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Rearranging the sugarcane holobiont via plant growth-promoting bacteria and nitrogen input

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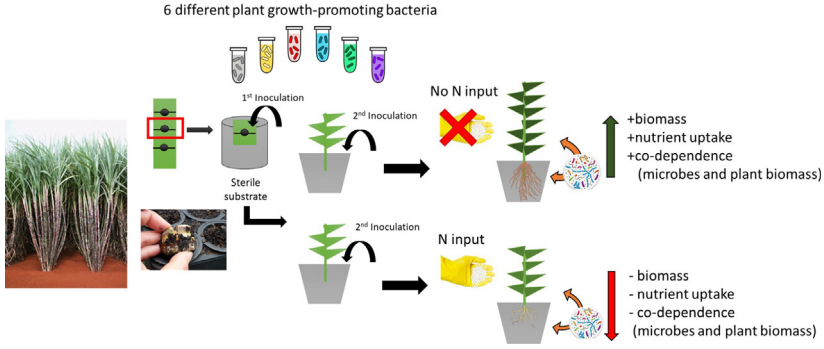
HIGHLIGHTS

- Inoculation shifted the sugarcane microbiome (rhizosphere and endosphere).
- N input decreased the importance of plant growth promoting microbes.
- Rhizosphere prokaryotes are associated with nutrient uptake in sugarcane.
- N input reduced the co-dependence between soil microbes and plant nutrients.
- *Leifsonia* abundance increased with N input as potential risk of ratoon stunting disease.

GRAPHICAL ABSTRACT



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ABSTRACT

The development and productivity of plants are governed by their genetic background, nutrient input, and the microbial communities they host, i.e. the holobiont. Accordingly, engineering beneficial root microbiomes has emerged as a novel and sustainable approach to crop production with reduced nutrient input. Here, we tested the effects of six bacterial strains isolated from sugarcane stalks on sugarcane growth and physiology as well as the dynamics of prokaryote community assembly in the rhizosphere and root endosphere under two N fertilization regimes. All six strains, *Paraburkholderia caribensis* IAC/BECa 88, *Kosakonia oryzae* IAC/BECa 90, *Kosakonia radicincitans* IAC/BECa 95, *Paraburkholderia tropica* IAC/BECa 135, *Pseudomonas fluorescens* IAC/BECa 141 and *Herbaspirillum frisingense* IAC/BECa 152, increased in shoot and root dry mass, and influenced the concentration and accumulation of important macro- and micronutrients. However, N input reduced the impact of inoculation by shifting the sugarcane microbiome (rhizosphere and root endosphere) and weakening the co-dependence between soil microbes and sugarcane biomass and nutrients. The results show that these beneficial microbes improved plant nutrient uptake conditioned to a reduced N nutrient input. Therefore, reduced fertilization is not only desirable consequence of bacterial inoculation but essential for higher impact of these beneficial bacteria on the sugarcane microbiome.

1. Introduction

Sugarcane is one of the most important crops in the world as a source of food (sugar), renewable energy (ethanol) and biomaterials.

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Nitrogen (N) availability is often a limiting factor for plant productivity, and therefore high rates of N fertilization are used to maximize sugarcane yield (Paungfoo-Lonhienne et al., 2014). However, intense N fertilization is often associated with environmental degradation since most of the applied N is lost rather than assimilated by the plant (Wang et al., 2016). It is becoming increasingly clear that sugarcane development, growth, health and productivity are determined not only by genetic makeup and nutrient input but also by the complex microbial communities hosted by the plant (Antunes et al., 2019; da Silveira et al., 2018), in line with the holobiont concept (Sánchez-Cañizares et al., 2017). Accordingly, engineering beneficial root microbiomes has been proposed as a novel and sustainable approach that could complement plant breeding and other management practices aimed at production with less nutrient input. While plant growth-promoting microbes and host-associated environments, such as the plant rhizosphere, root endosphere, and phyllosphere, have received extensive attention in recent years (Armada et al., 2018; Beans, 2017; Busby et al., 2017; da Silveira et al., 2018; Schlemper et al., 2018), a comprehensive mechanistic understanding of beneficial plant-microbe interactions and how they can be manipulated to improve crop productivity remains lacking. Such an understanding would enable the development and use of microbial inoculants that could eventually lead to a more productive and sustainable agricultural system.

Intensifying crop productivity while reducing fertilization is an important challenge, as losses of N due to leaching, run-off, and microbial assimilation can cause a variety of environmental problems, such as loss of biodiversity and increased production of greenhouse gases (Soares et al., 2016). However, little is known about the influence of bacterial inoculants on the assembly of plant rhizosphere and root endosphere prokaryotic communities. Here, we present an initial step towards understanding the effects of beneficial bacterial inoculation on the assembly of prokaryotic communities around and in the roots of sugarcane plants and, in turn, plant development.

In this study, six endophytic bacterial strains with proven plant growth-promotion capabilities isolated from sugarcane (da Silveira et al., 2018; Schlemper et al., 2018; Kuramae et al., 2020) were used to assess the effects of inoculation on the sugarcane rhizosphere and root endosphere prokaryotic communities. To evaluate the impact of N fertilization on community assembly, two N fertilization regimes were used. Prokaryotic community dynamics in both plant compartments were followed over time via 16S rRNA gene sequencing. Potential relationships between the changes in the rhizosphere and root endosphere prokaryotic communities and improved plant phenotypes were assessed by identifying prokaryotic genera associated with important plant physiological endpoints: biomass production; shoot nutrient concentrations, accumulation, and use efficiency; and activities of nitrate and nitrite reductase enzymes.

2. Materials and methods

2.1. Experimental design

Single bud sugarcane mini-stalks, from the variety IAC 95-5000, were obtained from plants at the same stage of development at harvesting. The mini-stalks were dry heat (52 °C – 30 min) disinfected to prevent disease occurrence. Disinfected stalks were placed in plastic pots (200 mL) containing autoclaved (121 °C for 1 h) commercial substrate (Tropstrato HA® Hortaliças) and transferred to a greenhouse with 60% shading with 12 h day/night ratio and 73% relative humidity (Supplementary Fig. S1). The experiment was setup during the months of November and December with a 16 °C and 34 °C minimum and maximum temperatures, respectively. Water supply was provided with distillated water once every 48 h. The substrate is a mixture of pine tree material and peat soil, treated at high temperature for elimination of plant pathogens. The mini-stalks were inoculated with a single bacterial strain (1st inoculation) and three weeks after the first inoculation, sugarcane plants were transferred to new pots containing 3 L of non-sterile soil with previously determined physico-chemical properties (Supplementary Table S1). The nutrients input calculations were based on fertility analysis of the soil and fertilization guidelines for sugarcane in Brazil (Supplementary Table S2). Nitrogen application was divided in two, 1/3 of N applied at transplanting and 2/3 after 30 days of transplanting. A second inoculation, with the same bacterial strains of first inoculation, was performed after plants were transferred to the pots containing soil. Sampling was performed at the moment of transplanting (T0), thirty (T30) and forty-five (T45) days after transplanting. The experimental design consisted of six bacterial strains plus a control (no inoculation) and two N fertilization (with and without N addition) with six replicates per time point.

