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## Abstract

The objective of this study was to evaluate the effects of plant growth-promoting bacteria (PGPB) strains of Azospirillum brasilense, Pseudomonas fluorescens and Rhizobium tropici on biomass yield, number of tillers, nitrogen accumulation and nutritive value of shoots of Megathyrsus (syn. Panicum) maximus cultivar BRS Zuri (Zuri Guinea grass). For that, one experiment was performed for 14 months to evaluate inoculation and re-inoculation with Azospirillum brasilense strains Ab-V5 and Ab-V6, Pseudomonas fluorescens strain CCTB 03 and of co-inoculation with Rhizobium tropici strain CIAT 899 + A. brasilense strain Ab-V6 combined with N-fertilizer (100 kg of N ha-1). Shoot dry weight yield (SDWY), number of tillers (NT), total N concentration (TNC), total N uptake (TNU) and nutritive value of Zuri Guinea grass was evaluated for eight cuts, and inoculation increased all parameters. In the NT, the treatments inoculated with PGPB were superior to the positive non-inoculated control receiving N-fertilizer, by up to 36%. For the accumulated of SDWY the treatment re-inoculated with P. fluorescens CCTB 03 after each cut was statistically superior tin 7% the positive control. The PGPB when combined N-fertilizer also increased SDWY, NT, the relative chlorophyll index, TNC, total N uptake, neutral detergent fiber, acid detergent fiber, crude protein and in vitro digestibility dry matter of Zuri Guinea grass. The results indicate that PGPB can represent a sustainable alternative for reducing the use of N-fertilizers. The lower 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.

**Keyword:** Megathyrsus, biological nitrogen fixation, crude protein, SPAD, tropical forage grass.

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## Abstract

*The objective of this study was to evaluate the effects of plant growth-promoting bacteria (PGPB) strains of Azospirillum brasilense, Pseudomonas fluorescens and Rhizobium tropici on biomass yield, number of tillers, nitrogen accumulation and nutritive value of shoots of Megathyrsus (syn. Panicum) maximus cultivar BRS Zuri (Zuri Guinea grass). For that, one experiment was performed for 14 months to evaluate inoculation and re-inoculation with Azospirillum brasilense strains Ab-V5 and Ab-V6, Pseudomonas fluorescens strain CCTB 03 and of co-inoculation with Rhizobium tropici strain CIAT 899 + A. brasilense strain Ab-V6 combined with N-fertilizer (100 kg of N ha$^{-1}$). Shoot dry weight yield (SDWY), number of tillers (NT), total N concentration (TNC), total N uptake (TNU) and nutritive value of Zuri Guinea grass was evaluated for eight cuts, and inoculation increased all parameters. In the NT, the treatments inoculated with PGPB were superior to the positive non-inoculated control receiving N-fertilizer, by up to 36%. For the accumulated of SDWY the treatment re-inoculated with P. fluorescens CCTB 03 after each cut was statistically superior tin 7% the positive control. The PGPB when combined N-fertilizer also increased SDWY, NT, the relative chlorophyll index, TNC, total N uptake, neutral detergent fiber, acid detergent fiber, crude protein and in vitro digestibility dry matter of Zuri Guinea grass. The results indicate that PGPB can represent a sustainable alternative for reducing the use of N-fertilizers. The lower 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:** *Megathyrsus*, biological nitrogen fixation, crude protein, SPAD, tropical forage grass.

## 1. INTRODUCTION

Standing out approximately 15 years ago among the largest meat producers and exporters in the world, Brazil has reached this level due to its large production capacity for beef cattle on pasture [1]. Among the areas destined to the sector, about 90% of the cultivated pastures are occupied with the genera *Urochloa* spp. and *Megathyrsus* spp. [2]. In the genus *Megathyrsus* (syn. *Panicum*), *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.

Most of the soils under pastures in the tropics have a degradation level that impairs the forage yield potential, especially because of mismanagement and lack of fertilization [4]. The area with pastures in Brazil is estimated to be about 180 million ha, with about 70% under some level of degradation [5]. This scenario is aggravated because naturally low-fertility soils are used extensively for pastures.

Nitrogen plays a key role in the maintenance or increase the potential yields of forage grasses due to its influence on plant biomass production [6]; [7], especially leaf size, stem morphology, and the development of tillers [8]. However, the high price of chemical N-fertilizers limits its broad use in extensive pastures. On the other hand, when used in excess, N-fertilizers result in contamination of surface and groundwaters with nitrate and increased greenhouse gases emissions (GGE) [9]. Therefore, the use of plant-growth promoting bacteria (PGPB), especially diazotrophs such as *Azospirillum* as inoculants of 'Marandu' palisade grass seeds could improve forage production, and the nutritional status [10], especially in low-fertility soils [11]; [12]; [13].Additionally, concerns about mitigation of the emissions of GHG are also applicable to the agribusiness sector [14]. If, on the one hand, cattle production is placed as a protagonist in the emission of GGH [15], initiatives such as the use of PGPB figure as an important alternative to increase production with sustainability [13]; [16]; [17]; [18]. PGPB may promote plant development by a variety of mechanisms, including biological $N_2$ fixation, production of phytohormones and phosphate solubilization [19]; [20]; [21]; [22]. For example, trials performed with *Urocholoa* spp., inoculation with *Azospirillum brasilense* resulted in a contribution estimated as 40 kg ha$^{-1}$ of N, in addition to important sequestration of $CO_2$, helping in the mitigation of GGE [13].

Nowadays, it is well established that priority should be given to the use of alternative strategies that promote improvements in animal production, especially management strategies that associate sustainability with profitability. Therefore, the use of PGPB as inoculants of forage grasses may represent an important management alternative to improve pasture production and quality, and animal production. The objective of this study was to evaluate the effects of elite strains of *A. brasilense,* (Ab-V5 and Ab-V6), *Pseudomonas fluorescens* CCTB 03 and *Rhizobium tropici* CIAT 899, previously identified in other crops [23]; [24]; [12]; [25]; [13]; [26], on growth and nutritive value of Zuri Guinea grasses.

## 2. MATERIALS AND METHODS

### *Experimental sites*

The experiments were carried out in the experimental area of the Faculty of Veterinary Medicine, at São Paulo State University (UNESP) in Araçatuba County, São Paulo State, Brazil (21°11'12" LS, 50°26'20" LW, 390 m above sea level) during 14 months (November to January of 2018/2020). The climate, according to Köppen, is Aw, characterized by hot and humid summers and warm and dry winters, with most of the rainfall distributed between November and March. The average annual temperature and rainfall are, respectively, 22 ºC and 1,206 mm, with an average maximum temperature of 31 ºC and average minimum of 19 ºC. Details on the climatic events during the experiment are shown in Figure 1.

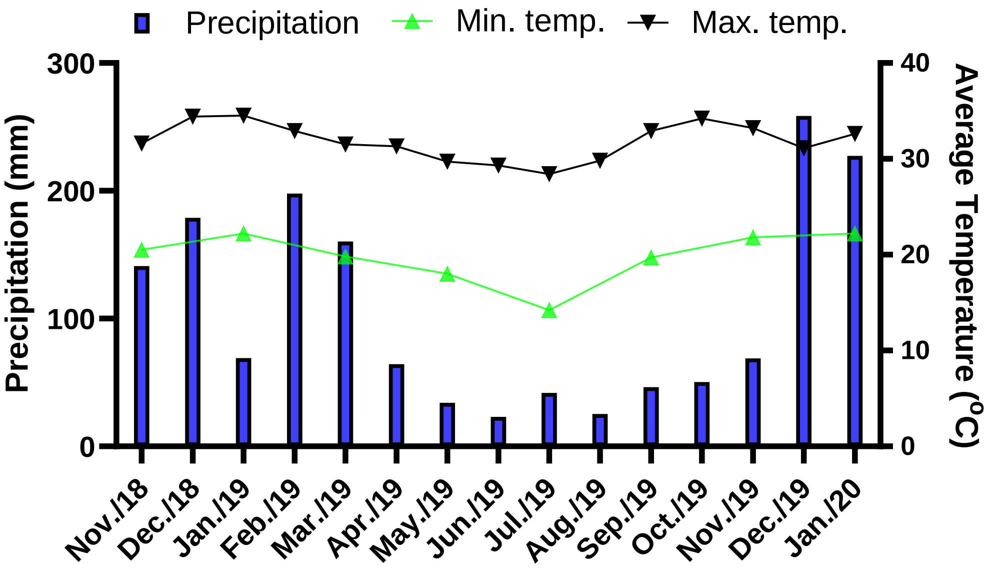


**Figure 1**. Maximum and minimum temperature (°C) and precipitation (mm) during the experiment (2018/2020).

The soil was classified as an Ultisol [27] and sampled at the 0-20 cm layer before the experimental installation for analysis of chemical properties and granulometric fractions, according to [28]. The results obtained were: 9 mg dm$^{-3}$ P (resin); 24 g dm$^{-3}$ O.M.; 4.9 pH ($CaCl_2$); K = 1.9 mmol$_c$ dm$^{-3}$; Ca = 13 mmol$_c$ dm$^{-3}$; Mg = 11 mmol$_c$ dm$^{-3}$; H + Al = 30 mmol$_c$ dm$^{-3}$; base sum (SB) = 25.9 mmol$_c$ dm$^{-3}$; cation exchange capacity (CEC) = 55.9 mmol$_c$ dm$^{-3}$; base saturation (V) = 46.3%. The need for liming was determined from the base saturation method to reach 70% of BS [29]. The limestone was distributed homogeneously on the soil surface and incorporated by plowing at a 0.2 m depth. Using the semi-solid NFb (N-free broth) culture medium the total population of diazotrophic microorganisms in the soil was estimated at 9.5x10$^4$ bacteria g$^{-1}$ of soil by the most-probable number method, according to [30]; [ 31].

