CO-INOCULATION WITH *Azospirillum brasilense* AND *Bradyrhizobium japonicum* IN SOYBEAN IN THE FIRST AND THIRD YEAR OF CULTIVATION †

[CO-INOCULACIÓN CON *Azospirillum brasilense* Y *Bradyrhizobium japonicum* EN EL CULTIVO DE LA SOJA EN ÁREAS DE PRIMER Y TERCERO AÑO DE CULTIVO]

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SUMMARY

Background. The use of microorganisms in agriculture, such as *Bradyrhizobium japonicum* and *Azospirillum brasilense* and soybean cultivation together with nitrogen fertilization, may be interesting alternatives to increase crop yield. The soybean with the use of *Azospirillum brasilense*, together with *Bradyrhizobium* bacteria, in a current approach aimed at agricultural, economic, social and environmental sustainability. **Objective**. To evaluate the influence of the application of different doses of *Azospirillum brasilense* on co-inoculation with *Bradyrhizobium japonicum* in the soybean crop, in the first and third year of cultivation. **Methodology.** The experiment was carried out in the municipality of Alta Floresta - MT, Brazil, in the harvest of 2016/2017. The treatments consisted of the combination of four doses (0, 2, 4, 8 mL kg^{-1} of seeds) of the commercial product Nitrogeo AZ®, composed of *Azospirillum brasilense*, with 4 replicates. For the standard inoculant the commercial product Nitrogeo Soja Turfa® was used *Bradyrhizobium japonicum*, with a dose of 3.2 g kg^{-1} of seeds. Height of first pod insertion, plant height, number of pods per plant, mass of one thousand grains, productivity and leaf content of nitrogen were analyzed. **Results.** There was an increasing in both grain yield and thousand grains weight in the treatments with *Azospirillum brasilense*. Higher plants with a higher number of pods were found in the area of the 3rd year of cultivation, compared to the area of the 1st year, however, the co-inoculant doses did not influence this variable. The highest grain yield was obtained with the use of 338 mL of commercial product per 50 kg of seeds. **Conclusion.** The use of these bacteria (*Azospirillum* with *Bradyrhizobium*) together contributes to increase productivity in the soybean crop.

Keywords: Seed treatment; Diazotrophic bacteria; Biological fixation of nitrogen

RESUMEN

Antecedentes. El uso de microorganismos en la agricultura, como *Bradyrhizobium japonicum* y *Azospirillum brasilense* y el cultivo de soja junto con la fertilización nitrogenada, pueden ser alternativas interesantes para aumentar el rendimiento de los cultivos. La soja con el uso de *Azospirillum brasilense*, junto con la bacteria *Bradyrhizobium*, en un enfoque actual dirigido a la sostenibilidad agrícola, económica, social y ambiental. **Objetivo**. Evaluar la influencia de la aplicación de diferentes dosis de *Azospirillum brasilense* en la coinoculación con *Bradyrhizobium japonicum* en el cultivo de soja, en el primer y tercer año de cultivo. El experimento se llevó a cabo en el municipio de Alta Floresta - MT, Brasil, en la cosecha de 2016/2017. Los tratamientos consistieron en la combinación de cuatro dosis (0, 2, 4, 8 mL kg^{-1} de semillas) del producto comercial Nitrogeo AZ®, compuesto de *Azospirillum brasilense*, con 4 repeticiones. Para el inoculante estándar, se usó el producto comercial Nitrogeo Soja Turfa® *Bradyrhizobium japonicum*, con una dosis de 3.2 g kg^{-1} de semillas. Se analizaron la altura de la inserción de la primera vaina, la altura de la planta, el número de vainas

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por planta, la masa de mil granos, la productividad y el contenido de nitrógeno en las hojas. **Resultados.** Hubo un aumento tanto en el rendimiento de grano como en el peso de mil granos en los tratamientos con *Azospirillum brasilense*. Se encontraron plantas más altas con un mayor número de vainas en el área del tercer año de cultivo, en comparación con el área del primer año, sin embargo, las dosis de co-inoculantes no influyeron en esta variable. El mayor rendimiento de grano se obtuvo con el uso de 338 mL de produto comercial por 50 kg de semillas. **Conclusión.** El uso de estas bacterias (*Azospirillum* con *Bradyrhizobium*) juntas contribuye a aumentar la productividad en el cultivo de soja.

