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Full Length Article

Sources and Doses of Nitrogen Associated with Inoculation with *Azospirillum brasilense* Modulate Growth and Gas Exchange of Corn in the Brazilian Amazon

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Abstract

The specific objective of the study was to evaluate effect of inoculation with *Azospirillum brasilense* and nitrogen (N) doses on vegetative growth and gas exchange in *Zea mays* L. The experimental design adopted was the completely randomized, in a 4 2 2 factorial scheme, in the following way: four doses of N (0 60 120 and 180 kg ha^{-1} of N), two sources of N (common urea and urease inhibitor-treated urea) and absence and presence of inoculation with *A. brasilense*, with four replications. The evaluations were made for vegetative growth of the plant (plant height, stem diameter, leaf area, number of leaves, dry mass of stem, root, leaves and aerial part and total dry mass) and photosynthesis, stomatal conductance, transpiration, internal carbon, relationship between internal and external carbon and content of chloroplast pigments. The application of N provided an improvement in plant growth, and, in general, the dose of 180 kg ha^{-1} N associated with *A. brasilense*, promoted an increase in stem diameter, photosynthesis, stomatal conductance, transpiration and internal carbon ratio of the corn. The treatment with urease inhibitor, greatly promoted the stem diameter, transpiration, *Ci/Ca* ratio and chlorophyll (Chl) a, b, total compared to urea treatment. The inoculation of the corn seeds with the bacteria and the use of N fertilization, regardless of the source, promoted an improvement in the vegetative growth of the hybrid, improving the vegetative growth and the physiological responses of corn when applied to the highest dose of 180 kg/ha N. © 2021 Friends Science Publishers

Keywords: Growth promoting bacteria; Nitrogen fertilization; Photosynthesis; Urease inhibitor

Introduction

Corn (*Zea mays* L.) is a cereal crop that belongs to the Poaceae family and to the genus *Zea*. It has a great economic importance on the world as its grains are used in animal and or human diets, and also as thickeners, adhesive and in the production of oils. Brazil is the world’s third biggest corn producer, after the USA and China, and the second largest exporter of Coêlho (2018). However, to achieve high yields, high doses of nitrogen (N) are necessary since the soil does not have an adequate supply to satisfy the needs of the crop (Galindo *et al.* 2016), making the N fertilization one of the most expensive input in the production process (Souza *et al.* 2019).

The indiscriminate use of mineral fertilizer may affect negatively soil fertility, and cause problems to the environment, such as soil acidification, environmental pollution and reduction of microbial activity; thus, plant-growth promoting bacteria appear as an alternative to reduce production costs and promote agricultural sustainability (Vijayalakshmi *et al.* 2019). Because N is fundamental in the metabolism by actively participating in amino acids, proteins, nucleic acids, amides and coenzymes (Munareto *et al.* 2018). It is the nutrient absorbed in greater quantity by corn and the one that most limits production, exercising functions in the essential components of the plant cell, involved in the increment of grain productivity. However, the Brazilian soils present, in their majority, low content of available N, making N fertilization an indispensable practice (Dartora *et al.* 2013). In the Brazilian Amazon region, farmers have adapted the recommendations for fertilizing corn grown in the south of the country, since research data for the crop is scarce, so the doses of fertilizers may have been overestimated and or underestimated.

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There is a need for studies that intensify the efficiency improvement in the use of N, aiming at a more sustainable production. An alternative is the use of diazotrophic bacteria of the genus *Azospirillum* due to their ability to reduce the use of chemical fertilizers, therefore improving the activities of soil microorganisms and enhancing the growth of the root system of plants (Vogel and Fey 2016). When associated with the rhizosphere, the bacteria provide biological nitrogen fixation by breaking down the N_2 molecule available in the atmosphere, making it assimilable to plants in the form of ammonia (Galindo *et al.* 2019), as well as it enhances the absorption of phosphorus, N and micronutrients, and promotes the production of auxins, cytokinins, gibberellins and ethylene (Marngar and Dawson 2017).

Despite the benefits of inoculation with *Azospirillum*, the bacteria cannot supply the N amount required for corn; therefore, the crop needs to be supplemented with N fertilizers, mainly in the form of urea (IFA 2019). The advantage of usinf urea as high N source is that it has, high solubility, ease of mixing with other sources and lower cost of N. However, it presents high losses due to ammonia volatilization (NH_3), particularly in countries with tropical regions such as in Brazil, where there is a prevalence of high temperatures (Frazão *et al.* 2014).

One of the alternatives to minimize N losses is the treatment of urea with a substance that inhibits the activity of the enzyme urease, the so-called N-(nbutil) triamide thiophosphate (NBPT). The addition of the inhibitor to urea reduces the NH_3 volatilization by around 60%, increasing the efficiency of use of N and the productivity of the crop (Cantarella *et al.* 2008). However, there is a lack of studies that define the necessary dose of N in combination with *Azospirillum* spp. to obtain maximum corn yield, so it is essential to determine the potential of using the bacteria in combination with NBPT in corn, therefore, evaluating the efficiency of the use of N in crop productivity (Galindo *et al.* 2019). Thus, the hypothesis of this work is that the inoculation with *A. brasilense* combined with the source and dose of N, improves the vegetative growth processes and the physiological behavior of corn hybrid. The specific objective of the study was to evaluate effect of inoculation with *A. brasilense* and N doses with common urea and urease inhibitor on growth and gas exchange in corn in the Brazilian Amazon.

