

Effects of Cropping Practice on *Bupleurum Chinense* Rhizosphere Microbial Community

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Abstract

It is of great importance to understand the effects of cropping practices of *Bupleurum chinense* on the properties of rhizosphere soil. Therefore, the chemical properties of rhizosphere soil and the rhizosphere microbiome were assessed in the field trial with *Bupleurum* and three cropping practices (continuous monocropping, *Bupleurum*-corn intercropping and *Bupleurum*-corn rotation). The results showed cropping practices changed the chemical properties of the rhizosphere soil and composition, structure and diversity of the rhizosphere microbial communities. Continuous monocropping of *Bupleurum chinense* not only decreased soil pH and the contents of NO_3^- -N and available K, but also decreased the alpha diversity of bacteria and beneficial microorganisms. However, *Bupleurum*-corn rotation improved soil chemical properties and reduced the abundance of harmful microorganisms. Soil chemical properties, especially the contents of NH_4^+ -N, soil organic matter (SOM) and available K, were the key factors affecting the structure and composition of microbial communities in the rhizosphere soil. These findings could provide a new basis for overcoming problems associated with continuous cropping and promote development of *B. chinense* planting industry by improving soil microbial communities.

Introduction

Bupleurum chinense (Apiaceae) is an important medicinal plant that has been used in China and other Asian countries for thousands of years. The plant has many important properties, such as anti-inflammatory [1], liver-protecting [2], anti-depressant [3], anti-tumor [4], and immunomodulatory [5] activities, and is widely used in the clinical treatment of fever, influenza, malaria, distending pain in the chest, menstrual disorders, and other symptoms. The active components of *Bupleuri radix* mainly include saponins [6], polysaccharides [7], essential oil [8], flavones [9], and coumarin [10]. These compounds are not only related to *Bupleurum* germplasm, but also closely related to the environment of the producing area and cropping practices [9,11].

In the recent decades, as a result of rapid development and large-scale cultivation, the planting area of *B. chinense* has expanded substantially. However, due to the limited available land and maximum economic benefits, the continuous cultivation of *B. chinense* is becoming increasingly popular. Studies have shown that long-term continuous cropping alters physical properties of the soil [12] and the soil microbial community structure, resulting in a diminished community of beneficial microorganism in soil, an increase in the pathogenic microorganisms, and a decline in the yield and quality of medicinal materials [13]. For example, the continuous planting of American ginseng [14] and *Sophora flavescens* [15] not only weakened soil microbial diversity and amassed fungal root pathogens, but also changed soil physical properties, resulting in decreased crop yield and quality.

Studies have shown that multiple cropping system [characterized by more than one crop grown together, either mixed in space (intercropping) or time (crop rotation)] can effectively alleviate the problem of monocropping. Intercropping, in which two or more crops are planted in the same field, can increase the absorption of trace elements, improve soil fertility [16] and reduce the risk of pests and diseases [17]. For example, the intercropping of turmeric, ginger and patchouli not only changed the soil physical properties and the microbial community structure, but also improved the quality of patchouli [18].

Unlike intercropping, crop rotation involves the systematic rotation of different types of crops in the same field. Crop rotation can balance soil nutrients, improve soil chemical properties, increase the abundance of beneficial microorganisms, and enhance disease resistance [19]. For example, the rotation *Pinellia ternata*-wheat improved soil properties, enriched beneficial microorganisms and inhibited pathogenic microorganisms [20].

Understanding the effects of cropping practices on the structure and diversity of microbial community in the rhizosphere soil and revealing a mechanism governing the dynamics of rhizosphere microbiome will provide a theoretical basis for the optimization of *B. chinense* cultivation technology. However, the effects of cropping practices on the physical properties and microbiome in the rhizosphere soil have not been studied in detail; this lack of knowledge affects the development of *B. chinense* planting industry.

The objective of the study was to investigate the effect of cropping practices on soil rhizosphere microbial community. A high-throughput Illumina MiSeq sequencing platform was used to determine the microbial community structures in the *B. chinense* rhizosphere soil in different cropping practices. Based on the MiSeq sequencing results, the characteristics of microbial community structures, and the main functional bacteria and fungi were analyzed in different cropping practices. Our study could provide a new basis for overcoming continuous-cropping obstacles and promote development of *B. chinense* planting industry.

Materials And Methods

Field experiment

The trial field was located at the *B. chinense* plantation base in Zhangqiu City, Shandong Province, China (117°22'54" E 36°35'27" N, altitude 524 m). The annual average sunshine was 2647.6 h, and the sunshine rate was 60%. The annual average temperature was 12.8°C, and the annual average precipitation was 600.8 mm. The soil type was brown soil.

The field experiment was conducted from June 2018 to October 2020. The study complies with with relevant institutional, national, and international guidelines and legislation. The same field management measures were adopted throughout, including hand weeding, no fertilization and no watering. After two consecutive years of planting and harvesting of *B. chinense* in the same field, three treatments were implemented in the third year, namely *B. chinense* continuous cultivation (BCC), *B. chinense* intercropped with corn (BIC) and growing corn after *B. chinense* (BCR) (Table 1).

Collection of soil samples

Rhizosphere soil samples under different cropping practices were collected by the five-point sampling method [46]. There were triplicate soil samples for each treatment. Firstly, the loose soil was shaken off from roots, and the soil closely adhering to the root system was sampled as rhizosphere soil by brushing off [28]. The collected soil was then placed in a sealed sterile bag and taken back to the laboratory. Each soil sample was divided into two subsamples: one was used for chemical analysis, and the other was stored at -20°C for microbial analysis.

