NITROGEN FERTILIZATION AND *Azospirillum brasilense* INOCULATION ON *Panicum maximum* CV. Mombasa †

[FERTILIZACION NITROGENADA E INOCULACIÓN CON *Azospirillum brasilense* EN LA FORRAJE *Panicum maximum* cv. Mombasa]

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SUMMARY

Background. Nitrogen fertilization of pastures is considered one of the main limiting factors and that most promote an increase in the production of forage biomass. For this reason, research that evaluates alternative techniques that can complement or supply the demand for N in forage becomes important. Recently, some researches have been studying the action of bacteria like Azospirillum brasilense, and their ability to make symbiotic associations with grasses, fixing atmospheric nitrogen and producing hormones that stimulate the growth of their roots. **Objective.** The aim of this work was to evaluate the development and production of *Panicum maximum* cv. Mombasa as a function of nitrogen (N) doses and inoculation with diazotrophic bacteria. **Methodology.** The experimental design was completely randomized (CRD), in 5 x 2 factorial scheme, in pots with capacity for 10 dm^3 of soil, with four replicates. Treatments consisted of the combination of N doses (0, 50, 100, 150, 200 kg ha^{-1}), using urea (45% N) as source, with and without inoculation with *Azospirillum brasilense*. The parameters evaluated were: SPAD index, leaf dry matter, leaf area, crude protein content (CP), root volume, root dry matter, stem dry matter and leaf/stem ratio. **Results.** Root volume, CP content, stem dry matter and green color index (SPAD) increased linearly as a function of the N doses. Only leaf/stem ratio and green color index responded to the inoculation with bacteria. Nitrogen fertilization directly contributes to promoting higher yields and fast establishment of the pasture. So, inoculation with *Azospirillum brasilense* leads to promising results in *Panicum maximum* cv. Mombasa, improving its leaf/stem ratio and SPAD index. However, it is not sufficient to promote higher forage production, so further research is needed with other grasses to confirm its efficiency, when associated with N. **Conclusion.** Nitrogen fertilization directly contributes to promoting higher yields and fast establishment of the pasture. Nitrogen doses between 100 and 150 kg ha^{-1} proved to be the most indicated for the development of *P. maximum* cv. Mombasa.

Keywords: Diazotrophic bacteria; forage; leaf dry mass; stem dry mass; urea.

RESUMEN

Antecedentes. La fertilización nitrogenada de los pastos se considera uno de los principales factores limitantes y que más promueven un aumento en la producción de biomasa forrajera. Por esta razón, la investigación que evalúa técnicas alternativas que pueden complementar o abastecer la demanda de N en el forraje se vuelve importante. Recientemente, algunas investigaciones han estado estudiando la acción de bacterias como *Azospirillum brasilense*, y su capacidad para establecer asociaciones simbióticas con los pastos, fijar el nitrógeno atmosférico y producir hormonas que estimulan el crecimiento de sus raíces. **Objetivo.** El objetivo de este trabajo fue evaluar el desarrollo y la producción de *Panicum maximum* cv. Mombasa en función de las dosis de nitrógeno (N) y la inoculación con bacterias diazotróficas. **Metodología.** El diseño experimental fue completamente al azar (CRD), en esquema factorial 5x2, en macetas con capacidad para 10 dm^3 de suelo, con cuatro repeticiones. Los tratamientos consistieron en la combinación de dosis de N (0, 50, 100, 150, 200 kg ha^{-1}), utilizando urea (45% de

