



Soil bacterial and fungal communities resilience to long-term nitrogen addition in subtropical forests in China

Xinlei Fu¹ · Yunze Dai^{1,2} · Jun Cui^{1,3} · Pengfei Deng¹ · Wei Fan¹ · Xiaoni Xu¹

Received: 18 October 2022 / Accepted: 11 April 2023
© The Author(s) 2023

Abstract Atmospheric nitrogen (N) deposition is predicted to increase, especially in the subtropics. However, the responses of soil microorganisms to long-term N addition at the molecular level in N-rich subtropical forests have not been clarified. A long-term nutrient addition experiment was conducted in a subtropical evergreen old-growth forest in China. The four treatments were: control, low N (50 kg N ha⁻¹ a⁻¹), high N (100 kg N ha⁻¹ a⁻¹), and combined N and phosphorus (P) (100 kg N ha⁻¹ a⁻¹ + 50 kg P ha⁻¹ a⁻¹). Metagenomic sequencing characterized diversity and composition of soil microbial communities and used to construct bacterial/fungal co-occurrence networks. Nutrient-treated soils were more acidic and had higher levels

of dissolved organic carbon than controls. There were no significant differences in microbial diversity and community composition across treatments. The addition of nutrients increased the abundance of copiotrophic bacteria and potentially beneficial microorganisms (e.g., Gemmatimonadetes, *Chaetomium*, and *Aureobasidium*). Low N addition increased microbiome network connectivity. Three rare fungi were identified as module hubs under nutrient addition, indicating that low abundance fungi were more sensitive to increased nutrients. The results indicate that the overall composition of microbial communities was stable but not static to long-term N addition. Our findings provide new insights that can aid predictions of the response of soil microbial communities to long-term N addition.

Project funding This work was supported by the National Science Foundation of China (No. 31770672 and 3137062) and the National Basic Research Program of China (No. 2010CB950602).

The online version is available at <http://www.springerlink.com>

Corresponding editor: Yanbo Hu.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11676-023-01675-6>.

✉ Xiaoni Xu
xnxu2007@ahau.edu.cn

¹ School of Forestry and Landscape Architecture, Anhui Agricultural University, Hefei 230036, Anhui, People's Republic of China

² Suzhou Polytechnic Institute of Agriculture, Suzhou 215008, Jiangsu, People's Republic of China

³ College of Life and Environment Sciences, Huangshan University, Huangshan 245041, Anhui, People's Republic of China

Keywords Long-term nitrogen addition · Old-growth subtropical forest · Metagenomics · Beneficial microorganisms · Co-occurrence network

Introduction

The global rate of anthropogenic nitrogen (N) addition (210 Tg N a⁻¹) is currently greater than the supply by natural processes (203 Tg N a⁻¹; Fowler et al. 2013) as human activities have disturbed the global N cycle (Gruber and Galloway 2008). Chronic N deposition has several effects on net primary productivity, biodiversity, the global carbon cycle, and climate (Erismann et al. 2013). The effects of increased N deposition on global biodiversity, especially on soil microorganisms in forest ecosystems (Wang et al. 2018a), have received increased attention. This is because soil microorganisms, which represent a high proportion of terrestrial biodiversity, affect major ecosystem processes, including nutrient cycles (Canfield et al. 2010), plant growth

(van der Heijden et al. 2008), and soil structure (Rillig and Mummey 2006).

Although numerous studies have examined the responses of soil microorganisms to long-term N addition, there remain unresolved issues. The effects of increased N on soil microbial diversity vary among forest ecosystems, with some studies showing that added N can have positive (Haas et al. 2018) or negligible (Contosta et al. 2015) effects. In contrast, N deposition has resulted in dramatic decreases in microbial diversity in cold temperate forests (Yan et al. 2018), in bamboo plantations (Li et al. 2016), and in boreal forests (Allison et al. 2007). The effects of added N on microbial communities also vary, with some studies showing that these communities are altered (Zeng et al. 2016) or remain unaltered (Li et al. 2019). Most studies of N addition have been conducted in boreal and temperate forest ecosystems (WallisDeVries and Bobbink 2017). In contrast, tropical and subtropical forests are often referred to as N-rich and phosphorus (P)-poor (Walker and Syers 1976; Vitousek and Farrington 1997). N and P are necessary for the development and reproduction of plants. These two nutrients are generally in a dynamic equilibrium in natural environments (Elser et al. 2007), but increases in one can induce a limitation in the other (Elser et al. 2007). Some studies have shown that adding N has little effect on plant productivity in N-saturated forests (Harrington et al. 2001; Cusack et al. 2011), but can alter soil C sequestration (Zak et al. 2017; Lu et al. 2021b). Plant productivity might be nutrient limited, while soil microbes always are carbon limited in natural ecosystems (Soong et al. 2020). Tropical and subtropical forests thus warrant increased research attention.

Bacteria and fungi coexist in the natural environment, and there is substantial nutrient and energy exchange between them (Benoit et al. 2015). The ecological functions of microorganisms can be performed via the formation of an effective interaction co-occurrence network rather than by a single species (Pande and Kost 2017). In these networks, the interactions between microbes are diverse and include both cooperative and competitive relationships (Faust and Raes 2012). Yuan et al. (2021) reported that climate warming increases the complexity of soil microbial networks. More intense microbial interactions after N addition have been reported by Li et al. (2021). These findings suggest that soil microbial interactions likely play a key role in forest management.

In this study, the effects of seven years of N addition on soil bacteria and fungi in a subtropical evergreen broad-leaved old-growth forest with rich in N but poor in P were reported, using metagenomic sequencing. The objectives were: (1) to clarify the effects of N and P addition on the diversity and community composition of soil microbes; (2) to identify the bacterial and fungal taxa sensitive to nutrient addition; and (3) to clarify the effects of adding N on microbial co-occurrence networks. The same data as that in Dai

et al. (2021) were used, but information on soil fungi was included, which are believed to be potentially more sensitive to N addition than bacteria (Wang et al. 2021). Further investigation of the responses of microorganisms to N addition at the species level was carried out using identifying biomarkers and creating bacterial and fungal co-occurrence networks, rather than only analyzing microbial community diversity.

Materials and methods

Study site description and experimental design

An N deposition experiment was conducted in a 200-year-old, evergreen broad-leaved forest on Xianyu Mountain (30° 01'47" N, 117° 21'23" E), Shitai County, Anhui Province, eastern China. The site has a humid subtropical monsoon climate, with an annual average temperature of 16 °C and precipitation of 1,626 mm (Dai et al. 2021). The dominant species in the stands include *Castanopsis eyrei* (Champ. ex Benth.) Hutch. and *Cyclobalanopsis glauca* (Thunberg) Oersted; the main associated species are *Symplocos* spp. and *Pinus massoniana* Lamb.; the main understory shrubs are *Camellia cuspidata* (Kochs) Wright ex Gard., *Loropetalum chinense* (R. Br.) Oliver, *Tricyrtis macropoda* Miq., and various species of ferns. The soil is a ultisol or red clay soil, according to the United States Department of Agriculture (USDA) Soil Taxonomy classification and has developed from a regolith of granites; the pH is acidic.

The field experiment was initiated in June 2011 and consisted of four treatments: control (CK, neither N nor P added), low N (LN, 50 kg N ha⁻¹ a⁻¹), high N (HN, 100 kg N ha⁻¹ a⁻¹), and combined N and P additions (HNP, 100 kg N ha⁻¹ a⁻¹ + 50 kg P ha⁻¹ a⁻¹). The experiment was conducted in a randomized block design. There were three replicates 20 m × 110 m blocks separated by a 10-m buffer, each containing four treatments and a total of 12 experimental plots (20 m × 20 m) separated by 10 m. From 2011 to 2018, NH₄NO₃ and Ca(H₂PO₄)₂ solutions dissolved in 20 L of water to simulate wet deposition, were applied bimonthly using a backpack sprayer. Each control plot received equivalent amounts (20 L) of water corresponding to an increase of 0.3 mm a⁻¹ precipitation.

Soil sampling and physicochemical analysis

After seven years of nutrient addition, soil samples were collected in July 2018. Within each plot, samples of the upper mineral soil (0–10 cm) were collected according to the five-point method using a steel auger after removing the litter layer and were evenly combined. Twelve samples were collected and transported to the laboratory and passed through

a 2-mm steel sieve to remove stones and roots. One portion of the samples was kept at 4 °C for chemical and physical properties analysis; another was kept at –80 °C for DNA sequencing.

