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RESEARCH ARTICLE

Opportunistic keystone diazotrophs from co-occurrence networks drive biological nitrogen fixation in peanut/cotton intercropping systems

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Highlights

- Intercropping and rhizosphere soils significantly enhanced BNF potentials by 7.8–323.0%.
- Heterogeneous selection primarily drove diazotrophic community assembly.
- Opportunistic diazotrophs dominated networks and acted as keystone taxa enhancing BNF.

Abstract

Legume-based intercropping enhances asymbiotic biological nitrogen fixation (BNF); however, the underlying mechanisms remain unclear, including the roles of soil keystone diazotroph taxa with varying niche breadths. A field experiment was conducted to evaluate soil BNF variations between rhizosphere and bulk soils in peanut/cotton intercropping systems and monocultures. BNF activities were measured by nitrogen fixation rates, nitrogenase activity, and *nifH* gene abundance. Phylogenetic null models, co-occurrence networks, and niche breadth analysis were applied to investigate the roles of diazotrophic keystone taxa and their ecological niches. Rhizosphere soils exhibited 7.8–125.5% higher BNF potentials than bulk soils, whereas intercropping systems showed 11.6–323.0% increases over monocultures for nitrogen fixation rate, nitrogenase activity, and *nifH* gene abundance (all $P<0.05$). Diazotrophic community composition and diversity differed significantly, with Proteobacteria (excluding Alphaproteobacteria) enriched in intercropping and rhizosphere soils, while Cyanobacteria and Firmicutes were less abundant. Deterministic processes, particularly heterogeneous selection, dominated community assembly in the rhizosphere (91.9%) and intercropping soils (86.3%). The co-occurrence networks consistently revealed more complex and interconnected communities in intercropping and rhizosphere soils that were dominated by opportunistic diazotrophs (78.8–85.9%), followed by specialists (10.2–18.5%) and generalists (1.38–3.80%). Keystone taxa, including opportunists such as *Azoarcus*, *Azohydromonas*, and *Steroidobacter*, and generalists like *Pseudomonas* and *Azotobacter*, correlated positively with microbial biomass carbon and nitrate nitrogen, contributing to enhanced BNF. Peanut/cotton intercropping enhances BNF by selectively enriching the keystone diazotrophic taxa with varying ecological roles, particularly opportunists and generalists. Such targeted intercropping strategies can optimize BNF, improve soil fertility, and promote sustainable agricultural production.

Keywords: biological nitrogen fixation, community assembly, habitat ecotypes, keystone diazotroph taxa

1. Introduction

Biological nitrogen fixation (BNF) is a fundamental ecological process that converts atmospheric nitrogen into bioavailable forms. This is particularly critical in agricultural systems, where available nitrogen enhances plant growth, improves soil fertility, and reduces reliance on synthetic fertilizers

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to promote sustainable farming practices. BNF provides approximately 97% of the "new" nitrogen input into natural ecosystems (Vitousek *et al*. 2013). BNF contributes between 22 and 53 kg N ha^{-1} yr^{-1} of nitrogen annually to farmland ecosystems, and symbiotic BNF, primarily driven by diazotrophs, plays a major role with regard to these inputs (Ma *et al.* 2019; Zhang *et al.* 2022).

BNF is particularly critical in legume-based systems, where symbiotic rhizobia-host plant interactions are considered the dominant nitrogen source. In legume/non-legume intercropping systems, the non-legume plants such as cereals deplete soil nitrogen, thereby stimulating legume nodules to enhance symbiotic BNF — a phenomenon described by the "nitrogen competition hypothesis" (Xiao *et al.* 2004; Fan *et al.* 2006). The rhizosphere serves as a crucial interface between plant roots and soil, and it provides a unique microhabitat in which microbial communities thrive and contribute significantly to the BNF process (Philippot *et al.* 2013; Brooker *et al.* 2015). Rhizosphere signaling, including root exudates, has been shown to mediate interspecific facilitation by enhancing rhizobia nodulation efficiency (Li *et al.* 2016). Furthermore, intercropped plant-derived metabolites, e.g., flavonoids and coumarins, have been shown to promote the growth of both free-living and symbiotic diazotrophs, thereby enhancing nitrogen fixation activity (Qiao *et al.* 2024). Despite these insights, research in this area has predominantly focused on symbiotic rhizobia in nodules, leaving free-living diazotrophs, which are critical for nitrogen fixation in non-legume crops, largely unexplored in intercropping systems.

Legume/non-legume intercropping enhances BNF by fostering synergistic interactions between plant species and promoting diverse microbial communities (Hu *et al.* 2021; Dang *et al.* 2024; Zhang *et al*. 2024). Free-living diazotrophs exhibit various survival strategies for adapting to diverse environments, occupy a broad spectrum of niches, and contribute to nitrogen fixation across various conditions and habitats. Based on their ecological adaptations, these microorganisms can be categorized as habitat generalists, opportunists, or specialists (Fridley *et al.* 2007). Generalists thrive across a wide range of conditions, while opportunists exploit particularly favorable conditions, and specialists are highly adapted to specific environments (Chen *et al.* 2021; Dehling *et al.* 2021). These strategic differences influence diazotrophic activity and their contributions to ecosystem functioning, which are also shaped by factors such as niche breadth and the co-occurrence patterns of diazotrophs that affect ecosystem services including BNF (Liu *et al.* 2022). The roles of microorganisms in BNF have been increasingly examined through bioinformatics and ecological modeling, which has facilitated the identification of keystone taxa that drive nitrogen fixation in various ecosystems (Hu *et al.* 2024; Jiang *et al.* 2024). However, knowledge gaps remain regarding how key microbial taxa, especially those with varying niche breadths such as generalists, opportunists, and specialists, contribute to BNF in intercropping systems. A broader understanding of these mechanisms is essential for elucidating the significance of diazotrophs and applying this knowledge to enhance sustainable agricultural practices.

The peanut/cotton intercropping system is a hallmark of sustainable agricultural practices in China, particularly in the Huang-Huai-Hai plain (Chi *et al.* 2019, 2023; Qiu *et al.* 2023; Lu *et al.* 2024). By intercropping a leguminous crop with a non-leguminous crop, this system enhances nitrogen availability through BNF while also influencing soil microbial dynamics. Given that free-living diazotrophs facilitate nitrogen fixation independently of host legumes and respond dynamically to environmental factors, their contribution to BNF warrants further investigation, particularly how soil heterogeneity selects for keystone diazotrophs with distinct niche strategies in the peanut/cotton intercropping system.

In this study, we examined the effects of peanut/cotton intercropping on soil BNF, with a particular focus on the roles of keystone diazotrophic taxa in the varying ecotypes and their differentiation between rhizosphere and bulk soils. We hypothesized that intercropping and rhizosphere environments will maintain higher BNF potentials and more distinct diazotrophic communities than monoculture and bulk soils, respectively, and that these environments selectively enrich key diazotrophs, particularly opportunistic species that contribute substantially to nitrogen fixation. To test these hypotheses, we conducted an experiment based on a 6-year field study that compared peanut/cotton intercropping and monoculture systems. The objectives of our study were to (1) quantify and evaluate the differences in BNF between intercropping and monoculture systems, as well as between rhizosphere and bulk soils, and (2) determine the effects of soil properties, diazotrophic diversity, assembly processes, and co-occurrence networks on BNF potential, in addition to identifying keystone taxa and their specific habitat ecotypes in a peanut/cotton intercropping system.

2. Materials and methods

2.1. Experimental design

The field experiment to examine the peanut (*Arachis hypogaea* L.)/cotton (*Gossypium hirsutum* L.) intercropping system commenced in 2017 at the Anyang Experimental Station, northern China (36°06′N, 114°21′E). The initial soil characteristics and climatic conditions of the experimental site are detailed in our previous publication (Zhang *et al.* 2025). A randomized complete block design with three replicates was employed, encompassing four treatments: monocropping of peanuts (MP), monocropping of cotton (MC), intercropping of peanuts (IP), and intercropping of cotton (IC). In both monocropping and intercropping plots, peanuts were planted with 40 cm row spacing and 20 cm plant spacing, while cotton was spaced 80 cm between rows with 90,000 plants ha^{-1}. In the intercropping system, strips of two rows of peanuts and two rows of cotton were established, with 60 cm spacing between the two crops. Nitrogen fertilizer was applied at 240 kg N ha^{-1} (urea, 46%) during planting and the early flowering stage of cotton, with the total amount divided equally between the two applications. Phosphate fertilizer (120 kg P_2O_5 ha^{-1} as triple superphosphate) and potassium fertilizer (120 kg K_2O ha^{-1} as potassium sulfate) were applied as base fertilizers before planting. Other field management practices adhered to local agricultural protocols.

