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Biological nitrogen fixation and urea-N recovery in 'Coastcross-1' pasture treated with *Azospirillum brasilense*

Abstract – The objective of this work was to quantify the inoculation effect of *Azospirillum brasilense* (Ab-V5 and Ab-V6 strains) on the forage yield, biological nitrogen fixation (BNF), and urea-^{15}N recovery of the forage grass 'Coastcross-1'. The experiment was carried out in a 2 (with or without inoculation) × 2 (without N fertilizer and with 100 kg ha^{-1} N per year as urea) × 7 (cuts) factorial arrangement. The natural ^{15}N abundance method was used to determine BNF; to determine urea-N recovery, ^{15}N-labeled urea was applied in microplots. Forage yield was higher in grasses subjected to inoculation, with 7.4 Mg ha^{-1} dry matter per year, for the treatment without N fertilizer, and 8.0 Mg ha^{-1} dry matter per year for the treatment with 100 kg ha^{-1} N per year, respectively, which shows an additive effect of inoculation and N fertilization. The contribution of BNF was 23.0 and 53.8 kg ha^{-1} per year for the unfertilized treatment, both in uninoculated and inoculated plants, respectively. Urea-^{15}N recovery was 13.7 and 16.5 kg ha^{-1} per year for uninoculated and inoculated treatments, respectively, corresponding to 13.7 and 16.5% of the urea-N applied. Inoculation with *A. brasilense* increases forage yield and the contribution of BNF to grass nutrition with N, as well as urea-N recovery by the forage grass.

Index terms: *Cynodon dactylon*, forage yield, natural abundance of ^{15}N.

Fixação biológica de nitrogênio e recuperação de N-ureia em pastagem de 'Coastcross-1' tratada com *Azospirillum brasilense*

Resumo – O objetivo deste trabalho foi quantificar o efeito da inoculação de *Azospirillum brasilense* (estirpes Ab-V5 e Ab-V6) na produção de forragem, na fixação biológica de nitrogênio (FBN) e na recuperação de ^{15}N-ureia pela gramínea forrageira 'Coastcross-1'. O experimento foi feito em arranjo fatorial 2 (com e sem inoculação) × 2 (sem fertilização nitrogenada e com 100 kg ha^{-1} de N por ano, na forma de ureia) × 7 (cortes). O método da abundância natural de ^{15}N foi utilizado para determinar a FBN; para a recuperação de N-ureia, aplicou-se ^{15}N-ureia marcada em microparcelas. As produções de forragem foram maiores nas gramíneas com inoculação, com 7,4 Mg ha^{-1} de matéria seca por ano, no tratamento sem N fertilizante, e 8,0 Mg ha^{-1} de matéria seca por ano no tratamento com 100 kg ha^{-1} de N por ano, o que mostra o efeito aditivo da inoculação e da fertilização nitrogenada. A contribuição da FBN foi de 23,0 e 53,8 kg ha^{-1} por ano para o tratamento sem fertilização de N, tanto nas plantas sem inoculação como naquelas com inoculação, respectivamente. A recuperação de ^{15}N-ureia foi de 13,7 e 16,5 kg ha^{-1} por ano, para os tratamentos sem e com inoculação, respectivamente, o que corresponde a 13,7 e 16,5% do N-ureia aplicado. A inoculação com *A. brasilense* aumenta a produção de forragem e a contribuição da FBN para a nutrição nitrogenada de 'Coastcross-1', assim como a recuperação de N-ureia pela gramínea.

Termos para indexação: *Cynodon dactylon*, produção de forragem, abundância natural de ^{15}N.

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Introduction

Grasses of the genus *Cynodon* show advantages for its use as a source of fiber for dairy cattle in Brazil, since they are perennial, and show a rapid establishment and high-forage production (Ziech et al., 2016). Among the cultivars of *Cynodon dactylon* (L.) Pers., 'Coastcross-1' stands out because it is well-adapted to the subtropical climate, it is responsive to fertilization and has a good leaf/stem ratio (Aguirre et al., 2014). In order to exploit the productive potential of this grass, farmers usually use high amounts of fertilizers, especially N, which raises the production costs (Ziech et al., 2016). However, much of the applied N is lost by leaching, volatilization of ammonia, and denitrification, with detrimental effects to the environment (Hungria el al., 2016).

