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The Effect of N Fertilization on Wheat under Inoculation with *Azospirillum brasilense*

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Additional information is available at the end of the chapter

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Abstract

The biological nitrogen fixation (BNF) process in wheat (*Triticum aestivum* L.) occurs by diazotrophic bacteria, particularly *Azospirillum brasilense*. However, researches are lacking on BNF efficiency to define how much mineral nitrogen (N) can be applied to achieve more sustainable high yields, and if urea with the urease enzyme inhibitor is less harmful, benefiting BNF in grasses (cereals). Therefore, the objective was to evaluate the effect of N sources (urea and Super N, urea with urease enzyme inhibitor N-(n-butyl thiophosphoric triamide) (NBPT) and N rates (0, 50, 100, 150, and 200 kg ha⁻¹) applied in topdressing associated with inoculation with *A. brasilense*, regarding the leaf N concentration, leaf chlorophyll index (LCI), accumulation of N in the straw and grains, the nitrogen utilization efficiency (NUE), recovery of the applied nitrogen (RAN), physiological efficiency (FE), agronomic efficiency (AE), and wheat grain yield in the Brazilian Cerrado (tropical savanna) region. The N sources provide similar N accumulations in straw and grains, and wheat grain yield. Inoculation with *A. brasilense* afforded higher N grain concentration (increase in protein content more sustainably) by applying less N fertilizer in topdressing. Inoculation with *A. brasilense* increased the AE, RAN, and NUE.

Keywords: *Triticum aestivum* L., nitrogen sources, biological nitrogen fixation, urease inhibitor, no-tillage system

1. Introduction

The management of nitrogen fertilization is performed in order to ensure adequate productivity, and depending on the N dynamics in the soil, large amounts of N are added to the soil,

raising production cost for the farmers. This applies to wheat (*Triticum aestivum* L.) that is an annual cycle plant, considered, among the winter season's cereal, one that has the greater economic importance with large grain yield capacity [1]. This cereal has great relevance in the diet and is cultivated in a wide range of environments and geographic regions. The cereal occupies over 17% of cultivable land in the world and represents approximately 30% of the world's grain production. In the period from 2012 to 2016, the annual average area of wheat cultivated worldwide was approximately 220 million hectares, reaching 734 million tons in the 2015/2016 harvest [2].

The final crop yield is defined according to the cultivar used, the amount of agricultural supplies and management techniques employed. The increasing use of the high yield potential of wheat has implicated in a more frequent use of agricultural supplies, among which nitrogen fertilization is shown to be important in defining the grain yield [3]. Several authors reported a positive response of nitrogen fertilization on the grain yield of wheat [4–6]. Therefore, there is a need to study wheat cultivars verifying their response in the uptake and utilization of nutrients in the soil and their performance and cultural practices in different environments [7].

Nitrogen fertilization includes one of the highest costs of the production process of non-leguminous crops [8]. Wheat, corn, and rice crops utilize approximately 60% of the N fertilizers produced in the world [9]. The use of N fertilizers must be carefully controlled to ensure good yield and manage N in the soil; N fertilizer increases production costs for farmers [10]. Also, both nitrogen fertilizer production and application contribute to the emission of gases (CO_2 and NO_2) that contribute to the increase of the greenhouse effect on the Earth. In a report developed by the International Fertilizer Industry Association and the United Nations Environment Program, 873 m³ of natural gas was used to produce 1 metric ton of nitrogen fertilizer synthesized by the Haber-Bosch process [11].

It is estimated that there may be a reduction in grain yield due to the volatilization of N-NH_3 at the rate of 10 kg ha⁻¹ of grains for each 1% N that is volatilized [12]. In this context, one possibility to increase the nitrogen fertilization efficiency is the use of the N-(n-butyl) thiophosphoric triamide (NPBT) inhibitor, which can delay the hydrolysis of urea and significantly reduce NH_3 losses depending on the weather, that is, heat and rain as well as the chemical characteristics of the soil [13, 14]. Due to the climate in Brazil, urea with urease enzyme inhibitor and conventional urea are equally effective in terms of N nutrition and grain yield of cereals. Studies in countries with milder weather have had different results [15].

Due to the high cost of fertilizers and awareness in support of sustainable agriculture and less pollution, in which the research is growing, another possibility would be to use inoculants containing bacteria that promote growth and increase the productivity of plants. Studies on biological nitrogen fixation (BNF) by *Azospirillum* species in grass have been carried out in Brazil. Until recently, no commercial inoculants with these bacteria are available in the country [16].

Although the plant genotype performs an essential role in the colonization of bacteria, existing cultivars with high and low potential of association [17]. Several studies have been published confirming that *Azospirillum* produces phytohormones that stimulate root growth in many

plant species. The components released by *Azospirillum brasilense* responsible for stimulating root growth are indole acetic acid (IAA), gibberellins, and cytokinins [18]. Inoculation with *Azospirillum* can improve the 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 as reported by Barassi et al. [19]. Increases in photosynthetic pigments such as chlorophyll a and b, and auxiliary photoprotective pigments, such as violaxanthine, zeaxanthine, atheroxanthine, lutein, neoxanthine, and beta-carotene, which result in greener plants without water-related stress, were verified by Bashan et al. [20].

In addition, 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 [21, 22]. According to Dobbelaere et al. [23], positive responses to inoculation with *A. brasilense* are obtained even when the crops are grown in soils with high N content, which indicates that the plant responses occur not only due to the fixed N₂ but also depending on the production of phytohormones growth promoters such as cytokinin, gibberellin, and indole acetic acid. Lemos et al. [24], studying five wheat cultivars, found a positive interaction between *A. brasilense* and N fertilization only for one wheat cultivar (CD 150). Increases in N fertilization efficiency associated with inoculation with *A. brasilense* were reported by Galindo et al. [14] but in the corn grain yields in the *Brazilian Cerrado*.

Considering the benefits attributed to several crops by inoculation with *A. brasilense*, with an emphasis on biological nitrogen fixation, greater development of the root system, and, consequently, greater uptake of water and nutrients, the inoculation can improve crop performance allowing greater efficiency of nitrogen fertilization. Thus, 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 of N minerals can be applied for BNF to be successful in increasing the 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 *A. brasilense* can increase the efficiency of N fertilization and N plant nutrition. The objective was to evaluate the effect of N sources and N rates associated, or not, with *A. brasilense* inoculation regarding the leaf N concentration, leaf chlorophyll index (LCI), accumulation of N in the straw and grains, the nitrogen utilization efficiency (NUE), recovery of the applied nitrogen (RAN), physiological efficiency (FE), agronomic efficiency (AE), and wheat grain yield in the *Brazilian Cerrado* (tropical savanna) region.