The following physicochemical characteristics were quantified: pH, water content (SWC), ammonium ($\text{NH}_4^+ - \text{N}$), nitrate ($\text{NO}_3^- - \text{N}$), organic carbon (SOC), total N (TN), total phosphorus (TP), dissolved organic carbon (DOC), dissolved organic N (DON), microbial biomass carbon (MBC), and N (MBN). pH was measured using a pH meter in 2.5:1 water/soil suspensions. SWC was determined by oven-drying fresh soil to a constant mass at 105 °C. Available N ($\text{NH}_4^+ - \text{N}$ and $\text{NO}_3^- - \text{N}$) were extracted from fresh soil samples (30 g) with 50 mL of 1 M KCl solution and measured with a flow injection autoanalyzer (FIASStar 5000, FOSS, Sweden). A 1-g air-dried sample was digested in a 10 mL $\text{H}_2\text{SO}_4 - \text{HClO}_4$ (3:1) solution, and TP in the extract was measured by a flow injection autoanalyzer based on molybdenum blue chemistry. SOC and TN were determined using a CHN autoanalyzer (EA 3000, EuroVector, Italy). The chloroform-fumigation-extraction method was used to calculate MBC and MBN (Vance et al. 1987). Fresh subsamples (30 g) were extracted using a 100-mL 0.5 M K_2SO_4 solution, shaken for 30 min, and filtered. The subsamples were fumigated with ethanol-free chloroform for 24 h at 25 °C in a vacuum desiccator; the other subsamples were treated without fumigation. DOC and DON were quantified using a TOC analyzer (Multi N/C 3100, Jena Analytik AG, Germany). δ_{MBC} (mg kg^{-1}) was estimated using the following:

$$\delta_{\text{MBC}} = \delta_{\text{CFE}}/0.45 \quad (1)$$

where δ_{CFE} refers to the difference in DOC content between fumigated and non-fumigated extracts. δ_{MBN} (mg kg^{-1}) was estimated using the following formula:

$$\delta_{\text{MBN}} = \delta_{\text{NFE}}/0.54 \quad (2)$$

where δ_{NFE} is the difference in DON content between fumigated and non-fumigated extracts (Wu et al. 1990).

DNA extraction, library construction, sequencing, assembly, and annotation

Metagenomic sequencing was used to analyze soil microbial communities which precludes the need to cultivate species (Chen and Pachter 2005), and overcomes limitations of amplicon sequencing (Sharpton 2014). Metagenomic sequencing permits nutrient-susceptible biomarkers to be precisely identified and to predict the ecological effects of N deposition. In this study, the taxonomic information from our metagenomes were used for analysis.

An E.Z.N.A. soil DNA Kit (D5625-02, Omega, Inc. USA) was used to extract genomic DNA from different samples. DNA was extracted using 50 μL of elution buffer, and stored at –80 °C until further analysis. The extracted DNA samples were submitted to LC-Bio Technology Co. Ltd. (Hangzhou, China) for sequencing.

A DNA library was constructed using the TruSeq Nano DNA LT Library Preparation Kit (FC-121-4001). DNA was fragmented for 30 min at 37 °C with dsDNA fragmentase (NEB, M0348S). Fill-in reactions and exonuclease activity produced blunt-end DNA fragments. An A-base was added to the blunt ends to facilitate ligation to the indexed adapters. The DNA fragments with ligated adapters were amplified by PCR via thermal-cycling conditions: initial denaturation at 95 °C for 3 min; eight cycles of denaturation at 98 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. Amplification of DNA fragments was performed with universal PCR primers (forward primer: 5'-AATGATACGGCGACC ACCGAGATCTACAC [i5] TCGTCGGCAGCGTCAGAT GTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and reverse primer: 5'-CAAGCAGAAGACGGCATAACGA GAT [i7] GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTAC-HVGGGTATCTAATCC-3'). After amplification, purification, quantification, and quality control, the DNA library was sequenced on an Illumina-Hiseq 4000-PE 150 platform (Illumina Inc., San Diego, CA, USA).

Raw sequencing reads were preprocessed to obtain clean reads before subsequent data analysis. Joint reads were removed using Cutadapt version 1.9 and low-quality reads truncated using fqtrim version 0.94 with a window size of 6 bp. The samples were assembled by SPAdes version 3.7.0 to generate a FASTQ file, and each sequence was referred to as a contig; these were used to predict coding regions (CDs) with MetaGeneMark version 3.26. CDs were clustered by CD-HIT version 4.6.1 to obtain unigenes. Abundance information was determined using the transcripts per million method based on the number of aligned clean reads against a database of gene sequences using Bowtie2 version 2.2.0 (Langmead and Salzberg 2012). DIAMOND version 0.7.12 with an e-value threshold of 1×10^{-5} was used to align the unigenes against the NCBI-nr version July 2016 database of non-redundant protein reference sequences to identify species. Based on taxonomic classification from the NCBI taxonomy database, specific species annotation information at each classification level was obtained using the least common ancestor algorithm (Huson et al. 2011).

Statistical analysis

Hill numbers, which are a unified family of diversity indices, were used to quantify soil bacterial and fungal diversity:

$${}^qD = \left(\sum_{i=1}^S p_i^q \right)^{1/(1-q)} \quad (3)$$

where qD denotes the Hill number, the exponent and superscript q is the order of the diversity, S the number of species, and p_i the relative abundance of species i in the samples (Jost 2006). When $q=0$, 0D is identical to the species richness. When $q=2$, 2D is equal to the inverse Simpson index and can be interpreted as the effective number of dominant species (Tuomisto 2011; Daly et al. 2018). This function is undefined for $q=1$, but 1D can be calculated by modifying q to 0.99999 (Alberdi and Gilbert 2019). 1D corresponding to the limit of the unity is the analog of the Shannon index and can be considered as the effective number of typical species. Additionally, ${}^{-1}D$ ($q=-1$) and 3D ($q=3$) represent rare and common species, respectively. The comparisons of differences among groups are examined by multiplicity-adjusted P -values to avoid increasing the rate of false positives using the “simboot” package in R software (Pallmann et al. 2012). Linear mixed models (LMM) were used to test the effect of treatments on soil properties. The treatments were included as fixed factors, and a block was included as a random factor using the function “lmer” in “lme4” the package in R (Bates et al. 2015; Piepho 2018; Piepho and Edmondson 2018). The residuals were examined by the Shapiro test to check assumptions of a one-way analysis of variation (ANOVA) (Kozak and Piepho 2018). *Post-hoc* tests between treatments were performed as all-vs-all comparisons using the package “emmeans” in R (Lenth et al. 2023), and statistical differences were deemed significant at $P < 0.05$. All data are presented as mean \pm standard deviation.

Non-metric multidimensional scaling (NMDS) is a rank-order-mapping method that makes no assumptions about the underlying data structure. The rank-order relationships between ordination distances and original distances are iteratively improved using an algorithm of successive approximations to obtain an optimized map of n individuals in a low-dimensional ordination space. In most cases, microbial data are nonlinear, and NMDS is the appropriate model for these data (Kenkel et al. 2002; Ramette 2007). In this study, NMDS based on the Bray–Curtis distance at the species level was used to investigate dissimilarity in the composition of bacteria and fungi among treatments. Permutational multivariate analysis of variance (i.e., PERMANOVA, 999 permutations) was conducted to evaluate statistical significance using the function “adonis” in the R package “vegan” (Oksanen et al. 2022).

Linear discriminant analysis (LDA) effect size (LEfSe) is an algorithm for high-dimensional biomarker discovery and explanation that can be used in metagenomic analyses. Because the relative abundance in metagenomic data frequently violate the main assumptions of typical parametric

tests, the non-parametric tests used by LEfSe are more reliable (Segata et al. 2011). In our study, the LEfSe algorithm was used (<https://huttenhower.sph.harvard.edu/galaxy>) to identify potential biomarkers based on various taxonomic levels (from supergroup to species) in soil microbes. Specifically, we used the non-parametric factorial Kruskal–Wallis rank-sum test to detect significant differential abundance, and LEfSe then estimated the effect size of each differentially abundant taxon and determined the taxa that were significantly affected by N addition. The factorial Kruskal–Wallis test was conducted using an alpha value of 0.05, and the threshold of the logarithmic LDA score was 2.0.

Four co-occurrence networks were created for the treatments by integrating information on the bacterial and fungal species in each group. Those with average relative abundances $> 0.1\%$ were retained from each group. Pearson correlation analysis between microbes was conducted at the species level using the package “psych” in R (Revelle 2022). Pearson correlation coefficients greater than 0.6 were considered statistically robust. Gephi version 0.9.2 was used to build the co-occurrence networks and determine their topological properties. The keystone microbes were recognized based on their within-module connectivity (Z_i) and among-module connectivity (P_i) values. We defined the nodes as network hubs (highly connected nodes within the entire network, $Z_i \geq 2.0$ and $P_i \geq 0.6$), module hubs (highly connected nodes within modules, $Z_i \geq 2.0$ and $P_i < 0.6$), connectors (nodes that connect modules, $Z_i < 2.0$ and $P_i \geq 0.6$), or peripherals (nodes connect in modules with few connections to the outside world, $Z_i < 2.0$ and $P_i < 0.6$).

Results

Changes in soil physicochemical properties

The pH was significantly lower ($P < 0.01$) and DOC higher ($P < 0.01$) in the nutrient addition treatments than in the controls (Table 1). However, there were no significant differences in SWC, SOC, TN, TP, DON, $\text{NH}_4^+ - \text{N}$, $\text{NO}_3^- - \text{N}$, MBC, MBN, and SOC/TN among treatments (Table 1).

Soil microorganisms

Bacterial community

High-quality sequences were clustered into 9230 bacterial species. The shapes of the soil bacterial Hill number curves for the different treatments were similar (Fig. 1a). The multiplicity-adjusted P -values from $q = -1$ to $q = 3$ were not significant, and some of them were far from the significance threshold ($P > 0.05$; Table S1).