Materials and Methods

Area characterization and soil analysis

The experiment was carried out in a greenhouse located at the Institute of Agricultural Sciences of the Federal Rural University of the Amazon, Belém, Brazil. Its geographic coordinates are 48°26'18.0" West longitude of Greenwich and 1°27'17.3" South latitude. According to the Köppen (1918), the climate is Afi-type, with an average rainfall of at least 60 mm. The Sandy-loam textured soil used in the experiment was collected at a depth of 0 to 20 cm, and classified as a dystrophic Yellow Latosol (Embrapa 2018). Soil, samples were taken for physical and chemical analysis of the soil at the Brazilian Institute of Analysis (IBRA), according to the methodology described by (Silva 1999). The soil analysis demonstrated the need to correct only the potassium content with the application of 60 kg ha^{-1} of potassium chloride (Table 1), following the recommendation of De Oliveira *et al* (2018). Climatic data from the experimental area were collected during the conduct of the experiment (Fig. 1).

Experimental design

The experimental design adopted was a completely randomized block, in a 4 × 2 × 2 factorial scheme, composed of four doses of N (0; 60; 120 and 180 kg ha^{-1} of N) following the recommendation of (Ritchie *et al.* 1993), two sources of N, common urea (with 45% N) and urease inhibitor-treated urea - NBPT (with 45% N) and presence and absence of *A. brasilense*, with four replications.

Source and sowing of seeds

The seed used in the experiment was the K9960 VIP3 *Zea mays* hybrid (classified as high productive potential, excellent stem health, high planting adaptability for tropical/ subtropical regions and early cycle), commonly adopted in the southeastern region of the state of Pará. It was donated by the company Juparanã. Early after collection, the soil was sieved in a 2-mm sieve, then homogenized in 256 kg of organic material from mango pruning residues for 768 kg of soil. The 25 × 32 cm (490.8 cm²) pots were filled with 16 kg of substrate, prepared by mixing the soil with organic matter in a 3:1 ratio, respectively.

Treatment application

For the treatments inoculation purpose, the seeds were homogenized together with the inoculant (200 mL diluted in water equivalent to 10% of the weight of the seeds, strains Ab-V5 and Ab-V6 in the concentration 2×10^8 CFU mL^{-1}), inoculated one hour before sowing (Leite *et al.* 2019). N doses with common urea and urea inhibitor were applied in topdressing once, performed at 10 DAE (days after plant emergence) when the plant started to develop its secondary root system following the recommendation of (Jadoski *et al.* 2016). The buckets received controlled daily irrigation to replace the water lost through evapotranspiration over the experimental period, and the soil water content was maintained closer to the field capacity, using the gravimetric method (Catuchi *et al.* 2011), which consists of replacing the irrigation depth based on daily weighing of the buckets. The control of weeds and pests was carried out daily and manually through mechanical plucking and manual picking, respectively.

Plant phenology and measurement

The evaluations were carried out on the full male flowering (V_T phenological stage), that is, at plant tasseling (Ritchie *et al.* 1993). At 50 days after germination, (period that stabilized the growth of the aerial part), the variables of vegetative growth and gas exchange were measured.

The measurements related to plant height (PH) and stem diameter (SD), were performed with the aid of a ruler and digital caliper, respectively; then, the plants were sectioned into different parts (leaves, root, stem + sheath), packed in 5-kg paper bags, identified and taken to the forced air circulation oven at 65 ± 2ºC for 72 h. Upon reaching constant weight, each part of the plant was weighed on an analytical scale to determine the dry mass of the root (RDM), dry mass of the leaf (LDM), dry mass of the stem (SDM), dry mass of the aerial part (APDM) (calculated as the sum of the dry mass of stem and leaf) and total dry mass (TDM). The number of leaves (LN) was obtained through manual counting, considering all the completely expanded leaves. For determination of the leaf area (LA), length (L) and width (W) were measured in the median part of all leaves of each of the plants, to obtain the initial LA according to (Sangoi *et al.* 2007). Using the proposal of Sangoi *et al.* (2007) the calculation of the leaf area was obtained using the following equation: LA (m^2) = 0.75 × Width × length of the leaf. Afterwards, the individual values of all the leaves were summed to obtain the value of total leaf area per plant.

Gas Exchange evaluation

Gas exchanges were determined on the second leaf, from the base (lower), and on the first fully mature leaf, from the apex (upper), on a day with clear sky (no clouds), representing the daytime period in which photosynthesis reaches maximum values, according to what was determined from the daytime curves of leaf exchanges, that is, between 08 h at 11 h. The net assimilation rate of CO_2 (*A*), stomatal conductance to water vapor (*gs*), intercellular CO_2 concentration (*Ci*) and leaf transpiration rate (*E*), were measured in the range from 08 h to 10 h 30 min in the maximum photosynthesis of the daytime curve, using a portable gas exchange model open flow system (LI-6400-02B, LI-COR Inc., Lincoln, NE, USA), under an external CO_2 concentration of 400 μmol mol^{-1} of air and under a flow of photosynthetically active radiation of 900 μmol m^{-2} s^{-1} of photons. Subsequently, the internal and external carbon (*Ci/Ca*) ratio was calculated.

Photosynthetic pigment determination

First, 100 mg of fresh leaf from each sample was weighed, then, placed in a mortar, containing 3 mL of 80% acetone, followed by maceration and filtering with paper towels. The supernatant was transferred to a volumetric flask, measuring the volume to 25 mL. After, the samples were read on a spectrophotometer at 663 nm (Chl a), 647 nm (Chl b) and 470 nm (carotenoids) and as white, only 80% acetone was used, with final concentrations of chlorophylls and carotenoids calculated according to the methodology recommended by Sims and Gamon (2002).

