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Mitigation of Mombasa Grass (*Megathyrsus maximus*) Dependence on Nitrogen Fertilization as a Function of Inoculation with *Azospirillum brasilense*

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ABSTRACT: Using biological inputs to improve the efficiency of nitrogen fertilizers represents an alternative for the cultivation of grasses in tropical regions. *Azospirillum brasilense* is a species of plant growth promoting bacteria widely studied and used in inoculants. Thus, this study aimed to evaluate the performance of Mombasa grass (*Megathyrsus maximus*) in association with *A. brasilense* and nitrogen (N) fertilization. The study was conducted under field conditions in Araguaína-Tocantins State, between December 2017 and May 2018. The treatments were arranged in randomized blocks, in a 5 × 2 factorial arrangement, with five doses of N fertilization (0, 25, 50, 75, and 100 kg ha^{-1}) combined with two inoculation treatments (inoculated and non inoculated), in four replicates. For the number of tillers and root production, the inoculation efficiency varied as a function of the supplied N doses. However, the percentage of leaf N was higher for inoculated plants regardless of the application of nitrogen. In the absence of nitrogen fertilization, it was possible to increase forage production by up to 36 % with inoculation.

Keywords: growth-promoting microbes, inoculant, biological nitrogen fixation, tropical pasture.

INTRODUCTION

In Brazil, meat and milk are produced mainly in pasture areas (Pezzopane et al., 2017) and there are currently 190 million hectares of pasture (Jank et al., 2014). *Urochloa* (*Brachiaria*) is the most cultivated genus, occupying nearly 77 % of the area (Guarda and Guarda, 2014). However, aiming at increased forage productions, the genus *Megathyrsus* (Carneiro et al., 2017), which already occupies 10 % of the Brazilian pasture area, has been widely used.

Megathyrsus maximus is recognized as one of the best tropical forage grass due to high yield (Mishra et al., 2008). However, it requires soils with good fertility, especially in relation to nitrogen (N) (Paciullo et al., 2017). This high demand has a negative impact on their cultivation since, at the same time as they seek to increase productivity, producers face challenges to achieve a sustainable and less dependent production regarding chemical inputs (Bounaffaa et al., 2018).

The practice of nitrogen fertilization considerably increases the cost of pasture production since its synthesis requires fossil fuel sources and most of the input is currently imported (Morais et al., 2012; Canto et al., 2016). Moreover, the benefits of nitrogen fertilization are only short term in highly weathered tropical soils, with accelerated loss due to leaching and volatilization, along with the risk of soil and water contamination by nitrate additions (Hungria et al., 2016; Pedreira et al., 2017). For this reason, it is important to develop agricultural practices to maintain or even increase production with greater sustainability (Di Salvo et al., 2018). In this context, the use of biological inputs to improve the efficiency of nitrogen fertilizers is an alternative to the cultivation of grasses in tropical regions, in addition to reducing environmental risks (Bounaffaa et al., 2018; Martins et al., 2018; Numan et al., 2018; Oliveira et al., 2018).

A bacterial species widely recognized for being a plant growth promoter (PGPB) and used as an inoculant is *Azospirillum brasilense* (Hungria et al., 2016; Herrera et al., 2018; Malinich and Bauer, 2018). Strains of *Azospirillum* can present both the ability to biologically fix atmospheric nitrogen and to synthesize phytohormones and solubilize phosphates (Döbereiner et al., 1976; Okon and Labandera-Gonzalez, 1994; Dobbelaere et al., 2003; Hungria et al., 2016). In addition, Rubin et al. (2017) and Fukami et al. (2017, 2018) mentioned stress reduction by biotic and abiotic factors, such as pathogens and drought, respectively.

Although studies on PGPB in grasses date back more than six decades, reference countries in these studies, such as Brazil, still present considerably modest use (Martins et al., 2018). Only in 2009, the first commercial strains began to be used in commercial inoculants with corn (*Zea mays*) and wheat (*Triticum aestivum*) (Hungria et al., 2010). In Brazil, the benefits of inoculation of *A. brasilense* on pasture are still poorly studied. In brachiaria, increases in biomass production and protein contents were confirmed in 2016 (Hungria et al., 2016) and the first commercial product was launched in 2018. Thus, the objective of this study was to evaluate the performance of Mombasa grass (*Megathyrsus maximus*) in association with *Azospirillum brasilense* and nitrogen fertilization.