Chemical properties

After air-drying, the pH value of the soil was measured using a pH meter (pHS-3S), and the contents of soil organic matter (SOM), available phosphorus (Ava-P), and available potassium (Ava-K) were determined by the method reported by Qu et al. [47]. Determination of NO_3^- -N and NH_4^+ -N in soil were determined by the method reported by Li et al [22].

DNA extraction

A PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) was used to extract DNA from soil samples. Each soil sample was extracted three times according to the PowerSoil kit manufacture's protocol. The extracted DNA was eluted using 100 μL sterile water, quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Canada) and stored at -20°C for further use.

Gene amplification and Illumina MiSeq sequencing

The V4-V5 region of soil bacteria 16S rDNA was amplified using the primers 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 926R 5'-CCGTCAATTCMTTGTAGTTT-3', whereas the fungal ITS1 region was amplified using F 5'-CTTGGTCATTTAGAGGAAGTAA-3', and R 5'-GCTGCGTTCTTCATCGATGC-3'. The bacterial and fungal PCR reactions were included denaturation at 94°C , followed by 22 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The target band was detected by 2% agarose gel electrophoresis and recovered by a QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA). Finally, paired-end sequencing of the amplicon library was performed on an Illumina MiSeq platform (TinyGene Bio-Tech Co., Ltd., China), according to the standard protocol.

Illumina data analysis

The raw fastq files were demultiplexed based on the barcode. Paired-end reads for all samples were run through Trimmomatic (version 0.35) to remove low-quality base pairs. Flash (Version 1.2.11) software was used to splice the paired reads into a sequence. Mothur (Version 1.33.3) was used to control the sequence quality to obtain the optimized sequence. Then, UPARSE software (usearch Version V8.1.1756, <http://drive5.com/uparse/>) was adopted for OTU (the operational taxonomic unit) clustering. Based on taxonomic information, statistical analysis of community structure was carried out at the phylum and genus levels.

Diversity analysis were performed using mothur soft (Version 1.33.3). On the basis of the above analysis, rarefaction curves, NMDS and community structure histograms were calculated and visualized by R (Version 3.6.0).

Results

The effect of cropping practices on the chemical properties

The chemical properties of *B. chinense* rhizosphere soil in different treatments were shown in Table 2. Compared with intercropping and crop rotation, soil pH and the contents of NO_3^- -N and Ava-K decreased after continuous planting of *B. chinense*, but the Ava-P content increased. The chemical parameters of rhizosphere soil differed significantly among the treatments, except the NO_3^- -N content.

Amplicon sequencing and rarefaction curves

To characterize the microbiome in the *B. chinense* rhizosphere soil in different cropping practices, nine samples were sequenced by Illumina MiSeq. The amplicon sequencing resulted in 450,038 effective reads of bacterial 16S rRNA genes and 437,141 effective reads of fungal ITS region. Based on 97% similarity, the OTUs of microbial community in the rhizosphere soil were obtained. The results were shown in supplementary Table 1.

To construct rarefaction curves, the dataset was flattened according to the minimum number of sample sequences. The rarefaction curves of nine rhizosphere soil samples were constructed based on the number of OTUs observed (Supplementary Fig 1). The rarefaction curves showed that the number of OTUs rose sharply and then gradually flattened out, indicating that the sequencing library reached saturation. Therefore, it could be used for analyzing the diversity of microorganism in the rhizosphere soil of *B. chinense*.

Alpha diversity of bacterial and fungal communities

The alpha diversity represents the measurement of within-community microbial diversity (Table 3). Theoretically, the larger the Shannon index or the smaller the Simpson index, the higher the community diversity. According to the Shannon index, the bacterial richness was highest (6.513) in the rhizosphere soil of the rotation of *B. chinense* and corn, followed by continuous monocropping (6.421) and intercropping of *B. chinense* and corn (6.328). The Simpson index analysis confirmed the above-mentioned diversity analysis. The Shannon index and Simpson index values for fungal communities in the rhizosphere of *B. chinense*-corn intercropping were 4.401 and 0.029, respectively, followed by those of rotation with corn (4.250 and 0.033, respectively), and the lowest diversity values were in *B. chinense* monocropping (4.201 and 0.049, respectively). The results showed that the rotation and intercropping of *B. chinense* with corn were the main factors affecting the diversity of, respectively, bacteria and fungi in the rhizosphere. In summary, the cropping practices had an important effect on the diversity of rhizosphere microorganisms.

Beta diversity of bacterial and fungal communities

In order to shed more light on the differences in microbial community structure, NMDS analysis was performed based on the Weighted UniFrac distance (Fig 1). When the sample distance was above 0.2 (e.g. 0.3 for fungi), the samples could be divided into three groups, according to the species composition in the *B. chinense* rhizosphere (Fig 2). There were similarities in the structure of microbial communities within the groups and significant differences in the structure among the groups, which indicated that the cropping practices in the same field was an important factor affecting the composition microbial communities in the *B. chinense* rhizosphere.

The composition and structure of the bacterial community

In order to clarify the microbial community structure in the *B. chinense* rhizosphere, two taxonomic levels (phylum and genus) were analyzed.

