Agronomic performance of second crop maize grown under different methods of inoculation with *Azospirillum brasilense*[1]

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10.1590/0034-737X202370060004

ABSTRACT

The objective of this work was to evaluate the agronomic performance of a second crop maize grown under different methods of inoculation with *Azospirillum brasilense*. The experiment was conducted in a randomized block design with 6 treatments and four replications. The treatments used were: no inoculation (T1); inoculation with *A. brasilense* via seed (T2); inoculation with *A. brasilense* via sowing furrow (T3); inoculation with *A. brasilense* via leaf (T4); two inoculations with *A. brasilense* via leaf (T5); and combination of inoculations via seed, sowing furrow, and leaf (T6). Physiological, morphological, production, and yield components of the second crop maize were evaluated. The data were subjected to analysis of variance at 0.05 probability level and when significant, the means were subjected to the Scott-Knott test at 0.05 probability level, using the R-bio statistical program. Inoculation via seed results in higher nitrate reductase enzyme activity. The inoculation with *A. brasilense* does not result in gains in grain yield for second crop maize; however, it improves the maize quality by increasing its gross protein contents.

Keywords: diazotrophic bacteria; biological nitrogen fixation; grain yield.

INTRODUCTION

Diazotrophic bacteria of the genus *Azospirillum* associated with maize crops perform the process of transformation of inorganic atmospheric nitrogen (N_2) through combinations with H^+, forming ammonia (NH_3^+) (Novakowiski *et al.*, 2011), and produce growth phytohormones, such as indole-acetic acid, cytokinins, gibberellins, and ethylene (Moreira *et al.*, 2010; Kappes *et al.*, 2013; Vasconcelos *et al.*, 2016).

The main barrier for the use of this technology in maize crops is the inconsistency of results. The symbiotic relation between diazotrophic bacteria and maize plants depends on many biotic and environmental factors (Roesch *et al.*, 2006).

The inoculation can be carried out using different methods (Braccini *et al.*, 2016). The main methods used for maize crops are inoculation via seed, sowing furrow, and leaf.

Inoculation with *Azospirillum brasilisense* is usually carried out in the seed treatment process. However, this practice became unviable because maize seeds are usually marketed with phytosanitary products, and treating the seed again with the bacterium is not interesting to the farmer (Morais *et al.*, 2016). In addition, insecticides and fungicides used in the seed treatment can compromise the bacterium viability.

Submitted on January 23rd, 2022 and accepted on June 03rd, 2023.
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Moreover, results of studies involving the different inoculation methods have presented high variability, increasing the need for more researches on this subject.

Considering the hypothesis that the type of contact of the bacterium with the plant directly affects the efficiency of establishing associative interactions, the objective of this work was to evaluate the agronomic performance of second crop maize grown under different methods of inoculation with *Azospirillum brasilense*.

MATERIAL AND METHODS

The experiment was conducted between March and July 2018 (second crop season), in the experimental field of the Federal University of Goiás (UFG), in the municipality of Jataí, Goiás (GO), Brazil (17°55'32''S, 51°42'32''W, and altitude of 685 m).

According to the Köppen classification, the climate of the region is Aw, with two well-defined seasons: dry (April-September) and rainy (October-March); the mean annual temperature is 22 °C and the mean annual rainfall depth is 1,800 mm (Alvares *et al.*, 2013). Figure 1 shows weather data measured during the experiment.

The soil of the experimental area was classified as a Typic Hapludox (Latossolo Vermelho distroferrico; of clay texture—585, 240, and 175 g dm^{-3} of clay, silt and sand, respectively (Dos Santos *et al.*, 2018). The chemical attributes of the 0–20 cm soil layer were: pH ($CaCl_2$): 5.1; Ca: 4.14 $cmol_c$ dm^{-3}; Mg: 2.12 $cmol_c$ dm^{-3}; Al: 0.05 $cmol_c$ dm^{-3}; H+Al: 4.0 $cmol_c$ dm^{-3}; K: 0.20 $cmol_c$ dm^{-3}; P Mehlich 1: 7.7 mg dm^{-3}; S: 4.0 mg dm^{-3}; B: 0.20 mg dm^{-3}; Cu: 7.1 mg dm^{-3}; Fe: 30 mg dm^{-3}; Mn: 50.1 mg dm^{-3}; Zn: 2.7 mg dm^{-3}; Na: 2.6 mg dm^{-3}; organic matter: 40.4 g dm^{-3}; CEC: 10.5 $cmol_c$ dm^{-3}; and base saturation: 61.5%.

