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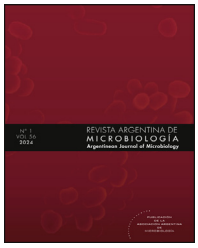

ORIGINAL ARTICLE

Effects of plant growth-promoting rhizobacteria on the productivity and nutrition of sugarcane under reduced urea fertilization

Julián Esteban Másmela-Mendoza *, Yohan Sebastián Mejía-Flórez, Julián Fernando Mateus-Rodríguez

Colombian Sugarcane Research Center (Cenicaña), Agronomy, Nutrition and Fertility Program, Experimental Station Km 26 Via Cali-Florida, Valle del Cauca, Colombia

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Abstract The practice of biofertilization enhances the sustainability of sugarcane cultivation. This study evaluated the ability of several bacterial strains to improve sugarcane growth and reduce its dependence on urea. Strains belonging to the genera *Gluconacetobacter*, *Azotobacter*, *Rhizobium*, and *Azospirillum* were efficient in biological nitrogen fixation, indole-3-acetic acid production, and inorganic phosphorus solubilization. *Azotobacter* and *Gluconacetobacter* efficiently solubilized rock phosphate, while *Azospirillum* was more effective with tricalcium phosphate. *Azospirillum brasilense* Ab12 showed viability in diluted vinasse, utilizing organic acids. Inoculation with *A. brasilense* strains Ab12 and Ab16 at 4×10^8 CFU/ml in pre-sprouted cuttings increased productivity in field-transplanted plants without chemical fertilization. The application of *Azospirillum* improved stem number, stem weight and length, cane yield per hectare, and sucrose content. In addition, it promoted greater tillering and enhanced the uptake of P, S, Fe, and Zn. Bacterial inoculants reduced the need for urea fertilization, showing a complementary effect on plant growth even with the full recommended urea dose. *Azospirillum*-based biofertilizers allowed the reduction of urea rates, with similar growth observed when using 25–50% of the standard dose. These results demonstrate the potential of *A. brasilense* as a biofertilizer to reduce dependence on synthetic inputs and improve the nutritional efficiency of sugarcane cultivation.
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* Corresponding author.
E-mail address: julianmasmela@gmail.com (J.E. Másmela-Mendoza).

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PALABRAS CLAVE
Azospirillum;
Promoción del crecimiento vegetal;
Biofertilizantes;
Urea;
Caña de azúcar

Efectos de rizobacterias promotoras del crecimiento vegetal en la productividad y nutrición de caña de azúcar con menor uso de urea

Resumen La práctica de biofertilización mejora la sostenibilidad del cultivo de caña de azúcar. Este estudio evaluó la capacidad de algunas cepas bacterianas para mejorar el crecimiento de la caña de azúcar y reducir su dependencia de la urea. Cepas de los géneros *Gluconacetobacter*, *Azotobacter*, *Rhizobium* y *Azospirillum* fueron eficientes en fijación biológica de N, producción de ácido 3-indolacético y solubilización de P inorgánico. *Azotobacter* y *Gluconacetobacter* solubilizaron eficientemente fosfatos de roca fosfórica, mientras que *Azospirillum* fue más eficaz con fosfato tricálcico. *Azospirillum brasilense* Ab12 mostró viabilidad en vinaza diluida, utilizando ácidos orgánicos. La inoculación de las cepas Ab12 y Ab16 de *A. brasilense* a 4×10^8 UFC/ml en esquejes pre-brotados, aumentó la productividad en plantas trasplantadas al campo sin fertilización química. La aplicación de *Azospirillum* mejoró el número de tallos, el peso y la longitud del tallo, las toneladas de caña por hectárea y el contenido de sacarosa. Además, promovió un mayor macollamiento y mejoró la absorción de P, S, Fe y Zn. Los inóculos bacterianos redujeron la necesidad de fertilización con urea, mostrando un efecto complementario en el crecimiento de las plantas incluso con la dosis completa recomendada. Los biofertilizantes de *Azospirillum* permitieron la reducción de las dosis de urea, observándose un crecimiento similar cuando se utilizó el 25-50% de la dosis. Estos resultados evidencian el potencial de *A. brasilense* como biofertilizante para reducir la dependencia de insumos sintéticos y mejorar la eficiencia nutricional del cultivo.
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Introduction

Sugarcane is an economically important crop used for producing sugar, ethanol, molasses, and agro-industrial by-products such as vinasse and bagasse[2,18]. In Colombia, sugarcane plays a key role in the country's economy, with 13 sugar and ethanol production plants nationwide and 242 000 ha planted to the crop, predominantly in the Cauca River Valley[2]. Colombia ranks seventh worldwide in sugarcane production, contributing 42.7 million tons, which represents 2.1% of global output[18]. Due to the crop's rapid growth, sugarcane requires high amounts of nutrients, particularly nitrogen (N). Increasing crop productivity while reducing costs and environmental impact is essential. However, a common practice is to apply mineral and synthetic fertilizers in excess to compensate for soil nutrient depletion[24]. The excessive use of chemical fertilizers has led to environmental and health concerns, including greenhouse gas emissions, eutrophication, and soil degradation due to reduced microbial diversity[17,35]. These issues, along with rising input costs, have driven interest in sustainable alternatives such as biofertilizers and organic fertilizers[17,24].

Biological inoculants or biofertilizers promote plant growth and productivity[1,24]. Plant growth-promoting rhizobacteria (PGPR) enhance nutrition by mobilizing and solubilizing macro- and micronutrients such as N, P, sulfur (S), and iron (Fe), and by improving plant responses to abiotic and biotic stress through mechanisms such as biological N fixation, siderophore production, phytohormone synthesis, phosphorus (P) solubilization, and mineralization of organic compounds[33,37]. *Azospirillum*, a prominent plant growth-promoting bacterial genus, has been used to enhance growth and productivity in sugarcane[15,21]. The positive effects of *Azospirillum* inoculants are attributed to two main biochemical processes: biological N fixation and the synthesis of phytohormones, such as auxins[14].

Sugarcane can form symbiotic relationships with diazotrophic or free-living N-fixing bacteria. The application of *Azospirillum* inoculants has been shown to enhance crop sustainability and improve growth and productivity parameters[15,23]. Other PGPR, such as *Bacillus*, *Gluconacetobacter*, *Herbaspirillum*, *Burkholderia* and *Pantoea*, have also been identified as beneficial for sugarcane growth[10,15,16,25].

Large quantities of vinasse, a by-product of ethanol production from sugarcane molasses, are generated during the distillation process[2]. While vinasse offers potential value within the circular economy of sugarcane cultivation[8], its application is limited by challenging physicochemical characteristics, including low pH, high organic matter content, and elevated biological oxygen demand as well as high concentrations of potassium (K) and calcium (Ca)[8]. Nevertheless, vinasse remains a valuable organic residue for crop fertilization. Thus, evaluating the compatibility of plant growth-promoting bacterial strains with vinasse is crucial for developing biofertilizers that use this by-product as a carrier or culture medium.

