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Nitrogen Supply and Host Genotype Shape Root-Zone Soil and Endosphere Bacterial Communities in Maize

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Received: 13 August 2026; Accepted: 12 October 2026; Published: 19 October 2026

ABSTRACT: Root-associated microbiomes can influence plant nutrient acquisition and adaptation to nutrient limitation, but how nitrogen availability shapes bacterial communities in different root compartments remains incompletely understood. We used 16S rRNA gene amplicon sequencing to characterize root-zone (RE) soil and root endosphere bacterial communities in four maize inbred lines previously classified as having contrasting responses to low-nitrogen (NL) conditions grown under low- and normal-nitrogen (NZ) conditions. Nitrogen supply was significantly associated with bacterial community composition in both the RE soil ($R^2 = 0.09$, $p = 0.001$) and root endosphere ($R^2 = 0.06$, $p = 0.011$) whereas genotype was significantly associated with community composition in both the RE soil ($R^2 = 0.15$, $p = 0.011$) and root endosphere ($R^2 = 0.18$, $p = 0.002$). The nitrogen $\times$ genotype interaction was significant in the RE soil ($R^2 = 0.18$, $p = 0.003$) but not in the root endosphere ($R^2 = 0.14$, $p = 0.127$). Nitrogen supply significantly affected Shannon diversity in the RE soil ($F = 6.20$, $p = 0.024$), whereas its effect on root endosphere Shannon diversity was not significant after exclusion of organelle-associated ASVs ($F = 0.266$, $p = 0.613$). NL conditions enriched several genera with reported plant growth-promoting and stress-related functions, including *Bacillus*, *Exiguobacterium*, *Acinetobacter*, *Kosakonia*, and members of the *Burkholderia-Caballeronia-Paraburkholderia* complex. By contrast, several actinobacterial taxa were preferentially enriched under NZ conditions. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) predicted differences in pathways related to nitrogen and carbon metabolism and environmental signal transduction. Conclusions: These findings indicate that nitrogen supply and host genotype are associated with variation in maize root-associated bacterial communities in a compartment-dependent manner, with nitrogen-associated changes more evident at the level of community composition than Shannon diversity. The low-nitrogen-associated taxa identified here provide candidates for future functional evaluation.

KEYWORDS: *Zea mays*; root microbiome; nitrogen limitation; root-zone soil; endosphere; 16S rRNA gene amplicon sequencing

1 Introduction

Nitrogen is an essential macronutrient and a major determinant of maize growth and productivity [1]. Modern maize production relies heavily on nitrogen fertilizers, but excessive nitrogen inputs increase production costs and contribute to nitrate leaching, soil acidification, and greenhouse gas emissions [2,3]. Improving nitrogen-use efficiency is therefore a central objective of sustainable maize production. Although nitrogen-use efficiency is strongly influenced by plant genetic mechanisms governing nitrogen uptake,

assimilation, and remobilization [4], root-associated beneficial microorganisms can enhance maize nitrogen uptake under reduced nitrogen availability [5].

Root-associated microbial communities are spatially organized into distinct compartments, including the rhizosphere and endosphere [6]. The rhizosphere is directly influenced by soil properties and root exudates, whereas the endosphere comprises microorganisms that overcome host barriers and colonize internal root tissues. Consequently, these compartments differ in microbial diversity, community assembly processes, and ecological functions [7]. Their contrasting microbial communities arise from the combined effects of the surrounding soil microbial pool, root-derived resources, host selection, and microbial adaptation to distinct ecological niches [8]. Rhizosphere communities are generally more diverse and more responsive to soil conditions and nutrient availability, whereas endophytic communities are subject to stronger host-mediated filtering [9]. Because nitrogen availability can alter maize root exudate composition and rhizosphere bacterial communities [10] while nitrogen fertilization can also modify the root endophytic microbiome [11], nitrogen supply may reshape root-associated bacterial communities in a compartment-dependent manner.

Maize genotypes vary markedly in their tolerance to nitrogen limitation, and part of this variation may be associated with differences in their ability to recruit and maintain beneficial microorganisms [12]. Host genotype is an important determinant of plant-associated microbiome assembly, and genetic variation among maize genotypes can influence root architecture, nutrient uptake, root exudation, and host-mediated microbial selection, thereby affecting the recruitment of root-associated microbial taxa [13,14]. In turn, bacteria involved in nitrogen transformation, phytohormone production, stress tolerance, and nutrient mobilization may contribute to plant performance under nitrogen-limited conditions [8]. Previous studies have therefore provided evidence that both nitrogen supply and maize genetic background can influence root-associated microbial communities. However, these factors have often been examined separately, and it remains unclear whether maize genotypes with contrasting tolerance to nitrogen limitation harbor distinct bacterial communities under different nitrogen regimes. Moreover, it is not well understood whether nitrogen-responsive bacterial communities are similarly regulated in the RE soil and root endosphere, which are subject to different environmental conditions and degrees of host-mediated selection. Consequently, the relationships among nitrogen supply, maize genotype, and root compartment in shaping bacterial community composition and functional potential remain insufficiently resolved.

In this study, we characterized RE soil and root endosphere bacterial communities in four maize inbred lines with contrasting NL tolerance. The low-nitrogen-tolerant genotypes were 16SD159 and M2325, whereas K17HL0169 and K17HL0871 were classified as low-nitrogen-sensitive genotypes. Plants were grown under NL and NZ conditions, and their bacterial communities were profiled using 16S rRNA gene amplicon sequencing. We assessed alpha and beta diversity, taxonomic composition, differentially abundant taxa, predicted functional pathways, and microbial co-occurrence networks. Our aim was to determine how nitrogen availability and root compartment influence bacterial community structure and whether these responses differ among maize genotypes with contrasting tolerance to nitrogen limitation. The present study specifically focuses on the RE soil and root endosphere compartments.

