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Inoculation of *Herbaspirillum seropedicae* in three corn genotypes under different nitrogen levels

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The biological nitrogen fixation (FBN) associated with maize culture becomes extremely important, even if only part of the need for N by culture may be supplied by this process. The aim of this study was to verify the behavior of three maize hybrids to inoculation of *Herbaspirillum seropedicae* under different nitrogen levels. The test was conducted under controlled conditions of greenhouse and the experimental design was completely randomized design adopted in factorial scheme 3 (hybrid corn) × 2 (plants inoculated and non-inoculated) × 2 (levels nitrogen), with four replicates. Plant height, stem diameter, chlorophyll content, length root, volume of root, content N in shoot and root were determined for 35 days after emergence, as well as the efficiency of use of N. The hybrid BRS 3035 stands out for most of the variables analyzed, producing most of the shoot dry mass, plants with greater height, diameter of stem, root volume and showing the highest rates of N use efficiency. The *H. seropedicae* was inoculated with promoted increase in the volume of root, root length, dry weight of shoot, chlorophyll content, content of N in aboveground and N use efficiency. Inoculation strain Z-94 of *H. seropedicae* plus 80 kg ha^{-1} de N increased the concentration of N in the aerial part of plants of corn by as much as 25% in the evaluated genotypes.

Key words: Inoculants, diazotrophic bacteria, nutrition, biological nitrogen fixation.

INTRODUCTION

In Brazil, corn is cultivated in almost all the national territory, with 90% of its production concentrated in South, Southeast and Central-west regions. The expected harvest for 2012/2013 is nearly 76 million tons of corn, according to the Brazil's state-owned National Food Supply Company-CONAB (CONAB, 2013). This crop is usually influenced by environmental stress problems, among which stands out the low fertility of soils, which frequently show nitrogen (N) deficiency as well. According to Fancelli and Dourado Neto (2008), such deficiency can reduce grain yield by 14 to 80%. The identification, selection and use of corn genotypes more tolerant to N deficiency and more efficient at acquiring this element constitute an important strategy (Reis Junior

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et al., 2008). Therefore, the search for genotypes forming more efficient associations with diazotrophic and growth-promoting bacteria must be considered. It is known that there are interactions between N and those bacteria in the assimilation and use of this nutrient by plants (Reis Junior et al., 2008). Huergo et al. (2008), Cassán et al. (2008) and Donate-Correa et al. (2004) observed for example that diazotrophic bacteria can stimulate plant growth for acting in the process of biological nitrogen fixation (FBN), in the increase of nitrate reductase activity when occurring endophytically and in the production of phytohormones such as auxin, gibberelin and citocianin.

Due to the large area occupied by cereals, nearly five times the area of leguminous plants, the FBN associated with these crops becomes extremely important, even if only part of their N needs can be supplied by FBN (Sala et al., 2007). Regarding FBN contribution, Alves (2007) reports N values up to 67% from FBN, when inoculated with *Herbaspirillum seropedicae* and yield increase of up to 34%, depending on the corn genotype and mineral-N dose.

There are variations between corn genotypes in the response to N fertilization (Alfoldi et al., 1992), and the interactions between corn the diazotrophic bacteria are dependent on plant genotypes and on the microorganisms involved in such associations (García de Salomone and Dobereiner, 1996). Nowadays, among the diazotrophic microorganisms found in associations with cereals and grasses, the species of *Herbaspirillum* constitute one of the most studied groups.

Although the genus *Herbaspirillum* comprise nine species, only four of them can fixate nitrogen: *H. seropedicae*, *Herbaspirillum rubrisubalbicans*, *Herbaspirillum frisingense* and *Herbaspirillum lusitanun*. Most of the inoculation experiments refer to *H. seropedicae*, because of its occurrence in a large number of studied plants (Alves, 2007).

