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Evaluation of methods for inoculating soybean seeds with *Bradyrhizobium* spp. and *Azospirillum brasilense* in the plant nodulation and productivity

Avaliação de métodos de inoculação de sementes de soja com Bradyrhizobium spp. e Azospirillum brasilense na nodulação das plantas e produtividade

Adriana Pereira da Silva[1], Alessandro Lucca Braccini[2], Pedro Henrique Felber[3], Danilo Cesar Volpato Marteli[4], Dáfila dos Santos Lima Fagotti[5], Rayssa Fernanda dos Santos[6]

ABSTRACT: Besides replacing the mineral fertilization, the biological nitrogen fixation promoted by bacteria from genus *Bradyrhizobium* provides enough nitrogen to enable high yields in the soybean crop. The objective of this study was to compare the standard inoculation of a product containing *Bradyrhizobium* spp., either applied alone or associated to *Azospirillum brasilense*, to the preinoculation by seed treatment in the plant nodulation and productivity of soybean crop grains. The trial was implanted in november, 2020 in four locations (Mandaguaçu/PR, Marialva/PR, Paranavaí/PR, and Itaquiraí/MS) with different edaphoclimatic features. The design with randomized blocks was used, with seven treatments and five repetitions. The evaluated variables were number and dry mass of nodules in the beginning of flowering, dry biomass of the aerial portion, N content and total N in the aerial portion and grains, and grain yield. The results obtained in the four tests enabled to conclude that the inoculation with *Bradyrhizobium* spp. + *Azospirillum brasilense* by seed treatment of leaf spraying with *Azospirillum* showed to be technically feasible in the soybean crop.

Key words: *Glycine max*, inoculant, coinoculation, biological nitrogen fixation, yield.

RESUMO: A fixação biológica de nitrogênio, promovida por bactérias do gênero *Bradyrhizobium*, além de substituir a adubação mineral, fornece nitrogênio suficiente para propiciar elevados rendimentos na cultura da soja. O objetivo do trabalho foi comparar a inoculação padrão de produto contendo *Bradyrhizobium* spp., aplicado isolado ou em associação com Azospirillum brasilense, com a pré-inoculação, via tratamento de sementes, na nodulação das plantas e produtividade de grãos da cultura da soja. O experimento foi implantado no mês de novembro de 2020 em quatro localidades (Mandaguaçu/PR, Marialva/PR, Paranavaí/PR e Itaquiraí/MS) com características edafoclimáticas distintas. Foi utilizado o delineamento em blocos casualizados, com sete tratamentos e cinco repetições. As variáveis avaliadas foram o número e massa seca de nódulos no início do florescimento, biomassa seca da parte aérea, teor de N e N-total na parte aérea e nos grãos e rendimento de grãos. Os resultados obtidos nos quatro ensaios permitiram concluir que a inoculação com *Bradyrhizobium* spp. + *Azospirillum brasilense*, via tratamento de sementes ou pulverização foliar do *Azospirillum*, mostrou-se tecnicamente viável na cultura da soja.

Palavras-chave: *Glycine max*, inoculante, coinoculação, fixação biológica de nitrogênio, rendimento.

Autor correspondente: Adriana Pereira da Silva
E-mail: apereira@rizobacter.com

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[1] Pós-Doutoramento em Agronomia (Solos), Universidade Estadual de Londrina (UEL), Londrina- PR.

[2] Doutorado em Fitotecnia (Produção Vegetal), Departamento de Fitotecnia, Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brasil.

[3] Mestrado em Produção Vegetal, Universidade Estadual de Maringá (UEM), Maringá- PR.

[4] Engenheiro agrônomo, Universidade Estadual de Maringá (UEM), Maringá- PR.

[5] Doutorado em Agronomia, Universidade Estadual de Londrina (UEL), Londrina- PR.

[6] Doutorado em Produção Vegetal, Universidade Estadual de Maringá, Maringá- PR.

1 INTRODUCTION

The soybean (Glycine max [L.] Merrill) is the main protein source used to formulate animal rations and the second greatest source of vegetal oil. It is destined with lesser expressiveness to human consumption and raw material for food products (USDA, 2013). In the 2020/2021 agricultural harvest, Brazil was consolidated as the largest soybean producer and exporter in the world, with a record production of 135.5 million metric tons, representing an 8.6% increase compared to the previous harvest, a cultivation area of approximately 38.5 million hectares, and average productivity of 3,523 kg/ha (CONAB, 2021). Nevertheless, the country still has potential to increase this crop productivity and production.

The use of high-quality seeds is among the main factors that influence a crop success (MARCOS FILHO, 2015). Thus, the use of seed treatment is a strong ally, protecting the seed and enabling the initial development of seedlings, even under adverse conditions (NAKAGAWA, 1999). According to Bonotto (2008), the seed treatment can be composed by insecticides, fungicides, inoculants, and some micronutrients, and its use has been considerably increasing in the last years due to its practicality, low cost, and better nutritional management.

The biological nitrogen fixation (BNF) promoted by bacteria from genus *Bradyrhizobium* has enabled a reduction in the production costs in the soybean crop. Then, besides replacing the mineral nitrogenated fertilization, the BNF enables a sufficient N input to obtain high grain productivities (HUNGRIA, et al., 2001). However, and especially in tropical regions, the efficiency of this process is influenced by several edaphoclimatic factors, besides the management practices, such as the seed treatment with fungicides before the inoculation (CAMPO et al., 2009; ZILLI, et al., 2009).

Considering the main current BNF limitations and potentials with the soybean and the benefits attributed to several crops by the inoculation with growth-promoting bacteria, such as *Azospirillum*, it is deduced that a coinoculation with both organisms might increase the crop performance, in an approach respecting the current demands for agricultural, economic, social, and environmental sustainability. However, it is necessary to conduct coinoculation trials with *Bradyrhizobium* and *Azospirillum* under the Brazilian climatic conditions (HUNGRIA, et al., 2013).

Therefore, the present study was developed intending to compare the standard inoculation of a product containing *Bradyrhizobium* spp., both applied alone and associated to *Azospirillum brasilense*, with a preinoculation by seed treatment in the plant nodulation and grain productivity in the soybean crop.

