YIELD, YIELD COMPONENTS AND NUTRIENTS UPTAKE IN ZURI GUINEA GRASS INOCULATED WITH PLANT GROWTH-PROMOTING BACTERIA

Gilmar Cotrin de Lima[1]; Mariangela Hungria[2]; Marco Antonio Nogueira[2]; Marcelo Carvalho Minhoto Teixeira Filho[3]; Adônis Moreira[2]; Reges Heinrichs[4]; Cecílio Viega Soares Filho[1*]

[1]São Paulo State University (UNESP), Araçatuba, SP, Brazil;

[2]Embrapa-Soja, CNPSo, Londrina, PR, Brazil;

[3]São Paulo State University (UNESP), Ilha Solteira, SP. Brazil;

[4]São Paulo State University (UNESP), Dracena, SP. Brazil.

Correspondence author: Cecílio Viega Soares Filho[1] (*E-mail:cecilio.soares-filho@unesp.br)

Abstract

The objective of this study was to evaluate the effects of strains of Azospirillum brasilense, Pseudomonas fluorescens and Rhizobium tropici on biomass yield and nutrients uptake of shoots and roots of Megathyrsus (syn. Panicum) maximus cultivar BRS Zuri (Zuri Guinea grass) inoculated with plant growth-promoting bacteria (PGPB). Treatments consisted of inoculation and re-inoculation with A. brasilense strains Ab-V5 and Ab-V6, P. fluorescens strain CCTB 03 and of co-inoculation with R. tropici strain CIAT 899 + A. brasilense Ab-V6, with or without N-fertilizer (100 mg dm^{-3}). Evaluations were performed on three cuts for the determination of root and shoot dry weight yield, morphological compositions, tiller mass, number of tillers, and nutrient uptake. Inoculation with bacteria in association with N-fertilizer increased N, NH_4^+, Ca, Fe, Mn and Zn accumulation in shoots and P and K uptake in roots. P. fluorescens and co-inoculation with R. tropici CIAT 899 + A. brasilense Ab-V6 increased the relative chlorophyll index in relation to the non-inoculated control. As expected, PGPB were not able to fully replace N-fertilization. However, when combined with N-fertilizer, the PGPB increased yield, the relative chlorophyll index, and the uptake of N, NH_4^+, Ca, Zn, Mn and Fe of Zuri Guinea grass. The results indicate that PGPB can represent a sustainable alternative for reducing the use of N-fertilizers. There were no effects of re-inoculation with PGPB on the nutrition or yield of Zuri Guinea grass, demonstrating that the determination of the method of application and periodicity of inoculation still require investigation.

Keywords: diazotrophic bacteria, inoculant, nitrogen, biological nitrogen fixation, tropical forage grass,

1.INTRODUCTION

The areas devoted to pasture cultivation in Brazil have increased over the last few decades, given that they constitute the basis for ruminant production in the country [1]. Brazil has 160 million hectares of pasture, under different edaphoclimatic conditions. The area supports 226 million head of cattle, representing 33% of the global number of heads, occupying the second position after India [2]. In the genus

Megathyrsus (syn. *Panicum*), the *M. maximus* species has been broadly cultivated in both tropical and subtropical regions, mainly due to its tolerance and adaptability to diverse edaphoclimatic conditions [3]. The Zuri Guinea grass (*M. maximus* cv. BRS Zuri) is one of the most important cultivars because of its agronomic and nutritional qualities. In addition to a rapid growth and high biomass yield, this forage grass uses its extensive root system to regrow over successive cycles.

Nitrogen (N) is often a limiting factor in plant growth and yield, especially in tropical forage grasses [4]. Fertilization represents an alternative to potentially reduce seasonal variations in warm-season grass quantities and may increase their quality; however, commercial fertilizers are the costliest input for warm-season grass forage yields. Fertilizer costs, with an emphasis on N-fertilizers, have increased over the last few decades, mainly in response to the increased costs of fossil fuels. Nitrogen is routinely the first nutrient applied to warm-season grass pastures because of its effect on forage production and its nutritional value. However, repeated fertilizations or high amounts of N alone may cause nutrient unbalances in soil and can ultimately have negative effects on forage production and on the nutritional value [5]. In addition, growing concerns about the development of more sustainable and less polluting agriculture have led to the search for alternatives to reduce the environmental impact of mineral fertilizers [6] without causing losses in productivity [7]; [8].

In this sense, the beneficial use of bacterial inoculants stands out as a viable alternative, especially under conditions of low soil fertility [9]; [10]; [11]; [12]. Some bacteria, known as plant growth-promoting bacteria (PGPB), can highly contribute to plant growth, by means of several processes, that can act in a single, cumulative or cascading manner [13], including biological nitrogen fixation [14], increased nutrient and water uptake [15]; the production and secretion of phytohormones and other signaling molecules, such as auxins [16], cytokinins [17], gibberellins [18] and salicylic acid [19]; [20]; phosphate solubilization [21], among others.

Priority should be given to the use of alternative strategies that promote improvements in animal production, especially management strategies that associate sustainability with profitability. Thus, the use of PGPB in forage grasses may represent an important management alternative for improved pasture production and quality, consequently, animal production. The objective of this study was to evaluate the effects of different species and inoculation procedures with *Azospirillum brasilense, Pseudomona fluorescens* and *Rhizobium tropici* previously identified as elite PGPB in other crops [22]; [23]; [10]; [12]; [24]; [25], on the nutrient uptake of shoots and roots and the shoot and root dry weight yields of Zuri Guinea grasses.

2. MATERIALS AND METHODS

Growth conditions and experimental design

The experiments were conducted with forage species *Megathyrsus* (syn. *Panicum) maximus* cv. BRS Zuri during spring and summer (November to March of 2017/2018) under greenhouse conditions (average temperature of 22°C and photoperiod of 14/10 h, day/night) in 8-L plastic pots, at São Paulo State University (UNESP) in Araçatuba County, São Paulo State, Brazil (21°8' LS, 50°25' LW, 415 m.

The pots were filled with ultisol [26] collected at a depth of 0-0.2 m with the following chemical

attributes: 23 mg dm^{-3} P (resin); 26 g dm^{-3} O.M.; 5.2 pH ($CaCl_2$); K = 2.9 $mmol_c$ dm^{-3}; Ca = 25 $mmol_c$ dm^{-3}; Mg = 17 $mmol_c$ dm^{-3}; H + Al = 28 $mmol_c$ dm^{-3}; base sum (SB) = 44.9 $mmol_c$ dm^{-3}; cation exchange capacity (CEC) = 72.9 $mmol_c$ dm^{-3}; base saturation (V) = 62% according to [27]. Using an NFb (N-free broth) culture medium in a semi-solid form we estimated the total population of diazotrophic microorganisms in the soil to be $9.5x10^4$ bacteria g^{-1} of soil by the technique of the largest probable number, according to [28]; [29].

The experimental design was a randomized complete block design with five replicates with repeated measures over time (three growth cycles). The main plots consisted of different treatments. The treatments were determined based on the inoculation of plant growth promoting bacterial (PGPB) strains, including (1) *Azospirillum brasilense* strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084), (2) *Pseudomonas fluorescens* CCTB 03 (=CNPSo 2719) and (3) co-inoculation with *Rhizobium tropici* CIAT 899 (=CNPSo 103, =SEMIA 4077), and *Azospirillum brasilense* Ab-V6, each with or without the application of N. All strains result from selection programs performed in Brazil and are used in commercial inoculants. *A. brasilense* Ab-V5 and Ab-V6 are used as inoculant for maize (*Zea mays* L.) [10], wheat (*Triticum aestivum* L.) [10], *Brachiaria* (*Urochloa* spp.) [24] and co-inoculation of soybean (*Glycine max*) [12] and common bean (*Phaseolus vulgaris* L.) [12]; *P. fluorescens* is used in maize [25], *R. tropici* in common bean [12]. In addition to the three treatments, we evaluated the effect of re-inoculation after each round of cutting, as well as two control treatments, one without inoculation and with the application of N (positive control) and one without N fertilization and without inoculation (negative control), totaling eleven treatments. All strains used are deposited in the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054). The inoculants were produced at the Laboratory of Soil Biotechnology of Embrapa Soja (Londrina, Paraná State, Brazil). at sowing. *A. brasilense* was prepared in DYGS medium [30], *P. fluorescens* in TSB medium [29], while *R. tropici* inoculum was produced in YM medium [29]. At sowing, the concentration of each bacterial inoculant was adjusted to 2 x 10^8 cells per mL. The soil from each pot following nutrient addition consisted of the following: $Ca(H_2PO_4)_2$, 200 mg dm^{-3} P; K_2SO_4, 150 mg dm^{-3} K and 61.53 mg dm^{-3} S; H_3BO_3, 0.5 mg dm^{-3} B; $CuSO_4$ 1.0 mg dm^{-3} Cu; H_2MoO_4, 0.1 mg dm^{-3} Mo; $MnSO_4$, 5 mg dm^{-3} Mn; $ZnSO_4$, 2.0 mg dm^{-3} Zn. After four days, the Zuri Guinea grass was sowed.

