



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

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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 ¹⁵N fertilizer recovery. Nitrogen losses were significantly reduced in *Ab* and *Ab+Na* treatments. At the molecular level, rhizosphere soil analysis showed a reduced abundance of microbial functional genes related to nitrification and denitrification (*amoA*, *nirK*, and *nosZ*) in inoculated treatments, indicating a lower potential for nitrogen losses. 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 combining microbial inoculants 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.

Keywords Diazotrophic bacteria · Functional gene abundance · Gas exchange · Rhizosphere modulation · Root exudates

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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 et al. 2019a, b). 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. 2019a, b; Martini 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 et al. 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 et al. 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 et al. 2021; Schwab et al. 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 and Podile 2019; Fukami et al. 2018). Root exudates are central to rhizosphere ecology, shaping microbial communities, influencing nutrient solubilization, and fostering mutualistic interactions (Cesari et al. 2019; Pereira et al. 2021). 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 and 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 and Phillips 2002; Hartmann and 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.

Materials and methods

Experimental design

The experiment, started in November 2021, 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.

Soil preparation, cultivation conditions and fertilization

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). The chemical composition of the lime was: CaO 39%, MgO 13%, neutralization power (NP) 100%, and relative total neutralization power (RNP) 91.5%.

Fertilization was based on soil analysis and expected yield targets, corresponding to the equivalent rates of 90 kg N ha⁻¹, 180 kg P₂O₅ ha⁻¹, and 80 kg K₂O ha⁻¹. The fertilizers used were: ammonium sulfate (20% N), single superphosphate (18% P₂O₅), and potassium chloride (60% K₂O). No pesticides were applied during the experiment; weed control inside pots was performed manually, without herbicide use.

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.

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). The Ab-V5 and Ab-V6 strains were used in combination, a standard widely adopted in research and commercial products in Brazil. *Nitrospirillum 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, 2018; Santos et al. 2021); and CNPSo 4060 [Na] (Dartora et al. 2016; Junior et al. 2008).

Applications were carried out using a CO₂-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.

¹⁵N-Labeled Fertilizer Application

Ammonium sulfate enriched with 2.683 atom % excess ¹⁵N ((¹⁵NH₄)₂SO₄; Sigma-Aldrich, St. Louis, MO, USA) was

applied to the pots at the equivalent rate of 90 kg N ha⁻¹. The labeled fertilizer was incorporated into the soil surface to minimize ammonia volatilization.

Plant and soil evaluations

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).

Organic acid exudation

Soil sampling for the determination of organic acids was performed at 200 days after emergence (DAE) of sugarcane seedlings, corresponding to the phenological stage of intense vegetative growth, as previously described (Camara 1993). For each experimental unit, soil was collected from the region immediately adhering to the roots, and was therefore considered rhizosphere soil. The soil adhering to the roots was collected and stored in sterile Falcon tubes, sealed with Parafilm to reduce volatilization losses, kept on ice during transport, and subsequently stored at -20 °C until analysis.

For extraction, 5.00 g of fresh soil were diluted in 25.0 mL of 0.005 N H₂SO₄ (soil-to-solution ratio 1:5, w/v) and allowed to rest for 24 h to facilitate desorption of acid compounds. Then, samples were centrifuged at 10,000 × g for 10 min at 10 °C, and the resulting supernatants were filtered through polyvinylidene fluoride (PVDF) membranes (0.22 µm, 13 mm; Millipore). Carboxylic acids were analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu chromatograph (model 10 A) equipped with a refractive index detector (RID-10 A) and an isocratic pump (LC-10AD), operating at a flow rate of 1.0 mL min⁻¹ with 0.005 N H₂SO₄ as the mobile phase. Separation was performed using a Bio-Rad Aminex HPX-87 H column (H⁺ form) at 65 °C, with temperature control provided by a CTO-10 A oven. 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). The units obtained were in g kg^{-1} of soil. Subsequently, these data were normalized, using the Z-score, due to the differences in concentration magnitudes for each acid, for graphic representation using a heat map.

Soil DNA extraction and quantification

Rhizospheric soil sampling for molecular analyses (DNA and qPCR) was performed at the same time (200 DAE). The soil adhered to the roots was removed using sterile spatulas and stored in sterile tubes on ice, and subsequently kept at $-20\text{ }^{\circ}\text{C}$ until DNA extraction in order to minimize microbial alterations and nucleic acid degradation. 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 and 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.

Quantitative Real-Time PCR (qPCR)

The abundances of total *Bacteria* and *Archaea* (each quantified using primers targeting the *16 S 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
16 S rRNA Archaea	519 F	5'-CAGCCGCCGCGGTA A 3'	95°C – 10 min	(Coolen et al. 2004; Stahl 1991)
	915R	5'-GTGCTCCCCCGCCAATTCCT 3'	40 cycles: 95°C – 30 s; 57°C – 30 s; 72°C – 50 s*.	
16 S rRNA Bacteria	Eub338	5'-CCTACGGGAGGCAGCAG-3'	95 °C–10 min;	(Muyzer et al.1993)
	Eub518	5'-ATTACCGCGGCTGCTGG-3'	40 cycles: 95 °C–30 s; 53 °C–40 s; 72 °C–40 s*.	
<i>nifH</i>	nifHF	5'-AAAGGYGGWATCGGYAARTCCACCAC-3'	95 °C–10 min;	(Rösch et al. 2002)
	nifHR	5'-TTGTTSGCSGCRATCATSGCCATCAT-3'	40 cycles: 95 °C–1 min; 55 °C–27 s.; 72 °C–1 min*.	
Arch-amoA	Arch-amoAF	5'-STAATGGTCTGGCTTAGACG-3'	95 °C–5 min;	(Francis et al. 2005)
	Arch-amoAR	5'-GCGGCCATCCATCTGTATGT-3'	40 cycles: 95 °C–40 s; 56 °C–30 s.; 72 °C–1 min*.	
Bac-amoA	amoA1F	5'-GGGGTTTCTACTGGTGGT-3'	95 °C–10 min;	(Rotthauwe et al. 1997)
	amoA2R	5'-CCCCTCKGSAAAGCCTTCTTC-3'	40 cycles: 95 °C–40 s; 56 °C–30 s; 72 °C–1 min*.	
<i>nirK</i>	NirK876	5'-ATYGGCWGAYGGGGA-3'	95 °C–10 min;	(Henry et al. 2004)
	NirK1040	5'-GCCTCGATCAGRTTRTGGTT-3'	40 cycles: 95 °C–15 s; 63 °C–30 s; 72 °C–30 s*.	
<i>nosZ</i>	nosZ2F	5'-CGCRACGGCAASAAGGTSMSST-3'	95 °C–10 min;	(Henry et al. 2006)
	nosZ2R	5'-CAKRTGCAKSGCRTGGGAAGAA-3'	40 cycles: 95 °C–40 s; 63 °C–30 s; 72 °C–40 s*.	

16 S 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. The sequences were deposited in the NCBI Sequence Read Archive under the accession number PRJNA1293782.

Biomass determination

Plants were harvested and separated into leaves, stalk, 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.

Isotopic analyses and nitrogen recovery

Total N and ¹⁵N enrichment in plant and soil samples were analyzed using an elemental analyzer (Flash EA, Thermo Scientific, Germany) coupled to an isotope ratio mass spectrometer (Delta V, Thermo Scientific, Germany). Data were expressed as atom % ¹⁵N excess and recovery percentage.

Nitrogen derived from fertilizer (Ndff, kg ha⁻¹) and recovery efficiency were calculated using the following equations:

1. $\text{Ndff (kg ha}^{-1}\text{)} = (x / y) \times \text{TN}$.
2. $^{15}\text{N recovery (\%)} = (\text{Ndff} / \text{NFR}) \times 100$.
3. $\text{Unrecovered FS (\%)} = 100 - \text{recovery FS}$.
4. $^{15}\text{N remaining (\%)} = \text{recovery FS} - \text{recovery sugarcane}$.
5. $\text{Unrecovered SS (\%)} = \text{remaining} - \text{recovery SS}$.

Where *x* and *y* represent the excess ¹⁵N in plant tissues or soil and the fertilizer source, respectively (natural abundance=0.368 atom %), *TN* is total N in each compartment, and *NFR* is the total N applied.

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 (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 126,391 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 16 S 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 composition 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 and 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, we extracted information on which microbial taxa were selected under each soil amendment treatment. This allowed to identify the microbiome profile, *sensu* Rtoni 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 acid, 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 and 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 2021) using RStudio (v1.3.1073), and graphs were prepared in GraphPad Prism (v9.5.1).

Results

N cycle functional genes (real-time PCR)

The abundance of the archaeal 16 S rRNA gene differed among treatments (Fig. 1A). The highest values were observed in the Control (9.59×10^6 gene copies g^{-1} soil)

Fig. 1 Absolute abundance of phylogenetic markers 16 S 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 ($p \leq 0.05$)

and in the combined inoculation of *Azospirillum brasilense* + *Nitrospirillum amazonense* (*Ab+Na*) (8.25×10^6 gene copies g^{-1} soil), which showed comparable archaeal abundances. In contrast, plants inoculated with *A. brasilense* (*Ab*) or *N. amazonense* (*Na*) alone exhibited significantly lower archaeal 16 S rRNA gene abundances, corresponding to reductions of 31.7% and 53.3%, respectively, relative to the control.