Table 1: Physico-chemical soil characteristics at 0-20 cm depth. The soil was sampled prior to corn sowing

Characteristics	Values	Unit
Total N	4.96	Mg ha^{-1}
pH	5.40	-
Organic matter	36.00	g dm^{-3}
Organic carbon	21.00	g dm^{-3}
Phosphate-P	0.13	g dm^{-3}
K^+	1.50	$mmol_c$ dm^{-3}
Ca^{2+}	48.00	$mmol_c$ dm^{-3}
Mg^{2+}	11.00	$mmol_c$ dm^{-3}
H+Al	29.00	$mmol_c$ dm^{-3}
Sulfur + Boron	124.00	$mmol_c$ dm^{-3}
Cation exchange capacity	153.00	$mmol_c$ dm^{-3}
Saturation of bases	68.00	%
Cu^{2+}	1.60	mg dm^{-3}
Fe^{3+}	37.00	mg dm^{-3}
Mn^{2+}	36.30	mg dm^{-3}
Zn^{2+}	52.00	mg dm^{-3}
Boron	0.59	mg dm^{-3}
Sulfur	16.00	mg dm^{-3}
Silt	99.00	g kg^{-1}
Clay	136.00	g kg^{-1}
Sand	765.00	g kg^{-1}

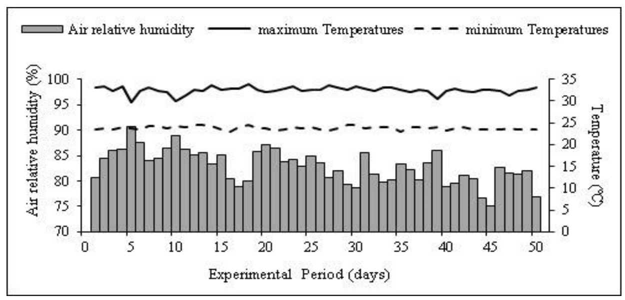

Fig. 1: Air relative humidity and maximum and minimum temperatures of the experimental area over the experimental period in 2019, in Belém, Pará, Brazil

Statistical analysis

The results of the analysis of growth and gas exchange were submitted to the tests of Shapiro-Wilks and Levene to verify the normality and homoscedasticity of the data, respectively. After meeting the basic assumptions, the analysis of variance was carried out, in which the unfolding was carried out, proving to be significant To assess the effect of different doses of N, fertilizers and inoculation on corn hybrid, analysis of variance was performed, with Tukey test at 5% probability, and adjusted for polynomial regression to differentiate whether there was a linear or nonlinear response to the N rates applied, using the Sisvar statistical software program (Ferreira 2019).

Table 2: Summary of analysis of variance, applied on growth characteristics of corn in accordance with the source (S), *A. brasilense* inoculation (I), and nitrogen doses (D)

Cause of variation	DF	Mean squares								
		SD	PH	LA	LN	LDM	APDM	TDM	SDM	RDM
Source (S)	1	0.070^{ns}	0.023^{ns}	0.001^{ns}	0.350^{ns}	0.003^{ns}	0.001^{ns}	0.001^{ns}	0.003^{ns}	0.030^{ns}
Inoculation (I)	1	0.665^{ns}	0.023^{ns}	0.001^{ns}	0.450^{ns}	0.009^{ns}	0.001^{ns}	0.001^{ns}	0.001^{ns}	0.009^{ns}
N dose (D)	3	6.672*	0.274*	0.001*	32.355*	0.155*	0.055*	0.036*	0.542*	0.189*
S × I	1	0.006^{ns}	0.002^{ns}	0.001^{ns}	0.056^{ns}	0.001^{ns}	0.001^{ns}	0.001^{ns}	0.001^{ns}	0.005^{ns}
S × D	3	0.255^{ns}	0.014^{ns}	0.001^{ns}	1.163^{ns}	0.002^{ns}	0.001^{ns}	0.001^{ns}	0.010^{ns}	0.005^{ns}
I × D	3	0.274^{ns}	0.027^{ns}	0.001^{ns}	4.916*	0.017*	0.004*	0.003*	0.028^{ns}	0.022^{ns}
S × I x D	3	0.605*	0.016^{ns}	0.001^{ns}	2.326^{ns}	0.007^{ns}	0.002^{ns}	0.001^{ns}	0.013^{ns}	0.009^{ns}
Blocks	3	0.504*	0.017^{ns}	0.001^{ns}	1.098^{ns}	0.009^{ns}	0.003^{ns}	0.002^{ns}	0.042*	0.008^{ns}
Residue	45	0.175	0.014	0.001	1.822	0.003	0.001	0.001	0.014	0.009
CV (%)	-	5.0	2.5	0.5	1.7	3.0	1.8	1.5	4.7	5.7

ns: not significant, *: significant at 5% probability by the F test, CV: coefficient of variation

Table 3: Summary of analysis of variance for gas exchange and pigments contents of corn leaves in accordance with the source (S), the inoculation of *A. brasilense* (I), and the nitrogen doses (D)

