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MONYCK JEANE DOS SANTOS LOPES

BIOPROMOTORES DE CRESCIMENTO EM *Brachiaria brizantha* cv. BRS PIATÃ: SELEÇÃO, ASPECTOS MORFOFISIOLÓGICOS, ANATÔMICOS E BIOQUÍMICOS

BELÉM
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Tese apresentada ao Programa de Pós-Graduação em Agronomia da Universidade Federal de Rural da Amazônia, como requisito para obtenção do título de Doutor em Agronomia.
Orientadora: Profª. Dra. Gisele Barata da Silva
Co-orientador: PhD. Moacyr Bernardino Dias-Filho

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20 de junho de 2017

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LISTA DE FIGURAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ABREVIATURAS

BRM-32111 - *Pseudomonas fluorescens*

BRM-32113 - *Burkholderia pyrrocinia*

MIX - *Pseudomonas fluorescens* + *Burkholderia pyrrocinia*

PGPR – Rizobactérias promotoras de crescimento em plantas

H ‑ Altura

TDM ‑ Massa seca total

SDM ‑ Massa seca da parte aérea

RDM – Massa seca radicular

RGR - Taxa de crescimento relativo

gs ‑ Condutância estomática

E ‑ Transpiração

A ‑ Fotossíntese

WUE – Eficiência do uso de água

LA - Área foliar

SLA ‑ Área foliar especifica

LApR - Taxa de aparecimento foliar

LER - Taxa de alongamento foliar

CER - Taxa de alongamento do colmo

NT - Número de perfilhos

EF- Folha expandida

NL – Número de folhas

NDF – Fibra detergente neutro

ADF – Fibra detergente ácido

RESUMO

O uso de microrganismos benéficos em pastagens é uma técnica potencialmente vantajosa para o manejo sustentável, reduzindo a adubação química na manutenção das pastagens ainda produtivas e na recuperação de áreas degradadas, em sistemas convencionais e integrados. Os objetivos deste estudo foram: determinar o melhor método de inoculação de microrganismos capazes de promover o crescimento da gramínea forrageira *Brachiaria* (Syn. *Urochloa) brizantha* cv. Piatã; examinar o seu efeito sobre aspectos morfológicos, anatômicos, fisiológicos, bioquímicos e nutricionais; e verificar o efeito dos biopromotores no crescimento dessa gramínea sob diferentes intensidades luminosas. A seleção foi *in vivo* com três métodos de inoculação: sementes, sementes e rega, e apenas por rega do solo. Os ensaios foram conduzidos em casa de vegetação e em viveiro sob pleno sol e sombra na Universidade Federal Rural da Amazônia (UFRA) (01º27'25"S, 48º26'36"W), em Belém, Pará, Brasil. Foram avaliados parâmetros de morfogênese, anatômicos, fisiológicos, bioquímicos e nutricionais. O uso de *Pseudomonas fluorescens* (BRM-32111) e *Burkhoderia pyrrocinia* (BRM-32113), coinoculadas pela rega do solo, modificou a anatomia (folha, colmo e raiz), incrementou as taxas fotossintéticas (14%), eficiência do uso da água (22%), índice Spad (37%), clorofila (122%), nitrato (100%), proteínas (80%), carboidratos totais (29%), amido (133%), N (30%), P (20%), Mg (20%) e Fe (180%), promovendo uma maior qualidade e produção de biomassa em *B. brizantha.* Também promoveram o crescimento em *B. brizantha* sob diferentes condições luminosas, incrementando em mais de 100% a produção de biomassa, em comparação as plantas controles com e sem adubo. O método de inoculação e a intensidade de luz foram fundamentais para determinar o potencial do microrganismo como promotor do crescimento em *B. brizantha*. Nosso estudo evidencia o potencial biofertilizante da coinoculação de *P. fluorescens* e *B. pyrrocinia* em *B. brizantha* cv. BRS Piatã sob diferentes intensidades de luz, possibilitando seu uso em sistemas convencionais e integrados, contribuindo para a obtenção de pastagens mais produtivas e de maior qualidade.

Palavras-chave: Inoculação. Forrageira. BRS Piatã. *Pseudomonas fluorescens. Burkhoderia pyrrocinia*. Biomassa.

ABSTRACT

The inoculation of beneficial microorganisms in forage grasses is a potentially advantageous technique for sustainable pasture management by decreasing the need for chemical fertilization to maintain pasture productivity and reclaim degraded pastures, under both conventional and integrated systems (i.e., subjected to shade). The aim of this study was to determine the most effective method of microorganism inoculation on *Brachiaria* (Syn. *Urochloa) brizantha* cv. BRS Piatã, by examining its effect on selected morphological, anatomical, physiological, biochemical and qualitative traits. In addition, we examined the impact of inoculation on the growth characteristics of this forage grass cultivar, grown under contrasting light conditions. Screening tests were carried out *in vivo,* for three methods of inoculation: in the seed, seed and soil, and soil. The experiment was conducted in a greenhouse, under full sun and shade at the Federal Rural University of Amazonia (UFRA) (01º27'25 "S, 48º26'36" W) in Belém, Pará, Brazil. *Pseudomonas fluorescens* (BRM-32111) and *Burkhoderia pyrrocinia* (BRM-32113), when co-inoculated by soil drenching, modified the anatomy (leaf, stem, root), increased the photosynthetic rate (14%), water use efficiency (22%), Spad index (37%), chlorophyll (122%), nitrate (100%), proteins (80%), total carbohydrates (29%), starch (133%), N (30%), P (20%), Mg (20%) and Fe (180%) contents and increased forage quality and biomass production of *B. brizantha* cv. BRS Piatã. Co-inoculation promoted growth of *B. brizantha* under contrasting light conditions, increasing biomass production by more than 100% relative to unfertilized- and fertilized-control (non-inoculated) plants. Our results imply that the method of inoculation and the light environment are important in determining the potential of microorganism for promoting plant growth. This study attests that co-inoculation of *P. fluorescens* and *B. pyrrocinia* are effective biofertilizers to increase *B. brizantha* productivity and forage quality under full sun and shaded environments.

Keywords: Inoculation. Forage grass. BRS Piatã. *Pseudomonas fluorescens*, *Burkholderia pyrrocinia.* Biomass.

SUMÁRIO

CONTEXTUALIZAÇÃO

O Brasil é um dos maiores produtores e exportadores de carne bovina do mercado mundial (USDA, 2017). A razão para isso é que a criação do rebanho bovino brasileiro é a pasto, o que torna a produção mais econômica. As pastagens brasileiras tem grande potencial produtivo, mas apenas 30% desse potencial esta sendo explorado (STRASSBURG et al., 2014). Uma das principais causas dessa baixa produtividade é o aumento de pastagens degradadas (DIAS-FILHO, 2011).

Para atender a crescente demanda mundial por produtos de origem animal, com menor impacto ambiental, é necessário aumentar a produtividade das pastagens ainda ativas e recuperar as que estão degradadas (DIAS-FILHO, 2011; PAGANO et al., 2017). Uma das formas de aumentar a produção nos pastos do Brasil, sem maior expansão das áreas, é pelo manejo da fertilidade do solo, mas seu uso é limitado, pois as adubações periódicas têm alto custo (DIAS-FILHO, 2011). Uma solução para uma produção menos onerosa e com menor impacto ambiental seria substituir os insumos químicos por biopromotores de crescimento do vegetal (PAGANO et al., 2017; TIMMUSK et al., 2017; DUCHENE et al., 2017).

Os biopromotores são microrganismos benéficos que promovem o crescimento vegetal por regular a rota metabólica dos fitormônios e tornar os nutrientes do solo disponíveis (DOORNBOS et al., 2012; PAGANO et al., 2017; TIMMUSK et al. 2017). O efeito positivo das rizobactérias promotoras de crescimento é relatado em várias Poaceae, como: arroz (REGAR & YADAV, 2017), cana-de-açúcar (SANTOS et al., 2017), milho (CALVO et al., 2017) e forrageiras (HUNGRIA et al., 2016; MARQUES et al., 2017).

No Brasil, cerca de 80% das áreas de pastagens são formadas por gramíneas do gênero *Brachiaria* (Syn. *Urochloa*), uma forrageira perene, com elevado crescimento, tolerância ao pastejo e alta disponibilidade de forragem (REIS et al., 2013; PAGANO et al., 2017). Dentre as cultivares de *B. brizantha* disponíveis no mercado, destaca-se a BRS Piatã, por apresentar menor alongamento da haste e sazonalidade na produção, maior acúmulo de forragem durante o período seco e desempenho animal, e ser mais tolerante à cigarrinha-das-pastagens em relação as outras cultivares dessa espécie (REIS et al., 2013).

No entanto, o alto potencial alelopático de *Brachiaria* (SOUZA FILHO et al., 2005; KATO-NOGUCHI et al., 2014) e as diferentes intensidades de luz nas pastagens em sistemas convencionais e integrados, podem interferir na interação mutualística entre plantas e

microrganismos benéficos (AGUILAR-CHAMA & GUEVARA, 2016; KONVALINKOVÁ & JANSA, 2016; VIMAL et al., 2017).

Estudos prévios com *Pseudomonas* sp. e *Burkolderia* sp. atestam seu potencial como promotor de crescimento em arroz (RÊGO et al., 2014, NASCENTE et al., 2016). Estas rizobactérias são conhecidas por aumentarem a síntese de auxina, absorção de nutrientes, teor de clorofila, taxa fotossintética e produção de biomassa (AHEMAD & KIBRET, 2014). Nossa hipótese é que *Burkhoderia pyrrocinia* e *Pseudomonas fluorescens* também são capazes de promover o crescimento em *Brachiaria brizantha* cv. BRS Piatã (Hochst. ex A. Rich.) Stapf., aumentando a produtividade das pastagens, em sistemas convencionais e integrados, de uma forma sustentável e promissora. Portanto, nosso objetivo geral é determinar o melhor método de inoculação de *B. pyrrocinia* e *P. fluorescens* e verificar seus efeitos no crescimento em *B. brizantha* cv. BRS Piatã. Para alcançar o objetivo, essa pesquisa foi dividida nos seguintes artigos:

Artigo 1: *Pseudomonas fluorescens* **and** *Burkholderia pyrrocinia* **growth improvement and physiological responses of** *Brachiaria brizantha*

Esse artigo apresenta o melhor método de inoculação de *P. fluorescens* e *B. pyrrocinia*, verificando a sua influência sobre as características morfológicas, fisiológicas e bioquímicas de *B. brizantha*.

Artigo 2: **Valor nutritivo, produção de biomassa e parâmetros anatômicos de** *Brachiaria brizantha* **com rizobactérias promotoras de crescimento (PGPR)**

Nesse artigo avaliam-se os efeitos da co-inoculação de *P. fluorescens* e *B. pyrrocinia* na anatomia, bioquímica, qualidade nutricional e produção de biomassa de *B. brizantha*.

Artigo 3: **Light and plant growth-promoting rhizobacteria (PGPR) effects on** *Brachiaria brizantha* **growth and phenotypic plasticity to shade**

Nesse artigo avaliam-se os efeitos da intensidade de luz e da inoculação de *P. fluorescens* e *B. pyrrocinia* no crescimento, plasticidade fenotípica e tolerância à sombra de *B. brizantha.*

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Pseudomonas fluorescens **and** *Burkholderia pyrrocinia* **growth improvement and physiological responses of** *Brachiaria brizantha*[1]

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

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. 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, *U. 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

[1] Este capítulo segue as normas de formatação da **Applied Soil Ecology**.

valuable source of practical information in the search of an eco-friendlier approach to increase pasture productivity.

Highlights

- Inoculation method defines the potential of microorganism for promoting plant growth.
- *P. fluorescens* and *B. pyrrocinia* increased *Brachiaria brizantha* biomass production.
- Their co-inoculation was an effective biofertilizer in *B. brizantha.*

Keywords: forage grass, *Urochloa*, plant growth-promoting rhizobacteria, fertilized, nitrate, nitrogen.

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 (Strassburg et al., 2014). 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 (Dias-Filho, 2011; Kelemu et al., 2011; Hungria et al., 2016; Marques et al., 2017).

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 (Doornbos et al., 2012; Timmusk et al., 2017). Another beneficial mechanism of growth-promoting microorganisms are 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 (Doornbos et al., 2012; Timmusk et al., 2017). Growth promotion, resulting

48 from the association of beneficial microorganisms, has been reported in wheat plants (Silveira
49 et al., 2016), forage grass (Marques et al., 2017) and *Sorghum bicolor* (Santos et al., 2017).
50 In Brazil, around 80% of pasture areas are formed by grasses of the *Brachiaria* (Syn.
51 *Urochloa*) genus, among which stands out *B. brizantha*, being the cultivar BRS Piatã one of the
52 major current options for pasture formation (Valle et al., 2014). Most of these pasture areas is
53 under low fertility soils, requiring chemical fertilization to produce satisfactorily and, in
54 particular, nitrogen fertilization to intensify pasture management (Dias-Filho, 2011; Hungria et
55 al., 2016; Marques et al., 2017). However, the efficiency of fertilizer use by plants, particularly
56 that of nitrogen, may vary greatly. This can create adverse environmental impacts by increasing
57 greenhouse gas emissions and eutrophication (Mantelin and Touraine, 2003; Keuter et al., 2014;
58 Pii et al., 2015).
59 The association between *B. brizantha* and diazotrophic bacteria could be highly
60 beneficial, for nitrogen fixation and subsequent transfer of the fixed nitrogen to the host plant,
61 increasing the sustainability of agriculture and reducing its impact on the environment.
62 However, *Brachiaria* genus is known to be very allelopathic (Souza Filho et al., 2005; Kato-
63 Noguchi et al., 2014). This may deter the establishment of beneficial microorganisms in the
64 rhizosphere (Doornbos et al., 2012). Therefore, research on the optimal inoculation method of
65 beneficial microorganisms is crucial for this grass genus.
66 Studies conducted at the Federal Rural University of Amazon, proved that diazotrophic
67 rhizobacteria identified as *Pseudomonas fluorescens* and *Burkholderia pyrrocinia*, isolated
68 from the rhizosphere soil, in Pará, Brazil, are growth promoters in rice plants (Rego et al., 2014;
69 Nascente et al., 2016). We hypothesize that these growth-promoting microorganisms are
70 capable of stimulating growth in *B. brizantha* cv. BRS Piatã, grown in low-fertility soils, being
71 an important strategy for the sustainable intensification of pasture production systems.
72 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 biochemical 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) (01º27'25 "S, 48º26'36" W) in Belém, Pará, Brazil. Seeds were sown in polyethylene pots (15 x 25 x 0.05 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 48h at 28◦C. The bacterial suspension was prepared in water and adjusted to A540 = 0.2 (10^8 CFU/mL).

