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The synergy effect of arbuscular mycorrhizal fungi symbiosis and exogenous calcium on bacterial community composition and growth performance of peanut (*Arachis hypogaea* L.) in saline alkali soil[§]

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Peanut (*Arachis hypogaea*. L) is an important oil seed crop. Both arbuscular mycorrhizal fungi (AMF) symbiosis and calcium (Ca^{2+}) application can ameliorate the impact of saline soil on peanut production, and the rhizosphere bacterial communities are also closely correlated with peanut salt tolerance; however, whether AMF and Ca^{2+} can withstand high-salinity through or partially through modulating rhizosphere bacterial communities is unclear. Here, we used the rhizosphere bacterial DNA from saline alkali soil treated with AMF and Ca^{2+} alone or together to perform high-throughput sequencing of 16S rRNA genes. Taxonomic analysis revealed that AMF and Ca^{2+} treatment increased the abundance of Proteobacteria and Firmicutes at the phylum level. The nitrogen-fixing bacterium *Sphingomonas* was the dominant genus in these soils at the genus level, and the soil invertase and urease activities were also increased after AMF and Ca^{2+} treatment, implying that AMF and Ca^{2+} effectively improved the living environment of plants under salt stress. Moreover, AMF combined with Ca^{2+} was better than AMF or Ca^{2+} alone at altering the bacterial structure and improving peanut growth in saline alkali soil. Together, AMF and Ca^{2+} applications are conducive to peanut salt adaption by regulating the bacterial community in saline alkali soil.

Keywords: *Arachis hypogaea* L., 16S rRNA, rhizosphere bacterial community, saline alkali soil, AMF, Ca^{2+}

Introduction

Peanut (*Arachis hypogaea* L.) is an important oil and cash crop cultivated worldwide (Xu *et al.*, 2020a). Irrational agricultural irrigation practices and an increasing population have aggravated the conflict between grain and oil arable land. Due to the limited arable land and the requirement for developing regional agriculture, peanuts have been cultivated in the saline alkali soil of northern China, such as the Yellow River delta coastal areas (Chakraborty *et al.*, 2016). Soil salinity severely affects peanut germination, growth, and productivity, since it can lead to ionic imbalance, osmotic stress, and oxidative damage to the plants (Yang and Guo, 2018; Xu *et al.*, 2019). Correspondingly, plants have evolved several complex regulatory strategies to withstand salt stress (Deinlein *et al.*, 2014; Mickelbart *et al.*, 2015; Damodharan *et al.*, 2018). The root external environment has been reported to influence peanut salt tolerance due to many stress-response plant growth promoting rhizobacteria (PGPRs) in rhizosphere soil that are beneficial to plant survival and adaptation to stresses (Dai *et al.*, 2019; Xu *et al.*, 2020b).

Calcium (Ca^{2+}) is not only an essential nutrient required for plant growth, but also a conserved second messenger in plant adaptations to environmental stress, especially to salt stress (Knight, 2000; Han *et al.*, 2019; Yu *et al.*, 2020a). Salt stress-elicited cytosolic free Ca^{2+} plays a vital role in the salt response through re-establishing cellular ionic, osmotic, and reactive oxygen species (ROS) homeostasis (Zhu, 2002). In *Arabidopsis*, cytoplasmic Ca^{2+} is sensed by the EF-hand Ca^{2+}-binding protein salt overly sensitive 3 (SOS3), further phosphorylating SOS2. Ca^{2+}-dependent SOS3-SOS2 phosphorylates the plasma membrane-localized Na^+/H^+ antiporter SOS1, which transports Na^+ from the cytoplasm to the apoplast to regulate ion homeostasis during salt stress (Quintero *et al.*, 2011; Ji *et al.*, 2013). The Ca^{2+}/CaM complex has been reported to regulate another Na^+ determiner vacuolar membrane Na^+/H^+ exchanger (NHX1), compartmentalizing Na^+ into vacuoles under salt stress (Yamaguchi *et al.*, 2005). In addition, Ca^{2+} is closely associated with abscisic acid (ABA) biosynthesis and signaling to regulate osmotic equilibrium under salt stress, and it is also associated with antioxidant enzyme activity, affecting the plant antioxidant system (Zhu, 2002; Han *et al.*, 2019). Thus, salt-elicited Ca^{2+} plays an important role in plants adapting to salt stress.

In plants, Ca^{2+} is mainly limited to uptake via root tips from the soil and then it is transferred to the shoot via the xylem. Insufficient Ca^{2+} in the soil will affect plant growth and stress tolerance (Cui *et al.*, 2019). Exogenous Ca^{2+} application de-

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creased the Na^+:K^+ ratio, improved the root and leaf cell plasma membrane permeability, and regulated the plant antioxidant system in foxtail millet salt adaption (Han *et al.*, 2019). In general, plants deal with Ca^{2+} deficiency and further regulate the ecosystem sustainability by establishing symbiosis with microbes, known as the arbuscular mycorrhizal fungi (AMF) association (Cui *et al.*, 2019). AMF symbiosis increases the Ca^{2+} content in peanut, while in contrast, Ca^{2+} application promotes the development of AMF symbiosis (Cui *et al.*, 2019). After the application of exogenous Ca^{2+}, AMF promoted plant growth under salt stress (Dagher *et al.*, 2020; Tisarum *et al.*, 2020). However, the synergy role and molecular mechanism of Ca^{2+} and AMF in the peanut salt adaptation is largely unknown.

The rhizosphere microbial community structure changes with the external environment and is involved in improving peanut salt tolerance (Naylor *et al.*, 2017; Ullah *et al.*, 2018; Xu *et al.*, 2020b). However, whether AMF and Ca^{2+} affect the rhizosphere microbial community, whether AMF and Ca^{2+} withstand salt stress by modulating rhizosphere bacterial community in peanut, and how AMF and Ca^{2+} synergistically regulate the salt stress response in the rhizosphere are unclear. Here, 16S rRNA sequencing and statistical analysis were used to investigate the peanut rhizosphere bacterial community diversity and its correlation with the application of AMF and Ca^{2+} in peanut salt adaption. This study mainly aimed to explore the synergy effect of AMF and Ca^{2+} on rhizosphere bacterial community modification in salt stress tolerance, to provide a theoretical basis and practical guidance for the rational planting of peanuts and the improvement of their stress tolerance in saline alkali soil.

Materials and Methods

Plant materials and growth conditions

Peanut cultivar Huayu25 was cultivated at the Dongying experimental station, China (118.66°E, 37.32°N). The physiochemical properties of the soil were as follows: pH 8.8, ECe 5.17 dS/m (which was obtained according to a previous study) (Yao *et al.*, 2016), organic content 15.8 g/kg, available nitrogen 36.98 mg/kg, available potassium 10.73 mg/kg, and available phosphorous 284.5 mg/kg. The soils contained approximately 2.5 g/kg salt. Before sowing, 15–15–15 (N-P_2O_5-K_2O) fertilizer at 1,200 kg/ha was incorporated into the soils as the negative control.

For the Ca^{2+} treatment groups, calcium superphosphate (40 kg/ha) together with 15–15–15 (N-P_2O_5-K_2O) fertilizer (975 kg/ha) were applied to the soils. For the AMF treatment groups, the seeds were inoculated with AMF (*Rhizophagus irregularis* SA : *Funneliformis mosseae* BEG95 = 1 : 1) by a seed coating agent (0.1 kg AMF was inoculated with 1 ha of seeds) and then they were sown into the soils incorporating 15–15–15 (N-P_2O_5-K_2O) fertilizer (975 kg/ha). The AMF was provided by Symbiom Ltd. in powder formulation with 252 spores/g for seed treatment (confirmed by a certificate of Symbiom Ltd. as the manufacturer). For the Ca^{2+} and AMF compound-treatment groups, calcium superphosphate (40 kg/ha) together with 15–15–15 (N-P_2O_5-K_2O) fertilizer (975 kg/ha) were applied to the soils, and the seeds were inoculated with AMF.

