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Research Article

Co-inoculation with *Bacillus thuringiensis* RZ2MS9 and rhizobia improves the soybean development and modulates soil functional diversity

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Abstract

Despite the beneficial effects of plant growth-promoting rhizobacteria on agriculture, understanding the consequences of introducing foreign microbes into soil taxonomic and functional diversity is necessary. This study evaluated the effects co-inoculation of soybean with *Bacillus thuringiensis* (Bt) RZ2MS9 and commercial rhizobia on the natural microbial community structure and functional potential. Our results indicated that soybean development was positively influenced by co-inoculation, plants exhibited greater height and a higher number of pods, and no reductions in productivity estimates. Soil prokaryotic diversity and community structure remained unchanged by Bt RZMS9 inoculation or co-inoculation with rhizobia 147 days after sowing. However, functional diversity was influenced by sole Bt inoculation, potentially due to community *quorum sensing* disruption by N-acyl homoserine lactone hydrolases. The genes enriched by co-inoculation were mostly related to soil phosphorus cycling, with *gcd* showing the most pronounced increase. The *nif*A genes increased when rhizobia alone were inoculated, suggesting that this pathway could be affected by Bt RZ2MS9 inoculation. This study demonstrates the synergistic activity of rhizobia and Bt RZ2MS9 on soybean development, without significantly interfering with natural microbial community, presenting a promising approach for sustainable crop management.

Keywords: bioproducts; metagenomics; microbial ecology; sustainable agriculture; tropical agriculture

Highlights

- RZ2MS9 and rhizobia co-inoculation improved soybean height and pod number.
- Soil prokaryotic taxonomic diversity and structure were unaffected by bacterial inoculation, but sole *Bacillus thuringiensis* RZ2MS9 impacted the bacterial functional diversity.
- Co-inoculation led to an increase in soil phosphorus cycling genes, with *gcd* showing the most significant rise.

Introduction

Bradyrhizobium spp. establish a symbiotic relationship with soybean plants, a phenomenon exploited by agricultural practices due to increased nitrogen fixation and grain yield, which reduces the reliance on inorganic nitrogen fertilizers (Hungria et al. 2015). This symbiosis, observed in many leguminous, provides an increase in nitrogen availability in various agroecosystems (Egamberdieva et al. 2020). Consequently, numerous studies using different *Bradyrhizobium* strains are conducted worldwide, screening for new beneficial rhizobia applicable to soybean crops in agriculture (Ulzen 2016 et al. 2016, Temesgen and Assefa 2020).

Growing evidence indicates that other beneficial soil bacteria can positively affect rhizobia performance (Korir et al. 2017). Soybean inoculation with different rhizobacteria strains, mainly species from the genera *Azospirillum*, *Bacillus*, and *Pseudomonas*, in consortium with rhizobia, has been reported to promote plant growth and enhance crop yield (Zeffa et al. 2020). Additionally, this approach increases seed germination, nodulation, and nitrogen fixation (Aung et al. 2013, Rechiatu et al. 2015, Ulzen et al. 2016).

Some studies have assessed the impact of combining inocula on plant growth, such as the co-inoculation of *Bradyrhizobium japonicum* and *Pseudomonas striata*. This combination resulted in a

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significant improvement in soybean growth and grain yield compared to the sole application of *B. japonicum* (Wasule et al. 2007). Additionally, the co-inoculation of *Bacillus* spp. with *B. japonicum* in soybean led to enhanced nodulation and nitrogen fixation, attributed to the formation of larger nodules (Sibponkrung et al. 2020).

Plant growth-promoting rhizobacteria (PGPR) can directly facilitate plant growth through various mechanisms, including the production of siderophores, synthesis of phytohormones such as auxins, cytokinins, and gibberellins, and solubilization of nutrient minerals (Masciarelli et al. 2014). Since different strains show different effects on plant physiology, and they also vary in their symbiotic effectiveness with different cultivars, there is a need to unravel the mechanisms involved in the PGPR interaction with plants (Temesgen and Assefa 2020). Furthermore, recent studies involving the co-inoculation of two or more PGPRs have shown improved crop morphology and physiological structure, driven by the combined action of different PGPRs, such as *Pseudomonas putida* KT2440 and *Sphingomonas* sp. OF178, *Azospirillum brasilense* Sp7, and *Acinetobacter* sp. EMM02 (Molina-Romero et al. 2017), *Pseudomonas stutzeri* (E25) and *Stenotrophomonas maltophilia* (Rojas-Solís et al. 2018), and *Azospirillum brasilense* and *Bradyrhizobium* spp. (Barbosa et al. 2021).

Bacillus thuringiensis (Bt) RZ2MS9, a PGPR isolated from guarana rhizosphere, has demonstrated significant effects on promoting soybean growth (Batista et al. 2018). Notably, the inoculation of Bt RZ2MS9 resulted in a substantial increase in the dry weight of both soybean shoots and roots compared to their noninoculated counterparts. Furthermore, Bt RZ2MS9 exhibits several plant growth-promoting traits, including the production of indole acetic acid (IAA), biological nitrogen fixation, and phosphate solubilization (Batista et al. 2018). One of the major Bt RZ2MS9 traits involved in the plant growth is the IAA production (Batista et al. 2021, Figueredo et al. 2023).

Although there is already substantial evidence of the benefits of using inoculants to promote the health and growth of plants, there is a growing interest in understanding the interaction of the inoculant with the soil microbiome. The soil microbiome comprises a complex and rich diversity of species, and the interactions among them play an essential role in plant health and productivity. As a result, there is increasing interest in research on beneficial PGPR strains and their diversity in soil for successful inoculation techniques (Philippot et al. 2013, Jiménez et al. 2020).

Trabelsi and Mhamdi (2013) highlighted the importance of evaluating impacts of microbial inoculants on soil microbial communities. They selected 17 studies significant on the theme, and resumed the impacts of inoculants on soil microbial community as nonconsistently changing the number and composition of the native taxonomic groups. Also, they highlighted the need for investigations of the complexity of the metabolic potentials of soil microbial communities. The studies available at the time used techniques with low power of discrimination of the soil microbial diversity such as denatirung gradiant gel electrophoresis (DGGE), Terminal restriction fragment length polymorphism (T-RFLP), and quantitative PCR (qPCR). More recently, Mawarda et al. (2020) revisited the theme, and after reviewing 108 studies, observed that 86% of them showed that inoculants modify soil microbial communities, highlighting the need for functional studies using multiomics exploration. At their review, most of the studies used 16S rRNA sequencing to investigate the bacterial soil community. The relevance of studies of genetic potential of soil microbial communities after inoculations using whole sequencing metagenomics is also featured as a challenge for future studies (Wang et al. 2024).

Thus, this study aimed to evaluate the impact of Bt RZ2MS9 and its co-inoculation with rhizobia on soybean growth, as well as on the diversity, community structure, and functional diversity and functional potential of soil natural communities in field conditions.

Materials and methods

Biological material

The PGPR Bt RZ2MS9 was first isolated from the rhizosphere of the Amazon tree guarana plants (*Paullinia cupanea* var. sorbilis) (Batista et al. 2018). It is stored in 20% glycerol at −80°C, at the Genetics of Microorganisms Laboratory, at ESALQ/USP, Piracicaba, SP, Brazil. Bt RZ2MS9 cultures were routinely obtained on Luria–Bertani (LB) medium (tryptone 10 g·l^{-1}, yeast extract 5 g·l^{-1}, and NaCl 10 g·l^{-1}) at 28°C with 150 rpm agitation.

