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RUBSON DA COSTA LEITE

ASSOCIAÇÃO ENTRE NITROGÊNIO E INOCULAÇÃO DE FORRAGEIRAS TROPICAIS COM BACTÉRIAS PROMOTORAS DE CRESCIMENTO EM PLANTAS

ARAGUAÍNA (TO)

2019

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal Tropical, da Universidade Federal do Tocantins, para obtenção do grau Mestre em Ciência Animal Tropical.

Orientador: Prof°. Dr· Antonio Clementino dos Santos

Co-Orientador: Prof° Dr. José Geraldo Donizzeti dos Santos

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal Tropical, da Universidade Federal do Tocantins, foi avaliada para obtenção do título de mestre em Ciência Animal Tropical, e aprovada em sua forma final pelo orientador e pela banca examinadora.

Orientador: Prof°. Dr· Antonio Clementino dos Santos

Co-Orientador: Prof° Dr. José Geraldo Donizzeti dos Santos

Aprovado em 20 /02 / 2019

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Examinador

Dedico inicialmente a DEUS. Meus pais, Lucas e Valdirene. Meus irmãos, Robson e Dagna. Minha namorada, Ana Cristina. Meu avô, José Tomé de Sousa Leite (In memoriam).

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Colossenses 3: 23

RESUMO GERAL

O uso de microrganismos associados às forrageiras surge como uma alternativa para reduzir o uso de insumos químicos, com notáveis benefícios ambientais e econômicos. Portanto, objetivou-se avaliar os benefícios decorrentes da associação entre nitrogênio com forrageiras tropicais inoculadas com bactérias promotoras de crescimento em plantas. Foram conduzidos dois experimentos em condições de campo no município de Araguaína-TO. O experimento 1 foi conduzido entre os meses de março de 2016 e março de 2017, com a forrageira marandu. Os tratamentos organizados em blocos ao acaso, em arranjo fatorial 4x2, com quatro repetições. Foram estudadas quatro doses de fertilizante nitrogenado em cobertura (0,0; 12,5; 25,0 e 50,0 kg/ha de N) combinadas com dois tratamentos de inoculação (inoculado e não inoculado), com avaliações realizadas em três períodos do ano (transição, seca e água). O experimento 2 ocorreu entre os meses de dezembro de 2017 e maio de 2018, com a forrageira mombaça. Os tratamentos organizados em blocos ao acaso, em arranjo fatorial 5x2, com cinco doses de fertilizante nitrogenado em cobertura (0,0; 25,0; 50,0; 75,0 e 100,0 kg/ha de N) combinadas com dois tratamentos de inoculação (inoculado e não inoculado), em quatro repetições. Plantas de capim Marandu inoculadas com *A. brasilense* apresentaram maior altura de planta, número de perfilhos e produção de forragem do que plantas não inoculadas, independentemente da dose de N. A inoculação do capim Marandu permitiu uma redução de 20% na adubação nitrogenada. Para o capim Mombaça, o número de perfilhos e produção de raízes, a eficiência de inoculação variou em função da dose de N fornecida. No entanto, a porcentagem de N foliar foi maior para plantas inoculadas, independentemente da aplicação do fertilizante nitrogenado. Em condições de ausência de adubação nitrogenada, foi possível aumentar a produção de forragem em 36% com a inoculação do capim Mombaça.

Palavras chave: *Azospirillum brasilense. Megathyrsus maximus. Urochloa brizantha.*

GENERAL ABSTRACT

The use of microorganisms associated with forages appears as an alternative to reduce the use of chemical inputs, with notable environmental and economic benefits. Therefore, the objective was to evaluate the benefits of the association between nitrogen and tropical forages inoculated with growth promoting bacteria in plants. Two experiments were conducted under field conditions in the municipality of Araguaína-TO. Experiment 1 was conducted between the months of March 2016 and March 2017, with the forage marandu. The treatments were arranged in randomized blocks, in a 4x2 factorial arrangement, with four replications. Four nitrogen fertilizer doses (0.0, 12.5, 25.0 and 50.0 kg/ha N) were combined with two inoculation treatments (inoculated and non-inoculated), with evaluations carried out in three periods of the year (transition, dry and wet season). Experiment 2 occurred between the months of December 2017 and May 2018, with the forage mombasa. The treatments were arranged in randomized blocks, in a 5x2 factorial arrangement, with five doses of nitrogen fertilizer (0.0, 25.0, 50.0, 75.0 and 100.0 kg/ha of N) combined with two inoculation treatments (inoculated and uninoculated), in four replicates. Marandu grass plants inoculated with *A. brasilense* presented higher plant height, number of tillers and forage production than non - inoculated plants, regardless of the dose of N. The inoculation of the Marandu grass allowed a 20% reduction in nitrogen fertilization. For Mombasa grass, number of tillers and root production, inoculation efficiency varied according to the N dose supplied. However, the percentage of leaf N was higher for inoculated plants, regardless of the nitrogen fertilizer application. In conditions of absence of nitrogen fertilization, it was possible to increase forage production by 36% with the inoculation of the Mombasa grass.

Keywords: *Azospirillum brasilense. Megathyrsus maximus. Urochloa brizantha.*

LISTA DE SIGLAS

N	Nitrogen
Mha	Millions of hectares
N_2	Atmospheric Nitrogen
BNF	Biological Nitrogen fixation
PGPB	Plant Growth Promoter bacterial

SUMÁRIO

INTRODUÇÃO GERAL

Diante de uma população crescente, existe a necessidade de aumento em torno de 70% na produção de alimentos até 2050, para isso são necessárias abordagens que minimizem o uso de insumos, maximizem a produtividade e sejam ambientalmente corretas (JEZ et al., 2016). Práticas sustentáveis em pastagens são importantes ferramentas para atender às demandas futuras por carne bovina (LOPES et al., 2018), especialmente no Brasil, onde estas constituem o meio mais econômico e usual na alimentação bovina (JANK et al. 2014).

A produtividade das pastagens brasileira é considerada baixa, associada a manejos inadequados e degradação, com destaque para a não reposição de nutrientes ao solo (DIAS FILHO, 2014). O estudo de microrganismos benéficos associados às culturas é menos dispendioso e mais sustentável do que a aplicação de fertilizantes químicos (MALINICH; BAUER, 2018). Portanto, o uso de microrganismos associados às forrageiras surge como uma alternativa para reduzir o uso de insumos químicos, com notáveis benefícios ambientais e econômicos (LOPES et al., 2017).

