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Co-Inoculation of *Azospirillum brasilense* and *Bacillus* sp. Enhances Biomass and Photosynthetic Efficiency in *Urochloa brizantha*

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Abstract: The synergism between plant growth-promoting bacteria species (PGPB) was evaluated regarding the effect of inoculation on productivity and the physiological aspects of *Urochloa brizantha*. The study included seven experimental groups arranged in a 3 × 2 + 1 factorial design consisting of three inoculants (*Azospirillum brasilense*, *Bacillus* sp. isolate EB-40, and *Bacillus* sp. isolate EB-40 + *A. brasilense* mixture), two application methods (seed and foliar spray), and controls. The MIX conjugate inoculation significantly increased plant height in all three harvests, with gains of 57%. At 60 and 90 days, MIX increased the number of tillers by 47% and the number of leaves by 61% compared to other treatments in all harvests. MIX also increased shoot dry mass in the second and third harvests, with improvements of 57–60% compared to the control. MIX improved the quantum efficiency of photosystem II and the ratio between variable and maximum chlorophyll fluorescence. Maximum fluorescence (Fm) was 11% higher in MIX-treated plants compared to the control, indicating increased potential photosynthesis. Variable fluorescence (Fv) efficiency improved by 22% for inoculation with *A. brasilense* and *Bacillus* sp. Our study reveals that *A. brasilense* plus the *Bacillus* sp. isolate EB-40 (MIX) has the potential to improve the resilience and productivity of *U. brizantha*.

Keywords: plant growth-promoting bacteria; *Urochloa brizantha*; chlorophyll fluorescence; photosynthesis; forage biomass

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1. Introduction

Complex interactions occur between soil microorganisms and associated plants [1]. Beneficial microbes interact directly by regulating host plant growth or indirectly through antagonistic activity against plant pathogens [2,3]. Protocooperation is a positive intra- or interspecific interaction where one species benefits from another, yet neither species requires the other for survival. Plant–microorganism interactions enhance plant growth, stress tolerance, and disease resistance while increasing nutrient availability, absorption, and biodiversity [4,5].

Plant growth-promoting bacteria (PGPB) are free-living microorganisms that associate with plant roots. These bacteria inhabit soil and the rhizosphere, often colonizing plant tissues [6]. The rhizosphere stimulates microbial proliferation, as plants release organic compounds and energy that promote microbial growth, enhancing plant–microorganism associations [7]. Certain microorganisms are incorporated into commercial soil additives due to their plant growth-promoting properties [8].

Azospirillum brasilense and *Bacillus* sp. isolate EB-40 species are plant growth-promoting bacteria that enhance plant development and productivity through atmospheric nitrogen fixation [8,9], the production and release of plant hormones [9,10], and the solubilization of inorganic phosphates [11]. Co-inoculation of nitrogen-fixing *A. brasilense* and phosphate-solubilizing *Bacillus* sp. can increase seed yield and grain protein content, functioning as an effective biological resource for sustainable development [12,13]. In pastures, the inoculation of PGPB strains demonstrates benefits through increased productivity, improved drought stress tolerance, and reduced soil and pasture degradation [9,14].

Urochloa brizantha cv. Marandu is the primary forage cultivar utilized for grazing in warm subhumid and humid climates [11]. *U. brizantha*, a forage grass native to Africa, has been widely cultivated in Brazil due to its high biomass production and adaptability to tropical climates [15]. This species is characterized by its allotetraploid nature and apomictic reproduction, which allows the stable transmission of favorable traits. Its high drought tolerance and ability to thrive in various soil conditions make it a valuable agricultural resource. Farmers cultivate this species in tropical soils deficient in nitrogen and phosphorus, conditions that induce plant stress and contribute to pasture degradation. Effective application of PGPB on Marandu palisade grass could expand this technology to tropical pasture cultivation, reducing fertilization costs and dependence on inorganic fertilizers [2].

Seed treatment with PGPB is a viable and economical method to apply this biotechnology [16]. The literature claims high efficacy in seed treatment with microorganisms, since the translocation mechanism of these organisms occurs in the soil [11,17]. Further investigation is needed to comprehensively evaluate the effectiveness of foliar PGPB application across diverse plant systems. This approach is particularly relevant for established forage plants or for repeated applications in perennial pastures. Here, we investigated the effects of inoculating *A. brasilense* and *Bacillus* sp. isolate EB-40, individually and mixed, on seeds or foliage and on the physiological responses and dry mass production of Marandu palisade grass.

2. Materials and Methods

2.1. Microorganisms and Propagation

The strain *Bacillus* sp. isolate EB-40 was previously identified and evaluated for its in vitro biotechnological potential [10,18,19]. The *A. brasilense* strain was isolated from a commercial nitrogen-fixing inoculant and the strain *Bacillus* sp. isolate EB-40 was isolated from the roots of the banana tree cultivar 'Prata Anã' in Minas Gerais State (Table 1). Both bacterial species were cultivated in a modified dextrose, yeast, glutamate, and sucrose (DYGS) medium [20], containing 2 g L^{-1} glucose, 2 g L^{-1} malic acid, 1.5 g L^{-1} bacteriological peptone, 2 g L^{-1} yeast extract, 1.5 g L^{-1} glutamic acid, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 20 g L^{-1} agar for a solid medium. The pH was adjusted to 6.8 with 10% KOH solution. All the reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Table 1. Microbial strains used for the inoculation of Marandu palisade grass.