2.2. Bacterial strains and inoculation procedure

Six bacterial strains previously isolated from sugarcane stalks (Cipriano et al., 2021) were selected based on their plant growth promotion traits: capability to solubilize phosphate, produce indole-3-acetic acid (IAA), siderophores and Hydrogen Cyanide (HCN), and amplification of *nifH* gene by PCR (Schlemper et al., 2018). The strains belong to Soil Microbiology Department at the Centre of Soil and Environmental Resources of the IAC (Campinas, Brazil) culture collection. Bacterial strains were grown in DYGS medium for 72 h, centrifuged for 10 min at 2000 $\times g$ and suspended in sterile distilled water. Bacterial suspension was diluted with sterile distilled water previously being used for inoculation and inoculum concentration was adjusted using optical density. In the first inoculation, 2 mL of bacterial suspension were applied on the top of the single bud sugarcane mini-stalk, whereas for the second inoculation 10 mL were applied directly to the base of the plant stem. For both inoculations, inoculum concentration was 10^8 bacterial cells/mL. Equivalent amounts of distilled sterile water were applied to the control treatment.

2.3. Evaluation of plant growth-promotion and plant nutritional status

At harvest, at T0, T30 and T45, shoots and roots were separated. Shoot and root dry weight were evaluated after air-drying at 60 °C to constant weight. Shoot macro and micronutrients of plants collected at T30 and T45 were determined according to Bataglia et al. (1983), nutrient content and nutrient-use efficiency index (UEI) according to Siddiqi and Glass (1981).

2.4. Enzyme extraction and enzyme activities determination

At T45, the second true leaf, according to the system of Kuijper (Casagrande, 1991), were collected and immediately frozen in liquid nitrogen and stored at −80 °C till enzyme activity determination. The activity of the enzymes nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) was performed, with some modifications, as described by Silveira et al. (2010).

2.5. Rhizosphere and root endosphere sampling

On every sampling date, we randomly selected a subset of three pots containing a sugarcane plant for each treatment and overturned them on a clean surface. Carefully, the plant was recovered and loose soil was aseptically removed from the root system by shaking and crumbling; leaving only a thin layer of soil (around 1 mm) attached to the root system (rhizosphere soil). Above ground plant parts were aseptically removed and the root system was placed inside a sterile 50 mL tube, which was placed on ice and transported to the laboratory. Upon arrival 30 mL of sterile sodium phosphate buffer containing Tween 20 (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0, 0.5% Tween 20) was added to the tubes containing the roots. Tubes were vortexed at maximum speed for 20 s, in order to release the rhizosphere soil

from the root system. To enhance rhizosphere soil recovery, roots were transferred aseptically to a new (sterile) 50 mL tube and another 20 mL of sodium phosphate buffer containing Tween 20 was added. Tubes were again vortexed at maximum speed for 20 s. Buffer containing rhizosphere soil from the two washing steps were combined into a single 50 mL tube, and centrifuged at 2000 ×*g*, for 10 min at 4 °C. After centrifugation, supernatant was discarded and tubes containing rhizosphere soil were stored at −80 °C. In order to sample the sugarcane root endosphere, after the second wash of the root system, roots were rinsed with an excess of tap water and placed in a new (sterile) 50 mL tube. Root segments were surface sterilized by sequential washing in 70% ethanol (v/v) for 1 min, sodium hypochlorite (2%, v/v – supplemented with 0.02% Tween-20) for 3 min, 70% ethanol (v/v) for 30 s and 3 successive rinses with sterilized distilled water. At the end of the sterilization procedure roots were transferred to a new (sterile) 50 mL tube and stored at −80 °C. Water from the last root rinse was kept and a 100 μL was inoculated in 10 mL of DYGS medium, in order to determine whether the sterilization procedure was successful. Success of the sterilization was evaluated by observing no microbial growth in the medium after 72 h. The microbes present in the root fraction were used as a proxy of root endosphere community.

2.6. DNA extraction and quantification

Total DNA was extracted from the rhizosphere soil as well as from sugarcane roots using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). Extractions were performed according manufacturer's instructions. Prior extractions, sugarcane roots were grinded with liquid nitrogen. Total DNA quantity and quality were measured using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA) and visualized on 1% (w/v) agarose gel under UV light after staining with Sybr® Safe DNA gel Stain (Life Technologies).

2.7. Rhizosphere and root endosphere prokaryotic community

The rhizosphere and root endosphere archaeal and bacterial communities were investigated by partial sequencing of the 16S rRNA gene (V3–V4 regions). Amplicons were generated by Polymerase Chain Reaction (PCR) containing 12 μL of 5× Phusion HF buffer, 200 μM of dNTPs, 0.4 μM of each primer, 1.2 U of Phusion Hot Start II DNA polymerase (Thermo Scientific, Wilmington, DE, USA), 0.3 μL of Bovine Serum Albumin (20 mg/mL) (Thermo Scientific) and 7 μL of template DNA (5 ng/μL). PCR reactions were performed in a total volume of 60 μL. Primer sequences and cycle conditions are shown in Table S1. Confirmation of amplification was determined by performing electrophoresis of PCR products on a 1.5% (w/v) agarose gel with TBE buffer. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific), and quantified using a Qubit 2.0 Fluorometer (Life Technologies). Purified and quantified PCR products were then mixed in equimolar amounts, which was then purified using the Agencourt AMPure XP beads (Beckman Coulter Inc., Atlanta, GA). Purified amplicons were sequenced using Illumina MiSeq (Illumina, San Diego, CA, USA) at the Argonne National Laboratory (Argonne, IL, USA). The raw sequence datasets were deposited in the NCBI SRA database under accession number ERP128043.

2.8. DNA extraction and shotgun sequencing of PGPB isolates

The six PGPB isolates were chosen for DNA extraction. The genomic DNA of each isolate was sequenced on a single PacBio P6C4 SMRT Cell (University of Maryland, Baltimore, Maryland, USA), which was assembled following the procedures in Kuramae et al. (2020) using Prokka v1.11 (Seemann, 2014) and annotated using the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al., 2008) and the EggNOG-mapper v1.0.3 using the EggNOG database v4.5.1 (Huerta-Cepas et al., 2017). Circular genome maps were drawn used CGView software (Stothard and Wishart, 2005).

2.9. Data analysis

Sequencing analyses were performed using a Snakemake workflow (Koster and Rahmann, 2012). The RDP extension to PANDASeq (Masella et al., 2012) named Assembler (Cole et al., 2014) was used to merge paired-end reads with a minimum overlap of 40 bp and at least a PHRED score of 25. Primer sequences were removed using Flexbar version 2.5 (Dodt et al., 2012). Reads were then clustered into Operational Taxonomic Units (OTUs), at 97% similarity, using VSEARCH version 1.0.16 (Rognes and Mahé, 2015) and the UCLUST smallmem algorithm (Edgar, 2010). Next, chimeric sequences were detected and discarded using the UCHIME algorithm (Edgar et al., 2011) implemented in VSEARCH. Finally, an OTU table was created and taxonomic information for each OTU added. Taxonomic information was determined using RDP Classifier version 2.10 (Cole et al., 2014). The resulting OTU table was filtered to remove: OTUs classified as Eukaryote (chloroplast and mitochondria), OTUs that were not assigned to either Bacterial or Archaeal domains, singletons and samples containing less than 1000 reads.

