

Alfalfa/maize intercropping has comparable productivity and lower environmental impact than nitrogen fertilizer application

Bing Zhang (✉ 1519161286@qq.com)

Jilin Agricultural University

Jamal Nasar

Siqi Dong

xue zhou

<https://orcid.org/0000-0002-8279-9375>


Qiang Gao

Research Article

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Abstract

Background and Aims

Due to the unique biological nitrogen fixation of legume crops, intercropping maize with legume/grass crops such as alfalfa becomes an effective way to reduce exogenous nitrogen fertilizer inputs. This study aimed to determine whether intercropping could be compared to nitrogen fertilizer application and identify potential differences.

Methods

The trial was laid out in the field since 2015 in a randomized complete block design. Treatments included maize monocropping without nitrogen (NOM) and with nitrogen (NM), maize/alfalfa intercropping without nitrogen (NOIM). In autumn 2019, crop and rhizosphere soil samples were collected to determine yield, rhizosphere soil properties and microbial indicators.

Results

Results demonstrated that NOIM and NM treatments had the similar effect on the enhancement of maize yield and yield composition factors and the improvement of soil physicochemical properties related to nitrogen and potassium. Additionally, NM treatment significantly increased the Chao1 and ACE indices by 28.1% and 29.49%, while the NOIM treatment significantly increased the Shannon index by 1.90%. The NM and NOIM treatments had significantly different pathways for increasing maize yield. Both NM and NOIM treatments increased the relative abundance of assimilatory nitrate reduction. And the NOIM treatment increased nitrogen fixation as well. At the same time, NM significantly increased nitrogen dissimilation by 23.98% and intercropping significantly increased denitrification by 12.81%.

Conclusion

Intercropping can be considered comparable to nitrogen fertilizer application in terms of yield and yield composition factors, rhizospheric soil physicochemical properties. Moreover, intercropping was found to be more conducive to the stability of rhizospheric soil bacteria and more environmentally friendly.

Introduction

The application of inorganic nitrogen (N) fertilizers in agriculture is a common practice to enhance soil health and crop yield, thus contributing to human sustenance in various regions (Smil 1999; Galloway et al. 2003). While N fertilizer application can increase soil organic carbon (SOC) and total N (TN) due to higher crop residue inputs, it can also result in organic matter mineralization, leading to nutrient losses and soil organic carbon depletion (Malhi and Lemke 2007). Long-term use of N fertilizer application can thus alter soil C and N turnover, resulting in decreased or increased soil fertility. Several studies have shown that N fertilizer application alters soil carbon content and C/N ratio (Marschner et al. 2001), soil pH (Zhang et al. 2014), and rhizospheric soil properties, which can lead to soil-borne diseases and affect arable land quality and crop yield. Rhizospheric soil physicochemical properties are significantly correlated with changes in the rhizospheric soil bacterial community structure (Bolton et al. 1985; Yang et al. 2016; Qin et al. 2017a). N fertilizer application has a significant impact on the rhizospheric soil bacterial community (Suding et al. 2005; Chu et al. 2007; Pan et al. 2014), increases soil organic carbon (SOC) (Xie et al. 2017; Taylor et al. 2021), modifies the root exudation behavior (Zhou et al. 2017a), and changes the rhizospheric soil bacterial community structure (Wang et al. 2018). Soil environmental factors, such as pH, can change the microbial community composition and distribution of bacterial phyla in the rhizosphere (Latati et al. 2013; Wang et al. 2018) and fertilizer application can change the bacterial taxa involved in the soil N cycle (Enwall et al. 2007; Fierer et al. 2011).

Long-term intensive cultivation has depleted soil resources, leading to the need for N fertilizer application to support crop growth. However, excessive and prolonged N fertilizer use can cause soil degradation and affect crop yield and quality. Intercropping legumes and grasses are an effective approach to biologically fix N by exploiting the root nodule symbiosis of legumes to provide N to grasses. This method can effectively address the problems associated with long-term N fertilizer application. In northeastern China, maize is a major cereal and feed crop, while alfalfa is a high-yielding and nutritious legume (Nasar and Alam 2018; Sun et al. 2018; Shao et al. 2020). Additionally, the extensive root system of alfalfa can help improve soil fertility and reduce wind erosion by providing year-round soil cover. Therefore, intercropping these two crops could be a promising approach to sustainable agricultural production in the region. Previous research has shown that maize/alfalfa intercropping is more beneficial than monocropping (Liu 2018). For instance, Sun et al. (2014, 2018) found that maize/alfalfa intercropping resulted in higher yields than monocropping. Furthermore, maize/alfalfa intercropping has been reported to be

advantageous for plant nutrient uptake and utilization, and soil fertility when compared to monocropping (Nasar et al. 2020). However, most studies have focused on the enhancement effect of intercropping over monocropping under the same experimental conditions, and a comparison between intercropping and N fertilizer application is lacking.

Therefore, a field trial was conducted in Lishu County, Jilin Province, to compare three treatments: (1) maize monocropping without N fertilizer application, (2) maize/alfalfa intercropping without N fertilizer application, and (3) maize monocropping with N fertilizer application. The study aimed to measure crop yield and its components, rhizospheric soil physicochemical properties, and rhizospheric bacterial in different treats and evaluate whether intercropping and N fertilizer application have comparable performance in actual cultivation. We hypothesize that, first, intercropping can achieve crop yield and rhizospheric soil properties comparable to N fertilizer application. Second, intercropping can enhance rhizospheric soil bacterial diversity, stabilize the rhizospheric soil bacterial community composition, and improve effective resistance to external shocks. Third, intercropping is more environmentally friendly than N fertilizer application.

Materials and methods

Site description

The field study was carried out in Lishu County, Jilin province, Changchun, China (Latitude: 43.21N; Longitude: 124.18E; Altitude: 160.2m). The area is classified as having a temperate continental monsoon climate, which has four distinct seasons and moderate semiarid characteristics, the soil type is black soil. The yearly rainfall ranges from 550 to 910 mm, and its average annual temperature is 3–5°C.

Experimental design and arrangement

The trial was laid out in the field since 2015 in a randomized complete block design including three treatments with four replications each, for a total of 12 plots. Treatments included (1) maize monocropping without N (NOM), (2) maize/alfalfa intercropping without N (NOIM), (3) maize monocropping with N (NM). Maize cultivars for Zhengdan 958 (*Zea mays L. cv.*) and alfalfa cultivars for Northeast Normal University variety (*Medicago sativa L.*). The area planted in maize monoculture was 24 m² (6 m x 4 m), with 26 cm plant spacing and 60 cm row spacing, planted with 6 rows of maize. Maize/alfalfa intercrop was planted on 72.9 m² (6 m x 12.15 m) with the same spacing and row spacing as the monocrop, 15 cm plant spacing and 30 row spacing for alfalfa, and 4 rows of maize and 6 rows of alfalfa in the intercrop pattern. Maize monocrop was planted with no N fertilizer application as maize intercrop.

In maize, the seeds were germinated at a field plant density of 60,000 plants hm⁻², and for alfalfa, at a seed rate of 15.0 kg hm⁻². Only the N fertilizer application treatment plot received N fertilizer (N 225.0 kg hm⁻²), while the all-treatment plots received standard amounts of phosphorus (120.0 kg hm⁻²) and kalium (60.0 kg hm⁻²). Urea (46% N), phosphorus pentoxide (46% phosphorus), and kalium oxide (60% kalium) were the fertilizer sources used. Measurements are taken as needed for additional agricultural tasks such timely weeding, insect and pest control, and irrigation.

Rhizospheric soil sampling

Rhizospheric soil samples were collected at harvest of fully mature plants in 2019. Rhizospheric soil samples were collected by removing the maize roots from the center row of each treatment, shaking off the non-rhizospheric soil, and collecting the soil attached to 1 mm of the maize rhizospheric system. All samples were kept in plastic bags, labeled appropriately, stored in cool ice boxes, and brought to the laboratory immediately. Samples were well mixed, sieved (2mm), divided into two sections and sealed. One section was used to extract rhizospheric soil DNA at -80°C and the other section was used to record rhizospheric soil physiochemical properties at 4°C.

