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Evaluation of *Urochloa decumbens* cv. Basilisk in Response to Nitrogen Fertilization and Inoculation With Diazotrophic Bacterium

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Abstract

Nitrogen fertilization provides a great response in pasture productivity and quality but, after applied to the soil, this element undergoes several transformations, what increase its losses. To minimize this problem, a promising alternative currently suggested is diazotrophic bacteria use, which can contribute to a greater use of nitrogen by plants. This study aimed to evaluate the effect of nitrogen doses with and without inoculation of seeds with *Azospirillum brasilense* on the structural characteristics, chemical composition, and mass production of *Urochloa decumbens* cv. Basilisk. The experimental design was completely randomized, arranged in a 2 × 5 factorial scheme, with four replications. Treatments consisted of forage seed inoculation or not with *Azospirillum* and five nitrogen doses (0, 50, 100, 150, and 200 kg ha^{-1}). The variables analyzed were plant height, number of tillers, shoot dry mass (SDM), root dry mass (RDM), SDM/RDM ratio, chlorophyll index, nutrient content in forage shoot, crude protein (CP), neutral detergent fiber (NDF), and nitrogen use efficiency. The inoculation of forage seeds with *A. brasilense* associated with nitrogen doses up to 100 kg ha^{-1} contributed positively to dry mass, plant-shoot nutrient content and bromatological composition of *U. decumbens* cv. Basilisk. The inoculation of seeds of *U. decumbens* cv. Basilisk, with *A. brasiliense*, is a viable alternative for partial substitution of nitrogen fertilization.

Keywords: *Azospirillum brasilense, Urochloa decumbens,* crude protein, neutral detergent fiber

1. Introduction

In Brazil, nearly 180 million hectares have been grown with pastures, mainly grasses of the genus *Urochloa* (Dias, 2011). The state of Mato Grosso do Sul owns an area of 16 million hectares under pasture, about 50% of which are degraded due to the low system productivity, which compromises the economic potential of livestock (Holsback, 2016).

An improved management of pastures during extended periods seems to be one of the main causes of soil fertility decline (Pereira et al., 2013). A poor input of nutrients and, hence, a decrease in soil organic matter stand out among the fertility decline factors. According to Alcântara et al. (2000), sustainable management of inputs and especially fertilization is the most viable alternative, aiming not only to recover areas already degraded but also to prevent the degradation of new areas.

Among the alternatives for recovering soil fertility in grazing areas, nitrogen fertilization is one of the most important practices, but it is also more difficult to manage (Vogel, Martinkoski, & Ruzicki, 2014) given the nitrogen dynamics in the soil, being a nutrient of low residual effect (Aguiar & Silva, 2005).

Nitrogen (N) is the main nutrient responsible for maintaining productivity, being responsible for leaf and stem morphological characteristics, development of tillers, among others (Taiz & Zeiger, 2009). Therefore, N fertilization is among the most important technologies able to improve plant productive potential, besides increasing dry matter production and enhancing forage quality (Juarez Lagunes, Fox, Blake, & Pell, 1999; Martha Júnior & Corsi, 2000; Teutsch, Fike, & Tilson, 2005). For Benett et al. (2008), increasing N doses up to 200 kg ha^{-1} in *U. brizantha* cv. *Marandu* pasture improved its bromatological composition by rising the contents of nitrogen and reducing the levels of neutral detergent fiber (NDF).

Another factor to be considered is the use of N by *Urochloa* species through N_2 fixation, in which these plants can associate with diazotrophic bacteria (Reis, 2007). Endophytic diazotrophic microorganisms can play an important role in the recovery and sustainability of ecosystems by incorporating atmospheric nitrogen (N_2) to the soil, besides producing and releasing plant growth regulators such as auxins, gibberellins, and cytokines. These substances are responsible for increasing the root system and thus improving mineral nutrition and water use by plants (Bazzicalupo & Okon, 2000).

The species *Azospirillum brasilense*, when associated with small doses of nitrogen in Poaceae species, shows high efficacy both for morphological aspects and for productivity (Vogel, Martinkoski, Martins, & Bichel, 2013). In Brazil, *Azospirillum* was successfully used to inoculate forage grasses (Vogel, Martinkoski, & Ruzicki, 2014).

The objective of this study was to evaluate the effect of nitrogen doses, with and without inoculation of the seeds with *Azospirillum brasilense*, on the structural characteristics, chemical composition, and mass production of *Urochloa decumbens* cv. Basilisk.

2. Method

The experiment was carried out in a greenhouse located in the municipality of Dourados, MS (Brazil) (22º11′43.7″ S and 54º56′08.5″ W, and 452-m altitude). The local climate is classified according to the Köppen's classification (1948) as Cwa—humid mesothermal.