Species	Remarkable Skills
Bacillus sp. isolate EB-40 (EB-40/GQ340516.1)	BNF, IAA, SIF
Azospirillum brasilense	BNF, IAA

Note: Biotechnological potential of the species regarding BNF—biological nitrogen fixation; IAA—production and release of indolacetic acid; and SFI—solubilization of inorganic phosphates.

2.2. Disc Diffusion Assay for Bacillus sp. and Azospirillum brasilense Antagonism

We assessed the antagonistic effect of *Bacillus* sp. isolate EB-40 on *A. brasilense* using the method from [18]. Pure *A. brasilense* culture in the exponential phase was inoculated across 90 × 150 mm plates containing solid DYGS medium [20]. After 24 and 48 h of growth at 30 °C, we applied 6 mm filter paper discs impregnated with 0.01 mL *Bacillus* sp.

isolate EB-40 inoculum to the plate surface. We repeated this process using plates with pure *Bacillus* sp. culture, applying discs impregnated with *A. brasilense* broth (cultured for 48 h at 30 °C) to the surface.

After 24 h of incubation at 30 °C, we observed the presence or absence of inhibition halos and measured their diameters. We classified inhibition zones as very strong (20–25 mm), strong (15–19 mm), moderate (11–14 mm), weak (9–10 mm), and no inhibition (<9 mm). All tests were performed in triplicate.

2.3. Cross-Streak Method for Assessment of Bacillus sp. and Azospirillum brasilense Antagonism

The inhibition test had a single strain inoculated onto a solid DYGS medium plate containing bromocresol purple (0.02 g L^{-1}) using a 0.01 mL loop and followed the protocol [21]. The inoculum was prepared in 0.85% NaCl solution according to MacFarland scale 1.

Inoculated plates were incubated for 24 h (*Bacillus* sp. isolate EB-40) or 48 h (*A. brasilense*), both at 30 °C. After incubation, we applied a chloroform layer to cover the entire bacterial range, then kept the plates closed for 10 min to inactivate all living cells.

Subsequently, the plates were left open for 10 min to evaporate the chloroform. Next, we applied suspensions of *A. brasilense* and *Bacillus* sp. (in 0.85% NaCl solution, McFarland scale 1) perpendicularly to the chloroform-inactivated strain using a 0.01 mL loop. We then incubated the plates at 30 °C for 24 h (*Bacillus* sp. isolate EB-40) or 48 h (*A. brasilense*). The inhibition test, performed in triplicate in a laminar flow hood, was evaluated by observing growth inhibition zones after incubation.

2.4. Experimental Design

The study took place in Montes Claros, Minas Gerais, Brazil (16°43′ S, 43°53′ W, 650 m altitude). We conducted the experiment in a greenhouse from June to October 2019. The minimum temperature varied from 14.8 °C to 20.8 °C (58.6 °F to 69.4 °F) and the maximum temperature ranged from 29.1 °C to 33.4 °C (84.4 °F to 92.1 °F) during the experiment [22]. The greenhouse cover reduced the full sunlight by 50%, resulting in 900 µmol m^{-2} s^{-1} photon flux density.

The study included seven experimental groups arranged in a 3 × 2 + 1 factorial scheme. The scheme consisted of three inoculants (*A. brasilense*, *Bacillus* sp. isolate EB-40, and *Bacillus* sp. isolate EB-40 + *A. brasilense* mix) and two application methods (seed and foliar spray). Each block contained all seven experimental groups. No treatments received nitrogen fertilization during the experiment.

2.5. Soil Properties and Fertilization

We used ferralsol collected from a 0 to 20 cm depth for the experimental plots. After sieving, we sterilized the soil in an autoclave (120 °C, 1.5 atm, 1 h) and placed it in 7 dm^3 pots. Soil analysis revealed pH 7.3; phosphorus, 10.25 mg dm^{-3}; potassium, 142 mg dm^{-3}; Ca, 7.60 mg dm^{-3}; Mg, 1.30 cmolc dm^{-3}; Al, 0 cmolc dm^{-3}; base saturation, 91%; fine sand, 8.30 dag kg^{-1}; coarse sand, 15.70 dag kg^{-1}; silt, 52 dag kg^{-1}; clay, 24 dag kg^{-1}; and organic matter, 2.34 dag kg^{-1}.

The pots were fertilized with single superphosphate at a dose equivalent to 36 mg dm^{-3} of phosphorus. We then planted 10 seeds per pot and thinned them to five Marandu palisade grass (*U. brizantha*) plants 15 days after planting.

2.6. Inoculant Preparation and Inoculation

After confirming the absence of antagonism between strains, we prepared liquid inoculants containing *Bacillus* sp. isolate EB-40 and *A. brasilense*. We cultivated *Bacillus* sp. in DYGS medium at 30 °C for 7 h and *A. brasilense* for 23 h, both under 120 rpm agitation. This yielded 2 × 10^8 colony-forming units (CFU) mL^{-1} for each strain. The bacteria mixture was created by combining equal volumes of *Bacillus* sp. and *A. brasilense* liquid cultures, each at 2 × 10^8 cells mL^{-1}, resulting in a final concentration of 1 × 10^8 cells mL^{-1} for both

species. We confirmed the cell count using optical density (OD) measurements at 600 nm with a spectrophotometer.

