RESEARCH ARTICLE

Differential responses of arbuscular mycorrhizal fungi to nitrogen addition in a near pristine Tibetan alpine meadow

Yong Zheng[1], Yong-Chan Kim[1], Xiao-Fang Tian[1], Liang Chen[1,2], Wei Yang[1,2], Cheng Gao[1], Ming-Hua Song[3], Xing-Liang Xu[3] & Liang-Dong Guo[1]

[1]State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; [2]University of Chinese Academy of Sciences, Beijing, China; and [3]Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing, China

Correspondence: Liang-Dong Guo, State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China.
Tel./fax: +86 10 64807510;
e-mail: guold@sun.im.ac.cn

Received 11 February 2014; revised 20 May 2014; accepted 20 May 2014. Final version published online 19 June 2014.

DOI: 10.1111/1574-6941.12361

Editor: Ian C. Anderson

Keywords
454 pyrosequencing; AMF; community composition; nitrogen deposition; Qinghai–Tibetan Plateau.

Abstract

Elucidating the responses of soil microbial abundance and community composition to nitrogen (N) addition is important for predicting ecosystem function under increased atmospheric N deposition. We examined the arbuscular mycorrhizal (AM) fungal community under three N forms (NH_4^+-N, NO_3^--N, and NH_4NO_3-N) and two N rates (1.5 and 7.5 g N m^{-2} $year^{-1}$) in an alpine meadow of the Qinghai–Tibetan Plateau. AM fungal extraradical hyphal density was significantly decreased by NH_4^+-N in May, but was not affected by N form nor N rate in August. N rate, but not N form, significantly affected AM fungal spore density; high N rate decreased spore density. No direct N addition effect was observed on AM fungal community; however, soil available phosphorus, pH, and NO_3^--N were considered as important factors that influenced AM fungal community composition. Structural equation model results showed that N rate, not N form, strongly affected soil characteristics, which directly influenced community compositions of plants and AM fungi, as well as spore density. Therefore, AM fungal community was influenced by N addition, primarily because of altered soil characteristics, and partially by a modified plant community, but not or just slightly by direct N addition effects in this alpine meadow ecosystem.

Introduction

Arbuscular mycorrhizal (AM) fungi, belonging to the phylum *Glomeromycota* (Schüßler *et al.*, 2001), form mutualistic associations with most land plant species and obtain carbon (C) from their plant partners in exchange for mineral nutrients (Smith & Read, 2008; Kiers *et al.*, 2011). Given that AM fungi are fundamental soil microbial components, they have been postulated to be the most crucial plant-derived C consumers (Staddon, 2005; Drigo *et al.*, 2010) and comprise *c.* 1.4 Pg (Pg = 10^{15} g) of dry biomass within roots globally (Treseder & Cross, 2006). AM associations functioning as vital links between above-ground and below-ground biotic communities are influenced by environmental changes in ecosystems (Staddon *et al.*, 2003; Antoninka *et al.*, 2011; van Diepen *et al.*, 2011; Liu *et al.*, 2012). Therefore, elucidating the responses of AM fungal abundance and community composition to simulated environmental pressure is critical in assessing the effect of anthropogenic disturbance on biodiversity and ecosystem functioning.

Nitrogen (N) addition alters plant community composition, diversity, productivity, and ecosystem functioning (Magnani *et al.*, 2007; Treseder, 2008; Bobbink *et al.*, 2010; Fornara & Tilman, 2012). Simultaneously, changes in N availability may affect soil microbial community that regulates many fundamental N transformation processes in terrestrial ecosystems (Veresoglou *et al.*, 2012). N addition affects AM fungal abundance, community composition, and diversity in agricultural (Antunes *et al.*, 2012; Lin *et al.*, 2012; Tian *et al.*, 2013), forest (Treseder *et al.*, 2007; Garcia *et al.*, 2008; van Diepen *et al.*, 2010, 2011), and grassland (Johnson *et al.*, 2003; Bradley *et al.*, 2006; Antoninka *et al.*, 2011; Chen *et al.*, 2014) soils.

AM fungal extraradical hyphae (ERH) assimilate N mainly in the form of NH_4^+ and possibly NO_3^-, but the

latter is not rapidly delivered to the plant (Tanaka & Yano, 2005; Veresoglou *et al.*, 2012; Ngwene *et al.*, 2013). Compared with NO_3^-, NH_4^+ form exhibits lower diffusion in soils; a preference for one of the two available N forms may indicate conformation to a particular adaptation strategy of a plant (Veresoglou *et al.*, 2011b), and AM fungal activity can be decreased under NH_4^+ supply condition (Ngwene *et al.*, 2010). Given that AM fungal growth can be influenced by host plants and even more by soil nutrient conditions (Sikes *et al.*, 2014), application of NH_4^+ and NO_3^- may result in altered AM fungal abundance and community composition. Although N addition shows negative, positive, and neutral effects on AM fungal abundance, community composition, and diversity under different amounts of N inputs in various ecosystems (Eom *et al.*, 1999; Egerton-Warburton *et al.*, 2007; Antoninka *et al.*, 2011; Liu *et al.*, 2012), high N fertilization (e.g. 27.2 g N m^{-2} $year^{-1}$) can weaken and even disrupt the relationship between AM fungi and plants, as well as alter AM functioning in semi-arid grasslands (Corkidi *et al.*, 2002; Bradley *et al.*, 2006; Wei *et al.*, 2013). However, information on the response of root-associated AM fungi to N addition in the alpine meadow ecosystem is limited.

Alpine meadow is the main vegetation type in the Qinghai–Tibetan Plateau, which occupies an area of *c.* 2.5 million km^2 with an average altitude of more than 4000 m above sea level, and is sensitive to climate change and anthropogenic activities (Zhang *et al.*, 2009; Tian *et al.*, 2014; Zheng *et al.*, 2014). Alpine meadow remains relatively undisturbed by humans because it is far from the main industrial areas. Qinghai–Tibetan Plateau receives lesser atmospheric N deposition (*c.* 0.46 g N m^{-2} $year^{-1}$) than heavily developed regions in China (*c.* 2.2–2.4 g N m^{-2} $year^{-1}$, Liu *et al.*, 2013). Therefore, the alpine meadow on this area is an ideal and important region to examine the consequences of spatio-temporal variation in available N quality and quantity, in relation to changing atmospheric N deposition using N addition experiments, on ecosystem stability and functioning. In 2005, a controlled N addition experiment that included three N forms (NH_4^+-N, NO_3^--N, and NH_4NO_3-N) and two N supply rates (1.5 and 7.5 g N m^{-2} $year^{-1}$) was established to investigate the effect of simulated N deposition on the alpine meadow ecosystem of the Qinghai–Tibetan Plateau. The result showed that N rate, but not N form, changes plant community composition (Song *et al.*, 2012), soil microbial biomass, and the abundance of N-cycling microorganisms, such as ammonia-oxidizers (Tian *et al.*, 2014). However, the influence of N form and N rate on AM fungal abundance and community composition in this alpine meadow ecosystem remains unknown.

In this study, we examined AM fungal abundance (ERH and spore density) and community composition in a 6-year N addition experiment as previously outlined. Our hypotheses were as follows: (1) experimental N addition decreases AM fungal abundance (i.e. hyphal length and/or spore density); (2) compared with the N form, the N supply rate will strongly alter the AM fungal community composition; and (3) soil factors, such as N availability, will have a substantial effect on AM fungal variables. We expect that this study will provide insights into the function of AM fungi under future increased N deposition in natural alpine meadow ecosystems.