Cause of variation	DF	Mean squares								
		A	*Ci*	*gs*	*E*	*Ci/Ca*	Chl a	Chl b	Chl total	Carotenoids
Source (S)	1	0.003^{ns}	4544.4^{ns}	0.47^{ns}	0.081^{ns}	0.003*	17819.00*	6728.60*	29.69*	663.94^{ns}
Inoculation (I)	1	0.202*	402.84^{ns}	10.72*	6.93*	0.001^{ns}	610.19^{ns}	14.32^{ns}	2.06^{ns}	1012.90^{ns}
N dose (D)	3	0.244*	2439.7^{ns}	10.943*	1.01^{ns}	0.002*	9045.40*	6970.60*	21.48*	10762.00*
S × I	1	0.006^{ns}	6807.1^{ns}	0.57^{ns}	7.37*	0.001^{ns}	17251.00*	11682.00*	1.04^{ns}	420.51^{ns}
S × D	3	0.022^{ns}	385.01^{ns}	2.05^{ns}	6.65*	0.001^{ns}	9539.200*	7078.90*	30.91*	10985.00*
I × D	3	0.020^{ns}	1222.6^{ns}	1.47^{ns}	0.31^{ns}	0.001^{ns}	2240.400^{ns}	3701.400*	10.69^{ns}	2331.500*
S × I x D	3	0.015^{ns}	3107.5^{ns}	0.68^{ns}	0.83^{ns}	0.001^{ns}	8301.100^{ns}	3161.500*	22.03*	462.01^{ns}
Blocks	3	0.101*	74001.0*	3.56^{ns}	14.47*	0.006*	1666.400^{ns}	590.57^{ns}	11.52^{ns}	322.18^{ns}
Residue	45	0.024	3313.1	2.24	1.60	0.001	2973.4	1013.8	7.01	621.89
CV (%)	-	5.5	20.9	18.8	8.9	6.9	2.2	1.2	1.9	1.0

ns: not significant, *: significant at 5% probability by the F test, CV: coefficient of variation

Results

The results of the analysis of variance (Tables 2 and 3) showed a significant effect by means of the F test (P <0.05) of N sources, inoculation with *A. brasilense* and N doses in the growth and the evaluated physiological parameters, except the internal concentration of carbon. The significant effect on the triple interaction (Source × Inoculation × Doses) was observed for stem diameter, Chl b and total Chl, indicating the dependence on the factors evaluated in the experiment.

Plant growth and biomass yield

A significant effect was found for the SD in the triple interaction (Source × Inoculation × Dose) (Table 2). The SD in the corn plants with application of urea without inoculation resulted in a quadratic behavior for the N doses, with the highest value of 17.22 mm obtained in the dose of 149.75 kg ha^{-1} N, while the urea with *A. brasilense* and urease-inhibitor in the absence and presence of the bacteria treatments obtained adjustment for the increasing linear equation, in which the maximum dose of 180 kg ha^{-1} N promoted the maximum values of 17.26, 17.05 and 17.44 mm in diameter, respectively. The inoculation of corn with the bacteria promoted a higher increase in stem diameter than that found in plants without inoculation (Table 4).

The plant height (AP) and leaf area (AF) had a significant effect on the Dose factor, with the best fit for the quadratic equation (Table 2). The increase in N doses positively influenced the phytotechnical values of the crop. It was observed in this study that the doses of 152.11 and 157.75 kg ha^{-1} N were those that provided the highest PH (122.50 cm) and LA (43.61 dm²), respectively (Table 5). For the variable number of leaves (NL), it was observed a significant effect on the Inoculation × Doses interaction, adjusting to the quadratic regression model (Table 2). From the information in (Table 6), it was found that the number of leaves for plants without inoculation reached a maximum value of 14.22 plant^{-1} in the estimated dose of 180 kg ha^{-1} N, while for the inoculated plants, the number of leaves reached a value of 13.72 plant^{-1} at the dose of 131.25 kg ha^{-1} N.

The attributes including LDM, APDM and TDM were significant for the Inoculation × Dose interaction (Table 2). The treatments with urea in the absence and presence of *A. brasilense* resulted in the best fit to the quadratic regression model, in which the LDM variable obtained a value of 27.03 g plant^{-1} at a dose of 182.37 kg ha^{-1} N and 25.57 g plant^{-1} at a dose of 148.37 kg ha^{-1} of N, respectively (Table 6).

For the APDM, maximum values of 73.56 and 68.89 g plant^{-1} were found at doses of 177.73 and 158.41 kg ha^{-1} of N with the source urea in the absence and presence of *Azospirillum*, respectively. While, the highest TDM values of 89.08 and 83.12 g plant^{-1} were obtained at doses of

Table 4: Unfolding of the Source × Inoculation × Dose interaction with regression equation and estimate of maximum technical efficiency, applied to the content of chlorophyll *b* and total chlorophyll, and SD in corn hybrid without and with inoculation with *A. brasilense*

