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Research Article

N enrichment affects the arbuscular mycorrhizal fungi-mediated relationship between a C_4 grass and a legume

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Abstract

Arbuscular mycorrhizal fungi (AMF) regulate soil nutrient cycling, directly supplying a host plant with nitrogen (N). AMF can also affect the outcome of interspecific interactions, but a mechanistic understanding of how soil N availability affects AMF-mediated interspecific relationships is currently lacking. We selected one dominant (*Bothriochloa ischaemum*; C_4 grass) and one subordinate (*Lespedeza davurica*; legume) species in a natural grassland climax community to investigate the mechanism by which AMF influence interspecific interaction (mixed and monoculture) under three levels of N addition (0, low, and high N addition). Under the non-N addition treatment, AMF preferentially supplied N to the roots of *B. ischaemum* at the expense of N uptake by *L. davurica*, resulting in inhibited AMF benefits for *L. davurica* shoot growth. Under the low N addition treatment, interspecific interaction via AMF promoted *L. davurica* growth. Compared to the non-N addition treatment, N addition largely mitigated the effects, both positive (for *B. ischaemum)* and negative (for *L. davurica*), of AMF-mediated interspecific interaction on plant N uptake via AMF. When soil N availability severely limited plant growth, preferential N supply to the C_4 grass by AMF was important for maintaining the abundance of the dominant species. When the N limitation for plant growth was alleviated by N addition, the interaction between AMF and soil microorganisms improved nutrient availability for the legume by stimulating activity of the enzyme responsible for soil organic matter mineralization, which is important for maintaining the abundance of the subordinate species. These data could influence strategies for maintaining biodiversity.

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Introduction

Soil nitrogen (N) availability is a common limiting factor for plant growth in terrestrial systems (Elser et al., 2007). One of the main benefits of arbuscular mycorrhizal fungi (AMF) for host plants is improved mineral nutrient levels; however, most studies have demonstrated the role of AMF in plant phosphorus (P) rather than N nutrition (Smith and Read, 2008; Smith and Smith, 2011; Veresoglou et al., 2012). There is some evidence for AMF-mediated N uptake by plants (Govindarajulu et al., 2005; Hodge and Fitter, 2010; Veresoglou et al., 2012; Thirkell et al., 2016), and Jach-Smith and Jackson (2020) have suggested that AMF supplies agronomically substantially amounts of plant N. Notably, N is the principal nutrient limiting net primary productivity in high-latitude and semi-arid ecosystems (LeBauer and Treseder, 2008; Harpole et al., 2011), so an increase in soil N availability is expected to promote vegetation productivity. However, the response of AMF-mediated plant N acquisition to increased N availability is still uncertain, and N addition could reduce (Jach-Smith and Jackson, 2020), stimulate (Tu et al., 2006), or have a negligible effect (Schroeder-Moreno et al., 2012) on plant N uptake via AMF.

Liebig's Law of the Minimum states that plant growth is not limited by total resources available, but is controlled by a single essential resource that is in limited supply (Liebig, 1842; van der Ploeg et al., 1999). Johnson et al. (2015) suggested that the relative availability of N and P had a substantial impact on plant mycorrhizal growth responses (MGRs), and Liebig's Law of the Minimum is a useful framework for connecting resource supply and demand to predictions about mycorrhizal functioning (Sterner and Elser, 2002; Johnson, 2010). The N concentration in AM fungal hyphae is 4–7 times and at least 10 times higher than that in plant shoot and root, respectively, so AMF requires much more N per unit biomass than their host plants (Hodge and Fitter, 2010). In the N-limited systems, AMF are, therefore, unlikely to have surplus N for transporting to their host plants because their own N requirements should be primarily met. Johnson et al. (2015) found that AMF are likely to promote the plant growth of a host plant in P-limited systems but suppressed that in N-limited systems. N addition to N-limited soils could alleviate N limitation, leading to enhanced aboveground plant growth, while N addition to N-rich soils is expected to induce and aggravate P limitation, which induces plants to allocate more resources to roots and AMF (Johnson et al., 2003, 2015). Although soil N availability is thought to largely influence the AM benefit for plant growth, little is known about the linkage between plant N acquisition via AMF and plant MGR under various N availability conditions.

The AMF can have a considerable influence on interspecific relationships (Van Der Heijden et al., 2003; Wagg et al., 2011; Weremijewicz et al., 2018). When dominant species in natural plant communities are highly AMF-dependent, AMF can reduce species diversity by increasing interspecific competition and decreasing intraspecific competition (Hartnett and Wilson, 2002; Gross et al., 2010). Previous researches with root organ cultures revealed that carbon (C) and nutrient exchange between host plant and AMF follows a "reciprocal reward" model, in which AMF preferentially provide mineral nutrients to plants that provide the most C or represent the strongest sinks (Lekberg et al., 2010; Hammer et al., 2011; Kiers et al., 2011). AMF can, therefore, potentially amplify interspecific and size-asymmetric competition between plants (Merrild et al., 2013; Weremijewicz et al., 2016). However, Walder et al. (2012) showed that the growth of flax (*Linum usitatissimum*; a C3 plant) and sorghum (*Sorghum bicolor*; a C4 plant) benefited from the interspecific relationship between flax and sorghum plants mediated by AMF under controlled conditions because flax invests less C in AMF but gained much more N and P from AMF relative to sorghum, which showed a strong asymmetry in terms of C and nutrient trade. Walder and van der Heijden (2015) proposed taking competition for surplus resources and sink strength into consideration. Therefore, the N acquisition of plant species associated with AMF might play an important role in regulating interspecific relationships. Furthermore, Van Der Heijden et al. (2008) and Jia et al. (2020) suggested that AMF can reduce the negative impact of increased N availability on plant communities. N addition strongly increased the dominance of grasses in the plant community and reduced legume biomass, while AMF substantially enhanced the biomass of all legume species and reduced the relative abundance of grasses (Jia et al., 2021). N enrichment in soil is expected to largely affect sink strength of plants via AMF and surplus resources for AMF and associated plants. It is of great value for grassland management to investigate the effect of N supplied to plant species associated through AMF on the interspecific plant relationships under various N availability conditions.

AMF are likely to rely on soil saprotrophic microbes mineralizing soil organic matter (SOM) to obtain mineral nutrients because AMF generally lack saprotrophic capability (Smith and Read, 2008; Kohler et al., 2015). AMF slowly release labile C for saprotrophic microbes through hyphal exudation and turnover (Jones et al., 2004; Drigo et al., 2010), likely facilitating C-limited saprotrophic microbes utilizing this energy subsidy and producing extracellular enzymes to mine SOM for nutrients (Hodge et al., 2001; Phillips et al., 2011; Cheng et al., 2012). Previous studies have confirmed that AMF can stimulate the soil microbial functional activity associated with N cycling (Morrison et al., 2017; Teutscherova et al., 2019). Therefore, this study takes the interaction between AMF and soil microorganisms into consideration in order to comprehensively investigate the impact of interspecific interactions on nutrient cycling, and soil enzyme activity was the proxy to indicate the outcome of the interaction between AMF and soil microorganisms.

In the present research, one dominant (*Bothriochloa ischaemum*; C4 grass) and one subordinate species (*Lespedeza davurica*; legume) in a natural grassland climax

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community in the Loess Plateau, China, were selected as research objects because mixed cultures of *B. ischaemum* and *L. davurica* have been shown to have an advantage in improving growth performance and resource utilization efficiency (Xu et al., 2016, 2018). Our study aimed to comprehensively investigate the effect of N addition on the interspecific relationship between the C_4 grass and the legume species mediated by AMF, and the linkage between plant N acquisition via AMF and interspecific relationships. We hypothesized that: (1) C3 legumes have better growth performance relative to C4 grasses under low soil N condition, and the AM benefit for legume growth is less affected by N addition compared to the benefit for C4 grass; (2) N addition alleviates soil N deficiency but exacerbates soil P limitation, which could potentially shift AMF-mediated interspecific relationships from competition to facilitation; and (3) When plant growth is limited by soil N availability, N supply to plant species connected by AMF is an important mechanism to regulate the interspecific relationship between C_4 grasses and legumes.