### *Experimental design and treatments*

The experiment was installed in November 2018 and sowing in plots of 3 x 4 m. The experimental design was a complete randomized block design with four replications. The treatments were seeds of *Megathyrsus* (syn. *Panicum) maximus* cv. BRS Zuri. The main plots consisted of different treatments. The treatments were determined based on the inoculation of plant-growth promoting bacterial (PGPB) strains: (1) *Azospirillum brasilense* strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084), (2) *Pseudomonas fluorescens* strain CCTB 03 (=CNPSo 2719) and (3) co-inoculation with *Rhizobium tropici* CIAT 899 (=CNPSo 103, =SEMIA 4077), and *Azospirillum brasilense* Ab-V6; all inoculant treatments received 100 kg of N ha$^{-1}$, as will be detailed. The 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.) [12], wheat (*Triticum aestivum* L.) [12], *Brachiaria* (*Urochloa* spp.) [13] and co-inoculation of soybean (*Glycine max*) and common bean (*Phaseolus vulgaris* L.) [25]; *P. fluorescens* is used in maize [26], *R. tropici* CIAT 899 in common bean [25]. 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 eight 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). *A. brasilense* was prepared in DYGS medium [21], *P. fluorescens* in TSB medium [31], while *R. tropici* CIAT 899 inoculum was produced in YM medium [31]. At sowing, the concentration of each bacterial inoculant was adjusted to $2 \times 10^8$ cells per mL.

Fifteen mL of each inoculant ($2 \times 10^9$ cells mL$^{-1}$) were used for each kg of seed, resulting in the supply of $3 \times 10^9$ cells kg$^{-1}$ of seed, as recommended for brachiarias [13]. Considering that 1 g of seeds corresponds to approximately 660 seeds, the concentration of bacteria was of about $4.5 \times 10^3$ 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. 10 kg ha$^{-1}$ of viable pure seeds were used. This is the usual inoculation procedure adopted by the farmers pastures.

Phosphate fertilization consisted of 100 kg $P_2O_5$ ha$^{-1}$. Potassium fertilization consisted of 60 kg $K_2O$ ha$^{-1}$. The fertilizer was distributed manually and incorporated at a 0-0.1 m depth. Fifteen days after emergence, 60 kg of N ha$^{-1}$ was applied in the form of urea in all treatments, except for the negative control. After the second cut, 40 kg of N ha$^{-1}$ were applied as urea the haul in the treatments, totaling 100 kg of N ha$^{-1}$.

The plants were cut when they were 0.7m tall. The $T_4$ (strains Ab-V5 + Ab-V6), $T_6$ (strain CCTB 03) and $T_8$ (strains Ab-V6 + CIAT 899) treatments inoculated with PGPB were reinoculated after each cut by spraying a known volume (300 mL) after the cuts, at which time the leaves began to develop again. The same concentration of $3 \times 10^9$ 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.

### *Plant harvest and measurements of productive and nutritional parameters*

In the fourteen months, the experimental cuts were performed on Dec./2018, Jan./2019, Feb./2019, Mar./2019, Apr./2019, Jun./2019, Dec./2019 and Jan./2020. For yield determination, only the useful area of the plot was considered, the 0.5 m around it was disregarded. Cuttings were performed eight times during the rainy and dry seasons to determine the plant dry mass when the best treatment reached 0.7 m in height (four-week intervals spring and summer), when shoots were harvested down to 0.2 m above the surface of the ground. The samples for shoot dry mass estimation were taken with a sampler device delimiting a 1 m$^2$ area and cutting the plants at 0.2 m height above the soil.

After each harvest, the shoots were identified, weighed and oven dried at approximately 65 °C until they reached a constant weight. Shoots were subsequently weighed to obtain shoot dry weight yield (SDWY). After drying, the samples were ground to pass a 1 mm screen in a Wiley type mill. Total N concentration was determined were determined according to [32] Crude protein was determined by multiplying N concentration by 6.25. The acid detergent fiber (ADF) and neutral detergent fiber (NDF) concentration was determined according to [33] and in vitro digestibility dry matter (IVDMS) were determined according to [34].

Before each cutting, plant height readings were taken with a millimeter ruler. The relative chlorophyll index (RCI) was determined using a SPAD502 Plus chlorophyll meter (SPAD - Soil and Plant Analysis Development) (Spectrum technologies, Plainfield, IL, USA). The RCI values were obtained by the average of ten readings performed in the middle third of newly expanded leaves (diagnostic leaves) of each experimental unit. The tiller number was counted before the forage was cut, in a circular area of 0.25 $m^2$ at the center of each plot. The material was then collected, and a second separation was performed on the grass leaves and stems to determine the mass of each component.

### ***Statistical analysis***

The data were tested for error normality and homogeneity of variances. The results were assessed using analysis of variance (ANOVA), F test ($p \leq 0.05$) and compared using the Scott-Knott test with a 5% probability using SAS (Statistical Analysis System, version 8.2) [35].

## 3. RESULTS AND DISCUSSION

It is worth mentioning that all inoculated treatments received the same amount of N-fertilizer than the positive control, as rhizospheric diazotrophic bacteria cannot supply all plant´s N demands, and the objective of the study was to maximize the biomass production.

### ***Shoot dry weight yields and numbers tillers and relative chlorophyll index***

The results obtained in the 14 months (2018/2020) of growth are presented in showing eight evaluation cuts. In the analysis of variance, the parameters of shoot dry weight yields (SDWY), and the SDWY accumulation, relative chlorophyll index (RCI), and tillers units were highly significant, indicating higher yields in the treatments inoculated with PGBP receiving N-fertilizer ($p \leq 0.05$).

For most of the evaluated parameters, the treatments in which the plants were inoculated with PGPB + N-fertilizer had a better performance than those of the positive control, with N-fertilizer and without inoculation. Therefore, and as expected for PGPB with grasses, that the results indicated that PGPB can promote plant growth [36], resulting in a synergistic effect between PGPB inoculation and N fertilization [36, 37].

Statistically significant differences in the second, third, fourth, fifth and eighth evaluation were found for the number of tillers (Figure 2). In the second evaluation, the number of tillers for treatments inoculated with *P. fluorescens* CCTB 03 (207 units $m^{-2}$) and co-inoculated with Ab-V6 + *R. tropici* CIAT 899 (214 units $m^{-2}$) were higher than the other treatments, including the positive control (166 units $m^{-2}$), leading to an increase of 29% in relation to this treatment. In the third evaluation, the co-inoculation with

Ab-V6 + *R. tropici* CIAT 899 (370 units $m^{-2}$) was superior than the other treatments and 25% higher than the positive control (296 units $m^{-2}$). In the fifth evaluation, the treatments inoculated with *A. brasilense* Ab-V5 + Ab-V6 (228 units $m^{-2}$) and Ab-V6 + *R. tropici* CIAT 899 (221 units $m^{-2}$) were superior to the other treatment including the positive control (186 units $m^{-2}$). Finally, in the eighth evaluation, PGPB were superior to the positive control, with increases of up to 36%.