Materials and methods

Experimental site and design

The experiment was carried out in an alpine meadow at the Haibei Alpine Meadow Ecosystem Research Station (HAMERS) of the Chinese Academy of Sciences (37°37′N, 101°12′E, 3 200 m a.s.l.). This area is located in the northeast of the Qinghai–Tibetan Plateau in Qinghai Province, China (Zheng *et al.*, 2012), with mean annual temperature of −2 °C and annual precipitation of 500 mm. The plant community at the experimental site is dominated by *Elymus nutans*, *Poa pratensis*, *Carex scabrirostris*, *Scirpus distigmaticus*, *Gentiana straminea*, *Gentiana farreri*, *Blysmus sinocompressus*, and *Potentilla nivea* (Yang *et al.*, 2013). The soil is classified as a Cambisol (IUSS Working Group WRB, 2007).

In 2005, an area of 80 m × 60 m was marked out and half of the area was used for a controlled N addition experimental regime to investigate the effect of simulated N deposition on the alpine meadow ecosystem in the HAMERS. In this experimental platform, six N addition treatments and an unfertilized control were utilized; each had three replicate plots of 2 m × 2 m (Tian *et al.*, 2014). The six N treatments were arranged in a factorial combination of three chemical N forms and two rates of N application. Ammonium-N (NH_4^+-N), nitrate-N (NO_3^--N), and a combination of NH_4^+-N and NO_3^--N, coded as Am, Ni, and AN, respectively, were the three N forms. $(NH_4)_2SO_4$, $NaNO_3$, and NH_4NO_3 were used for the Am, Ni, and AN treatments, respectively. The two rates of N addition were 1.5 and 7.5 g N m^{-2} $year^{-1}$, designated as rate 1 (N1) and rate 2 (N2), respectively, which were medium N addition rates compared with other similar studies on grassland (e.g. Zhang *et al.*, 2008; Stevens *et al.*, 2010). The seven treatments were Am1, Am2, Ni1, Ni2, AN1, AN2, and control (Ctrl., without N addition). Nitrogen fertilizers were added only once with the total amount on July 10, 2005 and then supplied

twice a year during the vigorous plant-growing season (July and August) of 2006–2011, with half of the total annual N added for each N amendment episode. N was applied in aqueous solution, and 5 L of solution was evenly sprayed into each plot. For the control plot, 5 L of water was supplied (Song *et al.*, 2012).

Soil sampling and plant variables

Plant-growing season ranges from May to September (Song *et al.*, 2012); thus, the soil samples were collected on May 16 and August 29, 2011, representing the early and late plant-growing seasons, respectively. Five soil cores (3.0 cm in diameter; 15 cm in depth) were collected randomly from each plot and mixed as a composite sample. A total of 42 soil samples (seven treatments × three replicates × two sampling times) were obtained, which were immediately packed on ice after collection and transported to the laboratory. After removing fine roots and visible organic debris (< 2 mm), soil samples were divided into two parts, with one part stored at −80 °C for DNA extraction (only samples collected in August) and AM fungal hypha measurement; the other part was air-dried for analysis of AM fungal spore density and soil properties. Soil characteristics were determined using the methods of Tian *et al.* (2014) and shown in Supporting Information, Table S1. Table S2 presents the plant community data.

AM fungal ERH and spore densities

ERH were extracted from 4.0 g of soil from each sample using the membrane filter method (Rillig *et al.*, 1999) and separated into AM and non-AM fungal hyphae based on their morphology and staining color (Miller *et al.*, 1995). AM hyphal length was measured using a grid-line intersect method by observing 135 fields of view for each filter under 200× magnification using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). AM fungal spores were extracted from 20.0 g of air-dried soil from each sample with distilled water using the wet-sieving and decanting method (Daniels & Skipper, 1982) and counted under 40× magnification (Nikon 80i).

DNA extraction and 454 pyrosequencing

Soil DNA was extracted from 0.25 g of samples using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the instructions of the manufacturer. The quality and quantity of the extracted DNA were determined by electrophoresis on a 1.0% agarose gel and spectroscopic analysis (NanoDrop Technologies, Wilmington, DE). The possible inhibitory effects of humic substances were tested by PCR, and then, 10-fold diluted DNA (*c.* 10 ng μL^{-1}) was used in all subsequent PCRs (Tian *et al.*, 2014).

Soil genomic DNA extracts for 454 pyrosequencing were amplified using a two-step PCR procedure. In the first PCR, GeoA-2 (Schwarzott & Schüßler, 2001) and NS4 (White *et al.*, 1990) were the primer sets used. The PCR mixture consisting of 2.5 μL of 10× PCR buffer (Mg^{2+} plus), 2 μL of deoxyribonucleoside triphosphate mixture (each 2.5 mM), 0.5 μL of each primer (10 μM), 1 μL of template, 1.5 U Taq DNA polymerase (TaKaRa, Dalian, China), and 1 μL of template DNA was combined with sterile deionized H_2O to a total volume of 25 μL. The thermal cycling condition was followed by initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 1 min, and extension at 72 °C for 1.5 min, and then a final extension at 72 °C for 10 min. PCR products obtained in the first amplification were diluted 50 times, and 1 μL of the resulting solution was used as the template for the second PCR amplifications using the primers of NS31 (Simon *et al.*, 1992) and AML2 (Lee *et al.*, 2008), which were linked to the sequencing adaptors A (5′-ATCTCAT CCCTGCGTGTCTCCGACGACT-3′) and B (5′-CCTATC CCCTGTGTGCCTTGGCAGTCGACT-3′), respectively. A 10-base pair (bp) barcode sequence was inserted between the A adaptor and the NS31 primer (Table S3). The PCR condition for the second PCR was similar to the first PCR, except for an annealing temperature of 58 °C. The PCR products were purified using PCR Product Gel Purification Kit (Axygen, Union City, CA) and then measured using a TBS 380 fluorescence spectrophotometer (Promega, Madison, WI). A total of 50 ng of DNA from each sample was pooled and adjusted to 10 ng μL^{-1}. The pooled products were subjected to pyrosequencing on a Roche Genome Sequencer FLX Titanium (454 Life Sciences, Branford, CT). The raw sequence data were submitted to the Sequence Read Archive of the National Center for Biotechnology Information, USA (No. PRJNA238405).

Bioinformatic analysis

The noise generated during the sequencing process was removed using the 'shhh.flow' command in Mothur 1.31.2 (Schloss *et al.*, 2009). The sequences that contained invalid primer sequence or DNA tag, ambiguous bases, homopolymers with > 8 bases, or with an average quality score < 25 were removed using the 'trim.seqs' command in Mothur. As the average read quality score dropped below 25 after the 420th bp, the remaining long sequences were cut to 400 bp to ensure read quality (Schloss *et al.*, 2009). Potential chimeras were

checked for the sampled sequences using the 'chimera.uchime' command in MOTHUR. The remaining sequences were clustered into different operational taxonomic units (OTUs) at a 97% similarity level (Lumini *et al.*, 2010) using the CD-HIT-EST (Li & Godzik, 2006). Representative sequences (the most abundant one) for each OTU were blasted against the NCBI nt database (Altschul *et al.*, 1990), and all non-AM fungal OTUs (identified based on the closest BLAST hit not annotated as 'glomeromycota') were removed from the data set. To account for the influences of the different read numbers on the analyses of AM fungal community diversity and composition, the number of sequences per sample was normalized to the smallest sample size using without-replacement method by the 'normalized.shared' command in MOTHUR (Schloss *et al.*, 2009). OTUs occurring as singletons (OTUs only observed once) were excluded from the data to reduce the effects of rare species and less-representative OTUs (Zhou *et al.*, 2011). To identify the obtained AM fungal OTUs, a neighbor-joining tree of all the representative OTU sequences obtained in this study and reference sequences from GenBank was constructed using the *p*-distance model with 1000 replicates to produce bootstrap values. The cumulative number of AM fungal OTUs was calculated using the 'specaccum' function in the Vegan package in R version 3.0.2 (Oksanen *et al.*, 2013).