Variable	N source	N dose (kg ha^{-1})				Equation	R²	Ymet	Nmet
		0	60	120	180				
Chl b	Urea without	0.0014	0.0013	0.0018	0.0006	$Y= -0.00000008x^2 + 0.00001x +0.0013$	0.60	0.0016	65.16
(mmol kg^{-1} MF)	Urea with	0.0014	0.0007	0.0006	0.0009	$y = 0.00000007x^2 – 0.00001x + 0.0013$	0.99	0.0009	71.42
	Inhibitor without	0.0008	0.0011	0.0020	0.0008	$y = -0.0000001x^2 + 0.00002x + 0.0007$	0.65	0.0017	100.00
	Inhibitor with	0.0009	0.0021	0.0019	0.0012	$y = -0.0000001x^2 + 0.00002x + 0.001$	0.97	0.002	100.00
Total Chl	Urea without	0.0026	0.0030	0.0042	0.0023	$y = -0.0000002x^2 + 0.00003x + 0.0025$	0.63	0.0036	75.00
(mmol kg^{-1} MF)	Urea with	0.0038	0.0027	0.0025	0.0040	$y = 0.0000002x^2 – 0.00003x + 0.0038$	0.97	0.0026	75.00
	Inhibitor without	0.0025	0.0046	0.0040	0.0035	$y = -0.0000002x^2 + 0.00004x + 0.0026$	0.82	0.0046	100.00
	Inhibitor with	0.0024	0.0048	0.0043	0.0032	$y = -0.0000002x^2 + 0.00005x + 0.0025$	0.92	0.0056	125.00
SD	Urea without	128.65	152.32	173.97	168.60	$y = -0.0002x^2 + 0.0599x + 12.74$	0.97	17.22	149.75
(mm)	Urea with	142.75	158.77	169.47	172.67	$y = 0.0167x + 14.585$	0.92	-	-
	Inhibitor without	137.30	167.97	155.25	170.57	$y = 0.0145x + 14.471$	0.54	-	-
	Inhibitor with	144.45	155.07	174.55	174.47	$y = 0.0183x + 14.571$	0.89	-	-

R² - coefficient of determination; Ymet – estimate value of the maximum technical efficiency; Nmet – nitrogen dose by the maximum technical efficiency

Table 5: Unfolding of the nitrogen dose effect, with regression equation and estimate of the maximum technical efficiency, (some growth attributes) in corn hybrid submitted to nitrogen doses

Variable	N dose (kg ha^{-1})				Equation	R²	Y_{met}	N_{met}
	0	60	120	180				
PH (cm)	93.59	107.93	124.62	120.18	$y = -0.0013x^2 + 0.3955x + 92.42$	0.95	122.5	152.11
LA (dm²)	29.02	36.90	44.16	43.86	$y = -0.0006x^2 + 0.1893x + 28.687$	0.98	43.61	157.75
SDM (g plant^{-1})	19.24	30.86	40.89	40.73	$y = -0.0008x^2 + 0.2715x + 18.818$	0.98	41.85	169.68
RDM (g plant^{-1})	8.40	11.03	13.86	14.06	$y = -0.0002x^2 + 0.0634x + 8.2601$	0.98	13.28	158.50

R² - coefficient of the determination; Ymet – estimate value of technical maximum efficiency; Nmet – nitrogen dose by the maximum technical efficienc

186.89 and 159.28 kg ha^{-1} of N with the application of urea in the absence and presence of the bacteria, respectively (Table 6). The SDM and RDM variables had a significant effect on the dose factor, with the best adjustment of the data to the quadratic equation, in which the doses of 169.68 and 158.50 kg ha^{-1} N provided the highest values of 41.85 and 13.28 g plant^{-1}, respectively (Table 5).

Gas exchange characteristics

The analysis of variance identified a significant effect ($p <0.05$) of the isolated factors Inoculation and Dose for the net assimilation rate of photosynthesis (A), with adjustment to the increasing linear regression model (Table 3). The application of N in topdressing in the absence of the bacteria promoted values that varied from 13.07 to 18.23 µmol m^{-2} s^{-1}, whereas in corn inoculated with *A. brasilense* the values ranged from 16.00 to 19.85 µmol m^{-2} s^{-1} at doses zero to 180 kg ha^{-1} of N, respectively (Table 6), verifying that the plants in the presence of *Azospirillum* showed greater photosynthetic activity.

***gs*:** This attribute had a significant effect on the isolated factors Inoculation and Dose (Table 3), adjusting to the linear regression model, in which the increase in the doses promoted an improvement in conductance, ranging from 0.126 to 0.156 µmol m^{-2} s^{-1} in corn plants with no inoculation and 0.142 to 0.158 µmol m^{-2} s^{-1} in plants with the presence of *Azospirillum* at the dose of 0 and 180 kg ha^{-1} N, respectively (Table 6).

***E*:** The experimental data on *E* were submitted to analysis of variance, where an effect was observed on the Source × Inoculation and Source × Dose interactions (Table 3). Through the unfolding of the Source x Inoculation interaction, it was verified that the urea with inoculation presented a mean of 3.48 mmol m^{-2} s^{-1}, followed by the urease-inhibitor in the absence (3.27 mmol m^{-2} s^{-1}) and presence (3.23 mmol m^{-2} s^{-1}) of the bacteria Azospirillum, while urea without inoculation resulted in a value of 2.85 mmol m^{-2} s^{-1} of *E* (Table 7). For the Source × Dose interaction, the results did not fit any mathematical model, with a mean of 2.97 and 3.20 mmol m^{-2} s^{-1} for corn plant with urea and 3.09 and 3.62 mmol m^{-2} s^{-1} with urea inhibitor at a dose of 0 and 180 kg ha^{-1} N, respectively (Table 8).

Internal and external carbon (Ci/Ca) ratio: In the internal and external carbon (Ci/Ca) ratio, the analysis of variance had a significant effect on the isolated factors Source and N Dose (Table 3). When fertilized with urea, it was found that the results did not fit any mathematical model, showing a mean of 0.471 µmol mol^{-1}, while for the urea inhibitor, there was a quadratic effect, where the lowest value for this characteristic was 0.47 µmol mol^{-1} at an estimated dose of 85.71 kg ha^{-1} N, followed by a growth up to the maximum dose (Table 8).

