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GM soybean nodulation and yield in response to glyphosate applications and co-inoculation

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ABSTRACT: Glyphosate is the main herbicide applied post-emergence to GM soybean. Previous research showed that glyphosate can affect the number and mass of nodules that affect yield. However, with the use of techniques to increase nodulation, the effect of glyphosate on the yield of GM soy may be lessened. The objective was to evaluate GM soybean nodulation and yield as a function of glyphosate applications and co-inoculation. Four experiments were carried out in one area with a history of co-inoculation. The treatments of glyphosate included: weed burned-down 10 days prior to sowing plus one post-emergence application, weed burned-down 10 days prior to sowing plus two post-emergence applications, one post-emergence application, two post-emergence applications and weed control carried out mechanically with a hoe. The treatments of inoculation involved three types: co-inoculation, inoculation and no inoculation. Glyphosate applications reduced nodulation in only one of the four experiments. Co-inoculation increased nodule number and dry weight by 105 and 168%, respectively, in one experiment of the 2018/19 crop year compared to no inoculation. In this study, glyphosate applications altered nodulation but did not influence grain yield.

Key words: *Azospirillum brasilense*; *Bradyrhizobium*; *Glycine max* L.; herbicide; post-emergence applications

Nodulação e produtividade da soja GM em resposta a aplicações de glifosato e co-inoculação

RESUMO: O glifosato é o principal herbicida aplicado em pós-emergência na soja GM. Pesquisas anteriores mostraram que o glifosato pode afetar o número e a massa de nódulos que afetam o rendimento. No entanto, com o uso de técnicas para aumentar a nodulação, o efeito do glifosato na produtividade da soja GM pode ser diminuído. O objetivo foi avaliar a nodulação e a produtividade da soja GM em função das aplicações de glifosato e coinoculação. Quatro experimentos foram realizados em uma área com histórico de coinoculação. Os tratamentos com glifosato incluíram: dessecação 10 dias antes da semeadura mais uma aplicação em pós-emergência, dessecação 10 dias antes da semeadura mais duas aplicações em pós-emergência, uma aplicação em pós-emergência, duas aplicações em pós-emergência e controle realizado mecanicamente. Os tratamentos de inoculação envolveram três tipos: coinoculação, inoculação e sem inoculação. As aplicações de glifosato reduziram a nodulação em apenas um dos quatro experimentos. A coinoculação aumentou o número de nódulos e o peso seco em 105 e 168, respectivamente, em um experimento da safra 2018/19, em comparação ao sem inoculação. Neste estudo, as aplicações de glifosato alteraram a nodulação, mas não influenciaram na produtividade de grãos.

Palavras-chave: *Azospirillum brasilense*; *Bradyrhizobium*; *Glycine max* L.; herbicida; aplicação em pós-emergência

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Introduction

The soybean crop [*Glycine max* (L.) Merr.] is the fourth most widely cultivated crop in the world (FAO, 2021). Brazil is the world largest producer, with an estimated production of 123.8 million tons and a cultivated area of 40.9 million hectares in the 2021/22 crop season (CONAB, 2022).

Genetically modified (GM) soybean accounts for 90% of production in Brazil, with about 35.1 million hectares cultivated (ISAAA, 2019). The reason GM soybean is the most widely cultivated is associated with glyphosate-resistance, which facilitates weed management when the crop is already established.

Glyphosate is the most widely used herbicide for weed control in the pre-sowing and post-emergence of GM soybeans. The main advantages are the lower cost of glyphosate compared to other herbicides and the broad-spectrum, killing most types of plants (Kanissery et al., 2019). Glyphosate blocks the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), interfering with the biosynthesis of important amino acids in susceptible plants (Duke, 2021). However, symbiont bacteria, which possess the EPSPS enzyme can be affected by glyphosate (De María et al., 2006).

