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GRAZING INTENSITY AFFECTS COMMUNITIES OF CULTURABLE ROOT-ASSOCIATED FUNGI IN A SEMIARID GRASSLAND OF NORTHWEST CHINA

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ABSTRACT

Intensive grazing by large herbivores is known to result in the degeneration of grassland worldwide. However, little is known about how grazing affects the fungi living in roots, especially those with septate hyphae. We investigated grazing intensity effects on culturable root-associated fungi (c-RAF) of three dominant plant species; *Artemisia capillaris*, *Lespedeza davurica*, and *Stipa bungeana*, in a semiarid grassland of northwest China. Plant roots were sampled from four grazing intensities at four sampling dates. The total colonization rate of c-RAF was not affected by grazing intensity, but the diversity of c-RAF displayed a hump-shaped trend with grazing intensities. Few c-RAF were present in all plant species, with most of them being confined to only one or two plant species. The 16 most commonly isolated species were selected to investigate their effects on plant growth and health. Three *Fusarium* species were most pathogenic c-RAF. Three dark-sterile species did not affect growth parameters, regardless of host plants. With *A. capillaris*, the occurrence of *Fusarium* spp. was highest in the control plots, while with *S. bungeana*, the occurrence tended to increase as the grazing intensity increased. Two dark-sterile species, including *Darksidea alpha*, in *L. davurica* roots increased with grazing intensities; in contrast, *D. alpha* decreased for *S. bungeana* with grazing intensities. Our results strongly suggest that in grasslands, grazing intensity can lead to changes in the colonization of specific root fungal taxa, and these fungal changes would potentially feed back to plant performance depending on the abundance of host plants. Copyright © 2017 John Wiley & Sons, Ltd.

KEY WORDS: grasslands; grazing system; endophytic fungi; dark septate endophytes; *fusarium*

INTRODUCTION

Fungi can establish associations with roots of plants, both on the surface and within inner tissues, and these fungi are broadly termed as root-associated fungi (RAF) (Rodriguez *et al*., 2009). The composition of RAF is strongly affected by the decomposition of organic carbon and secretion of exudates from plant roots (Berg & Smalla, 2009). In turn, RAF affect plant performance by influencing decomposition and nutrient cycles (Smith & Read, 2008) or by acting as mutualists or pathogens (Jumpponen, 1998). Therefore, studies on RAF and their relationships with their host plants will further our understanding on the ecology, and possibly the evolution, of the fungi and their hosts.

The beneficial effects of mycorrhizal associations, especially those of the non-septate arbuscular mycorrhizal fungi (AMF), on host plants have been well established (Smith & Read, 2008). However, for the majority of culturable RAF (c-RAF), that is, a number of ascomyceteous and non-mycorrhizal basidiomycetous fungi with septate hyphae, their effects on host plants are little explored. There is increasing evidence that several groups of c-RAF can enhance the tolerance of host plants to abiotic stresses such as heat and salt (Rodriguez *et al*., 2008; Qin *et al*., 2017) and to biotic stresses such as pathogens and herbivores (Hartley & Gange, 2009). For example, dark septate endophytes (DSE), a major group of c-RAF which are characterized by dark melanized hyphae and commonly occur in all climate regions (Mandyam & Jumpponen, 2005; Rodriguez *et al*., 2009), have been found to confer fitness benefits to host plants, especially in extreme environments (Newsham, 2011). In addition, some species of genus *Fusarium*, usually considered to be crop pathogens worldwide (Booth, 1971), have been found to enhance plant growth and salt tolerance (Rodriguez *et al*., 2008; Redman *et al*., 2011). In natural grasslands, however, the ecological function of c-RAF including those two groups of fungi in apparently healthy plants is still elusive.

In grasslands, large herbivores are key drivers of community dynamics (Bakker *et al*., 2006). Grazing by large herbivores affects plant performance not only through direct consumption of the aboveground parts of plants (Collins *et al*., 1998), but also through indirect effects on biotic soil conditions (Bardgett & Wardle, 2010). Grazing can lead to changes in the composition of soil fungal communities including the fungi living in plant roots (Bardgett & Wardle, 2010). For example, grazing typically leads to short-term increases in C excretion from roots which influences the

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colonization and composition of root fungal communities (Hamilton & Frank, 2001). In addition, grazing results in changes in the concentration of plant metabolites in root tissues, which alters interactions between roots and RAF (Dam *et al*., 2010). Grazing effects on interactions of plant roots and AMF have been studied (Mendoza *et al*., 2011; García *et al*., 2012); however, very little is known about how the intensity of grazing influences the colonization of c-RAF.

In the semiarid grassland of northwest China, more than one-third of the grassland in the area is severely degraded owing to many years of intensive grazing and other human activities (Zhou *et al*., 2011). In 2001, a grazing trial with four levels of intensity of sheep grazing was established in the semiarid grassland and had been maintained for 12 years prior to the start of our experiments. In the present study, we hypothesized that the effects of long-term grazing of different intensities on the growth of plants depend on the combined impacts of grazing on soil properties and the plant community. Thus, we expected that fungal colonization of roots and interactions of these fungi with their host plants would be affected. The aims of our study were to investigate (1) how the intensity of grazing affects the colonization and composition of c-RAF and (2) how these c-RAF interact with their host plants and affect plant performance in a grazed ecosystem.

MATERIALS AND METHODS

Study Site

The experimental site is located in a semiarid grassland of the Tian Shui Grassland Research Station, in Huan County, Gansu Province, northwest China (37.12°N, 106.82°E; 1650 m in elevation). The area is a typical semiarid ecosystem, and the climatic conditions are harsh throughout the year. The soil temperature (0–10 cm) ranged from −4.0 to 21.0 °C, and the annual rainfall was approximately 320 mm, with about 70% occurring from July to September during 2013 (Figure S1). The dominant plant species are *Artemisia capillaris* Thunberg (Asteraceae), *Stipa bungeana* Trin. (Poaceae), and *Lespedeza davurica* (Laxm.) Schindl (Fabaceae) (Hou *et al*., 2002).

In autumn 2001, experimental plots that had similar vegetation composition and cover were fenced to establish a grazing trial. There were four intensities of grazing, 0, 4, 8, and 13 sheep, representing stocking rates of 0, 2.7, 5.3, and 8.7 sheep/ha, respectively. Each grazing intensity was replicated three times using 0.5-ha plots. All plots were arranged in a completely randomized design. In spring of each year, "Tan" lambs weighing approximately 20 kg were bought from local farmers. Each plot was rotationally grazed three times per year, each time for 10 days, with a rotation interval of 30 days from June to September.

Root Sampling and Processing

The three dominant species, *A. capillaris*, *L. davurica*, and *S. bungeana*, were selected for this investigation. For each species, five apparently healthy individuals, at least 50 m apart from each other along a W-shaped sampling line, were sampled from each plot of the four grazing intensities in the middle of May, July, September, and November in 2013. A taproot with associated lateral roots (fibrous root system for *S. bungeana*) from each plant was excavated, placed in polythene bags, brought to the laboratory using an iced cooler, and processed within 96 h.