Table 1 Effects of nutrient addition on soil physicochemical properties with linear-mixed effects

Treatments	pH	SWC (%)	DOC (mg·kg ⁻¹)	DON (mg·kg ⁻¹)	SOC (g·kg ⁻¹)	TN (g·kg ⁻¹)	TP (g·kg ⁻¹)	NH ₄ ⁺ -N (mg·kg ⁻¹)	NO ₃ ⁻ -N (mg·kg ⁻¹)	MBC (mg·kg ⁻¹)	MBN (mg·kg ⁻¹)	SOC/TN
CK	4.1 ± 0.1 ^a	0.5 ± 0.0 ^a	300.5 ± 3.7 ^a	21.0 ± 1.8 ^a	48.2 ± 6.7 ^a	2.8 ± 0.1 ^a	0.3 ± 0.1 ^a	22.1 ± 8.3 ^a	6.5 ± 4.8 ^a	1448.0 ± 408.7 ^a	159.2 ± 9.5 ^a	17.5 ± 1.7 ^a
LN	3.8 ± 0.0 ^b	0.7 ± 0.1 ^a	444.1 ± 46.5 ^b	25.8 ± 9.3 ^a	75.2 ± 14.1 ^a	4.0 ± 1.1 ^a	0.3 ± 0.1 ^a	28.5 ± 16.9 ^a	14.7 ± 12.6 ^a	1235.5 ± 349.8 ^a	130.5 ± 35.7 ^a	19.3 ± 2.3 ^a
HN	3.6 ± 0.0 ^b	0.6 ± 0.1 ^a	436.5 ± 57.8 ^b	20.7 ± 2.1 ^a	68.0 ± 8.5 ^a	3.6 ± 0.7 ^a	0.2 ± 0.0 ^a	29.3 ± 9.1 ^a	15.7 ± 7.7 ^a	1366.7 ± 546.0 ^a	146.4 ± 59.3 ^a	19.0 ± 1.7 ^a
HNP	3.8 ± 0.1 ^b	0.6 ± 0.1 ^a	392.0 ± 29.7 ^b	22.2 ± 3.0 ^a	74.4 ± 24.0 ^a	3.9 ± 0.9 ^a	0.4 ± 0.1 ^a	23.7 ± 9.0 ^a	10.4 ± 6.1 ^a	1435.2 ± 866.9 ^a	143.0 ± 49.2 ^a	19.0 ± 1.9 ^a
P-value	**	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	NS

Different lowercase letters indicate significant differences ($P < 0.05$)

Values are mean ± SD of three replicates; within a row, values followed by the same letter do not differ significantly ($P > 0.05$)

* $P < 0.05$; ** $P < 0.01$; NS, not significant. Treatments: control (CK), low N (LN), high N (HN) and combined N and P (HNP)

The most abundant bacterial phyla across all samples were Proteobacteria (an average of 51.5% of all unigenes), followed by Acidobacteria (26.0%) and Actinobacteria (4.1%), which together comprised 81.5% of bacterial unigenes (Table S2). With the exception of Gemmatimonadetes, nutrient addition had no significant effect ($P > 0.05$) on bacterial phyla (Table S2). The addition of nutrients had similar effects on Hill numbers; the dissimilarity of the bacterial community at the species level did not significantly differ between the treatments and control according to PERMANOVA ($P = 0.36$; Fig. 2a).

LEfSe analysis was conducted to identify the taxa affected by the addition of nutrients; nine biomarkers belonging to two phyla were affected ($P < 0.05$, LDA > 2.0 ; Fig. 3a). Eight taxa of the phylum Gemmatimonadetes were enriched in the low N group, including class Gemmatimonadetes, the orders Gemmatimonadales and Gemmatimonadetes_noname, the family Gemmatimonadetes_noname, the genus *Gemmatimonadetes_noname*, and the species *Gemmatimonas_sp._AP64* and *Gemmatimonadetes_bacterium_KBS708*. The species *Acinetobacter_sp._CIP_64.2* in the phylum Proteobacteria was sensitive to combined N and P addition (Fig. 3a, Table S3).

Fungal community

A total of 355 fungal species were identified from the samples. Soil fungal Hill numbers at all values of q were lower in the controls than in the nutrient addition treatments (Fig. 1b). The addition of nutrients did not have any significant effect on Hill numbers (Table S1). The most abundant fungal phyla were Ascomycota (average of 67.2% of the unigenes) and Basidiomycota (23.4%), together comprising 90.6% of the fungal unigenes detected (Table S4). In addition, adding nutrients did not result in significant shifts in the dissimilarity of the fungal community ($P = 0.69$; Fig. 2b).

Seven nutrient-susceptible fungi of two phyla were identified by LEfSe analysis ($P < 0.05$, LDA > 2.0 ; Fig. 3b). The sensitive fungal taxa were in the order Dothideales, the family Aureobasidiaceae, and the genus *Aureobasidium* in the HN group; the genera *Chaetomium* and *Nosema* in the LN group; and the genera *Brettanomyces* and *Naumovozya* in the controls (Fig. 3b, Table S5).

Co-occurrence of bacteria and fungi by network analysis

To identify the responses of microbe interactions to nutrient additions, four co-occurrence networks were created for each treatment (CK, LN, HN, HNP) using bacterial and fungal species with relative abundances ($> 0.1\%$) in each group (Fig. 4). The average clustering coefficient of the networks were 0.016, 0.002, and 0.008 higher in the LN, HN,

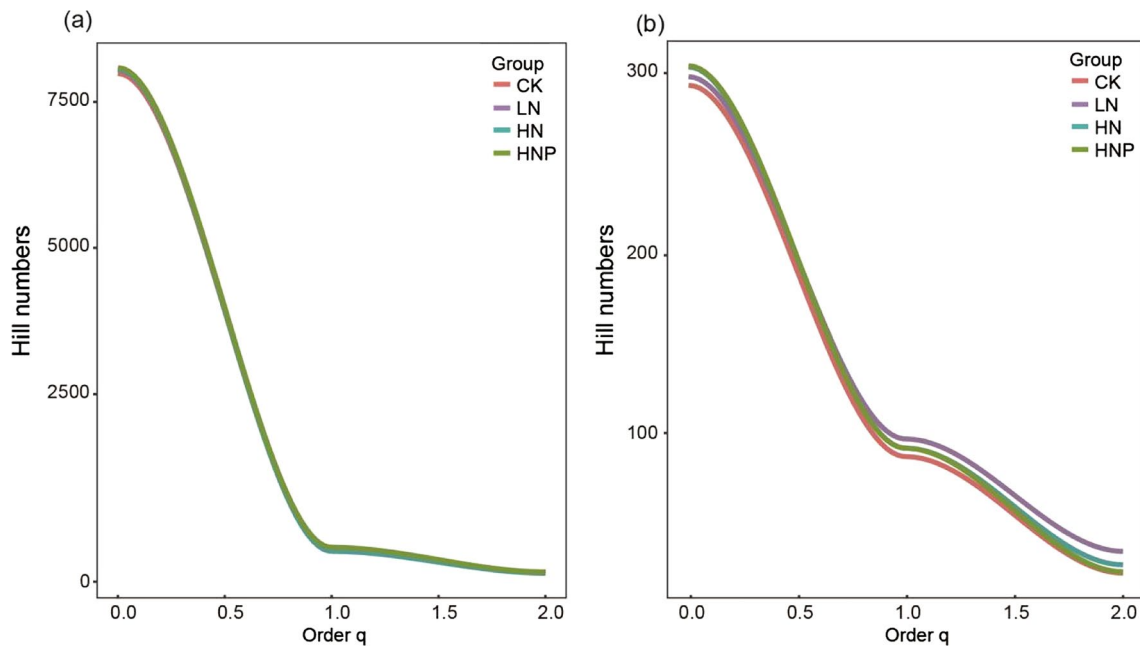


Fig. 1 Soil microbial diversity profiles based on abundance of each species; x-axis is the order q and y-axis the Hill numbers. **a** Bacterial diversity; **b** fungal diversity

