Enhancement of Soil Phosphorus Bioavailability by Arbuscular Mycorrhizae and Earthworms Through Regulating Soil Bacterial Community and Plant Nutrient Balance Under Salt Stress

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Abstract

Aims

Soil salinization is an important factor limiting plant phosphorus (P) uptake and crop production. This study aimed to investigate the effects of arbuscular mycorrhizal fungi (AMFs) and earthworms in enhancing soil P bioavailability by regulating soil salt ions and altering the soil bacterial community under salt stress.

Methods

Treatments with or without earthworms and with or without AMFs in a high-salinity soil were applied.

Results

The results showed that the maize biomass and plant P, Ca and Mg contents were significantly increased by earthworms and AMF inoculation, and the highest plant P, Ca and Mg contents were observed with earthworm application alone. Earthworms and AMFs significantly decreased the soil stable inorganic P (hydroxyapatite) proportion and increased the soil available dicalcium phosphate proportion. AMFs significantly increased soil phosphatase activity and inorganic P fraction contents. Earthworms and AMFs significantly increased soil bacterial Chao1 and phylogenetic diversity. Structural equation model analysis showed that the most important driver of soil P mineralization was soil bacterial diversity, followed by soil Ca^{2+} and total salt concentration. Network analysis suggested that the response of bacteria to soil Ca^{2+} but not salt concentration positively correlated with soil P availability. Earthworms and AMFs could stimulate certain bacteria harbouring the phoX alkaline phosphatase gene to increase soil phosphatase activity and soil P availability.

Conclusions

In conclusion, earthworms and AMFs could enhance soil P bioavailability by stimulating soil P-cycling bacteria to activate soil stable inorganic P and by improving the plant cation nutrient balance under salt stress.

Highlights

1. Arbuscular mycorrhizal fungi (AMFs) and earthworms could relieve the adverse effects of high salinity on maize P uptake and growth.
2. Earthworms strongly enhanced maize P utilization, which was significantly associated with increasing plant Mg and Ca uptake.
3. AMFs predominantly improved soil P availability by increasing soil phosphatase activity to activate stable inorganic phosphate (Ca_{10}-P).

4. AMFs and earthworms could increase soil bacterial diversity and stimulate P-cycling species to increase soil phosphatase activity and soil P availability.

1. Introduction

Phosphorus (P) limitation ofcrop productivity is of particular concern in saline soils due to the high ionic adsorption and alkaline pH (Bano and Fatima, 2009). High ionic strength can enhance the adsorption of soil P by clay minerals and Fe/Al oxides, which leads to a decrease in P activity (Arai and Sparks, 2007). In saline soils, excessive salt ions strongly interfere with plant phosphorus, magnesium and calcium uptake and result in a nutritional imbalance in maize (Farooq et al., 2015). Moreover, high salinity could have adverse effects on the preservation and mineralization of soil organic P by influencing organic matter inputs, destroying soil structure and decreasing soil microbial biomass and potential enzyme activities (Singh, 2016). A previous study demonstrated that for plants simultaneously subjected to soil salinity and soil P deficiency, the impact of the latter constraint on plant growth was predominant (Talbi Zribi et al., 2011). Therefore, understanding the links between soil P availability, soil ion alteration and plant nutrient uptake is important in saline soils.

Arbuscular mycorrhizal fungi (AMFs) symbiose with many crop plants and help their host plants acquire nutrients and resist salt stress (Porcel et al., 2012). AMFs could enhance soil organic P mineralization and increase soil P availability by releasing photosynthetic carbon to trigger soil phosphate-solubilizing bacterium growth (Zhang et al., 2016a). AMFs could release protons to mobilize insoluble P and extend the nutrient absorptive surface by the formation of extensive mycorrhizal hyphae (Smith and Smith, 2011). The existing information about mycorrhizal plants in saline soils focuses on the enhancement of plant nutrition acquisition and physiological tolerance to salt stress (Tigka and Ipsilantis, 2020; Wang et al., 2020). In contrast, scarce information is available on the possible role of AMFs in the soil microbial community in driving the P cycle or P transformation in saline soil. Hidriet al. (2019) reported that the bacterium *Bacillus subtilis* and AMFs could promote plant growth and contribute to increased plant nutrients and soil enzyme activities such as those of urease and alkaline phosphatase under salt stress. Therefore, how AMFs influence the soil bacterial community and their potential roles in enhancing P availability in saline soil need to be further studied.

Earthworms can influence soil P availability for plants and microorganisms by regulating soil P mobilization and sorption (Le Bayon and Binet 2006). The carboxyl groups in earthworm mucus can compete for P absorption sites, such as the cations Ca, Al and Fe, to increase soil P solubilization (Bianco and Defez 2010). The behaviour of earthworms, including excavation, excretion and limivorous action, can increase soil microbial activity and produce high phosphatase activity in vermicomposts to improve P cycling (Gusain and Suthar, 2020). All of the above reports about the roles of earthworms in P availability were conducted in non-saline soil. Just Oo et al. (2015) reported that vermicompost was effective in alleviating salinity, increasing soil total extractable phosphorus and improving crop growth. Our previous studies demonstrated that earthworms could increase soil P availability and improve the P absorption by maize byregulating the soil microbial community in saline soil (Zhang et al., 2016b; Zhang et al., 2018). However, the relations between the absorption of plant P and the cations affected by earthworms under salt stress are still not clear.

In non-P active soil, the availability of soil phytate-P and insoluble inorganic P (calcium phosphates) was effectively improved by earthworms and AMF interactions (Cao et al., 2015a; Cao et al., 2015b). Our previous studies showed that earthworms and AMFs cooperatively increased soil P availability in high-salinity soil (EC 8.56 dSm^{-1}, Zhang et al., 2018) and promoted maize P absorption in low-salinity soil (EC 4.41 dSm^{-1}, Zhang et al., 2016b). The mechanism through which earthworms and AMFs interact to affect maize P utilization in high-salinity soil needs to be further studied. Here, we explored the individual and combined roles of AMFs and earthworms in enhancing soil P availability and maize P uptake in high-salinity soil. Soil bacterial communities in the rhizosphere associated with earthworms and AMFs were assessed by Illumina MiSeq sequencing analysis. Correlations between maize P uptake, soil inorganic P fractions, soil salt ions, soil phosphatase activity and soil microbial diversity and composition were monitored. Our hypotheses were as follows: i) AMFs and earthworms relieve the negative effects of salt stress on maize P uptake by improving the plant nutrient balance and increasing soil P bioavailability; ii) AMFs increase soil P availability by enhancing soil phosphatase activity and regulating soil P-cycling bacteria to activate soil stable inorganic P; and iii) earthworms regulate maize cation utilization to enhance maize P utilization (Fig. 1).

2. Methods And Materials

2.1. Experimental design

A natural-salinity soil was collected from the top layer (0–25 cm) of farmland with summer maize and winter wheat rotation in Shandong Province, China (37°43' N, 117°34' E). The soil properties were as follows: total soluble salt concentration 6.33 g kg^{-1}, pH 8.24 (soil/H_2O 1/2.5), Olsen-P concentration 42.34 mg kg^{-1}, total nitrogen 0.91 g kg^{-1}, NH_4OAc-exchangeable potassium 215 mg kg^{-1}, and organic carbon concentration 5.60 g kg^{-1}. The air-dried soil was passed through a 2 mm sieve and sterilized with 25 K Gry γ-rays for 72 h to eliminate native microorganisms.

Four treatments were set up as follows: CK, high-salinity soil with maize planting; E, high-salinity soil with maize planting and earthworm addition; AM, high-salinity soil with maize planting and AMF inoculation; and E + AM, high-salinity soil with maize planting and earthworm and AMF addition. There were four replicates for each treatment. One germinated maize seed was sown in each pot. The mycorrhizal inoculum (*Funneliformis mosseae*, Beijing Academy of Agricultural and Forestry Sciences) was applied at 2.5 g kg^{-1} soil. Equal amounts of sterilized AMF inoculum were added to treatments without AMFs (CK and E). Ten similar adult earthworms (*Eisenia fetida*, 3.09 ± 0.08g fresh weight) were inoculated at the third-leaf stage of the maize. *Eisenia fetida* was chosen because it could be easily obtained and it was the only species that largely cultivated and applied to modify soil quality in China (Guo et al., 2019). Earthworms were incubated and adapted in the experimental soil for 14 days before addition. The surface and guts of earthworms were pre-treated to eliminate the impact of natural AMF propagules. All pots were maintained at 65% of the soil water-holding capacity by weighing and supplementing deionized water.

2.2 Soil and plant sample harvesting

The soil and plant samples were harvested 60 days after sowing. The shoots and roots were separately collected. Aliquotsof roots were frozen at -20°C for mycorrhizal colonization. The other plant samples were oven-dried for elemental analysis. To prevent the potential influence of exotic bacteria, the top 2 cm of surface soil was removed before collecting soil samples. The well-sieved and mixed soil was frozen for measuring phosphatase activity (-20°C) and soil DNA extraction (-70°C) or air-dried for detecting soil organic P fractions and salt ions. Earthworms were recovered, washed, and weighed.

2.3. Assays for root mycorrhizal colonization and plant nutrients

To determine the intensity of mycorrhizal colonization (M%) ofmaize roots, the cut root segments (1 cm) were stained with Trypan blue (McGonigle et al., 1990), and the MYCOCALC program was used to calculate M%. The dried and ground plant samples were digested with a mixture of HCl/HNO (3/1 v/v) and examined by inductively coupled plasma-optical emission spectroscopy for plant P, Ca and Mg contents.

