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ORIGINAL ARTICLE

Contribution of different arbuscular mycorrhizal fungal inoculum to *Elymus nutans* under nitrogen addition

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Abstract

Arbuscular mycorrhizal (AM) fungi are known to promote plant growth and nutrient uptake, but their role in nitrogen (N) uptake still remains unclear. Therefore, a pot experiment was set up to evaluate the impacts of N addition and AM inoculation (*Diversispora eburnea*, *Claroideoglomus etunicatum*, *Paraglomus occultum*, and their mixture) on AM root colonization, plant biomass, N and P nutrition in *Elymus nutans*. Our results showed that AM root colonization was unaffected by N addition but was significantly affected by different AM fungal species. *D. eburnea* and *C. etunicatum* showed significant higher root colonization than *P. occultum*. The *E. nutans* exhibited the highest biomass when inoculated with *D. eburnea* and significantly higher than non-mycorrhizal (the control) regardless of N addition. Under N addition treatment, *D. eburnea* significantly enhanced P content of roots, N content of shoots and roots, while AM mixture significantly enhanced shoot P content compared with non-mycorrhizal. However, N and P content in shoots and roots did not significantly vary among treatments when no N was added. In addition, inoculation with *C. etunicatum* and *P. occultum* showed no significant effect on plant biomass, N and P content regardless of N addition. In conclusion, this study revealed that the plant response to N addition depends on AM fungal species and also confirmed that significant functional diversity exists among AM fungal species.

Keywords Arbuscular mycorrhizal fungi · Nitrogen addition · *Elymus nutans* · Functional diversity

Introduction

Arbuscular mycorrhizae (AM) are symbiotic associations formed between plant roots and fungi of the subphylum Glomeromycotina (Spatafora et al. 2016). More than 80% of terrestrial plant species have been reported to have the ability to form an association with AM fungi (Smith and Read 2008). In this association, the host plant provides photosynthetic product for AM fungi, and in turn, the AM fungi enhance the absorption of soil nutrients to host plants (Smith and Read 2008). In addition to P uptake, AM fungi can transfer both inorganic and organic N to the host plants from the soil through the extraradical hyphal network (Sanders and Tinker 1971; Hodge et al. 2001; Tian et al. 2010; Corrêa et al. 2015; Hodge and Storer 2015). For instance, Toussaint et al. (2004) reported that 21% of the total N in AM roots came from extraradical hyphae of AM fungus *Rhizophagus intraradices* (syn. *Glomus intraradices*). Govindarajulu et al. (2005) and Jin et al. (2005) reported that about 30 and 50% of the total N found in mycorrhizal roots was delivered by AM fungi. Moreover, AM fungus *Rhizophagus aggregatus* (syn. *Glomus aggregatum/Rhizoglomus aggregatum*) contribute up to 75% of total N in maize roots in a pot experiment (Tanaka and Yano 2005). However, the AM contributions to N uptake may interact with many factors, such as plant type as well as the soil N and P availability (Johnson et al. 2010). Therefore, it is still not clear to what extent the AM contribute to the N nutrition for host plants under conditions with N supply.

In the past, several studies have revealed that AM fungi were quite sensitive to N enrichment (van der Heijden et al. 2008; van Diepen et al. 2011; Liu et al. 2012; Johnson et al. 2003) in both the field and pot experiments. On the average, N addition generally depressed AM fungal growth (Treseder

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2004; van der Heijden et al. 2008). For instance, a meta-analysis reported by Treseder (2004) showed that AM abundance was decreased by 15% under N enrichment conditions. The negative effect of N addition on AM was mainly because the plant becomes less N limited, thus decrease the carbon allocation to AM fungi. However, other studies have documented that N addition maintained or enhanced AM abundance (Dhillion and Amporpan 1992; Reynolds et al. 2005; Miransari 2011; Zheng et al. 2016). For example, Dhillion and Amporpan (1992) found that N addition stimulated AM root colonization of rice in a pot experiment. On the contrary, Reynolds et al. (2005) reported that AM root colonization was unaffected by N addition in pot experiment. In a field study conducted in Qinghai-Tibet Plateau, Zheng et al. (2016) found that AM fungal extraradical hyphal density was dramatically increased by N addition, whereas AM fungal spore density was unaffected. Such varying observations suggest that AM fungi fluctuate under N addition and thus necessitate need for further investigation.