Bactérias promotoras de crescimento em plantas (BPCP) podem ser encontradas na rizosfera em associação com sistemas radiculares de plantas, tanto na superfície radicular e em associações endofíticas (NIU et al., 2018). Essas rizobactérias podem conferir efeitos benéficos, como suprimir diversos patógenos de solo e promover o crescimento das plantas por métodos diretos e indiretos, como produção de fito-hormônios, mineralização e decomposição da matéria orgânica e melhorar a biodisponibilidade de diferentes nutrientes como ferro e fósforo (YANG et al., 2009; NUMAN et al., 2018). Algumas BPCP também provocam alterações físicas ou químicas relacionadas à defesa de plantas, processo referido como "resistência sistémica induzida" (YANG et al., 2009).

Apesar dos estudos com BPCP em gramíneas datarem de mais de seis décadas, países referência nesses estudos, como o Brasil, ainda têm uso modesto dessa tecnologia (MARTINS et al., 2018), sendo que apenas em 2009 as primeiras estirpes começaram a ser usadas em inoculantes comerciais com o milho (*Zea mays*) e o trigo (*Triticum aestivum*) (HUNGRIA et al., 2010).

No Brasil, os benefícios da inoculação em pastagens ainda são muito pouco estudados. Em braquiárias, incrementos na produtividade e mitigação do stress pela seca foram observados com uso de inoculante a base de *Azospirillum brasilense* (HUNGRIA et al., 2016; LEITE et al., 2018). Portanto, objetivou-se avaliar os benefícios decorrentes da associação entre nitrogênio com forrageiras tropicais inoculadas com bactérias promotoras de crescimento em plantas.

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CHAPTER 1

Productivity increase, reduction of nitrogen fertilizer use and drought stress mitigation by inoculation of Marandu grass (*Urochloa brizantha*) with *Azospirillum brasilense*

ABSTRACT

Among the forage species cultivated in South America, the genus Urochloa is the most used, and the cultivar Marandu of U. brizantha is the most widely planted in Brazil. The objective of this study was to evaluate forage performance in association with *Azospirillum brasilense*, combined with nitrogen (N) fertilisation. The study was conducted under field conditions in Araguaína, Tocantins, in the central region of Brazil, between March 2016 and March 2017. Four N fertiliser rates (0, 12.5, 25 and 50 kg/ha of N per cutting cycle) were combined with two inoculation treatments (inoculated and non-inoculated), with evaluations carried out in three periods of the year (transition, dry and wet seasons). Marandu grass plants inoculated with *A. brasilense* had greater plant height, number of tillers and forage production than non-inoculated plants, regardless of the N rate. Inoculation with *A. brasilense* allowed a 20% reduction in N fertilisation. Our results indicate that inoculation with *A. brasilense* in Marandu grass, as well as increasing forage production, can help to mitigate the stresses caused by the dry season.

Keywords: Growth-promoting bacteria. Nitrogen fixation. Tropical pastures.

1 INTRODUCTION

Brazil has the second-largest herd of cattle in the world, estimated at 219 million, and is responsible for 13.8% of world beef production (ABIEC, 2017). Pastures, comprising an area of nearly 190 Mha, are the most economical and usual means for cattle feeding in Brazil (JANK et al., 2014). However, the productivity of these pastures is mostly low because of pasture degradation and inadequate management, with non-replenishment of nutrients to the soil, contrary to practice in cropping areas (DIAS FILHO, 2014).

Although most Brazilian soils are responsive to applied nitrogen (N), its application increases production costs, and its effects are short-lived in tropical soils (CANTO et al., 2016). In addition, N fertilisers are manufactured from fossil fuels (MORAIS et al. 2012), and there are risks of contamination of soil and water by addition of nitrate, as well as emissions of greenhouse gases (PEDREIRA et al., 2017; SÁ et al., 2017).

In order to reduce the costs of N fertilisation, inoculation with bacteria capable of fixing atmospheric nitrogen (N_2) or of promoting plant growth by other mechanisms such as the production of phytohormones is presented as an important strategy for sustainability (HUNGRIA et al., 2016; LEITE et al., 2017; MARQUES et al., 2017).

In the search for positive results resembling those achieved with rhizobia–legume associations in which inoculation with elite strains can partially or fully replace N fertilisation, such as in soybean (Glycine max (L.) Merr.) (SATURNO et al., 2017), several studies have been carried out with forage grasses in association with bacteria of the genus Azospirillum. These bacteria, in addition to having the capacity for biological N_2 fixation (BNF), can contribute to the production of phytohormones and phosphate solubilisation enzymes (OKON; LABANDERA- GONZALEZ, 1994; BALDANI et al., 2014; HUNGRIA et al., 2016; FUKAMI et al., 2017; MARQUES et al., 2017; SOUZA et al., 2017). Furthermore, Rubin et al. (2017) and Fukami et al. (2017) highlighted the potential of Azospirillum to promote greater tolerance of plants to biotic and abiotic stresses such as drought. This would be particularly important in several regions of Brazil, where there is a long and well-defined drought period.

Among the forage species cultivated in South America, the genus Urochloa is the most used, and cv. Marandu is the most widely planted in Brazil, with high forage yield and good adaptation to the soils and tropical climatic conditions (MARCHI et al., 2017; LOPES et al., 2017; RODRIGUES et al., 2017). The characteristics and importance of this forage species for the Brazilian cattle-production chain justify the need to evaluate its performance in association with *Azospirillum brasilense* and N fertilisation.

2 MATERIAL AND METHODS

The study was conducted under field conditions in an experimental area of the Federal University of Tocantins, Campus Araguaína, School of Veterinary Medicine and Animal Science (809304.26 and 9213720.68 UTM; elevation 240m a.m.s.l.) between March 2016 and March 2017. The region is classified as a transition of the biomes Cerrado–Amazônia, with an Aw (hot and humid) climate, according to the Köppen International Classification (ALVARES et al., 2013), average annual precipitation 1863mm and average air humidity 78%. The soil of the experimental area is of sandy texture (Table 1), classified as a Quartzipsamment Entisol (USDA Soil Taxonomy).

