Variation of soil microbiome in licorice rhizosphere driven with inoculating dark septate endophytes under drought stress

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Research

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Abstract

Background

Dark septate endophytes (DSE) are facultative biotrophic ascomycetes that colonize plant roots either alone or with arbuscular mycorrhizal (AM) fungi. DSE may provide nutrients to their plant hosts and help them adapt to various abiotic and biotic stresses. DSE inoculation under drought stress increased the biomass, root exudates, and AM fungi in the licorice (*Glycyrrhiza uralensis* Fisch.) rhizosphere. We conducted a pot experiment to establish whether the responses of licorice to DSE inoculation under drought stress are caused by changes in the rhizosphere microbiome. Each pot was inoculated with either *Acrocalymma vagum* or *Paraboeremia putaminum*. One set of pots was inoculated with a sterile culture medium. All three DSE-treated and uninoculated pots were subjected either to a well-watered (70% field water capacity, FWC) or drought stress (30% FWC) water regime. Rhizosphere microbiome compositions were measured by Illumina MiSeq sequencing of the 16S and ITS2 rRNA genes.

Results

In total, 1,278 fungal and 1,583 bacterial operational taxonomic units (OUTs) were obtained at a 97% sequence similarity level. *Ascomycota* were the predominant fungi and *Proteobacteria*, *Actinobacteria*, *Chloroflexi* and *Firmicutes* were the predominant bacteria. DSE inoculation and water regime significantly influenced the rhizosphere microbiome composition. However, the effects of DSE on the fungal community were greater than those on the bacterial community. *Paraboeremia putaminum* exerted a stronger impact on the licorice rhizosphere microbiome than *Acrocalymma vagum* under drought stress. The observed changes in edaphic factors (water condition, soil organic matter, available N, available P, and available K) caused by DSE inoculation could be explained by the variations in rhizosphere microbiome composition. A network analysis indicated that DSE inoculation augmented the relative abundance of beneficial symbiotrophic fungi and growth-promoting bacteria but diminished the relative abundance of pathogens in the licorice rhizosphere.

Conclusions

The present study showed that the licorice rhizosphere microbial community differed between the DSE-inoculated and uninoculated plants. DSE had a stronger influence on the fungal than on the bacterial rhizosphere community under drought stress. These give us the guidance to develop biofertilizers with DSE consortia to enhance the cultivation of medicinal plants by shaping soil microbial community structure in dryland agriculture.

Background

Drought is a major abiotic stressor that limits plant growth and agricultural production in arid and semi-arid regions worldwide [1, 2]. Water deficit inhibits root growth and development and decreases plant nutrient and water uptake from the soil [3]. Under drought stress, plants modify their root exudate

abundance and composition [4]. This response alters the rhizosphere microbiome, which, in turn, strongly influences plant survival and growth and soil ecology [5]. Several studies have shown that plant adaptation to abiotic stress is closely associated with rhizosphere microorganisms [6, 7]. Beneficial endophytic fungal colonization can alter root growth, increase plant biomass, reduce water loss, and help host plants adapt to arid environments [8, 9]. Moreover, dark septate endophytes (DSE) enhance viral resistance and heavy metal tolerance in plants [10, 11].

DSE are ubiquitous root-colonizing fungi characterized by dark, septate hyphae and melanized microsclerotia. They are integral and functional parts of plant roots [12]. DSE inoculation facilitates plant growth, nutrient uptake, and biotic and abiotic stress tolerance [8, 11]. Previous studies demonstrated that rhizosphere-associated microbes indirectly affect plant growth by changing the rhizosphere microbial community composition and activity. These communities play key roles in soil nutrient cycling and structural formation [13, 14]. Fiorentino et al. [15] found that *Trichoderma* inoculation more strongly affected the eukaryotic community composition of low-N than N-fertilized soils. Changey et al. [16] found that arbuscular mycorrhizal (AM) fungal inoculation dramatically influenced the rhizosphere microbial community composition. Tian et al. [17] reported that *Glomus intraradices* inoculation increased certain beneficial bacterial species and decreased certain pathogenic fungi in the *Panax ginseng* rhizosphere. Han et al. [18] proposed that *Bacillus amyloliquefaciens* promotes cucumber growth by modulating its rhizosphere microbial community composition. The direct effects of beneficial rhizosphere microbes on plant growth have been extensively examined [19, 20]. Nevertheless, there is relatively little information on the impact of DSE inoculation on the rhizosphere microbial community and its network structure.

Licorice (*Glycyrrhiza uralensis* Fisch.) belongs to Fabaceae. It is widely distributed in arid and semi-arid regions worldwide. It is regarded as an “essential herbal medicine” in traditional Chinese medicine and has been prescribed and administered for > 1,000 y. Its active principles include glycyrrhizin and glycyrrhizic acid [21]. Licorice adapts very well to low-fertility soils and drought. In fact, it has been used in the ecological restoration of arid regions [22]. In its natural habitats, the licorice rhizosphere harbors actinobacteria, rhizobia, other soil bacteria, AM, and DSE with plant growth-promoting activity [23, 24]. An ester-linked phospholipid fatty acid (PLFA) analysis in our previous study showed that DSE (*Acrocalymma vagum* and *Paraboeremia putaminum*) inoculation increased AM and gram-negative bacterial abundance in the licorice rhizosphere under drought stress [8]. However, its effects on the composition of the microbial communities in the licorice rhizosphere remain unclear. Rhizosphere microbes play vital roles in plant growth, health, and abiotic stress tolerance. Here, we used Illumina MiSeq sequencing to reveal variations in the composition of the licorice rhizosphere microbial communities inoculated with DSE under drought stress. We hypothesized that DSE inoculation considerably alters the composition and network structure of the fungal and bacterial communities in the licorice plant rhizosphere. Furthermore, these changes are more pronounced under drought stress than well-watered conditions. Our objective was to test whether DSE inoculation alters the soil microbiome and increases drought tolerance in licorice.

Results

Characterization of Illumina sequencing data

We obtained 2,304,715 fungal and 2,098,398 bacterial sequences. Rarefaction curve analysis displayed high 16S rRNA gene sequencing depth and strong potential for observing community diversity in each licorice rhizosphere (Fig. 1). Rank abundance curves showed that all six treatments had high species evenness and homogeneity (Fig. 2). The sequencing results covered the biological information of most of the fungi and bacteria in the soil samples.

After filtering 1,266,009 low-quality sequences, 1,568,052 effective fungal and 987,120 effective bacterial sequences were clustered into 1,278 fungal and 1,583 bacterial operational taxonomic units (OTUs) at 97% sequence similarity. Of the 1,278 fungal OTUs, 196 occurred in all six treatments while 85, 94, 48, 21, 202, and 33 OTUs were found only in the uninoculated condition (NCK), *P. putaminum* inoculation (NPP), and *A. vagum* inoculation (NAV) under well-watered and in the three DSE inoculation treatments DCK, DPP, and DAV under drought stress, respectively (Fig. 3). Of the 1,583 bacterial OTUs, 1,082 were detected in all treatments and 31, 45, 71, 36, 64, and 38 existed only in NCK, NPP, NAV, DCK, DPP, and DAV, respectively (Fig. 3).

Rhizosphere microbial diversity and richness

Under well-watered, inoculation with either *P. putaminum* or *A. vagum* increased the soil Simpson fungus index compared to the uninoculated condition. The *A. vagum* inoculation decreased the soil fungus Ace and Chao1 indices. Compared with well-watered, drought stress decreased the diversity and richness of the fungal community colonizing the licorice rhizosphere. Compared to the uninoculated condition, *P. putaminum* inoculation increased the soil fungus Shannon, Ace, and Chao1 indices but decreased the soil fungus Simpson index. However, *A. vagum* inoculation increased the soil fungus Ace and Chao1 indices (Table 1).

DSE inoculation significant affected soil bacterial diversity and richness under all water regimes (Table 2). Under well-watered, DSE inoculation increased the soil bacteria Chao1 index but decreased the soil bacteria Simpson index compared with the uninoculated condition. Under drought stress, *P. putaminum* inoculation increased the soil bacteria Ace and Chao1 indices whereas *A. vagum* inoculation had no significant effect on soil bacterial community diversity and richness. Compared with the uninoculated condition, drought stress decreased the soil bacteria Simpson index.