Due to its high mobility in the phloem, glyphosate can move into the roots and accumulate in nodules (De María et al., 2006). The accumulation of glyphosate can inhibit the EPSPS enzyme of the bacteria, decreasing malate availability for bacteroids metabolism and biological nitrogen fixation (BNF) consequently (De María et al., 2006). Even glyphosate-resistant soybean can show reduced nodulation and BNF after glyphosate application (Zablotowicz & Reddy, 2004; Fan et al., 2017).

In the GM soybean cultivation, besides weed burned-down application in pre-sowing, it is common to have two glyphosate applications in post-emergence, due to weed escape. However, more glyphosate applications during the crop cycle intensify the negative effects on soybean nodulation (Chagas Junior et al., 2013). Therefore, these applications must be carefully done not to affect BNF.

The glyphosate application under unfavorable conditions, such as periods of water deficit (King et al., 2001) are detrimental to soybean nodulation. However, when application does not occur under these conditions, the effect of glyphosate on soybean nodulation may become minimal. Moreover, in areas that perform annual inoculation and co-inoculation, the population of *Bradyrhizobium* in the soil can be high, above 10^6 CFU g soil^{-1} (Hungria & Mendes, 2015). This can contribute to the formation of new nodules and recover BNF.

In Brazil, soybean is highly dependent on BNF, which contributes to more than 300 kg ha^{-1} of N for the crop (Hungria & Mendes, 2015). As soybean requires around 80 kg of nitrogen (N) per ton of grains (Saturno et al., 2017), it is estimated that BNF contributes to saving US$ 7 billion year^{-1} in fertilizers (Hungria et al., 2013). Thus, it is necessary to seek solutions that have less impact on bacteria and that increase the BNF of soybean crops.

The double inoculation *Bradyrhizobium* with *Azospirillum brasilense* is a technology that can increase nodulation and grain yield. In Brazil, the increment in the yield was observed to be in the 10 to 20% range (Ferri et al., 2017; Scheneider et al., 2017). The production of indole-3-acetic acid (IAA) by *Azospirillum brasilense* enhances communication between the plant and the bacteria (*Bradyrhizobium*) (Puente et al., 2018) and stimulates root growth (Rondina et al., 2020). These factors together contribute to increased nodulation, and consequently grain yield.

Although the decline in nodulation and soybean BNF in response to the glyphosate applications are reported in the literature (Zablotowicz & Reddy, 2004; Fan et al., 2017), in GM soybean cultivars, BNF can be recovered after some time (King et al., 2001). In addition, the co-inoculation *Bradyrhizobium* with *Azospirillum brasilense* can increase nodulation, offsetting the loss of nodules after glyphosate application. Thus, this research aimed at verifying GM soybean nodulation and yield as a function of glyphosate applications and co-inoculation.

Materials and Methods

Four independent field experiments were carried out in south Brazil during two crop years (2017/2018 and 2018/2019 seasons). Two experiments in the 2017/2018 crop year, with sowing in 10/30/2017 (Exp1) and 12/15/2017 (Exp2), and two in the 2018/2019 crop year, with sowing in 11/05/2018 (Exp3) and 12/12/2018 (Exp4). The experiments sown on 11/05/2018 and 12/12/2018 were in the same field as the experiments sown on 10/30/2017 and 12/15/2017.

The experimental field is part of the Department of Phytotechnics of the Universidade Federal de Santa Maria, situated at 29° 42′ S, 53° 42′ W and 116 m altitude. The soil in the area is classified as Ultisol based on the Soil Taxonomy categorization (Soil Survey Staff, 2014). Ranked according to the Köppen classification, the region experiences the Cfa type, subtropical and without a dry season and hot summer (Alvares et al., 2013). The physical and chemical properties of the soil are listed in Table 1.

