BIOLOGICAL NITROGEN FIXATION, SOURCES AND LEVELS OF N INCREASE THE MAIZE GRAIN YIELD IN CERRADO

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ABSTRACT - The objective of this study was to evaluate the effects of inoculation with *A. brasilense*, sources and levels of sidedress nitrogen (N) on agronomic traits of maize cultivated in two regions of Brazilian Cerrado during the summer season. The experiments were conducted during the 2011/12 growing season in Chapadão do Sul and Selvíria Cities, State of Mato Grosso do Sul, Brazil. The experimental design consisted of a randomized complete block design with a 2 × 2 × 5 factorial scheme (with and without inoculation with *A. brasilense,* two sources of N [regular urea and polymer coated urea], and 5 levels of sidedress N [0, 45, 90, 135, and 180 kg ha^{-1}], with 4 replicates for each location. The inoculation with *A. brasilense*, in the absence of sidedress N significantly increased the maize yield cultivated in Selvíria. However, the inoculation showed no effect on maize yield when cultivated in Chapadão do Sul. The polymer coated urea provided greater maize yield cultivated in Chapadão do Sul, however, no difference between sources of urea were observed in Selvíria. The greatest levels of sidedress N, without inoculation with *A. brasilense* showed the greatest maize yield in both locations.
Keywords: *Azospirillum brasilense*, nitrogen fertilization, policote®, polymer coated urea. "summer maize".

FIXAÇÃO BIOLÓGICA DE NITROGÊNIO, FONTES E DOSES DE N INCREMENTAM A PRODUTIVIDADE DE GRÃOS DE MILHO NO CERRADO

RESUMO - O objetivo deste trabalho foi avaliar os efeitos da inoculação com *A. brasilense*, fontes e doses de N em cobertura nas características agronômicas do "milho verão" cultivado em duas regiões do cerrado brasileiro. Os experimentos foram realizados durante a safra 2011/12 em Chapadão do Sul e Selvíria, Mato Grosso do Sul, em delineamento experimental em blocos casualizados com esquema fatorial 2 × 2 × 5 (com e sem inoculação com *A. brasilense*, duas fontes de N [ureia tradicional e uréia revestida de polímero] e 5 doses de N [0, 45, 90, 135 e 180 kg ha^{-1}] em cobertura, com 4 repetições. A inoculação com *A. brasilense*, na ausência de N em cobertura, aumentou significativamente a produtividade do milho cultivado em Selvíria. A ureia revestida de polímero proporcionou maior produtividade de milho em Chapadão do Sul. Os maiores níveis de N em cobertura, sem inoculação com *A. brasilense* proporcionaram as maiores produtividades de milho em ambos os locais.
Palavras-chave: *Azospirillum brasilense*, adubação nitrogenada, policote®, ureia revestida com polímero, "milho-verão".

Biological N fixation in maize (*Zea mays* L.) plants promoted by *Azospirillum. brasilense* has gained significant importance over the past years (Hungria et al., 2010; Sabundjian et al., 2016). Besides it can fixate N, *A. brasilense* possesses genes which produce plant growth substances that can promote positive influence on plant establishment, root development, and water and nutrient absorption (Cassán et al., 2014) and chlorophyll biosynthesis in the leaves (Inagaki et al., 2015).

Urea is the main source of N used worldwide. In Brazil, this product corresponds to approximately 60% of the total of N fertilizers commercialized, and its production is clearly favored by industries, due to its lower cost and easier production, when compared to other N sources (Soares et al., 2015). On the other hand, as a disadvantage, urea can suffer great N loss due to ammonia volatilization (Boaretto et al., 2013). To minimize such losses, many modifications on traditional urea have been evaluated (Sun et al., 2015), including the production of fertilizers with granule coated with resins or polymers (Zavaschi et al., 2014).

Recently, recommendations to reduce the application of N fertilizers are being questioned. There is a lack of information about the combination of inoculation with microorganisms technology and synthetic fertilizers (Kaneko et al., 2016).

Thus, the objective of this study was to evaluate the effect of inoculation with *A. brasilense*, sources and levels of sidedress N on agronomic traits of summer maize cultivated in two regions of Brazilian Cerrado.