Rhizosphere microbial community composition

A total of 1,278 fungal OTUs were found in the licorice rhizosphere and classified as *Ascomycota*, *Basidiomycota*, *Zygomycota*, *Glomeromycota,* and certain unknown fungi. *Ascomycota* and *Basidiomycota* were identified in all treatments. *Ascomycota* was the dominant fungal phylum and its relative abundance range was 89.9–99.6% across the various treatments (Fig. 4). A heatmap based on the top 50 abundant fungal genera revealed that the colonization of certain relatively abundant fungi

varied among treatments (Fig. 5). Of the 50 abundant fungal genera, five OTUs (*Aspergillus* OTU86, *Fusarium* OTU1748, *Gibberella* OTU1913, *Trichoderma* OTU8380 and *unclassified_f_Chaetomiacease* OTU936) were distributed mainly in NCK, four OTUs (*Acremonium* OTU454, *Chaetomium* OTU1508, *Phialophora* OTU1296 and *Stachybotrys* OTU1633) were distributed mainly in NPP, two OTUs (*Aspergillus* OTU86 and *Trichoderma* OTU8380) were distributed mainly in NAV, three OTUs (*Aspergillus* OTU86, *Trichoderma* OTU8380 and *unclassified_f_Microascaceae* OTU1963) were distributed mainly in DCK, four OTUs (*Chaetomium* OTU1508, *Pseudalescheria* OTU946, *unclassified_f_Chaetomiaceae* OTU936 and *unclassified_ k_Fungi* OTU1449) were distributed mainly in DPP, and two OTUs (*Aspergillus* OTU86 and *Trichoderma* OTU8380) were distributed mainly in DAV (Fig. 5).

A total of 1,583 bacterial OTUs were detected in the licorice rhizosphere. The dominant bacterial phyla were *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, *Acidobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Planctomycetes*, *Verrucomicrobia*, *Nitrospirae*, *Saccharibacteria,* and certain unknown bacteria. The relative abundance of these bacterial phyla varied among treatments (Fig. 4). Of the 50 dominant bacterial genera, nine OTUs (*Bacillus* OTU4110, *Devosia* OTU1666, *Nocardioides* OUT5635, *Oscillatoria* OTU6539, *Pseudarthrobacteria* OTU3533, *Sphingomonas* OTU5209, *norank-c-Acidobacteria* OTU1690, *norank-c-Gitt-GS-136* OTU3951 and *norank-o-JG30-KF-CM45* OTU6085) were distributed mainly in NCK, six OTUs (*Bacillus* OTU4110, *Pseudarthrobacteria* OTU3533, *Pseudomonas* OTU5762, *norank-c-Acidobacteria* OTU1690, *norank-f- Anaerolineaceae* OTU1186 and *norank-c-KD4-96* OTU3568) were distributed mainly in NPP, eight OTUs (*Bacillus* OTU4110, *Devosia* OTU1666, *Ensifer* OTU6341, *Paenibacillus* OTU4751, *norank-o-Acidmicrobiales* OTU4282, *norank-c- Acidobacteria* OTU1690, *norank-f-Anaerolineaceae* OTU1186 and *norank-o-JG30- KF-CM45* OTU6085) were distributed mainly in NAV, seven OTUs (*Bacillus* OTU4110, *Nocardioides* OTU5635, *Paenibacillus* OTU4751, *norank-c-Acidobacteria* 1690, *norank-o-Acidmicrobiales* OTU4282, *norank-c-Gitt-GS-136* OTU3951 and *norank-c-KD4-96* OTU3568) were distributed mainly in DCK, six OTUs (*Bacillus* OTU4110, *Lamia* OTU4301, *Pseudomonas* OTU5762, *norank-c-Acidobacteria* OTU1690, *norank-f-Anaerolineaceae* OTU1186 and *norank-c-KD4-96* OTU3568) were distributed mainly in DPP, and 11 OTUs (*Bacillus* OTU4110, *Lysinibacillus* OTU9205, *Microbacterium* OTU1007, *Nocardioides* OTU5635, *Paenibacillus* OTU4751, *Sporocytophaga* OTU2217, *norank-c-Acidobacteria* OTU1690, *norank- o-Acidmicrobiales* OTU4282, *norank-f-Anaerolineaceae* OTU1186, *norank-c-KD4-*96 OTU3568 and *norank-c-Gitt-GS-136* OTU3951) were distributed mainly in DAV (Fig.5).

Nonmetric multidimensional scaling (NMDS) ordination revealed that the rhizosphere fungal community composition significantly differed between the DSE inoculation and uninoculated treatments under well-watered (Fig. 6). Compared to the uninoculated condition, *P. putaminum* inoculation substantially affected fungal community composition under drought stress. No dramatic effect was observed in response to *A. vagum* inoculation (Fig. 6). DSE inoculation significantly affected the composition of the bacterial community under well-watered. The *A. vagum* and *P. putaminum* inoculation had different effects (Fig. 6). Under drought stress, *P. putaminum* inoculation significantly affected the bacterial community composition relative to the uninoculated condition. In contrast, *A. vagum* inoculation had no significant impact on the bacterial community composition (Fig. 6). Permutational multivariate analysis

of variance (PerMANOVA) indicated that the fungal (F = 7.435, R^2= 0.264, P = 0.001; F = 6.224, R^2= 0.331, P = 0.001) and bacterial (F = 6.125, R^2= 0.392, P = 0.001; F = 5.648, R^2= 0.440, P = 0.001) community compositions were significantly different between the well-watered and the drought stress treatments. Furthermore, drought stress and DSE inoculation more strongly affected the fungal than the bacterial community composition.

Edaphic factor and rhizosphere microbe preferences

An edaphic factor/microbe preference analysis showed that the fungi and bacteria colonizing the licorice rhizosphere significantly preferred the soil moisture, organic matter content, and available N, P, and K (Fig. 7). Of the 50 relatively abundant fungal OTUs, two (*Gibberella* OTU1913 and *Phialophora* OTU1296) had a significant positive preference for soil moisture but OTU575 (*Vermispora*) presented with a significant negative moisture preference. OTU308 (*Guehomyces*) showed negative available N preference. Three OTUs (*Guehomyces* OTU308, *unclassified_f_Lasiosphaeriaceae* OTU161, and *Schizothecium* OTU474) showed negative available P preferences. OTU1389 (*Humicola*) displayed a positive available K preference while OTU1539 (*Mycoarthris*) displayed a positive soil organic matter preference (Fig. 7). Of the 50 relatively abundant bacterial OTUs, OTU3533 (*Pseudarthrobacter*) and three OTUs (*Actinobacteria* OTU1174, *Streptomyces* OTU5992 and *Xanthomonadales* OTU7347) showed positive and negative moisture preferences, respectively. Two OTUs (*norank-f-Anaerolineaceae* OTU1186 and *Pseudomonas* OTU5762) positively correlated with available N. Seven OTUs (*Acidimicrobiales* OTU4282, *Gaiella* OTU 8721, *Iamia* OTU 4301, *Oscillatoria* OTU6539, *norank-f-Gemmatimonadaceae* OTU1174, *norank-f-Nitrosomonadaceae* OTU732 and *Streptomyces* OTU5992) had a significant negative correlation with available K. Three OTUs (*norank-c-Cynobacteria* OTU5893, *norank-f- Anaerolineaceae* OTU1186 and *norank-f-Sandaracinaceae* OTU476) and one OTU (*Lysinibacillus* OTU9205) showed positive and negative organic matter preferences, respectively (Fig.7).

Structure of various treatment-rhizosphere microbe networks

The various treatment-rhizosphere fungal networks are shown in Fig. 8. Under well-watered, *P. putaminum* inoculation increased the relative symbiotroph abundance but decreased the relative abundance of saprotroph and other fungi. In contrast, *A. vagum* inoculation increased the relative abundance of symbiotrophs and other fungi and decreased the relative abundance of pathotrophs and saprotrophs compared with the uninoculated condition. Drought stress decreased the relative abundance of symbiotrophs, saprotrophs, and other fungi but increased the relative abundance of pathotrophs compared with the well-watered condition. However, *P. putaminum* inoculation increased the relative abundance of symbiotrophs and other fungi and decreased the relative abundance of pathotrophs. Moreover, *A. vagum* inoculation increased the relative abundance of symbiotrophs, saprotrophs, and other fungi and decreased the relative abundance of pathotrophs compared with the uninoculated condition.

The network of different treatments-rhizosphere bacteria is shown in Fig. 8. *Bacillus,Pseudarthrobacter* OTU3533, and other bacteria had variable relative abundance under different treatments. *Oscillatoria* was only distributed under NCK. *Microbacterium* OUT1007 was distributed under NCK, NAV, DCK, and DAV. Inoculation with *A. vagum* decreased the relative abundance of *Oscillatoria* compared with the uninoculated condition. *Nocardioides* was only distributed under DCK and DAV. *Sporocytophaga* was only detected under DAV. *Arcnimonas* only occurred under NAV and DAV. *Ensifer* was found under NPP, NAV, and DPP. *Pedomicrobium* was only seen under NPP.