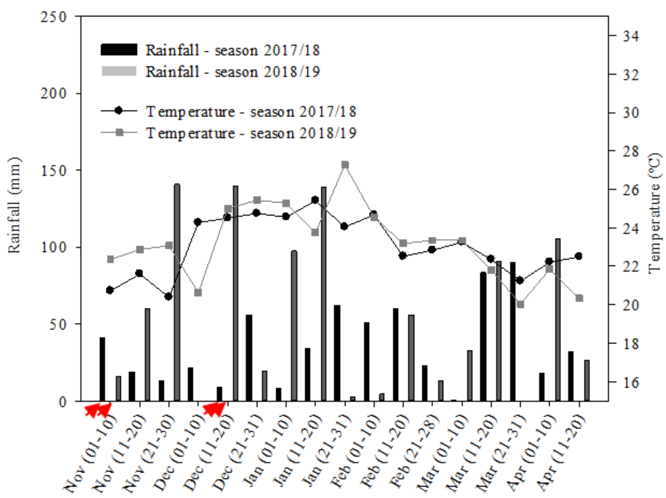
In the crop years (2017/18 and 2018/19), rainfall distribution was regular (Figure 1). However, the accumulated rainfall for the 2017/18 crop year was below the historical average for the region, with an accumulation of 572 mm. In the 2018/19 crop year, the accumulated precipitation was higher than the historical average, with 811 mm.

The randomized block experimental design was adopted for experiments in both seasons. The treatments were distributed in a 5 × 3 factorial, with four replications. The treatments of glyphosate included: (D+V2) - weed burned-down application 10 days prior to sowing plus one post-emergence application, (D+V2+V6) - weed burned-down 10 days prior to sowing plus two post-emergence applications, (V2) - one post-emergence application, (V2+V4) - two post-emergence applications, (Control) - weed control with no

Table 1. Physical and chemical soil analysis of the field experiments in the year 2017.

Layer (cm)	pH	OM	Clay	P	K	S	Ca	Mg	m	V
		(%)		(mg dm^{-1})			(cmol$_c$ dm^{-1})		(%)	
Field 1										
0-10	6.7	2.1	21.0	21.4	52.0	20.9	7.7	3.0	0.0	84.4
10-20	5.8	1.7	28.0	25.2	32.0	12.1	6.2	2.5	0.0	69.1
Field 2										
0-10	5.7	2.0	21.0	35.9	60.0	6.5	5.8	2.0	0.0	67.0
10-20	4.9	1.4	28.0	7.6	28.0	5.8	3.8	1.5	14.3	32.8

OM (organic matter), m (Al^{3+} saturation), V (base saturation).



* Red arrows = period of sowing of the experiments in the crops years 2017/18 and 2018/19.

Figure 1. Rainfall and temperature during the experiments. Santa Maria, RS, Brazil.

application of the glyphosate. The treatments of inoculation involved three types: co-inoculation with *Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* plus *Azospirillum brasilense*, inoculation with *Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens*, and no inoculation. The experimental plots were 7.75 × 2.25 m.

The applications of burned down and post-emergence included a glyphosate formulation composed of 370 g e.a/l of the di-ammonium salt, at a dose of 2.92 L ha^{-1} of the commercial product, equivalent to 1.08 kg e.a/ha. All the applications were carried on the aerial plants parts using an electric coastal sprayer, having four fan-type spray nozzles (XR 100.015), adjusted to 200 kPa pressure and 200 L ha^{-1} of the spray volume. Ten days prior to sowing of the experiments, the weed was burned down using glyphosate herbicide in the plots of treatments “D+V2” and “D+V2+V6”. The first application of glyphosate in post-emergence at the treatments “D+V2”, “D+V2+V6”, “V3” and “V2+V6” was V2 soybean development stage. The second application of glyphosate in post-emergence at the treatments “D+V2+V6” and “V2+V6” was V6 soybean development stage. In treatment with glyphosate application and without application “Control”, weed control was carried out mechanically with a hoe for no weed to escape.

