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

Rubson da C. Leite[A,D], *José G. D. dos Santos*[A], *Eduardo L. Silva*[A], *Cássio R. C. R. Alves*[A], *Mariangela Hungria*[B], *Robson da C. Leite*[C], and *Antonio C. dos Santos*[A]

[A]Federal University of Tocantins, Araguaína, TO, Brazil 65907-230.
[B]Brazilian Agricultural Research Corporation, Embrapa Soja, Londrina, PR, Brazil 86001-970.
[C]Federal University of Tocantins, Gurupi, TO, Brazil 77402-970.
[D]Corresponding author. Email: rubsonif@gmail.com

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.

Additional keywords: growth-promoting bacteria, nitrogen fixation, palisade grass, tropical pastures.

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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 (de 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 and 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 (de 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.

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 240 m 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 1863 mm 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).

The experiment was established in a randomised 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 20 m from each other), with four replicates. Each experimental plot had an area of 9.0 m^2 (3.0 m 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 and 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 2×10^8 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).

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

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

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

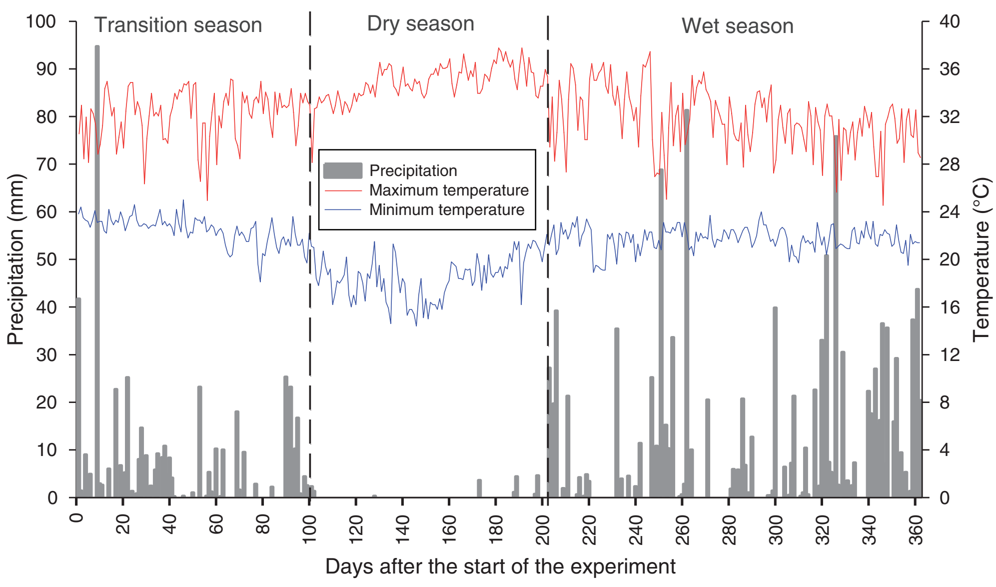

Fig. 1. Precipitation and maximum and minimum temperatures of the experimental area during the the experiment in Araguaína, TO, Brazil, 2017.

The parameters 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 1.0 m by 0.15 m 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°C for determination of dry root mass, in kg/ha. Annual forage accumulation was evaluated with a 1.0 m by 0.5 m metal frame, with a cut height of the residue of 20 cm, followed by drying in an oven at 55°C 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 analysed 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).

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. 2*a*). 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 favoured 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. 2*a*).

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. 2*b*).

Plant height was positively influenced by inoculation in all evaluated periods (Fig. 2*c*). 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. 2*c*).

At the start of the dry season the root mass of inoculated plants was 27% higher than of non-inoculated plants (Fig. 2*d*). 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. 2*e*). In the dry season, inoculated and non-inoculated plants were similar, whereas in the wet season, the inoculated plants had lower N concentration (Fig. 2*e*).

