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Effects of microbial organic fertilizers on *Astragalus membranaceus* growth and rhizosphere microbial community

Jian-Ping Liang¹, Zhi-Quan Xue¹, Zhen-Yu Yang¹, Zhi Chai², Jing-Ping Niu¹ and Zhi-Yong Shi^{1*} 

Abstract

Purpose: The application of excessive chemical fertilizers during the cultivation of *Astragalus membranaceus* leads to a decline in the quality of this medicinal plant as well as the soil's sustainable productivity. In this study, we developed a special microbial organic fertilizer for *A. membranaceus* and investigated its effects on plant growth and rhizosphere microbial communities.

Methods: The root biomass and main active components of *A. membranaceus* in different growth stages were measured to assess the impacts of microbial organic manure on plant growth. Meanwhile, 16S rRNA and ITS1 amplicons were amplified and high-throughput sequencing was performed to detect the dynamic impacts of microbial organic manure on rhizosphere microbial communities.

Result: The results demonstrated that microbial organic manure significantly increased wet and dry weights of *A. membranaceus* seedlings and the accumulation of two effective components (flavonoids and saponin) in bacterial fertilizer treatment groups are significant higher than the control group. Research on rhizosphere microbial flora shows that the number and polymorphism of bacteria and fungi were decreased after the application of special fertilizer during the rapid growth period of plant and then gradually increased with seedling growth. The community structure of bacteria was regulated after the application of special fertilizer, and the beneficial bacteria for plant growth are enriched. Functional profiles prediction showed that significant shifts in metabolic functions impacting KEGG pathways of the microbial fertilizer treatment groups are related to metabolism and biosynthesis.

Conclusion: The results indicate that the microbial organic manure can improve *A. membranaceus* growth by providing appropriate nutrients and regulating the rhizosphere microbial community which has good potential in ecological cultivation of *A. membranaceus*.

Keywords: *Astragalus membranaceus*, Microbial organic fertilizer, High-throughput sequencing, Rhizosphere microbial community, Diversity

Introduction

Soil microorganisms contribute to nutrient recycling, system stability, and anti-jamming capabilities and sustainable productivity of the soil's ecosystem (Heijden et al. 2010; Richard et al. 2017; Singh 2015). The application of natural microbial fertilizer, which is produced by

plant-growth-promoting bacteria (PGPB), is a potential alternative for a more sustainable and environmentally friendly agricultural development (Ahemad and Kibret 2014; Ferreira et al. 2019). Many previous studies have shown the positive effects of different microbial fertilizers on major crops, which have been proven to effectively increase the nutrient utilization of crops, thereby increasing plant production in a cost-effective and environmentally friendly way (Tabassum et al. 2017). They can also improve soil porosity, structural stability,

* Correspondence: shizy@sxau.edu.cn

¹College of Life Sciences, Shanxi Agricultural University, Taigu 030801, China
Full list of author information is available at the end of the article



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nutrient availability, and biological activity (Francis et al. 2010; Wang et al. 2011).

Astragalus membranaceus, a member of the Fabaceae family, is an important herb that has been used in traditional Chinese medicine (Liu et al. 2011). *Astragalus membranaceus* has significant effects in improving the immune system and cardiovascular system of the body, and it also has significant therapeutic effects in anti-aging, anti-virus, and anti-tumor (James and Ko 2007; Yang et al. 2013; Zhou et al. 2018). With over-harvesting of the wild *A. membranaceus* and high demand in the market, the available wild resources are becoming scarce. Thus, the cultivation of *A. membranaceus* continues to expand and the amount of chemical fertilizers and pesticides is large during the planning process. Unfortunately, these chemicals impart various negative effects on the agricultural ecosystem, including soil compaction and degradation, reduction of soil microbial diversity, ground-water resources pollution, and atmospheric contamination (Savci 2012; Sharma and Paliyal 2014; Yang et al. 2012).

As a type of Leguminosae plant, the roots of *A. membranaceus* usually interact with rhizobia in the soil, resulting in root nodule symbiosis which provides nutrients for plant growth and promotes the accumulation of active ingredients. Azotobacter fertilizer, which is mainly composed of native screened rhizobia, is an environmentally friendly and long-acting fertilizer for *A. membranaceus*. In the prophase experiment, we isolated two strains of rhizobia from the root nodule of *A. membranaceus*, *Rhizobium* sp. t16, and *Sinorhizobium* sp. t21, which demonstrate high nitrogen fixation activity (Zhiqian et al. 2016). Laboratory pot-experiment results showed that the two strains could significantly promote the growth of *A. membranaceus* seedlings (Zhiqian et al. 2016). However, future studies are necessary to determine the effects of organic microbial fertilizer in the field. The selection of inoculation method and carrier material of PGPB should be according to the viability of microorganisms, availability of plant, and feasible application. Solid carrier materials composed of nutrients and organic matter are considered the most suitable materials for field applications. (Tabassum et al. 2017). Meanwhile, returning stems and leaves to the field can affect soil structure and gas exchange and can also improve soil fertility. However, the effects of organic microbial fertilizer with stems and leaves powder as the carrier material for field plant growth and rhizosphere microbial communities remain unclear.

Therefore, we performed a field experiment to access the effects of special manure composed of stems, leaves powder, and two native strains of rhizobia for field plant growth and microbial community diversity. The purpose of this research was (i) to examine the effects of

microbial organic fertilizers on *A. membranaceus* growth and accumulation of the main medicinal components (ii) to assess the effects of microbial organic fertilizers on rhizosphere microbial community. We hypothesized that microbial organic fertilizers could promote *A. membranaceus* growth and affect the function and diversity of rhizosphere microbial communities.

Materials and methods

Site description

The field experimental site was located in Jingle County, Shanxi Province of China (38° 36' 51.47" N, 111° 94' 54.32" E). The farm area is located in a typical monsoon climate at an altitude of 2032 m, with a mean annual rainfall of 447.6 mm, humidity of 61%, and temperature of 7.3 °C. The soil basic physicochemical properties of the experimental site are as follows: total organic matter 4.57 g/kg, pH 8.88, total N 0.42 g/kg, total P 0.47 g/kg, total K 21.81 g/kg, available N 27.63 mg/kg, available P 7.21 mg/kg, and available K 81.24 mg/kg.

Preparation of the microbial organic manure

Two strains of nitrogen-fixing bacteria, *Rhizobium* sp. t16 and *Sinorhizobium* sp. t21, were isolated from the root nodule of *A. membranaceus* in Hunyuan County, Shanxi Province, and deposited in China General Microbiological Culture Collection Center, with the access number of CGMCC No. 9676 and 9677 (Zhiqian et al. 2016). Before the experiments, the bacteria were inoculated into Luria-Bertani liquid medium for fermentation at the temperature of 28 °C, and the concentration of bacteria was regulated at 10⁸ bacteria per mL.

The 3-year-old plants of *A. membranaceus* in the Jingle planning base were selected for the stems and leaves collection. The stems and leaves were harvested in May and August with 100 g for each plant. The plant tissue were washed with distilled water and dried at 70 °C and then crushed into powder via 60 mesh sieves for subsequent steps. Bacteria liquid was added at a powder to liquid ratio of 7:3. Different types of special manure containing the wet powder and bacteria solution were prepared for use.

Experimental design

The field experiment was conducted at May 2018 and three treatments using different combinations of bacterial solution and stem and leaf powder were performed based on randomized block design. The treatments were designed as follows: no fertilizer (control group), strain t16 bacterial solution + powder (group 4), strain t21 bacterial solution + powder (group 7), and t16 + t21 bacterial solution + powder (group 9). Five hundred milligrams of microbial fertilizer was applied to each group of experimental plot before sowing (1 m × 3 m)

and each treatment was conducted with three replications. The soil in the control group was only plowed before sowing. The row spacing and plant distance for sowing is 25 cm and 15 cm in each plot when sowing. This study includes 4 groups of experimental plots according to the experimental designs (CK, group 4, group 7, and group 9).