Material and Methods

Local and experiment management

Experiments were conducted during the agricultural year of 2011/12 on a Dystrophic red Latosol (Santos et al., 2013), in Chapadão do Sul and Selvíria cities, State of Mato Grosso do Sul, Brazil. Data of soil chemical analysis are displayed in Table 1.

In both locations, the climate is characterized as Aw, according to Koppen's classification. In Chapadão do Sul, located at latitude -18°41'33" S, longitude -52°40'45" W and height of 810 m (high altitude Cerrado biome), with an average annual precipitation of 1.500 mm and average temperature of 21ºC, the experiment was conducted under a non-irrigated system. The second experiment was installed in Selvíria, located at latitude 20º 22' S, longitude 51º 22' W and height of 335 m (low altitude Cerrado biome), with an average annual precipitation of 1.370 mm and average temperature of 23.5ºC. For Selvíria experiment, a supplemental irrigation using a central pivot was performed, according to Fancelli

Table 1. Chemical characteristics of the experimental areas (0-0.20 m).

Area	O.M.	pH ($CaCl_2$)	P (Resin)	S	K	Ca	Mg	H+Al	N Total (mineral)
	g dm^{-3}		----mg dm^{-3}---		----------mmol$_c$ dm^{-3}----------				g dm^{-3}
1	28	5.2	33	6	1.7	38	9	23	2.10
2	18	5.1	10	8	2.8	25	13	21	1.06

[1] Chapadão do Sul ; [2] Selvíria.* Abbreviations: O.M. – organic matter, P – phosphorus, S – sulfur, K – potassium, Ca – calcium, Mg – magnesium, H + Al - potential acidity, N – nitrogen. Methods of extraction: O.M – sodium dichromate; pH- $CaCl_2$; P – Resin; S – ammonium acetate; K – Melich 1; Ca e Mg – KCl 1 N, H+Al – SMP; N total – Kjeldhal.

and Dourado Neto (2000) recommendation for maize production. The data of climate conditions for both locations are displayed in Figure 1, Figure 2 and Table 2.

The maize was sowed in the production system soybean (summer) - millet (autumn) - maize (summer) for the previous five years, under a no-tillage system in both locations, performed in 28/10/2011 and 11/11/2011, using the hybrids 2B 604 Hx and AG 8088 VT PRO for Chapadão do Sul and Selvíria, respectively. The selected hybrids were selected according to its adaptation to each growing environment. The NPK fertilization consisted of 400 kg ha^{-1} of 08-24-12 and 08-28-16 in sowing furrow for Chapadão do Sul and Selvíria, according to Sousa and Lobato (2004) recommendation for maize crop in cerrado region.

All the seeds used in these trials were treated with insecticide (25 g i.a ha-1 of fipronil). Afterwards, the inoculation with *A. brasilense* was performed, using 200 g of peat inoculum per 25 kg of seeds. The emergence of plants occurred on 03/11/2011 and 17/11/2011 for Chapadão do Sul and Selvíria, respectively. Nitrogen sidedressing was applied in both locations during the physiological phase V_5, corresponding to 21/11/2011 and 05/12/2011, and the experiments were harvested on 06/04/2012 and 19/03/2012, for Chapadão do Sul and Selvíria, respectively.

Experimental design

Both experiments were arranged in a randomized complete block design with a 2 × 2 × 5 factorial scheme, where: two inoculations (with and without) of maize seeds with *Azospirillum brasilense* strains AbV_5 and AbV_6 (2.10^8 cells of *Azospirillum* g^{-1}), two N sources (urea and polymer coated urea), and 5 levels of sidedressing N (0, 45, 90, 135 and 180 kg ha^{-1}). The experimental areas consisted in 7 rows of maize with length of 11 and 6 m for Chapadão do Sul and Selvíria, respectively, with a space of 0.45 m between them. The 5 central rows of 5 m length were considered the useful areas for evaluation purposes.