Table 1. Chemical and physical parameters of the soil (layer 0–20 cm) from the experimental area, Araguaína, Brazil, 2017

pH ($CaCl_2$)	4.5	Potential acidity (H + Al) ($cmol_c$/kg)	9.9
Organic matter (g/kg)	14.2	Sum of bases (Ca + Mg + K) ($cmol_c$/kg)	2.0
Available P (mg/kg)	3.1	Cation exchange capacity ($cmol_c$/kg)	11.9
Available K (mg/kg)	2.0	Nitrogen in soil (g/kg)	0.4
Exchangeable Ca ($cmol_c$/kg)	1.4	Sand (%)	89.3
Exchangeable Mg ($cmol_c$/kg)	0.6	Silt (%)	0.5
Exchangeable Al ($cmol_c$/kg)	3.0	Clay (%)	10.0

Available phosphorus (P) and potassium (K) extraction with Mehlich-1; exchangeable calcium (Ca), magnesium (Mg) and aluminium (Al) extraction with KCl; H + Al extraction with calcium acetate

The experiment was established in a randomized block design of four N-fertilisation rates (0, 12.5, 25 and 50 kg/ha of N, applied as urea after each forage cut) and two inoculation treatments with *A. brasilense* (inoculated and non-inoculated, arranged at a distance of 20m from each other), with four replicates. Each experimental plot had an area of 9.0m2 (3.0m by 3.0 m). The inoculant contained elite strains Ab-V5 and Ab-V6 of *A. brasilense*, commercially used in Brazil for grasses (HUNGRIA et al., 2010, 2016) and co-inoculation of legumes (HUNGRIA et al. 2015).

A basal application of NPK was applied to all plots at 30 days after sowing, according to recommendations for cultivation (SOUSA; LOBATO, 2004) and soil fertility (Table 1) as follows: 20 kg N/ha (ammonium sulfate), 30 kg P/ha (single superphosphate) and 49 kg K/ha (potassium chloride).

Sowing was carried out in March 2016, using 12 kg/ha of viable pure seeds. Subsequently, soil was rolled to improve seed contact. At the time of sowing, seed homogenisation was performed by using inoculant applied at a rate of 200 mL/ha, comprising strains Ab-V5 and Ab-V6 of *A. brasilense* at the concentration of 2 x 10^8 colony forming units/mL.

At 62 days after sowing, when plots had achieved a cutting height of at least 40 cm, they were cut to a residual height of 20 cm and the N treatments were applied.

For the experimental period, data from the forage harvests were grouped according to seasons, defined as the transition (0–100 days), dry (100–200 days) and wet (200–365 days), established according to precipitation data collected in the experimental area (Fig. 1).

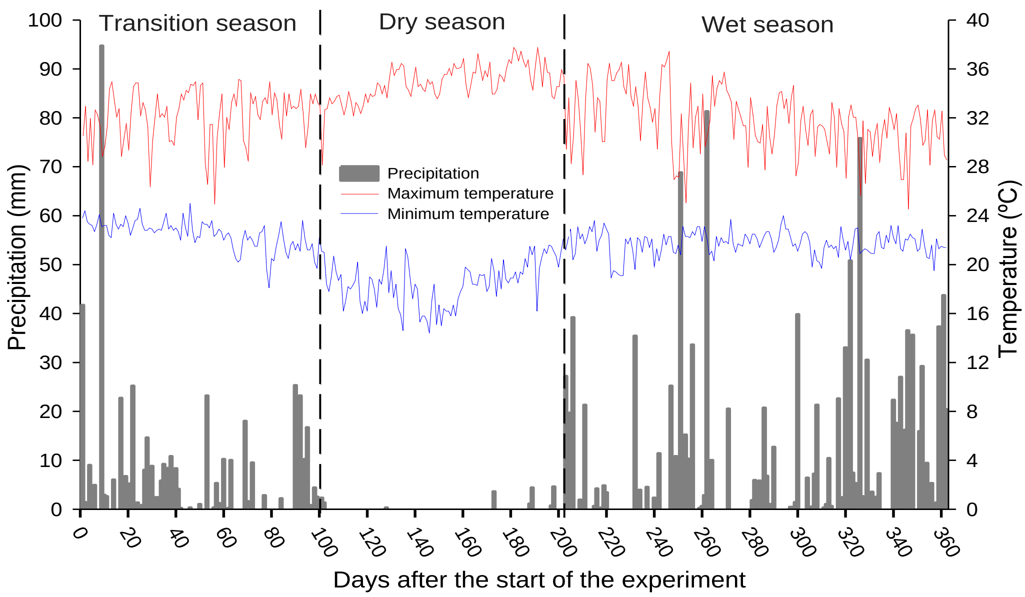

Figure 1. Precipitation, maximum and minimum temperatures of the experimental area during the period of conduction of the experiment. Araguaína-TO, 2017.

The variables evaluated were plant height, number of tillers, root mass, daily forage accumulation, forage N concentration and annual forage accumulation. Root mass and forage N concentration were grouped only in the dry season and wet season, and annual forage accumulation was evaluated as the sum of all cuts during the year. All other parameters were evaluated in the three defined periods of the year.

Plant height was determined with a graduated ruler, from the soil to the top of the plant. The number of tillers was counted by using a 1.0m by 0.15m metal frame. For root mass evaluation, two samples per plot were collected, using steel cylinders at depth 0–20 cm with 5 cm of distance of the cut clumps for the evaluation of productivity. After sampling, the material was placed in plastic bags for later washing and separation of roots from soil. The separated roots were weighed and oven dried at 55.8 ºC for determination of dry root mass, in kg/ha. Annual forage accumulation was evaluated with a 1.0m by 0.5m metal frame, with a cut height of the residue of 20 cm, followed by drying in an oven at 55.8 ºC for 72 h and subsequent weighing. Forage samples, after pre-drying and grinding in a 1-mm sieve, were digested with sulfuric acid and immediately distilled (Kjeldahl) for the percentage of N. Daily forage accumulation was estimated by dividing the yield of each cut by the number of days passed since the previous cut.

Data were initially tested for normality (Shapiro–Wilk test) and homoscedasticity. Inoculation treatment were analyzed by analysis of variance. The N-rate treatments were submitted to regression analysis, by evaluating the significance of slopes and determination coefficients to obtain the appropriate regression model, adopting a significance level of P = 0.05. All statistical procedures were performed with SISVAR 5.3 software (FERREIRA, 2011).

3 RESULTS

Analysis of variance demonstrated the effect of inoculation with *A. brasilense* on all parameters evaluated (Fig. 2). The only significant N rate inoculation interaction was for daily forage accumulation (Fig. 2a). For this parameter, there was a positive effect of inoculation in the transition season, when the inoculated plants accumulated 77 kg/ha.day of forage, whereas the non-inoculated plants accumulated 69 kg/ha.day. These values represent an increase of 12.5% for average daily yield provided by the inoculation.

In the dry season, there was a positive response of the inoculation in the absence of nitrogen fertilisation, at the dose of 50 kg/ha of N non-inoculated plants presented greater daily accumulation. These results indicate that, in the dry season, inoculation favored growth only in the absence of N fertilisation. In the wet season, inoculation resulted in an increase in daily forage accumulation only at the N rate of 50 kg/ha. The additional forage accumulation due to inoculation was 21.5 kg/ha.day, corresponding to a daily contribution of 23.4% to accumulation of forage (Fig. 2a).