2.2 Inoculation forms tested

- *Seed* (microbiolized seed): *U. 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).

2.3 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.

2.4 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, 10^8 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.

2.5 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 (MIX). The experimental design was completely randomized with five replications and five treatments. The all experiment was conducted in a greenhouse, with mean air temperature of 30 ± 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). 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 60ºC 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).

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).

2.4 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.

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.

The amino acid was determined using the 50 µl ethanolic extract, 50 µl Na-Citrate (1M + 0,2% Ascorbic Acid (100mL 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 12000g for 10s. Absorbance was measured at 570 nm. The calibration curve was made using Leucine (1mM) (Gibon et al., 2004). For total soluble proteins, each pellet was vigorously shaken in 1 mL absolute ethanol, incubated at 80 ºC for 20 min, and centrifuged at 12000g for 5 min., at 4 ºC. The supernatant was discarded and the pellet was shaken with 1 mL 0.2 M KOH. After heating for 60 min at 90 ºC, samples were cooled and centrifuged at 12,000g, for 5 min, at 4 ºC. 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).

2.5 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 x 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. pyrrocinia* (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 (Fig. 1 and 2). The beneficial effects of rhizobacteria on *B. brizantha* development could already be observed three days after inoculation (17 DASE) (Fig. 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/ADM 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 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 (Fig. 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 (Fig. 4a). For root dry mass production, this increment was above 770% and 262% relative to unfertilized- and fertilized-control plants, respectively (Fig. 4b).

When the rhizobacteria were inoculated individually, the increment in total biomass production was over 870% and 300%, respectively, relative to unfertilized- 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 (Fig. 4c). Biomass allocation data revealed that, except for fertilized-control plants, in all treatments there was a preferential allocation to leaves (Fig. 4d). The increment in total biomass production correlated positively to the SPAD index, LA, H, NT and *A* (Table 4).

3.3 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%), roots (60% and 16%) and total biomass (100% and 18%); amino acid in the root (135%); protein concentration in the leaves (33% and 12%), root (142% and 21) and total biomass (80% and 6%) (Fig. 5) and higher nitrogen concentration in leaves (30% and 11%), root (75% and 25%) and total biomass (27% and 19%) (Fig. 6). Higher concentrations of ammonium (leaf, root and total) and amino acids (leaves and total) were found in unfertilized-control plants (Fig. 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

219 growth, when these bacteria were inoculated in the seeds, may indicate that, during germination,
220 *B. brizantha* might be able to recognize microbial compounds, synthesizing substances capable
221 of inhibiting the beneficial effects of these rhizobacteria on plant growth promotion (Doornbon
222 et al., 2012). A similar mechanism of plant immune stimulation probably was also activated,
223 when plants were sequentially inoculated, both in the seed and by soil drench, inhibiting growth
224 promotion, could be related to the allelopathic potential of *Urochloa* (Souza Filho et al., 2005;
225 Kato-Noguchi et al., 2014). Under this condition, allelopathy can also affect the rhizosphere
226 microbial community, and may be the cause of the observed lower *Bacillus* spp. colonization
227 in *B. brizantha* rhizosphere (Araujo and Pedroso, 2013).

228 The amount allelopathic root exudates compounds may also vary during the plant´s
229 developmental stage (Doornbos et al., 2012). However, allelopathic compounds exudated by
230 *Brachiaria* roots are known to have no inhibitory effects at low concentrations (Souza Filho et
231 al., 2005). Thus, we can infer that the allelopathic compounds, detrimental to rhizobacteria,
232 possibly exuded by *B. brizantha* roots in our study, might have decreased over time, because
233 there was an increased growth of *B. brizantha* plants, inoculated by soil drench (Table 1).

234 Soil fertilization was antagonistic the bacterial activities on the growth of *B. brizantha*
235 (Table 2). On the other hand, inoculation with rhizobacteria increased growth in unfertilized
236 plants (i.e., exposed to nutrient limitation) (Table 2). It could be inferred that roots of
237 unfertilized plants modified rhizodeposition patterns, by secreting specific compounds,
238 resulting in an increased microbial biomass and activity around the roots (Doornbos et al., 2012;
239 Carvalhais et al., 2013). In maize plants, the nutritional status affects the root colonizing
240 bacterium, stimulating the repression of genes associated with protein synthesis, changing the
241 composition of root exudates, and influencing the physiology of associative bacteria
242 (Carvalhais et al., 2013). In temperate grasslands, Keuter et al., (2014) observed that
243 fertilization decreases non-symbiotic biological N fixation, through the inhibition of

244 nitrogenase. The higher N-fertilizer doses also reduce of the beneficial bacterial effects on the
245 growth in wheat (Saubidet et al., 2002; Silveira et al., 2016) and *Sorghum bicolor* plants (Santos
246 et al., 2017).

247 Our results show the potential of *P. fluorescens* and *B. pyrrocinia* for increasing plant
248 growth in *B. brizantha* cv. Piatã (Fig. 1). It seems that *P. fluorescens* and *B. pyrrocinia* probably
249 acted synergistically in co-inoculated plants, as plant growth was higher when they were
250 inoculated individually (Fig. 4). Increases in biomass production in *B. brizantha* were of over
251 20% and 14%, after seed inoculation with *Bacillus* (Araujo et al., 2012) and *Azospirilum*
252 *brasilense* (Hungria et al., 2016), and of over 100%, after root inoculation with endophytic
253 bacteria (Kelemu et al., 2011).

254 Tillering, root development and a high root /shoot dry mass ratio are important features
255 for an efficient pasture establishment. In the present study, these attributes increased in co-
256 inoculated plants as result of an increased nitrogen concentration in roots. Greater tillering and
257 root biomass were also reported for *Panicum virgatum* inoculated with *Burkholderia*
258 *phytofirmans* (Kim et al., 2012). Inoculated plants developed a higher leaf length, area, and
259 number, probably increasing their light capture ability. In addition, net photosynthesis,
260 evaluated on an area basis, was enhanced by inoculation. These improved responses might have
261 contributed to the increased relative growth rate and biomass production measured on those
262 plants. The positive effect of plant growth-promotion rhizobacteria on net photosynthetic is also
263 reported in rice (Nascente et al., 2016).

264 The relatively lower net photosynthetic rates of the co-inoculated plants, relative to
265 plants individually inoculated, could be attributed to a likely more advanced physiological stage
266 of these fast-growing, co-inoculated plants. In this regard, Wang et al., (2015) report a faster
267 decline, with plant age, in the rates of photosynthesis, transpiration and stomatal conductance
268 in *Panicum virgatum* inoculated with *Burkholderia phytofirman.* That is, *B. phytofirman*

accelerated development and maturation in *Panicum virgatum* seedlings, as well as induced earlier senescence and flowering in adult plants. According to Larcher (2006), gas exchange ability changes during plant development, tending to correlate negatively with the physiological stage. 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* (Hungria et al., 2016) and in wheat plants (Saubidet et al., 2002; Silveira et al., 2016). Higher Spad index, nitrogen content and biomass production were also reported in *Brachiaria* with bacterial endophytes, under low nutrient conditions (Kelemu et al., 2011).

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 (Pii et al., 2015), followed by nitrification. It is possible that the rhizobacteria alters the nitrate fluxes at the root plasma membrane (Saubidet et al., 2002; Pii et al., 2015), decreases the nitrate concentration at the root cell surface (rhizosphere), stimulating root development and increasing nitrate uptake capacity (Mantelin and Touraine, 2003).

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) (Corrêa et al., 2007; Costa et al., 2009). 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 (Thompson, 2004).

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 3). In addition, this might have contributed to higher net photosynthesis found in inoculated plants (Table 3). The higher biomass production of inoculated plants also related to greater leaf area and the number of tillers (Table 3), which, in turn, we could infer, was possibly favored by an enhanced auxin biosynthesis in inoculated plants. This, relationship will be investigated in future studies on changes in the plant hormones pathway of inoculated *B. brizantha* plants.

Furthermore, based on our results, it could be stated that the method of inoculation is key in determining the potential of microorganism for promoting plant growth. This is because inoculation by soil drench, at seedling stage, was more effective than inoculation only in the seed or both in the seed and by soil drench. Also, the fertilizer may have suppressed the beneficial bacterial effects on the growth of *B. brizantha*. Our results attested that *P. fluorescens* and *B. pyrrocinia* could promote growth in *B. brizantha* cv. Piatã, especially when co-inoculated. Our results point out to a potentially valuable source of practical information in the search of an eco-friendlier approach to increase pasture productivity.

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Tables

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

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

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

*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).

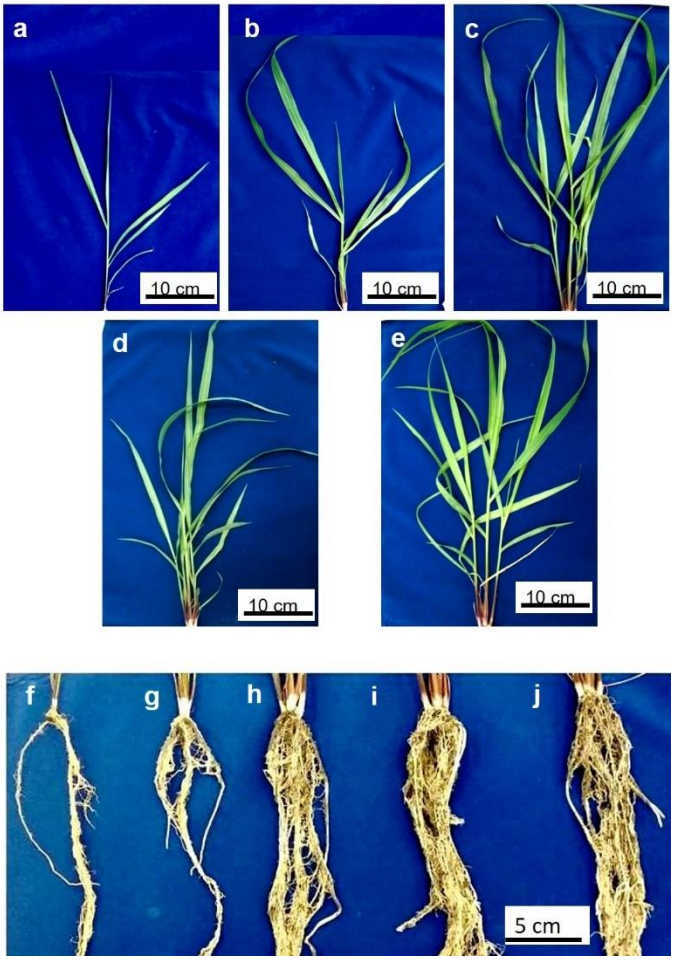
† C - = unfertilized-control; C + = fertilized-control; B = *Burkholderia pyrrocinia*; P = *Pseudomonas fluorescens*; MIX = B + P.

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	0.60*
N	0.89*

* P < 0.05.

447 **Figures**



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450 **Fig. 1** Shoot (a-e) and root (f-j) of *Brachiaria brizantha*, 21 days after inoculation (35 days after
451 seedling emergence). Unfertilized-control (a, f), fertilized-control (b, g), inoculated with
452 *Pseudomonas fluorescens* (BRM-32111) (c, h), *Burkholderia pyrrocinia* (BRM-32113) (d, i)
453 and co-inoculated with BRM-32111 + BRM-32113 (e, j).