In this context and faced with a consumer market that is increasingly demanding for food produced in a more sustainable way, alternatives are required to reduce and use better N fertilizers. Thus, the inoculation with associative bacteria has been investigated, especially with *Azospirillum brasilense*. In addition to its capacity to fix N_2, this bacteria produces phytohormones that are responsible for the greater development of the grass root system, increasing the density and length of root hairs, and number and volume of lateral roots (Moreira et al., 2010). Therefore, the inoculation of this bacteria may increase the nutrient uptake capacity, with a consequent higher recovery of the fertilization applied to pastures. Considering the large extent of pasture land in Brazil, totaling more than 175 million ha (Lapig, 2017), the impact of any increase in N-use efficiency could be very significant.

Researches using inoculation with *A. brasilense* in grasses are mostly carried out with annual crops (Hungria et al., 2010; Lana et al., 2012; Martins et al., 2018), while studies with perennial crops are rarer (Hungria et al., 2016).

The objective of this work was to quantify the inoculation effect of *Azospirillum brasilense* (Ab-V5 and Ab-V6 strains) on the forage yield, biological nitrogen fixation (BNF), and urea-^{15}N recovery of the forage grass 'Coastcross-1'.

Materials and Methods

The study was carried out from September 2014 to August 2015, at the Universidade Federal de Santa Maria (UFSM), located in the Central Depression of Rio Grande do Sul state, Brazil (29°41'S, 53°48'W, at about 90 m altitude). The climate is humid subtropical (Cfa), with 19.2°C average annual temperature; January and June are the hottest (24.9ºC) and coldest (13.6ºC) months, respectively. During the experimental period, the average monthly temperature and rainfall were 20.2ºC and 158.2 mm per month, respectively. Fourteen frosts were recorded, six in August 2014, seven in June 2015, and one in July 2015. Climatic data were obtained from the UFSM Meteorological Station, located approximately 700 m from the experimental area.

The soil is classified as an Argissolo Vermelho distrófico típico (Santos et al., 2018), that corresponds to a Hapludult (Soil Survey Staff, 2014), or Alisol (FAO, 2015). The 0–20 cm topsoil showed 280 g kg^{-1} clay and, before the start of the experiment, its main chemical characteristics were: 5.3, pH-H_2O; 7.6 mg dm^{-3} P-Mehlich 1; 116 mg dm^{-3} K; 8.2 cmol$_c$ dm^{-3} Ca; 3.4 cmol$_c$ dm^{-3} Mg; 3.2% OM; 0.2 cmol$_c$ dm^{-3} Al; 65.7% base saturation; and 1.7% Al saturation.

Before the experiment, the area was covered by a natural pasture grazed by dairy cattle. On July 20, 2014, 45 days before planting 'Coastcross-1' [*Cynodon dactylon* (L.) Pers., the soil acidity was corrected with 4.8 Mg ha^{-1} dolomitic limestone incorporated to the soil by harrowing. On August 27, one week prior to the 'Coastcross-1' planting, the basal fertilization was applied to provide 100 kg ha^{-1} P_2O_5 and 60 kg ha^{-1} K_2O, spread evenly over the soil surface and incorporated into the soil by light harrowing. Doses of limestone, P, and K were established based on the soil analysis and on the recommendation for warm season perennial grasses by Comissão de Química e Fertilidade do Solo, RS/SC (Tedesco et al., 2004).

The experiment was carried out in a randomized complete block design, in a 2×2 factorial arrangement (with or without inoculation of *Azospirillum brasilense*, and with or without N fertilization) in 5×3 m plots, with three replicates. 'Coastcross-1' was planted on 4 September using mature and rooted stolons obtained from an established grassland. The stolons were planted manually in 0.5×0.5 m spacing pits of about 10 cm depth. For the inoculation treatments, a spray mix containing *Azospirillum brasilense* (Ab-V5 and Ab-V6 strains) was applied directly over the seedling roots, before covering them with soil (Pereira et al., 2017). The spray mix was prepared with 0.5 L liquid inoculant (AzoTotal, Total Biotecnologia, Curitiba, PR, Brazil), containing $2.0x10^8$ CFU mL^{-1} of *Azospirillum brasilense*, in 199.5 L water, just before application. The spray mix was applied with a backpack sprayer at 200 L ha^{-1} (5 mL per pit). In the N-fertilized treatments, the rate of 100 kg ha^{-1} urea-N per year was split into five applications (20, 20, 30, 20, and 10 kg ha^{-1}). The first application was performed 20 days after planting (September 24, 2014), and the following ones were performed some days after the cuts of the pasture, as follows: after the 1st cut (November 25); 2nd (January 12, 2015); 3rd (February 04); and 4th (March 10).

