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ORIGINAL PAPER

Arbuscular mycorrhizal fungi abundance was sensitive to nitrogen addition but diversity was sensitive to phosphorus addition in karst ecosystems

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Abstract

Determining the effects of nitrogen (N) and phosphorus (P) application on arbuscular mycorrhizal fungi (AMF) communities is important for predicting AMF responses to nutrient deposition. The AMF parameters and soil properties were monitored in karst grassland after 2 years of N and P addition. Then, AMF abundance, diversity, and community composition significantly differed between seasons. AMF abundance was higher in July (summer) than in December (winter), whereas richness and Chao1 estimator values showed the opposite results. The numbers of the genera *Funneliformis* and *Sclerocystis* were significantly more abundant in December, but the proportions of *Scutellospora*, *Redeckera*, and *Diversispora* were significantly higher in July. N and NP treatments significantly increased AMF abundance; richness and Chao1 values in the P treatment were significantly higher than those of the control in July. AMF community composition changed substantially between December and July but did not respond to fertilization. AMF abundance was significantly correlated with total N (TN), while AMF richness was also significantly correlated with available P (AP) and pH. pH and nitrate N (NO_3^--N) strongly affected AMF community composition. These results suggested that P became more limiting with N fertilization, AMF investment increased access to more P, and richness was lower when certain AMF taxa (*Diversisporales*) increased in abundance during the growing season and under more P-limiting conditions. These results also suggested that N and P addition have specifically different effects on AMF abundance and diversity, and consequently potential effect on long-term vegetation composition and productivity.

Keywords Mycorrhiza · Hiseq sequencing · Grassland · N fertilizer · P fertilizer · Fragile ecosystems

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Introduction

Global atmospheric N deposition has substantially increased due to human activity (Camenzind et al. 2016; Galloway et al. 2004). Recent findings indicate that P levels under N enrichment are insufficient to balance increased N deposition, leading to severe P deficiency (Marklein and Houlton 2012). The availability of N and P, individually or in combination, has been widely recognized as a predominant factor limiting primary productivity in terrestrial ecosystems (Liu et al. 2012; Vitousek et al. 2010). Arbuscular mycorrhizal fungi (AMF) are of great ecological significance because they form obligate symbioses that provide P and N to 70–90% of plant species (Taylor et al. 2017). AMF can increase P acquisition, water uptake, and soil stability (Rillig 2004), and they are sensitive to N and P input (He et al. 2016). Cozzolino et al. (2010) reported that the addition of small quantities of P positively

influences AMF colonization when available soil P is low. N and P inputs are widely believed to affect (positively, negatively, or non-significantly) AMF abundance, diversity, and community composition by changing the soil microenvironment (Camenzind et al. 2014; Cozzolino et al. 2010; Xie et al. 2014; Zheng et al. 2014). However, N and P inputs in varied ecosystems with either nutrient saturation or limitation affect AMF communities differently (Delavaux et al. 2017; Matson et al. 1999). Therefore, the determination of the individual and combined effects of N and P on AMF communities is important for predicting AMF feedback to global N deposition, and also to improve our understanding of the effects AMF have on N and P processes.

N and P application might affect soil AMF abundance, diversity, and community composition in a number of ways. First, N and P addition may temporarily increase soil acidity, leading to increased losses of cations and decreased availability of P, thus indirectly affecting AMF abundance and community composition. Some AMF species are only found in acidic or alkaline soils, but others can inhabit both soils (Porter et al. 1987). Liu et al. (2013) found no significant correlation between N addition and soil pH in an N-saturated system. Therefore, in N-limited or alkaline soil, soil pH would be sensitive to N and P addition, thereby affecting AMF community composition (Nottingham et al. 2017). Other studies have shown that N and P applications can markedly change N and P availability (e.g., NH_4^+ or NO_3^-) (Egerton-Warburton et al. 2007; Wang et al. 2008). Plants invest less C to AMF in exchange for nutrients when the availability of N or P in the soil is sufficient for plant growth. Conversely, if N or P availability is limited, increased AMF abundance is expected (Johnson et al. 2003; Treseder 2004). Moreover, the season is an important factor affecting AMF community composition (Bohrer et al. 2004; Dumbrell et al. 2011; Santos-Gonzalez et al. 2007). Changes in temperature and moisture affect N and P availability, thereby altering AMF community composition and diversity (Dumbrell et al. 2011). Furthermore, the seasonal dynamics of AMF colonies within plant roots occurs in response to plant phenology (Bohrer et al. 2004). Higher AMF abundance coincides with the growing season when plant demand for additional N and P increases. To date, the effects of N and P applications on soil AMF community compositions have rarely been documented in different seasons and have focused primarily on AMF abundance (Bhadalung et al. 2005; Camenzind et al. 2014; Gavito et al. 2003; Liu et al. 2017; Johnson et al. 2003). With the increasing of N deposition, N addition may enhance P demand. AMF community compositions may respond differently to N and P addition. Therefore, it is necessary to determine changes in AMF community composition in response to N and P application in different seasons and across varied environmental conditions.

The effects of N and P application on AMF abundance and community depend on plant communities (Majewska et al. 2018; Turrini et al. 2017), soil type (Montiel-Rozas et al. 2017), and soil properties (Treseder and Allen 2002) in different ecosystems. Thus far, studies have generally been conducted on fertilization for more than 2 years (Bhadalung et al. 2005; Huang et al. 2016; Xue et al. 2017). However, short-term studies for less than 2 years are important because they can provide insights into the adaptation of soil microclimate and nutrients. In addition, P does not accumulate at the same rate as N owing to the lack of biological fixation and low atmospheric deposition (Huang et al. 2016; Walker and Syers 1976). Many experiments use the same N and P addition rates (Liu et al. 2013) or an N deposition rate close to atmospheric levels with low P addition (Camenzind et al. 2014; Tischer et al. 2015). It remains unclear how high N input and medium P input, or the interaction thereof, would influence AMF abundance, diversity, and community composition when N deposition dramatically increases, especially in grassland soils. Moreover, studies on the responses of AMF to N and P addition have been restricted to boreal, temperate, and tropical ecosystems thus far (Camenzind et al. 2016; Delavaux et al. 2017), and the effects of N and P fertilization on AMF are unknown in karst regions.