Data analysis

The abundance of a given AM fungal OTU is defined as the number of reads of that OTU in a sample. The frequency of a given AM fungal OTU is defined as the occurrence of that OTU in all samples. AM fungal OTU richness is defined as the number of OTUs in a sample. The Shannon diversity index calculated from OTU relative abundance was used to estimate AM fungal diversity for each sample (Fierer *et al.*, 2012).

A three-way repeated-measure ANOVA with the form and rate of N addition as between-subject effects and plant-growing season (i.e. sampling time) as within-subject effect was used to analyze the effects of N form, N rate, plant-growing season, and their interaction on the AM fungal ERH and spore densities. A two-way ANOVA was used to analyze the effects of N form, N rate, and their interaction on the AM fungal OTU richness and Shannon diversity index. Multiple comparisons of group means among treatments were carried out with Tukey's HSD test at $P < 0.05$ after one-way ANOVA indicated significant effect of treatment on AM fungal ERH density, spore density, OTU richness, and Shannon diversity index. Response ratios were used to indicate the response sensitivity of AM fungal ERH density, spore density, and soil N availability to N addition (Tian *et al.*, 2014). For example, the response ratio of spore density (R_S) was calculated as the ratio of spore density in the N addition plots (S_{N+}) to that in the Ctrl. (S_{N-}, mean value of three Ctrl. plots), that is $R_S = S_{N+}/S_{N-}$. To test the relationship between AM fungal ERH density or spore density variables and the soil N (NH_4^+-N and NO_3^--N) availability, Pearson's correlations between all these variables (i.e. response ratios) were performed.

Prior to the following analysis on AM fungal community composition, the abundance of every OTUs was Hellinger-transformed to down-weight the influence of rare OTUs. To evaluate the effects of N form, N rate, and their interaction on AM fungal community composition, permutational multivariate analysis of variance (PERMANOVA) using distance matrices was carried out using 'adonis' function in the Vegan package with 999 permutations (Oksanen *et al.*, 2013). Based on the calculated dissimilarities using the Bray–Curtis method (Clarke *et al.*, 2006), the distance matrices of AM fungal community and environmental (plant and soil) factors were subjected to multivariate analysis performed by redundancy analysis (RDA; length of gradient < 3), in which the Monte Carlo 999 permutation test was used to examine the relationship of AM fungal community composition and environmental factors at $P < 0.05$ level.

Structural equation model (SEM) was constructed to investigate the causal relationships among N form and N rate, plant community, soil, AM fungal community composition, and spore density. Prior to the SEM procedure, we investigated the interrelationships among the aforementioned variables by the Mantel test with the Bray–Curtis dissimilarity measurement using 'mantel' function in the Ecodist package (Goslee & Urban, 2007). Variables significantly related to at least one other variable in the Mantel test were retained for the following SEM construction. The *R* values derived from the Mantel test were used as input data to construct SEM. Based on *a priori* and theoretical knowledge, we assumed a conceptual model that N addition altered plant community and soil nutrient content, which in return affected AM fungal community composition and spore density. Maximum-likelihood estimation method was used to compare the SEM with the observation. Model adequacy was determined by chi-square tests, goodness-of-fit index (GFI), Akaike information criteria (AIC), and root square mean errors of approximation (RMSEA). Adequate model fits were indicated by a nonsignificant chi-square test ($P > 0.05$), high GFI (> 0.90), low AIC, and low RMSEA (< 0.05) (Grace, 2006). All statistical analyses were carried out in R 3.0.2 (R Core Team, 2013). SEM analyses were performed using AMOS 22.0.0 (Amos Development Corporation, Meadville, PA).

Results

AM fungal ERH density and spore density

Significant differences were observed in AM fungal ERH density and spore density between May and August ($P < 0.001$, Table 1). N form affected the ERH density ($P < 0.001$), and the effect depended on the N supply rate and plant-growing season (Table 1). N form did not affect ERH density in August, but significantly affected ERH density in May, with higher values in treatments of Ni1 and Ni2, followed by AN1 and AN2, Ctrl., Am1 and Am2 (Fig. 1a). No effect of N form was observed on AM fungal spore density, whereas significant interaction was detected between the N form and plant-growing season ($P = 0.003$, Table 1). Thus, higher spore density occurred in AN1 than the other treatments in August, but not in May (Fig. 1b). N supply rate did not affect ERH density (Table 1 and Fig. 1a), but significantly affected the spore density ($P < 0.001$, Table 1). Compared with the N1 rate, N2 seemed to decrease the spore density, independent of plant-growing season (Fig. 1b). Correlation analyses showed that the response ratio of spore density positively correlated with soil NH_4^+-N availability ($R^2 = 0.178$, $P = 0.010$, Fig. 2a), but negatively correlated with soil NO_3^--N availability ($R^2 = 0.299$, $P = 0.001$, Fig. 2b). No significant correlation relationships were found between AM fungal ERH and soil N availabilities (data not shown).

454 pyrosequencing analysis and identification of AM fungi

A total of 68 809 reads were retained from 92 554 raw reads after the denoising step, and 4550 reads were subsequently removed after the trimming step. Among the remaining 64 259 reads, 2904 potential chimeras were excluded from the data set. Thus, a total of 61 355 good (nonchimeric) reads were obtained and clustered into 645 OTUs at a 97% sequence similarity. Among these OTUs, 180 (22 914 reads) belonged to AM fungi, 26 (209 reads) to plants, 252 (37 805 reads) to animals, 70 (145 reads) to non-AM fungi, 52 (160 reads) to protozoa, and 45 (122 reads) to unidentified eukaryotes. For these 22 914 AM fungal reads, 83 singletons were removed and 97 AM fungal OTUs were obtained from 22 831 reads under non-normalization condition. However, to determine the influences of the different read numbers on the analysis of AM fungal community, AM fungal read numbers were further normalized to 241 when the number of AM fungal reads ranged from 241 to 2478 across all soil samples, resulting in a normalized data set containing 72 AM fungal OTUs (5023 reads). Among the 72 AM fungal OTUs, 18 singletons were removed from the data set. Finally, 54 AM fungal OTUs with more than two reads (5005 reads) were obtained. A total of 14.57 ($\pm$ 0.76) OTUs existed, with a range of 8–22 OTUs recovered from the 21 soil samples.

Among the 54 AM fungal OTUs, 27 (713 reads), 27 (713), 24 (716), 26 (714), 20 (716), 27 (717), and 23 (716) were recovered from the treatments of Am1, Am2, Ni1, Ni2, AN1, AN2, and Ctrl., respectively (Fig. 3a). For these 54 OTUs, 29 (95.9% of total reads) occurred in ≥ 3 samples (frequency $\geq 14.3\%$), defined as common OTUs, whereas the other 25 OTUs (4.1% of total reads) occurred in ≤ 2 samples (Fig. 3b). The nine most abundant OTUs (reads ≥ 100) accounted for 86.5% of the total AM fungal reads; the remaining 45 OTUs only accounted for 13.5% (Fig. 3c). Across all the 54 AM fungal OTUs obtained, 30 belonged to *Glomeraceae*, six to *Claroideoglomeraceae*, three to *Diversisporaceae*, 10 to *Gigasporaceae*, one to *Acaulosporaceae*, three to *Archaeosporaceae*, and one to *Ambisporaceae* (Fig. S1).

