Supporting Information

CHANGES IN THE MICROBIAL COMMUNITY IN MAIZE (*ZEA MAYS* L.) ROOT SPATIAL STRUCTURE FOLLOWING SHORT-TERM NITROGEN APPLICATION

Lanfang Bai[a], Yufen Wang[b], Yahua Li[b], Xiangqian Zhang[c*], Zhanyuan Lu[b,c*], Dejian Zhang[b], Fengcheng Sun[c], Xiaoqing Zhao[c]

a. Faculty of Agronomy College, Inner Mongolia Agricultural University, Hohhot, 010070, P.R. China.

b. Key Laboratory of Herbage & Endemic Crop Biotechnology, Ministry of Education, School of Life Science, Inner Mongolia University, Hohhot 010070, P.R. China

c. Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, Inner Mongolia Hohhot 010031, China.

* Corresponding Author:

X.Q., Zhang Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, No. 22 Zhaojun Road, Hohhot, Inner Mongolia Autonomous Region, 010031, P.R.China. zhangxiangqian_2008@126.com.

Z.Y., Lu, Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, No. 22 Zhaojun Road, Hohhot, Inner Mongolia Autonomous Region, 010031, P.R.China.

lzhy281@163.com

Contents included

(1) Materials and methods

1.Field trial and treatments

2. Sample collection

3. Testing method

4. Statistical analysis

(2) Table & Figures

(1) Materials and methods

1.Field trial and treatments

The field trials were established in Inner Mongolia Agriculture Academy of Sciences, Hohhot City, Inner Mongolia Province, China (40°45′N, 111°40′E, 1040 m above sea level). The land is brown loamy soil. From 1998 to 2017, the mean annual air temperature was 6.7°C and mean annual precipitation was 399.3 mm. The experimental area precipitation was 522.4 mm in 2018 maize growing season. Before seedings planted, the soil texture in the top 20 cm were measured right as shown in Table S1.

The experiment was a one-year short-term N application experiment which adopted a one-factor experimental design, with three treatments and three replicates. Three treatments were N application at rates of 0 (N0), 180 kg N ha^{-1} (N180), and 360 kg N ha^{-1} (N360), with N0 serving as the control. Resin-coated urea with N content of 45 % was selected as N fertilizer what commonly used by local farmers. The experimental variety was maize Xianyu 335, and was seeded manually on May 4th, 2018 at a target population of 75,000 plants ha^{-1}. Each treatment was composed of three replicate plots of 28 m^2 (8.0 m × 3.5 m). There is a buffer strip of 1 m between two adjacent plots. All fertilizers were applied as crop based fertilizer. Calcium super phosphate was used as phosphorus fertilizer at rates of 138 kg P ha^{-1}, potassium sulfate was used as potassium fertilizer at rates of 38.25 kg K ha^{-1}.

2. Sample collection

Samples from three different root spatial structures (bulk soil, rhizosphere soil, and endosphere) were collected on July 29, 2018 (filling period). For every plot, eight randomized soil cores were sampled and mixed to represent one field replicate. Four subsamples were collected fromevery treatment with five-point sampling method, which from mixing fifteen subsamples (five-point sampling×three replicates plot =fifteen) of three plots. The sampling tools were sterilized before sampling.

The depth of 5–20 cm was collected as bulk soil. The remaining bulk soil was sieved through a 1-mm sieve, air-dried and stored for soil chemical property and soil enzyme activities measurement. Using a sterile brush were sieved and taken, the tightly attached to the roots soil was brushed off as rhizosphere soil. Roots ultrasonically shaken with a PBS phosphate buffer for 3 min which as root sample.[1] All samples were collected in aseptic centrifuge tubes, flash freeze with dry ice and stored at −80°C before 16S rRNA and ITS sequencing. A total of seventy-two subsamples were collected (three treatments×three sample types (bulk soil, rhizosphere soil, and endophytic environment) × four replicates× two (bacteria and fungi) = seventy-two).

