Cps
Communications in
PLANT SCIENCES

http://cpsjournal.org
e-ISSN 2237-4027
Volume 12 (2022)

RESEARCH ARTICLE

Inoculation and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* in soybean crop with the use of soil bio-activator

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Submitted on June 07, 2021
Accepted on April 05, 2022
Early View on April 05, 2022
Final Publication on April 11, 2022

Authors declare no conflict of interest

KEYWORDS:
Glycine max (L.) Merrill
Growth promotion
Biological nitrogen fixation
Seaweed

ABSTRACT

In view of the high nitrogen availability required by soybean, inoculation with nitrogen fixing bacteria, such as *Bradyrhizobium japonicum*, is an economically viable option. Moreover, the co-inoculation of these microorganisms with plant growth promoting bacteria, such as *Azospirillum brasilense*, presents high efficiency compared to the isolated use of these microorganisms. Given the above, the study aimed to determine the effects of applying a soil bio-activator associated with the inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* bacteria, on morphometric, physiological and productive variables of soybean crop. The experiment was conducted in the field, in a randomized block design with four replicates. The treatments were: control; seed inoculation with Bradyrhizobium japonicum; seed inoculation with Azospirillum brasilense; application of soil bio-activator; seed inoculation with *B. japonicum* and *A. brasilense*; seed inoculation with *B. japonicum* and application of soil bio-activator; seed inoculation with *A. brasilense* and application of soil bio-activator; and seed inoculation with *B. japonicum* and *A. brasilense* and application of soil bio-activator. In the morphometric analysis, plants inoculated with *B. japonicum* presented a greater number of nodules compared to the control. The co-inoculation associated with use of soil bio-activator increased the chlorophyll relative content, at the beginning of the cycle, in 6.37% in relation to the control. In gas exchange analysis, the isolated use of soil bio-activator obtained higher intrinsic water use efficiency (*iWUE*) in V_4. No increases in relation to the control were observed in the other variables evaluated, rejecting the hypothesis that co-inoculation associated with the use of soil bio-activator would promote increases in morphometric, physiological and productive characteristics of the soybean crop.

Highlighted Conclusion

1. The use of soil bio-activator associated with inoculation and coinoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* does not provide increases in morphometric, physiological, and productive variables of the soybean crop.
2. Further studies are needed on the influence of soil bio-activators on the bacteria used in this study, enabling further verification of their applicability.

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is a legume with great expression both nationally and globally (Hirakuri and Lazzarotto 2014). Cultivated in several regions of Brazil, the crop is considered the main source of vegetable oils and proteins for human and animal consumption (Barbosa et al. 2013). According to CONAB (2020), the area allocated for soybean cultivation in the 2019/20 crop year was approximately 36.9 million hectares.

Considering the composition of its grains, soybean demands a large amount of nitrogen (Hungria et al. 2007). The absorption of this nutrient by the plants is optimized by the use of nitrogen fertilizers, however, it is understood that the process required to turn atmospheric nitrogen into assimilable nitrogen is complex (Martins et al. 2003). Furthermore, losses through leaching, denitrification and volatilization reach 50%, a condition that stimulates a reduction in the economic efficiency of the product (Hungria et al. 2001).

Therefore, biological nitrogen fixation (BNF) is the most ecologically and economically viable alternative for the crop (Zilli et al. 2006). Through genetic improvement, biotechnological advances have driven the selection of more efficient strains of nitrogen-fixing bacteria (BNF), which are able to provide enough nitrogen for the development of the crop (Oliveira Junior et al. 2010). Because of this, the use of mineral fertilization with nitrogen in soybean crops in Brazil has become dispensable (Döbereiner 1997).

In Brazil, the bacteria belonging to the genus *Bradyrhizobium* have been a major driving force for strengthening soybean production (Silva et al. 2011). These microorganisms come into contact with the roots, infecting them and promoting the formation of nodules, which enable the N_2 to be captured and converted into forms available to the plant (Taiz and Zeiger 2013). However, factors such as water stress, soil compaction, waterlogging, low fertility, soil acidity and the use of pesticides in seed treatments can limit the good performance of these bacteria (Campo and Hungria 1999; Sinclair et al. 2007).

Currently, there is the possibility of combining two different genera of bacteria for BNF in soybean crops. Co-inoculation, as so called, comes from the association between the genus *Bradyrhizobium* and *Azospirillum*, and can contribute positively to the grain yield of soybean crops (Sordi et al. 2017). Especially, diazotrophic bacteria of the genus *Azospirillum*, which have the ability to produce plant growth-promoting substances or stimulate their endogenous production (Rodrigues et al. 2012).

