

N-P fertilization did not reduce AMF abundance or diversity but alter AMF composition in an alpine grassland infested by a root hemiparasitic plant

Xuezhao Wang^{a, b}, Xiaolin Sui^b, Yanyan Liu^c, Lei Xiang^b, Ting Zhang^b, Juanjuan Fu^a, Airon Li^{b, **}, Peizhi Yang^{a, *}

^a Department of Grassland Science, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, Shaanxi, China

^b Department of Economic Plants and Biotechnology, Yunnan Key Laboratory for Wild Plant Resources, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

^c Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Ürümqi 830011, Xinjiang, China

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ABSTRACT

Fertilization has been shown to have suppressive effects on arbuscular mycorrhizal fungi (AMF) and root hemiparasites separately in numerous investigations, but its effects on AMF in the presence of root hemiparasites remain untested. In view of the contrasting nutritional effects of AMF and root hemiparasites on host plants, we tested the hypothesis that fertilization may not show strong suppressive effects on AMF when a plant community was infested by abundant hemiparasitic plants. Plants and soil samples were collected from experimental field plots in Bayanbulak Grassland, where N and P fertilizers had been applied for three continuous years for control against a spreading root hemiparasite, *Pedicularis kansuensis*. Shoot and root biomass of each plant functional group were determined. Root AMF colonization levels, soil spore abundance, and extraradical hyphae length density were measured for three soil depths (0–10 cm, 10–20 cm, 20–30 cm). Partial 18S rRNA gene sequencing was used to detect AMF diversity and community composition. In addition, we analyzed the relationship between relative abundance of different AMF genera and environmental factors using Spearman's correlation method. In contrast to suppressive effects reported by many previous studies, fertilization showed no significant effects on AMF root colonization or AMF species diversity in the soil. Instead, a marked increase in soil spore abundance and extraradical hyphae length density were observed. However, fertilization altered relative abundance and AMF composition in the soil. Our results support the hypothesis that fertilization does not significantly influence the abundance and diversity of AMF in a plant community infested by *P. kansuensis*.

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1. Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous components of the soil microbiome that form nutritional associations with a great majority of plants in various ecosystems (Smith and Read, 2008). AMF belong to the phylum Glomeromycota and generally form mutualistic symbioses with their host plants, providing

mineral nutrients to plants in exchange for photosynthates (Johnson, 2010). They play an important role in regulating plant productivity and maintaining plant diversity (Vogelsang et al., 2006; Wagg et al., 2011). Consequently, changes in AMF communities may strongly affect ecosystems. Thus, in order to optimize ecosystem management, it is important to improve our understanding of the factors that influence AMF communities.

Fertilizers have been commonly used to increase plant productivity. However, improper application of chemical fertilizers may change plant community structure and reduce species diversity (Bret-Harte et al., 2001; Clark and Tilman, 2008; Madan

* Corresponding author.

** Corresponding author.

E-mail addresses: airongli@mail.kib.ac.cn (A. Li), yangpeizhi@126.com (P. Yang).

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et al., 2007). Moreover, fertilization considerably decreases AMF diversity and abundance (Treseder and Allen, 2002). High levels of homogenous P supply have been shown to strongly suppress AMF colonization (Olsson et al., 1997), abundance, and diversity (Camenzind et al., 2014; Chen et al., 2014; Lin et al., 2012). In contrast, the effects of N fertilization on AMF are controversial. Some investigations have suggested that high levels of N fertilization have suppressive effects on AMF (Albizua et al., 2015; Verbruggen et al., 2013), whereas others have found no significant impact (Tian et al., 2013; Williams et al., 2013). This lack of consistency suggests that the effects of fertilization on AMF may be context dependent. Apart from fertilization, many other factors may play a role in shaping AMF communities in an ecosystem, including N and P availability ratios (Cheng et al., 2013; Williams et al., 2017), soil pH (Rousk et al., 2010), AMF species identity (Wang et al., 1993), and plant community composition (Smith and Read, 2008). How other factors interact with fertilization in shaping AMF community is not fully understood.

Root hemiparasitic plants are green plants that retain the ability to photosynthesize, but rely on host plants for optimal performance, extracting nutrients and other resources from host roots via specialized root structures called haustoria (Irving and Cameron, 2009). Root hemiparasitic plants often suppress host growth and productivity (Press and Phoenix, 2005). Furthermore, root hemiparasitic plants can alter plant community structure and diversity by changing competitive relationships between host and non-host plant species (Bao et al., 2015; Borowicz and Armstrong, 2012; Hedberg et al., 2005). Like AMF, root hemiparasitic plants form strong and direct nutritional associations with host plants and are ubiquitous components of many ecosystems (Press and Phoenix, 2005; Irving and Cameron, 2009). However, these two groups of organisms clearly have contrasting effects on host plants and shape plant community structure by different mechanisms. An interesting question is whether the presence of root hemiparasites alters how fertilization affects AMF communities.

Despite extensive research on how fertilizers alter plant community composition, AMF abundance and diversity, and root hemiparasitic plant performance (Borowicz and Armstrong, 2012; Gibson and Watkinson, 1991; Davies and Graves, 2000; Liu et al., 2017), few studies have examined how fertilizers affect soil AMF abundance and community composition in soils where root hemiparasitic plants occur. Because AMF and root hemiparasites affect host plant nutrition differently, we hypothesized that root hemiparasite infestation, which substantially deprives the host of nutrients, may offset the suppressive effects of fertilization, which are the result of high nutrient levels.

Pedicularis kansuensis Maxim. (Orobanchaceae) is a root hemiparasitic species widely distributed and rapidly spreading in the subalpine zone of western China. With an estimated spreading rate of 3.3×10^3 ha year⁻¹, this species has become a severe impediment to forage grass production for the local livestock industry (Liu et al., 2008; Sui et al., 2015). At present, no effective practice has been found to control this root hemiparasite. Our previous field experiments showed that N and P fertilization significantly reduce *P. kansuensis* biomass and change plant community structure (Liu et al., 2017). However, it remains unclear whether fertilization regimes affect AMF abundance and community structure.