Physiochemical properties of the rhizospheric soil

To prepare the soil samples for various physiochemical soil tests, they were air dried, minced, and put through a 2 mm screen. N was estimated by the Kjeldahl apparatus (Bremner 1986) and available nitrogen (AN) by the alkali-hydrolytic diffusion method (Nelson and Sommers 1982). Phosphorus (P) was determined by a colorimeter and available P by the sodium bicarbonate (NaHCO₃) extraction method (Olsen et al. 1954). Kalium (K) was determined by a flame photometer (Hitachi Z-2000, Tokyo) and available kalium (AK) by the air-dry soil extraction method with 1 M ammonium acetate (NH₄Ac), solution was filtered, and K was determined by a flame photometer (Hitachi Z-2000, Tokyo). Soil pH was measured by a pH meter (1:1 soil-water extraction). Organic matter in the soil was measured using the volumetric oxidation method (K₂Cr₂O₇). Total organic carbon (TOC) was estimated by wet combustion (chromic acid digestion). NH₄-N was determined

by extracting the sample with 2M KCl and $\text{NH}_4\text{-N}$ content was estimated by using the phenate method (Jeong et al. 2013). The $\text{NO}_3\text{-N}$ was extracted by CaSO_4 and analyzed by the phenol Di sulphonic acid method (Verma et al. 2013).

Determination of maize yield and yield composition factors

After maturity, the actual yield of maize was measured for each treatment and the height of the maize plant was measured with a tape measure. Stalk diameter, ear length and width were measured with vernier calipers. After harvest at full maturity, the number of rows per ear and the number of seeds per row were recorded by manual counting. Leaf traits were recorded at the co-growth stage for the number of leaves and leaf area. The number of leaves per plant was recorded by manual counting. For leaf area, three fully expanded leaves (top, middle and bottom) were selected and three readings were taken through each leaf, using an inch tape measure for length and width. However, in intercropping, alfalfa-side corn leaves were selected to measure leaf area. Leaf area was calculated using the following formula, with a coefficient value of 0.73 for corn leaf area (Derviş B.2013).

DNA extraction and sequencing

Total community DNA was extracted by using the soil DNA extraction kit Fast DNA SPIN Kit (MP Biomedicals, Santa Ana, USA) according to the manufacturer's manual. To get a deeper insight into bacterial community structure and function, part of the extracted DNA was subjected to high-throughput Illumina sequencing. A primer set targeting the V4-V5 hyper variable region of 16srRNA was used. The sequence of forward and reverse primers 515f/806r was (GCACCTAAYTGGGYDTAAAGNG and TACNVGGGTATCTAATCC) respectively. The sequencing was conducted in Personalbio Shanghai, P.R. Chian. The sequence libraries were generated by using the TruSeq Nano DNA LT Library Prep Kit and were quality checked on the Agilent Bioanalyzer using the Agilent High Sensitivity DNA Kit. After tagging each sample with a sequence adaptor, the samples were loaded onto the Illumina sequencer. The sequencing was performed based on 2×300bp paired-end.

QIIME 1.9.1 (Edgar 2010) were used for the downstream data analysis. Using adaptor sequences forward and reverse reads were stretched together. After removing the barcode and primer sequences quality filtering was performed. Ambiguous sequences not fulfilling the quality requirements were removed. UCHIME algorithms were used for chimeric sequences and were removed. The final valid sequences were used for taxonomic classification. Using VSEARCH (1.9.6), the final valid sequences were assigned to Operational Taxonomic Units (OTUs) at a 97% identity level. Bacterial functions were predicted by PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) upon KEGG (<https://www.kegg.jp/>) databases (Gavin M.D. et al. 2019).

Statistical analysis

The ACE, Chao1, Shannon and Simpsons indices were calculated using QIIME 1.9.1 and the significance of differences was verified by Kruskal-Wallis's rank sum test and Dunn's test as a post factor test for assessing the biodiversity of rhizospheric soil bacterial communities in intercropping versus N application treatments. Assessment of differences in rhizospheric soil bacterial community structure based on bray_curtis distance used for non-metric multidimensional scaling (NMDS) analysis was performed to assess the differences, and one-way analysis of variance (ANOSIM) test of variance was used to compare community structure under intercropping and N fertilizer application. Manhattan plot (MA) and Venn diagrams were performed using the R (4.1.3) software ggplot2 package to describe changes in the number of OTUs between intercropping and N fertilizer application treatments, while cumulative histograms were performed to count changes in relative abundance of species at the gate level between intercropping and N application treatments, and box plots were used to represent differences in maize yield under intercropping and N fertilizer application. The core species was obtained through the online analysis website microbiome Analyst (<http://www.microbiomeanalyst.ca/>) and a phylogenetic tree was constructed using MEGA (version 7.0.26) software to show the effect of intercropping versus N fertilizer application treatments on core species in bacteria. Intercropping and N fertilizer application yield increase pathways were analyzed using R (4.1.3) lavaan, haven, Hmisc, semPlot packages using structural equation modeling (SEM). Heatmapping of functional prediction genes associated with the N cycle was performed using the R (4.1.3) software pheatmap package, and significant differences ($p < 0.05$) between intercropping and N fertilizer application on K0 annotation results were determined using STAMP software, demonstrating the differences in functional prediction genes caused by intercropping versus N fertilizer application.

A molecular ecological network (MEN) was constructed based on random matrix theory, which can be used to explain the inter-ecological interactions between intercropping and rhizospheric soil bacterial communities of N fertilizer application treatments. The analysis was performed using the R (RMThreshold, igraph) package. In this study, molecular ecological networks were constructed using the RMT model after the data were normalized and Spearman correlations were estimated. The topological properties of the molecular ecological network, including the number of network nodes, the number of edges, the average degree (avgK), the average clustering coefficient (avgCC), the average path distance (GD), connectivity (Con), density, and modularity were analyzed using gephi software for visual presentation. Cohesion

and connectivity of the network were calculated. Cohesion measures the stability of the network, which can represent the positive and negative interrelationships between bacterial in the network (Herren and McMahon 2017; Mengting et al. 2021).

Results

Maize yield and yield composition factors

Statistical analysis of the yield data (Table 1) showed that the N0M treatment yielded 9508.55 (kg/hm²) and NM treatment yielded 10407.30 (kg/hm²), with a 15.70% yield increase compared to N0M ($p > 0.05$). Similarly, the N0IM treatment yielded 11387.45 (kg/hm²), with a 27.78% yield increase compared to N0M ($p < 0.05$). The yield stability was found to be higher in the N0IM treatment than the NM treatment (Fig S1). Further analysis of the yield composition factors (Table 2) revealed that compared to the N0M treatment, the NM treatment significantly increased leaf area (23.51%), ear length (27.22%), ear width (7.65%), No. of ear rows⁻¹ (26.42%), No. of grains rows⁻¹ (26.05%). On the other hand, the N0IM treatment significantly increased stem diameter (17.52%), leaf area (18.51%), ear width (6.93%), No. of ear rows⁻¹ (16.98%), No. of grains rows⁻¹ ($p < 0.05$). None of the eight yield component factors measured in this study were found to be significantly different between the NM and N0IM treatments. Based on these results, we conclude that intercropping can be comparable to N fertilizer application in terms of yield, yield stability, and yield composition factors. Moreover, the N0IM treatment showed higher improvement in terms of yield and yield stability, and there was no significant difference between the N0IM and NM treatments in terms of yield composition factors. These findings suggest that intercropping can be a viable alternative to N fertilizer application in sustainable agricultural production, especially in regions where soil resources are limited.

Table 1
Maize yields under different treatments.

Treatments	Yield (kg/hm ²)
N0M	9508.55 ± 303.93 b
N0IM	11387.45 ± 976.53 a
NM	10407.30 ± 482.48 ab

Significant differences between means at $p < 0.05$ in the LSD test are denoted by different lower-case letters. Each treatment was replicated four times: N0M for maize monocropping without N, N0IM for maize/alfalfa intercropping without N, and NM for maize monocropping with N.

Table 2
Physiological and agronomic indicators of maize in different treatments.

Treatments		Plant height (cm)	Stem diameter (mm)	No. of leaves Plant ⁻¹	Leaf area (cm ²)	Ear length (cm)	Ear width (mm)	No. of ear rows ⁻¹	No. of grains row ⁻¹
N0	Monocrop	251.72 ± 1.6a	31.10 ± 0.6b	9.00 ± 1.4a	283.35 ± 26.5b	15.17 ± 0.6b	49.53 ± 2.9b	13.25 ± 1.3b	29.75 ± 3.6b
	Intercrop	254.07 ± 2.7a	36.55 ± 2.5a	10.50 ± 1.3a	335.79 ± 38.5a	17.22 ± 2.0ab	52.96 ± 1.0a	15.5 ± 1.3a	35.75 ± 1.5a
N1	Monocrop	253.93 ± 5.0a	34.10 ± 2.4ab	10.75 ± 2.1a	349.96 ± 22.6a	19.30 ± 1.2a	53.32 ± 0.8a	16.75 ± 0.9a	37.50 ± 4.0a
	Intercrop increase rate (%)	0.93%	17.52%	16.67%	18.51%	13.51%	6.93%	16.98%	20.17%
Nitrogen increase rate (%)		0.88%	9.65%	19.44%	23.51%	27.22%	7.65%	26.42%	26.05%

Significant differences between means at $p < 0.05$ in the LSD test are denoted by different lower-case letters.

