

RESEARCH ARTICLE

Soil fungal community is more sensitive to nitrogen deposition than increased rainfall in a mixed deciduous forest of China

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ABSTRACT

Nitrogen (N) deposition and intensified rainfall can strongly affect soil microbial community, but compared with available studies on bacteria, those on soil fungi are quite limited. Here we carried out a field experiment in a mixed deciduous forest of China to study the influences of increased N deposition and rainfall on soil fungi by using quantitative PCR and high-throughput sequencing method. The results demonstrated that (1) N addition significantly increased fungal abundance and alpha diversity (richness, Shannon index and Invsimpson index), changed fungal community composition at OTU level, and marginally increased the relative abundance of Ascomycota and Zygomycota, while water addition showed no remarkable effects on fungal abundance, biodiversity and community composition. (2) N addition significantly increased the richness of saprotrophic fungi and pathogenic fungi, and the relative abundance of saprotrophic fungi, but water addition only slightly increased the abundance of pathogenic fungi. (3) Fungal composition dissimilarity closely correlated with the disparity of soil parameters as a whole. Soil $\text{NH}_4^+\text{-N}$ exhibited strong positive correlation with the richness of pathogenic fungi and mycorrhizal fungi, while both soil moisture and $\text{NH}_4^+\text{-N}$ tightly correlated with soil fungal abundance and alpha diversity indices. We concluded that in this N-limited but non-water-limited forest ecosystem, N deposition posed stronger effects on soil fungi than increased rainfall, partially mediated by changes in soil properties.

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1 Introduction

Global change is disrupting nearly all ecosystems on earth which would continue in the near future (Ladau et al., 2018). Nitrogen (N) deposition, as an important component of global change, is not an exception. Nitrogen deposition rate has dramatically increased worldwide over the last century (Galloway et al., 2004). As summarized by Zhao et al. (2018), increased N deposition has brought numerous negative effects on terrestrial ecosystem, such as decline in biodiversity and productivity, soil acidification, nutrient imbalance and forest degradation. Concurrent with increased N deposition, the average rainfall, in many mid-latitude regions, would also gradually increase (IPCC 2013). Increased rainfall has drawn broad attentions as it could alter the composition and structure of plant community and aboveground net primary productivity (ANPP), and increase the loss of soil nutrient through leaching (Zhao et al., 2018). Intensified rainfall could also stimulate soil respiration and greenhouse gases emissions, such as CO₂ and N₂O (Brown et al., 2012; Liu et al., 2016; Ni et al., 2019).

Fungi are ubiquitous in all kinds of ecosystems and play crucial roles in ecosystem functioning, such as decomposition and nutrient cycling (Thorn, 1997; Aguilar-Trigueros et al., 2015). According to their trophic modes, fungi can be classified into three groups: (1) saprophytic fungi obtaining nutrients by breaking down necromass or organic matter; (2) pathogenic fungi harnessing living hosts (including phagotrophs); and (3) mycorrhizal fungi (symbiotic fungi) receiving carbohydrates by exchanging resources with host plants (Nguyen et al., 2016). Saprophytic fungi decompose plant litter, transfer litter-derived C into soil, translocate soil-derived inorganic N up into the litter layer and assimilate carbon and other nutrients into fungal biomass, play a vital role in C and N cycle (Frey et al., 2003). While, pathogenic fungi, can infect certain plants, animals or other fungi (Gilbert and Webb, 2007; Dagenais and Keller, 2009; Looby and Treseder, 2018), causing diseases, and changing their community composition and structure. Mycorrhizal fungi, can form mutualistic symbiosis with most terrestrial plants and confer lots of benefits, such as improving plant uptake of mineral nutrients and water to exchange for carbohydrates (Smith and Read, 2008) and protecting host plant from biotic (Affokpon et al., 2011) and abiotic stresses (Chen et al., 2018; Wu et al., 2018).

In view of the important functions of soil fungi, many studies examined the impacts of N deposition on soil fungi. In general, simulated N deposition by N addition can increase soil fungal abundance (Zhou et al., 2016; Wang et al., 2018a) but decrease fungal alpha diversity, while fungal compositions at phylum level seemed to be resistant to N addition (Wang et al., 2018b). However, the effects of N deposition on soil fungi could be highly variable and inconsistent, as influenced by ecosystem types, soil conditions, N addition rate, duration of treatment, and other factors that can directly or indirectly alter the resource quantity and quality for soil fungi (Keiblinger

et al., 2010; Li et al., 2018). Similarly, the effects of increased rainfall and N–water interaction on fungal community are also largely elusive. Results of limited studies are highly variable: rainfall increment could have positive (McGuire et al., 2012), negative (Hawkes et al., 2011) or no effects (She et al., 2018) on fungal alpha diversity. For the impacts of elevated rainfall on soil fungal community composition, different studies also failed to reach a general conclusion (Hawkes et al., 2011; She et al., 2018). Moreover, increased rainfall could weaken the effects of N deposition on fungal alpha diversity (Zhang et al., 2018a), but no significant interactive impacts of N and water on fungal alpha diversity and community composition were detected in She et al. (2018).

In addition to general fungal biodiversity and community composition, the response of different fungal groups following N input has attracted more and more attentions. Latest research indicated that the relative abundance of Ascomycota responded positively to N addition (Zhou et al., 2016; She et al., 2018) and the proportion of saprotrophic fungi could also increase following N application due to increased resource (such as N and C) availability favoring fast-growing, copiotrophic fungi (Morrison et al., 2016). In addition, it was reported that application of inorganic fertilizer (N, P and K) increased the relative abundance of potentially pathogenic fungi (Hu et al., 2017; Wang et al., 2018a). By contrast, the richness and abundance of mycorrhizal fungi would decrease following high dose N input owing to the decline of dependence of plant on them under increased N availability, soil acidification and changes in aboveground plant community (Rousk et al., 2010; Huang et al., 2014; Chen et al., 2017; Barnes et al., 2018). On the other hand, saprophytic fungi can be affected by soil temperature and humidity conditions (Meier et al., 2010; Looby and Treseder, 2018), and are likely to benefit from enhanced rainfall (Barnes et al., 2018), which may be mediated by microclimate, soil moisture and other processes. Similarly, increased moisture caused by elevated rainfall can promote the growth, proliferating and dispersal of existing pathogenic fungi (Woods et al., 2005; La Porta et al., 2008) and the emergence of new pathogenic fungi favoring wet conditions.

