

Efficiency of Biostimulants for Alleviating Shade Effects on Forage Grass

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Abstract

Shade is considered an abiotic stress factor which reduce the primary metabolism and restricts normal growth in forage grass in integrated systems. Biostimulants are beneficial in promoting growth and protecting plants against environmental stresses. This is the first study that links biostimulants and the primary metabolism of plants grown under contrasting light intensities. We investigated how the use of biostimulants modifies the primary metabolism, reducing the deleterious effects of shading in the development of tropical forage grass

Brachiaria brizantha cv. BRS Piatã. The association of *Pseudomonas fluorescens* and *Burkholderia pyrrocinia*, inoculated by soil drench, were used as biostimulants. We measured leaf anatomy, plant growth, and biochemical parameters. The use of biostimulants increased the shade tolerance on *B. brizantha* by modifying leaf structure, increasing the chlorophyll content, and inducing the production of osmoregulants, such as carbohydrates and proteins. In turn, increasing the accumulation of primary metabolites, promoted root development, plant height, and leaf area, resulting in more vigorous plants with greater biomass production. These results suggested that forage grass can be protected from inhibitory effects of the shade stress by the biostimulants, and this would improve its survival and growth in integrated crop-livestock-forestry systems.

Keywords: *Pseudomonas fluorescens*, *Burkholderia pyrrocinia*, light, *Brachiaria* grass, PGPR

1. Introduction

Radiation is essential for the growth, development, and survival of plants. Shade, or low-light conditions, interfere with temperature, soil humidity, and biological interactions, being abiotic stress to plants (Kooke & Keurentjes, 2012; Valladares et al., 2016). Shade affects the regulation of plant growth, reduces the primary metabolism and restricts normal growth in forage grass in integrated systems (Pimentel et al., 2016). The increasing use of crop-livestock-forest systems requires shade-tolerant forage species, with adaptations to ensure plant survival and high biomass production (Dias-Filho & Andrade, 2019). Mechanisms of shade tolerance in plants include changes in morphology and biochemistry, which can limit their growth (Valladares et al., 2016; Mundim & Pringle, 2018). In forage grasses, shade tolerance requires changes in morphology and leaf anatomy which alter forage quality (Pimentel et al., 2016).

Plant biostimulants are substances or microorganisms that promote plant growth (Oosten et al., 2017; Malik et al., 2021; Pacheco et al., 2021). These biostimulants can be used as an alternative to increasing shade tolerance in pasture plants. Plant growth-promoting rhizobacteria (PGPR) act as biostimulants enhancing nutrient uptake, modulating hormones, and mitigating the effects of biotic and abiotic stress (Lopes et al., 2021; Riaz et al., 2021). Biostimulants can mitigate the deleterious effects of shade in *Solanum quitoense* Lam. (Casierra-Posada et al., 2013); heavy metals contamination of soil in *Triticum aestivum* L. (Singh et al., 2018); water stress in *Cicer arietinum* L. (Khan et al., 2019); and saline stress in *Arachis hypogaea* and *Zea mays* (Abbas et al., 2019). However, environmental factors, in addition to affecting plant growth, can also affect biostimulants efficiency, as the cost of maintaining symbiosis can outweigh its benefits, turning microorganisms into parasites, slowing plant growth (Odokonyero et al., 2016; Lopes et al., 2018). Therefore, it is necessary to know how growth-promoting rhizobacteria act on the primary metabolism of plants grown under particular stress.

This is the first study that links PGPR inoculation and the primary metabolism of plants grown under different light intensities. Primary metabolites, such as amino acids, proteins, carbohydrates, and organic acids, guarantee the maintenance of cell reproduction and plant

development (Mundim & Pringle, 2018, Wang et al., 2019). These osmoregulants increase plant tolerance against environmental stress conditions (Lopes et al., 2021), and shade reduced it is the concentration (Kooke & Keurentjes, 2012).

Changes in the light environment require the rearrangement of assimilates and photosynthetic metabolites to adapt the plant to its new environment (Valladares et al., 2016; Wang et al., 2019). This rearrangement can affect the morphological, physiological, and biochemical characteristics of plants, such as height, root growth, leaf area, and biomass production. In this study, physiological and biochemical parameters are integrated to elucidate how the interaction between plant and microbe influences plant metabolism. The aim is to understand how these factors are associated to mitigate the deleterious effects of shading on *Brachiaria* (Syn. *Urochloa*) *brizantha*. This information is basic for the selection of plant characteristics that will contribute to a better performance of forage species in tropical integrated crop-livestock-forestry systems.