The experimental design was completely randomized, in a 2 × 5 factorial, with four replications. The treatments were composed of two factors: inoculation with *Azospirillum brasilense* (presence or absence), and nitrogen doses (0, 50, 100, 150 and 200 kg ha^{-1}) in topdressing. Each plot consisted of a 5 dm^{-3} pot, totaling 40 experimental plots.

The soil used, classified as Quartzarenic Neosol (Santos et al., 2013), was collected in the city of Campo Grande, State of Mato Grosso do Sul. The soil was collected in the 0-20 cm layer, in an area under Cerrado vegetation. After collection, soil samples were air dried, crushed and sieved through a 2-mm mesh sieve to obtain air-dried fine soil (ADFS). Then, they were submitted to chemical and grain size analysis following the method of Claessen (1997). These analyses showed the following characteristics: pH ($CaCl_2$): 4.47; pH (water): 5.10; P (Mehlich-1): 3.84 mg dm^{-3}; K: 0.01 $cmol_c dm^{-3}$; Ca: 0.1 $cmol_c dm^{-3}$; Mg: 0.5 $cmol_c dm^{-3}$; H+Al: 0.73 $cmol_c dm^{-3}$; SB: 0.51 $cmol_c dm^{-3}$; CEC: 1.24 $cmol_c dm^{-3}$; BS: 41.13%; sand: 920 g kg^{-1}; silt: 10 g kg^{-1}, and clay: 60 g kg^{-1}.

The soil samples contained in the pots were submitted to two sequential incubations for a period of 30 days each, under humidity conditions equivalent to 60% of the total pore volume (TPV) occupied by water (Freire et al., 1980), controlled by daily weighing.

The first soil incubation was performed after soil liming for soil acidity correction. The liming was made to increase soil saturation to 50%, using a dolomitic limestone at a dosage of 3.5 Mg ha^{-1} (86% of PRNT, 31% CaO, and 21% MgO). After 30 days, implantation fertilization was performed in each pot applying phosphorus, potassium, sulfur, and micronutrients. These nutrients were applied in the form of PA salts corresponding to the following nutrient contents (in mg dm^{-3} soil): K (150); P (150); S (62); B (0.81); Cu (1.3); Zn (5.0); Mn (3.6); Fe (1.6), and Mo (0.15), whose sources were K_2SO_4, $(NH_4)_2HPO_4$, NH_4NO_3, KH_2PO_4, H_3PO_4, H_3BO_3, $MnSO_4·2H_2O$, $CuSO_4·5H_2O$, $(NH_4)_6Mo7O_{24}·4H_2O$, $FeSO_4·7H_2O$, $ZnSO_4·7H_2O$.

The forage *Urochloa decumbens* cv. Basilisk was sown after incubations, with fifteen seeds sown directly in the pots. The seeds were inoculated with *Azospirillum brasiliense* in the dosage of 30 g of peat inoculant for each kg of seeds and left for 15 minutes until material adherence. The dose used was 30 g kg^{-1} of seeds recommended by the product in grasses. Thinning was done ten days after sowing, leaving three forage plants per pot. Pots were maintained with moisture at 60% TPV (Freire et al., 1980). The plants were conducted up to 60 days after sowing.

Topdressing fertilizations with nitrogen were divided in four times. The first application was made at the time of sowing, and the other treatments were done at 10, 20, and 30 days after sowing (DAS).

The variables analyzed were: plant height, number of tillers, chlorophyll index, shoot dry mass (SDM), root dry mass (RDM), SDM/RDM ratio, nutrient content in forage shoot, crude protein (CP), neutral detergent fiber (NDF), and nitrogen utilization efficiency.

Plant height was measured with a graded ruler, from the soil to the curvature of the plant canopy. The number of tillers was evaluated by counting all the tillers of the plants per pot. The estimation of chlorophyll content was performed indirectly by the SPAD-502 (Soil Plant Analysis Development) reading, using a chlorophyll meter. The chlorophyll index per experimental plot was determined by the average of five readings per leaf.

After collecting the shoot, the root system was separated by washing the remaining soil in the pot after sampling, under running water and using sieves with a 2 mm mesh. After collection and washing, all the material was dried in an air circulation oven at 65 ºC for 72 hours until reaching constant dry mass (Malavolta, 2006). The material was then weighed for shoot dry mass (SDM) and root dry mass (RDM) determinations, as well as the SDM/RDM ratio. After weighing the samples were ground in a Willey type mill with 1 mm diameter sieves to determine the contents of N, P, K, Ca, and Mg, as described by Malavolta et al. (1997). Crude Protein (CP) was also determined according to procedures described by the Association of Official Analytical Chemists AOAC (1995), and Neutral Detergent Fiber (NDF) was determined as described by Van Soest (1994). The NDF was determined using TNT bags with a porosity of 100 gm^{-2}, using a fiber determiner (TE-149-Tecnal®).