The *Azospirillum brasilense* AbV_5 and AbV_6 strains used deposited at the "Culture Collection of Diazotrophic and Plant Growth Promoting Bacteria" of Embrapa Soja, Londrina, Paraná (Hungria et al., 2010), and the polymer used are an anionic type, soluble in water, and commercially named Policote®.

Evaluated characteristics

Final plant population: during the maize harvest, final plant population was determined based on the number of plants per two central lines of 5 m of length in each plot.

Plant dry matter: to measure plant dry matter, on plant blooming, 4 consecutive plants were collected from a pre-determined place of the useful area of each plot. The samples remained into a barn with natural ventilation, and in sequence it was shredded and transported to the laboratory. Samples were put into properly identified paper bags and placed in a forced air ventilation oven at 65ºC, until reached constant mass. The results were expressed in g $plant^{-1}$.

Leaf N concentration: for leaf N content measurements, the leaves of the main ear were collected from four consecutive plants per plot. The samples were dried in a forced air ventilation oven, at 65ºC for 72 hours. Afterwards, the samples were grounded in a Wiley type mill (1 mm sieve), and the N concentration was determined using a semi-micro Kjeldhal apparatus, after sulfuric digestion.

Grain productivity: At the end of experiments, the ears of two center lines of 5 m from each plot

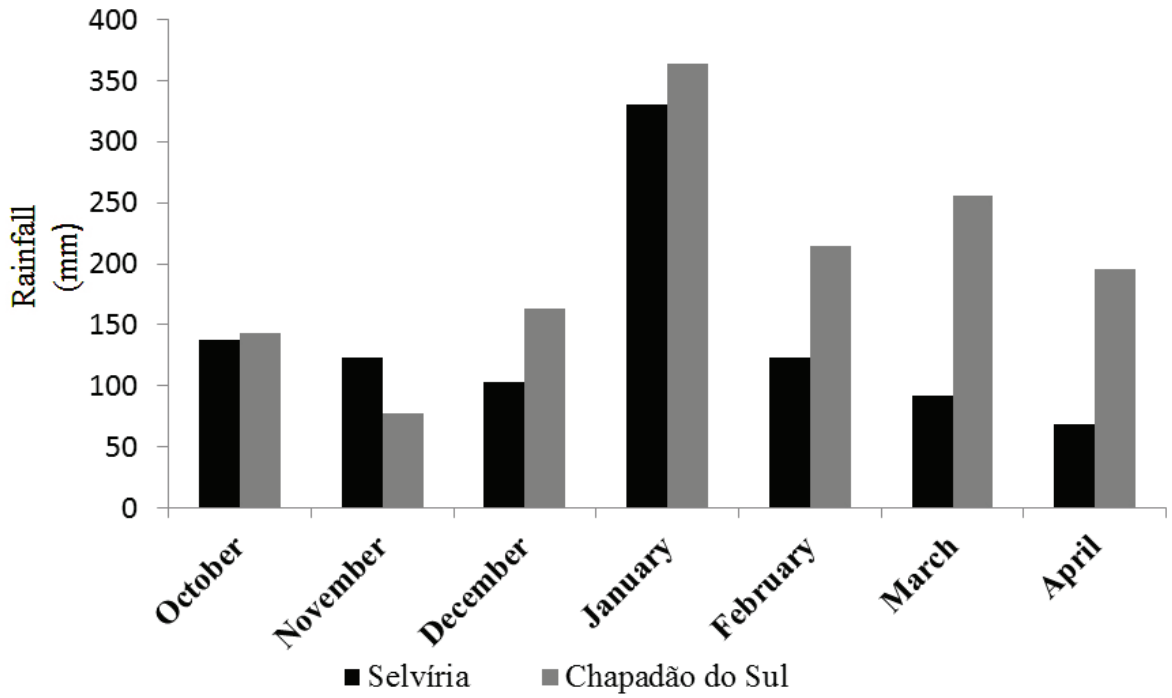

Figure 1. General rainfall data for the experimental areas of Chapadão do Sul and Selvíria-MS, Brazil, 2011/12.

