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Influence of seed co-inoculation with *Bradyrhizobium* species and *Azospirillum brasilense* on soybean development in Southern and Southeastern Brazil

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Bioinoculants are widely used in Brazil, based on efficient and low-cost. This study aimed to evaluate the agronomic efficiency of co-inoculation with *Bradyrhizobium* species and *Azospirillum brasilense* in soybean seeds. Field trials were established in four regions of Brazil using a randomized block design with 8 treatments and 4 replications as follows: T1 - Control, T2 - 200 kg ha^{-1} N, T3 - *Bradyrhizobium* spp. – peat; T4 - *Bradyrhizobium* spp. - liquid, T5 – *A. brasilense*, T6 - Standard co-inoculation *Bradyrhizobium* spp. + *A. brasilense*, T7 - Co-inoculation ratios: 1:1 *Bradyrhizobium* ssp. GRAP NOD L® + *A. brasilense* GRAP NOD AL®, T8 - Co-inoculation ratios: 1:1.5 *Bradyrhizobium* ssp. GRAP NOD L® + *A. brasilense* GRAP NOD AL®. The study results demonstrated the influence of co-inoculation 1:1.5, providing increments in shoots and root dry mass, nodules number and dry mass, pods number per plant, and yield when compared with the control and conventional N fertilization. The co-inoculation in the ratios 1:1 and 1:1.5, positively influenced the soybean development. These results confirmed that potential of the co-inoculation in incrementing soybean yield, which justify the recommendation of these treatments to reduce mineral N fertilization, improving good agronomic practices.

Key words: Rhizobia, *Glycine max*, BNF, PGPB, inoculation, plant nutrition, crop yield, plant-bacteria symbiosis, nitrogen fertilizer.

INTRODUCTION

Perhaps the biggest challenge for agricultural science nowadays is to fulfill the increasing global demand for

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crop production, targeting at a greater productivity and low environmental impact. It is expected that by 2050, the global agricultural production will need to be increased by 60 to 110% to meet the increasing population, diet shifts, meat, and dairy consumption, as well as biofuels demands (Ray et al., 2013).

Soybean (*Glycine max* (L.) Merrill.) is among the most important agricultural crops worldwide as it constitutes one of the largest sources of vegetable oil, human food, animal protein feed (containing about 40% protein) and biofuel in the world (Pagano and Miransari, 2016; Cabanos et al., 2021). The three major producers of soybean are Brazil, United States, and Argentina. In the 2020/2021 harvest, Brazil produced 123.829,5 million tons of soybeans, resulting in the world's largest grain production (Conab, 2022). However, in general, soybean requires a high N demand, in which approximately 80 kg of N are necessary to produce 1,000 kg of grains (Hungria et al., 2009, 2015). The use of nitrogen (N) fertilizers presents a series of issues. Approximately, more than 50% of the mineral N added to the crop is used by the plants; most of it is lost to the environment, threatening the ecosystem at local and global scales (Zhang, 2017).

Bioinoculants are efficient, low-cost and an environmentally friendly alternative. Inoculants based in plant growth-promoting bacteria (PGPB) and N_2 fixing bacteria have been widely used in Brazil, especially in the soybean crop. Soybean can establish a symbiosis with the diazotrophic bacteria *Bradyrhizobium* species*,* capable of fix the atmospheric N, into a process called Biological Nitrogen Fixation (BNF), in specialized organs usually located in the roots and called nodules, providing nearly all the N required by the plant (Alves et al., 2003; Hungria et al., 2006, 2009, 2015; Saranraj, 2021; Zilli et al., 2021).

Among PGPB bacteria, the non-symbiotic *Azospirillum* genus does not stand out due to its ability to fix atmospheric N_2, but most importantly to synthesize a variety of phytohormones, the indole-3-acetic acid (Fukami et al., 2017, 2018; Santos et al., 2021). This genus can promote plant growth and survival over mechanisms of tolerance of abiotic stresses, saline stress, osmotic adjustment, and plant defense expressing pathogenesis-related genes (Fukami et al., 2017, 2018; Santos et al., 2021).

Hungria et al. (2013) conducted a study showing the benefits of combining strains of *Azospirillum* species and *Bradyrhizobium* spp. in soybean and common bean crops. As a results, inoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense* improved nodulation and increased soybean yield by 16.1% when inoculated in-furrow, inoculation directly to the soil, and 14.1% in seed treatment (Hungria et al., 2013). After that, many studies were conducted in Brazil demonstrating that co-inoculation is a very useful biotechnological tool to improve yield, nodulation (consequently BNF) and soil sustainability (Hungria et al., 2013, 2015; Hungria and Mendes, 2015; Moretti et al., 2020; Rondina et al., 2020; Barbosa et al., 2021).

Therefore, the aim of this study was to evaluate the agronomic efficiency and feasibility of the co-inoculation with *Bradyrhizobium* spp. and *A. brasilense* in soybean seed treatment conducted in four different edaphoclimatic regions of Southern and Southeastern Brazil.