2.4 Assays for soil salt ions and soil inorganic P fractions

Soil soluble cations and anions were extracted with deionized water (soil/water 1/5 w/v) (Bao, 2000). Soil Na^+ was determined by flame photometry, whereas soil Ca^{2+} and soil Mg^{2+} were analysed by EDTA titration following a standard procedure. The concentration of soil Cl^- was determined by titration with 0.025 mol L^{-1} silver nitrate solutions using potassium chromate indicator (Bao, 2000). Soil HCO_3^- was determined by neutralization titration with double indicators, namely, phenolphthalein and bromophenol blue (Bao 2000). To study the P characteristics and transformation, soil inorganic P fractions, including calcium phosphate (Ca-P), nonoccluded aluminium phosphate (Al-P) and iron phosphate (Fe-P), and occluded phosphate (Oc-P), were analysed by sequential extractions (Jiang and Gu, 1989). Calcium phosphates, including dicalcium phosphate (Ca_2-P), octocalcium phosphate (Ca_8-P) and hydroxyapatite (Ca_{10}-P), were determined (Jiang and Gu, 1989). Briefly, 1 g of dry soil was first extracted by 0.25 mol L^{-1} $NaHCO_3$ for Ca_2-P determination; second, the $NaHCO_3$-extracted soil was dissolved in 0.5 mol L^{-1} NH_4OAc to measure Ca_8-P; third, the NH_4OAc-extracted soil was dissolved in 0.5 mol L^{-1} NH_4F to determine nonoccluded Al-P; fourth, 0.5 mol L^{-1} NaOH and 0.1 mol L^{-1} Na_2CO_3were added to the NH_4F-extracted soil to determine nonoccluded Fe-P; fifth, the extracted soil from the fourth step was added to 0.5 mol L^{-1} NaOH and digested with 10 ml of a $H_2SO_4/HClO_4/HNO_3$mixture (1/2/7 v/v/v) to determine Oc-P; and sixth, the extracted soil from the fifth step was extracted with 0.5 mol L^{-1} ($1/2H_2SO_4$) to determine Ca_{10}-P.All extracts were analysed for P fractions by the molybdate blue method (Murphy and Riley, 1962). The mobility and mineralization magnitude of inorganic P fractions was in accordance with their extraction order, i.e., Ca_2-P > Ca_8-P > nonoccluded Al-P > nonoccluded Fe-P > occluded P > Ca_{10}-P. Soil alkaline (pH 10) phosphatase was determined at 420 nm (Tabatabai, 1982).

2.5 Soil bacterial 16S rRNA analysis

Soil DNA extraction was conducted using an EZNA Soil DNA Kit (Omega Bio-Tek, Inc., USA). The 16 S RNA gene was amplified with the primer pair338 F (5'-ACTC CTACGGGAGGCAGCAG-3') and 806 R (5'-GGAC TACHVGGGTWTCTAAT-3') according to Herlemann et al. (2011).PCR was performed with SYBR® Premix Ex

Taq^{TM}II in a 50 µl reaction. Reaction procedures consisted of an initial step at 95°C for 5 min; 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 40 s; and a final extension at 72°C for 10 min.The purified PCR products were sequenced onthe Illumina MiSeq platform (Allwegene, Beijing, China).Taxonomic information foreach operational taxonomic unit (OTU, at 97% similarity) was assigned by the 16S rRNA Greengenes database. The software package QIIME 1.6.0 was used to process sequencing data and to calculate diversity (Chao1 and phylogenetic diversity indices) (Caporaso et al., 2010). A Circos diagram was createdwith Circos-0.67-7 to analyse the correspondence between the samples and bacterial species. The most differentially abundant taxa between treatments were analysed by the linear discriminant analysis effect size (LEfSe) method.

2.6 Data analyses

Significant differences between treatments were analysed by Tukey's test. Pearson's correlation analysis was performed using SPSS 17.0.Redundancy analysis (RDA) was performed by the Canoco 4.5 package. Soil variables were linked using a structural equation model (SEM) to determine the drivers of soil P availability (Tejada et al., 2006). The SEM model was built and modified using AMOS 21.0. Model adequacy was assessed according to nonsignificant χ2 tests ($p > 0.05$), a high comparative fit index (CFI ≥ 0.9) and a low root-mean-square error of approximation index (RMSEA < 0.1). Network analysis was performed to visualize the associations between soil properties and the soil bacterial community in Cytoscape 3.0. Pairwise similarities in the network were assessed by Spearman correlation coefficients.

3 Results

3.1. Plant biomass and nutrient uptake

The addition of earthworms, AMFs and their combination significantly increased maize biomass and the plant P content, indicating a positive effect of earthworms and AMFs on maize growth and P utilization under salt stress (Fig. 2). Compared to the CK treatment, treatments E, AM and E + AM significantly increased the plant Mg and Ca contents, with the highest values of plant Mg and Ca in the E treatment. These results indicated that earthworms and AMFs promoted maize cationic nutrient (Mg and Ca) uptake and that earthworms had an advantage in enhancing the utilization of Ca and Mg.

3.2. Individual soil P fractions and phosphatase activity

Analysis of the inorganic P fraction distribution showed that the largest proportions of soil inorganic P among all treatments were Ca-P, which accounted for 48–55%, and Al-P, which accounted for 18–21% (Fig. 3). Mycorrhizal inoculation (treatments AM and E + AM) significantly decreased the proportions of Ca_{10}-P (19–21%) and Al-P (12–15%) while increasing the proportion of Oc-P (60–90%) ($p < 0.05$). The proportion of Ca_2-P was significantly higher in the E + AM treatment than in the CK treatment ($p < 0.05$), indicating an increase in soil active P. Compared to those in the controls, the Ca_2-P, nonoccluded Al-P and nonoccluded Fe-P contents were significantly increased in all treatments (Table S1; $p < 0.05$). Treatments AM and E + AM significantly increased the soil Oc-P and Ca_8-P contents and soil alkaline phosphatase activity (Table S1 and Table 1; $p < 0.05$).

3.3. Changes in soil anions and cations

Compared to the CK, the AM and E + AM treatments significantly decreased the soil Cl^- concentration and increased the soil HCO_3^- concentration (Table 1; $p < 0.05$). The soil Na^+ concentration decreased in the AM treatment ($p < 0.05$, Table 1). The soil Ca^{2+} concentration was significantly decreased in all treatments, with the lowest value recorded in the AM treatment. The soil Mg^{2+} concentration was significantly higher with earthworm addition (E and E + AM) than without earthworm addition (Table 1; $p < 0.05$).

3.4. Mycorrhizal colonization and soil bacterial community

The intensity of mycorrhizal colonization of the roots was higher in treatments AM (13%-29%) and E + AM (25%-42%) than in the controls (0.2%-0.5%), indicating successful AMF colonization of maize roots. All initial earthworms were recovered, no significant change was found in their biomass at harvest. There was also no significant difference in their biomass between treatments E and E + AM. Metagenomic profiling analysis of Illumina-based 16S rRNA showed that treatments E, AM and E + AM increased the OTU numbers by 27%, 43% and 38%, respectively, compared with those in the controls (1128 OTUs). Good sequencing coverage was obtained. The rarefaction curves were shown in Fig. S1. The Chao1 and phylogenetic diversity indices were significantly increased in all treatments, indicating increased soil bacterial diversity (Table 1). Variations in the relative abundance of the dominant bacterial species (phyla) among all treatments are shown in Fig. 4. The phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were the dominant species associated with earthworms and AMF inoculation. The most abundant bacterial groups with significant differences at the phylum, class, order and family levels are summarized inTable S2. Treatments E, AM and E + AM significantly increased the relative abundance of the phylum*Chloroflexi* ($p < 0.05$). The AM treatment significantly increased phylum *Acidobacteria* abundance, and the E + AM treatment significantly decreased *Bacteroidetes* abundance ($p < 0.05$). At the class level, the E + AM treatment significantly increased the relative abundance of *Anaerolineae* and *Acidimicrobiia* ($p < 0.05$). Additionally, the AM treatment significantly increased the relative abundance of *Acidobacteria* and an unidentified species ($p < 0.05$). At the order level, earthworms and AMFs significantly decreased the relative abundance of *Flavobacteriales*, *Alteromonadales* and *Cytophagales* ($p < 0.05$). At the family level, earthworms and AMFs significantly increased the relative abundance of an unidentified species and *Bacillaceae* ($p < 0.05$). Earthworm presence significantly increased *Oceanospirillaceae* abundance ($p < 0.05$). As shown in Fig. 5, LEfSe analyses revealed significant sensitive bacterial species in all treatments. Overrepresented species included *Xanthomonadaceae*, *Xanthomonadales*, *Oceanospirillaceae*, *Arenimonas* and *Betaproteobacteria* in the E treatments. The predominant species included *Chloroflexi*, *Anaerolineales*, *Anaerolineae*, *Anaerolineaceae1* and *Anaerolineaceae2* in the AM and E + AM treatments.

3.5. Network analysis of soil P availability, salt ions and their associated bacteria

As shown in Fig. 6, the soil P availability and salt ions quantified in this study were significantly correlated with various bacterial species ($p < 0.05$). The network was divided into four groups: the decreases in soil total salt content and soil Ca^{2+} induced by the addition of earthworms and AMFs resulted in more increasing bacterial clusters (43) than decreasing bacterial clusters (10). The increases in soil P availability and soil

HCO_3^- concentration in all treatments resulted in more increasing bacterial clusters (49) than decreasing bacterial clusters (5). The bacteria associated with decreasing soil salt content (41) and Ca^{2+} (39) had the highest diversity, which generally increased with the addition of earthworms and AMFs. For instance, earthworms and AMFs elevated the abundance of the genera *Chloroflexi*, *Acidimicrobiales* and *Anaerolineaceae* (Table S3). The significant correlation relationships between soil chemical properties and their associated bacteria in the network indicated that the bacterial taxa were developed and shaped by a combination of factors.

