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Decoding microbial diversity in roots of rice plants under flooded conditions: influence of the host genotype, root compartment, and mycorrhizal association

Iratxe Busturia,[1] Héctor Martín-Cardoso,[1] Concha Domingo,[2] Antoni Garcia-Molina,[1] Blanca San Segundo[1,3]

AUTHOR AFFILIATIONS See affiliation list on p. 28.

ABSTRACT Rice is a vital staple for more than half of the world's population, uniquely adapted to grow under flooded conditions. Our current understanding of root-associated microbiomes in rice plants under flooded conditions (anaerobic rice) is, however, limited. While rice plants are capable of establishing symbiotic associations with arbuscular mycorrhizal (AM) fungi, also under flooded conditions, little is known about the impact that mycorrhizal associations might have on the root microbiome. Here, we used metagenomic sequencing to characterize the root-associated microbiomes of cultivated rice grown under flooded conditions. The root endosphere and rhizosphere compartments were found to harbor distinct microbial communities, with bacterial taxa comprising approximately 95% of the total microbial community. At the phylum level, the root bacteriome was primarily composed of Pseudomonadota (Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria), followed by Actinomycetota. The fungal microbiome was dominated by Ascomycota (Sordariomycetes, Eurotiomycetes, and Dothideomycetes) and Basidiomycota. Both root compartment and host genotype significantly influenced microbiome composition, with most genotype-specific recruitment occurring at the species level. These associations were further reshaped in plants pre-inoculated with the AM fungus *Rhizophagus irregularis*. Co-occurrence networks revealed a genotype-dependent effect in the complexity of microbial taxa in mycorrhizal rice plants. Key microbial species primarily involved in methane production and nutrient cycling (e.g., phosphate-solubilizing bacteria and nitrogen-cycling bacteria) residing in roots of flooded rice were identified. Functional analysis of gene abundance related to those microbial communities revealed compartmentalization, with nutrient-cycling bacteria being more prevalent at the endosphere. Overall, this study demonstrated that the rice genotype, root compartment, and mycorrhizal condition shape the root microbiome in rice plants, ultimately influencing holobiont dynamics in flooded rice fields.

IMPORTANCE Plant roots harbor a large diversity of microorganisms that strongly influence plant health and improve stress tolerance. Among them, root colonization by arbuscular mycorrhizal (AM) fungi enhances mineral nutrition and mitigates environmental stress. Although most rice cultivated worldwide is grown under anaerobic conditions (flooded fields), investigation of microbial communities inhabiting root-associated compartments of flooded rice, including AM fungi, has received only little attention. Here, we demonstrated that the rice genotype, root compartment, and mycorrhizal condition markedly influence the microbiome in roots of rice plants in flooded conditions, thus illustrating the potential of the rice plant to shape its root-associated microbiome. Differential root-associated microbiomes of commercial rice varieties include microbes primarily associated with methane production and nutrient cycling. Elucidation of factors influencing root-associated microbiomes in flooded rice systems,

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Address correspondence to Blanca San Segundo, blanca.sansegundo@csic.es, blanca.sansegundo@cragenomica.es, or Antoni Garcia-Molina, antoni.garcia@cragenomica.es.

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including their association with AM fungi, offers practical guidance for exploiting root-associated microbial communities for sustainable rice cultivation while reducing reliance on agrochemicals.

KEYWORDS methanogenic archaea, *Oryza sativa*, phosphate-solubilizing bacteria, nitrogen-cycling bacteria, *Rhizophagus irregularis*, root-associated microbiomes, shotgun metagenomic sequencing

In natural and agricultural environments, plants and soil microbes engage in complex dynamic interactions that might have different effects on the host plant. These associations can be beneficial to the host plant by facilitating nutrient uptake and improving plant health (1). The persistent microbiome associated with a plant in a particular environment is also expected to enhance plant resilience to abiotic and biotic stresses (2). Along with this, plants can no longer be viewed as autonomous entities but rather as more complex entities composed of the host plus the associated microbes.

Rice serves as a crucial staple food for a large part of the world's population. To meet the requirements of the increasing global population, there is a growing need to increase rice production (3). Over the past decades, there has been an enormous increase in the use of fertilizers and pesticides to optimize rice yield, which is also the principal cause of pollution in major rice-producing regions. However, the overuse of fertilizers contributes to soil degradation, eutrophication in aquatic ecosystems, loss of biodiversity, and imbalance in microbial community structures. Harnessing the plant microbiome offers new opportunities to improve plant nutrition and health for the development of sustainable rice cultivation systems (4–6).

Unlike other cereals, rice is usually grown under flooded conditions (or paddy fields). The rice plant must select microorganisms from the environment to assemble its own root microbiome, and, accordingly, the composition of the rice root microbiome is greatly influenced by flooding (7). Rice paddies create a unique anaerobic environment ideal for specific microorganisms, such as methanogenic archaea, which provide a source for methane production. Indeed, flooded rice fields contribute to a considerable portion of agricultural methane emissions (8).

Microbial communities involved in nutrient acquisition or nutrient cycling are particularly important for healthy plant growth (9). Cycling of nutrients, e.g., carbon (C), nitrogen (N), and phosphorus (P), represents an important part of the ecosystem function by facilitating the conversion of organic matter into accessible forms of essential elements. Regarding P, only a small fraction of P in agricultural soils is available for assimilation by plants due to immobilization of this element in different complexes (10). However, certain microorganisms can make P bioavailable to plants by hydrolyzing organic and inorganic P compounds into soluble forms. Phosphate-solubilizing microorganisms include diverse bacterial genera, referred to as phosphate-solubilizing bacteria (PSB), such as *Bacillus*, *Pseudomonas*, *Rhizobium*, *Enterobacter*, or *Burkholderia*, among others (10–15). Certain bacterial genera are also considered to be major drivers of N-cycling processes (herein referred to as NCyc bacteria) in aquatic ecosystems, such as *Rhizobium* species. Unfortunately, our understanding of bacteria involved in either Pi solubilization or nutrient cycling in paddy rice fields is still limited.

Beyond conventional agronomic management and genetic improvement, biologically based approaches can also be explored to improve plant stress adaptation in plants. Among them, the use of plant-associated microorganisms such as arbuscular mycorrhizal (AM) fungi is increasingly viewed as an important functional component in plant stress adaptation. AM fungi improve plant nutrient acquisition, mainly P and N, and enhance resistance to biotic and abiotic stress (16). Although AM symbiosis has been proven to have a great impact on plant growth and productivity in plants grown in aerobic soil, the benefits received by the AM symbiosis in flooded plants (anaerobic conditions) remain largely unexplored (6). Previous studies revealed that inoculation of rice plants with an AM fungus (e.g., *Rhizophagus irregularis* and *Funneliformis mosseae*) increases

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productivity and enhances disease resistance (17–19). Compared to aerobic conditions, flooding generally reduces the level of root colonization in rice. However, once the AM fungus has penetrated into the root, the AM symbiosis remains functional in flooded rice (20, 21). At present, however, it is unclear whether root colonization by an AM fungus has an impact on root-associated microbial communities. Ultimately, interactions between rice roots and soil microbes and microbe–microbe interactions would determine plant performance and microbial ecological functions in flooded rice fields.

Efforts carried out so far to decipher microbiome assemblages in rice demonstrated remarkable diversity and dynamics (6, 7, 22–29). Microbial communities in root-associated compartments—endosphere and rhizosphere—might also vary, depending on the geographical location, soil type, management practices, environmental factors, and/or host genotypes (4, 30). In soil-grown rice plants, a greater proportion of *Proteobacteria* and *Spirochaetes* occurred in the endosphere compared to the rhizosphere (31). Differences in the abundance of Beta- and Gammaproteobacteria (phylum Pseudomonadota) between the rhizosphere and root endosphere were also observed in rice varieties grown in different geographical locations in China, with Gammaproteobacteria being the most dominant taxa in the root endosphere (26). Remarkably, most studies so far carried out to investigate root-related microbiomes in rice have been conducted on plants grown under aerobic environments, while few studies have been conducted in rice plants growing in paddy fields (32). Furthermore, most studies on the characterization of rice microbiomes used metabarcoding amplicon sequencing (16S rRNA gene) (23, 25–27, 31, 33–35). Unlike amplicon sequencing, shotgun metagenomics captures total genomic DNA without target-specific PCR amplification, thereby providing more comprehensive information on the entire microbial community's taxonomic composition. Presumably, root microbial communities might have been selected by flooded rice cultivation; hence, understanding microbial diversity and functions of microbial communities in flooded rice ecosystems will help in developing strategies to enhance health and productivity in rice. This knowledge is also a requisite for the development of effective sustainable agricultural practices in rice cultivation.

To fill the gap in knowledge on microbial communities colonizing roots of rice plants under flooded conditions, we used shotgun metagenomics. The structure of root-associated microbial communities in the endosphere and rhizosphere communities of two temperate *japonica* rice varieties (Bomba and JSendra, commercial varieties) grown in paddy fields was systematically analyzed. A previous study performed in the same paddy field documented that these varieties are efficiently colonized by the AM fungus *Rhizophagus irregularis* (17, 19). Thus, pre-inoculation with an AM fungus followed by a short period of aerobic growth before transitioning the plants into their final flooded condition was found to allow efficient colonization of the host plant's roots (19). Accordingly, in this work, we investigated the effect of pre-inoculation of rice seedlings with the AM fungus *R. irregularis* on the microbial communities colonizing the root compartments in plants grown in paddy fields. Microbial taxa involved in P solubilization and N cycling in each root compartment and condition (e.g., *R. irregularis*-inoculated and mock-inoculated roots) were identified. A functional analysis of microbial genes identified in metagenomic sequencing data allowed us to infer metabolic functions of microbial communities in root compartments. Results obtained in this work demonstrate that the root compartment, genotype, and mycorrhizal status of the rice plant are key factors influencing root-associated microbiomes in flooded rice.

MATERIALS AND METHODS

Plant material, inoculation with *R. irregularis*, and field trials

The commercial rice varieties Bomba and JSendra (*O. sativa* and *japonica* cultivars) were used in this study. These varieties are typically grown in paddy fields in the major rice-producing regions in Spain. Rice seeds were germinated on petri dishes with sterile water for 7 days to ensure that all seeds germinated. The rice seedlings were then

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transplanted to seedling beds filled with AM/inert soil mixture (19). For inoculation of rice seedlings with the AM fungus, a commercial inoculum of *Rhizophagus irregularis* (MycAgro, Bretenière, France; http://www.mycagrolab.com) was used. The inoculum consisted of mineral inert solid particles and propagules of *R. irregularis* (spores, mycelium, and mycorrhizal root pieces; 10 propagules/g dry mass). No inoculum was added to the substrate for the control plants (referred to as mock-inoculated). The *R. irregularis*-inoculated and mock-inoculated seedlings were aerobically grown in nurseries (seedling beds) for 5 weeks and then transplanted to flooded fields as previously described (19). The rice plants continued growth under permanent flooding until harvest time.

Field trials were conducted in a traditional rice-producing region of eastern Spain (Valencia, 39°15′46.9″ N, 0°20′41.2″ W). The rice field had a long history of rice cultivation (e.g., more than 200 years). To minimize the risk of variations in environmental or geographical factors potentially affecting the root microbiome (e.g., soil type and management practices), the plants were grown in the same paddy field. Soil analysis was carried out via standard methodologies at VerchiLAB (https://verchilab.com/). Mock-inoculated and *R. irregularis*-inoculated plants were grown until maturity (May–September 2024). Three plots (each plot containing 30 plants) were established in the same field for each variety (Bomba, JSendra) and condition (*R. irregularis* and mock inoculated), maintaining a distance of 4 m between non-mycorrhizal and mycorrhizal plots (Fig. S1A). The experimental field was exclusively used for rice cultivation.

The soil structure and chemical properties of the experimental field are presented in Table S1 (a field photo of the soil is presented in Fig. S1B). To facilitate the establishment of the symbiotic association, the available phosphorus in the soil was low (14.2 mg/kg) (Table S1). Neither fertilizers nor pesticides were applied to the rice plants.

Sample collection

The rice plants were grown under continuous flooding conditions until harvesting time under the regular water management system used in the rice-growing region. At the harvest time, the standing water level was reduced to approximately 5 cm (from water depth of 10–12 cm), keeping the rice fields shallowly flooded during sample collection. The two rice varieties were harvested at the same maturation time, depending on the growth cycle of each variety. As Bomba has the shortest life cycle (about 7 days less), Bomba samples were harvested 7 days earlier than JSendra samples. Samples were collected from two niches: root endosphere and rhizosphere. For the metagenomic analysis of the root endosphere, three samples from each experimental plot were examined (each sample was derived from a pool of roots from two rice plants). A total of 36 endosphere samples and the corresponding 36 rhizosphere samples were examined (two genotypes × two conditions × three biological replicates × three plots). The roots were roughly shaken to remove non-adherent soil and washed with water to remove soil sediments and debris. For preparation of the endosphere samples, the root system was initially washed with abundant tap water to remove loosely attached soil. The roots were then subjected to successive washes in sterile water with gentle shaking to wash off the rhizosphere soil. Sonication or vortexing with glass beads was not performed for the preparation of the endosphere samples to avoid the fine lateral rice roots being damaged or lost. For preparation of the rhizosphere samples, the rhizosphere soil fraction was pelleted by centrifugation. Rhizosphere and root endosphere samples were pulverized before DNA extraction.