Therefore, this study on the interaction of rhizobacteria between *B. brizantha* ecophysiology can contribute to establishing management practices consistent with the productive potential of forage plants in low-light environments, ensuring the persistence in integrated systems, and sustainably increasing their productivity. We hypothesize that biostimulants can modify the primary metabolism of *B. brizantha* to mitigate the effects of shading. The aim was to investigate how the use of biostimulants modifies the primary metabolism, reducing the deleterious effect of shading in the development of tropical forage grass *Brachiaria brizantha*.

Therefore, this study on the interaction of rhizobacteria with the ecophysiology of *B. brizantha*, can contribute to establish management practices consistent with the productive potential of forage plants in low light environments, ensuring the persistence in integrated systems and increasing their productivity in a way sustainable.

Our hypothesis is that biostimulants can modify the primary metabolism of *B. brizantha* to mitigate the effects of shading. The objective was to investigate how the use of biostimulants modifies the primary metabolism, reducing the deleterious effect of shading on the development of the tropical forage grass *Brachiaria brizantha* cv. BRS Piata.

2. Method

2.1 Plant Material and Growth Conditions

The experiment was conducted in a semi-controlled environment in the Federal Rural University of Amazonia (01°27'25 "S, 48°26'36" W), Belém-Pará, Brazil. According to Koppen, the climate classification is Af (equatorial). During the experimental period, the mean air temperature was $32 \pm 2,8^{\circ}\text{C}$ and relative humidity was $73 \pm 3\%$ (mean \pm s.d.). Seeds of *Brachiaria* (Syn. *Urochloa*) *brizantha* cv. BRS Piatã were sown in polyethylene pots (15 x 25 x 0.5 cm) filled with Ferralsol soil (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 $\mu\text{molm}^{-2} \text{s}^{-1}$ and 1113 $\mu\text{molm}^{-2} \text{s}^{-1}$) obtained with black polyethylene screens.

2.2 Biostimulants

The rhizobacteria *Burkholderia pyrocinia* (BRM-32113) and *Pseudomonas fluorescens* (BRM-32111) were used as biostimulants are preserved in the *in vitro* collection of the Plant Protection Laboratory, at the Federal Rural University of the Amazon. The bacterial cultured, suspension was prepared and inoculated in plants by soil drench, according to Lopes et al. (2018).

2.3 Plant Growth Parameters

At 35 days after seedling emergence (DASE), the plants were collected and the growth parameters were measured (Hunt, 1990; Barbero et al., 2013). The height and culm length were determined and ten plants per treatment were harvested and separated into shoot and roots. The plant material was oven-dried at 60° until constant mass. Total dry mass was calculated by adding shoot dry mass and root dry mass. SPAD index (soil plant analysis development) was measured in the youngest fully expanded leaf blade (mean of five readings per leaf). A portable chlorophyll meter was used (SPAD-502. Konica Minolta Sensing, INC. Japan). Total leaf area per plant was estimated from specific leaf area and leaf mass results. Specific leaf area (the ratio of leaf area to leaf dry mass) was determined overleaf disks (5 disks per plant) of either 0.42 cm² or 2.28 cm², dried at 60°C until constant mass.

2.4 Anatomical Characteristics

The anatomical study was performed at the Laboratory of Plant Anatomy, in Museu Paraense Emílio Goeldi. At 35 DASE, leaf samples were collected and fixed in FAA solution 50% (ethanol: glacial acetic acid: formaldehyde). The samples were dehydrated in a graded ethanol series and embedded in hydroxyethyl methacrylate (Leica®, Germany). The blocks were cross-sectioned on a rotating microtome Leica RM 2265, with the section of leaves were 5 µm thick. Anatomical parameters measured were thickness of the adaxial and abaxial epidermis, and area of the bulliform cells, sclerenchyma, vascular bundles, bundle sheath, mesophyll chlorophyll, xylem, phloem, and the number of vascular bundles. The measurements were carried out under a BX61 Olympus microscope (Japan) and with the software Motic 2.0.

2.5 Biochemical Analysis

For measured photosynthetic pigments were used 10mg of fresh leaves and ethanol solutions. The absorbance of the samples was determined at 645 and 665 nm (Lopes et al., 2020). Total soluble carbohydrates and starch content were determined using the 50 mg of dry matter and the quantification of the total soluble carbohydrates at 490 nm, using glucose as a standard (Dubois et al., 1956). Ammonium content was determined in a sample of 50 mg of dry matter and quantification was carried out at 625 nm, with (NH₄)₂SO₄ as standard (Weatherburn, 1967). Nitrate content was measured in a sample of 100 mg of dry matter and quantification was carried out at 410 nm, with KNO₃ as standard (Cataldo et al., 1975). Amino acid and total soluble protein contents were determined using the 10 mg of fresh leaves. The absorbance was measured amino acid was at 570 nm. The calibration curve was made using Leucine (1mM) (Gibon et al., 2004). Quantification of the total soluble proteins was carried out at 595 nm, and albumin bovine was used as a standard (Bradford, 1976).