The field was plowed and irrigated twice one day before sowing to ensure the profile was fully moistened. The field experiment was carried out during the rainy season in 2019 and sowing was done on May 1st, and harvest was done on September 15th. The seeds were sown by hand, and each plot contained ten rows (50 cm distance between rows), with a spacing of 10 cm between plants within a row. Each treatment contained three biological replicates. Plants were irrigated twice per week with 25 mm of water each time in a previous study (Halilou *et al.*, 2016). Plant and soil samples were collected at the seedling stage (June 13th), flowering stage (July 11th), and podding stage (August 7th), respectively.

Growth and yield parameters of peanut

Six representative peanuts of each treatment were harvested at maturity. All samples were heated at 105°C for 30 min, then dried to a constant weight at 80°C, and weighed or counted separately in a previous study (Xu *et al.*, 2020b). In addition, the 100-pod weight, 100-seed weight, and the number of pods per plant of each treatment were also recorded.

The sample collection of rhizosphere compartments for 16S rRNA gene sequencing

Rhizosphere compartments consisted of the root surface soils and the soils around the roots (Ullah *et al.*, 2018). The whole peanut plant was dug out and the surface soils of the root system were carefully shaken off. Then the soils around the roots (soils tightly adhered to the root surface 1–10 mm) were brushed off gently with a sterile brush. The roots were cut off and placed in a centrifuge tube containing 40 ml PBS buffer (0.25 g of $NaH_2PO_4 \cdot H_2O$, 0.66 g of $Na_2HPO_4 \cdot 7H_2O$, 8 ml Silwet L-77, pH 7.0). The centrifuge tubes were high-speed centrifuged to remove plant debris, and then the supernatant was centrifuged at 5,000 × *g* for 15 min. The pellets were resuspend in PBS to obtain the root surface soil (Xu *et al.*, 2020b). Each treatment contained three biological replicates. The bacterial DNA was extracted by using a PowerSoil® DNA Isolation Kit (MoBio Laboratories).

16S rRNA gene sequencing

The quality and concentration of the extracted DNA was checked before being used for bacterial 16S rRNA gene sequencing by Beijing Biomarker (Ullah *et al.*, 2018; Xu *et al.*, 2020a). The V3 and V4 regions of the 16S rRNA genes were amplified using the specific primers 338F (forward primer, 5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′). A 2× Phanta Max Master Mix (P515, Vazyme) was used for the PCRs and then the Qiagen Gel Extraction Kit was used to purify the PCR product (Yu *et al.*, 2020b). The sequencing libraries were obtained by using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina) and 250 bp/300 bp paired-end reads were generated.

OTU cluster and bioinformatics analysis

The obtained paired-end reads were merged using FLASH

(version 1.2.7) and the raw tags were preliminarily screened according to the quality-controlled process of QIIME (Quantitative Insights into Microbial Ecology, v1.8.0) (Caporaso *et al.*, 2010). Chimera sequences were detected and further deleted via comparison with the reference Gold database (http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (Edgar *et al.*, 2011). The OTU was defined as the sequence that had a similarity threshold set ≥ 97% (Blaxter *et al.*, 2005).

Alpha and beta diversity analysis

To compute the alpha diversity, we calculated six indices: both Chao1 and ace estimate the species abundance; Sobs represents OTU numbers; Shannon and Simpson exhibit the species diversity; and Coverage reflects the sequencing depth. Rarefaction curves can evaluate the species richness and sequence depth, and the species accumulation curve was used to reflect community richness. The rank abundance curve reflects the species abundance and evenness (Bates *et al.*, 2013). The similarity or dissimilarities of the community structure among the soil samples were determined by beta diversity analysis, including principal component analysis (PCA), unweighted pair-group method with arithmetic mean (UPGMA), and analysis of similarities (ANOSIM) in a previous study (Wang *et al.*, 2012; Xu *et al.*, 2020a).

Linear discriminant analysis effect size (LEfSe) and metabolic function prediction analysis

LEfSe is a high dimensional biomarker algorithm for the analysis of biomarkers in soil groups (Segata *et al.*, 2011). LEfSe analysis was conducted at website (http://huttenhower.sph.harvard.edu/galaxy) with a factorial Kruskal-Wallis test among classes < 0.05 and the threshold on the logarithmic LDA score > 3.5. The metabolic functional features of the bacterial communities were predicted using the Cluster of Orthologous Groups (COG) database and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Langille *et al.*, 2013).

qPCR analysis of predominant bacterial community

The main bacterial phyla (Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, and Bacteroidetes) and beneficial genus (*Sphingomonas*) were measured by qPCR (Li *et al.*, 2018; Xu *et al.*, 2020a). qPCR analysis was performed by Bio-Rad CFX96 (Bio-Rad) with the following reaction mixture: 0.5 μl of primers (10 μM), 7.5 μl ChamQ SYBR Color qPCR Master Mix (Q411, Vazyme), and template DNA in a previous study (Yu *et al.*, 2019). The primers are listed in Table 1.

Soil enzyme activity measurement

The soil enzyme activities were measured according to a previous study (Xu *et al.*, 2020a). Invertase activity: 2 g of fresh rhizosphere soil was mixed with 15 ml of 8% sucrose, 5 ml of phosphate buffer at pH 5.5, and 5 drops of methylbenzene, and then cultured in a 37°C incubator for 24 h. After filtration, 1 ml filtrate was incubated with 3 ml of 3,5-dinitrosalicylic acid (DNS) in a boiling bath for 5 min. Finally, the absorbance at 508 nm was measured after the solution was diluted to 100 ml. Urease activity: 5 g of fresh rhizosphere soil was incubated with 1 ml of methylbenzene for 15 min. Then, 10 ml of 10% urea and 20 ml of citrate buffer (pH 6.7) were added and cultured at 37°C for 24 h. After filtration, 3 ml of filtrate was mixed with 4 ml of sodium phenoxide and 3 ml of sodium hypochlorite. Finally, the absorbance at 578 nm was measured after the solution was diluted to 50 ml. Catalase activity: 2 g of fresh rhizosphere soil was mixed with 40 ml of distilled water and 5 ml of 0.3% H_2O_2 with shaking at 120 rpm for 20 min. Then, 5 ml of 1.5 M H_2SO_4 was added to terminate the reaction. Finally, 25 ml of filtrate was titrated with $KMnO_4$. Soil acid phosphatase activity: 1 g of fresh rhizosphere soil was mixed with 4 ml modified universal buffer and 1 ml P-nitrobenzene sodium phosphate and cultured in a 37°C incubator for 30 min. Then, 1 ml of 2 M $CaCl_2$ and 4 ml of 0.2 M NaOH were added to terminate the reaction, followed by 90 ml of distilled water. After filtration, the absorbance at 405 nm was measured.