As shown in Fig 3A, 13 bacterial phyla were detected in the soil from different cropping practices. The dominant bacterial phyla in the *B. chinense* rhizosphere soil were Proteobacteria (27%), followed by Actinomycetes (22%), Acidobacteria (20%), and Chloroflexi (9%). The relative abundances of Proteobacteria and Actinobacteria increased significantly after 3 years of continuous cropping of *B. chinense*, but decreased after intercropping or rotation with corn. The relative abundance of Acidobacteria and Chloroflexi decreased after continuous cropping, but increased after intercropping and rotation.

At the genus level (Fig 4A), 34 bacterial genera were detected in the soil from different cropping practices. The dominant genera were Pseudarthrobacter (4%), Microvirga (2%), Gaiella (1%), Nitrospira (1%), and Pirellula (1%). Compared with the intercropping and rotation of *B. chinense* and corn, the relative abundance of Pseudarthrobacter and Gaiella increased after continuous cropping. However, the relative abundance of Microvirga and Nitrospira showed a downward trend after continuous cropping and intercropping.

The composition and structure of the fungal community

As shown in Fig 3B, five fungal phyla were detected in the soil from different cropping practices. The dominant fungal phyla were Ascomycota (78%), Basidiomycota (6%) and Zygomycota (5%). The relative abundance of Ascomycota decreased after continuous cropping and intercropping, but increased after rotation with corn. The relative abundance of Basidiomycetes increased after continuous cropping, but decreased after intercropping and rotation.

At the genus level (Fig 4B), 44 fungal genera were detected in the soil from different cropping practices. The dominant fungal genera in the rhizosphere soil were Gibberella (5%), Cercophora (5%), Fusarium (4%), Chaetomium (4%), Mortierella (4%), Preussia (4%), Cryptococcus (3%), Alternaria (3%), unclassified_Ascobolaceae (2%), Cladorhinum (2%), Paraphoma (1%), Knufia (1%), and Cladosporium (1%). After 3 years of continuous cultivation of *B. chinense*, the relative abundance of Cercophora, Cryptococcus, Alternaria, Paraphoma, and Cladosporium increased, but the relative abundance of Chaetomium, Mortierella, Preussia, and Cladorrhinum significantly decreased.

Correlation analysis of dominant bacteria and soil properties

Soil chemical properties were important explanatory factors that determined the clustering patterns of soil microbial communities in different cropping treatments [21]. The chemical properties of the *B. chinense* rhizosphere soil were significantly different under different cropping practices (Table 2). Hence, redundancy analysis (RDA) was conducted on the relative abundance of dominant bacteria and fungi at the genus level and soil chemical factors (Fig 5). The results showed that the cumulative variation explained by the soil chemical properties was 87.84% and 59.31% for bacteria and fungi, respectively, indicating that explanatory

variables had a significant influence on the structure of microbial communities. The effects of soil chemical properties on bacteria and fungi were in the order of $\text{NH}_4^+\text{-N} > \text{SOM} > \text{Ava-K} > \text{pH} > \text{NO}_3^-\text{-N} > \text{Ava-P}$ and $\text{NH}_4^+\text{-N} > \text{SOM} > \text{Ava-K} > \text{Ava-P} > \text{pH} > \text{NO}_3^-\text{-N}$, respectively (Fig 5). In conclusion, $\text{NH}_4^+\text{-N}$, SOM and Ava-K were the main chemical properties that affected the microbial abundance and composition in the *Bupleurum* rhizosphere soil.

Biomarker analysis

In order to identify the dominant microbial biomarkers in the *B. chinense* rhizosphere soil under different cropping practices, the linear discriminant analysis (LDA) effect size (LEfSe) was carried out (Fig 6). The LDA results identified 30, 33 and 55 bacterial biomarkers in continuous monocropping, intercropping and rotation with corn, respectively (Fig 6A). The most abundant bacterial family was Comamonadaceae from *B. chinense* continuous monocropping soil. *Rhizobium giardinii*, Desulfurellaceae and Burkholderiales were abundant in the rhizosphere of *B. chinense* intercropped with maize, whereas Methylobacteriaceae and Microvirga were significantly enriched in the rhizosphere of *B. chinense* in rotation with corn.

For the fungal community, we identified 92, 57 and 34 fungal biomarkers in continuous monocropping, intercropping and rotation with corn, respectively (Fig 6B). The relatively abundant biomarker fungal taxa included Dothideomycetes and Pleosporales in the *B. chinense* continuous monocropping, Chaetomiaceae, Mortierellaceae and Zygomycota in *B. chinense* intercropped with corn, and Nectriaceae, Chytridiomycetes and Rhizophlyctidales in *B. chinense* in rotation with corn.

Discussion

Soil chemical characteristics are the important indices for evaluating soil quality. Cropping practices influenced not only the chemical properties of soil, but also governed the composition of rhizosphere microorganisms [22]. Therefore, elucidating the changes in soil chemical properties can provide a basis for characterizing soil productivity under different cropping practices.

In our study, the contents of pH, $\text{NO}_3^-\text{-N}$ and Ava-K decreased after continuous cropping of *B. chinense*, but increased after intercropping and rotation with corn. Studies have shown that continuous monocropping systems have a negative impact on soil function and sustainability [23-24]. Soil nutrients such as SOM, Ava-P, Ava-K, $\text{NO}_3^-\text{-N}$ and $\text{NH}_4^+\text{-N}$ showed a decreasing trend after continuous cropping. However, rotation or intercropping with corn effectively alleviated this decline and imbalance in soil nutrients caused by continuous monocropping [22,25]. Our experiment, confirming this theory, contributed to the sustainable development of *B. chinense* planting industry through rotation of *B. chinense* with corn.