The network of various treatment-rhizosphere microbe functional group is shown in Fig. 8. Compared to the uninoculated treatment, under well-watered, DSE inoculation increased the relative abundance of beneficial and neutral fungi and bacteria and decreased the relative abundance of pathogenic fungi and bacteria. Relative to the uninoculated condition, under drought stress, *P. putaminum* inoculation decreased the relative abundance of pathogenic fungi and beneficial bacteria but increased the relative abundance of neutral fungi and neutral and pathogenic bacteria. The *A. vagum* inoculation increased the relative abundance of beneficial fungi and neutral bacteria but decreased the relative abundance of pathogenic fungi and bacteria.

Discussion

The plant rhizosphere houses a complex microbiome that includes bacteria, archaea, and fungi. These microorganisms affect plant survival, growth, and adaptability [25, 26]. Here, we used Illumina MiSeq sequencing to reveal the changes in microbial community diversity and network structure in the rhizosphere of licorice inoculated with DSE under drought stress. The relative abundance of microbes colonizing licorice rhizosphere differed among treatments. However, *Ascomycota*, *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, and *Firmicutes* were the dominant phyla under both well-watered and drought conditions. The predominance of *Ascomycota* in arid and semi-arid regions was previously reported [27, 28]. Dai et al. [29] found that *Actinobacteria*, *Proteobacteria*, *Saccharibacteria*, *Chloroflexi*, *Acidobacteria*, and *Cyanobacteria* were the predominant phyla in drought-treated and untreated peanut rhizosphere. Lundberg et al. [30] observed that *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Gemmatimonadetes*, and *Cyanobacteria* dominated in *Arabidopsis* rhizosphere. Barraza et al. [31] also reported that the bacterial community structure of the common bean roots, mainly composed by *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Firmicutes*. The major rhizosphere microbes vary widely among plant species. Nevertheless, *Actinobacteria* and *Proteobacteria* may be the most common bacterial phyla in plant rhizospheres.

Biotic and abiotic stressors alter rhizosphere microbe community structure and may augment or diminish certain microbial populations [32, 33]. He et al. [8] found that interactions between DSE inoculation and water regime markedly influenced the soil organic matter content. Compared to the uninoculated condition, *A. vagum* inoculation under drought conditions increased soil available N and P. In contrast, *P. putaminum* inoculation decreased soil organic matter and available N relative to the uninoculated condition. The microbes colonizing the licorice rhizosphere exhibited distinct preferences for various soil

factors. For instance, *Phialophora* OTU1296, *Gibberella* OTU1913, and *Pseudarthrobacter* OTU3533 showed a positive moisture preference whereas *Vermispora* OTU575, *Xanthomonadales* OTU7347, *Actinobacteria* OTU1174, and *Streptomyces* OTU5992 displayed a negative moisture preference. The *norank-f-Anaerolineaceae* OTU1186, *norank-f-Sandaracinaceae* OUT476, *Mycoarthris* OUT1539, and *norank-c-Cynobacteria* OTU5893 showed a positive soil organic matter preference while *Lysinibacillus* OTU9205 presented with a negative soil organic matter preference. These findings were consistent with those previously reported that microbial inoculation broadly influences plant rhizosphere microbial communities by altering soil chemical properties and indirectly affecting host plant growth [34, 35].

Here, NMDS ordination disclosed that *P. putaminum* inoculation had a significant effect on fungal community composition under drought stress. However, no significant difference was found between *A. vagum* inoculation and the uninoculated condition even though *A. vagum* inoculation increased the fungal community Ace and Chao1 indices in the licorice rhizosphere. The *P. putaminum* inoculation increased the relative abundance of *Basidiomycota* and *Zygomycota* but decreased the relative abundance of *Ascomycota* in the licorice rhizosphere under drought stress. Previous studies showed that the three aforementioned fungal phyla predominated in different ecological environments [36]. Certain *Ascomycota* including DSE form mycorrhizae in plant roots and enhance plant nutrient uptake and growth [37, 38]. Lin et al. [39] found that *Ascomycota*, *Basidiomycota*, and *Zygomycota* strongly tolerated heavy metal contamination. Moreover, *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Firmicutes*, and *Acidobacteria* predominated in the licorice rhizosphere under drought stress regardless of DSE inoculation. Compared to the uninoculated condition, inoculation with *P. putaminum* increased the relative abundance of *Acidobacteria*, *Chloroflexi*, and *Cyanobacteria* but decreased the relative abundance of *Actinobacteria*. The *A. vagum* inoculation increased the relative abundance of *Cyanobacteria* and *Bacteroidetes*. Khan et al. [40] reported that *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* were highly abundant in medicinal plant rhizosphere microbiomes in arid soil. *Proteobacteria*, *Acidobacteria*, and *Bacteroidetes* had high heavy metal tolerance [41]. Sanguin et al. [42] found that *Proteobacteria* enrichment increased the disease suppression capacity of the rhizosphere. Singh [43] reported that *Cyanobacteria* improved the soil environment and survived in arid soil by accumulating soil carbon and nitrogen. The *P. putaminum* inoculation increased *Chloroflexi* abundance which was consistent with a previous study on microflora in a biofertilizer soil [44]. Wu et al. [45] found that *Chloroflexi* does not produce oxygen during photosynthesis and inhibits nitrogen fixation. In contrast, *Acidobacteria* decompose cellulose and modulate soil pH. Hence, the enrichment of specific rhizosphere fungi and bacteria might enable plants to maintain active microbiomes that improve survival under drought stress [46].

It was unknown whether DSE-mediated changes in the rhizosphere microbial communities augment drought stress tolerance in licorice. Our previous study showed that DSE inoculation (*A. vagum* and *P. putaminum*) improve licorice growth and survival under drought conditions [8]. In the present study, a network structure analysis disclosed that both soil fungal and bacterial networks were characterized by high specialization and modularity. Drought stress markedly affected the relative abundance of various microbial functional groups. Compared to the uninoculated condition under drought stress, *P. putaminum*

and *A. vagum* inoculation increased symbiotrophic fungi by 78.2% and 34.6% and saprotrophic fungi by 0.4% and 12.1% and decreased pathotrophic fungi by 125.6% and 44.5%, respectively. *Microascaceae*, *Trichoderma*, and *Aspergillus* were the dominant fungi in DCK, *Chaetomium* and *Pseudalescheria* predominated in DPP, and *Trichoderma* and *Aspergillus* prevailed in DAV. The *Microascaceae* include both saprobes and plant pathogens. Certain species are intrinsically resistant to antifungal agents [47, 48]. *Trichoderma* is an effective biofertilizer, soil amendment, and biocontrol agent [49]. Certain *Aspergillus* species such as *A. flavus* are facultative plant pathogens under drought stress and can produce considerable amounts of aflatoxin [50]. In contrast, *A. niger* and *A. fumigatus* are metallotolerant [41]. *Chaetomiaceae* degrade cellulose in the soil and increase soil organic matter [51]. *Pseudallescheria* (*Scedosporium*) spp. are global pathogens that resistant most antifungal agents [52].

Compared to the fungal community, DSE inoculation and the water regime had less influence on the composition of the bacterial community colonizing the licorice rhizosphere. *Bacillus*, *Microbacterium*, *Nocardioides*, and *Pseudarthrobacter* predominated and constituted 78.6% of the total abundance under the DCK treatment. *Bacillus*, *Pseudarthrobacter*, and *Ensifer* occupied 54.6% of the total abundance under the DPP treatment. *Bacillus*, *Microbacterium*, *Nocardioides*, *Pseudarthrobacter*, *Sporocytophaga*, and *Arenimonas* comprised 81.3% of the total abundance under the DAV treatment. These bacteria have high drought stress tolerance and most of them promote plant growth [53–55]. *Bacillus* species are also effective biocontrol bacteria [56]. *Pseudarthrobacter* and *Sporocytophaga* efficiently degrade cellulose, crude oil, and multibenzene compounds [57, 58]. Compared to the uninoculated condition, *P. putaminum* and *A. vagum* inoculation increased the relative abundance of beneficial (2.5% and 56.3%) and neutral (86.4% and 4.1%) fungi but decreased the relative abundance of pathogenic fungi (178.5% and 50.7%) under drought stress. Compared to the uninoculated condition, *P. putaminum* inoculation increased the relative abundance (107.4% and 55.4%) of neutral and pathogenic bacteria but decreased the relative abundance (92.8%) of beneficial bacteria under drought stress. The *A. vagum* inoculation increased the relative abundance (45.8%) of neutral bacteria but decreased the relative abundance (128.6%) of pathogenic bacteria. Our results indicated that DSE inoculation alters the licorice rhizosphere microbiome community composition. It enriched beneficial and neutral fungi and reduced harmful fungi under drought stress. Hence, DSE inoculation may be an important modality for the improvement of plant growth and drought resistance [8, 59, 60].