Statistical analysis

ANOSIM analysis was performed using QIIME software with 999 displacement tests to determine whether the differences between the groups were statistically significant. All experiments were performed three times. Error bars in each

Table 1. Primers used in qPCR assay

Purpose	Name	Sequence	Reference
qPCR	Alphaproteobacteria F	ACTCCTACGGGAGGCAGCAG	Fierer *et al.* (2005)
	Alphaproteobacteria R	TCTACGRATTTCACCYCTAC	
	Gammaproteobacteria F	TCGTCAGCTCGTGTYGTGA	Karamipour *et al.* (2016)
	Gammaproteobacteria R	CGTAAGGGCCATGATG	
	Actinobacteria F	CGCGGCCTATCAGCTTGTTG	Fierer *et al.* (2005)
	Actinobacteria R	ATTACCGCGGCTGCTGG	
	Firmicutes F	ATTACCGCGGCTGCTGG	Fierer *et al.* (2005)
	Firmicutes R	ATTACCGCGGCTGCTGG	
	Acidobacteria F	GTYAACTCGGAGGAAGGT	Fierer *et al.* (2005)
	Acidobacteria R	CTGATCTGCGATTACTAGCGACTCC	
	Bacteroidetes F	GTACTGAGACACGGACCA	Fierer *et al.* (2005)
	Bacteroidetes R	ATTACCGCGGCTGCTGG	
	Sphingomonas F	MRGWCCAAAGATTTATCG	Leung *et al.* (1999)
	Sphingomonas R	CGGACCAAAGATTTATCG	

graph of qPCR and soil enzyme assays indicate the mean values ± SEM. Statistically significant differences between groups were determined using one-way ANOVA ($P < 0.05$; LSD and Duncan test) with Statistical Product and Service Solutions Statistics software (SPSS 23; IBM).

Availability of data and materials

The following information was supplied regarding data availability: Reads were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession SUB7540986 and bioproject PRJNA636625, and the database is accessible via the link https://www.ncbi.nlm.nih.gov/sra/PRJNA636625.

Results

Overall and alpha diversity analysis of sequence data in the peanut rhizosphere soils

Bacterial community structure diversity of the rhizosphere soils treated with AMF and Ca^{2+} in saline alkali soil was carried out by 16S rRNA gene sequencing. Bulk soil without plants was collected and designated as the bulk soil (BS) group. We combined the controlled rhizosphere soil groups at the seedling, flowering, and podding stages in saline alkali soil as the control rhizosphere soils sampled at the seedling (CSR), flowering (CFR), and podding (CPR) stages, respectively. Ca^{2+}-treated rhizosphere soil groups sampled at the seedling, flow-

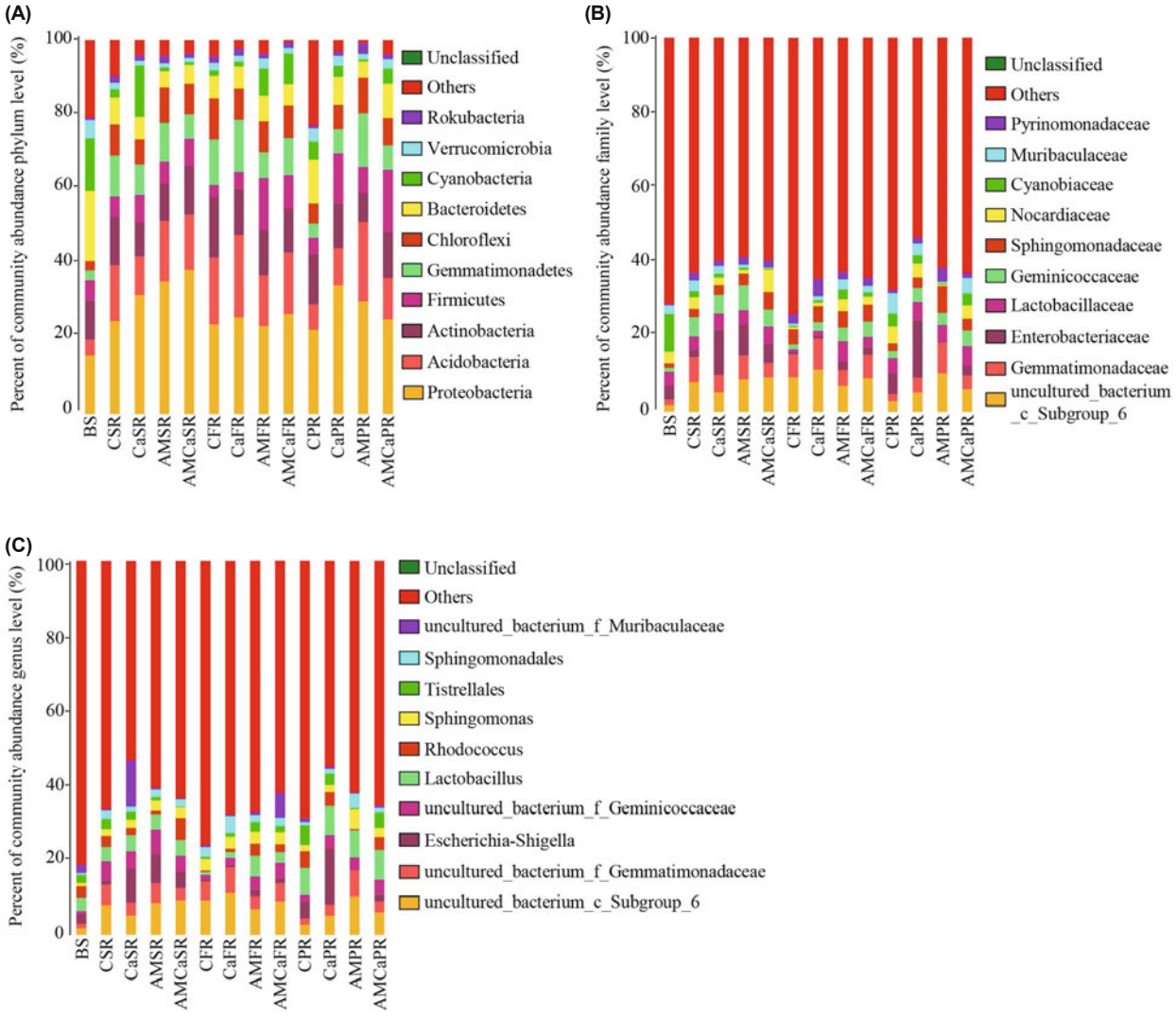

Fig. 1. Bacterial community structure of peanut rhizosphere soils. The bacterial community structure percent at the phylum level (A), family level (B), and genus level (C) in thirteen peanut rhizosphere soil groups. Both "unclassified" and "uncultured" in bacteria concerning unidentified species were obtained directly from the database via sequence alignment. Abbreviations: BS, bulk soil; CSR, controlled rhizosphere soils of the seedling stage; CaSR, Ca^{2+}-treated rhizosphere soils of the seedling stage; AMSR, AMF-inoculated rhizosphere soils of the seedling stage; AMCaSR, rhizosphere soils treated with AMF and Ca^{2+} and sampled at the seedling stage; CFR, controlled rhizosphere soils of the flowering stage; CaFR, Ca^{2+}-treated rhizosphere soils of the flowering stage; AMFR, AMF-inoculated rhizosphere soils of the flowering stage; AMCaFR, rhizosphere soils treated with AMF and Ca^{2+} and sampled at the flowering stage; CPR, controlled rhizosphere soils of the podding stage; CaPR, Ca^{2+}-treated rhizosphere soils of the podding stage; AMPR, AMF-inoculated rhizosphere soils of the podding stage; AMCaPR, rhizosphere soils treated with AMF and Ca^{2+} and sampled at the podding stage.

ering, and podding stages were designated as Ca^{2+}-treated rhizosphere soils sampled at the seedling (CaSR), flowering (CaFR), and podding (CaPR) stages, respectively, and AMF-inoculated rhizosphere soil groups sampled at the seedling, flowering, and podding stages were designated as AMF-inoculated rhizosphere of the seedling (AMSR), flowering (AMFR), and podding (AMPR) stages, respectively. The rhizosphere soils treated with AMF and Ca^{2+} and sampled at the seedling, flowering, and podding stages were defined as the AMF and Ca^{2+} inoculated rhizosphere of the seedling (AMCaSR), flowering (AMCaFR), and podding (AMCaPR) stages, respectively. The result showed that 1,222,767 sequences passed the quality screening and most of them were between 400 and 460 bp (Supplementary data Fig. S1). A total of 1,901 operational taxonomic units (OTUs) were obtained using > 97% sequence identification for taxonomic assignment evaluation, among which BS had the lowest OTU numbers (Supplementary data Fig. S2A).

