

Inoculation with *Azospirillum*, associated with nitrogen fertilization in maize¹

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ABSTRACT

The biological nitrogen fixation is an alternative to supply the nitrogen needed for maize. The objective of this study was to evaluate the development and yield of maize in response to inoculation with *Azospirillum* associated with nitrogen fertilization. We conducted two field experiments in the summer harvest, the first in the 2000/2001 crop year in the region of Marechal Cândido Rondon, under conventional tillage, and second in the 2002/2003 agricultural year in the region of Cascavel, under no tillage. The experimental design in both experiments was a randomized complete block, with four replications, 2x2x2 factorial, with two levels of nitrogen at sowing (zero and 20 kg ha⁻¹), two levels of inoculum (zero and 200 g ha⁻¹) and two levels of nitrogen in topdressing (zero and 100 kg ha⁻¹). There was evaluated the height of ear insertion, total plant height, leaf N content, shoot dry biomass and grain yield. The height of ear insertion and total plant height were not influenced by the factors under study. Nitrogen fertilization at sowing increased the leaf N content, causing the opposite effect when combined with inoculation. Inoculation with *Azospirillum* in the absence of nitrogen, provide productivity increases of 15.4% and 7.4% for 2000/2001 and 2002/2003 crops, respectively. The inoculation provided productivity similar to that obtained with 100 kg ha⁻¹ in topdressing in crop 2000/2001, while in association with the topdressing, reduced productivity and shoot dry biomass in crop 2002/2003.

Key words: *Zea mays* L., biological nitrogen fixation, leaf nitrogen content.

RESUMO

Inoculação com *Azospirillum*, associada à adubação nitrogenada, na cultura do milho

A fixação biológica de nitrogênio é uma alternativa para o suprimento de parte do nitrogênio necessário à cultura do milho. Com o objetivo de avaliar o desenvolvimento e produtividade do milho, em resposta à inoculação com *Azospirillum brasilense*, associada à adubação nitrogenada, foram conduzidos dois experimentos na safra de verão, o primeiro, no ano agrícola 2000/2001, no município de Marechal Cândido Rondon, sob plantio convencional, e, o segundo, no ano agrícola 2002/2003, no município de Cascavel, sob plantio direto. O delineamento experimental empregado foi em blocos casualizados, com quatro repetições, esquema fatorial 2x2x2, sendo dois níveis de adubação nitrogenada na semeadura (0 e 20 kg ha⁻¹); dois níveis de inoculante (0 e 200 g ha⁻¹) e dois níveis de nitrogênio em cobertura (0 e 100 kg ha⁻¹). Avaliaram-se altura de inserção da espiga, altura total das plantas, teor de nitrogênio foliar, biomassa seca de parte aérea e produtividade de grãos. As alturas de inserção da espiga e total das plantas não foram influenciadas pelos fatores em estudo. A adubação nitrogenada de base proporcionou incremento no teor de nitrogênio foliar, provocando efeito inverso, quando associada à inoculação. A inoculação com *Azospirillum*, na ausência de adubação nitrogenada,

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proporcionou incrementos na produtividade de 15,4 e 7,4%, para as safras 2000/2001 e 2002/2003, respectivamente. A inoculação proporcionou produtividade semelhante àquela obtida com 100 kg ha⁻¹ de N em cobertura, na safra 2000/2001, enquanto, em associação com a adubação em cobertura, reduziu a produtividade e a biomassa seca de parte aérea na safra 2002/2003.

Palavras-chave: *Zea mays* L., fixação biológica de nitrogênio, teor de nitrogênio foliar.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most cultivated crops in the world, and it is usually affected by problems of environmental stress, among which we may highlight low soil fertility, which, most of the times, has nitrogen deficiency.

Nitrogen is considered the mineral element that plants need in the greatest amount (Taiz & Zeiger, 2004), and it is important both to increase grain production and to rise protein content. Considering as well that cereals account for the production of most part of the food consumed by humans, clearly even if only part of nitrogen was provided by the association with fixing bacteria, nitrogen fertilizer would still be considerably economic (Döbereiner, 1992). Therefore, biological nitrogen fixation is an alternative to supply part of the nitrogen needed for maize culture.