2 Materials and Methods

2.1 Plant Materials and Experimental Design

Four maize (*Zea mays* L.) inbred lines, 16SD159, M2325, K17HL0169, and K17HL0871, were used in this study. All four inbred lines were obtained from the maize breeding program of Shanxi Agricultural University, Shanxi, China. Based on their agronomic performance and nitrogen-use efficiency under NL

conditions in previous evaluations conducted by our research group, 16SD159 and M2325 were classified as low-nitrogen-tolerant genotypes, whereas K17HL0169 and K17HL0871 were classified as low-nitrogen-sensitive genotype. These classifications were established prior to the present field experiment and were not independently re-evaluated using plant phenotypic or physiological measurements in the current trial. The field experiment was conducted during the summer of 2024 in Zhao County, Hebei Province, China, and root samples were collected at the flowering stage. Plants were grown under two nitrogen regimes, low nitrogen (NL) and normal nitrogen (NZ). Three biological replicates were included for each nitrogen $\times$ genotype treatment. Each replicate consisted of root tissues collected and pooled from multiple plants to obtain a representative sample. RE soil and endosphere (EP) samples were collected separately from the corresponding biological replicates.

In the NZ treatment, nitrogen was applied at 240 kg N ha^{-1} as urea (46% N), with equal applications at sowing and the V12 (large bell-mouth) stage. No additional nitrogen fertilizer was applied throughout the growing season in the NL treatment, and plants relied solely on the native soil nitrogen supply. Phosphorus ($90 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$) and potassium ($120 \text{ kg K}_2\text{O ha}^{-1}$) fertilizers were applied equally to all plots as basal fertilizers before sowing.

For initial sample collection, three compartments associated with the maize root system were considered: rhizosphere soil (RS), RE soil, and root endosphere (EP). RS represented the rhizosphere soil closely associated with the root surface, whereas RE represented the surrounding RE soil. EP represented the internal root compartment. The RS and RE samples were collected separately during root sampling. After removal of the soil fractions, roots were washed with sterile phosphate-buffered saline (PBS) to remove externally associated microorganisms and subsequently surface-sterilized for collection of EP samples. The roots were sequentially treated with 70% (v/v) ethanol for 30 s and 2% (w/v) sodium hypochlorite (NaClO) for 10 min, followed by three rinses with sterile water to remove residual sterilizing agents. During sample processing and quality assessment, several RS samples were found to be contaminated and were therefore excluded from further analysis. Following removal of the contaminated samples, the remaining RS dataset was incomplete and unbalanced across nitrogen treatments and maize genotypes and could not support the factorial comparisons conducted in this study. Therefore, RS samples were not included in the downstream analyses, and the present study focused on the RE and EP compartments.

2.2 Baseline Soil Physicochemical Properties

To characterize the background soil fertility of the experimental field, baseline soil samples were collected from the 0–20 cm layer at three locations (JC-1, JC-2, and JC-3) across the experimental field before sowing. Soil organic matter was determined by the potassium dichromate oxidation method, alkaline-hydrolyzable nitrogen by the alkaline hydrolysis diffusion method, available phosphorus by the Olsen method, available potassium by ammonium acetate extraction followed by flame photometry, and pH in a 1:2.5 soil:water suspension. Briefly, the soil had moderate organic matter content ($20.34 \pm 1.13 \text{ g kg}^{-1}$), relatively high alkaline-hydrolyzable nitrogen ($119.24 \pm 39.42 \text{ mg kg}^{-1}$) and available potassium ($442.00 \pm 32.42 \text{ mg kg}^{-1}$), moderate available phosphorus ($47.58 \pm 7.45 \text{ mg kg}^{-1}$), and an alkaline pH (8.11 ± 0.13) (mean $\pm$ SD, $n = 3$) (Table S1).

2.3 DNA Extraction

Total genomic DNA was extracted from approximately 0.25 g of each RE soil (RE) or root endosphere sample using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was quantified using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific,

Waltham, MA, USA), and DNA purity was assessed by measuring the A260/A280 and A260/A230 absorbance ratios with a NanoPhotometer NP80 spectrophotometer (Implen GmbH, Munich, Germany). DNA integrity was further verified by electrophoresis on a 1% agarose gel. Only high-quality DNA samples were used for subsequent PCR amplification and library construction. Purified DNA was stored at -80°C until sequencing.

2.4 16S rRNA Gene Amplification and Sequencing

The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR amplification was performed in a 25 μL reaction containing approximately 10 ng of template DNA and 0.2 μM of each primer under standard amplification conditions with 30 cycles. Negative controls without template DNA were included in each PCR run. PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA), quantified with a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and pooled at equimolar concentrations for library construction. Libraries were sequenced on an Illumina NovaSeq 6000 platform (paired-end 2×250 bp) by Novogene Co., Ltd. (Beijing, China).

