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A trade-off between space exploration and mobilization of organic phosphorus through associated microbiomes enables niche differentiation of arbuscular mycorrhizal fungi on the same root

Jiachao Zhou[1], Thomas W Kuyper[2] & Gu Feng[1*]

[1]College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, China;
[2]Soil Biology Group, Wageningen University & Research, Wageningen 6700 AA, The Netherlands

Ecology seeks to explain species coexistence, but experimental tests of mechanisms for coexistence are difficult to conduct. We synthesized an arbuscular mycorrhizal (AM) fungal community with three fungal species that differed in their capacity of foraging for orthophosphate (P) due to differences in soil exploration. We tested whether AM fungal species-specific hyphosphere bacterial assemblages recruited by hyphal exudates enabled differentiation among the fungi in the capacity of mobilizing soil organic P (P_o). We found that the less efficient space explorer, *Gigaspora margarita*, obtained less ^{13}C from the plant, whereas it had higher efficiencies in P_o mobilization and alkaline phosphatase (AlPase) production per unit C than the two efficient space explorers, *Rhizophagusintraradices* and *Funneliformis mosseae*. Each AM fungus was associated with a distinct *alp* gene harboring bacterial assemblage, and the *alp* gene abundance and P_o preference of the microbiome associated with the less efficient space explorer were higher than those of the two other species. We conclude that the traits of AM fungal associated bacterial consortia cause niche differentiation. The trade-off between foraging ability and the ability to recruit effective P_o mobilizing microbiomes is a mechanism that allows co-existence of AM fungal species in a single plant root and surrounding soil habitat.

arbuscular mycorrhizal fungi, carbon, space exploration, hyphosphere bacterial assemblages, organic phosphorus mobilization

INTRODUCTION

Understanding the assembly of biotic communities has been a basic question of ecology (Preston, 1948). Ecologists have tried to understand mechanisms involved in species coexistence (Coyte et al., 2021; Losos et al., 2003; Silvertown, 2004). Two classes of models have been invoked to explain co-existence: neutral models and niche models (Bell, 2000; Tilman, 1982; Yang et al., 2019). Neutral models are based on the assumption that all species are equivalent in demo-graphic rates (birth, death, dispersal, and speciation rates) (Chave, 2004), as a consequence of which biotic communities are structured through stochastic processes (Hubbell, 2005). Niche models emphasize competition for resources (space, time, and nutrients) among different species. Differentiation in the acquisition or use of these resources is crucial for co-existence (Silvertown, 2004; Tilman, 1982). Both classes of models have mostly been evaluated for larger organisms such as plants and animals. Mechanisms by which microbes coexist are still poorly understood. Explaining species coexistence poses additional challenges for organisms inhabiting dual niches (Vályi et al., 2016), both in the

*Corresponding author (email: fenggu@cau.edu.cn)

soil and inside the plant, such as arbuscular mycorrhizal (AM) fungi.

AM fungi have been recognized as model organisms for ecological research because of their widespread occurrence, key ecosystem services provision, and special life history (Duplessis et al., 2011; Johnson and Omland, 2004; Kiers et al., 2011; Maherali and Klironomos, 2007). Dumbrell et al. (2010) noted that both neutral and deterministic processes regulated AM fungal community composition along an environmental gradient, with a larger role for niche differentiation driven by soil factors than for neutral processes. As to AM fungal species coexistence, there is a further issue of spatial scale, as the balance between neutral and niche processes may be different in cases where we explain fungal species coexistence in a community with different plant species, as in the studies of Lekberg et al. (2007), Dumbrell et al. (2010), and Shi et al. (2014), or where we explain fungal species coexistence in the roots of one individual plant or soil. Jansa et al. (2008) suggested temporal niche differentiation to explain AM fungal species coexistence inside a single root. Kiers et al. (2011) proposed biological-market theory to explain the co-existence of different AM fungi inside one root. However, their experimental design with Petri dishes with only one mineral phosphorus (P) source did not allow niche differentiation in the external environment. Many researchers suggested that extraradical coexistence of AM fungal species could be explained by spatial niche differentiation, with species either being efficient or less efficient space explorers (Hart and Reader, 2005; Lekberg et al., 2007; Vályi et al., 2016). In agreement with that suggestion, coexisting species exhibited differences in hyphal length (Thonar et al., 2011). However, under a spatial niche differentiation model, it is difficult to explain how less efficient space explorers, which occupy only a small soil volume, can persist in the community.

In the mycorrhizosphere, there is strong competition, not only between plant roots and AM fungi, AM fungi and their associated bacteria, but also between different AM fungal species. Some AM fungal species such as *Rhizophagus intraradices* and *Funneliformis mosseae* are efficient space explorers and grow far beyond the root and extend the depletion zone for P considerably (Thonar et al., 2011), while other AM fungal species, e.g., *Gigaspora* spp., are less efficient space explorers and form most of their mycelium within 2 cm of the root surface, thereby seemingly contributing less to P acquisition (Thonar et al., 2014). These three above-mentioned AM fungal species coexisted in a single root system and surrounding soil habitat not only in greenhouse experiments but also in natural ecosystems (Bittebiere et al., 2020; Jansa et al., 2014). How do species that explore only a small soil volume manage to coexist with competitors if they acquire P over that smaller volume in the mycorrhizosphere? The answer to this question will help us

better understand mechanisms involved in species coexistence in assemblages of biotic communities, a basic question of ecology proposed many decades ago.

Phosphorus plays an important role in the functioning of the mycorrhizal symbiosis (Balzergue et al., 2011; Kiers et al., 2011; Nouri et al., 2014). Only orthophosphate anions in the soil solution can be directly taken up and utilized by plants and AM fungi (Li et al., 2022; McLaren et al., 2020). A possibility for coexistence of a less efficient space exploring fungal species is that its uptake system has a higher affinity (lower K_m) and lower C_{min} than other AM fungal species (Harrison and van Buuren, 1995; Thomson et al., 1990). However, the disparity in K_m and C_{min} is limited and likely insufficient to explain the coexistence of different AM fungal species. Although some other physiological differences were also observed (Boddington and Dodd, 1999; Morin et al., 2019; Redecker et al., 2000; Wyss et al., 2016), there was no direct evidence that these differences contributed to coexistence. An alternative explanation for species coexistence is that such seemingly less efficient fungi have larger physiological capabilities to acquire (organic) P from more recalcitrant sources through more effective desorption from mineral surfaces and/or higher production of phytases and phosphomonoesterases. Many forms of organic P (P_o), which often constitutes more than 50% of the total soil P pool, are potentially available P sources (Liang et al., 2017). If different AM fungi have differential access to these sources of P_o, co-existence will be possible as has been reported before for plants (Phoenix et al., 2020).

While AM fungi do not have the capability to directly acquire P_o (Morin et al., 2019; Tisserant et al., 2013), a cooperative relationship with phosphate-mineralizing bacteria (PMB) in the hyphosphere can make up for that lack of AM fungi to mobilize P_o directly (Zhang et al., 2016; Zhang et al., 2018a; Zhang et al., 2018b). Species of AM fungi could then coexist if they selectively associate and collaborate with PMB that differ in P_o acquisition capability and hence in the ability to deplete the (potentially available) soil P pool. Such symbioses between AM fungi and PMB could allow AM fungal species to coexist in the surrounding soil, if there is a trade-off between enlarging the spatial extent of the extraradical mycelium and the ability of mining sparingly soluble P by recruiting effective P_o mobilizing microbiomes. However, no evidence is available for this mechanism so far.