2. Material and methods

2.1. Location and soil-climatic conditions

The wheat experiment was conducted in 2015, in the field in an experimental area that belongs to the Paulista State University (UNESP), Engineering Faculty, located in Selvíria—MS/Brazil,

with the following geographical coordinates 20°22'S, 51°22'W and an altitude of 335 m. Soil in this experimental area was classified as dystroferic red oxisol with clay texture (with values of particle sizes as 420, 50, and 530 g kg⁻¹ of sand, silt, and clay, respectively), according to Embrapa [25], which has been cultivated with annual cultures over 27 years and with no-till-age system in the past 11 years. The area was under corn cultivation before sowing wheat. The annual average temperature was 23.5°C, annual average pluvial precipitation was 1370 mm, and annual average relative air humidity was between 70 and 80%. Weather data recorded during the experimental period are shown in **Figure 1**.

Glyphosate [1800 g ha⁻¹ of active ingredient (a.i.) and 2,4-D (670 g ha⁻¹ of a.i.)] herbicides were used for desiccation, applied 2 weeks prior to sowing wheat. Chemical attributes of the soil in the tillable layer were determined before the wheat experiment began. The methods proposed by Raij et al. [26] showed the following results: 13 mg dm⁻³ of P (resin), 6 mg dm⁻³ of S-SO₄, 23 g dm⁻³ of organic matter (OM), pH (CaCl₂) of 4.8, 2.6 mmol_c dm⁻³ of K⁺, 13.0 mmol_c dm⁻³ of Ca²⁺, 8.0 mmol_c dm⁻³ of Mg²⁺, 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% of base saturation. After soil chemical analysis, 2.5 t ha⁻¹ of dolomitic limestone (with 88% of relative total neutralizing power) was directly applied as topdressing for 80 days before the wheat was sown in 2015 in order to elevate base saturation to 70%, as recommended by Cantarella et al. [27].

2.2. Treatments and experimental design

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 with 45% N; five rates of N (0, 50, 100, 150,

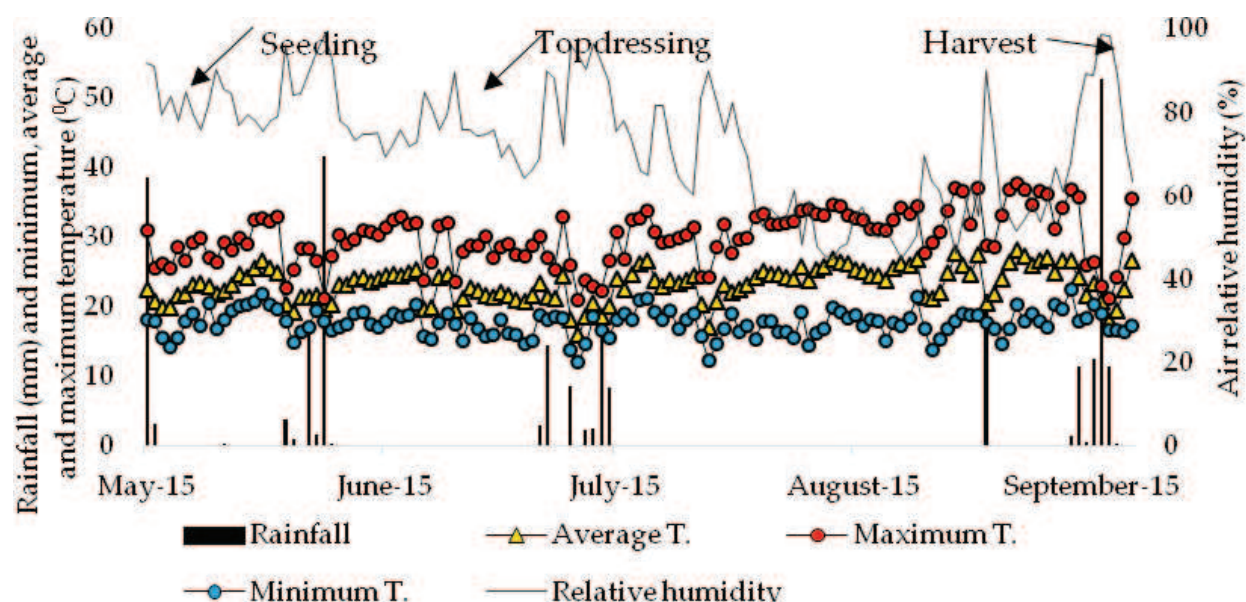


Figure 1. Rainfall, air relative humidity, and maximum and minimum average temperature obtained from the weather station located on the Education and Research Farm of FE/UNESP during wheat cultivation in the period from May to September 2015.

and 200 kg ha⁻¹) applied in topdressing at the growth stage 3.2 on Zadok scale; and two seed inoculation treatments—half of the tests were carried out with seeds inoculated with *A. brasilense*, whereas the other half did not have this inoculation. Each plot consisted of 5 m in length with 12 lines and an inter-row spacing of 0.17 m. The usable area of the plot was 8 center lines, excluding 0.5 m extremities. The plot size was 10.20 m².

2.3. Wheat crop management

Wheat seeds were inoculated with 300 mL of inoculant liquid of *A. brasilense* bacteria, AbV5 and AbV6 strains (guaranteed minimum analysis of 2×10^8 UFC mL⁻¹) per sack of 40 kg of wheat seeds. The inoculant was mixed with the seeds using a cement mixer, 1 h before planting, and after that, the seed treatments were carried out with carbendazim and thiram fungicides (45 and 105 g a.i. per 100 kg of seeds) and thiodicarb and imidacloprid insecticides (45 and 135 g a.i. per 100 kg of seeds).

Was applied 350 kg ha⁻¹ of a 08-28-16 formulation in the forms of urea, triple superphosphate and potassium chloride, respectively, at wheat sowing. The experiments were conducted in a no-tillage system. The area was irrigated by a central pivot sprinkler system. The water coverage was 14 mm over a period of around 72 h. The cultivar used was the CD 116 and sowing was done with an experimental machine on May 15, and 80 seeds per meter were being sown. Metsulfuron methyl (3.0 g a.i. ha⁻¹), a post-emergence herbicide, was applied 20 days after emergence (DAE) to control weeds like *Ipomoea grandifolia*, *Tridax procumbens*, and *Spermacoce latifolia*.

The seedling emergence was 6 days after sowing. Topdressing with nitrogen fertilization was performed at 35 DAE, manually distributing the fertilizer on the soil surface (no incorporation), and there was approximately 8 cm of sowing lines in order to avoid the contact of the fertilizer with the plants. The plants were harvested 110 days after wheat emergence.