Further statistical analyses were performed in R v4.0.2 (R Core Team, 2020) using phyloseq (McMurdie and Holmes, 2013), ade4 (Dray and Dufour, 2007), *metamicrobiomeR* (Ho et al., 2019) R packages. Initially, OTUs were merged at Genus level and transformed by CLR (Gloor et al., 2017). Between-class analysis (BCA) was performed to explore the dissimilarity between treatments over time. BCA allows assessing the amount of variability that can be explained by the applied treatment. Principal Component Analysis (PCA) was applied to each transformed dataset prior to the BCA (Kenkel, 2006) with Monte-Carlo 999 permutations to determine the significance of groups. In order to determine which specific bacterial treatments had a significant effect on the rhizosphere and root endosphere prokaryotic community, generalized additive model for location, scale, and shape (GAMLSS) was used in combination with a zero-inflated beta (BEZI) family. This approach allows to investigate the shifts in relative abundance of the microbiome while account for the random effects of different sampling time. P-values where adjusted using FDR, as implemented in the *metamicrobiomeR* package. The influence of the PGPB traits on the prokaryotic community was evaluated by co-inertia analysis (Dray et al., 2003). We also used the co-inertia analysis to evaluate how the changes in the rhizosphere and root endosphere microbiome are associated with the changes in the biomass, mineral nutrition and nutrient use efficiency of sugarcane. For all statistical analyses, statistical significance was accepted when $P < 0.05$, for the co-inertia analysis we also considered tendency when $P < 0.10$.

3. Results

3.1. Prokaryotic community structure in the rhizosphere and root endosphere of sugarcane

To assess the prokaryotic community in sugarcane, rhizosphere and root endosphere samples from the control (non-inoculated) treatment were analyzed. Communities specific to each compartment were identified by comparing the sugarcane rhizosphere and root endosphere (Supplementary Fig. S2). The centered log-ratio (CLR)-transformed data permitted the visualization of the proportion of prokaryotic genera of greater than average abundance in the control treatment. In general, a smaller proportion of the prokaryotic genera occurred with high abundance in the root endosphere (220) compared with the rhizosphere (264). In addition, the abundances of the genera specific to the root endosphere were approximately 6 log-fold higher than the average abundance in the sample (Supplementary Fig. S2A). Proteobacteria was the phylum with the highest number of bacteria genera dominating both the rhizosphere (109) and root endosphere (94) in the control treatment, followed by Actinobacteria (rhizosphere = 68; root

endosphere = 59), and Bacteroides (rhizosphere = 20; root endosphere = 17). Beyond these top 3 phyla, divergence was observed between the two compartments: the phylum with the fourth highest number of high-abundance genera was Acidobacteria (18) in the rhizosphere but Bacteroidetes (17) in the root endosphere; in fifth place was Chloroflexi (11) in the rhizosphere and Firmicutes (12) in the root endosphere (Supplementary Fig. S2A).

We also evaluated differences in the abundance between the two compartments for each prokaryotic genus (Supplementary Fig. S2B); 390 genera were more abundant in the root endosphere, whereas 263 genera were more abundant in the rhizosphere. However, a large proportion (63.3%) of the genera that were more abundant in the root endosphere had low abundance overall. Thus, the selection of highly abundant microbes was more specific in the root endosphere than in the rhizosphere.

Similarly, we investigated the influence of N application on the control sugarcane microbiome (Fig. 1, Supplementary Fig. S3). Compared with the control treatment without N application, N input increased the abundance of 21 different prokaryotic genera (13 Actinobacteria, 7 Proteobacteria, and 1 Firmicutes) and decreased the abundance of one genus of Bacteroidetes (*Mucilaginibacter*) (Fig. 1A). In the root endosphere, N input increased the relative abundance of 16 prokaryotic genera (9 Proteobacteria, 4 Actinobacteria, 2 Bacteroidetes, and 1 Chloroflexi) (Fig. 1B). This analysis also allowed us to evaluate community dynamics along the different time points (Fig. 1C–D). Once again, the selection of highly abundant microbes was more specific in the root endosphere than in the rhizosphere.

3.2. Treatment effects on sugarcane growth and mineral nutrition

We evaluated the effects of the factorial combination of inoculation and fertilization at each time point (T30 and T45). Growth promotion was evaluated by dry matter mass. We observed significant effects of bacterial isolates and N fertilization (Supplementary Tables S3–S7), and all bacterial treatments promoted plant growth.

3.2.1. Growth

At T0 (15 days after inoculation, in organic substrate), the inoculated seedlings exhibited higher production of dry matter from the aerial part than the control plants (plant that were not inoculated) (Supplementary Table S3). There was no difference in root dry matter production. Thirty days after transplanting into the soil (T30), the growth-promoting effects of the isolates on aboveground dry matter persisted, and inoculation with strain IAC/BECa 88 promoted greater root growth (Supplementary Table S3). At T45, growth promotion was evident throughout the plant. Nitrogen fertilization increased the production of dry matter from the roots of the plants and influenced the effects of bacterial inoculation on the concentration and accumulation (Supplementary Tables S4–S7) of most macro and micronutrients.

3.2.2. Mineral nutrition

At T30, N input reduced N and P content in shoot biomass by 12.8% and 13.9%, respectively, in plants inoculated with IAC/BECa 90 (Supplementary Table S4). Reductions of P and K content of 16.9% and 12.3%, respectively, were observed in plants inoculated with IAC/BECa 88 under N input. Plants inoculated with IAC/BECa 95 exhibited 41.1% less Fe and 19.7% less Cu under N input. Plants inoculated with IAC/BECa 141 contained 7.34% less N, 15.8% less P, and 15.7% less K under N input. By contrast, in the control treatment (non-inoculated), N input increased P and K content in shoot biomass by 8.6% and 17.7%, respectively. In addition, under N input, plants inoculated with IAC/BECa 95 had 11.0% higher N content, IAC/BECa 88 had 13.6% higher Cu content, and IAC/BECa 135 had 21.9% higher P content.

Compared with the control with no additional N input, the effects of N input at T30 varied depending on the inoculated strain: plants that received N input accumulated 26.7% less P when inoculated with IAC/BECa 90, 38.3% less Fe when inoculated with IAC/BECa 95, and 39.5% more P when inoculated with IAC/BECa 135.

At T45, additional N input continued to impact the effect of PGPB inoculation (Supplementary Table S6). Under N input, plants inoculated with IAC/BECa 90 showed 19.3% lower N content and 30.0% lower Cu content, and plants inoculated with IAC/BECa 135 exhibited 14.5% lower N content and 33.4% lower B content. For plants inoculated with IAC/BECa 88, IAC/BECa 95, or IAC/BECa 152, N input affected only the concentrations of micronutrients. Under N input, plants inoculated with IAC/BECa 88 contained 11.8% less Cu and 178.2% less Zn, plants inoculated with IAC/BECa 95 contained 26.5% less Zn, and plants inoculated with IAC/BECa 152 contained 17.9% less Fe, 26.4% less Cu, and 33.8% less B.

By contrast, compared with the control, plants inoculated with IAC/BECa 95 presented 13.5% higher N content under N input, whereas plants inoculated with IAC/BECa 90 exhibited 38.4% higher Fe content under N input.

When we assessed accumulated nutrients at T45, we found that additional N input resulted in increased accumulation of both macro- and micronutrients in shoot biomass, depending on the inoculated strain. N input combined with IAC/BECa 90 resulted in 29.6% more P and 33.9% more B; N input combined with IAC/BECa 95 resulted in 39.7% more P, 31.3% more N, and 39.2 more B; and N input combined with IAC/BECa 141 resulted in 28.9% more N, 24.7% more P, and 30.8% more Zn. Some accumulation of nutrients also occurred in the control treatment (no inoculation) under N input (39.8% increase in N and 20.0% increase in P accumulated in shoot biomass).

Despite the changes in nutrient concentration and accumulation, no significant effect of N input on the nutrient use efficiency of sugarcane plants (Supplementary Tables S4–S7) or the in vitro activity of nitrate reductase and nitrite reductase in sugarcane leaves (Supplementary Table S8) was observed.