Compared to scanty studies on the effects of changes in precipitation patterns on fungal community in grassland ecosystems with water deficiency, only few reports are from well-watered forest ecosystems. Forest ecosystems normally have higher biodiversity than arid or semi-arid grassland ecosystems, and high biodiversity is likely linked with high stability of ecosystem processes in response to environmental changes (Loreau and de Mazancourt, 2013); on the other hand, in well-watered ecosystems, the effects of increased water may be different from arid or semi-arid ecosystems in which water is one of the main limiting factors for productivity. Therefore, soil fungi in forest ecosystems may have different feedback to increased precipitation, which still need solid evidences. The last but not of least importance is that N or water application in most studies on forest ecosystems were

conducted in the understory, which may not faithfully reflect natural conditions. As well known, many N-related ecological processes occurred in canopy, such as N uptake, volatilization and transformation (Zhang et al., 2015). On the other hand, rainfall can be partitioned into interception, throughfall and stemflow by forest canopy. Throughfall is defined as the rainfall that drip from canopy surfaces or through gaps, while stemflow is the rainfall that drains from outlying crown components and then is converged to the stem (Rosier et al., 2015a). As summarized by Rosier et al. (2015a), throughfall and stemflow are differentially enriched with diverse substances, including micro- and macronutrients, heavy metals and dissolved or particulate organic matter, some of which are common allelochemicals. Therefore, canopy rainfall partitioning could play an important role in structuring soil microbial community through altering the amount of throughfall and soil characteristics, such as soil moisture, soil nutrients and concentration of allelochemicals (Rosier et al., 2015a, 2015b, 2016). Additionally, canopy rainfall partitioning may also change microbial density through influencing fine root distribution and the supplies of water and nutrients along root pathways (Rosier et al., 2015a).

Due to limited knowledge of the interactive effects of N deposition and rainfall regime on soil fungal community in forest ecosystem, we carried out a field trial to study the impacts of canopy addition of N and water on soil fungal community in a mixed forest of China. Our hypotheses are as follows: (1) Nitrogen deposition could significantly increase fungal abundance but decrease fungal alpha diversity and alter fungal community composition, while water addition would have no significant effects in the non-water-limiting forest ecosystem. (2) Nitrogen or water addition would have positive effects on the abundance and richness of pathogenic and saprotrophic fungi but negative effects on that of mycorrhizal fungi. (3) Impacts of N and water addition on soil fungal community would be partially mediated by changes in soil properties.

2 Materials and methods

2.1 Study site

The experiment was carried out in Jigongshan (JGS) National Nature Reserve (31°46'–31°52' N, 114°01'–114°06' E), Xinyang, Henan, China. The soil type is Ferri-Udic Argosols (yellow-brown sandy-loam soil) and the soil pH value is 5.0–6.0. Based on meteorological data over the past 60 years (1951–2011), the local mean annual temperature (MAT) is 15.2°C and the mean annual precipitation (MAP) is 1119 mm, of which 80% occurs in April–October (Zhang et al., 2015). The background N deposition rate in precipitation is 19.6 kg N ha⁻¹yr⁻¹, in which the NH₄⁺/NO₃⁻ ratio is close to 1 (Zhang et al., 2015). The dominant tree species at the JGS area were *Quercus acutissima* Carruth., *Quercus variabilis* Bl., and *Liquidambar formosana* Hance, and more information of JGS Reserve were described by Zhang et al. (2015).

2.2 Experimental design

This experiment was a completely randomized two-factor block design with canopy addition of N and water since 2013. There were four blocks in total, and each block included four circular plots with 17 m in radius. Within each block, each plot was randomly assigned with one of the four treatments: control (CK, ambient environment); canopy addition of N (CN) at 25 kg ha⁻¹ yr⁻¹; canopy addition of water (CW) equal to total amount of 336 mm precipitation (30% of MAP) every year; and canopy addition of both N and water (CNW). The interference among treatments was minimized as there was a buffer zone between any two plots (at least 20 m). A polyvinylchloride (PVC) board (1 m in depth) was inserted in the middle of the buffer zone.

Nitrogen was applied as NH₄NO₃ solution with a concentration of 3.85 mmol L⁻¹, both the solvent and water were collected from the local lake. Nitrogen and water were added during the growing season from April to October, and N was applied once a month (totally seven times per year), while water was added once a week (12 mm per week) for minimizing surface runoff. Nitrogen solution or water were added at almost 5 m above the forest canopy using canopy spraying system built in the center of each plot (Fig. 1). The composition, working principle and efficiency of the system were fully described by Zhang et al. (2015) and Shi et al. (2018).

2.3 Soil sampling and measurement of soil properties

Soil samples were collected in late November of year 2017 after 5-year experimental treatment. Ten soil cores (3 cm diameter, 10 cm depth) per plot were taken and mixed into one soil sample. The fresh soil samples were transported to laboratory on ice bag, passed through a 2-mm sieve, and divided into 2 subsamples. One was for the DNA extraction, the other for analysis of soil parameters.

Soil available phosphorus (AP) was determined by the Mo-Sb anti-spectrophotometry method after extraction with 0.5 M NaHCO₃ (Olsen et al., 1954). Soil pH was measured using PB-10 pH-meter (Sartorius, Göttingen, Germany) in a 1:2.5 (w/v) aqueous solution. Soil organic matter (SOM) was measured according to Walkley (1947). Total carbon (TC) and total N (TN) were determined using an element analyzer (Vario EL, Elementar, Germany) and C/N ratio was calculated based on TC and TN. Soil moisture content was measured gravimetrically by oven drying the fresh soil samples to constant weight at 105°C. Soil available N (AN) including NH₄⁺-N and NO₃⁻-N were extracted with 2 mol L⁻¹ KCl with a soil/water ratio of 1: 5 and measured using a continuous flow analyzer (SAN + + , Skakar, Breda, Holland). Soil N/P ratio was calculated based on AN and AP.

