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Bio-inputs promoting marandu grass growth: productivity, physiological response and nitrogen accumulation

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Abstract

Nutrient replenishment is one of the significant factors limiting the full development of extensive pasturelands. As a result, bio-inputs have been increasingly used under field conditions to improve plant growth in low-fertility soils. This study aimed to evaluate their effects on the productivity, physiological responses to and nitrogen (N) accumulation in marandu grass (*Urochloa brizantha* cv. marandu). The experiment was carried out using a randomized block design with four treatments and three replicates in a greenhouse under controlled conditions. In low-fertility soils, lower synthetic fertilizer inputs were simulated (40% N-P-K) (Control), and three bio-inputs were additionally applied (bio-inputs + 40% N-P-K): Azo (*Azospirillum brasilense* seed inoculation); HS+Herb (leaf application of humic substances combined with a cocktail consisting of *Herbaspirillum* species), and Coinoc (a combination of Azo and HS+Herb). We evaluated the plant biometrics (shoot and root dry matter, stem diameter and height) and physiological parameters (CO_2 assimilation rate, internal CO_2 concentration, transpiration, instantaneous carboxylation efficiency, and the maximum quantum yield of photosystem II), as well as the N accumulation 45 days after seedling emergence. Azo-treatment did not differ from the control in most of the variables studied. HS+Herb and Coinoc significantly ($p \leq 0.05$) increased the dry matter of shoots (+237% and +255%, respectively) and roots (+106% and 83%, respectively) compared to the control. These treatments (HS+Herb and Coinoc) significantly ($p \leq 0.05$) increased (almost double) the size and stem diameter, improved physiological parameters, and accumulated more N (up to 89%) compared to the control. Applying humic substance-based bio-inputs combined with the *Herbaspirillum* species cocktail was more efficient in stimulating the growth of marandu grass grown in soil of reduced fertility.

Keywords: *Azospirillum brasilense; Herbaspirillum* spp.; Plant growth-promoting bacteria; Microbial inoculants; Humic substances.
Abbreviations: HS_humic substances; PGPB_plant-growth promoting bactéria; P-rem_phosphorus retention capacity in soil; BNF_biological nitrogen fixation; V%_base saturation in soil; RNPT_relative neutralizing power total; DAE_days after emergence; MQY_maximum quantum yield of photosystem II; *A/Ci*_instantaneous carboxylation efficiency; *A*_net CO2 assimilation rate; *E*_transpiration rate; *Ci*_intercellular CO_2 concentration; PH_plant height; SD_stem diameter; SDM_shoot dry matter; RDM_root dry matter.

Introduction

Inadequate management practices, such as extensive cultivation, low technological investment, and scarce inputs for soil nutrient replacement, have often been adopted by Brazil's livestock farming (Polidoro et al., 2021; Satler et al., 2017). As a result, about 70% of the pastures are in some stage of degradation (Vasques et al., 2019; Hungria et al., 2016). In this context, technological practices that offer higher productivity with relatively lower investments are decisive for the competitive advantage of livestock due mainly to the constantly increased cost of mineral fertilizers and low nutrient-use efficiency by the crops.

Forage plants, such as marandu grass (*Urochloa brizantha* cv. marandu), occupy a large part of Brazilian pastures (Lopes et al., 2018). However, they are usually grown on low-fertility soils and have limited production, primarily due to their low levels of N-availability (Leite et al., 2021; Lopes et al., 2018; Pinheiro et al., 2018). In recent years, inputs based on humic substances (HS) and plant growth-promoting bacteria (PGPB) have emerged as promising biotechnologies to stimulate plant growth and increase the uptake efficiency of nutrients by multiple mechanisms (Kour et al., 2020; Pinheiro et al., 2020; Antunes et al., 2019; Leite et al., 2018; Olivares et al., 2017; Canellas et al., 2013).

Among the PGPB, the genera *Azospirillum* and *Herbaspirillum* seem to establish successful interactions for improved grass development, even when the way of inoculation and formulations are different (Dartora et al., 2016; Canellas et al., 2013). Substantial levels of biologically fixed N, the production of phytohormones, increased water and nutrient utilization, improved photosynthetic activity, inorganic phosphate solubilization, and attenuation of biotic and abiotic stresses are some of the effects reported after inoculation of these microorganisms (Kour et al., 2020; Olanrewaju et al., 2017). In addition, HS also have stimulatory effects on plants and can be used as a vehicle for bacterial inoculation and, therefore, can be applied in conjunction with PGPB to improve inoculation efficiency (Olivares et al., 2017; Canellas et al., 2013).