Analysis of root colonization by *R. irregularis*

To confirm root colonization by the AM fungus, root fragments were stained with cotton blue (0.1% cotton blue in acid lactic), destained, and examined microscopically as previously described (19). Estimation of AM colonization in root fragments (20 root fragments) was carried out according to the Trouvelot method (36). Root colonization by the AM fungus was verified for all inoculated groups in nursery-grown plants (at 5 and 10

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weeks after AM inoculation) and field-grown plants (at the harvest time). For molecular detection of *R. irregularis* in rice roots, DNA was extracted using MATAB as the extraction buffer (0.1 M of Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, and 2% MATAB, containing 1% PEG 6000 and 0.5% sodium sulfite) according to reference 37. *R. irregularis* was detected using PCR with specific primers for the 28S rRNA gene of *R. irregularis* (forward, 5′-TTCGGGTAATCAGCCTTTCG-3′; reverse, 5′-TCAGAGATCAGACAGGTAGCC-3′) (19). The PCR products were analyzed on 1% agarose gel, and their sequence was confirmed by DNA sequencing.

Metagenome sequencing

Shotgun sequencing was used to examine the root endosphere and rhizosphere microbiomes of rice roots. DNA from endosphere samples was extracted as described above. Extraction of DNA from rhizosphere samples was carried out using the Nucleo-Spin Soil Kit (Macherey-Nagel, Düren, Germany). A total of 36 endosphere samples (three experimental plots × three replicates for each plot × two genotypes × two conditions) and 36 rhizosphere samples (three plots × three replicates for each plot × two genotypes × two conditions) were examined.

For library preparation, the genomic DNA was randomly fragmented by sonication, and the obtained fragments were then end-repaired, A-tailed, and ligated with Illumina adapters, followed by PCR amplification using P5 and P7 primers (P5, 5'- AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3′; P7, 5′-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGATGACTATCTCGTATGCCGTCTTCTGCTTG-3′). The resulting PCR products were size selected, PCR amplified, and purified with an AMPure XP system. The libraries were examined for size distribution and quantified through Qubit and real-time PCR. Quantified libraries were sequenced (2 × 150 bp paired-end sequencing) on the Illumina platform at Novogene (Cambridge, UK) (depth of 6 Gb/library).

Bioinformatic and statistical analysis

Quality check of raw sequencing data was performed using FastQC (v0.12.1) (38), and adaptor sequences were trimmed with fastp (v0.24.0) (Q = 20, minimum read length = 30) (39). Trimmed and filtered reads were mapped to the *Oryza sativa* var. Nipponbare reference genome IRGSP-1.0 (https://plants.ensembl.org/Oryza_sativa/Info/Index) using Bowtie 2 (v2.5.4) (40). Positive hits with the *O. sativa* genome were removed to produce a final set of clean sequencing data. Metagenomes were assembled independently for each sample. The *R. irregularis* genome assembly ASM43914v3 (https://fungi.ensembl.org/Rhizophagus_irregularis_daom_181602_gca_000439145/Info/Index) and the *Xanthomonas oryzae* pv. *oryzae* and pv. *oryzicola* (assemblies ASM435582v1 and ASM837083v2, respectively; https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_004355825.1/ and https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_008370835.2/) were used to map and identify reads from the AM fungus and both pathovars of *Xanthomonas oryzae* in the various metagenomic data sets.

Taxonomy assignment of metagenomic sequencing reads was done with Kraken2 (v2.1.5) software and the Kraken core nt database (41). Bracken (v3.1) (Bayesian reestimation of abundance with KrakEN) was used to estimate the relative abundances of microbial taxa at the species level (42). Reads classified as bacterial and fungal reads were analyzed in R (v4.3.3) with functions of R packages (phyloseq v1.52.0, ggplot2 v4.0.0, tidyverse v2.0.0, vegan v2.7-1, microbiome v1.31.3, and pairwise Adonis v0.4.1) (43–47).

Clean reads from each sample were mapped to the contigs independently assembled with MEGAHIT (v1.2.9) (48) using Bowtie2 (v2.5.5) (49). Binning was then performed with MetaBAT2 (v2.17) (50) using default parameters on contigs longer than 1,500 bp. Protein-coding genes were predicted on assembled contigs with Prokka (v1.14.6) (51), and gene abundances were quantified by mapping reads to predicted genes using featureCounts (v2.1.1) (52). Predicted genes were functionally annotated using

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eggNOG-mapper (53), from which KEGG Orthology (KO) assignments were obtained and used for pathway enrichment and functional profiling analyses. Pathways with a false discovery rate (FDR) of <0.05 were considered significantly enriched.

Principal coordinate analysis (PCoA) was performed using the functions from the phyloseq R and microbiome packages using Bray–Curtis distances (44). Homogeneity of dispersion between groups of samples was tested with *betadisper* and a non-parametric test for significance with *permutest* functions. A Permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis distances using the *adonis2* function was performed to evaluate statistically significant differences between groups.

Shannon, Simpson, Chao1, and abundance-based coverage estimator (ACE) measurements were used to evaluate α-diversity indices in the bacterial and fungal communities across samples. Shannon and Simpson measurements not only reflect the number of species in the sample (richness) but also the abundance distribution of each species in the sample (evenness) (54, 55). Differences between samples were tested with a Kruskal–Wallis test followed by a post hoc analysis with Dunn's test adjusted by the Holm method. Hierarchical clustering heat maps were used to illustrate differences in the abundance of species across the different samples. Here, a $\log_2(x + 1)$ transformation was done, followed by the min–max normalization method (56). Sankey diagrams were produced using the Pavian R package (v1.2.1) (57).

Co-occurrence networks of the top 200 most abundant bacterial and fungal species were performed using the ggClusterNet R package (v2.0) (58). Networks were constructed based on Spearman's rank correlation coefficients. Only significant correlations ($P < 0.05$) were used for visualization and network analysis. Differential abundance analysis of microbiome data was carried out at the species level by Analysis of Compositions of Microbiomes with Bias Correction 2 (ANCOM-BC2) (v2.10.0) (59). ANCOM-BC2 is considered a conservative method for the analysis of differential abundance in microbiome studies designed to effectively control the FDR. Changes in relative abundance between two groups of samples with biological consistency were determined using multiple testing correction according to the Holm–Bonferroni method (adjusted $P < 0.05$). Statistical analysis of differential abundance at the species level within compartments was conducted according to linear mixed models for the effect of the variety, mycorrhizal treatment, and their interaction ($P < 0.05$), followed by Tukey's post hoc test for pairwise comparisons (adjusted $P < 0.05$).

RESULTS

Overall microbial community composition in root-associated compartments of rice plants

Shotgun metagenomics was employed to characterize the microbial community colonizing the root endosphere and rhizosphere of the rice varieties Bomba and JSendra (*japonica* rice cultivars) plants grown in paddy fields. Root colonization by AM fungi in this experimental field was confirmed (as discussed below).

A total data set of 272.84 Gb was generated from endosphere and rhizosphere samples. After trimming the reads, 198.9 Gb (1,313.29 million, 150-bp-end reads) passed the quality control parameters (Table S2). Removal of host reads yielded a final set of clean reads from the different samples, which were assembled independently (1,313,291,149 clean reads in total).

Taxonomic classification of metagenomic data sets was carried out using Kraken2 (v2.1.5). Bacterial taxa greatly exceeded fungal taxa across samples (Fig. S2). On average, 94%–96% of the total microbiota corresponded to bacteria, while fungal taxa represented 2%–3% of the microbial community. The complete list of species identified in each metagenomic data set, along with its abundance and the statistical analyses, is presented in Table S3.

α-Diversity using the Shannon index allowed the estimation of community diversity in bacterial and fungal populations in samples not inoculated with the AM fungus. The bacterial community in the endosphere of Bomba and JSendra displayed significantly

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higher Shannon indices compared to the rhizosphere, indicating higher species richness and evenness (Fig. 1A, left panel). Within the endosphere, the bacterial community in Bomba was more diverse than in JSendra, supporting the distinct capacity of rice genotypes to shape the bacterial community at this root level (Fig. 1A, left panel). Shannon diversity indices also indicated minimal differences between genotypes in the rhizosphere, revealing a high similarity in the bacterial community colonizing this compartment (Fig. 1A, left panel). As for the fungal community, Shannon indices were barely affected, indicating that neither the compartment nor the rice genotype could influence the fungal microbiome in the root endosphere (Fig. 1A, right panel). Simpson diversity index measurements indicated significantly higher values in the endosphere bacterial community than in the rhizosphere bacterial community, while no statistical differences were observed in ACE and Chao indices (Fig. S3A). However, an effect of the compartment was observed in ACE and Simpson indices in the fungal community, with higher values in the endosphere (Fig. S3B).

PCoA with Bray–Curtis distances was used for unsupervised examination of microbial β-diversity to verify (dis)similarity in microbial community structure between different samples. The principal coordinate 1 (PCo1, explaining 81.6%–81.5% of the total variance in bacterial and fungal communities, respectively) mainly separated root endosphere from rhizosphere communities (P = 0.001, PERMANOVA; F = 53.858 for bacteria, F = 19.644 for fungi) (Fig. 1B). For the bacterial communities, the PCo2 (11.4% total variance) further discriminated the rice genotype at the endosphere but not in the rhizosphere microbiome (Fig. 1B, left panel).

Taken together, these results suggest that rice plant genotype plays a key role in microbiome selection, exerting a stronger effect on bacterial communities than on fungal communities. However, the root compartment seems to have a much stronger effect on the microbial community assembly than the rice genotype.

Comparative study of the root endosphere and rhizosphere microbiomes in rice varieties

The distribution of taxa in our metagenomic data sets was assessed per compartment and genotype in samples that have not been inoculated with the AM fungus. The most prevalent classes in the bacterial community across the two compartments were Actinomycetes, Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria (Fig. 1C, left panel). However, the proportions varied among genotypes and compartments (Fig. 1C, left panel). For instance, the proportion of Actinomycetes at the endosphere was half of that at the rhizosphere in both genotypes, while Betaproteobacteria were 1.5- to 2.5-fold more abundant in the endosphere compartment. In the fungal community, the dominant taxa at the class level in all the samples were Sordariomycetes, followed by Eurotiomycetes and Dothideomycetes, with their relative abundance also varying among compartments (Fig. 1C, right panel). Thus, the representation of Sordariomycetes was half in the rhizosphere compared to the root endosphere in both rice varieties, while Eurotiomycetes were more abundant in the rhizosphere compartment (Fig. 1C, right panel). Between species, significant differences in abundances of 2,591 and 168 bacterial and 27 and 5 fungal species were observed in the endosphere and rhizosphere, respectively (adjusted $P \leq 0.05$, linear mixed model with Tukey's post hoc test; Fig. S4). For both bacteria and fungi, a cluster specific for the rhizosphere could be observed (Fig. S4). The endosphere presents not only a cluster with common species between varieties but also a cluster with variety-specific species. Our analysis supported the conclusion that compartment type is a major factor shaping the composition of microbial species in rice roots and that genotype-specific associations among microbial species occur. For a better understanding of microbial diversity in root-associated compartments of Bomba and JSendra plants, subsequent analyses were carried out at the species level using Sankey diagrams.

Sankey visualization revealed differences among bacterial and fungal taxa colonizing each root compartment of Bomba and JSendra roots. Kraken software was used for first

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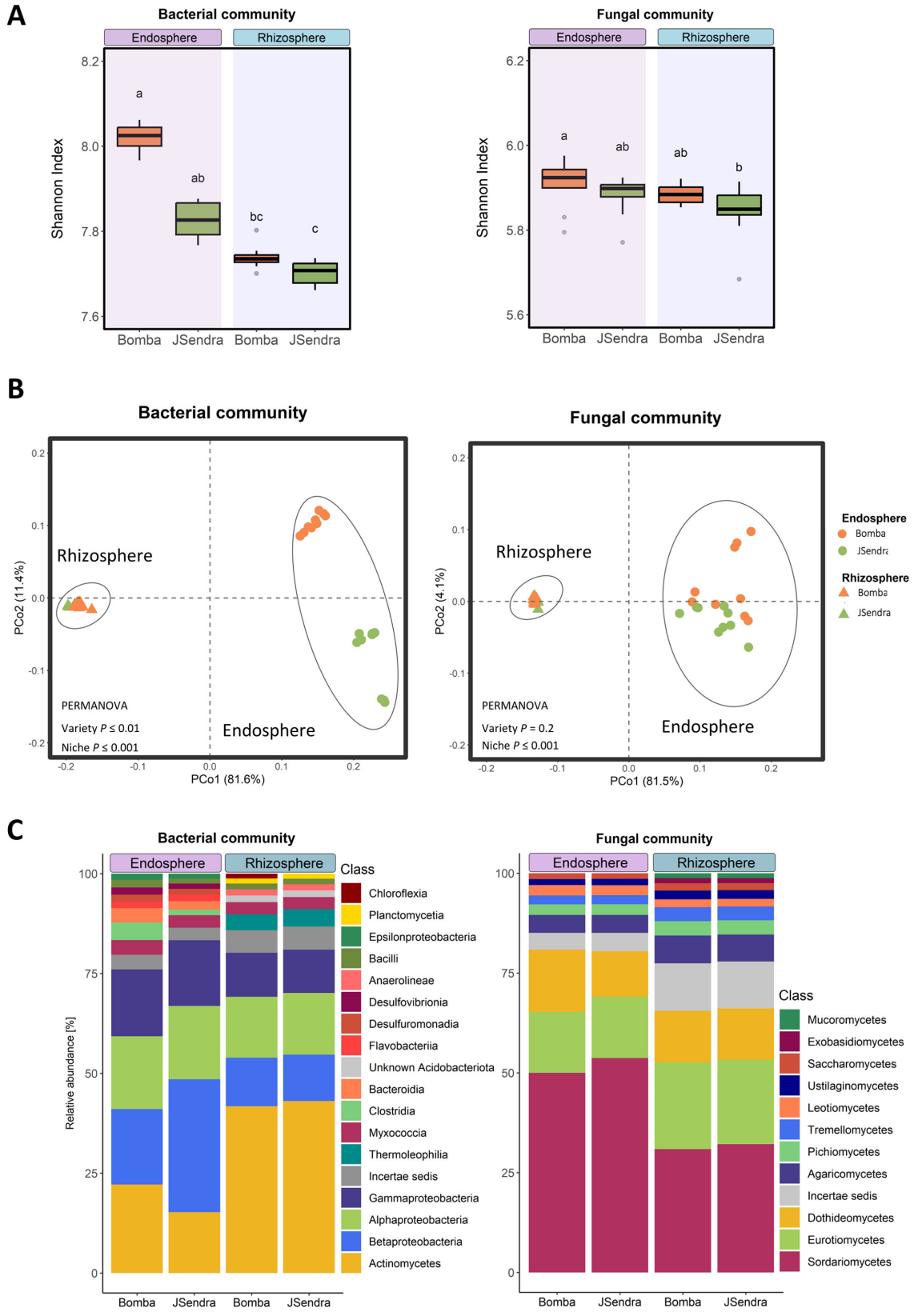


