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Nutritional characteristics of marandu grass (*Brachiaria brizantha* cv. marandu) subjected to inoculation with associative diazotrophic bacteria

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Due to the search for viable and cost-effective ways to provide nitrogen for forage grasses, inoculation with diazotrophs presents high potential as an alternative to plant nutrition by reducing the use of nitrogen fertilizers. The objective of this work was to evaluate the nutrition of marandu grass (*Brachiaria brizantha* cv. marandu) subjected to inoculation with associative diazotrophic bacteria grown in Cerrado soil (Brazilian Savanna). The experiment was conducted in a greenhouse with a completely randomized design using five treatments (three strains of associative nitrogen fixing bacteria [MTH2, MTB1 and Y2], an inoculant formed by combination of strains AbV5 and AbV6 (*Azospirillum brasilense*) and a control (100 mg dm^{-3} of N-urea]) and five replications, totaling 25 experimental units. The inoculation was performed by inserting a 5 mL aliquot of bacterial broth containing 10^{9} cells mL^{-1} in the soil near the root system of each plant. Three cuts were made at 30, 60 and 90 days after sowing. The variables: Falker chlorophyll index, nitrogen concentration in shoots and roots and crude protein in shoots of marandu grass were evaluated. The highest values for Falker chlorophyll index, nitrogen concentration, and crude protein were observed in the three cuts in plants that received N fertilization. The MTH2 strain positively influenced the nitrogen concentration in shoots and roots, and crude protein in shoots in the first and second cuts of marandu grass, whereas in the third cut, these parameters were influenced by nitrogen fertilization. In conclusion, the inoculation with associative diazotrophic bacteria may contribute positively to the nitrogen nutrition of marandu grass.

Key words: *Brachiaria brizantha*, Cerrado, diazotrophic microorganisms.

INTRODUCTION

The genus *Brachiaria* (*Urochloa* spp.) represents a milestone in Brazilian cattle breeding and occupies large areas of the Cerrado Biome (Brazilian Savanna) in central Brazil. The choice of species and cultivars was informed by desirable characteristics such as forage plants (Fagundes et al., 2006). Currently, the species of

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greatest importance are *Brachiaria decumbens*, *Brachiaria brizantha*, *Brachiaria ruziziensis* and *Brachiaria humidicola* (Sobrinho et al., 2010).

Extensive areas of Brazil are occupied by pastures; some of these areas are already degraded while some are in the degradation stage (Guarda and Guarda, 2014). Brazil has about 180 million hectares cultivated with pasture, and degradation affects approximately 70% of these areas (Hungria et al., 2016). This degradation is a direct consequence of inadequate nutritional management of these pastures; it has caused decrease in soil fertility, and making agriculture sustainability a challenge (Fonte et al., 2014; Lai et al., 2016).

Although the use of nitrogen fertilizers contributes to the increase in pasture production (Boddey et al., 2000; Palmer et al., 2014), their use have limited efficacy because of the complex steps in the nitrogen cycle which occurs in the soil, and which can result in the loss of the fertilizer after its application (Van Groenigen et al., 2015).

Among the factors that lead to the degradation of pastures, nitrogen limitation is considered one of the most important, since nitrogen is the major contributor to the quality and production of pastures (Boddey et al., 2004). However, the low economic return and environmental impacts caused by fertilizer use have raised the need to recover the pastures by sustainable means (Vaio et al., 2008).