Inoculation of *Azospirillum* spp. bacteria in grasses has caused significant increases in root growth, resulting in better benefit and use of fertilizer and water and, consequently, better plant growth (Baldani *et al.*, 1997). According to Okon & Vanderleyden (1997) at least part or, perhaps many of these effects caused by *Azospirillum* spp. on plants may be associated with the bacteria production of growth enhancing substances, including auxin, cytokinins and gibberellins, and not only the fixation of nitrogen.

Several authors have reported the positive effects of the interaction between *Azospirillum* spp. and maize, such as increases in dry matter production, grain yield and nitrogen accumulation in inoculated plants, mainly when unimproved genotypes were involved in the presence of low nitrogen availability (Okon & Vanderleyden, 1997; Hungria *et al.*, 2010).

There is also a wide variety of results in the literature regarding the relationship between inoculation and nitrogen fertilization. Barros Neto (2008), when working with maize subjected to nitrogen fertilization levels (100 and 150 kg ha⁻¹ of N) and inoculation of product based on *A. brasilense*, have found that inoculation provided an increase of 793 kg ha⁻¹ in grain yield compared to control. Reis Júnior *et al.* (2008) observed an increase in dry matter of maize roots inoculated with *Azospirillum* spp. and associated this higher yield in dry mass of inoculated plants with the production of growth enhancing

substances by bacteria. In the same way, Quadros (2009) have found increases of up to 3770 kg ha⁻¹ on the yield of dry weight of maize shoots, when seeds were inoculated with *A. brasilense* in relation to control.

Further studies on the interaction of nitrogen fertilization and its influence on the development of maize culture under inoculation with *Azospirillum* spp. are necessary. Thus, the objective of this work was to evaluate the development and yield of maize in response to inoculation with *Azospirillum* spp. associated with nitrogen fertilization, at sowing and at top dressing.

MATERIAL AND METHODS

Two experiments were conducted in the field on the summer harvest. The first in the agricultural year 2000/2001 in conventional tillage, with the BR 205 hybrid developed by Embrapa, in the region of Marechal Cândido Rondon. And the second in the agricultural year 2002/2003 in no-tillage system with the hybrid AG 6018 YG of Agrocères in the region of Cascavel, both in the western region of Paraná state, Brazil, in an eutroferic Oxisol with chemical characteristics that are shown on Table 1.

The experimental design used in both experiments was that of randomized blocks, with four replicates and treatments arranged in a 2x2x2 factorial scheme, with two levels of nitrogen fertilizer at sowing (0 and 20 kg ha⁻¹ of N) in the form of ammonium sulfate, two levels of liquid inoculant AZ39 - *Azospirillum brasilense* (0 and 200 g ha⁻¹) and two levels of nitrogen in topdressing (0 and 100 kg ha⁻¹), supplied as urea (45% N). Inoculation was performed by dissolving the inoculum in a solution containing 25% glucose mixed with the seeds and then made to sowing.

The application of nitrogen at sowing was made in furrow, and at topdressing, there was a broadcasting application at 35 days after plant emergence, at which time the plants had 4-6 leaves. Fertilization with phosphorus and potassium was carried out in the planting furrow according to the chemical analysis of soils. There were used triple superphosphate and potassium chloride, as fertilizer sources, applied at doses of 100 kg ha⁻¹ of P₂O₅ and 20 kg ha⁻¹ de K₂O in the experiment conducted in Marechal Cândido Rondon, while in the experiment carried out in Cascavel, were applied 120 kg ha⁻¹ of P₂O₅ and 60 kg ha⁻¹ of K₂O respectively.

Each experimental plot consisted of four rows spaced by 0.9 m and with 6 m length. To obtain the usable area, the two side lines and 0.5 m from each end of the plot were eliminated, totaling 9 m² per plot. The parameters assessed included: ear insertion height, plant total height, leaf N content, dry biomass production of shoots and grain yield.