Photosynthetic pigments

According to the analysis of variance for the content of Chl a, we found significant Source × Inoculation and Source × Dose interactions (Table 3). The Source x Inoculation interaction showed that the urea treatments with *Azospirillum* and urease-inhibitor in the absence and presence of the bacterium were statistically similar, differing

Table 6: Unfolding of the analysis of variance with regression equation and estimation of maximum growth attributes, gas exchange and carotenoids in corn hybrid grown in the absence and presence of *A. brasilense* and the nitrogen dose

Variable	*Azospirillum*	Dose (kg ha^{-1})				Equation	R^2	Ymet	Nmet
		0	60	120	180				
LN	Absence	11.75	13.50	13.25	14.25	$y = -0.00005x^2 + 0.0215x + 11.913$	0.83	14.22	180.00
	Presence	12.42	13.14	14.00	13.62	$y = -0.00008x^2 + 0.021x + 12.36$	0.93	13.73	131.25
LDM (g plant^{-1})	Absence	13.35	22.26	24.69	28.20	$y = -0.0004x^2 + 0.1459x + 13.729$	0.97	27.03	182.37
	Presence	17.35	20.81	27.52	25.73	$y = -0.0004x^2 + 0.1187x + 16.764$	0.89	25.57	148.37
APDM (g plant^{-1})	Absence	31.79	57.78	67.55	75.27	$y = -0.0013x^2 + 0.4621x + 32.499$	0.99	73.56	177.73
	Presence	40.63	51.73	72.77	66.68	$y = -0.0012x^2 + 0.3802x + 38.78$	0.89	68.89	158.41
TDM (g plant^{-1})	Absence	39.28	69.26	80.21	90.14	$y = -0.0014x^2 + 0.5233x + 40.181$	0.98	89.08	186.89
	Presence	49.93	62.32	87.83	79.95	$y = -0.0014x^2 + 0.446x + 47.606$	0.87	83.12	159.28
A (μmol. m^{-2}. s^{-1})	Absence	13.07	16.44	17.43	18.23	$y = 0.0274x + 13.829$	0.87	-	-
	Presence	16.00	17.18	20.20	19.85	$y = 0.0243x + 16.125$	0.84	-	-
gs (μmol. m^{-2}. s^{-1})	Absence	0.12	0.133	0.150	0.156	$y = 0.0002x + 0.1251$	0.95	-	-
	Presence	0.14	0.140	0.177	0.158	$y = 0.1543$	-	-	-
Carotenoids	Absence	0.001	0.002	0.001	0.001	$y = -0.00000007x^2 + 9E-06x + 0.0013$	0.99	0.001	64.28
(mmol kg^{-1} MF)	Presence	0.002	0.001	0.001	0.001	$y = -0.000005x + 0.0017$	0.98	-	-

R^2 - coefficient of determination; Ymet – estimated value of the maximum technical efficiency; Nmet – nitrogen dose by the maximum technical efficiency

Table 7: Summary of the mean analysis of the Source × Inoculation interaction, in accordance with the absence and presence of *A. brasilense* in corn seed, in transpiration (mmol m^{-2}s^{-1}) and chlorophyll *a* concentration (mmol kg^{-1} MF)

N source	*Azospirillum*	Transpiration	Chlorophyll *a*
Urea	Absence	2.85±0.55 Ab	0.0016±0.0008 Ba
	Presence	3.48±0.57 Aa	0.0021±0.0006 Aa
Inhibitor	Absence	3.27±0.70 Aa	0.0025±0.0011 Aa
	Presence	3.23±0.66 Aa	0.0021±0.0007 Aa

Columns with different capital letters between N source treatments (urea and inhibitor under the same inoculation treatment) and lower case letters between inoculation treatments (absence and presence of Azospirilum under the same N source) indicate significant differences by the Tukey test (P <0.05). Values described correspond to the average of 4 repetitions and Standard Deviation

Table 8: Unfolding of the Source × Dose interaction, with regression equation and estimate of the maximum technical efficiency, applied to the transpiration (*E*), internal and external carbon ratio (*Ci/Ca*), chlorophyll *a* (Chl *a*) and carotenoids, in hybrid seed of corn subjected to different sources of nitrogen

Variable	N source	Dose (kg ha^{-1})				Equation	R^2	Ymet	Nmet
		0	60	120	180				
E (mmol m^{-2} s^{-1})	Urea	2.97	3.48	3.003	3.20	$y = 3.1666$	-	-	-
	Inhibitor	3.09	2.84	3.36	3.62	$y = 3.2337$	-	-	-
Ci/Ca	Urea	0.47	0.44	0.48	0.488	$y = 0.4724$	-	-	-
	Inhibitor	0.54	0.42	0.54	0.53	$y = 0.000007x^2 – 0.0012x + 0.5225$	0.36	0.47	85.71
Chl a (mmol kg^{-1} MF)	Urea	0.002	0.002	0.002	0.002	$y = 0.0019$	-	-	-
	Inhibitor	0.002	0.003	0.002	0.002	$y = -0.00000008x^2 + 0.00002x + 0.0018$	0.43	0.003	125
Carotenoids (mmol kg^{-1} MF)	Urea	0.001	0.002	0.002	0.000	$y = -0.0000001x^2 + 0.00001x + 0.0014$	0.99	0.0016	50.00
	Inhibitor	0.002	0.001	0.001	0.001	$y = 0.00000005x^2 – 0.00001x + 0.0016$	0.97	0.0011	100

R^2 - coefficient of determination; Ymet – estimated value of the maximum technical efficiency; Nmet – nitrogen dose by the maximum technical efficiency

only from the urea treatment without inoculation, which achieved the values of 0.0021, 0.0025, 0.0021 and 0.0016 mmol kg^{-1} MF, respectively (Table 7). The Source × Dose interaction revealed that the application of the N source with urease-inhibitor resulted in the best fit of the quadratic equation, with the maximum value of 0.003 mmol kg^{-1} MF obtained at the dose of 125 kg ha^{-1} N. Meanwhile, the use of the urea source did not fit any mathematical model, with an average of 0.002 mmol kg^{-1} MF (Table 8).