Seeds underwent disinfection using 70% alcohol and 1% sodium hypochlorite for 5 min, followed by a 2 min rinse in sterile distilled water. We then immersed the seeds in inoculants (*A. brasilense*, *Bacillus* sp. isolate EB-40, or *A. brasilense* + *Bacillus* sp. isolate EB-40) according to experimental groups. The seeds were agitated in the inoculants for 10 min at a concentration equivalent to 300 mL ha^{-1} per 15 kg of seeds. Finally, we air-dried the seeds before planting.

For treatments receiving foliar spray inoculation, we applied bacteria 30 days after planting, coinciding with the standardization cut of all plots. We used a spray to apply the inoculant at a rate equivalent to 300 mL ha^{-1}. Irrigation maintained the soil at 60% field capacity throughout the experiment. The evaluation of agronomic variables occurred every 30 days following the uniform cut.

2.7. Assessment of Plant Growth, Physiology, and Root Parameters

We analyzed agronomic variables at 30, 60, and 90 days after the standardization cut. Before harvest, we measured plant height from the soil base to the tallest plant's top using a graduated ruler. We counted leaves (NL) and tillers (NTillers) on five entire plants per plot. To obtain shoot dry mass (SDM), we cut forage 10 cm above ground, weighed it, and dried it to determine dry matter content following AOAC (2005) Method 934.01.

Chlorophyll content was measured using a portable at-LEAF CHL STD chlorophyll meter (FT GREEN LLC, Wilmington, NC, USA). For each plot, we took readings from the middle third of the last ten fully expanded leaves. We used the average of these ten readings as the representative value for each plot.

Physiological variables were evaluated at the first harvest (30 days after standardization) using an ADC Lcpro-SD infrared gas analyzer (IRGA) (Analytical Development Co. Ltd., Hoddesdon, UK) under natural air circulation. Measured variables included photosynthetic rate (A) (μmol m^{-2} s^{-1}), stomatal water conductance (mol m^{-1} s^{-1}), transpiration rate (E) (mol H_2O m^{-2} s^{-1}), substomatal chamber CO_2 concentration (μmol mol^{-1}), and water use efficiency (WUE) (mol CO_2 mol H_2O^{-1}), calculated as the ratio of photosynthesis to transpiration. We conducted all evaluations on fully expanded leaves in the upper third of plants using natural light. To ensure homogeneous environmental conditions and accurate readings, we performed analyses between 8 and 11 am [23].

We analyzed photosystem II (PS II) photochemical efficiency using an Opti-Sciences Fluorometer (PSK model, Hudson, NH, USA) for light and dark conditions. We placed tweezers on the middle third of the first fully expanded leaf of the most developed basal tiller for 30 min of dark adaptation. This yielded minimum (F0) and maximum (Fm) fluorescence. We calculated variable fluorescence (Fv) as the difference between F0 and Fm. We then determined photochemical efficiency as the ratio of variable to maximum fluorescence (Fv/Fm) and electron transport rate (ETR μmol electrons m^{-2} s^{-1}) [24]. Ninety days after the uniform cut, we separated the roots from the soil using water. We measured the fresh root length with a graduated ruler. We then dried the roots in an air-forced circulation oven at 55 °C for 72 h to determine root dry mass (DRM).

2.8. Data Analysis

Statistical analysis employed analysis of variance (ANOVA) to compare data with the control group via Dunnett's test at a 5% significance level. Cases of significant interaction prompted further decomposition of means and the application of Tukey's test, also at 5% significance. The Expdes.pt package (version 1.2.2) in RStudio software (R Core Team, Vienna, Austria, 2021) facilitated all statistical analyses.

3. Results

3.1. Bacterial Species Compatibility

Analysis of potential antagonism between *Bacillus* sp. isolate EB-40 and *A. brasilense* revealed no inhibition halos using the disk diffusion method (Figure 1). Similarly, the cross-test method showed no growth inhibition among the tested bacteria (Figure 2). The absence of mutual growth inhibition supported the decision to proceed with the simultaneous application of *A. brasilense* and *Bacillus* sp. in subsequent tests.

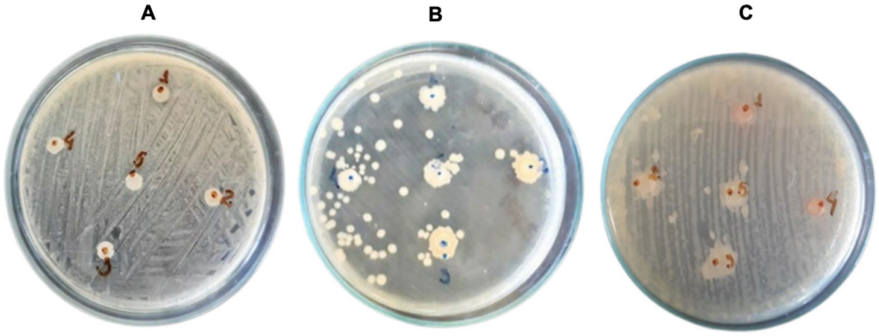

Figure 1. Disc diffusion assay confirming compatibility between *Bacillus* sp. isolate EB-40 and *Azospirillum brasilense*. (**A**) *Bacillus* sp. isolate EB-40 culture with discs impregnated with *A. brasilense* inoculum. (**B**) Twenty-four-hour *A. brasilense* culture with discs impregnated with *Bacillus* sp. isolate EB-40 inoculum. (**C**) Forty-eight-hour *A. brasilense* culture with discs impregnated with *Bacillus* sp. isolate EB-40 inoculum. The absence of inhibition halos demonstrates compatibility between the bacterial species.