Alpha diversity analysis was performed to analyze the community richness and diversity. The samples in the rarefaction curve did not approach the asymptote, indicating highly diversified bacterial communities in the peanut rhizosphere (Supplementary data Fig. S2B). The species accumulation curves showed that the growth rate of new species followed the increase in sample size, implying that the sequencing depth of all of the rhizosphere soils was high enough to observe community richness (Supplementary data Fig. S2C). Moreover, the rank abundance curves showed that all of the soil groups had a high species evenness and homogeneity (Supplementary data Fig. S2D). Other indices (Sobs, Shannon, Simpson, Chao1, and ace) were also calculated to detect the community richness and diversity (Supplementary data Table S1). Thus, the different soil samples of the peanut rhizosphere showed no significant differences in the alpha diversity analysis.

Peanut rhizosphere bacterial community structure taxonomic analysis

The bacterial community structure was analyzed at five levels (phylum, class, order, family, and genus). Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, and Gemmatimonadetes were the most abundant bacteria in all the soil groups at the phylum level, accounted for more than 70% of the total bacterial sequences (Fig. 1A). Further bacterial community structure analysis showed that bacterial abundance distributions were indeed altered with AMF and Ca^{2+} applications at different stages. The Proteobacteria were dramatically increased in the AMF or Ca^{2+} single- or compound-treated soils at the seedling stages, while Firmicutes increased at the podding stage. The AMF and Ca^{2+} compound-treatments had a superposition effect, increasing the numbers of Proteobacteria and Firmicutes to a greater extent compared with the single treatment (Fig. 1A). However, the abundance of Actinobacteria was lower in the AMF or Ca^{2+} single- or compound-treated soils compared with the control rhizosphere soils (Fig. 1A). Furthermore, Bacteroidetes were the dominant bacteria in the bulk soils, which was decreased in the peanut rhizosphere soils (Fig. 1A). At the class level, most of the bacterial community belonged to Gammaproteobacteria, Alphaproteobacteria, Bacteroidia, Subgroup_6, Actinobacteria, and Bacilli (Supplementary data Fig. S3A). The presence of Alphaproteobacteria and Bacilli increased in the AMF or Ca^{2+} single- or compound-treated soils at different stages, whereas the numbers of Clostridia decreased in these soils at the seedling and podding stages. Gammaproteobacteria only increased in AMF or Ca^{2+} single- or compound-treated soils at the seedling stage, and Bacteroidia decreased in all of the peanut rhizosphere soils relative to the bulk soils (Supplementary data Fig. S3A). Beneficial bacteria Lactobacillales and Sphingomonadales increased in the AMF or Ca^{2+} single- or compound-treated soils at the order level in peanut rhizosphere soils and the most dominant families were uncultured_bacterium_c_Subgroup_6 then Gemmatimona-

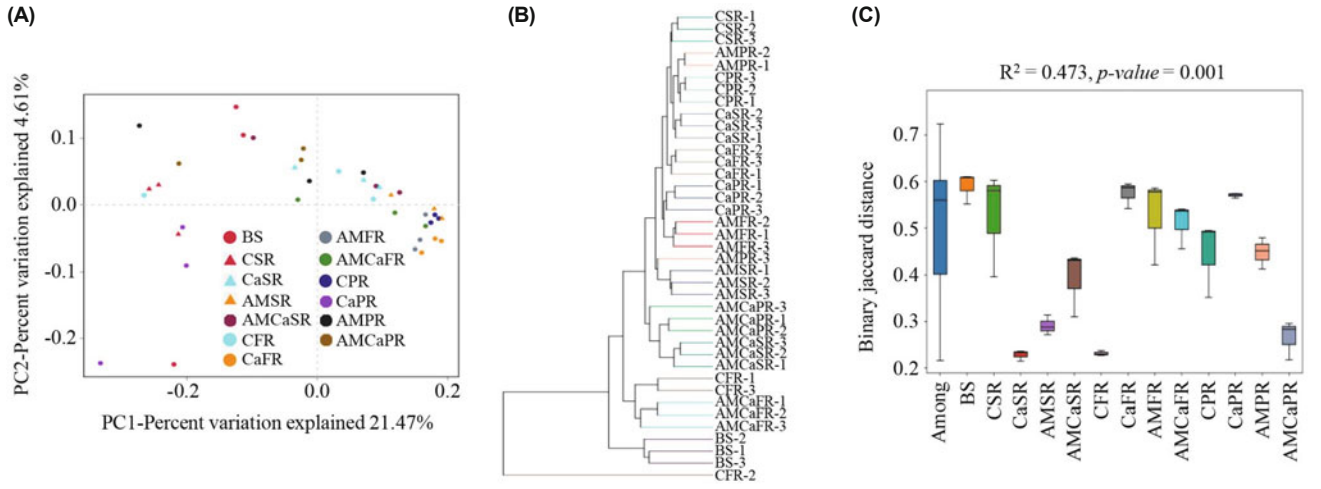

Fig. 2. Beta diversity analysis. (A) The principal component analysis (PCA). The same color points belong to the same soil group. (B) The unweighted pair-group method with arithmetic mean (UPGMA) analysis showing the degree of similarity of different soil bacterial community structures, which is clustered according to the similarity of their components in different soil groups. (C) The analysis of similarities (ANOSIM) showing the variation in the bacterial composition of rhizosphere or bulk soils.