3. Testing method

3.1 Yield determination

Fresh grass yield of maize were determined by manually harvesting from 4 rows in the center of each plot at a stubble height (20 cm) at September 15, 2018. Randomly took 10 corn plants and put them in an oven at 105 ℃ for 30 minutes, then dried them at 60 ℃ to a constant mass, weighed and calculated as biomass.

3.2 Soil properties and enzymatic activities

Because of the little amount of rhizosphere soil collected, soil texture of bulk soil were only

measured. According to Bao[2], we determined soil chemical properties. Soil pH was measured using potentiometric method. The total phosphorus (TP) content was determined using Sulfuric acid - perchloric acid digestion method. The available phosphorus (AP) content was determined based on soil extraction with an $NaHCO_3$ solution, followed by detection with an ultraviolet-visible spectrophotometer (Shun yu hen ping UV2800S, Shanghai, China). The total nitrogen (TN) content measured using Kjeldahl digestion. The alkali nitrogen (AN) content measured using the alkali solution diffusion method. The total carbon (TC) content measured using carbon and nitrogen analyzer (Analytik Jena AG multi N/C 2100, Thuringia, Germany). The total potassium (TK) and available potassium(AK) were determined by flame photometer (Jing ke fp6410, Shanghai, China).[3,4] Organic matter (OM) content measured using the potassium dichromate oxidation capacity method. The urease activity (UA) measured using phenol-sodium hypochlorite colorimetry. The invertase activity (IA) measured using colorimetry. The hydrogen peroxidase activity (HPA) was determined by potassium permanganate titration. The alkaline phosphatase activity (APA) measured using colorimetry of disodium phenyl phosphate.

3.3 High-throughput sequencing

We have subjected to DNA extraction using the soil kit (Omega bio-tek, Norcross, GA, USA). The quality of the extracted DNA was determined by electrophoresis on 1% agarose gel. Chloroplast excluding primers targeting the V5–V7 region of the bacterial 16S rRNA gene and ITS1 region of the fungal ITS gene was used for the bacterial 16S rRNA gene and fungal ITS gene amplification respectively. The PCR amplifications were implemented in 20 μL system containing 4 μL of 5×FastPfu buffe, 2 μL of 2.5 mm dNTPs, 0.8 μL of 5 μM each primer, 0.4 μL of FastPfu polymerase, and 10 ng of template DNA.[5] More information of primers used and PCR reaction condition are available in Table S2. PCR products were purified and recovered using agarose gels. All samples quantification, and then sequencing on the Illumina MiSeq PE300 platform (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China). The 16S rRNA and ITS gene raw sequencing data have been submitted to the NCBI's Sequence Read Archive (SRA) under accession number PRJNA833857.

3.4 Bioinformatics analysis

The raw fastq files were sequenced and quality-filtered with Trimmomatic-0.38 and Mothur 1.32.2, respectively. Then splicing by FLASH software. UPARSE software was used to perform Operational taxonomic units (OTUs) clustering according to 97% similarity (equivalent to 0.03 distance limit), and used UCHIME software to identified and remove chimeras.[6] RDP classifier algorithm was used analyzed the taxonomy of each 16S rRNA and ITS gene sequence. The Silva database (SSU123) was used to matched the 16S rRNA and ITS gene sequence based on OTU level, and the confidence threshold is set to 70%.[7]

4. Statistical analysis

Use linear mixed model (LMM) was used to analyze the effect of N fertilization and spatial structures on soil properties, enzymatic activities, maize yields and Alpha diversity of bacterial and fungal communities. Statistical analysis was carried out by SPSS Statistics 19.0 software (SPSS Inc., Armonk, NY, USA). The Majorbio i-sanger cloud platform (www.i-sanger.com) online analyzed the sequencing data. Principal co-ordinates analysis (PCoA) and Permutation multivariate

analysis of variance (PERMANOVA) were used to determine and assess the beta-diversity and significance of microbial beta diversity in different spatial structures under different N application rates which was performed on the basis of the calculated Bray-Curtis distance. Comparisons among treatments were multiple with LSD (Least—SignificantDifference) method. Linear discriminant analysis (LDA) effect size (LEfSe) was used to detect potential biomarkers, The alpha value of factorial Kruskal-Wallis test was set to 0.05, LDA score threshold was set to 2.0.[4] Heatmaps was carried out with the microbial community abundance, soil properties, and enzymatic activities to examine correlation between microbial and environmental variables. The variance inflation factor was used to screen for environmental factors before analysis, the threshold was set to 10.0.