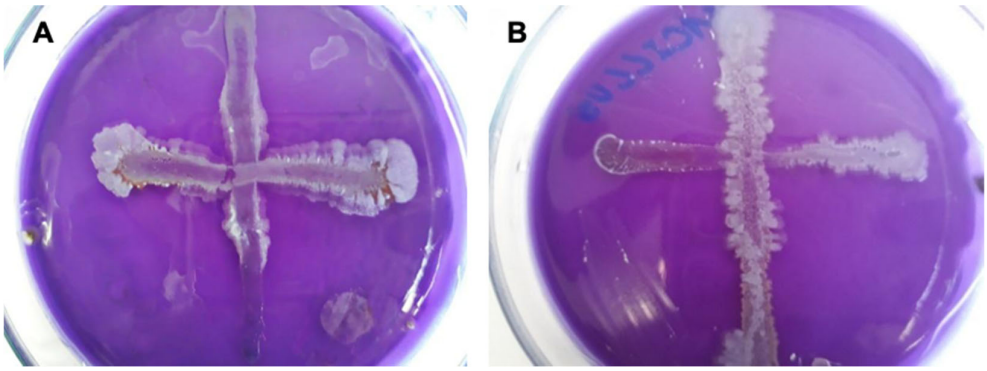

Figure 2. Compatibility between *Bacillus* sp. isolate EB-40 and *Azospirillum brasilense* confirmed by the cross-streak method. (**A**) No inhibition zone between *A. brasilense* (vertical streak, inoculated over *Bacillus* sp. isolate EB-40) and *Bacillus* sp. isolate EB-40 (horizontal streak, first inoculated). (**B**) Lack of inhibition between *Bacillus* sp. isolate EB-40 (vertical streak, inoculated over *A. brasilense*) and *A. brasilense* (horizontal streak, first inoculated), demonstrating compatibility.

3.2. Inoculating Bacillus sp. and Azospirillum brasilense Improves Palisade Grass Yield

The inoculum type and inoculation method did not interact to affect plant height, the number of tillers (NTillers), the number of leaves (NL), or shoot dry mass (SDM) ($p > 0.05$). The inoculation method (seed or foliar spray) had no significant effect ($p > 0.05$) on the tested variables during the three harvests (Table 2).

Table 2. Agronomic characteristics of Marandu palisade grass inoculated with *Azospirillum brasilense* and *Bacillus* sp. isolate EB-40.

Inoculants	Height (cm)	NTillers (NTillers Pot^{-1})	NL (NL Pot^{-1})	SDM (g Pot^{-1})
1st Harvest (30 Days)				
A. brasilense	53.99 b *	25.25 a *	67.50 b *	15.80 a *
Bacillus sp.	53.64 b *	22.13 a *	57.50 c *	15.46 a *
MIX	63.91 a *	28.38 a *	76.25 a *	16.45 a *
Control	27.5	15	43.75	8.09
CV (%)	6.47	5.42	2.83	7.64
2nd Harvest (60 days)				
A. brasilense	52.13 b *	26.75 b *	75.37 b *	16.30 b *
Bacillus sp.	41.63 c	20.50 c *	62.00 c *	13.69 c *
MIX	61.88 a *	30.25 a *	92.63 a *	18.10 a *
Control	38.5	14	36.25	7.30
CV (%)	4.95	7.71	4.15	4.60
3rd Harvest (90 days)				
A. brasilense	35.88 b *	27.75 b *	67.88 b *	15.88 a *
Bacillus sp.	31.00 c	21.38 b *	60.25 c *	14.10 b *
MIX	42.25 a *	30.38 a *	77.00 a *	16.60 a *
Control	26.5	14	33.75	7.09
CV (%)	9.70	8.92	7.00	5.88

Inoculants = *Azospirillum brasilense*, *Bacillus* sp. isolate EB-40, and MIX (*A. brasilense* + *Bacillus* sp. isolate EB-40). NL = number of sheets; Ntillers = number of tillers; SDM = shoot dry mass. Note: Means followed by the same letters in the column do not differ by the Tukey test at a 5% probability. Means followed by * show a statistical difference from the control with no inoculation based on Dunnett's test at 5% probability. CV (%): coefficient of variation.

Inoculation with the MIX increased the plant height of Marandu palisade grass in all three harvests, exhibiting gains of approximately 15%, 16%, and 15% over *A. brasilense*; 16%, 33%, and 27% over *Bacillus* sp. isolate EB-40; and 57%, 38%, and 37% over the control, respectively (Table 2). In the first harvest, the inoculation method did not affect NTillers, but at 60 and 90 days, MIX increased NTillers by 12% and 9% compared to *A. brasilense*, and 32% and 30% compared to *Bacillus* sp. Control comparisons showed increments of 47%, 46%, and 46% across the three harvests.

MIX application enhanced NL throughout all harvests, producing 12%, 19%, and 12% more leaves than *A. brasilense*; 25%, 33%, and 22% more than *Bacillus* sp.; and 43%, 61%, and 56% more than the control.

Inoculant types influenced SDM in the 2nd and 3rd harvests (Table 2). The MIX treatment increased SDM by 60% and 57% compared to the control at 60 and 90 days, respectively. In the second harvest, SDM was significantly higher in MIX than in *A. brasilense* and *Bacillus* sp. In the third harvest, MIX did not differ from *A. brasilense*, and both were superior to *Bacillus* sp.