MATERIALS AND METHODS

Sites description and field management

Four field experiments were performed, three in the state of Paraná, Southern Brazil, in the cities of Maringá, Ponta Grossa and Palmeira and one in the state of São Paulo, in the city Rio das Pedras.

At Maringá, the trials were performed by the State University of Maringá at Iguatemi Experimental Farm (23°25'^S, 51°57'^W, 540 m a.s.l.) in a Dystrophic Red-Argisol (Embrapa, 2013). The climate is classified as wet mesothermal (Cfa), with abundant rainfall in summer and dry winter, according to Köppen classification. The field trial was conducted in the 2013/2014 crop season, when the average maximum and minimum temperatures were 33.2°C in February 2014 and 17.6°C in October 2013, respectively. The accumulated precipitation during the experiment was 715.4 mm, the rainiest month was January 2014 (271.4 mm), and the driest month was November 2013 (54.2 mm).

At Ponta Grossa, the trial was performed between November 2013 and April 2014 on the Farm School of the State University of Ponta Grossa (25°05'29,05''^S and 50°03'43,0''^W), in a Dystrophic Cambisol (Embrapa, 2013). The area is located at 988 m a.s.l.), with maximum and minimum temperatures of 32.9°C in February and 10.8°C in April, respectively. The accumulated precipitation during experiment was of 909 mm, with the rainiest month being January (262.8 mm) and the driest April (93 mm) in 2014. The climate is classified as Cfb according to Köppen's classification.

At Palmeira, the trial was also conducted between November 2013 and April 2014 on the Campos Gerais Agricultural Experiment Station (25°25'32.14''^S, 50°3'0.41''^W, 885 m a.s.l.). The soil is classified as Haplicous Dystrophic Cambisol (Embrapa, 2013). The climate is classified as Cfb, Köppen - always humid, hot temperate, with no defined dry season and with frequent frosts in winter. The maximum and minimum temperatures during the experiment were 32.9°C in February and 10.8°C in April, respectively. The accumulated precipitation was 865.9 mm, being the rainiest month January (262.8 mm) and the driest April (81.5 mm).

At Rio das Pedras, the trial was conducted between November 2017 and March 2018 on the Furkan Farm (22°47'17.76''^S, 47°38'57.08''^W, 588 m a.s.l.). The soil in the area is classified as Oxisol (Dystrophic Red-Latosol, Empbrapa, 2013). According to Köppen classification, the regional climate is Cwa, tropical at altitude, with dry winters, average annual temperature of 22°C and average annual rainfall of 1,280 mm. During the experiment, the accumulated precipitation was of 788.1 mm, with the rainiest month being January (225.1 mm) and the driest February (71.8 mm). The maximum and minimum temperatures were 33.9°C in December and January and 13°C in November, respectively.

Before sowing, soil samples were randomly taken at each site, from the 0-10 or 0-20 cm layer, the chemical and physical characteristics were determined. The main chemical and physical soil properties for all the four locations are shown in Table 1. Fertilizers application was performed according to soil laboratory analysis recommendation of each location, but the N fertilization was performed accordingly with the experimental treatments

Table 1. Soil chemical and physical properties of each experimental areas, before the installation of field trials by each region, Maringá, Ponta Grossa, Palmeira e Rio das Pedras.

Experimental area	pH^a	H+Al	Al	Ca	Mg	K	SB^b	CEC^c	P^d	C^e	$Base^f$	Coarse sand	Thin sand	Clay	silt
		cmol dm^{-3}							mg dm^{-3}	g dm^{-3}	%				
Maringá	5.9	4.2	0.0	4.1	1.5	0.4	5.9	10.1	5.1	9.4	58.7	49.0	15.00	30.00	6.0
Ponta Grossa	5.2	-	0.0	4.0	1.2	0.1	-	10.6	14.4	-	-	-	-	-	-
Palmeira	4.6	-	0.6	2.2	4.0	0.5	-	-	13.8	33.0	-	31.2	-	40.00	28.8
Rio das Pedras	4.7	27.0	1.7	25.0	11.2	1.1	37.3	64.3	66.7	12.2	58.0	61.1	-	32.65	6.3

[a]pH in $CaCl_2$; [b]Sum of bases; [c]Cation exchange capacity; [d]Phosphorus (P) method Mehlich; [e]C detection by Walkley-Black method; [f]Base saturation index.
Source: Author

protocol. The crop management was the same as that suggested for a commercial crop based on local customary growers' good practices. The phytosanitary maintenance of the experimental locations was carried out when relevant, avoiding the application of any product that could interfere with the study.

Inoculants strains, soybean genotype and inoculation procedure

Seed inoculation with *B. japonicum* and *Bradyrhizobium diazoefficiens* was carried out with the commercial solid inoculant GRAP NOD+® (Agrocete Indústria de Fertilizantes Ltda.) containing the SEMIA 5079 (= CPAC 15) and SEMIA 5080 (= CPAC 7) strains (guaranteed concentration of 7×10^9 CFU mL^{-1}). Seeds were inoculated also with the liquid inoculant GRAP NOD L® (Agrocete Indústria de Fertilizantes Ltda.) with the same strains (guaranteed concentration of 5×10^9 CFU mL^{-1}). For the inoculation with *A. brasilense*, the commercial liquid inoculant GRAP NOD A® (Agrocete Indústria de Fertilizantes Ltda.) containing the Ab-V5 (= CNPSo 2083) and Ab-V6 (= CNPSo 2084) strains (guaranteed concentration of 2.0×10^8 CFU mL^{-1}) was used. All the aforementioned strains were obtained from the "Collection of Diazotrophic and Plant Growth Promoting Bacteria of Embrapa Soja" (WFCC # 1213, WDCM # 1054).

