Differences in the root-associated endophytic fungal community composition and structure of three medicinal licorices in Xinjiang, China

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Abstract: Endophytic fungi have played a very important role in influencing the quality and quantity of bioactive compounds of medicinal plants through specific fungus-host interactions. In medicinal licorices root, a total of 2,118,633 effective sequences and 1,063 effective operational taxonomic units (OTUs) with 97% identity were obtained by high-throughput sequencing. A total of 8 phyla and 140 genera were annotated, among them, the phylum Ascomycota and Basidiomycota, and the genera *Fusarium*, *Paraphoma* and *Helminthosporium* were significantly dominant. Moreover, Wilcoxon rank sum test showed that the Shannon index was significantly different distribution between *Glycyrrhiza uralensis* and *Glycyrrhiza inflata*, especially 0-20cm at the root depth, the Chao1 index in *Glycyrrhiza inflata* was

significantly affected by root depth, and there were significant differences in beta diversity between *Glycyrrhiza uralensis* and *Glycyrrhiza inflata*. Moreover, we explored the content of bioactive compounds (glycyrrhizic acid, liquiritin and total flavonoids) in roots of medicinal licorices based on high-performance liquid chromatography. Our results showed that the liquiritin content was not affected by the root depth (0-20cm, 20-40cm and 40-60cm), but was significantly affected by the main effect species (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, *Glycyrrhiza glabra*) ($P < 0.05$), and the content of liquiritin was accountable for the differences in the diversity of endophytic fungal community. Furthermore, distance-based redundancy analysis (db-RDA) showed that soil physicochemical properties (available potassium and ammonium nitrogen), and the root factor (liquiritin and water content) were the main contributing factors to the variations in the overall structure of endophytic fungal community in this study.

Keywords: bioactive compounds; endophytic fungal community; *Glycyrrhiz*a; Soil physicochemical

1. Introduction

Glycyrrhiza species are perennial herbs with widely grows in arid and semi-arid regions [1]. There are three different original plants of *Glycyrrhiza* stipulated in Chinese Pharmacopeia, namely dried root and rhizome of *Glycyrrhiza uralensis*, *Glycyrrhiza inflata* and *Glycyrrhiza glabra* its dried roots and rhizomes is one of the most commonly used herbs medicines in both Eastern and Western countries [2]. A

wide variety of bioactive compounds can be extracted from root [3], mainly include triterpene saponins, polysaccharides and flavonoids [4]. Glycyrrhizic acid, the richest content of triterpene saponins [5], is the important pharmacological bioactive compounds with anti-inflammatory [6], antiviral and immune regulation [7, 8] and other biological effects. Liquiritin is a major component of flavonoids that mainly exerts anti-inflammatory [9], antioxidant and antibacterial [10, 11]. Because of its medicinal and economic value, medicinal licorices plant has become the research direction of medicinal licorices to improve the content of licorice herbal medicine and understand its ecological characteristics.

The traditional view widely believes that the quality and quantity of the bioactive compounds extracted from medicinal plants are largely affected by the genetic background of the related plant, the ecological environment in which the plant lives, and soil nutrients [12, 13]. However, in recent years, some studies [14, 15] have shown that endophytic fungi have played a very important role in influencing the quality and quantity of bioactive compounds of medicinal plants through specific fungus-host interactions.

Endophytes, especially endophytic fungi, are one of the most important components in plant micro-ecosystems [16]. Endophytic fungi can form symbiotic relationships with host plants, on the one hand, which can present and grow in different healthy tissues of living plants, including stems [17], leaves [18] and roots [19]. Endophytic fungi, on the other hand, can extract carbohydrates and other nutrients from the host plant for their own growth [20]. In return, host plants may

receive benefits from endophytic fungi associations. First, endophytic fungi can promote the growth of host plants by increasing hormones, including Gibberellin, Indoleacetic acid, Abscisic acid, Zeatin [21]. Second, endophytic fungi can enhance the resistance of host plants to environmental stress by producing biologically bioactive compounds [22, 23], such as, endophytic fungi of wheat can promote plant growth and abiotic stress resistance [24]. Last but not least, endophytic fungi can promote the accumulation of secondary metabolites of the host plant [25], such as paclitaxel and deoxypodophyllotoxin, thereby affecting the quantity and quality of bioactive compounds of medicinal plants.