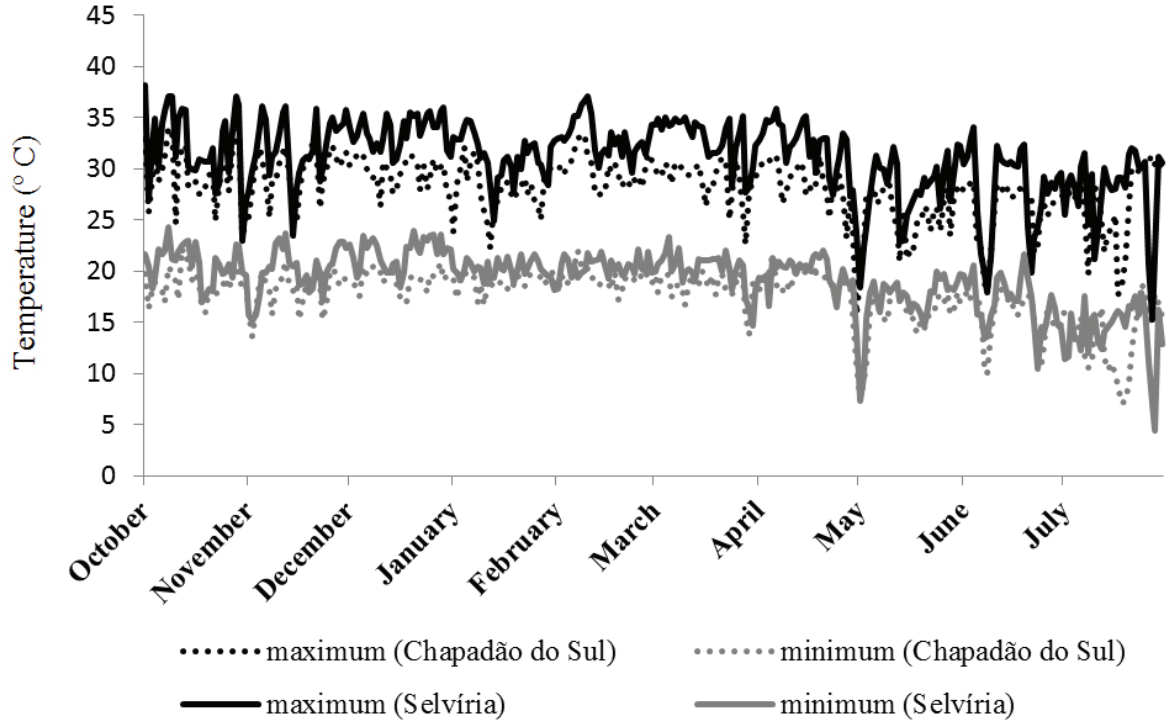

Figure 2. Maximum and minimum air temperatures observed for the experimental areas of Chapadão do Sul and Selvíria-MS, 2011/12.

Table 2. Rainfall close to nitrogen sidedressing for maize cultivated in Chapadão do Sul and Selvíria-MS, 2011/12.

Place	Last rainfall	Nitrogen fertilization	Rainfall
	Previous period	Date	Posterior period
Chapadão do Sul	14/11/2011 (25 mm)	21/11/2011	21/11/2011 (21 mm) 27/11/2011 (02 mm) 06/12/2011 (03 mm)
Selvíria[1]	01/12/2011 (7 mm)	05/12/2011	06/12/2011 (23 mm) 07/12/2011 (17 mm)

[1] Rain and irrigation.

were harvested, and submitted to mechanic trail, and the seed weight was determined. The maize yield results were calculated as kg ha^{-1} (corrected to 13% moisture).

Statistical analysis

The data were submitted to analysis of variance using the statistical software SISVAR 5.0 (Ferreira, 2011). According to the F test, (N levels, N sources and inoculation with *Azospirillum*), the data were submitted to linear and quadratic regression analysis and the Tukey test ($p \leq 0.05$) was used for mean comparisons.

Results and discussion

Final plant population and dry matter

The seed inoculation with *A. brasilense* reduced ($p < 0.05$) the final maize population cultivated in Chapadão do Sul. However, no effect was observed in maize cultivated in Selvíria (Table 3). The sources and levels of sidedress N did not change the final plant population in both locations (Table 3).