FIG 1 Influence of the root compartment and rice genotype on bacterial and fungal communities in rice. Plants (cv. Bomba and JSendra) were grown in rice fields under flooded conditions as previously described (19). For each variety, two sets of plants were grown in parallel. One set of plants was pre-inoculated

(Continued on next page)

Fig 1 (Continued)

with the AM fungus *R. irregularis* before transplanting to the rice field, whereas the other set of plants was mock-inoculated (19). Roots were collected at harvest time. For each compartment, the bacterial and fungal microbiomes were examined separately. (A) Shannon index (α-diversity) of bacterial and fungal communities in the root endosphere and rhizosphere of rice (cv. Bomba and JSendra) plants in non-inoculated plants. Different letters indicate statistically significant differences among samples (Dunn's test, $P < 0.05$). (**B**) Principal coordinate analysis of β-diversity, measured by Bray–Curtis distances of bacterial (left panel) and fungal (right panel) communities in the endosphere (circles) and rhizosphere (triangles) of non-inoculated Bomba and JSendra plants. A PERMANOVA based on Bray–Curtis distances was performed to evaluate statistically significant differences between groups. Ellipses were added manually for visual emphasis. (C) Relative abundance of microbial taxa at the class level in the root endosphere and soil rhizosphere of Bomba and JSendra plants (non-mycorrhizal condition). Taxa that exhibit a relative abundance of >1% are presented.

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taxonomic assignment, followed by Bracken software for genus and species assignment. Considering the bacterial microbiome, Pseudomonadota and Actinomycetota were the most abundant phyla in the endosphere of the two rice genotypes, followed by Bacillota and Bacteroidota (Fig. 2; Table S4). Pseudomonadota accounted for approximately 46.59% and 60.83% of all bacteria in the endosphere of Bomba and JSendra, respectively. In the endosphere of JSendra, Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria accounted for 29.58%, 16.35%, and 14.66 % of all microbes, while these classes showed a similar abundance in the endosphere of Bomba plants (16.25%, 15.7%, and 14.42%, respectively) (Table S4). *Hydrogenophaga* species (class Betaproteobacteria) were among the most abundant species in the endosphere of JSendra but not in Bomba (Fig. 2). *Hydrogenophaga* consists of bacteria capable of using hydrogen as an energy source and is related to hydrogen autotrophic denitrification (60). *Sulfurospirillum* sp. (class Epsilonproteobacteria) was identified among the most abundant species in the endosphere of Bomba but not in JSendra (Fig. 2). *Sulfurospirillum* species are generally found in contaminated environments and participate in carbon, sulfur, nitrogen, and halogen element cycling. They are considered candidates for bioremediation of environments polluted with chlorinated compounds, arsenic, or nitrate (61). It will be of interest to investigate whether root colonization by *Sulfurospirillum* provides an advantage to Bomba plants in growing in contaminated fields. Bacterial species in the Bacillota and Bacteroidota phyla were less represented in the endosphere of Bomba roots than in JSendra roots (Fig. 2; Table S4).

The most predominant fungal phyla colonizing the endosphere of rice roots were Ascomycota (80.5% and 80.15% of all fungi in Bomba and JSendra, respectively), followed by Basidiomycota (9.31% and 9.57% of all fungi in Bomba and JSendra, respectively) (Fig. 3; Table S4). Fungal species in the endosphere included potential pathogenic fungi for rice. For instance, *Alternaria alternata* was among the most abundant fungal taxa in the endosphere of Bomba roots but not in the endosphere of JSendra roots (Fig. 3). *Fusarium oxysporum*, *Nigrospora oryzae*, *Pyricularia oryzae*, *Pyricularia grisea*, and *Bipolaris oryzae* were identified in the endosphere of both varieties (Fig. 3). *Thermothelomyces* and *Thermothielavioides* species were also among the most abundant fungal species in the endosphere in both rice varieties. Compared with the bacterial microbiome, the host genotype appears to have a weaker effect on the fungal community in the endosphere in both Bomba and JSendra.

When examining the rhizospheric microbial communities, the bacterial community was dominated by taxa in the phyla of Pseudomonadota (accounting for 33.57% and 32.92% of bacteria in Bomba and JSendra plants, respectively) and Actinomycetota (41.51% and 42.75% of bacteria in Bomba and JSendra, respectively) (Fig. 4; Table S4). At the species level, distinct taxa were specifically enriched in the rhizosphere of one or another rice variety. For instance, the plant growth-promoting bacterium *Delftia acidovorans* (phylum Pseudomonadota) was identified among the most abundant species in the rhizosphere of Bomba but not in the rhizosphere of JSendra. *D. acidovorans* plays a role in bioremediation in rice paddies contaminated with heavy metals (62). The rhizosphere fungal community was dominated by Ascomycota in both rice varieties,

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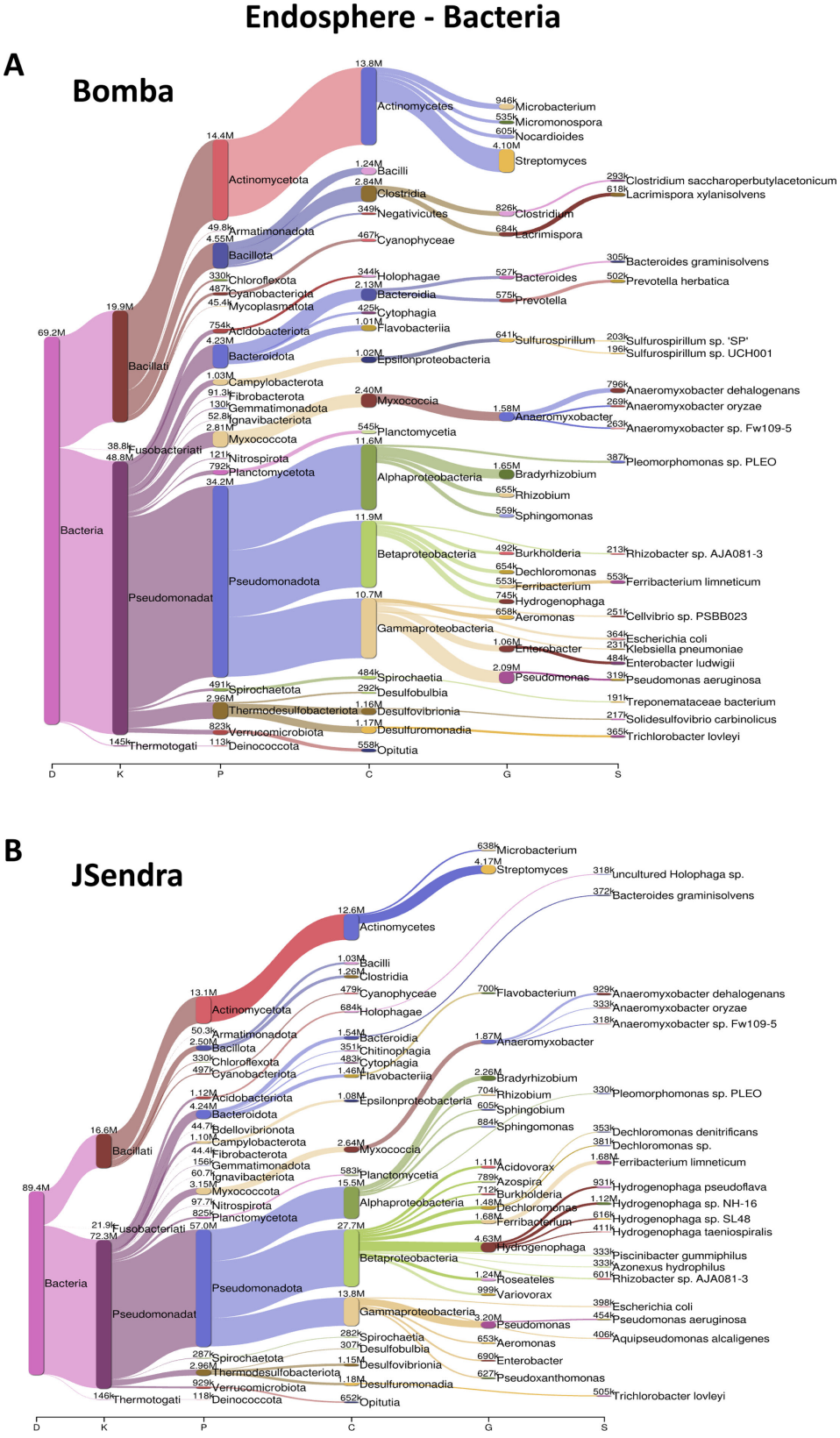


FIG 2 Most abundant bacterial taxa identified in the root endosphere of rice plants. Sankey diagrams for Bomba (A) and JSendra (B) plants are presented. The relative abundance of the top 20 taxa in each variety is presented, from the domain (on the left) to the species (on the right). The width of the bar is proportional to the abundance, whereas nodes depict the hierarchy levels of microbial taxa.

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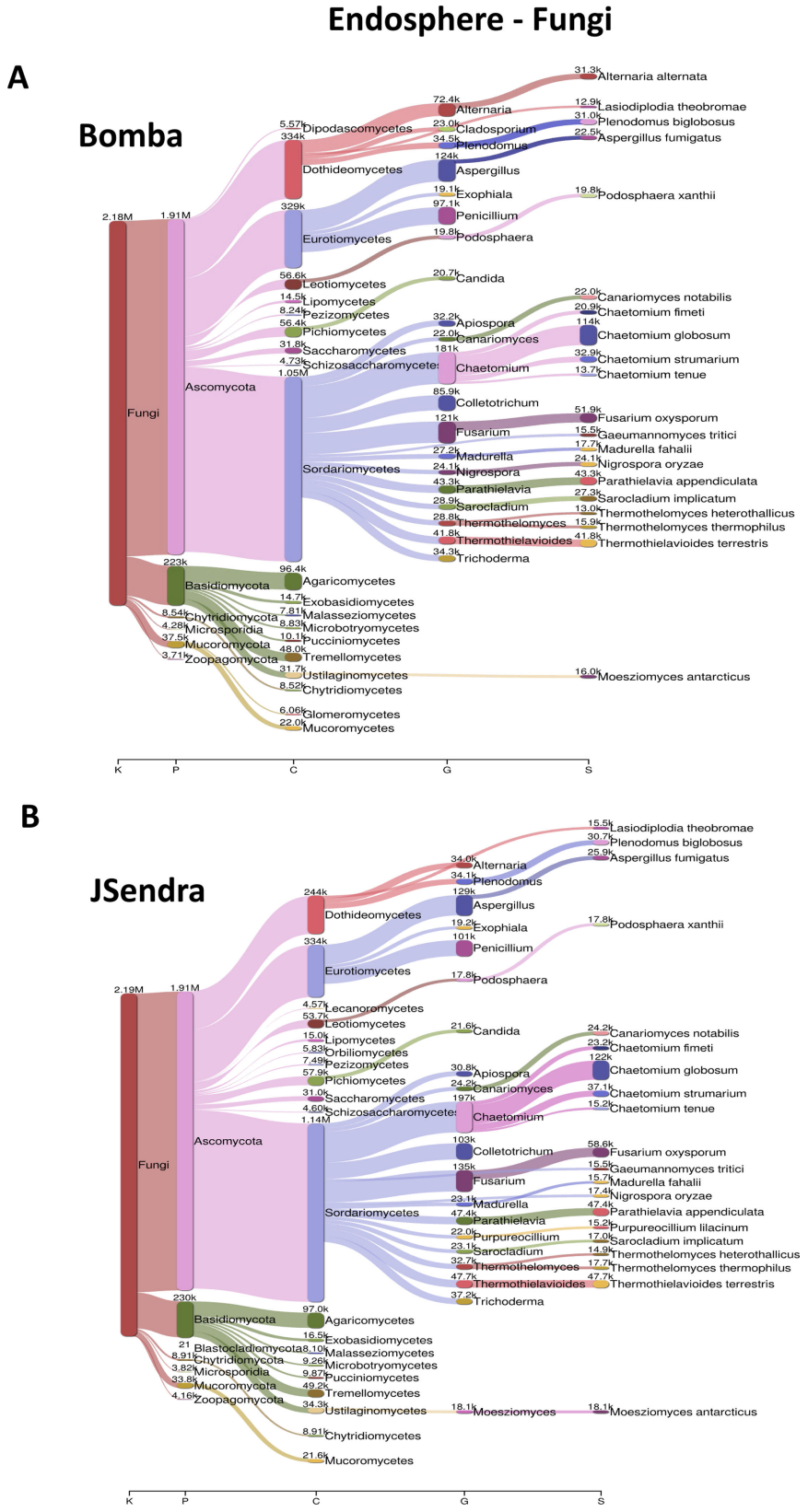


FIG 3 Most abundant fungal taxa identified in the root endosphere of rice plants. Sankey diagrams for Bomba (A) and JSendra (B) plants are presented. The relative abundance of the top 20 in each variety is presented, from the kingdom (on the left) to the species (on the right). The width of the bar is proportional to the abundance, whereas nodes depict the hierarchy levels of microbial taxa.

followed by Basidiomycota (Fig. S5). No major differences were detected in the taxonomic profile of the most abundant fungal species in the rhizosphere of rice varieties (Fig. S5).

