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Effect of *Pseudomonas fluorescens* and *Burkholderia pyrrocinia* on the Growth Improvement and Physiological Responses in *Brachiaria brizantha*

Monyck Jeane dos Santos Lopes[1], Moacyr Bernardino Dias Filho[2], Thomaz Henrique dos Reis Castro[1], Marta Cristina Corsi de Filippi[3], Gisele Barata da Silva[1]

[1]Plant Protection Laboratory, Institute of Agricultural Sciences, Federal Rural University of Amazonia (UFRA), Belém, Brazil
[2]Brazilian Agricultural Research Corporation (Embrapa), Belém, Brazil
[3]Brazilian Agricultural Research Corporation (Embrapa), Goiania, Brazil
Email: monyck_lopes@yahoo.com.br, gibarata@bol.com.br

Abstract

The use of beneficial microorganisms in forage grasses is a potentially advantageous technique for a more sustainable pasture management by decreasing the need for chemical fertilization. Our aims were to determine the best method of microorganism inoculation on *Brachiaria* (Syn. *Urochloa*) *brizantha* cv. BRS Piatã, compare the responses of inoculated plants of this forage grass with fertilized and unfertilized controls and examine its effect on some morphological, physiological and biochemical responses. On the first experiment, three inoculation methods were tested: in the seed, seed and soil, and soil, with *Pseudomonas fluorescens* (BRM-32111) and *Burkholderia pyrrocinia* (BRM-32113). In the second experiment, fertilized and unfertilized plants were either inoculated with BRM-32111, BRM-32113 and co-inoculated (BRM-32111 + BRM-32113). In a final experiment, *B. brizantha* was inoculated by soil drenching with BRM-32111, BRM-32113 and co-inoculated (BRM-32111 + BRM-32113), and compared to fertilized- and unfertilized-controls. The inoculation by soil drenching, at seedling stage, was more effective than inoculation only in the seed or both in the seed and by soil drenching. The fertilizer may have suppressed the beneficial bacterial effects on the growth of *B. brizantha*. *P. fluorescens* and *B. pyrrocinia* co-inoculated increased nitrate, protein, nitrogen concentration, Spad index (chlorophyll content), leaf area, number of tillers, net photosynthesis and total biomass production of *B. brizantha* plants. Our results point out to a potentially valuable source of practic-

al information in the search of an eco-friendlier approach to increase pasture productivity.

1. Introduction

Increasing global population coupled with shifting dietary preferences in emerging economies is leading to a substantial increase in the consumption of livestock products, mainly beef. Sustainable intensification of current pasturelands in developing counties is an important tool to meeting future demands for beef [1]. The use of plant growth-promoting microorganisms in pastures is a potentially advantageous technique, as a more economical and eco-friendlier approach to increase pasture productivity, when compared to the sole use of chemical fertilizers [2] [3] [4] [5].

Plant growth-promoting microorganisms are beneficial endophytic or rhizospheric microorganisms, able to colonize roots and directly promote growth by regulating the pathway of plant hormones, increasing the biosynthesis of auxin, cytokinin, gibberellin, or minimizing the ACC synthesis, the ethylene precursor, delaying plant senescence [6] [7]. Another beneficial mechanism of growth-promoting microorganisms is to increase the availability of essential nutrients for plant growth, such as nitrogen and phosphorus, and to promote induced resistance of plant defenses against diseases, pests, and abiotic stressors [6] [7]. Growth promotion, resulting from the association of beneficial microorganisms, has been reported in wheat plants [8], forage grass [5], maize [9] and *Sorghum bicolor* [10].

In Brazil, around 80% of pasture areas are formed by grasses of the *Brachiaria* (Syn. *Urochloa*) genus, among which stands out *B. brizantha*, being the cultivar BRS Piatã one of the major current options for pasture formation [11] [12]. Most of these pasture areas is under low fertility soils, requiring chemical fertilization to produce satisfactorily and, in particular, nitrogen fertilization to intensify pasture management [2] [4] [5]. However, the efficiency of fertilizer uses by plants, particularly that of nitrogen, may vary greatly. This can create adverse environmental impacts by increasing greenhouse gas emissions and eutrophication [13] [14] [15].

The association between *B. brizantha* and diazotrophic bacteria could be highly beneficial, for nitrogen fixation and subsequent transfer of the fixed nitrogen to the host plant, increasing the sustainability of agriculture and reducing its impact on the environment. However, *Brachiaria* genus is known to be very allelopathic [16] [17]. This may deter the establishment of beneficial microorganisms in the rhizosphere [6]. Therefore, research on the optimal inoculation method of beneficial microorganisms is crucial for this grass genus.

Studies proved that diazotrophic rhizobacteria identified as *Pseudomonas fluorescens* and *Burkholderia pyrrocinia*, isolated from the rhizosphere soil, in Pará, Brazil, when inoculated in seed, are growth promoters in rice plants, increased chlorophyll content, photosynthetic rate, nutrient uptake, and biomass production [18] [19]. We hypothesize that these growth-promoting microorganisms are capable of stimulating growth in *B. brizantha* cv. BRS Piatã, grown in low-fertility soils, being an important strategy for the sustainable intensification of pasture production systems. Therefore, the aim of this study was to determine the optimal inoculation method of plant growth promoting microorganisms, and examine their effect on some morphological, physiological, and biochemistry responses of *B. brizantha* cv. BRS Piatã.