In the assessment of ear insertion height and plant total height on the plot usable area, measurements were taken from the soil surface to the insertion of the ear and by the end of the stalk, respectively. In order to do so, a measurement ruler was used for surveying.

Leaf N content was determined using the method described by Tedesco *et al.* (1995), who recommends pick leaves of 10 plants per plot, at the stage of flowering of the culture, selecting the leaves in the opposite position and below the insertion of the ear.

Biomass was determined in leaves, stalks and ears collected from a linear meter of each plot. Before to be weighed the samples were dried in a forced circulation oven at 65 ° C to constant weight. Based on the mean moisture content of the sample, the dry biomass produced in each plot was calculated, then extrapolating to kg ha⁻¹.

To estimate the grain yield of the culture, all ears from the usable area of each plot were collected, and then threshing was performed with the aid of a manual thresher. The grains were exposed to the sun until they reached equilibrium moisture content (between 12 and 14%), and then grain yield in kg ha⁻¹ was determined.

Results were subjected to analysis of variance, applying the F test at 5% probability. Means were compared by Tukey test at 5% probability.

RESULTS AND DISCUSSION

Plant height and ear insertion height were not modified by N fertilization and inoculation at both sites (Table 2), which can be attributed to genetic characteristics of each hybrid, a result also found by Campos *et al.* (2000) when working with the application of the *Azospirillum* spp. based Graminante inoculant and nitrogen applications in sowing and topdressing in maize culture.

For the experiment conducted in Marechal Cândido Rondon, there was a significant effect of the interaction between nitrogen topdressing fertilization and inoculation on productivity. The shoot dry biomass was significantly influenced by the interactions between nitrogen topdressing fertilization and inoculation, as well as between nitrogen fertilization of base and topdressing (Table 2).

The unfolding of the interaction between nitrogen topdressing fertilization and inoculation on yield and dry biomass of shoots in Marechal Cândido Rondon is shown on Table 3. It was found that, both for shoot dry biomass and for yield, the inoculation with *Azospirillum* spp. provided similar means for the treatments with and without nitrogen topdressing fertilization, suggesting the possibility of replacement of nitrogen topdressing fertilization by the use of inoculation. Considering that nitrogen fertilizers represent about 40% of total production costs of maize (Machado *et al.*, 1998), such replacement would allow considerable savings in production costs.

It can also be noted on Table 3 that without the application of nitrogen topdressing fertilization, seed inoculation with *Azospirillum* significantly increased the production of shoot dry biomass in 652.3 kg ha⁻¹ and productivity in 891.9 kg ha⁻¹, with increases from 7.2 to 15.4% for these variables, respectively. These results exceed those obtained by Cavallet *et al.* (2000), who worked with the inoculation of maize seeds with a commercial product based on *Azospirillum* spp., and found an increase of 357 kg ha⁻¹ in productivity. Despite the increase provided by the inoculation, yield values obtained in this experiment were lower than the productivity of hybrid BR 205 which is 8700 kg ha⁻¹ for a population of 55,000 plants per hectare.

Increases in the production of shoot dry biomass and yield provided by inoculation in this study could be attributed to the stimulus that *A. brasilense* provides to the development of the root system, with increase in density and root hair length and on the volume and number of lateral roots, resulting in greater uptake ability and utilization of water and nutrients like reported by Huergo *et al.*, 2008.

Table 1 - Chemical characterization of the 0-20 cm layer of soil in the experimental areas

Marechal Cândido Rondon -PR Season 2000/2001										
M.O.	P	pH	H+Al	Al ³⁺	K ⁺	Ca ²⁺	Mg ²⁺	SB	CTC	V
g dm ⁻³	mg dm ⁻³	CaCl ₂				cmol _c dm ⁻³			%	
29.48	4.32	5.16	4.96	0	0.7	5.7	1.33	7.71	12.7	60.85
Cascavel - PR Season 2002/2003										
M.O.	P	pH	H+Al	Al ³⁺	K ⁺	Ca ²⁺	Mg ²⁺	SB	CTC	V
g dm ⁻³	mg dm ⁻³	CaCl ₂				cmol _c dm ⁻³			%	
36.86	4.22	5.07	5.76	0	0.6	6.3	2.3	9.10	14.9	61.24