Karst terrain typically develops on carbonate rocks (e.g., limestone, dolomite, or marble) and accounts for 15% of Earth's land surface. Furthermore, the world's largest karst regions occur in southwest China, occupying about 36% of the total land (Jiang et al. 2014). This region is characterized by poor stability, susceptibility to land degradation due to human disturbances, low-nutrient levels, and a decreased ability of self-recovery (Liu et al. 2015). This area experienced severe deforestation and agricultural expansion from 1949 to the end of the 1990s. Since the end of the 1990s, China launched the "Grain-for-Green" project, and most former croplands have been abandoned and transformed into woodland or grassland (Liu and Diamond 2005). The post-agricultural succession of grassland has been allowed to recover naturally for > 20 years. A large portion of soil N was lost during the cultivation period and was subjected to N-limited conditions in the early-stage grasslands of the karst region in southwest China (Zhang et al. 2015). Moreover, Niinemets and Kull (2005) reported a deficiency in bioavailable P rather than N in calcareous soils due to high pH. P availability may be another key factor that affects vegetation recovery in karst terrains (Hofmeister et al. 2002). Niinemets and Kull (2005) suggested that N and P co-limitations are common in diverse calcareous grasslands. AMF have been reported to play an important role in karst ecosystem protection and rocky desertification restoration (Wei 2012; Zhang et al. 2014). *Glomus* was found to be the dominant genus in a karst area of south China, and it should be a candidate for ecological restoration in such areas (Wei 2012). Liang et al.

(2015) found that karst soil of southwest China harbors abundant AMF resources and that AMF richness is closely correlated with plant richness, soil organic C, soil available P, and pH in this region. AMF richness in grasslands has been found to be higher than that in a shrub, secondary, and primary forest during vegetation restoration in degraded karst ecosystems (Liang et al. 2015). Selected AMF species might form stable cooperative relationships with plant species (Kiers et al. 2011). In addition, the higher light incidence in grasslands can improve the photosynthetic capacity and increase carbohydrates exported to roots for AMF. AMF diversity may play an important role in the post-agricultural succession of the grasslands. However, the consequences of nutrient deposition on AMF abundance, diversity, and community composition are not known for nutrient-limited grassland environments of karst regions.

A 2-year N and P fertilization experiment was performed in karst grassland to examine the effects of nutrient deposition on AMF abundance, diversity, and community composition. We hypothesized that the application of N and P might indirectly affect the AMF abundance, diversity, and community composition by changing soil pH and N and P availability. Therefore, we measured seasonal changes in AMF abundance, diversity, and community composition, as well as associated environmental factors (e.g., pH, NH_4^+-N, and NO_3^--N), under N and P addition to identify the key factors affecting AMF abundance, diversity, and community composition.

Materials and methods

Experimental description

The field experiment was conducted at the Huanjiang Observation and Research Station for Karst Ecosystems (107° 51′–108° 43′ E, 24° 44′–25° 33′ N), Chinese Academy of Sciences, Guangxi Province, southwest China. The region has a subtropical monsoon climate, and the average annual temperature and precipitation are 18.5 °C and 1380 mm, respectively. The wet season occurred from April to September and the dry season, from October to March. The average monthly temperatures in December 2016 and July 2017 were 9.9 °C and 25.0 °C, respectively. The average monthly precipitation values in December 2016 and July 2017 were 16.8 mm and 359.4 mm, respectively. The soil in this region was calcareous with high clay and Ca^{2+} content, which developed from a dolostone base. The study areas were characterized by a typical karst landscape with peak-cluster depression. Background N deposition in precipitation was about 37 kg N ha^{-1} $year^{-1}$ (Zhu et al. 2015).

The fertilization experiment was set up with 12 plots (5 × 4 m) in June 2014 and was conducted using a completely randomized block design with three replicates per treatment. All plots were surrounded by concrete slabs to prevent soil, water, and nutrients from being transferred among plots. Each concrete slab was 0.15 m wide. About 0.5 m of each concrete slab was inserted into the soil, leaving 0.2 m protruding above the ground. The entire experimental site with 12 plots was surrounded by a 3-m-wide buffer strip, which was similar in all treatments. There were four treatments: control (CK, no fertilization), N addition (N, 100 kg N ha^{-1} $year^{-1}$), P addition (P, 50 kg P ha^{-1} $year^{-1}$), and combined N and P addition (NP, 100 kg N ha^{-1} $year^{-1}$ + 50 kg P ha^{-1} $year^{-1}$). The high N rate and medium P rate were used for two reasons. First, our previous studies had found that karst grasslands were N-limited and that N deposition in this region has been increasing year by year. Thus, we intended to examine the effects of a high rate of N deposition (100 kg N ha^{-1} $year^{-1}$) on AMF. Second, N deficiency was greater than P deficiency in the karst grassland relative to the karst primary forest (Zhang et al. 2015). To simplify the experiment, and on the basis of the N/P ratio, a medium rate of P addition (50 kg P ha^{-1} $year^{-1}$) was used.

Since June 2015, 16.7 g N as NH_4NO_3 and 8.3 g P as $CaH_2PO_4{\cdot}H_2O$ alone or combined were mixed with 1.5 L of water and manually added each month with a sprayer over the grassland in each fertilization plot. Control plots were sprayed with 1.5 L of water. NH_4NO_3 and $CaH_2PO_4{\cdot}H_2O$ were dissolved in a 2-L sprayer. The solution was sprayed evenly on the plants at each plot, carefully ensuring the restriction of the movement of the solution across plots when spraying the sides. Additionally, the rainfall in this study area was very heavy, and there was a large amount of rainfall every month, so the nutrients applied in the treatment can be guaranteed to reach the soil, and a small part of the nutrient remains on the vegetation. The grassland areas were covered by naturally developed grass communities, with *Microstegium vagans* (31.7%), *Apluda mutica* L. (26.9%), and *Imperata cylindrica* (10.2%) as the dominant species in the CK treatment; *M. vagans* (66.1%) and *A. mutica* L. (20.2%) as the dominant species in the N treatment; *M. vagans* (47.8%) and *I. cylindrica* (38.0%) as the dominant species in the P treatment; and *M. vagans* (51.2%) and *A. mutica* L. (26.2%) as the dominant species in the NP treatment.

Sample collection and soil property measurements

Field sampling was conducted in December 2016 and July 2017. In each plot, soil samples (0- to 10-cm depth) were randomly collected with soil corers (diameter 2.5 cm) at five points according to the "S" type. The five soil samples from each plot were blended to obtain uniform composite soil after the removal of visible plant residue, roots, and stones. In the laboratory, portions of the fresh soil samples were used for exchangeable NH_4^+-N and NO_3^--N analyses. Another portion

of the soil samples was immediately flash-frozen, stored in liquid nitrogen, and preserved at − 80 °C in the laboratory for DNA extraction. The other samples were air-dried and sieved through a 2-mm mesh for physicochemical analysis. The main soil properties were pH, 7.31; soil organic C, 31.86 g kg^{-1}; total soil N (TN), 2.06 g kg^{-1}; total soil P (TP), 0.44 g kg^{-1}; and Ca, 0.73 g mL^{-1}; the aggregate sizes in the > 1 mm, 0.25–1-mm, and < 0.25-mm fractions were 90.08, 8.50, and 1.42, respectively.