Sampling

Collected samples included rhizosphere soil (soil directly associated with the roots), soil for physicochemical parameters and roots. Soil samples before sowing were collected at 20 cm deep below the surface in triplicate (CK0). Rhizosphere soil samples were collected in triplicate at 30 days after sowing (CK1, 4.1, 7.1, 9.1) and 90 days after sowing (CK2, 4.2, 7.2, 9.2). The definitions of different samples for subsequent analysis were as follows: the soil control sample, CK0, was collected at day 0 and corresponded to the sowing date of *A. membranaceus*. The soil samples collected at 30 days (CK1, 4.1, 7.1, 9.1), corresponding to the seedling stage (also the rapid growth period), including the germination stage (0–15 days). The soil samples collected at 90 days (CK2, 4.2, 7.2, 9.2) correspond to the stable growth period and the plant flowering period.

Rhizosphere soil samples were gathered by brushing off the remaining soil adhering to the roots with sterile brushes, mixed together to reduce errors, placed in 10 ml sterile centrifuge tubes and then immediately stored at -80°C for DNA extraction. Five seedlings of *A. membranaceus* were randomly collected from each group (30 days, 60 days, 90 days, and 120 days), placed in clean plastic, stored at icebox and taken back to the laboratory for growth parameters analysis (fresh weight, dry weight, root diameter, and effective components). Roots were cleaned with distilled water, absorbed moisture, measured the fresh weight, root diameter, dried at 70°C to constant quality and measured the dry weight.

The dried roots of *Astragalus* (120 days) were crushed and passed through a 0.25-mm sieve, and the main medicinal components of *Astragalus* were extracted by flash extraction method. With rutin as the control standard curve, the total flavonoids content was determined by ultraviolet spectrophotometry. A standard curve was prepared with astragaloside IV as a control, and the total saponin content was determined by vanillin-concentrated sulfuric acid colorimetry. Anhydrous glucose was used as a control standard curve, and the polysaccharide content was determined by the phenol-sulfuric acid colorimetric method (Chun-Hong et al. 2011; Xi et al. 2010; Zheng et al. 2011). The measured values were statistically analyzed using ANOVA ($p < 0.05$) with SPSS to detect the significant difference between groups.

DNA extraction

DNA of soil samples collected at day 0, day 30, and day 90 was extracted with an Omega E.Z.N.A soil DNA kit purchased from Feiyang BIOTECH Co., Ltd. (Guangzhou, China) according to the manufacturer's instructions (Dineen et al. 2010). The DNA was gathered with 0.8% agarose gel electrophoresis and then stored at -80°C for further analysis.

Illumina MiSeq sequencing

The V4 region of 16S rRNA amplicons for bacterial community analysis were amplified by universal primers 515F (5'-GTGCCAGCMGCCGCGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). For fungi amplicons, we amplified the internal transcribed spacer 1 (ITS 1) region with universal primers 1743F (5'-CTTG GTCATTTAGAGGAAGTAA-3') and 2043R (5'-GCTG CGTTCCTCATCGATGC-3'). Amplicons were generated using ExTaq HS (TaKaRa Bio Inc., Shiga, Japan) by polymerase chain reaction (PCR) under the following conditions: 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. All amplicons of bacteria and fungi were then sequenced by an Illumina MiSeq platform and MiSeq Reagent Kit v1 (Illumina, Inc., Santiago, CA, USA) at the Beijing Genomics Institute (Shenzhen, China).

Raw data generating from Illumina MiSeq platform were cleaned using python scripts as follows: (1) confirmation of barcode and adaptor completeness; (2) delete sequences which containing more than one ambiguous base (N); and (3) removal of sequences shorter than 100 bp. Cleaned pair-end reads were then connected into tags using the COPE software (Connecting Overlapped Pair-End, V 1.2.1) (Liu et al. 2012). Sequencing noise was removed using the pre.cluster tool in the MOTHUR software package v. 1.35.1 (Schloss et al. 2009). Chimera detection and removal were conformed using UCHIME v. 4.2 (Edgar et al. 2011). MOTHUR was also used to determine the Operational Taxonomy Unit (OTU) with a sequence identity threshold of 97% (Schloss et al. 2009). OTUs annotation was performed by local NCBI-BLAST v.2.9.0 and Usearch v.11.0 (Edgar 2018) and 16S rRNA training set 9 in the RDP database and ITS1 sequences in UNITE database were used as annotation references.

Statistical analysis

OTUs sequences and reads data were used for Alpha diversity analysis to assess the richness and diversity within the microbial communities. The Alpha diversity index was calculated by the MOTHUR package, including Simpson diversity, Shannon diversity, Chao 1 indices, as well as observed species and phylogenetic diversity

(Kemp and Aller 2004). Principal coordinate analysis (PCoA) was performed with QIIME (Caporaso et al. 2010) to measure dissimilarity at phylogenetic distances based on UniFrac analysis, and the results were visualized using KING (Crawford et al. 2009). Statistically significant differences between experimental and control groups were determined using the non-parametric Mann-Whitney U test and multivariate analysis of variance in R v.3.6.0 platform. Metagenome functional profiling of bacterial community was predicted with 16S rRNA reads clustered at a 97% identity threshold using PICRUSt (Langille et al. 2013) and the reference GreenGenes (version 13.5) database (DeSantis et al. 2006). Significantly enriched functional categories in different treatment groups were accessed using LEfSE (Segata et al. 2011) with a p value < 0.05 and a linear discriminant analysis (LDA) score >2.

Results

Effects of microbial organic fertilizer on the growth of *A.membranaceus*

The seedlings of *A. membranaceus* were harvested at 30 days, 60 days, 90 days, and 120 days after sowing. The fresh weight, taproot diameter, and dry weight of the roots were measured (Fig. 1a-c). The results showed no difference between the samples obtained 30 days after sowing. On the 60th and 90th days, there were higher values in the experimental group compared to the control group, but there was no statistical difference. On the 120th day, the fresh weight and dry weight of groups 4 and 9 were statistically higher than that of group CK and 7 ($p < 0.05$) (Fig. 1a). The results suggest that the first 30 days were necessary for the seeds to germinate into the main root; the root was short with low weight, and there were no obvious interactions with soil microorganisms. Lateral roots had grown by the 60th or 90th day of growth but were not developed enough to make a difference between the groups. On the 120th day, the number of lateral roots was large enough to support plant growth, indicating that the application of bacterial fertilizer to plants with well-developed roots might be more beneficial than application to other plants.

Determination of main medicinal components

The main medicinal components of *Astragalus* roots (sampled at 120 days after seedling) were measured, including the total flavonoids content, the total saponin content, and the polysaccharide content (Fig. 2). The results showed that the accumulation of flavonoids and saponin in bacterial fertilizer treatment group 7 and group 9 are higher than in the group CK, but the polysaccharides showed no difference between groups. Interestingly, the accumulation of flavonoids and saponin in treatment group 7 ($p < 0.05$) and group 9 ($p < 0.01$) is

both significantly higher than in group CK. However treatment group 4 showed no significant difference compared with the group CK (Fig. 2a-b).

Abundance and diversity of the bacterial and fungal microbiota

Illumina MiSeq sequencing was utilized to measure the microbial abundance and community diversity during the growth of *A. membranaceus*. A total of 103,396 high-quality 16S rRNA reads were generated from 9 sample sets for bacteria, with an average of 11,488 reads per sample (range: 10,332-12,214). Meanwhile, 370,055 high-quality ITS reads were obtained for fungi throughout the sequencing process, with an average of 41,117 reads per sample (range: 40,121-42,286) (Table 1). High-quality pair-end reads were then connected to tags and clustered into 982 OTUs for bacteria and 564 OTUs for fungi (both at 97% similarity). Finally, we obtained an average of 925 OTUs for bacteria (range: 880-949) and 334 for fungi (range: 311-365) after removing singletons. These sequences were subjected to further analysis.

