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Mechanistic understanding of interspecific interaction between a C4 grass and a C3 legume via arbuscular mycorrhizal fungi, as influenced by soil phosphorus availability using a ^{13}C and ^{15}N dual-labelled organic patch

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SUMMARY

Arbuscular mycorrhizal fungi (AMF) can improve plant nutrient acquisition, either by directly supplying nutrients to plants or by promoting soil organic matter mineralization, thereby affecting interspecific plant relationships in natural communities. We examined the mechanism by which the addition of P affects interspecific interactions between a C4 grass (*Bothriochloa ischaemum*, a dominant species in natural grasslands) and a C3 legume (*Lespedeza davurica*, a subordinate species in natural grasslands) via AMF and plant growth, by continuous ^{13}C and ^{15}N labelling, combined with soil enzyme analyses. The results of ^{15}N labelling revealed that P addition affected the shoot uptake of N via AMF by *B. ischaemum* and *L. davurica* differently. Specifically, the addition of P significantly increased the shoot uptake of N via AMF by *B. ischaemum* but significantly decreased that by *L. davurica*. Interspecific plant interactions via AMF significantly facilitated the plant N uptake via AMF by *B. ischaemum* but significantly inhibited that by *L. davurica* under P-limited soil conditions, whereas the opposite effect was observed in the case of excess P. This was consistent with the impact of interspecific plant interaction via AMF on arbuscular mycorrhizal (AM) benefit for plant growth. Our data indicate that the capability of plant N uptake via AMF is an important mechanism that influences interspecific relationships between C4 grasses and C3 legumes. Moreover, the effect of AMF on the activities of the soil enzymes responsible for N and P mineralization substantially contributed to the consequence of interspecific plant interaction via AMF for plant growth.

Keywords: arbuscular mycorrhizal fungi (AMF), interspecific plant interaction, mycorrhizal growth response, ^{15}N, P addition, plant nitrogen acquisition via AMF.

INTRODUCTION

Soil P availability is a common limiting factor for plant growth and productivity, as P plays a vital role in multiple plant processes, including physiological processes such as ATP synthesis and those occurring in the phospholipid bilayer (Abelson, 1999; Elser et al., 2007; Vitousek et al., 2010). Improved P availability is the most recognized benefit of arbuscular mycorrhizal fungi (AMF), which form arbuscular mycorrhizal (AM) symbiotic associations with approximately 74% of terrestrial plant species (Brundrett, 2009; Smith and Read, 2008; Wipf et al., 2019). By extending beyond the mineral nutrient depletion zones that surround the roots, AMF can provide host plants with as much as 80 and 25% of their P and N requirements, respectively (Smith and Smith, 2011). Mutualism between AMF and host plants commonly occurs in P-limited systems because AMF can effectively trade surplus P for plant photosynthates; however, when N supply is limited, AMF are unlikely to provide N to their host plants until their own needs have been met (Hodge and Storer, 2015; Johnson et al., 2015). Johnson et al. (2015) proposed that the relative availability of soil N and P determines whether the mycorrhizal benefits outweigh their costs, as previous studies have shown that the addition of P either has a negative influence on AM root colonization

or promotes the formation of arbuscules (Nouri et al., 2014; Smith and Smith, 1997).

Arbuscular mycorrhizal fungi (AMF) can determine the outcome of interspecific plant interactions and mediate plant community structure (Scheublin et al., 2007; Van Der Heijden et al., 2003). Plant traits and soil nutrient availability are the main factors that determine the effects of AMF on plant community structure and species composition (Lin et al., 2015). Plant species that belong to different plant functional groups respond differently to mycorrhizal inoculation. Compared with C3 grasses, which have fibrous and highly branched root systems with high nutrient uptake rates, C4 grasses generally have thicker root systems and rely more on AMF for the acquisition of soil nutrients (Wilson and Hartnett, 1998). Thus, mycorrhizal inoculation is expected to enhance the competitive capability of C4 grasses more than that of C3 grasses (Hartnett et al., 1993; Hetrick et al., 1994). N-fixing legume species generally have high P requirements for N fixation, leading to a high reliance on AMF for acquiring soil P. Therefore, mycorrhizal colonization promotes the competitive ability of N-fixing legume species (Scheublin et al., 2007; Wagg et al., 2011). Some studies have revealed that compared with legumes, C4 plants have a higher competitive ability via AMF, which is partly ascribed to their higher nutrient uptake via AMF (Meng et al., 2015; Wang et al., 2016). When dominant species in natural plant communities are highly dependent on AMF, AMF can reduce species diversity by increasing interspecific competition and decreasing intraspecific competition (Gross et al., 2010; Hartnett and Wilson, 2002). Walder and van der Heijden (2015) suggested that sink strength and competition for surplus resources, which are highly affected by soil nutrient conditions, are important factors that influence resource exchange between plants and common AMF symbionts. Notably, N is the key nutrient that limits net primary productivity in high-latitude and semi-arid ecosystems of the northern hemisphere (Harpole et al., 2011; LeBauer and Treseder, 2008). Previous studies have revealed that AMF can acquire substantial quantities of N from decomposing organic matter (Hodge et al., 2001; Hodge and Fitter, 2010), and can transfer this N to host plants (Fellbaum et al., 2012; Leigh et al., 2009). Increased soil P availability can decrease the positive effects of AMF on plant diversity and productivity (Collins and Foster, 2009). Specifically, the addition of P to nutrient-deficient soil can weaken the competitive ability of grasses relative to that of legumes for soil nutrient resources (Mendoza et al., 2016). However, the extent to which soil P availability influences interspecific plant interaction and plant N uptake via AMF is unclear. In particular, it is not known whether there is a direct relationship between N supply from AMF to associated plants and interspecific plant relationships, mediated by AMF, under various conditions of P availability.

Previous studies have shown that mixed cultures of dominant (*Bothriochloa ischaemum*) and subordinate species (*Lespedeza davurica*) in climax communities in natural grassland on the Loess Plateau, China, have high plant growth and resource-use efficiency, especially for N and P capture and absorption by *B. ischaemum* (Xu et al., 2016; Xu et al., 2018). Severe soil erosion in this region, owing to the destruction of native vegetation, has resulted in considerable losses of fertile topsoil, leading to the loss of soil C and nutrients (Fu et al., 2010; Zhang et al., 2012). Natural vegetation restoration of grasslands on the Loess Plateau is limited by the availability of N and P in the soil (Chen et al., 2020; Cui et al., 2018; Deng et al., 2019; Xiao et al., 2020). In the present study, a C4 grass (*B. ischaemum*) and a C3 legume (*L. davurica*) were selected to investigate the effect of P addition on N acquisition in associated plant species via AMF. The mechanism by which P addition affects the consequence of interspecific plant interaction via AMF for plant growth was also studied. We hypothesized that: (i) the addition of P would decrease the benefits of AM for the growth of the plants studied, partly attributed to the capability of plant N uptake via AMF; (ii) the interspecific interaction between the C4 grass and the C3 legume via AMF would have a positive effect on plant growth, and P addition would enhance the positive effects on the C3 legumes; and (iii) the interspecific interaction between the C4 grass and the C3 legume via AMF would promote N uptake in *B. ischaemum*, and P addition would mitigate the negative effect on N uptake in *L. davurica*.

RESULTS

Shoot biomass

Shoot growth was significantly affected by the culture system, P addition, mycorrhizal inoculation and the interaction between these factors (Table 1). Compared with non-mycorrhizal systems, AMF significantly increased the shoot biomass (Figure 1a and b; Table 2). In the mycorrhizal system, the shoot biomass in monocultures increased significantly with increasing P addition. Compared with the monoculture system, the mixed culture indicated a significant increase in the shoot biomass of *B. ischaemum* under the P30 treatment and that in *L. davurica* under the P30 and P100 treatments, but a significant decrease in the shoot biomass of *L. davurica* under the P0 treatment (Figure 1a).