Results

Mycorrhizal colonization

The mycorrhizal colonization of *B. ischaemum* in the monoculture under the high N treatment was significantly higher than that under the treatment without N addition and with low N treatment (Figure 1A). Compared with the monocultures, the mixed culture significantly decreased mycorrhizal colonization of *B. ischaemum* by 10.77% under the high N treatment.

Shoot growth performance

Shoot growth performances of both plant species were significantly affected by the culture system, N addition, mycorrhizal inoculation, and the interaction between these factors (Table 1). Compared with nonmycorrhizal systems, AMF significantly increased plant net photosynthetic rate (Pn; Table 2; Supplemental Figure S1), and shoot biomass, C, N, and P content (Table 2; Supplemental Figure S2). The shoot MGR is an indicator of AM effect on plant growth performance. In monocultures, the MGR of shoot biomass, C, N, and P content in *B. ischaemum* was highest under the high N treatment (Figure 2). However, N addition had a negligible effect on the MGR of shoot biomass and N content in *L. davurica*, but significantly decreased the MGR of shoot C and P content in *L. davurica* under the high N treatment.

Table 1 Three-way ANOVA (culture system (C) = monoculture or mixed culture; N addition (N) = 0, low N or high N; mycorrhizal inoculation (M) = mycorrhizal system or nonmycorrhizal system)

Factor	Shoot Growth Performance		
	Pillai's Trace	F-value	df
Culture System	0.273	6.432***	6
N Addition	1.062	19.626***	12
Mycorrhizal Inoculation	0.973	614.642***	6
C × N	0.130	1.203	12
C × M	0.181	3.794**	6
N × M	0.876	13.508***	12
C × N × M	0.221	2.152	12

The shoot growth performance comprises shoot biomass, shoot C, N, and P content, shoot N/P, and plant Pn.
Significant main effects and interactions are indicated: absence of *, not statistically significant; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

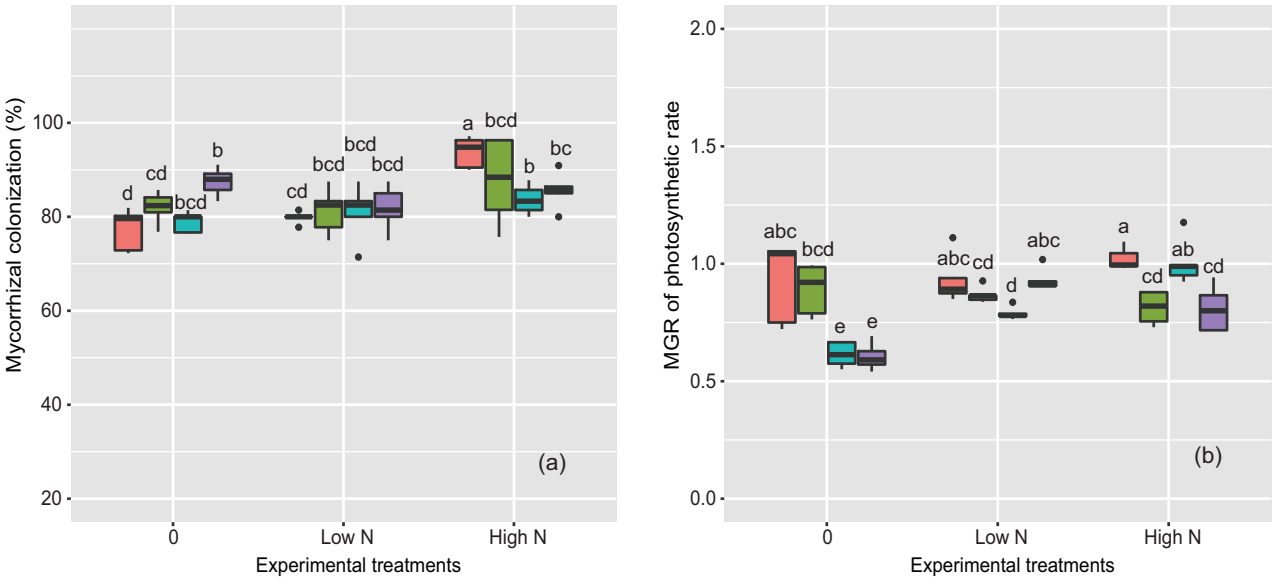

Figure 1 Effects of N addition and culture system on the mycorrhizal colonization and MGR of plant Pn. Note: In the box plot, the black boxes show the 25th and 75th quantiles, the whiskers are min–max values, and the horizontal line is the median. Statistical analysis was conducted using ANOVA followed by Tukey's honestly significant difference (HSD) mean-separation test and indicated by lowercase letters above the boxes ($P < 0.05$). Culture systems in mycorrhizal and nonmycorrhizal systems are indicated as: orange box, *B. ischaemum* in monoculture; green box, *B. ischaemum* in mixed culture; blue box: *L. davurica* in monoculture; purple box: *L. davurica* in mixed culture. Black dots are the outliers (values outside the range of ±1.5*IQR, where IQR is the interquartile range defined as the upper quartile minus the lower quartile) within the treatment.

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Table 2 Three-way ANOVA on shoot biomass, C, N, and P content, N/P, and Pn (culture system (C) = monoculture or mixed culture; N addition (N) = 0, low N or high N; mycorrhizal inoculation (M) = mycorrhizal system or nonmycorrhizal system) and two-way ANOVA on shoot N uptake from organic patch (culture system (C) = monoculture or mixed culture; N addition (N) = 0, low N or high N)

Analysis Object	Culture System	N Addition	Mycorrhizal Inoculation	C × N	C × M	N × M	C × N × M
Shoot Biomass	0.295	26.511***	342.826***	0.231	0.359	18.448***	0.329
Shoot C Content	1.531	11.527***	507.803***	0.007	2.010	6.220**	0.044
Shoot N Content	0.236	9.401***	214.441***	0.431	0.070	5.232***	0.627
Shoot P Content	7.421**	13.568***	676.775***	1.536	4.578*	7.619***	2.267
Shoot N/P	3.484	1.517	1.517	0.498	0.607	0.045	0.907
Shoot N Uptake from Organic Patch	11.838**	3.246*	—	2.529	—	—	—
Plant Pn	0.457	15.605***	191.255***	0.003	0.082	4.388*	0.321

F-ratios from two-way (shoot N uptake from organic patch) and three-way (shoot biomass, shoot C, N, and P concentration, shoot N/P, plant Pn) ANOVA represented. Significant main effects and interactions are indicated: absence of *, not statistically significant; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Compared with monoculture system, the mixed culture significantly decreased the MGR of shoot biomass in *L. davirica* under the treatment without N addition, and the MGR of shoot biomass in *B. ishcaemum* under the high N treatment, but significantly increased that in *L. davurica* under the low N treatment (Figure 2A). Additionally, in comparison with monoculture, the mixed culture significantly decreased the MGR of shoot C, N, and P content in *L. davurica* under the treatment without N addition; and the MGR of shoot P content and Pn in *B. ishcaemum* under the high N treatment, but significantly increased the MGR of shoot N and P content and Pn in *L. davurica* under the low N treatment (Figures 1, B and 2, B–D). Furthermore, in the mycorrhizal system, mixed culture significantly increased shoot N/P in *L. davurica* under the treatment without N addition by 66% relative to the corresponding treatment in the non-mycorrhizal system (Supplemental Figure S3).

Root–shoot ratios of growth performance

Compared with nonmycorrhizal systems, AMF significantly decreased root shoot ratio of biomass, C, N, and P content in *L. davurica* in monoculture under the non-N treatment; root shoot ratio of biomass, C, and P content in *B. ischaemum* in monoculture under the low N treatment; and root shoot ratio of biomass, N, and P content in *L. davurica* in mixed culture under the low N treatment (Table 3; Supplemental Figures S4 and S5; $P < 0.05$). In monocultures, N addition significantly decreased MGR of root shoot ratio of biomass, C, N, and P content in *B. ischaemum*; but significantly increased that in *L. davurica* (Figure 3). Compared with the monoculture, the mixed culture significantly increased the MGR of root shoot ratio of biomass, C, N, and P content in *L. davurica* under the non-N treatment; that of biomass, C, and P content in *B. ischaemum* under the low N treatment; and that of biomass, C, and N content in *L. davurica* under the high N treatment (Figure 3). However, the mixed culture significantly decreased the MGR of root shoot ratio of biomass, N, and P content in *L. davurica* under the low N treatment in comparison to the monoculture.