In the early studies on the associations between plants and *Azospirillum* spp., researchers believed that the benefits were derived only from biological nitrogen fixation (Dobbelaere *et al.*, 2001). However, later studies demonstrated that the positive effects provided by these nitrogen-fixing micro-organisms are due primarily to morphological and physiological changes in the roots of inoculated plants (Okon & Vanderleyden, 1997). Such changes in the root system are related to the production of growth enhancing substances by bacteria, since plant growth regulators (phytohormones), mainly the indole acetic acid (IAA), excreted by *Azospirillum* spp. play an important role in fostering the growth of plants in general (Bashan & Holguin, 1997).

Regarding the unfolding of the interaction between nitrogen fertilization in sowing and in topdressing for

shoot dry biomass (Table 4) for Marechal Cândido Rondon, it can be observed that the use of nitrogen topdressing fertilization provided a higher production of dry biomass in the absence of nitrogen in sowing. This finding may be associated with the soil management system adopted in this cultivation (conventional), which results in lower demand for nitrogen in the early stages of culture, unlike the no-tillage system, in which the high C/N ratio of previous culture residues may reduce nitrogen availability due to the immobilization of nitrogen in the soil (Pauletti, 1998).

For the experiment carried out in Cascavel, there was significant interaction among inoculation, N application at sowing and in topdressing for the variables yield, shoot dry biomass and leaf N content (Table 2). Results for such interactions are shown in Table 5.

Table 2 - Analysis of the variance of yield, shoot dry biomass, plant height, ear insertion height, and nitrogen content in the leaf tissue as a function of inoculation, nitrogen application at sowing and in topdressing

Marechal Cândido Rondon - PR Season 2000/2001					
	Yield	Biomass	Ear insertion height	Plant height	Leaf N content
Inoc	1059604.0	108112.5	0.0017	0.0001	0.0149
NB	204000.7	1128.13	0.0000	0.0001	0.0149
NC	3389757.0**	899140.50	0.0058	0.0001	0.1365
Inoc*NC	2229744.0**	2298368.00**	0.0026	0.0024	0.0149
Inoc*NB	339694.0	392055.13	0.0003	0.0008	0.0000
NC*NB	686499.0	2527876.13**	0.0048	0.0040	0.0158
Inoc*NC*NB	226296.2	299925.13	0.0053	0.0120	0.0000

Cascavel - PR Season 2002/2003					
	Yield	Biomass	Ear insertion height	Plant height	Leaf N content
Inoc	217571.4	482407.53	0.0000	0.0205	0.0052
NB	3233528.0**	10027361.53**	0.0205	0.0000	0.0553
NC	6575322.1**	15996582.03**	0.2574	0.0048	0.0124
Inoc*NC	2213800.2**	432682.53	0.0790	0.0011	0.3382**
Inoc*NB	887079.5**	5127202.53**	0.0081	0.0000	0.0979**
NC*NB	63750.3	1604288.28**	0.0052	0.0030	1.5269**
Inoc*NC*NB	772547.0**	1766730.03**	0.0443	0.0132	0.4027**

** Significant by F test to 1% probability.

Inoc – inoculation with *A. brasilense*; NB - nitrogen base; NC - nitrogen fertilization in topdressing..

Table 3 - Shoot dry biomass and maize yield as a function of inoculation and application of nitrogen in topdressing

	- Inoc	+ Inoc	Mean	- Inoc	+ Inoc	Mean
	Shoot dry biomass			Yield		
	kg ha ⁻¹					
NC ₀	9011.0 Bb	9663.3 Aa	9337.2	5770.4 Bb	6662.3 Aa	6216.4
NC ₁₀₀	9882.3 Aa	9462.5 Aa	9672.4	6956.0 Aa	6785.3 Aa	6870.6
Mean	9446.6	9562.9		6363.2	6723.8	

Means followed by same capital letter in the column, and regular letter on the row do not differ themselves by Tukey test at 5%.

