Microbiological Research 287 (2024) 127862

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Endophytic microbial diversity associated with commercial cultivar and crop wild relative banana variety could provide clues for microbial community management

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ARTICLE INFO

Keywords:
Calcutta 4 banana
Williams
Black Sigatoka disease
PGPB
Culturable methods
Metabarcoding *16 S*
Endophytic microbiota

ABSTRACT

Endophytes, microorganisms inhabiting internal plant tissues, play a pivotal role in plant growth and disease resistance. Moreover, previous studies have established that *Musa* plants derive disease protective functions from their microbiome. Notably, one of the crop wild relatives of banana, the Calcutta 4 variety, exhibits resistance to various phytopathogens such as *Pseudocercospora fijiensis* (*P. fijiensis*), while the Williams commercial cultivar (cv.) is highly susceptible. Therefore, this study aims primarily to characterize and compare the endophytic microbiota composition of Calcutta 4 and Williams banana plants when grown sympatrically. Alongside, differences in endophytic microbiome between plant sections (shoot or roots), growth phases (*in vitro* or greenhouse) and fitness factors such as the addition of plant growth-promoting bacteria *Bacillus subtilis* EA-CB0575 (T2 treatment) or infection by *P. fijiensis* (T3 treatment) were examined. Both culture-dependent and -independent techniques were used to evaluate these differences and assess the culturability of banana endophytes under varying conditions. Microbial cultures resulted in 331 isolates distributed across 54 genera when all treatments were evaluated, whereas *16 S* sequencing produced 9510 ASVs assigned in 1456 genera. Alpha and beta diversity exhibited significant differences based on plant section, with an increase in phylogenetic diversity observed in plants with pathogen infection (T3) compared to control plants (T1). Additionally, four differentially abundant genera associated with nitrogen metabolism were identified in T3 plants and seven genera showed differential abundance when comparing varieties. When culture-dependent and -independent methods were compared, it was found that isolates represented 3.7 % of the genera detected by culture-independent methods, accounting for 12–41 % of the total data depending on the treatment. These results are crucial for proposing management strategies derived from crop wild relatives to enhance the resilience of susceptible commercial varieties against fitness factors affecting crop development. Additionally, they help to decipher the pathogenic effects of *P. fijiensis* in banana plants and advance the understanding of how plant domestication influences the endosphere.

1. Introduction

Banana (*Musa* AAA) is an important crop for the global economy, being one of the most widespread monocultures, the most traded, and the second most consumed fruit around the world (Pathak, Mandavgane and Kulkarni, 2017; Soares et al., 2021). This fruit is indigenous to the Indo-Malaysian region, and is grown in many subtropical and tropical areas, particularly in some of the least developed and low-income food-deficit countries. It contributes not only to household food security as a staple but also generates income as a cash crop (FAO, 2023).

Colombia is the fifth-largest banana exporter in the world, lost the fourth position due to the requirements and restrictions in some major markets, adverse weather conditions, high cost of inputs, regulation of pathogen infections, transportation and fertilizers necessary to

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https://doi.org/10.1016/j.micres.2024.127862
Received 29 February 2024; Received in revised form 9 July 2024; Accepted 29 July 2024
Available online 2 August 2024
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guarantee a competitive and quality product (AUGURA, 2023; FAO, 2023). The cv. Williams, a parthenocarpic banana of Giant Cavendish type (AAA), is the most widespread cultivar in the country and one of the most widely grown in commercial plantations. However, it is highly susceptible to diseases such as black sigatoka (*P. fijiensis*, previously *Mycosphaerella fijiensis*), nematode *Radopholus similis*, banana bunchy top virus (BBTV), and, alarmingly it has been reported that Fusarium wilt (*F. odoratissimum* race 4, previously known as *Fusarium oxysporum* f. sp. cubense) has affected Colombian Williams plantations since 2019 (Marín et al., 2003; Kumar et al., 2015; García-Bastidas et al., 2020). More worryingly, several fungi, bacteria and viruses can impair the production of this cultivar as emergent threats or established and widespread diseases (de Souza-Pollo and de Goes 2020).

The Calcutta 4 (AA) variety, a crop wild relative, *Musa acuminata subsp. burmannica* (synonym of *Musa kattuvazhana*; Joe, Sreejith and Sabu, 2016); is one of the wild relatives of commercial bananas (Mertens et al., 2021). It is capable of sexual reproduction and resistant to black sigatoka disease, sigatoka leaf spot (Rodriguez et al., 2020; Soares et al., 2021), several races of *Fusarium* wilt (Liu et al., 2019) and partially resistant to *Radopholus similis* and other nematodes (Rodriguez et al., 2020). This variety, native to Myanmar, remains largely unknown, with its microbiota unstudied and its seeds stored at the Calcutta Botanical Garden, hence its name (Shephred 1999). Commonly used in breeding programs due to its resistance to many pathogens, it serves as a source of resistance genes and as parental variety for the generation of improved diploids followed by gene introgression into new cultivars (Orjeda 2000; Rodriguez et al., 2020; Soares et al., 2021). Nevertheless, it is not consumed as food due to the quantity of seeds per fruit and its flavor, rendering it commercially not-viable (Sánchez et al., 2016).

Chemical control in banana production is considered efficient, but problems can arise from its indiscriminate use, leading to detrimental effects on human and environmental health generating pathogen resistance (Dadrasnia et al., 2020; Churchill, 2011). Therefore, enhancing banana productivity requires alternative approaches, prompting a shift in cultivation methods from conventional crop management to certified and high-yielding organic alternatives. One of these alternatives involves using microorganisms to improve plant development, acting as PGPB (Plant Growth-Promoting Bacteria). They operate through direct or indirect mechanisms that enhance plant nutrition and health (García-Giraldo et al., 2022). Nowadays, PGPB strains have been applied in commercial banana plantations worldwide (Bubici et al., 2019; Gamez et al., 2019; Kavino et al., 2010; Yuan et al., 2015) and using bio-priming with these beneficial microorganisms could be a successful option to colonize plants with protective microorganisms, enhancing plant protection from *in vitro* or greenhouse stages (Mahmood et al., 2016). The introduction or preservation of endophytic microbes is also a potential method for plant growth and induction of defense responses in tissue-cultured banana plantlets (Xiang et al., 2023). In Colombia, research utilizing banana-isolated *Bacillus* strains from both the rhizosphere and phyllosphere have been conducted, leading to the development of bioproducts as biofertilizers and biopesticides as alternatives to reduce the traditional chemical applications (Cuellar-Gaviria et al., 2021; Franco-Sierra and Posada et al., 2020; García-Giraldo et al., 2022; Mosquera et al., 2014; Posada et al., 2016).

In recent years, research on plants as holobionts, encompassing not only the plant itself but also its associated microbial diversity and gene reservoir, has made significant progress, becoming a focal point in plant sciences and agronomy research. This area of study has provided insights into the roles of microorganisms inhabiting plants, highlighting differences between wild and cultivated plants or those subjected to various biotic and abiotic conditions (Omae and Tsuda, 2022; Pérez-Jaramillo et al., 2017). These microbial endophytic populations hold particular significance as they colonize internal plant tissues without causing harm and can be transmitted directly from seeds or parent plants. Also, they play multifaceted roles, serving as defensive barriers against pathogens, acting as biocontrol agents, or stimulating plant growth (Hardoim et al., 2015; Santoyo et al., 2016).

Traditional culture-dependent methods have historically dominated the study of microbial diversity, despite their limited scope. While cost-effective, they provide only a narrow perspective on plant microbial populations. Advanced methods like *16 S* metabarcoding and whole genome sequencing offer more thorough assessments, although dependent on available genomes and computing resources. Combining these approaches provide a comprehensive understanding of bacterial communities, aiding in community management and offering solutions to production challenges related to environmental conditions (Rizal et al., 2020; Wooley and Ye 2010; Lagier et al., 2012; Zampieri et al., 2021).

This project hypothesizes that the composition of endophytic microbiota differs between commercial banana cv. Williams and the crop wild relative Calcutta 4 variety. Furthermore, we hypothesize that fitness-affecting factors such as pathogen infection (*P. fijiensis*) or inoculation with the plant growth promoting bacteria *B. subtilis* EA-CB0575 also modulate plant microbiota composition. Importantly, this modulation may enable the identification of enriched groups suitable for isolating active principles for bioinoculants. Microbial communities were assessed using both culture-dependent and -independent techniques, shedding light on the association between plant-pathogen resistance and microbial composition in *Musa acuminata*. This study represents the first comparison of microbiota between the crop wild relative Calcutta 4 variety and the commercial cv. Williams, providing insights for the development of biotechnological strategies to manipulate the plant microbiome and suggesting avenues for producing bioproducts conducive to sustainable agriculture practices.

2. Materials and methods

2.1. Plant collection and management

2.1.1. Calcutta banana plants provenance

Calcutta 4 banana plants and seeds were collected at Montelindo Farm (Universidad de Caldas, Caldas-Colombia, 5.076280 N −75.671235 W) in areas with low human intervention, and transferred with their soil or whole fruit to Universidad EAFIT, Medellín - Colombia, for growing at greenhouse or for *in vitro* germination. Seeds were extracted from the fruit, disinfected (70 % ethanol for 2 min, 2 % hypochlorite for 10 min, 70 % ethanol for 3 min and sterile water between reagents), and stored at 22 °C until were planted *in vitro*. Murashige and Skoog medium (4.33 g/L of M524 basal salt mixture with vitamins PhytotechLab and 5 g/L of agar BD) was used for seed pre-germination at 22 °C under a 12:12 h light and dark cycle for 40 days. The resulting seedlings were then transferred to fresh MS medium in flasks and maintained under the previous conditions for 30 days until further experimentation. The Calcutta plants were cultivated in a controlled greenhouse in bags containing 2 kg of their natural soil.