MATERIALS AND METHODS

The study was conducted under field conditions in an experimental area of the Federal University of Tocantins - *Campus* Araguaína (Figure 1), School of Veterinary and Animal Science (810751.01; 9213652.69 UTM, altitude 240 m), between December 2017 and May 2018, using Mombasa grass. The region is classified as a transition of the biomes *Cerrado-Amazônia*, with Aw climate (hot and humid, with dry winters), according to the Köppen International Classification System (Alvares et al., 2013). The annual average rainfall of the area is 1,863 mm, and the average air humidity is 78 %. The soil of the experimental area presents sandy clay loam texture (Table 1) and is classified as *Latossolo Vermelho* (Santos et al., 2013), which corresponds to Oxisol (Soil Survey Staff, 2014).

A randomized complete block design in a 5 × 2 factorial arrangement was used, totaling ten treatments with four replicates each. Five doses of nitrogen fertilization (0, 25, 50, 75, and 100 kg ha^{-1}) combined with two inoculation treatments with *A. brasilense* (inoculated and non-inoculated) were studied. Each experimental plot had an area of 12.0 m^2 (3 × 4 m).

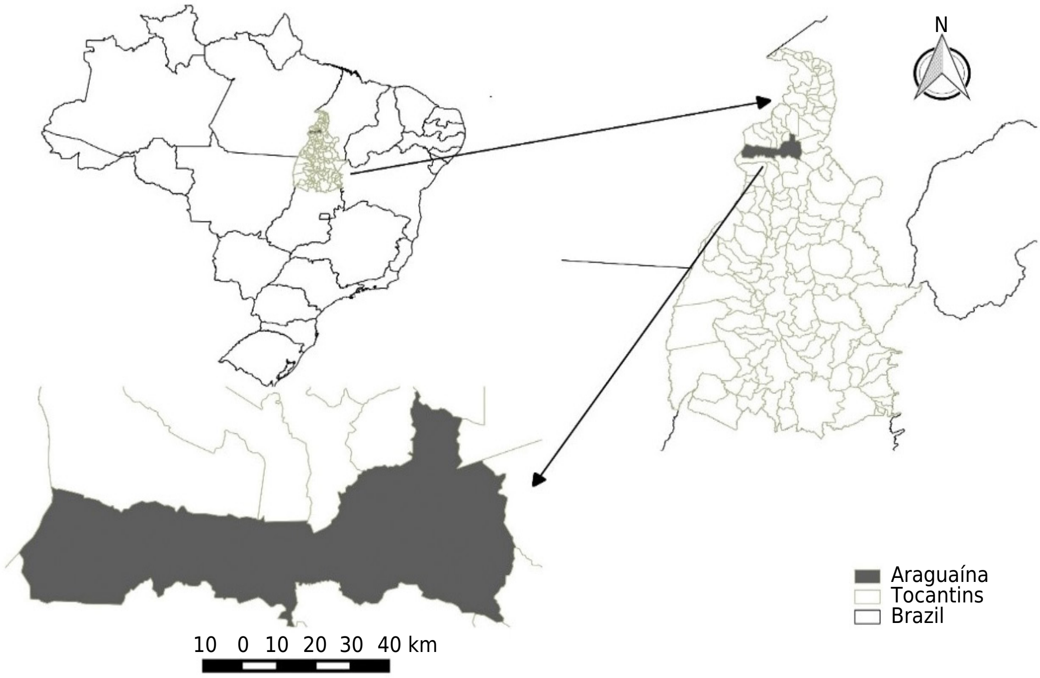

Figure 1. Location of the study area.

Table 1. Chemical and physical characterization of the soil (layer 0.00-0.20 m) of the experimental area. Araguaína-TO, 2018

Property	
pH($CaCl_2$)	6.0
Organic matter (g kg^{-1})	1.8
Available P (mg kg^{-1})	5.4
Available K (mg kg^{-1})	37.0
Ca^{2+} ($cmol_c$ kg^{-1})	4.5
Mg^{2+} ($cmol_c$ kg^{-1})	1.6
Al^{3+} ($cmol_c$ kg^{-1})	0.0
H+Al ($cmol_c$ kg^{-1})	2.4
SB ($cmol_c$ kg^{-1})	6.2
CEC ($cmol_c$ kg^{-1})	8.6
Base saturation (%)	72.0
Aluminium saturation (%)	0.0
Sand (g kg^{-1})	590
Silt (g kg^{-1})	90
Clay (g kg^{-1})	320

pH($CaCl_2$) at a ratio of 1:2.5 m/v; organic matter determined by Walkley-Black method; P and K available: extraction with Mehlich-1; Ca^{2+}, Mg^{2+}, and Al^{3+}: extraction with KCl 1 mol L^{-1}; H+Al: extraction with SMP. SB: sum of bases (Ca+Mg+K); Base saturation = (SB/CEC × 100); aluminium saturation = [Al/(Ca+Mg+K+Al) × 100]; clay: pipette method.