Fifteen mL of each inoculant (2 x 10^9 UFC mL) were used for each kg of seed before, resulting in the supply of 3 x 10^9 CFU kg^{-1} of seed, as recommended for brachiarias [24]. Considering that 1 g of seeds corresponds to approximately 660 seeds, the concentration of bacteria was of about 4.5 x 10^3 cells $seed^{-1}$. Seeds were soaked with the inoculants for 1 h, then dried for approximately 30 min in a cool and sun-sheltered location, after which they were seeded at 15 seeds per pot (experimental units). This is the usual inoculation procedure adopted by the farmers for all crops and pastures. According to the Brazilian legislation, experiments aiming at identifying elite microbial strains must include two non-inoculated controls, with and without chemical fertilizers. Therefore, these two controls were included, in our case with and without N-fertilizers [10]; [12]; [24].

The plants were thinned when they presented three fully expanded leaves, with five uniform plants maintained per pot. Only one inoculant treatments strains were reinoculated by spraying a known volume (300 mL) after the first and second cuts, at which time the leaves began to develop again. The same

concentration of 3 x 10^9 CFU $plant^{-1}$ was diluted to complete 300 mL with distilled water for spraying, that was performed directly onto the plant leaves. Re-inoculation was applied by foliar application because when the pasture grows, it covers completely the soil, and the only way of reintroducing the strains is by foliar spray. N-fertilization occurred only one via a solution from a graduated pipette four days before the forage was sown, for a total of 100 mg dm^{-3} of N (NH_4NO_3).

Plant harvest and measurements of productive and nutritional parameters

Two weeks after the emergence of Zuri Guinea grass, thinning was performed to keep five uniform plants per pot. Deionized water was used for irrigation. Evaluations were performed when the plants reached an average height of 0.6 m (four-week intervals), when shoots were harvested down to 0.1 m above the surface of the ground. Three growth cycles with four-week intervals were evaluated. After each harvest, the shoots were identified, weighed and oven dried at approximately 65°C until they reached a constant mass. Shoots were subsequently weighed on a precision balance to quantify the shoot dry weight yield (SDWY). After drying, the samples were ground to pass a 1 mm screen in a Wiley type mill and the foliar nutrient concentrations (N, P, K, Ca, Mg, S, B, Fe, Mn and Zn) were determined according to [31].

One day before each cutting took place, plant height readings were taken with a millimeter ruler. Relative chlorophyll indexes (RCI) were taken using a digital chlorophyllometer (Clorofilog). Plant height and RCI readings were carried out on two blades of newly expanded leaves from the five plants in each pot. The number of tillers per pot was also counted. The collected plant material was first separated into tillers and main plants, and later the tiller mass per pot was determined. The material was then collected, and a second separation was performed on the grass leaves and stems to determine the mass of each component.

The roots were collected at the end of the experiment and washed in running water using 2 mm mesh sieves until all soil was removed. To determine the root dry weight yield (RDWY) the samples were properly identified, bagged, and the material was dried as described above. After drying in forced ventilation at approximately 65°C to a constant mass, all root material collected was weighed on a precision balance to quantify the RDWY. The samples were ground to pass a 1 mm screen in a Wiley-type mill to determine the nutrient concentrations (N, P and K) in the roots according to [31].

Statistical analysis

For each response variable, the following linear mixed model was fitted to the data:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{e}$$

where **y** is the *r* x 1 vector of records for the response variable, **b** is the *p* x 1 vector of unobserved fixed effects, **X** is a *r* x *p* design matrix relating observations in **y** to fixed effects in **b**, **u** is the *n* x 1 vector of unobserved random effects, **Z** is a *r* x *n* incidence matrix relating observations in **y** to random effects in **u**, and **e** is the *r* x 1 vector of random residual effects. Fixed effects included the overall mean (i.e., intercept), cutting order, treatment and block. Random effects accounted for repeated measurements taken from the same plot, and were assumed $\mathbf{u} \sim MVN(\mathbf{0}, \mathbf{I}s^2_u)$ where s^2_u is the variance component attributed to the plots.

Residual effects were assumed $\mathbf{e} \sim MVN(\mathbf{0}, \mathbf{I}s^2_e)$ where s^2_e is the residual variance. The model was fitted with the *hglm* v2.2-0 package [32] in *R* v.3.5.3 [33]. All treatments were contrasted with the negative control, and significant differences were evaluated with a Wald t-test considering a = 0.05. The results were summarized using marginal means (i.e., average within a level of a factor corrected for all remaining effects in the model, analogous to least squares means in fixed effects regression), which were computed from the linear combination **Lb**, where **L** is a matrix containing contrasts between levels of a tested factor (e.g., treatment) and average values for levels of other factors (e.g., cutting and block). Standard errors for marginal means were computed as the square root of the diagonal elements of $\mathbf{L}(\mathbf{X}^T\mathbf{V}^{-1}\mathbf{X})^{-1}\mathbf{L}^T$ for $\mathbf{V} = \mathbf{I}s^2_u + \mathbf{I}s^2_e$.

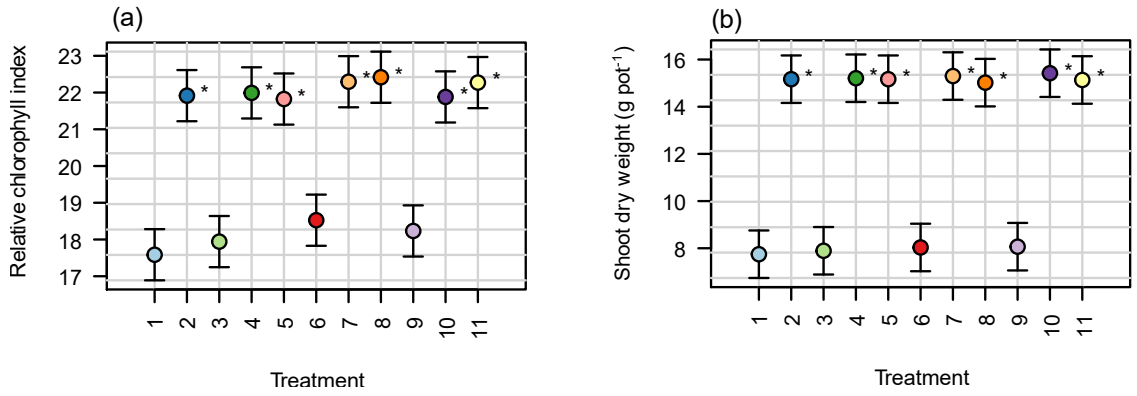
The data of shoot accumulation, roots dry weight, N root uptake, P root uptake and K root uptake were assessed using analysis of variance (ANOVA) with the F test ($p \leq 0.05$) and compared using the Scott-Knott test with a 5% probability.

3. RESULTS

It is worth mentioning that all inoculated treatments receiving the same amount of N-fertilizer than the positive control, as rhizospheric diazotrophic bacteria cannot supply all plant´s N demands, while the negative control is represented by non-inoculated non-fertilized plants.

Shoot and roots dry weight yields

In the analysis of variance for shoot dry weight yields (SDWY), and the SDWY accumulation, root dry weight yields (RDWY), relative chlorophyll index (RCI), tillers units and tillers dry mass was highly significant, indicating higher yields in the treatments receiving N-fertilizer ($p \leq 0.05$) (Figure 1 and Table 1). The values of SDWY ranged from 23.2 to 46.3 g pot-1, RDWY from 6.6 to 9.6 g pot-1, for the RCI of the Zuri Guinea grass, values ranged from 17.6 to 22.9, tillers units from 8.0 to 14.1 and tillers dry mass from 3.0 to 10.4 g pot-1. None of the inoculated treatments differed statistically from the positive control receiving N-fertilizer, and three of them had decreased SDWY (Table 1).



