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MARILEY DE CÁSSIA DA FONSECA

SYNERGISTIC EFFECTS OF FOLIAR AND SOIL INOCULATION WITH PLANT GROWTH-PROMOTING BACTERIA: ENHANCING SUGARCANE METABOLISM, PRODUCTIVITY, AND RHIZOSPHERE MODULATION

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.”

Arthur Schopenhauer

ABSTRACT

Sugarcane (*Saccharum spp.*) is a crop of great importance, playing a strategic role in the production of sugar, bioenergy, and biofuels. However, its intensive cultivation, particularly in tropical environments, faces challenges related to low nitrogen (N) use efficiency, high nutritional demand, and environmental impacts resulting from the application of synthetic fertilizers. In this context, the inoculation with plant growth-promoting bacteria (PGPB) has emerged as a promising alternative to increase productive efficiency, reduce fertilizer losses, and mitigate the adverse effects of environmental stress. This study uniquely investigated the effects of inoculation with *Azospirillum brasilense* (*Ab*), applied via foliar spraying, and *Nitrospirillum amazonense* (*Na*), applied to the soil, either individually or in consortium, on the physiological, nutritional, biochemical, and productive aspects of sugarcane. The experiments were conducted under greenhouse conditions and validated in the field. Results demonstrated that inoculation, particularly with *Ab* and the consortium (*Ab*+*Na*), enhanced the nutritional status of plants, especially by increasing N content. These improvements were reflected in favorable biochemical responses, such as reduced oxidative stress and enhanced photosynthetic parameters, water-use efficiency, and carboxylation efficiency, ultimately leading to higher stalk and sugar yields. In addition, inoculation modulated N dynamics within the soil–plant system by increasing N-fertilizer recovery, reducing losses through nitrification and denitrification, and enhancing the abundance of the *nifH* gene associated with biological N fixation. Changes in microbial community composition were also observed, with emphasis on the PGPB consortium, which promoted a more uniform structure functionally associated with improved nutrient-use efficiency. The findings of this study reinforce the potential of combined foliar (*Ab*) and soil (*Na*) inoculation as a sustainable strategy to intensify sugarcane production, promoting agronomic gains while contributing to reduced environmental impacts. The integration of physiological, nutritional, and microbial responses highlights the importance of biological management in the transition toward more efficient and resilient agricultural systems.

Keywords: *Azospirillum brasilense*; nitrogen cycle; *Nitrospirillum amazonense*; agricultural sustainability.

RESUMO

A cana-de-açúcar (*Saccharum spp.*) é uma cultura de grande importância, com papel estratégico na produção de açúcar, bioenergia e biocombustíveis. No entanto, seu cultivo intensivo, especialmente em ambientes tropicais, enfrenta desafios relacionados à baixa eficiência no uso de nitrogênio (N), elevada demanda nutricional e impactos ambientais decorrentes da aplicação de fertilizantes sintéticos. Nesse contexto, a adoção da inoculação com bactérias promotoras de crescimento de plantas (BPCP), tem emergido como alternativa promissora para aumentar a eficiência produtiva, reduzir perdas de fertilizantes e mitigar os efeitos adversos do estresse ambiental. Este trabalho investigou, de forma inédita, os efeitos da inoculação com *Azospirillum brasilense* (*Ab*), via foliar, e *Nitrospirillum amazonense* (*Na*), via solo, aplicados isoladamente ou em consórcio, sobre aspectos fisiológicos, nutricionais, bioquímicos e produtivos da cana-de-açúcar. Os estudos foram conduzidos em casa de vegetação e validados em campo. Os resultados demonstraram que a inoculação, especialmente com *Ab* e o consórcio (*Ab*+*Na*), promoveu melhorias no estado nutricional, com destaque para o aumento do teor de N nas plantas. Tais incrementos refletiram-se em respostas bioquímicas favoráveis, como a redução do estresse oxidativo e o aprimoramento de parâmetros fotossintéticos, eficiência de uso da água e da carboxilação, culminando em elevação da produtividade de colmos e de açúcar. Adicionalmente, a inoculação modulou a dinâmica do N no sistema solo-planta, aumentando a recuperação do N-fertilizante, reduzindo perdas por processos de nitrificação e desnitrificação e elevando a abundância do gene *nifH*, associado à fixação biológica de N. Alterações na composição da comunidade microbiana também foram observadas, com destaque para o consórcio de BPCP, que favoreceu uma estrutura microbiana mais uniforme e funcionalmente associada à melhoria da eficiência do uso de nutrientes. Os achados deste estudo reforçam o potencial da inoculação combinada via foliar (*Ab*) e via solo (*Na*) como estratégia sustentável para intensificação da produção de cana-de-açúcar, promovendo ganhos agronômicos e contribuindo para a redução de impactos ambientais. A integração de respostas fisiológicas, nutricionais e microbianas evidencia a importância do manejo biológico na transição para sistemas agrícolas mais eficientes e resilientes.

Palavras-chave: *Azospirillum brasilense*; ciclo do nitrogênio; *Nitrospirillum amazonense*; sustentabilidade agrícola.

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SUMMARY

GENERAL INTRODUCTION

Sugarcane (*Saccharum spp.*) ranks among the most important global agricultural crops, playing a fundamental economic and strategic role as a raw material for sugar, bioenergy, and biofuel production (Surendran *et al.*, 2016; dos Santos *et al.*, 2019; Martíni *et al.*, 2020). In Brazil, the crop was strongly boosted by the National Alcohol Program (Proálcool), launched in 1975, which positioned the country as a global leader and currently the world's largest producer of sugarcane (FAOSTAT, 2024).

Brazilian edaphoclimatic conditions, water resources, and large arable areas, favor crop expansion (Martinelli & Filoso, 2008). However, factors such as the frequent occurrence of water deficit and the naturally low fertility of tropical soils represent significant constraints to crop productivity (Fageria *et al.*, 2009; Pereira *et al.*, 2019; Holland *et al.*, 2018). As a semi-perennial species, sugarcane is often exposed to seasonal droughts, which compromise nutrient transport, reduce photosynthetic efficiency, affect antioxidant metabolism, and decrease carbohydrate accumulation in the stalks, with a direct impact on sugar yield (Morison & Morecroft, 2008; Ribeiro *et al.*, 2013; Sales *et al.*, 2015).

To meet the high nutritional demand of the crop, particularly for nitrogen (N), intensive applications of synthetic fertilizers are common. However, sugarcane's nitrogen use efficiency is generally low, with recovery rates ranging from 30% to 50% (Franco *et al.*, 2011; Vitti *et al.*, 2011; Yang *et al.*, 2019), leading to losses through leaching, volatilization, and denitrification (Malhi *et al.*, 2001; Otto *et al.*, 2017). This scenario not only limits agronomic gains but also contributes to environmental impacts, such as groundwater contamination by nitrate and the emission of nitrous oxide (N_2O), a potent greenhouse gas (Montzka *et al.*, 2011; Degaspari *et al.*, 2020).

In light of these challenges, there is growing interest in sustainable strategies that enhance input use efficiency, promote environmental conservation, and improve soil quality (dos Santos *et al.*, 2019; Fathollahzadeh *et al.*, 2019). In this context, the application of beneficial soil microorganisms has proven promising. Microorganisms play essential roles in biogeochemical cycles, improve nutrient availability through organic matter mineralization and mineral desorption, and can alleviate abiotic stresses by reducing the availability of toxic elements (Marschner *et al.*, 2011; Bünemann, 2015; Rajkumar *et al.*, 2012).

Among agronomically relevant microorganisms, plant growth-promoting bacteria (PGPB), such as *Azospirillum brasilense* and *Nitrospirillum amazonense*, stand out. Both are capable of biologically fixing atmospheric nitrogen and modulating root development through the synthesis of phytohormones such as indole-3-acetic acid (Dobbelaere *et al.*, 2003; Schwab *et al.*, 2018). By enhancing root system growth, these bacteria increase the plant's capacity to absorb water and nutrients, contributing to greater photosynthetic efficiency, carbohydrate production, and biomass accumulation (Silveira *et al.*, 2017; Hayat *et al.*, 2010).

In addition to their direct physiological effects, PGPB inoculation can alter root exudation patterns, thereby influencing the structure and functionality of the associated microbiota. Root exudates represent key carbon sources and act as chemical signals in the rhizosphere, recruiting microbial groups with specific functions such as nutrient solubilization, plant growth promotion, and pathogen suppression (Chaparro *et al.*, 2013; Mendes *et al.*, 2014; Pérez-Jaramillo *et al.*, 2017). Thus, PGPB application can trigger a cascading effect, activating plant metabolism, promoting the selection of beneficial microbial communities, and consequently enhancing crop resilience and productivity (Fukami *et al.*, 2018; Pereira *et al.*, 2020).

Beyond soil inoculation, the foliar application of certain groups of microorganisms has emerged as a practical and efficient alternative, capable of altering plant metabolism and indirectly influencing the rhizosphere (Cardozo *et al.*, 2022; Scudeletti *et al.*, 2023). Nevertheless, the physiological, microbial, and ecological effects of foliar-applied PGPB remain poorly understood in sugarcane systems.

Therefore, understanding how *A. brasilense* and *N. amazonense*, applied individually or in combination, affect plant metabolism, root exudation dynamics, and rhizosphere microbial community composition, as well as fertilizer-N recovery, may provide valuable insights for the development of more efficient and sustainable biological technologies. This thesis aims to integrate these different levels of response, physiological, nutritional, and ecological, in order to contribute to innovative strategies for sustainable sugarcane cultivation in tropical environments.

CHAPTER 1

COMBINING PLANT GROWTH-PROMOTING BACTERIA AS A TOOL TO IMPROVE THE METABOLISM AND PRODUCTIVITY OF SUGARCANE[1]

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Abstract

Sugarcane (*Saccharum spp.*) is a globally important crop, and strategies to minimize the negative impacts of its cultivation and enhance its development are highly relevant. Plant growth-promoting bacteria (PGPB) can sustainably foster plant growth in agricultural systems and mitigate adverse effects of stress on plants. This is the first study to investigate the combined use of *Azospirillum brasilense* (*Ab*) and *Nitrospirillum amazonense* (*Na*), two microorganisms widely applied in agricultural systems, aiming to elucidate their effects on the nutritional status, biochemical responses, and productive parameters of sugarcane. Greenhouse experiments were conducted under controlled water and temperature conditions with four treatments: application of *Ab, Na*, or *Ab* + *Na* (Mix) or no PGPB application (control). Sugarcane was cultivated until the middle of the rapid growth stage. To validate the results, the greenhouse trials were replicated under field conditions at two sites (Maracaí-SP and Pradópolis-SP). The results showed that the inoculation of sugarcane with plant growth-promoting bacteria (PGPB), particularly *Ab* and Mix, enhanced nutritional aspects, especially N content. These increases were significant under greenhouse ($p \leq 0.05$) and field conditions ($p \leq 0.10$). Additionally, inoculation reduced oxidative stress and improved photosynthetic parameters, such as net photosynthetic rate, water use efficiency, and carboxylation efficiency. These cascading effects contributed to significant gains in crop productivity, with an average increase in stalk yield of 10.9% for *Ab* and 12.2% for Mix across both environments. Similarly, there was an increase in sugar yield per hectare, with gains of 13.3% for *Ab* and 13.7% for Mix compared to the control. These findings highlight the potential of PGPB as a sustainable strategy to enhance crop

[1] Chapter written in accordance with the rules of Plant Physiology and Biochemistry.

productivity and resilience, contributing to environmentally balanced agricultural systems. Although the benefits of PGPB were evident, differences between *Ab* and Mix were not pronounced. Therefore, additional studies are needed to explore the potential of these combinations under adverse conditions when their effects could be more pronounced.

Keywords: Microorganisms, Oxidative stress; Plant development; Photosynthesis; Physiological parameters.

1.1 INTRODUCTION

Sugarcane (*Saccharum spp.*) is a globally economically important crop that is primarily used to produce sugar, biofuels and bioenergy (Surendran *et al.*, 2016; dos Santos *et al.*, 2019; Martíni *et al.*, 2020). In Brazil, the national biofuel program that was initiated in 1975 following the first Organization of Petroleum Exporting Countries (OPEC) crisis significantly expanded sugarcane cultivation (Loarie *et al.*, 2011; Heinrichs *et al.*, 2017; Linnenluecke *et al.*, 2018) making Brazil one of the world's largest producers.

Brazil offers ideal conditions for sugarcane cultivation due to its geographical location, abundance of water resources, high solar radiation, and vast areas suitable for planting (Martinelli and Filoso, 2008). Nonetheless, water deficits are a frequent problem during sugarcane crop development (Pereira *et al.*, 2019). Water stress negatively affects plant morphology, physiology, biochemistry, and molecular characteristics (Khoshru *et al.*, 2020). Sugarcane is a semi-perennial species, and seasonal droughts reduce nutrient transport (Morison and Morecroft, 2008), photosynthetic efficiency (Ribeiro *et al.*, 2013), and antioxidant metabolism (Sales *et al.*, 2015). The resulting reduction in carbohydrate accumulation compromises stalk production and sugar yield. Sugarcane productivity is also limited by soil quality. Most tropical soils are acidic and have low natural fertility (Fageria *et al.*, 2009). Continuous cultivation under these conditions leads to severe declines in soil quality, which reduce productivity (Crusciol *et al.*, 2017; Holland *et al.*, 2018; Bossolani *et al.*, 2020).

Numerous studies have focused on minimizing environmental risks, improving soil quality, and mitigating abiotic stresses in plants (dos Santos *et al.*, 2019;

Fathollahzadeh *et al.*, 2019; Pereira *et al.*, 2019). A promising option for promoting sustainability and improving both soil and plants is the use of microorganisms (Cao *et al.*, 2023). Microorganisms play a crucial role in biogeochemical cycles (Marschner *et al.*, 2011) and contribute to soil nutrient replenishment through mineral desorption and organic matter mineralization (Bünemann, 2015). Furthermore, some microorganisms release chelators and engage in redox reactions that reduce the availability of toxic elements to plants thereby minimizing abiotic stresses (Rajkumar *et al.*, 2012; Sheteiwy *et al.*, 2022).

Among agronomically relevant microorganisms, plant growth-promoting bacteria (PGPB) are a particularly efficient tool for improving nutrient acquisition, promoting plant development, and increasing productivity (dos Santos *et al.*, 2017; Fonseca *et al.*, 2022; Ibrahim and El-Sawah, 2022). PGPB play an important role in the stress tolerance of plants. These bacteria not only facilitate plant growth and survival under adverse conditions (Mesa-Marín *et al.*, 2018; Cesari *et al.*, 2019; Fonseca *et al.*, 2022) but also induce plant production of a variety of plant-beneficial compounds (Ankati and Podile, 2019). Among the plant growth-promoting bacteria (PGPB), *Azospirillum brasilense* stands out as one of the most commonly used bacteria for grasses, demonstrating significant benefits across several species (Cook *et al.*, 2022; Scudeletti *et al.*, 2023; Galindo *et al.*, 2024; Martin *et al.*, 2024).

On the other hand, *Nitrospirillum amazonense* (formerly *Azospirillum amazonense*), despite its high potential as a PGPB for grasses, has limited reports of its effectiveness under field conditions, particularly for sugarcane (Figueiredo *et al.*, 2017). Both bacteria are highly compatible with sugarcane and possess the ability to biologically fix atmospheric nitrogen (N) (Schwab *et al.*, 2018; Paludo *et al.*, 2024). While the individual effects of these bacteria have been extensively studied, little is known about their combined effects on the physiology and development of grass crops. Understanding the synergism between these two microorganisms could provide valuable insights and pave the way for innovative strategies to enhance crop performance. Plants associated with these PGPB exhibit enhanced photosynthetic efficiency, often attributed to the improved activity of key enzymes such as RuBisCO and PEPcase (Garg *et al.*, 2019). This increased enzymatic activity contributes to a more effective carbon fixation process, enabling the production of carbon skeletons that serve as fundamental precursors for the synthesis of various biomolecules essential for plant growth and development (Silveira *et al.*, 2017). Furthermore, these

PGPB are related with the production of siderophores, organic acids, and enzymes that increase plant resistance to stress conditions (Hayat *et al.*, 2010; Pereira *et al.*, 2019; Nader *et al.*, 2024).

Fonseca *et al.* (2022) reported that the PGPB *Bacillus subtilis* altered the nutritional and physiological parameters of sugarcane, which has significant implications for the development of more sustainable agricultural practices. *B. subtilis* mitigated the negative effects of water restriction, improving the net photosynthetic rate and stomatal conductance in sugarcane plants. Based on the established knowledge of the role of PGPB in promoting plant growth, this study was designed to test for the first time (field and greenhouse conditions) the hypothesis that inoculating sugarcane with *A. brasilense* and/or *N. amazonense* can significantly improve nutrient uptake, photosynthetic efficiency, reduce oxidative stress, and increase crop productivity and quality. It is expected that the combination of these bacteria (Mix) will result in greater gains compared to isolated inoculation. Furthermore, it is proposed that the interaction between plant growth-promoting bacteria and sugarcane improve plant metabolism and yield responses. This work aims to contribute to the advancement of more efficient and sustainable agricultural practices by integrating the use of PGPB in sugarcane fields to enhance productivity.

1.2 Materials and methods

1.2.1 Description of the experimental sites

This study was divided into two parts that followed the same experimental design. One part was conducted under greenhouse conditions at the School of Agronomic Sciences - UNESP, Botucatu, State of São Paulo, Brazil (latitude 22° 50’ 57’’ S, longitude 48° 25’ 59’’ W, and altitude of 815 m). The other part was carried out under field conditions at two sites (described below). The greenhouse was equipped with an internal air circulation system for heating and cooling, which maintained the temperature between 22°C and 32°C, and a relative humidity of 70%, conditions considered ideal for the development of sugarcane cultivation (Rodrigues, 1995). The maximum photosynthetic photon flux density reached approximately 1850 µmol photons m^{-2} s^{-1} at the leaf level. This was supplemented by 200 W LED lamps (Agroled 3500K + Deep Red 660nm; Master Plants, Atibaia, State of São Paulo, Brazil) to

maintain an optimal photoperiod of approximately 13 hours per day, promoting properly sugarcane growth (Rodrigues, 1995).

To validate the productivity effects observed in the greenhouse experiment, a field trial was carried out on second-cut ratoon cane at the Agroterenas Mill, Maracaí Unit, State of São Paulo, Brazil (site 1) (latitude: 22° 36' 51" S, longitude: 50° 40' 7" W, altitude of 421 m). The climate in the region is classified as humid subtropical (Cfa) according to the Köppen-Geiger climate classification system (Alvares *et al.*, 2013), with an average annual precipitation of 1502 mm. A second field trial was conducted at the São Martinho Group Mill, Pradópolis Unit, State of São Paulo, Brazil (site 2) (latitude: 21° 45' 40" S, longitude: 48° 04' 58" W, altitude of 620 m). The climate in this region is classified as Aw according to the Köppen-Geiger climate classification system (Alvares *et al.*, 2013), which corresponds to dry winters and hot, rainy summers, and has an average annual precipitation of 1580 mm. The soil chemical properties before the start of the study were determined at a depth of 0.0-0.2 m in the greenhouse experiment and 0.0-0.4 m in the field experiment, as shown in Table 1. The analyses were conducted according to the methodology described by van Raij *et al.* (2001).

Table 1 – Initial chemical characterization of the soil used in the greenhouse experiment, Botucatu-SP, the Agroterenas plant, Maracaí-SP (Site 1) and São Martinho Group, Pradópolis-SP (Site 2), including the following variables: soil pH (pH), soil organic matter (S.O.M.), resin-extractable phosphorus (P_{resin}), sulfur-sulfate ($S\text{-}SO_4^{2-}$), calcium (Ca), magnesium (Mg), potassium (K), aluminum (Al), potential acidity (H+Al), sum of exchangeable bases (SB), cation exchange capacity (CEC), base saturation (V%), and aluminum saturation (m%).

Greenhouse experiment – Botucatu-SP							
Soil layer (cm)	pH $CaCl_2$	S.O.M g dm^{-3}	P_{resin} ----- mg dm^{-3}----	$S\text{-}SO_4^{2-}$	Ca	Mg -------$mmol_c$ dm^{-3}--------	K
0-20	4.4	15.0	6.8	4.4	11	5.0	3.0
Soil layer (cm)	Al	H+Al	SB	CEC	V		m
	----------$mmol_c$ dm^{-3}----------				%		
0-20	2.5	24.3	19.2	43.5	44.1		14.0
Site 1 – Maracaí-SP							
Soil layer (cm)	pH $CaCl_2$	S.O.M g dm^{-3}	P_{resin} ----- mg dm^{-3}----	$S\text{-}SO_4^{2-}$	Ca	Mg -------$mmol_c$ dm^{-3}--------	K
0-20	5.8	10	29	2.9	23	7.2	2.2
20-40	5.5	8.1	30	2.9	18	6.8	1.3
Soil layer (cm)	Al	H+Al	SB	CEC	V		m
	----------$mmol_c$ dm^{-3}----------				%		

0-20	0.00	10	32	42	76	0.00
20-40	0.00	11	27	38	70	0.00
Site 2 – Pradópolis-SP						
Soil layer	pH	S.O.M	P_{resin}	$S\text{-}SO_4^{2-}$	Ca	Mg	K
(cm)	$CaCl_2$	g dm^{-3}	----- mg dm^{-3}----		-----$mmol_c$ dm^{-3}-----		
0-20	5.3	25	17	12	25	11	3.0
20-40	4.6	18	6.0	43	12	5.0	1.1
Soil layer	Al	H+Al	SB	CEC	V	m	
(cm)	----------$mmol_c$ dm^{-3}----------				%		
0-20	0.00	10	40	67	60	0.00	
20-40	0.00	11	18	56	32	0.00	

1.2.2 Experimental design

The experimental design of the greenhouse experiment was a completely randomized design, consisting of four treatments and four replications. In contrast, the field experiment followed a randomized block design with eight replications, as shown in Table 2. In the field experiments, the plots at site 1 (Agroterenas Mill, Maracaí Unit, State of São Paulo, Brazil) consisted of ten 10-m rows with an alternating row spacing of 1.5 m × 0.9 m. At site 2 (São Martinho Group Mill, Pradópolis Unit, State of São Paulo, Brazil), the plots consisted of ten 10-m rows with a uniform row spacing of 1.5 m.

Table 2 - Description of the treatments (greenhouse and field conditions).

Treatment	Description
Control*	Control treatment (no microorganism application)
Ab	Application of *Azospirillum brasilense*
Na	Application of *Nitrospirillum amazonense*
Mix	Application of *Ab* + *Na*

* Water Application

1.2.3 Experimental procedure

The greenhouse experiment was conducted in 50-L pots using the sugarcane variety CTC9001 and lasted approximately 200 days after emergence (DAE), during which the crop reached the mid-growth phenological stage, characterized by high metabolic activity. In the field experiments, sugarcane varieties CTC9001 (site 1) and CTC9005 (site 2) were used. The field experiments were conducted until the

phenological maturation stage (approximately 365 days) to obtain technological and productive parameters.

Prior to the establishment of the greenhouse experiment, all necessary soil corrections were performed. Soil was collected from a depth of 0.0-0.2 m, and its initial chemical characteristics are described in Table 1. Corrections were based on the initial chemical characterization, following standard recommendations for sugarcane cultivation. The amount of lime was determined to increase the soil base saturation to 70% using the method recommended for the State of São Paulo (Spironello *et al.*, 1997). The chemical characteristics of the lime were: calcium oxide (CaO) 39%, magnesium oxide (MgO) 13%, Neutralization Power (PN) 100%, and Relative Total Neutralization Power (PRNT) 91.5%.

After soil corrections, sugarcane seedlings were transplanted into pots for the first cultivation cycle. Fertilization was performed based on the initial chemical analysis of the soil at each site (Table 1) and the recommendations for sugarcane cultivation, considering the expected productivity, as described by (Spironello *et al.*, 1997). In the first year, under greenhouse conditions (plant cane), the equivalent of 90 kg N ha^{-1}, 180 kg P_2O_5 ha^{-1}, and 80 kg K_2O ha^{-1} were applied. In the second year (ratoon cane), the doses were adjusted to 120 kg N ha^{-1}, 30 kg P_2O_5 ha^{-1}, and 120 kg K_2O ha^{-1}. After the first harvest, a second growth cycle was conducted in the same pots, with straw from the previous cycle added to simulate the first ratoon. For greenhouse experiments, irrigation was performed based on the crop's needs, which were determined by using tensiometers to assess the soil matric potential as an indirect measure of water content (based on a soil water retention curve).

In the field experiments, at site 1 (ratoon cane), fertilization included 120 kg N ha^{-1}, 0 kg P_2O_5 ha^{-1}, and 120 kg K_2O ha^{-1}, while at site 2 (ratoon cane), 120 kg N ha^{-1}, 30 kg P_2O_5 ha^{-1}, and 120 kg K_2O ha^{-1} were applied. In both experiments, sugarcane plants were inoculated with *Azospirillum brasilense* (*Ab*) via foliar application (Scudeletti *et al.*, 2023) and with *Nitrospirillum amazonense* (*Na*) through soil drench application (Reis *et al.*, 2020), both carried out during the tillering stage. Considering the characteristics of *Ab*, foliar application was chosen due to its advantages, such as the absence of competition with native soil microbiota and reduced interference from the physicochemical properties of the edaphic environment (Zanettini and Puente, 2017; Cardozo *et al.*, 2022; Hernández *et al.*, 2024). Moreover, foliar spraying has proven to be a practical and effective strategy for *Ab* inoculation, comparable to

conventional methods such as in-furrow application or seedling immersion (Fukami *et al.*, 2016; Da Silva *et al.*, 2017). In contrast, *Na* was applied directly to moist soil using a directed jet, aiming to maximize contact between the microorganism and the rhizosphere, an environment where this bacterial genus exhibits greater functional effectiveness (Pereira *et al.*, 2019; Reis *et al.*, 2020).