To evaluate the recovery of urea-^{15}N by 'Coastcross-1', urea-^{15}N (1% excess ^{15}N atoms) was applied to 1.5 × 1.0 m unconfined microplots demarcated in the central area of each plot of the treatments with N fertilization. Urea-^{15}N was always applied in the same microplots, at the same dates, at an equivalent dose to urea-N. In the five applications, urea was distributed manually over the surface of the whole plot and microplot.

The forage yield of 'Coastcross-1' was estimated in seven cuts – in 2014 (on November 17), and in 2015 (on January 05, January 26, March 02, April 22, June 08, and August 07) –, when pasture reached approximately 25 cm height. In each cut, forage was collected 7 cm above the ground ,in 0.25 m^2 area randomly defined within the useful plot area. The samples were weighed, and the other plant species present in the pasture were removed. The forage samples were dried in a forced-air oven at 55ºC, until a constant mass was obtained to determine the dry matter (DM). The same sampling procedure was adopted in the microplots that received the urea-^{15}N. After sampling, the remaining pasture in the plots was cut to 7 cm height above the ground, and removed to standardize the plots.

After weighing, the dry forage samples were ground in a Wiley mill and, then, in a ball mill, with a 40-mesh sieve. In the ground material, the total N was determined with an elemental analyzer Flash EA 1112 (Thermo Finnigan, Milan, Italy). The isotopic abundance of ^{15}N was determined in the samples from the plots without N (with or without inoculation) and from the microplots receiving urea-^{15}N, using an isotope ratio mass spectrometer Delta V Advantage (Thermo Fisher Scientific, Bremen, Germany) (Gonzatto et al., 2016).

Samples from 0–15, 15–30, and 30–45 cm soil depths of each experimental block were taken in September 2014, in order to estimate the natural abundance of ^{15}N in nonfixing plants, considering the distribution of abundance of ^{15}N available to the plants in the soil profile (Urquiaga et al., 2012). The collected soil was air-dried and sieved in a 2 mm mesh. Plants considered non-N-fixing – radish (*Raphanus sativus* L.), beggarticks (*Bidens pilosa* L.), and nut grass (*Cyperus eragrostis* Lam.) – were cultivated in a greenhouse in 81 pots containing 200 g of soil moistened at 80% of the field capacity. Before sowing, the soil of each pot received the equivalent to 47 mg kg^{-1} P and 77 mg kg^{-1} K, as single superphosphate and potassium chloride, respectively. Sowing density per pot for radish and beggarticks was 10 seed each, and for nut grass 0.1 g seed. The plants were grown until they showed severe symptoms of N-deficiency and then cut close to the soil. Plants from each pot were oven-dried at 55°C, ground (40 mesh), and analyzed for N concentration and ^{15}N isotopic abundance, as previously described for 'Coastcross-1'.

The estimation of ^{15}N natural abundance in the non-N-fixing plants (reference plants) was performed by weighing the value of δ^{15}N at each soil layer (δ^{15}Nrp), in relation to the percentage of 'Coastcross-1' root in the different layers (% R) of the soil profile (0–45 cm), as follows:

$$\delta^{15}\text{Nreference plant} = \left(\delta^{15}\text{Nrp}\times\%\text{R}_{0-15}\right)+\left(\delta^{15}\text{Nrp}\times\%\text{R}_{15-30}\right) + \left(\delta^{15}\text{Nrp}\times\%\text{R}_{30-45}\right),$$

where δ^{15}N values were 10.8, 12.1, and 11.9‰, respectively, for the layers 0–15, 15–30 and 30–45 cm, and the %R values in the three layers were 62.4, 24.7, and 12.9%, respectively, according to Ribeiro et al. (2011). The statistical analysis indicated no difference for δ^{15}N values between the layers and the value of natural abundance of ^{15}N in the non-fixing plants (δ^{15}Nreference plant), which was 11.3‰.

The proportion of N in 'Coastcross-1' from the air (%Ndfa) by the BNF in treatments with or without inoculation, in the absence of N fertilization, was calculated using the equation proposed by Shearer & Kohl (1986):

$$\%Ndfa = \left(\frac{\delta^{15}\text{Nreference plant} - \delta^{15}\text{NCoastcross}}{\delta^{15}\text{Nreference plant}} \right) \times 100,$$

where δ^{15}NCoastcross is the value of the natural abundance of ^{15}N of the unfertilized 'Coastcross-1', either uninoculated or inoculated. Based on the values of %Ndfa and on the amount of N accumulated in 'Coastcross-1', the amount (kg ha^{-1}) of N accumulated coming from the BNF (named biologically fixed N) was calculated in the forage.