3.3. Treatment effects on the prokaryotic community structure of sugarcane

The BCA approach allowed us to identify the proportion of microbial variability related to different factors: (i) plant compartment, (ii) inoculation, (iii) nitrogen input, and (iv) time (Table 1). The proportion of variance explained by each one of these factors suggested a strong influence of time (31.4%), followed by plant compartment (17.97%), N input (5.5%), and lastly inoculation (4.61%). The latter was significant only if we accounted for the different factors within inoculation (N input, plant compartment, and time). The combination of all four factors explained 73.38% of the total variability in sugarcane prokaryotic community.

To investigate the effects of inoculation, we next examined the different plant compartments and the different treatments with N input.

3.4. Treatment effects on prokaryotic community structure in the sugarcane rhizosphere

Inoculation with the different PGPB strains shifted the rhizosphere microbiome, but N input reduced these effects (Fig. 2A–B). Without N input, the relative abundances of 172 prokaryotic genera changed, whereas under N input, this number decreased to 72. Without N input, the treatments formed two distinct clusters based on similarity of the prokaryotic rhizosphere: one cluster comprising strains IAC/BECa 141, IAC/BECa 135, and IAC/BECa 152 and one cluster comprising strains IAC/BECa 90, IAC/BECa 95, and IAC/BECa 88 (Fig. 2A). In the first cluster, *Duganella*, *Rhodovarius*, *Caulobacter*, *Prosthecomicrobium*, and one unclassified *Micrococcaceae* were the top five genera that increased in abundance (Table 2). In the second cluster, the top five genera that increased in abundance were *Caulobacter*, *Rhodovarius*, *Fimbriimonas*, one unclassified *Micrococcaceae*, and one unclassified *Sphingobacteriaceae* (Table 2).

PCA analysis showed how N addition moderated the impact of PGPB inoculation in the sugarcane rhizosphere. The center of the PCA plot

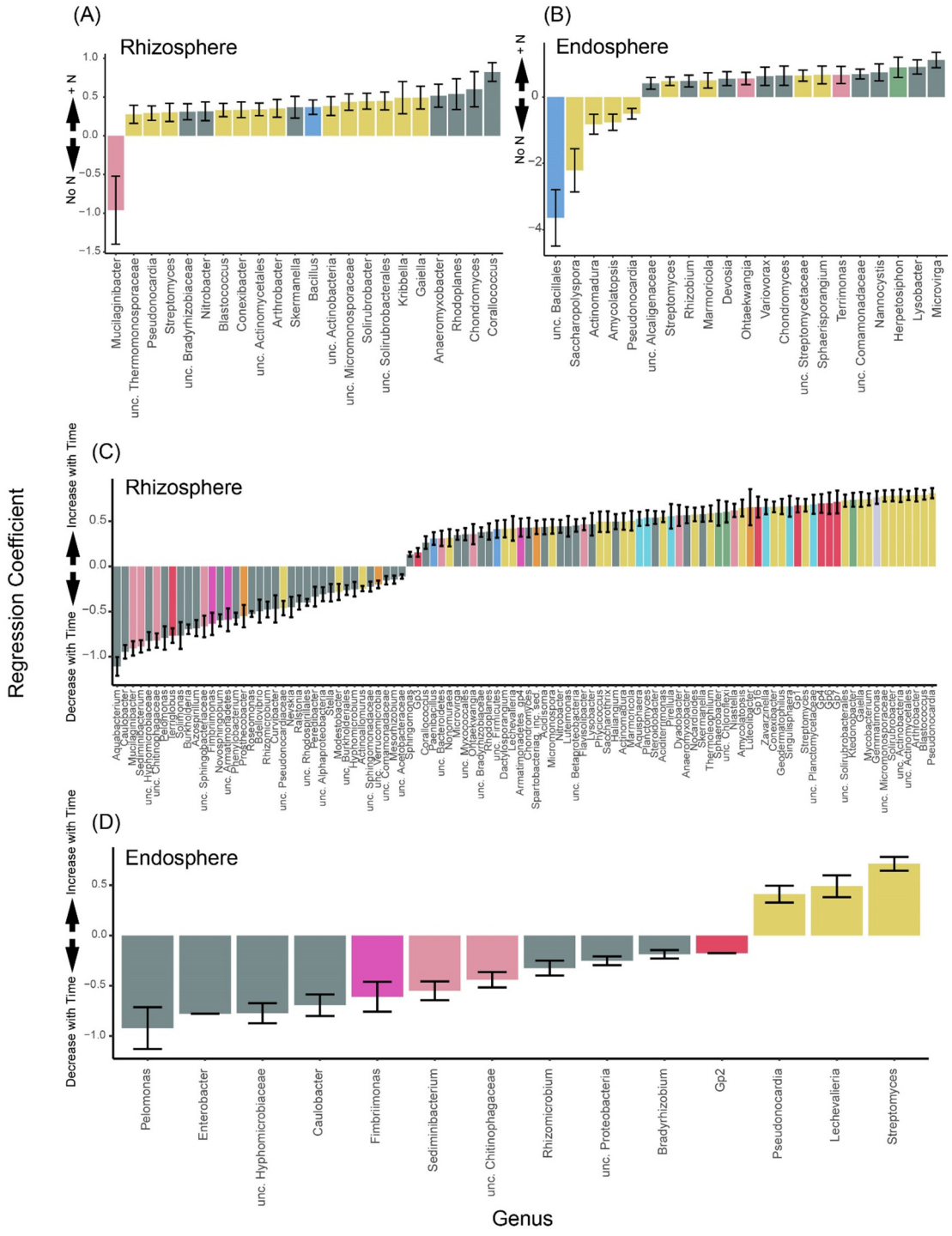

Fig. 1. Significant changes (regression coefficients) in sugarcane prokaryotic community profile according to effect of N input (A–B) and time (C–D) for both rhizosphere and endosphere.

Table 1
Proportion of variance attribute to each treatment variable according to between-class analysis (BCA).

Factor	Inertia (%)	P-value
Plant compartment	17.97	0.001
N input	5.50	0.001
Inoculation	4.61	0.130
Inoculation+N_{input}	11.86	0.002
Inoculation+ N_{input} + Plant compartment	30.92	0.001
Time	31.40	0.001
Inoculation+N_{input} + Plant compartment+Time	73.38	0.001

represents the control treatment (reference level), and any departure from the origin indicates larger differences from the control treatment without inoculation. Fig. 2C shows that IAC/BECa 135 and IAC/BECa 88 fostered distinct microbial community that became nearly identical under the additional N-input. Finally, Fig. 2E–F show the proportions of each of the bacteria genus in the sample, which indicates whether inoculation mainly affected the high-abundant or the low-abundant microbes. The majority of microbes affected by inoculation belonged to abundant groups (a value of zero on the CLR axis corresponds to the average abundance on log scale; any value above zero indicates that the prokaryote is more abundant than average in the sample). However,