Rarefaction curves of observed species and Simpson diversity indices were plotted for bacteria and fungi communities respectively (Fig. 3a-d). Although the rarefaction curve was not parallel to the x -axis, the Simpson diversity index reached saturation, indicating that most of the microbial diversity was captured in samples during the growth of *A. membranaceus*. Shannon index showed that bacterial diversity in treatment group 9 was higher than that in other groups both in the rapid growth period (6.07) and stable growth period (6.2) of *A. membranaceus*, and treatment group 7 had the lowest bacterial diversity among all the experimental groups. However, diversity indices between experimental and control groups showed no significant differences in bacterial and fungal diversity ($p > 0.05$) (Table 1).

Characterization of bacterial and fungal communities at phylum and genus levels

To examine the changes in the bacterial communities alongside plant growth, the relative abundance of bacterial taxa was compared at both phylum and genus levels between the experimental and control groups. There were eight bacterial phyla identified in the special manure group, including *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Chloroflexi*, *Nitrospirae*, *Bacteroidetes*, and *Planctomycetes*. Relative abundance analysis of bacterial phyla throughout the entire *A. membranaceus* growth process revealed that *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were the predominant bacterial phyla in both experimental and control groups (Fig. 4a). The proportion of *Proteobacteria* remained prominent throughout the growth period at an average level of 34% once it reached dominance.

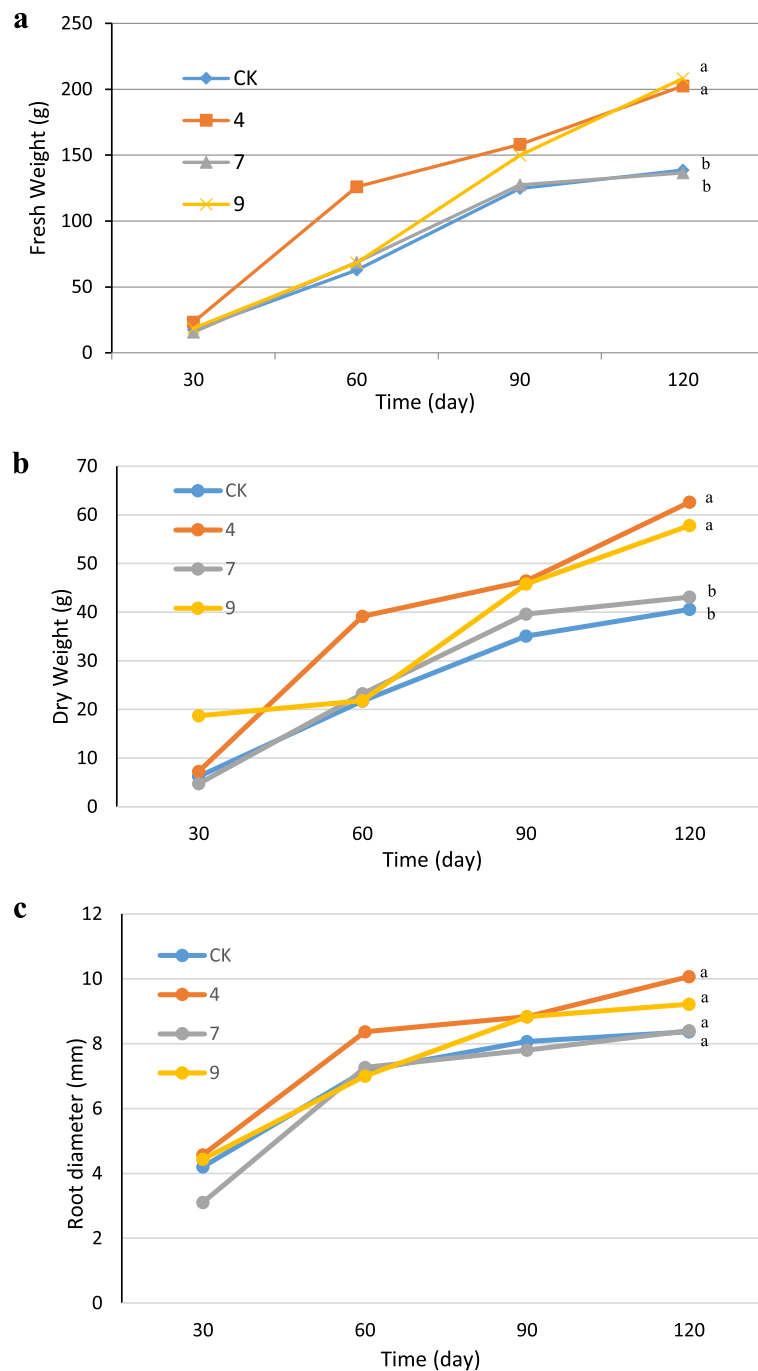


Fig. 1 The effect of different fertilizers on the growth of *Astragalus*. Note: **1a**, fresh weight of *Astragalus*; **1b**, dry weight of *Astragalus*; **1c**, root diameter of *Astragalus*. CK, without microbial fertilizer; 4, strain t16 + powder; 7, strain 21 + powder; 9, t16 + t21 + powder. Different lower case letters refer to significant differences between groups based on one-way ANOVA ($p < 0.05$)

Chloroflexi increased during plant growth, from the relative abundance of 3.16% before sowing (CK0) to 9.77% at the stable growth period (CK2). The abundance of *Actinobacteria* decreased from 28.52% before sowing (CK0) to 9.82% by day 30 (CK1) and then increased to 21.53% at day 90 (CK2). The observed decrease in the

stable growth period of *Actinobacteria* might be due to interspecific competition with *Proteobacteria* and *Chloroflexi*, which experienced a significant increase in the same period. The inhibitory effect on *Actinobacteria* is more obvious in the treatment groups, especially in group 7.