The experiment was conducted in a randomized block design with 6 treatments and four replications. The area of each plot was 13.50 m^2 (2.25 m width × 6 m length). The evaluation area consisted of 4 meters of the three central rows, which were spaced 0.45 m apart, considering as border one row on each side and 1 m at each end of the row.

The treatments used were: no inoculation (T1); inoculation with *Azospirillum brasilense* via seed, at the rate of 100 mL for each 25 kg of seeds (T2); inoculation with *A. brasilense* via sowing furrow, at the rate of 300 mL ha^{-1} (T3); inoculation with *A. brasilense* via leaf, at the rate 500 mL ha^{-1} applied at the maize V3 stage (presence of 3 fully expanded leaves) (T4); inoculation with *A. brasilense* via leaf, applied at the maize V3 stage (presence of 3 fully expanded leaves) and at the maize V6 stage (presence of 6 fully expanded leaves), both at the rate 500 mL ha^{-1} (T5); combination of inoculations via seed (100 mL for 25 kg of seeds), sowing furrow (300 mL ha^{-1}), and leaf (500 mL ha^{-1}) at the V3 stage (T6). The dose *Azospirillum brasilense* used in the seed treatment and via the sowing furrow was carried out in accordance with the recommendation of the company producing the inoculant (Koppert, 2023). The foliar applied dose was established based on reports of use by producers in the region.

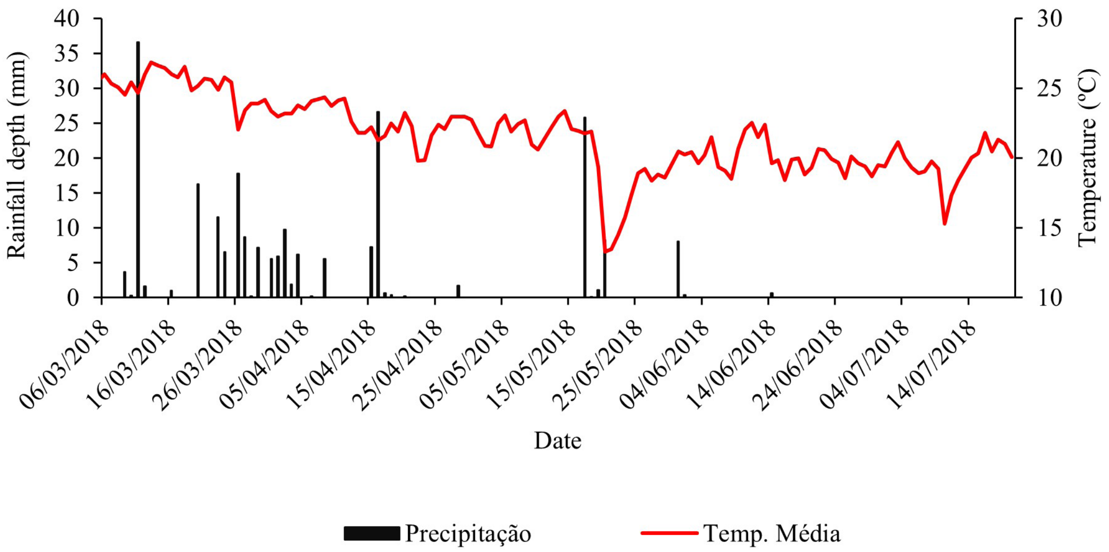

Figure 1: Rainfall depths (mm) and mean temperatures (°C) in the experimental area from March to July 2018.