Seeking new technologies capable of improving the microbiological characteristics of the soil in a sustainable way, studies have proven the efficiency of nanotechnology. The use of nanotechnology products aiming at soil bioactivation has been increasingly frequent, although these products do not contain biological agents in their composition, their application stimulates the exponential growth of beneficial microorganisms in the soil and plants (Castro et al. 2008; Caron et al. 2015).

These substances can promote benefits such as tolerance to pest, disease and nematode attack, water deficit and high temperatures, as well as improvements in the physical, chemical and microbiological properties of the soil. One of the main components of soil bio-activators is seaweed, which can positively interfere with plant physiology, making them more resilient and tolerant to stress conditions (Matysiak et al. 2011).

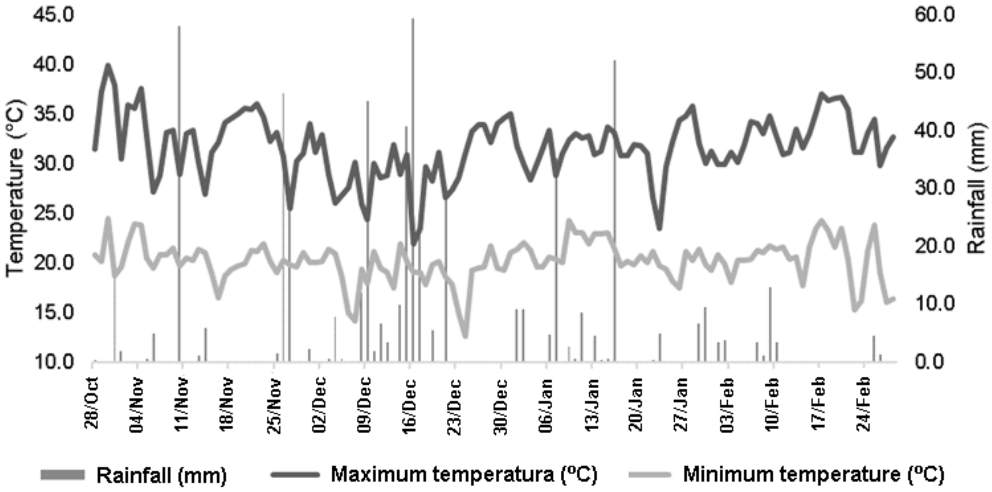
Thus, this work aimed to determine the effects of using soil bioactivator associated with inoculation and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* bacteria, on morphometric, physiological, and productive variables of the soybean crop.

MATERIAL AND METHODS

Characterization of the experimental area

The experiment was conducted in Marechal Cândido Rondon, coordinates 24º 33' 40'' S, 54º 04' 12'' W, with an altitude of 420 m. The soil is classified as Oxisoil (Santos et al. 2018).

The meteorological data corresponding to the period of the experiment were collected and recorded at the Climatological Station of UNIOESTE/INMET and are expressed in figure 1. The average temperature measured was 25.3°C, while the maximum and minimum temperatures reached 39.9°C and 12.6°C, respectively. The rainfall occurred during the crop cycle totaled 600 mm.



DOI: 10.26814/cps2022004 | 9 pages

Figure 1. Rainfall and air temperature variation at the site of the experiment execution in the period from October 2019 to February 2020.

For the chemical characterization of the soil used, five single samples were collected, which formed a composed soil sample, and then sent for chemical analysis. No liming of the soil was necessary, and the nutritional requirements of the crop were met with seeding fertilization (Moreira et al. 2017) (Table 1).

Table 1. Chemical characterization of the soil of the experimental area. Marechal Cândido Rondon, 2018.

Depth	P	MO	pH $CaCl_2$	H+Al	Al^{3+}	K^+	Ca^{2+}	Mg^{2+}	SB	CTC	V	Al
cm	mg dm^{-3}	g dm^{-3}	0,01 mol L^{-1}	------------------------$cmol_c$ dm^{-1}--------------------							----%----	
0-20	97.24	21.87	5.73	4.24	0.00	0.53	3.92	2.35	6.79	11.03	61.57	0.00
20-40	44.49	16.40	5.50	4.36	0.00	0.32	2.30	1.93	4.55	8.91	51.06	0.00

Experimental design

The experiment was conducted in a randomized block design, with four replicates. The treatments were: T1 - control; T2 - seed inoculation with *Bradyrhizobium japonicum*; T3 - seed inoculation with *Azospirillum brasilense*; T4 - application of soil bio-activator; T5 - seed inoculation with *B. japonicum* and *A. brasilense*; T6 - seed inoculation with *B. japonicum* and application of soil bio-activator; T7 - seed inoculation with *A. brasilense* and application of soil bio-activator; and T8 - seed inoculation with *B. japonicum* and *A. brasilense* and application of soil bio-activator.

