Revista Brasileira de Ciência do Solo

Division - Soil Use and Management | Commission - Soil Fertility and Plant Nutrition

Corn Yield and Foliar Diagnosis Affected by Nitrogen Fertilization and Inoculation with *Azospirillum brasilense*

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Received: September 25, 2015
Approved: October 16, 2015

How to cite: Galindo FS, Teixeira Filho MCM, Buzetti S, Santini JMK, Alves CJ, Nogueira LM, Ludkiewicz MGZ, Andreotti M, Bellote JLM. Corn Yield and Foliar Diagnosis Affected by Nitrogen Fertilization and Inoculation with *Azospirillum brasilense*. Rev Bras Cienc Solo. 2016;40:e0150364.

ABSTRACT: The biological nitrogen fixation (BNF) process in grasses is caused by diazotrophic bacteria, particularly *Azospirillum brasilense*. However, studies are lacking on BNF efficiency to define how much mineral nitrogen (N) can be applied to achieve more sustainable high yields. Furthermore, there should be an analysis of whether urea with the urease enzyme inhibitor NBPT is less harmful, benefiting BNF in grasses. The objective of this study was to evaluate the effect of N sources and N rates associated with inoculation with *Azospirillum brasilense* regarding foliar diagnosis and leaf chlorophyll index (LCI), agronomic efficiency (AE), and corn grain yield in the *Cerrado* (Brazilian tropical savanna) region. The experiment was conducted in a no-tillage system in a *Latossolo Vermelho Distroférrico* (Oxisol). A randomized block experimental design was used with four replications in a 2 × 5 × 2 factorial arrangement as follows: two N sources - urea and Super N, urea with urease enzyme inhibitor NBPT [N - (n-butyl thiophosphoric triamide)]; five N rates (0, 50, 100, 150, and 200 kg ha^{-1}) applied in topdressing; and two seed inoculation treatments, one with and one without *A. brasilense.* N rate positively influenced the LCI and concentrations of N, S, and Mn in leaves, and may increase the concentrations of P, Cu, and Fe; however, higher N rates can reduce AE. The N sources had similar effects, and therefore urea is recommended for N fertilization. Inoculation with *A. brasilense* decreased leaf concentration of Fe and increased LCI, leaf concentration of P, AE, and corn grain yield; the use of this diazotrophic bacterium is therefore viable even when high rates of N are applied.

Keywords: *Zea mays L.*, urease inhibitor, nitrogen rates, BNF, no-tillage system.

INTRODUCTION

Brazil is the world's third largest corn producer, despite the fact that, in general, Brazilian soil does not contain enough N for this crop to thrive. Nitrogen fertilization is one of the highest costs of the production process of non-leguminous crops (Nunes et al., 2015). Wheat, corn, and rice crops utilize approximately 60 % of the N fertilizer produced in the world (Espíndula et al., 2014). The use of N fertilizer must be carefully controlled to ensure good yield and manage N in the soil; N fertilizer increases production costs for farmers (Teixeira Filho et al., 2014).

Corn yield may be reduced because of NH_3-N volatilization. 10 kg ha^{-1} of grain is lost for each 1 % of N that is volatilized (Lara Cabezas et al., 2000). In the short term, urea is unlikely to be replaced by other sources of N because it has the lowest cost per kilogram of N.

The efficiency of N fertilization can be increased by the use an inhibitor, NBPT, which can slow urea hydrolysis and significantly reduce NH_3 losses, depending on weather conditions. Among urease inhibitors, NBPT [N - (n-butyl) thiophosphoric triamide] has provided the best results (Prando et al., 2013). Due to the climate in Brazil, urea with urease enzyme inhibitor and conventional urea are equally effective in terms of nutrition and yield of corn grains. Studies in countries with milder weather have had different results. In other words, urease-inhibiting action is not able to completely control the losses caused by NH_3 volatilization when urea is applied to the soil surface, considering that the effects of NBPT depend on the weather, that is, heat and rain, as well as the chemical characteristics of the soil (Cantarella et al., 2008). There is evidence of active urea transport by high affinity transporters (symport) located in the plasmatic membrane of the root epidermis cells, which would allow uptake of some urea applied before urease has acted and NH_3 has been formed, especially when urea concentration in the soil and soil pH are low (Liu et al., 2003).

Other factors related to the cost of N fertilizers are sustainability and pollution, areas in which research is being carried out. In addition, inoculants that contain bacteria can be used to promote growth and increase plant yield. In Brazil, many studies of biological nitrogen fixation (BNF) by *Azospirillum* in grass have been carried out. Until recently, no commercial inoculants with these bacteria were available in the country (Hungria, 2011).

Several studies have been published confirming that *Azospirillum* produces phytohormones that stimulate root growth in many plant species. The components released by *A. brasilense* responsible for stimulating root growth are indole-acetic acid (IAA), gibberellins, and cytokinins (Tien et al., 1979). The increase in root development caused by inoculation with *Azospirillum* is involved with several other effects. Increases in water and mineral uptake have been reported, as well as greater tolerance to stresses such as salinity and drought, resulting in a more vigorous and productive plant (Dobbelaere et al., 2001; Bashan et al., 2004). An improvement in leaf photosynthetic parameters, including chlorophyll content and stomata conductance, greater proline content in shoots and roots, improvement in water potential, an increase in water content in the apoplast, more elasticity of the cell wall, more biomass production, and greater plant size were reported by Barassi et al. (2008). Increases in photosynthetic pigments such as chlorophyll *a* and *b*, and auxiliary photoprotective pigments such as violaxantine, zeaxantine, ateroxantine, lutein, neoxantine, and beta-carotene, which result in greener plants without water related stress, were verified by Bashan et al. (2006). When studying BNF with ammonium release by diazatrophs in the root system of *Setaria viridis*, Pankievicz et al. (2015) verified an increase in root development and greater CO_2 fixation. When *A. brasilense* was introduced, plants grown in an environment with limited N developed in a manner similar to plants with sufficient N.

However, in most studies of seed inoculation with *Azospirillum* ssp., even though some benefits were observed, there was not always an increase in corn grain yield.

More experiments of this type should be carried out in order to evaluate the effect on plant nutrition. In addition, there are still few studies which define how much mineral N can be applied for BNF to be successful in increasing yield. It would be interesting to analyze urea with an NBPT urease enzyme inhibitor to verify whether it causes damage to BNF in grass.

The hypothesis of this study was that inoculation with *Azospirillum brasilense* increases the efficiency of N fertilization and plant nutrition. The objective was to evaluate the effect of N sources and N rates associated with inoculation with *Azospirillum brasilense* regarding foliar diagnosis and leaf chlorophyll index (LCI), agronomic efficiency, and corn grain yield in the Brazilian *Cerrado* region.

MATERIALS AND METHODS

The experiment was conducted in the 2013/14 and 2014/15 crop seasons in an experimental area belonging to the UNESP School of Engineering in Selvíria, MS, Brazil, at an altitude of 335 m. The soil in the experimental area was classified as a *Latossolo Vermelho Distroférrico* (Oxisol) with a clay texture (Santos et al., 2013). Annual crops have been grown there for 27 years, the last 10 years under a no-till system. Before corn was planted, the area was cropped to oat and wheat. The annual average temperature was 23.5 °C, annual average rainfall was 1,370 mm, and annual average relative humidity was between 70 and 80 %. Weather data recorded during the experimental period are shown in figure 1.

A randomized block experimental design in a 2 × 5 × 2 arrangement with four replications was used for both crops. There were two N sources - conventional urea with 45 % N and Super N, urea with a urease enzyme inhibitor, NBPT [N - (n-butyl thiophosphoric triamide, with 45 % N)]; five rates of N (0, 50, 100, 150, and 200 kg ha^{-1}) applied in topdressing; and two seed inoculation treatments - half of the tests were carried out with seeds inoculated with *A. brasilense*, while half did not have this inoculation. The experimental areas were composed of seven 6-m-long rows at a spacing of 0.45 m, with the five central rows being used for data collection, excluding 0.5 m on the edges.