The estimated N use efficiency was obtained by dividing the shoot dry mass per pot by the amount of nitrogen applied per pot, values expressed in g of dry mass per g of N added (Moll, Kamprath, & Jackson, 1982).

The data were submitted to analysis of variance and the means were compared by the F test ($p < 0.05$) to evaluate two variables using the ASSISTAT statistical software (Silva & Azevedo, 2016). Regression analysis was used for the N doses when the dose significance was verified. The following mathematical model for multiple linear regressions was used in this study.

$$y = \beta 0 + \beta 1 \cdot x1 + ... + \beta n \cdot xn + \varepsilon \quad (1)$$

Where, y is the response variable and xi (i = 1, 2... n) are the explanatory variables. β0 represents the value of y when the explanatory variables are null, the terms βi are called regression coefficients and the residue (ε) is the prediction error, *i.e.* the difference between the response variable actual and expected values, which is assumed to be normally distributed with mean zero and variance σ2 (Hair Jr., Anderson, Tatham, & Black, 2005).

3. Results and Discussion

An interaction between nitrogen doses and inoculation with *A. brasilense* ($p < 0.01$) was observed for structural characteristics and forage mass production (Figures 1a, 1b, 1c, 1d, and 1e). The variables plant height, number of tillers, shoot dry mass, and root dry mass were described by an increasing linear model (Figures 1a, 1b, 1c, and 1d). The dose of 200 kg ha^{-1} provided the highest values for these variables. This is due to the higher availability of N in the soil and its consequent absorption by the plants. A similar result was obtained by Hanisch, Balbinot Junior and Vogt (2017), who observed a linear increase of nitrogen fertilization on the availability of *U. brizantha* forage in the first evaluation year.

Nitrogen is the main nutrient responsible for the morphological characteristics of leaves and stems size and development of tillers (Taiz & Zeiger, 2009) and of the root system (Monteiro, 2010), since this element is part of the chlorophyll molecule, acting on the formation of substances such as proteins, enzymes, and nucleic acids (Gross, Von Pinho, & Brito, 2006).

The highest average plant height (60.75 cm), number of tillers (36.79 tillers per pot^{-1}), shoot dry mass (30.11 g pot^{-1}), and root dry mass (32.58 g pot^{-1}), were found in treatments inoculated with *Azospirillum*. These results respectively indicate increases of nearly 6%, 14%, 6%, and 11% if compared to the treatments without inoculation. For these treatments were seen the lowest averages of plant height (57.20 cm), number of tillers (32.25 tillers per pot^{-1}), shoot dry mass (28.51 g pot^{-1}), and root dry mass (29.29 g pot^{-1}). Similar results were found by Guimarães et al. (2011) which saw an increase approximately 8% in the number of leaves and 7% in the number of tillers inoculated in relation to the absence of the bacterium in pastures of *Urochloa*.

The results found with inoculation can be explained by the improvement in plant growth, mainly of the root system. According to Hungria, Campo, Souza, and Pedrosa (2010), the excretion of plant hormones by bacteria, especially indoleacetic acid, can promote plant growth and increase the absorption of nutrients and water. However, the highest efficiency of the *A. brasilense* bacterium is observed when associated with lower nitrogen doses (Figures 1a, 1b, 1c, and 1d). This result is due to the inactivation of the *Azospirillum* nitrogenase complex under conditions of high ammonia (NH_3) concentrations and low oxygen and carbon concentrations (Kavadia, Vayenas, & Aggelis, 2008).

Therefore, the competitive capacity of the diazotrophic bacteria is only when the soil conditions present low N availability in the environment (Silva, Antoniolli, Seminoti, & Voss, 2007). This study showed that inoculation associated with N doses had an effect on shoot (Figure 1c) and root (Figure 1d) dry mass production, being only observed in treatments receiving N doses up to 100 kg ha^{-1}.