Implementation and conduction of the experiment

The plant material used was the soybean cultivar NA 5909 RG, from Nidera®. The inoculants used were two commercial products, NITRO1000® GRAMINEAS, containing strains AbV5 and AbV6 of *A. brasilense*, at a concentration of 2.0 x 10^8 CFU per mL, and NITRO1000® SOJA, containing strains 5079 and 5080 of *B. japonicum*, at a concentration of 5.0 x 10^9 CFU per mL. The dose used was the one recommended by the company for both products, 100 mL for every 60,000 seeds.

The soil bio-activator used was the commercial product Vitasoil Nano Science®. The application mixture was prepared with 1 g of the product to 100 mL of non-chlorinated water, which was left to rest for 48 hours for activation. Three applications of 2 g ha^{-1} each were made, the first just after sowing, the second at the V_4 stage and the last at the V_8 stage, totaling 6 g ha^{-1}. For this, a motorized backpack sprayer was used, equipped with a fan spray nozzle under 30 psi pressure. The volume of solution applied was 160 L ha^{-1}.

The experimental units consisted of plots with 15 rows of 6 meters long, resulting in an area of 45 m^2. The implementation of the experiment was performed on October 28, 2019, with the aid of a manual wheeled seeder. A spacing of 0.50 m between rows and depth of 0.05 m was adopted, originating a stand of 18 plants per linear meter and a population of 360,000 plants per hectare. During sowing, to meet the nutritional demands of the crop and the expected productivity of 4,000 kg ha^{-1}, 200 kg ha^{-1} of NPK (02-20-18) fertilizer was applied in the furrow (Moreira et al. 2017).

After establishment, the plants were constantly monitored for pests, diseases, and invasive plants. At the time of the evaluations, three rows on both sides were excluded, as well as 1.0 m at the ends of the plots, forming a useful plot of 18 m^2. The harvest of the experiment was performed manually on February 28, 2020.

Evaluations

Morphometric evaluations. At the R_1 phenological stage, beginning of flowering, five plants per plot were collected and the height and basal diameter of the stem were evaluated. An Area Meter, model LI-3100C, was used to determine the leaf area. The number of nodules per plant was determined by counting the roots of five plants. To determine the mass of dry matter, the plants were sectioned into leaves + petioles, stem, roots and nodules, and were packed into kraft paper bags and dried in a forced air circulation oven at 65 °C ± 2 °C for 72 hours, then the samples were weighed on an analytical weighing scale.

Relative chlorophyll content and gas Exchange. Measurements of the relative chlorophyll content started at the V_3 phenological stage (formation of the third node) and performed fortnightly, until the R_6 stage (full grain), with the aid of the Konica Minolta Plus SPAD 520 device. Five plants per plot were evaluated, and the average value was obtained from measurements taken in the apical, middle and basal thirds of the plants.

The point gas exchange measurements were performed in the phenological stages V_4 and R_2 of the crop with the aid of a LI-COR® LI-6400XT portable gas exchange meter (IRGA), equipped with a light source model LI-6400-

02B. The readings were taken between 8 am and 11 am, in the central leaflet of the third node, considering the apex to the base of the plant.

Net CO_2 assimilation (*A*), stomatal conductance (*gs*), leaf transpiration (*E*), internal CO_2 concentration (*Ci*), instantaneous carboxylation efficiency (*ACi*), water use efficiency (*WUE*), and intrinsic water use efficiency (*iWUE*) were measured.

Components of production and productivity. At the R_8 stage, the components of production and grain yield were evaluated. For the determination, pods were collected from 10 plants per plot, evaluating the number of grains per pod and the number of pods per plant. After the trailing, the weight of one thousand grains was determined, which evaluation was performed in 8 repetitions of 100 grains, determining the sample mass that was later multiplied by 1000 and divided by the total number of seeds, according to the Rules of Seed Analysis - MAPA (2009). The remaining plants of the useful plot were harvested to estimate the productivity, expressed in kg ha^{-1}.

Statistical analysis. The data obtained were tabulated and submitted to analysis of variance using the F test ($p < 0.05$). When relevant, Tukey's test was applied for comparison of means ($p \leq 0.05$). Statistical analysis was performed using the Sisvar software (Ferreira 2014).