Conclusion

DSE inoculation and water regime markedly affected the composition and diversity of the microbial communities colonizing licorice rhizospheres, and such impact on fungal community was greater than bacterial community. Of the two DSE species, *P. putaminum* exerted a stronger influence on the licorice rhizosphere microbiome than *A. vagum* under drought stress. The edaphic factor changes caused by DSE inoculation and water regime partially account for the observed variations in licorice rhizosphere microbiome. DSE inoculation under drought stress enriched beneficial symbiotrophic fungi and growth-promoting bacteria but decreased the relative abundance of licorice rhizosphere pathogens. In this manner, it promoted licorice growth, strengthened pathogen resistance and drought tolerance, and

facilitate licorice survival under drought stress. These guide us to develop efficient and ecofriendly biofertilizers with symbiotic fungal consortia for the cultivation of medicinal plants based on the soil characteristics and the microbial community that it harbors in dryland agriculture.

Methods

Biological materials and growth substrates

The DSE strains, *Acrocalymma vagum* and *Paraboeremia putaminum*, were isolated from licorice roots naturally growing on the arid arable land of Northern China. They were deposited in the Laboratory of Endangered Species Breeding Engineering of the Institute of Medicinal Plant Development of the Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China [8]. Licorice seeds were provided by China National Traditional Chinese Medicine Corporation and stored at 4 °C.

The substrate used for the pot experiment was a 1:2 (w/w) mixture of sand (< 2 mm) and soil collected from the arid arable land of Northern China whereupon the licorice plants were naturally distributed. The physicochemical properties of the substrate were organic matter content, 21.57 mg g^{-1}; available nitrogen (N), 85.19 mg kg^{-1}; and available phosphorus (P), 7.90 mg kg^{-1}.

Experimental setup

The experiment had a randomized, complete factorial design with two factors: (1) DSE inoculation (including *Acrocalymma vagum* (AV) inoculation, *Paraboeremia putaminum* (PP) inoculation, and uninoculated control (CK)) and (2) water regime (including well-watered (WW) and drought stress (DS)). Each of the six treatments had five replicates and there were 30 pots in total [8].

Two licorice seedlings were transplanted to a sterile plastic pot (13 cm diameter × 12 cm height) filled with 800 g of nonsterile growth substrate. For the DSE inoculation treatments, 600 g of the growth substrate was poured into a pot, and 5-mm plugs were excised from the edges of actively growing DSE colonies on the culture media. The plugs were inoculated at 1-cm intervals near the licorice seedling roots. Then, 200 g of the growth substrate was added. For the uninoculated control, two plugs were excised from the fungus-free sterile medium and inoculated near the licorice seedling roots in each pot. The entire inoculation process was performed on a clean bench. All pots were maintained in a growth chamber under a 14 h/10 h photoperiod, 27 °C/22 °C (day/night), and 60% mean RH.

After 1 mo, half the DSE-inoculated and uninoculated pots were subjected to the WW treatment (70% field water capacity), while the balance were subjected to the DS treatment (30% field water capacity). Drought stress was applied according to the median soil moisture content recorded for the natural licorice habitat in Northern China. The soil moisture content in each pot was measured using a soil humidity recorder (L99-TWS-2; Hangzhou Loggertech Co. Ltd., Hangzhou, China). Lost water was replenished with sterile distilled water to maintain the desired field capacity determined by regular weighing. Seedlings were grown for 3 mo.

Rhizosphere soil sampling and physicochemical properties

The rhizosphere soil strongly adhering to the root surfaces was collected from each pot. Each soil sample was passed through a 2-mm sieve and divided into two subsamples. One was naturally dried at about 25 °C and its physicochemical properties were measured. The other was frozen at -80 °C until the subsequent microbial community composition analysis. A 0.2-g dried soil sample was digested in 10 mL of a 10:1:2 mixture of perchloric acid (12.7 M), sulfuric acid (18 M), and water in a Mars 6 microwave reaction system (CEM Corporation, Matthews, NC, USA) until a clear liquid was obtained. The soil organic matter content, available nitrogen (N), available phosphorus (P), and available potassium (K) were quantified by dichromate oxidization in the presence of sulfuric acid [61], alkaline hydrolysis diffusion, chlorostannous-reduced molybdophosphoric blue [62], and flame photometry [63], respectively.

Molecular analysis

The total genomic DNA from 0.25-g soil samples was extracted with a Powersoil® DNA Extraction kit (Mo Bio, Carlsbad, CA, USA). The DNA quality was tested by 0.1% (w/v) agarose gel electrophoresis. The DNA purity and concentration were measured with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Two universal primers 338F (5’-ACTCCTACGGGAGCAG CAG-3’)-806R (5’-GGACTACHGGGTWTCTAAT-3’) [64] and ITS1F (5’-CTTG GTCATTTAGGAAGTAA-3’)-ITS2R(5’-GCTGCGTTCTTCATCATGATGC-3’) [65] characterized the microbial communities by targeting the bacterial 16S rRNA genes *v3-v4* and the fungal ITS1 and ITS2 (internal transcribed spacer) regions, respectively. PCR was conducted in triplicate in a 20-μL reaction system containing 4 μL of 5× FastPfu Buffer (for 16s *v3-v4*) / 2 μL of 10× Buffer (for ITS), 2 μL dNTPs, 0.8 μL of the aforementioned forward and reverse primers, 0.4 μL FastPfu Polymerase (16S *v3-v4*) / 0.2 μL rTaq polymerase (ITS), 0.2 μL bovine serum albumin (BSA), and 10 ng template DNA. PCR amplification was conducted using the following thermal program: initial denaturation at 95 °C for 3 min followed by 28 denaturation cycles at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were detected by gel electrophoresis (2% (w/v) agarose), purified with an AxyPrep™ DNA Gel Extraction kit (Axygen BioSciences Inc., Union City, CA, USA), and quantified in a QuantiFluor™ dsDNA system fitted with a QuantiFluor™-ST fluorometer and a PCR tube adapter (Promega Corporation, Madison, WI, USA). The sample was sequenced using the paired end option (2 × 300 bp) of an Illumina MiSeq PE 300 platform (Illumina, San Diego, CA, USA) at the Environmental Genome Platform of Meiji Biomedical Technology Co. Ltd. (Shanghai, China).

Bioinformatics analysis

Raw fastq files were demultiplexed, quality-filtered, and merged by Trimmomatic and fast length adjustment of short reads (FLASH; Johns Hopkins University, Baltimore, MD, USA) [66]. Sequences that were < 50 bp long and had average quality score < 20 or ambiguous bases were removed. The filtered high-quality sequences were merged according to the overlap sequences between read pairs. Sequences with mismatches along the primer region were removed before the downward process. Non-chimeric

sequences were dereplicated and singletons were discarded. The filtered non-chimeric sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence level based on the UPARSE pipeline using USEARCH v. 8.0. The RDP Bayesian classifier algorithm was used to classify OTU representative sequences via the fungal (ITS) UNITE database v. 18.11.2018 and the Silva (SSU123) 16S rRNA reference database at confidence threshold = 0.7. The RDP then collated the functional gene database from GeneBank (Release 7.3; http://fungene.cme.msu.edu/) and obtained species annotation data. To eliminate potential bias caused by divergent sequence depth across samples, all samples were subsampled to the minimum sequencing depth. The dilution curve, the Venn map, and the community composition analysis were conducted in R (R Core Team, Vienna, Austria; version 3.4.0) based on the OTU count and associated taxonomy tables. The Alpha diversity index was calculated in mothur (v. 1.30.2). Other statistical analysis was performed in SPSS v. 22.0 (IBM Corp., Armonk, NY, USA) and the remaining graphs were generated with Origin v. 9.0 (OriginLab, Northampton, MA, USA) [67].

Abundance and diversity analyses

To characterize microbial diversity, the Chao1, Ace, Shannon, and Simpson indices were calculated based on the OTU data. Chao1 and Ace reflect community abundance while Shannon and Simpson indicate community diversity [68]. Rank abundance and rarefaction curves generated in QIIME estimate species evenness and evaluate species richness and sequence depth, respectively [69]. Fungal or bacterial OTU richness was defined as the number of fungal or bacterial OTUs per sample. The relative abundance of a specific fungal or bacterial OTU and class was defined as the ratio of corresponding sequences and class to the total reads PER sample. Each representative OTU sequence in this study was used for taxonomic identification at the phylum, class, order, family, and genus levels.