This study aimed to biochemically characterize bacterial strains from the sugarcane rhizosphere to evaluate microbial inoculants for promoting plant growth. The objectives were to assess native bacterial strains from the sugarcane rhizosphere in Colombia's Cauca River Valley in terms of their nutritional and fertility traits, including P solubilization, biomass production in vinasse, and their potential as

plant growth promoters under treatments without chemical fertilization and with different urea doses in greenhouse and field conditions.

Materials and methods

Isolation and molecular identification of bacterial strains

Bacteria were isolated from the rhizosphere soil of sugarcane crops in Colombia's Cauca River Valley using the method applied by Roa et al.[26]. Aliquots of serial dilutions of the samples were plated on selective and differential media for N-fixing bacteria. The semi-solid media used were: (i) N-free semi-solid minimal enrichment medium (NFb) for the isolation of *Azospirillum* spp.; (ii) Ashby medium for *Azotobacter* spp.: and (iii) LGI-P medium for *Gluconacetobacter* spp. (Supplementary Material, Table S1)[3]. The media were incubated at 30 °C for 5 days. When bacterial films formed on the subsurface of the culture media, portions of these biofilms were streaked onto Congo Red agar, DYGS agar, Ashby agar, and LGI-P agar.

Tests were conducted to quantify the biochemical capacities of the isolates to produce plant growth-promoting metabolites. Bacterial isolates were subjected to the acetylene reduction assay (ARA) to assess biological N fixation[12,26] and to a quantitative test of 3-indoleacetic acid (IAA) production as a precursor of auxins in tryptophan-containing medium[26]. Biochemical assays (IAA and ARA) focused on *Azospirillum*, *Azotobacter*, and *Gluconacetobacter* because these genera were the most predominant among our isolates and are also the most frequently reported sugarcane-associated PGPR in previous studies.

Molecular identification of the isolates was performed by sequencing the V1–V4 and V3–V4 regions of the 16S rRNA gene using primers 27F and 1492R. The resulting sequences were analyzed using BLAST against the NCBI 16S rRNA reference database, considering percentage identity, coverage, and e-value. Taxonomic assignments followed established thresholds for 16S rRNA similarity, where $\geq$98.7–99% identity with >95% coverage indicated species-level affiliation, and 95–98.7% identity indicated genus-level classification. To confirm the assignments, sequences were also compared using RDP Classifier and SeqMatch tools, and multiple sequence alignment was performed using the 30 most similar sequences obtained from BLAST. Phylogenetic trees were constructed using the Neighbor-Joining method and the Tamura-Nei model with 1000 bootstrap replicates in Geneious Prime (2024.0.5).

Inorganic phosphate solubilization assays

Details of the methodology are described in the supplementary material (Materials and Methods S1). For the quantitative evaluation of phosphate solubilization, we selected the bacterial isolates that previously showed the highest efficiency in biological N fixation and IAA production, as well as the ability to form visible solubilization halos in qualitative assays. Qualitative assays to measure P solubilization halos were conducted in NBRIP medium[20]. Quantitative tests were conducted using a randomized design with three replicates per isolate, with the experiment repeated twice over time, with bacterial genera and P source as treatments.

P solubilization was evaluated using tricalcium phosphate and phosphate rock as P sources. Soluble P (available phosphates) in the supernatant of NBRIP liquid medium was determined using the P-molybdenum blue (ascorbic acid) method according to Watanabe and Olsen[36], following the procedures described by Estrada-Bonilla et al.[11]. The NBRIP medium was supplemented with 5 g/l tricalcium phosphate and 5 g/l rock phosphate. *Azotobacter*, *Gluconacetobacter*, and *Azospirillum* strains were pre-cultured in DYGS broth[3,9] at 30 °C and 150 rpm for 24–48 h and adjusted to a McFarland standard of 4–5 ($\approx$1.2–1.5 $\times$ 10^9 CFU/ml). A 5 ml inoculum was transferred to 50 ml NBRIP broth (final concentration: 1.2 $\times$ 10^8 CFU/ml) and incubated at 30 °C and 150 rpm for 10–14 days. Samples were taken at 3, 6, and 9 days post-inoculation to determinate soluble P (ascorbic acid method), pH, and viable biomass (microdrop or total plate count). Soluble P was quantified using a standard curve (0.1–1.2 mg/l PO_4^{3-}) with absorbance measured at 712 nm using a Shimadzu® UV-1800 spectrophotometer. Phosphate solubilization was calculated as the difference in soluble P between inoculated cultures and the non-inoculated control[19,30].

Viability and growth of bacterial strains in vinasse medium

Microbial growth assays were conducted using vinasse as a culture medium. Inocula of *Azospirillum brasilense* strains Ab12 and Ab16 was first grown in DYGS[9] and BTB-2 broths[5] at 30 °C and 150 rpm for 48–72 h. Biomass was harvested by centrifugation (1700 *g*, 4 °C, 10 min), washed with sterile distilled water, and adjusted to 0.5 McFarland. A 5 ml aliquot was inoculated into 50 ml of sterilized vinasse broth, adjusted to pH 6.8–7.2 with KOH, and tested at six vinasse concentrations (5–50%). Strain growth was monitored on DYGS and Congo Red agars using the microdrop technique. A randomized factorial design with eight treatments and two replicates was implemented. Viable biomass was quantified after 3 days at 28 °C. Organic acid and glycerol consumption in vinasse was measured by HPLC (Module e2695, Waters®) at Cenicaña.

Mass production of bacterial inoculants for sugarcane growth promotion assays

For the greenhouse and open-field phases, we selected strains that showed the highest *in vitro* performance in key plant growth-promoting traits. Based on these criteria, *Azospirillum* strains exhibited superior profiles across all three traits compared with other genera. In addition, *Gluconacetobacter sacchari* strains were included as contrasting candidates since they displayed high phosphate-solubilizing potential *in vitro* but lacked nitrogen-fixing capacity. To produce bacterial inoculants, successive cultures were carried out in liquid media at increasing volumes. The streaking method was used to plate strains and obtain pure isolated colonies (28 °C, 5 days). Six strains were selected based on their high biological N fixation capacity,

IAA production, and inorganic P solubilization. *Azospirillum* strains were activated and cultured on Congo Red agar, while biomass production was performed in BTB-2 broth[3]. DYGS medium was used for *Gluconacetobacter* and YMA medium for *Rhizobium*. The colonies were suspended in sterile distilled water, and the initial inoculant was adjusted to McFarland 1. Biomass production in liquid medium followed a 1:10 ratio. For the first inoculum, 20 ml of bacterial suspension was cultured in 200 ml broth (30 °C, 3 days, 150 rpm). This first inoculum was then used to initiate a second mass production step consisting of 100 ml bacterial broth to produce 1 l culture medium in a 2 l Erlenmeyer flask (180 rpm, 30 °C, 4 days). At the end of the incubation, viable biomass was quantified by both plate count and microdrop techniques.