2 MATERIAL AND METHODS

The present trial was conducted in the 2020/21 harvest, in four trial areas. The first area was located at São Valentim Farm, in Mandaguaçu municipality, Northwestern Paraná state, on the latitude of 23º24'00" South and the longitude of 52º08'39" West of Greenwich, with average altitude of 480 m. The second area was located at Araucária Ranch, Marialva municipality/PR, on the following coordinates: 23º27'28" South and 51º47'41" West, with average altitude of 660 m. The third area was located at Diamante Farm, Paranavaí municipality/PR, on the coordinates of 23º01'11" South and 52º25'30" West and average altitude of 450 m. The last area was located at Mestiço Farm, in Itaquiraí municipality, Southern Mato Grosso do Sul state, with coordinates of 23º18'16" South and 54º12'40" West and average altitude of 360 m. The prevailing climate in the region is Cfa (humid mesothermal, with profuse rainfall in summer and dry winter with hot summer) type, according to the Köppen classification (ALVARES, et al., 2013).

The soil of the trial areas was classified according to Santos, et al. (2013) as dystroferric red latosol with clayish texture in Mandaguaçu/PR and Marialva/PR (Type 3 - approximately 60% of clay, respectively) and dystrophic red-yellow latosol with sandy texture in Paranavaí/PR and Itaquiraí/MS (Type 1 - approximately 15% of clay). Soil samples were collected from 0 cm to 20 cm deep to perform soil chemical (macro- and micronutrients) and granulometric analyses, which are presented in Table 1.

Table 1. Results of the soil chemical and granulometric analysis.

Mandaguaçu - PR												
Depth	**[1]P**	**[2]pH**		**H+Al**	**Al**	**K**	**[3]Ca**	**[3]Mg**	**SB**	**CTC**	**V**	**[4]C**
(cm)	**(mg/dm^3)**	**$CaCl_2$**	**SMP**		---------- **$cmolc/dm^3$** ----------						**%**	**g/dm^3**
0-20	12.42	5.30	5.90	5.23	0.0	0.65	4.08	1.17	5.90	11.13	53.01	21.34
	[5]$S\text{-}SO_4^{2-}$		**Zn**	**Fe**	**Cu**	**Mn**	**B**	**Total sand**		**Clay**		**Silt**
	---------- **mg/dm^3** ----------							---------- % ----------				
	32.37		4.22	35.37	13.22	82.93	0.26	30.0		64.0		6.0
Marialva - PR												
Depth	**[1]P**	**[2]pH**		**H+Al**	**Al**	**K**	**[3]Ca**	**[3]Mg**	**SB**	**CTC**	**V**	**[4]C**
(cm)	**(mg/dm^3)**	**$CaCl_2$**	**SMP**		---------- **$cmolc/dm^3$** ----------						**%**	**g/dm^3**
0-20	14.31	4.80	5.90	5.08	0.06	0.15	3.64	1.50	5.29	10.37	51.01	20.60
	[5]$S\text{-}SO_4^{2-}$		**Zn**	**Fe**	**Cu**	**Mn**	**B**	**Total sand**		**Clay**		**Silt**
	---------- **mg/dm^3** ----------							---------- % ----------				
	2.92		2.34	68.34	15.12	289.44	0.19	15.0		66.0		19.0
Paranavaí - PR												
Depth	**[1]P**	**[2]pH**		**H+Al**	**Al**	**K**	**[3]Ca**	**[3]Mg**	**SB**	**CTC**	**V**	**[4]C**
(cm)	**(mg/dm^3)**	**$CaCl_2$**	**SMP**		---------- **$cmolc/dm^3$** ----------						**%**	**g/dm^3**
0-20	9.61	5.51	5.90	2.27	0.0	0.27	1.27	0.37	1.91	4.18	45.69	6.06
	[5]$S\text{-}SO_4^{2-}$		**Zn**	**Fe**	**Cu**	**Mn**	**B**	**Total sand**		**Clay**		**Silt**
	---------- **mg/dm^3** ----------							---------- % ----------				
	1.81		1.97	14.60	0.77	53.00	0.10	90.10		7.00		2.90
Itaquiraí - MS												
Depth	**[1]P**	**[2]pH**		**H+Al**	**Al**	**K**	**[3]Ca**	**[3]Mg**	**SB**	**CTC**	**V**	**[4]C**
(cm)	**(mg/dm^3)**	**$CaCl_2$**	**SMP**		---------- **$cmolc/dm^3$** ----------						**%**	**g/dm^3**
0-20	12.73	5.40	5.90	2.14	0.0	0.10	1.03	0.65	1.78	3.92	45.41	8.11
	[5]$S\text{-}SO_4^{2-}$		**Zn**	**Fe**	**Cu**	**Mn**	**B**	**Total sand**		**Clay**		**Silt**
	---------- **mg/dm^3** ----------							---------- % ----------				
	0.67		0.96	102.24	0.66	19.32	0.08	85.00		12.00		3.00

[1]Mehlich Extractor 1; [2]CaCl2 at 0.01 mol/L; [3]KCl at 1 mol/L; [4]Walkley-Black Method; [5]Monocalcic Phosphate Method.

The detailed schedule of the employed treatments is presented in Table 2.

Table 2. Detailed schedule of the treatments of seed standard inoculation, coinoculation, and preinoculation with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense*, applied by seed treatment and leaf spraying in the soybean crop (Mandaguaçu/PR, Marialva/PR, Paranavaí/PR, and Itaquiraí/MS - 2020/2021).

Treatment	Description
1	No nitrogenated fertilizer and inoculation
2	Control with mineral N (200 kg of N/ha, being 50% in seeding and 50% in flowering)
3	Standard inoculation with the *Bradyrhizobium* registered at MAPA (Total Biotecnologia)
4	Standard coinoculation with the *Bradyrhizobium* + *Azospirillum* registered at MAPA (Total Biotecnologia)
5	Inoculation with Rizoliq TOP[1] + inoculation with Rizospirillum[2]
6	Inoculation with Rizoliq TOP in seed + Rizospirillum in leaf[4]
7	Inoculation with Rizoliq TOP + Rizospirillum + Premax[3], 10 days before seeding

[1]Rizoliq TOP[1] (*Bradyrhizobium japonicum*): concentration: 6 x 10^9 UFC/mL; dose: 100 mL/50 kg of soybean seeds;
[2]Rizospirillum (*Azospirillum brasilense*): strains Abv 5 and Abv 6; concentration: 2 x 10^7 UFC/mL;
[3]Premax: bacterial protector.
[4]The time of *Azospirillum brasilense* application on leaves was between stages V_4 and V_5.