According to Brazilian regulatory requirements, an inoculant to be registered ought to report the proof of its effectiveness claims assessed by an agronomic efficacy study. These regulatory studies include some treatments with the same active ingredient or formulation or application method as comparable standards or positive controls. Therefore, in compliance with Brazilian regulation, two commercial-registered inoculants were included, an *Azospirillum*-based inoculant containing the Ab-V5 and Ab-V6 strains (2.0×10^8 viable CFU mL^{-1}) and the *Bradyrhizobium*-based inoculant with the SEMIA 5079 and SEMIA 5080 strains (5.0×10^8 viable CFU mL^{-1}), both presented in a liquid formulation, with the same active ingredients of the ones from GRAP brand to be assessed by the co-inoculation doses treatments.

In each experimental site, the chosen soybean cultivars matched up with a range of environmental conditions of each region. Experiment in Maringá the cultivar used was BRS 360 RR, belonging to the early maturation group (6.2 of the North American classification). In Palmeira and Ponta Grossa, the soybean cultivar was BRASMAX ENERGIA RR, while in Rio das Pedras, the soybean variety used was M5917 IPRO (5.9 of the North American classification). All cultivars used have indeterminate growth habit. For seed inoculation, a plastic bag of 10 L per replication was chosen, containing 1.0 kg of soybean seeds.

After the application of the doses, the inoculant was homogenized with the seeds, for subsequent drying and sowing.

Treatments and experimental design

A randomized block design was used with 8 treatments and 4 replications. The experimental plots presented a total area of 24 m^2 (6.0 m × 4.0 m). Around each plot was left 1 m surround space to avoid contamination. The useful area was in the center of the plots, being 3.0 m × 5.0 m, totaling 15 m^2. Soon after seed treatment, sowing was performed in a groove with spacing of 0.5 m between rows and density of 15 seeds/linear m. The final plant density/linear m was 12 plants. The basic fertilization of sowing was made according to soil analysis of each area. The cultural treatments performed were those suggested for a commercial crop. Phytosanitary maintenance was performed when relevant. The treatments used in the experiments are described in Table 2.

The experimental treatments were conducted based on the criteria and requirements of the Normative Instruction No. 53/2013 and Normative Instruction No. 13/2011 and its annexes, from the Ministry of Agriculture, Livestock, and Food Supply of Brazil (MAPA). Co-inoculation with *Azospirillum*-based inoculant and *Bradyrhizobium*-based inoculant was chosen as the standard treatment, corresponding to a commercial and registered product in the MAPA. The standard commercial product dose was used as recommended by the manufacturer.

Plant sampling and harvesting

The first harvest of plants was carried out between the V7 - seven fully developed trifolios and R1 - one open flower anywhere on the main stem (Fehr et al., 1971), considering among five plants per plot, chosen at random, to carry out the following evaluations: shoot dry mass; root dry mass; nodules number; nodules dry weight in roots per plant. A cutting shovel was used to collect the roots and the collection was performed at 0.2 m depth and 0.15 m in diameter. The roots, nodules and soil were separated with the aid of a sieve with a 3 mm mesh running water for soil cleaning adhered to the plant parts. After drainage of excess water, the nodules were packed in paper bags and identified. To determine the dry weight, the shoot, roots,

Table 2. Treatments description used to evaluate the effect of coinoculation on soybean crop.

Treatment	Composition	Doses
T1 - Control*	Absence of mineral N fertilizer and inoculant	-
T2 - Mineral N fertilizer**	Nitrogen	200 kg ha^{-1}
T3 - GRAP NOD + (peat)	*Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* (SEMIA 5079 and SEMIA 5080)	60 g 50 kg^{-1} seed
T4 - GRAP NOD L (liquid)	*Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* (SEMIA 5079 and SEMIA 5080)	100 mL 50 kg^{-1}
T5 - GRAP NOD A (liquid)	*Azospirillum brasilense* (Ab-V5 and Ab-V6)	100 mL 50 kg^{-1}
T6- Standard co-inoculation	*Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* + *Azospirillum brasilense*	100 + 100 mL 50 kg^{-1}
T7 - Co-inoculation 1:1	*Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* + *Azospirillum brasilense*	100 + 100 mL 50 kg^{-1}
T8 - Co-inoculation 1:1.5	*Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* + *Azospirillum brasilense*	100 + 150 mL 50 kg^{-1}