Inoculant applications were performed using a CO_2-propelled compressed-air sprayer (Herbicate, Catanduva, SP, Brazil), operating at a working pressure of 1.8 BAR and calibrated to deliver 150 L ha^{-1} of solution. For the *Ab* application, the spray boom was equipped with TTI11004VP flat-fan nozzles (Jacto, Pompéia, SP, Brazil), ensuring uniform droplet distribution across the target surface. For the drench application, a Jacto AS-50DK applicator (Jacto, Pompéia, SP, Brazil) fitted with a jet-type nozzle was used, which is ideal for this type of operation. The working speed was 1 m s^{-1}, simulating the operational conditions of commercial agricultural sprayers. All applications followed the manufacturers' technical guidelines and were consistently carried out in the late afternoon (around 5:00 PM) to optimize efficacy. In the greenhouse experiments, harvest was conducted at 200 days after emergence (DAE), whereas in the field experiments, it occurred at physiological maturity. Several analyses were performed throughout crop development, as described in the following sections.

The concentration of *A. brasilense* (Ab-V5 and Ab-V6) and *N. amazonense* (BR 11145) in the inoculants was 1×10^8 CFU (Colony-Forming Units) mL^{-1}, and the applied dose was 300 mL ha^{-1}, based on the aforementioned CFU concentration. The strains were provided by EMBRAPA-CNPSo and are deposited in the Diazotrophic and Plant Growth-Promoting Bacteria Culture Collection at Embrapa Soybean (CNPSo Collection 2083 [Ab-V5], CNPSo 2084 [Ab-V6], (Hungria *et al.*, 2010, 2018; Santos *et al.*, 2021); CNPSo 4060, (Junior *et al.*, 2008; Dartora *et al.*, 2016).

1.2.4 Evaluations

1.2.4.1 Nutritional Analysis

For nutritional analysis, +1 leaves were collected at the mid-growth phenological stage. The central vein was discarded, and the middle third of the leaf was retained for analysis as described by Spironello *et al.* (1997). The macronutrients potassium (K), calcium (Ca), magnesium (Mg), and the micronutrients copper (Cu), iron (Fe),

manganese (Mn), boron (B) and zinc (Zn) were extracted by nitroperchloric digestion and quantified by atomic absorption spectrophotometer Perkin Elmer Analyst 200 (Norwalk, CT, USA) (Malavolta *et al.*, 1989). Phosphorus (P) and sulfur (S) were also extracted by nitroperchloric digestion, and determined by UV-VIS spectrophotometry (Shimadzu UV-2700, Kyoto, Japan) (Malavolta *et al.*, 1989). Nitrogen was extracted by using an H_2SO_4 (sulfuric acid) extraction solution and determined by the Kjeldahl distillation method (AOAC, 2016).

1.2.4.2 Photosynthetic Pigments

To quantify photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) in the leaf tissues, three discs were cut between the edge and the central vein of the +1 leaf using a paper punch (0.5 cm diameter) and weighed. The discs were incubated for 24 h in capped glass vials containing 2 mL of N, N-dimethylformamide (DMF) and wrapped in aluminum foil according to the methodology proposed by Lichtenthaler (1987) on the same day that leaves were collected for nutritional analysis. Absorbance readings were taken using a spectrophotometer Model Nova 2000 (Nova Instruments, Brazil), at wavelengths of 664, 647, and 480 nm, respectively. Absorbance was measured using 1 mL of extract and 1 mL of deionized water; a mixture of 1 mL of N-dimethylformamide (DMF) solution and 1 mL of deionized water was used as a “blank.” Pigment content was calculated following the methods of Wellburn (1994).

1.2.4.3 Gas exchange

Gas exchange was measured via nondestructive analysis with a portable gas exchange device (LI-6400 infrared gas analyzer (IRGA), LI-COR Biosciences Inc., Lincoln, NE, USA), from the middle region of the +1 leaf. The parameters of the instrument were as follows: 380-400 mol mol^{-1} atmospheric CO_2, 1200 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) supplied by LED lamps, 25-27 °C leaf chamber temperature and a relative humidity range of 60-70%. Gas exchange was measured throughout sugarcane plant development starting with the application of the treatments. Three readings were obtained during the tillering phase, and six readings were performed during the rapid growth phase (15, 30, 45, 60, 75, 90, 105, 120 and 135 days after application). The measured variables were the net photosynthesis rate (*A*; µmol CO_2 m^{-2} s^{-1}), stomatal conductance (*gs*; mol H_2O m^{-2} s^{-1}), internal CO_2

concentration (*Ci*; µmol mol^{-1}), leaf transpiration (*E*; mmol H2O m^{-2} s^{-1}), water use efficiency [*WUE* - *A/E* ratio; µmol CO_2 (mmol $H_2O)^{-1}$)], and carboxylation efficiency (*A/Ci* ratio; dimensionless). All measurements were taken between 8:00 and 11:00 h a.m.

1.2.4.4 Oxidative stress and antioxidant enzymes

The analysis of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), along with hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), was performed using fresh leaf tissue ground to a fine powder in a mortar under liquid nitrogen. For the extraction, one part of the pulverized tissue was combined with two parts of an extraction medium. The mixture was homogenized on ice for 20 seconds using an Ultra-Stirred homogenizer (BIOMT; 0.5-250 mL, 5-mm stainless steel rod; 50-60Hz). The resulting crude extract was processed for enzyme activity assessments following the methodology outlined by Silva *et al.* (2020).

For MDA quantification, the compound was extracted from fresh leaf tissue and reacted with thiobarbituric acid (TBA), according to Heath and Packer (1968). The absorbance of the TBA-MDA complex was measured at 532 nm using a spectrophotometer Model Nova 2000 (Nova Instruments, Brazil). The MDA concentration was determined from a standard curve prepared with 1,1,3,3-tetramethoxypropane and expressed in nmol MDA g^{-1} fresh weight (FW). The H_2O_2 content was assessed using the protocol of Alexieva *et al.* (2001), with the concentration calculated based on a standard curve of known H_2O_2 concentrations.

To measure SOD and CAT enzyme activities, the extraction buffer consisted of 200 mM potassium phosphate buffer (pH 7.8), 10 mM EDTA, 20 mM ascorbic acid, 1% polyvinylpyrrolidone (PVP-40, Sigma-Aldrich), and 1 mM 1,4-dithiothreitol (DTT, Sigma-Aldrich) (Giannopolitis and Ries 1977). After centrifugation at 12.000 × g at 4°C for 30 minutes, the supernatant was collected as the crude extract for the assays. SOD activity was determined through a photochemical method involving methionine (13 mM, Sigma-Aldrich), EDTA (100 nM), riboflavin (2 µM, Sigma-Aldrich), and nitroblue tetrazolium (NBT, 75 µM, Sigma-Aldrich) in 50 mM potassium phosphate buffer. The results were expressed in activity units (U) per mg of protein, as described by Giannopolitis and Ries (1977). CAT activity was assessed by monitoring the reduction of H_2O_2 (250 µM) over time. The activity was expressed in µmol min^{-1} mg^{-1} protein,

using a molar extinction coefficient of 39.4 mM^{-1} cm^{-1}, based on the methodology of Havir & McHale (1987).

1.2.4.5 Determination of total and active RuBisCO and PEPCase

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoenolpyruvate carboxylase (PEPCase) activities were determined using +1 sugarcane leaves collected simultaneously with the gas exchange analyses. The leaves were placed in Falcon tubes and transported in a drum with liquid nitrogen. The samples were then stored in an ultra-freezer at -80°C. Enzymatic determinations were performed using samples collected at the period of highest photosynthetic activity as indicated by the results of the gas exchange analyses.

RuBisCO activity was extracted by grinding the frozen plant material (leaves without veins) in liquid nitrogen and potassium phosphate buffer solution. The activity determination followed the methodology described by Reid *et al.* (1997). Spectrophotometric readings were taken at an absorbance of 340 nm over a period of 3 min (Reid *et al.*, 1997). The soluble protein content of the crude extract was determined following the methodology described by Bradford (1976) using readings taken at 595 nm on a spectrophotometer Model Nova 2000 (Nova Instruments, Brazil) and referring to a standard curve based on bovine albumin. After determining the protein concentration in each sample, RuBisCO activity was calculated and expressed as μmol min^{-1} mg $protein^{-1}$.

The extract used for RuBisCO determination was also used to assess PEPCase activity following the methodology described by Degl'Innocenti; (2002). Spectrophotometric readings were taken at 340 nm, and NADH oxidation was monitored for 1 min. Based on the protein content of the sample, PEPCase activity was expressed as μmol min^{-1} mg $protein^{-1}$.

The initial ($V_{initial}$) and total (V_{total}) activities of the photosynthetic enzymes were determined according to Sage *et al.* (1988), and their activation states were calculated as the ratio of $V_{initial}$ to V_{total} (%).

1.2.4.6 Sugarcane biometric parameters and productivity

In the greenhouse experiments, plant height was determined by measuring the distance from the soil surface to the auricular region of the +1 leaf following the numbering suggested by Kuijper (Van Dillewijn, 1952). Stalk diameter was measured

using a digital caliper (Model Absolute, Mitutoyo 500-197-30, Mitutoyo Sul Americana, Suzano, State of São Paulo, Brazil) at the third internode above the soil. To assess root dry matter, in the first year of the greenhouse experiment, samples were collected using an auger with a known volume, allowing for an estimate of total volume, considering the continuation of the experiment in the second year. In the second year, the roots were fully collected. The analysis of rhizome dry matter was conducted only in the second year, as it involves a destructive sampling method, and this organ plays a fundamental role in ratoon regrowth. However, the collection of stalks and leaves was performed in full during both years. To determine dry matter of the roots, rhizomes, stalks and leaves were separated into paper bags and subsequently dried in a forced-air (Marconi model MA035/5/10P, Piracicaba, State of São Paulo, Brazil) oven at 65°C until a constant weight was achieved. The dry matter was then weighed using an analytical balance (AY 220, Shimadzu).

In the field experiments, two rows of plants within the designated usable areas of the plots were selected. Within these rows, stalk height and stalk diameter were assessed in a random 1-m section employing the same methodologies used in the greenhouse experiments. The final count of stalks was established by counting the number of stalks in two central 5-m rows within each plot. In addition, 20 stalks per plot were weighed to ensure a more representative sample of each area (Prático, 2012). These measurements were used to estimate productivity in tons of stalks per hectare.

1.2.4.7 Technological Parameters

Technological evaluations were performed in a random 1-m section within two rows of plants in the usable area of the plot. The stalks within this 1-m section were harvested, topped at the apical bud height, and sent to the laboratory for the determination of technological parameters according to the Sugarcane Payment System by Sucrose Content and biannual updates from CONSECANA (described by Fernandes, 2011). The variables analyzed included apparent sucrose content in sugarcane (Pol) (%), juice purity (%), fiber (%), juice reducing sugars (RS), and total recoverable sugar (TRS) per hectare. Additionally, productivity in terms of tons of sugar per hectare and tons of stalks per hectare was evaluated.

1.2.5 Data analysis

For the greenhouse experiments, data were subjected to individual analysis of variance (ANOVA) using the F-test ($p \leq 0.05$); when significant differences were detected, treatment means were compared using the least significant difference (LSD) test ($p \leq 0.05$). For the field experiments, data were analyzed using individual ANOVA with the F-test ($p \leq 0.10$), and treatment means were compared using the LSD test ($p \leq 0.10$). Statistical analyses were performed using the software R (R Core Team, 2020), version 4.0.2 and its graphical interface, RStudio (RStudio Team, 2020), version 1.3.1073. (Team, 2020). Graphical representations of the results were generated with GraphPad Prism software (version 9.5.1; San Diego, CA, USA).

1.3 RESULTS

1.3.1 Nutritional analysis

1.3.1.1 Greenhouse experiment

In the greenhouse experiment (Table 3), sugarcane leaf N content was significantly higher in the treatments where PGPB was applied compared to the control. In the first year, the Mix, *Ab*, and *Na* treatments increased leaf N content by 21.7%, 12.6%, and 8.6%, respectively, relative to the control (19.8 g kg^{-1}), with *Ab* showing the highest increase. Leaf Mg content also increased, with Mix and *Ab* showing respective increases of 23.9% and 20.9% compared to the control (1.34 g kg^{-1}). Similarly, leaf B content rose by 29.3% in Mix and 36.5% in *Ab*, relative to the control (20.8 mg kg^{-1}). In the second year, leaf N content remained significantly higher in the PGPB treatments compared to the control (20.3 g kg^{-1}), with Mix and *Ab* showing increases of 27.1% and 13.3%, respectively. For leaf Mg content, a significant increase (17.7%) was observed only in *Ab*, compared to the control (1.24 g kg^{-1}). Additionally, leaf B content increased by 38.2% in *Ab* and 35.6% in Mix, relative to the control (22.5 mg kg^{-1}). No significant differences were observed for P, K, Ca, S, Fe, Cu, Zn, or Mn across treatments in either year of the greenhouse experiment.

Table 3 – Nutrient content of sugarcane leaves under greenhouse (Botucatu-SP) and field conditions (Site 1 = Maracaí-SP and Site 2 = Pradópolis-SP) as a function of treatment [Control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)].

Treat.	N	P	K	Ca	Mg	S	Fe	Cu	Zn	Mn	B
		---------- g kg^{-1} ----------						---------- mg kg^{-1} ----------			
Greenhouse 1st year – Botucatu-SP											
C	19.8c ± 0.58	1.64a ± 0.05	10.5a ± 0.26	4.01a ± 0.20	1.34b ± 0.06	1.53a ± 0.06	84.3a ± 7.06	10.2a ± 0.38	30.2a ± 1.11	154a ± 6.06	20.8b ± 0.78
Ab	22.3b ± 0.23	1.85a ± 0.08	11.6a ± 0.27	4.49a ± 0.18	1.62a ± 0.05	1.72a ± 0.10	78.3a ± 3.18	10.7a ± 0.61	32.7a ± 1.84	163a ± 6.16	28.4a ± 1.30
Na	21.5b ± 0.64	1.74a ± 0.05	11.1a ± 0.20	4.20a ± 0.08	1.56ab ± 0.05	1.78a ± 0.05	79.8a ± 5.56	10.2a ± 0.32	30.4a ± 1.20	172a ± 2.20	24.6ab ± 0.88
Mix	24.1a ± 0.43	1.77a ± 0.03	10.9a ± 0.50	4.23a ± 0.14	1.66 a ± 0.11	1.86a ± 0.07	80.9a ± 4.04	10.4a ± 0.41	29.9a ± 1.41	171a ± 4.36	26.9a ± 1.67
Greenhouse 2nd year – Botucatu-SP											
C	20.3c ± 1.09	1.68a ± 0.02	11.2a ± 0.52	4.64a ± 0.61	1.24b ± 0.04	1.04a ± 0.01	64.6a ± 5.57	9.60a ± 0.60	29.4a ± 0.60	168a ± 2.33	22.5b ± 0.26
Ab	23.0b ± 0.43	1.90a ± 0.05	12.2a ± 0.43	4.80a ± 0.19	1.46a ± 0.04	1.20a ± 0.06	66.3a ± 4.91	10.4a ± 0.40	34.1a ± 1.18	157a ± 6.33	31.1a ± 1.07
Na	21.4bc ± 0.74	1.82a ± 0.03	10.0a ± 0.59	4.38a ± 0.09	1.32ab ± 0.06	1.35a ± 0.17	65.8a ± 5.38	10.0a ± 0.20	28.4a ± 1.91	168a ± 7.77	23.9b ± 1.20
Mix	25.8a ± 0.23	1.79a ± 0.06	11.2a ± 0.15	4.00a ± 0.44	1.29ab ± 0.07	1.17a ± 0.07	62.6a ± 4.41	10.6a ± 1.31	29.6a ± 1.64	170a ± 1.67	30.5a ± 2.08
Site 1 – Maracaí-SP											
C	18.4b ± 0.42	2.10a ± 0.05	14.5a ± 0.38	4.24a ± 0.16	1.40a ± 0.03	1.97a ± 0.13	113a ± 16.7	4.65a ± 0.51	17.0a ± 1.02	52.8a ± 6.28	11.1c ± 0.97
Ab	20.1a ± 0.64	2.08a ± 0.01	14.5a ± 0.36	4.28a ± 0.33	1.43a ± 0.05	1.87a ± 0.10	99.4a ± 1.13	5.55a ± 0.67	14.8a ± 1.16	66.8a ± 7.38	12.9bc ± 0.94
Na	20.1a ± 0.42	2.11a ± 0.09	12.7b ± 0.89	4.63a ± 0.28	1.49a ± 0.08	1.61a ± 0.06	105a ± 5.62	6.00a ± 0.88	19.8a ± 2.50	61.9a ± 7.66	14.5ab ± 0.20
Mix	20.0a ± 0.36	2.19a ± 0.07	14.6a ± 0.62	4.19a ± 0.29	1.58a ± 0.11	1.84a ± 0.09	106a ± 6.90	5.40a ± 0.42	15.5a ± 1.62	80.0a ± 10.5	15.5a ± 0.30
Site 2 – Pradópolis-SP											
C	18.0b ± 0.55	1.87a ± 0.22	13.1a ± 0.42	2.71a ± 0.44	1.10b ± 0.11	1.75a ± 0.21	208a ± 18.9	4.54a ± 0.56	17.9a ± 2.41	58.7a ± 4.94	8.27b ± 0.87
Ab	20.0a ± 0.90	1.58a ± 0.06	12.4a ± 0.61	3.14a ± 0.31	1.27a ± 0.04	1.72a ± 0.20	166a ± 15.2	4.88a ± 0.53	17.1a ± 0.93	59.9a ± 5.23	11.0a ± 0.76
Na	19.8a ± 0.83	1.79a ± 0.12	13.6a ± 0.55	2.81a ± 0.24	1.30a ± 0.03	1.58a ± 0.26	195a ± 25.9	4.43a ± 0.08	21.2a ± 2.28	56.6a ± 5.58	10.7a ± 0.90
Mix	19.5a ± 0.54	1.71a ± 0.07	12.3a ± 0.74	3.06a ± 0.20	1.25a ± 0.10	1.53a ± 0.13	193a ± 16.8	5.33a ± 0.80	19.4a ± 1.46	48.2a ± 7.67	9.17ab ± 0.47

Different letters indicate significant differences between treatments by the LSD test at $p \leq 0.05$ ($n = 4$ for the greenhouse and $n = 8$ for field conditions)

1.3.1.2 Field experiment

In the field experiment (Table 3), at site 1 all treatments with PGPB significantly increased leaf N content. Compared with the control (18.4 g kg^{-1}), *Ab*, *Na*, and Mix increased leaf N content by 9.2%, 9.2%, and 8.7%, respectively. *Na*, and Mix increased leaf B content by 16.2%, 30.6%, and 39.6%, respectively, compared to the control (11.1 mg kg^{-1}). At Site 2, PGPB application also significantly increased sugarcane leaf N content. Compared to the control (18.0 g kg^{-1}), the *Ab*, *Na*, and Mix treatments increased leaf N content by 11.1%, 10%, and 8.3%, respectively. Additionally, leaf Mg content increased by 15.5%, 18.2%, and 13.6% in the *Ab*, *Na*, and Mix treatments, respectively, compared to the control (1.10 g kg^{-1}). Similarly, leaf B content increased by 33.0% in *Ab* and 29.4% in Mix, compared to the control (8.27 mg kg^{-1}).

1.3.2 Photosynthetic pigments

1.3.2.1 Greenhouse experiment

In the greenhouse experiments, PGPB significantly increased chlorophyll *a* content compared to the control (Figure 1). In the first year (Figure 1a), the *Ab* and Mix treatments increased chlorophyll *a* content by 5.85% and 8.7%, respectively, relative to the control (1487 mg g^{-1} FW). In the second year (Figure 1c), increases of 7.64% (*Ab*), 5.82% (*Na*), and 10.5% (Mix) were observed compared to the control (1596 mg g^{-1} FW). No significant differences were detected for chlorophyll *b* (Figure 1a,c) or total carotenoids (Figure 1b,d) among treatments.

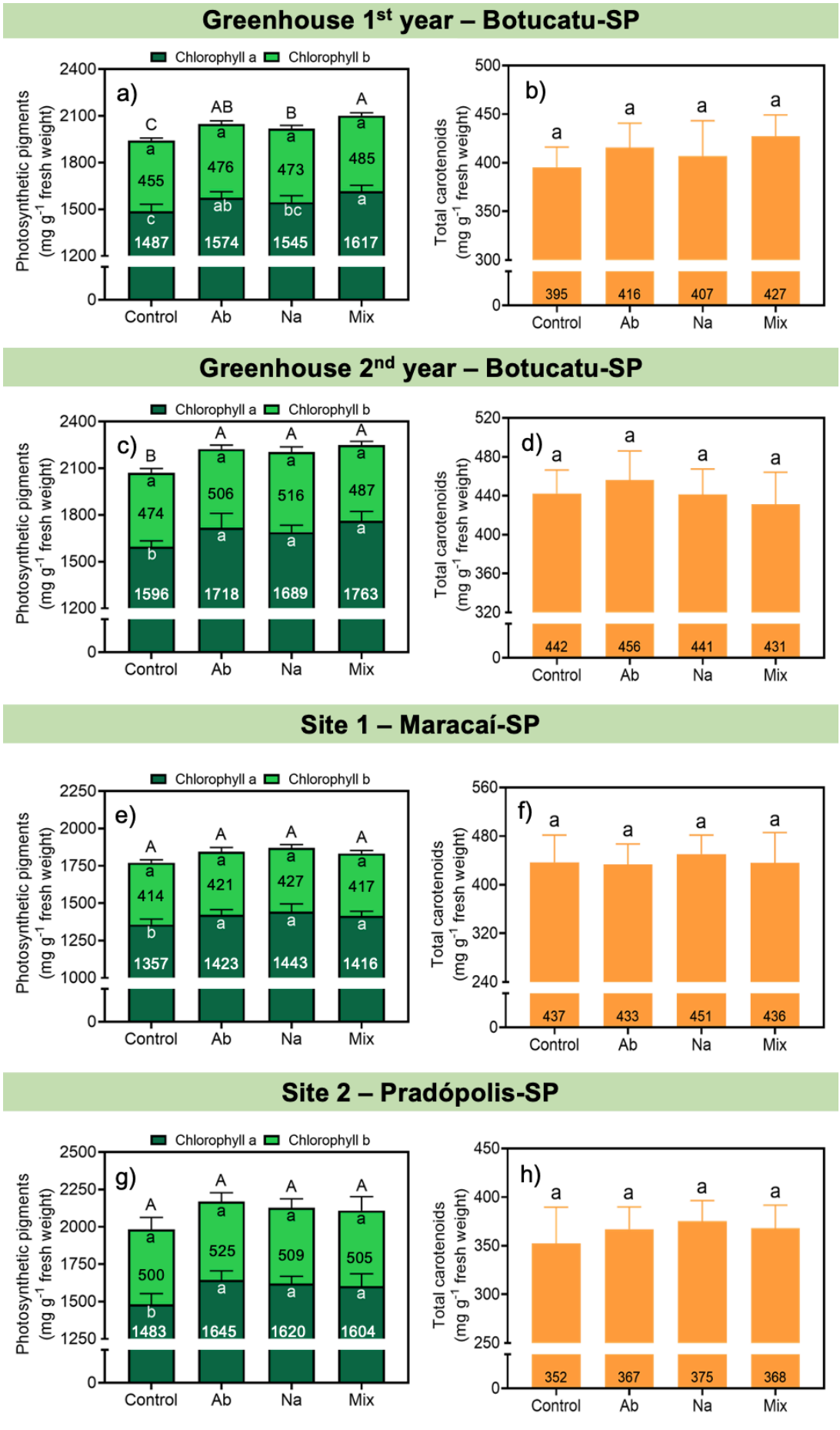

Greenhouse 1st year – Botucatu-SP
Chlorophyll a
Chlorophyll b
a)
Photosynthetic pigments (mg g-1 fresh weight)
C
AB
B
A
455
476
473
485
1487
1574
1545
1617
Control
Ab
Na
Mix
b)
Total carotenoids (mg g-1 fresh weight)
395
416
407
427
Greenhouse 2nd year – Botucatu-SP
c)
474
506
516
487
1596
1718
1689
1763
d)
442
456
441
431
Site 1 – Maracaí-SP
e)
414
421
427
417
1357
1423
1443
1416
f)
437
433
451
436
Site 2 – Pradópolis-SP
g)
500
525
509
505
1483
1645
1620
1604
h)
352
367
375
368

Figure 1. Effects of treatments [Control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on sugarcane leaf chlorophyll (a, c, e and g) and total carotenoid contents (b, d, f and h) in plants grown under greenhouse (a-d) and field conditions (e-h). Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences in total chlorophyll between treatments according to the LSD test at $p \leq 0.05$ for the greenhouse experiment and $p \leq 0.10$ for the field experiment. Error bars represent the standard error of the mean ($n = 4$ for the greenhouse and $n = 8$ for field conditions).

Following the trend observed for chlorophylls *a* and *b*, total chlorophyll content significantly increased in all PGPB treatments compared to the control in the greenhouse experiment. In the first year (1942 mg g^{-1} FW), *Ab*, *Na*, and Mix increased total chlorophyll content by 5.56%, 3.91%, and 8.24%, respectively. A similar trend was observed in the second year (2070 mg g^{-1} FW), with increases of 7.44% (*Ab*), 6.52% (*Na*), and 8.70% (Mix) relative to the control.

1.3.2.2 Field experiment

At both sites of the field experiment, PGPB treatments significantly increased chlorophyll *a* content (Figure 1e,g). At Site 1, compared to the control (1357 mg g^{-1} FW), *Ab*, *Na*, and Mix increased chlorophyll *a* by 4.86%, 6.34%, and 4.35%, respectively. At Site 2, the *Ab*, *Na*, and Mix treatments led to increases of 10.9%, 9.24%, and 8.2%, respectively, compared to the control (1483 mg g^{-1} FW). No significant differences were observed among treatments for chlorophyll *b* content (Figure 1e,g), total chlorophyll content (Figure 1e,g), or total carotenoid content (Figure 1f,h) at either site.