The spacing used between lines was 0.45 m, and the final stand was 12 plants set per linear meter in Paranavaí/PR and Itaquiraí/MS and 14 plants per meter in Mandaguaçu/PR and Marialva/PR, totaling the final population of approximately 266,666 and 311,111 plants/ha, respectively, in the four locations. The cultivar used in the trials was M 6410 IPRO (company Monsoy), with the technology Intacta RR2 PRO®, which present undetermined growth habit and belongs to the precocious maturation group (6.4).

The seeds were treated with the product Standak Top® (BASF) (pyraclostrobin, 25 g/L; methyl thiophanate, 225 g/L; fipronil, 250 g/L), at the dosage of 200 mL/100 kg of seeds, besides the respective inoculant dosages for each treatment.

The trial plots were constituted by nine lines of 6 m in length, spaced by 0.45 m between themselves, totaling 24.3 m^2, using a seeding depth of approximately 3 cm and a density of approximately 20 seeds per linear meter. The harvest footprint was constituted by four central rows, excluding the borders, that is, five outer lines, as well as 1.0 m of each extremity from the central lines.

The seeding occurred on November 16, 2020 in Marialva/PR and Paranavaí/PR and on November 17 and 19, 2020, respectively, in Itaquiraí/MS and Mandaguaçu/PR, employing the tillage system in all areas. The trial crops were installed and managed according to the Soybean Production Technology from the company Embrapa Soja (SEIXAS et al., 2020) for the microregion 202 (Northwestern PR and MS), and then 250 kg/ha of the formulate 4-30-10 were used for seeding, plus 100 kg/ha of KCl in covering (stage V_4), in Mandaguaçu/PR and Marialva/PR and 250 kg/ha of the formulate 4-30-10 and 165 kg/ha of MAP were used in the base, plus 120 kg/ha of KCl in covering (V_4), in Paranavaí/PR and Itaquiraí/MS.

Leaf spraying with the product Co-Mo Platinum® (Stoller) was performed at the dose of 100 mL/ha, in the development stages V_3/V_4 and R_1 (beginning of flowering).

The leaf applications with the product Rizospirillum (*Azospirillum brasilense*) were performed between the development stages V_4 and V_5, separately from the other products (only the inoculant in the carrier). These sprayings were performed using a backpack pump, with capacity for 20 liters. The insects, diseases and weed were controlled as necessary. The analyzed response variables are presented below:

Number of nodules per plant: the nodulation evaluation was performed in the beginning of flowering (stage R_1) in five randomly chosen plants per plot (HUNGRIA and ARAÚJO, 1994).

Nodule dry mass: in the following step, the formed nodules were taken to be dried in a stove at 65ºC until reaching a constant weight. The results were expressed in mg/plant (HUNGRIA and ARAÚJO, 1994).

Aerial portion dry biomass: in the next step, the nodules formed were dried in an oven at 65°C, until constant weight was obtained. The results were expressed in g/plant.

Determination of nitrogen (N) content and total N in the aerial portion and the grains: this was performed by using the Kjeldahl method to quantify the total nitrogen, according to the recommendation from the Association of Official Analytical Chemists (AOAC, 2016) and Vitti et al. (2001).

The results were expressed in mg of N/plant (N content and total N in the aerial portion), mg/kg (N content in the grains), and kg of N/ha (total N in the grains).

Grain yield: was determined by manual harvesting (maturation stage R9) of all plants in the useful area of the experimental units. Moisture was corrected to 13% and productivity was expressed in kg/ha for each location.

The trials were conducted by adopting the trial design in fully randomized blocks (FRB), with seven treatments and five repetitions, totaling 35 trial units (plots) in each location. The collected data were subjected to a variance analysis by the F test ($p \leq 0.05$) and, when significant, the averages were compared by the Tukey test, at the same significance level, by using the statistic analysis software (FERREIRA, 2011).

The count of diazotrophic microorganisms to determine the bacterial population in the no. of cells per mL or g was performed by estimating the more likely number (MLN), according to the methodology described by Döbereiner et al. (1995) for the four trial areas.

3 RESULTS AND DISCUSSION

3.1 SÃO VALENTIM FARM (MANDAGUAÇU/PR – 2020/2021)

The summary of the variance analysis of the data, enabled to infer that there were significant differences ($p \leq 0.05$) for the response variables evaluated in the present trial.

All inoculation, coinoculation, or preinoculation treatments (treatments 3 to 7) used with *Bradyrhizobium* spp., both alone or associated to *Azospirillum brasilense*, demonstrated an increase in the number of nodules evaluated in stage R_1 (Table 3) when compared to the absolute witness (negative control), where no inoculation and fertilization with nitrogenated fertilizer (treatment 1) was performed, or, then, they were performed just with a control with mineral N (treatment 2).

Table 3. Averages of the variables number of nodules per plant in the beginning of flowering (NNOD), mass of dry nodules per plant in the beginning of flowering (MNOD), dry biomass of the aerial portion of the plants (MSPA), nitrogen content in the aerial portion (TNPA), total nitrogen in the aerial portion of the plants (NTPA), nitrogen content in the grains (TNG), and total nitrogen in the grains (NTG) in response to the different seed treatments with standard inoculation, coinoculation, and preinoculation with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* applied by seed treatment and leaf spraying in the soybean crop (Mandaguaçu/PR - 2020/2021).

(Continua)

Treatment	NNOD	MNOD	MSPA	TNPA	NTPA	TNG	NTG
	(unit/plant)	(mg/plant)	(g/plant)	(g/kg)	(kg/ha)	(g/kg)	(kg/ha)
1	46.40 E	138.13 E	7.57 F	43.66 E	111.49 E	45.24 E	109.74 F
2	29.52 F	73.76 F	8.16 EF	46.79 DE	125.89 DE	47.79 D	124.64 E

(Conclusão)

Treatment	NNOD	MNOD	MSPA	TNPA	NTPA	TNG	NTG
	(unit/plant)	(mg/plant)	(g/plant)	(g/kg)	(kg/ha)	(g/kg)	(kg/ha)
3	58.32 D	158.56 D	8.55 DE	48.69 CD	139.39 CD	49.39 D	130.49 DE
4	81.20 B	199.75 B	10.25 B	51.13 BC	145.25 C	52.29 C	141.46 CD
5	99.80 A	222.08 A	11.15 A	58.18 A	183.19 A	60.96 A	187.18 A
6	70.36 BC	185.36 BC	9.12 CD	54.39 B	164.55 B	55.48 B	169.68 B
7	62.64 CD	171.82 CD	9.69 BC	52.81 B	149.59 BC	53.64 BC	150.36 C
Average	64.03	164.21	9.21	50.81	145.62	52.11	144.79
VC (%)	8.67	5.25	3.78	3.33	5.74	1.99	4.54

Averages followed by the same letter in the column do not significantly differ by the Tukey test at a 5% probability level.
unit/plant: unit per plant; mg/plant: milligrams per plant; g/plant: grams per plant; g/kg: grams per kilogram of nitrogen (N); and kg/ha: kilograms per hectare of nitrogen (N).

