agronomy

MDPI

Article

Growth Stimulation of Tropical Grass (*Megathyrsus maximus* Jacq.) by Humic Substances and *Herbaspirillum seropedicae*

Luciano P. Canellas [1,*], Natália A. Canellas [1], Rakiely M. Silva [1], Etelvino H. Novotny [2] and Fabio L. Olivares [1]

[1] Núcleo de Desenvolvimento de Insumos Biológicos para a Agricultura, Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes 28013-602, RJ, Brazil; nattyaguiar@gmail.com (N.A.C.); rakielym@gmail.com (R.M.S.); fabioliv@uenf.br (F.L.O.)

[2] Embrapa Solos, Rio de Janeiro 22460-000, RJ, Brazil; etelvino.novotny@embrapa.br

* Correspondence: lucianocanellas@gmail.com; Tel.: +55-22-27397198

Abstract: This study aimed to evaluate the effect of soluble humic substances and plant-growth-promoting bacteria on the vegetative growth of Mombaça grass. A greenhouse experiment was conducted to study the effects of foliar applications of humic substances (0, 12, 24, 48, and 96 mg C L^{-1}) on the growth of Mombaça fifteen days after germination. After determining the optimal concentration range, a field trial was carried out in which humic substances at the best concentration were applied simultaneously with *Herbaspirillum seropedicae* strain UENF-H19 fifteen days after germination in three replicates. The best growth of Mombaça in the greenhouse was obtained with 48 mg C of the humic substance L^{-1}, which promoted a shoot fresh weight 80% higher than in the control treatment. The increase was almost identical to that observed during the 50-week field experiment in plots treated with humic substances combined with *H. seropedicae*. The treated plants produced an 81% higher shoot fresh weight than the control, with no dry mass, nitrogen content, or crude protein change during the one-year evaluation period. Despite the efficiency of the selected microbial inoculants under controlled-environment experiments, the agronomical significance under field conditions remains a subject of debate and improvement. The present study demonstrates that combining *Herbaspirillum seropedicae* with humic substances (plural) could significantly increase pasture production under field conditions.

Keywords: biostimulants; plant-growth-promoting bacteria; tropical grass; sustainability

check for updates

Citation: Canellas, L.P.; Canellas, N.A.; Silva, R.M.; Novotny, E.H.; Olivares, F.L. Growth Stimulation of Tropical Grass (*Megathyrsus maximus* Jacq.) by Humic Substances and *Herbaspirillum seropedicae*. *Agronomy* **2024**, *14*, 2006. https://doi.org/10.3390/agronomy14092006

Academic Editors: Jiafa Luo and Zhongyang Huo

Received: 21 June 2024
Revised: 6 August 2024
Accepted: 19 August 2024
Published: 2 September 2024

1. Introduction

Plant biostimulants (PBs) are defined as any substance or microorganism applied to plants to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits, regardless of their nutrient content [1]. PBs are grouped as either "microbial" or "non-microbial". Humic substances (HSs) and plant-growth-promoting bacteria (PGPB) are among agriculture's most widely used biostimulants. HSs are defined as supramolecular associations of thousands of relatively small and heterogeneous molecules held together by hydrophobic forces (van der Waals, π-π, and C-π) and/or hydrogen bonds and weak electrostatic interactions [2]. Commercial PB HS-based products are obtained from various forms of coal, including leonardite. HSs are used as PBs because of their effects on plant physiology, including the up-regulation of primary and secondary metabolism, antioxidant defense, and nutrient uptake systems [3]. HSs can be used as delivery vehicles for beneficial microorganisms in crops because they are recalcitrant to catabolic action.

PBs that use microbes can increase crop growth through a combination of biological nitrogen fixation, the production of plant regulators, increased availability of soil nutrients, and disease control. Endophytic diazotrophic bacteria are a group of plant-growth-promoting microorganisms used as inoculants for non-leguminous plants. *Herbaspirillum* belongs to the phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*, family *Oxalobacteraceae*. *H. seropedicae* is one of the more efficient nitrogen fixers associated with gramineous plants. In addition to molecular methods, it is easily identified by its flagellar pattern and the use of specific carbon sources in a semi-solid JNFB medium [4]. The biofertilizer effect of *Herbaspirillum* spp. is derived from its ability to fix nitrogen and associate endophytically with plants of agricultural importance. In addition, it also solubilizes inorganic P and produces phytohormones (e.g., gibberellin and auxin) and other bioactive compounds [5].

PBs formulated based on HSs and PGPB have demonstrated the potential to improve nutrient absorption, optimize fertilizer utilization, and enhance crop production profitability in the context of reduced input and increased sustainability [6].

Over the past three decades, considerable attention has been devoted to elucidating the mechanisms underlying plant growth stimulation by HSs. These mechanisms encompass activating plasma membrane proton pumps, enhancing nutrient transporters, facilitating primary and secondary plant metabolic pathways, and eliciting plant defense mechanisms against stresses [3]. Moreover, supplementation with HSs has consistently yielded significant increases in pasture production, as evidenced by pot [4] and field trials [7–11].

Small-scale dairy farms, typically ranging from 5 to 100 hectares in size, constitute approximately 87% of Brazil's milk production [12]. Dairy farms bear significant social importance, serving as the primary income source for numerous rural families. However, the sector faces challenges from political and economic factors such as price volatility, labor shortages, and competition with imported dairy products. Recent escalations in the cost of cattle feed have not been met with corresponding increases in milk prices, often prompting farmers to curtail investments in fertilization practices as a cost-saving measure. Nitrogen is the most limiting factor for pasture growth in the tropics. Enhancing fertilizer efficiency and optimizing cost efficiency are crucial for the viability of the dairy sector.