3.6. Factors driving soil P availability

To investigate the predominant factor increasing soil P availability, a hypothesized SEM model (Fig. 9a) was established, and its fitting parameters were excellent (χ^2, 5.476; *p*, 0.602; CFI, 1.000; RMSEA, 0.000). Our SEM explained 76% of the variance in soil P availability (Fig. 9a). When considering both direct and indirect effects, soil bacterial diversity was the most important driver controlling soil P availability, followed by soil Ca^{2+} concentration (λ = -0.589) and salt concentration (λ = -0.529)(Fig. 9b). Soil P availability was directly affected by soil bacterial diversity and phosphatase activity and indirectly affected by soil Ca^{2+}, salt concentration and soil HCO_3^- (Fig. 9a). Soil P availability was positively driven by soil bacterial diversity, phosphatase activity and soil HCO_3^- but negatively affected by soil Ca^{2+} and salt concentration (Fig. 9b).

4. Discussion

Maize P uptake regulated by earthworms and mycorrhizae in saline soil

It is well known that high salinity decreases plant P uptake and adversely affects crop growth (Sima et al., 2019). In addition to being involved in membrane formation and intracellular metabolism, the increased plant inorganic phosphate ($H_2PO_4^-$) could enhance salt tolerance by strengthening the protoplasm's buffering capacity to alkaline pH ($KH_2PO_4 + KOH \rightarrow K_2HPO_4 + H_2O$) (Bental et al., 1988). Previous studies demonstrated that plant growth was significantly inhibited by salt stress, with less biomass under deficient P than sufficient P (Talbi Zribi et al., 2011; Sima et al., 2019). In this study, earthworms, mycorrhizae and their combination significantly increased maize biomass and plant P contents, indicating enhancement of maize P uptake in saline soil. There was a significant positive correlation between maize biomass and plant P content (r = 0.678, $p < 0.01$), suggesting that earthworms and mycorrhizae could increase plant P uptake to promote maize growth. Our previous study demonstrated that earthworms and AMFs could decrease the salt content, which could be attributed to the decrease in predominant salt ions (i.e., Cl^- and Na^+) induced by AMF inoculation (Table 1). Additionally, earthworms and AMFs could improve soil structure, which promotes salt leaching (Zhang et al., 2018; Oo et al., 2015). AMF inoculation significantly increased the amount of soil HCO_3^- along with the salt reduction. This may have been due to the release of CO_2 by mycorrhizal roots, resulting in the solubilization of $CaCO_3$ ($CaCO_3 + CO_2 + H_2O \leftrightharpoons 2Ca^{2+} + 2HCO_3^-$) during the desalting process (Akhter et al., 2003). Earthworms and AMFs increased the maize P content, which was significantly negatively correlated with the soil salt concentration (r = -0.547, $p < 0.05$, Fig. 7). These results indicated that the decrease in soil salt content induced by earthworms and AMFs could contribute to the P uptake by maize.

Magnesium plays a major role in plant phosphorus absorption and transport and enhances P use efficiency (Grzebis et al., 2018). Calcium could stabilize cell wall structure, regulate ion (K^+, Na^+ and Mg^{2+}) transport and selectivity and improve plant osmoregulation (Elkelish et al., 2019). Earthworms, mycorrhizae and their combination significantly increased the shoot Mg and Ca contents, which were significantly positively correlated with maize P content (Fig. 8, $p < 0.01$). Thus, we speculated that earthworms and mycorrhizae probably enhance maize P uptake by increasing Mg and Ca utilization. Tuffen et al. (2002) demonstrated that earthworm activity could enhance ^{32}P transfer by AMF mycelium to leek (*Allium porrum* L.) in non-saline soil. In this study, the E treatment resulted in the greatest magnitude of increase in maize P, Mg and Ca contents (Fig. 2), indicating that the addition of earthworms did not enhance the capacity of AMFs to transfer these soil nutrients to their host maize. However, earthworms addition increased the hyphal length density by 17.3% compared to that under AMF inoculation alone (Zhang et al., 2018). Therefore, we considered earthworm presence to help AMFs obtain P, Mg and Ca for their own growth instead of providing them to their host plant under salt stress. Consequently, earthworms played a predominant role in improving the P uptake and growth of AMF hyphae and their host plants by enhancing Mg and Ca utilization in saline soil.

Improvement by earthworms and mycorrhizae of soil P availability by activating stable inorganic P

The predominant form of soil P taken up for plant growth and productivity is free phosphate ions (HPO_4^{2-} and $H_2PO_4^-$) (Bucher, 2007). However, when applied to the soil, chemical phosphate fertilizer is largely immobilized into hard dissolved forms with low plant utilization efficiency, such as $CaHPO_4$, $Ca_3(PO4)_2$, $FePO_4$ and $AlPO_4$(Peak et al. 2012). Soil inorganic P fraction distribution analysis showed that the largest proportions of soil inorganic P across all treatments were Ca-P (48–55%) and Al-P (18–21%) (Fig. 3). This indicates that soil phosphates predominantly existed in the form of calcium phosphate in the high-salinity soil (Khan et al. 2009). During precipitation with Ca, soil P initially generates dicalcium phosphate (Ca_2-P), which can be easily absorbed by plants, and Ca_2-P can then be transformed into more stable forms, such as octocalcium phosphate (Ca_8-P) and hydroxyapatite (Ca_{10}-P), which have limited availability to plants (Shen et al., 2011). Earthworms and AMFs significantly decreased the proportion of soil hydroxyapatite (Ca_{10}-P) and increased the proportion of soil dicalcium phosphate (Ca_2-P) (Fig. 3).Simultaneously, the soil Ca_{10}-P proportion was significantly negatively correlated with the soil Ca_2-P proportion (Fig. 8, $p < 0.05$). These results indicate a transformation of the soil stable P form (Ca_{10}-P) into active or available P (Ca_2-P) driven by earthworm and AMF combination. Additionally, AMF inoculation significantly enhanced soil phosphatase activity and increased the soil inorganic P fractions (Ca_2-P, nonoccluded Al-P, Ca_8-P, nonoccluded Fe-P and Oc-P) (Table 1 and Table S1). Soil phosphatase activity was negatively correlated with the soil Ca_{10}-P proportion (Fig. 8, $p < 0.01$) and positively related to the increased soil inorganic P fractions ($r > 0.640$, $p < 0.01$). Therefore, we suggest that mycorrhizae could strengthen soil phosphatase activity to promote stable P (Ca_{10}-P) dissolution and mineralization. Thus, AMF appears to have a dominant role in activating hard dissolved P by increasing soil phosphatase activity under salt stress.

Enhancement of soil phosphatase activity by earthworms and mycorrhizae via altering soil bacteria

To overcome the limitation of bioavailable inorganic orthophosphate, plants and bacteria secrete numerous phosphatases to cleave orthophosphate from complex organic P substrates (Lidbury et al., 2017). In this study, the separate and combined addition of earthworms and AMFs significantly increased the Chao1 and phylogenetic diversity indices (Table 1), which were significantly positively correlated with soil alkaline phosphatase ($r > 0.656$, $p < 0.01$). These results suggested that earthworms and AMFs probably increased soil bacterial diversity to enhance soil phosphatase activity. To understand the drivers of soil P availability, an SEM was constructed to jointly investigate the multiple disparate pathways associated with P activation. The SEM provided evidence that soil bacterial diversity was the most important driver of soil P availability, followed by soil Ca^{2+} concentration and salt concentration (Fig. 9b). This indicated that low bacterial diversity was the key factor limiting soil P activation, while the increase in soil bacterial diversity induced by earthworms and AMFs resulted in increased soil P availability (Zhang et al., 2018). Network analysis could provide new insights into the relationships between soil nutrients and their associated bacteria under complex conditions (Zheng et al., 2018). Our network analysis showed that the change in bacterial diversity was largely dependent on the soil salt concentration and Ca^{2+} concentration (Fig. 6). Simultaneously, the response of bacteria toCa^{2+} concentration but not salt concentration had a significant positive correlation with soil P availability.

Identifying the physiological attributes of the dominant bacterial taxa is critical for understanding the microbial controls on some key soil processes, e.g., soil carbon and nutrient cycling (Manuel et al., 2018). Previous studies reported that abundant phyla, including *Acidobacteria* and *Chloroflexi*, harboured the phoX alkaline phosphatase gene and participated in organic phosphorus cycling in multiple soil environments (Ragot et al., 2017; Shao et al., 2020). Earthworms and AMFs significantly increased the abundance of the phylum *Chloroflexi*, and AMFs alone increased the abundance of the phylum *Acidobacteria*. The abundances of *Chloroflexi* and *Acidobacteria* were positively correlated with the soil phosphatase and soil Olsen-P contents (Fig. 7, $p < 0.05$). Thus, we speculated that earthworms and AMFs may stimulate the growth of *Chloroflexi* and *Acidobacteria* to increase soil phosphatase activity and thus enhance soil P availability. LEfSe analysis reveals some predominant phylotypes in smaller taxonomic categories. *Xanthomonadales* and *Oceanospirillaceae*species were significantly higher in the E treatment than in other treatments (Fig. 5), indicating that earthworms could stimulate these bacteria to participate in P degradation and accumulation (Martin et al., 2006; Sosa et al., 2017). Earthworms and AMF inoculation increased the abundance of *Chloroflexi* and *Anaerolineales* (Fig. 5), which are dominant in carbon-rich soil and associated with cellulose hydrolysis (Lian et al., 2017; Pinnell et al., 2014). Simultaneously, the earthworm and AMF combination decreased the abundance of *Cytophagale*, which is an efficient degrader of complex organic compounds in relatively low-carbon soil (Zhang et al., 2013). The shift inbacterial composition from the low-carbon type (*Cytophagales*) to the rich-carbon type (*Chloroflexi* and *Anaerolineales*) induced by earthworm and mycorrhizal activities suggested an improvement in saline soil nutrient conditions.