2.5 Sequence Processing and Taxonomic Annotation

Raw sequencing reads were processed using the DADA2 v1.36.0 package in R version 4.5.3 [15]. Primer sequences were removed using Cutadapt v4.9 before quality filtering. Reads were filtered using a maximum expected error threshold of 2, and reads were filtered with $\text{maxEE} = 2$ and truncated to 240 bp (forward) and 200 bp (reverse). Error models were then generated from the filtered reads, followed by dereplication, amplicon sequence variant (ASV) inference, paired-end read merging (minimum overlap of 12 bp), and chimera removal according to the DADA2 workflow. Representative ASV sequences were taxonomically assigned using the SILVA v138.1 reference database [16]. To minimize the contribution of plant-derived organelle sequences, ASVs annotated as Chloroplast at the order level or Mitochondria at the family level were excluded from the dataset. The same filtering procedure was applied to root endosphere (EP) samples. Because short 16S rRNA amplicons may not always fully discriminate chloroplast-derived sequences from genuine Cyanobacteria, the resulting organelle-filtered dataset was used for the primary diversity, community-composition figures.

2.6 Alpha Diversity Analysis

Alpha diversity was assessed using the Shannon diversity index, calculated with the phyloseq v1.52.0 and vegan v2.7.1 packages in R. Shannon diversity was analyzed separately for the RE soil (RE) and root endosphere (EP) using a two-way analysis of variance (ANOVA) with nitrogen treatment, maize genotype, and their interaction as fixed factors. When significant main effects or interactions were detected, Tukey's honestly significant difference (HSD) test was used for multiple comparisons. The assumptions of normality and homogeneity of variance were evaluated using the Shapiro-Wilk and Levene's tests, respectively. When these assumptions were not satisfied, a permutation-based two-way ANOVA was performed. Alpha diversity was visualized using the ggplot2 v3.5.2 package in R.

2.7 Beta Diversity Analysis

Beta diversity was evaluated using Bray-Curtis dissimilarities calculated from the ASV abundance table. Principal coordinate analysis (PCoA) was performed using the vegan package in R to visualize differences in bacterial community composition among root compartments, nitrogen treatments, and maize genotypes. The percentage of variation explained by each principal coordinate was reported for each ordination.

Differences in bacterial community composition were further evaluated using permutational multivariate analysis of variance (PERMANOVA; *adonis2* function, *vegan* package) based on Bray–Curtis dissimilarities with 999 permutations. Nitrogen treatment, maize genotype, and their interaction were included as fixed factors, and the pseudo-*F* statistic, R^2 , and *p* value were reported for each model term. Homogeneity of multivariate dispersion was assessed using the *betadis* function followed by a permutation test (PERMDISP, 999 permutations). Pairwise comparisons between NL and NZ within each genotype were performed using pairwise PERMANOVA, and *p* values were adjusted using the Benjamini–Hochberg (BH) method.

2.8 Taxonomic Composition Analysis

Bacterial community composition was summarized at the phylum and genus levels based on the taxonomic assignments of ASVs using the organelle-filtered dataset described in Section 2.5. The relative abundance of each taxon was calculated and visualized using the *ggplot2* package in R. Taxa with a mean relative abundance of <1% across all samples were grouped into the “Others” category for visualization.

2.9 Differential Abundance Analysis

Taxa that differed in relative abundance between nitrogen treatments were identified using linear discriminant analysis effect size (LEfSe v1.1.2) [17]. LEfSe analyses were performed separately for the RE soil and root endosphere (EP). Within each compartment, bacterial communities under low-nitrogen (NL) and normal-nitrogen (NZ) conditions were compared using nitrogen treatment as the sole grouping factor, pooling all four maize genotypes, in order to identify taxa associated with nitrogen treatment at the overall group level. Genotype effects were assessed separately using two-way ANOVA and two-way PERMANOVA. Statistical significance was assessed using the nonparametric Kruskal–Wallis test, followed by linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant taxon. No multiple-testing correction was applied, following the standard LEfSe workflow. Taxa with an LDA score greater than 2.0 and $p < 0.05$ were considered significantly enriched. An LDA threshold of 2.0 was selected because it is the commonly recommended cutoff in LEfSe analyses to identify biologically meaningful biomarkers.

2.10 Co-Occurrence Network Analysis

Genus-level co-occurrence networks were constructed separately for the RE soil and root endosphere under NL and NZ conditions using Spearman correlation coefficients. Each network was constructed using all biological replicates within the corresponding compartment and nitrogen treatment. Only correlations with $|r| \geq 0.6$ and $p < 0.05$ were retained. *p* values were not adjusted for multiple comparisons because the analysis was intended as an exploratory assessment of microbial co-occurrence patterns rather than a confirmatory test of individual microbial interactions. Network topological properties, including the numbers of nodes and edges and the mean degree, were calculated using the *igraph* package in R. Genera were ranked according to node degree, and highly connected genera were identified based on their observed network connectivity [18].

2.11 Functional Prediction

The functional potential of the bacterial communities was predicted from the ASV abundance table using PICRUSt2 v2.5.2 [19]. Predicted functions were annotated according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and pathway abundances were summarized at KEGG pathway level 3. Differentially enriched pathways between nitrogen treatments were identified using the Wilcoxon

rank-sum test, and p values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) method. Pathways with an adjusted p value < 0.05 were considered significantly enriched and were visualized using the ggplot2 package in R. Functional prediction was performed directly from the ASV abundance table using PICRUSt2, whereas the differential bacterial genera presented in the Results were identified independently by LEfSe analysis. The functional interpretation of predicted microbial pathways was considered in the context of previously reported links between microbial metabolic processes, mitochondrial function, and fungal pathogenicity [20].