Studies involving *H. seropedicae* are interesting, because, among the characteristics of this species, the capacity to colonize the interior and the aboveground part of plant tissues stands out (Baldani et al., 1997), settling within niches protected from oxygen, and being able to express its potential to FBN in the maximum degree (Kennedy et al., 2004). In Brazil, a few studies have focused on the interactions between corn genotypes, nitrogen fertilization and diazotrophic bacteria. In many cases, the absence of response to diazotrophic bacteria inoculation in grasses has been assigned to the use of inappropriate strains. There is a consensus that the plant genotype is the key factor to obtain the benefits from the FBN, combined with the selection of efficient strains (Reis et al., 2000). Despite many years of research, variable responses are still observed, which justifies and shows the importance of experiments in field and greenhouses.

Therefore, this study aimed to verify the behavior of corn hybrids in response to the inoculation of *H. seropedicae* under different N levels through a test conducted under greenhouse controlled conditions.

MATERIALS AND METHODS

The experiment was performed in a greenhouse, in the Agricultural Science College of the Federal University of Grande Dourados, Dourados-MS, Brazil, from December 2012 to January 2013 (22°12' S; 54°56'W; 452 m). The weather is classified as Cwa (humid climate, warm summers and dry winters) according to Köppen. The soil used, classified an Oxisol with very clayey texture, was collected at the layer of 0 to 20 cm. The results of the soil chemical analysis before the experiment were: pH ($CaCl_2$): 4.15; P: 26 mg dm^{-3}; K: 5.0 $mmol_C$ dm^{-3}; Ca: 9.0 $mmol_C$ dm^{-3}; Mg: 2.0 $mmol_C$ dm^{-3}; Al: 3.3 $mmol_C$ dm^{-3}; H+Al: 41.6 $mmol_C$ dm^{-3}; SB: 115.1 $mmol_C$ dm^{-3}; CEC: 531.1 $mmol_C$ dm^{-3}; and Base Saturation: 21.7%. Granulometric analysis showed 225 g kg^{-1} of sand, 125 g kg^{-1} of silt and 650 g kg^{-1} of clay.

A completely randomized experimental design was adopted in factorial scheme 3 (corn hybrids) × 2 (inoculated and non-inoculated plants) × 2 (levels nitrogen), with four replicates. The experimental units were comprised of 10 dm^{-3} plastic pots, filled with air dried soil, sieved through a 4-mm-grid sieve.

Considering the soil chemical analysis, very fine dolomitic limestone (TNP 100%) was applied 30 days before planting in order to increase base saturation to 50%. Due to the low fertility of the soil, foundation fertilization was applied to grant crop establishment. 200 kg ha^{-1} of P_2O_5 (270 mg dm^{-3}) and 60 kg ha^{-1} of K_2O (51 mg dm^{-3}) was mixed to the soil and applied in the form of single superphosphate and potassium chloride, respectively. Micronutrients were applied according to the crop demand, in the form of solution, using deionized water and salts per annum, according to Epstein and Bloom (2006). The nitrogen fertilization was performed in 80 dose kg ha^{-1} of N (54 mg dm^{-3}) in the form of urea (45%), applied in two times of 40 kg ha^{-1} of N (27 mg dm^{-3}). The first in the sowing and the second, by top-dressing, at 15 days after plant emergence.

Soil moisture was daily controlled, through weighing, to maintain the soil at 60% of the field capacity. Irrigation was performed with deionized water.

Corn seeds of simple hybrid P3646H (Pioneer), triple hybrid BRS3035 (Embrapa) and double hybrid Maximus (Syngenta) were used, being previously inoculated with the Z-94 strain of *H. seropedicae* (inoculant cells concentration around 10^9) in the peat-based formulation, produced by Embrapa Agrobiologia, Seropédica-RJ. The dose was 250 g of peat inoculants for each 10 kg of corn seeds. For inoculation, 60 mL of a sugar solution at 10% (mass/volume) were added to increase inoculant adhesion to seeds. The seeds germinated directly in the pots and the thinning was performed in only one plant in each experimental unit.