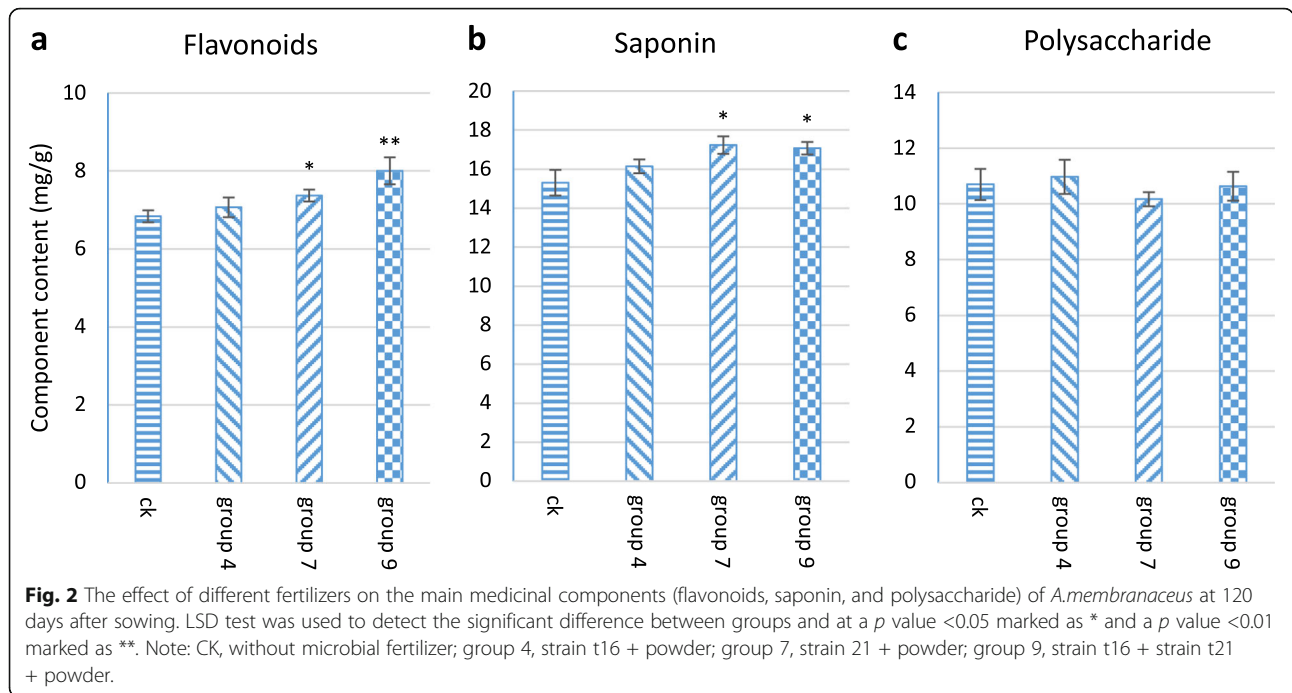
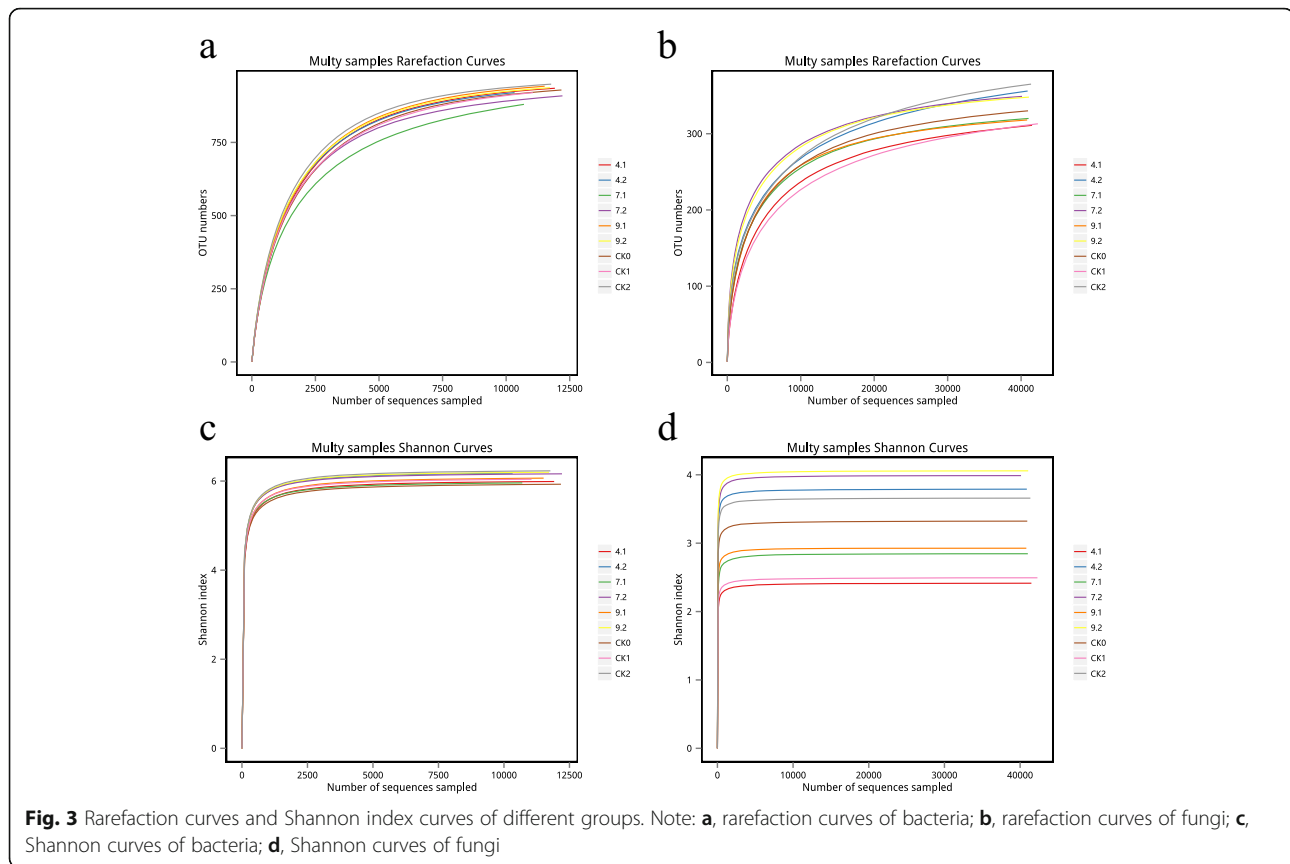


Table 1 Sample information, microbial diversity and sequence abundance

Sample	Treatment	Time (days after sowing)	Number of reads		Number of OTUs		Shannon index		Simpson index		Chao 1 index	
			Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
CK0	No fertilization	0	12174	40944	929	330	5.93	3.32	0.009	0.093	953.23	348.09
CK1	No fertilization	30	11052	42286	920	313	6.04	2.49	0.005	0.234	965.53	350.6
4.1	<i>Rhizobium</i> sp. t16 + <i>A. membranaceus</i> powder	30	11916	41490	935	311	5.99	2.41	0.007	0.266	955.39	336.11
7.1	<i>Sinorhizobium</i> sp. t21+ <i>A. membranaceus</i> powder	30	10700	41020	880	320	5.96	2.85	0.005	0.224	963.18	346.11
9.1	<i>Rhizobium</i> sp. t16 + <i>Sinorhizobium</i> sp. t21+ <i>A. membranaceus</i> powder	30	11514	40882	942	318	6.07	2.93	0.006	0.162	967.88	333.75
means±SD			11376±505	41130±260	919±27	316±3.8						
CK2	No fertilization	90	11771	41357	949*	365*	6.23	3.66	0.003	0.067	972.06	393.98
4.2	<i>Rhizobium</i> sp. t16 + <i>A. membranaceus</i> powder	90	10332	40888	920	356	6.18	3.79	0.004	0.054	953.36	388.69
7.2	<i>Sinorhizobium</i> sp. t21+ <i>A. membranaceus</i> powder	90	12214	40121	909	349	6.16	3.99	0.004	0.05	951.58	362.09
9.2	<i>Rhizobium</i> sp. t16 + <i>Sinorhizobium</i> sp. t21+ <i>A. membranaceus</i> powder	90	11723	41067	937	348	6.2	4.06	0.004	0.032	958	361.53
means±SD			11423±797	40692±410	922±11#	351±3.6						

*Compared with CK1, *p*<0.05

#Compared with CK2, *p*<0.05



At the genus level, uncultured bacteria were the dominant genera in all groups, ranged from 20.46 to 26.79% (Fig. 4b). During growth, the abundance of *Arthrobacter* decreased from 9.31% before sowing (CK0) to 2.33% at the stable growth period (CK2). In the treatment group 7, the abundance of *Arthrobacter* even dropped to 0.47% at the stable growth period (7.2). In contrast, the abundance of *Nitrospira* has a tendency to expand during the growth of *A. membranaceus*.

Both bacteria strain *Rhizobium* sp.t16 and *Sinorhizobium* sp.t21 were approximately estimated in the sequencing results. However, the reads which are identical with the two strains were low in all of the day 30 and day 90 samples, ranging from 102 reads/10700 reads to 508 reads/11723 reads in treatment groups and 27 reads/12174 reads to 94 reads/12174 reads for control group. But, the reads number of the two strains were still more in treatment groups than in control group.

Compared with bacteria, the fungal microbiota in *A. membranaceus* growth was less diverse. Only five fungal phyla were detected in experimental groups, compared with three fungal phyla in control group. *Ascomycota* and *Zygomycota* were the dominant phyla which widely existed in all groups, accounting for 52.99% and 41.17% of the average relative abundance of fungi at the phylum level (Fig. 5a). *Ascomycota* and *Zygomycota* contributed

34.62% and 62.83% in the rapid growth period, and 71.36% and 19.51% in the later stage, respectively.

At the genus level, *Mortierella* dominated the early period in experimental groups, accounting from 32.29 to 77.09%, and then decreased sharply at the stable growth period of plant (Fig. 5b). The abundance of *Gibberella* in experimental group 4.2 and 9.2 is significant less than in control group CK2. *Paraphoma* decreased sharply from 21.38% in group CK0 to an average of 1.63% in other groups with the growth of plant, which might be the result of inhibition of environmental fungi by root exudates (Badri and Vivanco 2009). *Chaetomium* was the second most abundant genus in the growth period, during which abundance increased from 3.75% on day 30 to 14.68% on day 90. The abundance of *Preussia* in experimental groups 4.2, 7.2, and 9.2 are all higher than that in the control group CK2 (1.36%), especially in group 4.2 (13.11%).