Furthermore, AM fungal species vary tremendously in functional traits, including colonization strategy, carbon fixation from plants, and nutrient utilization, which are sponsored by both genetic and environmental factors (Pearson and Jakobsen 1993; Koch et al. 2004; Munkvold et al. 2004; Kiers and van der Heijden 2006; Veresoglou et al. 2010; Mensah et al. 2015). These variations in fungal capabilities have been termed as "functional diversity" (Burleigh et al. 2002) and contribute greatly to variations in outcomes of symbioses. For instance, Munkvold et al. (2004) showed that *Funneliformis mosseae* (*syn. Endogone mosseae/Glomus mosseae*) , *F. caledonium* (syn. *Endogone macrocarpa/Glomus caledonium*), and *F. geosporum* (syn. *Endogone macrocarpa/Glomus macrocarpum/Glomus geosporum*) varied their response in improving plant growth and P uptake of cucumber. In addition to the genetic factor, soil nutrient availability is another factor influencing AM functional diversity. Johnson et al. (2015) found that AM symbioses ranged from commensalism or parasitism in P-rich soils to mutualism in P-limited soils. Likewise, a shift in AM functional traits in response to N addition was also observed in a recent field study (Jiang et al. 2018). The AM fungal functional diversity is considered to be very important for regulating ecosystem process and function (De Deyn et al. 2008). However, there is a lack in understanding how changing environment, such as N enrichment will influence AM functional diversity. Filling this gap is important for understanding how N enrichment impact AM symbiosis in terrestrial ecosystems.

Elymus nutans, which form an extensive symbiosis with AM fungi (Liu et al. 2012; Jiang et al. 2018), is widely distributed on the alpine meadow of the Qinghai-Tibet Plateau. It is a constructive plant species and often used for ecosystem restoration in this region (Chen et al. 2013). Its high abundance may be due to its high adaptability, resistance to various abiotic stresses, high yield, and nutrient, thus became one of the most forage grass in this area (Dou et al. 2017). However, in recent decades, the distribution of *E. nutans* has been greatly reduced due to anthropogenic activities as well as climate change (Chen et al. 2013). Most especially, the N deposition is increasingly obvious in the Qinghai–Tibetan Plateau, ranging from 4 to 13.8 kg N ha^{-1} $year^{-1}$ (Liu et al. 2013), which will further threaten the distribution of *E. nutans*. Therefore, we conducted a pot experiment to examine the influence of N addition and AM inoculation on growth of *E. nutans*. Our aims were to reveal (Antunes et al. 2006) how AM root colonization responds to inorganic N addition, (Bennett and Bever 2009) whether AM fungi contribute differently to the N uptake of *E. nutans* with and without N supply, and (Burleigh et al. 2002) whether AM functional diversity existed in plant growth and nutrient uptake under conditions with and without N supply.

Material and methods

Plant and AM fungal species

The study was conducted at Shangzhuang experimental station of China Agricultural University, Beijing in 2011. The seeds of *E. nutans* and soil (Mat-Gryic Cambisol) used for this study were collected from Qinghai-Tibet Plateau (37° 37′N and 101° 12′E, 3200 m above the sea level), China. Seeds were preserved at 4 °C until the commencement of the experiment and the soil samples were air-dried and sieved with 1 mm mesh for routine analysis. The soil chemical analysis before autoclaving showed pH of 6.5, total organic carbon 23.7 g kg^{-1}, total N 1.7 g kg^{-1}, total P 0.5 g kg^{-1}, available N 80.4 mg kg^{-1}. Thereafter, the soil was mixed with sand in ratio 1:2 (soil/sand, respectively) and then autoclaved for 2 h at 90 °C. This was later used as growing medium in this study. Five days prior to the commencement of the experiment, *E. nutans* seeds were sterilized using 70% alcohol for 3 min and rinsed three times using distilled water, then sowed in nursery flats to germinate. *Diversispora eburnea* (syn. *Glomus eburneum*), *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*), and *Paraglomus occultum* (syn. *Glomus occultum*) were used as AM inoculum in the present study, which were commonly isolated from field experiments and their use has also been documented in pot experiments (Munkvold et al. 2004; Mensah et al. 2015). AM fungal spores were extracted using the wet-sieving and decanting method and the number of spores was counted before inoculation (Gerdemann and Nicolson 1963; Daniels and Skipper 1982).

