitted to the Shapiro-Wilk normality test and then to ANOVA by the F-test ($p \leq 0.05$). Where there was a significant difference, the averages were compared by Tukey's test ($p \leq 0.05$) for the qualitative factor (inoculation) and polynomial regression analysis ($p \leq 0.05$) for the quantitative factors (doses of glyphosate and periods, when necessary). The reading time was considered as a subdivided plot. The analysis was performed using the software SISVAR® (Ferreira, 2014).

Results and Discussion

Several authors have shown that the leaf chlorophyll index measured with a portable chlorophyllometer has many advantages, mainly owing to the ease and speed of the results collection process, and since it is not a destructive method there is no need to collect plant material for a posteriori laboratory quantification (Cerovic et al., 2012). It is worth noting that the results show a direct correlation with the concentration of N in the plant, and therefore, its use has become very popular in the last decade (Uddling et al., 2007). It was verified that there was significant interaction ($p < 0.01$) between inoculation × reading periods of the chlorophyll index (Table 1).

The analysis of the significant interaction between inoculation and the periods of leaf chlorophyll index readings shows that the soybean plants submitted to inoculation presented higher values than those not inoculated at all the periods studied after application of the herbicide (Figure 1).

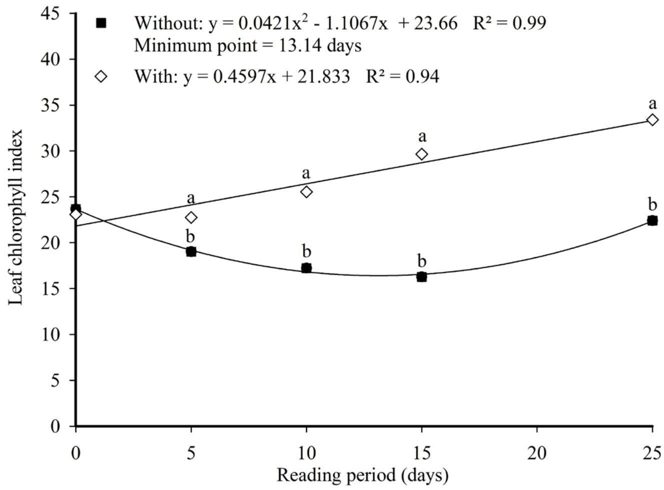
As for the periods, there was an adjustment to a quadratic equation for the values of leaf chlorophyll index in the absence of inoculation, with a minimum point around 13.14 days after the application of glyphosate (V_5 stage) and increasing thereafter. This is owing to the ability of the plant to recover after herbicide application, since it may have been degraded through the development of the crop, due to the fact that the isomorph of the EPSPs enzyme is not affected by the product. In the first days after the application of the herbicide, there may be a reduction in the physiological activity of the plant; however, after a few days it resumes its full function (Figueiredo et al., 2011; Silva et al., 2016).

When the leaf chlorophyll index values were submitted to rhizobial inoculation, the data were adjusted to an increasing linear equation, with chlorophyll content increasing during

Table 1. F-values and average leaf chlorophyll index based on the treatments used

Treatments	Leaf chlorophyll index
Inoculation (I)	
Without	19.71 b
With	26.89 a
Glyphosate [1] (G)	
0	23.13
1.0	22.70
2.0	23.37
4.0	24.01
Reading periods (RP)	
V_3	23.38
V_4	20.89
V_5	21.38
V_6	22.95
R_2	27.90
F-Test	
I	351.761^{**}
G	11.878^{ns}
RP	246.438^{**}
I × G	8.553^{ns}
I × RP	252.758^{**}
G × RP	3.425^{ns}
I × G × RP	5.489^{ns}
Average	23.30
CV (%)	10.38
DMS_I	0.75
RL_G	4.328^{*}
RQ_G	0.785^{ns}
RL_{EL}	91.038^{**}
RQ_{EL}	72.078^{**}

Means followed by the same letter in column do not differ by Tukey's test at 0.05 probability; **Significant $p < 0.01$; *Significant $0.01 < p < 0.05$; ^{ns}Not significant; [1](L ha^{-1}); RL_G and RL_{EL} - Linear regression for glyphosate doses and reading periods, respectively; RQ_G and RQ_G - Quadratic regression for glyphosate doses and reading periods, respectively



V_3 - 0 day; V_4 - 5 days; V_5 - 10 days; V_6 - 15 days; R_2 - 25 days; DMS: 1.69

Figure 1. Interaction of chlorophyllometer reading period within each inoculation level for leaf chlorophyll index

each subsequent change in phenological stage, demonstrating that the herbicide did not cause any negative interference with the measured values. Despite RR soybean being resistant to glyphosate, it may present an initial yellowing owing to the accumulation of the first phytotoxic metabolite from glyphosate, aminomethylphosphonic acid (AMPA), which is capable of reducing phytomass accumulation and plant development, as well as reducing the levels of leaf chlorophyll (King et al., 2001). In the present study, this chlorotic effect was observed to be more pronounced in the first days after the application of the herbicide in the absence of inoculation; in the inoculated condition, the effect did not occur. Zobiole et al. (2010), working with a transgenic soybean cultivar (BRS 242 RR), did not observe a change in the chlorophyll index through the glyphosate effect, corroborating the data obtained in this study.