Palabras clave: Tratamiento de semillas; Bacterias diazotróficas; Fijación biológica de nitrógeno

INTRODUCTION

The soybean (*Glycine max* L.) crop represents one of the pillars of the world agribusiness, as one of the main exploited species economically focused on grain production, with significant growth in the quantity produced. This potential is a result of the great capacity of insertion in the international market, mainly due to the trade of products of the soybean agroindustrial complex, besides the use of the grains as source of vegetable protein. This scenario, due to increased demand, allowed the culture to expand to several regions around the world (Hirakuri and Lazzaroto, 2014).

Currently, soybean production is one of the most important agricultural activities in the world. In Brazil, in the grain harvest of 2017/2018, reached 35.05 million hectares, with production close to 113.0 million tons (Conab, 2018).

However, one of the limitations to achieving high yields with soybean cultivation is related to N fertilization management. This is due to the fact that N plays an important role in several essential processes of the plant, as a constituent of the chlorophyll molecule, amino acids, nitrogen bases, coenzymes, enzymes and nucleic acids. This limits production (Zuffo et al., 2018), making it necessary to adopt alternative methods to meet the demand of N by plants and increase productivity.

Thus, one of the techniques that has proven to be efficient in soybean cultivation is the inoculation of seeds with *Bradyrhizobium* strains, in order to reach high yields, where the symbiosis can fix large amounts of atmospheric N. This process will favor soybean physiological activities such as photosynthesis and uptake of nutrients, which are fundamental for its growth (Braccini, 2012).

However, traditional practices of *Bradyrhizobium* inoculation can be threatened by global climatic changes, such as increasing periods of drought and high temperatures, and by factors such as incompatibility with pesticides due to the chemical treatment of the seeds before inoculation, causing interference in the biological fixation of N - BFN (Zilli et al., 2006).

In Brazil, soybean co-inoculation studies have been advocated with the use of *Azospirillum brasilense*, together with *Bradyrhizobium* bacteria, in a current approach aimed at agricultural, economic, social and environmental sustainability, as well as contributing to the main limitations of BFN, obtained through the traditional inoculation of soybean only with bacteria of the genus *Bradyrhizobium* (Hungria et al., 2013).

Growth promoting bacteria of the genus *Azospirillum* can act in the plant, mainly by the production of phytohormones, being able to increase the length of the root system and soil explored volume, thus influencing soybean nodulation and nutrient absorption efficiency (Gitti, 2015). However, the dose to be used in function of the planting area and the number of previous crops needs a better understanding in order to achieve better effectivity of the inoculation of both bacteria on crop yields.

The objective of this study was to evaluate the influence of the application of different doses of *Azospirillum brasilense* on co-inoculation with *Bradyrhizobium japonicum* in the soybean crop in first and third year of cultivation.

MATERIAL AND METHODS

The experiment was conducted during the 2016/17 harvest in two experimental areas (areas 1 and 2, corresponding to the first and third year of soybean cultivation, respectively), belonging to the Farm Agropecuária WS, located at coordinates 10°03'31 "S and 55°59'23" W, altitude of 283 meters, in the rural area of the municipality of Alta Floresta-MT, Brazil. The region is characterized by rainy tropical climate (type Am), with two well defined dry-rain climatic seasons, presenting annual precipitation of up to 3,100 mm, being the average of 2,950 mm (Alvares et al., 2014). These values are annual averages measured by climatological stations installed in the region.

