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Rhizobacteria inoculation in maize associated with nitrogen and zinc fertilization at sowing¹

Inoculação de rizobactérias no milho associada à adubação com nitrogênio e zinco na semeadura

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HIGHLIGHTS:

*Co-inoculation of *Azospirillum brasilense* and *Bacillus subtilis* contributes to increase maize growth.*

**Azospirillum brasilense* can enhance the capacity of zinc use in maize plants.*

Simultaneous utilization of biological and chemical sources can produce positive responses in maize.

ABSTRACT: Rhizobacteria have a wide range of plant growth-promoting mechanisms of action, making them an alternative and/or complementary biological input for chemical fertilizers. In this respect, the present study aimed to assess growth and nitrogen accumulation in maize plants as a function of *Azospirillum brasilense*, *Bacillus subtilis*, zinc and nitrogen application at sowing. The experiment with the 'AL Bandeirante' maize variety was conducted in a greenhouse, using a completely randomized design. The treatments were arranged in a 2 x 2 x 2 x 2 factorial scheme, with six repetitions, considering the presence and absence of *Azospirillum brasilense* (5 g kg⁻¹), *Bacillus subtilis* (5 mL kg⁻¹) and zinc (20 g kg⁻¹) in the seeds and addition or not of nitrogen (30 kg ha⁻¹) to the soil. The variables assessed were plant height, stem diameter, leaf, stem, shoot, root and total dry weight, and shoot nitrogen content. Nitrogen fertilization in the absence of zinc increased shoot and total dry weight as well as shoot nitrogen content. Zinc applied to the seeds improved the total and stem dry weight of maize plants in the absence of *Bacillus subtilis*. Inoculation with *Azospirillum brasilense* and *Bacillus subtilis* increased the stem diameter and shoot nitrogen content of maize plants when nitrogen fertilization was not performed at sowing. There was no isolated or interaction effect between factors for plant height and root dry weight.

Key words: *Zea mays*, *Azospirillum brasilense*, *Bacillus subtilis*, PGPR, co-inoculation

RESUMO: As rizobactérias possuem mecanismos de ação diversificados capazes de promover o crescimento vegetal fazendo com que se tornem um insumo biológico alternativo e/ou complementar aos fertilizantes químicos. Neste sentido, objetivou-se avaliar o crescimento e o acúmulo de nitrogênio em plantas de milho em função da aplicação de *Azospirillum brasilense*, *Bacillus subtilis*, zinco e nitrogênio na semeadura. O experimento com o milho variedade 'AL Bandeirante' foi realizado em casa de vegetação, no delineamento inteiramente casualizado. Os tratamentos foram dispostos em esquema fatorial 2 x 2 x 2 x 2, com seis repetições, considerando-se a ausência e presença de *Azospirillum brasilense* (5 g kg⁻¹), *Bacillus subtilis* (5 mL kg⁻¹) e zinco (20 g kg⁻¹) nas sementes e adição ou não de nitrogênio (30 kg ha⁻¹) ao solo. As variáveis avaliadas foram: altura da planta, diâmetro basal do colmo, massas secas das folhas, do colmo, da parte aérea, da raiz e total e nitrogênio acumulado na parte aérea. A adubação nitrogenada, na ausência de zinco, incrementou as massas secas da parte aérea, total e o acúmulo de nitrogênio na parte aérea. O zinco aplicado nas sementes aumentou as massas secas do colmo e total das plantas de milho, na ausência de *Bacillus subtilis*. A inoculação com *Azospirillum brasilense* e *Bacillus subtilis* aumentou o diâmetro basal do colmo e a quantidade de nitrogênio acumulada na parte aérea quando a adubação nitrogenada não foi efetuada na semeadura. Não se observou efeito isolado nem interação dos fatores para altura e massa seca da raiz das plantas.

Palavras-chave: *Zea mays*, *Azospirillum brasilense*, *Bacillus subtilis*, RPCPs, coinoculação

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INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are a group of microorganisms with recognized potential for beneficial responses in different plant species. Their reported mechanisms of action include biological nitrogen fixation, plant hormone production, nutrient solubilization, and plant pathogen control. In this respect, PGPR have been used as an alternative and/or complementary biological input for chemical and particularly nitrogen fertilizers, in order to reduce demand for these products, minimize their environmental impacts and lower production costs (Graças et al., 2015; Souza et al., 2015).