A burndown was carried out on March 07, 2018, before sowing the second crop maize, to control pre-existing plants in the area, using the herbicide Gramoxone 200 (Paraquat 200 g L^{-1}; Syngenta, Basel, Switzerland) at the rate of 2.0 L ha^{-1}.

The maize seeds were treated with the commercial product Standak Top (BASF, Ludwigshafen, Germany) at the rate of 200 mL for each 100 kg of $seeds^{-1}$. This product is composed of protective action fungicide (Pyraclostrobin 20 g L^{-1}), systemic insecticide (Thiophanate Methyl 225 g L^{-1}), and contact and ingestion insecticide (Fipronil 250 g L^{-1}), which are from the strobilurin, benzimidazole, and pyrazole groups, respectively.

The seeds for second crop maize were sown manually on March 08, 2018, using the hybrid Dekalb VT PRO 2 (Dekalb, USA), using 3.0 seeds per meter to obtain a final population of 66,666 plants ha^{-1}.

The liquid inoculant Azokop (Koppert Biological Systems, Berkel, The Netherlands) was used for the species *A. brasilense,* strain AbV5 + AbV6, at the bacterial concentration of 1.8×10^8 Colony Forming Units (CFU) mL^{-1}. The inoculation with *A. brasilense* via seed was carried out at sowing, after seed treatment with Standak Top. The inoculations via sowing furrow and via leaf were carried out with the aid of a CO_2- pressurized backpack sprayer equipped with a spray boom having a hollow cone jet, running at constant pressure with a solution flow of approximately 120 L ha^{-1}.

Soil fertilizer was broadcasted at sowing by using 50 kg ha^{-1} of nitrogen (N) (urea), 72 kg ha^{-1} of P_2O_5 (simple superphosphate), and 60 kg ha^{-1} of K_2O (potassium chloride). Topdressing was carried out at the maize V3 vegetative stage by broadcasting 50 kg ha^{-1} of N (urea). The fertilizer application and topdressing were based on results of the soil analysis and on the expected maize grain yield, according to Sousa & Lobato (2004).

The control of weed plants at post-emergence was carried out on March 16, 2018, using a combination of the herbicides atrazine (2.0 L ha^{-1}) and glyphosate (2.0 L ha^{-1}). The applications of phytosanitary products were carried out using a tractor sprayer, with a solution flow equivalent to 120 L ha^{-1}.

The number of plants of the initial population in the evaluation area of each plot was counted on March 31, 2018, and the results were extrapolated to plants ha^{-1}. The methodology for quantification of the nitrate reductase enzyme activity used was adapted from Jaworski (1971) with modifications proposed by Meguro & Magalhães (1982). The adaptation made in this protocol was the addition of isopropanol alcohol to facilitate the diffusion of the enzyme substrate within the plant tissue. To measure the N nitrate reductase enzyme activity, the last completely expanded leaf was collected from 10 plants per plot.Samples were collected between 10:00 am and 12:00 pm, thus ensuring adequate light and heat for an enzymatic activity to peak. Leaf collection was performed when corn plants were in the V3 vegetative stage.

Field evaluations of agronomic characteristics were carried out at the R1 reproduction stage (silk and pollination, visible style-stigma outside the ears).

Ten random plants were collected in the evaluation area of each plot and measured for plant height; first ear insertion height; culm diameter; and chlorophyll content (Falker), which was measured on leaves opposite and below the ear, using a chlorophyl meter (Clorofilog CFL 1030 Falker).

Ten leaves opposite and below the ear of plants in the evaluation area of each plot were collected, discarding the ends and central ribs. The middle parts of leaves were sent to a laboratory for quantification of mineral matter and gross protein content using the methodology described by AOAC (1990).

The final population and number of ears per plant were quantified by accounting all plants and ears in the evaluation area, on July 15, 2018, before the harvest, and the results were extrapolated to plants and ears per hectare.