When a coinoculation with *Bradyrhizobium* spp. + *Azospirillum brasilense* (treatment 5) was performed by seed treatment, there was usually an increase in the average mass of dry nodules at stage R_1 (Table 3), with such values being significantly higher than the ones in the absolute witness (treatment 1), as well as the ones in nitrogenated fertilization (treatment 2), using a dose of 200 kg/ha of N. Therefore, treatments 1 and 2 achieved the worst results in this evaluated variable (Table 3). These lower values are obviously related to the lack of inoculation in the respective treatments. Furthermore, and according to EMBRAPA Soja (2013), besides reducing the nodulation and the efficiency of the biological nitrogen fixation (BNF), the application of a nitrogenated fertilizer in seeding or covering at any plant development stage does not cause any increase in productivity for the soybean. Therefore, it is recommended to respect the limit of 20 kg/ha of N if the farmer will use any nitrogen-containing fertilizer formula, if this type will be more cost-efficient than nitrogen-less formulations.

When compared to other studies of the same type, the results obtained in this trial showed a proper nodulation, as a dry nodule mass ranging between 100 and 200 mg/plant would be sufficient to ensure the N supply required by a soybean plant for its normal development (HUNGRIA et al., 2007).

Considering the number of nodules per plant (NNOD), evaluated in R_1 (Table 3), the evaluated treatments involving an inoculation with *Bradyrhizobium* spp. alone (treatment 3) or in coinoculation with *Azospirillum brasilense*, either by seeds or leaf spraying (treatments 3 to 6), or even a preinoculation of seeds with 10-day antecedence before seeding with a product containing *Bradyrhizobium* spp. + *Azospirillum brasilense* + bacterial protector (treatment 7), presented higher average results when compared to the absolute witness and to the fertilization with mineral N (treatments 1 and 2, respectively).

The highlight was the treatment 5 (seed coinoculation with *Bradyrhizobium* spp. + *Azospirillum brasilense*), which presented results that were significantly higher ($p \leq 0.05$) than all other treatments (Table 3).

It is noted that the coinoculation treatments with *Bradyrhizobium* spp. and *Azospirillum brasilense* evaluated in this trial, either applied by seeds or leaf spraying (treatments 5 and 6, respectively), or then the 10-day preinoculation (treatment 7), have significantly increased the number and mainly the dry biomass of nodules per plant (MNOD), with values well above those considered as satisfactory by Hungria et al. (2007) for the soybean crop. The values of the number of nodules (NNOD) overcame the level of 60 nodules per plant for treatments 5 to 7, besides values higher than 170 mg per plant in the MNOD variable for the same treatments (Table 3).

These results are according to the ones reported by Câmara (2000), who revealed that plants with 10 to 30 nodules in flowering present enough conditions to achieve high fixated nitrogen contents and, consequently, high grain yield.

Thus, the results achieved until now corroborate the ones achieved in several other places in Brazil, indicating that the nitrogenated fertilization in soybean crop is unnecessary, provided a seed inoculation is performed (HUNGRIA, 1997; MENDES et al., 2000; BÁRBARO et al., 2006).

These results enable to infer that the coinoculation treatments with *Bradyrhizobium* spp. and *Azospirillum brasilense* under investigation in this trial, either by seed treatment (treatments 4 and 5) or leaf application of *Azospirillum* (treatment 6), as well as preinoculation with 10-day antecedence, have increase the nodulation parameters in the soybean plants.

In the evaluation of the dry biomass of the aerial portion in R_1 (Table 3), treatment 5 (coinoculation of *Bradyrhizobium* spp. and *Azospirillum brasilense*) provided significant increases in this variable when compared to the other treatments, as well as compared to the negative control (treatment 1) or the control with mineral N (treatment 2). Referring to the N content in the aerial portion (TNPA) and the total N in the aerial portion (NTPA) of the plants, treatment 5 (seed coinoculation with products containing *Bradyrhizobium* **spp.** and *Azospirillum brasilense*) once more presented a significant difference compared to the other treatments (Table 3).

Referring to the N content in the grains (TNG) and the total N in the grains (NTG), treatment 5 once more presented a significant difference compared to the other treatments (Table 3). In both variables, the lowest response was seen for treatment 1 (control with lack of nitrogenated fertilizer and inoculation). It is seen that treatments 4, 6, and 7 presented intermediate results, a bit lower than treatment 5, but still significant ($p \leq 0.05$).

When evaluating the grain yield (Figure 2), it is seen that treatment 5 (seed coinoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense*) presented the best productive performance, that is, this treatment presented a result significantly higher ($p \leq 0.05$) than the negative control (treatment 1), as well as all the other treatments evaluated in this trial.

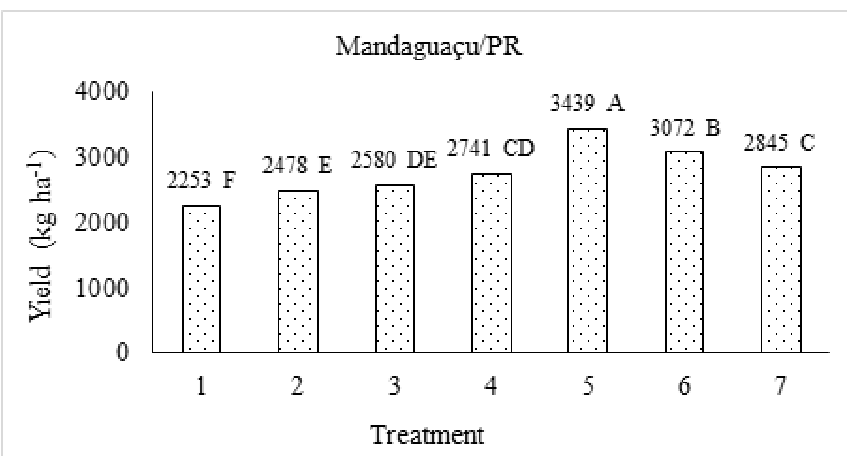

Figure 2. Grain yield in response to the different treatments of standard inoculation, coinoculation, and preinoculations of seeds with products containing *Bradyrhizobium* **spp.** and *Azospirillum brasilense*, applied by seed treatment and leaf spraying in the soybean crop (Mandaguaçu/PR - 2020/2021).