*All treatments received fertilization with 00-23-23. ** 50% in sowing and 50% in flowering.
Source: Author

and modules were placed in an oven at 65°C until reaching constant weight. Subsequently, the dry biomass was weighed. The determination of N concentration in shoots was performed using the Kjeldahl method (Sertsu and Bekele, 2000). The second harvest of plants was evaluated at full plant maturation phenological stage R8 - when 95% of pods had the typical color of mature pods (Fehr et al., 1971) when the grains were collected. After grains collection, moisture was determined, productivity (kg ha^{-1}) was estimated with moisture correction to 13%; the mass of a thousand grains was obtained through the evaluation of 8 sub-samples of 100 grains each and the mean was multiplied by 10, as determined by the Rules for Seed Analysis (BRASIL, 2009). The determination of N concentration in grains was performed using the Kjeldahl method (Sertsu and Bekele, 2000) as recommended by the Association of Official Analytical Chemists (AOAC, 1990) and Vitti et al. (2001), with certain modifications.

Statistical analysis

The raw dataset was first assessed by the Shapiro-Wilk test regarding the normality, then again by the Bartlett test about the homoscedasticity and homogeneity. When necessary, the data were transformed into log_{10} to meet the assumptions of the parametric tests. Means were assessed by ANOVA and Tukey´s test, considering inoculation treatments and experimental sites as fixed factors. Within all the effects analyzed it was considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

In the field trials conducted in four locations: Maringá, Palmeira, and Ponta Grossa at Paraná State, and Rio das Pedras at Sao Paulo State. The data analysis shows the average results of nine (9) variables: shoot dry mass, root dry mass, nodules number per plant, nodules dry mass, shoot N content, mass of one thousand grains, N content in the grains, and yield, in response to the isolated application of the inoculant GRAP NOD AL® or in association with the inoculant containing *Bradyrhizobium* subspecies, by the co-inoculation *Bradyrhizobium-Azospirillum* ratios of 1:1 and 1:1.5, in the soybean crop. It is possible to infer, through the analysis of variance presented in Table 3, that there were significant differences for all variables in the treatments and in the sites evaluated.

Influence of co-inoculation on nodule number and development

In general, when co-inoculation was performed by the association of the *Bradyrhizobium* spp. with *A. brasilense* via seed treatment (T6, T7 and T8), there was an increase in the nodules number per plant and in the average weight nodules with values significantly higher than the absolute control (T1), as well as the N fertilization (T2), using a dose of 200 kg ha^{-1} of N. (Table 4). For nodules number, all treatments involving inoculation using liquid or solid formulation via seed treatment, with isolated application or in co-inoculation, showed higher average results when compared with the control and with N fertilization alone (T1 and T2, respectively) for all experimental sites. The highest value 90.1 nodule per plant by the co-inoculation ratio 1:1.5 (T8), was observed at Maringá, statistically greater when compared with the other treatments. At Palmeira, Ponta Grossa and Rio das Pedras, the number nodules values observed was 43.3, 44.0, and 45.2 mg $plant^{-1}$, respectively. When co-inoculation was carried out via seed treatment (T6, T7 and T8), there was an increase in the average weight of nodules. At Palmeira, Ponta Grossa and Rio das Pedras, the nodules dry mass values observed were 325.8, 360, and 105.7 mg $plant^{-1}$, respectively. The highest value 499 mg $plant^{-1}$ by the co-inoculation ratio 1:1.5

Table 3. *P*-values of the analysis of joint variance for shoot and root dry mass, number and dry mass of nodules, N content in shoot and grains, number of pods, mass of 1,000 grains and yield of soybean plants that received inoculation treatments with *Bradyrhizobium* spp. and *Azospirillum brasilense* at Maringá, Palmeira and Ponta Grossa, Paraná (PR) State and at Rio das Pedras, Sao Paulo (SP) State, Brazil, in the 2013/2014 and 2017/2018 growing crop season, at PR and SP, respectively.

Factor	Degrees of freedom	Shoot dry mass	Nodule number plant $^{-1}$	Nodule dry mass	Shoot-N content	1,000 Grains mass	Yield	Grain-N content	Roots dry mass‡
	p-value								
Blocks/sites	3	0.604	0.427	0.061	0.778	0.030	0.253	0.366	0.081
Sites (S)	3	< 0.001	0.003	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	<0.001
Treatments (T)	7	< 0.001	< 0.001	< 0.001	0.012	< 0.001	< 0.001	< 0.001	<0.001
S × T	21	< 0.001	< 0.001	< 0.001	0.010	< 0.001	< 0.001	< 0.001	<0.001
CV (%)		10.5	14.0	16.5	1.48	2.24	4.95	1.59	6.39

‡Root dry mass was not determined in the Maringa site, Paraná State of Brazil; CV = coefficient of variation.
Source: Author

Table 4. Nodules number (nod plant^{-1}) and nodule dry mass (mg plant^{-1}) of soybean received inoculation or co-inoculation treatments with *Bradyrhizobium* spp. and *Azospirillum brasilense* at Maringá, Palmeira and Ponta Grossa, Paraná state and at Rio das Pedras, Sao Paulo state, Brazil, in the 2013/2014 and 2017/2018 seasons.

