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# Dynamics of arbuscular mycorrhizal fungal community structure and functioning along a nitrogen enrichment gradient in an alpine meadow ecosystem

**Shengjing Jiang[1], Yongjun Liu[1], Jiajia Luo[1], Mingsen Qin[1], Nancy Collins Johnson[2], Maarja Öpik[3], Martti Vasar[3], Yuxing Chai[1], Xiaolong Zhou[4], Lin Mao[4], Guozhen Du[4], Lizhe An[1] and Huyuan Feng[1]**

[1]Ministry of Education Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou 730000, China; [2]School of Earth Sciences and Environmental Sustainability, Northern Arizona University, Flagstaff, AZ 86011, USA; [3]Department of Botany, University of Tartu, Tartu 51005, Estonia; [4]State Key Laboratory of Grassland and Agro-Ecosystems, School of Life Sciences, Lanzhou University, Lanzhou 730000, China

Authors for correspondence:
*Yongjun Liu*
*Tel: +86 931 8912560*
*Email: yjliu@lzu.edu.cn*

*Huyuan Feng*
*Tel: +86 931 8912537*
*Email: fenghy@lzu.edu.cn*

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## Summary

- Nitrogen (N) availability is increasing dramatically in many ecosystems, but the influence of elevated N on the functioning of arbuscular mycorrhizal (AM) fungi in natural ecosystems is not well understood.
- We measured AM fungal community structure and mycorrhizal function simultaneously across an experimental N addition gradient in an alpine meadow that is limited by N but not by phosphorus (P). AM fungal communities at both whole-plant-community (mixed roots) and single-plant-species (*Elymus nutans* roots) scales were described using pyro-sequencing, and the mycorrhizal functioning was quantified using a mycorrhizal-suppression treatment in the field (whole-plant-community scale) and a glasshouse inoculation experiment (single-plant-species scale).
- Nitrogen enrichment progressively reduced AM fungal abundance, changed AM fungal community composition, and shifted mycorrhizal functioning towards parasitism at both whole-plant-community and *E. nutans* scales. N-induced shifts in AM fungal community composition were tightly linked to soil N availability and/or plant species richness, whereas the shifts in mycorrhizal function were associated with the communities of specific AM fungal lineages.
- The observed changes in both AM fungal community structure and functioning across an N enrichment gradient highlight that N enrichment of ecosystems that are not P-limited can induce parasitic mycorrhizal functioning and influence plant community structure and ecosystem sustainability.

## Introduction

The amount of biologically available nitrogen (N) is increasing dramatically in many ecosystems throughout the world due to human activities (Vitousek *et al.*, 1997). Increased N availability often increases plant productivity but reduces plant species diversity (Stevens *et al.*, 2004; Clark & Tilman, 2008) and causes local extinction of some susceptible functional groups (Suding *et al.*, 2005). In addition, N enrichment can also dramatically change the abundance and diversity of soil microbial communities (Ma *et al.*, 2016; Zhou *et al.*, 2016), induce dormancy and decrease the diversity of active microbes (Kearns *et al.*, 2016), and weaken plant–microbe interactions (Wei *et al.*, 2013). Due to the cascade of negative effects on biotic communities, global N enrichment is considered one of the greatest threats to ecosystem structure and function (Smith *et al.*, 1999).

Arbuscular mycorrhizal (AM) fungi in the subphylum Glomeromycotina (Spatafora *et al.*, 2016) are widespread root-associated microorganisms that contribute significantly to a range of ecosystem processes (van der Heijden *et al.*, 2015). It is generally accepted that AM fungi provide host plants with soil phosphorus (P) and N (Hodge *et al.*, 2010) as well as other benefits, such as protection against pathogens (Jung *et al.*, 2012), in exchange for plant-assimilated carbon (Selosse & Rousset, 2011). AM fungi also play key roles in regulating plant communities (van der Heijden *et al.*, 2008; Bever *et al.*, 2010; Zobel & Öpik, 2014), rhizosphere microbial communities (Vestergård *et al.*, 2008; Lioussanne *et al.*, 2010; Veresoglou *et al.*, 2012), soil structure (Wilson *et al.*, 2009; Leifheit *et al.*, 2015) and nutrient cycles (Bender *et al.*, 2015). Despite their importance for plant growth and ecosystem functioning, it is not well understood how changing environments, such as increasing terrestrial N deposition,

influence AM fungal community structure and function. Filling this knowledge gap will enable better predictions of the consequences of mycorrhizal fungal community change under global change scenarios.

Theoretical models predict that AM fungal community structure and function should be very sensitive to changes in carbon, N and P stoichiometry (Johnson, 2010). Numerous field studies have shown that increased N availability can reduce the abundance and diversity of AM fungal communities (Egerton-Warburton & Allen, 2000; van Diepen *et al.*, 2010; Camenzind *et al.*, 2014; Chen *et al.*, 2017; Williams *et al.*, 2017), but neutral or even positive effects have also been observed in some cases (e.g. Egerton-Warburton *et al.*, 2007; van Diepen *et al.*, 2011; Zheng *et al.*, 2014). These contradictory findings may be caused by the complex interactions of many factors, such as the relative availability of N and P (Johnson, 2010) and plant identity (Egerton-Warburton *et al.*, 2007; Johnson *et al.*, 2008). Although the abundance and composition of AM fungal communities are known to change with N enrichment, linking community changes in the field to mycorrhizal functioning remains a challenge (van der Heijden & Scheublin, 2007). Many experimental tests with artificially assembled communities have demonstrated that changes in community attributes of AM fungi, such as diversity or species composition, can change plant performance and ecosystem functioning (e.g. van der Heijden *et al.*, 1998; Vogelsang *et al.*, 2006; Maherali & Klironomos, 2007; Wagg *et al.*, 2011; Li *et al.*, 2012), suggesting that functioning of mycorrhizal (plant–fungal) interactions may be largely influenced by mycorrhizal fungal community structure.

While AM symbioses are generally considered to be mutually beneficial, rich evidence shows that the interactions between AM fungi and plants will shift from stronger mutualism to weaker mutualism or parasitism with increasing soil N and P availability (e.g. Bethlenfalvay *et al.*, 1983; Johnson, 1993; Hoeksema *et al.*, 2010; Johnson *et al.*, 2015). The functional equilibrium model predicts that N enrichment of P-limited soils will generate a strong mutualism due to the increased P-uptake value of mycorrhizas, whereas N enrichment of P-rich soils will reduce plant allocation to mycorrhizas and induce parasitism (Johnson, 2010; Revillini *et al.*, 2016). These predictions are well supported by a recent glasshouse study showing that the effect of N enrichment on the phenotype of AM symbioses ranged from commensalism or parasitism in P-rich soils to mutualism in P-limited soils (Johnson *et al.*, 2015). There is also evidence that N enrichment in N-limited soils will benefit AM symbionts (Johnson *et al.*, 2008), because N limitation could directly inhibit fungal growth (Treseder & Allen, 2002) and generate strong plant–fungus competition for N (Püschel *et al.*, 2016). Furthermore, it has been well documented that mycorrhizal performance varies among different combinations of plants and AM fungi (Helgason *et al.*, 2002; Klironomos, 2003; Uibopuu *et al.*, 2009), suggesting that N-induced shifts in species composition of both plant and AM fungal communities should also be taken into account when we predict how mycorrhizal function responds to N enrichment.

In this study, we simultaneously explored the responses of AM fungal community structure and function to 4 yr of N addition treatments in an alpine meadow ecosystem on the Tibetan Plateau, where both plant and AM fungal diversity are relatively high (Liu *et al.*, 2012) and ecosystem productivity is strongly limited by N availability (Xu *et al.*, 2015), but not P availability (X. L. Zhou, unpublished data). The abundance and composition of AM fungal communities inside roots were analyzed at the scale both of the whole plant community and of an individual plant species (*Elymus nutans*; a winner species after N enrichment). To assess the influence of AM fungi at the whole-plant-community scale, we applied a 4 yr mycorrhizal-suppression treatment (using the fungicide benomyl) that was nested within each N addition treatment, and mycorrhizal function was quantified in terms of changes in plant biomass and plant tissue N and P content. In addition, we established a glasshouse experiment with mycorrhizal fungal inoculation (entire AM fungal community collected from each field plot) and N addition (similar with the addition levels in field) to explore the mycorrhizal response of *E. nutans* in terms of biomass and tissue nutrient content. We hypothesized that: $H_1$, N enrichment would reduce AM fungal abundance and diversity, resulting in a significant variation in AM fungal community composition; $H_2$, AM function would shift from mutualism to parasitism across the N enrichment gradient, and that the functional shift would be associated with the N-induced changes in AM fungal community structure; and $H_3$, the predicted effects of N enrichment on AM fungi would be manifested at both the whole-plant-community scale and the single-plant-species scale.