2.6 Statistical Analyses

The experimental design was completely randomized in 2×2 factorial arrangements (non-inoculated *B. brizantha* and co-inoculated (*B. pyrrocinia* + *P. fluorescens*) x full-sun and shade), with ten replicates. Data were subjected to analysis of variance and means compared by the Duncan test ($P < 0.05$). Statistical analyses were performed by the STATISTICA (StatSoft, Inc., Tulsa, USA).

3. Results

Biostimulants were able to modify the metabolism of *B. brizantha*, changing its leaf structure so that the ambient light was used most efficient, resulting in more vigorous plants, with greater height and biomass production under full sun and shade ($F_{1,20} = 517.23$; $P < 0.01$) (Figure 1).

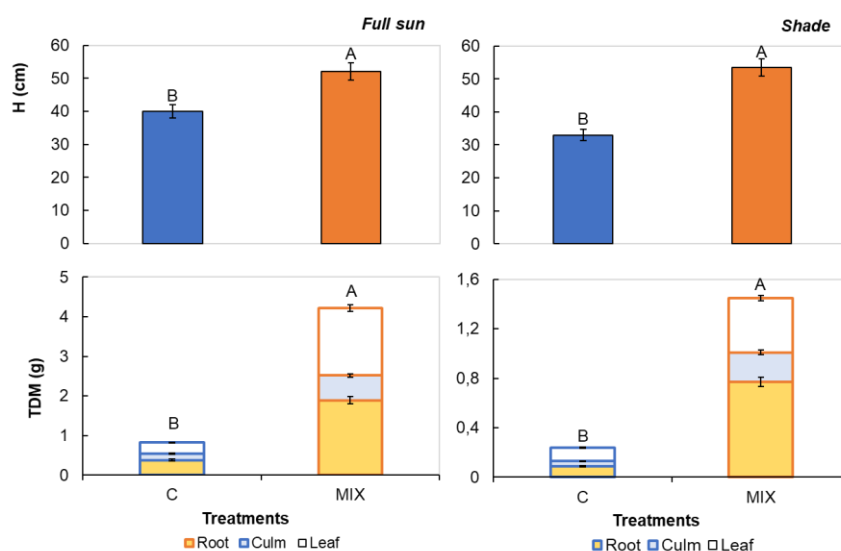


Figure 1. Effect of biostimulants and light on dry mass production (TDM) and plant height (H) of *Brachiaria brizantha*. Error bars represent standard errors of the mean ($n = 10$). Different letters are indicating significant differences ($P < 0.05$) among treatments (C = non-biostimulants; Mix = *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*)

Under full sun, inoculated plants showed similar biomass partition between leaves and roots. Under the shade, inoculated plants increase biomass allocation to roots (Figure 1). Under full sun and shade, the use of biostimulants also favored an increased in the SPAD index, chlorophyll concentration, especially chlorophyll b (Figure 2), and leaf area ($P < 0.01$) (Figure 3). Biostimulants reduced specific leaf area in *B. brizantha* under both light intensities ($F_{1,20} = 113.8$; $P < 0.01$) (Figure 3).

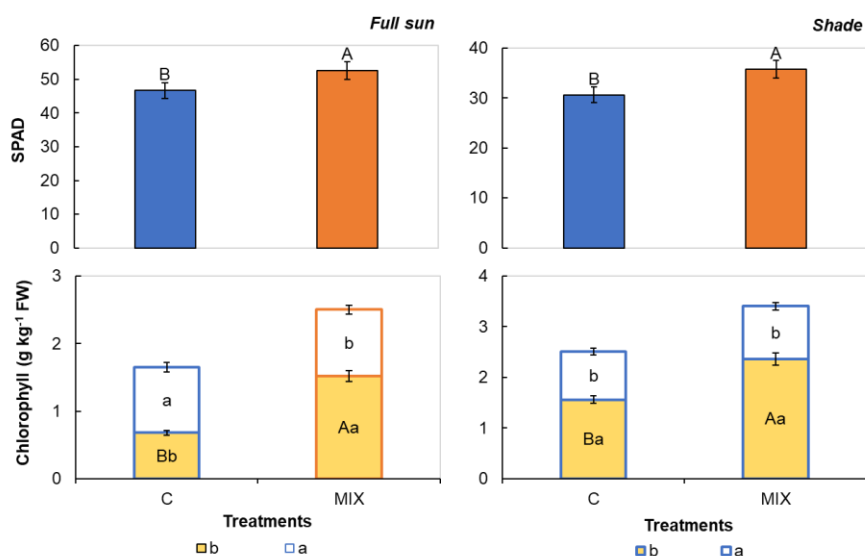


Figure 2. Effect of biostimulants and light on SPAD index and chlorophyll total (a + b) of *Brachiaria brizantha*. Error bars represent standard errors of the mean (n = 10). Different lowercase letters within columns indicate significant differences among shoot and root. Different upper-case letters are indicating significant differences (P < 0.05) among treatments (C = non-biostimulants; Mix = *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*)