Soil microbial diversity has an important impact not just on soil quality, function and sustainability [26], but also is a key factor in controlling pathogenic microorganisms [27]. Therefore, the loss of soil microbial diversity and function is one of the reasons for poor crop growth under continuous monocropping. In the present study, the cropping practices, had significant effects on the structure and composition of soil

microbiome. In *B. chinense* continuous monocropping, alpha diversity decreased, beta diversity increased, pathogenic microorganisms became more abundant and beneficial microorganisms declined. However, these changes could be alleviated by rotation or intercropping with corn. Similar results were also obtained in continuous monocropping of sugar beet [28] and *Coptis chinensis* Franch. [29], intercropping of potato with onion and tomato [30] and intercropping of black pepper and vanilla [26], as well as rotation of Brassica vegetables with eggplant [22] and *Pinellia ternata* with wheat [20].

Beta diversity showed that cropping practices had a great influence on the soil microbial community. In other words, the use of different cropping systems may lead to significant differences in the structure of microbial communities in soil [31]. Importantly, changes in microbial community structure and composition usually are associated with changes in plant metabolic capacity, biodegradation of organic pollutants, disease inhibition, and other functions [32].

In our study, the continuous monocropping of *B. chinense* strongly reduced the abundance of beneficial microorganisms, such as Microvirga, Haliangium, Chaetomium, Mortierella, Preussia, and Cladorrhinum. These rhizosphere microorganisms played an important role in plant growth and the inhibition of pathogenic microorganisms [33-38]. In contrast, some potentially pathogenic microorganisms, such as Cercophora [39], Alternaria, Paraphoma, Cladosporium [40], Monographella [41], Hydropisphaera [42], and Colletotrichum were significantly amplified. For example, Alternaria and Paraphoma can cause root rot of *B. chinense* [43-44], and Colletotrichum can infect leaves to produce disease spots [45]. These pathogenic microorganisms not only significantly reduce the relative abundance of beneficial bacteria in the field, but also affected the normal growth of *B. chinense*.

Conclusion

The cropping practices changed the chemical properties and the structure and composition of the microbial communities in the *B. chinense* rhizosphere soil. Soil chemical properties, especially the contents of $\text{NH}_4^+\text{-N}$, SOM and Ava-K, influenced the microbial structure and composition of the *B. chinense* rhizosphere soil. These findings could provide a new basis for overcoming the problems associated with continuous cropping and promoting development of *B. chinense* planting industry by improving soil microbial communities.

Declarations

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Conflict of interest

The authors declare they have no competing interests.

Authors contribution

Professor Gao Demin designed the experiments and revised the manuscript. Professor Cao Hailu, Liu Li, Geng Yanan, Fan Ya and Du Kan collected the rhizosphere soil of *Bupleurum chinense*. Liu Li and Feng Haiyang carried out the data analysis and wrote the manuscript. Professor Bu Xun assisted in microbial sequencing. All authors read the final manuscript.

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Tables

Table 1. Experiment setup of the field experiment

Treatment	Year		
	2018	2019	2020
BCC	<i>B. chinense</i>	<i>B. chinense</i>	<i>B. chinense</i>
BIC	<i>B. chinense</i>	<i>B. chinense</i>	<i>B. chinense</i> -corn
BCR	<i>B. chinense</i>	<i>B. chinense</i>	corn

Notes: *B. chinense*, *Bupleurum chinense*. BCC, continuous planting *B. chinense* for three years. BIC, *B. chinense* intercropped with corn in the third year. BCR, planting corn in the third year.

Table 2. Chemical properties of rhizosphere soil of *Bupleurum chinense* from different cropping practices

Note: Different lowercase letters indicate significant differences between different samples ($P < 0.05$). BCC stands for *Bupleurum chinense* continuous cultivation. BIC stands for *B. chinense* intercropped with corn, while BCR stands for growing corn after *Bupleurum chinense*. SOM, soil organic matter. Ava-K, available potassium. Ava-P, available phosphorus.

Table 3. Shannon and Simpson indices of rhizosphere microbial community of *Bupleurum chinense* under different cropping practices

Samples	pH	SOM mg/Kg	NO ₃ ⁻ -N mg/Kg	NH ₄ ⁺ -N mg/Kg	Ava-K mg/Kg	Ava-P mg/Kg
BCC	6.67±0.04 ^a	18.32±0.18 ^a	49.53±2.23 ^a	43.58±0.49 ^a	154.12±11.87 ^a	92.19±3.30 ^a
BIC	7.94±0.07 ^b	17.39±0.32 ^b	93.98±4.15 ^b	38.97±0.78 ^b	186.54±4.01 ^b	60.92±1.82 ^b
BCR	8.07±0.07 ^c	19.07±0.30 ^c	95.55±2.28 ^b	62.27±1.89 ^c	220.19±12.88 ^c	73.28±1.44 ^c

Sample	Bacteria		Fungi	
	Shannon	Simpson	Shannon	Simpson
BCC	6.421±0.019 ^a	0.006±0.001 ^a	4.201±0.218 ^a	0.049±0.020 ^a
BIC	6.328±0.046 ^{ab}	0.006±0.000 ^a	4.401±0.197 ^a	0.029±0.005 ^a
BCR	6.513±0.074 ^{ac}	0.004±0.000 ^b	4.250±0.192 ^a	0.033±0.008 ^a

Note: Shannon and Simpson represents the diversity of bacteria or fungi. Values are expressed as mean ± SD (n = 3). Different lowercase letters indicate significant differences between different samples ($P < 0.05$).

