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Combination of *Azospirillum* and *Bradyrhizobium* on Inoculant Formulation Improve Nitrogen Biological Fixation in Soybean

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Abstract

Co-inoculation in soybean with *Azospirillum brasilense* and *Bradyrhizobium* spp. is a consolidated practice to ensure nitrogen supply and maximize productivity and sustainability. This study aimed to evaluate the agronomic efficiency of the co-inoculation in soybean, via seed or in-furrow, of doses of the mixed inoculant containing *Bradyrhizobium* spp. and *A. brasilense* in the same formulation. The experiments were conducted under field conditions in four different edaphoclimatic regions to compare seven treatments: Control without nitrogen (N) and inoculant (T1), 200 kg ha^{-1} of mineral N fertilizer (T2), 100 mL of standard liquid inoculant of *Bradyrhizobium* spp. (T3) in 50 kg^{-1} seeds, 100 mL (T4) or 200 mL (T5) of the mixed liquid inoculant in 50 kg^{-1} seeds, 200 mL (T6) or 300 mL (T7) ha^{-1} of the mixed inoculant applied in-furrow. Nodulation characteristics (number and mass of nodules) and carbon and N metabolism (dry mass, N content, and N accumulation in the aerial part) were evaluated at the phenological stage R2 (full flowering—there is an open flower at one of the two uppermost nodes). At maturity (R8—full maturity—95% of the pods have reached their full mature color), yield, the mass of 1,000 grains, N content in the grains, and grain N export were measured. The data were submitted to analysis of variance using the F test and comparison of means was performed using the Duncan test (5%). Under field conditions, the mixed inoculant at doses of 200 mL 50 kg^{-1} seed or 300 mL ha^{-1} applied in-furrow were equivalent or superior to the standard inoculant for the total number of nodules, N content in the grains, grain N export, productivity, and mass of 1,000 grains. The use of the mixed inoculant containing *Bradyrhizobium* spp. and *A. brasilense* in the same formulation meets the demand of grain N export and resulted in increases of 28%, 18% and 7.4% in soybean productivity compared with control without nitrogen, mineral N fertilizer and standard liquid inoculant of *Bradyrhizobium* spp. respectively.

Keywords: *Glycine max* L., co-inoculation, seed treatment, in-furrow application, symbiosis, plant-growth-promoting bacteria (PGPB)

1. Introduction

Nitrogen (N) is one of the essential chemical elements for plant development and is required in larger quantities by crops. Plants can obtain N through the decomposition of organic matter, non-biological fixation, N fertilization, and biological N fixation (BNF); the last two are the ones that most contribute to N supply (Hungria et al., 2001; 2007). BNF is the process by which N present in the atmosphere (N_2) is converted into forms that plants can utilize. The reaction is catalyzed by the enzyme nitrogenase, found in all N-fixing bacteria. The symbiosis between N-fixing bacteria (belonging to several genera and species of bacteria, collectively known as rhizobia) and legumes such as soybean [*Glycine max* (L.) Merrill] is the most important. The process occurs in typical structures formed in the roots, the nodules (Hungria et al., 2013).

Brazilian research on BNF has adapted the N needs of soybean at levels close to 5,000 kg ha^{-1} (Hungria et al., 2015). The inoculants used have excellent quality and the country gained a considerable advantage from the benefits of the symbiotic process for more productive and sustainable agriculture. Currently, no Brazilian soybean farmer does not benefit from this technology, supplying more than 90% of the plant N demand in the field (because the soil always contributes a little) (Hungria et al., 2006, 2007). However, in several countries such as China, the United States, and Australia, the regulations are insufficient to encourage companies to improve the quality of their inoculants. This leads to low performance and subsequent abandonment of inoculants used soybean farmers, who have no benefits from using this biological input (De Souza et al., 2019).