Soil pH (1:2.5 soil/water ratio) was determined using a pH meter (Metter-Toledo 320). The exchangeable NH_4^+-N and NO_3^--N contents were analyzed using 2 M KCl extraction (10 g soil, 80 mL KCl^{-1}) and were measured using an autoanalyzer (FIAstar™ 5000; FOSS, Pål Anders, Sweden) (Hood-nowotny et al. 2010). TN was determined with an Element Auto-Analyzer (Vario MAX CN; Elementar, Hanau, Germany). TP was determined by acid digestion with a solution of H_2SO_4 + $HClO_4$. Available P (AP) was analyzed by the molybdenum blue colorimetric method (Carter and Gregorich 2006; Chen et al. 2018a, b).

Soil DNA extraction and real-time PCR

Soil DNA was extracted from 0.3 g of each soil sample using the Fast DNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). Extraction was conducted according to the manufacturer's instructions. Extracted DNA was diluted approximately tenfold with nuclease-free water for real-time polymerase chain reaction (qPCR) amplification.

AMF abundance was determined using an ABI Prism 7900 system (Applied Biosystems, Foster City, CA, USA). DNA was used as a template to amplify a region within AMF 18S SSU rRNA using AMV4.5NF (AAGCTCGTAGTTGA ATTTCG) and AMDGR (CCCAACTATCCCTATTAATC AT) primers (Sato et al. 2005). The 10-μL reaction volume contained 5 μL 2× SYBR Premix Ex TaqTM, 0.2 μL Rox (Takara Bio, Kusatsu, Japan), 0.3 μL (0.4 μmol L^{-1}) of both forward and reverse primers, 1 μL (5 ng μL^{-1}) of sample template DNA, and 3.2 μL of nuclease-free water. PCR runs started at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min. Standard curves used to quantify the AMF gene were created using a tenfold dilution series of plasmids containing the target gene. The R^2 values of the standard curve were > 0.993, and amplification efficiencies were around 80%.

Illumina HiSeq sequencing and bioinformatic analyses

PCR amplicons for HiSeq sequencing were conducted using nested PCR. AML1 (ATCAACTTTCGATGGTAGGA TAGA) and AML2 (GAACCCAAACACTTTGGTTTCC) primers were used to amplify an AMF 18S rRNA gene fragment (Lee et al. 2008). PCR was carried out using a 50-μL reaction system with 2.5 μL of template DNA, 25 μL of 2× PCR Ex Taq (Takara Bio), 1.25 μL of each primer (10 μmol L^{-1}), and deionized water to make up the volume. The PCR program consisted of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min, followed by a final extension period of 10 min at 72 °C. The first PCR product was diluted 50 times. The dilutions were used as template DNA in a second PCR performed using the designed primers AMV4.5NF and AMDGR as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min, ending with a final extension at 72 °C for 10 min. The barcode sequences (6 bp) were linked to the 5′-end of the primers to distinguish the sequences of each sample. Fifty-microliter reaction mixtures contained 2.5 μL of template DNA, 25 μL of 2× PCR Ex Taq (Takara Bio), 1.25 μL of each primer (10 μmol L^{-1}), and 20 μL of water. There were two technical replicates for each sample, which was subsequently pooled into one tube. Negative controls were included to determine contaminant DNA originating from DNA extraction kits (Schöler et al. 2017; Vestergaard et al. 2017). PCR products were assessed using the Illumina HiSeq × Ten 250-bp paired-end sequencing platform at Novogene (Beijing, China) after purification using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania).

AMF sequence data were processed following the UPARSE pipeline in USEARCH (v.10.0.240; Edgar 2010) and R (R Development Core Team 2018). Specifically, paired-end reads were spliced to produce contigs assigned to each sample based on their barcodes using QIIME. All contigs were merged using the "-fastq_mergepairs" USEARCH command, and primers were truncated with the "-fastx_truncate" USEARCH command. Reads were removed prior to analysis if they were shorter than 200 bp, contained any ambiguous bases, had an average quality score lower than 30, or had any nucleotide mismatches within the barcode or primer using the "-fastq_filter" USEARCH command. Then, AMF operational taxonomic unit (OTU)-representative sequences were picked out at 97% sequence similarity. OTU sequences were aligned to the SILVA SSU database. Sequences that failed to be aligned to the reference database were manually checked and verified with BLAST in NCBI, and sequences that did not belong to AMF were discarded. Subsequently, a phylogenetic tree was constructed in MEGA using the neighbor-joining algorithm. After dereplicating the sequences grouped together in the phylogenetic tree with similarities higher than 97%, a new group of AMF OTU representatives was manually picked out, and the normalized OTU table was calculated in USEARCH. After the sequence number of each rarefied sample reached 10,000, the α diversity was calculated in R using the "vegan" package.

Statistical analyses

Repeated measures analysis of variance (ANOVA) was carried out with function "LSD.test" of R package "agricolae" (de Mendiburu 2014) to reveal the differences in AMF abundance, richness, and Chao1 estimator values among different fertilization treatments or different sampling times in the same treatment ($p < 0.05$). Non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) were used to investigate the effect of seasonal changes and fertilization on AMF community compositions. NMDS and PERMANOVA were carried out in function "metaNMDS" and "adonis2" of R package "vegan" (Oksanen et al. 2017) using a distance matrix estimated following the methods of Bray-Curtis. Multivariate analysis of variance (MANOVA) was applied to determine the main and interactive effects of seasonal changes and fertilization on AMF abundance, richness, and Chao1 values in R function "aov." The "linear discriminant analysis effect size" (LEfSe) method was used to determine the AMF community composition differences between December and July (Segata et al. 2011). The LEfSe was carried out with default parameters in the online Galaxy tool developed by Huttenhower's group (http://huttenhower.sph.harvard.edu/galaxy/). A structural equation modeling (SEM) framework was applied to investigate the relationships among environmental factors, AMF abundance, and richness. SEM was carried out with the Amos 21.0 software package (Smallwaters Corporation, Chicago, IL, USA). The relationships between environmental factors and AMF abundance and richness were analyzed using Pearson correlation tests before the SEM procedure. The significance of environmental variables ($p < 0.05$) in the Pearson correlation was retained for the following SEM construction. We established the best relationship based on the model instructions. p-values, χ^2, goodness-of-fit indices (GFIs), and root mean square errors of approximation (RMSEA) were used to evaluate the structural equation model fit (Hu et al. 2014). The multivariate regression trees were performed with function "mvpart" of R package "mvpart" to determine the relationships between environmental factors and AMF community composition. All the other data and image preparation, if not specifically explained, were performed using R.

