Effects of Mixed Humus Soil and Straw Ash Substrate on Rhizosphere Bacterial Community and Growth of Hot Pepper

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Abstract [**Objectives**] To explore the effects of mixed humus soil and straw ash substrate on rhizosphere bacterial community and growth of hot pepper. [**Methods**] In this pot experiment, high-throughput sequencing was conducted to analyze bacterial communities in the rhizosphere soil of pepper plants treated with four different HA proportions. [**Results**] Pepper seedlings exhibited optimal growth in the 6∶4 (*w/w*) HA substrate. Bacterial structure and composition varied with the HA proportion. The relative abundance of the *Proteobacteria phylum* (ranging from 48.37% to 60.40%) was the highest across all treatments. Correlation analysis indicated that certain bacterial communities were closely related to the availability of soil nutrients and enzymatic activities. [**Conclusions**] This study elucidates the impact of HA proportion on rhizosphere bacterial communities and plant growth, laying a foundation for understanding the application of different mixed substrates and their effects on soil microbiology.

Key words Humus soil, Straw ash, Plantation, Hot pepper, Rhizosphere

0 Introduction

Hot pepper, as an annual vegetable crop of the *Capsicum* L. genus, has rich capsaicin and vitamins and possesses high nutritional value[1]. The planting area of hot pepper accounts for about 10% of the total vegetable planting area in China, ranking first in all kinds of vegetables[2]. Guizhou Province had the largest area of planting hot pepper in recent years, making hot pepper planting a major crop of Guizhou Province[3]. In recent years, pepper planting areas increase continuously due to the rapid development of pepper industry. However, the factors of affecting plant growth and pepper yield are extremely complex, including weather (*e.g.*, temperature, moisture, *etc.*), soil (including soil type, nutrient status and plantation substrates), fertilizer, rhizosphere microorganisms, and diseases, becoming the main obstacle to the sustainable, healthy, and efficient development of the pepper industry[4-6].

Among the effecting factors, fertilizer is a main factor influencing the hot peppers growth. Most of the previous studies on hot pepper effect of fertilization have focused on the nutritive element (such as nitrogen, phosphorous and potassium, *etc.*), organic matters, and pH and so on. Furthermore, fertilizer type is also a factor of attention[7]. With continuously understanding the function of the rhizosphere microbes in plant growth and development, the study that effect of fertilizer or plantation substrate type on the rhizosphere microbes is focused on. It has been reported that mixed fertilizer, which was composed of fermented yeast waste and silk worm excrement, improved the microbial diversity in rhizosphere, the plant growth and fruit quality of pepper[8]. The mixture of 75% organic and 25% chemical fertilizer improved the microbial diversity and increased the biomass of oilseed rape, changed bacterial community composition in the rhizosphere soil[9]. Most of soil bacteria implied in bio-control of phytopathogen or other application are potential beneficial. However, how different substrate proportions influence the rhizosphere bacterial community and growth of hot pepper is poorly understood.

Considering that different substrate proportions affect microbial traits and pepper growth, we aimed to explain the changes in rhizosphere bacterial communities of planting pepper's soils in mixed humus soil and straw ash substrate. We hypothesized that different substrate proportions (mixed humus soil and straw ash substrate, hereafter referred to as HA) would markedly influence microbial diversity, and change microbial community structures. This study is performed to compare the responses of bacterial diversities and community composition to HA of planting pepper; and determine the relationships between rhizosphere microbial communities and soil properties. We hoped to find the optimal HA proportion that is conducive to pepper growth and fitness. More importantly, this study provides insights into finding optimal proportion of other substrates promoting plant growth and fitness.

1 Materials and methods

1.1 Experimental material preparation Humus soil and straw ash were applied for fertilization experiments. The humus soil was bought from Liyintang Professional Planting Cooperative (Tengchong City, Yunnan Province, China). The straw ash was produced from rice straw which was collected from the local rice field. The humus soil and straw ash were mixed with four proportions of 10∶0, 6∶4, 4∶6, and 2∶8 (*w/w* and dry weight basis) to gain the mixed pot experimental soil (Table 1). Pepper seeds from natural plants were gained from the residents of Liping

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County in Guizhou Province, China. To eliminate a variety of pathogen on the surface, the pepper seeds were soaked in 55 ℃ warm-water for 20 min, during which continuous stirring was required, then the seeds were taken out and soaked in clean water for 10 h to ensure that they fully absorb water and expand. After drying the surface water, the seeds were wrapped in a wet cloth, and placed in the condition at 28 ℃ for germination three to five days, during which the seeds should be checked daily and stirred appropriately to ensure were heated evenly to gain higher germination rate, when about 70% of the seeds begin to germinate, they could be sown in two pots with nutrient soil, in a greenhouse with natural light. When the pepper seedlings grew up to 10 cm, they were prepared for the greenhouse pot experiment.

1.2 Greenhouse pot experiment On July 11 in 2024, the greenhouse pot experiment was carried out a vegetable field, located at Xiashui Village, Wudang District in Guiyang City, Guizhou Province of China (26°71′ N, 106°73′ E, altitude 1 200 m). The pepper seedlings with approximately 10 cm height and uniform growth were transplanted into the pots (10 cm × 10 cm) with 2 kg mixed humus soil and straw ash (HA), four proportion treatments were conducted for pepper pot plantation, namely CK (2 kg humic soil without straw ash), HAS (mixed 1.2 kg humus soil and 0.8 straw ash), HAF (mixed 0.8 kg humus soil and 1.2 straw ash),and HAT (mixed 0.4 kg humus soil and 1.6 kg straw ash) (Table 1), a pot with a plant, a treatment with 12 seedlings, which were subjected to natural light and appropriately watered.