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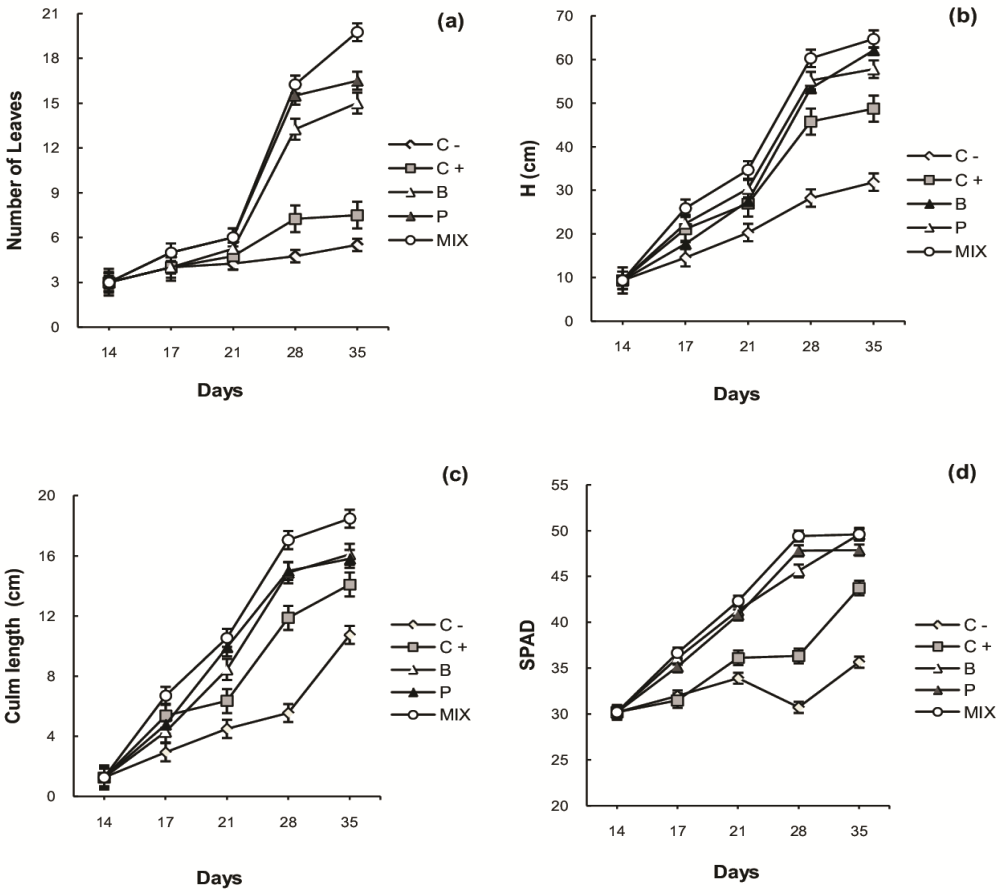
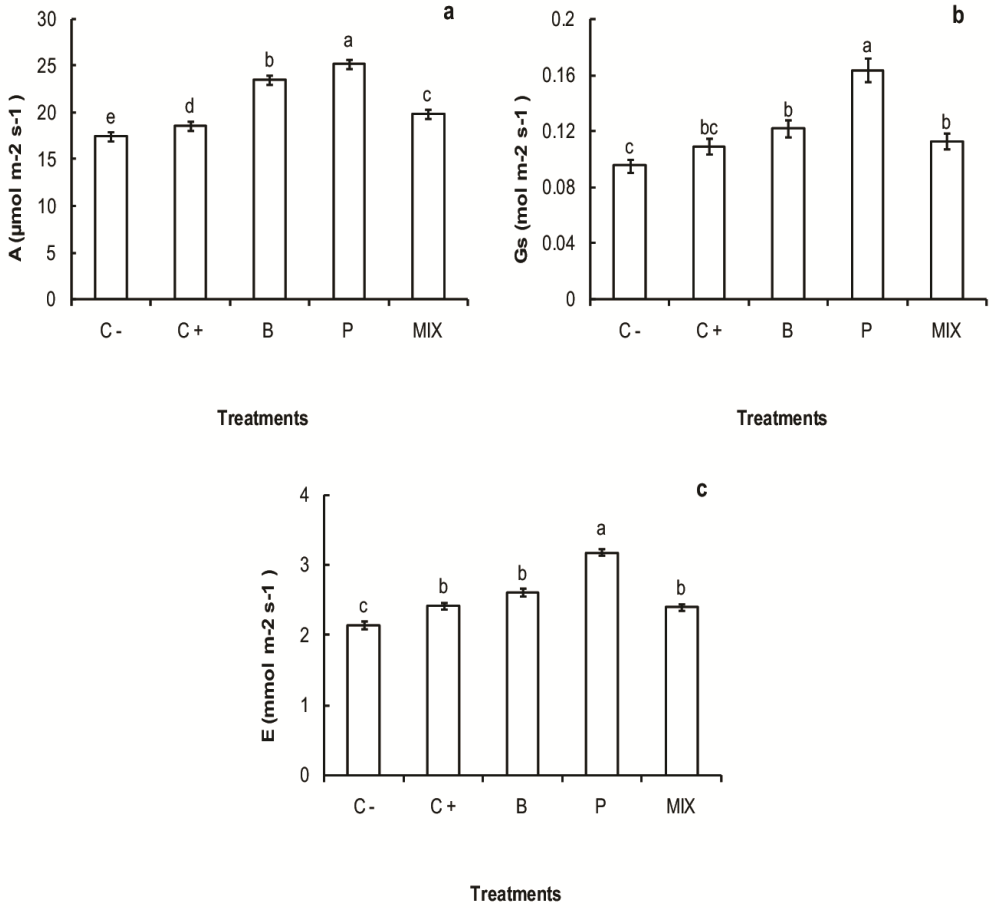


Fig. 2 Number of leaves per plant (a), plant height (H) (b), culm length (c) and SPAD index (d) of *Brachiaria brizantha* inoculated with growth-promoting rhizobacteria. Values are means ± SE (*n* = 5). Days = days after sowing. C - = unfertilized-control; C + = fertilized-control; B = *Burkholderia pyrrocinia;* P = *Pseudomonas fluorescens*; MIX = B + P.



478

479 **Fig. 3** *Brachiaria brizantha* responses to growth-promoting rhizobacteria. (a) Net
480 photosynthesis (*A*), (b) stomatal conductance *(gs)* and (c) transpiration (*E*). At 21 days after
481 inoculation (35 days after seedling emergence). Columns with different letters are significantly
482 different among treatments ($P < 0.05$, Duncan Test). C - = unfertilized-control; C + = fertilized-
483 control; B = *Burkholderia pyrrocinia*; P = *Pseudomonas fluorescens*; MIX = B + P.

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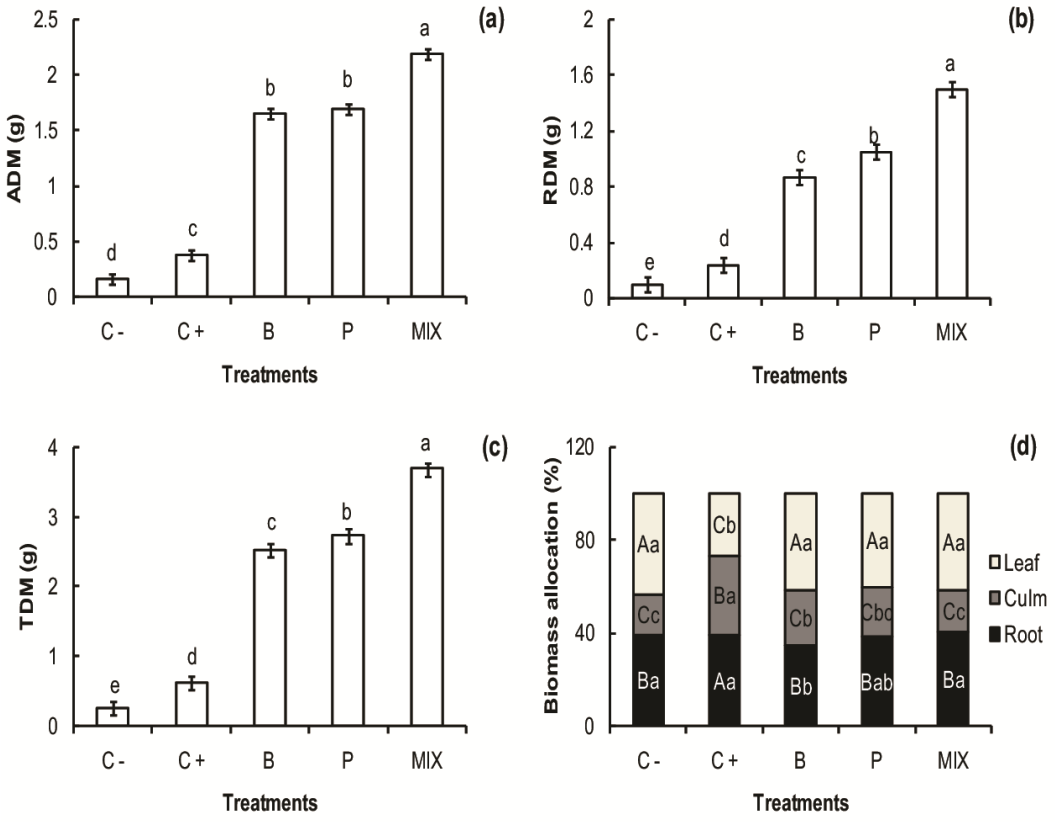

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491 **Fig. 4** *Brachiaria brizantha* responses to growth-promoting rhizobacteria. (a) Shoot dry mass
492 production (SDM), (b) root dry mass production (RDM), (c) total dry mass production (TDM)
493 and, (d) biomass allocation pattern. 21 days after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

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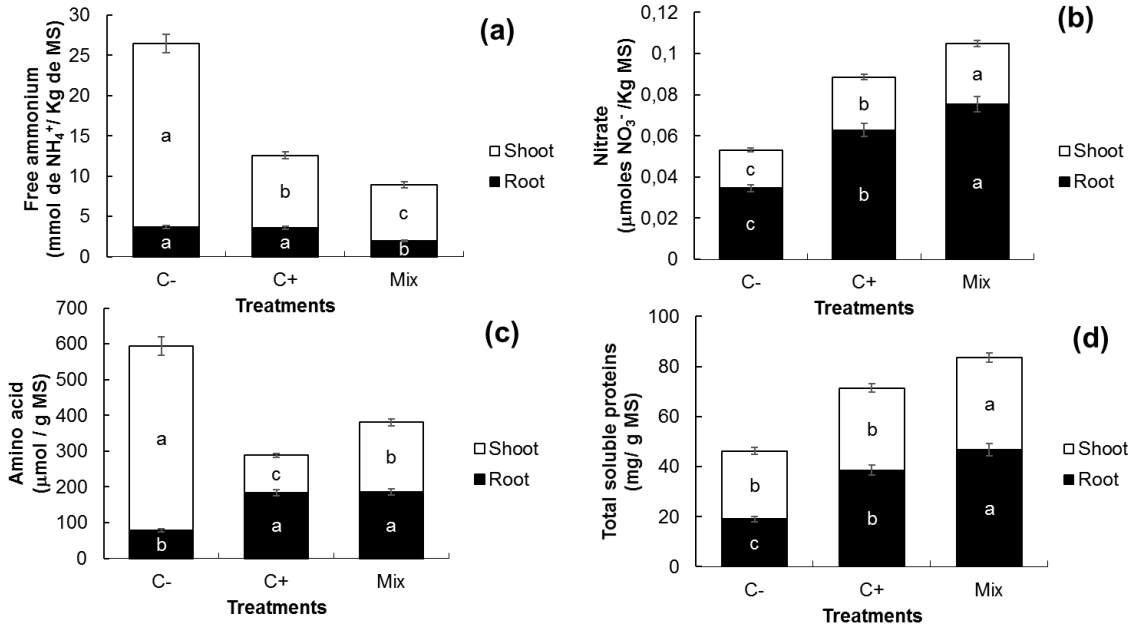

Fig. 5 *Brachiaria brizantha* responses to growth-promoting rhizobacteria. (a) Free ammonium, (b) nitrate, (c) amino acid and, (d) total soluble proteins. 21 days after inoculation (35 days after seedling emergence). Columns with different letters are significantly different among treatments ($P < 0.05$,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

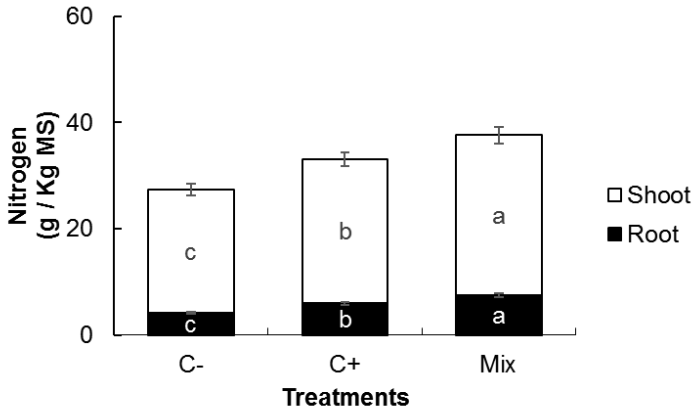

Fig. 6 Nitrogen concentration of *Brachiaria brizantha* inoculated with growth-promoting rhizobacteria. 21 days after inoculation (35 days after seedling emergence). Columns with different letters are significantly different among treatments ($P < 0.05$, Duncan Test). C - = unfertilized-control; C + = fertilized-control; Mix= *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*.

Valor nutritivo, produção de biomassa e parâmetros anatômicos de *Brachiaria brizantha* com rizobactérias promotoras de crescimento (PGPR)[2]

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Resumo

O uso de microrganismos promotores de crescimento é uma alternativa promissora e sustentável para aumentar a produtividade de pastagens. O objetivo foi avaliar o efeito da co-inoculação de *Pseudomonas fluorescens* (BRM- 32111) e *Burkolderia pyrrocinia* (BRM-32113) na anatomia, bioquímica, qualidade nutricional e produção de biomassa de *B. brizantha*. O delineamento experimental foi inteiramente casualizado com três tratamentos: plantas não inoculadas sem adubação (C-), com adubação (C +) e plantas coinoculadas com *P. fluorescens* e *B. pyrrocinia*. Foram avaliados parâmetros anatômicos, fisiológicos e de qualidade nutricional. A inoculação com rizobactérias modificou a anatomia foliar, do colmo e da raiz; incrementou o teor de clorofila (122%), taxas fotossintéticas (14%), eficiência do uso da água (22%), carboidratos totais (29%), amido (80%) e proteína bruta (37%); também aumentou os teores de N (30%), P (20%), Mg (20%) e Fe (180%), promovendo uma maior qualidade e produção de biomassa (400%) em *B. brizantha*. Por tanto, essas rizobactérias poderiam ser usadas como biofertilizantes para *B. brizantha*, contribuindo para a obtenção de forragem de maior produtividade e valor nutritivo.

Palavras-chave: biofertilizante, nitrogênio, fósforo, *Pseudomonas fluorescens*, *Burkolderia pyrrocinia*

[2] Este capítulo segue as normas de formatação da **Grass and Forage Science**.

INTRODUÇÃO

Na América Latina, o interesse crescente pelo sequestro de carbono e redução de gases do efeito estufa, requer uma melhor gestão nos sistemas de pastagem (Pagano *et al.* 2017). As pastagens tropicais são formadas principalmente por *Brachiaria* (Syn. *Urochloa*), uma gramínea forrageira perene, com elevado crescimento, tolerância ao pastejo e alta disponibilidade de forragem (Reis *et al.*, 2013; Pontes *et al.*, 2016; Pagano *et al.*, 2017). Dentre as cultivares de *B. brizantha* disponíveis no mercado, destaca-se a BRS Piatã, por apresentar menor alongamento da haste e sazonalidade na produção, maior acúmulo de forragem durante o período seco e desempenho animal, e ser mais tolerante à cigarrinha-das-pastagens em relação as outras cultivares dessa espécie (Reis *et al.*, 2013).

O uso de microrganismos promotores de crescimento seria uma solução sustentável e promissora para melhorar a tolerância ao estresse biótico e abiótico, nutrição e produção de pastagens (Pagano *et al.*, 2017; Timmusk *et al.*, 2017; Duchene *et al.*, 2017). Rizobactérias promotoras de crescimento (PGPR) contribuem para o desenvolvimento das plantas através de mecanismos diretos, estimulando a produção de fitormônios e atuando como biofertilizantes; e de mecanismos indiretos, aumentando a resistência das plantas a estresses bióticos e abióticos. As PGPR atuam como biofertilizantes por aumentar a disponibilidade de nutrientes no solo, a biomassa, área radicular e a capacidade de absorção de nutrientes da planta, podendo afetar os mecanismos bioquímicos e o estado nutricional, aumentando o crescimento e o rendimento das plantas (Richardson *et al.*, 2009; Calvo *et al.*, 2014; Pii *et al.*, 2015; Timmusk *et al.*, 2017).