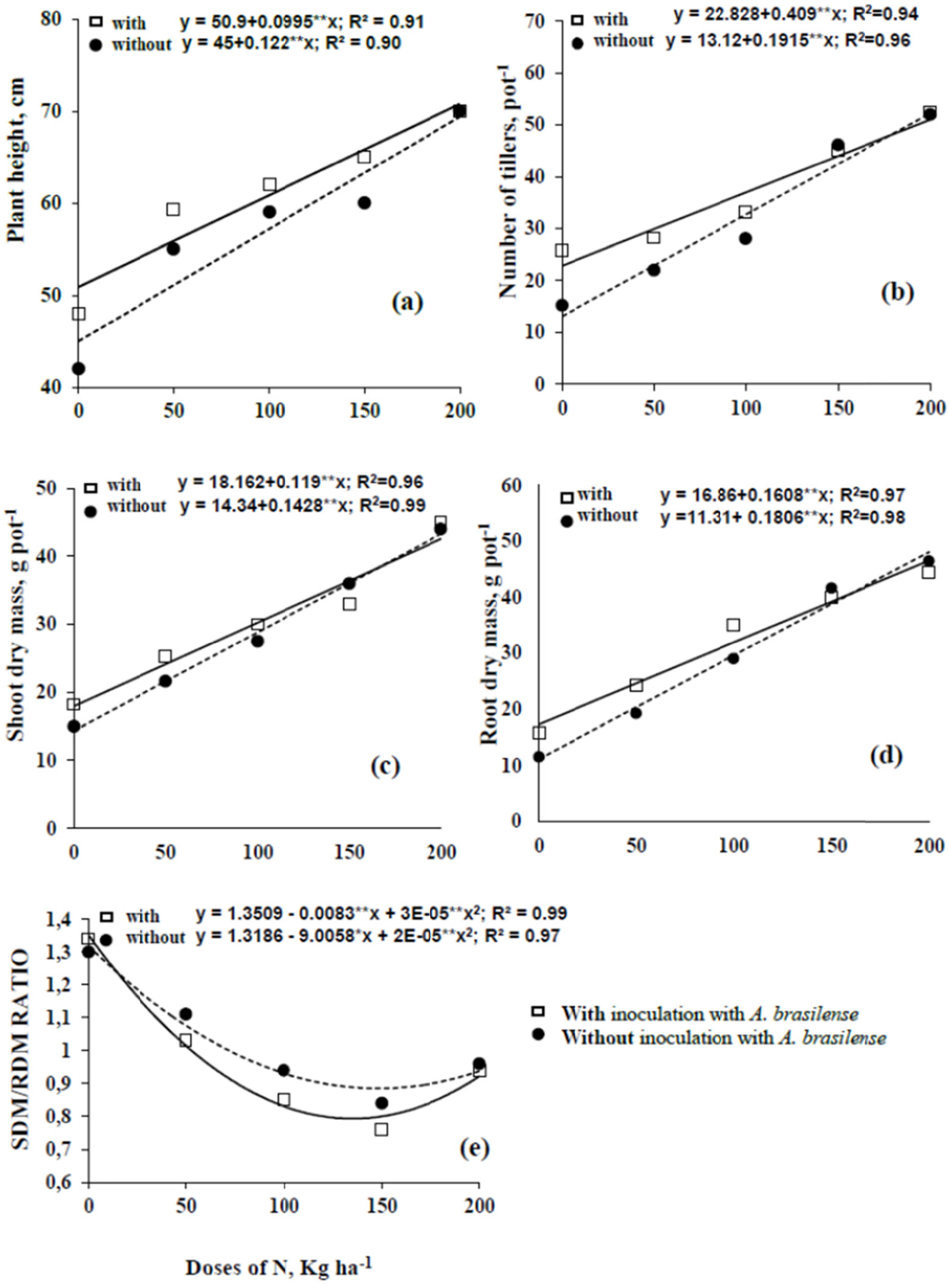

Figure 1. Structural features of *Urochloa decumbens* cv. Basilisk, according to the nitrogen doses with and without inoculation with *Azospirillum brasilense*. (a) plant height; (b) number of tillers; (c) Shoot dry mass (SDM); (d) root dry mass (RDM); (e) SDM/RDM ratio

Note. **: significant at 1% probability.

These observations corroborate the discussions made by Hungria, Nogueira, and Araujo (2016) in an experiment with two species of *Urochloa* in three different regions of Brazil over two years. The authors obtained a 22% increase in forage production in response to the addition of 40 kg ha^{-1} of N in combination with *Azospirillum.*

Kuss (2006) reported that the use of this bacterium helps increase production from 12% to 14%. Moreover, Vogel, Martinkoski, and Bichel (2013) verified that this bacterium has stood out in agricultural environment as a sustainable alternative to reduce nitrogen application via fertilizers.

The data for SDM/RDM ratio and N rates were observed to conform to the quadratic polynomial model (Figure 1E). The lowest SDM/RDM ratios were estimated at 0.78 and 0.90, at doses of 145 and 138.33 kg ha^{-1} of N, respectively, with and without inoculation. The association of plants with *Azospirillum brasilense* is more related to the promotion of plant development, mainly of the root system than to biological nitrogen fixation (BNF), although numerous reports point to this process viability (Oliveira et al., 2013).