1.3.3 Gas exchange

1.3.3.1 Greenhouse experiment

The net photosynthetic rate varied significantly among the treatments in both years of the greenhouse experiment. In the first year, 60 days after PGPB application (DAA), the *Ab* and Mix treatments exhibited a significantly higher net photosynthetic rate (Figure 2a) compared to the control, a result that was maintained until the final evaluation period (135 DAA). Regarding water-use efficiency (Figure 2b), *Ab* and Mix

showed positive effects from 15 DAA, while *Na* exhibited improvements starting at 45 DAA. The control, however, consistently maintained the lowest values during the longest evaluation period, in relation to *Ab* and Mix. Additionally, carboxylation efficiency (Figure 2c) varied among treatments, with positive effects observed from 15 DAA in all treatments that received PGPB. However, the Mix treatment exhibited the highest carboxylation efficiency throughout almost the entire evaluation period, consistently remaining superior to the control. The results for stomatal conductance, leaf transpiration, and internal CO_2 concentration are provided in the Supplementary material (Figure S1). At 30 DAA (Figure S1a), all PGPB treatments showed higher stomatal conductance values compared to the control. These differences persisted throughout the evaluation period, being more pronounced in the *Ab* and Mix treatments. For leaf transpiration (Figure S1b), results varied without a clear pattern among treatments. At 45 DAA, the control exhibited the highest transpiration rate, whereas at 75 DAA, this value was highest in the Mix treatment. Regarding internal substomatal CO_2 concentration (Figure S1c), all PGPB treatments showed lower values at 45 DAA. Differences between the control and Mix remained evident throughout the evaluation period.

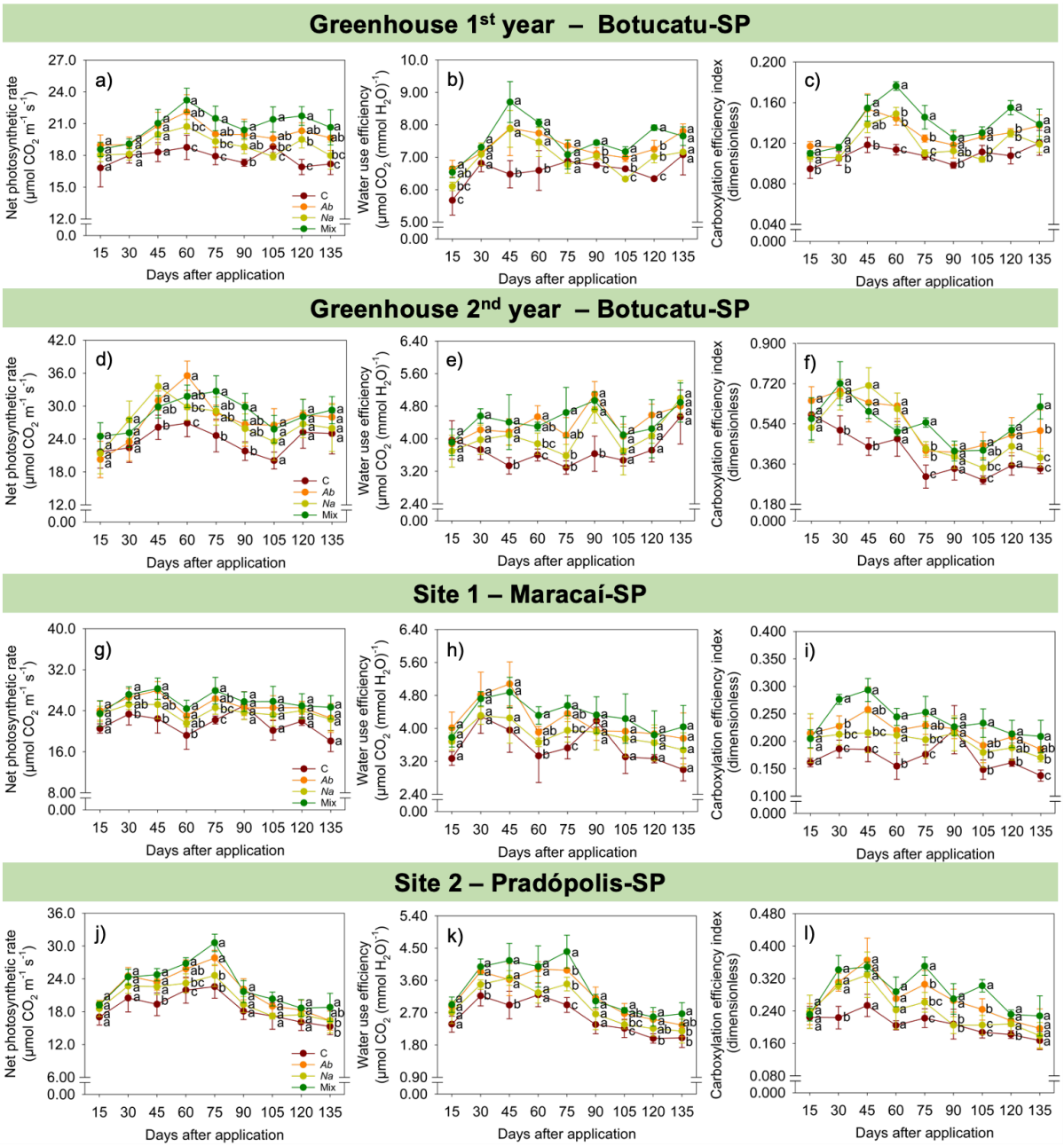


Figure 2. Effects of the treatments [Control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on the net photosynthetic rate (a, d, g, and j), water use efficiency (b, e, h and k), and carboxylation efficiency (c, f, i and l) measured in sugarcane leaves under greenhouse (a-f) and field conditions (g-l). Different lowercase letters indicate significant differences between treatments according to the LSD test at $p \leq 0.05$ in the greenhouse and $p \leq 0.10$ in the field. Error bars represent the standard error of the mean ($n = 4$ for greenhouse and $n = 8$ for field conditions).

In the second year of the greenhouse experiment, the net photosynthetic rate (Figure 2d) was significantly higher at 45 DAA in the *Ab* and *Na* treatments compared to the control. The Mix treatment exhibited the highest net photosynthetic rate between 60 and 75 DAA, whereas *Na* peaked at 45 DAA and *Ab* at 60 DAA. Water-use efficiency (Figure 2e) was significantly higher in all PGPB treatments at 45 DAA compared to the control, with a similar trend observed at 90 DAA. However, the Mix treatment maintained higher efficiency for a longer period, showing significant differences compared to the control at 45, 60, 75, and 90 DAA. Carboxylation efficiency, all PGPB treatments showed higher values than the control at 30 DAA (Figure 2f). The *Ab* and Mix treatments maintained higher levels throughout most of the evaluation period, with significant differences still observed at 135 DAA.

The results for stomatal conductance, leaf transpiration, and internal CO_2 concentration are provided in the Supplementary material (Figure S1). In the second year of the greenhouse experiment, significant differences in stomatal conductance were observed between the control and *Na* at 30 and 45 DAA, with *Na* showing higher values. At 75 DAA, the Mix treatment had the highest values among treatments, remaining superior to the control until 105 DAA. For leaf transpiration (Figure S1e), no significant differences were detected among treatments. Regarding internal substomatal CO_2 concentration, the control consistently showed the highest values throughout the evaluation period, particularly when compared to the *Ab* and Mix treatments.

1.3.3.2 Field experiment

Significant differences in photosynthetic parameters were observed in the field experiments. At Site 1, the net photosynthetic rate was higher in the *Ab* and Mix treatments between 30 and 75 DAA (Figure 2g). Regarding water-use efficiency (Figure 2h), the Mix treatment outperformed *Na* and the control at 60 and 75 DAA, while the *Ab* treatment showed a significant increase only at 75 DAA. For carboxylation efficiency (Figure 2i), the Mix treatment maintained higher values than the control throughout most of the evaluation period (30, 45, 60, 105, 120, and 135 DAA) and stood out among treatments at 30 DAA. At Site 2, the net photosynthetic rate was significantly higher in all PGPB treatments at 45 DAA, compared to the control (Figure 2j), with *Ab* and Mix maintaining higher values than the control until 75 DAA. Water-use efficiency remained lower in the control throughout most of the evaluation period,

particularly when compared to *Ab* and Mix (Figure 3k). The Mix treatment also stood out at 75 DAA, following the same trend observed for carboxylation efficiency (Figure 2l).

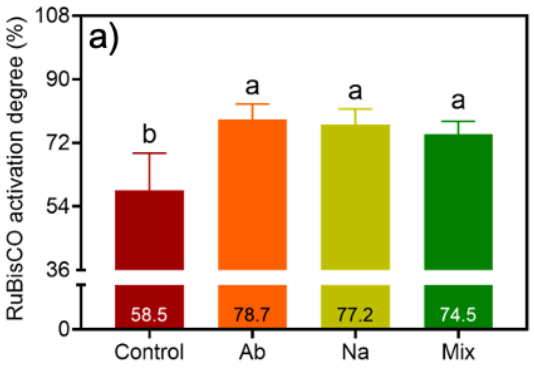
The results for stomatal conductance, leaf transpiration, and internal CO_2 concentration are available in the Supplementary Material (Figure S1). Stomatal conductance was significantly higher at 90 and 105 DAA (Figure S1g) and remained elevated in all PGPB treatments (Figure S1j) compared to the control, with *Na* and Mix showing the highest values at 135 DAA. Leaf transpiration showed no significant differences among treatments at Site 1 (Figure S1h), while at Site 2, it was higher in the control at 75 and 105 DAA (Figure S1k). Internal CO_2 concentration was reduced in the Mix treatment at 30 and 105 DAA (Figure S1i), while at 105 DAA, the control showed higher values than Mix.

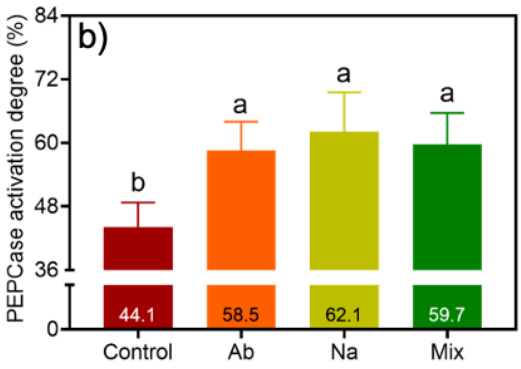
1.3.4 Degree of activation of the photosynthetic enzymes Rubisco and PEPCase

1.3.4.1 Greenhouse experiment

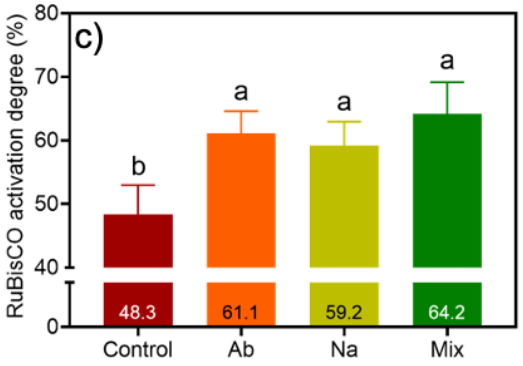
The application of microorganisms significantly increased the degree of activation of the photosynthetic enzymes RuBisCO and PEPCase, calculated as the ratio of initial to total activity. In the first year of the greenhouse experiment (Figure 3a), the *Ab*, *Na*, and Mix treatments increased RuBisCO activation by 34.5%, 32.0%, and 27.4%, respectively, compared to the control (58.5% activation degree). In the second year (Figure 3c), the degree of RuBisCO activation increased by 26.5% (*Ab*), 22.6% (*Na*), and 32.9% (Mix) compared to the control (48.3% activation degree).

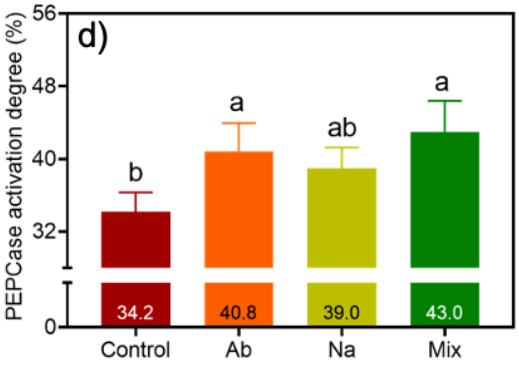
Greenhouse 1st year – Botucatu-SP



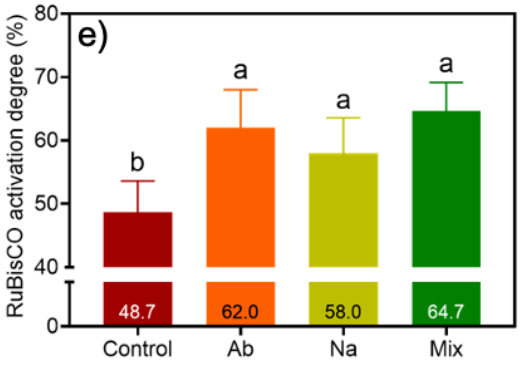


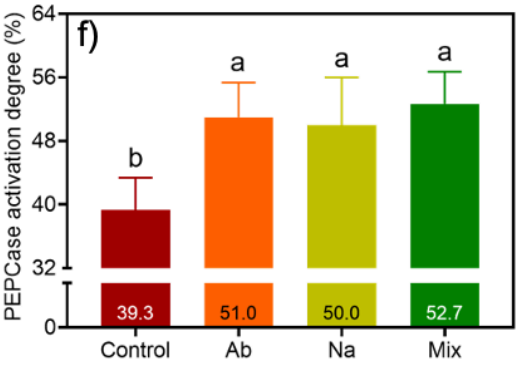
Greenhouse 2nd year – Botucatu-SP



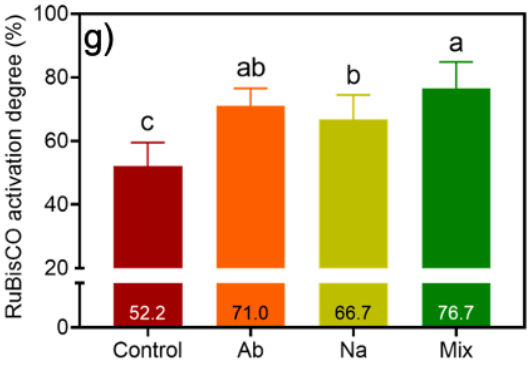


Site 1 – Maracaí-SP





Site 2 – Pradópolis-SP



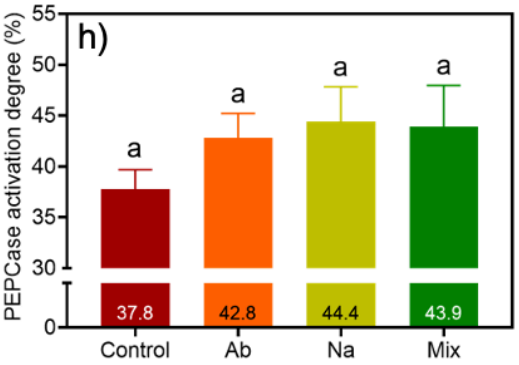


Figure 3. Effects of the treatments [Control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on the degree of activation (%) of RuBisCO (a, c, e and g) and PEPCase (b, d, f and h) in sugarcane leaves under greenhouse (a-d) and field conditions (e-h). Different lowercase letters indicate significant differences between treatments according to the LSD test at $p \leq 0.05$ in the greenhouse and $p \leq 0.10$ in the field. Error bars represent the standard error of the mean ($n = 4$ for greenhouse and $n = 8$ for field conditions).

The degree of activation of PEPCase (Figure 3b) was 32.7%, 40.8%, and 35.4% higher in *Ab*, *Na* and Mix, respectively, than in the control (44.1% of activation degree) in the first year. In the second year of the greenhouse experiment (Figure 3d), the PEPCase activation level in the control was 34.2%. The *Ab* and Mix treatments showed increases of 19.3% and 25.7%, respectively, compared to the control.

1.3.4.2 Field experiment

In the field experiments, all PGPB treatments significantly increased RuBisCO activation levels at Site 1 (Figure 3e) compared to the control (48.7%). The *Ab*, *Na*, and Mix treatments showed increases of 27%, 19.1%, and 32.9%, respectively. A similar trend was observed at Site 2 (Figure 3g), where the control had an average activation level of 52.2%, while the *Ab*, *Na*, and Mix treatments exhibited increases of 36%, 27.8%, and 46.9%, respectively. For PEPCase activation levels, all PGPB treatments at Site 1 (Figure 3f) showed significantly higher values than the control. The control presented an average activation level of 39.3%, while the *Ab*, *Na*, and Mix treatments increased it by 29.8%, 27.2%, and 34.1%, respectively. At Site 2, PEPCase activation levels did not differ significantly between treatments.

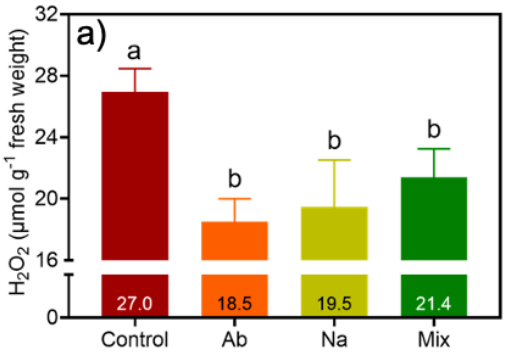
1.3.5 Oxidative stress

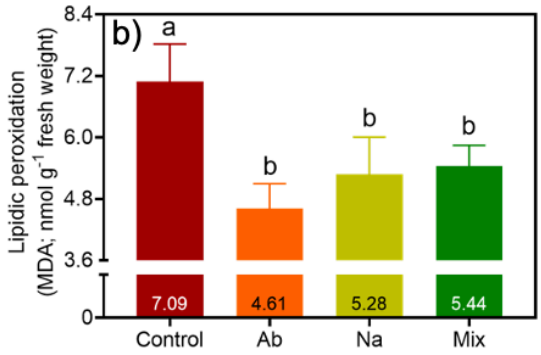
1.3.5.1 Greenhouse experiment

The application of *A. brasilense* and/or *N. amazonense* significantly influenced oxidative stress levels in sugarcane plants. In the greenhouse experiment, H_2O_2 concentration was significantly reduced by PGPB treatments. In the first year (Figure 4a), the control had the highest H_2O_2 concentration (27 μmol g^{-1} fresh weight), while the *Ab*, *Na*, and Mix treatments reduced it by 31.5%, 27.8%, and 20.7%, respectively.

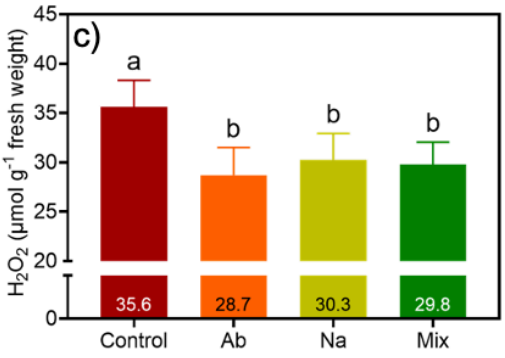
A similar trend was observed in the second year (Figure 4c), where the control exhibited an H_2O_2 concentration of 35.6 µmol g^{-1} fresh weight. Compared to the control, the *Ab*, *Na*, and Mix treatments showed reductions of 19.4%, 14.9%, and 16.3%, respectively.

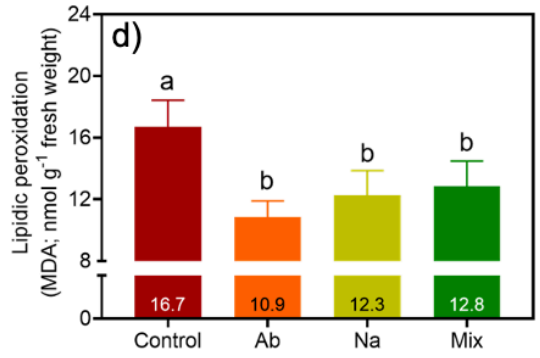
Greenhouse 1st year – Botucatu-SP



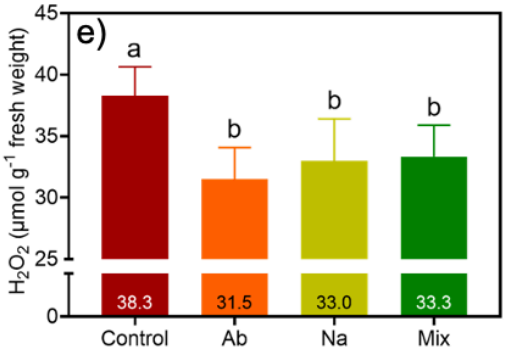


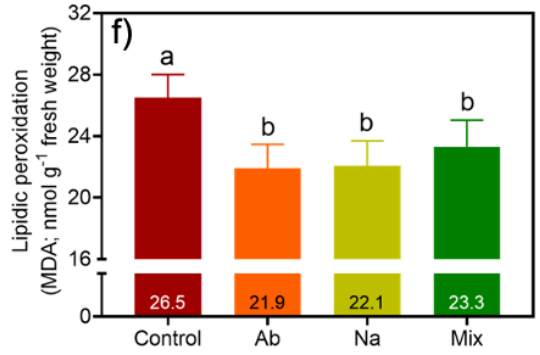
Greenhouse 2nd year – Botucatu-SP



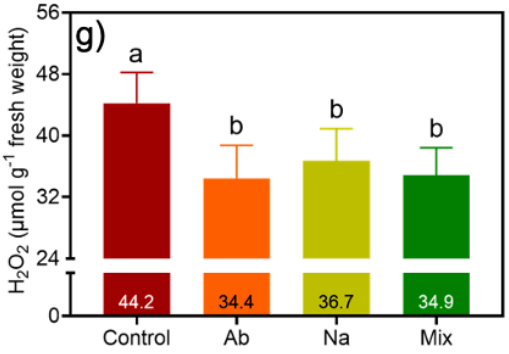


Site 1 – Maracaí-SP





Site 2 – Pradópolis-SP



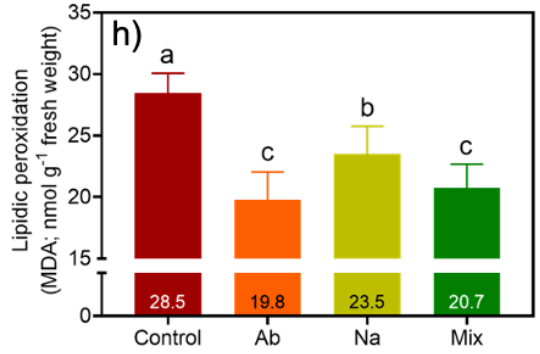


Figure 4. Effects of the treatments [Control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on hydrogen peroxide (H_2O_2) concentration (a, c e and g), and malondialdehyde (MDA) concentration (b, d, f and h) in sugarcane leaves under greenhouse (a-d) and field conditions (e-h). Different lowercase letters indicate significant differences between treatments according to the LSD test at $p \leq 0.05$ in the greenhouse and $p \leq 0.10$ in the field. Error bars represent the standard error of the mean ($n = 4$ for greenhouse and $n = 8$ for field conditions).

The concentration of MDA was also determined as an indicator of lipid peroxidation and oxidative stress. In the first year of the greenhouse experiment (Figure 4b), the MDA concentration was 35.0%, 25.5%, and 23.3% lower in *Ab*, *Na*, and Mix, respectively, than in the control (7.09 nmol g^{-1} fresh weight). In the second year (Figure 4d), the MDA concentration was 34.7%, 26.3%, and 23.4% lower in *Ab*, *Na*, and Mix, respectively, than in the control (16.7 nmol g^{-1} fresh weight).

1.3.5.2 Field experiment

Significant differences in oxidative stress levels were observed in the field experiments (Figure 4). At both sites, H_2O_2 concentrations were significantly higher in the control compared to the PGPB treatments. At Site 1, H_2O_2 concentrations were 17.8%, 13.8%, and 13.1% lower in *Ab*, *Na*, and Mix, respectively, compared to the control (38.3 µmol g^{-1} fresh weight). A similar trend was observed at Site 2, where H_2O_2 concentrations were reduced by 22.2% (*Ab*), 17% (*Na*), and 21% (Mix) relative to the control (44.2 µmol g^{-1} fresh weight). Lipid peroxidation, measured as malondialdehyde (MDA) concentration, was also significantly lower in PGPB treatments at both sites. At Site 1, MDA concentrations were 17.4%, 16.6%, and 12.1% lower in *Ab*, *Na*, and Mix, respectively, compared to the control (26.5 nmol g^{-1} fresh weight). At Site 2, MDA concentrations decreased by 30.5% (*Ab*), 17.5% (*Na*), and 27.4% (Mix), relative to the control (28.5 nmol g^{-1} fresh weight), with the lowest values observed in *Ab* and Mix.

1.3.6 Antioxidant metabolism activity

1.3.6.1 Greenhouse experiment

The application of *A. brasilense* and/or *N. amazonense* significantly influenced the activity of antioxidant enzymes in sugarcane plants (Figure 5). In the first year,

SOD activity was significantly lower in the control (50.3 Units SOD mg^{-1} protein) compared to all PGPB treatments. The *Ab* treatment increased SOD activity by 66.0%, followed by *Na* (25.6%) and Mix (30.4%) relative to the control. However, CAT activity did not show significant differences among treatments. In the second year, SOD activity remained lower in the control (193 Units SOD mg^{-1} protein), with increases of 13% (*Ab*) and 9.8% (*Na*) compared to the control. Conversely, CAT activity increased by 27.9% (*Ab*) and 16% (Mix) relative to the control (0.437 µmol min^{-1} mg^{-1} protein).