Table 1 Pot experiment of hot pepper treated by HA

Treatments	Total dry weight//kg	Humic soil//kg	Straw ash//kg	Proportion
CK	2	2.0	0	10∶0
HAS	2	1.2	0.8	6∶4
HAF	2	0.8	1.2	4∶6
HAT	2	0.4	1.6	2∶8

1.3 Collection of samples On August 22 in 2024, when the pepper grew for 40 d, the plants with roots and soil were removed from pots, after the loose soil was shaken off, the soil adhering to the seedling roots was defined as rhizosphere soil and collected into a sterile bag. Rhizosphere soil and plants of three peppers with uniform growth were considered as a replicate in same treatment when we sampled, three replicates a proportion treatment, a total of 12 soil and plant samples were put into sterile bags, respectively, which were placed in a foam box with ice and was promptly transported to laboratory for analysis. The soil of a sample was divided into two parts, one part was stored in −80 ℃ refrigerator for high-through sequencing, and the other was prepared for soil trait analysis.

1.4 Determination of soil physiochemical characteristics and enzyme activities Naturally air-dried soil from pepper rhizosphere was used for analysis of soil traits and enzyme activities. In this pot experiment, rhizosphere physiochemical characteristics of pepper plants included organic matter (OM), available nitrogen (AN), available potassium (AK), available phosphorus (AP) and pH, these soil traits except for pH were determined using soil test kits produced by Shandong Laiende Intelligent Technology Co., Ltd. (Shandong Province, China) on the basis of manufacturer's protocols. After the soil samples were processed, OM, AN, AP, and AK levels were tested in a nutrient speedometer (Shandong Laiende Intelligent Technology Co., Ltd., China), respectively.

The soil enzymatic activities in the hot pepper rhizosphere were analyzed using Guan's method[10]. The phenol-sodium hypochlorite photo-colorimetric method was applied to test the urease activity, which was presented as micrograms of ammonium nitrogen (mg NH_4^+-N) in 1 g of rhizosphere soil after 24-h reaction (μg NH_4^+-N/g/24 h). The 3,5-dinitrosalicylic acid photo-colorimetric method was used to test the sucrase activity, which was presented as glucose milligrams in 1 g of rhizosphere soil after 24-h reaction (mg glucose/g/24 h). 4-nitrophenyl phosphate disodium salt hexahydrate colorimetry was applied to analyze the acid phosphatase activity, which was defined as milligrams of phenol within 1 g of rhizosphere soil after a 24-h reaction (mg phenol/g/24 h).

1.5 Estimation of pepper growth and health After the plant height, single fresh weight, and root length were measured, the healthy indexes in hot pepper leaves were determined on the basis of Gao's method[11], these indexes included the activities of peroxidase (POD) and superoxide dismutase (SOD), and contents of proline and malondialdehyde (MDA).

1.6 Rhizosphere bacterial DNA extraction and high-through sequencing Rhizosphere bacterial DNA extraction and analysis were conducted by Sangon Biotechnology Co. Ltd, Shanghai of China. Firstly, 12 collected rhizosphere soil samples were subjected to remove the fine root and plant residues on the ultra-clean table, and then were placed into sterile bags, respectively. Finally, the total of 12 soil samples were put into a foam box with dry ice and transported to Sangon Biotech in Shanghai. According to the manufacture's protocols, A E.Z.N. ATM Mag-Bind Soil DNA Kit (OMEGA, M5635-02, USA) was applied to extract the rhizosphere DNA from each pepper soil sample. A Qubit 4.0 (Thermo, USA) was used to test the concentration of the extracted DNA in order to gain adequate amounts of high-quality DNA. Two universal 16S rRNA amplicon primers (341 forward 5′-CCTACGGGNGGCWGCAG-3′ and 805 reverse 5′-GACTACHVGGGTATCTAATCC-3′) were applied[12], and the V3-V4 region of pepper rhizosphere bacterial 16S rRNA was amplified using 2 × Hieff® Robust PCR Master Mix (YEASEN, 10105ES03, China) in the first amplification. Illumina Bridge PCR compatible primers were used to amplify DNA sequences in the second amplification. The PCR amplified products were checked via electrophoresis in 2% agarose gels, and then the concentration of gene libraries was quantified using Qubit 4.0 fluorimeter. After All DNA amplicons were pooled in equal proportion, sequencing was carried out on the Illumina MiSeq system (Illumina MiSeq, USA).

1.7 Bioinformatics and statistics After sequencing, the raw sequencing data from Illumina HiSeq system mentioned above were

converted to raw reads by Basa Calling analysis. The Cutadapt software v1.18 was applied to remove the adapters and sequencing primers[13], then PEAR (v0.9.8) was applied to assembly of paired-end reads. The bacterial (16S) reads of hot pepper rhizosphere were subjected to quality-control though removing the below 20 bp low quality sequences, splicing, de-noise, and de-chimerism procedures[14-15] using software Usearch (v11.0667) at the 97% similarity level[16-17]. In the end, 1 346 167 clean reads were obtained, with the ranges of the clean sequence varying from 350 to 465 bp. A total of 1 065 118 OTUs from 12 pepper rhizosphere samples were gained by clustering analysis. The RDP classifier (v138) was applied to align the OUT taxonomic classification against SILVA 16S database (http://www.arb-silva.de/). The taxonomic information of rhizosphere bacteria from hot pepper for each OUT was gained at different classification levels[18-19]. Bacterial functional communities from hot pepper rhizosphere based on the OTUs were analyzed by software Tax4Fun2 software v1.2.1, referred to genome database (Kyoto Encyclopedia of Genes and Genomes [KEGG]) of known metabolism function, the predictions of pepper rhizosphere bacterial metabolic functions were conducted[20], the abundance bar plot of bacterial functional communities at the metabolic pathway level 1 was also generated using Excel 2016.