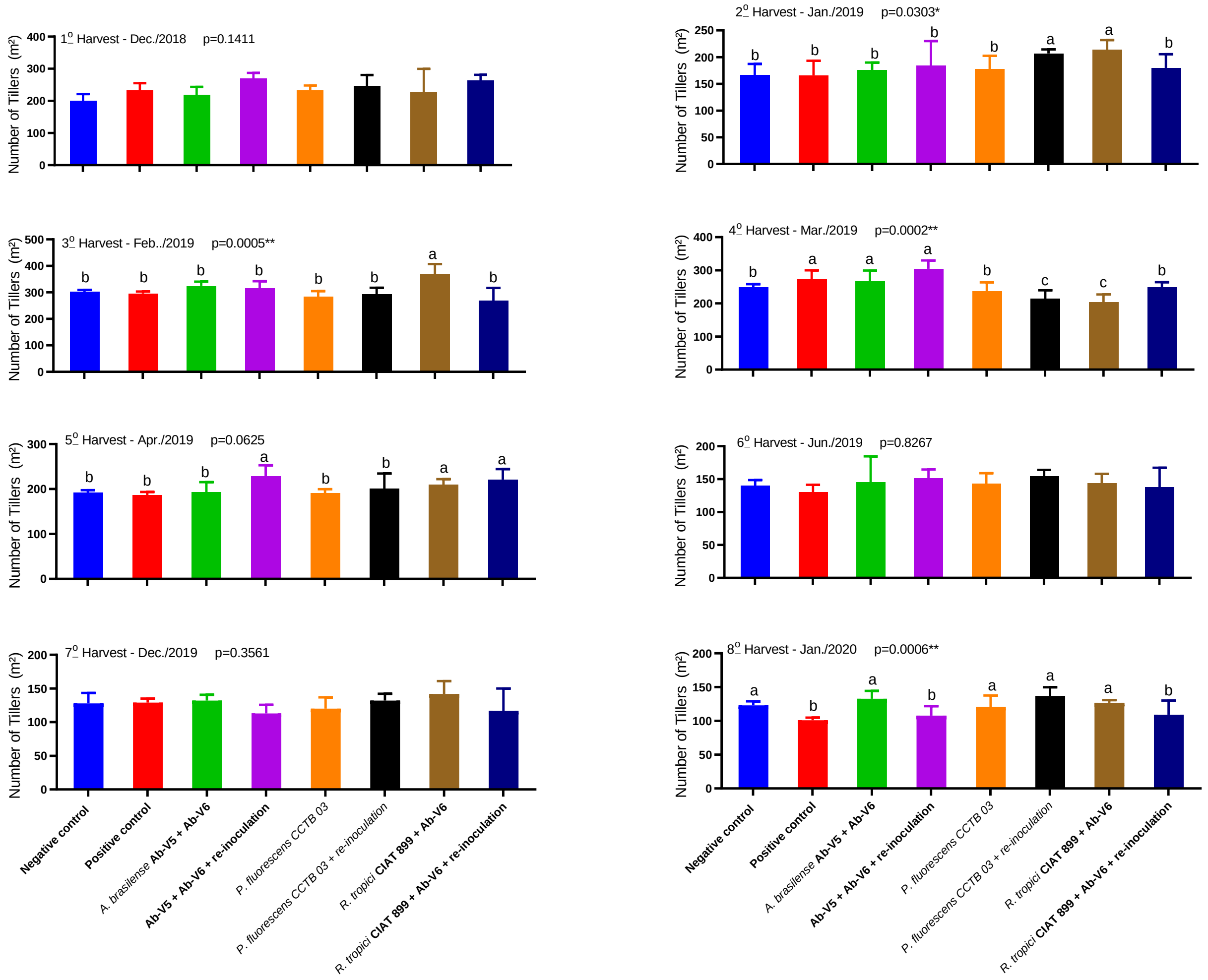


**Figure 2.** Number of tillers (units $m^{-2}$) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

For many PGPB, one main benefit results from the synthesis of phytohormones such as auxins as indoleacetic acid (IAA) and gibberellins. IAA has an important effect on root growth, resulting in increases in the absorption of water and nutrients, ensuring the efficient use of these resources [12]. 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 [38]; [8].

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 [23], and the same for *R. tropici* CIAT 899 [39].

In a previous study [40], increments of 9% in the number of leaves and of 12% in the number of tillers per plant in *U. brizantha* cv. 'Marandu' were found with the inoculation of *Azospirillum*. Evaluating tillering at a dose of 100 kg ha$^{-1}$ of N with *U. brizantha* cv. 'Xaraés' inoculated with *P. fluorescens* CCTB 03 and *Pantoea ananatis* AMG521 it was shown that the two bacteria promoted the greatest increase in the number of basal tillers, more than 100% in relation to the other treatments [18]. At the dose of 50 kg ha$^{-1}$ of N, there were no significant increases in the number of basal tillers by inoculation, except for the inoculation with *A. brasilense* Ab-V5, where the number was lower. Similar results were obtained in our study where the significant effects with the PGPB inoculation promoted an average increase of 28% in the tillering of the grass. However, other studies have shown lower increases, of 5% in SDWY [40], or lack of responses [42].

For the relative chlorophyll index (RCI), a significant effect of treatments was found in the first evaluation of Zuri grass (Figure 3). There was a decrease in the values from the first to the eighth evaluation, which is probably due to the lower N content in the plants' chlorophyll over 14 months of experimentation. In the first evaluation, treatments inoculated with PGPB did not differ statistically from the positive control. In the first five evaluations that corresponded to the summer and autumn seasons, the values varied from 30 to 45 and afterwards they decreased, presenting values below 30.

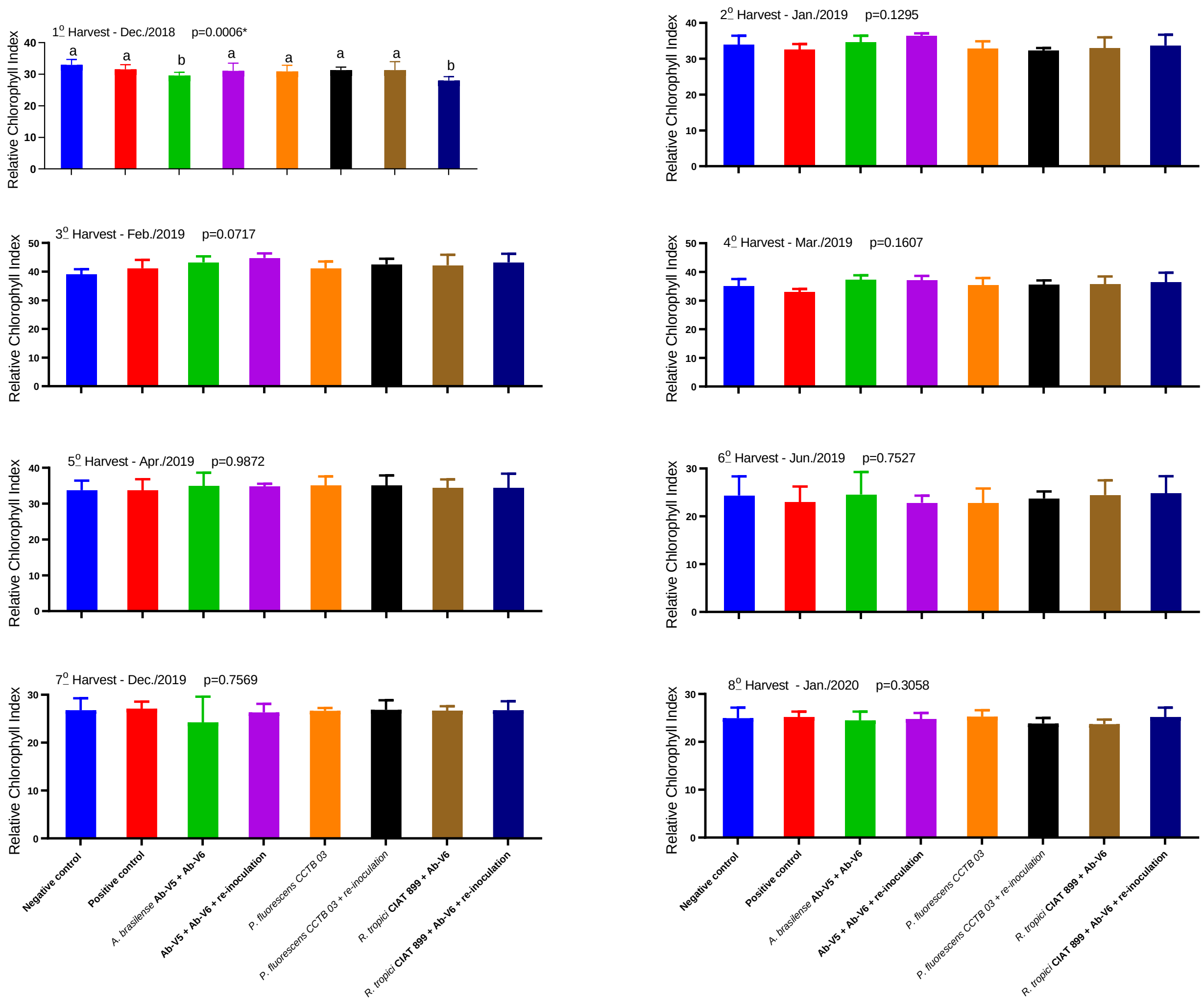


**Figure 3.** Relative chlorophyll index (RCI) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

The treatments in which plants were inoculated with PGPB and fertilized with N had higher RCI values relative to the unfertilized plants. According to [8], the photosynthetic capacity is optimized with 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 30 can be considered as indicative of good nutritional status of grass.