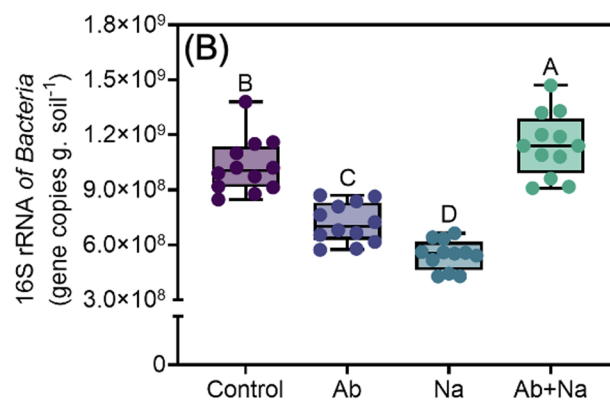
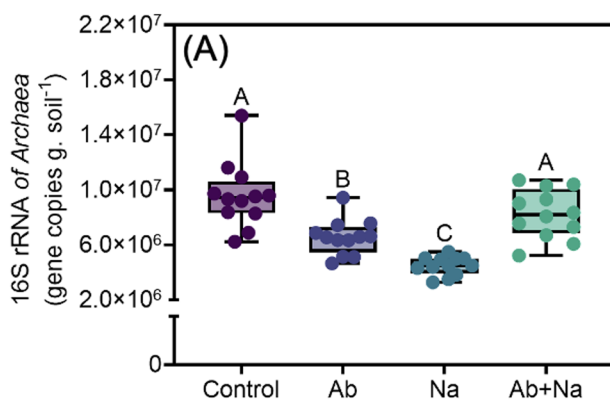
For the quantification of the bacterial 16 S rRNA gene (Fig. 1B), the highest absolute abundance was observed in the *Ab+Na* treatment, which was 11.7% greater than that of the Control (1.03×10^9 gene copies g^{-1} soil). In contrast, isolated inoculations resulted in lower bacterial 16 S rRNA gene abundances, with reductions of 30.1% in the *Ab* treatment and 47.2% in the *Na* treatment relative to the Control.

Regarding the *nifH* gene (Fig. 1C), the highest absolute 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).

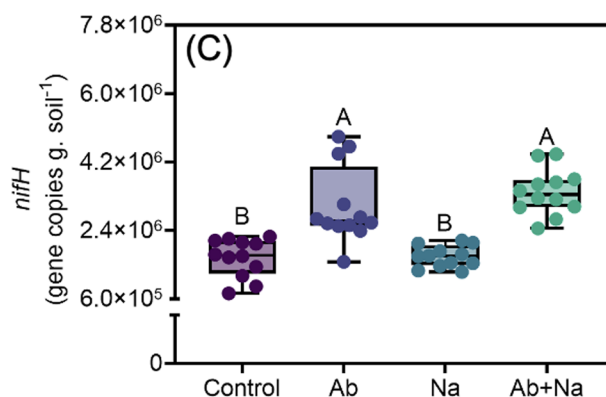
For the archaeal *amoA* gene (AOA), all treatments inoculated with PGPB showed reduced absolute abundance to the Control (Fig. 1D). The Control presented 3.57×10^6 gene copies g^{-1} soil, while the *Ab* and *Na* treatments showed an average decrease of 59.5%. The *Ab+Na* treatment also resulted in a 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 absolute abundance (Fig. 1E) compared to the Control (1.70×10^5 gene copies g^{-1} soil). With respect to the *nirK* gene (Fig. 1F), associated with the denitrification process, the highest absolute 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 (Fig. 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.

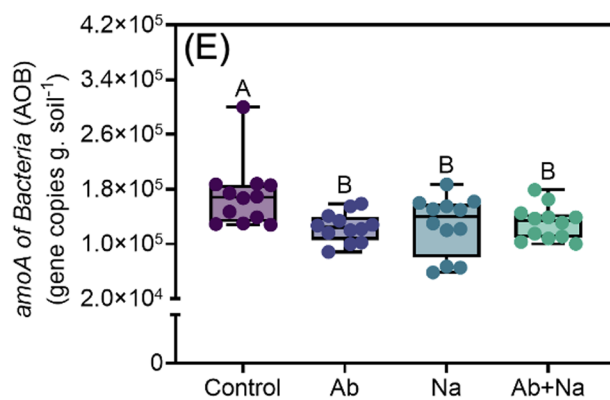
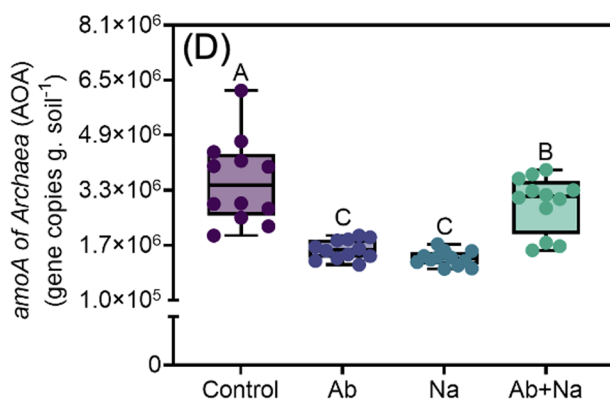
Phylogenetic markers



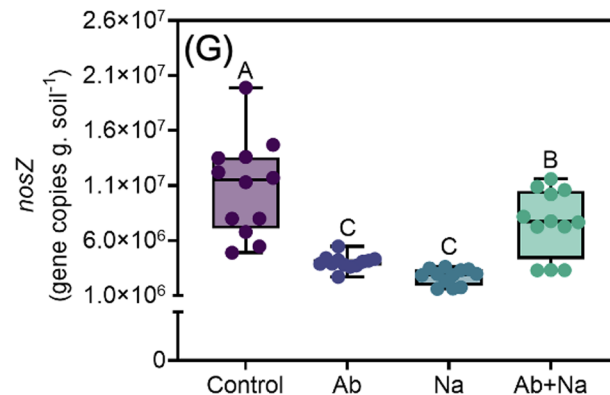
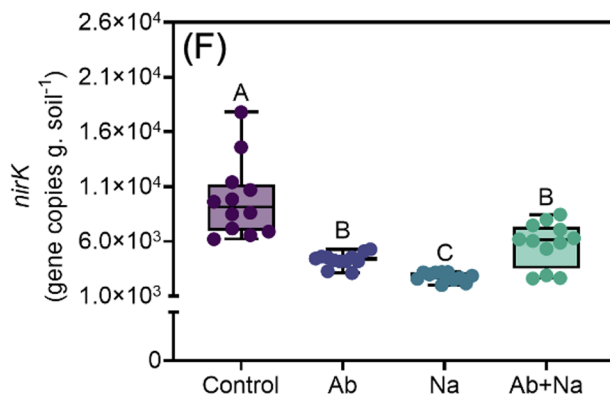
Nitrogen fixers



Nitrification



Denitrification



Gas exchange

Physiological parameters related to gas exchange were significantly influenced by the inoculation with plant growth-promoting bacteria (PGPB). Net photosynthetic rate (Fig. 2A) was significantly higher ($p \leq 0.05$) in all

treatments receiving microbial inoculation compared to the Control ($17.2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). The highest increase was observed in the *Ab+Na* treatment, with an enhancement of 22.1%, followed by *Azospirillum brasiliense* (*Ab*) with 16.9% and *Nitrospirillum amazonense* (*Na*) with 9.88%.