Since the 1960s, inoculant industries have been established in Brazil, exploiting the strains selected by research (Nogueira & Hungria, 2013). Initially, the most commonly used inoculants were of the peat type, whereas, in the last decade, liquid inoculants combining two of these four strains were launched: *Bradyrhizobium elkanii* SEMIA 587 and SEMIA 5019 (29w), and *B. japonicum* SEMIA 5079 (CPAC-15) and SEMIA 5080 (CPAC-7), this latter currently reclassified as *B. diazofficiens* (Zilli et al., 2006; Delamuta et al., 2013). Liquid inoculants have also been recommended for application in-furrow to avoid contact with other products applied via seed treatment (*e.g.*, fungicides, insecticides, and micronutrients such as cobalt, molybdenum, and nickel), which can drastically reduce the efficiency of traditional seed inoculation. Therefore, soybean cultivation is only economically viable due to inoculants with *Bradyrhizobium* spp. strains, which can provide more than 300 kg of N ha^{-1}. The inoculation should be performed annually to maximize the benefits provided by microorganisms, resulting in average increments in soybean yields of around 8% (Hungria et al., 2013). Besides the quality and type of inoculants, correct inoculation is of paramount importance for the success of the BNF. Most of the failures eventually detected in the inoculation process are due to the inadequate use of the biological input. The soybean farmers are often not aware of the correct inoculant usage, leading to the inefficient mixture of the inoculant on seeds, inoculation too far in advance of sowing, sowing in dry soil, under-dosing of the inoculant, and other errors that compromise the success of the inoculation (Fonseca, 2011).

To mitigate these negative processes, the application of liquid inoculants in-furrow was validated by research. The inoculation in the sowing furrow has more benefits than the application via seed that uses high doses. The number of viable bacterial cells is considerably increased, which benefits nodulation and making N available in periods of great demand, such as in the grain filling. Pre-treatment is another innovative process that brings the possibility of inoculating soybean seeds longer than 24 hours before planting, without losses compared to traditional inoculation (Zilli et al., 2010).

Co-inoculation or mixed inoculation of bacteria is a consolidated technology in Brazil, also in line with the current agricultural approach, which respects the demands for high yields but with agricultural, economic, social, and environmental sustainability. It consists in adding more than one microorganism known to be beneficial to plants, aiming to maximize their contribution. Thus, it combines inoculating soybean seeds with N-fixing bacteria rhizobia, a practice already well known by crop producers, with the use of *Azospirillum*, a bacterium known for its growth-promoting action in grasses (Bárbaro et al., 2009, 2011; Hungria et al., 2013; Embrapa, 2013).

The genus *Azospirillum* belongs to the group of plant growth-promoting bacteria, which has as main characteristics the capacity of BNF, the increase of nitrate reductase activity when they grow endophytically in plants, and the production of hormones such as auxins, cytokinins, gibberellins, and ethylene (Tien et al., 1979; Bottini et al., 1989; Strzelczyk & Kamper, 1994; Cassán et al., 2008; Huergo et al., 2008). According to Hungria (2011), *Azospirillum* spp. is marketed as an inoculant for wheat and corn with increases of 31 and 26% in grain yield, respectively. However, part of the N required by the plants must be supplied by mineral N fertilizer.

The mechanisms of action of *Bradyrhizobium* spp. and *Azospirillum* are different. In the latter, the benefits come from BNF, and as previously mentioned, however, its greater efficiency is due to the synthesis of phytohormones that regulate plant growth (Spaepen & Vanderleyden, 2015). Thus, a more voluminous root system provides better absorption and/or utilization of water and nutrients, as well as greater resistance to water stress (Fukami et al., 2017; Marques et al., 2017). Concerning nutrients, greater plant vigor is also observed (Ardakani et al., 2011). *Azospirillum brasilense* positively influences soybean nodules, improving their BNF efficiency (Groppa et al., 1998; Libório et al., 2020). Improvements in soybean agronomic traits were attributed to the positive influence of co-inoculation (Hungria et al., 2013; Embrapa, 2013; Hungria et al., 2015; Bárbaro et al., 2017, 2018a, 2018b; Dourado et al., 2018; Galindo et al., 2018). However, these results were not confirmed by Gitti et al. (2012) and Zuffo et al. (2015, 2016).

The presence of different bacterial species in a single biological product formulation can maximize the desired effects due to their synergistic interaction. This synergism results from the production of microbial metabolites under *in vitro* and *in vivo* conditions. Synergistic interactions between *B. japonicum* and *A. brasilense* are reported to be partially associated with quorum sensing communication mechanisms (Fagotti et al., 2019). Furthermore, signalling molecules in *Bradyrhizobium* cultures can enhance the expression of genes related to increased resistance to stress conditions (Boiero et al., 2007; Donati et al., 2013; Torres et al., 2018; Torres et al., 2021).

In this context, it is still necessary to ameliorate the development of inoculant formulations containing different species of bacteria and improved strains, and verify their agronomic efficiency under different doses and application modes.