Glyphosate [1,800 g ha^{-1} of active ingredient (a.i.) and 2,4-D (670 g ha^{-1} of a.i.)] herbicides were used for desiccation. Chemical properties of the soil in the tillable layer were determined before in 2013, before the corn experiment began. The methods proposed by Raij et al. (2001) showed the following results: 13 mg dm^{-3} of P (resin); 6 mg dm^{-3} of S-SO_4; 23 g dm^{-3} of organic matter (OM); pH($CaCl_2$) of 4.8; 2.6 $mmol_c$ dm^{-3} of K^+; 13.0 $mmol_c$ dm^{-3} of Ca^{2+}; 8.0 $mmol_c$ dm^{-3} of Mg^{2+}; 42.0 $mmol_c$ dm^{-3} of H+Al; 5.9 mg dm^{-3} of Cu; 30.0 mg dm^{-3} of Fe; 93.9 mg dm^{-3} of Mn; 1.0 mg dm^{-3} of Zn (DTPA); 0.24 mg dm^{-3} of B (hot water); and 36 % base saturation.

After soil chemical analysis, 2.5 Mg ha^{-1} of dolomitic limestone (with 88 % relative total neutralizing power) were directly applied as topdressing 65 days before the corn was sown in 2013 in order to elevate base saturation to 70 %, as recommended by Cantarella et al. (1997). 400 kg ha^{-1} of a 08-28-16 formulation were applied at sowing in both years of the experiment. This is equivalent to 32 kg ha^{-1} of N, 112 kg ha^{-1} of P_2O_5, and 64 kg ha^{-1} of K_2O, based on soil analysis and the requirement for corn growing.

The experiments were conducted in a no-tillage system. The area in both crops was irrigated by a central pivot sprinkler system. The water coverage was 14 mm over a period of around 72 h.

Corn seeds were inoculated with 200 mL of liquid *Azospirillum brasilense* bacteria AbV5 and AbV6 inoculant (guaranteed minimum analysis of 2 × 10^8 UFC mL^{-1}) per hectare. A cement mixer was used to mix the inoculant with the seeds 1 h before the seeds were planted. DKB 350 VT PRO triple hybrid seed corn (resistant to fall army worm *Spodptera frugiperda*) was used. The corn was mechanically sown on December 4, 2013 for the

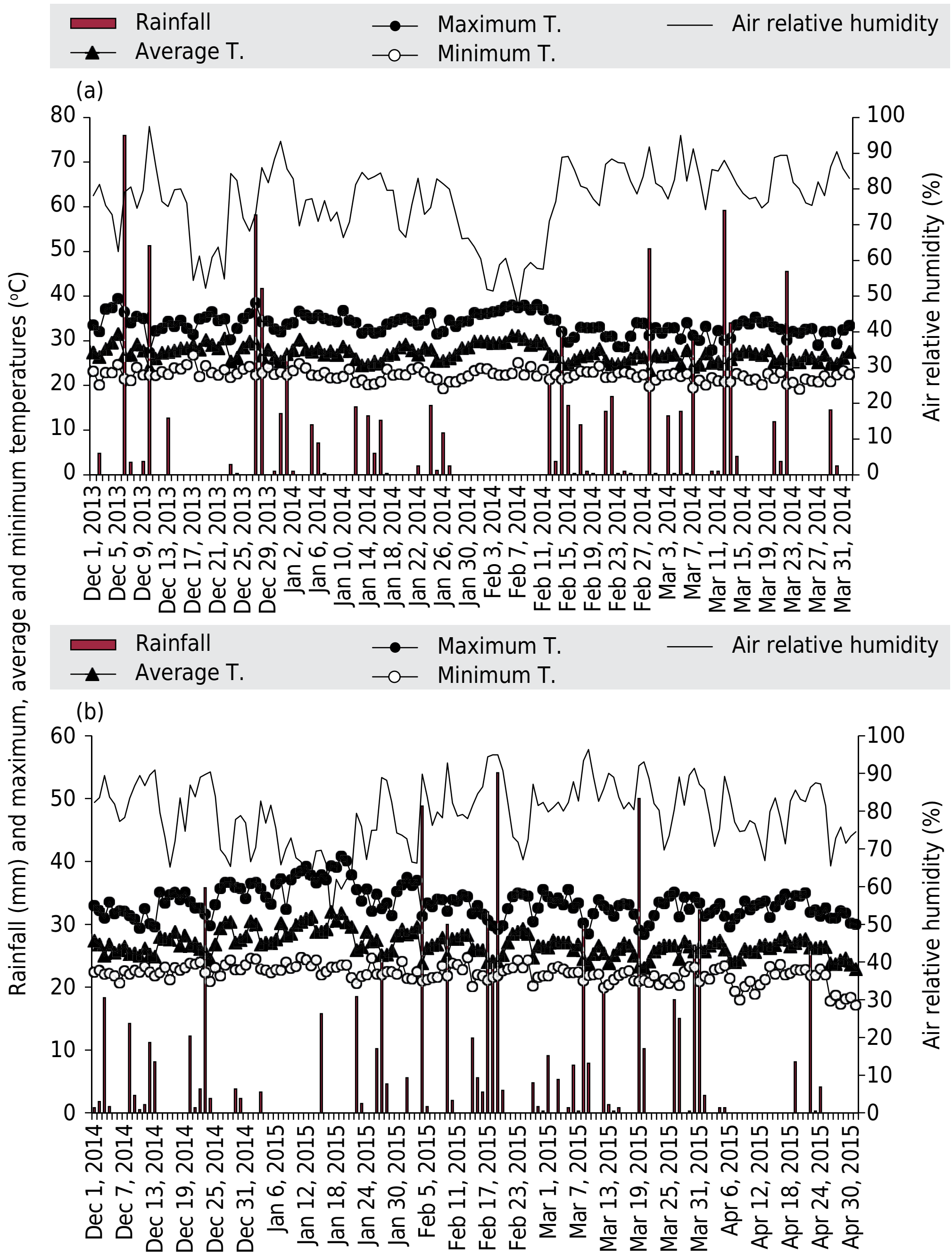

Figure 1. Rainfall, air relative humidity, and maximum, average, and minimum temperature obtained from the weather station located on the Education and Research Farm of FE / UNESP during corn cultivation in the period of December 2013 to April 2014 (a) and December 2014 to April 2015 (b).

2013/14 crop season and on December 2, 2014 for the 2014/15 crop season. Three seeds were planted per meter. Seedlings emerged five days after sowing, on December 4, 2013 and December 7, 2014. Two herbicides were applied to control weeds after the corn had sprouted: Tembotrione (84 g ha^{-1} of a.i.) and Atrazine (1,000 g ha^{-1} of a.i.). A vegetable oil adjuvant was added (720 g ha^{-1} of a.i.) to the herbicide tank mix on January 2, 2013 and December 28, 2014. Metomil (215 g ha^{-1} of a.i.) and Triflumurom (24 g ha^{-1} of a.i.) were applied to control insects on January 15, 2014 and January 11, 2015.

Nitrogen fertilizer was applied as topdressing without incorporation between the corn rows on January 8, 2014 and January 4, 2015 when the plants had six fully open leaves (V6). Fertilizer was manually distributed over the soil surface approximately 0.10 m from the rows in order to avoid contact between the fertilizer and the plants. The plants were harvested on March 28, 2014 and April 1, 2015, 108 and 120 days after corn emergence.