The interaction between inoculation type and method influenced ($p < 0.05$) the root dry mass of Marandu palisade grass (Figure 3). All inoculation types increased root dry mass compared to the control. For seed inoculation, MIX showed greater root growth promotion than *A. brasilense* and *Bacillus* sp. For foliar spray, no differences were observed among inoculation types. Seed inoculation yielded higher root dry mass than foliar spray for MIX (33%) and *A. brasilense* (15%). In contrast, *Bacillus* sp. inoculation increased root mass by 17% more with foliar spray than seed inoculation.

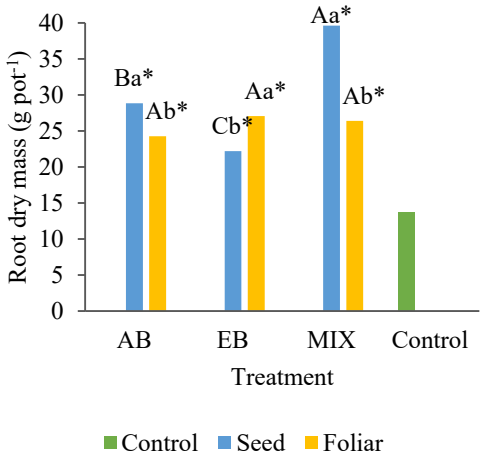

Figure 3. Root dry mass (G·pot^{-1}) of Marandu palisade grass inoculated with plant growth-promoting bacteria at 90 days after the standardization cut. Means with the same uppercase letter compare the inoculants (AB, EB, and MIX) within each inoculation method (seed and foliar spray) while means with the same lowercase letter compare inoculation methods within each inoculant type using Tukey's test at 5% probability. Means followed by * show a statistical difference from the control with no inoculation based on Dunnett's test at 5% probability. AB: *Azospirillum brasilense*; EB: *Bacillus* sp. isolate EB-40; MIX: *A. brasilense* + *Bacillus* sp. isolate EB-40.

The inoculant type and inoculation method independently influenced the root length of Marandu palisade grass, with no significant interaction ($p < 0.05$). The MIX treatment provided greater root length than *A. brasilense* (16% increase) and *Bacillus* sp. (17% increase), with a 135.6% increase over the control (Table 3). Seed inoculation increased root length by approximately 15% compared to foliar spray, regardless of the inoculant (Table 4). The gains over the control were 124% and 94% for seed and foliar inoculation, respectively. Inoculation with PGPB markedly increased palisade grass root mass compared to the control (Figure 4).

Table 3. Root lengths of Marandu palisade grass inoculated with plant growth-promoting bacteria.

Inoculants	Root Length (cm)
A. brasilense	51.13 b *
Bacillus sp.	50.75 b *
MIX	61.25 a *
Control	26.0
CV (%)	3.55

Inoculants = *Azospirillum brasilense*, *Bacillus* sp. isolate EB-40, and MIX (*A. brasilense* + *Bacillus* sp. isolate EB-40). Note: Means followed by the same letter within a column do not differ significantly according to Tukey's test at 5% probability. Means followed by * show a statistical difference from the control with no inoculation based on Dunnett's test at 5% probability. CV (%): coefficient of variation.

Table 4. Root lengths of Marandu palisade grass inoculated with plant growth-promoting bacteria in seeds or leaf spray.

Inoculation	Root Length (cm)
Seeds	58.25 a *
Leaf spray	50.5 b *
Control	26
CV (%)	3.55

Inoculation = *Azospirillum brasilense*, *Bacillus* sp. isolate EB-40, and MIX (*A. brasilense* + *Bacillus* sp. isolate EB-40). Note: Means within a column followed by the same letter are not significantly different according to Tukey's test at 5% probability. Means followed by * show a statistical difference from the control with no inoculation based on Dunnett's test at 5% probability. CV (%): coefficient of variation.

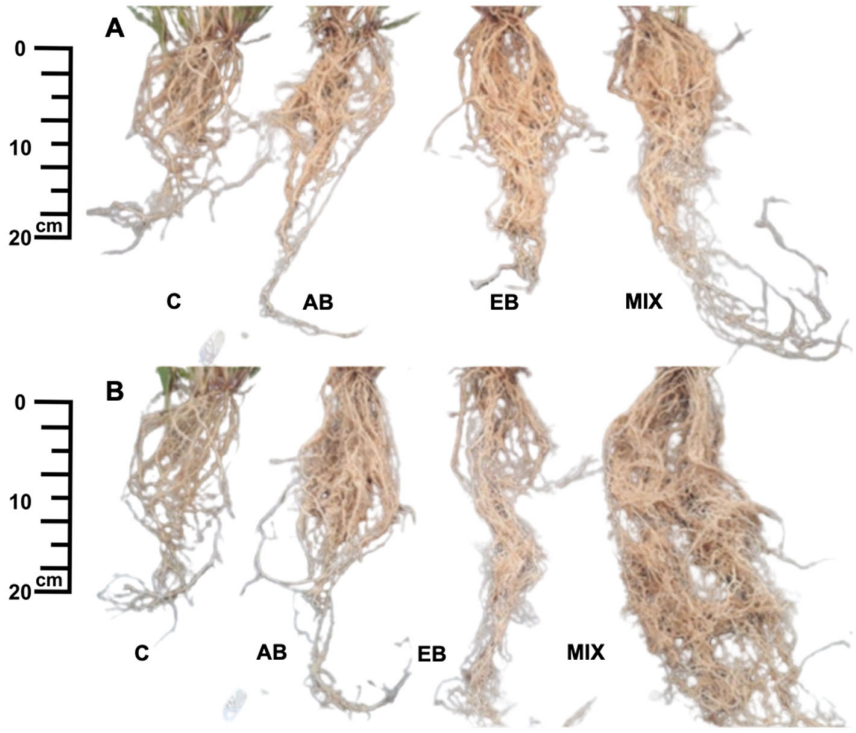

Figure 4. Root growth of Marandu palisade grass inoculated with plant growth-promoting bacteria. (**A**) Seed inoculation treatments. (**B**) Foliar spray inoculation treatments. C = control (non-inoculated); AB = *Azospirillum brasilense*; EB = *Bacillus* sp. isolate EB-40; and MIX = *A. brasilense* + *Bacillus* sp. isolate EB-40.