In maize crops, the use of *Azospirillum brasilense* and *Bacillus subtilis* has contributed to reducing the amount of industrial nitrogen fertilizer used as topdressing by 50 to 80% and heightened its effect when applied only at sowing (Lima et al., 2011; Dartora et al., 2016; Garcia et al., 2017; Mumbach et al., 2017; Picazevicz et al., 2017). These rhizobacteria can enhance zinc availability to crops by solubilizing this micronutrient, which is easily adsorbed by the soil, making it unavailable to plants before root development (Goteti et al., 2013; Mumtaz et al., 2017).

Isolated positive effects have been reported for nitrogen (Andrade et al., 2014), zinc (Preetha & Stalin, 2014), *Azospirillum brasilense* (Marini et al., 2015) and *Bacillus subtilis* (Araujo, 2008) in maize. However, studies that investigate a combination of these factors in this crop are still incipient. As such, the present study aimed to assess the isolated and/or combined effect of *Azospirillum brasilense*, *Bacillus subtilis*, nitrogen and zinc application on growth and nitrogen accumulation in the 'AL Bandeirante' maize variety.

MATERIAL AND METHODS

The experiment with the 'AL Bandeirante' maize variety was carried out in a greenhouse in the experimental area of the Universidade Federal do Acre (9° 57' S, 67° 52' W and altitude of 169 m), in Rio Branco, Acre state, Brazil, from February to April 2017. A completely randomized design was used in a 2 x 2 x 2 x 2 factorial scheme, considering *Azospirillum brasilense*, *Bacillus subtilis*, zinc and nitrogen as factors, each with two levels, namely the application or not of bacteria and addition or not of nitrogen to the soil, with six repetitions, totaling 96 experimental units. Each unit consisted of a white PVC pipe, 200 mm wide and 50 cm high, with a volume of 15.7 dm³.

The soil added to the pipes was taken from the surface layer (0-20 cm) of a fallow area. Granulometric analysis indicated that the soil contained 427 g kg⁻¹ of clay, 227 g kg⁻¹ of silt and 346 g kg⁻¹ of sand. The apparent density was 1.21 kg dm⁻³. Its initial chemical attributes were: pH (CaCl₂) = 3.7; organic matter = 41.0 g dm⁻³; P = 10.0 mg dm⁻³; K = 81.9 mg dm⁻³; Ca = 1.7 cmol_c dm⁻³; Mg = 1.0 cmol_c dm⁻³; Al = 1.4 cmol_c dm⁻³; H+Al = 7.2 cmol_c dm⁻³; sum of bases = 2.9 cmol_c dm⁻³; CEC = 10.1 cmol_c dm⁻³; base saturation = 28.9%. In order to increase base saturation to 50 to 60%, considered suitable for maize cultivation (Coelho, 2006), calcined lime (ENP 100%) was added to the soil in the experimental units and allowed to rest for 90 days for reaction purposes.

Given that the experiment was conducted in pipes with a limited amount of soil, fertility was improved by adding 100 mg kg⁻¹ of P in the form of single superphosphate (18% P₂O₅) and 100 mg kg⁻¹ of K in the form of potassium chloride (58% K₂O), which were mixed into the soil seven days before sowing. Thus, the chemical attributes of the soil at the onset of the experiment were: pH (CaCl₂) = 5.1; organic matter = 39.0 g dm⁻³; P = 27.0 mg dm⁻³; K = 198.9 mg dm⁻³; Ca = 4.5 cmol_c dm⁻³; Mg = 0.9 cmol_c dm⁻³; Al = 0.1 cmol_c dm⁻³; H+Al = 3.8 cmol_c dm⁻³; sum of bases = 6.0 cmol_c dm⁻³; CEC = 9.8 cmol_c dm⁻³; base saturation = 61.2%.

The sources of the rhizobacteria were commercial peat-based inoculants for *Azospirillum brasilense*, containing the AbV5 and AbV6 strains, and for *Bacillus subtilis*, a concentrated suspension consisting of the UFPEDA 764 isolate. In order to assess the quality of the biological products, the number of viable cells was estimated using serial dilution and plate count technique (Tortora et al., 2017), with selective medium for *Azospirillum brasilense* consisting of malic acid (5 g), dipotassium phosphate (0.5 g), iron sulfate (0.5 g), manganese (0.01 g), magnesium (0.2 g), sodium chloride (0.1 g), calcium (0.02 g), sodium molybdate (0.002 g), bromothymol blue (0.002 g), agar (20 g), potassium hydroxide (4 g) and distilled water (1 L). The culture medium for *Bacillus subtilis* was composed of peptone (5 g), beef extract (3 g), agar (15 g) and distilled water (1 L). The estimated number of viable cells for *Azospirillum brasilense* was 1.5 x 10⁸ per g of inoculant and 6.7 x 10⁸ per mL of inoculant for *Bacillus subtilis*. Urea (46% N) and zinc oxide (80% Zn) fertilizers were used as nitrogen and zinc sources, respectively.