Currently, sustainable approaches are used in the entire world in order to minimize degradation impacts and improve the production in the pasture systems (Fonte et al., 2014). Thus, the use of endophyte associative diazotrophic bacteria as a biological input is a technology that may be utilized to supply nitrogen to crops. This technology has gained strength in the recent years as an alternative for sustainable production (Gosal et al., 2012; Sarathambal et al., 2015). These bacteria have the ability to promote plant growth by producing phytohormones as auxin, gibberellin and others, in addition to N_2 fixation to colonize the root tissue and shoots of plants (Fibach-Paldi et al, 2012; Kochar and Srivastava, 2012). Among the species of nitrogen fixing bacteria associated with forage grasses are *Azospirillum brasilense* and *Azospirillum lipoferum* (Tarrand et al., 1978), *Azospirillum amazonense* (Magalhães et al., 1983), *Azospirillum halopraeferens* (Reinhold et al., 1987), *Azospirillum doebereinerae* (Eckert et al., 2001) and *Azospirillum Milinis* (Peng et al., 2006).

The association between diazotrophic bacteria and several cultures such as grasses (forage plants), corn (*Zea mays*), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and sugarcane (*Saccharum officinarum* L.) has been of interest because of the biotechnology potential which is evidenced in the increase of productivity, reduces production costs, and maintains and conserves environmental resources (Moreira et al., 2010).This is the case of Brachiaria which has a high degree of association with *A. brasilense* species (Okumura et al., 2013). This association was observed by Hungria et al. (2016). These authors reported that there was a higher biomass production in plants inoculated with *Azospirillum brasilense* treatment combined with nitrogen fertilization of 40 kg N ha^{-1}.

Thus, the objective of this work was to evaluate the nutrition of marandu grass (*Brachiaria brizantha* cv. marandu) subjected to inoculation with associative diazotrophic bacteria grown in Cerrado soil (Brazilian Savanna).

MATERIALS AND METHODS

The experiment was conducted in a greenhouse at the Federal University of Mato Grosso, Campus Rondonópolis (16°28'15" S and 54°38'08" W).

The soil was classified as a Dystrophic Oxisol (Embrapa, 2011), collected at 0 to 0.20 m depth in an area under Cerrado vegetation (Brazilian savanna). Chemical and particle size analysis were conducted in accordance with the methodology proposed by Embrapa (2011). The soil presented the following chemical and physical characteristics: pH ($CaCl_2$ a 0.01 mol L^{-1}) = 4.6; organic matter = 18.8 g dm^{-3}; P = 1.4 mg dm^{-3}; K = 66 mg dm^{-3}; Ca = 1.1 $cmol_c$ dm^{-3}; Mg = 0.4 $cmol_c$ dm^{-3}; Al = 0.5 $cmol_c$ dm^{-3}; sand = 325 g kg^{-1}; clay = 525 g kg^{-1}; silt = 150 g kg^{-1}.

The base saturation of the soil was raised to 50% with the incorporation of dolomitic limestone with 80.3% effective neutralizing power (similar to TNRP – total neutralization relative power), reacting for a period of 30 days for the correction of soil acidity. During the limestone reaction in the soil, moisture was kept by the gravimetric moisture method at 60% of the maximum soil water retention, which was determined in the laboratory with pots with a corresponding volume to that used in the experiment, in three repetitions (Santos et al., 2013). After the limestone incubation period, fertilization was performed applying 200 mg dm^{-3} of phosphorus (using triple super phosphate as a source), 150 mg dm^{-3} potassium (using potassium chloride as a source) and 40 mg dm^{-3} of sulfur (using calcium sulfate as a source). The fertilization with micronutrients was made with 1.5 mg dm^{-3} of boric acid, 2.5 mg dm^{-3} of copper chloride, 2.0 mg dm^{-3} of zinc chloride and 0.25 mg dm^{-3} of sodium molybdate.

The experiment was conducted in a greenhouse in a completely randomized design with five treatments [(three strains of associative nitrogen fixing bacteria (MTH2 – *Bacillus* sp., MTB1- similar to *Burkholderia* sp. and Y2 – *A. amazonense*), an inoculant formed by combination of strains AbV5 and AbV6 (*A. brasilense*) and a control (100 mg dm^{-3} of N-urea)] in five replications. Each plot consisted of vases with 8 dm^3 capacity, totaling 25 experimental plots. Fifteen seeds (per plot) were sown directly into the pots. Seven days after sowing, the plants were trimmed, leaving five plants per pot.