We applied the commercial peat bioinoculant Masterfix® Soja for the co-inoculation study, which contains the rhizobia *B. japonicum* and *Bradyrhizobium elkanii* (SEMIA 5079 e SEMIA 5019, respectively). Seed treatment was performed according to the instructions provided by the manufacturer. Finally, the field study was conducted with the commercial soybean cultivar Potencia BMX (Brasmax Genetica, Brazil), responsive to inoculant for biological nitrogen fixation (Braccini et al. 2016).

Experimental area characterization

The field experiment was conducted from December 2018 to April 2019 in an area of 1 ha of the Anhumas São Paulo Uty Research Station, in Piracicaba, SP (latitude 22° 50′ 26″ south, longitude 48° 1′ 20″ west), Brazil. The experiment was installed in an area previously planted with soybeans (summer). Chemical and physical characterization of the soil in which soybean was cultivated are presented in Supplementary Table 1.

Bio-inoculum preparation and seeds treatment

The Bt RZ2MS9 inoculum was prepared and transported at the same day to the experimental areas where seed bacterization was performed before seeding. The inoculum consisted of bacterial suspension in saline solution (~1.10^8 CFU·ml^{-1}), which was prepared by previously growing the bacterium in LB medium at 28°C with 150 rpm agitation and then measuring the optical density of the culture and adjusting the concentration. The inoculum dosage applied was 8 ml of the bio-inoculant for each 1 kg of seeds, which were dried in the shade before mechanical planting. To the negative control, the same procedure was performed, but using pure LB medium.

The inoculation with the commercial rhizobia product Masterfix® Soja was performed according to the manufacturer's instructions, diluting the peat product in saline solution to the final concentration of 1.10^8 CFU·ml^{-1} and directly applying the inoculant in the seeds. The material was also dried in the shade before seeding, which occurred 2 h after seed treatment for all inoculations tested.

Field experiment

The experiment was conducted in a strip design to have restricted areas of inoculant application in the field to avoid the spread among treatments in case of a smaller plot design. Replications were performed within each strip, with 20 sampling points being marked in the strips considering a 5-m border for both sides of treatments. The treatments were the control (nonbacterial inoculation), Bt (Bt RZ2MS9 inoculation) Bt_rhizobia (Bt RZ2MS9 +

Masterfix Soja co-inoculation), and rhizobia (Masterfix Soja inoculation) (Supplementary Fig. 1).

Mechanical seeding occurred on 28 November 2018, with soybean seeds planted at a depth of 3 cm along the experimental strips (40 rows wide, spaced by 45 cm, and 100 m in length). Prior to seeding, fertilizer Nutrisafra® 04-20-20 was applied. All treatments received the same crop treatment, which was performed with applications of the fungicide Approach®Prima (300 ml·ha^{-1}) and the insecticide Belt® (70 ml·ha^{-1}).

Effects of bacterial inoculation on soybean growth promotion and productivity

At the beginning of the flowering stage [R1—45 days after sowing (DAS)], we measured the plant height. Five plants were sampled per point, totalizing 100 plants per treatment measured from the base of the plant (on the ground) up to the apex of the main stem using a metric table, according to Rocha et al. (2015). Crop lodging was assessed for each sampling point based on the average erectness of the main stem of plants at R8 (full maturity), according to Antwi-Boasiako (2017). The rating system applies a scale from 1 to 5, with 1 = all plants erect, 2 = 25% of plants lodged, 3 = 50% of plants lodged, 4 = 75% of plants lodged, and 5 = all plants lodged.

The soybean harvest was carried out on 3 April 2019. Each harvesting strip had previously been marked along with the 20 sampling points, and they consisted of two rows of plants with 5 m each, which were evaluated for total grain yield and 100-seed weight. Five plants from each sampling point were kept for measurements of dry mass, stem diameter, pod number, and seeds per pod for production estimates.

Soybean seeds oil and protein content

The percentage of oil and protein content in soybean seeds was measured through near-infrared (NIR) spectroscopy (Jiang 2020). This analysis was performed at the Laboratory of Applied Biotechnology for Plant Breeding at Universidade Estadual Paulista, Jaboticabal—SP, Brazil. Data gathering was performed with whole soybean seeds from each treatment, divided into 20 biological replicates and 3 technical replicates in Bruker® FT-NIR TANGO spectroscopy equipment.

Soil sampling, DNA extraction, library construction, and data processing

Soil sample for metagenomic analysis was collected at 20 sampling points within each treatment strip, at each time considered [Before—before sowing; CropR1—during crop development at R1 stage (45 DAS); and After—21 days after total harvesting of soybean plants (147 DAS)], respecting a 5-m border at each side of the strips. For each sampling point, 0–20 cm of soil was collected with the help of a soil probe. The material was immediately transported to the Laboratory of Genetics of Microorganisms at ESALQ/USP, Piracicaba, SP, Brazil, and stored at −80°C until DNA extraction.

Soil collected was separated for DNA extraction as follows: for the time Before, 20 soil samples from the field area were grouped in one composite sample of 5 g and then in 4 compoused samples of 250 mg for DNA extractions. DNA extractions of samples from time CropR1 and After were performed for each treatment in 4 composite samples, mixed from 20 points of soil, each one with 5 g of soil.

Total DNA extraction was performed using the DNeasy PowerSoil® Kit (Qiagen). The quality of the DNA was assessed using agarose gel electrophoresis, and the quantification was performed using a NanoDrop One and a fluorometer Qubit 4.0 with the kit DNA High Sensitivity (ThermoFisher). Fragment sizes were assessed with the Bioanalyser DNA (Agilent Technologies), applying the kit DNA HS 2100 (Agilent Technologies). Library was prepared with Nextera DNA Flex kit (Illumina). Samples were then sequenced with an Illumina NextSeq 550 platform for 300 base pairs readings (2×151 bp) (Illumina).

FastQC and MultiQC were used to assess the quality of raw reads and to compile an integrated report, respectively. Sequence trimming was performed using Trimmomatic v0.33, where we set a minimum quality threshold of 20 phred (Bolger et al. 2014). Post-trimming, the taxonomic classification of the sequences was carried out using Kraken2 v2.1.3 (Wood and Salzberg 2014), leveraging the RefSeq NCBI Standard database provided at https://benlangmead.github.io/aws-indexes/k2, dated 5 June 2023. The paired function was employed in Kraken2 for this classification.

The functional annotation of filtered metagenomic sequences was performed using bash scripts in linux, with SUPERFOCUS (Silva et al. 2016) against the SEED Subsystem database (Overbeek et al. 2014). To identify genes associated with PGPR, we employed the PGPg_finder pipeline (Pellegrinetti et al. 2024), referencing the PLaBAse database (Patz et al. 2021), specifically utilizing the mgPGPT-db in FASTA format. In this process, metagenomic sequences were first paired using the PEAR software (Zhang et al. 2014), and then converted into protein sequences with Prodigal (Hyatt et al. 2010). These protein sequences were subsequently aligned using the DIAMOND program (Buchfink et al. 2015) and processed in R to generate an abundance table.

Data analysis

Field experiment data were statistically evaluated with ANOVA, followed by Tukey tests to compare the means obtained for each treatment. All analysis was performed in R software (R Core Team 2017), and the significance level adopted in all tests was .05.