2.1.2. Williams banana plant provenance

Williams banana meristems were obtained from Meristemos de Colombiaś Company (Rionegro, Colombia) and kept at 30°C under laboratory conditions (22 °C, 12:12 h light and dark and mean relative humidity of 60 %) until the experiments were conducted. Nine-months-old Williams banana plants from *in vitro* culture were also acquired from the same supplier. Subsequently, they were transplanted into 2 kg bags filled with a commercial mixture of soil (dark soil, rice husk and organic material in a ratio of 1:1:0.5) for greenhouse evaluations, according to banana producers recommendations and the processes they carry out in the production area. These plants were acclimatized under environmental conditions (23–30 °C and mean relative humidity of 60 %) until the experiments were conducted in a controlled greenhouse at EAFIT University.

2.2. Samples collection

2.2.1. Plant material collection for endophytes isolation

Fifty plants of each variety/cultivar were grown at the greenhouse stage, and fifteen were selected for bacterial isolation. A sample composed of a combination of 10 g from each of the five selected plants was used as an experimental unit (totaling 50 g per experimental unit, 10 g per plant). Three experimental units were utilized for isolation.

For the isolation of endophytic bacteria from cv. Williams plants at the *in vitro* level, pots containing 45 meristems each were used, with one pot serving as an experimental unit. Ten grams of the plant material from each pot were randomly selected for bacterial isolation. Three experimental units were employed for this purpose.

To isolate the microbiota from the wild relative Calcutta banana, 500 pre-germinated seeds were acquired, and 50 germinated plants were randomly chosen to obtain 10 g of banana tissues. To ensure temporal replication, the entire experiment was conducted twice, once in August and again in September of 2018.

2.2.2. Isolation of endophytes

The native endophytic microbiota of greenhouse and *in vitro* plants from crop wild relative Calcutta 4 and cv. Williams was determined by collecting sections of leaves, pseudo-stem and roots. These tissues were disinfected and diluted in 1:10 ratio using KH_2PO_4 (pH 7.4), then macerated using an Ultraturrax (ULTRA-TURRAX® Tube Drive 2004IK3645000). Serial dilutions were prepared for culturing on five solid media: AIA (Chaudhary et al., 2013), PIA (Jimenez et al., 1999); YDC (Scortichini et al., 2001); TSA 50 % (Merck, USA) and R2A (Reasoner and Geldreich, 1985). Microbial morphotypes obtained from all media were purified through consecutive growth in their respective media and preserved in TSB supplemented with 20 % Glycerol at −80 °C. Individual morphotypes were deposited in the Collection "Microorganisms isolated from banana Williams and Calcutta 4 plants" at the Biodiversity Information System (SiB) at Colombian Humboldt Institute (Villegas-Escobar and Londoño 2023), following the guidelines of the framework contract for access to genetic resources and their derived products; permit number ARG166 of the Colombian Ministry of Environment.

2.2.3. Collection 16 S sequencing

Total DNA extraction of all morphotypes was conducted using the Ultraclean Microbial DNA kit (Qiagen, Germany). DNA quality was assessed through agarose electrophoresis (1 %) and spectrometric quantification with NanoDrop™ 2000 (Thermo Scientific, USA). Amplification of the 16 S rRNA gene was performed by PCR (Mix: 0.02 U/μL of Taq polymerase 5 U/mL; 0.2 μM of primers (8 F and 1492 R); 0.2 mM of dNTPs-Mix; 1.5 mM of $MgCl_2$; 1X of Taq buffer, 100 ng of DNA and PCR-grade water in a 50 μL reaction volume. The PCR conditions included an initial denaturation at 94 °C for 3 min, followed by 30 cycles of amplification (denaturation at 94 °C for 30 seconds, annealing at 60 °C for 20 seconds, and extension at 72 °C for 90 seconds) and a final extension at 72 °C for 5 minutes using a T1000 ThermoCycler (BioRad, USA)). Subsequently, sequencing was performed using the Sanger method with primer 907 R at Macrogen (Seul, Korea). Chromatograms were basecalled using Tracy v0.59 (Rausch et al., 2020), trimmed with Cutadapt (Martin, 2011) and then converted to a Qiime2 artifact for taxonomy assignment.

2.2.4. Diversity estimation for culturable microbiota

The local and global diversity of endophytic bacteria in Calcutta 4 and Williams tissues were estimated using PAST 4.01 (Paleontological Statistics software, Hammer et al., 2001). Individual rarefaction analyses were performed for each treatment (1. Calcutta 4 greenhouse plants (CG), 2. Williams greenhouse plants (WG), 3. Calcutta 4 *in vitro* plants (CI) and 4. Williams *in vitro* plants (WI)), testing the maximum cultivable taxa and then generating a matrix with the genera versus the four treatments and their occurrence in each one, determined by the number of identified similar morphotypes and isolates within each treatment. Analysis of alpha diversity for treatments and genera was implemented using the Shannon, Simpson (1-D), Chao 1 and Evenness indexes. Additionally, beta diversity was estimated using the Whittaker index (Jurasinski et al., 2009).

2.3. Culture-independent evaluation of banana microbiota

50 banana plants of Calcutta 4 and Williams varieties were grown from the laboratory conditions (from seeds for Calcutta 4 plants and from meristems from cv. Williams), and after 90 days, 25 of them were transferred to seedbeds in Urabá, Colombia (longitude and latitude: 7.7945192 N, −76.649334 W).

Plants were grown sympatrically under the following conditions: 29–33 °C, watering every other day, and mean relative humidity of 84 %. Three treatments were evaluated: Treatment 1 (T1) consisted of 3-month-old plants without PGPB inoculation or without artificial pathogen infection (serving as the control treatment), and they were placed under a controlled plastic chamber to minimize interaction possibilities with other treatment plants. Treatment 2 (T2) comprised plants inoculated with the PGPB *B. subtilis* EA-CB0575 during laboratory growth and were re-inoculated monthly at the seedbed. Treatment 3 (T3) involves plants artificially infected with the pathogen *P. fijiensis* during lab growth and monthly re-infected at the seedbed.

Plant fertilization and planting conditions followed the recommendation of the banana producers, involving the application of DAP (5 g per plant at sowing), 15–15–15 (5 g per plant once per month), and Wuxal (100 g in 20 L solution, applied three times per week for two weeks). Each treatment involved twenty-five plants, cultivated in plastic bags filled with 5 kg of river sand and rice husk in the greenhouse (1:1 ratio). Of these plants, fifteen plants were designated for DNA extraction and subsequent NGS analysis, while the remaining ten were used to assess physiological variables such as total dry weight, leaf number, shoot length, and pseudostem diameter

2.3.1. PGPB inoculation of banana plants (T2)

The PGPB *B. subtilis* EA-CB0575 (part of the microorganisms' collection at Universidad EAFIT), used in Treatment 2 (T2), was isolated from the rhizosphere of *Musa* AAA cv. Valery in Urabá, Colombia (Posada et al., 2016) and cultured on trypticase soy agar 50 % (TSA, Oxoid) for 24 h at 30 °C. A single colony was inoculated into a 2 L Erlenmeyer containing 500 mL of TSB and incubated for 24 h at 150 rpm and 30 °C. Subsequently, cells were harvested by centrifugation at 2490 g for 15 min, and the resulting pellet was suspended in sterile distilled water to OD_{600}= 1.0, equivalent to 1×10^8 colony-forming units (CFU)/mL. For the initial inoculation, Williams banana plants from meristem culture in MS medium were immersed in the bacterial suspension for 1 h and then planted in MS medium for 30 days. Subsequently, they were re-immersed in the bacterial suspension for another hour before being transferred to greenhouse trays filled with a peat: sterile soil (1:1 ratio). These plants were then grown under conditions 29–33 °C and 70 % ± 5 % relative humidity for 30 days. Calcutta 4 seeds were pre-germinated in MS medium and then immersed in the bacterial suspension for inoculation. Control plants were immersed in sterile distilled water instead. Upon transfer to seedbeds at the crop location, PGPB inoculation was repeated monthly by spraying 100 mL of bacterial suspension containing 1×10^8 CFU/mL onto the soil. Plants of the control were sprayed with sterile water.

2.3.2. Pathogen culture and plant infection (T3)

P. fijiensis ascospores, used in Treatment 3 (T3), were isolated from banana plant leaves following the methodology reported by Gutiérrez-Monsalve et al., (2015). The ascospores were cultured in PDA (Oxoid) for 14 days at 30 °C and then fragmented using glass beads in sterile distilled water. Subsequently, 3 mL of fragmented mycelium were

inoculated in 50 mL of 2 % liquid Sabouraud with 2 % dextrose medium (Merck, USA), supplemented with 200 μg/mL chloramphenicol, and incubated for 10 days at 30 °C and 150 rpm. The fungal biomass was adjusted to 10^5 fragment/mL using glass beads, and 0.05 % Tween 20 (v/v) and 0.05 % CMC were for inoculum stabilization in 2 % Sabouraud liquid medium (v/v) (Merck, USA).

Leaf number one of both cultivar plants was infected with the fungal suspension at the seedbed, and subsequently harvested to assess disease severity through necrotic area evaluation and severity grading according to Stover scale (Stover, 1971).