Mombaça grass is recognized as one of the most prolific tropical forages, boasting a substantial potential for biomass production with an approximate 13% protein content [13]. The inoculation of this grass with *Herbaspirillum seropedicae*, an endophytic diazotrophic bacterium, has positively impacted crop yield [14]. Nevertheless, the observed responses are intricately linked to the specific genotype of the plant [15]. Extensive examination of a formulation incorporating *H. seropedicae* and humic acids has been conducted across various crops cultivated in soils characterized by low fertility [16]. The study and development of PBs has been approached by utilizing various methodological approaches and strategies, including microarray and physiological analyses and transcriptome, genomic, phenomic, molecular, proteomic, chemical, and metabolomic methods [17]. However, field experiments are necessary to legitimize and support the development of this biotechnology. Nonetheless, there remains a scarcity of studies providing empirical data on grass inoculation with both HSs and endophytic diazotrophic bacteria.

This work aimed to test if it could improve field biomass production by applying HSs in combination with endophytic diazotrophic bacteria. We evaluated the growth of Mombaça grass treated with different HS concentrations in a greenhouse followed by a field trial with and without inoculation with *H. seropedicae* in the presence of the best HS concentration.

2. Materials and Methods

This study was divided into two experiments. First, the best concentrations of the HSs were evaluated in a greenhouse, followed by a field trial to evaluate the application of humic substances and an *H. seropedicae* suspension in a rotative pasture system.

A preliminary study was undertaken in a greenhouse employing an entirely randomized design with four repetitions to determine the optimal humic concentration for enhancing pasture growth. The treatments comprised five dilutions of commercial humic substances from leonardite (Monty's Liquid Carbon®, Louisville, KY, USA) in water, namely 0, 12, 24, 48, and 96 mg C L^{-1}, along with a control group devoid of the HS. Each pot containing 700 dm^3 of Neossolo Flúvico, classified according to the Brazilian System of Soil Classification or Inceptisol as per the US Soil Survey, was seeded with 15 mg of seeds.

The soil used in the experiment was obtained from the surface layer (0–20 cm) collected in the vicinity of Lagoa de Cima, Campos dos Goytacazes, State of Rio de Janeiro, Brazil, situated at coordinates 21°44′24.6″ S 41°32′07.8″ W. The collected soil was air-dried and sieved using 2.0 mm to obtain air-dried, fine soil. For soil chemical characterization, the following analyses were performed according to the Embrapa Soil Analyses Handbook [18]: pH in water, in a 1:2.5 soil:liquid suspension; exchangeable Al, Ca, Mg, and Na extracted with 1 mol L^{-1} KCl, in a 1:10 ratio, with Al determined by titration with 0.025 mol L^{-1} NaOH, Ca and Mg by atomic absorption spectrometry (ICE 3000 Thermo Fisher, A.A.S., Waltham, Massachusetts, USA), and Na by flame photometry (Tecnal, São Paulo, Brazil); available K and P by extraction with Mehlich1 (HCl 0.05 mol L^{-1} + H_2SO_4 0.0125 mol L^{-1}) in a 1:10 ratio, measured by flame photometry and colorimetry, respectively; and H + Al (potential acidity) using 0.5 mol L^{-1} $Ca(OAc)_2$, adjusted at pH 7.0, in a 1:15 ratio, titrated with 0.0606 mol L^{-1} NaOH [15]. The soil chemical characteristics are presented in Table 1.

Table 1. Baseline chemical attributes of the soil used in both experiments.

pH *	P	K^+	Na^+	Al^3	$H^+ + Al^{3+}$	Ca^{2+}	Mg^{2+}	CEC
	g kg^{-1}				cmol$_c$ dm^{-3}			
4.6	4.3	15.0	0.2	0.1	3.2	0.8	1.04	5.5

* pH in H_2O; CEC: cation exchange capacity.

The soil water holding capacity was measured by a simplified methodology [19], and the pots were irrigated with 200 mL of water every two days until the end of the experiment.

The humic suspension was applied as leaf spraying 15 days after emergence using a pre-compression spray device of 1.25 L PCP-1P. The volume of the humic suspension applied for each treatment was 100 mL. After 30 days of application, the first cut occurred 10 cm from the ground. Two additional cuts were conducted at 60 and 90 days after application.

Field Experiment

A field experiment was conducted in 2022–2024 at the same place used for soil sampling. According to the Koppen Classification System, the climate is Aw Tropical type, with rainy summers and dry winters, with the temperature of the coldest month exceeding 18 °C. The annual mean precipitation, temperature, and relative air humidity are 1023 mm, 24 °C, and 77%, respectively. The average temperatures and precipitation obtained during the study period are shown in Figure 1.