Soil microbiome diversity, considering taxonomy and functions, was analyzed using the microeco package v.1.1.0 in R software (v.4.2.1) (Liu et al. 2021). For alpha diversity, we evaluated observed genus richness, Shannon's diversity index, and Simpson's diversity index, all at the genus level. These indices were statistically compared using ANOVA followed by the Tukey honestly significant difference (HSD) test. All P values were set with a 95% confidence interval, and differences were considered significant when $P < .05$. For beta diversity, we employed NMDS (nonmetric multidimensional scaling) and PCoA (Principal Coordinates Analysis) based on the Bray–Curtis distance matrix of the soil samples, with statistical validation through PERmutational multivariate analysis of variance (PERMANOVA). PCoA was used when NMDS stress was insufficient. We also generated taxonomic summary bar charts to display the relative abundance ratio (%) at the phylum level, emphasizing the top 12 taxa. Any taxa not within this top 12 were grouped under "others." Genus differential abundance was determined using the paired comparison using Welch's t-test in the STAMP v2.1.3, setting an alpha level of 0.05 and focusing on the taxonomic at the genus level. We assessed differential taxonomy across different phases of the experiment, separately examining the R1 phase and the experiment's concluding phase.

Concerning the PGPR genes, we assessed the differential abundance of genes using the same methodology as for taxonomic differential abundance. We identified genes specific to each phase (R1 and subsequent phases) for each inoculation. The differential PGPR gene abundance was evaluated in STAMP similarly as described earlier to differential genus abundance.

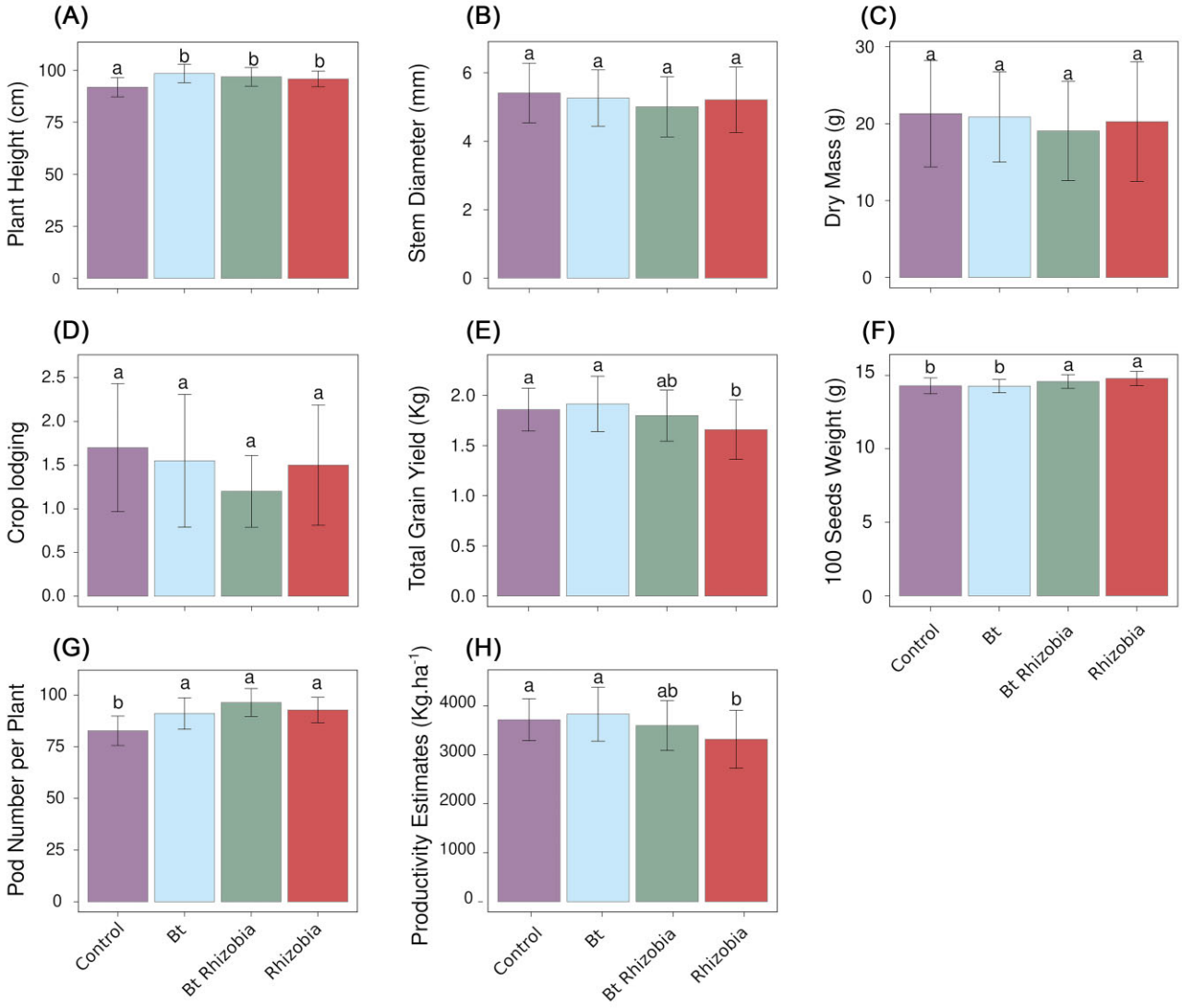

Figure 1. Effects of bacterial inoculation on soybean growth and productivity parameters. The average plant height (A) measured at the beginning of the flowering stage, stem diameter at harvest (B), dry mass at harvest (C), crop lodging (D), total grain yield (E), 100-seed weight (F), pod number per plant (G), and productivity estimates (H) of harvested soybean plants from two rows of 5 m for each treatment. Control (no inoculation), Bt (Bt RZ2MS9 inoculation), Bt_rhizobia (co-inoculation of Bt RZ2MS9 and Masterfix® Soja), and rhizobia (only Masterfix® Soja inoculation). Different lowercase letters above the bars indicate statistical differences by ANOVA followed by a Tukey post hoc test at $P < .05$ between mean values. Masterfix® Soja contains the rhizobia *B. japonicum* and *B. elkanii* (SEMIA 5079 e SEMIA 5019, respectively).

Results

Co-inoculation of soybean with Bt RZ2MS9 and rhizobia

The control exhibited significantly lower plant height compared to other treatments. Bt showed the highest plant height values followed by Bt_rhizobia and rhizobia (Fig. 1A). The pod numbers were also higher in all inoculated treatments comparing with the control (Fig. 1G). The data did not show a significant variation in stem diameter (Fig. 1B), shoot dry mass (Fig. 1C), and plant lodging (Fig. 1D).

Regarding the total grain yield, we observed a slightly higher grain yield for the Bt compared to both the control and the Bt_rhizobia. Interestingly, rhizobia had a lower grain yield (Fig. 1E), but a higher average weight of 100 seeds (Fig. 1F), which indicates that it produces less but bigger grains.

Average total grain yield was used to estimate productivity in kg·ha^{-1}. Although, Bt did not differ statistically from the control or Bt_rhizobia in productivity, Bt promoted an increased ~10% of productivity (Fig. 1H). No effect of inoculations was observed on oil and protein content from the seeds, with all treatments presenting very similar results (Supplementary Fig. 2).

Bt_rhizobia co-inoculation had both the positive effects of rhizobia on pod number and higher 100-seed weight, but not lower productivity estimates nor lower total grain yield, showing the potential of this co-inoculation.