Experiment design

The experimental design was a randomized complete block design with two factors. The first factor was AM fungal inoculum, containing five treatments: *D. eburnea*, *C. etunicatum*, *P. occultum*, 1:1:1 mixture of each AM fungal species and no mycorrhizal addition. The second factor (N additive) contained two treatments: N addition (equal to 200 kg N ha^{-1} $year^{-1}$) and no N addition. There are eight replications in each treatment, resulting to 80 pots (capacity: 30 cm × 20 cm × 15 cm) in total. We added ca. 1000 spores in 100 ml sterilized water for each single AM fungal inoculation treatment in each pot, ca. 330 spores in 33 ml sterilized water for each AM fungal species to give a total of ca. 1000 spores for the three-species AM fungal mixture (1:1:1) treatment, and 100 ml sterilized water (no AM fungal spores) for the no mycorrhizal treatment. Subsequently, 15 plant seedlings were transplanted from nursery flats with an average height of 3 cm. Pots were watered every 2 days. The positions of the pots were adjusted at random in the greenhouse and rotated every 2 weeks.

We added 20 ml of modified Hoagland nutrient solution to all treatments at 2-week interval after transplanting (Hoagland and Arnon 1950). The solution consisted of: 0 or 60 mM NH_4NO_3; 2.5 mM K_2SO_4; 0.5 mM $Ca(H_2PO_4)_2$; 2 mM $CaSO_4$; 25 μM H_3BO_3; 2 μM $MnSO_4$; 2 μM $ZnSO_4$; 0.5 μM $CuSO_4$; and 0.5 μM $(NH_4)_6 Mo_7O_4$. The total amount of N given to each of the pot equalled to 200 kg N ha^{-1} $year^{-1}$ for N addition treatment and 0 kg N ha^{-1} $year^{-1}$ for no N addition treatment.

Harvest

Plants were harvested 5 months after transplanting and separated into shoots and roots. Shoots were oven dried at 85 °C to constant mass and weighed. Fresh root samples were weighed and divided into two subsamples. One subsample was stored at 4 °C for AM root colonization observation. The other subsample was oven dried at 85 °C to constant mass, weighed, and used to calculate the dry mass to fresh mass ratio. The total dry root weight was calculated by multiplying the fresh root mass with the dry mass to fresh mass ratio. The total biomass of *E. nutans* was calculated as the sum of the dry biomass of roots and shoots.

AM root colonization

Fifty fine root fragments of each sample were cleared in 10% KOH, stained with acid fuchsin, and examined under magnification × 200 (Phillips and Hayman 1970). The AM root colonization was then quantified by the magnified line-intersect method (McGonigle et al. 1990).

N and P content

Dried plant shoots and roots were ground in a ball mill and sieved. Plant material was then digested with $HClO_3$ and H_2O_2 at 140 °C for 7 h. Root and shoot N concentrations were determined using the standard micro-Kjeldahl (Mckenzie and Wallace 1954); P concentrations were determined using vanadomolybdo phosphoric acid yellow color methods (Jackson 1973). The N content and P content were then calculated by multiplying plant biomass by the concentration of each nutrient and divided by the individual number.

Statistical analysis

Mycorrhizal growth response (MGR) was calculated as a simple response ratio as described in Johnson et al. (2010): $MGR = \log_e (AW/NW)$, where AW = the mean biomass of mycorrhizal plants and NW = mean biomass of a non-mycorrhizal control treatment. Two-way ANOVAs were used to test the effects of N addition and AM inoculum on AM root colonization, MGR, shoot biomass, root biomass, total biomass, root/shoot ratio, N and P content in shoots and roots. All data were tested for normality and homogeneity of variance before subjecting them to two-way ANOVA. The post hoc HSD analysis was then performed to test a significant difference among the treatments using the SPSS 20.0 software, with the significance level of 0.05.