Estudos prévios demonstraram que a co-inoculação de *Pseudomonas fluorescens* e *Burkolderia pyrrocinia* promovem o crescimento em *Brachiaria brizantha* (pesquisa exploratória conduzida pelo primeiro autor). Nossa hipótese é que essas rizobactérias são capazes de elevar tanto a quantidade, como a qualidade de forragem de *B. brizantha*, podendo

ser usadas como biofertilizantes. Por tanto, nosso objetivo foi avaliar o efeito da co-inoculação de *B. pyrrocinia* e *P. fluorescens* na anatomia, bioquímica, qualidade nutricional e produção de biomassa em *B. brizantha* cv. BRS Piatã.

MATERIAL E MÉTODOS

O experimento foi conduzido no Laboratório de Proteção de Plantas (LPP) e em casa de vegetação na Universidade Federal Rural da Amazônia (UFRA) (01º27'25"S, 48º26'36"W) em Belém, Pará, Brasil. O experimento foi inteiramente casualizado com três tratamentos: plantas controles não inoculadas sem fertilizante (C-), com fertilizante (C+) e coinoculadas com *Pseudomonas fluorescens* (BRM-32111) e *Burkholderia pyrrocinia* (BRM-32113), com dez repetições cada, cada repetição correspondente a uma planta. As rizobactérias foram repicadas em placas de Petri com meio de cultura 523 (Kado and Hesket, 1970) e incubadas durante 48 horas a 28◦C. A suspensão bacteriana foi preparada com água estéril e ajustada a 540 nm = 0.2 (10^{-8} UFC/mL).

Foram semeadas dez sementes por vaso de polietileno (15 x 25 x 0.05 cm) em solo de baixa fertilidade (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}). As plantas controles não inoculadas com fertilizante foram adubadas com 5 mg dm^{-3} de N, 14 mg dm^{-3} de P_2O_5 e 10 mg dm^{-3} de K_2O. A suspensão bacteriana (5 mL, 10^8 CFU/mL) foi inoculada por rega do solo 14 dias após a emergência da planta (DAEP). O experimento foi conduzido em casa de vegetação, com radiação fotossinteticamente ativa de 900 μmol m^{-2} s^{-1}, temperatura média do ar de 30 °C e umidade relativa de 74%.

A taxa de assimilação líquida de CO2 (*A*) foi determinada 35 DAEP, com um analisador de gás infravermelho (IRGA) (LI-6400XT, LICOR, Lincoln, NE), com 400 μmol m^{-1} de CO_2 e 1000 μmol $m^{-2}s^{-1}$ de radiação fotossintética ativa (obtida por uma fonte de luz artificial acoplada

à câmara IRGA). A eficiência de uso da água (WUE) foi calculada pela razão entre a fotossíntese e a transpiração. O teor de clorofila foi estimado com um medidor portátil (SPAD - soil plant analysis development; SPAD-502. Konica Minolta Sensing, INC. Japão).

Aos 35 DAEP, as amostras de folhas, colmo e raízes foram coletadas e fixadas em solução de FAA 50% (etanol: ácido acético glacial: formaldeído). O estudo anatômico foi realizado no Laboratório de Anatomia Vegetal, Departamento de Botânica, Museu Emilio Goeldi, MPEG. As amostras foram desidratadas em uma série de etanol graduada e emblocadas em metacrilato de hidroxietilo (Leica®, Alemanha). Os blocos foram seccionados transversalmente em um micrótomo rotativo Leica RM 2265. As secções foram coradas com 0,05% de azul de toluidina em tampão acetato, pH 4,3 (O'brien et al., 1964) e montadas em resina sintética Entellan®.

As observações foram realizadas no microscópio Olympus BX61 (Japão) com câmera digital acoplada conectada a um computador com software Motic 2.0. As medidas foram realizadas usando o programa ImageJ para análise de imagem (Cappellari et al., 2015). Os parâmetros anatômicos medidos foram: folhas - espessura da epiderme adaxial e abaxial, área das células buliformes, do xilema, do floema, do esclerênquima, dos feixes vasculares, da bainha do feixe e do mesófilo clorofiliano; colmo – espessura da epiderme, área e número de feixes vasculares; e raiz - epiderme, exoderme, parênquima, córtex, endoderme, periciclo, cilindro vascular, números de metaxilema e protoxilema e diâmetro do xilema. Para as avaliações anatômicas, o delineamento experimental foi inteiramente casualizado, com dez repetições por tratamento, cada repetição correspondente a uma planta, composto do valor médio de três lâminas com seis seções cada.

Aos 35 DAEP, mensurou-se a altura (H) e as mudas foram coletadas, separando parte aérea (folha e colmo) e raiz. O material vegetal coletado foi seco em estufa com temperatura de

60 ºC até atingir massa constante. Para avaliar a produção total de biomassa, somou-se a massa seca da parte aérea e raiz de cada planta. A área radicular foi estimada usando o programa de imagem ImageJ (Tajima and Kato, 2011; Schneider *et al.* 2012). Os nutrientes N, C, P, K, Mg, Ca, B, Fe, Zn e Cu foram determinados por espectrometria de emissão óptica com plasma acoplado indutivamente (ICPOES). A fibra em detergente neutro e a fibra em detergente ácido (base na matéria seca) foram determinadas utilizando o procedimento descrito por Van Soest *et al*., (1991). O teor de N foi analisado de acordo com o Kjeldahl. A proteína bruta foi calculada multiplicando o teor de N (%) por 6.25. A extração dos pigmentos fotossintéticos foi em etanol segundo Porra *et al.* (1989) e quantificado de acordo com Lichtenthaler (1987). Os carboidratos solúveis totais e o amido foram determinados de acordo com Dubois *et al*. (1956).

Os dados foram submetidos a análise de variância e as variáveis com valores significativos de F foram comparadas pelo teste de Duncan ($P < 0,05$). O software usado foi o STATISTICA 7 (StatSoft, Inc., Tulsa, EUA)

RESULTADOS

Parâmetros anatômicos

O limbo foliar das plantas inoculadas com rizobactérias apresentou epiderme adaxial com maior espessura (80%), e o aumento das áreas de células buliformes (30%), bainha dos feixes (20%), feixes vasculares e mesófilo clorofiliano (10%) (Tabela 1; Figura 1 (a - c)). As plantas adubadas apresentaram maior epiderme abaxial, área de esclerênquima e número de feixes (Tabela 1; Figura 1 (a - c)).

O colmo das plantas com rizobactérias apresentou incremento em média de 900% na quantidade de feixes vasculares em relação as plantas controles sem adubo e de 102% comparado com as plantas controle adubadas (Tabela 1; Figura 1). Com floema voltado para

sua periferia e o xilema em oposição, também apresentou maior número de feixes. As plantas com rizobactérias foram as únicas com anel esclerenquimático, apresentando 2-5 camadas de células e medula oca (Figura 1f).

As rizobactérias modificaram a anatomia das raízes das plantas, incrementando em mais de 50% a exoderme, periciclo, parênquima e cilindro vascular, de 30% o protoxilema e o diâmetro das células do xilema e de 10% a endoderme em comparação as plantas controles (Tabela 1; Figura 1 (g - i)).

Qualidade nutritiva

Nas folhas das plantas inoculadas os maiores incrementos foram nos teores de N (30% e 11%) e Fe (180% e 150%) em comparação as plantas controle sem e com adubo (Tabela 2). Também apresentaram um incremento médio de 20% do P, Na, e Mg em relação as plantas controle; e de 23% em Zn e 9% em Cu, em comparação as plantas controle sem adubo. Nas raízes das plantas inoculadas houve maior concentração de N, P, K, Na, Mg, Fe e Mg do que as plantas controles (Tabela 2).

A quantidade de NDF não diferiu entre os tratamentos, e a de ADF foi maior nas plantas controles com adubo (Tabela 2). O teor de proteína bruta (CP) foi maior nas plantas inoculadas, com incremento em 37% em comparação as plantas controle sem adubo e 18% as plantas adubadas (Tabela 2).

Spad, trocas gasosas e parâmetros bioquímicos

As plantas com rizobactérias apresentaram maiores concentrações de clorofila com incremento médio de 143% em clorofila a, 95% em clorofila b e 122% no total (Figura 2 a). O índice Spad aumentou em 37% em relação as plantas controles sem adubo e 11% em comparação as plantas controles adubadas (Figura 2 b). As rizobactérias também

incrementaram em média de 14% a taxa fotossintética (*A*) e em 22% a eficiência momentânea do uso da água (WUE) em relação as plantas controles (Figura 2 c, d).

O uso das rizobactérias incrementou a concentração de amido nas folhas (127% e 180%), nas raízes (46% e 73%) e o total (80% e 133%), em comparação com as plantas controles sem e com adubo (Figura 3 a). Plantas inoculadas também apresentaram maior concentração de carboidratos solúveis totais, com incremento de 45% nas folhas, 25% nas raízes e 29% nos totais, em comparação as plantas controles (Figura 3 b).

Crescimento e produção de biomassa

As rizobactérias promoveram maior altura (mais de 100% e 30%), área radicular (131% e 122%), produção de biomassa foliar (470%, e 450%), do colmo (480% e 79%), da raiz (330% e 200%) e total (mais de 400% e 200%), em comparação as plantas controle sem e com adubo (Figura 4).

DISCUSSÃO

A co-inoculação com *P. fluorescens* (BRM-32111) e *B. pyrrocinia* (BRM-32113) modificou a anatomia, a bioquímica e o acúmulo de nutrientes, promovendo um maior desenvolvimento em *B. brizantha* (Figura 5). Essas rizobactérias provavelmente aumentaram a disponibilidade de nutrientes na rizosfera, através da fixação assimbiótica de nitrogênio; solubilização de fósforo, por ácidos orgânicos e fosfatases; sequestro de ferro por produção de sideróforos e liberação de micronutrientes de fontes insolúveis, além de estimular os sistemas de transporte iónico na raiz (Vancheron *et al.*, 2013; Calvo *et al.*, 2014; Pii *et al.*, 2015).

A maior absorção de nutrientes pelas plantas inoculadas, também foi em resultado das alterações que as rizobactérias causaram nas raízes, com o incremento da exoderme, reduzindo o refluxo de íons para solução do solo, o que resultou em maior concentração de K e Mg nas

raízes; maior periciclo, resultante provavelmente do aumento de auxina, facilitando a difusão de oxigênio da base para o ápice, e maior desenvolvimento de xilema, protoxilema e área radicular, aumentando a absorção e fluxo de água e nutrientes (Richardson *et al.*, 2009; Vacheron *et al.*, 2013).

No presente estudo, o aumento dos teores de nutrientes nas plantas inoculadas, sobretudo o nitrogênio, como também a maior área da bainha do feixe vascular e do mesófilo clorofiliano nas folhas, aumentou o teor dos pigmentos fotossintéticos (clorofila a, b e total). Por sua vez, em decorrência do maior desenvolvimento do sistema vascular da folha, colmo e raiz, aumentou o transporte de água e solutos, resultando em uma maior eficiência fotossintética, incrementando a concentração de carboidratos totais e de amido, que tem alta digestibilidade sendo fonte de energia aos animais. O uso de rizobactérias promotoras de crescimento também aumentou a clorofila, taxas fotossintéticas e eficiência do uso da água em *Phaseolus coccineus* (Stefan *et al.*, 2013); pigmentos fotossintéticos em *Mentha piperita* (Cappellari *et al*., 2015) e carboidratos e proteína em *Zea mays* L. (El-Ghany *et al.*, 2015), *Arachis hypogaea* (Mathivanan *et al.*, 2017) e *Coriandrum sativum* L. (Warwate *et al.*, 2017).

É possível supor que os benefícios resultantes da inoculação também aumentariam a tolerância de *B. brizantha* ao pastejo e a ambientes com limitação de recursos hídricos. Isso por que as plantas inoculadas, além de apresentarem a raiz e o sistema vascular mais desenvolvido, maiores taxas fotossintéticas e acúmulo de amido, também tiveram maior eficiência no uso de água, e incremento em áreas de células buliformes, que são responsáveis por enrolar as folhas, reduzindo a área de transpiração (Reis *et al.*, 2013). Além disso, as plantas inoculadas apresentaram o colmo com anel esclerenquimático, o que as tornariam mais resistentes ao acamamento (Reis *et al.*, 2013). A inoculação com rizobactérias também modificou a anatomia, promovendo maior capacidade de adaptação, por aumentar o desenvolvimento vascular em

703 *Triticum aestivum* L. (El-Afry *et al.,* 2012), e a densidade de pelos radiculares em
704 *Handroanthus impetiginosus* (Larraburu and Llorente, 2015).

705 Em nosso estudo, a inoculação com rizobactérias incrementou os macro e
706 micronutrientes nas folhas (N, P, Na, Mg, Fe e Cu) e raízes (N, P, K, Na, Mg, Fe, Cu e Mn),
707 melhorando a translocação e mobilização de nutrientes, aumentando a qualidade nutricional de
708 *B. brizantha*. O maior teor de nitrogênio também resultou no incremento da proteína bruta (CP),
709 que é um indicador da qualidade da forragem, a qual deve ser superior a 7%, para não reduzir
710 o consumo voluntário e a digestibilidade pelo gado (Reis *et al.,* 2013; Ball *et al.,* 2015). Essa
711 maior nutrição nitrogenada nas plantas inoculadas, também foi verificada nas avaliações do
712 índice Spad, que deve apresentar valores superior a 40 (Reis *et al.,* 2013). O aumento na CP e
713 biomassa total também ocorreu em *B. brizantha* cv. BRS Piatã (Orrico Júnior *et al.*, 2013), cv.
714 Marandu (Pontes *et al.*, 2016) e em *Panicum maximum* (Paciullo *et al*., 2016) adubadas com
715 nitrogênio.