3 Results

3.1 Sequencing Data Quality and Overview

A total of 65 maize root-associated samples were subjected to 16S rRNA gene amplicon sequencing (Table S2). After quality filtering, denoising, paired-end read merging, and chimera removal, 4,948,258 high-quality reads were retained, corresponding to an average of 76,127 reads per sample. These reads were resolved into 115,786 amplicon sequence variants (ASVs). The samples originated from three root-associated compartments: rhizosphere soil (RS), the RE soil (RE), and the root endosphere (EP). Sequencing depth ranged from 7425 to 107,360 reads per sample, and the number of detected ASVs ranged from 120 to 2864. Rhizosphere soil (RS), RE soil (RE), and root endosphere (EP) samples were initially collected during the experiment. During sample processing and quality assessment, several RS samples were identified as contaminated and were excluded from subsequent analyses. As a consequence, the remaining RS dataset was incomplete and unbalanced across nitrogen treatments and maize genotypes and could not support the same factorial comparisons performed for the RE and EP datasets. Therefore, the RS samples were excluded from downstream comparative analyses. After quality control and sample exclusion, the final dataset comprised 47 samples, including 24 RE samples and 23 EP samples (Table S2), which were used for subsequent diversity, community composition, differential abundance, functional prediction, and network analyses.

3.2 Alpha Diversity

Alpha diversity of bacterial communities was assessed using the Shannon index. Overall, bacterial communities in the RE soil exhibited greater within-sample diversity than those in the root endosphere (Fig. 1A,B). Shannon index values were predominantly distributed between 5.0 and 7.0, whereas those of EP samples ranged from 1.0 to 5.5, indicating lower within-sample diversity in the endosphere compartment. in the RE soil compartment.

Two-way ANOVA based on the organelle-filtered dataset showed that nitrogen treatment significantly affected Shannon diversity in the RE soil (RE; $F = 6.20$, $p = 0.024$), whereas its effect on root endosphere (EP) Shannon diversity was not significant ($F = 0.266$, $p = 0.613$; Table S3). These results indicate that nitrogen-associated differences in Shannon diversity were detected in RE but not in EP after exclusion of organelle-associated ASVs.

3.3 Beta Diversity and Community Structure

Principal coordinate analysis based on Bray-Curtis dissimilarities was used to visualize differences in bacterial community composition between nitrogen treatments in the RE soil and root endosphere. Samples from the two nitrogen treatments generally occupied different regions of the ordination space in both compartments (Fig. 2; Supplementary Fig. S1). These ordination patterns provide a visual representation of community-level variation, and the significance of nitrogen and genotype effects was further evaluated using PERMANOVA.

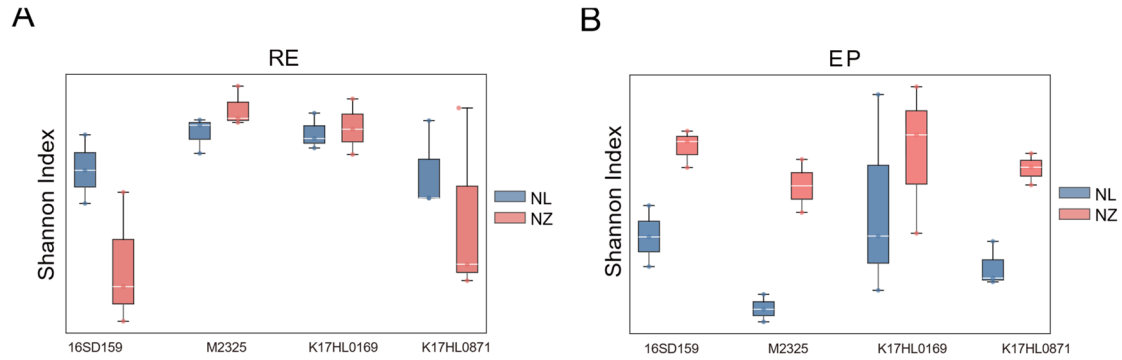


Figure 1: Nitrogen availability differentially affects bacterial alpha diversity in maize root compartments. Shannon diversity indices of bacterial communities in the RE soil (RE; **A**) and root endosphere (EP; **B**) of four maize inbred lines grown under NL and NZ conditions. The low-nitrogen-tolerant genotypes were 16SD159 and M2325, whereas K17HL0169 and K17HL0871 were classified as low-nitrogen-sensitive genotypes. Points represent individual biological replicates. Boxes indicate the interquartile range, and center lines indicate the median. And whiskers extend to 1.5× IQR. Statistical significance was evaluated using two-way ANOVA followed by Tukey-adjusted *post hoc* comparisons.