To evaluate the difference of pepper rhizosphere bacteria in different treatments of HA proportions, the bacterial diversity, community composition, and function predictions were analyzed. Based on the OTUs, Mothur (v1.43.0) was used to calculate the bacterial α-diversity[21], in this experiment, the ACE (Abundance-based Coverage Estimator), Shannon and library coverage indices were applied to evaluate the bacterial community richness, diversity and sequencing depth in rhizosphere bacteria, and individually visualized using R ggplot2 package. Analysis of variance (one-way ANOVA) and least significant difference (*LSD*) test were applied to assess the difference of bacterial α-diversity in HA proportion treatments. The rarefaction data of Shannon index were analyzed using software Mothur (v1.43.0) and visualized by R (v3.6.0)[21]. Venn Diagram R package (v1.6.20) was used to draw Venn diagram of rhizosphere bacterial shared and unique OTU number in different HA proportion[22]. Bacterial community structural model was evaluated based on weighted Unifracdistance calculating all OTUs and ordinated by Non-metric Multidimensional Scaling (NMDS)[23]. According to the OTUs in different HA proportion treatments, the relative abundances of pepper rhizosphere bacterial communities at different classification levels were calculated, the abundance bar plot of bacterial community composition at phylum level was conducted using Excel 2016, the bacterial community with relative abundance greater than 1% were defined as dominant phylum, while those lower than 1% were defined others. At genus level, the bacterial community dot-rot heatmap of hot pepper rhizosphere in different HA proportion treatments was generated by R gplot package (v3.0.1.1) based on the bacterial community relative abundance, bacterial genera with abundances greater than 1% were presented as enriched species, those with abundances lower than 1% were presented as others which were not showed in the dot-rot heatmap. A relationship heatmap between rhizosphere bacterial communities at genus level and pepper indices was made using R gplots package[24]. Additionally, the averages of pepper soil property, seedling growth and healthy parameters were calculated using Excel 2016 for each HA proportion treatment, and difference and error analysis were conducted using software SPSS (v23.0, SPSS Inc., Chicago, IL, USA).

2 Results and analysis

2.1 Rhizosphere soil traits under mixed humus soil and straw ash (HA) treatment Pepper rhizosphere traits varied with different mixed HA proportions (Table 2). Compared with CK (without straw ash), HAS, HAF, and HAT treatments significantly increased ($P<0.05$) the pH, while no significant difference between HAS and HAF was observed. The OM and AN levels in the pepper rhizosphere declined with the decreasing proportion of mixed HA, the both values in HAT treatment significantly declined ($P<0.05$) by 50.49% and 59.46% contrast to CK treatment, respectively. Inversely, soil AP content enhanced with the decreasing proportion of mixed HA, it significantly increased ($P<0.05$) by 39.32% and 84.49% in HAF and HAT treatments compared with CK, while there was no significant difference between CK and HAS treatments. Soil AK levels in all treatments were in higher than AP, but they had the same trend in HAF and HAT treatments, the value in HAS was lower than CK, though no significant difference between CK and HAS treatments was detected. The enzymatic activities (sucrase, acid phosphatase, and urease) in the pepper rhizosphere generally declined with the decreasing proportion of mixed HA except acid phosphatase activity in HAS was lower than that in HAF, and they were significantly decreased ($P<0.05$) 4.51 mg glucose/g/24 h, 7.41 mg phenol/g/24 h, and 60.60 μg NH_4^+-N/g/24 h in HAT treatment contrast to CK, respectively.

2.2 Growth and health of hot pepper under mixed humus soil and straw ash treatments There were various responses in pepper growth and health parameters to the different proportions of mixed HA (Fig. 1). These findings suggested that appropriate mixed HA proportion improved the plant growth of hot pepper, which could be also showed by the pepper pictures after the seedlings treated for 40 days (Fig. 1A). With the decreasing proportions of mixed HA, pepper plant height and fresh weight were first increased and then declined, they were the highest (32.42 cm and 11.31 g/plant, respectively) in HAS treatment, followed by HAF treatment, the lowest was in HAT, but no significant difference ($P\geqslant0.05$) between CK and HAT was found (Fig. 1B, C). Compared with CK, the longest root length in HAS and HAT treatments significantly enhanced ($P<0.05$) 3.63 and 1.70 cm, though it also increased in HAF treatment, there was no significant difference ($P\geqslant0.05$) between CK and HAF (Fig. 1D).

The pepper health parameters were evaluated by antioxidant enzymatic activities of peroxidase (POD) and superoxide dismutase (SOD), and the contents of malondialdehyde (MDA) and proline in pepper leaf (Fig. 2). Compared with CK, the four health indexes in HAS, HAF, and HAT treatments significantly

declined to various degrees except for POD activity and MAD content in HAT, in which the decreasing trend in HAF was the most significant ($P<0.05$), followed by HAS (Fig. 2A-D). It suggested that application of appropriate HA proportion was beneficial to improve the fitness of hot pepper plant.