Plant height, stem diameter and chlorophyll content in leaves were measured at 35 days after plant emergence. Plant height was obtained by measuring the distance from base to the apical meristem of the stem, with a ruler; stem diameter was obtained using a digital caliper at the height of 2 cm from the base of the stem; and chlorophyll content in leaves (SPAD reading) was measured with a chlorophyll meter (Minolta-Model 502). Plants were then divided into root and shoot parts. All the material was sequentially washed in detergent solution (3 mL L^{-1}), running water, HCl solution (0.1 mol L^{-1}) and deionized water. Root length was determined with a ruler and root volume by the volumetric flask method, in which roots were immersed in a known volume of deionized water in a graduated cylinder, and the volume was obtained by the difference between initial and final volumes in the container. Samples were then packed in paper bags and dried in an oven with forced air circulation at 65ºC for 72 h. After drying, the material was weighed, ground in a Wiley mill and then subjected to

Table 1. Summary of variance analysis for plant height (ALT), stem diameter (DIA), root volume (VR), root length (CR), shoot dry mass (MSPA), root dry mass (MSR), chlorophyll content (CLO), shoot nitrogen content (TNPA), root nitrogen content (TNR) and nitrogen use efficiency (EUN) for three hybrids of corn subjected to different nitrogen levels and inoculated with *Herbaspirillum seropedicae*. Dourados, MS (2013).

Source of variation	GL	Mean square									
		ALT	DIA	VR	CR	MSPA	MSR	CLO	TNPA	TNR	EUN
Hybrid (H)	2	83.01*	7.30*	885.89*	34.39	51.30*	3.94	114.16*	14.77	55.95*	1.36*
Nitrogen (N)	1	485.14*	53.93*	7600.33*	143.52*	224.42*	41.45*	2328.26*	2473.07*	1181.88*	12.26*
Inoculation (I)	1	0.14	7.39	5292.00*	150.52*	33.41*	12.19	75.25*	107.71*	24.73	1.17*
H*N	2	30.54	0.47	294.64	23.39	7.23	0.48	39.07	28.23	13.04	0.79
H*I	2	7.21	11.84*	192.56	18.89	2.55	12.53	2.67	65.63*	4.25	0.06
N*I	1	1.54	3.43	0.33	63.02	4.30	3.49	0.15	157.26*	24.73	0.06
H*N*I	2	47.09	1.93	143.39	141.89	0.77	0.72	3.88	81.53	62.31	0.66
Residue	36	11.16	2.08	84.50	15.32	3.33	5.01	3.36	8.22	10.05	0.12
CV (%)		6.39	8.91	12.41	6.58	24.07	9.18	7.50	13.13	19.35	17.41

* – Significant by Tukey test to 5% probability. CV- coefficient of variation.

Table 2. Effect of nitrogen doses on plant height (ALT), stem diameter (DIA), root volume (VR), root length (CR), shoot dry mass (MSPA), root dry mass (MSR), chlorophyll content (CLO), shoot nitrogen content (TNPA) and root nitrogen content (TNR) of corn plants cultivated in greenhouse and harvested 35 days after sowing. Dourados, MS (2013).

Nitrogen (kg ha^{-1})	ALT (cm)	DIA (mm)	VR (cm^3/plant)	CR (cm)	MSPA (g)	MSR (g)	CLO (SPAD)	TNPA (g kg^{-1})	TNR	EUN (mg g^{-1})
0	49.10^b	15.14^b	61.50^b	57.75^b	5.43^b	23.47^b	17.49^b	14.66^b	11.42^b	1.52^b
80	55.45^a	17.26^a	86.66^a	61.20^a	9.75^a	25.33^a	31.42^a	29.02^a	21.34^a	2.54^a

Means followed by the same letter in the row do not differ statistically between themselves by Tukey test at 5% probability.

sulfur digestion to determine N contents in root and shoot, according to the methodology described in Embrapa (2009). The N use efficiency, ratio between total dry mass produced and the total N accumulation in the plant, was calculated according to Siddiqi and Glass (1981).

The results were subjected to variance analysis and the means compared through Tukey test at 5% of probability, using the statistical program SISVAR (Ferreira, 2000).

RESULTS AND DISCUSSION

Significant effects ($p \leq 0.05$) were observed in the interaction Hybrid (H) × Inoculation (I) for stem diameter and shoot N content, while the interaction Nitrogen (N) × Inoculation (I) influenced for shoot N content. No other interaction had significant effects and the results are presented independently for hybrid, inoculation and nitrogen (Table 1).