In this study, our overall aim was to test whether fertilization suppresses AMF when plant communities are heavily infested by root hemiparasitic plants. Specifically, we asked three questions: (1) Does fertilization have a suppressive effect on AMF spore abundance, hyphae length density or root colonization in a plant community heavily infested by *P. kansuensis*? (2) Does fertilization affect the diversity or community composition of AMF in a plant community heavily infested by *P. kansuensis*? (3) Do other factors

such as soil depth, soil pH or plant biomass affect AMF abundance and community composition under these fertilization regimes? To answer these questions, we collected plant and soil samples from field experimental plots that had been fertilized with N or P for three continuous years to control against heavy infestation by *P. kansuensis*. Answering these questions will not only help identify the factors that influence AMF communities, but also contribute to an improved understanding of how these biotic and abiotic factors interact to shape AMF communities.

2. Materials and methods

2.1. Study site and experimental design

Sampling was carried out in the research field at Bayanbulak subalpine grassland (42°53.1'N, 83°42.5'E, 2500 m), where N and P fertilizers had been applied for three continuous years to test their control effect against *P. kansuensis*. Bayanbulak Grassland is located in the southern Tianshan Mountains of the Xinjiang Uygur autonomous region of China (Gong et al., 2010). It is a typical subalpine meadow with *Stipa purpurea* and *Festuca ovina* as dominant plant species. The annual temperature is -4.8 °C, with the lowest monthly mean occurring in January (-27.4 °C) and the highest in July (11.2 °C) (Li et al., 2012). The mean annual precipitation is 276.2 mm.

Fertilization treatments have been conducted since 2014 (Liu et al., 2017). In 2013, one 100 m × 100 m study site was chosen based on two criteria: heavy *P. kansuensis* infestation and similarity to the surrounding plant community. Sixteen blocks (4 m × 3 m) were randomly assigned for fertilization tests. A 2 m buffer zone was maintained between blocks to avoid nutrient diffusion into adjacent blocks. Four fertilization treatments were included: (1) non-fertilized control (CK), (2) N applied at 3 g N m⁻² yr⁻¹ as urea (N₃), (4) N applied at 9 g N m⁻² yr⁻¹ as urea (N₉), and (4) P applied at 10 g Ca(H₂PO₄)₂·H₂O m⁻² yr⁻¹. Each treatment had four replicates in a completely randomized design. Fertilizers were divided into two equal parts to increase fertilization efficiency while reducing ammonia volatilization from urea. The treatments were applied twice a year, mid-June and mid-July in 2014, 2015, and 2016, respectively.

2.2. Plant and soil sampling

Plant and soil samples were collected in September 2016 in a similar manner as described by Liu et al. (2017), except that a circle quadrat 30 cm in diameter was used instead of a 1 m × 1 m square quadrat to facilitate the assessment of above-ground biomass per unit area for each plant functional group (grasses, forbs, legumes, and *P. kansuensis*). Briefly, a portable circle quadrat frame was randomly landed on plots that had not been previously sampled in each block. For each fertilization regime, four replicate quadrats were sampled, resulting in 16 sampling quadrats in total. Above-ground plant tissues within the circle quadrat were clipped at soil surface, sorted into different functional groups, and weighed separately for dry shoot biomass after drying at 80 °C for 48 h. Soil cores were taken using a soil auger (8 cm in diameter and 10 cm in depth; Eijkelkamp Agrisearch Equipment, the Netherlands) at three depths (0–10 cm, 10–20 cm, and 20–30 cm) from the center of each circle quadrat. Soil cores were stored in plastic bags and transported back to the lab immediately after collection. After extraction from soil, roots were carefully brushed, and washed with water. Roots from the same soil core were pooled together as it was not possible to separate them into different functional groups. Subsamples were taken for AMF colonization assessment and other analyses. The remainder was oven-dried at 80 °C for 48 h and weighed. Dry weight (DW) of the subsamples used for checking

AMF colonization and other analyses were calculated from the ratio between fresh weight, dry weight of the remainder and fresh weight of the subsamples. Total root DW for each soil core was presented as a sum of dry weight of all extracted roots. Soil was sieved through a 1-mm mesh to remove root fragments and other debris. Soil subsamples for molecular analyses were stored at -80°C before DNA extraction. A small amount of soil subsamples was taken from the remainder for water content measurement before air-drying at room temperature for soil nutrient analysis, extraction of AMF spores and extraradical hyphae.

2.3. Measurement of AMF colonization, spore abundance and extraradical hyphae length density

To assess AMF colonization in each treatment, a weighed subsample of root material (diameter < 1 mm, about 100 root segments) was taken, cleared in 10% KOH and put in a 5% lactic acid solution, then stained with 0.05% Trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The percent of root length hyphae, arbuscules/coils and vesicles were quantified by a gridline intersect method (Giovannetti and Mosse, 1980). Spores of AMF in soil samples were extracted by a wet sieving and decanting method (Brundrett et al., 1994). Briefly, 10 g of dry soil sample was suspended in water and filtered through a set of sieves with pore sizes ranging from 0.15 to 0.038 mm, then centrifuged in 50% sucrose solutions at 2000 rpm for 2 min to separate the spores from the soil particles. Extraradical hyphae length density was determined as described by Jakobsen et al. (1992). Twenty-five fields of view were examined under a microscope at $200\times$ for AMF hyphae determination as described by Miller et al. (1995).

2.4. Soil chemical analysis

The soil moisture was measured by oven-drying the weighed samples at 105°C for 24 h and calculating water content with a fresh weigh and a corresponding dry weigh. Soil pH was determined by a soil-to-water ratio of 1:2 using a pH meter (Mettler–Toledo 320, Mettler Toledo Instruments Co. Ltd., Greifensee, Switzerland). Soil organic matter was determined using a modified Walkley-Black chromic acid wet oxidation method (Wang et al., 2012). Total N concentrations were analyzed with the elemental analyzer (Vario EL III Element Analyzer, Elementar, Hanau, Germany) following the burning method at 850°C and 1180°C , respectively. Soil ammonium ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$) were measured with a Lachat Flow Injection Analyzer Quikchem 8500 S2 (Lachat instruments, Hach company, United States). Soil total P concentrations were determined with the HCl-extractable fraction method (Rodrigues et al., 2016). Soil available P was measured using the molybdate-blue colorimetric method (Mehlich, 1984).

2.5. Soil DNA extraction

Soil DNA was extracted from 0.25 g soil sample using Power-Soil™ DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. Total DNA was extracted from 48 soil samples (3 samples from different soil depths for each of the 16 sampling quadrats from the four fertilization regimes). The concentration and purity of extracted DNA was checked on Nano-Drop 2000. All extracted DNA was stored at -80°C until further use.