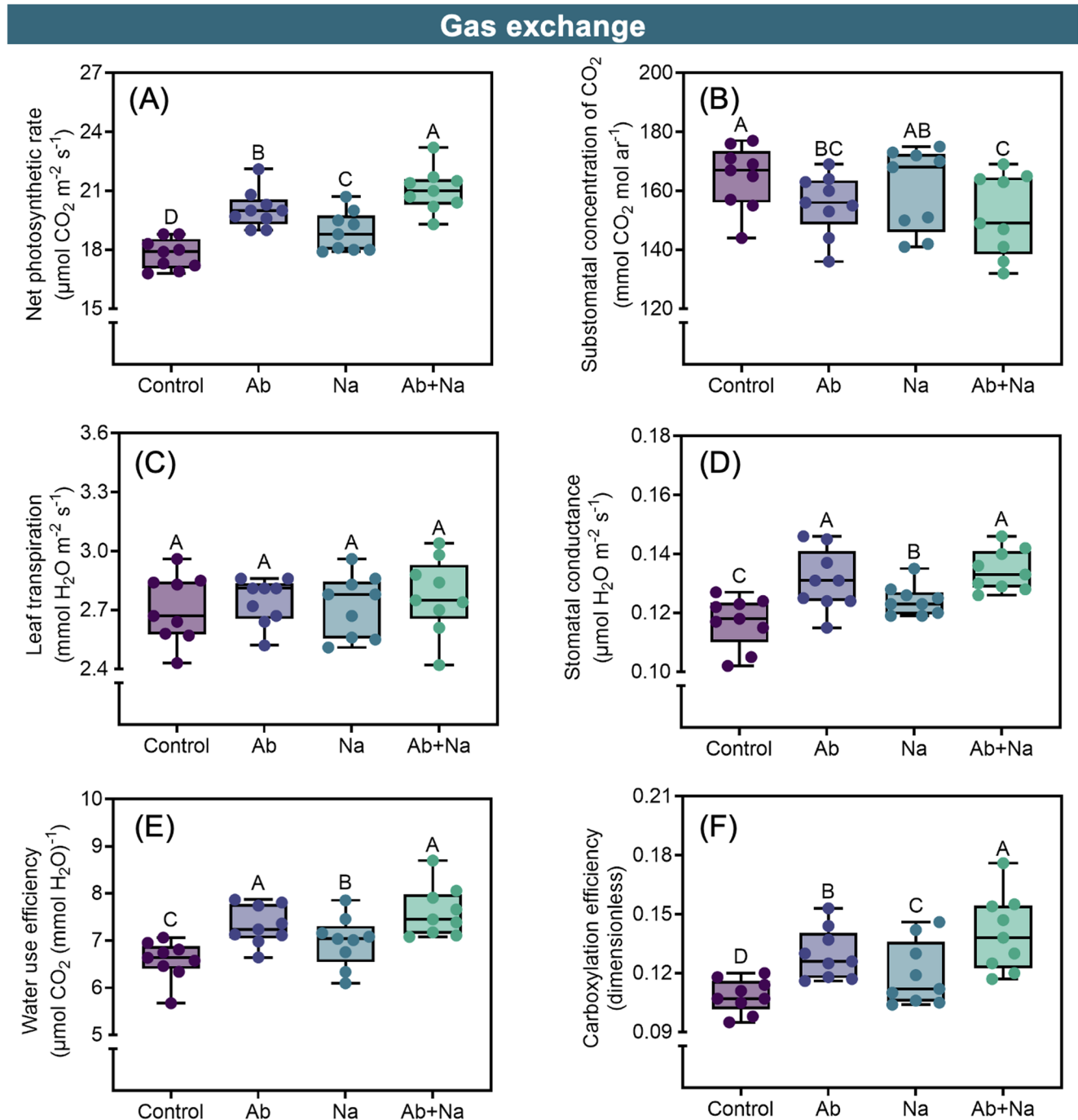


Fig. 2 Net photosynthesis rate (A), internal sub-stomatal CO_2 concentration (B), leaf transpiration (C), stomatal conductance (D), water use efficiency (E), and carboxylation efficiency (F) in sugarcane leaves grown in a greenhouse under different treatments: Control (no microorganism application), *Ab* (*Azospirillum brasiliense*), *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 CO₂ concentration in the substomatal cavity (Fig. 2B) was highest in the Control (165 mmol CO₂ 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 CO₂ due to enhanced photosynthetic activity. On the other hand, transpiration rate (Fig. 2C) showed no statistically significant differences among treatments.

Stomatal conductance (Fig. 2D) was higher in all treatments inoculated with microorganisms. The Control showed an average value of 0.117 $\mu\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$, 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 (Fig. 2E) was also significantly higher in the PGPB treatments ($p \leq 0.05$) compared to the Control (6.59 $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{H}_2\text{O}$). The *Ab+Na*, *Ab*, and *Na* treatments promoted increases of 15.6%, 11.1%, and 5.92%, respectively.

Carboxylation efficiency (Fig. 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.

Fate of ¹⁵N fertilizer in the soil-plant system, nitrogen accumulation, and plant biomass

Plant biomass was significantly influenced by the application of microorganisms (Fig. 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.

The distribution of fertilizer-derived ¹⁵N among plant compartments varied in response to the applied treatments (Fig. 3B), with significant differences observed for some compartments. In the roots, all treatments involving microbial inoculation showed greater ¹⁵N recovery compared to the Control. The Control treatment exhibited 5.95% of fertilizer-derived ¹⁵N 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 ¹⁵N recovery, with 10.2% of fertilizer-derived ¹⁵N 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 ¹⁵N recovered, corresponding to a 13.5% increase compared to the Control, which exhibited 19.2% fertilizer-derived ¹⁵N. No significant differences were observed among treatments for ¹⁵N recovery in the stalk and in the soil. Consistent with the increases in fertilizer-derived ¹⁵N, the *Ab+Na* treatment also showed the highest total biomass accumulation, supporting the physiological link between nitrogen availability and plant growth.

Regarding fertilizer ¹⁵N 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 ¹⁵N (Fig. 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 ¹⁵N 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 (Fig. 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 stalk, 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).

Quantification of organic acids

Organic acids were quantified in the rhizospheric soil and expressed as g kg⁻¹ of dry soil prior to data standardization. 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 Fig. 4, where data were normalized using Z-score exclusively for visualization.

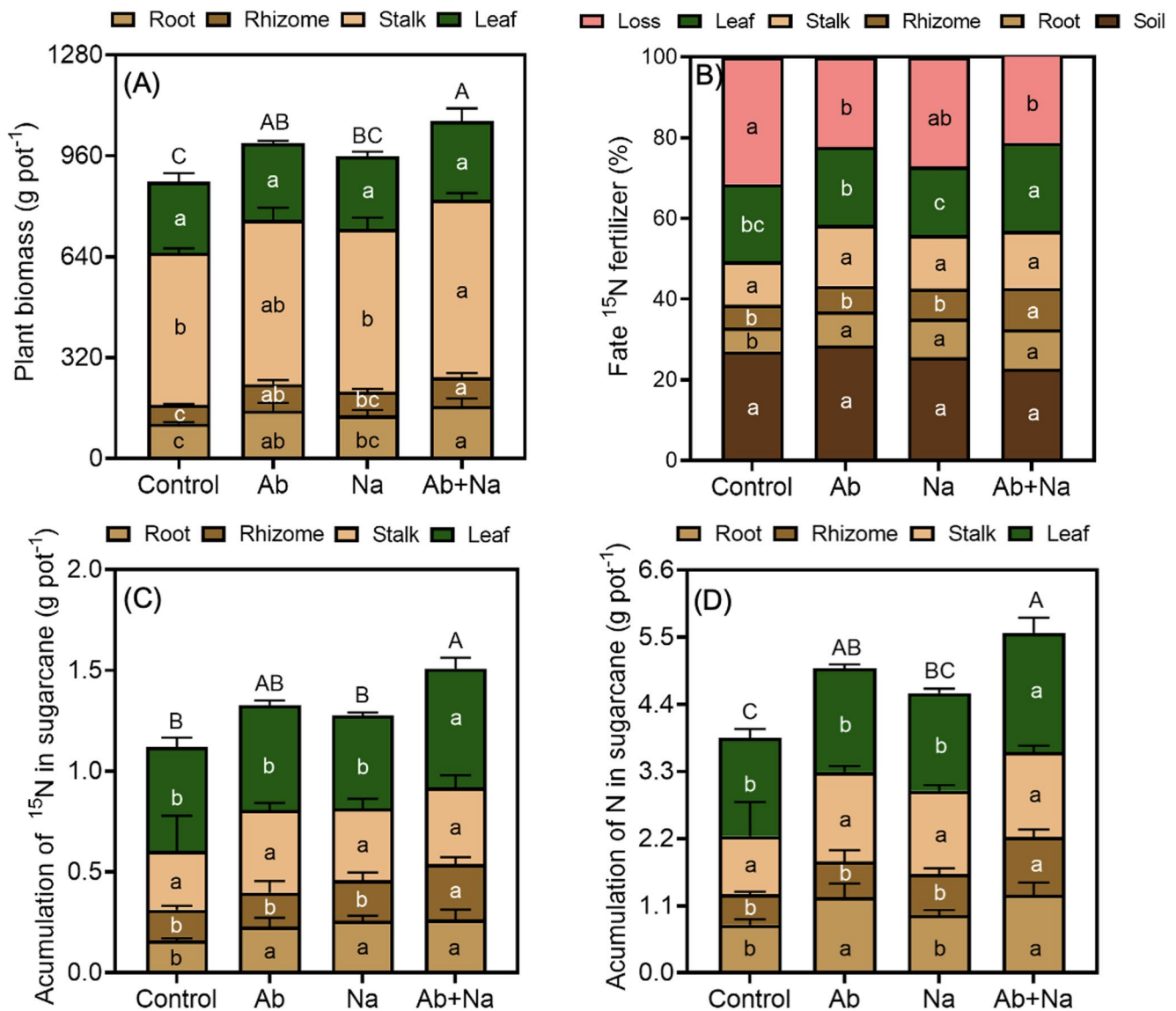


Fig. 3 Plant biomass (A) Fate of the ¹⁵N-labeled fertilizer in the soil and plant compartments (B); accumulation of ¹⁵N in sugarcane (C) and accumulation of N total in sugarcane (D), under different treatments: Control (no microorganism application), Ab (*Azospirillum brasilense*), Na (*Nitrospirillum amazonense*), and combination of Ab+Na. Differ-

ent lowercase letters indicate significant differences between treatments, while uppercase letters above the bars indicate significant differences among treatments for the total value of each variable, as determined by the LSD test ($p \leq 0.05$)

Microbial community composition

The Non-metric Multidimensional Scaling (NMDS) analysis revealed marked differences in the soil microbial community composition among the evaluated treatments (Fig. 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 composition 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 composition.