2. Materials and Methods

2.1. Study Site, Plant and Soil

The experiment was conducted at the Plant Protection Laboratory and greenhouse of the Federal Rural University of Amazonia (UFRA) (01˚27'25"S, 48˚26'36"W) in Belém, Pará, Brazil. Seeds were sown in polyethylene pots (15 × 25 × 0.5 cm) filled with low-fertility soil (Ferralsol, pH, 4.2; organic matter, 18.80 g·dm^{-3}; P, 2 mg·dm^{-3}; K, 4 mg·dm^{-3}; Ca, 0.2 mmolc·dm^{-3}; Ca + Mg, 0.3 mmolc·dm^{-3}; Al, 1.4 mmolc·dm^{-3}) and kept under greenhouse conditions.

2.2. Preparation of Inoculum

Pseudomonas fluorescens (BRM-32111) and *Burkholderia pyrrocinia* (BRM-32113) are currently stored and preserved in the *in vitro* collection of the Plant Protection Laboratory, at the Federal Rural University of the Amazon. The bacterial isolates were cultured in solid 523 medium for 48 h at 28˚C. The bacterial suspension was prepared in water and adjusted to A540 = 0.2 (10^8 CFU).

2.3. Inoculation forms Tested

- Seed (microbiolized seed): *B. brizantha* seeds were sterilized with 70% ETOH and 2% NaClO, both for 1 minute, washed in sterile water for 1 minute, and placed on sterile filter paper, for 1 hour. Before sowing, the seeds were steeped in the suspensions for for 24 hours, at 28˚C and at constant agitation.
- Seed and Soil: microbiolized seed + soil drenched.
- Soil (soil drenched): 5 mL of suspension of each treatment, bacterial isolates (10^8 CFU) drenched the trial soil at 14 days after seedling emergence (DASE).

2.4. Experiment I

The treatments consisted of two microorganisms, BRM-32111 and BRM-32111 with three inoculation forms and a control. The experimental design was completely randomized with five replications. At 21 DASE, seedlings were harvested and separated into shoot (leaf blades and culms) and roots. Plant material was oven dried (60˚C) until constant mass. Total dry mass (TDM) was calculated by

adding shoot dry mass (SDM) and root dry mass (RDM). The experiment was repeated three times with similar results.

2.5. Experiment II

The treatments consisted of fertilized and unfertilized non-inoculated *B. brizantha* plants, or fertilized plants, inoculated with BRM-32111, BRM-32113 and co-inoculated with BRM-32111 + BRM-32113 (MIX). The experimental design was completely randomized with eight treatments and five replications. Fertilized plants were fertilized with 5 mg dm-3 of N, 14 mg·dm^{-3} of P_2O_5, and 10 mg·dm^{-3} of K_2O. Suspension of bacterial isolates (5 mL, 108 CFU), water drenched the trial soil at 14 DASE. At 21 DASE, seedlings were harvested to determine biomass production. The experiment was repeated three times with similar results.

2.6. Experiment III: Growth Promotion Effects of Microorganism on *B. brizantha*

The treatments consisted of non-inoculated *B. brizantha* fertilized-(positive) and unfertilized-controls (negative), inoculated with BRM-32111, BRM-32113 and co-inoculated with BRM-32111 + BRM-32113 (MIX). The experimental design was completely randomized with five replications and five treatments. All experiment was conducted in a greenhouse, with mean air temperature of 30°C ± 2.5°C and relative humidity of 74% ± 4% (mean ± s.d.), respectively.

Plant growth parameters were calculated according to Hunt (1990) and Barbero *et al.*, (2013) [20] [21]. At 35 DASE, plants were harvested to determine biomass production. We calculated root /shoot dry mass ratio (RDM/SDM). Leaf area (LA) was determined over leaf disks of either 0.42 cm^2 or 2.28 cm^2, dried at 60°C until constant mass. The biomass allocation pattern was estimated as the leaf, culm and root mass ratios (respectively, the ratio between total leaf, culm, and root dry mass per plant and total dry mass per plant).

Five evaluation periods (14, 17, 21, 28 and 35 DASE) were used to determine the number of leaves (NL), height (H), culm length (CL) and chlorophyll content (SPAD index-soil plant analysis development), estimated by a portable chlorophyll meter (SPAD-502. Konica Minolta Sensing, INC. Japan). Relative growth rate (change in total mass per total dry mass of plant per day, RGR) was calculated for harvests at 14 and 35 DASE. Morphogenetic and structural parameters, calculated according to Gomide and Gomide (2000), were: leaf appearance rate (ratio between the difference in the number of initial and final leaves the number of evaluation interval days, LApR), leaf elongation rate (ratio between the difference of the initial and final lengths of the expanded sheets and the number of days of the evaluation interval, LER), number of leaves per plant (NL) and number of tillers per plant (NT) [22].