This study aimed to evaluate the agronomic efficiency of co-inoculation, via seed or in-furrow, of doses of a mixed inoculant containing *Bradyhizobium* spp. and *Azospirillum brasilense* in soybeans grown in four different edaphoclimatic regions during the crop year of 2015/2016.

2. Material and Methods

The experiments were installed in the crop year 2015/2016 under field conditions in four different edaphoclimatic experimental areas in Colina-SP, Selvíria-MS, Maringá-PR, and Rio Verde-GO. The geographic coordinates, climate classification, minimum and maximum average temperature, rainfall, and soil classification of each experimental area are presented in Table 1.

Table 1. Geographic coordinates, climate classification, average minimum and maximum temperature, rainfall and soil classification where each field experiments were performed during the 2015/2016 crop season

Site	**Coordinates[a]**	**Elevation (m)**	**Climate Classification[a]**	**Average minimum temperature (ºC)**	**Average maximum temperature (ºC)**	**Rainfall (mm)**	**Soil classification**
Colina (SP)	20°43′05″S 48°3′38″W	568	*Cwa*	18.7	31.3	828.6	Dystrophic Red Latosol (Oxisol)
Selvíria (MS)	20°20′42.8″S 51°24′01″W	335	*Aw*	19.0	31.0	1,313	Dystrophic Red Latosol (Oxisol)
Maringá (PR)	23°20′56″S 51°04′31″W	540	*Cfa*	20.0	28.5	1,065	Dystrophic Red Ultisol
Rio Verde (GO)	17°47′52″S 50°55′40″W	742	*Aw*	19.6	30.6	1,230	Dystrophic Red Latosol (Oxisol)

Note. [a] Köppen-Geiger: *Cwa*: Humid subtropical with dry winter and hot summer; *Aw*: tropical with dry winter; *Cfa*: humid subtropical without dry season and hot summer.

Before installing the experiment, soil samples (0-20 cm) were harvested from the experimental areas for further chemical and physical analyses. *Bradyrhizobium* and associative *Diazotrophic* bacteria were counted from the soil (0-10 cm) before sowing. Bacteria counts were performed in the Laboratory of Agricultural Microbiology FCAV/UNESP, Jaboticabal/SP, according to the recommendations of Dobereiner et al. (1995), and are shown in Table 2.

Table 2. Soil chemical characterization and granulometry in 0-20 cm depth and rhizobia and diazotrophic population in the 0-10 cm depth where each field experiments were performed during the 2015/2016 crop season

Site	pH[a]	Organic matter	P	K[c]	K[b]	Ca[b]	Mg[b]	S[d]	CEC	Base saturation	Granulometry			Rhizobia	Diazotrophic[b]
											Clay	Silt	Sand		
		g dm^{-3}	- mg dm^{-3} -	------------ $mmol_c$ dm^{-3} -----------						----------------- % --------------				---------- CFU g^{-1} ----------	
Colina-SP	5.2	22.5	18.5[b]	-	3.04[b]	18.7[b]	12.9	3.57	62.0	55.7	150	45.0	804	2.14×10^7	1.1×10^6
Selvíria-MS	5.3	20.0	31.0[c]	-	1.9[b]	17.0[b]	11.0	9	71.9	42.0	410	34	556	2.04×10^7	2.4×10^6
Maringá-PR	5.2	18.1	4.70[c]	-	6.1[b]	30.8[b]	21.8	8.26	85.0	69.1	546	15.5	438	7.23×10^2	9.5×10^5
Rio Verde-GO	5.5	34.9	15.2[c]	255[c]	-	44.0[b]	13.0	-	109.0	55.6	362	174	464	2.3×10^4	7.2×10^2

Note. [a]pH in water; [b]Mehlich-3 extractor; [c]Resin extractor; [d]Calcium phosphate.

The soybean cultivars used in this study, the fertilization management practices, planting date, population density, and harvest date are presented in Table 3. The field trial management practices such as seed treatment with pesticides, weed, pest, and disease control were adopted to achieve the maximum productive potential of the crop in each region according to Embrapa (2013) technical recommendations for soybean cultivation.