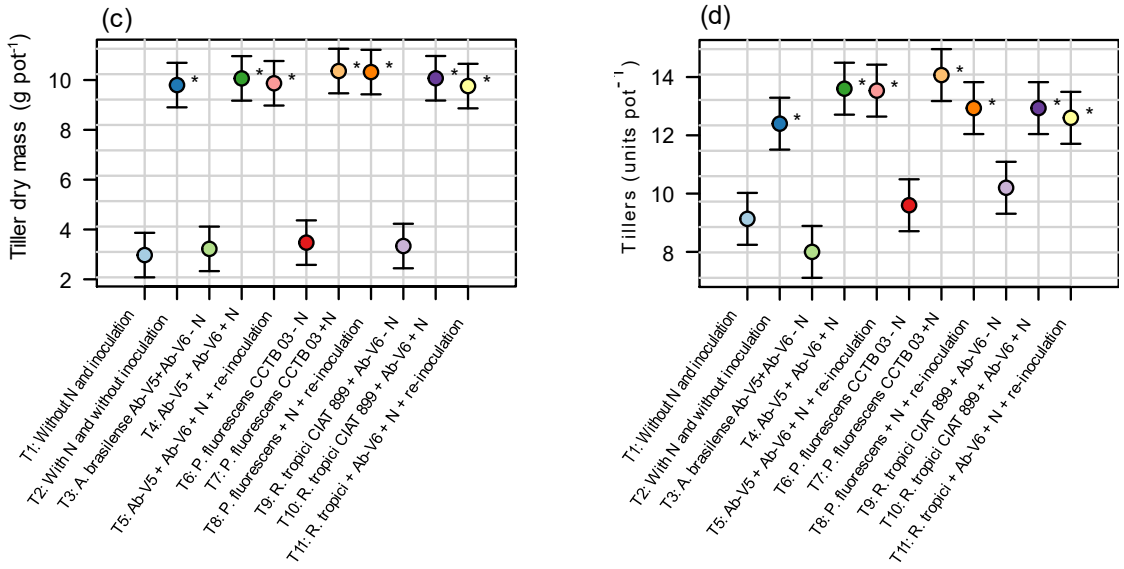


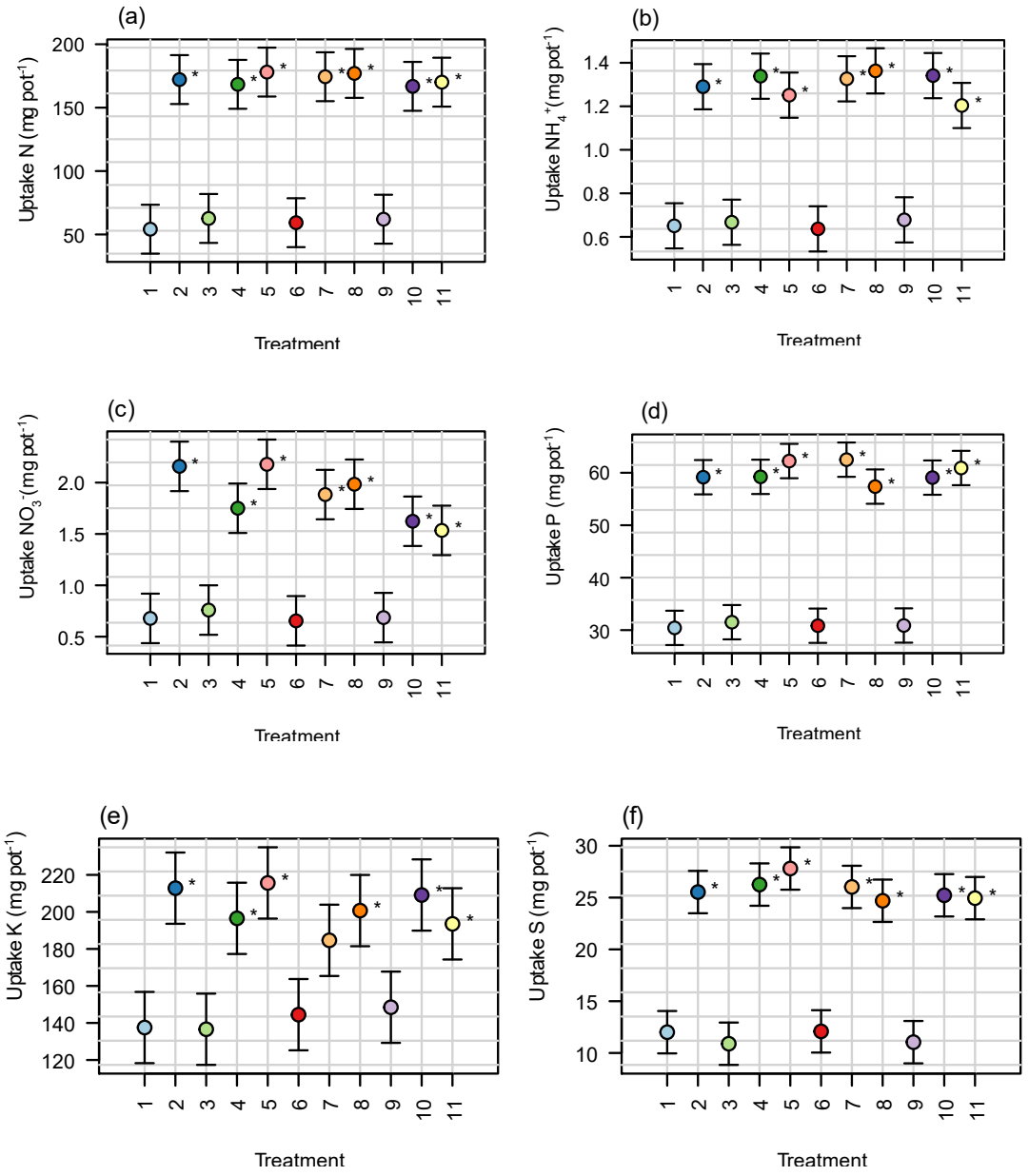
Figure 1. Relative chlorophyll index (a), shoot dry weight yield (g pot^{-1}) (b), tiller dry mass (g pot^{-1}) (c), number of tillers (units) (d) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5+Ab-V6 – N, T4= *A. brasilense* Ab-V5+Ab-V6 + N, T5= *A. brasilense* Ab-V5+Ab-V6 + N + re-inoculation, T6= *P. fluorescens* CCTB 03 – N, T7= *P. fluorescens* CCTB 03 + N, T8= *P. fluorescens* CCTB 03 + N + re-inoculation, T9= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 – N, T10= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 + N and T11= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 + N + re-inoculation. Per-treatment marginal means estimated from mixed models for all response variables investigated in the present study. Error bars represent the standard errors of the means. All marginal means followed by asterisks (*) differed significantly ($p < 0.05$) from the negative control (treatment 1).

Table 1 Shoot dry weight yield (SDWY) accumulation (g pot^{-1}) and root dry weight yield (RDWY) (g pot^{-1}) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici*.

Treatments	SDWY accumulation (g pot^{-1})	RDWY (g pot^{-1})
Negative control (without N and inoculation)	23.24 b	7.03c
Positive control (with N and without inoculation)	45.50 a	9.55 a
A. brasilense Ab-V5+Ab-V6 - N	23.68 b	6.77 c
A. brasilense Ab-V5+Ab-V6 + N	45.62 a	9.18 a
A. brasilense Ab-V5+Ab-V6 + N + re-inoculation	45.50 a	8.37 a
P. fluorescens CCTB 03 - N	24.10 b	7.23 c
P. fluorescens CCTB 03 + N	45.90 a	7.90 b
P. fluorescens CCTB 03 + N + re-inoculation	45.06 a	9.11 a
R. tropici CIAT899 + *A. brasilense* Ab-V6 - N	24.20 b	6.64 c
R. tropici CIAT899 + *A. brasilense* Ab-V6 + N	46.26 a	7.99 b
R. tropici CIAT899 + *A. brasilense* Ab-V6 + N + re-inoculation	45.40 a	9.47 a
P value	<0.01	<0.01

Means followed by lowercase letters differ for treatments as determined by the Scott-Knott test (P <0.05).

Statistically significance differences in the -ammonium, nitrate, P, K, S, Ca and Mg accumulations in the shoots of Zuri Guinea grass were observed in the experiment (Figure 2).