3.3. Enhanced Physiological Responses of Palisade Grass From Bacillus sp. and Azospirillum brasilense Inoculation

Inoculation with *Bacillus* sp. isolate EB-40 and *A. brasilense*, alone or in combination (MIX), increased the chlorophyll content of Marandu palisade grass compared to the control, except for *Bacillus* sp. at 30 and 60 days. The chlorophyll content was influenced by the interaction between the inoculant type and inoculation method ($p < 0.05$) at 30, 60, and 90 days. Seed inoculation with MIX resulted in the highest chlorophyll content (Figure 5). At 30 and 60 days, seed inoculation with *A. brasilense* or MIX exhibited higher chlorophyll levels than *Bacillus* sp. At 90 days, MIX seed inoculation outperformed *A. brasilense* and *Bacillus* sp. For foliar spray, MIX performed better at 30 days, while *A. brasilense* was superior at 90 days. No differences were observed among foliar treatments at 60 days. Seed inoculation with MIX resulted in higher chlorophyll levels than foliar spray at 60 and 90 days (Figure 5).

The inoculant type significantly influenced ($p < 0.05$) the physiological responses of Marandu palisade grass (Table 5), except for substomatal CO_2 concentration and stomatal conductance, which averaged 207.23 $\mu mol \cdot mol^{-1}$ and 0.055 $mol \cdot m^{-1} \cdot s^{-1}$, respectively. Plants inoculated with MIX exhibited a 66% higher photosynthetic rate (A) compared to the control and were 31% and 43% higher than *A. brasilense* and *Bacillus* sp., respectively. *Bacillus* sp. did not differ from the control for A.

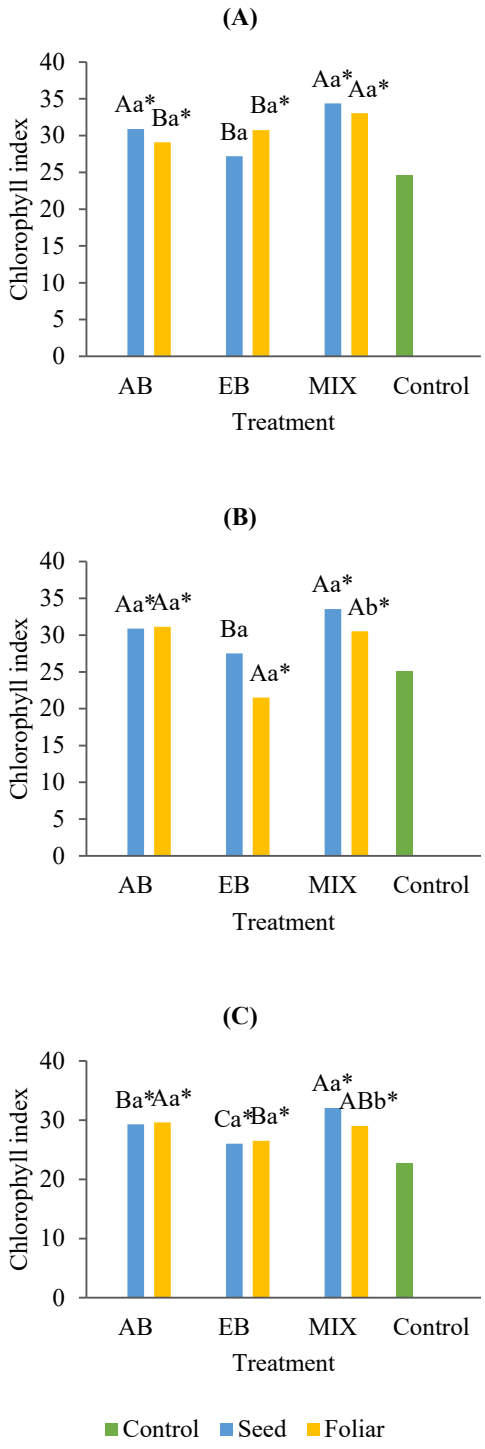

Figure 5. Chlorophyll content in Marandu palisade grass leaves inoculated with growth-promoting bacteria via seed and foliar spray at (**A**) 30, (**B**) 60, and (**C**) 90 days. Means with the same uppercase letter compare the inoculants (AB, EB, and MIX) within each inoculation method (seed and foliar spray) while means with the same lowercase letter compare inoculation methods within each inoculant type using Tukey's test at 5% probability. Means followed by * show a statistical difference from the control with no inoculation based on Dunnett's test at 5% probability. AB: *Azospirillum brasilense*; EB: *Bacillus* sp. isolate EB-40; MIX: *A. brasilense* + *Bacillus* sp. isolate EB-40.

Table 5. Photosynthetic parameters of Marandu palisade grass inoculated with plant growth-promoting bacteria.