RESULTS AND DISCUSSION

Plants inoculated with *Bradyrhizobium japonicum* had the highest number of nodules compared to the control and the treatments with inoculation of *B. japonicum* associated with the application of soil bio-activator and co-inoculation associated with the application of soil bio-activator (Table 2). However, the number of nodules present in the roots, analyzed in isolation, may not represent the effective quality of the inoculation, since the viability of nodules can vary depending on the cycle and genotypic characteristics of the plant (Pereira et al. 2016). Thus, in some cases, despite the plants presenting an adequate number of nodules, these may not be active, characterized by parasitic behavior and not nitrogen supplier (Pereira et al. 2019).

Table 2. Averages of leaf area (LA), stem diameter (SD), plant height (PH), number of nodules per plant (NN), root dry mass (RDM), nodule dry mass (NDM) and leaf dry mass (LDM) as a function of treatments.

Treatments	LA		SD		PH		NN		RDM		NDM		LDM	
	cm^2		mm		cm		Unity per plant		g per plant		mg per plant		g per plant	
Control[2]	5,788.36	a[1]	6.84	a	68.44	a	9.67	b	1.541	a	55.00	a	4.103	a
Bra	4,492.98	a	6.93	a	66.03	a	18.87	a	1.617	a	80.75	a	3.613	a
Azo	5,181.02	a	6.79	a	65.89	a	15.20	ab	1.871	a	68.50	a	3.670	a
BS	5,509.65	a	7.20	a	64.93	a	13.67	ab	2.029	a	72.00	a	4.675	a
Bra + *Azo*	5,742.11	a	6.81	a	68.55	a	15.67	ab	1.679	a	79.25	a	4.419	a
Bra + SB	5,130.00	a	6.60	a	71.56	a	10.73	b	1.605	a	43.00	a	3.950	a
Azo + SB	5,671.70	a	6.92	a	65.79	a	11.80	ab	1.956	a	81.50	a	4.168	a
B + *A* + SB	5,425.32	a	6.61	a	68.39	a	9.73	b	1.955	a	52.00	a	4.496	a
CV (%)	11.52		6.96		6.38		23.28		13.61		30.57		12.93	
LSD	1466.4883		1.1285		10.2040		7.2709		0.5751		0.0482		1.2694	

(1) Averages followed by the same letter in the column for the comparison between different treatments do not differ by Tukey's test ($p \leq 0.05$). (2) The treatments were: control (Control), inoculation of *Bradyrhizobium japonicum* (Bra), inoculation of *Azospirillum brasilense* (Azo), application of soil bio-activator (SB), co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense*, inoculation of *Bradyrhizobium japonicum* associated with the application of soil bio-activator (Bra + SB), inoculation of *Azospirillum brasilense* associated with the application of soil bio-activator (Azo + SB) and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* associated with the application of soil bio-activator (B + A + SB).

According to Hungria et al. (2007), for efficient biological nitrogen fixation, plants should have, at flowering, between 15 and 30 nodules, or 100 to 200 mg of dry nodules per plant. Based on these data, it is possible to observe that nodulation was deficient in some treatments in this study, since the plants analyzed presented between 9.67 and 18.87 nodules and 43 to 81.50 mg of dry nodules per plant. It is worth noting, that the lack of water during the first weeks of the crop in the field may have been a determinant factor (Figure 1), impairing the activity of the nitrogenase enzyme and decreasing the availability of oxygen in the bacteroid zone by reducing the synthesis of leghemoglobin (Hungria and Vargas 2000). This condition may have limited the production in quantity and accumulation of nodule mass, necessary for the proper establishment and development of the crop.

Given the possibility of the soil bio-activator to induce the multiplication of beneficial soil microorganisms, the lower number of nodules in the treatments with inoculation of *B. japonicum* and co-inoculation both associated with the application of soil bio-activator may be related to competition between the nitrogen-fixing bacteria and the microorganisms present in the soil (Zuffo et al. 2015). The competition for essential resources for the establishment of the bacteria in the plant roots, as well as the influence of genetic and edaphoclimatic factors, can significantly limit the responses of the crop submitted to inoculation, compromising the growth, development and production of soybean plants (Franciscon et al. 2014; Mundim et al. 2018).

