Characterization of Nitrogen-Fixing Diazotrophs Associated with Sugarcane Root Systems

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ABSTRACT

Background: Biological nitrogen fixation (BNF) by root-associated diazotrophs offers a sustainable alternative to synthetic nitrogen fertilizers in sugarcane, a crop with high nitrogen demand and substantial environmental footprint from conventional fertilization.

Objective: This study aimed to isolate, identify, and functionally characterize diazotrophic bacteria colonizing the rhizosphere and root endosphere of sugarcane, and to evaluate their plant growth-promoting potential.

Methods: Root and rhizosphere samples were collected from three sugarcane varieties across two agro-climatic zones. Diazotrophs were isolated on nitrogen-free semi-solid media (NFb, LGI, JNFb), characterized morphologically and biochemically, identified via 16S rRNA and nifH gene sequencing, and screened for nitrogenase activity (acetylene reduction assay), IAA production, phosphate solubilization, siderophore production, ACC deaminase activity, and biofilm formation. Data were analyzed using one-way ANOVA with Tukey's post-hoc test ($p < 0.05$).

Results: Forty-two isolates were recovered, clustering into six genera dominated by *Gluconacetobacter*, *Herbaspirillum*, and *Azospirillum*. Endophytic isolates showed significantly higher nitrogenase activity and IAA production than rhizospheric isolates, while phosphate solubilization and siderophore production were more prevalent among rhizospheric strains.

Conclusion: Sugarcane roots harbor a functionally diverse diazotrophic community with strong biofertilizer potential; endophytic strains in particular merit further field-scale evaluation for reducing chemical nitrogen inputs in sugarcane cultivation.

Keywords: Biological nitrogen fixation; Diazotrophic bacteria; Sugarcane (*Saccharum officinarum* L.); Endophytic bacteria

1. Introduction

Sugar cane (*Saccharum officinarum L.*) is one of the most important crops in terms of economics due to its role in sugar and bioethanol production as well as the many farmers who depend on it for their livelihoods. The significant biomass that this crop produces is why there is such a high need for nitrogen, and this has usually been met through the use of synthetic fertilizers, which raises costs and also results in leaching of nitrates and greenhouse gases as well as damages to the soil.

Biological nitrogen fixation (BNF) is important for salt management as it uses diazotrophic bacteria to produce ammonia from air nitrogen with the help of nitrogenase, which makes BNF essential for sustainable nitrogen management. Sugarcane is Jack of all trades among grasses because it has been known for ages for its association with endophytes and rhizosphere diazotrophic bacteria such as *Gluconacetobacter* diazotrophicus, *Herbaspirillum* spp., *Azospirillum* Many of these can supply a large amount of nitrogen to plants under favorable conditions.

Root microbiome includes various plant growth-promoting functions apart from nitrogen fixation like phytohormone synthesis, phosphate solubilization, iron acquisition through siderophores, and the induction of systemic stress tolerance by ACC deaminase. Therefore, understanding the taxonomy and functionality of the root-dwelling nitrogen-fixing bacteria is crucial for developing effective and locally adapted biofertilizer products. In this study, we isolated and characterized the diazotrophic bacteria from the root system of sugarcane through a combined morphological, biochemical, molecular, and functional approach to evaluate their potential role in sustainable nitrogen management.

Materials and Methods

Study Area and Sample Collection

Field trials were conducted at two commercial sugarcane-growing sites representing distinct agro-climatic zones. Root and adhering rhizosphere soil were collected from three widely cultivated sugarcane varieties at the tillering stage. Samples were obtained in triplicate per site-variety combination, transported in sterile containers at 4°C, and processed within 24 hours. Roots designated for endophyte isolation were surface-sterilized sequentially with ethanol and sodium hypochlorite, with sterility confirmed by plating the final rinse water.

Isolation of Diazotrophs

Rhizosphere soil suspensions and macerated surface-sterilized root tissue were inoculated into semi-solid nitrogen-free NFb, LGI, and JNFb media and incubated at 30°C. Pellicle-forming cultures were serially sub-cultured to purity on the corresponding solid media, and purified isolates were preserved in glycerol stocks at −80°C.