Table 2 Soil characteristics of pepper plants under different complex ratio of humic soil and plant ash ($\bar{x}\pm$SE)

Treatments	pH	OM //g/kg	AN//mg/kg	AP//mg/kg	AK//mg/kg	Sucrase mg glucose/g/24 h	Acid phosphatase mg phenol/g/24 h	Urease mg NH_4^+-N/g/24 h
CK	5.61 ±0.02 c	105.52 ±4.82 a	69.81 ±1.17 a	60.78 ±3.15 c	415.33 ±13.03 c	9.33 ±0.43 a	9.81 ±0.44 a	97.01 ±6.27 a
HAS	6.61 ±0.01 b	87.82 ±3.65 b	65.54 ±0.50 b	66.16 ±3.09 c	411.30 ±7.68 c	8.14 ±1.98 a	4.79 ±0.11 b	69.51 ±3.57 b
HAF	6.63 ±0.05 b	81.24 ±2.21 b	63.21 ±6.06 b	84.68 ±1.28 b	453.27 ±16.76 b	6.96 ±0.33 b	5.14 ±0.37 b	43.55 ±2.11 c
HAT	6.84 ±0.03 a	52.24 ±0.21 c	28.30 ±0.19 c	112.13 ±5.62 a	519.95 ±13.03 a	4.82 ±0.25 c	2.40 ±0.62 c	36.41 ±0.62 d

NOTE The data in this table are the means plus standard errors (SE) of three repetitions by one-way ANOVA. Organic material (OM), available nitrogen (AN), available phosphorus (AP), available potassium (AK). Least significant difference (LSD) was applied to test the difference among four complex ratios of humus soil and plant ash, namely the humus soil to straw ash ratios (w/w) were 10∶0 (CK), 6∶4 (HAS); 4∶6 (HAF), and 2∶8 (HAT). Means ± SE plus different lowercase in the same row show significant difference at $P<0.05$ under different complex ratio treatments of humus soil and straw ash (HA).

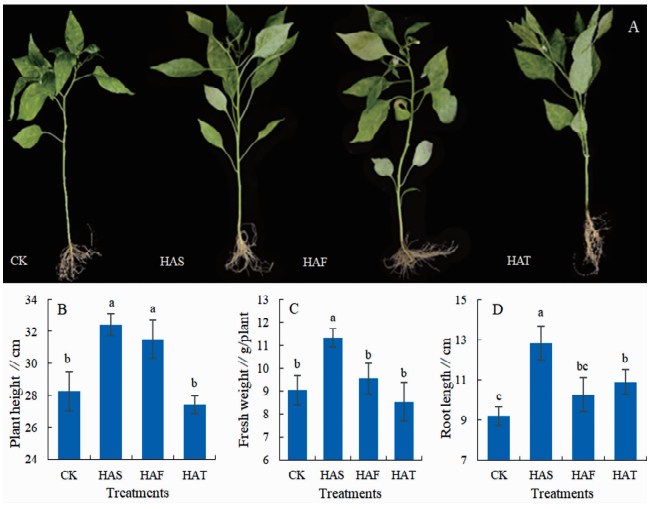
2.3 Sequencing results and bacterial diversity in rhizosphere Rhizosphere bacterial rarefaction curve of Shannon index approached a saturated plateau (Fig. 3A), suggesting that soil sequencing data from the pepper rhizosphere could represent the most of bacterial species in 12 soil samples. The average of library coverage indexes ranged from 0.988 to 0.993, and there were no significant differences ($P\geqslant0.05$) across the four treatments (Fig. 3E), further implying that sequence data in all samples was reliable and enough for the following analysis. Venn diagram showed the number of bacterial OTUs from pepper rhizosphere shared by the four HA treatments was 3 014, the trend of rhizosphere unique OTUs in all HA treatments: CK > HAS > HAF > HAT (Fig. 3B). In this study, the bacterial richness and diversity in hot pepper rhizosphere based on OTU numbers were presented. ACE and Shannon indexes were not significantly different ($P\geqslant0.05$) among four HA treatments except there was significant difference ($P<0.05$) in ACE index between HAS and HAT treatments (Fig. 3C, D). Non-metric Multidimensional Scaling (NMDS) based weighted Unifrac-distance was applied to the heterogeneity and similarity of bacterial community among HA proportion treatments (Fig. 3F). The results showed that rhizosphere bacterial communities were significantly (Stress, 0.026 76) differed among four treatments along the NMDS1, implying that different HA proportions had significant effect on rhizosphere bacterial structure of the hot pepper.

2.4 Bacterial community composition in rhizosphere By comparing the composition of pepper rhizosphere bacterial communities in HA proportion treatments, at the phylum level, 12 bacterial phyla with relative abundance greater than 1% were used to conduct bar plot (Fig. 4A), the enriched bacterial phylum was Proteobacteria with the relative abundance ranging from 48.37% to 60.40%, the abundant trend: CK < HAS < HAF < HAT, followed by Acidobacteriota and Actinobacteriota with the relative abundance variating from 18.22% to 12.10% and from 10.11% to 6.98%, respectively, and their abundance trends were opposite to that of Proteobacteria. With the decreasing of HA proportion, the relative abundance of bacterial phylum Chloroflexi, Bacteroidota, and Patescibacteria first enhanced and reached the highest (5.40%, 5.01%, and 3.18%, respectively) in HAS treatment, and then declined to the lowest (3.74%, 3.85%, and 2.00%, respectively) in HAT treatment. However, bacterial Gemmatimonadota had the highest relative abundance (5.05%) in HAF treatment and the lowest (3.02%) in HAT, with the abundant trend: HAT < CK < HAS < HAF.

