***New Phytologist* Supporting Information**

Article title: A core microbiome in the hyphosphere of arbuscular mycorrhizal fungi has functional significance in organic phosphorus mineralization

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The following Supporting Information is available for this article:

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Fig. S1 Details of the experimental sites and experimental system. **a** Geographical location of the three experimental sites in China. **b** The in-growth tubes were buried to a depth of 20-30 cm in soil and 15 cm away from the plants. PVC tubes (12 cm in diameter, 5 cm in length) were sealed with 30 μm mesh (permitting AM fungal hyphae but not roots to grow into) or 0.45 μm membrane (excluding AM fungal hyphae and roots to grow into) in the two ends, respectively. **c** Schematic drawing of the in-growth tubes. **d** Schematic drawing of the two-compartment cultivation system (microcosm) used in the pot experiment. The microcosm is divided into a root compartment and a hyphal compartment by two 30 μm nylon meshes and a buffer zone. **e** The hyphae accumulated on the surface of the membranes. AM fungal hyphae were manually collected using a stereoscope, according to their different characteristics from other fungi. AM fungal hyphae are colorless and transparent, and have no septum. The collected hyphae were washed five times in washing buffers (0.01 M Phosphate buffer saline, pH = 7) to remove the adhered soil.

Fig. S2 The ratio of AM fungal to total fungal DNA copy number in hyphal samples across three sites. The higher the ratio, the greater the proportion of AM fungi in our hyphal samples. Control indicates the pure AM fungal hyphal samples collected from AM fungal inoculum. Values are means (n = 8) and bars represent standard errors.

Fig. S3 Rarefaction curve of OTUs for individual sample across the different datasets: **a** AM fungal 18S rRNA gene dataset in Experiment 1, **b** hyphosphere and bulk soil bacterial 16S rRNA gene dataset in Experiment 1, **c** hyphosphere and bulk soil bacterial 16S rRNA gene dataset in Experiment 2.

Fig. S4 Mycorrhizal colonization (**a**) of host plants and hyphal length density (**b**) in the in-growth tubes at each site. Values are means (n = 8) and bars represent standard errors.

Fig. S5 Correlation matrix (Spearman) of phosphatase activities and soil properties in the tubes across the three sites. Positive correlations are displayed in red and negative

correlations in blue. Color intensity is proportional to the correlation coefficients. Correlations significant at $P < 0.05$ were not marked with a cross.

Fig. S6 Box plot showing Shannon-Wiener diversity indices (**a**) and observed species indices (**b**) for three communities (AM fungi, hyphosphere and bulk soil bacteria). For each community, the asterisk indicates significant difference among three sites. The asterisk on the solid line represented the significant difference between bacteria in the hyphosphere and bulk soil. Boxes show first quartile, median and third quartile. Whiskers extend to the most extreme points within 1.5 × box length, and the points are values that fall outside the whiskers. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, no significance.

Fig. S7 Bipartite networks display experimental site-sensitive OTUs (*ss*OTUs) in bulk soil (**a**) and hyphosphere (**b**) bacterial communities. Circles represented individual bacterial OTUs that are positively and significantly associated ($P < 0.05$) with one or more of sites (associations given by connecting lines). OTUs were colored according to their phyla assignment. **c** Qualitative taxonomic composition of experimental site-insensitive OTUs (non-*ss*OTUs) is reported as proportional OTUs numbers per class.

Fig. S8 *hs*OTUs are largely conserved at the class levels across three experimental sites. Phylogenetic characterization was conducted using a neighbor-joining model of 82 *hs*OTUs. Circles represent non-*ss*OTUs and triangles represent *ss*OTUs. Taxonomic information at the class and genus level are provided. Histograms show the relative abundances (as counts per million, CPM) of *hs*OTUs at three sites. Dot plots show the correlations between relative abundances of *hs*OTUs and phosphatase activities (only significant positive correlation are shown, $P < 0.05$). Point size indicates the correlation coefficient.

Fig. S9 Co-occurrence networks visualizing the significant pairwise correlations ($r > 0.8$, $P < 0.01$) between OTU pairs for bacteria in the hyphosphere, bacteria in the bulk soil and AM fungi. The color of nodes indicates the associations of OTUs with different experimental sites. The number of nodes, number of edges, average degree, and *ss*OTUs are given below the specific networks.

Fig. S10 The 16S rRNA gene copy number of the hyphosphere bacteria across three sites.

Fig. S11 Mycorrhizal colonization (**a**) of host plants and hyphal length density (**b**) in the hyphal compartments under each soil type in Experiment 2. Values are means ($n = 8$) and bars represent standard errors. Different letters indicate significant differences among four soil types under the same inoculation treatment ($P < 0.05$).

Fig. S12 Difference of P contents and phosphatase activities in the hyphal compartment (HCs) inoculated or not inoculated with AM fungi in Experiment 2. (**a**) inorganic P, (**b**) organic P, (**c**) alkaline phosphatase (ALP) activity and (**d**) acid phosphatase (ACP) activity in the HCs. Data are means (n = 6) + standard error. HN indicates acidic red soil collected from Hunan site, SX indicates loessial soil collected from Shaanxi site, XJ indicates grey desert soil collected from Xinjiang site, and DB indicates black soil collected from Jilin site. Different letters indicate significant differences among four soil types ($P < 0.05$). The asterisk indicates significant differences between the hyphal compartments with and without the presence of AM fungi. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. S13 Profiling of hyphosphere bacterial communities in Experiment 2. **a** Histogram diagram showing the relative abundances of major orders of hyphosphere and bulk soil bacteria across four soil types. **b** Box plot showing Shannon-Wiener diversity indices for bulk soil and hyphosphere bacterial communities. The asterisk indicates significant difference between the bulk soil and the hyphosphere. **c** VENN plot showing the number of OTUs enriched in the hyphosphere at each soil type. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. S14 Qualitative taxonomic composition of module 4 (M4) in Experiment 1 and module 30 (M30) in Experiment 2 is reported as proportional OTUs numbers per class.

Table S1 Physicochemical properties of the soil used in Experiment 1 and 2.

Table S2 The comparison between the hyphosphere core microbiome in our study and the root-associated core microbiome in previous studies. “Y” represents “yes” under “Also core in”. Further details of the maize and cotton root-associated core microbiome can be found in Walters *et al*. (2018) and Zhang *et al*. (2022).

Methods S1 Details of the experimental set-up.

Experiment 1: field sampling experiment

The field sampling experiment was carried out in three long-term soil fertility experiments at Qiyang, Hunan Province; Yangling, Shaanxi Province; and Wulumuqi, Xinjiang Province, China, representing moist, semiarid, and desert climatic conditions. These three experimental sites belonged to The National Long-term Monitoring Network of Soil Fertility and Fertilizer Effects, which was founded in 1990. To characterize the complex interactions between AM fungi and hyphosphere bacteria *in situ*, we buried in-growth tubes to collect AM fungal hyphae *in situ*. For each site, all growth tubes were buried at the beginning of sowing and excavated at crop maturity in 2018, i.e. 90 days after being buried. Further details regarding soil properties, crop rotation and climate conditions can be found in Zhang *et al*. (2009), Khan *et al*. (2018), and Peng *et al*. (2021).

The complex problem of studying AM fungal-bacterial interactions under field conditions was exemplified by how to retrieve the AM fungal hyphae from the soil. A mixture of soil (< 30 μm) and glass beads has been used previously to collect hyphae (Chen *et al*., 2001; Zhang *et al*., 2018). But this approach will change soil physicochemical properties, and cannot explore the effects of soil types. Therefore, we established two types of in-growth tubes (sealed with 30 or 0.45 μm mesh) combined with the membrane to collect hyphae for this experiment (Fig. S1c, d). The membranes with the above size of pores are usually used to study nutrient (e.g., N and P) mobilization and utilization by the interaction between AM fungi and soil bacteria (Hodge *et al*., 2001; Zhang *et al*., 2018).