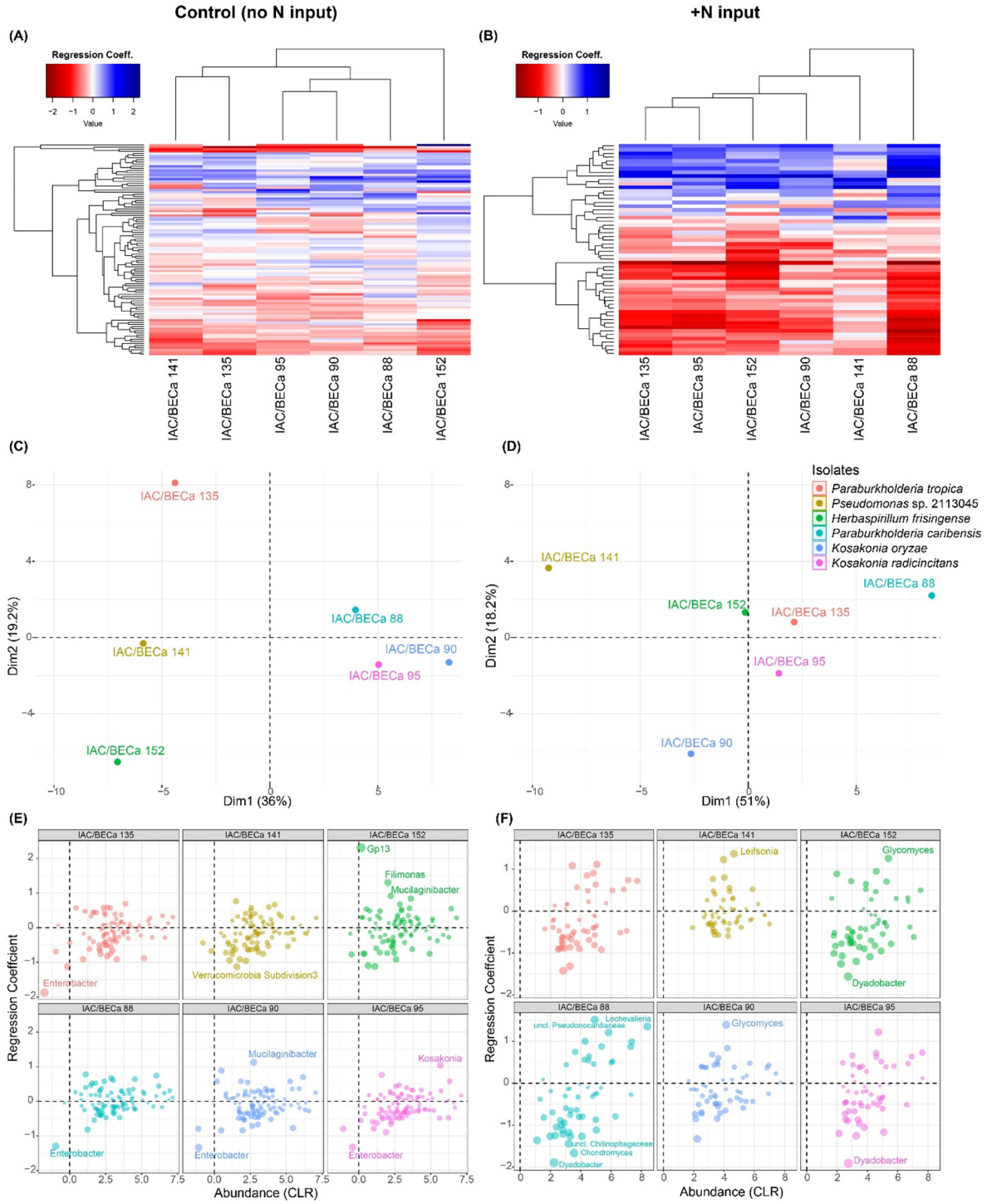

Fig. 2. Impact of PGPB inoculation on the sugarcane rhizosphere with and without N input. (A–B) Heatmaps of the regression coefficients showing the significant shifts (positive in blue and negative in red); (C–D) PCA of the overall variability of the regression coefficient as induced by each inoculum; (E–F) relationship between the regression coefficient and abundance (CLR – centered log-ratio transformed data).

under N-input the high abundant microbes became even more dominant in the rhizosphere community.

3.5. Treatment effects on prokaryotic community structure in the root endosphere of sugarcane

Similar to the effects observed in the rhizosphere, PGPB impacted the root endosphere microbiome, and N input reduced this impact (Fig. 3A–B). Without N input, changes in the relative abundance of 99 prokaryotic genera were observed, whereas when N was added, this number decreased to 56. Without N input, strains IAC/BECa 90, 95, and 88 formed a cluster distinct from the cluster comprising strains IAC/BECa 141 and 135, whereas strain IAC/BECa 152 had the most distinct microbiome profile (Fig. 3A). The addition of N to the soil completely shuffled the clusters and resulted in smaller differences between the microbiomes. The microbial community in the treatment with strain IAC/BECa 135 was much more closely related to that of the treatment with strain IAC/BECa 95 than without N input. In addition,

Table 2
Top five bacteria (genus level) that changed in abundance in the sugarcane rhizosphere in response to inoculation with different IAC/BECa strains without and with N input.

Change	IAC/BECa 88 (*Paraburkholderia caribensis*)	IAC/BECa 90 (*Kosakonia oryzae*)	IAC/BECa 95 (*Kosakonia radicincitans*)	IAC/BECa 135 (*Paraburkholderia tropica*)	IAC/BECa 141 (*Pseudomonas* sp.)	IAC/BECa 152 (*Herbaspirillum frisingense*)
Without N input						
Positive	*Burkholderia*	*Caulobacter*	*Glycomyces*	*Caulobacter*	*Duganella*	*Caulobacter*
	Prosthecobacter	*Rhodovarius*	*Caulobacter*	*Luteolibacter*	*Rhodovarius*	*Herbaspirillum*
	Lechevalieria	*Fimbriimonas*	*Lechevalieria*	*Rhodovarius*	*Caulobacter*	*Rhodovarius*
	Actinophytocola	Unc. *Micrococcaceae*	*Sphingobium*	*Prosthecomicrobium*	*Prosthecomicrobium*	*Mucilaginibacter*
	Catenulispora	Unc. *Sphingobacteriaceae*	*Prosthecomicrobium*	*Duganella*	Unc. *Micrococcaceae*	*Pedobacter*
Negative	*Sediminibacterium*	Unc. *Armatimonadetes*	*Dyadobacter*	*Roseateles*	*Aquabacterium*	*Rhizomicrobium*
	Kosakonia	*Planctomyces*	*Actinoallomurus*	*Ralstonia*	*Actinoallomurus*	*Actinoallomurus*
	Solimonas	*Nevskia*	*Pseudoxanthomonas*	*Saccharibacteria* gen. Inc. sed.	*Solimonas*	*Curvibacter*
	Hyphomicrobium	*Chryseobacterium*	*Sediminibacterium*	*Nitrososphaera*	*Nevskia*	*Sediminibacterium*
	Methanomassiliicoccus	*Curvibacter*	*Dongia*	*Bacillus*	*Acidobacteria* Gp1	Unc. *Rhodospirillales*
With N input						
Positive	*Glycomyces*	*Glycomyces*	*Glycomyces*	*Glycomyces*	*Leifsonia*	*Sphingobium*
	Lechevalieria	*Sphingobium*	*Sphingobium*	*Leifsonia*	*Sphingobium*	*Glycomyces*
	Sphingobium	*Leifsonia*	*Lechevalieria*	*Catenulispora*	*Dyella*	*Leifsonia*
	Saccharothrix	*Catenulispora*	*Novosphingobium*	*Lechevalieria*	*Novosphingobium*	*Novosphingobium*
	Catenulispora	*Lechevalieria*	*Mycobacterium*	*Burkholderia*	*Glycomyces*	*Massilia*
Negative	*Sorangium*	Unc. *Thermoplasmata*	Unc. *Thermoplasmata*	Unc. *Thermoplasmata*	*Nitrososphaera*	Unc. *Thermoplasmata*
	Dyadobacter	*Methanomassiliicoccus*	*Methanomassiliicoccus*	*Methanomassiliicoccus*	Unc. *Thermoplasmata*	*Methanomassiliicoccus*
	Terrimonas	*Nitrososphaera*	*Dyadobacter*	*Nitrososphaera*	*Methanomassiliicoccus*	*Nitrososphaera*
	Methanomassiliicoccus	*Sorangium*	*Nitrososphaera*	*Dyadobacter*	*Corallococcus*	*Corallococcus*
	Unc. *Thermoplasmata*	*Terrimonas;*	*Terrimonas*	Unc. Alphaproteobacteria	*Anaeromyxobacter*	*Sphaerobacter*

under N input, the treatment with IAC/BECa 152 clustered with IAC/BECa 135 and IAC/BECa 95, indicating a strong loss of selection power due to N addition. The treatment with strain IAC/BECa 90 was also grouped in the same cluster, leaving the treatment with strain IAC/BECa 141 as the outlier in the dendrogram (Fig. 3B) and the most distinct community.