Overall, these analyses revealed species-level variations in microbial assemblages, mainly in bacterial community structures, and between root compartments, which were also dependent on the rice genotype. The observed differences demonstrated a preferential recruitment of microbes from the rhizosphere into the rice root endosphere.

The arbuscular mycorrhizal symbiosis shapes the bacterial and fungal communities in rice

In this study, we investigated whether root colonization by the AM fungus *R. irregularis* has an effect on microbiome assembly in roots in rice plants in paddy fields. Recently, we reported that pre-inoculation of Bomba and JSendra seedlings with *R. irregularis* and growth in nurseries under aerobic conditions during the early vegetative stage prior to transplanting them into flooded fields allow the fungus to enter into the root (nursery stage) and ensure functioning of the AM symbiosis in plants under flooded conditions (19). Furthermore, inoculation with the AM fungus increased grain yield and blast resistance in the Bomba and JSendra varieties (19).

Before conducting metagenome analysis, root colonization by AM fungi of rice plants in paddy fields was confirmed. Histological analysis of cotton blue-stained roots revealed typical AM structures (i.e., intra-cellular hyphae, arbuscules, and some vesicles) in both nursery-grown seedlings and paddy field-grown plants inoculated with *R. irregularis* (Fig. S6A and B). To note, in paddy field-grown plants, AM fungal colonization was also observed in the roots of mock-inoculated plants, likely reflecting natural mycorrhization by indigenous AM fungi in the field environment (Fig. S6C). However, vesicles (lipid storage structures) were more frequently observed in roots of *R. irregularis*-inoculated plants compared with naturally colonized (e.g., mock-inoculated) plants. However, the overall percentage of root colonization in plants that have not been inoculated with *R. irregularis* and then grown in paddy field-grown plants remained low (Fig. S6D, left panel), which is in agreement with results previously reported in rice plants under permanent flooded conditions or alternate wetting and drying conditions (63, 64). Whereas histological staining of rice roots does not discriminate among different AM fungi, PCR analysis using *R. irregularis*-specific primers enabled unambiguous detection of *R. irregularis* in rice plants that had been pre-inoculated and then grown in paddy fields (Fig. S6D, right panel).

Metagenomic reads were mapped to the *R. irregularis* reference genome ASM43914v3. When comparing with mock-inoculated plants, *R. irregularis* reads increased from 7,436.44 to 13,362.5 in Bomba (adjusted $P \leq 0.05$, linear mixed model with Tukey's post hoc test) and from 10,532.94 to 12,631.39 in JSendra. In AM-inoculated plants, *R. irregularis* reads accounted for 0.07% and 0.05% of the total metagenome reads in Bomba and JSendra, respectively, whereas in mock-inoculated plants, *R. irregularis* reads accounted for 0.043% and 0.046% of the total reads in Bomba and JSendra, respectively (Table S2). Reads mapping against the *R. irregularis* genome in the rhizosphere accounted for less than 0.01% of the total microorganisms. No other indigenous mycorrhizal fungi could be detected in our metagenomic data, which is understandable, given the low number of *R. irregularis* reads retrieved from our metagenome data sets. Although this increase was significant only in Bomba, our initial analysis supported that *R. irregularis* inoculation shaped the endosphere microbiome in a variety-dependent manner ($P \leq 0.05$ interaction variety and mycorrhizal state, PERMANOVA), with significant effects observed in both varieties in pairwise comparisons (mock-inoculated vs *R. irregularis*-inoculated endosphere, adjusted $P \leq 0.05$). Together, microscopic analysis and molecular analyses confirmed root colonization by *R. irregularis* under flooded conditions. Combined with previous reports of increased yield and blast resistance (19), these results support the establishment of a functional symbiosis.

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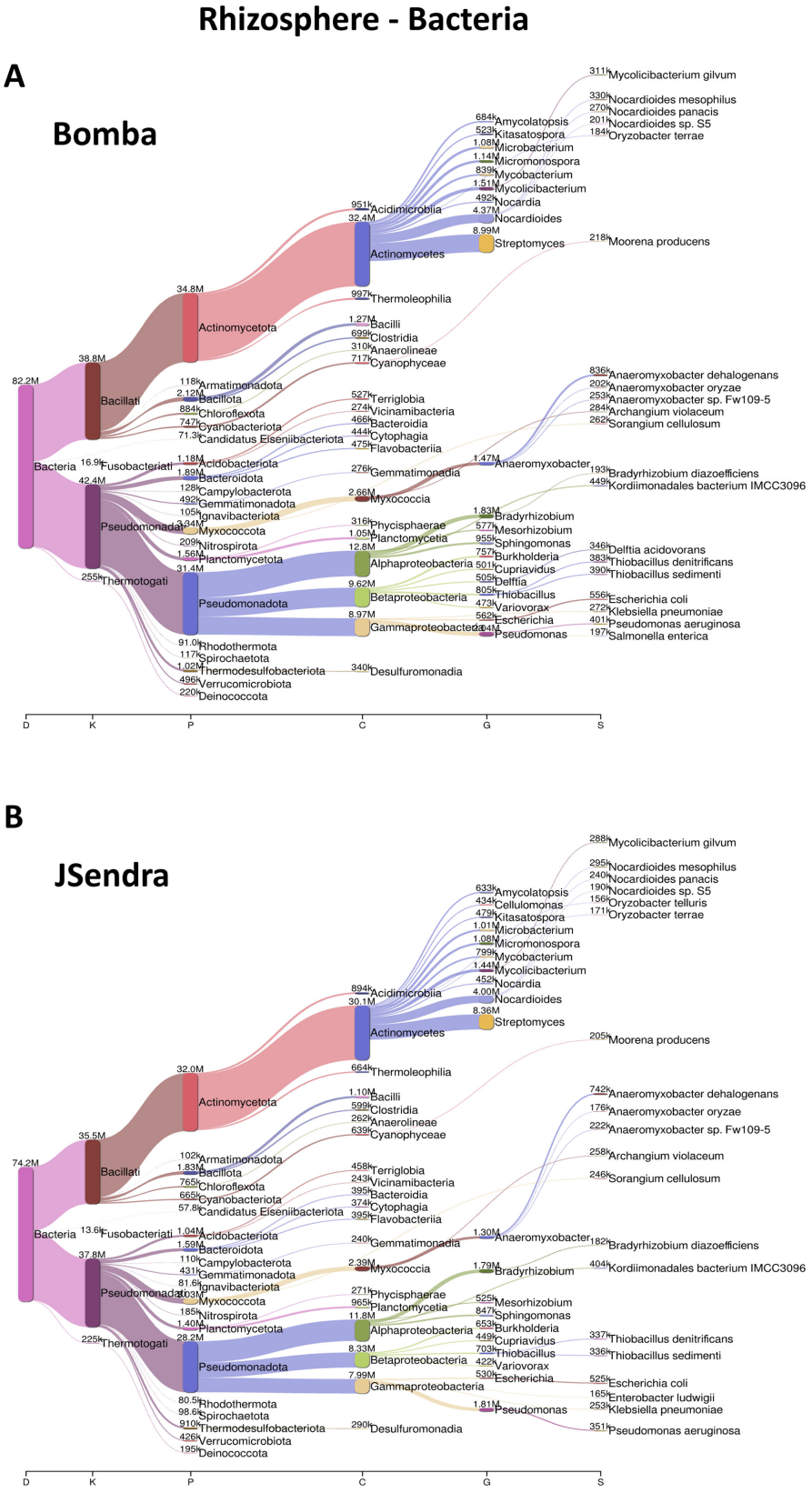


FIG 4 Most abundant bacterial taxa identified in the rhizosphere of rice plants. Sankey diagrams for Bomba (A) and JSendra (B) plants are shown. The relative abundance of the top 20 taxa in each variety is presented, from the kingdom (on the left) to the species (on the right). The width of the bar is proportional to the abundance, whereas nodes depict the hierarchy levels of microbial taxa.

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Next, we approached the characterization of root-associated microbiomes in roots of mycorrhizal rice plants grown in paddy fields. An exploratory analysis within inoculated plants was initially performed, and the PCoA discriminated between rhizosphere from endosphere bacterial and fungal populations. Here, PCo1 explained 88.3% and 81% of the variability, respectively ($P = 0.001$, PERMANOVA; $F = 45.297$ for bacteria, $F = 18.447$ for fungi), and only separated varieties for the endosphere bacteria (PCo2 5%) (Fig. 5A).

At the class level, the taxonomic analysis of root compartments of mock-inoculated and *R. irregularis*-inoculated plants showed similar composition, but the rice genotype and root compartment had an effect on the bacterial and fungal community in

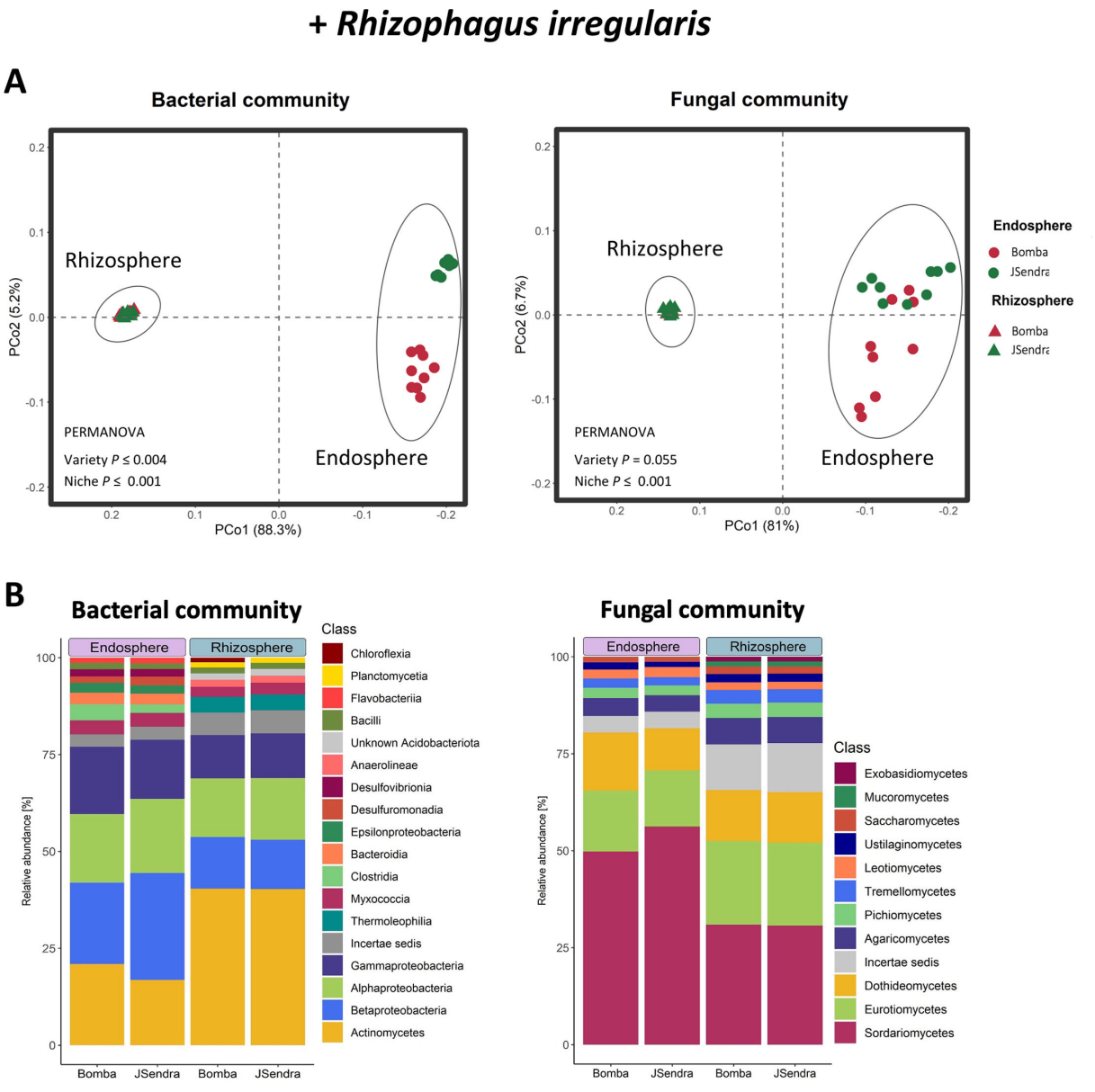


FIG 5 Effect of inoculation with the AM fungus *R. irregularis* on the bacterial and fungal communities in rice roots. Plants (cv. Bomba and JSendra) were grown in rice fields under flooded conditions. (A) Principal coordinate analysis of β-diversity, measured by Bray–Curtis distances of bacterial (left panel) and fungal (right panel) communities in the endosphere (circles) and rhizosphere (triangles) of *R. irregularis*-inoculated Bomba and JSendra plants. A PERMANOVA based on Bray–Curtis distances was performed to evaluate statistically significant differences between groups. Ellipses were added manually for visual emphasis. (B) Taxonomic composition of the bacterial (left panel) and fungal (right panel) communities in *R. irregularis*-inoculated roots of Bomba and JSendra plants. Different colors indicate different taxa at the class level.