2.4. Research evaluations and statistical analysis

The 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 N leaf concentration (leaf diagnosis) was performed by collecting 20 leaf flags in the flowering of wheat plants, according to the methodology described in Cantarella et al. [27]. The N determination was carried out as described by Malavolta et al. [28]. The N concentrations in the grains and straw (above the soil) of wheat were also measured during the harvest occasion (at the end of the crop cycle), in 10 plants per useful area of the plot, according to the methodology described by Cantarella et al. [27]. By means of these nutrient concentrations and dry matter of plants, nutrient accumulation in the grains and straw was calculated and extrapolated to kg ha⁻¹. The wheat was harvested from the plants in the useful area of each plot and the grain yield was calculated after mechanical threshing. Data were transformed into kg ha⁻¹ and corrected for 13% moisture (on a wet basis).

The accumulation values of N were obtained by the N concentrations in the plant and the dry matter production (DM). With the data of dry mass and accumulation of N, the following indices were calculated:

1. Nitrogen utilization efficiency = total dry matter in kg/accumulation of N in g, in kg of DM/kg of accumulated N [29],
2. Recovery of the applied nitrogen = accumulation of N in kg with fertilization-accumulation of N in kg without fertilization/N rate applied in kg \times 100, in percentage [30],
3. Physiological efficiency (FE) = biological yield (straw and grains) without fertilization in kg/N accumulation with fertilizer (straw and grains) in kg-N accumulation without fertilization (straw and grains) in kg, in kg of DM/kg of accumulated N [30], and
4. Agronomic efficiency (AE) = grain yield with fertilization in kg-grain yield without fertilization in kg/N rate in kg, in kg of DM/kg of applied N [30].

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 *A. brasilense* with those that had not been inoculated. Regression equations were fitted for the effect of N rates using the Sisvar program [31].

3. Results and discussions

LCI increased linearly with increasing doses of N (**Table 1, Figure 2**). The increase in LCI values, as a consequence of the N rates, resembles those reported by Theago et al. [6], who observed an increase in LCI in wheat up to the dose of 200 kg ha⁻¹, and Teixeira Filho et al. [5] up to 147 kg ha⁻¹ of N. This behavior is due to the increase in chlorophyll concentration, promoted by the greater availability of total N in the tissues. This relationship is attributed mainly to the fact that 50–70% of the total leaf N is integral to enzymes that are associated with chloroplasts [32].

Regarding the sources, the Super N provided greater readings of LCI compared to urea (**Table 1**). However, inoculation with *A. brasilense* did not influence LCI, unlike that reported by Galindo et al. [14], working with the N rates (0, 50, 100, 150 and 200 kg ha⁻¹ in topdressing) and the sources urea and Super N in the corn crop.

The increase of N rates influenced N leaf concentration (**Table 1**). There was an adjustment of the quadratic function, with maximum concentration point obtained of the N rate at 181 kg ha⁻¹ approximately (**Figure 2**). The increase of N concentration in the leaf tissue was expected since by providing more nutrient quantity, the uptake of the crop would be greater, with reflection in the concentration in the leaf and in the aerial part, even in a consolidated system of no tillage in the area of study, over 10 years, which could supply the need of this nutrient due to the decomposition of the straw and as a result of the crop sequence after wheat was the maize crop, the wheat crop response to N rates was more evident.

Similar results were obtained by Theago et al. [6], who observed a linear increase in foliar N concentration with the increment of the doses in coverage (0, 50, 100, 150, and 200 kg ha⁻¹). The increase of N concentration in the wheat leaf, due to the increase of N rates, was also reported by Teixeira Filho et al. [33], with a quadratic response up to the dose of 100 kg ha⁻¹

	LCI	N leaf concentration (g kg ⁻¹)	N straw	N grains
N rates (kg ha ⁻¹)				
0	50.92	38.89	24.12	44.86
50	52.31	44.20	31.39	78.35
100	54.50	47.39	37.08	92.22
150	55.23	48.46	38.59	77.67
200	56.16	49.95	54.89	96.10
N sources				
Urea	52.66 b	46.08 a ^ψ	38.87 a	78.35 a
Super N	54.99 a	45.47 a	35.56 a	77.33 a
L.S.D. (5%)	1.31	1.33	4.91	9.81
Inoculation				
With <i>Azospirillum</i>	54.26 a	45.24 a	34.82	79.20 a
Without <i>Azospirillum</i>	53.39 a	46.31 a	39.60	76.48 a
L.S.D. (5%)	1.31	1.33	4.91	9.81
Overall mean	53.82	45.78	37.21	77.84
C.V. (%)	4.64	5.55	25.26	19.04

Selvíria—MS, Brazil, 2015.

^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 1. Leaf chlorophyll index (LCI), N leaf concentration, N accumulated in straw and grains of wheat in function of N rates, and sources and inoculation with *Azospirillum brasilense* in wheat crops.

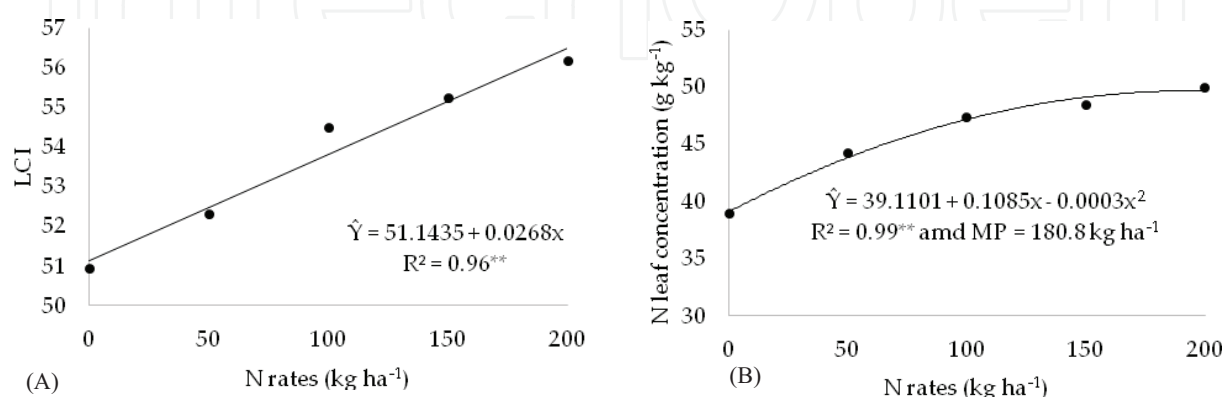


Figure 2. LCI (A) and N leaf concentration (B) in wheat crops in the function of N rates. Selvíria—MS, Brazil, 2015.

of N, and Teixeira Filho et al. [34], with a point of maximum concentration of N being reached with the application estimate of 163 kg ha^{-1} of N. It should be noted that the concentration of N in the leaf tissue, even in the absence of nitrogen fertilization (0 kg ha^{-1}), was above that as recommended by Cantarella et al. [27], ranging from $20\text{--}34 \text{ g kg}^{-1}$.