## Materials and Methods

### Study site and field experimental design

This study was carried out in an alpine meadow on the eastern Tibetan Plateau of China (33°40′N, 101°51′E, *c.* 3500 m above sea level (asl); Supporting Information Fig. S1), where mean annual temperature is 1.2°C, mean annual precipitation is 672 mm, the soil type is Cambisol (FAO taxonomy), and the vegetation is dominated by *Kobresia capillifolia* and some grasses, such as *E. nutans*. This experimental meadow is managed by the Research Station of Alpine Meadow and Wetland Ecosystems of Lanzhou University, China. The research site has been fenced and is only grazed every winter from November to April since 2011. Total N deposition in this region is estimated to range from 14.26 to 18.65 kg $ha^{-1}$ $yr^{-1}$ (Lü & Tian, 2007).

The N addition experiment was established in April 2011. Four levels of N addition (including 0, 5, 10 and 15 g N $m^{-2}$ $yr^{-1}$; hereafter referred to as N0, N5, N10 and N15, respectively) with six replicates were distributed in 24 10 × 20 $m^2$ plots (1 m buffer strips) using a randomized block design (Fig. S1). The N addition gradient was generated with different amounts of $NH_4NO_3$ fertilizer applied annually (spread manually on drizzly days) at the beginning of the growing season (usually in May).

To understand how AM functioning responds to N addition in the field, on 11 June 2011, we established a mycorrhizal suppression experiment within the setup of the N addition experiment. We selected 16 plots within four blocks to conduct this

experiment (Fig. S1). Two $1 \times 2\ m^2$ subplots with 1 m buffer strips were nested in each plot: one subplot was treated with fungicide monthly throughout the growing season (8 g benomyl in 5 l water $m^{-2}$ $month^{-1}$ from May to September every year), and the other received an equal amount of water every time (as a control). Benomyl was chosen because it has been shown to effectively suppress AM fungal colonization but has small effects on plants and other soil microorganisms (Hartnett & Wilson, 1999; O'Connor *et al.*, 2002), and benomyl application has been used successfully to test the functions of AM fungal communities in field studies (e.g. O'Connor *et al.*, 2002; Callaway *et al.*, 2004; Yang *et al.*, 2014).

### Sample collection and vegetation analysis in the field

Samples were collected on 20 June, 25 July and 30 August 2014 (the fourth year of treatments). On each sampling date, five soil cores (diameter, 3.8 cm; depth, 25 cm) were taken randomly from each plot and mixed into a composite sample, resulting in a total of 72 samples. Fine live roots were separated carefully from each soil sample, washed and used for DNA extraction and determination of root AM fungal colonization. The remaining soil samples were air-dried, sieved (2 mm) and used to measure soil properties (including soil moisture, pH, total N, organic carbon, available N and available P) using the same methods described by Liu *et al.* (2012), and densities of AM fungal spores and hyphae. To explore the effect of N addition on the AM fungal community at the individual-plant-species level, we sampled a dominant plant species, *E. nutans*, on the third sampling date. Eight *E. nutans* individuals were chosen randomly from each plot, excavated wholly and the fine roots of four individuals were pooled as one sample (24 samples in total) for DNA extraction and determination of root AM fungal colonization; the remaining four individuals were used to measure shoot and root biomass. To determine the suppression effect of benomyl application on AM fungi, we randomly collected three soil cores from each subplot on the third sampling date, and the fine live roots were collected and used for determination of root AM fungal colonization.

Vegetation was described in two $0.5 \times 0.5\ m^2$ quadrats in each plot or one quadrat in each subplot in mid-August 2014. All plant species were identified and the numbers of individuals of each species were recorded. Subsequently, all plants in each quadrat were clipped to the soil surface to measure shoot biomass. Concurrently with the above-ground vegetation sampling, five soil cores (diameter, 3.8 cm; depth, 25 cm) in each plot and two soil cores in each subplot were taken randomly, and the live plant materials below ground were collected carefully to measure root biomass. All plant biomass samples were dried at 80°C for 48 h and weighed, and the dried plants were used then to analyze tissue N and P concentrations using the methods described by Liu *et al.* (2012).

### Mycorrhizal inoculation of *E. nutans* in the glasshouse

Because the benomyl treatment cannot fully inhibit mycorrhizal fungal colonization in the field (Hartnett & Wilson, 1999), we conducted a glasshouse microcosm experiment with *E. nutans* and field inocula to investigate the functional implication of AM fungal community change induced by N addition. We used a mixture (1 : 1, v/v) of soil collected from a nearby meadow and sand as the soil substrate in the microcosms. The soil substrate was sieved (2 mm), steam-sterilized twice (1 d interval, 121°C for 25 min each), air-dried and used to fill 144 plastic pots with 1 kg of substrate in each pot. Mycorrhizal fungal inocula were the fresh soils containing mycorrhizal roots, AM fungal mycelia and spores that were collected (based on five soil cores) from each plot on 25 July 2014, for a total of 24 inocula. Each inoculum was replicated three times, whereby 100 g of living-soil inoculum was added in each pot to create a mycorrhizal treatment (M). In addition, three pots were treated similarly but with the corresponding sterilized soil inoculum to create a nonmycorrhizal treatment (NM). To correct for differences in communities of other soil microbes, the three NM pots received 80 ml of soil sievate from the corresponding living-soil inoculum produced by blending 150 g inoculum in 500 ml water and passing through a 38 μm sieve, and the mycorrhizal pots received the same volume of water. Surface-sterilized seeds of *E. nutans* were pregerminated and sown in each pot, and eight 5-d-old seedlings were retained in each pot. To ensure that soil N concentrations were similar to those of corresponding plots in the field, extra N was added as $NH_4NO_3$ according to the transformed formula: 20 mg N $kg^{-1}$ soil added in a pot = 5 g N $m^{-2}$ $yr^{-1}$ fertilized in the field. Plants were grown in a glasshouse with controlled temperature (day: 20°C, night: 16°C) and a *c.* 170 μmol photons $m^{-2}$ $s^{-1}$ artificial illumination for a day length of 16 h. All pots were watered every 3 d and the pot positions were randomized every 2 wk. After 12 wk, all plant individuals in each pot were harvested and used for measuring shoot and root biomass and AM fungal colonization. Plant biomass and tissue N and P concentrations were measured using the same methods as described above, and all variables were represented by the means of the three replicated pots.

### Analyses of AM fungal colonization, spore density and extraradical hyphal length density

Root samples were cleared in 10% (w/v) KOH at 70°C for 25 min, acidified in 2% (v/v) HCl for 30 min and stained with 0.05% (w/v) Trypan blue at 70°C for 10 min. The percentage of root length colonized by AM fungi (%RLC), by arbuscules (%AC) and by vesicles (%VC) were quantified using the magnified intersection method (McGonigle *et al.*, 1990). AM fungal spores in soil were extracted by wet sieving and sucrose centrifugation (Brundrett *et al.*, 1994), and the spore density was calculated. Extraradical hyphae were extracted from 5 g of soil and stained with trypan blue using the methods described by Brundrett *et al.* (1994). AM fungal hyphae were distinguished from other fungal hyphae according to morphology and staining color (Miller *et al.*, 1995; see Fig. S2), and the hyphal length density was quantified by the grid-line intersect method (Brundrett *et al.*, 1994).