Soil microbiome diversity and structure analysis

Independent of the treatment, the alpha and beta taxonomic diversity measurements did not show significant variations, suggesting that the diversity of soil natural communities of Bacteria and Archaea was resistant to changes due to inoculation of Bt or rhizobia, in the time frame evaluated (Figs 2A and B and 3A and B). Similarly, functional diversity followed a comparable pattern, with no significant differences in functional richness (Fig. 2C), with sig-

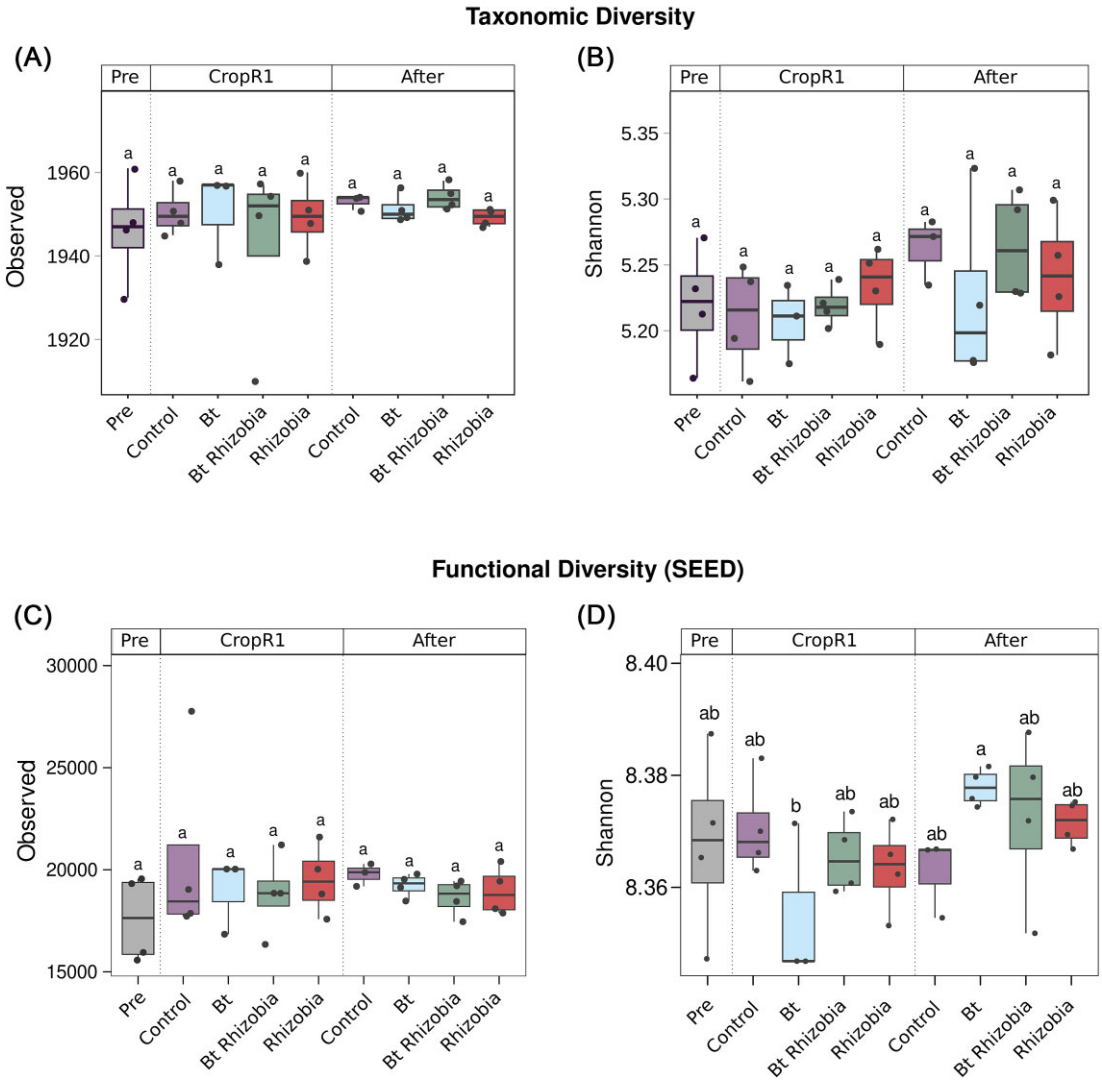

Figure 2. Alpha taxonomic diversity of soil bacterial community according to the observed features (A), and Shannon indexes (B), and alpha functional diversity of SEED features (C) and Shannon functional diversity of SEED features (D) before crop cycle (Pre), at R1 stage (Crop R1—45 DAS), and After (147 DAS), for the treatments: control (no inoculation), Bt (Bt RZ2MS9 inoculation), Bt_rhizobia (co-inoculation of Bt RZ2MS9 and Masterfix® Soja), and rhizobia (only Masterfix® Soja inoculation). Boxplots with different letters above the boxes denote means that are significantly different by ANOVA followed by a Tukey post hoc test at $P < .05$. Masterfix® Soja contains the rhizobia *B. japonicum* and *B. elkanii* (SEMIA 5079 e SEMIA 5019, respectively).

nificant changes only when comparing functional Shannon diversity based on SEED features annotation of Bt 45 DAS (CropR1) and Bt 147 DAS (After) (Fig. 2D). This result indicates a functional effect on the community of inoculating Bt, on the first 45 days of inoculation, lost after plant removal and 147 DAS. Additionally, PCoA showed no significant differences in the functional structure among treatments in both the CropR1 and postharvest phases, indicating that the functional changes were restricted to a few functions (Fig. 3C and D).

The effect of Bt inoculation on soil functional Shannon diversity between CropR1 and After was evaluated using the complete functional annotation of the data. The function "quorum sensing and biofilm formation" was one of the functions significantly increased in CropR1 at 45 DAS but was reduced to the levels of the control natural community on After time, by 147 DAS (Fig. 4A). In CropR1, this function was also significantly increased in soil community when Bt inoculation was compared to Bt + rhizobia, rhizobia alone, and the control natural community (Fig. 4B). More specifically, within this functional class, the annotation for "N-acyl_homoserine_lactone_hydrolase" showed an increase.

The analysis of the relative abundance of the bacterial and archaeal taxa at the phylum level showed high homogeneity among treatments, even when considering the time variable (Before, CropR1, and After). Taxonomic classification further revealed 59 phyla, 117 classes, 250 orders, 560 families, and 2029 genera. The dominant phyla were Proteobacteria (syn. Pseudomonadota) (46.56%), Actinobacteria (syn. Actinomycetota) (45.64%), Planctomycetes (Syn. Planctomycetota) (1.65%), Firmicutes (syn. Bacillota) (1.36%), Bacteroidota (1.01%), Euryarchaeota (0.91%), and Acidobacteria (0.88%) (Supplementary Fig. 3).

Differential taxonomy and function abundance

Considering the differential abundance of taxa, significant alterations in the soil microbial community in response to the inoculants were found, especially in Bt_rhizobia at CropR1. It was characterized by the presence of distinct genera such as *Agromyces*, *Capillimicrobium*, *Luteitalea*, and *Anaeromyxobacter*, each known for beneficial plant interactions. This diversity suggests a synergistic effect of Bt and rhizobia, potentially enhancing soybean growth