The soil filling in the tubes was collected from the top 20 cm in the experimental sites. In order to minimize the disturbance to the soil, we did not sterilize it. The soil was passed through 2 mm sieve to remove root fragments, and then packed into tubes. The soil bulk density varied from 1.2 to 1.5 g^{-1} cm^3 across three sites, and we took the mean (1.35 g^{-1} cm^3). To make the results at the three sites comparable, we choose the Loessial soil (SX site) as the benchmark, the weight of the soil that needs to be added to the tubes is calculated based on the soil bulk density. The weight of the soil in the tubes at the other two sites (HN and XJ) remained the same as for the SX site.

We imbedded the membranes in each tube to induce the growth of the hyphae on the membrane (Fig. S1c). The membranes were sterilized by gamma irradiation (25 kGy, ^{60}Co gamma rays). The principle is that the AM fungal hyphae enter the tube, and after

encountering the membrane, the hyphae grow and accumulate against the membrane, which is convenient for collection. Firstly, we sealed one end of the tube with 30 or 0.45 μm mesh, then added one sixth amount (150 g) of the soil in the tube, gently pressed the soil with a rod, and made the soil surface flat and centered. Then we put the sterilized Poly tetra fluoro ethylene membrane (10 cm in diameter, Shanghaixinya corporation, China) in the tube, and again added 150 g of the soil. We repeated the above procedures until all five membranes were placed into the tube. Finally, we pressed the soil and made the surface flat, and covered it with a 30 or 0.45 μm mesh (Fig. S1c). In the tubes, the membranes were all tightly attached to the soil. The in-growth tubes were buried in close proximity to the roots in the field (a depth of either 20-30 cm in soil 15 cm away from a plant; Fig. S1b). And the 30 μm mesh outsides the tubes did not hinder the growth of the hyphae into the tubes. The length of the in-growth tube was only 5 cm, previous research has shown that AM fungal hyphae could grow up to 12 cm from the root surface (Li *et al*., 1991a). Therefore, the extraradical hyphae of AM fungi colonizing the roots can successfully grow into the tubes and reach the membrane surface. Moreover, the effects of the different pore sizes in the membranes on bacterial migration and P diffusion can be neglected in this in-growth tube and has been demonstrated to be negligible, so the bacteria can also access the tube freely (Li *et al*., 1991b; Zhang *et al*., 2018; Zhang *et al*., 2020).

We used a stereoscope to manually collect the hyphae accumulated on the surface of membranes and measured the ratio of AM fungal and all fungal gene copy number to validate that the vast majority of hyphae we chose were AM fungal hyphae (Fig. S2a). The 0.45 μm membrane prevented AM fungal hyphae penetration, but allowed soil solution flow and bacterial migration (Li *et al*., 1991b; Zhang *et al*., 2018; Zhang *et al*., 2020). Importantly, hyphal exudates can act on the soil bacteria through the membrane. As the mesh was tightly combined with soil before AM fungal mycelium arrived, the hyphae on the mesh had a close contact with the fine soil particles (Fig. S1c, d). Therefore, the bacteria on the surface of membrane living hyphae were consistent with the microbiome on the soil living hyphae. And the mycelium segments collected from the membrane can represent the mycelium growing in soil.

Experimental 2: the microcosm experiment

Soil

Soil was collected from locations within each of the same fields used in Experiment

1, plus a black soil collected from Changchun, Jilin. The collected soil was air dried, sieved (2 mm) and the following nutrients were added to one kg of soil: 200 mg N (NH_4NO_3), 200 mg K (K_2SO_4), 50 mg Mg ($MgSO_4 \cdot 7H_2O$), 5 mg Zn ($ZnSO_4 \cdot 7H_2O$), 5 mg Mn ($MnSO_4 \cdot H_2O$), and 2 mg Cu ($CuSO_4 \cdot 5H_2O$). The soil was sterilized by gamma irradiation (25 kGy, ^{60}Co gamma rays) at the Beijing Atomic Energy Research Institute to eliminate indigenous microorganisms and mycorrhizal propagules before use.

Microcosms

We used a compartmented microcosm system that separated the growing spaces of root systems and the extraradical mycelium of AM fungi (Fig. S1e). The microcosms were constructed using polyvinyl chloride (PVC) plates and consisted of root compartments and hyphal compartments. The air buffer zone consisted of the PVC plate with the 30 μm mesh glued on both sides (Fig. S1e), which allowed AM fungal hyphae to pass through but prevented root penetration.

Host plants

Maize (*Zea mays* L., cv. Zhengdan 958) was used as the host plant. The seeds were surface-sterilized with 10% (v/v) H_2O_2 for 10 min and 70% (v/v) ethanol for 3 min and then rinsed eight times with sterile deionized water. After imbibing water at 27°C in the dark for 2 d, the seedlings were sown into the microcosms.

AM fungal and bacterial inoculant

The AM fungal strain *Rhizophagus intraradices* EY108 was selected as inoculant, and was purchased from the International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (West Virginia University, Morgantown, WV). The inocula was propagated for 8 months in a mixture of zeolite and river sand (5:1, w:w) using maize and plantago as the host plants in the greenhouse. The inocula consisted of substrate containing spores (approximate 8 spores g^{-1} substrate), mycelium and fine root segments. The filtrate of each soil type was obtained by suspending 30 g of each soil in 300 mL of sterile water and filtration through six-layer quantitative filter paper, respectively. This allowed the passage of common soil microbes but effectively retained AM fungal spores and hyphae. A total of 10 mL of each soil filtrate was added to the hyphal compartments as the original microflora.

Experimental design and procedure

The microcosm experiment contained the following treatments: four soil types, including acidic red soil, loessial soil, grey desert soil and black soil, and with or without AM fungal inocula. Each treatment had six replicates, which were arranged in

a randomized block design in a glasshouse. At planting, 600 g soil for each root compartment was carefully added to the root compartment, and then 60 g AM fungal inoculum was added to each root compartment. Finally, three pre-germinated seeds were sown, and the remaining 250 g soil was added to the root compartments. 900 g soil for each hyphal compartment was added. The control treatments (NM) received the same amount of sterilized inoculum. Seven days after sowing, the seedlings were thinned to one in each microcosm. During the experiment, soil moisture was kept at 18-20% (w/w, c. 70% of field moisture capacity) as determined gravimetrically by weighing the pots every 2 days and adding water as necessary. Plants in these microcosms were grown in the glasshouse at China Agricultural University in Beijing from 1 May to 9 July 2019.

DNA extraction and PCR

To identify the AM fungal taxa in Experiment 1, an approximately 334 bp region of 18S rRNA gene was amplified with a two-step PCR (Yang *et al.*, 2018). In the first round, GeoA-2 (5′-CCAGTAGTCATATGCTTGTCTC-3′) and AML2 (5′-GAACCCAAACACTTTGGTTTCC-3′) primer pairs were used (Schwarzott & Schussler, 2001; Lee *et al.*, 2008). The PCR amplification was carried out in a total volume of 25 μL, including 12.5 μL 2×Taq Plus Master Mix, 3 μL BSA, 6.5 μL ddH_2O, 1 μL of each primer (5 μM), and 1 μL template DNA. The thermal cycling was followed by an initial denaturation at 94℃ for 2 min, 35 cycles of denaturation at 94℃ for 30 s, annealing at 59℃ for 1 min, and extension at 72℃ for 2 min, followed by a final extension at 72℃ for 7 min. The products of the first amplification were diluted for 10 times with sterilized deionized water and 1.0 μL diluted solution was used as the template for the nested PCR. The second round of PCR was performed with AMDGR (5′-TTGGAGGGCAAGTCTGGTGCC-3′) and NS31 (5′-CCCAACTATCCCTATTAATCAT-3′) primer pairs (Simon *et al.*, 1992; Helgason *et al.*, 1998). Both forward and reverse primers were augmented with 8 bp long barcodes unique to each sample. The conditions of the nested PCR were similar to the first PCR, except for 35 cycles of denaturation at 94℃ for 30 s, annealing at 58℃ for 1 min, and extension at 72℃ for 1 min.