Physiochemical properties of the rhizospheric soil

The statistical analysis of the rhizospheric soil physicochemical properties of the N0M, N0IM, and NM treatments (Table 3) revealed that only the N0IM treatment significantly increased N (12.61%), AN (13.20%), K (7.32%), and AK (8.22%), compared to the N0M treatment ($p < 0.05$). The rhizospheric soil physicochemical properties measured in this study were not significantly different between the NM and N0IM

treatments. In conclusion, the results indicate that intercropping can be comparable to N fertilizer application in terms of rhizospheric soil physicochemical properties. Furthermore, the NOIM treatment demonstrated higher improvement in N and K-related properties compared to the NOM treatment.

Table 3
Physicochemical properties of rhizospheric soil under different treatments.

Treatments		N (%)	AN (mg·kg ⁻¹)	P (ppm)	AP (mg·kg ⁻¹)	K (ppm)	AK (mg·kg ⁻¹)	TOC (%)	OM (g·kg ⁻¹)	pH	NH ₄ (mg·kg ⁻¹)	NO ₃ (mg·kg ⁻¹)
N0	Monocrop	1.79 ± 0.11b	74.12 ± 4.63b	0.11 ± 0.01a	18.99 ± 1.46a	9.53 ± 0.28b	83.3 ± 2.74b	0.61 ± 0.16a	10.53 ± 2.82a	5.56 ± 0.28a	21.91 ± 1.01a	24.38 ± 1.16a
	Intercrop	2.01 ± 0.09a	83.90 ± 3.94a	0.10 ± 0.01a	17.54 ± 1.78a	10.22 ± 0.24a	90.14 ± 2.38a	0.59 ± 0.08a	10.18 ± 1.36a	5.59 ± 0.18a	21.81 ± 3.70a	24.93 ± 2.80a
N1	Monocrop	1.90 ± 0.10ab	79.22 ± 4.37ab	0.11 ± 0.02a	19.21 ± 2.91a	9.99 ± 0.46ab	87.81 ± 4.51ab	0.68 ± 0.12a	11.79 ± 2.09a	5.42 ± 0.17a	23.14 ± 1.17a	25.77 ± 1.32a
	Intercrop	12.61%	13.20%	-7.42%	-7.62%	7.32%	8.22%	-3.40%	-3.40%	0.40%	-17.14%	2.24%
Nitrogen increase rate(%)		6.58%	6.89%	1.12%	1.15%	4.83%	5.42%	11.95%	11.95%	-2.52%	5.63%	5.69%

Significant differences between means at $p < 0.05$ in the LSD test are denoted by different lower-case letters.

Rhizospheric soil bacterial community diversity and richness

In the present study, various indices were used to characterize α -diversity of the rhizospheric soil bacterial community, including the Chao1 index, ACE index, Shannon index, and Simpson index (Fig. 1). The ACE and Chao1 index were primarily used to reflect the community's richness, while the Shannon and Simpson index determined the community's homogeneity. Statistical analysis of the α -diversity indices showed that the NM treatment significantly increased the Chao1 index by 28.10% and the ACE index by 29.49% compared to the NOM treatment ($p < 0.05$). Furthermore, the NOIM treatment significantly increased the Shannon index by 1.90%. However, when combining the four α -diversity indices measured in this study, there was no significant difference between the NM and NOIM treatments. Based on these findings, we conclude that intercropping can be comparable to N fertilizer application in terms of rhizospheric soil bacterial α -diversity. Specifically, the NOIM treatment showed improvement in the Shannon index, while the NM treatment exhibited an increase in the Chao1 and ACE indices. Overall, these results suggest that intercropping could be a promising approach for improving the diversity of the rhizospheric soil bacterial community.

Rhizospheric soil bacterial community structure

The NMDS analysis showed that samples from different treatments formed distinct clusters in the sorted space (Fig. 2). The Anosim difference test analysis further confirmed that there were significant differences among NOM, NOIM, and NM treatments, with the NM treatment having a greater effect on the rhizospheric soil bacterial community structure than the NOIM treatment (as indicated by the greater R-value). Further analysis at the OTU level (Fig S2) revealed that the abundances of 514 OTUs changed significantly in the NM treatment compared to the NOIM treatment, with 245 OTUs showing significantly higher abundances and 269 OTUs showing significantly lower abundances. Similarly, 397 OTUs showed significant changes in abundances in the NOIM treatment, with 245 OTUs showing significantly higher abundances and 175 OTUs showing significantly lower abundances. There were 397 significant changes in OTUs abundance in the NOIM treatment, of which 245 OTUs abundances were significantly higher and 175 OTUs abundances were significantly lower. Interestingly, only three OTUs showed significant changes in abundances that were common to both NM and NOIM treatments, while the rest of the OTUs with significant changes in abundances due to NM and NOIM treatments were unique to each treatment. In conclusion, the effects of intercropping and N fertilizer application on the structure of the rhizospheric soil bacterial community were significantly different. Specifically, N fertilizer application had a greater effect on the rhizospheric soil bacterial community structure than intercropping. Furthermore, the differences in OTUs abundances between the NM and NOIM treatments were relatively major, with only a few OTUs showing common changes in abundances between these two treatments.

Taxonomic composition of core species and bacterial communities

We analyzed the differences in relative abundance at the phylum level between treatments (Fig S3). Among the high-abundance phyla, the NM treatment showed decreased abundance of Chloroflexi, Acidobacteria, and Gemmatimonadetes, and increased abundance of Proteobacteria and Actinobacteria compared to the NOM treatment. In the NOIM treatment, the phyla Actinobacteria and Chloroflexi decreased in abundance, while Proteobacteria, Acidobacteria and Gemmatimonadetes increased in abundance. In the low-abundance phyla, the NM treatment did not contain the Tenericutes, FCPU425 and WS1 phyla, but had an additional FBP phylum. The NOIM treatment, on the other hand, had the addition of Rokubacteria and FBP phyla. We also screened and analyzed the core species of the different treatments (Fig. 3). The results showed that Actinobacteria, Chloroflexi, Gemmatimonadetes, WPS-2 and Proteobacteria phyla were distributed by 19 core species in NOM, NOIM, and NM treatments. Compared to the NOM treatment, the NM treatment reduced the total abundance of core species by 4.04% and the NOIM treatment reduced the total abundance of core species by 3.42% compared to NOM, but there was no significant difference ($p > 0.05$). Analysis of specific core species revealed that the NM treatment significantly affected four core species compared to NOM, which significantly reduced the abundance of otu105446 (67.29%), otu1902 (46.31%), and otu8332 (21.47%), and significantly increased the abundance of otu42839 (204.86%). The NOIM treatment significantly affected five core species, significantly reducing otu68588 (47.78%), otu82453 (37.90%), otu8332 (21.42%), otu86921 (42.97%), and significantly increasing otu22568 (86.64%). Between the NM and NOIM treatments, the core species otu105446, otu82453 and otu86921 showed significant differences ($p < 0.05$). In conclusion, our findings suggest that intercropping and N fertilizer application have different effects on species in terms of rhizospheric soil bacterial community composition and core species. The NM treatment had a greater effect on rhizospheric soil bacterial community structure than the NOIM treatment, and the effect of N fertilizer application on rhizospheric soil bacterial community structure was significantly greater than that of intercropping.

Molecular ecological network analysis of rhizospheric soil bacterial community

To evaluate the differences in the stability of the rhizospheric soil bacterial community between the NM and NOIM treatments, this study employed molecular ecological network (MEN) analysis to construct a network diagram of the bacterial community between the two treatments (Fig. 4a). The topological index analysis indicated that the average path distance of the MEN graphs for both treatments was 3.415–3.657, which was in proximity to the logarithm of the number of network nodes (Table 4). These results indicate that the rhizospheric bacterial communities of both treatments exhibited typical small-world characteristics, implying that nodes in the network were closely related to each other, and any external disturbances were rapidly reflected in the entire network.