The Y2 strain was cultivated in LGI medium (Döbereiner et al., 1995) and the other strains were cultivated in Dygs medium (Rodrigues Neto, 1986) under 100-rpm stirring, at 30°C, for 24 h. An aliquot containing 3 mL of bacterial broth (10^8 cells mL^{-1}) was applied to the soil around the root area of each plant. The appropriate number of cells was obtained using the most probable number method (Andrade and Hamakawa, 1994). Three cuts were made in the shoots at 30-days interval. The first cut was made 30 days after seedling emergence. The first and second cuttings were made at 5 cm from the base of the plant; the third cut was made close to the base of the plant, as described by Bonfim-Silva and Monteiro (2007).

Nitrogen and potassium fertilization was applied again after each cut in all experimental plots using the same doses and sources

Table 1. Treatment averages, F values and estimates of orthogonal contrasts of the Falker chlorophyll index for treatments within cuts of marandu grass submitted to inoculation with associative diazotrophic bacteria in Cerrado soil.

Treatment	Cuts		
	1º	2º	3º
	Average		
MTH2	40.16	21.14	24.48
MTB1	39.80	22.06	23.30
Y2	42.54	21.84	23.30
A. brasilense	39.64	20.40	22.22
Control	44.08	30.64	35.40
Contrasts	F_c		
C1	0.006**	0.000***	0.000***
C2	0.355ns	0.322ns	0.255ns
C3	0.460ns	0.553ns	0.389ns
C4	0.086ns	0.889ns	1.000ns
	Estimate		
C1	3.545	9.280	12.075
C2	-1.193	-1.280	-1.473
C3	-1.010	-0.810	1.180
C4	-2.740	0.220	0.000

C1 = Control vs. strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ns, ** and *** - Not significant, significant at 1 and 0.1%, respectively, by F test.

shown above. 3 mL of broth corresponding to bacterial inoculation treatments, also inoculated with the same number of cells (10^8 cells mL^{-1}) was added. Chlorophyll index, the nitrogen concentration in shoots and roots, and crude protein in the shoots of marandu grass were analyzed. The chlorophyll index analysis was performed indirectly using a Falker chlorophyll meter clorofiLOG® model CFL1030. The shoot and root dry matter was performed using oven method. To determine the dry mass of shoot and root, the material was packaged in paper bags separately (shoots and roots) and placed to dry in an oven at 65°C for 72 h. Then, the dry matter values were obtained by weighing the material in a semi-analytical weight. The concentration of nitrogen and the crude protein was analyzed by the Kjeldahl method described by Malavolta et al. (1997).

Data were subjected to ANOVA, and the averages were analyzed by orthogonal contrasts and F-test using the SISVAR software (Ferreira, 2011). The contrasts applied to analyze treatments within each cut consisted of: (C1) = Nitrogen fertilization vs. strains of diazotrophic bacteria; (C2) = *Azospirillum brasilense* vs. other diazotrophic strains (MTH2, Y2 and MTB1); (C3) = MTH2 vs. Y2 and MTB1; (C4) = Y2 vs MTB1. The contrasts applied to analyze the cuts in each of the treatments consisted of: (C1) = First cut vs. second and third cuts; (C2) = Second cut vs. third cut.