Statistical methods

Two-way analysis of variance (ANOVA) was used to disclose the effects of DSE inoculation, water regime, and their interactions on fungal and bacterial OTU diversity. Data shown in the figures are means of ≥ 3 replicates. Variations among treatment means were compared using Tukey's honestly significant difference (HSD) tests at $P < 0.05$. Non-metric multidimensional scaling (NMDS) was used to visualize compositional dissimilarities in the rhizosphere fungal and bacterial communities. The metaMDS command in the vegan package v. 2.4-1 was used [70]. To evaluate the effects of DSE inoculation on the rhizosphere fungal and bacterial communities, permutational multivariate analysis of variance (PerMANOVA) was run using the adonis command in the vegan package with 999 permutations [70]. Rarefaction curves for the bacterial and fungal OTUs were calculated using the specaccum function in the vegan package [70]. The edaphic factors such as water condition, soil organic matter, available N, available P, and available K/microbe preference analysis was performed according to Yao et al. [71] and Huang et al. [72].

To visualize the structure of the rhizosphere bacterial and fungal networks among treatments, a network was drawn on the basis of genera with abundance > 200 for OTU-level matrices. To this end, the Prefuse Force Directed OpenCL Layout in CYTOSCAPE v. 3.4.0 was used [73].

To identify the beneficial, harmful, and neutral fungi and bacteria, a network analysis was conducted on the abundance of the genera that significantly differed among treatments [74, 75]. The online FUNGuild application (http://www. stbates.org/ guilds/app.php) was used to assign trophic status to frequent OTUs [74]. The trophic status was assigned with different certainty levels (possible, probable, or highly probable) based on a combination of the aforementioned effects. The community designating the nutrient type as a compound nutrient type was included in “other fungi”. The community identified as a compound multifunctional method was unified into “other pathogens/saprophytic fungi” under the nutrient type [74, 75].

Abbreviations

AM, arbuscular mycorrhizae; AV, *Acrocalymma vagum*; BSA, bovine serum albumin; CK, uninoculated control; DS, drought stress; DSE, dark septate endophyte; OTU, operational taxonomic unit; PLFA, phospholipid fatty acid; PP, *Paraboeremia putaminum*; WW, well-watered; NCK, non-inoculation under well-watered condition; NPP, inoculation with *Paraboeremia putaminum* under well-watered condition; NAV, inoculation with *Acrocalymma vagum* under well-watered condition; DCK, non-inoculation under drought stress; DPP, inoculation with *Paraboeremia putaminum* under drought stress; DAV, inoculation with *Acrocalymma vagum* under drought stress.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The Illumina MiSeq sequence datasets are available at the NCBI Sequence Read Archive BioProject ID PRJNA664875

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CH and WQW designed the experiment; CH and JLH collected the samples; CH, JLH and XEL performed the laboratory work; and CH, WQW and XEL analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Diversity and richness index of licorice rhizosphere soil fungi

Samples	Shannon	Simpson	Ace	Chao1
DCK	1.63±0.41c	0.35±0.08a	324.22±26.51d	285.46±38.25e
DPP	3.62±0.48a	0.07±0.03d	721.28±59.33a	675.46±75.47a
DAV	1.63±0.15c	0.37±0.06a	430.36±43.16c	352.79±39.38d
NCK	2.81±0.27b	0.16±0.04c	590.11±67.24b	536.27±40.08b
NPP	2.32±0.43bc	0.28±0.02b	558.51±33.29b	522.83±39.48b
NAV	2.08±0.31bc	0.31±0.06ab	494.84±42.57bc	435.04±37.39c

Note: Different letters in the same column indicate significant differences between different samples ($P < 0.05$). NCK, non-inoculation under well-watered condition; NPP, inoculation with *Paraboeremia putaminum* under well-watered condition; NAV, inoculation with *Acrocalymma vagum* under well-watered condition; DCK, non-inoculation under drought stress; DPP, inoculation with *Paraboeremia putaminum* under drought stress; DAV, inoculation with *Acrocalymma vagum* under drought stress.

Table 2 Diversity and richness index of licorice rhizosphere soil bacteria

Samples	Shannon	Simpson	Ace	Chao1
DCK	6.45±0.29ab	0.0067±0.003c	4150.99±429.98b	4142.55±480.04bc
DPP	6.78±0.05a	0.0045±0.001c	5203.69±651.62a	4906.27±211.29a
DAV	6.34±0.31ab	0.0066±0.002c	4289.02±341.35b	4068.12±477.16bc
NCK	5.78±0.13b	0.0251±0.008a	4404.73±226.99b	3795.56±358.63c
NPP	6.43±0.21ab	0.0102±0.002b	5009.08±441.12ab	4485.35±85.86b
NAV	6.54±0.25ab	0.0055±0.002c	5259.61±630.63a	4322.72±508.26b

Note: Different letters in the same column indicate significant differences between different samples ($P < 0.05$). NCK, non-inoculation under well-watered condition; NPP, inoculation with *Paraboeremia putaminum* under well-watered condition; NAV, inoculation with *Acrocalymma vagum* under well-watered condition; DCK, non-inoculation under drought stress; DPP, inoculation with *Paraboeremia putaminum* under drought stress; DAV, inoculation with *Acrocalymma vagum* under drought stress.

Figures

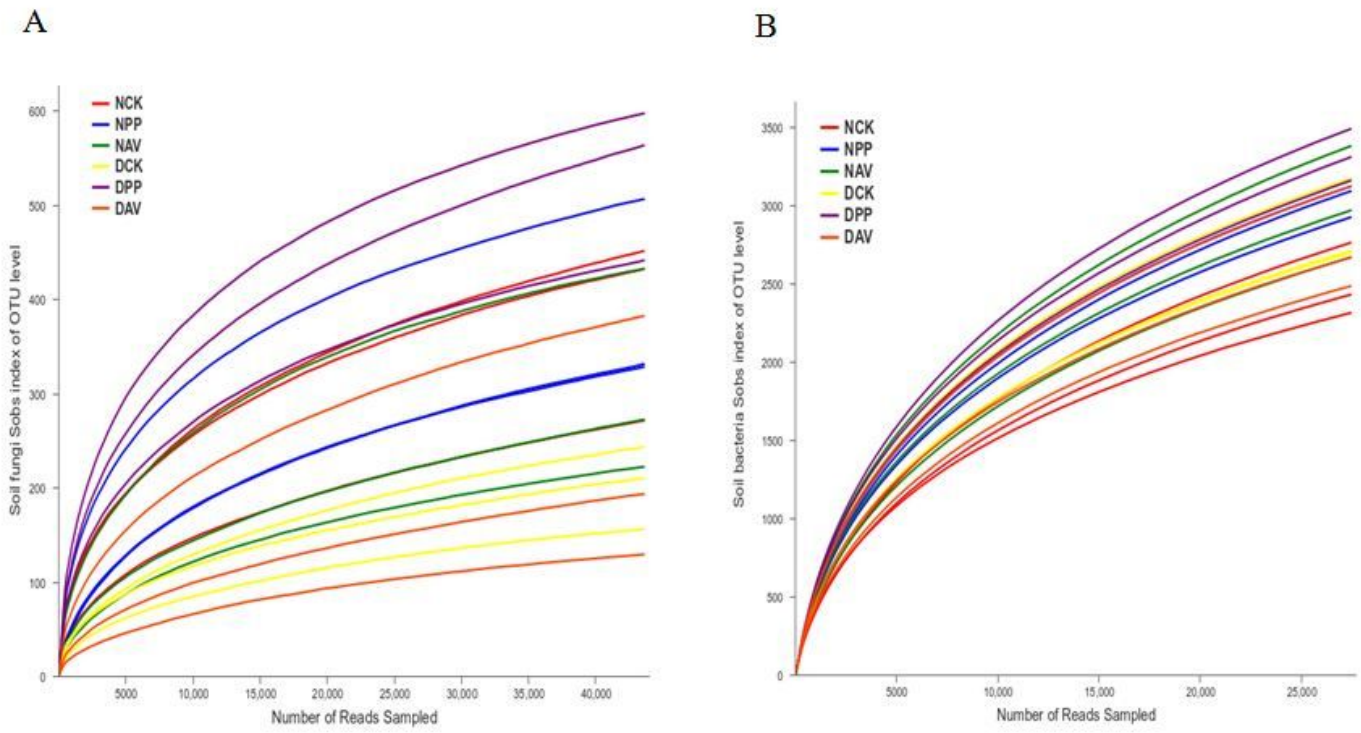

Figure 1

Rarefaction curves for the fungal (A) and bacterial (B) operational taxonomic units (OTUs) in licorice rhizosphere. NCK, non-inoculation under well-watered condition; NPP, inoculation with Paraboeremia putaminum under well-watered condition; NAV, inoculation with Acrocalymma vagum under well-watered condition; DCK, non-inoculation under drought stress; DPP, inoculation with Paraboeremia putaminum under drought stress; DAV, inoculation with Acrocalymma vagum under drought stress.

