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Inoculation with plant growth-promoting bacteria mitigates the negative impacts of 2 $^{\circ}$ C warming on the photosynthesis, growth, and nutritional value of a tropical C₄ grassland under field conditions



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HIGHLIGHTS

G R A P H I C A L A B S T R A C T

- Future warming will impact managed ecosystems such as tropical pastures
- Warming of 2 °C decreased plant water status, productivity, and forage quality
- Inoculation of plant growth-promoting bacteria improved photosynthesis, productivity, and forage quality
- When combined, inoculation mitigated the negative warming impacts
- Inoculation is a sustainable strategy to enhance climate resilience of tropical pastures

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ABSTRACT

Human-induced climate change is causing Earth's temperature to rise, and models indicate a persistent increase in the next years. Temperature is one of the most important factors regulating the carbon flux of natural and managed ecosystems. In the last decades, the use of plant growth-promoting bacteria in C₄ grasses has emerged as an important alternative to alleviate the negative impacts of abiotic factors on plant metabolism, growth, and

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Pseudomonas fluorescens Solutions for mitigation T-FACE Tropical forage Tropical climate Warming forage nutritional quality. In this study, we investigated the effects of warming $(+2 \,^{\circ}C)$ on the photosynthesis, plant water status, growth, and nutritional quality of a managed pasture of Brachiaria (syn. Urochloa) Mavuno inoculated or not with Azospirillum brasilense and Pseudomonas fluorescens. We evaluated two levels of temperature (ambient and elevated) under two levels of inoculation (inoculated and non-inoculated) in a multifactorial design. Our results showed that inoculation stimulated root growth and increased photosynthetic rates through higher stomatal conductance and improved photosystem II performance, presumably resulting in higher productivity, crude protein content, and forage digestibility with reduced lignin and fiber fraction. Warming increased non-photochemical quenching and electron transport rate in the wet season, but decreased midday maximum quantum efficiency of PSII photochemistry during dry season, relative water content, productivity, and forage quality and digestibility. When inoculated plants developed under a warmer atmosphere, the positive effects of inoculation completely counteract the negative impacts of warming on photosynthesis, growth, nutritional quality, and digestibility, resulting in a pasture with reduced lignin content and improved heating dissipating capacity and digestibility. Our results demonstrated that A. brasilense and P. fluorescens co-inoculation is a sustainable option to fully mitigate the negative impacts of elevated temperature on Mavuno grass pastures. These findings highlight the potential of microbial inoculants in enhancing forage resilience and productivity under climate stress.

1. Introduction

Anthropogenic climate change is the most significant threat to food security and crop production due to its impact on crop yields and pasture systems (Anderson et al., 2020; Muluneh, 2021). In recent decades, Earth's surface temperature has rapidly increased due to increased emissions of greenhouse gases (GHG) such as carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O) (IPCC, 2021). Currently, the global average temperature is 1.45 °C warmer than pre-industrial temperatures averages (WMO, 2024). Consequently, the frequency, duration, intensity, and spatial distribution of extreme weather events such as floods (Tabari et al., 2020; Robinson et al., 2021), droughts (Rodell and Li, 2023), and heatwaves (Perkins-Kirkpatrick and Lewis, 2020; Geirinhas et al., 2021; Robinson et al., 2021), have been increasing worldwide. Moreover, climate models predict that temperature and extreme weather events will continue to rise in the next years (Domeisen et al., 2023; Suarez-Gutierrez et al., 2023), potentially affecting agriculture and livestock (Goulart et al., 2023; Silva et al., 2023), especially in countries that are highly dependent on rainfed pastures, such as Brazil. To avoid more severe climate impacts, including increased frequency and intensity of droughts, heat waves, and heavy rainfall, some international agreements, such as the Paris Agreement (established at the UN Climate Change Conference, COP21), aim to keep the global average temperature increase well below 2 °C above pre-industrial levels, with a strong commitment to limit the rise to 1.5 °C by the end of this century.

Temperature is an essential variable for plant metabolism, growth, and productivity. However, the impacts of warming on plants are highly dependent on their intensity, timing and duration, species, soil characteristics, and interactions with other biotic and abiotic factors (Sage and Kubien, 2007). For most warm-season and tropical crops, the optimal temperature range for photosynthesis is typically between 7 °C and 40 °C (Moore et al., 2021). Therefore, when warming occurs under this optimum range, the effects on plant growth, photosynthetic rates, and productivity are positive (Sage and Kubien, 2007; Moore et al., 2021). However, when warming exceeds the optimum temperature range, the activity of photosynthetic enzymes is impaired (Sharkey, 2005), increasing the production of reactive oxygen species (ROS) (Sharkey and Schrader, 2006), leading to damages in the integrity of lipids, DNA, and membranes (Choudhury et al., 2017). Even small temperature increases can exceed a plant's optimum range in tropical conditions because tropical regions are already exposed to high temperatures. Additionally, warming also increases vapor pressure deficit, which impacts plant water relations and water status (Schönbeck et al., 2022; Habermann et al., 2024), potentially resulting in xylem cavitation and plant death, especially under water scarcity conditions (Vilagrosa et al., 2012). Moreover, elevated temperature is recognized as an important antiquality factor for pasture nutritional quality (Moyo and Nsahlai,

2021), as warming stimulates the lignification of plant tissues (Lee et al., 2017). Therefore, future warming can significantly impact managed agroecosystems by modifying photosynthetic efficiency, plant growth, and carbon dynamics. These changes require innovative approaches to maintain ecosystem productivity in the face of rising temperatures.

Utilizing beneficial microorganisms represents an innovative strategy to increase ecosystem resilience and productivity in response to climate change. In recent decades, the inoculation of plant growthpromoting bacteria (PGPB), such as Azospirillum brasilense and Pseudomonas fluorescens, in C₄ grasses has emerged as an important strategy to increase the sustainability and resilience of C₄ pastures (Hungria et al., 2016, 2021; Fukami et al., 2018a). The PGPB are bacterial species that reside in the rhizosphere and can promote plant growth through various mechanisms such as biological nitrogen (N) fixation, the synthesis of root growth-promoting hormones like ethylene, auxin, cytokinin, and gibberellins (Fukami et al., 2018a; Orozco-Mosqueda et al., 2023), solubilization of inorganic phosphorous (Turan et al., 2012), and synthesis of antioxidant enzymes (Upadhyay et al., 2012; Fukami et al., 2017, 2018b). Consequently, PGPB can improve plant productivity (Timmusk et al., 2023; Hungria et al., 2016, 2021), partially replace the use of Nfertilizers (Hungria et al., 2022), and stimulate root growth, potentially improving water and nutrient absorption (Ardakani et al., 2011; Hungria et al., 2021). While limited evidence suggests that PGPB can enhance the nutritional quality and digestibility of forages (Mishra et al., 2008; Sá et al., 2019; Duarte et al., 2021), there is a substantial body of research highlighting that inoculation of PGPB can mitigate the adverse effects of abiotic stresses. Studies have demonstrated that PGPB inoculation can alleviate the impacts of drought (Cohen et al., 2015; Freitas et al., 2022), heat stress (Mitra et al., 2021), and heavy metal toxicity (Wang et al., 2022). Therefore, PGPB inoculation shows potential to enhance plant stress tolerance mitigating the negative impacts of future warming on plants. However, the precise interactions between elevated temperatures and PGPB remain unclear, and there is a critical need to understand whether the impacts of PGPB inoculation are consistent in field conditions.