2.6. PCR amplification

DNA samples were diluted to $10\text{ ng }\mu\text{L}^{-1}$ in DEPC water before PCR amplification. Partial sequences of AMF 18S rRNA genes were amplified from the extracted soil DNA via a nested PCR method,

with GeoA2–AML2 as the first primer pair and NS31–AMDG the second. First round PCR was carried out at a final volume of $25\text{ }\mu\text{L}$ which contained $2\text{ }\mu\text{L}$ diluted DNA, $1\text{ }\mu\text{L}$ concentration of primers, $8.5\text{ }\mu\text{L}$ double-distilled H_2O and $12.5\text{ }\mu\text{L}$ premix (Tsingke, Beijing) with the following cycling conditions: 95°C for 3 min, $30\times$ (94°C for 1 min, 58°C for 50 s and 72°C for 1 min) and 72°C for 10 min. The PCR product was diluted with double-distilled H_2O (1:50) and $2\text{ }\mu\text{L}$ of the diluted DNA was used as template for second round PCR amplification. Conditions for second round PCR were the same as first round PCR. All PCR products were examined on 1% (w/v) agarose gels with ethidium bromide staining to confirm the product integrity. Second round PCR products were purified with the DNA Gel Extraction Kit (Tsingke, Beijing) to obtain the predicted DNA fragments. The purified PCR products from all samples were mixed and further sequenced by the Second-generation sequencer-Illumina Miseq sequencer at Chengdu Institute of Biology, Chinese Academy of Science.

Sequence trimming and quality control were conducted using QIIME V1.9.0 (<http://qiime.org/tutorials/tutorial.html>) following the suggested pipeline (Caporaso et al., 2010). Sequences <200 bp or with average quality scores <30 were removed (Edgar et al., 2011), and instances of exact barcode matching, two-nucleotide mismatch during primer matching, and reads containing ambiguous characters were removed. Usearch V8.0 was used to detect and remove chimeric sequences. A total of 813,301 high-quality and chimera-free reads with an average length of 300 ± 50 bp were collected by sequencing, and there were 9857 sequences for each sample, which were sufficient to capture the majority of AMF information, as shown by the plateaus observed in the curves. Operational taxonomic units (OTU) were defined using a 97% similarity cutoff. The raw sequence data have been deposited in the Sequence Read Archive of the National Center for Biotechnology Information, USA (NCBI; Accession no. SRP136946).

2.7. Statistical data analysis

One-way analysis of variance (ANOVA) and Least Significant Difference (LSD) multiple range test were used for analyzing the effects of fertilization or soil depth on plant biomass, total root colonization and vesicle colonization of AMF, soil AMF spore density and extraradical hyphae length density. Data for percentage root length colonized by vesicles were transformed by natural logarithm (\ln) before the analyses in order to improve normality and homogeneity of residuals. Spearman rank correlations were used to calculate the relationships between environmental variables (soil properties and plant biomass) and AMF community structure at genera levels. Statistical analysis of ANOVA and correlations were carried out using the Statistical Product and Service Solution (SPSS) software (version 19.0, IBM Corporation, Armonk, New York, USA).

AMF α -diversity can be represented by evenness, richness and Shannon diversity. According to Bonfim et al. (2016), Shannon index: $H' = -\sum(P_i \log[P_i])$, where $P_i = n_i/N$, n_i = number of individuals of the species i , and N = total number of individuals of all species; Pielou's evenness index: $J = H'/\log(S)$, in which H' is value obtained by the Shannon index and S is the total number of species (da Silva et al., 2015); richness was determined by the total number of species identified in fertilizer treatment, whereas mean species richness was obtained by the mean of the number of species obtained per sample (Melo et al., 2018).

To explore the relationship between AMF OTU compositions and treatments, and the environmental variables (including soil depth and fertilizer treatment) were fitted as vectors onto the nonmetric multidimensional scaling (NMDS) plot using function "envfit" in the R version (R Development Core Team, 2011). Redundancy

analysis (RDA, length of gradient <4) was applied with 9999 permutations and combinations of different soil depths and plants to infer relationships between AMF community composition in soil and soil chemical parameters using CANOCO 4.5 (<http://www.canoco5.com/>).

3. Results

3.1. Soil properties

Soil properties varied between different soil depths. Soil available N (sum of nitrate and ammonium N), available P, and organic matter content were all significantly higher at 0–10 cm ($P < 0.01$) than at other depths. The soil pH at 0–10 cm was reduced significantly more in P-fertilized soil ($P < 0.01$) than in the non-fertilized control. Soil pH was generally lower at 0–10 cm ($P < 0.05$), whereas it was higher at 20–30 cm ($P < 0.05$) (Table 1).

3.2. Plant biomass in different treatments

Shoot biomass of grasses and forbs increased slightly (not statistically significant) after N and P fertilization, whereas *P. kansuensis* biomass decreased significantly after high N and P fertilization (by 95.61% and 52.63%, respectively). After three years of fertilization, legume biomass was high in P-fertilized quadrats, whereas it was negligible in other quadrats (Table 2). Roots were mainly distributed in the 0–10-cm soil layer regardless of fertilization regime ($P < 0.01$). P fertilization significantly increased root biomass at 0–10 cm ($P = 0.011$) (Table 2).

3.3. Effects of fertilization on AMF colonization, soil spore abundance, and extraradical hyphae length density

Hyphae and vesicles were the most commonly observed AMF structures in sampled roots. However, arbuscules were very patchy in all samples (less than 1%). The total and vesicle colonization of N_9 and P fertilization groups at 0–10 cm were significantly higher ($P < 0.01$) than at other soil layers. Vesicle and total colonization levels for fertilized groups at 10–20 cm were lower than for the non-fertilized

control at the same soil depth (Fig. 1A and B). Soil spore abundance of AMF increased gradually under the fertilization gradient (Fig. 1C), especially in the P-fertilized group ($P < 0.01$). After P fertilization, soil spore numbers increased by 67.24%, 66.86% and 74.82% in three soil layers respectively. Hyphae length density increased by 59.74% at 0–10 cm in the P-fertilized group ($P < 0.05$) (Fig. 1D).