N sources did not differ in N concentration, indicating that Super N was not efficient for N nutrition, even in the area with remaining straw of corn or wheat (**Table 1**). Similar results were verified by Meira et al. [35], using ammonium sulfonitrate, ammonium sulfate, and urea sources for the N concentration of irrigated wheat in the *Cerrado*. However, Teixeira Filho et al. [5] verified higher N concentrations in leaf tissue when the sources of ammonium sulfonitrate and ammonium sulfate compared to urea were used.

On the other hand, Megda et al. [36] (ammonium sulfonitrate, ammonium sulfate, and urea), in the corn crop, verified that the ammonium sulfonitrate source had a higher N concentration in the leaf, compared to the other sources, unlike the results obtained in the present research. It should be noted that Super N acts on the inhibition of the urease enzyme, whereas the ammonium sulfonitrate has in its composition dimethylpyrazolophosphate (DMPP) molecules that act to inhibit nitrification. This makes the fertilizer less susceptible to leaching, since there is a longer residence time of N, as ammonium in the soil, and, under conditions of tropical climate and high temperatures, presents different responses to Super N. Also, 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 is low [37].

According to Pankiewicz et al. [38], there is a greater development and growth of the root system of the *Setaria viridis* grass inoculated with *A. brasilense* as a function of associative fixers, with greater CO_2 fixation and less accumulation of photoassimilated carbon in the leaves, which would favor the plant with greater growth in aerial part, greater accumulation of water, and conditions of less stress caused by the greater accumulation and metabolism of carbon, thus, increasing the concentration of nutrients in the plant. On the other hand, Bashan et al. [22] reported that the production of plant hormones, mainly indole acetic acid by bacteria of the genus *Azospirillum*, plays an essential role in the promotion of plant growth and, according to Hungria et al. [39], can improve the uptake of several macro and micronutrients, increasing the efficiency of the use of the available nutrients; however, this result was not verified in the present work, which raises the question of the difference of efficiency of the inoculation in grass crops.

Analyzing the split between with or without *A. brasilense* inoculation and N rates for N accumulation in the straw (**Table 2**), there was no difference between inoculation and not with *A. brasilense* for any of the N rates. With the increase in N rates, there was a linear increase in the N accumulation in straw (**Figure 3**), regardless of inoculation or not with *A. brasilense*. Dobbelaere et al. [23] also reported positive responses to inoculation with *A. brasilense* even when the crops are grown in soils with high N content available, which indicates that the plant responses occur not only due to the fixed N_2 but mainly depending on the production of phytohormone growth promoters such as cytokinin, gibberellin, and indole acetic acid.

Inoculation	N rates (kg ha ⁻¹)				
	0	50	100	150	200
With	22.38 a ^ψ	30.29 a	35.28 a	34.20 a	51.97 a
Without	25.86 a	30.29 a	38.88 a	42.99 a	57.81 a
L.S.D. (5%)	10.98				

Selvíria—MS, Brazil 2015.
^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 2. Inoculation with *A. brasilense* and N rates interaction in the N accumulation in wheat straw.

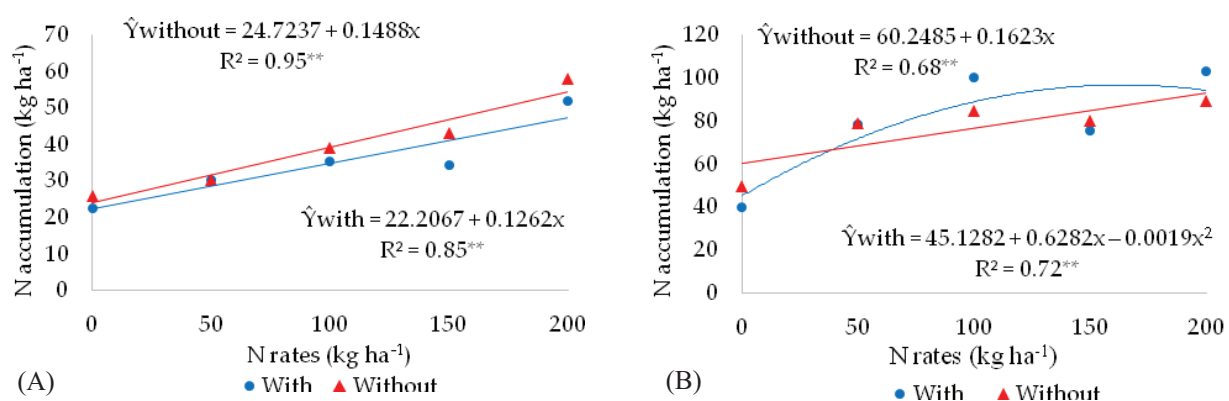


Figure 3. N rates and inoculation with *A. brasilense* interaction in the N accumulation in straw (A) and in grains (B) of wheat. Selvíria—MS, Brazil 2015.

The greater accumulation of nutrients provided by the increase of the N rates associated or not with the inoculation with *A. brasilense* is a very significant result considering that conservation practices benefit the productive systems and environmental conservation, such as no-tillage system, whose straw will provide nutrients to subsequent crops, recycling nutrients, and minimizing losses and environmental pollution, such as nutrient leaching.

N is the nutrient that most interferes in the development and productivity of crops, especially grasses. This mineral nutrient is found in higher concentrations, in vegetative tissues and grains, which characterizes it as being the element most demanded by the wheat plant. Thus, the higher availability of this nutrient in the plants favored the development of the root system, which, by exploiting a larger volume of soil, may have an uptake of a greater amount of nutrients and water, reflecting the accumulation in aerial part, both in the straw and in the grains. Since N is involved in the synthesis of proteins, chlorophyll, coenzymes, phytohormones, nucleic acids, and secondary metabolites (Amines, amides, amino sugars, purines, pyrimidines and alkaloids) [40].

The interaction between N rates and inoculation with *A. brasilense* was significant for N accumulation in grains; however, there was no difference between with or without *A. brasilense* inoculation for any of the N rates (Table 3). However, in treatments inoculated with *A. brasilense*,

Inoculation	N rates (kg ha ⁻¹)				
	0	50	100	150	200
With	40.13 a ^ψ	78.03 a	99.90 a	75.13 a	102.81 a
Without	49.59 a	78.66 a	84.54 a	80.20 a	89.40 a
L.S.D. (5%)	21.94				

Selvíria—MS, Brazil, 2015.

^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 3. Inoculation with *A. brasilense* and N rates interaction in the N accumulation in wheat grains.

there was an adjustment to the quadratic function for the dose of 165.3 kg ha⁻¹ of N, whereas in the treatments without inoculation with this bacterium, the adjustment was linearly increasing for N in the grains (**Figure 3**) but reaching lower N concentrations in the highest rate of N.