The harvest was carried out on July 20, 2018 (134 days after emergence) and 10 sample ears were collected in the evaluation area of each plot to evaluate the following agronomic parameters: ear length; ear diameter (middle part of the ear); number of rows per ear; number of grains per row; number of grains per ear; cob diameter; and grain length.

Grain yield was obtained after a mechanical threshing followed by measurement of grain weight of all harvested ears in the evaluation area of the plots. The 1,000-grain weight was obtained using the methodology described in Mapa (1992).

The R-bio statistical program (Bhering, 2017) was used for the statistical analysis of the data. The data were subjected to analysis of variance by the F test at 0.05 probability level. The means referring to the methods of inoculation with *A. brasilense* were compared by the Scott-Knott test at 0.05 probability level.

Table 1: Analysis of variance (F values) for sources of variation (SV; blocks and treatments) for the variables: culm diameter (CD), ear insertion height (EIH), plant height (PH), mineral matter (MM), gross protein (GP), Falker chlorophyll index (FCI), and nitrate reductase enzyme activity (NRA)

SV	CD	EIH	PH	MM	GP	FCI	NRA
	Mm	cm	cm	g	%		NO_{2^-} H^{-1} g^{-1} FM
Blocks	5.29*	0.47 ^{ns}	1.93 ^{ns}	1.75 ^{ns}	1.06 ^{ns}	3.70*	2.55 ^{ns}
Treatments	0.87 ^{ns}	1.02 ^{ns}	0.73 ^{ns}	0.26 ^{ns}	3.86 *	0.38 ^{ns}	37.39**
Means	25.06	137	248	9.22	13.02	78.05	0.167
CV%	3.19	3.53	1.76	7.35	1.4	3.24	20.02

** significant at 0.01 probability ($p < 0.01$); * significant at 0.05 probability ($0.01 < p < 0.05$); ^{ns} Not significant ($p > 0.05$); CV = coefficient of variation; FM = fresh matter.

RESULTS AND DISCUSSION

The data analysis showed that the air temperatures were close to that considered adequate for maize crops during the experiment, with mean temperatures varying from 15 to 27 °C (Figure 1). According to Kappes *et al.* (2014) the ideal conditions for a good maize crop development includes mean daily temperatures varying from 18 to 25 °C.

The total rainfall depth during the experiment was 227 mm. According to Fancelli & Dourado Neto (2004), the satisfactory water depths for a good maize crop development are between 350 and 500 mm. Therefore, the pluviometry results were below those considered ideal for the crop.

Table 1 shows that there was significant difference for the variables: gross protein content and nitrate reductase enzyme activity.

The Scott-Knott test separated the treatments into two groups for gross protein. The inoculation with *Azospirillum brasilense* via sowing furrow and the two inoculations via leaf resulted in higher gross protein contents when compared to the other treatments (Table 2).

Morais *et al.* (2016) found that inoculation via sowing furrow was as efficient as inoculation via seed and reported that the advantage of inoculation via furrow is its practicality in the field as it does not require a new seed treatment, and the decrease in incompatibility of pesticides used in the seed treatment, which can cause toxicity to bacteria.

The treatments were separated into five groups for nitrate reductase enzyme activity (Table 3). The seed inoculation with *A. brasilense* seed resulted in higher nitrate reductase enzyme activity when compared to the other treatments.

The inoculation via furrow (T3) and inoculation via seed, sowing furrow, and leaf (T6) were the only ones belonging to a same group.

Aliasgharzad *et al.* (2014) evaluated wheat crops inoculated with four strains of *Azospirillum* (*A. lipoferum* AC45-II, *A. brasilense* AC46-I, *A. irakense* AC49-VII, and *A. irakense* AC51-VI) under water deficit conditions and found that all bacterial strains significantly increased the nitrate reductase enzyme activity when compared to the control treatment with no inoculation. Contrastingly, Cadore *et al.* (2016) evaluated maize crops under the same edaphic conditions to those of the present study and found no effect of inoculation of seeds with *A. brasilense* on nitrate reductase enzyme activity.