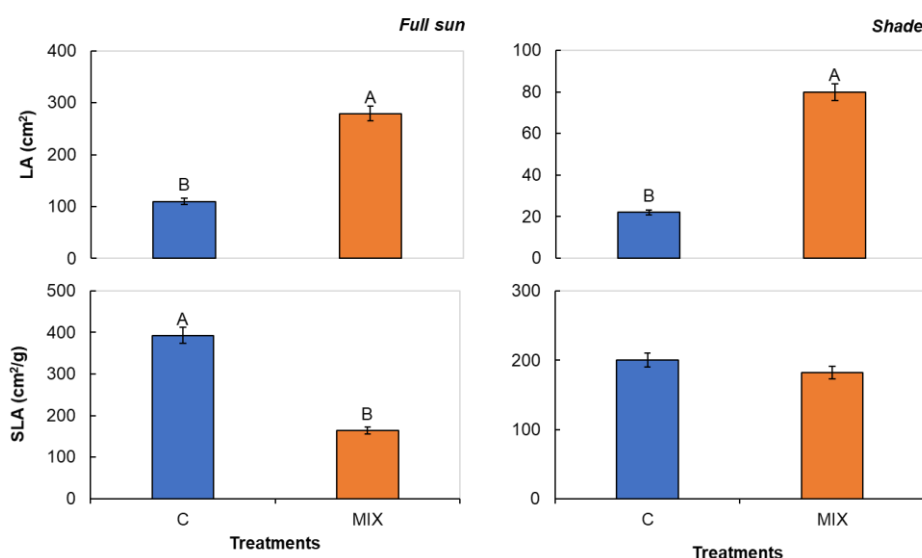


Figure 3. Effect of biostimulants and light on leaf area (LA) and specific leaf area (SLA) of *Brachiaria brizantha*. Error bars represent standard errors of the mean (n = 10). Different letters are indicating significant differences (P < 0.05) among treatments (C = non-biostimulants; Mix = *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*)

Biostimulants modified leaf anatomy. In the cross-sections of leaf blades of inoculated plants, it was possible to observe increased leaf thickness (Figure 4; Table 1). Inoculation also resulted in larger vascular bundles area, sheath cells, vascular sheath, and chlorophyll

mesophyll, regardless of the light intensity in which plants were grown ($P < 0.01$) (Table 1).

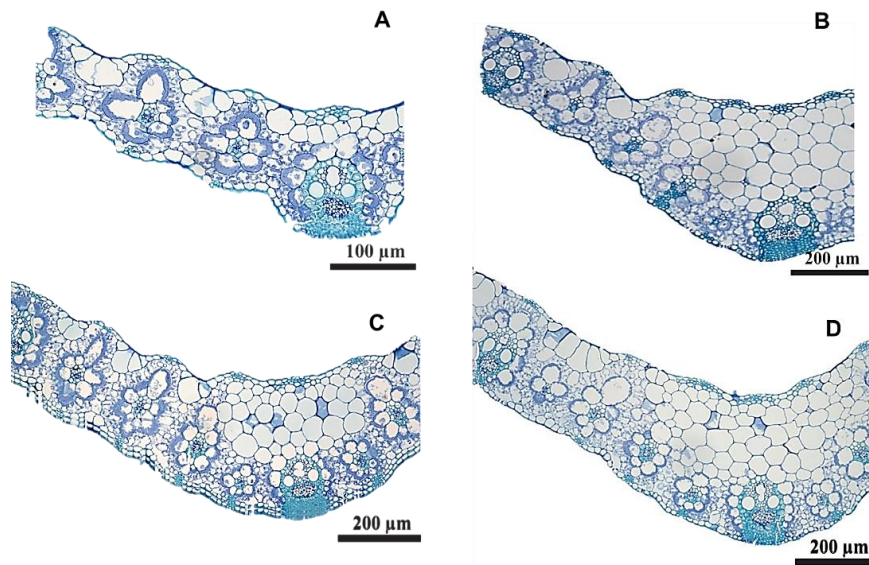


Figure 4. *Brachiaria brizantha* cross sections leaf. Plants under shade (a,b) and full sun (c,d). Non-biostimulants ((a,c) and biostimulants plants (b, d)

Under both full sun and shade, inoculated plants showed higher carbohydrates ($F_{1,20} = 122.8$; $P < 0.001$) and starch content ($F_{1,20} = 463.6$; $P < 0.01$) (Figure 5). Inoculated full-sun plants showed higher carbohydrates and starch concentration in the shoot (Figure 5), while in inoculated shade plants carbohydrate partition was similar between shoot and root and starch concentration was higher in shoots (Figure 5).