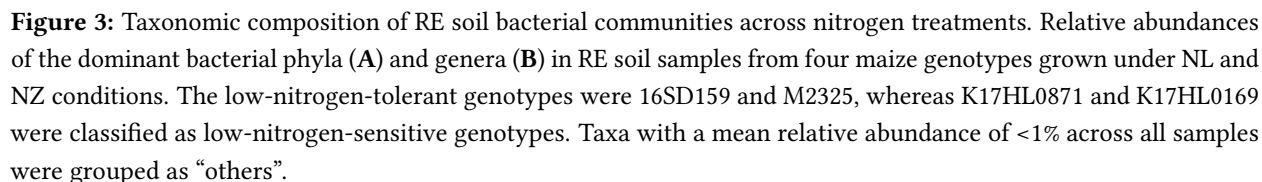
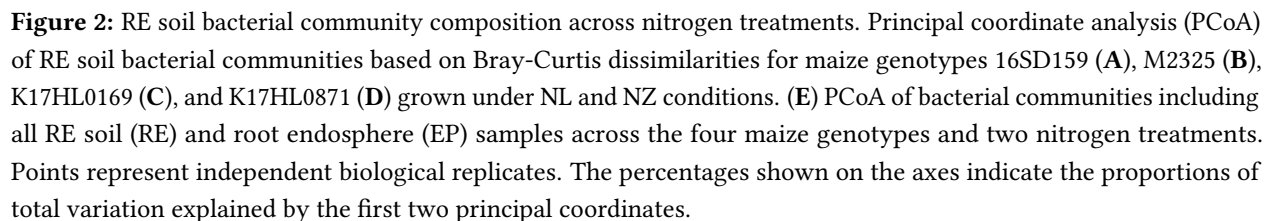
To evaluate the potential influence of organelle-associated sequences, the PERMANOVA was repeated after excluding ASVs annotated as Chloroplast or Mitochondria. The RE results were highly similar to those of the baseline analysis, with significant effects of nitrogen treatment, genotype, and their interaction. In EP, the nitrogen effect remained significant after exclusion of organelle-associated ASVs ($R^2 = 0.06$, pseudo- $F = 1.59$, $p = 0.011$), whereas the genotype effect became significant ($R^2 = 0.18$, pseudo- $F = 1.50$, $p = 0.002$), and the nitrogen × genotype interaction remained non-significant ($R^2 = 0.14$, pseudo- $F = 1.14$, $p = 0.127$; Table S3). Thus, the nitrogen-associated difference in EP community composition was retained after organelle-associated ASVs were excluded, whereas the significance of the genotype effect was sensitive to this filtering step.

3.4 Taxonomic Composition of Bacterial Communities

Bacterial community composition differed markedly between the RE soil and root endosphere. At the phylum level, Proteobacteria dominated the RE community, accounting for approximately 60.2% of the total relative abundance, followed by Firmicutes, Bacteroidota, Acidobacteriota, and Actinobacteriota (Fig. 3A). In the EP compartment, Proteobacteria were also dominant, accounting for 57.5% of the mean relative abundance, followed by Actinobacteriota (17.4%) and Bacteroidota (6.8%) (Supplementary Fig. S2A). These results indicate distinct taxonomic profiles between the RE soil and root endosphere communities.

At the genus level, the RE community was characterized primarily by *Pseudomonas*, *Bacillus*, the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* complex, *Sphingobium*, *Pantoea*, and *Sphingomonas* (Fig. 3B). By contrast, the EP community was dominated by *Streptomyces*, *Sphingobium*, the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* complex, and *Lechevalieria* (Supplementary Fig. S2B).

Relative abundance patterns also differed between nitrogen treatments. In the RE compartment, *Pseudomonas* was relatively more abundant under NZ conditions, whereas *Bacillus* was more abundant under NL conditions. In the EP compartment, *Lechevalieria* and *Sphingobium* showed higher relative abundances under NZ conditions. Overall, these patterns indicate that root compartment and nitrogen treatment were associated with differences in the taxonomic composition of maize root-associated bacterial communities.



3.5 Differentially Abundant Bacterial Genera

LEfSe analysis identified bacterial genera that differed in relative abundance between the NL and NZ treatments, using an LDA score threshold of 2.0 and $p < 0.05$ (Fig. 4A). Because genotypes were pooled within each nitrogen treatment, these taxa represent exploratory associations with nitrogen treatment at the overall group level rather than evidence of consistent nitrogen responses across genotypes. Genera enriched under NZ conditions included *Actinoplanes*, *Lacunisphaera*, *Sphaerisporangium*, *Opitutus*, *Lacibacter*, *Promicromonospora*, *Actinocorallia*, *Pseudoxanthomonas*, and *Hydrogenophaga*. By contrast, genera enriched under NL conditions included *Bacillus*, *Acinetobacter*, *Exiguobacterium*, *Kosakonia*, *Steroidobacter*, *Brevibacillus*, *Terribacillus*, and the *Burkholderia-Caballeronia-Paraburkholderia* complex. Many of the taxa enriched under NZ conditions belonged to actinobacterial lineages, whereas the NL-enriched group contained several genera commonly detected in the RE soil.