Greenhouse evaluation of bacterial inoculants

An augmented randomized factorial design consisting of five treatments was used with three replicates (Supplementary Material, Table S2). Each replicate consisted of 35 seedlings, for a total of 105 seedlings per treatment. One-month-old seedlings were obtained from 60-cm mini-cuttings of Cenicaña variety CC-8592. Cuttings were planted in boiler ash substrate. Each seedling was grown in pots containing 600 g dry-weight compost-soil substrate (4:1; 75% soil, 25% compost).

Five treatments were established: (1) unfertilized control with water; (2) *A. brasilense* strains Ab12 and Ab16 (2×10^8 CFU/ml, BTB-2 broth); (3) *Rhizobium* strains R10 and R64 ($5–9 \times 10^6$ CFU/ml, YMA broth); (4) *G. sacchari* strains Gs5 and Gs9 ($9.6 \times 10^7–1.2 \times 10^8$ CFU/ml, DYGS broth); and (5) mineral fertilization with 100 kg N ha^{-1} applied 45 days after planting. All inoculants were applied at 50 ml per plant, and treatments 2–4 were evaluated without chemical fertilization. The roots of each plant were drenched with 50 ml of the corresponding inoculant, resulting in final concentrations ranging from 4.16×10^5 to 3.3×10^7 CFU/g of dry substrate. Inoculants were applied twice weekly for four weeks, totaling eight applications per seedling.

Random samples of 12 healthy seedlings per replicate were collected 45 days after applying the treatments. The following variables were measured: dry root, stem, and leaf biomass (60 °C, 72 h); leaf area index (three plants); and root length per replicate.

Field evaluation of *Azospirillum*, *Gluconacetobacter*, and *Rhizobium* inoculants

The field experiment was conducted at Cenicaña, located in San Antonio, Valle del Cauca, Colombia (3°21′41″ N, 76°17′49″ W), in plots near Lot 19A. The experimental area covered 576 m^2 and is characterized by a multiannual average temperature of 23.2 °C and annual precipitation of 1167 mm. The site corresponds to a semi-dry agroecological zone with slight moisture deficit and fine silty clay-type ''Cantarina'' soil (12.03% sand, 43.42% clay, 44.53% silt), pH of 6.9, 1.60% organic C, 3.04% organic matter, and exchangeable cations: 20.5 cmol Ca, 13.1 cmol Mg, 0.38 cmol K, and 0.44 cmol Na per kg in horizon A.

After two months of growth, seedlings produced in the greenhouse were transplanted with their entire root ball (rhizosphere soil). Seedlings were inoculated with bacterial inoculant prior to transplanting. The increase in the community of free-living N-fixing bacteria due to inoculation was quantified using the most probable number method (Supplementary Materials and Methods S2).

An augmented randomized factorial design was used with three replicates per treatment and 20 plants per replicate, for a total of 60 individuals per each of the six treatments. The experiment was conducted in a 9.6×60 m plot (576 m^2), composed of 36 furrows (two per plot). Twelve seedlings were transplanted per furrow, spaced 80 cm apart, with 165 cm between furrows.

Bacterial inoculants were applied four times over a two-month period at 15-day intervals, using the concentrations and treatments described in Supplementary Table S2, at a rate of 100 ml per plant. No mineral or synthetic fertilizers were applied to inoculated plots. In contrast, the N-fertilized control plots received urea (100 kg N ha^{-1}) applied 45 days after transplanting.

Field measurements of growth and productivity

Harvesting was performed 10 months after transplanting. The following variables were measured: stem weight, length, and diameter; number of stems per stool; cane weight per stool; and sucrose percentage per stem. Stem length and diameter were recorded from a subsample of 12 stems per plot. Stem weight and stem number per stool were used to calculate and extrapolate cane yield in tons per hectare (TCH). Sucrose content (g per stem) and sucrose percentage were determined from the juice of five stems per plot using the ICUMSA Method GS5-1[13], which involves VIS-polarimetry after clarification with lead acetate, in combination with refractometry.

Effect of urea dose and bacterial inoculation on sugarcane growth

A randomized complete block design with two factors (bacterial inoculant and urea dose) was used, each with four levels and their combinations (Supplementary Table S3). Urea application was split into three stages: 30% at 1 month after planting, 35% at 2.5 months, and the remaining 35% at 3.7 months after bud emergence, following the N uptake curve of variety CC 05-430.

Azospirillum inoculant was applied at five levels: 0% (no inoculation), 25% (2.5×10^7 CFU/ml), 50% (5×10^7 CFU/ml), 75% (7.5×10^7 CFU/ml), and 100% (1×10^8 CFU/ml). The inoculant was produced in BTB-2 broth[5] with an initial 1:10 inoculation ratio and incubated for 72 h at 30 °C. A bacterial suspension (1×10^8 CFU/ml) was applied at 200 ml per plant, corresponding to the full-dose (100%) treatment.

Each experimental unit consisted of eight seedlings per replicate, with three replicates per treatment. Seedlings of the Cenicaña variety CC 05-430 were grown in plastic containers filled with a soil-to-compost substrate (3:1). After six months of growth, plant development variables were measured, including number of stems per stool, plant height, stalk weight, stem diameter, root dry weight, and leaf N,

P, and K contents. Macronutrients (P, K) and micronutrients were analyzed using standard foliar analysis techniques described by the Soil Chemistry Laboratory of Cenicaña (Supplementary Materials and Methods S3).

Statistical analysis

Data normality and homoscedasticity were tested using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Variables with a normal distribution were analyzed by analysis of variance (ANOVA) ($p \leq 0.05$). Duncan's and Tukey's post hoc tests were used to determine differences among treatment levels. When no significant differences were detected by ANOVA among treatments with plant growth-promoting bacteria, Student's *t*-tests for two independent samples were applied. All statistical analyses were performed using SPSS (version 2024, IBM Corp.).

Results

Culturable microbial community in the sugarcane rhizosphere and biochemical capacities related to plant growth promotion

A total of 95 bacterial isolates were identified from the sugarcane rhizosphere, corresponding to three phyla, four classes, eight orders, nine families, and 12 genera based on 16S rRNA sequencing. The most representative genera were *Azospirillum*, *Azotobacter*, and *Gluconacetobacter* (Supplementary Fig. S1). Genera such as *Rhizobium*, *Pseudomonas*, *Paenibacillus*, and *Kosakonia* were also identified among the isolates. Based on percentage identity, coverage, and e-value, isolates such as *A. brasilense* Ab12 and Ab16 were assigned to species level (>99% identity and >97% coverage with reference sequences), while other isolates, including *G. sacchari* Gs5 and *G. diazotrophicus* Gd24, showed high sequence similarity.