Net photosynthesis (A), stomatal conductance (gs) and transpiration (E) were measured 35 days after seedling emergence, on one young, fully expanded blade

per plant, with an infrared gas analyzer (IRGA) (LI-6400XT; LICOR, Lincoln, NE). Measurements were made under CO_2 of 400 μmol·m^{-1} and a constant photosynthetic active radiation of 1000 μmol·m^{-2}·s^{-1} (obtained by an artificial light source coupled to the IRGA chamber).

2.7. Biochemical Assays

For determination of free ammonium, nitrate, amino acid, total soluble proteins, and mineral analysis of nitrogen (N) we selected the treatment that promoted the greatest growth in *B. brizantha* (co-inoculated with BRM-32111 + BRM-32113) and two non-inoculated controls.

For determination of the free ammonium, 50 mg of dry matter incubated with 5 mL of sterile distilled water at 100˚C for 30 min, and was centrifuged at 2.000 g for 5 min at 20˚C and the supernatant was removed. The quantification of the free ammonium was carried out at 625 nm in accordance with Weatherburn (1967), with $(NH_4)_2SO_4$ as standard [23].

For determination of nitrate, 100 mg of dry matter was incubated with 5 ml of sterile distilled water at 100˚C for 30 min. The homogenized mixture was centrifuged at 3.000 g for 15 min at 25˚C, and the supernatant was removed. The quantification of the nitrate was carried out at 410 nm in accordance to Cataldo *et al.*, (1975), with KNO_3 as standard [24].

The amino acid was determined using the 50 μl ethanolic extract, 50 μl Na-Citrate (1 M + 0.2% Ascorbic Acid (100 mL NaCitrate + 0.2 g Asc. Acid)) and 100 μl Ninhydrin solution (1%). The mixture was incubated at 95˚C for 20 min. and centrifuged at 12,000 g for 10 s. Absorbance was measured at 570 nm. The calibration curve was made using Leucine (1 mM) (Gibon *et al.*, 2004) [25]. For total soluble proteins, each pellet was vigorously shaken in 1 mL absolute ethanol, incubated at 80˚C for 20 min, and centrifuged at 12,000 g for 5 min., at 4˚C. The supernatant was discarded and the pellet was shaken with 1 mL 0.2 M KOH. After heating for 60 min at 90˚C, samples were cooled and centrifuged at 12,000 g, for 5 min, at 4˚C. Quantification of the total soluble proteins was carried out at 595 nm in accordance with Bradford (1976), with albumin bovine as standard. Shoot mineral analysis of nitrogen (N) was determined by inductively coupled plasma optical emission spectrometry (ICPOES) [26].

2.8. Statistical Analysis

All data were subjected to analysis of variance and variables with significant F values were compared by Duncan test ($P < 0.05$). The LN, H, SL and SPAD were analyzed by ANOVA in a factorial arrangement (evaluation period × treatment). Post hoc contrasts were calculated for assessing differences between controls and inoculated plants for LN, H, SL, SPAD, MST, LA, A, gs and E. Parametric correlation analysis was calculated between SPAD, LA, H, NT, N or A versus MST. The statistical package STATISTICA for Windows release 7 (StatSoft, Inc., Tulsa, USA) was used for all computations of the data.

3. Results

3.1. Inoculation forms Tested

No increase in biomass production could be observed when microorganisms were inoculated solely in the seeds or seed + soil drench (Table 1). When inoculation was performed by soil drench *P. fluorescens* (BRM-32111) and *B. pyrocinia* (BRM-32113) increased biomass production ($F_{1,36} = 1989,23$; $P < 0.01$) by 242% and 112%, respectively (Table 1).

3.2. Effects of Fertilizers and PGPR on *B. brizantha*

No increase in biomass production could be observed when microorganisms were inoculated on fertilized plants (Table 2). However, inoculation of unfertilized plants with BRM-32113, BRM-32111 or Mix (BRM-32111 + BRM-32113) increased biomass production by 95%, 227% and 327%, respectively, relative to non-inoculated unfertilized-control plants ($F_{1,32} = 3123,7$; $P < 0.01$) (Table 2).

3.3. Growth Promotion Effects of Microorganism on *B. brizantha*

The total number of leaves ($F_{1,75} = 537.6$; $P < 0.01$), plant height ($F_{1,75} = 2387.2$; $P < 0.01$), culm length ($F_{1,75} = 241.5$; $P < 0.01$) and Spad index ($F_{1,75} = 963.9$; $P < 0.01$) were higher in inoculated plants (Figure 1 and Figure 2). The beneficial effects of rhizobacteria on *B. brizantha* development could already be observed three days after inoculation (17 DASE) (Figure 2).

Leaf area was increased ($F_{1,20} = 186.9$, $P < 0.01$) by inoculation. This increment was above 700% relative to unfertilized-control plants and 108% relative to fertilized-control plants (Table 3). The RDM/SDM ratio was higher in co-inoculated plants (Table 3). The RGR ranged from 0.1 to 0.17, being higher in co-inoculated plants (Table 3).