Treatment	Maringá-PR		Palmeira-PR		Ponta Grossa-PR		Rio da Pedras-SP	
	nod plant^{-1}	mg plant^{-1}	nod plant^{-1}	mg plant^{-1}	nod plant^{-1}	mg plant^{-1}	nod plant^{-1}	mg plant^{-1}
T1[1]	12.6 ± 0.72^{f}	91.1 ± 6.86^{ef}	18.9 ± 0.97^{b}	114 ± 6.07^{c}	13.1 ± 0.42^{b}	121 ± 6.44^{c}	13.9 ± 0.97^{b}	27.9 ± 3.5^{b}
T2[2]	18.4 ± 2.11^{ef}	57.2 ± 7.58^{f}	18.3 ± 0.41^{b}	172 ± 14.9^{c}	19.4 ± 0.44^{b}	182 ± 15.8^{c}	23.3 ± 1.41^{b}	43.0 ± 4.12^{ab}
T3[3]	36.3 ± 2.45^{cd}	215 ± 4.95^{cd}	41.6 ± 2.29^{a}	270 ± 25.2^{b}	36.5 ± 2.72^{a}	286 ± 47.7^{b}	38.4 ± 4.07^{a}	66.7 ± 11.1^{ab}
T4[4]	45.8 ± 1.29^{bc}	274 ± 17.5^{bc}	44.6 ± 1.01^{a}	290 ± 9.12^{ab}	47.3 ± 1.07^{a}	307 ± 9.67^{b}	45.2 ± 3.97^{a}	87.2 ± 4.09^{ab}
T5[5]	26.4 ± 0.41^{de}	117 ± 2.85^{ef}	44.8 ± 1.69^{a}	320 ± 40.8^{ab}	21.0 ± 1.02^{b}	171 ± 20.5^{c}	23.4 ± 2.63^{b}	56.5 ± 12.0^{ab}
T6[6]	30.8 ± 0.44^{d}	152 ± 3.88^{de}	42.6 ± 1.67^{a}	362 ± 11.1^{a}	45.1 ± 1.77^{a}	384 ± 11.7^{a}	47.1 ± 3.43^{a}	100 ± 5.60^{ab}
T7[7]	51.1 ± 0.64^{b}	344 ± 6.33^{b}	41.2 ± 2.19^{a}	335 ± 17.5^{ab}	43.7 ± 2.32^{a}	355 ± 18.6^{ab}	46.7 ± 3.16^{a}	105 ± 14.6^{a}
T8[8]	90.1 ± 3.32^{a}	499 ± 17.6^{a}	44.7 ± 0.75^{a}	322 ± 17.5^{ab}	47.4 ± 0.79^{a}	341 ± 18.5^{ab}	48.8 ± 3.38^{a}	113 ± 9.59^{a}

[1]Absence of mineral nitrogen fertilizer and inoculant. [2]200 kg N ha^{-1}: 50% sowing and 50% flowering. [3]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 7 x 10^{9} CFU ml^{-1} - peat. [4]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 5 x 10^{9} CFU ml^{-1} - liquid. [5]*Azospirillum brasilense* (Ab-V5, Ab-V6), at of 2 × 10^{8} CFU ml^{-1} - liquid. [6]Standard commercial *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [7]Co-inoculation rate 1:1 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [8]Co-inoculation rate 1:1.5 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 150 mL 50 kg^{-1}).
*Means (± error) followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability (n = 4). ns = not significant. nd = not determined.
Source: Author

(T8) was observed at Maringá, statistically greater when compared with the other treatments. The results obtained in this assay are in accordance with other studies and presented adequate nodulation for the N supply. The N required by a soybean plant for its normal development would be supplied by the number of nodules per plant from 15 to 30 and the mass of nodules between 100 and 200 mg plant^{-1} (Hungria et al., 2007). It is important to highlight that the use of microorganisms that participate in BNF in crops such as soybean, for example, provided better

Table 5. Shoot and root dry mass (g plant^{-1}) of soybean that received inoculation or co-inoculation treatments with *Bradyrhizobium* spp. and *Azospirillum brasilense* at Maringá, Palmeira and Ponta Grossa, Paraná state and at Rio das Pedras, Sao Paulo state, Brazil, in the 2013/2014 and 2017/2018 seasons.