In monocultures of *B. ischaemum*, the shoot mycorrhizal growth response (MGR) under the P0 and P30 treatments was significantly higher than that under the P100 treatment, whereas the corresponding values decreased significantly with the addition of P in the *L. davurica* monoculture (Figure 1c). Compared with the monoculture system, the mixed culture significantly increased the shoot MGR in *B. ischaemum* under the P30 treatment and that in *L. davurica* under the P100 treatment, with a significant

Table 1 Multivariate analysis of variance

	Shoot growth		
Factors	Pillai's trace	*F* value	d.f.
Culture system	0.286	6.861***	6
P addition	1.209	26.479***	12
Mycorrhizal inoculation	0.974	653.720***	6
C × P	0.848	12.772***	12
C × M	0.212	4.620***	6
P × M	1.216	26.890***	12
C × P × M	0.868	13.287***	12

Culture system: C, monoculture or mixed culture; P, addition of P (P0, P30 or P100); M, mycorrhizal inoculation, mycorrhizal system or non-mycorrhizal system. Shoot growth comprised of shoot biomass, shoot C, N and P concentration, and plant P_n.
Significant main effects and interactions are indicated: NS, not statistically significant;
*$P \leq 0.05$;
**$P \leq 0.01$;
***$P \leq 0.00$.

decrease in shoot MGR for *L. davurica* under the P0 treatment (Figure 1c).

Plant N uptake via AMF and mycorrhizal colonization

The plant uptake of C from organic patches via hyphae was much lower than that of N (Figures S2 and S8). Therefore, it is clear that the AMF, *Funneliformis mosseae*, required N as a decomposition product and could not directly transfer organic C from the organic patch to the host plant. Notably, the addition of P significantly increased the shoot N uptake by *B. ischaemum* via AMF, but significantly decreased that by *L. davurica* in their respective monocultures (Figure 1d). Compared with the monocultures, in the mixed culture the shoot N uptake was significantly increased, by 29.8%, in *B. ischaemum* via AMF under the P30 treatment. Although shoot N uptake was significantly decreased in *L. davurica* via AMF under the P0 and P30 treatments, it was significantly increased, by 16.9%, under the P100 treatment. Additionally, the mycorrhizal colonization of *B. ischaemum* in the mixed culture and that of *L. davurica* in both the monoculture and the mixed culture significantly decreased upon the addition of P (Figure S3; Table 3). Compared with the monoculture systems, the mixed culture resulted in a 10.8% increase in the mycorrhizal colonization of *L. davurica* under the P0 treatment and a 12.5% decrease in that of *B. ischaemum* under the P100 treatment.

Shoot N and P

Mycorrhizal inoculation significantly influenced shoot N concentration, and the shoot P concentration was significantly affected by the culture system, P addition and mycorrhizal inoculation (Figure S4; Table 2). In monocultures, the P100 treatment significantly increased the mycorrhizal response (MR) of shoot N concentration and content in *B. ischaemum*, relative to those in the P0 treatment, whereas P addition had a negligible effect on these variables for *L. davurica* (Figure 2a and b). Compared with the monoculture system, the mixed culture system significantly increased the MR of shoot N concentration in *B. ischaemum* and *L. davurica* under the P0 and P30 treatments, respectively (Figure 2a). Moreover, it significantly decreased the MR of shoot N content in *B. ischaemum* and *L. davurica* under the P100 and P0 treatment, respectively (Figure 2b).

In monocultures, the P30 treatment significantly decreased the MR of shoot P concentration and content in *L. davurica*, and the P100 treatment significantly increased these variables relative to those in the P0 treatment (Figure 3a and b). Furthermore, the P30 treatment significantly decreased the MR of shoot P concentration in *B. ischaemum* relative to that in the P0 treatment (Figure 3a and b). Compared with the monoculture system, the mixed culture system significantly increased the MR of shoot P concentration and content of *B. ischaemum* and *L. davurica* under the P30 treatment, but significantly decreased those in *B. ischaemum* under the P100 treatment and those in *L. davurica* under the P0 and P100 treatments (Figure 3a and b).

Plant C and photosynthesis

Shoot C concentration was significantly affected by P addition, mycorrhizal inoculation and the interaction between these factors (Figure S5; Table 2). Compared with non-mycorrhizal systems, P addition significantly decreased the MR of shoot C concentration in both plant species (Figure 4a). Compared with the monoculture system, the mixed culture system significantly decreased the MR to shoot C concentration for both plant species under the P0 treatment. Compared with non-mycorrhizal systems, AMF significantly enhanced the net photosynthetic rate (P_n) (Figure S5; Table 2). In monocultures, P addition significantly decreased the MR of P_n for *B. ischaemum*, whereas that for *L. davurica* increased significantly with P addition (Figure 4b). Compared with the monoculture system, the mixed culture system significantly decreased the MR of P_n in both plant species under the P30 treatment, but significantly decreased that under the P100 treatment.

Effect of soil biochemical factors on plant growth traits

Redundancy analysis (RDA) showed that the differences in the mycorrhizal effect on shoot growth traits in *B. ischaemum* under the P30 treatment and those in *L. davurica* under the P0 and P100 treatments between monoculture and mixed culture systems were mainly influenced by the MR of soil available P concentration and alkaline phosphatase (Figure 5a, Table S1). Moreover, the mycorrhizal effects on shoot C, N and P concentration were mainly driven by the MR of alanine aminopeptidase,