There was a significant effect of treatments for SDWY in the first, second, fourth and sixth evaluations (Figure 4). In the first evaluation, the treatments inoculated with PGPB were superior but did not differ from the positive control, probably due to the initial growth vigor of the grass. In the second evaluation, the treatments inoculated with *P. fluorescens* CCTB 03 were superior to the others, but did not show statistical difference from the positive control. Although in the re-inoculated treatment with *P. fluorescens* CCTB 03 after cutting the grass did not differ from the positive control, it produced 7% more SDWY. In the fourth evaluation cut of Zuri Guinea grass, the treatments re-inoculated with PGPB after cutting were superior to the positive control, producing 29% more SDWY. In the sixth cut, the treatment inoculated with *A. brasilense* Ab-V5 + Ab-V6 was superior to the others and in relation to positive control it produced 42% more SDWY.

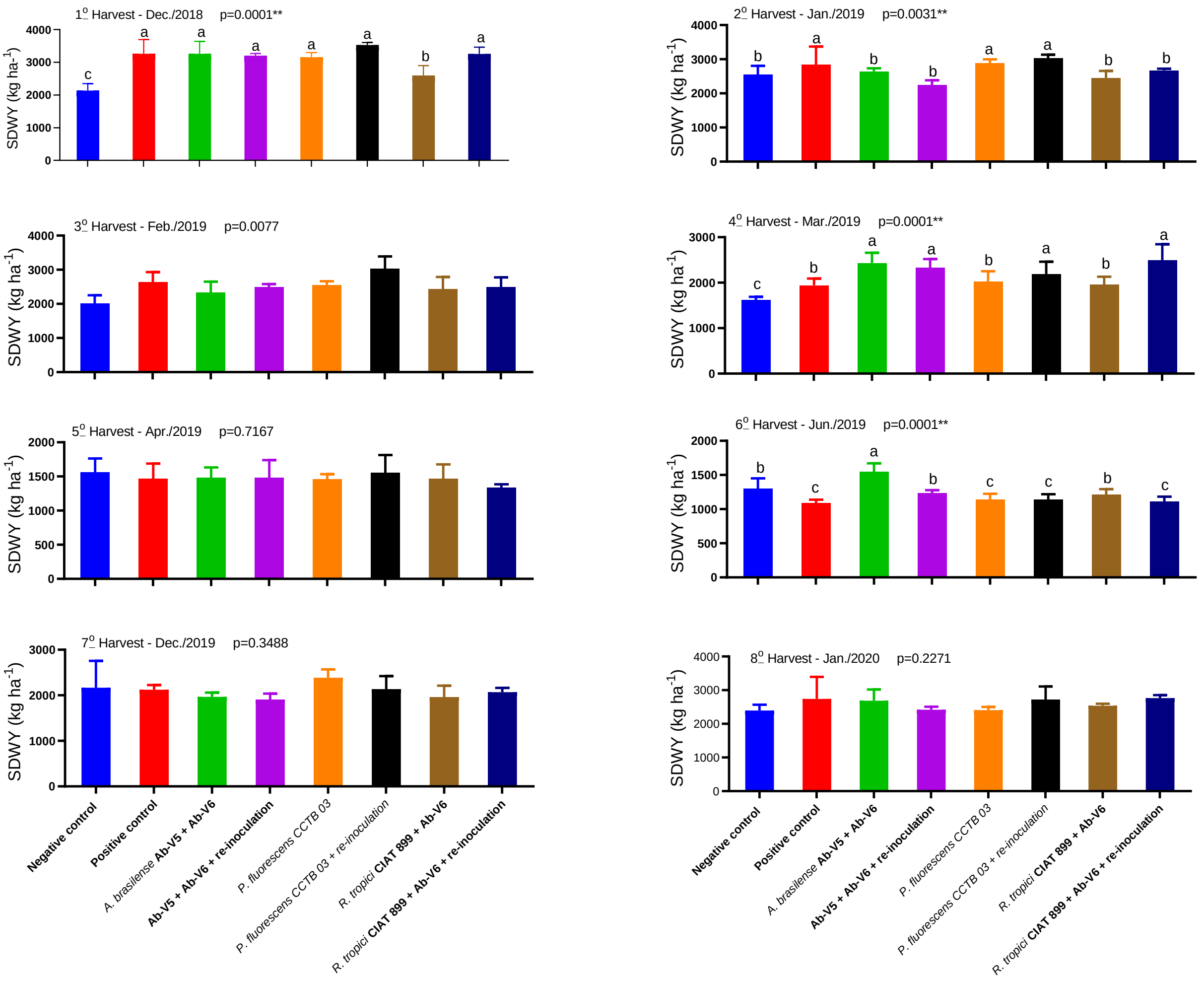


**Figure 4.** Shoot dry weight yield (SDWY) (kg ha$^{-1}$) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

According to Resende and collaborators [2], he effects of inoculation with *A. brasilense* strains (Ab-V5 + Ab-V6) and with *R. tropici* + Ab-V6 in maize, increases in height and consequently in production of dry mass of the aerial part of the plants were found in relation to the control treatment without inoculation.

Evaluating the agronomic response of triticale culture to different forms of application of strains of *Azospirillum*, Sipione and collaborators [41] did not find positive effects of leaf inoculation for height, stem dry matter and shoot dry matter.

PGPBs have the ability to promote growth and make nutrients available to plants, biologically fix nitrogen, solubilize phosphate, increase plant resistance to biotic and abiotic stresses and produce metabolites essential for growth [44]; [45]; [46]; [47].

The co-inoculation of *Azospirillum* Ab-V6 and *R. tropici* CIAT 899 has shown successful results for growth of maize, and a main driven effect could be the induction of plant systemic resistance to tolerance of abiotic stresses [24]. 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 [47];[48]. In Gramineae, strains of *Azospirillum* (Ab-V5 and Ab-V6) contribute as plant growth promoters [25] mainly by the synthesis of (IAA) [8]; [49]; [24], while *Rhizobium* could also phytohormone in non-legumes [50]; [51]; [37]. For example, Itzigsohn *et al*. [52] 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 [53]. This justifies the significant results by PGPB in the present work, since the soil used was of low fertility although chemical correction was carried out.

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) [54]. 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, higher dry and green mass productions was verified in comparison to plants receiving only N fertilization and emphasized that such increases were the result of the release of phytohormones [55].

The results obtained in the present experiment are in agreement with previouss results [56] obtained with Coastcross-1 grass (*Cynodon dactylon*), inoculated with *Azospirillum* strains Ab-V5 and Ab-V6 and fertilized 100 kg ha$^{-1}$ of N, with positive effects on the production of forage of the aerial part when compared to the non-inoculated treatments. Other studies [13] also reported beneficial effects on the shoot aerial part of *Urochloa* spp. Grasses, combining strains of *Azospirillum* Ab-V5 and Ab-V6 and 40 kg ha$^{-1}$ of N, with the bacteria promoting increases in production from 17.4 to 29.5%. The results reported [40] also evaluate the seed inoculation effects of *A. brasilense* strains Ab-V5 and Ab-V6 on the shoot of *U. brizantha* cv. ‘Marandu’ and found that the overall increase in shoot dry mass due to inoculation was of 13% and 6% in the first and second years, respectively, compared with the control. Other authors have also observed increases in shoot dry mass yield in plants inoculated PGPB [57]; [58]; [59], demonstrating its general, non-specific potential as plant-growth promoter.