2.4 Soil DNA extraction and real-time quantitative PCR (qPCR)

Soil DNA was extracted from 0.25 g freeze-dried soil sample



Fig. 1 Photography of the canopy spraying system.

using Power-Soil[®] DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA USA) according to the manufacturer's instructions. The quantity and quality of DNA samples were measured with NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA USA).

The fungal 18S gene copy numbers of all samples were determined by CFX Manager (Bio-Rad Laboratories Inc., Hercules, CA USA) with the forward primer 5'-CGGCTACCA-CATCCAAGGAA-3' and the reverse primer 5'-GCTGGAAT-TACC GCGGCT-3'. The standard curve was generated from a clone with the 18S gene correct insert in 10-fold serial dilutions. The 10 μ L PCR reaction mixture contained 5.0 μ L 2 \times SG Green qPCR Mix (Kapa Biosystems, Wilmington, MA USA), 0.2 μ L (20 μ M) of each primer, 3.6 μ L ddH₂O and 1.0 μ L DNA template. The reaction conditions comprised an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 62.5°C for 20 s, and 72°C for 10 s. Four technical replicates were performed for each sample. The fungal 18S gene copy number was calculated according to a regression equation to convert the cycle threshold (Ct value) to the known number of gene copies in standards.

2.5 Illumina HiSeq sequencing and bioinformatics

We conducted PCR amplification with tagged primer pair ITS1F/ITS2 (targeting at ITS1 region for Illumina sequencing) (Rajala et al., 2012). The PCR reaction system was 50 μ L, which contained 5.0 μ L 10 \times Ex *Taq* Buffer (Mg²⁺ plus), 4.0 μ L dNTP mixture, 0.5 μ L Ex *Taq* (5U μ L⁻¹) (TaKaRa, Dalian, China), 1.5 μ L (10 μ M) of each primer, 36.5 μ L sterilized water and 1.0 μ L DNA template. The PCR amplification program were as follows: 98°C for 3 min; 30 cycles at 98°C for 10 s, 54°C for 30 s, 72°C for 30s; followed by 72°C for 10 min. Each sample was amplified in duplicates (technical repetition to minimize the stochastic error) and then mixed into one representing corresponding sample.

PCR products were separated through a 1.5% agarose (Biowest, Nuaille, France) gel in 1 \times TAE, bands were excised, and purified with GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA USA). The amount of DNA in the purified PCR products were measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA USA) and mixed at equimolar concentrations, then used to construct DNA library using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA USA) following the standard workflow. Qualified library was sequenced on the Illumina HiSeq2500 (PE250) platform at Beijing Fixgene Technology Co., Ltd (Beijing, China).

The initial quality filtering and assembling of paired-end reads were performed by Beijing Fixgene Technology Co., Ltd. Quality control was achieved by NGSTQCToolkit v 2.3.3. Main procedures were as follows: (1) Remove the low-quality reads (Q value < 20); (2) Delete reads containing adapters (short reads). Paired-end reads were assembled by FLASH v.2.2.0 (Magoč and Salzberg, 2011). Raw sequences data have been submitted to Sequence Read Archive database of NCBI under the BioProject ID PRJNA561473. Sequences with maxhomop > 8, maxambig > 0 or shorter than 200 bp were removed. The detection and deletion of chimeras and OTU clustering at a 97% similarity cutoff were achieved in Usearch v.9.0.2132_i86linux32 (Edgar, 2010). OTUs < 5 sequences were discarded. Taxonomic assignment was performed by blasting the representative sequence of each OTU against UNITE database v.7.2 (release date 12.01.2017) (Nilsson et al., 2019) using the BLASTN search algorithm (Altschul et al., 1997). Only OTUs with representative sequences belong to fungi with similarity \geq 90%, e value < e⁻⁵⁰, alignment length > 150 bp and singleton with identity > 98% were used in subsequent analysis.

We classified fungal OTUs into functional groups according to FUNGuild database (Nguyen et al., 2016). We only focused on taxa with confidence levels "highly probable" or "probable"

and unique trophic guilds which belong to saprotrophic fungi, pathogenic fungi or mycorrhizal fungi. Saprotrophic fungi included undefined saprotroph, wood saprotroph, soil saprotroph, plant saprotroph, litter saprotroph and dung saprotroph; pathogenic fungi referred to plant pathogen, animal pathogen and fungal parasite; mycorrhizal fungi consisted of ectomycorrhizal (EcM) fungi and arbuscular mycorrhizal (AM) fungi.

2.6 Statistical analysis

All statistical analyses were performed in R (R Development Core Team). To avoid bias caused by different sequencing depth, samples were resampled to the minimum 27 061 sequences per sample, and then 'Hellinger' transformed to calculate fungal alpha diversity indices (species richness, Shannon and InvSimpson). The alpha diversity indices were calculated using the function 'diversity' in R package 'vegan' (Oksanen et al., 2013). To analyze the effects of N and water addition and their interactions on soil properties, fungal abundance and alpha diversity indices, two-way analysis of variance (ANOVA) was performed, followed by Duncan's multiple range test. The significant difference was accepted at $P < 0.05$. To assess the impacts of N, water addition and their interactions on fungal community composition, two-way permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2010) were performed with the "Bray-Curtis dissimilarity" using the function 'adonis2' in R package 'vegan' (Oksanen et al., 2013) with 9999 permutations. To analyze the relationship between soil parameters (available phosphorus (AP), pH, soil organic matter (SOM), total carbon (TC), total N (TN), soil moisture, NH_4^+ -N, NO_3^- -N, available N (AN), C/N ratio, N/P ratio) and fungal abundance, alpha diversity indices or the richness of each functional group, we performed Pearson correlation analysis. Mantel test was conducted to analyze the disparity of soil factors and the dissimilarity of fungal community composition. The disparity of soil factors was evaluated by 'Euclidean distance' and the

dissimilarity of fungal community composition was determined by 'Bray-Curtis distance', both were implemented by 'vegdist' function in R package 'vegan' (Oksanen et al., 2013). To identify the contribution of each soil parameters, we conducted variation partition analysis using 'varpart' function in R package 'vegan' (Oksanen et al., 2013).