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

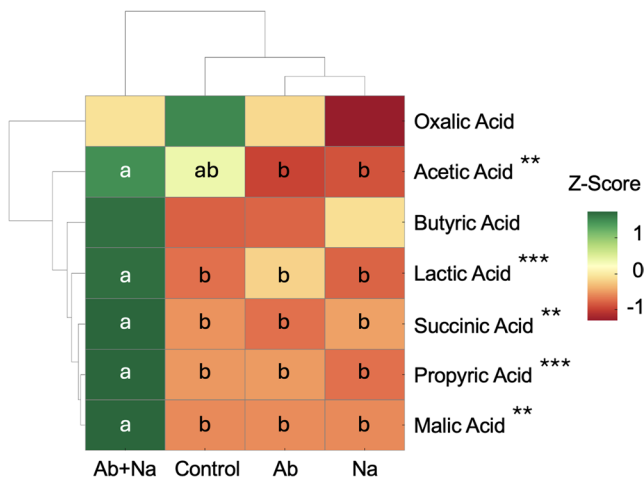


Fig. 4 Heat map representing the quantification of organic acids under the influence of different treatments. The evaluated treatments were Control (no microorganism application), *Ab* (*Azospirillum brasilense*), *Na* (*Nitrospirillum amazonense*), and combination of *Ab*+*Na*. Original quantification was performed using HPLC and expressed as g kg⁻¹ of dry soil before Z-score transformation. Statistical significance was determined using the LSD test ($p \leq 0.05$). Symbols denote statistical differences between treatments: $p \leq 0.05$ (**) and $p \leq 0.001$ (***)

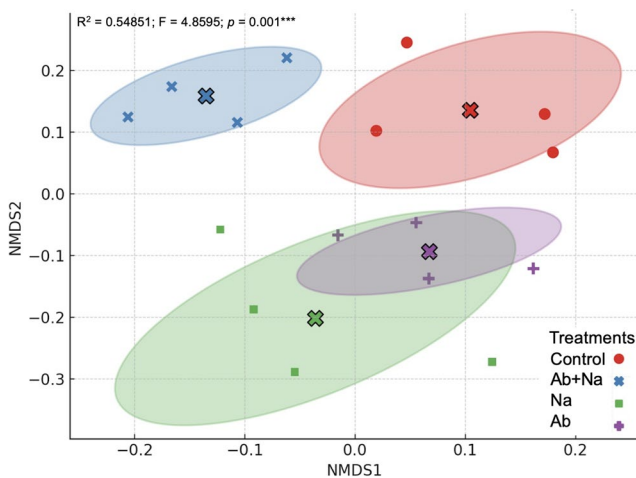


Fig. 5 Non-metric Multidimensional Scaling (NMDS) analysis depicting the microbial community composition under different treatments: Control (no microorganism application), *Ab* (inoculation with *Azospirillum brasilense*), *Na* (*Nitrospirillum amazonense*), and the combination of *Ab*+*Na*. The pairwise significance between treatments is presented in Supplementary Table S4

Phycoccus), 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* (Fig. 6A). Notably, both *Azospirillum* and *Nitrospirillum* genera were not detected in the rhizosphere soil of non-inoculated sugarcane (control treatment).

Interestingly, the foliar inoculation of *A. brasilense* (Fig. 6B) induced a big decrease in the abundance of the genus *Pseudarthrobacter* (from 30% in the control

to 19.1%). This decrease was followed by an increase in several different genera of bacteria. A total of 15 genera belonging to the top 100 most abundant microbes in the control treatment increased in abundance as a result of the inoculation of *A. brasilense* (*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 altered the rhizosphere microbiome by decreasing the dominance of the most abundant bacterial genera and increasing the relative contribution of low-abundance (rare) taxa that were at very low levels in the control treatment. Figure 6C represents the shifts in the composition of the sugarcane rhizosphere microbiome as a result of inoculating *N. amazonense*; it 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%), *Sot phingomonas* (4.63% to 5.27%), *Conexibacter* (3.30% to 4.07%), and *Streptomyces* (0.80% to 1.13%) increased in abundance. These shifts indicate changes in the competitive balance within the microbial community following inoculation, favoring the establishment of previously low-abundance taxa. Below the top 100 most abundant genera, inoculation triggered a pronounced turnover within the rare biosphere, with 54 low-abundance genera disappearing and 64 previously undetected genera becoming established. This indicates that inoculation strongly reshaped the rare fraction of the microbiome, even though the most dominant taxa showed more moderate changes. In summary, Inoculation of *N. amazonense* affected a total of 124 bacterial genera and reshaped the community of rare bacterial genera reducing the abundance of the most dominant genus.

The combined strategy of applying foliar spaying and soil inoculation (Fig. 6D) also affected the bacterial community in the rhizosphere of sugarcane. Interestingly, the most abundant taxa (e.g., Actinobacteria, Firmicutes and Proteobacteria) exhibited smaller relative shifts across treatments,

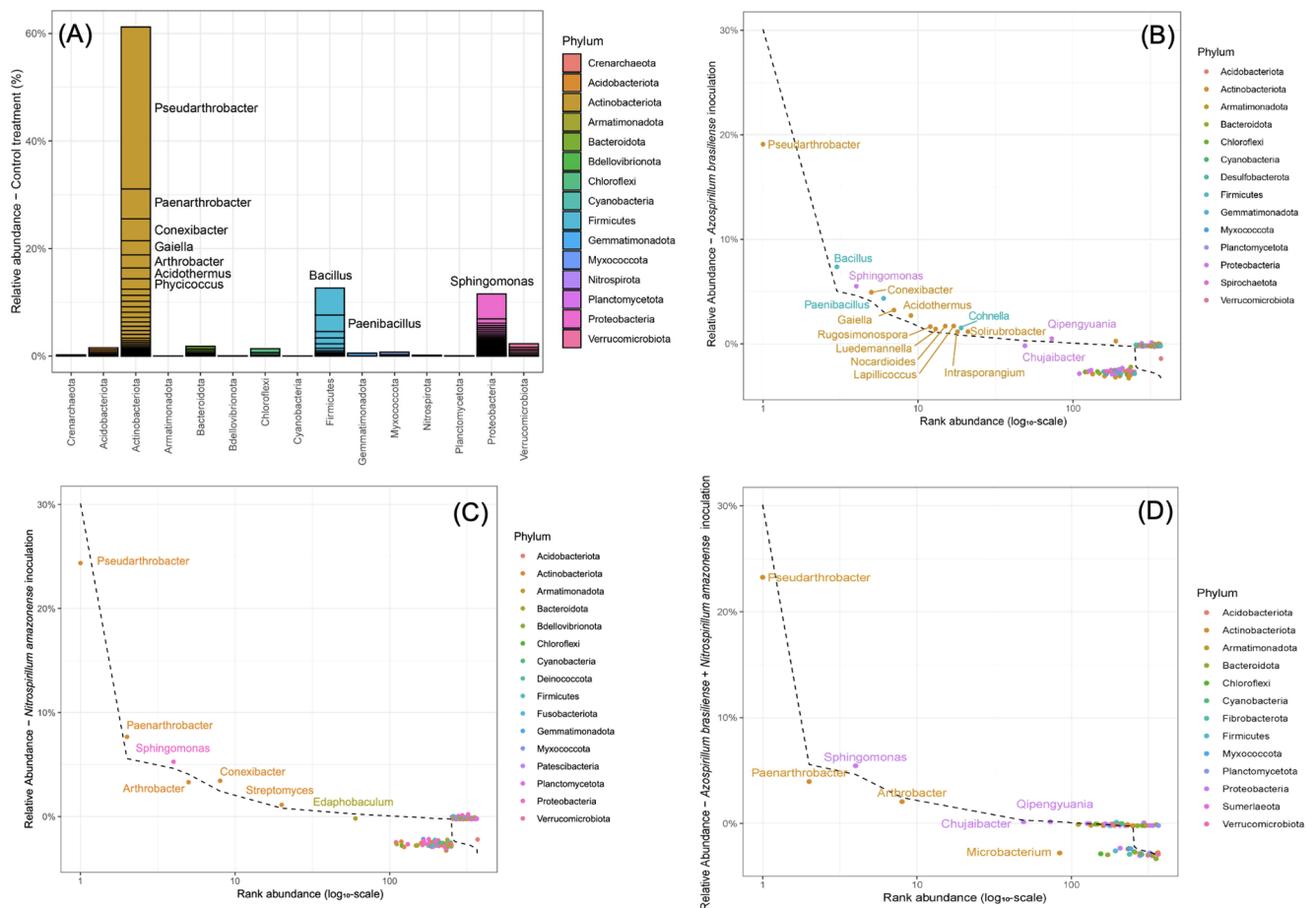


Fig. 6 Relative abundance (GJAM estimates) of archaeal and bacterial communities (Phylum level) in the sugarcane rhizosphere. Panel A shows the estimated relative abundance in the control treatment (no inoculation). We label all the taxa with a relative abundance bigger than 1.5%. Panels B, C, and D depict the relative abundance estimates under inoculation with *Azospirillum brasilense* (B), *Nitrospirillum amazonense* (C), and the combination of *A. brasilense*+*N. amazo-*

nense (D), respectively, compared to the control (indicated by the 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

while less abundant taxa responded more strongly. 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 (Fig. 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. brasilense* 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 joint modeling framework, GJAM enabled us to estimate conditional associations between microbial genera and multiple plant-related variables, including nitrogen fate (losses, recovery, and accumulation), plant performance traits (biomass and height), concentrations of root-exuded organic acids, and the abundance of functional

and phylogenetic marker genes (Fig. 7). Based on similarity in these association profiles, the microbial community was clustered into three major groups (C1–C3). Cluster C1 groups 30 genera predominantly characterized by negative associations with plant performance variables (e.g., total biomass, rhizome biomass, and plant height), indicating that these taxa tend to co-occur with lower plant growth conditions. Cluster C2 represented a large group of 94 genera showing strong negative associations with root-exuded organic acids, while exhibiting weaker or non-significant associations with plant biomass traits. Finally, Cluster C3 consisted of two subgroups: one with weak or non-significant associations across most plant variables, and another subgroup containing 46 genera that displayed consistently positive associations with both plant biomass and organic acid concentrations, suggesting that these microbes tend to co-occur under conditions of enhanced plant growth and rhizosphere metabolic activity. Altogether, our GJAM results indicate that root-exuded organic acids are a key mediator of rhizosphere structuring in sugarcane, as reflected by the clustering patterns: taxa in Cluster C3 showed positive associations with both biomass traits and organic acid profiles, whereas taxa in Clusters C1 and C2 were negatively associated with growth performance or root exudation, respectively. These patterns support a plant-driven filtering process that shapes microbial assembly and may contribute to growth-promoting interactions. 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.