Therefore, there are positive effects of PGPB inoculation in the production of SDWY and in tillering of Zuri guinea grass, which promoted increases in production when compared to treatments without inoculation (positive control), since bacteria secrete substances that increase root growth, plants have greater support and conditions for productivity and growth. The results show s that PGPBs alone do not replace N-fertilizers in grasses, but when associated, they promote greater absorption and use of the N available in the soil [60]; [61], showing a possible synergistic effect between inoculation x nitrogen fertilization [35]. In Zuri guinea grass the results were more expressive at the dose of 100 kg ha$^{-1}$ of N-fertilizer.

For the accumulated of SDWY in the eight evaluation cuts of Zuri guinea grass, the treatment reinoculated with *P. fluorescens* CCTB 03 (19332 kg ha$^{-1}$) after each cut was statistically superior to the positive control (18103 kg ha$^{-1}$) (Figure 5), with 7% increase in relation to positive control. However, it did not differ statistically from the treatments Ab-V5 and Ab-V6 (18345 kg ha$^{-1}$) and Ab-V6 + *R. tropici* CIAT 899 (18199 kg ha$^{-1}$) that were reinoculated after each cut. This higher rate of SDWY accumulation is possibly related to the increase in the activity of photosynthetic enzymes and N assimilation enzymes [12]. Similar results were previously obtained [62], with inoculated *U. brizantha* cv. 'Marandu'. The authors pointed out the inoculation as a sustainable alternative to increase forage production. The increase in production may be related to the greater release of phytohormones essential for growth, which improve the absorption of macro and micronutrients [63].

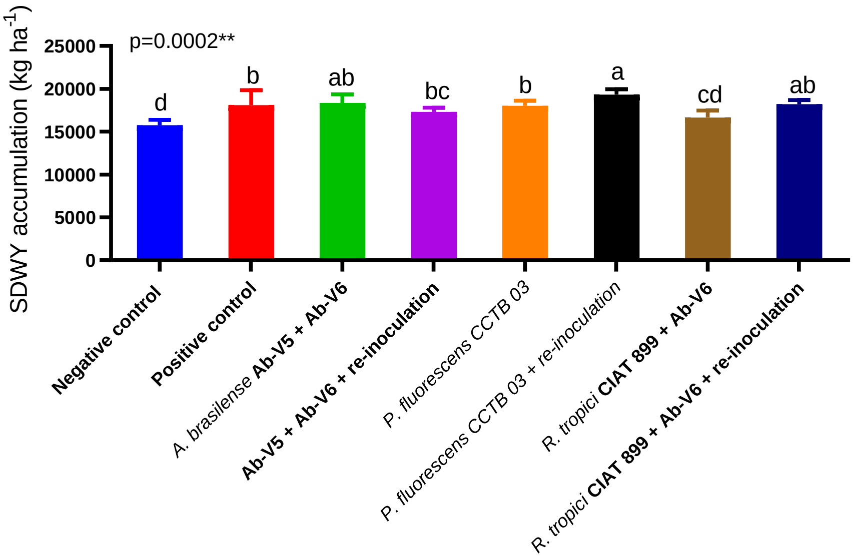


**Figure 5.** Shoot dry weight yield (SDWY) accumulation (kg ha$^{-1}$) in Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

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 resulted in improved SDWY accumulation. These results disagree with [64], who concluded that the re-inoculation of *A. brasilense* in Coastcross-1 grass after the first year of cultivation was not necessary, and also with [65], who 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 nutrient with the greatest accumulation were N, important nutrients for forages [66]; [67]. The increases in N accumulation, 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*, probably also by a contribution of biological nitrogen fixation [21]; [68]. In addition, *Azospirillum* may influence the activity of glutamine synthetase in grass roots, impacting plant N nutrition and growth [69]; [70].

Re-inoculation after cutting is a practice that proved to be feasible to provide SDWY accumulation for PGPB and, therefore, deserves to be better studied if it can be alternated with each cut or even with strategic applications during the year.

## Total N concentration and total N Uptake in shoots

Statistically significance differences in the total N concentration in the shoots of Zuri Guinea grass were observed in the experiment (Figure 6). Plants inoculated with *A. brasilense* Ab-V5 + Ab-V6 and *P. fluorescens* CCTB 03 and Ab-V6 + *R. tropici* CIAT 899 at sowing after the eight cuttings had the best performance in terms the total N concentration, with an average increase of 38%, 44% and 45%, respectively, in relation to positive control. The total N concentration for the treatments re-inoculated after cutting showed no statistical difference in relation to the positive control.

Regarding the nutrient concentration, leaf N increased with the evaluations demonstrating that biological nitrogen fixation was effective. However, results obtained by [40] evaluating the seed inoculation effects of *A. brasilense* strains Ab-V5 and Ab-V6 on the total N concentration of *U. brizantha* cv. 'Marandu' disagree have reported no effects due to the inoculation with *Azospirillum*. Increases in the other total N concentration might be related to the improvement of plant effectiveness in nutrient uptake, since there is a physical response to the increase of the root absorptive surface, including an increase of specific uptake mechanisms via transporters, which are mainly proteins depending on N to be formed. The total N concentration was within the range considered adequate for this forage species [71].

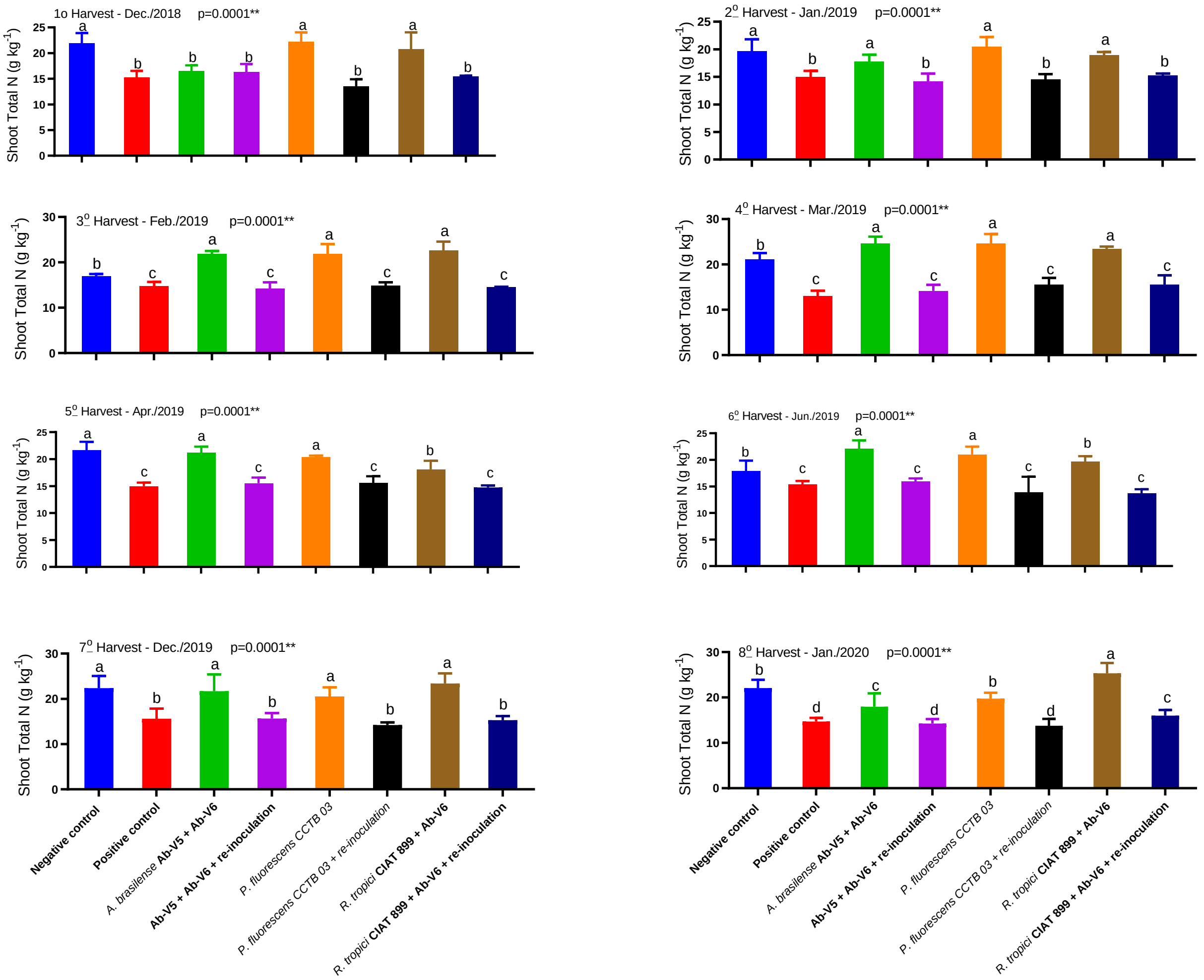


**Figure 6.** Total N concentration (g kg$^{-1}$) in the shoots of Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

In relation to the total N uptake, plants fertilized with N and inoculated with *P. fluorescens* CCTB 03 at sowing after the cuttings presented the highest value, of 43% more and differing statistically from the positive control (Figure 7). The negative control was statistically lower than the other treatments. The treatment inoculated with *A. brasilense* Ab-V5 + Ab-V6 accumulated 37% more, differing statistically from the positive control.

