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Application of *Azospirillum brasilense* and nitrogen in topdressing in corn hybrids

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ABSTRACT

Corn (*Zea mays* L.) is the most cultivated cereal in the world. The high yields obtained for this crop are mainly linked to nitrogen requirements. Technologies that aim to reduce costs with nitrogen fertilizers, with less environmental impact, such as diazotrophic bacteria, should be considered an advantageous alternative for this crop. The present study aimed to evaluate two corn hybrids, BioGene 7046® and Dekalb 310®, with three topdressing nitrogen doses (0.35 and 70 kg ha^{-1} of N) associated with four application methods of *Azospirillum brasilense* (without inoculation, seed inoculation, foliar inoculation, and the combination of inoculation via seeds and leaves). A randomized block design with four replications arranged in a 2x3x4 factorial scheme was used to evaluate variables related to growth and development, physiological analysis of nitrate reductase enzyme activity, and yield variables and their components. The use of *Azospirillum brasilense* did not affect the development of corn plants and did not increase grain yield. In the edaphoclimatic conditions in which this research was developed, nitrogen fertilization at doses of 35 and 70 kg ha^{-1} benefit nutrition, development, and corn yield components but are insufficient to increase grain yield.

Keywords: Diazotrophic bacteria, Growth promoting bacteria, *Zea mays* L.

Aplicação de *Azospirillum brasilense* e nitrogênio em cobertura em híbridos de milho

RESUMO

O milho (*Zea mays* L.) é o cereal mais cultivado no mundo. As altas produtividades obtidas para esta cultura estão ligadas principalmente às exigências ao nitrogênio. Tecnologias que visam à redução dos custos com fertilizantes nitrogenados, com menor impacto ao meio ambiente como a utilização de bactérias diazotróficas devem ser consideradas uma alternativa vantajosa para esta cultura. Objetivou-se com este trabalho avaliar dois híbridos de milho BioGene 7046® e Dekalb 310® com 3 doses de nitrogênio em cobertura (0,35 e 70 kg ha^{-1} de N) associado com 4 métodos de aplicação de *Azospirillum brasilense* (sem inoculação, inoculação nas sementes, inoculação foliar e a combinação de inoculação via sementes e folhas). Utilizou-se o delineamento de blocos ao acaso com 4 repetições com arranjo fatorial 2x3x4 avaliando-se, as variáveis relacionadas ao crescimento e desenvolvimento, análise fisiológica da atividade da enzima redutase do nitrato e variáveis do rendimento e seus componentes. O uso de *Azospirillum brasilense* não teve efeito no desenvolvimento das plantas de milho e não incrementou a produtividade de grãos. Nas condições edafoclimáticas em que foi desenvolvida essa pesquisa a adubação nitrogenada nas doses de 35 e 70 kg ha^{-1} beneficiam a nutrição, o desenvolvimento e os componentes da produção do milho, porém não são suficientes para proporcionar incrementos na produtividade de grãos.

Palavras-chave: Bactérias diazotróficas, Bactérias promotoras de crescimento, *Zea mays* L..

1. Introduction

Corn (*Zea mays* L.) is one of the most important cereals cultivated and consumed in Brazil and the world, not only for its economic value but also for its importance in human and animal nutrition (Riva Neto et al., 2021). An alternative that all corn cropping systems seek is the acquisition of nitrogen by plants in a more sustainable way (Dartora et al., 2013). Currently, most grass species production systems depend on nitrogen fertilization (Prado and Campos, 2018), whose fertilizers come from sources obtained through non-renewable energy processes, mainly in high-level agriculture technology. This dependence makes Brazil one of the countries that import fertilizers globally, with special emphasis on nitrogen and potassium (Anda, 2021).

Nitrogen fertilization is essential to obtain high corn grain yield levels, as this species is extremely demanding in this macronutrient. According to Coelho (2010), 20 to 30 kg ha^{-1} of N is needed for each ton of grain produced. As nitrogen fertilization is indispensable, alternative sources that can reduce nitrogen application must be studied. One of the alternatives that have been studied for some time is the ability of biological nitrogen fixation by diazotrophic bacteria in non-legume plants (Hungria et al., 2010).