Results

AM root colonization

E. nutans roots in all AM inoculation treatments were colonized by AM fungi, and generally, the AM colonization levels in non-mycorrhizal treatments were near zero (Fig. 1a). AM root colonization was significantly affected by AM inoculation, but unaffected by N addition and their interaction (Table 1). AM root colonization in *D. eburnea* and *C. etunicatum* plants was fairly high (20–30%) and significantly higher than that in *P. occultum* (< 10%) regardless of N addition (Fig. 1a). However, AM root colonization in *P. occultum* and AM mixture was quite lower than that in *D.eburnea* and *C. etunicatum* (Fig. 1a).

Plant biomass and root/shoot ratio

Two-way ANOVA analysis indicated that the shoot, root, and total biomass of *E. nutans* were significantly influenced by AM fungal inoculation, N addition, and their interaction (Table 1). The *E. nutans* exhibited highest biomass when inoculated with *D. eburnea*, and significantly higher than non-

Table 1 Two-way ANOVA examining the effects of nitrogen (N) addition and AM fungal (AMF) inoculation on shoot biomass, root biomass, total biomass, root/shoot ratio, shoot N content, root N content, shoot P content, root P content, AM root colonization, and mycorrhizal growth response (MGR) of *E. nutans*

Variables	N			AMF			N×AMF		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Shoot biomass	1	46.93	< 0.01	4	51.29	< 0.01	4	14.82	< 0.01
Root biomass	1	49.96	< 0.001	4	48.45	< 0.001	4	20.26	< 0.001
Total biomass	1	57.27	< 0.001	4	58.68	< 0.001	4	20.38	< 0.001
Root/shoot ratio	1	0.22	0.64	4	5.62	0.001	4	1.82	0.13
Shoot N content	1	22.4	< 0.001	4	10.45	< 0.001	4	6.6	< 0.001
Root N content	1	37.3	< 0.001	4	9.8	< 0.001	4	7.9	< 0.001
Shoot P content	1	3.3	0.07	4	5.6	0.001	4	2.3	0.07
Root P content	1	5.5	0.02	4	6.7	< 0.001	4	3.2	0.02
Root colonization	1	0.01	0.95	3	18.6	< 0.001	3	1.33	0.28
MGR	1	107.46	< 0.001	3	31.34	< 0.001	3	3.23	0.03

mycorrhizal control regardless of N addition (Fig. 2a–c). Inoculation with AMF mixture caused a great increase in plant biomass under N addition, but the case was not true when no N was added (Fig. 2a–c). However, inoculation with *C. etunicatum* and *P. occultum* showed no significant effect on shoot, root, and total biomass regardless of N addition (Fig. 2a–c). Root/shoot ratio was significantly affected by AM inoculation (Table 1), and *D. eburnea* improved the root/shoot ratio by 96.9% compared with non-mycorrhizal plants under N addition (Fig. 2d).

Plant N and P content

The N and P contents of *E. nutans* were significantly affected by AM inoculation, N addition, and their interaction (Table 1). Under N addition treatment, *D. eburnea* significantly enhanced P content of roots, N content of shoots and roots, while AM mixture significantly enhanced shoot P content compared with non-mycorrhizal control (Fig. 3a, b, Fig. 4a, b). However, none of these AM inoculations caused any significant increase in plant N and P content when no N was added (Fig. 3a, b, Fig. 4a, b).

Plant mycorrhizal growth response

Plant MGR was significantly affected by AM inoculation, N addition, and their interaction (Table 1). Under N addition, the plant MGR was significantly higher in *D. eburnea* and AMF mixture than in *P. occultum* and *C. etunicatum* (Fig. 1b). *D. eburnea* exhibited the highest plant MGR and significantly higher than *P. occultum* (Fig. 4b) when no N was added. In addition, all plant MGR was significantly enhanced by N addition compared with control except for *P. occultum* inoculation (Fig. 1b).

Discussion

Several studies (glasshouse and field) have reported that N addition showed negative effects on AM root colonization (Sigüenza et al. 2006; van der Heijden et al. 2008; Liu et al. 2012). Thus, it was proposed that AM fungal growth should be depressed due to a reduction in allocation of C from host plants under high N availability (Treseder and Allen 2002). In contrary to previous studies, N addition did not significantly influence AM root colonization in this study, which was