Concentrations of N, P, K, Ca, Mg, S, Cu, Fe, Mn, and Zn were measured in corn plant leaves. The middle third of 20 ear leaves in the female flower from plants were collected using the method described by Cantarella et al. (1997). The leaf chlorophyll index (LCI) was determined indirectly after application of the treatments and when the plants were in the flowering stage in 10 plants per plot through readings in the leaf below the ear (in the middle third of each leaf). The corn was harvested from the plants in the useful area of each plot and grain yield was calculated after mechanical threshing. Data was transformed into kg ha^{-1} and corrected for 13 % moisture (wet basis). The agronomic efficiency of the treatments was determined:

AE = (grain yield with fertilizer - grain yield without fertilizer) / amount of N applied.

The results were subjected to analysis of variance and the Tukey test at 5 % probability to compare the averages of N sources and plants that had been inoculated with *Azospirillum brasilense* with those that had not been inoculated. Regression equations were fitted for the effect of N rates using the Sisvar program. SAS software was used for Pearson correlation analyses.

RESULTS AND DISCUSSION

The increase in N rates influenced concentrations of N, P, and S in leaves (Tables 1 and 2). A quadratic function for N concentration was fitted in the 2013/14 and 2014/15 crops. The maximum concentration was obtained from 155 kg ha^{-1} of N in 2013/14 and 185 kg ha^{-1} of N in 2014/15 (Figures 2a and 2b). Mar et al. (2003), Soratto et al. (2010), and Costa et al. (2012) also verified that N rates had a positive linear effect on leaf tissue. It should be noted that N concentration was considered adequate (27-35 g kg^{-1}), except for the control treatment (Cantarella et al., 1997).

Concentration of P was influenced by N rates in the 2013/14 crop (Table 1) when the quadratic function was fitted. Maximum concentration occurred at 144 kg ha^{-1} (Figure 2c). Kappes et al. (2013a) also verified a linear increase in P concentration with an increase in N rates in topdressing. The root system develops better when N fertilizer is used, which improves diffusion between the phosphate and roots in the soil. This leads to greater nutrient uptake, which is reflected in P concentration in leaves. Average P concentrations were found to be adequate (between 2.0 and 4.0 g kg^{-1}) (Cantarella et al., 1997). This result can be attributed to dissolved P from the phosphate fertilizer and adequate nutrient contents in the soil. Casagrande and Fornasieri Filho (2002) had similar results: they evaluated N rates of 0, 30, 60, and 90 kg ha^{-1} and verified that leaf P concentration at flowering in corn plants was lower (though still considered adequate) when N fertilizer was not applied than in treatments that received phosphate fertilization. This is because the root system develops better at higher N rates, which favors contact by diffusion of phosphate with corn roots.

The N rate had a linear influence on S concentration in both years of the study (Figures 2d and 2e). Soratto et al. (2010) likewise affirmed that S concentration increased up to the estimated maximum application rate of 65.8 kg ha^{-1} of N, regardless of the source used. Casagrande and Fornasieri Filho (2002) also verified that S concentration in corn leaves increased along with N rates when N was applied in the form of urea. However, S concentrations in all treatments were within the range considered adequate for the crop (Cantarella et al., 1997), which was 1.5 to 3.0 g kg^{-1}. The ratios between N and S concentrations were between 12 and 15 to 1, within the range indicated by Arnon (1975). This promoted maximum production potential for dry matter and protein weight.

Concentration of K was not influenced by N rates in topdressing. Casagrande and Fornasieri Filho (2002) and Kappes et al. (2013a) also found that N rates did not influence K concentration in corn leaves. Average K concentrations (Table 1) were below recommended values (Cantarella et al., 1997), which range from 17.0 to 35.0 g kg^{-1}. Adequate K

Table 1. Means and Tukey test concerning nitrogen, phosphorus, potassium, calcium, and magnesium leaf concentrations of corn affected by rates and sources of nitrogen, with or without inoculation by *Azospirillum brasilense* (2013/14 and 2014/15)

	N		P		K		Ca		Mg	
	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15
	g kg^{-1}									
N rate (R) (kg ha^{-1})										
0	26.50	24.51	3.61	3.48	13.50	15.42	2.61	2.56	1.00	1.04
100	30.82	29.28	4.04	3.43	13.83	15.63	2.80	2.17	1.07	1.00
150	30.89	32.08	4.15	3.36	14.83	15.42	2.96	2.23	1.06	1.02
200	30.58	31.50	3.98	3.30	14.33	16.25	2.90	2.22	1.09	0.98
N source (S)										
Urea	29.20 a	28.87 a	3.88 a	3.51 a	13.73 a	15.42 a	2.68 a	2.35 a	1.04 a	1.04 a
Super N	29.68 a	29.27 a	3.96 a	3.40 a	14.07 a	15.58 a	2.79 a	2.26 a	1.04 a	0.96 a
LSD (5 %)	0.89	1.19	0.11	0.26	1.79	1.33	0.36	0.24	0.09	0.09
Inoculation (I)										
With *Azospirillum*	29.63 a	28.97 a	3.98 a	3.67 a	13.87 a	14.92 a	2.76 a	2.39 a	1.05 a	1.04 a
Without *Azospirillum*	29.25 a	29.18 a	3.86 b	3.24 b	13.93 a	16.08 a	2.71 a	2.22 a	1.03 a	0.96 a
LSD (5 %)	0.89	1.19	0.11	0.26	1.79	1.33	0.36	0.24	0.09	0.09
Overall mean	29.44	29.07	3.92	3.45	13.90	15.50	2.73	2.31	1.04	1.00
CV (%)	5.81	7.84	5.29	14.20	24.66	16.47	25.04	19.96	16.19	17.76
F Test										
R	15.398**	21.357**	12.462**	1.184^{ns}	0.519^{ns}	0.503^{ns}	1.310^{ns}	1.411^{ns}	0.969^{ns}	0.371^{ns}
S	1.162^{ns}	0.465^{ns}	2.066^{ns}	0.792^{ns}	0.142^{ns}	0.064^{ns}	0.440^{ns}	0.488^{ns}	0.000^{ns}	2.937^{ns}
I	0.733^{ns}	0.127^{ns}	4.880*	11.614**	0.006^{ns}	3.132^{ns}	0.087^{ns}	2.219^{ns}	0.159^{ns}	3.244^{ns}
R × S	1.016^{ns}	0.580^{ns}	2.608^{ns}	0.351^{ns}	0.043^{ns}	0.903^{ns}	1.439^{ns}	0.632^{ns}	1.599^{ns}	2.070^{ns}
R × I	0.163^{ns}	0.379^{ns}	1.348^{ns}	1.485^{ns}	1.098^{ns}	0.535^{ns}	0.613^{ns}	0.281^{ns}	0.309^{ns}	0.220^{ns}
S × I	0.014^{ns}	0.018^{ns}	0.906^{ns}	0.746^{ns}	3.280^{ns}	1.023^{ns}	1.644^{ns}	0.020^{ns}	0.008^{ns}	3.088^{ns}
R × S × I	0.338^{ns}	1.261^{ns}	0.835^{ns}	0.685^{ns}	0.644^{ns}	1.382^{ns}	0.434^{ns}	1.593^{ns}	1.190^{ns}	2.096^{ns}

Means followed by the same letter in the column do not differ by the Tukey test at 5 %. **, * and ^{ns}: significant at $p<0.01$, $0.01<p<0.05$, and not significant, respectively.

concentrations were expected to be obtained in corn since K fertilizer was applied, and K soil contents in the experimental area were within the appropriate range of availability. Potassium is not part of any cellular compound in the plant (Malavolta et al., 1997) and 100 % of K that comes from crop residues is released (Calonego et al., 2005), which implies that it should be readily available to the plant. Either there was not enough K from these sources or the K demand of this triple hybrid is lower than that of varieties studied in the 1990s that were used to establish the range for the diagnosis.