The rates N influenced all the studied variables of corn plants (Table 1). There was a significant effect of nitrogen fertilization on plant height (Table 2). The increment in height of plants observed in this study is associated with the elongation of the stem promoted by N due to the fact that, according to Marschner (1995) the application of high doses of N in the early stages of development of cereals promotes increased production of hormones growth promoters (giberilinas and auxins, cytokinins) responsible for the processes of division and cell expansion. Studying the effects of nitrogen application in the culture of corn, Silva et al. (2003) found a positive response at the time of plants with increased doses of nitrogen. This shows that plants properly nourished with N may have higher vegetative development.

The basal stem diameter was influenced positively by increasing the dose of N (Table 2). It is noted that greater stem diameter is directly related to the increase in production, once it operates in soluble solids storage that will be used later to the formation of the grains (Fancelli and Dourado Neto, 2008).

The volume of root, root length and root dry mass increased with the dose of N (Table 2), corroborating the results of Taylor and Arkin (1981) and Glass (1990), which reported a change in growth of roots in soil fertility function. In addition, favors the growth of the root system, the plant, providing conditions for greater absorption of water and nutrients (Rao et al., 1992).

The chlorophyll content in the leaves of corn was higher in fertilized plants with 80 kg ha^{-1} of N (Table 2). Read results of chlorophyll that reinforces nitrogen fertilization improved nutritional nitrogen level in maize, due to the fact that the amount of this pigment correlate positively with N content in the plant (Booij et al., 2000).

Table 3. Behavior of three corn hybrids regarding plant height (AP), stem diameter (DIA), root volume (VR), shoot dry mass (MSPA), chlorophyll content (CLO), root N content (TNR) and N use efficiency (EUN), cultivated in greenhouse and harvested at 35 days after sowing. Dourados, MS (2013).

Hybrid	ALT (cm)	DIA (mm)	VR (cm^3/plant)	MSPA (g)	CLO (SPAD)	TNR (g kg^{-1})	EUN (mg g^{-1})
Maximus	50.40^b	15.62^b	67.68^b	5.91^b	26.77^a	18.44^a	1.79^b
P3646H	51.62^b	16.04^{ab}	72.31^b	7.38^b	25.06^b	15.91^{ab}	1.94^b
BRS3035	54.81^a	16.95^a	82.25^a	9.47^a	21.53^c	14.79^b	2.36^a

Means followed by the same letter in the row do not differ statistically between themselves by Tukey test at 5% probability.

Table 4. Effect of inoculation with *Herbaspirillum seropedicae* on root volume (VR), root length (CR), shoot dry mass (MSPA), chlorophyll content (CLO) and shoot N content of corn plants cultivated in greenhouse and harvested at 35 days after sowing. Dourados, MS (2013).

Inoculation	VR (cm^3/plant)	CR (cm)	MSPA (g)	CLO (SPAD)	TNPA (g kg^{-1})	EUN (mg g^{-1})
Without	63.58^b	57.70^b	6.75^b	23.20^b	20.34^b	1.87^b
With	84.58^a	61.25^a	8.42^a	25.71^a	23.34^a	2.19^a
Increase (%)	33.02	61.52	24.74	10.81	14.74	17.11

Means followed by the same letter in the row do not differ statistically between themselves by Tukey test at 5% probability.

In Mello 2012, also found increase in chlorophyll content in the leaves of corn with the doses of N. Kappes et al. (2013) observed that the application of 90 kg ha^{-1} of N in coverage provided greater foliar chlorophyll content in maize plants. The results confirm the role of nitrogen in plant metabolism, directly connected to the biosynthesis of chlorophylls (Andrade et al., 2003), and it is important at the initial stage of plant growth and development, during which the absorption is more intense.

The content of N in aboveground, content of N in root, absorption efficiency and utilization of N by plants of corn was superior at 80 kg ha^{-1} of N (Table 2). Carvalho et al. (2013) evaluating maize cultivars on the efficiency of absorption and utilization of N in contrasting levels of nitrogen consisted of N doses effect on dry matter production of the aboveground N content on the shoot and root, and on the efficiency of use of paragraph in literature several papers corroborating to those found in this study (Carvalho et al., 2013; Kappes et al., 2011, 2013).