The coinoculation of *Bradyrhizobium* spp. and *Azospirillum brasilense* tested in this study, either by seed treatment or leaf spraying with *Azospirillum*, as well as preinoculation with 10-day antecedence referring to the crop seeding (treatment 7) might ensure a larger bacterial population at the germination time, so proving an increase in the cell number, which enables a profuse and efficient nodulation formation at the plant crown, favoring the biological nitrogen fixation more rapidly, and thus, consequently, enabling higher soybean yields (Figure 2). Therefore, the biological interaction between genera *Bradyrhizobium* spp. and *Azospirillum brasilense* efficiently replaces the use of nitrogenated mineral fertilization in the soybean crop.

The inoculant application via seed treatment has proven to be technically feasible in soybean crops, especially when co-inoculating *Bradyrhizobium* spp. and *Azospirillum brasilense*.

3.2 ARAUCÁRIA RANCH (MARIALVA/PR – 2020/2021)

The variation coefficients (VC) were relatively low for most evaluated features, except for the number of nodules per plant (NNOD) and the dry mass of nodules per plant at stage R_1 (MNOD), which presented values above 10%.

A significant increase was seen in the number of nodules per plant (NNOD) or the dry mass of nodules (MNOD) evaluated at stage R_1 (Table 4) in all the evaluated treatments, that is, since the standard inoculation (treatment 3) and passing by the coinoculation treatments (treatments 4 to 7), when compared to the treatments where no inoculation was performed (treatments 1 and 2). The single exception was between treatments 3 and 1 in the variables NNOD and MNOD, where there was no significant difference ($p > 0.05$) between these two treatments.

Table 4. Averages of the variables number of nodules per plant in the beginning of flowering (NNOD), mass of dry nodules per plant in the beginning of flowering (MNOD), dry biomass of the aerial portion of the plants (MSPA), nitrogen content in the aerial portion (TNPA), total nitrogen of the aerial portion of the plants (NTPA), nitrogen content in the grains (TNG), and total nitrogen in the grains (NTG) in response to the different seed inoculation, coinoculation, and preinoculation treatments with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* applied by seed treatment and leaf spraying in the soybean crop (Marialva/PR - 2020/2021).

Treatment	NNOD	MNOD	MSPA	TNPA	NTPA	TNG	NTG
	(unit/plant)	(mg/plant)	(g/plant)	(g/kg)	(kg/ha)	(g/kg)	(kg/ha)
1	55.00 E	172.11 D	10.21 E	38.64 E	135.75 F	33.52 D	87.82 E
2	28.00 F	98.69 E	11.06 E	41.15 DE	158.69 EF	43.24 C	116.62 D
3	60.36 DE	199.23 D	12.90 D	43.21 CD	179.91 DE	44.44 C	122.64 CD
4	86.04 BC	299.54 BC	16.84 B	50.30 B	245.97 B	52.68 AB	147.22 AB
5	110.64 A	393.48 A	20.15 A	53.49 A	306.57 A	55.58 A	159.42 A
6	74.08 CD	266.17 C	14.68 C	45.18 C	203.01 CD	45.79 C	126.27 CD
7	96.24 AB	346.01 AB	15.68 BC	48.01 B	217.64 BC	48.95 BC	135.12 BC
Average	72.91	253.60	14.50	45.71	206.79	46.31	127.87
VC (%)	10.01	12.00	4.64	2.78	7.29	6.63	5.97

Averages followed by the same letter in the column do not significantly differ by the Tukey test at a 5% probability level. unit/plant: unit per plant; mg/plant: milligrams per plant; g/plant: grams per plant; g/kg: grams per kilogram of nitrogen (N); and kg/ha: kilograms per hectare of nitrogen (N).

The treatment 2 that presented the worst nodulation results (NNOD and MNOD), that is, the control with nitrogenated fertilization and no seed inoculation (Table 4). These results are according to what was reported in the Soybean Production Technologies of (SEIXAS et al., 2020), which states that, though nitrogen (N) is the mineral element required in highest quantity by the soybean crop, most of the N obtained by the plants comes from the symbiotic fixation, which occurs with bacteria from genus *Bradyrhizobium*. Furthermore, it is settled that N must be supplied by inoculation and that doses higher than 20 kg of N per hectare might be harmful to the bacterioid development and inhibit the plant nodulation and, consequently, negatively interfere with the Biological Nitrogen Fixation (BNF).

According to Oliveira Junior et al. (2015), there is great discussion on the possible benefits of using mineral N in soybean, but most results obtained under field conditions show that N application in seeding or covering by soil and/or leaves does not produce significant productivity results. However, according to Seixas et al. (2020), in cases of use of NPK formulations containing MAP (9% to 10% of N and 50% to 55% of P_2O_5) as P source, the application of N doses higher than 20 kg/ha must be avoided, aiming to properly set the BNF.

In the plant nodulation variables (NNOD and MNOD) evaluated in flowering, treatment 5 (coinoculation with *Bradyrhizobium* spp. and *Azospirillum brasilense* by seed treatment) presented the best results when compared to the negative control (treatment 1), to the fertilization with mineral N (treatment 2), and to the other treatments with inoculation with *Bradyrhizobium* alone (treatment 3) or seed coinoculation (treatments 4 and 6) and preinoculation (treatment 7) (Table 4).

The same thing occurred with treatment 4 (standard coinoculation with a product registered at MAPA), which was statistically higher than the absolute witness, the nitrogenated fertilization, and the standard inoculation with *Bradyrhizobium* spp. by seeds (treatments 1 to 3, respectively) (Table 4). These results are according to the ones reported by Câmara (2000), who revealed that plants with 10 to 30 nodules in flowering present enough conditions to obtain high fixated nitrogen contents and, consequently, high soybean grain yield (Figure 3).

Referring to the evaluation of the dry biomass of the aerial portion (MSPA) at stage R_1 (Table 4), treatment 5, closely followed by treatment 4 (Table 4), were those that promoted the highest significant increase ($p \leq 0.05$) in such variable when compared to the other treatments.