According to the classification proposed by Moreira et al. (2017), the soil presented an average organic matter content (Table 1), reinforcing the hypothesis that the soil bio-activator may have contributed to the improvement of the chemical composition of this soil, considerably increasing the organic matter content and, consequently, increasing the availability of nutrients for plants (Pavinato and Rosolem 2008). If so, this nutritional increase in the soil may have reflected negatively on nodulation, not directly affecting productivity (Summerfield et al.1985).

The association with *A. brasilense* may have stimulated the production of phytohormones and induced the growth of root hairs on plant roots, providing a better use of the nutrients present in the soil (Neto et al. 2013). The inoculation of *A. brasilense* in soybean may also have promoted the anticipation of nodulation, increasing the utilization of nitrogen by plants and intensifying plant growth (Bárbaro et al. 2009; Mauad et al. 2010). In this context, Bulegon et al. (2016) tested the effect of co-inoculation of *B. japonicum* and *A. brasilense* and found no significant gains in nodulation with *Azospirillum brasilense*, corroborating with the results obtained in this study.

Taking into account the efficient biological nitrogen fixation by *B. japonicum* and the synthesis of phytohormones essential for plant development by *A. brasilense*, the co-inoculation, even with the lack of water at the beginning of the cycle, may have provided a better start for soybean plants. On the other hand, the isolated use of the soil bio-activator, through the beneficial action of the algae extract, may have provided increased water use at the beginning of the cycle, resulting in more uniform plants (Smiderle 2019).

The treatment with co-inoculation increased the number of grains per pod when compared to the treatment with isolated application of soil bio-activator (Table 3). Thus, it is worth noting that plants submitted to the isolated use of soil bio-activator may have had less availability of assimilable nitrogen than inoculated plants, resulting in plants with slower development and a reduction in the number of branches and in the production of nodes, where the reproductive buds develop, reflecting negatively on the number of pods per plant, even though there was no difference between treatments for this variable (Board and Settimi 1986). This hypothesis may justify the reduction in the number of grains per pod in these plants, since both production components are correlated, that is, factors that decrease the number of pods per plant also decrease the number of grains per pod (Farias 2007).

Table 3. Average number of pods per plant (PP), number of grains per pod (GPo), number of grains per plant (GP), weight of one thousand grains (TGW) and productivity as a function of treatments.

Treatments	PP	GPo	GP	TGW	Productivity
	unity	unity	unity	g	kg ha^{-1}
Control[2]	64.57 a[1]	2.72 ab	175.87 a	151.65 a	2,298.19 ab
Bra	58.23 a	2.65 ab	154.43 a	152.17 a	2,193.33 ab
Azo	60.80 a	2.65 ab	160.58 a	150.91 a	2,337.92 ab
BS	69.93 a	2.54 b	177.62 a	147.55 a	2,506.53 a
Bra + *Azo*	74.03 a	2.84 a	208.01 a	148.73 a	1,886.11 b
Bra + SB	58.90 a	2.61 ab	153.29 a	150.37 a	2,255.56 ab
Azo + SB	72.63 a	2.72 ab	196.23 a	151.32 a	2,158.47 ab
B + *A* + SB	72.03 a	2.73 ab	197.17 a	150.05 a	2,273.19 ab
CV (%)	14.36	4.15	14.36	2.33	11.92
LSD	22.6152	0.2644	60.6194	8.3224	644.6084

[1] Averages followed by the same letter in the column for the comparison between different treatments do not differ by Tukey's test ($p \leq 0.05$). [2] The treatments were: control (Control), inoculation of *Bradyrhizobium japonicum* (Bra), inoculation of *Azospirillum brasilense* (Azo), application of soil bio-activator (SB), co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense*, inoculation of *Bradyrhizobium japonicum* associated with the application of soil bio-activator (Bra + SB), inoculation of *Azospirillum brasilense* associated with the application of soil bio-activator (Azo + SB) and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* associated with the application of soil bio-activator (B + A + SB).

Considering that the crop was affected by an intense attack of bed bugs during the formation and filling of grains, the application of soil bio-activator may have contributed beneficially and stimulated the production of phenolic compounds by the plants. These structures, in turn, have attraction and repulsion effects, and can act by protecting against the attack of pests and pathogens (Borella and Pastorini 2010). In view of the above, it was observed that the treatment with isolated application of the soil bio-activator, despite the low nodulation capacity, showed higher productivity compared to the co-inoculated plants (Table 3). Furthermore, the isolated use of soil bio-activator provided an increase in productivity of 208.34 kg ha^{-1} in relation to the control, even though there were no statistical differences between these treatments.