In all the analyses involving fungal alpha diversity indices, the abundance and richness of each functional group, and soil parameters, Dixon's Q test was performed at 95% confidence level in order to discover outliers (Dean and Dixon, 1951), and some data, including NO_3^- -N, AN and N/P, were sqrt transformed to satisfy the assumption of normality.

3 Results

3.1 Changes of soil parameters after canopy addition of N and water

For all soil parameters, only pH value was significantly decreased by canopy N addition, while no any significant changes were induced by water addition (Table 1).

3.2 Effects of canopy addition of N and water on fungal abundance

Nitrogen input significantly increased fungal abundance, while, water addition had no significant influence; moreover, there was a significant N-water interactions: N addition increased fungal abundance in case of no water addition only, but no significant effects with water addition (Table 2).

3.3 The overall output of DNA-sequencing

After filtering out chimeras, 2 199 448 sequences were kept and clustered into 3238 OTUs; After discarding OTUs with less than 5 sequences, there were 2969 OTUs (2 198 412 sequences). Following blasting against UNITE database,

Table 1 Effects of canopy addition of nitrogen and water on soil parameters.

	Treatments				Significance		
	CK	CN	CW	CNW	N (F, P)	W (F, P)	N × W (F, P)
AP (mg/kg)	22.86±6.35(a)	18.24±3.90(a)	25.95±1.40(a)	35.74±4.88(a)	0.530, 0.485	1.258, 0.291	0.146, 0.711
pH	4.49±0.07(a)	4.29±0.04(b)	4.57±0.07(a)	4.27±0.04(b)	16.664, 0.003	0.067, 0.802	0.952, 0.355
SOM (g/kg)	33.16±3.64(a)	35.55±2.76(a)	33.78±3.86(a)	34.43±1.31(a)	0.000, 0.989	0.364, 0.561	0.657, 0.439
TC (%)	3.33±0.62(a)	3.28±0.31(a)	2.96±0.28(a)	3.25±0.17(a)	0.029, 0.868	1.257, 0.288	0.001, 0.971
TN (%)	0.19±0.01(ab)	0.18±0.00(b)	0.20±0.01(a)	0.18±0.00(ab)	3.017, 0.116	2.019, 0.189	0.623, 0.450
C/N	17.50±2.53(a)	18.50±1.24(a)	14.47±1.01(a)	18.29±0.84(a)	1.021, 0.339	4.372, 0.066	0.058, 0.816
Moisture	18.14±0.83(a)	20.70±0.73(a)	19.66±1.19(a)	23.37±0.93(a)	4.343, 0.067	0.835, 0.385	0.641, 0.444
NH_4^+ -N (mg/kg)	3.63±0.56(a)	4.54±0.47(a)	3.89±0.44(a)	3.89±0.22(a)	0.583, 0.465	0.295, 0.600	1.101, 0.321
NO_3^- -N (mg/kg)	7.00±0.86(a)	8.93±0.86(a)	5.70±0.53(a)	4.22±2.91(a)	2.452, 0.152	0.227, 0.645	0.143, 0.714
AN (mg/kg)	10.63±1.13(a)	13.46±1.12(a)	9.59±0.92(a)	8.11±3.01(a)	2.790, 0.129	0.128, 0.729	0.035, 0.856
N/P ratio	0.53±0.09(a)	0.81±0.11(a)	0.37±0.02(a)	0.23±0.29(a)	4.232, 0.070	1.715, 0.223	0.002, 0.968

CK, control; CN, canopy addition of N; CW, canopy addition of water; CNW, canopy addition of N and water. Data are presented as means± standard error (SE). N, N addition; W, water addition; N×W, the interaction. Significance of treatment effect was determined by two-way ANOVA. Different letters in brackets next to SE in the same row indicate significant differences ($P < 0.05$) by Duncan's multiple range test. Significant effect ($P < 0.05$) is highlighted in bold.

Table 2 Impacts of canopy addition of N and water on fungal abundance and alpha diversity indices.

	18S gene copies (*10 ⁸ /g dry soil)	Richness	Shannon	InvSimpson
CK	2.8±0.3(b)	617.0±17.5(b)	5.8±0.05(b)	147.7±7.1(c)
CN	7.9±0.8(a)	718.0±19.1(a)	6.0±0.03(a)	214.9±7.5(a)
CW	5.5±1.3(ab)	658.0±21.6(ab)	5.9±0.05(ab)	173.2±18.2(bc)
CNW	6.2±0.7(a)	698.3±15.4(a)	6.0±0.03(a)	202.0±7.3(ab)
Significance				
N (F, P)	9.338, 0.014	11.316, 0.008	7.475, 0.023	15.196, 0.004
W (F, P)	0.246, 0.632	0.256, 0.625	0.236, 0.639	0.261, 0.622
N×W (F, P)	5.596, 0.042	2.093, 0.182	1.442, 0.260	2.441, 0.153

CK, control; CN, canopy addition of N; CW, canopy addition of water; CNW, canopy addition of N and water. Data are presented as means± standard error (SE). N, N addition; W, water addition; N×W, the interaction. Significance of treatment effect was determined by two-way ANOVA. Different letters in brackets next to SE in the same column indicate significant differences ($P < 0.05$) by Duncan's multiple range test. Significant effect ($P < 0.05$) is highlighted in bold.

certain OTUs were further excluded with representative sequences: (1) not belong to fungi; (2) e value $> e^{-50}$; (3) alignment length < 150 bp; (4) identity $< 90\%$; (5) singleton with identity $< 98\%$. Screened by strict criteria, a total of 1412 OTUs (1770382 sequences) were retained.

Among the 1412 fungal OTUs, the unclassified OTUs accounted for 2.3% and their relative abundance was 7.8% (138195/1770382). While other OTUs covered 6 phyla, among which, the dominant phyla were Ascomycota, Basidiomycota and Zygomycota (Fig. 2).