These bacteria can act on plant growth by producing substances promoting development (auxins, gibberellins, and cytokinins) which provide better root growth [41] and, therefore, help in greater uptake of water and nutrients [42], resulting in a more vigorous and productive plant [16, 22]; to be free-living organisms with endophytic characteristics, it is possible to perform some of the metabolic and vital use of nutrients in the plant, which would then be made available to reflect in increased concentrations in the grains.

Galindo et al. [14] studied N rates (0, 50, 100, 150, and 200 kg ha⁻¹, in topdressing), N sources (Super N and urea), with and without inoculation with *A. brasilense* in maize, and found positive influence of inoculation on nutrient concentration in leaf tissue, which may be indicative of the phytohormonal effect cited in the literature, confirming that *Azospirillum* produces phytohormones that stimulate root growth of several plant species and that this greater development of the roots may imply several other effects such as increases in the water and nutrient uptakes and greater tolerance to stresses such as salinity and drought, resulting in a more vigorous and productive plant [22]. In addition, Barassi et al. [19] reported improvement in leaf photosynthetic parameters, including chlorophyll content and stomatal conductance, higher proline content in shoots and roots, improvement in water potential, increase in water content of apoplast, greater cell wall elasticity, and higher production of plant biomass.

The increase in N rates influenced significantly the number of kernels per spike and spikes per meter (**Table 4**). For the number of kernels per spike, the data adjusted the quadratic function with maximum point in 151 kg ha⁻¹ of N (**Figure 4**). On the other hand, the N rates influenced the number of spikes per meter, adjusted to the quadratic function, up to the dose of 110 kg ha⁻¹ of N (**Figure 4**). These results explain why grain yield was influenced positively by the increase of N rates, independently of the source of N and inoculation with *A. brasilense* (**Figure 4**).

For the hectoliter weight and 100-kernel weight, there was no influence of N rates (**Table 4**). Similar results were obtained by Teixeira Filho et al. [33] and Theago et al. [6] that did not verify the influence of the N rates on the mentioned parameters in the irrigated wheat crop. Nunes et al. [8] and Souza et al. [43], who found no influence of N rates in topdressing for 100-kernel

	Number of kernels per spike	Number of spikes per meter	Hectoliter weight (kg 100 L ⁻¹)	100-kernel weight (g)	Grain yield (kg ha ⁻¹)
N rates (kg ha⁻¹)					
0	34.40	69.17	87.23	4.20	1906
50	35.75	76.50	86.83	4.10	3027
100	42.48	84.42	87.42	4.19	3363
150	37.88	73.75	86.14	4.13	3167
200	38.91	74.50	85.96	4.12	3263
N sources					
Urea	38.60 a ^ψ	75.50 a	87.14 a	4.17 a	2930 a
Super N	37.17 a	75.83 a	86.29 a	4.13 a	2960 a
L.S.D. (5%)	2.70	5.90	1.40	0.06	212
Inoculation					
With <i>Azospirillum</i>	39.04 a	76.00 a	86.61 a	4.14 a	3007
Without <i>Azospirillum</i>	36.73 a	75.33 a	86.82 a	4.15 a	2883
L.S.D. (5%)	2.70	5.90	1.40	0.06	212
Overall mean	37.88	75.67	86.71	4.15	2945
C.V. (%)	13.65	14.92	3.61	3.36	16.08

Selv3ria—MS, Brazil, 2015.

^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 4. Number of kernels per spike, number of spikes per meter, hectoliter weight, 100-kernel weight, and wheat grain yield in function of N rates and sources and inoculation with *Azospirillum brasilense* in wheat crop.

weight in an area with high availability of N. According to Frank & Bauer (1996), in the period between the emergence phase of the seedlings and the differentiation of the floral primordium, N deficiency reduces the mass of 100-kernel weight. Therefore, the results obtained can be explained by the fact that N deficiency was not observed in the plants in any of the treatments by the fact of the irrigated wheat crop and because the number of kernels per spike increased as a function of increasing N rates, thereby providing increment on the competition for photo assimilates inside the spike [5] but not to the point of reducing the mass of grains. This can be proved by the high hectoliter weight to be obtained (>78 kg 100 L⁻¹) in the experiment, which classified (by individual analysis) the type 1 wheat with the best quality [10].

Conventional urea and urea with NBPT provided similar results for number of kernels per spike, number of spikes per meter, hectoliter weight, 100-kernel weight, and, consequently, for wheat grain yield (Table 4) in agreement with Megda et al. [36] and Theago et al. [6]. Prando et al. [44], evaluating the sources of urea, urea + NBPT, and coated urea, in another climatic condition (temperate climate), also did not observe changes in grain yield. It is worth noting that N sources may have presented similar behavior due to irrigation during the entire