Response of AM fungal community to N addition

N rate, but not N form, showed significant effect on AM fungal OTU richness (Table 1). Compared with the Ctrl., N amendment treatments potentially increased the OTU

Table 1. Summary of ANOVAS results indicating the effects of the forms and rates of nitrogen (N) addition and plant-growing season (within-subject effect) on arbuscular mycorrhizal fungal extraradical hyphal (ERH) density, spore density, OTU richness, and Shannon diversity index

		ERH density		Spore density		OTU richness (August data)		Shannon diversity index (August data)	
Source of variation	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
N form	2, 15	22.6	< 0.001	1.25	0.298	2.231	0.150	1.974	0.181
N rate	1, 16	0.298	0.744	30.6	< 0.001	7.259	0.020	4.196	0.063
N form × N rate	2, 12	6.89	< 0.001	1.19	0.332	0.176	0.841	0.547	0.592
Season	1, 34	5.07	< 0.001	85.8	< 0.001	na	na	na	na
Season × N form	2, 30	18.1	< 0.001	6.68	0.003	na	na	na	na
Season × N rate	1, 32	0.082	0.921	1.03	0.368	na	na	na	na
Season × N form × N rate	2, 24	4.75	0.004	3.573	0.015	na	na	na	na

na, the data are not available; OTU, operational taxonomic unit.

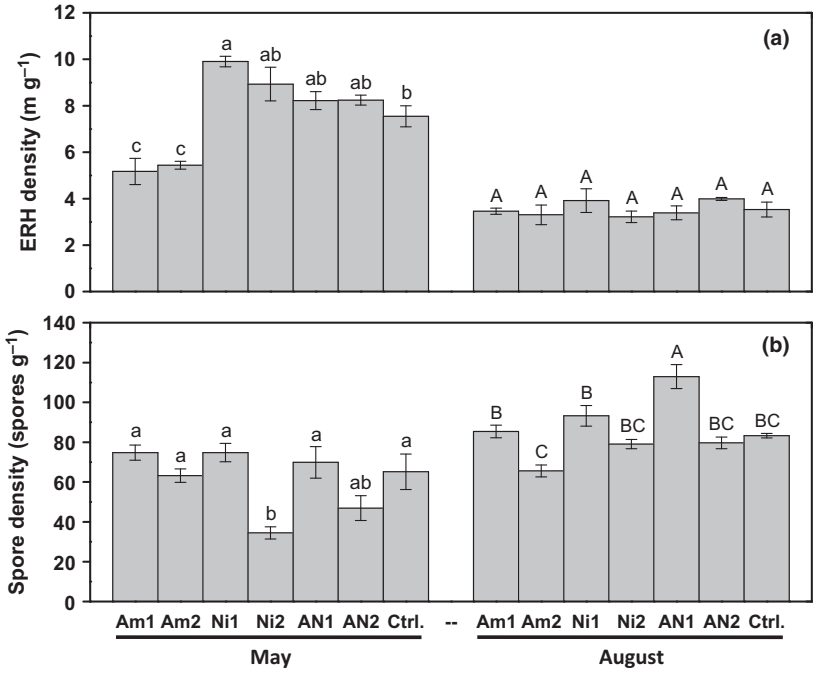


Fig. 1. AM fungal extraradical hyphal (ERH) density (a) and spore density (b) under different treatments of nitrogen (N) form and N supply rate in May and August in an alpine meadow. Bars (mean ± SE) without shared lowercase (comparisons within May) and capital (within August) letters indicate significant difference at $P < 0.05$. Am1, NH_4^+-N, 1.5 g N m^{-2} year^{-1}; Am2, NH_4^+-N, 7.5 g N m^{-2} year^{-1}; Ni1, NO_3^--N, 1.5 g N m^{-2} year^{-1}; Ni2, NO_3^--N, 7.5 g N m^{-2} year^{-1}; AN1, NH_4NO_3-N, 1.5 g N m^{-2} year^{-1}; AN2, NH_4NO_3-N, 7.5 g N m^{-2} year^{-1}; Ctrl., no N addition.

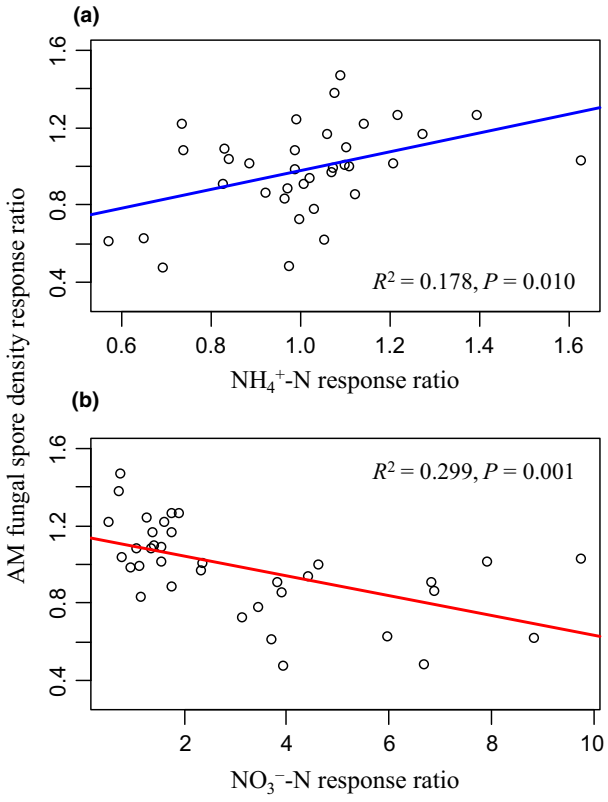


Fig. 2. Relationships of response ratio of AM fungal spore density with response ratios of soil available NH_4^+-N (a) and NO_3^--N (b).

richness, and lower rate N1 (i.e. Am1, Ni1, and AN1) significantly enhanced the OTU richness (Fig. 4a). No significant effects of N form and N rate were observed on the Shannon diversity index of AM fungi by two-way ANOVA analyses (Table 1). However, compared with the Ctrl., N additions at N1 (Am1, Ni1, and AN1) could significantly increase the AM fungal Shannon diversity index as revealed by the multiple comparisons (Fig. 4b).

PERMANOVA analysis showed that the AM fungal community composition was neither affected by N form ($F = 1.24$, $P = 0.255$) nor N rate ($F = 0.95$, $P = 0.528$), except for a significant interaction between N form and N rate ($F = 1.95$, $P = 0.020$). In addition, RDA indicated that the AM fungal community composition did not show noticeable difference among treatments ($R^2 = 0.094$, $P = 0.380$). However, the AM fungal community composition was related to soil available phosphorus (P) ($R^2 = 0.419$, $P = 0.009$), pH ($R^2 = 0.308$, $P = 0.035$), and NO_3^--N content ($R^2 = 0.294$, $P = 0.039$; Fig. 5).

Relationships among N addition, plant, soil, and AM fungal variables

Mantel test results showed that the AM fungal community composition was related to plant community composition ($r = 0.202$, $P = 0.029$) and soil factors ($r = 0.275$, $P = 0.022$), and AM fungal spore density was related to N supply rate ($r = 0.271$, $P = 0.002$) and soil factors ($r = 0.333$, $P = 0.012$; Table S4). Subsequently, the final SEM using Mantel r values (relation coefficients) as input