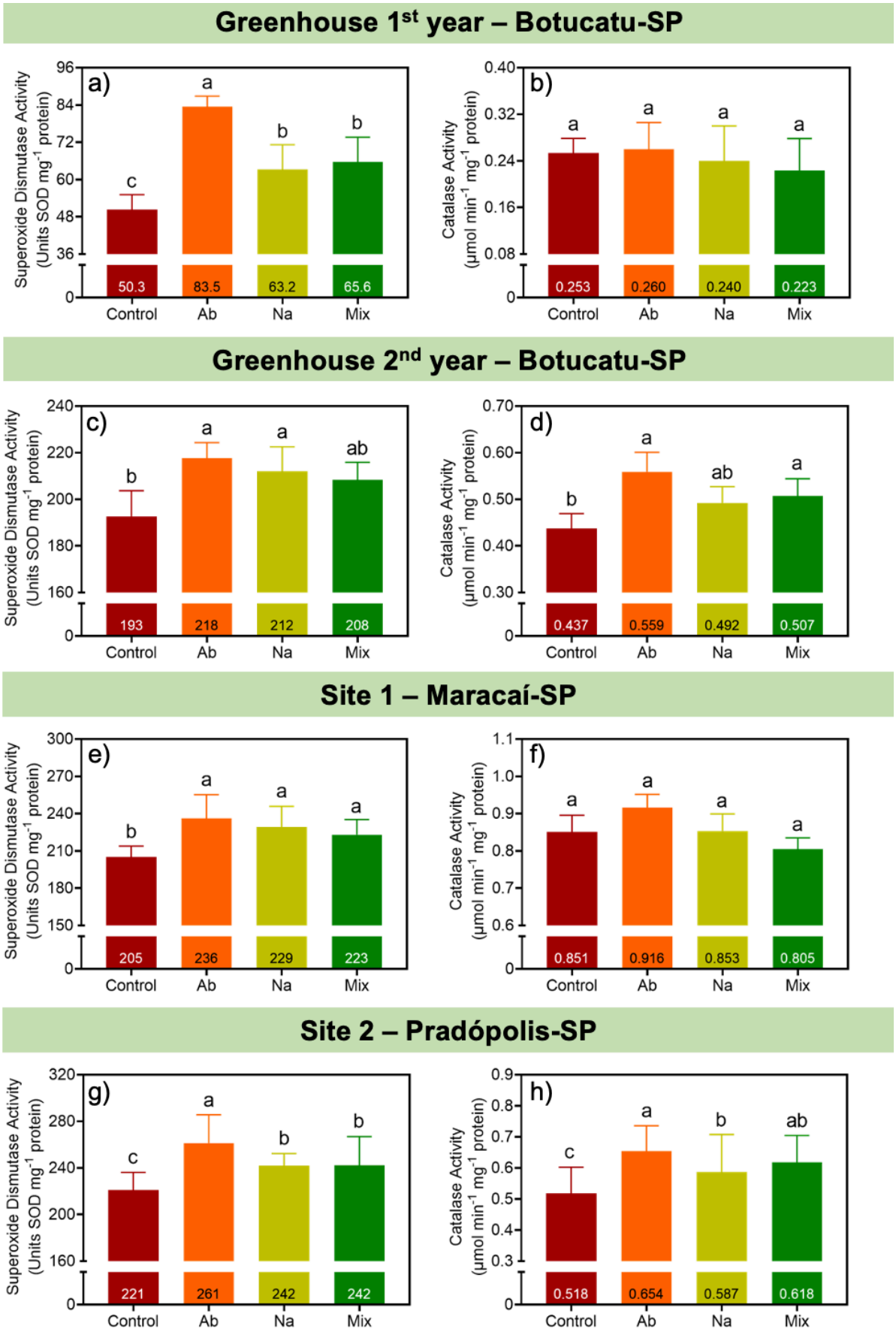

Greenhouse 1st year – Botucatu-SP
a)
Superoxide Dismutase Activity (Units SOD mg-1 protein)
96
84
72
60
48
36
0
c
a
b
b
50.3
83.5
63.2
65.6
Control
Ab
Na
Mix
b)
Catalase Activity (µmol min-1 mg-1 protein)
0.40
0.32
0.24
0.16
0.08
0
a
a
a
a
0.253
0.260
0.240
0.223
Control
Ab
Na
Mix
Greenhouse 2nd year – Botucatu-SP
c)
Superoxide Dismutase Activity (Units SOD mg-1 protein)
240
220
200
180
160
0
b
a
a
ab
193
218
212
208
Control
Ab
Na
Mix
d)
Catalase Activity (µmol min-1 mg-1 protein)
0.70
0.60
0.50
0.40
0.30
0
b
a
ab
a
0.437
0.559
0.492
0.507
Control
Ab
Na
Mix
Site 1 – Maracaí-SP
e)
Superoxide Dismutase Activity (Units SOD mg-1 protein)
300
270
240
210
180
150
0
b
a
a
a
205
236
229
223
Control
Ab
Na
Mix
f)
Catalase Activity (µmol min-1 mg-1 protein)
1.1
1.0
0.9
0.8
0.7
0.6
0
a
a
a
a
0.851
0.916
0.853
0.805
Control
Ab
Na
Mix
Site 2 – Pradópolis-SP
g)
Superoxide Dismutase Activity (Units SOD mg-1 protein)
320
280
240
200
160
0
c
a
b
b
221
261
242
242
Control
Ab
Na
Mix
h)
Catalase Activity (µmol min-1 mg-1 protein)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
c
a
b
ab
0.518
0.654
0.587
0.618
Control
Ab
Na
Mix

Figure 5. Effects of the different treatments [Control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on superoxide dismutase (SOD) activity (a, c, e and g), catalase (CAT) activity (b, d, f and h) in sugarcane leaves under greenhouse (a-d) and field conditions (e-h). Different lowercase letters indicate significant differences between treatments according to the LSD test at $p \leq 0.05$ in the greenhouse and $p \leq 0.10$ in the field. Error bars represent the standard error of the mean ($n = 4$ for greenhouse and $n = 8$ for field conditions).

1.3.6.2 Field experiment

Significant differences in antioxidant metabolism activity levels between the treatments were also observed in the field experiments. At site 1, SOD activity (Figure 5e) was 15.1%, 11.7%, and 8.8% higher in *Ab*, *Na*, and Mix, respectively, than in the control (193 Units SOD mg^{-1} protein). However, catalase activity (Figure 5f) did not differ between the treatments. At site 2, SOD activity (Figure 5g) was 18.1%, 9.5%, and 9.5% higher in *Ab*, *Na*, and Mix, respectively, than in the control (221 Units SOD mg^{-1} protein). However, CAT activity (Figure 5h) showed important differences between treatments. The control showed lower CAT activity (0.518 µmol min^{-1} mg^{-1} protein), while *Ab, Na and Mix* showed significant increase of 26.3%, 13.3% and 19.3%, respectively.

1.3.7 Biometric variables under greenhouse conditions and field conditions

1.3.7.1 Greenhouse experiment

In the first year of greenhouse cultivation (Table 4), plant height, stalk diameter, and the dry matter of roots, stalks, and leaves varied significantly among treatments. Sugarcane plants were significantly taller in *Ab*, *Na*, and Mix than in the control, with average plant height in the control reaching 123 cm. Compared to the control, *Ab*, *Na*, and Mix increased plant height by 13.8%, 18.7%, and 21.1%, respectively. However, stalk diameter increased significantly only in Mix, with a 17.8% increase compared to the control (18.5 mm). Root dry matter accumulation was highest in Mix, followed by *Ab* and *Na*, with all treatments showing significantly greater values than the control (55.5 g), with increases of 50.5% (Mix), 37.8% (*Ab*), and 23.4% (*Na*). Similarly, stalk dry matter accumulation was significantly higher in Mix, with a 16.8% increase relative

to the control (241 g). Leaf dry matter accumulation also increased significantly in Mix, with an 11.6% increase compared to the control (112 g).

Table 4. Effects of the different treatments [control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*) and Mix (*Ab* + *Na*)] on plant height, stalk diameter, root dry matter (RDM), stalk dry matter (SDM), leaf dry matter (LDM) and rhizome dry matter (RzDM) of sugarcane in the greenhouse experiments, and stalk height, stalk diameter, number of stalks per meter, and weight of 20 stalks, at the two sites of the field experiment (site 1, Maracaí-SP; site 2, Pradópolis-SP).

Greenhouse 1st year – Botucatu-SP						
Treat.	**Plant height**	**Stalk diameter**	**RDM**	**SDM**	**LDM**	**RzDM**
	cm	mm	g plant^{-1}			
C	123b ± 5.33	18.5b ± 0.23	55.5 c ± 1.11	241b ± 3.54	112 b ± 6.90	n/a
Ab	140a ± 2.46	19.2b ± 0.43	76.5 ab ± 6.05	261ab ± 9.72	122 ab ± 2.02	n/a
Na	146a ± 4.10	18.8b ± 0.30	68.5 b ± 4.14	259b ± 8.78	115 b ± 3.82	n/a
Mix	149a ± 3.71	21.8a ± 0.55	83.5 a ± 5.91	282a ± 5.24	125 a ± 10.0	n/a
Greenhouse 2nd year – Botucatu-SP						
Treat.	**Plant height**	**Stalk diameter**	**RDM**	**SDM**	**LDM**	**RzDM**
	cm	mm	g plant^{-1}			
C	64.9b ± 1.79	24.5a ± 1.19	89a ± 14.3	119c ± 2.91	168a ± 4.33	186a ± 4.81
Ab	78.1a ± 1.30	24.8a ± 0.32	106a ± 9.62	152a ± 1.45	183a ± 3.33	200a ± 5.61
Na	75.9a ± 3.72	24.0a ± 0.87	84.5a ± 6.13	141ab ± 2.67	176a ± 7.02	183a ± 15.9
Mix	70.5ab ± 3.38	24.4a ± 0.97	108a ± 13.2	135b ± 7.02	181a ± 6.23	214a ± 7.84
Site 1 – Maracaí-SP						
Treat.	**Plant height**	**Stalk diameter**	**Number of stalks m^{-1}**	**Weight of 1 stalk**		
	cm	mm	-	kg stalk^{-1}		
C	270b ± 2.72	26.2a ± 0.68	11.5a ± 0.35	1.31a ± 0.02		
Ab	289a ± 4.50	27.4a ± 0.60	11.9a ± 0.83	1.42 a ± 0.13		
Na	280ab ± 3.23	26.9a ± 0.39	11.8a ± 0.43	1.39 a ± 0.03		
Mix	292a ± 10.1	28.0a ± 0.63	11.4a ± 0.59	1.46 a ± 0.02		
Site 2 – Pradópolis-SP						
Treat.	**Plant height**	**Stalk diameter**	**Number of stalks m^{-1}**	**Weight of 1 stalk**		
	cm	mm	-	kg stalk^{-1}		
C	217b ± 8.07	24.7a ± 0.34	13.0 a ± 0.35	0.95 a ± 0.01		
Ab	236a ± 3.57	24.0a ± 0.30	13.5 a ± 0.54	1.03 a ± 0.05		
Na	228ab ± 6.28	24.5a ± 0.17	13.0 a ± 0.54	1.00 a ± 0.02		
Mix	234a ± 4.30	25.2a ± 0.52	14.4 a ± 0.43	0.99 a ± 0.05		

Different letters indicate significant differences between treatments, by the LSD test at $p \leq 0.05$ ($n = 4$) in the greenhouse experiments and LSD test at $p \leq 0.10$ ($n = 8$) in the field conditions.

n/a: not applicable - There was no rhizome collection in the first year. The experiment was only dismantled at the end of the second cultivation.

In the second year (Table 4), plants were tallest in *Ab* and *Na*, compared to the control (64.9 cm), showing an increase of 20.3% and 16.9%, respectively. Stalk dry matter was significantly higher in *Ab* (an increase of 27.7%) than in the control, which

had the lowest dry matter (119 g). *Na* and Mix had intermediate values of stalk dry matter (with an increase of 18.49% and 13.45%, respectively) that were significantly greater than that in the control. Stalk diameter, leaf dry matter, root dry matter, and rhizome dry matter (evaluated only in the second year) did not differ significantly between the treatments.

1.3.7.2 Field experiment

At Site 1 (Table 4), stalk height varied significantly among treatments. The control had the shortest stalks, with an average height of 270 cm, while the *Ab* and Mix treatments showed increases of 7.04% and 8.15%, respectively. However, stalk diameter, the number of stalks per meter, and the weight of stalks did not differ significantly among treatments. Similarly, at Site 2 (Table 4), stalk height was significantly lower in the control (217 cm) compared to the *Ab* and Mix treatments, which showed increases of 8.76% and 7.83%, respectively. As in Site 1, no significant differences were observed for stalk diameter, the number of stalks per meter, or the weight stalks.

1.3.8 Technological parameters under field conditions

The technological variables Pol, purity, fiber content, RS, and TRS did not differ significantly between the treatments at either field site. These results are detailed in Table S1 in the Supplementary Material.

1.3.9 Stalk and sugar yield under field conditions

At Site 1, stalk and sugar yields were significantly higher in all PGPB treatments compared to the control (Figure 6a,b). Stalk yield increased by 9.52%, 7.93%, and 9.52% in *Ab*, *Na*, and Mix, respectively, compared to the control (126 tons of stalks ha^{-1}). Similarly, sugar yield (Figure 6b) was 13.2%, 9.91%, and 12.4% higher in *Ab*, *Na*, and Mix, respectively, than in the control (24.4 tons of sugar ha^{-1}). At Site 2, stalk and sugar yields per hectare increased significantly only in *Ab* and Mix compared to the control (Figure 6c,d). The average stalk yield per hectare was 12.2% and 14.9% higher in *Ab* and Mix, respectively, than in the control (81.8 tons ha^{-1}). Similarly, sugar yield

was 13.4% and 14.8% higher in *Ab* and Mix, respectively, than in the control (14.2 tons ha^{-1}). These results demonstrate that the beneficial effects of PGPB on plant metabolism generated a cascading effect, leading to significant increases in crop productivity. On average, stalk yield increased by 10.9% (*Ab*) and 12.2% (Mix) across both environments. Similarly, sugar yield increased by 13.3% (*Ab*) and 13.7% (Mix) compared to the control.

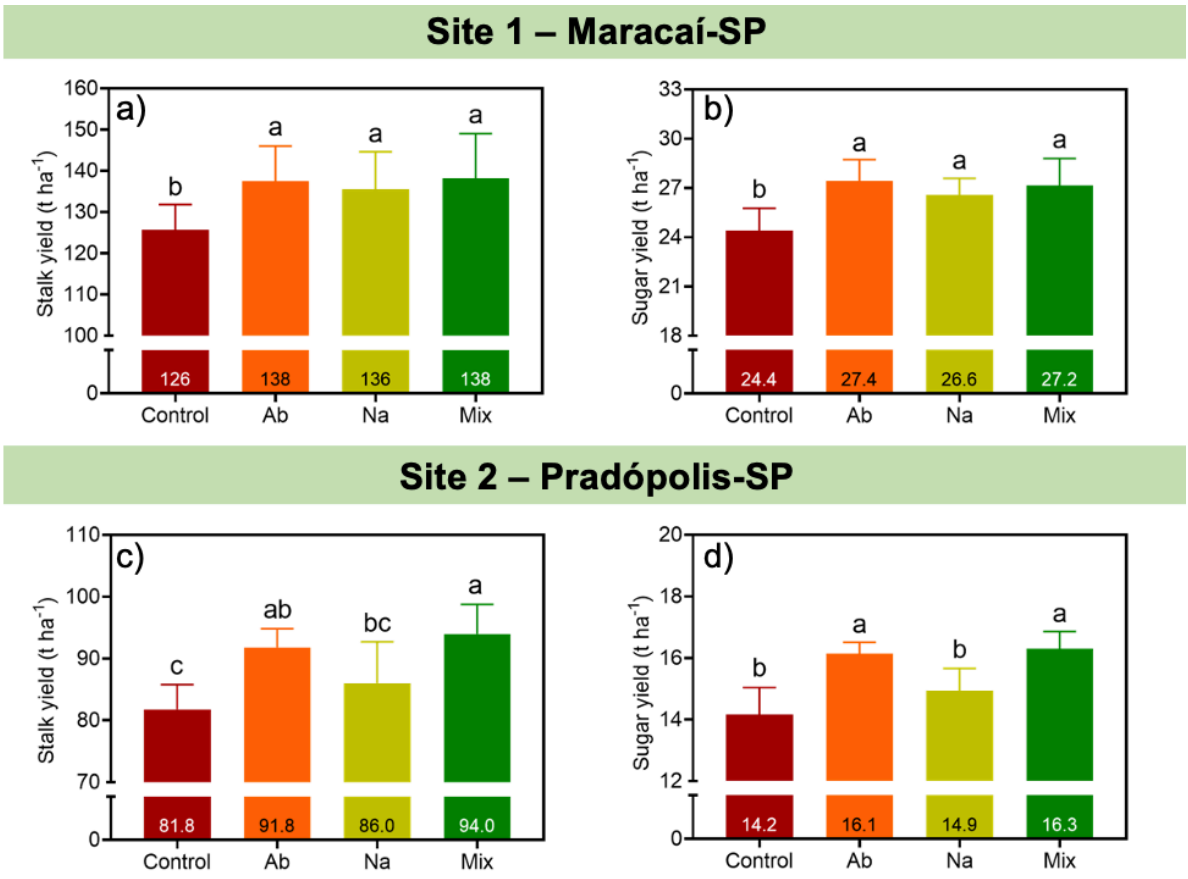


Figure 6. Effects of the treatments [control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on stalk yield (a, c) and sugar yield (b,d) per hectare at the two field experiment sites: Maracaí-SP (site 1) and Pradópolis-SP (site 2). Different letters indicate significant differences between treatments according to the LSD test at $p \leq 0.10$. Error bars represent the standard error of the mean ($n = 8$).

1.4 DISCUSSION

In sugarcane cultivation, the increasing demand for cost-effective and sustainable strategies to boost productivity and longevity highlights beneficial microorganisms as a promising solution. To the best of our knowledge, this is the first report to investigate the combined use of two widely applied microorganisms in agricultural systems, *Azospirillum brasilense* and *Nitrospirillum amazonense,* with the aim of elucidating their effects on sugarcane's nutritional status, as well as its physiological, biochemical, and productivity responses.

1.4.1 PGPB inoculation improves leaf contents of nutrients in sugarcane

The results from both greenhouse and field experiments demonstrated the positive effects of applying plant growth-promoting bacteria (PGPB) on the nutritional content of sugarcane leaves, particularly nitrogen (N). These increases can be attributed to the ability of PGPB to enhance nutrient availability in the soil and improve plant uptake, likely through mechanisms such as biological nitrogen fixation, nutrient solubilization, and modulation of plant metabolism (Fukami et al., 2018a; Schwab et al., 2018; Scudeletti et al., 2023).

In Table 3, it is possible to observe significant differences in leaf N content between treatments. In the first year, under greenhouse conditions, the Mix treatment showed the highest increase (21.7%), followed by *Azospirillum brasilense* (*Ab*, 12.6%) and *Nitrospirillum amazonense* (*Na*, 8.6%). This pattern remained consistent in the second year, with Mix and Ab standing out compared to the control. In field experiments, the results corroborated those observed in the greenhouse, with increased leaf N content across all PGPB treatments, ranging from 8.3% to 11.1% at both field sites.

The improvement in N content reflects greater efficiency in the supply of this essential element, which is critical for plant growth and development. Both microorganisms are diazotrophic and capable of promoting nitrogen fixation, thus influencing total N content in plants (Fukami et al., 2018b; Cipriano et al., 2021). *A. brasilense* possesses genes associated with biological nitrogen fixation (BNF), while *N. amazonense* is officially registered as a nitrogen-fixing bacterium in Brazil (Schwab *et al.*, 2023). These microorganisms stimulate root system growth by

producing phytohormones and Siderophores (Spaepen and Vanderleyden, 2015; Fukami et al., 2018b), contributing to increased water and nutrient uptake (Pii *et al.*, 2015; Cipriano *et al.*, 2021).

In addition to N, magnesium (Mg) and boron (B) levels were also positively influenced by the treatments, though the effects varied across experiments. In the greenhouse experiment (Table 3), the significant increase in Mg content in *Ab* and Mix suggests that these PGPB may influence processes related to the solubilization or mobility of this nutrient in the soil. Meanwhile, the higher B concentration in leaves treated with *Ab* and Mix aligns with reports indicating that growth-promoting bacteria can release organic compounds that enhance micronutrient availability (Masood *et al.*, 2019; Jalal *et al.*, 2024).

Notably, Mg content also increased significantly at Site 2 for all PGPB treatments, while B levels rose in *Na* and Mix at Site 1 and in *Ab* and *Na* at Site 2. These variations among treatments suggest that the response of microorganisms may depend on edaphoclimatic conditions, as these living organisms can exhibit varying sensitivity to specific environmental factors (Lopes *et al.*, 2021). However, further studies are required to evaluate these variations in greater depth.

1.4.2 Application of the combination of *A. brasilense* and *N. amazonense* enhances photosynthesis in sugarcane

The increased assimilation of nutrients, particularly N and Mg, may have directly influenced the concentrations of photosynthetic pigments in sugarcane leaves, as these nutrients are integral structural components of the chlorophyll molecule (Tantray *et al.*, 2020; Li *et al.*, 2021). In photosynthesis, chlorophylls are responsible for capturing light, which excites electrons used to produce reducing and energetic compounds (NADPH and ATP) during the Calvin cycle reactions (Croft *et al.*, 2017; Bossolani *et al.*, 2022). Although higher chlorophyll concentrations may indicate a potential positive effect on photosynthesis, several other factors can negatively impact this physiological process (Teskey *et al.*, 1995). Thus, direct measurements of photosynthesis and the enzymes involved in this process can provide clearer insights.

To better understand the impact of microbial inoculation on photosynthesis in sugarcane plants, various analyses were conducted using an IRGA to observe fluctuations in photosynthesis during the crop cycle (Figure 2). Overall, all PGPB

treatments showed superior photosynthetic performance compared to the control, with improvements in the net photosynthetic rate, water use efficiency, and carboxylation efficiency. These improvements were greatest in the *Ab* and Mix. Previous studies have suggested that PGPB can enhance photosynthesis in various crops, such as wheat and maize, by improving nutrient absorption and increasing root growth (Harish *et al.*, 2022). It is well established that PGPB contribute to plant development by producing phytohormones such as auxins, cytokinins, and gibberellins, which promote root development and improve water and nutrient absorption (Fukami et al., 2018b).

The increase in root area enables more efficient soil exploration in search of environmental resources. This greater nutrient availability may have influenced the increase in the photosynthetic rate, as nutrients such as N and Mg are essential for photosynthesis, protein synthesis, chlorophyll production, and the activity of photosynthetic enzymes (Kumagai *et al.*, 2014; Tantray *et al.*, 2020; Li *et al.*, 2021). When nutrient availability increases, plants can fix more CO_2 and use water more efficiently, consistent with the observed increase in stomatal conductance and lower CO_2 concentration in the sub-stomatal chamber of sugarcane leaves in the Mix treatment during most of the assessed periods. Although peaks in photosynthesis, water use efficiency, and carboxylation efficiency were observed in all treatments at different points throughout the crop cycle, likely due to changes in environmental conditions and the response of the genetic material to those changes (Schultz *et al.*, 2017), microbial inoculation clearly produced better results. The combination of *A. brasilense* and *N. amazonense* (Mix treatment) proved to be a viable strategy for enhancing the photosynthetic metabolism of sugarcane throughout the study period under both field and greenhouse conditions.

Photosynthesis in C4 plants is governed by the enzymes PEPCase and RuBisCO (Taiz *et al.*, 2017). The presence of PEPCase in the cytosol of mesophyll cells in sugarcane allows the plant to utilize a CO_2 concentration strategy. Initially, PEPCase fixes CO_2 into a four-carbon compound, oxaloacetate, through carboxylation. This compound is then transported to the bundle sheath cells, where RuBisCO operates more efficiently, minimizing photorespiration (Taiz *et al.*, 2017). This CO_2 concentration mechanism enhances both photosynthetic efficiency and water use efficiency in C4 plants. Consequently, the quantification of these enzymes can provide precise information to support the results obtained by IRGA.

Our results showed that PGPB application increased the degrees of activation of RuBisCO and PEPCase in sugarcane leaves (Figure 3). The higher concentrations of N and Mg in the plant tissue may have contributed to these enhanced enzymatic activities, as these elements play crucial roles not only in photosynthesis but also the composition and activation of these enzymes (Taiz *et al.*, 2017). N is a key component of RuBisCO's structure and function (Tantray *et al.*, 2020) and forms part of the enzyme's active site (Kumagai *et al.*, 2014). Additionally, RuBisCO contains a magnesium ion (Mg^{2+}) that stabilizes the formation of the transition state during carboxylation and is essential for catalytic activity (Parry *et al.*, 2008; Li *et al.*, 2021). Interestingly, N and Mg were the nutrients in sugarcane leaves that were most influenced by the application of PGPB. These results, especially those in the Mix treatment, further support the hypothesis that improved nutrition can result in higher RuBisCO activation levels. While total enzyme activity provides a good indication of improvements in photosynthetic metabolism, measuring the degree of activation offers more accurate insights into the plant's actual metabolic state.

1.4.3 Oxidative stress is reduced in sugarcane inoculated with PGPB

In addition to enhancing photosynthetic activity in sugarcane plants, the inoculation of sugarcane with PGPB reduced oxidative stress (Figure 4). Most plant stress originates from failures in the photosynthetic process (Hajiboland, 2014), which lead to increased concentrations of reactive oxygen species (ROS). The concentration of H_2O_2 serves as a marker of oxidative stress in plants (Dumanović *et al.*, 2021). Under both greenhouse and field studies (across two sites), the PGPB treatments resulted in lower sugarcane leaf H_2O_2 concentrations compared to those in the control. These decreases were particularly evident in the treatments with *A. brasilense* (*Ab* or Mix). The reduction in H_2O_2 levels suggests enhanced antioxidant efficiency, likely due to the upregulation of antioxidant enzyme activity. This is consistent with studies indicating that *A. brasilense* improves plant tolerance to oxidative stress by reducing H_2O_2 levels (Fukami et al., 2018b; c).

Malondialdehyde (MDA) is a well-established marker of lipid peroxidation and oxidative stress. Across both years of the greenhouse experiment and both field sites, the PGPB treatments consistently resulted in lower sugarcane leaf MDA concentrations compared to those in the control. The reduction in MDA levels indicates

decreased lipid peroxidation and, consequently, reduced oxidative damage to the cell membranes of plants treated with PGPBs. Studies have suggested that inoculation with *A. brasilense* (either alone or in combination with *N. amazonense*) can lower MDA levels, protecting plants against oxidative damage and improving cell membrane integrity (Dumanović *et al.*, 2021).