### Molecular identification of AM fungi colonizing roots and bioinformatic analysis

Ninety-six root samples, including 72 mixed and 24 *E. nutans* root samples collected in the field, were used for molecular analysis. For each sample, DNA was extracted from 100 mg of randomly selected root fragments using a Plant DNA Extraction Kit according to the manufacturer's instructions (Tiangen Biotech, Beijing, China). The extracted DNA was used to amplify the 18S rRNA gene with the universal fungal primers GeoA2 and Geo11 (Schwarzott & Schüßler, 2001), following the PCR conditions described by Liu *et al.* (2012). All samples were amplified successfully, and the PCR products of mixed roots from each plot were combined across the three sampling dates. A total of 48 PCR products (24 for mixed roots and 24 for *E. nutans* roots) were subjected to a nested PCR with the fusion primers NS31 (Simon *et al.*, 1992) (454-adaptor A + 8-bp barcode + NS31, forward primer) and AML2 (Lee *et al.*, 2008) (454-adaptor B + AML2, reverse primer). The reactions were carried out in a 20 μl reaction volume containing 10 ng of template DNA, 0.25 mM dNTPs, 0.2 μM of each primer and 2 U of the FastPfu DNA polymerase (TransGen Biotech, Beijing, China) on a GeneAmp PCR system 9700 (Applied Biosystems), with the following PCR conditions: 94°C for 2 min, 30 cycles of 94°C for 30 s followed by 58°C for 1 min and 72°C for 1 min, and a final elongation step at 72°C for 10 min. PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen, USA) and quantified using the QuantiFluor system (Promega). Equimolar concentrations of 48 tagged samples were sequenced on a Roche Genome Sequencer FLX system using Titanium Series reagents (Roche Applied Science).

The bioinformatic analysis of sequence data was implemented following the methods described by Davison *et al.* (2012) and Grilli *et al.* (2015). Briefly, the reads were retained only if they carried the correct barcode and forward primer sequences, and had average quality score ≥ 25 and length ≥ 170-bp (excluding barcode and primer sequences). The filtered sequences were checked for chimeras using UCHIME v.7 (Edgar *et al.*, 2011) with the Maarj*AM* database of Glomeromycotina as a reference (Öpik *et al.*, 2010), and all potentially chimeric sequences were removed from the dataset. The remaining 479 947 reads were analyzed by BLAST v.2.5 (Camacho *et al.*, 2009) against the Maarj*AM* database (Öpik *et al.*, 2010) and assigned to virtual taxa (VTs) with the following criteria: a sequence similarity level ≥ 97%, alignment length ≥ 95% of the query sequence and *e*-value < 1e-50. Sequences that had no match against the Maarj*AM* database were subjected to BLAST search against the International Nucleotide Sequence Database (INSD) using the same parameters, except with a similarity threshold of 90% and an alignment length ≥ 90% of the query sequence. After filtering, the remaining sequences were clustered at 97% similarity and sequences in clusters of four or more sequences were aligned with all representative sequences of VTs available in the Maarj*AM* database (March 2016) using MAFFT v.7 (Katoh & Standley, 2013); a neighbor-joining phylogenetic analysis was then performed using TOPALI v.2.5 (Milne *et al.*, 2009) to determine whether putatively Glomeromycotina sequences represented novel VTs. In total, 412 795 Glomeromycotina sequences (77.5% of all trimmed sequences) were identified, and the representative sequences of each AM fungal VT obtained in this study have been deposited in the GenBank database under accession numbers KY349825–KY349922.

To determine which Glomeromycotina genera or families our VTs belonged to, we aligned the representative sequences of our VTs and the representative sequences of major AM fungal genera using CLUSTALW and constructed a maximum-likelihood (ML) phylogenetic tree using MEGA6 with the Tamura three-parameter model and 1000 bootstrap replications (Tamura *et al.*, 2013). Each VT obtained in this study was grouped into a corresponding genus according to the phylogenetic tree, and if a VT could not be placed in a known genus, we regarded it as a new genus-like clade. The VT/genus compositions of AM fungal communities were calculated on the basis of the numbers of sequencing reads of each VT/genus in each sample.

### Statistical analysis

We calculated mycorrhizal responses in terms of plant biomass and tissue contents of N or P to quantify the changes in AM fungal function under different N treatments. The mycorrhizal growth response (MGR) of plants was calculated according to the following formulas (Veiga *et al.*, 2011): if $NM_{mean} < AM$, $MGR = (1 - (NM_{mean}/AM)) \times 100$; if $NM_{mean} > AM$, $MGR = ((AM/NM_{mean}) - 1) \times 100$; where '$NM_{mean}$' is the mean of total plant biomass in benomyl-treated subplots or in nonmycorrhizal pots for each N addition level, and 'AM' is the total plant biomass in each control subplot with an N treatment or in each mycorrhizal inoculation treatment in the glasshouse experiment. A positive MGR means that plants benefited from AM fungi in terms of biomass, whereas a negative MGR indicates that plant biomass was suppressed by AM fungi. Similarly, we calculated the mycorrhizal N-uptake response (MNR) and P-uptake response (MPR) of plants according to the same formulas with the biomass replaced by total N or P contents of plants.

Before analysis, the raw data of those variables which were measured at three sampling dates or in two replicated quadrats in each plot were pooled, using the means to represent the status of each variable during the whole growing season or in each plot. All measured data were tested for normality and data were $\log_e(x+1)$-transformed when needed; moreover, we calculated the Moran's *I* autocorrelations coefficients using the 'Moran.I' function from R library 'APE' (Paradis *et al.*, 2004) with a matrix of plot/subplot distance weights and our measured variables. We did not observe significant spatial autocorrelations in almost any of the tested variables; thus, the effects of spatial autocorrelations were not taken into account in the following analyses.

The effects of N and/or benomyl treatments on soil, plant and AM fungal variables in the field experiment were analyzed using linear mixed-effects models ('lme' and 'anova.lme' functions from R package 'NLME', Pinheiro *et al.*, 2016), with N and/or benomyl treatments as fixed effects and block as a random effect. Similarly, the effects of N addition and mycorrhizal inoculation

on plant and AM fungal variables in the glasshouse experiment were also determined using linear mixed-effects models, with N and mycorrhizal inoculation as fixed effects and block as a random effect (because the inocula were collected from each plot in the field). Based on the linear mixed-effects models, the differences of each variable between treatments were tested by *post-hoc* pairwise comparisons (Tukey method) using 'glht' function in R package 'MULTCOMP' (Hothorn *et al.*, 2015). The effects of N addition on the variables of plant mycorrhizal response (MGR, MNR and MPR) in benomyl and inoculation experiments were also analyzed using linear mixed-effects models, and *t*-tests at the 95% confidence level were used to test if the mycorrhizal response variables were significantly different from the null expectation of zero. Relationships between AM fungal variables and soil or plant variables were tested using Pearson correlation.

Dissimilarities in species composition of plant or AM fungal communities among samples were analyzed by nonmetric multidimensional scaling (NMDS) with the Bray–Curtis dissimilarity index using the 'metaMDS' function from R package 'VEGAN' (Oksanen *et al.*, 2015). The effect of N addition on community composition was analyzed by permutational multivariate analysis of variance (PERMANOVA; 'adonis' function from R package 'VEGAN') with constraining permutations within blocks, and we performed permutational multivariate analyses of dispersion (PERMDISP; 'betadisper' and 'permutest' functions from R package 'VEGAN') to verify that significant PERMANOVA results stemmed from structure differences, not unequal dispersion of variability, among treatments (all $P > 0.24$ for our community composition data). To correlate community composition with environmental variables, the soil and/or plant variables were fitted onto the corresponding two-dimensional NMDS ordination plots using the 'envfit' function from R package 'VEGAN'. Relationships between any two communities (e.g. plant and AM fungal communities; Bray–Curtis distance) were examined using Mantel tests ('mantel' function from 'VEGAN' package). To explore the relationships between AM fungal community structure and mycorrhizal functions, we used Mantel tests to measure the correlations between VT composition (Bray–Curtis distance) and MGR, MNR or MPR (Euclidean distance). The relationships between VT/genus abundance (VT/genus with > 1% abundance were tested) and MGR, MNR or MPR were also tested using Pearson correlation.

## Results

### Effects of N addition on soil and plant properties in the field

Four years of N addition increased soil available N concentration ($F_3 = 11.1$, $P < 0.001$) and soil N : P ratio ($F_3 = 4.8$, $P = 0.02$; Table S1), reduced plant species richness and shifted the species composition of the plant community (PERMANOVA: $F_3 = 2.14$, $P = 0.005$) towards dominance of *E. nutans* (Table 1) and AM plants (Table S2). At both plant community and *E. nutans* scales, shoot biomass and tissue N concentration increased with N addition, whereas the root : shoot biomass ratio decreased and the tissue N : P ratio increased (Table 1).