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N) como fuente, con y sin inoculación con *A. brasilense*. Los parámetros evaluados fueron: índice SPAD, materia seca de la hoja, área de la hoja, contenido de proteína cruda (PC), volumen de la raíz, materia seca de la raíz, materia seca del tallo y relación hoja / tallo. **Resultados.** El volumen de la raíz, el contenido de PC, la materia seca del tallo y el índice de color verde (SPAD) aumentaron linealmente en función de las dosis de N. Solo la relación hoja/tallo y el índice de color verde respondieron a la inoculación con bacterias. La fertilización nitrogenada contribuye directamente a promover mayores rendimientos y un rápido establecimiento del pasto. Entonces, la inoculación con *A. brasilense* conduce a resultados prometedores en *P. maximum* cv. Mombasa, mejorando su relación hoja/tallo y su índice SPAD. Sin embargo, no es suficiente para promover una mayor producción de forraje, por lo que se necesita más investigación con otros pastos para confirmar su eficiencia, cuando se asocia con N. **Conclusión.** La fertilización nitrogenada contribuye directamente a promover mayores rendimientos y un rápido establecimiento del pasto. Las dosis de nitrógeno entre 100 y 150 kg ha^{-1} resultaron ser las más indicadas para el desarrollo de *P. maximum* cv. Mombasa.

Palabras clave: Bacterias diazotróficas; forraje; masa seca de la hoja; masa seca del tallo; urea.

INTRODUCTION

Tropical forage species used as animal feed constitute an important factor for the national livestock production, because cattle are mostly raised in pastures, which is one of the systems with lowest costs for beef and milk production (Costa *et al.*, 2010).

The productive potential of grasses is defined by the genetic traits of the species. However, inadequate management practices and phytosanitary problems, caused by pests and diseases, reduce their animal support capacity, thus evidencing the need for nitrogen (N) fertilization, because N supply promotes increments in yield and crude protein content (CP) and better digestibility for the animal (Lupatini *et al*., 2013).

For these reasons, pasture fertilization, especially with N, is among the limiting factors that most stimulate pasture biomass production, because the N from soil organic matter mineralization is not able to meet the demand of grass species (Novakowiski *et al*., 2011), so it needs to be complemented by topdressing application.

Thus, due to the importance of N for forage grasses, studies aiming at alternative techniques capable of complementing or supplying N to grass species are important. In addition, it is particularly necessary for extensive cattle farming that they are sustainable and less harmful to the environment, if compared to areas with reduced yield and high degradation, which are currently very common in several livestock farming regions in the Brazilian Amazon.

In this context, some studies have been conducted with microorganisms, assessing the action of bacteria such as *Azospirillum brasilense*, which are able to establish symbiotic associations with grasses, fixing atmospheric N and producing hormones that stimulate the growth of their roots (Hungria, 2011). The use of *Azospirillum* spp. inoculated in seeds of *Urochloa brizantha* cv. Marandu associated with N fertilization has been found to have good results in final leaf length, but studies related to the behavior of *Azospirillum* spp. combined with N doses are still scarce, requiring further scientific research.

The present study aimed to assess the development and biomass production of *Panicum maximum* cv. Mombasa as a function of application of N doses combined with inoculation of *Azospirillum brasilense*.

MATERIAL AND METHODS

The study was carried out between February and July 2016 in a protected environment of the Plant Science Laboratory (LabFITO) of the Mato Grosso State University, *Campus* of Alta Floresta, located at 9º 51' 41.83'' S latitude, 56º 04' 09.61'' W longitude and altitude of 283 m.

The experiment was conducted using plastic pots with capacity for 10 dm^3, in a greenhouse. The soil used to fill the pots was classified as Dystrophic RED YELLOW LATOSOL (Santos *et al*., 2013), sampled in the 0-0.20 m layer, to determine chemical and granulometric characteristics according to the procedure recommended by Embrapa (2009), and had the following results: clay: 379 g kg^{-1}; sand: 521g kg^{-1} and silt: 100 g kg^{-1} (sandy clay); pH in water: 5.5; P (Mehlich^{-1}): 4.8 mg dm^{-3}; K: 0.14 cmolc dm^{-3}; Ca: 2.32 cmolc dm^{-3}; Mg: 0.68 cmolc dm^{-3}; (H+Al): 2.60 cmolc dm^{-3}; SB: 3.14 cmolc dm^{-3}; V: 54.7% and CEC: 5.7 cmolc dm^{-3}.

For soil correction, dolomitic limestone (RNV 95%) was applied 60 days before sowing the forage, to increase base saturation to 60% (Embrapa, 2009). Basal fertilization consisted of 200 mg dm^{-3} of P, 150 mg dm^{-3} of K (Malavolta, 1981), using single superphosphate (18% P_2O_5) and potassium chloride (60% K_2O) as sources, respectively.