3.4 Influences of canopy addition of N and water on fungal alpha-diversity and community composition

Canopy N addition significantly increased fungal alpha-

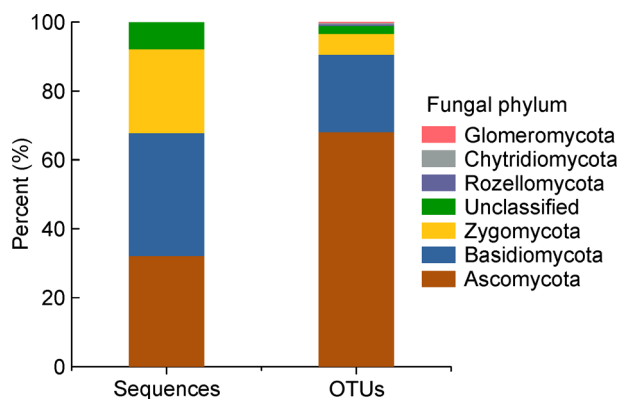


Fig. 2 The proportional distributions of sequences and derived fungal OTUs from all soil samples

diversity: richness (number of OTUs), Shannon and InvSimpson index. However, water addition showed no significant effect on any fungal alpha diversity indices. In addition, no significant interactions were detected between N and water in influencing fungal alpha diversity (Table 2).

For fungal community composition at OTU level, effect of N addition was significant, but not for water addition; nevertheless, at phylum level, both N and water showed no significant effects. Meanwhile, there were also no significant interactions of N and water no matter at OTU level or phylum level (Table 3).

For the relative abundance of the three dominant fungal phyla, N addition marginally increased the relative abundance of Ascomycota and Zygomycota; in contrast, Basidiomycota relative abundance tended to be decreased by N addition, but statistically insignificant. However, water addition had no significant influences on the relative abundance of all three fungal phyla (Fig. 3).

3.5 Effects of canopy addition of N and water on the richness and abundance of different functional groups (saprotrophic fungi, pathogenic fungi and mycorrhizal fungi)

Nitrogen addition significantly increased the richness of saprotrophic fungi and pathogenic fungi (Fig. 4A), moreover, marginal or significant interactions of N and water were observed in affecting the richness of saprotrophic fungi and pathogenic fungi: water addition weakened the positive effects of N addition. However, for the richness of mycorrhizal fungi, N, water and their interaction all showed no significant effects (Fig. 4A).

Table 3 Effects of canopy addition of nitrogen and water on fungal community composition.

	df	Composition at OTU level		Composition at phylum level	
		F-value	P-value	F-value	P-value
N	1	1.631	0.004	1.296	0.234
W	1	0.921	0.630	0.351	0.980
N × W	1	1.029	0.363	0.904	0.495

F-values and P-values are presented, determined by two-way PERMANOVA using 'adonis2' function in R package 'vegan' (Oksanen et al., 2013). N, nitrogen addition; W, water addition; N × W, the interaction. Significant effect ($P < 0.05$) is highlighted in bold.

Simultaneously, N input showed significant impacts on saprotrophic fungal abundance, while water addition marginally increased pathogenic fungal abundance, but both N and water did not significantly change mycorrhizal fungal abundance (Fig. 4B). No significant interactions of N and water were observed (Fig. 4B).

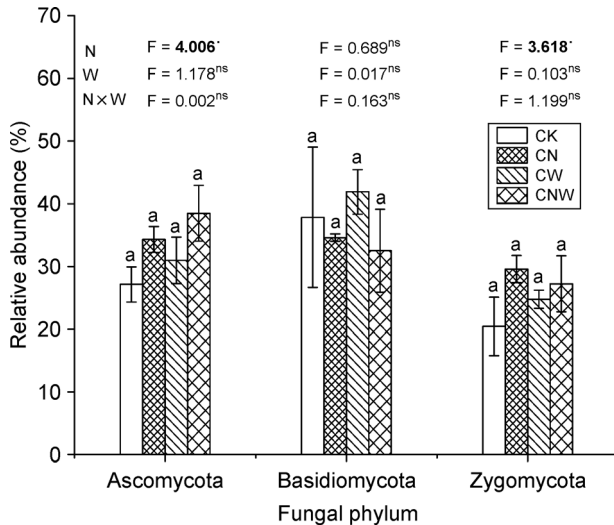
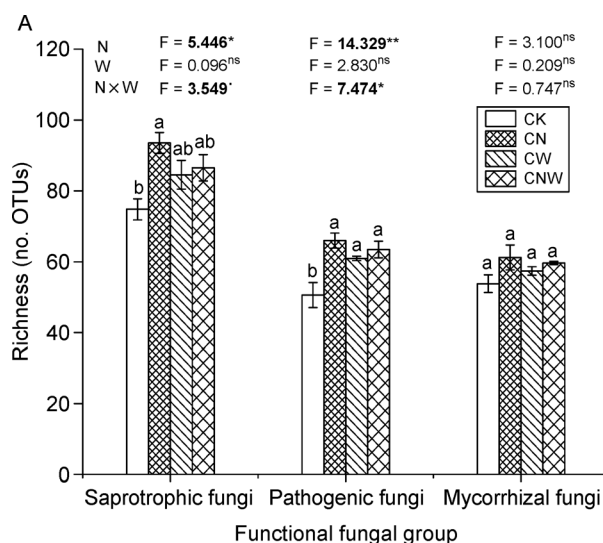


Fig. 3 Impacts of canopy addition of N and water on the relative abundances of three dominant fungal phyla (Ascomycota, Basidiomycota and Zygomycota). CK, control; CN, canopy application of N; CW, canopy addition of water; CNW, canopy addition of N and water. N, N application; W, water addition; N × W, the interaction. F values were presented and significance of treatment effect was determined by two-way ANOVA. ** $P < 0.01$; * $P < 0.05$; • $P < 0.1$; ns, not significant. Different letters above the columns indicate significant difference between corresponding treatments by Duncan's multiple range test ($P < 0.05$).