Furthermore, the modularity analysis results showed that both the NM and NOIM treatments reduced the modularity of the molecular ecological network compared to the NOM treatment. However, the NOIM treatment was more modular and had a more stable network structure compared to the NM treatment (Table 4). To further analyze the NM and NOIM treatments' network stability structure, the study conducted an analysis of network cohesion, and the results are shown in Fig. 4b. The analysis indicated that the NM treatment did not significantly affect the total cohesion of the rhizospheric bacterial molecular ecological network, including both positive and negative cohesion, compared to the NOM treatment ($p > 0.05$). However, the NOIM treatment significantly reduced 7.57% of the total cohesion ($p = 0.043$) and 7.18% of the positive cohesion ($p = 0.043$) of the molecular ecological network. Additionally, the NM treatment significantly increased the molecular ecological network's total cohesion and positive cohesion by 14.63% total cohesion and 14.56% positive cohesion ($p = 0.021$) and significantly decreased the negative cohesion by 14.69% ($p = 0.021$) compared to NOIM treatment. In conclusion, the results suggest that intercropping fosters a more stable ecological network concerning the stability facilitation of rhizospheric soil bacterial networks, which is advantageous in resisting external environmental shocks.

Table 4
Molecular ecology network (MEN) properties of rhizosphere soil bacteria communities under different treatments.

	Network size/nodes number	Connectivity/total links	Average degree (avgK)	Average clustering coefficient (avgCC)	Average path distance (GD)	Density (D)	Transitivity (Trans)	Modularity
NOM	479	13348	55.733	0.811	3.657	0.117	4	0.658
NOIM	478	14210	59.456	0.78	3.642	0.125	4	0.634
NM	476	17265	70.634	0.827	3.415	0.153	3	0.555

Direct and indirect effects of environmental factors and bacterial community on maize yield

To evaluate the direct and indirect effects of rhizospheric soil physicochemical properties and bacterial communities on maize yield, we used structural equation modeling (SEM) in this study (Fig. 5). Our results revealed that rhizospheric soil physicochemical properties, including AP and AN, directly affected maize yield. Meanwhile, N forms (NH_4 and NO_3) indirectly impacted maize yield by affecting bacterial community structure. The path coefficient analysis indicated that AN had the most significant direct effect on crop yield (path coefficient = 0.757), followed by rhizospheric soil bacterial community structure (path coefficient = 0.476), while AP had a direct negative effect on maize yield (path coefficient = -0.485). Furthermore, the intercropping treatments significantly influenced the rhizospheric soil physicochemical properties, such as AN and AK content, and thereby indirectly affected the diversity of the rhizospheric soil bacterial community. In conclusion, the intercropping treatments affected the rhizospheric soil physicochemical properties, which directly or indirectly regulated maize yield by affecting the rhizospheric soil bacterial community structure and, consequently, the final maize yield.

N-related biological pathways and functional prediction

In this study, we used PICRUST to predict the functions of rhizospheric soil bacterial communities associated with the N cycle (Fig. 6). Our results showed that different treatments significantly impacted 41% of the predictive functions associated with N cycle ($p < 0.05$) (Fig. 7). Compared to NOM, the NM treatment significantly increased gene abundance for nirB related to dissimilatory nitrate reduction, but significantly decreased the abundance of genes related to denitrification (napA and napB). The effects on assimilatory nitrate reduction were inconsistent, with an increased abundance of nasA and decreased abundance of nirA. On the other hand, the NOIM treatment significantly increased gene abundance for N fixation-related genes (nifH, nifD, nifK), denitrification-related genes (norL), and assimilatory nitrate reduction-related genes (nasB). NM treatment also significantly increased the abundance of assimilatory nitrate reduction-related genes (nasA), but significantly decreased the abundance of denitrification-related genes (napA and norC) and N fixation-related genes (nifD, nifK, nifH) compared to the NOIM treatment. Dissimilatory nitrate reduction-related genes showed an inconsistent pattern of change, with significantly increased nirB gene abundance and significantly decreased nirD gene abundance under the NM treatment. In conclusion, our results show that intercropping and N fertilizer application significantly impact the functional prediction of rhizospheric soil bacterial communities associated with the N cycle, specifically in terms of assimilatory nitrate reduction, dissimilatory nitrate reduction, denitrification, and N fixation-related genes.

Discussion

Intercropping as a sustainable alternative to excessive nitrogen fertilizer application: evidence from maize yield and yield composition factors

Our study compared the maize yield and yield components of intercropping treatments with those of N fertilizer application treatments. We found that both the N fertilizer application and intercropping treatments resulted in higher maize yield compared to the monocropping without N fertilizer application (Table 1). Notably, the intercropping treatment demonstrated a significant increase in yield ($p < 0.05$) and exhibited good yield stability (Fig S1). Furthermore, we observed no significant difference between the N fertilizer application and intercropping treatments in terms of yield components (Table 2) ($p < 0.05$). Intercropping systems can enhance crop productivity through the efficient use of available resources, as evidenced by previous studies (Pelzer et al. 2016; Nasar et al. 2019). The root interactions between the crops in intercropping systems can lead to changes in crop root intervals, resulting in either competitive or facilitative root interactions (Ghosh et al. 2006). The movement of nutrients from legumes to cereals may also be directly linked to these changes in transport (Ehrmann and Ritz 2014). Consistent with our findings, earlier studies have reported that intercropping can significantly improve overall crop growth and yield through efficient use of natural resources (Latati et al. 2013; Xiao et al. 2013; Brooker et al. 2015). Our study provides evidence that intercropping can be comparable to N fertilizer application in terms of maize yield, with the added benefit of being potentially higher in terms of yield component factors.

Intercropping as a sustainable alternative to excessive nitrogen fertilizer application: evidence from rhizospheric soil physicochemical properties

In this study, it was observed that the NM treatment did not have any significant impact on the physicochemical properties of rhizospheric soils, as compared to the NOM treatment (Table 3). This finding is contrary to the results of conventional studies, where optimization of N fertilizer application helped increase soil nutrient content (Verma et al. 2014). We speculate that the fertilizer application rate might be the reason why N fertilizer application did not have a significant effect on the physicochemical properties of rhizospheric soils. Specifically, conventional N fertilizer application rates were used in this study, and N fertilizer application rates after optimal management were not employed. This may have resulted in changes in crop N concentration and increased uptake of total N at the same treatment level, mainly due to different N fertilizer application rates (Neugschwandtner and Kaul 2015). Another possible reason is that the study only focused on one year of data. Future research will integrate the results of multi-year trials to confirm this speculation. In contrast, the NOIM treatment significantly affected the rhizospheric soil physicochemical indicators related to N and K (N, TN, K, TK) as compared to the NOM treatment.

The increased soil nutrient content could be attributed to various reasons, including the secretion of plant roots that help dissolve nutrients from unavailable forms into plant-available forms (Shi et al. 2011). Furthermore, the function of the rhizospheric soil bacterial community, as well as enzyme activity, can facilitate the conversion of organic forms of nutrients into effective inorganic forms for crop growth (Liu 2018). Another reason for the increased soil nutrient content may be the N fixation capacity of legumes, which helps to improve the soil fertility status (Shao et al. 2022).