Inoculants	A ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	E ($molH_2O \cdot m^{-2} \cdot s^{-1}$)	WUE ($molCO_2 \cdot molH_2O^{-1}$)
A. brasilense	5.52 b *	1.66 ab *	3.27 b *
Bacillus sp. isolate EB-40	4.62 b	1.39 b	3.24 b
MIX	8.09 a *	1.84 a *	4.43 a *
Control	2.75	1.08	2.55
CV (%)	30.29	19.96	18.76

Inoculants = *Azospirillum brasilense*, *Bacillus* sp. isolate EB-40, and MIX (*A. brasilense* + *Bacillus* sp. isolate EB-40). Note: Means followed by the same letter within a column do not differ significantly according to Tukey's test at 5% probability. Means followed by * show a statistical difference from the control with no inoculation based on Dunnett's test at 5% probability. A: photosynthesis rate; E: transpiration rate; WUE: water use efficiency; CV (%): coefficient of variation.

MIX inoculation resulted in a higher transpiration rate (E) than *Bacillus* sp. but not *A. brasilense*, with a 53% increase over the control. *Bacillus* sp. did not differ from *A. brasilense* or the control for E (Table 5). The water-use efficiency (WUE) was highest in the MIX treatment, outperforming the control and pure inoculations. MIX inoculation increased WUE by 73% compared to the control and by approximately 36% over individual inoculations. Only *Bacillus* sp. exhibited a WUE similar to the control (Table 5).

There was no interaction between inoculant type and inoculation method for chlorophyll fluorescence parameters, nor an effect of the inoculation method. The electron transport rate (ETR) reached a maximum of 63.40 µmol electrons$\cdot m^{-2} \cdot s^{-1}$ in the MIX treatment, which also provided the highest photosynthetic rates (Table 6). MIX did not differ from *A. brasilense* but exhibited higher ETR than *Bacillus* sp. and the control, with a 119% increase over the control. *A. brasilense* increased ETR by 93% compared to the control.

Table 6. Chlorophyll fluorescence assessment for Marandu palisade grass inoculated with plant growth-promoting bacteria.

Inoculants	ETR (µmols of Electrons$\cdot m^{-2} \cdot s^{-1}$)	F_m	F_v
A. brasilense	55.89 a *	2400.88 b	1853.63 b *
Bacillus sp.	37.29 b	2443.50 ab	1990.63 b *
MIX	63.40 a *	2622.88 a *	2089.25 a *
Control	28.9	2363.75	1633.25
CV (%)	12.46	6.37	6.71

Inoculants = *Azospirillum brasilense*, *Bacillus* sp. isolate EB-40, and MIX (*A. brasilense* + *Bacillus* sp. isolate EB-40). Note: Means followed by the same letter within a column do not differ significantly according to Tukey's test at 5% probability. Means followed by * show a statistical difference from the control with no inoculation based on Dunnett's test at 5% probability. ETR: electron transport rate; Fm: maximum fluorescence; Fv: variable fluorescence; CV (%): coefficient of variation.

Inoculation with MIX provided higher maximum fluorescence (Fm) than the control, while pure inoculations with *A. brasilense* or *Bacillus* sp. did not differ from the control. However, no significant difference was observed between MIX and *A. brasilense*. Plants inoculated with MIX showed an 11% higher Fm compared to the control (Table 6).

The MIX inoculation also resulted in higher variable fluorescence (Fv) than pure inoculations with *A. brasilense* or *Bacillus* sp. and the control (Table 6). The application of MIX increased Fv by 22% compared to the control and by 9% and 11% over *A. brasilense* and *Bacillus* sp., respectively.

4. Discussion

The co-inoculation analysis between the EB-40 isolate of *Bacillus* sp. and *A. brasilense* did not show inhibition halos or growth inhibition (Figures 1 and 2), which suggests a compatible interaction that supports their simultaneous application. This mutual compatibility

of bacterial strains, such as *Bacillus* sp. and *A. brasilense*, is essential for their survival and function in consortia, as demonstrated by the improved performance of *Bacillus velezensis* consortia when antagonistic interactions are inhibited [25]. Compatibility ensures that the beneficial characteristics of each microbial species, such as biofilm formation and root colonization, are not compromised by antagonistic interactions.

Although the inoculation method (seed or foliar spray) did not significantly affect the parameters of plant height, number of tillers, number of leaves, or shoot dry mass (SDM) ($p > 0.05$) in *U. brizantha*, the type of inoculum did, especially when the mixed inoculum was used. The MIX treatment consistently outperformed other inoculants, including *A. brasilense* and *Bacillus* sp., as well as the control, across multiple growth and yield metrics. MIX led to significant increases in plant height across all three harvests, with gains of 15–57% at 60 and 90 days. It also increased tiller number by 9–47% and leaf number by 12–61% compared to other treatments across all harvests. SDM in the second and third harvests, in the presence of MIX, resulted in improvements of 57–60% over the control (Table 2).

The simultaneous use of PGPB *A. brasilense* and *Bacillus* sp. can improve nutrient absorption from soil, especially N and P, favoring biomass production and establishment through increased height, dry mass, and tillering rate [14]. A study on *Urochloa* spp. showed that *A. brasilense* inoculation increased shoot biomass by 16.8% on average, N content by 11.7–20.7%, and improved root architecture through phytohormone synthesis, contributing to plant growth and nutritional status [11]. Previous studies also confirmed the positive effects of *A. brasilense* inoculation on *Urochloa ruziziensis* and *U. brizantha* genotypes [14,26]. As N and P are macronutrients required in high quantities by C4 grasses, plant response to their availability is substantial [27].