2.2. Soil sampling and physicochemical analyses

In August 2022, soil samples were collected during the podding stage of peanuts and the bolling stage of cotton. The five-point approach was used to collect bulk soil samples at a depth of 0–20 cm. Six random plants were selected from each plot, and rhizosphere soil was carefully scraped from the roots. The six samples from each plot were combined to form a composite sample, yielding a total of 24 soil samples (4 treatments×2 niches×3 replicates). The fresh soil samples were homogenized, passed through a 2-mm sieve and divided into three subsamples: one stored at 4°C for labile property analysis, another air-dried for stable physio-biochemical analyses, and the third stored at –80°C for molecular assays. Soil properties (Appendix A), including pH, ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), microbial biomass carbon (MBC), nitrogen (MBN), dissolved organic carbon (DOC), soil organic carbon (SOC), total nitrogen (TN), available phosphorus (AP), and available potassium (AK), were measured for each sample as detailed in Appendix B.

2.3. BNF potential analyses

Nitrogen fixation rate The BNF rates of the soil samples were determined using the $^{15}N_2$ isotope dilution technique (Fan *et al.* 2019; Zhou *et al.* 2023). Briefly, 2 g of fresh soil was placed in a 12 mL sealed headspace vial. The vials were sealed with rubber septa and screw caps, followed by evacuation to remove the ambient air. Synthetic gas composed of 20% (v/v) O_2 and 80% (v/v) $^{15}N_2$ (99 atom% ^{15}N) was immediately introduced into the vials, while unlabeled N_2 was used in the control vials. All vials were incubated in the dark at 28°C for 22 days. After incubation, the soil samples were freeze-dried and ground to a fine powder. The ^{15}N atom% in the soil samples was then analyzed using an elemental analyzer coupled to a stable isotope mass spectrometer (Vario Isotope Cube-Isoprime, Elementar). The BNF rates (μg kg^{-1} d^{-1}) were calculated using the following equation:

$$\text{Nitrogen fixation rate}=(^{15}\text{N atom\%Sample}-^{15}\text{N atom\%Control})\times\text{TN}\times\frac{1}{t}$$

where ^{15}N atom%Sample and ^{15}N atom%Control represent the ^{15}N atom excess (%) in labeled and unlabeled soil samples, respectively; TN is the total nitrogen content after incubation (μg kg^{-1}), and t is the incubation period (d).

Nitrogenase activity The acetylene reduction assay (ARA) method of Hu *et al.* (2023) was used to quantify the nitrogenase activity in soil samples. A fresh 10 g portion of soil was weighed and placed into a 100 mL serum vial, which was then sealed tightly with a rubber stopper. Using a gas-tight syringe, 10 mL of air was extracted from the flask, followed by the injection of 10 mL of acetylene gas (C_2H_2). Following a 24-h incubation at 28°C, the gas phase was analyzed using a gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). Data are reported as mean values from four replicate cultures. Nitrogenase activity (nmol g^{-1} h^{-1}) was calculated using the following formula:

$$\text{ARA}=K\times\frac{T+X}{T}\times\frac{Y}{760}\times\frac{Z}{22.4\times10^{-6}}\times\frac{1}{W}\times\frac{1}{t}$$

where K is the ratio of ethylene peak height to acetylene peak height, T is the absolute temperature (K), X is the ambient temperature (°C) during the measurement, Y is the atmospheric pressure (mmHg) at the time of measurement, Z is the volume of acetylene injected (mL), W is the weight of the soil sample (g), and t is the reaction time after acetylene injection (h).

Quantitative polymerase chain reaction (qPCR) for the *nifH* gene DNA was extracted from 0.5 g of soil using the FastDNA™ Spin Kit for Soil (MP Biomedicals, USA), following the manufacturer's protocol. The quality of the extracted DNA was determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, USA).

The quantification of the *nifH* gene, which is indicative of diazotrophic community abundance, was performed on a Bio-Rad fluorescence detection system (Bio-Rad Laboratories, USA). Amplification was carried out with the primer pair PolF (5′-TGGGAYCCSAARGCBGACTC-3′) and PolR (5′-ATSGCCATCATYTCRCCGGA-3′), as previously described by Poly *et al.* (2001). The qPCR reaction mixture comprised 10 μL of 2× SYBR Premix *Ex Taq* Perfect Real Time (TaKaRa Biomedical Technology Co., Ltd., Japan), 0.5 μL of each primer (10 μmol L^{-1}), 1.0 μL of DNA template, and 8.0 μL of ddH_2O. The thermal cycling conditions were as follows: 30 s at 95°C for initial denaturation, 40 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C with fluorescence acquisition. A melting curve analysis was performed using a linear PCR product and a 10-fold serial dilution of plasmid harboring the *nifH* gene. The amplification efficiency for the *nifH* gene was >98%.

2.4. *nifH* gene amplicon sequencing and analysis

After the qPCR analysis, the same extracted DNA was used for further amplification and sequencing of the *nifH* gene. The *nifH* gene was amplified using the primer pair PolF/PolR in a 25 μL reaction volume consisting of KAPA2G Robust HotStart ReadyMix PCR (KAPA Biosystems, Wilmington, USA), 1 μL of each primer (5 μmol L^{-1}), 5 μL of template DNA, and nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. PCR amplifications were performed in triplicate, and the resulting products were pooled. The purified amplicons were then used to prepare sequencing libraries according to the manufacturer's protocol, using the NEB Next Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). The quality of the libraries was verified, and they were subsequently sequenced on the Illumina MiSeq PE300 platform (Allwegene Company, Beijing, China) using paired-end sequencing.

The raw sequencing data were first processed using the software FLASH (v. 1.2.11) to merge the paired-end reads (Magoč and Salzberg 2011). Barcodes, primers, and low-quality reads were trimmed and filtered using QIIME 2 (https://qiime2.org) with the DADA2 plugin to remove noise, correct errors, and eliminate chimeric sequences. This process resulted in high-quality sequences that were subsequently denoised to generate amplicon sequence variants (ASVs).

Taxonomic classification of the ASVs was performed using the FunGene database (http://fungene.cme.msu.edu/) through the Ribosomal Database Project naïve Bayesian classifier. To equalize sequencing depth and ensure a robust comparative analysis, the sequences were rarefied to a uniform depth of 4,433 reads per sample. The raw sequencing data were submitted to the NCBI Sequence Read Archive under accession number PRJNA1083480.

2.5. Community assembly and co-occurrence network analysis

The neutral community model (NCM) was applied to assess the role of stochastic processes in the assembly of diazotrophic communities in both rhizosphere and bulk soils in the monocropping and intercropping systems (Chen *et al.* 2019). This model estimates species dispersal and establishment probabilities by evaluating two key parameters: m (immigration rate) and R^2 (goodness of fit). To further distinguish between deterministic and stochastic processes, the beta-nearest taxon index (β-NTI) was calculated using a null model in the "picante" R package, which provides insights into phylogenetic turnover (Stegen *et al.* 2013). A β-NTI value above 2 or below –2 indicates a dominance of deterministic processes (heterogeneous or homogeneous selection, respectively), while values between –2 and 2 suggest a stronger influence of stochastic processes. To partition these stochastic processes, the Raup-Crick index based on Bray-Curtis dissimilarity (RC_{Bray}) was calculated, which indicates either dispersal limitation ($RC_{Bray}>0.95$), homogeneous dispersal ($RC_{Bray}<–0.95$), or undominated processes such as ecological drift ($|RC_{Bray}|<0.95$).

Diazotrophic co-occurrence networks were constructed using the ASVs present in over 75% of the samples. Pairwise correlations among these ASVs were calculated with Pearson's correlation ($r>0.90$, $P<0.01$), and reliable correlations were identified using Random Matrix Theory (RMT) (Feng *et al.* 2022). Network topological features, including the numbers of nodes and edges, average degree, clustering coefficient, modularity, average path length, geodesic efficiency, and connectedness, were calculated using the R packages "ggClusterNet" and "igraph." (Wen *et al.* 2022). Keystone nodes were identified based on within-module connectivity (Zi) and among-module connectivity (Pi), with keystone species classified as network hubs (Zi>2.5, Pi>0.62), module hubs (Zi>2.5, Pi<0.62), or connectors (Zi<2.5, Pi>0.62) (Shi *et al.* 2016).