3.4. AMF community composition and diversity at different soil depths

In total, 43 AMF OTUs were detected, belonging to 7 genera within 6 families: Ambisporaceae (3 OTUs within *Ambispora*), Archaeosporaceae (2 OTUs within *Archaeospora*), Diversisporaceae (3 OTUs within *Diversispora*), Gigasporaceae (2 OTUs within *Gigaspora*), Glomeraceae (24 OTUs within *Funneliformis*, *Glomus*, *Rhizophagus* and three new genus-like clades), Paraglomeraceae (1 OTU within new genus-like clade). Eight OTUs were only named to class. The most abundant genus was *Glomus*, accounting for 57.66% of all AMF genera, followed by *Diversispora* (33.19%), *Gigaspora* (2.57%), *Archaeospora* (1.3%). The relative abundance of *Ambispora*, *Funneliformis* and *Rhizophagus* was much lower. Relative abundances of individual genera varied greatly at different soil depths and were significantly correlated to fertilization regimes (Fig. 2A and B).

Fertilization did not significantly affect AMF α -diversity, as indicated by similarities in Shannon diversity index, richness of OTUs, and Pielou's evenness in the same soil layers under different fertilization regimes (Fig. 3). However, soil depths showed a significant impact on Shannon index and particularly richness of AMF. In all treatments, AMF richness at 0–10 cm was higher ($P < 0.05$) than that at deep layers. When more N fertilizer was applied, the Shannon index of AMF was higher at 0–10 cm than at 20–30 cm ($P = 0.046$).

3.5. Relationship between AMF community structures and soil properties

The effect that changes in soil properties had on relative abundance varied depending on both AMF genus and fertilization regime. The relative abundance of *Glomus*, *Archaeospora* and

Table 1
Soil characteristics at different depths under different fertilization treatments.

	Soil depths	CK	N_3	N_9	P
Total N (%)	0–10 cm	0.3 ± 0.01ABa	0.4 ± 0.02Aa	0.3 ± 0.03Ba	0.3 ± 0.03Ba
	10–20 cm	0.3 ± 0.04Aa	0.3 ± 0.01Ab	0.3 ± 0.02Aa	0.3 ± 0.03Aa
	20–30 cm	0.3 ± 0.05Aa	0.3 ± 0.02Ab	0.3 ± 0.01Aa	0.2 ± 0.04Aa
Total P (g/kg)	0–10 cm	2.3 ± 0.11Aa	2.5 ± 0.09Aa	2.5 ± 0.07Aa	2.7 ± 0.20Aa
	10–20 cm	2.2 ± 0.08Aa	2.3 ± 0.05Aab	2.4 ± 0.10Aa	2.2 ± 0.16Aa
	20–30 cm	2.2 ± 1.12Aa	2.2 ± 0.09Ab	2.0 ± 0.07Ac	2.3 ± 0.13Aa
Organic matter contents (%)	0–10 cm	8.0 ± 0.25ABa	7.6 ± 0.45ABa	6.7 ± 0.47Ba	8.1 ± 0.50Aa
	10–20 cm	5.7 ± 0.44Ab	5.7 ± 0.08Ab	5.6 ± 0.32Ab	5.5 ± 0.17Ab
	20–30 cm	3.7 ± 0.38Ac	3.8 ± 0.28Ac	3.8 ± 0.14Ac	3.9 ± 0.19Ac
Available N (mg kg ⁻¹)	0–10 cm	65.2 ± 4.00Aa	65.6 ± 3.23Aa	79.2 ± 9.30Aa	66.9 ± 2.30Aa
	10–20 cm	44.6 ± 4.38Ab	38.9 ± 1.80Ab	37.6 ± 1.13Ab	43.3 ± 2.41Ab
	20–30 cm	20.1 ± 3.95Ac	24.1 ± 4.37Ac	22.3 ± 3.30Ab	27.1 ± 3.95Ac
Available P (mg kg ⁻¹)	0–10 cm	38.6 ± 9.12Ba	16.6 ± 6.12Ba	28.7 ± 7.59Ba	175.5 ± 6.75Aa
	10–20 cm	21.4 ± 3.77Bab	17.4 ± 2.43Ba	17.9 ± 2.10Ba	42.9 ± 6.86Ab
	20–30 cm	14.1 ± 2.75Ab	18.4 ± 6.46Aa	15.0 ± 5.18Aa	24.8 ± 9.17Ab
Moisture (%)	0–10 cm	35.4 ± 6.09Aa	35.7 ± 8.02Aa	35.4 ± 4.41Aa	50.9 ± 5.24Aa
	10–20 cm	38.2 ± 3.65Aa	33.6 ± 3.87Aa	35.6 ± 3.18Aa	43.1 ± 3.31Aab
	20–30 cm	31.5 ± 0.66Aa	32.7 ± 4.29Aa	38.6 ± 3.12Aa	34.2 ± 2.90Ab
pH	0–10 cm	7.7 ± 0.06Ab	7.8 ± 0.08Ab	7.8 ± 0.11Ab	7.5 ± 0.06Bb
	10–20 cm	8.1 ± 0.11Aa	8.1 ± 0.08Aa	8.1 ± 0.12Aab	7.9 ± 0.07Ba
	20–30 cm	8.1 ± 0.10Aa	8.2 ± 0.09Aa	8.2 ± 0.10Aa	8.1 ± 0.04Aa

Data are means ± SE ($n = 4$). CK, non-fertilized control; N_3 , fertilized with low N level at a rate of 3 g N m⁻² yr⁻¹ as urea; N_9 , fertilized with high N level at a rate of 9 g N m⁻² yr⁻¹ as urea; P, fertilized with 10 g Ca (H₂PO₄)₂·H₂O m⁻² yr⁻¹. Different uppercase letters indicate difference between different treatments within the same soil layer; different lowercase letters indicate difference between different soil layers of the same treatment. Significant differences across treatments within each variable were determined using Fisher's least significant difference (LSD) test ($P < 0.05$) after one-way ANOVA and are indicated by dissimilar letters.

Table 2
Shoot and root biomass of different plant functional groups under various fertilization treatments.