The five root samples of each plant species within the same replicate plot of each grazing intensity were merged into a composite sample prior to processing. The roots were washed thoroughly with running tap water, then twice more with distilled water to remove debris. Lateral roots and narrow sections of taproots for *A. capillaris* and *L. davurica* and fibrous roots for *S. bungeana* were used to isolate fungi. Root segments were surface-sterilized with a short wash in 75% ethanol and 1% sodium hypochlorite for 2 min, rinsed three times in sterile water, air-dried on sterile filter paper, and then cut into 1- to 2-mm-long segments (Skipp *et al*., 1986). Ten randomly selected root segments were placed in each of six petri dishes containing potato dextrose agar (containing 200 mg/l penicillin and streptomycin, ABPDA). Thus, this resulted in 10 segments per dish × 6 dishes per replicate × 3 replicates × 4 grazing intensities × 4 sampling dates × 3 plant species, for a total of 8640 root segments examined. All the petri dishes were sealed with parafilm (Pechiney Plastic Packaging, Chicago, IL) and incubated at 22 °C. The petri dishes were initially examined after 3–4 days using a stereo dissecting microscope, and subsequently up to 10 days, and developing colonies were subcultured onto PDA dishes.

Identification of Fungal Isolates

Following the isolation of fungi present in the roots, the isolates were initially grouped based on their colony characteristics including color, shape, texture, and growth rate on PDA. Fungal identification was based on morphology, notably the mechanism of spore production and the spore characteristics (Nelson *et al*., 1983; Barnett & Hunter, 1987). Sterile isolates that were unidentified were sorted into different morphotypes based on their colony appearance (Lacap *et al*., 2003).

Some of the isolates that were difficult to distinguish using only morphological characteristics, such as some *Fusarium* spp., were further identified based on rDNA ITS sequence analysis. For DNA extraction, small amounts of mycelia were collected from purified cultures of the isolates with a sterile applicator stick, and the genomic DNA was extracted from lyophilized samples using the E.Z.N.A. Fungal DNA Kit (Omega Bio-Tek, Doraville, GA) according to the manufacturer's protocol. The ITS regions were amplified using the primer pairs ITS4 (5′TCCTCCGCTTA TTGATATGC3′) and ITS1 (5′TCCGTAGGTGAACCTG CGG3′) (White *et al*., 1990). The 25 μl reaction mixtures, containing 10 pmol of each primer, 0.625 U of *Taq* DNA polymerase (Takara Corp., Dalian, China), 0.25 mM dNTP, 1× polymerase chain reaction (PCR) buffer, and 20 ng of

template DNA, were amplified in a GenAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) using the following protocol: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. Amplified products were directly sequenced by GENEWIZ Inc. (Beijing, China) using the primers from the original PCR reactions to sequence in both directions. Sequences obtained were compared with the GenBank database using BLAST, and closely matching sequences (similarity >97%) were included in our phylogenetic analysis to infer species. The phylogenetic analyses were performed using the neighbor-joining method with MEGA version 6.0 (Tamura *et al*., 2013), and the reliability of the phylogenic relationships were validated with bootstrap analysis (1000 reps) (Figure S2).

Pathogenicity Tests

Sixteen c-RAF species that had high isolation frequency were selected to test their effects on the growth and health of the three plant species. The pathogenicity tests included two procedures involving two different substrates: a petri dish experiment in which pre-germinated seeds were placed onto PDA close to the margin of fungal cultures and a pot trial in which seeds were sown in sterilized soil inoculated with fungi. The petri dish experiment examined seedling susceptibility to the tested fungi at a high density of inoculum (Kirkpatrick & Bazzaz, 1979). A 5-mm-diameter plug was taken from the colony margin, placed in the center of each of three 12-cm petri dishes containing PDA, and incubated at 22 °C for 7 to 10 days depending on the growth rate of each fungal species. Seeds of each species were surface-sterilized by submersion in 75% ethanol for 1 min and then 1% sodium hypochlorite for 5 min, rinsed three times with sterile distilled water, and pre-germinated on moist, sterilized filter paper at 22 °C for 24 h to ensure that all seeds in this experiment would germinate. For *L. davurica*, seeds were scarified using a flamed scalpel to break dormancy prior to pre-germination. Seeds were examined under a stereoscopic microscope, and those with an emerging radicle were selected for testing. Ten seeds of each species that had started to germinate were placed equidistant from each other at the margins of each fungal culture or on PDA dishes without fungal cultures as controls. All petri dishes were sealed with parafilm, and stacks of three replicates per treatment were bound together and placed in an incubator at 22 °C.

The seedlings were examined after 2 weeks of incubation. Disease severity ratings were categorized as 0 = healthy, 1 = primary root tip necrotic but firm, 2 = primary root tip rotted and soft, 3 = dead seedling, and 4 = dead seed (Wen *et al*., 2015). The disease index (DI) was calculated as follows:

$$\%\mathrm{DI} = \frac{\sum(x \times t)}{4 \times \sum t} \times 100$$

Where: x is the numerical value of disease severity rating and *t* is the number of individuals in a rating category.

In the pot trials, the effects of selected c-RAF species on seedling survival and other growth parameters of the three plant species were tested. Fungal isolates were grown on PDA dishes for 2 weeks at 22 °C in the dark. With those isolates that had sporulated, inoculum was prepared by adding 10 ml sterile distilled water to a colony, scraping conidia from the agar surface with a sterile scalpel, and adjusting the concentration to approximately 5×10^6 conidia/ml with a hemocytometer. For fungal isolates that failed to sporulate, mycelial suspensions were prepared as follows (Tamura *et al*., 2008). Fungal isolates were cultured with shaking in 50-ml potato dextrose broth medium at 22 °C for several days, and the resulting mycelium was homogenized with a blender (Nippon Seiki, Nagaoka, Japan) after removal of the culture medium. The concentration of hyphal suspensions was also adjusted to approximately 5×10^6 mycelial segments/ml with a hemocytometer. Similar to the petri dish experiment above, seeds were surface-sterilized with 1% sodium hypochlorite for 5 min and then rinsed three times with sterile distilled water. However, seeds in pot trials were not pre-germinated to simulate the intact status of seed. Surface-sterilized seeds were dipped in sterile distilled water (control) or in the conidial or mycelial suspensions for 2 h and then transferred to 15-cm-diameter plastic pots containing soil that had been sterilized by autoclaving at 120 °C for 5 h. Fifteen seeds were sown in each pot at a depth of 0.5 cm and watered daily. A completely randomized design with three replicates was used. The percentage of seedling emergence was determined 3 weeks after sowing (after which no new seedlings emerged). After 5 weeks, seedling survival was determined, and seedling growth was evaluated. Plant weights were determined after drying at 80 °C for 72 h. Pieces of roots of diseased plants from each treatment were plated onto PDA to verify the presence of the fungi tested to confirm Koch's postulates.

Calculations and Statistical Analysis

Colonization rate was calculated as the number of colonies of segments divided by the total number of incubated segments (Wilberforce *et al*., 2003). The frequency of a particular taxon was calculated as the number of colonies of that taxon divided by the total number of incubated segments. The relative frequency was calculated as the number of colonies of a particular taxon divided by the total number of colonies of all taxa (Su *et al*., 2010). The c-RAF diversity was evaluated using the Shannon–Weiner diversity index ($H' = \sum_1^k P_i \times \ln P_i$, where k is the total species number of each replicate and P_i is the relative frequency of c-RAF species of each replicate) (Su *et al*., 2010).