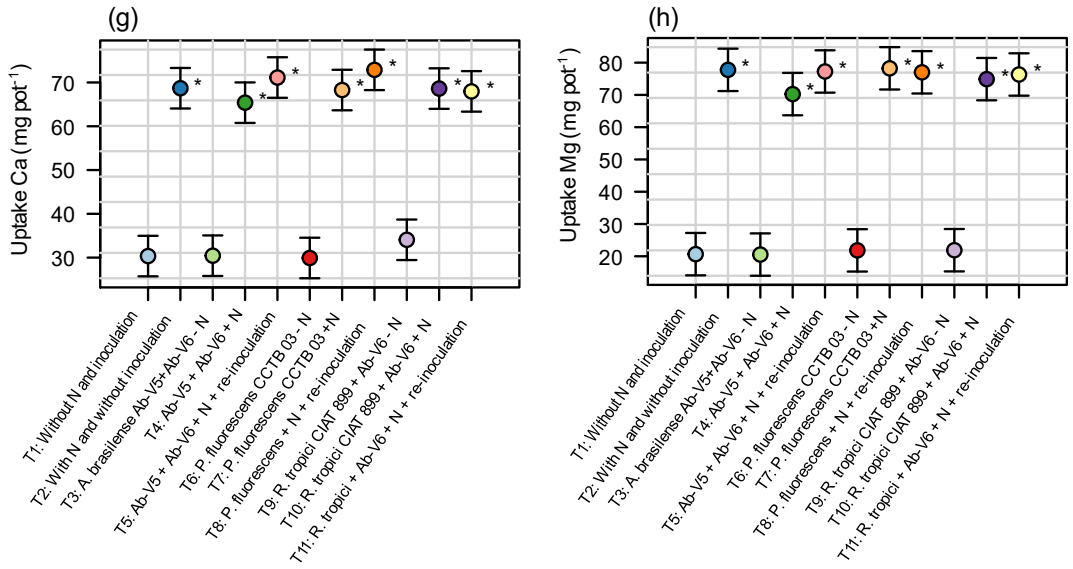


Figure 2. Uptake N (mg pot^{-1}) (a), uptake NH_4^+ (mg pot^{-1}) (b), uptake NO_3^- (mg pot^{-1}) (c), uptake P (mg pot^{-1}) (d), uptake K (mg pot^{-1}) (e), uptake S (mg pot^{-1}) (f), uptake Ca (mg pot^{-1}) (g), uptake Mg (mg pot^{-1}) (h) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5+Ab-V6 – N, T4= *A. brasilense* Ab-V5+Ab-V6 + N, T5= *A. brasilense* Ab-V5+Ab-V6 + N + re-inoculation, T6= *P. fluorescens* CCTB 03 – N, T7= *P. fluorescens* CCTB 03 + N, T8= *P. fluorescens* CCTB 03 + N + re-inoculation, T9= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 – N, T10= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 + N and T11= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 + N + re-inoculation. Per-treatment marginal means estimated from mixed models for all response variables investigated in the present study. Error bars represent the standard errors of the means. All marginal means followed by asterisks (*) differed significantly ($p < 0.05$) from the negative control (treatment 1).

Plants inoculated with *A. brasilense* Ab-V5 + Ab-V6 and *P. fluorescens* CCTB 03 at sowing and then reinoculated after the first and second cuttings had the best performance in terms of N accumulation, with 418 and 416 mg pot^{-1} of N, respectively. Additionally, plants that were inoculated with *A. brasilense* Ab-V5 + Ab-V6 together with N fertilization accumulated 2.72 mg pot^{-1} of NH_4^+, and for both variables, N fertilization together with inoculation was statistically higher than the the positive control (2.44 mg pot^{-1}). Although not significantly different from the negative control treatment, the plants inoculated with *A. brasilense* Ab-V5 + Ab-V6, *P. fluorescens* CCTB 03 or *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 had 17.2%, 9.7% and 13.9% increased N accumulation, respectively (Figure 2).

In relation to the NO_3^- accumulations, plants fertilized with N and inoculated with *A. brasilense* Ab-V5 + Ab-V6 at sowing and re-inoculated after the first and second cuttings presented the highest value, of 5.06 mg pot^{-1}, however, not differing statistically from the positive control. The unfertilized treatments were statistically lower than the other treatments

The accumulations of P, K, S, Ca and Mg in the shoots of the Zuri Guinea grass in all inoculated treatments receiving N-fertilizer, as well as in the positive control were statistically higher to those not

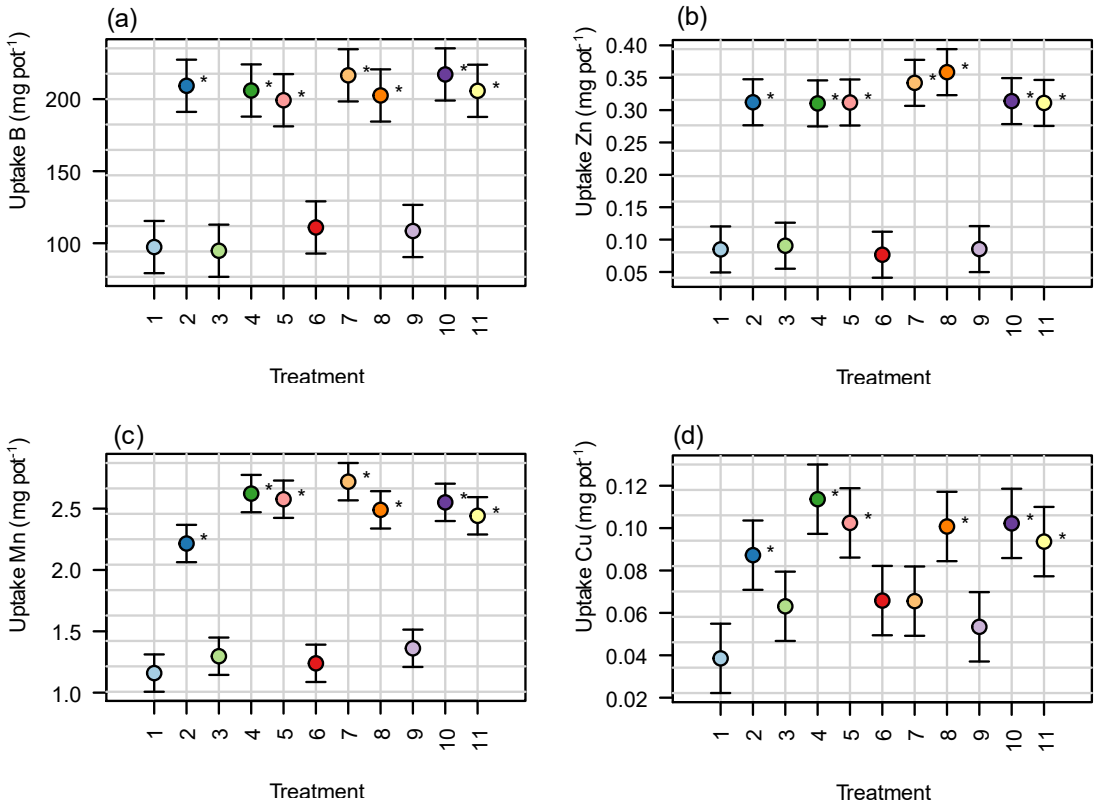
fertilized and similar to each other (Figure 2).

For the K accumulations in the shoots again the control without N-fertilizer was statistically lower. The positive control and plants inoculated with *A. brasilense* with N presented the highest accumulation of K ($p \leq 0.05$), with 480 and 490 mg pot^{-1}, respectively (Figure 2).

The accumulation of Ca was higher in the treatment re-inoculated with strains of *P. fluorescens* CCTB 03 after the first and second cuttings, of 70 mg pot^{-1}. However, plants that were inoculated with *A. brasilense* or *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 had a 12.5% increase in Ca accumulation relative to the positive control.

For the Mg accumulation it should be noted that the treatment without N fertilization were lower than the other treatments. The treatments in which the plants were inoculated with *P. fluorescens* showed the highest accumulation of Mg at 80 mg pot^{-1}, which was a 4.8% increase in accumulation relative to the positive control.

For the accumulation of B the plants fertilized with N were statistically higher to those not fertilized and similar to each other, and there were no significant differences between the unfertilized treatments (Figure 3). The treatments in which the plants were inoculated with *R. tropici* CIAT 899 and *A. brasilense* Ab-V6 N showed the highest accumulation of B, with 220 mg pot^{-1}, which was a 6.3% increase in accumulation relative to the positive control.



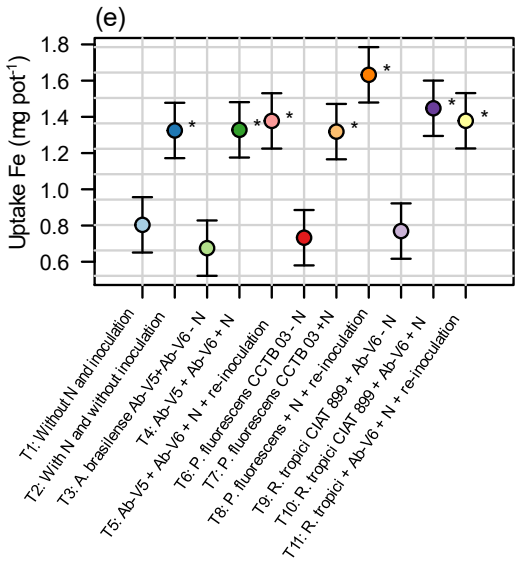


Figure 3. Uptake B (mg pot^{-1}) (a), uptake Zn (mg pot^{-1}) (b), uptake Mn (mg pot^{-1}) (c), uptake Cu (mg pot^{-1}) (d), uptake Fe (mg pot^{-1}) (e) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5+Ab-V6 – N, T4= *A. brasilense* Ab-V5+Ab-V6 + N, T5= *A. brasilense* Ab-V5+Ab-V6 + N + re-inoculation, T6= *P. fluorescens* CCTB 03 – N, T7= *P. fluorescens* CCTB 03 + N, T8= *P. fluorescens* CCTB 03 + N + re-inoculation, T9= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 – N, T10= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 + N and T11= *R. tropici* CIAT899 + *A. brasilense* Ab-V6 + N + re-inoculation. Per-treatment marginal means estimated from mixed models for all response variables investigated in the present study. Error bars represent the standard errors of the means. All marginal means followed by asterisks (*****) differed significantly ($p < 0.05$) from the negative control (treatment 1).