The experimental design was completely randomized, with 10 treatments and four replicates, in 5 x 2 factorial scheme, formed by the combination of five doses of N (0, 50, 100, 150 and 200 kg ha^{-1}) in the form of urea (45 % N), in the presence and absence of *A. brasilense*.

Seeds were inoculated just before sowing, using the liquid commercial product NitroGeo AZ, containing *A. brasilense* at concentration of 2x108 CFU mL^{-1}, AbV5 and AbV6 bacterial strains, with dose of 100 mL of the product for every 25 kg of seeds.

The forage species used was *Panicum maximum* cv. Mombasa, and 10 pelleted seeds were planted in each pot. Thinning was carried out at 30 days after sowing (DAS) in order to leave three plants per experimental unit (pot).

Topdressing fertilization in the 40 sampling units was applied at 51 DAS, using 22 mg dm^{-3} of N (50 kg ha^{-1} of N) per pot, in the form of urea dissolved in 200 mL of water. At 60 DAS, a standardizing cut was performed in the sampling units.

Treatments were applied 30 days after the standardizing cut (90 DAS), and the quantity of N in each treatment was diluted in 1600 mL of water, applying 200 mL of the solution per pot.

In total, three cuts were performed along the experimental period, when 75% of the plants were 0.90 m tall, maintaining their height at 0.30 m from soil surface (Pietroski *et al*., 2015).

Before each cut for evaluation, SPAD index was read in a SPAD-502™ chlorophyll meter (Soil and Plant Analysis Development) to determine the green color index of the leaves. Readings were taken in all replicates of each treatment, in five laminae of recently expanded leaves per plant, with five readings per leaf, totaling 300 readings per treatment.

All the fresh matter from each cut was collected and placed in plastic bags to separate the material into two parts: leaf lamina and stems, and each fraction was weighed on semi-analytical scale (0.01 g) to obtain the leaf/stem ratio. Leaves from each cut were measured for area (cm^2) using LICOR 3100™ leaf area meter. Then, all the material was separately placed in Kraft paper bags and dried in a forced air circulation oven at 65 ºC until constant weight, in order to obtain the dry matter.

The dry matter was ground in Wiley-type mill according to the procedure described by Embrapa (2009) to obtain N contents, which were multiplied by the conversion factor 6.25 to estimate the crude protein content of the plant material, following the methodology described by Mariotti *et al.* (2008).

After the last cut, the root system was collected to determine root dry matter, using the same procedure carried out for the shoots. Root volume was determined by the graduated cylinder method and its results were expressed in cm^3, according to the procedures proposed by Bouma *et al*. (2000).

The results were compared by analysis of variance (ANOVA), and F test was applied to the factors and their interactions. When significant, the quantitative factor (N doses) was subjected to polynomial regression analysis and the qualitative factor (inoculation) to Tukey test at 0.05 probability level, using the statistical software R (R Development Core Team, 2018).

RESULTS AND DISCUSSION

Based on the results for the application of increasing N doses associated with inoculation of *A. brasilense* in *P. maximum* cv. Mombasa (Table 1), it was observed that seed inoculation with the bacterial strain caused differences only in leaf/stem ratio and SPAD index. N doses positively influenced all variables analyzed.

There was no increase in leaf and stem dry matter yields with the inoculation of *Azospirillum* spp. This is possibly due to factors such as genetic traits of the plants and environmental conditions to which they were subjected (Sala *et al*., 2007). According to the variations in these conditions, bacteria may induce physical and biochemical changes in plants, through the production of phytohormones which act in the increase of the root system, promoting its growth. Thus, characteristics such as temperature and water availability may have favored plant development in the treatment without inoculation to the point of causing no significant difference between this condition and the inoculated treatment.