The rhizobacteria increased NT and the mean EF (Table 3). The L/C ratio was higher in plants co-inoculated and unfertilized-control plants (Table 3). LApR

Table 1. Inoculation method of plant growth-promoting rhizobacteria on the biomass production of *Brachiaria brizantha*. Shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM).

Inoculation	Isolates	Biomass (mg)		
		SDM	RDM	TDM
Seed	*Pseudomonas* spp.	54 ± 1.03c	29.6 ± 0.89c	83.6 ± 1.71c
	Burkholderia spp.	50.9 ± 1.77c	28.2 ± 0.80cd	79.1 ± 2.46c
Seed + Soil	*Pseudomonas* spp.	55.7 ± 1.01c	26.8 ± 0.97d	82.5 ± 1.08c
	Burkholderia spp.	53.6 ± 0.51c	29.8 ± 0.93c	83.4 ± 1.36c
Soil	*Pseudomonas* spp.	202.2 ± 0.81a	89.2 ± 1.25a	291.4 ± 1.89a
	Burkholderia spp.	123.2 ± 1.21b	57.6 ± 1.44b	180.8 ± 1.93b
	Control	54.6 ± 1.67c	30.8 ± 0.77c	85.4 ± 1.14c

*Significant at the 0.05 probability level. Data are means ± SE. Means followed by different letters in each column are significantly different ($P < 0.05$, Duncan Test).

Table 2. Effects of fertilization and plant growth-promoting rhizobacteria on the biomass production of *Brachiaria brizantha.* Shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM).

Treatments		Biomass (mg)		
		SDM	RDM	TDM
Fertilized	*Pseudomonas* spp.	104.6 ± 0.68d	47.8 ± 0.37d	150.8 ± 0.91d
	Burkholderia spp.	103.1 ± 0.71d	48 ± 0.32d	150.8 ± 0.58d
	Mix	102.8 ± 0.73d	47.8 ± 0.42d	150.4 ± 1.23d
	Control	102.6 ± 0.76d	47.6 ± 0.75d	152.2 ± 1.39d
Unfertilized	*Pseudomonas* spp.	203.8 ± 1.35b	87 ± 0.83b	290.8 ± 2.18b
	Burkholderia spp.	116.4 ± 1.03c	56.6 ± 0.87c	173 ± 1.82c
	Mix	282.4 ± 2.11a	96.6 ± 0.93a	379 ± 2.87a
	Control	58.4 ± 0.81e	30.4 ± 0.67e	88.8 ± 1.24e

*Significant at the 0.05 probability level. Data are means ± SE (n = 5). Means followed by different letters in each column are significantly different (P < 0.05, Duncan Test).

Table 3. Leaf area (LA·cm^2), root dry mass (RDM-g·day^{-1})/shoot dry mass (SDM) ratio, relative growth rate (RGR), number of tillers (NT), expanded leaf length (EF-cm), leaf appearance rate (LApR-L^{-1}·day^{-1}) and leaf elongation rate (LER-cm·day^{-1}) of *Brachiaria brizantha* with growth-promoting rhizobacteria.

Treatments	LA	RDM /SDM	RGR	NT	E	LApR	LER
C−	19.02 ± 1.96e	0.65 ± 0.02b	0.01 ± 0.0004e	0 ± 0d	22.74 ± 0.59d	0.19 ± 0.02e	0.90 ± 0.08e
C+	73.70 ± 13.27d	0.64 ± 0.02b	0.03 ± 0.0003d	1 ± 0c	37.28 ± 0.56c	0.27 ± 0.02d	1.40 ± 0.10d
B	187.20 ± 9.57b	0.54 ± 0.01c	0.12 ± 0.0042c	2 ± 0b	45.66 ± 0.50ab	0.49 ± 0.05c	1.84 ± 0.10c
P	153.51 ± 7.74c	0.62 ± 0.02b	0.13 ± 0.0017b	2 ± 0b	43.54 ± 0.72b	0.66 ± 0.04b	2.53 ± 0.19b
MIX	334.05 ± 25.58a	0.68 ± 0.02a	0.17 ± 0.0036a	4 ± 0a	46.72 ± 0.53a	0.71 ± 0.05a	2.79 ± 0.24a

*Significant at the 0.05 probability level. Data are means ± SE (*n* = 5). Means followed by different letters in each column are significantly different (P < 0.05, Duncan Test). †C− = unfertilized-control; C+ = fertilized-control; B = *Burkholderia pyrrocinia*; P = *Pseudomonas fluorescens*; MIX = B + P.

and LER from inoculated plants were higher than those of the control plants, either fertilized, or unfertilized (Table 3). The photosynthetic rate ($F_{1,20}$ = 364.8, P < 0.01), stomatal conductance ($F_{1,20}$ = 84.24; P < 0.01) and transpiration ($F_{1,20}$ = 72.70, P < 0.01) were higher in inoculated plants (Figure 3).