PCA analysis confirmed the impact of N addition on the community selected by the inoculum. Fig. 3C shows that IAC/BECa 88, IAC/BECa 90, and IAC/BECa 95 produced nearly identical impacts on the rhizosphere, likely due to their genetic similarity, especially between IAC/BECa 90 (*Kosakonia oryzae*) and IAC/BECa 95 (*Kosakonia radicincitans*). However, when N was added to the soil, the overall departure from the control decreased, with greater concentration on the first axis (Fig. 3D). Finally, Fig. 3E–F show the proportions of each of the bacterial genera in the sample, which indicates whether inoculation mainly affected the more abundant microbes. The majority of microbes affected by inoculation belonged to abundant groups.

In the root endosphere, we found that without additional N fertilization, IAC/BECa 95 (*Kosakonia radicincitans*) increased the abundance of the *Kosakonia* genus (strongest increase in abundance) (Table 3) and IAC/BECa 152 (*Herbaspirillum frisingense*) increased the abundance of the *Herbaspirillum* genus (fourth strongest increase in relative abundance). However, under N input, those genera lost importance and dropped below the top five strongest shifts (Table 3). For IAC/BECa 95, *Kosakonia* dropped to fourth place, with *Glycomyces* occupying first place. *Glycomyces* also became more relevant in the root endosphere when sugarcane was inoculated with IAC/BECa 152, and *Herbaspirillum* disappeared from the top five (Table 3). In addition, the genus *Leifsonia* was strongly increased in the root endosphere of sugarcane when N was added in combination with IAC/BECa 90, IAC/BECa 135, or IAC/BECa 152.

3.6. Influence of genome-based growth-promotion traits of the six strains on the prokaryotic community structure of sugarcane

The genomes of strains IAC/BECa 88, IAC/BECa 90, IAC/BECa 95 and IAC/BECa 141 were sequenced, assembled, and annotated (Supplementary Table S9). The genomes of IAC/BECa 135 (*Paraburkholderia tropica*) and IAC/BECa 152 (*Herbaspirillum frisingense*) were sequenced and annotated previously (Kuramae et al., 2020). Supplementary Fig. S3 depicts the assembly for each one of the six PGPB strains. IAC/BECa 88 (*Paraburkholderia caribensis*) assembly resulted in one chromosome and two chromids with lengths between 2.01 and 3.68 Mb. IAC/BECa 135 (*P. tropica*) assembly resulted in one chromosome and four chromids, and assembly for IAC/BECa 90 (*Kosakonia oryzae*), IAC/BECa 95 (*Kosakonia radicincitans*), IAC/BECa 141 (*Pseudomonas* sp.), and IAC/BECa 152 (*Herbaspirillum frisingense*) resulted in one chromosome. We quantified the number of genes in each strain's genome corresponding to functions related to plant growth promotion to identify functional similarities in their potential to promote plant growth (Fig. 4). Only strains IAC/BECa 90 (*Kosakonia oryzae*) and IAC/BECa 95 (*Kosakonia radicincitans*) presented genes for nitrogen fixation. Strains IAC/BECa 88 (*Paraburkholderia caribensis*) and IAC/BECa 135 (*Paraburkholderia tropica*) presented the highest number of genes related to indole acetic acid (IAA) production, but all strains contained genes to perform that task. All strain genomes also possessed genes for phosphate solubilization and regulation.

Strong co-dependence (Table 4) of the changes in the rhizosphere and the growth-promotion traits of each genome strain were observed with ($Rv = 0.66$, $P = 0.049$) and without ($Rv = 0.83$, $P = 0.023$) nutrient input, but no significant co-dependence for the root endosphere microbiome ($Rv = 0.54$; $P = 0.35$; $Rv = 0.55$; $P = 0.14$). In addition, we found that the shifts in prokaryotes induced by inoculation were associated with changes in mineral nutrition, nutrient use efficiency, and enzyme activity in sugarcane leaves (Table 4). However, we only identified a significant effect of the control treatment (without N input), in which the changes in the rhizosphere had significant co-dependence with nutrient accumulation after 30 days ($Nutr_{acc}$ T30) and sugarcane biomass after 45 days (Biom T45). We also observed a significant association between the changes in the root endosphere and sugarcane biomass after 30 days of inoculation. Interestingly, for the treatment with additional N input, only the association between the rhizosphere and root endosphere was significant.

4. Discussion

We explored the holobiont perspective to evaluate the impacts of both PGPB strains and N input on sugarcane over time. We demonstrated that N input reduced the impact of PGPB inoculation on plant growth and the soil microbial community. However, our study goes beyond merely identifying shifts in the soil microbiome as a result of PGPB inoculation. We also evaluated the functional profiles of these microbes, namely the