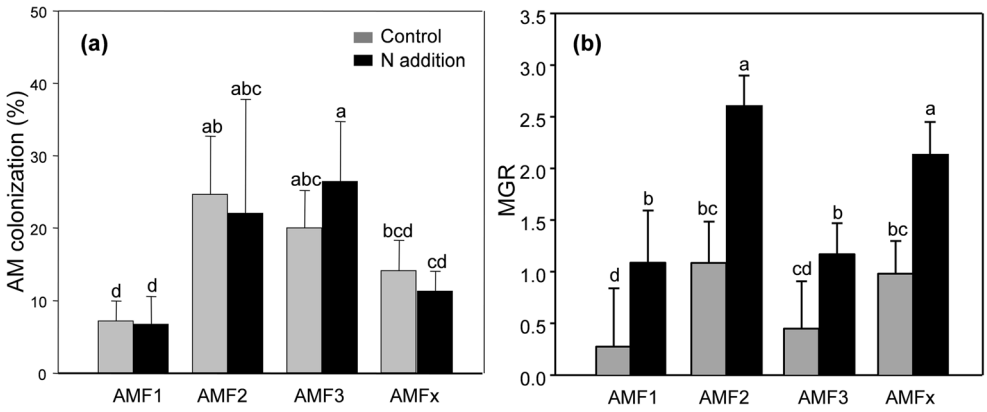

Fig. 1 AM root colonization (**a**) and mycorrhizal growth response (MGR, **b**) among treatments. Bars without shared letters indicate significant difference according to the post hoc HSD analysis ($p < 0.05$). Bars are standard deviations of the means ($n = 8$). Abbreviations: AMF1, *Paraglomus occultum*; AMF2, *Diversispora eburnea*; AMF3, *Claroideoglomus etunicatum*; AMFx, mixture of *P. occultum*, *D. eburnea*, and *C. etunicatum*

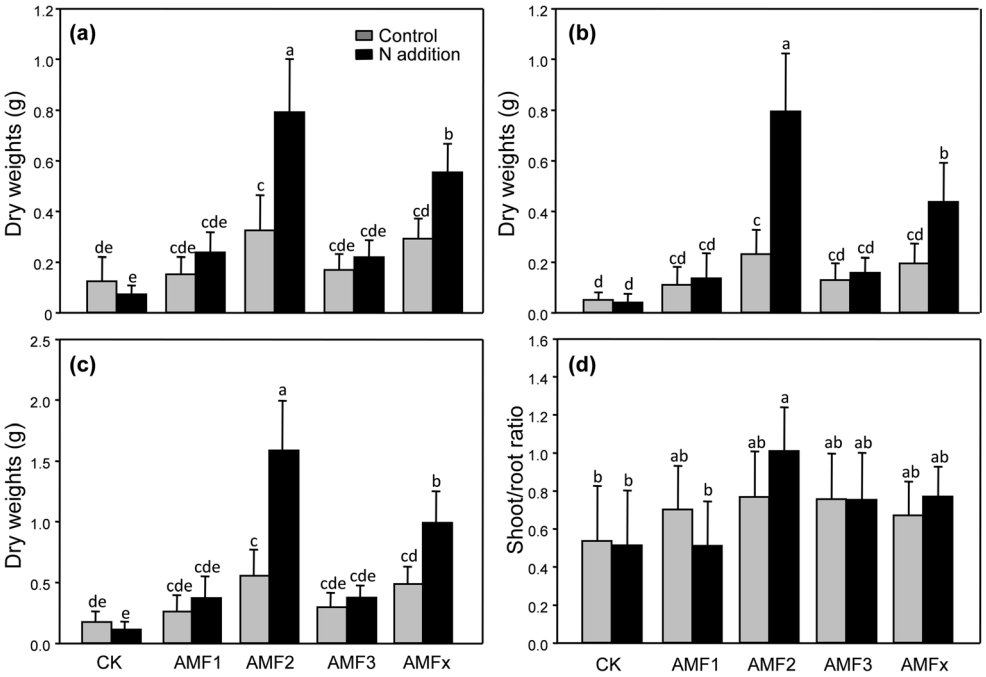

Fig. 2 Dry weights of shoots (**a**), roots (**b**), whole plants (**c**), and root/shoot ratio (**d**) of *Elymus nutans* among treatments. Bars without shared letters indicate significant difference according to the post hoc HSD analysis ($P < 0.05$). Bars are standard deviations of the means ($n = 8$). Abbreviations: CK, control; AMF1, *Paraglomus occultum*; AMF2, *Diversispora eburnea*; AMF3, *Claroideoglomus etunicatum*; AMFx, mixture of *P. occultum*, *D. eburnea*, and *C. etunicatum*

consistent with a few glasshouse studies (Subramanian and Charest 1997; Vázquez et al. 2001). For instance, Vázquez et al. (2001) reported that high N availability did not affect AM colonization in roots of *Medicago sativa* L. Different AM fungal species, plant species, or the soil nutrient availability may have brought about these discrepancies (Hart and Reader 2002; Munkvold et al. 2004).