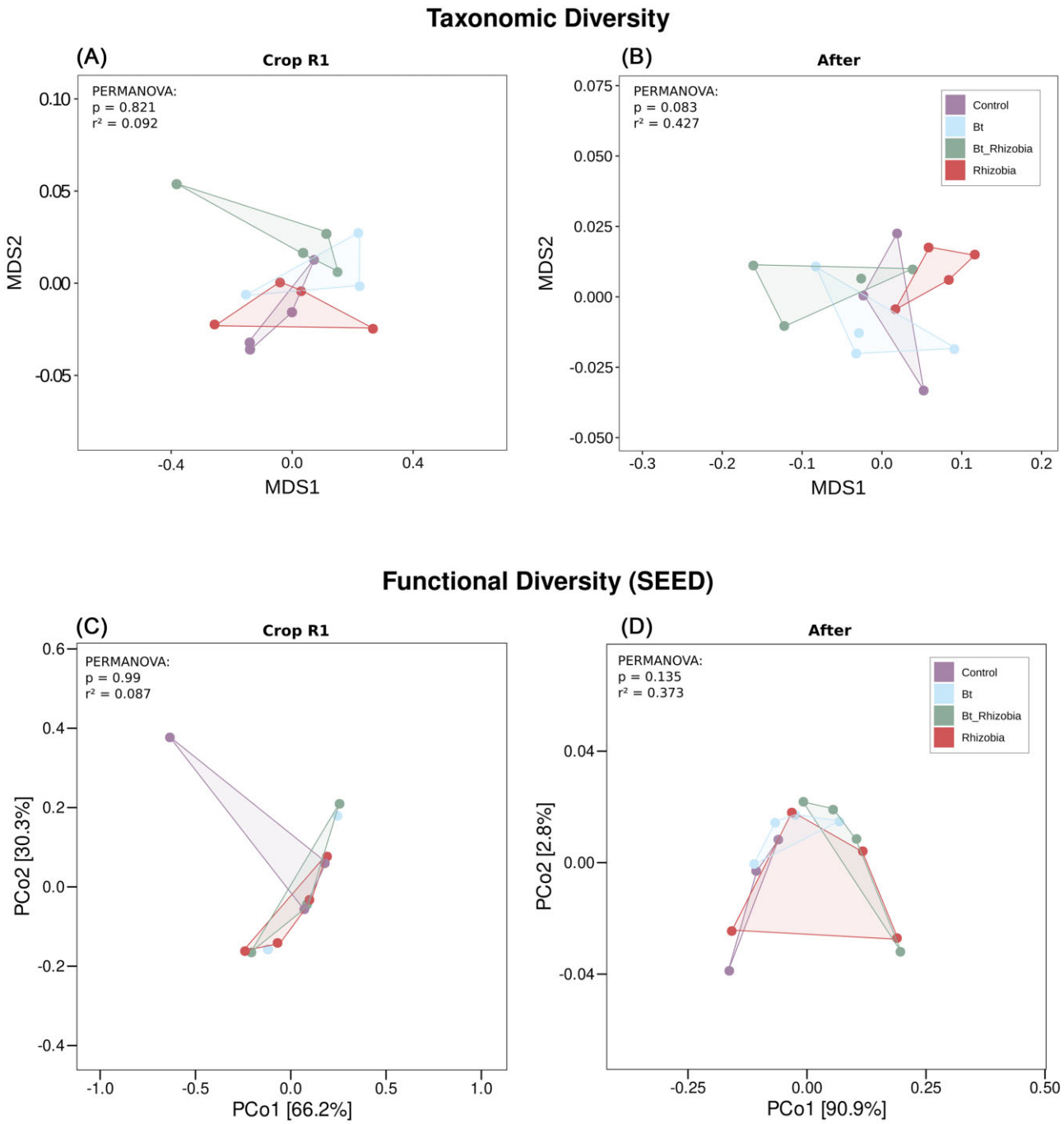

Figure 3. Comparison of soil taxonomic bacterial diversity with NMDS plots, grouping samples in ellipses among inoculation treatments in times R1—45 DAS (A), and 21 days after harvest or 147 DAS (B), and functional diversity based on SEED feature annotations using PcoA grouping samples in ellipses among inoculation treatments at R1—45 DAS (C), and 21 days after harvest or 147 DAS (D). Treatments are control (no inoculation), Bt (Bt RZ2MS9 inoculation), Bt_rhizobia (co-inoculation of Bt RZ2MS9 and Masterfix® Soja), and rhizobia (only Masterfix® Soja inoculation). Masterfix® Soja contains the rhizobia *B. japonicum* and *B. elkanii* (SEMIA 5079 e SEMIA 5019, respectively).

and health. Comparing Bt, it exhibited a different microbial profile, with an increased presence of genera such as *Gemmata* and *Frigoriglobus*. Meanwhile, the rhizobia resulted in an increase in beneficial bacteria such as *Streptomyces*, *Sorangium*, and *Anaeromyxobacter*, some of their species known for their roles in promoting plant growth and soil health (Fig. 5).

From After samples, the microbial community in the Bt_rhizobia exhibited a diverse range of differential genera, including *Capillimicrobium*, *Gottfriedia*, *Arthrobacter*, *Nitrospira*, and *Nordella*. This diversity contrasts with the control, which maintained a more limited range of genera such as *Mycobacterium*, *Nocardia*, *Gemmatirosa*, and *Gemmatimonas*. The presence of *Nitrospira*, a known nitrifier, along with *Arthrobacter* and other beneficial microbes in the Bt_rhizobia group, suggests enhanced nitrogen cycling and other plant growth-promoting activities in the soil, which are crucial for soybean health and yield (Fig. 5).

Regarding the presence of plant-growth promoting functional potential, we observed that at CropR1, control soil samples were

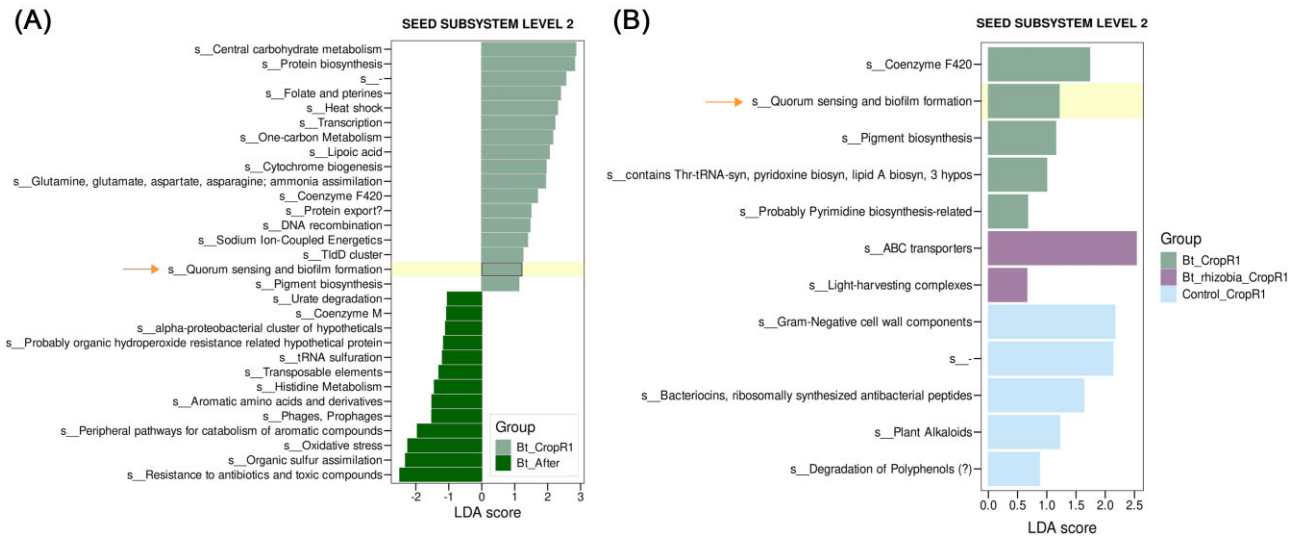

Figure 4. Shifts in functional annotations of SEED category level 2 comparing (A) Bt (Bt RZ2MS9 inoculation) in CropR1 (45 DAS) and After (147 DAS) and (B) Bt (Bt RZ2MS9 inoculation) compared to Bt_rhizobia (co-inoculation of Bt RZ2MS9 and Masterfix® Soja) and the control on CropR1. Only significant annotations are shown ($P < .05$).