Embrapa Soja and the Federal University of Paraná teamed up and conducted laboratory research and field tests with bacteria of the genus *Azospirillum* in corn for some years. According to the criteria of the Brazilian legislation for inoculants, established by the Ministry of Agriculture, Livestock and Food Supply (MAPA), strains of *Azospirillum* that presented greater promotion of plant growth, greater survival in the soil, and greater adaptation to the techniques used in corn cultivation. The strains selected and authorized by MAPA are of the *Azospirillum brasilense* species and are AbV4, AbV5, AbV6, and AbV7 (Hungria, 2011).

The main method of application (inoculation) of *Azospirillum* is via seed treatment to facilitate multiplication and establishment in the rhizosphere, as these two characteristics are essential for a good plant-bacteria interaction to acquire the benefits provided by bacteria diazotrophs (Baldani et al., 1986; Kappes et al., 2017). However, as in seed treatment, there is not only the addition of the inoculant but also insecticides and fungicides, among other phytosanitary products, many are incompatible with these microorganisms and may reduce the number of viable cells, thus compromising the inoculation.

As bacteria of the genus *Azospirillum* are facultative endophytic diazotrophs, foliar application becomes an alternative and is part of the application methods of *Azospirillum brasilense*, aiming at a better establishment of these bacteria and better acquisition of their benefits. This is possible since when these bacteria are applied to the leaves, they are housed in the sheath, and later, with the rains, they can be carried to the stem and soil, coming into contact with the roots.

There are reports in the literature that each crop hybrid presents a different response to inoculation, suggesting that, depending on the genotype, the benefit of inoculation can be observed in different parts of the plant, such as grains, shoots, or stems (Quadros et al., 2014). Thus, the present study aimed to evaluate the behavior of two corn hybrids with nitrogen doses applied in topdressing and application methods of *Azospirillum brasilense* in the development and grain yield of the corn crop.

2. Material and Methods

The experiment was carried out in the 2013/2014 harvest, in the municipality of Jataí, located in the Southwest region of Goiás, with an average altitude of 700 m. According to the Koopen classification, the climate of the region is Aw-type, with a well-defined dry season (April-September) and a rainy season (October-March) with average annual temperature and precipitation of 22°C and 1,800 mm. During the experiment, the total precipitation was 887.4 mm, and the temperature ranged from 16.7 to 35.7 ^{o}C.

The soil in the experimental area was classified as a Latossolo Vermelho distroférrico, clayey texture with clay, silt and sand contents of 490, 100, and 410 g dm^{-3}, respectively. Soil chemical attributes in the 0 – 20 cm layer were: pH ($CaCl_2$): 5.3; Ca: 1.22 $cmol_c$ dm^{-3}; Mg: 0.68 $cmol_c$ dm^{-3}; Al: 0.07 $cmol_c$ dm^{-3}; H+Al: 5.3 $cmol_c$ dm^{-3}; K: 0.13 $cmol_c$ dm^{-3}; Melich 1= P: 1.9 mg dm^{-3}; Cu: 9.6 mg dm^{-3}; Fe: 28 mg dm^{-3}; Mn: 34.4 mg dm^{-3}; Zn: 0.4 mg dm^{-3}; Na: 1.6 mg dm^{-3}; O.M.: 34.2 g dm^{-3}; CEC pH 7.0: 7.3 $cmol_c$ dm^{-3} and base saturation (%): 27.8. The area was being cultivated under no-tillage (succession with soybean in the first crop and sorghum in the second crop) in the harvests of 2008/2009, 2009/2010, 2010/2011, and 2011/2012; in the 2012/2013 harvest, the area was left fallow and managed with a discing harrow to incorporate 2.73 Mg ha^{-1} of dolomitic limestone with an ECCE of 86%.