2.4. Culture-independent endophyte community characterization

2.4.1. DNA extraction and quantification

The total endophytic DNA of the plants was extracted as follows: Fifteen grams of sections from Calcutta 4 and Williams banana plants (including leaves, roots or pseudostem) were disinfected according to the protocol outlined in the isolates collection section, which had been previously standardized for banana samples. The sections were then macerated using liquid nitrogen for DNA extraction employing the DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturer's recommended protocol, with additional steps implemented to enhance DNA concentration. After the spin filters were removed, 4 mL of 5 M NaCl were added and the solution was inverted. Subsequently, 200 mL of cold 96 % ethanol were added, and the tubes were inverted before being centrifuged at 16,200 g for 5 minutes at room temperature. The supernatant was then discarded. To remove residual ethanol, the samples were subjected to a Vacuum Concentrator (Eppendorf, model V-HV) at 30°C for 20 minutes. The resulting pellet was resuspended in sterile 10 mM Tris-HCl buffer at pH 8.5 and stored at −80°C. The DNA concentration was assessed using 1 % agarose gel electrophoresis, a Nanodrop™ spectrophotometer (Thermo Fisher Scientific, USA), and a Qubit® 3.0 Fluorometer (ThermoScientific, USA)

2.4.2. Next-generation sequencing, bioinformatic pipeline and taxonomy assignment

DNA samples from the endophyte banana community with a concentration greater than 10 ng/μL were sent to the BaseClear facility (Leiden, Netherlands) for normalization, library preparation, and next-generation sequencing (NGS) of the hypervariable V3-V4 region from *16 S* rRNA gene, using primers 341 F/805 R, in combination with chloroplast and mitochondrial blocking primers. The raw data was quality checked with FastQC (Andrews et al., 2012) and MultiQC (Ewels et al., 2016). Subsequently, *16 S* rRNA amplicon sequence variants (ASVs) were inferred using the DADA2 pipeline within Qiime2 v. 20208 (Bolyen et al., 2019). Forward and reverse reads were truncated at 280 and 250 bp, respectively, and the 10 first bases of all reads were trimmed. The pseudo-pooling method was enabled, and all other parameters were run by default. Mitochondria or chloroplast ASV sequences were filtered using VSEARCH (Rognes et al., 2016) and the references of the *16 S* and *18 S* genes from *Musa acuminata* cv. Williams (NCBI accession codes: LC609628 and LC612097). ASVs with an identity percentage greater than 90 % were excluded from downstream analyses.

Database curation was conducted using q2-RESCRIPt plugin with SILVA v138.1, GTDB bacterial and archaeal 214.1 releases (Quast et al., 2012, Parks et al., 2022). Taxonomy assignment for both isolates and ASVs was performed using independent naïve bayes (NB) classifiers per region and VSEARCH consensus assignment with databases of primer sets 341 F-805R and 8 F-907R. Subsequently, both assignments were merged with RESCRIPt (Robeson et al., 2021). Phylogenetic analyses were conducted both *de novo* and by saté-enabled phylogenetic placement (SEPP). For *de novo* analyses, the following methods were employed: q2 align-to-tree-mafft-iqtree, q2 align-to-tree-mafft-fasttree and q2 align-to-tree-mafft-RAxML (Katoh et al., 2002; Nguyen et al., 2015; Stamatakis, 2014). SEPP analysis (Mirarab et al., 2012) was performed using SILVA 128 reference and default parameters.

2.4.3. Diversity estimations, statistics and differential abundance analysis for uncultured samples

Alpha diversity was estimated using Shannon as richness and evenness estimator, Simpson E and Lladser as evenness estimators (Lladser et al., 2011) and Fisher-alpha and Faith's phylogenetic diversity as richness estimators, following the recommendations of Hagerty et al., (2020). These metrics were calculated using q2-alpha, q2-alpha-phylogenetic for Faith Phylogenetic Diversity (Faith's PD) and differences were tested using Kruskal-Wallis test (Kruskal, 1952) implemented in the q2-alpha-significance plugin. Rarefaction depth was set at 9200 reads when necessary.

Beta-diversity was estimated using Bray-Curtis and DEICODE Aitchison distance indexes with q2-diversity-beta and q2-DEICODE (Cameron et al., 2019). Principal Coordinate Analysis (PCoA) was then performed for all beta diversity indices evaluated. Then, differences were tested using Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson 2001) and Analysis of similarities (ANOSIM). PCoA results were plotted in R with qiime2R (Bisanz, 2018). Additionally, differentially abundant taxa at the genus level were identified using q2-composition ANCOM-BC (Lin et al., 2020). Finally, to further explore relationships among ASVs, a co-occurrence network analysis was conducted using the Qiime2 plugin for SCNIC, with $R > 0.35$ (Shaffer et al., 2022).

2.4.4. Representativeness of isolate 16 S rRNA gene sequence in metabarcoding data

To calculate the representation of the cultured collection in NGS sequencing data, the regions corresponding to the V3-V4 region from 16 S gene from the culture collection amplicons, along with the sequence of *B. subtilis* EA-CB0575 (KC170988), were extracted using q2 extract-reads and 341 F-805R primer set. The resulting sequences were then utilized as the database input for VSEARCH searching, with ASVs representative sequences as queries. Searches were conducted using the Marine Biological Lab identity definition, which considers gap openings, as suggested by Steen et al. (2019). An ASV was considered to be represented by the culture collection when VSEARCH (Rognes et al., 2016) reported a match with an identity score greater than 0.97 with a strain sequence.

Next, features from each category (represented or not represented by the culture collection) were collapsed with q2-feature-table group by summing their frequency per treatment. Then, the proportion of the 16 S sequencing data represented within the culture collection was calculated by dividing the sum of the frequencies of represented ASVs and the total frequency for each treatment, and then multiplied by 100.

3. Results

3.1. Culturable banana microbiota shows differences between crop wild relative and commercial plants

The culturable endophytic microbiota from banana plants Calcutta 4 and cv. Williams were isolated and stored in the SiB Microorganism's Collection at Universidad EAFIT, under code 226 EA-ED isolates (Villegas-Escobar and Londoño 2023). In this study, a total of 331 morphotypes were isolated, subsequently sequenced, and identified to determine the community composition at different stages of plant growth (*in vitro* and greenhouse levels) or in different plant sections (roots section, and pseudostems and leaves comprising the shoot section). Genbank accession codes for successfully sequenced strains are PP330421 to PP330712. A total of 54 genera were identified throughout the experiment and only two isolates could not be identified due to the growth issues, and 11.04 % of isolates remained unclassified (Supplementary Table 1).

3.1.1. Culturable endophytic microbiota of plant sections

The shoot microbiota of crop wild relative Calcutta 4 banana plants

showed 108 isolates in 27 genera. *Bacillus* was the most prevalent genus, with a relative occurrence of 22.22 % *Bacillus* and 12.03 % *Bacillus_*A. This was followed by *Stenotrophomonas* (8.33 %), *Pseudomonas* (6.48 %), *Agrobacterium* (5.55 %) and *Herbaspirillum*, *Klebsiella* and *Microbacterium,* each accounting for 4.62 %. In contrast, 112 isolates representing 25 genera were identified from the shoots of Williams plants. Notably, *Stenotrophomonas* (24.10 %) and *Pseudomonas_E* (17,85 %) were the predominant genera in this cultivar and section. Additionally, fourteen genera (*Agrobacterium*, *Allorhizobium, Bordetella, Brevibacillus, Chryseobacterium, Herbaspirillum, Klebsiella, Kosakonia, Lactococcus, Leucobacter, Micrococcus, Mucilaginibacter, Pantoea* and *Sphingobium)* were exclusively isolated from the shoot section of Calcutta 4 plants, while twelve genera (*Arthrobacter, Brachybacterium, Exiguobacterium_A, Fictibacillus, Methylobacterium, Paenarthrobacter, Peribacillus, Rossellomoea, Staphylococcus, Sutclifiella, Sutclifiella_A, Telluria*) were found solely in the shoot section of Williams plants.

The root sections of Williams plants exhibited 49 isolates across 18 genera, whereas the root sections of Calcutta 4 plants presented 62 isolates in 20 genera. Seven genera (*Cupriavidus, Gottfriedia, Niallia, Novosphingobium, Ochrobactrum, Serratia* and *Variovorax)* were exclusively isolated from the root section of Calcutta 4 plants, while another seven genera (*Acinetobacter, Intrasporangium, Kitasataspora, Neobacillus, Neorhizobium, Phyllobacterium* and *Stutzerimonas)* were solely isolated from the root section of Williams plants. Genera such as *Bacillus, Bacillus_A, Chryseobacterium, Curtobacterium, Microbacterium, Micrococcus, Paenibacillus, Peribacillus, Priestia, Pseudomonas, Ralstonia, Rhizobium_B, Sphingomonas, Stenotrophomonas* and *Xanthomonas* were prevalent in both varieties/cultivars, with some even being shared between plant sections (see Fig. 1).

3.1.2. Culturable endophytic microbiota across different culture media

Four culture media were used to isolate bacteria from banana varieties. When TSA medium was used, 30 isolates (9 genera) were obtained from Williams plants, whereas 46 isolates (21 genera) were obtained from Calcutta 4 plants (see Figure S1 and Supplementary Table 1). The predominant genera observed were *Bacillus* (28.57 %) and *Stenotrophomonas* (28.57 %) for Williams banana plants, and *Bacillus* (6.52 %) and *Ralstonia* (6.52 %) for Calcutta 4 plants. The TSA medium yielded the most diverse collections. A few unique isolates were obtained on TSA medium, including *Gottfriedia, Niallia, Ochrobactrum* and *Paenarthrobacter*. For R2A medium, 47 isolates (21 genera) were obtained from Williams plants, while 28 (13 genera) were obtained from Calcutta 4 plants. This medium facilitated the isolation of exclusive genera, such as *Arthrobacter, Brevibacillus, Fictibacillus, Methylobacterium, Neorhizobium, Phyllobacterium, Rossellomoea, Stutzerimonas* and *Variovorax*.

Using YDC medium for microorganism isolation resulted in 30 isolates (8 genera) for Williams and 36 isolates (18 genera) for Calcutta 4 plants. *Stenotrophomonas* and *Bacillus* were the most prevalent genera, accounting for 30 % and 20 % respectively, in the Williams cultivar. The diversity of isolates was higher in Calcutta 4 than in Williams, with *Bacillus* and *Bacillus_A* being the most represented genera (16.66 % and 22.22 %, respectively). *Curtobacterium* isolates were exclusively obtained on YDC medium. When AIA medium was used, 42 isolates (17 genera) were obtained from the Calcutta variety, while 32 isolates (20 genera) were obtained from the Williams cultivar. Ten distinct genera (*Acinetobacter, Exiguobacterium, Intrasporangium, Kitasatopora, Intrasporangium, Mucilaginibacter, Neobacillus, Sphingobium, Sutcliffiella_A and Telluria*) were only isolated using this medium.