Biomass production was higher in inoculated plants ($F_{1,20}$ = 2289.5, P < 0.01), with an increment of shoot dry mass of more than 930%, relative to unfertilized-control plants, and over 334% relative to fertilized-control plants (Figure 4(a)). For root dry mass production, this increment was above 770% and 262% relative to unfertilized- and fertilized-control plants, respectively (Figure 4(b)).

When the rhizobacteria were inoculated individually, the increment in total biomass production was over 870% and 300%, respectively, relative to unferti-
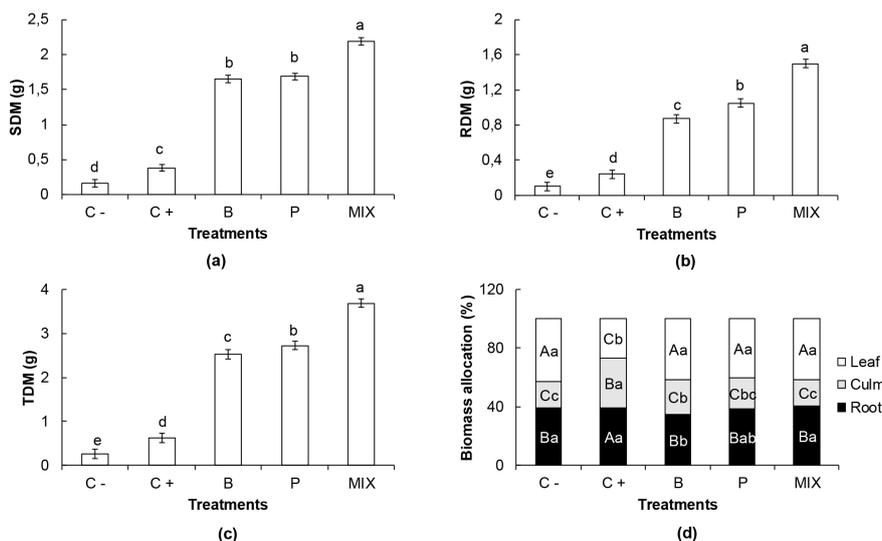
Figure 1. Shoot (a)-(e) and root (f)-(j) of *Brachiaria brizantha*, 21 days after inoculation (35 days after seedling emergence). Unfertilized-control (a) (f), fertilized-control (b) (g), inoculated with *Pseudomonas fluorescens* (BRM-32111) (c) (h), *Burkholderia pyrrocinia* (BRM-32113) (d) (i) and co-inoculated with BRM-32111 + BRM-32113 (e) (j).
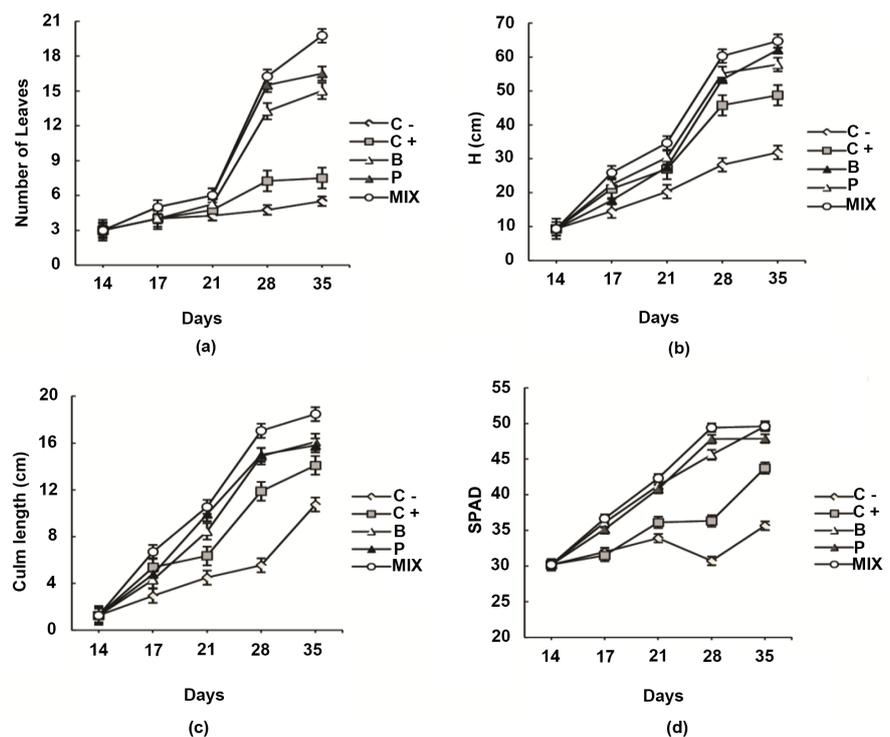
Figure 2. Number of leaves per plant (a), plant height (H) (b), culm length (c) and SPAD index (d) of *Brachiaria brizantha* inoculated with growth-promoting rhizobacteria. Values are means ± SE (*n* = 5). Days = days after seedling emergence. C− = unfertilized-control; C+ = fertilized-control; B = *Burkholderia pyrrocinia;* P = *Pseudomonas fluorescens*; MIX = B + P.
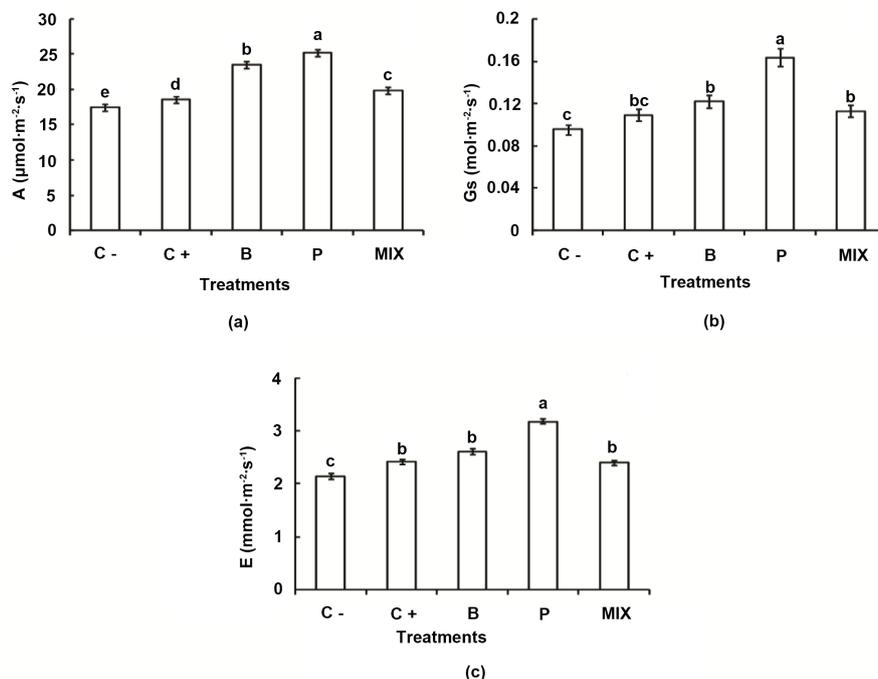
Figure 3. *Brachiaria brizantha* responses to growth-promoting rhizobacteria. (a) Net photosynthesis (*A*), (b) stomatal conductance *(gs)* and (c) transpiration (*E*). At 21 days after inoculation (35 days after seedling emergence). Columns with different letters are significantly different among treatments (P < 0.05, Duncan Test). C− = unfertilized-control; C + = fertilized-control; B = *Burkholderia pyrrocinia*; P = *Pseudomonas fluorescens*; MIX = B + P.

