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Evaluation of *Azospirillum brasilense* dose response on fresh and dry matter of shoot and root of corn plants

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ABSTRACT

Zea mays (corn) is one of the leading crops focused on producing food and feed for humans and animals. The use of diazotrophic bacteria such as *Azospirillum brasilense* demonstrates a positive effect on nitrogen availability, a primordial element for the development of this plant. This study aimed to evaluate the gain of the fresh and dry matter of shoots and roots in a corn cultivar inoculated with *A. brasilense* at different doses. The experiment was carried out in pots containing soil. The corn seeds, cultivar Agroceres® 5055, were inoculated with *A. brasilense* at doses between 100 to 250 mL 20 kg^{-1}. It was observed that inoculation with *A. brasilense* positively affected fresh and dry matter gain of both shoot and roots between doses of 100-200 mL 20 kg^{-1} compared to the control. As for plant height in two stages, V6 and V8, there was no positive effect with the inoculant compared to the control. *Azospirillum brasilense* significantly affected shoot and root matter gain in the corn cultivar Agroceres® 5055, thus ensuring better development of corn plants.

Keywords: Nitrogen, Corn, Nitrogen fixing bacteria, *Azospirillum*.

Avaliação da dose resposta sobre massa fresca e massa seca, aérea e radicular, de milho inoculado com *Azospirillum brasilense*

RESUMO

Zea mays (milho) é uma das principais culturas agrícolas, voltada à produção de alimentos para humanos e de rações para animais. O uso de bactérias diazotróficas como *Azospirillum brasilense* demonstra efeito positivo na disponibilidade do Nitrogênio, elemento essencial para o desenvolvimento dessa cultura. Esse estudo teve por objetivo avaliar o cultivo de milho inoculado com *A. brasilense* em diferentes doses e o ganho de massa fresca e seca da parte aérea e radicular. O experimento foi realizado em vasos contendo solo e sementes do cultivar Agroceres® 5055 com inoculante *A. brasilense* nas doses entre 100 a 250 mL 20 kg^{-1}. Foi observado que a inoculação com *A. brasilense* apresentou efeito positivo sobre ganho de massa fresca e seca tanto da parte aérea quanto radicular entre as doses 100-200 mL 20 kg^{-1} quando comparado ao controle. Já para altura de plantas em dois estádios V6 e V8 não foi verificado efeito positivo com o inoculante comparado ao controle. *Azospirillum brasilense* demonstrou efeito significativo quanto ao ganho de massa da parte aérea e radicular no cultivar Agroceres® 5055, com isso garantindo melhor desenvolvimento de plantas de milho.

Palavras-chave: Nitrogênio, Milho, Bactéria fixadora de Nitrogênio, *Azospirillum*.

1. Introduction

Corn (*Zea mays*) is one of the most important crops in the world, as food for humans and animals is produced from the grain (Gavilanes et al., 2020). The main corn-producing countries are Brasil, the United States, China, Mexico, France, Argentina, and India. Brasil is the fourth largest exporter of this grain in the world; in the 2020/2021 harvest, 252.3 million tons of corn grain were produced (Conab, 2021; Pereira et al., 2021). The increase in production over the cultivated area can be carried out by choosing the adequate NPK formulation, breeding and management of crops, soil quality, and the use of microorganisms (inoculants) (Zeffa et al., 2019). Dependence on chemical fertilizers is still highly worrying since the excess of the various NPK fertilizers can cause eutrophication of water bodies, causing a biological imbalance in the natural environment. According to Ladha et al. (2016), a viable option against the indiscriminate use of fertilizers of synthetic origin is the use of diazotrophic bacteria that positively affect symbiotic nitrogen fixation.

Among the diazotrophic bacteria, we can mention the most important groups *Arthrobacter*, *Azobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Gluconacetobacter*, *Herbaspirillum*, *Pseudomonas,* and *Azospirillum* (Steenhoudt and Vanderleyden, 2000; Pankaj and Sharma, 2018). *Azospirillum brasilense* is circumscribed in a special group of bacteria that have a high capacity to mobilize nitrogen contained in the soil in a non-labile way, in the environment, and the production of growth-promoting substances (Fibach-Paldi et al., 2012; Domingues Neto et al., 2013; Santos et al., 2017).