	Shoot Biomass (g/m ²)				Root Biomass (g/m ²)		
	Grasses	Forbs	<i>P. kansuensis</i>	Legumes	0–10 cm	10–20 cm	20–30 cm
CK	275.3 ± 40.81A	68.3 ± 18.57A	11.4 ± 2.47A	—	121.1 ± 29.49Ba	29.7 ± 10.81Ab	14.7 ± 2.30Ab
N ₃	339.7 ± 51.15A	91.0 ± 11.23A	7.7 ± 1.63AB	—	159.3 ± 20.79Ba	50.7 ± 17.86Ab	11.6 ± 1.64Ab
N ₉	324.5 ± 91.01A	83.5 ± 32.55A	0.5 ± 0.00C	—	228.2 ± 55.62ABa	46.2 ± 8.52Ab	23.3 ± 6.96Ab
P	285.6 ± 46.35A	58.9 ± 10.83A	5.4 ± 1.58BC	34.1 ± 0.01	272.2 ± 26.32Aa	33.6 ± 10.9Ab	26.4 ± 9.61Ab

Data are means ± SE ($n = 4$). CK, non-fertilized control; N₃, fertilized with low N level at a rate of 3 g N m⁻² yr⁻¹ as urea; N₉, fertilized with high N level at a rate of 9 g N m⁻² yr⁻¹ as urea; P, fertilized with 10 g Ca (H₂PO₄)₂·H₂O m⁻² yr⁻¹. For shoot biomass, different uppercase letters indicate difference between different treatments within the same kind of plant. For root biomass, different uppercase letters indicate difference between different treatments within the same soil layer; different lowercase letters indicate difference between different soil layers of the same treatment. Significant differences across treatments within each variable were determined using Fisher's least significant difference (LSD) test ($P < 0.05$) after one-way ANOVA and are indicated by dissimilar letters.

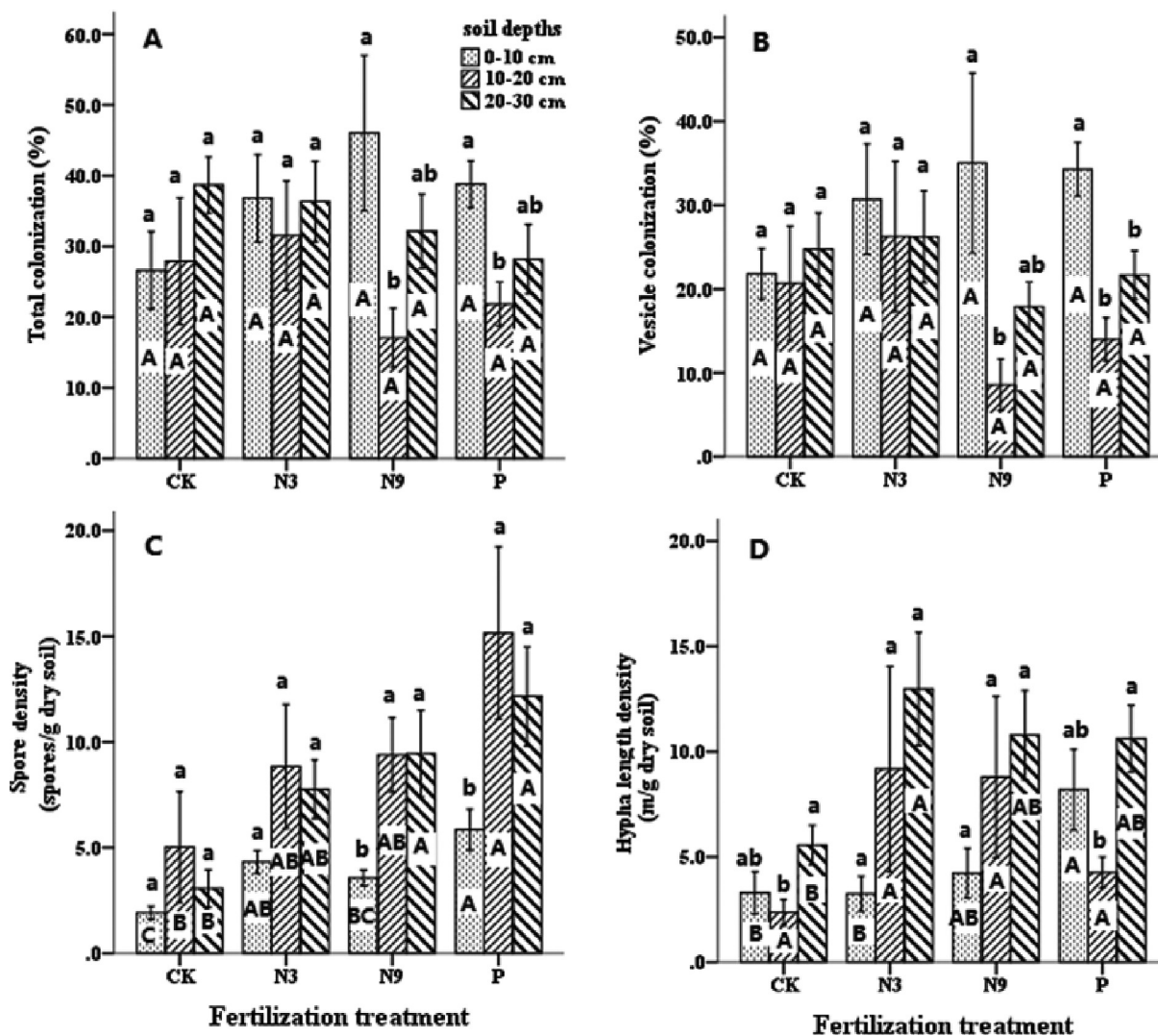


Fig. 1. Total colonization (A) and vesicle colonization (B) by AMF in roots, AMF spore density (C) and extraradical hyphae length density (D) varied across different fertilization regimes. Data are means ± SE ($n = 4$). CK, non-fertilized control; N₃, fertilized with low N level at a rate of 3 g N m⁻² yr⁻¹ as urea; N₉, fertilized with high N level at a rate of 9 g N m⁻² yr⁻¹ as urea; P, fertilized with 10 g Ca (H₂PO₄)₂·H₂O m⁻² yr⁻¹. Different uppercase letters indicate difference between different treatments within the same soil layers; different lowercase letters indicate difference between different soil layers of the same treatment. Significant differences across treatments within each variable were determined using Fisher's least significant difference (LSD) test ($P < 0.05$) after one-way ANOVA and are indicated by dissimilar letters.