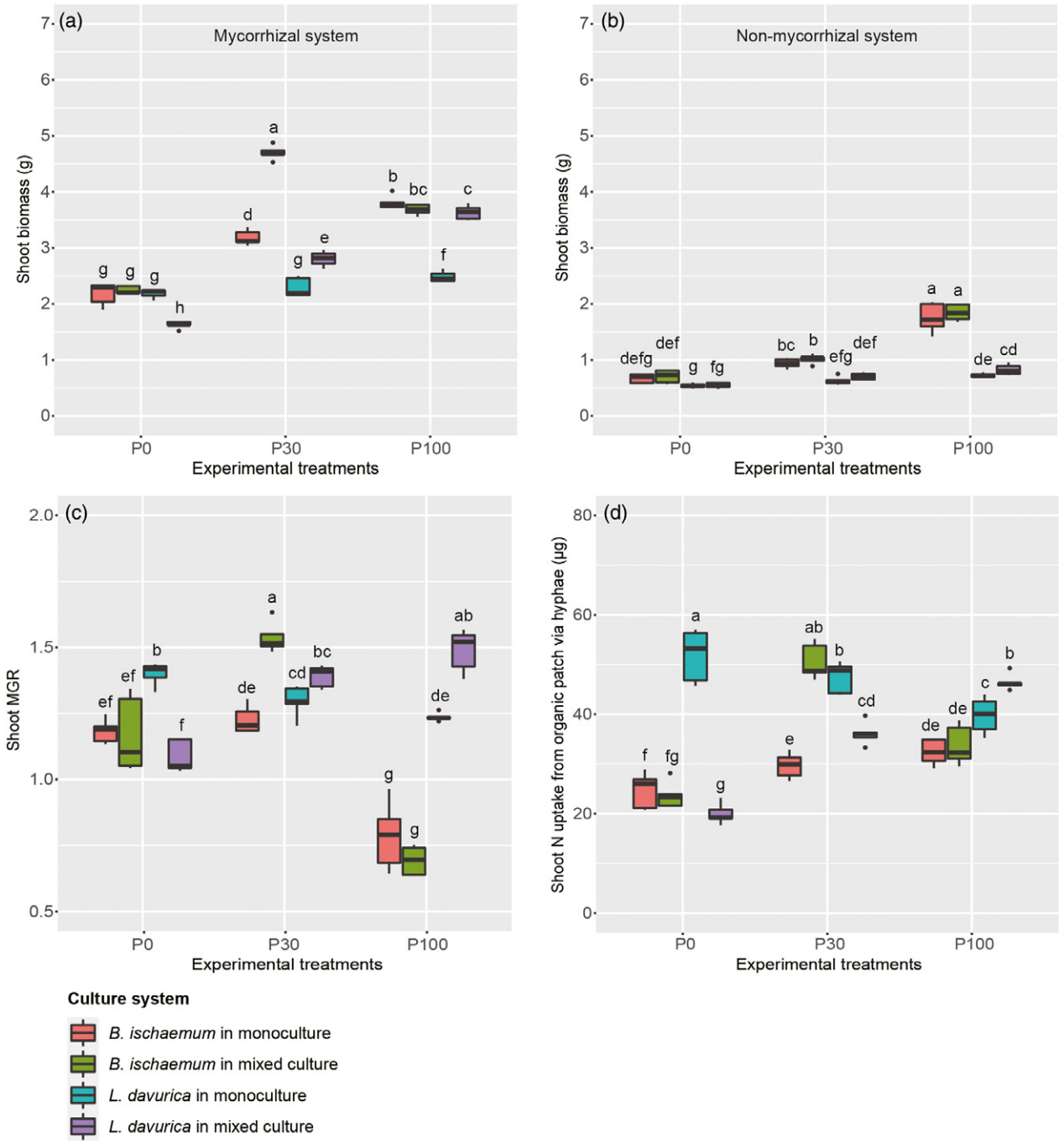

Figure 1. Effects of P addition and culture system on: (a) shoot biomass in the mycorrhizal system; (b) shoot biomass in the non-mycorrhizal system; (c) shoot mycorrhizal growth response (MGR); and (d) shoot N uptake from the organic patch via hyphae. In the box plot, the black boxes indicate the 25th and 75th quantiles, the whiskers indicate the minimum–maximum values and the horizontal line is the median value. Different lowercase letters above the boxes indicate significant differences between the treatments ($P < 0.05$). Culture systems in mycorrhizal and non-mycorrhizal systems are indicated as follows: orange, *Bothriochloa ischaemum* in monoculture; green, *B. ischaemum* in mixed culture; blue, *Lespedeza davurica* in monoculture; and purple, *L. davurica* in mixed culture.

β-cellobiosidase, nitrate N and β-xylosidase, whereas the mycorrhizal effect on shoot biomass and P_n was mainly affected by the MR of soil available P concentration and alkaline phosphatase (Figure 5b).

Structural equation modelling (SEM) explained 98.0% of the variance in the shoot growth of mycorrhizal plants (Figure 6, Table S2). The shoot biomass was directly affected by shoot C accumulation, P_n and soil available N:

Table 2 Analysis of variance on shoot growth performance

	Shoot biomass		Shoot C concentration		Shoot N concentration		Shoot P concentration		Net photosynthetic rate	
Factors	*F* value	d.f.	*F* value	d.f.	*F* value	d.f.	*F* value	d.f.	*F* value	d.f.
Culture system	8.350**	1	0.894NS	1	0.038NS	1	4.018*	1	4.705*	1
P addition	48.904***	2	142.578***	2	0.108NS	2	10.778***	2	22.323***	2
Mycorrhizal inoculation	549.569***	1	324.32***	1	4.08*-	1	27.034***	1	193.757***	1
C × P	5.105**	2	0.317NS	2	0.006NS	2	2.516NS	2	9.09***	2
C × M	4.494*	1	0.774NS	1	0.009NS	1	2.337NS	1	1.915NS	1
P × M	11.618***	2	210.555***	2	0.022NS	2	2.213NS	2	6.069**	2
C × P × M	4.252*	2	1.271NS	2	0.008NS	2	2.616NS	2	10.346***	2

Culture system: C, monoculture or mixed culture; P, addition of P (P0, P30 or P100); M, mycorrhizal inoculation, mycorrhizal system or non-mycorrhizal system. *F* values and degrees of freedom (d.f.) from a three-way (shoot biomass, shoot C, N and P concentration, plant net photosynthetic rate) ANOVA are represented. Significant main effects and interactions are indicated: NS, not statistically significant;
*$P \leq 0.05$;
**$P \leq 0.01$;
***$P \leq 0.001$.

P, and was indirectly affected by soil available P concentration and soil N-acquiring enzyme activity. The shoot N uptake from organic patches via hyphae was directly affected by soil N-acquiring enzyme activity.

DISCUSSION

Effects of P addition on the benefits of AM for plant growth in monoculture

The soil used in this study was collected from a natural grassland on the Loess Plateau of China, where natural vegetation restoration is limited by soil P availability (Chen et al., 2020; Cui et al., 2018; Deng et al., 2019; Xiao et al., 2020). Smith and Smith (2011) proposed that improved P nutrition is the most important benefit of AM symbiosis for host plants. Correspondingly, our study revealed that AMF increased the P content in both the C4 grass (*B. ischaemum*) and the C3 legume (*L. davurica*), thereby enhancing their growth. Specifically, we found that the addition of high quantities of P greatly decreased the benefit of AM for shoot growth of *B. ischaemum* and *L. davurica*. Johnson et al. (2015) reported that the relative availabilities of N and P determine the mycorrhizal benefit for host plant growth. Compared with the N requirements of their host plants, AMF have higher N requirements for their own growth, which is why they are unlikely to supply host plants with biologically relevant quantities of N under N-limited conditions (Hodge and Fitter, 2010; Johnson, 2010). However, AMF can efficiently provide surplus P for host plants under P-limited conditions (Johnson et al., 2015; Koide, 1991). Therefore, Johnson et al. (2015) have suggested that the mutualism between AMF and host plants is likely to occur in P-limited systems, whereas commensalism or parasitism is more probable in N-limited systems. In the present study, the addition of high quantities of P to the mycorrhizal system largely decreased the shoot N/P of *B. ischaemum* and *L. davurica* in monocultures, indicating the alleviation of P limitation.

The results of ^{15}N labelling revealed that the addition of P significantly increased the shoot N uptake in *B. ischaemum* via AMF under a monoculture. Previous studies have revealed that AMF can acquire substantial quantities of N from decomposing organic material (Hodge et al., 2001; Hodge and Fitter, 2010) and can transfer it to their host plants (Fellbaum et al., 2012; Leigh et al., 2009). In the present study, the addition of P largely increased soil P bioavailability (Figure S10), which further alleviated soil microbial metabolic P limitation (Figure S9). Specifically,

Table 3 Analysis of variance on shoot N uptake from the organic patch via hyphae and mycorrhizal colonization

	Shoot N uptake from organic patch via hyphae		Mycorrhizal colonization	
Factors	*F* value	d.f.	*F* value	d.f.
Culture system	1.223NS	1	0.936NS	1
P addition	8.186***	2	38.663***	2
C × P	9.300***	2	5.422**	2

Culture system: C, monoculture or mixed culture; P, addition of P (P0, P30 or P100). *F* value and degrees of freedom (d.f.) from a two-way (shoot N uptake from organic patch via hyphae and mycorrhizal colonization) ANOVA are represented. Significant main effects and interactions are indicated: NS, not statistically significant;
*$P \leq 0.05$;
**$P \leq 0.01$;
***$P \leq 0.001$.