The doses of the commercial biological products and micronutrient added per kg of seeds were 5 g for *Azospirillum brasilense*, 5 mL of *Bacillus subtilis* and 20 g of zinc. In order to ensure greater adherence to the seeds, better distribution and a more uniform and homogeneous peat inoculant and zinc oxide mixture, the seeds were moistened with a 10% sugar solution (Brandão Júnior & Hungria, 2000) at a dose of 12 mL kg⁻¹ of seeds. For treatments in which 5 mL of the *Bacillus subtilis* biological product was applied, either in isolation or combined, 7 mL of the 10% sugar solution per kg of seeds was added to standardize the amount of liquid included. After inoculant and zinc oxide application, five equidistant seeds were sown in each experimental unit at a standard depth of 3 cm. Nitrogen was mixed into the soil at sowing, at a depth of approximately 3 cm and dose equivalent to 30 kg ha⁻¹.

Thinning was performed at phenological growth stage V3, leaving only the most vigorous plant in each experimental unit. The plants were irrigated regularly to maintain a moisture content of approximately 75% of field capacity. Temperature and relative humidity during the experiment were monitored by a datalogger installed inside the greenhouse, with average values of 29 °C and 79%, respectively.

Assessments were carried out when at least 50% of the plants were in the tasseling stage (VT), with the bottom-most branch of the tassel completely visible. The following growth-related variables were evaluated: plant height, stem diameter, leaf, tassel, stem, root and total dry weight. Plant height was measured from the ground to the tip of tassel, using a tape

measure, and stem diameter with a pachymeter, at the base of the plant. Leaf, stem, tassel and root dry weight were determined by separating the different plant parts and drying them in an oven at 65 °C until constant weight. Shoot dry weight was calculated by adding the values obtained for leaf, stem, and tassel phytomass, and total dry weight by adding the shoot and root dry weight values. Shoot nitrogen content was quantified using the semi-micro Kjeldahl method described by Tedesco et al. (1995).

The results were initially analyzed to determine the presence of discrepant data (Grubbs, 1969), error normality (Shapiro & Wilk, 1965) and homogeneity of variance (Bartlett, 1937). The data were submitted to analysis of variance using the F-test. In the event of significant interactions ($p \leq 0.05$) between two or more factors, these were unfolded to evaluate the effect of one factor for different levels of the others. Analysis of variance was carried out using the Sisvar statistical software (Ferreira, 2011).

RESULTS AND DISCUSSION

The rhizobacteria promoted different responses in the dry weight of maize plants when combined with zinc. *Azospirillum brasilense* increased leaf dry weight in the presence of this element and no significant effect was observed when only the microorganism or micronutrient were applied to the seeds (Table 1). Santini et al. (2018) reported an increase in the zinc demand of maize plants stimulated by inoculation with *Azospirillum brasilense*, indicating that the micronutrient had a greater effect in the presence of these rhizobacteria. By contrast, *Bacillus subtilis* reduced stem dry weight but did not affect total dry weight when associated or not with zinc (Table 2).

The increase in leaf dry weight (Table 1) with combined application of zinc and *Azospirillum brasilense* to the maize seeds may be related to the effect of the bacteria in enhancing the plant's capacity to use the zinc made available through fertilization. Root system modifications (lengthening, lateral root and root hair formation) are associated with the efficiency of *Azospirillum brasilense* in improving water and nutrient use

Table 1. Leaf dry weight (g) of 'AL Bandeirante' maize plants as a function of interaction between *Azospirillum brasilense* and zinc

<i>Azospirillum brasilense</i>	Zinc		CV (%)
	Absence	Presence	
Absence	24.44 Aa	24.15 Ab	11.89
Presence	23.94 Ba	26.30 Aa	

Means followed by the same lowercase letters in the columns and uppercase in the rows do not differ ($p > 0.05$) according to the F-test

Table 2. Stem (SDW) and total dry weight (TDW) of 'AL Bandeirante' maize plants as a function of the interaction between *Bacillus subtilis* and zinc

Variable	<i>Bacillus subtilis</i>	Zinc		CV (%)
		Absence	Presence	
SDW (g)	Absence	35.77 Aa	38.22 Aa	11.66
	Presence	37.19 Aa	34.90 Ab	
TDW (g)	Absence	82.11 Ba	87.48 Aa	10.90
	Presence	85.29 Aa	82.30 Aa	