The main objective of this study was to assess the mechanism by which coexisting AM fungal species might exhibit a trade-off between traits that allow occupation of a large soil volume, by extending the depletion zone and foraging far outside the roots, and traits that allow enhanced mining of P_o from more recalcitrant sources through recruiting more efficient PMBs. We hypothesized that (i) the bacterial assemblages recruited by different AM fungal species possessed different capacities for soil P_o miner-

alization, and that the species that formed most of its mycelium close to the root had the ability to access more P_o; (ii) through AM fungal species-specific recruitment of bacterial assemblages in the hyphosphere, coexistence of different AM fungal species is possible. We synthesized a community with three AM fungal species, *F. mosseae*, *R. intraradices*, and *Gigaspora margarita*. *F. mosseae*, *R. intraradices*, both members of the Glomeraceae, are efficient in space exploration, whereas the less related *G. margarita*, belonging to the Gigasporaceae, is a less efficient explorer of space. As investigating individual hyphospheres under multispecies inoculation conditions is impossible, we employed a split-root culture system and inoculated a single maize plant without or with three different AM fungal species simultaneously.

RESULTS

Lower carbon flux to and extraradical biomass of *Gigaspora* compared with the two other species

AM fungi significantly improved biomass, P concentration, and P content of host plants by 19%, 63%, and 91%, respectively (Table S1 in Supporting Information). Hyphal compartments (HCs) in the inoculated treatments showed significantly higher $\delta^{13}C$ abundance compared with the non-mycorrhizal (NM) treatment (the $\delta^{13}C$ signal of the latter treatment was approximately $-20‰$, similar to soil organic matter), indicating that photosynthetic carbon was transferred to the hyphal compartment and that no root exudates moved to the hyphal compartments. Further differences between hyphosphere and bulk soil or between the different AM fungi can also be attributed to the effects of hyphae. The higher $\delta^{13}C$ abundance was therefore caused by the AM fungal mycelium, compounds exuded by the mycelia and bacteria that used those fungal exudates. There were significant differences between AM fungal species. Hyphal compartments with both *Rhizophagus* and *Funneliformis* showed a higher $\delta^{13}C$ abundance than those with *Gigaspora* (Figure 1A), indicating more carbon in fungal biomass, more carbon in hyphal exudates and/or more carbon in bacteria that used these hyphal exudates. Based on DNA copy number as a proxy for biomass of each AM fungal species, *Rhizophagus* and *Funneliformis* produced more extraradical biomass than *Gigaspora* (Figure 1B). We noted about 100 copies of AM fungi in the NM treatment, which was considered as a background value for the methods (Kiers et al., 2011).

Higher mineralization of P_o in compartments with *Gigaspora* than with the two other species due to recruitment of more efficient PMB

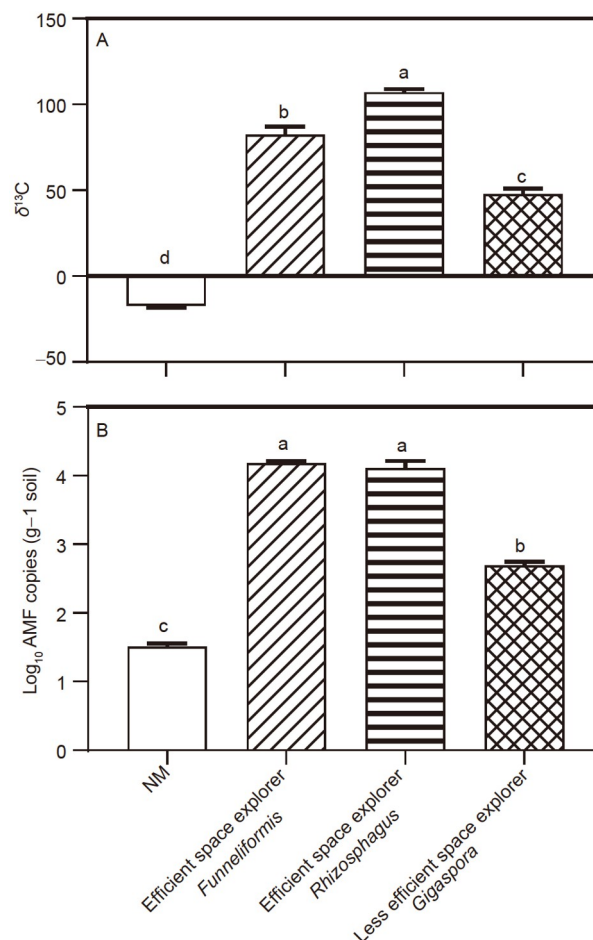All three AM fungi caused a significant decrease in labile P_o

Figure 1   Carbon allocation to different AM fungal species. A, $\delta^{13}C$ abundance in HC soil after $^{13}CO_2$ labelling for one week, which indicates the total carbon exported to different AM fungal species. B, The $\log_{10}$ AM fungal gene copies, which indicate the biomass of different AM fungal species. NM refers to non-mycorrhizal control and *Funneliformis*, *Rhizophagus*, and *Gigaspora* refer to three differently efficient spaces exploring AM fungal species in the community. Values are the means of three replicates, and the error bar means the SD. Different lower-case letters mean significant differences at $P<0.05$.

(Figure 2A), compared with the NM control. *Gigaspora* caused the largest decrease of P_o, as shown by the lowest soil P_o concentration, whereas the two other species showed a smaller decrease that did not differ significantly from each other (Figure 2A). Consistent with the decrease in soil P_o, all three AM fungal species significantly increased alkaline phosphatase (AlPase) activity compared with the NM control. *Gigaspora* had the highest AlPase activity, consistent with the largest effect on decreasing soil P_o (Figure 2B). In addition, the efficiency of P_o mobilization and AlPase production (P_o mineralized and AlPase increases by per unit $\delta^{13}C$) was higher with *Gigaspora* compared with *Rhizophagus* and *Funneliformis* (Figure 2C).