These positive effects significantly contribute to environmental and economic sustainability, leading to less dependence on mineral fertilizers and increasing productivity and efficiency in agriculture (Boleta et al., 2020; Coniglio et al., 2019). However, the versatility by which these bio-inputs can be applied, the selection of better functional PGPB strains, synergistic biostimulatory interactions among bio-inputs, as well as the different inoculation methods adopted raise questions about which is the best option to employ to increase forage yield in low-fertility soils (Sani and Young, 2022; Prasad et al., 2019; Canellas et al., 2015).

These bio-inputs have been developed with isolated application forms and remarkable potential has been confirmed, but little is known about their combined use and synergism, especially for forage grasses in soils with low fertility (Prasad et al., 2019). Moreover, understanding the biochemical and physiological mechanisms governing the grass-PGPB interaction is advancing (Prasad et al., 2019). Moreover, little essential information is available about the effect on marandu grass productivity, which is widely used with Brazilian livestock.

We investigated how bio-inputs based on beneficial bacteria and humic substances that are delivered in different ways (seed, leaf, and combined) could impact productivity (height, stalk diameter, the dry matter of shoots and roots), physiological parameters (gas exchange and maximum quantum yield of photosystem II) and the N accumulation components of marandu grass when grown in soil of reduced fertility.

Results and discussion

The marandu grass plants submitted to the application of biological inputs were taller and more architectured in shoots and roots. The PGPB can produce phytohormones that modulate cell division and expansion (Gênero et al., 2020), which is essential in yield response. The bio-input applications promoted significant increases ($p \leq 0.05$) in plant height (PH) and stem diameter (SD), with values ranging from 21.50 to 41.92 cm and 1.78 to 4.20 mm, respectively (Fig 1A and 1B). The treatments Azo, HS+Herb, and Coinoc enabled the respective increases of 49%, 237%, and 255% for shoot dry matter (SDM) and 25%, 106%, and 83% for root dry matter (RDM) (Fig 1C and 1D), respectively. Treatment Azo did not differ from the control ($p > 0.05$). While products based on strains of *Azospirillum* ssp. are already marketed and well-accepted to stimulate forage grasses growth (Brito et al., 2019; Leite et al., 2019; Hungria et al., 2016; Reis et al., 2001), there are few studies related to strains of *Herbaspirillum* spp. with *Urochloa* ssp. (Pinheiro et al., 2018), especially under conditions of low soil fertility. The great majority of extensive pasturelands in Brazil is planted with *Urochloa* ssp. (Guarda and Guarda, 2014). We have shown that better growth promotions with HS+Herb and Coinoc benefit forage production (Fig 2).

We evaluated the mechanisms associated with forage production, such as photosynthesis-related parameters. The gas exchange results (Fig 2) are within the range observed in other studies (Daniel et al., 2018; Bulegon et al., 2017) and were positively influenced by bio-inputs. The net CO_2 assimilation rate (A) and instantaneous carboxylation efficiency (A/Ci) were up to 54 and 64 %, respectively, higher for the plants that received the application of the bio-inputs (Fig 2A and 2D). Such results point to higher efficiency in CO_2 consumption. A slight increase in transpiration rate (E) and a lower internal CO_2 concentration (Ci) were also found in these plants (Fig 2B and 2C). However, no significant difference was observed for resistance to CO_2 diffusion by stomata (data not shown), i.e., the influence of bio-inputs in stomatal conductance was unclear in the present study.

The application of the bio-inputs also increased the maximum quantum yield of PSII (MQY) (Fig 3), which indicates a biostimulant effect and lower susceptibility to abiotic stresses, in addition to an improvement in the functioning of the photosynthetic apparatus (MQY > 0.75 = high photochemical efficiency; MQY < 0.75 = PSII inhibition), and supports the results related to gas exchange (Fig 2). Increased overall photosynthetic efficiency (MQY) may be either by nitrogen acquisition via biological nitrogen fixation (BNF) and/or by increased efficiency nutrient absorption. In this regard, Ramos et al. (2020) draws attention to a possible higher activity of the biochemical pathway associated with photosynthetic carbon assimilation. Positive correlations between MQY and the production parameters (SDM, RDM, PH and SD) show a positive effect of bio-inputs (Fig 6).