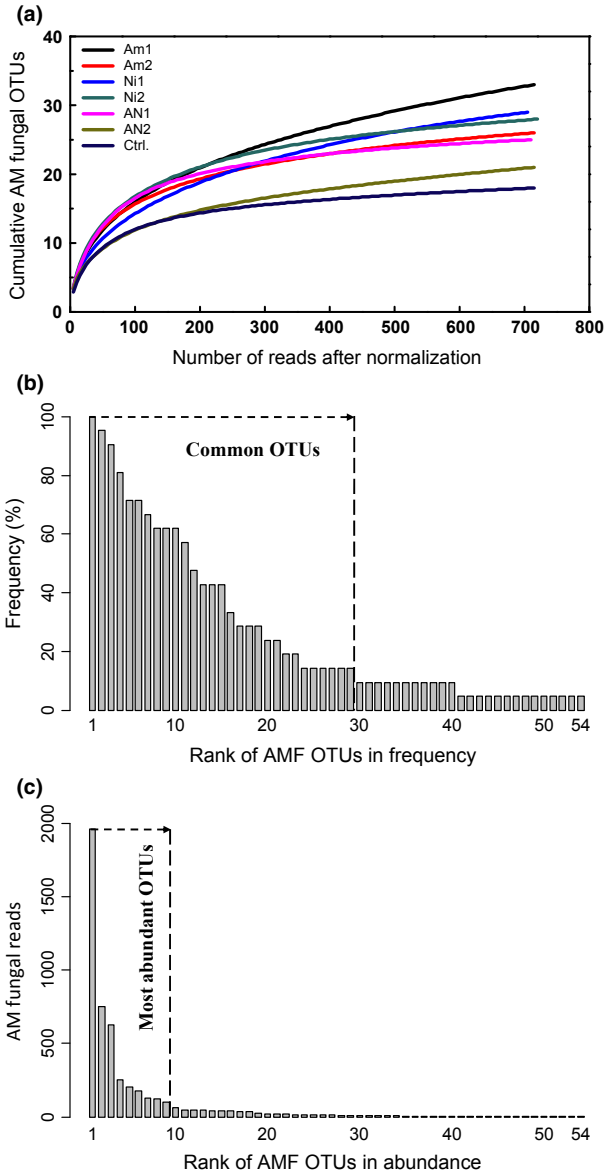


Fig. 3. Rarefaction curves for observed AM fungal OTUs among the different treatments (a); rank of frequency (b) and of reads (c) occurred in all obtained 54 AM fungal OTUs. The 29 common OTUs and nine most abundant OTUs were highlighted by dashed line in (b) and (c), respectively. Am1, NH_4^+-N, 1.5 g N m^{-2} $year^{-1}$; Am2, NH_4^+-N, 7.5 g N m^{-2} $year^{-1}$; Ni1, NO_3^--N, 1.5 g N m^{-2} $year^{-1}$; Ni2, NO_3^--N, 7.5 g N m^{-2} $year^{-1}$; AN1, NH_4NO_3-N, 1.5 g N m^{-2} $year^{-1}$; AN2, NH_4NO_3-N, 7.5 g N m^{-2} $year^{-1}$; Ctrl., no N addition.

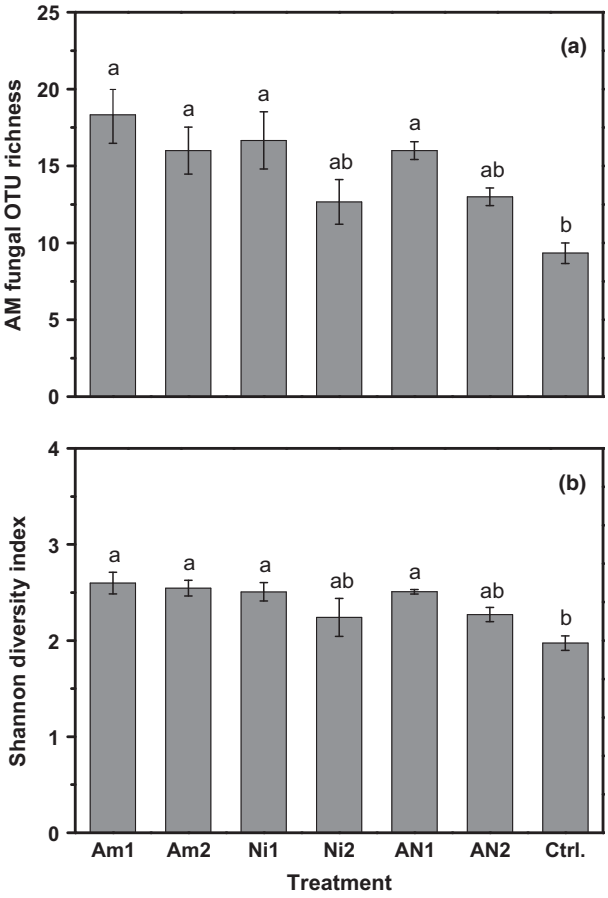


Fig. 4. AM fungal OTU richness (a) and Shannon diversity index (b), under different N addition treatments. Bars (mean ± SE) without shared letters indicate significant differences at $P < 0.05$ level. Am1, NH_4^+-N, 1.5 g N m^{-2} $year^{-1}$; Am2, NH_4^+-N, 7.5 g N m^{-2} $year^{-1}$; Ni1, NO_3^--N, 1.5 g N m^{-2} $year^{-1}$; Ni2, NO_3^--N, 7.5 g N m^{-2} $year^{-1}$; AN1, NH_4NO_3-N, 1.5 g N m^{-2} $year^{-1}$; AN2, NH_4NO_3-N, 7.5 g N m^{-2} $year^{-1}$; Ctrl., no N addition.

adequately fitted the data describing the interaction pathways among plant community composition, soil, AM fungal community composition, and spore density in response to N form and/or N rate ($\chi^2 = 6.867$, d.f. = 6, $P = 0.333$, GFI = 0.989, AIC = 36.87, RMSEA = 0.026; Fig. 6a). The final SEM explained 9.2% and 13.5% of the variation in AM fungal community composition and spore density, respectively (Fig. 6a). N form showed an influence on plant community composition ($\lambda = 0.201$, $P = 0.002$), but not on soil properties, AM fungal community composition, or spore density (all $P > 0.05$). By contrast, N rate showed direct influences on plant community ($\lambda = -0.360$, $P < 0.001$), soil ($\lambda = 0.426$, $P < 0.001$), and AM fungal spore density ($\lambda = 0.158$, $P = 0.027$), but not on AM fungal community composition. Strong influences of soil properties were also detected on plant community composition ($\lambda = 0.493$, $P < 0.001$), AM fungal community composition ($\lambda = 0.234$, $P < 0.001$), and spore density ($\lambda = 0.266$, $P < 0.001$, Fig. 6a). Overall, compared with N form, N rate was more likely to strongly affect soil characteristics, which subsequently influenced plant community composition, AM fungal community composition, and spore

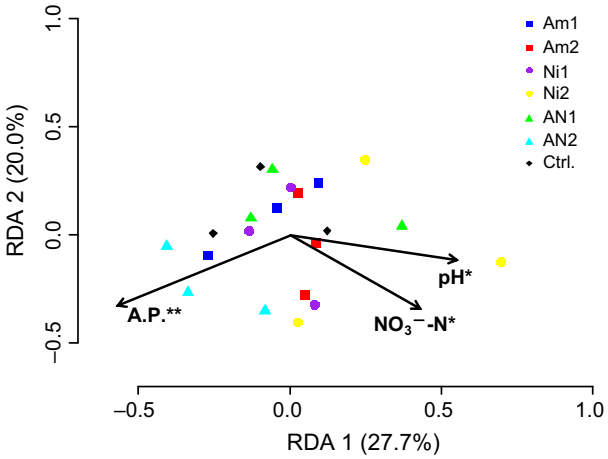
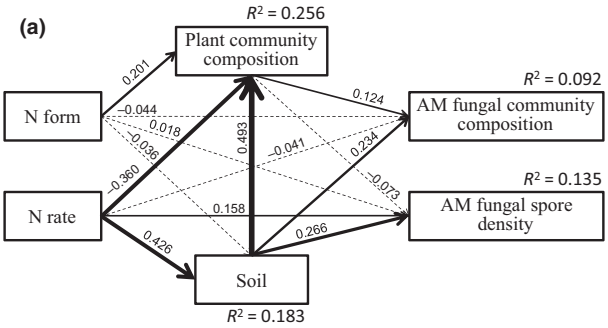


Fig. 5. RDA biplot showing relationship between AM fungal community composition and nitrogen (N) additions (R^2 = 0.094, P = 0.380). The eigenvalues of the first and second axes were 2.461 and 1.831, respectively. Significant variables of soil available phosphorus (A.P., R^2 = 0.419, P = 0.009), pH (R^2 = 0.308, P = 0.035), and soil NO_3^--N content (R^2 = 0.294, P = 0.039) in 'envfit' (based on 999 permutations) were presented as vectors on the ordination graph. Am1, NH_4^+-N, 1.5 g N m^{-2} $year^{-1}$; Am2, NH_4^+-N, 7.5 g N m^{-2} $year^{-1}$; Ni1, NO_3^--N, 1.5 g N m^{-2} $year^{-1}$; Ni2, NO_3^--N, 7.5 g N m^{-2} $year^{-1}$; AN1, NH_4NO_3-N, 1.5 g N m^{-2} $year^{-1}$; AN2, NH_4NO_3-N, 7.5 g N m^{-2} $year^{-1}$; Ctrl., no N addition.