Forage was sowed in December 2017 and fertilization (119 kg ha^{-1} of P_2O_5) followed the recommendations of the crop (Sousa and Lobato, 2004) (Table 1). For the treatments with inoculation, seeds were homogenized together with the inoculant (200 mL diluted in water - equivalent to 10 % of the seed weight, strains Ab-V5 and Ab-V6 at the concentration 2 × 10^8 CFU mL^{-1}). The climatic data of the area were collected during the experimental period (Figure 2).

At 45 days after sowing, uniform cuts and nitrogen fertilization (urea) were applied to each treatment, which was repeated later at each cut. The cutting season and evaluation occurred at every 30 days after the previous cut (40-cm residue height).

The following variables were evaluated: plant height, number of tillers, daily forage accumulation, forage mass, percentage of nitrogen in forage, and root mass.

Plant height was obtained with a graduated ruler, measuring the soil at the average height of the forage canopy. The number of tillers was obtained by manual counting, with a metallic frame of 1.0 × 0.15 m; later, the data were converted to m^2. Daily forage accumulation was performed dividing the production of each cut by the number of days passed from the previous cut. Forage mass production was evaluated using a 1.0 × 1.0 m metal frame, with a cut from the 40-cm residue height, followed by drying in an oven at 55 °C for 72 h and subsequent weighing. Samples of forage, after drying and grinding in 1-mm sieves, were digested in sulfuric acid, sequentially distilled (Kjeldahl) and titrated (Boaretto et al., 2009) to determine the percentage of N in the forage.

To obtain root mass, two samples per plot were collected, using metal cylinders in the layer of 0.00-0.20 m, 5 cm of the clump. After sampling, the material was placed in plastic bags for later washing and separation of soil roots. The separated roots were weighed and oven dried at 55 °C for 72 h.

The experiment was conducted during three cycles, with the data grouped into period averages, except for root mass, which comprised the forage production during the three harvests.