Intercropping had less impact on community structure and fostered a more stable ecological network

In this study, both NM and N0IM treatments significantly increased the α -diversity index in terms of α -diversity (Fig. 1), but there was no significant difference between NM and N0IM ($p < 0.05$). However, in terms of rhizospheric soil bacterial community composition (Fig. 2), there was a significant difference between N0M, N0IM, and NM treatments. Moreover, compared to N0M, NM and N0IM treatments significantly affected OTUs differently (Fig S2), with NM treatment causing the greatest change. These findings are consistent with the results of Zhang et al. (2022) who showed that soil tillage and N fertilizer application significantly affect the structure and diversity of rhizospheric soil bacterial communities (Zheng et al. 2021). The dominant phylum in all rhizospheric soil samples in this study was found to be (Fig S3), which is the most common bacterial phylum in agricultural rhizospheric soils (Qin et al. 2017b; Li and Wu 2018). The NM and N0IM treatments had different effects on high- and low-abundance phyla, with two phyla producing the same effect in the high-abundance phylum (40%) and one phylum in the low-abundance phylum (20%). Tenericutes, FCPU425, and WS1 phyla were not detected in NM treatment, and FBP phylum was added compared to N0M. Li et al. (2019) observed significant changes in bacterial community in rhizospheric soil using soil N effectiveness as a variable, where Tenericutes was found to be the major taxon of phytopathogenic organisms. Tu et al. (2022) also found that N fertilizer application reduced the relative abundance of Tenericutes. Moreover, macrogenomics analysis by Sun et al. (2016) predicted that Tenericutes has prominent nucleic acid degradation ability and has a greater correlation with elements such as N, which may be the reason for the decrease in Tenericutes abundance after N fertilizer application. On the other hand, the N0IM treatment added Rokubacteria and FBP phyla, and Wang et al. (2022) also found that the intercropping system increased the abundance of Rokubacteria phylum. The analysis of core species revealed that the total abundance of core species in N0M, NM, and N0IM treatments was not significantly different ($p < 0.05$), and the most abundant core species belonged to the Proteobacteria phylum. Proteobacteria members from different phyla play a crucial role in global nutrient cycling of carbon, N, and sulfur (S) due to their morphological and metabolic diversity (Moon et al. 2018). However, there was a significant difference ($p < 0.05$) between NM and N0IM treatments on core species, with 66.67% of OTUs belonging to the Chloroflexi phylum, which is also an active phylum in agroecosystems (Liu 2018). These changes in rhizospheric soil bacterial community structure are attributed to potential mechanisms such as interspecific root interactions and root exudation (Qin et al. 2017a), which alter soil enzyme activity and soil physicochemical properties, and thus the rhizospheric soil bacterial community structure (Qin et al. 2017b; Zhou et al. 2017b; Guo et al. 2020), it can also be attributed to N fertilizer application, and evidence from related studies suggests that N fertilizer application increases or decreases the rhizospheric soil bacterial community (Fierer et al. 2011; Wang et al. 2018). Molecular ecological network analysis results showed that although both NM and N0IM treatments reduced the modularity of the molecular ecological network compared to N0M, the N0IM treatment had a higher modularity index and a more stable ecological network structure compared to the NM treatment. The results of Zhu and Hai et al. (2020) showed that both N fertilizer application and intercropping reduced the stability of the molecular ecological network, respectively. It was also demonstrated that intercropping of grasses and legumes produced a complex modularity of the bacterial community network, which improved bacterial collaboration and crop growth vigor (Hui 2022), which supports the findings of our study. We also assessed network cohesion indices for rhizospheric soil bacterial communities, which can indicate the intensity of cooperation and competition between species in the community as well as the stability of bacterial networks using negative cohesion. A larger negative cohesion results in a more stable network structure (Herren 2017). Our results found that the NM treatment did not have a significant effect on negative cohesion indices compared to N0M, while the N0IM treatment had a greater negative cohesion index ($p < 0.05$). Overall, the molecular ecological network environment of intercropping is more stable, and even better negative cohesion of the rhizospheric soil bacterial community can be obtained. In general, the effects of intercropping on community structure were smaller and more stable.

Intercropping had significantly different pathways for increasing maize yield, and was more environmentally friendly

Our study results revealed that intercropping and N fertilizer application directly or indirectly influenced maize yield through rhizospheric soil physicochemical properties and rhizospheric soil bacterial communities. Moreover, intercropping significantly affected maize yield by significantly altering rhizospheric soil physicochemical properties (AN, AK), and indirectly by influencing rhizospheric soil community structure. Similarly, in a maize and soybean intercropping experiment, Searle et al. (1981) observed that intercropping and N fertilizer

application were distinct modes of yield promotion, with N fertilizer application leading to increased soil N and intercropping stimulating maize growth indirectly.

Our findings also revealed that the NM treatment significantly increased the abundance of functional genes related to dissimilatory nitrate reduction and reduced the abundance of functional genes related to denitrification compared to N0M. Li et al. (2022) noted that different specific genes were affected differently, indicating that the effect of N fertilizer application on nitrate reduction genes was not unique. Nitrate reductase, which is involved in the first step of nitrate assimilation (Datta and Sharma 1999), is predominantly produced by soil-beneficial rhizospheric soil bacteria such as Rhizobia, Azotobacter, and Clostridium (Nason et al. 1971; Raymond et al. 2004). The abundance of these beneficial rhizospheric soil bacteria also affects nitrate reduction genes, indicating a complex process. The N0IM treatment resulted in a significant increase in the abundance of functional genes related to N fixation, while decreasing the abundance of genes related to denitrification and assimilatory nitrate reduction. This finding is consistent with previous studies, which have shown that intercropping rhizospheric soil bacteria and root secretions can mediate a new process of N fixation stimulation and high crop yield, controlled primarily by Nitrosomonas and Nitrobacter bacteria. These processes significantly enhance the abundance of N fixation-related functional genes in intercropping systems. In contrast, the NM treatment significantly increased the abundance of functional genes related to assimilatory nitrate reduction, while significantly decreasing the abundance of genes related to denitrification and N fixation compared to the N0IM treatment. This finding is in line with previous research, which has found that N fertilizer application can inhibit the ability of biological N fixation. However, the high abundance of functional genes related to N fixation in the N0IM treatment may be attributed to the large amount of N fixation provided by rhizobia of legume crops.

At the same time, we found that in terms of functional prediction, N fertilizer application could improve the N assimilation, and intercropping could improve the soil fertility and crop yield by improving N fixation and N assimilation. But N fertilizer application could significantly increase the N dissimilation by 23.98%. Intercropping only significantly improved denitrification by 12.81%. Both denitrification and N dissimilation will lead to the loss of soil N to the atmosphere, thus causing environmental pollution (Chen et al. 2013). Intercropping was more environmentally friendly.

Conclusions and perspectives

In conclusion, this study demonstrated that intercropping is comparable to N fertilizer application in terms of yield and yield composition factors, rhizospheric soil physicochemical properties, and bacterial α -diversity indexes. However, intercropping was found to be more conducive to the stability of rhizospheric soil bacteria and more environmentally friendly. Further research is needed to explore the long-term effects of intercropping on soil health and the sustainability of crop production systems.

Declarations

Data availability

The sequence files were submitted to the NCBI Sequence Read Archive repository (<http://www.ncbi.nlm.nih.gov/sra>) and are accessible with the Ascension Numbers: Bio Project PRJNA963179.

Statements and Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Bing Zhang: Experiment investigation, Sampling and analysis, Writing-Original draft preparation. Jamal Nasar: Experiment investigation, Sampling and analysis, Writing-Original Draft. Siqi Dong: Experiment investigation, Sampling and analysis. Xue Zhou: Writing-Reviewing and Editing, Supervision, Funding acquisition. Qiang Gao: Funding acquisition.

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Figures

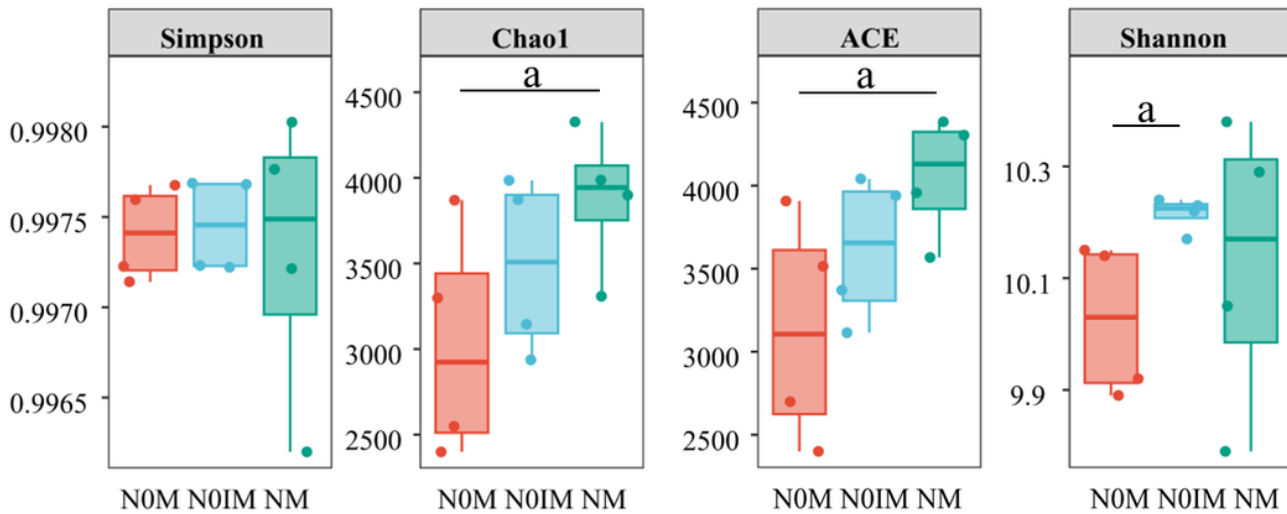


Figure 1

The α -diversity of rhizospheric soil bacterial communities under different treatments. Each treatment was replicated four times: NOM for maize monocropping without N, NOIM for maize/alfalfa intercropping without N, and NM for maize monocropping with N. Significant differences between means at $p < 0.05$ in the LSD test are denoted by different lower-case letters.