**Table 1** Plant properties for the entire plant community and for *Elymus nutans* under different nitrogen addition treatments in the field (mean ± SE, $n = 6$) and the significance of N addition on each variable. N0, N5, N10 and N15 represent fertilizer applications of 0, 5, 10 and 15 g N $m^{-2}$ $yr^{-1}$, respectively

| | Plant-community scale | | | | | | | *Elymus nutans* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Species richness | Shoot biomass ($kg\ m^{-2}$) | Root biomass ($kg\ m^{-2}$) | Root : shoot biomass ratio | Tissue N ($mg\ g^{-1}$) | Tissue P ($mg\ g^{-1}$) | Tissue N : P ratio | Relative abundance (%) | Shoot biomass (g per plant) | Root biomass (g per plant) | Root : shoot biomass ratio | Tissue N ($mg\ g^{-1}$) | Tissue P ($mg\ g^{-1}$) | Tissue N : P ratio |
| N0 | 32.7 ± 0.5a | 0.4 ± 0.1b | 2.4 ± 0.4 | 6.0 ± 0.8a | 12.3 ± 1.2bc | 1.7 ± 0.4 | 9.1 ± 1.8b | 7.1 ± 1.4b | 0.92 ± 0.08c | 0.5 ± 0.03 | 0.55 ± 0.05a | 9.6 ± 0.5b | 0.9 ± 0.1 | 11.1 ± 1.3ab |
| N5 | 23.8 ± 1.4b | 0.6 ± 0.1a | 2.2 ± 0.2 | 3.8 ± 0.5b | 10.5 ± 0.8c | 1.3 ± 0.1 | 8.7 ± 1.2b | 18.6 ± 1.5a | 1.12 ± 0.06bc | 0.5 ± 0.03 | 0.43 ± 0.02b | 9.6 ± 0.5b | 1.0 ± 0.1 | 9.8 ± 0.7b |
| N10 | 24.3 ± 0.8b | 0.6 ± 0.1a | 2.4 ± 0.1 | 3.7 ± 0.3b | 14.3 ± 0.6ab | 1.7 ± 0.3 | 9.7 ± 1.7b | 24.0 ± 3.7a | 1.48 ± 0.15ab | 0.6 ± 0.03 | 0.41 ± 0.03b | 14.3 ± 1.0a | 1.0 ± 0.2 | 16.3 ± 2.6a |
| N15 | 23.7 ± 1.2b | 0.6 ± 0.1a | 1.9 ± 0.1 | 3.3 ± 0.4b | 16.8 ± 1.2a | 1.2 ± 0.2 | 15.7 ± 2.0a | 25.0 ± 2.8a | 1.50 ± 0.18a | 0.5 ± .0.08 | 0.37 ± 0.04b | 17.3 ± 1.6a | 1.1 ± 0.1 | 16.3 ± 1.5a |
| Summary of N effect | | | | | | | | | | | | | | |
| *F*-value | **18.07** | **16.09** | 1.05 | **6.41** | **9.35** | 1.61 | **4.93** | **12.10** | **7.99** | 1.13 | **5.85** | **21.97** | 0.39 | **4.76** |
| *P*-value | **< 0.001** | **< 0.001** | 0.40 | **0.005** | **0.001** | 0.23 | **0.014** | **< 0.001** | **0.002** | 0.37 | **0.007** | **< 0.001** | 0.76 | **0.016** |

Significant differences of each variable among treatments are indicated by dissimilar letters and significant N effects are highlighted in bold.

### Changes in AM fungal abundance and community structure with N addition in the field

The %RLC in both mixed and *E. nutans* root samples, and the % AC and %VC in mixed roots declined under conditions of high N-fertilization (Fig. 1a–c); surprisingly, the hyphal length density ($F_3 = 1.44$, $P = 0.272$) and spore density ($F_3 = 0.36$, $P = 0.786$) of AM fungi in soil did not vary across the N addition gradient (Fig. S2).

Ninety-eight AM fungal VTs within 12 genera of seven families were detected (mixed roots: 93 VTs, *E. nutans* roots: 67 VTs; Table S3; Fig. S3), of which VT325 (*Rhizophagus* sp.; 22.2% of total AM fungal reads) and VT113 (related to *R. irregularis*; 41.7%) were the most frequently detected VTs in mixed and *E. nutans* roots, respectively. *Rhizophagus* was the most abundant genus in both mixed roots (49.4%) and *E. nutans* roots (78.9%), followed by *Glomus* (mixed roots: 39.2%; *E. nutans* roots: 11.8%) (Fig. S4a,b). N enrichment did not change the abundance of *Rhizophagus* in either root systems (both $P > 0.28$), while the abundance of *Glomus* was marginally lower under N-fertilized conditions (mixed roots: $F_3 = 2.8$, $P = 0.076$; *E. nutans* roots: $F_3 = 5.35$ $P = 0.016$; Fig. S4c,d). Enriched soil N also reduced (e.g. *Glomus* VT371 in mixed roots and *Rhizophagus* VT247 in *E. nutans* roots) or increased (e.g. *Glomus* VT199, related to *G. hoi*, in mixed roots and *Claroideoglomus* VT276 in *E. nutans* roots) the abundance of some VTs in both root systems (Table S3).

Nitrogen addition increased both total VT richness ($F_3 = 7.60$, $P = 0.003$) and *Rhizophagus* VT richness ($F_3 = 7.74$, $P = 0.002$) in mixed roots but not in *E. nutans* roots (both $P > 0.50$; Fig. 1d, e); however, the VT richness of *Glomus* varied across the fertilization gradient in *E. nutans* roots ($F_3 = 4.68$, $P = 0.017$) but not in mixed roots ($F_3 = 2.51$, $P = 0.099$; Fig. 1f). NMDS analysis and PERMANOVA showed that VT compositions of AM fungal communities in both mixed ($F_3 = 2.11$, $P = 0.009$) and *E. nutans* ($F_3 = 1.95$, $P = 0.035$) roots were affected by N addition (Fig. 2), but in *E. nutans* roots the VT composition did not differ between unfertilized conditions and low levels of N addition (Fig. 2b). Vector fitting revealed that the factors most strongly related to AM fungal community in mixed roots were soil available N ($r^2 = 0.32$, $P = 0.017$), plant species richness ($r^2 = 0.27$, $P = 0.048$) and the root : shoot biomass ratio ($r^2 = 0.26$, $P = 0.049$), whereas only soil available N ($r^2 = 0.25$, $P = 0.046$) was correlated with the fungal community in *E. nutans* roots (Fig. 2). Furthermore, we did not detect significant correlation between the AM fungal VT composition in mixed roots and that in *E. nutans* roots (Mantel test: $r = -0.006$, $P = 0.478$), and neither fungal community was associated with plant species composition (mixed roots: $r = -0.05$, $P = 0.703$; *E. nutans* roots: $r = 0.039$, $P = 0.329$).

### Effects of N addition on plant responses to mycorrhizal fungi in the field

Four years of benomyl application reduced the %RLC by half in all N treatments (Fig. 3a), suggesting that our fungicide application effectively suppressed root colonization by AM fungi. We did not detect any significant effects of benomyl on plant species richness or biomass variables (all $P > 0.1$; Fig. 3d–f), but in comparison to the paired control subplots, benomyl increased plant biomass allocation to roots under higher N-fertilized conditions (Fig. 3g). In addition, benomyl application increased plant N concentration and reduced plant P concentration (Fig. 3h,i), resulting in a significant increase in plant N : P ratio (Fig. 3j).

The MGR of the plant community was affected by N addition ($F_3 = 5.38$; $P = 0.021$), with a quadratic relationship ($R^2 = 0.37$, $P = 0.019$), and the highest MGR under the N5 treatment but the lowest under the N15 treatment (Fig. 4a). The MNR was negative under both unfertilized (N0) and N15 treatments, whereas it was neutral under low or intermediate levels of N addition (Fig. 4b). The MPR also showed a quadratic relationship with N addition ($R^2 = 0.42$, $P = 0.012$), and was positive under N5 but negative under the N15 treatment (Fig. 4c).