Previous reports have shown that the combined foliar application of humic acids and *Herbaspirillum seropedicae* was found to boost plant growth in other grasses, such as maize (*Zea mays*) (Olivares et al., 2017) and sugarcane (interspecific *Saccharum* hybrids) (Canellas et al., 2013). This study confirms the applicability of microbial technologies as a feasible tool to promote marandu grass growth and development (Fig 2, 3, and 4). The positive results of this study may be due to the mode of application of the biological inputs. The previous studies combining humic acids and endophytic diazotrophic bacteria (such as *H. seropedicae*) showed delivery as foliar spray was more effective compared with on furrows (Da Silva et al., 2017) or via seed applications (Dartora et al., 2016). *Herbaspirillum* spp. may express maximum BNF potential due to their endophytic habit, which gives them a more efficient and protected colonization against biotic and abiotic constraints (Bashan and De-Bashan, 2010).

Furthermore, the two foliar applications (15 and 30 DAE) of the bio-inputs containing *H. seropedicae* may have culminated in the better establishment of bacterial population endophytically in the tissues of the marandu grass to overcome the growth promotion provided by *Azospirillum brasilense* (a bacterium that associates mainly in the rhizosphere) (Di Salvo et al., 2018). Additionally, previous reports have confirmed a better performance of PGPB in low-fertility soils (Antunes et al., 2019; Schultz et al., 2014). HS go far beyond just an inoculation vehicle for bacterial strains, and their effects on the activation of H^+-ATPases, auxin secretion, abiotic and biotic protection and increased root volume and lateral root emission have been widely studied (Canellas et al.. 2020; Da Silva et al., 2021; De

Table 1. Chemical and particle size characterization of the original soil before experimentation (acidity correction, fertilization, and application of treatments).

Depth	pH	pH	P	K	Ca	Mg	Al	H	H+AL	SB	T	t
(cm)	(H_2O)	(KCl)	-(mg dm^{-3})-		-------------------------(cmol$_c$ dm^{-3})-----------------------							
0-20	4.1	3.9	1.46	26.5	0.23	0.08	0.8	4.7	5.50	0.38	5.88	1.18
V	m	P-rem	MO	S	B	Fe	Cu	Mn	Zn	Clay	Silt	Sand
----(%)----		(mg L^{-1})	(%)	----------------(mg dm^{-3})--------------						--------(%)--------		
6.46	67.8	26.3	0.74	24.8	0.55	48.7	0.15	1.74	0.66	42.7	2.9	54.4

pH in water: potentiometry in soil-water solution 1:2.5; available phosphorus: extracted by Mehlich^{-1} + spectroscopy); extractable aluminum: determined by titration with NaOH 0.025 mol L^{-1}; calcium and magnesium: extracted with 1 mol L^{-1} KCl solution and determined by atomic absorption spectrophotometry; exchangeable potassium and sodium: extracted with Mehlich^{-1} extractor and determined by flame photometry; SB = Ca + Mg + K + Na; T = SB + (H + Al); t = SB + Al; V% = SB x 100/T; m% = Al x 100/t; ; P-rem = phosphorus retention capacity in soil; MO: soil C content x 1.724. available micronutrients: extracted by Mehlich^{-1}; Particle size analysis: sand, silt, and clay content.