In both experiments the seeds inoculation were performed using peat inoculant. The peat may have been accumulated in the seeding machine, changing the seed distribution and, consequently, the final plant population, as observed by Novakowiski et al. (2011). The number of plants per unit of area is the first and most important component that influences on maize grain yield, indicating, therefore, that further studies are needed to evaluate the effect of peat accumulation in the seeding machines on final plant population.

No effect was observed in plant dry matter content with the inoculation of *A. brasilense*. When the polymer coated urea was used, no effect in dry matter of maize was evidenced in Chapadão do Sul. However, in Selvíria, a significant ($p < 0.05$) increase in dry matter content was observed in response to its application (Table 3). The positive effect of polymer coated urea on dry matter content of maize from Selvíria, can be explained by the lower loss of N through volatilization, compared with conventional urea (Zavaschi et al., 2014). Another hypothesis is that the slower release of N from coated urea promoted the maintenance of N in NH_4^+ form in the soil during the periods close to N sidedressing, directly affecting the N metabolism, since plants spend less energy to metabolize N compounds from NH_4^+ than do from nitrate (Hachiya et al., 2012).

The maize cultivation conditions in Selvíria was better due to supplemental irrigation using

Table 3. Final maize plants` population and dry matter, cultivated in Chapadão do Sul and Selvíria-MS, 2011/12.

Treatments	Final plant population (plants ha^{-1})		Plant dry matter (g plant^{-1})	
	Chapadão do Sul	Selvíria	Chapadão do Sul	Selvíria
Inoculation				
Without	69.72 a	56.44	62.92	72.60
With	63.13 b	54.67	67.42	81.25
Sources				
Urea	65.97	55.00	63.84	74.95 b
Coated urea	66.88	56.61	66.50	78.90 a
N levels (kg ha^{-1})				
0	68.13	53.89	65.86	73.79[1]
45	66.04	57.64	65.18	80.38
90	65.17	54.72	66.78	82.06
135	64.83	53.47	61.84	73.24
180	67.95	58.06	66.18	75.15
F Test				
Inoculation (I)	9.52 ($p<0.05$)	3.15	2.30	2.61
Source (S)	0.89	0.42	2.18	3.03 ($p<0.05$)
Dose (D)	2.08	1.24	0.94	3.76 ($p<0.05$)
I × S	3.31	0.27	2.12	0.23
I × D	1.84	2.23	2.14	2.87
S × D	0.80	0.27	1.92	0.67
I × S × D	1.83	0.24	0.51	1.62
CV (%)	9.05	10.33	16.35	21.54

Means followed by different letters differ from each other, at a 5% probability, according to Tukey test. [1] $\hat{y} = 74.9727 + 0.1162\ x - 0.0007\ x^2$ ($R^2 = 0.46$).

the central pivot. On the other hand, in Chapadão do Sul, shoot dry matter showed no response to N supply, possibly because of the severe drought stress during the plant growing physiological phase (Figure 1). This probably happened because plant stress situations resulted in a decrease of its photosynthetic activity and increased the respiration rate, reducing the carbon supply which is used to fixate N in Calvin cycle (Reis et al., 2015).

Leaf N concentration and grain productivity

A significant interaction ($p < 0.05$) of inoculation with *Azospirillum* and sidedress N was observed for N content in leaves of maize grown

in Chapadão do Sul (Table 4). The plots without *Azospirillum* showed greater N content, except for the treatments without N fertilization (Table 5). A possible hypothesis to explain the interaction of inoculation with *A. brasilense* and sidedress N levels (Table 5) is the water stress occurred in Chapadão do Sul during the experimental period. In the symbiosis between plant and diazotrophic bacteria, the plants provide energy to the bacteria, which in turn release N to the plant. During the water stress, the plant shared energy with *Azospirillum*, but it was not able to convert it to N for corn.