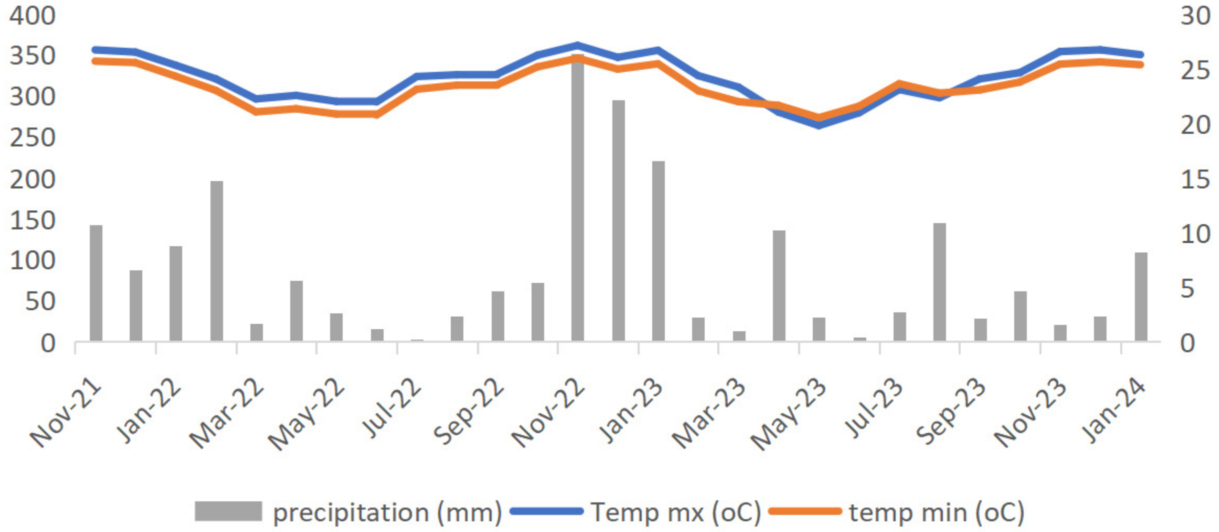


Figure 1. Mean max and min temperature (°C) and precipitation (mm) during the study period.

The experimental area has a history of use of more than 15 years with the native grass "*grama pernambuca*" in extensive management. In July 2022, after cleaning and preparing the soil for the implementation of the experiment, sowing was performed (*Megathyrsus maximus* Jacq. cultivar *Mombaça*) using 4 kg ha^{-1} of encrusted seed. Before sowing, the area was plowed and mechanically harrowed, applying the equivalent of 2 tons of dolomitic limestone, 100 kg P_2O_5 natural rock phosphate, 50 kg K_2O (KCl), and 10 tons of organic compost per hectare.

The experiment was conducted in an entirely randomized design with three replicates and two treatments, namely application and not (control) of humic substances (2.5 mL L^{-1}) combined with the suspension of *Herbaspirillum seropedicae* (final concentration of 5×10^8 cells mL^{-1}) fifteen days after germination in a volume of 20 L per parcel of 25 m × 35 m (875 m^2) using an electric knapsack sprayer. The exact amount of pure water was applied to the control parcels. The visual representation of field parcels is shown in Figure 2.

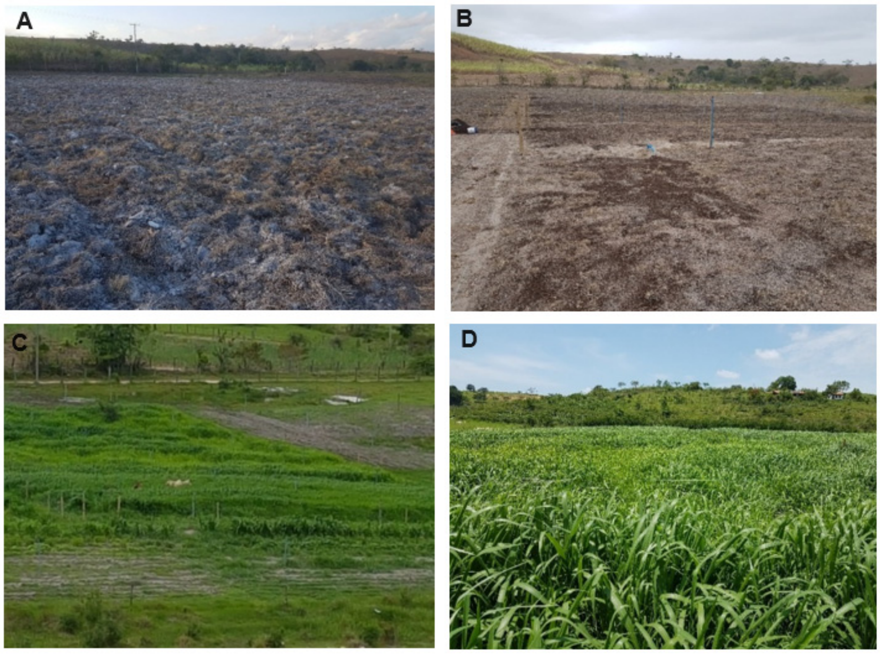


Figure 2. Visual representation of field parcels: (**A**) plowing and limestone incorporation; (**B**) compost fertilization at the soil surface; (**C**,**D**) general view of parcels.

The bacteria strains belong to the Bacteria Culture Collection of Laboratório de Biologia Celular e Tecidual (LBCT-UENF). *Herbaspirillum seropedicae* strain UENF-H19 was activated from the stock by growing the pre-inoculum in 5 mL of Nutrient Broth medium (HIMEDIA, Kennett Square, PN, USA) in a rotatory shaker at 30 °C and 150 rpm for 48 h. Afterward, a 50 µL suspension was transferred to a 250 mL flask containing 75 mL of the same liquid medium and growth conditions described herein. The final individual bacterium density was adjusted to 5×10^8 cells mL^{-1}.

The Total Organic Carbon of the HSs was evaluated using a TOC analyzer (Shimadzu, Tokyo, Japan). Diffuse reflectance infrared Fourier transform (DRIFT) spectra were recorded with a Shimadzu Prestige 21 (Shimadzu, Tokyo, Japan), equipped with a diffuse reflectance accessory, accumulating up to 100 scans with a resolution of 4 cm^{-1}. Before DRIFT analysis, the dry samples were finely ground with an agate mortar and diluted with a KBr powder (1/30, *w*/*w*). The solid-state ^{13}C nuclear magnetic resonance spectra were obtained with cross-polarization and magic angle spinning (^{13}C CPMAS-NMR). The spectra were acquired with a Bruker AVANCE 300 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 4 mm Wide Bore M.A.S probe and operating at a ^{13}C resonating frequency of 75.475 MHz. The powdered HS was spun at 13 kHz within a 4 mm Zirconia rotor and

Kel-F caps. In total, 4000 scans were collected over an acquisition time of 25 ms and a recycle delay of 2.0 s. The Bruker Topspin 1.3 software was used to collect and analyze the data. All free induction decays (FIDs) were transformed by applying a 4 k zero-filling and a line broadening of 100 Hz.