Additionally, given the dominance of the *Pseudarthrobacter* genus, we highlight its associations with the abovementioned variables in Fig. 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 associations indicate that, despite its prevalence in the sugarcane rhizosphere, *Pseudarthrobacter* does not exhibit close links with plant physiological traits or organic acid profiles, but instead shows stronger statistical associations with microbial nitrogen-cycling markers and nitrogen loss pathways. These patterns highlight its potential ecological role within the soil microbial network, although functional validation would be required to confirm such contributions.

A closer look at the microbes significantly associated to plant growth also indicates that both rare and dominant microbes in sugarcane rhizosphere are positively related to plant biomass (Fig. 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

Fig. 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 summarize the shifts in the abundance of the different microbes in response to the inoculation treatments. White color represent non-significant associations

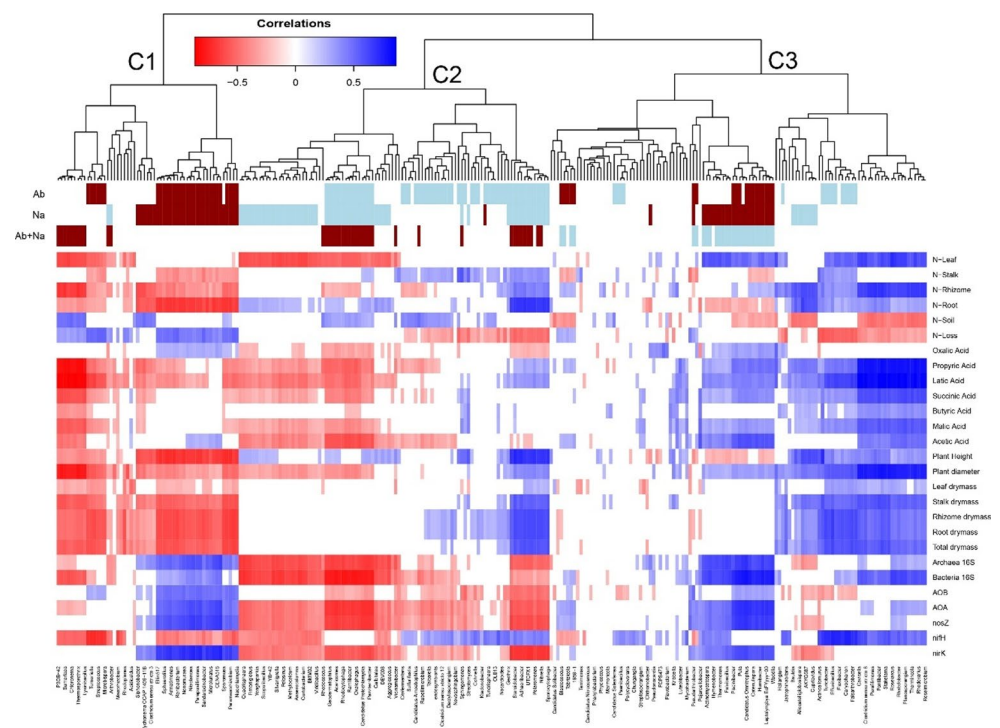


Fig. 8 GJAM estimates of associations between the genus *Pseudarthrobacter* and the crop productivity variables (nitrogen fate, plant performance, root exudates) and the abundance of phylogenetic markers

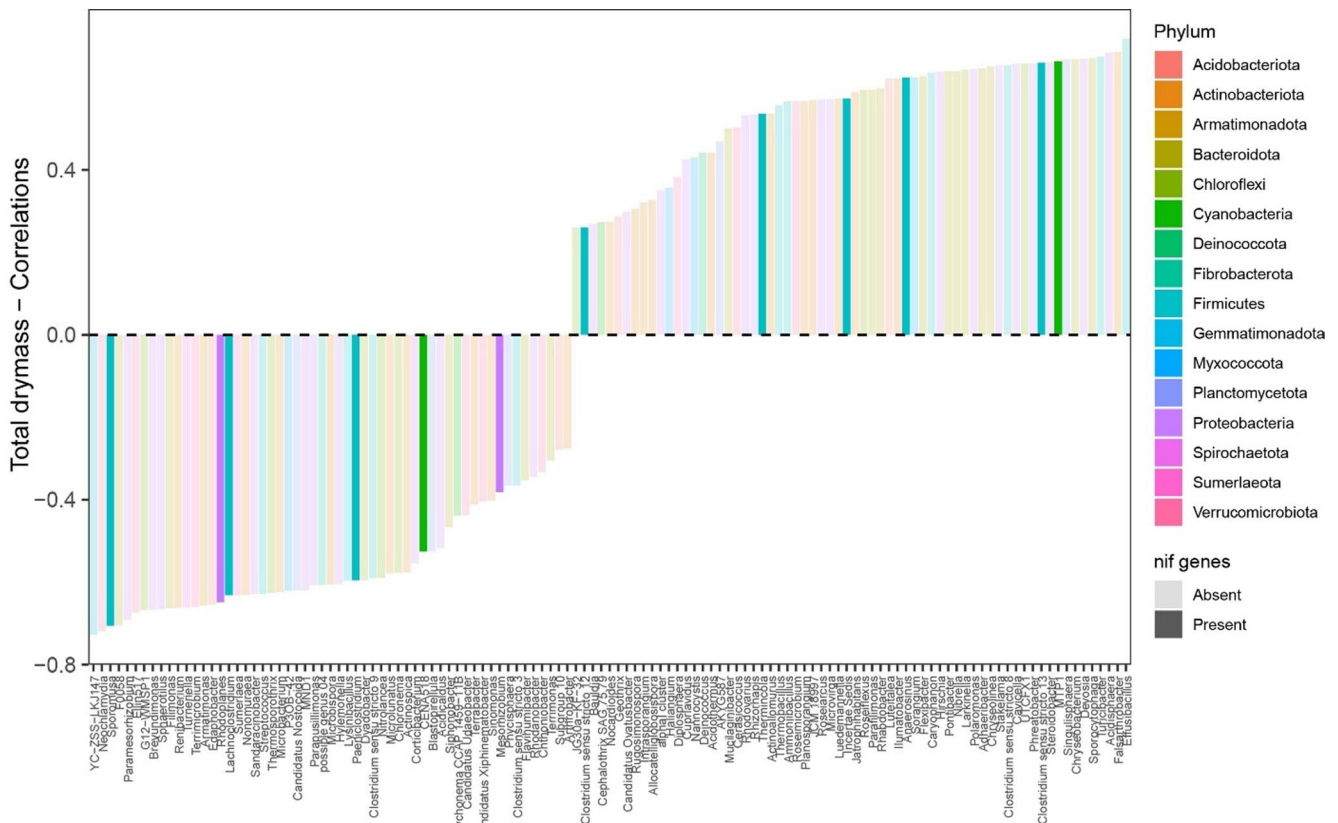
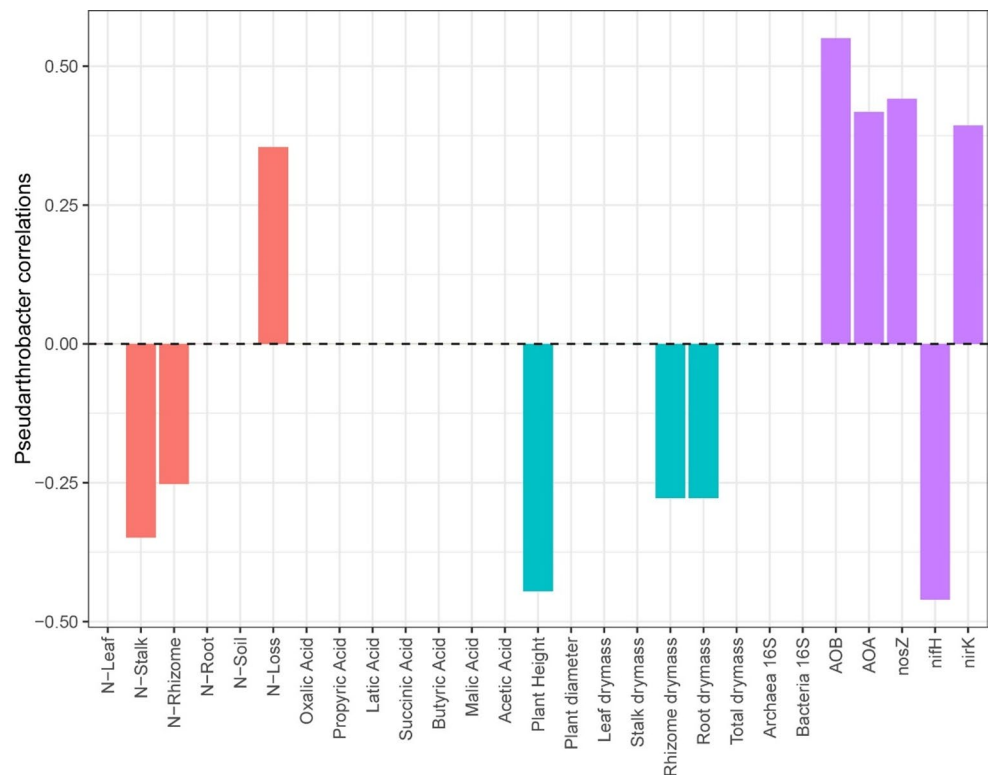


Fig. 9 GJAM estimates of associations between the sugarcane total drymass and the soil bacteria in sugarcane rhizosphere. Non-Shaded bar correspond to microbes with the genes related to nitrogen fixation (*nifH*, *nifD*, and *nifK*) as suggested by PICRUST2

nitrogen (*Oxyphotobacteria Incertae Sedis* MTP1, *Clostridium sensu stricto* 13, *Anaerostipes*, *Lachnospiraceae Incertae Sedis*, *Thermincola*, and *Clostridium sensu stricto* 12). However, six other microbes potentially able to fix nitrogen in soil were negatively associated with total growth of sugarcane (*Mesorhizobium*, CENA518, *Paenibacillus*, *Lachnospirillum*, *Rhodospirillum*, *Sporosarcina*).