Three-way ANOVA was used to test the significance of variation in total colonization and c-RAF diversity with plant species, grazing intensity, and sampling date as the main effects. *Post hoc* tests were performed using Tukey's tests at $p < 0.05$. One-way ANOVA with Tukey's tests ($p < 0.05$) was used to examine the effect of grazing on the isolation frequency of fungal species. For data from the

pathogenicity tests, statistical differences in comparison with the control were evaluated by independent sample *t*-tests at $p < 0.05$. Prior to ANOVA, data were checked for normality and homogeneity and were transformed whenever necessary to meet the assumptions of the analysis. Means are reported with standard errors. The previous statistical analyses were performed using GenStat version 17.1 (VSN International Ltd., UK).

The permutational multivariate analysis of variance (PerMANOVA) was used to evaluate the effects of grazing intensity, sampling date, and plant species on the composition of c-RAF. Given the predominant role of plant species in affecting root fungal composition, the distance-based redundancy analysis (db-RDA) was used to further assess the effects of grazing intensity and sampling date on fungal assemblages for each plant species. The relationship between soil chemical properties and fungal colonization was explored by coupling principle coordinate analysis (PCoA) ordination of the fungal colonization and vector analysis. Vectors included soil pH, organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), NH_4^+-N, and NO_3^--N (unpublished data) and only shown in the PCoA biplot when it was statistically significant ($p < 0.05$). All multivariate statistical analysis was conducted in R program (R Core Team, 2016). The PerMANOVA was done with the *Adonis* function in vegan package, and the db-RAD and PCoA were carried out with the *capscale* function in vegan package, with 4999 permutations.

RESULTS

Fungal Colonization

In total, 6639 fungal colonies were obtained from 8640 root segments of the 720 plants sampled from four grazing intensities at four sampling dates. The total colonization of c-RAF was significantly affected by plant species and sampling date (Table I). The total colonization of c-RAF showed a hump-shaped trend with sampling date for the three species (Figure 1a–c). There was an interactive effect of plant species and sampling date on the colonization of c-RAF (Table I). The total colonization rate of c-RAF in July was significantly higher than that in May and November for *L. davurica* and *S. bungeana* (Figure 1b, c). Grazing intensity had no significant effects on the total colonization of c-RAF (Table I).

Table I. Results from three-way ANOVA for the effects of plant species, grazing intensity, and sampling date on the total colonization rate and Shannon–Weiner index (H') of culturable root-associated fungi

Source of variation	df	CPS		H'	
		F	*p*	*F*	*p*
Plant species (PS)	2	36.08	**<0.001**	52.03	**<0.001**
Grazing intensity (GI)	3	0.22	0.885	35.90	**<0.001**
Sampling date (SD)	3	22.00	**<0.001**	27.51	**<0.001**
PS × GI	6	3.56	**0.003**	8.26	**<0.001**
PS × SD	6	3.40	**0.004**	5.35	**<0.001**
GI × SD	9	1.05	0.404	1.65	0.112
PS × GI × SD	18	0.84	0.653	1.67	0.059

The total colonization rate was expressed as the number of colonies per root segment (CPS). *F* values (*F*) and *p* values (*p*) were given. Significant *p* values ($p < 0.05$) in bold.

Composition of Culturable Root-Associated Fungi

The isolates were classified into 53 taxa, 45 of which belonged to Ascomycota (41), Basidiomycota (2), and Zygomycota (2) (Table II). The sterile isolates were grouped into different morphotypes based on their colony appearance (Table II). Pleosporales (15) and Hypocreales (10) were the orders contributing the most taxa to the c-RAF assemblages (Table II). *Fusarium*, *Penicillium*, *Trichoderma*, and *Chaetomium* were the common genera, which occurred in all three plant species. Four species, *Fusarium tricinctum*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Setophoma terrestris*, which had high isolation frequencies, were obtained from roots of all three plant species (Table II). Seven c-RAF species were isolated from the two of the three plant species, and the remaining 42 fungal taxa were obtained from only one of the three plant species (Table II). The H' of c-RAF was significantly affected by plant species, grazing intensity, and sampling month (Table I). H' of the three plant species from different sampling months showed a similar hump-shaped trend with grazing intensity, with the highest diversity occurring at the grazing intensity of 2.7 sheep/ha (Figure 1d–f).

The PerMANOVA showed that the composition of c-RAF was significantly affected by plant species, grazing intensity, and sampling date (Table III). There were interactive effects of plant species, grazing intensity, and sampling date on the composition of c-RAF (Table III). The db-RDA showed that the composition of c-RAF of *A. capillaris* differed among grazing intensities, but was not affected by sampling date (Figure 2a). By contrast, the interaction of grazing intensity and sampling date affected the composition of c-RAF for *L. davurica* and *S. bungeana* (Figure 2b, c). Interestingly, root fungal communities of *L. davurica* and *S. bungeana* from the treatment of 8.7 sheep/ha were distinctly different from the other three grazing intensities (Figure 2b, c).

Occurrence of Fusarium and Dark-Sterile Species

The isolation frequency of *Fusarium* spp. from roots of *A. capillaris* in the control was significantly higher than that in the other three grazing intensities (Figure 3a). The isolation frequency of *Fusarium* spp. in the grazing intensity of 8.7 sheep/ha was significantly lower than that in the other three grazing intensities for *L. davurica* (Figure 3b). The frequency of *Fusarium* spp. in the grazing intensity of 8.7 sheep/ha was significantly higher than that in the control and 2.7 sheep/ha for *S. bungeana* (Figure 3c). The frequency of dark-sterile morphospecies 1 (MSP-1) and 2 (MSP-2) tended to increase as the grazing intensity increased for *A.*

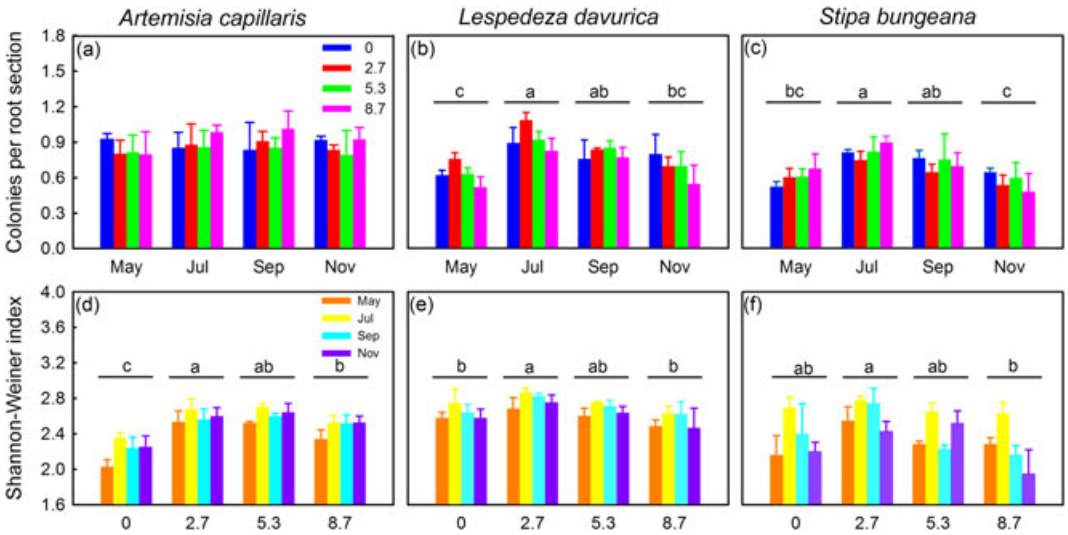

Figure 1. Mean colonization rate (expressed as the number of colonies per root section) and Shannon–Weiner index of culturable root-associated fungi in *Artemisia capillaris* (a, d), *Lespedeza davurica* (b, e), and *Stipa bungeana* (c, f) at four grazing intensities (0, 2.7, 5.3, and 8.7 sheep/ha) in the middle of May, July, September, and November in 2013. Data are represented as the mean of three replicates, and the bars indicate standard errors. Within each panel, different small case letters indicate significant differences using Tukey's tests at $p < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

capillaris (Figure 3d). The frequency of dark-sterile morphospecies 4 (MSP-4) and *D. alpha* isolated from *L. davurica* increased significantly with increasing grazing intensities (Figure 3e). The frequency of *D. alpha* isolated from *S. bungeana* decreased significantly as the grazing intensity increased (Figure 3f).