According to the expected results, the PIA medium was used for the most selective isolation. From Williams plants, 3 genera and 22 isolates were obtained, with *Pseudomonas* being the most prevalent genus (36.33 %). From Calcutta 4, 7 genera and 17 isolates were obtained, with *Pseudomonas* being the most predominant genus (41.17 %), followed by *Stenotrophomonas* (23.53 %), *Burkholderia* (11.76 %), and *Bordetella, Brevibacterium, Lactococcus* and *Serratia* each at 5.88 %. This medium exhibited lower diversity and richness compared to the other evaluated media (Figure S1). However, it facilitated the isolation of exclusive genera such as *Bordetella, Brevibacterium, Burkholderia* and *Serratia,* all of which were isolated only from Calcutta 4. Notably, *Pseudomonas* and *Stenotrophomonas* showed a high occurrence in samples for both types of banana plants when using this medium.

3.1.3. Culturable endophytic microbiota across different collections

Four microbiota collections were obtained: CI (Calcutta 4 grown *in*

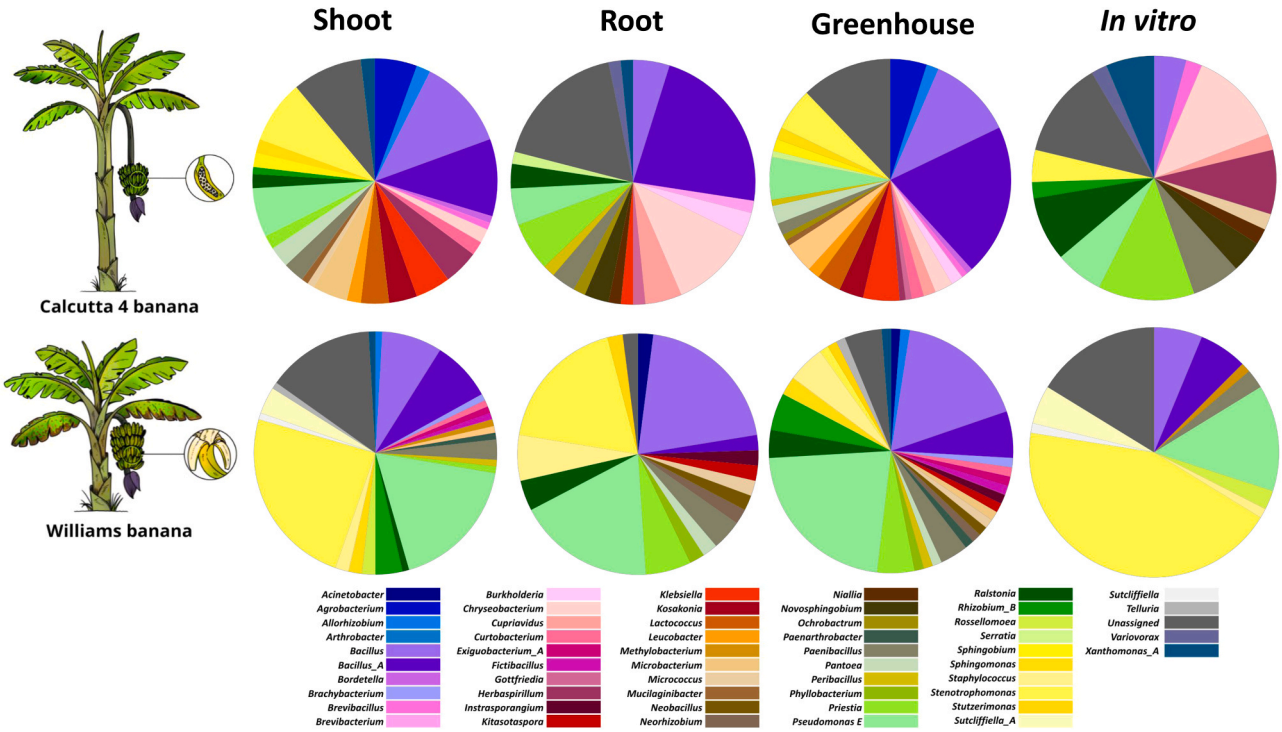

Fig. 1. Microbiota isolated from shoots and roots of crop wild relative Calcutta 4 and cv. Williams at greenhouse and *in vitro* level.

vitro), CG (Calcutta 4 grown in greenhouse), WI (Williams grown *in vitro*) and WG (Williams grown in greenhouse). Their alpha and beta diversities were assessed based on the richness and evenness of their isolates (see Supplemental Table S2).

The highest diversity, based on occurrence data, was observed in isolates from crop wild relative *in vitro* grown Calcutta 4 plants (CI) (Simpson index 0.94 and Evenness 0.97), with 47 isolates classified into 17 genera. The most prevalent genera were *Chryseobacterium* and *Priestia,* each accounting 12.77 %, followed by *Ralstonia* and *Herbaspirillum,* both at 8.45 %, and *Paenibacillus and Xanthomonas,* each at 6.38 %. When the abundance was analyzed (CFU/g), the genera *Paenibacillus*, *Ralstonia*, and *Chryseobacterium* exhibited high populations with $2.7*10^6$, $1.9*10^6$ and $1.8*10^6$ CFU/g of tissue, respectively. *Micrococcus* and *Stenotrophomonas* showed low abundance in the samples (see Supplementary Table 1).

The collection isolated from the crop wild relative Calcutta 4 plants grown in the greenhouse (CG) ranked second in diversity (Simpson index 0.92 and Evenness 0.66), consisting of 123 isolates classified under 28 genera. *Bacillus_A* was the most abundant genus accounting for 20.32 % of the isolates, followed by unassigned taxa (12.19 %), *Bacillus* (11.38 %), *Pseudomonas,* and *Stenotrophomonas* (5.60 %), and *Agrobacterium* (4.89 %) (Fig. 1). *Bacillus, Leucobacter, Bacillus_A and Cupriavidus* exhibited high abundances in samples, with abundance values of $1.6*10^7$; $1.5*10^7$; $1.3*10^7$; and $5.1*10^6$ (CFU/g) respectively, while *Bordetella, Herbaspirillum, Burkholderia, Serratia* and *Peribacillus* had the lowest abundances (Supplementary Table S1).

The third most diverse collection was isolated from commercial Williams plants grown in greenhouse (WG) (Simpson index 0.91 and Evenness 0.67) with 30 genera identified across 81 total isolates. The genera *Pseudomonas*_E representing 22.22 %, and *Bacillus,* accounting 17.28 %, were the most prevalent, followed by *Bacillus*_A (6.17 %) and others in lower proportions. *Staphylococcus, Bacillus,* and *Pseudomonas_E* exhibited higher abundances, with values of $2.9*10^6$, $2.2*10^6$ and $1.35*10^6$ CFU/g respectively, while *Curtobacterium*, *Ficitbacillus* and *Exiguobacterium* showed lower abundances, around of $3.3–5*10^3$ CFU/g.

The least diverse collection was isolated from Williams plants grown *in vitro* (WI) (Simpson index 0.76 and Evenness 0.57), comprising only 11 genera and 80 isolates. *Stenotrophomonas* accounted for 51.25 % of isolates, followed by *Pseudomonas* (21.25 %), *Bacillus* (15 %), and *Sutcliffiella (*6.25 %) (Fig. 1). *Stenotrophomonas, Pseudomonas and Sutcliffiella* were highly abundant, with *Stenotrophomonas* being the most prevalent genus with the highest abundance ($2*10^6$ CFU/g), while *Pseudomonas* showed moderate presence ($2.7*10^5$ CFU/g). Genera *Rossellomoea* and *Paenibacillus* exhibited low abundances ($5*10^2$ and $2.2*10^3$ CFU/g).

For all culturable microbiota collections, rarefaction curves reached a stationary phase, indicating sufficient sampling effort of morphotypes (Supplementary Table S2). The Whittaker index for beta diversity across collections was 1.56, indicating differences between the collections. Genera such as *Bacillus, Bacillus_A, Curtobacterium, Micrococcus, Microbacterium, Paenibacillus, Pantoea, Peribacillus, Priestia, Pseudomonas_E, Ralstonia, Rhizobium_B, Sphingomonas, Stenotrophomonas* and *Xanthomonas_A* were isolated from the two plant varieties and at different stages. Thus, these prominent genera can be designated as the “generic” or “core” culturable microbiome of bananas.

Genera such as *Agrobacterium*, *Allorhizobium*, *Bordetella, Brevibacillus, Brevibacterium, Burkholderia, Chryseobacterium*, *Cupriavidus*, *Gottfriedia*, *Herbaspirillum, Klebsiella, Kosakonia, Lactococcus, Leucobacter, Mucilaginibacter, Niallia, Novosphingobium, Ochrobactrum, Serratia, Sphingobium* and *Variovorax* were exclusively isolated from the crop wild relative Calcutta 4 variety. On the other hand, genera including *Acinetobacter, Arthrobacter, Brachybacterium, Exiguobacterium, Fictibacillus, Intrasporangium, Kitasatospora, Methylobacterium, Neobacillus, Neorhizobium, Paenarthrobacter, Phyllobacterium, Rossellomoea, Staphylococcus, Stutzerimonas, Sutcliffiella, Sutcliffiella_A* and *Telluria* were solely isolated from William bananas (Fig. 1).