### Effects of N addition on responses of *E. nutans* to mycorrhizal fungi in the glasshouse

Mycorrhizal fungal colonization was well established in all inoculated *E. nutans* (mean %RLC: 34.7%, %AC: 18.3%, %VC: 5.2%), but both %RLC ($F_3 = 5.36$; $P = 0.01$) and %VC ($F_3 = 6.19$; $P = 0.006$) declined gradually across the N addition gradient (Fig. 5a–c).

Total biomass of *E. nutans* was not affected by either N addition or mycorrhizal inoculation (both $P > 0.11$). However, mycorrhizal inoculation reduced shoot biomass in fertilized soil (Fig. 5d), but increased root biomass under unfertilized conditions and low N addition (Fig. 5e). The root : shoot biomass ratio of mycorrhizal plants was considerably higher than that of nonmycorrhizal plants and gradually decreased with increasing N inputs (Fig. 5f). Furthermore, mycorrhizal inoculation did not affect any plant nutrient variables (all $P > 0.32$; Fig. 5g–i), but showed significant interactive effects with N addition on plant P and N : P ratio (both $P \leq 0.002$; Fig. 5h,i). The MGR, MNR or MPR of *E. nutans* were positive in unfertilized soils, but shifted towards negative in soils with higher N availability, and all showed similarly negative relationships with N addition (all $R^2 \geq 0.39$, $P < 0.001$; Fig. 4d–f).

### Relationship between AM fungal community structure and mycorrhizal function

At the whole-plant-community scale, we did not observe significant relationships between AM fungal community composition of all VTs in mixed roots with any of the plant mycorrhizal response variables obtained in the field (Mantel test; all $P > 0.10$), but MGR and MNR correlated significantly with the community composition of abundant VTs (mean relative abundance > 1%; Mantel $r = 0.25$, $P = 0.043$) and of *Rhizophagus* VTs (Mantel $r = 0.26$, $P = 0.036$), respectively. The MGR of the plant community correlated positively with both the relative abundance of *Glomus* ($r = 0.58$, $P = 0.018$) and the VT richness of *Glomus* ($r = 0.45$, $P = 0.049$), while MNR and MPR were marginally positively related to the VT richness of *Rhizophagus*

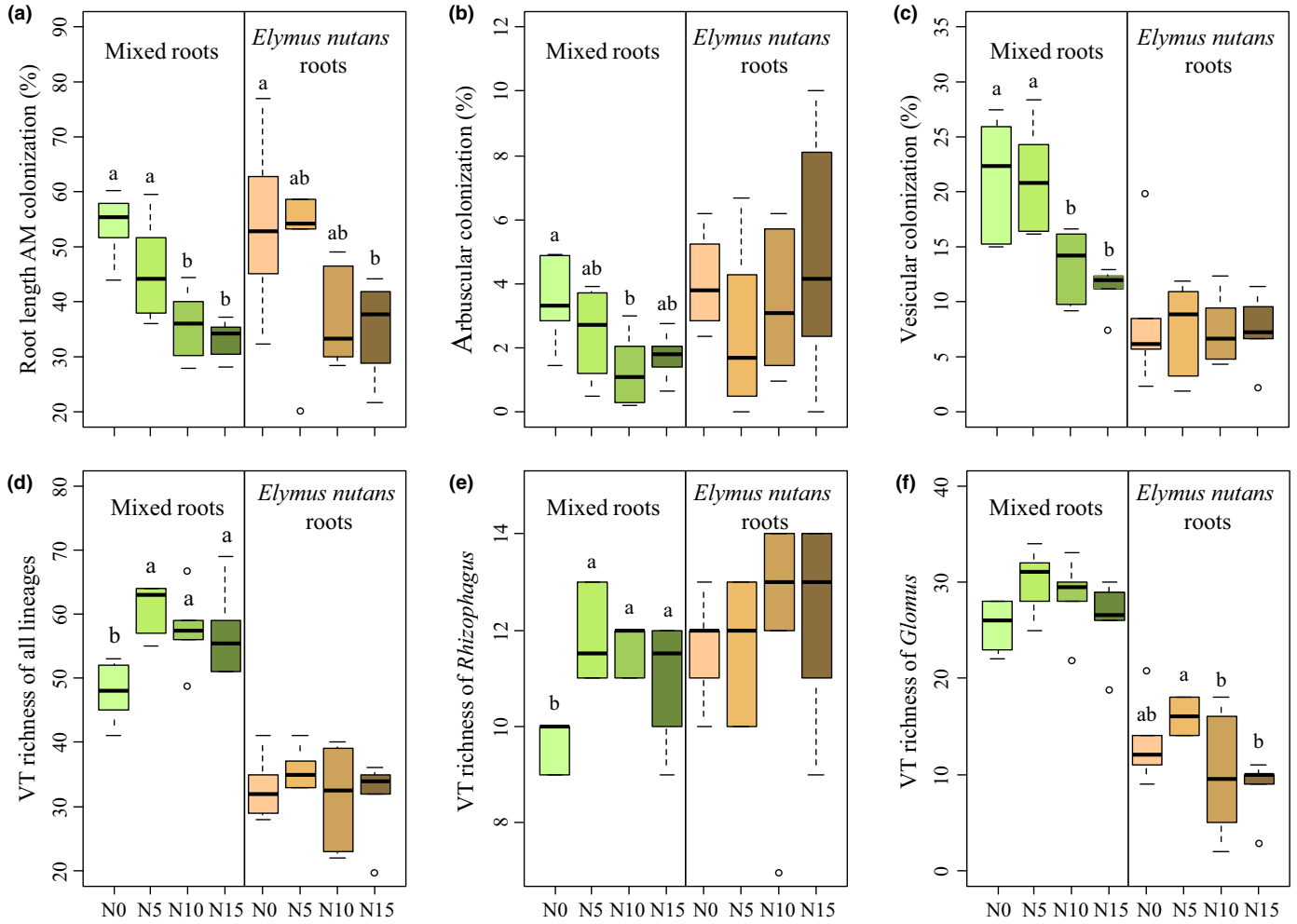


**Fig. 1** Total (a) root length colonization (%RLC), (b) arbuscular colonization (%AC) and (c) vesicular colonization (%VC) by arbuscular mycorrhizal (AM) fungi, (d) virtual taxon (VT) richness of all lineages, (e) VT richness of *Rhizophagus* and (f) VT richness of *Glomus* of AM fungal communities in mixed and *Elymus nutans* roots across an N addition gradient ($n = 6$). Median (central black line), quartile (box), maximum and minimum (whiskers) and outlying values (circles) are shown. N0, N5, N10 and N15 represent fertilizer applications of 0, 5, 10 and 15 g N $m^{-2}$ $yr^{-1}$, respectively. Significant differences of each variable of the same root system among treatments are indicated by dissimilar letters above boxes.

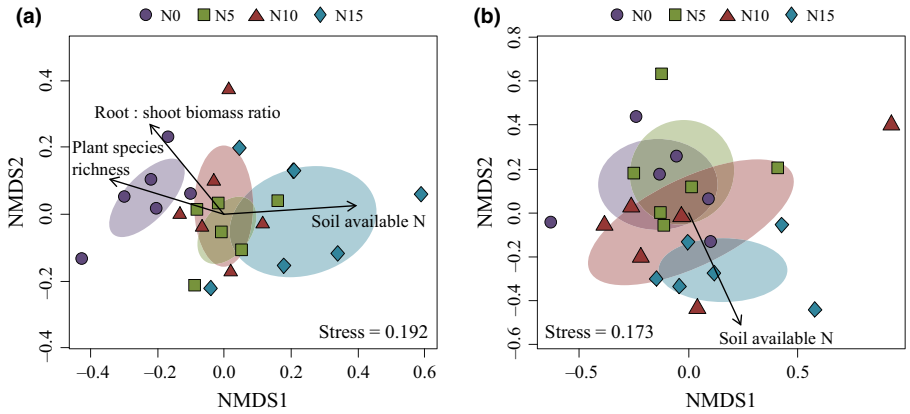


**Fig. 2** Nonmetric multidimensional scaling analysis (NMDS) (Bray–Curtis distance) of community composition of arbuscular mycorrhizal (AM) fungal virtual taxa colonizing (a) mixed plant roots and (b) *Elymus nutans* roots among N treatments in the field experiment. N0, N5, N10 and N15 represent fertilizer applications of 0, 5, 10 and 15 g N $m^{-2}$ $yr^{-1}$, respectively. Ellipses with different colors indicate 95% confidence intervals for each treatment. Significant plant and/or soil variables that are correlated with each community ordination are shown.