In high altitude Cerrado (Chapadão do Sul), there was an interaction ($p < 0.05$) of levels and sources of sidedress N (Table 6) regarding the leaf N concentration. Polymer coated urea appeared to present superior N nutritional status. From each unit (kg ha^{-1}) of N applied, an increment of 0.038 and 0.042 g kg^{-1} of leaf N concentration was observed for conventional and polymer coated urea, respectively. In this study, the polymer coated urea increased the N accumulation in the leaves by around 11%, when compared with the conventional urea.

For Selvíria conditions, there was increase ($p < 0.05$) in leaf N concentration in response to combination of inoculation and levels of N (Table 7). Increases on leaf N content due to N fertilization were obtained only in the treatments without inoculation with *A. brasilense*. For each 1 kg of N applied per hectare there was an increase of 0.038 g kg^{-1} of leaf N content. For the plots without sidedress N, there was a positive effect of inoculation with *A. brasilense*, improving plant nutritional status, providing greater leaf N concentration. Thus, when applying N fertilizers at a greatest level (180 kg ha^{-1}), the greatest leaf N concentration was observed, without inoculation with *A. brasilense*. Radwan et al. (2004) observed, in controlled conditions, that increasing N in the nutritive solution, negatively interfered on the auxins levels by two strains of the genus *Azospirillum*. Thus, it is possible that the root systems might have less development in greater N levels, when inoculated with *Azospirillum*, therefore being unable to uptake N in deeper soil layers.

Even with the development of maize in Chapadão do Sul being strongly affected by the period of drought during the vegetative phase, N sidedressing promoted increase ($p < 0.05$) on maize yield (Table 4). However, in these conditions, the inoculation with *Azospirillum* was not able to increase yield. It is important to emphasize, that the amounts of organic matter and total mineral N in Chapadão do Sul (Table 1) was able to supply part of the demand of N of the corn plants, limiting the benefits of biological N fixation by *Azospirillum* (Sangoi et al., 2015) .

For maize cultivated in Selvíria, although the greatest yield was obtained with 180 kg ha^{-1} of sidedress N wihout *Azospirillum* inoculation, representing an increase of 57% when compared to the control treatment, it is only 14% greater than the ones observed in the plots inoculated with *Azospirillum*, without N sidedress (Table 8), that showed an increase in the yield of 2.700 kg ha^{-1}, when compared with no inoculated seeds and without N sidedress. Braccini et al. (2012) reported benefits of inoculation with *A. brasilense* combined with lower N levels than the usual recommended for maize, confirming the results obtained in this study. However, the decrease of the yield and leaf N concentration observed in treatments inoculated with *Azospirillum* in great levels of N supply was not observed in other studies, such as Hungria et al. (2010), Marks et al. (2013), and Cunha et al. (2014).

Polymer coated urea provided greater maize yield in Chapadão do Sul, showing an average increment of 300 kg ha^{-1} in comparison with conventional urea (Table 4). Nonetheless, there was no significant effect between urea and polymer coated urea on maize yield cultivated in Selvíria. Gagnon et al. (2011) reported that in three years of maize cultivation, under a situation of milder temperatures, the polymer coating of urea provided significant increase on maize yield. It is interesting

Table 4. Nitrogen content of leaves and grain productivity of the maize cultivated in Chapadão do Sul and Selvíria-MS, 2011/12.

Treatments	N content of leaves (g kg^{-1})		Grain productivity (kg ha^{-1})	
	Chapadão do Sul	Selvíria	Chapadão do Sul	Selvíria
Inoculation				
Without	24.73	29.06	7.717	9.514
With	22.63	28.63	7.965	9.668
Sources				
Urea	23.52	28.46 b	7.867 b	9.327
Polymer coated urea	23.83	29.22 a	8.163 a	9.856
N levels (kg ha^{-1})				
0	20.12	26.62	7.142[1]	8.480
45	21.90	27.34	7.041	9.706
90	23.54	29.70	8.076	9.673
135	25.41	30.26	8.449	9.728
180	27.41	30.28	8.494	10.370
F test				
Inoculation (I)	24.73 ($p<0.01$)	0.42	0.73	0.20
Source (S)	1.01	3.28 ($p<0.05$)	3.52 ($p<0.05$)	2.33
Dose (D)	69.04 ($p<0.01$)	13.08 ($p<001$)	14.31 ($p<0.01$)	9,73 ($p<0.01$)
I × S	0.05	2.23	0.26	0.79
I × D	4.15 ($p<0.01$)	8.55 ($p<0.01$)	0.32	5.40 ($p<0.01$)
S × D	6.29 ($p<0.01$)	0.91	0.42	0.28
I × S × D	1.96	2.21	1.19	0.09
CV (%)	5.96	7.34	13.04	13.99

Means followed by different letters differ from each other, at a 5% probability, according to Tukey test.