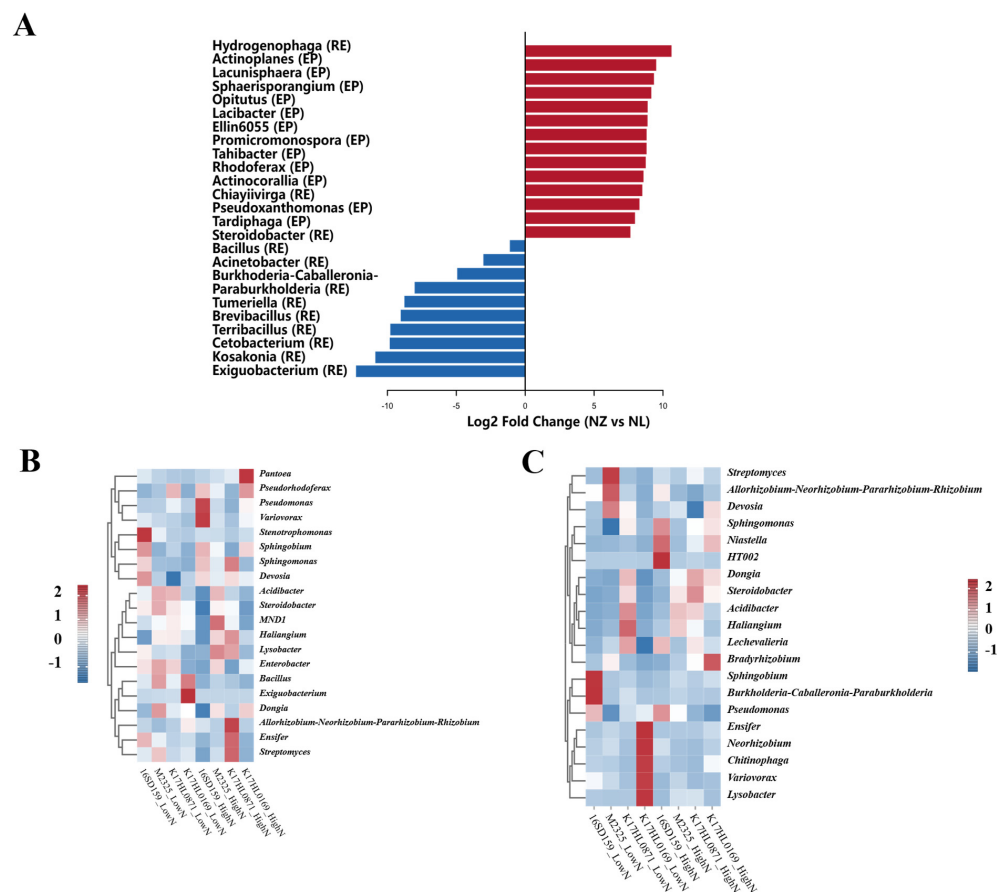


Figure 4: Nitrogen-responsive bacterial genera and their abundance patterns across maize genotypes. (A) Taxa associated with nitrogen treatment bacterial genera identified by LEfSe across all four maize genotypes. Differentially abundant bacterial genera identified by LEfSe (LDA > 2.0, $p < 0.05$). Bars show the log2 fold change [$\log_2(\text{NZ/NL})$] calculated from the mean relative abundance of each selected genus. Positive values indicate enrichment under NZ conditions, whereas negative values indicate enrichment under NL conditions. The root compartment in which each genus was identified is indicated in parentheses. (B,C) Heatmaps showing the normalized relative abundances of dominant bacterial genera across maize genotypes and nitrogen treatments in the RE soil (B) and root endosphere (C). Red indicates higher relative abundance, whereas blue indicates lower relative abundance. Dendrograms indicate hierarchical clustering of bacterial genera.

Heatmap analysis further revealed genotype- and treatment-associated abundance patterns within the two compartments (Fig. 4B,C). In the EP compartment, *Streptomyces* was relatively abundant in 16SD159 under NL conditions, whereas *Bradyrhizobium* was enriched in K17HL0169 under NZ conditions. *Variovorax* and *Lysobacter* were primarily associated with K17HL0169 under NL conditions, while the abundance of *Sphingomonas* varied among genotypes.

In the RE compartment, *Exiguobacterium* and *Bacillus* were particularly abundant in K17HL0169 under NL conditions. By contrast, *Pantoea* and the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* complex generally showed higher relative abundances under NZ conditions. Together, the LEfSe and heatmap analyses highlighted bacterial genera showing exploratory associations with nitrogen treatment and patterns of variation across root compartments and maize genotypes.

3.6 Predicted Functional Potential

PICRUSt2-based functional prediction followed by KEGG pathway analysis identified nine pathways associated with nitrogen-responsive differences in bacterial community composition (Fig. 5A). Seven pathways remained significant after false discovery rate correction, whereas methane metabolism and xenobiotic degradation were significant only before multiple-testing correction.