Morphological, Biochemical, and Molecular Characterization

Colony morphology, Gram reaction, cell morphology, and motility were recorded, along with catalase, oxidase, citrate utilization, urease, and carbohydrate utilization tests. Genomic DNA was extracted using a standard CTAB protocol; the 16S rRNA gene and nifH gene were amplified by PCR using universal primer pairs, sequenced, and compared against reference databases. Phylogenetic relationships were inferred using the neighbour-joining method.

Functional Characterization

Nitrogenase activity was quantified by the acetylene reduction assay (ARA) using gas chromatography. Ammonia production, IAA production (Salkowski method), phosphate solubilization (Pikovskaya's medium), siderophore production (CAS assay), ACC deaminase activity, and biofilm formation (crystal violet assay) were determined using standard colorimetric and plate-based protocols.

Statistical Analysis

All assays were performed in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test using R software (v4.3), with statistical significance set at $p < 0.05$.

Results

A total of 42 diazotrophic isolates were recovered, comprising 24 rhizospheric and 18 endophytic strains. Molecular identification grouped isolates into six genera: *Gluconacetobacter*, *Herbaspirillum*, *Azospirillum*, Bacillus, Enterobacter, and Pantoea (Table 3, Figure 3). Colony and cell morphology varied by genus, with Gram-negative motile rods predominating among *Gluconacetobacter* and *Herbaspirillum* isolates (Table 2).

Nitrogenase activity, measured by ARA, ranged from 0.8 to 18.6 nmol ethylene mg protein^{-1} h^{-1}, with endophytic *Gluconacetobacter* and *Herbaspirillum* isolates showing significantly higher activity than rhizospheric isolates ($p < 0.05$; Table 5). IAA production was likewise highest among endophytic isolates, whereas phosphate solubilization index and siderophore production were significantly greater among rhizospheric Bacillus and Enterobacter isolates (Table 4). Biofilm formation, associated with root colonization efficiency, was strongest in *Herbaspirillum* and *Azospirillum* isolates.

Discussion

The recovery of *Gluconacetobacter*, *Herbaspirillum*, and *Azospirillum* as dominant genera corroborates prior reports of sugarcane's characteristic association with these diazotrophs and reinforces the crop's exceptional capacity to host efficient nitrogen-fixing endophytes. The significantly higher nitrogenase activity observed in endophytic isolates likely reflects the more stable, microaerobic, carbon-rich environment of root tissue relative to the more variable and competitive rhizosphere, consistent with established endophyte-rhizosphere functional contrasts.

The complementary distribution of traits, nitrogen fixation and IAA production concentrated in endophytes, versus phosphate solubilization and siderophore production in rhizospheric strains, suggests that multi-strain consortia rather than single-strain inoculants may maximize agronomic benefit. Such consortia could substantially reduce reliance on synthetic nitrogen fertilizer while improving nutrient use efficiency and stress resilience under variable field conditions.

Key limitations of this study include its reliance on culturable diversity, which underestimates the full diazotrophic community, and its site-restricted sampling. Future work integrating metagenomics, transcriptomics, and microbiome engineering approaches would allow more comprehensive characterization of unculturable taxa and in planta gene expression dynamics, supporting the rational design of climate-resilient, field-validated sugarcane biofertilizers.

Conclusion

This study demonstrates that sugarcane root systems harbor a taxonomically and functionally diverse community of diazotrophic bacteria, with endophytic strains showing particularly strong nitrogen-fixing and phytohormone-producing potential, and rhizospheric strains contributing complementary nutrient-mobilizing traits. These findings support the development of multi-strain diazotrophic biofertilizers as a viable component of sustainable nitrogen management in sugarcane, while underscoring the need for field-scale validation and long-term agronomic trials prior to commercial deployment.