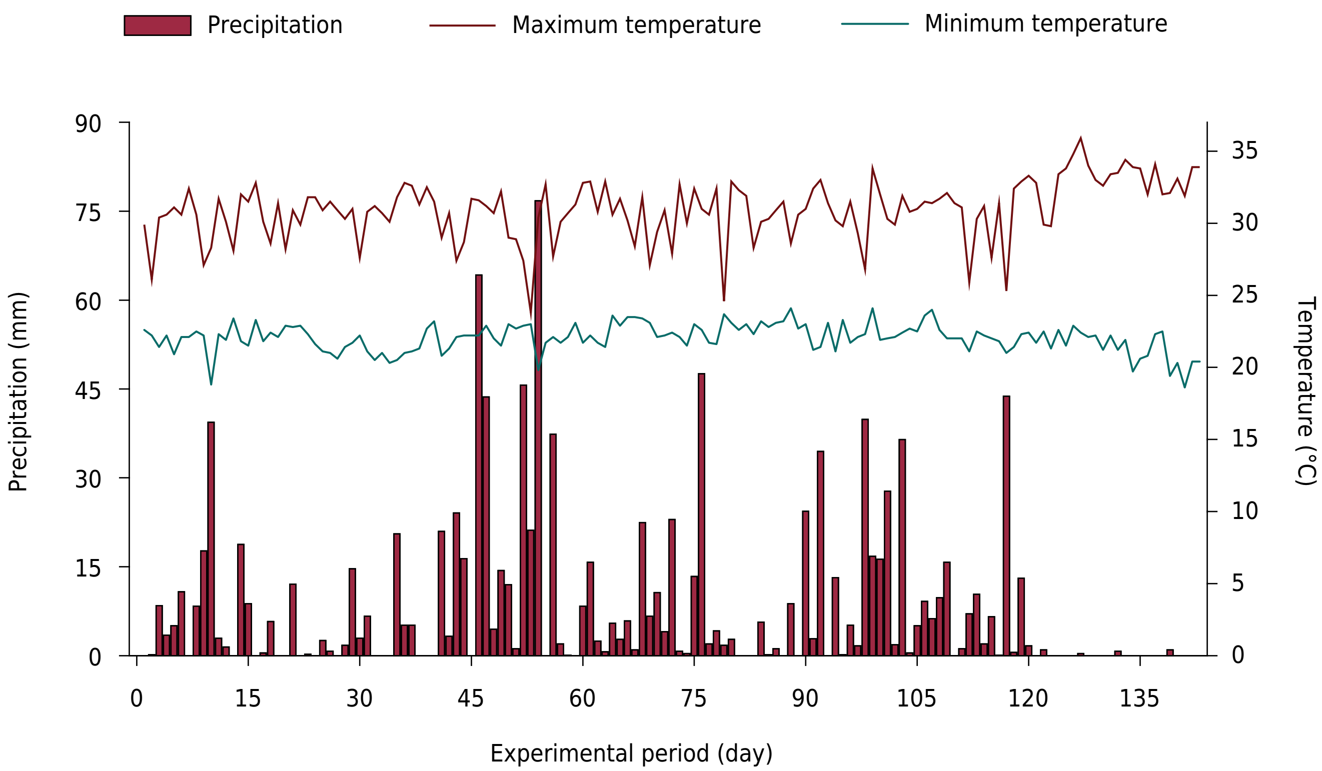

Figure 2. Precipitation and maximum and minimum temperatures of the experimental area during the period of conduction of the experiment in Araguaína-Tocantins State in the year of 2018.

All data were initially tested for normality by the Shapiro-Wilk method and homoscedasticity. The F test was applied for the qualitative data (inoculation) and, when these were significant, the Tukey test was performed at 5 % probability. For the quantitative data (N doses), regression analysis was performed, evaluating the significance of betas and determination coefficients to obtain the appropriate regression model. All statistical procedures were performed using SISVAR 5.3 software (Ferreira, 2011).

RESULTS

The results of the analysis of variance showed interaction ($p<0.05$) between inoculation factors and nitrogen doses for the number of tillers, daily forage accumulation, forage mass, and root mass (Figure 3).

Plant height had no influence on inoculation with *A. brasilense* regardless of whether or not it was supplied with nitrogen. The non-inoculated and inoculated plants presented average heights of 96 and 94 cm, respectively (Figure 3a).

Regarding the number of tillers, the highest values as a function of inoculation were observed at the following N doses: 25, 50, and 100 kg ha^{-1}, which resulted in increases of the order of 41, 52, and 58 %, respectively (Figure 3b). For doses 0 and 75 kg ha^{-1} of N, there was no difference ($p>0.05$) considering inoculation presence and absence.

As for daily forage accumulation in the absence of N, inoculated plants presented daily forage accumulation of 52 kg ha^{-1}, while in the non-inoculated plants the daily accumulation was 37.8 kg ha^{-1}, which represented an increase of 36 % (Figure 3c).

Reflecting on the daily forage accumulation behavior, the forage mass during the three evaluation cycles was significantly different ($p<0.05$) between inoculated and non-inoculated plants only in the absence of N fertilization (Figure 3d). Considering the three evaluation cycles in the absence of N, non-inoculated plants produced 3,653 kg ha^{-1} of dry mass, while inoculated plants accumulated 4,680 kg ha^{-1}.

The percentage of nitrogen in forage was significant for the inoculation factor, with behavior independent of the N supply and the applied dose. Inoculation resulted in increases in the N contents (Figure 3e). On average, non-inoculated plants presented 1.8 % of foliar N, while inoculated plants presented 2 %, representing an increase of 9 %.

For root mass, the response of plants to inoculation varied as a function of the dose of N (Figure 3f). In the absence of N, the root mass of non-inoculated plants was 3,635 kg ha^{-1}, while in the inoculated plants it was 7,144 kg ha^{-1}, representing an increase of 96 % in root production. When N was supplied to the plants, only the dose 75 kg ha^{-1} of N had an effect ($p<0.05$) with root mass of 2,263 and 4,714 kg ha^{-1} for non-inoculated and inoculated plants, respectively, representing an increase of 108 % in root production.

Regarding the behavior of the plants as a function of the applied dose of N, all variables presented an adjustment to the proposed models (Figure 4). Plant height of inoculated plants had an adjustment to the positive linear model (Figure 4a). In the absence of N, the plants were 0.91 m high and, from that value, there was an increase of 0.07 cm in plant height for each dose of N, resulting in 0.98 m in the highest dose of N. On the other hand, non-inoculated plants presented an adjustment to the quadratic model in the absence of N, presenting plant height of 0.87 m, showing maximum efficiency at the dose of 71 kg ha^{-1} of N, with plants with a height of 1.01 m.