To identify the bacterial taxa in both experiments, the V3-V4 regions of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Sakurai *et al.*, 2008). Both forward and reverse

primers were augmented with 5-8 bp long barcodes unique to each sample. The PCR amplification was performed in a total volume of 25 μL, containing 12.5 μL 2×Taq Plus Master Mix, 1 μL of each primer (5 μM), 1 μL DNA template, 3 μL BSA and 6.5 μL ddH_2O. The PCR program consisted of an initial denaturation at 94°C for 5 min; 28 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C and 60 s of elongation at 72°C; and 7 min of final elongation at 72°C.

Bioinformatic processing

The raw data were first screened, and sequences were removed from consideration if they were shorter than 230 bp, had a low-quality score (≤ 20), contained ambiguous bases or did not exactly match to primer sequences and barcode tags, and separated using the sample-specific barcode sequences. Qualified reads were clustered into operational taxonomic units (OTUs) at a similarity level of 97% use Uparse algorithm of Vsearch (v2.7.1) software (Edgar, 2013). The Ribosomal Database Project (RDP) Classifier tool was used to classify all sequences into different taxonomic groups against SILVA132 database (Quast *et al.*, 2013). QIIME (v1.8.0) was used to calculate the richness and diversity indices based on the OTU information.

Real time q-PCR analysis of 16S rRNA, AM fungal and total fungal gene

To determine the copy numbers of hyphopshere bacteria, AM fungi and total fungi in our hyphal samples, the DNA extracted from hyphal samples was quantified in triplicate by real-time q-PCR using q-PCR analyzer TIB8600 (Triplex Bioscience (China) Co., Ltd., Xiamen, China) with 16S rRNA, AM fungal and total fungal gene specific primers. The primer sets were 515F (5′-GTGCCAGCMGCCGCGGTAA-3′)/907R (5′-CCGTCAATTCMTTTRAGTTT-3′), AMV4.5NF (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG [GC clamp] AAGCTCGTAGTTGAATTTCG-3′)/AMDGR (5′-CCCAACTATCCCTATTAATCAT-3′) and 5.8s (CGCTGCGTTCTTCATCG)/ITS1f (TCCGTAGGTGAACCTGCGG) targeting for bacteria, AM fungi and all fungi, respectively (Sato *et al*., 2005; Sakurai et al., 2008; Rousk *et al*., 2010). SYBR Green real time q-PCR Master Mix (TOYOBO, Japan) was used and conducted under the following reaction conditions: initial denaturation at 95℃ for 5 min; 40 cycles consisting of denaturation at 95℃ for 15 s, annealing at 60℃ for 30 s, and elongation at 72℃ for 1 min. Fluorescence of SYBR green was detected after every cycle. The dissolution curve was collected when the whole reaction ended in 0.5℃ increments from 65℃ to 95℃. No amplification was detected in the negative controls. The plasmid was sequenced for verification before

263 constructing a standard curve for absolute quantification of gene copy. The standard
264 curve was prepared in triplicate using five serial 10-fold dilutions, and quantification
265 calculated by determining the starting copy number by considering the concentration
266 of the plasmid and number of base pairs (vector plus primer).

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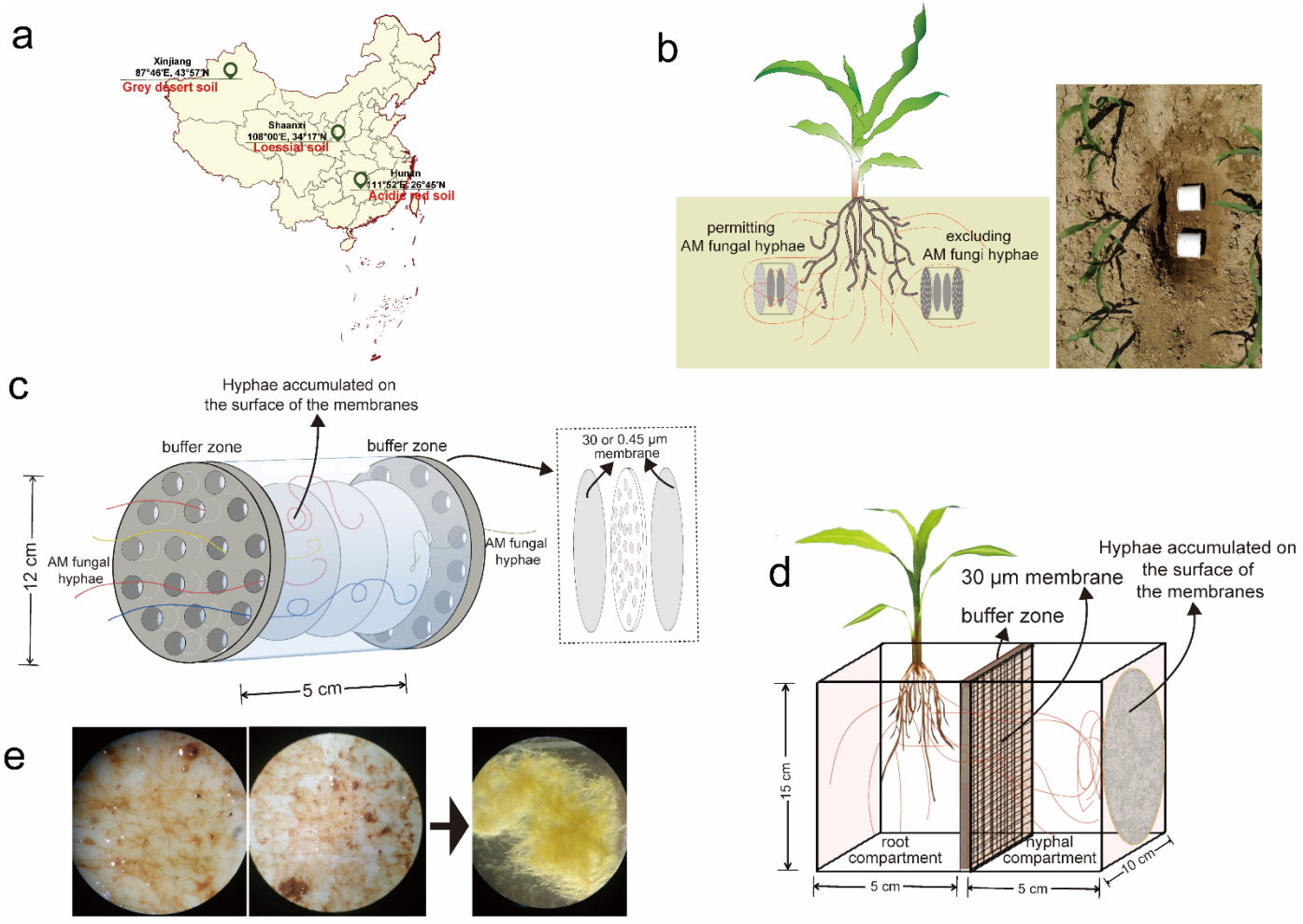
a
Xinjiang
87°46'E, 43°57'N
Grey desert soil
Shaanxi
108°00'E, 34°17'N
Loessial soil
Hunan
111°52'E, 26°45'N
Acidic red soil
b
permitting
AM fungal hyphae
excluding
AM fungi hyphae
c
Hyphae accumulated on
the surface of the membranes
buffer zone
buffer zone
30 or 0.45 μm
membrane
12 cm
AM fungal
hyphae
AM fungal
hyphae
5 cm
d
30 μm membrane
Hyphae accumulated on
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buffer zone
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root
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compartment
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5 cm
e