The inoculation treatments involved mixing the soybean seeds with the inoculant containing the *Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium diazoefficiens* SEMIA 5080 strains in 5 × 10^9 CFU mL^{-1} concentration. In the co-inoculation treatments, the seeds were mixed with the inoculants containing the *Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium diazoefficiens* SEMIA 5080 strains at a concentration of 5 × 10^9 CFU mL^{-1}, and *Azospirillum brasilense* strains Ab-V5 and Ab-V6, at a concentration of 2 × 10^8 CFU mL^{-1}. Furthermore, it should be noted that before the implementation of the experiment, the area was cultivated for five consecutive years with soybeans and received co-inoculation in all crops. A general characterization of the experimental fields was performed at the time of experiment implementation, which indicated a soil population of *Bradyrhizobium* spp. and *Azospirillum brasilense* of 4.3 × 10^6 CFU g soil^{-1} and 3 × 10^3 PMN g soil^{-1}, respectively.

In the winter (off-season period) of years 2017 and 2018, cover crops were cultivated for no-till soybean. Forage turnip and wheat were the cover crops used in the 2017/2018 crop season and black oat in the 2018/2019 crop season. All cover crops were sown in the winter of each year and slashed in early October. The soybean sowing was carried with 0.45 m row spacing and 30 seeds m², employing the undetermined growth habit cultivar NS 5959 IPRO.

At the R2 soybean development stage (Fehr & Caviness, 1977) four plants were collected per plot to assess the number and dry weight of nodules. The collection of plants with the root was done with a shovel in the central row of the plot, a soil volume of 0.008 m³ was collected with dimensions of 0.2 m width, 0.2 m length and 0.2 m depth, with the plant centred in the middle. The roots were then washed under running water and separated from the shoot. Nodules were detached from the root and placed on a 2 mm mesh sieve for counting. Subsequently, they were dried in a forced air circulation oven at 65 °C for 72 hours and the dry weight was calculated.

When physiological maturation was complete at the R8 (Fehr & Caviness, 1977) soybean development stage, a specific plot of 6 m² useful area was harvested and the grain yield was evaluated. To determine the grain yield, all the plants from the useful area of each experimental unit were threshed and weighed in an analytical balance, the content correction of the seed moisture at 13%.

The four experiments were independent, and the types of inoculation and glyphosate applications were considered as fixed factors. The data were submitted to Shapiro-Wilk and Bartlett test for verification of normality and homogeneity of variance, respectively. After ANOVA assumptions were met, the data of response variables, number of nodules, dry weight of nodules and grain yield were submitted to analysis of variance with $p < 0.05$, by using the F test. When significant difference was identified, the Scott-Knott test was performed, with $p < 0.05$.

Results and Discussion

In the four experiments carried out during crop years (2017/18 and 2018/19), there was no significant interaction ($p > 0.05$) between the glyphosate applications and types of inoculation for variables number of nodules, nodule dry weight and yield. The two factors affected the variables only in an isolated way.

Glyphosate applications significantly affected the number of nodules ($p \leq 0.01$) and dry weight of nodules ($p \leq 0.01$) of GM soybean only in the experiment 1. All glyphosate treatments caused a decrease in the number and dry weight of nodules compared to the control without application (Table 2). Soybean produced an average of 94.5 nodules and 0.21 g of nodule dry weight when glyphosate was applied, approximately 37% less than the control, which produced 129 nodules and 0.29 g of nodule dry weight (Table 2).

Glyphosate applications had a low impact on the number and dry mass of nodules in GM soybean measured at R2 stage, 60 and 49 days after emergence, respectively, in the first and second sowing dates. In only one of the four field trials did glyphosate negatively affect nodules (Table 2). However, the amount and dry weight of nodules found in glyphosate-treated soybean plants were high, about 94.5 nodules and 0.21 g dry weight per plant. This may justify the fact that grain yield was not depressed (Figure 2).

According to Hungria et al. (2007), at the flowering period, a well-nodulated soybean plant should show about 15 to 30 nodules or 0.1 to 0.2 g dry weight of nodules. In all four experiments, all treatments, including without inoculation, showed a high number of nodules and dry weight (Table 2). The fact that the area had a high *Bradyrhizobium* naturalized population (4.3×10^6 CFU g soil^{-1}) due to successive co-inoculated soybean crops may have contributed to higher nodulation, and consequently to the low effect of glyphosate applications.