The interaction between elevated temperatures and PGPB is still poorly understood. PGPB inoculation could potentially enhance the beneficial effects of warming or conversely, partially or fully mitigate its negative impacts. To our knowledge, there are no field experiments investigating the effects of warming (+2 °C) on a managed pasture of *Brachiaria* (synonymous *Urochloa*) Mavuno grass and its interactions with *A. brasilense* and *P. fluorescens* inoculation. This study addresses this gap by using a T-FACE (Temperature Free-Air Controlled Enhancement) system to apply canopy warming conditions throughout the year, allowing plants to grown under field conditions without limitations on root development (Kimball, 2005). Mavuno grass, a hybrid between *Brachiaria brizantha* (A.Rich.) Stapf x *Brachiaria ruziziensis* (R.Germ.& Evrard) Crins cv. Mixe Drwn 12, is a C₄, tussock-forming tropical perennial grass widely used as fodder in Brazil (Sá et al., 2019). The *Brachiaria* genus represents 85 % of the cultivated pasture area in Brazil (Jank et al., 2014). This hybrid is known for its high productivity (Pereira et al., 2021) and drought resistance, supposedly by featuring a well-developed root system and fast metabolic recovery from water deficits (Habermann et al., 2021). Previous studies have demonstrated that inoculation with *A. brasilense* and *P. fluorescens* benefits Mavuno grass growth (Sá et al., 2019; Duarte et al., 2021). This study aims to evaluate whether these benefits are sustained or modified under warming conditions in the field, focusing on key aspects such as photosynthesis, plant water status, growth, and nutritional quality.

A. brasilense is a Gram-negative species of the Azospirillaceae family, known for fixing atmospheric N (N2), producing growth-promoting phytohormones, solubilizing soil inorganic phosphorus, and inducing tolerance to abiotic stress (Fukami et al., 2018a). Over the past decades, it has been used to partially replace N-fertilizers in various crops including sugarcane (Saccharum spp.), maize (Zea mays L.), wheat (Triticum aestivum L.), rice (Oryza sativa L.), and pastures (Hungria et al., 2016, 2021, 2022; Galindo et al., 2022; Scudeletti et al., 2023). On the other hand, P. fluorescens, a Gram-negative species of the Pseudomonadaceae family, is known for its biocontrol properties and synthesis of growth-promoting molecules (Bernd et al., 2014; Sandini et al., 2019; Hungria et al., 2021; Guimarães et al., 2022). Both species are commercially available in Brazil, either as standalone treatment or in combination. The objective of this study was to evaluate the effects of elevated temperature on the photosynthesis, productivity, and nutritional quality of Mavuno grass, and to explore the mitigating effects of microbial inoculation on these adverse impacts. Specifically, the goals include: a) assessing the impact of +2 °C warming (maximum UN Paris Agreement threshold) on the photosynthetic efficiency, growth, and nutritional quality of Mavuno grass; b) evaluating the effects of PGPB inoculation on the photosynthetic efficiency, growth, and nutritional quality of Mavuno grass under ambient and elevated temperature conditions; and c) investigating the interaction between warming and microbial inoculation on the photosynthetic efficiency, growth, and nutritional quality of Mavuno grass. We hypothesized that the inoculation will alleviate the negative effects of canopy warming on photosynthesis, growth, nutritional quality, and digestibility of Mavuno grass forage, thereby improving the resilience of managed pasture system to climate change.

2. Material and methods

2.1. Study area

The study was conducted at the University of São Paulo, Ribeirão Preto campus, Brazil (21° 9'42.56" S, 47° 50' 47.12" W) (Fig. 1) from January 2023 to February 2024, totalizing 405 days of experiment. The climate in this region is classified as B2rB'4a' (moist meso-thermal with small water deficiency) (Thornthwaite, 1948), with a Rhodic Ferralsol soil with clay texture (IUSS Working Group WRB, 2015).

2.2. Soil preparation, planting, and pasture management

Before planting, we collected four soil subsamples in each plot (0–20 cm deep) and combined them to form one composite sample per plot. Based on the soil chemical analysis data, we addressed any variations in soil pH between plots by applying dolomite limestone to adjust the soil pH to 5.5. One day before sowing, nutritional differences between plots were adjusted by applying differential amounts of zinc sulfate hepta-hydrate, boric acid, potassium chloride, and triple superphosphate. Immediately before sowing, all plots received 1000 kg ha⁻¹ of nitrogen (N), phosphorus (P), and potassium (K) as composed fertilizer N-P-K (04–14-08), providing 40 kg ha⁻¹ of N, 140 kg ha⁻¹ of P and 80 kg ha⁻¹ of K (Raij et al., 1997). We used a power rotary tiller in all stages of soil preparation.



V Trop-T-FACE Facility

Fig. 1. The geographic location of the Trop-T-FACE facility, designed to investigate the effects of warming and inoculation on Mavuno grass. The site is located on the University of São Paulo (USP) campus in Ribeirão Preto, São Paulo, Brazil.

On January 15, 2023, seeds of *Brachiaria* Mavuno (a hybrid between *Brachiaria brizantha* (A.Rich.) Stapf × *Brachiaria ruziziensis* (R.Germ.& Evrard) Crins cv. Mixe Drwn 12) were broadcast-sown at a rate of 12 kg ha⁻¹ in 16 plots, each measuring of 4×4 m (16 m²). The seeds were covered with 2 cm of soil. In half of the plots, seeds were previously inoculated with a mixture containing two bacteria species: two strains of *Azospirillum brasilense* [CNPSo 2083 (=Ab-V5) and CNPSo 2084 (=Ab-V6)] and one strain of *Pseudomonas fluorescens* [CNPSo 2719 (=CCTB 03)] and a commercial protector (Pastomax Protege®), to protect bacteria from desiccation. The final concentration of each strain was 5×10^8 CFU mL⁻¹. On the seeding day, we applied 4 mL of each *A. brasilense* strain +8 mL of *P. fluorescens* + 4 mL of commercial protector per kilogram of seeds. After the inoculation, seeds were dried in the shade under ambient conditions and immediately sowed in inoculated plots.

We chose this season for planting due to the high incidence of rainfall and elevated temperatures, which stimulate seedling growth and pasture establishment (Supplementary File 1). During the experiment, plants were rainfed and subjected to seasonal changes in soil moisture to simulate the natural conditions in which pastures are often found in this region. The warming treatment (item 2.4) was initiated right after sowing and maintained 24 h per day until the end of the experimental period. Throughout the experiment, we performed four standardization clippings at 30 cm aboveground to simulate grazing and maintain plants at an adequate height for the warming system. After each standardization cut, we conducted N fertilization with urea as described by Pereira et al. (2021) to recover soil N and sustain maximum regrowth. In the second half of September 2023, the Midwest and Southeast regions of Brazil experienced an unprecedented heatwave, which increased air temperature up to 39.4 °C for two weeks. Consequently, as a precautionary measure, we conducted additional fertilization in all plots on day 269 after sowing with N-P-K (20-0-20) (150 kg ha⁻¹) to help pasture recovery.

2.3. Meteorological data acquisition

The climate conditions were monitored using an automatic weather station, WS-GP2 (Delta-T Devices, UK), located in the central area of the experimental site. During the experiment, air temperatures (T_{air}) were higher than expected based on the historical 30-year average, particularly in the latter half of the experimental period (see Supplementary File 1). For instance, in September, October, November, and December of 2023, the average T_{air} was 4.2 °C, 2.9 °C, 2.9 °C, and 3.1 °C warmer than the historical average, respectively, due to multiple heatwave events. Consequently, the experimental period was 1.5 °C warmer than anticipated and our additional experimental warming occurred during an already warm year. The average T_{air} , average maximum T_{air} , and average minimum T_{air} were 23.7 °C, 34.1 °C, and 14.3 °C, respectively. The total precipitation was 1688 mm, with the majority of rainfall events occurring during the summer (Supplementary File 1).