Plant N uptake via AMF

There was no significant linear relationship between plant uptake of C from the organic patch and that of N. The plant uptake of C from organic patches via hyphae was far lower than that of N (Supplemental Figure S6). Additionally, the shoot N uptake by *B. ischaemum* and *L. davurica* via AMF in the monoculture was highest under the treatment without N addition and lowest under the low N treatment (Figure 4, A and B). However, the root N uptake by *B. ischaemum* in the monoculture was highest under the high N treatment, while N addition significantly decreased that by *L. davurica*. Compared with the monocultures, the mixed culture significantly increased root N uptake by *B. ischaemum* under the treatment without N addition and low N treatment by 29.82% and 23.88%, respectively. The mixed culture significantly decreased shoot and root N uptake by *L. davurica*, and this negative effect was alleviated by increased N addition. Furthermore, there were significant linear relationships between plant N uptake via AMF and plant N content (Supplemental Figure S7).

Soil biochemical factors affecting plant growth traits

The redundancy analysis (RDA) revealed that the difference of the MGR of shoot growth traits in *B. ischaemum* in between monoculture and mixed culture under the high N treatment ($P < 0.05$) was mainly driven by the MGR of soil *N*-acetyl-glucosaminidase activity, while that in *L. davurica* in between monoculture and mixed culture under the non-N and low N treatment ($P < 0.05$) was mainly influenced by the MGR of soil alkaline phosphatase and available P content (Figure 5A; Supplemental Table S1). Moreover, the MGR of shoot biomass, shoot P content, and plant photosynthetic rate were mainly affected by the MGR of soil available P concentration and alkaline phosphatase activity,

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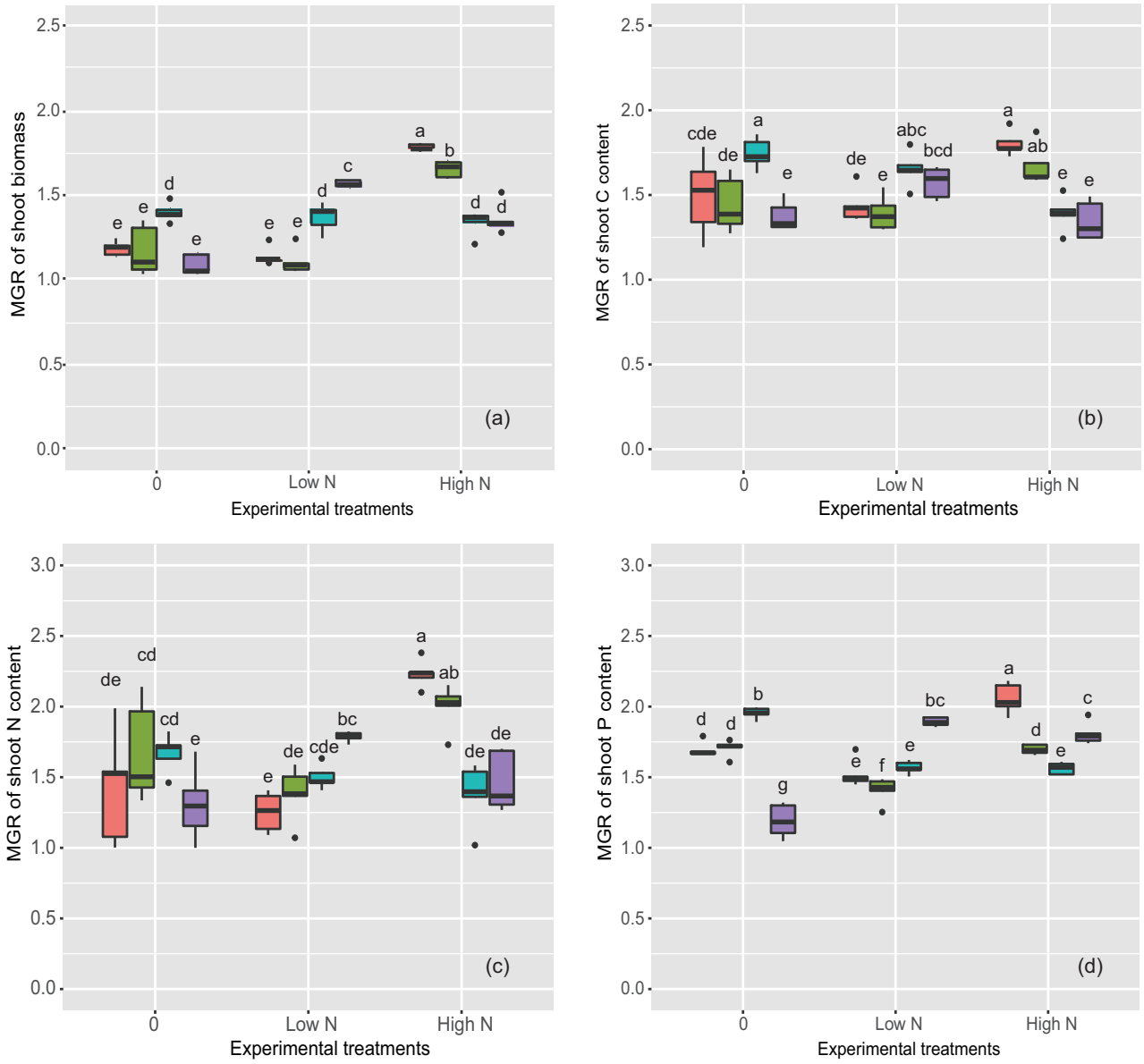

Figure 2 Effects of N addition and culture system on the MGR of shoot biomass, C, N, and P contents. Note: In the box plot, the black boxes show the 25th and 75th quantiles, the whiskers are min–max values, and the horizontal line is the median. Statistical analysis was conducted using ANOVA followed by Tukey's HSD mean-separation test and indicated by lowercase letters above the boxes ($P < 0.05$). Culture systems in mycorrhizal and nonmycorrhizal systems are indicated as: orange box, *B. ischaemum* in monoculture; green box, *B. ischaemum* in mixed culture; blue box: *L. davurica* in monoculture; purple box: *L. davurica* in mixed culture. Black dots are the outliers (values outside the range of ±1.5*IQR, where IQR is the interquartile range defined as the upper quartile minus the lower quartile) within the treatment.

whereas the MGR of shoot C and N content were mainly influenced by soil *N*-acetyl-glucosaminidase and β-xylosidase activity (Figure 5B).

Structural equation modeling (SEM) explained the 92% of the variance in the shoot biomass of the mycorrhizal plants (Figure 6). Plant photosynthesis, which directly influences shoot C content (Supplemental Table S2; $P < 0.001$), was directly determined by soil dissolved inorganic N concentration (Supplemental Table S2; $P < 0.01$). Shoot biomass was directly affected by shoot C content (Supplemental Table S2; $P < 0.001$) and enzyme activity related to C cycling (β-glucosidase, β-cellobiosidase, and β-xylosidase; Supplemental Table S2; $P < 0.01$).