Effects of organic microbial fertilizer on the regulation of bacterial and fungal community structure

To understand the bacterial community structure, a heatmap was generated to reveal differences in genera between experimental and control groups (Fig. 6). At the rapid growth period of *A. membranaceus*, the genera structure in treatment group 7.1 and control group CK1

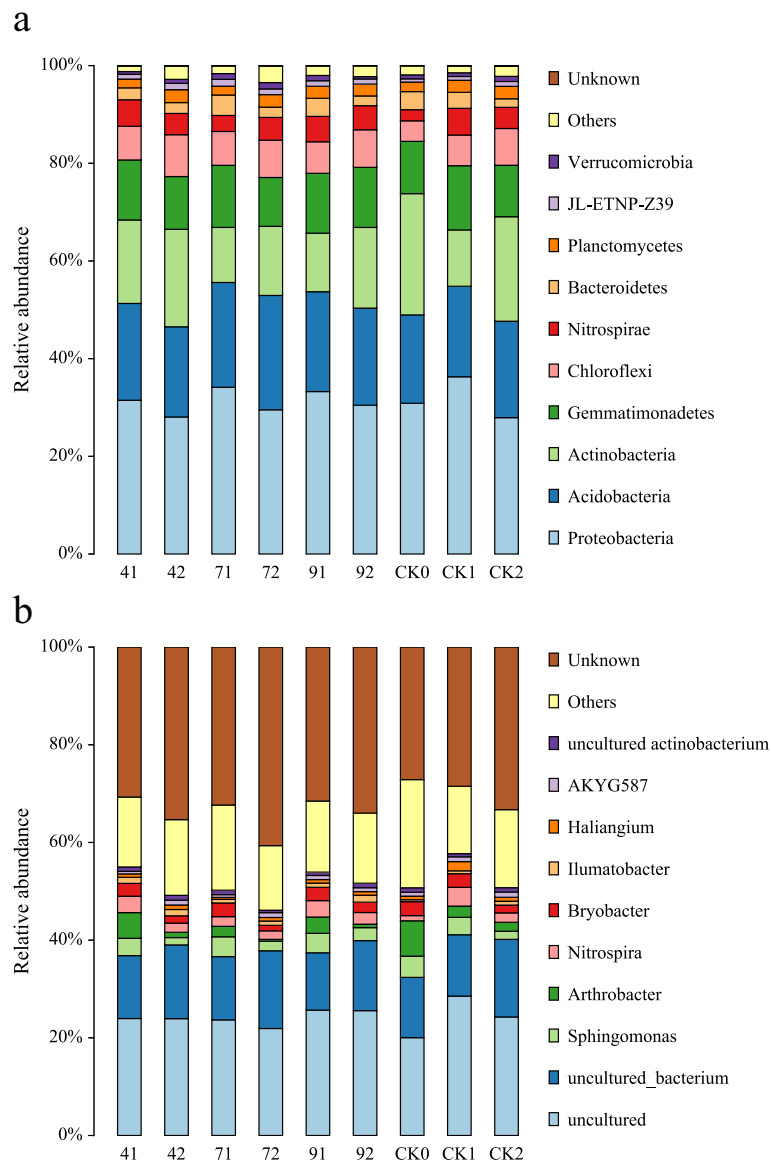


Fig. 4 Relative abundance of bacteria at phyla (a) and genus (b) levels of different groups. X-axis shows the samples collected before sowing (CK0), 30 days after sowing (CK1, 4.1, 7.1, 9.1), and 90 days after sowing (CK2, 4.2, 7.2, 9.2)

has the most significant difference, while in the stable growth period it is between the group 9.2 and the control group CK2. The dominant strains are different in each group based on the plant growth periods, and there are also differences in each treatment groups. Meanwhile, PCoA and cluster analysis of the bacterial microbiota were performed to evaluate the similarities between bacterial communities in all groups. Both weighted (PC1 variance = 37.12%, PC2 variance = 18.07%; Fig. 7a) and unweighted (PC1 variance = 50.89%, PC2 variance = 17.37%; Fig. 7b) PCoA was performed. The PCoA result based on weighted UniFrac values revealed an overlap of bacterial communities between rapid and

stable growth periods of *A. membranaceus*, but cluster analysis indicated a clear difference between the two periods based on the weighted UniFrac metric (Fig. 8). The result indicated that the bacterial communities in treatment group 7.1 and control group CK1 had the most significant difference in rapid growth period of *A. membranaceus*. In the stable growth period, it was treatment group 9.2 and control group CK2 which had the significant difference in bacterial communities.

Multivariate analysis was performed to estimate the similarities of fungal microbiota communities during the different growth period (PC1 variance = 67.56%, PC2 variance = 10.97%; Fig. 9a). PCoA result of fungal