The latter two species had a significantly higher number of 16S rDNA copies in the hyphosphere compared with NM and *Gigaspora* (Figure 3A), indicating that the decline in P_o
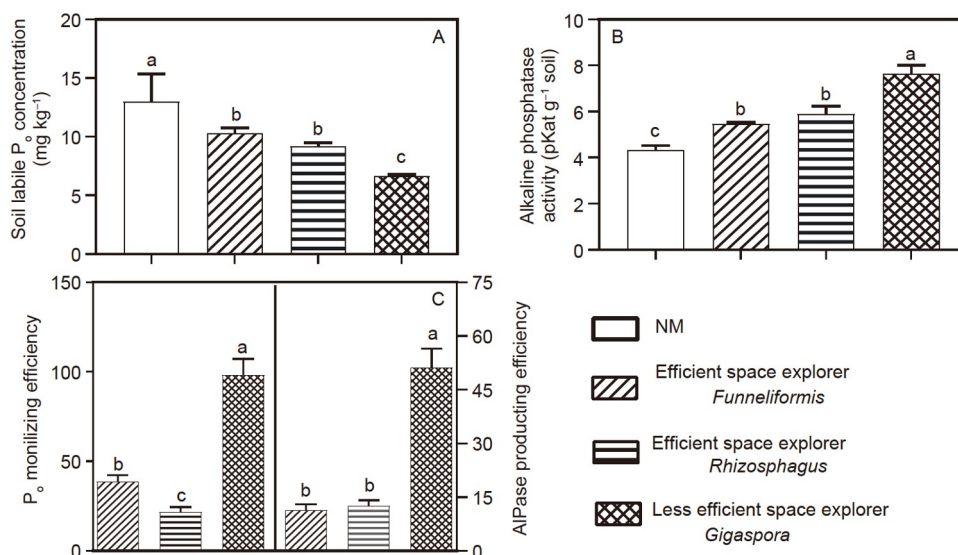
Figure 2 P_o mineralizing capability of different AM fungal species. (A) Soil labile P_o concentration, (B) alkaline phosphatase activity, (C) P_o mobilization and AlPase production efficiency index of different AM fungi. NM refers to non-mycorrhizal control and *Funneliformis*, *Rhizophagus*, and *Gigaspora* refer to three differently efficient spaces exploring AM fungal species in the community. Values are the means of three replicates, and the error bar means the SD. Different lower-case letters mean significant differences at $P<0.05$.

was not due to the number of bacteria per se but to the numbers and/or the efficiency of specific bacteria. Consistent with the positive effect on AlPase activity and P_o mineralization, the mycorrhizal treatments showed a higher number of *alp* gene copies compared with the NM treatment (Figure 3B). The *alp* gene copies number and relative abundance in hyphosphere of *Gigaspora* were higher than that of the two other species, indicating preferential recruitment of PMB by *Gigaspora* (Figure 3B and C). Expressed as bacterial gene copies recruited per unit AM fungal copies, *Gigaspora* showed twice as many total hyphosphere bacteria (as assessed by 16S rDNA copies) and 650 times as many PMB (as assessed by *alp* gene copies) compared with *Rhizophagus* and *Funneliformis* (Figure 3D). Hyphosphere AlPase activity per unit of 16S rDNA copies was also the highest in *Gigaspora*, due to higher AlPase activity and lower 16S rDNA copy (Figure 3D). There was no difference in AlPase activity per unit of *alp* gene copies between different AM fungal species (Figure 3E). There was a significant negative correlation ($r=-0.92$, $P<0.0001$) between soil P_o concentration and AlPase activity in the hyphosphere (Figure S1A in Supporting Information), implying that higher AlPase activity caused enhanced P_o mineralization. Consistent with this interpretation, there were also significant positive correlations ($P<0.001$, in both cases) between AlPase activity and *alp* gene copies (Figure S1B in Supporting Information), and between the species richness of bacteria harboring the *alp* gene and soil AlPase activity (Figure S1C in Supporting Information). Taken together, our results indicated that both the abundance and community structure of the PMB drove the increase in AlPase activity

after AM fungal inoculation.

Distinct communities of PMB were observed in the hyphosphere of different AM fungal species. The $\delta^{13}C$ signal of bacterial DNA indicated that carbon compounds exuded by the AM fungal extraradical mycelium had been consumed by the hyphosphere microbiome. Based on *alp* gene, *Pseudomonas* was the dominant taxon at generic level (Figure 4A), especially in the hyphosphere of *Gigaspora*. The community composition of ^{13}C-labelled bacterial assemblages showed differences among the three fungal species. Through nonmetric multidimensional scaling (NMDS) analysis, the community structure of the hyphosphere microbiome, assessed through *alp*, differed significantly ($P<0.01$) among AM fungal species. The closely related *Funneliformis* and *Rhizophagus* recruited closer communities of PMB (Figure 4B).

The hyphosphere microbiome showed significantly different physiological profiles

The CO_2 production of hyphosphere soils incubated with different substrates indicated the characteristics of community-level physiological profiles (CLPPs) of the hyphosphere microbiome. There were small differences among the three AM fungal species. On average inoculation with *Rhizophagus* resulted in a microbiome with the lowest use of sugars and the highest use of carboxylates (Figure 5A). All hyphosphere microbiomes used orthophosphate to the same extent, while there were significant differences between AM fungal species with the use of phytate. The hyphosphere microbiomes produced more CO_2 than the NM control after
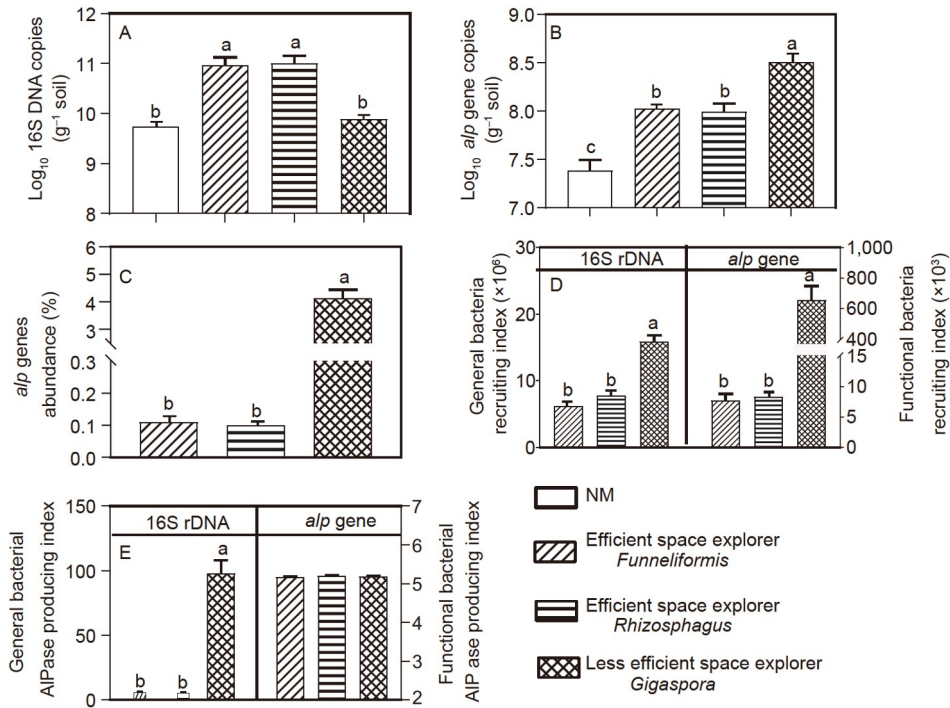
Figure 3 Hyphosphere general and P_o mineralizing functional bacterial traits. Hyphal compartment soil 16S rDNA (A), *alp* gene copy number (B) and abundance (C), the 16S rDNA and *alp* gene recruited by per AM fungal copy (D), and the AlPase activity released by per unit 16S rDNA and *alp* gene copies (E). NM refers to non-mycorrhizal control and *Funneliformis*, *Rhizophagus* and *Gigaspora* refer to three differently efficient spaces exploring AM fungal species in the community. Values are the means of three replicates, and the error bar means the SD. Different lower-case letters mean significant differences at $P<0.05$.

phytate addition (Figure 5B). The microbiome from *Gigaspora* produced most CO_2, while no difference was observed between the two other species when phytate was added, indicating functionally similar but taxonomically different bacterial communities of both members of the Glomeraceae (compare Figure 5B with 4B).