The soil of the experimental area was classified, according to Embrapa (2013), as Dystrophic Red-Yellow Latosol (LVAd). Before the implementation of the experiment, soil samples were collected at 0.20 m depth for chemical analysis, in which the following characteristics were obtained: **Area 1:** V% (base saturation) = 40.19; pH (H_2O) = 5.6; P (Mehlich Method) = 3.7 mg dm^{-3}; K = 39.1 mg dm^{-3}; Ca = 1.45 $cmol_c$ dm^{-3}; Mg = 0.58 $cmol_c$ dm^{-3}; H+Al = 3.17 $cmol_c$ dm^{-3}; and **Area 2:** V% (base saturation) = 50.54; pH (H_2O) = 6.1; P (Mehlich Method) = 5.0 mg dm^{-3}; K = 35.8 mg dm^{-3}; Ca = 2.3 $cmol_c$ dm^{-3}; Mg = 0.91 $cmol_c$ dm^{-3}; H+Al = 3.23 $cmol_c$ dm^{-3}. The physical analysis showed the following granulometric composition: area 1: sand = 590 g kg^{-1}; silte = 107 g kg^{-1}; clay = 303 g kg^{-1}, and area 2: sand = 340 g kg^{-1}; silt = 146 g kg^{-1}; clay = 514 g kg^{-1}.

The liming was carried out in previous years during soil correction for rice seeding, where in the first year area, 1.17 Mg of dolomitic limestone was applied, aiming at raising base saturation to 60%. For sowing fertilization, 300 kg ha^{-1} of the formulation 00-30-10 and 100 kg ha^{-1} of KCl were applied for both areas, that is, 0 kg ha^{-1} of N; 90 kg ha^{-1} of P_2O_5; 90 kg ha^{-1} of K_2O, according to the recommendations of Souza and Lobato (2004) for the soybean crop.

In the first area, soybean cultivation was carried out for the first time, and previously there was pasture for 15 years. Soon after, rice was cultivated, followed by soy. The other area was different from this, as it had been cultivated for three years with soybeans, but with the same previous history, that is, pasture and later rice cultivation.

Land preparation before sowing in the first year area was performed in a mechanized way, with two heavy field operation, passing the plow twice, revolving the soil to 20 cm of depth. In the second operation, too passing the plow twice were performed, using the leveling grid to 10 cm of depth. In both cases, disk plow was used. In the third year of cultivation, there was no revolving of the soil, because the crop was cultivated under no-tillage system. When the no-till system was implemented, Brachiaria ruziziensis was previously cultivated. This grass was sown for the production of vegetal mass that would be used to cover the soil in the next crop.

In each area (cultivation time), the plots were distributed in bands of 5.85 m in width, corresponding to 13 lines spaced at 0.45 m. The plots, 5.85 m wide and 10 m long, were installed in the different bands corresponding to the co-inoculation doses. The three central lines were considered to have a useful area, therefore, the useful area of each plot had 29.25 m^2.

The experimental design used was of bands, with 8 treatments and 4 replicates. Thus, the organization of the experiment occurred as follows: the treatments originated from the two areas with different cultivation times with soy (1 and 3 years) and the 4 doses of *Azospirillum*. That would add up to 8 treatments. The design used was in a range, with areas as the main factor and doses as a secondary factor. Each plot had 13 lines (planter width) and for the useful area, only the 4 central lines were considered. The harvest was carried out manually and the platna were collected in the three central lines, each 5 meters long that is, 15 linear meters.

The liquid co-inoculant applied was Nitrogeo AZ®, indicated for grasses, composed of the bacteria *Azospirillum brasilense* strains AbV5 and AbV6. The doses used were 0, 2, 4, 8 mL kg^{-1} of seeds.

The cultivar M 8210 IPRO was used for both cultivation areas (first and third year). As inoculant, *Bradyrhizobium japonicum* was supplied with the commercial product NitroGeo SojaTurfa®, solid, turfous in the dose of 3.2 g kg^{-1} of seeds.