Use Cytoscape (version 3.5.1) for non-random collinearity analysis.[3] The CoNet plug-in in Cytoscape was used to perform a network analysis of potential associations between microbial communities at the genus level. We used only top 30 most abundant microorganisms as network analysis of association between species revealed, and then perform zero-value data filtering.[8] Correlations among the microbial by the spearman scores with relative absolute value of 0.6, and $P < 0.01$. The correlation data obtained is marked with positive or negative correlation according to the values of the data. At the same time, the species at the genus level are classified to the phylum level, and then imported into the Cytoscape software. Use Cytoscape plug-in network analyzer to calculate the topological properties, the degree of each node in the network, transitivity and others.

A structural equation model (SEM) construction and analysis were carried out using AMOS 17.0 (SPSS, Chicago, IL, USA). We inserted these variables such as spatial structure, microbial alpha diversity (Shannon index), soil enzyme activities, and nitrogen application rate into the software. The hypothetical models were valid represented by Chi-square ($P > 0.05$), Akaike information criteria (AIC), $X^{2/}$DF (chi-square/DF < 3) and the approximation root mean square error (RMSEA < 0.05) (Bai et al., 2021).

(2) **Table & Figures**

Table S1. Soil physicochemical properties in the top 20 cm before seedings planted

Type	pH	TP (g kg^{-1})	AP (mg kg^{-1})	TC (g kg^{-1})	OM (g kg^{-1})	TK (g kg^{-1})	AK (mg kg^{-1})	TN (g kg^{-1})	AN (mg kg^{-1})
Soil property	7.62	0.81	16.11	0.02	21.93	0.34	116.99	1.07	58.8

Table S2 Information of primers used in this study

		Primers	Sequence (5'- 3')	Target subfragment	subfragment length (bp)	PCR condition
Bacteria[9]	First round	799F	5'-AACMGGATTAGATACCCKG-3'	V5-V7	593	95 °C 3 mins, 27 cycles of 95 °C 30 s, 55 °C 30 s, and 72 °C 45 s, and finally 72 °C 10mins.
		1392R	5'-ACGGGCGGTGTGTRC-3'			
	Second round	799F	5'-AACMGGATTAGATACCCKG-3'	V5-V7	394	
		1392R	5'-ACGGGCGGTGTGTRC-3'			
Fungi[10]		ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	ITS1	300	95 °C 3 mins, 27 cycles of 95 °C 30 s, 55 °C 30 s, and 72 °C 45 s, and finally 72 °C 10mins.
		ITS2R	5'-GCTGCGTTCTTCATCGATGC-3'			

Table S3. Effects of N application rate on the changes of alpha diversity (Shannon index) of bacterial and fungal communities

Type		N0	N180	N360	P	F
Bulk Soil	Bacterial community	5.85A	5.75B	5.89A	0.02	6.53
	Fungal community	4.28A	4.26A	4.18A	0.97	0.03
Rhizosphere	Bacterial community	5.60C	5.65B	5.75A	<0.01	34.05
	Fungal community	4.46A	4.25AB	4.11B	0.04	5.52
endosphere	Bacterial community	4.60AB	4.83A	4.17B	0.08	3.38
	Fungal community	3.42A	3.76A	2.48A	0.15	2.34

Abbreviation: Data are presented as means±SD. Different letters in row indicate significant differences based on One-way ANOVA ($P < 0.05$).