Superoxide dismutase (SOD) is a crucial antioxidant enzyme that protects cells from damage caused by reactive oxygen species (ROS). Our results showed that SOD activity was significantly higher in all treatments that received PGPB application, with the *Ab* treatment standing out, particularly in the first year under greenhouse conditions and at field site 2. (Figures 5a and 5g). Studies have shown that *A. brasilense* can induce the production of antioxidant enzymes, enhancing plant resistance to oxidative stress (Fukami *et al.*, 2018c). The increase in SOD activity may be attributable to greater nutrient availability and the microbial production of phytohormones, which stimulate the plant's antioxidant defenses (Koza *et al.*, 2022). In addition, catalase (CAT) is another important antioxidant enzyme that decomposes H_2O_2 into water and oxygen, thus protecting cells from oxidative stress (Sharma and Ahmad, 2014). In the first year of the greenhouse experiment and in the field experiment at site 1 (Figures 5b and 5f), CAT activity did not differ significantly between the treatments. However, in the second year of the greenhouse experiment and in the field experiment at site 2 (Figures 5d and 5h), CAT activity was significantly higher in the *Ab* and Mix treatments than in the control. The increases in CAT activity in sugarcane plants in the *Ab* and Mix treatments indicate an enhanced antioxidant response and improved tolerance to oxidative stress. Studies have indicated that inoculation with *A. brasilense* can enhance CAT activity, helping plants cope with oxidative stress caused by adverse conditions (Fukami *et al.*, 2018c). Our results suggest that inoculation with *A. brasilense* provides more significant benefits to antioxidant metabolism than inoculation with *N. amazonense*. However, the combination of these two microorganisms had superior effects on photosynthetic parameters, indicating that the combined use of these technologies should not be disregarded.

1.4.4 PGPB inoculation improves sugarcane productivity by improving photosynthesis and alleviating oxidative stress

In summary, sugarcane inoculation with plant growth-promoting bacteria (PGPB) significantly improved the photosynthetic performance of the plants, reduced the accumulation of reactive oxygen species (ROS), and enhanced the activity of antioxidant enzymes. Since much of plant stress arises from compromised photosynthesis, the benefits of PGPB inoculation in boosting photosynthesis efficiency play a crucial role in alleviating stress. Additionally, the reduction in ROS production minimizes the degradation of proteins, DNA, RNA, and, most importantly, phospholipid membranes. Preserving these membranes is vital to maintaining cellular selectivity and compartmentalization (Matoso *et al.*, 2021). With improved photosynthetic metabolism and enhanced anti-stress mechanisms, plants exhibit a greater capacity to accumulate biomass (Rampazzo *et al.*, 2018). These cascading effects result in increased productivity, as observed in our results.

Among the treatments evaluated, those involving *A. brasilense* (*Ab* and Mix) were particularly effective in enhancing sugarcane productivity across both sites (Figure 6). However, the *Nitrospirillum amazonense* (*Na*) treatment displayed yield variations, suggesting that factors such as soil type, nutrient availability, and/or environmental stressors likely influenced the differential responses observed, as microorganisms may respond differently to various stimuli. This variability highlights the need for further investigations to optimize the use of these technologies under varying edaphoclimatic conditions.

As expected, inoculation did not affect sugarcane technological parameters such as fiber content, purity, reducing sugars (RS), and total recoverable sugars (TRS) (Table S1 - supplementary material). Therefore, the observed increase in sugar yield per hectare was solely attributed to the higher stalk production, without alterations in the metabolic pathways of sugar synthesis (Scudeletti *et al.*, 2023). The ultimate goal of this research is to maximize sugarcane productivity, measured as tons of fresh stalks with higher sugar accumulation, in a sustainable manner. The consistent yield increases observed for the *Ab* and Mix treatments across both sites underscore their reliability and potential for large-scale application. Although the benefits of using PGPB have been evident, the differences between the *Ab* and Mix treatments were not pronounced. However, under adverse conditions, such as challenging climatic or soil scenarios, the differences between the treatments could be more pronounced, as the use of these PGPB improved physiological parameters, which could contribute to greater resilience and, consequently, increased crop productivity in a sustainable

manner. Therefore, more studies are needed to assess the use of these microorganisms under different cultivation conditions.

1.5 CONCLUSION

This study highlights that the inoculation of sugarcane with plant growth-promoting bacteria (PGPB) offers significant benefits to crop metabolism. The application of *Azospirillum brasilense* (*Ab*), either individually or in combination with *Na* (Mix), resulted in substantial improvements, including higher nutrient content in the leaves (especially nitrogen), greater photosynthetic efficiency, reduced oxidative stress, and increased yield of stalks and sugar per hectare compared to the control.

The variations in responses observed with the use of these strains, applied individually or in combination, underscore the need to refine these technologies to enhance their effectiveness in diverse environments. Although the benefits of using PGPB were evident, the differences between the *Ab* and Mix treatments were not pronounced. However, under adverse conditions, these differences may become more apparent, as the physiological changes promoted by these bacteria provide greater resilience for plants to adapt to environmental stresses. Thus, further investigations are needed to maximize the benefits provided by these bacteria, both individually and in combination, under various cultivation conditions.

This work reinforces the essential role of innovative biological technologies in advancing sustainable agriculture, contributing to overcoming global challenges and promoting more balanced and environmentally responsible production systems.

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SUPPLEMENTARY MATERIAL

Table S1 – Effect of different treatments [control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on technological parameters of cane juice: POL, purity, fiber content, reducing sugars (RS), and total recoverable sugars (TRS), at two locations (Site 1, Maracaí-SP; Site 2, Pradópolis-SP).

Site 1 – Maracaí-SP					
Treat.	POL (%)	Purity (%)	Fiber (%)	RS (%)	TRS kg ton^{-1}
C	19.4a ± 0.55	90.2a ± 0.29	13.7a ± 0.44	0.45a ± 0.01	158a ± 3.94
Ab	19.6a ± 0.44	90.1a ± 0.57	12.9a ± 0.05	0.46a ± 0.02	162a ± 3.58
Na	19.9a ± 0.28	90.6a ± 0.22	13.5a ± 0.21	0.44a ± 0.01	162a ± 2.32
Mix	19.7a ± 0.38	90.0a ± 0.70	13.2a ± 0.19	0.46a ± 0.19	161a ± 3.07
Site 2 – Pradópolis-SP					
Treat.	POL (%)	Purity (%)	Fiber (%)	RS (%)	TRS kg ton^{-1}
C	21.2a ± 0.25	84.3a ± 1.64	14.0a ± 0.27	0.73a ± 0.07	173a ± 1.93
Ab	21.6a ± 0.30	87.3a ± 1.68	14.1a ± 0.29	0.65a ± 0.06	174a ± 2.17
Na	21.2a ± 0.26	87.0a ± 1.10	13.9a ± 0.37	0.66a ± 0.04	172a ± 2.84
Mix	21.3a ± 0.30	86.2a ± 1.58	14.1a ± 0.20	0.68a ± 0.05	172a ± 2.02

Different letters indicate significant differences between treatments by the LSD test $p \leq 0.10$ ($n = 8$).

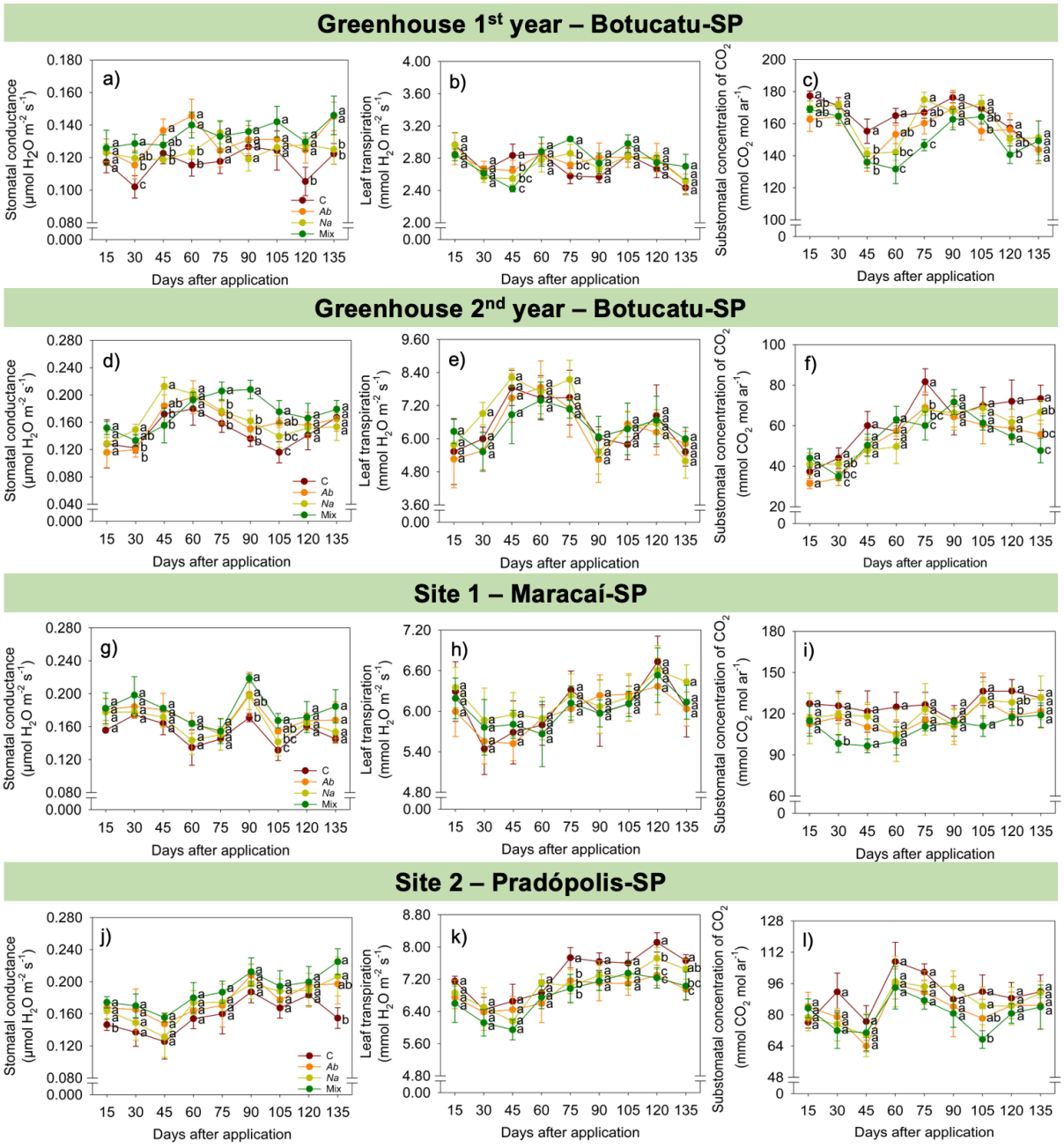

Figure S1 – Effect of treatments [control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)] on stomatal conductance (a, d, g, and j), leaf transpiration (b, e, h, and k), and sub-stomatal CO_2 concentration (c, f, i, and l), measured in sugarcane leaves under greenhouse and field conditions. Lowercase letters indicate significant differences between treatments, according to LSD test at $p \leq 0.05$ in the greenhouse and $p \leq 0.10$ in the field. Error bars represent the standard error of the mean ($n = 4$ for greenhouse and $n = 8$ for field conditions).

CHAPTER 2

SYNERGISTIC EFFECTS FROM COMBINING FOLIAR AND SOIL INOCULATION OF PLANT GROWTH PROMOTING MICROBES: ENHANCED SUGARCANE PHYSIOLOGY AND RHIZOSPHERE MODULATION VIA ORGANIC ACID EXUDATION AND NITROGEN - CYCLING[2]

Abstract

Sugarcane (*Saccharum officinarum* L.) is a strategic crop for the energy sector, but its intensive cultivation requires sustainable management practices. Plant growth-promoting bacteria (PGPB) such as *Azospirillum brasilense* and *Nitrospirillum amazonense* are known for their ability to fix atmospheric nitrogen and stimulate growth in grasses. This study evaluated the effects of inoculation with *A. brasilense* (*Ab*) and *N. amazonense* (*Na*), applied individually or in combination (*Ab*+*Na*), on sugarcane physiology, nitrogen use efficiency, root exudation, and rhizospheric microbial communities. The experiment was conducted under controlled greenhouse conditions with four treatments: Control (no inoculation), *Ab*, *Na*, and *Ab*+*Na*. The *Ab*+*Na* treatment promoted the highest photosynthetic rate, carboxylation efficiency, and water use efficiency. These physiological responses were associated with greater nitrogen accumulation in roots, rhizomes, and leaves, as well as increased total biomass and ^{15}N fertilizer recovery. Nitrogen losses were significantly reduced in *Ab* and *Ab*+*Na* treatments. At the molecular level, inoculated plants exhibited reduced abundance of *amoA*, *nirK*, and *nosZ* genes, involved in nitrification and denitrification, respectively. However, gene abundances were higher in the *Ab*+*Na* than in the individual inoculations, likely due to greater organic acid exudation stimulating soil organic matter mineralization. Only *Ab* and *Ab*+*Na* increased *nifH* abundance, indicating enhanced biological nitrogen fixation, predominantly associated with *A. brasilense*. The *Ab*+*Na* treatment also led to a distinct rhizospheric microbial profile and promoted more uniform microbial community structure. These findings suggest that foliar microbial consortia improve plant nitrogen efficiency and modify rhizosphere functioning through coordinated physiological and microbial mechanisms. Foliar inoculation with compatible PGPB offers a promising strategy for sustainable sugarcane intensification.

[2] Chapter written in accordance with the rules of Soil Biology and Biochemistry

Keywords: Diazotrophic bacteria; Functional gene abundance; Gas exchange; Rhizosphere modulation; Root exudates.

2.1 INTRODUCTION

The growing global demand for food, renewable energy, and industrial inputs presents significant challenges to agricultural sustainability. Projections indicate that the global population will reach approximately 9.7 billion by 2050 (FAO, 2018), thereby intensifying the need for agricultural systems capable of increasing productivity while minimizing environmental impacts (Godfray *et al.*, 2010; Santos; Nogueira; Hungria, 2019). In this context, sugarcane (*Saccharum officinarum* L.) emerges as a strategic crop for the energy sector, serving as a major source of biofuels and industrial biomass (Dos Santos *et al.*, 2019; Martíni *et al.*, 2020; Surendran et al., 2016). However, the high nutrient demands and long growth cycle of sugarcane require innovative agronomic strategies to optimize resource use efficiency and reduce dependence on synthetic fertilizers, among which the use of plant-beneficial microorganisms stands out as a promising alternative (Cao *et al.*, 2023).

Microorganisms play essential roles in agroecosystems by driving nutrient cycling, modulating rhizosphere interactions, and improving soil health (Bünemann, 2015; Marschner; Crowley; Rengel, 2011). Among them, plant growth-promoting bacteria (PGPB) have been extensively studied for their ability to enhance nutrient uptake and stimulate plant development through multiple mechanisms (Cheng *et al.*, 2025; Freitas *et al.*, 2023; Jalal; Júnior; Teixeira Filho, 2024). *Azospirillum brasilense* and *Nitrospirillum amazonense* are particularly relevant due to their nitrogen-fixing capacity and growth-promoting effects in grasses. These species establish both endophytic and epiphytic interactions with host plants, modulating root architecture and physiological performance (Galindo *et al.*, 2022; Santos; Nogueira; Hungria, 2021; Schwab; Terra; Baldani, 2018).

The beneficial effects of PGPBs are mediated by mechanisms such as phytohormone synthesis (notably indole-3-acetic acid), nutrient mobilization, and modulation of root exudation patterns (Ankati; Podile, 2019; Fukami; Cerezini; Hungria, 2018a). Root exudates are central to rhizosphere ecology, shaping microbial communities, influencing nutrient solubilization, and fostering mutualistic interactions (Cesari *et al.*, 2019b; Pereira *et al.*, 2020). Moreover, PGPB can improve nitrogen use

efficiency (NUE) either by enhancing root surface area for uptake or by altering soil microbial processes that regulate nitrogen availability, such as nitrification and denitrification (Kuypers *et al.*, 2018; Hartmann & Tringe, 2019). Studies employing ^{15}N isotopic techniques have shown that microbial inoculation enhances nitrogen recovery from fertilizers, with microbial consortia often outperforming single-strain inoculants in terms of efficiency and stability (Pascale *et al.*, 2020).

Although inoculation is traditionally performed via seed or soil, foliar application has gained attention as an alternative that may influence the rhizosphere both indirectly, by altering plant physiology and exudation patterns, and directly, via microbial runoff reaching the soil surface (Dakora & Phillips, 2002; Hartmann & Tringe, 2019). However, the ecological and physiological consequences of this route of inoculation remain underexplored, particularly in sugarcane systems.

In this context, the present study aimed to evaluate the effects of foliar application of *Azospirillum brasilense* combined or not with soil-drench application of *Nitrospirillum amazonense*, on sugarcane physiological performance, root exudation of organic acids, and rhizospheric microbial community composition. In addition, we investigated how microbial inoculation modulates genes involved in nitrogen cycling and the efficiency of nitrogen recovery using a ^{15}N tracer. This study seeks to elucidate the extent to which foliar inoculation of PGPBs can simultaneously benefit plant metabolism and soil microbial ecology, providing key insights for the development of biologically based technologies in sugarcane cultivation. The knowledge generated may contribute to the formulation of agricultural practices that reconcile high crop performance with long-term sustainability and environmental stewardship.

2.2 MATERIAL AND METHODS

2.2.1 Experimental Design

The experiment was conducted under controlled greenhouse conditions at the School of Agricultural Sciences, São Paulo State University (UNESP), Botucatu, Brazil (22°50’57” S, 48°25’59” W, 815 m altitude). A completely randomized design was adopted, comprising four treatments: (i) control (no microbial application), (ii) *Azospirillum brasilense* (*Ab*), (iii) *Nitrospirillum amazonense* (*Na*), and (iv) a combination of both strains (*Ab*+*Na*), with four replicates each.

2.2.2 Soil Preparation and Cultivation Conditions

The soil used in the experiment was collected from the 0 - 20 cm layer and chemically characterized (Supplementary Table S1). Corrections were based on the initial chemical characterization, following standard recommendations for sugarcane cultivation. The amount of lime was determined to increase the soil base saturation to 70% using the method recommended for the State of São Paulo (Spironello *et al.*, 1997). Nutrient inputs were adjusted to reach an expected sugarcane yield, totaling 90 kg N ha^{-1}, 180 kg P_2O_5 ha^{-1}, and 80 kg K_2O ha^{-1}.

Plants of the sugarcane variety CTC9001 were grown in 50 L pots. Irrigation was managed according to crop water requirements and monitored with tensiometers, based on soil water retention curves. The greenhouse was equipped with an air circulation system maintaining temperatures between 22°C and 32°C and relative humidity at approximately 70% (Rodrigues, 1995). The experiment was conducted for approximately 200 days after emergence (DAE), until the crop reached the mid-vegetative growth stage, a phase of high metabolic activity.

2.2.3 Microbial Inoculations

Sugarcane plants were inoculated with *A. brasilense* (*Ab*), strains Ab-V5 and Ab-V6, via foliar spraying (Freitas *et al.*, 2023; Scudeletti *et al.*, 2023) to minimize competition with the native soil microbiota and reduce interference from the physicochemical properties of the edaphic environment (Cardozo *et al.*, 2022; Hernández *et al.*, 2024) and with *N. amazonense* (*Na*), strain BR 11145, via soil application, an environment in which this bacterial genus exhibits greater functional efficacy (REIS *et al.*, 2020). Both inoculations were performed during the tillering stage, using a concentration of 1×10^8 CFU mL^{-1}. All strains were provided by EMBRAPA-CNPSo and are deposited in the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja (Collection CNPSo 2083 [Ab-V5], CNPSo 2084 [Ab-V6] (Dartora *et al.*, 2016; Hungria *et al.*, 2010; Hungria; Ribeiro; Nogueira, 2018; Santos; Nogueira; Hungria, 2021); and CNPSo 4060 [Na] (Dartora *et al.*, 2016; Junior *et al.*, 2008).

Applications were carried out using a CO_2-propelled airbrush sprayer (Herbicate, Catanduva, SP, Brazil), calibrated to deliver 150 L ha^{-1} at a pressure of 1.8 BAR. A flat fan nozzle (TTI11004VP; Jacto, Pompéia, SP, Brazil) was used to ensure uniform droplet coverage. Spraying was performed at 17:00 to minimize evaporation and maximize absorption. The applied dose was 300 mL ha^{-1}.

2.2.4 ^{15}N-Labeled Fertilizer Application

Ammonium sulfate enriched with 2.683 atom % excess ^{15}N ($(^{15}NH_4)_2SO_4$; Sigma-Aldrich, St. Louis, MO, USA) was applied to the pots at the equivalent rate of 90 kg N ha^{-1}.

2.2.5 Plant and Soil Evaluations

2.2.5.1 Nutrient Analysis

Leaf samples were collected from the +1 position during vegetative growth. The mid-section of the lamina was separated after removing the central vein, as per (Spironello *et al.*, 1997). Macronutrients (K, Ca, Mg) and micronutrients (Cu, Fe, Mn, Zn, B) were extracted using a nitric-perchloric digestion and quantified by atomic absorption spectrophotometry (Analyst 200, Perkin Elmer, Norwalk, CT, USA). Phosphorus and sulfur were analyzed by UV - VIS spectrophotometry (Shimadzu UV-2700, Kyoto, Japan) after the same digestion method. Total nitrogen was extracted via Kjeldahl digestion and determined by titration (AOAC, 2016).

2.2.5.2 Organic Acid Exudation

Carboxylic acids were analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu chromatograph (model 10A) equipped with a refractive index detector (RID-10A) and an isocratic pump (LC-10AD), operating at a flow rate of 1.0 mL min^{-1} with 0.005 N H_2SO_4 as the mobile phase. Separation was performed using a Bio-Rad Aminex HPX-87H column (H^+ form) at 65°C, with temperature control provided by a CTO-10A oven. Samples were previously diluted in 0.005 N H_2SO_4 solution and kept at rest for 24 h to allow acid extraction. They were then centrifuged at 12,000 rpm at 10°C, and the supernatant was filtered through a polyvinylidene fluoride (PVDF) membrane (0.22 µm, 13 mm; Millipore). Chromatograms were

analyzed by comparing retention times and peak areas with those of standard organic acids prepared at predefined concentrations. Identification and quantification were performed using the N2000 Chromatography Data System software, based on the corresponding peak areas (Ribani *et al.*, 2004)

2.2.5.3 Soil DNA Extraction and Quantification

Soil total DNA was extracted using the PowerSoil DNA Extraction Kit (MoBio Laboratories) according to the manufacturer's instructions. DNA quality was initially verified via 1% agarose gel electrophoresis stained with GelRed and run in sodium borate buffer (Brody; Kern, 2004). Additionally, the DNA quantification and purity checks were performed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), considering the 260/280 nm absorbance ratio.

2.2.5.4 Quantitative Real-Time PCR (qPCR)

The abundances of total *Bacteria* and *Archaea* (each quantified using primers targeting the *16S rRNA* gene), nitrogen-fixing microorganisms (*nifH* gene), ammonia-oxidizing Bacteria (*amoA* gene, referred to as AOB), ammonia-oxidizing Archaea (*amoA* gene, referred to as AOA), and denitrifying microorganisms (*nirK* and *nosZ* genes) were quantified via quantitative PCR (qPCR) using group-specific primers. Standard curves were constructed from 10^1 to 10^9 gene copies. Reactions were run on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA), and copy numbers were expressed per gram of dry soil. Primer sequences, amplification conditions are provided in Table 1.

Table 1. Description of primers, standards DNA and amplification conditions used in qPCR analysis.

Target genes	Primers	Sequences	Amplification conditions	Reference
16S rRNA Archaea	519F	5' CAGCCGCCGCGGTA A 3'	95◦C - 10 min 40 cycles: 95◦C – 30 s; 57◦C – 30 s; 72◦C – 50 s*.	(Coolen *et al.*, 2004; Stahl, 1991)
	915R	5' GTGCTCCCCCGCCAATTCCT 3'		
16S rRNA Bacteria	Eub338	5'-CCTACGGGAGGCAGCAG-3'	95°C - 10 min; 40 cycles: 95°C - 30 s; 53°C - 40 s; 72°C - 40 s*.	(Muyzer; De Waal; Uitterlinden, 1993)
	Eub518	5'-ATTACCGCGGCTGCTGG-3'		
nifH	nifHF	5'-AAAGGYGGWATCGGYAARTCCACCAC-3'	95°C - 10 min; 40 cycles: 95°C - 1 min; 55°C - 27 s.; 72°C - 1 min*.	(Rösch; Mergel; Bothe, 2002)
	nifHR	5'-TTGTTSGCSGCRTACATSGCCATCAT-3'		

Arch-amoA	Arch-amoAF	5'-STAATGGTCTGGCTTAGACG-3'	95°C - 5 min; 40 cycles: 95°C - 40 s; 56°C - 30 s.; 72°C - 1min*.	(Francis *et al.*, 2005)
	Arch-amoAR	5'-GCGGCCATCCATCTGTATGT-3'		
Bac-amoA	amoA1F	5'-GGGGTTTCTACTGGTGGT-3'	95°C - 10 min; 40 cycles: 95°C - 40 s; 56°C - 30 s; 72°C - 1 min*.	(Rotthauwe; Witzel; Liesack, 1997)
	amoA2R	5'-CCCCTCKGSAAAGCCTTCTTC-3'		
nirK	NirK876	5'-ATYGGCWGAYGGGGA-3'	95°C - 10 min; 40 cycles: 95°C - 15 s; 63°C - 30 s; 72°C - 30 s*.	(Henry *et al.,* 2004)
	NirK1040	5'-GCCTCGATCAGRTTRTGGTT-3'		
nosZ	nosZ2F	5'-CGCRACGGCAASAAGGTSMSSGT-3'	95°C - 10 min; 40 cycles: 95°C - 40 s; 63°C - 30 s; 72°C - 40 s*.	(Henry *et al.*, 2006)
	nosZ2R	5'-CAKRTGCAKSGCRTGGGAAGAA-3'		

2.2.5.5 16S rRNA Gene Sequencing

Amplicon sequencing analyses were conducted to characterize prokaryotic communities (*Bacteria* and *Archaea* together) based on the *16S rRNA* gene. The V4 region of the *16S rRNA* gene was amplified using modified primers 515FB (5'-GTGYCAGCMGCCGCGGTAA) and 806RB (5'-GGACTACNVGGGTWTCTAAT) (Caporaso *et al.*, 2011). PCR amplification was performed separately for each sample, followed by purification using an amplification purification kit (Quantabio, Beverly, MA, USA). Libraries were prepared using the SP Reagent Kit v1.5 (Illumina, San Diego, CA, USA), and sequencing was conducted according to the manufacturer's protocol on an Illumina MiSeq platform with 2 × 250 bp paired-end reads.