Treatment	Maringá-PR		Palmeira-PR		Ponta Grossa-PR		Rio da Pedras-SP	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
	g plant^{-1}							
T1[1]	10.0 ± 2.39^{e}	nd	22.5 ± 0.98^{b}	3.54 ± 0.17^{b}	23.8 ± 1.04^{b}	3.75 ± 0.19^{b}	21.9 ± 1.35^{d}	1.21 ± 0.06^{d}
T2[2]	17.3 ± 1.18^{de}	nd	27.3 ± 0.83^{b}	3.53 ± 0.32^{b}	29.0 ± 0.88^{b}	3.74 ± 0.34^{b}	27.9 ± 0.60^{cd}	1.43 ± 0.12^{cd}
T3[3]	27.8 ± 0.76^{bc}	nd	51.3 ± 1.51^{a}	6.16 ± 0.04^{a}	54.3 ± 1.60^{a}	6.53 ± 0.03^{a}	33.1 ± 1.15^{bc}	1.95 ± 0.11^{abc}
T4[4]	30.7 ± 0.60^{bc}	nd	52.8 ± 2.89^{a}	6.01 ± 0.01^{a}	56.0 ± 3.07^{a}	6.37 ± 0.01^{a}	33.1 ± 1.05^{bc}	1.95 ± 0.12^{abc}
T5[5]	23.3 ± 0.65^{cd}	nd	52.7 ± 1.08^{a}	6.15 ± 0.03^{a}	55.9 ± 1.15^{a}	6.44 ± 0.05^{a}	27.5 ± 0.75^{cd}	1.76 ± 0.13^{bcd}
T6[6]	24.7 ± 0.19^{cd}	nd	54.5 ± 2.43^{a}	6.33 ± 0.14^{a}	57.8 ± 2.57^{a}	6.71 ± 0.15^{a}	41.5 ± 4.20^{ab}	2.22 ± 0.11^{ab}
T7[7]	33.8 ± 0.33^{b}	nd	55.2 ± 1.38^{a}	6.36 ± 0.10^{a}	58.5 ± 1.46^{a}	6.74 ± 0.11^{a}	41.9 ± 1.45^{ab}	2.52 ± 0.05^{a}
T8[8]	46.2 ± 6.31^{a}	nd	54.7 ± 1.57^{a}	6.37 ± 0.12^{a}	58.0 ± 1.66^{a}	6.75 ± 0.13^{a}	43.2 ± 2.95^{a}	2.25 ± 0.19^{ab}

[1]Absence of mineral nitrogen fertilizer and inoculant. [2]200 kg N ha^{-1}: 50% sowing and 50% flowering. [3]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 7 × 10^{9} CFU ml^{-1} - peat. [4]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 5 × 10^{9} CFU ml^{-1} - liquid. [5]*Azospirillum brasilense* (Ab-V5, Ab-V6), at of 2 × 10^{8} CFU ml^{-1} - liquid. [6]Standard commercial *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [7]Co-inoculation rate 1:1 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [8]Co-inoculation rate 1:1.5 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 150 mL 50 kg^{-1}).
*Means (± error) followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability (n = 4). ns = not significant. nd = not determined.
Source: Author

results than mineral nitrogen fertilization itself. The application of mineral N in areas that have been inoculated with microorganisms tend to reduce the efficiency of nodulation and consequently decrease the efficiency of BNF (Embrapa Soja, 2013).

Effect of co-inoculation on root and shoot biomass and development

Shoot and root dry mass were higher in the plants inoculated or co-inoculated with *Bradyrhizobium* spp. and *A. brasilense* when compared with plants without inoculation (T1 and T2) (Table 5). Some bacteria have a function of promoting plant growth. In general, the main mechanism involved is the production and release of growth hormones, such as auxins. Under these conditions, the plant tends to increase root production and explore higher soil volume. Thus, the plant also becomes more efficient in absorbing nutrients (Hungria et al., 2015). In Palmeira and Ponta Grossa, the data observed for shoot were 53.5 and 56.8 g plant^{-1} and 6.2 and 6.6 g plant^{-1} for root. Maringá and Rio das Pedras demonstrated that shoot dry mass response to T8 - co-inoculation rate was 1:1.5 with *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *A. brasilense* (100 + 150 mL 50 kg^{-1}) with 46.2 and 42.2 g plant^{-1}, respectively. Significant biomass increases were observed when compared with the control (T1) and conventional mineral N fertilization (T2) (Table 5). In Maringa and Rio das Pedras, the single inoculation with *Bradyrhizobium* subspecies (T3 and T4) or co-inoculation (T6 and T7) showed intermediate results regarding the shoot dry mass, ranging from 23.3 to 33.8 g plant^{-1} at Maringa and 27.5 to 41.9 g plant^{-1} at Rio das Pedras. These results agreed somehow with the other variables analyzed, that is, the nodules number in the root, as well as the nodules dry mass. The increase in the dry mass of shoots may be related to the efficiency of the inoculation and the co-inoculation of *Bradyrhizobium* spp. and *A. brasilense*, applied via seed treatment, with all the necessary care not to reduce the viability of the bacteria.

Influence of inoculation on N uptake in shoot and grains

Shoots and grains in Palmeira and Ponta Grossa, did not differ significantly for N concentration. Regarding N concentration in shoots and grains in Maringá under T8 - co-inoculation rate 1:1.5 with *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *A. brasilense* (100 + 150 mL 50 kg^{-1}), a significant difference was observed compared to the other treatments, especially the control (T1)

Table 6. Mean shoot and grains N concentration (g kg^{-1}) of soybean that received inoculation or co-inoculation treatments with *Bradyrhizobium* spp. and *Azospirillum brasilense* at Maringá, Palmeira and Ponta Grossa, Paraná state and at Rio das Pedras, Sao Paulo state, Brazil, in the 2013/2014 and 2017/2018 seasons.