Table S4. Highest degree microbial sensitively responding to nitrogen level

Typle	Nitrogen level	Highest degree microbial	degree	Clustering
Bacterial	N0	g__Ralstonia	24	0.87
	N180	g__Rubrobacter	23	0.76
		g__Streptomyces	23	0.87
	N360	g__norank_o__Gaiellales	20	0.79
		g__norank_o__Gemmadaceae	20	0.79
Fungal	N0	g__Humicola	18	0.71
	N180	g__Gibberella	16	0.61
	N360	g__Humicola	17	0.69

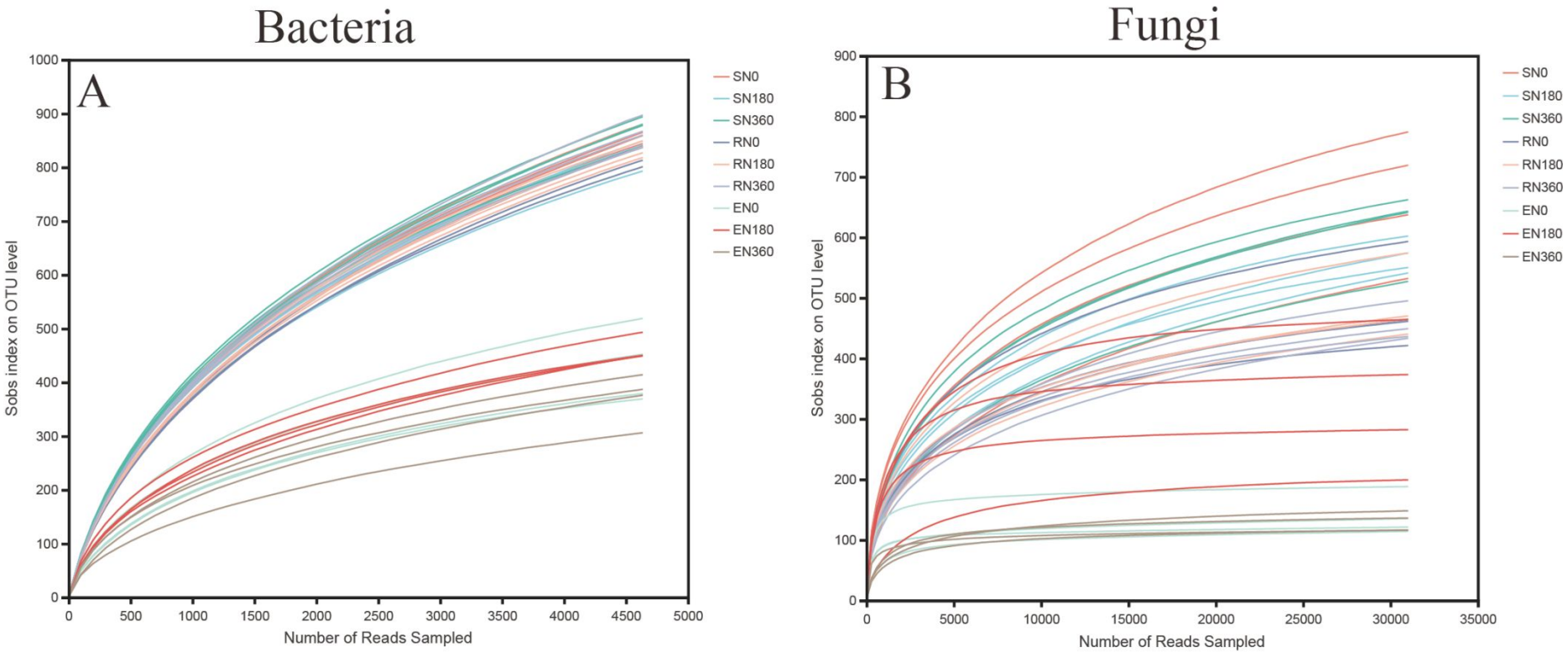


Figure.S1 Rarefaction curves of the bacterial (A) and fungal (B) communities in different fertilization treatments.