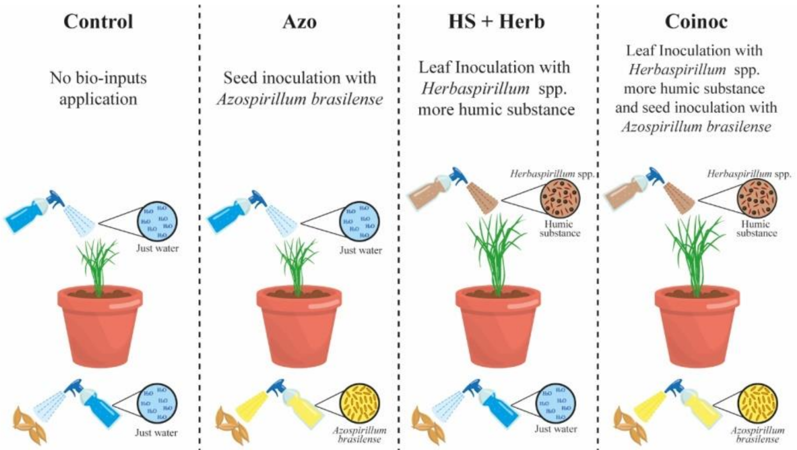

Figure 1. Schematic representation of the application of candidate bio-inputs to stimulate marandu grass growth. Control = no bio-input application; Azo = inoculation via seed with *Azospirillum brasilense* (strains Ab-V5 and Ab-V6); *HS+Herb* = inoculation of *Herbaspirillum* spp. (strain H-UENF2019 of *H. seropedicae* and HCC101 of *H. rubrisubalbicans* using humic substances (40 mg L^{-1} of C) as inoculation vehicle); Coinoc = combined treatment (Azo + (HS+Herb)). When a particular bio-input was not applied in some of the study treatments, deionized water was applied in the same way to balance the experiment.

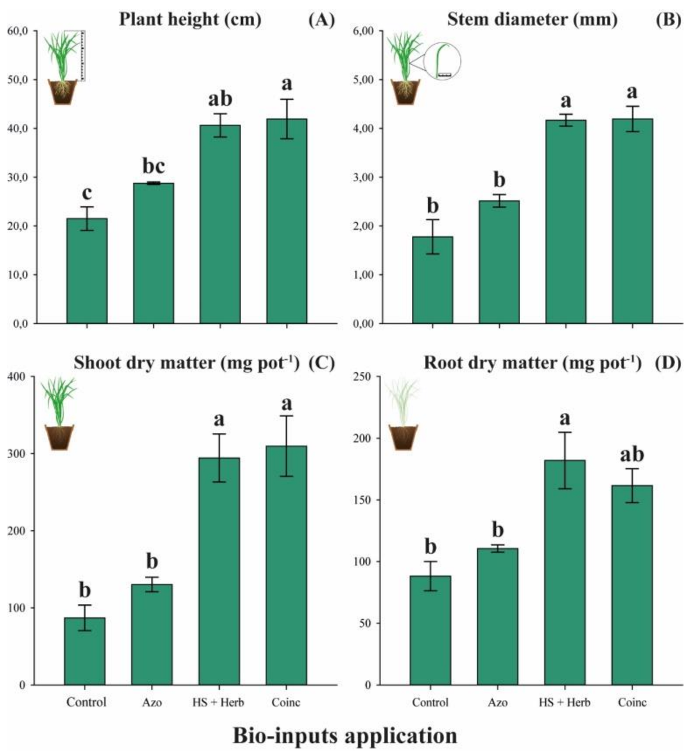

Figure 2. Plant height (PH), diameter of the stem (SD), dry mass of the aerial part (SDM), and roots (RDM) (respectively, figures 2A, 2B, 2C, and 2D), of marandu grass plants submitted to the application of bio-inputs that differed in composition and mode of application, at 45 days after emergence. Means followed by the standard error followed by the same capital letter do not differ by Tukey's test for $p \leq 0.05$.