3.6 Relationships between soil factors and fungal abundance, alpha diversity, composition dissimilarity and the richness of each functional fungal group

Pearson correlation analysis demonstrated that soil moisture significantly positively correlated with fungal richness, Shannon index and InvSimpson index. Likewise, soil $\text{NH}_4^+\text{-N}$ had a significant positive correlation with fungal abundance, InvSimpson index, and marginally positively correlated with fungal richness and Shannon index (Table 4).

In addition, there was a significant positive correlation between fungal community composition dissimilarity and the disparity of all soil factors indicated by Mantel test (Table 5). Eighteen percent the variation in fungal community composition can be explained by changes of soil factors as a whole, among which soil N/P ratio, AP, soil moisture, and pH value could individually explain 5%, 4%, 3%, and 1%, respectively (Table 5).

Pearson correlation analysis also demonstrated that soil moisture and the richness of saprotrophic fungi and pathogenic fungi were weakly correlated, meanwhile, the correlation between soil $\text{NH}_4^+\text{-N}$ content and the richness of saprotrophic fungi, pathogenic fungi and mycorrhizal fungi were marginally significant, significant and highly significant, respectively (Table 6). In addition, soil N:P ratio slightly positively correlated with mycorrhizal fungal richness (Table 6).

4 Discussion

To understand the effects of multiple climate factors on soil fungal community under natural conditions can help predicting the response of terrestrial ecosystem to future climate

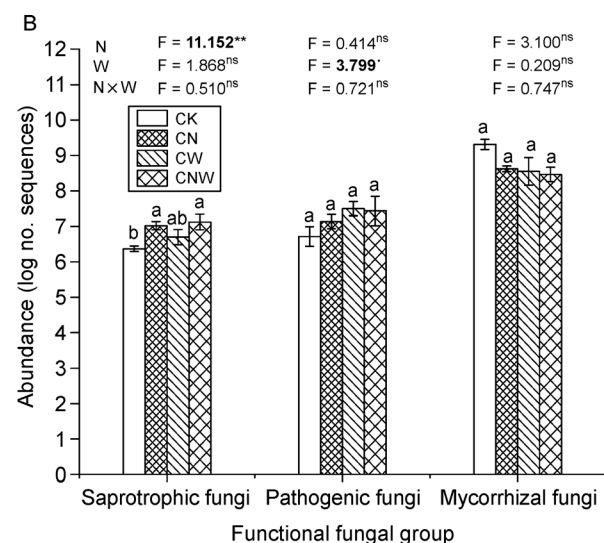


Fig. 4 Effects of canopy addition of N and water on the richness (A) and abundance (B) of three functional fungal groups. CK, control; CN, canopy application of N; CW, canopy addition of water; CNW, canopy addition of N and water. N, N application; W, water addition; N × W, the interaction. F values were presented and significance of treatment effect was determined by two-way ANOVA. ** $P < 0.01$; * $P < 0.05$; • $P < 0.1$; ns, not significant. Different letters above the columns indicate significant difference between corresponding treatments by Duncan's multiple range test ($P < 0.05$).

Table 4 Pearson correlation analysis between soil properties and fungal abundance and alpha diversity indices at OTU level.

	df	Abundance		Richness		Shannon		InvSimpson	
		r	P	r	P	r	P	r	P
AP (mg/kg)	14	-0.051	0.574	0.060	0.413	0.157	0.281	0.170	0.264
pH	14	-0.182	0.750	-0.185	0.754	-0.097	0.639	-0.293	0.865
SOM (g/kg)	14	-0.166	0.730	-0.162	0.726	-0.249	0.824	-0.034	0.550
TC (%)	14	-0.164	0.728	-0.281	0.854	-0.364	0.917	-0.149	0.710
TN (%)	14	-0.320	0.887	-0.297	0.868	-0.324	0.889	-0.215	0.789
C/N	14	0.005	0.493	-0.150	0.710	-0.247	0.822	-0.021	0.530
Moisture	14	0.417	0.054	0.536	0.016	0.529	0.018	0.649	0.003
NH ₄ ⁺ -N (mg/kg)	14	0.597	0.007	0.407	0.059	0.383	0.072	0.459	0.037
NO ₃ ⁻ -N (mg/kg)	14	0.172	0.263	0.217	0.209	0.222	0.205	0.288	0.139
AN (mg/kg)	14	0.297	0.132	0.289	0.139	0.287	0.141	0.370	0.079
N/P ratio	14	0.176	0.257	0.127	0.320	0.065	0.406	0.120	0.329

Correlation coefficients and *P*-values are presented, significant effect ($P < 0.05$) is highlighted in bold.

Table 5 Mantel test of fungal community composition dissimilarity with the disparity of soil matrices and variation partition analysis of fungal community composition dissimilarity.

	Mantel test		Variation partition analysis (explained)
	r	P	
All	0.361	0.027	18%
AP (mg/kg)	0.168	0.149	4%
pH	0.258	0.096	1%
SOM (g/kg)	0.242	0.092	0%
TC (%)	0.330	0.072	0%
TN (%)	0.247	0.096	0%
C/N	0.248	0.099	0%
Moisture	0.327	0.036	3%
NH ₄ ⁺ -N (mg/kg)	0.039	0.351	0%
NO ₃ ⁻ -N (mg/kg)	-0.119	0.727	0%
AN (mg/kg)	-0.090	0.666	0%
N/P ratio	0.032	0.302	5%

Explained proportions by soil matrices, correlation coefficients and *P*-values are presented, significant effect ($P < 0.05$) is highlighted in bold. The disparity of soil factors was evaluated by 'Euclidean distance' and the dissimilarity of fungal community composition was determined by 'Bray-Curtis distance', both were implemented by 'vegdist' function in R package 'vegan' (Oksanen et al., 2013). Variation partition analysis was conducted using 'varpart' function in R package 'vegan' (Oksanen et al., 2013).

scenarios and has far-reaching significance. We studied the influences of 5-year canopy N and water addition on soil fungal abundance, community composition and different functional groups in a mixed deciduous forest of China. The results showed that fungal abundance and alpha diversity were increased, and community composition (at OTU level) was altered by N deposition but not by rainfall increment (Tables 2 and 3). Meanwhile, soil fungal community composition, at phylum level (Table 3), was resistant to increased N and rainfall; but the relative abundance of certain fungal phyla can be marginally altered (Fig. 3). Nitrogen addition significantly increased the richness of pathogenic fungi and saprotrophic fungi and the abundance of saprotrophic fungi (Fig. 4). While, water addition only slightly increased pathogenic fungal abundance (Fig. 4B). Most importantly, counteractive N–water interactions were detected in shaping

fungal abundance (Table 2), the richness of pathogenic fungi and saprotrophic fungi (Fig. 4A). As expected, soil parameters played a role in shaping fungal community composition and the richness of each functional group (Tables 4–6).