716 O fósforo (P) é um dos nutrientes mais limitantes para a formação e manutenção da
717 produtividade de pastagens (Dias-Filho, 2011; Mota *et al.,* 2017). Mesmo quando os
718 fertilizantes fosfatados são adicionados aos solos, a oferta de P inorgânico pode sofrer
719 limitação, pois parte desse fósforo pode ser prontamente ligado às partículas do solo (Dias-
720 Filho, 2011; Duchene *et al.*, 2017). No presente estudo, plantas inoculadas apresentaram maior
721 teor de P, o que sugere que as rizobactérias aumentaram a disponibilidade de P no solo. É
722 possível supor, que a inoculação, além de contribuir para o maior desempenho da pastagem,
723 também contribuiria para a melhoria da nutrição animal, diminuindo a necessidade da
724 suplementação de P na dieta animal, o qual é comum em pastagens tropicais (Dias-Filho, 2011).
725 A inoculação com micorriza também promoveu o crescimento em *B. decumbens*, mas não foi
726 capaz de elevar a concentração de P dessa forrageira (Mota *et al.*, 2017).

A qualidade da forragem também é determinada pela quantidade de fibras, como a fibra detergente neutra (NDF) e a fibra detergente ácida (ADF), que são carboidratos estruturais que formam a parede celular da planta e incluem celulose, hemicelulose, lignina e pectina. (Ball *et al.*, 2015). A qualidade da forragem também está relacionada com a anatomia foliar, pois quanto maior a espessura da epiderme, principalmente a abaxial, e a área de esclerênquima, mais resistente a forrageira será à fermentação dos microrganismos do rumem, diminuindo a digestibilidade (Paciullo *et al*., 2016; Tsuzukibashi *et al.*, 2016), o que foram menores nas plantas com rizobactérias do que as com adubo. Como forragens com teores de fibras (NDF e ADF) superiores a 60% tem baixa digestibilidade, resultando em menor consumo e desempenho animal (Ball *et al.*, 2015), é possível sugerir que as plantas inoculadas teriam maior potencial de consumo durante o pastejo.

No presente estudo, as rizobactérias atuaram como biofertilizantes em *B. brizantha*, por estimular a nutrição, modificar a fisiologia, contribuindo para acúmulo de metabólitos, resultando em maior altura e massa de forragem, o que também seria benéfico em campo, porque aumentaria a massa por bocado e a ingestão diária total de forragem pelos animais (Reis *et al.*, 2013). O uso de rizobactérias aumentou a concentração de N e biomassa em *Brachiaria* spp. (Hungria *et al.*, 2016) e promoveu incremento de nutrientes, produção de biomassa e crescimento em *Triticum aestivum* L. (Rana *et al.*, 2012); *Zea mays* (Dhawi *et al.*, 2015; Armada *et al.*, 2015; Calvo *et al*., 2017); *Brassica oleracea* (Kumari *et al.*, 2017).

Nossos resultados demonstraram que a co-inoculação de *P. fluorescens* and *B. pyrrocinia* foi mais eficiente do que a adubação química em *B. brizantha*. A inoculação modificou a anatomia foliar, do colmo e da raiz; incrementou o teor de clorofila, taxas fotossintéticas, eficiência do uso da água, carboidratos totais, amido e proteína; também aumentou os teores de N, P, Na, Mg e Fe nas folhas e raízes, promovendo uma maior qualidade

e produção de biomassa em *B. brizantha* (Figura 5). Por tanto, essas rizobactérias poderiam ser usadas como biofertilizantes em pastos formados por *B. brizantha*, contribuindo para a obtenção de pastagens de maior produtividade e valor nutritivo.

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885 **Tabelas**

886 **Tabela 1**. Parâmetros anatômicos (μm or μm^2) de *Brachiaria brizantha* não inoculada sem
887 fertilizante (C-), com fertilizante (C+) e coinoculada com *Pseudomonas fluorescens* (BRM-
888 32111) e *Burkholderia pyrrocinia* (BRM-32113) (Mix).

Parâmeteros		**C -**	**C +**	**Mix**
Folha	Epiderme adaxial	11,54 b	11,86 b	20,24 a
	Epiderme abaxial	16,60 c	25,49 a	23,33 b
	Células buliformes (área)	12828,99 b	12703,61 c	16594,07 a
	Xilema (área)	8100,35 c	8869,52 b	9116,19 a
	Floema (área)	3147,13 a	2160,41 c	2830,56 b
	Esclerênquima (área)	1171,01 b	1472,41 a	800,97 c
	Feixes vasculares (área)	3058,87 b	2686,55 c	3200,26 a
	Bainha dos feixes (área)	18862,34 c	21484,33 b	24316,82 a
	Mesófilo clorofiliano (área)	193524,01 b	188700,81 c	211559,31 a
Colmo	Epiderme	13,47 c	18,46 a	14,07 b
	Feixe vascular (área)	1989,95 c	10089,77 b	20364,01 a
	Número de feixes	33,57 c	42,48 b	69,12 a
Raiz	Epiderme	14508,01 a	13037,02 b	12734,13 c
	Exoderme	18873,81 b	14179,94 c	23063,50 a
	Córtex	209883,81 a	145267,23 c	153069,71 b
	Endoderme	12954,78 c	14006,01 b	14693,67 a
	Periciclo	25278,37 b	23272,28 c	37372,27 a
	Cilindro vascular	338050,4 a	190090,8 b	337155,31 a
	Números de metaxilema	16,52 a	8,24 c	15,30 b
	Números de protoxilema	31,51 b	23,95 c	36,11 a
	Diâmetro do xilema	30041,11 c	38490,77 b	45667,51a

889 *Significativo ($P < 0.05$, Duncan Test)
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Tabela 2 Concentração de macro e micronutrients (g/kg), fibra em detergente neutron (FDN - g kg^{-1} of DM), fibra em detergente ácido (FDA - g kg^{-1} of DM) e proteina bruta (PB - g kg^{-1} of DM) de *Brachiaria brizantha* não inoculada sem fertilizante (C-), com fertilizante (C+) e coinoculada com *Pseudomonas fluorescens* (BRM-32111) e *Burkholderia pyrrocinia* (BRM-32113) (Mix).

Parameters	*Folha*			*Raiz*		
	C-	**C+**	**Mix**	**C-**	**C+**	**Mix**
N	23.19 c	27.08 b	30.08 a	4.14 c	5.96 b	7.49 a
P	1.07 c	1.17 b	1.22 a	0.07 b	0.08 b	0.17 a
K	24.39 c	23.66 b	22.71 c	8.12 c	9.83 b	14.74 a
Na	0.6 c	0.77 b	0.81 a	1.16 c	1.52 b	1.96 a
Ca	5.94 c	6.88 a	6.79 b	1.12 b	1.53 a	1.09 b
Mg	4.56 c	5.76 b	5.86 a	0.66 c	0.75 b	1.08 a
Fe	191.02 c	212.50 b	542.69 a	2176.9 c	2350.5 b	3143.6 a
Zn	97.12 c	149.13 a	123.37 b	80.86 a	78.98 b	67.03 c
Cu	13.29 c	14.14 b	14.61 a	3.91 c	5.62 a	4.96 b
Mn	143.02 c	169.24 a	145.06 b	53.26 c	61.05 b	74.57 a
FDN	260	251	260			
FDA	130 b	150 a	132 b			
PB	157.6 c	183.3 b	215.8 a			

*Significantivo ($P < 0.05$, Duncan Test).

Figuras

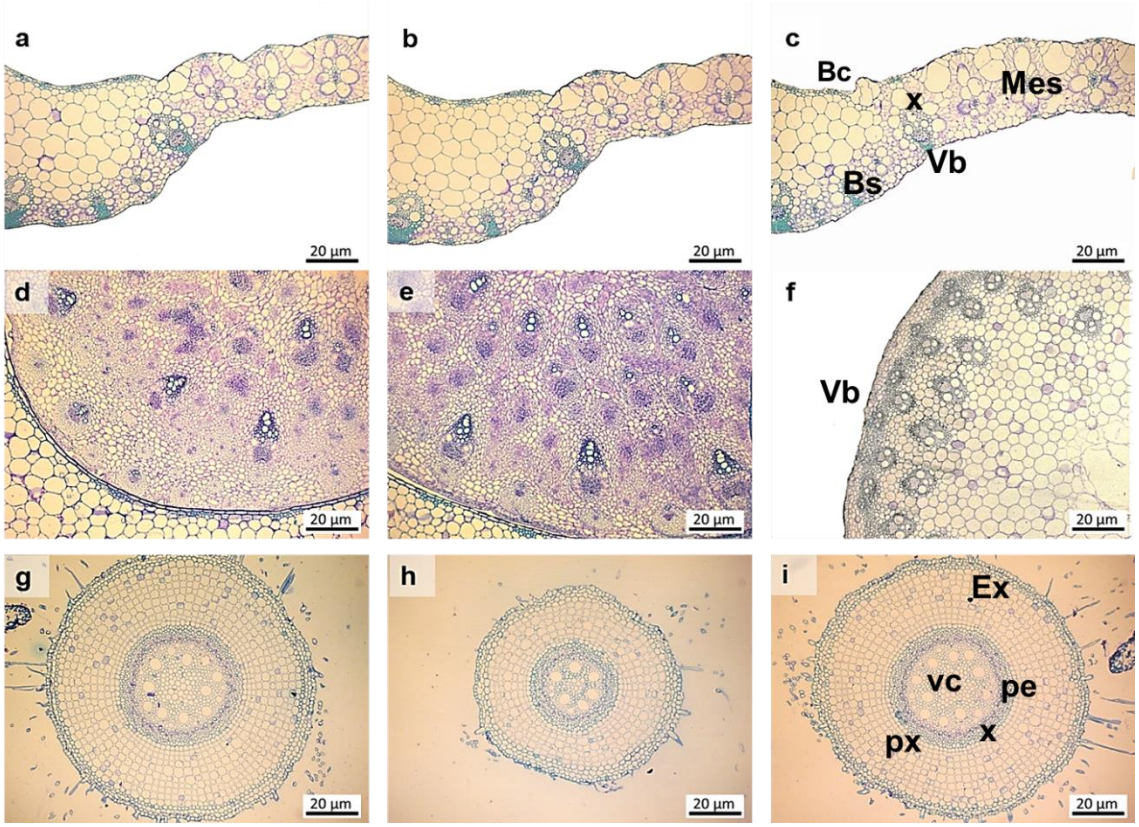


Figura 1 Secção transversal de folha (a-c), colmo (d-f) e raiz (g-i) de *Brachiaria brizantha* não inoculada sem fertilizante (a, d, g), com fertilizante (b, e, h) e coinoculada com *Pseudomonas fluorescens* (BRM-32111) e *Burkholderia pyrrocinia* (BRM-32113). 21 dias após a inoculação (35 dias após a emergência das plantas). Célula buliforme (Bc), bainha do feixe (Bs), feixe vascular (Vb), xilema (X), mesófilo (Mes), exoderme (Ex), periciclo (pe), cilindro vascular (vc) e protoxilema (px).

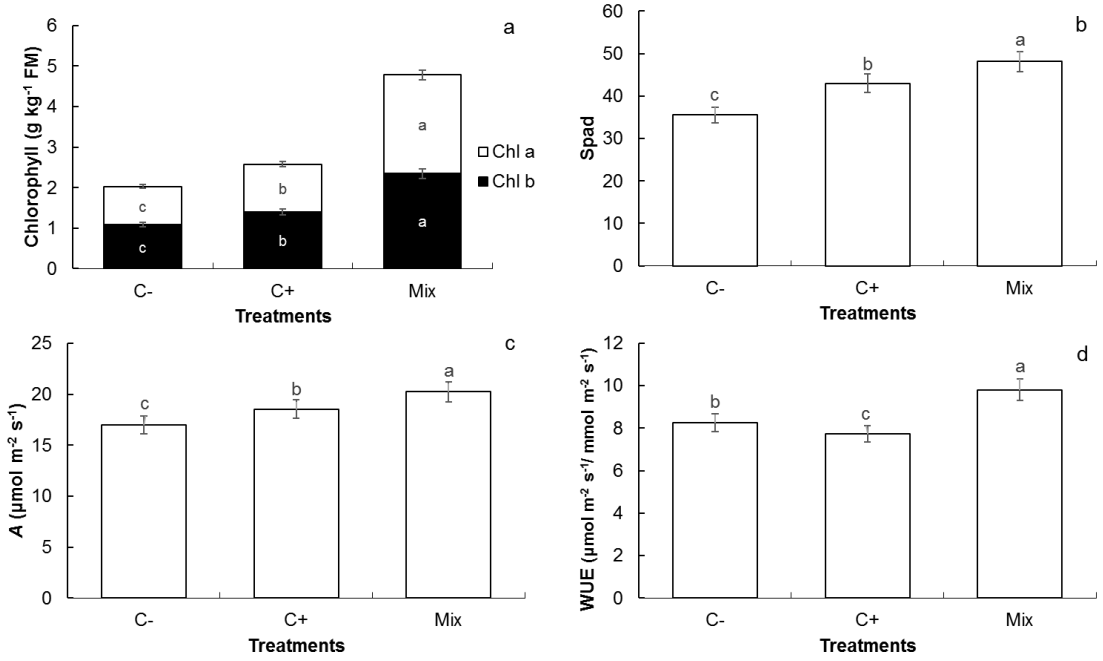


Figura 2 Clorofila (a), índice Spad (b), fotossíntese líquida (*A*) (c) e eficiência do uso da água (WUE) de *Brachiaria brizantha* não inoculada sem fertilizante (C-), com fertilizante (C+) e coinoculada com *Pseudomonas fluorescens* (BRM-32111) e *Burkholderia pyrrocinia* (BRM-32113) (Mix). Médias seguidas da mesma letra na coluna não diferem (P <0,05, teste de Duncan).