Results

Soil AMF abundance, species richness, and Chao1 estimator values

In total, there were 221 OTUs for all sequences after chimeras and singletons were discarded. Soil AMF abundance ranged from 7.20×10^7 to 1.92×10^8 copies g^{-1} dry soil.

Table 1 Effects of seasonal changes, N addition, P addition, and their interaction on the richness, Chao1 estimator values, abundance, and community composition of arbuscular mycorrhizal fungi

Items	Richness	Chao1	Abundance	Community composition
s[a]	17.912***	16.118**	20.422***	3.432**
n[b]	0.076	0.032	14.352**	1.573
p[c]	7.533*	7.977*	0.890	1.357
$s \times n$[d]	1.462	1.343	7.535*	1.213
$s \times p$[e]	0.680	0.913	0.708	0.935
$n \times p$[f]	0.027	0.221	0.051	0.718
$s \times n \times p$[g]	1.740	1.842	0.134	0.816

[a] Effect of seasonal changes

[b] Effect of N addition

[c] Effect of P addition

[d] Interaction effect of seasonal changes and N addition

[e] Interaction effect of seasonal changes and P addition

[f] Interaction effect of N and P addition

[g] Interaction effect of seasonal changes and N and P addition

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

AMF abundance was significantly affected by seasonal changes, N addition, and interaction effects of seasonal changes and N addition, while AMF richness and Chao1 values were significantly affected by seasonal changes and P addition (Table 1).

Specifically, AMF abundance in the N and NP treatments was significantly higher in July (summer) than in December (winter). Among the fertilization treatments, AMF abundance in the P treatment plots was significantly less than that in the N and NP treatment plots in July. The highest AMF abundance occurred in the N-supplemented plots, and the lowest occurred in the P-supplemented plots (Fig. 1a). Conversely, soil AMF species richness and Chao1 values followed similar seasonal variations, which were higher in December than in July. AMF richness and Chao1 estimator values of the P treatment were significantly higher than those of the control in July. There was no significant difference in AMF abundance, species richness, or Chao1 values for any of the treatments in December (Fig. 1b, c).

Soil AMF community composition

AMF communities at order, family, and genus level are detailed in Fig. 2. AMF communities belonged to *Paraglomerales*, *Glomerales*, and *Diversisporales*. The most abundant order was *Glomerales*, and its abundance was higher in December (95.38%) than in July (91.07%) (Fig. 2a). As shown in Fig. 2b, AMF communities could be grouped into six main families, and the most abundant family was *Glomeraceae*. AMF communities could be

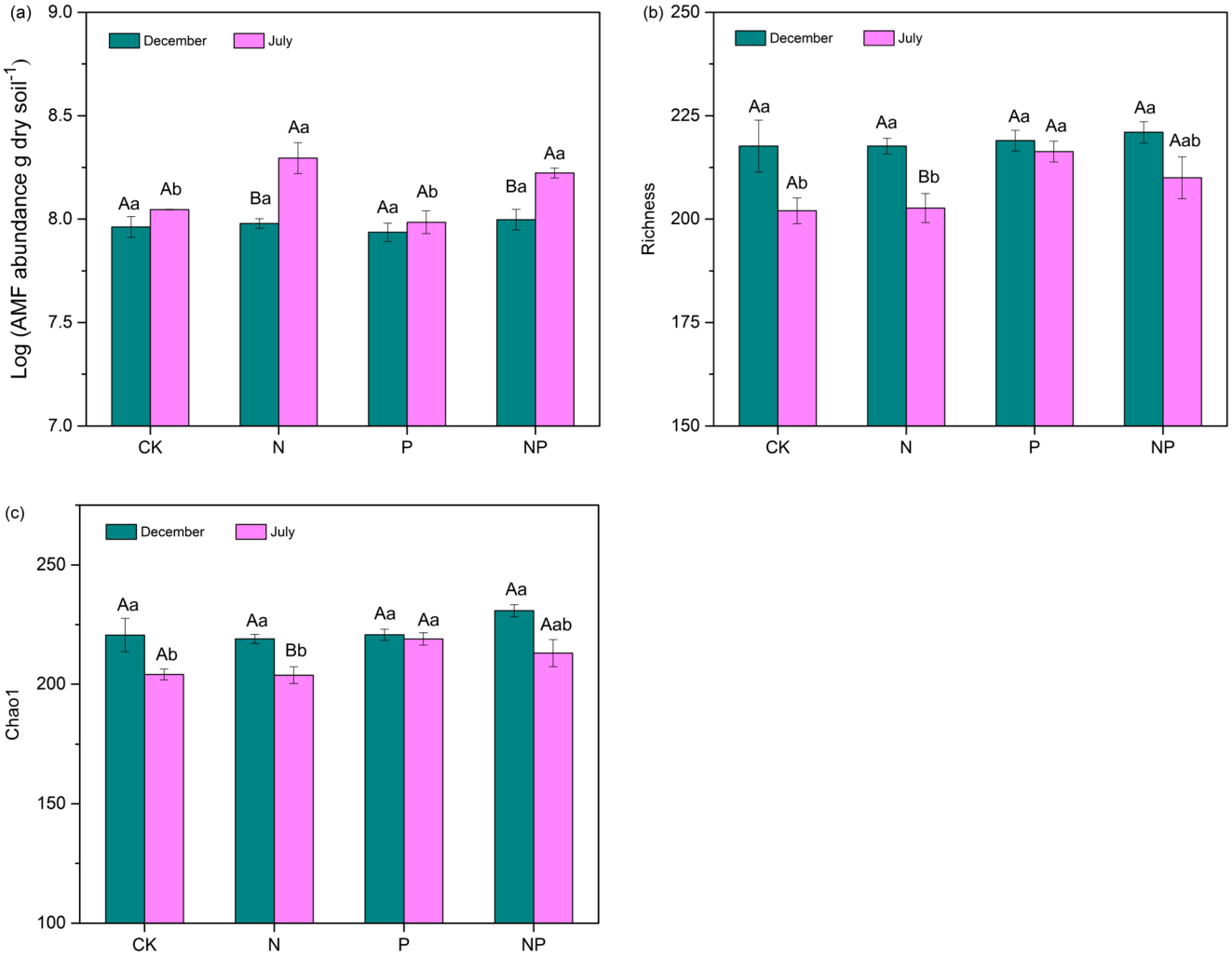

Fig. 1 Effect of N, P, and NP treatments on arbuscular mycorrhizal fungi properties in December 2016 and July 2017. **a** Soil arbuscular mycorrhizal fungi abundance, **b** richness, and **c** Chao1 values. CK, N, P, and NP indicate no fertilization, the addition of 100 kg N ha^{-1} year^{-1}, the addition of 50 kg P ha^{-1} year^{-1}, and combined N and P addition of 100 kg N ha^{-1} year^{-1} plus 50 kg P ha^{-1} year^{-1}, respectively. Different lowercase letters indicate significant differences between treatments at the same sampling time ($p < 0.05$); different uppercase letters indicate significant differences at different sampling time for the same treatment ($p < 0.05$). Bars show standard error of the mean ($n = 3$)

further identified as belonging to 10 main genera, including *Septoglomus*, *Scutellospora*, *Sclerocystis*, *Rhizophagus*, *Paraglomus*, *Glomus*, *Funneliformis*, *Diversispora*, *Claroideoglomus*, and *Acaulospora* (Fig. 2c).