Effects of Soil Chemical Properties on Fungal Colonization

Principle coordinate analysis was used to reveal relationships between soil chemical properties and fungal colonization. Together, metric multidimensional scaling1 (MDS1) and 2 (MDS2) accounted for 49.65 and 17.59 of the total variation (Figure 4). Along the MDS1, the variation was mainly formed by the total colonization. In contrast, MDS2 variation was mainly introduced by the colonization of *Fusarium* and dark-sterile species. Among all the soil variables examined, only SOC, soil NH_4^+-N, and NO_3^--N were associated with fungal colonization ($p < 0.05$) (Figure 4). The colonization of *Fusarium* species had a positive correlation with SOC, but a negative correlation with soil NH_4^+-N and NO_3^--N (Figure 4). By contrast, the colonization of dark-sterile species was positively correlated with soil NH_4^+-N and NO_3^--N, but negatively correlated with SOC (Figure 4).

Pathogenicity Tests

Although isolated from asymptomatic roots, the majority of the selected fungal species caused symptoms on developing seedlings of the three plant species following exposure of pre-germinated seeds to fungal cultures on PDA dishes (Figure 5). Pre-germinated seeds exposed to the three *Fusarium* species were quickly colonized by hyphae and a tuft of mycelium formed which completely inhibited the development of seedlings (Figure S3). Another 10, 7, and 8 fungal species caused seedlings of *A. capillaris*, *L. davurica*, and *S. bungeana* to be stunted or even collapsed, respectively (Figure 5). In contrast, three dark-sterile species, that is, *D. alpha*, MSP-1, and MSP-4, did not cause any symptoms on developing seedlings (Figure 5). MSP-2 did not cause symptoms on seedlings of *L. davurica* and *S. bungeana* but caused slight browning on root tips of *A. capillaris* (Figure 5).

None of the fungi tested significantly affected seedling emergence in pot trials (Figure S4). *F. oxysporum* and *F. tricinctum* significantly reduced seeding survival of the three plant species (Figure S5). *Fusarium redolens* significantly reduced seedling survival of *A. capillaris* and *S. bungeana* (Figure S5). *R. solani* and *S. terrestris* significantly reduced seeding survival for *A. capillaris* (Figure S5). The majority of the tested fungi did not affect shoot growth of the three plant species, except for *F. oxysporum* and *F. tricinctum* that caused shoots of *A. capillaris* to be shorter in comparison with the control (Figure S6). In contrast, three *Fusarium* species, *F. tricinctum*, *F. oxysporum*, and *F. redolens* significantly reduced root growth of the three plant species as compared with the control (Figure S7). In addition, *R. solani* infection resulted in shorter roots for *A. capillaris* and *L. davurica* (Figure S6), and *S. terrestris* significantly reduced root growth for *A. capillaris* (Figure S7). Significantly lower biomass in comparison with the control was observed for seedlings of the three plant species inoculated with *F. redolens* and *F. tricinctum* (Figure 6). Additionally, the presence of *F. oxysporum* resulted in a lower biomass for seedlings of *A. capillaris* and *L. davurica* (Figure 6a, b). Inoculation with *R. solani* and *S. terrestris* also resulted in lower biomass of *A. capillaris* as compared with the control (Figure 6a). Four dark-sterile species, that is, *D. alpha*, MSP-1, MSP-2, and MSP-4, did not affect growth parameters of the three plant species (Figure 6).

Table II. Number and taxonomic affiliation of culturable root-associated fungi isolated from roots of *Artemisia capillaris*, *Lespedeza duvarica*, *Stipa bungeana*

Phylum	Order	Species	RF (%)			Mean RF (%)
			A. capillaris	*L. duvarica*	*S. bungeana*	
Ascomycota (41)	Capnodiales (3)	*Cladosporium globisporum*	3.07	—	—	1.16
		Cladosporium herbarum	4.82	—	—	3.63
		Cladosporium perangustum	—	1.97	—	0.65
	Dothideales (1)	*Aureobasidium* sp.	0.48	—	—	0.18
	Eurotiales (6)	*Aspergillus niger*	—	0.64	—	0.21
		Paecilomyces varioti	—	1.47	—	0.48
		Penicillium atrovenetum	—	—	5.57	1.63
		Penicillium chrysogenum	0.24	—	—	0.09
		Penicillium soppii	—	—	1.34	0.39
		Penicillium urticae	—	3.34	—	1.10
	Hypocreales (10)	*Acremonium* sp.	3.26	2.29	—	1.99
		Fusarium equiseti	—	—	1.19	0.35
		Fusarium oxysporum	10.35	8.25	6.60	8.56
		Fusarium redolens	—	5.13	1.75	2.20
		Fusarium tricinctum	12.62	7.51	5.93	8.98
		Stachybotrys sp.	0.28	1.92	—	0.63
		Trichoderma atroviride	—	—	4.23	1.24
		Trichoderma gamsii	—	9.21	—	3.03
		Trichoderma hamatum	—	—	6.19	1.81
		Trichothecium sp.	—	4.35	—	1.43
	Microascales (2)	*Periconia macrospinosa*	—	—	7.12	2.08
		Chalaropsis sp.	—	3.30	—	1.08
	Pleosporales (15)	*Alternaria alternata*	6.25	6.64	—	4.55
		Alternaria tenuissima	4.14	—	—	1.57
		Bipolaris sorokiniana	—	—	9.29	2.71
		Bipolaris sp.	—	—	1.55	0.45
		Curvularia sp.	4.38	—	2.99	2.53
		Darksidea alpha	—	6.26	6.50	3.96
		Dictyosporium sp.	—	0.27	—	0.09
		Didymella glomerata	—	3.71	—	1.22
		Epicoccum nigrum	—	3.80	—	1.25
		Paraphoma chrysanthemicola	0.20	—	—	0.08
		Pithomyces sp.	0.16	—	—	0.06
		Setophoma terrestris	7.88	5.63	6.66	6.78
		Stemphylium botryosum	—	—	2.37	0.80
		Ulocladium sp.	—	0.14	—	0.05
		Morphospecies 5	6.33	—	—	2.39
	Sordariales (4)	*Chaetomium ancistrocladum*	9.20	—	7.17	5.57
		Chaetomium interruptum	—	—	4.02	1.17
		Chaetomium murorum	5.10	—	—	1.93
		Chaetomium truncatulum	—	3.48	—	1.14
Basidiomycota (2)	Cantharellales (1)	*Rhizoctonia solani*	9.63	6.23	6.71	7.65
	Moniliellales (1)	*Moniliella* sp.	—	0.09	—	0.03
Zygomycota (2)	Mucorales (2)	*Mucor hiemalis*	0.20	—	—	0.08
		Rhizopus sp.	—	—	1.19	0.35
Unclassified (8)		Morphospecies 1	6.37	—	—	2.41
		Morphospecies 2	2.83	—	—	1.07
		Morphospecies 3	—	2.47	—	0.81
		Morphospecies 4	—	6.70	—	2.20
		Morphospecies 6	—	2.29	—	0.75
		Morphospecies 7	0.16	—	—	0.06
		Morphospecies 8	—	—	5.42	1.58
		Morphospecies 9	—	5.54	—	1.82

Relative frequencies (RFs) of fungal taxa from each plant species were given. The relative frequency was calculated as the number of colonies of a particular taxon divided by the total number of colonies of all taxa. Morphospecies 1–5 were dark in color, and morphospecies 6–8 were white in color.