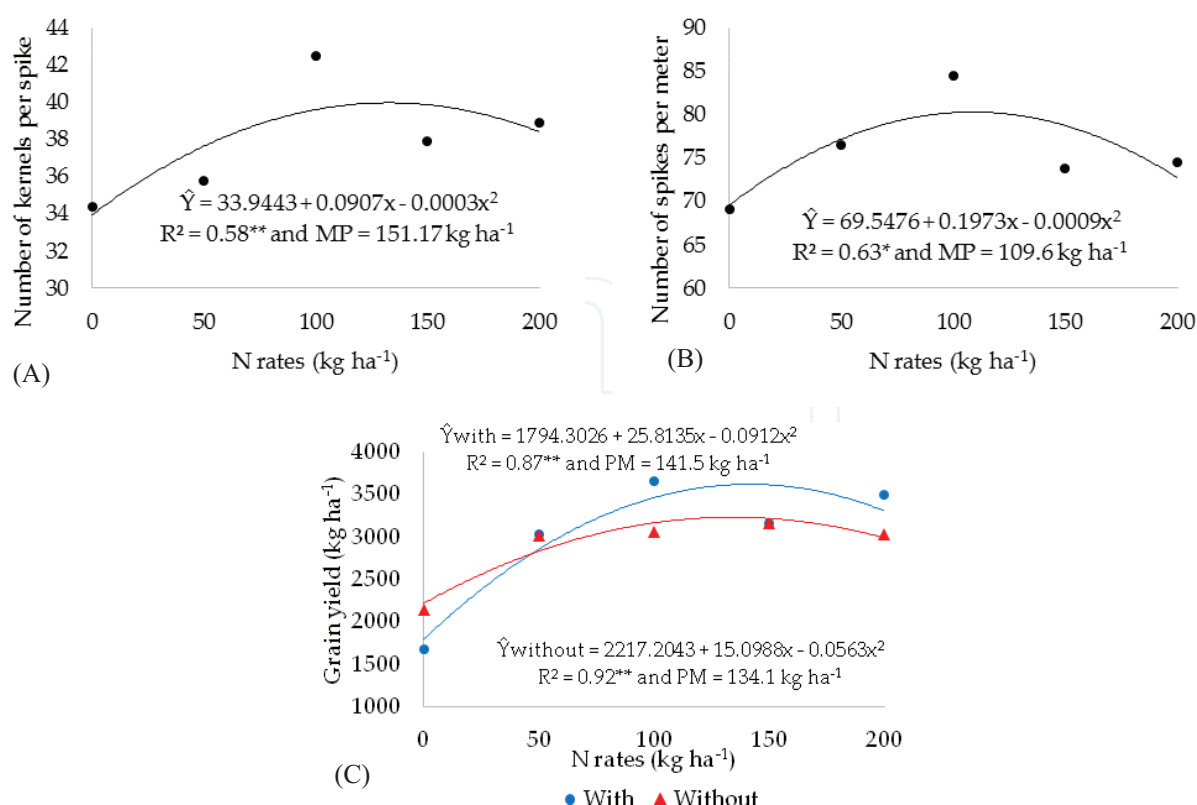


Figure 4. Number of kernels per spike (A) and number of spikes per meter (B) in the function of N rates and N rates and inoculation with *A. brasilense* interaction in the wheat grain yield (C). Selvíria—MS, Brazil 2015.

crop cycle, which would have reduced volatilization losses, mainly in the form of NH_3 , which occur at a higher proportion in the first 4 days after urea application.

As regards inoculation with *A. brasilense*, this alone did not significantly interfere in the number of kernels per spike, number of spikes per meter, hectoliter weight, and 100-kernel weight (Table 4). Most probably, there was no effect of this inoculation on these production components because the number of kernels per spike is mainly determined by the genetic characteristics of the cultivar, and from the other evaluations mentioned above, it is due to the cultivation of irrigated wheat, fertilization, and adequate plants stand and soil fertility.

There was a significant interaction between N rates and inoculation with *A. brasilense* for wheat grain yield. At the dose of 100 kg ha⁻¹ of N, the inoculation provided higher productivity than the uninoculated treatment (Table 5). Grain yield adjusted the quadratic function for N rates in the treatments with and without inoculation with *A. brasilense*, with a positive response up to 142 and 134 kg ha⁻¹ of N, respectively (Figure 4). However, in relation to the control (without N), the optimal N dose of the treatment inoculated with this diazotrophic bacterium provided a higher grain yield of 391 kg ha⁻¹ in relation to the best N rate of the treatment without inoculation; that is, this increase was higher by 7%.

The N rates mentioned above for obtaining the maximum grain yield were high since the wheat was cultivated on corn straw (high C/N ratio), that is, part of the applied N was immobilized by the decomposing/mineralizing of microorganisms.

Inoculation	2015				
With	1671 a ^ψ	3036 a	3663 a	3167 a	3497 a
Without	2141 a	3018 a	3063 b	3166 a	3029 a
L.S.D. (5%)	474				

Selvíria—MS, Brazil, 2015.

^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 5. Inoculation with *A. brasilense* and N rates interaction in the wheat grain yield.

The *A. brasilense* inoculation associated with the N rates in the 140 kg ha⁻¹ range provided the maximum grain yield of the wheat crop; in contrast, in the absence of inoculation, the magnitude of response to N rates was higher. Worth mentioning that the estimated grain yield of the inoculated treatments was higher, numerically, most of the rates tested in both crops, even using N rates considered high, showing the benefit of inoculation with *A. brasilense* in irrigated wheat crop. In turn, Galindo et al. [45] verified that the co-inoculation with *A. brasilense*, and Co + Mo application via seeds promote higher grain yield and profitability with the soybean crop in the *Brazilian Cerrado*, being technically and economically viable.

With regard to grain yield, several authors also reported a positive response to N fertilization on wheat [4, 7, 46]. In similar climatic conditions for wheat crop in the low-altitude *Cerrado region*, it was reported that the maximum grain yield was 78 [33], 90 [7], and 120 kg ha⁻¹ of N [5]. This difference in rates of N that provides maximum wheat grain yield is due to different N requirements of cultivars as well as the variation in soil and climatic conditions of these researches. Anwar et al. [47] found that maximum spikes m⁻², grains per spikes, thousand grains weight, and grain yield (4061 kg ha⁻¹) were produced by 125 kg ha⁻¹ of N for two wheat cultivars in Pakistan.

Lemos et al. [24] studied five wheat cultivars (CD 104, CD 108, CD 119, CD 120, and CD 150), with and without inoculation and associated with nitrogen rates, and found that response to inoculation with *A. brasilense* in wheat crop occurs satisfactorily when held in conjunction with the nitrogen fertilization, as observed in this study at a dose of 100 kg ha⁻¹ N (**Table 4**). However, *A. brasilense* alone is not effective enough to replace entire nitrogen fertilization but is associated with N fertilization, which makes it possible to achieve the highest yields of irrigated wheat grains in *Brazilian Cerrado*.

On the other hand, Ferreira et al. [48], working with foliar application of *A. brasilense* and nitrogen rates in the wheat crop in the *Brazilian Cerrado*, observed that inoculation had no effect on grain yield. Similarly, Nunes et al. [8] studied inoculation with *A. brasilense* in soils with high and low availability of N, and Galindo et al. [49] in research with application times by the leaf of *A. brasilense* with the application of 100 kg ha⁻¹ N found no effect of inoculation in the production components and grain yields of wheat in the *Brazilian Cerrado*.

It is noteworthy that bacteria of *Azospirillum* genus are native from soil [50], so it is possible that these were at a high population in the soil under study and, therefore, cancel or minimize the effect of inoculation. Moreover, the affinity of the cultivar with the strains of

this bacteria diazotrophic may vary and determine the success or failure of *A. brasilense* inoculation.

The NUE was negatively affected by the increment of N rates with adjustment to the linear function decreasing (**Table 6, Figure 5**). This result can be attributed to the losses of N portrayed clearly in the literature. The increase of N rates culminates in greater losses and less use by the crops, since there is a limit in the nutritional demand of the plant, that is, the plants uptake certain amount of nutrients for a given time; thus, the N that is applied and is not taken can be lost, decreasing the efficiency of fertilization with the higher rates of N, as portrayed in the literature by the law of decreasing increments. The results are similar to those reported by Silva et al. [51], studying N rates (0, 100, 200, and 300 kg ha⁻¹) in the Marandu palisadegrass and Sant'Ana et al. [52], working with common bean crop in the rates of 0, 30, 60, 120, and 240 kg ha⁻¹ of N in topdressing.