2.4. Warming treatment

Warming treatment (+2 °C above ambient temperature) was applied under field conditions by a Temperature Free-Air Controlled Enhancement (T-FACE) system (Supplementary file 2) during the entire experimental period as described by Kimball (2005) and Kimball et al. (2008). The T-FACE system is a component of the Trop-T-FACE facility that also includes a Free-air Carbon dioxide Enrichment system (FACE), located at the University of São Paulo, Ribeirao Preto Campus, Sao Paulo, Brazil (Fig. 1). In half of the plots of the T-FACE, plants were warmed by six 750 W IR heaters (FTE-750-240, Salamander, Mor Electric Heating, Comstock Park, MI, USA) placed at 0.8 m above the plant canopy in a hexagonal arrangement. Each heater was installed in an aluminum reflector Salamander ALEX (Mor Electric Heating, Comstock Park, MI, USA). For heating intensity control, T-FACE used a proportionalintegrative-derivative (PID) control system installed in a datalogger model CR1000 with an AM16/32B Multiplexer (Campbell Scientific, Logan, UT, USA). All plots were equipped with an infrared radiometer SI-1H1-L20 (Apogee Instruments, Logan, UT, USA) responsible for monitoring canopy temperature (Tcanopy). In summary, the T-FACE warming system increases the T_{canopy} of elevated temperature (eT) plots by +2 °C above the T_{canopy} of the ambient temperature (*aT*) plots. This is achieved by the infrared thermometers SI-1H1-L20, which continuously monitor the temperatures of both aT and eT plots, sending this information to a control unit. The control unit processes the data and adjusts the voltage supplied to the infrared heaters to maintain the +2 °C differential. The system dynamically regulates temperature in real-time: when the temperature difference between warmed and non-warmed plots is small, the heating intensity is low; when the difference is large, the heating intensity increases, continuously adjusting heater intensity to maintain the setpoint of a $+ 2 \degree C$ differential. Once the *eT* plot reaches the +2 °C target, the system maintains the heating intensity constant until the temperature of the *aT* plot changes, ensuring precise control. We installed the same aluminum structure and 'dummy' heaters in non-warmed plots. The warming treatment was applied within a circular area with a diameter of 2 m within each 16 m² plot (Supplementary File 2). Therefore, the T-FACE system warmed the pasture canopy to levels equivalent to those projected under the maximum threshold scenario of global warming outlined in the Paris Agreement.

We observed that throughout the experimental period, the average T_{canopy} under eT was approximately 1.82 ± 0.4 °C higher than under aT plots (see Supplementary File 1). The monthly average T_{canopy} values for aT and eT plots during the entire experimental period can be found in Supplementary File 1. This difference between the set point and observed warming can be attributed to factors such as wind speed, evapotranspiration, and vegetation density (Kimball, 2005; Kimball et al., 2008). During the heatwave event in September 2023, we recorded the maximum T_{canopy} of 45.3 °C and 48.2 °C in the aT and eT plots, respectively. The minimum T_{canopy} , recorded in June 2023, under aT and eT was 3.6 °C and 6.2 °C, respectively.

2.5. Experimental design

We tested two levels of temperature (ambient (aT) and elevated (eT)) and two levels of inoculation (inoculated (iN) plants and non-inoculated (nI) plants) in a multifactorial experimental design with four randomized blocks (n = 4). We combined both factors and levels in four treatments per block: non-inoculated plants under ambient temperature (nIaT), inoculated plants under ambient temperature (iNaT), noninoculated plants under elevated temperature (nIeT), and inoculated plants under elevated temperature (iNeT).

2.6. Leaf gas exchange

Leaf gas exchange measurements were conducted using two crosscalibrated portable photosynthesis system model LI-6800 (Li-Cor, Lincoln, NE, USA) equipped with a round 6 cm² cuvette. Measurements were taken from the central region of three fully expanded leaves per plot from 9:00 am to 1:00 pm. Leaves were placed into the cuvette and maintained until the stabilization of gas exchange parameters which took 10 to 30 min depending on plant, day of measurement, and treatment factors. We measured the gas exchange of one block per morning and the results were expressed as the four-day average of consecutive measurements, totaling four blocks. To perform the measurements, we controlled the following environmental conditions within the cuvette: $[CO_2]$ was set to 400 ppm, flux was set to 600 µmol m⁻² s⁻¹, relative air humidity was set to 65 %, leaf temperature to 30 °C, and radiation was set to 2000 μ mol m⁻² s⁻¹. We measured the net photosynthesis rate (A), stomatal conductance (gs), transpiration rate (E), and calculated the water-use efficiency (WUE) using the ratio A/g_s . The measurements were conducted on days 54, 142, 200, 262, 310, and 370 after sowing.

2.7. Chlorophyll fluorescence

The maximum quantum efficiency of PSII photochemistry (Fv/Fm) was measured in the adaxial leaf surface of six fully expanded leaves per plot at predawn (between 5 and 6 am) and midday periods (between 1 and 2 pm) using an OS-30p Chlorophyll Fluorometer (OptiScience, Hudson, NH, USA). The leaf region in which measurements were conducted was dark-adapted for 30 min using dark adaptation clips. To calculate the Fv/Fm ratio, we determined the variable fluorescence (Fv), where Fv = Fm-Fo; Fo is the minimum Chl fluorescence yield in the dark-adapted state and Fm is the maximum fluorescence after the light saturation pulse. Fv/Fm was measured on days 51, 116, 142, 199, 261, 277, 297, and 385 after sowing.

Alongside with gas exchange measurements conducted on days 142 and 370, chlorophyll fluorescence parameters were also measured by the portable photosynthesis system LI-6800 (Li-Cor, Lincoln, NE, USA) equipped with a multiphase flash fluorometer (6800-01 A). Lightadapted measurements were conducted together with gas exchange determination under radiation of 2000 μ mol m⁻² s⁻¹. We set the dark mode rate, light mode rate, and flash mode rate to 50 Hz, 1 kHz, and 250 kHz, respectively. We used the multiphase flash with a red target of 10,000 μ mol m⁻² s⁻¹. We set the dark pulse to 25 μ mol m⁻² s⁻¹ (FarRed target). Dark-adapted measurements were conducted in the same leaves used for light-adapted measurements with actinic light off at predawn after an overnight period. We measured the photochemical quenching (qP) according to the equation qP = (Fm' - Fs)/(Fm' - Fo'), where Fo' and Fm' are the minimum and maximum fluorescence in light-adapted samples, respectively, and Fs is the fluorescence at the dynamic equilibrium state of light-adapted samples. Non-photochemical quenching (NPQ) was calculated as NPQ = (Fm - Fm')/Fm', where Fm is the maximum fluorescence of dark-adapted samples. The maximum efficiency of PSII photochemistry in the light (Fv'/Fm') was calculated according to the equation: Fv'/Fm' = (Fm' - Fo')/Fm', where Fv' is the variable fluorescence in light-adapted samples. The proportion of open reaction centers (qL) was calculated as qP Fo'/Fs, while the proportion of closed reaction centers was calculated as 1-qL. Electron transport rate (ETR) was determined as ETR = 0.5 \times PSII yield \times PAR \times 0.84 μ equivalents $m^{-2} s^{-1}$.

2.8. Leaf water status

We collected three fully expanded leaves per plot to determine leaf relative water content (*RWC*). From each leaf, we excised 10 leaf discs with a diameter of 1.5 cm. Samples were taken from the central region of the leaf. Immediately, the discs were weighed to obtain the fresh weight (FW). Subsequently, the samples were submerged in distilled water and kept at 5 °C, in the absence of light. After 24 h, the discs had excess water removed and were weighed to obtain the turgid weight (TW). Later, the discs were dried in an oven at 60 °C until reaching a stable weight, thus obtaining the dry weight (DW). The calculation of *RWC* was performed according to Smart and Bingham (1974). *RWC* measurements were taken during the midday period (12m to 1:30 pm) on days 64, 116, 199, 366, and 373 after sowing.

2.9. Productivity

Plant material was collected at the same time of standardization clippings and data were used to estimate the annual productivity (ton ha⁻¹ year⁻¹). To estimate the forage productivity, we placed one quadrat of 1 m² per plot and harvested all plant material 30 cm above the ground. Leaves and stems were separated and dried in an oven at 60 °C until reaching a stable weight to obtain the dry weight. Using the dry mass of leaves and stems we calculated the leaf/stem ratio and using the total dry mass sampled 30 cm above the ground (stems + leaves) we estimated the productivity.

2.10. Root biomass

Root biomass was directly assessed by collecting soil core samples using a soil probe (Sondaterra, Brazil) with a volume of 424 cm^3 in each of the two soil depths (0–20 cm and 20–40 cm) on day 405 after sowing. From each plot, two subsamples were obtained, with the soil core probe consistently placed at 10 cm from the root crown, specifically at the 9 o'clock position relative to the plant (where 12 o'clock is North). This consistent sampling position aimed to minimize variability in the presence of roots in the soil core sample. The soil samples were then sieved (0.5 mm sieve) and washed in 20 % alcohol. Roots were dried in an oven at 60 °C until a constant mass to determine the root biomass in both depths.