The sowing of area 1 and 2 was performed mechanically using a vacuum seeder with 12 plants per linear meter, with an initial population density of 266,000 plants ha^{-1}, with a line spacing of 0, 45 m.

Desiccation with herbicide was performed when the soybean was at the R8.2 stage (Fehr and Caviness, 1977) and, after seven days, the harvest was carried out, where only the useful area was harvested. The edges of the plots were neither harvested nor considered for the purpose of evaluating treatments.

The variables analyzed were: N-plant content, 50 fully-developed leaves of the third trifolium without petiole were harvested at the stage R2 (beginning of flowering), after which the leaves were dried in a forced circulation oven at 65 °C 72 h, then milling the leaves in Willey-type Mill and subsequent determination followed the recommendations of Malavolta et al. (1989). The height of insertion of the first pod (cm), height of plant (cm), and number of pods per plant were determined with ten plants chosen at random, which represent the condition of the plot as a whole. The yield and mass of 1000 grains were determined by manual threshing of all the plants harvested in the useful area of each plot. After the threshing and sifting, four samples of 1000 grains of each plot were counted and the grain mass that was transformed into kg ha^{-1} was determined. The mass of 1000 grains and productivity had their values converted to weight with 13% water.

The data were submitted to analysis of variance, and for the qualitative factor (areas) the Tukey test was applied at 5% probability and for the quantitative factor (dose of co-inoculant) the study was performed using statistical software R (R Development Core Team, 2018).

RESULTS AND DISCUSSION

In Table 1, it can be observed that first pod insertion height, plant height and number of pods per plant were not significant ($p>0.05$) to co-inoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense*. In addition, no significant interaction between the factors was observed. These results corroborate with those obtained by Zuffo et al. (2015) who observed that seed inoculation as well as foliar application with *A. brasilense* individually or in co-inoculation with *B. japonicum* also did not present significant influence on the variables evaluated.

A. braziliense inoculation doses not influenced first pod insertion height. Differences occurred only between areas, where the area of the 3rd year of cultivation presented plants with higher insertion height of the first pod in relation to the area of 1st year cultivation (Table 1).

The height of insertion of the first pod is an important characteristic for the adjustment of cutting height of the harvester, aiming to reach the maximum efficiency during this activity, since it is a production step that cause more grain losses. This is due to the fact that plants with insertion of the first pod inferior to 0.12 m for flat areas, and 0.15 m for areas with slope can cause grain losses (Cruz et al., 2016). The highest height of first pod insertion observed in plants cultivated in the area with longer cultivation time is due to the greater fertility of this area, which allowed the greater vegetative development. This area had high fertility because, in addition to having its acidity corrected, with liming, it was still being cultivated for three years, a fact that would potentially indicate greater fertility in relation to areas whose cultivation was carried out only a year ago.

Although there was a difference between the areas, it should be noted that the height of first pod insertion in all treatments is above the minimum considered to obtain a harvest with low grain loss rates.

The third year of cultivation also provided plants with higher stature relative to the 1st year area (Table 1), however, co-inoculant doses did not influence this variable. These higher values verified in the 3rd year area can also be attributed to the previous crop with pasture in the area, which provided good soil cover, due to its great capacity of phytomass production, and greater nutrient cycling, lower temperature oscillation of the soil and thus providing greater growth of the same, as Franchini et al. (2014) argued. Despite extracting nutrients, the forage is used as a cover plant and is not grazed. Thus, it leaves all the dry mass produced in the area, thus recycling nutrients.

The non-difference between the doses of *A.braziliense* inoculation for height of insertion of first pod and height of plants, corroborates the data of Rodrigues et al. (2012), in a study of seed treatment of *Vigna unguiculata* with *A. brasilense*. Although bacteria of the genus *Azospirillum* perform BNF and can produce growth promoting compounds or stimulate endogenous plant production of these compounds. This fact was not observed in the study with the soybean in the Cerrado of Minas Gerais, Brazil, with both microorganisms transmitted via seeds.