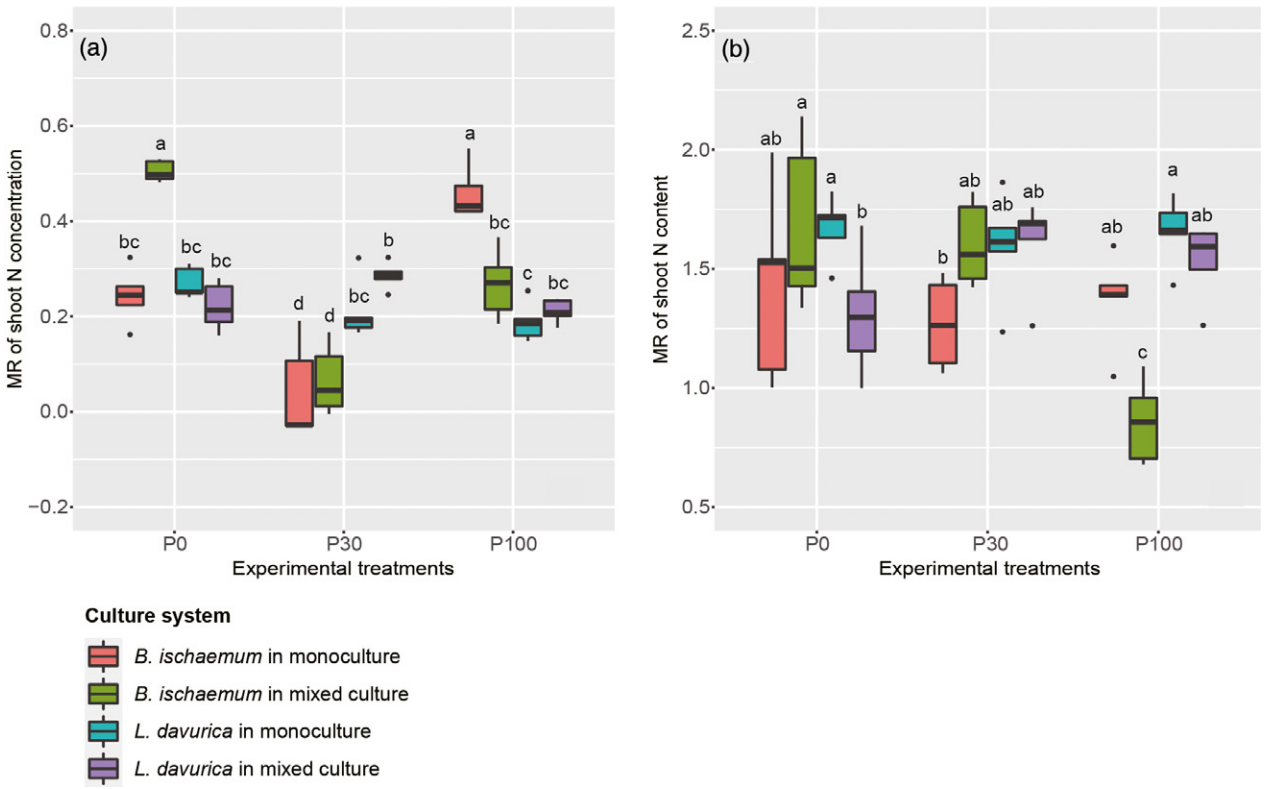

Figure 2. Effects of P addition and culture system on the mycorrhizal response (MR) of: (a) shoot N concentration; and (b) shoot N content. In the box plot, the black boxes indicate the 25th and 75th quantiles, the whiskers indicate the minimum–maximum values and the horizontal line is the median value. Different lowercase letters above the boxes indicate significant differences between the treatments ($P < 0.05$). Culture systems in mycorrhizal and non-mycorrhizal systems are indicated as follows: orange, *Bothriochloa ischaemum* in monoculture; green, *B. ischaemum* in mixed culture; blue, *Lespedeza davurica* in monoculture; and purple, *L. davurica* in mixed culture.

the vector angle approached 45° under the P100 treatment (Figure S9), indicating that soil microbial N and P metabolism were relatively balanced after the addition of high quantities of P (Moorhead et al., 2013). This result demonstrates that N supply by AMF to host plants is not directly related to the benefit of AM for host plant growth under P-limited conditions. In P-limited systems, the addition of P promoted the growth of *B. ischaemum* by alleviating P limitation, leading to the increased N demand of *B. ischaemum*. As mentioned above, when the level of soil N is sufficient, AMF can efficiently trade surplus N for host plant photosynthate (Johnson, 2010; Johnson et al., 2015). Although AMF increased the N supply for host plants with an increase in soil P availability, the benefit of AM for host plant growth was limited by the capability of plant P uptake through AMF.

In contrast, the shoot N uptake in *L. davurica* via AMF in monoculture decreased significantly with increasing P addition. As a legume species, *L. davurica* is able to simultaneously form a tripartite symbiotic relationship with *Rhizobium* and AMF. Previous studies have revealed that compared with non-symbiotic plants, in which growth is reliant on mineral N, N_2-fixing legumes require more P for their growth (Schulze and Drevon, 2005; Sulieman and Tran, 2015). There is evidence that nodule P concentrations are higher than shoot and root P concentrations, indicating that nodules are strong N sinks and that soil P availability is important for legume symbiosis processes (Bargaz et al., 2012; Schulze and Drevon, 2005). Therefore, soil P availability can enhance biological N fixation by promoting nodule formation and functioning (Bargaz et al., 2018; Vardien et al., 2016), which would reduce the N uptake requirement of *L. davurica*.

Effects of AMF on interspecific plant interaction at different levels of soil P availability

In the treatment without P addition, the interaction between *B. ischaemum* and *L. davurica* via AMF had a negligible effect on the AM benefit for plant growth of *B. ischaemum*, but repressed AM benefit for shoot growth of *L. davurica*. This result indicates that interspecific plant interactions mediated by AMF exacerbated the nutrient limitation for legume growth because more plant biomass is allocated to the root (Brouwer, 1984; Tilman and Cowan,

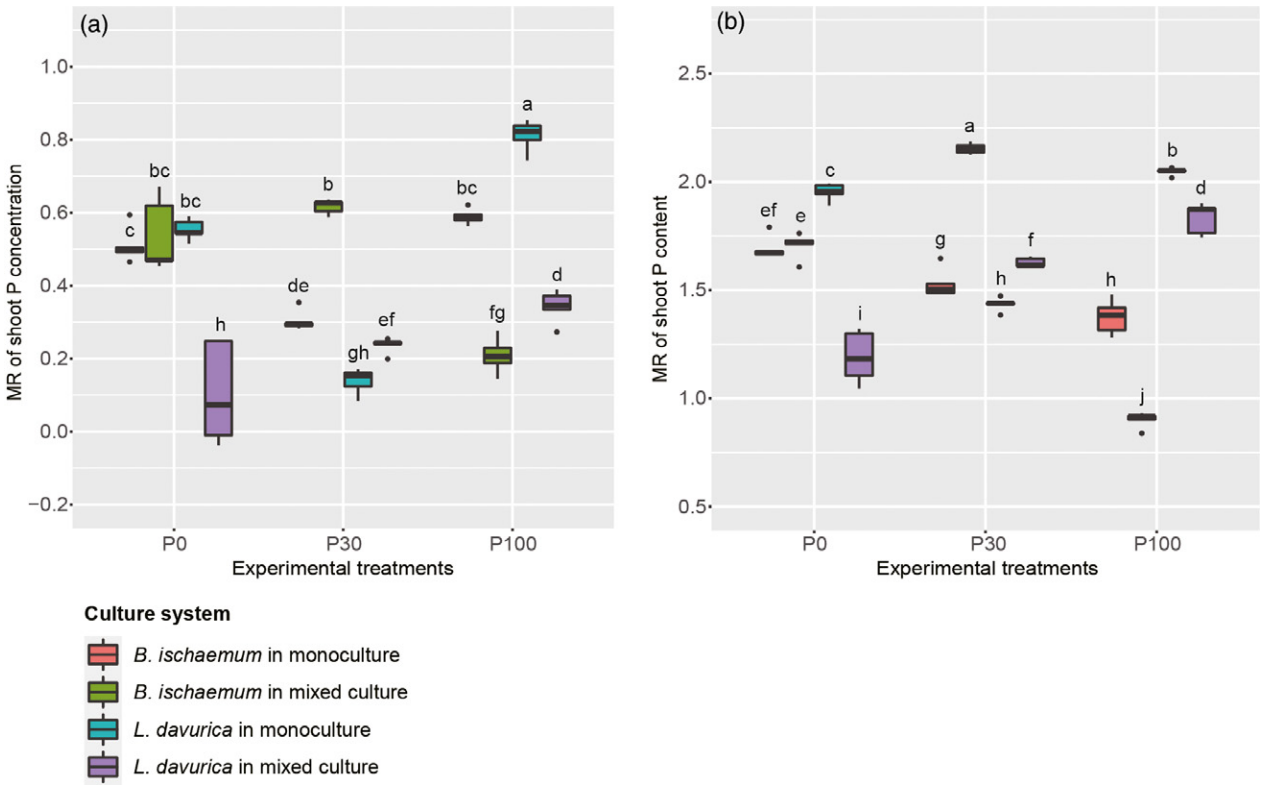

Figure 3. Effects of P addition and culture system on the mycorrhizal response (MR) of: (a) shoot P concentration; and (b) shoot P content. In the box plot, the black boxes indicate the 25th and 75th quantiles, the whiskers indicate the minimum–maximum values and the horizontal line is the median value. Different lowercase letters above the boxes indicate significant differences between the treatments ($P < 0.05$). Culture systems in mycorrhizal and non-mycorrhizal systems are indicated as follows: orange, *Bothriochloa ischaemum* in monoculture; green, *B. ischaemum* in mixed culture; blue, *Lespedeza davurica* in monoculture; and purple, *L. davurica* in mixed culture.