2.11. Forage nutritive value and digestibility

The aboveground biomass collected from each standardization clipping was dried in an oven at 60 °C, powdered, and used for the determination of forage nutritional value and digestibility. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) content were measured according to the protocol proposed by Mertens (2002), using a fiber determination equipment model TE-149 (Tecnal, Piracicaba, BR). The crude protein content (CP) was determined using a nitrogen distiller model TE-0363 (Tecnal, Brazil), and CP values were calculated using the eq. CP = N × 6.25 (AOAC, 1990). Lignin content assessment involved subjecting the ADF fraction to acid hydrolysis with 72 % sulfuric acid (Silva and Queiroz, 2002). In vitro dry matter digestibility (IVDMD) was assessed following the method outlined by Tilley and Terry (1963), adapted for artificial rumen conditions by ANKOM® utilizing the "Daisy incubator" device from Ankom Technology (in vitro true digestibility).

2.12. Statistical analysis

We conducted a factorial analysis of variance (ANOVA) containing two factors (inoculation and temperature) with two levels each: inoculation (inoculated and non-inoculated plants) and temperature (ambient and elevated temperature) to test for the isolated effects of inoculation (I) and temperature (T) and for the interaction between both factors (I \times T) (P < 0.05). We used the Shapiro-Wilk test to analyze data normality. Data was log-transformed when necessary to satisfy ANOVA assumptions. We conducted Student t-tests to compare two average values when necessary (P < 0.05). Map from Fig. 1 was created using OGIS 3.34 software. We performed correlations between the parameters and determined the Pearson correlation coefficient (r) for each correlation. In this study, we considered a strong correlation when r > 0.7 and a moderate correlation when r was between 0.5 and 0.7. These analyses were conducted using GraphPad Prism version 10.3.1 for macOS (GraphPad Software, Boston, Massachusetts USA). Hierarchical cluster analysis was performed using the Euclidean distance coefficient and the single linkage method to connect the groups. Principal Component Analysis (PCA) was performed using a covariance matrix to investigate the interrelationships between variables and explain them in terms of their inherent dimensions (components). A two-dimensional plot (Biplot) was generated, representing observation points and vectors corresponding to the response variables. Hierarchical cluster and PCA analyses were investigated using the Python programming language (version 3.9.7; Python Software Foundation). To conduct the multivariate analyses, we used the average values of parameters measured throughout the experiment, such as gas exchange, fluorescence parameters, water status, nutritive value, and digestibility. For productivity and root dry mass, we used the final values.

3. Results

3.1. Leaf gas exchange

Overall, *iN* improved the gas exchange parameters of Mavuno grass during the experimental period (Fig. 2). Throughout the experimental period, we observed that regardless of the temperature level, the *iN* increased *A* by up to 15 % (P < 0.05) (Fig. 2A), an average gain of approximately 5 µmol m⁻² s⁻¹ of carbon fixed by the inoculated plants (Fig. 2A). On day 142 after sowing, g_s and *E* increased (P < 0.05) by 18 % and 22 %, respectively, under *iN* plots, regardless of the temperature level (Fig. 2B, C). Still, this g_s response was not consistent throughout the experiment. *E* also increased (P < 0.05) under *iN* regardless of the temperature level on day 370. *WUE* was not modified by treatments in any sampling or season averages (Fig. 2D). In summary, PGPB inoculation had a more significant effect on photosynthesis, stomatal conductance, and transpiration during the wet season, but water use efficiency was mainly influenced by seasonal changes rather than the PGPB inoculation treatment.

3.2. Chlorophyll fluorescence

During the predawn period, we observed that $Fv/Fm_{predawn}$ increased (P < 0.05) under *eT* regardless of the inoculation on days 116 and 142 (Fig. 3A). However, on day 199, both *iN* and *eT* increased (P < 0.05) $Fv/Fm_{predawn}$, resulting in a 7.7 % higher $Fv/Fm_{predawn}$ under *iNeT* when compared to *nIaT* (*Student t-test comparison*) (Fig. 3A). No significant impacts of treatments on $Fv/Fm_{predawn}$ were observed during the other samplings (Fig. 3A). During the midday period, the *iN* treatment regardless of the temperature level increased Fv/Fm_{midday} on day 116, but this response was not present in other samplings (Fig. 3B). During the dry season, *eT* decreased Fv/Fm_{midday} regardless the inoculation by approximately 6 % on days 199 and 261 after sowing (Fig. 3B).

Inoculation improved (P < 0.05) ETR by 15 % regardless of the temperature level on day 142 (Table 1). On day 370, both factors interacted, resulting in an increased ETR of 19.8 %, 20.8 %, and 21.8 % under *iNaT*, *nIeT*, and *iNeT*, respectively, compared to the *nIaT* treatment

(Table 1). We also observed that plants grown under *eT* regardless of the inoculation showed an increase (P < 0.05) of approximately 13 % and 20 % in NPQ on days 142 and 370, respectively (Table 1). In the first sampling, qP increased (P < 0.05) due to *iN* effects regardless of the temperature level. This increase was accompanied by a higher (P < 0.05) qL and consequently, a lower (P < 0.05) proportion of closed reaction centers (1-qL). In the second sampling date, qP increased (P < 0.05) under *eT* regardless of the inoculation presence (Table 1). Overall, we observed that *iN* promoted the most positive and significant (P < 0.05) effects on chlorophyll fluorescence parameters, whilst *eT* promoted mixed impacts depending on the time of day and season (Fig. 3, Table 1).

3.3. Plant water status

We observed that *eT* regardless of the inoculation decreased the *RWC* up to 8.4 % during the experiment (Fig. 4). On day 373 after sowing, an interaction between I \times T resulted in a similar *RWC* value between *nIaT* and *iNeT* (*Student t-test comparison*) (Fig. 4). The lowest *RWC* values was observed during periods of low precipitation, such as 199 and 366 days after sowing, reaching approximately 80 % under *eT* (Fig. 4).

3.4. Aboveground and belowground dry mass

While *iN* increased productivity by approximately 20 %, *eT* decreased biomass productivity by 28 %, resulting in intermediary but similar productivity between *nIaT* and *iNeT* treatments (*Student t-test comparison*) (Fig. 5A). For root dry mass, we observed that warming partially offset the increment in root dry mass promoted by *iN* between 0 and 20 cm of depth (Fig. 5B). No effects of treatments were detected between 20 and 40 cm of depth (Fig. 5C). Overall, inoculation and warming promoted antagonistic effects on aboveground productivity and root dry mass of Mavuno grass (Fig. 5A).

3.5. Forage nutritional quality and dry matter digestibility

Overall, warming and inoculation promoted antagonistic effects on



Fig. 2. Leaf gas exchange measured in leaves of *Brachiaria* Mavuno during the experimental period. (A) A = net photosynthesis rate. (B) g_s = stomatal conductance. (C) E = transpiration rate. (D) WUE = water use efficiency. Grey background indicates the dry season. White background indicates the wet season. Treatments: non-inoculated plants under ambient temperature (*nIaT*), inoculated plants under ambient temperature (*iNaT*), non-inoculated plants under elevated temperature (*nIeT*), and inoculated plants under elevated temperature (*iNeT*). Each bar is the average value of four replicates (n = 4). Error bars show the standard error of the mean. ANOVA results are shown for each factor: I: inoculation isolated effect, T: temperature isolated effect, and I × T: interaction between inoculation and temperature. ns = non-significative effects of any factor. *P < 0.05, **P < 0.01, ****P < 0.001,



Fig. 3. Maximum quantum efficiency of PSII photochemistry (Fv/Fm) measured in leaves of *Brachiaria* Mavuno at predawn (A) and midday (B). Grey background indicates the dry season. White background indicates the wet season. Treatments: non-inoculated plants under ambient temperature (*nIaT*), inoculated plants under ambient temperature (*iNaT*), non-inoculated plants under elevated temperature (*nIeT*). Each point is the average value of four replicates (n = 4). Error bars show the standard error of the mean.