Figure 4. *Brachiaria brizantha* responses to growth-promoting rhizobacteria. (a) Shoot dry mass production (SDM), (b) root dry mass production (RDM), (c) total dry mass production (TDM) and, (d) biomass allocation pattern. 21 days after inoculation (35 days after seedling emergence). Columns with different letters are significantly different among treatments (P < 0.05, Duncan Test). Different upper-case letters within columns indicate significant differences among plant organs (P < 0.05, Duncan Test). C− = unfertilized-control; C + = fertilized-control; B = *Burkholderia pyrrocinia*; P = *Pseudomonas fluorescens*; MIX = B + P.

lized- and fertilized-control plants. The maximum gain in total biomass production was achieved by co-inoculation (MIX), which accounted for an increment of over 1300%, relative to unfertilized-control plants and nearly 500%, when compared to fertilized-control plants (**Figure 4(c)**). Biomass allocation data revealed that, except for fertilized-control plants, in all treatments there was a preferential allocation to leaves (**Figure 4(d)**). The increment in total biomass production correlated positively to the SPAD index, LA, H, NT and A (**Table 4**).

3.4. Biochemical Effects of Microorganism on *B. brizantha*

Relative to unfertilized- and fertilized-control plants, co-inoculated plants showed higher nitrate concentration in leaves (130% and 20%) and roots (60% and 16%); amino acid in the root (135% only relative to unfertilized); protein concentration in the leaves (33% and 12%) and root (142% and 21%) (**Figure 5**) and higher nitrogen concentration in leaves (30% and 11%) and root (75% and 25%) (**Figure 6**). Higher concentrations of ammonium (leaf, root and total) and amino acids (leaves and total) were found in unfertilized-control plants (**Figure 5**).

4. Discussion

P. fluorescens and *B. pyrrocinia* fostered the highest growth in *B. brizantha* cv. Piatã, when inoculated by soil drench, during seedling stage (**Table 1**). Failure to promote plant growth, when these bacteria were inoculated in the seeds, may indicate that, during germination, *B. brizantha* might be able to recognize microbial compounds, synthesizing substances capable of inhibiting the beneficial effects of these rhizobacteria on plant growth promotion [6]. A similar mechanism of plant immune stimulation probably was also activated, when plants were sequentially inoculated, both in the seed and by soil drench, inhibiting growth promotion, could be related to the allelopathic potential of *Brachiaria* (Syn. *Urochloa*) [16] [17]. Under this condition, allelopathy can also affect the rhizosphere microbial community, and may be the cause of the observed lower *Bacillus* spp. colonization in *B. brizantha* rhizosphere [27].