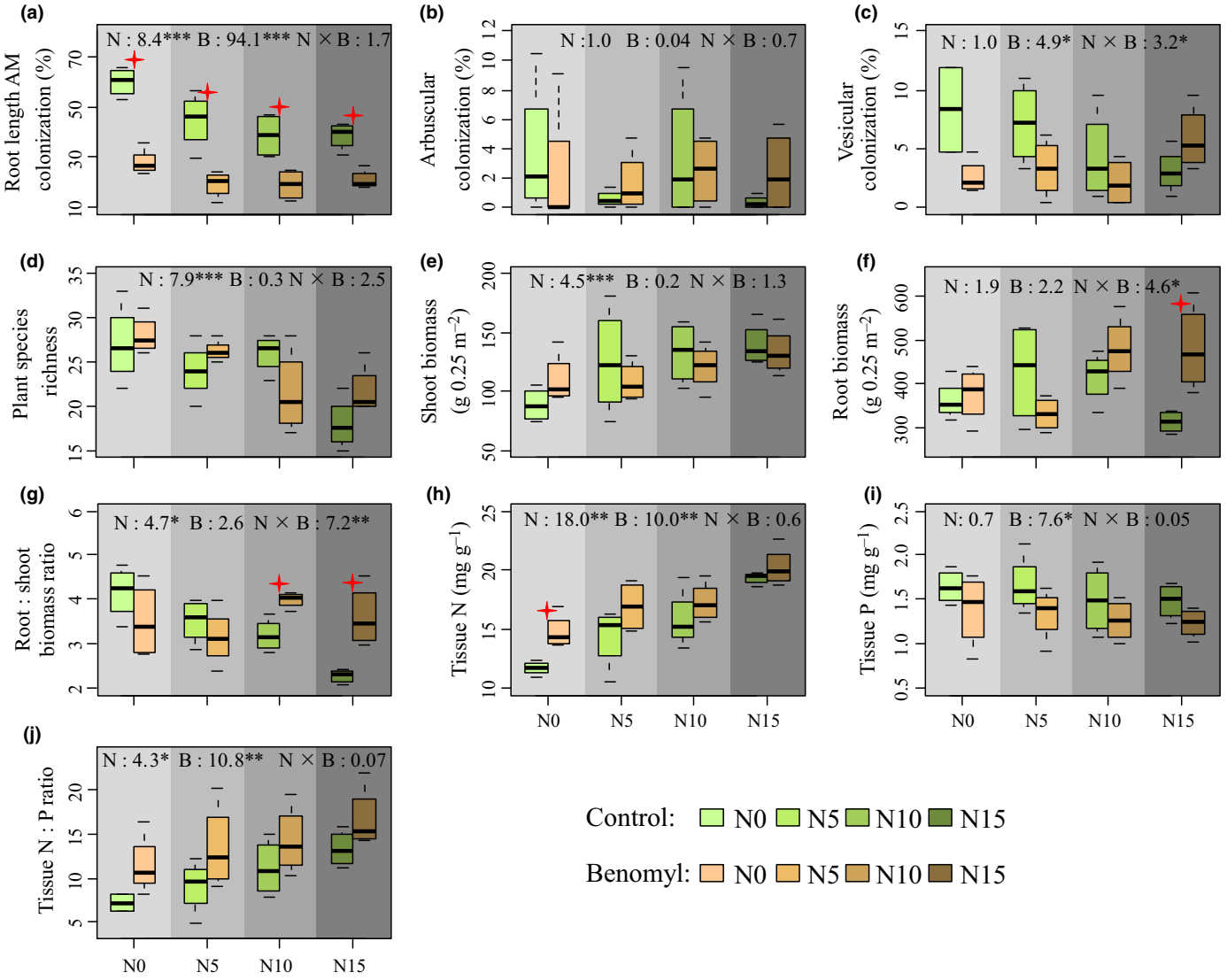


**Fig. 3** Changes in (a) arbuscular mycorrhizal (AM) fungal total root length colonization (%RLC), (b) arbuscular colonization (%AC), (c) vesicular colonization (%VC), (d) plant species richness, (e) plant shoot biomass, (f) root biomass, (g) root : shoot biomass ratio, (h) tissue N concentration, (i) tissue P concentration and (j) tissue N : P ratio after 4 yr of nitrogen and benomyl treatments in the field ($n = 4$). Median (central black line), quartile (box), and maximum and minimum (whiskers) are shown. N0, N5, N10 and N15 represent fertilizer applications of 0, 5, 10 and 15 g N $m^{-2}$ $yr^{-1}$, respectively. The red stars indicate significant differences between the control and benomyl treatment at a same N addition level according to *t*-tests ($P \leq 0.05$). The effects of N addition (N), benomyl application (B) and their interaction on each variable were analyzed using linear mixed-effects models, and the ANOVA summary of *F*-values and *P*-values are shown: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

($r = 0.45$, $P = 0.081$) and of all lineages ($r = 0.47$, $P = 0.065$), respectively. Moreover, the relative abundance of *Rhizophagus* VT325 was negatively associated with both MGR ($r = -0.55$, $P = 0.027$) and MNR ($r = -0.53$, $P = 0.034$). For *E. nutans*, however, neither the community composition (Mantel test; all $P > 0.30$) nor the VT richness (all $P > 0.10$) of all AM fungi, abundant VTs, *Glomus* VTs or *Rhizophagus* VTs identified in the field were related with any of the mycorrhizal response variables measured in the glasshouse.

## Discussion

By measuring the AM fungal community structure and function at the scale of the entire plant community as well as for a single plant species, we found that N enrichment, especially high levels of N addition, reduced AM fungal abundance, changed AM fungal diversity and community composition (Figs 1, 2), and shifted the mycorrhizal symbiosis towards parasitism (Fig. 4). Furthermore, we found that the mycorrhizal responses of the plant community in terms of biomass and nutrient uptake were significantly correlated with the diversity and community composition of abundant VTs or specific AM fungal lineages colonizing mixed roots, suggesting that N-induced changes in community structure of AM fungi might impact mycorrhizal functioning. These findings support our research hypotheses and highlight the negative influence of N enrichment on AM mutualism in an alpine meadow ecosystem.

Nitrogen-induced declines in AM fungal abundance have been observed in many natural ecosystems (e.g. Treseder, 2004; van