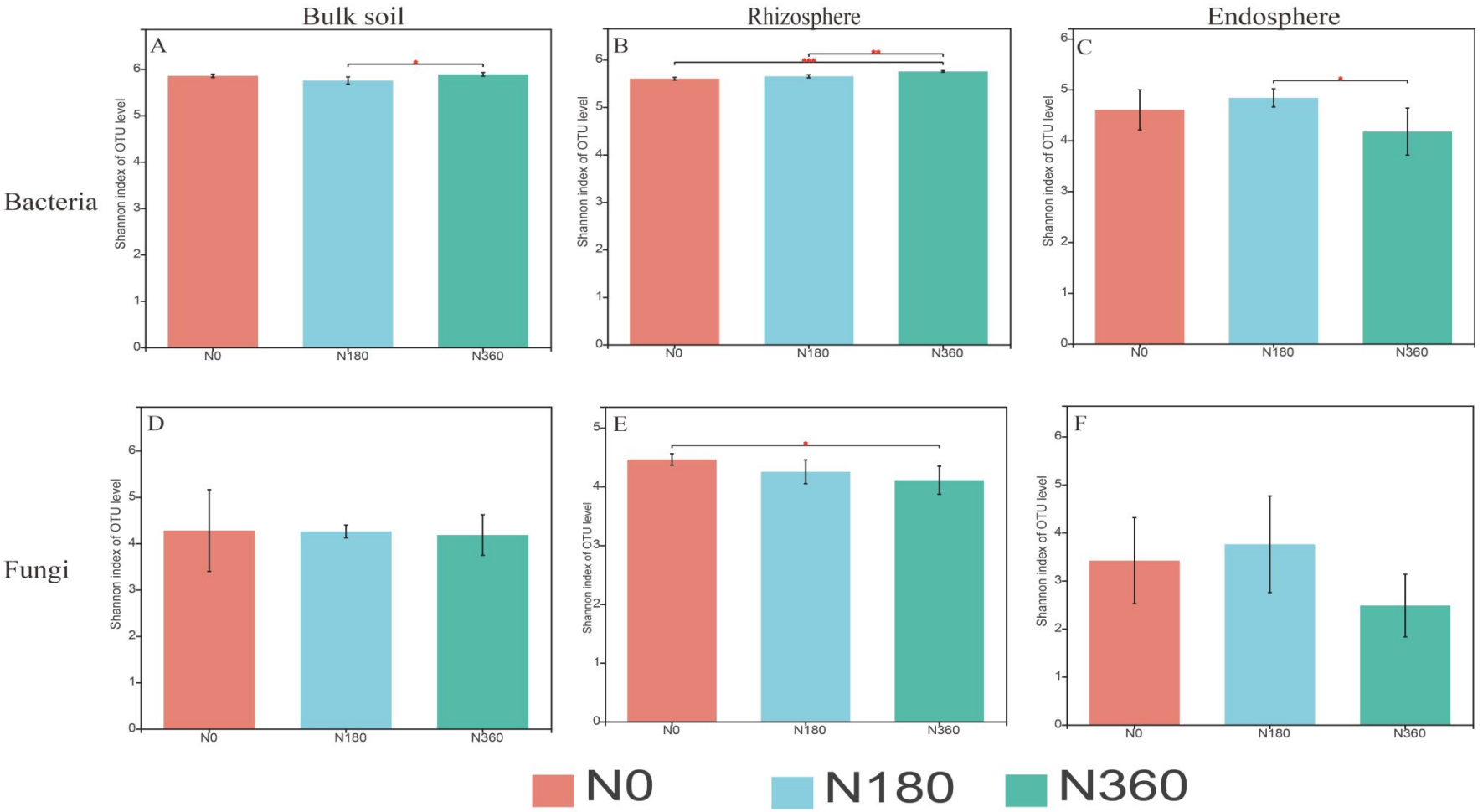


Figure.S2 Bacterial (A-C) and fungal (D-F) Shannon diversity in bulk soil, rhizosphere, and endosphere of maize in different nitrogen treatment. There are significant differences of the two set of tags. * $P< 0.05$, ** $P < 0.01$, and *** $P \leq 0.001$.