IAA production and biological nitrogen fixation efficiency differed among genera ($p < 0.001$) and among isolates ($p < 0.0001$) (Supplementary Table S4). *Azotobacter* strains produced higher IAA concentrations in tryptophan-supplemented medium (99–140 μg/ml) compared with *Azospirillum* (24–57 μg/ml) and *Gluconacetobacter* (46–65 μg/ml). *Azospirillum* isolates were the most efficient in enzymatic acetylene reduction (73–80%) compared with *Gluconacetobacter* isolates (27–58%). Results of acetylene reduction assays differed among *Gluconacetobacter* species, being positive for *G. diazotrophicus* and negative or indeterminate for *G. sacchari* isolates.

Inorganic phosphorus solubilization by bacterial strains

Fifteen strains were characterized qualitatively and quantitatively for phosphorus solubilization by measuring solubilization halos and soluble phosphate concentrations. Five genera were evaluated. All strains formed solubilization halos on NBRIP agar with indices ranging from 1.39 to 2.30. Solubilization indices varied among genera, with mean values of 2.08 ± 0.18, 2.28 ± 0.19, and 1.88 ± 0.22 (n = 3) for *Gluconacetobacter*, *Azotobacter*, and *Azospirillum*, respectively. Strains *G. sacchari* Gs5, *A. tropicalis* At98, *A. chroococcum* Ac109, and *G. diazotrophicus* Gd24 solubilized higher phosphate concentrations from phosphate rock compared with tricalcium phosphate at 6 and 9 days post-incubation (Supplementary Fig. S2 and Table 1). In contrast, *Rhizobium* and *A. brasilense* strains produced higher levels of soluble phosphate when grown with tricalcium phosphate during days 3, 6, and 9 of incubation. Soluble phosphate concentrations differed between strains up to 9 days post-incubation ($p = 0.017$, n = 3). The most efficient solubilizers were *Azotobacter* and *Gluconacetobacter*, which maintained soluble phosphate release over time in phosphate rock assays, likely due to utilization of the slow-release P fraction of the mineral fertilizer (Supplementary Fig. S3).

For *Gluconacetobacter* Gs5, P solubilization increased at 6 days in NBRIP + phosphate rock medium and decreased at 9 days, suggesting consumption of released phosphate during stationary growth. In contrast, *Azotobacter* Ac109 maintained stable solubilization values over time.

Supernatant pH in NBRIP medium differed between tricalcium phosphate and phosphate rock assays at 3, 6, 9, and 12 days of incubation for *Azotobacter* and *Gluconacetobacter* (Student's *t*-test, $p = 0.001$). The pH decreased to 4.06–7.52 in NBRIP + tricalcium phosphate medium and to 2.86–3.36 in NBRIP + phosphate rock medium (Supplementary Table S5). The solubilization mechanism involved acidification of the medium through hydrogen ion and organic acid production, intensified when slow-release phosphate sources were used. After 3 and 12 days of incubation, strains reached stationary phase with populations ranging from 3.5×10^8 to 1.7×10^9 CFU/ml.

Viability and growth of bacterial strains in vinasse

Azospirillum isolates remained viable and capable of reproduction in vinasse prior to thermal concentration through multiple-effect evaporation (Supplementary Fig. S4). Vinasse concentration significantly affected *Azospirillum* viability ($p = 0.001$). A 30% (v/v) vinasse supplemented with 5 g/l phosphate rock enhanced viable cell numbers, reaching $2.6 \pm 0.6 \times 10^8$ CFU/ml. Vinasse diluted to 30–50% also promoted *Azospirillum* biomass growth.

A. brasilense Ab12 remained viable and reproduced in both 10% and 30% vinasse broths, reaching $2.4 \pm 0.09 \times 10^9$ CFU/ml in 10% broth at 24 h – an increase from the initial inoculum of $7.02 \pm 0.02 \times 10^7$ CFU/ml. Viable populations ranged from $1.58 \pm 0.11 \times 10^8$ to $3.8 \pm 0.17 \times 10^8$ CFU/ml in 30% broth. However, the strain was not viable in 50–90% vinasse after 24 h incubation (Supplementary Fig. S5). After 3 days, *Azospirillum* and *Gluconacetobacter* isolates consumed glycerol, propionic, trans-aconitic, and acetic acids from 10% and 30% vinasse during incubation (Supplementary Fig. S6).

Plant growth of sugarcane 45 days after bacterial inoculation

Application of *Azospirillum* and *Gluconacetobacter* inoculants significantly increased dry biomass ($p = 0.045$) 45 days

Table 1 Soluble phosphate concentrations in the supernatant of NBRIP medium after 6 days of incubation for strains grown with two phosphate sources. Different letters indicate significant differences according to Student's *t*-test (two independent samples, based on two assays over time).

Strain	Source of phosphate	Phosphate (μg/ml)
Azotobacter At96	Tricalcic phosphate	17.025 ± 2 b
	Phosphate rock	92.245 ± 0.28 a
Azotobacter Ac109	Tricalcic phosphate	24.15 ± 3.17 b
	Phosphate rock	63.05 ± 0.29 a
Gluconacetobacter Gs5	Tricalcic phosphate	14.54 ± 12 b
	Phosphate rock	88.79 ± 3.47 a
Gluconacetobacter Gd24	Tricalcic phosphate	2.82 ± 0.41 b
	Phosphate rock	54.93 ± 0.77 a
Rhizobium Rm10	Tricalcic phosphate	33.83 ± 5.9 a
	Phosphate rock	10.08 ± 3.13 b
Azospirillum Ab12	Tricalcic phosphate	16.71 ± 1.51 a
	Phosphate rock	6.63 ± 0.84 b

after application (Supplementary Fig. S7). Bacterial inoculation also enhanced the culturable community of N-fixing bacteria in the rhizosphere, indicating a bioaugmentation effect (Supplementary Table S6).

Plant growth and yield of sugarcane in the field after bacterial inoculation

Field application of *A. brasilense* and *Gluconacetobacter* strains resulted in a 21% yield increase compared with water-treated controls ($p = 0.002$) (Fig. 1). Yields (tons cane per hectare, TCH) were 97.94 ± 5.26 for *Azospirillum*, 95.61 ± 4.2 for *Gluconacetobacter*, 93.27 ± 4.86 for urea-treated, and 80.9 ± 4.56 for unfertilized plots ($p = 0.002$). Higher yields in biofertilizer-treated plants were mainly due to increased stem number, weight per plant and higher viable populations of plant growth-promoting bacteria.

Stem weight per stalk was significantly higher in plants inoculated with *A. brasilense* ($p = 0.008$) compared with uninoculated treatments (Fig. 2). *Azospirillum* increased stalk weight to 16.84 ± 1.0 kg, compared with urea (14.11 ± 0.84 kg) and unfertilized plants (13.32 ± 0.74 kg) ($p = 0.024$). Mean stem weight in *Azospirillum* Ab12–Ab16 treatments was 1.82 ± 0.035 kg, higher than water (1.64 ± 0.049 kg) or urea controls (1.64 ± 0.176 kg) (Student's *t*-test, $p = 0.045$), representing a 10.97% increase.