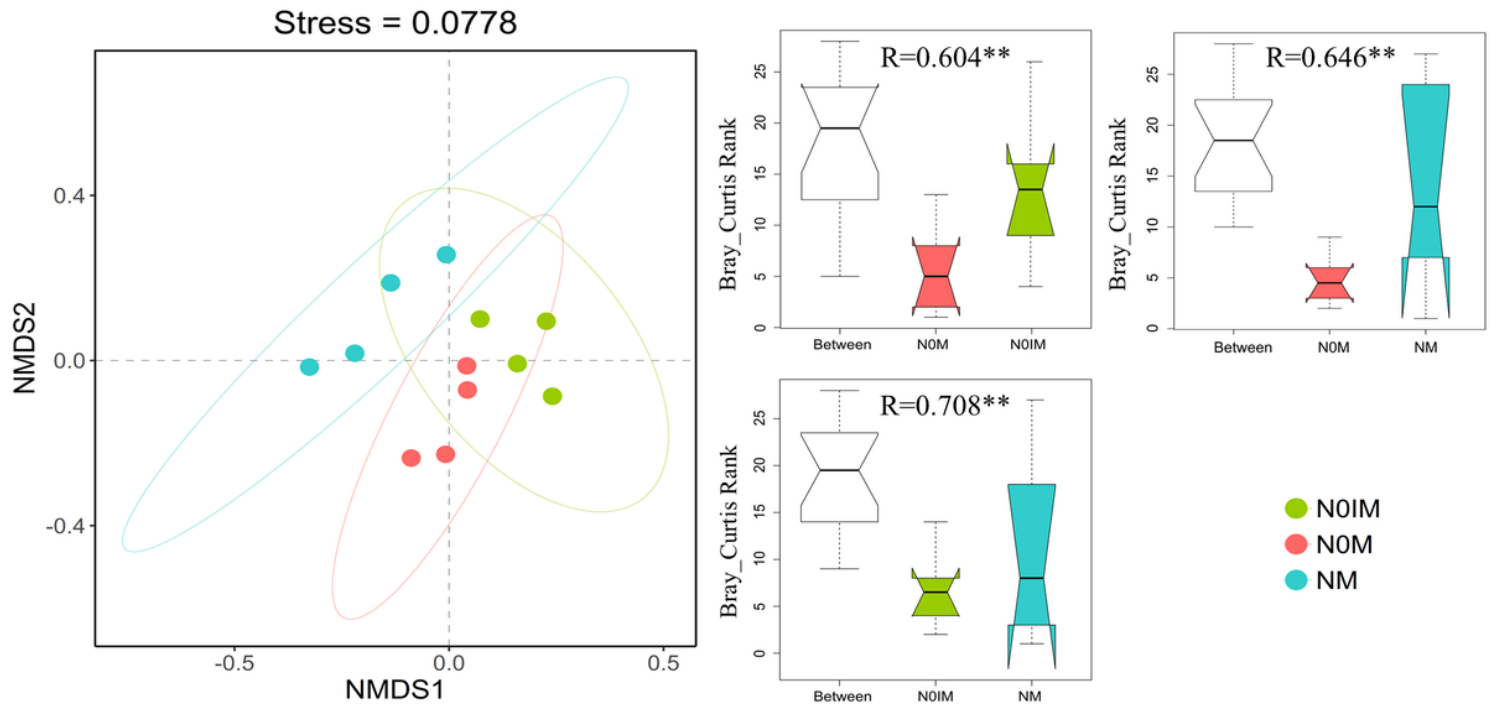


Figure 2

Beta-diversity of rhizospheric soil bacterial communities in different treatments. The results of non-metric multidimensional scaling (NMDS) analysis display patterns of beta-diversity between the control and treatments. Ellipses with a 95 percent level of confidence are displayed around each group. The Anosim analysis results are characterized by a boxplot. Asterisks denote for significant probability levels (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

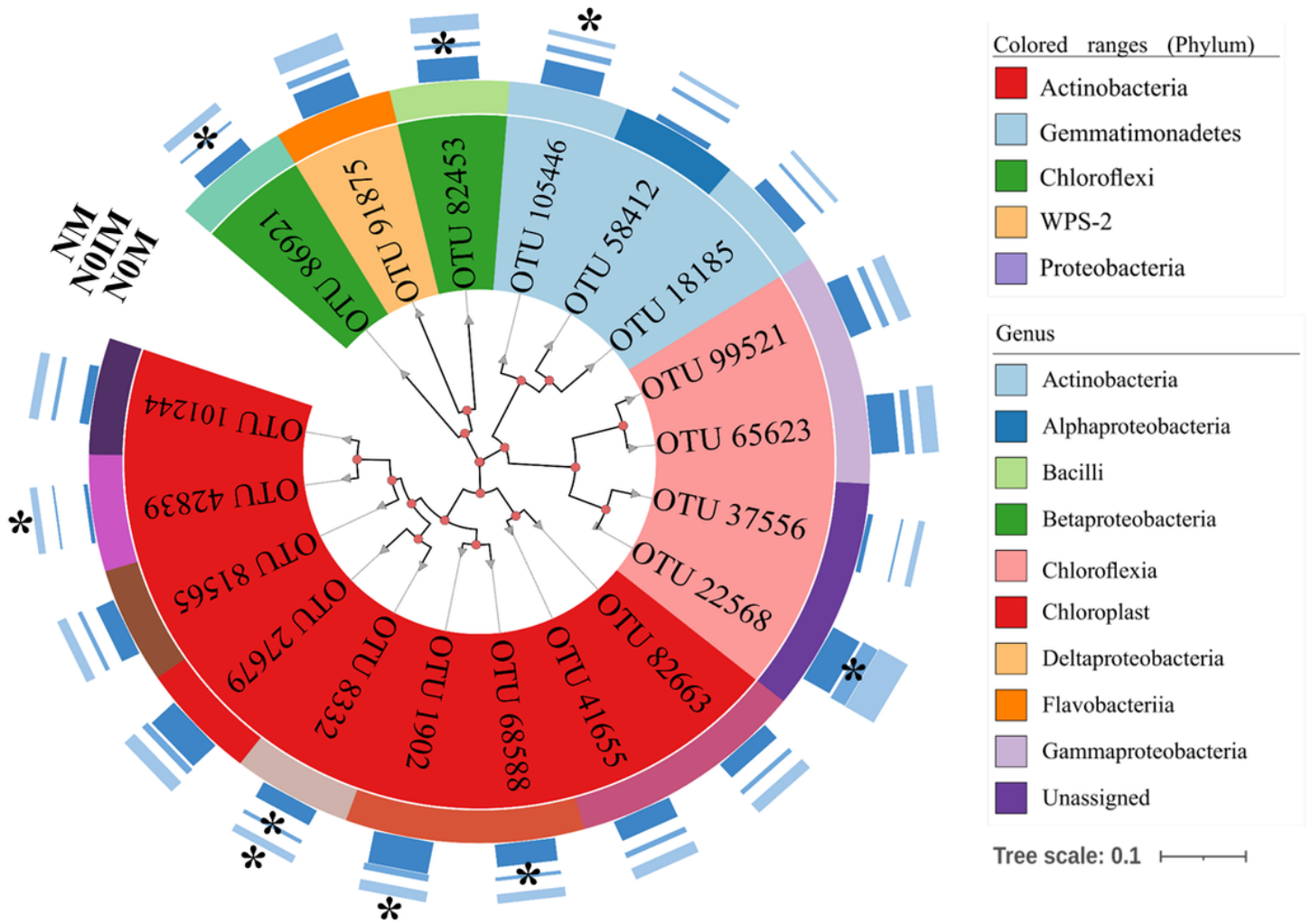


Figure 3

Taxonomic information of core species in different treatments. The stacked bar chart displays the relative abundance of the core species in each treatment. Each treatment was replicated four times: NOM for maize monocropping without N, N0IM for maize/alfalfa intercropping without N, and NM for maize monocropping with N. Asterisks denote for significant probability levels (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