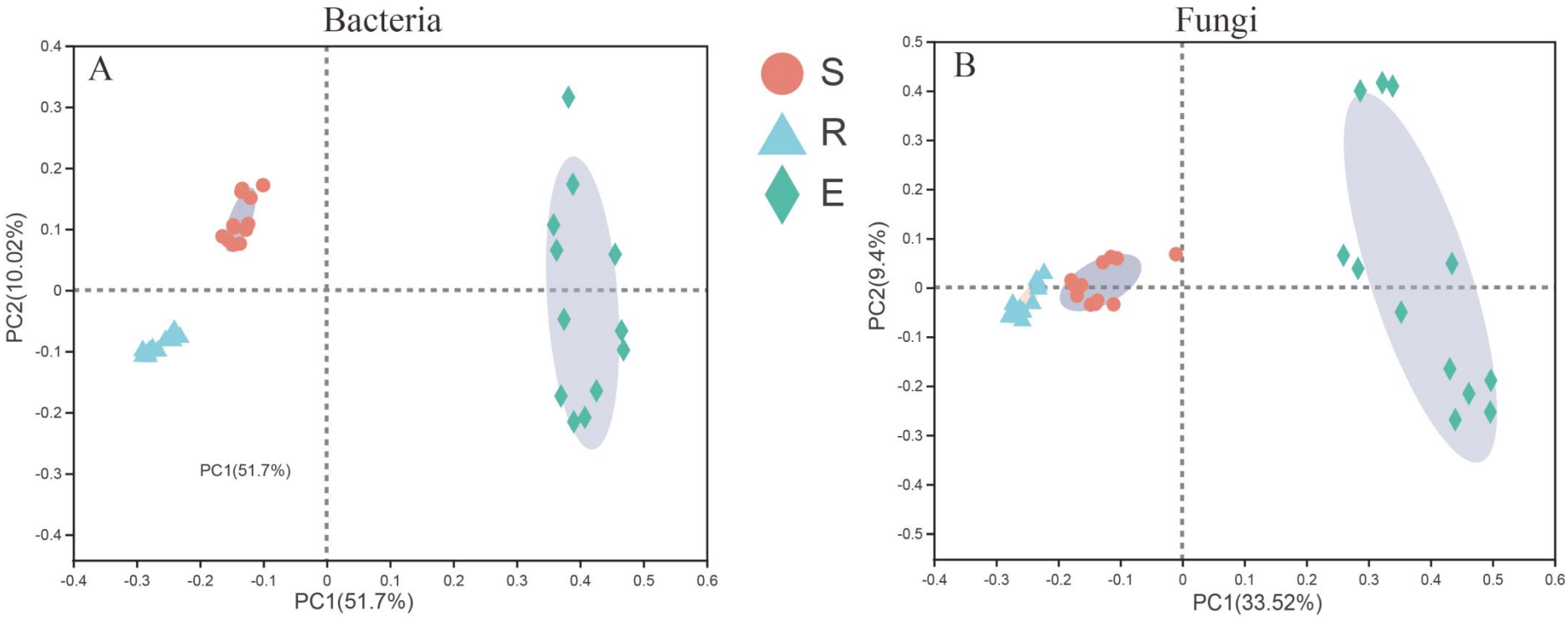


Figure.S3 Beta-diversity of the bacterial (A) and fungal (B) communities in bulk soil (S), rhizosphere (R), and endosphere (E) of maize visualized by principal co-ordinates analysis (PCoA) based on bray-Curtis distance metrics at the OTU level.