The lower the value of the SDM/RDM ratio, the greater the root dry mass, which confers a higher root surface area, and consequently the water and nutrients absorption capacity. According to Didonet, Lima, Candaten, and Rodrigues (2000), the increase of the root system in inoculated plants can also provide greater longevity to the green tissues and, hence, a period of photosynthetic activity occurs, which results in greater N assimilation compared to the non-inoculated plants.

Chlorophyll contents and N efficiency were significantly different ($p < 0.01$) as a function of N doses and inoculation (Figures 2a and 2b), with the highest values of chlorophyll corresponding to treatments with the highest N doses (Figure 2a). These data corroborate those obtained by Jordão et al. (2010). The predictability of these results is justified by the fundamental role of N in plant metabolism, participating directly in the biosynthesis of proteins and chlorophylls. The highest values of chlorophyll index were observed in inoculated treatments, especially when associated with the lowest N doses (Figure 2a). According to Monteiro (2010), values of 20 to 25 indicate forage with N deficiency. Values above 40 suggest good nitrogenous nutrition of the grass.

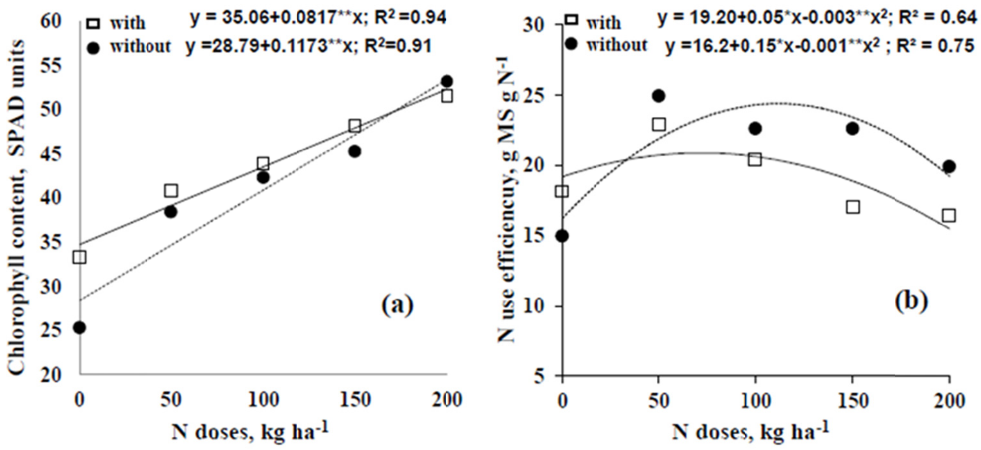

Figure 2. (a) Chlorophyll content and (b) Nitrogen Utilization Efficiency (NUE) of *Urochloa decumbens* cv. Basilisk, as a function of nitrogen doses and inoculation with *Azospirillum brasiliense*

Note. **: significant at 1% probability.

Bashan et al. (2006) reported an increase in several photosynthetic pigments, such as chlorophyll a, b, and auxiliary photopigments, due to the presence of *Azospirillum* spp., justifying its positive effect on the chlorophyll index. This pigment is directly associated with the photosynthetic activity potential, and the plant nutritional state is usually associated with chlorophyll amount and quality (Zorateli et al., 2003).

The data for N use efficiency (NUE) is observed to conform to the quadratic polynomial model (Figure 2b). Deriving the equations, the most efficient nitrogen dose to be applied to the soil was estimated around 105 kg ha^{-1}, with an efficiency of 23.91 with no inoculation. In the treatments inoculated, however, the estimated efficiency was 21.06 at the dose of 79 kg ha^{-1} of N, resulting in dry mass accumulations with lower investment in nitrogen fertilization.

A significant interaction was observed between N doses and inoculation for the contents of nitrogen, potassium, copper, iron, and manganese (Figures 3a, 3b, 3c, 3d, and 3e). Foliar N data in the absence of inoculation were adjusted to linear regression. However, the N foliar content presented a quadratic response to N application in inoculated treatments, with its maximum point estimated at 184.28 kg ha^{-1} N, with foliar N content of 31.97 g kg^{-1}. If the same N dose (184.28 kg ha^{-1}) is considered without inoculation the estimated leaf N content will be 27.94 g kg^{-1}, which generates an increase of approximately 15% in N content, when combined with *Azospirillum*.