RESULTS AND DISCUSSION

The mean values of orthogonal contrasts showed that there was a significant interaction between cuts and treatments for all the variables studied. In the deployment of the interaction of treatments within each cut, there was a significant effect for contrast 1 (C1) for all cuts. The first cut Y2 and MTH2 inoculation treatments showed mean values of 42.54 and 40.16 for the chlorophyll index. These values were close to the value observed in the control with nitrogenous fertilizer (44.08), which corresponds to the maximum production obtained. In other cuts, there were reductions in the chlorophyll index for all treatments (Table 1). The results observed for contrasts C1, C2 and C3, compared to nitrogen fertilization with inoculated treatments, indicate an influence of this treatment on the concentration of nitrogen in marandu grass leaves, allowing higher readings compared to the inoculated treatments. However, when the maximum values of chlorophyll index observed in marandu leaves that received nitrogen fertilizer was considered (44.08, 30.64 and 35.40), the Y2 strain with a value of 42.54 took 96.5% in the first cut. In the second and third cuts, the values reached 72.0% and 69.1% with the MTB1 and MTH2 strains respectively. It indicates that these strains have the potential to provide some of the nitrogen required for the development of marandu. Besides,the reading of the chlorophyll index is a parameter that can be used to evaluate the nutritional status of plants in relation to nitrogen (Argenta et al., 2001).

In the course of this study, when the chlorophyll content was assessed a better response in nitrogen fertilization was observed, this corroborates the observations reported by Abreu and Monteiro (1999) and Cabral et al. (2013), where the highest values of chlorophyll were observed in plants that received nitrogen fertilization. Viana et al. (2014) evaluated the effect of nitrogen fertilization on *Panicum maximum* cv. Tanzania grass and observed that this culture responded positively to the nitrogen doses applied. The values of chlorophyll content ranged from 35.53 to 47.85 at doses of 0 and 80 g dm^{-3} of nitrogen, respectively, and according to Maranhão et al. (2009), the chlorophyll content, in addition to indirectly measuring the nitrogen status of plants, may also be used as an indicator of the crude protein content of marandu grass. In the interaction of the cuts within each treatment, the highest values for chlorophyll index were observed in the first cut in all treatments. However, upon evaluating the other cuts, there was a significant effect for the third cut (Table 2).

The result observed for the first contrast (C1) indicates that marandu grass responded positively to the inoculation with associative diazotrophic bacteria, as well as to applied nitrogen doses, indicating the contribution of all nitrogen sources in the period related to the first cut in relation to others. Thus, the inoculation of associative diazotrophic bacteria may provide an increase in root surface, thus increasing the nitrogen that can be exploited in the soil and enabling the reduction of the use of nitrogen fertilizers (Okon and Vanderleyden, 1997; Gosal et al., 2012).

Table 2. Treatments averages, F values and estimates of orthogonal contrasts of the Falker chlorophyll index for cuts within treatments of marandu grass submitted to inoculation with associative diazotrophic bacteria in Cerrado soil.

Cut	Treatment				
	MTH2	**MTB1**	**Y2**	***A. brasilense***	**Control**
	Average				
1º	40.16	39.80	42.54	39.64	44.08
2º	21.14	22.06	21.84	20.40	30.64
3º	24.48	23.30	23.30	22.22	35.40
Contrasts	**Fc**				
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.037*	0.432ns	0.356^{ns}	0.251^{ns}	0.004**
	Estimate				
C1	17.350	17.120	19.970	18.330	11.060
C2	-3.340	-1.240	-1.460	-1.820	-4.760

C1: First cut vs. second and third cuts; C2: Second cut vs. third cut. ^{ns}, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

In contrast C2, the third cut in treatment with MTH2 strain was stressed, indicating that this strain was able to associate positively with marandu grass in comparison to other treatments with inoculation. In the third cut, the treatment with nitrogen fertilization was also stressed. It showed a positive response of this forage crop in relation to the application of mineral nitrogen. According to Olivares et al. (1997), the endophytic association allows these microorganisms to be free of competition with other groups of edaphic organisms present in the rhizosphere of plants, enabling a better use of the biologically fixed nitrogen.

The nitrogen-fixing bacteria provide this nutrient to the host plant, so that the greater its availability, the greater the amount of chlorophyll in leaves. Guimarães et al. (2011a) evaluated *Brachiaria decumbens* grass inoculated with *Azospirillum* spp. and observed a positive effect of these bacteria on the reading of the chlorophyll index in this grass, which was equivalent to 82% of the readings observed for the treatment with nitrogen fertilization (considered as maximum production), confirming that the association between the plant and bacteria may result in an adequate supply of nitrogen.