Table 3. The cultivar, fertilization, sowing date, plant population and harvest date in each field experiments were performed during the 2015/2016 crop season

Site	Soybean cultivar	Fertilization (kg ha^{-1})	Sowing date	Plant population (plants ha^{-1})	Harvest date
Colina-SP	5D634 RR	200 (04-20-20)	Dec/03/2015	320,000	Mar/18/2016
Selvíria-MS	BMX Potência RR	330 (Single Superphosphate)	Dec/20/2015	378,000	Apr/01/2016
Maringá-PR	BMX Potência RR	350 (0-20-20)	Oct/28/2015	266,700	Mar/13/2016
Rio Verde-GO	NS 7209 IPRO	350 (0-30-15)	Dec/19/2015	320,000	Apr/05/2016

The evaluated treatments were: control without N and inoculant application (T1), 200 kg ha^{-1} of mineral N fertilizer applied half at planting plus a half at the V4 phenological stage (T2), 100 mL of standard liquid inoculant for soybean containing *B. elkanii* (strain Semia 5019) and *B. japonicum* (strain Semia 5079) at 5×10^9 CFU mL^{-1} (Masterfix® L Soja, Stoller do Brasil Ltda) applied via seed treatment (50 kg^{-1} seed) (T3), 100 mL (T4) or 200 mL (T5) of the mixed liquid inoculant containing *B. japonicum* (strain Semia 5079, 1×10^9 CFU mL^{-1}) and *A. brasilense* (strains AbV5 and AbV6, 1×10^7 CFU mL^{-1}) in the same formulation (Dual Force®, Stoller do Brasil Ltda) applied via seed treatment (50 kg^{-1} seed), and 200 mL (T6) or 300 mL (T7) ha^{-1} of the mixed inoculant applied in-furrow.

The distribution of the experimental plots (5 rows spaced at 0.45 m wide and 10 meters long) followed a randomized block design with four repetitions.

At the onset of flowering (phenological stage R2), ten plants per experimental plot were harvested. The parameters evaluated were: total number of nodules present in the main root and secondary roots (number of nodules plant^{-1}), total dry mass of nodules (mg plant^{-1}), and dry mass of the aerial part (g plant^{-1}). Subsequently, the N content in the dry mass of the aerial part (g kg^{-1}) was determined using the Kjeldahl method (Vitti et al., 2001), and the accumulation of N in the aerial part was calculated (mg N plant^{-1}).

At maturity (R8), the three central rows (functional area of each plot) were harvested to estimate productivity (kg ha^{-1}). The mass of 1,000 grains was determined by weighing eight subsamples of 100 grains and multiplying the average by 10 (MAPA, 2009). The total N content of the grains was also evaluated by the Kjeldahl method (Vitti et al., 2001) and the grain N export was calculated.

The data from each area were submitted to analysis of variance using the F test and the means were compared by the Duncan test (5%) using the R program (R Core Team, 2021).

3. Results and Discussion

The soils had many soybean-nodulating bacteria, with average values of 2.16×10^7 CFU g^{-1} soil in Colina-SP and Selvíria-MS, 7.23×10^2 CFU g^{-1} soil in Maringá-PR, and 2.3×10^4 CFU g^{-1} soil in Rio Verde-GO. With this condition, even plants in the control treatment (T1) without inoculation formed nodules (Table 4). Chueiri et al. (2005) reported that in new areas or areas of high acidity, applying twice the dose of inoculants is recommended because the bacteria survival is affected by soil acidity and competition between native and introduced strains. Higher inoculant doses are expected to compensate for the loss of viable cells in low pH soils (Silva et al., 2011). Krasova-Wade et al. (2006) observed that by raising the rhizobial density at inoculation, the competition of inoculated strains against the native rhizobial population increased. However, Campos (1999) found no benefit from inoculant doses in established no-tillage areas. Annual reinoculation is reported to increase soybean grain yields with average gains of 4.5% even in soils with high *Bradyrhizobium* spp. cells in the Cerrado (Vargas & Hungria, 1997).