When compared to non-inoculated strains, the inoculated ones showed good efficiency as for BNF. The better response in the presence of *Azospirillum* is due to the characteristics of this bacterium, because, in addition to the N_2 fixation, it improves the root system, increasing the efficiency of nitrogen fertilization.

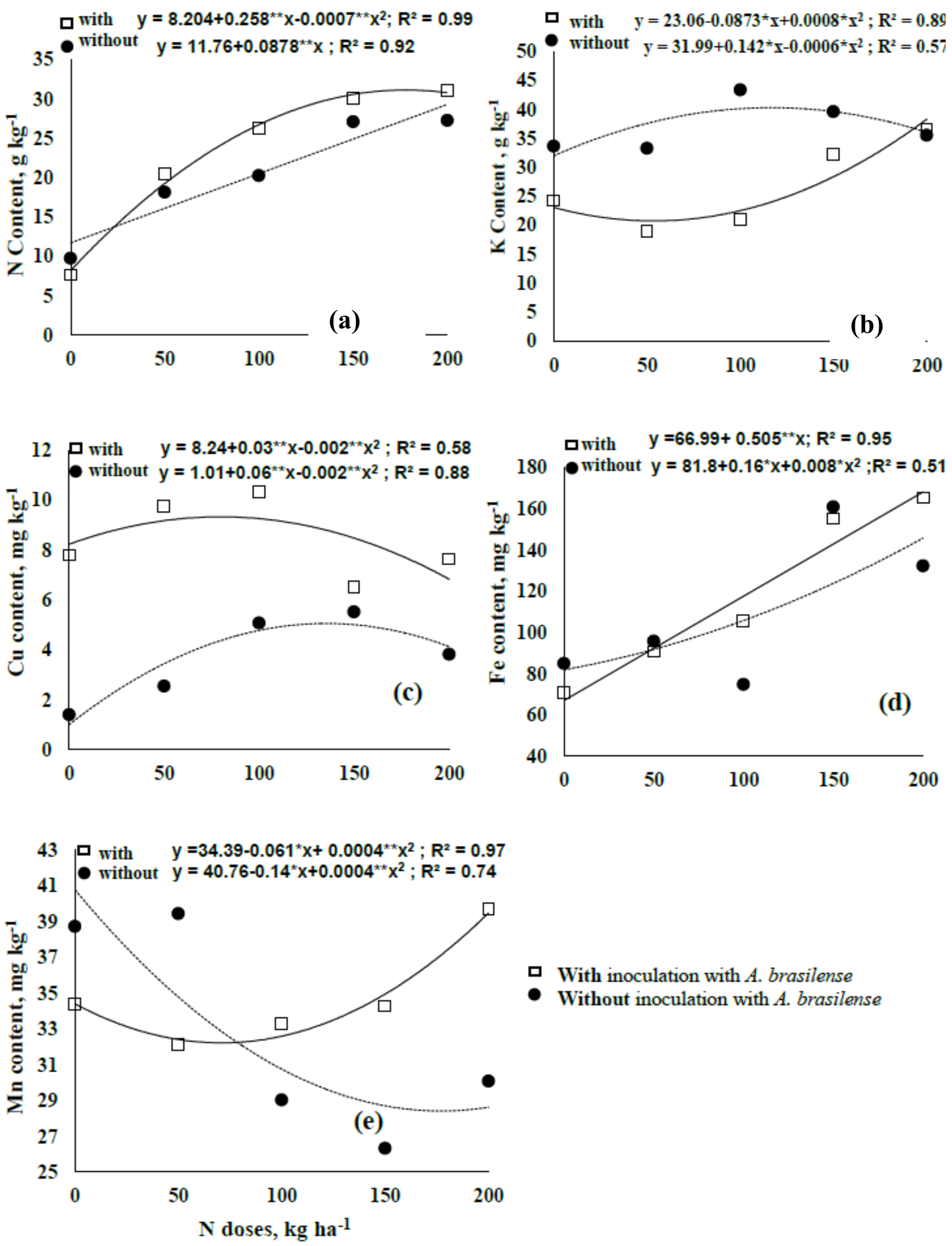

Figure 3. Nitrogen (a), potassium (b), copper (c), iron (d), and manganese (e) leaf contents in *Urochloa decumbens* plants as a function of nitrogen doses with and without inoculation with *Azospirillum brasilense*

A quadratic adjustment was observed for the K content without inoculation (Figure 3b), with an estimated maximum point at the dose of 118.67 kg ha^{-1} of N, with a content of 40.44 g kg^{-1}. This increase in K content is due to the high correlation between N and K, where there is synergism between applied N and leaf K (Primavesi et al., 2006). When seeds were inoculated, the minimum point was estimated at the dose of 54.56 kg ha^{-1} of N, with a content of 20.70 g kg^{-1} in treatments without inoculation.