3.2. Inoculation and Black Sigatoka infection assay in greenhouse

Calcutta 4 and Williams banana plants were inoculated with *B. subtilis* EA-CB0575, a plant growth promoting bacterium, to assess its potential to enhance plant growth compared to plants without *Bacillus* application. It was found that Williams banana plants exhibited significantly increased shoot diameter and shoot length compared to the control group without *Bacillus* inoculation, showing increases of 19.89 % and 45.23 %, respectively. However, the number of leaves was reduced by 26.34 % compared to the control (Figure S2). In contrast, the inoculation had no significant effect on the number of leaves and diameter for Calcutta 4 banana plants, but there was a 45.30 % increase in shoot length after inoculation. Plants infected with the pathogen (T3) did not show significant differences in growth responses compared to the control group (Figure S2). Nevertheless, successful *P. fijiensis* infection was observed in greenhouse conditions, with Williams plants reaching an advanced stage on the Stover severity scale of around 4.45. Williams plants inoculated with *B. subtilis* EA-CB0575 (T2) reached a severity stage of 3.09 due to the presence of *P. fijiensis* spores in the banana production zone, which are naturally found throughout the year. Calcutta 4 plants infected with *P. fijiensis* did not show any signs of infection, likely due to the high resistance of this variety to Black Sigatoka disease, as reported by previous studies (Rodriguez et al., 2020; Soares et al., 2021). Control plants exhibited a low severity stage due to the natural inoculum of Black Sigatoka disease present in the environment, even though they were isolated inside a controlled chamber (Figure S2).

3.3. Culture-independent method shows key groups in banana endomicrobiome

Out of the 48 libraries prepared for studying the endophytic microbiota of banana plants, only 36 were successfully sequenced using the V3-V4 region of the *16 S* rRNA gene (Bioproject code: PRJNA1046476, metadata available on Supplementary Table 3). The evaluations comprised n= 17 samples from the aerial section (shoot and leaves) and n= 19 from the root section, with n= 12 representing the crop wild relative Calcutta 4 variety and n= 24 for samples of the Williams cultivar. Additionally, n= 12, 14 and 10 samples were used for evaluating plants with treatments T1, T2 and T3, respectively. Unfortunately, samples from the Calcutta 4 variety were most affected by technical failures. Nevertheless, other factors such as plant tissue and PGPB/artificial infection remained evenly distributed. After removing plastids and mitochondrial sequences, a total of 749,856 total reads were used and 9510 ASVs retained for downstream analyses (Supplementary Table 4).

3.3.1. General taxonomy assignment of endophyte community

Taxonomic assignments using GTDB databases performed outperformed SILVA and NCBI RefSeq. Metrics for these assessments of the tested databases are available in Figure S3. General taxonomy assignment at the phylum level for the 36 sequenced libraries is depicted in Fig. 2. Pseudomonadota emerged as the most abundant phylum within the endophyte community, followed by Actinomycetota and Bacteroidota (constituting 51.4 %, 17.9 % and 13.6 % of the sequences, respectively). Additionally, a notable abundance of phyla such as Patescibacterota, Acidobacteriota and superphylum Planctomycetota-Verrucomicrobiota-Chlamydiota (PVC) is observed in the root section. Meanwhile, Bacillota predominates in the aerial section (Fig. 2). Three archaeal phyla were detected: Thermoproteota, Halobacteriota and Nanoarchaeota. ASVs were assigned to 1456 genera, with the top 10 most conspicuous genera being *Arthrobacter_K, Sphingomonas, Streptomyces, Pantoea, Trinickia, Chitinophaga, Arachidicoccus, Bacillus, Acinetobacter* and *Acidocella* (comprising 7.31 %, 3.12 %, 3.06 %, 2.61 %, 2.35 %, 2.19 %, 1.95 %, 1.58 %, 1.5 % and 1.49 % of sequences, respectively).

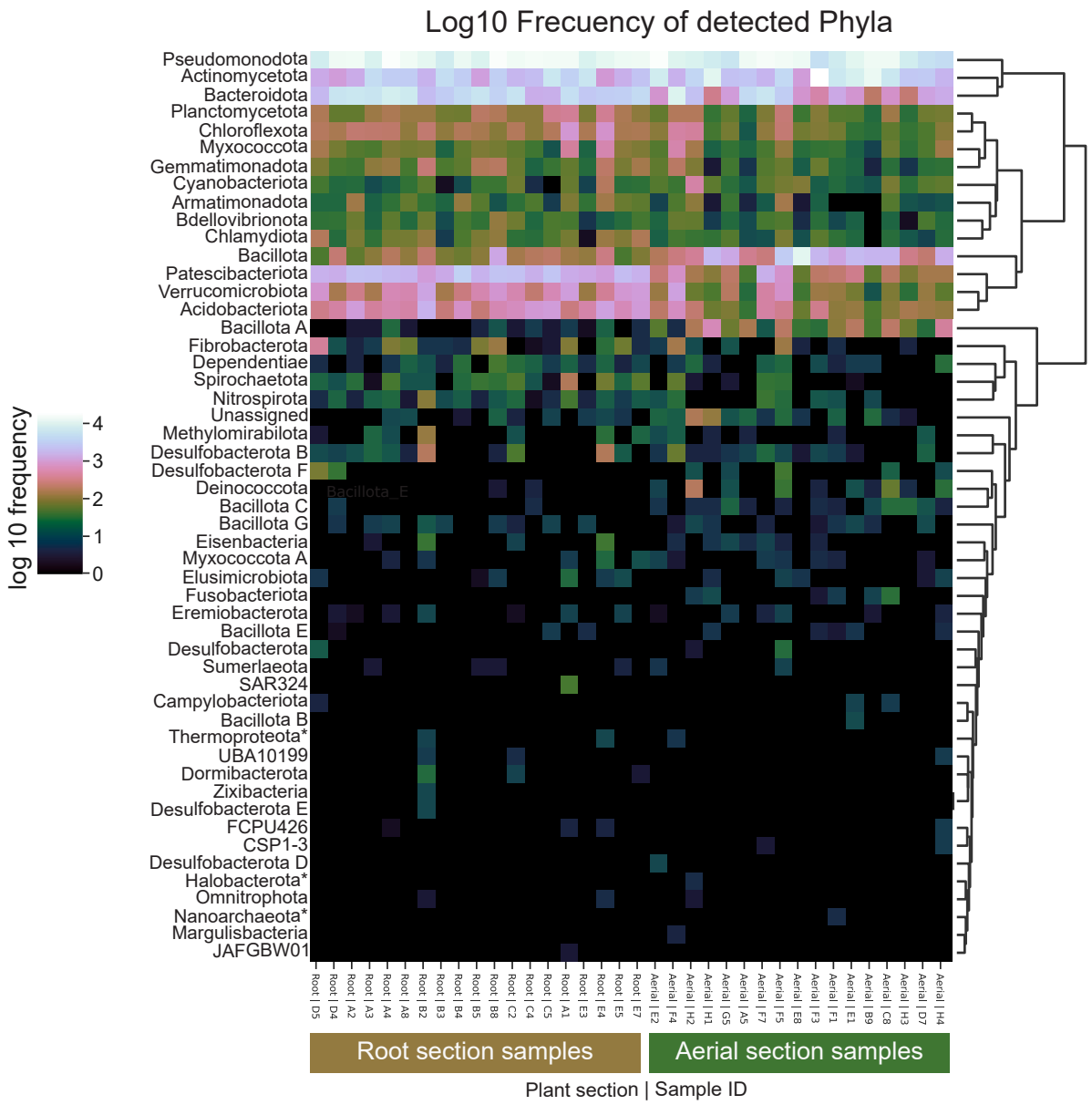

Fig. 2. Log-10 frequency heatmap at phylum level using GTDB classification.

3.3.2. *Alpha diversity shows strong influence of section in endophyte diversity*

Rarefaction was conducted at 9300 for all libraries. Alpha and beta diversity indices were calculated for three factors (variety, section/tissue and PGPB/pathogen application, see Table 1). All diversity metrics exhibited significant differences when compared across plant sections. Root samples displayed significantly higher richness according to the evaluated metrics (Faith phylogenetic diversity, p-value < 0.03; Fisher-alpha, p-value < 0.001). Additionally, alpha diversity metrics reflecting evenness, such as Lladser, Shannon and Simpson E, also showed significant differences between plant sections (p-value < 0.001). Furthermore, richness metrics were significantly different between plants infected with *P. fijiensis* (T3) or inoculated with *B. subtilis* EA-CB0575 (T2) and plants without either treatment (T1) (Faith's PD, Shannon and Fisher-alpha; p-value < 0.04).

Evenness metrics such as Shannon and Simpson E also exhibited significant differences between infected and non-infected plants (p-value < 0.001). Both beta-diversity metrics assessed demonstrated clear and significant differentiation between roots and aerial samples (Aitchison-DEICODE and Bray-Curtis; PERMANOVA and ANOSIM, p-value < 0.001). Additionally, Aitchison-DEICODE and Bray-Curtis distances revealed differentiation (Aitchison-DEICODE, PERMANOVA p-value < 0.05) between Calcutta 4 and Williams banana plants (Fig. 3), though this differentiation was not observed when evaluating the ANOSIM test of Bray-Curtis distance. Similarly, T1, T2 and T3 plants exhibited significant differences (PERMANOVA test, p-value < 0.05) but were not significantly different when assessed with ANOSIM. Finally, the top variants contributing to variation according to DEICODE include *Acidocella, Streptomyces, Arthrobacter K, Cutibacterium, Sphingomonas,* and *Trinickia* (Fig. 3).