The N percentage in 'Coastcross-1', derived from urea-^{15}N fertilizer (%Ndff) in treatments with N fertilization with or without inoculation, was calculated according to the following equation:

$$\%Ndff = \frac{\text{atom}\%^{15}\text{Nexcess in the forage}}{\text{atom}\%^{15}\text{Nexcess in the fertilizer}} \times 100.$$

The N amount of 'Coastcross-1' from urea-N (Ndff, kg ha^{-1}) was obtained from the %Ndff and the amount of total N accumulated in the forage in each evaluation. The percentage of urea-N recovered by Coastcross-1 ($R^{15}N$, % of N added) in each evaluation was calculated using the following equation:

$$R^{15}N(\%) = \frac{\text{Ndff}\left(\text{kg ha}^{-1}\right)}{\text{amount of N applied}\left(\text{kg ha}^{-1}\right)} \times 100.$$

For each cut, the sum of the applied amounts until the moment was considered, discounting the values that had already been recovered in previous cuts.

The recovery of urea-N was also calculated by the apparent recovery method (NAR):

$$NAR(\%) = \frac{(\text{NatN-Natc})}{\text{NaptN}} \times 100$$

where NatN is the amount of N accumulated in treatments with N fertilization, with or without inoculation; Natc is the amount of N accumulated in the treatment without N fertilizer and without inoculation; and NaptN is the amount of N applied in the treatments with N fertilization (100 kg ha^{-1} N per year).

The data were tested by the analysis of variance using the Mixed procedure in the statistical package SAS (SAS Institute Inc., Cary, NC, USA). In the analysis of %Ndfa and N biologically fixed, treatments without N fertilization were considered. For $R^{15}N$ and Ndff, treatments with urea-N fertilization were considered and tested for the interaction between inoculation × forage cuts. For the other variables, the interaction inoculation × N fertilization × forage cuttings was tested. When a significant effect of the factors and/or interaction between them was detected, the means were compared by Student’s t-test, at 5% probability. The covariance matrices used were chosen by the lowest value of AIC (Akaike’s information criterion) (Bozdogan, 1987). The errors were subjected to the normality test. Pearson’s correlation analysis was applied to check the association between variables.

Results and Discussion

Seven cuts, at 47-day interval, were made during the evaluation period. There was an interaction between inoculation, N fertilization and cuts ($p<0.0001$) for the forage yield (Table 1). Differences were detected in all cuts, as well as in the total forage yield of 'Coastcross-1'. In the inoculated and treatments without N fertilization, there was a superior forage yield in six out of seven cuts than yields of the uninoculated treatments. In fertilized treatments with 100 kg N ha^{-1} per year, there was a greater forage yield of the inoculated grass in five out of seven cuts. It is noteworthy that, in the first evaluation, inoculated 'Coastcross-1' that did not receive N fertilizer had the highest-forage yield and, in following four cuts, it had a similar production to inoculated 'Coastcross-1' fertilized with 100 kg ha^{-1} N per year.

Grasses of the genus *Cynodon*, such as 'Coastcross-1', respond well to N fertilization (Aguirre et al., 2014). Our results show that this grass is also responsive to inoculation with *A. brasilense*. Primavesi et al. (2004) evaluated 'Coastcross-1' without N fertilization receiving 125 kg ha^{-1} N per year as urea, split into five applications, as in the present study, and obtained yields of 4.0 and 7.1 Mg ha^{-1} dry matter per year, respectively; the values are close the production of nonfertilized uninoculated 'Coastcross-1' and nonfertilized inoculated 'Coastcross-1', obtained in the present study, respectively. Thus, the inoculation, when not associated with N fertilization, is equivalent to the application of more than 100 kg ha^{-1} N, since the total forage yield of 'Coastcross-1' inoculated and without N fertilization was higher than that of the uninoculated and fertilized treatment with 100 kg ha^{-1} N per year.

Regarding the N concentration in 'Coastcross-1' (Table 2), there was an interaction only between N fertilization and cuts (p=0.0405). In the comparison of means, higher values were found in the fourth and seventh cuts, in the grass fertilized with 100 kg ha^{-1} N per year. The lack of inoculation effect on the N concentration was also reported by Lana et al. (2012), who evaluated the N concentration in leaves of maize without N fertilization. However, when 100 kg ha^{-1} N were applied as topdressing, they registered a decline of 6.23% in the N concentration in the leaves of the inoculated plants, in comparison to the uninoculated ones receiving the same N fertilization. In turn, Hungria et al. (2016) evaluated *Brachiaria ruziziensis* fertilized with 40 kg ha^{-1} N, in three different locations, and obtained, in the first year of evaluation, 9.2% mean increases of N concentration in the biomass of plants subjected to inoculation with *A. brasilense*. It is clear that further research is needed to elucidate the inoculation influence of *A. brasilense* on the N concentration in grasses, since the responses vary.