1989). Specifically, the effect of interspecific plant interactions via AMF on the AM benefit for legume growth was mainly influenced by the AM effect on soil available P concentration and alkaline phosphatase activity. Alkaline phosphatase can catalyse the hydrolysis of phosphomonoesters, generating phosphate ions from the substrate (Zalatan et al., 2008). Notably, AMF generally lack saprotrophic capabilities and are therefore likely to stimulate soil microbial activity in order to compensate for their poor organic nutrition capability (Smith and Read, 2008; Tedersoo and Bahram, 2019). The input of easily available C to the soil stimulates soil microbial metabolic activity, thus triggering soil organic matter decomposition, which is termed the priming effect (Kuzyakov et al., 2000). AMF hyphae preferentially grow towards organic matter patches, and slowly release bioavailable C to surrounding soil at relatively low concentrations, which might lead to a greater priming effect on decomposition than is provided by roots (Cheng et al., 2012; Drigo et al., 2010; Hodge et al., 2001). However, the presence of AMF can also repress soil microbial activity through competition with free-living saprotrophs for limited shared resources (Leifheit et al., 2015). Our results revealed that interspecific plant interactions via AMF repressed the activities of the soil enzymes responsible for P cycling in the soil of *L. davurica* (Figure S11), and resulted in a decrease in plant P content and an increase in plant N/P content, confirming that interspecific plant interactions via AMF aggravated P limitation instead of N limitation for legume growth.

The addition of low quantities of P significantly enhanced the positive interaction between the two plant species via AMF, as interspecific plant interaction via AMF greatly improved the AM benefit for shoot growth of *B. ischaemum*, which was mainly driven by the AM effects on soil P bioavailability (including enzyme activity involved in P cycling and soil available P concentrations). In particular, interspecific plant interactions via AMF greatly promoted the activity of soil enzymes involved in P cycling (Figure S11), leading to an increase in shoot P concentrations in *B. ischaemum*. A previous study reported a strong cooperation between AMF and the soil phosphate-solubilizing bacterium *Rahnella aquatilis* for the mineralization of organic P (in the form of phytate) (Zhang et al., 2016). Additional P allocation to the leaves is critical for promoting plant growth, as P is an essential nutrient for energy transfer as well as stem strength and photosynthesis

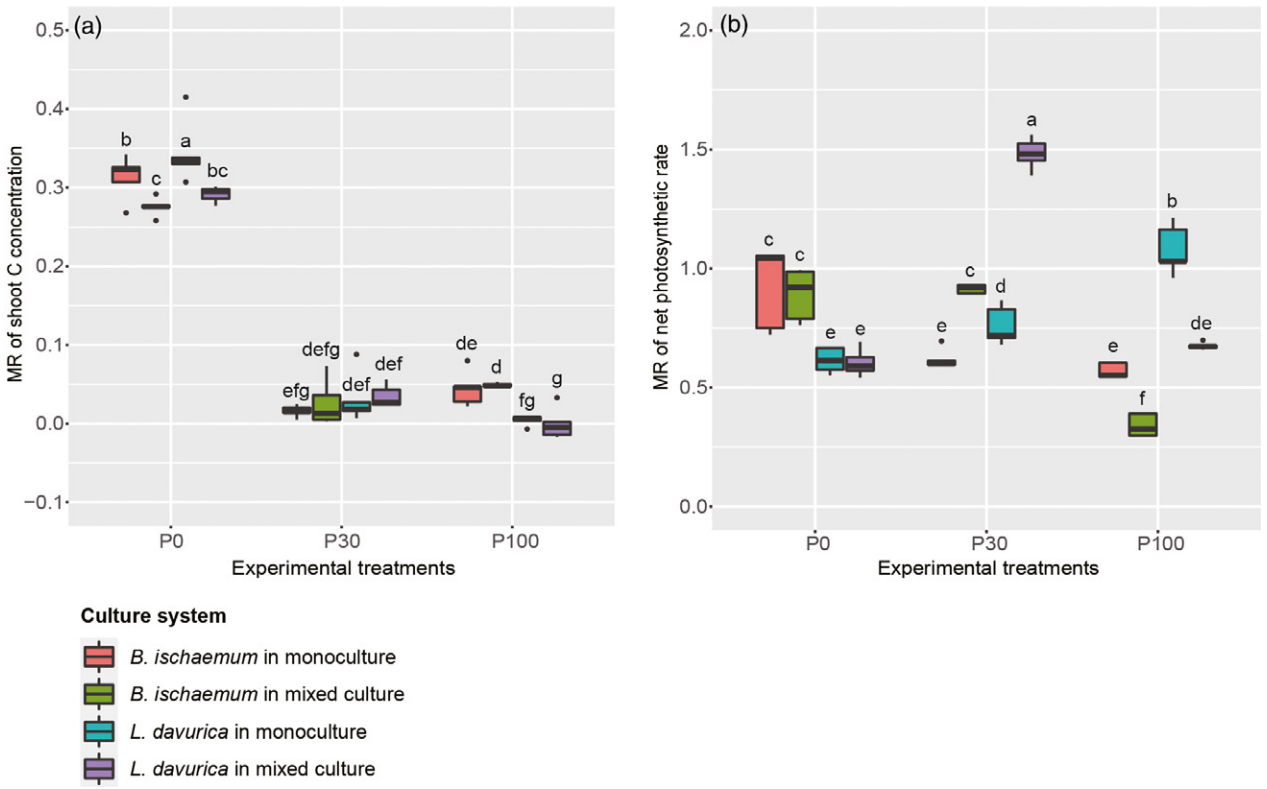

Figure 4. Effects of P addition and culture system on the mycorrhizal response (MR) of: (a) shoot C concentration; and (b) net photosynthetic rate. In the box plot, the black boxes indicate the 25th and 75th quantiles, the whiskers indicate the minimum–maximum values and the horizontal line is the median value. Different lowercase letters above the boxes indicate significant differences between the treatments ($P < 0.05$). Culture systems in mycorrhizal and non-mycorrhizal systems are indicated as follows: orange, *Bothriochloa ischaemum* in monoculture; green, *B. ischaemum* in mixed culture; blue, *Lespedeza davurica* in monoculture; and purple, *L. davurica* in mixed culture.

maintenance in plants (Meena, 2020; Valkama et al., 2016; Wright et al., 2003).

Under treatments without P addition or with the addition of low quantities of P, ^{15}N labelling highlighted that interspecific plant interactions promoted N uptake via AMF by *B. ischaemum* at the expense of that by *L. davurica*, consistent with the effect of interspecific plant interactions on plant growth. Generally, in mixed culture systems, C4 plants are stronger competitors than legumes (Hauggaard-Nielsen and Jensen, 2001; Wang et al., 2016). Some *in vitro* studies on root organ cultures have revealed that the roots that are able to supply the highest quantity of C to AMF take up the greatest quantity of mineral nutrients from AMF, leading to the hypothesis that C and nutrient exchange between AMF and host plants involves 'reciprocal rewards' (Fellbaum et al., 2014; Kiers et al., 2011; Lekberg et al., 2010). Compared with *L. davurica*, the C4 grass *B. ischaemum* inherently has a higher photosynthetic capability and is able to provide more C for its associated AMF, granting it a higher competitive ability to obtain N from AMF. Additionally, the legume *L. davurica* can simultaneously form tripartite symbiotic relationships with *Rhizobium* and AMF. The nodules in the roots of *L. davurica* can fix atmospheric N in the rhizosphere and increase the N availability for host plant growth. In the mixed culture system of the legume and the C4 plant, *Rhizobium* promoted the N-fixation efficiency of the legume, and AMF enhanced the N uptake via hyphae in the C4 plant (Meng et al., 2015; Wang et al., 2016).