Our results indicated that AM root colonization was significantly different among AM fungal species; *D. eburnea* and *C. etunicatum* exhibited significantly higher root colonization than *P. occultum*. The variation in AM root colonization among different AM fungal species has been observed in large number of studies (Klironomos 2003; Munkvold et al. 2004; van der Heijden et al. 2008). Some AM fungal species form extensive inner hyphae within roots (Antunes et al. 2006), whereas others prefer to develop extraradical hyphae in soil (Denison and Kiers 2011). In consistent with this study, Kelly (1999) reported that *Trifolium incarnatum* exhibited higher AM root colonization when inoculated with *D. eburnea* and *C. etunicatum* than *P. occultum*in. The different colonization strategy is proposed to be taxonomically based (Hart and Reader 2002). Glomeraceae isolates reported by Hart and Reader (2002) had high root colonization but low soil colonization, while Gigasporaceae isolates showed the opposite trend. Moreover, Merckx et al. (2012) reported that plants prefer to form symbiosis relationship with AM fungal species from Diversisporales and Glomerales than Paraglomerales.

The role of AM fungi in N uptake has previously been discussed in many field and glasshouse studies, and the effect was observed to vary from negative to positive (Hodge et al. 2001; Tian et al. 2010; Schroeder-Moreno et al. 2011; Liu et al. 2012). Our results indicated that inoculation with *D. eburnea*

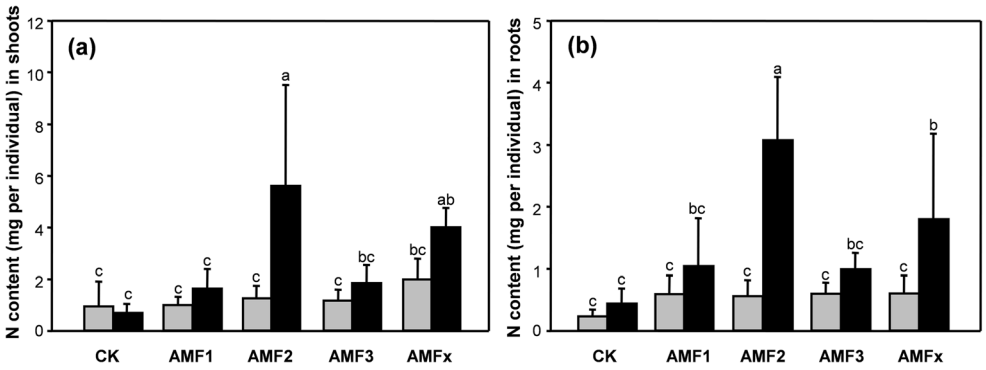

Fig. 3 Nitrogen (N) content of *Elymus nutans* in shoots (**a**) and roots (**b**) among treatments. Bars without shared letters indicate significant difference according to the post hoc HSD analysis ($P < 0.05$). Bars are standard deviations of the means ($n = 8$). Abbreviations: CK, control; AMF1, *Paraglomus occultum*; AMF2, *Diversispora eburnea*; AMF3, *Claroideoglomus etunicatum*; AMFx, mixture of *P. occultum*, *D. eburnea*, and *C. etunicatum*