In the dry season, the number of tillers in the inoculated treatments was 28% higher than in non-inoculated treatments, and in the wet season it was 12% higher. There was no significant effect in the transition season (Fig. 2b).

Plant height was positively influenced by inoculation in all evaluated periods (Fig. 2c). In the transition season, inoculation resulted in an increase of 4% in relation to the non-inoculated treatment regardless of the rate of N. For the dry season, the increase was 16% and in the wet season 11%.

From the start of the dry season, daily pasture accumulation rates on non-inoculated treatments decreased by 17% relative to the transition season, whereas inoculated treatments decreased by only 7%. The recovery in the wet period was 13% for the non-inoculated plants and 9% for the inoculated plants. Consequently, the greater plant height of Marandu grass indicates the beneficial effects provided by inoculation in mitigating the effects of water stress (Fig. 2c).

At the start of the dry season the root mass of inoculated plants was 27% higher than of non-inoculated plants (Fig. 2d). At the end of the wet season, this ranking was reversed, with the inoculated plants showing root mass 15% lower than the non-inoculated plants.

In relation to the forage N concentration, plants responded differently to inoculation in the evaluated periods (Fig. 2e). In the dry season, inoculated and non-inoculated plants were similar, whereas in the wet season, the inoculated plants had lower N concentration (Fig. 2e).

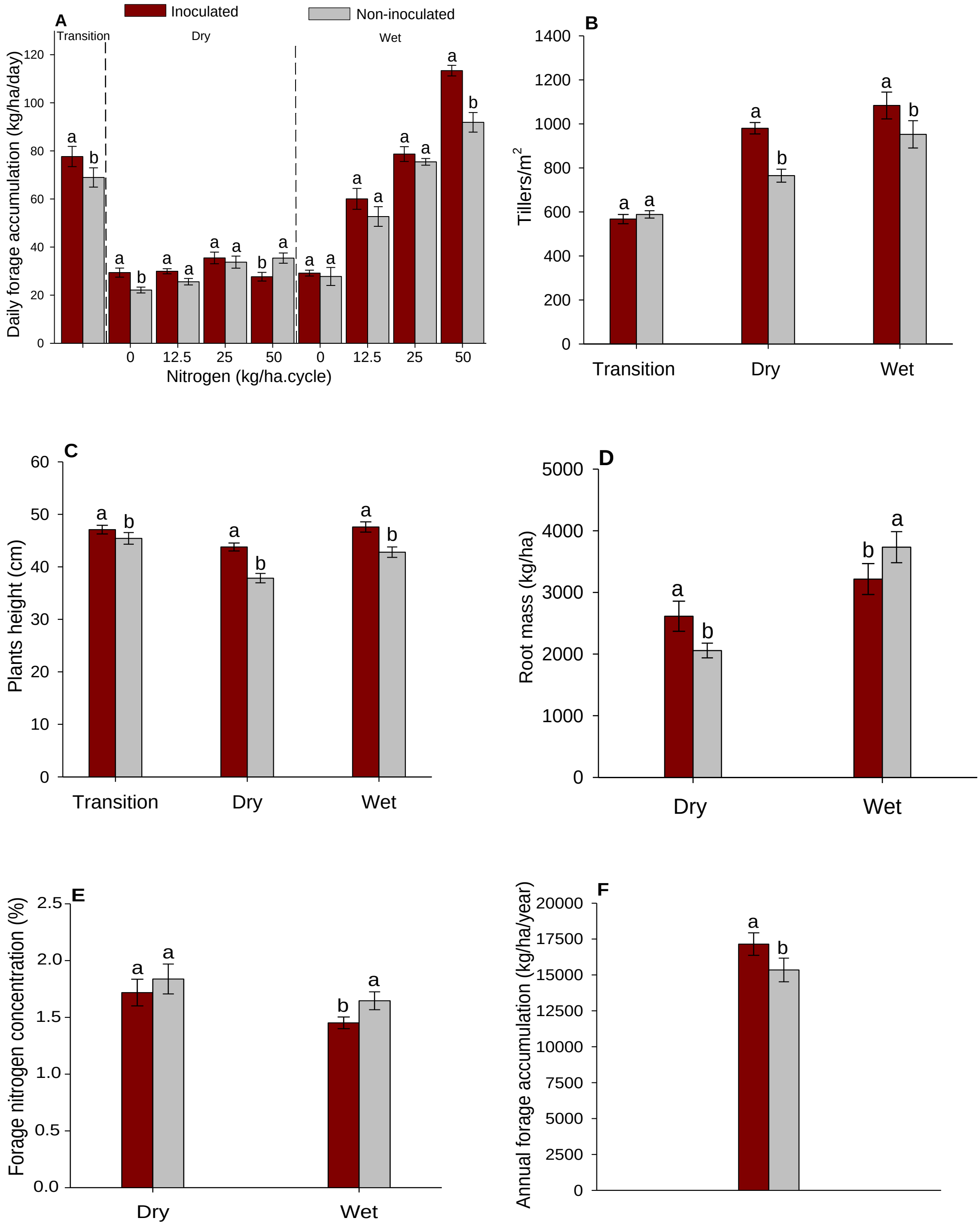

Figure 2. Daily forage accumulation (A), number of tillers (B), plant height (C), root mass (D), forage nitrogen concentration (E) and annual forage accumulation (F) of *Urochloa brizantha* cv. Marandu in relation to inoculation with *Azospirillum brasilense* and nitrogen fertilization (0, 12.5, 25 and 50 kg/ha of N). Values followed by the same letter for each variable and period do not significantly differ by the Tukey test at 5%.

Annual forage accumulation was increased by inoculation (Fig. 2f). Inoculated plants had total annual forage accumulation of 17 t/ha.year, and non-inoculated 15 t/ha.year, averaged across N rates.

Plants showed different responses to N in each period (Table 2). In the transition season, the highest daily accumulation rate for inoculated plants was at 50 kg N/ha, which provided an accumulation of 99 kg/ha.day, representing an increase of 60% compared with nil N fertiliser. For non-inoculated plants, there was a significant effect of N fertiliser, and plants accumulated, on average, 68 kg/ha.day. In the absence of N fertiliser, inoculated plants showed 32% higher accumulation than non-inoculated plants.

Table 2. Daily forage accumulation (DFA), plant height (PH), number of tillers (NT), forage nitrogen content (FNC) and forage mass (FM) of Marandu grass, in relation to inoculation with *Azospirillum brasilense* and nitrogen fertilization.