Concerning the experiment evaluation, immediately after germination, three points of 0.5 m^2 were marked in each plot, spaced 7.5 m from the plot boundary and 10 m apart from each sampling area, and used to manually cut the grass at a height of 20 cm from the ground when the plant reached 0.90–1.0 m. A sprinkler irrigation system was set up, and a 5 mm water layer was used weekly until the first cut 75 days after germination. After collecting the points used to evaluate pasture growth, four dairy cows were allowed to enter and remained on the plot limited by an electric fence for six hours a day, six days a week. The initial weights of the cows were 312, 347, 300, and 365 kg. The seventh day was used for replacement fertilization using calculations from NPK reposition builds considering average nutrient extraction by pasture and production [20,21] followed by irrigation with 3 mm of water. The rotation between plots was carried out during the one year corresponding to the evaluation period of the experiment. The grass was sown on 15 July 2022, and the first inoculation was carried out on 7 September 2022. A second inoculation was carried out in April 2023 at the beginning of the dry period. The first rotational grazing cut and start was on 15 November 2022.

The data collected during the experiment were submitted to analysis of variance (ANOVA) using statistical R software version 4.1.2. A regression analysis was employed to assess the impact of treatments on pasture production over time, utilizing the Matlab program (On line version). The LSD test ($p < 0.05$) was used to compare differences between means. All data were the average of three replicates.

3. Results

The HSs showed typical chemical features of the leonardite core, as shown by the ^{13}C NMR spectral distribution (Figure 3). The main feature is a high aromaticity with a predominance of unsubstituted C-aryl (125 ppm), accompanied by a high acidity due to the strong presence of carboxyl C at 174 ppm. The presence of alkyl C, primarily long chains $(CH_2)_n$, was also notable, with a broad signal centered at 22 ppm and a small resonance signal at 56 ppm, indicative of the presence of methoxylic or N-alkyl groups.

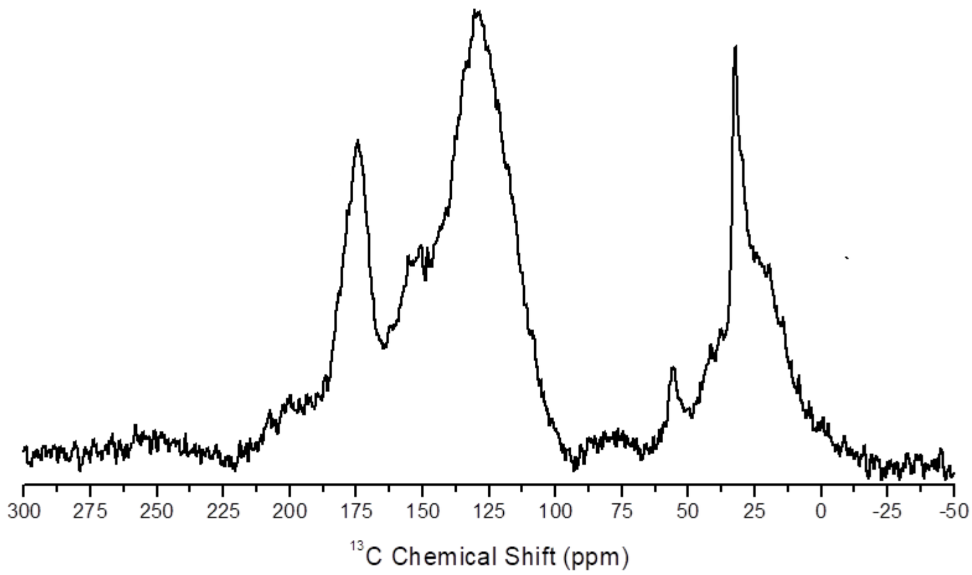


Figure 3. CP/MAS^{13}C NMR spectrum of humic substances used in the experiments.

After the emergence of Mombaça grass, the application of the HSs promoted plant growth according to the concentrations, showing a bell-shaped curve at low concentrations with significant polynomial model response (Figure 4). The best growth was obtained by diluting 2.5 mL of the humic product in 1 L of water. The dilution of 2.5 mL L^{-1} C promoted shoot fresh weight 80% higher than that obtained in the control treatment at the first cut (Figure 4B). In the second cut, the dilution of 1.25 mL L^{-1} promoted 20% of shoot fresh weight concerning the control, while in the third cut, the minor dilution (0.62 mL L^{-1}) also promoted plant growth, resulting in significant fresh weight, representing an increase of 87% compared to the control treatment (Figure 2B). At the end of the experiment, the incremental concentrations of 0.62, 1.25, and 2.25 mL L^{-1} had a positive effect when applied 15 days after germination, resulting in a significant accumulation in the shoot fresh weight (4.40, 4.72, and 7.11 g) and representing an increase of 21, 29, and 95% of shoot fresh weight, respectively (Figure 4A).