Treatment	Maringá-PR		Palmeira-PR		Ponta Grossa-PR		Rio das Pedras-SP	
	Shoot-N	Grain-N	Shoot-N	Grain-N	Shoot-N	Grain-N	Shoot-N	Grain-N
	g.kg^{-1}							
T1[1]	43.0 ± 1.11c	63.8 ± 0.^{64e}	35.7 ± 0.85^{ns}	58.3 ± 0.35^{ns}	40.2 ± 0.95^{ns}	61.8 ± 0.37^{ns}	49.2 ± 1.39 ab	53.5 ± 0.61^{ns}
T2[2]	45.8 ± 0.10^{abc}	70.7 ± 0.12^{b}	37.3 ± 0.39	58.6 ± 0.23	42.0 ± 0.44	62.1 ± 0.24	48.5 ± 2.31 ab	54.7 ± 0.80
T3[3]	48.4 ± 0.17^{abc}	67.3 ± 0.17^{d}	36.5 ± 0.61	58.3 ± 0.64	41.1 ± 0.69	61.8 ± 0.68	49.9 ± 2.47 ab	55.4 ± 0.62
T4[4]	47.6 ± 0.17^{abc}	69.9 ± 0.21^{bc}	38.4 ± 1.31	57.6 ± 0.14	43.1 ± 1.47	61.0 ± 0.15	45.2 ± 1.44 b	54.6 ± 0.41
T5[5]	45.0 ± 0.06^{bc}	66.3 ± 0.23^{d}	36.8 ± 0.74	58.2 ± 0.54	41.3 ± 0.83	61.7 ± 0.58	51.5 ± 1.82 a	54.5 ± 0.56
T6[6]	46.8 ± 0.10^{abc}	68.2 ± 0.19^{cd}	37.1 ± 0.57	57.5 ± 0.52	41.7 ± 0.64	61.0 ± 0.55	47.8 ± 2.15 ab	54.6 ± 0.78
T7[7]	49.2 ± 0.19^{ab}	71.7 ± 0.24^{ab}	38.4 ± 0.70	58.0 ± 0.38	43.1 ± 0.78	61.5 ± 0.40	50.0 ± 2.98 ab	54.1 ± 0.56
T8[8]	51.2 ± 0.89^{a}	73.2 ± 0.57^{a}	38.6 ± 1.00	58.2 ± 0.16	43.4 ± 1.12	61.7 ± 0.18	45.3 ± 1.65 b	54.2 ± 0.86

[1]Absence of mineral nitrogen fertilizer and inoculant. [2]200 kg N ha^{-1}: 50% sowing and 50% flowering). [3]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 7×10^{9} CFU ml^{-1} - peat. [4]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 5 × 10^{9} CFU ml^{-1} - liquid. [5]*Azospirillum brasilense* (Ab-V5, Ab-V6), at of 2 × 10^{8} CFU ml^{-1} - liquid. [6]Standard commercial *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [7]Co-inoculation rate 1:1 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [8]Co-inoculation rate 1:1.5 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 150 mL 50 kg^{-1}). *Means (± error) followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability (n = 4). ns = not significant. nd = not determined.
Source: Author

(Table 6). No difference was observed between treatments inoculated or co-inoculated and N fertilization for shoot N concentration (Table 6). N concentration in the grains was not diferent between the two co-inoculation rates tested. It is highly likely that combinations of bacteria applied via seed treatment can provide an increase in the agronomic characteristics of soybean, which are directly related to the greater N_2 fixation provided by the action of these microorganisms. The use of inoculants containing bacteria of the genus *A. brasilense* has shown beneficial effects in this association, since the bacterium has the capacity to produce phytohormones that determine greater development of the root system. More developed root system results in an increase in the amount of associated nodules and in the amount of N fixed and made available to the plant (Fukami et al., 2018; Santos et al., 2021). Thus, the results obtained so far corroborate those obtained in several other places in Brazil indicating that with the inoculation of seeds using these microorganisms the yield efficiency of the crop can be as interesting as the application of nitrogen fertilization (Hungria, 1997; Mendes et al., 2000; Bárbaro et al., 2006).

Soybean yield

The results of the mass of 1,000 grain and yield in Maringá, in response to the isolated application or in association of different inoculants and doses in the soybean crop are shown in Table 7. It is observed that there was a significant difference between treatments regarding the weight of one thousand grains. The T8 - co-inoculation rate 1:1.5 with *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *A. brasilense* (100 + 150 mL 50 kg^{-1}), promoted the greatest increase in this variable, when compared with the others, with a mass of 127 g. Thus, when the seeds were co-inoculated with doses of 1:1.5, the mass of one thousand grains was superior to the absolute control (T1) (Table 7). At Palmeira, the harvest was performed in 2013, soybean yield data are shown in Table 6. The control and the conventional N fertilization (T1 and T2) recorded the significantly lower yield when compared with the other. Ponta Grossa showed similar results, the co-inoculation with *Bradyrhizobium* spp. + *A. brasilense* (T6, T7 and T8) prompt yield with 3,255 kg ha^{-1}, significantly higher in average by 117% than the treatments single inoculation, 2,781 kg ha^{-1}. At Maringa and Rio das Pedras, the evaluation of grain yield showed that T8 - co-inoculation rate 1:1.5 with *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *A. brasilense* (100 + 150 mL 50 kg^{-1}), presented the best performance, in which this treatment significantly

Table 7. Mass of 1,000 grains (g) and yield (kg ha^{-1}) of soybean plants that received inoculation or co-inoculation treatments with *Bradyrhizobium* spp. and *Azospirillum brasilense* at Maringá, Palmeira and Ponta Grossa, Paraná state and at Rio das Pedras, Sao Paulo state, Brazil, in the 2013/2014 and 2017/2018 seasons.