Niche differentiation between AM fungal species

Based on 15 parameters that were assessed in our study to describe the niches of the AM fungal species, cluster analysis showed that the amount of niche overlap was the largest for both members of the Glomeraceae (Figure 6). Visual inspection of Figure 6 and Figure S2 in Supporting Information shows that niche differentiation of the three AM fungal species was mainly due to differences related to the acquisition of P_o (AlPase activity, depletion of P_o, and bacterial use of phytate).

DISCUSSION

This study aimed to understand how different species of AM fungi coexist in a single plant root and surrounding soil. With respect to the coexistence of several species of AM fungi in a single habitat, studies provided evidence for spatial and

temporal niche differentiation (Dumbrell et al., 2010; Lekberg et al., 2007; Vályi et al., 2016). Coexistence could also be an outcome in case of a tradeoff between the ability to (rapidly and extensively) colonize roots and to extensively colonize the soil (Hart and Reader, 2005). Here we add a further niche dimension that contributes to AM fungal species coexistence, where some species scavenge a large soil volume for labile orthophosphate and other species acquire P from P_o from more strongly bound pools from a smaller soil volume. This mechanism suggests that some AM fungal species may be more than just scavengers and have some ability to be miners, which is ultimately due to AM fungal species-specific associations with different bacterial assemblages that differ in their ability to mineralize P_o. Coexistence of different AM fungal species would be more likely under conditions where a large fraction of P_o is mineral-associated and where disturbance is sufficiently rare not to benefit the species with the highest soil volume colonizing ability. Observations that members of the Gigasporaceae are more sensitive to the application of mineral P fertilizer and soil disturbance (Verbruggen and Toby Kiers, 2010) are consistent with our model. Further experiments along gradients of P availability and forms are needed to assess the conditions for competitive exclusion and coexistence.

Previous studies have shown that AM fungi recruit or are associated with hyphal-wall associated PMB to transform P_o
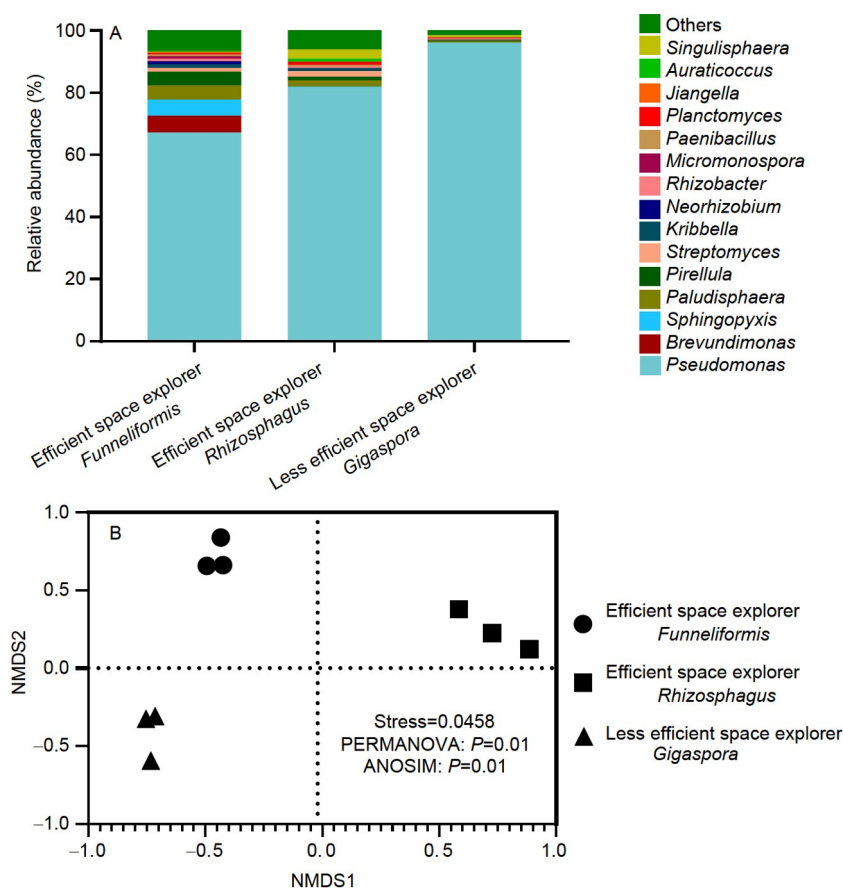
Figure 4 *alp* gene harbouring bacterial community structure at the genus level (A) and NMDS analysis plot (B). NM refers to non-mycorrhizal control and *Funneliformis*, *Rhizophagus* and *Gigaspora* refer to three differently efficient spaces exploring AM fungal species in the community. The values shown in (A) and (B) are the means of three replicates.

into bioavailable orthophosphate as the AM fungi lack genes coding for external phosphatases (Emmett et al., 2021; Tisserant et al., 2013; Zhang et al., 2018a; Zhou et al., 2020). We offer the following lines of evidence that AM fungi select specific PMB in their hyphosphere: (i) each AM fungal species recruited its own distinct *alp*-gene harboring microbiome (Figure 4B); (ii) a significantly higher CO_2 production in the CLPPs test with *Gigaspora* when incubated with phytate compared with the two other species, while no difference was observed when they were incubated with orthophosphate (Figure 5B). Our results show that, although the capability to mobilize P_o is present in the hyphosphere of each AM fungal species, the less efficient space explorer, *Gigaspora*, is more efficient than the two members of the Glomeraceae, which are more efficient in exploring a large soil volume (Figures 2, 3 and 5). As a result, niche differentiation due to differential P acquisition capabilities among coexisting fungi was achieved (Figure 7; Figure S2 in Supporting Information). Distinguishing how different AM fungal species recruit their PMBs from soil microbial communities is the next challenge.