2.6. Determination of niche breadth and habitat classification

The ecological niche breadths of diazotrophic ASVs were quantified using the Levins methods, implemented through the R package "spaa". To assess the significance of these niche breadth values, the occurrence frequencies of ASVs across habitats were randomized 1,000 times using the "EcolUtils" package, generating a null distribution (Salazar 2022). Species within both the entire diazotrophic community and co-occurrence networks were then categorized into three ecological strategies based on their niche breadth relative to this distribution: generalists (niche breadth exceeding the 95% confidence interval, indicating broad ecological amplitude), specialists (below the lower 95% interval, indicating high ecological specialization), and opportunists (within the interval, exhibiting intermediate characteristics) (Pandit *et al.* 2009; Chen *et al.* 2021).

2.7. Statistical analyses

Statistical analyses were performed using R v. 4.2.0. Data normality was assessed with the Shapiro-Wilk test, and log or Hellinger transformations were applied as needed to stabilize the variance. Analysis of variance (ANOVA) followed by Tukey's HSD or the Tukey-Kramer test was used to compare soil physicochemical properties, BNF potentials, and diazotroph relative abundance among the treatments. The Wilcoxon rank-sum test in the "edgeR" package, with FDR-adjusted P-values (<0.05), was used for pairwise comparisons of diazotrophic community composition. Non-metric multidimensional scaling (NMDS) and permutational ANOVA, using Bray-Curtis distance matrices, were conducted with the "vegan" package to visualize and test community composition differences. Differences in the β-NTI index and niche breadth among treatments were analyzed with Welch's t-test. Random forest (RF) modeling and Pearson's correlation analysis, using the "randomForest," "rfPermute," and "psych" packages, were employed to determine the influence of soil parameters on BNF potentials. Partial least squares path modeling (PLS-PM) was implemented using the "plspm" R package to further explore the direct and indirect effects of soil factors on BNF potentials. An *a priori* model was established based on ecological theory and the observed variables identified from the RF and Pearson correlation analyses (Appendix C). Specifically, soil properties comprised 11 measured physicochemical indices as manifest variables (Appendix A); community diversity was represented by the NMDS1 axis derived from Bray-Curtis dissimilarities; community assembly was quantified by integrating the β-NTI and RC_{Bray} indices; and the relative abundances of keystone taxa categorized into varying ecotypes were included as separate input matrices. The model was implemented using the "plspm" package and iteratively refined by removing non-significant paths (bootstrap-adjusted $P<0.05$), and model validity was assessed using the goodness-of-fit index (GOF) index, with GOF>0.6 considered acceptable.

3. Results

3.1. BNF potentials

The BNF potentials, including N fixation rate, nitrogenase activity, and *nifH* gene abundance, differed significantly between rhizosphere and bulk soils across the cropping systems. On average, the BNF potentials ranged from 34.4 to 113.9 μg N kg^{-1} soil d^{-1} for N fixation rate, 793.3 to 1,099.6 nmol g^{-1} soil h^{-1} for nitrogenase activity, and 7.10^5 to 1.09^7 copies g^{-1} soil for *nifH* gene abundance (Fig. 1). Rhizosphere soils exhibited higher BNF potentials than bulk soils, with increases of 71.8% in N fixation rate, 7.8% in nitrogenase activity, and 125.5% in *nifH* gene abundance ($P<0.05$; Appendix D). Similarly, intercropping treatments

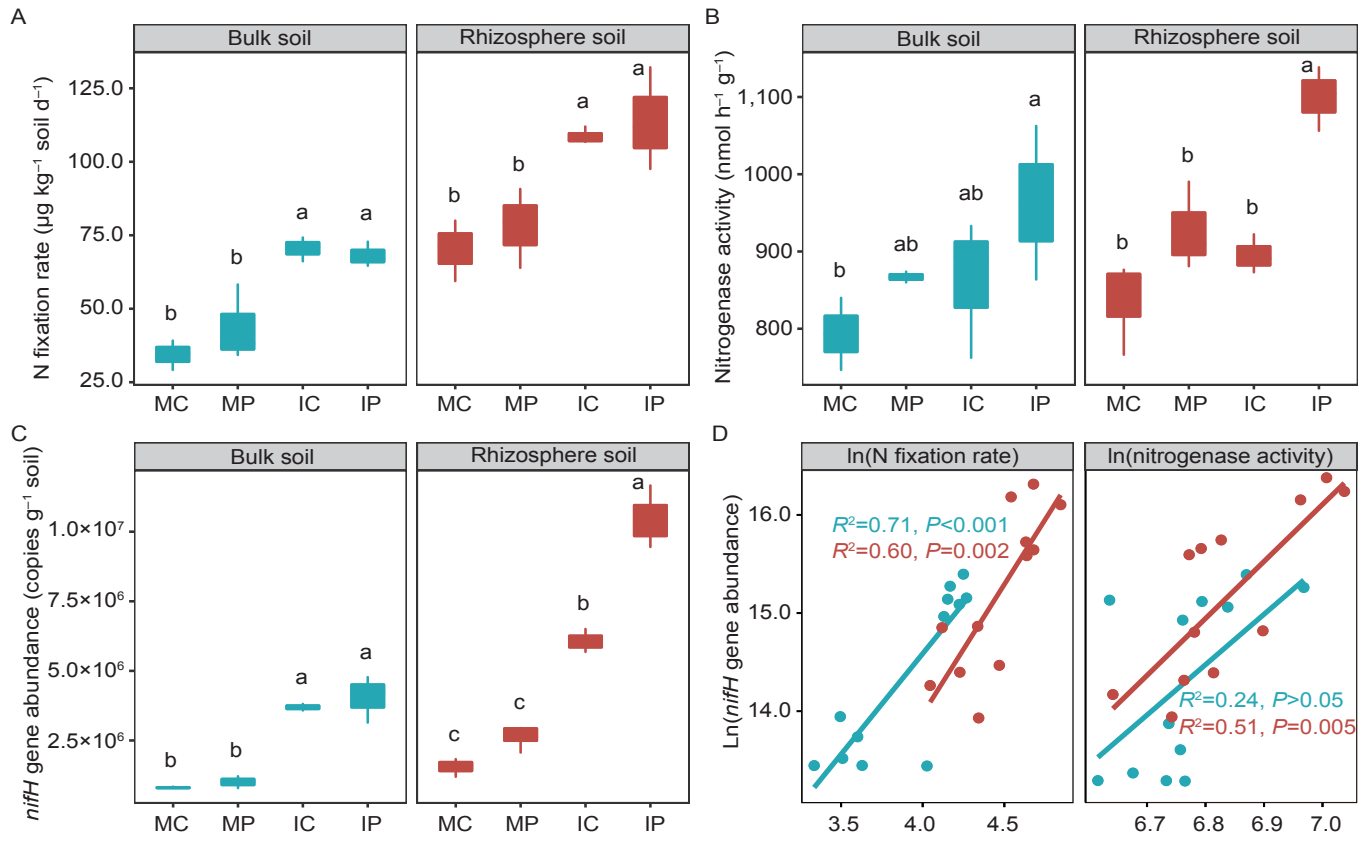


Fig. 1 Biological nitrogen fixation (BNF) potentials in rhizosphere and bulk soils from peanut/cotton monoculture and intercropping systems. A, soil N fixation rates. B, nitrogenase activity. C, *nifH* gene copy number. D, linear correlations corresponding to peanut/cotton monoculture and intercropping. MC, monoculture of cotton; MP, monoculture of peanut; IC, intercropping of cotton; IP, intercropping of peanut. Vertical bars denote standard errors of the mean values of each index (n=3). Different lowercase letters beside the vertical bars indicate significant differences among treatments at P<0.05 (least significant differences test). The regressions in rhizosphere and bulk soils are represented in red and blue, respectively.

outperformed monoculture treatments, showing increases of 58.8% in N fixation rate, 11.6% in nitrogenase activity, and 323.0% in *nifH* gene abundance (P<0.05). Specifically, the BNF potentials were the highest in IP, followed by the IC, MP, and MC systems; however, the IC system exhibited greater increases in N fixation rates (48.1% for IP *vs*. MP; 71.3% for IC *vs*. MC) and *nifH* gene abundance (303.7% for IP *vs*. MP; 355.8% for IC *vs*. MC) than the IP system in both rhizosphere and bulk soils. Strong linear correlations were observed between *nifH* gene abundance, nitrogen fixation rate, and nitrogenase activity in both soil types (Fig. 1-D).