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*), improved physiological performance by enhancing carbon fixation processes, including increases in net photosynthetic rate, carboxylation efficiency, and water use efficiency. These physiological improvements translated into greater above- and belowground biomass production and higher nitrogen use efficiency, accompanied by reduced fertilizer-derived nitrogen losses and increased recovery of ^{15}N in plant tissues. Furthermore, the *Ab+Na* treatment stimulated the exudation of organic acids in the rhizosphere and reshaped the microbial community, reducing the dominance of abundant taxa while enriching rare taxa commonly associated with beneficial functional traits.

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.

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 (*g_s*), reduced sub-stomatal CO_2 concentration (*C_i*), and improved water use efficiency (*WUE*). These effects were most prominent in the *Ab+Na* treatment, as verified by Fonseca et al. (2025). Such improvements in metabolic pathways suggest that PGPB inoculation stimulates carbon assimilation and promotes a more favorable water-carbon balance (Oliveira et al. 2025). Previous studies have shown that *A. brasilense* can modulate plant growth through phytohormone production (e.g., auxins, gibberellins, cytokinins) (Cassán et al. 2014; Fukami et al. 2018; Zaheer et al. 2022), which may help to explain the responses observed

here, although hormonal levels were not quantified in our experiment.

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 (Fonseca et al. 2025). 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; Pankiewicz et al. 2021).

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 potential suppression of ammonium-to-nitrate conversion and a subsequent reduction in nitrous oxide formation. Such patterns are indicative of a microbiome less prone to driving nitrogen losses through leaching or volatilization, a pattern consistent with reduced potential 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 and Scow 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 gene 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. These organic acids can selectively stimulate the growth of microbial taxa that are able to metabolize them, altering community composition. Consequently, the increased

N availability could support the potential activity of nitrifiers and denitrifiers, despite the overall suppressive trend relative to the Control (Canarini et al. 2019; Kuypers et al. 2018).

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

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, the higher *nifH* abundance observed in the *Ab* and *Ab+Na* treatments reflect an overall increase in diazotrophic functional potential in the rhizosphere in response to inoculation, rather than the contribution of a single diazotrophic genus.

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 treatment showed higher exudation of some organic acids compared to the individual inoculations. This pattern likely reflects a compensatory strategy for nutrient acquisition, as non-inoculated plants depend exclusively on rhizosphere-mediated mechanisms to mobilize and acquire nutrients. Similar increases in organic acid exudation under nutrient demand have been described in non-inoculated plants grown in low-N or low-P environments. In contrast, inoculated plants exuded lower amounts of these specific acids, consistent with reports that associative diazotrophs can reduce root carbon allocation to metabolically costly nutrient acquisition pathways (Dakora and Phillips 2002).

Rhizospheric microbiota: composition and functional feedback

The NMDS ordination revealed consistent and significant shifts in soil microbial community composition

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, these taxa have been documented in the literature to share functional traits related to phytohormone synthesis, exudate-mediated interactions, and competitive niche occupation (Fukami et al. 2018; Mendes et al. 2013), although such functions were not directly assessed in the present study. In addition, *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 interactions 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-abundant microbial genera responded to the treatments 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, bacteria known for their plant growth-promoting properties (Chan and Katznelson 1961; Fincheira and Quiroz 2018). Its dominance across all control plots suggests strong selective pressure by sugarcane root exudates or soil conditions that favor its proliferation. Similar patterns were observed in canola, where a *Pseudarthrobacter* strain was part of the core rhizosphere microbiota and positively correlated with yield (Floc'h et al. 2020). 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 beneficial 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.

Interestingly, our analysis did not detect the presence of the soil inoculum (*N. amazonense*) which suggests that our soil inoculum promoted the plant growth but was unable to colonize the rhizosphere of sugarcane. Previous studies already showed the some potential plant growth promoting microbes might be able to deliver its benefits without being effective colonizers of the plant rhizosphere (Armada et al. 2016). Our rank abundance analysis further indicates that sugarcane does not typically recruit this bacterial group into its rhizosphere. Plants can preferentially recruit specific microbial groups, sometimes in a genotype-dependent manner (Wippel et al. 2021; Rotoni et al. 2024), a concept relevant for interpreting rhizosphere selection patterns. These observations highlight that the success of microbial inoculation depends not only on the properties of the microbes, but also on the plant's ability to recruit and sustain them in the rhizosphere. In addition, the successful competition of the inoculants versus the soil microbiome also should be taken into account when selecting microbial consortia intended for field application.

An integrated view of plant-microbe-soil interactions

The results demonstrate a mechanistic cascade triggered by microbial inoculation. This reflects a general physiological principle whereby increased photosynthetic efficiency enhances carbon assimilation and can raise nitrogen demand to sustain metabolism and growth. This demand is met not only by improved nitrogen uptake from fertilizers but also by enhanced root function and metabolic activation in inoculated plants. Elevated photosynthetic activity in inoculated plants increases total carbon assimilation and can support enhanced rhizodeposition. However, increased carbon fixation does not necessarily lead to higher exudation of all classes of organic compounds. Instead, carbon allocation to specific exudate classes depends on the plant's nutritional status and ecological function of the metabolites involved. In this context, inoculation stimulated photosynthesis yet reduced the need for carbon-intensive organic acids linked to nutrient mobilization, indicating a shift in the qualitative composition of exudates rather than a uniform increase in their release. This sequence reinforces a functional feedback loop between plant physiology, root exudation, and the composition 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).

Interactions and Drawbacks of this study

Despite the consistent outcomes and integrative nature of this study, some methodological limitations should be acknowledged. The rhizosphere microbiome was characterized at a single sampling time, which constrains the understanding of temporal community dynamics across the crop cycle and under varying environmental conditions. Nevertheless, the microbiome assessment was integrated with physiological, biochemical, isotopic, and molecular approaches (exudates, functional genes, and ^{15}N), increasing interpretative strength and partially mitigating this limitation. In addition, the experiment was conducted under greenhouse conditions, which may limit direct extrapolation to field environments; however, controlled experiments remain essential to isolate biological effects and constitute a necessary step before large-scale agronomic validation.

Future directions include multi-environment validation under real agricultural conditions, evaluation across additional crop species, and the incorporation of omics-based and temporal approaches (e.g., metatranscriptomics, metabolomics) to deepen the mechanistic understanding

of microbial modulation, root exudation, and nitrogen use. Overall, despite these limitations, the study advances by integrating physiological, biochemical, isotopic, and microbiological dimensions, contributing to a mechanistic understanding of nitrogen-use efficiency under complementary microbial inoculation.

Conclusions

Our integrated evaluation of plant physiological traits, nitrogen dynamics, and rhizosphere microbial responses demonstrates that combining foliar *Azospirillum brasilense* with soil-applied *Nitrospirillum amazonense* enhances plant-microbe interactions in sugarcane more effectively than individual inoculations. Rather than acting through a single pathway, the combined strategy coordinated plant performance and diazotrophic activity with shifts in rhizospheric functional potential. These findings indicate that the use of complementary inoculation routes can broaden the spectrum of plant-microbe interactions involved in nutrient acquisition and nitrogen cycling, which is agronomically relevant for sugarcane grown under fertilizer-based systems. This work supports the development of microbial consortia and delivery strategies tailored to improve nitrogen use efficiency in sustainable production systems. While our results are encouraging, we emphasize that they represent short-term responses under controlled conditions. Future studies comparing different developmental stages, environments, and long-term field systems will be important to verify the stability, scalability, and mechanisms underlying the combined effects observed here.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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References