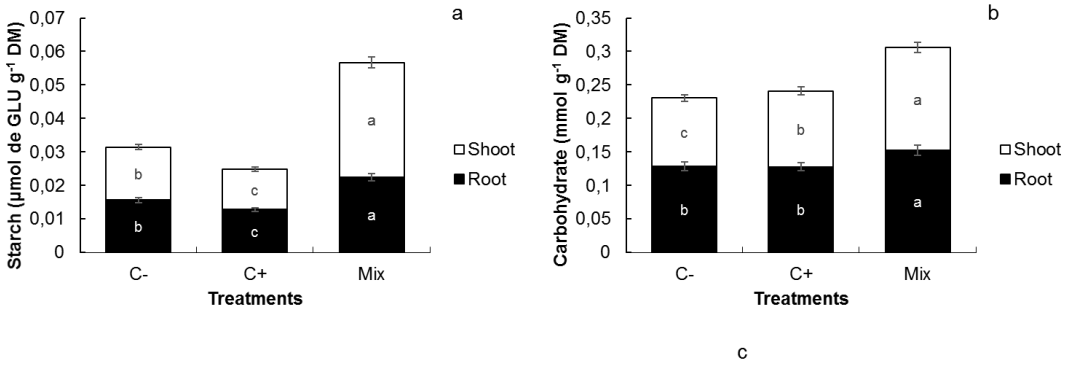
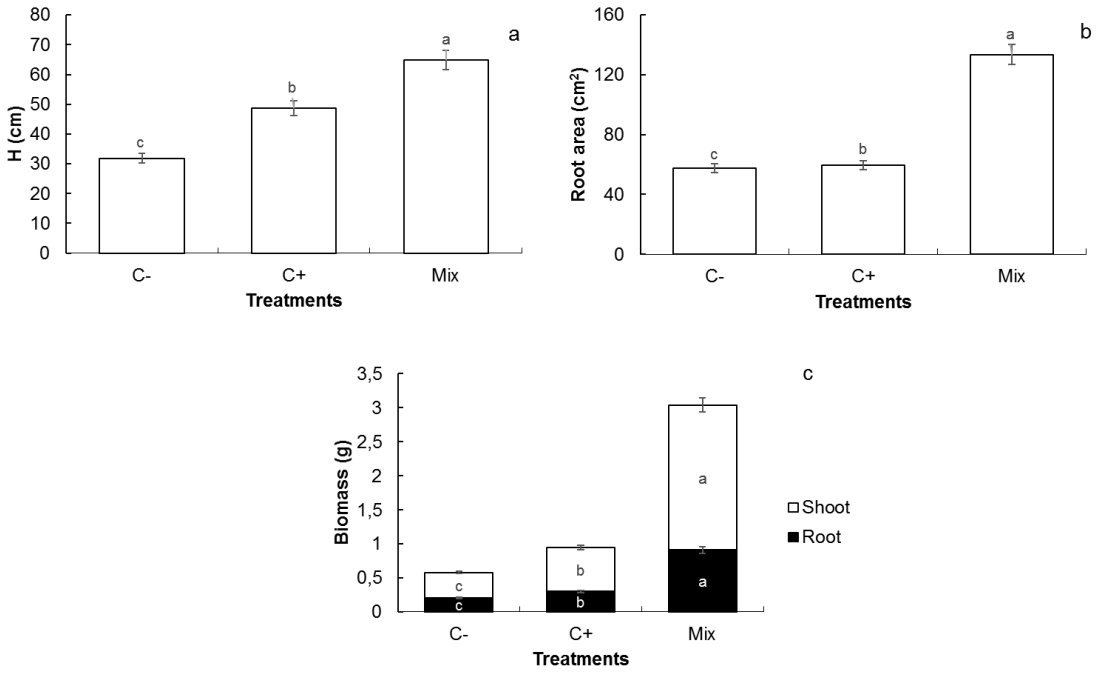


Figura 3 Amido (a) e carboidratos totais (b) de *Brachiaria brizantha* não inoculada sem fertilizante (C-), com fertilizante (C+) e coinoculada com *Pseudomonas fluorescens* (BRM-32111) e *Burkholderia pyrrocinia* (BRM-32113) (Mix). Médias seguidas da mesma letra em cada coluna não diferem (P <0,05, teste de Duncan).



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940 **Figura 4** Altura (H) (a), área radicular (b) e produção de biomassa (c) de *Brachiaria brizantha*
941 não inoculada sem fertilizante (C-), com fertilizante (C+) e coinoculada com *Pseudomonas*
942 *fluorescens* (BRM-32111) e *Burkholderia pyrrocinia* (BRM-32113) (Mix). Médias seguidas da
943 mesma letra na coluna não diferem (P <0,05, teste de Duncan).

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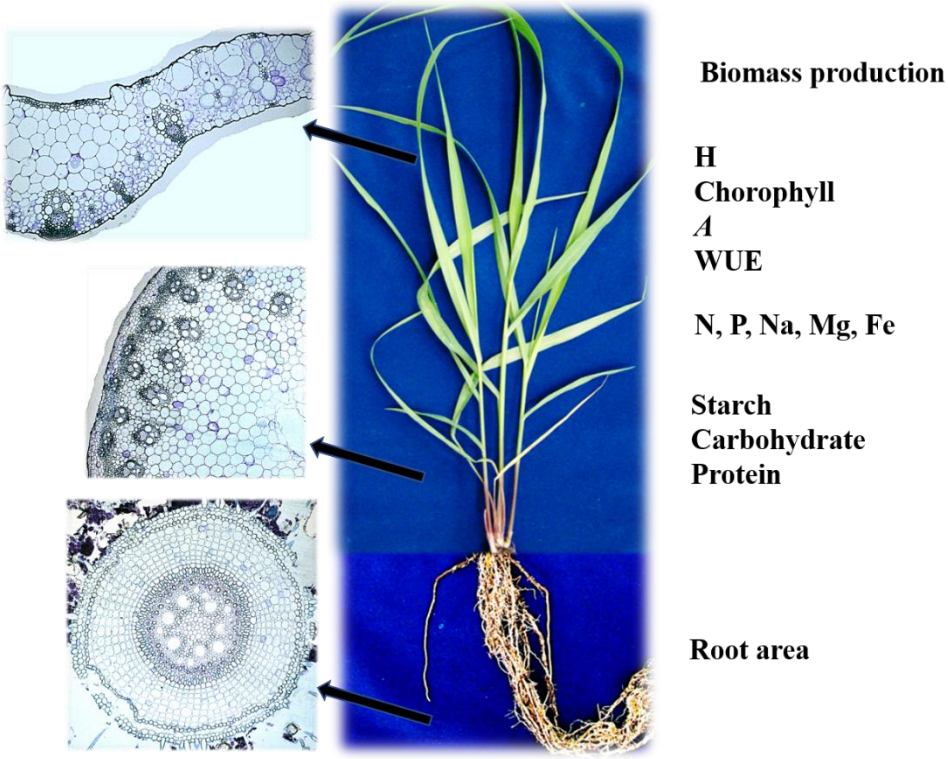

Figura 5 *Brachiaria brizantha* coinoculada com rizobactérias promotoras de crescimento em plantas (PGPR), 21 dias após a inoculação (35 dias após a emergência das plantas). *Pseudomonas fluorescens* (BRM-32111) e *Burkholderia pyrrocinia* (BRM-32113) modificaram a anatomia (folha, colmo e raiz), incrementaram a altura (H), taxa fotossintética (*A*), eficiência do uso da água (WUE), concentração de clorofila, N, P, Na, Mg, Fe, amido, carboidrato, proteína, área radicular, aumentando a qualidade de forragem e produção de biomassa em *B. brizantha*.

Light and plant growth-promoting rhizobacteria (PGPR) effects on *Brachiaria brizantha* growth and phenotypic plasticity to shade[3]

Monyck Jeane dos Santos Lopes, Moacyr Bernardino Dias Filho, Thomaz Henrique dos Reis Castro, Gisele Barata da Silva

Abstract

This is the first report on the effect of light intensity and plant growth-promoting rhizobacteria (PGPR) on the growth of a tropical forage grass, being a relevant study to improve pasture management in conventional farming and integrated crop-livestock-forestry systems. In this study, our aim was to evaluate the effects of light intensity and *Burkholderia pyrrocinia* and *Pseudomonas fluorescens* inoculation on *Brachiaria brizantha* cv. BRS Piatã growth, phenotypic plasticity and shade tolerance. The experiment was conducted in a semi-controlled environment. Seedlings of *B. brizantha* were allocated to full sun and shade. *P. fluorescens* and *B. pyrrocinia* were inoculated individually or co-inoculated by soil drench, 14 days after seedling emergence. We evaluated morphogenesis, structural and growth parameters. Irrespective of the light regime, co-inoculated plants had greater leaf area and SPAD index (chlorophyll content). Increase in total biomass production in co-inoculated plants was over 100% and 300%, under full sun and shade, respectively. Co-inoculated *P. fluorescens* and *B. pyrrocinia* increased shade tolerance in *B. brizantha*, improving plant performance. Co-inoculation promoted growth in *B. brizantha* under both sun and shade, indicating its potential as a bio-fertilizer in conventional and integrated systems, especially in silvipastoral systems, where light availability to pasture growth may be limited.

Keywords: shade, forage grass, *Pseudomonas fluorescens*, *Burkholderia pyrrocinia*

[3] Este capítulo segue as normas de formatação da **Grass and Forage Science**.

INTRODUCTION

Integrated crop-livestock-forestry systems are efficient land-management alternatives for restoring degraded pastures, increasing food security and promoting carbon sequestration (Dias-Filho, 2011; Moraes *et al.*, 2014; Paciullo *et al.*, 2016; Santos *et al.*, 2016). However, as the forage grass species planted in integrated systems may be subjected to light restriction, imposed by agricultural crops or tree species, it is necessary to know their agronomic performance under shade, to define their potential of use in these systems (Dias-Filho, 2000; Paciullo *et al.*, 2011; Gomez *et al*., 2012; Pimentel *et al.*, 2016).

Phenotypic plasticity relates to the species adaptability to changes in the environment, such as variations in solar radiation, temperature, soil water and nutrient availability (Gomez *et al*., 2012; Valladares *et al.*, 2016). Shade tolerant plants can adjust their morphophysiology, such as biomass allocation pattern, chlorophyll content and leaf area and thickness to maximize light capture (Paciullo *et al.*, 2016; Valladares *et al.*, 2016)

In conventional and integrated systems, forage grass production is usually limited by low soil fertility (Paciullo *et al.*, 2011; Paciullo *et al.*, 2016; Pimentel *et al.*, 2016). The use of plant-growth-promoting rhizobacteria (PGPR) could be an alternative for reducing the use of chemical fertilizers, with clear environmental and economic benefits (Nadeem *et al.*, 2014; Paredes and Lebeis, 2016). The beneficial effect of PGPR on forage grasses has been reported for *Azospirilum brasilense* on *B. briazantha* (Hungria *et al.* 2016), and *Azotobacter*, *Azospirillum* and *Herbaspirillum* on *Axonopus affinis*, *Paspalum notatum*, *Andropogon lateralis* and *Aristida laevis* (Marques *et al*., 2017).

Beneficial microorganisms are known to improve nutrient uptake, phosphorus solubilization, phytohormone production and disease resistance by elicited induced systemic resistance or systemic acquired resistance. Also, beneficial microorganisms modify the

phenotypic plasticity of plants, by mitigating the negative impact of abiotic stresses (Goh *et al.*, 2013; Vacheron *et al*., 2013; Paredes and Lebeis, 2016; Vimal *et al.*, 2017), including light limitation (Konvalinková and Jansa, 2016).

Previous studies with *Pseudomonas* sp. and *Burkolderia* sp. attested their growth promote potential in rice (Rêgo *et al.*, 2014; Nascente *et al*., 2016) and *Brachiaria brizantha* (exploratory research conducted by the first author at the Federal Rural University of Amazonia). These rhizobacteria are known to increase the auxin synthesis, nutrient uptake (e.g., nitrogen, phosphorus, and iron), chlorophyll content, photosynthetic rate, and biomass production (Ahemad and Kibret, 2014; Nascente *et al*., 2016).

Although light is known to interfere in mycorrhiza-plant mutualism (Saner *et al.,* 2011; Aguilar-Chama and Guevara, 2016; Konvalinková and Jansa, 2016), we could not find any published study on the effect of light intensity and use of rhizobacteria on the growth and phenotypic plasticity of a tropical forage grass species.

In Brazil, *Brachiaria* is the most important grass genus for pasture formation (Reis *et al*., 2013; Santana *et al*., 2016; Santos *et al.,* 2016), which stands out *B. brizantha* (Hochst ex. A. Rich.) cv. BRS Piatã (Reis *et al*., 2013). We hypothesized that *Burkhoderia pyrrocinia* and *Pseudomonas fluorescens* inoculation can increase *B. brizantha* growth under limited light conditions, by increasing the shade tolerance of this grass cultivar. In this study, our aim was to evaluate the effects of light intensity and *B. pyrrocinia* and *P. fluorescens* inoculation on the growth, phenotypic plasticity and shade tolerance of *B. brizantha* cv. BRS Piatã.

MATERIALS AND METHODS

Study site, plant material and inoculant

The experiment was conducted in a semi-controlled environment in the nursery seedling production unit of the Federal Rural University of Amazonia (UFRA) (01º27'25 "S, 48º26'36" W) in Belém, Pará, Brazil. The regional climate here according to Koppen classification is Af (equatorial). During the experimental period the mean air temperature and relative humidity were 32±2,8°C and 73±3% (mean ± s.d.), respectively.

Seeds of *B. brizantha* cv. BRS Piatã were sown in polyethylene pots (15 x 25 x 0.05 cm) filled with soil (Ferralsol - top soil from a second growth forest - 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. Plants were grown under full-sun and artificial shade (0% and 47% of shade, or 2100 $\mu molm^{-2}$ s^{-1} and 1113 $\mu molm^{-2}$ s^{-1}, at canopy height, measured at 11 am, local time, on a cloudless day) obtained with black polyethylene screens (solar radiation transmissivity of ca. 50%) placed 160 cm above the canopy height.

We used *P. fluorescens* (BRM-32111) and *B. pyrrocinia* (BRM-32113), supplied by the Plant Protection Laboratory of the Federal Rural University of Amazonia *in vitro* collection, and originally selected from the rhizosphere of rice plants. The rhizobacteria were cultured in Petri dishes with a solid culture medium 523 (agar, casein hydrolysate, magnesium sulfate anhydrous, potassium phosphate monobasic, sucrose and yeast extract) (Kado and Heskett, 1970) and incubated for 48h at 28°C. The bacterial suspension was prepared in sterile water and adjusted to 550 nm (10^8 CFU). Soil-drenched inoculation was carried out 14 days after seedling emergence, each pot received a 5mL bacterial suspension. The experiment was repeated three times with similar results.