3.2. Composition and diversity of diazotrophic communities

The composition and diversity of soil diazotroph communities differed significantly across treatments (Fig. 2). Among the 947 identified ASVs, Proteobacteria dominated (65.33–89.00%), followed by Cyanobacteria (5.82–19.50%), Firmicutes (4.12–12.87%), Verrucomicrobia (1.20–2.11%), and Chloroflexi (0.01–0.12%). At the class level, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Bacilli, Nostocophyceae, and Cyanophyceae collectively accounted for 97.98% of the diazotrophic community (Fig. 2-A and B; Appendix E). Diazotrophic communities exhibited significant separation between the monoculture and intercropping systems, as well as between the rhizosphere and bulk soils (Fig. 2-C). In rhizosphere soil, 74 ASVs were depleted and 120 ASVs were enriched, compared with bulk soil, and the cotton/peanut intercropping systems showed totals of 39 depleted and 56 enriched ASVs, compared with monocultures (Fig. 2-D). Most of the species in the phylum Proteobacteria were enriched in the intercropping systems, whereas Alphaproteobacteria were more abundant in monocultures. Conversely, Cyanobacteria and Firmicutes were generally depleted during intercropping, except for a cluster of Nostocophyceae that was enriched (Fig. 2-E). In the rhizosphere, compared with the bulk soil, the phylum Proteobacteria was more enriched, whereas the phyla Cyanobacteria and Firmicutes were less abundant (P<0.05; Fig. 2-E).

3.3. Assembly processes of diazotrophic communities

The assembly mechanisms of diazotrophic communities in rhizosphere and bulk soils under peanut/cotton monoculture and intercropping systems were analyzed using the NCM and phylogenetic null models (Fig. 3). The NCM analysis indicated a moderate stochastic influence that explained an average of 44.6% of the community variation (Fig. 3-A–D). Consistently, the phylogenetic null model indicated that deterministic processes, particularly heterogeneous selection,

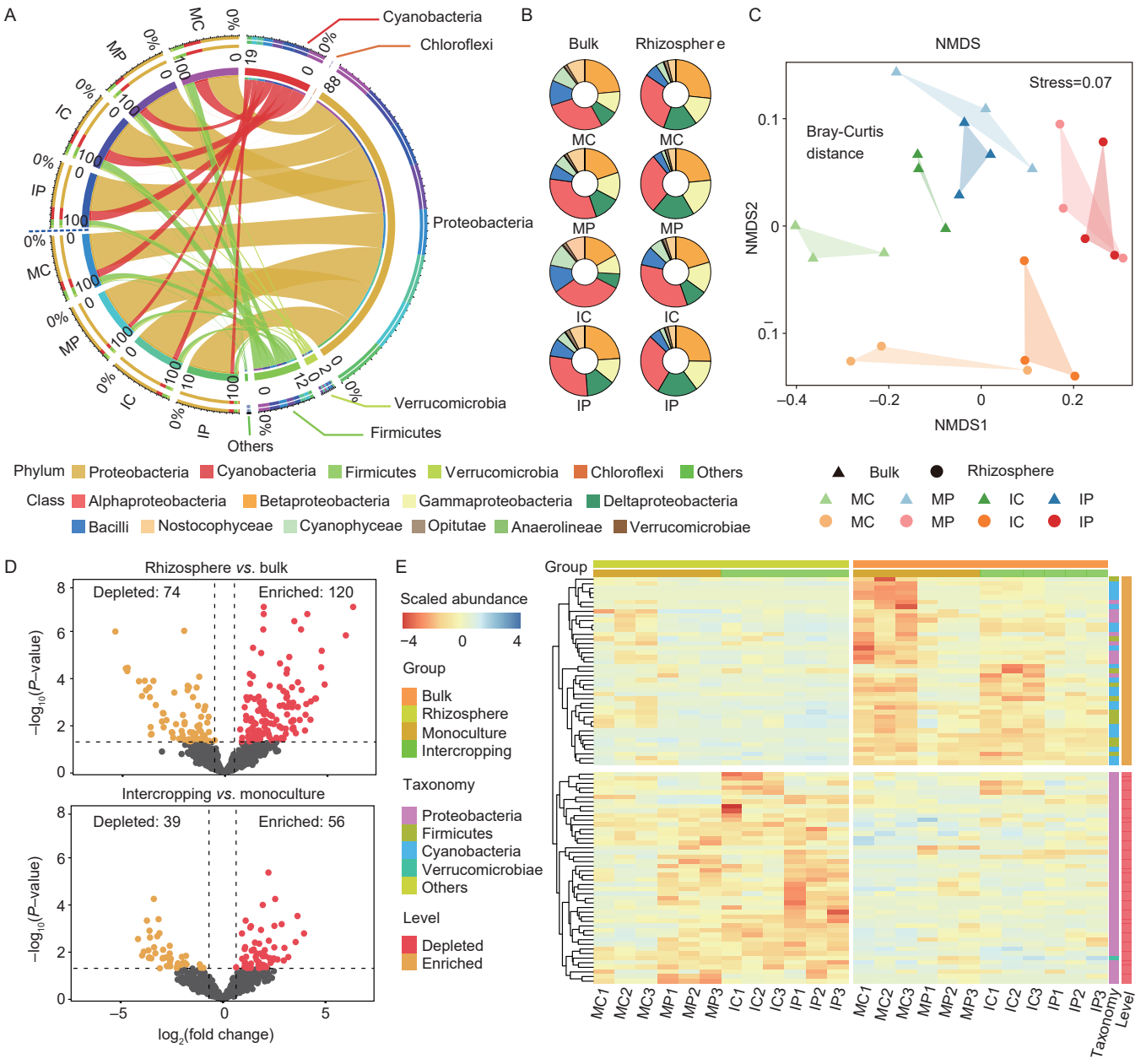


Fig. 2 Compositions and structures of the soil diazotrophic communities in rhizosphere and bulk soils from peanut/cotton monoculture and intercropping systems. A, phylum-level relative abundances of the diazotrophic community. B, class-level proportions of the diazotrophic community. C, nonmetric multidimensional scaling (NMDS) analysis of the soil diazotrophic communities. D and E, volcano and heatmap plots illustrating abundant differential ASVs (>0.01% abundance) among distinct groups, as determined by a Wilcoxon and Tukey-Kramer test, respectively, at a 95% confidence interval (P<0.05; n=3). The left semicircles in (A) indicate the distribution of diazotrophs in the treatments, where the bulk and rhizosphere treatments were divided by the dashed lines into upper and lower parts in the circos diagram, and the right semicircles indicate the relative abundances and proportions of diazotrophic phyla. The lengths of the bars on the inner and outer rings represent the relative abundance (percentage) and distribution proportion of the diazotrophs. The black dotted threshold lines in (D) represent absolute fold change=2 or P=0.05. MC, monoculture of cotton; MP, monoculture of peanut; IC, intercropping of cotton; IP, intercropping of peanut.

predominantly shaped the diazotrophic communities, particularly in rhizosphere soils (91.9%) and intercropping systems (86.3%). In contrast, stochastic processes had comparably stronger impacts on the communities in bulk soils and monoculture systems, accounting for approximately 30% of the variation, and were influenced by ecological drift (12.1%), homogenizing dispersal (9.1%), and dispersal limitation (6.8%). This was further supported by the β-NTI data, where distributions in rhizosphere soils and intercropping systems generally remained within the threshold of −2 to 2 (Fig. 3-E and F).