Table 2: Gross protein content (%) in second crop maize plants grown under different methods of inoculation with *Azospirillum brasilense*

Treatments	Means
T3. Inoculation via sowing furrow	13.35 a
T5. Two inoculations via leaf	13.13 a
T2. Inoculation via seed	12.96 b
T1. With no inoculation	12.94 b
T6. Inoculation via seed, sowing furrow, and leaf	12.91 b
T4. Inoculation via leaf	12.88 b

Means followed by same letter are not statistically different from each other by the Scott-Knott test at 0.05 probability level.

Table 3: Nitrate reductase enzyme activity in second crop maize plants grown under different methods of inoculation with *Azospirillum brasilense* in µmoles of NO_{2-} H^{-1} g_{-1} FM

Treatments	Means
T2. Inoculation via seed	14.54 a
T5. Two inoculations via leaf	11.36 b
T1. With no inoculation	7.4 c
T3. Inoculation via furrow	5.0 d
T6. Inoculation via seed, furrow, and leaf	4.95 d
T4. Inoculations via leaf	2.09 e

Means followed by same letter are not statistically different from each other by the Scott-Knott test at 0.05 probability level. FM = fresh matter.

Marschner (1995) reported that nitrate reductase enzyme activity is induced by the substrate, i.e., by the nitrogen content inside the plant. Silva *et al.* (2011) found a variation of 8.2 to 14.82 µmoles NO_{2-} h^{-1} g^{-1} MF in nitrate reductase enzyme activity as the nitrogen rates were increased up to 100 kg ha^{-1}, which represents an increase in enzymatic activity due to increases in nitrogen contents.

Table 4 shows that there was no difference for initial and final populations, number of ears per plant, cob diameter, ear diameter, and ear length.

Repke *et al.* (2013) found no effect of inoculation with *A. brasilense* via seed in maize crops for all variables analyzed, reinforcing that the factors that affect the responses of inoculation are not yet fully understood.

Regarding the inoculation via leaf, Costa *et al.* (2015) reported that the lack of response can be connected to the fact that this treatment is commonly applied only at 20 days after sowing when the plants do not have enough time to express the inoculation effect. Morais *et al.* (2016) highlighted that high inoculant concentrations have an inhibitory effect due to the imbalance in the soil microbial population by removing microorganisms that can have beneficial associations with the maize rhizosphere.

Considering the components of production (number of grain rows, number of grains per row, grain length, 1,000 grain weight, and grain yield) of the second crop maize plants, there was no significant difference for none of the variables (Table 5).

The 1,000-grain weight is an important component for second crop maize that was not affected by the methods of inoculation with *A. brasilense*, which is consistent with the results reported by Kappes *et al.* (2017), who evaluated the inoculation with *A. brasilense* via leaf and found no significant effect on grain weight. Similarly, the inoculation methods presented no effect on the second crop maize grain yield. According to Cruz *et al.* (2008), production components affect ear weight and, consequently, grain yield. Therefore, grain yield was not affected due to the lack of effects of the inoculation methods on production components.

The average productivity achieved was 6,261 kg ha^{-1}. The average productivity is within the expected range depending on the time the corn was sown. The lack of response to inoculation can also be associated with the organic matter content in the soil in the experimental area, which according to the classification described in Sousa & Lobato (2004) is considered adequate. Added to this, the nitrogen available via fertilization may also have minimized the

Table 4: Analysis of variance (F values) for sources of variation (SV; blocks and treatments) for the variables: culm diameter (CD), ear insertion height (EIH), plant height (PH), mineral matter (MM), gross protein (GP), Falker chlorophyll index (FCI), and nitrate reductase enzyme activity (NRA)

SV	IP	FP	NEH	CD	ED	EL
	Plants ha^{-1}	Plants ha^{-1}		mm	mm	cm
Blocks	0.68 ^{ns}	0.57 ^{ns}	1.09 ^{ns}	1.18 ^{ns}	2.88 ^{ns}	2.87 ^{ns}
Treatments	0.66 ^{ns}	1.11 ^{ns}	1.36 ^{ns}	0.64 ^{ns}	0.43 ^{ns}	0.13 ^{ns}
Means	65.045	54.937	54.783	28.69	49.41	14.94
CV%	8.87	5.77	7.27	2.94	2.41	4.2

** significant at 0.01 probability ($p < 0.01$); * significant at 0.05 probability ($0.01 < p < 0.05$); ^{ns} Not significant ($p > 0.05$); CV = coefficient of variation.