Plant growth parameters

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 and the number of days of the evaluation interval ~~days~~, LApR), leaf elongation rate (ratio between the difference of the initial and final lengths of the expanded laminae and the number of days of the evaluation interval, LER), culm elongation rate (ratio between the difference of the initial and final lengths of culm and the number of days of the evaluation interval, CER), number of leaves per plant (NL) and number of tillers per plant (NT). We also determined ~~the~~ plant height (H), culm length (CL) and we measured the chlorophyll content (SPAD index - soil plant analysis development) in the youngest fully expanded leaf blade. Each SPAD index value was the mean of five readings per leaf. We used a portable chlorophyll meter (SPAD-502. Konica Minolta Sensing, INC. Japan).

At 35 days after seedling emergence, five plants per treatment 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 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).

Specific leaf area (SLA, the ratio of leaf area to leaf dry mass) was determine over leaf disks of either 0.42 cm^2 or 2.28 cm^2, dried at 60ºC until constant mass. Total leaf area per plant was estimated from SLA and leaf mass results. We also calculated root dry mass/shoot dry mass ratio (RDM/SDM) and leaf area ratio (ratio of leaf area per total dry mass per plant, LAR). Relative growth rate (change in total mass per total dry mass of plant per day, RGR) was calculated for harvests at 14 and 35 days after seedling emergence. The allometric coefficient

K was calculated as the ratio of the growth rate of root and shoot. All plant growth parameters were calculated according to Hunt (1990) and Barbero *et al.* (2013).

Statistical analyses

The experimental design was completely randomized in a 4 × 2 factorial arrangements (non-inoculated *B. brizantha*, inoculated with BRM-32111, inoculated with BRM-32113 and co-inoculated (BRM-32111 + BRM-3213) x full-sun and shade), with five replicates. Data were subjected to analysis of variance and, when appropriate, the means were compared by Duncan test at 5%. The assumption of homogeneity of variances and normality were tested for each ANOVA and, when necessary, data were log, or square root transformed. Transformed values were back transformed for presentation. Statistical analyses were performed by the statistical package STATISTICA (StatSoft, Inc., Tulsa, USA).

RESULTS

When the bacteria were inoculated individually, plants under full-sun showed the highest growth with *P. fluorescens* (BRM-32111), and with *B. pyrrocinia* (BRM-32113), when grown under shade (Figure 1). The maximum increase in height ($F_{3,32}$ = 38.05; $P < 0.001$) resulted from the activity of the BRM-32113, under full-sun (18%), and of BRM-32111, under shade (13%) (Figure 2). Leaf appearance ($F_{3,32}$ = 11.64; $P < 0.001$) and elongation ($F_{3,32}$ = 57.14; $P < 0.001$), culm elongation ($F_{3,32}$ = 24.97; $P < 0.001$), number of leaves ($F_{3,32}$ = 43.02; $P < 0.001$) and leaf length ($F_{3,32}$ = 5.98; $P < 0.05$), were higher for BRM-32113, in full-sun plants, and, in most cases, for BRM-32111, in shaded plants (Table 1).

Higher values of specific leaf area (SLA) ($F_{3,32}$ = 245.64; $P < 0.001$), and leaf area ratio (LAR) ($F_{3,32}$ = 347.52; $P < 0.001$), were obtained in non-inoculated plants, in the shade. Under full sun, the highest SLA and LAR values were found in plants inoculated with BRM-32113 (Figure 3). Leaf area increased by 122% in full sun plants, inoculated with BRM-32113 and by

20% in shade plants, inoculated with BRM-32111 (Figure 4). When inoculated individually, the SPAD index increased ($F_{3,32} = 6.18$; $P < 0.05$) by 4%, in full sun plants and by 7%, in shade plants (Figure 4).

When the rhizobacteria were inoculated individually, biomass production ($F_{3,32} = 616.61$; $P < 0.001$) and relative growth rate ($F_{3,32} = 119.97$; $P < 0.001$) were increased in full-sun plants by 30% (BRM-32113) and, under shade, by more than 100% (BRM-32111) (Table 2; Figure 5). Under shade, inoculation favored an increased biomass allocation to roots (Figure 6). Under full sun, the root to shoot biomass ratio ($F_{3,32} = 42.71$; $P < 0.05$) and allometric index (K) ($F_{3,32} = 57.16$; $P < 0.0001$) were higher in non-inoculated plants and in plants inoculated with BRM-32111. The reason for that was a preferential biomass allocation to the roots, while in plants inoculated with BRM-32111, biomass was allocated mainly to leaves (Table 2; Figure 6).

Co-inoculation promoted growth of B. *brizantha* under both full-sun and in the shade (Figure 1). Plant height under co-inoculation increased by 28% and 65%, under full-sun and shade, respectively, relative to non-inoculated plants (Figure 2). Higher leaf appearance and elongation rates, culm elongation, number of leaves and leaf length occurred in co-inoculated plants, regardless of light regime (Table 1).

Tillering was observed only in plants grown under full sun, being 50% higher in co-inoculated plants (Table 1). The increase in the culm length and in the leaf and culm elongation rates, in response to shade, was higher in co-inoculated plants (Table 1). The co-inoculated plants had a 130% larger leaf area~~,~~ in full sun plants and a 200% larger leaf area in shade plants (Figure 4). The SPAD index was higher in co-inoculated plants, increasing by 13% in full sun plants and by 17% in shade plants.

Co-inoculation increased biomass production, irrespective of the light intensity (Fig. 5). Under full sun co-inoculation increased leaf (139%), culm (86%) and root (88%) biomass, resulting in an increase of over 100%, in total biomass relative to non-inoculated plants (Table 2; Fig. 5). Under shade, co-inoculation increased leaf (300%), culm (500%) and root (670%) biomass, resulting in an increase of about 300%, in total biomass, relative to non-inoculated plants (Table 2; Fig. 5).

Co-inoculation also increased relative growth rate by 110%, under full sun, and 500%, in shaded plants (Figure 5). Shaded plants showed higher values of root to shoot biomass ratio and allometric index (K), particularly when co-inoculated (Table 2). Under full sun, co-inoculated plants allocated proportional amounts of biomass between leaves and roots (Fig. 6).

DISCUSSION

When inoculation was performed individually, the benefit varied with the type of bacteria and light intensity. Growth of plants inoculated only with *B. pyrrocinia* was positively related to light intensity. A possible cause for this response was a likely higher demand in photoassimilates for the symbiosis of this microorganism (Aguilar-Chama and Guevara, 2016; Konvalinková and Jansa, 2016). A contrasting response occurred in plants inoculated only with *P. fluorescens*, which fostered higher growth mainly under restricted light intensities.

It could be inferred that the higher radiation intensity might have modified the quantity and chemistry composition of root-exudates, affecting rhizodeposition processes and disturbing rhizosphere functioning (Haichar *et al*., 2008; Venturi and Keel, 2016; Vimal *et al*., 2017). This could have interfered in the quorum sensing, inhibiting the mutual interaction under full sun (Goh *et al.,* 2013; Venturi and Keel, 2016). This response supports the concept that light can interfere with mutualistic interaction, which in turn, varies per the host, and beneficial microorganisms (Konvalinková and Jansa, 2016). Under limited light conditions, microbial root

symbionts can create additive costs, resulting in decreased plant fitness, as observed in *Vatica albiramis* (Saner *et al.*, 2011) and in *Datura stramonium* (Aguilar-Chama and Guevara, 2016), where growth promotion is positively related to light intensity, occurring only at higher light.

When co-inoculated, *B. pyrrocinia* and *P. fluorescens* promoted growth of *B. brizantha*, irrespective of light intensity. The rhizosphere microbiota is known to extend the adaptive capacity of plants to environmental stresses (Venturi and Keel, 2016). The overall beneficial effect of the interaction between microorganisms and plant growth, under contrasting light environments, has also been reported in studies with mycorrhizal growth promoters (Casierra-Posada *et al.*, 2013; Aguilar-Chama and Guevara, 2016; Konvalinková and Jansa, 2016).

In rice, from which the rhizobacteria used in this study were originally isolated, growth is higher with *B. pyrrocinia* inoculation than with *P. fluorescens* inoculation (Rêgo *et al*., 2014; Nascente *et al.*, 2016), or with co-inoculation with these rhizobacteria (Rêgo *et al*., 2014). The synergistic effect of *B. pyrrocinia* and *P. fluorescens*, promoting growth in *B. brizantha*, was possibly a response of increased nutrient flow (Casierra-Posada *et al.*, 2013, Paredes and Lebeis, 2016), phytohormone production, such as auxin or cytokinin, or decreased plant ethylene levels, through the action of the enzyme ACC deaminase (Ahemad and Kibret, 2014; Vimal *et al.*, 2017).

It can be inferred that co-inoculation increased phenotypic plasticity in various key traits as a strategy to mitigate the effect of shade in *B. brizantha*. This was achieved by increasing plant height, culm and leaf elongation (i.e., etiolation) (Goh *et al.*, 2013; Martins *et al*., 2014; Valladares *et al.*, 2016), hence increasing total plant biomass. Since tree shading (Santos *et al.*, 2016) and crop shading (Neves Neto *et al*., 2015) are known to reduce forage production in *B. brizantha* cv. BRS Piatã pastures, it could be inferred that co-inoculation would be a suitable strategy to counteract limited pasture growth in integrated systems.

Specific leaf area (SLA) and leaf area ratio (LAR) vary with light, temperature, moisture and nutrient availability (Barbero *et al.*, 2013; Daniagry and Dang, 2014). Increased SLA and LAR under low light is a strategy to improve light capture in *B. brizantha* (Dias-Filho, 2000), and this response was observed in non-inoculated plants in our study. Our results also indicate that the ability of *B. brizantha* to modify SLA and LAR under low light can be influenced by plant growth-promoting rhizobacteria. A possible cause for this response is the potential ability of the rhizobacteria to supply the needed soil nutrient resources for shaded *B. brizantha*. This is attained through increased root growth, making it unnecessary for the shaded plants to increase SLA and LAR to those levels observed in uninoculated plants. This indirect effect could make inoculated plants more tolerant to additional stresses such as grazing, increasing pasture performance and productivity (Pimentel et al., 2016).

Considering that the greater the SPAD index, the higher the chlorophyll and nitrogen content of *B. brizantha* leaves (Martucello *et al.*, 2009), presumably co-inoculated rhizobacteria also acted synergistically to increase the nitrogen content of leaves. This is because plant growth-promoting rhizobacteria can also affect plant nutrient-acquisition processes, influencing nutrient availability in the rhizosphere, improving the plant nutritional status, thus increasing pasture growth and productivity (Pii *et al.*, 2015). Higher SPAD values correlating positively with chlorophyll and nitrogen contents, were also found in *B. brizantha* with higher levels of nitrogen fertilization (Lima *et al.*, 2016).

The general increase in root biomass under co-inoculation and the biomass allocation patterns to full-sun and shade plants observed in this study, probably increased the efficiency of use of water and nutrients. The observed responses of co-inoculated plants would be useful to increase the fitness of *B. brizantha* in water- limited environments. In grazed pastures, resources use efficiency ensures the persistence of plants over time and longevity of pastures

(Dias Filho, 2000; Martucello *et al.,* 2009; Pimente*l et al.*, 2016). Also, the increased relative growth rate, tillering, leaf area and biomass production in co-inoculated plants, would be a desirable trait in intensively managed systems, such as rotational grazing systems, reducing grazing intervals.

In conclusion, our results attest the potential of co-inoculated *P. fluorescens* and *B. pyrrocinia* to increase biomass production in B. *brizantha* and the expression of those plant characters that may enhance persistence under reduced light availability. In addition, these findings set up the basis for additional exploratory studies, particularly on the ability of this beneficial interaction to remain fully active under grazing, in integrated crop-livestock-forestry systems.

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No conflict of interest

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TABLES

Table 1 Light intensity and plant growth-promoting rhizobacteria (PGPR) on number of tillers (NT), number of leaves per plant (NL), expanded leaf length (EF - cm), culm length (C - cm), leaf appearance rate (LApR - $L^{-}.day^{-1}$), leaf elongation rate (LER - $L^{-}.day^{-1}$) and culm elongation rate (CER - $L^{-}.day^{-1}$) of *Brachiaria brizantha*

TRATAMENTS		NT	NL	EF	C	LApR	LER	CER
Full sun	Non-inoculated	2 c	11 d	24.83 d	10.80 c	0.37 c	0.88c	0.43 b
	BRM-32111	2 c	13 c	27.88 c	11.75 b	0.45 b	0.84d	0.42 b
	BRM - 32113	2 b	16 b	33.6 b	12.05 a	0.45 b	0.96 b	0.44 b
	MIX	3 a	17 a	36.5 a	12.01 a	0.51 a	1.32 a	0.60 a
Shade	Non-inoculated	0	5 d	21.91 c	10.35 d	0.10 c	0.61c	0.26 d
	BRM - 32111	0	6 b	27.77 b	13.83 b	0.20 b	1.45b	0.63 b
	BRM - 32113	0	5 c	28.2 b	11.66 c	0.18 b	1.41 b	0.56 c
	MIX	0	6 a	34.07 a	15.60 a	0.3 a	2.05 a	0.88 a

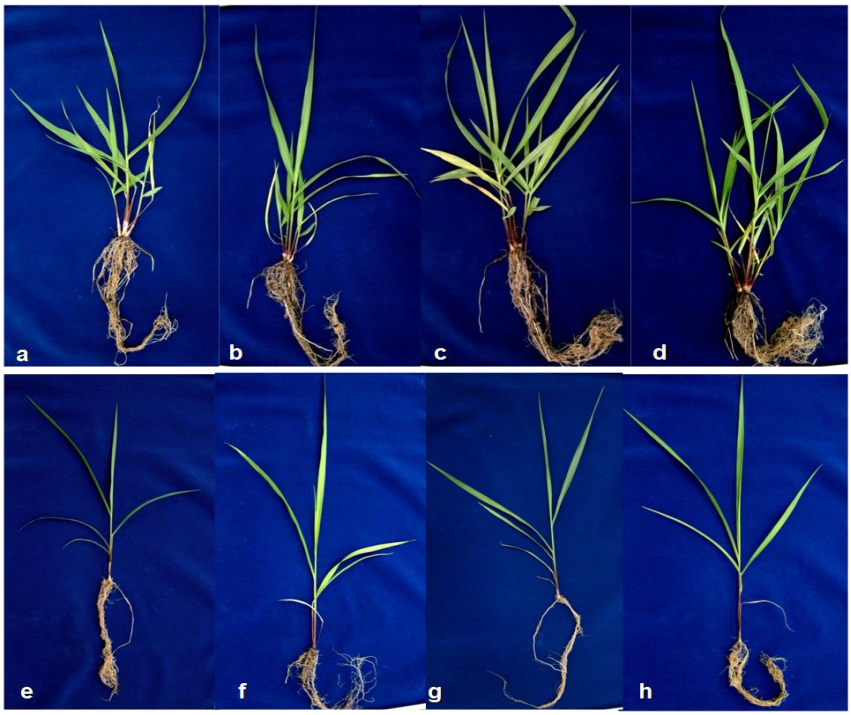
Means followed by different letters in each column and within each light intensity are significantly different ($P < 0.05$, Duncan Test). BRM-32111 = *Pseudomonas fluorescens*; BRM-32113 = *Burkholderia pyrrocinia*; MIX= BRM-32111 + BRM-32113.