After the addition of high quantities of P, interspecific plant interactions via AMF had a negligible effect on the AM benefit for the plant growth of *B. ischaemum* but had a positive effect on the growth of *L. davurica*. In particular, interspecific plant interaction via AMF increased the soil N and P bioavailability for *L. davurica*. The activity of soil enzymes responsible for P cycling (A_P) was repressed by interspecific plant interactions (Figure S11), which partially confirmed that the increased soil available P concentration in the mixed culture was a consequence of decreased plant P uptake from the soil (Figure S10). Previous studies have noted that AMF stimulates soil microbial activity associated with N cycling (Morrison et al., 2017; Teutscherova et al., 2019). In the present study, although interspecific plant interactions via AMF stimulated the activities of soil enzymes involved in N cycling (leucine aminopeptidase and *N*-acetyl-glucosaminidase, Table S3), it enhanced N

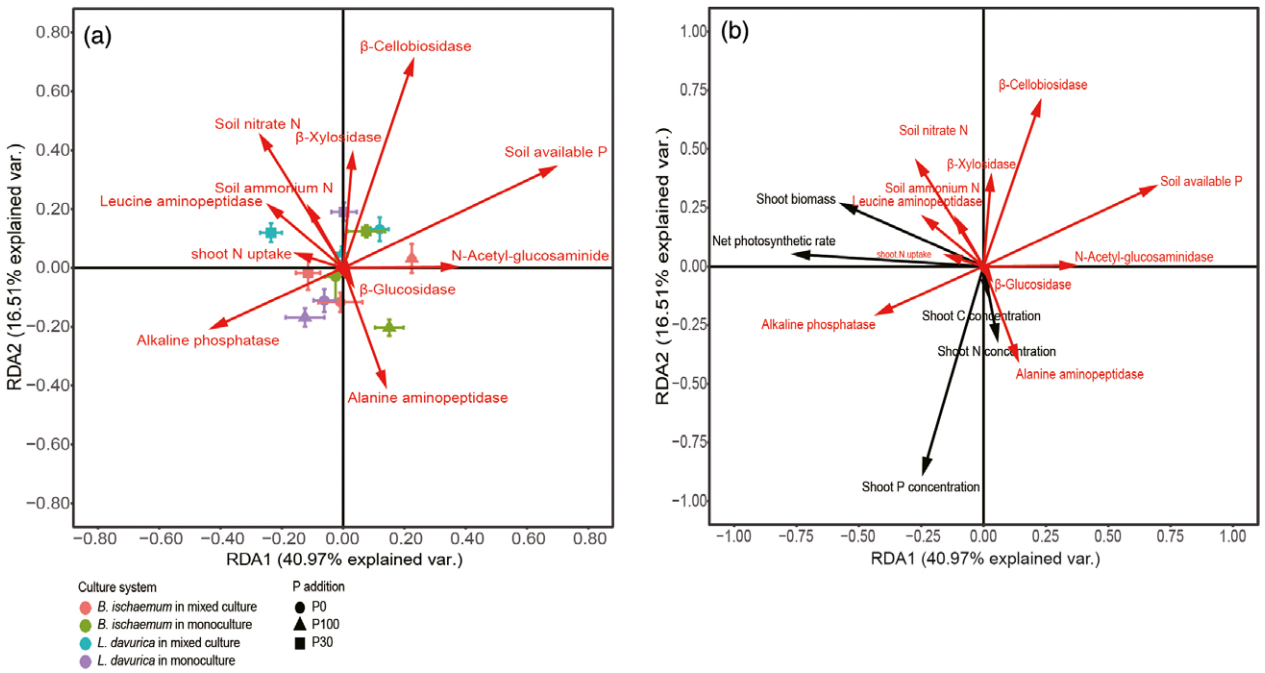

Figure 5. Relationships between the mycorrhizal effect on shoot growth traits (mycorrhizal growth response, MGR; mycorrhizal response, MR, of shoot C, N and P concentration; and net photosynthetic rate, P_n), soil biochemical properties (MR of soil biochemical properties) and shoot N uptake via hyphae explored by redundancy analysis (RDA). The relationships between the samples (centroids of the response variables) and the explanatory variables (MR of soil biochemical properties and shoot N uptake via hyphae) are shown in Figure 6(a) and the relationships between the response variables (MGR, MR of shoot C, N and P concentration, and P_n) and explanatory variables (MR of soil biochemical properties and shoot N uptake via hyphae) are illustrated in Figure 6(b). Arrows indicate the lengths and angles between explanatory and response variables and reflect their correlations; black arrows represent response variables and red arrows represent explanatory variables. Soil samples with different symbols and different colours correspond to different P-addition treatments and different combinations of plant species in distinct culture systems. Circles indicate treatments without P addition, triangles indicate treatments with low P addition and squares indicate treatments with high P addition. Orange, *Bothriochloa ischaemum* in monoculture; green, *B. ischaemum* in mixed culture; blue, *Lespedeza davurica* in monoculture; and purple, *L. davurica* in mixed culture.

bioavailability in the soil of the legume while decreasing it in the soil of the C4 grass. Thus, the effect of AM on plant N uptake should be taken into consideration when exploring the mechanisms by which AMF affect interspecific plant interactions.

Interestingly, the addition of high quantities of P greatly promoted the N supply from AMF to *L. davurica*, but greatly decreased the N supply via AMF to *B. ischaemum*, indicating that the beneficial effects of N acquisition via AMF can vary with changes in soil nutrient conditions. Numerous studies have found that compared with grasses, legumes have a higher demand for P, and that P addition can weaken the ability of grasses to compete with legumes for resources, which has a positive effect on maintaining abundant legumes in grasslands (Bobbink, 1991; Bolan et al., 1987; Mendoza et al., 2016; Zhao et al., 2019). As a legume species, *L. davurica* requires much higher quantities of N for growth than are required by *B. ischaemum*. The addition of a large quantity of P further increased the N demand of this legume for growth. Walder and van der Heijden (2015) suggested that sink strength and competition for surplus resources are important factors that affect resource exchange in AM symbiosis. The much greater sink strength of N in the legume relative to that in the C4 grass contributed to the greater N uptake through AMF by the legume. A previous study showed that P addition can increase the competitive ability of legumes but decreases that of grasses in an intercropping system, relative to those in monocultures (Bi et al., 2019). The present study revealed that the relative competitive capability of *B. ischaemum* and *L. davurica* via AMF was consistent with their N acquisition capabilities via AMF, which highlights that N allocation by AMF to associated plants is an important mechanism influencing the interspecific relationship between C4 grasses and legumes in grasslands.

Conclusions

Increased soil P availability decreased the benefit of AMF for plant growth under P-limited conditions. P addition largely increased shoot N uptake via AMF by the C4 grass but decreased that by the C3 legume. Under severely P-limited conditions, the growth of the C3 legume was repressed by interspecific plant interactions via AMF as a result of P deficiency. After the addition of low quantities of P, interspecific plant interactions via AMF promoted the growth of the C4 grass as a result of alleviated P limitation. Compared