Table 1: Sampling locations and sugarcane varieties

Site	Agro-climatic zone	Variety	Growth stage	Replicates
Site A	Sub-tropical	Co 0238	Tillering	3
Site A	Sub-tropical	CoJ 64	Tillering	3
Site B	Tropical	Co 86032	Tillering	3

Table 2: Morphological and biochemical characteristics of bacterial isolates

Genus	Gram reaction	Cell shape	Motility	Catalase	Oxidase
Gluconacetobacter	Negative	Rod	+	+	-
Herbaspirillum	Negative	Curved rod	+	+	+
Azospirillum	Negative	Vibrioid	+	+	+
Bacillus	Positive	Rod (spore-forming)	+	+	-
Enterobacter	Negative	Rod	+	+	-
Pantoea	Negative	Rod	-	+	-

Table 3: Molecular identification and phylogenetic classification

Isolate code	Closest 16S rRNA match	Identity (%)	nifH detected	Source
SC-R3 / SC-R7	*Gluconacetobacter* diazotrophicus	99.1	Yes	Rhizosphere
SC-E2 / SC-E5	*Herbaspirillum* spp.	98.7	Yes	Endophytic
SC-R11 / SC-R14	*Azospirillum* spp.	98.9	Yes	Rhizosphere
SC-E9	Bacillus subtilis	99.3	No	Endophytic
SC-R18	Enterobacter cloacae	98.4	Yes	Rhizosphere
SC-E13	Pantoea agglomerans	98.6	No	Endophytic

Table 4: Plant growth-promoting traits of diazotrophic isolates

Genus	IAA (µg/mL)	P-solubilization index	Siderophore (%)	ACC deaminase	Biofilm
Gluconacetobacter	24.6	1.2	Low	+	Moderate
Herbaspirillum	31.2	1.1	Low	+	Strong
Azospirillum	28.4	1.8	Moderate	+	Strong
Bacillus	12.1	2.6	High	+	Weak
Enterobacter	18.3	2.4	High	+	Moderate
Pantoea	15.7	2.0	Moderate	-	Weak

Table 5: Statistical comparison of functional characteristics (mean ± SD)

Trait	Rhizospheric isolates	Endophytic isolates	p-value
Nitrogenase activity (nmol C_2H_4 mg^{-1} h^{-1})	6.4 ± 1.8	13.9 ± 2.7	<0.001
IAA production (µg/mL)	17.9 ± 3.1	27.8 ± 4.0	0.002
Phosphate solubilization index	2.1 ± 0.4	1.3 ± 0.3	0.01
Siderophore production (% CAS units)	High	Low-Moderate	0.03

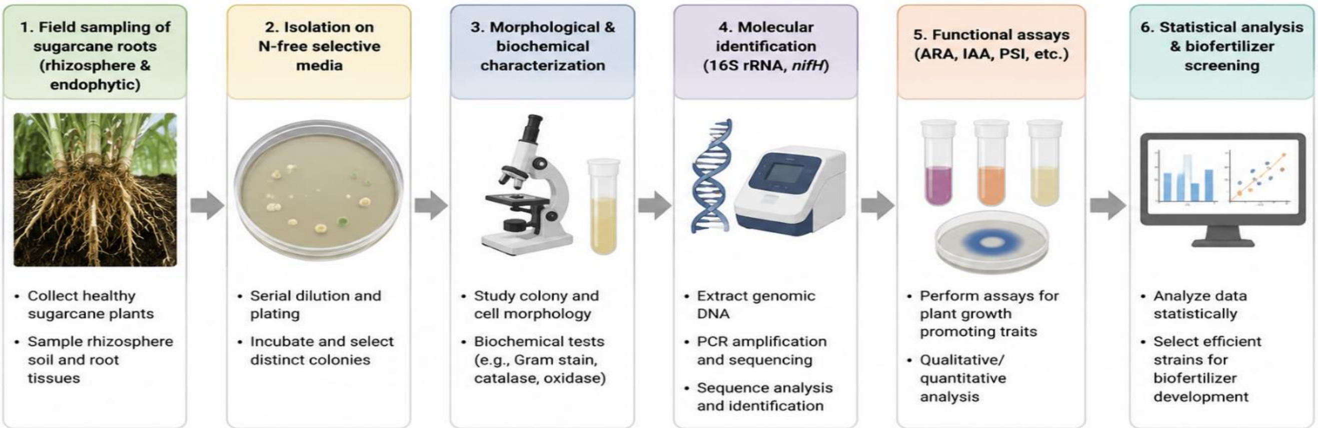

Fig 1: Overall experimental workflow, from field sampling to statistical analysis