Unlike, the results for the vegetative development observed in the present study, Correa et al. (2008) and Barassi et al. (2008) argue that plants co-inoculated with *A. brasilense* present higher biomass production and higher plants. The response to inoculation with *A. brasilense* depends on environmental conditions. When they are favorable to bacteria, they perform better and this reflects in the better development of the inoculated plants.

Regarding the number of pods per plant (Table 1), it was verified that there was a difference only between the cultivated areas, where the third year area produced plants with the highest number of pods. The best results observed in the 3rd year area may be related to the conditions most favorable to the crop, in terms of soil temperature, humidity, carbon sources and soil structuring, due to the due to the non tillage of the field and the cover provided by the vegetal residues on the surface, produced by pasture in the previous crop (Menezes et al., 2008).

Regarding the leaf content of N (Table 2), no significant difference was observed between the areas and between the inoculant doses, as well as the interaction between them. According to Malavolta (2006), the ideal range for N foliar contents is 55 to 58 g kg^{-1}, however in this study, the levels found were in the range of 60.7 to 61.3 g kg^{-1} indicating good nutrition of plants with respect to N. Nonato (2016) concluded that co-inoculation with *A. brasilense* provided an increase in leaf N contents, in addition to other nutrients, such as P and K.

Studies carried out by Hungria et al. (2013) show that in treatments inoculated only with *A. brasilense*, differences occurred in the accumulation of foliar N, thus providing greater root growth, which results in a larger area of absorption. Therefore, it can be inferred that the association between *Azospirillum* and *Bradyrhizobium* may not necessarily result in increased N-leaf content, however, in case of water or mineral deficits, these bacteria have the capacity to identify signals perceived by plants, triggering responses of with the plant, resulting in increased tolerance to different stress conditions (Coelho et al., 2017).

The lack of a significant effect of the use of *A. brasilense* on the parameters, first pod insertion height, plant height and N foliar content can be attributed to the efficiency of *B. japonicum* bacteria in atmospherical N fixation within the root nodules, and in competition with the other bacteria, including the genus *Azospirillum* (Zuffo et al., 2014). Thus, it appears that the use of co-inoculation did not influence these two variables, indicating the non-influence of Azospirillum on the action of *Bradyrhizobium*.

For the mass of 1000 grains soybean no significant difference between the first and third year of cultivation occurred; however, there was influence of the *A. braziliense* inoculant dose (Table 2). The response of the 1000 grain mass showed a quadratic behavior with higher response obtained with 1.73 doses of the commercial product Nitrogeo AZ®, which is equivalent to 6.94 mL kg^{-1} seeds of the prizobiumoduct (Figure 1).

Table 1. Values of F, coefficient of variation (CV), minimum significant difference (DMS, Tukey 5%) and mean values for first pod insertion height (IH), plant height (PH) and number of pods per plant (NPP) , as a function of inoculation with *Bradyrhizobium japonicum* used in co-inoculation with *Azospirillum brasilense*, in the first and third year of cultivation. 2016/2017.

	IH	PH	NPP
	------------(cm)--------------		unit)
Areas			
1° year	19,90 b	70,81 b	43,34 b
3° year	20,64 a	80,60 a	51,86 a
F Values	47,68 **	291,993 **	81,23 **
DMS	2,34	1,82	3,00
Dose inoculant (I)			
0	20,12	76,36	44,28
100	19,66	76,66	45,66
200	21,53	74,05	48,19
400	19,76	75,77	52,28
F value	$0,94^{ns}$	$0,34^{ns}$	$2,71^{ns}$
F Linear Regression	-	-	-
F Quadratic Regression	-	-	-
Interaction A*D			
F value	$1,34^{ns}$	$0,31^{ns}$	$3,2^{ns}$
CV 1 (%)	1,50	2,14	5,62
CV 2 (%)	12,46	7,44	12,68
CV 3 (%)	7,79	12,14	8,02

Means followed by the same lowercase letter in the column do not differ by Tukey test (p≤0.05); **, * and ^{ns} correspond respectively to 1 and 5% significance and not significant, by the test F.