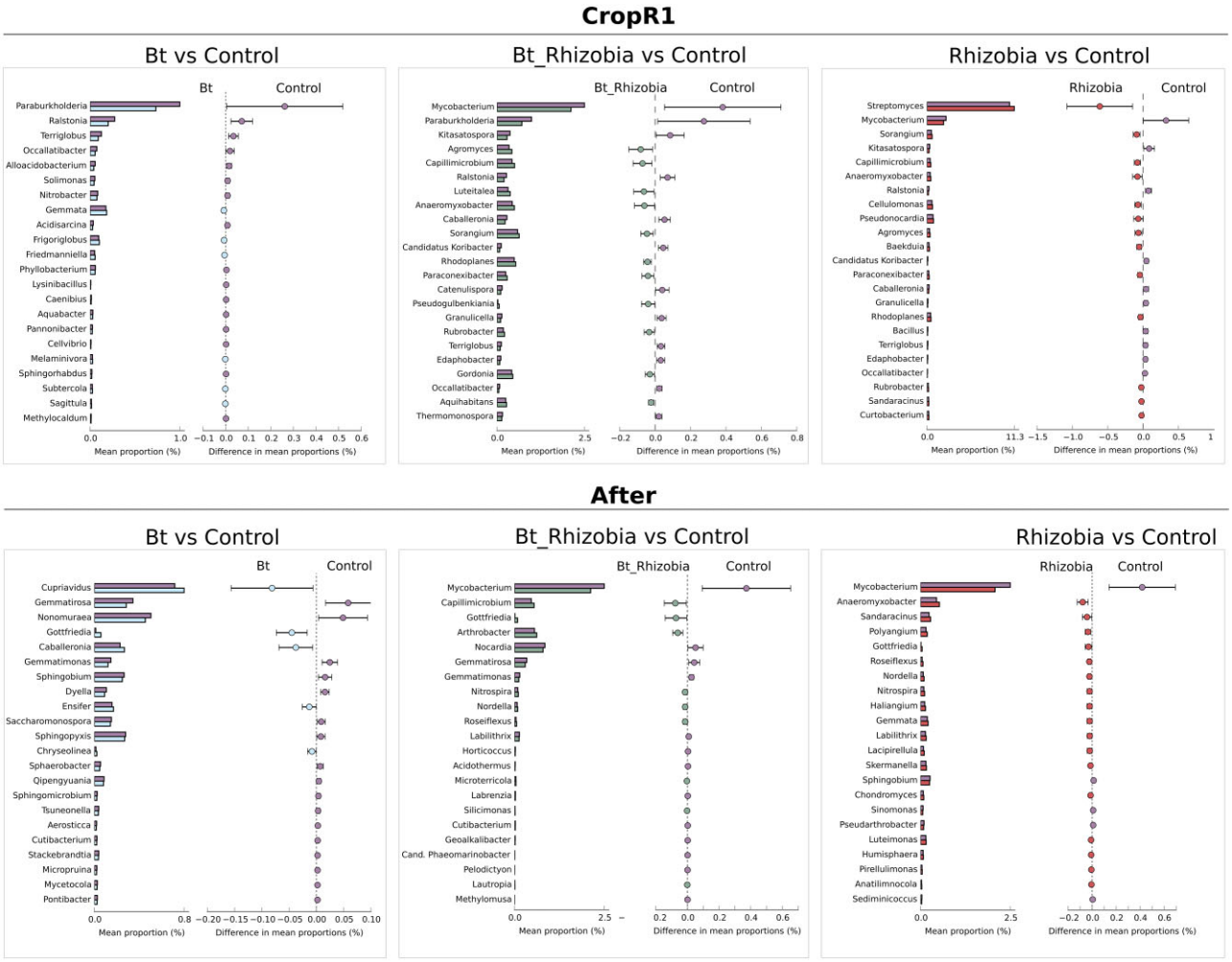

Figure 5. Differential abundance of bacterial taxa during the soybean growth cycle. The upper panels display the microbial taxa differential abundance at the CropR1 stage (45 DAS), while the lower panels show the results after harvest (147 DAS). Each panel contrasts the microbial community composition associated with three different treatments: control (no inoculation), Bt (Bt RZ2MS9 inoculation), Bt_rhizobia (co-inoculation of Bt RZ2MS9 and Masterfix® Soja), and rhizobia (only Masterfix® Soja inoculation). Error bars represent the standard error of the mean proportion, based on three to four biological replicates per treatment. Masterfix® Soja contains the rhizobia *B. japonicum* and *B. elkanii* (SEMIA 5079 e SEMIA 5019, respectively).