Nitrogen rates did not influence P in leaf tissue in the 2014/15 crop; and N rates did not influence concentrations of Ca, Mg, and K in either year (Table 1). It should be noted that Ca had average concentrations within the value considered to be adequate (Cantarella et al., 1997), which ranges from 2.5 to 8.0 g kg^{-1}, whereas the average concentration for Mg was below the adequate value (Cantarella et al., 1997), which ranges from 1.5 to 5.0 g kg^{-1}.

The sources of N did not differ in regard to concentrations of N, P, K, Ca, Mg, and S in leaves, indicating that Super N was not an efficient source for nutrition with N, even in the area with residual oat or wheat straw (Tables 1 and 2). When ammonium sulfate nitrate was applied, it led to greater N concentrations than ammonium sulfate and starea sources (Soratto et al., 2010); however, it did not differ from urea, and S concentration was not influenced by the source, as found in the current study. In contrast, when studying ammonium sulfate nitrate, ammonium sulfate, and urea as

Table 2. Means and Tukey test concerning sulfur, cooper, iron, manganese, and zinc leaf concentrations of corn affected by of rates and sources of nitrogen, with or without inoculation with *Azospirillum brasilense* (2013/14 and 2014/15)

	S		Cu		Fe		Mn		Zn	
	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15
	g kg^{-1}		mg kg^{-1}							
N rate (R) (kg ha^{-1})										
0	2.08	2.03	11.25	38.00	147.75	102.67	60.25	62.92	20.33	18.92
50	2.10	2.23	11.83	38.75	147.08	125.42	59.83	61.92	16.25	19.67
100	2.21	2.28	13.92	38.17	151.75	119.46	66.17	64.13	17.58	18.75
150	2.18	2.39	14.08	46.33	151.50	127.67	67.00	67.83	17.17	19.67
200	2.21	2.41	13.67	35.33	147.08	125.50	64.17	68.08	16.92	19.08
N source (S)										
Urea	2.15 a	2.39 a	13.03 a	40.67 a	150.30 a	119.48 a	63.53 a	64.55 a	17.60 a	19.43 a
Super N	2.16 a	2.27 a	12.87 a	37.97 a	147.77 a	120.80 a	63.43 a	65.40 a	17.70 a	19.00 a
LSD (5 %)	0.08	0.13	0.94	4.92	17.93	9.02	3.37	3.27	1.95	1.26
Inoculation (I)										
With *Azospirillum*	2.24 a	2.10 b	13.23 a	37.17 a	133.87 b	110.88 b	64.53 a	66.58 a	16.93 a	16.80 b
Without *Azospirillum*	2.07 b	2.43 a	12.67 a	41.47 a	164.20 a	129.40 a	62.43 a	63.37 a	18.37 a	21.63 a
LSD (5 %)	0.08	0.13	0.94	4.92	17.93	9.02	3.37	3.27	1.95	1.26
Overall mean	2.16	2.27	12.95	39.32	149.03	120.14	63.48	64.98	17.65	19.22
CV (%)	6.83	10.68	13.88	23.95	23.02	14.36	10.16	9.64	21.11	12.57
F Test										
R	2.240^{ns}	4.785**	6.377**	2.317^{ns}	0.058^{ns}	4.222**	3.162*	2.460^{ns}	2.146^{ns}	0.376^{ns}
S	0.022^{ns}	1.302^{ns}	0.129^{ns}	1.233^{ns}	0.082^{ns}	0.087^{ns}	0.004^{ns}	0.276^{ns}	0.011^{ns}	0.483^{ns}
I	18.547**	27.645**	1.490^{ns}	3.128^{ns}	11.726**	17.274**	1.592^{ns}	3.959^{ns}	2.219^{ns}	60.099**
R × S	0.834^{ns}	0.498^{ns}	0.451^{ns}	1.545^{ns}	0.307^{ns}	1.190^{ns}	0.806^{ns}	2.154^{ns}	0.656^{ns}	0.033^{ns}
R × I	0.654^{ns}	1.531^{ns}	0.137^{ns}	1.287^{ns}	0.706^{ns}	0.931^{ns}	1.096^{ns}	0.967^{ns}	0.692^{ns}	0.507^{ns}
S × I	3.490^{ns}	2.012^{ns}	0.129^{ns}	0.205^{ns}	0.178^{ns}	0.148^{ns}	0.020^{ns}	0.566^{ns}	0.529^{ns}	0.003^{ns}
R × S × I	1.109^{ns}	0.392^{ns}	0.090^{ns}	1.324^{ns}	0.235^{ns}	0.431^{ns}	1.836^{ns}	0.514^{ns}	0.298^{ns}	0.067^{ns}

Means followed by the same letter in the column do not differ by the Tukey test at 5 %. **, * and ^{ns}: significant at p<0.01, 0.01<p<0.05, and not significant, respectively.

N sources in corn growing, Meira et al. (2009) verified that ammonium sulfate nitrate led to a greater N concentration in leaves than other sources, which is different from the results obtained in this study.

Super N inhibits the urease enzyme, while ammonium sulfate nitrate contains DMPP (dimethylpirazolphosphate) and inhibits nitrification. This makes the fertilizer less susceptible to leaching because N remains in the soil for longer periods in the form of ammonium (Meira et al., 2009). In a tropical climate and at high temperatures, ammonium sulfate nitrate has different responses than Super N. A small amount of urea may be taken up before the action of urease and NH_3 formation, which might explain the similar results obtained from different N sources (Liu et al., 2003).

Plants grown from seeds that were inoculated with *A. brasilense* had significantly different leaf P and S concentrations from those that were not inoculated. Inoculation led to greater P concentrations in leaves in the 2013/14 and 2014/15 crop seasons, and greater S concentrations in 2013/14. For 2014/15, plants that had been inoculated had lower S concentration (Table 2). For N, K, Ca, and Mg, inoculation did not seem to have a significant effect (Table 1).

The root system of *Setaria viridis* grass inoculated with *A. brasilense* developed better and grew more because of associative fixation, with more CO_2 fixation and less accumulation of