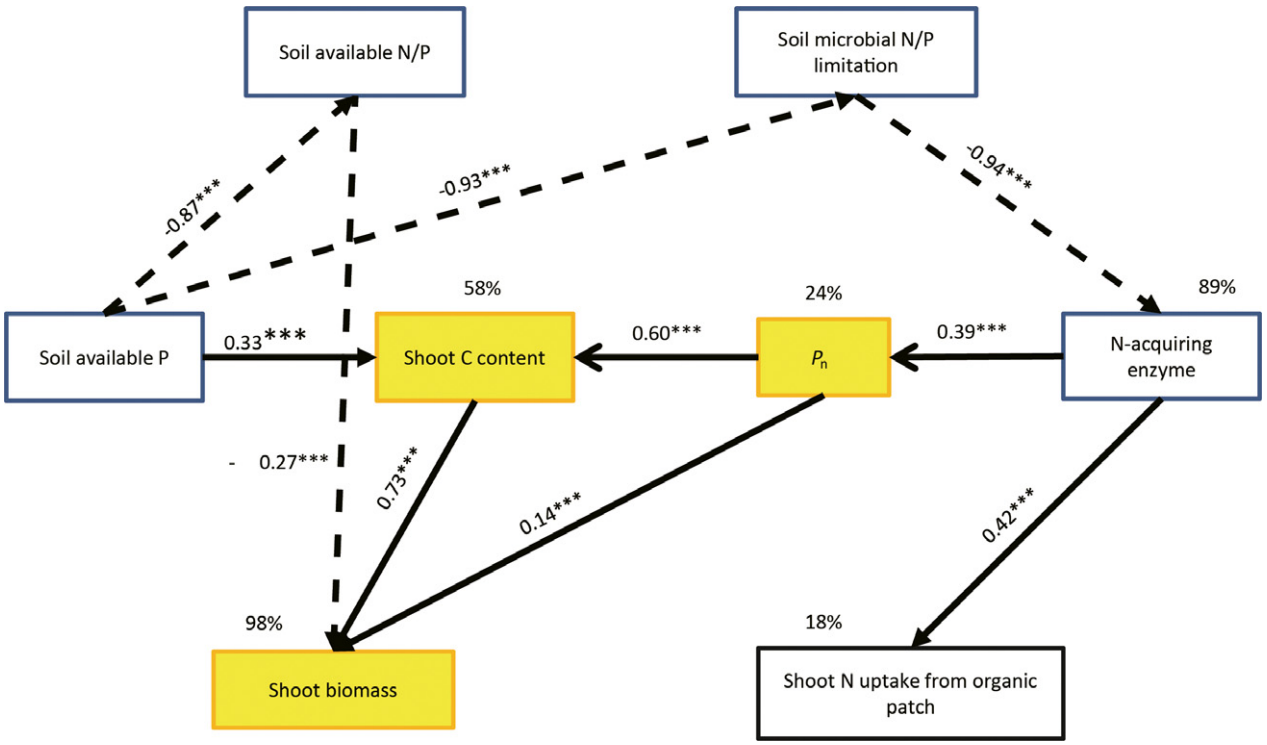

Figure 6. Structural equation modelling (SEM) of the effects of soil biochemical properties on the shoot growth performance of mycorrhizal plants. Both models were satisfactorily fitted to data based on the χ^2, *P* value, goodness of fit index (GFI) and root mean square error of approximation (RMSEA) analyses: $\chi^2 = 21.45$; d.f. = 15; $P = 0.132$; GFI = 0.961; and RMSEA = 0.046. Solid and dashed arrows indicate the positive and negative effects on the fitted SEM, respectively. Numbers on the arrows are standardized path coefficients (equivalent to correlation coefficients). Variances explained by the model (R^2) are shown next to each endogenous variable. The yellow boxes highlight plant growth traits and distinguishes them from soil biochemical properties.

with the C3 legume, the C4 grass had a greater competitive capability via AMF under limited soil-P conditions. However, the addition of high quantities of P greatly promoted the N supply from AMF to the C3 legume in mixed culture, relative to that in the monoculture, stimulating the growth of the C3 legume. Our study suggests that the capability of plant N uptake via AMF is an important mechanism for influencing the interspecific relationship between C4 grasses and legumes. Furthermore, the effect of AMF on the activity of the soil enzymes responsible for N and P mineralization was found to play a vital role in interspecific plant interactions via AMF for plant growth.

EXPERIMENTAL PROCEDURES

Growth substrate and organic patch material

The soil used in this experiment was collected from the upper soil layer (the first 20 cm) of a natural grassland in the Loess Plateau of China (36°51′30″N, 109°19′23″E). Detailed physicochemical properties of the soil sampled are provided in Appendix S1. The growth substrate was prepared by mixing autoclaved soil (121°C, 1 h) and thoroughly washed sand in a 1:1 ratio (w:w).

Organic patch material was produced using a method adapted from Hodge et al., (1998). *Lolium perenne* L. was labelled with both ^{13}C and ^{15}N, and the labelled shoot material was harvested, oven dried and milled (<2 mm). It was then mixed with fresh soil (1:10 w:w) to produce the organic patch material. Notably, the shoot material contained 38.4% C (1.124 atom% ^{13}C) and 1.26% N (9.233 atom% ^{15}N). The detailed method for the preparation of the organic patch material is described in Appendix S1.

Experimental design

A three-compartment growth microcosm was constructed using plastic boxes was used for the growth chamber labelling experiment (Figure 7). Each root compartment (RC) consisted of two identical subcompartments (length × width × height = 10 × 8 ×13 cm), which were separated by a 25-μm nylon mesh. Each subcompartment of the RCs contained 1.4 kg of the growth substrate. A 25-μm nylon mesh bag (10 × 1 × 5 cm) with 11 g of organic patch material was placed in the hyphal compartment (HC; length × width × height = 20 × 2 × 13 cm), 3 cm above the bottom of the RC. The growth chamber labelling experiment was conducted under a rain shelter with open sides in order to block rainwater but provide natural sunlight and temperature for plant growth. The experimental area had a mean annual precipitation, temperature and sun duration of 674.3 mm, 13.28°C and 1993.7 h, respectively. The growth chamber labelling experiment was conducted with three distinct culture systems (i.e. two monocultures and one mixed culture), and it included two AMF inoculation treatments (one non-inoculated treatment and one AMF- inoculated treatment) and three levels of P addition (P0, 0 mg kg^{-1} P; P30, 30 mg kg^{-1} P; and P100, 100 mg kg^{-1} P). Each treatment had five replicates, and a total of 90 experimental microcosms were established (i.e. three culture systems × two AMF inoculation treatments × three P addition levels × five replicates).

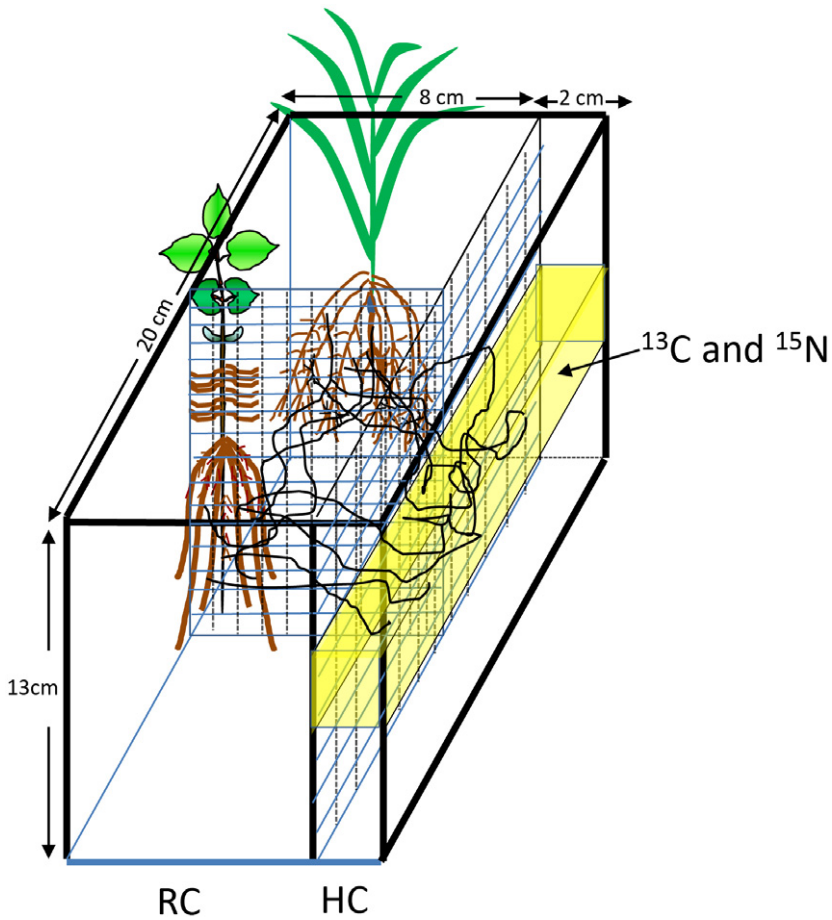

Figure 7. Schematic diagram of the compartmented microcosms used in the present study. The root compartment (RC) was made using a 25-μm nylon mesh bag that was evenly separated by a 25-μm nylon mesh wall into two compartments. A 25-μm nylon mesh bag containing organic patch material was placed in the hyphal compartment (HC). The plants used were *Bothriochloa ischaemum* (B) and *Lespedeza davurica* (L), either as a pair of conspecific plants (B:B and L:L) in the monoculture model or in combination (B:L) in the mixed-culture model.