4.1 Changes in soil properties following canopy N and water addition

In this study, soil pH value was most significantly decreased by 5-year canopy N addition (Table 1), in agreement with many previous reports (Lu et al., 2014; Tian and Niu, 2015). Possible mechanisms are summarized by Zhao et al. (2018): (1) NH₄⁺ ions are absorbed by plants leading to H⁺ release; (2) NH₄⁺ ions are oxidized to nitrates step by step, producing H⁺; (3) The loss of base cations and metal cations could abate soil buffer capacity against acidification. In addition, in the meta

Table 6 Relationships between soil properties and the richness of three functional groups.

	df	Saprotrophic fungi		Pathogenic fungi		Mycorrhizal fungi	
		r	P	r	P	r	P
AP (mg/kg)	14	-0.013	0.519	0.102	0.354	-0.219	0.784
pH	14	-0.051	0.574	-0.200	0.771	-0.351	0.900
SOM (g/kg)	14	-0.117	0.667	-0.195	0.766	-0.056	0.579
TC (%)	14	-0.204	0.776	-0.412	0.944	-0.044	0.561
TN (%)	14	-0.118	0.669	-0.348	0.907	-0.218	0.782
C/N	14	-0.154	0.715	-0.256	0.831	0.132	0.319
Moisture	14	0.396	0.064	0.385	0.070	0.323	0.120
NH ₄ ⁺ -N (mg/kg)	14	0.339	0.099	0.450	0.040	0.679	0.003
NO ₃ ⁻ -N (mg/kg)	14	0.270	0.156	-0.018	0.527	0.102	0.359
AN (mg/kg)	14	0.312	0.120	0.097	0.361	0.294	0.144
N/P ratio	14	0.122	0.326	0.019	0.472	0.367	0.089

Correlation coefficients and *P*-values are presented, significant effect ($P < 0.05$) is highlighted in bold.

analysis of Tian et al. (2018), the content of soil total N in temperate forests of China were not significantly increased by N addition, this paper also confirmed this (Table 1). This could be attributed to low dosage and short duration of N addition, and increased N leaching (Tian et al., 2018) and raised emissions of N oxides (Butterbach-Bahl et al., 1997).

4.2 Impacts of N and water addition on soil fungal abundance and community composition

In the present study, N addition significantly increased fungal abundance, confirming our first hypothesis in line with Zhou et al. (2016) and Wang et al. (2018a). The possible mechanisms were: (1) Increased N availability directly released N limitation for supporting soil fungal growth; (2) N input may change plant growth and indirectly alter resource quantity and quality for soil fungi (Keiblinger et al., 2010; Li et al., 2019). Moreover, N enrichment significantly increased fungal richness (Table 2), in contravention to our first hypothesis, this may be ascribed to the ecosystem type and the N addition rate, as in the meta analysis of Wang et al. (2018b), the effect size (characterized by the difference between the mean log e-transformed outcome in the treatment group and that in the control group) of fungal Chao1 index (representing fungal richness) was positive (increased) in tundra but negative (decreased) in desert/shrublands under N addition, but no studies in forests were included; on the other hand, in case of N addition $< 5 \text{ g N m}^{-2} \text{ yr}^{-1}$, the effect of N input on fungal Chao1 index was positive; when the rate $> 5 \text{ g N m}^{-2} \text{ yr}^{-1}$, the effect became negative. The N addition rate in this study was $25 \text{ kg ha}^{-1} \text{ yr}^{-1} < 5 \text{ g N m}^{-2} \text{ yr}^{-1}$, so increased fungal richness by N addition was understandable. However, the results of a study conducted in forest ecosystems (Entwistle et al., 2013) showed that N addition had no significant effects on fungal Chao1 richness, this may be partially explained by the experimental duration: the duration of Entwistle et al. (2013) was more than 15 years, while the duration of our experiment was 5 years. When the duration was less than 5 years or more than 10 years the effect could

be nonsignificant (Wang et al., 2018b). In addition, InvSimpson index and Shannon index were also increased by N addition in our study (Table 2). Yet, N addition significantly decreased fungal Shannon index in forest ecosystems in the synthesized analysis of Wang et al. (2018b), but only 5 papers were incorporated, so the generality of the results may be limited, as they also pointed out that soil conditions, treatment duration, N addition rate and changes in soil organic carbon under N addition all influenced the effect size of N input on microbial Shannon index.

Furthermore, N input significantly altered fungal community composition at OTU level (Table 3), confirming the results of Morrison et al. (2016), who found that fungal composition was fundamentally changed by more than 20 years of N addition in a temperate hardwood forest, while contradicted to the work of McHugh et al. (2017) in a semiarid grassland. Besides difference in ecosystem types, experimental duration and N addition rate probably can explain the different outcomes: the experimental duration in our study was 5 years and in Morrison et al. (2016), it was more than 20 years, while it lasted for only 2 years in McHugh et al. (2017); in addition, N addition rates ($\leq 8 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) of McHugh et al. (2017) were very low compared with our experiment ($25 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) and that of Morrison et al. (2016) ($\geq 50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$).