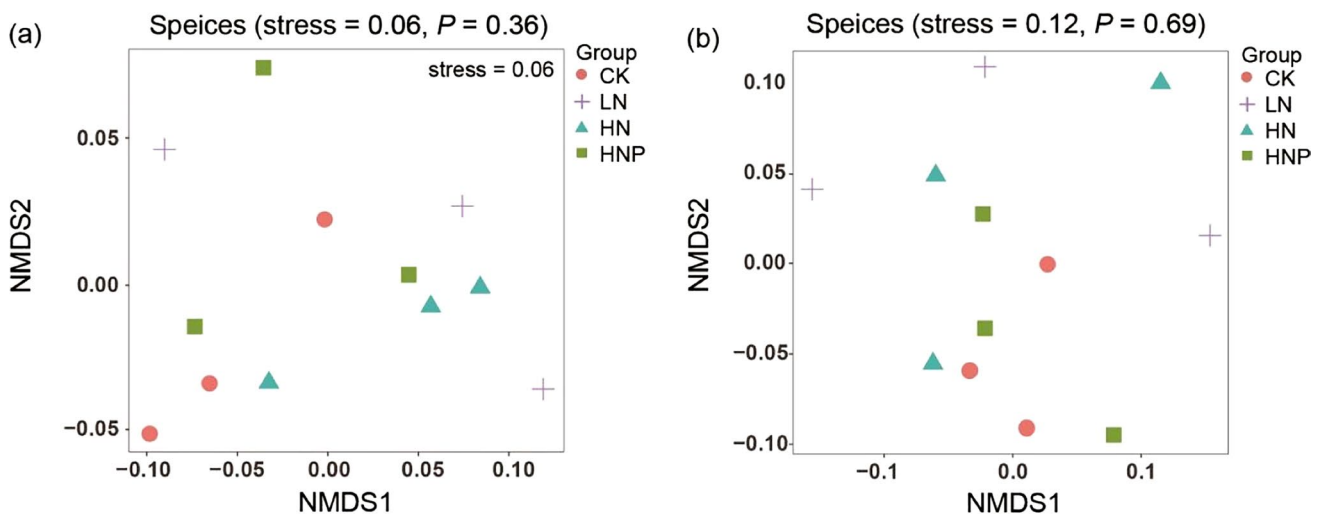


Fig. 2 NMDS ordination plot showing the dissimilarity of the composition of microbial species between different treatments based on Bray-Curtis (a, stress = 0.06; b, stress = 0.12) distance matrices; **a** dissimilarity of the bacterial community; **b** dissimilarity of the fungal community

and HNP group than in the controls, respectively (Fig. 4, Table 2). The low N treatment had the highest number of edges (1,861; 37.8%), the highest average edges per node value (7.85; 34.4%), and the lowest average path length value (11.469; 4.8% lower compared with the controls; Fig. 4, Table 2). In the four networks, the percentage of inter-kingdom edges was higher than the percentage of edges within fungal nodes, which was higher than the percentage of edges within bacterial nodes (Fig. 4, Table 2). The low

and high N addition treatments increased the percentage of positive edges to 63.3% and 62.1%, respectively, and the combined N and P treatment decreased the percentage of positive edges to 51.6% (Fig. 4, Table 2).

To explore the topological roles of microbial taxa in the networks, four categories were established according to their Z_i and P_i values: peripherals, module hubs, network hubs, and connectors. Most nodes were peripherals in each network (Fig. 5). Three module hubs were discovered

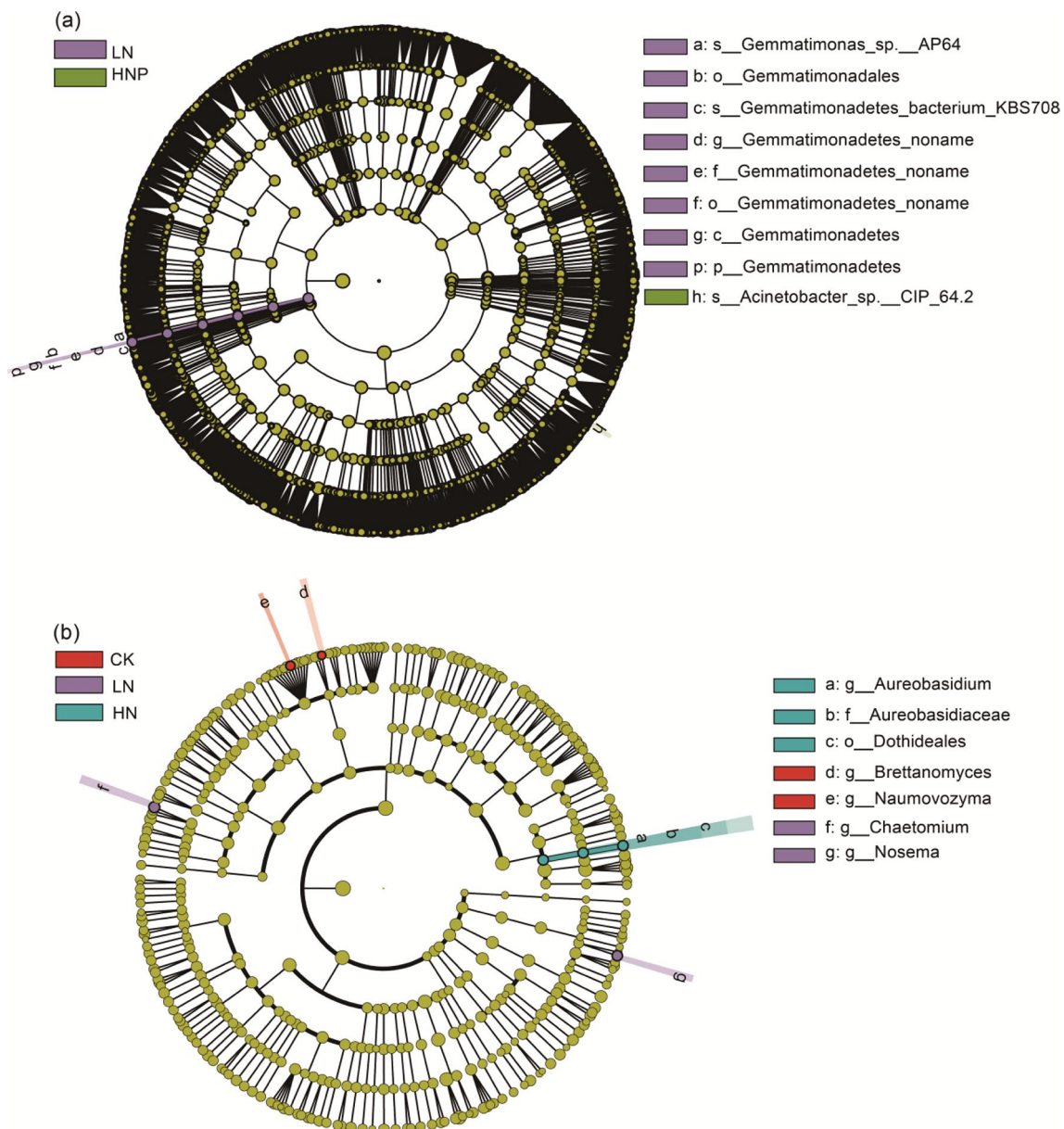


Fig. 3 LEfSe cladograms showing the soil microbial biomarkers sensitive to nutrient addition; **a** bacterial biomarkers; **b** fungal biomarkers. The cladograms show the supergroup, phylum, class, order, family, genus, and species levels from inside to outside; the nodes

indicate taxa belonging to the taxonomic level. Taxa with significantly greater relative abundance in one group are shown by different colored nodes; taxa with no significant changes in the treatments are represented by yellow nodes

in the networks, which originated from various groups: *Mycosphaerella pini* within Ascomycota in LN, *Melanospichium pennsylvanicum* within Basidiomycota in HN, and *Zymoseptoria tritici* within Ascomycota in HNP, and were considered potential keystone taxa because of their key roles in the networks (Fig. 5). The relative abundances of these module hubs were relatively low, 0.3%, 0.4% and 0.6% in LN, HN, and HNP, respectively. No network hubs or connectors were detected.

Discussion

Responses of soil physicochemical properties to nutrient addition

The pH at a depth of 10 cm in the nutrient treatments decreased substantially after seven years relative to the controls, indicating that long-term addition of nutrients will lead to soil acidification. This finding is consistent with some