For the accumulation of N in 'Coastcross-1' grass (Table 3), there was an interaction between inoculation, N fertilization and cuts (p<0.0001). Differences were found in all cuts, and also in the total N accumulated by 'Coastcross-1'. The effect of inoculation on the N accumulation in 'Coastcross-1', without N, was evident, with higher values for all evaluations, than in uninoculated 'Coastcross-1' without N fertilizer. When combined with N fertilization, there was also an effect of inoculation, and in six out of seven evaluations, 'Coastcross-1' inoculated and fertilized with 100 kg ha^{-1} N per year showed higher values than grass receiving the same fertilization but without inoculation. For the total accumulated N in the year, the values were higher for grass fertilized with 100 kg ha^{-1} N per year and inoculated, followed by inoculated 'Coastcross-1' without N fertilization.

The total N accumulation by the grass was highest when it was fertilized with 100 kg ha^{-1} N per year and inoculated with *A. brasilense*; however, the increase of N accumulation was mainly due to increase of biomass accumulation (Table 1) and not to the N concentration. Inoculation of 'Coastcross-1' with *A. brasilense*, when grass was not fertilized with N, was equivalent to the

Table 1. Forage yield of 'Coastcross-1' (*Cynodon dactylon*) subjected to inoculation with *Azospirillum brasilense* and N fertilization in seven cuts[(1)].

Treatment	Forage yield (Mg ha^{-1} dry matter)							Total
	1 (Nov. 14)	2 (Jan. 15)	3 (Jan. 15)	4 (Mar. 15)	5 (Apr. 15)	6 (June 15)	7 (Aug. 15)	
0 N	0.5D	0.9C	0.3C	0.6B	0.8B	0.3B	0.5B	3.9D
I-0 N	1.4A	1.6B	0.9B	0.9A	0.9AB	0.7A	1.0A	7.4B
100 N	0.8C	1.6B	1.2A	0.5B	0.8B	0.3B	0.9A	6.1C
I-100 N	1.2B	2.2A	1.1A	0.9A	1.0A	0.7A	0.9A	8.0A
CV (%)	7.3	5.7	4.2	3.9	1.8	7.0	4.2	2.0

[(1)]Means followed by equal letters, in the same column, do not differ by Student's t-test, at 5% probability. 0 N, uninoculated 'Coastcross-1', without N fertilization; I-0 N, inoculated 'Coastcross-1', without N fertilization; 100 N, uninoculated 'Coastcross-1' subjected to 100 kg ha^{-1} N per year; I-100 N, inoculated 'Coastcross-1' subjected to 100 kg ha^{-1} N per year. The rate of 100 kg ha^{-1} N of treatments was split into five applications (20, 20, 30, 20, and 10 kg ha^{-1}). CV, coefficient of variation.

Table 2. Nitrogen concentration in 'Coastcross-1' (*Cynodon dactylon*) subjected to inoculation of *Azospirillum brasilense* and N fertilization in seven cuts[(1)].

N level (kg)	N concentration (%)							Mean
	1 (Nov. 14)	2 (Jan. 15)	3 (Jan. 15)	4 (Mar. 15)	5 (Apr. 15)	6 (June 15)	7 (Aug. 15)	
0	1.9	1.6	2.1	1.8B	2.5	2.8	2.4B	2.2
100	2.1	1.6	2.2	2.0A	2.6	2.8	2.8A	2.3
CV (%)	6.3	2.7	2.7	2.8	3.0	2.9	4.4	

[(1)]Means followed by equal letters, in the same column, do not differ by Student's t-test, at 5% probability. CV, coefficient of variation.

application of more than 100 kg ha^{-1} N per year. In addition to the productive gains, the environmental importance of this response is highlighted, since the reduction of N application through chemical fertilizers decreases the possibility of leaching losses, volatilization of ammonia, and denitrification and, consequently, of environmental pollution (Hungria et al., 2016).