The experimental design was randomized blocks, with four replications, arranged in a 2x3x4 factorial scheme. The first factor was two corn hybrids H1: Dekalb 310® YieldGard VT PRO™ (DKB 310) and H2: BioGene 7046® (BG 7046), which were selected because they stood out for their grain yield in an experiment with ten hybrids conducted in the second harvest in 2013 under different doses of nitrogen in topdressing and inoculation with *Azospirillum brasilense* in the seeds. In the second factor, the treatments corresponded to D1: without application of nitrogen in topdressing, D2: application of 35 kg ha^{-1} of N in topdressing 21 days after emergence (V4 – V6), and D3: 70 kg ha^{-1} of N in topdressing 21 days after emergence (V4 – V6). The third factor corresponds to the four methods of application of *Azospirillum brasilense* M1: without *Azospirillum brasilense* application, M2: inoculation of *Azospirillum brasilense* in the seeds at a dose of 100 mL of inoculant for 25 kg of seeds, M3: inoculation of *Azospirillum brasilense* via foliar application at a dose of 200 mL ha^{-1} 19 days after the emergency (V4 – V6), and M4: a combination of the two previous methods: seed inoculation plus inoculation via foliar application of *Azospirillum brasilense*.

In all treatments, 30 kg ha^{-1} of N was applied at sowing, and the inoculant used was the species *Azospirillum brasilense* strain AbV5 + AbV6 with a bacterial concentration of 2.0 x 10^8 CFU per mL. Nitrogen application in topdressing was performed manually, and the source used was ammonium sulfate (20% N), where each plot received the amount according to its treatment.

To implement the experiment, 10 days before sowing, the glyphosate herbicide (360 g L^{-1} a.i.) was applied at a dose of 5 L ha^{-1} to control spontaneous vegetation. Sowing fertilization was carried out one day before sowing, according to the soil analysis results and the corn crop's expected yield, according to Souza and Lobato (2004). It was applied 30 kg ha^{-1} of N, 180 kg ha^{-1} of P_2O_5, and 80 kg ha^{-1} of K_2O (source 04-30-16, ammonium sulfate, and potassium chloride) in the sowing furrow with a tractor seed drill.

After fertilization, sowing was carried out manually on November 8, 2013, distributing two seeds per every 0.33 m, with 0.45 m spacing between rows.

After 11/15/2013, emergency stage (ES), the seedlings were thinned, leaving only one seedling to obtain the final population of 66,666 plants ha^{-1}. The plots consisted of four rows of 6 m in length. Two central rows of each plot were used for data collection, disregarding 0.66 m at the ends. The seeds used contained only the standard seed treatment, which consists of industrial treatment with a fungicide based on Fludioxonil 25 g L^{-1} a.i. + Metalaxyl-M 100 g L^{-1} a.i. and insecticides based on Deltamethrin 25 g L^{-1} a.i.+ Pirimifos Methyl 500 g L^{-1} a.i. + Thiamethoxan 35% a.i. 0.12 L 60,000 seeds^{-1}. Phytosanitary treatments were conducted when necessary.

Data collection began by quantifying the activity of the nitrate reductase enzyme (NRA) at 28 DAE, between V6 - V8, after the topdressing application of nitrogen, and at 45 DAE, between V16 - V18 (pre-tasseling), NRA was again quantified.

The methodology for quantification of the nitrate reductase enzyme activity used was adapted from Jaworski et al. (1971) with modifications proposed by Meguro and 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 NRA, the last completely expanded leaf of three different plants within each useful area was collected in just three blocks, with one block being disregarded due to the large number of samples generated in the laboratory. Samples were collected between 10:00 am and 12:00 pm, thus ensuring adequate light and heat for an enzymatic activity to peak.

In the flowering period, field evaluations of agronomic characteristics were initiated. Five plants were taken at random in the useful area of each plot, and the height of each plant was measured in meters from the soil surface to the flag leaf. The first ear insertion height was measured in meters from the soil surface to the first ear insertion. Stem diameter was measured with a digital caliper graduated in millimeters taking the second internode above the base of the plant. The total chlorophyll content was quantified with the Falker chlorofiLOG CFL 1030 device, where the reading was performed in the basal third of the opposite leaf and below the ear in each plant.