Means followed by the same lowercase letters in the columns and uppercase in the rows do not differ ($p > 0.05$) according to the F-test

in plants by increasing the root surface area and, consequently, root soil contact. (Bashan & Bashan, 2010). Additionally, rhizosphere acidification due to organic acid synthesis by this microorganism species (Carrillo et al., 2002; Richardson et al., 2009) contribute to lowering the pH of the surrounding soil, which may have influenced the bioavailability of zinc to plants. Although the soil used exhibited a pH of 5.1 considered ideal in terms of zinc availability and is therefore not a limiting factor in the availability of this micronutrient to plants, the presence of *Azospirillum brasilense* may have improved the use of zinc applied to the seeds, since the same increase in leaf dry weight was not observed in the absence of this rhizobacteria.

According to Goteti et al. (2013) and Mumtaz et al. (2017), bacteria from the genus *Bacillus* act as zinc solubilizers via the synthesis of chelating agents and organic acids and contribute, in general, to improving zinc availability to plants. However, in the present study, zinc application increased the total dry weight of maize plants in the absence of *Bacillus subtilis* (Table 2). Thus, the rhizobacteria may have improved solubilization of the micronutrient supplied via fertilization and that already present in soil organic matter, resulting in an antagonistic response to plant growth, as observed in the reduction of stem dry weight (Table 2). In this case, the bacteria may have supplied the plants with sufficient zinc to make additional application of the micronutrient at sowing unnecessary.

The responses to *Bacillus* use to improve the bioavailability of insoluble zinc sources such as ZnO to plants are similar to those observed by Hussain et al. (2015), who demonstrated the zinc-solubilizing potential of these bacteria, but differ in terms of the positive effects on growth-related variable in maize (shoot and root length and dry weight). However, the present study demonstrated the importance of zinc to plant growth since its application increased leaf dry weight in the presence of *Azospirillum brasilense* (Table 1) and total dry weight in the absence of *Bacillus subtilis* (Table 2). Similar results were obtained by Prado et al. (2007) for initial growth of maize when zinc (ZnO) was applied to seeds.

In regard to the effect of nitrogen-zinc interaction on plant growth (Table 3), total dry weight rose only when zinc was applied to the seeds or nitrogen fertilizer added to the soil, with both elements promoting similar improvements in this variable. However, nitrogen only increased shoot dry weight and shoot nitrogen content in the absence of zinc.

Combined nitrogen and zinc application at sowing typically produces positive effects in maize plants since both are essential to growth in this species. However, this was not observed

Table 3. Shoot (SHDW) and total dry weight (TDW) and shoot nitrogen content (SNC) of 'AL Bandeirante' maize plants as a function of the interaction between zinc and nitrogen

Variable	Nitrogen	Zinc		CV (%)
		Absence	Presence	
SHDW (g)	Absence	69.57 Ab	73.71 Aa	11.07
	Presence	75.19 Aa	71.90 Aa	
TDW (g)	Absence	80.36 Bb	86.19 Aa	10.90
	Presence	87.05 Aa	83.58 Aa	
SNC (mg)	Absence	1135.91 Ab	1236.61 Aa	2.08
	Presence	1279.46 Aa	1163.79 Ba	

Means followed by the same lowercase letters in the columns and uppercase in the rows do not differ ($p > 0.05$) according to the F-test

in the present study (Table 3). The combination of the soil characteristics, sources and doses of the elements used, and their respective application methods were likely insufficient to produce the same promising results obtained in other studies (Asif et al., 2013; Iqbal et al., 2016).

The effects on stem diameter and shoot nitrogen content were the result of interaction between *Azospirillum brasilense*, *Bacillus subtilis* and the nitrogen fertilizer (Table 4). Positive responses were observed in plants whose seeds were inoculated with both rhizobacteria in the absence of nitrogen fertilization. Due to the effect promoted by *Azospirillum brasilense* and *Bacillus subtilis*, stem diameter increased from 9.0 to 13.8% and shoot nitrogen content from 13.1 to 27.4%. Thus, the increases observed indicate synergy between the two microorganism species in promoting improvements in these variables, demonstrating that, in this case, combined inoculation with *Azospirillum brasilense* and *Bacillus subtilis* in maize reduced the need for nitrogen fertilization at sowing (Table 4).