- INOC, + INOC - absence and presence of inoculant; NC₀, NC₁₀₀ - doses of nitrogen topdressing fertilization.

As for productivity, it can be noted that, when inoculation with *Azospirillum* spp. was performed, there was no significant difference between applying or not nitrogen at sowing (Table 5), which indicates that in the absence of nitrogen at sowing, the practice of seed inoculation can come to meet part of the nitrogen demand in the initial stage of culture. Quadros (2009), in an experiment under tillage with the intent to assess the inoculation of maize genotypes with *Azospirillum* spp. associated with nitrogen fertilization, has also reported that inoculation can supply part of the nitrogen needed for culture, since inoculation along with the addition of 50 kg ha⁻¹ of N in the base, provided grain yield equivalent to the application of 130 kg ha⁻¹ of N (50 kg ha⁻¹ at the base and 80 kg ha⁻¹ in topdressing). Dalla Santa *et al.* (2008), when working with wheat, also found that the isolated use of inoculation with *Azospirillum* spp. resulted in increased grain yield of 23.9% compared to control.

It is observed that in the absence of nitrogen application in sowing the inoculation of *Azospirillum* spp. gave a mean yield of 7262.5 kg ha⁻¹, and where there was no inoculation, 6764.6 kg ha⁻¹ (Table 5), reaching, therefore, significant increase in productivity, of about 7.4%, when inoculation was used alone, once again

Table 4 - Shoot dry biomass of maize plants as a function of nitrogen application at sowing and in topdressing

	NB ₀	NB ₂₀	Mean
Shoot dry biomass			
kg ha ⁻¹			
NC ₀	9062.0 Bb	9612.3 Aa	9337.2
NC ₁₀₀	9959.4 Aa	9385.4 Ab	9672.4
Mean	9510.7	9498.8	

Means followed by same capital letter in the column, and regular letter on the row do not differ among themselves by Tukey test at 5%.

NB₀, NB₂₀ - doses of nitrogen sowing fertilization; NC₀, NC₁₀₀ - doses of nitrogen topdressing fertilization.

confirming the beneficial effects of diazotroph bacteria inoculation to maize crop. Despite this increase in relation to control, the yield values obtained in this experiment are inferior to 8319 kg ha⁻¹ estimated for the hybrid AG 6018 YG for a population of 55 to 60 thousand plants per hectare.

Nitrogen topdressing fertilization, associated with inoculation, has reduced productivity and shoot dry biomass production (Table 5). These results agree with those obtained by Quadros (2009), on the other hand, Barros Neto (2008) found that the variation of the amount of nitrogen applied in topdressing (100 kg ha⁻¹ and 150 kg ha⁻¹) had no effect on maize yield, whose seeds were inoculated with pre-commercial product based on *Azospirillum brasilense*.

In this case, it is possible to consider that the incorporation of higher amount of nitrogen fertilizer to soil, due to topdressing fertilization, has undermined the effect of inoculation. According to Hartmann (1988), the efficiency of biological fixation in *Azospirillum* spp. is rapidly reduced or even inhibited in the presence of higher concentrations of nitrogen compounds in the soil, especially ammonium, which causes the rapid inhibition of nitrogenase activity in bacteria, responsible for the conversion of the atmosphere nitrogen (N₂) to a form that is acceptable by the plants (Lopes, 2007). This process of control and regulation of nitrogenase complex activity by modulator environmental factors (concentration of assimilated nitrogen and oxygen) becomes necessary due to the high demand for energy involved in the process of reducing atmospheric nitrogen (N₂) by diazotrophic organisms, some 28 moles of ATP per mole of ammonia produced (Baldani *et al.*, 2009).