For leaf and stem dry matter production, under increasing N doses (Figures 1a and 1b), the forage responded with a quadratic behavior, showing maximum values of leaf and stem dry matter production at the doses of 135 and 127 kg ha^{-1} of N, respectively. Differently from the present study, Freitas *et al*. (2007) found no effect of N doses up to 400 kg ha^{-1} on the same forage species. By contrast, Costa *et al.* (2006) recommend the application of N doses between 50 and 300 kg ha^{-1} $year^{-1}$ in *Panicum* species, and 50 kg ha^{-1} of N is considered as the minimum dose to avoid pasture degradation, while higher doses are indicated to increase pasture and animal yields. Thus, the N doses of 135 and 127 kg ha^{-1} observed in the present study agree with results found by these authors, because they are similar and within the range suggested by them.

Increment in dry matter yield, due to the application of increasing N doses, occurs especially because of the production of new cells and plant growth, affecting the size of leaves and stems. All of these are characteristics and functions performed by this macronutrient in grass species, such as *P. maximum* (Pietroski *et al*., 2015).
Root dry matter production data fitted to a quadratic model as a function of the N doses applied (Figure 1c), and plants reached maximum root dry matter accumulation at N dose of 118.75 kg ha^{-1}.

Root volume increased linearly as a function of the N doses applied, indicating that the tested doses were not able to lead to maximum production of roots (Figure 1d), although an important increase was observed in root system volume with the N application. Corroborating these results, Sarmento *et al*. (2008) tested N doses up to 450 kg ha^{-1} and obtained maximum root density of *P. maximum* with 200 kg ha^{-1}, indicating greater root growth until this level, which was the maximum dose tested in the present study.

According to White and Veneklaas (2012), a possible explanation for low root production is that, when leaves are cut, there may be a reduction in carbon assimilation. Thus, the assimilation performed may not have been sufficient to meet the demand of the leaves, exporting carbon from the roots to the shoots.

The effect of N application on the leaf area of this species is demonstrated in Figure 1e, which shows that under this condition there is a quadratic behavior, with maximum response obtained at the dose of 143.96 kg ha^{-1}, resulting in leaf area of 7,184 cm^2 pot^{-1}. Eichler *et al*. (2008), in agreement with these results, also found increments in Mombasa grass leaf area up to N doses of 714 kg ha^{-1}, with production of 9,896 cm^2 pot^{-1}, which is equivalent to a N application that is 459.9% higher in order to reach a leaf area that is 37.73% higher, compared to the present study. Thus, these results demonstrate the good development exhibited by the forage under the treatments applied. Increase in leaf area is due to the direct action of this macronutrient in the process of cell division and expansion, besides participating in the chlorophyll molecule, leading to fast plant growth and higher leaf area index and accumulation of carbohydrates (Quadros and Andrade, 2006).

Table 1. F values, coefficient of variation, Minimal significant difference (MSD – Tukey 5%) and mean values of leaf dry matter (LDM), stem dry matter (SDM), root dry matter (RDM), leaf area, green color index (SPAD), root volume (RV) and crude protein content (CP) in *P. maximum* cv. Mombasa, subjected to N doses with and without inoculation of *Azospirillum brasilense*. Alta Floresta - MT, Brazil.

	LDM	SDM	RDM	RV	Leaf area per pot	Green color index	CP
Doses of N (N)	---------- (g pots^{-1}) ----------			cm^3	mm^2		%
0	18.1	0.3	21.3	57.0	2.803	65.3	3.8
50	28.2	0.9	28.8	58.6	4.088	68.3	4.2
100	38.3	1.5	45.4	98.6	7.184	92.6	6.2
150	34.3	1.4	29.4	76.7	6.377	110.2	8.6
200	34.7	1.0	32.2	85.5	6.088	111.1	8.3
Inoculant (I)							
With	31.9	1.0	31.4a	79.1a	5.456	91.1a	6.2a
Without	29.6	1.0	31.4a	71.5a	5.160	88.2b	6.2a
LSD	2.9	0.3	7.1	16.2	534.6	2.7	0.4
	-- F values --						
N	23.4**	6.8**	5.0 **	3.9*	38.0**	221.9**	79.6**
Inoculant	2.5 ns	0.1 ns	0.0 ns	0.9 ns	1.2 ns	4.4*	0.0 ns
N x I	0.4 ns	0.9 ns	0.3 ns	0.8 ns	0.8 ns	0.2 ns	0.9 ns
Cv (%)	15.07	49.67	35.0	33.41	15.5	4.69	11.28

Averages followed by the same letter do not differ from each other at the 5% level by the Tukey test. ns, * and ** mean, respectively, not significant and significant at the 1% and 5% probability level by the F test.