Although MIX inoculum has shown superior results for *U. brizantha*, the efficacy of inoculants can vary based on plant species and environmental conditions. For example, different inoculants have shown varying success in other crops, such as legumes and rice, where specific combinations or sequential inoculations have been shown to increase growth [28,29]. Additionally, the preparation and application of inoculants can influence results, as seen in soil inoculation studies [30]. These findings suggest that while MIX is effective for *U. brizantha*, other crops may require tailored inoculant strategies for optimal growth. Seed inoculation with MIX resulted in a 33% increase in root dry matter compared to foliar spray, while *A. brasilense* showed a 15% increase with seed inoculation compared to foliar spray.

Seed inoculation increased root length (Table 4), likely due to direct contact with the developing root system, minimizing microbial cell loss. Exogenous auxins during initial plant formation enhance vascular tissue formation rate, increasing xylem and phloem vessel numbers when inoculating seeds with *A. brasilense* [31]. Corn seeds inoculated with *Azospirillum* sp. isolate BRM 63574 exhibited increased total length, diameter, volume, root dry mass, shoot dry mass, and total dry mass compared to the control [32]. The genus *Azospirillum* sp. promotes increased root length and volume and enhanced plant growth primarily through phytohormone production, particularly auxins [33]. These hormones also increase xylem vessel diameter and total cross-sectional area.

In contrast, *Bacillus* sp. inoculation was more effective with foliar spray, increasing root mass by 17% more than seed inoculation. The efficacy of *Bacillus* sp. in the foliar spraying of *U. brizantha* is in line with findings from other studies in which microbial inoculants improved plant growth parameters [34]. *Bacillus* sp. and *A. brasilense* also improved nutrient uptake and modified root system architecture, which may lead to increased root biomass [35,36]. Although seed inoculation generally shows greater efficacy in promoting root growth, the choice between foliar and seed methods depends on specific bacterial strains and environmental conditions [37].

Seed inoculation with MIX was more effective than foliar spray, especially at later growth stages (60 and 90 days) in *U. brizantha*, suggesting that the inoculation method plays a critical role in maximizing the benefits of PGPB. The type of inoculant significantly influenced the physiological responses of *U. brizantha*, with MIX inoculation showing

superior results in photosynthetic rate, transpiration rate, and water use efficiency (WUE) compared to other treatments (Table 5). This suggests that the combination of different bacterial strains may improve the physiological performance of *U. brizantha* more effectively than individual strains. MIX inoculation increased the photosynthetic rate by 66% relative to the control and improved WUE by 73%, indicating a synergistic effect of the combined strains on the growth and efficiency of *U. brizantha*.

The study of chlorophyll fluorescence parameters in relation to microbial inoculants reveals significant information about plant physiology and photosynthetic efficiency. This suggests that a synergistic effect can improve photosynthetic parameters more effectively than applications of a single inoculant. The MIX treatment achieved an ETR of 63.40 µmol of electrons, significantly higher (119%) when compared to the control (Table 6). Maximum fluorescence (Fm) was 11% higher in MIX-treated plants, indicating increased photosynthetic potential (Table 6). Variable fluorescence (Fv) increased by 22% compared to the control and 9% and 11% in *A. brasilense* and *Bacillus* sp., respectively, suggesting greater electron transport efficiency [38].

Co-inoculation with PGPB, especially *A. brasilense* in MIX, is an option to increase forage production, as the bacteria-supplied N stimulates multiple aspects of plant growth and production [39]. PGPB fix N, increase root expansion, and enhance nutrient uptake [11]. With more N available, plants improve chlorophyll synthesis and photosynthesis, providing more photoassimilates for cell division and expansion in shoots and roots. Consequently, inoculated plants exhibit vigorous vegetative growth, as *Azospirillum* sp. can stimulate photosynthetic pigment production, promoting greater photosynthetic efficiency in palisade grass.

Microbial inoculants, including MIX, increase photosynthetic efficiency by improving light harvesting and utilization, as evidenced by increased chlorophyll fluorescence parameters. These improvements contribute to improved crop resilience and productivity, supporting the potential of microbial inoculants in sustainable agriculture. However, it is important to consider the broader context of plant–microbe interactions and the impact of environmental conditions such as soil compaction and nutrient availability [40].

5. Conclusions

Co-inoculation of *Azospirillum brasilense* and *Bacillus* sp. isolate EB-40 enhanced biomass accumulation in Marandu palisade grass while modulating photosynthesis physiology. The bacterial mix increased chlorophyll content, photosynthetic rate, and stomatal conductance, indicating more physiologically active plants with accelerated growth, irrespective of inoculation method. Seed inoculation increased root mass and length, presenting a stress mitigation alternative.

These findings highlight the potential of using multiple growth-promoting bacterial strains simultaneously to boost plant growth and photosynthetic performance by facilitating efficient electron utilization through higher pigment content. This study opens new avenues for developing innovative biological products to enhance pasture productivity through large-scale grass inoculation with the *A. brasilense* and *Bacillus* sp. isolate EB-40 consortium. Furthermore, it is important to highlight that the use of PGPB may contribute to a reduction in mineral fertilizer application, promoting more sustainable agricultural practices. To realize the full potential of this bacterial consortium for sustainable agriculture, further tests are necessary to optimize the inoculant formula for upscaling commercial production.

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