For the accumulation of Zn the plants inoculated with *P. fluorescens* presented the highest accumulation of Zn, 0.35 mg pot^{-1}, representing a statistically significant increase of 13.7% relative to the positive control. The Cu accumulation of the plants inoculated with *A. brasilense* Ab-V5 + Ab-V6 standed out at 0.12 mg pot^{-1}, representing an increase of 40.0%, although not statistically different, compared to the N-fertilized control treatment (Figure 3d).

The Mn accumulation in the plants inoculated with PGPB were statistically higher than the others, including the positive control. The Mn accumulation in the plants inoculated with *A. brasilense* Ab-V5 + Ab-V6, *P. fluorescens* CCTB 03 or *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 was of 2.7, 2.8 and 2.6 mg pot^{-1}, respectively, representing increases of 17.5%, 23.5% and 19.5%, respectively, relative to the positive control (Figure 3c).

For the Fe uptake the plants inoculated with *P. fluorescens* presented the highest accumulation of Fe,1.6 mg pot^{-1}, which was statistically higher and represented an increase of 14.3% relative to the positive control (Figure 3e).

Nutrient accumulation in roots

The P and K uptake (or accumulation), but N in roots were significantly affected by the treatments as (Table 2). Although not statistically different, *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 accumulated 147 mg pot^{-1} of N, an increase of 34.9% relative to the positive control; the same was verified for the P content (22 versus 14 mg pot^{-1}). As for the K in roots, the inoculation with *A. brasilense* Ab-V5 + Ab-V6 accumulated 40.4% more K than the positive control.

Table 2. Uptake N root (mg pot^{-1}), uptake P root (mg pot^{-1}) and uptake K root (mg pot^{-1}) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici*.

Treatments	N root (mg pot^{-1})	P root (mg pot^{-1})	K root (mg pot^{-1})
Negative control (without N and inoculation)	109.00	18.00 b	70.00 a
Positive control (with N and without inoculation)	109.00	14.00 b	47.00 b
A. brasilense Ab-V5+Ab-V6 - N	121.00	21.00 a	66.00 a
A. brasilense Ab-V5+Ab-V6 + N	113.00	15.00 b	47.00 b
A. brasilense Ab-V5+Ab-V6 + N + re-inoculation	114.00	17.00 b	54.00 b
P. fluorescens CCTB 03 - N	114.00	20.00 a	61.00 a
P. fluorescens CCTB 03 + N	128.00	15.00 b	64.00 a
P. fluorescens CCTB 03 + N + re-inoculation	138.00	15.00 b	51.00 b
R. tropici CIAT899 *+ A. brasilense* Ab-V6 - N	118.00	21.00 a	56.00 b
R. tropici CIAT899 *+ A. brasilense* Ab-V6 + N	123.00	16.00 b	48.00 b
R. tropici CIAT899 *+ A. brasilense* Ab-V6 + N + re-inoculation	147.00	22.00 a	49.00 b
P value	0.152	0.036	0.004

Means followed by lowercase letters differ for treatments as determined by the Scott-Knott test ($P < 0.05$)

The Supplementary Material shows the soil means chemical properties at the beginning and end of the experiment. The data show that there was a decrease in the chemical properties of the organic matter soil, K, Mg, H + Al, S, cation exchange capacity, base saturation, B, Cu, Fe, Mn and Zn (Table 3 and Table 4).

4. DISCUSSION

For most of the evaluated parameters, the treatments in which the plants were inoculated exclusively with plant growth-promoting bacteria (PGPB) had a lower performance than those that received a combination of PGPB + N fertilizer Therefore, and as expected for PGPB with grasses, the results showed that the bacteria alone cannot replace N fertilizers, but that they do promote greater uptake and utilization of the available N in the soil [34], resulting in a synergistic effect between PGPB inoculation and N fertilization [35].

In general, there was no significant difference between inoculation with bacteria and control positive treatments for most of the variables analyzed, what can be attributed to soil chemical conditions that were decreased at the end of the experiment (Table 3). However, PGPB promoted increases in yields when

compared to the non-inoculated control without N-fertilizer, since we observed positive effects of PGPB inoculation on SDWY, tillers dry mass and RCI of Zuri grass.

For many PGPB, one main benefit results from the synthesis of phytohormones such as auxins as indoleacetic acid (IAA) and giberellins. IAA has an important effect on root growth, resulting in increases in the absorption of water and also of nutrients, ensuring the efficient use of these resources [10]. Auxins and gibberellins act on the growth and elongation of stalks, leaves and roots, and induce changes in the expansion, division and cellular stretching of the meristematic regions, where plant growth occurs [36]; [37]. In this study, some of the strains have been reported as able to synthesize phytohormones. The inoculant strains *A. brasilense* strains Ab-V5 and Ab-V6 are well known by the synthesis of IAA [22], and the same for *R. tropici* CIAT 899 [38].

Increases in leaf and stem production of forage plants results in higher SDWYs and, consequently, higher amounts of carbon (C) are hijacked to increase the productivity and for storage in the soil via the roots. Well-managed forage plants with high biomass production can sequester a considerable amount of C [39]. Reported by [24] the sequestration of 9.27 Mt e-CO_2 in pasture areas inoculated with *A. brasilense* strains Ab-V5 and Ab-V6 and destined for forage biomass yields.

The main reported mechanisms of action of the genus *Pseudomonas* improving plant growth are the solubilization of phosphate, and the promotion of phytohormones (including IAA) [40]. In the case of *P. fluorescens* CCTB 03, we have identified that the strain possesses the capacity of synthesis of IAA and of P solubilization *in vitro* (unpublished data). By evaluating the effects of inoculation with *P. fluorescens* on *Pennisetum clandestinum* during the winter, [41] verified higher dry and green mass productions by the plant compared to plants receiving only N fertilization and emphasized that such increases were the result of the release of phytohormones.

The co-inoculation of *Azospirillum* Ab-V6 and *R. tropici* CIAT 899 has been successfully used in Brazil for the common bean crop [12], and also promoted growth of maize, and a main driven effect could be the induction of plant systemic resistance to tolerance of abiotic stresses [23]. The approach of co-inoculation consists on the combination of microorganisms that can contribute with different biological processes, resulting in a synergistic effect, tending to surpass the productive results obtained when these organisms are used in an isolated form [42]; [43]. In Gramineae, strains of *Azospirillum* (Ab-V5 and Ab-V6) contribute as plant growth promoters [12] mainly by the synthesis of (IAA) [37]; [44]; [22], while *Rhizobium* could also participate phytohormone in non-legumes [45]; [46]; [38]. For example, [47] found that the inoculation of *Azospirillum* spp. in natural pastures had a beneficial potential, especially in regions with hydric deficits and low soil fertility, due to the larger root biomasses that increases the soil exploration capacity [48].

The results obtained in this study agree with those obtained by [49], in which, when evaluating the production of Coastcross-1 grass inoculated with *Azospirillum* Ab-V5 and Ab-V6 and fertilized with 100 kg N ha^{-1} observed increased shoot production in comparison to non-inoculated plants. [24] also observed beneficial effects of PGPB on biomass yield when evaluating 26 cuts of *Brachiaria* (*Urochloa)* spp., with mean increase of 5.4% with the application of 40 kg N ha^{-1}, and of 22.1% when combining the same dose of N with inoculation with *A. brasilense* Ab-V5 and Ab-V6. In general, rhizobia are broadly used in microbial inoculants for legumes, but not for grasses. However, there are rhizobial strains that can also be effective PGPB for grasses. [23] evaluated the effects of co-inoculation of *R. tropici* and Ab-V6 in maize,

and reported increases in in plant growth relative to the control treatments without inoculation; in addition, an important effect on the increase in salinity tolerance was observed. In addition, the combination of *A. brasilense* and *R. tropici* in this study resulted in a further 2% SDWY over the positive control. The treatments in which plants were inoculated with PGPB and fertilized with N had higher RCI values relative to the unfertilized plants. According to [50], the photosynthetic capacity is optimized with a higher availability of N, as this nutrient is the main constituent of the chlorophyll molecule. Thus, the RCI can be used to predict the nutritional status of N in plants by reading the amount of green pigments in the forage leaves, and RCI values over 20 can be considered a good nutritional status of grass. [51], using a chlorophyll apparatus for RCI readings in *Brachiaria brizantha* cv. Marandu inoculated with *A. brasilense* (Ab-V5 and Ab-V6) obtained similar results than in this study, with an average value for the *P. fluorescens* CCTB 03 inoculated treatment group of 22.5 RCI.