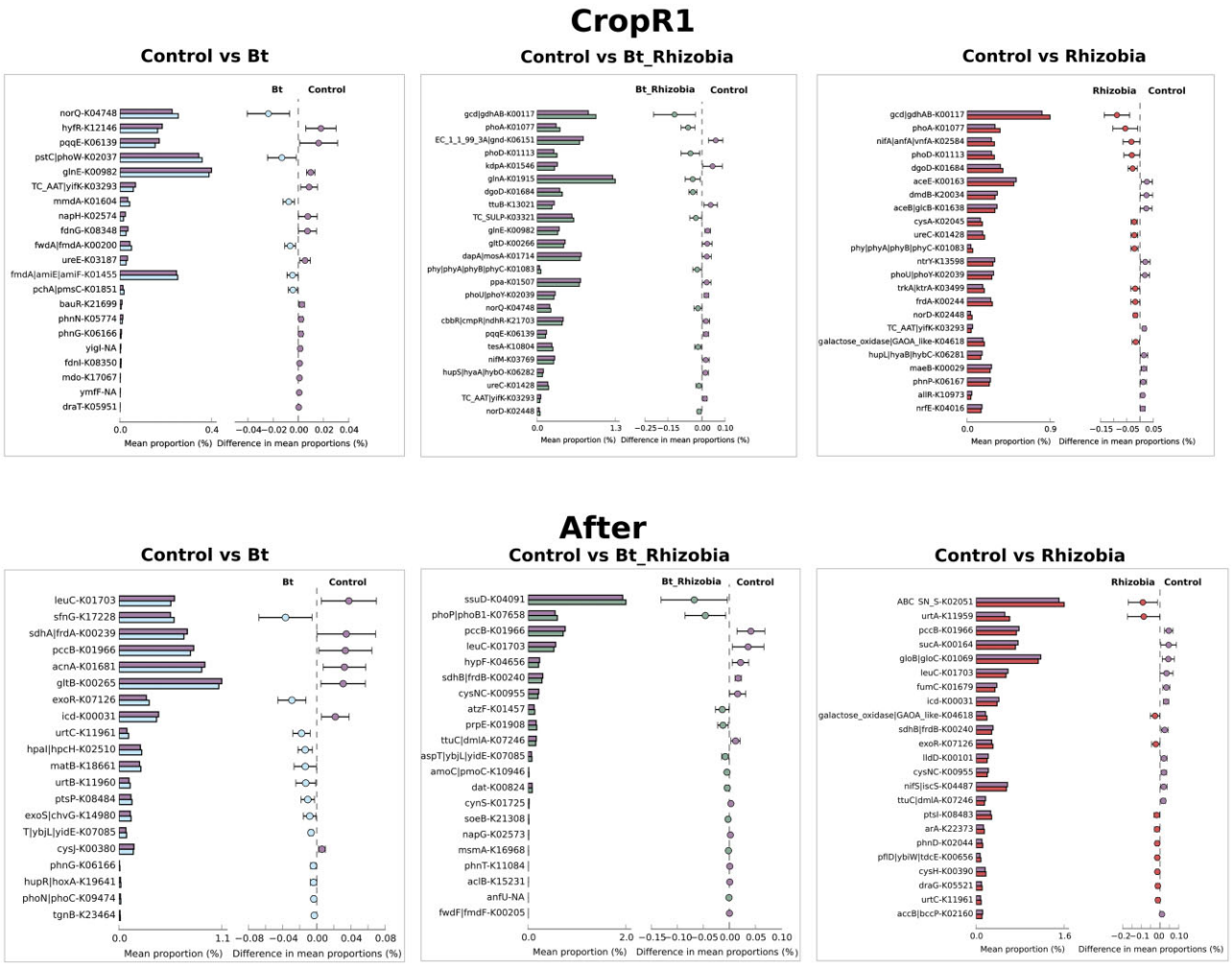

Figure 6. Comparative analysis of functional gene abundance. The upper panels display results from the CropR1 growth stage (45 DAS), and the lower panels show data collected after harvest (147 DAS). Error bars denote the standard error across three to four biological replicates comparing the treatments: control (no inoculation), Bt (Bt RZ2MS9 inoculation), Bt_rhizobia (co-inoculation of Bt RZ2MS9 and Masterfix® Soja), and rhizobia (only Masterfix® Soja inoculation). Masterfix® Soja contains the rhizobia *B. japonicum* and *B. elkanii* (SEMIA 5079 e SEMIA 5019, respectively).

characterized by genes associated with stress response (*hyf*R, *pqq*E), nitrogen metabolism (*ure*E), and various central metabolic pathways (*gln*E, *yif*K), reflecting the native functional capabilities of the soil microbiota. Upon Bt, a distinct enrichment of genes related to nutrient transport (*pst*C), modulation of the nitrogen cycle (*nor*Q), and carbon processing (*mmd*A, *fud*A) was observed. The Bt_rhizobia treatment further diversified the functional gene profile, with an abundance of genes implicated in phosphate mobilization (*pho*A, *pho*D), nitrogen assimilation (*gln*A), and carbohydrate metabolism (*dgo*D), alongside genes linked to environmental stress resilience (*phy*). Similarly, rhizobia promoted genes beneficial for phosphate mobilization and nitrogen fixation (*nif*A, *pho*A, *pho*D), as well as those involved in sulfur assimilation (*cys*A) and urea hydrolysis (*ure*C) (Fig. 6).

Following the harvest, a persistent alteration in the soil metagenome was evident. Control samples continued to show an abundance of genes central to metabolic integrity and nutrient cycling. In contrast, Bt-inoculated soils exhibited genes that could potentially influence postharvest nitrogen cycling (*sfn*G), microbial community structure through biofilm regulation (*exo*R), and phosphate transport (*pst*P). Notably, the Bt_rhizobia treatment demonstrated a wide array of functional genes, including those related to complex organic compound degradation (*ssu*D), response regulation (*pho*P), and atrazine degradation (*atz*F), suggesting a long-term effect on the soil's capacity for self-renewal and environmental detoxification. Rhizobia treatment maintains an abundance of genes that may enhance nitrogen utilization (*urt*A) and provide environmental stress resilience (K04618), possibly aiding in soil restoration for future crop cycles.

Discussion

Inoculants and plant growth promotion

Soybean [*Glycine max* (L.) Merr.] stands out as one of the globally predominant crops employing inoculants, primarily relying on a variety of bacteria from the genus *Bradyrhizobium* (Santos et al. 2019). Also, the co-inoculation of rhizobia in consortium with other PGPR significantly improved soybean growth and grain yield compared to the sole application of rhizobia (Wasule et al. 2007). The bacterial strain studied here, Bt RZ2MS9, has already demonstrated positive results when inoculated in soybean and maize, as well as their ability to colonize maize endophytically (Batista et al. 2018, Almeida et al. 2021). It is possible that this bacterium is also endophytic in soybean, which could explain its role in promoting plant height growth. Ferrarezi et al. (2022) showed previ-

ously the effect of this strain on maize rhizobiome in field condition. Considering the potential of this strain as a bioinoculant, this study presents the first evaluation of the co-inoculation of rhizobia and Bt RZ2MS9 and its effects on soybean, as well as on the soil bacterial diversity and functional potential in field conditions.

In PGPR, mechanisms related to the plant growth-promoting effect involve biological processes such as IAA production, phosphate solubilization, and urease activities, exerting a direct impact on the nutrient and water uptake by the plant (Khan et al. 2019). A previous study with Bt RZ2MS9 demonstrated its capability to produce IAA in the presence of L-tryptophan (Batista et al. 2021, Figueredo et al. 2023), possibly attributed to the strain's ability to utilize L-tryptophan as a physiological precursor (Spaepen et al. 2007). Several strains of *B. thuringiensis* have been used to promote plant growth, and the findings of this study align with previous reports (Vidal-Quist et al. 2013, Tagele et al. 2019, Viljoen et al. 2019, Jo et al. 2020).

In previous studies, soybean inoculation with Bt RZ2MS9 resulted in increased plant growth (Batista et al. 2018). The average shoot length of treatments inoculated and co-inoculated with this strain was greater than the control, but there was no significant effect on shoot dry mass, stem diameter, or productivity. Even though rhizobacteria from the genus *Bacillus* are commonly observed to interact positively with plants, different species and strains may have varying effects on other aspects of plant growth. The PGPR can produce phytohormones, improve drought resistance, and suppress pathogens, but some of these attributes may not be directly correlated with significant increases in grain yield under field conditions (Elkoca et al. 2010; Tsigie et al. 2011). Experiments involving different plant species and varying environmental conditions may reveal different plant growth-promoting features and productivity results.

Similarly, Bai et al. (2020) evaluated Bt A5-BRSC inoculation on the development of okra. Their results showed a significant increase in seed germination, shoot height, root length, leaf diameter, vigor index, fruit weight, seed weight, and total fresh weight as well as dry weight of inoculated plants in comparison to the control. Hungria et al. (2013) observed an increase of 420 kg·ha^{-1} (16.1%) in soybean production co-inoculated with *B. japonicum* and *A. brasilense* compared to control treatment inoculated only with *B. japonicum*. However, Zuffo et al. (2016) reported no significant differences in productivity in soybean co-inoculated with *B. japonicum* and *A. brasilense*, and the control group only inoculated with the former bacterium. A study with *Bacillus subtilis* in co-inoculation with *B. japonicum* in soybean by Atieno et al. (2012) showed increased soybean nodulation and biomass traits. Thus, what is not clear is the impact of co-inoculation on soybean grain yield (Zeffa et al. 2020).

Interestingly, we observed an increase in pod number in all inoculated treatments: Bt, Bt_rhizobia, and only rhizobia, compared to the control. Bioinoculants may influence soil nutrient availability to the plant, thereby impacting grain production, and such differences are observed based on the type of formulation used for crop inoculation (Maitra et al. 2021). The increase in pod number follows a 100-seed weight increase in the treatments that had rhizobia applied (alone or in co-inoculation). This shows that rhizobia stimulates both pods and grain weight increase, also observed by Azfal et al. (2010). Bt alone, however, only promoted an increase in pod number, indicating that rhizobia biological fixation is mostly the reason of the changes.