(b)

	Direct path		Indirect path		Total effects	
	N form	N rate	N form	N rate	N form	N rate
Soil	−0.036	0.426	0	0	−0.036	0.426
Plant community composition	0.201	−0.360	−0.015	0.182	0.186	−0.178
AM fungal community composition	−0.044	−0.041	0.010	0.111	−0.034	0.070
AM fungal spore density	0.018	0.158	−0.028	0.132	−0.010	0.290

Fig. 6. (a) SEM showing the causal relationships among N addition, plant community composition, soil, AM fungal community composition, and spore density. The final model fit the data well: maximum likelihood, χ^2 = 6.867, d.f. = 6, P = 0.333; GFI = 0.989; AIC = 36.87; RMSEA = 0.026. Bold and dashed lines indicate significant and nonsignificant pathways, respectively. Width of the bold line indicates the strength of the relationships. R^2 values represent the proportion of variance explained for each variable. (b) Direct, indirect, and total effect coefficients of N form and N rate on soil, plant, and AM fungal variables in this SEM.

density. In addition, plant community composition affected AM fungal community composition (λ = 0.124, P = 0.038), but not spore density (Fig. 6a). These results showed that the effect of N rate on soil properties was mainly by direct path (Fig. 6b). The effect of N form on plant community composition was also by direct path, and N rate effect was both by direct and by indirect paths. Furthermore, the N rate effect on AM fungal spore density was through direct and indirect paths (Fig. 6b). No strong total N addition effect on AM fungal community composition, except for a relatively apparent N rate effect by indirect path, was observed (Fig. 6b).

Discussion

Influence of N addition on AM fungal ERH and spore densities

A significant effect of N form was observed on AM fungal ERH density (P < 0.001, Table 1), and distinctly lower ERH values were found in Am treatments vs. Ni treatments in the early plant-growing season (May), but not in the late growing season (August; Fig. 1a). Similarly, Ngwene *et al.* (2013) reported that the growth of AM fungal ERH is decreased by the supply of NH_4^+-N. This result may be attributed NH_4^+-N, which exhibits lower diffusion in soils compared with NO_3^--N (Sigunga *et al.*, 2002) and may result in a lagged and/or more sustainable N nutrition to plant, decreasing the mutualistic relationship between AM fungi and plants. Therefore, AM fungi will obtain less C allocation from the host and subsequently show lower ERH density. Lower values of ERH density were detected in the NH_4^+ treatments (5.31 m g^{-1}) compared with the control treatment (7.55 m g^{-1}) in May (Fig. 1a). In August, more N (especially NH_4^+-N; Tian *et al.*, 2014) is available for plant growth compared with May. This phenomenon results in a higher C demand in N-fed plant roots that leads to the reduction of C allocation for fungal growth (Treseder *et al.*, 2007; Ngwene *et al.*, 2013) and thus lower ERH density than those in May. Ngwene *et al.* (2013) proposed that N export from the AM fungal hyphae to the plant root and shoot may be greater following NO_3^--N uptake. The host plants, in return, will provide more C to AM fungi and thus increase the ERH density under Ni treatment compared with Am treatment (Fig. 1a). This finding supports that the reciprocal reward strategies by plant and fungal partners guarantee a 'fair trade' of N against C (Fellbaum *et al.*, 2012).

N supply rate did not show significant effects on AM fungal ERH density both in May and August (Table 1 and Fig. 1a). Our results are not unprecedented because investigations of N addition on AM fungal ERH show negative, neutral, and even positive effects according to the summa-

rization by van Diepen *et al.* (2010). The neutral effect observed in this study may be due to the AM fungi, which altered their C allocation patterns to favor intraradical structures rather than external hyphae (Treseder *et al.*, 2007). No clear effect of N form on AM fungal spore density was observed in the present study (Table 1), which is supported by a previous study, in which the spore density was not significantly affected by different N fertilization forms (i.e. NH_4^+ and NO_3^-; Ngwene *et al.*, 2013). However, the higher N supply rate (N2) was more likely to decrease spore density compared with the lower N supply rate (N1), regardless of the plant-growing season (Fig. 1b). Decreased AM spore abundance by N fertilization has been documented in other ecosystems (Egerton-Warburton & Allen, 2000; Bhadalung *et al.*, 2005; Antoninka *et al.*, 2011). Given that the higher available N : P ratios were detected in N2 relative to N1 (Table S1), our results corroborated the previous finding, which illustrated that AM fungal spore densities were altered by soil N : P (Egerton-Warburton *et al.*, 2007). Moreover, the significantly different NO_3^--N content between N1 and N2 treatments mainly accounted for their distinct N : P (Table S1), and higher NO_3^--N will result in lower spore density in this study (Fig. 2b).

N addition effects on AM fungal richness and diversity index

In this study, AM fungal OTU richness was significantly affected by N rate, but not N form (Table 1). Specifically, the N additions (except for Ni2 and AN2) significantly increased the AM fungal richness and Shannon diversity index (Fig. 4a and b). Our results clearly indicated that N addition showed positive effects on the AM fungal richness and diversity. A moderate N addition (6.4 g N m^{-2} $year^{-1}$) increased AM fungal OTU richness compared with the control treatment and high N addition in another alpine meadow ecosystem (Liu *et al.*, 2012). However, Eom *et al.* (1999) observed a neutral N addition (10 g N m^{-2} $year^{-1}$) effect on AM fungal richness and diversity in a tallgrass prairie ecosystem. A medium N addition can support a higher soil microbial diversity as opposed to reduction in microbial diversity under high N input (Zhang *et al.*, 2008). Although intrinsic differences (e.g. mean annual temperature and precipitation) are observed among different ecosystems, our results further confirmed that AM fungal richness could be increased by adding moderate N (1.5–7.5 N m^{-2} $year^{-1}$) in alpine meadow ecosystems.

Factors influencing the AM fungal community composition

The RDA results demonstrated that the AM fungal community composition was not significantly directly influenced by N form and/or N rate, which also supported by the result of PERMANOVA analysis ($P > 0.05$). The soil properties of available P, pH, and NO_3^--N contents were important factors influencing AM fungal community composition (Fig. 5). AM fungal community composition was structured by soil pH and N availability by Egerton-Warburton *et al.* (2007). Alterations in cation/anion balance, as influenced by N addition, clearly have the potential to directly affect soil pH (despite nonsignificance found in this study) and inorganic N availability (Table S1). Therefore, the effects of N addition on AM fungal spore density, richness, and community composition were likely indirect, predominantly mediated through soil, and possibly secondarily by plants (Fig. 6). By contrast, Liu *et al.* (2012) previously reported that N inputs can affect AM fungal community composition mainly by an indirect plant-mediated path. This finding may be due to the concomitant addition of P fertilizer; the N : P ratios in the N addition treatments were much lower (0.3–0.5) in their study than in our study site (7.9–14), as well as in a typical southern European grassland (8.7–11; Mamolos *et al.*, 2005). A strong P limitation will result in the path we observed of N–soil–AM fungi that has a stronger function than the path of N–plant–AM fungi observed by Liu *et al.* (2012). In the absence of P addition, soil microbial communities are independent from plant species, but no such distinction can be observed under P fertilization (Veresoglou *et al.*, 2011a). Therefore, our results will strengthen the importance of soil properties as determinants of AM fungal community (Jansa *et al.*, 2014). In addition, our results agree with a recent study, which proposed that adding N weakens plant–microorganism interactions by a downregulating effect resulting from the nutrient availability in soil (Wei *et al.*, 2013).