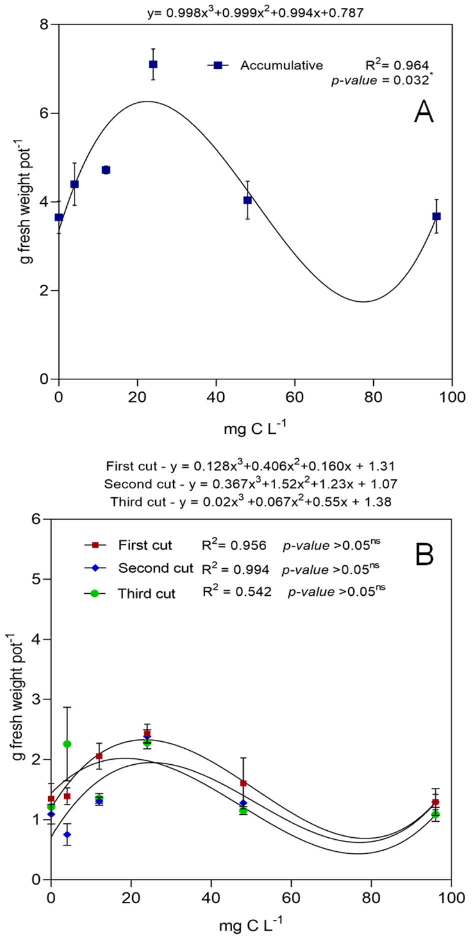


Figure 4. (**A**) Production of fresh-weight grass with six different commercial concentrations of humic substances (HSs) (0.0, 0.62, 1.25, 2.5, 5, and 10 mL L^{-1}). (**B**) Production of grass fresh weight per pot in three successive harvests. ns: not significant.

Field Trial

The inoculation of grass parcels (25 × 35 m) with HSs and *Herbaspirillum seropedicae* UENF-H19 at 2.5 mL L^{-1} and 5×10^8 cells mL^{-1} significantly promoted the production of shoot fresh weight compared to control. The plant growth promotion varied from 71% to 116% concerning control plants, with 84% higher in the dry months (April to September) and 116% in the rainy season (October to March) (Figure 3 and Supplementary Tables S2 and S3). The general increase of Mombaça grass shoot fresh weight was 91% higher in the inoculate parcels in comparison with the control (640 g 0.5 m^2 vs. 340 g

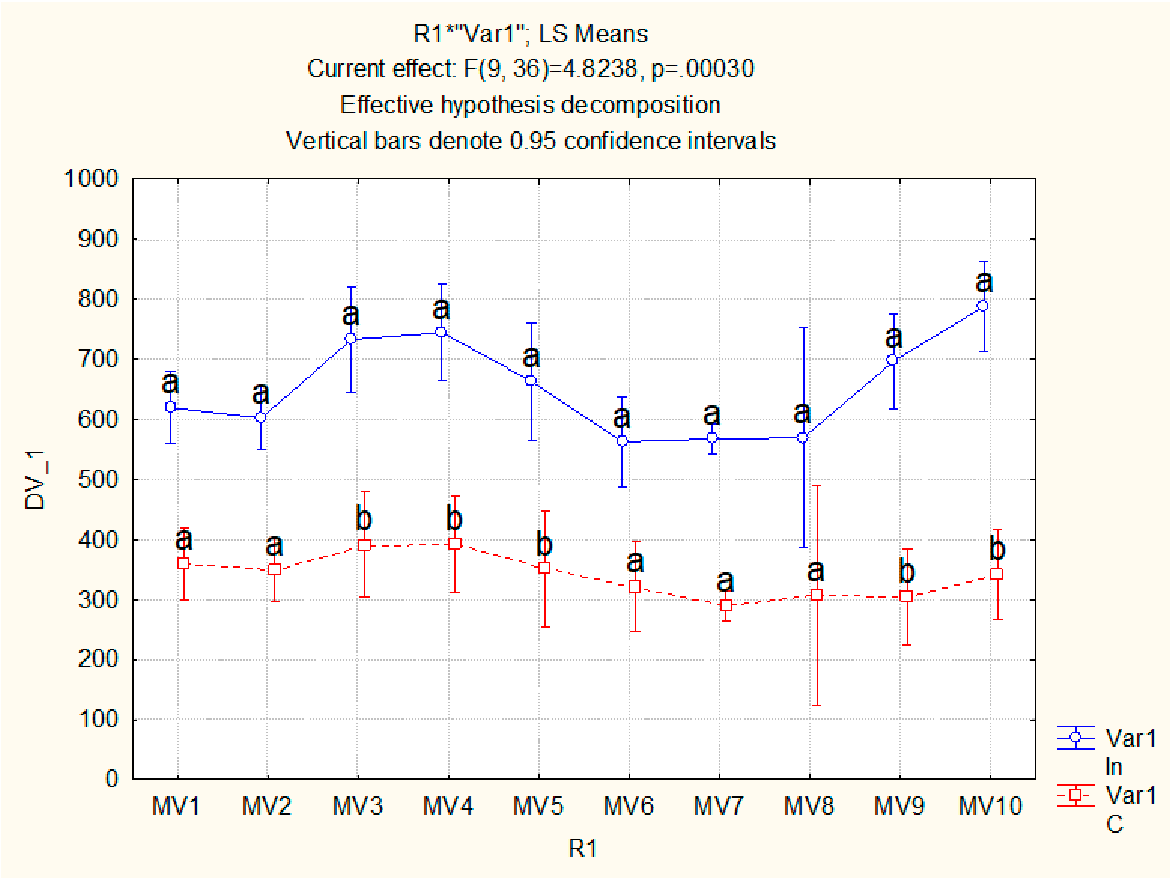
0.5 m^2), corresponding to 12.8 tons ha^{-1} of shoot fresh weight while the control produced equivalent to 6.8 tons ha^{-1} (Figure 4). No differences were found in the dry weight and crude protein content, with values of 27% and 13%, respectively (Supplementary Table S4). The cows grazing in the experimental parcel increased their life weights at the average rate of 2.6 kg per month, equivalent to 86 g per day (Supplementary Table S5).