Leaf Cu content increased when 69 and 150 kg ha^{-1} of N were applied, with maximum levels of 9.19 and 5.51 mg kg^{-1} respectively, with and without inoculation (Figure 3c). The leaf Fe contents increased linearly with the increase of nitrogen fertilization in the inoculated treatments. A quadratic effect was found for the treatments with no inoculation, with a minimum point estimated at the dose of 99.12 kg ha^{-1} of N, with leaf content of 105.41 mg kg^{-1} of Fe (Figure 3d). The increase of N doses had a quadratic effect on Mn leaf contents, generating minimum points of 32.03 and 28.58 mg kg^{-1}, at the doses of 76.75 and 174.50 kg ha^{-1} of N, respectively, with and without inoculation (Figure 3e).

The effect of inoculation on the forage nutrient content considering the dose of 100 kg ha^{-1} of N is observed to cause an increase of 32%, 80%, 11%, and 5%, respectively, for the contents of N, Cu, Fe, and Mn, when compared to non-inoculated treatments. The lowest levels of K were observed for inoculated treatments.

Werner et al. (1996) suggested content ranges of N, K, Cu, Fe, and Mn for a good *U. decumbens* growth and development, being of 12 to 20 g kg^{-1}; 12 to 25g kg^{-1}; 4 to 12 mg kg^{-1}; 50 to 250 mg kg^{-1}, and from 40 to 250 mg kg^{-1}, respectively. Considering these values, despite changes in nutrient content in the forage shoot due to nitrogen fertilization and inoculation, K and Fe contents are in the appropriate range for any of the N doses and in both situations (with and without inoculation).

Regardless bacterium presence or absence, an application of at least 50 kg ha^{-1} N is necessary for forage plants in order to reach at least a minimum N content, as indicated by Werner et al. (1996). An amount of 100 kg ha^{-1} N has to be added without inoculation so that plants could reach an appropriate content of Cu. However, inoculated treatments showed Cu levels within the appropriate range, regardless of the N doses. By contrast, Mn contents found were below those indicated by the authors, regardless of the N doses and bacteria presence or absence.

The P content only showed a significant difference between the doses, with adjustment of the data to the quadratic model (Figure 4), with an estimated maximum point in the dose of 115 kg ha^{-1} of N, with a content of 5.62 g kg^{-1} of P. However, leaf contents are considered adequate in all treatments when compared to the range of 0.8 to 3.0 g of P kg^{-1} indicated by Werner et al. (1996) to diagnose the nutritional status of forage grasses. Thus, phosphate fertilization performed was enough to feed the plant with P, even if the initial soil levels were low.

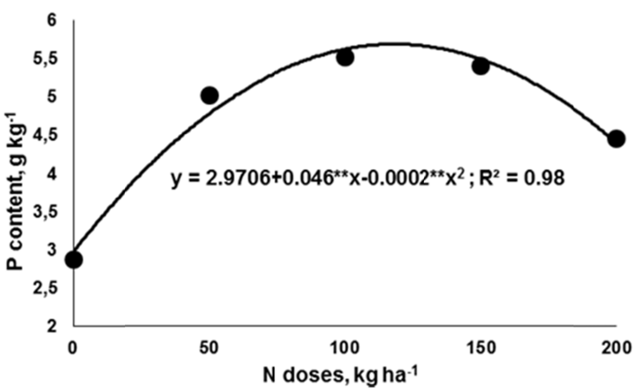

Figure 4. Phosphorus leaf content in *Urochloa decumbens* plants as a function of nitrogen doses with and without inoculation with Azospirillum brasilense

No significant difference was found among treatments for contents of Ca, Mg, and Zn, which reached mean foliar contents of 3.86 g kg^{-1}, 3.25 g kg^{-1}, and 20.32 mg kg^{-1} respectively, but being considered adequate for forage development (Werner et al., 1996).

The concentration of nutrients in plant tissue is important since it implies in mineral nutrition and development of forage-eating animals. For a 350-kg calf with daily weight gain of 0.5 kg, the minimum requirement in terms of forage composition is 11.2 g kg^{-1} N; 0.5 g kg^{-1} P; 6.0 g kg^{-1} K; 1.2 g kg^{-1} Ca; 1.0 g kg^{-1} Mg; 1.5 g kg^{-1} S; 10 mg kg^{-1} Cu; 30 mg kg^{-1} Mn, and 30 mg kg^{-1} Zn (National Randsandarch Council, 2000). According to these values, with the exception of the Zn content, the contents found in the forage fully satisfy the needs of the animals kept under grazing.