The positive effect of N application up to a dose of 144.32 kg ha^{-1} can also be explained by the fact that N fertilization accelerates plant growth up to saturation, influencing the size of leaves and stems, tillering and consequently shoot growth. These results agree with those reported by Marcelino *et al*. (2001), who observed increase in dry matter production due to the increment in N doses up to saturation.

The SPAD index, which is an index related to the green color intensity of leaves, fitted to an increasing linear model (Figure 1f), indicating that it increased with the increment in N doses. In addition, bacterial inoculation led to higher means of SPAD index, which increased by 3.18% compared to the treatment which did not receive *A. brasilense* (Table 1). Similar results were also reported by Pietroski *et al*. (2015), who applied increasing N doses in Mombasa grass and observed higher intensity of green color on the leaves, attributing this behavior to the increase in chlorophyll concentration in the leaves.

Approximately 70% of the total N present in the leaves is part of chloroplasts and chlorophyll (Marenco and Lopes, 2005). Nitrogen is a nutrient that participates in the synthesis and structure of chlorophyll molecules, so that the increase in its supply to plants will lead to increase in chlorophyll contents and, consequently, increase in the green color, which in the present study caused an increasing linear behavior as the applied N doses increased.

Another important fact to be highlighted is that the leaves, when well-nourished with N, have higher capacity to assimilate CO_2 and synthesize carbohydrates during photosynthesis, increasing biomass accumulation in the plants. Thus, inoculation with *A. brasilense* contributes to chlorophyll synthesis in the leaves, evidenced by the higher SPAD index in inoculated plants, demonstrating physiological response to the growth-promoting bacteria (Hungria, 2011).

A linear increase was observed in the crude protein content (CP) (Figure 1g), indicating a trend of increasing response of this variable as the N doses increased. Pietroski *et al*. (2015) observed increments in crude protein content with the increase in the applied doses, which demonstrates the physiological importance of this nutrient in plants, resulting in greater vegetative growth because it is a constituent of amino acids, the basic units for protein formation.

In study with nitrogen fertilization through the soil in Mombasa grass, Mazza *et al*. (2009) also observed significant effect of N doses on crude protein content and commented that these contents tend to increase with the N doses applied. However, increase in CP contents caused by increase in N doses may be gradually reduced over time, for the formation of new shoots (Quadros and Andrade, 2006).

Geron *et al.* (2011) reports that the minimum CP content the forage should have to allow optimal animal development is 6 to 8%. This value was observed in the present study only for doses above 90.56 kg ha^{-1}, indicating that it is necessary to supply at least this amount of nutrient in order to obtain adequate CP contents, so that the forage can adequately nourish the animals and allow higher weight gain.

According to Marschner (1995), reduction in the response from a certain dose is due to the action of N. It modifies the composition of the plants, causing drastic reduction in the contents of two main reserve carbohydrates (fructan and starch) and, in contrast to such reduction in carbohydrates, lignin contents in grasses may increase because the amino acids phenylalanine and tyrosine are the precursors in the synthesis of lignin, which would result in leaves with lower protein content.

Analysis of the significant interaction between N doses and Inoculant for the leaf/stem ratio is presented in Table 2 and Figure 2. By analyzing the effect of inoculation at each dose (Table 2), it is possible to observe that only in the absence of N application and at dose of 50 kg ha^{-1} there was significant difference between treatments with and without inoculation for leaf/stem ratio; without N application, higher leaf/stem ratio occurred when inoculation was not carried out, whereas the opposite occurred for the dose of 50 kg ha^{-1}.