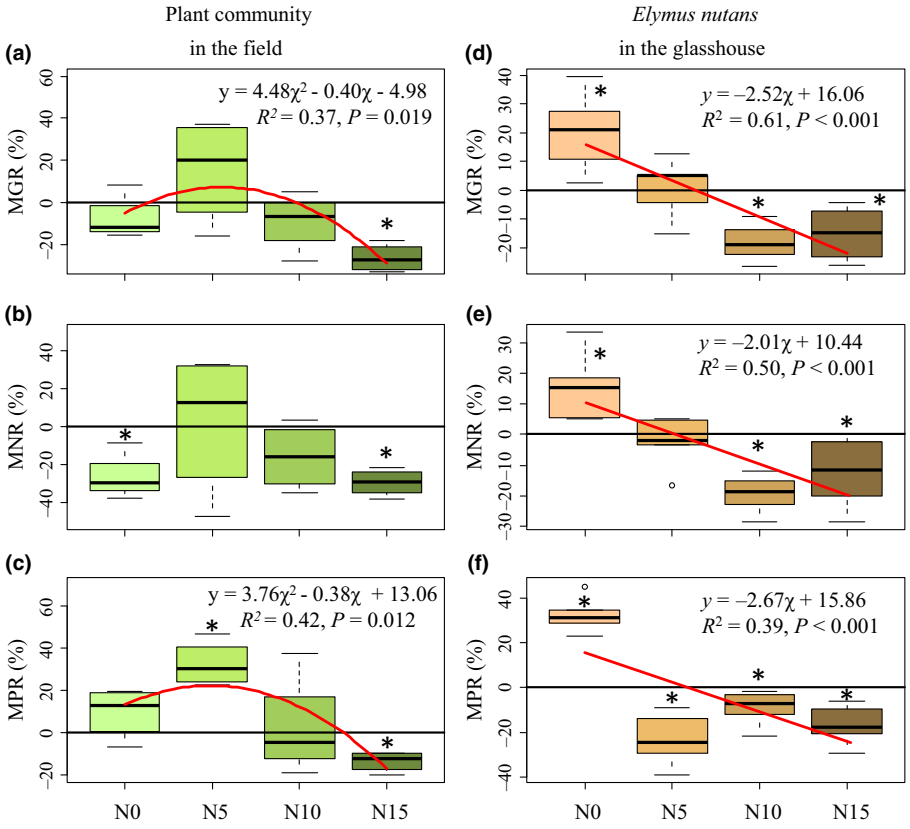


**Fig. 4** Mycorrhizal growth response (MGR), N uptake response (MNR) and P uptake response (MPR) of (a–c) plant communities in the field ($n = 4$) and of (d–f) *Elymus nutans* in the glasshouse ($n = 6$) under different N treatments. Median (central black line), quartile (box), maximum and minimum (whiskers) and outlying values (circles) are shown. N0, N5, N10 and N15 represent fertilizer applications of 0, 5, 10 and 15 g N $m^{-2}$ $yr^{-1}$, respectively. An asterisk indicates a significant difference between the mycorrhizal response and zero according to *t*-tests: *, $P \leq 0.05$. The solid red lines represent the fitted linear or quadratic regressions ($P \leq 0.05$ are shown) between the raw data of mycorrhizal response variables and the level of N addition.

Diepen *et al.*, 2010; Chen *et al.*, 2017). Our observed reduction of AM fungal colonization with N addition supports this and indicates that N enrichment of systems that are not P-limited reduces plant carbon allocation to mycorrhizal fungi (Johnson, 2010). However, our N addition treatments did not reduce the abundance of AM fungal extraradical hyphae in the soil, suggesting that the intraradical structures of AM fungi might be more sensitive to N enrichment than the extraradical hyphae. Abundance of extraradical hyphae was also insensitive to N-fertilization in some other studies in N-limited ecosystems (Treseder *et al.*, 2007; Zheng *et al.*, 2014); in addition, annual and seasonal variation in the responsiveness of extraradical hyphae to N addition has been observed in some temperate grassland systems (Johnson *et al.*, 2003). Because the core function of extraradical mycelium of AM fungi is absorbing nutrients from soil (Smith & Smith, 2011), we can speculate that AM fungi in N-enriched conditions would allocate sufficient energy to extraradical hyphae to ensure their own nutrient acquisition from soil or for competing for resources with plants and other organisms.

Contrary to our first expectation, N fertilization increased the AM fungal VT richness at the whole-plant-community scale (Fig. 1d), even though plant species richness declined with N enrichment. These results do not support other studies showing a decline in AM fungal richness with increasing soil N availability (Egerton-Warburton & Allen, 2000; Camenzind *et al.*, 2014; Chen *et al.*, 2017; Williams *et al.*, 2017) and positive relationships between AM fungal and plant species richness (Landis *et al.*, 2004; Liu *et al.*, 2012; Hiiesalu *et al.*, 2014). However, our findings are in line with a study in another alpine meadow on the Tibetan Plateau, where addition (1.5–7.5 g N $m^{-2}$ $yr^{-1}$) of different N forms (ammonium, nitrate or combined) generally increased AM fungal diversity in the soil (Zheng *et al.*, 2014). In typical alpine meadows on the Tibetan Plateau, sedges are the dominant plants, which are replaced by grasses with increasing soil N availability (Liu *et al.*, 2012). This is also observed in our study site, where N fertilization shifted the dominant plant species from a sedge, *K. capillifolia* that is rarely colonized by AM fungi, to a grass, *E. nutans* (Table S2). Under such conditions, it is possible that more host plants for AM fungi in fertilized plots could provide increased opportunities for colonization of newcomers (total richness of AM fungal VT in fertilized treatments was 7.7–12.8% higher than that in the unfertilized treatment; Table S3). Also, in a single plant species, we did not detect significant reduction of AM fungal richness across the N addition gradient, although community composition changed and, thus, some fungal taxa had been replaced by others. This suggests that many AM fungal taxa are adapted to an N-enriched environment.

Four years of N treatments, especially the high level of N addition, substantially shifted the community composition of AM fungi colonizing roots at the scale of the whole plant community as well as for a single plant species (Fig. 2). Changes in AM fungal community structure caused by nutrient enrichment are often attributed to shifts in plant community composition (Liu *et al.*, 2012) and variation in soil characteristics (Wu *et al.*, 2011). This

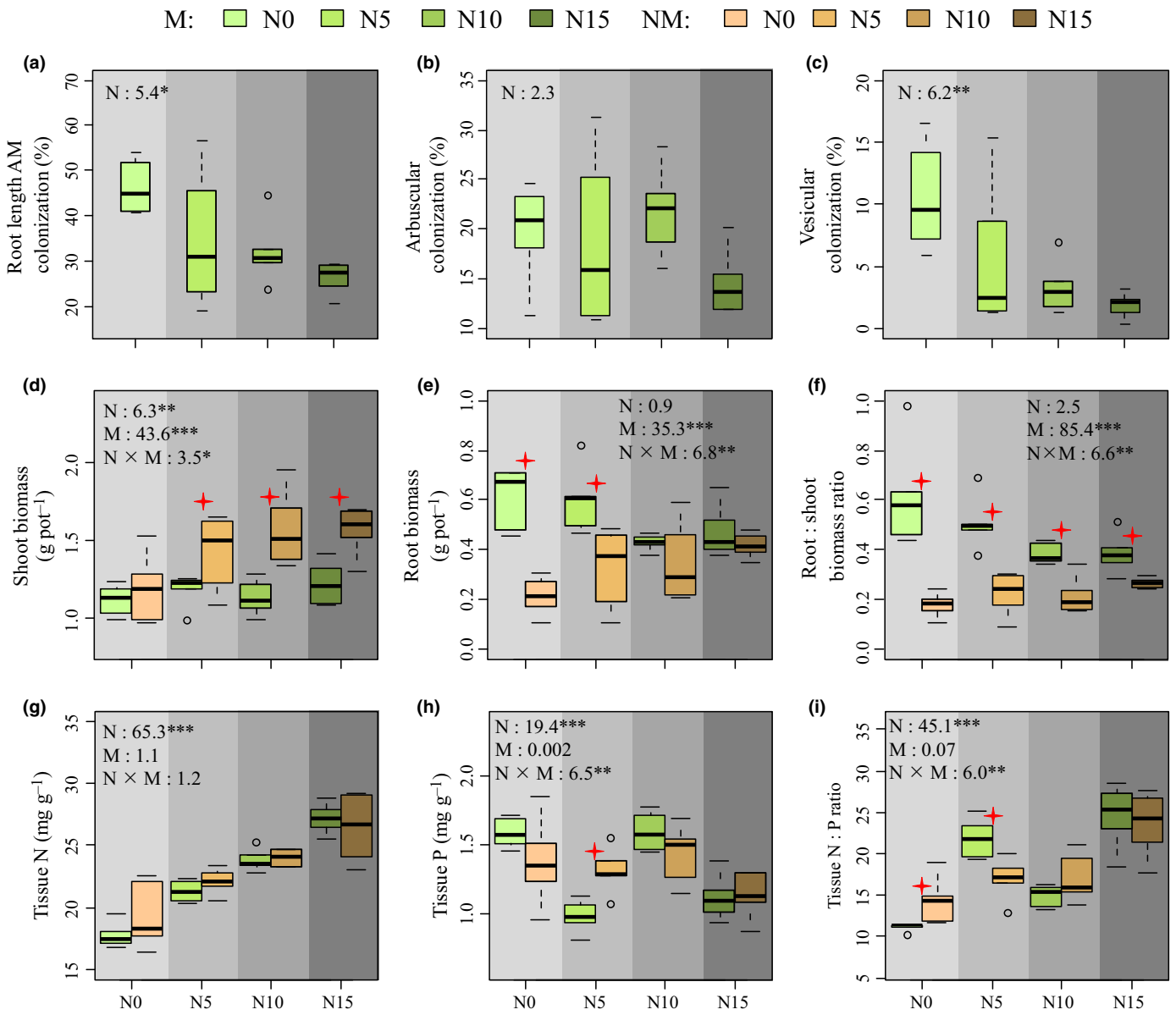


**Fig. 5** Total (a) root length arbuscular mycorrhizal (AM) fungal colonization (%RLC), (b) arbuscular colonization (%AC), (c) vesicular colonization (%VC), (d) shoot biomass, (e) root biomass, (f) root : shoot biomass ratio, (g) tissue N concentration, (h) tissue P concentration and (i) tissue N : P ratio under mycorrhizal inoculation (M) and nonmycorrhizal (NM) treatments at different N addition levels in the glasshouse ($n = 6$). Median (central black line), quartile (box), maximum and minimum (whiskers) and outlying values (circles) are shown. N0, N5, N10 and N15 represent fertilizer applications of 0, 5, 10 and 15 g N $m^{-2}$ $yr^{-1}$, respectively. Red stars indicate significant differences between M and NM treatments at a same N addition level according to *t*-tests ($P \leq 0.05$). The effects of N addition (N), mycorrhizal inoculation (M) and their interaction (N × M) on each variable were analyzed using linear mixed-effects models, and the ANOVA summary of *F*-values and *P*-values are shown: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