The fact that the highest nitrogen content was recorded in plants whose seeds were inoculated with both *Azospirillum brasilense* and *Bacillus subtilis* without added N demonstrates that the combined effect of these microorganism species was greater than their individual use in terms of increasing the availability of biologically fixed nitrogen and/or improving the use of nitrogen available in the soil. This is because, as reported by Graças et al. (2015) and Souza et al. (2015), these PGPR have a diazotrophic mechanism of action and solubilize the nutrients present in soil organic matter. On the other hand, inoculation of only one of the bacterial species without applying nitrogen fertilizer resulted in no differences in nitrogen content. Mazzuchelli et al. (2014) also reported a synergistic effect of simultaneous *Azospirillum brasilense* and *Bacillus subtilis* application on stem diameter in the absence of nitrogen fertilization. This demonstrates that inoculation with these rhizobacteria may provide enough nitrogen to plants to ensure the improvements obtained without the need to incorporate additional sources of nitrogen to the soil.

The increase in nitrogen content in maize caused by *Bacillus subtilis* inoculation combined with nitrogen fertilization in the absence of *Azospirillum brasilense* indicates that the former rhizobacteria was efficient in improving the plants' ability to use the nitrogen added to the soil (Table 4). Due to the root colonization potential of this bacterial species,

Table 4. Stem diameter (SD) and shoot nitrogen content (SNC) of 'AL Bandeirante' maize plants as a function of the interaction between *Azospirillum brasilense*, *Bacillus subtilis* and nitrogen

Variable	Rhizobacteria		Nitrogen		CV (%)
	<i>Azospirillum brasilense</i>	<i>Bacillus subtilis</i>	Absence	Presence	
SD (mm)	Absence	Absence	18.18 AaA	17.67 AaA	819
	Absence	Presence	16.78 AbB	17.72 AaA	
	Presence	Absence	17.22 AbA	18.10 AaA	
	Presence	Presence	19.09 AaA	17.51 BbA	
SNC (mg)	Absence	Absence	1157.29 AaA	1183.06 AaA	2.08
	Absence	Presence	1055.45 Bab	1215.22 AaA	
	Presence	Absence	1188.05 AbA	1289.21 AaA	
	Presence	Presence	1344.24 AaA	1199.02 AaA	

Means followed by the same lowercase letters in the columns, uppercase in the rows, underlined uppercase in the columns for the first and third means and underlined lowercase in the columns for the second and fourth means, do not differ ($p > 0.05$) according to the F-test

the synthesis and concentration of substances resulting from their metabolism in the rhizosphere, such as plant hormones responsible for root growth and development, may have contributed to optimizing the absorption and use of nitrogen fertilizer. Lima et al. (2011) also found a higher plant nitrogen content when *Bacillus subtilis* was combined with the 120 and 160 kg ha⁻¹ of N, although these doses were higher than that used in this study (30 kg ha⁻¹). The authors attributed this result to the accumulation of cytokinin produced by *Bacillus subtilis* in the rhizosphere.

No isolated and/or combined effect on plant height and root dry weight was observed for the factors evaluated. As such, for these variables, neither the rhizobacteria (*Azospirillum brasilense* and *Bacillus subtilis*) nor the chemical fertilizers (zinc oxide and urea) interfered sufficiently to cause significant differences.

Considering the effects of *Azospirillum brasilense*, *Bacillus subtilis*, zinc and nitrogen on the 'AL Bandeirante' maize plants, these biological and chemical sources are recommended as a strategy to obtain vigorous and potentially more productive plants at lower economic and operating costs. Furthermore, the wide variety of maize varieties and cultivars, as well as the different physical, chemical, and biological characteristics of soil, mean that these practices could probably provide more significant and relevant contributions under conditions different from those used in this experiment.

CONCLUSIONS

1. Simultaneous application of *Azospirillum brasilense* and zinc to seeds increases leaf dry weight in 'AL Bandeirante' maize.
2. Zinc application to seeds increases total dry weight in 'AL Bandeirante' maize when not combined with *Bacillus subtilis*.
3. Nitrogen fertilization of the soil in the absence of zinc application to seeds improves shoot and total dry weight as well as shoot nitrogen content in 'AL Bandeirante' maize.
4. Seed inoculation with *Azospirillum brasilense* and *Bacillus subtilis* in the absence of nitrogen fertilization increases stem diameter and nitrogen content in 'AL Bandeirante' maize.
5. Combined application of *Bacillus subtilis* to seeds and nitrogen fertilization of the soil in the absence of *Azospirillum brasilense* enhances nitrogen accumulation in 'AL Bandeirante' maize plants.

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