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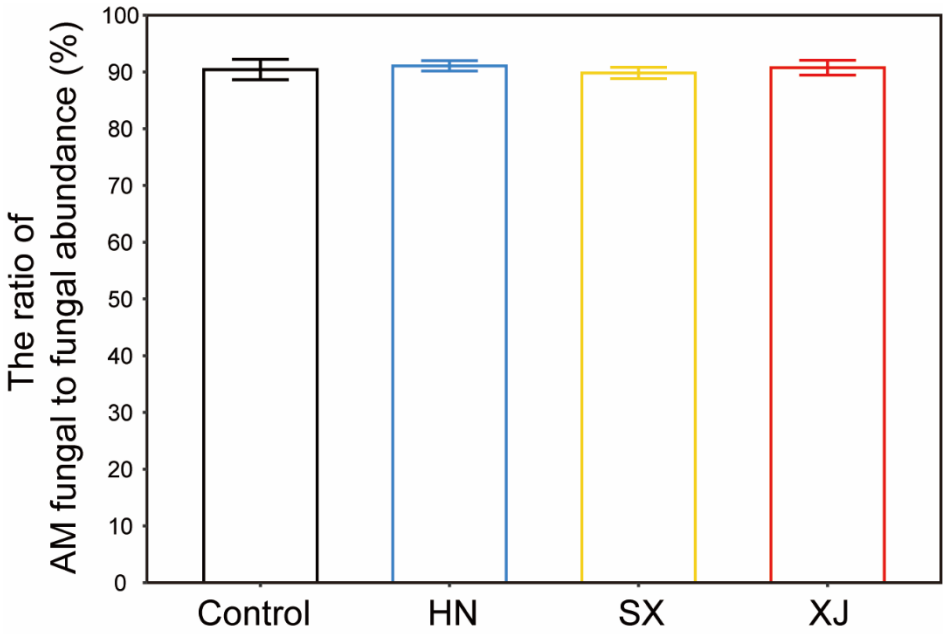

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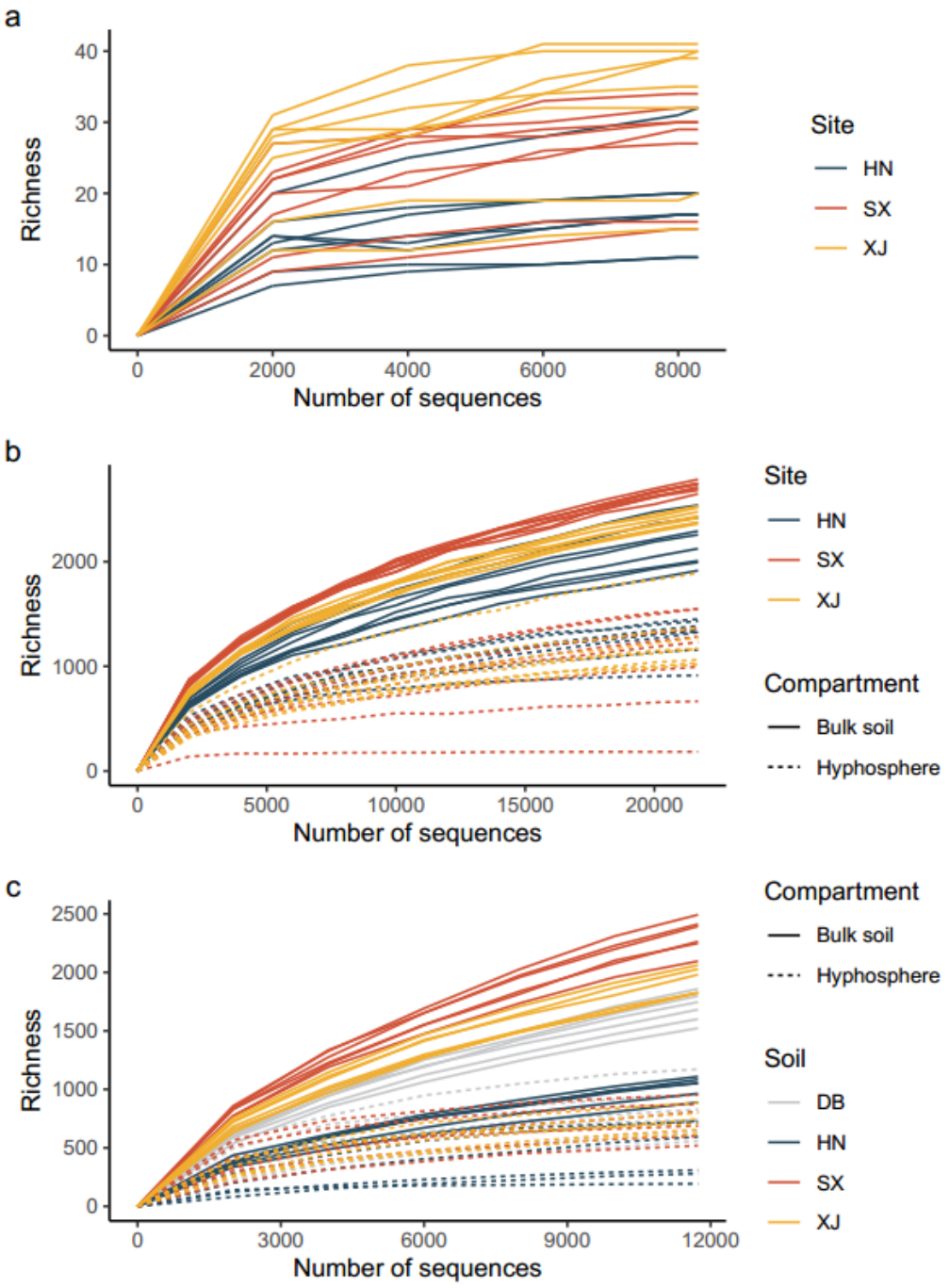

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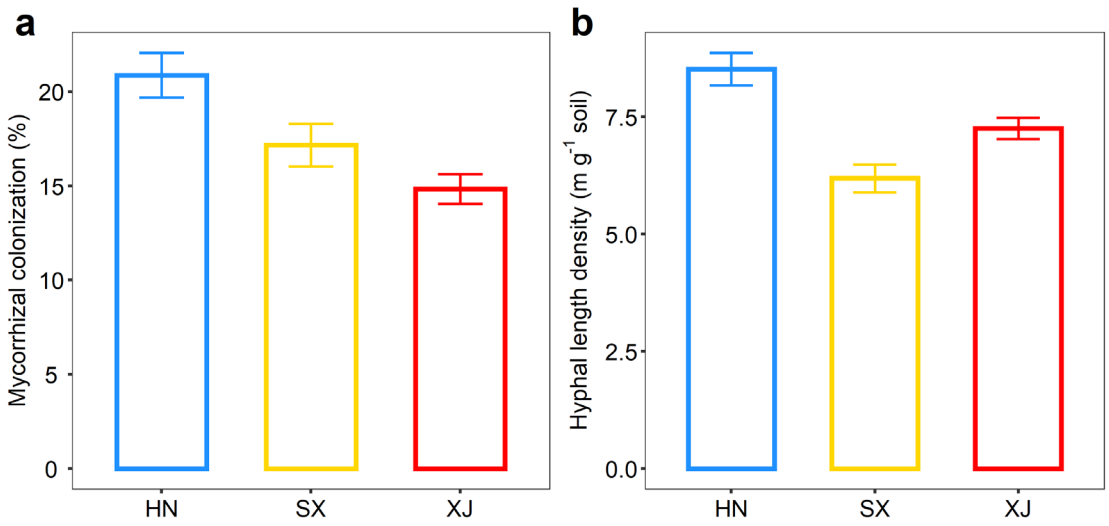