The decrease of nodulation of the soybean in response to the glyphosate applications was verified by Zablotowicz & Reddy (2004) and Fan et al. (2017). However, in GM soybean cultivars, BNF can be recovered after some time (King et al., 2001). Chagas Junior et al. (2013) observed that the application of two glyphosate formulations reduced the number of nodules of GM soybean at 45 days after application, but at 60 days after application, no difference was observed.

The decrease of the number of nodules after glyphosate application in the experiment 1 was not enough to affect grain yield (Figure 2). Reis et al. (2014) showed that the decrease in nodules number by the glyphosate formulations

Table 2. Number of nodules per plant (NN) and nodule dry weight (g plant^{-1}) (NDW) measured at R2 stage, 60 and 49 days after emergence, respectively, in the first and second sowing dates in response to the types of inoculation and glyphosate applications in four field experiments carried in south Brazil during two crop years (2017/2018 and 2018/2019 seasons).

Season 2017/18	Exp.1*		Exp2.	
Inoculation types	NN	NDW	NN	NDW
Co-inoculation	98 b	0.22 a	97 b	0.23 a
Inoculation	113 a	0.23 a	119 a	0.22 a
No inoculation	94 b	0.21 a	105 b	0.18 b
Glyphosate applications				
Control	129 a	0.29 a	98 a	0.20 a
Burned-down[1] + one post-emergence[2]	88 b	0.19 b	102 a	0.19 a
Burned-down + two post-emergence[23]	88 b	0.19 b	100 a	0.20 a
One post-emergence	101 b	0.23 b	119 a	0.23 a
Two post-emergence	101 b	0.21 b	116 a	0.24 a
Season 2018/19	**Exp.3**		**Exp.4**	
Inoculation types	NN	NDW	NN	NDW
Co-inoculation	133 a	0.67 a	119 a	0.59 a
Inoculation	100 b	0.46 b	109 a	0.62 a
No inoculation	65 c	0.25 c	101 a	0.65 a
Glyphosate applications				
Control	88 a	0.46 a	122 a	0.61 a
Burned-down + one post-emergence	82 a	0.38 a	111 a	0.69 a
Burned-down + two post-emergence	104 a	0.44 a	113 a	0.60 a
One post-emergence	112 a	0.46 a	95 a	0.54 a
Two post-emergence	110 a	0.38 a	108 a	0.65 a

*Experiment. [1]Burned-down 10 days prior to sowing. [2]V2 and [3]V6, soybean development stage. Means followed by distinct letters in the column differ statistically by the Scott-Knott test, at $p \leq 0.05$.