Table 4. Nodule number (nº $plant^{-1}$) and nodule dry mass (mg $plant^{-1}$) of soybean with different treatments of co-inoculation (seed or in-furrow) with a liquid mixed inoculant containing *Bradyrhizobium* and *Azospirillum* in field trials during the 2015/2016 in Colina-SP, Selvíria-MS, Maringá-PR and Rio Verde-GO, Brazil

Treatment	Colina-SP		Selvíria-MS		Maringá-PR		Rio Verde-GO		Average	
	nº $plant^{-1}$	mg $plant^{-1}$	nº $plant^{-1}$	mg $plant^{-1}$	nº $plant^{-1}$	mg $plant^{-1}$	nº $plant^{-1}$	mg $plant^{-1}$	nº $plant^{-1}$	mg.$plant^{-1}$
T1 Control	27.4c[3]	225.0b	58.1a	379.6b	34.4f	29.6f	19.2^{ns}	185.9^{ns}	34.8c	205.0b
T2 200 kg ha^{-1} N	17.1d	137.5c	43.9b	208.4b	49.8e	45.3e	16.1	138.2	31.7c	132.3c
T3 Seed: 100 mL Liquid Brady[1]/50 kg^{-1}	30.3b	337.5a	57.7a	379.3a	60.8cd	63.8c	18.5	171.3	41.8b	238.0ab
T4 Seed: 100 mL Azo + Brady[2]/50 kg^{-1}	30.2b	318.8a	62.4a	416.1a	55.3de	52.0d	17.0	143.5	41.2b	232.6ab
T5 Seed: 200 mL Azo + Brady/50 kg^{-1}	29.2bc	325.0a	61.0a	467.9a	82.1b	79.8b	15.2	124.0	46.9a	249.2a
T6 In-furrow: 200 mL Azo + Brady/ha^{-1}	32.3a	318.8a	67.6a	447.3a	68.0c	70.6bc	19.3	154.1	46.8a	247.7a
T7 In-furrow: 300 mL Azo + Brady/ha^{-1}	29.3b	331.3a	64.1a	491.6a	91.8a	101.4a	15.4	140.4	50.1a	266.2a
p value	0.0001	0.0001	0.0026	0.0994	<0.001	<0.001	0.7061	0.5448	<0.001	<0.001

Note. [1] Liquid Brady: Standard liquid inoculant with *Bradyrhizobium elkanii* (strains Semia 5019) and *Bradyrhizobium japonicum* (strains Semia 5079) at a concentration of 5×10^9 CFU mL^{-1}.

[2] Azo + Brady: Liquid mixed inoculant developed for this study with *Bradyrhizobium japonicum* (strains Semia 5079) at a concentration of 1×10^9 CFU mL^{-1} and *Azospirillum brasilense* (strains AbV5 & AbV6) at a concentration of 1×10^7 CFU mL^{-1}.

[3] Means values of 4 replicates. Values in each column followed by the same letter do not differ statistically from each other (Duncan. p < 5%; n.s. non-significant).

Tables 4 and 5 show the average results evaluated in the phenological stage R2. The F test detected high statistical significance ($p \leq 0.01$) for most of the variables analyzed. Nodule mass, N content in the aerial part, mass of the aerial part, and N accumulation did not meet the assumption of homogeneity of residuals between the experiments, not validating the joint analysis of experiments for these parameters (Banzatto & Kronka, 2006).

At the beginning of flowering, the total number of nodules was highest when the mixed inoculant was applied at 200 mL ha^{-1} (Colina-SP and Selvíria-MS) or 300 mL ha^{-1} (Maringá-PR) in-furrow. In the joint analysis of the total number of nodules per plant, the treatments with 200 mL ha^{-1} (seed or in-furrow) and 300 mL ha^{-1} applied in-furrow did not differ statistically from each other but surpassed the treatment with 100 mL 50 kg^{-1} seed with both the standard and the mixed inoculants (Table 4). These results corroborate the reports of Groppa et al (1998) and Libório et al. (2020) that point out favorable influences of *A. brasilense* on soybean nodules.

N fertilization reduced the dry nodule mass in the Colina-SP and Selvíria-MS areas, which was expected due to the contribution of mineral N. In Maringá-PR, the lowest mass of nodules per plant was observed in control (without inoculant and mineral N) due to the lower number of nodules in these plants. A study of in-furrow co-inoculation with several soybean cultivars revealed an increased number of nodules from 36.9 in control to 44.4 nodules per plant on average with the co-inoculation (Libório et al., 2020). In general, the supply of 200 kg ha^{-1} of mineral N showed inferior results regarding the number and mass of nodules (Table 4). Compared with other studies, the nodulation observed in this work proved to be adequate, since the number of nodules (between 15 and 30) and the nodule mass (between 100 and 200 mg $plant^{-1}$) is sufficient to ensure the supply of N required by a soybean plant for its normal growth and development (Hungria et al., 2007; Brandelero et al., 2009, Bárbaro et al., 2009).