4. Discussion

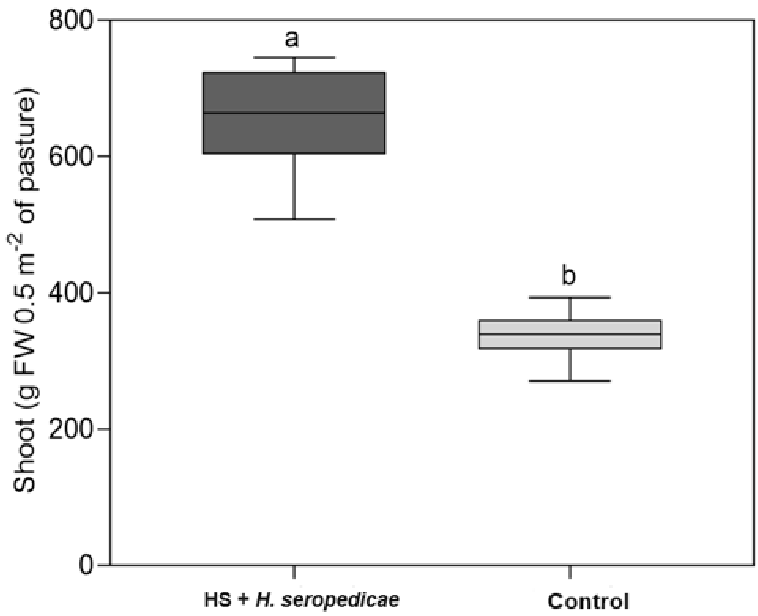
Studies on the effect of HSs on pasture species are scarce. Field studies applying a commercial product to grass showed variable growth and nutrient uptake results across various soil types, grass species, and age [7,8,16]. Here, we present the results of HSs applied with PGPB for one year of field tropical pasture (Mombaça) growth in soil with very low natural fertility. As HSs' effects and growth benefits closely depend on the application rate, we performed a preliminary experiment in a greenhouse to establish the concentration–growth curve. Despite the low R, the value was significant for the cubic model ($p < 0.05$), showing a Gaussian curve, i.e., low plant growth promotion at low concentrations and an inhibition effect at relatively high concentrations (Figure 2). Typically, there is an upper limit at which maximal growth benefits are seen, with a depressive or lack of effect at rates that exceed this. Asli and Neumann [22] suggested that high concentrations (1 g L^{-1}) promote the accumulation of humic substances at cell wall pores, restricting root water and nutrient uptake. However, more realistic concentrations do not permanently block the plant surface, and the root application of a humic acid extracted from leonardite increased the root hydraulic conductivity significantly [23]. The fouling of root pores is a transient phenomenon and disappears with time due to conformational changes induced by HSs' interactions with the root surface, but it is enough to cause mild stress that activates plant adaptative responses and growth [24]. However, the most plausible reason for the typical quadratic-shaped curve is the typical hormone-like behavior found in HSs [25], where the hormonal plant balance is changed, inducing molecular, biochemical, and anatomical responses in optimal concentrations with drastic deleterious effects in relatively high hormone concentrations. HSs are claimed to induce auxin- and gibberellin-like effects on plants and increase plasma membrane proton pump (H+-ATPase) activities, promoting cell elongation and enhancing ion transport across the cell membrane [3]. The hormone-like activity was unequivocally demonstrated by the gene reporter assay using different plant constructs [26,27].

After determining an optimal response range for Mombaça grass, we set up a field experiment in soil with very low natural fertility (Table 1), applying the HSs together with a selected strain of *H. seropedicae*, a notorious diazotrophic endophytic bacteria with a broad spectrum of beneficial action on plant growth that includes, in addition to biological nitrogen fixation, phosphate solubilization, the alleviation of abiotic stress, and phytohormone production [5]. The synergic effect of HSs and PGPB has been previously demonstrated in several plant crops and includes a heterogeneous and highly rough surface that favors the anchoring of bacteria and biofilm formation, hydrophobicity, and photoprotection [16]. The net result is increased plant colonization and crop yield.

The effect of combined inoculation on Mombaça shoot growth during one year of experimentation using a rotative pasture system was very significant (Figure 5B). The cows demonstrated a sustained increase in live weight throughout the experimental period at an equivalent rate of 84 g day^{-1} (Supplementary Table S5).



(**A**)



(**B**)

Figure 5. (**A**) Monthly production of shoot fresh weight of Mombaça during the rotational grazing system. Tree parcels with 875 m^2 (35 × 25 m) were inoculated or not (control) with 20 L of commercial humic substances (2.5 mL L^{-1}) and 5 × 10^8 cells mL^{-1} of *Herbaspirillum seropedicae* UENF-H19. Different letters represent significant differences by the LSD test ($p < 0.05$). (**B**) General average of Mombaça shoot fresh weight in the parcels treated or not (control) with humic substances and *Herbaspirillum seropedicae*. Different letters represent significant differences by the LSD test ($p < 0.05$). The raw data are shown in Supplementary Tables S2 and S3.

In this discussion, we would like to highlight two specific aspects. First, we did not observe a decline in grass production after a high-yielding first grass cut, which is a well-known phenomenon in grass physiology [28], probably due to the repositioning system of fertilizer and irrigation system activation. This represents a positive impact on grass production in a sustainable way. The second aspect addresses the benefits of achieving sustainable production by optimizing natural and renewable resources [29]. The advantages of combining HSs and PGPB include preserving and improving the natural capital, optimizing resource efficiency, and promoting system efficiency. Grass production can be inside a circular economy strategy, reducing fertilizer use, optimizing nutrient cycles, and minimizing water use. Combined with two applications of humic substances and *H. seropedicae*, it has proven to enhance plant growth without modifying crude protein or dry mass. The benefits described here are exceptional, achieving up to a 91% increase in green biomass production in the field, and these benefits are susceptible to potential directions of future research for exploiting the properties responsible for this result. The impacts of HSs on the soil microbiome and the structure and function of soil microorganisms involved in nutrient cycling are strong candidates, as observed previously on grass treated with a coal-derived HS [28].