By analyzing the values of the SPAD index (Table 4), obtained by readings taken every two weeks, it was possible to observe a correlation with the data obtained for the variable number of nodules per plant, since both results may have been influenced by the availability of nutrients in the soil. According to Sant'Ana et al. (2010), the SPAD index has a direct relationship with the chlorophyll content of the leaves. This content, in turn, is used to indicate the concentration of nitrogen in plants, since this nutrient is part of the composition of chlorophyll molecules (Rajcan et al. 1999).

Table 4. Averages of the SPAD index measured at 15, 30, 45, 60, 75, and 90 days after emergence (DAE) of the plants as a function of the treatments.

Treatment	15 DAE	30 DAE	45 DAE	60 DAE	75 DAE	90 DAE
Control(2)	39.72 c(1)	41.73 a	41.67 ab	43.17 a	43.72 a	38.69 a
Bra	41.66 ab	41.73 a	42.29 ab	44.88 a	44.01 a	37.40 ab
Azo	40.97 abc	41.16 a	42.42 ab	42.83 a	40.05 a	27.90 c
BS	40.99 abc	40.34 ab	41.83 ab	42.99 a	44.19 a	33.00 bc
Bra + *Azo*	40.52 bc	41.19 a	42.51 ab	44.11 a	43.91 a	34.98 ab
Bra + SB	41.77 ab	37.92 b	40.52 b	43.27 a	42.79 a	36.78 ab
Azo + SB	40.93 abc	41.35 a	42.96 ab	43.98 a	43.87 a	37.35 ab
B + *A* + SB	42.25 a	40.44 ab	43.10 a	43.67 a	44.31 a	33.15 bc
CV (%)	1.50	3.00	2.53	3.08	5.63	6.64
LSD	1.4641	2.9031	2.5292	3.1903	5.7915	5.4959

(1) Averages followed by the same letter in the column for the comparison between different treatments do not differ by Tukey's test ($p \leq 0.05$). (2) The treatments were: control (Control), inoculation of *Bradyrhizobium japonicum* (Bra), inoculation of *Azospirillum brasilense* (Azo), application of soil bio-activator (SB), co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense*, inoculation of *Bradyrhizobium japonicum* associated with the application of soil bio-activator (Bra + SB), inoculation of *Azospirillum brasilense* associated with the application of soil bio-activator (Azo + SB) and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* associated with the application of soil bio-activator (B + A + SB).

The main function of chlorophyll is to capture light for photosynthesis, converting light radiation into chemical energy and, consequently, influencing the growth and adaptability of plants to adverse environmental conditions (Fonseca et al. 2012). Thus, it was found that in the evaluation performed 15 days after emergence (DAE) of the plants, the treatment with co-inoculation associated with the application of soil bio-activator showed higher SPAD index values compared to the control and the treatment with co-inoculation. Thus, the soil bio-activator may have maximized the beneficial effects of co-inoculation, optimizing the absorption of water and nutrients from the soil and increasing the synthesis of chlorophyll, increasing the use of incident light on the plants, causing greater photosynthetic activity of these plants compared to the other treatments (Ferrazza and Simonetti 2010; Brito et al. 2020).

At 30 DAE, lower SPAD index values were observed in plants inoculated with *B. japonicum* with application of soil bio-activator compared to control, co-inoculated, inoculated with *B. japonicu*m, *A. brasilense* and *A. brasilense* with application of soil bio-activator.

The decrease in chlorophyll content of plants inoculated with *B. japonicum* combined with the application of soil bio-activator may be related to the competition between the inoculated bacteria and beneficial microorganisms for the soil's natural resources, which ensure the viability and survival of the microbiota, reflecting the inability of plants to fix nitrogen correctly (Becker et al. 2012). However, this antagonistic behavior was not observed in plants inoculated with *A. brasilense* in association with the application of soil bio-activator, since *A. brasilense* can establish a relationship with the plant more quickly compared to *B. japonicum*, which requires a high energy expenditure for the formation of nodules in the roots (Senthilkumar et al. 2011).

In the readings taken 45 DAE, the treatment with co-inoculation combined with the application of soil bio-activator showed higher SPAD index values compared to the treatment with inoculation of *B. japonicum* and application of soil bio-activator. Taking into account the beneficial effects provided by *A. brasilense*, as well as in the previous analysis, the bacterium may have stimulated the production of chlorophyll in the leaves, increasing the accumulation of nitrogen in the plants (Hungria 2011). There was no statistical difference between treatments in the evaluations performed 60 and 75 DAE.