Sucrose content per stem was higher in *Azospirillum* treatments (268.08 ± 10 g) than in urea controls (225.21 ± 12 g) ($p \leq 0.05$), representing a 19.03% increase (Table 2). Although sucrose percentage differences among treatments were not statistically significant, inoculated plants showed a 0.53% increase in juice sucrose content. This modest increase may still be relevant for sugar mills, so both total and percentage sucrose are reported to assess agronomic and industrial effects of inoculation.

Cane stem length differed significantly among treatments ($p < 0.005$). Plants harvested 10 months post-inoculation were taller than those treated only with water or synthetic N fertilizers (Supplementary Fig. S8). Plants inoculated with *A. brasilense* Ab12 and Ab16 reached 235.87 ± 5.11 cm, compared with 225.10 ± 4.65 cm for water controls ($p = 0.003$), representing a >10% increase. *Rhizobium*-treated plants were also taller than those fertilized with urea ($p = 0.023$; 232.07 ± 4.59 cm).

Stem diameter, fiber, purity, and °Brix did not differ significantly among treatments (Supplementary Table S7), although inoculation slightly increased juice purity and °Brix compared to urea-fertilized plants.

Urea dose and *Azospirillum* inoculant application

Bacterial inoculant and urea doses significantly affected the number of stems per stalk ($p = 0.0001$) (Fig. 3). Three applications of 200 ml/plant of *Azospirillum* inoculant (1×10^8 CFU/ml) increased tillering by more than 1.5 stems per stalk compared with unfertilized and 25% urea treatments (4.15 g/plant). Responses were like those of 50% and 100% urea treatments. Tillering was mainly influenced by inoculant dose rather than urea dose. Low urea doses alone did not increase tillering, but when combined with *Azospirillum*, stem number increased during establishment.

Stalk weight differed among treatments with varying urea and inoculant doses ($p < 0.0001$) (Table 3 and Supplementary Fig. S8). *Azospirillum* (1×10^8 CFU/ml) increased stalk weight compared with non-inoculated and unfertilized plants. Biomass increased with higher inoculant concentrations. Similar stalk weights were obtained with 50–100% urea (4.15–16.6 g/plant) and inoculant applications. Optimal results were observed with 50–75% urea doses (8.3–12.45 g/plant). Both low (25%) and high (100%) urea doses produced similar stalk weights, suggesting a dose–response curve linked to N deficiency or excess. Combining urea (25–50%) with *Azospirillum* achieved comparable results. For instance, 4.15 g urea/plant could be partially substituted by three inoculant applications, and 12.45 g urea by 8.3 g urea plus 600 ml inoculant (5×10^7 CFU/ml) in three applications.

Root dry weight at 6 months differed among treatments ($p = 0.009$). Applying 25–75% urea doses (4.15–12.45 g/plant) plus inoculant (5–7.5 × 10^7 CFU/ml)

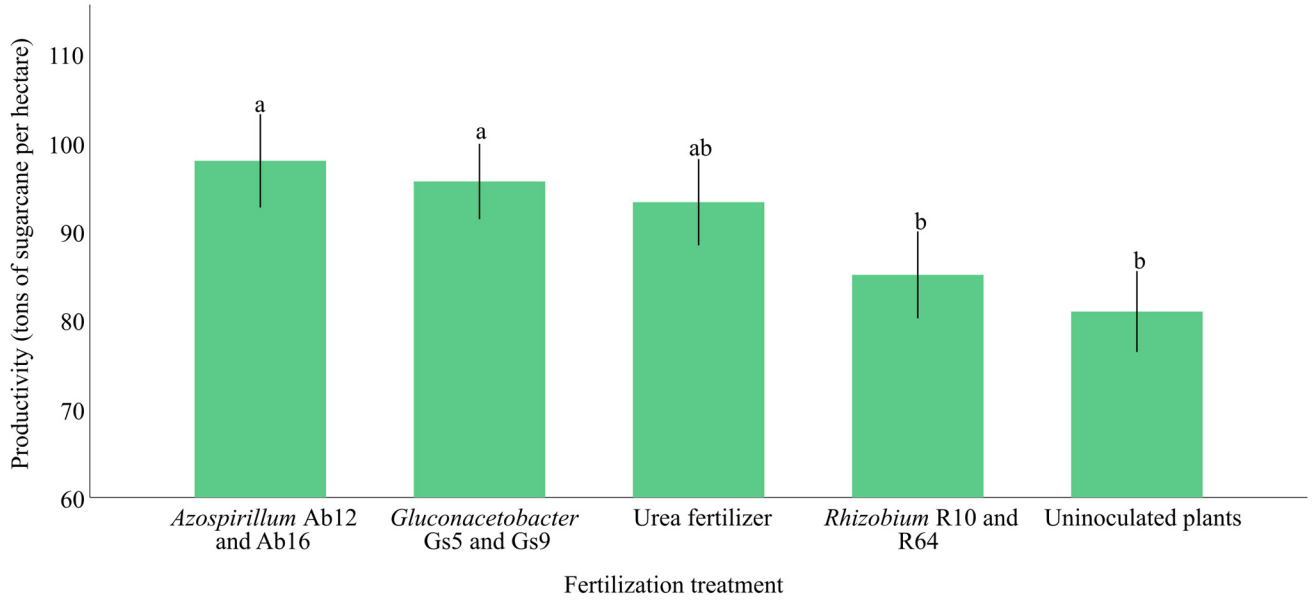

Figure 1 Sugarcane yield (tons per hectare) following the application of bacterial strains native to Colombia's Cauca River Valley. Different letters indicate statistically significant differences according to Tukey's post hoc test (n = 3).