Originally, *A. brasilense* was developed by Embrapa Soja in partnership with the Federal University of Paraná in Londrina, state of Paraná, Brasil. Data released by Embrapa in 1997 showed that the inoculation of *A. brasilense* increased average weight gain between 25-30% for corn plants and 8-11% for wheat plants. Not restricted to these two crops, *A. brasilense* has been applied to several other crops, such as cotton, sugarcane, coffee, brachiaria, potato, and cereals (Leite et al., 2018; Coniglio et al., 2019; Barbosa et al., 2020; Kargapolova et al., 2020; Barbosa et al., 2021).

In this sense, there are numerous advances in agronomic traits in management practices and the various corn cultivars available on the market. That is why researchers and producers seek to increase yield and profitability in this large crop, dependent on nitrogen, one of the main requirements during the physiological development of corn plants. Its availability and the morphological traits of the rhizosphere in the corn root system will guarantee yield gains, with well-developed plants, greater fresh and dry matter, resistance to water deficit and pest attack (Detoni et al., 2017; Gotosa et al., 2019; Galeano et al., 2019). This study aimed to evaluate the inoculation of seeds of the corn, cultivar Agroceres® 5055, on different doses of inoculant with *Azospirillum brasilense* as a microorganism, and with that to determine the gain of the fresh and dry mass of the shoot and root.

2. Material and Methods

The study was carried out on the Nova Pontal II farm, located in the municipality of Porteirão, Goiás State, Brazil, (17°52'06.1"S and 50°01'23.9"W). According to Koppen-Geiger, the climate classification is Cfa, which indicates an oceanic climate, or maritime temperate, with rainfall distributed throughout the year (Figure 1) (Cardoso et al., 2014). The corn cultivar used was Agroceres® 5055, early cycle, tall, good root system, good stalk quality, high ear insertion, excellent stay green, semi-hard grain type, commonly used in grain production and silage with the whole plant.

For seed inoculation, the commercial product Azototal® (AgriStore brand) (1.5 L organic inoculant) (*A. brasilense* concentration: $2x10^{11}$ CFU L^{-1}) was used, supplied by the company Total Biotecnologia in partnership with Embrapa, Brazil. After inoculation, the seeds were inserted into the soil 3 cm deep with the aid of a meter. The germination test was performed *in vitro*, with a germination rate of 98.5%. The germination test was carried out with four replications with 50 seeds each, totaling 200 seeds distributed on sheets of Germitest® paper moistened with distilled water, in the proportion 2.5 times the weight of the paper. After sowing, the rolls containing seeds were transferred to B.O.D at 20 °C in the presence of light for 8 h and 16 h without light. The count was performed on the 10th day, where germinated seeds were evaluated as described by Stefanello and Goergen (2019) adapted. The germination rate equates to 5/m^2 (seeds arranged per m^2), resulting in a population of 55.000 plants per hectare.

The experiment was carried out in 14 L pots, 28 cm in diameter, filled with Argisol. The physicochemical characterization of the soil is shown in Table 1. Then, the soil was sieved and corrected by applying lime. Forty pots were used, divided into four blocks, with eight repetitions, arranged in a completely randomized design, varying according to the dose applied concerning the stipulated amount of seeds (Figure 2**).** The experiment was installed in March 2020, where the treatment with corn seeds was initially carried out, with planting in intervals of 14 days, with no Nitrogen fertilization at planting.