3.4. Co-occurrence networks, keystone taxa, and contributions of the various ecotypes

The co-occurrence networks of diazotrophic communities

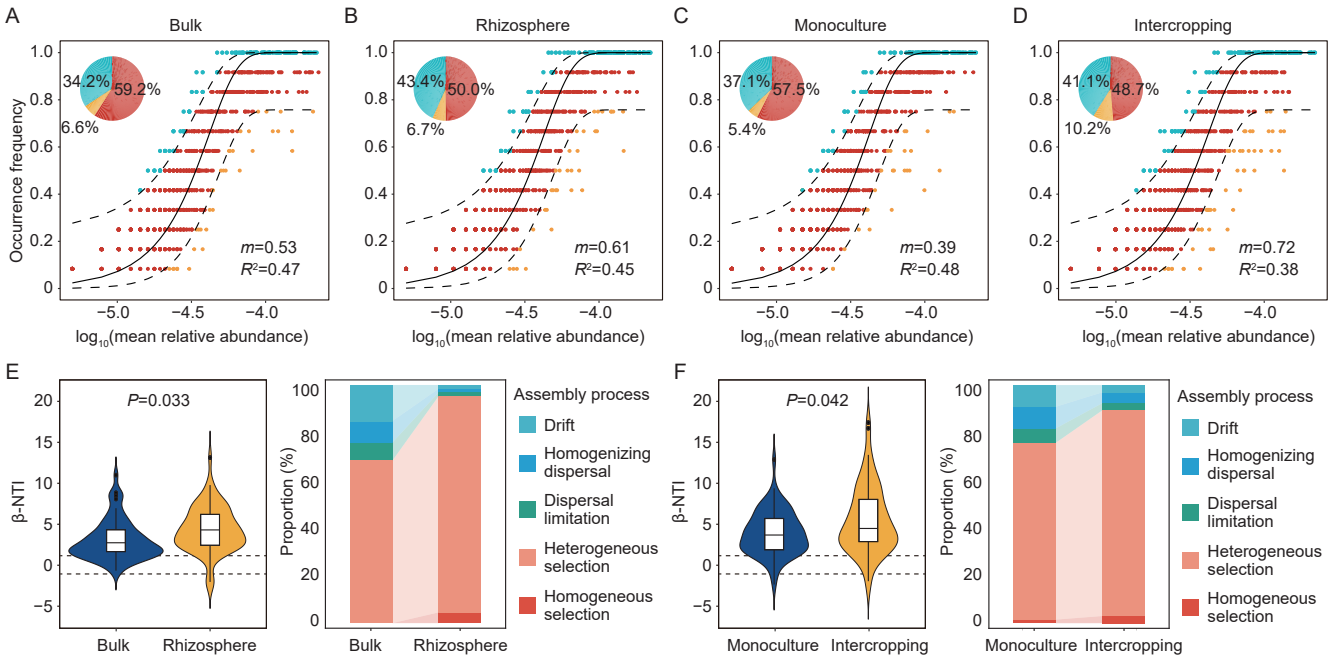


Fig. 3 Diazotrophic community assembly processes in rhizosphere and bulk soils from peanut/cotton monoculture and intercropping systems. A, bulk soil fit to neutral community model (NCM). B, rhizosphere soil fit to NCM. C, monoculture system fit to NCM. D. intercropping system to NCM. E and F, β-nearest taxon index (β-NTI) based on the phylogenetic null model. The ASVs represented by blue dots showed a higher frequency of occurrence than the model predicts, and those represented by orange dots were the opposite. The ASVs predicted by the 95% confidence interval of the model are shown as red dots, *m* is the migration rate between populations, and R^2 indicates the fit to this model. βNTI was determined by Welch's *t*-test at a 95% confidence interval (P<0.05, n=15); |β-NTI|<2 indicated a stochastic process and |β-NTI|>2 indicated a deterministic process.

were analyzed to explore distinct interactions among the treatments (Fig. 4-A–D). Compared with bulk soils and monocultures, networks in rhizosphere soils and intercropping systems exhibited more nodes and edges; higher average degree, geodesic efficiency, and connectedness; and shorter average path distances, indicating a more complex and tightly knit community structure (Table 1). In addition, the rhizosphere and intercropping networks had higher average clustering coefficients and modularity, with 14.4–32.9% reductions in negative associations, suggesting larger, more positively interacting clusters of diazotrophs within ecological modules. This was evidenced by the nearly doubling of keystone taxa, confirmed by module hubs, connectors, and network hubs within the co-occurrence networks, under the rhizosphere and intercropping patterns, compared with the bulk soils or monoculture systems (Fig. 4-E–H).

Diazotrophs in bulk soils and intercropping systems had significantly wider niche breadths than those in rhizosphere soils and monocultures (Fig. 4-I–J and L–M; Appendix F). Across all communities and within co-occurrence networks, opportunists contributed the most (78.8–85.9%), followed by specialists (10.2–18.5%), and generalists (1.38–3.80%). The contribution of specialists increased by 27.5% in rhizosphere soils, compared with bulk soils, and the contribution of generalists in intercropping systems was nearly three times that in monocultures.

Even though opportunists were the key players across treatments, their classifications varied. In bulk soils, keystone opportunists mainly belonged to the classes Cyanophyceae (*Trichormus* genus) and Bacilli (*Bacillus* genus), whereas in the rhizosphere and intercropping systems, they predominantly belonged to Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria, including the genera *Steroidobacter*, *Azoarcus*, *Azohydromonas*, and *Geobacter* (Fig. 4-K and N). More importantly, a group of Alphaproteobacteria, including the genus *Azospirillum*, was identified as keystone specialists in rhizosphere soils; a group of Gammaproteobacteria, including the genera *Pseudomonas* and *Azotobacter*, was identified as keystone generalists in the intercropping system; and the relative abundances of most of these specialists or generalists exceeded 0.1% (Appendices G and H).

3.5. Associations between soil BNF, diazotrophic community, and soil properties

The influence of soil properties and diazotrophic communities on BNF potentials differed between bulk and rhizosphere soils as well as among the monoculture and intercropping systems (Fig. 5; Appendix I).

In bulk soils, MBC, NH_4^+-N, AK, and keystone opportunists were the primary positive factors influencing BNF potentials (Fig. 5-A). The PLS-PM results confirmed a strong impact of soil properties (direct coefficient=0.51, indirect coefficient=0.13) and a moderate influence of keystone opportunists (direct coefficient=0.23, indirect coefficient=−0.09; Fig. 5-E). Among the keystone opportunists for bulk soils, the genera *Nostoc* (class Nostocophyceae, regression weight=0.479) and *Bacillus*