Rhizophagus showed strong negative correlations with soil depth and soil pH ($P < 0.05$), but showed positive correlations with soil available N, organic content and root biomass ($P < 0.01$). In contrast, *Diversispora* and *Gigaspora* showed the opposite pattern. For these genera, soil depth and soil pH were positively correlated

($P < 0.001$), whereas soil available N, organic content, and root biomass were negatively correlated ($P < 0.001$). The relative abundance of *Glomus* and *Rhizophagus* increased with total N ($P < 0.05$), but *Diversispora* and *Gigaspora* decreased as soil total N increased. The relative abundance of *Archaeospora* was positively

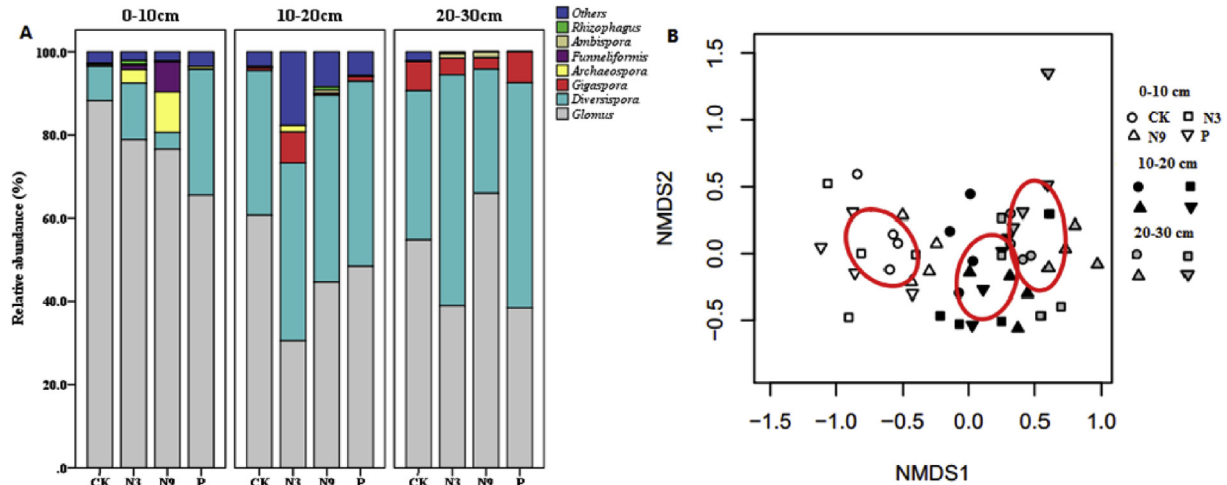


Fig. 2. Relative abundance of AMF taxa (A) and non-metric multidimensional scaling (NMDS) ordination of AMF OTU composition in different soil layers (B). Notes: AMF taxa not named to any genera were presented as *others*. CK, non-fertilized control; N₃, fertilized with low N level at a rate of 3 g N m⁻² yr⁻¹ as urea; N₉, fertilized with high N level at a rate of 9 g N m⁻² yr⁻¹ as urea; P, fertilized with 10 g Ca (H₂PO₄)₂·H₂O m⁻² yr⁻¹.

correlated with soil total P ($P < 0.05$), whereas the relative abundance of *Gigaspora* decreased with increasing total P and available P ($P < 0.05$). Relatively more *Funneliformis* was detected in the topsoil layer than in deeper layers. Interestingly, a positive correlation between biomass of *P. kansuensis* and the relative abundance of *Diversispora* was observed ($P < 0.05$) (Table 3).

Based on RDA analyses, different environmental variables had differential effects on soil AMF community ($P = 0.001$) (Fig. 4). The main factors that influenced AMF composition, regardless of soil depth, were soil pH, organic content, available nitrogen and root biomass (Fig. 4A). At 0–10 cm, no significant influence was detected by any of the tested factors on AMF community composition (Fig. 4B). For soil at 10–20 cm, the main factors that shaped AMF communities were soil pH and soil water content (Fig. 4C). For soil at 20–30 cm, AMF community composition was significantly influenced by soil total N, biomass, and the relative abundance of *P. kansuensis* ($P < 0.05$), which, combined, explain 64% of the variation observed (Fig. 4D).

4. Discussion

Fertilization did not affect AMF colonization significantly (Fig. 1A and B). In this study, fertilization was applied at a rate of 3 or 9 g m⁻² N as urea (corresponding to 1.4 or 4.25 g m⁻² N), or

10 g m⁻² P as Ca (H₂PO₄)₂·H₂O (corresponding 4.6 g m⁻² P). Similar fertilization rates have been reported to cause significant suppression of AMF colonization in croplands (Liu et al., 2016; Bakhshandeh et al., 2017), but not in an alpine meadow ecosystem (Liu et al., 2012). However, when we examined the soil available P and N of our experimental plots, we found that due to relatively high background soil nutrient levels, the moderate fertilization in this study resulted in soil N and P levels comparable to those of heavily fertilized treatments described by Liu et al. (2012), where AMF colonization was strongly suppressed. The root hemiparasite *P. kansuensis* may have offset, or at least delayed the suppressive effects of fertilization on AMF colonization by causing host nutrient deprivation. In a previous study on the same experimental plots, we observed that the response of plant biomass to fertilization was delayed (Liu et al., 2017). It would be interesting to test whether a suppressive effect of fertilization on AMF colonization can be detected as we continue the fertilization experiments for another couple of years. We observed that sampled roots had very patchy arbuscules. A previous field experiment that investigated the effects of long-term N and P fertilization (8 years) on AMF in an alpine meadow ecosystem also observed very few arbuscular structures. For these studies, AP was high. According to Kobae et al. (2017), this may inhibit arbuscular formation.