Considering the number of tillers as a function of the applied nitrogen doses, only the inoculated plants presented adjustment to the positive linear regression model, with an

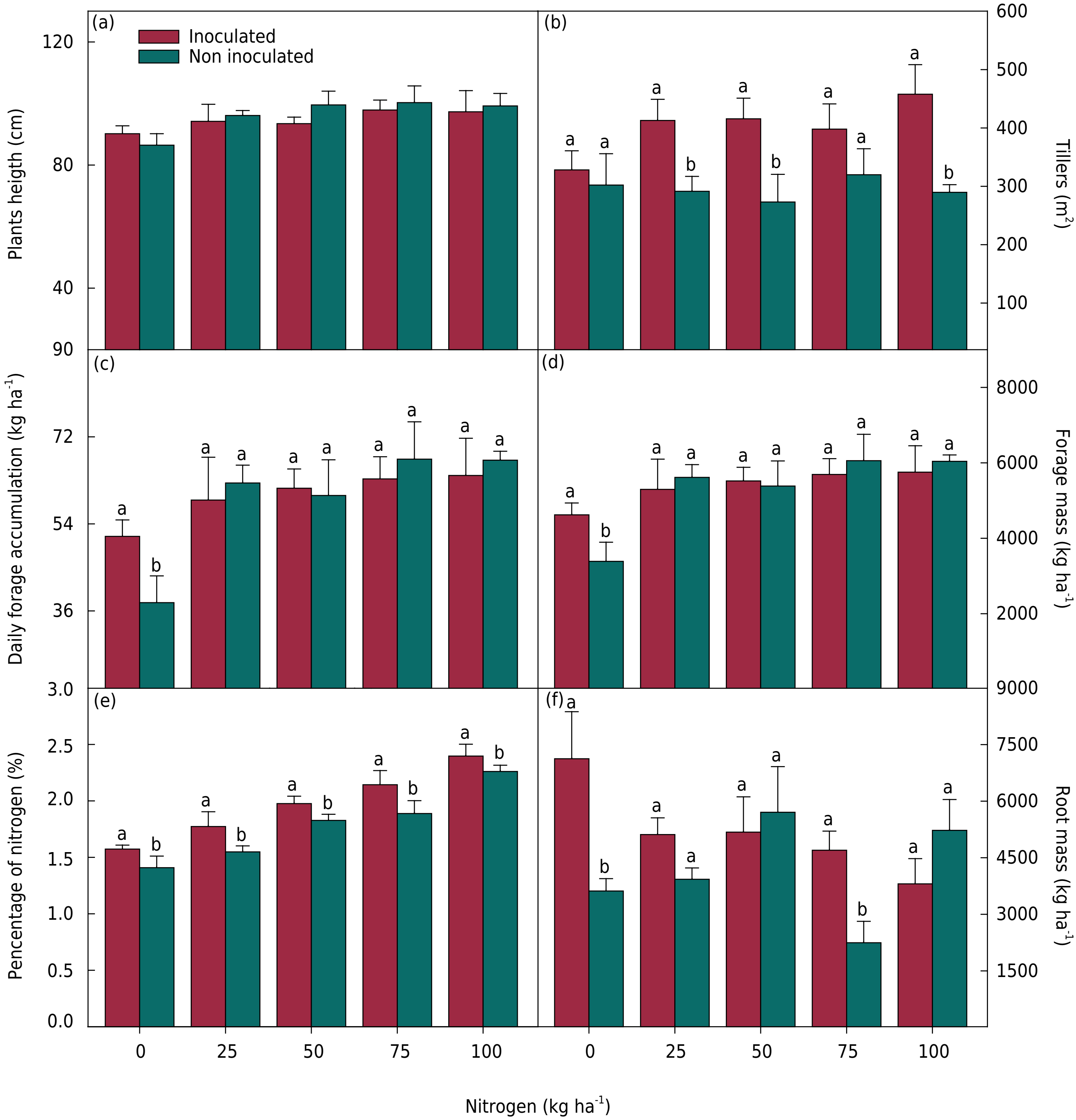

Figure 3. Plants height (a), number of tillers (b), daily forage accumulation (c), forage mass (d), percentage of nitrogen (e), and root mass (f) of Mombasa Grass in relation to inoculation with *A. brasilense* and nitrogen fertilization. Values followed by the same letter for the doses do not significantly differ by the Tukey test at 5 %.

increment of 0.99 tiller m^{-2} per kg of N, producing 454 tiller at the dose of 100 kg ha^{-1} of N (Figure 4b). Non-inoculated plants had an average value of 296 tillers m^{-2}, regardless of the N supply and the applied doses.

Considering the daily forage accumulation, plants presented an adjustment to the quadratic model regardless of the inoculation (Figure 4c). For inoculated plants, the maximum efficiency dose was 88 kg ha^{-1} of N, with a production of 64 kg ha^{-1}. For non-inoculated plants, the maximum efficient dose was 76 kg ha^{-1} of N with a daily accumulation of 67 kg ha^{-1}.