Recruitment of hyphae-associated microbiomes through

fungal exudates might incur a C cost for the fungus, as mycorrhizal fungi are generally considered to be C-limited. Exudation by AM fungi has been reported by Toljander et al. (2007), Drigo et al. (2010), Kaiser et al. (2015), and Wang et al. (2016) possibly associated with enhanced nutrient acquisition. The extent to which these exudates select for specific consortia of bacteria has been less clear. While it has been suggested that C and P exchange may enable co-operation between AM fungus and PMB (Zhang et al., 2016), the question whether these co-existing fungi can detect, discriminate, and select the best PMB from the soil microbial pool is largely unresolved. Our results demonstrated specific recruitment of bacteria with the capability to access mineral-bound organic P by the fungus that is less efficient in space exploration. Firstly, the less efficient explorer recruited a more efficient PMB community which exhibited the highest AlPase activity and mobilized most P_o per unit δ^{13}C-hyphal exudates (Figure 2). Secondly, it also recruited more abundant PMB and *alp* gene copy numbers per unit fungal DNA copy number (a proxy for extraradical AM fungal biomass) (Figures 3 and 4). Thirdly, the PMB recruitment index (ratio of *alp* gene copies to 16S rDNA
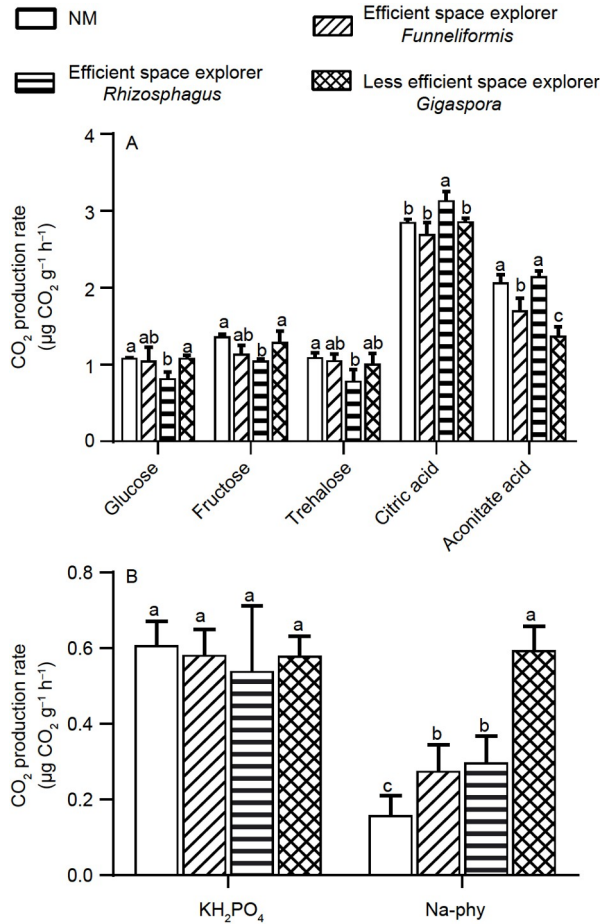
Figure 5 The community-level physiological profiles of the microbiome associated with different AM fungi. The CO_2 production rate after the addition of carbon (A) or phosphate (B) substrates. NM refers to non-mycorrhizal control and *Funneliformis*, *Rhizophagus* and *Gigaspora* refer to three differently efficient spaces exploring AM fungal species in the community. Values are the means of three replicates, and the error bar means the SD. Different lower-case letters mean significant differences at $P<0.05$.

Figure 6 Cluster analysis of all the standardized data. All the data were standardized. NM refers to non-mycorrhizal control and *Funneliformis*, *Rhizophagus* and *Gigaspora* refer to three differently efficient spaces exploring AM fungal species in the community. ** mean significant differences at $P<0.01$.

copies) of the less efficient space explorer was 40 times higher than that of the two other fungi (Figure 3C). More than that, the partners recruited by the less efficient explorer conducted P_o mobilization more efficiently. Although there was no difference in the abundance of the functional gene (*alp* genes) (Figure 3E), higher P_o mineralization ability was observed with the microbiome of *Gigaspora* than that of two members of the Glomeraceae because of its higher abundance of *alp* gene copies and AlPase efficiency (Figures 2 and 3). A possible mechanism is that *Gigaspora* allocated more C to its associated PMB (Figures 1 and 3D). Hyphal compartments in the inoculated treatments showed significantly higher ^{13}C abundance compared with the NM treatment. There were also significant differences between AM fungal species, with *Rhizophagus* inoculation resulting in the highest ^{13}C abundance and *Gigaspora* inoculation in the lowest (Figure 1A). Q-PCR showed that all inoculated treatments had almost two to three orders of magnitude
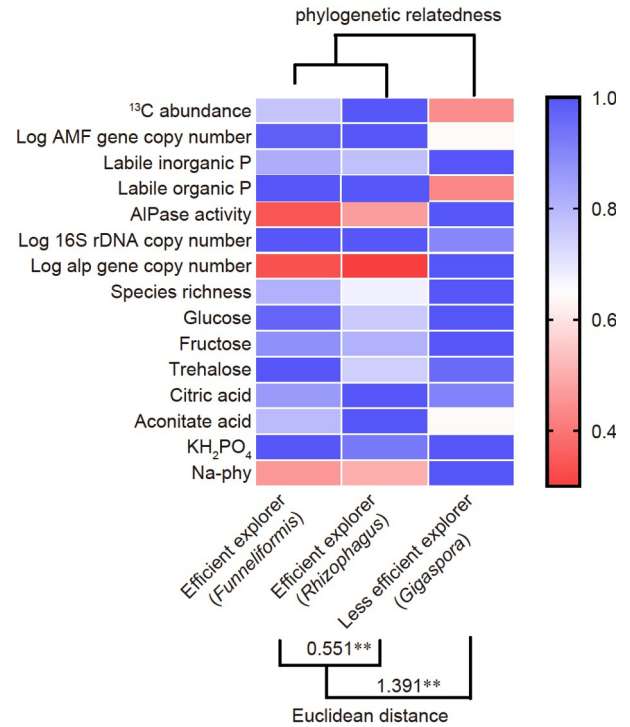
higher numbers of DNA copies than those of control treatments (Figure 1B). Consistent with the ^{13}C signal, samples with *Gigaspora* contained lower AM fungal DNA copy number than samples with *Rhizophagus* and *Funneliformis* (Figure 1B). ^{13}C abundance per AM fungal gene copy was 10 times higher in *Gigaspora* hyphospere than in the hyphosphere of the two other species (Figure 2). These results suggest a trade-off between space exploration and ability to recruit PMB, a trade-off that enabled species to coexist. The *alp*-carrying bacterial community of *Gigaspora* was dominated by *Pseudomonas*, which was consistent with Ragot et al. (2015), suggesting that *Pseudomonas* spp. might be key players in hyphosphere PMB community. The mechanism through which *Gigaspora* recruited more *Pseudomonas*, should be further studied.