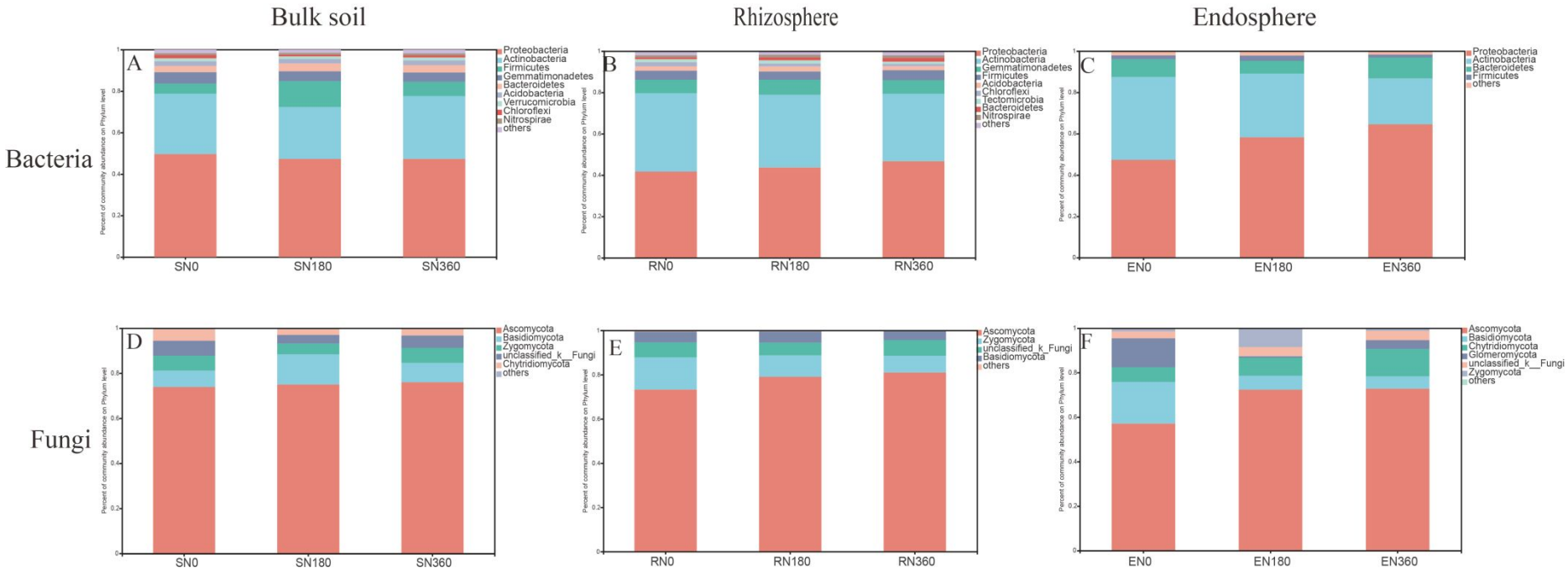
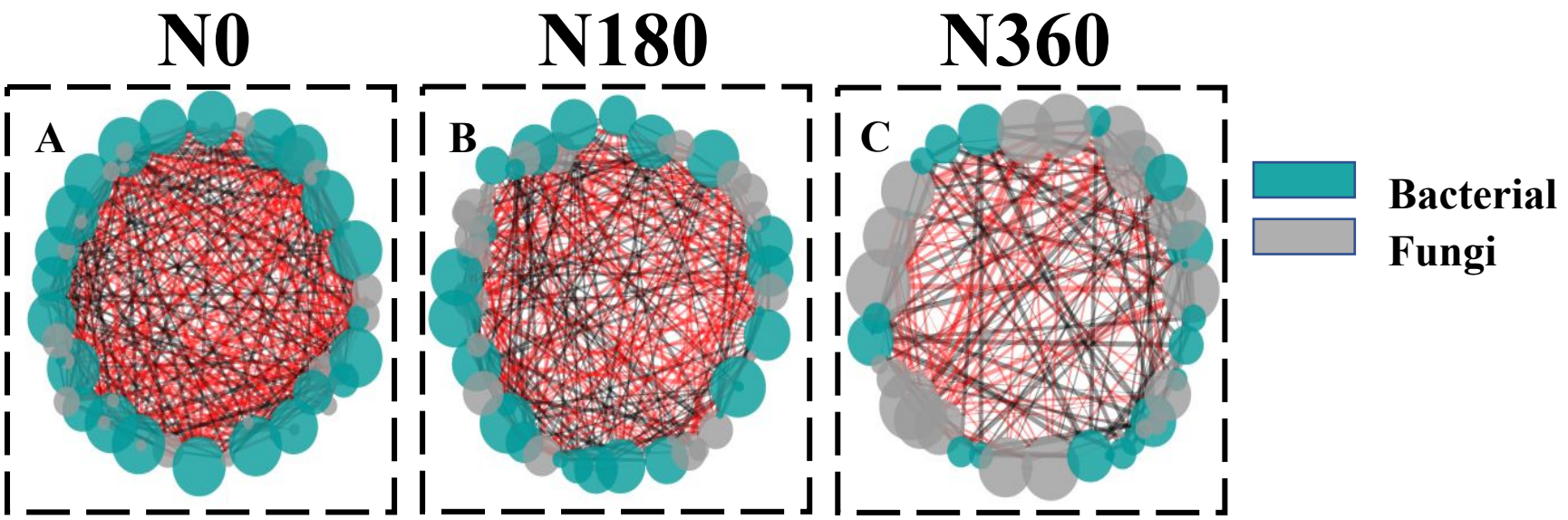