Figures

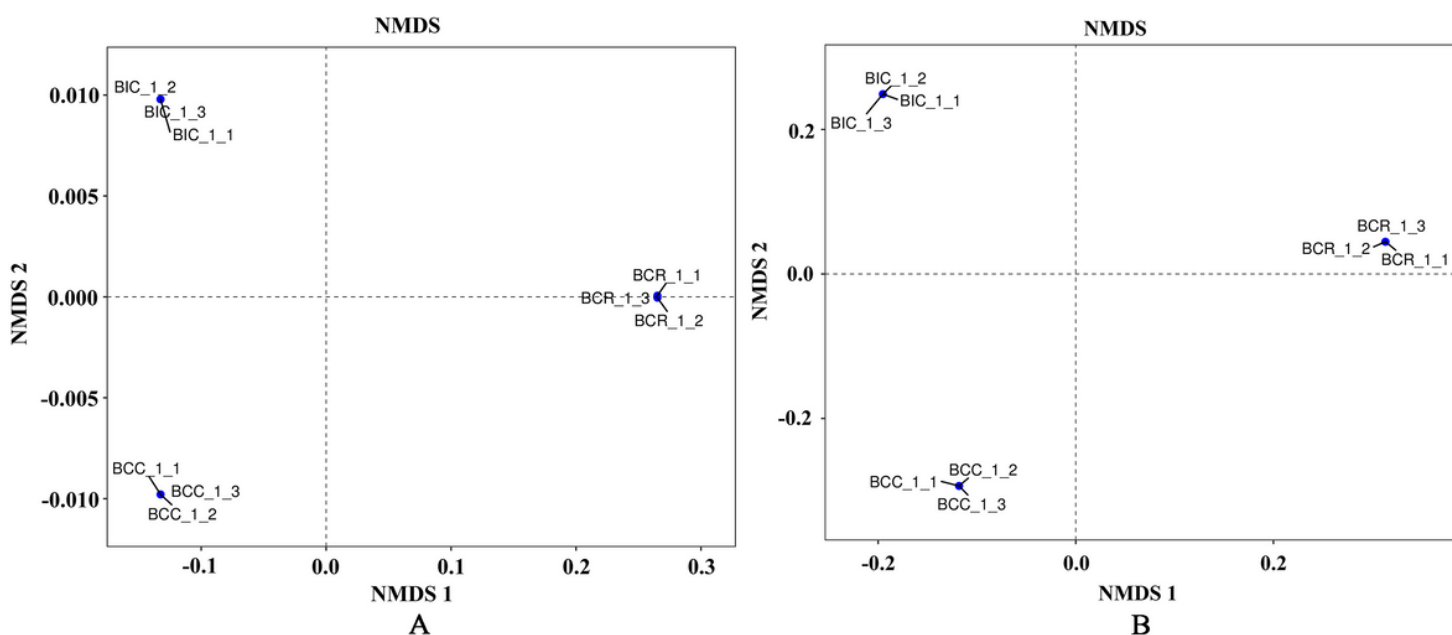


Figure 1

Based on the unweighted UniFrac distance, the heat map analysis of rhizosphere soil microbiome community structure of *Bupleurum chinense* from various cropping practices. (A) Bacteria, (B) Fungi. BCC, *Bupleurum chinense* continuous cultivation. BIC, *Bupleurum chinense* intercropped with corn. BCR, growing corn after *Bupleurum chinense*. The following soil samples in the figures are all the same.