At the genus level, rhizosphere 27 bacteria with average relative abundances greater than 1% in any HA treatment were analyzed and generated a dot-rot heatmap (Fig. 4B). norank_Caulobacteraceae and *Qipengyuania* abundances were the highest in CK, while the lowest were in HAF. The relative abundances of 11 bacterial genera (including norank_Acidobacteriales, *Sphingomonas*, norank_Subgroup_2, *Pseudolabrys*, norank_Micropepsaceae, *Bryobacter*, Candidatus_Solibacter, norank_Gaiellales, *Acidothermus*, *Arthrobacter*, and unclassified_Xanthobacteraceae) were the highest in CK and were the lowest in HAT treatment. The relative abundances of five bacterial genera (norank_Elsterales, norank_JG30-KF-AS9, *Rhodanobacter*, *Acidibacter*, and norank_Chitinophagaceae) were the most enriched in HAS treatment, however the lowest abundance of them were in different HA treatments, for example norank_*Elsterales* had the lowest abundance in HAF, while the relative abundance of norank_JG30-KF-AS9 was the lowest in HAT, the lowest abundance of bacteria *Rhodanobacter* was in CK, *Acidibacter* and norank_Chitinophagacea were the lowest in HAT. The relative abundances of six bacterial genera (including unclassified_Sphingomonadaceae, norank_Gemmatimonadaceae, norank_Alphaproteobacteria, Micropepsis, Ellin6067, and *Gemmatimonas*) were the highest in HAF treatment, the lowest abundance of bacterium unclassified_*Sphingomonadaceae* was in CK, other five of six bacteria had the lowest abundance in FAT treatment. Two bacterial genera *Arenimonas* and *Devosia* have the highest relative abundance in FAT treatment, and the lowest in CK. These results implied that the composition of pepper rhizosphere bacterial communities differed among different HA proportions.

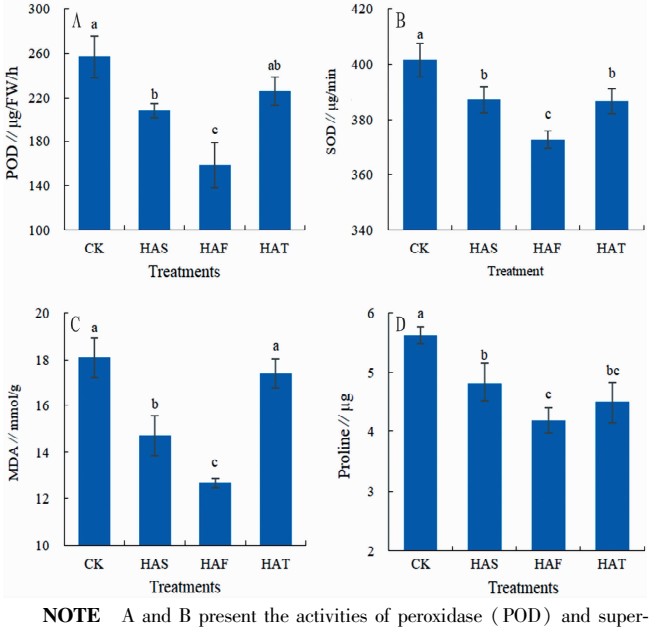
2.5 Bacterial functional prediction To explore bacterial functional differences among four HA treatments, metabolic pathway functional disparities of pepper rhizosphere bacterial communities at the level-1 were predicted (Fig. 5). Bacterial functions correlated with metabolism pathways were enriched, the top eight included metabolic pathways, microbial metabolism in diverse environments, the ATP-binding cassette (ABC) transporters, biosynthesis of secondary metabolites, biosynthesis of antibiotics, two-compo-

nent system, cellular community-prokaryotes, and carbon metabolism. The sum relative abundance of these eight bacterial functional communities changed from 44.14% to 44.19%, and their individual relative abundances were not significantly different across all HA proportion treatments. This finding implied that different HA proportions did not lead to disparities of bacterial functional communities in all treatments.



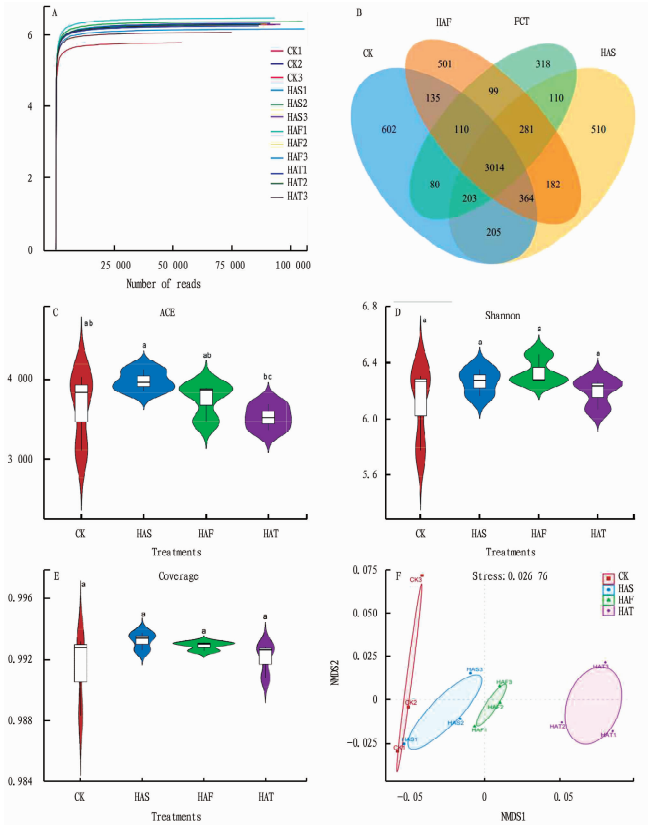
NOTE A shows the growth pictures of pepper plants after treated for 40 d; B, C, and D represent the plant height, fresh weight per plant, and root length of pepper under different HA treatments, respectively. The bar plots plus error line shows the mean ± SE, different lowercase are significant difference at $P < 0.05$.