- Ankati S, Podile AR (2019) Metabolites in the root exudates of groundnut change during interaction with plant growth promoting rhizobacteria in a strain-specific manner. *J Plant Physiol* 243:153057. <https://doi.org/10.1016/j.jplph.2019.153057>
- AOAC (2016) Official methods of analysis of AOAC international. AOAC International, Rockville, MD
- Armada E, Probanza A, Roldán A, Azcón R (2016) Native plant growth promoting bacteria *Bacillus Thuringiensis* and mixed or individual mycorrhizal species improved drought tolerance and oxidative metabolism in *Lavandula dentata* plants. *J Plant Physiol* 192:1–12. <https://doi.org/10.1016/j.jplph.2015.11.007>
- Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B* 57:289–300. <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>
- Brody JR, Kern SE (2004) Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36:214–216. <https://doi.org/10.2144/04362BM02>
- Bünemann EK (2015) Assessment of gross and net mineralization rates of soil organic phosphorus – A review. *Soil Biol Biochem* 89:82–98. <https://doi.org/10.1016/j.soilbio.2015.06.026>
- Busse HJ (2016) Review of the taxonomy of the genus *Arthrobacter*, emendation of the genus *Arthrobacter* sensu lato, proposal to reclassify selected species of the genus *Arthrobacter* in the novel genera *glutamicibacter* gen. nov., *paeniglutamicibacter* gen. nov., *pseudoglutamicibacter* gen. nov., *paenarthrobacter* gen. nov. And *pseudarthrobacter* gen. nov., And emended description of *Arthrobacter roseus*. *Int J Syst Evol Microbiol* 66:9–37. <https://doi.org/10.1099/ijsem.0.000702>
- Callahan BJ, McMurdie PJ, Holmes SP (2017) Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J* 11:2639–2643. <https://doi.org/10.1038/ismej.2017.119>
- Camara GMS (1993) Ecofisiologia da cultura da cana-de-açúcar. In: GMS Camara and EAM Oliveira (eds) *Produção de cana-de-açúcar*. ESALQ, Piracicaba, Brazil, pp 31–64
- Canarini A, Kaiser C, Merchant A, Richter A, Wanek W (2019) Root exudation of primary metabolites: mechanisms and their roles in plant responses to environmental stimuli. *Front Plant Sci* 10:157. <https://doi.org/10.3389/fpls.2019.00157>
- Cao M, Narayanan M, Shi X, Chen X, Li Z, Ma Y (2023) Optimistic contributions of plant growth-promoting bacteria for

- sustainable agriculture and climate stress alleviation. *Environ Res* 217:114924. <https://doi.org/10.1016/j.envres.2022.114924>
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 108:4516–4522. <https://doi.org/10.1073/pnas.1000080107>
- Cardozo P, Di Palma A, Martin S, Cerliani C, Esposito G, Reinoso H, Travaglia C (2022) Improvement of maize yield by foliar application of azospirillum *Brasilense* Az39. *J Plant Growth Regul* 41:1032–1040. <https://doi.org/10.1007/s00344-021-10356-9>
- Cassán F, Vanderleyden J, Spaepen S (2014) Physiological and agronomical aspects of phytohormone production by model plant-growth-promoting rhizobacteria (PGPR) belonging to the genus *azospirillum*. *J Plant Growth Regul* 33:440–459. <https://doi.org/10.1007/s00344-013-9362-4>
- Cassán F, Coniglio A, López G, Molina R, Nieves S, de Carlan CLN, Donadio F, Torres D, Rosas S, Pedrosa FO (2020) Everything you must know about *azospirillum* and its impact on agriculture and beyond. *Biol Fertil Soils* 56:461–479. <https://doi.org/10.1007/s0374-020-01463-y>
- Cesari A, Paulucci N, López-Gómez M, Hidalgo-Castellanos J, Plá CL, Dardanelli MS (2019) Restrictive water condition modifies the root exudates composition during peanut–PGPR interaction and conditions early events, reversing the negative effects on plant growth. *Plant Physiol Biochem* 142:519–527. <https://doi.org/10.1016/j.plaphy.2019.08.015>
- Chan ECS, Katznelson H (1961) Growth interactions of *Arthrobacter globiformis* and *Pseudomonas* sp. in relation to the rhizosphere effect. *Can J Microbiol* 7:759–767. <https://doi.org/10.1139/m61-090>
- Cheng Y, Shi X, Narayanan M, Li Z, Li Y, Ma Y (2025) Enhancing nutrient uptake efficiency in *Zea mays*: the role of plant growth-promoting bacteria in modifying root characteristics and mobilizing soil nutrients. *J Sci Food Agric* 105:5030–5039. <https://doi.org/10.1002/jsfa.14233>
- Clark JS, Nemergut D, Seyednasrollah B, Turner PJ, Zhang S (2017) Generalized joint attribute modeling for biodiversity analysis: median-zero, multivariate, multifarious data. *Ecol Monogr* 87:34–56. <https://doi.org/10.1002/ecm.1241>
- Coolen MJL, Hopmans EC, Rijpstra WIC, Muijzer G, Schouten S, Volkman JK, Damsté JSS (2004) Evolution of the methane cycle in Ace lake (Antarctica) during the holocene: response of methanogens and methanotrophs to environmental change. *Org Geochem* 35:1151–1167. <https://doi.org/10.1016/j.orggeochem.2004.06.009>
- Dakora FD, Phillips DA (2002) Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant Soil* 245:35–47. <https://doi.org/10.1023/A:1020809400075>
- Dartora J, Guimarães VF, Menezes CRJ, Freiburger MB, Castoldi G, Gonçalves EDV (2016) Maize response to inoculation with strains of plant growth-promoting bacteria. *Rev Bras Eng Agríc Ambient* 20:606–611. <https://doi.org/10.1590/1807-1929/agriambi.v20n7p606-611>
- dos Santos SG, Chaves VA, da Silva Ribeiro F, Alves GC, Reis VM (2019a) Rooting and growth of pre-germinated sugarcane seedlings inoculated with diazotrophic bacteria. *Appl Soil Ecol* 133:12–23. <https://doi.org/10.1016/j.apsoil.2018.08.015>
- Douglas GM, Maffei VJ, Zaneveld J, Yurgel SN, Brown JR, Taylor CM, Langille MGI (2019) PICRUST2: an improved and extensible approach for metagenome inference. *bioRxiv* 672295. <https://doi.org/10.1101/672295>
- FAO (2018) The future of food and agriculture: alternative pathways to 2050. FAO, Rome
- Fincheira P, Quiroz A (2018) Microbial volatiles as plant growth inducers. *Microbiol Res* 208:63–75. <https://doi.org/10.1016/j.micres.2018.01.002>
- Floc'h JB et al (2020) Bacterial communities of the Canola rhizosphere: network analysis reveals a core bacterium shaping microbial interactions. *Front Microbiol* 11:568270. <https://doi.org/10.3389/fmicb.2020.01587>
- Fonseca MC, Bossolani JW, de Oliveira SL, Moretti LG, Portugal JR, Scudelletti D, de Oliveira EF, Crusciol CAC (2022) *Bacillus subtilis* inoculation improves nutrient uptake and physiological activity in sugarcane under drought stress. *Microorganisms* 10:809. <https://doi.org/10.3390/microorganisms10040809>
- Fonseca M, Bossolani JW, Alves Filho I, de Oliveira SL, Galeriani TM, Andreote FD, Crusciol CAC (2025) Combining plant growth-promoting bacteria as a tool to improve the metabolism and productivity of sugarcane. *Plant Physiol Biochem* 109980
- Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci U S A* 102:14683–14688. <https://doi.org/10.1073/pnas.0506625102>
- Freitas GS, Moreira A, Prudencio MF, Heinrichs R, Nogueira MA, Hungria M, Soares Filho CV (2023) Foliar spray inoculation with plant growth promoting bacteria associated with nitrogen doses in *Megathyrus Maximus* Cv. BRS Zuri Agron 13:1040. <https://doi.org/10.3390/agronomy13041040>
- Fukami J, Cerezini P, Hungria M (2018) *Azospirillum*: benefits that go far beyond biological nitrogen fixation. *AMB Express* 8:73. <https://doi.org/10.1186/s13568-018-0608-1>
- Galindo FS, Rodrigues WL, Fernandes GC, Boleta EHM, Jalal A, Rosa PAL, Buzetti S, Lavres J, Teixeira Filho MCM (2022) Enhancing agronomic efficiency and maize grain yield with *azospirillum Brasilense* inoculation under Brazilian Savannah conditions. *Eur J Agron* 134:126471. <https://doi.org/10.1016/j.eja.2022.126471>
- Geisseler D, Scow KM (2014) Long-term effects of mineral fertilizers on soil microorganisms – A review. *Soil Biol Biochem* 75:54–63. <https://doi.org/10.1016/j.soilbio.2014.03.023>
- Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C (2010) Food security: the challenge of feeding 9 billion people. *Science* 327:812–818. <https://doi.org/10.1126/science.1185383>
- Hartman K, Tringe SG (2019) Interactions between plants and soil shaping the root Microbiome under abiotic stress. *Biochem J* 476:2705–2724. <https://doi.org/10.1042/BCJ20180615>
- Henry S, Baudoin E, López-Gutiérrez JC, Martin-Laurent F, Brauman A, Philippot L (2004) Quantification of denitrifying bacteria in soils by *NirK* gene targeted real-time PCR. *J Microbiol Methods* 59:327–335. <https://doi.org/10.1016/j.mimet.2004.07.002>
- Henry S, Bru D, Stres B, Hallet S, Philippot L (2006) Quantitative detection of the *NosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *NosZ* genes in soils. *Appl Environ Microbiol* 72:5181–5189. <https://doi.org/10.1128/AEM.00231-06>
- Hernández AG, da Silva EP, Ferreira PAAA, Lovato PE, de Oliveira GP, Soares CRFS (2024) Inoculation methods and agronomic efficiency of *azospirillum Brasilense* strain Az39 for corn crop under different Brazilian edaphoclimatic conditions. *Rev Bras Ciênc Agrár* 19:e3609. <https://doi.org/10.5039/agraria.v19i1a3609>
- <https://doi.org/10.1128/aem.63.12.4704-4712.1997>
- <https://doi.org/10.1128/aem.59.3.695-700.1993>
- <https://doi.org/10.1016/j.plaphy.2025.109980>
- Hungria M, Campo RJ, Souza EM, Pedrosa FO (2010) Inoculation with selected strains of *azospirillum Brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant Soil* 331:413–425. <https://doi.org/10.1007/s1104-009-0262-0>
- Hungria M, Ribeiro RA, Nogueira MA (2018) Draft genome sequences of *azospirillum Brasilense* strains Ab-V5 and Ab-V6, commercially used in inoculants for grasses and legumes in Brazil.