The dry mass (g $plant^{-1}$), N content (g kg^{-1}), and accumulation of N in the aerial part (mg $plant^{-1}$) are presented in Table 5 for Colina-SP, Maringá-SP, and Rio Verde-GO. For Selvíria-MS data not available. The application of 200 mL (via seed or in-furrow) and 300 mL (in-furrow application) of the mixed inoculant provided greater gains in total dry mass in Colina-SP and increased N content in the aerial part in Maringá-PR. Bárbaro et al. (2009) observed no significant differences between the control (without inoculant) and with *Bradyrhizobium*-based inoculation in the aboveground mass of the cultivar MG BR 46 (Conquista) in the Colina-SP region. Conversely, Brandelero et al. (2009) observed a significant correlation between soybean productivity, nodulation, and leaf dry matter.

Table 5. N content in shoot (g kg^{-1}), shoot dry mass per plant (g $plant^{-1}$) and total nitrogen in shoot (mg $plant^{-1}$) of soybean with different treatments of co-inoculation (seed or in-furrow) with a liquid mixed inoculant containing *Bradyrhizobium* and *Azospirillum* in field trials during 2015/2016 in Colina-SP, Maringá-PR and Rio Verde-GO, Brazil

Treatment	Colina-SP			Maringá-PR			Rio Verde-GO			Average		
	g kg^{-1}	g $plant^{-1}$	mg $plant^{-1}$	g kg^{-1}	g $plant^{-1}$	mg $plant^{-1}$	g kg^{-1}	g $plant^{-1}$	mg $plant^{-1}$	g kg^{-1}	g $plant^{-1}$	mg $plant^{-1}$
T1 Control	46.8^{ns}	8.8b	412c	62.7f	11.3f	711g	49.3a	2.3ab	115ab	52.9	7.5	413
T2 200 kg ha^{-1} N	48.2	9.1b	437bc	71.4e	13.8e	988f	48.0ab	1.9b	91.0b	55.9	8.3	505
T3 Seed: 100 mL Liquid Brady[1]/50 kg^{-1}	50.5	10.2a	517a	80.3c	17.9d	1439d	47.5b	2.5a	121a	59.4	10.2	692
T4 Seed: 100 mL Azo + Brady[2]/50 kg^{-1}	46.7	8.9b	417c	76.1d	16.2d	1230e	47.5b	1.8b	87.1b	56.7	9.0	578
T5 Seed: 200 mL Azo + Brady/50 kg^{-1}	46.4	10.5a	489ab	88.0b	25.5b	2247b	49.0ab	2.1ab	103ab	61.1	12.7	946
T6 In-furrow: 200 mL Azo + Brady/ha^{-1}	49.5	10.2a	503ab	82.5c	21.9c	1805c	47.8ab	2.3ab	109ab	59.9	11.5	806
T7 In-furrow: 300 mL Azo + Brady/ha^{-1}	46.7	10.6a	493ab	94.8a	29.3a	2780a	48.3ab	2.3ab	108ab	63.3	14.0	1127
p value	0.39321	0.0024	0.0124	<0.001	<0.001	0.0000	0.1134	0.1438	0.1212			

Note. [1] Liquid Brady: Standard liquid inoculant with *Bradyrhizobium elkanii* (strain Semia 5019) and *Bradyrhizobium japonicum* (strain Semia 5079) at a concentration of 5×10^9 CFU mL^{-1}.

[2] Azo + Brady: Liquid mixed inoculant developed for this study with *Bradyrhizobium japonicum* (strain Semia 5079) at a concentration of 1×10^9 CFU mL^{-1} and *Azospirillum brasilense* (strains AbV5 & AbV6) at a concentration of 1×10^7 CFU mL^{-1}.

[3] Means values of 4 replicates. Values in each column followed by the same letter do not differ statistically from each other (Duncan. p < 5%; n.s. non-significant).

Table 6 shows the results for N content in the grains (g kg^{-1}) and grain N export by soybean (kg ha^{-1}). The N content in the grains was significantly higher with the application of the mixed inoculant in Colina-SP and Maringá-PR compared to the application of standard inoculant and control without N, respectively. Several contributions are attributed to the interaction among plants and bacteria of the genus *Azospirillum* such as increases in chlorophyll, N, and proline contents in the aerial part and roots; improved stomatal conductance, greater plant height, biomass production, grain production, and root development; and tolerance to water stress (Hungria, 2011; Yadav et al., 2012). In Selvíria-MS and Rio Verde-GO, of the treatment unaffected N content in the grains.