[1] $y = 7.018{,}05 + 9{,}139\ x$ ($R^2 = 0{,}85$).

Table 5. Interaction of inoculation with *A. brasilense* and sidedress N levels, for N content of maize leaves, cultivated in Chapadão do Sul-MS, 2011/12.

Inoculation	Sidedress N levels (kg ha^{-1})				
	0	45	90	135	180
	N content of leaves (g kg^{-1})				
Without[1]	19.97	23.38 a	24.78 a	26.45 a	29.03 a
With[2]	20.26	20.43 b	22.03 b	24.38 b	24.13 b

Means followed by different letters in the column differ from each other, at a 5% probability, according to Tukey test.
[1] $\hat{y} = 20.4860 + 0.0471$ x ($R^2 = 0.98$); [2] $\hat{y} = 19.6293 + 0.033$ x ($R^2 = 0.95$).

Table 6. Interaction of sidedress N sources and levels, for N content of maize leaves, cultivated in Chapadão do Sul, 2011/12.

Sources	Sidedress N levels (kg ha^{-1})				
	0	45	90	135	180
	N content of leaves (g kg^{-1})				
Urea[1]	20.12	20.94 b	24.46 a	25.78	26.32 b
Coated urea[2]		22.87 a	22.62 b	25.05	28.51 a

Means followed by different letters in the column differ from each other, at a 5% probability, according to Tukey test.
M. [1] $\hat{y} = 20.073 + 0.038$ x ($R^2 = 0.93$); [2] $\hat{y} = 20.043 + 0.042$ x ($R^2 = 0.91$).

Table 7. Interaction of inoculation with *A. brasilense* and sidedress N levels, for N content of maize leaves, cultivated in Selvíria, 2011/12.

Inoculation	Sidedress N levels (kg ha^{-1})				
	0	45	90	135	180
	N content of leaves (g kg^{-1})				
Without[1]	24,97 b	28.37	29.09	30.63	32.24 a
With	28,28 a	26.32	30.32	29.84	28.33 b

Means followed by different letters in the column differ from each other, at a 5% probability, according to Tukey test. [1] $\hat{y} = 25.6757 + 0.038$ x ($R^2 = 0.95$).

Table 8. Interaction of inoculation with *Azospirillum brasilense* and sidedress N levels concerning maize grain productivity, cultivated in Selvíria-MS, 2011/12.

Inoculation	Sidedress N levels (kg ha^{-1})				
	0	45	90	135	180
	Grain productivity (kg ha^{-1})				
Without [1]	7.130 b	9.346	9.409	10.518 a	11.162 a
With	9.830 a	10.065	9.937	8.938 b	9.572 b

Means followed by different letters differ from each other at a 5% probability according to the Tukey test.
[1] $y = 7.664{,}45 + 20{,}5549$ x ($R^2 = 0{,}90$).

to note that the experiment performed in Chapadão do Sul showed milder temperatures compared to Selvíria (Figure 2). In this case, the polymer coating showed a positive effect on yield in comparison to the conventional urea.

Conclusions

• Inoculation with *A. brasilense* without N in cover, promoted greater productivity of maize grains in Selvíria. However, the combination with 135 and 180 kg ha^{-1} N on cover negatively affected the productivity.

• Coated urea increased the N content in maize leaves in both Selvíria and Chapadão do Sul (45 and 180 kg ha^{-1}), reflecting greater grain yield in Chapadão do Sul.

The greatest grain yields were obtained using the maximum dose of N in cover (180 kg ha^{-1}), without *Azospirillum* inoculation, regardless of the source of N used.

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