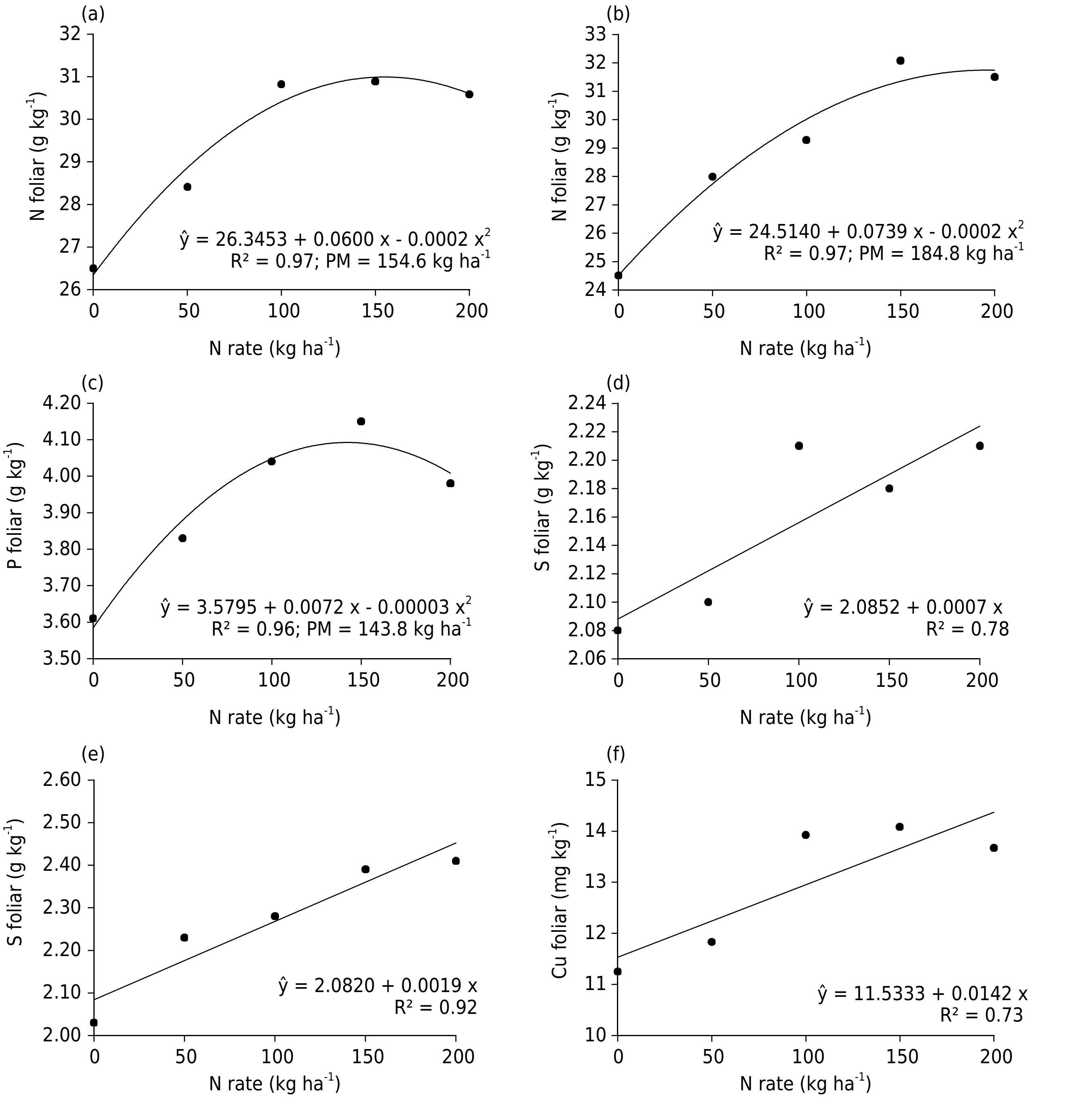

Figure 2. Concentrations of leaf nitrogen in 2013/14 (a) and 2014/15 (b), leaf phosphorus in 2013/14 (c), leaf sulfur in 2013/14 (d) and 2014/15 (e), and leaf cooper in 2013/14 (f) of corn affected by nitrogen rates.

photo-assimilated C in the leaves. This leads to greater growth of plant shoots, more water accumulation, and less stress from C accumulation and metabolism, and more nutrients are taken up by the plant (Pankievicz et al., 2015). Bashan et al. (2004) noted that bacteria of the *Azospirillum* genus produce plant hormones such as indole-acetic acid (IAA), which play an essential role in plant growth. They can improve the uptake of several macro and micronutrients, increasing plant efficiency in using available nutrients, which can help to explain an increase in P and S concentration in leaf tissue (Hungria et al., 2010). Another possible explanation for the increase in P concentration in leaves is related to the ability of some endophytic bacteria to promote plant growth through phosphate dissolution (Collavino et al., 2010).

Inoculation of bacteria with the ability to dissolve phosphate in the soil means there may be dissolved or precipitated P in a form which the plant is not able to take up (non-labile P), and this may result in greater nutrient concentration in the shoots, with better plant development and productive capacity (Canbolat et al., 2009; Dias et al., 2009).

Sulfur oxidation is one of several microbiological and biochemical transformation processes by bacteria that live in the soil. This may cause an increase in SO_4^{2-} availability to plants and a decrease in soil pH (Moreira and Siqueira, 2002; Stamford et al., 2005). This may increase the solubility of inorganic P compounds and reduce sulfate, culminating in SO_4^{2-} losses in the soil. This would probably lead to the uptake and concentration of these nutrients in leaves, corroborating the results observed in this study.

Nitrogen also affected cationic micronutrients. Increasing N rates led to increases in Cu concentration in leaves in the 2013/14 crop, Fe in the 2014/15 crop, and Mn in the 2013/14 and 2014/15 crops (Table 2) (Figures 2f, 3a, 3b, and 3c). Only Zn concentration in leaves was not altered by N rates in either harvest. This result was observed since N influences growth and development of the corn crop. Nitrogen induces the plant to develop its root system, which culminates in more utilization of the soil volume by the roots and greater nutrient uptake. Note that Oxisols generally have high Cu, Fe, and Mn contents, as in this study. The application of lime before the experiment raised soil pH. Nitrogen fertilizer can lead to acidification in the surface layer of the soil, which may have increased the availability of these micronutrients and consequently increased the uptake of these cationic micronutrients.

The adequate concentration of copper in leaves is 6-20 mg kg^{-1}; for Fe, 30-250 mg kg^{-1}; for Mn, 20-200 mg kg^{-1}; and for Zn, 15-100 mg kg^{-1} (Cantarella et al., 1997). All of these micronutrients had average concentrations. Neither macronutrient concentrations nor micronutrient concentrations (Cu, Fe, Mn and Zn) were influenced by the different sources of N (Table 2).

Inoculation with *A. brasilense* influenced leaf Fe and Zn concentrations (Table 2). Inoculation led to lower Fe values in both crops. The effect on Zn concentration was similar in the 2014/15 crop, in which inoculation with *A. brasilense* led to a reduction in Zn concentration. Some bacteria can produce and secrete molecules with low molecular weight (siderophores) which have a high affinity with Fe (Gray and Smith, 2005; Souza et al., 2013). These bacteria are capable of providing enough Fe to the plant when the amount of Fe in the soil is small. Fe may be sequestered by the soil, which would decrease its uptake by plants, and consequently decrease the concentration of this micronutrient in leaf tissue. It is also possible that Fe and Zn are precipitated when more P is taken up in leaf tissue since these nutrients have an antagonistic effect in the soil and the plant (De Muner et al., 2011). Inoculation with *A. brasilense* leads to more P uptake in leaf tissue.

This negative effect on Fe and Zn concentrations in leaves may also occur because these nutrients are immobilized by bacteria, which reduces their availability to plants (Moreira and Siqueira, 2002; Stamford et al., 2005), or because of a possible change in the form of Fe and Zn in the soil.

The increase in N rates had a positive linear effect on LCI in both crops (Table 3; Figures 3d and 3e). Nitrogen affects the LCI because N is one of the components of the chlorophyll molecule. It is reasonable to expect that an increase in N rates also increases leaf chlorophyll content. There have been several studies on N rates in topdressing of corn crops in which N had a positive linear correlation with LCI. Costa et al. (2012) used up to 200 kg ha^{-1} of N with urea as the N source and Kappes et al. (2013b) used 0, 60, 90, and 120 kg ha^{-1} of N with urea as an N source in topdressing. Note that the LCI values are relatively high even in the control crops. Costa et al. (2012) verified LCI values ranging from 39.9 to 71.2, and Kappes et al. (2013b) verified LCI values ranging from 51.1 to 68.5.

The different sources of N did not have a discernible effect on LCI and AE in the 2014/15 crop (Table 3), which, in part, is because there are similar concentrations of leaf nutrients obtained with urea and Super N. This may be because the highly active urease enzyme caused NBPT to act inefficiently. Some straw remained in the soil from the previous year's wheat crop and the year was exceptionally hot (Figure 1). Another possible explanation is the uptake of a small part of the urea applied before urease began to have an effect and form NH_3 (Liu et al., 2003).