Regarding the other variables studied, it is observed that the data were only significant for the inoculation factor (Table 2).

In the presence of inoculation with the rhizobia, higher average values were obtained for the analyzed variables, except for the number of nodes per plant and the number of branches, which did not present significant results; these variables are factors intrinsic to the cultivar and spatial distribution of the plants, similar to the number of branches, since some soybean cultivars have a compensatory effect (Cruz et al., 2016), adapting in different environments and systems (Monteiro et al., 2016).

For the leaf N content, the inoculated plants presented higher values owing to the efficiency of biological nitrogen fixation (BNF), which results in the production of ureides (allantoin and allantoic acid) in the nodules. These are sent to the aerial part, where they are dissociated into other forms of N, contributing to the proper physiological and biochemical functioning of the plant (Fagan et al., 2007; Canatto et al., 2013). Interestingly, BNF via inoculation led to an increase in the values observed for plant height, number of leaves, and total plant dry mass, when compared with those of the non-inoculated plants. This is also due to the greater accumulation of N in the aerial part, which is required for numerous processes of growth and development of the plant, as well as being a structural part of molecules such as chlorophyll, which

Table 2. F-values and averages for N leaf content, plant height, number of nodes, branches and leaves, and total dry matter of the aerial part based on the treatments used

Treatments	N leaf ($g\ kg^{-1}$)	Plant height (cm)	Number of nodes	Number of branches	Number of leaves	Total dry mass of the aerial part ($g\ plant^{-1}$)
Inoculation (I)						
Without	36.49 b	63.03 b	16.75	9.09	37.94 b	17.65 b
With	45.48 a	78.66 a	16.75	10.12	59.40 a	34.28 a
Glyphosate [1] (G)						
0	40.68	71.44	16.75	8.81	48.31	26.60
1	38.21	72.14	17.00	10.06	48.37	26.78
2	39.67	69.93	16.63	10.12	48.06	24.38
4	45.33	69.87	16.65	9.43	49.93	26.12
F-Test						
I	14.87**	66.20**	0.01^{ns}	2.24^{ns}	68.79**	128.25**
G	1.74^{ns}	0.34^{ns}	0.10^{ns}	0.8^{ns}	0.11^{ns}	0.56^{ns}
I × G	4.65^{ns}	0.84^{ns}	0.22^{ns}	1.10^{ns}	0.25^{ns}	0.81^{ns}
Mean	40.98	70.84	16.75	9.60	48.67	25.97
CV (%)	16.10	7.67	9.64	15.20	15.04	15.99
DMS	4.84	3.99	1.18	1.42	5.34	3.05
RL_G	3.07^{ns}	0.60^{ns}	0.09^{ns}	0.15^{ns}	0.21^{ns}	0.16^{ns}
RQ_G	2.02^{ns}	0.01^{ns}	0.01^{ns}	2.1^{ns}	0.10^{ns}	0.65^{ns}

Averages followed by the same letter in column do not differ by Tukey's test at 0.05 probability; **Significant $p < 0.01$; *Significant $0.01 < p < 0.05$; ^{ns}Not significant; [1]($L\ ha^{-1}$); RL_G and RQ_G - Linear regression and quadratic regression for glyphosate doses, respectively

act directly in the production of photoassimilates that are used in the metabolic activities of the plant.

Regarding the application of glyphosate, no significant effect was observed on any agronomic parameter studied, which indicates that, for cultivars that have the resistance gene to glyphosate, there was no damage due to applications during the crop cycle. Another factor that influences the absence of negative effects of the herbicide on the BNF efficiency of the symbiotic bacteria is that most of the applied glyphosate that reaches the soil is strongly adsorbed in the colloids, contributing to its inactivation and unavailability to the plants (Figueiredo et al., 2011; Ferreira et al., 2013).

The glyphosate-tolerant soybean expresses two versions of the EPSPs enzyme, one native to the species and the other introduced from *Agrobacterium* sp., which is tolerant to the herbicide (Bervald et al., 2010). Thus, there is no accumulation of chiquimate-3-phosphate in the plant and no reduction in biomass or other components of agronomic interest in resistant plants (Gomes et al., 2015). Some authors consider that the symbiotic bacterium does not have the same tolerance to the herbicide, which may reduce its efficiency; however, it is also reported that both the plant and the bacterium have the capacity to recover after a few days of application (King et al., 2001).

Glyphosate is one of the most used herbicides in agriculture, and several authors consider it to be one of the least toxic herbicides for nodulation and subsequent BNF (Böhm & Rombaldi, 2010; Santos, 2015). Some studies indicate that soil microorganisms possess the ability to adapt to the application of this herbicide, obtaining, over time, low sensitivity to its presence, and growing suitably for symbiosis even at higher product concentrations (Galli & Montezuma, 2005; Andrighetti et al., 2014).

Conclusions

1. There was no change in the leaf chlorophyll index of soybean inoculated with *Bradyrhizobium* after the application of glyphosate.

2. Glyphosate, even at the highest dose (4 L ha^{-1}), did not interfere with N leaf content and other agronomic parameters, regardless of inoculation.

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