Table 2 Light intensity and plant growth-promoting rhizobacteria (PGPR) on the leaf (LDM - g), culm (CDM - g), root (RDM - g) and root/shoot dry mass production, and allometric coefficient K (growth rate of root and shoot ratio) of *Brachiaria brizantha*

TRATAMENTS		LDM	CDM	RDM	Root/shoot	K
Full sun	Non-inoculated	0.80 c	0.35 c	1.01 c	0.88 a	0.91 a
	BRM-32111	0.92 c	0.28 d	1.01 c	0.85 a	0.87 a
	BRM - 32113	1.32 b	0.42 b	1.08 b	0.63 c	0.64 c
	MIX	1.91 a	0.65 a	1.90 a	0.75 b	0.76 b
Shade	Non-inoculated	0.11 d	0.04 c	0.10 d	0.43 c	0.48 c
	BRM - 32111	0.29 b	0.14 b	0.37 b	0.90 c	0.96 b
	BRM - 32113	0.20 c	0.10 c	0.23 c	0.83 c	0.91 b
	MIX	0.44 a	0.24 a	0.77 a	1.16 a	1.21 a

Means followed by different letters in each column and within each light intensity are significantly different ($P < 0.05$, Duncan Test). BRM-3211 = *Pseudomonas fluorescens*; BRM-3213 = *Burkholderia pyrrocinia*; MIX= BRM-3211 + BRM-3213.

1338 **FIGURES**



1339 **Figure 1** *Brachiaria brizantha* cv. Piatã under full-sun (a-d) and shade (e-h). 21 days after
1340 inoculation (35 days after seedling emergence). Non-inoculated plant (a, e), inoculated with
1341 *Pseudomonas fluorescens* (BRM-32111) (b, f), inoculated with *Burkholderia pyrrocinia*
1342 (BRM-32113) (c, g) and co-inoculated with BRM-32111 + BRM-32113 (d, h).

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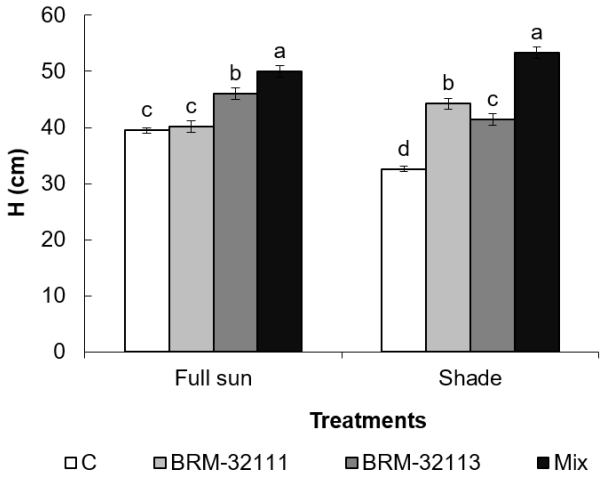

1345

1346 **Figure 2** Light intensity and plant growth-promoting rhizobacteria (PGPR) on plant heigth (H)
1347 of *Brachiaria brizantha*. Columns with different letters are significantly different among PGPR
1348 inoculation ($P < 0.05$, Duncan's test). Mean ± s.e. ($n = 5$). C = Non-inoculated; BRM-32111 =
1349 *Pseudomonas fluorescens*; BRM-32113 = *Burkholderia pyrrocinia*; MIX= BRM-32111 +
1350 BRM-32113.

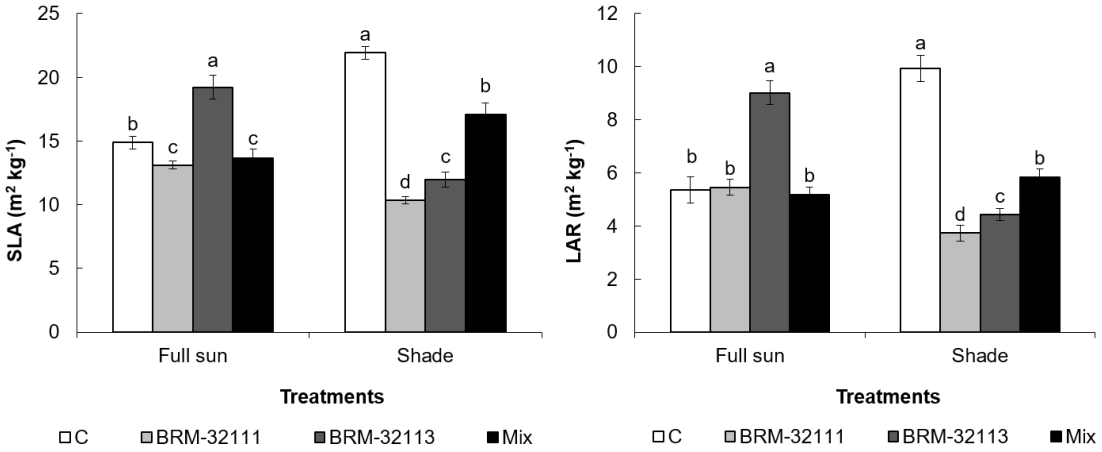

Figure 3 Light intensity and plant growth-promoting rhizobacteria (PGPR) on specific leaf area (SLA) and leaf area ratio (LAR) of *Brachiaria brizantha*. Columns with different letters are significantly different among PGPR inoculation ($P < 0.05$, Duncan's test). Mean ± s.e. ($n = 5$). C = Non-inoculated; BRM-32111 = *Pseudomonas fluorescens*; BRM-32113 = *Burkholderia pyrrocinia*; MIX= BRM-32111 + BRM-32113.

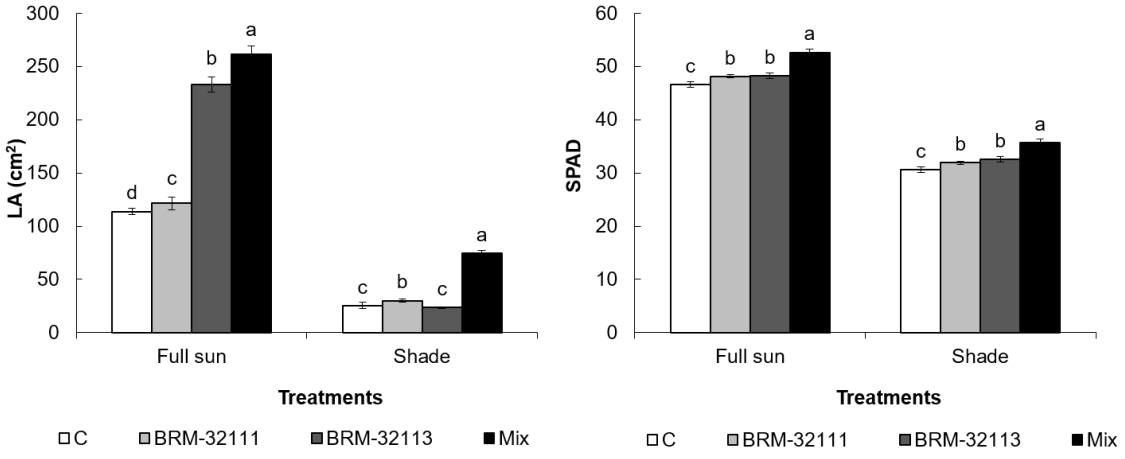

Figure 4 Light intensity and plant growth-promoting rhizobacteria (PGPR) on leaf area (LA) and SPAD index (chlorophyll content) of *Brachiaria brizantha*. Columns with different letters are significantly different among PGPR inoculation ($P < 0.05$, Duncan's test). Mean ± s.e. ($n = 5$). C = Non-inoculated; BRM-32111 = *Pseudomonas fluorescens*; BRM-32113 = *Burkholderia pyrrocinia*; MIX= BRM-32111 + BRM-32113

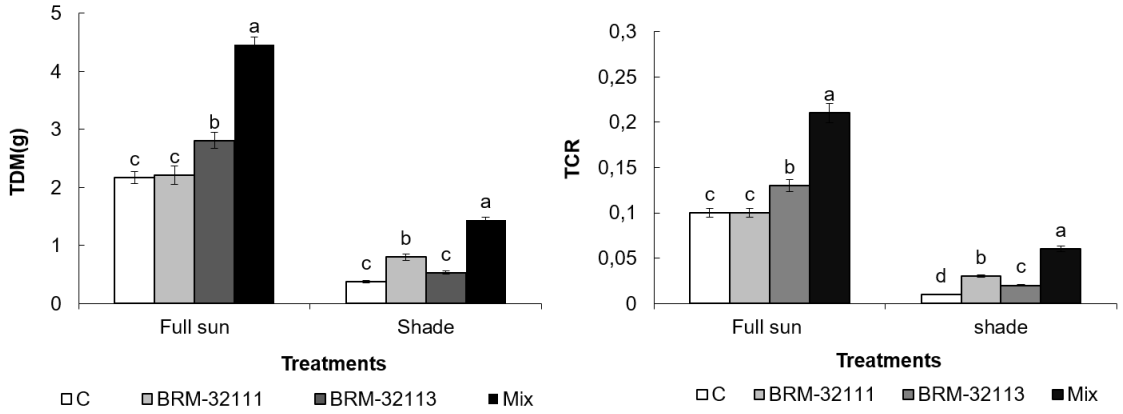

Figure 5 Light intensity and plant growth-promoting rhizobacteria (PGPR) on total dry mass production (TDM) and relative growth rate (RGR) of *Brachiaria brizantha*. Columns with different letters are significantly different among PGPR inoculation ($P < 0.05$, Duncan's test). Mean ± s.e. ($n = 5$). C = Non-inoculated; BRM-32111 = *Pseudomonas fluorescens*; BRM-32113 = *Burkholderia pyrrocinia*; MIX= BRM-32111 + BRM-32113.

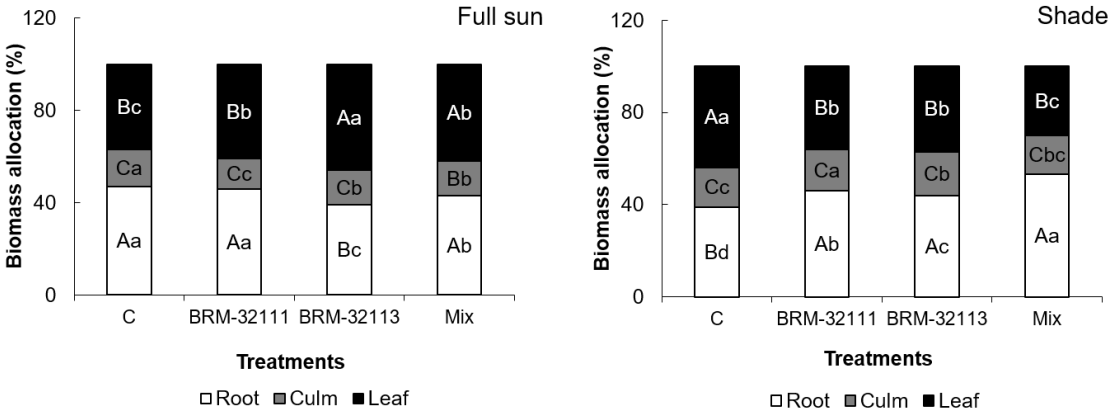

Figure 6 Light intensity and plant growth-promoting rhizobacteria (PGPR) on biomass allocation patter of *Brachiaria brizantha*. Columns with different lower case letters are significantly different among treatments ($P < 0.05$, Duncan Test). Different upper-case letters within columns indicate significant differences among plant organs ($P < 0.05$, Duncan's test). Mean ± s.e. ($n = 5$). BRM-32111 = *Pseudomonas fluorescens*; BRM-32113 = *Burkholderia pyrrocinia*; MIX= BRM-32111 + BRM-32113.

CONCLUSÕES GERAIS

O método de inoculação e a intensidade de luz foram fundamentais para determinar o potencial de *P. fluorescens* e *B. pyrrocinia* em promover o crescimento em *Brachiaria brizantha* cv. Piatã. A coinoculação dessas rizobactérias, pela rega do solo, modificou a anatomia (folha, colmo, raiz), incrementou as taxas fotossintéticas, eficiência instantânea do uso da água, índice Spad (clorofila), nitrato, proteínas, carboidratos totais, amido, N, P, Na, Mg e Fe, promovendo uma maior qualidade e produção de biomassa em *B. brizantha.* Também promoveram o crescimento em *B. brizantha* sob diferentes condições luminosas, incrementando em mais de 100% a produção de biomassa, em comparação as plantas controles com e sem adubo. Esse estudo evidencia o potencial biofertilizante da coinoculação de *P. fluorescens* e *B. pyrrocinia* em *B. brizantha* sob diferentes intensidades de luz, possibilitando seu uso como biofertilizante em sistemas convencionais e integrados, contribuindo para a obtenção de pastagens mais produtivas e com maior valor nutritivo.