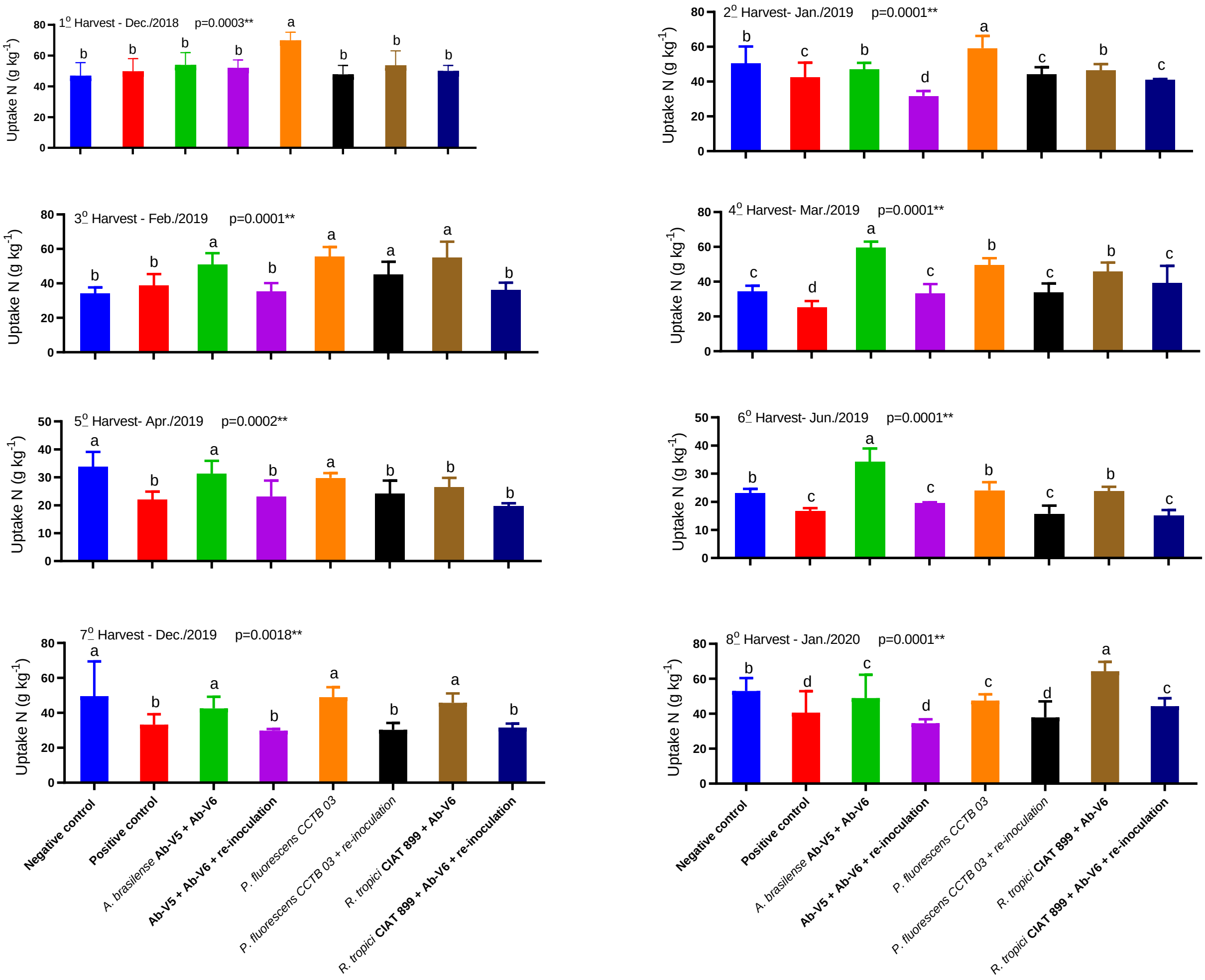


**Figure 7.** Total N uptake (g $kg^{-1}$) in the shoots of Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

The total N concentration and total N accumulation in the shoots of Zuri Guinea grass showed positive effects when the plants were inoculated with PGPB, probably explained by the increase in root volume and consequently greater absorption of water and nutrients [67]; [66]. The benefits were mainly attributed to the strains of *Azospirillum* and *Pseudomonas*. We may suppose that the effects could be attributed to improvement in root growth, enhancing the absorption of water and nutrients by plants [69], in addition to the BNF and activity of glutamine synthetase by *Azospirillum* [69]; [70], as mentioned before.

Evaluating the effects of inoculation [13]; [72] of strains of *A. brasilense* (Ab-V5 + Ab-V6) in *U. brizantha* and *U. ruzizienses* resulted in increments of 4 to 15% in the total N accumulation, when compared to the positive control. In a previous report [39] of the inoculation of Zuri Guinea Grass also increased up to 8.8% the N accumulation, while in our study the values were of up to 40%.

Considering the potential for increasing biomass production of Zuri Guinea grass, *Azospirillum* and *Pseudomonas* are promising PGPB. For example, *Azospirillum* inoculation associated with 40 kg $ha^{-1}$ of N as topdressing 30 days after sowing corresponded to an extra application of 40 kg $ha^{-1}$ of mineral N [25]. This is an important issue to be considered for the reduction of the environmental impacts of mineral N fertilizers because of more efficient use. Besides being converted into proteins in plant biomass, the more efficient use of mineral N by plants inoculated with *Azospirillum* reduces nitrate losses to groundwater by leaching or greenhouse gas emissions to the atmosphere via denitrification. Thus, the use of more environmentally-friendly tools for pasture establishment is important for maintaining the sustainability of livestock activities, as it improves the land efficiency and nutrient use, helps soil and water conservation and contributes for the sequestration of C in either plant shoot biomass [13] or in the soil as roots.

## Nutritive value in Zuri Guinea grass

Statistically significant differences in the neutral detergent fiber (NDF) in the shoots of Zuri Guinea grass were observed in the experiment (Figure 8). The treatments inoculated with PGPB did not differ from the positive control in the eight evaluations.

Animal consumption of dry matter and digestibility are related to NDF and ADF, respectively. In this way the ADF indicates the percentage of highly indigestible material, therefore low values of ADF indicate greater energy and high digestibility, and forages with low content of NDF have higher consumption rate; therefore, contents of NDF greater than 60% in the dry matter of the food are detrimental to consumption, with lower values being desirable [73]. The NDF values of the present study were above 60%, being in a range not recommended for a good rate of forage consumption by the animals [74].