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mycorrhizal roots (Fig. 5B; Fig. S7). Here, significant differences in 2,417 and 829 bacterial and 212 and 70 fungal species were found between mock- and *R. irregularis*-inoculated Bomba and JSendra plants, respectively (adjusted $P \leq 0.05$, linear mixed model with Tukey's post hoc test) (Table S3). Venn diagrams were applied to illustrate heterogeneity among root-associated compartments. Most bacterial and fungal species were found to be shared in root compartments of rice plants (Bomba and JSendra) across conditions (e.g., mock-inoculated and *R. irregularis*-inoculated roots), thus confirming co-existence of species in the bacterial and fungal communities (Fig. S8).

Analysis of compositions of microbiomes (ANCOM) (65) was used to identify differences between mycorrhizal and non-mycorrhizal roots in bacteria residing in the endosphere and rhizosphere in each genotype at the species-level resolution. The endosphere of mycorrhizal Bomba plants was enriched in *Kosakonia*, *Arcobacter*, *Acinetobacter*, *Shewanella*, and *Dickeya* species (Fig. 6A, orange bars), whereas the endosphere of non-mycorrhizal Bomba plants was enriched in *Klebsiella pasteurii and Streptococcus koreensis* (adjusted $P \leq 0.05$) (Fig. 6A, blue bars). Regarding JSendra plants, *Arcobacter* and *Shewanella* species were the most differentially abundant species in the endosphere of mycorrhizal roots (adjusted $P \leq 0.05$) (Fig. 6B, orange bars), while *Pseudomonas*, *Citrobacter*, *Phytobacter*, or *Enterobacter* species were enriched in non-mycorrhizal JSendra roots (adjusted $P \leq 0.05$) (Fig. 6B, blue bars).

In the context of bacterial taxa contributing the most to the dissimilarity between mycorrhizal and non-mycorrhizal in the root rhizosphere, *Klebsiella*, *Limnobacter*, and *Trichormus* (as well as *Sulfurospirillum* and *Rheinheimera* species, among others), were found to preferentially colonize the rhizosphere of mycorrhizal Bomba plants (Fig. 7A, orange bars), whereas *Rickettsia*, *Planktothricoides*, *Streptococcus*, and *Flavobacterium* were highly enriched in the rhizosphere of mycorrhizal JSendra plants (Fig. 7B, orange bars). Specific bacterial signatures in the rhizosphere of non-mycorrhizal roots included *Prevotella*, *Streptococcus*, and *Rickettsia* species in Bomba plants (Fig. 7A, blue bars) and *Xanthomonas* and *Streptomyces* species in JSendra plants (Fig. 7B, blue bars). Overall, differences in bacterial and fungal communities in root compartments of mock-inoculated and *R. irregularis*-inoculated rice plants appear to rely more on the relative abundance rather than on the presence/absence of distinct species.

However, similar taxonomic composition does not necessarily imply similar ecological structures. Therefore, co-occurrence network analyses were conducted to evaluate whether microbial interactions and community organization of the top 200 most abundant taxa differed among treatments (Fig. 8). Network topology metrics were subsequently compared to assess changes in complexity, connectivity, and potential ecological associations. In both bacteria and fungi, a genotype-dependent performance was observed when mycorrhiza was inoculated. On the one hand, the endosphere of JSendra shows an increased number of connections (Table S5) in mycorrhizal conditions, resulting in a denser network (Fig. 8). In contrast, Bomba displayed the opposite pattern. At rhizosphere levels, in both genotypes, the average degree decreased but increased the modularity (Table S5). Similar results were obtained for the fungal community. The contrasting network responses observed between genotypes suggest that, despite the high taxonomic overlap among treatments, mycorrhizal inoculation selectively influences the abundance and ecological relevance of particular microbial taxa. To identify the microorganisms potentially underlying these network rearrangements, differential abundance analyses were subsequently conducted.

Regarding pathogenic species colonizing the rice roots, an examination of metagenomic data sets revealed the presence of *X. oryzae* in the endosphere of Bomba and JSendra roots, its abundance being higher in the latter one (Fig. S9). Two pathovars of *Xanthomonas oryzae* are responsible for devastating diseases of rice, bacterial leaf blight (BLB, caused by *X. oryzae* pv. *oryza* [*Xoo*]) and bacterial leaf streak (BLS, caused by *X. oryzae* pv. *oryzicola* [*Xoc*]). Both bacteria were detected at similar rates when mapping them to the clean reads (Table S6). At present, however, no incidence of BLB nor BLS has been reported in the rice-growing region in which rice plants were grown (Valencia,

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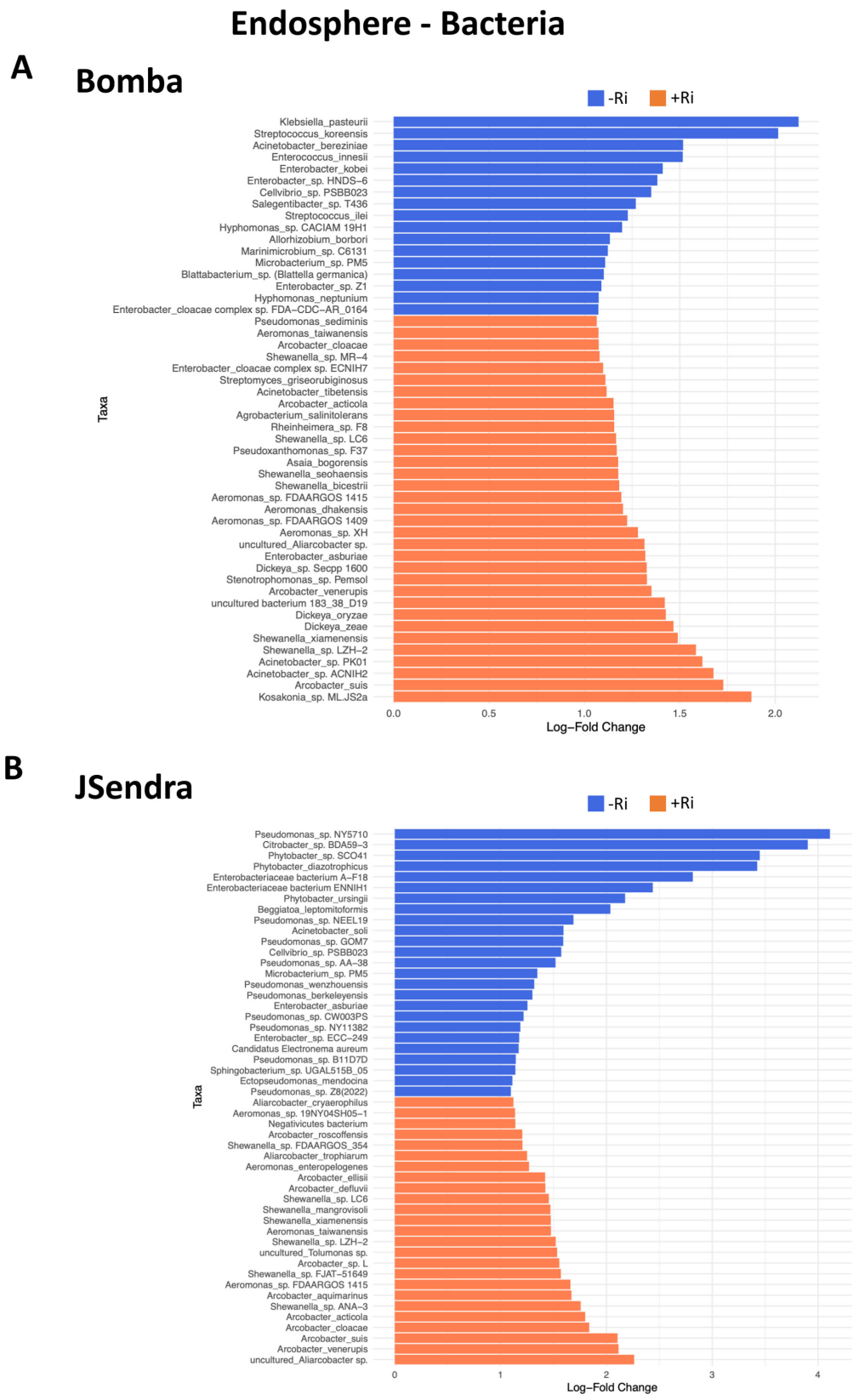


FIG 6 Specific bacterial signatures in mycorrhizal and non-mycorrhizal endospheres at the species level. ANCOM analysis was used to identify the 50 most differentially abundant bacterial species in the root endosphere of non-mycorrhizal and

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Fig 6 (Continued)

mycorrhizal Bomba (A) and JSendra (B) plants. Significantly enriched bacterial species at $P < 0.05$ (adjusted using the Holm–Bonferroni method) are presented for each comparison. Blue, bacterial species enriched in the endosphere of non-mycorrhizal roots; orange, bacterial species enriched in the endosphere of mycorrhizal roots. Bars represent the relative abundance of each bacterium within the kingdom Bacteria.

eastern region of the Iberian Peninsula). Importantly, *X. oryzae* abundance decreased in mycorrhizal roots relative to non-mycorrhizal roots in both rice varieties. This observation suggests that the AM symbiosis might be beneficial for the host plant in preventing *Xoo* or *Xoc* invasion of rice plants and an aspect that deserves further investigation.

Collectively, this analysis demonstrated that inoculation with the AM fungus *R. irregularis* alters the bacterial root microbiome of rice plants grown under flooded conditions. This effect can be observed at both the endosphere and rhizosphere.

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Methanogens in root-associated microbiomes of rice plants

After exploring the overall composition of microbiomes in rice roots, we put the focus on species related to functional activities. Our metagenomic data revealed the presence of several species of methanogenic archaea in the rhizosphere and root endosphere of rice plants (Table S7). Species of the *Methanosarcina* genus, namely, *Methanosarcina siciliae* and *Methanosarcina barkeri*, were the most abundant methanogens in root compartments in both Bomba and JSendra plants, in both mycorrhizal and non-mycorrhizal roots (Fig. 9A). Compared to the endosphere, an overall homogeneous composition of methanogenic archaea was observed at rhizospheres in both genotypes (Fig. 9A). The genus, (i) identified predominantly in the endosphere of Bomba plants (*Methanocella arvoryzae*, *Methanosarcina barkeri*, *Methanosarcina horonobensis*, *Methanosarcina mazei*, and *Methanosarcina thermophila*), (ii) less abundant in the endosphere of JSendra plants (*M. barkeri* and *Methanosarcina flavescens*), (iii) less represented in the endosphere in both varieties (*Methanothrix soehngenii*), or (iv) altered at the endosphere by the mycorrhizal status (*Methanospirillum* sp.), displayed similar patterns at the rhizosphere (Fig. 9B; details on the abundance of reads in metagenomic data sets are presented in Table S8). We reasoned that the distinct composition could impact methanogenic activities under paddy fields. To this end, metagenomic shotgun data sets were employed to infer the relative abundance of marker genes of methanogenesis, i.e., methyl-coenzyme M reductases (*mcrA*/*B*/*G*) (66). As depicted in Fig. 9C, *mcrA* accumulated high abundances at the rhizospheres in both genotypes, regardless of the AM state of the plants.

Together, these results suggest that, although the interplay between host genotype, root compartment, and AM fungus plays a key role in shaping the structure of the methanogenic community in the rice root, functional convergence may be expected to maintain comparable methanogenic activities of the microbiome at the rhizosphere.

Nutrient-cycling bacteria in root-associated microbiomes of rice varieties

To have a better comprehension of differences in microbial composition and function across genotypes and conditions (e.g., non-inoculated and *R. irregularis*-inoculated roots), a KEGG enrichment analysis was applied. Significant pathway enrichment (adjusted $P < 0.05$) was only observed in the rhizosphere of both varieties. Mycorrhizal roots of JSendra plants were enriched in pathways related to energetic metabolism and bacterial growth (lipoic, propanoate, and oxocarboxylic acid metabolisms; flagellar assembly and bacterial chemotaxis) (Fig. 10A). In contrast, the rhizosphere of Bomba showed enriched pathways related to nutrient transport, response to chemical signals, and synthesis/degradation of arginine and aromatic compounds (Fig. 10A). Interestingly, the KO terms assigned to these pathways are related to N metabolism and the transport of other nutrients (Table S9).

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FIG 7 Specific bacterial signatures in mycorrhizal and non-mycorrhizal rhizospheres at the species level. ANCOM analysis was used to identify the 50 most differentially abundant bacterial species in the rhizosphere of non-mycorrhizal and mycorrhizal Bomba (A) and JSendra (B) plants. Significantly enriched bacterial species at $P < 0.05$ (adjusted using the Holm–Bonferroni

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Fig 7 (Continued)

method) are presented for each comparison. Blue, bacterial species enriched in the rhizosphere of non-mycorrhizal roots. Orange, bacterial species enriched in the rhizosphere of mycorrhizal roots. Bars represent the relative abundance of each bacterium within the Bacteria kingdom.