Conclusion

The present study indicated that earthworms and AMFs could interact to relieve the negative effects of salt stress on maize P uptake and growth. Earthworms predominantly enhanced maize P uptake by increasing plant Ca and Mg utilization, and AMFs played a dominant role in activating hard dissolvable P (Ca_{10}-P) by

increasing soil alkaline phosphatase activity. Soil bacterial diversity was the most important driver controlling saline soil P availability under the combined regulation of earthworms and AMFs. Earthworms and AMFs stimulated certain bacteria (*Acidobacteria* and *Chloroflexi*) harbouring the phoX alkaline phosphatase gene to enhance soil phosphatase activity. This study clearly proved that earthworms and AMFs could strengthen soil P bioavailability by improving soil bacterial diversity and altering soil salt ions and their associated bacterial species under salt stress.

Declarations

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References

1. Arai Y, Sparks DL (2007) Phosphate reaction dynamics in soils and soil components: A multiscale approach. Adv agron 94:135–179
2. Akhter J, Mahmood K, Malik K, Ahmed S, Murray R (2003) Amelioration of a saline sodic soil through cultivation of a salt-tolerant grass *Leptochloa fusca*. Environ Conserv 30(2):168–174
3. Bano A, Fatima M (2009) Salt tolerance in *Zea mays* (L.) following inoculation with *Rhizobium* and *Pseudomonas*. Biol Fert Soils 45:405–413
4. Bental M, Oren-Shamir M, Avron M, Degani H (1988) ^{31}P and ^{13}C-NMR Studies of the Phosphorus and Carbon Metabolites in the Halotolerant Alga, *Dunaliella salina*. Plant Physiol 87:320–324
5. Bianco C, Defez R (2010) Improvement of phosphate solubilization and medicago plant yield by an indole-3-acetic acid over producing strain of Sinorhizobium meliloti. Appl Environ Microbiol 76:4626–4632
6. Bucher M (2007) Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytol 173:11–26
7. Cao J, Huang Y, Wang C (2015a) Rhizosphere interactions between earthworms (*Eisenia fetida*) and arbuscular mycorrhizal fungus (*Funneliformis mosseae*) promote utilization efficiency of phytate phosphorus in maize. Appl Soil Ecol 94:30–39
8. Cao J, Wang C, Huang Y (2015b) Interactive impacts of earthworms (*Eisenia fetida*) and arbuscular mycorrhizal fungi (*Funneliformis mosseae*) on the bioavailability of calcium phosphates. Plant Soil 396(1–2):45–57
9. Caporaso JG, Kuczynski J, Stombaugh J et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336
10. Elkelish AA, Alnusaire TS, Soliman MH et al (2019) Calcium availability regulates antioxidant system, physio-biochemical activities and alleviates salinity stress mediated oxidative damage in soybean

seedlings. J Appl Bot Food Qual 92:258–266

11. Farooq M, Hussain M, Wakeel A, Siddique KH (2015) Salt stress in maize: effects, resistance mechanisms, and management. A review. Agron Sustain Dev 35:461–481
12. Grzebis W, Szczepaniak W, Barlog P et al (2018) Phosphorus sources for winter oilseed rape (*Brassica napes* L.) during reproductive growth - magnesium sulfate management impact on P use efficiency. Arch Agron Soil Sci 64(12):1646–1662
13. Guo YF, Liang AZ, Zhang Y, Zhang SX, Chen XW, Jia SX, Zhang XP, Wu DH (2019) Evaluating the contributions of earthworms to soil organic carbon decomposition under different tillage practices combined with straw additions. Ecol Indic 105:516–524
14. Gusain R, Suthar S (2020) Vermicomposting of duckweed (*Spirodela polyrhiza*) by employing *Eisenia fetida*: Changes in nutrient contents, microbial enzyme activities and earthworm biodynamics. Bioresource Technol 311:123585
15. Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF (2011) Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J 5:1571–1579
16. Hidri R, Mahmoud OMB, Debez A, Abdelly C, Barea JM, Azcon R (2019) Modulation of C:N:P stoichiometry is involved in the effectiveness of a PGPR and AM fungus in increasing salt stress tolerance of Sulla carnosa Tunisian provenances. Appl Soil Ecol 143:161–172
17. Jiang BF, Gu YC (1989) A suggested fractionation scheme of inorganic phosphorus in calcareous soils. Sci Agr Sinica 22(3):58–66
18. Khan AA, Jilani G, Akhtar MS, Naqvi SS (2009) Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. J Agric Biol Sci 1:48–58
19. Le Bayon RC, Binet F (2006) Earthworms change the distribution and availability of phosphorous in organic substrates. Soil Biol Biochem 38:235–246
20. Lian TX, Jin J, Wang GH et al (2017) The fate of soybean residue -carbon links to changes of bacterial community composition in Mollisols differing in soil organic carbon. Soil Biol Biochem 109:50–58
21. Lidbury IDEA, Fraser T, Murphy ARJ et al (2017) The 'known' genetic potential for microbial communities to degrade organic phosphorus is reduced in low-pH soils. MicrobiologyOpen 6(4):474
22. Manuel DB, Angela MO, Tess EB, Alberto BG, David JE, Richard DB, Fernando TM, Brajesh KS, Noah F (2018) A global atlas of the dominant bacteria found in soil. Science 359(6373):320–325
23. Martin HG, Ivanova N, Kunin V et al (2006) Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. Nat biotechnol 24(10):1263–1269
24. Murphy J, Riley JP (1962) A modified single solution method for determination of phosphate in natural waters. Anal Chim Acta 27:31–36
25. Oo AN, Iwai CB, Saenjan P (2015) Soil properties and maize growth in saline and nonsaline soils using cassava-industrial waste compost and vermicompost with or without earthworms. Land Degrad Dev 26(3):300–310
26. Peak D, Kar G, Hundal L, Schoenau J (2012) Kinetics and mechanisms of phosphorus release in a soil amended with biosolids or inorganic fertilizer. Soil Sci 177:183–187

27. Pinnell LJ, Dunford E, Ronan P, Hausner M, Neufeld JD (2014) Recovering glycoside hydrolase genes from active tundra cellulolytic bacteria. Can J Microbiol 60(7):469–476
28. Porcel R, Aroca R, Ruiz-Lozano JM (2012) Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. Agron Sustain Dev 32(1):181–200
29. Ragot SA, Kertesz MA, Meszaros E, Frossard E, Bunemann EK (2017) Soil phoD and phoX alkaline phosphatase gene diversity responds to multiple environmental factors. FEMS microbiol ecol 93:fiw212
30. Shao TY, Zhao JJ, Liu AH, Long XH, Rengel Z (2020) Effects of soil physicochemical properties on microbial communities in different ecological niches in coastal area. Appl soil ecol 150:103486
31. Shen J, Yuan L, Zhang J et al (2011) Phosphorus dynamics: from soil to plant. Plant physiol 156(3):997–1005
32. Sima NAK, Samani NR, Ebadi A, Ghaffari MR (2019) Effects of calcium and phosphorus enrichment on yield and physiological characteristics of Salicornia persica under different salinity levels. J Plant Nutr 42(9):971–981
33. Singh K (2016) Microbial and enzyme activities of saline and sodic soils. Land Degrad Dev 27(3):706–718
34. Smith SE, Smith FA (2011) Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. Annu Rev Plant Boil 62:227–250
35. Sosa OA, Repeta DJ, Ferrón S, Bryant JA, Mende DR, Karl D, DeLong EF (2017) Isolation and characterization of bacteria that degrade phosphonates in marine dissolved organic matter. Front microbiol 8:1786
36. Talbi Zribi O, Abdelly C, Debez A (2011) Interactive effects of salinity and phosphorus availability on growth, water relations, nutritional status and photosynthetic activity of barley (*Hordeum vulgare* L.). Plant Biol 13(6):872–880
37. Tuffen F, Eason WR, Scullion J (2002) The effect of earthworms and arbuscular mycorrhizal fungi on growth of and 32P transfer between Allium porrum plants. Soil Biol Biochem 34(7):1027–1036
38. Tabatabai MA (1982) Soil enzymes. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis, part 2. American Society of Agronomy, Madison, pp 903–947
39. Tejada M, Garcia C, Gonzalez J, Hernandez M (2006) Use of organic amendment as a strategy for saline soil remediation: influence on the physical, chemical and biological properties of soil. Soil Biol Biochem 38:1413–1421
40. Tigka T, Ipsilantis I (2020) Effects of sand dune, desert and field arbuscular mycorrhizae on lettuce (*Lactuca sativa* L.) growth in a natural saline soil. Sci Hortic 264:109191
41. Wang J, Zhai L, Ma J et al (2020) Comparative physiological mechanisms of arbuscular mycorrhizal fungi in mitigating salt-induced adverse effects on leaves and roots of Zelkova serrata. Mycorrhiza 30:341–355
42. Zhang L, Xu M, Liu Y, Zhang F, Hodge A, Feng G (2016a) Carbon and phosphorus exchange may enable cooperation between an arbuscular mycorrhizal fungus and a phosphate-solubilizing bacterium. New Phytol 210(3):1022–1032