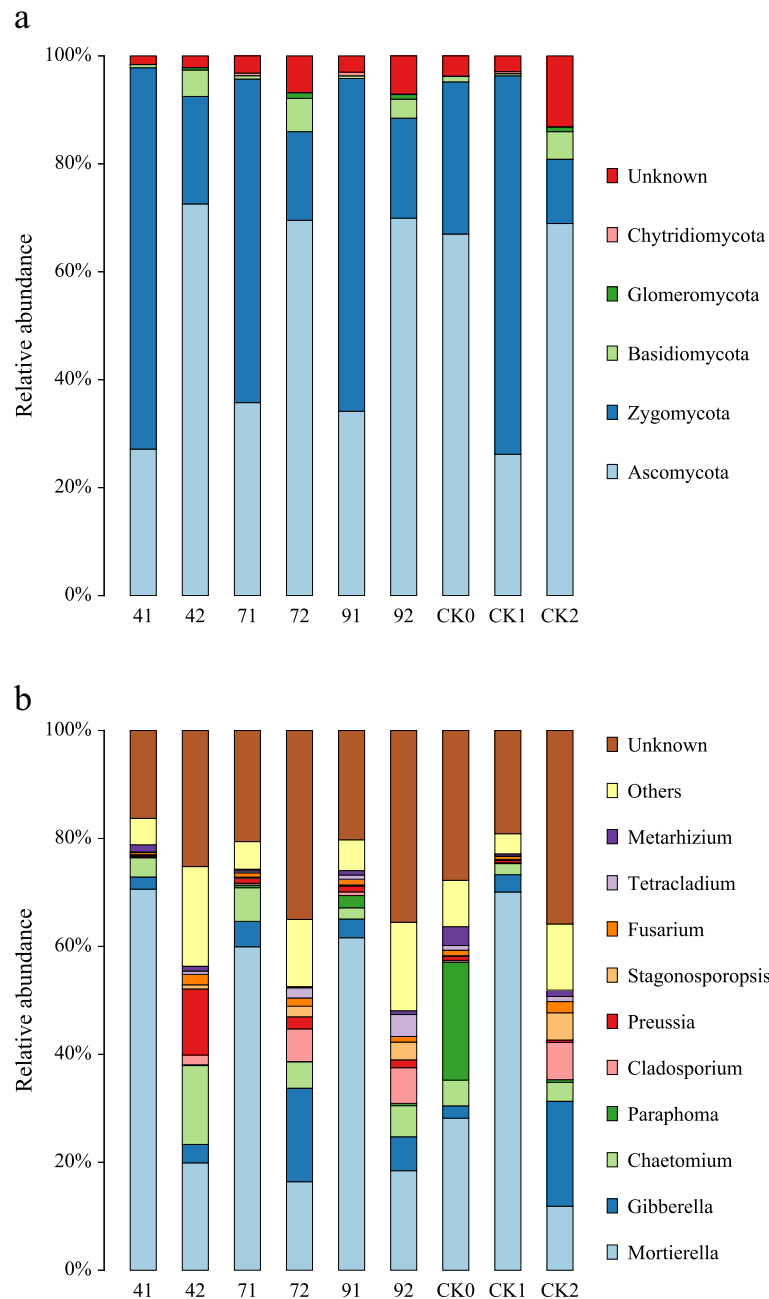


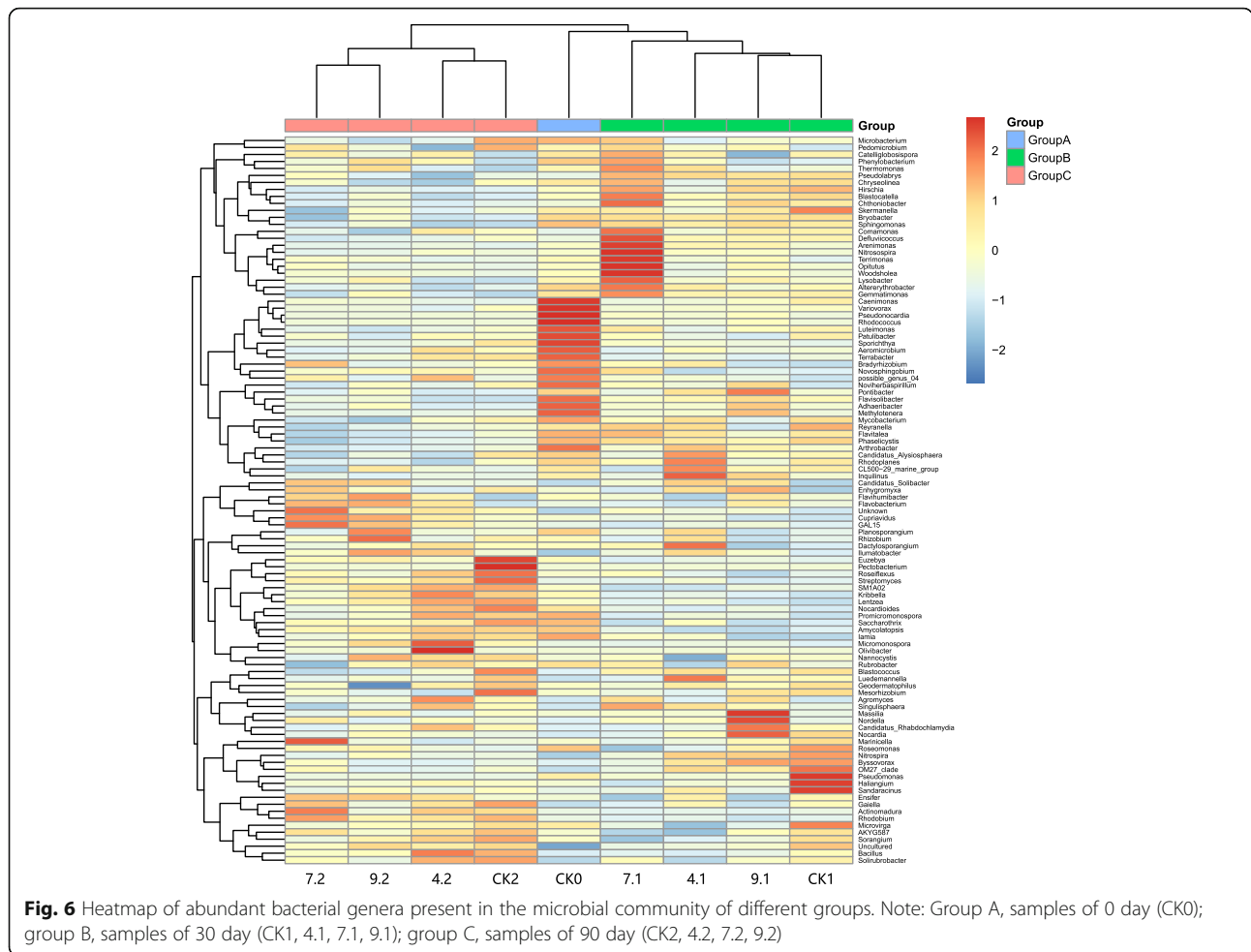
Fig. 5 Relative abundance of fungi at phyla (a) and genus (b) levels in different groups. X axis shows the samples collected before sowing (CK0), 30 days after sowing (CK1, 4.1, 7.1, 9.1), and 90 days after sowing (CK2, 4.2, 7.2, 9.2)

microbiota communities based on unweighted UniFrac index indicated that samples collected at the same time point were gathered together rather than between different treatment groups (Fig. 9b).

Metagenome functional content prediction of bacterial community

In order to compare the potential functions of the bacterial community between different treatments, functional

content was predicted from amplicon data using PICRUSt and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs. When assessed with LEfSE at LDA > 2 and *p* value < 0.05, the microbiome of treatment groups were significantly enriched in eight functional categories compared to the control group (Table 2). These enriched functional categories were related to amino acid metabolism (e.g., alanine, aspartate and glutamate metabolism), biosynthesis of other secondary metabolites (e.g., tropane,



piperidine, and pyridine alkaloid biosynthesis), metabolism of cofactors and vitamins (e.g., ubiquinone and other terpenoid-quinone biosynthesis), lipid metabolism (e.g., fatty acid biosynthesis), translation (e.g., translation factors), and carbohydrate metabolism (e.g., pentose phosphate pathway).

Discussion

Effects on the plant growth and accumulation of active ingredients

In the present study, we first developed the special manure of bacterial fertilizer and utilized it for cultivation of *A. membranaceus* in the field. The results suggest that the fertilizer can promote plant growth in the field which is consistent with numerous studies of nitrogen-fixing bacteria (Chi et al. 2010; Gage 2004; Gopalakrishnan et al. 2015; Rincon-Rosales et al. 2009; Zhiquan et al. 2016) as well as recent results from our laboratory using another type of bacterial fertilizer (Liang et al. 2019).

The main effective components of the *A. membranaceus* roots, including flavonoids, saponin, and

polysaccharide were measured to evaluate the effect of bacterial manure on the accumulation of active ingredients in host. The result revealed that rhizobial bacteria of the fertilizer (strain *Rhizobium* sp. t16 and *Sinorhizobium* sp. t21) mainly interact with the plant root to increase the biosynthesis of secondary metabolites and the simultaneous inoculation of these two rhizobia will make this effect more obvious which indicates a special interaction between the two nitrogen bacteria. Previous studies have shown that inoculation with plant growth-promoting rhizobium could increase secondary metabolite content in marigold and *Mentha piperita* (Cappellari et al. 2017; Cappellari et al. 2013; Santoro et al. 2016), and our experiments have obtained consistent results. The molecular mechanism of the two nitrogen fixing bacteria in promoting the accumulation of secondary metabolites in host still needs further study.

Effects on the microbial abundance and community structure

Furthermore, the rhizosphere microbial abundance and community diversity during the plant growth were