	NUE	RAN	FE	AE
	(kg kg ⁻¹)	(%)	(kg D.M. kg ⁻¹ of N accumulated)	(kg grains kg ⁻¹ N applied)
N rates (kg ha⁻¹)				
0	–	–	–	–
50	38.34	78.82	50.27	22.41
100	30.07	62.08	48.90	14.57
150	13.92	31.08	55.24	8.40
200	15.30	39.52	39.00	6.79
N sources				
Urea	29.65 a	56.87	51.63 a ^ψ	14.14
Super N	19.16 b	48.87	45.08 a	11.95
L.S.D. (5%)	6.51	12.78	16.97	3.05
Inoculation				
With <i>Azospirillum</i>	26.15 a	63.11	42.01 a	16.58
Without <i>Azospirillum</i>	22.67 a	42.64	54.70 a	9.50
L.S.D. (5%)	6.51	12.78	16.97	3.05
Overall mean	24.41	52.87	48.35	13.04
C.V. (%)	17.25 [#]	15.33 [#]	19.44 [#]	24.24 [#]

Selv3ria—MS, Brazil 2015.

[#]Corrected data following equation $(x + 0.5)^{0.5}$.

^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 6. Nitrogen utilization efficiency (NUE), recovery of the applied nitrogen (RAN), physiological efficiency (FE), and agronomic efficiency (AE) in the function of N rates and sources and inoculation with *Azospirillum brasilense* in the wheat crop.

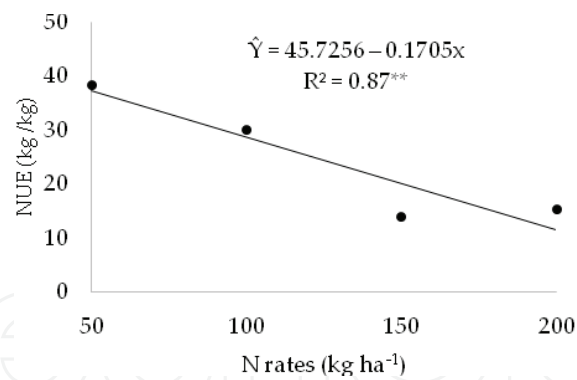


Figure 5. Nitrogen utilization efficiency (NUE) in wheat crop in the function of N rates. Selvíria—MS, Brazil 2015.

In relation to the N sources, urea presented higher NUE compared to Super N, differently from what was expected due to the possibility of mitigation of volatilization of the ammonia provided by Super N fertilizer (urea with urease enzyme inhibitor NBPT) (**Table 6**). However, Dupas et al. [53] evaluated in the *Brazilian Cerrado* the dry-matter yield, RAN, and NUE of palisade grass in response to sources of N (ammonium nitrate, ammonium sulfate, ammonium sulfate-nitrate, urea, urea with urease inhibitor NBPT, polymer-coated urea, and control) in seven harvests (100 kg ha⁻¹ N applied after each harvest) and reported for RAN and NUE that the use of N fertilizers that minimizes N loss, such as urea with urease inhibitor NBPT, and polymer-coated urea, was very promising, especially for minimizing the environmental impact of N fertilization. However, they found no difference in DMY due to N sources.

The inoculation with *A. brasilense* did not influence the NUE, although it gave 15.4% greater efficiency compared to the non-inoculated treatments (**Table 6**), which again may be indicative of the phytohormonal effect cited in the literature, confirming that *Azospirillum* produces phytohormones that stimulate root growth of several plant species and that this greater development of the roots may be implied in several other effects, such as increases in the water and nutrient uptakes like N.

The interaction between N rates and inoculation, and N sources and inoculation, was significant for RAN. Analyzing the split between N rates and inoculation, at 50 and 100 kg ha⁻¹ of

Inoculation*	N rates (kg ha ⁻¹)			
	50	100	150	200
With	96.29 a ^ψ	79.21 a	33.11 a	43.83 a
Without	61.34 b	44.95 b	29.05 a	35.21 a
L.S.D. (5%)	25.56			

Selvíria—MS, Brazil 2015.

*Corrected data following equation $(x + 0.5)^{0.5}$.

^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 7. Inoculation with *A. brasilense* and N rates interaction in the recovery of the applied nitrogen (RAN) in wheat crop.

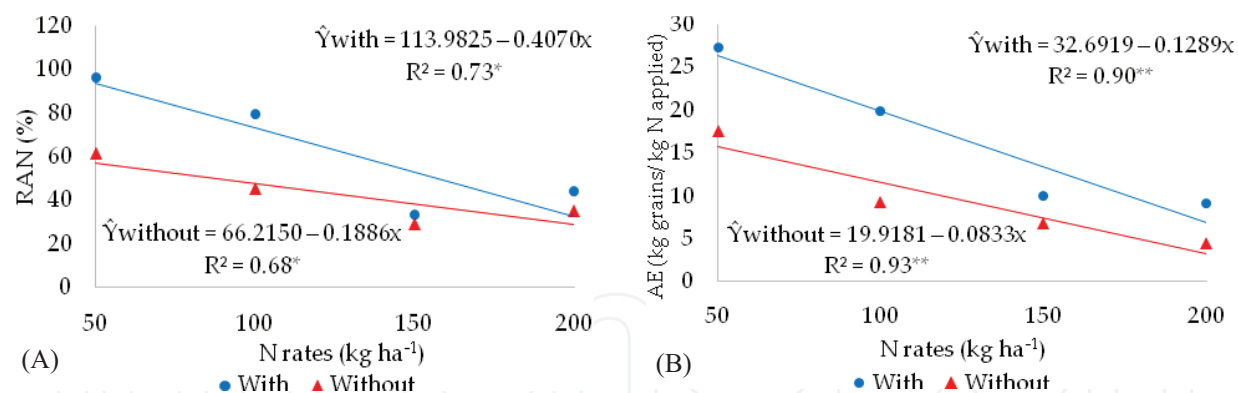


Figure 6. N rates and inoculation with *A. brasilense* interaction in the recovery of the applied nitrogen (RAN) (A) and agronomic efficiency (AE) (B) in wheat crop. Selvíria—MS, Brazil, 2015.

N applied in topdressing, the treatments inoculated provided higher values in the recovery of the applied N (**Table 7**, **Figure 6**). There was adjustment to the linear decreasing function, regardless of inoculation or not with *A. brasilense* (**Figure 6**). In the unfolding between N sources and inoculation, in the absence of inoculation with *A. brasilense*, urea provided higher RAN compared to Super N. When the N source used was Super N, inoculated treatments provided higher RAN compared to those not inoculated (**Table 8**), that is, this compensated the lower efficiency of Super N.

FE was not influenced by N rates, N sources, and inoculation with *A. brasilense* (**Table 6**), which is explained by the adequate growth of the plant (dry-matter accumulation) even when no N was supplied due to adequate N leaf concentration in all treatments (**Table 1**), as previously mentioned.