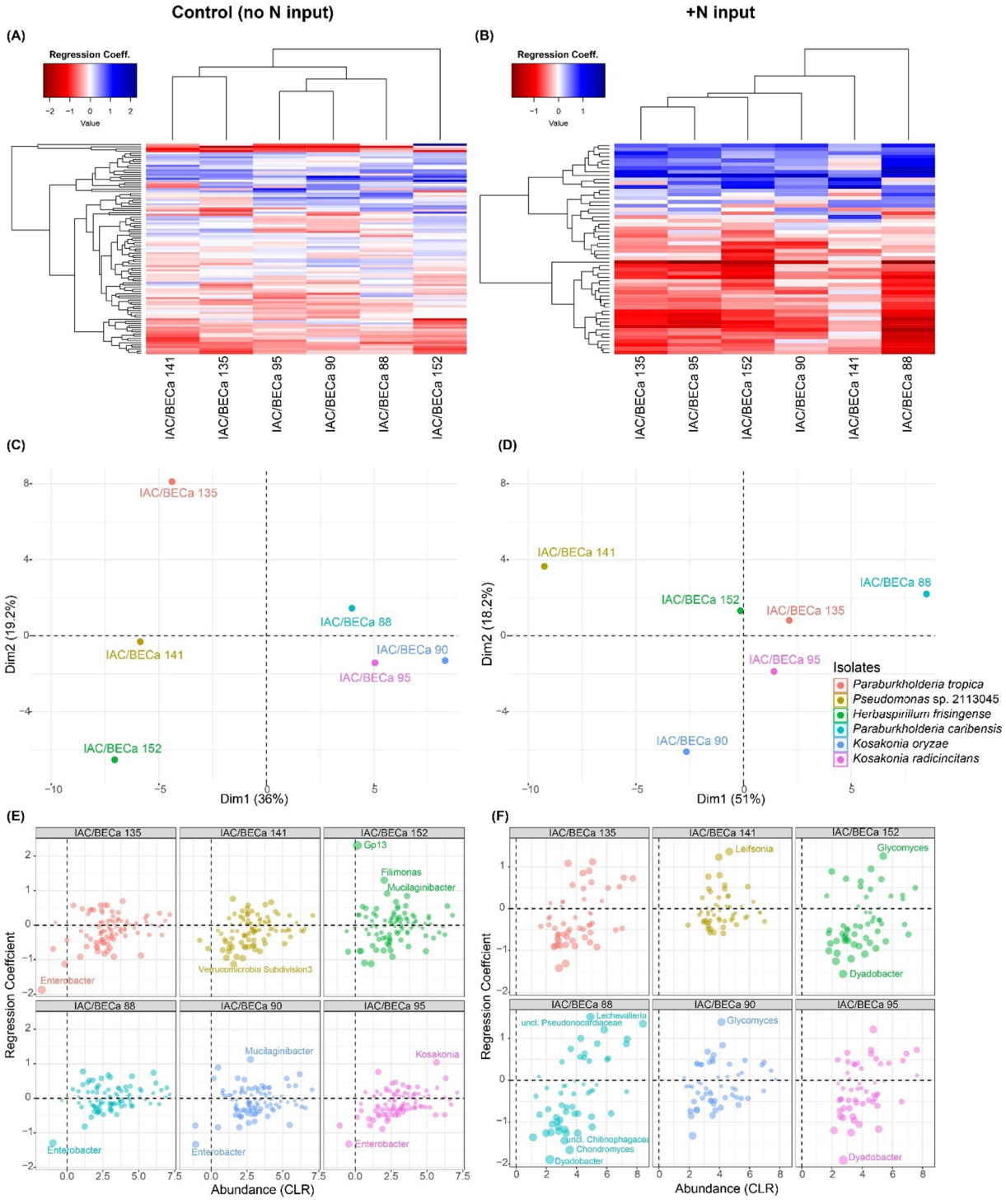

Fig. 3. Impact of PGPB inoculation on the sugarcane root endosphere with and without N input. (A–B) Heatmaps of the regression coefficients showing the significant shifts (positive in blue and negative in red); (C–D) PCA of the overall variability of the regression coefficient as induced by each inoculum; (E–F) relationship between the regression coefficient and abundance (centered log-ratio - CLR transformed data).

numbers of genes corresponding to functions related to growth promotion that could influence the soil microbiome. These analyses represent a general attempt to find microbes that will promote plant growth. Selecting microbes based solely on their impact on plant traits runs the risk of interfering with the soil microbiome and attracting other potentially harmful microbes. Brazil is the largest producer of sugarcane (CONAB, 2019) but has relatively low use of nitrogen fertilizer (40 and 80 kg ha^{-1} for plant cane and second ratoon cane, respectively) (Antunes et al., 2019) compared with countries such as India and the United States (150 and 200 kg ha^{-1} of N). Therefore, it is important not only to evaluate the gains in plant growth and plant production due to fertilization but also to account for the impact on the sugarcane microbiome (rhizosphere and root endosphere).

We found that PGPB strains IAC/BECa 90, IAC/BECa 135, IAC/BECa 141, and IAC/BECa 152 increased the abundance of bacteria of the genus *Leifsonia*. Although some species of *Leifsonia* could act as plant-growth promoter (Kang et al., 2014), this bacteria genus

Table 3
Top five bacteria (genus level) that changed in abundance in the sugarcane root endosphere in response to inoculation with different IAC/BECa strains without and with N input.

Shift	IAC/BECa 88 (*Paraburkholderia caribensis*)	IAC/BECa 90 (*Kosakonia oryzae*)	IAC/BECa 95 (*Kosakonia radicincitans*)	IAC/BECa 135 (*Paraburkholderia tropica*)	IAC/BECa 141 (*Pseudomonas* sp.)	IAC/BECa 152 (*Herbaspirillum frisingense*)
Without N input						
Positive	*Duganella*	*Mucilaginibacter*	*Kosakonia*	*Microvirga*	*Duganella*	*Acidobacteria* Gp13
	Unc. *Proteobacteria*	*Duganella*	*Pleomorphomonas*	Unc. *Streptomycetaceae*	*Nocardioides*	*Filimonas*
	Pelomonas	*Lechevalieria*	*Lechevalieria*	*Dokdonella*	*Microvirga*	*Mucilaginibacter*
	Microvirga	*Fimbriimonas*	*Mucilaginibacter*	*Rhodovarius*	Unc. *Streptomycetaceae*	*Herbaspirillum*
	Lysobacter	*Rhodovarius*	*Novosphingobium*	*Mucilaginibacter*	*Lysobacter*	Unc. *Streptomycetaceae*
Negative	*Enterobacter*	*Enterobacter*	*Enterobacter*	*Enterobacter*	*Verrucomicrobia* Subdivision3	*Rhizomicrobium*
	Unc. *Rhodospirillales*	Unc. *Armatimonadetes*	*Verrucomicrobia* Subdivision3	Unc. *Armatimonadetes*	*Sediminibacterium*	Unc. *Armatimonadetes*
	Unc. *Microbacteriaceae*	*Verrucomicrobia* Subdivision3	*Acidobacteria Gp13*	*Actinomadura*	Unc. *Armatimonadetes*	*Pseudonocardia*
	Verrucomicrobia subdivision3	*Acidobacteria* Gp13	Unc. *Armatimonadetes*	Unc. *Cytophagaceae*	Unc. *Sphingobacteriaceae*	*Pleomorphomonas*
	Terriglobus	*Burkholderia*	*Bdellovibrio*	*Amycolatopsis*	Unc. *Rhodospirillales*	*Amycolatopsis*
With N input						
Positive	Unc. *Pseudonocardiaceae*	*Glycomyces*	*Glycomyces*	*Glycomyces*	*Leifsonia*	*Glycomyces*
	Lechevalieria	Unc. *Actinomycetales*	*Lechevalieria*	Unc. *Pseudonocardiaceae*	*Dokdonella*	*Leifsonia*
	Glycomyces	Unc. *Pseudonocardiaceae*	*Actinomadura*	*Mycobacterium*	*Chryseobacterium*	*Kosakonia*
	Actinosynnema	*Actinomadura*	*Kosakonia*	*Leifsonia*	*Kosakonia*	*Dokdonella*
	Saccharothrix	*Leifsonia*	*Amycolatopsis*	*Amycolatopsis*	*Mycobacterium*	Unc. *Actinomycetales*
Negative	*Dyadobacter*	*Dyadobacter*	*Dyadobacter*	*Dyadobacter*	Unc. *Comamonadaceae*	*Dyadobacter*
	Chondromyces	Unc. *Burkholderiales*	*Pseudolabrys*	*Pseudolabrys*	*Variovorax*	Unc. *Brucellaceae*
	Unc. *Chitinophagaceae*	*Chondromyces*	*Shinella*	*Devosia*	*Lysobacter*	*Variovorax*
	Unc. *Cryomorphaceae*	*Shinella*	*Devosia*	*Dongia*	*Acidobacteria* Gp6	Unc. *Comamonadaceae*
	Sorangium	*Azospirillum*	Unc. *Chitinophagaceae*	Unc. *Chitinophagaceae*	*Spartobacteria* gen. Inc. sed.	*Lysobacter*

might indicate the presence of *Leifsonia xyli* which might not be harmful (Kang et al., 2016) but brings the risk of ratoon stunting disease (Monteiro-Vitorello et al., 2004). While we did not detect any symptoms of plant disease in sugarcane during the experiment, successive cycles of inoculation with this strain could raise the risk for sugarcane cultivation. Moreover, the increase in *Leifsonia* abundance was stronger when the soil received N input (greater than in the control that did not receive inoculation). Therefore, the potential risk of ratoon stunting disease from combining PGPB strains with N input warrants closer attention.