Fig. 1 Growth of pepper plants under different complex ratio of HA



NOTE A and B present the activities of peroxidase (POD) and superoxide dismutase (SOD) in pepper leaf, respectively; C and D present the contents of malondialdehyde (MDA) and proline in pepper leaf, respectively. The bar plots plus error line shows the mean ± SE, different lowercase are significant difference at $P < 0.05$ (one-way ANOVA and *LSD* test).

Fig. 2 Health of pepper plants under different HA complex ratio

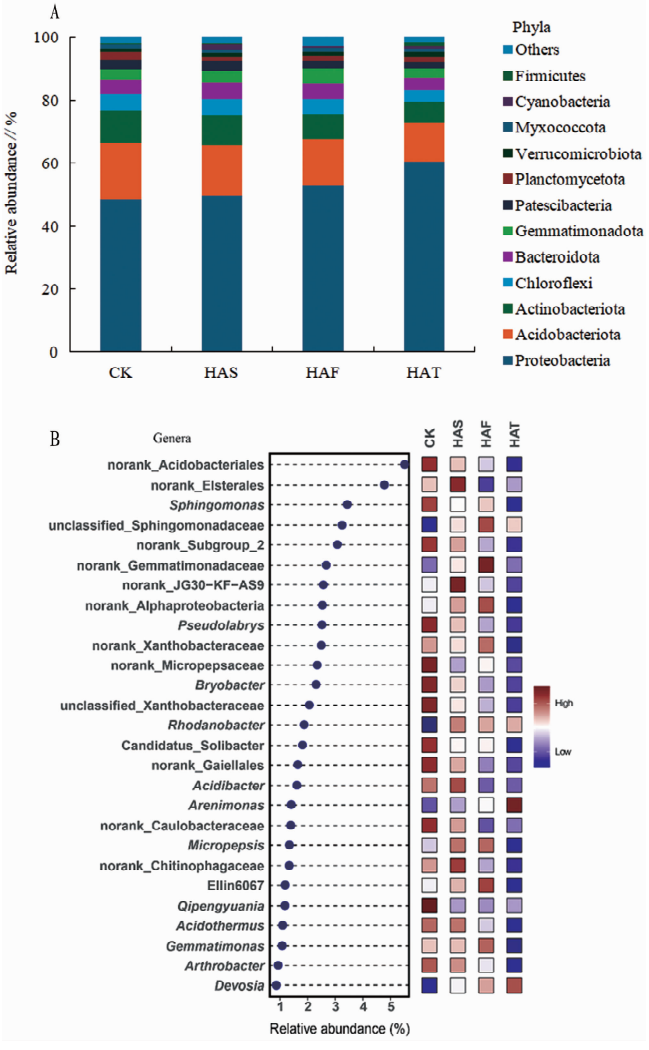


NOTE A and B show the rarefaction and Venn diagrams of reads; C, D, and E show α-diversity indexes of ACE, Shannon, and coverage of soil bacteria from hot pepper rhizosphere under four treatments, respectively; F represents non-metric multidimensional scaling (NMDS).

Fig. 3 Sequence results and diversity of soil bacteria from hot pepper under different complex ratio of HA

2.6 Relationship between pepper indicators and rhizosphere bacteria To present the relationship between pepper corresponding indexes and rhizosphere bacteria, the correlation heatmap was visualized based on the parameters of rhizosphere traits and enzymatic activities, pepper growth and health, and the relative abundances of rhizosphere bacterial genera (Fig. 6). The results discovered that rhizosphere bacteria were more closely associated with soil traits and enzymatic activities than with pepper growth and health. For example, the relative abundances of six bacterial genera (including *Sphingomonas*, *Arthrobacter*, norank_Acidobacteriales, *Pseudolabrys*, unclassified_Xanthobacteraceae, and norank_*Gaiellales* were significantly positively related ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) to soil OM and AN contents, the activities of soil sucrase, acid phosphatase and urease, reversely, significantly negatively related ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) to soil AP and AK contents. The abundances of three bacterial genera norank_LWQ8 and norank_Chitinophagaceae, and *Acidothermus* were significantly positively correlated ($P < 0.05$) with soil OM and AN contents and sucrase activity, while significantly negatively correlated ($P < 0.05$ or $P < 0.01$) with soil AP and AK contents. Norank_Vicinamibacterales had significant positive correlations ($P < 0.05$) with soil OM and AN contents and significant negative

correlations ($P<0.05$ or $P<0.01$) with soil AP and AK contents. norank_Xanthobacteraceae had significant positive correlations ($P<0.05$ or $P<0.01$) with soil OM and AN contents and



NOTE A shows the composition of soil bacteria with relative abundance greater than 1% in any HA proportion treatment at the phylum level; B presents the dot-rot heatmap of soil bacteria with relative abundance lower than 1% at the genus level.