Variable	Period	Inoculation	Model	Equation	Coefficient of determination
DFA	TS	YES	L*	61.06+0.75x	0.98
	DS	NO	L**	23.19+0.27x	0.85
	WS	YES	L*	34.59+1.63x	0.98
		NO	L**	34.47+1.25x	0.92
NT	DS	NO	L**	655.60+4.98x	0.92
	WS	YES	L*	758.84+14.83x	0.97
		NO	L*	641+14.23x	0.99
PH	TS	YES	L**	43.81+0.14x	0.91
	DS	NO	L**	35.17+0.12x	0.92
	WS	YES	L**	44.03+0.16x	0.94
		NO	L*	38.81+0.18x	0.98
FNC	DS	YES	L**	1.29+0.0073x	0.90
		NO	L*	1.37+0.012x	0.97
	WS	YES	L**	1.32+0.017x	0.81
		NO	L**	1.45+0.017x	0.95
FM		YES	L*	10377+309x	0.98
		NO	L**	9584+263x	0.92

L: linear regression model; *, ** Significant at 1% and 5% probability, respectively; TS: transition season. DS: dry season and WS: wet season; YES: inoculated; NO: non-inoculated.

During the dry season, the non-inoculated plants showed higher daily accumulation rates at the highest N rate, producing 37 kg/ha.day. In the absence of N, inoculation resulted in an increase of 33% in forage daily accumulation.

In the wet season, the inoculation treatments showed similar positive linear responses to increased N fertilisation, but with different magnitudes (Table 2). For the inoculated plants, at the rate of 50 kg/ha of N, the daily forage accumulation was

116.1 kg/ha.day, which represented an increase of 241% compared with nil N. Non-inoculated plants at the same N rate produced 97 kg/ha.day, 181% higher than in the absence of N fertilisation.

With regard to tiller density in response to N fertiliser in the dry season, the non-inoculated treatment had 905 tillers/m^2 at the highest N rate, whereas the inoculated treatment had an average of 980 tillers/m^2 and was not significantly affected by N rate. Even in the absence of N fertiliser, the inoculated plants had a higher tiller density than the non-inoculated plants at 50 kg N/ha. In the wet season in the absence of N, tiller densities were 758 and 641 tillers/m^2 for inoculated and non-inoculated treatments, respectively. When 50 kg N/ha was applied, tiller densities were 1500 tillers/m^2 for inoculated and 1352 tillers/m^2 for non-inoculated treatments.

Plant height in the transition season was not significantly affected by N fertiliser for non-inoculated plants (mean 45 cm), but there was a significant effect in the inoculated treatment, which produced plants up to 51 cm in height at 50 kg N/ha (Table 2). In the dry season, inoculated plants were not significantly affected by N-fertiliser rate, with mean plant height of 47.5 cm. However, there was a significant effect for non-inoculated plants, with 41 cm height at 50 kg N/ha (Table 2). Inoculated plants in the dry season were therefore taller than non-inoculated plants at the highest N rate. In the wet season, in the absence of N fertilisation, inoculated plants were 15% taller than non-inoculated plants, and at 50 kg N/ha, they were 6% taller. Plants that were inoculated and receiving 25 kg N/ha had similar height to those without inoculation and receiving 50 kg N/ha.

Forage N concentration was decreased by inoculation but increased by N fertilisation. In the dry season, inoculated plants had 6% lower N concentration at nil N and 19% lower at 50 kg N/ha. In the wet season, the respective values were 9% and 6%.

For annual forage accumulation in the absence of inoculation, maximum yield was 23 t/ha at 50 kg N/ha, which represented a productivity increase of 13 t/ha relative to the treatment without N. Similar results were observed for the inoculated plants, but with an even higher maximum yield of 26 t/ha, an increase of 10 t/ha relative to the treatment without N.

4 DISCUSSION

Increases in plant height and number of tillers in grasses inoculated with *A. brasilense* have been mainly attributed to the production of phytohormones (HUNGRIA et al., 2016; PEDREIRA et al., 2017). Auxins, the main phytohormones released by *A. brasilense* to the host plants, promote root and shoot growth and have the capacity to regulate plant height (DOBBELAERE et al., 2003; TAIZ; ZEIGER, 2009; FUKAMI et al., 2017). According to Souza et al. (2017), the auxin IAA (indole-3-acetic acid) promotes root growth and stimulates the differentiation in the meristematic tissues that depends on hormonal concentration. Among the benefits of Azospirillum, apparently IAA production is quantitatively the most important for grass growth (VANDE BROEK; VANDERLEYDEN, 1995; FUKAMI et al., 2017).

In our study, plant height, number of tillers and root mass of plants inoculated with *A. brasilense* were significantly increased under drought conditions; consequently, inoculation is presented as an alternative to minimize impacts in Brazilian pastures under these conditions.

The greater development of roots allows better water and nutrient absorption, causing an increase in biomass production and chlorophyll concentration, and promoting tolerance to environmental stresses such as drought (SOUZA, 2014; SOUZA et al., 2017; FUKAMI et al., 2018). In addition to these benefits, plant-growth-promoting bacteria can provide increased water retention in the soil through production of an extracellular matrix containing oligosaccharides and polysaccharides that increase water-retention capacity (RUBIN et al., 2017).

According to Pedreira et al. (2017), the dry season in Brazil is characterized by an intense period of water deficit (Fig. 1), which reduces growth and increases plant mortality. Those authors suggest inoculation with *A. brasilense* in pastures to minimize the effects of climatic constraints, attributing the increased stress tolerance to improved root development. Indeed, this was confirmed by our results.

In the dry season, the non-inoculated seedlings showed variation in N concentration of 1.37–1.97%, similar to values found for Urochloa brizantha (1.3–2.0) (SOUSA; LOBATO, 2004). The inoculated plants showed a range of 1.29–1.65% N, with the minimum value being slightly below adequate for the grass. However, use of stable isotopes (^{15}N) would be necessary to confirm the contribution of BNF. We did not perform ^{15}N analyses because we assumed that the main benefit of strains Ab-V5 and Ab-V6 of *A. brasilense* would be attributed the production of phytohormones (HUNGRIA et al., 2016; FUKAMI et al., 2017).

Livestock carrying capacity is related, among other factors, to the dry-matter-production capacity of the pastures (HUNGRIA et al., 2016). In the present study, inoculation with Azospirillum resulted in an increase in forage yield per year of 8–14%, depending on N-application rate, or 11% if 15 kg N/ha is taken as standard. Therefore, to produce an amount of forage similar to the inoculated plants, it would be necessary to have a larger area, of 1.1 ha. It would be possible to reduce the current pasture area in Brazil by inoculation with *A. brasilense* from 190 Mha (JANK et al., 2014) to 169 Mha, without any reduction to animal production.