Given the prevalence of microbiomes modulating plant nutrition, we next interrogated differences in processes related to nutrient metabolism in the two rice genotypes in response to *R. irregularis* inoculation. The heat map presented in Fig. 10B summarizes the abundance of genes involved in pathways related to N, P, S, Fe, and Zn metabolism in Bomba and JSendra roots (non-inoculated with *R. irregularis*). Phosphorus and nitrogen-cycling processes stand out for having a higher number of active processes than the rest, highlighting nitrogen fixation, dissimilatory nitrate reduction, denitrification for N cycling (NCyc), phosphonate degradation, phosphate transport and regulation for P cycling. To have a closer look at these processes, genes related to them were studied. Figure 11 recapitulates the differential abundance of NCyc bacteria and PSB in root compartments of Bomba and JSendra plants grown in paddy fields, both mycorrhizal and non-mycorrhizal plants. Details on the relative abundance of NCyc bacteria and PSB bacterial species in the different samples are presented in Table S10. Briefly, the two main clusters of the heat map depicted rhizosphere-specific (cluster I) and endosphere-specific (cluster II) bacteria that showed almost identical abundance in both genotypes (Fig. 11). Note that cluster I (rhizosphere) mainly encompassed specialized NCyc bacteria and PSB, while cluster II (endosphere) included mainly bacteria capable of operating as both NCyc bacteria and PSB. The rest of the clusters consisted of bacteria that were more prominent within the endosphere, depending on the genotype (JSendra, cluster III; Bomba, cluster IV), that also can work as NCyc bacteria and PSB (Fig. 11). Two-thirds of the total bacteria were allocated at the endosphere, highlighting the relevance of the diversifying bacteria specialized in nutrient cycling within the root. However, a distinct recruitment of NCyc bacteria and PSB per genotype was envisaged. AM colonization led to lowered abundance for the genotype-specific species in clusters III and IV, a fact consistent with the minor dependence on the microbiome to obtain N and P under this state. These patterns allowed us to propose a selection for endosphere-resident bacteria based on versatility to cycle for both P and N, whereas rhizosphere bacteria could be more specialized for one or another mineral. These findings reinforce the scenario whereby AM-root colonization led to diminished bacteria contributing to plant nutrition.

In line with the prevalent presence of microbiomes related to N and P cycling in bacteria at the endosphere, higher fractions of genes participating in NCyc bacteria and PSB were found at this compartment compared to the rhizosphere in both JSendra and Bomba (Fig. 12A and B; Table S11). Indeed, approximately 60% and 50% of NCyc and PSB genes were most relevant in the endosphere (Fig. 12A and B), confirming that most pathways for nutrient cycling assisted by the microbiome mainly occur at this level. In addition, our data analysis validated the singularity of JSendra to recruit higher abundances of NCyc bacteria and PSB, as genes of both classes were detected at higher abundances in the endosphere of JSendra than in that of Bomba. Concerning NCyc genes, this is the case of the glutamate dehydrogenase (*gdhA*), the nitric oxide reductases (*nosD*/*F*/*L*/*Z*), the nitrite reductases (*nirB*/*Q*/*S*), the nitrate reductases (*napC*, *nasE*, and *narI*), and ureases (*ureB*/*F*) (clusters III and V, Fig. 12A). Interestingly, the most predominant PSB genes in the JSendra endosphere included features participating in P solubilization and mineralization via phosphonate metabolism (*phnG*/*F*/*I*/*J*/*K*/*L*/*M*/*N*) (cluster III, Fig. 12B). Again, the abundance of most of these transcripts tended to decrease upon AM colonization and may be attributable to a minor necessity to recruit an additional microbiome due to the beneficial effect of AM fungi on P acquisition. Collectively, these observations suggest an improved nutritional state in JSendra that, ultimately, could explain a better performance of this cultivar compared to Bomba in paddy fields.

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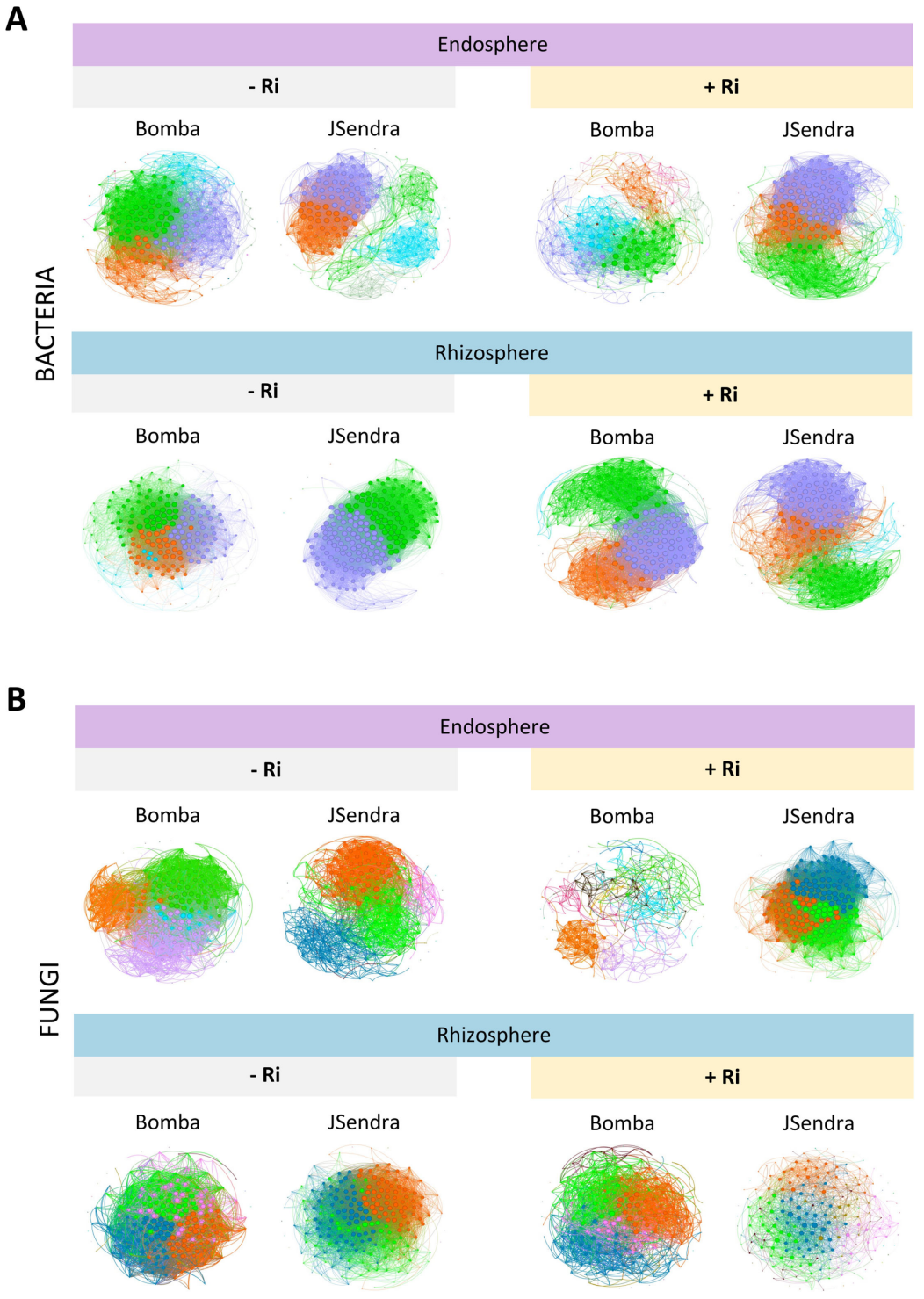


FIG 8 Co-occurrence networks of top 200 most abundant bacteria (A) and fungi (B) for JSendra and Bomba endosphere and rhizosphere, under mycorrhizal and non-mycorrhizal conditions (−Ri, +Ri). Nodes represent individual species, with node size proportional to the degree metric of each node. Edges (lines connecting nodes) indicate significant co-occurrence associations between pairs. Nodes are grouped into clusters or modules representing densely interconnected sub-networks as determined by the Fruchterman–Reingold algorithm. Colors represent different modules. Nodes with a higher number of shared or direct connections are tended to be positioned closer together. Spatial proximity reflects the strength and density of associations among species.

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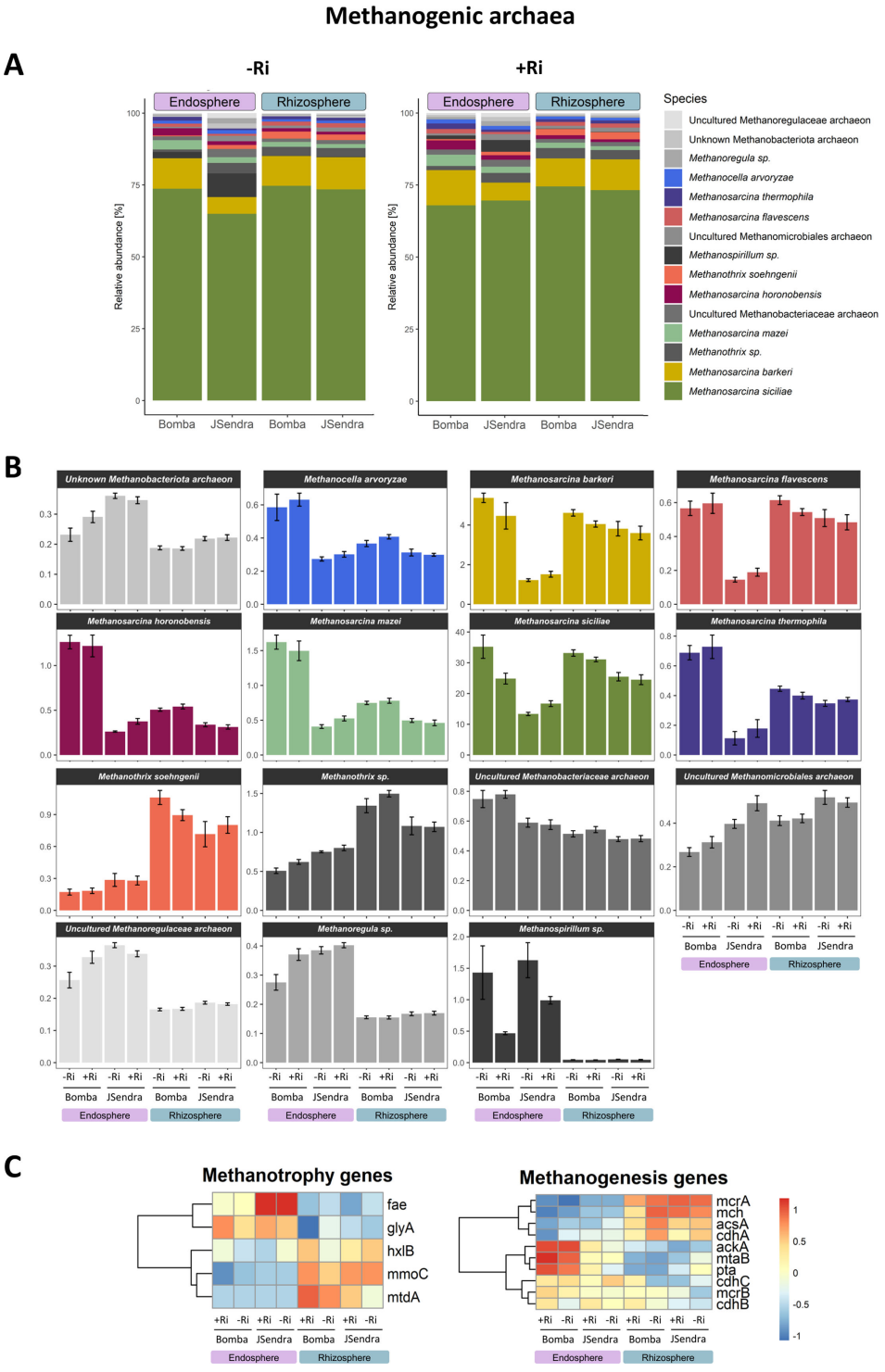


FIG 9 Relative abundance of methanogenic archaea in the endosphere and rhizosphere of mycorrhizal and non-mycorrhizal rice plants. (A) Relative abundance within methanogenic archaea at the species level in the root endosphere and soil rhizosphere of Bomba and JSendra plants (mycorrhizal and non-mycorrhizal conditions in each variety). (B) Relative abundance of each methanogenic archaea within the Archaea kingdom. (C) Heat maps with hierarchical clustering according to the Ward D2 algorithm for the normalized abundance of selected genes involved in methanogenesis.