Referring to TNPA and NTPA, treatment 5 (seed coinoculation with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense*) presented a significant difference compared to the other employed treatments (Table 4). The lowest results in both variables were seen for treatment 1 (negative control), followed by treatment 2 (control with mineral N). No significant difference was seen between treatments 4 and 7 (standard coinoculation by seeds and preinoculation with *Bradyrhizobium* spp. + *Azospirillum brasilense* + bacterial protector, respectively) or between treatments 3 and 6 (standard inoculation with *Bradyrhizobium* spp. or coinoculation with *Bradyrhizobium* spp. by seeds and *Azospirillum brasilense* by leaf spraying, respectively).

Referring to TNG and NTG, treatment 5 presented results higher than the other treatments (Table 4), but without a significant difference ($p > 0.05$) from treatment 4.

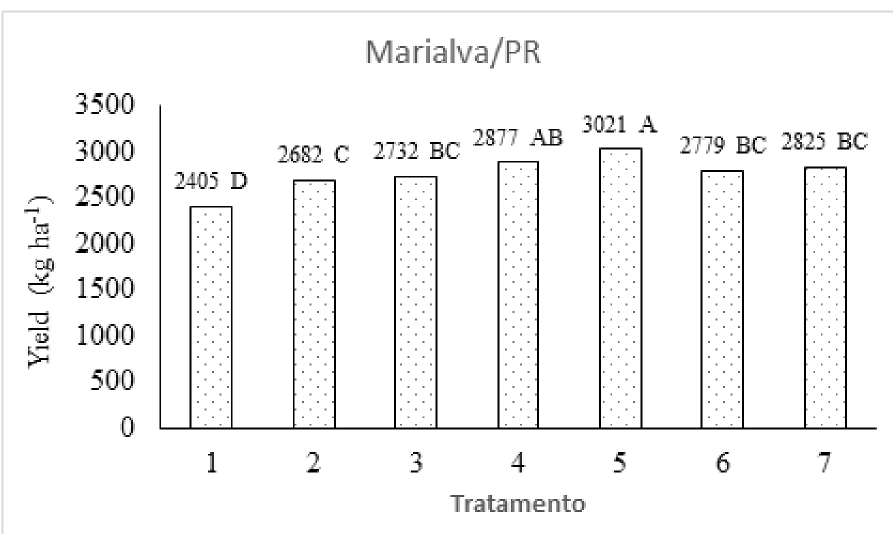

Figure 3. Grain yield in response to the different seed inoculation, coinoculation, and preinoculation treatments with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* applied by seed treatment and leaf spraying in soybean crop (Marialva/PR - 2020/2021).

In the variable REND (Figure 3), treatment 5 (coinoculation with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense*) promoted the highest productivity in the soybean crop, nevertheless not differing from treatment 4 (standard coinoculation), that is, they presented results significantly ($p \leq 0.05$) higher than the negative control (treatment 1) and the fertilization with mineral N (treatment 2).

No significant differences were seen between the other treatments (2, 3, 6, and 7), in this same response variable (REND).

3.3 DIAMANTE FARM (PARANAVAÍ/PR – 2020/2021)

Generally,the variation coefficient (VC) were relatively low for most evaluated features, except for the number of nodules per plant (NNOD), which presented a value above 15%.

All employed treatments, either with standard inoculation (treatment 3) or then, coinoculation (treatments 4 to 7) showed a significant increase in the number of nodules per plant (NNOD) or the mass of dry nodules (MNOD) evaluated at stage R_1 (Table 5) when compared to treatments where no inoculation was performed (treatments 1 and 2). The only exception was between treatments 3 and 1 in the variable NNOD, where there was no statistical difference ($p > 0.05$).

Table 5. Averages of the variables number of nodules per plant in the beginning of flowering (NNOD), mass of dry nodules per plant in the beginning of flowering (MNOD), dry biomass of the aerial portion of the plants (MSPA), nitrogen content in the aerial portion (TNPA), total nitrogen of the aerial portion of the plants (NTPA), nitrogen content in the grains (TNG), and total nitrogen in the grains (NTG) in response to the different seed standard inoculation, coinoculation, and preinoculation treatments with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* applied by seed treatment and leaf spraying in the soybean crop (Paranavaí/PR - 2020/2021).

Treatment	NNOD	MNOD	MSPA	TNPA	NTPA	TNG	NTG
	(unit/plant)	(mg/plant)	(g/plant)	(g/kg)	(kg/ha)	(g/kg)	(kg/ha)
1	16.68 CD	56.66 D	6.87 F	43.50 F	92.30 F	46.24 E	102.18 F
2	12.12 D	53.39 D	9.16 E	46.53 E	121.01 E	50.68 D	121.57 E
3	18.92 CD	65.80 C	10.45 D	49.37 D	136.56 DE	53.55 CD	133.60 D
4	29.48 B	77.57 B	11.16 CD	54.45 B	178.01 B	59.54 B	157.06 B
5	44.72 A	85.59 A	15.15 A	57.06 A	210.41 A	64.79 A	172.64 A
6	22.60 BC	73.12 B	12.91 B	51.20 CD	149.81 CD	54.68 C	140.27 CD
7	24.60 BC	75.54 B	12.00 BC	52.87 BC	165.47 BC	56.45 C	144.93 C
Average	24.16	69.67	11.10	50.71	150.51	55.13	138.89
VC (%)	17.10	4.30	4.28	2.41	7.35	2.65	3.73

Averages followed by the same letter in the column do not significantly differ by the Tukey test at a 5% probability level.
Unit/plant: unit per plant; mg/plant: milligrams per plant; g/plant: grams per plant; g/kg: grams per kilogram of nitrogen (N); and kg/ha: kilograms per hectare of nitrogen (N).

In the variable number of nodules per plant (NNOD) evaluated at R_1, all the coinoculation treatments (4 to 7), presented higher average results, with treatment 5 presenting the best result when compared to the negative control, fertilization with mineral N, and standard inoculation (Table 5).

The same thing occurred with treatment 4 (standard coinoculation with product registered at MAPA), which was statistically higher than the treatments 1 to 3. Nevertheless, there was no significant difference ($p > 0.05$) between treatment 4 and treatments 6 (*Bradyrhizobium* spp. by seeds + *Azospirillum* *brasilense* by leaves) and 7 (*Bradyrhizobium* spp. + *Azospirillum brasilense* + bacterial protector in seed preinoculation, 10 days before the seeding).