The increasing reliance on chemical inputs (fertilizers, pesticides, nutrients, etc.) in farming systems represents a significant risk to various environmental and human health concerns. These include threats to crop productivity, soil fertility, the nutritional value of farm produce, the management of pests and diseases, the well-being of agroecosystems, and the health of humans and animals. Microbial inoculants are employed as an alternative to conventional inorganic fertilizers. Nevertheless, the results observed in the field are variable and, on occasion, inconsistent. The endophytic compartment is less susceptible to abiotic stress and biotic competition than the rhizosphere or root surface. Despite the evidence accumulated for biological nitrogen fixation in plants treated with Herbaspirillum under controlled environment experiments, the agronomical significance of bioinoculants for N-nutrition under field conditions remains a subject of debate and improvement [29]. The present study demonstrates that combining *Herbaspirillum seropedicae* with HSs could significantly increase pasture production under field conditions. The results supported the use of this technique to optimize tropical pasture production systems as a low-cost technology.

5. Conclusions

Applying HSs in combination with *Herbaspirillum seropedicae* at the start of the growing season induced an overall positive effect on shoot fresh weight yield in the field and pot experiments with Mombaça grass. The observed effects in the greenhouse experiment were confirmed by field trials without changes in dry matter or crude protein content. Grass inoculation contributed to more efficient use of fertilizers and water.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14092006/s1, Table S1: Raw data of greenhouse experiment with Mombaça grass shoot growth (g fresh weight pot^{-1}) due to different humic substance concentrations (mL L^{-1}), Table S2: Raw data of field experiment with Mombaça grass shoot growth (g fresh weight/0.5 m^2) on rotative pasture system; Table S3: Average shoot fresh weight production per plot (g/0.5 m^2) in a rotative pasture system. Data from Table S2 (n = 3); Table S4: Shoot dry matter and crude protein; Table S5: Evolution of life weight of cows used for parcel grazing during one year of the experiment.

Author Contributions: Conceptualization, L.P.C. and F.L.O.; investigation, L.P.C., N.A.C., and E.H.N.; formal analysis, R.M.S.; writing—original draft preparation, L.P.C.; writing—review and editing, F.L.O. and E.H.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (F.A.P.E.R.J.); Montys Co., USA, funded the A.P.C.

Data Availability Statement: Data are contained within the article or Supplementary Materials.

Conflicts of Interest: The authors declare that this study received funding from Montys Co. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