The analysis of variance showed an interaction of Source × Inoculation × Dose (Table 3) for the variables Chl b and total Chl. Chl *b* showed the best fit to the quadratic model, in which urea fertilization with the absence and presence of bacterial inoculation reached the maximum value of 0.00161 and 0.0009 mmol kg^{-1} MF at the estimated doses of 65.16 and 71.42 kg ha^{-1} N, respectively, while the application of the source with urease inhibitor in the absence and presence of *Azospirillum* promoted the value of 0.00170 and 0.00200 mmol kg^{-1} MF in the estimated dose of 100 kg ha^{-1} N, respectively. The total Chl concentration was adjusted to the quadratic model, where it was observed that the application of urea with the absence and presence of *Azospirillum* resulted in a maximum content of 0.00363 and 0.0026 mmol kg^{-1} MF in the estimated dose of 75 kg ha^{-1} N, respectively, and the use of the urease inhibitor in the absence and presence of the bacteria obtained the maximum value of 0.00460 and 0.00563 mmol kg^{-1} MF at the doses of 100 and 125 kg ha^{-1} N, respectively (Table 4).

In the case of carotenoids content, the analysis of variance indicated significant Inoculation × Dose and Source × Dose interactions (Table 3), and the treatment related to N fertilizer with absence of *A. brasilense*, adjusted to the quadratic regression model, in which the maximum concentration of 0.001 mmol kg^{-1} MF was estimated at a dose of 64.28 kg ha^{-1} N (Table 6). However, plants grown in soil fertilized with N inoculated with the bacteria showed a decreasing linear response, where the increase in N fertilization decreases the concentration of carotenoids per plant, reaching the value of 0.002 moll kg^{-1} MF with the lowest N dose. For the Source × Dose interaction, it presented the best fit to the quadratic model, with the highest value of 0.0016 and 0.0011 mmol kg^{-1} MF obtained in the doses of 50 and 100 kg ha^{-1} N for the source's urea and urease-inhibitor, respectively (Table 8).

Discussion

Increase in growth by inoculation of corn with *A. brasilense* may be associated with the production of growth hormones such as auxins, gibberellins and indoleacetic acid, excreted by the bacteria, which besides promoting the seed germination process, stimulates the plant growth through cell elongation (Vogel and Fey 2019). Similar trends were described by Costa *et al.* (2015) and Marini *et al.* (2015), who found a larger stem diameter in corn inoculated with *Azospirillum*, in comparison to the absence of the bacteria. The increment in the N fertilization, focusing on the 180 kg ha^{-1} N dose associated with *A. brasilense* inoculation greatly increased stem diameter. The increase in stem diameter in corn is associated with the increase in production, since it allows the storage of soluble solids that will later be used during the grain formation phase (Fancelli and Dourado Neto 2000), mainly under stress condition that compromises the rate of production or translocation of photo-assimilates (Dartora *et al.* 2013).

Nitrogen fertilization at the correct dose and satisfying the nutritional requirements of the plants plays a fundamental role in the vegetative growth as well as in the production, as a result. The greater vegetative mass in plants fertilized with N and inoculated with *A. brasilense*, may be associated with the positive effect of N in some physiological processes of the plant, in addition to the greater N fixation and its stimulating action on plant growth (Kordi and Ghanbari 2019). Morais *et al.* (2015) identified that application of doses of 100 and 200 kg ha^{-1} N, promoted an increase in all vegetative attributes of corn, as justified by the importance of N for the crop, which is an essential macronutrient, responsible for amino acids, proteins, nitrogenous bases and nucleic acids biosynthesis. The results showed that the highest dose of 180 kg ha^{-1} N was responsible for the highest accumulation of total dry matter in corn not inoculated with *Azospirillum*; in contrast, plants inoculated with the bacteria obtained maximum values in lower doses of N, where the highest value of TDM of 89,08 and 83,12 g plant^{-1} were obtained in doses of 186,89 and 159,28 kg ha^{-1} de N with application of urea in the absence and presence of the bactéria, respectively. Because N is the main component of the chlorophyll molecule, amino acids and proteins, it allows the crop to grow until it reaches full maturity (Marngar and Dawson 2017), thus, the greater availability of N promotes an increase in the production of dry mass of corn (Bianchet *et al.* 2015). Early studiesinvolving inoculations between plants and *Azospirillum* reported that the benefit was essentially derived from biological N_2 fixation; however, in later studies they identified a positive effect on the morphological and physiological changes in the roots of the inoculated plants (Okon and Vanderleyden 1997; Dobbelaere *et al.* 2001). Except for the growth-promoting hormone excretion, N increased absorption of water and nutrients (Reis *et al.* 2008), resulting in a greater dry mass production and assimilation of nutrients by inoculated plants.