Discussion

Effect of AMF on the interspecific plant interaction between C4 grass and C3 legume

Our study revealed that AMF largely promoted the growth performance of a C_4 grass (*B. ischaemum*) and C3 legume

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Table 3 Three-way ANOVA on root shoot ratio of biomass, C, N, and P content (culture system (C) = monoculture or mixed culture; N addition (N) = 0, low N or high N; mycorrhizal inoculation (M) = mycorrhizal system or non-mycorrhizal system) and two-way ANOVA on root N uptake from organic patch and mycorrhizal colonization (culture system (C) = monoculture or mixed culture; N addition (N) = 0, low N or high N)

Analysis Object	Culture System	N Addition	Mycorrhizal Inoculation	C × N	C × M	N × M	C × N × M
Root Shoot Ratio of Biomass	4.099*	12.265***	2.796	3.325*	4.587*	1.895	2.667
Root Shoot Ratio of C Content	1.689	2.220	9.816**	1.166	4.767*	2.387	0.236
Root Shoot Ratio of N Content	12.750**	17.133***	7.252**	1.019	0.004	0.270	2.310
Root Shoot Ratio of P Content	7.466**	1.619	20.738***	6.384**	7.588**	3.314*	0.063
Root N Uptake from Organic Patch	7.535**	0.579	—	0.510	—	—	—
Mycorrhizal Colonization	2.128NS	11.149***	—	3.809*	—	—	—

*F-ratios from two-way (root N uptake from organic patch and mycorrhizal colonization) and three-way (root biomass, root C, N, and P content, and root N/P) ANOVA represented. Significant main effects and interactions are indicated: absence of , not statistically significant; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

(*L. davurica*). AMF benefit the growth performance of host plants through at least two mechanisms. First, AMF could directly provide P and inorganic N for host plants, which alleviates soil nutrient limitation for plant growth and promotes plant growth (Hodge and Fitter, 2010; Smith and Smith, 2011). In this study, AMF significantly decreased soil available P and inorganic N concentrations (Supplemental Figures S8 and S9), indicating that AMF promoted plant N and P uptake from soil. Second, AMF strongly stimulated soil C-, N-, and P-acquiring enzyme activities compared to the non-mycorrhizal systems (Supplemental Figure S10), leading to the accelerated mineralization of SOM and increased soil bioavailable N and P, which further alleviated soil microbial metabolic P limitation (Supplemental Figure S13). Although AMF generally lack saprotrophic capabilities, AMF can input C into the soil via hyphae to stimulate microbial activity related to N cycling and cooperate with soil P-solubilizing bacteria related to organic P (phytate) mineralization (Smith and Read, 2008; Zhang et al., 2016; Morrison et al., 2017).

Under the non-N addition condition, interactions between *B. ischaemum* and *L. davurica* via AMF inhibited the AM benefit for shoot growth of *L. davurica*, but largely facilitated that for root shoot ratio of *L. davurica*. This result indicated that the interspecific interaction via AMF exacerbated nutrient limitation for plant growth of *L. davurica* because *L. davurica* allocated more biomass to its roots, which is a plant growth strategy when confronting environmental stress conditions (Brouwer, 1984; Tilman and Cowan, 1989). Our RDA revealed that the effect of interspecific interaction via AMF on the AM benefit for shoot growth of *L. davurica* was mainly driven by the AM effects on soil AP activity and available P content. Specifically, AP can catalyze the hydrolysis of a phosphomonoester, generating a phosphate ion from the substrate (Zalatan et al., 2008), which is important for maintaining soil P bioavailability. In this study, interspecific interaction via AMF largely repressed AM benefit for AP activity in soil of *L. davurica*, leading to aggravated P limitation for plant growth of *L. davurica*.

The results of ^{15}N labeling showed that compared with monocultures, AMF in the mixed cultures largely increased N supply to roots of *B. ischaemum* at the expense of largely reduced N supply to *L. davurica* via AMF under the non-N addition condition. Numerous studies reported that a legume is generally a weak competitor relative to the C4 plant in the mixed culture system of C4 plant and legume (Hauggaard-Nielsen and Jensen, 2001; Wang et al., 2016), which is consistent with the present results. Some in vitro studies with root organ cultures revealed that those roots that are able to supply the most C to AMF garner the greatest amount of mineral nutrients from AMF, leading to the hypothesis that C and nutrient exchange between AMF and host plants involves "reciprocal rewards" (Lekberg et al., 2010; Kiers et al., 2011; Fellbaum et al., 2014). Compared with *L. davurica*, *B. ischaemum*, which is a C_4 grass, has an inherently higher photosynthetic capability, leading to a higher competitive ability to obtain N from AMF. Additionally, *L. davurica* as a legume species can form tripartite symbiotic relationships with rhizobium and AMF simultaneously. Nodules formed by interaction between rhizobium and roots of legume are able to fix atmospheric N in rhizosphere and increase N supply to host plant. Hence, *L. davurica* relies less on AMF for N uptake relative to *B. ischaemum*. Similar to our results, previous studies found that in a mixed culture of a legume and C4 plant, rhizobia improved the N fixation efficiency of the legume, and the interaction between the legume and C4 plant via AMF improved N uptake via hyphae by the C4 plant (Meng et al., 2015; Wang et al., 2016).

Effects of N addition on the interspecific plant interaction between C_4 grass and C3 legume via AMF

The N addition largely increased plant biomass of both plant species in monoculture of mycorrhizal system. The results of SEMs showed that shoot biomass accumulation in mycorrhizal systems was driven by soil N availability and soil C mineralization. In this study, N addition largely improved soil N

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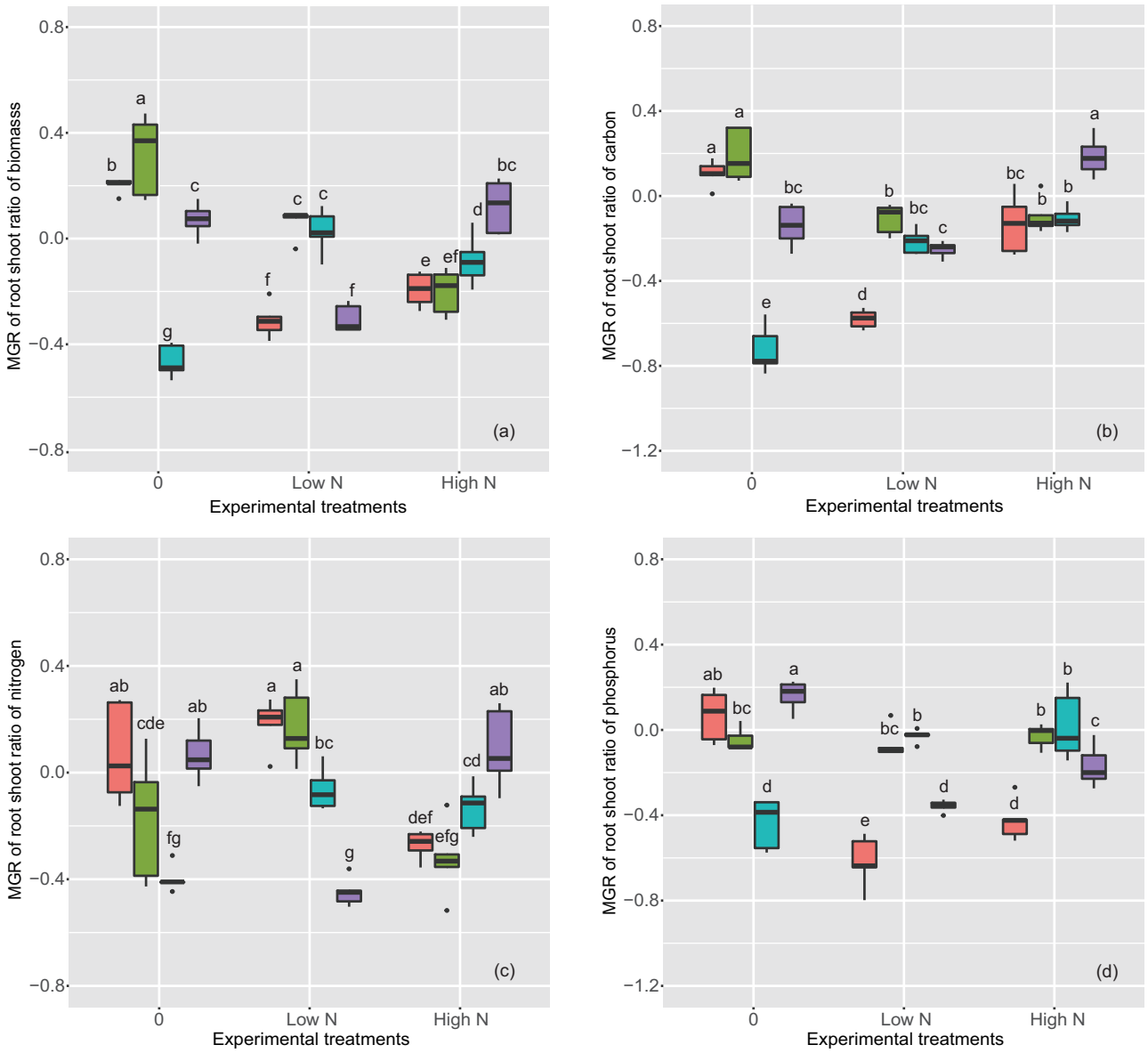

Figure 3 Effects of N addition and culture system on the MGR of root shoot ratio of biomass, C, N, and P contents. Note: In the box plot, the black boxes show the 25th and 75th quantiles, the whiskers are min–max values, and the horizontal line is the median. Statistical analysis was conducted using ANOVA followed by Tukey's HSD mean-separation test and indicated by lowercase letters above the boxes ($P < 0.05$). Culture systems in mycorrhizal and nonmycorrhizal systems are indicated as: orange box, *B. ischaemum* in monoculture; green box, *B. ischaemum* in mixed culture; blue box: *L. davurica* in monoculture; purple box: *L. davurica* in mixed culture. Black dots are the outliers (values outside the range of ±1.5*IQR, where IQR is the interquartile range defined as the upper quartile minus the lower quartile) within the treatment.

availability, which promoted microbial growth and metabolism by alleviating N limitation (Supplemental Figure S13). Furthermore, N-addition increased enzyme activities involved in C mineralization to meet the high C requirements of the soil microorganisms (Supplemental Figure S10), which is supported by previous research (Yao et al., 2009; Yang and Zhu, 2015; Ma et al., 2020).