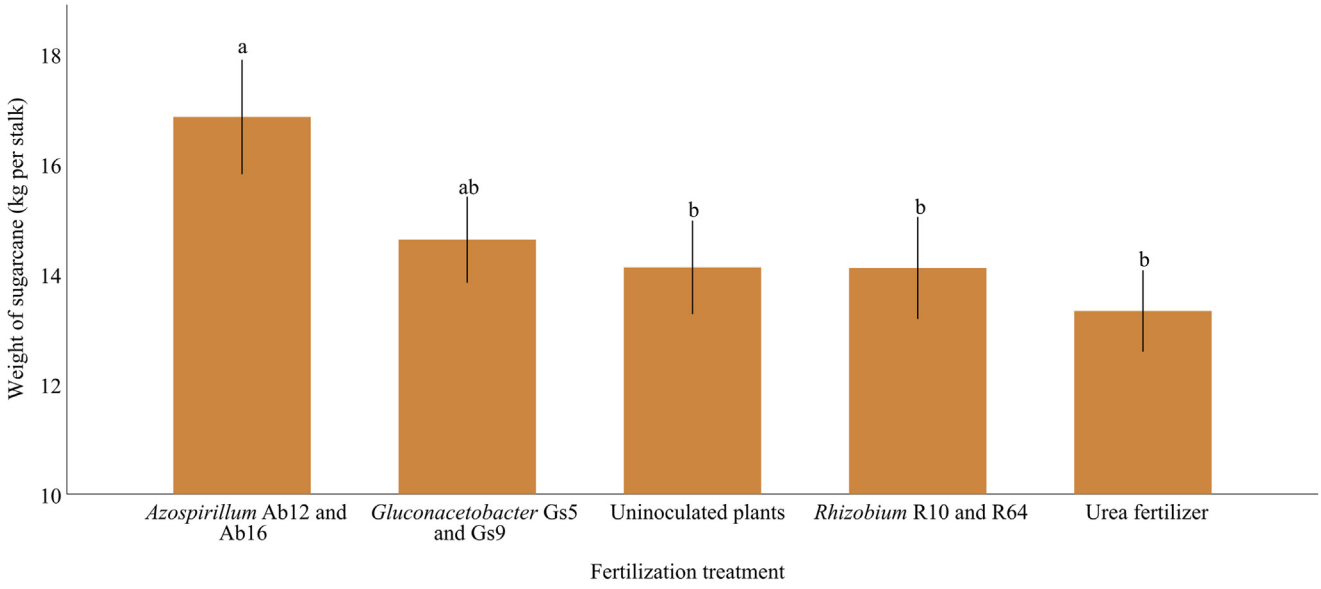

Figure 2 Weight per sugarcane stalk (kg) after inoculation with *Azospirillum*, *Gluconacetobacter*, and *Rhizobium* strains. Different letters indicate statistically significant differences according to Tukey's test (n = 3). Values represent mean ± SD.

Table 2 Sugarcane production under different field treatments with plant growth-promoting bacteria.

Number	Treatment	Weight of sugarcane per stem (kg)	Sucrose per stem (g)	Percentage sucrose in cane (%)
1	Negative control (plants irrigated only with water)	1.64 ± 0.049 a	233.6 ± 21 a	14.2 ± 0.97 a
2	*Azospirillum* strains Ab12 and Ab16	1.82 ± 0.035 b	268.1 ± 10 b	14.73 ± 0.29 a
3	*Rhizobium* strains R10 and R64	1.76 ± 0.114 a	248.4 ± 25 a	14.02 ± 0.52 a
4	*Gluconacetobacter* strains Gs5 and Gs9	1.64 ± 0.11 a	232.2 ± 8.7 a	14.25 ± 0.58 a

a,b Statistically significant differences using the Student's t-test comparing the response variable with any of the treatments. Same letters mean that there were no differences using this test for two independent samples (n = 3).

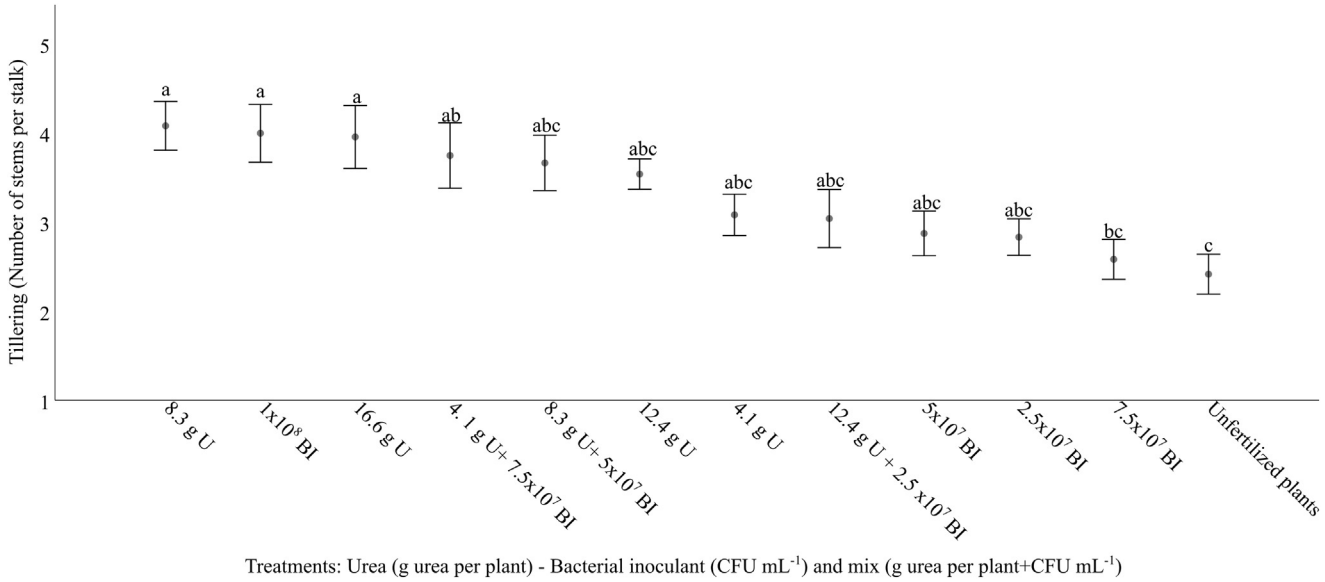

Figure 3 Sugarcane tillering under treatments with urea (U) and *Azospirillum* bacterial inoculant (BI). Bars represent the standard error (n = 4). Different letters indicate statistically significant differences according to Tukey's post hoc test.

Table 3 Agronomic response of sugarcane after 6 months of growth under treatments with different urea doses and concentrations of *Azospirillum* biological inoculant.

Treatment	*Azospirillum* inoculant (CFU/ml)	Urea dose per plant (g)	Stems per stalk (no.)	Weight per stalk (kg)	Root weight (kg)
Unfertilized plants	Negative	Negative	2.42 c	1.71 d	0.15 bc
50% *Azospirillum*	5×10^7	Negative	2.87 abc	1.92 cd	0.13 c
75% *Azospirillum*	7.5×10^7	Negative	2.58 bc	2.11 cd	0.13 c
25% *Azospirillum*	2.5×10^7	Negative	2.83 abc	2.24 bcd	0.16 abc
25% urea	Negative	4.15	3.08 abc	2.60 abcd	0.16 abc
100% *Azospirillum*	1×10^8	Negative	4.0 a	2.74 abc	0.17 abc
75% urea + 25% bacterial inoculant	2.5×10^7	12.45	3.04 ab	2.77 abc	0.16 abc
100% urea	Negative	16.6	3.95 a	2.79 abc	0.18 abc
50% urea + 50% bacterial inoculant	5×10^7	8.3	3.66 ab	2.99 ab	0.24 a
25% urea + 75% bacterial inoculant	7.5×10^7	4.15	3.75 ab	3.178 ab	0.23 ab
75% urea	Negative	12.45	3.54 abc	3.05 ab	0.24 a
50% urea	Negative	8.3	4.08 a	3.12 a	0.17 abc

Statistically significant differences according to ANOVA and Duncan's post-hoc test. Different letters indicate significant differences between subgroups, while same letters mean there were no differences using this test (n = 8 randomized plants per replicate).

increased root dry weight compared with unfertilized or low-urea treatments (Table 3).