Table 1

Chlorophyll fluorescence parameters obtained from *Brachiaria* Mavuno leaves grown under field conditions at two temperature levels and inoculation. ETR = electron transport rate. NPQ = non-photochemical quenching. Fv'/Fm' = maximum efficiency of PSII photochemistry in the light. qP = photochemical quenching. qL = proportion of open reaction centers. 1-qL = proportion of closed reaction centers. Each value represents the average of 4 replicates (n = 4) followed by its respective standard error of the mean. The result of the two-way ANOVA analysis for each sampling and variable is shown in the last column. I: Isolated effect of inoculation. T: Isolated effect of temperature. IxT: Interaction between factors. * = P < 0.05, ** = P < 0.01. ns = no significant effects. Treatments: non-inoculated plants under ambient temperature (*iNaT*), non-inoculated plants under elevated temperature (*nIeT*), and inoculated plants under elevated temperature (*iNeT*).

Variable	nIaT	iNaT	nIeT	iNeT	ANOVA
142 days after sow	ving				
ETR	134.4 ± 8	169.7 ± 5	156.4 ± 3	165.1 ± 8	I **
NPQ	2.87 ± 0.11	$\textbf{2.42} \pm \textbf{0.25}$	3.19 ± 0.01	$\textbf{2.94} \pm \textbf{0.14}$	T *
Fv`/Fm`	0.42 ± 0.01	0.41 ± 0.01	0.42 ± 0.01	0.41 ± 0.01	ns
qP	0.40 ± 0.03	0.46 ± 002	0.44 ± 0.01	0.49 ± 0.01	I *
qL	0.28 ± 0.02	0.36 ± 0.03	0.31 ± 0.01	0.36 ± 0.01	I *
1-qL	$\textbf{0.72} \pm \textbf{0.01}$	$0.64\pm0.0.03$	0.69 ± 0.01	$\textbf{0.64} \pm \textbf{0.01}$	I *
370 days after sowing					
ETR	206.1 ± 11	251.5 ± 7	254 ± 12	256.6 ± 8	I * T * IxT *
NPQ	1.73 ± 0.24	1.2 ± 0.06	2.3 ± 0.14	2.3 ± 0.21	Т *
Fv`/Fm`	0.62 ± 0.02	0.63 ± 0.01	0.62 ± 0.03	0.59 ± 0.03	ns
qP	0.42 ± 0.03	0.47 ± 0.02	0.49 ± 0.01	0.51 ± 0.03	T *
qL	0.22 ± 0.3	0.25 ± 0.02	0.27 ± 0.02	0.31 ± 0.04	ns
1-qL	$\textbf{0.78} \pm \textbf{0.03}$	0.75 ± 0.02	0.73 ± 0.02	$\textbf{0.69} \pm \textbf{0.04}$	ns

the nutritional quality and digestibility of Mavuno grass (Fig. 6). Leaf/ stem ratio was not modified by treatments in the first three growing cycles but increased under eT regardless of the inoculation level in the last standardization clipping (Fig. 6A). iN improved CP by either mitigating the negative impacts of eT or independently increasing CP levels by up to approximately 38 % (Fig. 6B). Similar to CP results, the increases in NDF and ADF promoted by eT were completely counteracted by *iN*, resulting in similar (P > 0.05) NDF and ADF values between *nIaT* and iNeT (Student t-test comparison) or decreased NDF and ADF under iN regardless of the temperature level (Fig. 6C, D). Lignin was not modified by treatments in the first growing cycle. However, lignin content decreased under iN regardless of the temperature level by approximately 22 % and 18 % in the second and third growing cycles, respectively. In the fourth growing cycle, an interaction between $I \times T$ mitigated the increase in lignin promoted by eT, resulting in similar lignin values between nIaT and iNeT (Student t-test comparison) (Fig. 6E).

In the first growing cycle, we observed that inoculation completely mitigated the decrease in IVDMD promoted by *eT*, resulting in similar (P > 0.05) IVDMD values between *nIaT* and *iNeT* (*Student t-test comparison*). In the second growing season, an interaction between I × T resulted in

similar (P > 0.05) IVDMD values between *nIaT* and *iNeT* (*Student t-test comparison*). However, in the third growing cycle, IVDMD increased under *iN* regardless of the temperature level by approximately 8 %. In the fourth growing cycle, IVDMD increased under *iN* depending on the temperature level (Fig. 6F).

Our correlation analysis (Supplementary File 3) showed significant (P < 0.05) correlations between the parameters (Fig. 7). We found positive and strong (r > 0.7) correlations between $g_s \times A$, $E \times A$, $E \times g_s$, RDM0–20 × Fv/Fm_{predawn}, ADM × Fv/Fm_{midday}, qP × ETR, qL × qP, IVDMD × CP, NDF × ADF, lignin × ADF, and lignin × NDF. Negative and strong (r > 0.7) correlations were found between 1-qL × ETR, qL × ETR, 1-ql × qP, 1-qL × qL, lignin × ADM, ADF × CP, NDF × CP, lignin × CP, IVDMD × ADF, IVDMD × NDF, and IVDMD × lignin (Fig. 7).

The hierarchical cluster analysis indicated that temperature and inoculation grouped the plants into two clusters according to the inoculation factor. The inoculated plants were grouped regardless of the temperature condition and were in a distinct cluster from the non-inoculated plants (Fig. 8). Under the *nIaT*, increases in 1-qL were highly related to this treatment. At the same time, variables such as *A*, NPQ, ETR, qP, and RDM0–20, showed a low relationship with this



Fig. 4. Relative water content (*RWC*) measured in leaves of *Brachiaria* Mavuno during the experimental period. Treatments: non-inoculated plants under ambient temperature (*nIaT*), inoculated plants under ambient temperature (*iNaT*), non-inoculated plants under elevated temperature (*nIeT*), and inoculated plants under elevated temperature (*iNeT*). Each bar is the average value of four replicates (n = 4). Error bars show the standard error of the mean. ANOVA results are shown for each factor: I: inoculation isolated effect, T: temperature isolated effect, and I × T: interaction between inoculation and temperature. ns = non-significative effects of any factor. *P < 0.05, **P < 0.01, ***P < 0.001.

treatment. Under *iNaT*, increases in parameters such as ETR, RDM0–20, CP, IVDMD, ADM, and *A* showed a high relationship with this growth condition and a low relationship with NDF, ADF, and lignin (Fig. 8). On the other hand, increases in parameters such as NDF, ADF, lignin, and NPQ were highly related to *nIeT* treatment. Meanwhile, *iNeT* was highly related to increases in the parameters ETR, *E*, qP, qL, and IVDMD (Fig. 8).

PCA was performed to visualize the relationships between measured variables and to examine the distribution of treatments in relation to these variables (Fig. 9). The first two principal components (Dim1 and Dim2) accounted for 59.8 % of the total variance in the studied parameters with Dim1 explaining 34.3 % and Dim2 explaining 25.5 % of the variance. The parameter 1-qL contributed the most to explaining the variance and was strongly associated with the *nIaT* treatment, while it showed an antagonistic relationship with qP, RDM0–20, ETR, and qL. These last four variables were more closely linked to the *iNeT* treatment, along with *A* and *E*, which also demonstrated positive interrelations. In

contrast, CP, IVDMD, ADM, Fv/Fm midday, g_s, and Fv'/Fm' were highly associated with plant responses under *iNaT*. The increases in CP, IVDMD, and ADM were negatively correlated with lignin, NDF, and ADF, which were strongly linked to plants under *nIeT* treatment (Fig. 9).

4. Discussion

In this study, we demonstrated that inoculation of *Brachiaria* Mavuno with *A. brasilense* and *P. fluorescens* effectively mitigated warming effects in plants grown under elevated temperature conditions (+2 °C), supporting our hypothesis. Inoculated plants maintained higher gas exchange and photosynthetic rates, and promoted enhanced growth compared to non-inoculated plants. These findings suggest that the inoculation of plant growth-promoting bacteria not only mitigated the negative impacts of warming on plant performance but also enhanced the nutritional quality of tropical forage. Our results highlight the potential of inoculation as a sustainable strategy to enhance climate resilience in tropical pastures, particularly during atypically warm years.