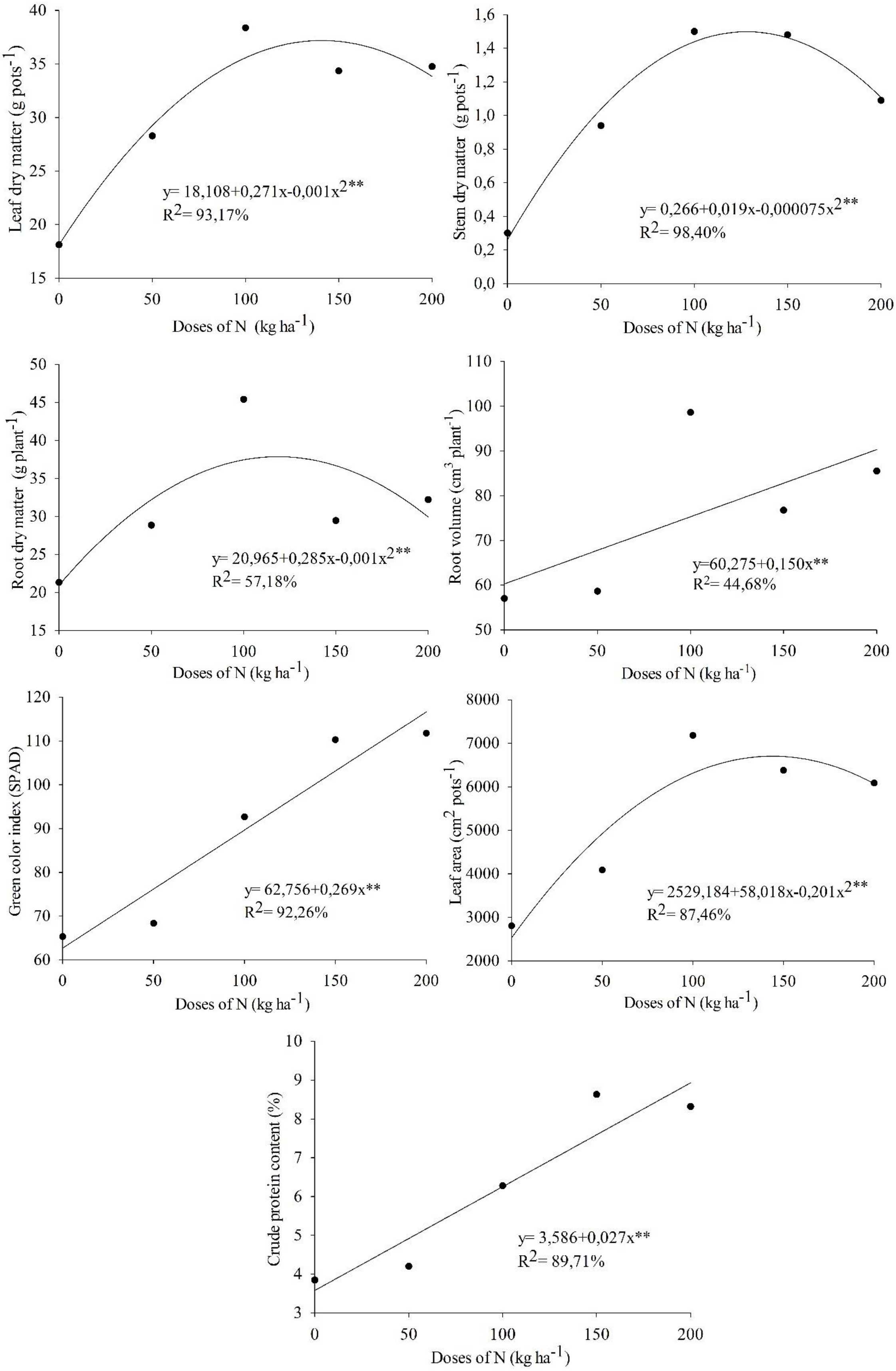

Figure 1. Leaf dry matter (LDM) (a), stem dry matter (SDM) (b), root dry matter (RDM) (c), root volume (d), green color index (e), leaf area (f) and crude protein content (g) in *Panicum maximum* cv. Mombasa as a function of nitrogen doses. Alta Floresta – MT, Brazil.