Similarly, there have been studies that compared urea coated with polymers to conventional urea. There were no significant differences; yield was the same for urea and urea coated with slow-release polymers for corn grown in an Oxisol with a clay texture (Queiroz et al., 2011). Valderrama et al. (2011) compared the effect of traditional urea with urea coated with soluble polymers and found that encapsulated urea had no advantage over conventional urea in corn grown in an Oxisol with a clay texture. Meira et al. (2009) studied corn using ammonium sulfate, ammonium sulfate nitrate, and urea as N sources, and Goes et al. (2014) used urea, ammonium sulfate, and ammonium nitrate. Neither observed significant differences in grain yield. Since different sources of N had no effect on the analyses carried out, urea is the most advantageous source because it has a better cost- benefit ratio (Queiroz et al., 2011).

Different N sources and different N rates had a significant effect on AE in the 2013/14 crop (Table 3). Urea was more efficient than Super N at an application rate of 50 kg ha^{-1} (Table 4),

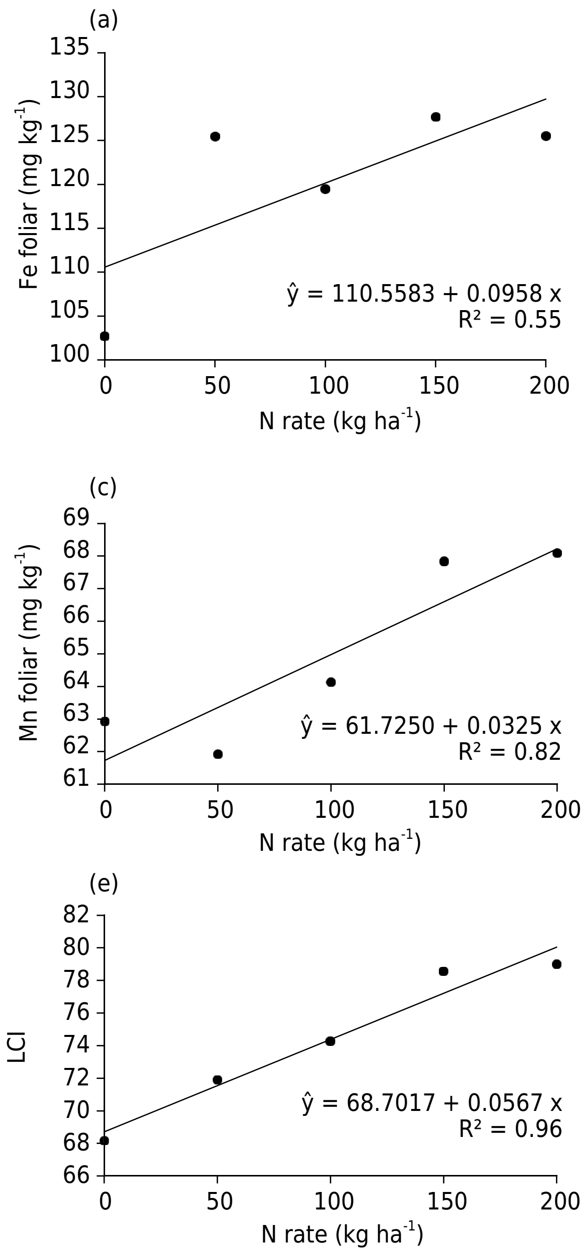

Figure 3. Concentrations of leaf iron in 2014/15 (a), leaf manganese in 2013/14 (b) and 2014/15 (c), and the leaf chlorophyll index (LCI) of corn affected by nitrogen rates in 2013/14 (d) and 2014/15 (e).

Table 3. Mean and Tukey test concerning leaf chlorophyll index (LCI), agronomic efficiency (AE), and grain yield of corn affected by rates and sources of nitrogen, with or without inoculation with *Azospirillum brasilense* (2013/14 and 2014/15)

	LCI		AE		Grain yield	
	2013/14	2014/15	2013/14	2014/15	2013/14	2014/15
			kg grain kg^{-1} N applied		kg ha^{-1}	
N rate (R) (kg ha^{-1})						
0	54.63	68.16	-	-	8215	5611
50	57.07	71.88	18.69	11.48	8714	5553
100	61.03	74.28	12.09	14.44	9213	6323
150	62.15	78.55	8.23	10.96	9126	6863
200	62.56	78.99	5.03	10.09	9186	7265
N source (S)						
Urea	59.32 a	73.42 a	14.03	8.17 a	8936 a	6178 a
Super N	59.66 a	75.32 a	7.99	15.32 a	8845 a	6468 a
LSD (5 %)	1.88	2.70	6.36	8.06	451	802
Inoculation (I)						
With *Azospirillum*	61.68 a	81.70 a	15.95	19.34 a	9254	6972
Without *Azospirillum*	57.30 b	67.04 b	6.08	4.15 b	8528	5674
LSD (5 %)	1.88	2.70	6.36	8.06	451	802
Overall mean	59.49	74.37	11.01	11.74	8891	6323
CV (%)	6.03	6.95	54.51#	64.90#	11.33	28.31
F Test						
R	11.259**	9.402**	7.407*	0.146^{ns}	2.891*	2.847*
S	0.137^{ns}	2.043^{ns}	3.723^{ns}	3.113^{ns}	0.164^{ns}	0.528^{ns}
I	22.322**	120.780**	9.259**	17.064**	10.398**	10.509**
R × S	0.119^{ns}	0.707^{ns}	8.031**	1.223^{ns}	1.140^{ns}	0.410^{ns}
R × I	0.563^{ns}	0.624^{ns}	6.181*	1.233^{ns}	2.750*	2.671*
S × I	1.104^{ns}	0.036^{ns}	0.187^{ns}	0.481^{ns}	0.020^{ns}	0.005^{ns}
R × S × I	1.053^{ns}	0.540^{ns}	0.972^{ns}	0.710^{ns}	0.640^{ns}	1.645^{ns}

Means followed by the same letter in the column do not differ by the Tukey test at 5 %. **, * and ^{ns}: significant at p<0.01, 0.01<p<0.05, and not significant, respectively. #: data fitted by following equation $(x+0.5)^{0.5}$.

Table 4. Sources, inoculation, and nitrogen rate interaction for agronomic efficiency (AE) of corn (2013/14)

	N rate			
	50	100	150	200
	kg ha^{-1}			
N source#				
Urea**	26.67 a	13.70 a	10.57 a	5.18 a
Super N^{ns}	10.72 b	10.48 a	5.90 a	4.87 a
LSD (5 %)	12.72			
Inoculation#				
With *Azospirillum***	26.05 a	19.60 a	11.18 a	6.95 a
Without *Azospirillum*^{ns}	11.33 b	4.58 b	5.28 a	3.10 a
LSD (5 %)	12.72			

Means followed by the same letter in the column do not differ by the Tukey test at 5 %. **, * and ^{ns}: significant at p<0.01, 0.01<p<0.05, and not significant, respectively. #: data fitted by following equation $(x+0.5)^{0.5}$.

possibly because Super N reduces the availability of N for a period of time. Urea releases N into the soil faster. This N is very important for plant nutrition, since the corn was planted over black oat straw, which has a high C/N ratio. This can cause partial microbial immobilization of applied N.

The AE decreased linearly only when urea was used to increase N in topdressing (Figure 4a). This result can be attributed to the loss of N, as clearly described in the literature. Greater N rates result in greater losses and less utilization by the crops since plant nutritional demand is limited. Plants are able to absorb a certain quantity of nutrients in a certain time; the N that is applied and is not taken up can be lost, decreasing the efficiency of fertilization with higher N rates, as stated in the literature as the law of diminishing returns.

Plants that were inoculated with *A. brasilense* had greater LCI, AE, and grain yield than those that were not inoculated, for both crop seasons (Table 3). Kappes et al. (2013c) and Quadros et al. (2014) found that plants that were inoculated with *A. brasilense* had improved LCI. The authors found that the LCI was higher in the treatments with diazotrophs than in the treatments without inoculation, corroborating the data from the current study.