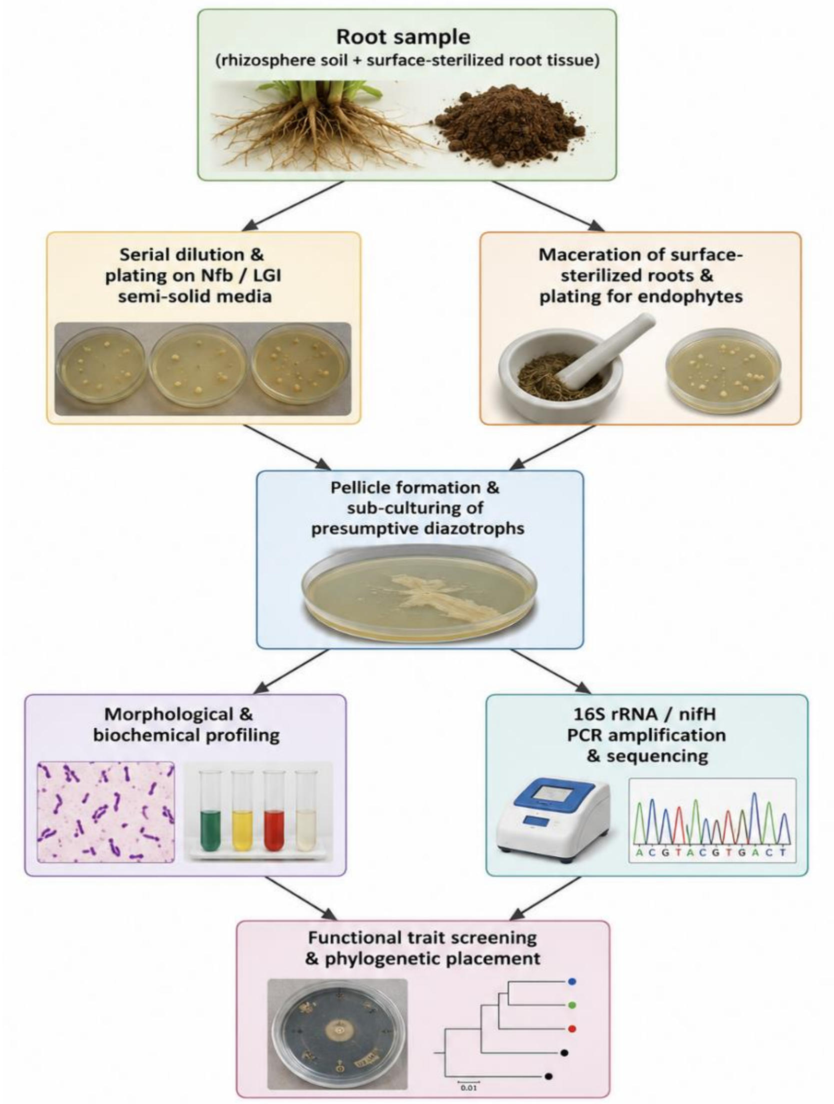

Fig 2: Isolation and characterization workflow of rhizospheric and endophytic diazotrophs