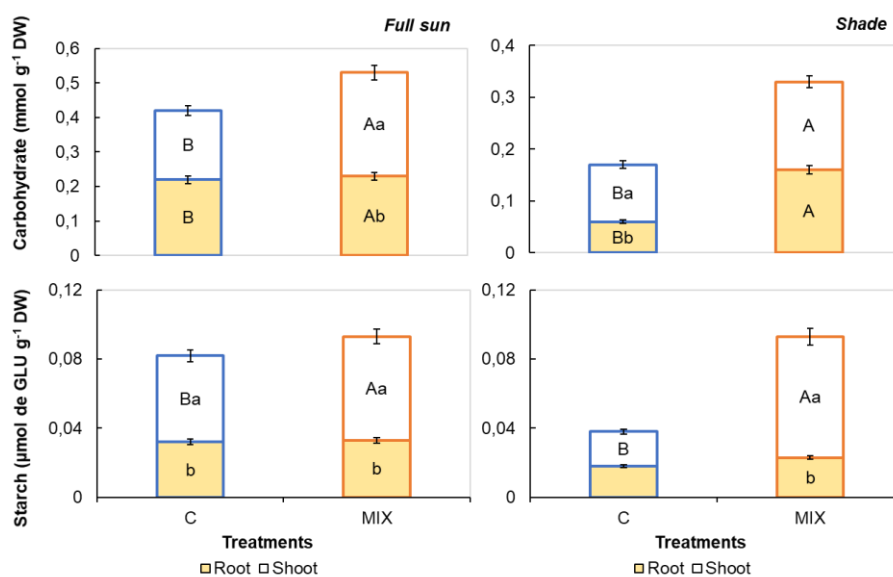


Figure 5. Effect of biostimulants and light on carbohydrate and starch of *Brachiaria brizantha*. Error bars represent standard errors of the mean ($n = 10$). Different lowercase letters within columns indicate significant differences among shoot and root. Different

upper-case letters are indicating significant differences ($P < 0.05$) among treatments (C = non-biostimulants; Mix = *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*)

Table 1. Biostimulants and light intensity on anatomical parameters of *Brachiaria brizantha*.

Parameters	Non-biostimulants	Biostimulants
<i>Full sun</i>		
Thickness (μm)	48,48 b	100,86 a
Adaxial epidermis (μm)	2,29 b	5,24 a
Abaxial epidermis (μm)	2,36 b	5,09 a
Bulliform cells area (μm^2)	112,51 b	319,76 a
Xylem area (μm^2)	359,56 b	489,98 a
Phloem area (μm^2)	338,25 b	411,91 a
Sclerenchyma area (μm^2)	90,81 b	234,36 a
Vascular bundles area (μm^2)	86,42 b	245,38 a
Bundle sheath area (μm^2)	422,40 b	950,94 a
Mesophyll chlorophyll area (μm^2)	104,68 b	889,87 a
<i>Shade</i>		
Thickness (μm)	38,14 b	87,59 a
Adaxial epidermis (μm)	1,09 b	4,54 a
Abaxial epidermis (μm)	2,86 b	4,87 a
Bulliform cells area (μm^2)	54,41 b	57,97 a
Xylem area (μm^2)	4,76 b	26,95 a
Phloem area (μm^2)	1,97 b	6,10 a

Sclerenchyma area (μm^2)	2,33 b	17,16 a
Vascular bundles area (μm^2)	5,42 b	65,72 a
Bundle sheath area (μm^2)	24,47 b	130,87 a
Mesophyll chlorophyll area (μm^2)	32,55 b	58,46 a

Irrespective of inoculation and light regime *B. brizantha* showed higher ammonium concentration in roots, and more nitrate and amino acids in the shoot (Figure 6). Inoculated full-sun plants had higher ammonium content in roots, while nitrate was equally distributed between shoots and roots (Figure 6). In shade, the inoculated plants had lower ammonium and a higher nitrate concentration, and two metabolites concentration was higher in the shoot (Figure 6). Inoculated plants, under both sun and shade environments, had lower amino acids and a higher protein concentration, both with a greater allocation to shoots ($F_{1,20} = 24.69$; $P < 0.01$ and $F_{1,20} = 13.7$; $P < 0.01$, respectively), as shown in Figure 7.