Our results demonstrated that inoculation significantly enhanced the photosynthetic performance of Mavuno grass, as evidenced by gas exchange and chlorophyll fluorescence analysis (Figs. 2, 3, Table 1). The observed improvements in photosynthesis align with previous studies where the inoculation of plant growth-promoting bacteria has promoted chlorophyll accumulation through higher absorption of N and P, resulting in a more efficient photosynthetic apparatus. For instance, Guimarães et al. (2023) showed that in Megathyrsus maximus (Poaceae, C₄), depending on the cultivar, inoculation of A. brasilense and P. fluorescens increased the content of N, P, Mg, S, B, Fe, Ca, Mn, and Zn. Similarly, in maize, studies using radioactive ⁵²Mn²⁺ demonstrated that A. brasilense facilitated Mn uptake and assimilation, leading to enhanced photosynthesis (Housh et al., 2022). This effect was presumably due to the increased Mn in the leaves, an essential cofactor in the oxygenevolving complex. In our study, we observed that both metabolic and stomatal factors likely contributed to improved photosynthesis, as indicated by the strong positive correlation between stomatal conductance \times net photosynthesis rate and transpiration rate \times net photosynthesis rate (Fig. 7). Higher stomatal conductance allows for greater CO₂ diffusion into the mesophyll, enhancing photosynthetic rates and overall plant growth (Jiang et al., 2006).

Our results demonstrated that the performance of photosystem II (PSII) was affected by inoculation and warming (Fig. 3, Table 1). Both factors modified the maximum quantum efficiency of PSII photochemistry during predawn and midday on certain sampling dates. The



Fig. 5. Aboveground productivity (A) and root dry mass between 0 and 20 cm (B) and 20–40 cm of depth (C) obtained from samples collected at the end of the experimental period. Treatments: nI (non-inoculated plants), iN (inoculated plants), aT (ambient temperature), and eT (elevated temperature, +2 °C above ambient). Each bar is the average value of four replicates (n = 4). Error bars show the standard error of the mean. ANOVA results are shown for each factor: I: inoculation isolated effect, T: temperature isolated effect, and I × T: interaction between inoculation and temperature. ns = non-significative effects of any factor. *P < 0.05, **P < 0.01, ***P < 0.001.



Fig. 6. Forage quality and digestibility parameters obtained from samples collected during the experimental period. Treatments: non-inoculated plants under ambient temperature (*nIaT*), inoculated plants under ambient temperature (*iNaT*), non-inoculated plants under elevated temperature (*nIeT*), and inoculated plants under elevated temperature (*iNeT*). CP = crude protein content. NDF = neutral detergent fiber. ADF = acid detergent fiver. IVDMD = in-vitro dry matter digestibility. Each bar is the average value of four replicates (n = 4). Error bars show the standard error of the mean. ANOVA results are shown for each factor: I: inoculation isolated effect, T: temperature isolated effect, and I × T: interaction between inoculation and temperature. ns = non-significative effects of any factor. *P < 0.05, **P < 0.01, ***P < 0.001.

maximum quantum efficiency of PSII photochemistry estimates the maximum efficiency at which light absorbed by PSII is used for the reduction of quinone Q_A during photosynthesis after a dark-adaptation period, with values typically remaining around 0.78–0.84 for non-stressed leaves (Baker, 2008; Saccon et al., 2022). The PSII reaction center, mainly the protein subunit D1, is one of the most susceptible structures to ROS in the thylakoid membrane, especially in conditions of high irradiance and other stressful conditions such as drought or heat stress (Nath et al., 2013). Therefore, a set of molecular mechanisms evolved to maintain the integrity of P680 during the diurnal course and dynamically adjust the amount of energy absorbed or dissipated as heat by the non-photochemical process (Ruban, 2016). Throughout the day,

both processes - non-photochemical quenching (Ruban, 2016) and the D1 repair cycle (Nixon et al., 2010) - continuously function to maintain a high PSII quantum yield, especially under the intense conditions of midday when temperature and irradiance are elevated (Saccon et al., 2022). However, any abiotic or biotic factors can disrupt the balance between D1 degradation and the repair cycle (Saccon et al., 2022; Arief et al., 2023). In this study, we demonstrated that *iN* slightly increased the maximum quantum efficiency of PSII photochemistry at predawn and midday (Fig. 3), suggesting its role in enhancing D1 turnover and partially mitigating the impacts of midday stress. Warming also improved the maximum quantum efficiency of PSII photochemistry at predawn but had a negative effect during the dry season at midday,



Fig. 7. Heatmap of Pearson correlation coefficient (r) between parameters obtained from plants of *Brachiaria* Mavuno. The range from -1.0 to 1.0 represents the degree of correlation between the variables, where 1.0 (blue) indicates a strong positive correlation, -1.0 (red) indicates a strong negative correlation, and 0 (white) indicates no correlation. Squares without a number indicate that the value is in the thousandths place. The *P*-values of each correlation can be found in Supplementary File 3. *A* = net photosynthesis rate. g_s = stomatal conductance. *E* = transpiration rate. *WUE* = water use efficiency. Fv/Fm predawn = maximum quantum efficiency of PSII photochemistry measured at predawn. RWC = relative water content. ADM = aboveground productivity. RDM0–20 = root dry mass between 0 and 20 cm of depth. RDM20–40 = root dry mass between 20 and 40 cm of depth. CP = crude protein content. NDF = neutral detergent fiber. ADF = acid detergent fiver. IVDMD = in-vitro dry matter digestibility. ETR = electron transport rate. NPQ = non-photochemical quenching. Fv'/Fm' = maximum efficiency of PSII photochemical quenching. qL = proportion of open reaction centers. 1-qL = proportion of closed reaction centers. Asterisks (*) indicate statistically significant correlations: *P < 0.05, **P < 0.01, ***P < 0.001.

demonstrating eT interaction with drought (Fig. 3). The presence of midday negative impacts, with no concomitant effects on the maximum quantum efficiency of PSII photochemistry at predawn, suggests that plants can recover after an overnight period from transient stresses under eT during the diurnal cycle.

The positive effects of *iN* on PSII functioning were particularly evident under low rainfall conditions (with an accumulated rainfall of 19.7 mm in the sampling month on day 142), but less intense in the wet season on day 370 (with a monthly accumulated rainfall of 115.3 mm) (Supplementary file 1, Table 1). On day 142, photochemical quenching and the proportion of open reaction centers increased under *iN* regardless of the temperature level during the dry season (Table 1), suggesting that under *iN* a greater proportion of captured energy is being used in the

electron transport chain. A higher proportion of open reaction centers indicates that *iN* promotes a higher degree of Q_A in the oxidized state (Baker, 2008). This suggests that the electron transport chain is operating efficiently due to the *iN* effect, ensuring an efficient Q_A turnover and sustained photochemical processes (Murchie and Lawson, 2013; Saccon et al., 2022). Moreover, the proportion of closed reaction centers decreased under *iN* regardless of the temperature level (Table 1). The proportion of closed reaction centers is a proxy for the redox state of the plastoquinone pool (Baker, 2008). This result suggests that the electron transport chain of inoculated plants is working efficiently without excessive buildup of reduced Q_A , resulting in a favorable redox balance. As a result, electron transport rate also increased under *iN* regardless of the temperature level during the dry season (Table 1). Electron transport