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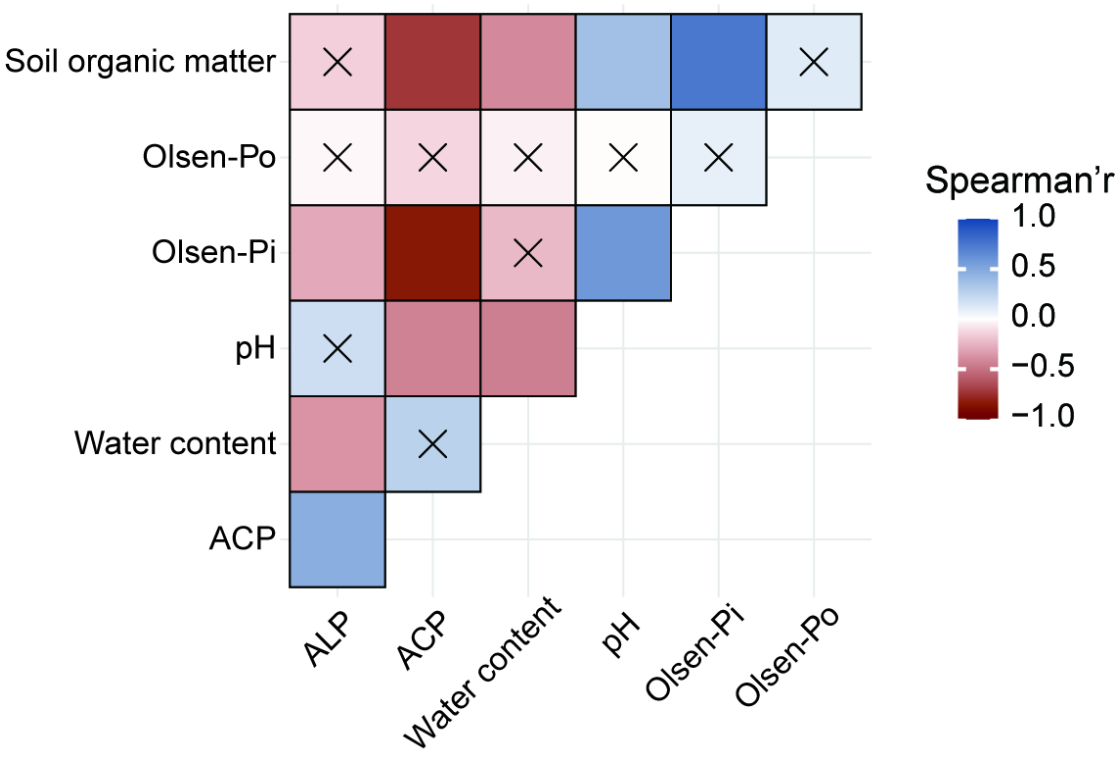

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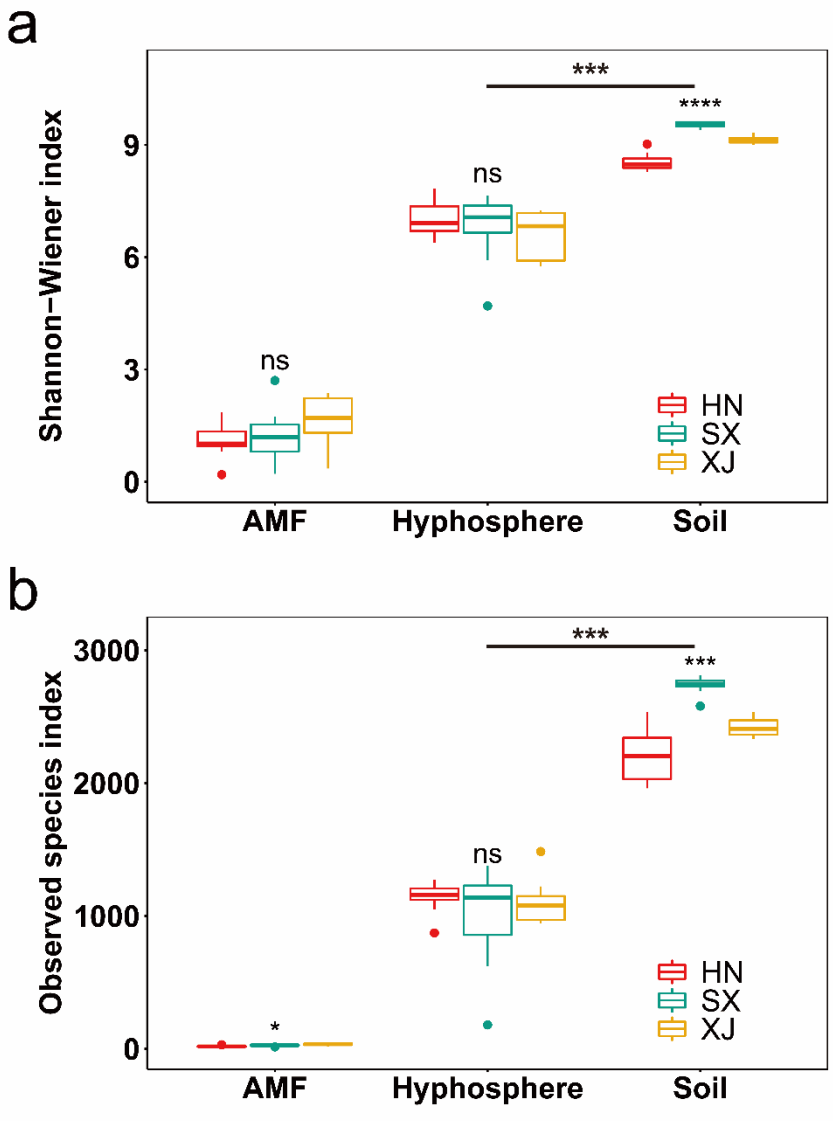

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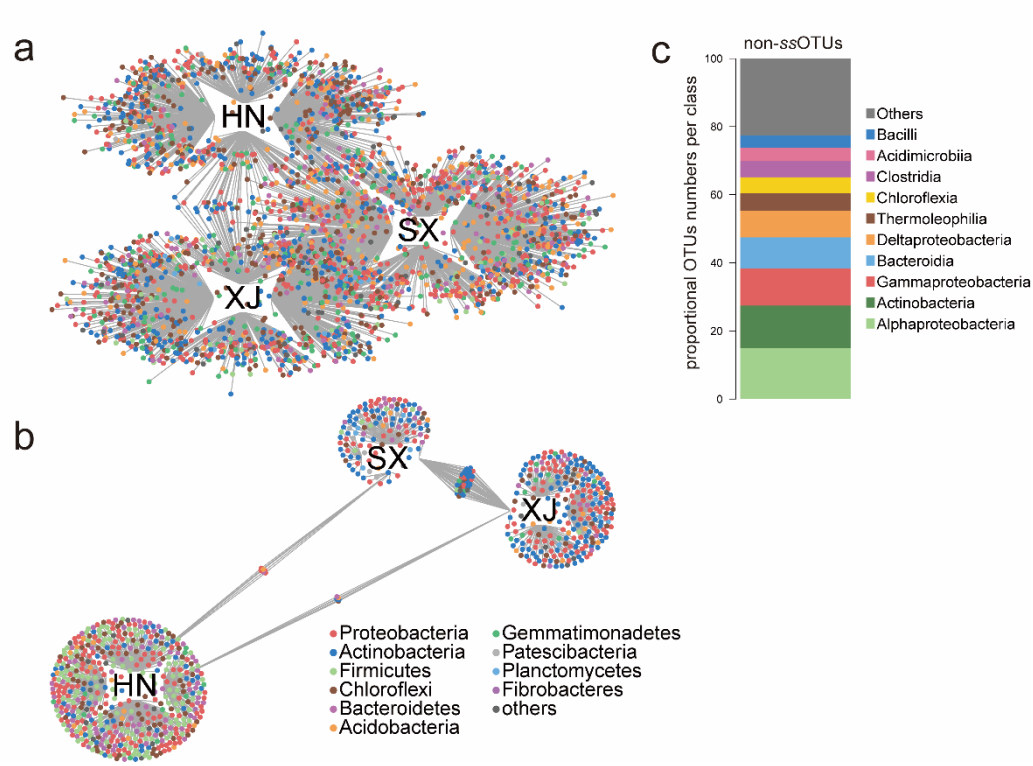