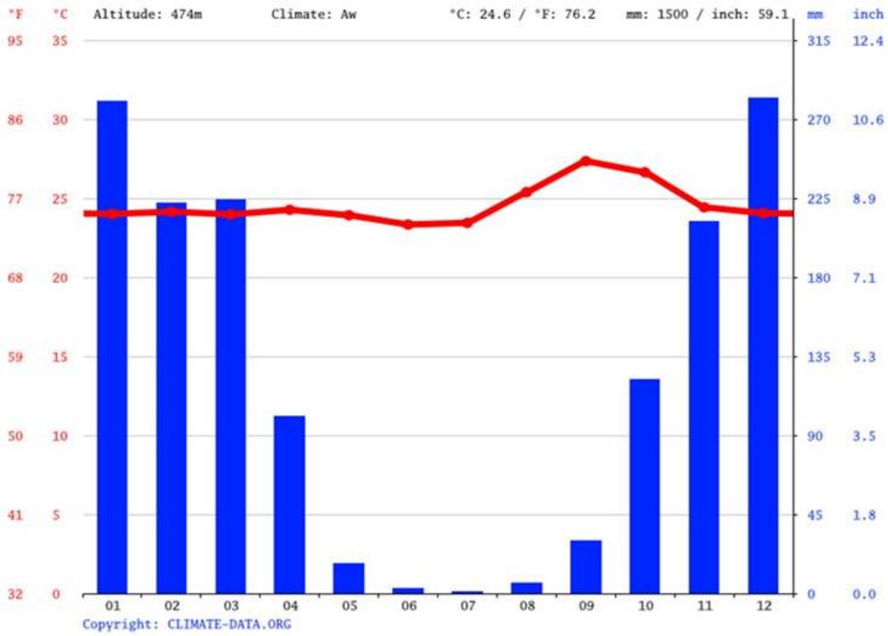

Figure 1. Average monthly rainfall for the municipality of Porteirão, Goiás, Brazil. Source: Climate Data, 2020.

Table 1. Chemical and physicochemical properties of the soil used in the experiment.

Parameters	$pH_{(CaCl2)}$	Ca	Mg	Ca + Mg	Al	H + Al	Cu	Mn
	5.16	5.10	1.25	6.35	0.00	4.32	6.06	66.37
P	K Cmolc dm^{-3}	K Mg dm^{-3}	Na Mg dm^{-3}	S	B	CEC*	Fe	Zn
35.15	0.58	227.52	1.97	5.97	0.27	11.24	42.62	5.40
OM** (%)	Sat Al***	IB***	Ca/Mg (%)	Ca/CEC	Mg/CEC	Clay	Silt	Sand
3.47	0.00	61.38	4.13	45.01	11.12	46.38	9.35	44.27

*Cation Exchange Capacity. **OM: Organic matter. ***IB: Interchangeable bases. Source: Authors, 2022.

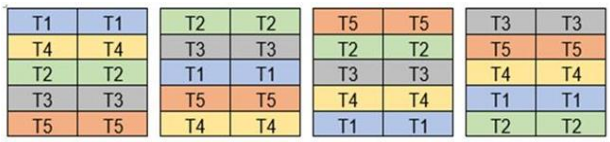

Figure 2. Experimental test and description of treatments with corn seeds inoculated with *Azospirillum brasilense* doses and control treatment. T1: Control. T2: Inoculation with 100 mL 20 kg^{-1}. T3: Inoculation with 150 mL 20 kg^{-1} (recommended dose). T4: Inoculation with 200 mL 20 kg^{-1}. T5: Inoculation with 250 mL 20 kg^{-1}.

Upon germination, there was thinning of excessive seedlings, thus leaving one plant per pot. An application of urea with a dosage of 300 kg ha^{-1} was carried out in the treatments in April 2020, when most plants were at the V4 development stage. During this period, an attack by *Spodoptera frugiperda* was observed, where an out-of-plan application was necessary with Dipel WP® insecticide (registration with the Ministry of Agriculture: no. 00858901), a microbiological product (*Bacillus thuringiensis var. Kurstaki*, HD-1 strain, 16.000 CFU mg L^{-1}, minimum of 25 billion viable spores per gram (32.0 g kg^{-1})) in its entirety, not causing any influence on the development of inoculated *A. brasilense*, at a dosage of 250 g ha^{-1} in May, date in which the plants were at the V6 development stage. Throughout its cycle, the crop was irrigated uniformly daily at a rate of 13 mm m^2, equivalent to (13 L hectare).