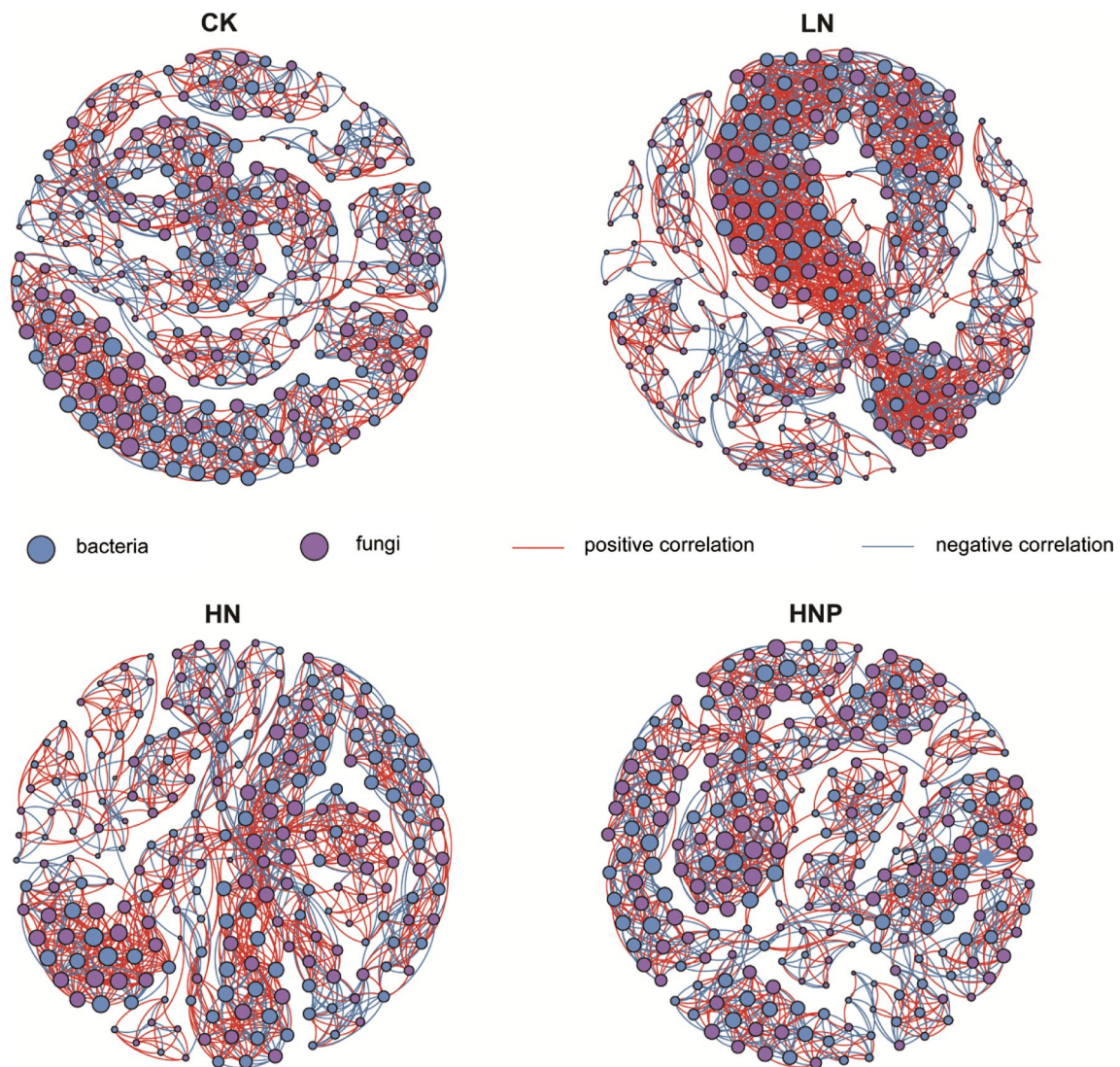


Fig. 4 The networks reflecting the effects of nutrient addition on the co-occurrence patterns between bacteria and fungi at the species level. The size of nodes is proportional to the relative abundance of

species, with blue nodes representing bacteria and purple nodes representing fungi. Red links indicate positive correlations between two nodes, and blue links negative correlations

studies showing that N deposition lowers the pH in N-rich tropical (Wu et al. 2019) and subtropical soils (Lu et al. 2021a). N enrichment contributes to reductions in pH as a result of the following processes: (1) release of H^+ when NH_4^+ transforms into NO_3^- via nitrification; (2) release of H^+ when plants absorb NH_4^+ ; (3) loss of base cations resulting from the exchange of NH_4^+ ; and (4) leaching of NO_3^- (Chen et al. 2016). In the present study, the addition of N significantly increased the quantity of dissolved organic carbon (DOC) in the upper 10-cm layer. Similar to our results, Fang et al. (2014) found that DOC in surface layers were strongly increased by the addition of N. This may be because added N promotes plant growth, leading to increased organic matter inputs to soil through litter-fall and root exudates that favor the accumulation of DOC

(Portillo-Estrada et al. 2013). In addition, a lower pH may reduce the capacity of soils to stabilize SOC as a result of reductions in the (negative) surface charge of minerals (Fisore et al. 2008), causing more organic carbon to be converted into DOC. The levels of soil $NH_4^+ - N$ and $NO_3^- - N$ were not affected by the addition of nutrients in our experiment, possibly because NH_4NO_3 is absorbed by plants and microbes or lost through denitrification, volatilization, and/or leaching.

Response of microbial communities to nutrient addition

In this study, the predominant bacterial phyla were Proteobacteria, Acidobacteria, and Actinobacteria, and the major fungal phyla were Ascomycota and Basidiomycota. These

Table 2 Topological indices of bacterial and fungal networks in the four treatments: control (CK), low N (LN), high N (HN), and combined N and P (HNP)

Treatments	Average clustering coefficient	Average path length	Network density	Number of nodes	Number of edges	Average edges per node	Bacterial nodes
CK	0.733	12.050	0.051	231	1350	5.84	110 (47.6%)
LN	0.749	11.469	0.067	237	1861	7.85	110 (43.0%)
HN	0.735	11.729	0.052	227	1334	5.88	113 (48.5%)
HNP	0.741	12.429	0.049	241	1427	5.92	102 (46.9%)
Treatments	Fungal nodes	Positive edges	negative edges	Edges within bacterial nodes	Edges within fungal nodes	Edges between bacterial and fungal nodes	Modularity
CK	121 (52.4%)	734 (54.3%)	616 (45.6%)	304 (22.5%)	373 (27.6%)	673 (49.9%)	0.813
LN	117 (57.0%)	1179 (63.4%)	682 (36.7%)	457 (24.6%)	501 (26.9%)	903 (48.5%)	0.711
HN	128 (51.5%)	828 (62.1%)	506 (37.9%)	322 (24.1%)	354 (26.6%)	658 (49.3%)	0.787
HNP	135 (53.1%)	736 (51.6%)	691 (48.4%)	317 (22.2%)	401 (28.1%)	709 (49.7%)	0.835

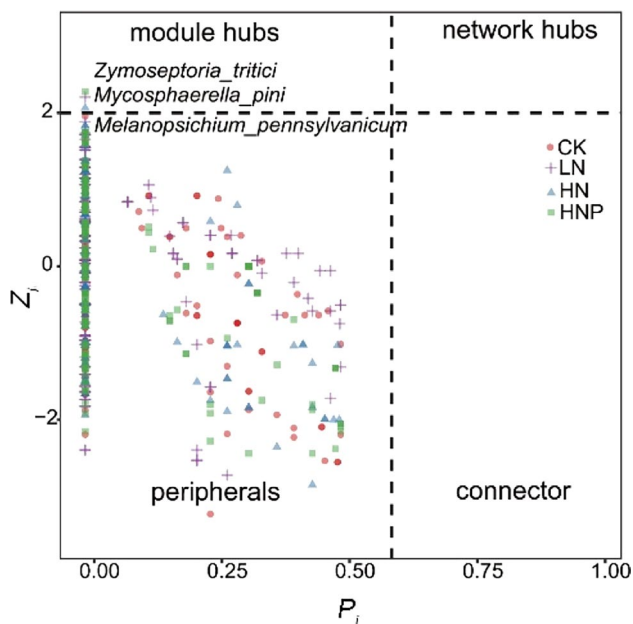


Fig. 5 Distribution of bacterial and fungal species based on their network roles which was used to identify keystone species within networks. Network nodes were categorized as peripherals, module hubs, network hubs, or connectors. $x - P_i$ represents among-module connectivity, and the $y - Z_i$ represents within-module connectivity. Network hubs: $Z_i \geq 2.0$, $P_i \geq 0.6$; module hubs: $Z_i \geq 2.0$, $P_i < 0.6$; connectors: $Z_i < 2.0$, $P_i \geq 0.6$; peripherals: $Z_i < 2.0$, $P_i < 0.6$

phyla are widely distributed in acidic forest soils (Wang et al. 2018b). The diversity and community composition of these microbes were not affected by the addition of nutrients. In other regions, such as temperate forests in northeastern China, N input reduced soil bacterial richness and altered microbial community structure (Yan et al. 2018). One possible explanation for these contrasting findings might be that the responses of microorganisms to N deposition in N-rich

subtropical forest soils differed from those in N-deficient soils. Compared with previous experiments carried out on N-rich soils, such as N addition ($150 \text{ kg N ha}^{-1} \text{ a}^{-1}$) in the Dinghushan Nature Reserve (Wang et al. 2018b), the amounts of added N ($100 \text{ kg N ha}^{-1} \text{ a}^{-1}$ for the highest level) applied in our study might not be enough to cause significant changes in soil microbe communities. Some studies reported differences in microbial composition between primary and secondary forests (Li et al. 2019) and between old-growth and young forests (Dai et al. 2021), and this might result in a greater resilience of microbes in old-growth forests to nutrient additions. Furthermore, seasonal and interannual differences in microbial communities were greater than the effects of added N for years to decades (Gutknecht et al. 2012; Contosta et al. 2015), indicating that microbial communities might have adapted to their historical environment, and long-term experiments over decades might be required to observe shifts in microbes.

Microbes sensitive to nutrient addition

Nine bacterial biomarkers in the phyla Gemmatimonadetes and Proteobacteria were sensitive to nutrient addition according to LEfSe analysis. However, no changes in overall microbial community composition were detected. Bacteria of the phylum Gemmatimonadetes are frequently detected in soils, with their relative abundance ranging from 0.2 to 6.5% (DeBruyn et al. 2011), which is consistent with the abundance of Gemmatimonadetes in our study (0.5%). Members of Gemmatimonadetes have been described as copiotrophic organisms, ones living in nutrient-rich environments (Li et al. 2022), and they were enriched by the addition of N in our study. Some studies have shown that taxa of the phylum Gemmatimonadetes can be antagonistic against plant pathogens and have thus

been suggested to be beneficial bacteria (Yin et al. 2013; Fernández-González et al. 2020; Liu et al. 2020). *Acinetobacter*_sp._CIP_64.2 in the class Gammaproteobacteria in the phylum Proteobacteria was enriched in the HNP treatment. Gammaproteobacteria comprise N₂-fixing rhizobia, as well as fast-growing copiotrophic bacteria (Leewis et al. 2016).