- Genome Announc 6:e01011–e01018. <https://doi.org/10.1128/genome.00393-18>
- Jalal A, Júnior EF, Teixeira Filho MCM (2024) Interaction of zinc mineral nutrition and plant growth-promoting bacteria in tropical agricultural systems: a review. *Plants* 13:571. <https://doi.org/10.3390/plants13050571>
- Júnior FBR, Machado CTT, Machado AT, Sodek L (2008) Inoculation of azospirillum Amazonense in two maize genotypes under different N treatments. *Rev Bras Ciênc Solo* 32:1139–1146
- Kuypers MMM, Marchant HK, Kartal B (2018) The microbial nitrogen-cycling network. *Nat Rev Microbiol* 16:263–276. <https://doi.org/10.1038/nrmicro.2018.9>
- Levene H (1960) Robust tests for equality of variances. In: Olkin I (ed) *Contributions to probability and statistics*. Stanford University Press, Stanford, pp 278–292
- Marschner P, Crowley D, Rengel Z (2011) Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis – model and research methods. *Soil Biol Biochem* 43:883–894. <https://doi.org/10.1016/j.soilbio.2011.01.005>
- Martini AF, Valani GP, Boschi RS, Bovi RC, Simões da Silva LFF, Cooper M (2020) Is soil quality a concern in sugarcane cultivation? A bibliometric review. *Soil Tillage Res* 204:104751. <https://doi.org/10.1016/j.still.2020.104751>
- Mendes R, Garbeva P, Raaijmakers JM (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol Rev* 37:634–663. <https://doi.org/10.1111/1574-6976.12028>
- Mimmo T, Del Buono D, Terzano R, Tomasi N, Viganì G, Crecchio C, Cesco S (2014) Rhizospheric organic compounds in the soil–microorganism–plant system: their role in iron availability. *Eur J Soil Sci* 65:629–642. <https://doi.org/10.1111/ejss.12158>
- Muyzer G, De Waal EC, Uitterlinden A (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700
- Oliveira CEDS, Sena Oliveira TJS, Jalal A, Fernandes GC, Bastos AC, da Silva MR, Sant’Ana GR, Aguiar JV, De Camargos LS, Zoz T (2025) Inoculation and co-inoculation of lettuce and arugula hydroponically influence nitrogen metabolism, plant growth, nutrient acquisition and photosynthesis. *Front Plant Sci* 16:1547821. <https://doi.org/10.3389/fpls.2025.1547821>
- Pankiewicz VCS, do Amaral FP, Ané J-M, Stacey G (2021) Diazotrophic bacteria and their mechanisms to interact and benefit cereals. *Mol Plant Microbe Interact* 34:491–498. <https://doi.org/10.1094/MPMI-11-20-0316-FI>
- Parks DH, Tyson GW, Hugenholtz P, Beiko RG (2014) STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30:3123–3124. <https://doi.org/10.1093/bioinformatics/btu494>
- Pascale A, Proietti S, Pantelides IS, Stringlis IA (2020) Modulation of the root Microbiome by plant molecules: the basis for targeted disease suppression and plant growth promotion. *Front Plant Sci* 10:1741. <https://doi.org/10.3389/fpls.2019.01741>
- Pereira LB, Gambarini VMO, de Menezes AB, Ottoboni LMM, Vicentini R (2021) Responses of the sugarcane rhizosphere microbiota to different levels of water stress. *Appl Soil Ecol* 159:103817. <https://doi.org/10.1016/j.apsoil.2020.103817>
- Philippot L, Raaijmakers JM, Lemanceau P, Van der Putten WH (2013) Going back to the roots: the microbial ecology of the rhizosphere. *Nat Rev Microbiol* 11:789–799. <https://doi.org/10.1038/nrmicro3109>
- Quast C, Pruesse E, Yilmaz P et al (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41:D590–D596. <https://doi.org/10.1093/nar/gks1219>
- R Core Team (2021) R: a Language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
- Reis V, Rios FA, Braz GBP, Constantin J, Hirata ES, Biffe DF (2020) Agronomic performance of sugarcane inoculated with nitrospirillum Amazonense (BR11145). *Rev Caatinga* 33:918–926. <https://doi.org/10.1590/1983-21252020v33n406rc>
- Ribani M, Bottoli CBG, Collins CH, Jardim ICSF, Melo LFC (2004) Validação Em métodos cromatográficos e eletroforéticos. *Quim Nova* 27:771–780. <https://doi.org/10.1590/S0100-40422004000500017>
- Rodrigues JD (1995) Fisiologia Da cana-de-açúcar. Inst. Biociência, Unesp, Botucatu
- Rösch C, Mergel A, Bothe H (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl Environ Microbiol* 68:3818–3829. <https://doi.org/10.1128/AEM.68.8.3818-3829.2002>
- Rotoni C, Leite MF, Pijl A, Kuramae EE (2022) Rhizosphere Microbiome response to host genetic variability: a trade-off between bacterial and fungal community assembly. *FEMS Microbiol Ecol* 98:fiac061. <https://doi.org/10.1093/femsec/fiac061>
- Rotoni C, Leite MF, Wong LC, Stürmer SL, Pijl A, Kuramae EE (2024) Cultivar governs plant response to inoculation with single isolates and the Microbiome associated with arbuscular mycorrhizal fungi. *Appl Soil Ecol* 197:105347. <https://doi.org/10.1016/j.apsoil.2024.105347>
- Rothauwe J-H, Witzel K-P, Liesack W (1997) The ammonia monooxygenase structural gene AmoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* 63:4704–4712
- Santos MS, Nogueira MA, Hungria M (2019b) Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. *AMB Express* 9:205. <https://doi.org/10.1186/s13568-019-0932-0>
- Santos MS, Nogueira MA, Hungria M (2021) Outstanding impact of azospirillum Brasilense strains Ab-V5 and Ab-V6 on the Brazilian agriculture: lessons that farmers are receptive to adopt new microbial inoculants. *Rev Bras Ciênc Solo* 45:e0200128. <https://doi.org/10.36783/18069657rbcs20200128>
- Schwab S, Terra LA, Baldani JI (2018) Genomic characterization of nitrospirillum Amazonense strain CBAmC, a nitrogen-fixing bacterium isolated from surface-sterilized sugarcane stems. *Mol Genet Genomics* 293:997–1016. <https://doi.org/10.1007/s00438-018-1439-0>
- Scudeletti D, Crusciol CAC, Momesso L, Bossolani JW, Moretti LG, De Oliveira EF, Tubaña BS, de Almeida Silva M, de Castro SGQ, Hungria M (2023) Inoculation with azospirillum Brasilense as a strategy to enhance sugarcane biomass production and bioenergy potential. *Eur J Agron* 144:126749. <https://doi.org/10.1016/j.eja.2023.126749>
- Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). *Biometrika* 52:591–611. <https://doi.org/10.1093/biomet/52.3-4.591>
- Spironello A, van Raij B, Penatti CP, Cantarella H, Morelli JL, Orlando Filho J, Landell MGD, Rosseto R (1997) Cana-de-açúcar. In: van Raij B et al (eds) *Recomendações de adubação e calagem Para o Estado de São Paulo*, 2nd edn. IAC, Campinas, pp 237–239
- Stahl DA (1991) Development and application of nucleic acid probes in bacterial systematics. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. Wiley, New York, pp 205–248
- Surendran U, Ramesh V, Jayakumar M, Marimuthu S, Sridevi G (2016) Improved sugarcane productivity with tillage and trash management practices in semi arid tropical agro ecosystem in India. *Soil Tillage Res* 158:10–21. <https://doi.org/10.1016/j.still.2015.10.009>

- Vives-Peris V, Gómez-Cadenas A, Pérez-Clemente RM (2018) Salt stress alleviation in citrus plants by plant growth-promoting rhizobacteria *Pseudomonas Putida* and *Novosphingobium* Sp. Plant Cell Rep 37:1557–1569. <https://doi.org/10.1007/s00299-018-2328-z>
- Wang Z, Leite MF, Jiang M, Kuramae EE, Fu X (2023) Responses of soil rare and abundant microorganisms to recurring biotic disturbances. Soil Biol Biochem 177:108913. <https://doi.org/10.1016/j.soilbio.2022.108913>
- Wipfel K, Tao K, Niu Y, Zgadzaj R, Kiel N, Guan R, Garrido-Oter R (2021) Host preference and invasiveness of commensal bacteria in the Lotus and Arabidopsis root microbiota. Nat Microbiol 6:1150–1162. <https://doi.org/10.1038/s41564-021-00941-9>
- Zaheer MS, Ali HH, Iqbal MA, Erinle KO, Javed T, Iqbal J, Hashmi MIU, Mumtaz MZ, Salama EAAA, Kalaji HM (2022) Cytokinin production by *azospirillum* Brasilense contributes to increase in growth, yield, antioxidant, and physiological systems of wheat (*Triticum aestivum* L). Front Microbiol 13:886041. <https://doi.org/10.3389/fmicb.2022.886041>

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