Table 2. F values, coefficient of variation (CV), minimum significant difference (DMS - Tukey 5%) and mean values for a thousand grain mass (GM), N (TN) content and productivity (P), as a function of inoculation with *Bradyrhizobium japonicum* used in co-inoculation with *Azospirillum brasilense*, in first and third year cultivation areas. 2106/2017.

	TN	GM	P
	(g kg^{-1})	**(g)**	**kg ha^{-1}**
Areas			
1° year	60,70 b	209,81	4.478,47
3° year	61,38 a	210,03	4.512,24
F value	0,50 ^{ns}	0,007 ^{ns}	0,17 ^{ns}
DMS	3,04	8,19	261,48
Dose inoculant (I)			
0	60,72	188,63	4209,90
1/2	61,19	198,69	4223,09
1	60,90	226,31	4841,84
2	61,36	226,06	4706,60
F value	0,14 ^{ns}	96,68**	22,24**
F Linear Regression	-	222,11**	38,13**
F Quadratic Regression	-	40,66**	9,31
Interaction A*D			
F value	0,69 ^{ns}	1,34 ^{ns}	0,16 ^{ns}
CV 1 (%)	4,43	3,47	5,17
CV 2 (%)	3,56	2,63	4,36
CV 3 (%)	3,64	1,49	4,08

Means followed by the same lowercase letter in the column do not differ by Tukey test ($p \leq 0.05$); **, * and ^{ns} correspond respectively to 1 and 5% significance and not significant, by the test F.

Braccini et al. (2016) in a study with *B. japonicum* and *A. brasilense* inoculation via seed treatment, verified increases in the physiological characters, as well as increases in the grain mass of the soybean when compared to the control (without inoculation). According to Nonato (2016), due to the greater root growth that phytohormones provide, a number of secondary benefits of the inoculation have been observed, such as improvements in water and nutrient absorption, greater tolerance to water stresses and pathogens. Thus resulting in more productive plants, since they have better balance of nutrients and water, in this way they become more vigorous. Thus, factors such as these may have resulted in an increase in the mass of 1000 grains.

For productivity, there was no difference between cultivation areas, but there was a difference between *A. braziliense* inoculant dose (Table 2). The grain yield showed a quadratic response to the doses of co-inoculant used, and the highest response was obtained with 1.69 doses of Nitrogeo AZ®, corresponding to the application of 6.76 mL kg^{-1} seeds of commercial product (Figure 2). These results indicate the viability of the co-inoculation of the soybean seeds with both *B. japonicum* and *A. brasilense* to increase the productivity of the crop.

Hungria et al. (2011) verified that there was a 7.1% increase in soybean yield by co-inoculation with the bacterium *A. brasilense* compared to the treatment inoculated with *Bradyrhizobium* alone and 16.1% with respect to the uninoculated treatment. In the works by Müller et al. (2012) and Bárbaro et al. (2009), there was no difference in grain yield between co-inoculation and *Bradyrhizobium* inoculation. The latter was more productive when compared to treatments inoculated with *Azospirillum* alone and the control with split fertilization of 200 kg ha^{-1} of N. These results demonstrate that the *response* to co-inoculation with *A. brasilense* depends on the edaphoclimatic conditions to wich the crop is submitted. Of all the factors that are responsible for these results, the main ones are soil fertility and moisture.