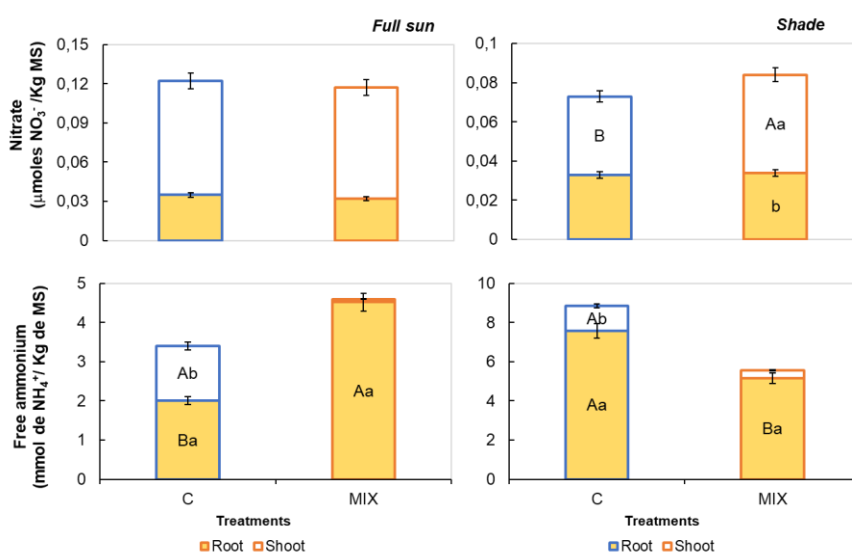


Figure 6. Effect of biostimulants and light on nitrate and free ammonium of *Brachiaria brizantha*. Error bars represent standard errors of the mean ($n = 10$). Different lowercase letters within columns indicate significant differences among shoot and root. Different upper-case letters are indicating significant differences ($P < 0.05$) among treatments (C = non-biostimulants; Mix = *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*)

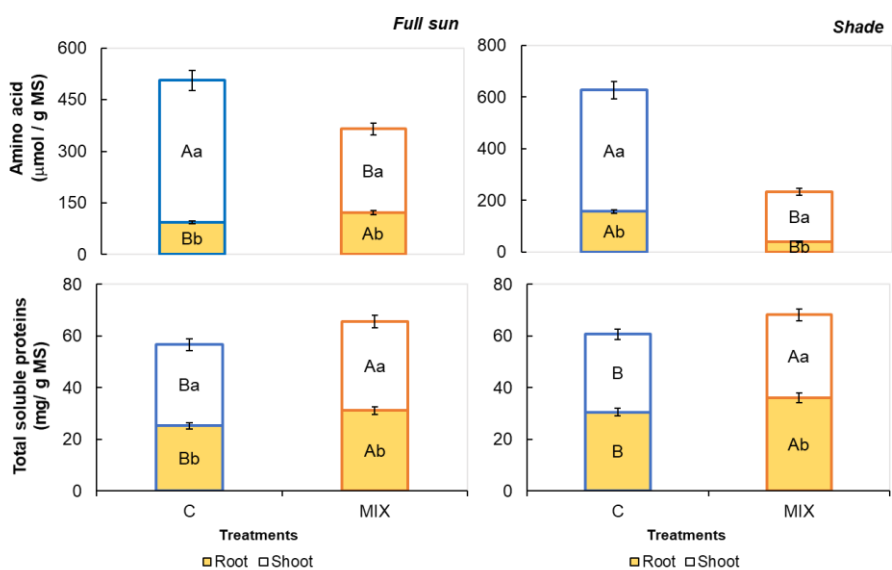


Figure 7. Effect of biostimulants and light on amino acid and proteins of *Brachiaria brizantha*. Error bars represent standard errors of the mean (n = 10). Different lowercase letters within columns indicate significant differences among shoot and root. Different upper-case letters are indicating significant differences ($P < 0.05$) among treatments (C = non-biostimulants; Mix = *Burkholderia pyrrocinia* + *Pseudomonas fluorescens*)

Shade reduced the growth and development of *B. brizantha*, but the use of biostimulants mitigated the effect of shading on plant development (Table 2). Regardless of light intensity, the use of biostimulants increased biomass production, which was linearly correlated ($P < 0.05$) with SPAD index, leaf area, height, chlorophyll b, primary metabolites, and mesophyll chlorophyll area (Table 2).