Fig. 4 Composition of soil bacteria from hot pepper rhizosphere under different complex ratio of HA

acid phosphatase activity, but significantly negative correlation ($P<0.05$) with rhizosphere AP content. In addition, the abundances of bacterial genera *Arenimonas*, norank_Beggiatoaceae, and *Devosia* were significantly negatively associated with soil OM and AN contents, and the activities of soil sucrase, acid phosphatase and urease, conversely, significantly positively associated with soil AP and AK contents. However, pepper leaf proline content was significantly positively related to the abundances of certain bacteria (such as norank_Acidobacteriales, unclassified_Xanthobacteraceae, norank_Subgroup_2, *Bryobacter*, and norank_Caulobacteraceae), but significantly negatively related to the abundances of *Devosia*, unclassified_Sphingomonadaceae, *Rhodanobacter* and MND1. Additionally, significant negative relationships ($P<0.05$ or $P<0.01$) between the abundances of norank_Acidobacteriales, norank_Gaiellales, norank_Subgroup_2, norank_Micropepsaceae, norank_Caulobacteraceae and leaf MDA content, between the

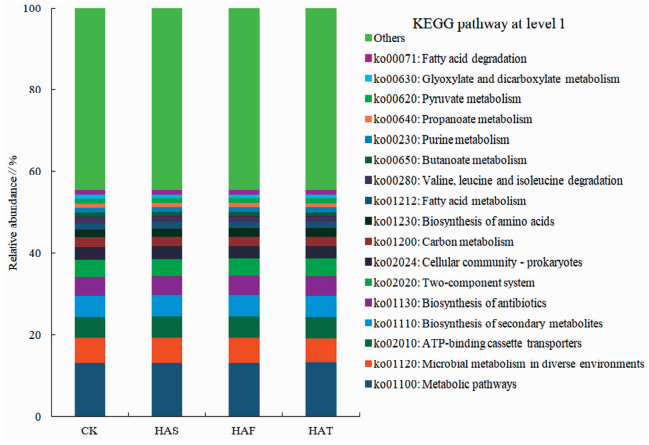


Fig. 5 Bacterial functional composition in rhizosphere of hot pepper under the different complex ratio of HA with the relative abundance greater than 1% at pathway level 1

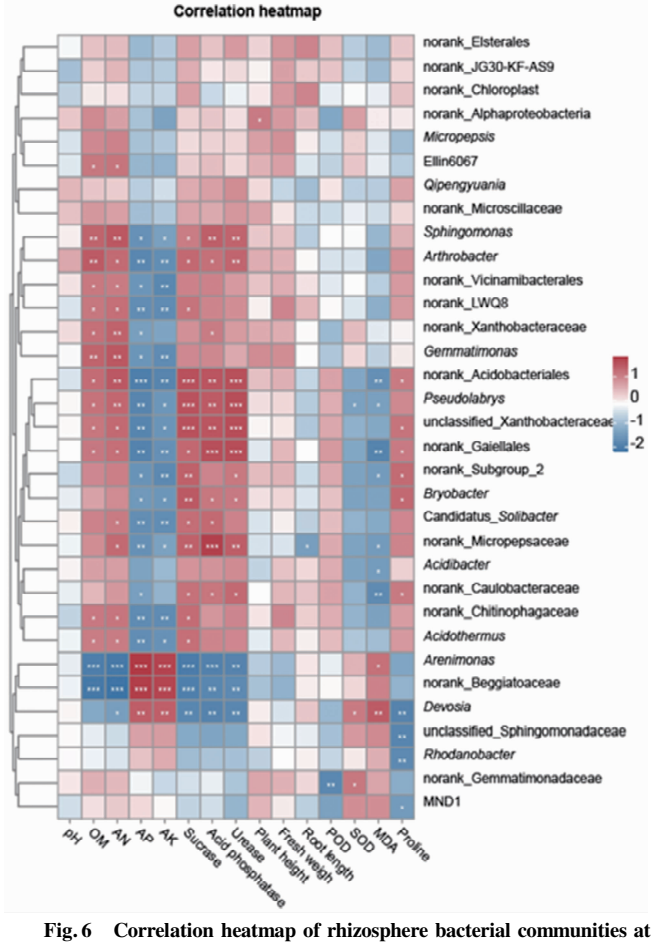


Fig. 6 Correlation heatmap of rhizosphere bacterial communities at genus level and indicators of soil and pepper plants ($n=12$)

abundance of norank_Gemmatimonadaceae and leaf POD were detected. significant positive relationships ($P<0.05$ or $P<0.01$) between the abundances of *Arenimonas*, *Devosia* and leaf MDA content, between the abundances of *Devosia*, norank_Gemmatimo-

nadaceae and leaf SOD activity were found. These findings suggested that some bacterial communities (*e. g.* norank_Acidobacteriales, *Pseudolabrys*, and unclassified_Xanthobacteraceae as well) were not only highly associated soil nutrient availability, but also closely associated with the health of hot pepper plants.