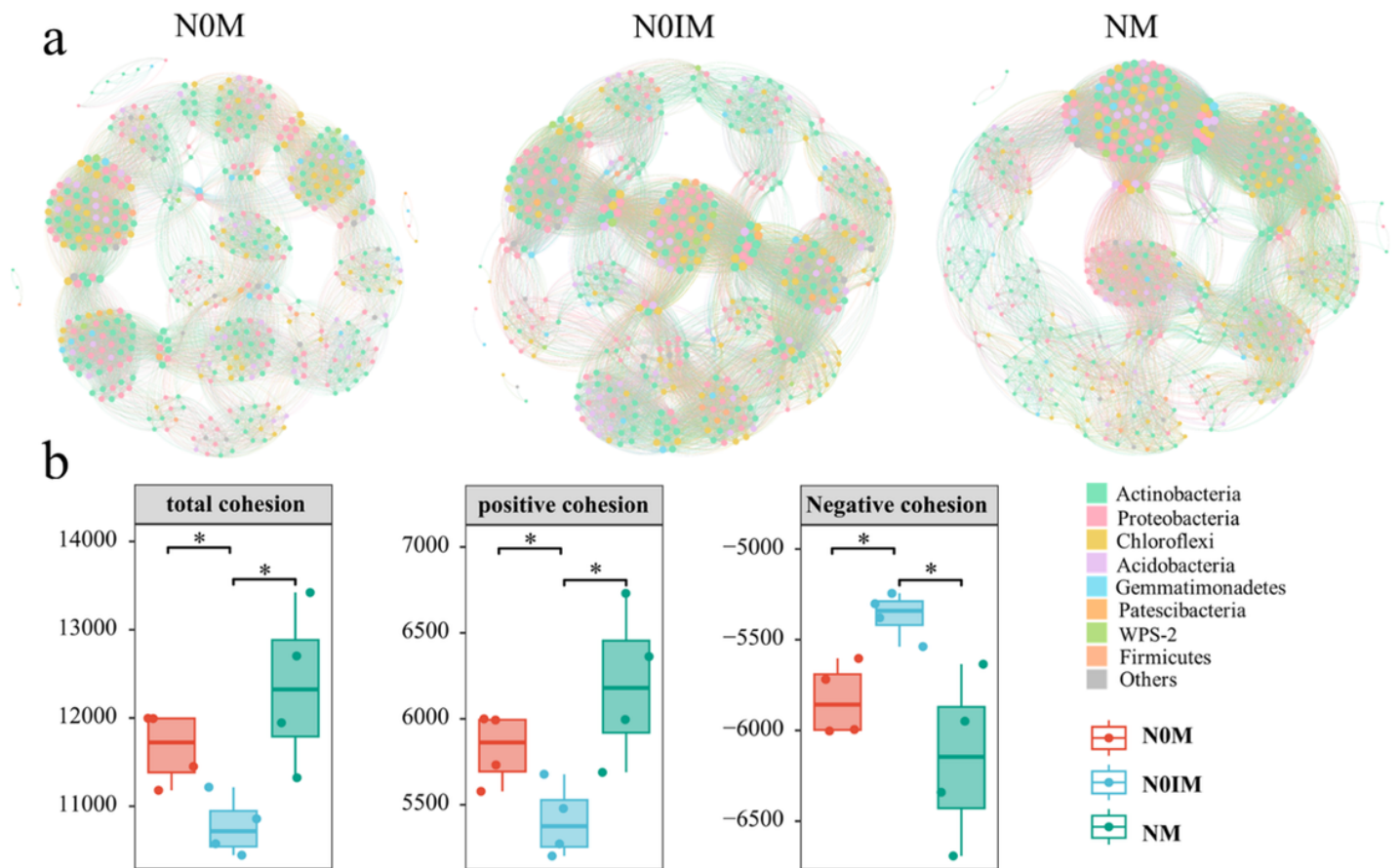


Figure 4

The molecular ecological network of rhizospheric soil bacterial communities in different treatments (a) and boxplot of network cohesion (b). The analysis diagram illustrates the co-occurrence patterns among bacterial species in different treatments, with each node representing a bacterial species, and each edge indicating a significant correlation between two species. The boxplot of network cohesion demonstrates the distribution of network cohesion, with each treatment indicated by a different color. The higher the negative cohesion value, the more stable the network. Asterisks denote for significant probability levels (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

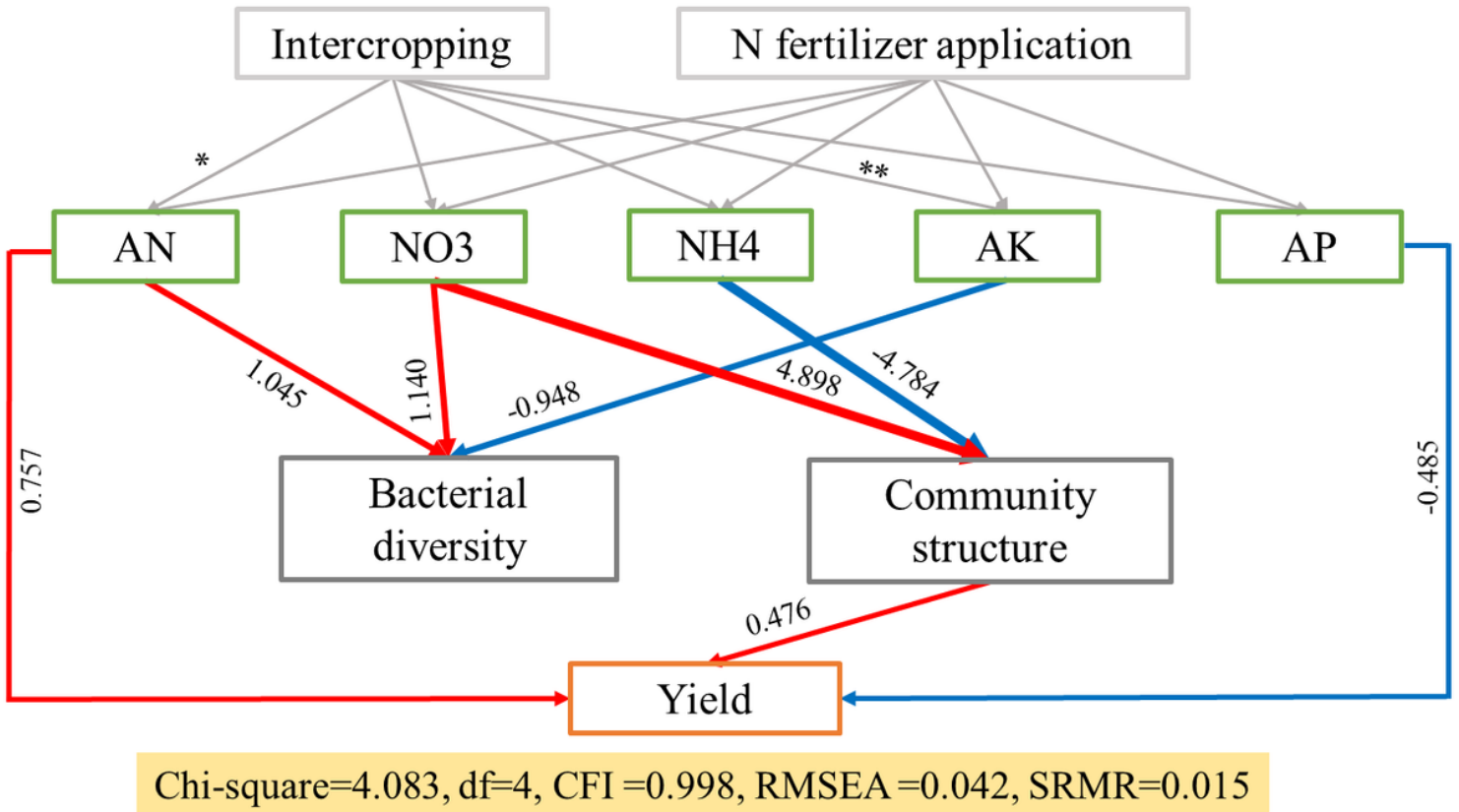


Figure 5

Structural equation modeling (SEM) showing the direct and indirect effects of rhizospheric soil physicochemical properties, rhizospheric soil bacterial community diversity and structure on maize yield. Red and blue arrows indicate the positive and negative effects and numbers adjacent to arrows are standard path coefficients. Asterisks denote for significant probability levels (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