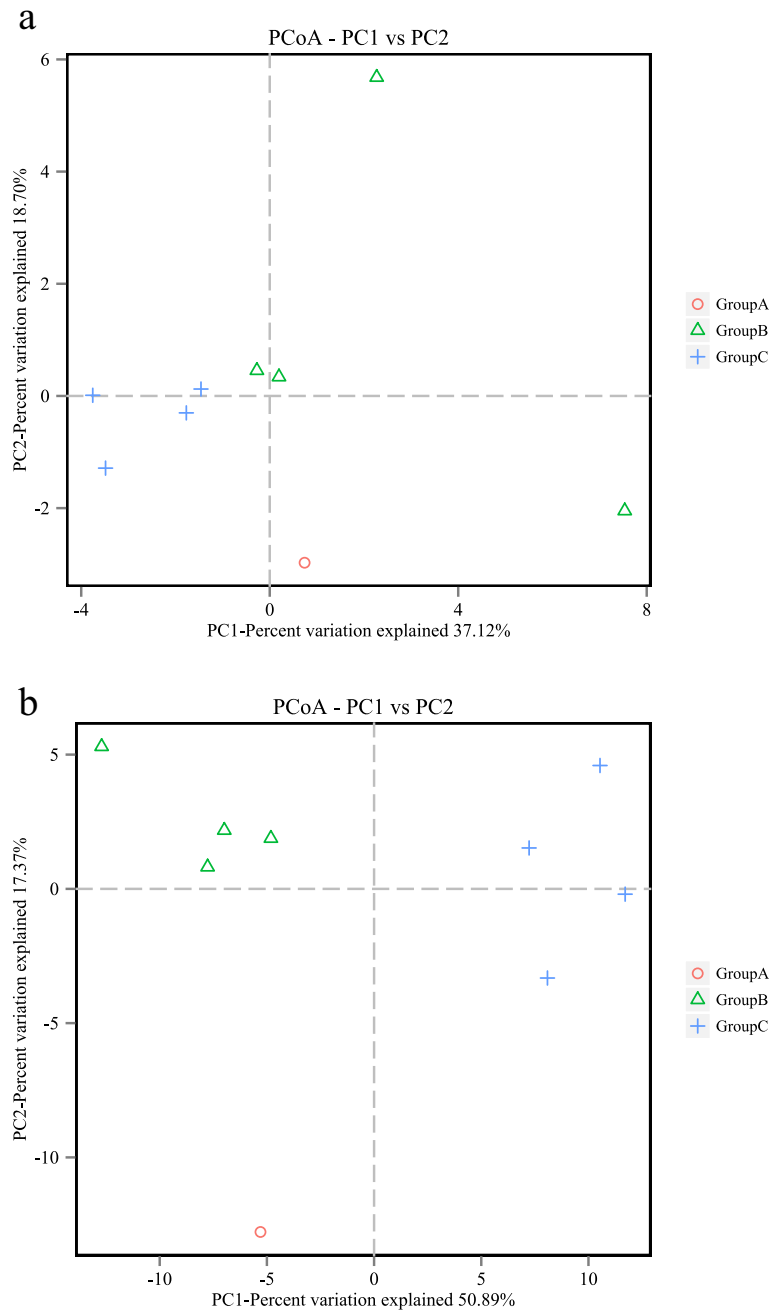
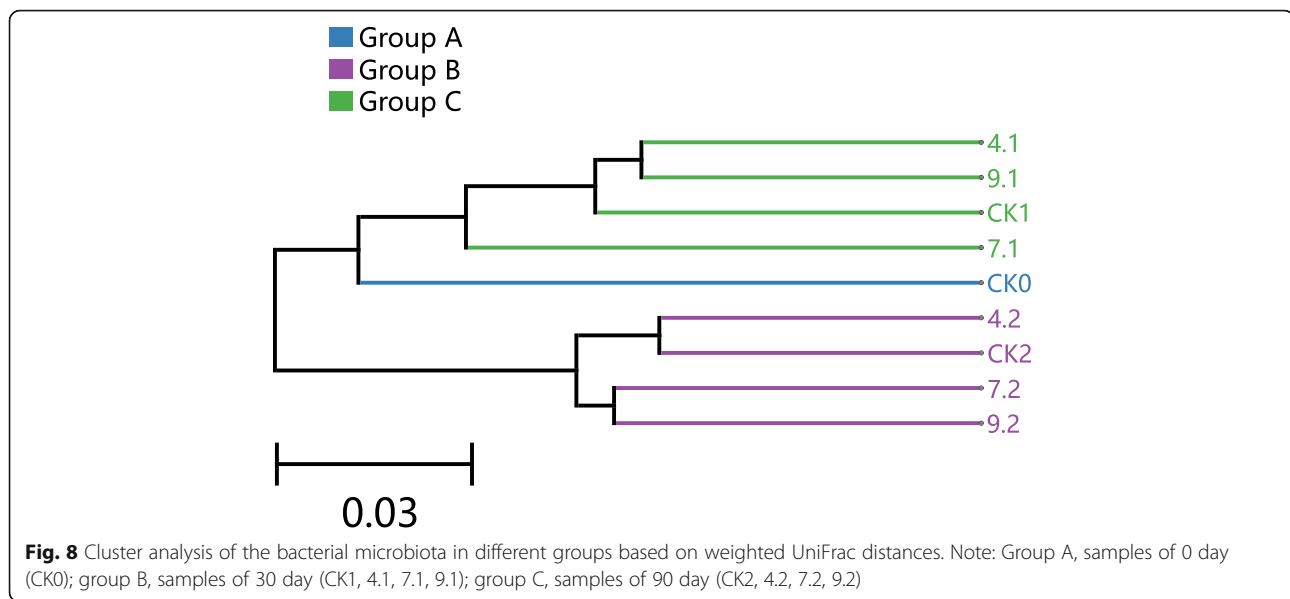


Fig. 7 Principal coordinate analysis of bacterial communities based on (a) weighted and (b) unweighted UniFrac metrics of samples in different groups. Note: Group A, samples of 0 day (CK0); group B, samples of 30 day (CK1, 4.1, 7.1, 9.1); group C, samples of 90 day (CK2, 4.2, 7.2, 9.2)

measured by Illumina MiSeq sequencing data. Diversity indices demonstrated an increase in bacterial community diversity with organic microbial fertilizer application, consistent with a previous report (Tabassum et al. 2017). However, this increase was not significant in this experiment. With the growth of plants, the abundance of indigenous bacteria in soil was inhibited, and some new strains have gained the dominant position. The dominant strains of the treatment group were also

different compared with the control group. These results suggest that bacteria were suppressed during germination then promoted when seedlings unearthed, presumably due to root development, soil porosity, and sufficient oxygenation. Temperature, humidity, and biological interactions between microorganisms or between microbial and plant could inhibit microorganisms which have less tolerance to the environmental challenges, unlike adaptable microbes that thrive



during *A. membranaceus* growth process (Tabassum et al. 2017). The result indicated that interspecific competition could contribute to the decline of the microbial community diversity, allowing adaptation of the microorganisms to changing environment for survive and eventually thrive (O’Sullivan et al. 2015). In the present study, the community diversity of fungi remained stable during both experimental and control groups, indicated that the fungal species could adapt to both environments and tolerate the transition between different periods.

In addition, the results of the relative abundance of rhizosphere bacteria throughout the entire *A. membranaceus* growth process shows three predominant phyla, *Proteobacteria*, *Actinobacteria*, and *Acidobacteria*, which is consistent with previous reports (Liu et al. 2015; Marsh et al. 2014). At genus level, the results showed that *Pseudomonas*, *Lactobacillus*, *Corynebacterium*, *Bacillus*, and *Acinetobacter* were the dominant bacteria during the host growth. *Acinetobacter* and *Pseudomonas* genera are able to degrade biosurfactants and hydrocarbons (Hamme et al. 2003). *Bacillus* species are confirmed an important role during the stable growth period of plant by secreting microbial inhibitors and plasminogen. *Bacillus subtilis* can produce antibacterial and bacteriolytic substances, such as subtilisin, organic acids, which can inhibit the growth and reproduction of pathogenic bacteria (Wang et al. 2019). *Bacillus subtilis* can also produce proteases, lipases, amylases, cellulases that promote the biochemical process during the *A. membranaceus* growth, which contribute to the accumulation of secondary metabolites, such as amino acids, organic acids, and aglycones (Yuan et al. 2019). Thus, these bacteria may play an important role in inhibiting

the growth of pathogenic bacteria and promoting plant growth and accumulation of secondary metabolites through the secreted metabolites of these organisms during the stable growth period of *A. membranaceus*.

The abundance of the bacteria strain *Rhizobium* sp.t16 and *Sinorhizobium* sp.t21 were detected. Interestingly, their abundance in rhizosphere soil did not increase significantly after application of bacterial fertilizer. The results revealed that both rhizobial bacteria of the fertilizer mainly interact with the plant root to form special nodules to function in nitrogen fixation; thus, the distribution in the soil is relatively low and does not change significantly following application of fertilizer.

In this study, we also observed time point effects on the differentiation of bacterial community structure along with the plant growth, suggesting an interaction between plant development and the dynamics of rhizosphere bacteria community. The bacterial communities were relatively stable during the rapid growth period, but fluctuated considerably during the stable growth. Combining this with the physiological indices of *A. membranaceus* seedlings, we hypothesized that the nutrients consumed by the seedlings during the rapid growth period were mainly stored in seeds, and only a small part was applied to the soil, so there was no significant difference in the physiological characteristics of the seedlings of each group. Contrarily, during the stable growth period, the nutrient requirement rapidly increased. On one hand, special fertilizer could provide nutrients in time because it contains nutrients suitable for the growth of *A. membranaceus* (Liang et al. 2019). On the other hand, bacteria interact with root tissues by forming nodules on lateral roots, playing a role in nitrogen fixation and providing nutrients for plants.