For Guimarães et al. (2011b), upon evaluating the effect of *Azospirillum* spp. inoculation on the productive characteristics of *Brachiaria brizantha* cv. marandu, there was a greater reading of the chlorophyll content in the treatment with nitrogen fertilization. However, among the inoculated treatments, the AZ18 strain had a value equivalent to 80% of that observed for plants that received N fertilization. This evidence shows that the inoculation with diazotrophic bacteria has the potential to partially replace nitrogen fertilization in low fertility soils (Itzigsohn et al., 2000).

In the outspread of treatments within the cuts for shoot nitrogen concentration, there was a significant effect in the treatment whose plants were inoculated with the MTB1 strain and in nitrogen fertilization in the third cut (Table 3). The nitrogen concentrations in this study were higher in the first cut compared to other cuts. This increase may be justified by the fact that young plants grow in relation to the expansion of leaf surface, where there are high concentrations of nitrogen that tend to decrease due to leaf senescence of forages over time (Muller et al., 2005).

Among the inoculation treatments with diazotrophic bacteria, the highest nitrogen concentrations in the first and second cuts were found in plant shoots inoculated with the MTB1 strain. This demonstrates that there is an association between the host plant and this strain when it is present in the rhizosphere of plants, because the rhizosphere of plants is considered a conditioner dynamics of microorganisms in the soil and can even interfere with the establishment of bacteria when inoculated in plants (Reis Junior et al., 2006).

In the third section, the concentration of nitrogen in the leaves of the inoculated treatments associate of diazotrophs showed an average of 47.3% of the maximum observed in plants corresponding to control with nitrogen fertilization (17.92). This shows that treatment with diazotrophs has economic value (compared to nitrogenous fertilizers), reduced negative effect on the environment and a contributing part of the nitrogen that is globally established by FBN (Reed et al., 2011)

Batista and Monteiro (2007), working with nitrogen levels from 1.0 to 33.0 mmol L^{-1}, observed that the influence of the concentration of this mineral in the blades of newly expanded leaves of marandu grass, ranges from 16.0 to 31.0 g kg^{-1} in a sample of the first cut and 9.5 to

Table 3. Treatment averages, F values, and estimates of orthogonal contrasts for the nitrogen concentration of the shoot of marandu grass in the outspread of treatments within each cut.

Treatment	Cut		
	1º	2º	3º
	Average (g kg^{-1})		
MTH2	22.68	7.2	8.12
MTB1	25.76	9.8	8.68
Y2	24.08	8.96	8.68
A. brasilense	19.04	3.36	5.88
Control	24.64	9.8	17.92
Contrasts		F_c	
C1	0.253^{ns}	0.108^{ns}	0.000^{***}
C2	0.002^{**}	0.001^{**}	0.100^{ns}
C3	0.182^{ns}	0.194^{ns}	0.737^{ns}
C4	0.384^{ns}	0.663^{ns}	1.000^{ns}
		Estimate	
C1	1.750	2.470	10.080
C2	-5.133	-5.293	-2.613
C3	-2.240	-2.180	-0.560
C4	1.680	0.840	0.000

C1 = Control vs strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ^{ns}, ** and *** - Not significant, significant at 1 and 0.1%, respectively, by F test.

17.0 g kg^{-1} in the second cut of the plants, and these results corroborates with the values found in this study, in both the first and second cut in all treatments observed for treatment with MTB1 and fertilized plants with nitrogen. The plants that received nitrogen fertilization had higher concentrations of this nutrient in the third cut, confirming the results obtained by Silva et al. (2005), according to which the nitrogen concentration in the shoots of marandu grass increased in function of nitrogen fertilizer doses. It is important to consider that even with the reduced concentration of nitrogen in the inoculation treatments in the second and third cuts, when the accumulation percentage of the inoculation treatments were compared with the maximum concentration observed in the control treatment, there were an approximation of 74.7 and 43.75%, respectively.