Table 2. Biostimulants and light on percentage increase of *Brachiaria brizantha*

Parameters	Full sun	Shade
Leaf dry mass	507	264
Culm dry mass	294	500
Root dry mass	397	755
Total dry mass	402	476
Leaf area	154	265
Height	30	62
SPAD index	13	17
Chlorophyll b	123	51
Carbohydrate shoot	50	54
Carbohydrate root	-	167
Starch shoot	20	250
Free ammonium root	126	-
Nitrate shoot	-	25
Total soluble proteins shoot	10	7
Total soluble proteins root	24	20

4. Discussion

The *B. pyrrocinia* and *P. fluorescens* acted as biostimulants, promoted the highest in nitrogen content, increasing the chlorophyll concentration, primary metabolites content, such as sugars and proteins, resulting in more vigorous and productive plants under light intensity, and also increased shade tolerance in *B. brizantha* (Figure 8).

Environmental stress can affect the survival, diversity of microbes, and plant growth-promoting microbes potential to improve plant growth and stress tolerance (Lopes et al. 2021). This is observed in *Brachiaria* inoculated with *Sarocladium implicatum*. According

to Odokonyero et al. (2016), this interaction was not beneficial, *S. implicatum* has a higher metabolic cost, does not attenuate the effects of water stress, reduces the biomass and carbohydrates content, affecting the quality and production in *Brachiaria*.

Furthermore, shade reduces the products of photosynthesis, concentrations of metabolites, plant growth, and production of biomass mainly on C4 forage plants, such as *B. brizantha* (Gobbi et al., 2011; Pimentel et al., 2016), *Phalaris aquatica* (Ciavarella et al., 2000) and *Trichloris crinite* (Cavagnaro & Trione, 2007).

Our results support the idea of functional stability in the forage grass – biostimulants interaction facing disturbances. In the present study, it was observed that the use of bioinoculants was beneficial to increase growth, physiological plasticity, and shade tolerance in *B. brizantha*.

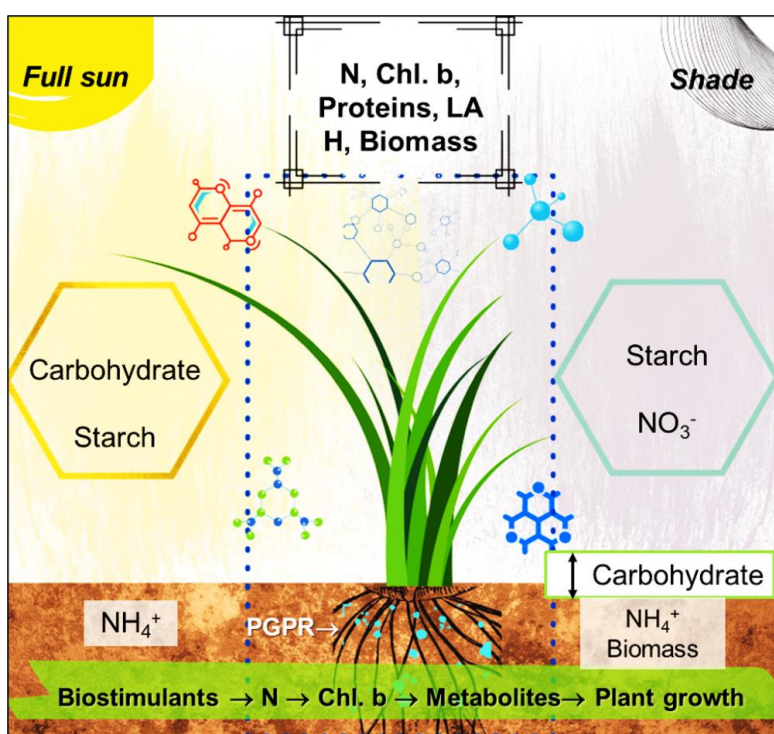


Figure 8. Summary of main key mechanisms of biostimulants (PGPR) on *Brachiaria brizantha* growth under full sun and shade. Nitrogen (N); leaf area (LA); plant height (H); nitrate (NO_3^-); ammonium (NH_4^+); Chlorophyll b (Chl. b)

Biostimulants improved morphological adaptations to increase shade tolerance in *B. brizantha*, such as the increase in the leaf area, and ecophysiological mechanism which allows the increase in the photosynthetic surface to absorb light (Taiz & Zeiger, 2017; Gobbi et al., 2011; Valladares et al., 2016). In the present study, the use of bioinoculants increased leaf thickness and biomass by about 500% in full-sun plants and over 200% in shade plants, reducing the specific leaf area in both light environments (Table 2).