Figure.S4 Relative abundance of the taxonomic composition of bulk soil, rhizosphere, and endosphere bacterial (A-C) and fungal (D-F) community at phylum level, respectively. Nitrogen fertilization treatments were: 0 (N0), 180 (N180), and 360 (N360) kg N ha $^{-1}$.



Topological indices of each network in Figure

Item	N0	N180	N360
Clustering coefficient	0.70	0.73	0.36
Network density	0.24	0.21	0.17
Number of nodes	58	57	51
Netework heterogeneity	0.58	0.53	0.62
Netework centralization	0.15	0.17	0.18

Figure.S5 The networks visualize fertilization treatment (including 0 (N0), 180 (N160), and 360 (N360) kg N ha^{-1}) effects on the co-occurrence pattern between bacterial, and fungal taxa at genus level. The node size corresponded to the degree of connection, and the nodes filled in blue are bacterial taxa, in gray are fungal taxa. The red and black lines represent the positive correlation and the negative correlation, respectively, and the thickness of edges to the size of the spearman correlation.

REFERENCES

(1) Edwards, J.; Johnson, C.; Santos-Medellın, C.; Lurie, E.; Podishetty, N. K.; Bhatnagar, S.; Eisen, J. A.; Sundaresan, V. Structure, variation, and assembly of the root-associated microbiomes of rice. *Proceedings of the National Academy of Sciences, USA*. **2015**, *112*, 911-920.

(2) Bao, S. D. Soil agricultural chemistry analysis. 3rd ed. -China Agriculture Press.; Beijig, 2005; pp 5-200.

(3) Zhou, Y. J.; Li, J. H.; Friedman, C. R.; Wang, H. F. Variation of soil bacterial communities in a chronosequence of rubber tree (Hevea brasiliensis) plantations. *Front Plant Sc.i.* **2017**, *8*, No: 849.

(4) Bai, L. F.; Zhang, X. Q.; Li, B. Z.; Sun, F. C.; Zhao, X. Q.; Wang, Y. F.; Lu, Z, Y.; Zhang, D. J.; Fang, J. Fungal communities are more sensitive to nitrogen fertilization than bacteria in different spatial structure of silage maize under shore-term nitrogen fertilization. *Appl Soil Ecol*. **2022**, *170*, No. 104275.

(5) Li, B. Z.; Zhang, Q. Q.; Chen, Y. H.; Su, Y. L.; Sun, S. Y. Different crop rotation systems change the rhizosphere bacterial community structure of Astragalus membranaceus (Fisch) Bge. var. *mongholicus* (Bge.) Hsiao. *Appl Soil Ecol*. **2021**, *166*, No. 104003.

(6) Edgar, R. C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat Methods*. **2013**, *10*, 996–998.

(7) Christian, Q.; Elmar, P.; Pelin, Y.; Jan, G.; Timmy, S.; Pablo, Y.; Jörg, J.; Frank Oliver, G. The silva ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. **2013**, *41*, 590-596.

(8) Hu, H. W.; He, J. Z. Manipulating the soil microbiome for improved nitrogen management. *Microbiology Australia*. **2018**, *39*, 24-27.

(9) Bates, S. T.; Berg-Lyons, D.; Caporaso, J. G.; Walters, W. A.; Knight, R.; Fierer, N. Examining the global distribution of dominant archaeal populations in soil. *ISME J*. **2011**, *5*, 908-917.

(10) Rousk, J.; Baath, E.; Brookes, P. C.; Lauber, C. L.; Lozupone, C.; Caporaso, J. G.; Knight, R.; Fierer, N. Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISEM J*. **2010**, *4*, 1340-1351.