The use of *Azospirillum brasilense* has shown beneficial effects in the referred association to *Bradyrhizobium* spp., as these free-living bacteria are able to produce phytohormones that determine a higher root system development and, therefore, the possibility of exploiting a broader soil volume, as shown by Piccinin et al. (2011) and Braccini et al. (2012) in wheat and corn crops. Thus, the results obtained until now corroborate the ones obtained by several researchers in several places in Brazil (HUNGRIA, 1997; MENDES et al., 2000; BÁRBARO et al., 2006; BÁRBARO et al., 2020).

Treatments 1 and 2 also achieved the lowest results referring to the mass of dry nodules per plant (MNOD), while treatment 5 presented the highest nodule mass (Table 5), significantly differing from all the other treatments, including the standard inoculation (treatment 3). The low MNOD values in treatments 1 and 2 might be explained because of the lack of inoculation in both treatments and the soil feature of the Caiuá Sandstone (approximately 90% of sand) in this area located at Paranavaí municipality/PR.

Referring to the evaluation of the dry biomass of the aerial portion (MSPA) at stage R_1 (Table 5), treatment 5 provided the highest significant increase ($p \leq 0.05$) in such variable when compared to the other treatments.

In the variables N content in the aerial portion (TNPA) and total N in the aerial portion (NTPA) of the plants (Table 5), treatment 5 presented the highest average values.

Similarly, to what was seen for variables TNPA and NTPA, the best results in the evaluation of the N content in the grains (TNG) and the total N in the grains (NTG) (Table 5) were also achieved in treatment 5, statistically differing from all the other treatments.

For the variable grain yield (REND) (Figure 4), treatment 5 too promoted the best productive performance in the soybean crop.

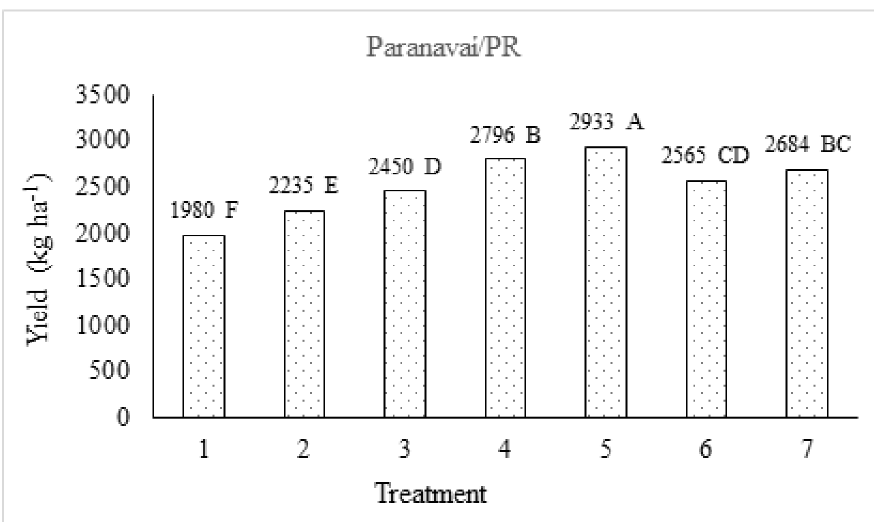

Figure 4. Grain yield in response to the different seed standard inoculation, coinoculation, and preinoculation treatments with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* applied by seed treatment and leaf spraying in the soybean crop (Paranavaí/PR - 2020/2021).

By the average test employed, it is seen that it is not possible to distinguish significant differences ($p > 0.05$) in the variable productivity between treatments 4 and 7 or even between treatments 6 and 7. These results indicate that products containing bacteria from genus *Bradyrhizobium* spp. associated to *Azospirillum brasilense* presented intermediate results compatible among themselves.

The low productivity results seen in this trial are related to the water stress occurred during the harvest at Paranavaí/PR region, with low rainfall indexes (389 mm in total), especially in November/2020 and February and March/2021, exactly during the crop postflowering, that is, at the reproductive stages more critical for water scarcity, as attested by Silva et al. (2015). This water deficit condition even promoted a faster plant maturation and the anticipation of the crop harvest compared to a more favorable climatic condition.

3.4 MESTIÇO FARM (ITAQUIRAÍ/MS – 2020/2021)

It is possible to infer that there were significant differences ($p \leq 0.05$) for all the response variables evaluated by the variance analysis in the trial area located at Itaquiraí municipality/MS. The variation coefficients (VC) were relatively low for most evaluated parameters, so indicating a good precision in the results obtained in this trial, except for the dry biomass of the aerial portion (MSPA) and the total N of the aerial portion of the plants (NTPA), where a VC above 10% was seen.

It is seen that both the number of nodules (NNOD) and the dry mass of nodules (MNOD) per plant were higher in treatment 5 compared to all the other employed treatments, particularly compared to the treatments 1, 2 and 3 (Table 6).

Nevertheless, both NNOD and MNOD were considered as relatively low in practically all treatments. These data are related to the soil type and texture, as this municipality located at Mato Grosso do Sul state presents sandier soils cultivated with soybean for little time (7 years), and, therefore, with lower bacterial populations settled in the soil (Table 6).

The employed treatments enabled to achieve the nodule number considered as satisfactory by Câmara (2000). Nevertheless, when compared to other studies of the same type, the results obtained in this trial only presented a proper nodulation for treatments 4 and 5, according to Hungria et al. (1997), a nodule mass ranging between 100 and 200 mg per plant would be adequate to ensure the N supply required by a soybean plant for its normal development.

Table 6. Averages of the variables number of nodules per plant in the beginning of flowering (NNOD), mass of dry nodules per plant in the beginning of flowering (MNOD), dry biomass of the aerial portion of the plants (MSPA), nitrogen content in the aerial portion (TNPA), total nitrogen of the aerial portion of the plants (NTPA), nitrogen content in the grains (TNG), and total nitrogen in the grains (NTG) in response to the different seed standard inoculation, coinoculation, and preinoculation treatments with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* applied by seed treatment and leaf spraying in the soybean crop (Itaquiraí/MS - 2020/2021).