Nevertheless, water addition had no significant effects on fungal abundance, in support of first hypothesis. The response could be resulted from the fact that water is not a limiting factor in our study, as speculated by Hawkes et al. (2011) that fungal responses to precipitation may be depending on the relative availability of water for metabolism, so that fungi only responded positively to rainfall in drier soils where water is limited. Similarly, fungal richness, Shannon and InvSimpson index did not respond to water input (Table 2), supporting our first hypothesis, but incompatible with the work of Hawkes et al. (2011) carried out in a grassland ecosystem, which can be ascribed to the difference in ecosystem types. Additionally, water addition also showed no impacts on fungal community composition (Table 3), confirming our first hypothesis, and supported Jumpponen and Jones (2014) that soil

fungal communities are compositionally resilient to altered rainfall regime.

At phylum level, neither Ascomycota nor Basidiomycota significantly altered their relative abundance under N addition as indicated by the meta-analysis of Wang et al. (2018b), but in our study, the relative abundance of Ascomycota was marginally increased by N addition (Fig. 3), which was in accordance with the study of She et al. (2018) carried out in a desert shrubland. This could be explained by the copiotrophic-oligotrophic theory originated from bacteria, now applied to fungi (Ho et al., 2017; Yao et al., 2017). Bacteria or fungi can be ecologically classified into copiotrophic and oligotrophic taxa. Copiotrophic taxa prefer labile soil organic C pools and flourish in habitats with high resource availability, in contrast, oligotrophic taxa are likely to outcompete copiotrophs and dominate in nutrient-poor environment due to their higher substrate affinities (Fierer et al., 2007). Ascomycota could roughly be classified into copiotrophic group (Ho et al., 2017; Yao et al., 2017), so the increase in its relative abundance under N addition was not surprising. In addition, it was reported that the relative abundance of oligotrophic Basidiomycota decreased following N enrichment (Entwistle et al., 2013), yet, in our study, Basidiomycota relative abundance tended to decrease but not significant may have been masked by large variations within treatment (Fig. 3).

4.3 Response of different functional fungal groups to N and water addition

In this study, the richness of pathogenic fungi was significantly increased following N enrichment (Fig. 4A), in support of our second hypothesis, and consistent with previous reports that application of inorganic fertilizer can increase the proportion of potential pathogenic fungi and may bring negative impacts on forest health (Paungfoo-Lonhienne et al., 2015; Hu et al., 2017). The richness and abundance of saprotrophic fungi (Fig. 4A, B), as expected, also responded positively to N addition. The thriving of saprotrophic fungi following N addition demonstrated the increased importance of decomposer and could have profound effects on N and C dynamics. Moreover, significant or marginal N–water interactions were detected on the richness of pathogenic fungi or saprotrophic fungi (Fig. 4A). The significant N–water interactions were also observed on soil fungi by Zhang et al. (2018b). It is possible that water addition enhances N loss through leaching and runoff.

In addition, the abundance of pathogenic fungi marginally positively responded to water addition (Fig. 4B), confirming the study of Barnes et al. (2018) on root-associated fungi, because increased moisture could be beneficial to the growth, reproduction and dispersal of existing pathogenic fungi (Woods et al., 2005; La Porta et al., 2008).

4.4 Soil properties mediated the effects of N and water addition on soil fungal community

We found that there were significant or marginal positive

correlations between fungal alpha diversity index and soil NH_4^+ content or soil moisture (Table 4). We speculate that N addition increased fungal alpha diversity probably via raising soil N availability and releasing N limitation. Meanwhile, the study of He et al. (2017), conducted in a subtropical forest, also showed the positive relationship between soil moisture and the Shannon index of fungal communities, which can be explained by the fact that fungi preferentially live in large soil pores, which are filled at high moisture but become empty at low moisture, and fungal community responded rapidly to soil moisture fluctuation (Kaisermann et al., 2015). Simultaneously, fungal community composition dissimilarity was positively correlated with the disparity of soil factors as a whole (Table 5). Furthermore, marginal or significant positive correlations between soil moisture or soil NH_4^+ content and the richness of saprotrophic fungi and pathogenic fungi were also recorded (Table 6), which are readily comprehensible, because high soil moisture and resource availability can be favorable to the growth and propagation of certain saprotrophic fungi and pathogens (Woods et al., 2005; La Porta et al., 2008; Richardson, 2009). Besides, mycorrhizal fungal richness showed close relationship with soil NH_4^+ content and N/P ratio (Table 6), we speculate that N input mitigated N-limitation for mycorrhizal fungi and imbalanced the nutrient supply of the ecosystem, increasing the importance of available P (Vitousek et al., 2010). These results together supported our third hypothesis.

An experiment conducted in a boreal forest ecosystem found that both N addition and season had significant impacts on fungal alpha diversity indices (Chao1 and Shannon), and their interaction was also observed in influencing fungal Shannon diversity (Yan et al., 2017). In addition, fungal community composition, at the same N addition level, was significantly different between summer and autumn. Simultaneously, the response of soil fungal community to increased rainfall also can be affected by seasonality, for example, Hawkes et al. (2011) showed that fungal communities were more abundant, diverse, and consistent under drought conditions, but less abundant, less diverse, and more variable during wetter periods and under rainfall increment treatments. In the following study, we should take the frequency and duration of watering, and seasonal and inter-annual variations into account.

5 Conclusion

In the present study we investigated the effects of increased N deposition and rainfall on the soil fungal biodiversity and community composition in forest ecosystem in a more natural way. The results demonstrated that soil fungal community composition could be altered by N addition at OTU level but showed high resistance at phylum level; however, the relative abundances of certain fungal phyla can be changed, the copiotrophic fungi can benefit from nutrient enrichment, but the relative abundance of oligotrophic fungi would be decreased. On the other hand, water addition generally

showed no remarkable effects on fungal biodiversity and community composition. The results also indicated that increased N deposition and rainfall contributed to the thriving of pathogenic fungi and saprotrophic fungi, which can bring series of influences on forest health and ecosystem functions, and research in these area should be strengthened. Further study should take plant community data, frequency and duration of watering, and seasonal and inter-annual variations into account to better understand the response of soil fungi to climate change in forest ecosystems.

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Conflict of interest

The authors declare no conflict of interest.

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