Table 4. Correlation coefficient (r) of the correlations between SPAD, leaf area (LA), plant height (H), number of tillers (NT), photosynthesis (A) and nitrogen concentration (N) versus total dry mass (TDM) of *Brachiaria brizantha*.

Parameters	*r*
SPAD	0.84*
LA	0.95*
H	0.90*
NT	0.96*
A	0.78*
N	0.89*

*P < 0.05.
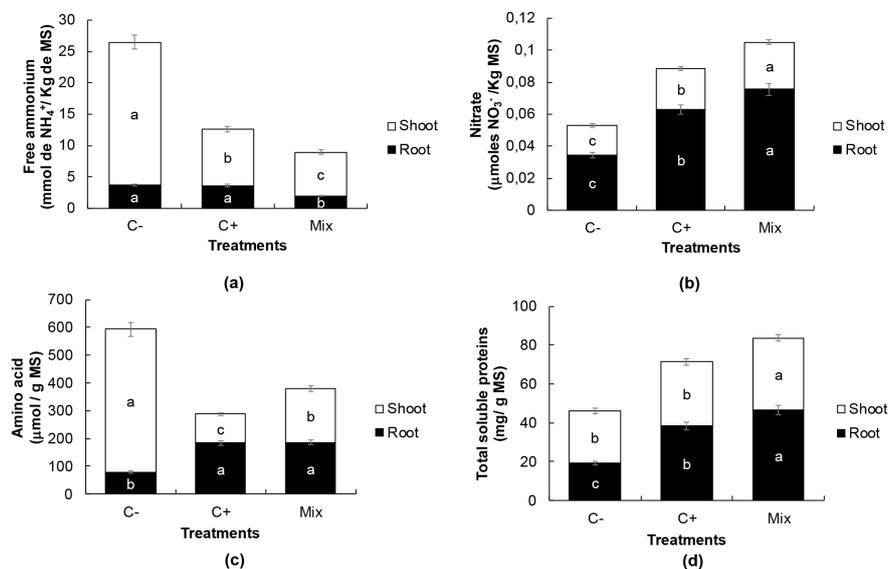
Figure 5. *Brachiaria brizantha* responses to growth-promoting rhizobacteria. (a) Free ammonium, (b) nitrate, (c) amino acid and, (d) total soluble proteins. 21 days after inoculation (35 days after seedling emergence). Columns with different letters are significantly different among treatments (P < 0.05, Duncan Test). C− = unfertilized-control; C+ = fertilized-control; Mix = *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*.
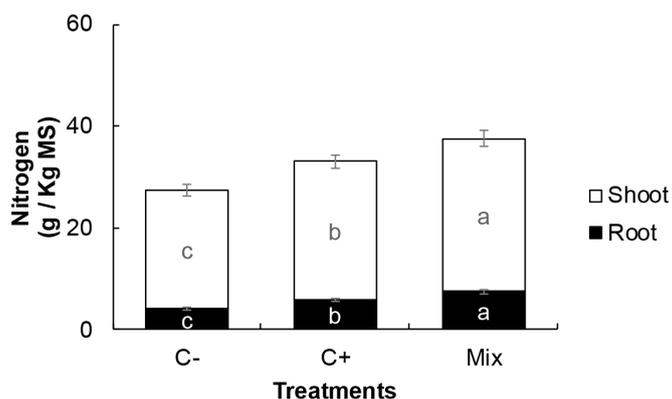
Figure 6. Nitrogen concentration of *Brachiaria brizantha* inoculated with growth-promoting rhizobacteria. 21 days after inoculation (35 days after seedling emergence). Columns with different letters are significantly different among treatments (P < 0.05, Duncan Test). C− = unfertilized-control; C+ = fertilized-control; Mix= *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*.

The amount allelopathic root exudates compounds may also vary during the plant's developmental stage [6]. However, allelopathic compounds exudated by *Brachiaria* roots are known to have no inhibitory effects at low concentrations [16]. Thus, we can infer that the allelopathic compounds, detrimental to rhizobacteria, possibly exuded by *B. brizantha* roots in our study, might have decreased over time, because there was an increased growth of *B. brizantha* plants, inoculated by soil drench (Table 1).

Soil fertilization was antagonistic the bacterial activities on the growth of *B. brizantha* (Table 2). On the other hand, inoculation with rhizobacteria increased growth in unfertilized plants (*i.e.*, exposed to nutrient limitation) (Table 1). It could be inferred that roots of unfertilized plants modified rhizodeposition patterns, by secreting specific compounds, resulting in an increased microbial biomass and activity around the roots [6] [28]. In maize plants, the nutritional status affects the root colonizing bacterium, stimulating the repression of genes associated with protein synthesis, changing the composition of root exudates, and influencing the physiology of associative bacteria [28]. In temperate grasslands, Keuter *et al.*, (2014) observed that fertilization decreases non-symbiotic biological N fixation, through the inhibition of nitrogenase [14]. The higher N-fertilizer doses also reduce of the beneficial bacterial effects on the growth in wheat [8] [29] and *Sorghum bicolor* plants [10].