When evaluating the N accumulation in maize plants, Martins et al. (2018) obtained similar values in the comparison between maize without N fertilization inoculated with *A. brasilense*, strains Ab-V5 and Ab-V6, and uninoculated maize receiving 100 kg ha^{-1} N. Our study corroborates the findings of these authors, who show the effect of *A. brasilense* inoculation on plants, when it is not combined with N fertilization. In plants without N fertilization, the authors found 41.1% N increase when seed were inoculated. When they associated the inoculation with the fertilization with 100 kg ha^{-1} N, the increase of N accumulation in the plants was 71.4%, in comparison with the uninoculated plants receiving the same N dose. For 'Coastcross-1', the increase of N accumulation caused by inoculation was 91.4 and 26.3% for plants without N fertilization and receiving 100 kg ha^{-1} N per year, respectively. This is in contrast to the results of Martins et al. (2018), who reported that the effect of inoculation on the N accumulated by plants was more pronounced when inoculation was not associated with N fertilization.

The BNF in 'Coastcross-1' was observed in uninoculated and inoculated plants (Table 3). This is confirmed by the lower-delta $\delta^{15}N$ values in uninoculated (average 8.1‰) 'Coastcross-1' and in inoculated (average 7.3‰) plants, in comparison to reference plants (11.3‰). Differences between delta $\delta^{15}N$ values in uninoculated and inoculated

Table 3. Nitrogen accumulation, natural abundance of ^{15}N ($\delta^{15}N$), percentage contribution of BNF for N nutrition (%Ndfa), and biologically fixed N, in 'Coastcross-1' (*Cynodon dactylon*) subjected to inoculation with *Azospirillum brasilense* and N fertilization in seven cuts[(1)].

Treatment	Cuts							Total
	1 (Nov. 14)	2 (Jan. 15)	3 (Jan. 15)	4 (Mar. 15)	5 (Apr. 15)	6 (June 15)	7 (Aug. 15)	
	N accumulation in 'Coastcross-1' (kg ha^{-1})							
0 N	9.4D	13.3C	6.6D	10.7B	19.5C	9.6C	12.7C	81.8D
I-0 N	28.6A	25.3B	18.9C	17.1A	22.5B	21.4A	22.8B	156.6B
100 N	16.3C	26.5B	26.8A	10.2B	22.5B	7.5D	24.6AB	134.4C
I-100 N	24.1B	33.2A	22.8B	17.8A	25.6A	19.6B	26.7A	169.8A
CV (%)	6.2	2.7	2.0	2.9	3.4	2.5	4.9	3.5
	$\delta^{15}N$ (‰)							Mean
0 N	10.3	6.6	8.0A	9.1A	8.7A	7.0A	7.1	8.1
I-0 N	10.1	6.5	7.0B	8.0B	6.8B	6.0B	6.5	7.3
CV (%)	1.7	2.7	2.4	2.1	2.3	2.7	2.6	
	%Ndfa							Mean
0 N	7.0	41.7	28.9B	19.1B	22.4B	38.0B	37.2B	27.8
I-0 N	10.1	41.8	37.8A	28.5A	39.9A	46.5A	42.1A	35.2
CV (%)	18.9	3.9	4.8	6.8	5.2	3.8	4.1	
	Biologically fixed N (kg ha^{-1})							Total
0 N	0.6B	5.6B	1.9B	2.1B	4.4B	3.7B	4.7B	23.0B
I-0 N	2.8A	10.6A	7.2A	4.8A	8.9A	9.9A	9.6A	53.8A
CV (%)	20.4	4.4	7.7	10.2	5.3	5.2	4.9	3.7

[(1)]Means followed by equal letters, in the same column, do not differ by Student's t-test, at 5% probability. 0 N, uninoculated 'Coastcross-1' without N fertilization; I-0 N, inoculated 'Coastcross-1', without N fertilization; 100 N, uninoculated 'Coastcross-1' plus 100 kg ha^{-1} N per year; I-100 N, inoculated 'Coastcross-1' plus100 kg ha^{-1} N per year. The rate of 100 kg ha^{-1} N of treatments was split into five applications (20, 20, 30, 20, and 10 kg ha^{-1}). CV, coefficient of variation.

'Coastcross-1' were also observed in four of the seven cuts performed.

The %Ndfa was significantly ($p<0.0001$) influenced by the interaction between inoculation and cuts. A difference was detected in five out of seven cuts, with higher values in inoculated 'Coastcross-1', showing the potential of BNF without N fertilization. The superiority for %Ndfa, observed in most evaluations in the inoculated grass, contradicts the assertion made in the report by Hungria et al. (2010), in which the benefits of inoculation with *A. brasilense* strains Ab-V5 and Ab-V6 would not be related to BNF, but mainly to the growth promotion ability. Therefore, the occurrence of BNF by *A. brasilense* was observed when in association with 'Coastcross-1' without N fertilization.