Positive effect of inoculation was obtained in the absence of nitrogen in topdressing, with a higher production of shoot dry biomass and productivity in relation to treatment without inoculation. However,

Table 5 - Yield, shoot dry biomass and leaf N content of maize plants as a function of inoculation and nitrogen application at sowing and topdressing - Cascavel

	- Inoc	+ Inoc	Mean	- Inoc	+ Inoc	Mean	- Inoc	+ Inoc	Mean
YieldShoot dry biomass									
kg ha ⁻¹									
NB ₀	6764.6 Bb	7262.5 Aa	7013.6	9710.4 Ba	9697.2 Bb	9703.8	2.07 Bb	2.24 Aa	2.16
NB ₂₀	7733.3 Aa	7565.3 Aa	7649.3	11062.4 Aa	10584.9 Ab	10823.6	2.34 Aa	2.11 Ba	2.22
Mean	7249.0	7413.9		10386.4	10141.0		2.20	2.18	
NC ₀	6532.6 Bb	7223.6 Ba	6878.1	9279.1 Bb	9834.1 Ba	9556.6	2.16 Aa	2.25 Aa	2.20
NC ₁₀₀	7965.3 Aa	7604.7 Ab	7785.0	11493.7 Aa	10447.6 Ab	10970.6	2.24 Aa	2.10 Ba	2.17
Mean	7249.0	7414.2		10386.4	10140.8		2.20	2.18	

Means followed by same capital letter in the column, and regular letter on the row do not differ among themselves by Tukey test at 5%.

Caption: signs - INOC, + INOC represent the absence and presence of inoculation, NB₀, NB₂₀ represent nitrogen sowing fertilization doses, NC₀, NC₁₀₀ represent nitrogen topdressing fertilization doses.

positive responses are not always obtained of increased production as a function of seed inoculation with *Azospirillum* spp., as reported by Campos *et al.* (1999) for wheat and oat crops and Campos *et al.* (2000) for maize. By the other hand, it is agreed that plant genotype is the key factor to obtain the benefits derived from biological nitrogen fixation, combined with the selection of efficient strains.

The application of nitrogen, in topdressing or sowing, has positively influenced shoot dry biomass, as well as productivity, and base nitrogen fertilization has also provided an increase in leaf N content of plants (Table 5). These results highlight the significant effects of nitrogen on the development of maize crop, because nitrogen provides a significant increase in leaf area and in the production of dry matter weight, resulting in greater grain yield (Escosteguy *et al.*, 1997).

Increased leaf N content, found in this study, may have enhanced the effects of nitrogen in plant development, since the formation of grain in maize culture is closely related to the translocation of sugars (Crawford *et al.*, 1982) and nitrogen (Karlen *et al.* 1988) of vegetative organs, especially from the leaves, to the grains. Moreover, a higher content of nitrogen in leaves is associated with increased production of pigments and proteins necessary for photosynthesis and photochemical and carboxylate processes performance (Field & Mooney, 1986; Andreeva *et al.*, 1998), increasing the photosynthetically active leaf area of plant directly associated with crop yield.

On the other hand, the associations between inoculation and nitrogen fertilization, base or topdressing, have caused a reduction in leaf N content. Machado *et al.* (1998), have found similarities of leaf N contents, by comparing the isolated use of nitrogen topdressing fertilization (100 kg N ha⁻¹) in maize in relation to the use associated with the inoculation of diazotrophs.

The contribution of biological nitrogen fixation in grasses or food products, such as maize and wheat, in fertilization programs is an issue that needs further study. Research results are not always positive, as stated by Moreira & Siqueira (2002), increased yield in response to inoculation are generally about 5 to 30%, however it is not clear whether the expectation is overestimated or not, considering the tendency of not publishing results.

CONCLUSION

In both seasons, the ear insertion height and total plant height were not affected by the factors under study.

For the season 2000/2001, the increases in the production of shoot dry biomass and grain yield provided by the inoculation were 7.2 and 15.4%, respectively.

For the season 2002/2003, inoculation with *Azospirillum* spp. caused an increase of 7.4% in yield, compared with control, without nitrogen at sowing and without inoculation. In addition, nitrogen topdressing fertilization, associated with the use of inoculation, has reduced yield and the shoot dry biomass production.

Nitrogen fertilization at sowing has increased leaf N content; however, when complemented by inoculation, it caused a reduction of such a variable.

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