Fig. 8. Heat map of the hierarchical clustering of parameters obtained during the experimental period. Treatments: non-inoculated plants under ambient temperature (*nIaT*), inoculated plants under ambient temperature (*iNaT*), non-inoculated plants under elevated temperature (*nIaT*), and inoculated plants under elevated temperature (*iNeT*). The range from 0.0 to 1.0 indicates similarity between the variables, where 1.0 is the highest similarity and 0.0 is the highest dissimilarity. A = net photosynthesis rate. $g_s =$ stomatal conductance. E = transpiration rate. WUE = water use efficiency. Fv/Fm predawn = maximum quantum efficiency of PSII photochemistry measured at predawn. RWC = relative water content. ADM = aboveground productivity. RDM0–20 = root dry mass between 0 and 20 cm of depth. RDM20–40 = root dry mass between 20 and 40 cm of depth. CP = crude protein content. NDF = neutral detergent fiber. ADF = acid detergent fiver. IVDMD = invitro dry matter digestibility. ETR = electron transport rate. NPQ = non-photochemical quenching. Fv'/Fm' = maximum efficiency of PSII photochemistry in the light. qP = photochemical quenching. qL = proportion of open reaction centers. 1-qL = proportion of closed reaction centers.

rate quantifies the total flux of electrons through PSII, which are utilized by electron sinks from linear electron flow (such as CO_2 assimilation and photorespiration, the latter being suppressed in C_4 plants), as well as alternative pathways including the Mehler reaction, cyclic electron flow at PSI and PSII, and nitrate assimilation (Suorsa, 2015). Overall, these findings indicate an improvement in PSII function under *iN*, leading to higher assimilation rates, as evidenced by the strong correlation between net photosynthesis rate and photochemical quenching, electron transport rate, and maximum quantum efficiency of PSII photochemistry (Fig. 7).

During the wet season, both iN and eT individually increased

electron transport rate, and when combined, their interaction further increased electron transport rate to a higher value than those achieved under *iN* or *eT* alone (Table 1). In addition to the increase in photochemical quenching under *eT* regardless of the inoculation presence, these results suggest that the effect of *eT* on Mavuno grass PSII is improved by increased water availability. Earlier studies conducted with the tropical forage grass Mombaça grass (*Megathyrsus maximus*) also demonstrated the positive effects of 2 °C warming on plant nutrient status and productivity only under well-watered conditions (Viciedo et al., 2021). In our study, non-photochemical quenching increased under *eT* regardless of the inoculation in both seasons (Table 1). Non-



Fig. 9. Two-dimensional scatter plot of Principal Component Analysis (PCA) based on parameters collected during the experimental period. Non-inoculated plants under ambient temperature (*nIaT*), inoculated plants under ambient temperature (*iNaT*), non-inoculated plants under elevated temperature (*nIeT*), and inoculated plants under elevated temperature (*iNeT*). The range from 0.0 to 1.0 indicates similarity between the variables, where 1.0 is the highest similarity and 0.0 the highest dissimilarity. *A* = net photosynthesis rate. *g_s* = stomatal conductance. *E* = transpiration rate. *WUE* = water use efficiency. Fv/Fm predawn = maximum quantum efficiency of PSII photochemistry measured at predawn. RWC = relative water content. ADM = aboveground productivity. RDM0–20 = root dry mass between 0 and 20 cm of depth. RDM20–40 = root dry mass between 20 and 40 cm of depth. CP = crude protein content. NDF = neutral detergent fiber. ADF = acid detergent fiver. IVDMD = in-vitro dry matter digestibility. ETR = electron transport rate. NPQ = non-photochemical quenching. Fv'/Fm' = maximum efficiency of PSII photochemistry in the light. qP = photochemical quenching. qL = proportion of open reaction centers. 1-qL = proportion of closed reaction centers.

photochemical quenching estimates the proportion of energy that is being dissipated as heat from PSII and represents one of the most important protective mechanisms in avoiding the overexcitation of PSII (Ruban and Wilson, 2020). The non-photochemical quenching process is complex and involves the conversion of the carotenoids violaxanthin to zeaxanthin in the xanthophyll cycle, changes in the regulatory protein PsbS, and the acidification of the thylakoid lumen (Demmig-Adams et al., 2014; Ruban and Wilson, 2020). Therefore, this response is presumably associated with the acclimation of this species under a warmer atmosphere, a response that has been observed in *Stylosanthes capitata* (Fabaceae, C₃) pasture (Martinez et al., 2014; Habermann et al., 2019). However, it is important to note that although the non-photochemical quenching mechanism protects the PSII, the excessive energy dissipation as heat may reduce the amount of energy available for C fixation, potentially limiting growth under these conditions.

In fact, warming reduced aboveground productivity, but this negative effect was completely offset by inoculation (Fig. 5A). Biomass accumulation is a complex process resulting from cumulative physiological changes and translocation processes occurring throughout the entire growing season (Demura and Ye, 2010). Under *aT*, *iN* increased annual productivity by approximately 20 %, which corroborates the findings reported by Hungria et al. (2016) where the inoculation of two *A. brasilense* strains enhanced the productivity of *Brachiaria brizanta* (cv. Marandu) and *Brachiaria ruziziensis*. These results are likely linked with the improved performance of PSII (Fig. 3, Table 1), carbon fixation (Fig. 2A), root growth (Fig. 5B), and soil N availability under *iN* plots, which led to greater availability of substrates for investment in aboveground growth, root development, and protein synthesis. On the other hand, we demonstrated that warming had adverse effects on productivity, presumably due to the negative effects of *eT* on PSII, either by reducing the maximum quantum efficiency of PSII photochemistry at midday (Fig. 3B) or by increasing energy loss as heat (Table 1).

Infrared warming induces a phenomenon known as "parallel moisture manipulation", where the soil moisture in warmed plots decreases due to an increase in vapor pressure deficit, which intensifies soil evapotranspiration, resulting in secondary effects on plants (Kimball, 2005). Although we did not monitor soil moisture levels in this study, relative water content (Fig. 4) serves as a proxy for plant water status, being positively correlated with soil moisture (Ali et al., 2023; Levinsh, 2023). Cell turgidity is essential for cell expansion, photosynthesis, enzymatic activity, ionic balance, growth, and productivity (Ali et al., 2023). Without sufficient turgor pressure, cells are unable to expand properly, resulting in impaired growth, wilting, and overall reduced plant development (Ali et al., 2023). We hypothesize that the smaller relative water content under eT (Fig. 4) is part of the negative impacts that resulted in smaller productivity at *nIeT* plots, as corroborated by the positive correlations between relative water content \times the maximum quantum efficiency of PSII photochemistry at midday and aboveground dry mass production \times relative water content (Fig. 7). However, *iN* fully counteracted the eT impacts on productivity, resulting in similar aboveground biomass between *nIaT* and *iNeT* treatments (Fig. 5A). This result is likely linked to the improved photosynthetic performance under iN plots and root growth, which mitigated the warming effects (Fig. 5B).

In this study, iN increased root biomass in the topsoil profile (0-20 cm of depth), but this effect was partially offset by warming. The enhanced root growth observed is a well-documented response to inoculation with A. brasilense, which is known to promote root development through the production of indole-3-acetic acid (IAA) and gibberellins (Saharan and Nehra, 2011; Hungria et al., 2021). This root expansion is critical for improved soil exploration, which enhances plant's resilience to environmental stresses like water deficits (Lynch et al., 2021). Our findings are supported by documented effects of A. brasilense on root architectural traits such as increased root elongation, branching, root hair density, and root biomass accumulation (Lopes et al., 2011; Hungria et al., 2021, 2022). Moreover, the role of P. fluorescens in increasing root branching and root dry mass (Hungria et al., 2021; Guimarães et al., 2023) further supports our observations of improved root biomass in iN plots, suggesting that co-inoculation with both taxa enhances the plant's capacity to absorb water and nutrients under stress conditions. Mechanistically, this root growth may be attributed to the increased availability of substrates resulting from enhanced photosynthesis (Fig. 2A, Table 1) and to the synthesis of nitric oxide by A. brasilense, which plays a signaling role in auxin pathways that promote lateral root formation, as seen in tomato (Lycopersicon esculentum) plants (Creus et al., 2005). Similarly, P. fluorescens synthesizes cyclodipeptides that regulate auxin-controlled genes in Arabidopsis thaliana (Ortiz-Castro et al., 2020). Here, we demonstrated that the coinoculation not only stimulated root biomass but also increased the tolerance of Mavuno grass to elevated temperatures. This underscores the potential for microbial inoculants to play a vital role in sustainable agricultural practices under changing climatic conditions.