The seeds that germinated within a week were transplanted into the RCs on 25 April 2017. On the day of transplantation, the seedlings in each subcompartment were inoculated with a 10-g AMF inoculum of *F. mosseae* (Bank of Glomales in China, No. BGC BJ109) or with a 10-g inoculum sterilized at 121°C for 30 min as a non-mycorrhizal control. In addition, a 25 ml of filtered washing of the AMF inoculum without arbuscular mycorrhizal propagules (see Appendix S2) was added to each RC subcompartment to equalize the starting microbial communities (Hodge et al., 2001; Koide and Li, 1989). A week after transplantation, 40 ml of modified 1/2 N and ½ P Hoagland solution (for the composition, see Appendix S2) was added to each RC subcompartment once a week for 4 weeks. Four weeks after transplantation, organic patch materials were added to each HC. After seedling emergence, the seedlings in each RC subcompartment were thinned to five healthy plants. These plants were watered manually with distilled water every day. The addition of P started on 15 June 2017, and a KH_2PO_4 solution was applied to each compartment at a rate of 0, 3.75 or 12.5 mg kg^{-1} P once a week for 8 weeks.

Photosynthetic characteristic measurements and sample collection

Five leaves of the plants in each RC subcompartment were selected in early August 2017 for measuring their photosynthetic characteristics. P_n, intercellular CO_2 concentration, transpiration rate and stomatal conductance were measured with an open gas-exchange system (LI-6400XT; LI-COR Biosciences, https://www.licor.com) between 8:30 and 11:30 AM. The parameter settings for these measurements are given in Appendix S3.

Plant samples were collected in early September 2017 and separated into shoots and roots. The shoots were cut at the soil surface, and the roots were washed with tap water before determining their fresh weight. Each root sample was divided into two subsamples: one was weighed, and the other was stored in a formalin–acetic acid–alcohol solution (FAA; 37% formaldehyde: glacial acetic acid:95% ethanol = 9:0.5:0.5, v:v:v) to determine mycorrhizal colonization (Gavito and Miller, 1998). The harvested shoot samples and weighed root subsamples were immediately placed in an oven at 105°C for 30 min and then dried at 80°C until a constant weight, to determine their dry weights. Plant biomass was calculated as the dry weight of the five whole plants in each RC subcompartment. Subsequently, these samples were milled into a fine powder with a ball mill to further measure chemical characteristics. The top 2 cm of soil in each RC subcompartment were discarded to eliminate any possible surface effects. The remaining soil was homogenized to obtain a uniform matrix and sieved through 0.25- and 1-mm sieves for subsequent physicochemical analysis.

Biochemical analysis

The fixed root samples were cleaned with 10% KOH (w:v) at 90°C for 1 h, followed by acidification with HCl for 5 min. Thereafter,

the samples were stained with 0.05% trypan blue (w:v) at 90°C for 30 min and incubated in a decolouring solution (lactic acid:glycerol = 1:1, v:v) (Philips & Hayman, 1970). Magnified cross sections were used to determine mycorrhizal colonization (McGonigle et al., 1990).

Total C, N and P contents of the plant samples were determined using the H_2SO_4-$K_2Cr_2O_7$ oxidation method (Mebius, 1960), the Kjeldahl method (Bremner and Mulvaney, 1982) and using persulphate oxidation followed by colorimetric analysis (Schade et al., 2003), respectively. The ^{13}C and ^{15}N atom% of the plant samples were determined using an elemental analyser with a continuous-flow isotope ratio mass spectrometer (EA-ConFlo-IRMS, Flash 2000 HT and MAT 253; ThermoFisher Scientific, https://www.thermofisher.com). The detailed procedure used to determine soil biochemical characteristics is described in Appendix S4.

Statistical analysis

Plant C and N uptake was calculated from a ^{13}C and ^{15}N dual-labelled organic patch, based on the isotope method as follows:

$$\text{Plant C or N uptake via AMF} = T \times \left[\frac{(A_i - A_u)}{A_p}\right],$$

where T is the total C or N content of plant samples under inoculated treatments, A_i is the ^{13}C or ^{15}N atom% excess of plant samples under inoculated treatment, A_u is the ^{13}C or ^{15}N atom% excess of plant samples under the corresponding non-inoculated treatment and A_p is the ^{13}C or ^{15}N atom % excess in the organic patch.

The shoot MGR was calculated as described by Johnson (2010):

$$\text{MGR} = \ln(\text{AM}/\text{NM}),$$

where AM is the plant biomass in the AMF inoculum treatment and NM is the plant biomass in the corresponding non-inoculated treatment.

The MR of plant nutrient and photosynthesis was calculated as follows:

$$\text{MR} = \ln(\text{AM}/\text{NM}),$$

where AM is the plant P_n and nutrients in the AMF inoculum treatment and NM is the plant P_n and nutrients in the corresponding non-mycorrhizal control treatment. Plant nutrients included shoot C, N and P concentrations and contents.

Kolmogorov–Smirnov and Levene tests were used to test the normality and homogeneity of variances, respectively. The data were checked and transformed appropriately to normalize any skewed distribution before the statistical analyses. Multivariate analysis of variance (MANOVA) was used to analyse the effect of culture system, P addition, mycorrhizal inoculation and the interaction among these factors on shoot growth (including shoot biomass, C, N and P concentrations, and P_n). Three-way ANOVA was used to analyse the effect of the culture system, P addition, mycorrhizal inoculation and the interaction among these factors on individual shoot growth trait (including shoot biomass, C, N and P concentrations, and P_n), and two-way ANOVA was used to investigate the effect of the culture system and P addition on mycorrhizal colonization and shoot N uptake from the organic patch via hyphae. RDA was used to elucidate the relationships between the effect of AM on shoot growth traits and on soil biochemical properties. Monte Carlo tests were used to test for significant associations between shoot growth traits in the mycorrhizal system and soil biochemical properties. RDA was conducted using the VEGAN package in R 3.6.1 (R Core Team, 2019). The SEM procedure was started by specifying a conceptual model of hypothetical relationships based on *a priori* and theoretical knowledge (Figure S1). Changes in soil available P concentration and available N/P induced by P addition were assumed to affect shoot biomass and C content. Moreover, changes in soil available P concentration and available N/P were expected to indirectly influence soil N-acquiring enzyme activity, which can directly affect plant P_n and shoot N uptake via hyphae. Finally, shoot N uptake via hyphae was assumed to directly influence shoot biomass. In the SEM analysis, the model-implied variance–covariance matrix was compared with the observed variance–covariance matrix, and the data were fitted to the models using the maximum-likelihood estimation method. The model was established and run using IBM® SPSS® AMOS™ 21.0 (IBM, https://www.ibm.com). The box plots and RDA figures were generated using the GGPLOT2 package in R.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

DATA AVAILABILITY STATEMENT

All data can be found within the article and its supporting information.

SUPPORTING INFORMATION

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

Figure S1. Illustration of all plausible interaction pathways in the plant–soil–mycorrhizal fungi system studied.

Figure S2. Effects of P addition and culture system on shoot C uptake from the organic patch via hyphae.

Figure S3. Effects of P addition and culture system on the mycorrhizal colonization.

Figure S4. Effects of P addition and culture system on the shoot N and P concentrations, and N/P in mycorrhizal and non-mycorrhizal systems.

Figure S5. Effects of P addition and culture system on the shoot C concentration and net photosynthetic rate in mycorrhizal and non-mycorrhizal systems.

Figure S6. Effects of P addition and culture system on the root N and P concentrations, and N/P in mycorrhizal and non-mycorrhizal systems.

Figure S7. Effects of P addition and culture system on the root C concentration in mycorrhizal and non-mycorrhizal systems.

Figure S8. Effects of P addition and culture system on the root C uptake from the organic patch via hyphae in mycorrhizal and non-mycorrhizal systems.

Figure S9. Effects of P addition and culture system on the soil available N/P and vector angle in mycorrhizal and non-mycorrhizal systems.

Figure S10. Effects of P addition and culture system on the soil ammonium N, nitrate N and available P concentrations in mycorrhizal and non-mycorrhizal systems.

Figure S11. Effects of P addition and culture system on the activities of soil C-acquiring, N-acquiring and P-acquiring enzymes in mycorrhizal and non-mycorrhizal systems.

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

Table S2. Results of structural equation modelling (SEM) of the effects of soil biochemical properties on the shoot growth performance of mycorrhizal plants, as illustrated in Figure 6.

Table S3. Ecoenzymes included in this study.

Methods S1. Soil physiochemical properties, organic patch material and plant seeds.

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

Methods S3. Parameter settings for photosynthetic characteristic measurements.

Methods S4. Additional details on the biochemical analysis.

Methods S5. Additional details on the calculation of parameters.

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