DISCUSSION

In this study, we investigated quantitative differences in culturable root fungal communities of three dominant plant species in relation to grazing intensity and explored the possible ecological roles of the component fungi by performing pathogenicity test. A wide diversity of fungi, nearly all with septate hyphae, was present in surface-sterilized root pieces.

Table III. Results from permutational multivariate analysis of variance for the effects of plant species, grazing intensity, and sampling date on the compositional dissimilarity of fungal assemblages

Source of variation	Df	MeanSqs	*F*	*P*
Plant species (PS)	2	9.355	162.621	<**0.001**
Grazing intensity (GI)	3	0.307	5.333	<**0.001**
Sampling date (SD)	3	0.245	4.256	<**0.001**
PS × GI	6	0.466	8.102	<**0.001**
PS × SD	6	0.260	4.525	<**0.001**
GI × SD	9	0.086	1.498	**0.027**
PS × GI × SD	18	0.064	1.103	0.26

Mean Sqs, *F* model (*F*), and *p* values (*p*) were given. Significant *p* values ($p < 0.05$) in bold.

Although the total colonization of c-RAF was not affected by the intensity of grazing, the frequency of colonization of some fungal taxa showed species-specific trends with respect to grazing intensity.

Fungal Colonization

Colonization rate is an indicator of the number of root fungi in host plants (Novas *et al*., 2007). In this study, the overall colonization rate was not affected by the intensity of grazing, which contrasts with previous reports on AMF in which grazing had either positive (Pietikäinen *et al*., 2009) or negative (Wearn & Gange, 2007) effects on the colonization of AMF. Plant roots can harbor a wide diversity of septate fungal communities, and different fungal taxa can spread between roots (Rodriguez *et al*., 2009). Thus, the reduction or disappearance of root colonization by certain fungi due to grazing effects could be quickly counteracted by the proliferation of other root fungal taxa that have similar mechanisms of accessing host photosynthates or similar host entry and colonization sites (Lockwood, 1992).

Diversity of Culturable Root-Associated Fungi

Our results demonstrated that roots of the three plant species harbored a wide diversity of fungal species, ranging from those known as pathogens like *Fusarium* spp. to saprophytic fungi like *Alternaria* spp. Interestingly, the diversity of c-RAF showed a hump-shaped relationship with grazing intensity and peaked at the treatment of 2.7 sheep/ha. This finding was similar to a previous report on AMF in which higher numbers of AMF spores were found in lightly and moderately grazed plots in a meadow steppe of northeast China (Ba *et al*., 2012). Root fungal communities are largely structured by the limiting resources in plant roots (Berg & Smalla, 2009). Light and moderate grazing can improve the photosynthetic capability of plants, leading to more resources being deposited in roots (Zhao *et al*., 2008), and increasing the opportunities for a larger number of c-RAF to colonize the roots. Further, it is suggested that moderate grazing can contribute to increased plant diversity (Sasaki *et al*., 2009). In the area in which our grazing trial is located, plant diversity was found to be higher in the treatments of 2.7 and 5.3 sheep/ha than in the control and the 8.7 sheep/ha treatment (unpublished data). Thus, increased plant diversity

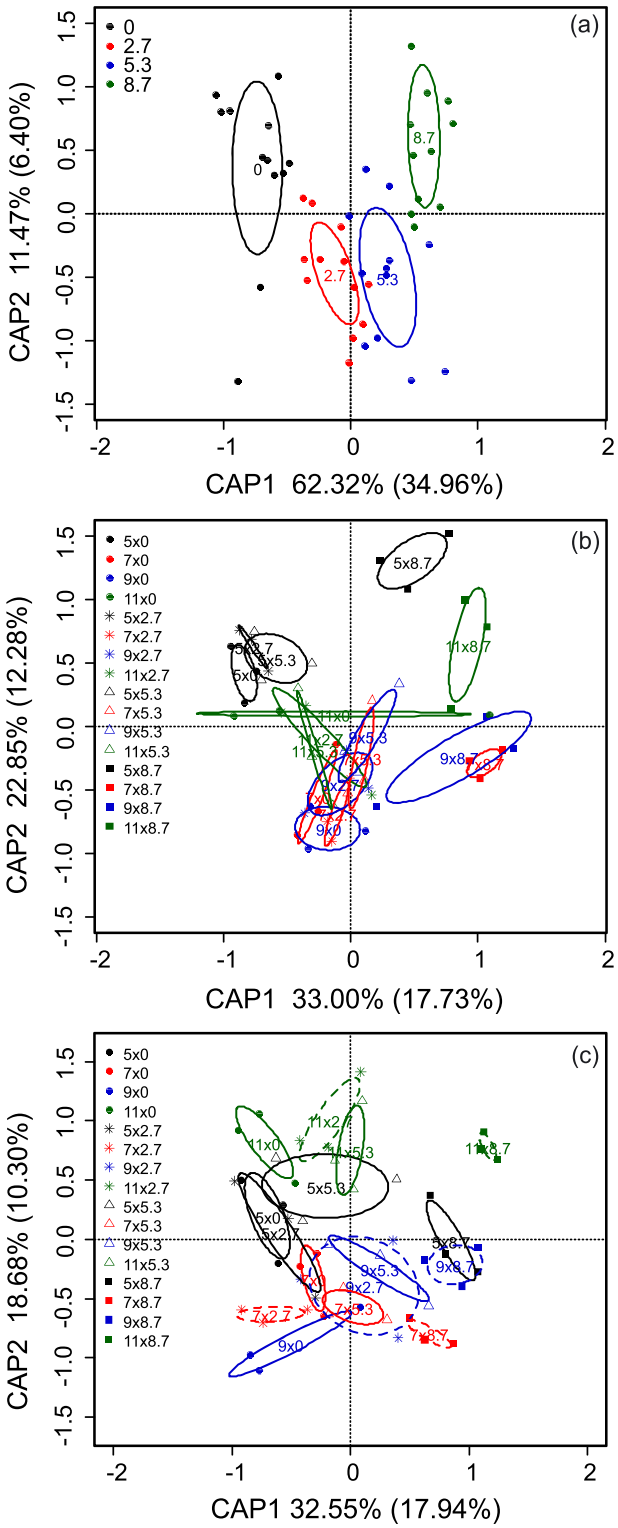

Figure 2. The distance-based redundancy analysis ordination plots of culturable root-associated fungi of *Artemisia capillaris* (a), *Lespedeza davurica* (b), and *Stipa bungeana* (c), showing the relative differences in community composition among four grazing intensities (0, 2.7, 5.3, and 8.7 sheep/ha) at four sampling months (5, 7, 9, and 11). The constrained variation explained in the constrained analysis of principal coordinates was showed in the axis labels with the explained total variation in the parentheses. [Colour figure can be viewed at wileyonlinelibrary.com]

may also lead to diversity in nutrient availability, such as different forms of carbon or variation in carbon allocation in