In the evaluation performed 90 DAE, the control presented higher SPAD index values compared to the plants inoculated with *B. japonicum*, *A. brasilense*, co-inoculation, application of soil bio-activator and co-inoculation with application of soil bio-activator. In this period, the crop was in the phenological stage R_6, finishing the grain filling process (Farias et al. 2007). According to Imsande and Schmidt (1998), from the moment the pod formation begins, the plants stop mobilizing nitrogen for the vegetative portion and start directing it to the grains, justifying the lower SPAD index values obtained in this evaluation compared to others previously performed.

When considering the higher chlorophyll content in the leaves of control plants at this phase of development, it is understood that there was a lower mobilization of nitrogen to the grains and pods, negatively interfering in the productivity of the soybean crop (Hungria et al. 2001). Furthermore, at the end of the cycle, the high levels of nitrogen in the leaves allow the plants to remain green for longer, making it more difficult to identify the maturation point and to perform the mechanized harvest operation.

In the gas exchange evaluation performed at the V_4 phenological stage (Table 5), there was no statistical difference for the parameters net CO_2 assimilation rate (*A*), water use efficiency (*WUE*), and instantaneous carboxylation efficiency (*ACi*).

Table 1. Averages of net CO_2 assimilation rate (*A*), stomatal conductance (*gs*), internal CO_2 concentration (*Ci*), leaf transpiration (*E*), water use efficiency (*WUE*), intrinsic water use efficiency (*iWUE*) and instantaneous carboxylation efficiency (*ACi*) at the V_4 phenological stage as a function of treatments.

Treatments	*A*	g_s	*Ci*	*E*	*WUE* (*A/E*)	*iWUE* (*A/gs*)	*ACi* (*A/Ci*)
	µmol CO^2 m^{-2} s^{-1}	mol H_2O m^{-2} s^{-1}	µmol CO^2 mol^{-1}	µmol H_2O m^{-2} s^{-1}	µmol CO^2 (µmol H_2O) m^{-2} s^{-1}	µmol CO_2 (µmol H_2O) m^{-2} s^{-1}	(µmol m^{-2} s^{-1}) (µmol mol^{-1})$^{-1}$
Control[(2)]	25.42 a[(1)]	1.20 a	296.48 ab	7.48 ab	3.51 a	22.05 b	0.0850 a
Bra	23.53 a	0.97 ab	292.82 ab	7.48 ab	3.30 a	25.47 b	0.0819 a
Azo	23.48 a	0.98 ab	291.15 ab	7.38 ab	3.33 a	27.04 b	0.0782 a
BS	22.91 a	0.83 b	285.37 b	6.87 b	3.30 a	34.59 a	0.0755 a
Bra + *Azo*	23.42 a	1.03 ab	297.08 ab	7.58 ab	3.23 a	25.24 b	0.0829 a
Bra + SB	22.42 a	0.95 ab	298.45 a	7.69 ab	2.97 a	22.88 b	0.0759 a
Azo + SB	24.78 a	1.13 ab	299.41 a	7.63 ab	3.38 a	21.76 b	0.0826 a
B + *A* + SB	25.84 a	1.22 a	298.17 a	7.87 a	3.43 a	22.00 b	0.0873 a
CV (%)	13.18	23.47	3.19	10.07	13.57	22.87	13.38
LSD	4.0196	0.3088	11.9200	0.9575	0.5695	7.2928	0.0138

(1) Averages followed by the same letter in the column for the comparison between different treatments do not differ by Tukey's test (p≤0.05). (2) The treatments were: control (Control), inoculation of *Bradyrhizobium japonicum* (Bra), inoculation of *Azospirillum brasilense* (Azo), application of soil bio-activator (SB), co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense*, inoculation of *Bradyrhizobium japonicum* associated with the application of soil bio-activator (Bra + SB), inoculation of *Azospirillum brasilense* associated with the application of soil bio-activator (Azo + SB) and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* associated with the application of soil bio-activator (B + A + SB).

The values of stomatal conductance (*gs*) were higher in the control and in the plants submitted to co-inoculation associated with the application of soil bio-activator. Using the stomatal conductance as an indicator of the opening of the stomata, these results indicate that there was a greater capacity for gas diffusion, influencing the greater ability of the plant to assimilate carbon, even though these were not statistically different (Lawlor and Tezara 2009). This greater capacity for assimilation is confirmed in the higher values of internal CO_2 concentration (*Ci*), indicating that there was no limitation in the carbon available to be assimilated, and leaf transpiration (*E*) showing high input and output of gases (Raschke 1979).