It is worth mentioning that the inoculated treatments fertilized with N tended to have no significant effect after the first cut, demonstrating that the effects of bacteria and N fertilization were more pronounced at the plant establishment stage. There were also effects of grass exposure during periods of low light intensity in the rainy summer. All these factors could be related to the addition of N-fertilizer only at the beginning of the experiment.

Re-inoculation of PGPB in permanent pastures is a difficult task. The effects of re-inoculation are not well defined yet, as well as the method of re-introducing the strains, as PGPB are rhizospheric bacteria, and the soil is covered by the grass, such that foliar application would represent practically the only viable strategy. In this study, re-inoculation did not result in improve SDWY and RDWY. These results agree with [49], who concluded that the re-inoculation of *A. brasilense* in Coastcross-1 grass after the first year of cultivation was not necessary. [52] also concluded that the re-inoculation of Mavuno grass with PGPB did not present significant results for shoot and roots yields. In general, nutrient accumulation in the shoots and roots of Zuri Guinea grass was positively affected by inoculation with PGPB. The nutrients with the greatest accumulation were N and K, important nutrients for forages [53]; [54]. The increases in nitrogen accumulation, as well as N, nitrate and ammonium compounds, benefited mainly by the inoculation with the *Azospirillum* and *Pseudomonas*, might be attributed mainly to the synthesis of phytohormones, improving root biomass and, in the case of *Azospirillum*, and also by a contribution of biological nitrogen fixation [20]; [55]. In addition, *Azospirillum* may influence the activity of glutamine synthetase in grass roots, impacting plant N nutrition and growth [56]; [57]. The present study demonstrated (in absolute values) a greater nitrate accumulation relative to ammonium. These are important values because the use of absorbed N can vary according to the proportion of NO_3^-/NH_4^+. To be used, nitrate needs to be reduced in an energy-dependent process that is mediated by nitrate reductase and nitrite reductase enzymes, whereas ammonium does not require this step to be assimilated [37]. Despite this high energy demand for nitrate utilization, plant growth is better when supplied with nitrate compared to ammonium [58].

Although the differences were not significant relative to the other bacteria or to the positive control treatment, inoculation with *P. fluorescens* had the highest P accumulation in the first cuttings, which was higher than the other treatments. These data might be attributed to the reports that *P. fluorescens* may increase the available P through the mineralization of organic phosphates from phosphatases or the solubilization of inorganic phosphates and organic acids [59]. [60] reported that each strain of *P. fluorescens*

secretes different amounts of organic acid, which directly influences phosphate solubilization and promotes plant growth. As we mentioned before, *P. fluorescens* CCTB 03 has the capacity of phosphate solubilization *in vitro* (unpublished data).

In general, the accumulation of micronutrients was positively affected when the plants were inoculated with PGPB. The bacteria significantly increased the accumulation of Mn, Fe and Zn relative to the control treatment with N. The higher accumulation of these nutrients might be attributed to increases in root biomass, allowing higher uptake to these nutrients. Other reported microbial mechanisms could also be involved, such as the synthesis of siderophores, but they have not been investigated yet in the strains used in this study. For example, *Pseudomonas* can produce siderophores, that bind to Fe with a high affinity [61]; [62], allowing the utilization of this nutrient for its growth, and also conferring competitiveness advantage in relation to other microorganisms [63]. Some plants can take advantage of the bacterial Fe-siderophore complex, making it available to plant growth [64]. Zn is one of the most limiting micronutrients to forage grass yields, participating in important processes as photosynthesis, synthesis of tryptophan, and processes to maintain the integrity of bio membranes [65]. Increases in Zn content of inoculated plants *P. fluorescens* CCTB 03 could also result from higher root biomass.

As expected PGPB were not able to replace N fertilization. However, when combined N-fertilizer, the PGPB increased yield, the relative chlorophyll index, and the uptake of N, NH_4^+, Ca, Zn, Mn and Fe of Zuri Guinea grass. This result indicates that PGPB can be a sustainable alternative for reducing the use of N-fertilizers. There were no effects of re-inoculation with PGPB on the nutrition or yield of Zuri Guinea grass, demonstrating that the determination of the method of application and periodicity of inoculation still require investigation.

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6. REFERENCES

[1] Gilabel, A.P., Nogueirol, R.C., Garbo, A.I. & Monteiro, F.A. The role of sulfur in increasing guinea grass tolerance of copper phytoxicity. *Water Air soil Pollut*. **225,** 1806 (2014). https://doi.org/10.1007/s11270-013-1806-8

[2] Cook, R. Beef2 Live. In:http://beef2live.com/story-world-beef-productionranking-countries-0-106885. (2017). Acess 05.03.2019.

[3] Silva, E.B. *et al.* Availability and toxicity of cádmium to forage grasses grown in contaminated soil. *Int. J. Phytoremediat.* **18,** 847-852 (2016). https://doi.org/10.1080/15226514.2016.1146225

[4] Santos, J.H.S., De Bona, F.D. & Monteiro, F.A. Growth and productive responses of tropical grass *Panicum maximum* to nitrate and ammonium supply. *Rev. Bras. Zootecn.* **42,** 622-628 (2013). http://dx.doi.org/10.1590/S1516-35982013000900003

[5] Soares Filho, C.V. *et al.* The impact of organic biofertilizer application in dairy cattle manure on the chemical properties of the soil and the growth and nutritional status of *Urocholoa* grass. *Communications in soil science and plant analysis*. **49**, 358-370 (2018). https://doi.org/10.1080/00103624.2018.1427261

[6] Costa, R.R. *et al*. Efficiency of inoculant with *Azospirillum brasilense* on the growth and yield of second-harvest maize. *Pesqu. Agropecu. Trop.* **45**, (3): 304-311 (2015). https://dx.doi.org/10.1590/1983-40632015v4534593

[7] Lal, R. Soil carbon sequestration for sustaining agricultural production and improving the environment with particular reference to Brazil. *Journal Sustainable Agric.* **26,** 23-42 (2005). https://dx.doi.org/10.1300/J064v26n04_04

[8] Sá, J.C. *et al.* Carbon depletion by plowing and its restoration by no-till cropping systems in oxisols of sub-tropical and tropical agro-ecoregions in Brazil. L*and Degrad. Dev*. **26,** 531-543 (2015). https://doi.org/10.1002/ldr.2218

[9] Carvalahais, L.C. *et al.* Linking plant nutritional status to plant-microbe interactions. *PLoS One.* **8**, (7): e68555 (2013). https://doi.org/10.1371/journal.pone.0068555

[10] Hungria, M., Campo, R.J., Souza, E.M. & Pedrosa, F.O. Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant and Soil.* **331**, (1/2): 413-425(2010). https://doi.org/10.1007/s11104-009-0262-0

[11] Hungria, M., Mendes, I.C. & Mercante, F.M. A fixação biológica do nitrogênio como tecnologia de baixa emissão de carbono para as culturas do feijoeiro e da soja. Embrapa Soja, Londrina (2013).

[12] Hungria, M., Nogueira, M.A. & Araujo, R.S. Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability. *Biol. Fertil. Soils***. 49,** 791–801 (2013) https://doi.org/10.1007/s00374-012-0771-5

[13] Bashan, Y. & De-Bashan, L.E. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. *Adv Agronomy.* **108,** 77–136 (2010). https://doi.org/10.1016/S0065-2113(10)08002-8

[14] Marques, A.C. *et al.* Biological nitrogen fixation in C_4 grasses of different growth strategies of South

America natural grasslands. *Appl Soil Ecology*. **113,** 54-56 (2017). https://doi.org/10.1016/j.apsoil.2017.01.011

[15] Ardakani, M.R., Mazaheri, D., Mafakheri, S. & Moghaddam, A. Absorption efficiency of N, P, K through triple inoculation of wheat (*Triticum aestivum* L.) by *Azospirillum brasilense*, *Streptomyces* sp., *Glomus intraradices* and manure application. *Physiol Mol Biol Plants.* **17**,(2): 181–192 (2011). https://doi.org/10.1007/s12298-011-0065-7

[16] Spaepen, S. & Vanderleyden, J. Auxin signaling in *Azospirillum brasilense*: a proteome analysis. Wiley, Hoboken. (2015). https://doi.org/10.1002/9781119053095.ch91

[17] Tien, T.M., Gaskins, M.H. & Hubbell, D.H. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of Pearl Millet (*Pennisetum americanum* L.). A*ppl Environ Microbiol*. **37**, (5): 1016–1024 (1979).