Treatment	NNOD	MNOD	MSPA	TNPA	NTPA	TNG	NTG
	(unit/plant)	(mg/plant)	(g/plant)	(g/kg)	(kg/ha)	(g/kg)	(kg/ha)
1	15.68 E	68.52 D	4.68 CD	40.64 C	55.44 E	44.15 D	114.54 E
2	12.04 F	52.89 E	4.09 D	48.01 BC	62.71 DE	50.04 C	132.53 D
3	18.80 D	75.66 CD	5.17 CD	48.75 B	72.53 CDE	52.55 BC	140.74 D
4	24.36 B	115.22 B	5.96 BC	51.30 B	84.92 BC	54.87 B	164.03 C
5	31.88 A	137.91 A	6.70 B	50.20 B	80.51 BCD	53.94 BC	153.71 C
6	20.28 CD	89.17 C	7.08 B	64.63 A	122.14 A	60.95 A	196.57 A
7	22.96 BC	106.08 B	8.80 A	54.87 B	96.10 B	56.71 AB	177.23 B
Average	20.86	92.21	6.07	51.20	82.05	53.31	154.19
VC (%)	7.11	7.97	10.99	7.18	12.24	4.43	3.48

Averages followed by the same letter in the column do not significantly differ by the Tukey test at a 5% probability level.
Unit/plant: unit per plant; mg/plant: milligrams per plant; g/plant: grams per plant; g/kg: grams per kilogram of nitrogen (N); and kg/ha: kilograms per hectare of nitrogen (N).

However, the results obtained in the plant nodulation were not related to the dry biomass of the aerial portion (MSPA), as treatment 7 was the most effective one in this variable, with values significantly higher than the other evaluated treatments (Table 6). There was no significant difference ($p > 0.05$) among the coinoculation treatments 4, 5, and 6.

These results are according to those obtained by Anghinoni et al. (2017), who verified that the preinoculation of soybean seeds subjected to the industrial treatment, and with a 10-day antecedence before seeding, did not affect their agronomic performance.

The results obtained in the N contents in the aerial portion (TNPA) and the total N in the aerial portion of the plants (NTPA) (Table 6) enabled to infer that the most effective treatment in these both evaluated parameters was treatment 6. On the other hand, there was no significant difference between the other treatments, except for treatment 1 for the variable TNPA. Referring to NTPA, a great ambiguity is seen when comparing the averages by the Tukey test, so enabling to conclude that, in this variable, there was statistical difference just between treatments 6 and 7, with the highest values, and the absolute witness (treatment 1), which presented the worst result.

Referring to the variables TNG and NTG (Table 6), it is seen again that the highest values were verified in treatment 6, being closely followed by treatment 7. No significant difference was seen between these both treatments (6 and 7), just in TNG.

Referring to the total N in the grains (NTG), there was a statistical difference between the best treatment (6) and the second best one (7), as well as between treatment 7 and treatments 4 and 5, which presented intermediate results in this same variable (Table 6).

The highest grain productivity was obtained in treatment 6, followed by treatment 7 (Figure 5). There was no significant difference among treatments 3, 4 and 5.

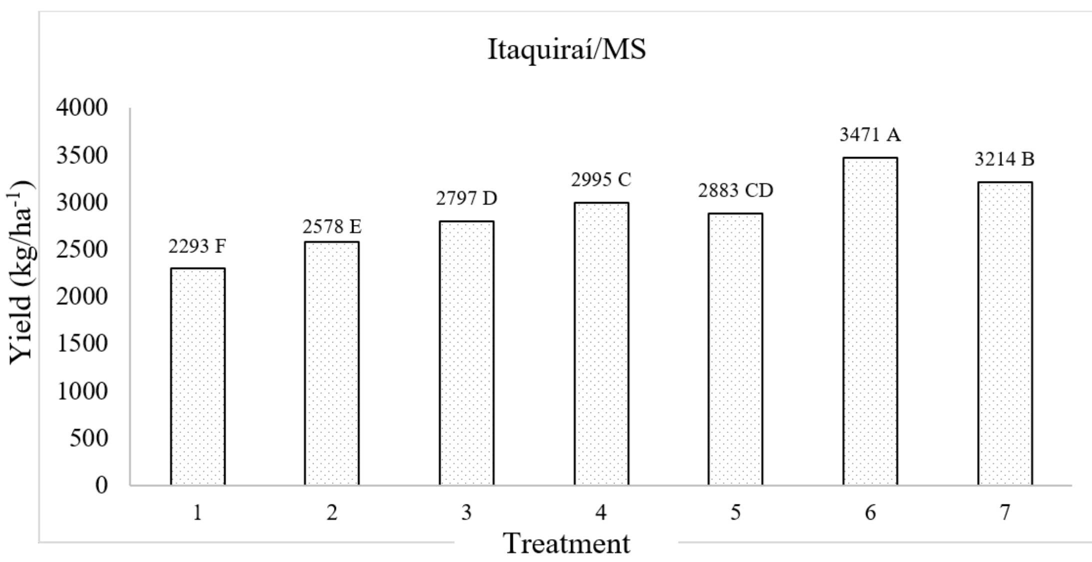

Figure 5. Grain yield in response to the different seed standard inoculation, coinoculation, and preinoculation treatments with products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* applied by seed treatment and leaf spraying in the soybean crop (Itaquiraí/MS - 2020/2021).

Corroborating the results obtained in the present study, Santos et al. (2021) verified that the success in obtaining high productivities in inoculated soybean is related to the pesticide compatibility with the used microbial inoculants, that is, with environmentally correct bioproducts. On the other hand, the lack of response of the productivity components to the seed inoculation under field conditions, in sandier soils with low organic matter and nutrient (especially mineral N), is probably due to the quality of the used inoculant, as well as to the mode of application of the referred products.

4 FINAL CONSIDERATIONS

The results achieved in the present trial indicate that the application of products containing *Bradyrhizobium* spp. and *Azospirillum brasilense* by seed treatment or leaf spraying of *Azospirillum brasilense* showed to be technically feasible in the soybean crop.

The use of products with *Bradyrhizobium* spp. and *Azospirillum brasilense*, particularly applied in seed treatment, has increased the plant nodulation, the production of dry biomass of the aerial portion, the N contents, and the grain yield in soybean.

The practice of soybean seed preinoculation with *Bradyrhizobium* spp. + *Azospirillum brasilense* + bacterial protector, with a 10-day antecedence before the crop seeding has also provided satisfactory results in all the analyzed response variables, with results equal to or higher than the treatment with standard coinoculation.

In the four evaluated areas, the treatments evaluated with inoculants containing *Bradyrhizobium* spp. and *Azospirillum brasilense* presented yield results higher than the absolute control with no inoculation and higher than or equal to the standard coinoculation, representing 70%-100% of positive results.

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