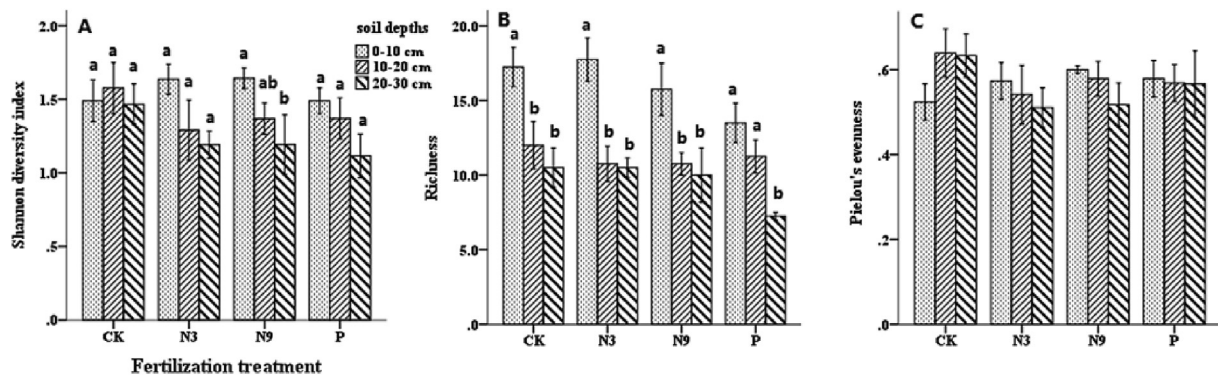


Fig. 3. Mean Shannon diversity index (A), Richness of AMF OTUs (B), Pielou's evenness (C). Data are means \pm SE ($n = 4$). CK, non-fertilized control; N₃, fertilized with low N level at a rate of 3 g N m⁻² yr⁻¹ as urea; N₉, fertilized with high N level at a rate of 9 g N m⁻² yr⁻¹ as urea; P, fertilized with 10 g Ca (H₂PO₄)₂·H₂O m⁻² yr⁻¹. Different lowercase letters indicate difference between different soil layers of the same treatment. Significant differences across treatments within each variable were determined using Fisher's least significant difference (LSD) test ($P < 0.05$) after one-way ANOVA and are indicated by dissimilar letters.

Table 3
Spearman's correlation coefficient with the relative richness of AMF genera and environmental factors.

Soil AMF genus	Correlation to soil variables and plant biomass													
	Layers	pH	TN	TP	AN	AP	Organic content	Water content	Root biomass	Shoot biomass	Grasses biomass	Forbs biomass	<i>P. kansuensis</i> biomass	Legume biomass
<i>Glomus</i>	-0.477***	-0.527***	0.434**	0.119	0.478***	0.142	0.412**	0.254	0.439**	0.055	0.101	0.088	-0.245	-0.0404
<i>Archaeospora</i>	-0.657***	-0.418**	0.099	0.317*	0.582***	0.226	0.558***	0.046	0.586***	0.071	0.026	0.064	-0.130	0.0109
<i>Diversispora</i>	0.494***	0.505***	-0.368**	-0.143	-0.500***	-0.040	-0.446**	-0.152	-0.518***	-0.114	-0.150	-0.167	0.321*	0.0652
<i>Gigaspora</i>	0.772***	0.494***	-0.319*	-0.300*	-0.701***	-0.358*	-0.675***	-0.279	-0.579***	0.105	0.117	0.095	-0.052	0.1021
<i>Ambispora</i>	0.024	0.235	0.054	0.103	-0.047	0.044	0.087	-0.084	-0.040	0.040	0.068	0.171	-0.029	-0.1232
<i>Funneliformis</i>	-0.336*	-0.242	0.194	0.268	0.263	-0.037	0.254	0.100	0.268	-0.148	-0.120	-0.054	-0.020	-0.1779
<i>Rhizophagus</i>	-0.398**	-0.292*	0.285*	0.200	0.312*	-0.080	0.378**	0.059	0.362*	-0.198	-0.278	0.094	0.007	-0.1548

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; mean AMF genera are in bold; negative correlations are in italic; positive correlations are in bold.

AMF colonization levels varied among different soil depths after high N and P fertilization treatments; specifically, AMF colonization was higher in topsoil than in soil at 10–20 cm (Fig. 1A and B). Previous research found that when phosphorus concentration exceeded 7.5 g m^{-2} , AMF colonization of maize roots was lower in the topsoil (0–20 cm) than in the subsoil (20–40 cm) (Wang et al., 2017). But Kabir et al. (1998) found that AMF colonization was highest at a soil depth of 0–15 cm. The inconsistency of these results may be related to the heterogeneity of P distribution in the soil or to plant root distribution. In this study, when soil was fertilized with more N or P, plant roots grew well and were mainly distributed at 0–10 cm. This may have significantly increased AMF colonization at this soil depth.

Fertilization affected soil spore abundance and hyphae length density. Spore abundance and hyphae length density were higher in soil fertilized with P ($4.6 \text{ g P m}^{-2} \text{ yr}^{-1}$) than in the non-fertilized control. This was inconsistent with other studies. Liu et al. (2012) found that when soil was fertilized with $30 \text{ g m}^{-2} \text{ yr}^{-1}$ of $(\text{NH}_4)_2\text{HPO}_4$ (corresponding to 6.4 g N and $7 \text{ g P m}^{-2} \text{ yr}^{-1}$), soil spore abundance reached its peak, but hyphae length density of AMF decreased along a fertilization gradient. Johnson et al. (2003) indicated that in N-enriched (more than 10 g m^{-2}) plots, spore abundance and hyphae length density were lower compared to that in ambient plots, but when N and P were applied together, spore abundance was significantly high. Sheng et al. (2013) found that with $3.5 \text{ g m}^{-2} \text{ P}$, spore abundance increased, but the hyphae length density decreased. In our study, soil spore abundance and hyphae length density increased after fertilizer was added, especially under P fertilization conditions. Because AMF form mutualistic associations with plants and obtain C resources from host plants in exchange for mineral elements, under fertilization conditions, plants may be less dependent on AMF for nutrients. In this case, C allocation to AMF may decrease, causing the abundance of AMF to decrease. In our research, the presence of root hemiparasitic plants led to nutrient stress for host plants. As a result, in nutrient-poor soil, the growth of host plants and the abundance of AMF were suppressed. However, when fertilizer was applied, this situation improved. The reasons are as follows. On the one hand, root hemiparasitic plants consume lower levels of nutrients from host plants; on the other hand, the competition for nutrients between AMF and root hemiparasites is alleviated. When this occurs, there will be no surplus of nutrients even under the fertilization condition. This may explain why fertilization did not inhibit, but, on the contrary, appeared to promote AMF abundance. Strigolactones, plant hormones that enhance the germination of AMF spores and promote AMF hyphae branching (Mori et al., 2016), have been shown to increase in response to P deficiency (Al-Babili and Bouwmeester, 2015; Andreo-Jimenez et al., 2015). In our study, the presence of root hemiparasitic plants, which often cause nutrient (including P) deprivation in their hosts, may have increased excretion of strigolactones in host plants and thereby promoted AMF propagation.