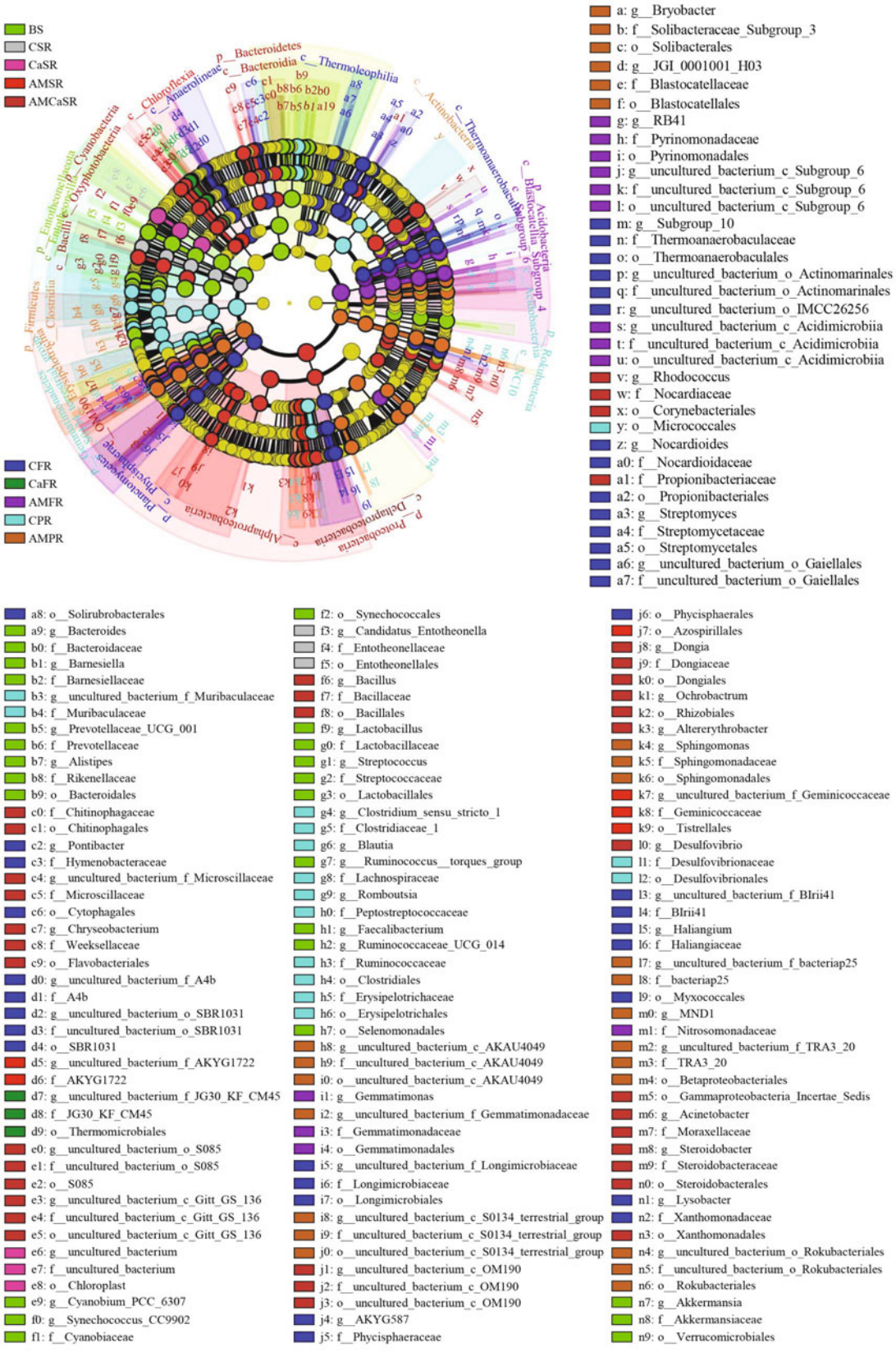

Fig. 3. Specific phylotypes of the peanut rhizosphere responded to AMF and Ca^{2+} in saline alkali soil. Specific phylotypes of the peanut rhizosphere bacterial community responded to AMF and Ca^{2+} with linear discriminant analysis (LDA) scores > 3.5. The inner-circle to the outer-circle indicates phylogenetic levels from phylum to genus. The node size corresponds to the average relative abundance of the taxa.

daceae, followed by Enterobacteriaceae and Lactobacillaceae (Fig. 1B; Supplementary data Fig. S3B).

A thorough investigation at the genus level showed that many sequences named as "uncultured" or "unclassified" were unidentified species but were present in large numbers in the rhizosphere soils, demonstrating that the peanut soils remained a challenging reservoir of biodiversity that needed further study (Fig. 1C). AMF or Ca^{2+} application enhanced the quantities of *Sphingomonas* at three diverse stages but only increased the numbers of *Escherichia-Shigella* at the seedling stage (Fig. 1C; Supplementary data Table S2). Thus, the application of AMF and Ca^{2+} synergistically causes changes in the distribution of the bacterial abundance and the enrichment of some specific bacterial species in saline alkali peanut rhizosphere soils.

Beta diversity analysis of the bacterial community

Beta diversity analysis was performed to check the similarities and dissimilarities among the different soil groups, including principal component analysis (PCA), unweighted pair-group method with arithmetic mean (UPGMA), and analysis of similarities (ANOSIM). PCA analysis showed that the bacterial community structures of the different soil groups were diverse from each other, and the first two principal components (PC1 and PC2) of PCA explained 21.47% and 4.61%, respectively (Fig. 2A). The UPGMA analysis revealed that the bacterial community structures were diverse in different soil groups, whereas three duplicate samples in the same soil group tended to cluster together (Fig. 2B). ANOSIM analysis showed a significant difference in the bacterial community structures (Fig. 2C, $R^2 = 0.473$, p-value = 0.001) of the different rhizosphere soil groups. Beta diversity analysis exhibits

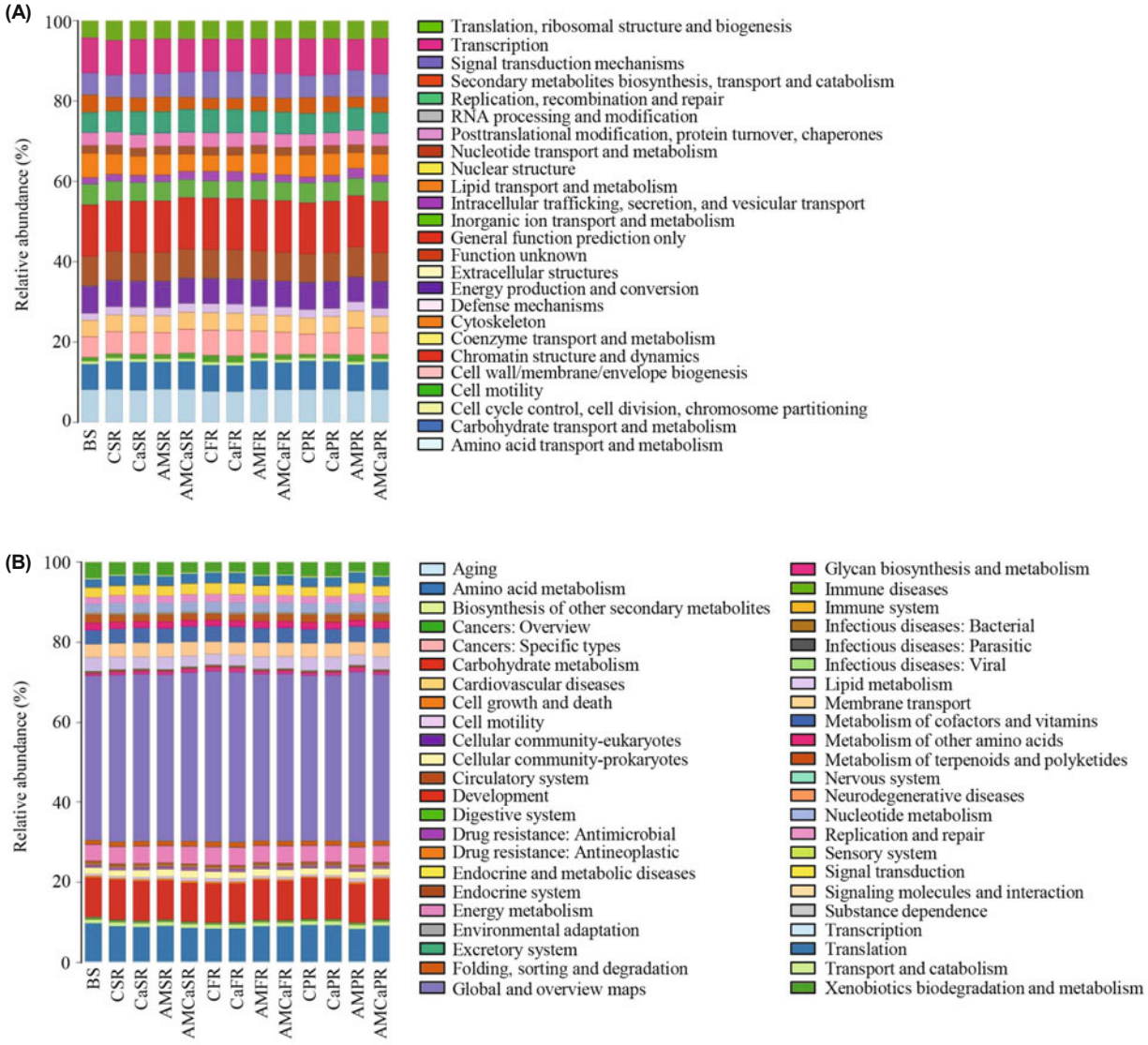

Fig. 4. Metabolic functional features of thirteen rhizosphere soils. (A) Bar chart showing the relative abundance of predicted functional groups in various peanut rhizosphere or bulk soil groups in the context of the Cluster of Orthologous Groups (COG) database. (B) The Kyoto Encyclopedia of Genes and Genomes (KEGG) database showing the relative abundance of predicted functional groups in various peanut rhizosphere or bulk soil groups.