Table 6. N content in grain (g kg^{-1}) and grain N export (kg ha^{-1}) of soybean with different treatments of co-inoculation (seed or in-furrow) with a liquid mixed inoculant containing *Bradyrhizobium* and *Azospirillum* in field trials during 2015/2016 in Colina-SP, Selvíria-MS, Maringá-PR and Rio Verde-GO, Brazil

Treatment	Colina-SP		Selvíria-MS		Maringá-PR		Rio Verde-GO		Average	
	g kg^{-1}	kg ha^{-1}	g kg^{-1}	kg ha^{-1}	g kg^{-1}	kg ha^{-1}	g kg^{-1}	kg ha^{-1}	g kg^{-1}	kg ha^{-1}
T1 Control	62.9b	231.8c	62.7^{ns}	239.8b	79.6e	133.5g	58.8^{ns}	132.9c	66.0d	184.5e
T2 200 kg ha^{-1} N	68.5a	264.4abc	63.4	267.8ab	86.9d	165.4f	60.0	143.0c	69.7bc	210.2d
T3 Seed: 100 mL Liquid Brady[1]/50 kg^{-1}	59.1c	232.2c	68.5	302.2a	92.2c	205.7d	59.5	179.7b	69.8bc	229.9c
T4 Seed: 100 mL Azo + Brady[2]/50 kg^{-1}	65.9ab	275.9ab	63.0	252.3ab	89.1d	181.7e	58.3	193.8ab	69.1c	225.9c
T5 Seed: 200 mL Azo + Brady/50 kg^{-1}	67.8a	297.6a	63.0	276.0ab	97.5b	230.8b	60.0	199.2a	72.1ab	250.9ab
T6 In-furrow: 200 mL Azo + Brady/ha^{-1}	65.7ab	256.9bc	66.8	265.6ab	94.6c	218.0c	59.0	209.7a	71.5ac	237.5bc
T7 In-furrow: 300 mL Azo + Brady/ha^{-1}	65.9ab	280.5ab	66.0	278.8ab	101.9a	270.7a	59.3	206.0a	73.3a	259.0a
p value	0.0005	0.0023	0.6319	0.1639	0.0000	0.0000	0.6566	<0.001	<0.001	<0.001

Note. [1] Liquid Brady: Standard liquid inoculant with *Bradyrhizobium elkanii* (strain Semia 5019) and *Bradyrhizobium japonicum* (strain Semia 5079) at a concentration of 5×10^9 CFU mL^{-1}.

[2] Azo + Brady: Liquid mixed inoculant developed for this study with *Bradyrhizobium japonicum* (strain Semia 5079) at a concentration of 1×10^9 CFU mL^{-1} and *Azospirillum brasilense* (strains AbV5 & AbV6) at a concentration of 1×10^7 CFU mL^{-1}.

[3] Means values of 4 replicates. Values in each column followed by the same letter do not differ statistically from each other (Duncan. p < 5%; n.s. non-significant).

Table 7 shows the results for productivity and mass of 1,000 grains. The highest soybean yields were observed with the application of 200 mL 50 kg^{-1} seed of the mixed inoculant in Colina-SP and Selvíria-MS, and 300 mL ha^{-1} in-furrow in Maringá-PR. In Rio Verde-GO, the application of the mixed inoculant differed statistically from the treatments with standard inoculant and mineral N fertilizer regardless of the dose and application mode. The joint analysis of the areas shows higher productivity for applying the mixed inoculant in-furrow when compared to seed, regardless of the dose used. The application of the standard or the mixed inoculant via seed generated similar productivity results (Table 7).

Several studies have shown positive influences of co-inoculation on soybean agronomic traits (Bárbaro et al., 2009; Hungria et al., 2013; Hungria et al., 2015; Bárbaro et al., 2017; Bárbaro et al., 2018a; Bárbaro et al., 2018b; Dourado et al., 2018; Galindo et al., 2018). However, Gitti et al. (2012), and Zuffo et al. (2015, 2016) do not support these authors.