2.2.5.6 Biomass Determination

Plants were harvested and separated into leaves, stems, rhizomes, and roots. Samples were oven-dried at 65°C until constant weight (MA035/5/10P, Marconi, Piracicaba, Brazil), weighed on an analytical balance (AY 220, Shimadzu), and ground in a Wiley mill for further analysis. Soil samples were dried at 40°C for 48 h, ground in a ball mill, and sieved through a 100-mesh (0.0059 mm) screen for isotopic analysis.

2.2.5.7 Isotopic Analyses and Nitrogen Recovery

Total N and ^{15}N enrichment in plant and soil samples were determined using an elemental analyzer (Flash EA, Thermo Scientific, Germany) coupled to an isotope ratio mass spectrometer (Delta V, Thermo Scientific, Germany). The results were expressed as atom % ^{15}N excess and percentage recovery. Nitrogen derived from

fertilizer (*Ndff*, kg ha^{-1}) and recovery efficiency were calculated using the following equations:

(1) $Ndff$ (%) = (a/b)

(2) $Ndff$ (kg ha^{-1}) = [$Ndff$ (%) / 100] × TN

(3) ^{15}N *recovery* = [$Ndff$ (kg ha^{-1}) / NR] ×100

(4) Soil residual ^{15}N (kg ha^{-1}) = [Ndff (%) / 100] × TN

(5) Soil residual ^{15}N (%) = [Soil residual ^{15}N (kg ha^{-1}) / NR] × 100

In these calculations, *Ndff* corresponds to the nitrogen in the plant derived from fertilizer, *a* and *b* correspond to the atom % ^{15}N excess in the product (plant or soil) and in the fertilizer, respectively; *TN* is the total nitrogen content (kg N ha^{-1}); ^{15}N recovery refers to the percentage of fertilizer N recovered by the plant; *NR* is the applied N rate (kg N ha^{-1}); and Soil residual ^{15}N indicates the amount of labeled fertilizer remaining in the soil (Trivelin *et al.*, 1994).

2.2.6 Data Analysis

The sequences for prokaryotic (based on *16S rRNA*) communities were processed using the DADA2 pipeline (Callahan, 2017) in the R environment (R Core Team, 2021). First, the primers from the demultiplexed data were removed. After, the data were trimmed and filtered to remove low-quality sequences and, the denoising inference step was performed (based on the learn error step). After that, the forward and reverse sequences were merged, and the chimeric sequences were removed based on the “consensus” method. Next, the taxonomic inference was performed using the SILVA database (v. 138; Quast *et al.* 2012) for the *16S rRNA* data. Finally, the prokaryotic matrix was filtered to remove sequences classified as mitochondria and chloroplast and rarefied to 126391 sequences per sample (Supplementary Material, Table S2 and Figure S1).

Following DADA2, the resulting amplicon sequence variant (ASV) table and representative sequences were used as input for PICRUSt2 analysis (Douglas *et al.*, 2019). PICRUSt2 infers the functional potential of microbial communities based on 16S rRNA gene sequences, enabling the prediction of gene family abundances such as KEGG orthologs. This approach allowed us to identify putative functional profiles of the

sugarcane-associated microbiome, providing insights into the metabolic roles and ecological contributions of microbial taxa within sugarcane rhizosphere.

Microbial community structure was assessed using non-metric multidimensional scaling (NMDS) based on ASV-level Bray-Curtis dissimilarities. Differences among groups were evaluated using PERMANOVA (999 permutations) and pairwise PERMANOVA tests. Differential taxonomic composition was assessed using STAMP (Parks *et al.*, 2014), with genus and phylum-level matrices as input. *p-values* were determined by the two-sided Tukey-Kramer test, followed by false discovery rate (FDR) correction using the Benjamini-Hochberg method (Benjamini; Hochberg, 1995).

The generalized joint attribute modelling (GJAM) package (Clark *et al.*, 2017) was used to estimate the effects of treatments on rhizosphere selection and relate the microbial community with the additional plant variables. By using a two-way factorial design, we were able to disentangle two effects: (i) the microbial community selection because of the foliar spraying; (ii) the rhizosphere selection for the soil inoculation; and (iii) the combined effects of foliar spraying and soil inoculation. GJAM model allowed us to identify main effects and interactions (the synergistic effects of the combined inoculations).

GJAM model the compositional nature of microbiome data by doing the following: (i) a latent variable from a censored model that captures the variability of the relative abundances for the different microbial taxa (in our case, Genus level); (ii) the sequencing depth (total number of reads per sample) is used as a measure of sequencing effort to determine the precision of the estimates for each taxa abundance; (iii) the sparse nature of the data is also accounted for and the different types of zeros are now considered as negative values. In the current study, we keep the negative values as information on the absence of the different microbes. Therefore, the negative estimates in the abundance of certain microorganisms in the different treatments is used as metric on the certainty about the absence of specific taxa.

Based on that, we focused on sugarcane rhizosphere recruitment of soil microbes, exploring the impact of the different inoculation strategies on the microbial community. As a Bayesian approach GJAM uses Gibbs sampling to estimate the regression coefficients and for that we used 20,000 iterations with a 5,000 as a cut-off. From the final model, information were extracted on which microbials are selected according to each soil amendment treatment. This allowed to identify the microbiome profile, sensu Rotoni *et al.* (2022), which corresponds to the different taxa of microbes

(bacteria and archaea). Besides as a data integration approach, GJAM allowed us to evaluate the joint effects on soil fertility and crop productivity and explore the associations between microbes and the plant performance (Nitrogen Accumulation, Organic Acid Exudation, and Biomass Production). We explored the associations between plant performance and microbiome. To represent the shifts in the microbial community abundance as a result of the treatments we used a rank-abundance plot.

The data (qPCR, gas exchange, organic acidic, Fate of ^{15}N Fertilizer in the Soil-Plant System, Nitrogen Accumulation, and Biomass Production) were subjected to the Shapiro-Wilk test for normality (Shapiro; Wilk, 1965) and the Levene's test for homoscedasticity (Levene, 1960), both at a 5% significance level ($p \leq 0.05$). ANOVA was performed, and means were compared using Fisher's LSD test at 5% probability. Analyses were conducted in R (v4.0.2; R Core Team, 2020) using RStudio (v1.3.1073), and graphs were prepared in GraphPad Prism (v9.5.1).

2.3 RESULTS

2.3.1 N cycle functional genes (real-time PCR)

The results show that the abundance of the archaeal 16S rRNA gene (Figure 1A) was highest in the Control (9.59×10^6 gene copies g^{-1} soil) and *Azospirillum brasilense* + *Nitrospirillum amazonense* (*Ab*+*Na*) (8.25×10^6 gene copies g^{-1} soil) treatments. In contrast, the treatments with isolated inoculation of *Azospirillum brasilense* (*Ab*) and *Nitrospirillum amazonense* (*Na*) exhibited reductions of 31.7% and 53.3%, respectively, compared to the Control.

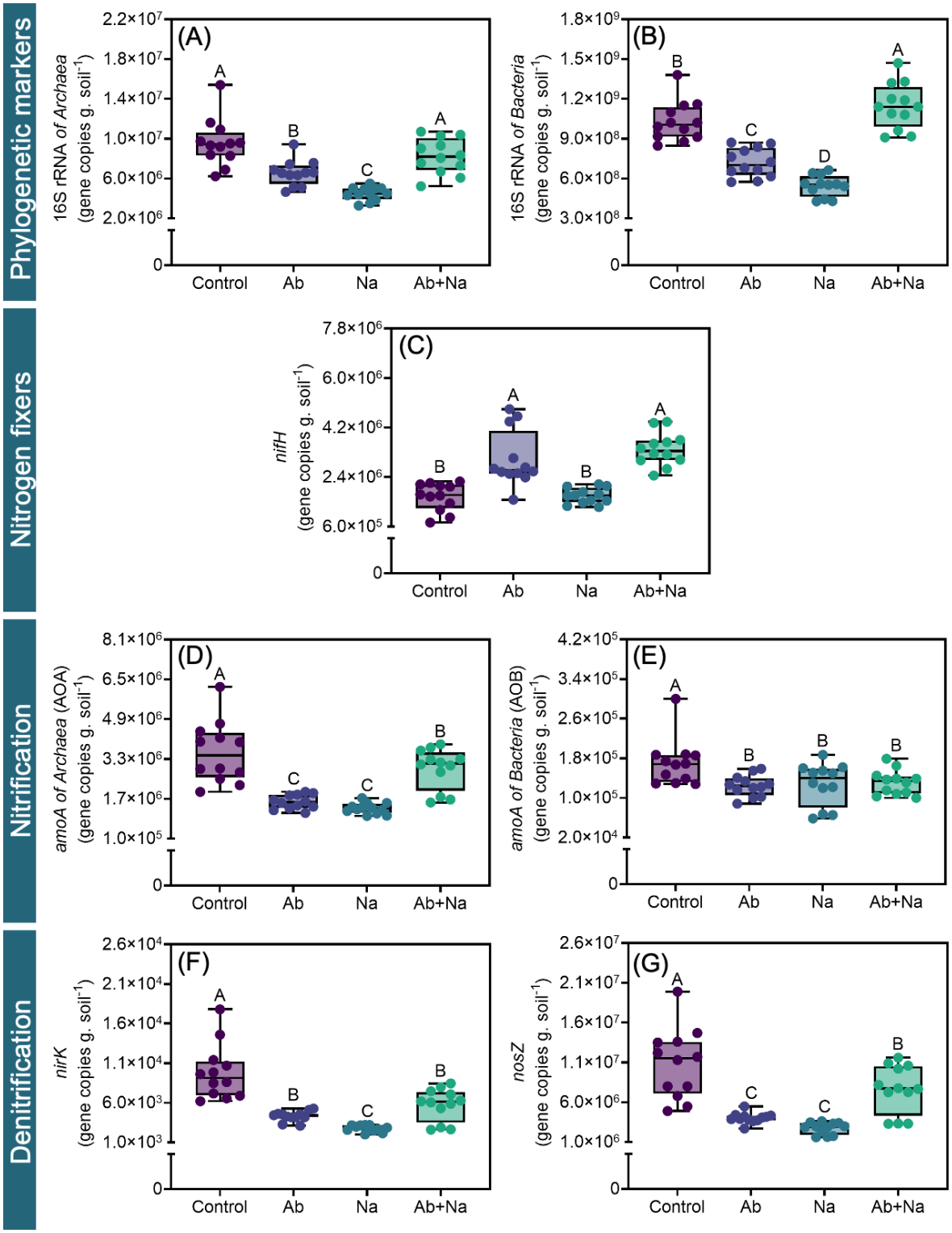

Figure 1. Abundance of phylogenetic markers *16S rRNA* of *Archaea* (A) and *Bacteria* (B), as well as functional genes *nifH* (C), *amoA* of *Archaea* - AOA (D), *amoA* of *Bacteria* - AOB (E), *nirK* (F), and *nosZ* (G) in rhizospheric soil under the influence of different treatments: Control (no microorganism application), *Ab* (*Azospirillum brasilense*), *Na* (*Nitrospirillum amazonense*), and combination of *Ab* + *Na*. Statistical significance was determined using the LSD test ($p \leq 0.05$).

For the quantification of the bacterial 16S rRNA gene (Figure 1B), the *Ab*+*Na* treatment exhibited the highest abundance, which was 11.7% greater than that of the Control (1.03×10^9 gene copies g^{-1} soil). In contrast, isolated applications led to a decrease of 30.1% in the *Ab* treatment and 47.2% in the *Na* treatment compared to the Control.

Regarding the *nifH* gene (Figure 1C), the highest abundance was observed in the *Ab* and *Ab*+*Na* treatments ($p \leq 0.05$), with an average increase of 91% compared to the Control (1.69×10^6 gene copies g^{-1} soil).

For the archaeal *amoA* gene (AOA), all treatments inoculated with PGPB showed reduced abundance relative to the Control (Figure 1D). The Control presented 3.57×10^6 gene copies g^{-1} soil, while the *Ab* and *Na* treatments showed an average decrease of 59.5%. The *Ab*+*Na* treatment also resulted in a significant reduction, albeit intermediate, with an 18.5% decrease compared to the Control. For the bacterial *amoA* gene (AOB), microbial treatments resulted in a 25% lower abundance (Figure 1E) compared to the Control (1.70×10^5 gene copies g^{-1} soil). With respect to the *nirK* gene (Figure 1F), associated with the denitrification process, the highest abundance was found in the Control (9.83×10^3 gene copies g^{-1} soil). The *Ab* and *Ab*+*Na* treatments presented, on average, a 49% reduction, while the *Na* treatment exhibited the lowest abundance, 71.6% lower than the Control.

Finally, for the *nosZ* gene (Figure 1G), which is involved in the final step of denitrification, the Control maintained the highest abundance (1.08×10^7 gene copies g^{-1} soil). The *Ab* and *Na* treatments exhibited a significant average reduction of 68.8% ($p \leq 0.05$), whereas the *Ab*+*Na* treatment resulted in intermediate values, with a 29.4% reduction compared to the Control.

2.3.2 Gas Exchange

Physiological parameters related to gas exchange were significantly influenced by the inoculation with plant growth-promoting bacteria (PGPB). Net photosynthetic rate (Figure 2A) was significantly higher ($p \leq 0.05$) in all treatments receiving microbial inoculation compared to the Control (17.2 μmol CO_2 m^{-2} s^{-1}). The highest increase was observed in the *Ab*+*Na* treatment, with an enhancement of 22.1%, followed by

Azospirillum brasilense (*Ab*) with 16.9% and *Nitrospirillum amazonense* (*Na*) with 9.88%.

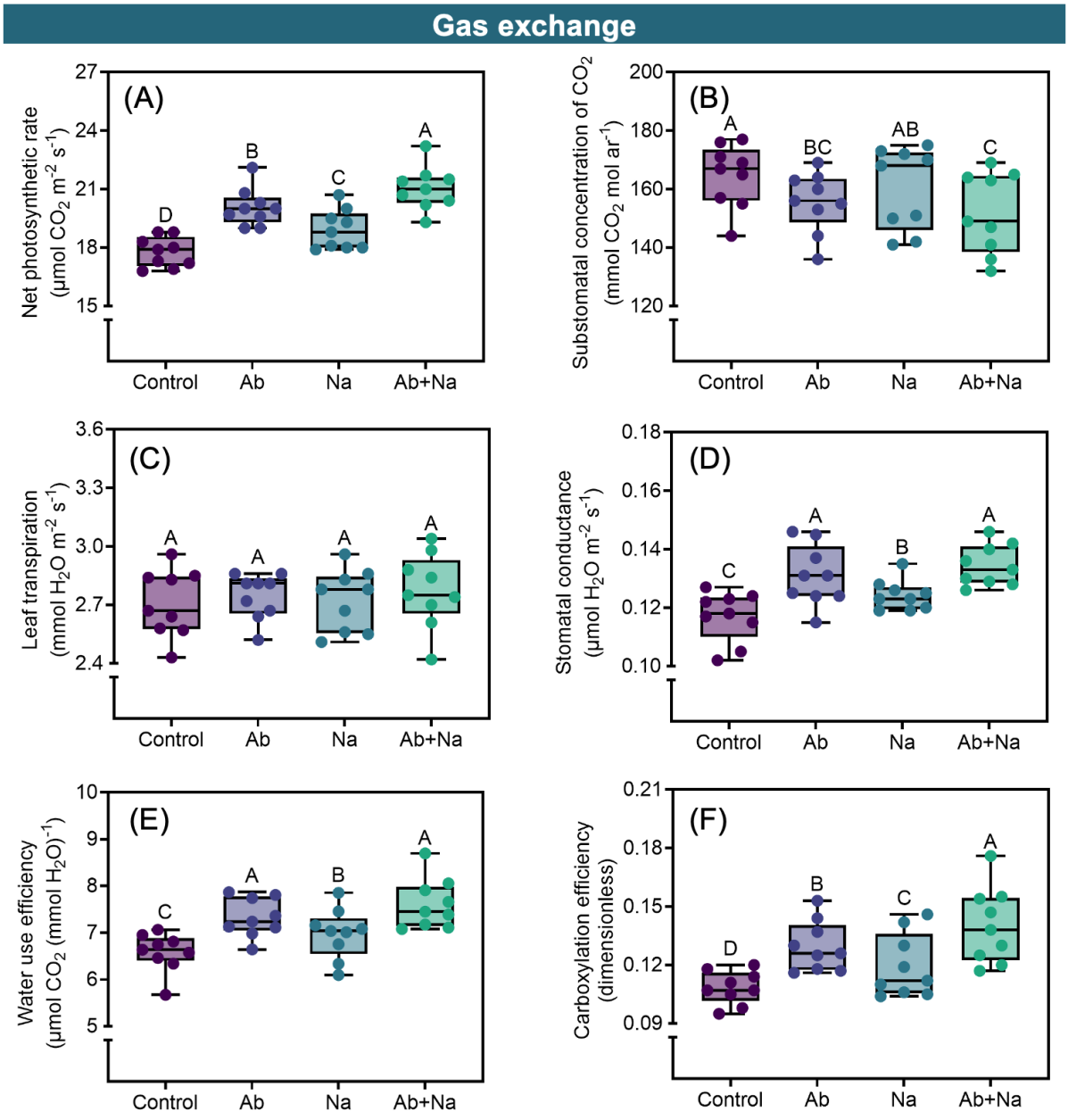

Figure 2. Net photosynthesis rate (A), internal sub-stomatal CO_2 concentration (B), leaf transpiration (C), stomatal conductance (D), water use efficiency (E), and carboxylation efficiency (F) in sugarcane leaves grown in a greenhouse under different treatments: Control (no microorganism application), *Ab* (*Azospirillum brasilense*), *Na* (*Nitrospirillum amazonense*), and combination of *Ab* + *Na*. Measurements were taken in nine readings over 135 days after treatment application (DAA). Different lowercase

letters indicate significant differences between treatments, as determined by the LSD test ($p \leq 0.05$).

The internal CO_2 concentration in the substomatal cavity (Figure 2B) was highest in the Control (165 mmol CO_2 mol^{-1} air), suggesting a lower carbon fixation rate. The *Ab+Na* and *Ab* treatments significantly reduced this parameter ($p \leq 0.05$) by 7.88% and 5.45%, respectively, indicating a greater consumption of CO_2 due to enhanced photosynthetic activity.

Stomatal conductance (Figure 2D) was higher in all treatments inoculated with microorganisms. The Control showed an average value of 0.117 µmol H_2O m^{-2} s^{-1}, while the *Ab+Na* and *Ab* treatments exhibited increases of 14.5% and 12%, respectively. The *Na* treatment showed an intermediate value, with an increase of 5.98%.

Water use efficiency (Figure 2E) was also significantly higher in the PGPB treatments ($p \leq 0.05$) compared to the Control (6.59 µmol CO_2 $mmol^{-1}$ H_2O). The *Ab+Na*, *Ab*, and *Na* treatments promoted increases of 15.6%, 11.1%, and 5.92%, respectively. On the other hand, transpiration rate (Figure 2C) showed no statistically significant differences among treatments.

Carboxylation efficiency (Figure 2F) varied significantly among treatments. The Control exhibited the lowest value (0.108, dimensionless), while the *Ab+Na*, *Ab*, and *Na* treatments promoted increases of 29.6%, 20.4%, and 10.2%, respectively.

2.3.4 Fate of ^{15}N Fertilizer in the Soil-Plant System, Nitrogen Accumulation, and Biomass Production

Biomass production was significantly influenced by the applied treatments (Figure 3A). Root dry matter (DM) was higher in the *Ab* and *Ab+Na* treatments compared to the Control (111 g pot^{-1}), with increases of 37.8% and 50.5%, respectively. Rhizome DM also increased relative to the Control (60.4 g pot^{-1}), with enhancements of 37.6% in the *Ab* treatment, 23.5% in the *Na* treatment, and 50.5% in the *Ab+Na* treatment. Total DM was greater in the *Ab* and *Ab+Na* treatments, resulting in increases of 14% and 22%, respectively, compared to the Control (878 g pot^{-1}). On the other hand, no significant differences were observed in leaf DM among treatments.

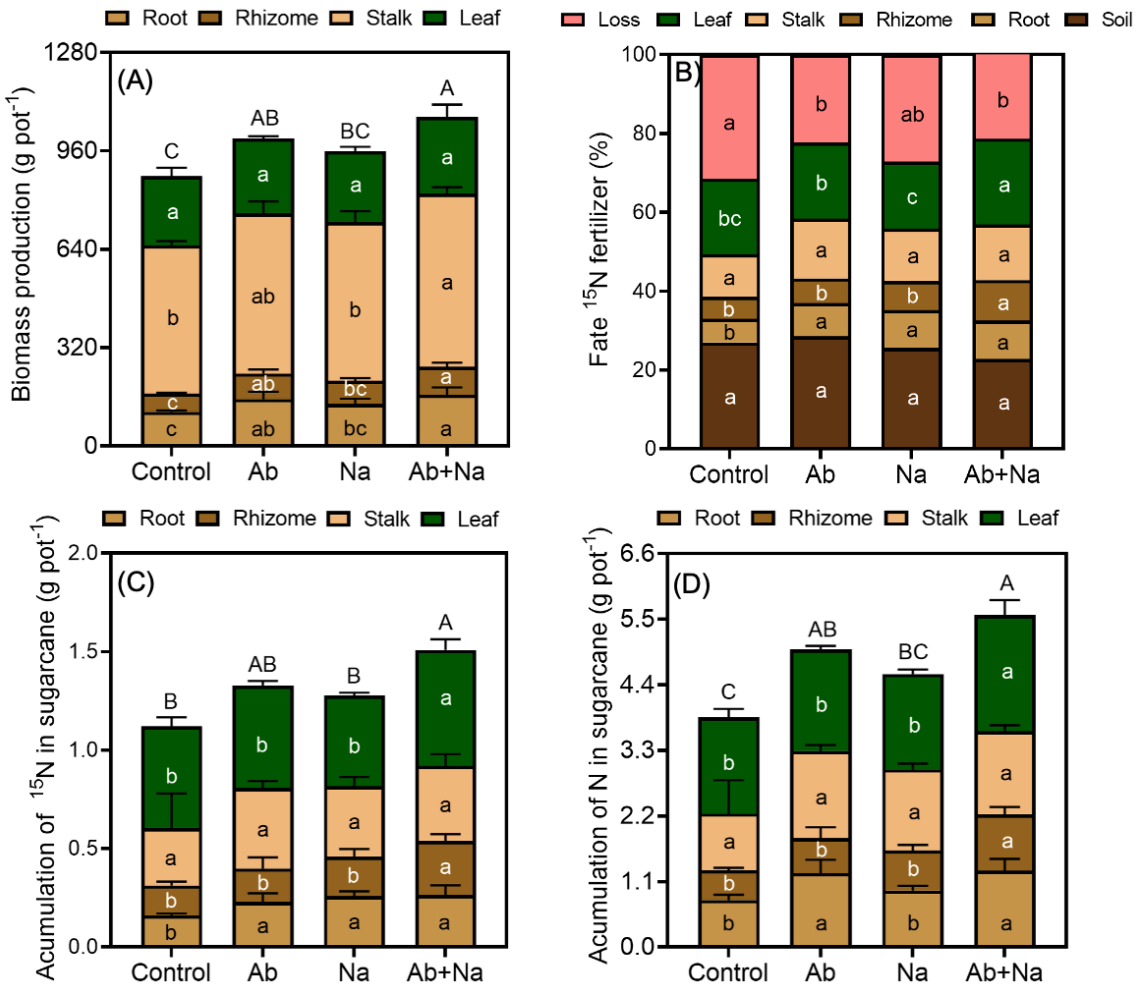

Figure 3. Biomass production (A) Fate of the ^{15}N-labeled fertilizer in the soil and plant compartments (B); accumulation of ^{15}N in sugarcane (C) and accumulation of N total in sugarcane (D), under different treatments: Control (no microorganism application), *Ab* (*Azospirillum brasilense*), *Na* (*Nitrospirillum amazonense*), and combination of *Ab* + *Na*. Different lowercase letters indicate significant differences between treatments, as determined by the LSD test ($p \leq 0.05$).

The distribution of fertilizer-derived ^{15}N varied significantly among the different plant compartments as a function of the applied treatments (Figure 3B). In the roots, all treatments involving microbial inoculation showed greater ^{15}N recovery compared to the Control. The Control treatment exhibited 5.95% of fertilizer-derived ^{15}N recovered in the roots, whereas the microbial treatments showed an average increase of 55.7% relative to this value.

In the rhizomes, only the *Ab*+*Na* treatment resulted in a significant increase in ^{15}N recovery, with 10.2% of fertilizer-derived ^{15}N recovered, representing an 81.9% increase compared to the Control (5.62%). In the leaves, the *Ab*+*Na* treatment also stood out, with 21.8% of fertilizer-derived ^{15}N recovered, corresponding to a 13.5% increase compared to the Control, which exhibited 19.2% fertilizer-derived ^{15}N. No significant differences were observed among treatments for ^{15}N recovery in the stems and in the soil.