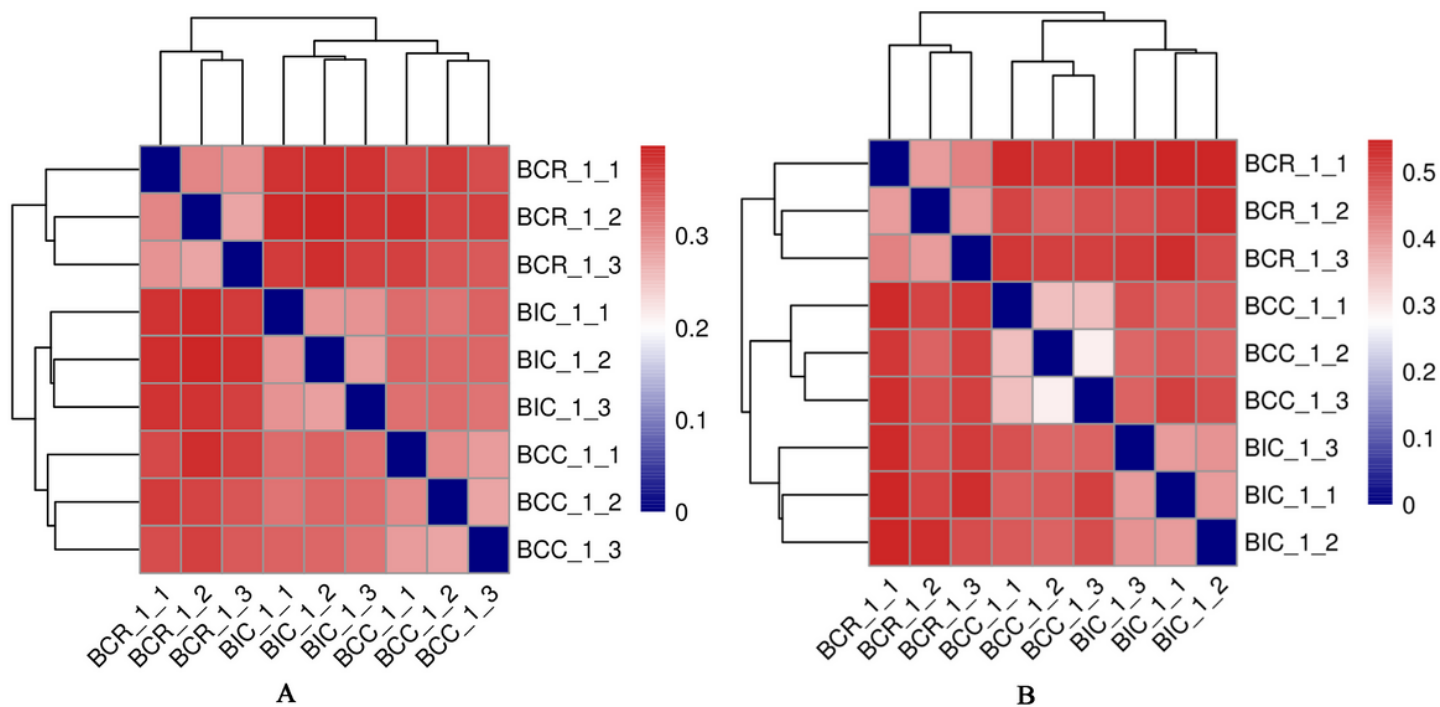


Figure 2

The non-metric multidimensional scale (NMDS) map of the unweighted UniFrac distance represents the microbial community structure. (A) Bacteria, (B) Fungi.

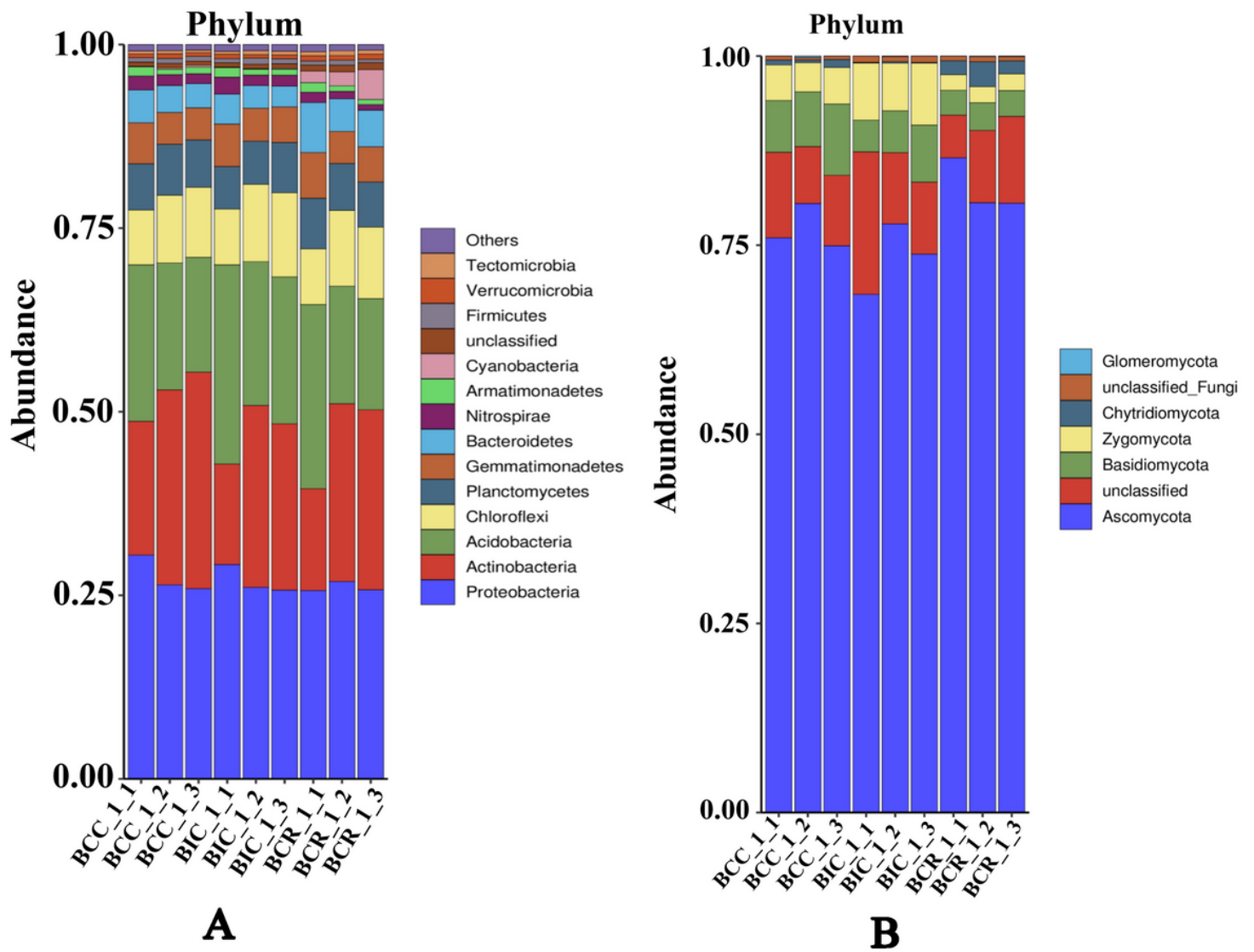


Figure 3

Relative abundance of species under various cropping practices at the microbial phylum levels. (A) Bacteria, (B) Fungi.