The average value of 27.8% for %Ndfa measured in uninoculated 'Coastcross-1' indicates an important contribution of BFN to pasture in natural conditions, without *A. brasilense* inoculation. This BNF is possibly related to the presence of native diazotrophic bacteria associated with 'Coastcross-1'. Silva et al. (2010) evaluated elephant grass (*Pennisetum purpureum* Schum.) and brachiaria [*Brachiaria decumbens* Stapf. and *B. humidicola* (Rendle) Schweick], and detected the presence of *Azospirillum* spp. in *B. decumbens* and elephant grass, *Herbaspirillum* spp. in the two brachiaria, and *Gluconacetobacter diazotrophicus* in elephant grass. The mean %Ndfa found by these authors was 23.6%, which is close to the mean value found in 'Coastcross-1' uninoculated of the present study. Marques et al. (2017) isolated bacteria of the genera *Azospirillum, Herbaspirillum*, and *Azotobacter* from the rhizosphere and grass roots of native grasses grown in the soil of the same region of the present study. A higher %Ndfa was observed for *Aristida laevis* grass, at approximately 36%, and lower %Ndfa for *Axonopus affinis*, at 22%, values that are close to those found in 'Coastcross-1' inoculated (35.2%) and uninoculated (27.8%) plants, respectively. These results show the occurrence of BNF by bacteria naturally associated with grasses in the soils of the region, and inoculation may contribute to raise these rates.

The biologically fixed N was significantly ($p<0.0001$) influenced by the interaction between inoculation × cuts (Table 3). Inoculation with *A. brasilense* increased the amount of biologically fixed N in 'Coastcross-1' in all cuts and, consequently, it increased the total amount of biologically fixed N by pasture (53.8 vs 23.0 kg ha^{-1} N). Then, the gain in the inoculated 'Coastcross-1' was 30.8 kg, which represented 133.9% increase in comparison to those of uninoculated grass. These results confirm that the inoculation increased the BNF, and that, despite a possible competition with native bacteria, the inoculation with *A. brasilense* was effective.

The highest-N accumulation from BNF in inoculated 'Coastcross-1' is due to the sum of the effects of a higher production of 'Coastcross-1' biomass (Table 1) and to the highest %Ndfa (Table 3) found in most of the cuts. Besides BNF, the increase of N accumulation in the inoculated plants may be associated with the performance of bacteria as plant growth promoters. According to Hungria et al. (2010), *A. brasilense* stimulate the plant root development due to phytohormones, allowing of a greater absorption of nutrients and water. This results in more productive plants (Table 1) and higher accumulation of N from the soil, which explains the additional 44 kg N accumulated in inoculated 'Coastcross-1' that did not come from the BNF.

Based on the atom ^{15}N excess (%), which did not differ between uninoculated and inoculated treatments, the total N derived from the labeled fertilizer (Ndff) was calculated (Table 4). There was no interaction of inoculation × cuts ($p=0.0793$), but a single effect only of inoculation on total Ndff ($p=0.0411$). The total urea-N recovered by 'Coastcross-1' was higher in the inoculated plants. The values for both inoculated and uninoculated 'Coastcross-1' are higher than those observed by Martha Júnior et al. (2009) in *Panicum maximum* 'Tanzania', under different rates of N fertilization (40, 80, and 120 kg ha^{-1}), recovered in the upper layer of the pasture (above 40 cm), of 1.2, 3.0, and 4.7 kg ha^{-1}, respectively. In addition to the effect of inoculation, the split urea-N application may have contributed to increase the recovery, in comparison with that observed by Martha Júnior et al. (2009), since these authors did not split the N application.

For the percentage recovery of applied labeled fertilizer ($R^{15}N$), the interaction inoculation versus cuts was significant ($p=0.0080$). There was a significant difference for four out of seven evaluations, with higher values for the inoculated 'Coastcross-1'. The higher recovery of urea-N is correlated with a higher-forage yield (Table 1) ($r=0.72353$, $p<0.0001$). According to Hungria et al. (2010, 2016) and Moreira et al. (2010), when associated with grasses, *A. brasilense*, especially Ab-V5 and Ab-V6 strains (Hungria et al., 2010), act as plant growth-promoting bacteria. This fact can explain the higher productivity of inoculated grasses in the present work, which is possibly due to a greater root development that resulted in a higher recovery of the applied N at four of the harvests. One of the main causes for the low $R^{15}N$ may have been the loss of N from ammonia volatilization (NH_3) with the use of urea. Cantarella et al. (2001) measured NH_3 volatilization, after applying 100 kg ha^{-1} urea-N to 'Coastcross-1', and found 27% loss of applied N. Importantly, this -loss value may be underestimated due to the method used to measure volatilized NH_3 (Miola et al., 2014).