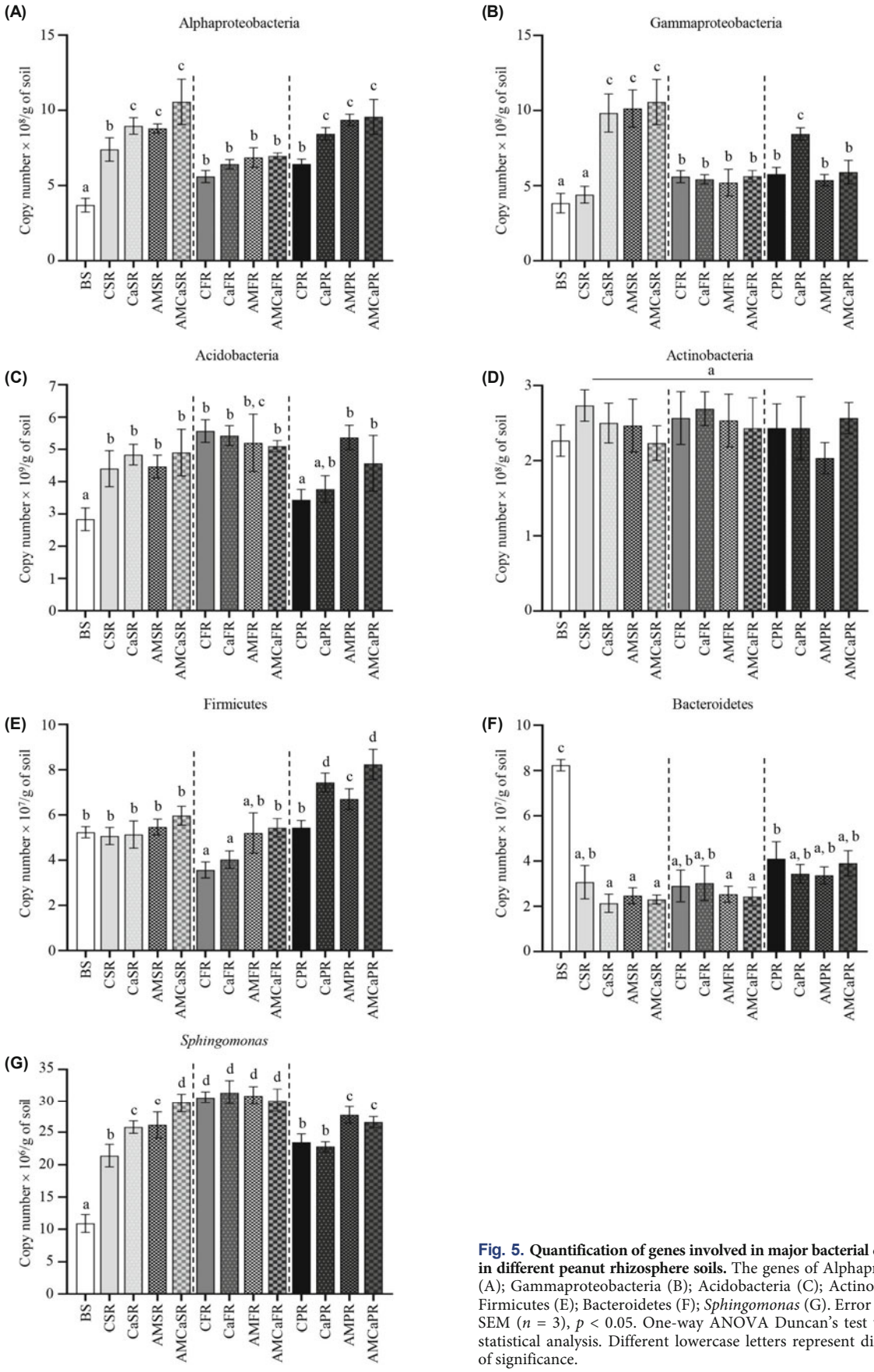

Fig. 5. Quantification of genes involved in major bacterial communities in different peanut rhizosphere soils. The genes of Alphaproteobacteria (A); Gammaproteobacteria (B); Acidobacteria (C); Actinobacteria (D); Firmicutes (E); Bacteroidetes (F); *Sphingomonas* (G). Error bars indicate SEM (n = 3), $p < 0.05$. One-way ANOVA Duncan's test was used for statistical analysis. Different lowercase letters represent different levels of significance.

a significant separation of the bacterial community structures in different rhizosphere soils, suggesting that the AMF and Ca^{2+} applications do indeed alter the peanut rhizosphere bacterial abundance distribution in saline alkali soil.

Specific phylotypes of the rhizosphere bacterial community responded to AMF and Ca^{2+} in saline alkali soil

Linear discriminant analysis (LDA) effect size (LEfSe) was employed to identify specific phylotypes of the peanut rhizosphere bacterial community from the phylum to genus level responding to AMF and Ca^{2+} (Fig. 3; Supplementary data Fig. S4; LDA ≥ 3.5). In the bulk soil (BS), LEfSe analysis confirmed the significant enrichment of Bacteroidetes (from phylum to genus) found by community structure taxonomic analysis in Fig. 1 (Fig. 3). In peanut rhizosphere soils with different treatments, the specific phylotypes were diverse. At the seedling stage, Entotheonellales (from order to genus, namely, Entotheonellales, Entotheonellaceae, and *Candidatus_Entotheonella*) were uniquely enriched in CSR, and Chloroplast (order) dominated in CaSR, while Tistrellales (from order to genus, namely, Tistrellales, Geminicoccaceae, and *uncultured_bacterium_f_Geminicoccaceae*) and Proteobacteria (phylum) were specifically elevated in AMSR and AMCaSR, respectively (Fig. 3). At the flowering stage, Planctomycetes (phylum) and Anaerolineae (class) dominated in CFR, and Acidobacteria (from phylum to genus, namely, Acidobacteria, Subgroup_6, uncultured_bacterium_c_Subgroup_6, uncultured_bacterium_c_Subgroup_6, and *uncultured_bacterium_c_Subgroup_6*) were predominant in CaFR, while Thermomicrobiales (order) were enriched in AMFR (Fig. 3). Additionally, at the podding stage, Gemmatimonadetes (phylum) were specifically dominate in CPR, and Sphingomonadales (from order to genus, namely, Sphingomonadales, Sphingomonadaceae, and *Sphingomonas*) were more abundant in AMPR (Fig. 3). The enrichment of the specific bacteria was diverse in different soil groups, which may be associated with their survival abilities after the application of AMF and Ca^{2+} in saline alkali soil.