Anatomical leaf changes in inoculated plants increased light capture and shade tolerance. The greater efficiency in capturing light, followed by better photochemical use of inoculated

plants, reflected an increase of around 400% in total biomass production, irrespective of light intensity (Table 2). Inoculated plants showed an increase in bulliform cells. This characteristic would increase *Brachiaria* resistance to water stress (Gobbi et al., 2011). These changes in the leaf internal structure affected the metabolic routes that determine the amount of carbon fixed in the chloroplasts and sugar allocation and transport, resulting in higher carbohydrate and starch concentration in these plants. In *Brachiaria decumbens* cv. Basilisk, shade also increased specific leaf area, which was granted to a reduction in leaf thickness, vascular bundles, and bulliform cell areas (Gobbi et al., 2011).

A significant increase in the chlorophyll content in plant biostimulants, mainly chlorophyll b, optimized the absorption of photosynthetically active radiation. This is because chlorophyll b absorbs energy at wavelengths greater than chlorophyll a in plants under shade (Gonçalves et al., 2001; Taiz & Zeiger, 2017). The increase in chlorophyll has also been reported in *Trichloris crinita* grown under shade (Cavagnaro & Trione, 2007), *Triticum aestivum* L. (Singh et al., 2018), and *Cicer arietinum* L. with biostimulants (Khan et al., 2019).

Biostimulants are also beneficial in mitigating other effects of abiotic stresses, by increase the photosynthetic pigments, carbohydrates, proteins concentrations, and total biomass (Lopes et al., 2021), as related in *Cicer arietinum* L. (Khan et al., 2019) and *Triticum aestivum* L. (Singh et al., 2018) both under drought. Our results showed that the biostimulants attenuated the effect of shading by the accumulation of sugars, resulting in a higher concentration of starch, carbohydrate, and nitrogen, increasing biomass production.

In the present study, plant biostimulants also had a higher SPAD index, suggesting higher nitrogen content. Nitrogen is an essential nutrient to forage growth, influences leaf area development and the carbohydrates allocation, can direct carbohydrate to the secondary metabolism, synthesizing it in lignin, an indigestible compound to animals (Gobbi et al., 2011). The nitrogen absorbed is metabolized in the roots or xylem transported to the shoot, being preferentially absorbed in the form of nitrate (NO_3^-) and ammonium (NH_4^+) (Taiz & Zeiger, 2017). In this study, regardless of the treatment, ammonium was assimilated in the roots, while nitrate was transported to shoot in *B. brizantha*. Once assimilated, nitrogen was converted into other organic compounds, which resulted in an increase in amino acids and proteins in plants inoculated with biostimulants.

Carbohydrate accumulation in the shoot of inoculated plants, in this study, may have acted as a means to activate the gene expression related to photosynthetic activities (Odokonyero et al., 2016; Khan et al., 2019). To survival under stress, the plant can use mechanisms, as the increase in carbohydrates which acts as an osmotic regulator and increases starch content, which is an energy reserve (Oosten et al., 2017; Hennion et al., 2019). Therefore, the higher accumulation of primary metabolites in inoculated plants also mitigated the effects of shade. The increase in carbohydrate and starch contents are also beneficial in forage grasses to improve palatability, protein content and dry matter consumption, allowing higher weight gain and milk production (Ciavarella et al., 2000; Barbero et al., 2013).

Sugars also regulate root elongation, modeling the plasticity of root architecture in response to environmental factors (Caretto et al., 2015; Hennion et al., 2019). Previous research shows

that shade decreases total dry mass production and reduces the amount of carbon allocated to roots (Pimentel et al., 2016). In the present study, the increase in root biomass observed in inoculated shade plants suggests that the use of biostimulants potentiated water and nutrients absorptions, improving plant growth under shade. Bioinoculants also increased root biomass in *Cicer arietinum* L. (Khan et al., 2019). The fact that inoculated *B. brizantha* allocated more biomass to roots, could make them more tolerant to grazing, increasing pasture longevity (Pimentel et al., 2016; Lopes et al., 2018).

5. Conclusions

The use of bioinoculants increased the shade tolerance on *B. brizantha* by modifying leaf structure, increase the chlorophyll content, and inducing the production of osmoregulators, such as carbohydrates and proteins. In turn, increasing the accumulation of primary metabolites, promoted root development, plant height, and leaf area, resulting in more vigorous plants with greater biomass production. These results suggested that the use of biostimulants can reduce the deleterious effect of shading in forage grass, which would improve its survival and growth in integrated crop-livestock-forestry systems. Studies of multiple resource limitations (e.g., combined nutrient, drought, and flooding) are still essential and may improve our understanding and management of pasture ecosystems.

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