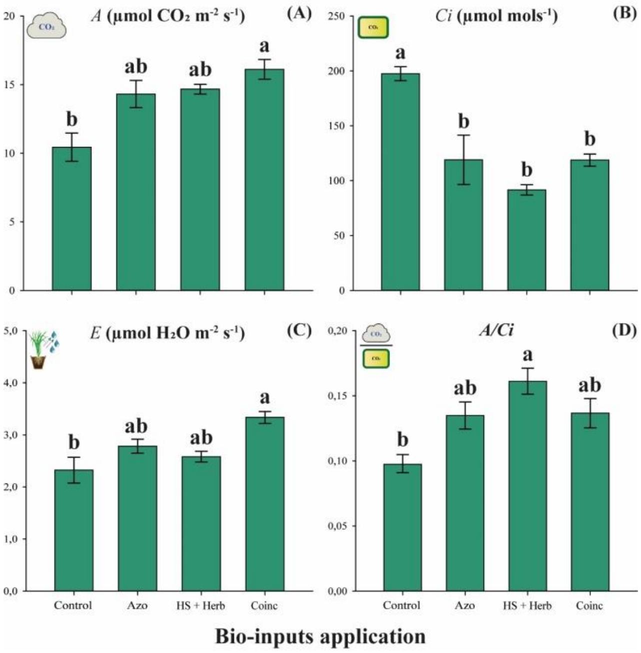

Figure 3. Net CO_2 assimilation rate (*A*), Intercellular CO_2 concentration (*Ci*), transpiration rate (*E*), and instantaneous carboxylation efficiency (*A/Ci*) (respectively, figures 3A, 3B, 3C, and 3D) of marandu grass plants submitted to the application of bio-inputs that differed in composition and mode of application, at 45 days after emergence. Means followed by the standard error followed by the same capital letter do not differ by the Tukey test for $p \leq 0.05$.

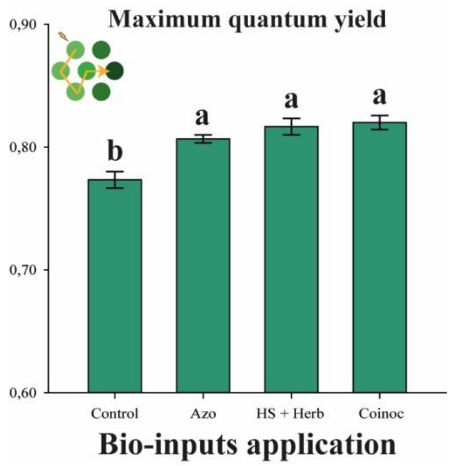

Figure 4. Maximum quantum yield of photosystem II (MQY) of marandu grass plants submitted to the application of bio-inputs differed in composition and mode of application 45 days after emergence. Means followed by the standard error followed by the same capital letter do not differ by Tukey's test for $p \leq 0.05$.

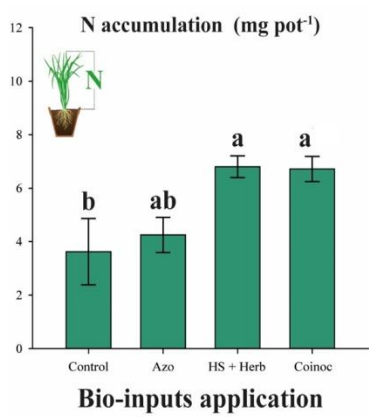

Figure 5. Accumulation of nitrogen in marandu grass shoots plants submitted to the application of bio-inputs that differed in composition and mode of application at 45 days after emergence. Means followed by the standard error followed by the same capital letter do not differ by Tukey's test for $p \leq 0.05$.

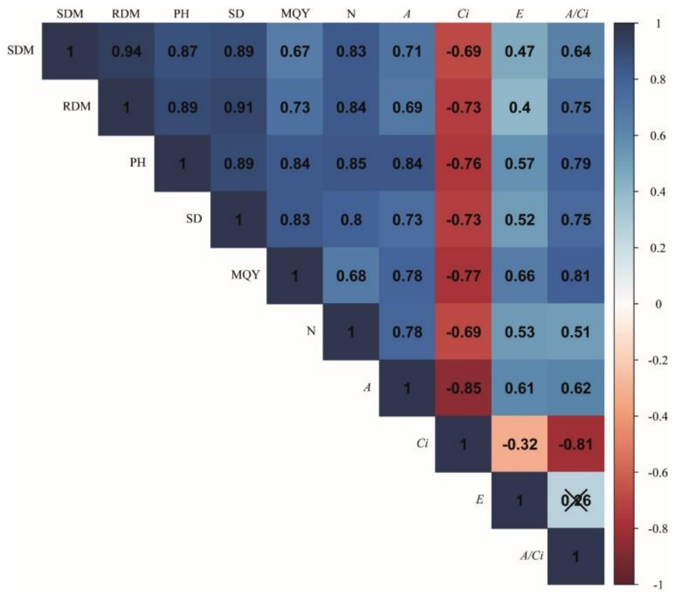

Figure 6. Heat map showing Pearson's correlation between yield (SDM = Shoot dry matter; RDM: Root dry matter; PH = Plant height; SD = Stem Diameter), physiological (MQY = maximum quantum yield; *A* = net CO_2 assimilation rate; *Ci* = intercellular CO_2 concentration; *E* = transpiration rate; *A/Ci* = instantaneous carboxylation efficiency) and N accumulation in marandu grass shoots. Cross-tagged regions indicate non-significance between related data.