According to Kerbauy (2004), the lower values of stomatal conductance observed in plants that received only the treatment with soil bio-activator may indicate the low quantity of water available in the soil. However, the intrinsic water use efficiency (iWUE) was higher in plants submitted to this treatment, indicating that the evapotranspirative demand of these plants in relation to stomatal conductance was lower, providing greater use of available water by plants (El-Sharkawy et al.1985).

In the evaluation of gas exchange at the phenological stage R_2 (Table 6), there was no statistical difference for the parameters net CO_2 assimilation rate (*A*), stomatal conductance (*gs*), internal CO_2 concentration (*Ci*), leaf transpiration (*E*) and intrinsic water use efficiency (*iWUE*). The values of water use efficiency (*WUE*) were statistically higher in the control.

Table 2. Averages of the evaluations performed at the phenological stage R_2 of net CO_2 assimilation rate (*A*), stomatal conductance (*gs*), internal CO_2 concentration (*Ci*), leaf transpiration (*E*), water use efficiency (*WUE*), intrinsic water use efficiency (*iWUE*) and instantaneous carboxylation efficiency (*ACi*) as a function of treatments.

Treatments	*A*	g_s	*Ci*	*E*	*WUE* (*A/E*)	*iWUE* (*A/gs*)	*ACi* (*A/Ci*)
	µmol CO^2 m^{-2} s^{-1}	mol H_2O m^{-2} s^{-1}	µmol CO^2 mol^{-1}	µmol H_2O m^{-2} s^{-1}	µmol CO^2 (µmol H_2O) m^{-2} s^{-1}	µmol CO_2 (µmol H_2O) m^{-2} s^{-1}	(µmol m^{-2} s^{-1}) (µmol mol^{-1})$^{-1}$
Control[(2)]	23.20 a[(1)]	1.17 a	295.62 a	6.58 a	3.67 a	21.76 a	0.0787 ab
Bra	23.95 a	1.35 a	294.29 a	7.25 a	3.41 ab	21.42 a	0.0822 a
Azo	21.78 a	0.99 a	287.71 a	6.42 a	3.59 ab	27.03 a	0.0761 ab
BS	22.88 a	1.32 a	299.70 a	6.79 a	3.43 ab	19.60 a	0.0769 ab
Bra + *Azo*	20.57 a	1.20 a	299.15 a	6.73 a	3.15 ab	20.81 a	0.0690 b
Bra + SB	20.87 a	1.00 a	291.17 a	6.77 a	3.14 ab	23.62 a	0.0716 ab
Azo + SB	20.96 a	1.08 a	286.20 a	6.81 a	3.18 ab	24.51 a	0.0737 ab
B + *A* + SB	20.42 a	1.04 a	288.06 a	6.76 a	3.06 b	24.67 a	0.0712 ab
CV (%)	13.26	34.14	4.19	13.86	12.78	37.40	13.31
LSD	3.6657	0.4960	15.5741	1.1893	0.5394	10.8811	0.0126

(1) Averages followed by the same letter in the column for the comparison between different treatments do not differ by Tukey's test ($p \leq 0.05$). (2) The treatments were: control (Control), inoculation of *Bradyrhizobium japonicum* (Bra), inoculation of *Azospirillum brasilense* (Azo), application of soil bio-activator (SB), co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense*, inoculation of *Bradyrhizobium japonicum* associated with the application of soil bio-activator (Bra + SB), inoculation of *Azospirillum brasilense* associated with the application of soil bio-activator (Azo + SB) and co-inoculation of *Bradyrhizobium japonicum* and *Azospirillum brasilense* associated with the application of soil bio-activator (B + A + SB).

The water use efficiency (*WUE*) evaluates the ability of the plant to reduce water loss and to absorb enough CO_2 for photosynthesis (Taiz and Zeiger 2013). The plants submitted to *B. japonicum* inoculation showed statistically superior values for instantaneous carboxylation efficiency (*ACi*), indicating that there was a greater use of the assimilated CO_2 by the plants in this treatment. The increase in instantaneous carboxylation efficiency (*ACi*) is a reflection of the increase in internal CO_2 concentration (*Ci*) and stomatal conductance (*gs*), even though these were not statistically different, and are directly related to temperature, amount of light and availability of CO_2 in the leaf mesophyll (Silva et al. 2015). Thus, low intercellular CO_2 concentrations imply a reduction in the passage of this component into the cells, resulting in the use of CO_2 from respiration to maintain a minimum rate of photosynthesis (Taiz and Zeiger 2013).

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