Forty-three OTUs were detected in this subalpine grassland ecosystem, suggesting relatively high AMF biodiversity (Chen et al., 2014; Santos et al., 2006; Vandenkoornhuyse et al., 2002). *Glomus* was a dominant genus under all fertilization regimes. This was consistent with previous reports on the dominance of this genus in many ecosystems (Lin et al., 2012; Oehl et al., 2004; Santos et al., 2006). It has been suggested that *Glomus* spp. are more capable of colonizing via fragments of mycelium when compared with other AMF species (Biermann and Linderman, 1983). Furthermore, Giovannetti et al. (1999) suggested that *Glomus* spp. can easily form anastomoses among mycelia and therefore have the ability to re-establish an interconnected network after mechanical disruption. Interestingly, the distribution of *Glomus* and *Archaeospora* differed

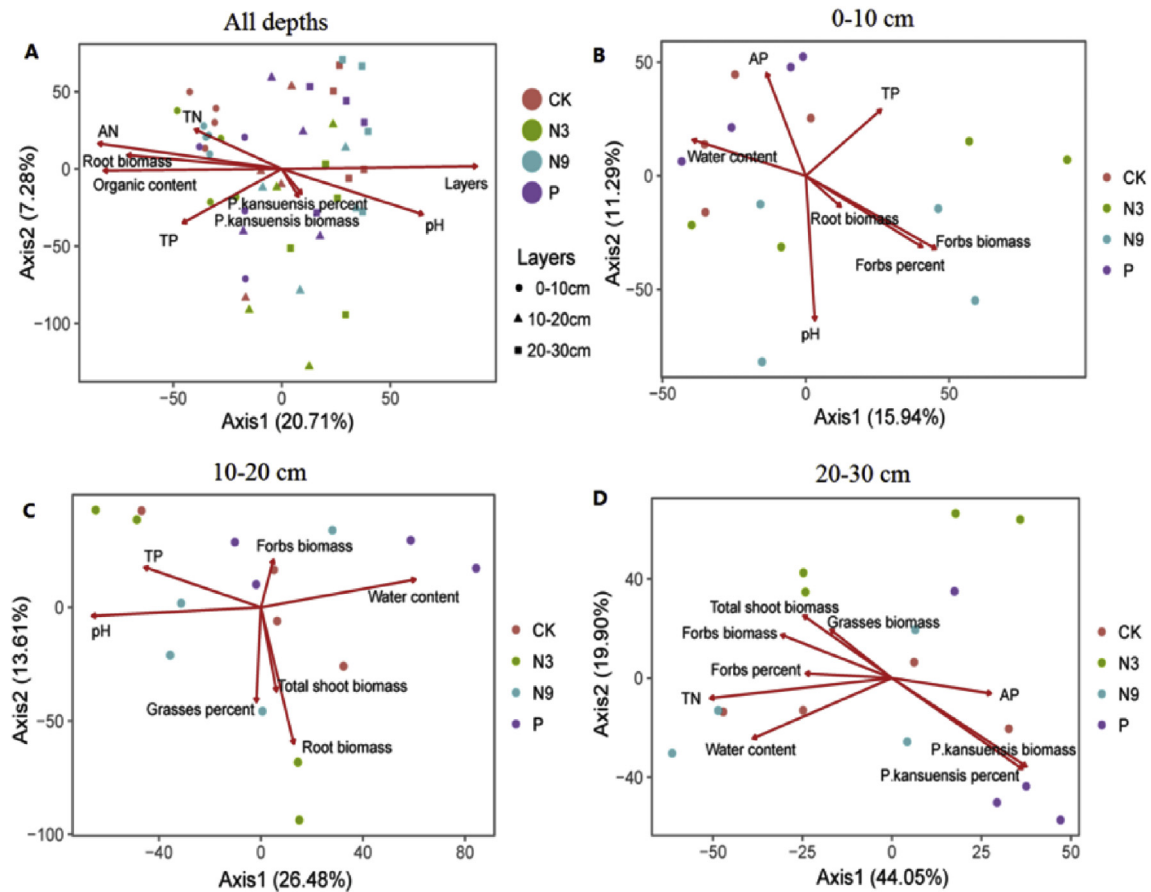


Fig. 4. Redundancy analysis (RDA) relating AMF community structure to soil properties and plants biomass at different soil depths. AMF community structure in all depths (A) and three soil depths separately (B–D) under different treatments. The percentages in the axis labels indicate the variance explained by that axis.

from that of *Diversispora* and *Gigaspora*. The former were mainly distributed in topsoil, especially in N-fertilized quadrats, whereas the latter mainly occurred in deeper soil, where available N was low. These results suggest that 3-year N fertilizer practice could change soil AMF community.

AMF diversity was not affected by fertilization. This does not agree with previous studies that showed that soil AMF diversity decreases after fertilization (de Pontes et al., 2017; Tian et al., 2013; Wang et al., 2011). The influence of soil fertilizer on the diversity of AMF remains controversial, and the inconsistency of these results may be attributed to the application rate of fertilization (Mathimaran et al., 2005), soil properties (Verbruggen et al., 2012) and host plant species (Gosling et al., 2013). In our study, nitrogen and phosphate fertilizer application did not exert an overall significant effect on AMF diversity. One possible explanation is the existence of root hemiparasitic plants. Fertilization allows host plants to absorb essential nutrients from soil, thereby lowering the nutrient stress caused by hemiparasitic plants. However, root hemiparasitic plants continue to absorb nutrients from host plants. AMF and hemiparasitic plants compete for nutrients and host plants still need AMF to provide the mineral nutrients for their growth. Hence, fertilization does not significantly influence AMF diversity. AMF species richness was higher in topsoil than in deep soil (Fig. 3B). Hence, shallow and deep soil layers are both necessary to fully evaluate AMF diversity. This phenomenon corresponds to a study by Taniguchi et al. (2012), in which AMF richness decreased at lower soil depths, even while more than five phylotypes were observed at depths up to 100 cm. The soil surface and seasonal

experience swings, result in different soil temperature and moisture at depth (Brady and Weil, 2002). Both soil moisture (Schimel et al., 1999) and soil temperature (Zogg et al., 1997) have been found to influence soil AMF richness (Chaparro et al., 2012).

5. Conclusions

This is the first study to investigate the influence of fertilization on AMF communities under field conditions where root hemiparasitic plants exist. In contrast to suppressive effects reported by many previous studies, fertilization showed no significant effects on AMF root colonization or AMF species diversity. Instead, we observed that soil spore abundance and extraradical hyphae length density increased markedly. Further investigations are required to unravel the underlying mechanisms of this phenomenon.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.pld.2018.05.001>.

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