43. Zhang W, Cao J, Zhang S, Wang C (2016b) Effect of earthworms and arbuscular mycorrhizal fungi on the microbial community and maize growth under salt stress. Appl soil ecol 107:214–223
44. Zhang W, Wang C, Lu T, Zheng Y (2018) Cooperation between arbuscular mycorrhizal fungi and earthworms promotes the physiological adaptation of maize under a high salt stress. Plant soil 423(1–2):125–140
45. Zhang YN, Li YL, Wang L, Tang YS, Chen JH, Hu Y, Fu XH, Le YQ (2013) Soil microbiological variability under different successional stages of the Chongming Dongtan wetland and its effect on soil organic carbon storage. Ecol Eng 52:308–315
46. Zheng W, Zhao Z, Gong Q, Zhai B, Li Z (2018) Responses of fungal–bacterial community and network to organic inputs vary among different spatial habitats in soil. Soil Biol Biochem 125:54–63

Tables

Table 1 Soil salt ions, alkaline phosphatase activity and t bacterial diversity indices of the salinity soil inoculated with earthworms and/or AMF.

Indices	Treatments				Significance due to		
	CK	E	AM	E+AM	E	AM	E*AM
Cl^- (mg kg^{-1})	174.0±1.9a	160.8±4.8ab	152.3±8.8b	158.0±0.8b	ns	*	ns
HCO_3^- (mg kg^{-1})	285.2±15.2c	305.4±4.4bc	334.2±4.2a	329.8±4.1ab	ns	***	ns
Na^+ (mg kg^{-1})	574.5±4.2a	574.5±3.4a	561.0±12.6b	571.3±2.4ab	ns	*	ns
Ca^{2+} (mg kg^{-1})	178.2±18.3a	157.5±3.9b	117.6±11.3c	147.5±8.5b	ns	***	***
Mg^{2+} (mg kg^{-1})	76.01±14.71b	125.7±28.5a	96.79±5.27b	137.2±7.9a	***	ns	ns
Alkaline phosphatase (mg g^{-1} h^{-1})	0.47±0.02c	0.57±0.02bc	0.74±0.03a	0.65±0.03ab	ns	**	**
Chao1 diversity	1438±4b	1555±32a	1635±11a	1573±22a	ns	**	ns
Phylogenetic diversity	93.66±0.96b	101.2±1.5a	105.2±1.0a	104.5±1.4a	*	***	**

Data are the means of four replicates ± SE and were compared using Tukey's tests. The values with the same lower case letter are not significantly different. CK is the soil without mycorrhizae and earthworms; E is the soil with earthworms inoculation; AM is soil with mycorrhizae addition; E+AM is the soil inoculated mycorrhizae and earthworms. *p <0.05, **p <0.01, ***p <0.001, ns indicates not significant.

Figures

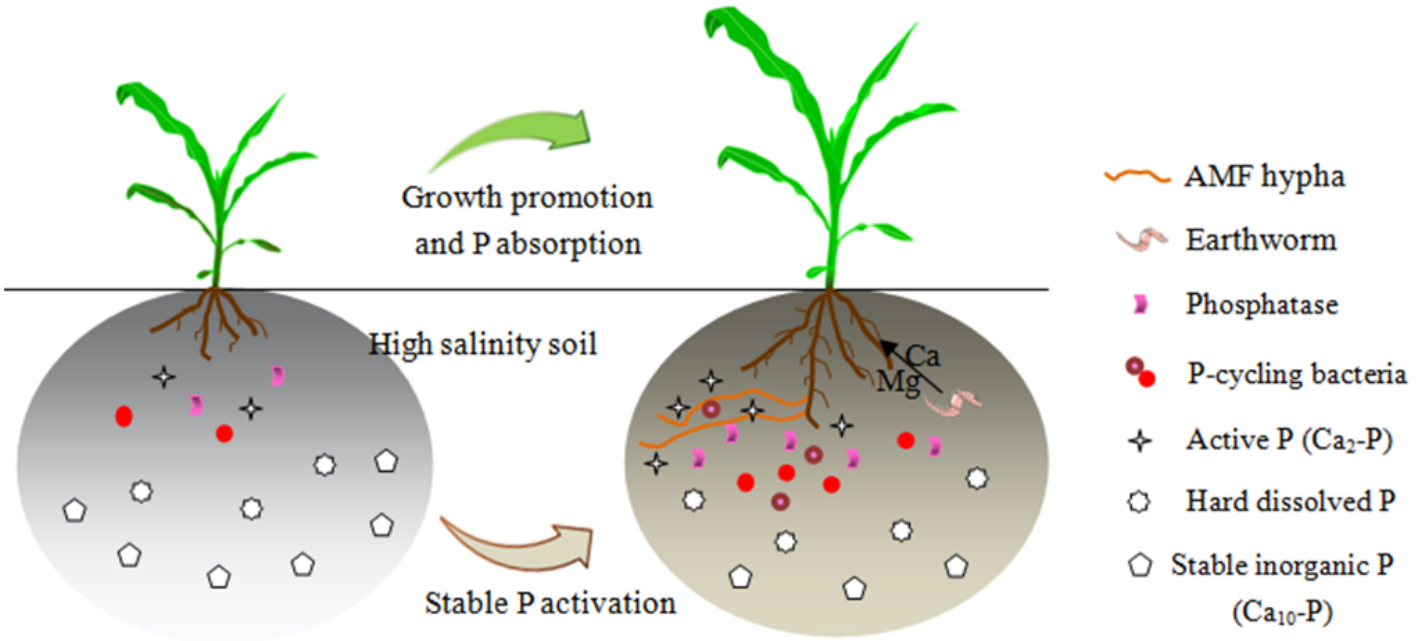


Figure 1

Schematic representation of the cooperative effect of earthworms and arbuscular mycorrhizael fungi (AMF) on P cycling processes in a high salinity soil. We hypothesized that the cooperation of earthworms and AMF could increase soil P availability and promote maize P absorption and growth (a); AMF increased the soil P availabity by increasing soil phosphatase activity to activate stable inorganic phosphate (hydroxyapatite, Ca10-P) transforming into plant-available P form (Dicalcium phosphate, Ca2-P) (b); Earthworms improved mazie cations (Ca and Mg) utilization to enhance the maize P uptake (c); Earthworms and AMF could increase soil bacterial diversity and P-cycling bacteria to increase soil phosphatase activity and soil P availability (d).

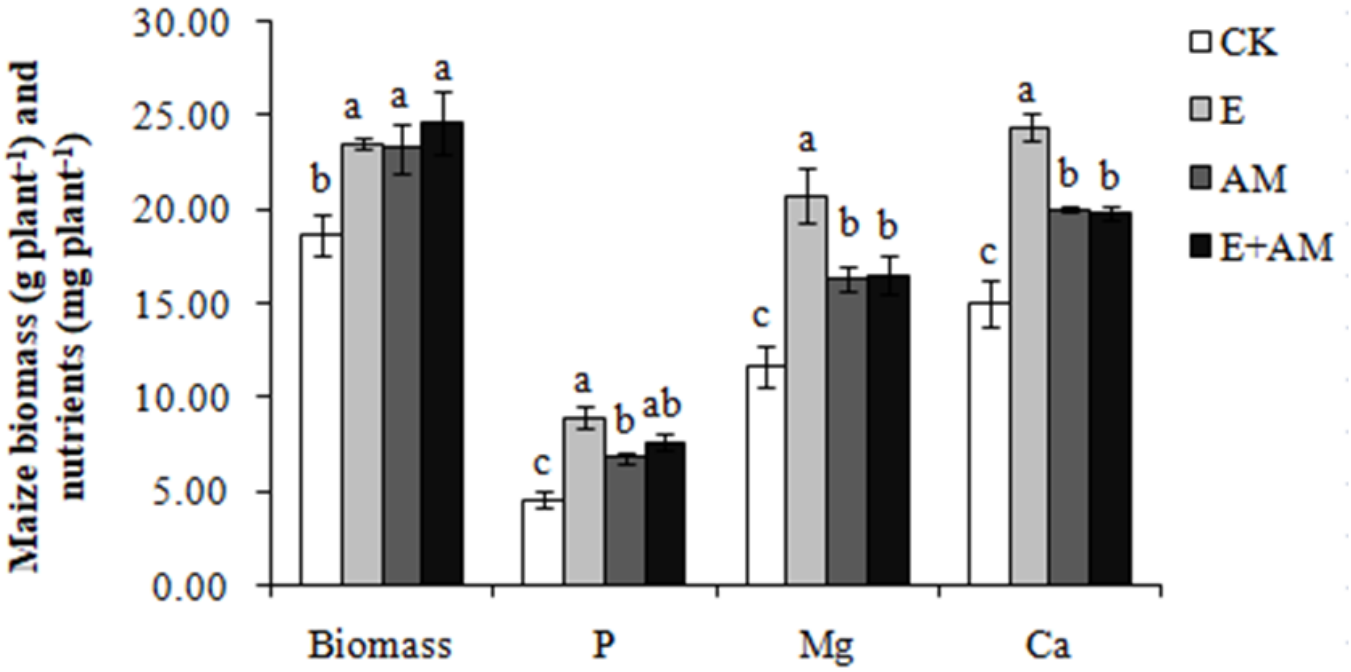


Figure 2

Maize biomass and nutrients uptake in the salinity soil inoculated with earthworms and/or mycorrhizae. Data are the means of four replicates ± SE and were compared using Tukey's tests. The values with the same lower case letter are not significantly different. CK is the soil without mycorrhizae and earthworms; E is the soil with earthworms inoculation; AM is soil with mycorrhizae addition; E+AM is the soil inoculated mycorrhizae and earthworms.