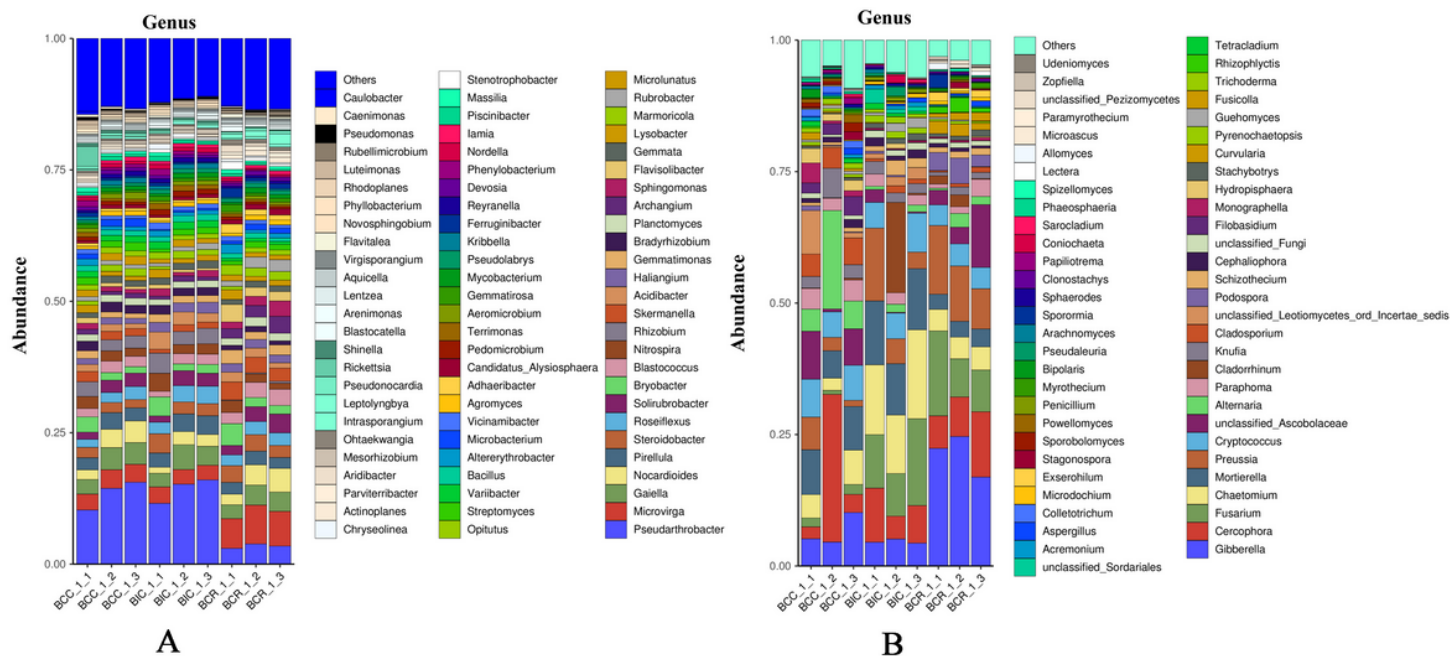


Figure 4

Relative abundance of species from various cropping practices at the microbial genera levels (A) Bacteria, (B) Fungi.