Inoculation of Marandu grass plants provided greater annual forage accumulation (Fig. 2f), reaching an increase of 11% in relation to non-inoculated plants during the year. Because degraded pastures show drastically reduced productive capacity (DIAS FILHO, 2014), we suggest that the inoculation practice could represent an important component in efforts to reverse the degradation of Brazilian pastures.

By evaluating the inoculation of native pastures with *A. brasilense*, Itzigsohn et al. (2000) concluded that the practice of inoculation with these bacteria has the potential to increase forage production and reduce environmental damage caused by the use of fertilisers. Similarly, from inoculation of *A. brasilense* on Coastcross grass (Cynodon dactylon (L.) Pers.), Aguirre et al. (2018) found an increase in forage yield and better pasture establishment. Overall, our results for U. brizantha support the results of Itzigsohn et al. (2000) and Aguirre et al. (2018).

In our study, considering the regression analyses for all evaluated parameters, inoculation with *Azospirillum* allowed an estimated reduction of 20% in the need for N fertiliser. Therefore, considering the area of Brazil planted with Marandu grass (50.0 Mha), the inoculation would represent an economy of 505 000 t N, considering the average fertilisation rate of 50 kg/ha (SOUSA; LOBATO, 2004).

Finally, it should be considered that the residual benefits of pasture inoculation with *A. brasilense* can be observed in subsequent or associated crops, for example, as reported for maize (Zea mays L.) grown in no-till, inoculated pasture (LEITE et al., 2017). The benefits reported in our study suggest that the inoculation of pastures as a practice, in addition to reducing costs and increasing productivity, contributes to environmental sustainability.

5 CONCLUSIONS

Marandu grass plants inoculated with *A. brasilense* had greater plant height, number of tillers and forage production than non-inoculated plants, regardless of the N rate.

Inoculation with *A. brasilense* allowed a 20% reduction in N fertilisation. Our results indicate that inoculation with *A. brasilense* in Marandu grass, as well as increasing forage production, can help to mitigate the stresses caused by the dry season.

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CHAPTER 2

Mitigation of mombasa grass dependence on nitrogen fertilization as function of inoculation with *Azospirillum brasilense*

ABSTRACT

Using biological inputs to improve the efficiency of nitrogen fertilizers represents an alternative for the cultivation of grasses in tropical regions. A species of growth promoting bacterium plant widely studied and used in inoculants is *Azospirillum brasilense*. 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-TO, between December 2017 and May 2018. The treatments were arranged in randomized blocks in a 5x2 factorial arrangement, with five doses of N fertilizer in the cover (0; 25; 50; 75 and 100 kg/ha of N) 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 N dose supplied. However, the percentage of leaf N was higher for inoculated plants regardless of the application of N fertilizer. In conditions of absence of N fertilization, it was possible to increase forage production in 36 % with inoculation.

Keywords: Diazotrophic bacteria. inoculant. *Megathyrsus maximus*. tropical pasture.

1 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; GUARDA, 2014). However, in search of increased forage production, 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 grasses due to good and 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 in 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 imported (MORAIS et al., 2012; CANTO et al., 2016). Moreover, the benefits of nitrogen fertilization are only short term in 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 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; BAUER, 2018). Strains of Azospirillum can present both the ability to biologically fix atmospheric nitrogen and to synthesize phytonutrients and solubilize phosphates (DÖBEREINER et al., 1976; OKON; 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.

In view of the benefits of the association of these microorganisms, the objective of this study was to evaluate the performance of Mombasa grass in association with *Azospirillum brasilense* and nitrogen fertilization.

2 MATERIAL AND METHODS

The study was conducted under field conditions in an experimental area of Araguaína, Tocantins, Brazil (Fig. 1), in coordinates 7º05'32'' S and 48º12'23'' W, between December 2017 and May 2018, using Mombasa grass. The region is classified as transition of the biomes Cerrado-Amazônia, with Aw climate (hot and humid, with dry winters), according to the Köppen International Classification (ALVAREZ 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 an Oxisols Udox (USDA classification system).

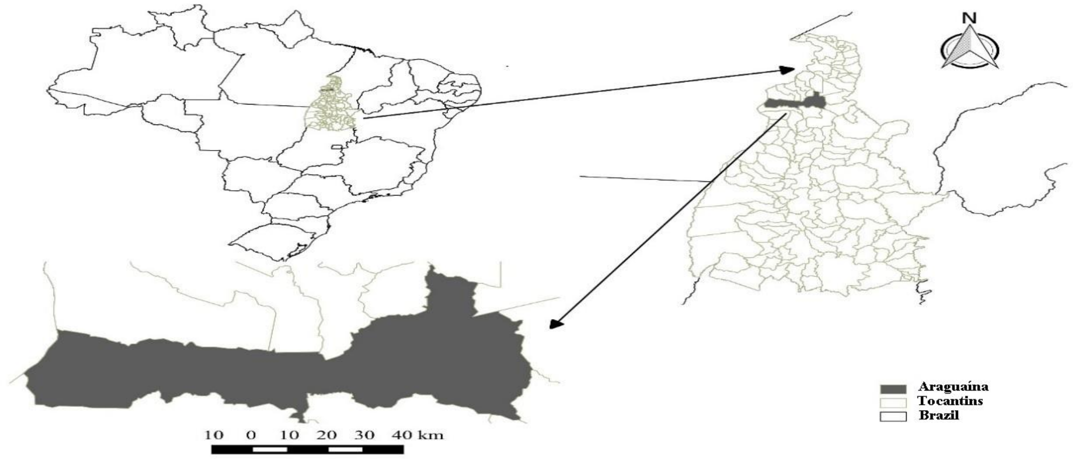

Figure 1. Location of the study area.

Table 1. Chemical and physical characterization of the soil (layer 0-20 cm) of the experimental area. Araguaína-TO, 2018.

pH ($CaCl_2$)	6.0	Potential acidity (H + Al) ($cmol_c$/kg)	2.4
Organic matter (g/kg)	1.8	Sum of bases (Ca + Mg + K) ($cmol_c$/kg)	6.2
Available P (mg/kg)	5.4	Cation exchange capacity ($cmol_c$/kg)	8.5
Available K (mg/kg)	37.0	Base saturation (%)	72.0
Exchangeable Ca ($cmol_c$/kg)	4.5	Sand (%)	59.0
Exchangeable Mg ($cmol_c$/kg)	1.6	Silt (%)	9.0
Exchangeable Al ($cmol_c$/kg)	0.0	Clay (%)	32.0

Available phosphorus (P) and potassium (K) extraction with Mehlich-1; exchangeable calcium (Ca), magnesium (Mg) and aluminium (Al) extraction with KCl; H + Al extraction with calcium acetate.