3.3.3. *Differential abundance results*

The Log-Fold Change of taxa identified as differentially abundant (DA) by ANCOM-BC across all factors is presented in Fig. 4. The highest diversity of classified taxa was observed at the genus level, which also exhibited the highest count of Unique labels and Taxonomic Entropy (level 6, supplemental Figure 3). While characterizing the differences between the rhizosphere and phyllosphere falls beyond the scope of this

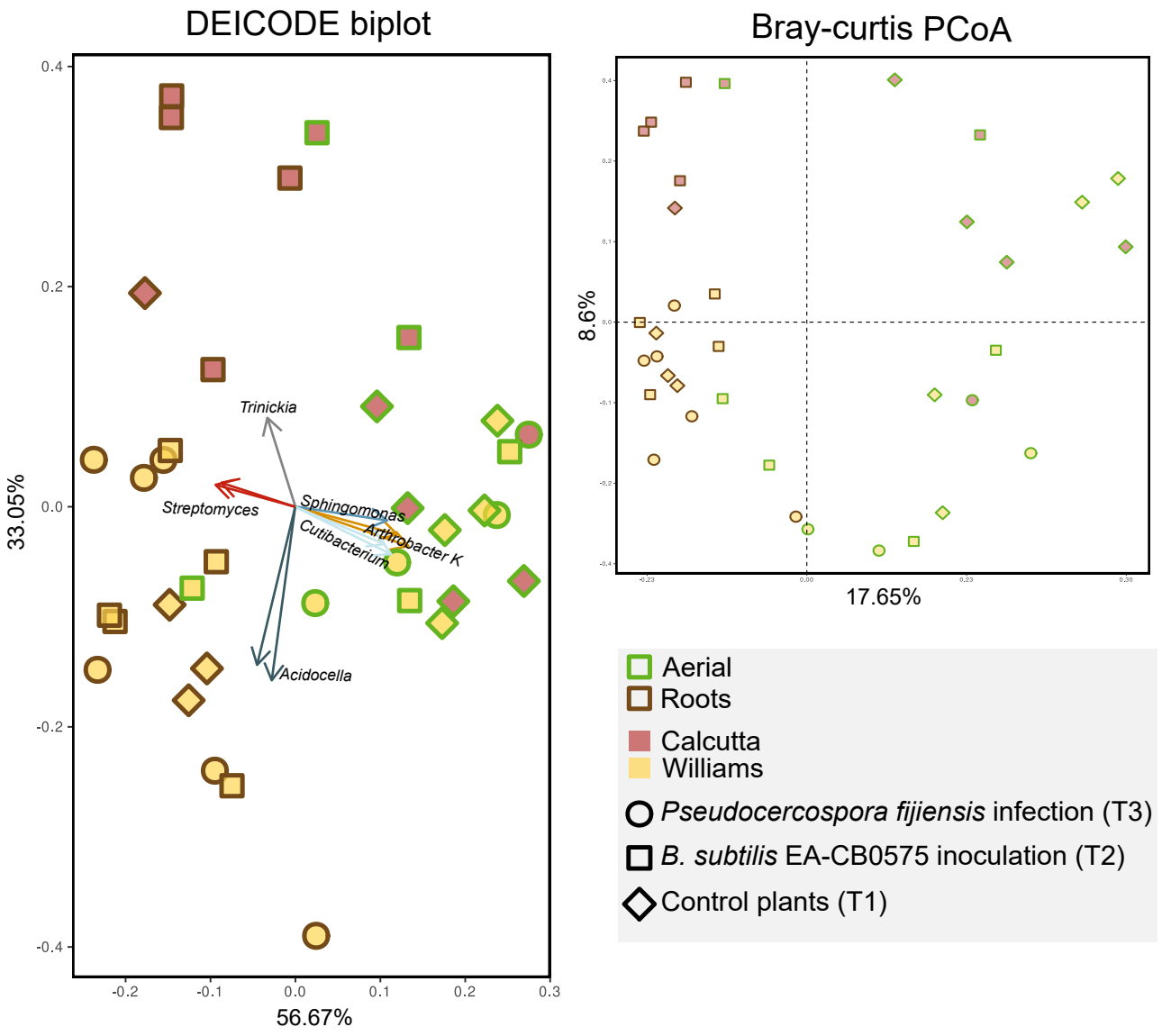

Fig. 3. PCoA of DEICODE-Aitchison and Bray-Curtis distance.

study, ANCOM-BC identified at least 70 genera as differentially abundant, primarily in the root section (Fig. 4). Specifically, 7 genera were identified as DA between varieties: in cv. Williams, *Acidocella*, JABFSM01, JALHMI01, UBA1547, whereas in Calcutta 4, *Kosakonia, Noviherbaspirillum*, and *Sphingomonas* were identified. Furthermore, during infection with *P. fijiensis* (T3), 4 genera were detected as DA: *Azonexus, Panacibacter, Gallionella*, and *Chryseolinea*, alongside unidentified members of families Burkholderiaceae_B, Rhodocyclaceae, and Xanthobacteracae. In plants inoculated with *B. subtilis* EA-CB0575 (T2), 3 genera were found DA: *Flavipsychrobacter, Qipengyuania,* and JALHOQ01. Additionally, 2 genera were identified as DA in plants without any treatment (T1): *Neorhizobium* and *Crossiella*. Finally, SCNIC detected 347 modules with R > 0.35. The top 10 module-forming genera were *Rhizomicrobium* (26 modules), *Chitinophaga* (25), *Sphingomonas* (24), 66–474 (21), *Rhodanobacter* (18), *Flavipsychrobacter* (18), *Dyella* A (18), *Dongia* (17), *Streptomyces* (16), and *Methylobacterium* (16).

3.3.4. *Representation of culture collection in NGS data*

The proportion of NGS data represented by the culture collection was higher for the aerial community than for the rhizosphere community (41 % vs 12 %), and it equally represents both varieties (Fig. 5). Among the 537 ASVs (5.6 %) represented by strains, features matching *B. subtilis* EA-CB0575 at a 0.996 identity score accounted for 2 % in plants without infection or inoculation, 0.2 % in infected plants, and 0.07 % for plants with PGPB inoculation. Furthermore, the proportion of *B. subtilis* EA-CB0575 was higher in Calcutta-4 (2 %) than in Williams plants (0.16 %) and was elevated in the aerial section (1.72 %) compared to the root section (0.02 %). The cultivated organisms belong to 54 genera (excluding the Unassigned category), representing 3.7 % of the genus categories detected in the NGS data. Differentially abundant genera represented in the cultured collection include *Methylobacterium, Curtobacterium, Sphingomonas*, and *Kosakonia.*

4. Discussion

Evidence from various studies conducted over the last 30 years suggest that plants coexist and coevolve with endophytic microorganisms, forming what is known as the holobiont, a term first coined by Margulis (1991) and redefined by Zilber-Rosenberg and Rosenberg (2008). This concept has gained popularity in recent years, with increasing attention in the fields of agroecology, plant biology, and evolution (Cesaro et al., 2021). Derived studies offer opportunities for applying plant microbiota to aid plants in adapting to both biotic and abiotic stresses (Munir et al., 2022; Sandrini et al., 2022). Moreover, as

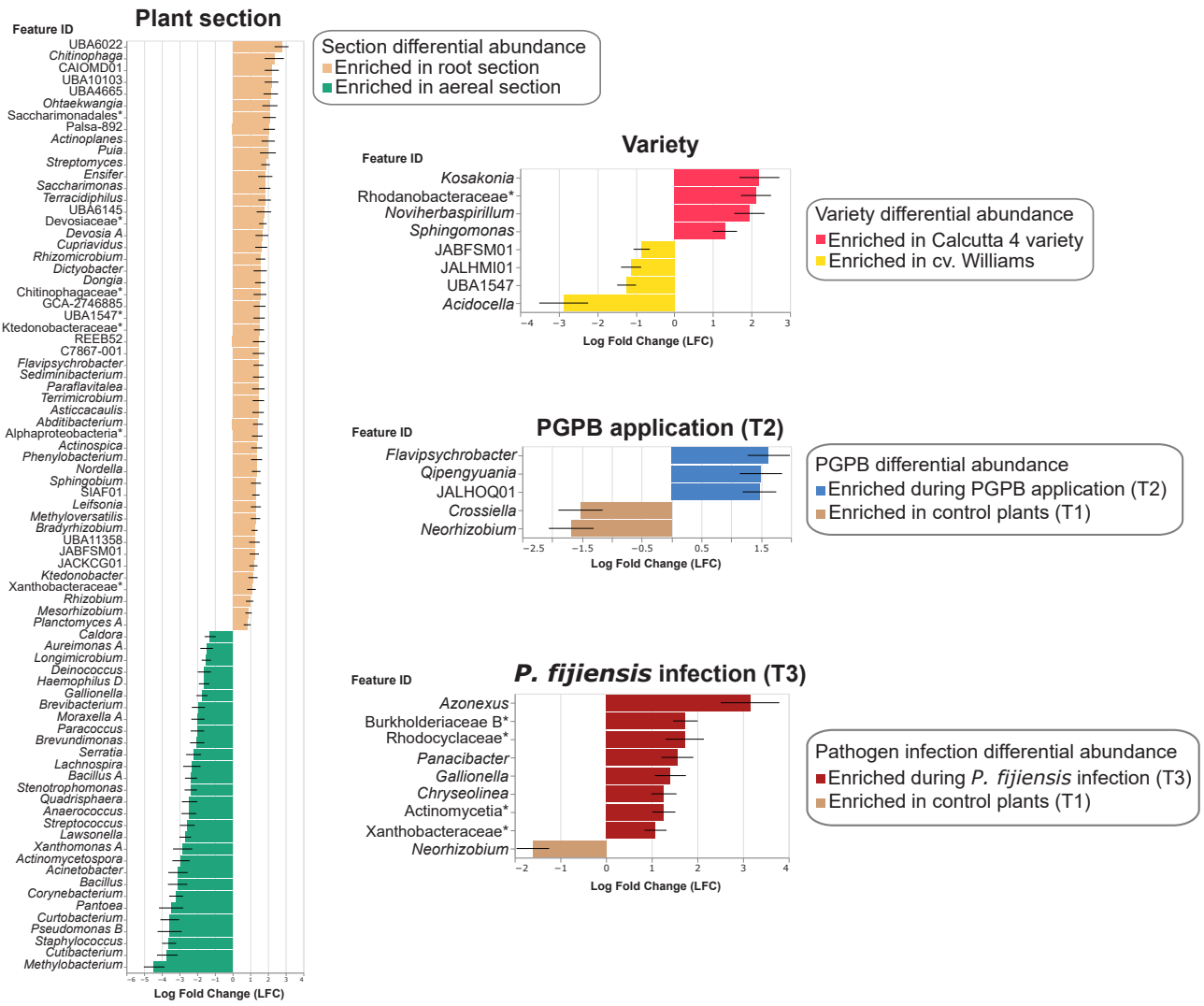

Fig. 4. Log-Fold Change barplot of differentially abundant genera inferred by ANCOM-BC.

demonstrated by Lucaciu et al., (2019) and Liu et al. (2019), the intimate relationship between plants and their microbiota, emphasizes the crucial role of diverse microbial communities in plant survival.