Metabolic functional features prediction of the peanut rhizosphere bacterial community

Then, the function of the bacterial population metabolism was further predicted based on the known microbial genomic data. The Cluster of Orthologous Groups (COG) database was used first, and some categories such as replication, recombination and repair, and signal transduction mechanisms,

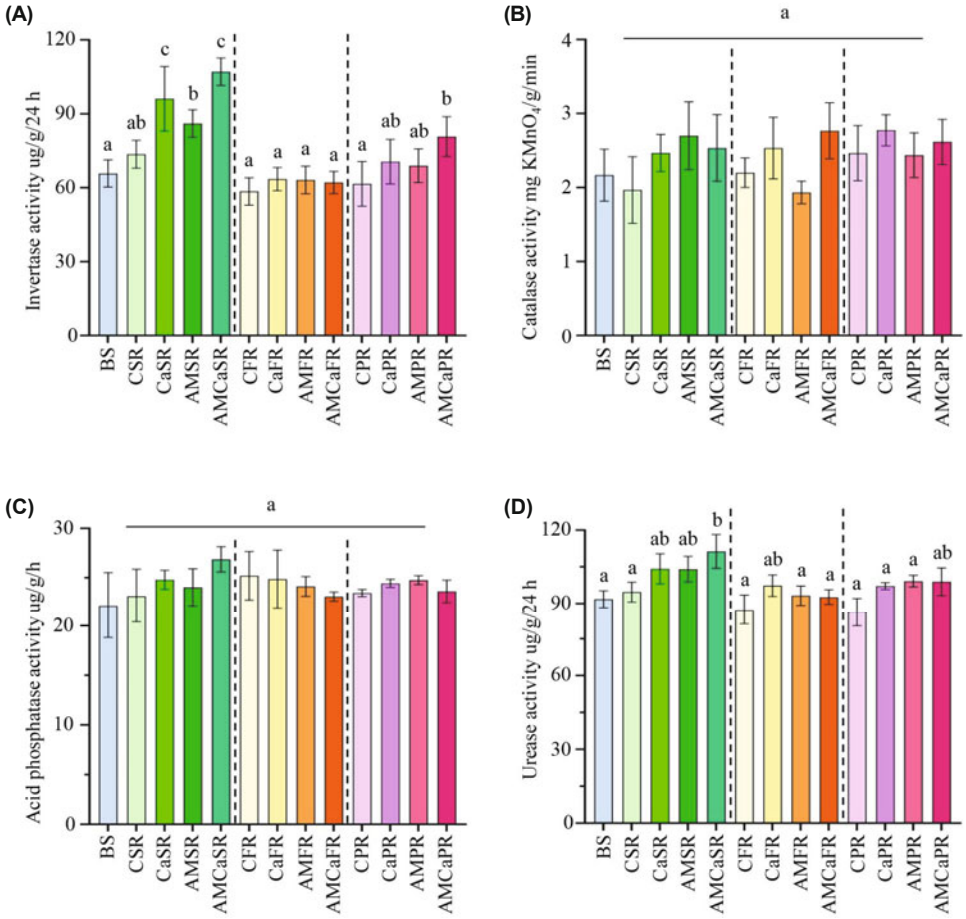

Fig. 6. Soil enzyme activities of the peanut rhizosphere soils and the bulk soils. Soil (A) invertase, (B) catalase, (C) acid phosphatase, and (D) urease activities in diverse soil groups. Error bars indicate the SEM (n = 3). One-way ANOVA Duncan's test was used for statistical analysis. Different lowercase letters represent different significance levels.

Table 2. Effects of AMF and Ca^{2+} applications on the morphology and yield of peanut in saline-alkali soil

Treatment	Plant morphology					Yield			
	Main stem height	Lateral branch length	Primary branch number	Secondary branch number	Main stem leaf number	100 pods weight (g)	100 seeds weight (g)	The number of total pods	Kernel percentage
CK	20.1 ± 1.2a	23.1 ± 0.9a	5.5 ± 0.6b	4.2 ± 0.2b	14.3 ± 0.7b	212.5 ± 1.1a	81.0 ± 0.6a	14.7 ± 0.2a	67.7 ± 0.2a
Ca^{2+}	21.3 ± 1.6a	24.3 ± 1.1a	4.5 ± 0.5a	3.5 ± 0.6a	12.7 ± 1.0a	219.3 ± 1.2ab	93.2 ± 0.8b	15.0 ± 0.3a	68.6 ± 0.1a
AMF	30.0 ± 1.3c	32.6 ± 1.7b	5.7 ± 0.2b	4.0 ± 0.3b	14.8 ± 0.7c	238.2 ± 1.4c	97.9 ± 0.5b	17.2 ± 0.5ab	71.0 ± 0.6b
Ca^{2+} + AMF	32.9 ± 1.3d	39.2 ± 0.8c	5.2 ± 0.5b	4.2 ± 0.2b	14.8 ± 1.2c	248.7 ± 0.9cd	101.8 ± 0.6c	19.7 ± 0.3b	71.7 ± 0.3b

Notes: Mean values marked followed by different letters differ significantly at $P < 0.05$ (Duncan test).

were higher in soils with AMF and Ca^{2+} at the seedling and podding stages (Fig. 4A; Supplementary data Table S3), which may be related to stress tolerance in saline alkali soil.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was also utilized to predict the bacterial metabolic functional features. Some growth-related groups (cell growth and death; cell motility) were enriched in the AMF- and Ca^{2+}-applied soils at the seedling and podding stages (Fig. 4B; Supplementary data Table S4). Interestingly, some defense-related functional groups in the AMF- and Ca^{2+}-applied soils were also higher than that of the control at diverse stages, such as environmental adaptation (at the podding stage), signal transduction (at the seeding and podding stages) and xenobiotics biodegradation and metabolism (at the flowering stage) (Fig. 4B; Supplementary data Table S4). Thus, the application of AMF and Ca^{2+} may be beneficial to peanut stress tolerance, at least in part, by regulating stress-related functional groups of the bacterial community in saline alkali soil.

qPCR of specific bacterial groups

To further verify the 16S rRNA gene sequencing data, qPCR assays were employed to analyze the changes in the abundance distribution of the major bacterial phyla (Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, Bacteroidetes) and AMF- and Ca^{2+}-induced bacterium *Sphingomonas* in all of the soil groups. Specific primers for identification of the above bacteria are listed in Table 1. The numbers of phylum Alphaproteobacteria and beneficial bacterium *Sphingomonas* were significantly higher in AMF-and Ca^{2+}-applied peanut rhizosphere soils as shown in the taxonomic analysis in Fig. 1 (Fig. 5). The Bacteroidetes were especially enriched in the bulk soils, and gradually decreased in the peanut rhizosphere soils (Figs. 1A and 5). Most of these results were consistent with the 16S rRNA gene sequencing analysis. Taken together, Alphaproteobacteria and *Sphingomonas* were the predominant bacteria in the AMF- and Ca^{2+}-applied saline alkali soil, which may act as PGPRs for salt resistance mechanisms in peanuts.

Peanut rhizosphere soil extracellular enzyme activities responded to AMF and Ca^{2+}

The soil extracellular enzyme activities of various soil samples were analyzed to determine whether they were altered with the changes of the soil bacterial communities in AMF- and Ca^{2+}-applied saline alkali soil. We found that catalase activity was roughly identical in all of the soil groups, whereas the average invertase activity in AMF or Ca^{2+} single- or compound-treated soils was increased by 30.56%, 16.95%, and 45.50% compared with the CSR at the seedling stage, respectively, and AMF symbiosis combined with exogenous Ca^{2+} was better than AMF or Ca^{2+} alone at improving invertase activity (Fig. 6A and B). These findings suggest that bacterial life activities tend to be more vigorous to some extent in AMF- and Ca^{2+}-applied saline alkali soil. Moreover, soil phosphatase activity and urease activity were also examined. We found that urease activity was slightly elevated in AMF and Ca^{2+} compound-applied saline alkali soil compared with that of the control, whereas acid phosphatase activity had no significant difference among the diverse peanut rhizosphere soils (Fig. 6C and D). Soil invertase and urease activities were associated with the soil carbon and nitrogen cycle, respectively, and thus the higher soil invertase and urease activities in the AMF- and Ca^{2+}-applied soils may be beneficial to promote peanut growth and alleviate salt stress in saline alkali soil by improving the soil quality to some extent.