At the end of the crop cycle, the final plant population was quantified, counting all the plants, including the lodged and broken plants, and all the ears were harvested, considering the two central rows as useful areas, excluding 0.66 m at each end of the plot.

For sampling, eight ears of plants present in the useful area of the plot were used to determine length, number of grains per row, and number of rows in the ear. The average length in centimeters of the ear was determined using measurements from the base to the apex with the aid of a ruler. The number of grains per row was obtained by counting the number of grains in a row in the eight ears sampled, and for the number of rows, all rows of the sampled material were counted.

For the 1000-grain weight, the methodology used was in accordance with Brasil (2009). The grain yield was obtained by harvesting the ears in the useful area, where they were threshed, and the grains were weighed, and later the water content in the grains was evaluated using a G600 portable grain moisture meter. The data were corrected for 13% water content, and results were extrapolated to an area of one hectare and expressed in Mg ha^{-1} of grains. The effects of the factors of the hybrids, doses of nitrogen in topdressing, and methods of application of *Azospirillum brasilense* were analyzed through analysis of variance and the F test. The means were compared by the Tukey test (5%).

3. Results and Discussion

In the data evaluation, a triple interaction was observed between the sources of variation, corn hybrids, nitrogen doses, and inoculation of *Azospirillum brasilense*, only for nitrate reductase activity (NRA) in the stage between V16 – V18. The interaction for the nitrogen doses in topdressing within the hybrids for the NRA between V16 – V18 is shown in table 1. A significant difference was observed in the dose of 70 kg ha-1 N concerning the treatment that did not receive nitrogen fertilization in the combination of hybrid DKB 310 and inoculation with Azospirillum brasilense via seeds. Combining hybrid BG 7046 with inoculation with Azospirillum brasilense via foliar spray and 35 and 70 kg ha-1 of N in topdressing increased NRA concerning the plants without nitrogen.

The Azospirillum brasilense application methods within corn hybrids (DKB 310 VT PRO™ and Biogene 7046) for the variable nitrate reductase enzyme activity at the V16 - V18 stage is shown in Table 2. There is a significant difference in the method with inoculation in the seeds, compared with the absence of inoculation in the combination of hybrid DKB 310 and dose of 70 kg ha^{-1} of N. In the combination of hybrid BG 7046 and the 70 kg ha^{-1} dose of N, the inoculation method in the leaves and inoculation in the seeds and leaves were superior to the method with inoculation only in the seeds, but none of them differed from the treatment with no inoculation.

García de Salamone and Döbereiner (1996), when studying corn genotypes and different *Azospirillum* species, noted both increased and decreased nitrate reductase activity and attributed such responses to the dependence of inoculation on the plant genotype and the bacterial species and strains employed and the interaction between them. The higher nitrate reductase activities can be considered either by biological nitrogen fixation (BNF) or by stimulating higher nitrate uptake by bacteria of the genus *Azospirillum* (Bashan and Levanony, 1990). There was an interaction between N doses and *Azospirillum brasilense* application methods on corn grain yield (Table 3).

Table 1. Interaction among nitrogen doses in top dressing, corn hybrids, and application methods of *Azospirillum brasilense* for nitrate reductase activity at V16 - V18 stages in µmoles of NO_2-H^{-1} g^{-1} FM.

Doses	DKB 310 VT PRO™ and seed inoculation	Biogene 7046 and inoculation on leaves
0 kg ha^{-1} of N	2.114 b	0.902 b
35 kg ha^{-1} of N	2.564 ab	3.045 a
70 kg ha^{-1} of N	4.164 a	3.076 a
MSD	1.946	1.946

Means followed by the same letter in the column do not differ by the Tukey test at 5% probability. [1/]MSD: Minimum significant difference.

Table 2. Interaction among *Azospirillum brasilense* application methods, corn hybrids, and nitrogen doses for nitrate reductase activity at V16 - V18 stages in µmoles of NO_2-H^{-1} g^{-1} FM.