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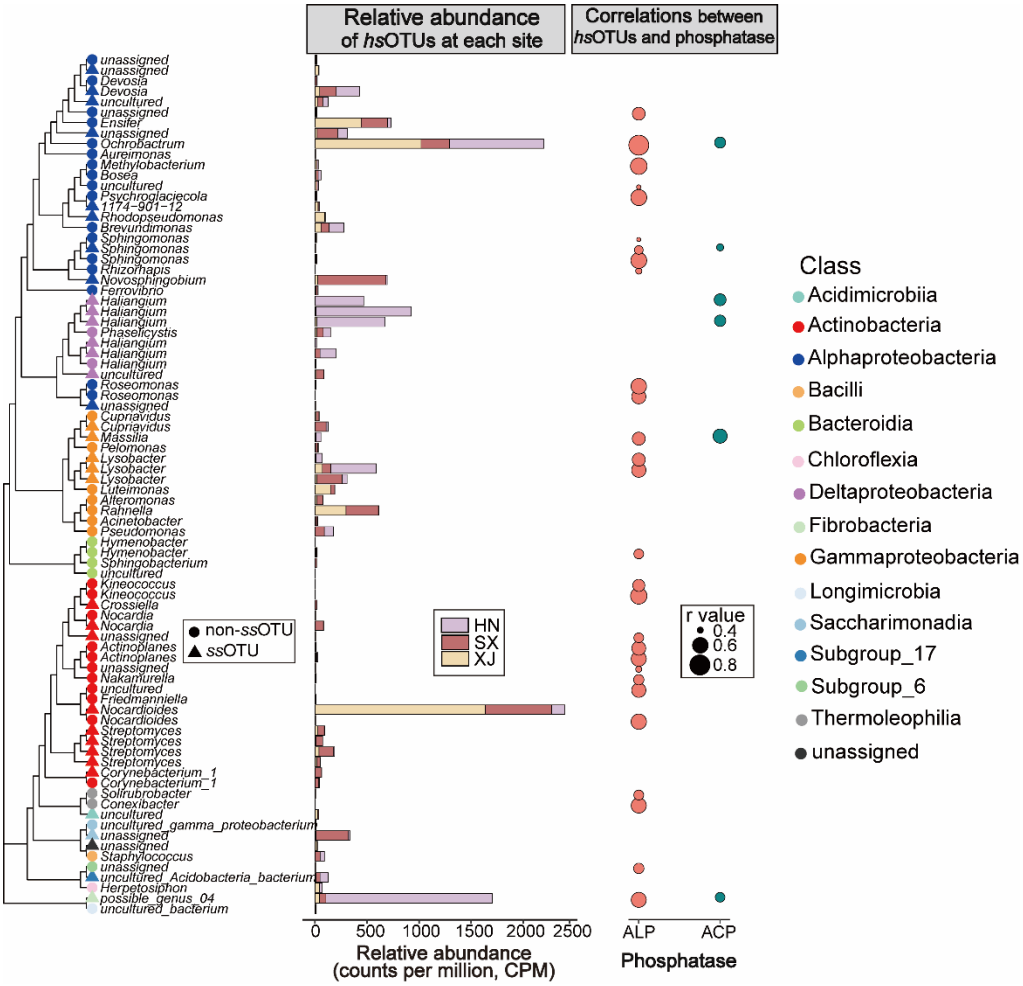

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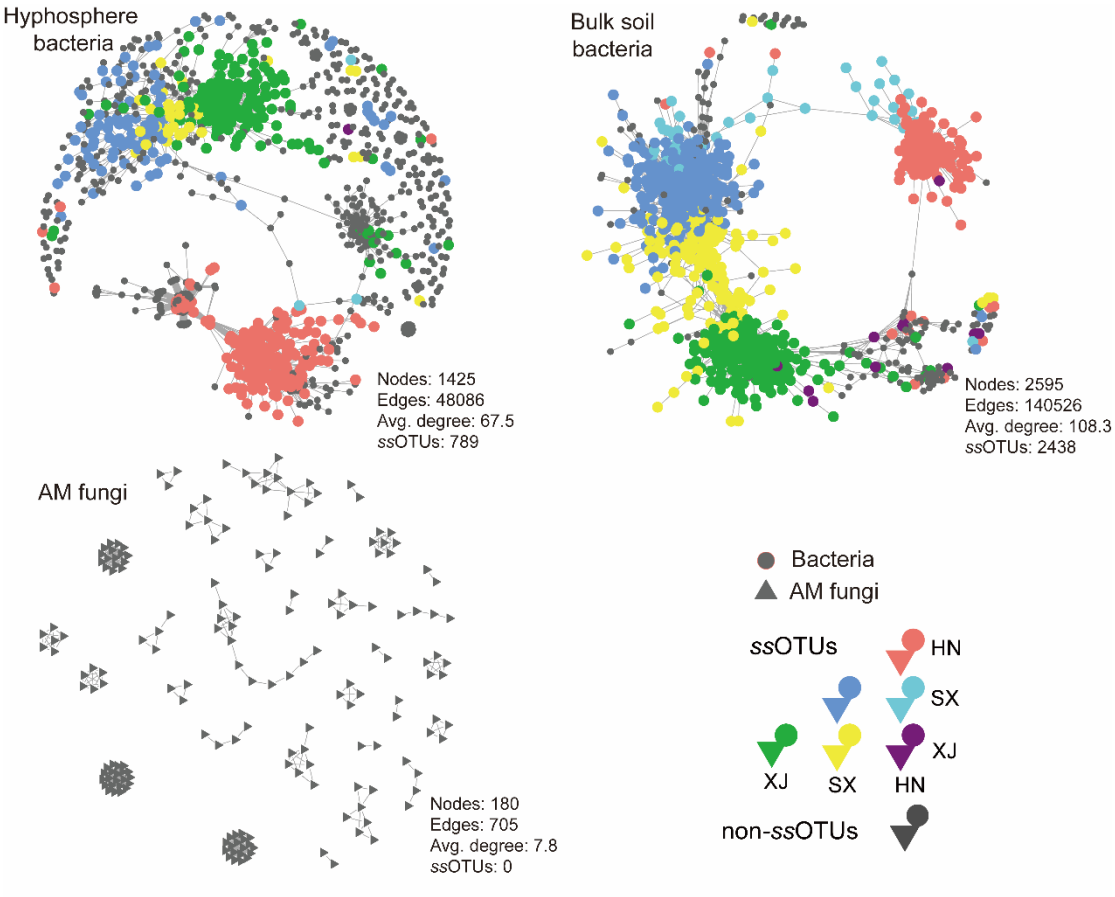