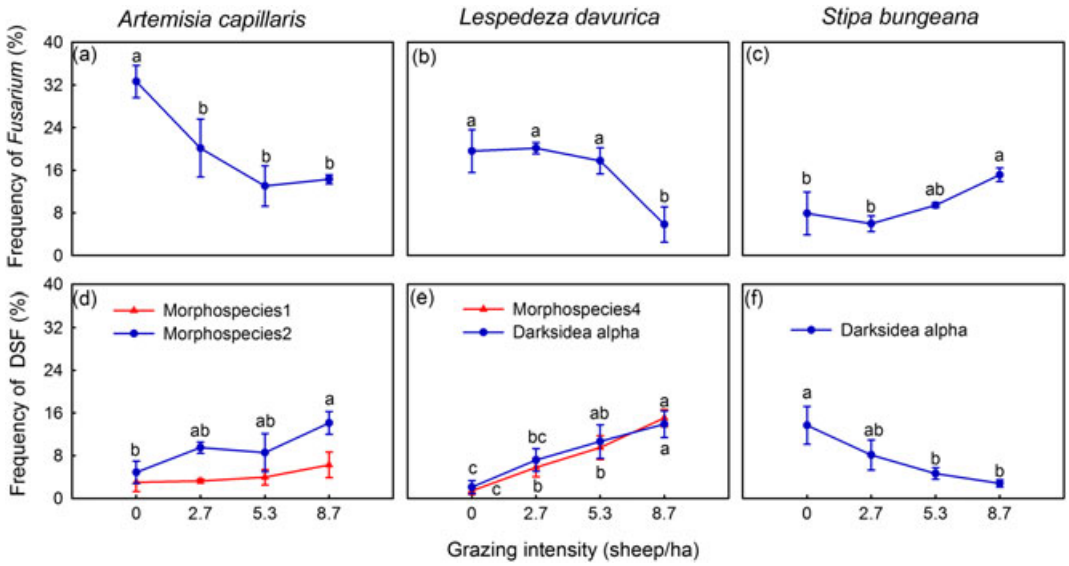

Figure 3. Frequencies of *Fusarium* species and dark-sterile fungi isolated from roots of *Artemisia capillaris* (a, d), *Lespedeza davurica* (b, e), and *Stipa bungeana* (c, f) from different grazing intensities. Data are represented as the mean of three replicates, and the bars indicate standard errors. Different small case letters indicate significant differences among grazing intensities using Tukey's tests at $p < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

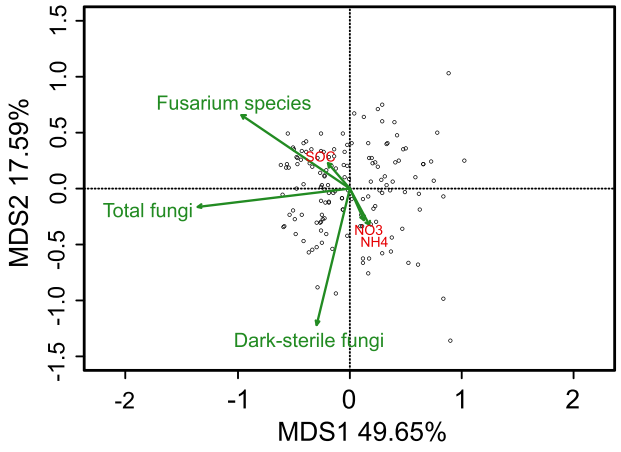

Figure 4. The ordination of principle coordinate analysis showing the correlation of colonization of total fungi, *Fusarium* species, and dark-sterile fungi with soil chemical properties. The selected variables included soil pH, organic carbon, total nitrogen, total phosphorus, NH_4^+-N, and NO_3^--N and only shown in the principle coordinate analysis biplot when it was statistically significant ($p < 0.05$). Direction of arrow indicates the parameters associated with colonization of fungal communities, and the length of the arrow indicates the magnitude of the association. [Colour figure can be viewed at wileyonlinelibrary.com]

time and space, which provides opportunities for more c-RAF species to proliferate (Hooper & Vitousek, 2008).

Composition of Root-Associated Fungi

In general, the composition of c-RAF was dominated by Ascomycota, which was consistent with the results of many previous studies (Yin *et al*., 2008; Angelini *et al*., 2012). Pleosporales and Hypocreales were the orders contributing the most species to the c-RAF assemblages. This result was similar to previous studies in many semiarid plants (Porras-Alfaro & Bayman, 2007; Jin *et al*., 2013). There was little overlap in root fungal communities between different plant species, with the exception of four most common species, *F. tricinctum*, *F. oxysporum*, *R. solani*, and *S. terrestris*, which occurred in all three plant species. This finding was in agreement with the previous assumption that endophytic communities are usually species-specific to a host plant (Errasti *et al*., 2010; Li *et al*., 2007). The db-RDA analysis showed that root fungal communities of *L. davurica* and *S. bungeana* from the treatment of 8.7 sheep/ha were distinctly different from that of other three grazing intensities. This is mainly because the colonization of *Fusarium* species and/or dark-sterile species in the treatment of 8.7 sheep/ha differed greatly from the other three grazing treatments. For example, the colonization rate of *Fusarium* species in *L. davurica* was 5.8% in the treatment of 8.7 sheep/ha, compared with 19.6, 20.1, and 17.8% in the treatments of 0, 2.7, and 5.3 sheep/ha, respectively.

Pathogenicity Test

Although isolated from asymptomatic plants, the majority of fungal species negatively affected seedling growth following pathogenicity test on PDA dishes. However, only few of them, like the most pathogenic *Fusarium* species, had adverse effects on growth parameters in pot trials, suggesting that dosage and substrate are important factors determining the outcome of plant–fungus interactions (Keim *et al*., 2014). Many members of the genus *Fusarium* are important plant pathogens worldwide (Booth, 1971). In this study, three *Fusarium* species, *F. oxysporum*, *F. tricinctum*, and *F. redolens*, were the most pathogenic fungi, regardless of substrate type. By contrast, three dark-sterile species, including *D. alpha*, did not produce symptoms on developing seedlings or affect other growth parameters. Another dark-sterile species MSP-2 did not affect growth parameters for *L. davurica* and *S. bungeana*, but had a slight effect on seedlings of *A. capillaris* in the petri dish trial.