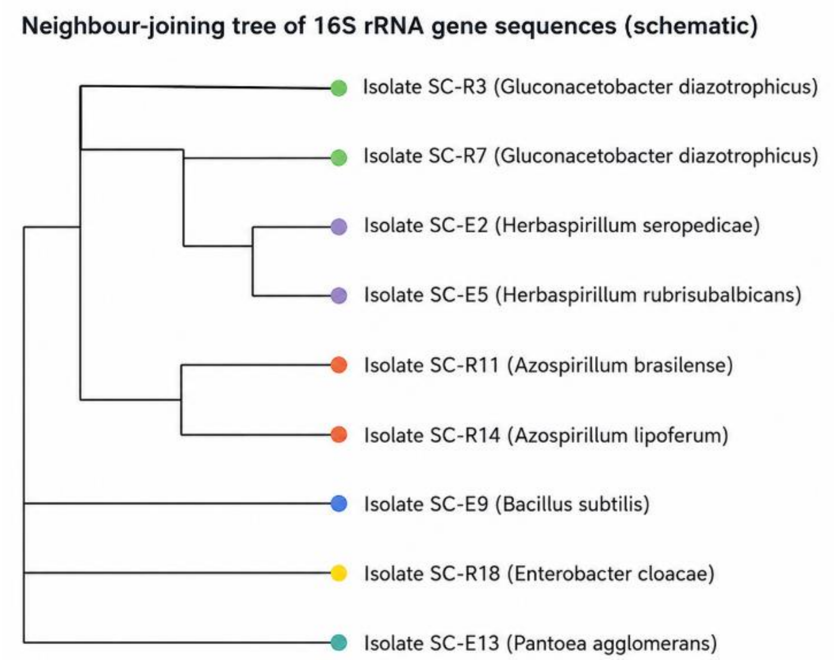

Fig 3: Schematic neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of representative isolates

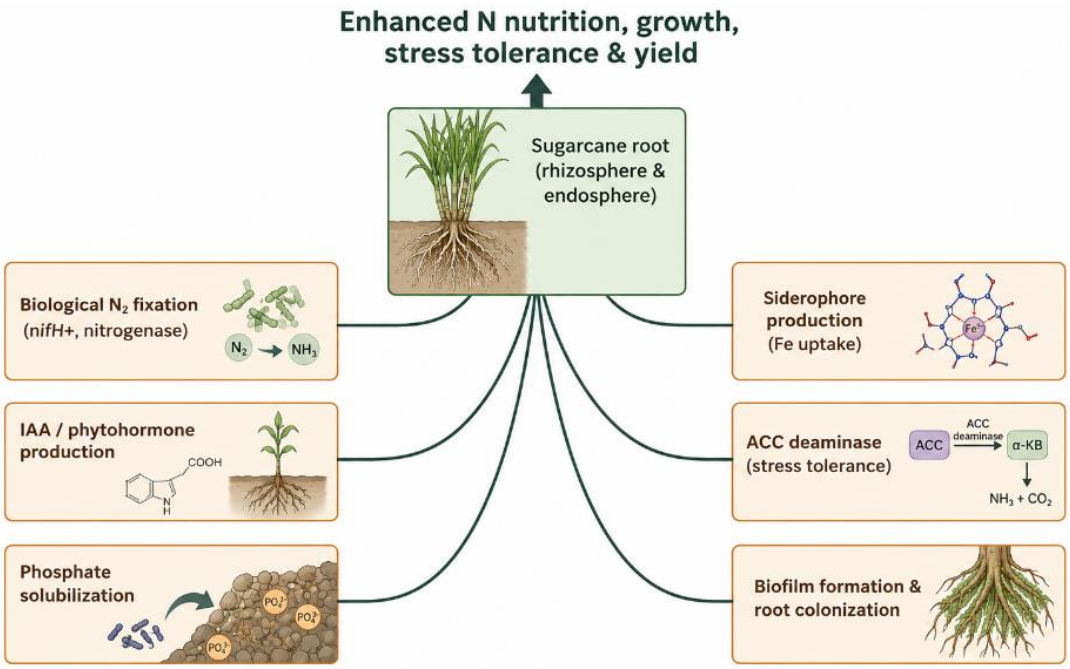

Fig 4: Proposed mechanisms of plant growth promotion by sugarcane-associated diazotrophs

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