AMF community composition was significantly affected by seasonal changes, but N and P addition had no significant effect on it (Table 1). NMDS showed that community composition differed between December and July (Fig. 3). Regardless of treatment, the numbers of the genera *Scutellospora*, *Redeckera*, and *Diversispora* were higher in July, whereas *Sclerocystis* and *Funneliformis* were more abundant in December (Fig. 4a). *Scutellospora*, *Redeckera*, and *Diversispora* belong to Diversisporales. Both *Sclerocystis* and *Funneliformis* belong to *Glomerales* (Fig. 4b).

Driving factors for AMF diversity and community composition

SEM analysis generated a model that fit the causal hypothesis well ($x^2 = 4.38$, df = 6, GFI = 0.95, RMSEA < 0.001) and explained 41% and 56% of the variance in AMF abundance and richness, respectively. According to the path coefficients (λ) for the casual models, TN showed the largest positive and negative effect on AMF abundance and richness, respectively. AMF richness was significantly correlated with AP and pH, while AP was significantly correlated with TP and pH. Moreover, TP was significantly correlated with TN (Fig. 5). According to the multivariate regression tree analysis, pH and NO_3^--N were the main factors affecting AMF community composition (Fig. 6).

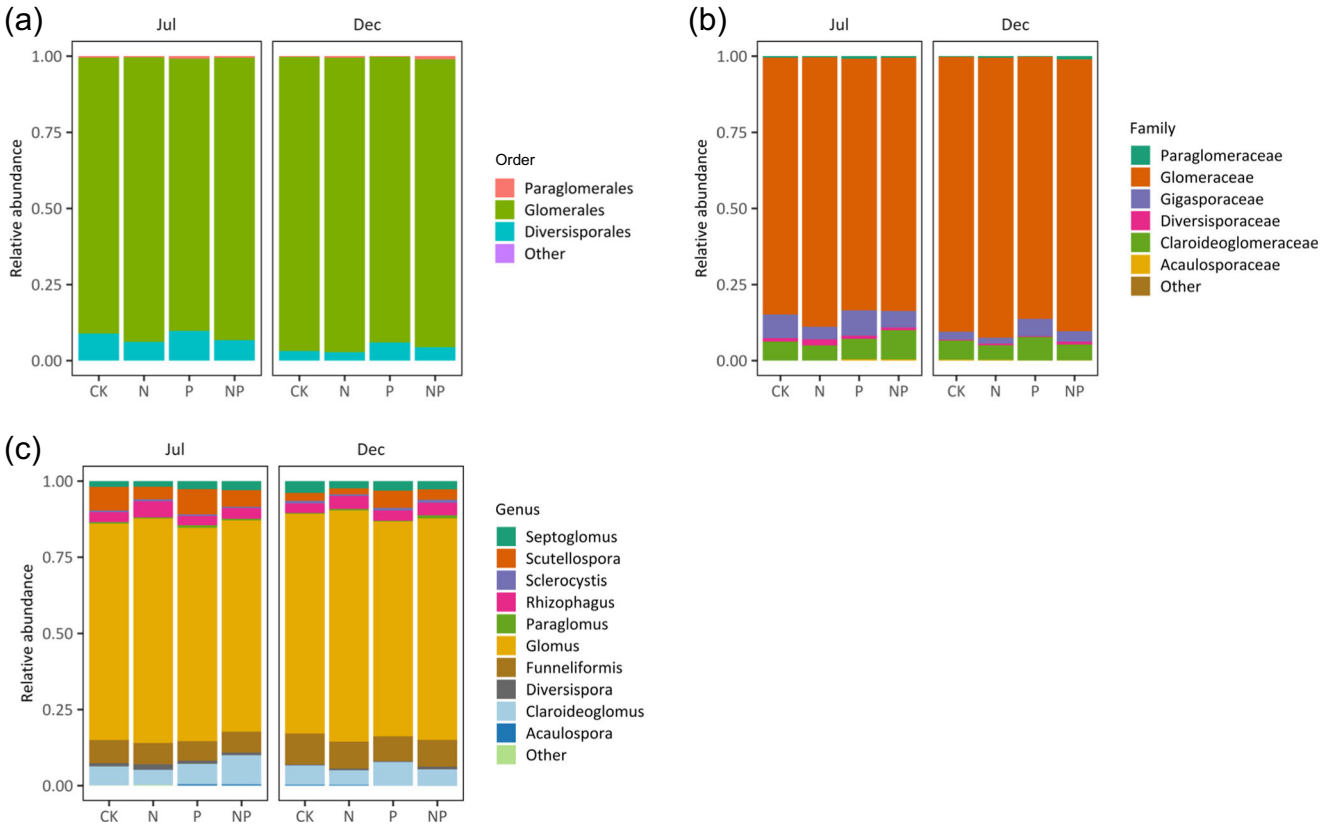

Fig. 2 Soil arbuscular mycorrhizal fungi community compositions at **a** order, **b** family, and **c** genus level under different treatments in December 2016 and July 2017; CK, N, P, and NP represent no fertilization, N addition of 100 kg N ha^{-1} $year^{-1}$, P addition of 50 kg P ha^{-1} $year^{-1}$, and combined N and P addition of 100 kg N ha^{-1} $year^{-1}$ plus 50 kg P ha^{-1} $year^{-1}$, respectively. Other: the sum of order, family, and genus occupying < 0.5% of the total population