In summary, our results showed that N form affected AM fungal ERH density only in the early plant-growing season. The higher rate of N addition (N2, 7.5 g N m^{-2} $year^{-1}$) tended to decrease spore density compared with the lower N rate (N1, 1.5 g N m^{-2} $year^{-1}$) regardless of N form, and the spore density correlated well with soil available N. Compared with the control treatment, the lower rate of N addition (N1) significantly increased AM fungal richness and Shannon diversity index. Furthermore, the AM fungal community composition was not directly influenced by N form or N rate, and the effects were predominantly indirectly mediated through soil and also partially by plants. Soil properties, including available P, pH, and soil NO_3^--N concentrations, were the key factors influencing AM fungal community composition. We suggest that AM fungal abundance and community composition can be influenced by increasing N addition, mainly due to altered soil characteristics and possibly by

changes in the plant community, but not or only slightly by the direct effects of N addition in the Qinghai–Tibetan Plateau alpine meadow ecosystem.

Acknowledgements

The authors would like to thank Dr. Jennifer KM Walker of Hawkesbury Institute for the Environment, University of Western Sydney for English improvements and Dr. Xin-Yu Zhang of the Institute of Microbiology, Chinese Academy of Sciences for support on bioinformatics analysis. We also acknowledge the valuable feedback provided by the editor and three anonymous referees during the revision of the manuscript. This study was financially supported by the National Natural Science Foundation of China (Grant nos 41001149, 31070434, and 31270503). The authors declared that no conflict of interest exists.

References

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Antoninka A, Reich PB & Johnson NC (2011) Seven years of carbon dioxide enrichment, nitrogen fertilization and plant diversity influence arbuscular mycorrhizal fungi in a grassland ecosystem. *New Phytol* **192**: 200–214.

Antunes PM, Lehmann A, Hart MM, Baumecker M & Rillig MC (2012) Long-term effects of soil nutrient deficiency on arbuscular mycorrhizal communities. *Funct Ecol* **26**: 532–540.

Bhadalung NN, Suwanarit A, Dell B, Nopamornbodi O, Thamchaipenet A & Rungchuang J (2005) Effects of long-term NP-fertilization on abundance and diversity of arbuscular mycorrhizal fungi under a maize cropping system. *Plant Soil* **270**: 371–382.

Bobbink R, Hicks K, Galloway J *et al.* (2010) Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. *Ecol Appl* **20**: 30–59.

Bradley K, Drijber RA & Knops J (2006) Increased N availability in grassland soils modifies their microbial communities and decreases the abundance of arbuscular mycorrhizal fungi. *Soil Biol Biochem* **38**: 1583–1595.

Chen YL, Zhang X, Ye JS, Han HY, Wan SQ & Chen BD (2014) Six-year fertilization modifies the biodiversity of arbuscular mycorrhizal fungi in a temperate steppe in Inner Mongolia. *Soil Biol Biochem* **69**: 371–381.

Clarke KR, Somerfield PJ & Chapman MG (2006) On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray-Curtis coefficient for denuded assemblages. *J Exp Mar Biol Ecol* **330**: 55–80.

Corkidi L, Rowland DL, Johnson NC & Allen EB (2002) Nitrogen fertilization alters the functioning of arbuscular mycorrhizas at two semiarid grasslands. *Plant Soil* **240**: 299–310.

Daniels BA & Skipper HD (1982) Methods for the recovery and quantitative estimation of propagules from soil. *Methods and Principles of Mycorrhizal Research* (Schenck NC, ed.), pp. 29–37. American Phytopathological Society, St. Paul, MN, USA.

Drigo B, Pijl AS, Duyts H *et al.* (2010) Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO_2. *P Natl Acad Sci USA* **107**: 10938–10942.

Egerton-Warburton LM & Allen EB (2000) Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. *Ecol Appl* **10**: 484–496.

Egerton-Warburton LM, Johnson NC & Allen EB (2007) Mycorrhizal community dynamics following nitrogen fertilization: a cross-site test in five grasslands. *Ecol Monogr* **77**: 527–544.

Eom AH, Hartnett DC, Wilson GWT & Figge DAH (1999) The effect of fire, mowing and fertilizer amendment on arbuscular mycorrhizas in tallgrass prairie. *Am Midl Nat* **142**: 55–70.

Fellbaum CR, Gachomo EW, Beesetty Y, Choudhari S, Strahan GD, Pfeffer PE, Kiers ET & Bücking H (2012) Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. *P Natl Acad Sci USA* **109**: 2666–2671.

Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA & Knight R (2012) Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *ISME J* **6**: 1007–1017.

Fornara DA & Tilman D (2012) Soil carbon sequestration in prairie grasslands increased by chronic nitrogen addition. *Ecology* **93**: 2030–2036.

Garcia MO, Ovasapyan T, Greas M & Treseder KK (2008) Mycorrhizal dynamics under elevated CO_2 and nitrogen fertilization in a warm temperate forest. *Plant Soil* **303**: 301–310.

Goslee SC & Urban DL (2007) The ecodist package for dissimilarity-based analysis of ecological data. *J Stat Softw* **22**: 1–19.

Grace JB (2006) *Structural Equation Modeling and Natural Systems*. Cambridge University Press, Cambridge, UK, New York, NY.

IUSS Working Group WRB (2007) *World Reference Base for Soil Resources 2006, First Update 2007*. World Soil Resources Reports No. 103. FAO, Rome.

Jansa J, Erb A, Oberholzer H-R, Šmilauer P & Egli S (2014) Soil and geography are more important determinants of indigenous arbuscular mycorrhizal communities than management practices in Swiss agricultural soils. *Mol Ecol* **23**: 2118–2135.

Johnson NC, Rowland DL, Corkidi L, Egerton-Warburton LM & Allen EB (2003) Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. *Ecology* **84**: 1895–1908.

Kiers ET, Duhamel M, Beesetty Y *et al.* (2011) Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* **333**: 880–882.

Lee J, Lee S & Young JPW (2008) Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiol Ecol* **65**: 339–349.

Li WZ & Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**: 1658–1659.

Lin XG, Feng YZ, Zhang HY, Chen RR, Wang JH, Zhang JB & Chu HY (2012) Long-term balanced fertilization decreases arbuscular mycorrhizal fungal diversity in an arable soil in north China revealed by 454 pyrosequencing. *Environ Sci Technol* **46**: 5764–5771.

Liu YJ, Shi GX, Mao L, Cheng G, Jiang SJ, Ma XJ, An LZ, Du GZ, Johnson NC & Feng HY (2012) Direct and indirect influences of 8 yr of nitrogen and phosphorus fertilization on Glomeromycota in an alpine meadow ecosystem. *New Phytol* **194**: 523–535.

Liu XJ, Zhang Y, Han WX *et al.* (2013) Enhanced nitrogen deposition over China. *Nature* **494**: 459–462.

Lumini E, Orgiazzi A, Borriello R, Bonfante P & Bianciotto V (2010) Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach. *Environ Microbiol* **12**: 2165–2179.

Magnani F, Mencuccini M, Borghetti M *et al.* (2007) The human footprint in the carbon cycle of temperate and boreal forests. *Nature* **447**: 848–850.

Mamolos AP, Vasilikos CV & Veresoglou DS (2005) Vegetation in contrasting soil water sites of upland herbaceous grasslands and N : P ratios as indicators of nutrient limitation. *Plant Soil* **270**: 355–369.