The protein and oil content of soybean seeds in this study did not vary among treatments. However, Sheteiwy et al. (2021) tested the effect of co-inoculation of *Bacillus amyloliquefaciens* and mycorrhiza on soybeans under drought stress and they observed an increase of protein and oil content in seeds from inoculated plants cultivated under drought stress compared with to the control. Yasmin et al. (2020) observed the same results of increased oil and protein content when testing the co-inoculation effects of *Pseudomonas pseudoalcaligenes* and *B. subtilis* in soybean under salinity stress. Therefore, the inoculation of both bacteria tested in this study under the same conditions of salinity and irrigation may not have shown a potential protective effect of these rhizobacteria against drought and salinity stresses, which can be assessed with different experimental conditions. Besides, Barbosa et al. (2021) showed that other variables, such as soybean growth habit, climate, soil texture, and management system, affect co-inoculation results, and thus they should be considered in determining the inoculation strategy to be applied. Thus, further experimentation considering different experimental conditions or plant species can reveal other potential benefits from the use of Bt RZ2MS9 in co-inoculation strategies.

Inoculants and soil prokaryotic community structure

One important factor that affects the efficacy of soil microbial inoculants is the competition of inoculated microorganisms with the native soil microbiota (Kaminsky et al. 2019). In this study, inoculation with Bt RZ2MS9 and rhizobia exhibited minimal interference on the native soil taxonomic diversity. For bacterial composition, the phyla Proteobacteria (syn. Pseudomonadota) and Actinobacteria (syn. Actinomycetota) were predominant in all soil samples. Both phyla are commonly found in soil and can be associated with plant growth promotion by mechanisms such as facilitating the degradation of aminocyclopropane carboxylate and contributing to the suppression of root diseases (Jorquera et al. 2012, Zhang et al. 2019). Analysis of percentage abundance and beta diversity over time did not reveal clear impacts of either sole inoculations or co-inoculation on bacterial taxonomic diversity.

Considering that community beta diversity in this study was not impacted by the inoculations over time, the application of Bt RZ2MS9 and rhizobia seems to be safe for environmental application, from the taxonomic perspective. Further testing is needed to reach a final conclusion, including longer time frames and various environmental conditions. Moreover, changes in soil bacterial community structure due to the inoculation of a Bt strain were reported by Jo et al. (2020), and such effect also occurred after 6 weeks of inoculation, consistent with the findings reported here. In this study, soil sampling for diversity analysis during crop development occurred 45 DAS the inoculated seeds, and sampling after harvest occurred 147 DAS. This emphasizes the importance of future analysis on the long-term impacts of Bt RZ2MS9 inoculation on soil bacteria diversity.

Even though we did not see a change in the structure of the soil microbial community, some taxa were differently affected by the inoculation treatments. For example, the genus *Ralstonia* was the only one with reduced relative abundance in treatments inoculated with Bt RZ2MS9 or rhizobia, or the combination of both, in CropR1, compared to the control. Also, the genus *Gottfriedia* was the only consistently enriched in relative abundance in the soil, apart the inoculation performed, in the After moment, compared to the control. *Ralstonia* is found in soils and includes various species of Gram-negative, non-spore-forming bacteria, some of them are plant pathogens (Peeters et al. 2013). *Gottfriedia* is

a genus previously known as part of *Bacillus*, with many species agronomic relevant (Gupta et al. 2020). The two genera can potentially act as bioindicators of inoculation since they are sensible to the presence of the inoculants studied. Bioindicators can be used as a metric in determining soil functionality, useful to measure soil quality, restoration, and resilience, concerning both agriculture and the environment (Bhaduri et al. 2022). The mechanisms explaining the increase in relative abundance of *Ralstonia* and the decrease in *Gottfriedia* can be various, such as competition and collaboration with soil native microbes, and to understand the ecology of the inoculants is necessary to discuss soil microbial ecology.

Another genus that showed a consistent responsive behavior to inoculants was *Mycobacterium*. Mycobacteria, a diverse and ubiquitous group of Actinobacteria, includes species that are significant pathogens and are prevalent in a wide range of habitats, including soil and aquatic environments (Walsh et al. 2019). In all situations where rhizobium was inoculated, either alone or in combination with Bt RZ2MS9, *Mycobacterium* consistently showed a reduction in relative abundance during both evaluated time points. In contrast, the inoculation of Bt RZ2MS9 alone did not induce significant changes in *Mycobacterium* relative abundance, indicating a specific responsiveness to rhizobium.

Inoculants and microbiota functional potential

The functional diversity measured using the Shannon index on the functional annotation shows a decrease in Bt CropR1 (45 DAS) compared to Bt in After (147 DAS). Among the functions that change between the two time points, the relative abundance of N-acyl homoserine lactone hydrolase increases in Bt CropR1, the period where the total functional diversity is at its lowest. This enzyme hydrolyzes the ester bond of the homoserine lactone ring of N-acyl-L-homoserine lactones, key bacterial *quorum sensing* regulator, rendering the signaling molecules incapable of binding to their target transcriptional regulators and thus blocking microbial quorum sensing (Kim et al. 2005). Bt RZ2MS9 carries out the gene *aiiA*, encoder of acyl homoserine lactonase (Bonatelli et al. 2020). Bt inoculation can potentially disrupt *quorum sensing* in the soil bacterial community, thereby reducing the Shannon functional diversity at 45 DAS. However, soil functional diversity returned to natural levels at 147 DAS, demonstrating the microbial resilience. The wide distribution of N-acyl homoserine lactone-degrading enzymes in *B. thuringiensis* is well documented (Lee et al. 2002), and its *quorum quenching* action was previously observed in co-inoculation with PGPR (Rosier et al. 2021), but when compared to isolated bacteria. This study is the first to report that Bt inoculation in soil can influence functional diversity and that functional diversity can return to previous levels after days to weeks of bacterial inoculation.

The genetic markers related to plant growth promotion that are enriched after inoculating Bt RZ2MS9 do not show equal enrichment when rhizobia is co-inoculated. This disparity may be attributed to stronger interactions between rhizobia and the soil's natural community. Conversely, functions related to phosphorus (alkaline phosphatase) and carbon (galacto dehydratase) cycling are enriched in soils inoculated with rhizobia alone or combined with Bt RZ2MS9. The soils inoculated with rhizobia, isolated or in association with Bt, had genes enriched in relative abundance, compared to the control. Most of these genes were directly related to phosphorus metabolism (*pho*A and *pho*D), but the highest increase was of a quinoprotein glucose dehydrogenase (*gcd*). Soil microbes solubilize mineral phosphates by secreting gluconic acid, among other acids. The gluconic acid is produced from glucose by quinoprotein glucose dehydrogenase (EC1.1.5.2, GDH) (An et al. 2016).

The inoculation of Bt RZ2MS9 and rhizobia promoted some parameters involved in soybean growth in height, whether applied alone or in co-inoculation. The native soil prokaryotic microbiome showed no significant influence on both microbial diversity and community structure, but Bt inoculation influenced functional diversity. The genera *Agromyces*, *Capillimicrobium*, *Luteitalea*, and *Anaeromyxobacter* consistently increased in relative abundance after the co-inoculation of Bt RZ2MS9 and rhizobia. These genera potentially serve as bioindicators of the presence of inoculants. The genes enriched after co-inoculation were mostly related to phosphorus cycling in the soil. The most pronounced increase was observed in the *gcd* gene, indicating the release of gluconic acid and phosphorus solubilization as a potentially relevant pathway to promote plant nutrition and growth. The *nif*A genes increased only when rhizobia were inoculated alone, highlighting the need for a better understanding of the impacts of co-inoculation with Bt RZ2MS9 on nitrogen fixation outside plant nodules. Microbial interactions in soil are complex, and despite the inoculation of foreign bacteria does not harm community structure and diversity, it can influence specific native microbial relationships and affect functional diversity.

Author contributions

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Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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Data availability

The metagenome sequences have been deposited in the Zenodo database and are referenced as Fonseca de Souza et al.

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