Regarding fertilizer ^{15}N losses, the *Ab* and *Ab*+*Na* treatments exhibited the lowest losses. The Control treatment presented average losses of 31%, while the *Ab* and *Ab*+*Na* treatments reduced these losses by 30.8% compared to the Control, reaching values close to 21.8%.

The accumulation of ^{15}N (Figure 3C) in the roots was significantly higher in all treatments that received inoculation with PGPB, resulting in an average increase of 55.3% compared to the control treatment, which presented a mean value of 0.161 g pot^{-1}. However, when assessing ^{15}N accumulation in rhizomes, leaves, and the whole plant, only the treatment with *Ab*+*Na* showed values superior to the control, with average increases of 81.6%, 13.3%, and 34.8%, respectively, compared to the control means of 0.518, 0.152, and 1.12 g pot^{-1} for rhizomes, leaves, and total plant accumulation.

Total nitrogen accumulation in the plant (Figure 3D) in the roots was higher in the *Ab* and *Ab*+*Na* treatments, with an average increase of 60% compared to the Control (0.788 g $plot^{-1}$). In the rhizomes, the highest nitrogen accumulation was observed in the *Ab*+*Na* treatment, with an 89.4% increase compared to the Control (0.499 g $plot^{-1}$). A similar trend was observed for nitrogen accumulation in the leaves, with a 20.4% higher value in the *Ab*+*Na* treatment compared to the Control (1.62 g $plot^{-1}$). For nitrogen accumulation in the stems, no significant differences were detected among treatments. However, total nitrogen accumulation in the plants was significantly higher in the *Ab* (29.3% increase) and *Ab*+*Na* (44.3% increase) treatments compared to the Control (3.86 g $plot^{-1}$).

2.3.5 Quantification of Organic Acids

Organic acids were quantified in the rhizospheric soil. The *Ab*+*Na* treatment exhibited the highest concentrations for most of the compounds evaluated. This

treatment stood out in particular for the concentrations of acetic, lactic, succinic, propionic, and malic acids when compared to the other treatments, as shown in Figure 4.

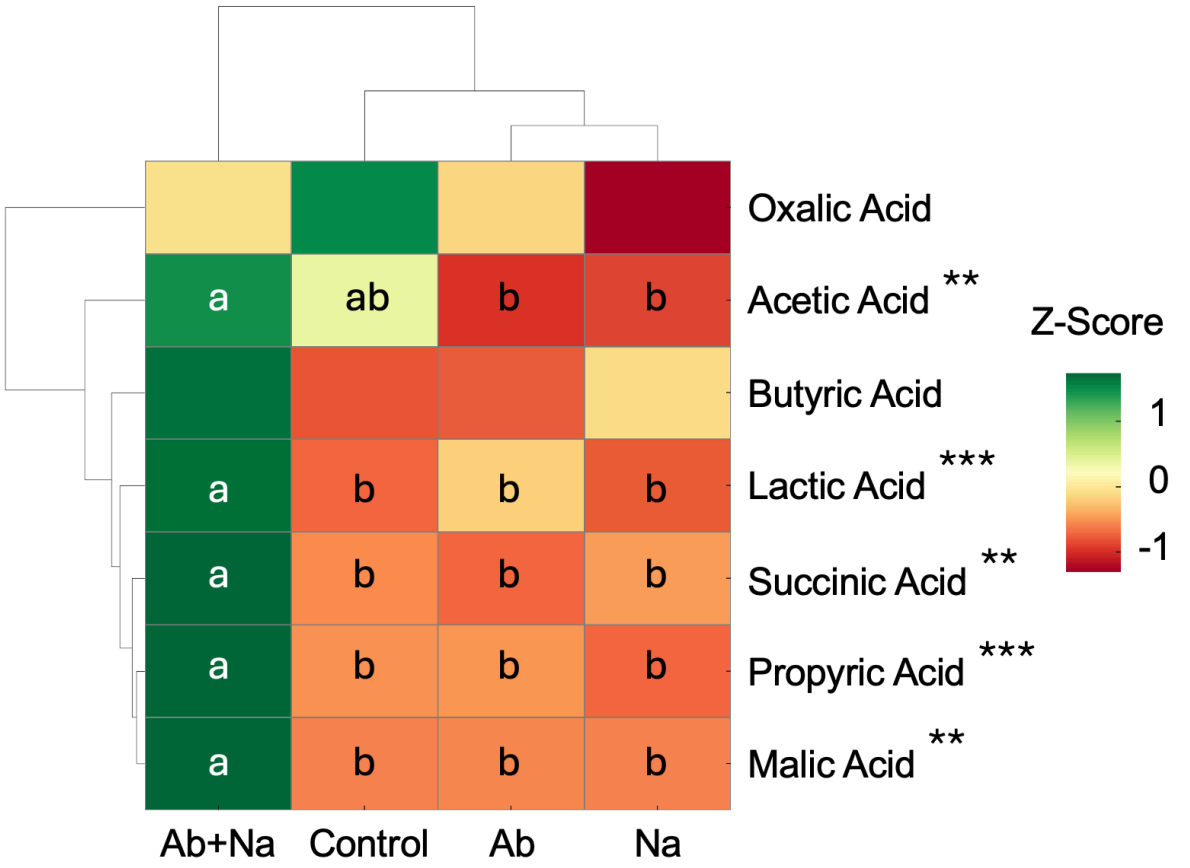

Figure 4. Heat map representing the quantification of organic acids under the influence of different treatments. The evaluated treatments were Control (no microorganism application), *Ab* (*Azospirillum brasilense*), *Na* (*Nitrospirillum amazonense*), and combination of *Ab* + *Na*. Color tones indicate values standardized by the Z-Score. Statistical significance was determined using the LSD test ($p \leq 0.05$). Symbols denote statistical differences between treatments: $p \leq 0.05$ (**) and $p \leq 0.001$ (***).

2.3.6 Microbial Community Structure

The Non-metric Multidimensional Scaling (NMDS) analysis revealed marked differences in the soil microbial community structure among the evaluated treatments (Figure 5). The clear separation between groups indicates that microbial inoculation significantly influenced both the composition and activity of the microbial community. The *Ab+Na* treatment exhibited a distinct microbial structure compared to the other

treatments, resulting in a unique profile. In contrast, the *Na* and *Ab* treatments showed partial overlap. The Control treatment, which did not receive microbial inoculation, also displayed a clear separation, reinforcing the notion that inoculation alters the microbial dynamics in the soil. The dispersion of samples within each treatment suggests that the *Ab+Na* treatment promoted a more homogeneous effect on microbial community structure.

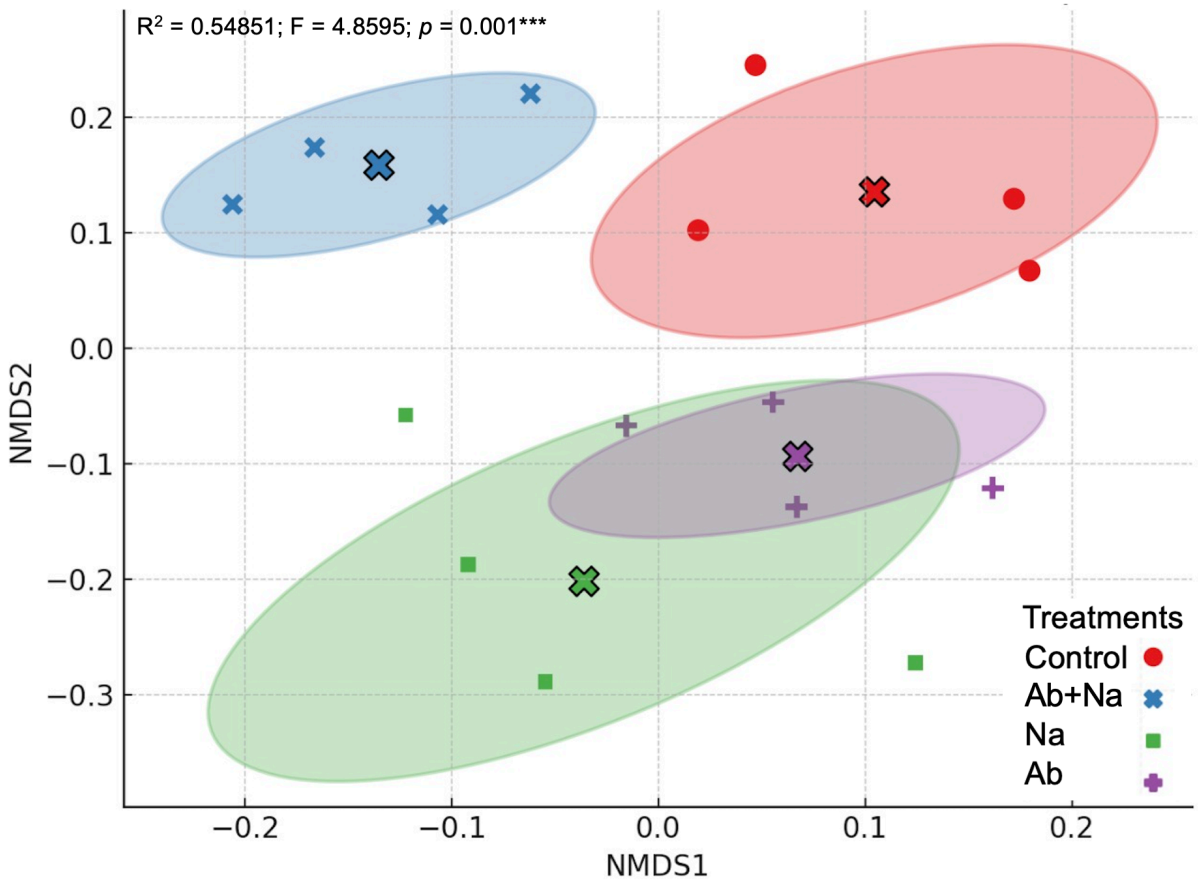

Figure 5. Non-metric Multidimensional Scaling (NMDS) analysis depicting the microbial community structure under different treatments: Control (no microorganism application), *Ab* (inoculation with *Azospirillum brasilense*), *Na* (*Nitrospirillum amazonense*), and the combination of *Ab* + *Na*. The pairwise significance between treatments is presented in Supplementary Table S3.

Using GJAM estimates of microbial relative abundance, we constructed the abundance plot for the control treatment which served as the basis for showing the significant changes in the sugarcane, as a function of treatments. In the control treatment, the most dominant group of bacteria belongs to the phylum Actinobacteriota

(*Pseudoarthrobacter*, *Paenarthrobacter*, *Conexibacter*, *Gaiella*, *Arthrobacter*, *Acidothermus*, and *Phycicoccus*), followed by the phylum Firmicutes (dominated by the genera *Bacillus* and *Paenibacillus*), and the third most dominant phylum is Proteobacteria largely represented by a single genus *Sphingomonas* (Figure 6). Notably, both *Azospirillum* and *Nitrospirillum* genera were not detected in the rhizosphere soil of non-inoculated sugarcane (control treatment).

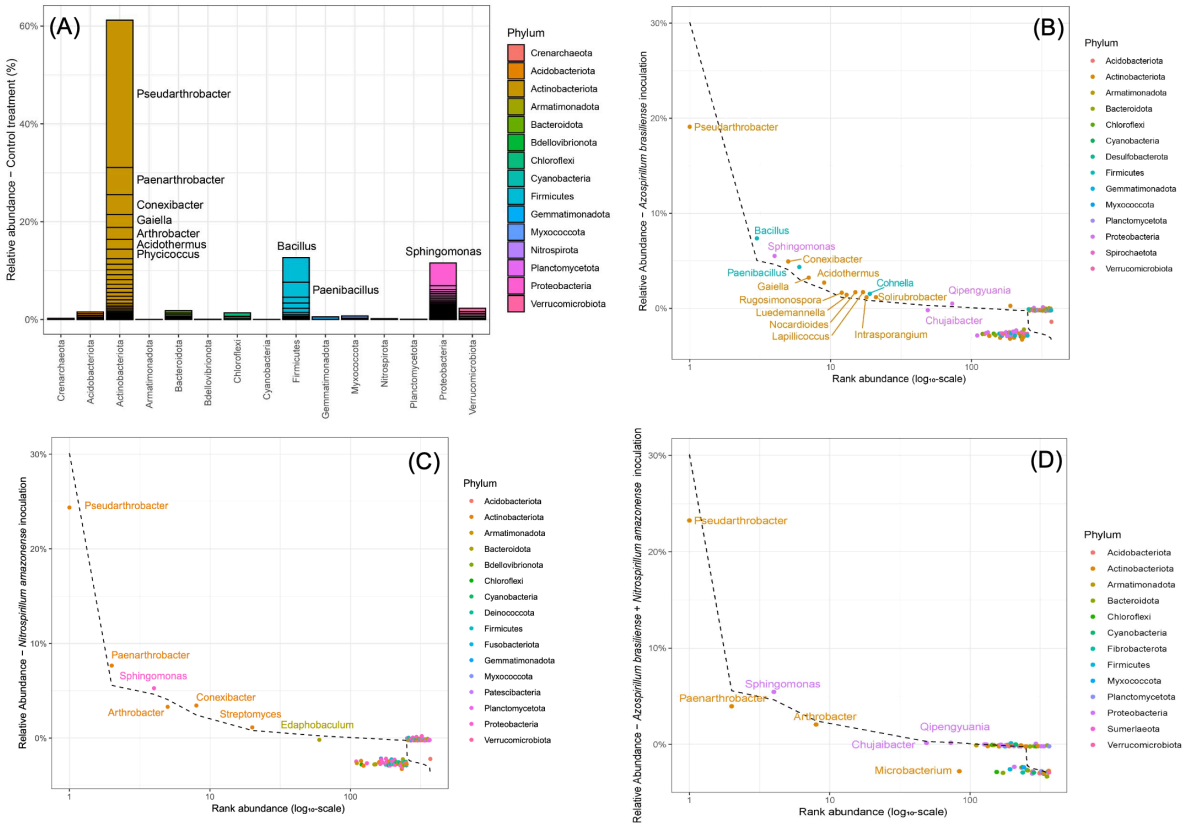

Figure 6. Relative abundance (GJAM estimates) for the sugarcane rhizosphere in the control treatment (A) for the archaeal and bacterial communities (Phylum level). We label all the taxa with a relative abundance bigger than 1.5%. GJAM estimates of relative abundance for the sugarcane rhizosphere microbiome when subjected to the inoculation of *Azospirillum brasiliense* (B), *Nitrospirillum amazonense* (C) and *A. brasiliense* + *N. amazonense* (D), comparison with the control treatment (no inoculation, dashed line). Negative abundance estimate expresses the likelihood of the microbe to be absent from the treatment (the more negative the estimated abundance the more likely the microbe is to be absent). We label all the taxa that significantly differed from the abundance in the control treatment that are bigger than zero in both treatments.

Interestingly, the foliar inoculation of *A. brasiliense* (Figure 6B) induced a big decrease in the abundance of the genus *Pseudarthrobacter* (from 30% in the control to 19.1%; Figure 8). This decrease was followed by an increase in several different genera of bacteria. A total of 16 genera belonging to the top 100 most abundant microbes in the control treatment increased in abundance as a result of the inoculation of *A. brasiliense* (*Bacillus, Sphingomonas, Conexibacter, Paenibacillus, Gaiella, Acidothermus, Rugosimonospora, Luedemannella, Nocardioides, Lapillicoccus, Intrasporangium, Cohnella, Solirubrobacter, Chujaibacter*, and *Qipengyuania*). Below the top 100 we observed a total of 48 genera that were initially rare (with near-zero relative abundance) showed a decline and likely disappeared from the rhizosphere (as indicated by negative abundance estimates). Meanwhile, 58 previously low-abundance genera became more likely to colonize the sugarcane rhizosphere after inoculation, showing a positive shift compared to the control.

The different inoculation strategies changed the sugarcane rhizosphere. We observed a reduction of dominance for the followed by an increase of the rare microbiome (below top 100 most abundant microbes). Figure 6C represents the shifts in sugarcane rhizosphere as induced by the *N. amazonense* soil inoculation reduced the abundance of *Pseudarthrobacter*, whose relative abundance dropped from 30.1% to 24.36%. Other dominant genera such as *Arthrobacter* (3.44% to 2.44%) and *Edaphobaculum* also declined, with the latter showing a negative abundance estimate, indicating a very low probability of presence after inoculation. At the same time, less dominant but potentially beneficial genera like *Paenarthrobacter* (5.57% to 7.66%), *Sphingomonas* (4.63% to 5.27%), *Conexibacter* (3.30% to 4.07%), and *Streptomyces* (0.80% to 1.13%) increased in abundance. These shifts suggest a redistribution of microbial niches in response to inoculation. We also observed changes in the microbial community below the rank 100, with the removal of 54 bacterial genera from the low abundant microbes being replaced by 64 other bacterial genera not detected in the control treatment. In summary, Inoculation of *N. amazonense* affected a total of 124 bacterial genera and reshaped the community of rare microbiome by reducing the abundance of the most dominant genus.

The combined strategy of applying foliar sparying and soil inoculation (Figure 6D) also affected the bacterial community in the rhizosphere of sugarcane. Interestingly the effects were less prominent in the most abundant microbes. The

combined inoculation strategy reduced the abundance of the of *Pseudarthrobacter* (30.1% to 23.25%), *Paenarthrobacter* (5.57% to 3.95%), and Arthrobacter (2.44% to 2.06%). The combined inoculation strategy also induced an increase in the abundance of *Sphingomonas* (4.63% to 5.45%). Interestingly, below the top 100 most abundant microbes, we observed a general increase in the likelihood of those microbes appearing in the microbial community (Figure 9). In total, we observed that Only 24 genera showed significantly lower values of relative abundance than in the control indicating a general increase in the importance of the rare microbial community in the rhizosphere of sugarcane.

In summary, GJAM-based relative-abundance estimates reveal that the dual inoculation strategy (foliar spraying of *A. brasiliense* with soil application of *N. Amazonense*) resulted in a smaller impact on the most dominant bacterial genera in the sugarcane rhizosphere (notably *Pseudarthrobacter* from 30.1% to 23.25%, *Paenarthrobacter* from 5.57% to 3.95%, and *Arthrobacter* from 2.44% to 2.06%) while enhancing the relative abundance of certain Proteobacteria (e.g., *Sphingomonas* increased from 4.63% to 5.45%). Interestingly, we observed no significant effect of the inoculation treatment in the abundance of the genera belonging to the inoculated microbes. Altogether, the integrated foliar-soil approach rebalancing dominant taxa and substantially enriching the diversity and presence likelihood of the rare microbial community.

As a data integration approach, GJAM also allowed to observe the associations between the soil microbiome and the plant variables (nitrogen fate, plant performance, root exudates) and the abundance of phylogenetic markers (Figure 7). We found three major groups of microbes that differed in the way they respond to plant biomass and organic acids exudated by sugarcane. Cluster C1 groups 30 bacterial genera mainly characterized by negative associations with the majority of the plant performance variables. Cluster C2 represented a large group of 94 bacterial genera with a still strongly negative association with root exudates but less relevance in the plant biomass. Finally, cluter C3 combines two subcluster containing microbes with weaker associations with both groups of variables, but there is also another subcluster that contain 46 bacterial genera with strongly positive association with the plant biomass and the organic acids found in root exudates. Altogther, our model shows that the sugarcane is also playing a strong role in modulating the rhizosphere microbiome via root exudates and that a large community of soil microbes is potentially contributing to

the plant growth. It is also interesting to note that several bacterial genera that are positively associated with plant growth are also increased in abundance as a effect of the inoculation treatments indicating a capacity of the different strategies of PGPB inoculation in fostering a specific rhizosphere microbiome that will help in plant growth.

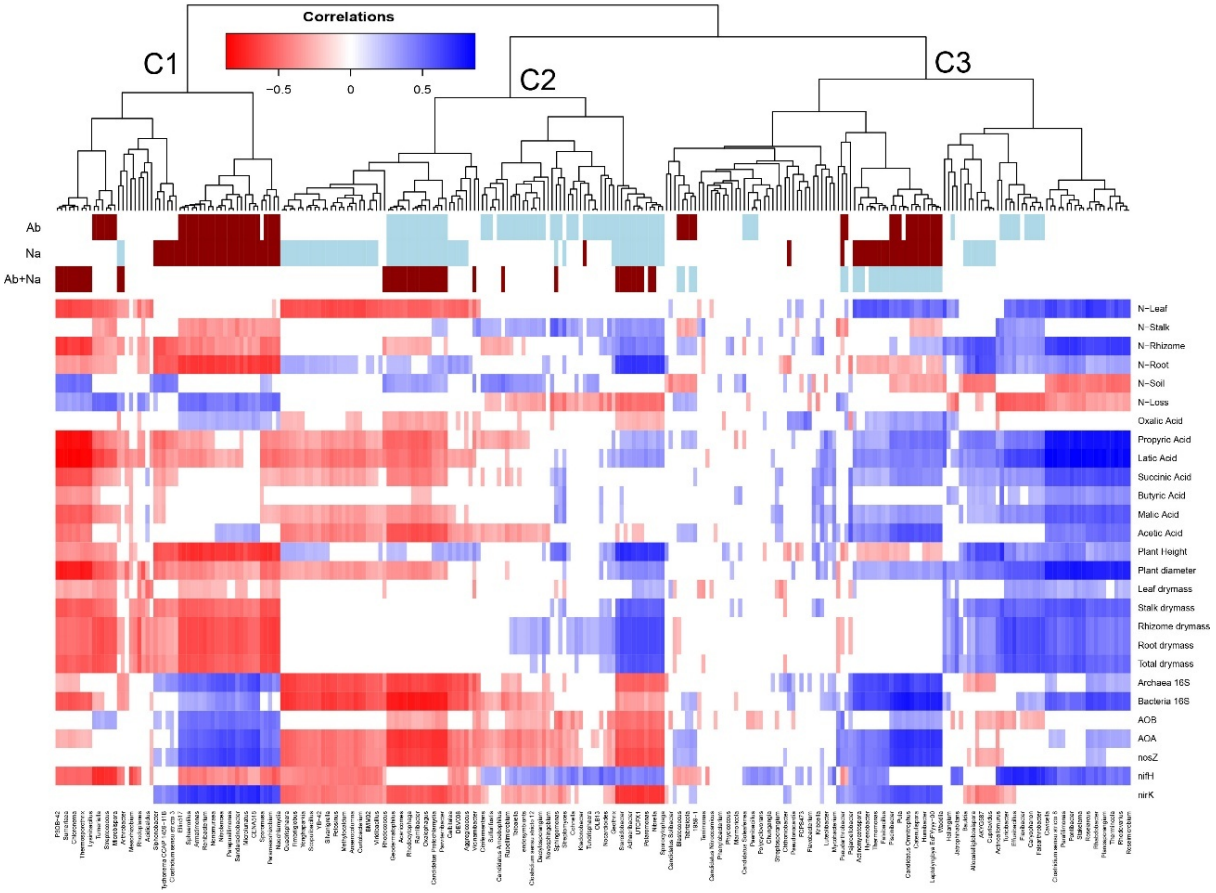

Figure 7. GJAM estimates of associations between the microbes and the crop productivity variables (nitrogen fate, plant performance, root exudates) and the abundance of phylogenetic markers. Red and blue colors indicate negative and positive associations, respectively, between the relative abundance of the microbes and the crop productivity variables. Dark red and light blue colors summarizes the shifts in the abundance of the different microbes in response to the inoculation treatments. White color represent non-significant associations.

Additionally, give the dominance of the *Pseudarthrobacter* genus, we highlight its associations with the abovementioned variables in Figure 8. Our analysis suggested that this genus is negatively associated with nitrogen accumulation in sugarcane stalk and rhizome, plant height, rhizome and root drymass, and the abundance of *nifH*

genes. On the other hand, we observed significant positive associations with nitrogen loss, and the abundance of the functional genes *AOB*, *AOA*, *nosZ*, and *nirK*. Interestingly, the *Pseudarthrobacter* genus does not seem to respond to the changes in the organic acids produced by sugarcane (no significant correlation). Altogether, these results suggest that as a dominant microbe in sugarcane rhizosphere, is more strongly involved in shaping the functional soil microbiome and also contributing for nitrogen losses.