The corn hybrids showed significant response ($p \leq 0.05$) to plant height, stem diameter, root volume, shoot dry mass, chlorophyll content, root N content and N use efficiency (Table 1). The hybrid BRS 3035 was statistically higher ($p \leq 0.05$) hybrids P3646H and Maximus for plant height, stem diameter, root volume, dry weight of shoot and N use efficiency (Table 3). Opposite results were found for the other variables. Chlorophyll and root N contents for the hybrid Maximus were higher than those for P3646H and BRS 3035 (Table 3), showing that the former is more efficient in N absorption, while the hybrid BRS 3035 is more efficient in the use of nitrogen. Fernandes et al. (2005), studying six corn cultivars, observed significant differences in N use efficiency by the plants. Reis Junior et al. (2008) also observed differences in dry mass accumulation and N use efficiency between the studied corn hybrids. Araujo et al. (2013) confirms the distinction of response between maize cultivars in terms of dry matter production and N content of increment in the aerial part of plants. Such differences between corn hybrids, regarding N use efficiency, are due to the genetic variations between them (Alfoldi et al., 1992).

Inoculation with the Z-94 strain of *H. seropedicae* influenced root volume, root length, shoot dry mass, chlorophyll content, shoot N content and N use efficiency (Table 1). Plants inoculated with this strain showed an increase on the order of 33.02% in root volume and 61.52% in root length, compared to the control group, non-inoculated (Table 4 and Figure 1). This effect is probably due to the auxin production by the bacteria, which stimulates the growth of secondary roots, thus increasing the specific area for water and nutrients absorption by plants (Radwan et al., 2004). Similar results were found by Quadros (2009), reporting that the root volumes for corn hybrids P32R48 and D2B587, in the treatments inoculated with *Azospirillum*, were approximately 60 and 80% higher than those in non-inoculated treatments. Canellas et al. (2013) found increased root area of corn plants when inoculated with *H. seropedicae* in combination with humic substances.

Shoot dry mass and, the content of N in aboveground, the absorption efficiency of N and N utilization increased in the order of 24.74, 14.74 21.95 and 17.11%, respectively with the inoculation of *H. seropedicae* relative to the control (Table 4), evidencing the beneficial effects of the bacteria in nitrogen assimilation by plants of corn. Corroborating with the results, Alves (2007) verified that inoculation with *Herbaspirillum* spp. contributed with up to 28% of the N absorbed by corn plants. In greenhouse conditions, Guimarães et al. (2007) working

Table 5. Stem diameter (DIA) and shoot nitrogen content (TNPA) of three corn genotypes inoculated and non-inoculated with *Herbaspirillum seropedicae.* Dourados, MS (2013).

Hybrid	DIA (mm)		TNPA (g kg^{-1})	
	Inoculation		Inoculation	
	Without	With	Without	With
Maximus	14.55^{bB}	16.70^{abA}	23.40^{aA}	21.90^{aA}
P3646H	16.61^{aA}	15.46^{bA}	18.72^{bB}	22.84^{aA}
BRS 3035	16.27^{abA}	17.63^{aA}	18.91^{bB}	25.27^{aA}

The lowercase letters separate the inside of the lines and the upper case separates the averages within each column. Same letters do not differ by Tukey test at 5% probability.

Table 6. Shoot nitrogen content (TNPA) in corn plants in response to nitrogen fertilization and inoculation with *Herbaspirillum seropedicae.* Dourados, MS (2013).

Nitrogen	TNPA (g kg^{-1})	
	Inoculation	
	Without	With
0	14.98^{bA}	14.35^{bA}
80 kg ha	25.71^{aB}	32.33^{aA}

The lowercase letters separate the inside of the lines and the upper case separates the averages within each column. Same letters do not differ by Tukey test at 5% probability.

with rice plants inoculated with strain Z-94 of *H. seropedicae*, observed increases of up to 34% in the total nitrogen of the aerial part, in relation to the absolute witness. Similar data were obtained by Dobbelaere et al. (2001), when working with bacteria of the genus *Azospirillum.* These authors report that the highest content of N in plants inoculated is a result both of the FBN, as mechanisms to promote root growth, which can increase the ability of plants to absorb this nutrient. Similar results were also obtained by Ferreira et al. (2010, 2011) and Guimarães et al. (2010) when working with rice plants inoculated with *H. seropedicae*.