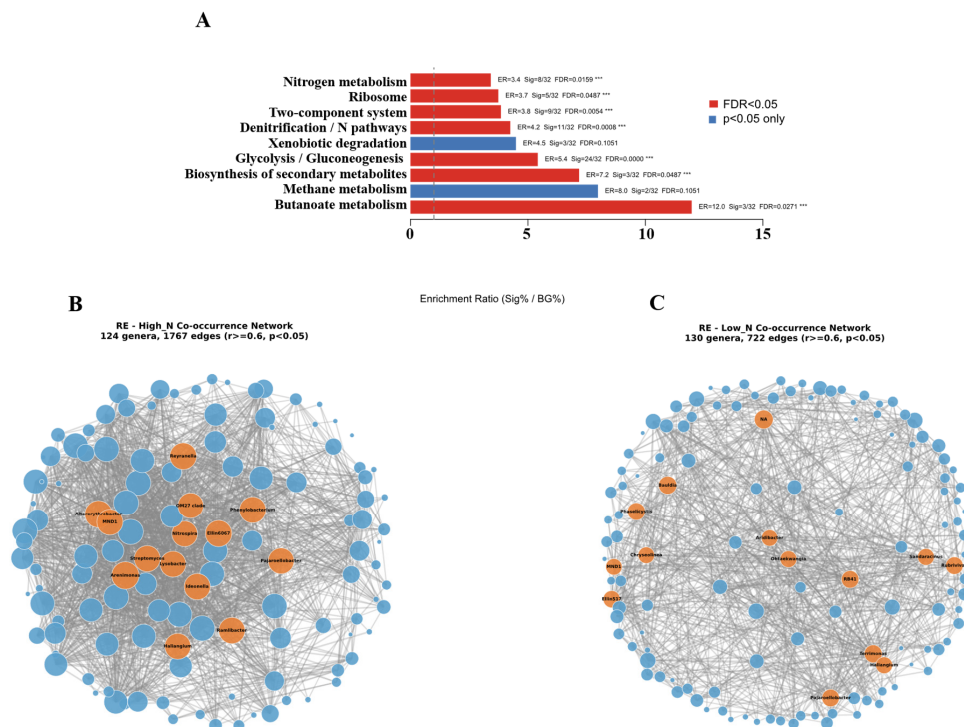


Figure 5: Predicted functional pathways and RE soil bacterial co-occurrence networks across nitrogen treatments. (A) KEGG pathways associated with nitrogen-responsive differences in bacterial community composition, as predicted using PICRUSt2. Red bars indicate pathways that remained significant after false discovery rate correction, whereas blue bars indicate pathways that were significant before, but not after, multiple-testing correction. Three asterisks (***) indicate highly significant differences after false discovery rate (FDR) correction (adjusted $p < 0.001$). (B,C) Genus-level co-occurrence networks of RE soil bacterial communities under NZ (B) and NL (C) conditions. Nodes represent bacterial genera, and edges represent significant Spearman correlations with $|r| \geq 0.6$ and $p < 0.05$. Node size represents degree centrality. Orange nodes indicate highly connected genera, whereas blue nodes represent all remaining genera.

The pathways retained after correction included nitrogen metabolism, denitrification-related functions, glycolysis/gluconeogenesis, ribosome-related functions, two-component systems, butanoate metabolism, and the biosynthesis of secondary metabolites. Butanoate metabolism showed the highest enrichment ratio. These results indicate differences in predicted functional potential between nitrogen treatments, rather than directly measured microbial metabolic activities.

3.7 Co-Occurrence Network Analysis

Genus-level co-occurrence networks were constructed using Spearman correlations to compare observed bacterial co-occurrence patterns between nitrogen treatments and root compartments (Fig. 5B,C, Supplementary Fig. S3 and Tables S4–S7). In the EP compartment, the NL network contained 37 nodes and 144 significant correlations, with a mean degree of 7.78. Under NZ conditions, the network expanded to 79 nodes and 480 correlations, and the mean degree increased to 12.15. The RE networks contained more nodes and edges and showed higher mean degree than the corresponding EP networks. Under NL conditions, the RE network comprised 130 nodes and 722 correlations, with a mean degree of 11.11. Under NZ conditions, the number of nodes was similar at 124, whereas the number of correlations increased to 1767 and the mean degree increased to 28.50. Thus, the NZ networks contained more observed correlation-based associations and exhibited higher mean connectivity than the corresponding NL networks in both compartments. The genera identified as highly connected genera also differed among compartments and treatments. In the EP networks, highly connected genera included *Bradyrhizobium*, *Sphingobium*, *Pseudomonas*, and *Altererythrobacter*. In the RE networks, potential hub genera included *Nitrospira*, *Lysobacter*, *Streptomyces*, *Phenylobacterium*, and *Altererythrobacter*. These results show that nitrogen availability was associated with differences in the observed topology and taxonomic composition of bacterial co-occurrence networks, particularly in the RE soil.

4 Discussion

This study shows that root compartment, nitrogen availability, and maize genotype jointly shape root-associated bacterial communities. The RE soil contained greater bacterial diversity and more extensive patterns of observed co-occurrence associations than the root endosphere. This difference is consistent with the contrasting ecological conditions of the two compartments. The RE soil is continuously influenced by soil microorganisms, root exudates, and nutrient gradients, whereas bacteria entering the endosphere must pass through physical, chemical, and immune barriers imposed by the host [21,22]. Nitrogen treatment caused relatively modest changes in Shannon diversity but was associated with clearer changes in community composition. Similar differences between alpha- and beta-diversity responses to nitrogen supply have been reported in maize and barley [23].

The strength and structure of the nitrogen and genotype effects differed between the two root compartments. PCoA suggested separation among samples according to nitrogen treatment and genotype. Consistent with these patterns, PERMANOVA based on the organelle-filtered dataset detected significant effects of nitrogen availability on bacterial community composition in both RE and EP ($p = 0.001$ and 0.011 , respectively). Genotype was also significantly associated with community composition in both RE and EP ($p = 0.011$ and 0.002 , respectively), while the nitrogen $\times$ genotype interaction was significant in RE ($p = 0.003$) but not in EP ($p = 0.127$). These results indicate that nitrogen availability and genotype were associated with bacterial community composition in both compartments, whereas the nitrogen $\times$ genotype interaction was detected only in the RE community. Although the four genotypes showed different bacterial profiles, the two low-nitrogen-tolerant genotypes did not consistently form a group distinct from the two sensitive

genotypes. The observed differences may therefore represent general genotype effects rather than microbial features specifically associated with NL tolerance. In addition, the present experiment did not include direct measurements of plant physiological or agronomic traits related to NL tolerance, such as biomass, nitrogen accumulation, or nitrogen-use efficiency. Therefore, the present data cannot establish a direct relationship between the observed bacterial community patterns and NL tolerance. NL conditions enriched several bacterial genera that include plant growth-promoting strains, including *Bacillus*, *Exiguobacterium*, *Acinetobacter*, *Kosakonia*, and members of the *Burkholderia*–*Caballeronia*–*Paraburkholderia* complex. *Bacillus* strains can improve maize growth and nutrient-use efficiency under nutrient limitation [24], while *Burkholderia* and *Paraburkholderia* isolates can promote maize biomass production and nitrogen and phosphorus use efficiency [25]. Some root-associated *Kosakonia* strains can also fix nitrogen and may contribute to biological nitrogen inputs in maize [26]. These genera may represent candidates for further investigation. Nevertheless, genus-level enrichment does not demonstrate that the detected bacteria benefited the plants. Functional traits can vary substantially among species and strains within the same genus. The candidate bacteria should be isolated and tested individually or in defined microbial communities to determine their effects on maize growth, nitrogen uptake, and nitrogen-use efficiency. In contrast, several actinobacterial genera, including *Actinoplanes*, *Promicromonospora*, and *Actinocorallia*, were enriched under NZ conditions, suggesting that different nitrogen levels favor bacterial groups with distinct ecological strategies.