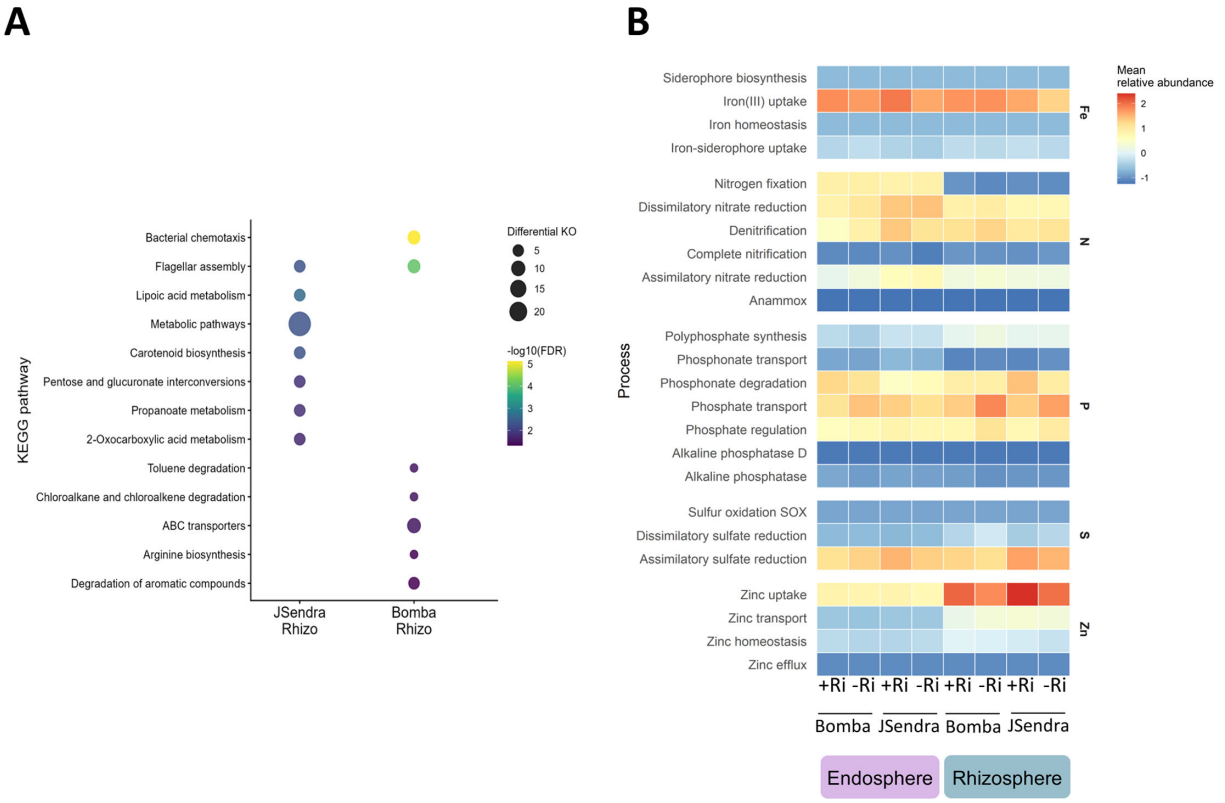


FIG 10 Functional profiling of microbial community. (A) KEGG pathway enrichment analysis showing the metabolic pathways significantly enriched in the rhizosphere of *R. irregularis*-inoculated roots relative to non-inoculated roots ($P < 0.05$, adjusted using the Holm–Bonferroni method). (B) Mean relative abundance of annotated genes involved in N, P, S, Fe, and Zn-related processes.

DISCUSSION

The present study provides a comprehensive microbial diversity characterization of the root-associated compartments in rice plants growing in flooded fields, a widely used system for rice cultivation whose microbiome is sparsely documented. Two agronomically relevant varieties, Bomba and JSendra (temperate *japonica* rice), well adapted to Mediterranean climate conditions, were assayed. Furthermore, changes in the microbial community structure driven by the AM symbiosis in rice roots have been identified. Contrary to the conventional assumption that rice grown in flooded fields does not form symbiotic relationships with AM fungi, recent studies demonstrate that AM fungi can colonize rice roots in flooded rice (17, 19). It is also known that the AM symbiosis increases rice productivity and confers resistance to infection by the rice blast fungus *M. oryzae* (17, 19) or the bacterial pathogen *Xoo* responsible for bacterial leaf blight in rice (18). Deciphering how microbial communities colonize the root compartments in rice genotypes of agronomic relevance and how AM fungi shape the rice root microbiome provides a framework for advancing potential applications of AM fungi in rice cultivation.

Microbial community structure in roots of flooded rice

Our results show that the root compartment, rather than the plant genotype, is the strongest factor influencing root-associated microbial communities in flooded rice. In both genotypes, the relative abundance of Pseudomonadota (e.g., Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria) was higher in the endosphere than in the rhizosphere, whereas Actinomycetes were more abundant in the rhizosphere. Fungal taxa in the phylum Ascomycota dominated the root-associated compartments of rice plants, of which Sordariomycetes were more abundant in the endosphere than in the

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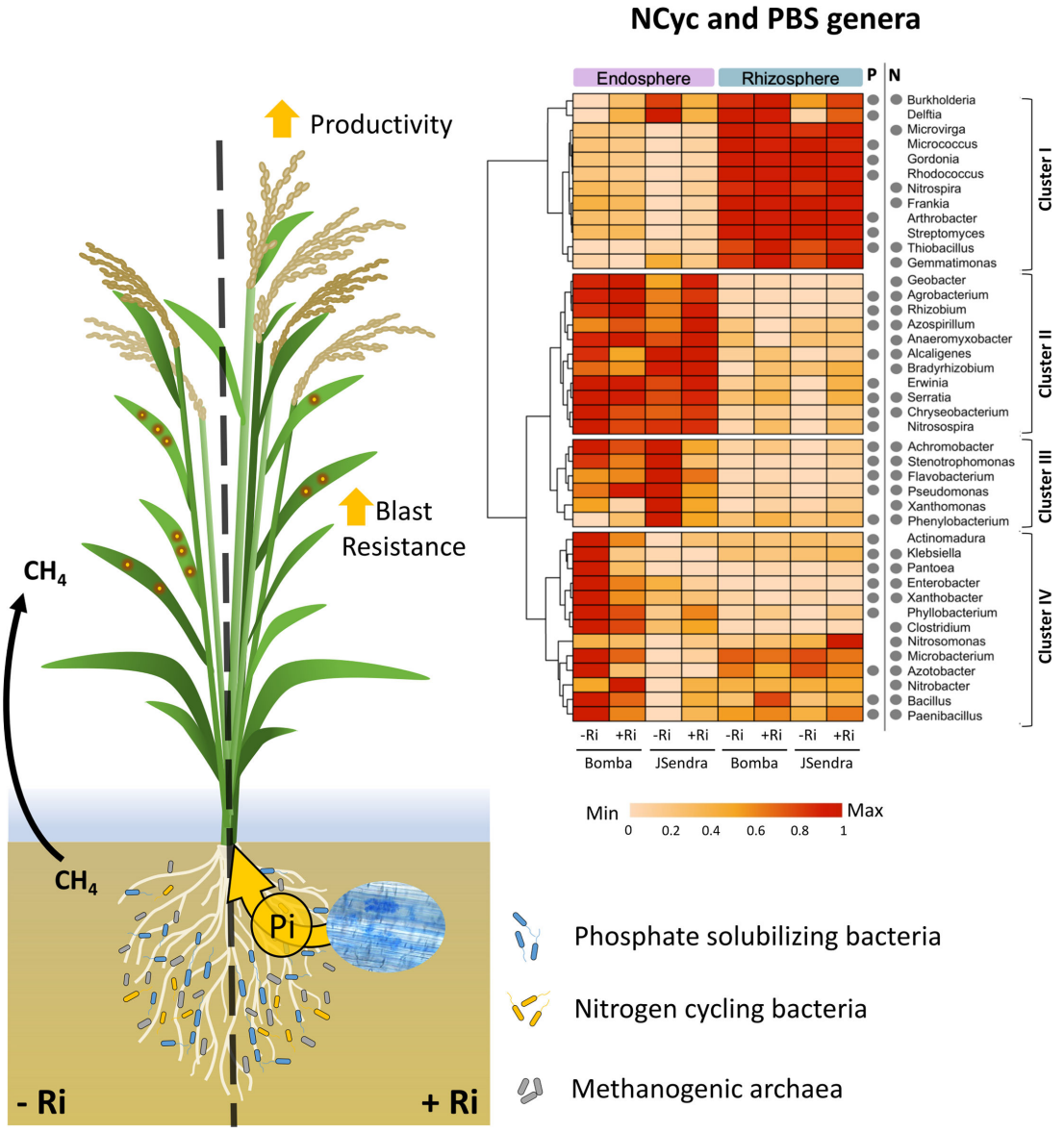


FIG 11 NCyc and PSB genera found in the endosphere and rhizosphere of rice plants. The heat map (right panel) shows enrichment of NCyc and PSB genera in root compartments of Bomba and JSendra plants, under mycorrhizal and non-mycorrhizal conditions. The left panel depicts a rice plant showing root-associated microbial communities investigated in this work (e.g., NCyc, PSB, and methanogenic archaea). PSB associates with AM fungi and promotes the mineralization of organic P, which is omnipresent in the soil, into inorganic P that is easily assimilated by AM fungi, thereby increasing Pi availability to the host plant. Variations in these microbial communities can be linked to the rice genotype (Bomba and JSendra), the root compartment (endosphere and rhizosphere), and the mycorrhizal status of the rice plant. These factors also influence methanogenic archaea colonizing the rice roots, which are responsible for methane emission from rice fields. *R. irregularis* intervention in flooded rice cultivation was previously shown to increase Pi uptake, grain yield, and resistance to the rice blast fungus *M. oryzae* in the aerial part of the rice plant in flooded field conditions (same paddy fields as in this study) (19). N, nitrogen; NCyc, nitrogen-cycling bacteria; P, phosphate; PSB, phosphate-solubilizing bacteria.

rhizosphere, and Eurotiomycetes were more prevalent in the rhizosphere. The differences between compartments were even more remarkable for bacterial classes in minority. The microbial species identified in this study in rice roots, most probably, reflect microbial taxa that have been shaped along the plant's life cycle in paddy fields. In other studies, Gammaproteobacteria (e.g., Enterobacteriaceae and Pseudomonadaceae families), Firmicutes (e.g., *Bacillus* genus), Actinobacteria, and Bacteroidetes phyla were described as common inhabitants in the rice root endosphere, whereas the Ascomycota phylum

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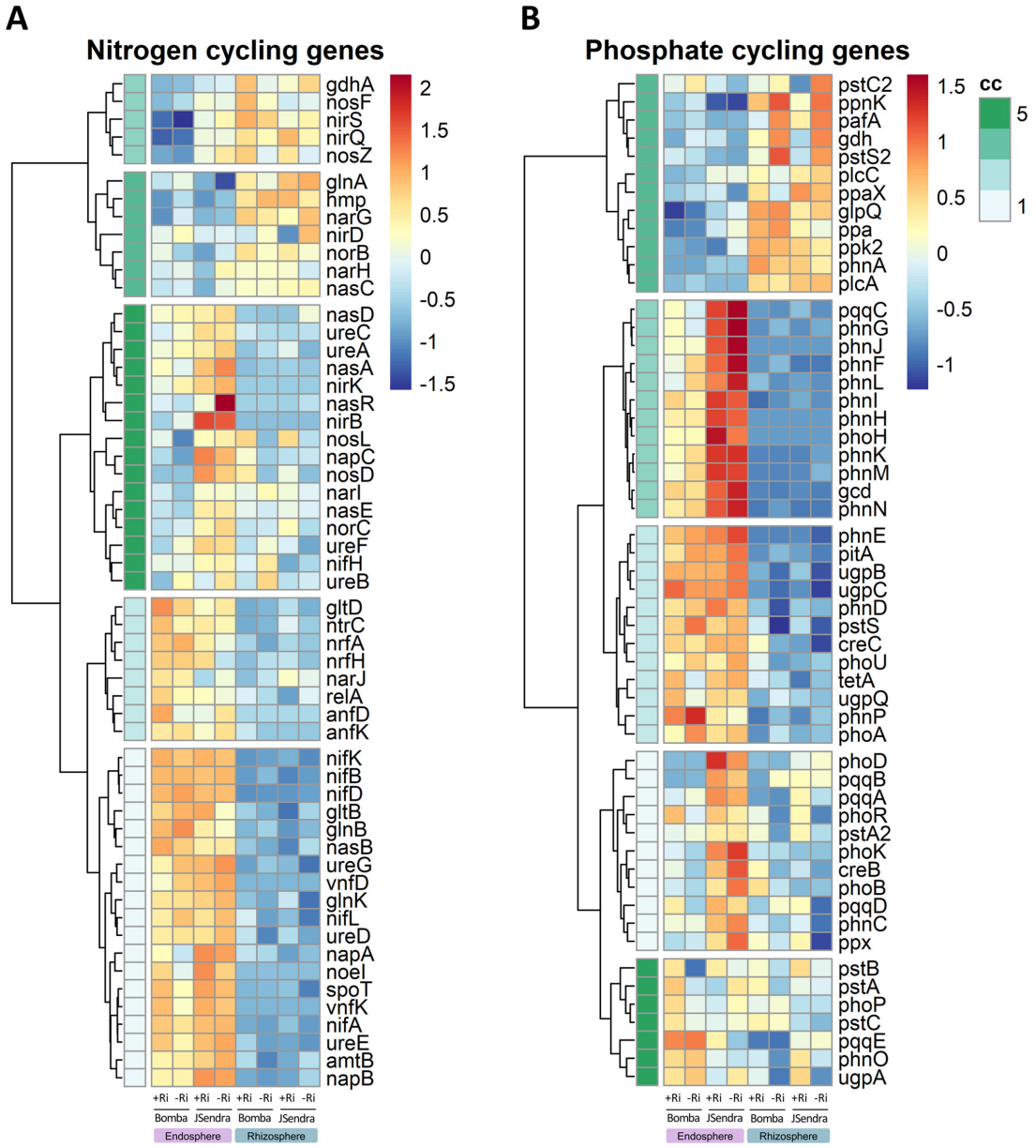


FIG 12 Differences in abundance of genes involved in nutrient cycling. Heat maps were drawn using hierarchical clustering according to the ward D2 algorithm of the relative abundance of selected genes for nitrogen cycling (A) and phosphate cycling (B) derived from the microbial communities in the endosphere and rhizosphere of non-AM and AM Bomba and JSendra rice plants.

was found to be the most prominent fungal population in the rice endosphere (67–70). On the other hand, several studies have indicated that the bacterial community in the rice rhizosphere is largely represented by Proteobacteria (mainly Alphaproteobacteria, Betaproteobacteria, and Deltaproteobacteria classes), Acidobacteria, and Actinobacteria (7). The observed preferential colonization of rice roots by distinct microbial species supports the capacity of the plant to actively recruit certain microbial taxa from the rhizosphere. Selection of specialized microbial taxa would explain higher α-diversity, as measured by the Shannon index, in the endosphere than in the rhizosphere compartment. In this respect, the rhizosphere has historically been regarded as the compartment with a higher microbial diversity (relative to the endosphere compartment). However, there are numerous studies in which the endosphere compartment exhibited higher diversity than the rhizosphere. This is the case for specialized plants and plants under

unique ecological niches and/or specific tissue environments (71, 72). Certain environmental stresses, such as salt stress, have also been shown to increase microbial diversity in the root endosphere, a situation in which a higher proportion of microbes are allowed to enter the plant tissue (73). In other studies, it was reported that the cultivation medium used to grow orchid plants (which are different from natural soil conditions) determines microbial diversity in rhizocompartments, further supporting the effect of the environment on the microbial ecology in root compartments (74).