Regarding SPAD readings, inoculation increased chlorophyll contents in leaves in 10.81% compared to the control (Table 4), demonstrating the efficiency of this microorganism to increment the contents of chlorophyll in the leaf, and correlating with the increase in the nitrogen content in the plant. According to Chapman and Barreto (1997), this is attributed to the fact that more than 50% of the total nitrogen of the leaves being members of compounds of the chloroplast and chlorophyll from the leaves. Concordant results found in Lima (2010) and Canelas et al. (2013) reported positive effect of inoculation with *Bacillus subtilis* and *H. seropedicae,* respectively, under the chlorophyll content in the leaves of corn, confirming the effect of inoculation with these bacteria in the development of corn and in promoting greater photosynthetic capacity of the plant.

There was positive interaction between hybrids (H) and inoculation (I) with *H. seropedicae* on the stem diameter and shoot N content. It is observed that the hybrid P3646H showed higher stem diameter, differing statistically ($p<0.05$) from the hybrids BRS 3035 and Maximus, while the latter was superior to the others in the shoot N accumulation, when not inoculated with the bacteria. With inoculation, the corn hybrid BRS 3035 was superior to the others for both the increase in stem diameter and shoot N accumulation, even with no statistical difference ($p\leq0.05$) between hybrids for shoot N content (Table 5). The stem diameter of the hybrid P3646H and the shoot N content of hybrids BRS 3035 and Maximus were not affected by the inoculation with *H. seropedicae*, with significant difference ($p\leq0.05$) only for stem diameter of the hybrid Maximus and shoot N content of hybrids P3646H and BRS 3035 (Table 5).

There was positive interaction between inoculation with *H. seropedicae* and the N levels only for shoot N content. It is observed that the inoculation with the Z-94 strain of *H. seropedicae* plus 80 kg ha^{-1} of N caused an increase in the shoot N content of corn plants in the order of 25. 74% relative to the control composted with 80 kg ha^{-1} (Table 6). Regarding the inoculated and non-fertilized control, the increase was greater than 100%. It should be pointed out that commonly a greater contribution of inoculation, associated with nitrogen fertilization, is verified. According to Baldani et al. (1996), the inoculation of *Herbaspirillum* combined with small doses of N is proven to be more efficient for the system plant/bacteria when compared to the isolated use of bacteria. This is due to the fact that the wealth of organic compounds excreted, deposited and/or exudates in the rhizosphere by plant in the presence of small doses N produces intense activities and microbial interactions that allow these bacteria to effect settlement, namely, provides signals to the micro-organisms. Dobbelaere et al. (2002) found that the effect of *Azospirillum brasilense* strain Sp inoculation of 245 and *Azospirillum irakense* KBC1 strain was higher when associated to doses of nitrogen. In inoculation experiments conducted under greenhouse conditions, using the strains M130 (*Burkholderia* sp.), ZAE94 (*H. seropedicae*) and M209 (*Burkholderia* sp.), it was observed that there was a contribution ranging from 11 to 20% in the accumulated N in the dry mass of rice plants (Baldani et al., 2000). Dalla Santa et al. (2004), in corn experiments, using the strains RAM-7 and RAM-5 of *Azospirillum* sp., observed that the use of these strains was able to reduce by 40% the amount of nitrogen fertilization recommended.

Conclusions

The hybrid BRS 3035 stands out for most of the variables analyzed, producing most of the shoot dry mass, plants with greater height, diameter of stem, root volume and showing the highest rates of N use efficiency.

The inoculation with the Z-94 strain of *H. seropedicae* promoted increase in root volume, root length, shoot dry mass, chlorophyll content, shoot N content and N use efficiency.

The inoculation with the Z-94 strain of *H. seropedicae* plus 80 kg ha^{-1} of N increased the N content in the shoot of corn plants by up to 25% in the analyzed genotypes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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