In all the cuts within each treatment, the first and second cuts had significant effects in the treatment with nitrogen fertilization, whereas for treatments with inoculation of diazotrophic bacteria, only the first cut had no significant effect (Table 4).

Costa et al. (2009), in studies with marandu grass subjected to doses and sources of nitrogen, observed that in the highest nitrogen doses, the concentrations of this nutrient in the plant were 18.86, 20.96 and 27.73 g kg^{-1} for the years 2004, 2005 and 2006, respectively, with an increasing linear effect over time. These results relate to this study, for they show that the nitrogen concentration tends to increase to the extent that this nutrient is available in a greater quantity to the plant. However, the nitrogen concentration decreased in the second cut in MTH2, *A. brasilense* and nitrogen control treatments increased the values in the third cut.

Cabral et al. (2013), in studies with marandu, convert and decumbens grasses, observed that for the first cut, in the absence of nitrogen fertilization, marandu and convert grasses had a higher concentration of nitrogen, inferring that these forages have a higher potential in the supply of nitrogen to ruminal microorganisms in the early stages of the forage grass development.

For the variable crude protein, the outspread of treatments within the cuts had a significant effect of diazotrophic bacteria strains on the first and second cuts. In the first cut, compared to nitrogen fertilization, the MTB1 strain showed an increase of 4.54%. The MTH2 and Y2 strains had crude protein values of about 92% and 97%, respectively, compared to the concentration of crude protein observed in the plants fertilized with mineral nitrogen (corresponding to the maximum production obtained). Higher concentrations of crude protein in the third cut were found in plants that received nitrogen fertilization (Table 5).

According to Minson (1990) and Van Soest (1994), pastures with levels below 7% of crude protein in dry matter are considered deficient for ruminants. Values below this level were observed in this study in the second cut for all treatments; in the third cut, it was satisfactory only for nitrogen fertilization. However, it corroborates the results obtained by Nicodemo et al. (2004), in which the authors found concentrations of crude protein ranging from 5.3 to 10.4% in pasture samples of *Brachiaria brizantha*.

Studying the crude protein content and the dry matter production of Brachiaria grass under nitrogen doses, Chagas and Botelho (2005) reported that the concentration of crude protein of this forage increased as the nitrogen rate increased, also in the analysis performed in the first cut.

In the outspread of cuts within each treatment, there were significant effects on the first cut in all treatments, and on the first and second cuts for nitrogen fertilization.

Similar to the results obtained in the nitrogen concentration in marandu leaves, there was a decrease in the second cut to MTH2, *A. brasilense* and nitrogen control treatments, increasing again at the third cut (Table 6).

In the first cut, treatment with MTB1 strain obtained a crude protein concentration similar to control, and only in the third cut the value of crude protein between these treatments was reduced. The lower production of crude protein observed in the inoculation in other treatments in the second and third cuts may have occurred due to a lower nitrogen accumulation in this phase, with a lower absorption of nitrogen and consequently a lower content of amino acids, proteins, nucleic acids, hormones and

Table 4. Treatment averages, F values, and estimates of orthogonal contrasts of nitrogen concentration in the shoot of marandu grass subjected to different cut and the interaction within each treatment.

Cut	Treatment				
	MTH2	MTB1	Y2	*A. brasilense*	Control
	Average (g kg^{-1})				
1º	22.68	25.76	24.08	19.04	24.64
2º	7.2	9.8	8.96	3.36	9.8
3º	8.12	8.68	8.68	5.88	17.92
Contrasts	F_c				
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.633^{ns}	0.561^{ns}	0.884^{ns}	0.194^{ns}	0.000***
	Estimate				
C1	15.020	16.520	15.260	14.420	10.780
C2	-0.920	1.120	0.280	-2.520	-8.120

C1: First cut vs second and third cuts; C2: Second cut vs third cut. ^{ns}, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

Table 5. Treatment averages, F values and estimates of orthogonal contrasts of crude protein in the shoot in the outspread of interactions of treatments within each marandu grass cut submitted to inoculation with associative diazotrophic bacteria cultivated in Cerrado soil.