Azevedo et al., 2019; Olivares et al., 2017; Alves et al., 2015), enhancing the growth promotion effect provided by *Herbaspirillum* spp.

Either by biostimulation (an increase of the amount of soil volume explored by the roots, an increase in plant nutrient uptake, and utilization efficiencies) or by biofertilization (BNF), the plants that received the bio-input application accumulated nitrogen at 17%, 89%, and 86% (respectively for Azo, HS+Herb, and Coinoc) more than the control (Fig 4). Breda et al. (2019) concluded that *H. seropedicae* displays a more intensive process of assimilation of absorbed N compared to *A. brasilense*. The distinction in the growth promotion process (biofertilization or biostimulation) associated with the present study is unclear. Better N nutrition can contribute to hormonal and physiological changes (De Souza et al., 2016). It has been shown that the growth (SDM, RDM, PH, and SD) and physiological (mainly A, QMY, and Ci) parameters are positively and significantly affected by N accumulation. Photosynthetic rate and yield measures are related to N supply, especially in C4 species (Brown, 1978; Tsutsumi et al., 2017; Makino and Ueno, 2018). Nitrogen dissipates excess light energy, protecting the photosystem from possible oxidative damage and, therefore, providing a higher yield of PSII (Chen et al., 2018).

Materials and methods

Procedures for soil: sampling, preparation and characterization

A greenhouse experiment was carried out under controlled conditions using, as a substrate for marandu grass growth, a tropical Oxisol (Soil Survey Staff, 1999), representative of a degraded pasture area (20° 45' S, 41° 29' W) in the Atlantic Forest biome in the southeastern region of Brazil. First, the single soil samples of the degraded area were combined in composite samples, homogenized, dried, and passed through a 2.0 mm mesh sieve. This process was followed by the chemical and granulometric characterization of the soil (Table 1) (Teixeira et al., 2017).

The base saturation method was used for soil correction to raise its value to 50% after 21 days of liming (RNPT = 113%). To obtain a sterile substrate free of native microorganisms and to better express the effect of the biological inputs used as a study factor, the soil was twice autoclaved (1.1 atm, 121ºC for 20 minutes). The doses of N, P, K, and S were applied at 40% of that recommended by Novais et al. (1991) (40, 120, 60, and 40 mg dm^{-3}, respectively). Parcel distribution of N fertilization was applied in two periods, 15 and 30 days after the emergence of seedlings (DAE). The micronutrients B, Cu, Fe, Mn, Mo, and Zn were applied in respective doses of 0.81, 1.33, 1.55, 3.66, 0.15, and 4.00 mg dm^{-3} (100% of that recommended by Novais et al. (1991).

Plant material, experimental design, and treatments

The brachiaria genotype used was *Urochloa brizantha* cv. marandu was previously selected based on biomass production efficiency and compatibility with N-fixing organisms. The experimental design was a randomized block design with four treatments and four replications. The seeds were germinated in trays. Seedlings were selected based on homogeneity in vigor and size and transferred to plastic pots (0.8 dm3) with 10 DAE of the seedlings. The treatments consisted of the use of biological inputs that differed in composition and mode of application (Fig 1).

The soil of the experimental units (pots) was maintained at 60% of the maximum water storage capacity and 45 DAE experimental time. Evaluations of plant biometric and physiological parameters were performed on the last day of the experiment. The greenhouse temperature was recorded daily, with a variation of 26-38ºC (34.7ºC on average) for the maximum and 19-23ºC (20.9ºC on average) for the minimum.

Preparation and application of bio-inputs

Marandu grass seeds were inoculated with strains Ab-V5 and Ab-V6 of *Azospirillum brasilense* (15 mL kg^{-1} and shade-dried) with a guarantee of $2{\times}10^8$ colony forming units (mL^{-1}).