After the addition of low N amounts, interspecific interactions via AMF stimulated the AM benefit for the shoot growth of *L. davurica*. Notably, interspecific interactions via AMF alleviated nutrient limitation for plant growth of *L. davurica* because mixed cultured largely decreased AM benefit for root shoot ratio of *L. davurica* in comparison to monoculture. Mixed cultures largely improved soil N availability in both plant species. The inoculation effect of rhizobium on legume N fixation is highly dependent on soil nutrient status (Wang et al., 2016). Specifically, extremely low and high soil N availability can both lead to inhibited legume N fixation. Previous studies reported that co-inoculation with rhizobium and AMF substantially promoted plant growth of soybean (*Glycine max*) under the low N addition treatment relative to the treatments without N addition and with high N addition (Wang et al., 2011, 2016). Moreover, N fixed in the soil by legume can be

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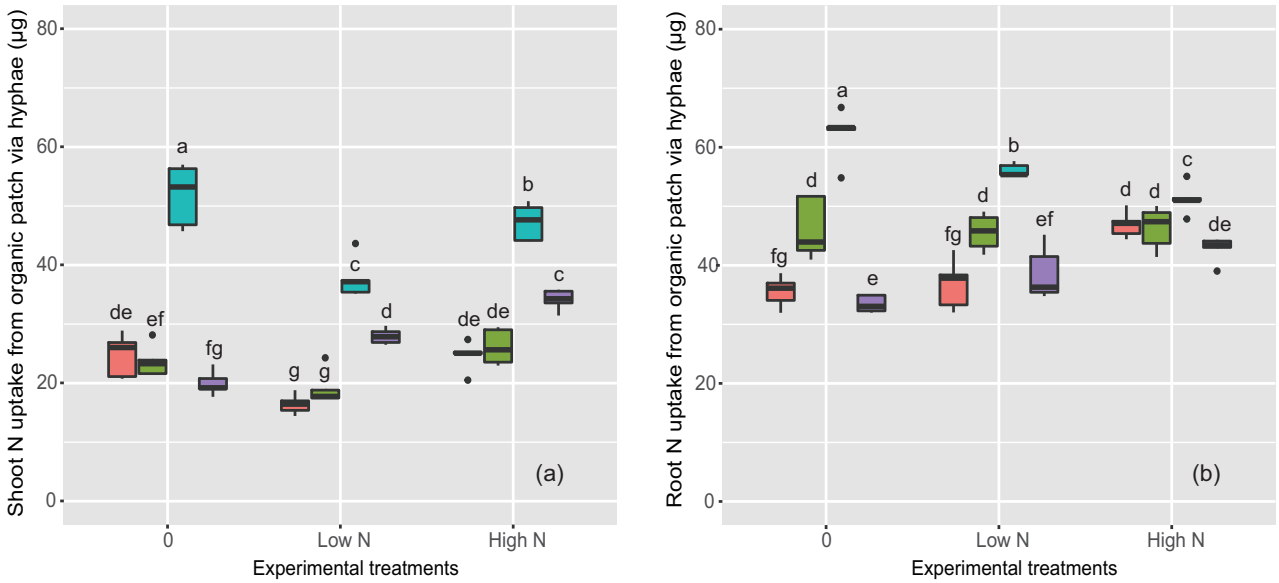

Figure 4 Effects of N addition and culture system on the shoot and root uptake of N from organic patches via AM fungal hyphae. In the box plot, the black boxes show the 25th and 75th quantiles, the whiskers are min–max values, and the horizontal line is the median. Statistical analysis was conducted using ANOVA followed by Tukey's HSD mean-separation test and indicated by lowercase letters above the boxes ($P < 0.05$). Culture systems in mycorrhizal and nonmycorrhizal systems are indicated as: orange box, *B. ischaemum* in monoculture; green box, *B. ischaemum* in mixed culture; blue box: *L. davurica* in monoculture; purple box: *L. davurica* in mixed culture. Black dots are the outliers (values outside the range of ±1.5*IQR, where IQR is the interquartile range defined as the upper quartile minus the lower quartile) within the treatment.

absorbed by neighboring nonlegumes via root or AM fungal hyphae, which can deplete N availability in the rhizosphere of legume and further stimulate the N fixation (Moyer-Henry et al., 2006; Sierra and Nygren, 2006; Meng et al., 2015).

In this study, mixed cultures largely improved shoot N content of *L. davurica* relative to monoculture, so that interspecific interactions via AMF alleviated N limitation for plant growth of *L. davurica*. Although AMF still preferentially supplied *B. ischaemum* with N at the cost of reduced N supply to mixed cultured *L. davurica*, the addition of low N amounts mitigated the sink strength of N in *B. ischaemum* from AMF, leading to the decreased negative effects of mixed culture on plant N uptake by *L. davurica* via AMF. Second, increased plant photosynthesis rate of *L. davurica* induced by increased N uptake promoted C input into soil, which stimulated C-limited saprotrophic microbes to produce enzymes involved in C and P mineralization, further increasing soil available P content by the priming effect on SOM decomposition (Kuzyakov et al., 2000; Blagodatskaya and Kuzyakov, 2008). Previous studies have suggested that AMF could increase soil P bioavailability by promoting soil acidification (Li et al., 2007; Qiao et al., 2015).

The addition of high N amounts largely stimulated the AM benefit for the plant growth of *B. ischaemum*. The addition of high N amounts significantly increased the shoot N/P and root N concentration in *B. ischaemum*, but significantly decreased shoot P concentration in *B. ischaemum*, indicating that soil N availability was sufficient for plant growth of *B. ischaemum*, but soil P availability became the limiting factor for plant growth. AMF tended to be more beneficial for host plant growth when plants were under P-limited and N-sufficient conditions because AMF is inherently more N-limited and fewer P-limited than their host plants (Johnson, 2010; Smith and Smith, 2011; Johnson et al., 2015). The N addition had a negligible effect on the AM benefit for the shoot growth of *L. davurica*. As a legume species, *L. davurica* requires much more N for plant growth than *B. ischaemum* which is a C4 grass species. The shoot biomass of *L. davurica* increased with N addition, confirming that the plant growth of *L. davurica* was limited by soil N availability under the treatment without N addition and low N treatment. The plant N/P of *L. davurica* remained constant with N addition, indicating that the plant growth of *L. davurica* was still limited by soil N availability under the high N treatment.