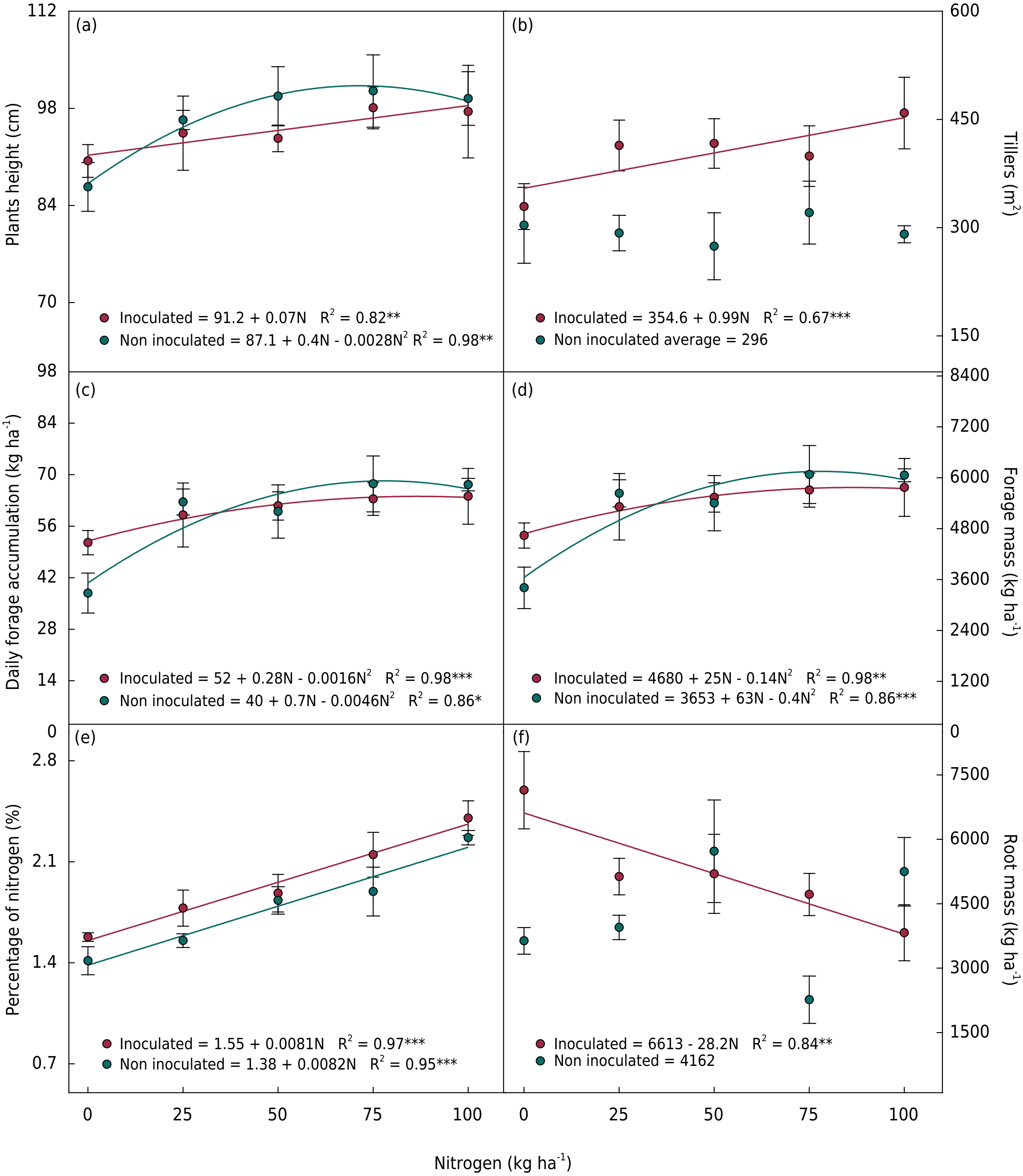

Figure 4. Plant height (a), number of tiller (b), daily forage accumulation (c), forage mass (d), percentage of nitrogen (e), and root mass (f) of mombaça grass at doses of nitrogen for inoculated and non-inoculated plants.

Total forage production (forage mass) presented an adjustment to the quadratic model, regardless of the inoculation (Figure 4d). For inoculated plants, there was production of 5,796 kg ha^{-1} at the maximum efficient dose of 83 kg ha^{-1} of N. Non-inoculated plants had a production of 6,213 kg ha^{-1} at the maximum efficient dose of 80 kg ha^{-1} of N.

For the percentage of nitrogen in forage, inoculated and non-inoculated plants presented adjustment to the linear model (Figure 4e). Inoculated and non-inoculated plants had 1.55 and 1.38 % of foliar N, respectively, in the absence of nitrogen fertilizer and both showed an increase of 0.008 % for each kg of N.