3 Discussion

In this study, it characterized the rhizosphere bacterial community composition, diversity, soil traits and pepper growth and health under HA proportion treatments by high-throughput sequencing and pepper parameter analysis. Humus soil is a type of soil composed of high concentration humus, and humus is a macromolecular organic complex generated by plants, animal residues and biological decomposition through complicated microbial and chemical process[25-26]. Humus soil is commonly applied in agriculture and horticulture or other fields because it is beneficial to improve soil quality and enhance plant growth[27-28]. However, humus soil is acidic (pH usually from 5.0 to 5.9), and the pepper growing soil is light acidic or neutral, so regulators (such as quicklime, plant ash and wood ash or others) mixed with humus soil could be applied to pepper agricultural production. In our experiment, different mixed HA proportion treatments improved rhizosphere soil pH, AP and AK contents, decreased the rhizosphere AN and OM contents and the three enzymatic activities.

The effects of different substrate ratios on the growth of pepper seedlings were different. Gao *et al.*[29] reported that the optimal proportion for chili seedlings growth is determined as a ratio of 5∶4∶1, comprising coir bran, river sand, and farm manure, respectively. The study of Zhu *et al.*[30] showed that the cultivated soil added appropriate amount of carbonized rice husks had an obvious effect on promoting the development of the root system of pepper seedlings, the root number, average root length and the longest root length increased significantly compared with the soil without carbonized rice husks. In Ye's experiment[31], the substrate ratio of turf to vermiculite was at 2∶1, added 2 kg/m^3 compound fertilizer, the results exhibited that this combination resulted in the highest comprehensive growth and health for pepper seedlings. In this study, as the HA proportion was at 6∶4, the values of plant height, fresh weight and root length of pepper seedlings were individually highest (Fig. 1), while 4∶6 mixed HA proportion was benefit for the seedling health, because HAF (at 4∶6 ratio of HA) treatment exhibited the significant lowest values of leaf POD and SOD activities, MDA and proline contents (Fig. 2). These findings agreed with earlier studies[32-34], which synergistic treatment of mixed humic substrate and conditioner had shown positive effects on crop growth and health.

In this pot experiment, different mixed HA ratio treatments considerably changed the composition of pepper rhizosphere bacterial communities (Fig. 4), while there was no obvious shift in bacterial functional composition (Fig. 5). Humus soil is enriched in humic acid, previous studies revealed that application of appropriate mixed humic acid and fertilizer strongly changed microbial community composition in relative abundances[35]. In current study, the relative abundance of enriched bacterial phyla Proteobacteria increased with decreasing HA ratio, while Acidobacteriota and Actinobacteriota were reverse, other phyla altered in different HA treatments but no obvious regular trend (Fig. 4A). These three bacterial phyla also were found to be three top dominant communities from Dafang knit pepper and Irish pepper rhizosphere, which were planted in a field of Dafang County of Guizhou Province, in southwest of China[36], and an experimental filed of Taian City, Shandong Province, in northeast of Chian[37], respectively. In the genus level, the relative abundance of some bacteria (such as *Sphingomonas*, *Bryobacter*, and *Qipengyuania* so on) decreased to varying degrees because of mixture in straw ash compared with CK, while certain bacterial genera increased in relative abundance when humus soil was mixed with different proportion straw ash (Fig. 4B). It implied that various bacterial genera responded differently to HA. However, mixture of humic soil and rice straw ash did not significantly alter rhizosphere bacterial ACE and Shannon indexes exception for significant difference in ACE index between HAS and HAT treatments (Fig. 3), which were consistent with previous studies[36-37].

The availability of nutrients and enzymatic activity in the rhizosphere soil are closely linked with soil microorganisms indirectly influencing the health and growth of plants[38-39]. In the pot experiment, relationship analysis revealed that soil OM, AN exhibited significant positive linkage with 13 bacterial genera, in which six bacterial genera were significantly positively related to soil three enzymatic activities, these genera included *Sphingomonas*, *Arthrobacter*, *Gemmatimonas*, *Pseudolabrys*, *Acidothermus* as well as unclassified_ Xanthobacteracea, norank_ Acidobacteriales and Gaiellales (Fig. 6). It implied that these bacteria maybe can secret extracellular sucrase, acid phosphatase, and urease into rhizosphere soil, and then improve the enzymatic activity increasing, thereby, soil nutrient availability might be enhanced[40]. Certainly, the improvement of soil enzymatic activity was also related to other factors such as temperature, plant species, and soil moisture so on[41]. However, soil AP and AK levels were significantly negatively interplayed with these 13 bacterial genera above, it might be because soil more AP and AK were absorbed by pepper seedlings than soil AN. Inversely, compared with 13 genera, the relative abundance of three bacterial genera including Arenimonas, *Devosia*, and norank_Beggiatoaceae showed opposite relationship trends with soil nutrients and enzymatic activities above, except of unsignificant correlation between soil OM and Devosia (Fig. 6). It suggested that some bacteria had negative interplay with certain element availability and enzyme activities, inverse trend might exist.

4 Conclusions

In this pot experiment, the growth and fitness of Liping hot pepper varied with the difference of HA proportions. Additionally, the structure and composition of pepper rhizosphere bacteria also shifted accordingly. Generally, HAS treatment (mixture of 1.2 kg humic soil and 0.8 kg straw ash) had more benefit for the growth of the pepper plants among four treatments. Relationship analysis uncovered that some rhizosphere bacteria were closely related to the availability of some soil nutrients and enzyme activities.

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