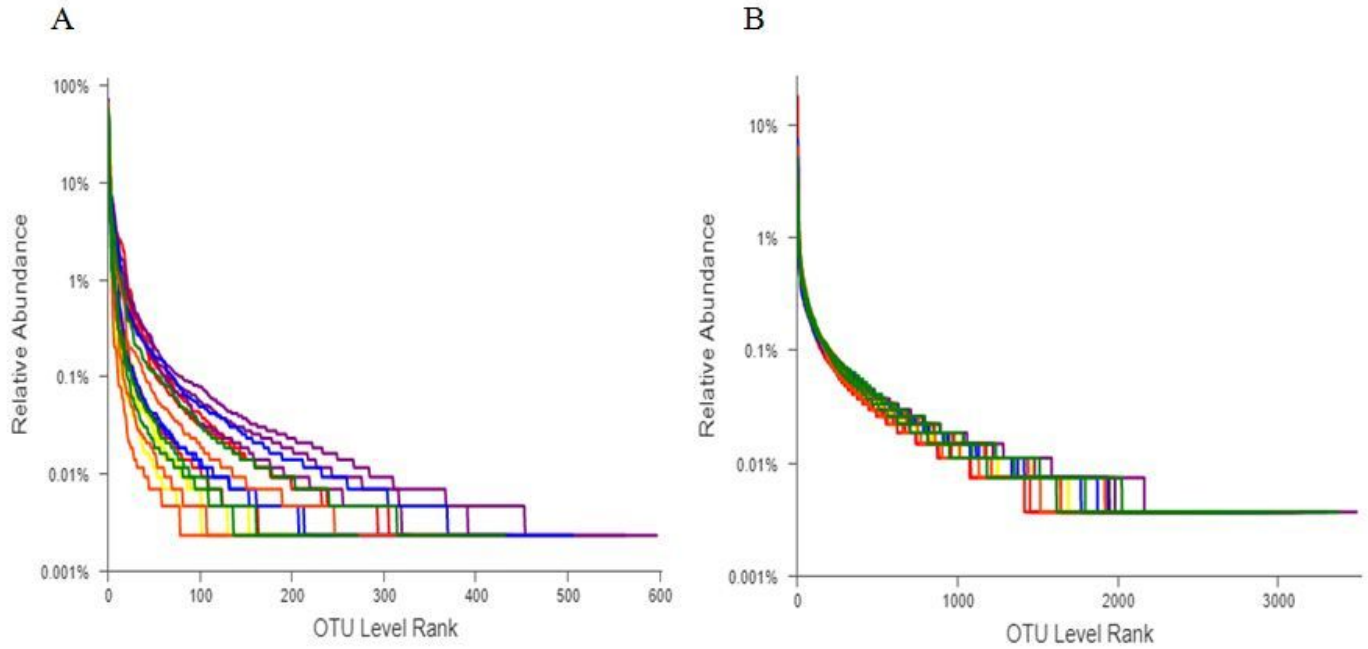

Figure 2

Ranking by the abundance of the fungal (A) and bacterial (B) operational taxonomic units (OTUs) in licorice rhizosphere.

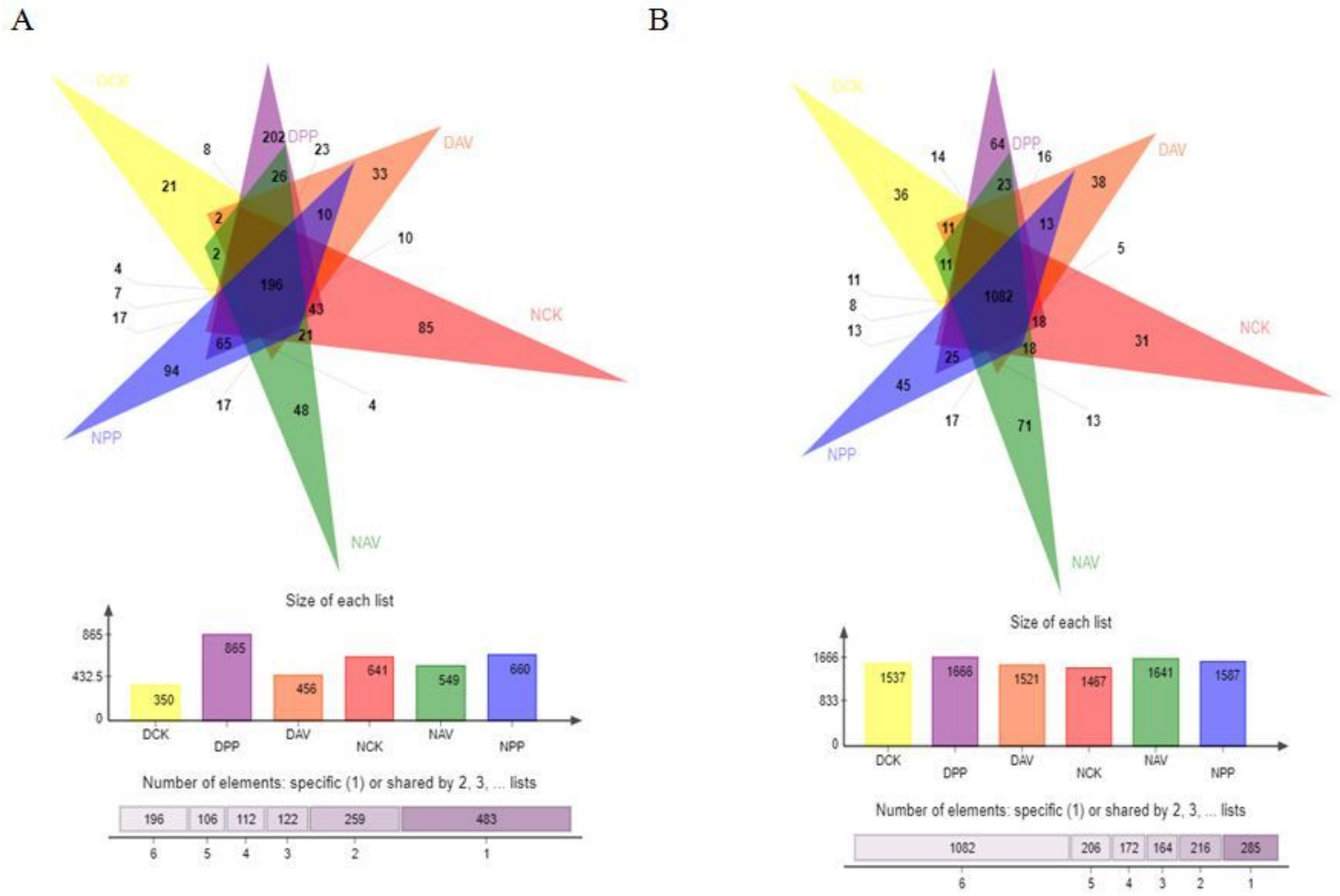

Figure 3

Venn diagram showing differences in composition of fungal (A) and bacterial (B) OTUs inoculated with different DSE species under well-watered and drought stress, respectively. Each part in the Venn diagram represents one (group) treatment, and the number of circles and circles overlapped represents the number of OTUs shared between the samples (group), while the number without overlapped represents the number of OTUs unique to the samples (group). NCK, non-inoculation under well-watered condition; NPP, inoculation with Paraboeremia putaminum under well-watered condition; NAV, inoculation with Acrocalymma vagum under well-watered condition; DCK, non-inoculation under drought stress; DPP, inoculation with Paraboeremia putaminum under drought stress; DAV, inoculation with Acrocalymma vagum under drought stress.