Methods	DKB 310 and 70 kg ha^{-1} of N	BG 7046 and 70 kg ha^{-1} of N
Without inoculation	1.956 b	2.466 ab
Inoculation via seed (S)	4.164 a	0.824 b
Inoculation via foliar spray (L)	2.397 ab	3.076 a
Inoculation via seeds and foliar spray (S+L)	2.715 ab	3.048 a
[1/]DMS	2.142	2.142

Means followed by the same letter in the column do not differ by the Tukey test at 5% probability. [1/]MSD: Minimum significant difference.

Table 3. Interaction between *Azospirillum brasilense* application methods and N doses for grain yield in Mg ha^{-1}

Methods	0 kg ha^{-1} of N	35 kg ha^{-1} of N	70 kg ha^{-1} of N	MSD
Without inoculation	6.11 b A	7.63 a AB	7.18 ab A	1.22
Inoculation via seed (S)	6.32 b A	7.80 a A	7.08 ab A	1.22
Inoculation via foliar spray (L)	6.97 ab A	6.39 b B	7.88 a A	1.22
*Inoculation S+L	6.64 a A	7.02 a AB	7.54 a A	1.22
[1/]MSD	1.34	1.34	1.34	

Means followed by the same uppercase letter in the column and lowercase letter in the rows do not differ by the Tukey test at 5% probability. * Inoculation via seeds + inoculation via foliar spray. [1/]Minimum significant difference.

No effect of inoculation was found for grain yield, although the dose of 35 kg ha^{-1} of N in topdressing and inoculation with *Azospirillum brasilense* in the seeds differed from the inoculation with *Azospirillum brasilense* in the leaves. However, the difference above may be an isolated effect since both did not differ from the treatment without inoculation. Unlike what was observed in the present research, the results obtained by Bulla and Balbino Junior (2012) did not show the interaction between inoculation and N doses for corn grain yield, reinforcing the absence of a response pattern in the research found in the literature involving this theme.

The lack of response to inoculation can be explained due to the numerous factors that can interfere with this symbiosis relationship, such as: cultivar, microbial community in the soil, genetic characteristics of the strains, N availability in the soil, plant residues deposited in the soil by the predecessor crop in the no-tillage system, and forms of *Azospirillum* application (Braccini et al., 2012; Quadros et al., 2014). Similarly, Pandolfo et al. (2015) also obtained no grain yield increase when corn seed inoculated with *Azospirillum brasilense* was evaluated.

There was a difference between corn hybrids for NRA at the V6 - V7 stage, plant height (PH), ear insertion height (EIH), total chlorophyll (CLO), lodged plants (LP), cob length (CL), number of grain rows (NGR), grains per row (GPR), 1000-grain weight (1000W), and yield (YLD) (Table 4). For NRA at the V6-V8 stage, EIH, LP, CL, and 1000W, the hybrid DKB 310 had higher means than BG 7046. However, for PH, SPAD, NGR, GPR, and YLD, hybrid BG 7046 had higher means than DKB 310.

All these differences between the hybrids are due to their genetic variations since the environmental conditions were the same for both (Borém et al., 2017). There were differences between N doses for total chlorophyll, stem diameter, ear length, number of grain rows, and lodged plants (Table 5). The doses of 35 and 70 kg ha^{-1} of N increased total chlorophyll, stem diameter, ear length, and number of grains per row concerning the treatment without N fertilization.

The increase in total chlorophyll and stem diameter by N availability is explained by the fact that nitrogen is a constituent of the chlorophyll molecule and is associated, among other plant functions, with vegetative growth (Cruz et al., 2008; Taiz et al., 2017).

According to Oliveira et al. (2012), the events determining the length and number of rows per ear coincide with the second week after emergence, a phase in which the formation of the ear primordia begins. After this phase, a lack of water and nutrients, especially N, can affect these components.