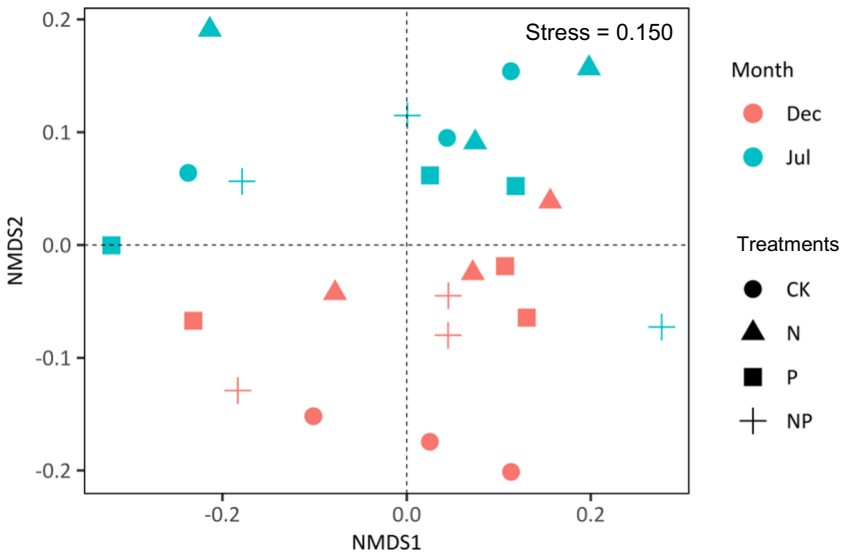

Fig. 3 Non-metric multidimensional scaling (NMDS) ordinations of arbuscular mycorrhizal fungi community composition based on OTUs in 24 soil samples under four treatment regimes in December 2016 and July 2017. CK, no fertilization; N, 100 kg N ha^{-1} $year^{-1}$; P, 50 kg P ha^{-1} $year^{-1}$; NP, 100 kg N ha^{-1} $year^{-1}$ plus 50 kg P ha^{-1} $year^{-1}$

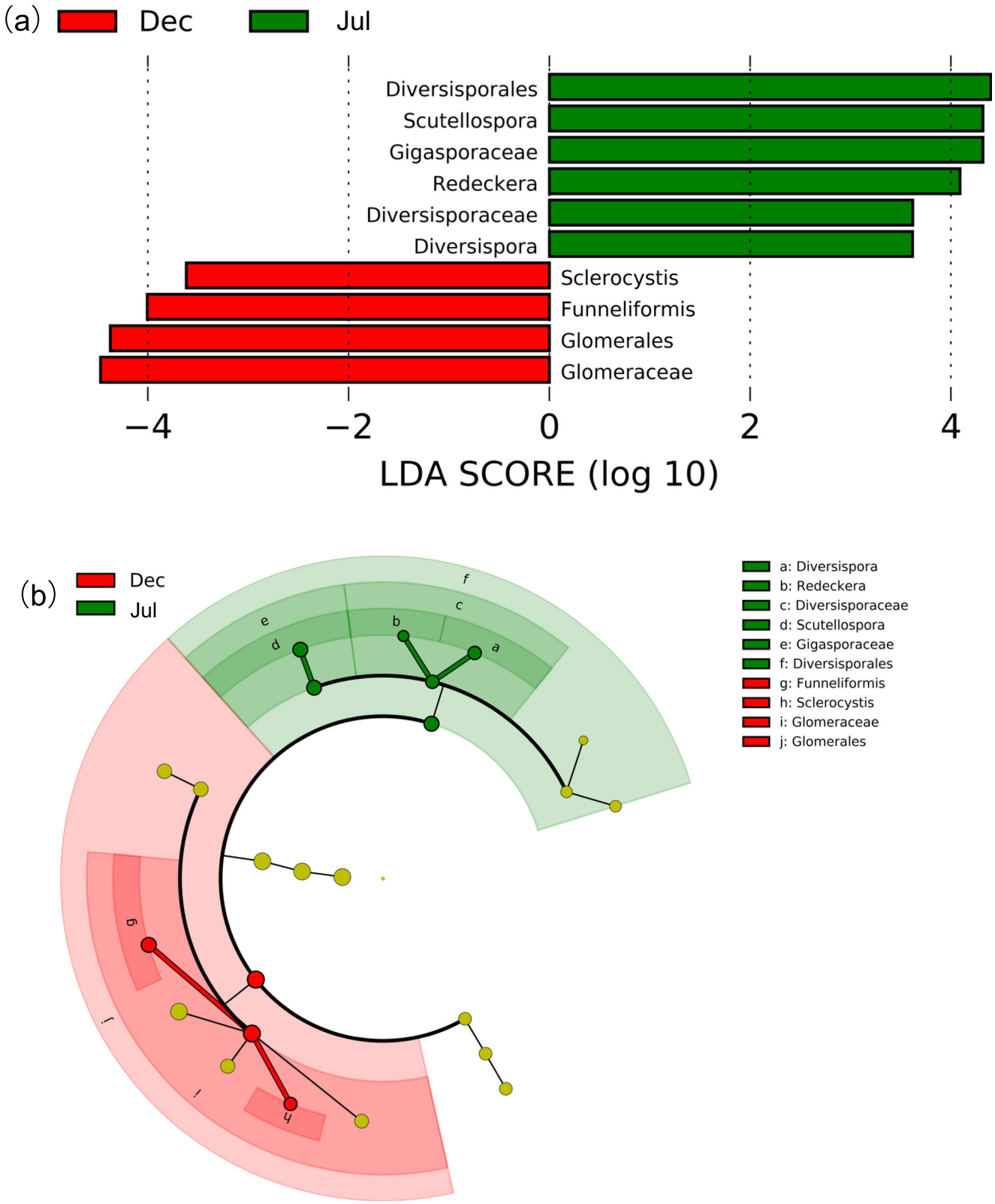

Fig. 4 Amplicon sequencing results using the "linear discriminant analysis effect size" (LEfSe) analysis between December and July. **a** Responses of the proportions of putative functions determined using LEfSe analysis and **b** differences in soil arbuscular mycorrhizal fungi community composition. The figure illustrates the LDA score of arbuscular mycorrhizal fungi features. Only significant features with an LDA score larger than 2.0 were obtained. The arbuscular mycorrhizal fungi lineages (in green) were significantly more abundant in July, while the arbuscular mycorrhizal fungi lineages (in red) were significantly more abundant in December

Discussion

Seasonal variation in AMF abundance, richness, Chao1 values, and community composition

There was no significant change in AMF abundance in December (Fig. 1a). This could have been caused by a shift in plant phenology, including plant-derived labile nutrients, litter, and root exudates (Wang et al. 2018; Zubek et al. 2016). Typically, plants produced new roots and stimulated an increased AMF mycelial network that extended into the soil for nutrient uptake during the growing season (Bohrer et al. 2004; Johnson et al. 2010). In the present study, rains and available nutrients (NH_4^+-N and NO_3^--N) were lower in winter than in summer (Fig. S1b, c). Yang et al. (2010) showed that soil water availability could explain the change in AMF biomass by affecting dissolved nutrients in the soil. Plants grow slowly over the winter and demand fewer nutrients compared to the growing season; therefore, we predicted that AMF colonization would be low in December. Meanwhile, some microorganisms may have been dormant or non-viable due to low soil temperature and moisture during winter. Moreover, soil fungivores prefer consuming plant roots over fungal hyphae in the growing season, and their feces are used by AMF for extracting nutrients; both of these factors increase AMF abundance. Conversely, less plant roots are available for fungivores in the winter, leading to the consumption of fungal hyphae and in turn, decreased AMF abundance (Ngosong et al. 2014; Thomsen and Hart 2018).