[18] Bottini, R., Fulchieri, M., Pearce, D. & Pharis, R.P. Identification of gibberellins A1, A3, and iso-A3 in cultures of *Azospirillum lipoferum*. *Plant Physiology.* **90,** 45–47 (1989). https://doi.org/10.1104/pp.90.1.45

[19] Sahoo, R.K. *et al.* Phenotypic and molecular characterization of native *Azospirillum* strains from rice fields to improve crop productivity. *Protoplasma*. **251**, (4):943–953 (2014). https://doi.org/10.1007/s00709-013-0607-7

[20] Fukami *et al*. Revealing different strategies of quorum sensing in *Azospirillum brasilense* strains Ab-V5 and Ab-V6. *Archives of Microbiology*, **200**, (1), 47-56, (2018). https://doi.org/ 10.1007/s00203-017-1422-x).

[21]Rodriguez, H., Gonzalez, T., Goire, I. & Bashan, Y. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. *Naturwissenschaften.* **91,** 552–555 (2004). https://doi.org/10.1007/s00114-004-0566-0

[22] Fukami J, Ollero FJ, Megías M, Hungria M (2017) Phytohormones and induction of plant-stress tolerance and defense genes by seed and foliar inoculation with *Azospirillum brasilense* cells and metabolites promote maize growth. AMB Express 7:153-166 https://dx.doi.org/10.1186%2Fs13568-017-0453-7

[23] Fukami, J. *et al.* Co-inoculation of maize with *Azospirillum brasilense* and *Rhizobium tropici* as a strategy to mitigate salinity stress. *Functional Plant Biology.* **45**, 328-339 (2017) https://doi.org/10.1071/FP17167

[24] Hungria, M., Nogueira, M.A. & Araújo, R.S. Inoculation of *Brachiaria spp*. with the plant growth-promoting bacterium *Azospirillum brasilense*: An environment-friendly component in the reclamation of degraded pastures in the tropics. *Agriculture, Ecosystems and Environment.* **221,** 125–131 (2016). https://doi.org/10.1016/j.agee.2016.01.024

[25] Sandini, I.E, Pacentchuk. F., Hungria, M., Nogueira, M.A., Cruz, S.P., Nakatani, A.S., Araujo, R.S. (2019) Seed inoculation with *Pseudomonas fluorescens* promotes growth, yield and reduces nitrogen application in maize. Int J Agric Biol https://doi:10.17957/IJAB/15.1210

[26] Santos, H.G. *et al*. Brazilian system of soil classification (5th ed.). Brasilia, DF. Embrapa. (2018).

[27] Van Raij. *et al*. Chemical analysis for fertility evaluation of tropical soils. P.284. Campinas: Instituto Agronômico. (2001).

[28] Döbereiner, J., Marriel, I. & Nery, M. Ecological distribution of *Spirillum lipoferum*. *Can. J. Microbiol***. 22,** 1464–1473 (1976) https://doi.org/10.1139/m76-217

[29] Hungria, M. & Araujo, R.S. Manual de métodos empregados em estudos de microbiologia agrícola. EMBRAPA**,** Brasília (1994). Available at: https://www.embrapa.br/busca-de-publicacoes/-/publicacao/199952/manual-de-metodos-empregados-em-estudos-de-microbiologia-agricola>

[30] Fukami, J.; Cerezin, P.; Hungria, M. *Azospirillum*: benefits that go far beyond biological nitrogen fixation. *AMB Express*, **8**, (1), 73, (2018). https://doi.org/10.1186/s13568-018-0608-1

[31] Malavolta, E., Vitti, G.C. & Oliveira, S.A. Avaliação do estado nutricional das plantas: princípios e aplicações. In: Potafós. Princípios e aplicações. 2ed. Piracicaba, São Paulo (1997).

[32] Ronnegard, L., Shen, X., Alam, M. hglm: A Package for Fitting Hierarchical Generalized Linear Models. *The R Journal*. **2**, 20-28 (2010).

[33] R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria https://www.r-project.org (2017).

[34] Roesch, L.F., Camargo, F.O., Selbach, P.A., Sá, E.S. Reinoculação de bactérias diazotróficas aumentando o crescimento de plantas de trigo. *Ciência Rural*. **35,** 1201-1204 (2005). http://dx.doi.org/10.1590/S0103-84782005000500035

[35] Lana, M.C., Dartora, J., Marini, D. & Hann, J.E. Inoculation with Azospirillum, associated with nitrogen fertilization in maize. *Revista Ceres*. **59**, (3):399-405 (2012). http://dx.doi.org/10.1590/S0034-737X2012000300016

[36] Duca, D. *et al.* Indole-3-acetic acid in Plant-microbe interactions. *Antonie van Leeuwenhoek*. **106,** 85-125 (2014) https://doi.org/10.1007/s10482-013-0095-y

[37] Taiz, L. & Zeiger, E. Fisiologia vegetal. Artemed, Porto Alegre (2013).

[38] Tullio LD *et al*. Revealing the roles of *y4wF* and *tidC* genes in *Rhizobium tropici* CIAT 899: Biosynthesis of indolic compounds and impact on symbiotic properties. *Archives of Microbiology***, 201**, n.2, 171-183, (2019). https://doi.org/10.1007/s0023-018-1607-y

[39] Cerri, C.C. *et al.* Greenhouse gas mitigation options in Brazil for land-use change, livestock and 43 agriculture. *Sci. Agri.* **67,** 102–116 (2010). http://dx.doi.org/10.1590/S0103-90162010000100015

[40] Muleta, D., Assefa, F., Börjesson, E. & Granhall, U. Phosphate-solubilising rhizobacteria associated with *Coffea arabica* L. in natural coffee forests of southwestern Ethiopia. *Journal of the Saudi Society of Agricultural Sciences*. **12,** 73-84(2013). https://doi.org/10.1016/j.jssas.2012.07.002

[41] Criollo, P., Obando, M., Sánchez, L. & Bonilla, R. Efecto de bacterias promotoras del crecimiento vegetal (PGPR) asociadas a *Pennisetum clandestinum* en el altiplano cundiboyacense‖. *Revista Corpoica – Ciencia y Tecnologia Agropecuaria*. **13**, (2): 189-195 (2012). https://doi.org/10.21930/rcta.vol13_num2_art:254

[42] Bárbaro, I.M. *et al.* Técnica alternativa: co-inoculação de soja com *Azospirillum* e *Bradyrhizobium* visando incremento de produtividade. http://www.infobibos.com/Artigos/2008_4/coinoculacao/index.htm (2008).

[43] Ferlini, H.A. Co-Inoculación en Soja (*Glicyne max*) con Bradyrhizobium japonicum y *Azospirillum brasilense.* International Business Comunity Related to Animal Production (2006). http://www.engormix.com/co_inoculacion_soja_glicyne_s_articulos_800_AGR.htm

[44] Ilyas, N. & Bano, A. *Azospirillum* strains isolated from roots and rhizosphere soil of wheat (*Triticum aestivum*.) grown under different soil moisture conditions. *Biology Fertility Soil.* **46,** 393-406 (2010). https://doi.org/10.1007/s00374-009-0438-z

[45] García-Fraile, P. *et al. Rhizobium* promotes non-legumes growth and quality in several production steps: towards a biofertilization of edible raw vegetables healthy for humans. *PLoS One.* **7**, (5): e38122 (2012). https://doi.org/10.1371/journal.pone.0038122

[46] Yanni, Y.G. & Dazzo, F.B. Occurrence and ecophysiology of the natural endophytic *Rhizobium*–rice association and translational assessment of its biofertilizer performance within the Egypt Nile delta.

Biological nitrogen fixation. (Ed. FJ de Bruijn) 747–756 (2015). https://doi.org/10.1002/9781119053095.ch111

[47] Itzigsohn, R. *et al.* Plant-growth promotion in natural pastures by inoculation with *Azospirillum brasilense* under suboptimal growth conditions. *Arid Soil Research*. **13,** 151-158 (2000). https://doi.org/10.1080/089030600263076

[48] Malik, K.A. *et al.* Association of nitrogen-fixing, plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice. *Plant and Soil*. **194,** 37-44 (1997). https://doi.org/10.1023/A:1004295714181

[49] Aguirre, P.F. *et al.* Forage yield of Coastcross-1 pastures inoculated with *Azospirillum brasilense*. *Acta Sci., Ani Sci*. **40,** e36392. (2018). http://dx.doi.org/10.4025/actascianimsci.v40i0.36392

[50] Larcher, W. Ecofisiologia vegetal. Rima, São Carlos. (2000).