Seven nutrient-sensitive fungal biomarkers were detected in this study. The rare genera *Brettanomyces* and *Naumovozyma* within the Saccharomycetales family in the phylum Ascomycota were relatively enriched in the controls. Yang et al. (2015) found that the relative abundance of *Naumovozyma* were negatively related to the number of bacterial-feeding nematodes, which have been reported to stimulate N mineralization and promote plant growth (Ferris and Matute 2003; Irshad et al. 2011). Therefore, we speculate that in soils without the addition of nutrients, the growth of bacterivorous nematodes might be suppressed by promoting the enrichment of *Naumovozyma* fungi. The relative abundances of the genus *Chaetomium* of the phylum Ascomycota, normally found in soil, increased under low N addition. Several studies have confirmed the antagonistic activities of *Chaetomium* to pathogenic microorganisms, such as their ability to resist *Venturia inaequalis*, *Verticillium dahliae*, *Puccinia recondite*, *Pythium ultimum*, and *Fusarium nivale* (Aggarwal et al. 2004; Fernández-González et al. 2020). Members of the genus *Nosema* of the phylum Microsporidia, which are widespread in the environment, were abundant in the low N treatment, but the effects of increases in *Nosema* abundance in forest ecosystems remain unclear. The relative abundances of several N-susceptible taxa in the order Dothideales, the family Aureobasidiaceae, and the genus *Aureobasidium* were markedly higher under high N additions than in the other treatments. Among the fungi identified, the order Dothideales contained only one family Aureobasidiaceae, one genus *Aureobasidium*, and four species (*A. melanogenum*, *A. pullulans*, *A. namibiae*, and *A. subglaciale*), which have been reported to be sources of important metabolites, including pullulan, polymalic acid, and extracellular polysaccharides. They are thus thought to be key for enhancing resistance to pathogenic microorganisms (Di Francesco et al. 2020).

Our findings suggest that N addition can promote the accumulation of copiotrophic bacteria. Several potentially beneficial microorganisms were enriched by nutrient additions, which might promote the development of resistance of soil to disease and resilience to environmental changes. These biomarkers sensitive to nutrient addition however, are low-abundance taxa and long-term experiments are required to determine whether they have an effect on microbial communities.

Response of microbial co-occurrence networks to nutrient addition

Most linkages between species were positive in our four networks, which is consistent with the results of previous studies in natural ecosystems (Shi et al. 2019), as many microbes show high niche overlap and engage in diverse interactions, including cross-feeding, syntrophic interactions, mutualistic interactions, commensalism, co-aggregation, and co-colonization (Faust and Raes 2012). In this study, low N and high N additions increased the percentage of positive edges in the network, and the combined N and P addition increased the percentage of negative edges compared with the control. In general, positive connections (positive edges between nodes) predict cooperative relationships, including cross-feeding, co-aggregation, and co-colonization, and reflect common environmental requirements and shared dispersal barriers (Berry and Widder 2014), whereas negative connections mostly indicate competition for limited resources, different environmental niches, and spatial isolation. The addition of N increased the number of mutualistic microorganisms because it weakened competitive advantage and promoted the development of various trophic levels. In contrast, N and P addition increased competition among microorganisms. We also found that inter-kingdom (between bacteria and fungi) species created more associations than intra-kingdom (within bacteria or fungi) species, indicating that inter-kingdom species constructed mutualistic communities more easily in this study.

More links within fungal nodes than within bacterial nodes were identified in our study, and the three module hubs were all fungi. This might indicate that fungi with hyphae could communicate more effectively within a module, whereas the smaller size and shorter generation times of bacteria resulted in decreased levels of communication (Klein et al. 2016). The mycelia developed by members of Ascomycota and Basidiomycota, pervasive in soils, might explain why the three species from the two phyla were identified as module hubs.

Previous work generally focused on high-abundance microorganisms, but whether these taxa play the most important roles in microbial communities remains unclear. A high number of high- and low-abundant taxa were identified via metagenomic sequencing. Three module hubs were detected, identified as keystone taxa within the networks in the nutrient addition treatments, and their relative abundance ranged from 0.3 to 0.6%, indicating that less-abundant taxa might be more sensitive to the addition of nutrients. Similarly, Coyte et al. (2015) found that rare microbes were keystone species in networks. The number of such species in a network is known to be positively related to network stability. The presence of the three key taxa indicated that

the nutrient addition treatment increased the stability of the network.

High average clustering coefficient values, links, links per node, and the short average path lengths of the low nitrogen network indicated that interactions among microorganisms were more complex than in the other treatments. Complexity often results in stability in ecological systems (MacArthur 1955), and macroecological research has shown that network stability increased with complexity (Montoya et al. 2006). A number of studies have indicated that complex soil microbial networks could reduce the incidence of disease (Rybakova et al. 2017; Yang et al. 2017; Fernández-González et al. 2020). Therefore, we predict that low N addition could increase the complexity of microbial networks, which might promote resistance to environmental changes and inhibit soil-borne diseases.

Although our findings enhance understanding of the responses of soil microbes to N deposition in subtropical forests, seven years of the experiment is insufficient for detecting the long-term effects of N deposition on microbes; experiments conducted over longer periods are needed to clarify the responses of microbes to N deposition.

Conclusions

Metagenomic sequencing provided insights into the responses of soil bacteria and fungi to nutrient enrichment. Long-term N addition enriched copiotrophic bacteria and increased the abundances of several potentially beneficial microbes, which might increase their resistance to diseases. Low N addition increased the complexity of microbial networks. Microorganisms in subtropical old-growth forest soils are adapted to changes induced by adding nitrogen, which could explain why there were no notable differences in the diversity and community composition of soil microbes after nutrient addition at our site. Additional studies are needed to validate our hypotheses.

Author contributions XX conceived the study and designed the experiment. Material preparation, data collection and analysis were performed by XF, YD and WF. The first draft of the manuscript was written by XF and was revised and edited by XX, PD and JC. All authors read and approved the final manuscript.

Data availability The raw data of the sequences had been submitted to NCBI (BioProject ID: PRJNA882189). To review our unreleased data please log in to Submission Portal's DataView Interface: <https://dataview.ncbi.nlm.nih.gov>.

Declarations

Conflict of interest 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.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Aggarwal R, Tewari AK, Srivastava KD, Singh DV (2004) Role of antibiosis in the biological control of spot blotch (*Cochliobolus sativus*) of wheat by *Chaetomium globosum*. *Mycopathologia* 157:369–377. <https://doi.org/10.1023/B:MYCO.0000030446.86370.14>
- Alberdi A, Gilbert MTP (2019) A guide to the application of Hill numbers to DNA-based diversity analyses. *Mol Ecol Resour* 19:804–817. <https://doi.org/10.1111/1755-0998.13014>
- Allison SD, Hanson CA, Treseder KK (2007) Nitrogen fertilization reduces diversity and alters community structure of active fungi in boreal ecosystems. *Soil Biol Biochem* 39:1878–1887. <https://doi.org/10.1016/j.soilbio.2007.02.001>
- Bates D, Mächler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. *J Stat Softw* 67:1–41. <https://doi.org/10.18637/jss.v067.i01>
- Benoit I, van den Esker MH, Patyshakuliyeva A, Mattern DJ, Blei F, Zhou MM, Dijksterhuis J, Brakhage AA, Kuipers OP, de Vries RP, Kovács ÁT (2015) *Bacillus subtilis* attachment to *Aspergillus niger* hyphae results in mutually altered metabolism: interaction of *Aspergillus niger* and *Bacillus subtilis*. *Environ Microbiol* 17:2099–2113. <https://doi.org/10.1111/1462-2920.12564>
- Berry D, Widder S (2014) Deciphering microbial interactions and detecting keystone species with co-occurrence networks. *Front Microbiol* 5:219. <https://doi.org/10.3389/fmicb.2014.00219>
- Canfield DE, Glazer AN, Falkowski PG (2010) The evolution and future of earth's nitrogen cycle. *Science* 330:192–196. <https://doi.org/10.1126/science.1186120>
- Chen K, Pachter L (2005) Bioinformatics for whole-genome shotgun sequencing of microbial communities. *PLoS Comput Biol* 1:106–112. <https://doi.org/10.1371/journal.pcbi.0010024>
- Chen D, Li JJ, Lan ZC, Hu SJ, Bai YF (2016) Soil acidification exerts a greater control on soil respiration than soil nitrogen availability in grasslands subjected to long-term nitrogen enrichment. *Funct Ecol* 30:658–669. <https://doi.org/10.1111/1365-2435.12525>
- Contosta AR, Frey SD, Cooper AB (2015) Soil microbial communities vary as much over time as with chronic warming and nitrogen