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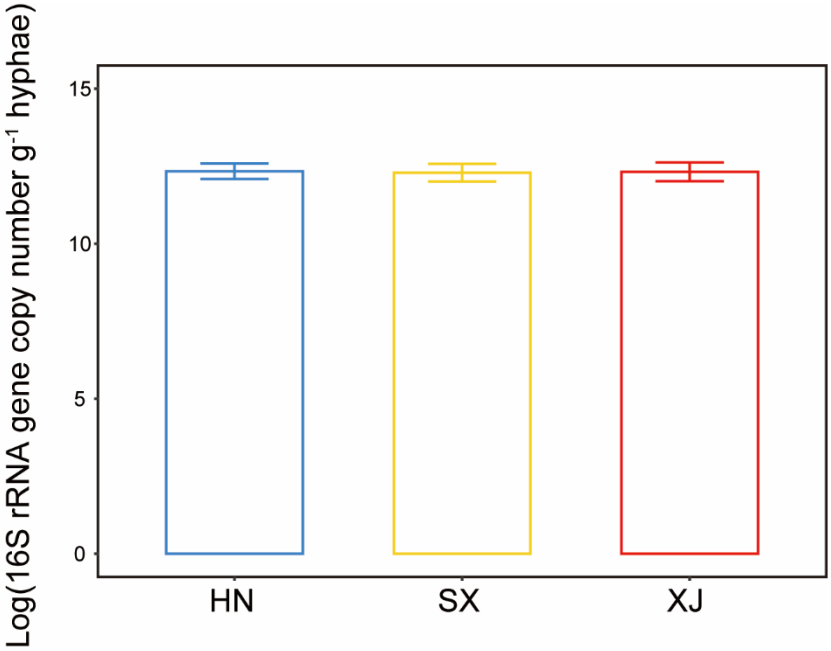

Fig. S11 Mycorrhizal colonization (**a**) of host plants and hyphal length density (**b**) in the hyphal compartments under each soil type in Experiment 2. Values are means (n = 8) and bars represent standard errors. Different letters indicate significant differences among four soil types under the same inoculation treatment ($P < 0.05$).

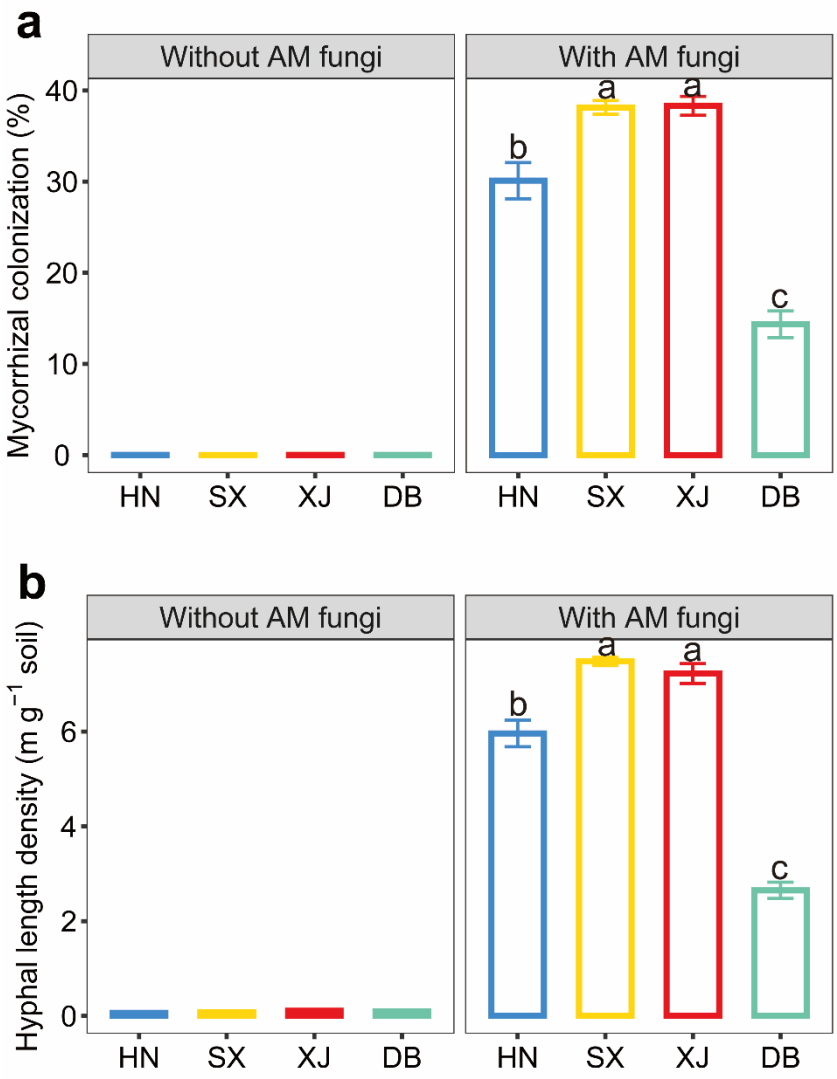

Fig. S12 Difference of P contents and phosphatase activities in the hyphal compartment (HCs) inoculated or not inoculated with AM fungi in Experiment 2. (**a**) inorganic P, (**b**) organic P, (**c**) alkaline phosphatase (ALP) activity and (**d**) acid phosphatase (ACP) activity in the HCs. Data are means (n = 6) + standard error. HN indicates acidic red soil collected from Hunan site, SX indicates loessial soil collected from Shaanxi site, XJ indicates grey desert soil collected from Xinjiang site, and DB indicates black soil collected from Jilin site. Different letters indicate significant differences among four soil types ($P < 0.05$). The asterisk indicates significant differences between the hyphal compartments with and without the presence of AM fungi. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, no significance.

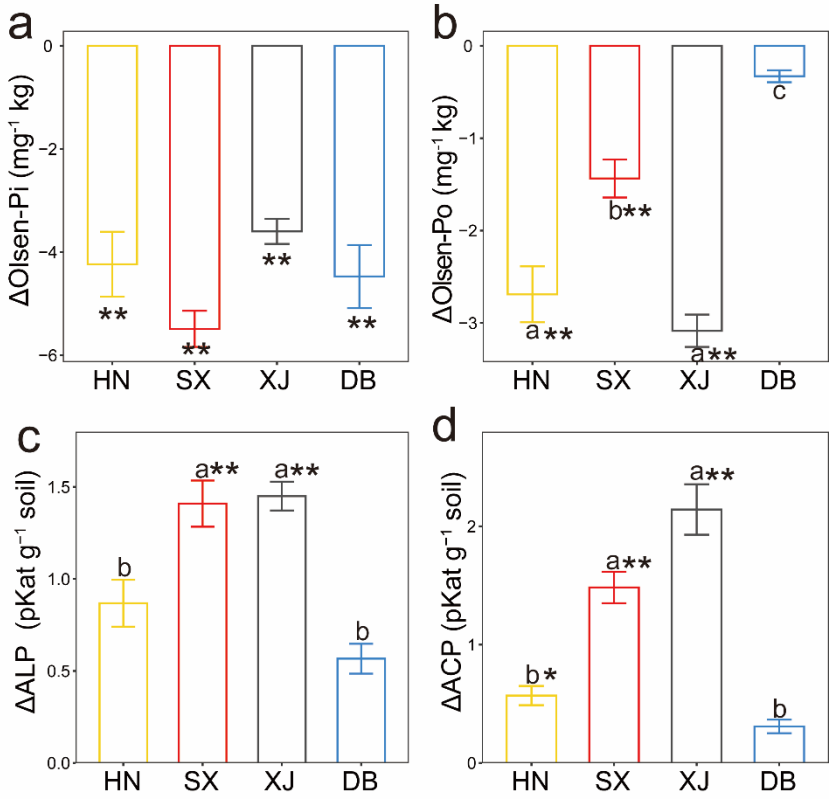

Fig. S13 Profiling of hyphosphere bacterial communities in Experiment 2. **a** Histogram diagram showing the relative abundances of major orders of hyphosphere and bulk soil bacteria across four soil types. **b** Box plot showing Shannon-Wiener diversity indices for bulk soil and hyphosphere bacterial communities. The asterisk indicates significant difference between the bulk soil and the hyphosphere. **c** VENN plot showing the number of OTUs enriched in the hyphosphere at each soil type. Boxes show first quartile, median and third quartile. Whiskers extend to the most extreme points within 1.5 × box length, and the points are values that fall outside the whiskers. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