Following Hungria et al. (2010), the seeds were previously disinfected for this procedure.

The inoculant based on the *Herbaspirillum seropedicae* strain H-UENF2019 and *Herbaspirillum rubrisubalbicans* strain HCC101 was prepared by diluting 200 mL of bacteria (grown medium) in 800 mL of HS at pH 7.0, at a 40 mg L^{-1} of C (Pinheiro et al., 2018). The applications occurred at 15 and 30 DAE, in the late afternoon (between 6 and 7 pm), with the aid of a manual sprayer with an adjustable nozzle, aiming for total wetting of the leaves corresponding to 4.25 mL $plant^{-1}$. The strains H-UENF2019 and HCC101 were obtained from the Laboratory of Cell and Tissue Biology collection from the Universidade Estadual do Norte Fluminense Darcy Ribeiro and selected from previous studies. The bacteria were grown in a liquid DYGS medium for 24h at 30° C under agitation at 180 rpm to reach the optimum bacterial suspension density (10^8 per mL) (Döbereiner et al., 1995).

The HS was isolated from vermicompost in a 1:9 (vermicompost: water) ratio. This vermicompost contained pH (H_2O): 6.29; C: 60.4 g kg^{-1}; P: 987.50 mg dm^{-3}; and 2.57, 7.71, 8.25, 1.80, 18.52, 20.32 cmolc dm^{-3} of K, Ca, Mg, H + Al, SB, and CTC. The HS concentration was based on the C content. Chemical fractionation of these substances was also performed according to a method adapted from Swift (1996) to know their composition, which contained 67% and 33% fulvic and humic acids, respectively.

Physiological evaluations

A portable infrared gas analyzer (IRGA), model LI 6400 XT Portable Photosynthesis System (LI-COR, Lincoln, NE, USA), with a fixed light source at 1000 mmol m^{-2} s^{-1} of photosynthetic photon flux intensity, was used to obtain data on the net CO_2 assimilation rate (A), transpiration rate (E), and internal CO_2 concentration (Ci) of marandu grass plants. This procedure was performed on fully expanded leaves between 7:00 and 9:00 am. These results calculated the instantaneous carboxylation efficiency by the ratio A/Ci (Konrad et al., 2005). Concomitantly, a portable modulated light fluorometer (PSI FluorPen, model FP 100, Brasov, Czech Republic) was used to determine the maximum quantum yield of PSII (MQY) after adaptation of the leaves to the dark for 15 minutes.

Productive components and N accumulation

Total plant height was determined with a graduated ruler, while stem diameter was measured with a digital pachymeter. The shoots and roots were collected individually, stored in paper bags, and placed in an oven at 65 °C for 72 hours (until a constant weight was reached) to determine dry mass. Samples were ground to determine the N concentration in the tissues (Carmo et al., 2000). The N accumulation was calculated by multiplying the N concentration by the total dry mass contained in each pot.

Data analysis

The normality of the data was tested by the Shapiro-Wilk test, which confirmed normal distribution. They were also submitted to ANOVA in a simple design (1 factor = bio-inputs) and passed the F test. When they achieved significance, the means were compared by Tukey's test (ExpDesp package; $p \leq 0.05$). A Pearson correlation was developed from a heat map (corrplot package; $p \leq 0.05$) relating the data of the productive, physiological, and N accumulation parameters. All the cited procedures were performed in the R (ExpDes) program (R Development Core Team, 2018).

Conclusions

The application of bio-inputs stimulated the growth of marandu grass, and the positive results in low-fertility soil indicate that these bio-inputs are a promising tool for productivity increase with relatively lower chemical inputs.

Leaf application of HS combined with *Herbaspirillum seropedicae* strain H-UENF2019 and *Herbaspirillum rubrisubalbicans* strain HCC101 isolated or combined with *Azospirillum brasilense* Ab-V5/Ab-V6 inoculated via seed provided higher growth and biomass yield, and improved gas exchange and photochemical efficiency.

Marandu grass plants accumulated more N in bio-inputs treatments based on HS and the Herbaspirillum seropedicae strain H-UENF2019 and Herbaspirillum rubrisubalbicans strain HCC101 applied with or without Azospirillum brasilense Ab-V5/Ab-V6 inoculated via seed by biofertilization and/or biostimulation.

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