- additions. *Soil Biol Biochem* 88:19–24. <https://doi.org/10.1016/j.soilbio.2015.04.013>
- Coyte KZ, Schluter J, Foster KR (2015) The ecology of the microbiome: networks, competition, and stability. *Science* 350:663–666. <https://doi.org/10.1126/science.aad2602>
- Cusack DF, Silver WL, Torn MS, McDowell WH (2011) Effects of nitrogen additions on above- and belowground carbon dynamics in two tropical forests. *Biogeochemistry* 104:203–225. <https://doi.org/10.1007/s10533-010-9496-4>
- Dai YZ, Wang HL, Chen MY, Wang DD, Cao XQ, Chu BY, Xu XN (2021) Response of soil bacterial communities to nitrogen and phosphorus additions in an age-sequence of subtropical forests. *iForest Biogeosci For* 14:71–79. <https://doi.org/10.3832/ifor3655-013>
- Daly A, Baetens J, De Baets B (2018) Ecological diversity: measuring the unmeasurable. *Mathematics* 6:119. <https://doi.org/10.3390/math6070119>
- DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM, Radosevich M (2011) Global biogeography and quantitative seasonal dynamics of Gemmatimonadetes in soil. *Appl Environ Microbiol* 77:6295–6300. <https://doi.org/10.1128/AEM.05005-11>
- Di Francesco A, Di Foggia M, Zajc J, Gunde-Cimerman N, Baraldi E (2020) Study of the efficacy of *Aureobasidium* strains belonging to three different species: *A. pullulans*, *A. subglaciale* and *A. melanogenum* against *Botrytis cinerea* of tomato. *Ann Appl Biol* 177:266–275. <https://doi.org/10.1111/aab.12627>
- Elser JJ, Bracken MES, Cleland EE, Gruner DS, Harpole WS, Hillebrand H, Ngai JT, Seabloom EW, Shurin JB, Smith JE (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol Lett* 10:1135–1142. <https://doi.org/10.1111/j.1461-0248.2007.01113.x>
- Erisman JW, Galloway JN, Seitzinger S, Bleeker A, Dise NB, Petrescu AMR, Leach AM, de Vries W (2013) Consequences of human modification of the global nitrogen cycle. *Philos Trans R Soc B Biol Sci* 368:20130116. <https://doi.org/10.1098/rstb.2013.0116>
- Fang HJ, Cheng SL, Yu GR, Xu MJ, Wang YS, Li LS, Dang XS, Wang L, Li YN (2014) Experimental nitrogen deposition alters the quantity and quality of soil dissolved organic carbon in an alpine meadow on the Qinghai–Tibetan Plateau. *Appl Soil Ecol* 81:1–11. <https://doi.org/10.1016/j.apsoil.2014.04.007>
- Faust K, Raes J (2012) Microbial interactions: from networks to models. *Nat Rev Microbiol* 10:538–550. <https://doi.org/10.1038/nrmicr02832>
- Fernández-González AJ, Cardoni M, Gómez-Lama Cabanás C, Valverde-Corredor A, Villadas PJ, Fernández-López M, Mercado-Blanco J (2020) Linking belowground microbial network changes to different tolerance level towards Verticillium wilt of olive. *Microbiome* 8:11. <https://doi.org/10.1186/s40168-020-0787-2>
- Ferris H, Matute MM (2003) Structural and functional succession in the nematode fauna of a soil food web. *Appl Soil Ecol* 23:93–110. [https://doi.org/10.1016/S0929-1393\(03\)00044-1](https://doi.org/10.1016/S0929-1393(03)00044-1)
- Fissore C, Giardina CP, Kolka RK, Trettin CC, King GM, Jurgensen MF, Barton CD, McDowell SD (2008) Temperature and vegetation effects on soil organic carbon quality along a forested mean annual temperature gradient in North America. *Glob Change Biol* 14:193–205. <https://doi.org/10.1111/j.1365-2486.2007.01478.x>
- Fowler D, Coyle M, Skiba U, Sutton MA, Cape JN, Reis S, Sheppard LJ, Jenkins A, Grizzetti B, Galloway JN, Vitousek P, Leach A, Bouwman AF, Butterbach-Bahl K, Dentener F, Stevenson D, Amann M, Voss M (2013) The global nitrogen cycle in the twenty-first century. *Philos Trans R Soc B Biol Sci* 368:20130164. <https://doi.org/10.1098/rstb.2013.0164>
- Gruber N, Galloway JN (2008) An earth-system perspective of the global nitrogen cycle. *Nature* 451:293–296. <https://doi.org/10.1038/nature06592>
- Gutknecht JLM, Field CB, Balser TC (2012) Microbial communities and their responses to simulated global change fluctuate greatly over multiple years. *Glob Change Biol* 18:2256–2269. <https://doi.org/10.1111/j.1365-2486.2012.02686.x>
- Haas JC, Street NR, Sjödin A, Lee NM, Högberg MN, Näsholm T, Hurry V (2018) Microbial community response to growing season and plant nutrient optimisation in a boreal Norway spruce forest. *Soil Biol Biochem* 125:197–209. <https://doi.org/10.1016/j.soilbio.2018.07.005>
- Harrington RA, Fownes JH, Vitousek PM (2001) Production and resource use efficiencies in N- and P-limited tropical forests: a comparison of responses to long-term fertilization. *Ecosystems* 4:646–657. <https://doi.org/10.1007/s10021-001-0034-z>
- Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC (2011) Integrative analysis of environmental sequences using MEGAN4. *Genome Res* 21:1552–1560. <https://doi.org/10.1101/gr.120618.111>
- Irshad U, Villenave C, Brauman A, Plassard C (2011) Grazing by nematodes on rhizosphere bacteria enhances nitrate and phosphorus availability to *Pinus pinaster* seedlings. *Soil Biol Biochem* 43:2121–2126. <https://doi.org/10.1016/j.soilbio.2011.06.015>
- Jost L (2006) Entropy and diversity. *Oikos* 113:363–375. <https://doi.org/10.1111/j.2006.0030-1299.14714.x>
- Kenkel NC, Derksen DA, Thomas AG, Watson PR (2002) Multivariate analysis in weed science research. *Weed Sci* 50:281–292. [https://doi.org/10.1614/0043-1745\(2002\)050\[0281:RMAIWS\]2.0.CO;2](https://doi.org/10.1614/0043-1745(2002)050[0281:RMAIWS]2.0.CO;2)
- Klein T, Siegwolf RTW, Körner C (2016) Belowground carbon trade among tall trees in a temperate forest. *Science* 352:342–344. <https://doi.org/10.1126/science.aad6188>
- Kozak M, Piepho HP (2018) What's normal anyway? Residual plots are more telling than significance tests when checking ANOVA assumptions. *J Agron Crop Sci* 204:86–98. <https://doi.org/10.1111/jac.12220>
- Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9:357–359. <https://doi.org/10.1038/nmeth.1923>
- Leewis MC, Uhlik O, Leigh MB (2016) Synergistic processing of biphenyl and benzoate: carbon flow through the bacterial community in polychlorinated-biphenyl-contaminated soil. *Sci Rep* 6:22145. <https://doi.org/10.1038/srep22145>
- Lenth RV, Bolker B, Buerkner P, Giné-Vázquez L, Herve M, Jung M, Love J, Míguez F, Riebl H, Singmann H (2023) emmeans: estimated marginal means, aka least-squares means. <https://CRAN.R-project.org/package=emmeans>. Accessed 17 Mar 2023
- Li Q, Song XZ, Gu HH, Gao F (2016) Nitrogen deposition and management practices increase soil microbial biomass carbon but decrease diversity in Moso bamboo plantations. *Sci Rep* 6:28235. <https://doi.org/10.1038/srep28235>
- Li P, Shen CC, Jiang L, Feng ZZ, Fang JY (2019) Difference in soil bacterial community composition depends on forest type rather than nitrogen and phosphorus additions in tropical montane rainforests. *Biol Fertil Soils* 55:313–323. <https://doi.org/10.1007/s00374-019-01349-8>
- Li BB, Roley SS, Duncan DS, Guo JR, Quensen JF, Yu HQ, Tiedje JM (2021) Long-term excess nitrogen fertilizer increases sensitivity of soil microbial community to seasonal change revealed by ecological network and metagenome analyses. *Soil Biol Biochem* 160:108349. <https://doi.org/10.1016/j.soilbio.2021.108349>
- Li WJ, Li Y, Lv J, He XM, Wang JL, Teng DX, Jiang LM, Wang HF, Lv GH (2022) Rhizosphere effect alters the soil microbiome composition and C, N transformation in an arid ecosystem. *Appl Soil Ecol* 170:104296. <https://doi.org/10.1016/j.apsoil.2021.104296>
- Liu ZX, Liu JJ, Yu ZH, Yao Q, Li YS, Liang AZ, Zhang W, Mi G, Jin J, Liu XB, Wang GH (2020) Long-term continuous cropping of soybean is comparable to crop rotation in mediating microbial