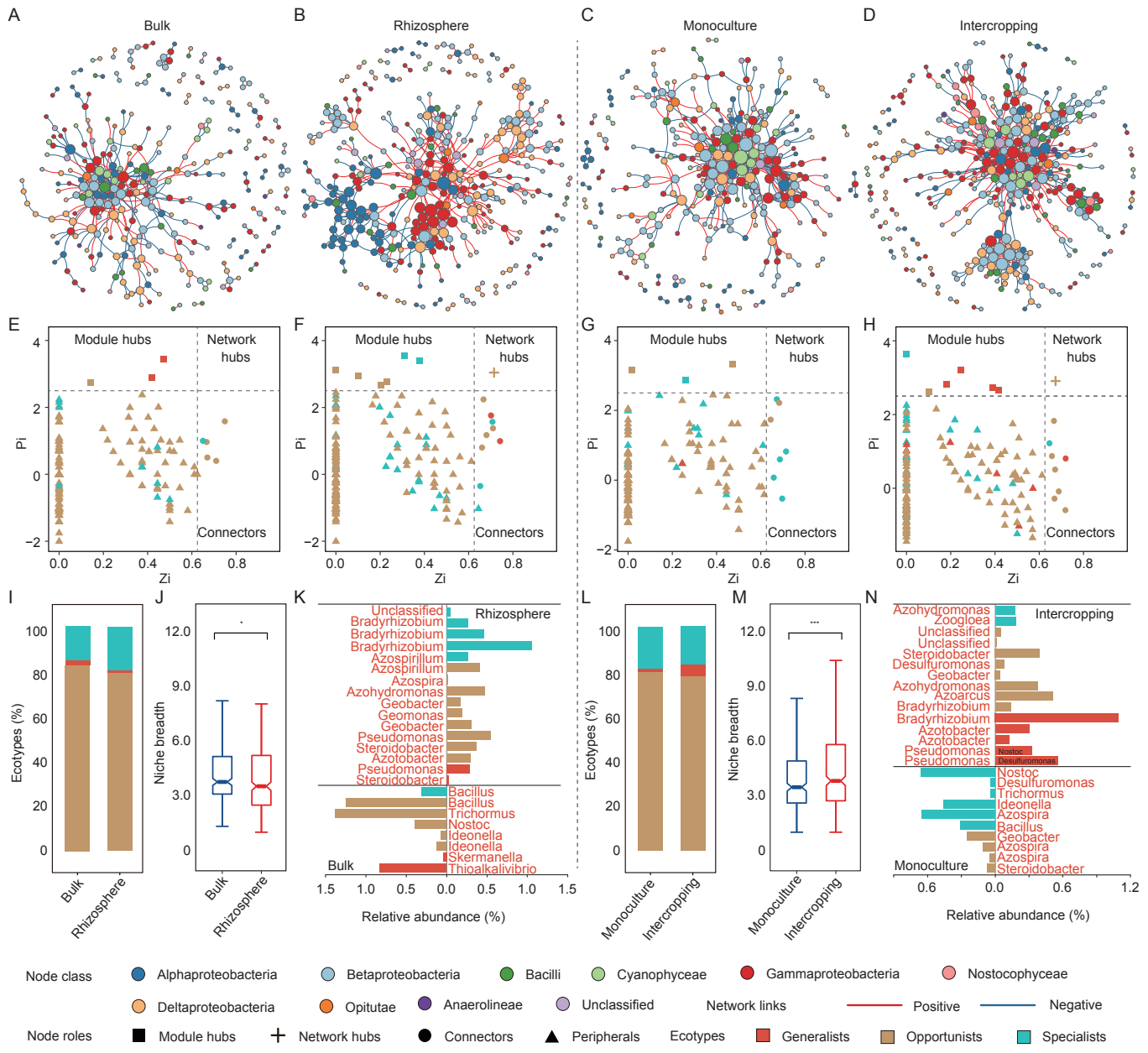


Fig. 4 Co-occurrence networks and keystone species of diazotrophs in rhizosphere and bulk soils from peanut/cotton monoculture and intercropping systems. A, bulk soils. B, rhizosphere soils. C, monoculture pattern. D, intercropping pattern. The size of each node in the networks is proportional to the number of degrees. E–H, keystone species in each network identified by within-module connectivity (Zi) and among-module connectivity (Pi). The nodes were categorized and shaped at the following levels: module hubs (Zi>2.5 and Pi<0.62), connectors (Zi<2.5 and Pi>0.62), network hubs (Zi>2.5 and Pi>0.62), and normal ASVs named peripherals (Zi<2.5 and Pi<0.62). The nodes are colored at the ecotype level and classified as habitat generalists, opportunists, and specialists. I and L, relative contributions of distinct habitat ecotypes in each network. J and M, niche breadth of the diazotrophs in each network. K and N, relative abundances of keystone diazotrophs at the genus level. *, P<0.05; ***, P<0.001.

(class Bacilli, regression weight=0.432) emerged as the dominant positive opportunistic contributors (Appendices H and I). In rhizosphere soils, key abiotic factors like NO_3^--N, MBN, pH, and MBC, along with biotic factors including beta diversity, keystone specialists, and opportunists, significantly influenced BNF (Fig. 5-B). PLS-PM indicated a strong positive direct effect of these key soil properties (direct coefficient=0.67) and keystone opportunists (direct coefficient=0.30) on BNF, with keystone specialists having a negative influence (direct coefficient=−0.17; Fig. 5-F; Table 2). Opportunistic keystone taxa such as *Pseudomonas* (class Gammaproteobacteria, regression weight=0.398) and *Steroidobacter* (class Gammaproteobacteria, regression weight=0.228) were positive contributors to BNF, whereas specialists such as *Bradyrhizobium* (class Alphaproteobacteria, regression weights=−0.270 to −0.291) acted as negative influencers in the rhizosphere (Appendices H and I).

In the monoculture systems, the key factors affecting BNF included soil pH, DOC, MBC, MBN, keystone specialists,

Table 1 Topological properties of co-occurrence networks of diazotrophic communities

Network index	Bulk	Rhizosphere	Monoculture	Intercropping
Number of nodes	287	363	304	374
Number of edges (positive/negative)	374 (42.5%/57.4%)	607 (75.4%/24.5%)	532 (42.1%/57.9%)	714 (56.5%/43.5%)
Average degree	2.519	3.344	3.5	3.818
Average clustering coefficient	0.096	0.173	0.125	0.145
Modularity	0.726	0.774	0.623	0.695
Average path distance	5.529	5.301	4.821	4.724
Geodesic efficiency	0.219	0.234	0.245	0.255
Connectedness	0.394	0.579	0.568	0.631

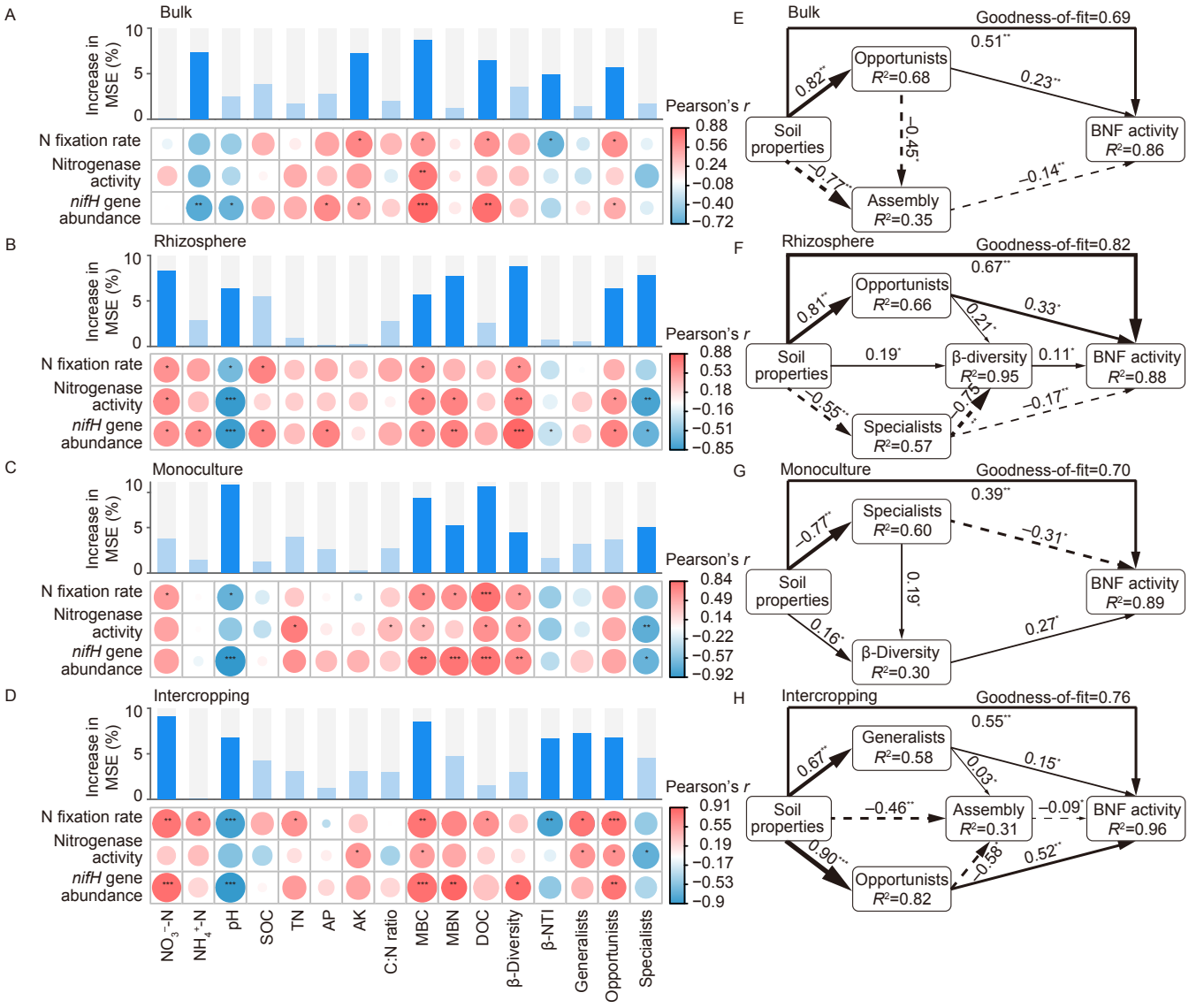


Fig. 5 Relationships between soil properties and diazotrophic community, and their potential contributions to soil biological nitrogen fixation (BNF) potentials in rhizosphere and bulk soils of peanut/cotton monoculture and intercropping systems. A–D, correlations between soil properties, diazotrophic community diversity, assembly processes, and keystone taxa categorized by ecotypes, along with their predictor importance (calculated *via* the random forest model) for BNF potentials. E–H, partial least squares-path model (PLS-PM) illustrating the direct and indirect effects of important predictors identified by the random forest model on soil BNF potentials. Path coefficients are indicated next to the arrows, with line thickness proportional to coefficient values. Asterisks denote statistical significance at *, $P<0.05$, **, $P<0.01$; ***, $P<0.001$.