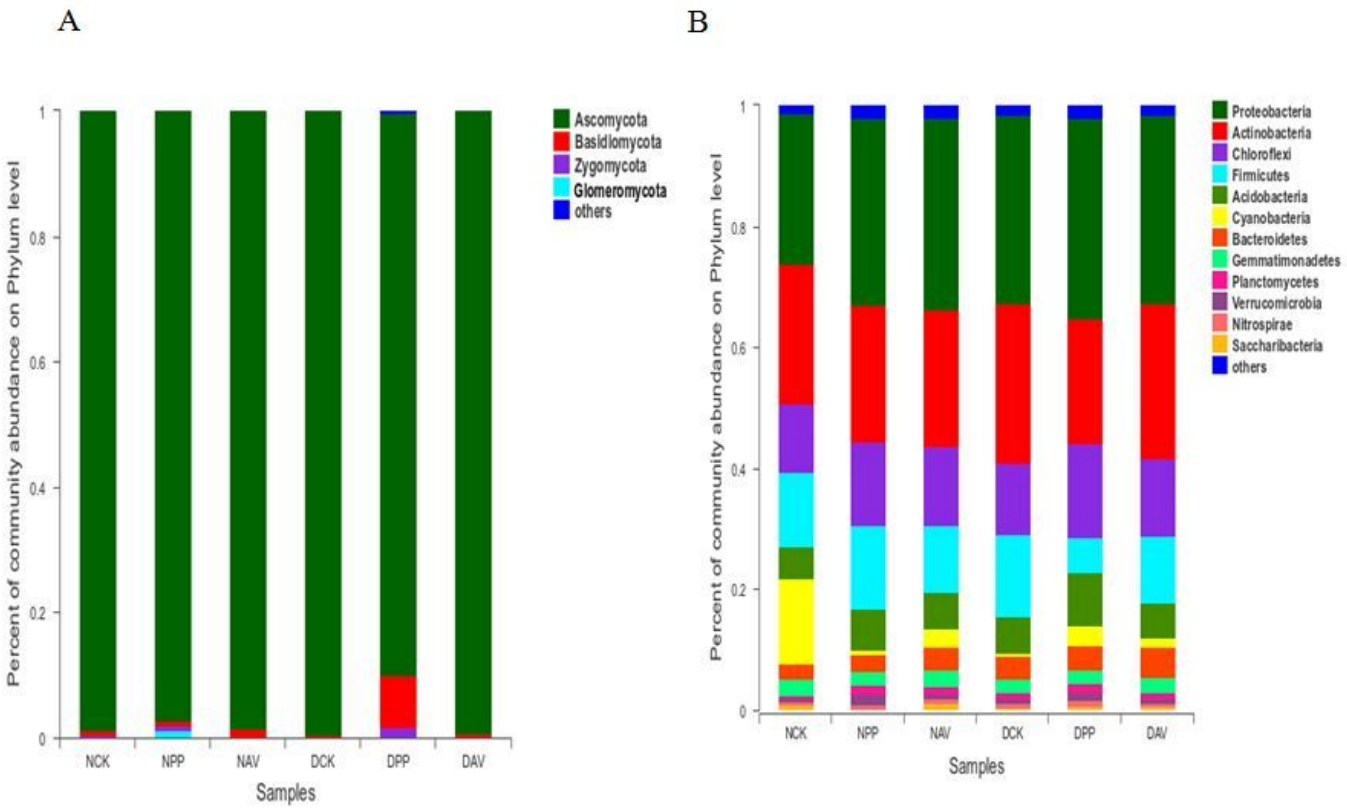

Figure 4

Relative abundance of fungi (A) and bacteria (B) at the phyla level in licorice rhizosphere. NCK, non-inoculation under well-watered condition; NPP, inoculation with Paraboeremia putaminum under well-watered condition; NAV, inoculation with Acrocalymma vagum under well-watered condition; DCK, non-inoculation under drought stress; DPP, inoculation with Paraboeremia putaminum under drought stress; DAV, inoculation with Acrocalymma vagum under drought stress.

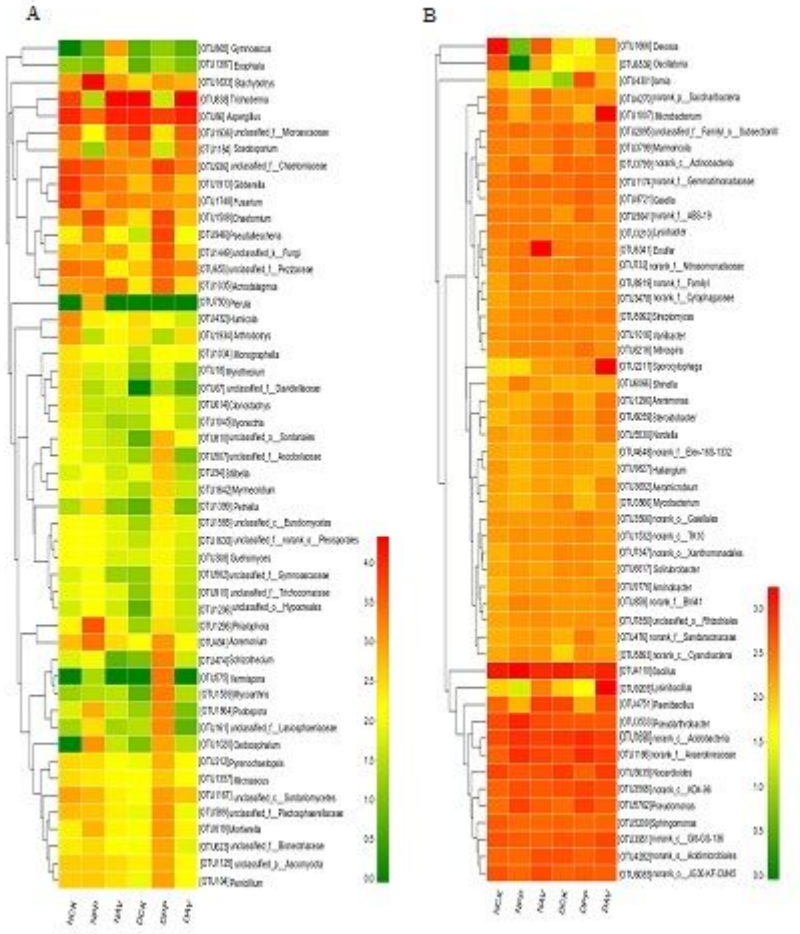

Figure 5

Heatmap depicting the distribution of relatively abundant fungal (A) and bacterial (B) operational taxonomic units (OTUs) in licorice rhizosphere. The color of each heat map cell indicates the relative abundance of the corresponding fungal and bacterial OTUs. Cluster analysis was performed based on Bray–Curtis similarities. The horizontal represents the treatment information, the vertical represents the clustering situation of different samples, the right represents the species and OTUs annotation, and the left represents the species clustering tree. NCK, non-inoculation under well-watered condition; NPP, inoculation with Paraboeremia putaminum under well-watered condition; NAV, inoculation with Acrocalymma vagum under well-watered condition; DCK, non-inoculation under drought stress; DPP, inoculation with Paraboeremia putaminum under drought stress; DAV, inoculation with Acrocalymma vagum under drought stress.