Treatment	Cut		
	1º	2º	3º
	Average (g kg^{-1})		
MTH2	14.17	4.50	5.07
MTB1	16.10	6.12	5.42
Y2	15.05	5.60	5.42
A. brasilense	11.90	2.10	3.67
Control	15.40	6.12	11.20
Contrasts	F_c		
C1	0.253^{ns}	0.108^{ns}	0.000***
C2	0.002**	0.001**	0.100^{ns}
C3	0.182^{ns}	0.194^{ns}	0.737^{ns}
C4	0.384^{ns}	0.663^{ns}	1.000^{ns}
	Estimate		
C1	1.093	1.543	6.300
C2	-3.208	-3.308	-1.633
C3	-1.400	-1.362	-0.350
C4	1.050	0.525	0.000

C1 = Control vs strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ^{ns}, ** and *** - Not significant, significant at 1 and 0.1%, respectively, by F test.

Chlorophyll (França et al., 2007; Lavres Junior and Monteiro, 2003).

The decrease in crude protein observed in this study may be related to the fact that in tropical forage grasses, with advancing maturity, an early lignification of the tissues occurs and consequently there are changes in the cytoplasm of the plant with a decrease in protein and other nutrients due to the gradual increase of cell wall constituents (Coward-Lord, 1972). Pronounced decreases in crude protein content as the interval between cuts increases have been reported for various grasses under a tropical climate (Ruggieri et al., 1995). In the interaction of treatments within each cut for the concentration of nitrogen in roots, there was a significant effect of the inoculant in the first and second cuts, with a significant effect on the third cut for nitrogen fertilization (Table 7).

Nitrogen fertilization, as shown in the C1 contrast, was able to supply the nutritional requirement of marandu grass, providing the nitrogen needed for the crop during its development. For the treatments with diazotrophic bacteria, as seen in the C2 contrast, strains showed a substantial contribution to marandu grass nutrition, effectively providing nitrogen to the crop. The nitrogen accumulation in grasses because of a fasciculated root system has an advantage over the pivoting system of legumes to extract water and nutrients from the soil. Thus, even if part of the nitrogen is provided by the association with fixing bacteria, the economy of nitrogen fertilizer can be considered equal to that seen in legumes self-sufficient in nitrogen (Döbereiner, 1992).

Changes in the chemical composition of forage plants can be verified as maturation occurs. Thus, there is the decrease of components digestible by animals, increasing the fiber content and therefore decreasing the nutritional value of the feed (Cano et al., 2004). Batista

Table 6. Treatment averages, F values and estimates of orthogonal contrasts of crude protein in the shoot in the outspread of interactions of treatments of cuts within each marandu grass cut submitted to inoculation with associative diazotrophic bacteria cultivated in Cerrado soil.

Cut	Treatment				
	MTH2	**MTB1**	**Y2**	***A. brasilense***	**Control**
	Average (g kg^{-1})				
1º	14.17	16.10	15.05	11.90	15.40
2º	4.50	6.12	5.60	2.10	6.12
3º	5.07	5.42	5.42	3.67	11.20
Contrasts	**F_c**				
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.633^{ns}	0.561^{ns}	0.884^{ns}	0.194^{ns}	0.000***
	Estimate				
C1	9.387	10.325	9.537	9.012	6.737
C2	-0.575	0.700	0.175	-1.575	-5.075

C1: First cut vs second and third cuts; C2: Second cut vs third cut. ^{ns}, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

Table 7. Treatment averages, F values and estimates of orthogonal contrasts for the nitrogen concentration in the roots of marandu grass by the outspread of interaction of treatments within each cut.