The results obtained for N concentration in leaves, even in control crops (without N application), did not seem to be related only to biological N_2 fixation, but also to growth promotion mechanisms that can increase the plant's capacity to take up N from the soil (Dobbelaere et al., 2001). This happens because crops that have been inoculated with *A. brasilense* have greater CO_2 fixation, improving the ability of some mutant strains to increase BNF and positively influence the C metabolism of C4 plants, which is closely related to the N assimilation metabolism in the plant. However, it is essential to study the effect of different *A. brasilense* strains and corn genotypes in order to deepen discussion in regard to this subject (Pankievicz et al., 2015).

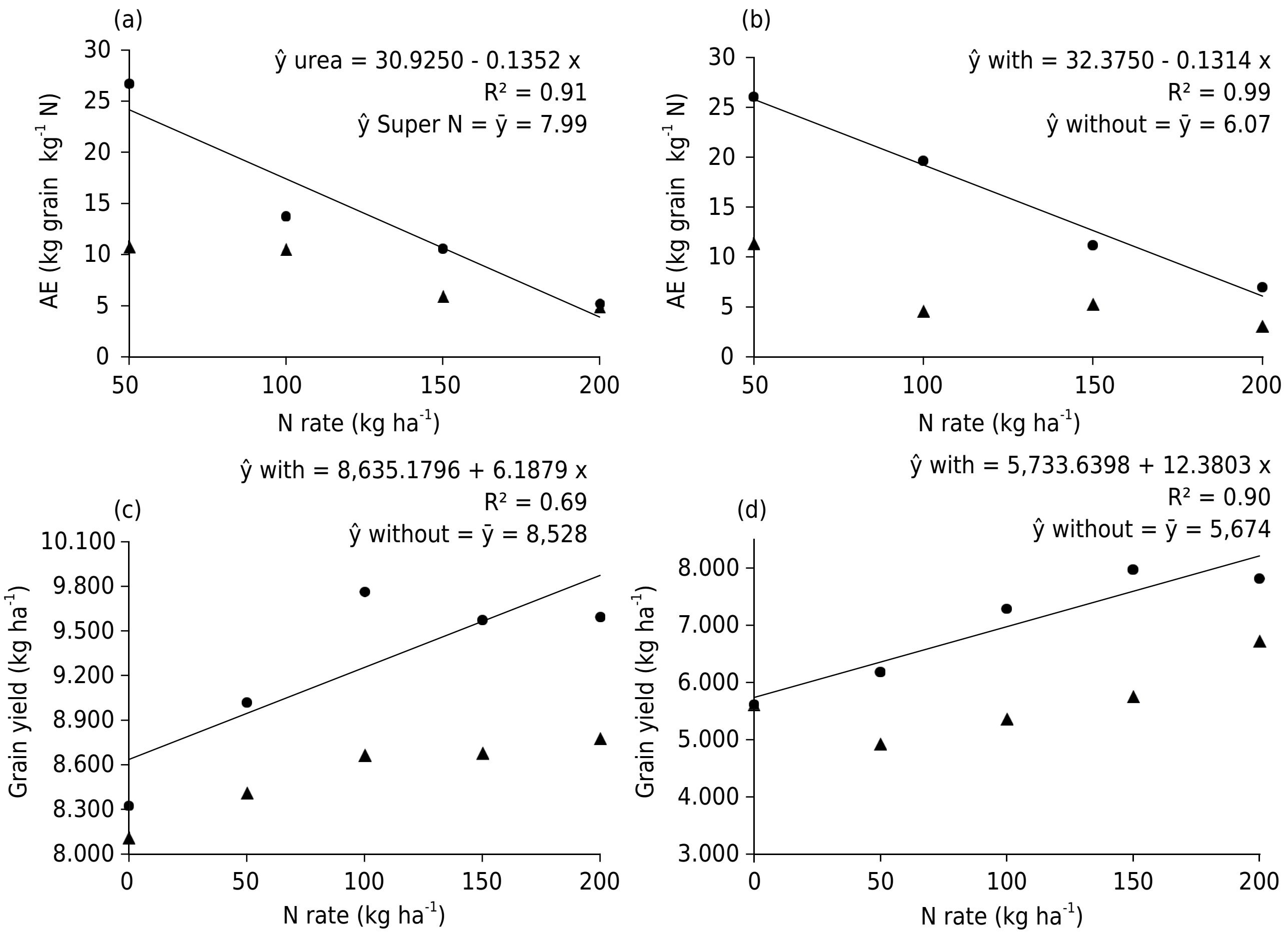

Figure 4. Agronomic efficiency (EA) of corn in 2013/14 (a) and 2014/15 (b) in regard to rate and source of nitrogen (N) interaction within inoculation, and corn grain yield in 2013/14 (c) and 2014/15 (d) in regard to rate of nitrogen (N) interaction within inoculation.

Nitrogen rates and inoculation with *A. brasilense* had a significant effect on AE in the 2013/14 crop at N rates of 50 and 100 kg ha^{-1}. Inoculated treatments had higher AE than non-inoculated treatments, which is a very interesting result (Table 4), since it indicates that less N was lost when plants had been inoculated with this diazotroph. The AE linear function decreased only with *A. brasilense* at higher N rates (Figure 4b), which can be explained by higher AE obtained with the rates of 50 and 100 kg ha^{-1} of N, as mentioned earlier. When Pankievicz et al. (2015) studied BNF with release of ammonium by diazotrophs, they verified that the root system of *Setaria viridis* grew more and developed faster and that there was more CO_2 fixation when the plant had been inoculated with *A. brasilense*. Plants grown in an environment with limited nitrate developed in a similar manner to those grown with enough N, elucidating the ability of some mutant strains to increase BNF. Agronomic efficiency was greater in the inoculated treatments with lower N rates. This may have been because conditions in the experimental area were favorable to microbial immobilization of applied N.

Agronomic efficiency responds positively to inoculation with *A. brasilense* even when the crops are grown in soil that contains large amounts of available N (Dobbelaere et al., 2003). This indicates that plant respond not only to fixed N_2, but also to the production of phytohormones that promote growth, such as cytokinin, gibberellin, and indole-acetic acid. This phenomenon may affect corn root development, which, according to Novakowiski et al. (2011), would improve the efficiency of utilization of residual N, water uptake, and uptake of other nutrients, directly increasing the agronomic efficiency of corn crops that have been inoculated with *A. brasilense*, as observed in the current study.

There was significant interaction among N rates, inoculation, and grain productivity for both crop seasons. The treatment of inoculation with *A. brasilense* at an application rate of 100 kg ha^{-1} of N in 2013/14 was superior to the treatment without inoculation (Table 5), while the treatments of inoculation with *A. brasilense* in 2014/15 were superior to the treatments without inoculation with rates of 100 and 150 kg ha^{-1} of N (Table 5). Note that corn yield in both crop seasons was always greater in plants that had been inoculated with this diazotroph. In both harvests, the linear function only increased in treatments of inoculation with *A. brasilense* because of the increase in N rates (Figures 4c and 4d), once more demonstrating the feasibility of this technology.

Positive results for grain yield did not often occur with the use of *Azospirillum*. Kappes et al. (2013c) studied N rates and *A. brasilense* inoculation in first crop corn. According to these authors, inoculation led to a non-significant increase of 9.4 % in grain yield. However, Cavallet et al. (2000) obtained a significant increase in grain yield of 17 % when N was applied in topdressing of a corn crop with seeds that were inoculated with *Azospirillum* spp. Bulla and Balbinot Júnior (2012) observed a 4.5 % increase in corn grain yield in plants that had been inoculated with *A. brasilense*, as an average of five different N rates that were applied in topdressing, with average grain yield greater than 12,500 kg ha^{-1}. Similar results were obtained by Novakowiski et al. (2011), in which the corn yield with *A. brasilense* inoculation was superior to the control, even with an increase in the amount of N applied. Increases in corn yield were also obtained by Hungria et al. (2010) and, depending on the *A. brasilense* strain used, the yield increase was from 24 % (or 662 kg ha^{-1}) to 30 % (or 823 kg ha^{-1}). The average increase in grain yield due to inoculation in 2013/14 was 727 kg ha^{-1}, or twelve 60 kg sacks, that is, an 8.5 % increase in grain yield. The average increase in grain yield due to inoculation in 2014/15 was 1,298 kg ha^{-1}, or 22 60 kg sacks, that is, a 22.9 % increase in grain yield (Table 3).