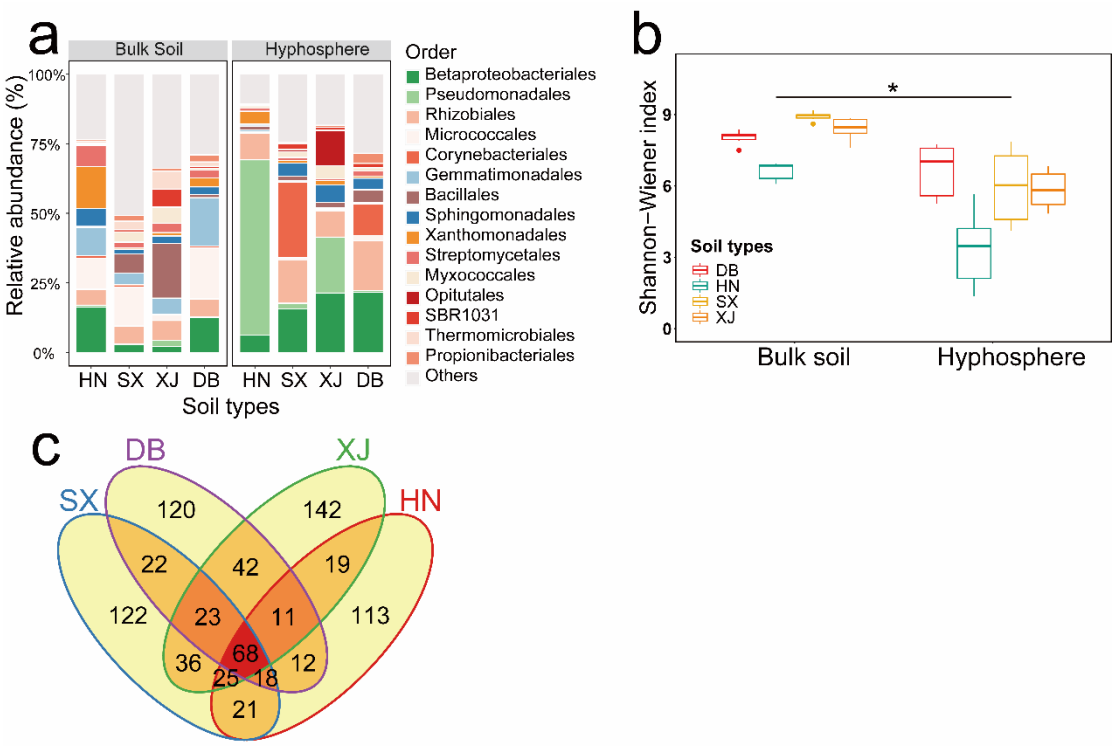

Fig. S14 Qualitative taxonomic composition of module 4 (M4) in Experiment 1 and module 30 (M30) in Experiment 2 is reported as proportional OTUs numbers per class.

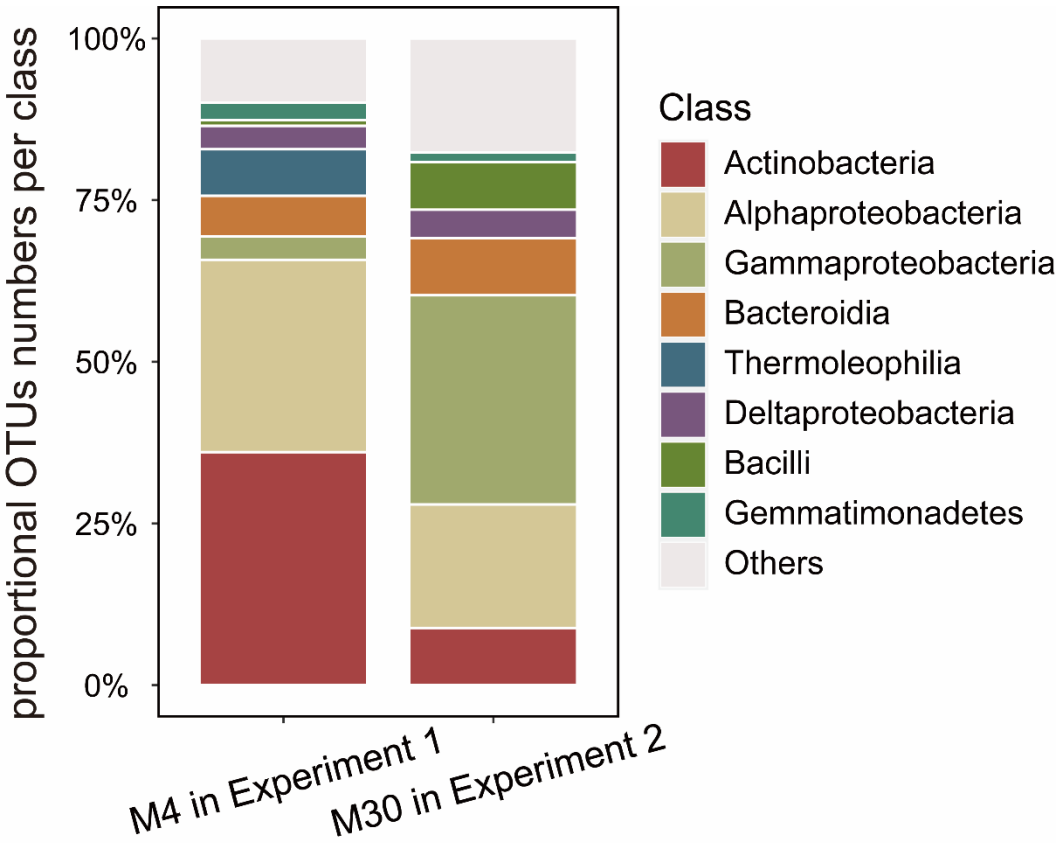

Table S1 Physicochemical properties of the soil used in Experiment 1 and 2.

	Soil types			
	Acidic red soil	**Loessial soil**	**Grey desert soil**	**Black soil**
Origination	Hunan Province (111°52′ E, 26°45′N)	Shaanxi Province (108°00′E, 34°17′N)	Xinjiang Province (87°46′E, 43°57′N)	Jilin Province (43°54′E, 125°18′ N)
Used in Experiment 1 or Experiment 2	Experiment 1 and 2	Experiment 1 and 2	Experiment 1 and 2	Experiment 2
Soil classification (FAO)	Haplic Calcisols	Calcaric Regosol	Ferralic Cambisol	Haplic Phaeozem
Olsen inorganic P (mg kg^{-1})	10.11	24.97	11.98	48.89
Olsen organic P (mg kg^{-1})	6.89	6.78	5.98	15.77
Soil pH	6.55	7.94	8.00	7.78
Soil organic matter (g kg^{-1})	5.51	11.12	7.68	18.72

Table S2 The comparison between the hyphosphere core microbiome in our study and the root-associated core microbiome in previous studies. "Y" represents "yes" under "Also core in". Further details of the maize and cotton root-associated core microbiome can be found in Walters *et al*. (2018) and Zhang *et al*. (2022).

Taxonomy			Also core in:	
Phylum	Class	Order	maize	cotton
Actinobacteria	Actinobacteria	Corynebacteriales		
		Frankiales		
		Pseudonocardiales	Y	Y
Proteobacteria	Alphaproteobacteria	Rhizobiales	Y	Y
Firmicutes	Bacilli	Bacillales		Y
Bacteroidetes	Bacteroidia	Chitinophagales		
		Cytophagales		
Chloroflexi	Chloroflexia	Chloroflexales		
Proteobacteria	Deltaproteobacteria	Myxococcales		
	Gammaproteobacteria	Alteromonadales		
		Betaproteobacteriales	Y	Y
		Enterobacteriales		
		Xanthomonadales	Y	

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