Table 4. Nitrate reductase activity (NRA), plant height (PH), first ear insertion height (EIH), total chlorophyll (CLO), lodged plants (LP), cob length (CL), number of grain rows (NGR), number of grains per row (GRP), 1000-grain weight (1000W), and grain yield (YLD) of corn hybrids.

Hybrids	NRA (V6 - V8)	PH (m)	EIH (m)	CLO (SPAD)	LP (%)	CL (cm)	NGR (un)	GPR (un)	1000W (g)	YLD (Mg ha^{-1})
DKB 310	3.27	2.35	1.47	51.99	28.69	16.31	16.54	31.96	289.44	6.75
BG 7046	2.52	2.49	1.30	57.31	19.64	15.56	17.35	34.33	278.14	7.35
[1/]CV (%)	46.14	2.99	4.96	5.03	83.8	5.48	3.38	5.48	5.85	14.43

The highest mean differs from the lowest mean in the column by the F-test. [1/]CV: Coefficient of variation.

Table 5. Total chlorophyll (CLO), stem diameter (SD), cob length (CL), number of grains per row (GPR), and lodged plants (LP) of corn plants with nitrogen doses in topdressing.

Doses	CLO (SPAD)	SD (mm)	CL (cm)	GPR (un)	LP (%)
0 kg ha^{-1} of N	53.019 b	21,692 b	15.50 b	32.25 b	31.71 a
35 kg ha^{-1} of N	55.062 a	22,484 a	16.06 a	33.41 a	21.87 ab
70 kg ha^{-1} of N	55.875 a	23,121 a	16.25 a	33.78 a	18.91 b
[1/]MSD	1,645	0.645	0.44	1,087	12.13
[2/]CV(%)	5.03	4.80	5.48	5.48	83.8

Means followed by the same letter in the column do not differ by the Tukey test at 5% probability. [1/]MSD: Minimum significant difference; [2/]CV: Coefficient of variation.

As the water conditions were the same for all treatments, the variations observed for these components were probably influenced by N doses, as Bicudo et al. (2009) reported when working with fertilization levels in corn in the second crop in São Paulo state. The 70 kg ha^{-1} of nitrogen in topdressing provided a lower rate of lodged plants concerning the treatment without nitrogen.

According to Cruz et al. (2003), the percentage of lodging is a complex phenomenon, and its manifestation is linked to genetic factors, interrelated with climate, soil, crop management adopted, source and drain relationship, and balance with potassium and nitrogen. In contrast, results obtained by Melero et al. (2013) showed greater plant growth with increasing nitrogen doses, making them more susceptible to lodging.

The results obtained in this research show that more studies need to be conducted on the subject since the discrepancy of responses of the corn crop to the application of *Azospirillum* has become more and more evident with the new works found in the literature. In general, what can be observed is that the use of this practice has not yet proved efficient as to the possibility of reducing the supply of nitrogen to the corn crop via fertilizers.

4. Conclusions

Inoculation with *Azospirillum brasilense* does not promote changes in corn plants capable of resulting in gains in grain yield. Nitrogen fertilization at doses of 35 and 70 kg ha^{-1} benefits the nutrition, development, and yield components of corn. Nitrogen fertilization in topdressing is necessary for corn plants cultivated under the edaphoclimatic conditions of Jataí-GO.

Authors' Contribution

Rafael Cadore contributed to the evaluation, interpretation of data, and manuscript preparation. Antônio Paulino da Costa Netto contributed to the orientation and critical supervision of the manuscript regarding the intellectual content. Edésio Fialho dos Reis collaborated in data analysis, interpretation, and statistical review. Simério Carlos Silva Cruz collaborated in the analysis, data interpretation, and statistical review.

Alex Oliveira Smaniotto contributed to the field experiment setup, cultural treatment, and data collection. Taynara Peres de Lima contributed to the installation of the field experiment, laboratory analysis, crop management, and data collection. Marieli Rossato contributed to the setup of the field experiment, lab analysis, crop management, and data collection. Ana Claudia Alves D'Abadia contributed to the setup of the field experiment, lab analysis, crop management, and data collection.

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