Table 5: Analysis of variance (F values) for sources of variation (SV; blocks and treatments) for the variables: number of grain rows (NR), number of grains per row (NGR), grain length (GL), 1,000-grain weight (1000GW), and grain yield (GY)

SV	NR	NGR	GL	1000GW	GY
			mm	g	kg ha^{-1}
Blocks	0.34 ^{ns}	5.25*	5.83**	18.91**	8.99**
Treatments	0.40 ^{ns}	0.54 ^{ns}	0.47 ^{ns}	2.46 ^{ns}	0.99 ^{ns}
Means	17.57	30.20	10.27	216.16	6.261
CV%	2.99	4.84	3.34	3.76	8.39

** significant at 0.01 probability ($p < 0.01$); * significant at 0.05 probability ($0.01 < p < 0.05$); ^{ns} Not significant ($p > 0.05$); CV = coefficient of variation.

occurrence of a positive response to the practice of inoculation. According to Quadros *et al.* (2014), the absence of effect of inoculation with *A. brasilense* on production components and grain yield of second crop maize may be due to edaphoclimatic conditions. The climate conditions in the present study were limited for a good development of maize (Figure 1), as the rainfall in the period that the crop was in the field was below that required, which may have compromised the associative relationship established between the bacterium and the maize plants.

Another important factor for establishing associative interactions between bacteria and host plants is described by Miguel & Moreira (2001). They evaluated biological N fixation and found different dynamics of strains according to the pH; all strains grew better in pH 6.0, reaching large numbers of colony forming units, and the strain INPA 03-11B presented a smaller growth under pH 5.0 than under pH 6.0 and 6.9. The soil pH in the present study was 5.1, which probably hindered the establishment of the bacteria in the soil.

According to Vargas & Suhet (1980), seed treatment with insecticides and fungicides can cause toxicity to bacteria, with irreversible damages. This toxicity decreases the number of viable cells and compromises the associative interactions established between the bacterium and the maize plants.

The results showed that, despite the greater practicality of inoculation via sowing furrow and via leaf when compared to inoculation via seed, the methods of inoculation with *A. brasilense* were not efficient in increasing the production components and grain yield of second crop maize under the edaphoclimatic conditions of the present study. These results are consistent with those reported by Santini *et al.* (2018), who evaluated inoculations with *A. brasilense* via seed, soil, and leaf.

The results found in this research only confirm the idea that more scientific investigations still need to be carried out on this subject so that it is possible to reach a more precise recommendation related to this practice for the farmer.

Some works available in the literature reinforce this issue. For example, Sangoi *et al.* (2015) state that even in situations of low and medium investment in mineral nitrogen fertilization, inoculation does not present good results in terms of enabling the elimination of N input. Mumbach *et al.* (2017), studying the inoculation with *Azospirillum brasilense* in the corn crop, associated with nitrogen fertilization, found a reduction in productivity in the treatments that received the inoculation, but without the supply of mineral N. However, according to these authors, the application of 50% of the recommended dose of N in coverage, that is, 45 kg ha^{-1} of N, when associated with inoculation, did not reduce corn productivity, showing that this technique can indeed allow savings for the producer regarding the investment made in mineral N.

CONCLUSION

The inoculation of second crop maize with *Azospirillum brasilense* does not result in gains in grain yield; however, it improves the maize quality by increasing its gross protein contents.

ACKNOWLEDGEMENTS, FINANCIAL SUPPORT AND FULL DISCLOSURE

The authors thank the Federal University of Jataí for the financial support. The authors report no conflict of interest.

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