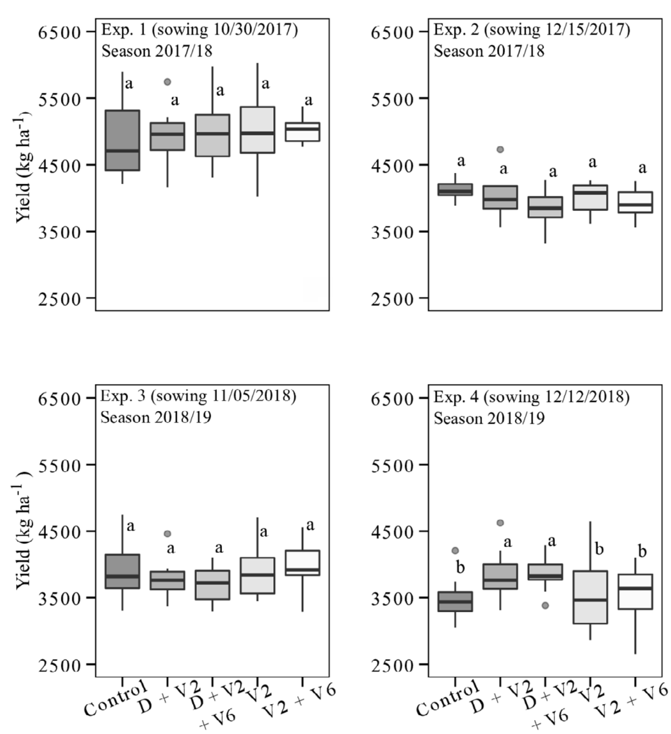


Figure 2. Soybean yield box plot in response to the glyphosate applications in four field experiments carried out in south Brazil during two crop years (2017/2018 and 2018/2019 seasons). D, weed burned-down 10 days prior to sowing. V2 and V6, soybean development stage. Different letters indicate statistical differences by the Scott-Knott test, at $p \leq 0.05$.

was insufficient to affect the yield, as the nodule dry weight was not affected. Elmore et al. (2001) evaluating glyphosate application on 13 GM soybean cultivars concluded that the herbicide did not affect the yield of any of the cultivars evaluated. Forte et al. (2019), applying twice the dose of glyphosate recommended (2160 g a.i. ha^{-1}) not found a negative effect on soybean grain yield.

It is important to highlight in both seasons, the conditions were favourable for the good development of soybeans. Rainfall distribution was regular during all soybean development (Figure 1) and the average yield of the experiments was above that found in the region.

Soybean grain yield was significantly affected ($p \leq 0.05$) by glyphosate applications in the experiment 4. In this experiment, there was a higher soybean yield for the treatments with the burnt weed 10 days before sowing plus the post-emergence applications. In the other experiments, glyphosate applications did not affected GM soybean yield (Figure 2).

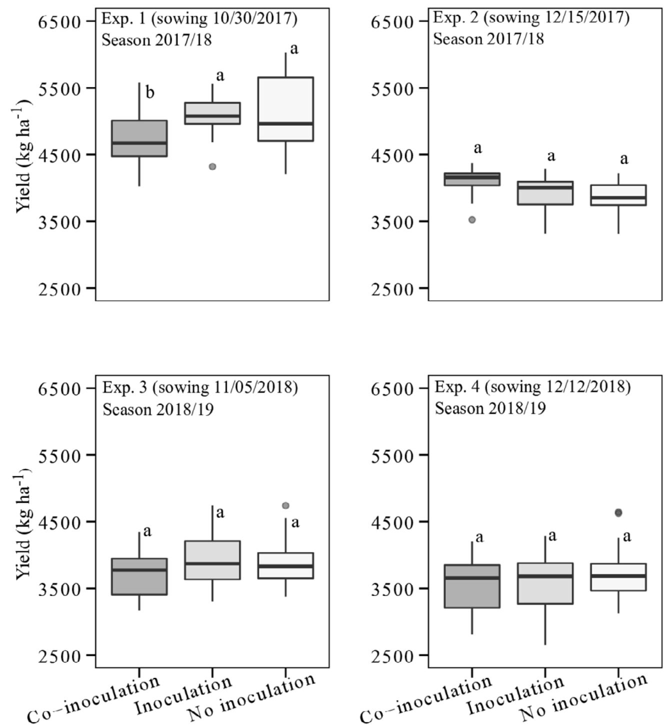
The types of inoculation significantly affected the number of nodules per plant in three of four experiments (Table 2). Nodule dry weight was also significantly affected by types of inoculation with ($p \leq 0.01$) and ($p \leq 0.05$), respectively, in the experiments 2 and 3. Co-inoculation decreased the number of nodules of soybean in the two experiments of the 2017/18 season compared to inoculation with *Bradyrhizobium* isolated. However, in the experiment 3, co-inoculation increased the number and dry weight of nodules by 105% and 63%, compared to no inoculation and inoculation with *Bradyrhizobium*, respectively (Table 2).