Leaf N ($p = 0.024$), P ($p = 0.049$), and K ($p = 0.022$) contents differed significantly among treatments (Supplementary Table S8). Higher urea doses increased leaf N compared with bacterial inoculants, while *Azospirillum* enhanced leaf P levels. K was higher in urea-fertilized plants. Treatments combining *Azospirillum* with urea increased leaf S ($p = 0.045$), Fe ($p = 0.042$), and Zn ($p = 0.0001$), suggesting improved nutrient uptake due to enhanced root growth.

Discussion

Plant growth-promoting bacteria in the sugarcane rhizosphere

Previous studies by Roa et al.[26,27] reported that the genera *Azospirillum*, *Azotobacter*, *Gluconacetobacter*, and *Rhizobium*, isolated from the sugarcane rhizosphere, can fix atmospheric nitrogen (N_2) into ammonium and to synthesize IAA as a precursor of auxins. *Azospirillum* inoculants

can act as biofertilizers that provide essential nutrients to stimulate plant growth through various direct and indirect mechanisms, studied both in vitro and in vivo[4,6,7].

The hypothesis of multiple mechanisms or cumulative effects suggests that each instance of *Azospirillum* inoculation may involve one or several mechanisms, such as plant regulators (e.g., auxins, gibberellins, cytokinins), nitrogen fixation, phosphorus solubilization, siderophore production, or mitigation of environmental stressors[4,6]. Studying *in vitro* biochemical capacities helps evaluate a bacterial strain's potential to produce secondary metabolites.

In bacterial inoculants, both viable cells and metabolites in the culture supernatant contribute to their activity, particularly those accumulated during the stationary phase of fermentation[7]. The main change induced by *Azospirillum* inoculation is the formation of secondary (lateral and adventitious) roots, together with an increase in the number of root hairs and total root volume, resulting in modifications in root architecture attributed to IAA biosynthesis[7].

Initial taxonomic assignments of the bacterial isolates were based on partial 16S rRNA gene sequencing (V1–V4 and V3–V4 regions), which provided genus-level resolution and, in some cases, species-level affiliation. For instance, isolates Ab12 and Ab16 showed >99% identity with *A. brasilense* reference sequences, while Gs5 was assigned to *G. sacchari* under the same criteria. To confirm these results and ensure strain-level traceability, whole-genome sequencing was subsequently conducted for representative isolates preserved in the Cenicaña culture collection, as reported by Pardo-Díaz et al.[25]. Genome-based taxonomic analysis using digital DNA-DNA hybridization (dDDH) revealed a value of 86.6% between strain Ap18 and *Azospirillum argentinense* Az39^T, and 92.4% between Ap92 and *G. sacchari* LMG 19747^T. These findings clarified the correspondence between the isolates identified by 16S rRNA sequencing and those analyzed at the whole-genome level. Specifically, Ap18 represents the same isolates previously designated as Ab12 and Ab16, initially classified as *A. brasilense* based on 16S rRNA similarity but more accurately resolved as *A. argentinense* through genome sequencing. In contrast, Ap92 consistently retained its identity as *G. sacchari*, corresponding to isolates Gs5 and Gs9. This integration of 16S rRNA and whole-genome analyses provides robust evidence for the taxonomic identity and traceability of the strains employed in greenhouse and field experiments.

The integration of our phenotypic assays with the genome-based characterization reported by Pardo-Díaz et al.[22] provides a consistent framework for interpreting the functional potential of the strains used in this study. Beyond taxonomic resolution, genome analysis revealed that *A. argentinense* Ab12-Ab16-Ap18 harbors the canonical nifHDK operon, directly linked to nitrogen fixation, which supports our in vitro evidence of high nitrogenase activity and explains its positive effect on plant growth under reduced nitrogen fertilization. Similarly, the genome of Ap92, characterized by high completeness and robust assembly quality, confirms its stable taxonomic assignment and supports its consistency in other plant growth-promoting traits. These results highlight the complementarity of 16S-based identification, biochemical assays, and whole-genome evidence in ensuring both taxonomic traceability and functional validation of microbial inoculants for sugarcane cultivation.

Phosphorus solubilization by nitrogen-fixing bacteria

Inorganic phosphorus (P) solubilization is a characteristic of nitrogen-fixing bacterial strains from the sugarcane rhizosphere in Colombia's Cauca River Valley. This capacity in *Azospirillum* strains may involve gluconic acid production when cultured with glucose or fructose and insoluble P sources[27,28,33,34]. Phosphorus solubilization occurs via medium acidification by organic acids and proton release, even in the absence of plant root exudates[4]. The application of *Azospirillum* Ab12 and related strains has been shown to enhance leaf P content, in agreement with previous findings in other studies[31–34].

In vitro P solubilization by our bacterial strains is influenced by growth conditions, including incubation time and carbon source. *Azotobacter* and *Gluconacetobacter* strains showed higher solubilization efficiencies when grown on phosphate rock in NBRIP medium. These processes depend on the type of soil P source and the use of slow-release fertilizers such as calcium phosphate and minerals like fluorapatite and carbonate apatite. Solubilization mechanisms involve organic acid secretion, soil acidification, and phosphatase activity, which enhance the bioavailability of inorganic P (Pi)[36,37].

Use of vinasse by bacterial strains for growth and reproduction

Azospirillum and *Gluconacetobacter* isolates remain viable and reproduce in vinasse at concentrations of 30% and 50%, while metabolizing organic acids such as glycerol, propionic acid, trans-aconitic acid, and acetic acid. Vinasse is commonly used in sugarcane fertigation and as a KCl supplement[8]. Given its potential alternative applications-such as its use as a culture medium or carrier in biofertilizer production-assessing the compatibility of plant growth-promoting bacterial strains with vinasse is essential.

Enhanced sugarcane growth due to the application of *Azospirillum* Ab12 and Ab16 inoculants

The use of microbial inoculants as a complementary or alternative practice to mineral fertilization enhances plant growth. Sugarcane biomass can be significantly increased through mass application of *Azospirillum* Ab12 compared with treatments without synthetic fertilization, as well as with the full recommended urea dose. Intensive inoculation in pre-sprouted sugarcane cuttings during the emergence, germination, and tillering phases, followed by field planting, was effective. *Azospirillum* Ab12 enhanced multiple agronomic variables such as stalk weight, tillering (number of stems per stalk), tons of cane per hectare (TCH), and sucrose content (g per stem). Different greenhouse and field studies have demonstrated the positive effects of *Azospirillum* biological inoculants on plant growth and productivity in sugarcane, particularly by increasing the number of stems

per stalk, stalk diameter, and height during the tillering stage[1,11,21–23,31,32]. The potential applications of *Azospirillum* Ab12 include the transplantation of inoculated seedlings in plantations undergoing ratoon renewal, as well as its use in organic sugarcane systems.