Miller RM, Reinhardt DR & Jastrow JD (1995) External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. *Oecologia* **103**: 17–23.

Ngwene B, George E, Claussen W & Neumann E (2010) Phosphorus uptake by cowpea plants from sparingly available or soluble sources as affected by nitrogen form and arbuscular-mycorrhiza-fungal inoculation. *J Plant Nutr Soil Sci* **173**: 353–359.

Ngwene B, Gabriel E & George E (2013) Influence of different mineral nitrogen sources (NO_3^--N vs. NH_4^+-N) on arbuscular mycorrhiza development and N transfer in a *Glomusintraradices* – cowpea symbiosis. *Mycorrhiza* **23**: 107–117.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH & Wagner H (2013) Vegan: community ecology package. R package version 2.0-10. http://CRAN.R-project.org/package=vegan.

R Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rillig MC, Field CB & Allen MF (1999) Soil biota responses to long-term atmospheric CO_2 enrichment in two California annual grasslands. *Oecologia* **119**: 572–577.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing mothur: open-Source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**: 7537–7541.

Schüßler A, Schwarzott D & Walker C (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. *Mycol Res* **105**: 1413–1421.

Schwarzott D & Schüßler A (2001) A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AM fungal spores. *Mycorrhiza* **10**: 203–207.

Sigunga DO, Janssen BH & Oenema O (2002) Ammonia volatilization from vertisols. *Eur J Soil Sci* **53**: 195–202.

Sikes B, Maherali H & Klironomos J (2014) Mycorrhizal fungal growth responds to soil characteristics, but not host plant identity, during a primary lacustrine dune succession. *Mycorrhiza* **24**: 219–226.

Simon L, Lalonde M & Bruns TD (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. *Appl Environ Microbiol* **58**: 291–295.

Smith SE & Read DJ (2008) *Mycorrhizal Symbiosis*, 3rd edn. Academic Press, London, UK.

Song MH, Yu FH, Ouyang H, Cao GM, Xu XL & Cornelissen JHC (2012) Different inter-annual responses to availability and form of nitrogen explain species coexistence in an alpine meadow community after release from grazing. *Glob Change Biol* **18**: 3100–3111.

Staddon PL (2005) Mycorrhizal fungi and environmental change: the need for a mycocentric approach. *New Phytol* **167**: 635–637.

Staddon PL, Thompson K, Jakobsen I, Grime JP, Askew AP & Fitter AH (2003) Mycorrhizal fungal abundance is affected by long-term climatic manipulations in the field. *Glob Change Biol* **9**: 186–194.

Stevens CJ, Thompson K, Grime JP, Long CJ & Gowing DJG (2010) Contribution of acidification and eutrophication to declines in species richness of calcifuge grasslands along a gradient of atmospheric nitrogen deposition. *Funct Ecol* **24**: 478–484.

Tanaka Y & Yano K (2005) Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied. *Plant, Cell Environ* **28**: 1247–1254.

Tian H, Drijber RA, Zhang JL & Li XL (2013) Impact of long-term nitrogen fertilization and rotation with soybean on the diversity and phosphorus metabolism of indigenous arbuscular mycorrhizal fungi within the roots of maize (*Zea mays* L.). *Agric Ecosyst Environ* **164**: 53–61.

Tian XF, Hu HW, Ding Q, Song MH, Xu XL, Zheng Y & Guo LD (2014) Influence of nitrogen fertilization on soil ammonia oxidizer and denitrifier abundance, microbial biomass, and enzyme activities in an alpine meadow. *Biol Fertil Soils* **50**: 703–713.

Treseder KK (2008) Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. *Ecol Lett* **11**: 1111–1120.

Treseder KK & Cross A (2006) Global distributions of arbuscular mycorrhizal fungi. *Ecosystems* **9**: 305–316.

Treseder KK, Turner KM & Mack MC (2007) Mycorrhizal responses to nitrogen fertilization in boreal ecosystems: potential consequences for soil carbon storage. *Glob Change Biol* **13**: 78–88.

van Diepen LTA, Lilleskov EA, Pregitzer KS & Miller RM (2010) Simulated nitrogen deposition causes a decline of intra- and extraradical abundance of arbuscular mycorrhizal fungi and changes in microbial community structure in northern hardwood forests. *Ecosystems* **13**: 683–695.

van Diepen LTA, Lilleskov EA & Pregitzer KS (2011) Simulated nitrogen deposition affects community structure of arbuscular mycorrhizal fungi in northern hardwood forests. *Mol Ecol* **20**: 799–811.

Veresoglou SD, Mamolos AP, Thornton B, Voulgari OK, Sen R & Veresoglou DS (2011a) Medium-term fertilization of grassland plant communities masks plant species-linked effects on soil microbial community structure. *Plant Soil* **344**: 187–196.

Veresoglou SD, Sen R, Mamolos AP & Veresoglou DS (2011b) Plant species identity and arbuscular mycorrhizal status modulate potential nitrification rates in nitrogen-limited grassland soils. *J Ecol* **99**: 1339–1349.

Veresoglou SD, Chen BD & Rillig MC (2012) Arbuscular mycorrhiza and soil nitrogen cycling. *Soil Biol Biochem* **46**: 53–62.

Wei CZ, Yu Q, Bai E, Lü XT, Li Q, Xia JY, Kardol P, Liang WJ, Wang ZW & Han XG (2013) Nitrogen deposition weakens plant–microbe interactions in grassland ecosystems. *Glob Change Biol* **19**: 3688–3697.

White TJ, Bruns TD, Lee SB & Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ & White TJ, eds), pp. 315–321. Academic Press, San Diego, CA.

Yang W, Zheng Y, Gao C, He XH, Ding Q, Kim YC, Rui YC, Wang SP & Guo LD (2013) The arbuscular mycorrhizal fungal community response to warming and grazing differs between soil and roots on the Qinghai-Tibetan Plateau. *PLoS ONE* **8**: e76447.

Zhang NL, Wan SQ, Li LH, Bi J, Zhao MM & Ma KP (2008) Impacts of urea N addition on soil microbial community in a semi-arid temperate steppe in northern China. *Plant Soil* **311**: 19–28.

Zhang LM, Wang M, Prosser JI, Zheng YM & He JZ (2009) Altitude ammonia-oxidizing bacteria and archaea in soils of Mount Everest. *FEMS Microbiol Ecol* **70**: 208–217.

Zheng Y, Yang W, Sun X, Wang SP, Rui YC, Luo CY & Guo LD (2012) Methanotrophic community structure and activity under warming and grazing of alpine meadow on the Tibetan Plateau. *Appl Microbiol Biotechnol* **93**: 2193–2203.

Zheng Y, Yang W, Hu HW, Kim YC, Duan JC, Luo CY, Wang SP & Guo LD (2014) Ammonia oxidizers and denitrifiers in response to reciprocal elevation translocation in an alpine meadow on the Tibetan Plateau. *J Soils Sediments* **14**: 1189–1199.

Zhou JZ, Wu LY, Deng Y, Zhi XY, Jiang YH, Tu QC, Xie JP, van Nostrand JD, He ZL & Yang YF (2011) Reproducibility and quantitation of amplicon sequencing-based detection. *ISME J* **5**: 1303–1313.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A neighbour-joining tree constructed based on a 400-bp fragment of arbuscular mycorrhizal (AM) fungal 18S rRNA gene sequences.

Table S1. Selected soil basic characteristics.

Table S3. Barcode sequence in each sample of different treatments in the raw sequence data used in this study.

Table S4. Mantel tests among distance matrices of N addition, plant community composition, soil, and AM fungal properties.

Table S2. Aboveground biomass (kg m^{-2}) of 20 plant species in nitrogen addition plots of an alpine meadow on the Qinghai–Tibetan Plateau.