Seasonal changes significantly affected AMF abundance, richness, Chao1 values, and community composition (Table 1). The AMF richness and Chao1 values showed the opposite results compared to AMF abundance (Fig. 1). This was similar to the findings of Dumbrell et al. (2011), wherein AMF species composition and α diversity levels differed between cooler (winter) and warmer (summer) periods. Other studies have shown that AMF diversity was sensitive to soil temperature (Gavito et al. 2003). Thus, the lower temperature might have restricted microenvironmental changes during the winter, which stimulated AMF community compositional changes and increased the spatial turnover of species (Dumbrell et al. 2011). In the present study, the numbers of the genera *Sclerocystis* and *Funneliformis* were significantly higher in December than in July (Fig. 4), suggesting that *Sclerocystis* and *Funneliformis* grow and outcompete other rare taxa during the winter. In addition, it is actually the decreased photosynthesis that limits C availability during winter, while the C supply increases within the growing season (Dumbrell et al. 2010). Consequently, the higher AMF species richness in December probably reflects a more competitive scenario in terms of the capture of limited resources (Tilman 1987).

Effects of N and P addition on AMF abundance, richness, Chao1 values, and community composition

The effects of N and P application or deposition on AMF abundance and community composition have been demonstrated (Bi et al. 2003; Liu et al. 2013; Treseder and Allen

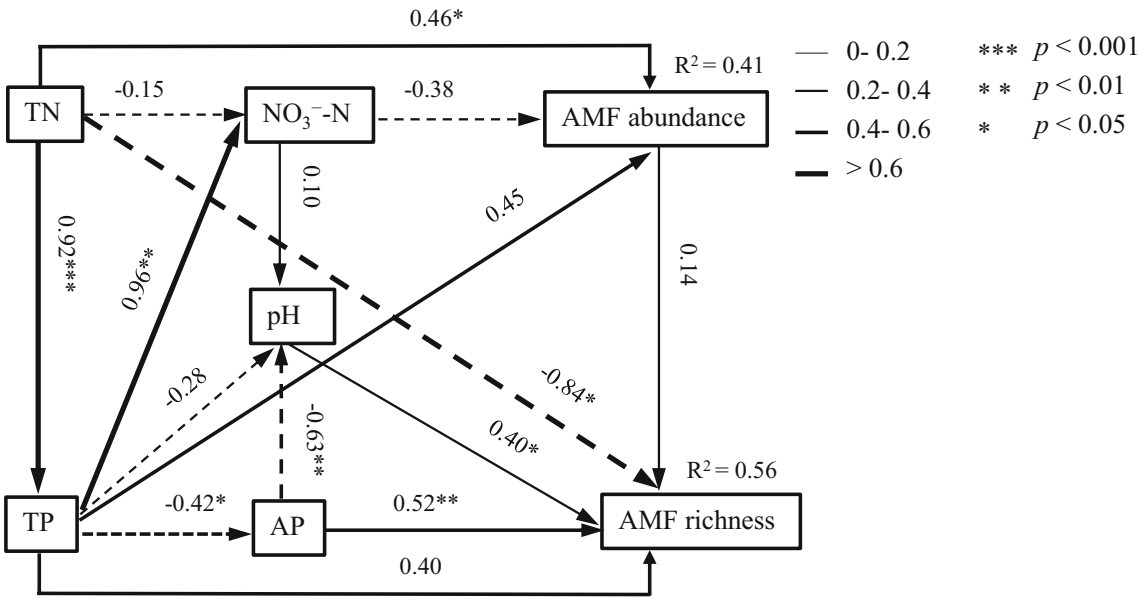

Fig. 5 Structural equation model (SEM) showing the direct and indirect effects of TN, TP, NO_3^--N, AP, and pH on arbuscular mycorrhizal fungi abundance and richness. TN, total N; NO_3^--N, nitrate N; TP, total P; AP, available P. The width of the arrows indicates the strength of the standardized path coefficient. The solid lines indicate positive path coefficients; dashed lines, negative path coefficients; and R^2 values, the proportion of variance explained for each endogenous variable ($n = 24$)

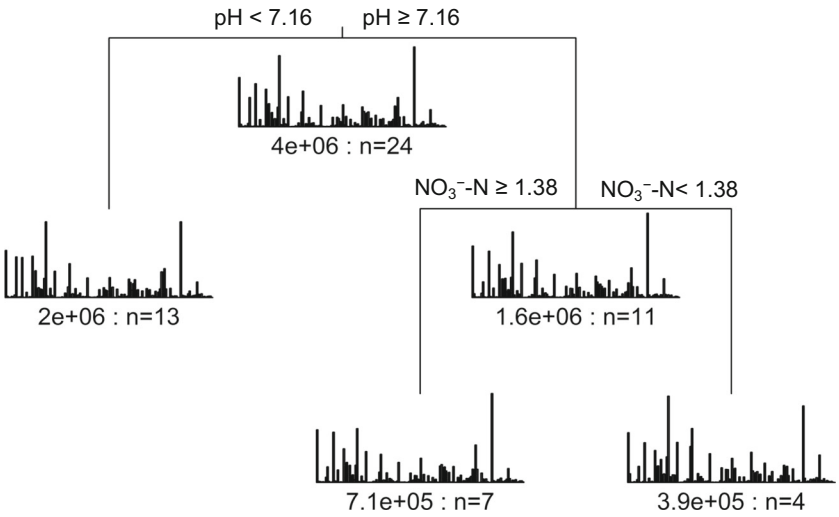

Fig. 6 The relationships between environmental factors and arbuscular mycorrhizal fungi community composition. The relationships were determined using multivariate regression tree analysis at the OTU level. NO_3^--N, nitrate N. The bar plots and the numbers show the average relative abundances of OTUs in each split group and the sample number within each group, respectively