[51] Guimarães, S.L. *et al.* Nutritional characteristics of marandu grass (*Brachiaria brizantha* cv. marandu) subjected to inoculation with associative diazotrophic bacteria. *African Journal of Microbiology Research*. **10**, (24): 873-882 (2016). https://doi.org/10.5897/AJMR2016.7951

[52] Sá, G.C.R., Carvalho, C.L.M., Moreira, A., Hungria, M., Nogueira, M.A., Heinrichs, R., Soares Filho, C.V. (2019) Biomass yield, nitrogen accumulation and nutritive value of Mavuno grass inoculated with plant growth-promoting bacteria. Communications in Soil Science and Plant Analysis 50(15):1931-1942 https://doi.org/10.1080/00103624.2019.1648498

[53] Boer, C.A. *et al.* Ciclagem de nutrientes por plantas de cobertura na entressafra em um solo de cerrado. *Pesq. agropec. Bras.* **42**,(9): 1269-1276 (2007). https://dx.doi.org/10.1590/S0100-204X2007000900008

[54] Torres, J.L. *et al.* Decomposição e liberação de nitrogênio de resíduos culturais de plantas de cobertura em um solo de cerrado. *Rev. Brasi. Ciênc. Solo*. **29**, (4): 609-618. (2005). http://dx.doi.org/10.1590/S0100-06832005000400013

[55] Gupta, K., Dey, A. & Gupta, B. Plant polyamines in abiotic stress responses. *Acta Physiol Plant***. 35**, (7): 2015–2036 (2013). https://doi.org/10.1007/s11738-013-1239-4

[56] Machado, A.T., Sodek, L., Döbereiner, J. & Reis, V.M. Efeito da adubação nitrogenada e da inoculação com bactérias diazotróficas no comportamento bioquímico da cultivar de milho Nitroflint. *Pesq. Agropec. Bras*. **33,** 961-970 (1998).

[57] Unno, H. *et al.* Atomic Structure of Plant Glutamine Synthetase. *The Journal of Biological Chemistry*. **281**, (39): 29287-29296 (2006). https://doi.org/10.1074/jbc.M601497200

[58] Barker, A.V. & Mills, H.A. Ammonium and nitrate nutrition of horticultural crops. *Horticultural Review.* **2,** 395–423 (1980). https://doi.org/10.1002/9781118060759.ch8

[59] Duijff, B.J., Gianinazzi-Pearson, V. & Lemanceau, P. Involvement of the outer membrane lipo polysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. *New Phytol*. **135,** 325–334 (1997) https://doi.org/10.1046/j.1469-8137.1997.00646.x

[60] Vyas, P. & Gulati, A. Organic acid production in vitro and plant growth promotion in maize under control environment by phosphate-solubilizing *fluorescent Pseudomonas*. *BMC Microbiology*. **9,** 174 (2009). https://doi.org/10.1186/1471-2180-9-174.

[61] Gray, E.J. & Smith, D.L. Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biology Biochemical*. **37**, 395-412 (2005). https://doi.org/10.1016/j.soilbio.2004.08.030

[62] Vandendergh, P.A. & Gonzalez, C.F. Methods for protecting the growth of plants employing mutant siderophore producing strains of *Pseudomonas putida*. *United States Patent Number* **4**, 479-936 (1984).

[63] Dobbelaere, S., Vanderleyden, J. & Okon, Y. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Crit. Rev. in Plants Sci.* **22,** 107-149 (2003). https://doi.org/10.1080/713610853

[64] Crowley, D.E., Reid, C.P. & Szaniszlo, P.J. Utilization of microbial siderophores in iron acquisition by oat. *Plant Physiology*. **87,** 680–685 (1988). https://doi.org/10.1104/pp.87.3.680

[65] Malavolta, E. Manual de nutrição de plantas. Agronômica Ceres, São Paulo (2006).

APPENDIX

Treatments	P – resin mg/dm³	O. M. g/dm³	pH CaCl2	K mmolc/dm³	Ca mmolc/dm³	Mg mmolc/dm³	H+Al mmolc/dm³	Base Sum mmolc/dm³
First Soil Analysis	23.0	26.0	5.20	2.90	25.0	17.0	28.0	44.9
Negative control (without N and inoculation)	29.2	19.4	5.48 a	0.86 a	23.8	18.2	20.0 b	42.8
Positive control (with N and without inoculation)	41.8	20.4	5.44 a	0.64 b	28.0	20.2	20.8 b	48.8
A. brasilense Ab-V5+Ab-V6 - N	30.8	20.0	5.48 a	0.92 a	24.8	18.8	20.4 b	44.5
A. brasilense Ab-V5+Ab-V6 + N	35.6	20.2	5.36 b	0.72 b	24.2	16.6	23.6 a	41.5
A. brasilense Ab-V5+Ab-V6 + N + reinoculation	35.8	21.0	5.42 a	0.70 b	24.6	17.4	21.2 b	42.7
P. fluorescens CCTB 03 - N	38.2	22.0	5.44 a	1.00 a	24.8	18.2	21.2 b	44.0
P. fluorescens CCTB 03 + N	34.4	21.4	5.32 b	0.62 b	24.0	16.2	21.6 b	40.8
P. fluorescens CCTB 03 + N + reinoculation	42.2	20.2	5.28 b	0.68 b	26.0	16.2	23.4 a	42.8
R. tropici CIAT899 + *A. brasilense* Ab-V6 - N	33.8	19.8	5.42 a	0.94 a	24.6	18.0	20.4 b	43.5
R. tropici CIAT899 + *A. brasilense* Ab-V6 + N	43.6	20.4	5.4a	0.68 b	25.6	17.2	21.6 b	43.4
R. tropici CIAT899 + *A. brasilense* Ab-V6 + N + reinoculation	37.6	20.2	5.42 a	0.60 b	25.4	16.4	20.8 b	42.4
P value	0.727	0.684	0.007	0.001	0.333	0.157	0.042	0.409

Table 3 - Soil chemical attributes at the start and last of the experiment.

Means followed by lowercase letters differ for treatments as determined by the Scott-Knott test (P <0.05).

Table 4 - Soil chemical attributes at the start and last of the experiment.

Treatments	S mg/dm³	CTC mmolc/dm³	Base Saturation %	B mg/dm³	Cu mg/dm³	Fe mg/dm³	Mn mg/dm³	Zn mg/dm³
First Soil Analysis	19.0	72.9	62.0	0.55	1.2	111.0	9.9	3.5
Negative control (without N and inoculation)	9.8	62.8	68.0 a	0.24 b	1.0	60.0	3.6 c	1.7 b
Positive control (with N and without inoculation)	9.6	69.6	70.2 a	0.30 a	1.0	64.2	4.1 c	2.3 a
A. brasilense Ab-V5+Ab-V6 - N	7.0	64.9	68.4 a	0.25 b	1.0	60.0	3.8 c	1.8 b
A. brasilense Ab-V5+Ab-V6 + N	5.2	65.1	64.0 b	0.28 a	1.1	63.2	4.0 c	2.3 a
A. brasilense Ab-V5+Ab-V6 + N + reinoculation	5.4	63.9	66.8 a	0.26 b	1.1	69.4	4.9 b	2.4 a
P. fluorescens CCTB 03 - N	9.8	65.2	67.6 a	0.27 a	1.1	63.6	4.0 c	2.0 b
P. fluorescens CCTB 03 + N	5.2	62.4	65.0 b	0.25 b	1.1	61.4	4.1 c	2.2 a
P. fluorescens CCTB 03 + N + reinoculation	5.4	66.2	64.0 b	0.29 a	1.1	71.0	4.2 c	2.5 a
R. tropici CIAT899 + *A. brasilense* Ab-V6 - N	6.0	63.9	68.2 a	0.27 a	1.1	63.2	4.0 c	1.9 b
R. tropici CIAT899 + *A. brasilense* Ab-V6 + N	3.8	65.0	66.6 a	0.29 a	1.1	67.8	5.2 a	2.4 a
R. tropici CIAT899 + *A. brasilense* Ab-V6 + N + reinoculation	6.6	63.2	66.8 a	0.28 a	1.0	75.4	6.3 a	2.3 a
P value	0.574	0.629	0.019	0.020	0.718	0.270	0.001	0.043

Means followed by lowercase letters differ for treatments as determined by the Scott-Knott test (P <0.05).