There were substantial interspecific differences between C_4 grasses and legumes in terms of plant nutrient uptake, where C_4 grasses had higher N and P use efficiency (grams of plant biomass produced per gram of N uptake) than legumes (Rao et al., 1995, 1997; Gubsch et al., 2011; Roscher et al., 2018). A previous study found that N addition substantially increased the N concentration of *B. ischaemum*, leading to a 75% increase in biomass (Xu et al., 2016), while N addition only increased the biomass of *L. davurica* by 10% and had a negligible effect on plant N and P concentrations (Xu et al., 2018), supporting our experimental results. Our results revealed that shoot N uptake of both plant species via AMF was highly linked to plant N accumulation. Previous studies have confirmed that AMF can obtain

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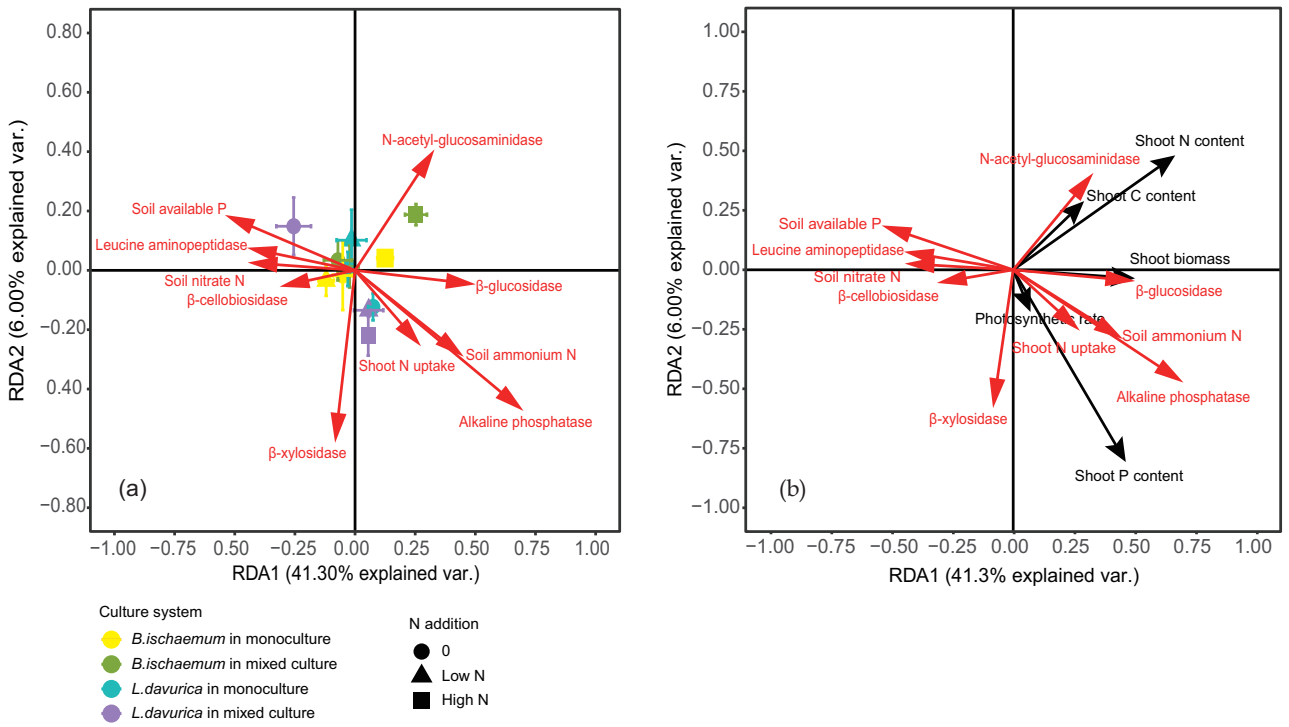

Figure 5 Relationships between MGR of shoot growth traits and MGR of soil biochemical properties or shoot N uptake via hyphae explored by RDA. The relationships between samples (centroids of the response variables) and explanatory variables (MGR of soil biochemical properties, shoot N uptake) are shown in (A), while the relationships between response variables (MGR of shoot growth traits) and explanatory variables (MGR of soil biochemical properties, shoot N uptake) are illustrated in (B). In (A), cross hairs on the circles, triangles, and squares represent standard deviation. Arrows indicate the lengths and angles between explanatory and response variables and reflect their corresponds. The black arrows and black fonts represent response variables, and the red arrows and red fonts reveal explanatory variables. Soil samples represented with different symbols and different colors correspond to different N addition treatments and different combinations of plant species in distinct culture systems, respectively. Circle, treatment without N addition; triangle, treatment with low N addition; square, treatment with high N addition. Yellow, *B. ischaemum* in monoculture; green, *B. ischaemum* in mixed culture; blue, *L. davurica* in monoculture; purple, *L. davurica* in mixed culture. Var. on the axis refers to variance.

substantial amounts of N from decomposing organic materials (Hodge and Fitter, 2010), and transfer them to their host plant (Leigh et al., 2009; Fellbaum et al., 2012). Additionally, there was no linear relationship between excess ^{13}C and ^{15}N in plant tissues, and no ^{13}C enrichment was detected in plant tissues, confirming that AMF acquire inorganic N from decomposed organic matter (Näsholm et al., 1998; Rains and Bledsoe, 2007; Hodge and Fitter, 2010).

After the addition of high N amounts, interspecific interactions via AMF inhibited the AM benefit for shoot growth of *B. ischaemum*. Notably, the interspecific interaction via AMF significantly decreased the AM benefit for shoot P content in *B. ischaemum* but largely increased that in *L. davurica*, indicating that P limitation for plant growth of *B. ischaemum* was aggravated by interspecific interaction via AMF. Specifically, compared with monocultures, the interaction between AMF and the soil microbial community in the mixed culture inhibited soil C-acquiring and P-acquiring enzyme activity in *B. ischaemum* (Supplemental Figure S12), leading to the decreased P availability in soil of *B. ischaemum*. Our study, to some extent, revealed that the interaction between AMF and the soil microbial community might play an important role in regulating soil nutrient cycling and bioavailability, further affecting interconnected plant growth performance.

The addition of high amounts of N largely mitigated the positive effects of the interspecific interaction via AMF on plant N uptake via AMF by *B. ischaemum* and negative effects of the interspecific interaction via AMF on that by *L, davurica*., compared to the treatment without N addition and low N treatment. Merrild et al. (2013) suggested that interspecific and size-asymmetric competition between plants may be amplified, rather than relaxed, by AMF that transfer P to large plants that provide the most C, rendering small plants P deficient. However, Walder et al. (2012) found that resource exchanges in AM symbiosis can be independent of any reciprocal regulation, and Walder and van der Heijden (2015) proposed taking competition for surplus resources and sink strength into consideration. In this study, legume N fixation via nodules can be inhibited by high N addition, which leads to plants in mixed culture more relying on AMF for acquiring N (Varin et al., 2009; Wang et al., 2011). Our results suggested that soil N availability can largely affect the sink strength of the host for AMF-derived N. In

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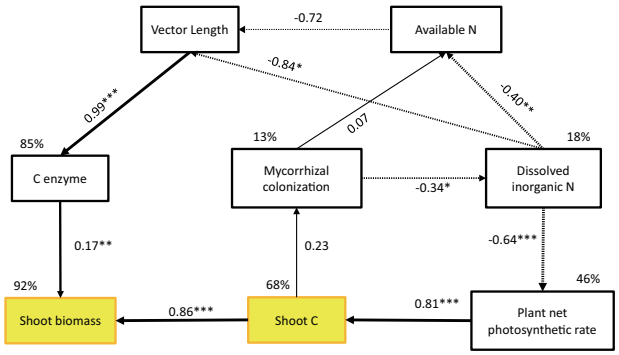

Figure 6 SEMs of the effects of soil biochemical properties and mycorrhizal colonization on the shoot growth performance (yellow boxes) of mycorrhizal plants. The model was satisfactorily fitted to data based on the χ^2 *P*-value, goodness of fit index (GFI), and the root means square error of approximation (RMSEA) analyses ($\chi^2 = 17.863$, df $= 12$, $P = 0.120$, GFI $= 0.959$, RMSEA $= 0.041$). Solid and dashed arrows represent the positive and negative effects on the fitted SEM, respectively. The arrow widths indicate the strength of the casual relationship (the standardized path coefficients on arrows). Variances explained by the model (R^2) are shown next to each endogenous variable. Numbers at arrows are standardized path coefficients (equivalent to correlation coefficients). Percentages close to endogenous variables indicate the variance explained by the model (R^2). Statistic significances are indicated: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

addition, Van Der Heijden et al. (2008) found that N addition largely increased grass biomass and reduced legume biomass, whereas AMF reduced this negative effect on plant communities induced by N enrichment. When soil is N sufficient and severely P deficient, the growth of C_4 grass could be sacrificed to maintain the growth of neighboring legumes through interaction via AMF, which might be an important mechanism for maintaining ecological diversity.