Some components of the main communities here identified in the endosphere have been previously reported as beneficial species in rice. For instance, *Chaetomium* species (Sordariomycetes) are generally considered as biological control agents against rice diseases, also with applications in rice straw degradation (75). Members of *Pseudomonas* and *Bacillus* genera were also identified in this study, for which roles in stimulating induced systemic resistance to pathogens in rice have been described (76). Other root endophytes identified in this work have been shown to promote rice growth, primarily through the production of phytohormones, such as cytokinin, indole-3-acetic acid (IAA), or gibberellins (e.g., *Burkholderia* and *Pantoea* as producers of IAA) (68). It will then be of interest to further explore if specific beneficial microbes identified in roots of Bomba and JSendra plants can regulate plant growth and stress resilience in paddy fields.

In addition to the root compartment, our results show that the host genotype can further determine the composition of the root microbiomes. Microbial community variations between Bomba and JSendra plants were more evident at the species level. Microbial taxa identified in Bomba and JSendra roots, two widely grown varieties in the western Mediterranean region, would represent well-adapted microbial species to these rice varieties capable of establishing associations with rice plants in paddy fields. Differences between our results and previous reports on microbial communities associated with rice roots (7, 25–27) might well be attributed to the host genotypes examined, the biogeography and cultivation systems used to grow the rice varieties, and/or environmental factors. Here, it is worth mentioning that considerable effort has been devoted to characterizing the root microbiomes of wild rice species, mainly in the rhizosphere, and determining whether microbial communities differ between wild and domesticated rice (34, 35, 77–82). These studies demonstrated that wild and domesticated rice have different microbiome compositions in the rhizosphere, including bacteria and fungi. Thus, while shedding light on drivers of assemblage of root microbial communities in flooded rice, our study evidences the urgency to conduct further characterization of microbiomes in domesticated and wild rice genotypes in major rice-growing regions.

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Differences in bacterial assemblages associated with methane production and nutrient solubilization in roots of rice plants under flooded conditions

Beyond identifying differentially abundant taxonomic groups in root compartments of rice plants and knowing the ability of the host plant to select its microbiome, we searched for *bona fide* microorganisms that are functionally relevant to the conditions in which rice is cultivated, that is, flooded conditions. We focused on taxonomic groups involved in methane production and nutrient recycling.

In the flooded anaerobic environment, methanogens use carbon from rice exudates or the soil organic carbon as metabolic substrates for methane production. Although most of the methane from paddy fields is produced by the activity of methanogenic archaea in the rice rhizosphere, certain methanogenic archaea (e.g., *Methanospirillum* and *Methanobacterium* genera) have previously been detected inside the rice roots (31, 83). According to our metagenomic analyses, *Methanosarcina* species emerged as the prevalent methanogenic archaea taxa in root compartments in Bomba and JSendra plants. These species might then contribute to methane emission in paddy fields. Furthermore, the *mcrA* gene, the most widely used functional and phylogenetic marker to track methanogen populations in rice paddy fields, was mainly detected

at the rhizosphere (Fig. 9C). *Methanosarcina* species utilize this functional marker for methanogenesis in flooded rice fields (84).

Regarding methanogenic microbes in the root endosphere, we found that this community was shaped by the rice genotype (e.g., the endosphere of Bomba roots had increased abundance in *M. horonobensis*, *M. thermophila*, *M. flavescens*, and *M. barkeri* compared to JSendra). In JSendra, the root endosphere contained more *Methanoregulaceae archeon* and *Methanobacteriota archeon* species. Despite Bomba and JSendra having a differential composition of methanogenic microbes in the root endosphere, these differences might not have a remarkable impact on methane production. Clearly, reducing methane emissions from paddy rice fields is vital, and knowing the population of methanogenic microbes in rice in paddy fields, specifically in the rhizosphere compartment, will help in this direction.

Our analyses also showed a compartment-specific signature for the bacterial community involved in nutrient cycling, particularly NCyc bacteria and PSB, in the two rice genotypes. For instance, the N-fixing *Rhizobium*, a symbiotic bacterium that improves nutrient uptake and growth in leguminous plants, also occurs as endophytes in rice roots (85, 86). *Flavobacterium anhuiense* contributes to potassium solubilizing (87), and *Pseudomonas sediminis* and *Agrobacterium salinitolerans* are salt-tolerant bacteria related to nutrient mobilization and inhibiting the growth of certain pathogenic fungi (88, 89). It is generally assumed that the rhizosphere compartment is dominated by broader nutrient-cycling microbial communities and that this community is essential for ecosystem functioning (7). The results presented here support that rhizospheric bacterial taxa are more specialized to either NCyc bacteria or PSB, while those in the endosphere could participate in both P and N cycling. This last pattern could also be observed for other genotype-specific endosphere bacteria. Accordingly, we propose an economization strategy whereby host plants have developed mechanisms to prioritize associations with bacteria with higher versatility to mobilize nutrients to ultimately maximize the benefits of the colonization. Importantly, the cultivar JSendra displayed higher abundance of genes related to P mobilization in the endosphere when compared to Bomba. Our finding supports the possibility that, in paddy fields, JSendra had acquired a refined capacity to recruit microbiomes that confer an enhanced nutritional state. This discovery paves the path for subsequent studies to follow up on the causality of the microbiome of Bomba and JSendra on agronomical performance in these varieties in paddy fields (e.g., productivity and tolerance to biotic and abiotic stresses).

Earlier studies in wild and domesticated rice also focused on microbes involved in nutrient cycling (35, 82). N-cycling genes were found to be more abundant in the wild rice rhizosphere microbiomes than in domesticated rice (82). The authors concluded that management practices in rice cultivation and directional selection in breeding programs (i.e., modern varieties are highly dependent on nitrogen fertilizers) have reduced the need to establish associations with microbes that normally provide nitrogen to rice plants. Unraveling the diversity of microbes in the roots of wild rice and the impact that rice domestication has triggered on the rice root microbiomes is a requisite to maximize the success of sustainable rice production by reducing fertilizer use and greenhouse gas emissions.

Variations in the root microbiome in mycorrhizal rice plants in flooded conditions

Root colonization by AM fungi in aerobic rice has long been documented (63, 90). It is also known that, under flooded conditions, the rice plant benefits from mycorrhiza in terms of increasing grain yield and blast resistance, with no need of extensive colonization of the root system by the AM fungus (17, 19). Presumably, the host plant can regulate levels of root colonization by AM fungi to prevent overcolonization and excessive symbiosis costs.

In terms of functionality, mycorrhiza inoculation resulted in enriched pathways related to carbon and nitrogen, such us lipoic acid metabolism, 2-oxocarboxylic acid

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metabolism, and pentose and glucuronate interconversion. Additionally, mycorrhized inoculated rhizospheres were enriched with ABC transporters, suggesting an increase in nutrient acquisition, as well as enriched in arginine biosynthesis, related to N metabolism and PGPR in other plant species (plant growth promoting rhizobacteria). These results, taken together with the emergence of processes related to chemotaxis and bacterial movement, suggest that mycorrhiza increases bacterial functions related to energy and carbon metabolism, nutrient cycling, and response to plant chemical signals in the rhizosphere.

Microbial assemblages described in this study in rice roots appear to be influenced by the genotype and the mycorrhizal status of the plant. In particular, the lowering of genotype-specific bacteria involved in phosphate solubilization and nutrient cycling PSB, along with the abundance of cognate genes, would be in line with the need to maintain appropriate Pi levels in the rice roots. Earlier studies reported a cooperative relationship between AM fungi and PSB in soil, making P more available to the plant and/or increasing tolerance to stress (24, 91–93). Given the beneficial consequences of AM colonization, we contemplate three plausible and potentially overlapping scenarios: (i) AM fungi could efficiently compete with the other potential colonizers; (ii) the homeostatic state upon AM colonization might alter or interfere with the plant signaling mechanisms to attract microbiota; or (iii) the host might actively focus the recruiting strategy on AM fungi as the most advantageous case.

Venn analyses revealed a substantial overlap in both bacterial and fungal taxa among treatments, indicating that *R. irregularis* inoculation had a limited impact on overall community membership. However, co-occurrence network analyses showed marked differences in network topology between inoculation conditions and rice genotypes. These findings suggest that mycorrhizal inoculation primarily influences the ecological organization of microbial communities rather than their taxonomic composition. In particular, the contrasting responses observed between JSendra and Bomba indicate that the effect of *R. irregularis* on microbial interactions is strongly genotype dependent. Therefore, changes in community structure appear to be driven less by taxon turnover and more by shifts in the relative importance, associations, and abundance of specific microbial taxa, as could be also observed with ANCOM analysis.

A priori, the root microbial composition would reflect the outcome of complex interactions involving the host plant and microbe–microbe interactions, and root colonization by an AM fungus might influence root colonization by distinct microbes, including microbes that can be beneficial to the host plant. Species whose abundance was modulated in mycorrhizal roots included *Rhizobium* species, which were enriched in mycorrhizal roots of JSendra relative to non-mycorrhizal roots. The abundance of *X. oryzae* decreased in mycorrhizal roots relative to non-mycorrhizal roots (Fig. S9). *Pyricularia* species were also identified in metagenomic data from rice roots, namely, *P. oryzae* and *P. grisea* (Table S2). Based on metagenomic data, however, the abundance of *Pyricularia* species did not vary significantly among genotypes, compartments, or mycorrhizal conditions. *P. oryzae* (syn. *Magnaporthe oryzae*) is a destructive disease in all rice-growing regions, while *P. grisea* is closely related and morphologically similar to *P. oryzae* (94). Although *P. oryzae* primarily infects leaves, nodes, and panicles in rice, it can also infect the roots (95, 96). Similarly, *Bipolaris oryzae*, the fungus that causes brown spot disease in rice, was also identified in rice roots but with no changes in abundance among samples. These observations indicated that specific microbial species, either beneficial or pathogenic species, are selected for root colonization in mycorrhizal rice.

Conclusions

This study systemically evaluated changes in the structure and function of the endosphere and rhizosphere microbial communities in cultivated rice varieties grown under flooded conditions. Furthermore, this work revealed changes in the root-associated microbial community in response to inoculation with the AM fungus *R. irregularis*.

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The information presented here advanced our understanding of factors influencing root-associated microbial communities in rice varieties cultivated in paddy fields. These results provide a theoretical basis for understanding microbiome dynamics in flooded rice. Identifying the drivers of microbial community changes in rice roots of flooded rice offers practical guidance for exploiting microbiomes and transition to sustainable rice cultivation while reducing reliance on agrochemical use.

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AUTHOR AFFILIATIONS

[1]Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Campus Universitat Autònoma de Barcelona (UAB), Bellaterra (Cerdanyola del Vallés), Barcelona, Spain
[2]Departamento del Arroz and Centro de Genómica, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Spain
[3]Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

AUTHOR ORCIDs

Iratxe Busturia http://orcid.org/0009-0007-8234-7866
Héctor Martín-Cardoso http://orcid.org/0000-0002-0002-9001
Concha Domingo http://orcid.org/0000-0002-7204-7591
Antoni Garcia-Molina http://orcid.org/0000-0002-7790-9736
Blanca San Segundo http://orcid.org/0000-0001-7409-3172

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Iratxe Busturia, Formal analysis, Investigation, Methodology | Héctor Martín-Cardoso, Formal analysis, Investigation, Methodology | Concha Domingo, Investigation | Antoni Garcia-Molina, Investigation | Blanca San Segundo, Conceptualization, Resources, Supervision

DATA AVAILABILITY

The original data presented in this study are publicly available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) at the EMBL-EBI under accession number PRJEB105165.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (AEM01671-26-s0001.docx). Fig. S1 to S9.
Supplemental tables (AEM01671-26-s0002.xlsx). Tables S1 to S11.

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