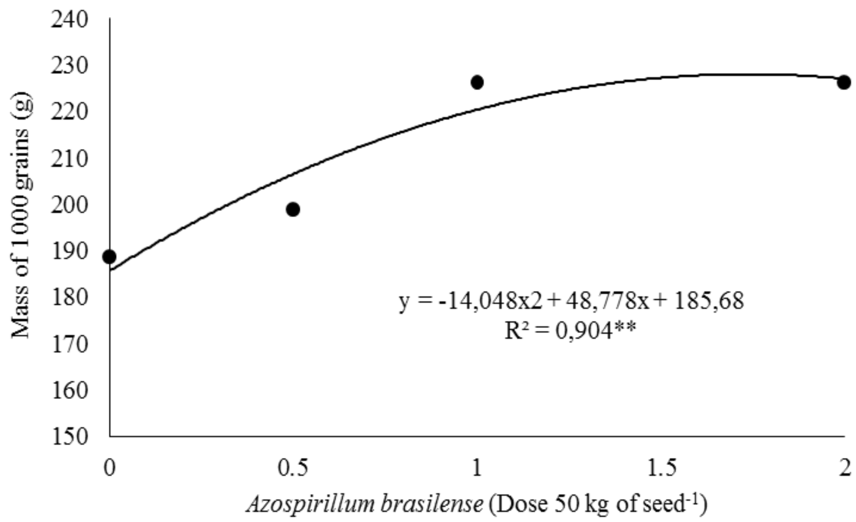

Figure 1. Mass of one thousand grains (GM) (g), of soybean as a function of doses *Azospirillum brasilense* in areas of first and third year of cultivation. 2016/2017. All plants were inoculated with *Bradyrhizobium japonicum.*

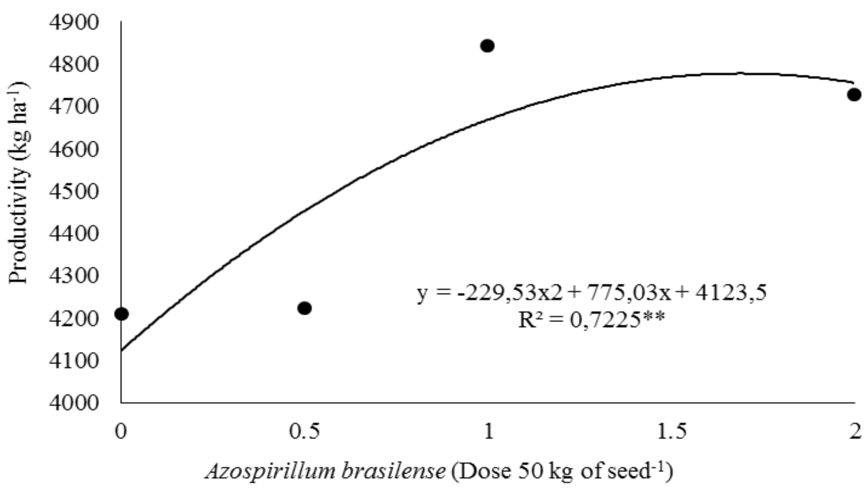

Figure 2. Productivity (P) (g) of soybean as a function of doses of *Azospirillum brasilense* and *Bradyrhizobium japonicum* in areas of first and third crop years. 2016/2017. All plants were inoculated with *Bradyrhizobium japonicum.*

Thus, the positive response of soybean to the co-inoculation with *A. brasilense* on some agronomic characteristics considered, especially productivity, underscores the significant role of diazotrophic bacteria in the performance of this crop, at the same time demonstrates the potential of using the technology studied.

CONCLUSIONS

The area of third year of cultivation promoted increase in the height of insertion of the first pod, height of plants and number of pods per soybean plant. The co-inoculation of the seeds with *Azospirillum brasilense* provides greater grain mass and soybean grain yield, with the doses of 6.94 and

6.76 mL kg^{-1} seeds being commercially available.

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Conflict of interest. The authors declare that there are no competing interests. The Faculty of Biological and Agrarian Sciences of the State University of Mato Grosso, Brazil, approved the present study for its development.

Compliance with ethical standards. This article does not contain studies carried out with human beings, therefore, there was no need for approval by the Research Bioethics Committee of the Faculty of Biological and Agrarian Sciences of the State University of Mato Grosso, Brazil, for its development.

Data availability. Data are available with the corresponding author (yama@unemat.br) upon reasonable request.

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