Some authors found different results. For instance, Pandolfo et al. (2015) did not observe a positive effect from inoculation when they studied N rates in topdressing (0, 25, 50, 75, 100, and 125 kg ha^{-1}) and *A. brasilense* inoculation in corn crops. Still, according to these authors, the fact that corn did not respond to *A. brasilense* inoculation in the study environment confirmed the observations of a survey by Okon and Labandera-Gonzales

(1994) in experiments conducted over 20 years, in which they verified that in 30 to 40 % of cases, inoculation did not result in grain yield increases. Hungria (2011) observed that the effects of inoculation of corn seeds on grain yield depend on the genetic characteristics of the plant and strain, in addition to environmental conditions.

Pearson linear correlation analysis revealed a positive correlation between the concentrations in leaf tissue of N and P (2013/14 and 2014/15 crops), P and S (2013/14 crop), N and S (2013/14 and 2014/15 crops), and Ca and Mg (2013/14 and 2014/15 crops), and a negative correlation between P and S (2014/15 crop), regardless of N rates and inoculation with *A. brasilense* (Table 6).

For the 2013/14 crop, the Pearson linear correlation between leaf concentrations of Cu and N, Cu and P, Mn and N, Fe and N, Mn and P, and Cu and Mn was significant and positive (Table 6). In the 2014/15 crop, the correlation was positive between the concentrations of Fe and S, Fe and Cu, Mn and Mg, Zn and S, Zn and Cu, and Zn and Fe, and negative between Fe and Ca, and Zn and P, regardless of the N source and inoculation with *A. brasilense* (Table 6).

Several studies have reported that increasing N rates in topdressing has a positive effect on corn grain yield (Gomes et al., 2007; Pavinato et al., 2008; Lana et al., 2009; Souza et al., 2011; Goes et al., 2014; Kappes et al., 2014). This reinforces the results obtained here, especially when plants were also inoculated with *A. brasilense*. The highest grain yields were obtained when N was supplied at higher rates. This can be explained by the high N demand of the hybrid being studied and because the corn was grown after grass with a high C/N ratio. Desiccation and mechanical decomposition of the residue took place fewer than 15 days before the corn was planted. This is evidence of the immobilization of N by the straw.

Regardless of the N source and *A. brasilense* inoculation, the Pearson correlation was positive between LCI and grain yield (2013/14 and 2014/15). In the 2013/14 crop, there was a negative correlation between grain yield and Fe concentration in leaves (Table 6). For the 2014/15 crop, the LCI had a negative correlation with Fe and Zn concentrations in leaves and a positive correlation with Mn concentration in leaf tissue. Grain yield had a negative correlation with K and Ca concentrations in leaves (Table 6).

The results obtained demonstrate benefits in terms of plant nutrition and corn grain yield. More research on the beneficial effects of inoculation with *A. brasilense* while using N fertilization should be carried out. This technology has many potential uses in the field because it is affordable, it is not toxic, and it increases the yield of corn crops even when large amounts of N are applied.

Table 5. Inoculation and nitrogen rate interaction for corn grain yield (2013/14 and 2014/15)

Inoculation	N rate				
	0	50	100	150	200
	kg ha^{-1}				
	2013/14				
With *Azospirillum***	8,324 a	9,018 a	9,762 a	9,573 a	9,593 a
Without *Azospirillum*^{ns}	8,107 a	8,410 a	8,665 b	8,678 a	8,778 a
LSD (5 %)	1,009				
	2014/15				
With *Azospirillum***	5,610 a	6,183 a	7,284 a	7,972 a	7,810 a
Without *Azospirillum*^{ns}	5,612 a	4,923 a	5,361 b	5,754 b	6,721 a
LSD (5 %)	1,792				

Means followed by the same letter in the column do not differ by the Tukey test at 5 %. **, * and ^{ns}: significant at p<0.01, 0.01<p<0.05, and not significant, respectively.

Table 6. Pearson correlation coefficients among leaf nutrient concentrations and corn grain yield in the 2013/14 and 2014/2014 crop season in a *Latossolo Vermelho Distroférrico* (Oxisol)

	LCI	N	P	K	Ca	Mg	S	Cu	Fe	Mn	Zn	Grain yield
							2013/14					
LCI	1	0.0601	0.1772	-0.1187	-0.0937	-0.0180	0.1757	0.0077	-0.1625	-0.0597	-0.1301	0.5076**
N		1	0.5760**	0.0997	0.0325	0.1144	0.4232**	0.5792**	0.0231	0.4139**	-0.1953	-0.1953
P			1	-0.0896	0.2182	0.1476	0.3818**	0.4837**	0.0730	0.4084**	-0.1928	-0.1252
K				1	0.0348	0.1417	-0.2138	0.1873	0.0609	0.1379	-0.0889	0.0546
Ca					1	0.5278**	0.0665	0.1115	-0.0463	0.1836	0.0090	-0.1427
Mg						1	0.1661	0.0052	-0.1715	0.0867	-0.0328	-0.1095
S							1	0.1884	-0.1583	0.2282	-0.1169	0.0410
Cu								1	0.1074	0.4439**	-0.1249	-0.2408
Fe									1	0.2137	0.0866	-0.3267*
Mn										1	-0.0046	-0.1986
Zn											1	-0.1814
Grain yield												1
							2014/15					
LCI	1	0.3617**	0.3038*	-0.0199	-0.0006	0.0441	-0.2047	-0.1295	-0.2549*	0.3744**	-0.5906**	0.3917**
N		1	-0.0859	0.1260	-0.1798	-0.0439	0.5158**	0.1657	0.4049**	0.2100	0.0192	0.2459
P			1	0.0704	-0.0831	0.0311	-0.3963**	0.0453	-0.1205	-0.1856	-0.2760*	0.0249
K				1	-0.1998	0.0123	-0.1518	-0.0484	0.1995	-0.1510	-0.2370	-0.2596*
Ca					1	0.5507**	-0.0011	-0.1388	-0.3532**	0.2022	-0.1007	-0.2584*
Mg						1	-0.1432	0.0153	-0.1644	0.3902**	-0.2317	-0.0728
S							1	0.1628	0.4648**	0.1492	0.5856**	0.0265
Cu								1	0.2748**	-0.1773	0.2716**	0.1226
Fe									1	-0.0981	0.2966*	0.0097
Mn										1	-0.0216	0.2089
Zn											1	-0.1865
Grain yield												1

**, * and ns: significant at $p<0.01$, $0.01 < p < 0.05$, and not significant, respectively. LCI: leaf chlorophyll index.

CONCLUSIONS

Super N and urea had a similar effect on macro and micronutrient concentrations in leaves, LCI, AE, and corn grain yield. Urea is recommended as an N source because it has a better cost-benefit ratio.

Greater nitrogen rates positively influenced the LCI and N, S, and Mn concentrations in leaves, and may increase P, Cu, and Fe concentrations; however, an increase in N rates can reduce AE.

Nitrogen rates of up to 200 kg ha^{-1} improve grain yield for corn that has been inoculated with *A. brasilense*, regardless of the N source utilized.

Inoculation with *A. brasilense* decreased Fe concentration in leaves and increased the LCI, the P concentration in leaves, AE, and corn grain yield.

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