The treatments were evaluated with the removal of plants between the V6 and V8 stages. On the 30th of May, the plants at the V8 development stage were removed. Shoot fresh mass (SFM) and root fresh mass (RFM) of all plants were determined in a semi-analytical precision digital balance. After determination of the fresh mass, the treatments were submitted to drying in an oven with forced air circulation at a temperature of 60 ºC ± 2 ºC until they reached constant mass. After drying, the shoot dry mass (SDM) and the root dry mass (RDM) were determined. Results were expressed as a percentage (%) of mass as described by Gonzaga et al. (2020) adapted. The evaluated characteristics were subjected to analysis of variance by the F test, and the means of the treatments were subjected to the regression analysis test at 5% of significance through the statistical program Sisvar 5.6 (Ferreira, 2019).

3. Results and Discussion

The germination test was not performed by submitting the seeds to different doses of *A. brasilense*; however, a high germination rate of 98% was obtained by the classic *in vitro* method on Germitest® paper. Alternative methods with inoculating microorganisms in seeds also show excellent germination results, as Vogel and Fey (2019) proposed that inoculation with this diazotrophic microorganism resulted in a germination rate of 97% in corn with a significant difference against control (93%). However, the researchers did not mention the genotype of the corn cultivar used; our germination rate was superior to their study.

The fresh and dry matter of shoots (Figures 3A and 3B) showed a gain of 97.57% and 96.56%, respectively. The best doses for SFM were 100 and 150 mL 20 $kg^{-1,}$ and for SDM, 150 and 200 mL 20 kg^{-1}. For the roots (Figure 3C and 3D) for the RFM, the model used was the quadratic with 99.08%, and for the RDM, the model best adapted to the data was the cubic with 99.38%, respectively. The best treatments were 150 and 200 mL 20 kg^{-1} for RFM and 200 mL 20 kg^{-1} for RDM for the cultivar Agroceres® 5055 inoculated with *A. brasilense*. Our results demonstrate a gain in the fresh and dry mass yield of shoots and roots in the evaluated maize cultivar. Similar results were observed in studies carried out by Roberto et al. (2010) using seeds of the corn cultivar Pioneer 30K75 and Domingues Neto et al. (2013) with the single hybrid cultivar P3646H both treated with *A. brasilense*, where they showed an increase in shoot fresh mass and root dry mass with inoculation with *A. brasilense* with high efficiency at the beginning of the development of maize plants. These authors add that the gain in shoot and root mass is due to the colonization of bacteria in the corn stalk and root system.

Although our findings showed significant doses of fresh mass gain in both organs evaluated, Roberto et al. (2010) did not observe a positive effect on these variables with inoculation with *A. brasilense*. Similar results were observed by Cavallet et al. (2000), where no positive effect was observed with the use of *A. brasilense* on plant height and the number of rows of grain per ear and by Campos et al. (2000) on the number of plants and ears, plant height and grain yield in maize cultivars.

Tien et al. (1979) found that some compounds, such as the hormones indole-acetic acid, gibberellins, and cytokinins, were observed in high concentrations in roots that received growth stimulation provided by inoculation with *A. brasilense*. The effects of inoculation with this diazotrophic bacterium also provide greater absorption of water and nutrients, tolerance to water deficit and saline stress, presenting a plant of high vigor and production (Dobbelaere et al., 2001; Bashan; Holguin, 2004, Domingues Neto et al., 2013; Galindo et al., 2019).