Our results attest the potential of *P. fluorescens* and *B. pyrrocinia* for increasing plant growth in *B. brizantha* cv. Piatã (Figure 1). It seems that *P. fluorescens* and *B. pyrrocinia* probably acted synergistically in co-inoculated plants, as plant growth was higher when they were inoculated individually (Figure 4). Increases in biomass production in *B. brizantha* were of over 20% and 14%, after seed inoculation with *Bacillus* [30] and *Azospirilum brasilense* [4], and of over 100%, after root inoculation with endophytic bacteria [3].

Tillering, root development and a high root/shoot dry mass ratio are important features for an efficient pasture establishment. In the present study, these attributes increased in co-inoculated plants as result of an increased nitrogen concentration in roots. Greater tillering and root biomass were also reported for *Panicum virgatum* inoculated with *Burkholderia phytofirmans* [31]. Inoculated plants developed a higher leaf length, area, and number, probably increasing their light capture ability. In addition, net photosynthesis, evaluated on an area basis, was enhanced by inoculation. These improved responses might have contributed to the increased relative growth rate and biomass production measured on those plants. The positive effect of plant growth-promotion rhizobacteria on net photosynthetic is also reported in rice [19].

The relatively lower net photosynthetic rates of the co-inoculated plants, relative to plants individually inoculated, could be attributed to a likely more advanced physiological stage of these fast-growing, co-inoculated plants. In this regard, Wang *et al.* (2015) report a faster decline, with plant age, in the rates of photosynthesis, transpiration and stomatal conductance in *Panicum virgatum* inoculated with *Burkholderia phytofirman* [32]. That is, *B. phytofirman* accelerated development and maturation in *Panicum virgatum* seedlings, as well as induced earlier senescence and flowering in adult plants [32]. According to Larcher (2006), gas exchange ability changes during plant development, tending to correlate negatively with the physiological stage [33]. As the co-inoculated plants showed a higher number of tillers and relative growth rate, we could assume these plants were in a more advanced physiological stage.

In co-inoculated plats, the amino acids had rapid conversion into proteins,

increasing nitrogen concentration and Spad index (chlorophyll content). This increased the development of photosynthetic organs, enhancing leaf length, leaf appearance rate, leaf area and biomass allocation to the leaves. These are desirable characteristics for forage grasses, since leaf blades are the preferred nutrient source for ruminants, for their higher protein content and digestibility. The inoculation with *Azospirilum brasilense* also promoted greater nitrogen uptake and biomass production in *B. brizantha* [4] and in wheat plants [8] [29]. Higher Spad index, nitrogen content and biomass production were also reported in *Brachiaria* with bacterial endophytes, under low nutrient conditions [3].

The increase in nitrate and nitrogen concentrations observed in the tissues of the inoculated plants is probably a response of organic matter mineralization by the rhizobacteria [15], followed by nitrification. It is possible that the rhizobacteria alters the nitrate fluxes at the root plasma membrane [15] [29], decreases the nitrate concentration at the root cell surface (rhizosphere), stimulating root development and increasing nitrate uptake capacity [13].

In forage grass, nitrate fertilization increases the protein contents and biomass production, but in excess can be toxic to cattle (0.35 to 0.45 dag/kg) [34] [35]. In our study, levels of nitrate in the leaves of co-inoculated plants did not reach toxic levels. In addition, the increase in nitrate concentration in *B. brizantha*, could improve its resistance to spittlebug attacks, as a higher nitrate concentration in the xylem is known to impair spittlebug nymphal development [36].

Our results showed that inoculation of rhizobacteria by soil drench, at seedling stage, enhanced beneficial morphological and physiological characteristics, and revealed a direct positive effect of plant growth-promoting rhizobacteria on biomass production of *B. brizantha* cv. Piatã, cultivated on a low-fertility soil. Because, nitrogen concentration and Spad index (chlorophyll content) was highly and positively correlated to total biomass production (Table 4). In addition, this might have contributed to higher net photosynthesis found in inoculated plants (Table 4). The higher biomass production of inoculated plants also related to greater leaf area and the number of tillers, which, in turn, we could infer, was possibly favored by an enhanced auxin biosynthesis in inoculated plants (Table 4). This, relationship will be investigated in future studies on changes in the plant hormones pathway of inoculated *B. brizantha* plants.

5. Conclusion

The inoculation of *P. fluorescens* and *B. pyrrocinia* by soil drenching, at seedling stage, was more effective for promoting growth in *B. brizantha* cv. Piatã. The fertilizer may have suppressed the beneficial bacterial effects on the growth of *B. brizantha*. *P. fluorescens* and *B. pyrrocinia* co-inoculated increased nitrate, protein, nitrogen concentration, Spad index (chlorophyll content), leaf area, number of tillers, net photosynthesis and total biomass production of *B. brizantha* plants. Our results point out to a potentially valuable source of practical information in the search of an eco-friendlier approach to increase pasture productivity.

Acknowledgements

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