Beyond the impacts of *iN* and *eT* on plant physiology and growth, our results showed a consistent antagonistic effect of both factors on forage nutritive value (Fig. 6). A desired diet for ruminants includes forages with sufficient yield, high leaf/ stem ratio, adequate protein content, reduced fiber levels, and high digestibility (Chase, 2021). Here, we demonstrated that *iN* enhanced crude protein content and completely counteracted the negative effects of *eT* on this parameter (Fig. 6B). Proteins are essential for ruminant nutrition, providing nitrogen and amino acids for the growth of rumen microorganisms, which optimize fermentation and nutrient utilization (Waghorn and Clark, 2004). Furthermore, an adequate protein supply is critical for milk and meat production, as well as for the immune system and overall animal health (Waghorn and Clark, 2004; Chase, 2021; Dias et al., 2022). This

outcome is likely linked to the higher N availability resulting from biological N fixation in the *iN* plots, as crude protein content is calculated based on the assumption that proteins contain, on average, 16 % of N in their composition (AOAC, 1990).

In this study, iN decreased forage fiber fraction (neutral and acid detergent fiber) and completely mitigated the increase induced by eT(Fig. 6C,D). Neutral detergent fiber fraction includes all hemicellulose, cellulose, and lignin of the forage, and higher values are associated with lower voluntary intake because the forage occupies more volume in the rumen, decreasing the animal consumption of food (Ball et al., 2001; Chase, 2021). Acid detergent fiber fraction includes all cellulose and lignin of the forage material and higher values are associated with decreased digestibility of plant material (Ball et al., 2001; Chase, 2021). Moreover, iN decreased lignin content and fully offset the increase caused by *eT* (Fig. 6E). Lignin is an indigestible hydrophobic polymer that forms bound with cellulose and hemicellulose, reducing the accessibility of digestive enzymes and microorganisms of the rumen to carbohydrates and cellular content (Moore and Jung, 2001). Corroborating our results (Fig. 6E), evidence indicates that elevated temperature increases the lignification process of cells, resulting in higher lignin forage content (Lee et al., 2017). Therefore, *iN* enhanced forage nutritive value under a warmer atmosphere, highlighting its potential as an effective strategy for supporting future food security.

The changes in forage chemical composition resulted in a modified pattern of in vitro dry matter digestibility (Fig. 6F). Digestibility directly affects the amount of energy that animals obtain from forage, consequently impacting animals' growth, health, meat production, and food security. Therefore, a high level of forage digestibility is associated with increased meat and milk production (Chase, 2021). Ruminants account for 29 % of global meat production and are the primary source of milk (Gerber et al., 2013). As expected, in vitro dry matter digestibility decreased due to eT effects, presumably due to the increased levels of neutral detergent fiber, acid detergent fiber, and lignin (Fig. 6C, D, E), as evidenced by the strong and negative correlation between in vitro dry matter digestibility \times acid detergent fiber, neutral detergent fiber, and lignin (Fig. 6). iN increased in vitro dry matter digestibility presumably due to the increased crude protein content (Fig. 6B) and reduced acid detergent fiber, neutral detergent fiber, and lignin content (Fig. 6C, D, E) as suggested by the correlations between these parameters (Fig. 7). When combined under *iNeT* treatment, *iN* completely offset the impacts of eT and increased the final in vitro dry matter digestibility under a warmer atmosphere (Fig. 6F). Here, we showed that changes in forage nutritive value and digestibility were independent of leaf/ stem ratio slightly changes during the growing season (Fig. 6), suggesting that the chemical composition of plant material, instead of proportion of leavesto stem, was the main factor in determining pasture nutritive value and digestibility. Moreover, forages with high nutritive quality are associated with fewer emissions of CH4 because plant material takes less time to be digested by ruminants (Lee et al., 2017). This is significant because CH4 has a global warming potential that is 28 to 36 times greater than that of CO₂ (U.S. Environmental Protection Agency (EPA), 2015). CH₄ is produced in the rumen by methanogenic Archaea as a byproduct of feed fermentation and currently accounts for 25 % of global anthropogenic CH4 emissions (U.S. Environmental Protection Agency (EPA), 2015). According to Ellis et al. (2007), a 10 % increase in the NDF is expected to rise daily CH4 emission by 20 %. Therefore, our results indicate that iN can act not only by decreasing N₂O emissions but also by reducing the amount of CH₄ emitted by animals.

Overall, cluster analysis and PCA elucidate the contrasting responses of plant traits to inoculation and warming, revealing that microbial inoculation not only enhances photosynthetic performance but also improves biomass quality by reducing structural compounds that hinder digestibility (Figs. 8, 9). This highlights the detrimental impact of environmental stress on forage quality and emphasizes the potential of microbial inoculants to mitigate these effects. The interconnection of photosynthetic, growth, and nutritive quality parameters with *iNeT* underscores the beneficial effects of inoculation combined with warming, indicating enhanced photosynthetic capacity and improved root growth that may support better overall plant growth. Moreover, the *iNaT* treatment showed strong associations with crude protein content, in vitro dry matter digestibility, and aboveground dry mass, reflecting an advantageous balance between biomass production and nutritional quality. The negative correlation of these variables with lignin, neutral detergent fiber, and acid detergent fiber, which were prevalent under the *nIeT* treatment, suggests that the absence of inoculation during warming led to increased lignification and reduced forage digestibility (Figs. 8, 9).

Although significant progress has been made over the past decades in understanding the mitigation effects of inoculation on plants under abiotic stress in controlled conditions, field studies remain scarce (Bashan and de-Bashan, 2010). Notably, no studies on field warming have been reported in the literature. Our study highlights the significant role of microbial inoculation in enhancing the resilience of Brachiaria Mavuno to elevated temperatures under field conditions. As observed here and in other studies (Bashan and de-Bashan, 2010; Fukami et al., 2018a), the stimulation of plant growth and mitigation of abiotic stress through inoculation is not attributed to a single mechanism but rather to a combination of several mechanisms, collectively known as the "Multiple Mechanisms Theory". In this study, the co-inoculation with A. brasilense and P. fluorescens effectively mitigated the adverse effects of warming through improved photosynthetic performance, increased root biomass, and enhanced nutritional quality of the forage. These findings underscore the potential of microbial inoculants as sustainable strategies for maintaining forage productivity and quality in the context of climate change.

5. Conclusion

We concluded that the co-inoculation of Azospirillum brasilense and Pseudomonas fluorescens significantly improved the photosynthetic efficiency, root growth, productivity, and forage nutritional quality and digestibility of Brachiaria Mavuno pastures. Inoculation effectively mitigated the negative impacts of warming on plants, suggesting that inoculation of plant growth-promoting bacteria is a sustainable strategy to offset global warming impacts on tropical pastures. Plants acclimated to warming conditions primarily by dissipating heat from PSII through non-photochemical quenching. These findings highlight the potential of microbial inoculants in enhancing forage resilience and productivity under climate stress. To further elucidate the role of inoculation of plant growth-promoting bacteria in mitigating elevated temperature effects, long-term studies using field warming technologies are necessary. Integrating microbial inoculants into pasture management can support sustainable agricultural practices and improve food security in the face of climate change.

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CRediT authorship contribution statement

Eduardo Habermann: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. Beatriz Neroni Riul: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. Fábio Henrique Moscardini Nóbile: Writing – review & editing, Validation, Methodology, Investigation, Data curation. Ramon Martins Santana: Writing – review & editing, Validation, Methodology, Investigation, Data curation. Ramon Martins Santana: Writing – review & editing, Validation, Methodology, Investigation, Data curation. Kamilla Silva Oliveira: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. Bruno de Souza Marques: Writing – review & editing, Validation, Methodology, Investigation, Data curation. Eduardo Augusto Dias de Oliveira: Writing – review & editing, Validation, Methodology, Investigation, Data curation. Roberto Botelho Ferraz Branco: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Kátia Aparecida de Pinho Costa:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Mariangela Hungria:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Data curation. **Marco Antônio Nogueira:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Data curation. **Carlos Alberto Martinez:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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