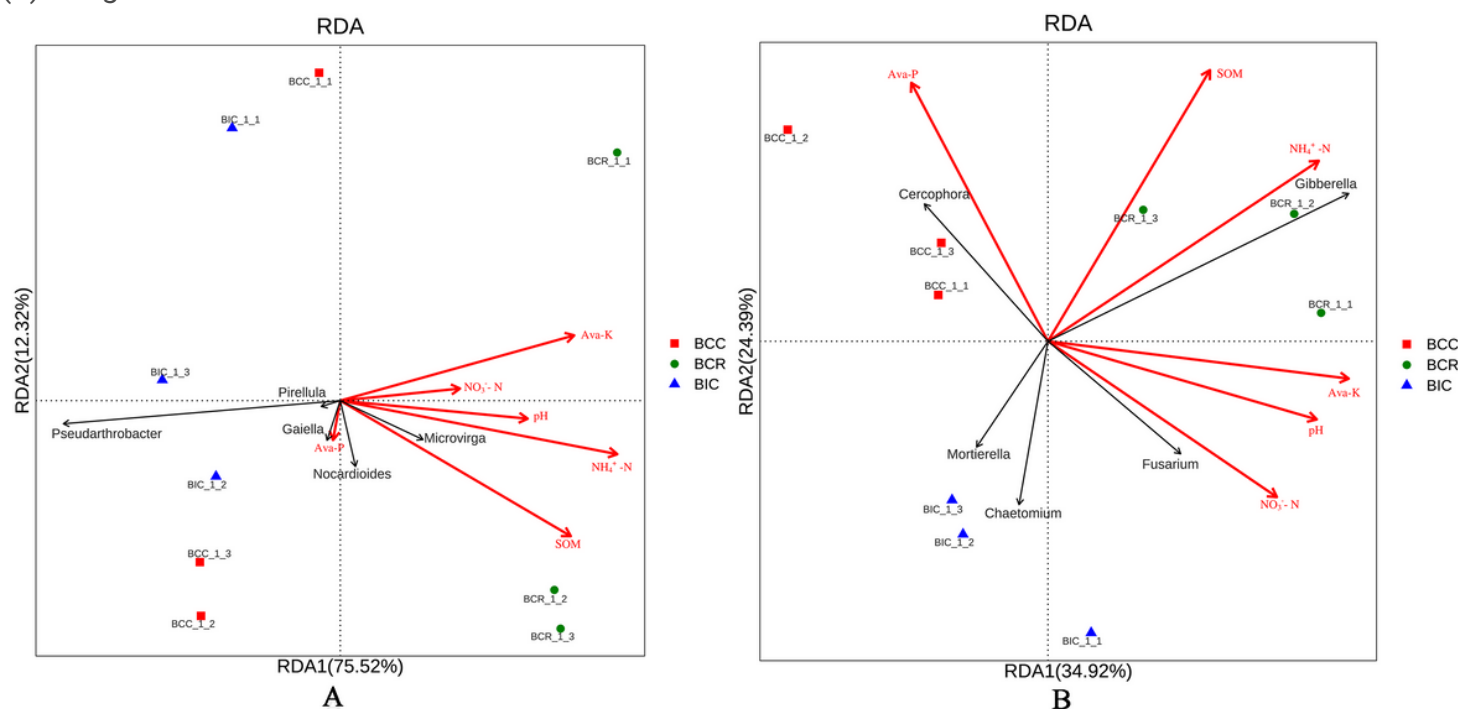


Figure 5

Redundancy analysis (RDA) on dominant bacterial genera (A) and dominant fungal genera (B) constrained by soil chemical properties

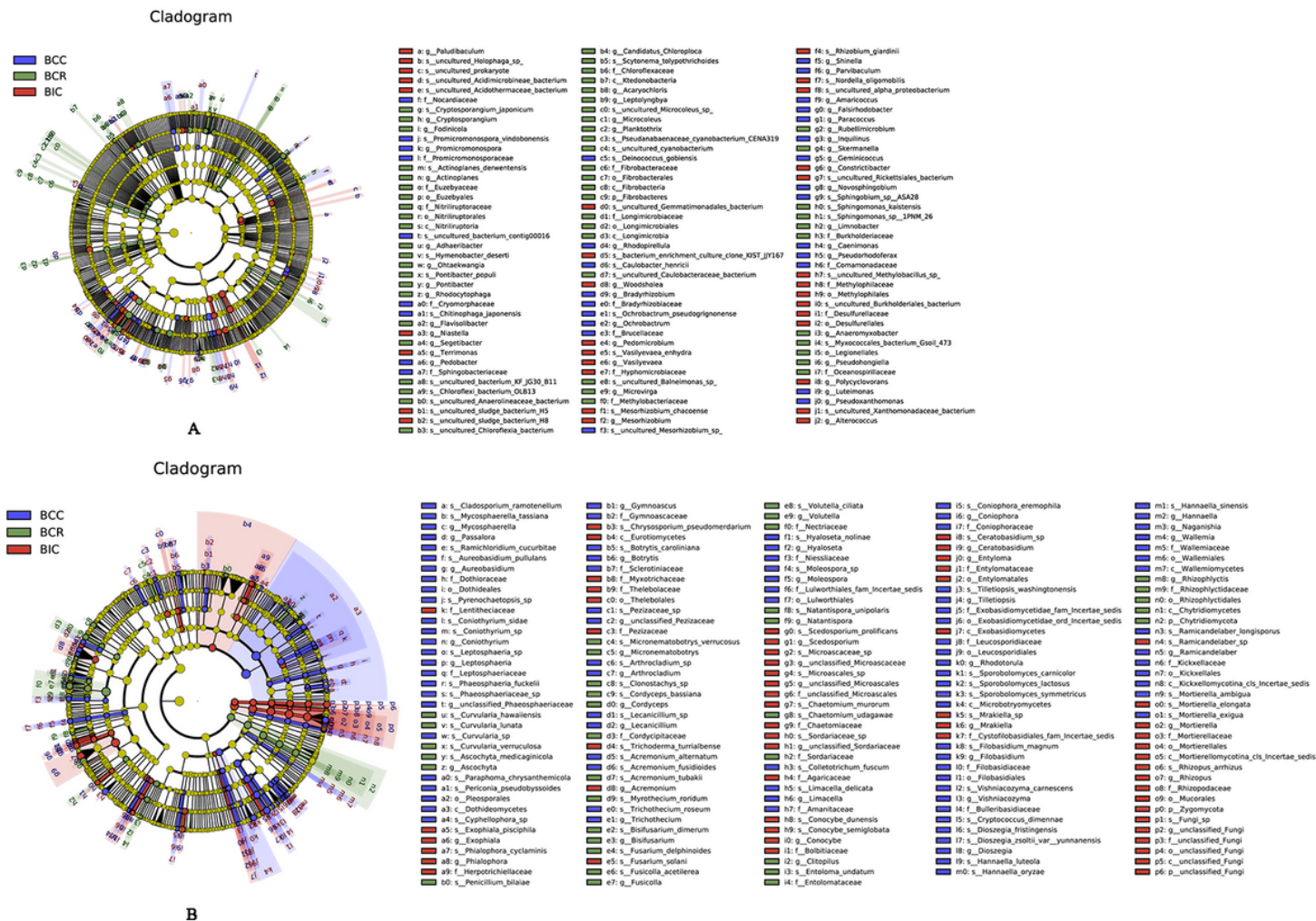


Figure 6

LEfSe analysis of community differences between groups of *Bupleurum chinense* in different cropping practices. (A) Bacteria, (B) Fungi.

Supplementary Files

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