For root mass, only inoculated plants presented regression fit in the negative linear model, with a production of 6,614 kg ha^{-1} in the absence of N and reduction of 28 kg for each kg of N provided to the plants (Figure 4f). Non-inoculated plants presented average root mass of 4,162 kg ha^{-1}.

DISCUSSION

Faced with a growing population, food production will have to be increased by up to 70 % by 2050, therefore, approaches that minimize the use of agricultural inputs, maximize productivity and are environmentally friendly are needed (Jez et al., 2016). The study of beneficial microorganisms associated with crops becomes necessary because it is less expensive and more sustainable than the application of chemical fertilizers (Malinich and Bauer, 2018).

In this study, the number of tillers was influenced by the inoculation with *A. brasilense,* indicating another possibly attributed effect in response to phytohormones. The hormonal relationships involved in tiller production and development involves equilibrium between auxin and cytokinin (Fioreze and Rodrigues, 2012), where auxin can modulate the concentration of cytokinin, which is synthesized in roots and transported to other parts of the plant in order to overcome dormancy of axillary buds (Valério et al., 2009). In addition to the production of auxins (Fukami et al., 2017), *Azospirillum* has also been used in the synthesis of cytokinin-like substances (Strzelczyk et al., 1994).

The adaptability to pasture is influenced by the ability of the plant to produce new tillers (Hodgson, 1990), and increased cutting intensity impairs the development of tillers (Portela et al., 2011). As an alternative to intense tiller death caused by pasture, inoculation with *A. brasilense* emerges as a strategy to increase tillering in tropical grass under pasture conditions. Although Pedreira et al. (2017) did not observe an increase in tillering when inoculating in brachiaria grass (*Urochloa brizantha* cv. Marandu), in the present study, there was a significant increase as a function of inoculation for all doses of N, except in the absence of N.

Nitrogen fertilization increases the production and development of tillers in Mombasa grass (Freitas et al., 2012) and, as an alternative to the reduction of tillers, Pontes et al. (2017) recommended increased nitrogen fertilization rates. However, the results of the present study demonstrated that inoculation was a sustainable alternative to increase the number of tillers. Another advantage of the number of tillers increase is the mitigation of erosion problems in pastures since the greater number of tillers would cause less soil exposure, improving soil conservation and minimizing the impact of raindrops, avoiding the disintegration of the particles (Araújo, 2015).

There was no difference in forage production between inoculated and non-inoculated plants considering the nitrogen supply conditions. Aguirre et al. (2018), when working with coast-cross grass (*Cynodon dactylon*) inoculated with *A. brasilense,* found similar results. However, it is important to emphasize the good soil fertility of the studies, considering that a different behavior can be observed in low fertility soils, as observed in the work of Leite et al. (2018). These authors worked on soil with low fertility and found greater benefits of inoculation in Marandu grass inoculated with *A. brasilense*, when nitrogen was supplied. Abiotic variables such as soil pH, soil nature, organic matter, and moisture content, climatic fluctuations, agricultural pesticides, and even fertilizers can make PGPB contributions vulnerable (Shameer and Prasad, 2018). In pastures that are more adapted to low fertility soils, such as brachiaria, there was a 15 % increase in forage mass production and 25 % in N in plants receiving 40 kg of ha^{-1} N (Hungria et al., 2016).

By evaluating the inoculation of native pastures with *A. brasilense*, Itzigsohn et al. (2000) concluded that inoculation practices have the potential to increase forage production and to reduce environmental damages caused by the insertion of fertilizers, without causing a negative impact on the environment. Ching-Jones et al. (2016), when evaluating inoculation of African star grass seeds (*Cynodon nlemfuensis*) with bacteria of the genus *Azospirillum*, obtained productivity similar to the nitrogen fertilization (78 kg ha^{-1}).

When comparing forage production of non-inoculated plants with nitrogen supply at the recommended dose (50 kg ha^{-1} of N $cycle^{-1}$) (Sousa and Lobato, 2004), there was 38 % reduction due to non-supply of N. According to Paciullo et al. (2017), a very relevant aspect in relation to the production of tropical forage is that these plants are severely limited by the availability of N. By comparing inoculated plants without N with the fertilization in recommended doses for the culture, there was 20 % reduction in production. These results demonstrated that in situations of non-supply of N, the inoculation practice would mitigate the absence of nitrogen fertilizer for the forage yield. These are relevant results, since a great part of the producers in tropical and subtropical regions do not apply periodic fertilizations in the pasture (Dias-Filho, 2014).