Some of the PGPB strains also increased the abundance of the genus to which they belong, suggesting some capacity of these strains to remain in the soil during plant growth. In the rhizosphere, strain IAC/BECa 152 (*Herbaspirillum frisingense*) increased the abundance of the genus *Herbaspirillum* compared with the control, and IAC/BECa 88 (*Paraburkholderia caribensis*) increased the abundance of the genus *Burkholderia*, albeit only in the treatments without additional N input. *Paraburkholderia caribensis* is a synonym of *Burkholderia* and is difficult to distinguish by 16S rRNA gene amplicon sequencing (Kaur et al., 2017). In the root endosphere, without additional N input, inoculation with IAC/BECa 152 increased the abundance of the genus *Herbaspirillum*, while inoculation with IAC/BECa 95 (*Kosakonia radicincitans*) increased the abundance of the genus *Kosakonia*. However, when N was added, those genera lost importance and did not appear among the top five

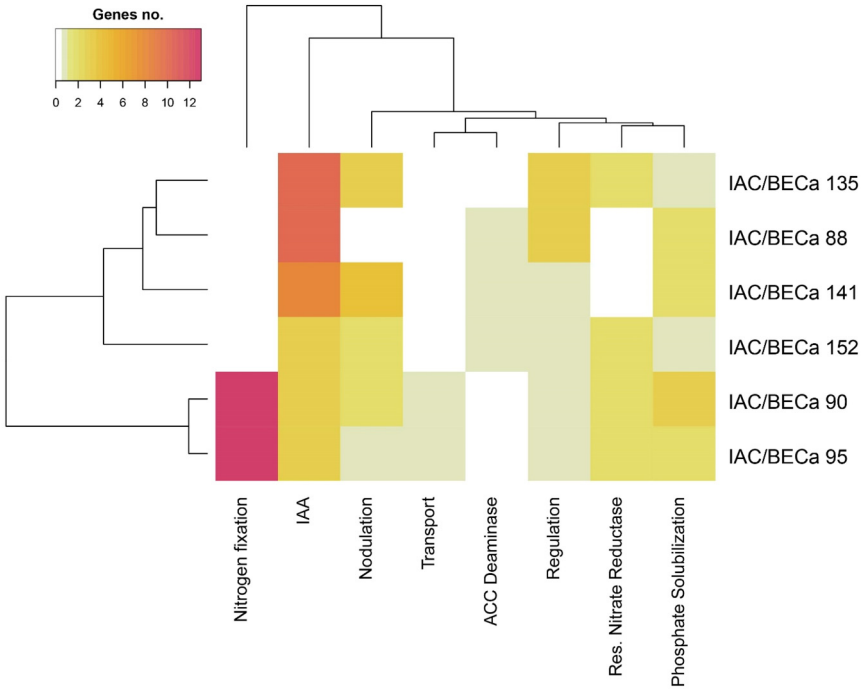

Fig. 4. Heatmap of the number of genes related to group functions of nitrogen fixation, indole acetic acid production (IAA), nodulation, transport, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, regulation, respiratory nitrate reductase, and phosphate solubilization in different genomes.

Table 4
Co-dependence of the microbiome shifts in the rhizosphere and root endosphere with sugarcane biomass and enzyme activity in sugarcane leaves.

		RhN0	PGPB traits	Biom T30	Nutr T30	$Nutr_{Acc}$ T30	$Nutr_{IEU}$ T30	Biom T45	Nutr T45	$Nutr_{Acc}$ T45	$Nutr_{IEU}$ T45	Enz
Control (No N input)	RhN0	–	0.83**	0.57	0.44	0.67**	0.30	0.72*	0.41	0.44	0.17	0.40
	RtN0	0.87**	0.55	0.69**	0.27	0.54	0.55	0.66	0.42	0.56	0.29	0.47
		RhN	PGPB traits	Biom T30	Nutr T30	$Nutr_{Acc}$ T30	$Nutr_{IEU}$ T30	Biom T45	Nutr T45	$Nutr_{Acc}$ T45	$Nutr_{IEU}$ T45	Enz
+N input	RhN	–	0.66**	0.67	0.45	0.54	0.52	0.45	0.54	0.36	0.34	0.36
	RtN	0.92**	0.54	0.62	0.32	0.39	0.57	0.44	0.63	0.47	0.36	0.25

Rh = rhizosphere microbiome; Rt = root endosphere microbiome; N = additional N input; N0 = no additional N; PGPB traits: Plant Growth Promotion traits; Biom = biomass variables (shoot dry matter, root dry matter, total dry matter, and number of tillers); Nutr = macro- and micronutrient concentrations in plant tissue (N, P, K, Ca, Mg, S, Fe, Mn, Cu, Zn, and B); $Nutr_{Acc}$ = macro- and micronutrients accumulated in plant biomass (N, P, K, Ca, Mg, S, Fe, Mn, Cu, Zn, and B); $Nutr_{IEU}$ = nutrient use efficiency of macro- and micronutrients (N, P, K, Ca, Mg, S, Fe, Mn, Cu, Zn, and B); Enz = in vitro activity of nitrate reductase (NR) and nitrite reductase (NiR) in sugarcane leaves; T30 = 30 days after transplanting; T45 = 45 days after transplanting.
** $P<0.05$.
* $P<0.10$.

most relevant shifts in the root endosphere. Unfortunately, the size of the 16S rRNA gene amplicon is not sufficient to provide enough genetic information in next-generation sequencing to allow us to identify the microbes found in high abundance in each treatment at the species level; nonetheless, we observed that additional N input reduced the relevance of the inoculum. Only some of the strains were likely to remain in the soil after inoculation, but all contributed to promoting various plant traits. Overall, when the strains were inoculated in the plants, they mainly followed two paths: (i) promoting plant growth without increasing in abundance in the microbiome or (ii) becoming part of the microbiome and persisting throughout the following crop cycle. These two paths suggest different strategies for the use of growth-promoting microbes. In the first strategy, the native microbiome is resilient to the presence of the PGPB without compromising the growth-promotion capacity of the PGPB; in the second, the PGPB successfully invades the microbiome and continues to deliver plant growth promotion in the following crop cycles (Mawarda et al., 2020).

5. Conclusions

Nitrogen input not only reduced the impact of inoculation on the rearrangement and modulation of the sugarcane microbiome (rhizosphere and root endosphere) but also weakened the co-dependence between the soil microbes and sugarcane biomass and nutrients. The contributions of beneficial microbes to improved plant nutrient use efficiency support the feasibility of intensifying crop productivity while reducing fertilization. Our findings provide additional evidence for the use of PGPB by demonstrating that reduced fertilization is not only desirable but also essential for greater impact on the sugarcane microbiome.

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CRediT authorship contribution statement

R.P.F.I, M.A.C, S.A.A, A.P.D.S. and E.E.K designed research; M.R.D and R.P.F.I. conducted the experiment; M.R.D., S.A.A, and R.P.F.I. obtained the data; M.F.A.L., M.H performed the statistical and bioinformatic analyses; M.F.A.L., M.R.D, A.P.D.S. and E.E.K wrote the paper. All authors reviewed the manuscript.

Declaration of competing interest

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

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