Intensive application of *Azospirillum* inoculants during the sprouting stage of sugarcane cuttings enhances tillering capacity by increasing the number of stems per stalk[21,31,32]. This early-stage intervention contributes significantly to crop establishment and productivity, with improvements primarily associated with greater stem proliferation rather than increased stalk diameter or height[1,31,32].

The high frequency and viable cell concentrations of *Azospirillum* inoculations in our study were chosen to test whether repeated applications could overcome the weak responses observed in previous studies under local production conditions in Valle del Cauca, Colombia. Two treatments ($\sim 10^6$ CFU/ml) often failed to improve yields, possibly due to high soil organic matter, strong native microbial competition, elevated urea/DAP inputs, and vegetative propagation of sugarcane. Using eight applications at 10^8 CFU/ml, however, produced marked gains: up to 19% more sucrose per stalk, 20% more biomass, and ~20.5% higher yield compared to unfertilized plants. Likewise, Scudeletti et al.[32] reported that *Azospirillum* inoculants applied at $1.5–2 \times 10^{11}$ CFU/ha during early developmental stages promote greater plant height, internode length, and stem density. In our study, while lower concentrations (2.5×10^7 CFU/ml) showed limited impact, repeated applications at higher concentrations (1×10^8 CFU/ml) significantly promoted shoot biomass. These results emphasize the importance of inoculant dose in eliciting positive plant growth responses.

Cost–benefit analysis in the study area showed conventional fertilization (urea + DAP) costs of approximately USD $457 per hectare, whereas inoculant-based treatments ranged between USD $423–679, depending on urea reduction. The added cost of the biofertilizer (~USD $250) is partly offset by savings in synthetic fertilizers (up to ~USD $197) and yield increases of 10–20% in tons of sugarcane per hectare. The current biofertilizer price reflects small-scale production; optimization of microbial scale-up processes, product formulation, and the possibility for farmers or sugar mills to propagate their own inoculants could substantially reduce costs, increasing adoption feasibility[7,17]. Furthermore, reducing urea inputs by 25–50% also lowers greenhouse gas emissions (N_2O), contributing to climate-smart agriculture in sugarcane systems[17,24].

Sucrose is another important productivity variable in sugarcane. Increases in sucrose percentage per stem were observed in plots inoculated with *Azospirillum*. This response has also been described by Aguado-Santacruz et al.[1] in plots inoculated with biofertilizers applied to roots or shoots for three months after mineral fertilizer application. It is important to note that some results reached statistical significance only at the threshold level. While these values confirm a measurable inoculation effect, they also highlight the need for careful interpretation of the magnitude and variability among replicates. The agronomic relevance of these findings is particularly noteworthy, given that field trials were conducted without mineral fertilization, suggesting potential application in organic sugarcane production or in sustainable systems aimed at reducing urea inputs. Nevertheless, further studies across different soil types, climatic conditions, and management regimes are necessary to validate these observed trends.

Relationships between study area, minimal urea use, and *Azospirillum* biofertilizer application

The positive effects of *A. brasilense* strains Ab12 and Ab16 without chemical mineral fertilization can be attributed to the favorable chemical properties of the soil, such as high organic matter content, labile carbon, neutral pH, and clay content. Additionally, the experimental soils exhibited a rich diversity of bacterial communities (unpublished data). Fertile soils can naturally support high productivity without mineral fertilization due to their physicochemical conditions and microbial biodiversity[28,29]. The effects of *A. brasilense* depend on factors such as soil type, plant species, edaphoclimatic conditions, and management practices[31,32]. Inoculation with *Azospirillum* is more effective in clayey soils under adequate irrigation, and nitrogen-fixing bacterial biofertilizers are generally applied between 30 and 60 days after planting, prior to N fertilization. However, applying *Azospirillum* to pre-sprouted seedlings is an effective strategy to bioaugment bacterial populations and establish strong root-associated microbial communities early on[28,29].

The mixture of *Azospirillum* Ab12 inoculants together with reduced urea doses can replace part of the applied nitrogen and benefit sugarcane growth. This complementary practice – using *Azospirillum* Ab12 inoculants while reducing urea doses by 25–50% – can have positive additive effects on crop tillering and stalk weight. Misra et al.[17] reported that *Azospirillum* biofertilizer application can reduce nitrogen fertilization requirements by 25%. Schultz et al.[30,31] reported that inoculation with nitrogen-fixing bacterial strains of the genera *Herbaspirillum*, *Paraburkholderia*, *Nitrospirillum*, and *Gluconacetobacter*, combined with chemical N fertilization, can promote sugarcane stem production.

Application of the *A. brasilense* Ab12 strain can enhance sugarcane root weight, although it does not necessarily increase leaf N content. Inoculation with plant growth-promoting bacteria does not always result in higher inorganic N inputs or increased soil nitrogen through biological fixation[15]. Instead, their effects on productivity are often linked to mechanisms such as phytohormone production (e.g., auxins) and phosphorus solubilization[14,30,31]. Similarly, combined effects of bacterial inoculants on sugarcane productivity have been observed with mixtures of *Paraburkholderia* and urea, leading to a 10% increase in tons of cane per hectare[23,24]. The use of biofertilizers offers benefits for both yield and quality of sugarcane, while potentially reducing N fertilizer doses and providing economic advantages by lowering input costs[31,32].

While biological N fixation by *Azospirillum* has been linked to increased root nitrogenase activity and higher leaf N levels in grasses and pastures, in our study, it did not result in increased leaf N. Instead, these results suggest that nitrogen fixed by the bacteria contributes to reduced fertilizer requirements in field crops[4]. These findings indicate that other metabolic capabilities of *Azospirillum*, such

as indole-3-acetic acid production, may play a more significant role in promoting plant growth. Root growth stimulation enhances water absorption and nutrient acquisition, including dissolved nitrogen ions that support biomass formation[6]. Higher concentrations of Fe, S, and Zn in plants inoculated with *Azospirillum* may be associated with secondary root formation, thus improving water and nutrient uptake.

Conclusions

Inoculation of sugarcane with *A. brasilense* strains Ab12 and Ab16 emerge as a sustainable strategy to reduce dependence on synthetic fertilizers and enhance plant growth. Mass inoculation schemes – with regular applications during the nursery stage or at the emergence and germination of buds, followed by transplanting with rhizosphere soil – could optimize key agronomic parameters such as tillering, stem weight, and sucrose content.

Future research should focus on scaling up inoculation protocols, optimizing application frequencies, and evaluating their long-term effects on yield stability and sucrose accumulation. From an environmental perspective, integrating biofertilizers with reduced urea inputs could mitigate nutrient losses, improve soil health, and contribute to the development of more sustainable sugarcane production systems.

Ethical approval

This article does not contain any studies conducted by the authors involving human participants or animals.

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Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version available at https://doi.org/10.1016/j.ram.2026.100716.

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