mechanism may be operating in our case, because the changes in AM fungal community composition in mixed roots were strongly associated with both plant species richness and soil available N concentration (Fig. 2). Furthermore, it has been demonstrated that AM fungal communities at the local scale are codetermined by (host and/or abiotic) environmental filtering and biotic interactions (Vályi *et al.*, 2016), and that fertilization can considerably increase the importance of competitive interaction in structuring AM fungal communities (Verbruggen & Kiers, 2010; Liu *et al.*, 2015). Thus, in our case, the N-induced changes in AM fungal community composition might also be associated with increasing competition for host niches and/or for nutrients among fungal taxa (Liu *et al.*, 2015). Nonetheless, it is clear that the communities of AM fungi in *E. nutans* roots, in contrast to those in mixed plant roots, were insensitive to low levels of N addition (Fig. 2), indicating that the dose (5 g N $m^{-2}$ $yr^{-1}$) of N application did not reach the threshold to cause a significant shift in community composition of AM fungi colonizing this nitrophilic species. Further research is needed to address whether this phenomenon is common for other plant species or only for nitrophilic species.

As predicted, most of the plant mycorrhizal responses in terms of biomass and nutrient uptake in both field and glasshouse

experiments shifted from positive or neutral to negative across the N addition gradient (Fig. 4), suggesting that high levels of N enrichment can induce parasitic interactions between plants and AM fungal communities. These results support previous findings based on mycorrhizal inoculation in glasshouse studies (e.g. Johnson, 1993; Johnson *et al.*, 2015) and provide the first observational evidence showing that N-induced AM fungal parasitism could also be operating in natural plant communities. However, it is surprising that mycorrhizal fungi did not increase productivity and N uptake of plant communities in our unfertilized plots, but a low level of N enrichment clearly increased the positive effect of mycorrhizal fungi on plant performance, especially on plant N and P uptake (Fig. 4). These findings could be related to N-induced shifts of dominant plants from less- or non-AM to AM host plants in the field (see Discussion above and Table S2), and also to the alleviation of N competition between plants and mycorrhizal fungi (Püschel *et al.*, 2016) in our unfertilized plots (Fig. 3h).

It has been suggested that N enrichment is likely to generate AM mutualism under P-limited conditions but commensalism or parasitism in P-rich conditions (Johnson, 2010). Our findings from the field support this prediction because our study site is highly N-limited and P fertilization does not significantly increase plant productivity (X. L. Zhou, unpublished data). In our glasshouse experiment, however, strong mutualism in the N0 treatment but parasitism in the N10 and N15 treatments indicates that AM functioning for the nitrophilic *E. nutans* might depend primarily on N availability. This idea is supported by a recent glasshouse experiment showing that N fertilization reduced AM mutualism in barley regardless of soil P status (Williams *et al.*, 2017). Given that AM fungi could increase plant N content via acquisition of N from decomposing organic material (Hodge & Fitter, 2010), future research should determine whether N nutrition is the primary benefit of AM for nitrophilic plants in the alpine meadow ecosystem. Furthermore, it is interesting that mycorrhizal fungal inoculation of *E. nutans* increased plant biomass allocation to roots, but this effect gradually weakened along the N addition gradient (Fig. 5d–f). These findings agree with a recent study showing that plants preferentially allocate more carbon to roots associated with mutualistic AM fungi, and that the patterns of plant allocation to mycorrhizal roots were highly dependent upon the relative availability of the limiting resource (Ji & Bever, 2016).

A widespread paradigm in ecology is that the structure of biological communities determines their functional properties, and root-associated AM fungi may also follow this rule (van der Heijden *et al.*, 1998; Maherali & Klironomos, 2007). Our experimental design cannot verify a causal relationship between AM fungal community structure and function, but our experimental manipulations with N addition and benomyl application allow us to explore the relationship between AM fungal community structure and function under natural conditions. In the field, we observed positive relationships between AM fungal VT richness of all lineages or some specific lineages (*Glomus* and *Rhizophagus*) with the mycorrhizal influence on plant growth and nutrient uptake, corroborating previous studies showing that increasing AM fungal species richness could result in higher plant productivity and more efficient exploitation of soil nutrients (van der Heijden *et al.*, 1998; Maherali & Klironomos, 2007). Moreover, we also detected significant correlations between mycorrhizal response variables and community composition as well as the relative abundance of specific AM fungi. These findings clearly show a tight linkage between AM fungal community structure and function, and suggest that N-induced changes in the community assembly or the abundance of some specific AM fungi might result in shifts in mycorrhizal function. Nonetheless, because benomyl application cannot totally suppress AM fungal colonization, and can also affect some other fungi including root endophytes (Almario *et al.*, 2017) and plant pathogens (M. S. Qin, unpublished data), new field-based experimental approaches that more accurately measure mycorrhizal responses of plants and links with AM fungal community composition need to be developed. Inoculation of entire AM fungal communities collected from the field is an alternative approach to explore mycorrhizal functioning, but it is difficult to connect AM fungal community structure and function because AM fungal communities colonizing roots under glasshouse conditions may be different from those in the field.

In summary, we have demonstrated that N enrichment changes AM fungal community structure and induces parasitic mycorrhizal interactions in an alpine meadow ecosystem. Our study provides the first field-based evidence that N-induced AM parasitism could be occurring at the scale of the entire plant community as well as for a single plant species. Considering the ecological importance of AM symbiosis in nature, more research is needed to address how and to what extent N-induced AM parasitism could affect plant diversity, productivity, nutrient cycling and ecosystem stability, especially in ecosystems that are experiencing high N fertilizer application and anthropogenic N deposition. Furthermore, future studies are needed to assess how N enrichment influences the community structure and function of other root-associated fungi, such as endophytes and pathogens, to improve our understanding of plant–microbe interactions under environmental change conditions.

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## Author contributions

Y.L. and H.F. designed the study with the help of L.A. and G.D.; S.J., J.L. and M.Q. performed the field and laboratory work helped by Y.C., X.Z. and L.M.; S.J., Y.L. and M.V. analyzed the data; Y.L. and S.J. wrote the manuscript with extensive discussion with N.C.J. and M.Ö., and all authors contributed to revisions.

## ORCID

Yongjun Liu http://orcid.org/0000-0002-2909-1730
Maarja Öpik http://orcid.org/0000-0001-8025-7460

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** The study site and experimental design of this study.

**Fig. S2** Soil fungal hyphae observed in this study and the densities of AM fungal extraradical hyphae and spores.

**Fig. S3** Neighbor-joining phylogenetic tree of representative sequences of each AM fungal virtual taxon (VT) obtained in this study.

**Fig. S4** Relative abundance of detected AM fungal genera and the most two abundant genera in mixed roots and *Elymus nutans* roots.

**Table S1** Soil characteristics in different nitrogen addition treatments in the field

**Table S2** Plant species composition in different nitrogen addition treatments and the AM status of each plant species

**Table S3** Taxonomic information of all detected AM fungal virtual taxa (VTs) and their relative abundance in mixed roots and *Elymus nutans* roots in each nitrogen addition treatment

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