- abundance, diversity and community composition. *Soil Tillage Res* 197:104503. <https://doi.org/10.1016/j.still.2019.104503>
- Lu XK, Mao QG, Wang ZH, Mori T, Mo JM, Su FL, Pang ZQ (2021a) Long-term nitrogen addition decreases soil carbon mineralization in an N-rich primary tropical forest. *Forests* 12:734. <https://doi.org/10.3390/f12060734>
- Lu XK, Vitousek PM, Mao QG, Gilliam FS, Luo YQ, Turner BL, Zhou GY, Mo JM (2021b) Nitrogen deposition accelerates soil carbon sequestration in tropical forests. *Proc Natl Acad Sci* 118:e2020790118. <https://doi.org/10.1073/pnas.2020790118>
- MacArthur R (1955) Fluctuations of animal populations and a measure of community stability. *Ecology* 36:533. <https://doi.org/10.2307/1929601>
- Montoya JM, Pimm SL, Solé RV (2006) Ecological networks and their fragility. *Nature* 442:259–264. <https://doi.org/10.1038/nature04927>
- Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Solymos P, Stevens MHH, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, Caceres MD, Durand S, Evangelista HBA, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill MO, Lahti L, McGlenn D, Ouellette MH, Cunha ER, Smith T, Stier A, Braak CJFT, Weedon J (2022) Vegan: community ecology package. <https://CRAN.R-project.org/package=vegan>. Accessed 15 Mar 2023
- Pallmann P, Schaarschmidt F, Hothorn LA, Fischer C, Nacke H, Priesnitz KU, Schork NJ (2012) Assessing group differences in biodiversity by simultaneously testing a user-defined selection of diversity indices. *Mol Ecol Resour* 12:1068–1078. <https://doi.org/10.1111/1755-0998.12004>
- Pande S, Kost C (2017) Bacterial unculturability and the formation of intercellular metabolic networks. *Trends Microbiol* 25:349–361. <https://doi.org/10.1016/j.tim.2017.02.015>
- Piepho HP (2018) Letters in mean comparisons: what they do and don't mean. *Agron J* 110:431–434. <https://doi.org/10.2134/agronj2017.10.0580>
- Piepho HP, Edmondson RN (2018) A tutorial on the statistical analysis of factorial experiments with qualitative and quantitative treatment factor levels. *J Agron Crop Sci* 204:429–455. <https://doi.org/10.1111/jac.12267>
- Portillo-Estrada M, Korhonen JFJ, Pihlatie M, Pumpanen J, Frumau AKF, Morillas L, Tosens T, Niinemets Ü (2013) Inter- and intra-annual variations in canopy fine litterfall and carbon and nitrogen inputs to the forest floor in two European coniferous forests. *Ann for Sci* 70:367–379. <https://doi.org/10.1007/s13595-013-0273-0>
- Ramette A (2007) Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol* 62:142–160. <https://doi.org/10.1111/j.1574-6941.2007.00375.x>
- Revelle W (2022) Psych: procedures for psychological, psychometric, and personality research. <https://CRAN.R-project.org/package=psych>. Accessed 16 Mar 2023
- Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure. *New Phytol* 171:41–53. <https://doi.org/10.1111/j.1469-8137.2006.01750.x>
- Rybakova D, Mancinelli R, Wikström M, Birch-Jensen AS, Postma J, Ehlers RU, Goertz S, Berg G (2017) The structure of the *Brassica napus* seed microbiome is cultivar-dependent and affects the interactions of symbionts and pathogens. *Microbiome* 5:104. <https://doi.org/10.1186/s40168-017-0310-6>
- Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C (2011) Metagenomic biomarker discovery and explanation. *Genome Biol* 12:R60. <https://doi.org/10.1186/gb-2011-12-6-r60>
- Sharpton TJ (2014) An introduction to the analysis of shotgun metagenomic data. *Front Plant Sci* 5:1–14. <https://doi.org/10.3389/fpls.2014.00209>
- Shi Y, Fan KK, Li YT, Yang T, He JS, Chu HY (2019) Archaea enhance the robustness of microbial co-occurrence networks in Tibetan Plateau soils. *Soil Sci Soc Am J* 83:1093–1099. <https://doi.org/10.2136/sssaj2018.11.0426>
- Soong JL, Fuchslueger L, Marañon-Jimenez S, Janssens IA, Penuelas J, Richter A (2020) Microbial carbon limitation: the need for integrating microorganisms into our understanding of ecosystem carbon cycling. *Glob Change Biol* 26:1953–1961. <https://doi.org/10.1111/gcb.14962>
- Tuomisto H (2011) Commentary: do we have a consistent terminology for species diversity? Yes, if we choose to use it. *Oecologia* 167:903–911. <https://doi.org/10.1007/s00442-011-2128-4>
- van der Heijden MGA, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol Lett* 11:296–310. <https://doi.org/10.1111/j.1461-0248.2007.01139.x>
- Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. *Soil Biol Biochem* 19:703–707. [https://doi.org/10.1016/0038-0717\(87\)90052-6](https://doi.org/10.1016/0038-0717(87)90052-6)
- Vitousek PM, Farrington H (1997) Nutrient limitation and soil development: experimental test of a biogeochemical theory. *Biogeochemistry* 37:63–75. <https://doi.org/10.1023/A:1005757218475>
- Walker TW, Syers JK (1976) The fate of phosphorus during pedogenesis. *Geoderma* 15:1–19. [https://doi.org/10.1016/0016-7061\(76\)90066-5](https://doi.org/10.1016/0016-7061(76)90066-5)
- WallisDeVries MF, Bobbink R (2017) Nitrogen deposition impacts on biodiversity in terrestrial ecosystems: mechanisms and perspectives for restoration. *Biol Conserv* 212:387–389. <https://doi.org/10.1016/j.biocon.2017.01.017>
- Wang C, Liu DW, Bai E (2018a) Decreasing soil microbial diversity is associated with decreasing microbial biomass under nitrogen addition. *Soil Biol Biochem* 120:126–133. <https://doi.org/10.1016/j.soilbio.2018.02.003>
- Wang H, Liu SR, Zhang X, Mao QG, Li XZ, You YM, Wang JX, Zheng MH, Zhang W, Lu XK, Mo JM (2018b) Nitrogen addition reduces soil bacterial richness, while phosphorus addition alters community composition in an old-growth N-rich tropical forest in southern China. *Soil Biol Biochem* 127:22–30. <https://doi.org/10.1016/j.soilbio.2018.08.022>
- Wang JQ, Shi XZ, Zheng CY, Suter H, Huang ZQ (2021) Different responses of soil bacterial and fungal communities to nitrogen deposition in a subtropical forest. *Sci Total Environ* 755:142449. <https://doi.org/10.1016/j.scitotenv.2020.142449>
- Wu J, Joergensen RG, Pommerening B, Chaussod R, Brookes PC (1990) Measurement of soil microbial biomass C by fumigation-extraction—an automated procedure. *Soil Biol Biochem* 22:1167–1169. [https://doi.org/10.1016/0038-0717\(90\)90046-3](https://doi.org/10.1016/0038-0717(90)90046-3)
- Wu JP, Liu WF, Zhang WX, Shao YH, Duan HL, Chen BD, Wei XH, Fan HB (2019) Long-term nitrogen addition changes soil microbial community and litter decomposition rate in a subtropical forest. *Appl Soil Ecol* 142:43–51. <https://doi.org/10.1016/j.apsoil.2019.05.014>
- Yan GH, Xing YJ, Wang JY, Zhang Z, Xu LJ, Han SJ, Zhang JH, Dai GY, Wang QG (2018) Effects of winter snowpack and nitrogen addition on the soil microbial community in a temperate forest in northeastern China. *Ecol Indic* 93:602–611. <https://doi.org/10.1016/j.ecolind.2018.05.048>
- Yang XJ, Wang X, Wang K, Su LX, Li HM, Li R, Shen QR (2015) The nematocidal effect of Camellia seed cake on root-knot nematode *Meloidogyne javanica* of banana. *PLoS ONE* 10:e0119700. <https://doi.org/10.1371/journal.pone.0119700>
- Yang DL, Pan LX, Peng P, Dang XQ, Li CF, Li T, Long MX, Chen J, Wu YJ, Du HH, Luo B, Song Y, Tian R, Luo J, Zhou ZY, Pan

- GQ (2017) Interaction between SWP9 and polar tube proteins of the microsporidian *Nosema bombycis* and function of SWP9 as a scaffolding protein contribute to polar tube tethering to the spore wall. *Infect Immun* 85:e00872-e916. <https://doi.org/10.1128/IAI.00872-16>
- Yin CT, Hulbert SH, Schroeder KL, Mavrodi O, Mavrodi D, Dhingra A, Schillinger WF, Paulitz TC (2013) Role of bacterial communities in the natural suppression of *rhizoctonia solani* bare patch disease of wheat (*Triticum aestivum* L.). *Appl Environ Microbiol* 79:7428–7438. <https://doi.org/10.1128/AEM.01610-13>
- Yuan MM, Guo X, Wu LW, Zhang Y, Xiao NJ, Ning DL, Shi Z, Zhou XS, Wu LY, Yang YF, Tiedje JM, Zhou JZ (2021) Climate warming enhances microbial network complexity and stability. *Nat Clim Change* 11:343–348. <https://doi.org/10.1038/s41558-021-00989-9>
- Zak DR, Freedman ZB, Upchurch RA, Steffens M, Kögel-Knabner I (2017) Anthropogenic N deposition increases soil organic matter accumulation without altering its biochemical composition. *Glob Change Biol* 23:933–944. <https://doi.org/10.1111/gcb.13480>
- Zeng J, Liu XJ, Song L, Lin XG, Zhang HY, Shen CC, Chu HY (2016) Nitrogen fertilization directly affects soil bacterial diversity and indirectly affects bacterial community composition. *Soil Biol Biochem* 92:41–49. <https://doi.org/10.1016/j.soilbio.2015.09.018>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.