Treatments with 200 mL (via seed or in-furrow) and 300 mL (in-furrow application) of the mixed inoculant led to the highest values for the mass of 1,000 grains. The increase in productivity and the gain in the mass of 1,000 grains demonstrate the beneficial effects of the mixed inoculant compared to the standard liquid inoculant and mineral N fertilizer. Considering the average of the four experiments, an additional gain of 205 kg ha^{-1} (3.4 sacks) or 7.1% was obtained by the co-inoculation compared to the treatment solely with *Bradyrhizobium* in the seed. This difference was statistically significant in each of the four areas and the joint analysis. These data corroborate the work of Hungria et al. (2013), which confirmed the agronomic efficiency of inoculation with *Bradyrhizobium* in the seed and *A. brasilense* in-furrow in soybean in the field.

Braccini et al. (2016) found that inoculation via seed and the association of *B. japonicum* with *A. brasilense* in-furrow improved physiological parameters and promoted increases in soybean grain yield compared with the control. Bárbaro et al. (2009) found that although the means of traditional inoculation, co-inoculation, and control presented numerical differences in yield values, these were not significant but do not exclude the possibility of an economic significance of the treatment involving co-inoculation. Rego et al. (2018) reported that the co-inoculation of soybean seeds with *B. japonicum* and *A. brasilense* promotes plant development, and grain yield and seed quality. Similarly, Dalolio et al. (2018) suggested that using *Azospirillum* spp. in soybean represents an economically viable strategy that enhances environmental benefits.

Table 7. Grain yield (kg ha^{-1}) and 1000-grains mass (grams) of soybean with different treatments of co-inoculation (seed or in-furrow) with a liquid mixed inoculant containing *Bradyrhizobium* and *Azospirillum* in field trials during 2015/2016 in Colina-SP, Selvíria-MS, Maringá-PR and Rio Verde-GO, Brazil

Treatment	Colina-SP		Selvíria-MS		Maringá-PR		Rio Verde-GO		Average	
	kg ha^{-1}	grams	kg ha^{-1}	grams	kg ha^{-1}	grams	kg ha^{-1}	grams	kg ha^{-1}	grams
T1 Control	3,685c	166.3ab	3,787b	148.5ab	1,681d	126.3f	2,261c	158.8b	2,853d	150.0c
T2 200 kg ha^{-1} N	3,862bc	164.7bc	4,230ab	146.3b	1,904c	131.3e	2,382c	158.5b	3,095c	150.2c
T3 Seed: 100 mL Liquid Brady[1]/50 kg^{-1}	3,930abc	160.0c	4,419a	146.3b	2,231b	135.0c	3,022b	167.8a	3,400b	152.3bc
T4 Seed: 100 mL Azo + Brady[2]/50 kg^{-1}	4,190ab	163.5bc	3,998ab	150.5ab	2,040c	133.0d	3,324a	170.0a	3,388b	154.3b
T5 Seed: 200 mL Azo + Brady/50 kg^{-1}	4,391a	170.9a	4,400a	159.5ab	2,369b	139.6b	3,319a	169.5a	3,619a	159.9a
T6 In-furrow: 200 mL Azo + Brady/ha^{-1}	3,910abc	165.8ab	3,991ab	161.3a	2,304b	138.3b	3,553a	169.8a	3,440b	158.8a
T7 In-furrow: 300 mL Azo + Brady/ha^{-1}	4,254ab	168.5ab	4,225ab	161.5a	2,659a	144.7a	3,476a	168.8a	3,653a	160.9a
p value	0.0418	0.0053	0.1082	0.0556	<0.001	<0.001	<0.001	0.0020	<0.001	<0.001

Note. [1] Liquid Brady: Standard liquid inoculant with *Bradyrhizobium elkanii* (strain Semia 5019) and *Bradyrhizobium japonicum* (strain Semia 5079) at a concentration of 5×10^9 CFU mL^{-1}.

[2] Azo + Brady: Liquid mixed inoculant developed for this study with *Bradyrhizobium japonicum* (strain Semia 5079) at a concentration of 1×10^9 CFU mL^{-1} and *Azospirillum brasilense* (strains AbV5 & AbV6) at a concentration of 1×10^7 CFU mL^{-1}.

[3] Means values of 4 replicates. Values in each column followed by the same letter do not differ statistically from each other (Duncan. p < 5%; n.s. non-significant).

4. Conclusions

The use of the mixed inoculant containing *Bradyhizobium* spp. and *A. brasilense* in the same formulation, via seeds (200 mL 50 kg^{-1} seed) or in-furrow (300 mL ha^{-1}), is recommended because it maximizes phytotechnical parameters of soybeans, especially grain yield.

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