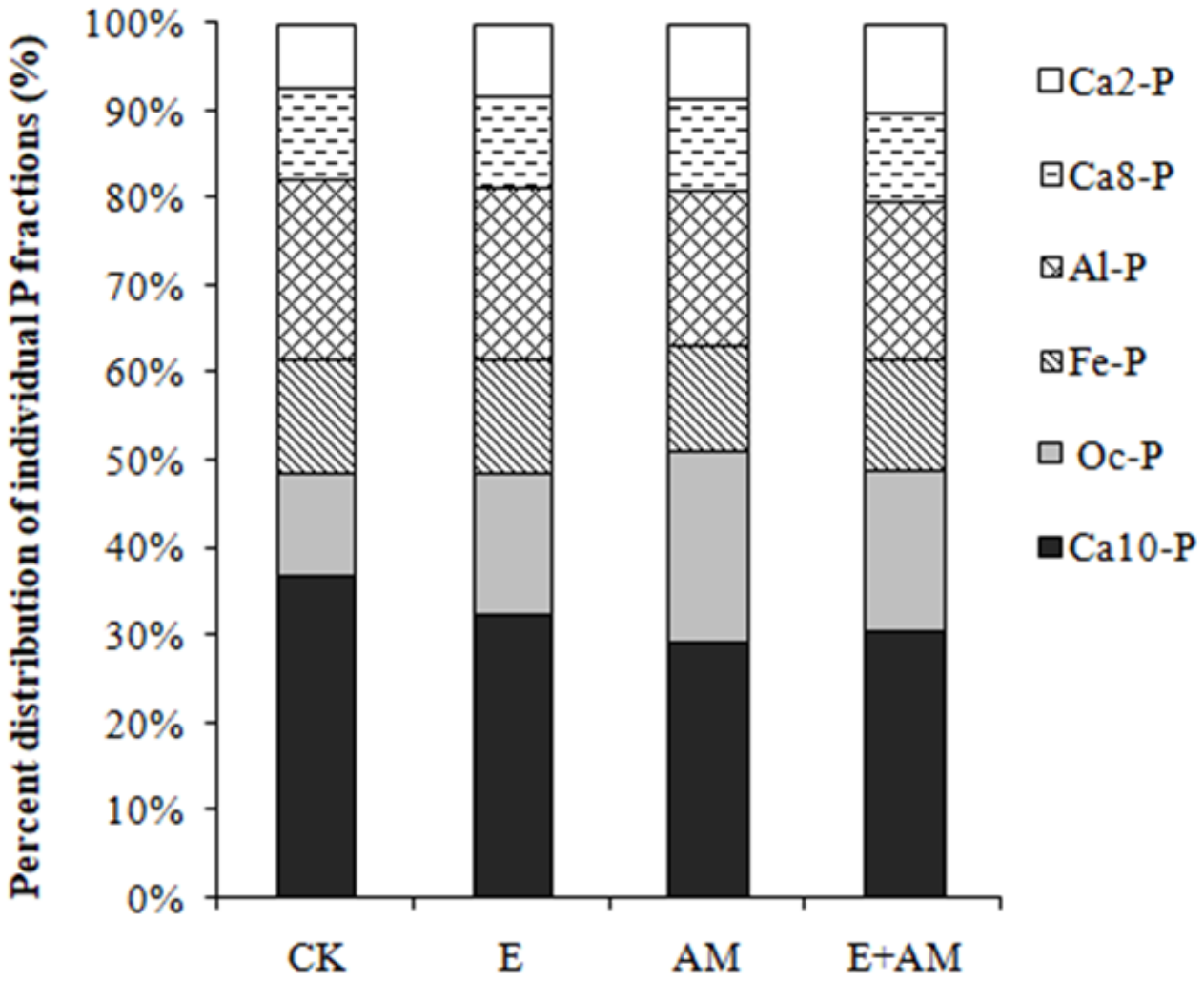


Figure 3

Percent distribution of inorganic P fractions among different treatments in a salinity soil. Dicalcium phosphate (Ca2-P), octocalcium phosphate (Ca8-P), non-occluded aluminum phosphate (Al-P), non-occluded iron phosphate (Fe-P), occluded phosphate (Oc-P) and hydroxyapatite (Ca10-P) were analyzed and listed. CK is the soil without mycorrhizae and earthworms; E is the soil with earthworms inoculation; AM is soil with mycorrhizae addition; E+AM is the soil inoculated mycorrhizae and earthworms.

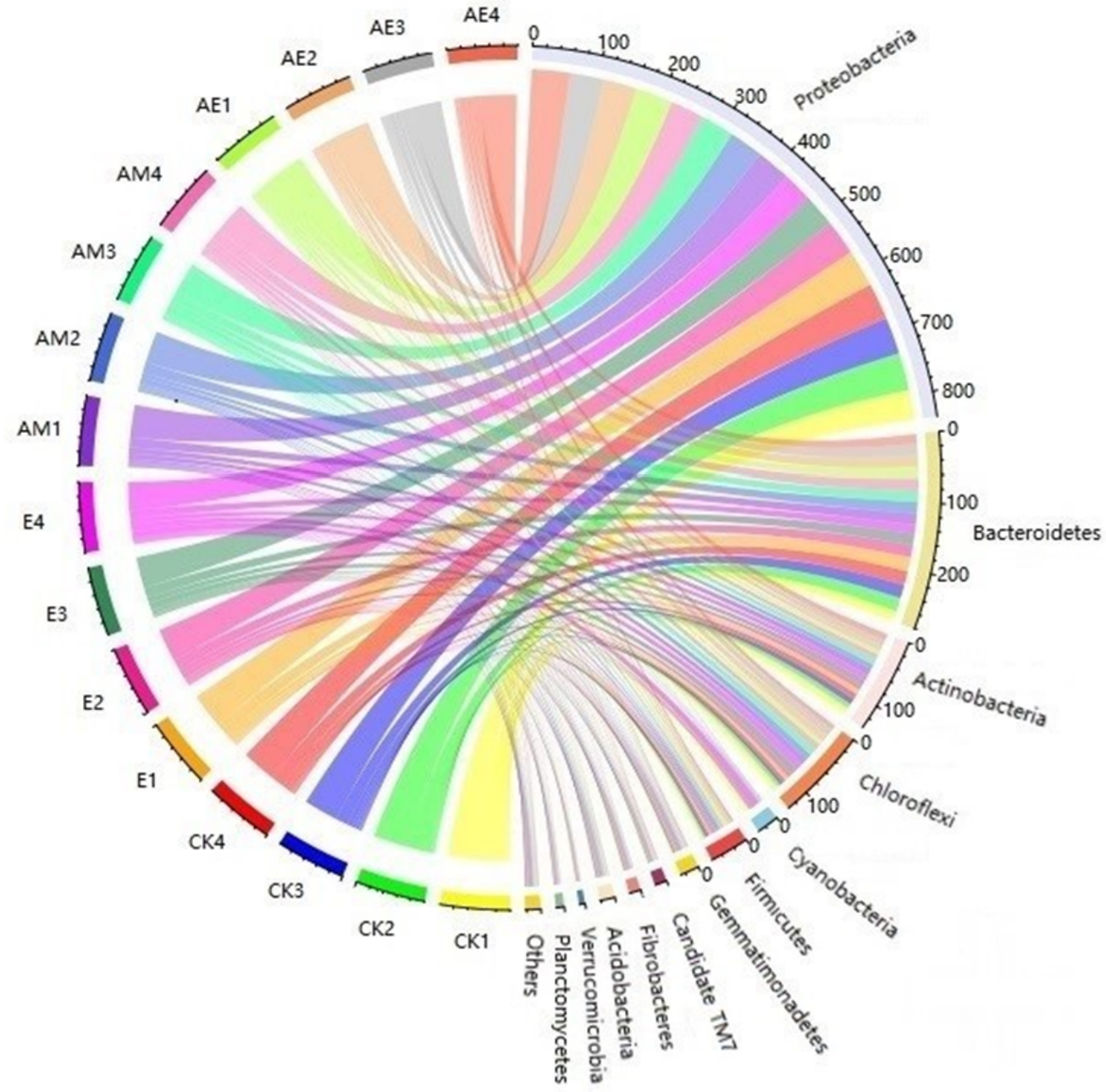


Figure 4

Variation of the dominant bacterial species at phylum level. The right half circle shows the species composition in the sample. The colour of the outer ribbon indicates the phlum group from which it comes. The width of the inner ribbon represents the relative abundance of the species in the corresponding sample. The colour of the left half circle represents the sample from which it comes. CK is the salinity soil without mycorrhizae and earthworms; E is the salinity soil with earthworms inoculation; AM is salinity soil with mycorrhizae addition; AE is the salinity soil inoculated mycorrhizae and earthworms.