Effects of AMF and Ca^{2+} applications on growth performance and yield of peanut in saline alkali soil

The results of Table 2 showed that the main stem height and the lateral branch length increased after AMF and Ca^{2+} applications, while the numbers of branches and stem leaves were not significantly different relative to the controls in saline alkali soil. In addition, the 100-pod weight, 100-seed weight, the number of total pods per plant, and kernel percentage, all showed an upward trend in the AMF and Ca^{2+} single- or compound-treated soil groups. For Ca^{2+} application, AMF symbiosis, or AMF cotreated with Ca^{2+}, the 100 pod weights of HY25 were increased by 3.20%, 12.09%, and 17.04%, respectively, and the 100 seed weights was increased by 15.06%, 20.86%, and 25.68%, respectively. Kernel percentage and the number of total pods per plant increased after AMF and Ca^{2+} compound-treatment (Table 2). Therefore, AMF and Ca^{2+} applications indeed promote growth and increase the pod yield of peanut in saline alkali soil.

Discussion

AMF symbiosis and Ca^{2+} application can promote plant growth and alleviate salt stress (Sanders *et al.*, 2002; Cui *et al.*, 2019; Tisarum *et al.*, 2020). Consistent with that, the application of AMF and Ca^{2+} in saline alkali soil increased the main stem height, lateral branch length, and pod yield of peanut to some extent (Table 2). It deserves to be mentioned that the effect of AMF combined with Ca^{2+} on peanut growth was better than that of AMF or Ca^{2+} alone (Table 2). Various

studies have emerged implicating the rhizosphere to be involved in salt stress tolerance (Egamberdieva *et al.*, 2017; Xu *et al.*, 2020b); however, whether AMF and Ca^{2+} synergistically regulate the salt stress response via partly modifying the rhizosphere microbial community structure in peanut is unclear. In this study, 16S rRNA gene sequencing was used to examine the response of the rhizosphere bacterial community to Ca^{2+} and AMF. Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, and Gemmatimonadetes were the most abundant phyla in all the soil groups, similar to what has been reported elsewhere (Lundberg *et al.*, 2012). Further bacterial community structure analysis and qPCR assays showed that the application of Ca^{2+} and AMF altered the peanut rhizosphere bacterial abundance distribution and the AMF and Ca^{2+} compound-treatments had a superposition effect on the bacterial community structural changes (Figs. 1 and 5). Beneficial bacteria Proteobacteria, Firmicutes, and *Sphingomonas* were more abundant in AMF or Ca^{2+} single- or compound-treated soils than in other soils (Fig. 1). Proteobacteria dramatically increased at the seedling stages, Firmicutes increased at the podding stage, while *Sphingomonas* was enriched at all three stages, indicating that AMF or Ca^{2+} may exert different roles in diverse stages, which needs further study.

It has been well documented that several factors influence the rhizosphere bacterial community structure, such as soil type, plant species, environmental conditions, agricultural management, and practices (Kavamura *et al.*, 2018). Fertilizers can directly influence the rhizosphere microbial communities through the provision of nutrients, improvement of soil properties, alteration of soil characteristics such as pH, and adjustment of root architecture to further alter microbial niche colonization sites (Zhang *et al.*, 2017; Kavamura *et al.*, 2018). AMF can also exert different selections on soil microbial communities depending on the plant species they are associated with whether directly through the excretion of carbohydrates (Toljander *et al.*, 2007; Qin *et al.*, 2016) or indirectly through the alteration of root exudates in a qualitative or quantitative way (Camprubi *et al.*, 1995). However, information about how Ca^{2+} application and AMF symbiosis affects the structure of the peanut rhizosphere bacteria community is scarce. Proteobacteria can degrade aliphatic and aromatic compounds into simpler forms and survive in some extreme conditions (Kostka *et al.*, 2011). Firmicutes are enriched in contaminated sites and play various roles in bioremediation and stress tolerance (Doolotkeldieva *et al.*, 2018). *Sphingomonas* were found to be involved in the decomposition of aromatic compounds, nitrogen fixation and denitrification, and the carbon cycle (Xie and Yokota, 2006; Peng *et al.*, 2008; Li *et al.*, 2018). These results reveal that AMF and Ca^{2+} applications can induce changes in soil bacterial community structure and attract more beneficial bacteria, which may participate in the nitrogen cycle, carbon cycle, and the degradation of toxic substances in these soils. These AMF and Ca^{2+}-induced beneficial bacteria may help peanuts maintain their physiological functions and adapt to salt stress in saline alkali soil.

Soil enzymes are bioactive proteins involved in soil nutrient cycling and they reflect the soil fertility (Dindar *et al.*, 2015). Invertase activity and catalase activity are correlated with microbial biomass and soil respiration intensity, indicating the soil microbial life activities (Yu *et al.*, 2016; Liang *et al.*, 2019). In addition, invertase activity is related to the soil carbon cycle (Li *et al.*, 2018); urease is involved in the nitrogen cycle and is correlated with soil quality (Gu *et al.*, 2019); and phosphatase participates in converting soil organic phosphate into inorganic phosphate, which can improve the soil-available phosphorus supply capacity (Yadav and Tarafdar, 2007). Here, the invertase activity in AMF or Ca^{2+} single- or compound-treated soils was significantly higher compared to the control at the seedling stage (Fig. 6A), suggesting that the bacterial life activities tend to be more vigorous after AMF and Ca^{2+} applications at the seedling stage in saline alkali soil. Furthermore, higher urease activity was only detected in AMF and Ca^{2+} compound-treated soils compared with that in the other soil groups (Fig. 6D), which may be partly related to the increased abundance of the nitrogen-fixing bacterium *Sphingomonas* in these soils (Fig. 5G). These results suggest that AMF and Ca^{2+} applications can improve soil fertility in a salt condition, implying again that AMF combined with Ca^{2+} was better than AMF or Ca^{2+} alone.

Metabolic functional prediction can reflect the potential functional features of soil bacterial communities under diverse conditions. KEGG database analysis showed that the percentages of reads categorized to growth-related groups (cell growth and death; cell motility) were enriched in the AMF- and Ca^{2+}-applied soils at the seedling and podding stages, suggesting that the bacterial life activities tend to be more vigorous after AMF and Ca^{2+} applications, which was consistent with the results of higher soil invertase activity (Figs. 4B and 6A; Supplementary data Table S4). Stress response categories such as environmental adaptation and signal transduction were also predicted to be higher in rhizosphere soils treated with AMF and Ca^{2+} (Fig. 4B; Supplementary data Table S4), which can confer high tolerance levels to salt stress. A previous study showed that some PGPRs can promote plant growth and salt stress tolerance by degrading xenobiotics and pollutants in soils (Ilangumaran and Smith, 2017; Xu *et al.*, 2020a). Thus, the more vigorous xenobiotics biodegradation and metabolism in AMF- and Ca^{2+}-applied soils may be beneficial to salt stress tolerance (Fig. 4B). Therefore, besides the previously reported mechanisms, the application of AMF and Ca^{2+} may also improve peanut stress tolerance by regulating stress-related functional groups of the bacterial community in saline alkali soil.

Taken together, AMF symbiosis combined with exogenous Ca^{2+} was better than AMF or Ca^{2+} alone at prompting plant growth, improving the soil quality, and modifying the bacterial community structure in the peanut salt adaption. Exploring salt response mechanisms and further enhancing peanut salt tolerance by regulating the AMF and Ca^{2+} applications and allocation may be good strategies to improve peanut production in saline alkali soil.

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Conflict of Interest

The authors declare no conflict of interest.

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