and beta diversity (Fig. 5-C). The PLS-PM model showed moderate effects of soil properties (direct coefficient=0.39) and keystone specialists (direct coefficient=−0.31) on BNF (Fig. 5-G; Table 2). Keystone specialists, such as the genera *Azospira* (class Betaproteobacteria, regression weight=−0.265) and *Desulfuromonas* (class

Table 2 Direct, indirect, and total effects of the soil parameters on BNF potentials in the PLS-PM analysis[1)]

	Effects	Soil properties	Generalists	Opportunists	Specialists	β-Diversity	Assembly
Bulk	Direct	0.51	–	0.23	–	–	–0.14
	Indirect	0.13	–	–0.09	–	–	0
	Total	0.64	–	0.14	–	–	–0.14
Rhizosphere	Direct	0.67	–	0.30	–0.17	0.11	–
	Indirect	0.05	–	0.02	–0.03	0	–
	Total	0.72	–	0.32	–0.20	0.11	–
Monoculture	Direct	0.39	–	–	–0.31	0.27	–
	Indirect	0.09	–	–	0.03	0	–
	Total	0.48	–	–	–0.28	0.27	–
Intercropping	Direct	0.55	0.15	0.52	–	–	–0.09
	Indirect	0.10	–0.02	0.04	–	–	0
	Total	0.65	0.13	0.56	–	–	–0.09

[1)] BNF, biological nitrogen fixation; PLS-PM, partial least-squares path model.

Deltaproteobacteria, regression weight=−0.211), were significant negative contributors to BNF potentials (Appendices H and I). In intercropping systems, the most significant predictors of BNF potentials were NO_3^--N, MBC, soil pH, keystone generalists, opportunists, and diazotrophic community assembly (Fig. 5-D). PLS-PM revealed that keystone ecotypes, particularly opportunists (coefficient=0.52) and generalists (coefficient=0.15), had a strong positive effect on BNF, whereas the diazotrophic community assembly had a weaker and negative influence (coefficient=−0.09; Fig. 5-H; Table 2). Notable keystone ecotypes included opportunists such as *Azoarcus* (class Betaproteobacteria, regression weight=0.442) and *Azohydromonas* (class Betaproteobacteria, regression weight=0.338) as well as generalists such as *Pseudomonas* and *Azotobacter* (class Gammaproteobacteria, regression weights=0.067–0.397), which were associated with enhanced BNF in the intercropping systems (Appendices H and I).

4. Discussion

4.1. Enhanced BNF potentials in rhizosphere and intercropping soils

Our results revealed significant differences in BNF potentials between the rhizosphere and bulk soils, as well as among cropping systems. The consistently higher BNF potentials observed in rhizosphere than in bulk soils emphasize the role of this particular system as a hub for microbial activity and nitrogen fixation (Hu *et al.* 2021). This enhancement can be attributed to the greater availability of root exudates, such as carbohydrates, amino acids, and plant hormones, which provide essential substrates and promote chemical signaling in diazotrophic communities (Li *et al.* 2016; Zhang *et al.* 2024).

Intercropping systems significantly outperformed monocultures in promoting the BNF potential, consistent with the findings of a global analysis (Li *et al.* 2024). Intercropped cotton exhibited a greater increase in both N fixation rate and *nifH* gene copy numbers than intercropped peanut, which was unexpected given the well-documented role of legumes in enhancing BNF (Zhou *et al.* 2023; Qiao *et al.* 2024). This can be attributed to the synergistic effect of nitrogen transfer and microbial adaptation, which disproportionately benefits cotton as the non-legume in the intercropping system. Nitrogen transfer from legumes to non-legumes through root interactions significantly enhances nitrogen uptake and growth in non-leguminous crops, resulting in a more pronounced increase in BNF markers, such as the *nifH* gene (Jing *et al.* 2023). In addition, microbial communities associated with non-leguminous plants in intercropping systems may become more active or diverse, further enhancing the BNF potential. This is facilitated by the evolutionary adaptations of nitrogen-fixing bacteria, such as *Bradyrhizobium* strains, which can efficiently fix nitrogen in both legume and non-legume hosts (De Matos *et al.* 2021).

These findings underscore the potential of intercropping to significantly enhance BNF in legume-based systems and non-legume crops, such as cotton. The differences between intercropped peanuts and cotton illustrate the complexity of plant-microbe interactions in shaping BNF, which emphasizes the need to optimize intercropping strategies to improve soil nitrogen fertility. This complexity is discussed further in the following sections on microbial communities.

4.2. Shifts in the composition and assembly of the diazotrophic community in rhizosphere and intercropping soils

The compositions and structures of diazotrophic communities were significantly influenced by both ecological niche (rhizosphere *vs*. bulk soil) and cropping system (intercropping *vs*. monoculture). Proteobacteria dominated the diazotrophic communities, particularly within the rhizosphere, where their relative abundance was higher than in bulk soil. This dominance was accompanied by reduced abundances of Cyanobacteria and Firmicutes in the rhizosphere, which may be attributed to the metabolic versatility and ability of Proteobacteria to rapidly respond to the nutrient-rich conditions provided by root exudates. Alphaproteobacteria and Betaproteobacteria are known for thriving in environments with high concentrations of root-derived organic compounds, which are abundant in the rhizosphere (Philippot *et al.* 2013; Hu *et al.* 2023). The reduced presence of Cyanobacteria in the rhizosphere, compared with the bulk soil, may be due to their preference for open environments with lower nutrient concentrations, where their photosynthetic abilities can be fully utilized (Edwards *et al.* 2015).

The significant enrichment of Betaproteobacteria and

Gammaproteobacteria within diazotrophic communities in intercropping systems suggests that these taxa are particularly well-suited to the complex interactions and resource-partitioning characteristics of such systems. Betaproteobacteria, which can respond rapidly to fluctuating nutrient levels, thrive in the diverse and temporally variable environments created by intercropping (Solanki *et al.* 2020). Gammaproteobacteria, which include numerous effective nitrogen fixers, are likely to be supported by the specific root interactions prevalent in intercropped soils (Zhang *et al.* 2022). The observed reduction in Alphaproteobacteria, despite their important role in nitrogen fixation, indicates that this shift may reflect the competitive exclusion principle, where more adaptable or faster-growing taxa such as Betaproteobacteria and Gammaproteobacteria outcompete Alphaproteobacteria in these niche environments (Fierer *et al.* 2007; Philippot *et al.* 2024). Furthermore, the depletion of Cyanobacteria and Firmicutes, along with the enrichment of Nostocophyceae, shows that intercropping selectively favors diazotrophic groups with advantageous traits, such as symbiotic relationships and efficient nitrogen fixation in variable environmental contexts (Edwards *et al.* 2015).

The assembly of diazotrophic communities in this study was predominantly driven by deterministic factors, as shown by both the NCM and phylogenetic null model analyses. Heterogeneous selection was dominant in the rhizosphere and the intercropping systems. The rich organic matter and diverse chemical microenvironments of the rhizosphere create specific ecological niches that favor certain microbial taxa, thereby amplifying the effects of heterogeneous selection. This aligns with the findings of previous studies, which showed how rhizosphere conditions significantly influence microbial community structure, thus promoting the proliferation of taxa adapted to these environments (Berg and Smalla 2009). In intercropping systems, the selective colonization of specific microbial taxa likely reflects adaptations to the unique ecological niches created by plant-plant interactions and resource allocation (Brooker *et al.* 2015; Duchene *et al.* 2017). The diversity and functionality of rhizosphere microbial communities in intercropping systems are closely linked to the types and configurations of the plant species involved (Shu *et al.* 2024). In contrast, in bulk soil and monoculture systems, stochastic factors appear to be more prominent, and ecological drift, homogenizing dispersal, and dispersal limitation contribute approximately 30% of the variation in community assembly. This aligns with the “neutral theory” of ecology, which predicts that in environments lacking strong selective pressures, stochastic processes and historical contingencies of community assembly dominate the formation of microbial communities (Wang *et al.* 2021).