In addition, during the pathogenicity test on PDA dishes, many of the fungal species including those exhibiting host

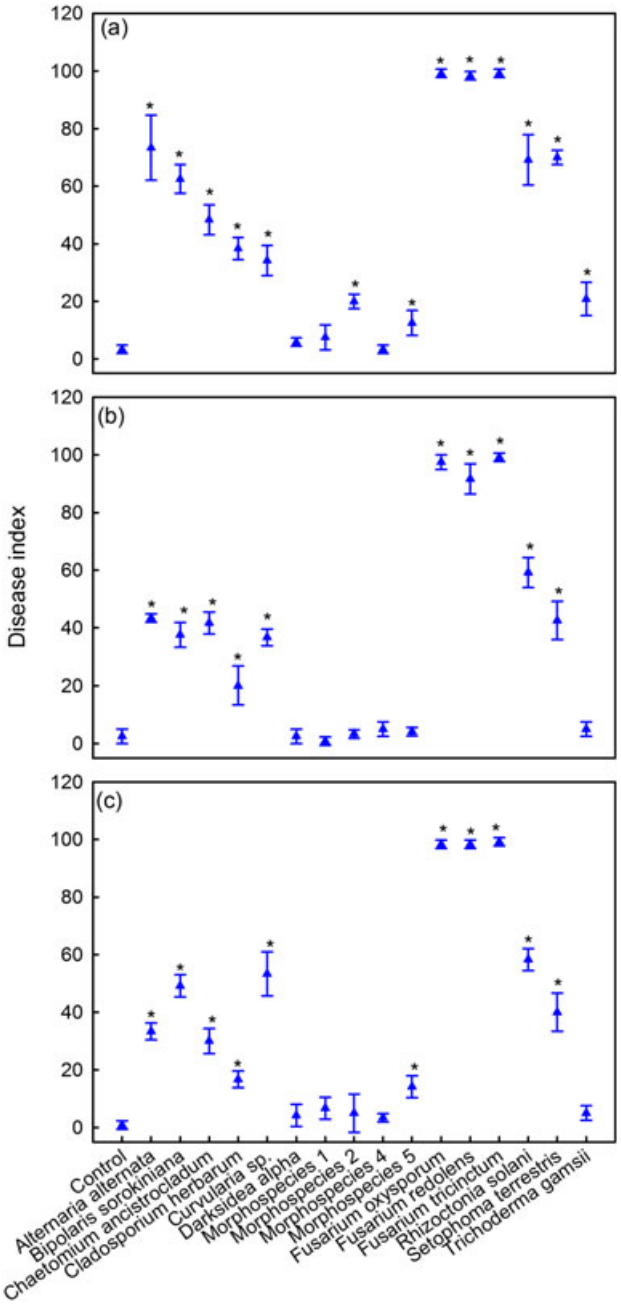

Figure 5. Disease index of tested fungi on seedlings of *Artemisia capillaris* (a), *Lespedeza davurica* (b), and *Stipa bungeana* (c) after 2 weeks of growth on potato dextrose agar plates. Data are represented as the mean of three replicates, and the bars indicate standard errors. Significant differences to the control ($p < 0.05$) are indicated by asterisks above the bars. [Colour figure can be viewed at wileyonlinelibrary.com]

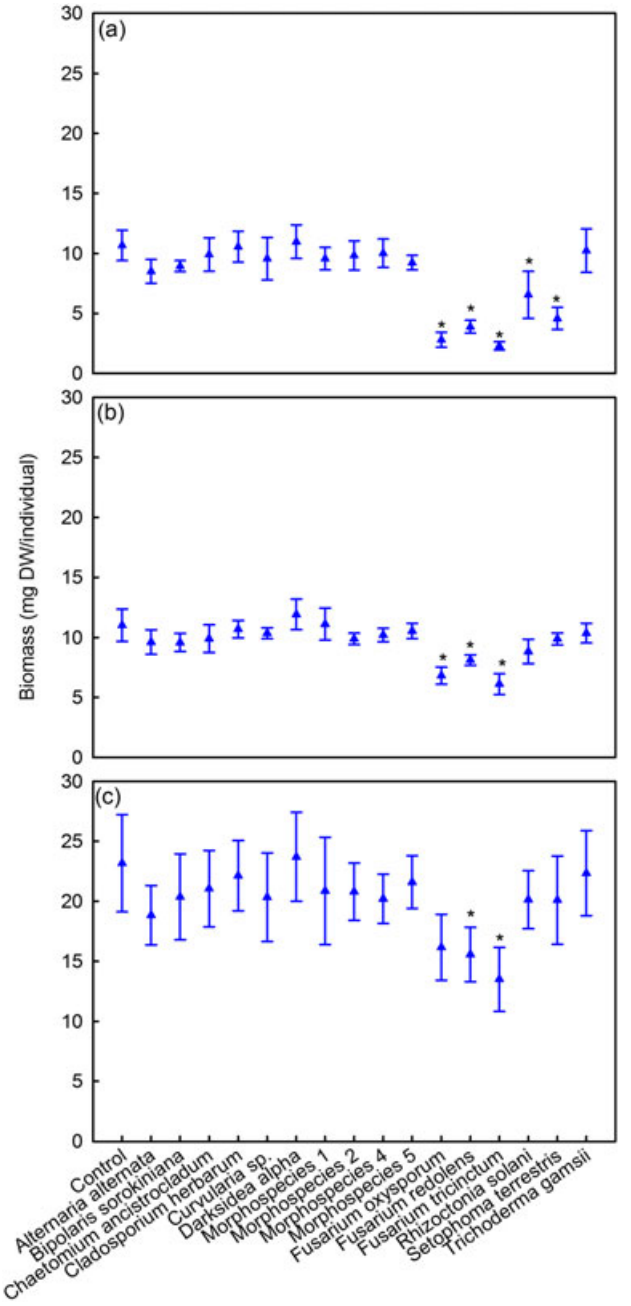

Figure 6. Effects of tested culturable root-associated fungi on bimass (dry weight) of *Artemisia capillaris* (a), *Lespedezadavurica* (b), and *Stipa bungeana* (c) grown in pots containing sterilized soil in a greenhouse for 5 weeks. Data are represented as the mean of three replicates, and the bars indicate standard errors. Significant differences to the control ($p < 0.05$) are indicated by asterisks above the bars. [Colour figure can be viewed at wileyonlinelibrary.com]

specificity in the field can invade seedlings of all three plant species, suggesting that the specificity could disappear when growing conditions are changed. This phenomenon of the disappearance of host specificity was also demonstrated in perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) when seedlings were grown axenically on water agar in petri dishes, and those fungi showing marked host specificity in the field could penetrate and grow in roots of non-host species (Skipp & Christensen, 1989). Our findings suggest that many of the fungi living in plant roots can invade and grow in various host plants, even though most fungi exhibited host preferences and were only isolated from certain plant species.

Ecological Roles of Fusarium and Dark-Sterile Species

One of the main findings we report here is that the occurrence of some fungal taxa showed species-specific trends with respect to grazing intensity. For example, the frequencies of *Fusarium* spp. in the control were significantly higher than those in the other three grazing intensities for

A. capillaris but showed an increasing trend as the grazing intensity increased for *S. bungeana*. In contrast, the frequencies of dark-sterile species, including *D. alpha*, increased significantly for *L. davurica* as the grazing intensity increased. Therefore, understanding the species-specific variation in the occurrence of the two groups of fungi among grazing intensities may shed light on the ecological functioning of root fungal communities in grazed ecosystems.

In natural grasslands, fungal pathogens are predicted to maintain plant species co-existence by lowering the abundance of dominant plant species (Petermann *et al*., 2008; Allan *et al*., 2010). A previous study in our plots revealed that *A. capillaris* was the most dominant species in the semiarid grassland of northwest China and had the highest abundance in the control plots (Chen *et al*., 2017). In that study, *A. capillaris* plants grown in unsterilized soil from the control plots had smaller and blacker root systems than those grown in sterilized soil. Subsequent isolations and pathogenicity tests showed that *F. tricinctum* was a key factor causing the root blackening of *A. capillaris*. However, in the study described here, we did not find any symptoms in field-sampled roots of *A. capillaris* from the control plots, although the isolation frequencies of *Fusarium* spp. were still highest in roots from the control plots. Many environmental factors, such as soil temperature and moisture, are responsible for the growth, survival, and function of the pathogenic fungi (Wong *et al*., 1984). The area in which our grazing trial is located is a typical semiarid ecosystem and is characterized by dry climatic conditions throughout the year, which may therefore lower the growth and function of the pathogenic *Fusarium* spp. In addition, latent pathogens can live within plants for some time as symptomless endophytes (Photita *et al*., 2004). Thus, we inferred that the pathogenic *Fusarium* spp. isolated from the symptomless roots may have the potential to reduce the abundance of *A. capillaris* to maintain species co-existence, although they did not cause symptoms on roots of *A. capillaris* in the field.