Numerous evaluations have been conducted on plant microbiota using classical methods, but Next-Generation Sequencing (NGS) has significantly enhanced and expedited the analysis of plant-associated microorganisms (Lucaciu et al., 2019; Bertani et al., 2016). Presently, a synergistic approach combining classical and molecular techniques appears to offer a more comprehensive understanding of the plant holobiont (Lagier et al., 2015; Martellacci et al., 2019). A previous study compared culture-dependent and -independent methods in analyzing banana endophytic microbiota (Thomas and Sekhar, 2017), revealing that while a metataxonomic survey detected 269 genera, only seven (2.6 %) were isolated through culture-based methods.

We isolated 54 genera, representing 3.7 % of all genera detected through culture independent methods, 5.6 % of ASVs, and 12–41 % of count data obtained through culture-independent methods. Among the isolated genera were well-known banana endophytes such as *Bacillus, Pseudomonas*, and *Stenotrophomonas* (Nakkeeran et al., 2021), among others. Similarly, a comparison of culturable and unculturable microbiota in two *Populus* species found that isolates accounted for 50 % of ASV sequences and 5 %-56 % of count data, depending on the studied factor (Carper et al., 2021). The study also noted an influence of host species on isolated recovery. Likewise, our findings indicated a higher diversity of isolates from crop wild relative Calcutta 4 compared to cv. Williams collections, suggesting that wild relatives of banana crops could serve as valuable sources of representative isolates. Culturable assessments revealed variations in recovered genera depending on plant variety, section, and growth stage, suggesting that endophyte culturability may be highly influenced by the source plant material.

Numerous studies have been conducted to identify the unculturable microbiome present in banana plants (Birt et al., 2022; Köberl et al., 2015; Rossmann et al., 2012; Singh et al., 2023; Thomas and Sekhar, 2017). However, a comparison between the microbiota of the commercial banana cv. Williams and its wild crop relative *Musa acuminata* subsp. burmannica, Calcutta 4, had not yet been assessed. Previous research on this subject has highlighted significant variations in microbiota composition among plants, varieties/cultivars, and under different conditions such as pH levels, soil types, growth stages, phenological stages, and agronomic management practices in banana farms (Köberl et al., 2015; Rossmann et al., 2012). According to Kauskhal et al. (2020), core genera for the banana variety Sukari Ndizi AAB include *Bacillus, Pseudomonas*, and some free-living diazotrophs such as *Azospirillum* and *Bradyrhizobium*(Kaushal et al., 2020). Rossmann et al. (2012) evaluated different banana varieties in Uganda farms, while Köberl et al. (2015) focused on the banana cultivar *M. acuminata* Colla (AAA group), specifically Gros Michel. Both evaluations identified Gammaproteobacteria as the most prevalent group, comprising

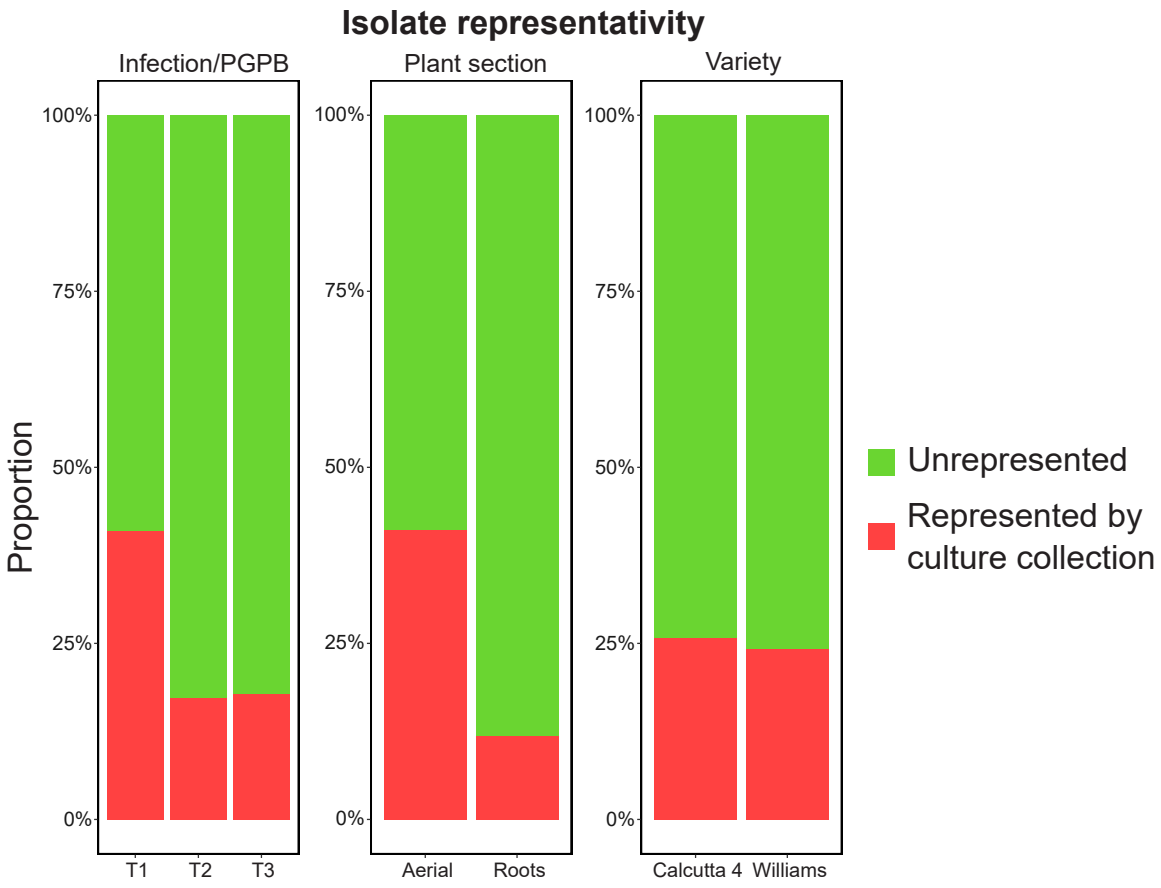

Fig. 5. Stacked barplot of strain representativity in culture-independent dataset.

one-third of the total groups, in bananas. These findings are consistent with our research, where Pseudomonadota emerged as the most abundant phylum. Another study conducted in Colombia, using the same source of commercial Williams banana plant material and employing meta-taxonomic evaluations with the 16 S *rRNA* V3-V4 region, indicated that the core microbiota for Williams banana plants primarily consists of Proteobacteria and Firmicutes (García-Giraldo et al., 2022). However, our study revealed a highly complex and diverse phylum composition of the banana microbiome, as previously highlighted by other works on banana endophytes (Birt et al., 2022; Thomas and Sekhar, 2017). This complexity extends beyond well-known banana endophyte phyla such as Pseudomonadota or Bacillota, encompassing superphyla like PVC, three archaeal phyla, among others.

A recent study investigating banana endophytes and their wild crop relatives highlighted pronounced differences between compartments and host varieties within *Musa* species, indicating a substantial influence of host genotype on endophytic community function (Singh et al., 2023). However, alpha diversity values do not exhibit significant differences when comparing different varieties or species (Birt et al., 2022; Singh et al., 2023). In our study, we observed significant diversity discrepancies driven by plant compartments. Furthermore, no significant differences in alpha diversity were detected when comparing Calcutta-4 variety and cv. Williams. Nonetheless, distinct genera exhibited differential abundance when comparing different varieties.

Previously, *Kosakonia radicincitans* was found to carry several phylosymbiosis genes (Singh et al., 2023), suggesting a strong association of this genus with *Musa* spp. In our study, this genus exhibited differential abundance in crop relative Calcutta 4 plants in comparison to Williams. Additional research indicates that *Kosakonia* may possess nitrogen-fixing abilities in certain plants (Singh et al., 2022), aid plants in resisting abiotic stresses (Petrzik et al., 2021), and inhibit pathogens such as *Ralstonia syzygii* (Suhaimi et al., 2016) and *Fusarium* spp. through ACC deaminase activity (Munakata et al., 2021; Liu et al., 2019). Similarly, the genera *Sphingomonas* and *Noviherbaspirillum* also displayed differential abundance in Calcutta 4 plants in comparison to Williams. *Sphingomonas* is renowned for its capacity to degrade environmental pollutants and has been identified as a core member of the banana microbiota, reported to exhibit antagonistic activity against certain bacterial strains and fungi, fix nitrogen, and produce gibberellins (Birt et al., 2022; Taffner et al., 2020; Singh et al., 2022; Khan et al., 2014). *Noviherbaspirillum*, on the other hand, has been associated with healthy banana plant soil due to its denitrification potential and ability to enhance plant uptake of ferric-siderophore complexes (Fu et al., 2019; Ishii et al., 2017; Peta et al., 2021). However, further research is needed to determine whether any of the aforementioned genera offer protection against *P. fijiensis*. In conjunction with prior studies by Singh et al. (2023), these findings underscore the importance of crop wild relatives as sources of novel microbiome-based bioproducts.