A higher net photosynthesis in plants with the bacterium can be attributed to the process of N fixation and the secretory function of growth-regulator hormone (Kordi and Ghanbari 2019). Saikia *et al.* (2007) and Barassi *et al.* (2008), in a study of corn plants inoculated with *Azospirillum* observed an improvement in the photosynthetic parameters of the leaves, therefore, collaborating with the results of this work. The increasing doses of N may have promoted the increase in carboxylation, due to the need for the carbonic chain for the assimilation of nitrate (Dos Anjos Soares *et al.* 2013). Moreover, considering that corn was at full growth requires higher levels of carbon and N, the increase in N availability enabled the increase in carboxylation (Jadoski *et al.* 2016). In addition, the rise in the concentration of N in the soil may have favored cell division and expansion, giving rise to a greater photosynthetically active area in the corn leaf (Marngar and Dawson 2017), since it is the N constituent of the components of the photosynthetic process, the increase results in an increase in the rate of carbon assimilation (Braz *et al.* 2019).

The improvement in stomatal conductance in corn plants inoculated with *A. brasilense* (Barassi *et al.* 2008) occurred due to a higher concentration of CO_2 in the intercellular spaces and the rate of leaf *E* (Rodrigues *et al.* 2014). According to Jadoski *et al.* (2016), stomatal conductance increased as the dose of N applied at 46 DAE increased due to the greater carboxylation and the translocation of photo-assimilates promoted by the increment in the photosynthesis. Our results revealed approximately constant values, regardless of the factors analyzed (Tables 7 and 8), which presumably occurred because of the absence of water limitation during the experimental period since the test was maintained in the field capacity, thus, the corn plants expressed the maximum transpiration demand. Bulegon *et al.* (2016), reported that the soybean crop, the plant expressed the maximum transpiration demand under adequate water conditions.

The activities of *A. brasilense* in the root system and conducting vessels allow the high activity of the hormone auxin, thus allowing a greater vegetative growth in corn plants and a greater absorption and transport of water, supporting hydration and biochemical activity in plant tissues (Hungria *et al.* 2010; Masciarelli *et al.* 2013; Filippou *et al.* 2014; Cassán and Diaz-Zorita 2016). Taiz and Zeiger (2013) showed that rubisco is an enzyme considerable found in leaves, representing about 40% of the total soluble proteins; therefore, concentrations of carbon dioxide in intercellular spaces may infer in an indication of malfunction of the enzyme. In addition, a higher rate of net CO_2 assimilation observed in this work is attributed to the increase in the concentration of CO_2 in the intercellular spaces due to the increase in the N doses which ranged from 14.53 at zero doses (control) to 19.04 μmol m^{-2} s^{-1} at the dose of 180 kg ha^{-1} of N. Rodrigues *et al*. (2014) attributes that higher rates of net CO_2 assimilation, stomatal conductance and leaf transpiration improve intercellular concentration of CO_2 in leaves. Farquhar and Sharkey (1982) reported that an increase in the concentration of CO_2 in the sub-stomatal chamber does not always provide an increase in the net CO_2 assimilation rate by the plant, which is defined as the maximum carboxylation efficiency. Jadoski *et al.* (2016) found that the increase in the amount of carbon in corn plants occurred due to the full growth and development of corn, and thus requiring higher CO_2 and N rates to compensate for the increase in carboxylation.

The increase in chlorophyll contents (Chl *a*, *b* and total) was promoted by the increment in the N metabolism, as it is the fundamental nutrient in plants, directly participating in the protein and chlorophyll biosynthesis. As a result, the application of N increases the chlorophyll concentration in corn plants (Morais *et al.* 2015). The excess of N available to the plant is harmful, observing that in the highest dose of N (180 kg ha^{-1} N) there was a reduction by 33% in Chl *a* contents compared to the dose of 125 kg ha^{-1} N, possibly due to the directing of N for the formation of dry mass of plants, causing a dilution in the concentration of the nutrient (Larrosa *et al.* 2009). In addition, the decrease in the concentration of carotenoids with the increase in the N, with the highest value of 0.0016 and 0.0011 mmol kg^{-1} MF obtained in the doses of 50 and 100 kg ha^{-1} of N for the sources urea and urease inhibitor, respectively, may have reduced the concentration of the chlorophylls, since the carotenoids in the leaves have the function of protecting the chlorophylls against degradation (Abdelgawad *et al.* 2015).

According to Dwyer *et al.* (1995), over-assimilated N accumulates in NO_3^-, and in this way, N is not associated with the chlorophyll molecule, which partly explains the decrease observed in the concentration of this photosynthetic pigment as the availability of N increases (Takebe and Yoneyama 1989). In addition, the reductions in chlorophyll concentration indicate a reduction in the photosynthetic capacity of corn, which is related to the action of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), the protein most widely distributed in the plant kingdom in leaves (Taiz and Zeiger 2013).

Conclusion

The inoculation of corn seed with *A. brasilense* and N doses promoted an increase SD, LN, LDM, APDM e TDM, in addition to improving *A*, *gs* and *E*. In general, the dose of 180 kg ha^{-1} de N promoted increases in growth and gas exchange variables. The dose of 120 kg ha^{-1} de N com *A. brasilense* favored the LN, LDM, APDM and improvement in *A* and *gs*. The application of N with the urease inhibitor resulted in the largest SD, *Ci/Ca* ratio, *E* and Chl a.

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Author Contributions

This work was carried out in collaboration with all authors. Authors JGP, RSO, GDPA and CFON elaborated the study, performed the statistical analysis, drafted the protocol and wrote the first draft of the manuscript. Authors MGN and DJPS wrote the manuscript. The authors, JSST, WRLLF and LCS analyzed the data, improving the final version of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

All authors declare no conflict of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper

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