The high abundance of Cyanobacteria-associated sequences in the root endosphere requires cautious interpretation. Bacterial 16S rRNA primers can amplify plant chloroplast-derived sequences because chloroplasts are evolutionarily related to Cyanobacteria, and plant-derived sequence contamination is a recognized issue in maize root microbiome studies [27,28]. In the present study, RE and EP samples were processed using the same sequence-quality filtering, denoising, taxonomic assignment, and organelle-associated sequence filtering procedures. Re-examination of the EP ASV annotations showed that 583,411 of 584,947 Cyanobacteria-associated reads (99.74%) were annotated as Chloroplast. This finding indicates that the apparent Cyanobacteria-associated signal in EP should not be interpreted directly as evidence of a high abundance of endophytic Cyanobacteria. Accordingly, ASVs annotated as Chloroplast or Mitochondria were excluded before the primary diversity and community-composition analyses. Based on the organelle-filtered dataset, nitrogen significantly affected Shannon diversity in RE ($p = 0.024$) but not in EP ($p = 0.613$). Nitrogen also significantly affected community composition in both RE and EP ($p = 0.001$ and 0.011 , respectively), while genotype was significant in both compartments ($p = 0.011$ and 0.002 , respectively). The nitrogen $\times$ genotype interaction was significant in RE ($p = 0.003$) but not in EP ($p = 0.127$). Thus, the filtered results indicate that nitrogen-associated changes in community composition were retained in both compartments, whereas the nitrogen-associated difference in EP Shannon diversity was not significant after organelle-associated ASVs were excluded. A methodological limitation of the present study is that the effectiveness of root surface sterilization was not independently validated by culturing the final rinse water or performing tissue-imprint assays. Although roots were subjected to a standardized surface-sterilization procedure before EP DNA extraction, residual rhizoplane microorganisms cannot be completely excluded and may have contributed to the observed EP community profiles. This limitation is particularly relevant when interpreting differences between the RE soil and endosphere communities. Therefore, the EP results should be interpreted with this potential source of technical confounding in mind. Future studies should incorporate final-rinse plating and/or tissue-imprint assays to directly verify sterilization efficiency.

PICRUSt2-based functional prediction suggested differences in the predicted functional potential of bacterial communities between nitrogen treatments, including pathways related to nitrogen metabolism,

carbon metabolism, environmental sensing, and secondary-metabolite biosynthesis. These results should be interpreted strictly as hypothesis-generating observations rather than evidence of active microbial processes. PICRUSt2 infers gene content from 16S rRNA gene profiles based on reference genomes rather than directly measuring microbial genes, gene expression, enzyme activity, or metabolic flux [29]. Its accuracy depends on the availability of closely related reference genomes and may be limited by strain-level variation, and comparisons with metagenomic data have shown that 16S-based functional inference may not reliably identify all differentially represented functions [30]. Therefore, the predicted pathway differences represent inferred functional potential rather than evidence that the corresponding microbial processes were present or active in the samples. Metagenomic, metatranscriptomic, enzymatic, or metabolomic analyses will be required to validate these predictions.

The co-occurrence networks contained more nodes and edges and showed higher mean degree in the RE soil than in the endosphere, consistent with the greater diversity and ecological openness of the RE soil. Observed network connectivity was also higher under NZ than under NL conditions in both compartments. These patterns indicate that nitrogen availability was associated with differences in the observed organization of bacterial co-occurrence networks. However, correlations do not necessarily represent direct microbial interactions. They may result from shared environmental preferences, indirect relationships, differences in taxon abundance, or the compositional nature of sequencing data [18,31]. Network connectivity can also be affected by sample size and the number of taxa included. Thus, highly connected taxa should be regarded as candidates for further study rather than confirmed ecological drivers.

5 Conclusions

This study demonstrates that the RE soil (RE) and root endosphere (EP) harbor distinct bacterial communities, with greater bacterial diversity in RE. After excluding organelle-associated ASVs, nitrogen supply significantly affected bacterial community composition in both RE and EP, while maize genotype was also significantly associated with community composition in both compartments. In contrast, nitrogen supply significantly affected Shannon diversity in RE but not in EP. These findings indicate that nitrogen supply and host genotype are associated with variation in maize root-associated bacterial communities in a compartment-dependent manner. The low-nitrogen-associated taxa identified here provide candidates for future functional evaluation.

Acknowledgement: Not applicable.

Funding Statement: This study was supported by the Sub-project of the National Key Research and Development Program of China (Grant No. 2016YDF0100103), the National Government Procurement Service (Project Nos. 19211100, 19221930, 19230621, and 22260315), and the biological breeding project of Shanxi Agricultural University (Project No YZGC2026128).

Author Contributions: Xin Liu and Yuan Ren conceived and supervised the work; Yuan Ren, Qijian Tian and Jin Zhang conducted the experiments, analyzed the data and prepared the figures; Yuan Ren drafted the manuscript, together with Qijian Tian and Jin Zhang. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The sequencing data is available in the National Center for Biotechnology Information: PRJNA1540471.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary Materials: The supplementary material is available online at <https://www.techscience.com/doi/10.32604/phyton.2026.090808/s1>.

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