By the apparent recovery method (NAR), the recovery of urea-N applied in 'Coastcross-1', showed a positive effect of inoculation ($p=0.0016$) on the inoculated 'Coastcross-1', with 57.3% of urea-N recover, whereas uninoculated plants recovered only 41.2% urea-N. The apparent urea-N recovery values were 200.7 and 245.2% higher than those obtained by the isotopic method - total Ndff (Table 3), for uninoculated and inoculated plants, respectively. According to Harmsen & Garabet (2003), when there are high-NAR values, $R^{15}N$ tended to be lower than NAR because of the occurrence of mineralization-immobilization turnover, which decreased the ^{15}N content of the mineral N pool; this phenomenon, referred to as "pool substitution", may be enhanced by an increased uptake efficiency of soil N by fertilized crops, or by an increased mineralization in fertilized treatments.

The highest value for apparently recovered urea-N in inoculated plants reinforces the assertion that the association with *A. brasilense* increases the N uptake from N-fertilizers. The value obtained for uninoculated 'Coastcross-1', 41.2 kg ha^{-1} N per year, is similar to that estimated by Primavesi et al. (2004), who evaluated 'Coastcross-1' under increasing N doses. Based on the regression analysis, these authors reached 44.5 kg ha^{-1} N apparently recovered by the plants at 100 kg N ha^{-1} N per year as urea. Observing the great difference between the isotope recovery method ($R^{15}N$) and the apparent one (NAR), it can be stated that the calculation by the apparent method overestimated the recovery of the applied N fertilizer. According to Martha Júnior et al. (2009), this is due to the fact that the apparent N fertilizer recovery method assumes that the soil-N transformations and

Table 4. Atom ^{15}N excess, recovery of urea-^{15}N ($R^{15}N$), and N derived from labeled fertilizer (Ndff), in 'Coastcross-1' (*Cynodon dactylon*) subjected to inoculation of *Azospirillum brasilense* and N fertilization in seven cuts[(1)].

Treatment	Cuts							Mean
	1 (Nov. 14)	2 (Jan. 15)	3 (Jan. 15)	4 (Mar. 15)	5 (Apr. 15)	6 (June 15)	7 (Aug. 15)	
				Atom ^{15}N excess (%)				
100 N	0.1269	0.0681	0.1817	0.1508	0.0833	0.0274	0.0363	0.0964
I-100 N	0.1156	0.0588	0.1891	0.1629	0.1163	0.0292	0.0361	0.1011
CV (%)	13.9	14.7	8.4	15.4	13.0	10.2	6.1	
				Ndff (kg ha^{-1})				Total
100 N	2.5	1.8	4.9	1.5	1.9	0.2	0.9	13.7B
I-100 N	2.8	2.0	4.3	2.9	3.0	0.6	1.0	16.6A
CV (%)	6.6	2.7	4.8	6.0	4.3	10.0	7.5	7.8
				$R^{15}N$ (%)				Mean
100 N	12.4B	4.8	7.4	1.9	2.1B	0.2B	1.0B	4.3
I-100 N	14.0A	5.3	6.6	3.7	3.4A	0.7A	1.2A	5.0
CV (%)	5.1	4.3	8.9	5.4	6.0	9.5	6.1	

[(1)]Means followed by equal letters, in the same column, do not differ by Student's t-test, at 5% probability. 100 N, uninoculated 'Coastcross-1' plus 100 kg ha^{-1} N per year; I-100N, inoculated 'Coastcross-1' plus 100 kg ha^{-1} N per year. The rate of 100 kg ha^{-1} N of treatments was split into five applications (20, 20, 30, 20, and 10 kg ha^{-1}). CV, coefficient of variation.

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the physiological plant processes are similar both in the presence and absence of N fertilization, which does not occur in the reality. This way, the use of the isotopic method is of utmost importance to estimate the real recovery of the applied N.

Conclusions

1. The inoculation of *A. brasilense* strains Ab-V5 and Ab-V6 in 'Coastcross-1' pasture, upon planting, increases the biomass production and nitrogen accumulated in the plants.
2. Biological nitrogen fixation (BNF) in 'Coastcross-1' occurs possibly by native bacterial occurrence, which increases with the inoculation with *A. brasilense*.
3. The inoculation of *A. brasilense* improves the urea-N recovery by 'Coastcross-1'.

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