A randomized complete block design was used in a 5x2 factorial scheme, totaling ten treatments with four replicates each. Five doses of nitrogen fertilization in the cover (0, 25, 50, 75, 100 kg/ha of N) 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 m x 4 m).

Forage was sowed in December 2017 and fertilization (119 kg/ha of P_2O_5), followed the recommendations of the crop (SOUSA; 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 $2x10^8$ CFU/mL). During the experimental period the climatic data of the area were collected (Fig. 2).

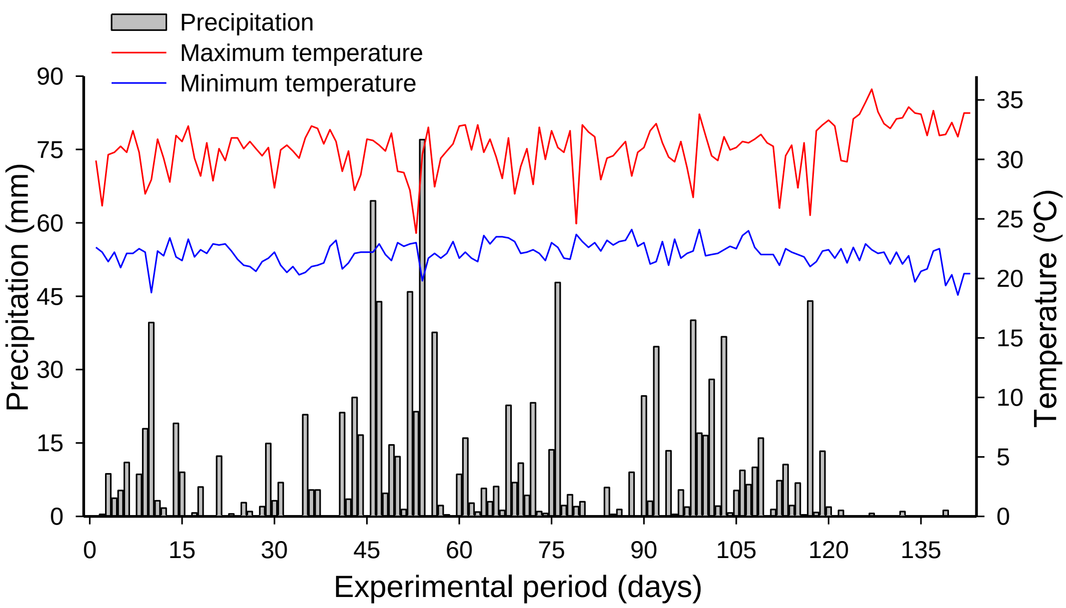

Figure 2. Precipitation and maximum and minimum temperatures of the experimental area during the period of conduction of the experiment. Araguaína-TO, 2018.

At 45 days after sowing, uniformity cuts and the nitrogen fertilization (urea) were applied to each treatment, which was repeated later at each cut. The cutting season and evaluation occurred 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 m x 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 m x 1.0 m metal frame, with a cut from the 40 cm residue height, followed for drying in an oven at 55 ºC for 72 h and subsequent weighing. Samples of forage, after drying and ground in 1 mm sieves, were digested in sulfuric acid, sequentially distilled (Kjeldahl) and titrated to determine the percentage of N in the forage.

In order to obtain root mass, two samples per plot were taken, using metal cylinders in the depth of 0 to 20 cm, to 5 cm of the cut clumps for productivity evaluation. 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 the root mass variable, which comprised the forage production during the three harvesting.

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, a 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.

3 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 variables (Fig. 3).