In contrast, in Williams variety plants, only one described genus, *Acidocella*, was found to be differentially abundant, along with three placeholder taxa (UBA1547, JALHMI01, JABFSM01). *Acidocella* genus possesses metabolic potential for producing organic low molecular weight compounds to reduce ferric iron (Degli Esposti et al., 2023), and has demonstrated promise for bioremediation of cycloalkanes and oil-contaminated sites, as well as production of antibacterial metabolites (Eze et al., 2021; Chan et al., 2021). The taxon UBA1547 belongs to Patescibacteriota (or CPR), with relatives within the *Microsaccharimonas* family that are highly dependent on their host due to the lack of many biosynthetic pathways (Lemos et al., 2019). Additionally, this genus has been observed to increase in abundance in sorghum after its own exudate application, suggesting a relationship with plants (Seitz et al., 2024). As for JALHMI01 and JABFSM01, both taxa have rarely been

detected as endophytes, thus their roles remain unclear. JALHMI01 belongs to the PALSA-1355 family, originally detected in a palsa from Swedish peatlands but subsequently found in lichen substrate in Chile (Leiva et al., 2021). JABFSM01 belongs to the GWC2–71–9 family (Gemmatimonadales), primarily found in soils and aquatic environments (Mujakić et al., 2023). In previous studies, an unidentified member of Gemmatimonadales was found to be a core member of the banana microbiome (Birt et al., 2022), which, in conjunction with our results, underscores the importance of further characterizing undescribed members of the banana microbiome.

Azonexus, *Panacibacter* and *Gallionella,* which were differentially abundant in T3 plants, are recognized for their denitrifying capacities through different mechanisms (Chang et al., 2021; Deng et al., 2021; Huang et al., 2024). Furthermore, *Azonexus* is known as a diazotrophic endophyte in sugarcane (Singh et al., 2022) and for its potential phosphate accumulation (Pelevina et al., 2023). *Gallionella* performs autotrophic nitrate-dependent Fe(II) oxidation which is boosted by organic acids and can use both ammonia and nitrate as its nitrogen source (Deng et al., 2021; Kiskira et al., 2017). *Chryseolinea*, also differentially abundant in infected plants, increases its abundance in complex nitrogen sources (livestock manure) (Fan et al., 2023, Shi et al., 2023, Dong et al., 2024), high malic acid concentrations (Ulbrich et al., 2022) and has been associated with lignocellulose biodegradation and humification (Ren et al., 2023). Some *P. fijiensis* strains release ammonia during their colonization of plants as a means to modify the medium pH (Carreón-Anguiano et al., 2023). The aforementioned bacterial genera could be using this nitrogen bonanza as a growth booster but it is unclear if they also benefit or act against *P. fijiensis.* Furthermore, a significant increase in alpha diversity was observed in infected plants (Table 1) suggesting a change in the community structure due to the infection. Similarly, previous studies of *Fusarium* wilt in bananas (Liu et al., 2019), found a higher diversity in plants near or infected by the pathogen. Previous experiments in plants exposed to detrimental conditions have shown the recruiting of sets of microorganisms to alleviate specific adverse consequences and to survive, a phenomenon known as “cry for help” (Beltran-Garcia et al., 2021), which could explain differences observed here. Further studies are needed to fully understand the impact of *P. fijiensis* infection on the banana microbiome.

Additionally, two genera (*Qipengyuania* and *Flavipsycrobacter*) and a placeholder taxon (JALHOQ01) were marked as differentially abundant in T2 plants. *Qipengyuania* genus has been found to decrease after inoculation of nitrogen-fixing bacteria in water yam (Liswadiratanakul et al., 2023). *Flavipsychrobacter* has been found as part of the rhizosphere microbiota in barley with enriched GO terms in response to abiotic stress, protein-containing complex assembly and bioluminescence (Alegria Terrazas et al., 2022). Finally, JALHOQ01 belongs to phylum Bdellovibrionota, Bdellovibrio and like organisms (BALOs), which can degrade biofilms from Gram-positive bacteria and harvest their nutrients (Brescia et al., 2023). Hypothetically, this group might be promoting plant growth through an unexplored approach, as they have the potential to control susceptible bacterial populations, alter microbial community structure, and contribute to biogeochemical cycling (Williams and Chen, 2020). Lastly, *Neorhizobium* and *Crossiella* were differentially abundant in T1 plants. *Neorhizobium* has been found as differentially abundant in *Musa* BB varieties (Singh et al., 2023). *Crossiella* has been recognized to increase after conventional phosphorus fertilization (Deng et al., 2021). Similar to infection, PGPB increased phylogenetic diversity when compared to control plants, but in a lesser way. These results are key to the understanding of plant and PGPB interactions.

Co-occurrence and DEICODE results suggest *Streptomyces* and *Sphingomonas* as key genera as they influence data composition and their relationships with other taxon, both have been identified as core members of the banana microbiome (Birt et al., 2022). SCNIC results suggest a tight relationship between the rhizosphere inhabitants. Additionally, *Flavipsychrobacter* interrelationships and its increase in T2 plants, suggest that PGPB inoculation might increase community complexity. Additionally, some *Acidocella* members were determined as top variants in DEICODE analysis. Further studies are needed to determine the true effects of these relationships and influences, and how they impact or promote banana plant growth.

Related to PGPB abundance after inoculation, the proportion of features matching *B. subtilis* EA-CB0575 was lower in inoculated plants, which was counterintuitive, as we expected to find a higher abundance of *B. subtilis* after inoculation. A limitation of this study is the exclusive use of the 16 S *rRNA* marker, as it is known that it lacks enough resolution to identify individual strains of *Bacillus* sp. (Rooney et al., 2009). Further studies are needed to trace exactly how this PGPB population changes after inoculation.

Additionally, GTDB was chosen because it allowed a detailed classification of ASVs and isolates (Figure S3). This database aims to standardize Bacterial and Archaeal taxonomy through genome phylogeny. It offers comprehensive *16 S* rRNA reference sequences and serves as an up-to-date database, aligning with established references like Greengenes and SILVA. Additionally, GTDB’s efforts enable researchers to accurately classify uncultured microbes, but also contextualize them ecologically and evolutionarily based on their closest phylogenetic relatives (Robeson et al., 2021). Such as REEB32, a placeholder taxon from the SM23–39 family (Chlamydia), differentially abundant in the root community. SM23–39 family have the genetic potential to synthetize chorismate (Davison and Hurst, 2023), perform acetogenic fermentation and use the arginine deiminase (ADI) pathway to produce ATP (Köstlbacher et al., 2021). Nevertheless, the ecological roles of plant-associated chlamydia are still unknown and a matter of ongoing research (Collingro et al., 2020). Further efforts are necessary to isolate and describe many of the place-holder genera here found, in order to understand their role as banana endophytes.

This study highlights the importance of combining both culture-dependent and -independent approaches, for a thorough understanding of microbial dynamics and their pivotal impact on plant fitness in varied environmental contexts. As they delve into the identity and relative abundance of microbial organisms associated with plants under diverse conditions, including phenological stages, biotic or abiotic stress, PGPB inoculation, or soil type (Liu and Howell 2021; Vescio et al., 2021).

The evaluations were conducted using soil traditionally employed during the greenhouse phase, which is crucial for the growth of export-grade bananas in Colombia. We adhered to instructions and recommendations provided by expert staff from the Banana Research Center, Cenibanano, to ensure optimal plant growth and development during the pre-field stages. Nevertheless, there are limitations in the evaluations that should be addressed in future trials. For instance, it is essential to include controls to establish the plant microbiota while using the same soil where Calcutta 4 seeds were originally planted for germination or meristems where developed. This may potentially influence the resulting microbiota. Furthermore, it is suggested to research into the soil’s and substrates effect on the microbiota of the evaluated plants, identifying their impacts during different phenological stages of the cultivation (Wang et al., 2022). The absence of studies in this area presents an opportunity for further investigation, aiming to complement existing research and develop more sustainable practices for banana cultivation on a global scale.

Finally, both cultivable and non-cultivable approaches for evaluating microbiota could potentially benefit from the use of culture media that simulate the conditions of the crop endosphere. This could yield insights into the present microbiota and has the potential to enhance the culturability of this niche, integrating both types of results. Ultimately, this study represents a crucial stride in comprehending microbial composition and identifying candidate-specific groups that may confer a "protective" effect on commercial crops. These taxa could potentially exhibit a correlation with plant fitness and could be transferred from resistant plants to susceptible ones, utilizing the concept of microbiota donor and

recipient. Alternatively, synthetic microbial communities (SynComs) (Marín et al., 2021) could be engineered based on both culturable and non-culturable approaches, thereby paving the way for the production of bioinoculants aimed at modulating the microbiomes of economically significant crops.

Funding

This work was supported by convening between the EAFIT University "project code C145I0006900000880–1 with AUGURA (Banana Growers Association of Colombia) and MINCIENCIAS (previously, Colciencias) postdoctoral grant 784 of 2017.

CRediT authorship contribution statement

Sebastián Zapata: Writing – review & editing, Methodology. **María Cadavid:** Writing – review & editing, Writing – original draft, Visualization, Validation, Data curation. **Luis A. Arteaga-Figueroa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Isabel Adarve-Rengifo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Luisa Fernanda Posada Uribe:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Javier Alvarez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

Data will be made available on request.

Acknowledgments

We are very grateful to professor Bernardo Villegas Estrada from Universidad Nacional de Colombia, Manizales for donating Calcutta 4 banana material. We also want to give special thanks to the Apolo Scientific Computing Center who provided the computational resources for this work, to the technical support of AUGURA's Team and to the academic staff from EAFIT University, who facilitated the infrastructure and resources to achieve this work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127862.

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