Treatment	Maringá-PR		Palmeira-PR		Ponta Grossa-PR		Rio da Pedras-SP	
	g	Kg ha^{-1}	g	Kg ha^{-1}	g	Kg ha^{-1}	g	Kg ha^{-1}
T1[1]	99.6 ± 1.74^{e}	1757 ± 237^{f}	135 ± 1.32^{ns}	1964 ± 51.3^{b}	138 ± 1.79^{b}	2081 ± 54.4^{c}	165 ± 1.61^{b}	3398 ± 73.5^{d}
T2[2]	103 ± 0.38^{de}	2531 ± 66.1^{e}	141 ± 0.80	2013 ± 4.32^{b}	150 ± 0.85^{a}	2133 ± 4.58^{c}	169 ± 2.46^{ab}	3892 ± 82.4^{c}
T3[3]	118 ± 0.32^{bc}	3512 ± 41.7^{c}	141 ± 1.58	2625 ± 35.8^{a}	146 ± 1.41^{a}	2782 ± 37.9^{b}	170 ± 1.94^{ab}	4036 ± 194^{abc}
T4[4]	121 ± 0.65^{abc}	4097 ± 32.2^{b}	140 ± 1.04	2610 ± 34.1^{a}	148 ± 1.10^{a}	2767 ± 36.1b	171 ± 4.56^{ab}	4089 ± 81.1^{abc}
T5[5]	108 ± 1.52^{d}	2826 ± 34.3^{de}	140 ± 0.62	2635 ± 64.2^{a}	148 ± 0.66^{a}	2793 ± 68.1b	170 ± 2.32^{ab}	3959 ± 104^{bc}
T6[6]	116 ± 0.35^{c}	3145 ± 92.9^{d}	140 ± 0.28	2854 ± 22.8^{a}	148 ± 0.39^{a}	3265 ± 2.42a	172 ± 2.66^{a}	4294 ± 97.6^{ab}
T7[7]	123 ± 0.48^{ab}	4326 ± 27.5^{b}	140 ± 1.08	2891 ± 57.3^{a}	153 ± 2.80^{a}	3269 ± 6.07a	171 ± 2.23^{ab}	4265 ± 105^{ab}
T8[8]	127 ± 1.34^{a}	4712 ± 49.6^{a}	140 ± 1.18	2894 ± 46.1^{a}	146 ± 1.44^{a}	3232 ± 35.1a	173 ± 1.56^{a}	4306 ± 105^{a}

[1]Absence of mineral nitrogen fertilizer and inoculant. [2]200 kg N ha^{-1}: 50% sowing and 50% flowering). [3]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 7 × 10^{9} CFU ml^{-1} - peat. [4]*Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080), at 5 × 10^{9} CFU ml^{-1} - liquid. [5]*Azospirillum brasilense* (Ab-V5, Ab-V6), at of 2 × 10^{8} CFU ml^{-1} - liquid. [6]Standard commercial *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [7]Co-inoculation rate 1:1 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 100 mL 50 kg^{-1}). [8]Co-inoculation rate 1:1.5 *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *Azospirillum brasilense* (100 + 150 mL 50 kg^{-1}). *Means (± error) followed by the same letter in the column do not differ from each other by the Tukey test at 5% probability (n = 4). ns = not significant. nd = not determined.
Source: Author

surpassed the control (T1). This treatment showed an average yield of 4,712 kg ha^{-1} at Maringá and 4,288 kg ha^{-1} at Rio das Pedras which surpassed the control (T1) by more than 100% in the two cases, as well as the application of N mineral (T2). In this way, co-inoculation via seeds at co-inoculation rate 1:1.5 with *Bradyrhizobium* spp. (SEMIA 5079, SEMIA 5080) + *A. brasilense* (100 + 150 mL 50 kg^{-1}) clearly reflect the ability of bacteria to promote greater development of soybean plants, with a positive impact on grain yield and, thus, minimizing production costs with economy of N fertilizers. These results agree with those reported by Câmara (2000), who revealed that plants with 10 to 30 nodules at flowering present sufficient conditions to obtain high levels of fixed N and, consequently, high grain yield. Thus, in general, it is possible to infer that the application allows to potentiate the BNF in the soybean crop, promoting greater growth of the soybean root system, with consequent increase in grain yield.

Conclusion

The co-inoculation with *Bradyrhizobium* spp. and *A. brasilense* in the treatment of soybean seeds carried out in four different edaphoclimatic regions of southern and southeastern Brazil demonstrated agronomic efficiency increasing soybean yield.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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