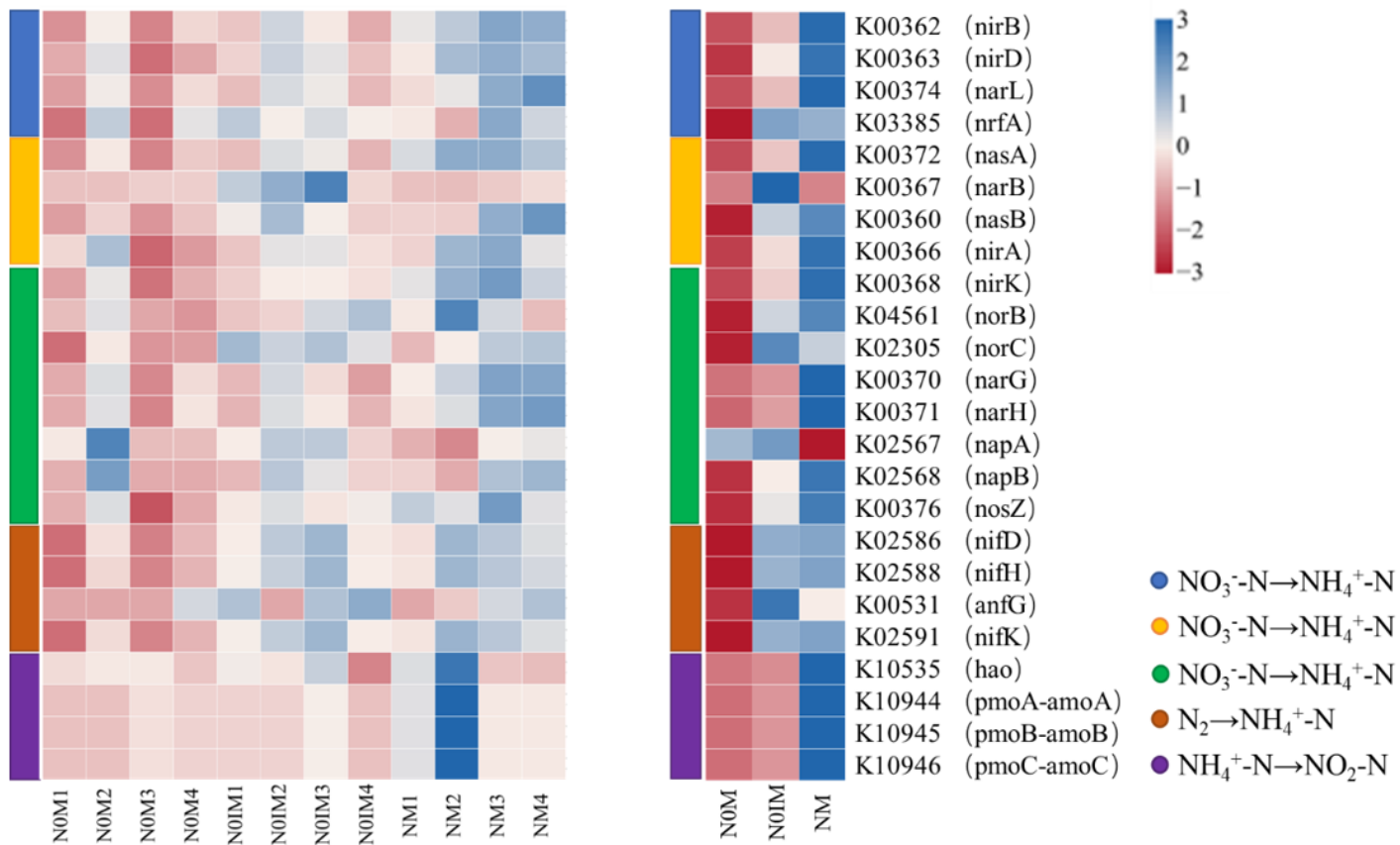


Figure 6

Analysis of KEGG metabolic pathways in the N cycle of rhizospheric soil bacterial communities in different treatments As shown in the picture, each treatment was applied four times: N0M for maize monocropping without N, N0IM for maize/alfalfa intercropping without N, and NM for maize monocropping with N.

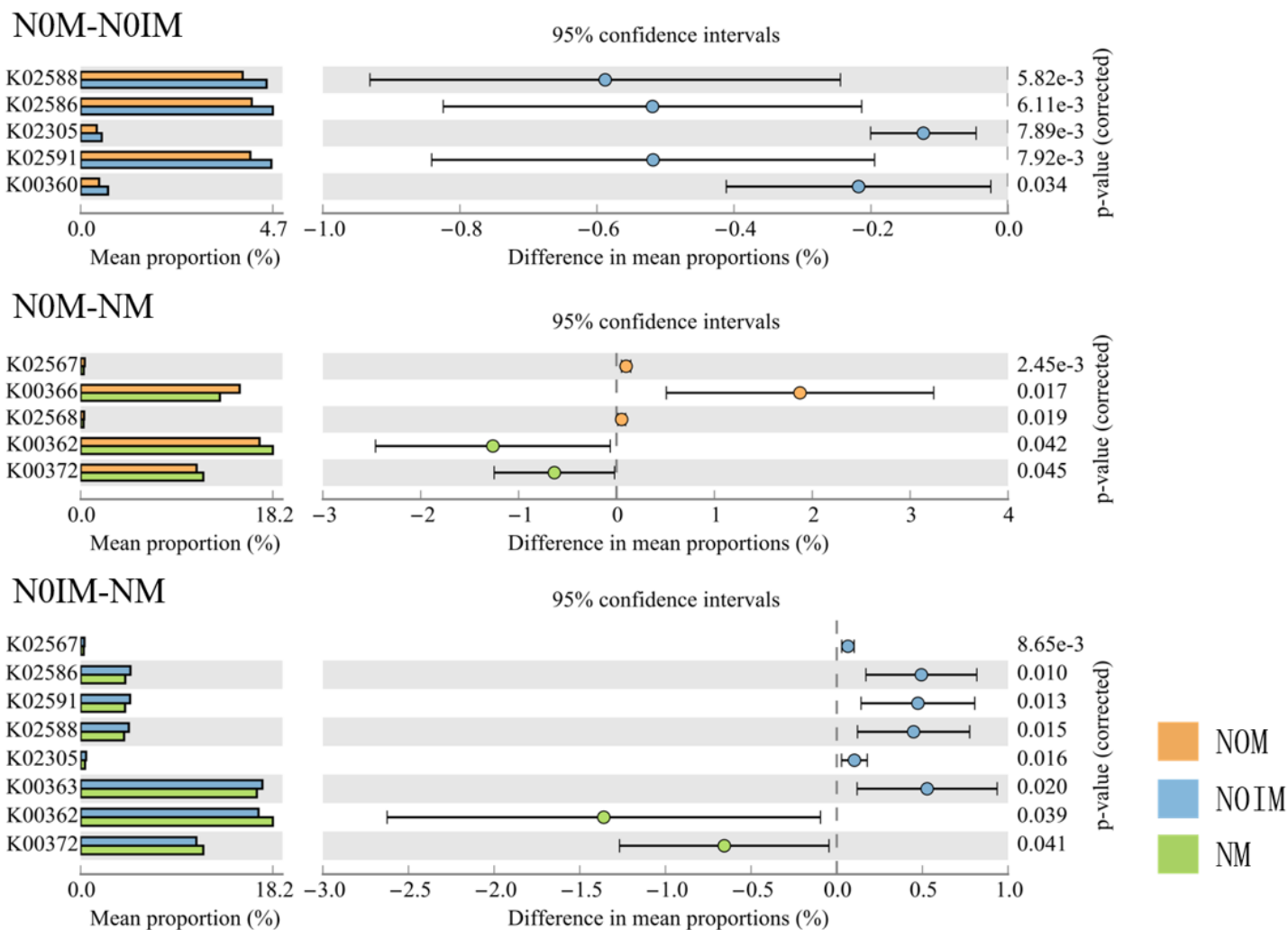


Figure 7

Analysis of differences in KEGG N cycling pathways among treatments ($p < 0.05$). As shown in the picture, each treatment was applied four times: NOM for maize monocropping without N, NOIM for maize/alfalfa intercropping without N, and NM for maize monocropping with N.

Supplementary Files

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- [Supplementarymaterial.docx](#)