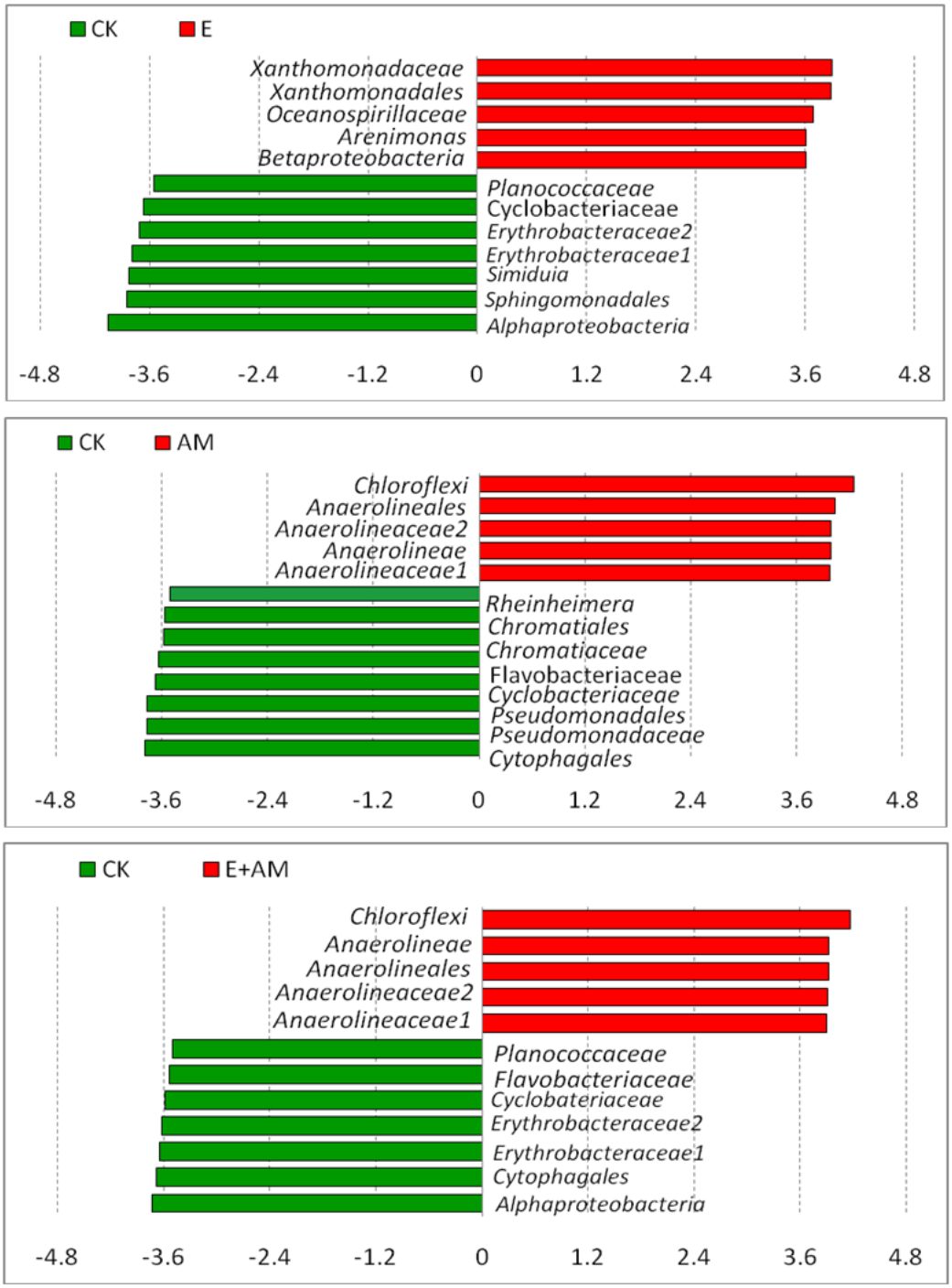


Figure 5

Supervised comparison identifies differential abundance of bacteria using LEfSe (LDA, linear discriminant analysis >3.5). CK is the salinity soil without mycorrhizae and earthworms; E is the salinity soil with earthworms inoculation; AM is salinity soil with mycorrhizae addition; E+AM is the salinity soil inoculated mycorrhizae and earthworms.

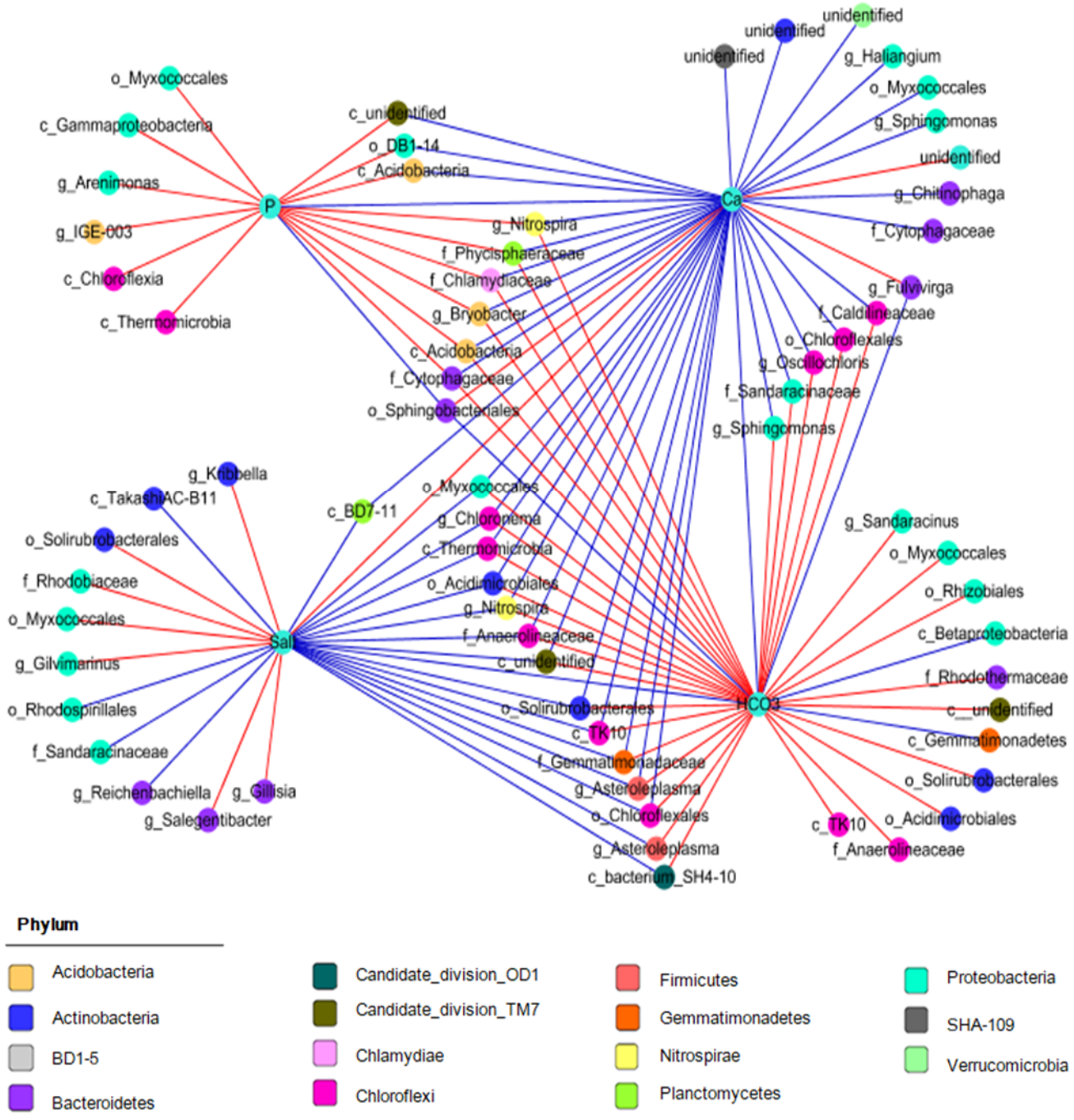


Figure 6

Network analysis showing the co-occurrence of soil salt, available P, ions and their potential response bacteria. The nodes with the same colour indicate bacterial species belonging to the same phylum. A connection represents a significant positive (red line) or negative (blue line) correlation ($r \geq 0.7$, $p < 0.05$). Data of soil salt and available P were referenced to Zhang et al., 2018.