2002). However, AMF properties in different soils, climates, and plant communities have been found to differ (Jumpponen et al. 2005). We found that AMF abundance dramatically increased with the application of N and NP in July compared to the control, and it was similar between the treatment of P and the control (Fig. 1a). A meta-analysis reported that the effects of N input on AMF abundance differ between N-rich and N-limited ecosystems (Treseder 2004). The AMF abundance generally declined with N enrichment. However, karst grassland is N-limited due to severe deforestation and agricultural expansion by human activities. When alleviating N limitation by high N input, a higher plant photosynthetic rate would result in increased AMF C allocation in exchange for nutrients (Delavaux et al. 2017; Treseder 2004). Furthermore, high N input could be insufficient to balance the N:P ratio and result in soil deficiency of P. In this case, plant dependence on AMF for additional P would increase (Delavaux et al. 2017). These results suggested that high N input resulted in P deficiency and more AMF investment to acquire limiting P. A previous study suggested that soil available P promotes AMF colonization at lower concentrations but inhibits colonization at higher concentrations (Xie et al. 2014). This suggests that P levels limit plant growth in karst grasslands and that P input might stimulate AMF species richness to improve P acquisition. In our study, N application had a significant effect on AMF abundance, but P application had a significant effect on AMF richness and Chao1 values (Table 1). These findings suggested that AMF abundance was sensitive to N addition, while AMF richness and Chao1 values were sensitive to P addition after 2 years of N and P addition.

N addition, P addition, and their interaction yielded no significant effect on AMF community composition (Table 1). In contrast, Bhadalung et al. (2005) found that three unidentified species of *Glomus* were highly sensitive to NP fertilization, while other AMF species were slightly sensitive to the fertilization in 27-year long-term NP fertilization plots. We found soil AMF communities to be dominated by *Glomus* (72.88%) genera (Fig. 2c). Chen et al. (2018a, b) reported that in the restoration of disturbed habitats in Hong Kong, *Glomus* (10–24%) was the most abundant genus, although significant proportions (51–83%) of AMF could not be identified to the genus level due to limitations in the reference database. Meanwhile, analysis of the Tibetan alpine steppe rhizosphere found the most dominant genera to be *Glomus*, *Diversispora*, and *Claroideoglomus*, representing approximately 34.5%, 33.3%, and 21.8% of the total clones, respectively (Zhang et al. 2016). These results suggested that the dominant AMF species differed between the karst region and other regions and that *Glomus* spp. in karst regions have an increased ability to colonize adverse environments with low nutrients and high Ca^{2+} compared to other regions. Thus, grassland N and P application under different climate conditions and soil properties exert different influences on AMF composition (Gai et al. 2006; Johnson et al. 2003; Oehl et al. 2017).

Key factors driving AMF abundance, richness, and community composition changes

AMF abundance, diversity, and community composition may change with nutrient addition as a result of alterations in soil properties (Zheng et al. 2014). Based on SEM, TN was found to be the main positive predictor of AMF abundance but was the main negative factor of AMF richness (Table 2). Hence, increased TN levels under N addition might increase AMF abundance but decrease AMF richness (Delavaux et al. 2017; Johnson et al. 2003; Zheng et al. 2014). TN was significantly correlated with TP, which then directly affected AMF abundance and richness (Fig. 5). Because N and P nutrition are closely coupled, evidence suggested that the balance between N and P was a more important factor in governing fungal colonization than the effect of N or P alone (Miller et al. 2002; Sylvia and Neal 1990).

Table 2 Direct, indirect, and total effects of environmental factors on arbuscular mycorrhizal fungi abundance and richness using a structural equation model (SEM)

Observed outcomes (λ)					
	TN[a]	NO_3^--N[b]	TPc	APd	pH
Direct	0.46	− 0.38	0.45	0	0
Indirect	0.15	0	− 0.36	0	0
Total	0.61	− 0.38	0.09	0	0
Direct	− 0.84	0	0.40	0.52	0.40
Indirect	0.22	0.09	− 0.25	− 0.25	0
Total	− 0.62	− 0.09	0.15	0.27	0.40

[a] Total N

[b] Nitrate N

[c] Total P

[d] Available P

AMF community composition was significantly affected by pH and NO_3^--N (Fig. 6). Similarly, Zheng et al. (2014) observed that AMF community composition was mainly affected by available P, pH, and NO_3^--N content in the Qinghai–Tibetan Plateau alpine meadow ecosystem, whereas Egerton-Warburton et al. (2007) found that soil N, N:P, and host plant were the key drivers of AMF community composition by a cross-site test in five grasslands. pH has been reported as an important factor in regulating AMF diversity (Aliasgharzad et al. 2010; Zheng et al. 2014). In the present study, AMF richness was significantly correlated with pH (Fig. 5). It has also been reported that fertilization induces soil acidification on a global scale (Tian and Niu 2015). Changes in pH may regulate P availability (Brady and Weil 2008). AP was significantly higher in the treatments of P and NP than in the control in July (Fig. S1), and AP was significantly correlated with soil pH (Fig. 5). These findings supported our hypothesis that N and P addition would induce a shift in AMF richness via a change in soil nutrient availability and pH levels. However, AMF abundance and richness were less susceptible to varied soil pH than to TN and AP (Table 2). These results indicated that soil nutrient availability had a greater effect on AMF abundance and diversity than pH.

Sampling time significantly affected AMF abundance, richness, and Chao1 values, as well as community composition. Temperature and precipitation were higher in July than in December in the study region. Warming and precipitation have been found to significantly affect plant phenology and nutrient availability (Brundrett 1991; da Silva et al. 2014; Sun et al. 2013). N addition could increase exchangeable NH_4^+-N and NO_3^--N especially in July, which increased the AMF abundance in our study (Fig. S1b, c). This supports findings by Sun et al. (2017), according to which nutrient sources, temperature, and water regimes interactively affect labile organic matter. Consequently, N and P application at different times may have affected the availability and quantity of dissolved nutrients, and thus, may have indirectly driven the distribution of soil AMF abundance and richness.

Conclusions

This study showed that sampling time had a significant effect on AMF abundance, diversity, and community composition. Higher AMF abundance was found in July (summer) whereas higher AMF diversity was found in December (winter). *Diversisporales* was significantly richer in July while *Glomerales* was more abundant in December. Additionally, AMF abundance was significantly affected by the N addition while AMF diversity was significantly affected by the P addition. The N and P addition showed no significant effect on AMF community composition. AMF abundance closely linked to TN, but AMF richness was also significantly affected by AP and pH. These findings suggested that the 2-year N and P application affected soil nutrient availability and induced a change in soil pH, which directly affected AMF richness. Proper P addition is important to maintain the balance of the system with the increasing of N deposition. Future studies should focus on the variation in AMF abundance with high N deposition, and on AMF diversity when P became more limiting with high N deposition in post-agricultural succession in the early-stage karst region grasslands, ensuring that seasonal and nutrient availability variation are taken into account.

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