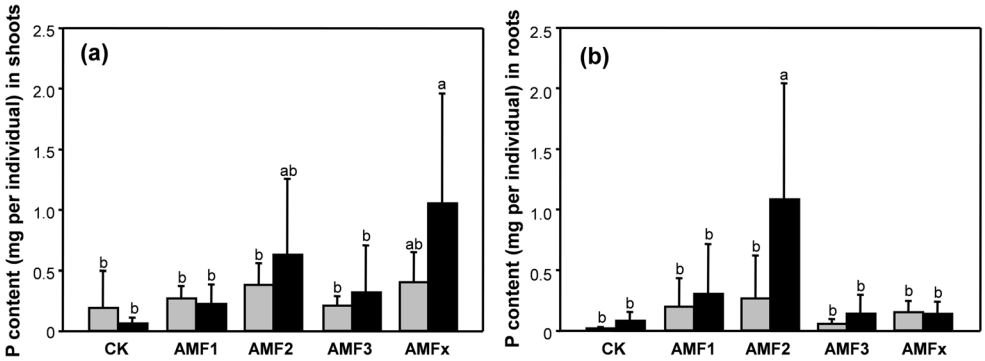

Fig. 4 Phosphoru (P) content of *Elymus nutans* in shoots (**a**) and roots (**b**) among treatments. Bars without shared letters indicate significant difference according to the post hoc HSD analysis ($P < 0.05$). Bars are standard deviations of the means ($n = 8$). Abbreviations: CK, control; AMF1, *Paraglomus occultum*; AMF2, *Diversispora eburnea*; AMF3, *Claroideoglomus etunicatum*; AMFx, mixture of *P. occultum*, *D. eburnea*, and *C. etunicatum*

did not significantly enhance plant N content under no N addition but caused a dramatic increase in plant N content under N addition. Similarly, Jackson et al. (2002) reported that shoot N content of *Lactuca sativa* was unaffected by *R. intraradices* with low N supply but significantly improved with high N supply in glasshouse experiment. It was reported that AM fungi have a high N demands; therefore, they are only likely to transfer significant N to the plant when their N demand had been satisfied (Hodge and Fitter 2010). It will then be under conditions of high N availability that the N transfer should occur.

Recent research had shown that different AM fungal species can induce highly variable plant growth response, ranging from beneficial to detrimental. The MGRs under our experimental conditions ranged from highly positive (> 2.5) to neutral (near 0), indicating different contributions of different AM fungal species to plant growth. Moreover, *D. eburnea* was demonstrated to significantly enhance N and P nutrition of *E. nutans*, while other AM fungal species seemed to have no influence on the N and P nutrition in present study. Similarly, Munkvold et al. (2004) used a more systematic approach and tested 21 AM isolates and showed that there is high functional diversity among AM fungal species with respect to P uptake and plant growth. In addition, Kelly reported that plant biomass and P content of *Trifolium incarnatum* were significantly increased by *D. eburnea* but unaffected by *P. occultum* (Kelly 1999). In another glasshouse study, *P. occultum* did not significantly enhance biomass of *Medicago sativa* and was characterized as "low performance isolates" (Mensah et al. 2015). Our results corroborate these findings and showed that *D. eburnea* was more effective than *C. etunicatum* and *P. occultum* with respect to plant growth and nutrition. Given that AM fungi differed substantially in their colonization strategy (Hart and Reader 2002), they may also differ their ability to acquire and supply nutrients to plants. It was proposed that functional diversity among AM fungal species may allow them to promote host plant growth more effectively. Therefore, a complementary effect of AM mixture on the plant growth and nutrition was expected in present study. However, although AM mixture contributes greatly to plant growth, N and P nutrition, the effect did not exceed that of *D. eburnea*. The possible explanation may be because our pot experiment was carried out under relatively homogenous conditions, or the competition among AM fungal species can be strong enough to result in the exclusion of one or two AM fungal species from host plant roots (Bennett and Bever 2009), which may have contributed to the lack of complementary effect.

In conclusion, the effects of N addition and AM inoculation on AM root colonization, plant growth, N and P content were examined in glasshouse. Although the AM root colonization level was unaffected by N addition, it was significantly higher in *D. eburnea* and *C. etunicatum* than *P. occultum*. The *E. nutans* exhibited highest biomass when inoculated with *D. eburnea*, and significantly higher than non-mycorrhizal (the control) regardless of N addition. Under N addition treatment, *D. eburnea* significantly enhanced P content of roots, N content of shoots and roots, while AM mixture significantly enhanced shoots P content compared with non-mycorrhizal. However, N and P content in shoots and roots was not significantly affected by AM inoculation when no N was added. In addition, inoculation with *C. etunicatum* and *P. occultum* showed no significance on plant biomass yield, N and P content regardless of N addition.

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