Similarities and differences in the PMB communities and their function were consistent with the fungal phylogeny (Zhou et al., 2020). Previous studies have equally demonstrated that phylogenetically closely related species are more similar in morphological and physiological traits, and this similarity apparently extends to the bacterial assemblages that these species select (Chen et al., 2017; Maherali and Klironomos, 2007; Slingsby and Verboom, 2006). *Rhizophagus* and *Funneliformis*, both members of the Glomeraceae, harbored functionally similar PMB communities with respect to P_o mineralization capability (Figures 4 and 5),
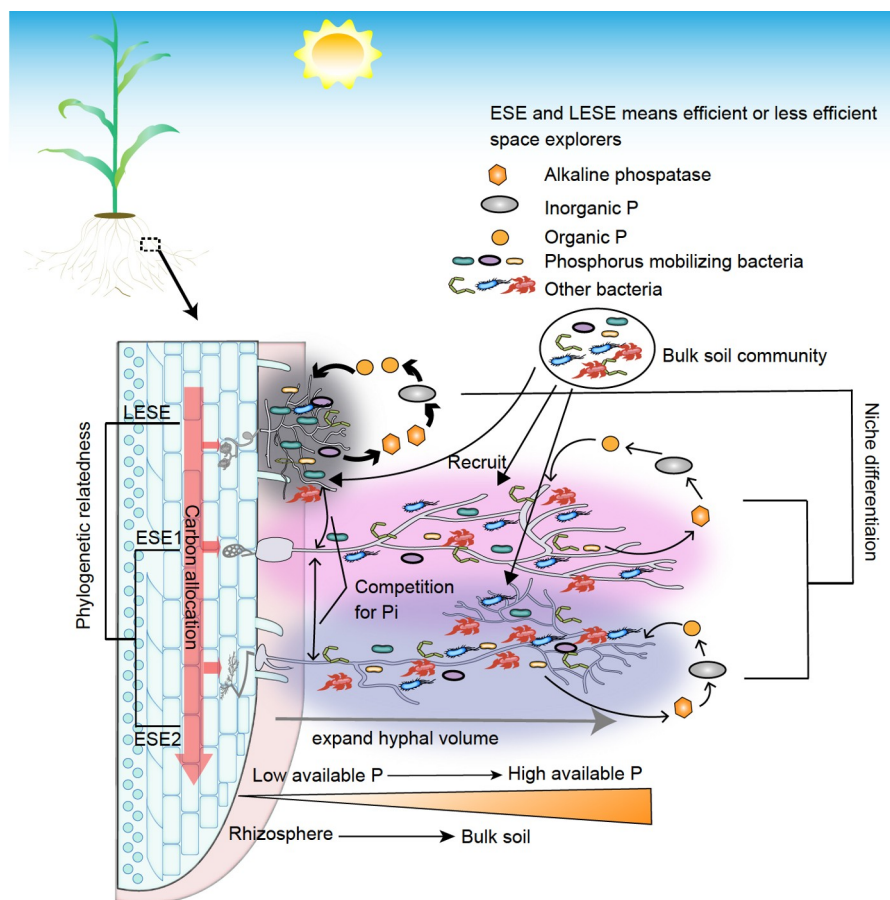
Figure 7   The summary graph of this study. Mazie was selected as the experimental host plant. Three AM fungal species, *Funneliformis*, *Rhizophagus*, and *Gigaspora* co-colonized the root. *Funneliformis* and *Rhizophagus* expanded their hyphae more than 10 cm and were classified as efficient space explorers (ESE, ESE1, and ESE2, respectively), and *Gigaspora* formed most of their hyphae around the root and was classified as inefficient space explorer (LESE). The rhizosphere was in a P-limited condition compared with bulk soil. There was a strong competition of inorganic P (P_i) not only between AM fungi and root, but also between different AM fungal species. To adapt to this environment, two ESE species allocated more carbon to explore the space of bulk soil far away from the root and where was rich in available P_i. The LESE species only formed their hyphae near the root, where is limited in P_i and allocated more carbon to recruit more powerful P_o mineralizing bacteria. All these three AM fungal species recruited their specific hyphosphere microbiome, and the microbiome of LESE hyphosphere was in bigger abundance of phosphorus solubilizing bacteria and was more efficient in organic P mineralization. This trade-off in carbon allocation to space exploring and PMB recruiting might help the niche differentiation of different AM fungal species.

even though these bacterial communities were taxonomically different (Figure 4B). These differences in bacterial community composition between both members of Glomeraceae suggest that even in taxonomically related AM fungal species bacterial microbiome differentiation can contribute to fungal species coexistence. Possible additional mechanisms for AM fungal species coexistence include differences in the production of specific exudates, potential benefits with strongly adsorbed mineral P sources, and/or other benefits by the specific hyphosphere microbiomes unrelated to P acquisition. These mechanisms need to be further explored in subsequent experiments. Our findings support the phylogenetic conservatism hypothesis that implies that more related species show larger functional similarities (Cavender-Bares et al., 2004; Maherali and Klironomos, 2007; Webb, 2000).

Our data cannot resolve the question about the origin of this close association between AM fungal species and a specific element of the hyphosphere microbiome (Emmett et

al., 2021). While it could be possible that every AM fungus recruits its assemblage of the microbiome from a common microbial pool in bulk soil, we consider it more likely that this association constitutes a case of co-evolution of the AM fungi and their hyphosphere bacterial assemblages. AM fungi are unable to produce phytases (Morin et al., 2019; Tisserant et al., 2013) and tight associations between specific hyphosphere PMBs and individual AM fungal species might therefore compensate for the lack of phytases (Zhang et al., 2016; Zhang et al., 2020). Genomic sequencing showed that, compared with *Rhizophagus*, *Gigaspora* possesses a larger secretome, indicating that it might release more carbon to the hyphosphere (Morin et al., 2019). In addition, *Gigaspora* also encodes more phosphate-related genes than the other two Glomeraceae fungal species (Venice et al., 2020). Further direct studies are needed to explain the mechanism through which AM fungi recruit their distinct PMB community, as the bacterial communities were able to use the

same carbon sources at comparable rates, however, exudates likely constitute a more complex cocktail than the few simple compounds tested by us.

In conclusion, we found that different AM fungal species recruited distinct P_o mobilizing bacterial assemblages. *Gigaspora*, the fungal species that is the less efficient explorer of soil space, recruited more powerful P_o mobilizing bacterial partners than *Rhizophagus* and *Funneliformis*. This trade-off between space exploration and recruitment of an efficient P_o mobilizing microbiome enabled niche differentiation and contributed to the coexistence of AM fungal species in a single plant root and surrounding soil habitat (Figure 7). The phylogenetic relatedness of AM fungi not only influenced its ecosystem function but also shaped the community and function of their recruited microbes.

MATERIALS AND METHODS

Soil

A slightly acidic soil (Inceptisol according to the USDA classification system) from Tai'an, Shandong Province, China ($36^o10'N$, $117^o09'E$) was used. Physicochemical properties of the soil are described in Table S2 in Supporting Information. The soil was air-dried and sieved (2 mm). In order to easily extract fungal hyphae from the soil, we used a fine soil-sand mixture medium in the hyphal compartments. Approximately 1 kg of air-dried soil was placed into a 5 L bucket to which 3–4 L tap water was added and the soil was then completely brought into suspension by stirring. The suspension was poured through a sieve with mesh width of 30 μm. This procedure was repeated 3 times on each 1 kg soil portion. The sieved soil suspension was collected in another bucket and allowed to settle until the water above the soil layer had become clear and could be siphoned off using a flexible tube. The remaining sludge was transferred to a shallow, heat-resistant dish and dried at 60°C until the material had become solid. The sand was also washed 9–10 times to remove nutrients. The remaining sand was dried at 60°C and sieved through 1 mm mesh. The 2 mm and 30 μm sieved soil and 1 mm sieved sand were sterilized (25 kGy, ^{60}Co γ-rays) in the Beijing Atomic Energy Research Institute (Beijing, China) to eliminate indigenous microorganisms and AM fungal propagules. Nutrients were added to 1 kg soil as indicated in Table S3 in Supporting Information. Previous studies have demonstrated that AM fungi recruit a hyphosphere microbiome that stimulates the mineralization of P_o (Zhang et al., 2016; Zhang et al., 2018b). We, therefore, added 100 mg phytin kg^{-1} soil (TCI, Japan) (which equals 20 mg P kg^{-1} soil) to the hyphal compartment as a P_o source. In addition, the nitrification inhibitor 3,4-dimethyl pyrazole phosphate (DMPP, EuroChem Agro GmbH, Germany) was added, at a rate of 1% (w/w) of the N applied.