Conclusions

In this study, we investigated the interaction between soil N enrichment and AMF on plant growth performance of C4 grass and legume. Soil N enrichment largely improved the AM benefit for plant growth performance when P limitation for plant growth was induced. When plant growth was severely limited by soil N availability, N supply to plant species connected by AMF was an important mechanism for regulating the interspecific relationship between C_4 grasses and legumes. Specifically, interspecific interaction between C4 grasses and C3 legumes via AMF largely enhanced plant N uptake via AMF by C4 grasses but highly reduced that by C3 legumes. When the N limitation for plant growth was alleviated by N addition, the AM benefit for plant N uptake did not play a dominant role in mediating the interspecific relationship anymore. Instead, the interaction between AMF and soil microorganisms affecting the mineralization of soil organic C and P plays a vital role in regulating interspecific relationships; the growth of C_4 grass could be sacrificed to maintain the growth of neighboring legumes interconnected by AMF, which is an interesting ecological strategy for maintaining biological diversity.

Materials and methods

Growth substrate, fungal inoculum, and plant material

Soil, which was used to produce growth substrate, was collected from the upper 20-cm-deep layer of a natural grassland climax community in the Loess Plateau, China (36°51′30″N, 109°1923″E). The soil (pH 8.45), which was classified as a Calcic Cambisol (IUSS Working Group WRB, 2015) with a silty loam texture, contained 1.68 g kg^{-1} organic C, 0.22 g kg^{-1} total N, 0.55 g kg^{-1} total P, and 5.04 mg kg^{-1} available P. The growth substrate was produced by mixing autoclaved (121°, 1 h) soil and sand (purchased from Weihe sand company in Yangling, China, and thoroughly washed with tap water before autoclaving) in a 1:1 (w/w) ratio.

The method from Hodge et al. (1998) was adapted to produce organic patch material in this experiment. In brief, perennial ryegrass (*Lolium perenne*) was labeled with both ^{13}C and ^{15}N during the growth period. After that, the labeled shoot material, which contained 38.4% C (13.614‰ $\delta^{13}C$) and 1.26% N (24108‰ $\delta^{15}N$), was harvested, oven dried, and finely milled (<2 mm). The organic patch material was produced by mixing labeled shoot material with fresh soil (1:10, w/w). The detailed methods for producing organic patch material are presented in Supplemental Method S1.

The AM inoculum of *Funneliformis mosseae* (bank of Glomale in China, No. BGC BJ109) consisted of a sandy substrate containing spores (~40 spores/g), mycelium, and a colonized root fragment. The seeds of *B. ischaemum* (C_4 grass) and *L. davurica* (C_3 legume), which were surface-sterilized with 10% (v/v) hydrogen peroxide before germination, were collected from the natural grassland of the climax community on the Loess Plateau, China (36°51′30″N, 109°1923″E). The detailed method of seed germination is presented in Supplemental Method S1.

Experimental design

Microcosms, which were constructed out of plastic boxes and consisted of three compartments (Figure 7; were used for planting C4 grasses and legumes. The root compartment (RC) was divided by a 25-μm nylon mesh into two identical compartments (length × width × height = 10 × 8 × 13 cm). Each compartment in the RCs was filled with 1.4 kg of autoclaved growth substrate. A 25-μm nylon mesh bag filled with 11 g of organic patch material was placed in the hyphal compartment (HC; length × width × height = 20 × 2 × 13 cm). This microcosm experiment was conducted under a large transparent plastic shed to provide natural conditions for plant growth at the Institute of Soil and Water Conservation, Chinese Academy of Sciences, and Ministry of Water Research, Yangling, China. The experimental site has a temperate continental monsoon climate, with a mean annual temperature of 13.3°C, a mean annual precipitation of 674.3 mm, and a mean annual sunshine duration of 1,993.7 h. Three plant culture systems (two monocultures [the same plant species in both compartments of RCs: *B. ischaemum* or *L. davurica*] and a mixed

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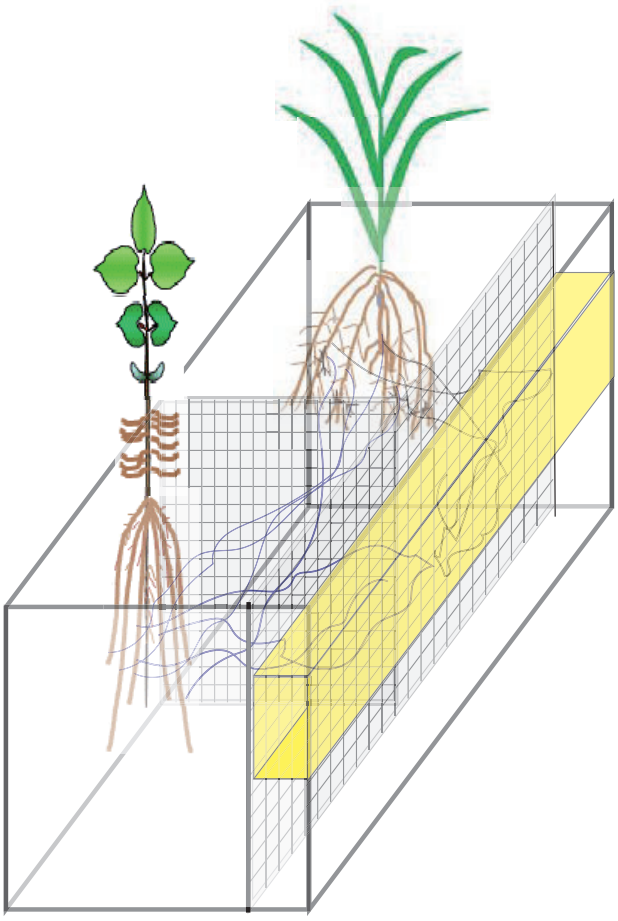

Figure 7 Schematic diagram of the compartmentalized microcosm design. The microcosm consisted of two identical compartments in the RC (length × width × height = 20 × 8 × 13 cm), and HC (length × width × height = 20 × 2 × 13 cm). The compartments in the microcosm were separated by 25-μm nylon mesh which is permeable for fungal hyphae but can prevent roots from penetrating. Five plants of *B. ischaemum* (B) or *L. davurica* (L) were planted in the two compartments of RC (the same plant species grown in the two compartments of RC as monoculture and each plant species grown in each compartment of RC as mixed culture). The 25-μm nylon mesh bag containing dual ^{13}C:^{15}N labeled organic patch material (yellow box) was placed in HC.

culture: *B. ischaemum* in one compartment of RCs and *L. davurica* in the other compartment); two AM fungal inoculation levels, including one with AM fungal inoculation and the other one without AM fungal inoculation; and three N addition levels (0 [0 mg N kg^{-1}], low N [25 mg N kg^{-1}], and high N [50 mg N kg^{-1}]) were set up in this microcosm experiment. Finally, 90 ($3 \times 2 \times 3 \times 5$) experimental microcosms were established in total with five replicates of samples in each treatment.

The germinated seeds were transplanted into the corresponding compartment in RCs on April 15, 2017. Ten grams of the AM fungal inoculum (*F. mosseae*) or 10 g of sterilized (121°C, 30 min) inoculum as a nonmycorrhizal control was added to each compartment on the same day as transplantation. To equalize the starting microbial communities, each RC compartment received a 25-mL filtered AMF inoculum wash without AMF propagules (see detailed information in Supplemental Method S2; Koide and Li, 1989; Hodge et al., 2001). One week after transplantation, each RC compartment received 40 mL of modified 1/2 N and 1/2 P Hoagland's nutrient solution (see recipe in Supplemental Method S2) once a week for 4 weeks, and the microcosms were watered with deionized water daily. Four weeks after transplantation, a 25-μm nylon mesh bag containing organic patch material was placed in the HC. After seedling emergence aboveground, the seedlings in each compartment were thinned to five healthy plants. The NH_4NO_3 solution was applied to each RC compartment at a rate of 0 mg N kg^{-1}, 25 mg N kg^{-1}, and 50 mg N kg^{-1} once a week for 8 weeks from June 15, 2017 (plant growth for 60 d).