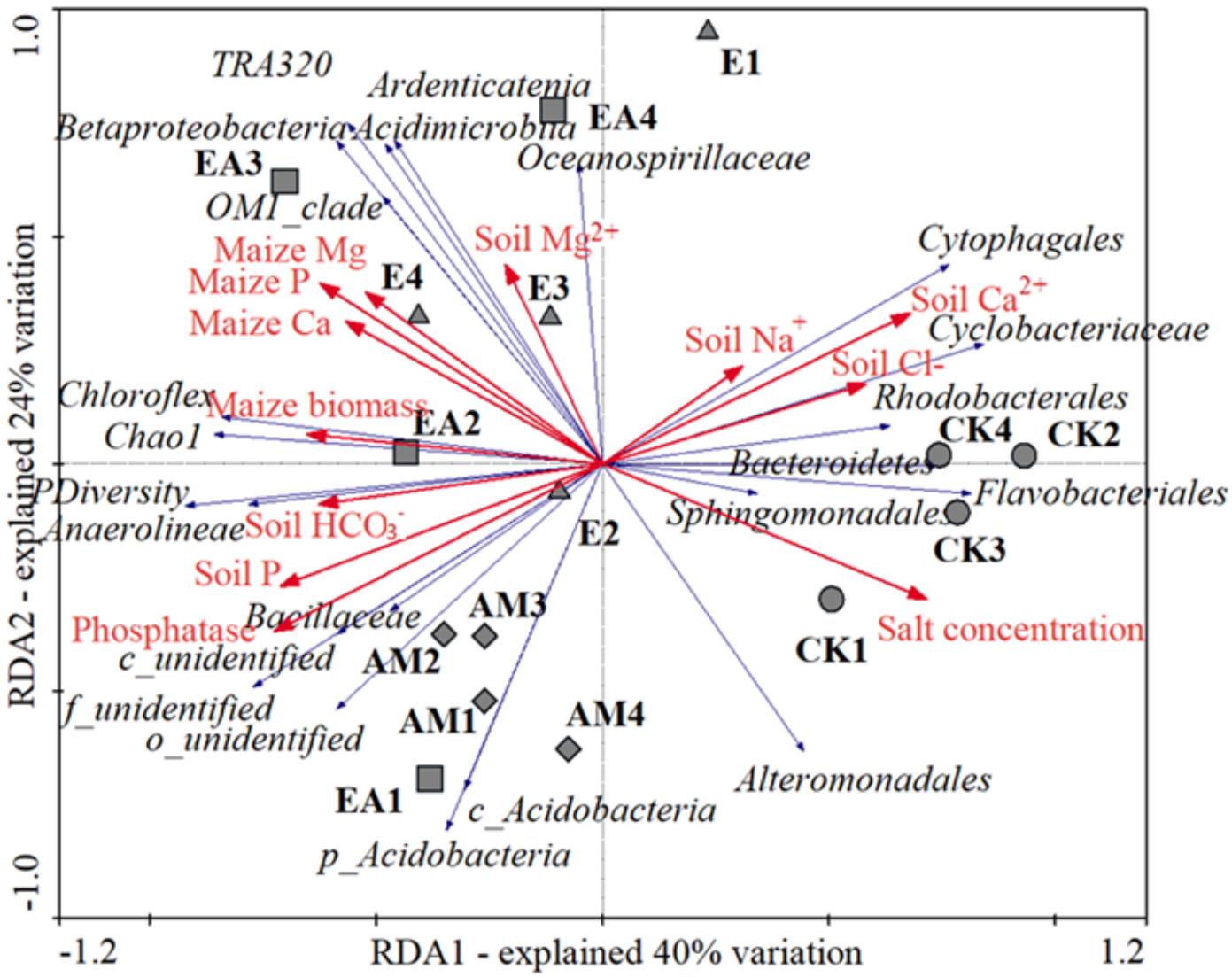


Figure 7

Two-dimensional similarities revealed by RDA plots for the relationship between environmental parameters and predominant bacteria with significant difference among all amendment treatments. CK is the salinity soil without mycorrhizae and earthworms; E is the salinity soil with earthworms inoculation; AM is salinity soil with mycorrhizae addition; EA is the salinity soil inoculated mycorrhizae and earthworms. Data of soil salt concentration and soil P (Olsen-P) were referenced to Zhang et al., 2018. p_ indicates species at phylum level, o_ indicates species at order level, c_ indicates species at class level, f_ indicates species at family level.

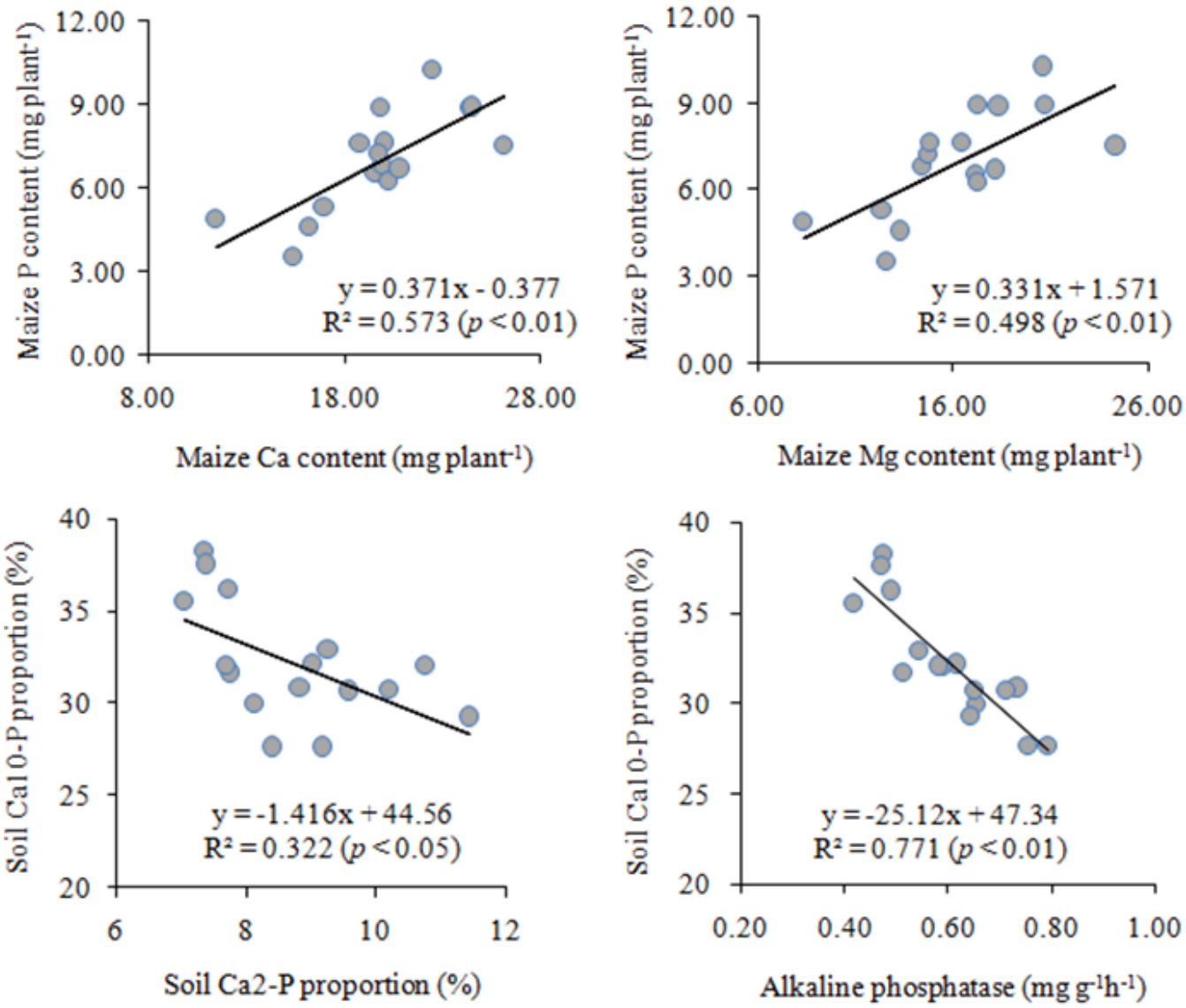
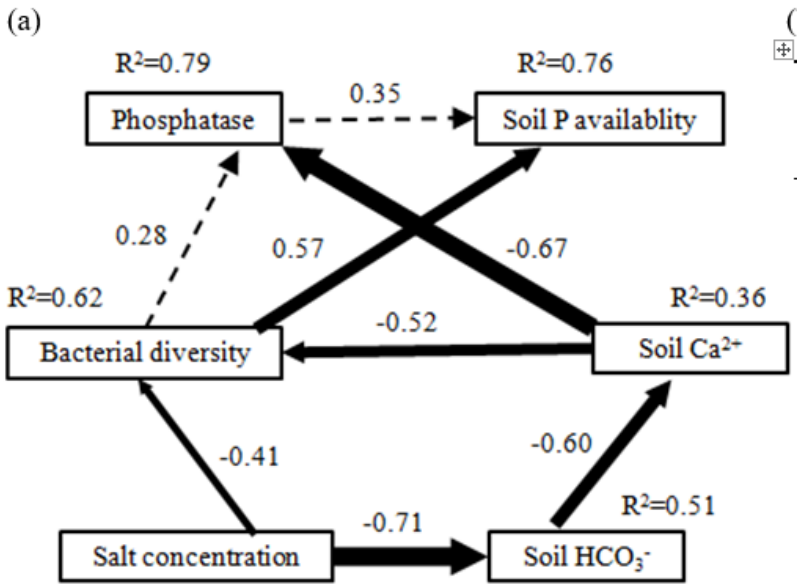


Figure 8

Correlations of the maize P content with the contents of maize Ca and Mg, relations between soil alkaline phosphatase and soil hydroxyapatite (Ca10-P) proportion, and beween soil Ca10-P proportion and soil dicalcium phosphate (Ca2-P) proportion.



(b)

Index	Direct path	Indirect path	Total effect
Bacterial diversity	0.574	0.099	0.673
Soil Ca^{2+}	-	-0.589	-0.589
Salt concentration	-	-0.529	-0.529
phosphatase	0.354	-	0.354
Soil HCO_3^-	-	0.353	0.353

Figure 9

Structural equation model (SEM) analysis of the effects of soil salt concentration and ions, soil bacterial diversity and phosphatase on soil P availability (a, b). The arrow width indicates the strength of the causal effect. Solid and dashed lines indicate the pathways with and without significant relationships respectively. The proportion of variation explained (R2) appears above response variables. Data of soil salt and available P were referenced to Zhang et al., 2018

Supplementary Files

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