Soybean grain yield was significantly affected by types of inoculation ($p \leq 0.05$) in the experiment 1. In this experiment, the treatments with co-inoculation, inoculation and no inoculation provided a soybean grain yield of 3889, 4126 e 4299 kg ha^{-1}, respectively. Co-inoculation caused a decrease of about 7 and 10% compared to inoculation with *Bradyrhizobium* and no inoculation, respectively (Figure 3).

Literature results showed co-inoculation can encourage early nodulation (Cerezini et al., 2016), raise the number of nodules of soybean (Ferri et al., 2017; Rondina et al., 2020) and increments of grain yield (Hungria et al., 2013; Ferri et al., 2017; Barbosa et al., 2021). However, co-inoculation caused different results for each of the experiments, ranging from negative for nodule number and yield in the crop year (2017/18), no effect and positive in the crop year (2018/19) for number and nodule dry weight.

The lower soybean yield for co-inoculation was not expected. However, there was a reduction in the experiment that was sown after the forage turnip crop. In the other experiments where co-inoculation was not negative, the previous cover crops were grasses. However, the causes of this effect have not been clarified.

In fields with a history of inoculation in soybean, in general, the population of *Bradyrhizobium* in the soil is high and can reach 10^6 bacteria per g of soil (Hungria & Mendes, 2015).



Different letters indicate statistical differences by the Scott-Knott test, at $p \leq 0.05$.

Figure 3. Soybean yield box plot in response to the different types of inoculation in four field experiments carried out in south Brazil during two crop years (2017/2018 and 2018/2019 seasons).

The experimental field showed an average *Bradyrhizobium* population of 4.3×10^6 CFU g $soil^{-1}$. Thus, even in the treatment without inoculation, the number and dry weight of nodules were high in the soybean roots (Table 2). In a soil with a naturalized *Bradyrhizobium* population of 2.871×10^4 CFU g^{-1} soil, Luca & Hungria (2014) observed good nodulation of soybean plants in the control treatment so they found no difference in the number and dry mass of nodules between the control and the inoculated.

The naturalized strains of *Bradyrhizobium* have the ability to nodulate roots and fix nitrogen, and when the fixed nitrogen is sufficient to meet the demand of the plant, inoculation may not result in increases in crop yield (Hungria et al., 2007). In the experiments, except for the experiment 1, in which co-inoculation was negative, there was no difference between the non-inoculated, inoculated and co-inoculated treatments for yield (Figure 3).

The lower soybean yield for co-inoculation was not expected. However, there was a reduction in the experiment that was sown after the forage turnip crop. In the other experiments where co-inoculation was not negative, the previous cover crops were grasses. In maize crop, Lima (2020) found that inoculation with *Azospirillum brasilense* in the forage turnip/maize rotation system caused negative result on grain yield. Portugal et al. (2017) found that inoculation with *A. brasilense* caused a negative result in corn productivity components when the cover crops used were not grasses. However, the causes of this effect have not been clarified.

Greenhouse experiments showed a negative effect of glyphosate application on N_2 fixation and growth of GM soybean (Fan et al., 2017). However, in field experiments, the effect of glyphosate applications can be considerably less. In fields cultivated with soybeans and with annual inoculation, the population of bacteria in the soil is high, allowing for greater nodule formation (Hungria & Mendes, 2015).

Conclusion

Glyphosate applications altered the nodulation in only one and, not influence grain yield. Co-inoculation can potentially enhance soybean nodulation, although a variety of responses are noted in different environments, thus necessitating further studies.

Compliance with Ethical Standards

Author contributions: Conceptualization: TNM, MMF; Data curation: MMF, GMB, GAA, EG; Formal analysis: MMF; Investigation: MMF, GMB, GAA, EG; Methodology: MMF, TNM; Project administration: MMF, TNM; Resources: MMF, TNM; Supervision: MMF, TNM; Validation: MMF, TNM, GMB, GAA, EG; Visualization: MMF, TNM, EG; Writing – original draft: MMF; Writing – review & editing: MMF.

Conflict of interest: The authors declare there are no conflicts of interest.

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