When evaluating the production of non-inoculated plants without N, with the inoculation of plants, it would be possible to reduce the area from 1 to 0.73 ha, without loss of productivity. With an average consumption of an animal unit (AU) (450 kg of live weight) of 17 kg day^{-1} of dry matter (NRC, 2000), the daily production of forage without nitrogen fertilization would contain 2.2 AU ha^{-1} during the evaluated period of 90 days. The area with inoculated plants would contain 3.0 AU ha^{-1}, representing an increase of 36 % in the daily stocking rate of animals. Although these values are modest when a 100-hectare property is considered and does not perform periodic fertilization of N, a common factor in a tropical region (Dias-Filho, 2014), it would allow an increase of 70 AU in the property.

Inoculated plants without N supply had a production of 4,638 kg ha^{-1} of dry mass during the three evaluation cycles, while the non-inoculated plants would require N in the dose 18 kg ha^{-1} per cycle, totaling 54 kg ha^{-1} of N during the experimental period to reach the same production of the inoculated plants. Considering that 1.0 kg of N-fertilizer is equivalent to 4.5 kg of CO_2-equivalents (Hungria et al., 2013), this saving of nitrogen fertilization could prevent the emission of 244 kg ha^{-1} of CO_2-equivalents, configuring the inoculation as a practice to contribute to the sustainability of the environment, allied to higher productions in comparison to non-inoculated plants without nitrogen fertilization.

With the inoculation of plants, the percentage of nitrogen in the forage varied from 1.5 to 2.4 % of N as a function of the doses of nitrogen fertilizer, values that are adequate for 1.5 % of Mombasa grass (Sousa and Lobato, 2004), being slightly below the appropriate levels in the absence of *Azospirillum*, which ranged from 1.3 to 2.2 %.

When evaluating the plants in the absence of nitrogen fertilizer, there was an increase of 12 % in the percentage of leaf nitrogen with the inoculation of plants. To reach the same percentage of foliar N found in inoculated plants and without nitrogen application, non-inoculated plants would require the application of 21 kg ha^{-1} of N. Studying the contribution of microorganisms in the biological fixation of nitrogen in forages, Marques et al. (2017) indicated that these microorganisms, among them the genus *Azospirillum*, colonize the root system of grasses contributing to the nitrogen nutrition of these species. In addition, *Azospirillum* inoculation may increase the efficiency of nitrogen fertilizer use, which has recently been demonstrated for Ab-V5 and Ab-V6 strains in corn (Martins et al., 2018).

The beneficial effects of *A. brasilense* have often been related to the synthesis and release of phytohormones for host plants (Dobbelaere et al., 2003; Hungria et al., 2016), stimulating their growth (Rashotte et al., 2003; Taiz and Zeiger, 2009). In fact, the *A. brasilense* strains used in this study, Ab-V5 and Ab-V6, mainly synthesize acetic acid (Fukami et al., 2017), and the release of this phytohormone into the rhizosphere should be the factor responsible for the greater mass of roots, with emphasis on the low doses of N. There was, however, no effect on plant height.

The root mass of inoculated plants in the absence of N reached 96 % increment in comparison to non-inoculated plants, indicating the high efficiency of *A. brasilense* in promoting root growth of the plants, also justifying the higher forage production in the absence of N. In addition, increased root development allows a greater area of water and nutrient absorption, reflecting on biomass production, besides promoting tolerance to environmental stresses such as drought (Souza et al., 2017).

The good development of forage roots becomes very interesting in countries such as Brazil, which has a very expressive drought during the year (Pedreira et al., 2017) that reduces the production of pastures.

The genus *Azospirillum* is found throughout the world under a wide range of environmental and soil conditions, being closely associated with the growth and productivity of many crops of commercial interest (Herrera et al., 2018). In 1976, researchers reported the forage *Megathyrsus maximus* as the grass species with the highest incidence of *Azospirillum lipoferum* in its rhizosphere (Döbereiner et al., 1976).

Aguirre et al. (2018) evaluated the inoculation in coast-cross grass for two consecutive years and found benefits of inoculation in the forage in the second year of the study. The results of the present study represent the initial period after forage implantation and the next step will be to investigate if additional benefits can be obtained by the re-inoculation of Mombasa grass with *Azospirillum*.

CONCLUSIONS

For the number of tillers and root production, the inoculation efficiency varied as a function of the N dose supplied. However, the percentage of leaf N was higher for inoculated plants regardless of the application of N fertilization.

In the absence of nitrogen fertilization, it was possible to increase forage production by up to 36 %, with inoculation.

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Writing – Original Draft: Rubson da Costa Leite, Robson da Costa Leite, and Leonardo Bernardes Taverny de Oliveira.

Writing – Review & Editing: Antonio Clementino dos Santos, José Geraldo Donizetti dos Santos, and Mariangela Hungria.

Visualization: Rubson da Costa Leite.

Supervision: Antonio Clementino dos Santos and Mariangela Hungria.

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