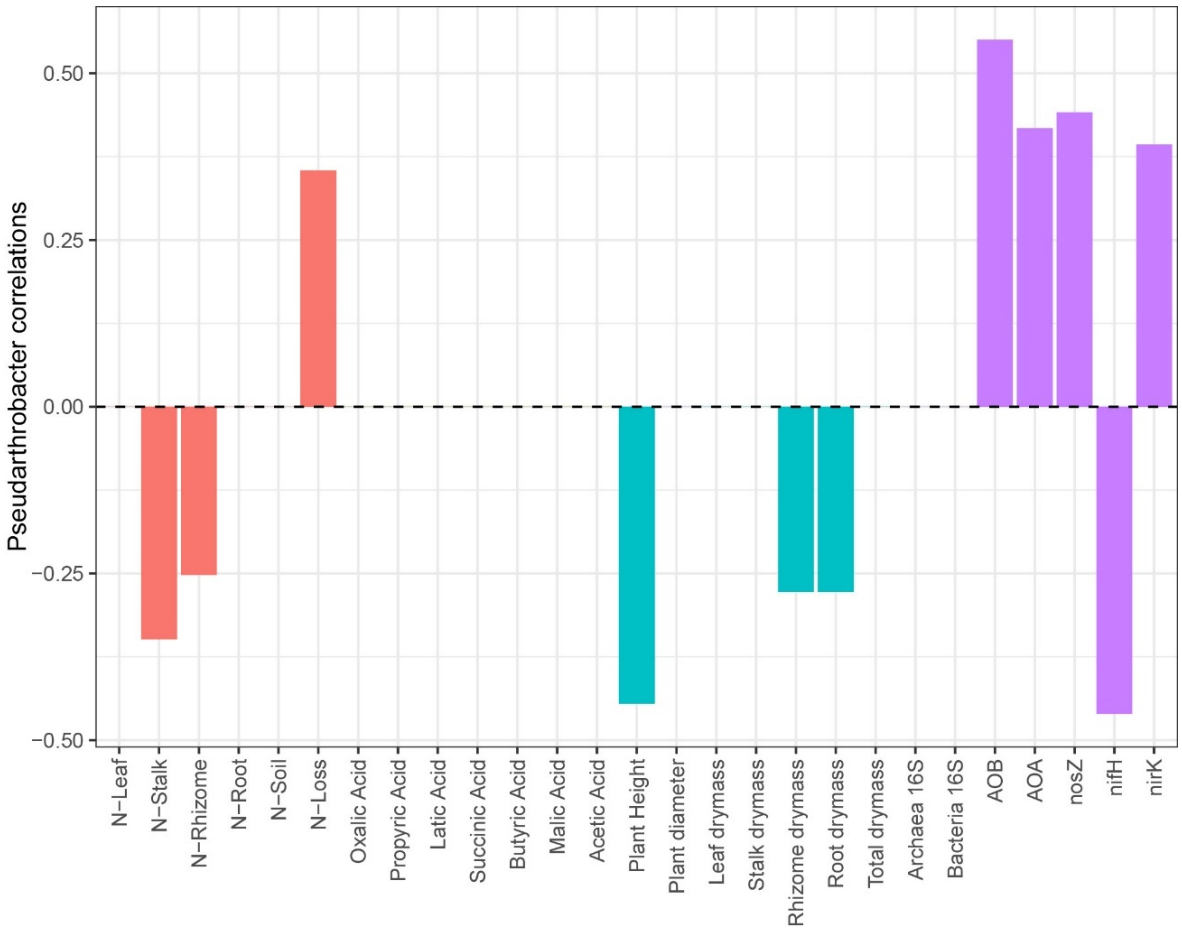

Figure 8. GJAM estimates of associations between the genus *Pseudarthrobacter* and the crop productivity variables (nitrogen fate, plant performance, root exudates) and the abundance of phylogenetic markers.

A closer look at the microbes significantly associated to plant growth also indicates that both rare and dominante microbes in sugarcane rhizosphere are positively related to plant biomass (Figure 9). Interestingly, when we looked for those microbes we observed that from a total of 66 bacterial genera positively associated

with sugarcane total drymass, we observed that only 6 of them are potentially able to fix nitrogen (*Oxyphotobacteria Incertae Sedis* MTP1, *Clostridium sensu stricto* 13, *Anaerosinus*, *Lachnospiraceae Incertae Sedis*, *Thermincola*, and *Clostridium sensu stricto* 12). However, other six microbes potentially able to fix nitrogen in soil where negatively associated with total growth of sugarcane (*Mesorhizobium*, CENA518, *Paeniclostridium*, *Lachnoclostridium*, *Rhodoplanes*, *Sporomusa*).

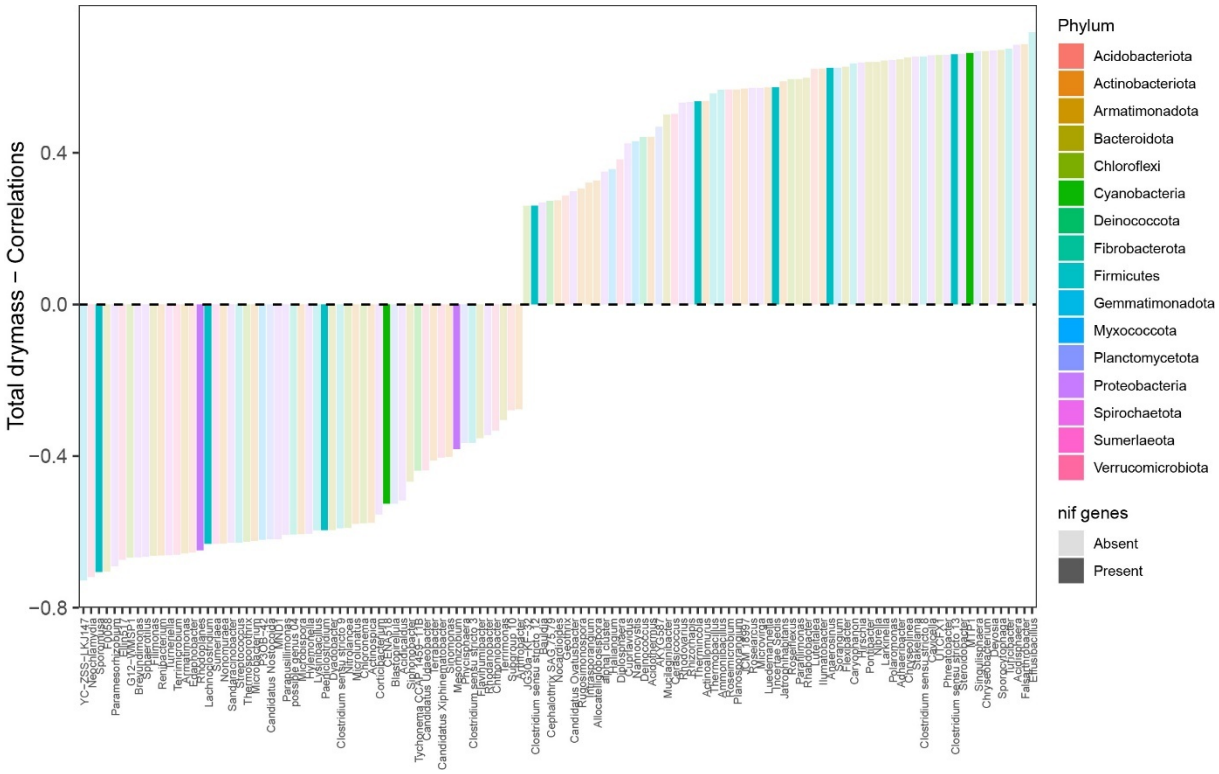

Figure 9. GJAM estimates of associations between the sugarcane total drymass and the soil bacteria in sugarcane rhizosphere. Non-Shaded bar correspond to microbes with the genes related to nitrogen fixation (*nif*H, *nif*D, and *nif*K) as suggested by PICRUST2.

Altogether, our results demonstrated that inoculation of sugarcane with plant growth-promoting bacteria (PGPB), especially the combination of *A. brasilense* and *N. amazonense* (*Ab*+*Na*), led to significant improvements in plant physiology, nitrogen use efficiency, and biomass production compared to the control. These treatments enhanced net photosynthetic rate, water use efficiency, and carboxylation efficiency, while reducing nitrogen losses and increasing the recovery of fertilizer-derived nitrogen in plant tissues. The *Ab*+*Na* treatment also resulted in higher concentrations of organic

acids in the rhizosphere and a unique microbial community structure, with a shift away from dominant taxa toward a more diverse and potentially beneficial rare microbiome.

2.4 DISCUSSION

The foliar application of plant growth-promoting bacteria (PGPB), particularly *Azospirillum brasilense* and its combination with *Nitrospirillum amazonense*, induced a cascade of coordinated responses at multiple biological levels in the plant-soil-microorganism system. These effects were evident across physiological, nutritional, molecular, and ecological parameters, reinforcing the multifaceted role of PGPB in promoting plant growth and nutrient efficiency.

2.4.1 Photosynthesis and Biomass Accumulation: Microbial Modulation of Plant Physiology

Gas exchange analyses revealed that inoculated plants exhibited enhanced photosynthetic performance, characterized by higher net photosynthesis (*A*), increased stomatal conductance (*gs*), reduced sub-stomatal CO_2 concentration (*Ci*), and improved water use efficiency (*WUE*). These effects were most prominent in the *Ab+Na* treatment. Such physiological gains suggest that PGPB inoculation stimulates carbon assimilation and promotes a more favorable water-carbon balance *(Oliveira et al.*, 2025). This enhanced efficiency is likely associated with microbial modulation of hormonal pathways, *A. brasilense* is well known for producing auxins (especially indole-3-acetic acid), gibberellins, and cytokinins, which enhance root development and nutrient uptake (Cassán; Vanderleyden; Spaepen, 2014; Fukami; Cerezini; Hungria, 2018a; Zaheer *et al.*, 2022).

The observed improvements in photosynthetic efficiency translated into increased biomass accumulation, particularly in roots, rhizomes, and total plant dry mass in the *Ab* and *Ab+Na* treatments. Enhanced root growth is consistent with the action of PGPB-derived auxins that stimulate lateral root proliferation and improve soil exploration capacity (Cassán *et al.*, 2020; Fonseca *et al.*, 2022; Pankievicz *et al.*, 2021). The positive correlation between photosynthetic parameters (e.g., A/Ci) and nitrogen accumulation in metabolically active tissues (leaves and rhizomes) reinforces

the hypothesis that microbial inoculation increases nitrogen demand for protein biosynthesis associated with photosynthetic machinery (Flexas *et al.*, 2006).

2.4.2 Nitrogen Recovery and Transformation in the Soil-Plant System

The increased nitrogen accumulation in roots, leaves, and rhizomes under *Azospirillum brasilense* (*Ab*) and *Ab*+*Na* treatments was accompanied by higher recovery of ^{15}N-labeled fertilizer and lower nitrogen losses. These results reflect improved nitrogen uptake efficiency and reduced environmental losses via leaching and gaseous emissions. This is further supported by qPCR data, which revealed a reduced abundance of key genes involved in nitrification (*amoA* from both ammonia-oxidizing archaea and bacteria) and denitrification (*nirK* and *nosZ*) in the inoculated treatments.

Notably, the lower abundance of these genes suggests a suppression of ammonium-to-nitrate conversion and a subsequent reduction in nitrous oxide formation. Such suppression is indicative of a microbiome less prone to driving nitrogen losses through leaching or volatilization, a pattern consistent with reduced nitrifier and denitrifier activity (Philippot et, al. 2013). This effect may be attributed to more efficient ammonium uptake by both the inoculated plants and the introduced microorganisms, leading to a lower concentration of free ammonium in the soil. Consequently, the inoculation appears to shift nitrogen dynamics in favor of plant assimilation and microbial immobilization, rather than transformation into mobile or gaseous forms (Geisseler, D., & Scow, K. M. 2014). These findings reinforce the role of PGPB in enhancing nitrogen retention and functional sustainability within the soil-plant-microbe system.

Although all PGPB treatments showed suppression of these genes compared to the Control, the *Ab*+*Na* treatment consistently presented higher abundances than *Ab* or *Na* alone. A plausible explanation is that the greater exudation of organic acids in the *Ab*+*Na* treatment, particularly acetic, lactic, succinic, and malic acids, may have stimulated microbial mineralization of soil organic matter (SOM), increasing nitrogen availability in the rhizosphere. This enhanced N availability could, in turn, support the activity of nitrifiers and denitrifiers, despite the inoculants' direct suppressive effect (Canarini *et al.*, 2019; Kuypers *et al.*, 2018).

This complex interaction between improved plant absorption, stimulated SOM mineralization, and modulated microbial gene expression reflects the integrated nature of PGPB-induced nitrogen cycling dynamics.

2.4.3 Biological Nitrogen Fixation and Organic Acid Exudation

The abundance of the *nifH* gene increased significantly only in the *Ab* and *Ab+Na* treatments, confirming the functional activation of associative nitrogen fixation pathways. The *Na* treatment did not differ from the Control in *nifH* abundance, suggesting limited stimulation of diazotrophs when applied alone. Thus, it is likely that the increased *nifH* abundance observed in the *Ab+Na* treatment was predominantly due to the presence and activity of *A. brasilense*.

This hypothesis is further supported by the enhanced exudation of organic acids observed in the *Ab+Na* treatment, which may have created favorable conditions for diazotrophic activity by providing labile carbon sources and modulating rhizospheric pH (Canarini *et al.*, 2019). Organic acids not only facilitate nutrient solubilization but also serve as chemoattractants and substrates for beneficial microbial populations (Canarini *et al.*, 2019; Mimmo *et al.*, 2014).

Interestingly, the Control showed greater exudation of some acids than the individual inoculations, which may reflect a compensatory mechanism under nutrient-limited conditions. In contrast, inoculated plants likely required lower carbon expenditure to acquire nutrients due to enhanced microbial efficiency (Dakora & Phillips, 2002).

2.4.4 Rhizospheric Microbiota: Structure and Functional Feedback

The NMDS ordination revealed consistent and significant shifts in soil microbial community structure in response to PGPB inoculation. The clear separation between the inoculated treatments and the Control indicates that the introduced microorganisms modulated the rhizospheric environment, selectively restructuring the native microbiota. Notably, the combination of *Azospirillum brasilense* and *Nitrospirillum amazonense* (*Ab+Na*) resulted in a distinct microbial profile, indicating a synergistic and more pronounced effect on community composition than the single-strain

applications. This suggests a stronger and more uniform microbial reshaping effect compared to single-strain inoculations.

While both *Ab* and *Na* treatments showed partial overlap in the ordination space, suggesting shared functional capacities such as phytohormone synthesis, exudate stimulation, and niche competition (Fukami *et al.*, 2018; Mendes *et al.*, 2013), the *Ab+Na* treatment promoted a more homogeneous microbial community, as evidenced by reduced intra-group dispersion. This spatial cohesion may reflect the convergence of microbial assemblages toward a functionally enriched and ecologically stable state, possibly driven by enhanced and diversified root exudation patterns under the combined inoculation.

Moreover, the higher variability observed in the Control group suggests a lack of selective microbial filtering, allowing greater stochasticity in community assembly. In contrast, the *Ab+Na* treatment appears to impose a selective pressure that stabilizes the microbiome, fostering taxa involved in nutrient mineralization, biosynthesis of bioactive compounds, pathogen suppression, and overall plant growth promotion (Vives-Peris *et al.*, 2018). This indicates that beyond modifying microbial composition, PGPB, especially when applied in consortia, can drive ecosystem-level microbial organization, optimizing the rhizospheric interface for plant-microbe symbiosis and functional resilience.

The GJAM analysis allowed us to evaluate the sugarcane rhizosphere microbial community by jointly modeling both dominant and low-abundance taxa together with the variables of plant performance. Our results suggested that both dominant and low-abudant microbial genera responded to the treaments different inoculation strategies. In line with previous studies (Wang *et al.*, 2023), we highlighted that rare taxa (below top 200) strongly shifted as a result of inoculation treatments, suggesting an important roles in shaping community function and plant-microbe interactions. Altogether, we revealed the importance of considering how different inoculation strategies will impact both dominant and rare microbial groups.

Our GJAM analysis revealed that *Pseudarthrobacter* was the most dominant genus in the sugarcane rhizosphere under control conditions, accounting for over 30% of the estimated relative abundance. This genus, recently reclassified from *Arthrobacter* (Busse, 2016), includes numerous soil-dwelling, gram-positive bacteria known for their plant growth-promoting properties (Chan; Katznelson, 1961; Fincheira; Quiroz, 2018). However, inoculation strategies targeting biological nitrogen fixation

caused a notable reshaping of this microbial landscape. All treatments, including foliar, soil, and combined applications, significantly reduced the abundance of *Pseudarthrobacter*, with the most pronounced decline observed in the combined strategy (from 30.1% to 23.25%). This reduction was accompanied by a general increase in less dominant genera and a substantial enrichment of the rare microbiome, particularly under the combined inoculation treatment. These shifts suggest that while *Pseudarthrobacter* may be a core and potentially member of the sugarcane microbiome, its competitive dominance can be modulated through microbial inoculants, potentially opening ecological niches for a broader range of beneficial taxa. The implications for microbial balance and functional redundancy in the rhizosphere merit further exploration, especially considering *Pseudarthrobacter*'s putative role in sugarcane growth and influence in the functional genes of the nitrogen cycle.

2.4.5 An Integrated View of Plant-Microbe-Soil Interactions

The results demonstrate a mechanistic cascade triggered by microbial inoculation, in which enhanced photosynthetic efficiency leads to increased carbon assimilation and greater nitrogen demand. This demand is met not only by improved nitrogen uptake from fertilizers but also by enhanced root function and metabolic activation in inoculated plants. The elevated photosynthetic activity promotes higher root exudation, especially of organic acids, which alters the composition of rhizospheric compounds. These exudates, in turn, selectively stimulate microbial taxa involved in nitrogen cycling and other key soil processes. This sequence reinforces a functional feedback loop between plant physiology, root exudation, and the structure and function of the soil microbiome. Notably, the consortium treatment (*Ab*+*Na*) intensified these effects, supporting the hypothesis that microbial combinations can elicit synergistic responses across biological domains, plant, soil, and microbial community, beyond those induced by single strains (Pascale *et al.*, 2020; Vives-Peris *et al.*, 2018).

2.5. CONCLUSION

The foliar application of *Azospirillum brasilense* and the soil drench application of *Nitrospirillum amazonense*, particularly in combination, promoted coordinated enhancements in plant physiology, nitrogen uptake, and rhizospheric microbial

processes in sugarcane. Inoculated plants exhibited improved photosynthetic efficiency, greater nitrogen accumulation, and higher biomass production, with the most pronounced effects observed in the *Ab*+*Na* and *Ab* treatments.

^{15}N isotope tracing revealed that these treatments also enhanced fertilizer nitrogen recovery and reduced N losses, supported by the downregulation of nitrification (*amoA*) and denitrification (*nirK*, *nosZ*) gene abundances. Although the *Ab*+*Na* treatment maintained a higher abundance of these genes than the individual inoculations, this response likely reflects increased rhizodeposition of organic acids, particularly acetic, lactic, and malic acids, which may have stimulated microbial turnover of native soil organic matter and thus increased nitrogen mineralization and availability.

The abundance of the *nifH* gene, a key marker of biological nitrogen fixation, was significantly elevated in the *Ab* and *Ab*+*Na* treatments. Since the *Na* treatment did not differ from the Control in *nifH* abundance, it is plausible that the increased nitrogen fixation potential in the *Ab*+*Na* was mainly driven by *A. brasilense*, reinforcing its primary role in associative and potentially endophytic N_2 fixation in sugarcane.

Additionally, microbial community structure was significantly altered by the inoculations, especially under the *Ab*+*Na* treatment, which promoted a more homogeneous and functionally enriched rhizospheric microbiome. These results highlight that foliar-applied PGPB, when combined with soil-applied counterparts, can trigger systemic responses in the plant that influence root exudation patterns, thereby shaping microbial community composition and functional gene expression.

Together, these findings provide strong evidence that microbial consortia, applied through complementary routes, can orchestrate integrated plant-microbiome interactions that enhance nitrogen cycling and sugarcane productivity. This supports the strategic use of multifunctional inoculants in sustainable and resource-efficient production systems.

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SUPPLEMENTARY MATERIAL

Table S1 – Bioinformatics steps for processing the 16S rRNA sequences.

Seq_Name	Sample_ID	input	filtered	denoisedF	denoisedR	merged	nonchim	mitochondria, chloroplast, rarefaction
NGS576-1	S01	232972	182023	175571	175843	163045	158943	126391
NGS576-2	S02	218303	172440	166180	166235	154638	151204	126391
NGS576-3	S03	245535	190808	184470	184766	171216	167839	126391
NGS576-4	S04	234896	182448	176674	177009	166420	163017	126391
NGS576-5	S05	232724	181889	176021	176082	164791	161018	126391
NGS576-6	S06	189090	145422	139713	139998	129146	126749	126391
NGS576-7	S07	228801	177824	171307	171770	159139	155973	126391
NGS576-8	S08	203940	159932	154049	154226	143210	140431	126391
NGS576-9	S09	203337	154442	149003	149437	138322	135280	126391
NGS576-10	S10	218935	171383	165413	165808	154128	151098	126391
NGS576-11	S11	219496	173975	168207	168719	158477	154652	126391
NGS576-12	S12	241187	188204	182744	182718	171830	167913	126391
NGS576-13	S13	208618	157081	151062	151224	138943	135970	126391
NGS576-14	S14	239833	186318	180569	180657	168231	163676	126391
NGS576-15	S15	212803	167023	161077	161517	149144	146302	126391
NGS576-16	S16	242519	187208	181276	181473	168833	165514	126391

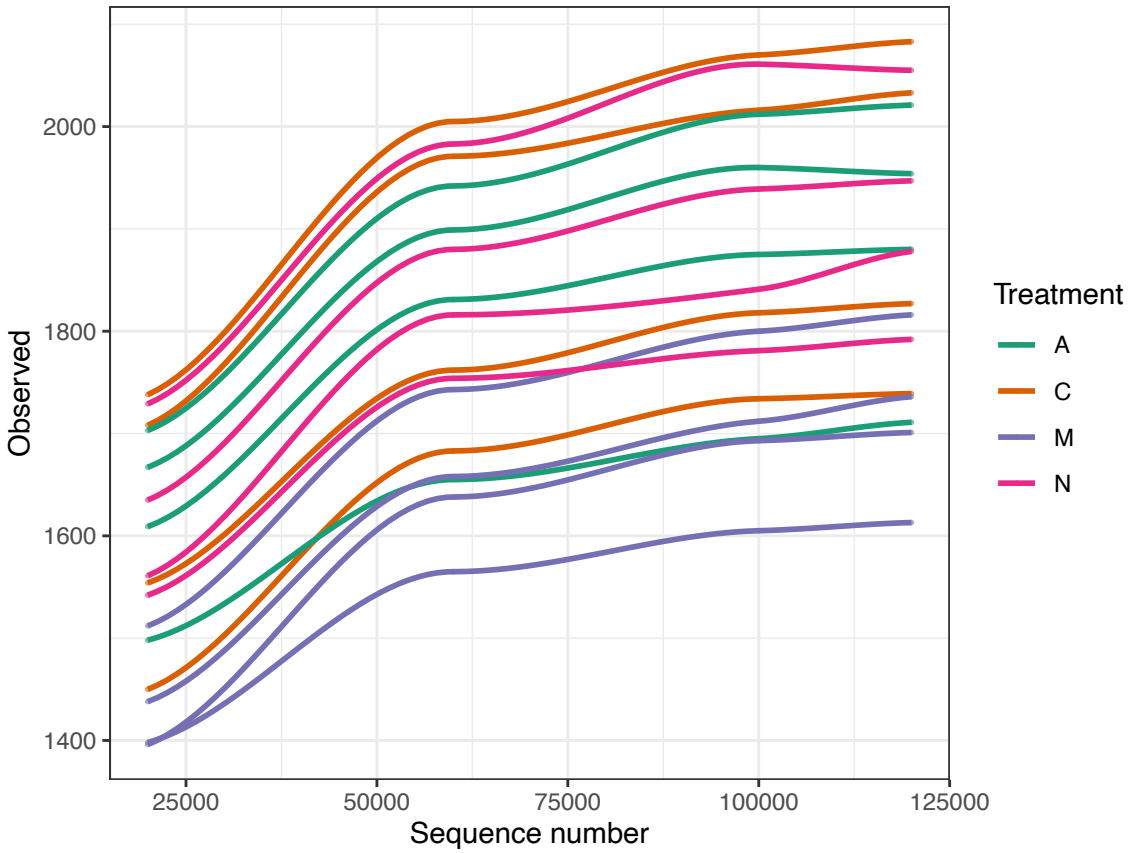

Figure S1 – Rarefaction curve.

Table S2 – Nutrient content of sugarcane leaves under greenhouse as a function of treatment [Control (C), *Azospirillum brasilense* (*Ab*), *Nitrospirillum amazonense* (*Na*), and Mix (*Ab* + *Na*)].

Treat.	N	P	K	Ca	Mg	S	Fe	Cu	Zn	Mn	B
	---------- g kg^{-1} ----------						---------- mg kg^{-1} ----------				
C	19.8 c	1.64 a	10.5 a	4.01 a	1.34 b	1.53 a	84.3 a	10.2 a	30.2 a	154 a	20.8 b
Ab	22.3 b	1.85 a	11.6 a	4.49 a	1.62 a	1.72 a	78.3 a	10.7 a	32.7 a	163 a	28.4 a
Na	21.5 b	1.74 a	11.1 a	4.20 a	1.56 ab	1.78 a	79.8 a	10.2 a	30.4 a	172 a	24.6 ab
Ab+Na	24.1 a	1.77 a	10.9 a	4.23 a	1.66 a	1.86 a	80.9 a	10.4 a	29.9 a	171 a	26.9 a

Different letters indicate significant differences between treatments by the LSD test at $p \leq 0.05$ ($n = 4$)

Table S3 – Pairwise comparison of microbial community structures among treatments based on PERMANOVA results (F, R^2, and *p-value*).

Groups measure	F	R^2	*p* - value	*p – value* adjusted
Control vs *Ab*	3.646692	0.3780252	0.022	0.036
Control vs *Ab+Na*	2.710628	0.3111863	0.035	0.042
Control vs *Na*	4.899214	0.4495016	0.020	0.036
Ab vs *Ab+Na*	8.152536	0.5760477	0.024	0.036
Ab vs *Na*	2.066731	0.2562043	0.052	0.052
Ab+Na vs *Na*	8.649605	0.5904327	0.022	0.036

FINAL CONSIDERATIONS

The results obtained in this thesis robustly demonstrate the high potential of plant growth-promoting bacteria (PGPB) as innovative biotechnological tools for the sustainable intensification of sugarcane production. The inoculation with Azospirillum brasilense via foliar application and Nitrospirillum amazonense via soil application, especially when applied in combination, promoted a series of physiological, nutritional, and microbial benefits that, in an integrated manner, resulted in significant increases in crop productivity and nitrogen use efficiency.

This body of evidence reinforces the effectiveness of PGPB inoculation as a robust agronomic strategy to enhance the productive performance of sugarcane, while simultaneously contributing to improved utilization of synthetic fertilizers and the mitigation of environmental impacts associated with the intensive cultivation of this species. Furthermore, the findings of this thesis expand the understanding of the complex interactions among plants, the rhizospheric microbiome, and nitrogen cycling processes, providing a solid scientific foundation for the adoption and refinement of more efficient and resilient agricultural practices aligned with the principles of bioeconomy and sustainability.

In light of these promising results, it is recommended that research efforts continue and expand to evaluate the application of these technologies under different edaphoclimatic conditions and management systems, aiming to improve inoculation strategies and maximize the benefits provided by PGPB to sugarcane cultivation and, potentially, to other agriculturally important crops.

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