References

1. du Jardin, P. Plant biostimulants: Definition, concept, main categories and regulation. *Sci. Hortic.* **2015**, *196*, 3–14. [CrossRef]
2. Piccolo, A. The supramolecular structure of humic substances: A novel understanding of humus chemistry and implications in soil science. *Adv. Agron.* **2002**, *75*, 57–134.
3. Nardi, S.; Carletti, P.; Pizzeghello, D.; Muscolo, A. Biological activities of humic substances. In *Biophysico-Chemical Processes Involving Natural Nonliving Organic Matter in Environmental Systems*; Part 1. Fundamentals and impact of mineral-organicbiota interactions on the formation, transformation, turnover, and storage of natural nonliving organic matter (NOM); Senesi, N., Xing, B., Huang, P.M., Eds.; Wiley: Hoboken, NJ, USA, 2009; Volume 2, pp. 305–339.
4. Baldani, J.I.; Baldani, V.L.D.; Seldin, L.; Döbereiner, J. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *Int. J. Syst. Evol. Microbiol.* **1986**, *36*, 86–93. [CrossRef]
5. Estrada, G.A.; Baldani, V.L.D.; De Oliveira, D.M.; Urquiaga, S.; Baldani, J.I. Selection of phosphate-solubilizing diazotrophic *Herbaspirillum* and *Burkholderia* strains and their effect on rice crop yield and nutrient uptake. *Plant Soil* **2013**, *369*, 115–129. [CrossRef]
6. Canellas, L.P.; Olivares, F.L.; Canellas, N.A.; Jindo, K.; Rosa, R.C.C.; Piccolo, A. Challenge of transition: The history of a case study involving tropical fruits polyculture stimulated by humic acids and plant-growth promoting bacteria. *Chem. Biol. Technol. Agric.* **2022**, *9*, 76. [CrossRef]
7. Pinheiro, P.L.; Passos, R.R.; Peçanha, A.L.; Canellas, L.P.; Olivares, F.L.; Mendonça, E.S. Promoting the growth of *Brachiaria decumbens* by humic acids (HAs). *Austr. J. Crop Sci.* **2018**, *12*, 1114–1121.
8. Gerk, J.; Meyer, U.; Römer, W. Phosphate, Fe and Mn uptake of N_2 fixing red clover and ryegrass from an Oxisol as affected by P and model humic substances application. 1. Plant parameters and soil solution composition. *J. Plant Nut. Plant Sci.* **1995**, *158*, 261–268. [CrossRef]
9. Verlinden, G.; Coussens, T.; De Vliegher, A.; Baert, G.; Haesaert, G. Effect of humic substances on nutrient uptake by herbage and on production and nutritive value of herbage from sown grass pastures. *Grass For. Sci.* **2010**, *65*, 133–144. [CrossRef]
10. Little, K.R.; Rose, M.T.; Jackson, W.R.; Cavagnaro, T.R.; Patti, A.F. Do lignite-derived organic amendments improve early-stage pasture growth and key soil biological and physicochemical properties? *Crop Past. Sci.* **2014**, *65*, 899–910. [CrossRef]
11. Espie, P.; Ridgway, H. Bioactive carbon improves nitrogen fertiliser efficiency and ecological sustainability. *Sci. Rep.* **2020**, *10*, 3227. [CrossRef]
12. IBGE—Instituto Brasileiro de Geografia e Estatística. Produção de Leite no Brasil. Available online: https://www.ibge.gov.br/explica/producao-agropecuaria/leite/br2022 (accessed on 12 January 2024).
13. Sales, M.F.L.; Valentim, J.F. *Capim Mombaça—Formação E Manejo de Pastagens No Acre*; Embrapa: Rio Branco, Brazil, 2002.
14. Olivares, F.L.; James, E.K.; Baldani, J.I.; Dobereiner, J. Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirilium*. *New Phytol.* **1997**, *135*, 723–737. [CrossRef]
15. Pankievicz, V.C.S.; Amaral, F.P.; Santos, K.F.D.N.; Agtuca, B.; Xu, Y.; Schueller, M.J.; Arisi, A.C.M.; Steffens, M.B.R.; de Souza, E.M.; Pedrosa, F.O.; et al. Robust biological nitrogen fixation in a model grass–bacterial association. *Plant J.* **2015**, *81*, 907–919. [CrossRef]
16. Olivares, F.L.; Busato, J.G.; Paula, A.M.; Lima, L.S.; Aguiar, N.O.; Canellas, L.P. Plant growth promoting bacteria and humic substances: Crop promotion and mechanisms of action. *Chem. Biol. Technol. Agric.* **2017**, *4*, 30. [CrossRef]
17. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in Plant Science: A Global Perspective. *Front. Plant Sci.* **2017**, *7*, 2049. [CrossRef] [PubMed]
18. EMBRAPA—Empresa Brasileira de Pesquisa Agropecuária. *Manual de Métodos de Análises de Solo*, 2nd ed.; Centro Nacional de Pesquisa de Solos: Rio de Janeiro, Brazil, 1997; 212p.
19. Nelson, J.T.; Adjuik, T.A.; Moore, E.B.; VanLoocke, A.D.; Ramirez Reyes, A.; McDaniel, M.D. A Simple, Affordable, Do-It-Yourself Method for Measuring Soil Maximum Water Holding Capacity. *Commun. Soil Sci. Plant Anal.* **2024**, *55*, 1190–1204. [CrossRef]
20. Oliveira, P.A.; Bernardi, A.C.C.; Alves, T.C.; Pedroso, A.F. Evolução na recomendação de fertilização de solos sob pastagens: Eficiência e sustentabilldade na pecuária. In *Embrapa Southeast Livestock*; Empresa Brasileira de Pesquisa Agropecuária—Embrapa: São Carlos, Brazil, 2015.
21. Siri-Prieto, G.; Bustamante, M.; Picasso, V. Impact of nitrogen and phosphorous on biomass yield, nitrogen efficiency, and nutrient removal of perennial grasses for bioenergy. *Biom. Bioenergy* **2020**, *136*, 105526. [CrossRef]
22. Asli, S.; Neumann, P. Rhizosphere humic acid interacts with root cell walls to reduce hydraulic conductivity and plant development. *Plant Soil* **2010**, *336*, 313–322. [CrossRef]
23. Olaetxea, M.; Mora, V.; Bacaicoa, E.; Baigorri, R.; Garnica, M.; Fuentes, M.; Casanova, E.; Zamarreño, A.M.; Iriarte, J.C.; Etayo, D.; et al. ABA-regulation of root hydraulic conductivity and aquaporin gene- expression is crucial to the plant shoot rise caused by rhizosphere humic acids. *Plant Physiol.* **2015**, *16*, 2587–2596. [CrossRef]
24. Olaetxea, M.; Mora, V.; Baigorri, R.; Zamarreño, A.M.; García-Mina, J.M. The Singular molecular conformation of humic acids in solution influences their ability to enhance root hydraulic conductivity and plant growth. *Molecules* **2021**, *26*, 3. [CrossRef]

25. Trevisan, S.; Botton, A.; Vaccaro, S.; Vezzaroa, A.; Quaggiotti, S.; Nardi, S. Humic substances affect Arabidopsis physiology by altering the expression of genes involved in primary metabolism, growth and development. *Environ. Exp. Bot.* **2011**, *74*, 45–55. [CrossRef]
26. Silva, R.M.; Peres, A.N.A.; Peres, L.E.P.; Olivares, F.L.; Sangi, S.M.; Canellas, N.A.; Spaccini, R.; Cangemi, S.; Canellas, L.P. Humic substances isolated from recycled biomass trigger jasmonic acid biosynthesis and signalling. *Plants* **2023**, *12*, 3148. [CrossRef] [PubMed]
27. Suárez-Eiroa, B.; Fernán Fernández, E.; Méndez-Martínez, G.; Soto-Oñate, D. Operational principles of circular economy for sustainable development: Linking theory and practice. *J. Clean. Prod.* **2019**, *214*, 952–961. [CrossRef]
28. Little, K.R. Commercial Lignite Coal-Derived Amendments for Improved Pasture Growth and Soil Health. Ph.D. Thesis, Monash University, Melbourne, VIC, Australia, 2015.
29. Matteoli, F.P.; Olivares, F.L.; Venancio, T.M.; Rocha, L.O.; Irineu, L.E.S.S.; Canellas, L.P. Herbaspirillum. In *Beneficial Microbes in Agro-Ecology: Bacteria and Fungi*; Amaresan, N., Kumar, M.S., Annapurna, K., Kumar, K., Sankaranarayanan, A., Eds.; Academic Press: Cambridge, MA, USA; Elsevier: London, UK, 2020; pp. 493–505.