Photosynthetic characteristic measurements and sample collection

The Pn, intercellular CO_2 concentration, transpiration rate, and stomatal conductance were measured from five selected leaves in each RC with an open gas-exchange system (LI-6400XT, Li-Cor Inc., Lincoln, NE, USA) between 08:30 and 11:30 in the morning from late August (plant growth for 125 d). The parameter settings during photosynthetic characteristic measurements are shown in Supplemental Method S3.

The samples were harvested in early September 2017 (plant growth for 140 d). The plant samples were separated into shoots and roots; the shoots were cut at the soil surface and the roots were washed thoroughly with tap water; the fresh weight of the latter was determined. Each root sample was divided into two subsamples: one of the subsamples was weighed, and the other was stored in a formalin–acetic acid–alcohol (37% formaldehyde: glacial acetic acid: 95% ethanol, 9:0.5:0.5, v:v:v) solution to determine the mycorrhizal colonization. The harvested plant shoot samples and weighed root subsamples were immediately placed in an oven at 105°C for 30 min and dried at 80°C to achieve a constant weight. The shoot and root dry weights were determined, and the dry weights of the whole root system were calculated based on the fresh weight ratio of the whole root system to the weighed root subsample. Plant biomass was represented as the sum of all five plants in each RC compartment. After that, these samples were ground in a ball mill to a fine powder for further measurement of the chemical characteristics. The top 2 cm of the soil in each RC compartment was discarded, and the remaining soil was homogenized and sieved through 0.25- and 1-mm sieves for the analysis of soil physiochemical properties.

Biochemical analysis

The fixed root samples were cleared with 10% KOH (w/v) at 90°C for 1 h and acidified with HCl for 5 min. After that, the samples were stained with 0.05% trypan blue (w/v) at 90°C for 30 min and decolorized in decoloring solution (lactic acid:glycerol = 1:1, v:v; Phillips and Hayman, 1970). Magnified cross-sections were used to measure mycorrhizal

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colonization (McGonigle et al., 1990). In each root sample, 20 cm of the root and 100 intersections between grid lines and roots were used to assess a sample.

The C concentration of the plant samples was determined using the H_2SO_4–$K_2Cr_2O_7$ oxidation method. The N concentration of the plant samples was determined by the Kjeldahl method (Bremner and Mulvaney, 1982), and the P concentration of the plant samples was measured by persulfate oxidation followed by colorimetric analysis (Schade et al., 2003). The ^{13}C and ^{15}N atom percentage of the plant samples were determined using an elemental analyzer with a continuous flow isotope ratio mass spectrometer (EA-ConFlo-IRMS, Flash 2000 HT and MAT 253; Thermo Fisher Scientific Inc., Waltham, MA, USA). The description of methods for the determination of soil biochemical characteristics is presented in Supplemental Method S4.

Statistical analysis

Plant C and N uptake from ^{13}C and ^{15}N dual-labeled organic patches were calculated by the isotope method as follows:

$$\text{Plant C and N uptake via CMNs} = T \times ((Ai - Au) / Ap)$$

where T is the total C or N content of plant samples in AMF-inoculated treatments, Ai is the atom percentage excess ^{13}C or ^{15}N of plant samples in inoculated treatments, Au is the atom percentage excess ^{13}C or ^{15}N of plant samples in corresponding uninoculated treatments, and Ap is atom percentage excess ^{13}C or ^{15}N in organic patch material.

^{13}C:^{15}N dual-labeled organic substrates can be used to test if the host plant acquires the N via AM fungal hyphae as intact organic forms (Näsholm et al., 1998; Nordin et al., 2001). Forms of organic N can be taken up intact when there is a significant linear relationship between excess ^{13}C and ^{15}N in the plant tissue. The slope of the regression can then be compared to the ^{13}C:^{15}N ratio of the substrate tracer, thus allowing a conservative estimate of N assimilated as an intact amino acid (Näsholm et al., 1998; Rains and Bledsoe, 2007).

The MGR of plant growth traits were calculated as described in Johnson (2010):

$$MGR = \log(e)(AM/NM)$$

where AM is the plant growth trait in the AM fungal inoculum treatment and NM is the plant growth trait in the corresponding nonmycorrhizal control treatment. The plant growth traits included shoot and root biomass, C content, N content, P content, and plant Pn.

Kolmogorov–Smirnov and Levene's tests were applied to test for normality and homogeneity of variances. For statistical analysis, data were checked and transformed appropriately to normalize the skewed distribution before analysis. Multivariate analysis of variance (MANOVA) was applied to provide an overview on the effect of the culture system, N addition, mycorrhizal inoculation, and the interactions among these factors on shoot and root growth performance. Analysis of variance (ANOVA) was applied to obtain detailed information about the effect of culture system, N addition, mycorrhizal inoculation, and the interaction among these factors on individual shoot growth traits (shoot biomass, C, N, and P content, and N/P, shoot N uptake via hyphae, plant Pn) and root growth traits (root biomass, C, N, and P content, and N/P, root N uptake via hyphae, mycorrhizal colonization; $P < 0.05$). To simplify presented results, ANOVA was applied to investigate the effect of culture system and N addition on MGR of plant growth traits. Separate linear regressions were conducted to estimate the effects of N uptake from organic patches via AMF on shoot and root N content, respectively. RDA enables us to explore the relationships between the effect of AMF on plant growth performance, the benefit of AMF for plant N uptake, and the effect of AMF on soil biochemical properties, and to further determine the contributions of the AM effects on plant N uptake and soil biochemical properties to that on plant growth performance. RDA was conducted using the "vegan" package in the R version 3.6.1 software (R Core Team, 2019). We started the SEM procedure with the specification of a conceptual model of hypothetical relationships which was based on a priori and theoretical knowledge (Supplemental Figure S12). We assumed that soil bioavailable N concentration not only directly affected shoot biomass, but also indirectly influenced shoot C content and biomass through altering soil, soil C enzyme activities, and plant Pn. Furthermore, shoot C availability (shoot C content and plant Pn) can affect mycorrhizal colonization. In the SEM analysis, the model-implied variance–covariance matrix was compared against the observed variance–covariance matrix, and data were fitted to the models using the maximum likelihood estimation method. Adequate model fits are indicated by a non-significant v2 test ($P > 0.05$), low Akaike Information Criteria, and low root square mean errors of approximation (<0.05; Grace, 2006). The model was established and run using IBM SPSS AMOS version 21.0 (SPSS Inc., Chicago, IL, USA). The boxplots, RDA figures, and linear regression figures were generated using "ggplot2" package in R software.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of N addition and culture system on the plant Pn in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S2. Effects of N addition and culture system on the shoot biomass, and C contents in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S3. Effects of N addition and culture system on the shoot N and P contents, and N/P in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S4. Effects of N addition and culture system on the root shoot ratio of biomass, and C contents in mycorrhizal and nonmycorrhizal systems.

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Supplemental Figure S5. Effects of N addition and culture system on the root shoot ratio of C and N contents in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S6. Effects of N addition and culture system on the plant uptake of C from organic patches via AM fungal hyphae.

Supplemental Figure S7. Linear correlation between the shoot N uptake from organic patch via hyphae and the shoot N content, and linear correlation between the root N uptake from organic patch via hyphae and the root N content.

Supplemental Figure S8. Effects of N addition and culture system on the soil ammonium N and nitrate N concentrations in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S9. Effects of N addition and culture system on the soil available P concentrations and soil available N/P in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S10. Effects of N addition and culture system on the activities of soil enzymes in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S11. Effects of N addition and culture system on the vector length and vector angle in mycorrhizal and nonmycorrhizal systems.

Supplemental Figure S12. Illustration of all plausible interaction pathways in the studied plant–soil–mycorrhizal fungi system.

Supplemental Table S1. Summary of the significance of the variation in the shoot growth traits explained by global model, all axes, and individual explanatory variables via Monte Carlo permutation test.

Supplemental Table S2. Results of SEMs of the effects of soil biochemical properties and mycorrhizal colonization on the shoot growth performance of mycorrhizal plants as illustrated in Figure 3.

Supplemental Table S3. Ecoenzymes included in this study.

Supplemental Methods S1. Organic patch material and plant seeds.

Supplemental Methods S2. Microbial filtrate and Hoagland's nutrient solution.

Supplemental Methods S3. Parameters settings of photosynthesis characteristic measurements.

Supplemental Methods S4. Laboratory analysis.

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Conflict of interest statement. The authors declare no conflict of interest.

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