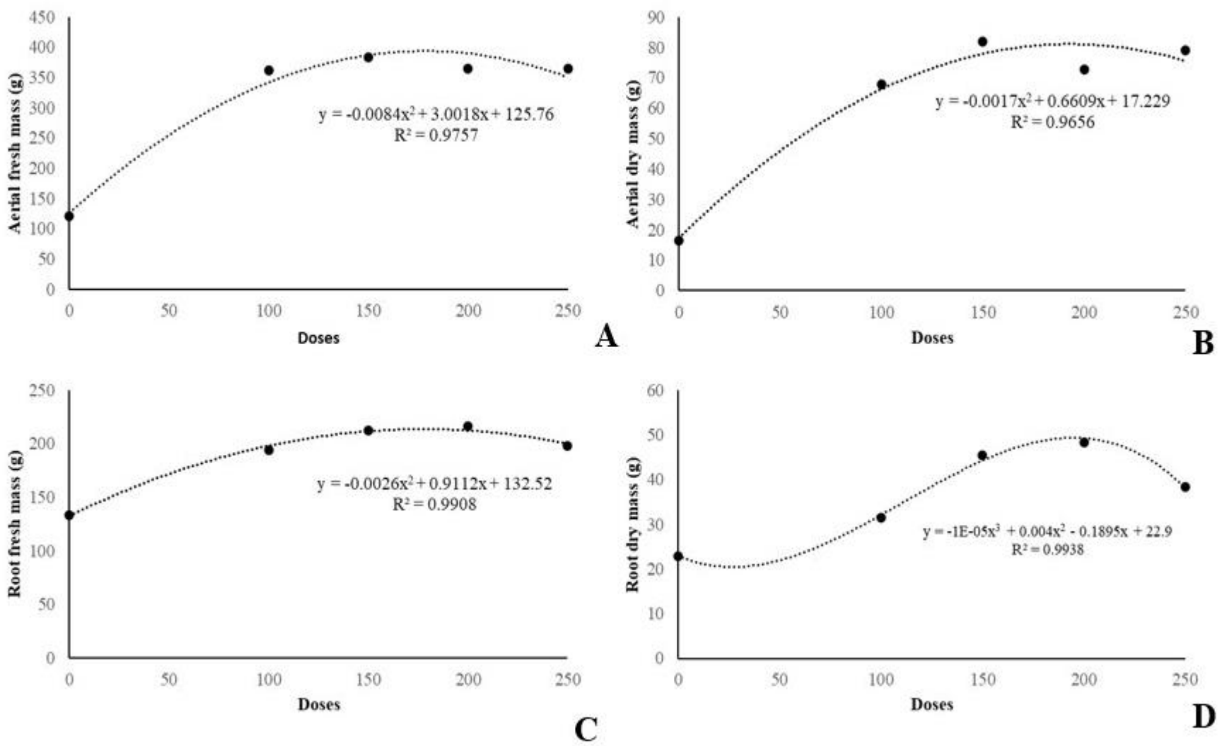

Figure 3. Shoot fresh mass (A), shoot dry mass (B), root fresh matter (C), and root dry mass (D). Source: Authors, 2022. CV% = 6.75, 8.22, 17.73, and 8.54 in for A, B, C, and D, respectively.

Figure 4 shows the treatments and control of the corn plants evaluated in this study. Qualitatively, greater development of shoots and roots was observed, in addition to fresh leaf mass, corroborating the quantitative data previously evaluated. As for plant height, in the V6 stage, R^2 = 96.76% was observed, whereas, at the V8 stage, the corn plants presented R^2 = 93.30% (Figures 5A and 5B), respectively. In this study, no differences were observed between plant heights in V6 and V8; however, the best concentration was 150 mL 20 kg^{-1}.

Promising results corroborating with this study were also observed by Zeffa et al. (2019), who observed an intensification in the growth and development of maize genotypes with the inoculation of Ab-V5 *A. brasilense*; the same was observed by Pereira et al. (2021) where they inoculated *A. brasilense* and *Bradyrhizobium japonicum* promoting the development of maize plants when compared to the control. Domingues Neto et al. (2013) in corn plants of the single hybrid P3646H did not observe any difference between the average height and diameter of the stem of the plants with the control up to 49 days. At 63 days, some treatments showed an increase in stem diameter between 14-16% compared to the control applying *A. brasilense* by the foliar route. Similar results were observed by Cavallet et al. (2000) with corn seeds inoculated with *A. brasilense,* where there was no positive effect on this morphological characteristic.