The interaction between inoculation and rates was also significant in AE, when the rates of 50, 100, and 200 kg ha⁻¹ of N were applied; the inoculated treatments with *A. brasilense* had higher AE compared to the non-inoculated treatments, which is a very good result (**Table 9**) because it indicates that there were smaller losses of N with this diazotrophic bacteria inoculation. There was adjustment to the linear decreasing function of AE with and without *A. brasilense* as a function of N rates (**Figure 6**). Pankiewicz et al. [38], studying FBN with ammonia release by associative fixers, verified increase and development of the *S. viridis* root system and greater CO₂ fixation through inoculation with *A. brasilense* in such a way that plants cultivated in a nitrate-limited environment similarly developed under N sufficient conditions, elucidating the ability of some mutant strains to increase BNF and positively interfering with the carbon metabolism of C4 plants, which has a close relationship with the assimilatory metabolism of N in the plant. Therefore, considering that in the experimental area, there was a favorable condition to the microbial immobilization of the applied N, and this explains why there was a higher AE of the treatments inoculated as a function of the N rates.

The interaction between N sources and inoculation in AE was significant. In treatments inoculated with *A. brasilense*, urea and Super N provided higher AE compared to uninoculated treatments. In the absence of inoculation, urea provided higher AE compared to Super N (**Table 10**).

Sources [‡]	Inoculation	
	With	Without
Urea	63.36 aA ^ψ	50.39 aA
Super N	62.86 aA	34.89 bB
L.S.D.	18.08	

Selvíria—MS, Brazil 2015.
[‡]Corrected data following equation $(x + 0.5)^{0.5}$.
^ψMeans followed by the same lowercase letters in the column and same uppercase letters in the line do not differ by Tukey at 0.05 probability level.

Table 8. Inoculation with *A. brasilense* and N sources interaction in the RAN in wheat crop.

Inoculation [‡]	N rates (kg ha ⁻¹)			
	50	100	150	200
With	27.30 a	19.92 a	9.98 a ^ψ	9.13 a
Without	17.53 b	9.22 b	6.83 a	4.44 b
L.S.D. (5%)	6.09			

Selvíria—MS, Brazil 2015.
[‡]Corrected data following equation $(x + 0.5)^{0.5}$.
^ψMeans followed by the same letters in the column do not differ by Tukey at 0.05 probability level.

Table 9. Inoculation with *A. brasilense* and N rates interaction in the AE in the wheat crop.

Sources [‡]	Inoculation	
	With	Without
Urea	16.42 aA ^ψ	11.85 aB
Super N	16.74 aA	7.16 bB
L.S.D.	4.31	

Selvíria—MS, Brazil, 2015.
[‡]Corrected data following equation $(x + 0.5)^{0.5}$.
^ψMeans followed by the same lowercase letters in the column and same uppercase letters in the line do not differ by Tukey at 0.05 probability level.

Table 10. Inoculation with *A. brasilense* and N sources interaction in the AE in wheat crop.

Regarding AE, according to Dobbelaere et al. [23], positive responses to inoculation with *A. brasilense* are obtained even when the crops are cultivated in soils with high levels of available N, which indicates that the plant responses do not only occur due to the fixed N₂ but mainly as a function of the production of growth-promoting phytohormones such as cytokinin,

gibberellin, and indole acetic acid. This fact could possibly have affected the root development of wheat, which according to Novakowski et al. [54] would improve the efficiency of utilization of residual N, water, and other nutrients uptake, directly reflecting a greater agronomic efficiency of the wheat crop with inoculation with *A. brasilense*, as verified in the present work.

N sources did not differ in AE (**Table 6**), which is due in part to the similar concentrations of foliar nutrients obtained with urea and Super N and can be explained by the non-efficacy of NBPT action due to high activity of the urease enzyme as a function of the straw of the predecessor cultures and the high temperatures that are recorded (**Figure 1**). Another possible explanation would be the uptake of a small part of the urea applied before the action of urease and NH_3 formation [37]. Comparisons between several nitrogen fertilizers were made by several authors, and, in general, with satisfactory conditions of soil moisture, no differences have been found in the efficiency of these sources such as grain yield of wheat in the Cerrado for sources of N ammonium sulfonitrate, uran, and urea [36] between urea and ammonium sulfonitrate in the no-tillage system [55] and between urea, urea + NBPT, and coated urea [56].

The efficiency of the use of N sources by annual crops, such as wheat, is low, around 50%, and the causes for this low value are related to the inadequate dose and timing of application associated with volatilization, leaching, as well as degradation, immobilization, and soil erosion [57] and differs with cultivars [58]. Thus, N fertilization strategy should aim to improve the synchronization between the season of application and the season of greater demand for the plant, in order to maximize N uptake and grain yield [59]. The improvement of N use and recovery efficiencies is desirable to increase productivity, reduce production costs, and maintain environmental quality [44].

It is worth noting that the tendency of agriculture is to seek to enrich food from the nutritional point of view, that is, to increase the availability of nutrients in the parts that will be used as food for humans and animals such as wheat grains. This research demonstrated that inoculation with *A. brasilense* associated with nitrogen fertilization in topdressing is beneficial not only to N nutrition and wheat yield but also to increase the nutritional quality of the grains more sustainably, like the protein content of this important cereal. Therefore, as the inoculation is a low-cost technique, easy to apply and use, non-polluting, and which falls within the desired sustainable context in actuality, the trend is that this technology can be increasingly used in wheat crops.

4. Conclusions

Urea provides higher N utilization efficiency, while the Super N obtains greater LCI and recovery of the applied nitrogen, being the last one only when inoculated. However, the N sources provide similar N accumulations in straw and grains yield of wheat; thus, it is recommended to use urea at the best cost-benefit ratio.

N leaf concentration, LCI, and N straw accumulation increase with the nitrogen fertilization increment, regardless of the N source or *A. brasilense* inoculation.

The increment in N rates in association with *A. brasilense* inoculation increases the N grain concentration up to 165 kg ha⁻¹ N, whereas without this inoculation occurred a linear increase with lower maximum N grain concentration. That is, the inoculation afforded higher N grain concentration applying less nitrogen fertilizers in topdressing. Therefore, it can increase more sustainably the protein content in the wheat grain.

With *A. brasilense* inoculation, the increment in N rates increases the wheat yield up to 142 kg ha⁻¹ N, whereas without this inoculation increases occurred up to 134 kg ha⁻¹. However, even at the highest doses, the inoculation afforded higher grain yield.

Inoculation with *A. brasilense* increased the agronomic efficiency, apparent N recovery, and N utilization efficiency. This research demonstrated that inoculation with *A. brasilense* associated with nitrogen fertilization in topdressing is beneficial to N nutrition and wheat yield, increasing nitrogen fertilization efficiency.

For further increasing the efficiency of nitrogen fertilization, new researches of complementary inoculation with *A. brasilense* during the vegetative phase of the plant would be interesting.

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