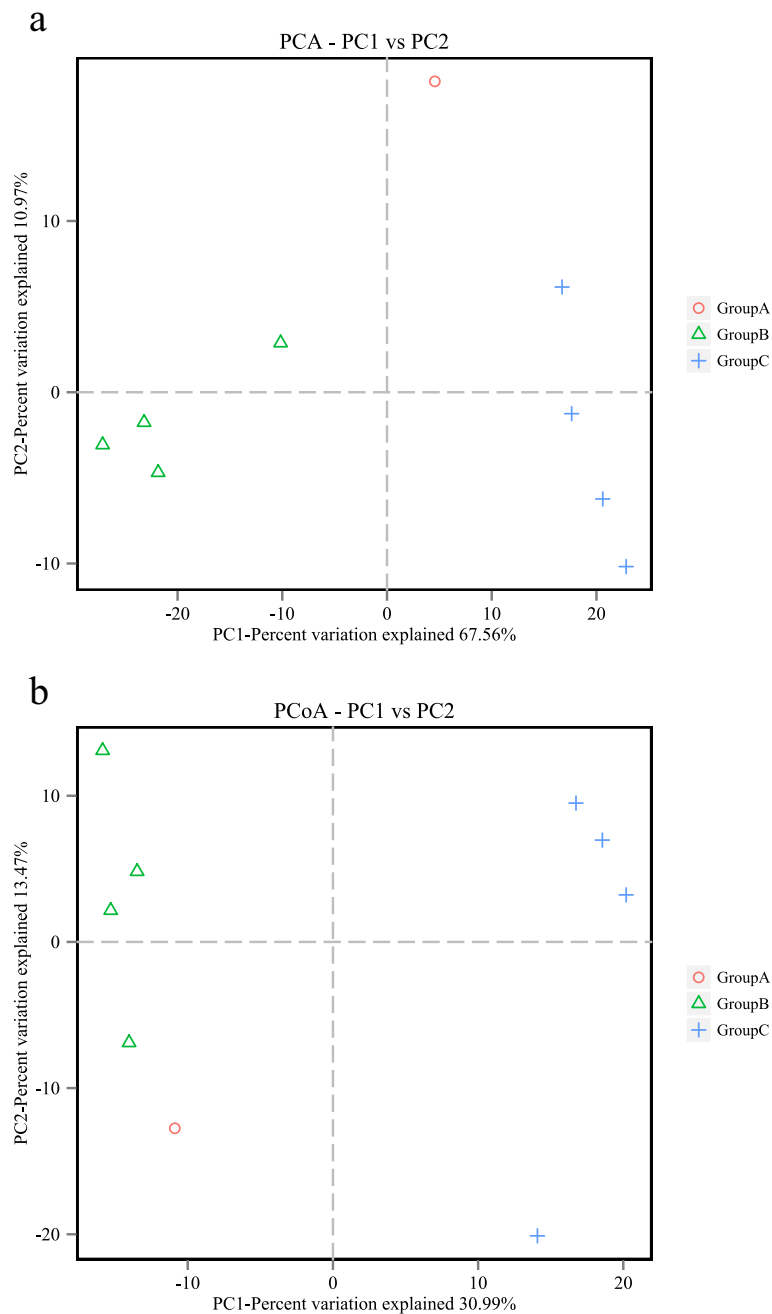


Fig. 9 Principal coordinate analysis of fungal communities based on (a) weighted and (b) unweighted UniFrac metrics of samples in different groups. Note: The values on the coordinate axis indicate the difference of the component in different groups. Group A, samples of 0 day (CK0); group B, samples of 30 day (CK1, 4.1, 7.1, 9.1); group C, samples of 90 day (CK2, 4.2, 7.2, 9.2)

Meanwhile, plants secrete organic components through the root tips to regulate the microbial community structure of soil's rhizospheres, and play a role in promoting growth (Badri and Vivanco 2009).

The functional content prediction of rhizosphere bacterial community was also conformed in our study. The functional approaches showed that significant shifts of metabolic functions impacting KEGG

pathways in the organic microbial fertilizer treatment groups are related to metabolism and biosynthesis. These results provide a new perspective for further research on the relationship between microbiome and plants. More importantly, these functions of the bacterial community may play an important role in promoting the main medicinal components accumulation of *A. membranaceus*.

Table 2 Functional profile of the microbial community in different groups

KO functional categories		Group 4 vs. CK		Group 7 vs. CK		Group 9 vs. CK	
Level 2	Level 3	LDA	P value	LDA	P value	LDA	P value
Amino acid metabolism	Alanine _ aspartate and glutamate metabolism	2.23	0.01	—	—	2.49	0.03
Biosynthesis of other secondary metabolites	Tropane, piperidine, and pyridine alkaloid biosynthesis	2.58	0.02	—	—	2.19	0.01
Metabolism of cofactors and vitamins	Ubiquinone and other terpenoid-quinone biosynthesis	2.12	0.01	—	—	—	—
Lipid metabolism	Fatty acid biosynthesis	—	—	2.15	0.04	—	—
Carbohydrate metabolism	Pentose phosphate pathway	2.14	0.03	—	—	2.21	0.02
Amino acid metabolism	Amino acid related enzymes	—	—	2.87	0.04	2.89	0.04
Translation	Translation factors	—	—	2.16	0.03	2.42	0.02
Metabolism of cofactors and vitamins	Pantothenate and CoA biosynthesis	—	—	—	—	2.01	0.01

Note: KEGG pathways were inferred from the 16S rRNA gene sequence using PICRUSt. Significantly enriched functional categories in different treatment groups were accessed using LEfSE (LDA > 2; P value < 0.05). Group 4, strain t16 bacterial solution; group 7, strain t21 bacterial solution; group 9, strain t16 + t21 bacterial solution

In addition to the bacterial community, the present study was also aimed to assess the fungal community structure. Shannon index results indicated that regulation of fungal community structure by the special fertilizer is also related to the growth stage of the plant. Microbial diversity of fungi in rhizosphere soil was inhibited during germination stage. During stable growth, there was a gradual development of the root system, with secretion of diverse compounds such as malic acid, sugars, amino acids, vitamins, various secondary metabolites, proteins, and mucilage to the soil to recruit beneficial microorganisms, which resulted in increased diversity of fungi in the soil's rhizospheres.

Therefore, our study suggested that the special fertilizer developed in this research can significantly promote *A. membranaceus* growth and medical active components accumulation by regulating the community structure of rhizospheres in soil in different growth stages. This special fertilizer shows potential value in application for many crops, especially traditional Chinese medicinal herbs, due to its low cost and easy use. Moreover, our study implies a potential effect of the special fertilizer in restoring and rehabilitating degraded cropland soils by altering rhizosphere microbial communities.

Conclusions

In this study, a special bacterial fertilizer composed of stems and leaves powder of *A. membranaceus* and two native strains of rhizobia was applied in the planting of *A. membranaceus*, and the bacterial and fungal diversity was studied using high-throughput sequencing. The special bacterial fertilizer promoted plant growth and the main medicinal components accumulation by providing appropriate nutrients, promoting nitrogen-fixing activity, and regulating the rhizosphere microbial community. The special manure developed in this study has a lot of

potential value for fertilizing crops as well as rehabilitating degraded cropland soils by altering the rhizosphere microbial communities.

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Authors' contributions

JL and ZS designed the study and wrote the manuscript. ZX, ZY and JN performed the experiments. ZX and ZS analyzed the data. ZC revised the manuscript. The authors read and approved of the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This article does not contain any studies with human participants or laboratory animals.

Consent for publication

Not applicable

Competing interests

The authors declare that there are no conflicts of interests.

Author details

¹College of Life Sciences, Shanxi Agricultural University, Taigu 030801, China.

²Shanxi University of Traditional Chinese Medicine, Jinzhong 030619, China.

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