Host plant

Maize (*Zea mays* L., cv. Xianyu 335) seeds were surface-sterilized with 10% (v/v) H_2O_2 (Wang et al., 2013), and germinated on moist filter paper for 2 days at 26°C in the dark. The seeds were then transferred to 40 cm×25 cm moist filter paper for 7 cm days (12 h light, 12 h dark, 26°C) to allow the roots to expand. Seedlings of similar size and with 10 roots (including taproot) were selected, and one plant was transplanted into each microcosm.

Experimental design

The AM fungal community consisted of three AM fungal species, *viz*. *R. intraradices* (EY108), *F. mosseae* (MD118), both belonging to the Glomeraceae, and *G. margarita* (JA101A*)*, belonging to the Gigasporaceae (Morton, 1993). The inocula were purchased from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM). *R. intraradices* and *F. mosseae* extend their hyphae more than 10 cm out of the root, and are considered efficient space explorers, while *G. margarita* extends its hyphae around 2 cm and is considered a less efficient space explorer (Thonar et al., 2011). For the NM control, we added sterilized AM fungal inoculum of the three species instead. Each treatment was replicated six times. Through PCR test, no cross contamination between different AM fungal species was observed (Figure S3 in Supporting Information, primers were described in Table S4 in Supporting Information, Jansa et al., 2003).

Microcosms

To test the microbiome associated with the extraradical mycelium of each individual AM fungal species that simultaneously colonized the same root system, we used a split-root and compartmented microcosm system that allowed separating the growing spaces of plant root systems and the extraradical mycelium of the AM fungal species (Figure S4 in Supporting Information). The microcosms were constructed using PVC plates, and consisted of nine compartments. The three middle compartments were separated by PVC plates and were used for split-root growth (root compartment, RC). The outer compartments (hyphal compartment, HC) were separated from the RCs by a 1 cm buffer zone (Figure S4 in Supporting Information). The buffer zone consisted of two 30 μm mesh sheets that allowed AM fungal hyphae to pass through, but prevented root penetration. Each HC was separated into two parts of which HC_a was filled with 30 μm sieved soil mixed with river sand and HC_b was filled with 2 mm sieved soil. The microcosms received the following amounts of soil-river sand mixture or soil: 900 g soil-river sand mixture (450 g soil+450 g river sand) in HC_a

and 900 g in each RC and HC$_b$. The soil or river sand mixture was added very carefully to each compartment to maintain equal bulk density in RC and HC.

AM fungal and bacterial inoculant

The three AM fungi were propagated on a mixture of maize (Nongda 108) and *Plantago depressa* Willd. in zeolite and sand for five months; spore density was about 20 spores g^{-1} substrate. In order to keep the same RC original microbiota, 5 mL of the filtrate of the two other AM fungal inocula was added to each RC together with the inoculation with the one target AM fungal inoculum. For the non-mycorrhizal control, 5 mL of the filtrate of the three AM fungal inocula was added to each RC. Five milliliters of soil filtrate was added to the RC, HC$_a$, and HC$_b$ as the original hyphal compartment microbiota. The filtrate of inoculum or soil was obtained by suspending 30 g of unsterilized inoculum or soil in 300 mL sterile water and filtration through six-layer quantitative filter paper (properties similar to Whatman Grade 43) (Wang et al., 2013), which allowed passing of common soil microbes, but effectively retained spores and hyphae of AM fungi.

Experimental procedure and $^{13}CO_2$ pulse labelling procedure

Plants were grown in a greenhouse at China Agricultural University in Beijing at 24°C/30°C (night/day). Soil gravimetrical moisture was kept at 18%–20% (w/w, ~70% water holding capacity) with deionized water added to weight every 2 days during the experiment. To trace the transfer of plant-derived C from mycorrhizal hyphae to hyphosphere microbes, $^{13}CO_2$ stable-isotope pulse labelling was conducted in the greenhouse for 7 days before harvest. Seven weeks after sowing, the maize plants were subjected to $^{13}CO_2$ (99% of ^{13}C atom) pulse labelling in an airtight Plexiglas growth chamber (Figure S4 in Supporting Information). The carbon isotope ratios of soil samples were determined using a DeltaPlusXP mass spectrometer (Thermo Fisher Scientific, Germany) coupled with an elemental analyzer (FlashEA 1112; CE Instruments, UK) in the continuous flow mode at the Stable Isotope Laboratory of the College of Resources and Environmental Sciences, China Agricultural University, Beijing, China. The elemental analyzer combustion temperature was 1,020°C. The carbon isotopic ratios were reported in the delta notation relative to the V-PDB (Vienna-Pee Dee Belemnite) standard using the following equation:

$$\delta^{13}C = -\left(R_{\text{sample}} / R_{\text{standard}}\right) \times 1000,$$

where $\delta^{13}C$ is the carbon isotope ratio of the sample in parts (‰), and R_{sample} and R_{standard} are the $^{13}C/^{12}C$ ratios of the sample and standard, respectively. The SD for the $\delta^{13}C$ measurements was <0.15‰ (Deniro and Epstein, 1978).

Harvest and sample analysis

The plant and soil samples were harvested one week after labelling. The microcosms were dismantled and separated into root and hyphal compartments. To prevent contamination of the surface soil in the hyphal compartment with exotic bacteria, we removed the top 2 cm of soil to reduce the potential influences on hyphosphere soil samples. In order to determine the effect of AM fungi on plant growth, the shoot was separated from the root and dried after which biomass and P concentration were determined according to Thomas et al. (1967). HC$_a$ soil was collected and stored at −20°C for hyphae collection. HC$_b$ soil was collected and a part of it stored at room temperature, while another part was stored at 4°C for assessment of chemical properties. In addition, samples of three HC$_a$ and HC$_b$ from the control were mixed as the samples of the non-mycorrhizal treatment. Soil P$_o$ was extracted by NaHCO$_3$, and measured according to Schoenau and Huange (1991). Alkaline phosphatase (AlPase) activity in HC$_b$ was measured following Neumann (2006).

Genomic DNA from HC$_b$ was extracted using the FastDNA SPIN Kit (MP Biomedicals LLC, USA) following the manufacturer's instructions. To determine the DNA-based AM fungal biomass, we used AM fungi genera primers for measuring the three fungi by real-time qPCR in a q-TOWER q-PCR analyzer (Jena, Germany). With the same instrument, we measured the gene copy number of AM fungi, 16S rDNA and AlPase-encoding gene (*alp*) DNA with 18S rDNA, 16S rDNA and *alp* gene-specific primers (Table S4 in Supporting Information) (Biddle et al., 2008; Fraser et al., 2015; Sato et al., 2005). SYBR Green real-time qPCR Master Mix (TOYOBO, Japan) mix was used and conducted under the following reaction conditions: 5 min of initial denaturation at 94°C; 40 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 60°C, and 1 min of elongation at 72°C. Fluorescence of SYBR green was detected after every cycle. The melting curve was collected when the whole reaction ended in 0.5°C increments from 65°C to 95°C. The amplification of the PCR reactions had an efficiency of 1.99, where 2 is the highest quality representing doubling each amplification cycle (Tellmann, 2006) and an error value of 0.002 calculated as the mean squared error of the standard curve. No amplification was detected in the negative controls.