Table 2. Analysis of the significant interaction between nitrogen doses and inoculation for leaf/stem ratio in *Panicum maximum* cv. Mombasa. Alta Floresta - MT, Brazil.

Inoculant	---------------------- Doses of N (kg ha^{-1}) ----------------------				
	0	50	100	150	200
With	28.32 b	15.21 b	23.60 a	10.18 a	15.98 a
Without	70.89 a	28.02 a	21.04 a	15.62 a	19.05 a
F values	133.39**	12.17**	0.48 ns	2.18 ns	0.69 ns
LSD		7.53			

Means followed by the same letter in the column do not differ from each other at the 5% level by the Tukey test. ns, * and **, mean, respectively, not significant and significant at 1 and 5% by the F test.

Figure 2 presents the behavior of N doses in the treatments with and without inoculation. Without inoculation, a quadratic behavior occurred, with minimum point at the dose of 133.77 kg ha^{-1} and, in the presence of inoculation, the doses had a decreasing linear behavior, indicating that with the increase in the dose there is a reduction in leaf/stem ratio. The values in all treatments were above the critical limit considered by Pinto *et al*. (1994), which is 1, and this critical level takes into account the quantity and quality of the forage produced. Rodrigues *et al*. (2008), testing N doses in *Brachiaria brizantha* cv. Xaraés, also observed a quadratic behavior with minimum points at N doses of 139.33 and 168.7 mg dm^{-3}.

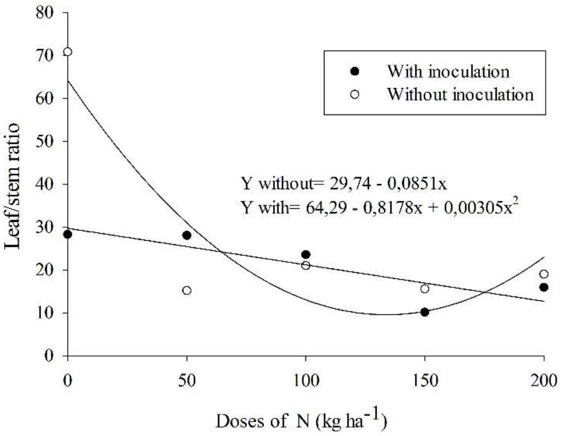

Figure 2. Analysis of the significant interaction between nitrogen doses and inoculation of *Azospirillum brasilense* for leaf/stem ratio in *Panicum maximum* cv. Mombasa. Alta Floresta – MT, Brazil.

Lower leaf/stem ratio indicates greater growth of the forage, thus justifying the reduction in this ratio as N doses increased and also the minimum point at dose of 133.77 kg ha^{-1}, close to the dose which leads to maximum forage production. Nitrogen acts in the zones with highest responses in elongation of the plants, causing greater leaf production and higher physiological activity. The accelerated growth provides shading to leaves at lower positions and buds, stimulating the plant to promote stem elongation in order to access light (Sbrissia and da Silva, 2001). Still according to these authors, the relevance of leaf/stem ratio varies according to the forage species, being lower in species with soft stem and less lignification, as occurs when pastures are well managed.

CONCLUSIONS

Inoculation with *Azospirillum brasilense* leads to promising results in *Panicum maximum* cv. Mombasa, improving its leaf/stem ratio and SPAD index. However, it is not sufficient to promote higher forage production, so further research is needed with other grasses to confirm its efficiency, when associated with N.

Nitrogen fertilization directly contributes to promoting higher yields and fast establishment of the pasture.

Nitrogen doses between 100 and 150 kg ha^{-1} are the most recommended for the development of *Panicum maximum* cv. Mombasa under pot conditions in a greenhouse.

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Conflict of interest. The authors declare that there are no competing interests. The Faculty of Biological and Agrarian Sciences of the State University of Mato Grosso, Brazil, approved the present study for its development.

Compliance with ethical standards. This article does not contain studies carried out with human beings, therefore, there was no need for approval by the Research Bioethics Committee of the Faculty of Biological and Agrarian Sciences of the State University of Mato Grosso, Brazil, for its development.

Data availability. Data are available with the corresponding author (yama@unemat.br) upon reasonable request.

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