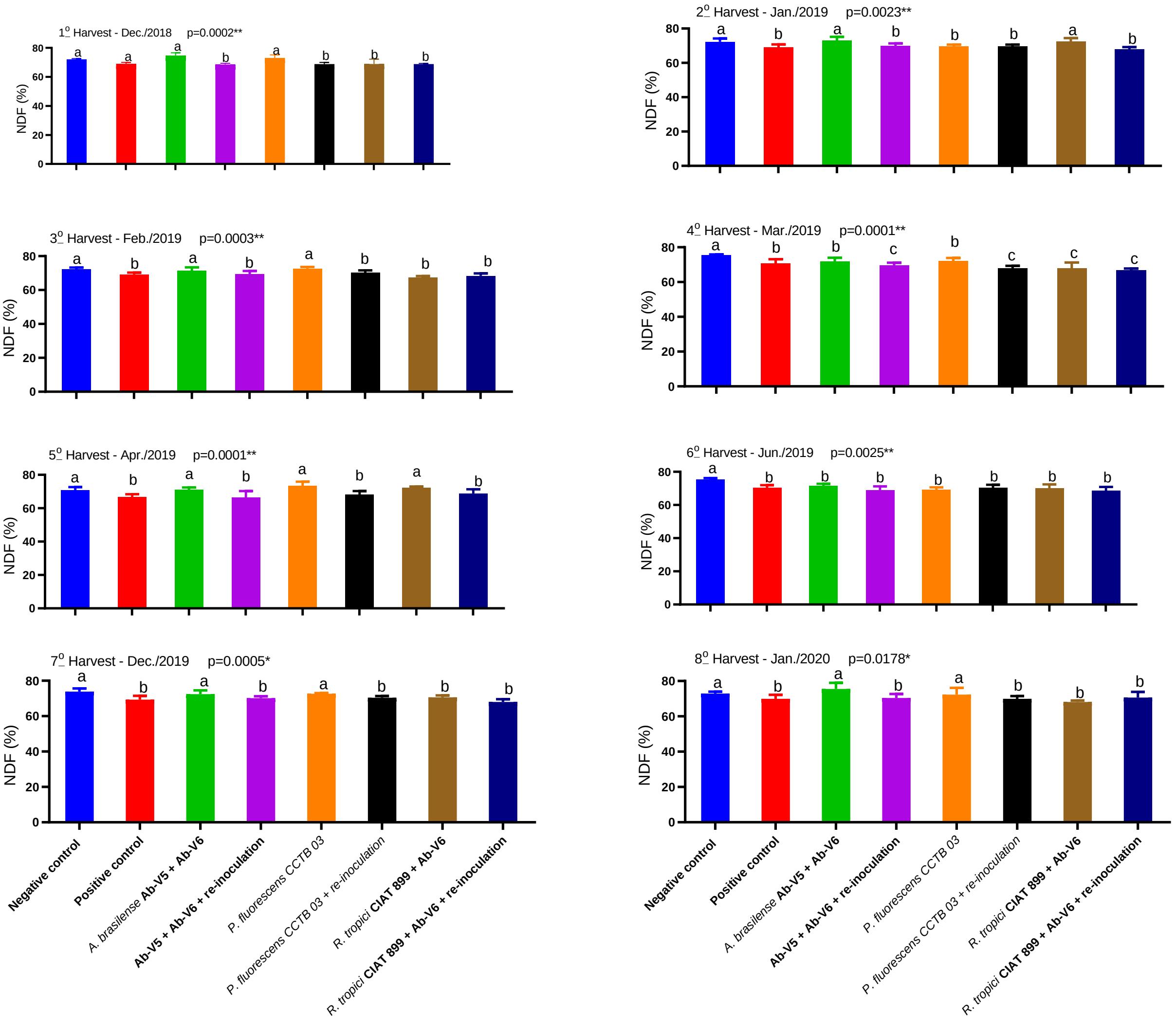


**Figure 8.** Neutral detergent fiber (NDF) (%) in the shoots of Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

The percentages of acid detergent fiber (ADF) in the aerial part of the Zuri Guinea grass showed significance in the analysis of variance for the treatments in the second, fourth, sixth and eighth assessment cut (Figure 9). The co-inoculated treatment with Ab-V6 + *R. tropici* CIAT 899 showed the lowest ADF content in the cuts in relation to the positive control.

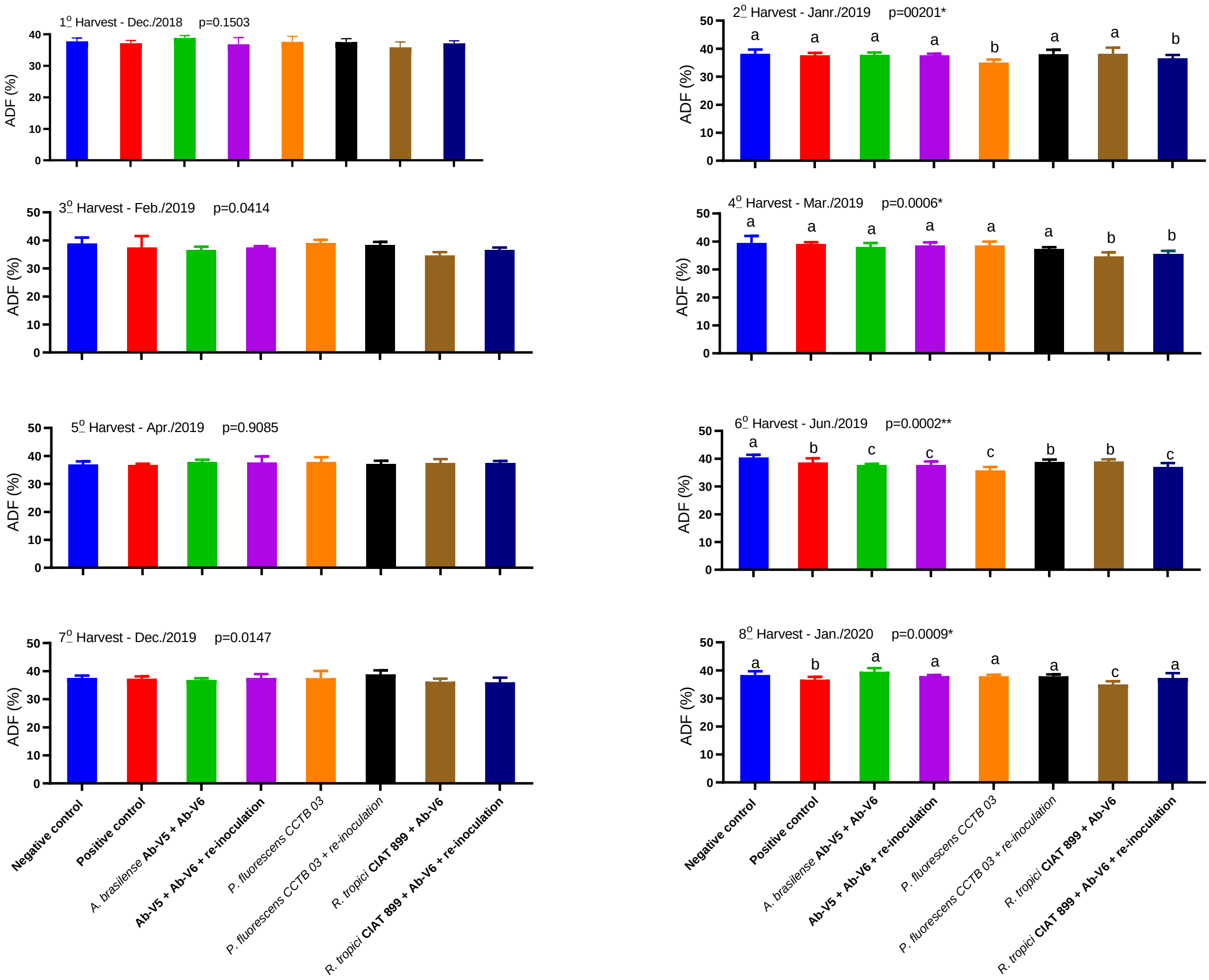
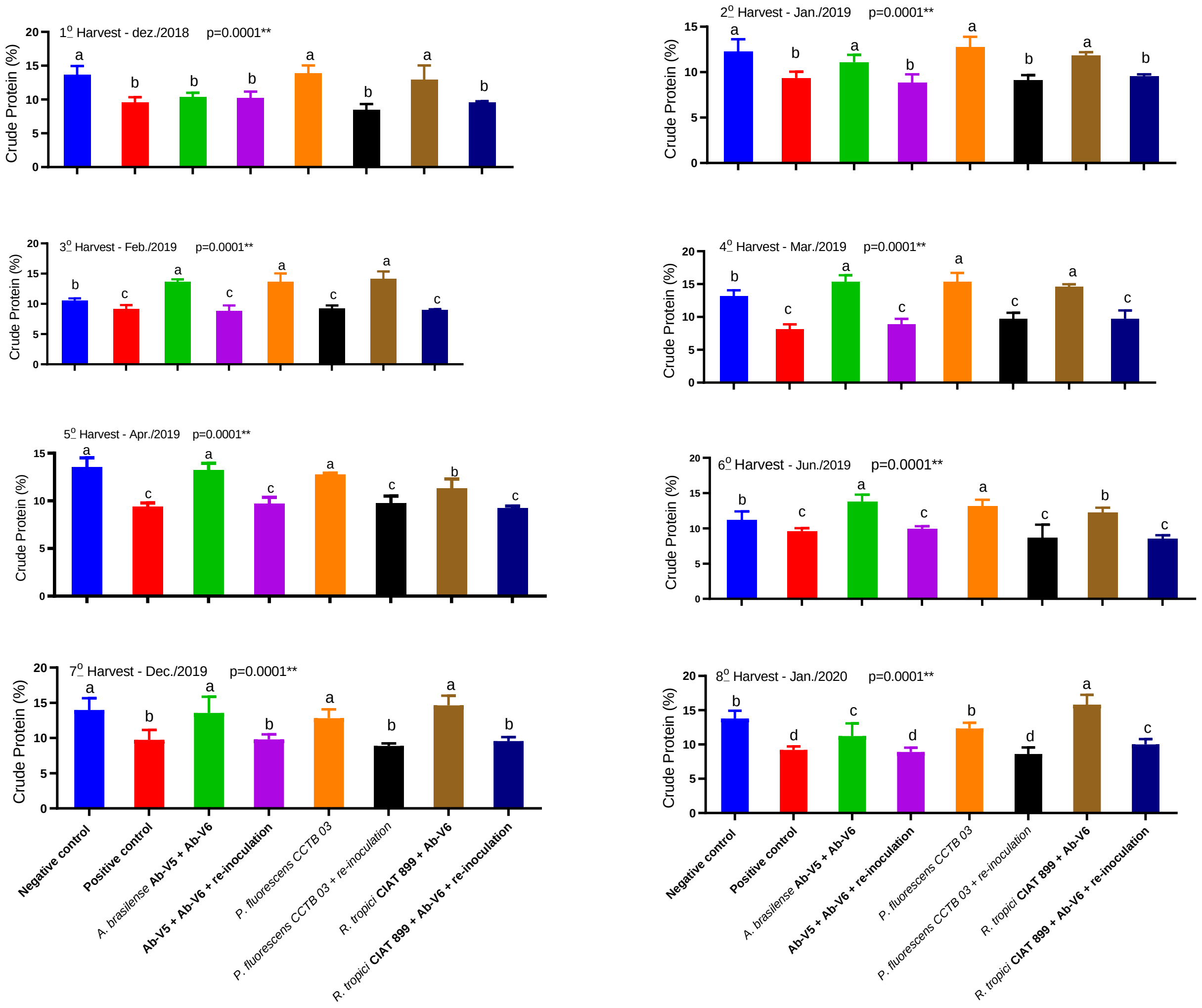