4.3. Roles of diazotrophic keystone taxa and habitat ecotypes in rhizosphere and intercropping soils

Co-occurrence network analysis revealed that the diazotrophic communities in rhizosphere soils and intercropping systems exhibited more complex and efficient interactions than those in bulk soils and monoculture systems. The greater numbers of nodes and edges and strengthened connectivity in these environments suggest more interconnected microbial communities, which support efficient nutrient cycling and functional redundancy. This complexity contributes to the resilience of microbial networks, and allows them to maintain ecosystem functions even under environmental disturbance (Luo *et al.* 2023). The elevated clustering coefficient, modularity, and keystone nodes (nearly twice the number of keystone taxa, including module hubs, connectors, and network hubs) indicated higher functional specialization within these communities, which reflects the distinct microhabitats created by plant roots and their exudates that selectively enrich specific diazotrophic taxa (Banerjee *et al.* 2018). These findings align with the notion that complex microbial networks are better equipped to sustain ecosystem functions under varying environmental conditions (Wahdan *et al.* 2023; Yang *et al.* 2023).

Diazotrophs in bulk soils and intercropping systems exhibited wider niche breadths than those in rhizosphere soils and monoculture systems. This suggests that diazotrophs in bulk soils and intercropping systems must adapt to a broader range of conditions, resulting in more generalist and opportunistic species (Liu *et al.* 2022; Jiang *et al.* 2024). Conversely, the narrower niche breadths in rhizosphere soils and monoculture systems reflect more specialized adaptation to stable niches with consistent resources. The higher proportion of specialists observed in the rhizosphere supports this concept, suggesting an advantage for taxa adapted to the specific conditions provided by plant roots (Nuccio *et al.* 2020). The nearly threefold increase in generalists in intercropping systems, compared with monocultures, underscores the greater ecological complexity and resource diversity in intercropping, which promotes a broader range of diazotrophic strategies and enhances functional diversity (Dehling *et al.* 2021; Hu *et al.* 2024).

The observed variations in the classification of keystone taxa across different soil treatments suggest that the ecological roles and adaptive strategies of diazotrophs are highly context-dependent (Shi *et al.* 2016; Nuccio *et al.* 2020; Xu *et al.* 2022). In bulk soils, the dominance of Cyanophyceae and Bacilli among opportunistic keystone taxa may be attributed to the wider and more variable environmental conditions typical of bulk soils, in which these taxa can exploit a wide range of resources and thrive under less competitive conditions. The high abundances of *Trichormus* and *Bacillus* in these environments underscore their capacity to rapidly colonize and utilize available niches, and they frequently serve as the pioneer species that initiate community assembly (Zhou *et al.* 2016). In monoculture systems, the predominance of specialists, such as *Azospira* and *Ideonella*, suggests that these taxa have evolved to efficiently exploit the limited range of available ecological niches, ensuring their survival and persistence in such stable environments (Wang *et al.* 2020). In contrast, the predominance of Betaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria as opportunistic keystone taxa in the rhizosphere and intercropping systems reflects the more specialized and competitive environments created by plant roots and their exudates. These taxa are well known for their

versatile metabolic capabilities and the ability to establish symbiotic relationships with plants, which likely confers a competitive advantage in nutrient-rich and consequently competitive environments (Hu *et al.* 2021; Dang *et al.* 2024). Moreover, the identification of Alphaproteobacteria (e.g., *Bradyrhizobium* and *Azospirillum*) as keystone specialists in the rhizosphere further highlights the importance of mutualistic relationships between plants and diazotrophs in these systems. These taxa are critical for nitrogen fixation and plant growth promotion, and their specialization allows them to dominate the highly competitive rhizosphere environment, which has been noted in studies highlighting the crucial roles of such specialists with regard to ecosystem functioning and stability (Banerjee *et al.* 2018; De Matos *et al.* 2021; Zhang *et al.* 2024). In contrast, the presence of Gammaproteobacteria (e.g., *Pseudomonas* and *Azotobacter*) as keystone generalists in intercropping systems suggests that these taxa play a crucial role in maintaining ecosystem stability by performing a wide range of functions in different plant species and soil conditions (Xu *et al.* 2022; Jiang *et al.* 2024).

These findings underscore the complex interplay between microbial community structure and ecological function in the rhizosphere and intercropping systems, and emphasize the potential of targeted management practices for optimizing BNF and soil health. Notably, our study confirmed that opportunists consistently represented the largest proportion of keystone taxa across different treatments, and their roles varied based on the environmental context. This adaptability emphasizes the crucial role of opportunists in maintaining the resilience and functionality of microbial communities in diverse agricultural systems.

4.4. Ecological linkages between BNF potentials, diazotrophic community, and soil properties

The complex interactions between soil properties, diazotrophic communities, and BNF activities across different agricultural practices and soil niches underscore the importance of both abiotic and biotic factors in modulating BNF potential (Dang *et al.* 2024; Li *et al.* 2024).

In bulk soils, BNF potential is primarily driven by abiotic factors, such as MBC, NH_4^+-N, and AK, while keystone opportunists, such as *Nostoc* and *Bacillus*, play supportive roles. These taxa help to maintain the BNF potential, but their overall impact remains moderate, suggesting that, despite their resource availability, bulk soils lack the complexity needed to independently sustain high levels of BNF (Philippot *et al.* 2013). In contrast, the nutrient-rich environment of the rhizosphere, which is shaped by root exudates and plant-microbe interactions, fosters a more complex and synergistic relationship between abiotic factors and microbial communities. This rhizosphere environment supports higher BNF potentials by providing abundant nitrogen sources (e.g., NO_3^-–N) and labile organic matter (e.g., MBC and MBN), which serve as energy and nutrient substrates for the diazotrophic communities, thereby enhancing nitrogen fixation efficiency (Hu *et al.* 2021; Wu *et al.* 2024). Keystone opportunists, such as *Pseudomonas* and *Steroidobacter*, are particularly effective in this competitive setting, and they use available resources to enhance nitrogen fixation (Wolińska *et al.* 2018). In addition, the reduced influence of keystone specialists, such as *Bradyrhizobium*, suggests that these taxa are less competitive in the rhizosphere because they rely on specific symbiotic relationships (Lu *et al.* 2024).

In monoculture systems, factors such as soil pH, DOC, MBC, and MBN contribute to BNF but have generally weaker impacts. Keystone specialists such as *Azospira* and *Desulfuromonas* negatively affect BNF, likely because of the homogeneity and lower complexity of monoculture environments, which limit microbial diversity and functional redundancy, thus restricting BNF potential (Wang *et al.* 2020; Shu *et al.* 2024). In contrast, the BNF potential in intercropping systems was significantly enhanced by fostering heterogeneous soil environments with elevated NO_3^-–N and MBC (Dang *et al.* 2024), along with more keystone generalists (e.g., *Pseudomonas*) and opportunists (e.g., *Azoarcus*, *Azohydromonas*) that dominate the modular co-occurrence networks. These heterogeneous conditions — rather than resource abundance alone — promote microbial niche partitioning and facilitate nitrogen fixation through free-living pathways (Hu *et al.* 2024; Dittmann *et al.* 2025). The complexity of intercropping systems creates diverse microhabitats that support a wider range of diazotrophic strategies, which optimizes BNF more effectively than monocultures (Duchene *et al.* 2017; Qiao *et al.* 2024).

Therefore, rhizosphere soils and intercropping systems effectively leverage the interplay between biotic and abiotic factors to create an environment that is conducive to enhanced nitrogen fixation. This is consistent with recent findings on the importance of soil microorganisms and interspecific interactions in boosting BNF and crop productivity (Hu *et al.* 2021; Shu *et al.* 2024). However, while this study offers valuable insights, it falls short of providing direct evidence to validate the proposed mechanisms. Future research should focus on directly testing the contributions of factors such as root exudates, core microbiomes, and other potential drivers of BNF in intercropping systems.

5. Conclusion

This study demonstrates that peanut/cotton intercropping systems and rhizosphere soils play a critical role in enhancing the BNF potential through a complex interplay of biotic and abiotic factors. Our findings underscore the importance of leveraging the diversity and adaptability of diazotrophic communities, particularly keystone opportunists such as *Azoarcus*, *Azohydromonas*, *Steroidobacter*, and *Pseudomonas*, in sustaining high soil BNF levels. These results highlight the potential of intercropping systems to promote soil health and agricultural productivity by recruiting beneficial microbial taxa. In addition, insights from ecological theories and advanced bioinformatics indicate that future studies should leverage experimental approaches such as culturomics and synthetic biology to validate the nitrogen-fixing functions of key microbial taxa. Such methods will be essential for translating these findings into practical strategies for sustainable agriculture.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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