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

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),

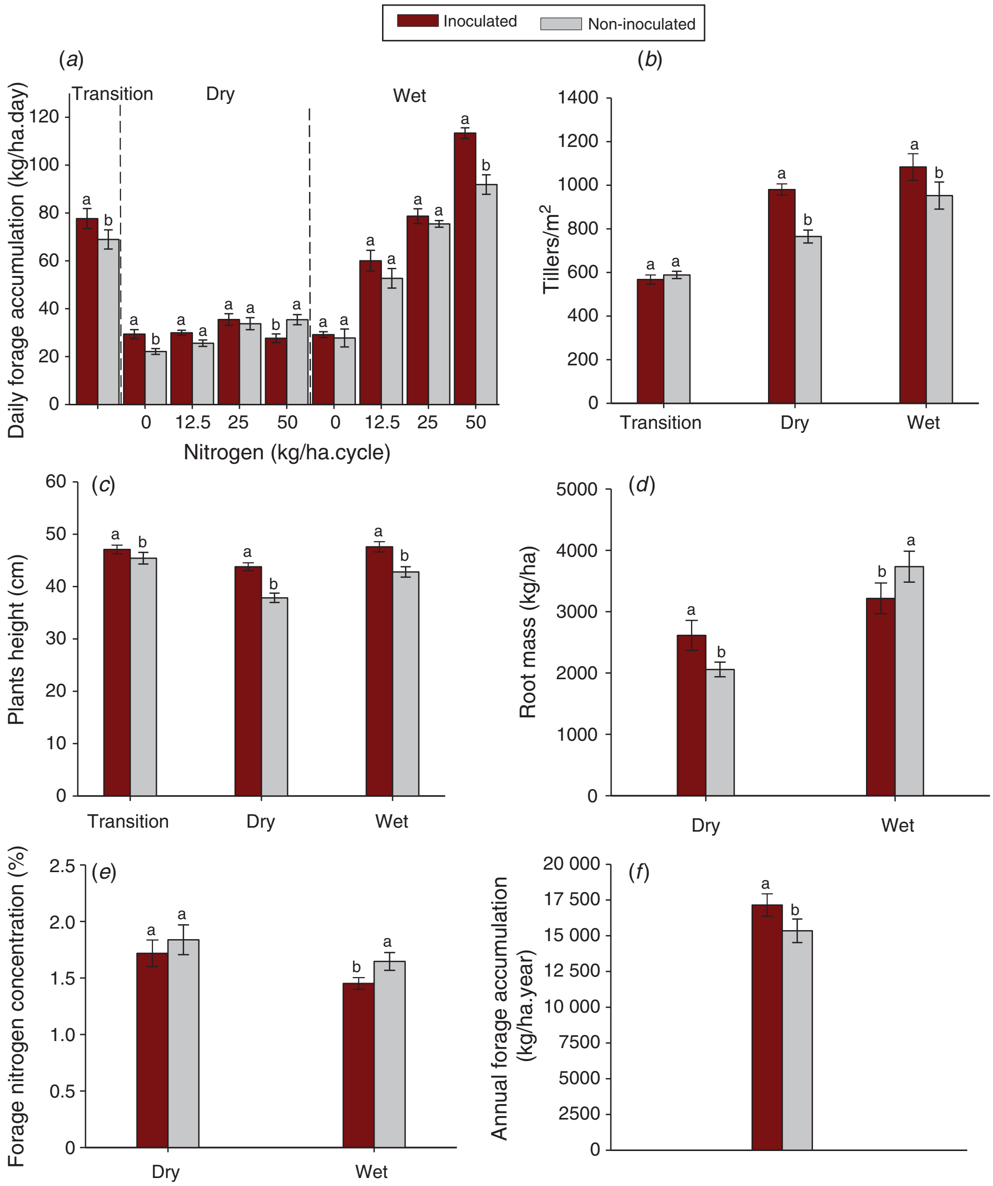

Fig. 2. (*a*) Daily forage accumulation, (*b*) number of tillers, (*c*) plant height, (*d*) root mass, (*e*) forage nitrogen concentration, and (*f*) annual forage accumulation of *Urochloa brizantha* cv. Marandu in relation to inoculation with *Azospirillum brasilense* and nitrogen fertilisation (0, 12.5, 25 and 50 kg/ha). For each variable, period or N treatment, means of inoculation treatments with the same letter are not significantly different by the Tukey test at $P = 0.05$.

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

Table 2. Relationships between measured parameters and nitrogen application rate (kg/ha.N cycle) for daily forage accumulation, number of tillers per m^2, plant height, forage nitrogen concentration and annual forage accumulation of Marandu grass
*$P < 0.05$; **$P < 0.01$; relationships that were not significant at $P = 0.05$ are not presented

Variable	Period	Inoculation	Regression model	Equation	Coefficient of determination
Daily forage accumulation (kg/ha.day)	Transition	Yes	Linear**	$61.06 + 0.75x$	0.98
	Dry	No	Linear*	$23.19 + 0.27x$	0.85
	Wet	Yes	Linear**	$34.59 + 1.63x$	0.98
		No	Linear*	$34.47 + 1.25x$	0.92
No. of tillers per m^2	Dry	No	Linear*	$655.60 + 4.98x$	0.92
	Wet	Yes	Linear**	$758.84 + 14.83x$	0.97
		No	Linear**	$641.00 + 14.23x$	0.99
Plant height (cm)	Transition	Yes	Linear*	$43.81 + 0.14x$	0.91
	Dry	No	Linear*	$35.17 + 0.12x$	0.92
	Wet	Yes	Linear*	$44.03 + 0.16x$	0.94
		No	Linear**	$38.81 + 0.18x$	0.98
Forage nitrogen concentration (%)	Dry	Yes	Linear*	$1.29 + 0.0073x$	0.90
		No	Linear**	$1.37 + 0.012x$	0.97
	Wet	Yes	Linear*	$1.32 + 0.017x$	0.81
		No	Linear*	$1.45 + 0.017x$	0.95
Annual forage accumulation (kg/ha.year)		Yes	Linear**	$10\,377 + 309x$	0.98
		No	Linear*	$9584 + 263x$	0.92

plants, but with an even higher maximum yield of 26 t/ha, an increase of 10 t/ha relative to the treatment without N.

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 and 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 and 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 minimise 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 characterised 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 minimise 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 and 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. 2*f*), 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 and 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.

Conflict of interests

The authors declare no conflicts of interest.

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