Treatment	Cut		
	1º	**2º**	**3º**
	Average (g kg^{-1})		
MTH2	2.26	0.72	0.81
MTB1	2.57	0.98	0.86
Y2	2.40	0.89	0.86
A. brasilense	1.90	0.33	0.58
Control	2.46	0.98	1.79
Contrasts	**F_c**		
C1	0.253^{ns}	0.108^{ns}	0.000***
C2	0.002**	0.001**	0.100^{ns}
C3	0.182^{ns}	0.194^{ns}	0.737^{ns}
C4	0.384^{ns}	0.663^{ns}	1.000^{ns}
	Estimate		
C1	0.175	0.247	1.008
C2	-0.513	-0.529	-0.261
C3	-0.224	-0.218	-0.056
C4	0.168	0.084	0.000

C1 = Control vs strains of diazotrophic bacteria; C2 = *A. brasilense* vs other strains of diazotrophic bacteria; C3 = MTH2 vs Y2 and MTB1; C4 = Y2 vs MTB1. ^{ns}, ** and ***- Not significant, significant at 1 and 0.1%, respectively, by F test.

and Monteiro (2006) demonstrated that the nitrogen concentration in the root tissue of marandu grass ranged from 29.5 g to 2.4 kg^{-1} in the nitrogen dose interval of 14-462 mg L^{-1} in the nutrient solution. In addition, in the study conducted by Maranhão et al. (2009) on the production and chemical composition of two Brachiaria cultivars fertilized with nitrogen, grass marandu showed a greater production of root, proving to be more resistant by

Table 8. Treatment averages, F values, and estimates of orthogonal contrasts for the nitrogen concentration in the roots of marandu grass by the outspread of interaction of cuts within each treatment.

Cut	Treatment				
	MTH2	MTB1	Y2	*A. brasilense*	Control
	Average (g kg^{-1})				
1º	2.26	2.57	2.40	1.90	2.46
2º	0.72	0.98	0.89	0.33	0.98
3º	0.81	0.86	0.86	0.58	1.79
Contrasts	F_c				
C1	0.000***	0.000***	0.000***	0.000***	0.000***
C2	0.633^{ns}	0.561^{ns}	0.884^{ns}	0.194^{ns}	0.000***
	Estimate				
C1	1.502	1.652	1.526	1.442	1.078
C2	-0.092	0.112	0.028	-0.252	-0.812

C1: First cut vs second and third cuts; C2: Second cut vs third cut. ^{ns}, *, ** and *** - Not significant, significant at 5, 1 and 0.1%, respectively, by F test.

exploring the soil more efficiently.

For the all the cuts within treatments, the nitrogen fertilization stood out in the first and second cuts, while the inoculation treatments presented a significant effect only in the first cut. Among the inoculation treatments, MTB1presented the highest nitrogen concentrations in the roots for the all cuts, exceeding the first cut and the values obtained in the roots of plants fertilized with mineral nitrogen (Table 8).

The nitrogen-fixing bacteria in association with plant roots, including forage, may assist in root growth and, therefore, plant growth by promoting substances such as phytohormones (Kuklinsky-Sobral et al., 2004; Pedraza, et al., 2004; Radwan et al., 2004). Thus, endophytic diazotrophic bacteria associated to plants may represent a promising alternative in promoting plant growth and soil management and, consequently, environmental quality.

With an area of 200 million hectares under pastures in Brazil, the appearance of areas in some stage of degradation, whether by soil management or use of fertilizer, especially nitrogen, is common. This may generate negative impacts on the environment (Boddey et al., 2006).

Thus, the use of diazotrophic bacteria associated to pastures aims to minimize the impacts caused by the indiscriminate use of nitrogen fertilizers, including contributing to sustainability in agriculture and livestock.

Conclusion

Associative diazotrophic bacteria contribute positively to the nitrogen nutrition of Marandu grass.

Conflict of interests

The authors have not declared any conflict of interest.

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