**Figure 9**. Acid detergent fiber (ADF) (%) in the shoots of Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

Statistically significant differences in the crude protein (CP) in the shoots of Zuri Guinea grass were observed in the experiment (Figure 10). The treatments inoculated with PGPB differed from the positive control in the eight evaluation cuts.



1º Harvest - dez./2018 p=0.0001**

2º Harvest - Jan./2019 p=0.0001**

3º Harvest - Feb./2019 p=0.0001**

4º Harvest - Mar./2019 p=0.0001**

5º Harvest - Apr./2019 p=0.0001**

6º Harvest - Jun./2019 p=0.0001**

7º Harvest - Dec./2019 p=0.0001**

8º Harvest - Jan./2020 p=0.0001**

**Figure 10.** Crude protein (CP) (%) in the shoots of 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, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test ($p \leq 0.05$).

Evaluating the effect of inoculation by *A. brasilense* on seeds of *U. brizantha* cv. 'Marandu' associated with the use of N, according to [75] found no significant effects of inoculation for the CP and NDF content of the grass, showing results that differ from those of the present study. According to [76], when evaluating the effect of inoculation with *P. fluorescens* and N levels on the CP content in corn, also did not find significant effects of inoculation, whether combined with N fertilization or not. In the present study, a response of up to 44% increase in CP content was found for bacteria and especially for *P. fluorescens* CCTB 03.

Statistically significant differences in the *in vitro* digestibility of dry matter in the shoots of Zuri Guinea grass were observed in the experiment (Figure 11). The treatments inoculated with PGPB differed from the positive control in the evaluations.

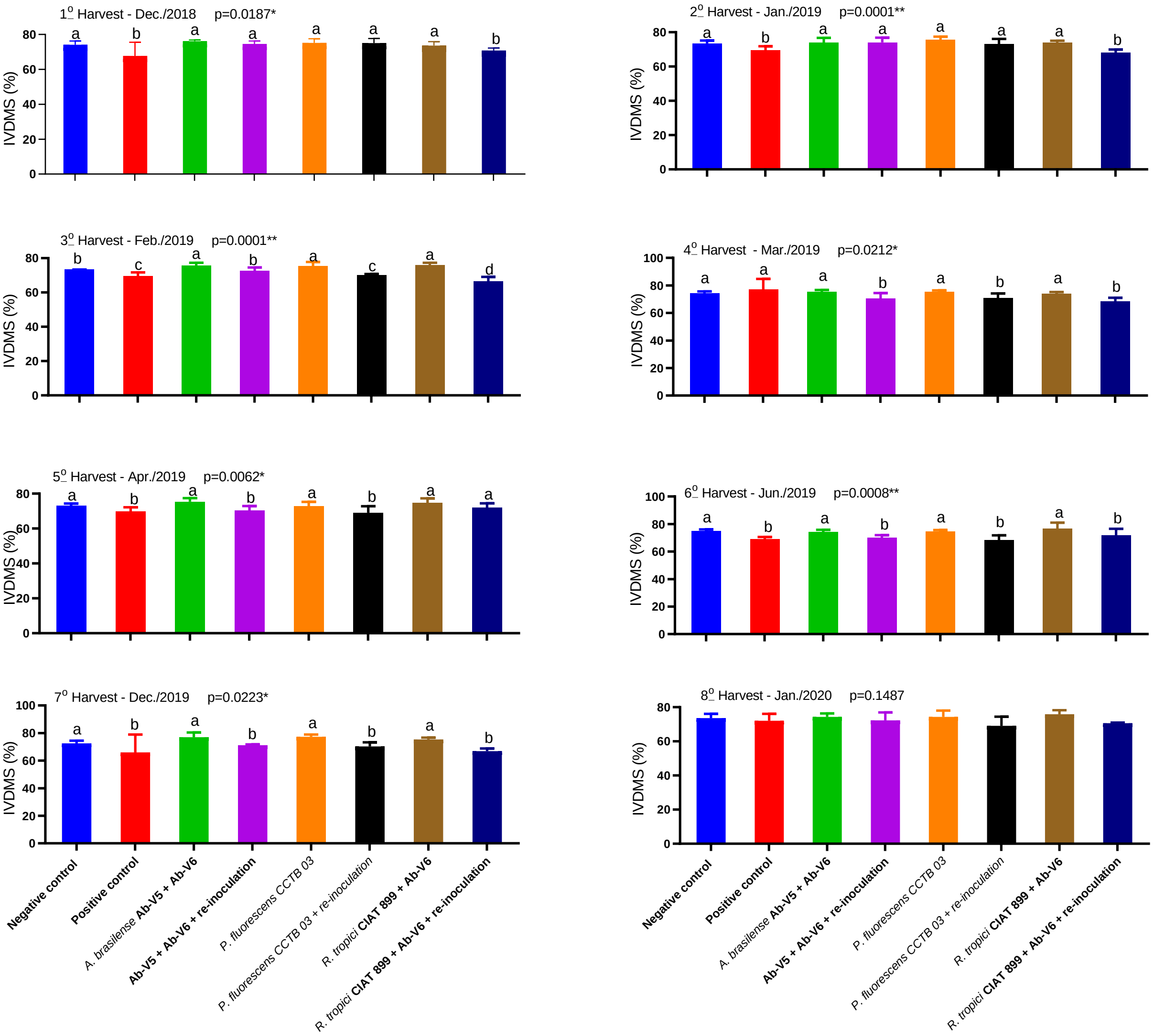


**Figure 11.** In vitro digestibility of dry matter (IVDMS) (%) in the shoots of Zuri Guinea grass inoculated with strains *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium tropici.* T1= Negative control (without N and inoculation), T2= Positive control (with N and without inoculation), T3= *A. brasilense* Ab-V5 + Ab-V6, T4= *A. brasilense* Ab-V5 + Ab-V6 + re-inoculation after cut, T5= *P. fluorescens* CCTB 03, T6= *P. fluorescens* CCTB 03 + re-inoculation after cut, T7= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 and T8= *R. tropici* CIAT 899 + *A. brasilense* Ab-V6 + re-inoculation after cut. Error bars represent the standard errors of the means. Averages followed by lowercase letters differ for treatments by the Scott-Knott test (P≤0.05).

The results of IVDMS are superior to those previously reported [62], who assessed the viability of *A. brasilense* associated with N fertilization in the chemical composition of 'Marandu' grass at different times of the year, and did not find significant effects of inoculation on the levels of IVDMS in summer and winter.

## 5.Conclusions

The PGPB when combined N-fertilizer, increased yield, tillers units, the relative chlorophyll index, the nutrient concentration total N, the uptake total N, NDF, ADF, CP and IVDMS of Zuri Guinea grass. This result indicates that PGPB can be a sustainable alternative for reducing the use of N-fertilizers. The lower 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.

## 6. ACKNOWLEDGMENT

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