Significant interactions were observed between inoculation and N doses in the forage bromatological composition ($p < 0.01$) (Figures 5a and 5b). A quadratic adjustment was found for crude protein (CP), with a maximum point at the dose of 148.50 kg ha^{-1} of N (19.46% CP) and 110.36 kg ha^{-1} of N (17.06% CP), with and without inoculation, respectively (Figure 5a).

Starting from the dose of 50 kg ha^{-1} N, CP levels were adequate for animal feed, being higher than the 7% considered by Van Soest (1994). According to Reis et al. (2009), values lower than 7% may limit consumption, reduce digestibility, and cause a negative nitrogen balance.

Values below 7% were observed in the present study only in the absence of nitrogen fertilization, in both situations (with and without inoculation). Thus, the importance of N availability is evident since both natural soil fertility conditions and native rhizobia strains were not sufficient to guarantee adequate CP levels. According to Paris et al. (2005), the use of nitrogen fertilization tends to increase the nutritive value of the plant, mainly by increasing the amount of soluble nitrogen in organic and inorganic forms.

Neutral detergent fiber (NDF) contents above 60% are negatively correlated to forage consumption (Van Soest, 1994). In the present study, NDF contents decreased linearly as a function of N doses in non-inoculated treatments (Figure 5b). Thus, higher N doses were shown to be more efficient in reducing NDF contents. When inoculated, a quadratic adjustment was found, with a minimum point of 101.19 kg of N per ha^{-1} for a content of 59.13% NDF. Cecato et al. (2001) emphasized the influence of N applications on NDF contents since it promotes an increase in the concentration of fibrous tissues, reducing forage quality.

Thus, the evaluation of the forage nutritive value suggested nitrogen fertilization of 100 kg ha^{-1} of N associated with the inoculation of the forage seeds with *Azospirillum* to increase CP and reduce NDF, which coincides with the range of N contents suitable for forage, which is 12 to 20 g kg^{-1} (Werner, Colozza, & Monteiro, 2001).

The use of nitrogen fertilization associated with *Azospirillum brasilense* is observed to present promising results, promoting a significant contribution to the structural variables, dry mass, nutrient contents, and bromatological composition of *U. decumbens* cv. Basilisk and inoculation may be a viable alternative to substitute part of the nitrogen fertilization.

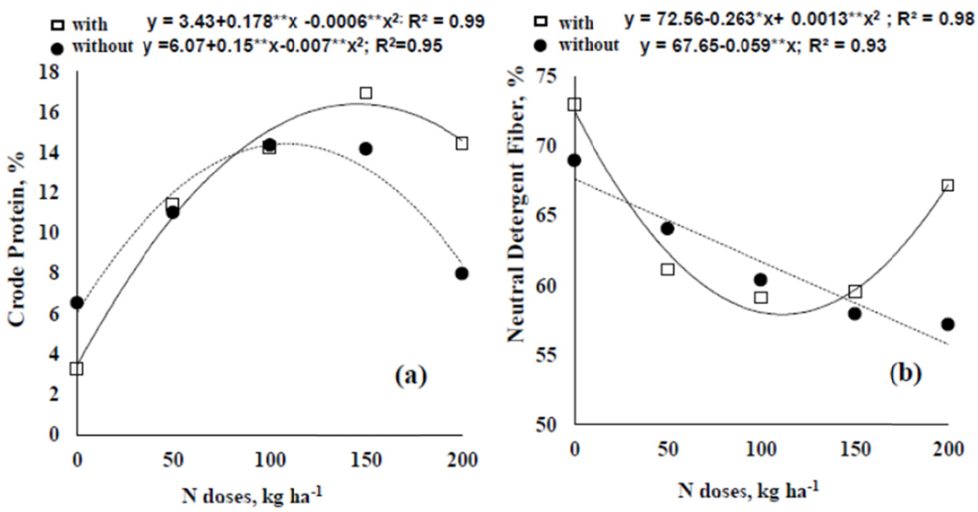

Figure 5. Crude protein content (a) and neutral detergent fiber (b) in *Urochloa decumbens* plants as a function of nitrogen doses with and without inoculation with *Azospirillum brasilense*

4. Conclusion

Seed inoculation with *Azospirillum brasilense* associated with nitrogen dose up to 100 kg ha^{-1} showed a positive contribution to forage structural characteristics such as dry mass, shoot nutrient content, and bromatological composition of *Urochloa decumbens* cv. Basilisk.

Inoculating seeds of *Urochloa decumbens* cv. Basilisk with *Azospirillum brasiliense* is a feasible alternative for partial substitution of nitrogen fertilization.

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