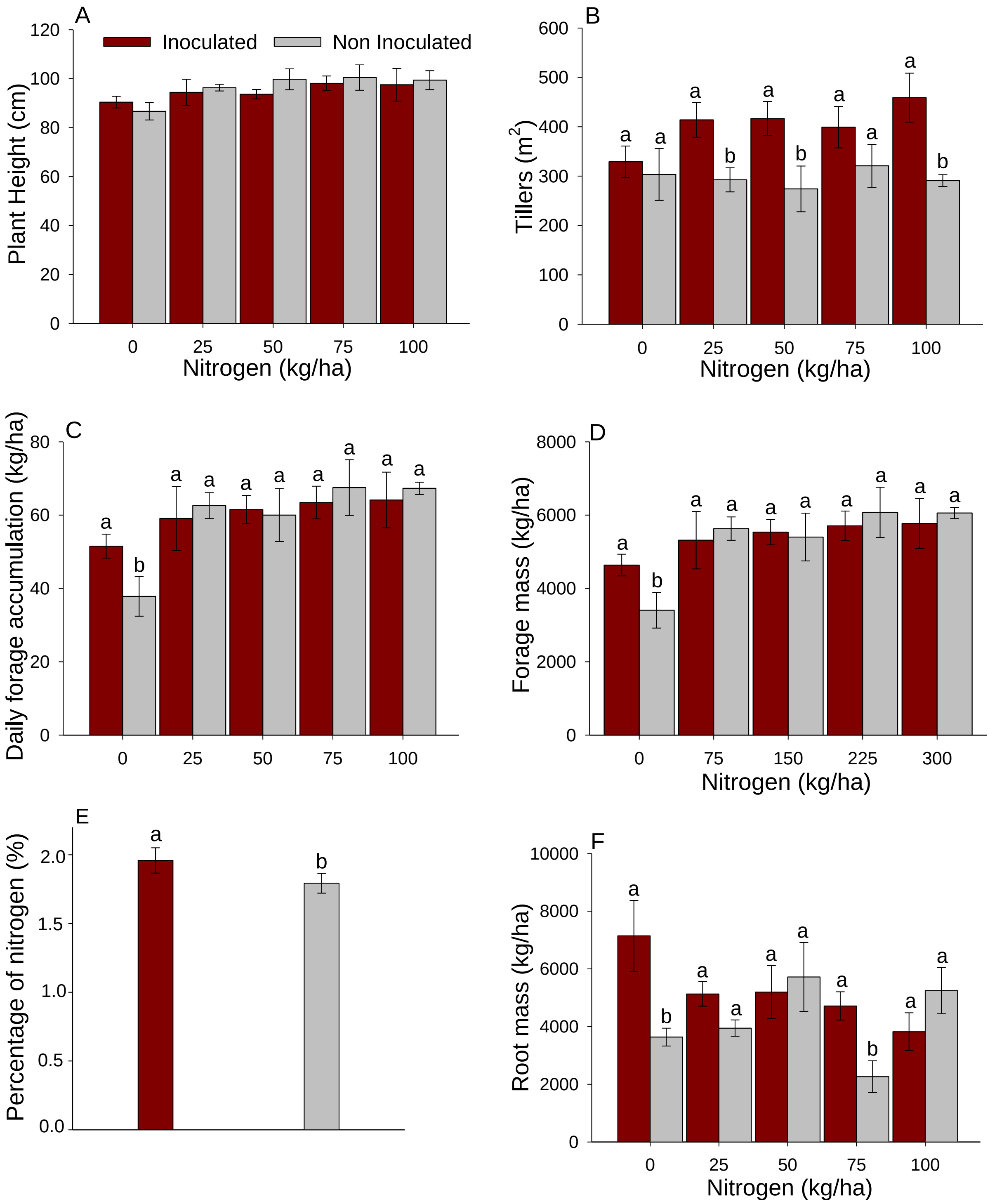

Figure 3. Height of plants (A), number of tillers (B), daily forage accumulation (C), forage mass (D), percentage of leaf nitrogen (E), and root mass (F) of Mombasa

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 %.

The percentage of nitrogen in forage was significant for the inoculation factor, with behavior independent of the N supply and applied dose. However, plant height had no influence on inoculation with *A. brasilense* regardless of whether or not it was supplied with nitrogen. The plants were inoculated at a mean height of 96 cm and inoculated at 94 cm (Fig. 3A).

In relation to number of tillers, the highest values as a function of inoculation were observed at the 25, 50, and 100 kg/ha doses of N, resulting in increases of the order of 41, 52 and 58%, respectively (Fig. 3B). For doses 0 and 75 kg/ha 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 had of 52 kg/ha while in the non-inoculated plants the daily accumulation was 37.8 kg/ha, which represented an increase of 36 % (Fig. 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 (Fig. 3D). Considering the three evaluation cycles in the absence of N, non-inoculated plants produced 3,653 kg/ha of dry mass, while inoculated plants accumulated 4,680 kg/ha.

In relation to the N content in leaves, inoculation resulted in increases in N content (Fig. 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 (Fig. 3F). In the absence of N, root mass of non-inoculated plants was 3,635 kg/ha, while in the inoculated plants it was 7,144 kg/ha, representing an increase of 96% in root production. When N was supplied to the plants, only the dose 75 kg/ha of N had an effect ($p<0.05$) with root mass of 2,263 kg/ha and 4,714 kg/ha 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 (Table 2). Plant height of inoculated plants had an adjustment to the positive linear model. In the absence of N, the plants were 91 cm high and, from that value, there was an increase of 0.07 cm in plant height for each dose of N, resulting in 98 cm 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 87 cm, showing maximum efficiency at the dose of 71 kg/ha of N, with plants with a height of 101 cm.

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

Table 2. Plant height (PH), number of tiller (NT), daily forage accumulation (DFA), forage mass (FM), percentage of leaf nitrogen (% N) and root mass (RM) of Mombasa Grass inoculation with *A. brasilense* and nitrogen fertilization.

Variable	Factor	Model	Equation	Coef. deter.
PH	I	L**	PH = 91.2 + 0.07N	82
	NI	Q*	PH = 87.1 + 0.4N – 0.0028N^2	98
NT	I	L***	NT = 354.6 + 0.99N	67
	NI	SA	NT = Average = 296	--
DFA	I	Q*	DFA = 52 + 0.28N – 0.0016N^2	98
	NI	Q***	DFA = 40 + 0,7N – 0.0046N^2	86
FM	I	Q*	FM = 4680.1 + 8.4N- 0.016N^2	98
	NI	Q***	FM = 3653.2 + 21.3N – 0.04N^2	86
% N	I	L*	%N = 1.55 + 0.0081N	97
	NI	L*	%N = 1.38 + 0.0082N	95
RM	I	L**	RM = 6613.7 – 28.2N	84
	NI	SA	RM = Average = 4162.4	--

I: inoculated plants; NI: non-inoculated plants; L: linear model; Q: quadratic model; Coef. deter.: coefficient of determination; *, **, ***: significant at 1, 5 and 10 %, respectively; NS: not significant.

Considering the daily forage accumulation, plants presented an adjustment to the quadratic model regardless of the inoculation (Table 2). For inoculated plants, the maximum efficiency dose was 88 kg/ha of N, with production of 64 kg/ha. For non-inoculated plants, the maximum efficient dose was 76 kg/ha of N with a daily accumulation of 67 kg/ha.

Total forage production presented an adjustment to the quadratic model, regardless of the inoculation (Table 2). For inoculated plants, there was production of 5,796 kg/ha at the maximum efficient dose of 268 kg/ha of N during the three cycles. Non-inoculated plants had a production of 6,213 kg/ha at the maximum efficient dose of 240 kg/ha of N in the three cycles.

For the percentage of nitrogen in forage, inoculated and non-inoculated plants presented adjustment to the linear model (Table 2). 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 production of 6,614 kg/ha in the absence of N and reduction of 28 kg for each kg of N provided to the plants (Table 2). Non-inoculated plants presented root mass of 4,162 kg/ha.

4 DISCUSSION

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, where auxin can modulate the concentration of cytokinin, which is synthesized in roots and transported to overcome dormancy of axillary buds (TAIZ; ZEIGER, 1991). In addition to the production of auxins (FUKAMI et al., 2017), *Azospirillum* has also been reported to synthesize 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). 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 increase in tillering when inoculating in Brachiaria grass (*Urochloa brizantha* cv. Marandu), in our 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, our results 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. 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; 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/ha of N (HUNGRIA et al., 2016).

In the absence of nitrogen fertilization, it was possible to increase forage production by 36 % with inoculation, improving animal support capacity and allowing better performance. 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 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) with the inoculation technique.

When comparing forage production of non-inoculated plants with nitrogen supply at the recommended dose (50 kg/ha of N per cycle) (SOUSA; 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 and non-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).

With the inoculation of plants, the percentage of nitrogen in 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; 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 percentage of leaf nitrogen with the inoculation of plants. To reach the same percentage of foliar N found in inoculated plants and without nitrogen cover, non-inoculated plants would require the application of 21 kg/ha 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 maize (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; 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* bacteria 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 (DOBEREINER 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 our 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*.

5 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 fertilizer.

In conditions of absence of N fertilization, it was possible to increase forage production in 36 % with inoculation.

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