The negative results observed by colleagues were possible of a microbiological nature, where in the soil we can find groups of *Pseudomonas* and that when in direct contact with *A. brasilense* cells, they can produce lethality using a type 6 antibacterial secretion system as discussed by Maroniche et al. (2018). In the findings by these colleagues, the diffusible metabolites of *Pseudomonas* spp. on *A. brasilense* in King's B medium showed strong inhibitory effects mediated mainly by siderophores. Comparing this among other studies with corn, the benefit of inoculation with *A. brasilense* depends on factors such as the cultivar, selected strains, interrelationships between both, and biological factors such as climate, soil, and rainfall (Sala et al., 2007; Domingues Neto et al., 2013).

Figure 4. Witness rooting and corn treatments at different doses of *Azospirillum brasilense*. Source: Authors, 2022.

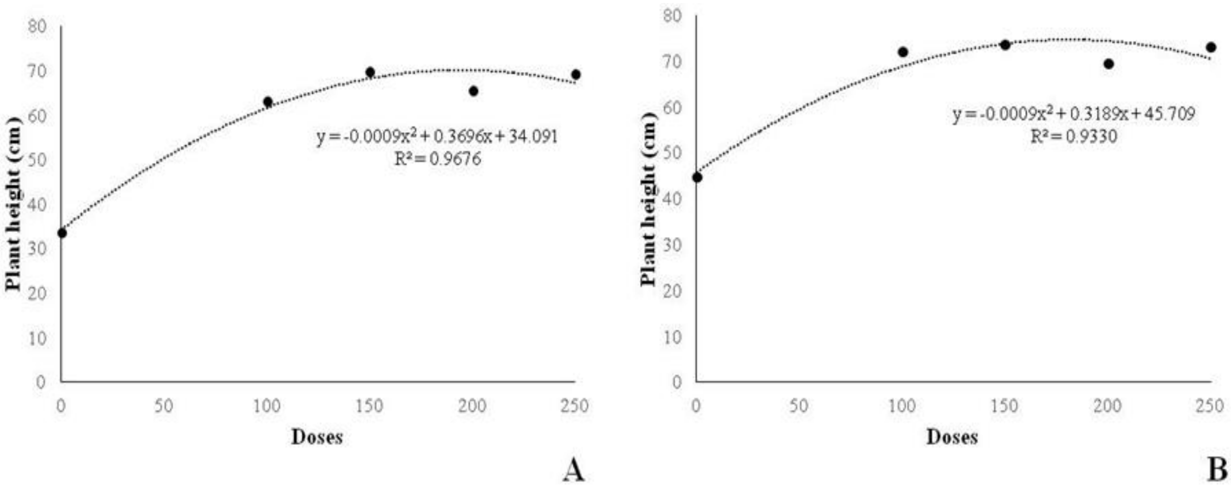

Figure 5. V6 stadium plant height parameter (**A**) and V8 stadium plant height (**B**). Source: Authors, 2022.

4. Conclusions

The inoculation of corn seeds of the cultivar Agroceres® 5055 with *Azospirillum brasilense* increased the biomass of the shoot and roots, promoting a larger area of explored soil. With this, it is concluded that the use of this microorganism is an alternative form as a substitute for Nitrogen fertilization, with efficiency in the production of growth-promoting substances, and that it also physiologically favors greater plant absorption, observed by greater root development, suggesting an increase in the use of available nitrogen source both from fertilization and fixed in the soil.

Authors' Contribution

Wellington Elias do Amaral Júnior: experiment setup, data collection, and writing article; Flávia Rodrigues Esteves: data collection and writing article; Antonio Carlos Pereira de Menezes Filho: data interpretation, orientation, and translation of the article; Matheus Vinicius Abadia Ventura: statistical analysis, data interpretation, orientation, and review.

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