In addition, beneficial fungi such as AMF can facilitate plant performance by promoting plant uptake of soil nutrients (Reynolds *et al*., 2003). *L. davurica* is a highly preferred legume species for grazing livestock in grasslands, and its abundance has decreased greatly due to many years of intense grazing. In a previous study by Chen *et al*. (2017), a high percentage of roots of *L. davurica* were colonized by AMF, suggesting that AMF may generate positive effects on the performance of *L. davurica* due to the inferior performance of this species in the field. In the present study, we found that the occurrence of dark-sterile species, including the DSE fungus *D. alpha*, increased greatly as the grazing intensity increased. *D. alpha* is an important member of DSE fungi in semiarid areas (Knapp *et al*., 2015). Previous studies have reported that some DSE fungi facilitate host plants either via N uptake or against root-infecting pathogens (Barrow & Osuna, 2002; Newsham, 2011). In this study, we found that the occurrence of dark-sterile species was positively correlated with soil NH_4^+-N and NO_3^--N. Therefore, it seems likely that *D. alpha*, together with the other three dark-sterile species, may also function like the AMF to promote plant performance when plant abundance in the field is low (Inderjit & Van der putten, 2010). Additional studies are needed to test whether the dark-sterile fungi are promising candidates that function like AMF and how they interact with pathogens to enhance host performance.

Methodological Considerations

Although this study reported how the intensity of grazing affects root fungal communities and explored possible roles of individual fungal species in a grazed ecosystem, there are several limitations that must be acknowledged. One major concern relates to the method used to evaluate root fungal communities. In this study, we used traditional techniques to isolate c-RAF and identified them by morphological characteristics, and if they were sterile or hard to distinguish only by morphology, we also used molecular sequence data to aid the identification of taxa. This method has become commonplace in many endophyte studies (Su *et al*., 2010; Ghimire *et al*., 2011), especially in determining the interaction of individual species with host plants. However, all isolations were carried out using the rich medium PDA which may have biased the study for the detection of those c-RAF that are able to quickly emerge and grow on this type of medium. Also, colonies could potentially arise from a single hypha or from a dense concentration of hyphae present in a single root piece, and thus, total number of colonies does not necessarily reflect the amount of each fungus present in the roots of the plants. Therefore, in the future, culture-independent approaches like high-throughput sequencing technology are required to provide comprehensive surveys of fungal communities and to better understand how the intensity of grazing affect the composition and diversity of RAF.

Another important concern relates to the environmental variables that used for explaining the variation of colonization of c-RAF. Soil chemical properties are considered to be important in determining the composition of soil fungal communities (Wang *et al*., 2014). In this study, of the variables tested, only SOC, soil NH_4^+-N, and NO_3^--N had weak associations with the colonization of c-RAF. This is in part because fungi in plant roots could be directly affected by the decomposition of organic carbon and secretion of exudates from plant roots (Berg & Smalla, 2009) or determined by traits of root systems (Newsham *et al*., 1995). Alternatively, other unmeasured environmental factors such as temperature and precipitation are also related to the proliferation of soil fungal communities (Bell *et al*., 2008; Castro *et al*., 2010). In this study, we failed to measure soil temperatures and soil moisture content of each plot during each sampling time, but meteorological data from a nearby station (2 km away from the experimental plots) suggested that soil temperature may play a significant role in determining the colonization of c-RAF (the colonization of c-RAF was highest in July, which coincided with highest temperature during the year).

CONCLUSIONS

To our knowledge, this study is the first to have examined how the intensity of grazing affects the colonization and composition of culturable root fungal communities in a grazed ecosystem. In conclusion, we found that a wide diversity of fungi, nearly all with septate hyphae, was present in surface-sterilized root pieces. The majority of c-RAF can invade and grow in multiple host plants, even though in field conditions, most of them exhibited host preference. Although the total colonization of c-RAF was not affected by the intensity of grazing, the occurrence of some fungal taxa showed species-specific trends with grazing intensity in relation to the field abundance of the host plants. For example, the occurrence of pathogenic fungi, like *Fusarium* spp., was common in plants where the abundance of a plant species was high in the field, whereas the dark-sterile fungi tended to occur in plants where plant abundance was low. Our study strongly suggest that the intensity of grazing can result in changes in the root colonization of specific fungal taxa, and these fungal species would feed back to plant performance depending on the field abundance of host plant species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Mean monthly precipitation (bars) and soil temperatures (points) at the Tian Shui Grassland Research Station, Huan County, Gansu Province, in 2013

Figure S2. Neighbor-joining tree showing relationships between ITS sequences of selected culturable root-associated fungi from the three dominant plant species and close BLAST matches from GenBank. Numerical values represent bootstrap percentiles from 1000 replicates. Nodes with less than 50% bootstrap support have been collapsed into polytomies. ACR, roots of *Artemisia capillaris*; LDR, roots of *Lespedeza davurica*; SBR, roots of *Stipa bungeana*

Figure S3. Symptoms of pre-germinated seeds of *Lespedeza davurica* inoculated with various fungi after 2 weeks of growth on potato dextrose agar (PDA) plates. A = control; B = seeds inoculated with *Fusarium oxysporum*; C–G indicates five scales of disease severity. C = healthy; D = primary

root tip necrotic but firm; E = primary root tip rotted and soft; F = dead seedling; G = dead seed

Figure S4. Effects of tested culturable root-associated fungi on seedling emergence of *Artemisia capillaris* (a), *Lespedeza davurica* (b), and *Stipa bungeana* (c) grown in pots containing sterilized soil in a greenhouse for 5 weeks. Data are represented as the mean of three replicates, and the bars indicate standard errors

Figure S5. Effects of tested culturable root-associated fungi on seedling survival of *Artemisia capillaris* (a), *Lespedeza davurica* (b), and *Stipa bungeana* (c) grown in pots containing sterilized soil in a greenhouse for 5 weeks. Data are represented as the mean of three replicates, and the bars indicate standard errors. Significant differences to the control ($p < 0.05$) are indicated by asterisks above the bars

Figure S6. Effects of tested culturable root-associated fungi on shoot length of *Artemisia capillaris* (a), *Lespedeza davurica* (b), and *Stipa bungeana* (c) grown in pots containing sterilized soil in a greenhouse for 5 weeks. Data are represented as the mean of three replicates, and the bars indicate standard errors. Significant differences to the control ($p < 0.05$) are indicated by asterisks above the bars

Figure S7. Effects of culturable root-associated fungi on root length of *Artemisia capillaris* (a), *Lespedeza davurica* (b), and *Stipa bungeana* (c) grown in pots containing sterilized soil in a greenhouse for 5 weeks. Data are represented as the mean of three replicates, and the bars indicate standard errors. Significant differences to the control ($p < 0.05$) are indicated by asterisks above the bars