The characteristics of AM fungal hyphae associated microbiome as assessed by CLPPs

We analyzed CLPPs by MicroResp^{TM} method (The James Hutton Institute, Dundee Scotland, UK) (Campbell et al., 2003) following the manual. We used five carbohydrates (glucose, fructose, trehalose, citric acid, aconitic acid) to

represent major compounds in hyphal exudates of AM fungi (Toljander et al., 2007; Zhang et al., 2016; Zhang et al., 2018a) and two P forms (potassium dihydrogen phosphate and sodium phytate) to represent inorganic and organic phosphates in soil. The measurements of CO_2 production rate after adding these substrates to the harvested soil samples indicate the physiological and metabolic capability of the AM fungus-associated microbiomes in using these substrates.

Extraradical mycelium collection, DNA extraction, density gradient centrifugation and q-PCR analysis

The extraradical hyphae of different AM fungi were collected using wet-sieving. AM fungal DNA and hyphosphere genomic DNA were extracted using the FastDNA SPIN Kit (MP Biomedicals LLC) following the manufacturer's instructions. To determine the active PMB that was stimulated by the hyphal exudates, density gradient centrifugation was conducted and identified using a qPCR test using the primers described in Table S4 in Supporting Information.

Alkaline phosphatase encoding gene (*alp*)-based MiSeq sequencing

Fractions with a buoyant density of approximately 1.58 were quality-checked, and then the DNA samples were sent to the Shanghai Personal Biotechnology Company (Shanghai, China) for functional-gene (*alp*) sequencing on an Illumina PE300 system. The *alp* gene was amplified using the primers set: *phoD*-F733 and *phoD*-R1083 described in Table S4 in Supporting Information. The DNA samples from the NM control soil were used as references for the original soil microbiome. In addition, ^{13}C-labeled samples were used as the active hyphosphere microbiome that was directly influenced by hyphal exudates.

Processing of sequencing data

The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline was employed to process the sequencing data (Caporaso et al., 2010). Briefly, raw sequencing reads with exact matches to the barcodes were assigned to respective samples and identified as valid sequences. The low-quality sequences were filtered using the following criteria: sequences that had a length of <150 bp, sequences that had average Phred scores of <20, sequences that contained ambiguous bases, and sequences that contained mononucleotide repeats of >8 bp were discarded. Paired-end reads were assembled using FLASH. After chimaera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST. A representative sequence was selected

from each OTU using default parameters. The OTUs classification was conducted by BLAST searching the representative sequences set against the Greengenes Database (DeSantis et al., 2006) using the best hit. An OTU table was further generated to record the abundance of each OTU in each sample and the taxonomy of these OTUs. Any OTU containing less than 0.001% of total sequences across all samples were discarded. To minimize the difference in sequencing depth across samples, an average, a rounded rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under 90% of the minimum sequencing depth for further analysis.

Calculations of indices that indicate hyphosphere C and P processes

In order to indicate the capability of each fungal species to mobilize soil P_o or to produce AlPase activity per unit C consumed, we calculated the ratio of P_o mobilizing efficiency and AlPase producing efficiency index by following formula:

(P_o concentration$_{NM}$−P_o concentration$_{AMF}$)/($\delta^{13}C_{AMF}$−$\delta^{13}C_{NM}$).

As the soil bulk density and the total weight of soils in different treatments were the same, the reduction of labile P_o concentration in soil reflects the mobilization.

AlPase producing efficiency index was calculated similarly as:

(AlPase activity$_{AMF}$−AlPase activity$_{NM}$)/($\delta^{13}C_{AMF}$−$\delta^{13}C_{NM}$).

The *alp* gene abundance was calculated as the ratio of ^{13}C labelled *alp* gene copies against ^{13}C labelled 16S rDNA copies to indicate the preference of AM fungal species in recruiting PMB.

The general and functional bacterial recruitment indexes were calculated to indicate the efficiency of different AM fungal species in recruiting bacteria.

We calculated the ratio of AlPase activities or decrease of P_o content against ^{13}C labelled 16S rDNA and *alp*-gene copies to indicate the capabilities in producing active AlPase or in mobilizing soil P_o based on equal amounts of general bacteria and *alp*-gene copies.

Calculation of niche differentiation

Shannon-Wiener index was used for calculation of niche breadth. The formula is as follows: $B = -\sum_{i=1}^{s} P_{ij} \ln P_{ij}$, $B \in [0, \ln s]$. Where B is the niche breadth Shannon-Wiener index; Pi is the performance (CO_2 production, in µg C-CO_2 g^{-1} h^{-1}) of the microbiome on carbon source i or Pi source i. When $B=0$, the microbiome uses only one C source and hence has the narrowest niche; when $B=1.61$, all five C sources are equally used (Levins, 1968). The Simpson index of evenness was

used for detecting the distribution of populations in organic P among all resources. The formula was modified from Levins (1968) as follows: $B=N_{Po}/N$, $B\in[0, 1]$. When $B=1$, the microbiome has the narrowest niche and the largest niche specialization.

The PONS (proportional of niche similarity) (Feinsinger et al., 1981) was calculated following the formula:

$$PONS_{ij}=1-0.5\sum|P_{ih}-P_{jh}|,$$

where P_{ih} is the percentage of utilization of h resource position by species i in all its resources; P_{jh} means the proportion of species j in the same resource position.

Statistical analysis

Shoot biomass, P concentration, and P content were analyzed separately for the NM or AM treatment. A posteriori comparison was made using t-tests ($P<0.05$) by SPSS v16.0.

Original and calculated data from different HCs were compared to determine the difference between different AM fungal species. Data from the NM control were also compared with these AM fungal species to determine fungal species-specific effects. One-way ANOVA with $P<0.05$ was employed to assess significance. Before ANOVA, DNA copy number, a proxy for extraradical mycorrhizal biomass in soil, was $\log_{10}$ transformed. Likewise, the data for the relative abundance of $\delta^{13}C$ in HC soil, and taxonomic group (phyla and classes) abundance were arcsin transformed.

Bray-Curtis distances of *alp* genes in NMDS were calculated by QIIME software, and then analyzed by vegan package in R (v 4.0.2). The significance of the data was estimated using PERMANOVA and ANOSIM with $P<0.05$ by vegan package in R (v 2.4.2).

Finally, the data that were pre-standardized in dividing by the largest value of each index were used to conduct cluster analysis. The cluster analysis with Euclidean distance was employed to detect differences between different AM fungal species.

Availability of data and materials

The Illumina MiSeq sequence datasets are available at the NCBI Sequence Read Archive BioProject ID PRJNA718990. All other data are available in the main text or supplementary materials.

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SUPPORTING INFORMATION

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