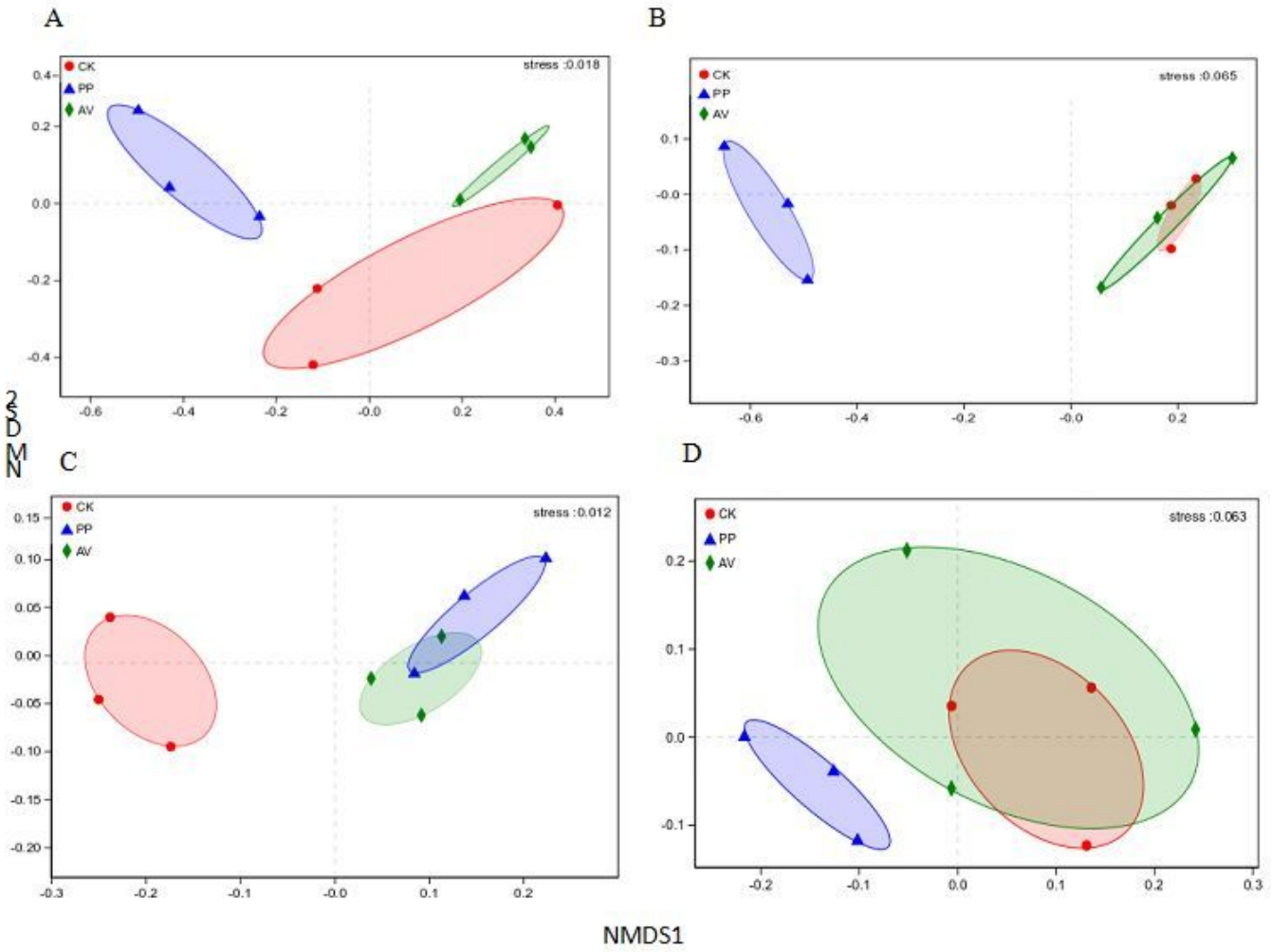

Figure 6

Non-metric multidimensional scaling (NMDS) ordination of the community composition of fungi and bacteria in licorice rhizosphere under different water regimes. A, fungal community under well-watered condition; B, fungal community under drought stress; C, bacterial community under well-watered condition; D, bacterial community under drought stress. CK, non-inoculation; PP, inoculation with Paraboeremia putaminum; AV, inoculation with Acrocalymma vagum.

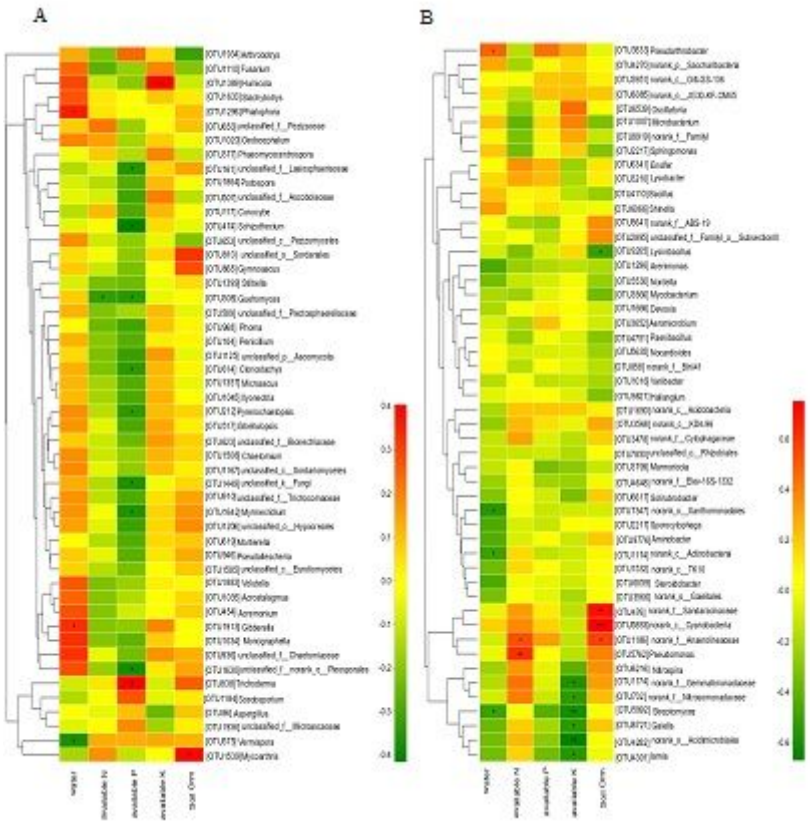

Figure 7

Preferences observed in soil factor-fungus (A) and soil factor-bacterium (B) associations.

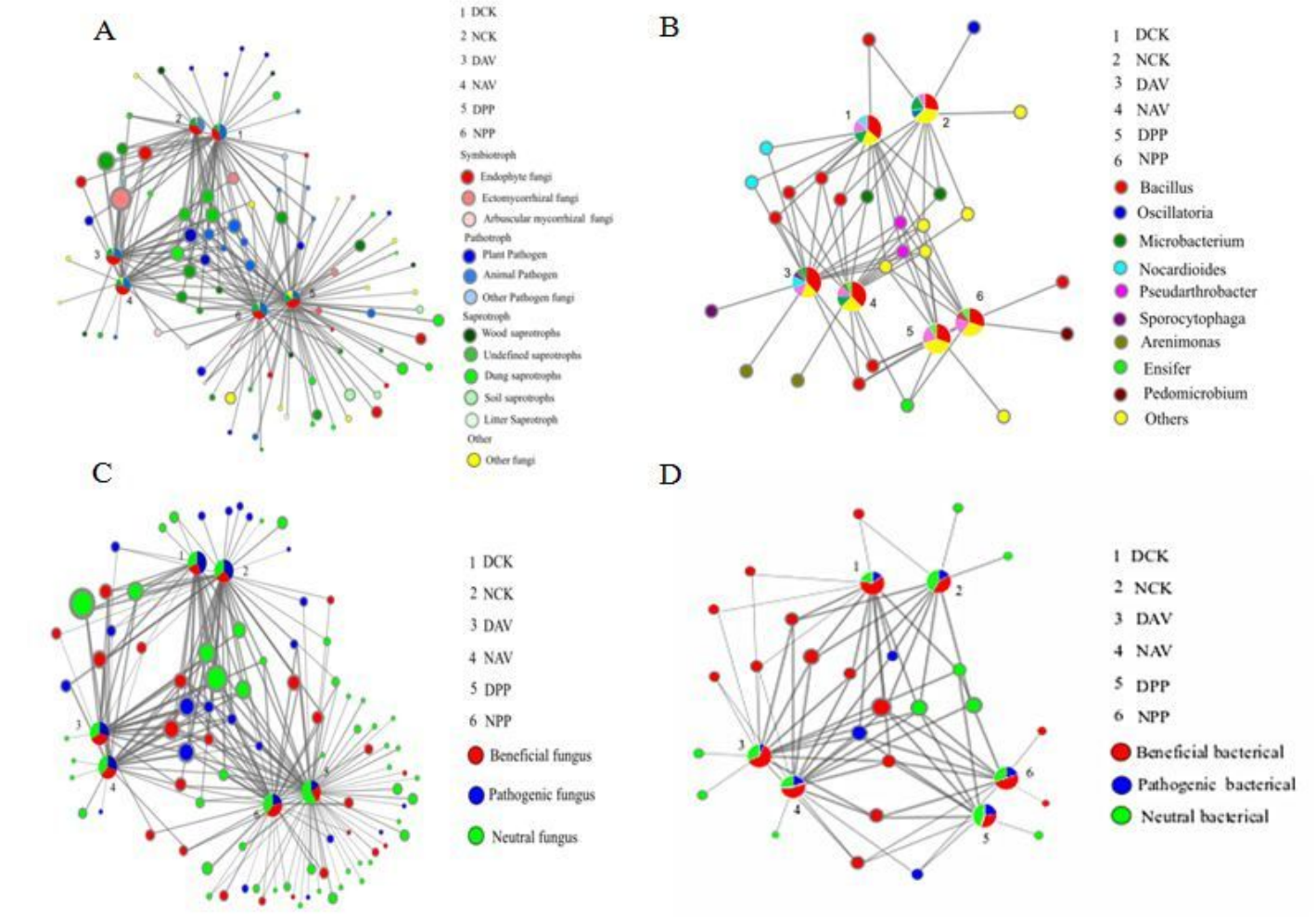

Figure 8

Architecture of the treatment-soil microorganism network. A, visualization of the fungi network at class level; B, visualization of the bacterial network at genus level; C, visualization network of beneficial, harmful and neutral fungi for licorice plants; D, visualization network of beneficial, harmful and neutral bacteria for licorice plants. The size of nodes in A, B, C, D roughly represents the relative abundance of fungal and bacterial operational taxonomic units.