Endophytic fungi have great biodiversity and are widely distributed in various terrestrial and aquatic plants species [26], and numerous studies have shown that endophytic fungi can be isolated from various plants species, ranging from important cash crop species [27] such as soybean, to medicinal plant species [28, 29], such as *Dendrobium Officinzle* and *Sceletium Tortuosum*. However, it should be noted that, with the rapid development of high-throughput sequencing technology and bioinformatics, a large number of undiscovered fungi have been discovered [30]. Previous studies based on high-throughput sequencing technology have speculated that there are as many as 5.1 million fungal species, most of which are involved in plant-endophytic interactions [31]. At present, only a small part of endophytic fungi are isolated and identified, and most of the endophytic fungi in medicinal plant cannot be purely cultured on the existing medium [32]. Therefore, it is necessary to detect the endophytic fungi community in medicinal plants by adopting non-culture methods.

Modern molecular technology, especially Illumina high-throughput sequencing technology, is an emerging technology in recent years, which can comprehensively and accurately detect the diversity of endophyte communities in medicinal plants [33, 34]. The high-throughput sequencing technique of next-generation sequencing is a more robust and accurate microbial community characterization technique compared to 18S rDNA-based non-culture methods and conventional culturing methods.

Numerous studies [35] have shown that the host genetic background (genotype or species) determine the composition of endophytic fungi. Meanwhile, soil fertility and ecological environment directly affect the content of bioactive compounds of medicinal plants, which will indirectly affect the composition and community structure of endophytic fungi [16]. However, for now, there is little information about the composition of endophytic fungi in the root of medicinal licorices at different genetic backgrounds (species), and soil environmental factors affecting the community structure of endophytic fungi in the root of medicinal licorice are still unclear. Therefore, in this study, we investigated the distribution and composition of endophytes fungal species of three medicinal licorices at three root depths through high-throughput sequencing and explored their relationship with host plants' bioactive compounds and soil physicochemical properties. The results will enhance researchers' understanding about the environmental and host factors that influence endophytic fungi and the friendly relationship between endophytic fungi and medicinal plants, thus providing reference information for licorice growing for commercial medicinal purposes.

2. Materials and Methods

2.1 Sample collection

The roots and rhizosphere soils samples (soil depths were 0-20cm, 20-40cm and 40-60cm, respectively) of three medicinal licorices (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata* and *Glycyrrhiza glabra*) were collected from August to September in 2019 from specimens growing at 3 distinct sites in 3 eco-regions in Xinjiang province, China; the geographical location of sampling points and soil physical and chemical properties are shown in Table 1. In addition, to ensure that the experiment was representative, we randomly selected three medicinal licorices plants in good growth condition from each geographical location according to the five-point sampling method, and all samples were cut with sterile scissors. The roots of each plant were divided into three sections: upper (0-20cm), middle (20-40cm), and lower (40-60cm), and the roots of each section are equally divided into two parts: one part was placed into a ziplocked bag for the determination of the bioactive compounds in the root, while the other part was placed into a sterile bag and quickly transported on a piece of ice to the laboratory in preparation for the microbe determination. All the samples were labeled by combination with letters and numbers, with the first letter representing the species (W, G and D: *Glycyrrhiza uralensis*, *Glycyrrhiza glabra* and *Glycyrrhiza inflata*, respectively), the second letter representing the root depth (1, 2 and 3: 0-20cm, 20-40cm, 40-60cm), and the third number representing the replicate number. For example, W.1.3 represents the third repetition of *Glycyrrhiza uralensis* at

0-20cm.

2.2 Surface sterilization

At the same time, to eliminate the interference of other microorganisms, the surface of roots was sterilized in the laboratory by first rinsing soil from the roots under running water followed by washing with sterile distilled water. The roots were then soaked in 75% alcohol for 30 s for surface disinfection, and then washed five times with sterile distilled water before soaking in 5% sodium hypochlorite for 5 min. Finally the roots were washed five times with sterile distilled water and air-dried under sterile conditions [36]. To confirm that the surface sterilization process was successful, the last rinse solution was inoculated onto a potato dextrose agar (PDA) plate and cultured at 28°C for 72 h. No fungi growth confirmed that the surface sterilization was successful [37]. All root samples were labeled and immediately placed on ice and then stored at liquid nitrogen prior to total DNA extraction.

2.3 Soil physicochemical

Soil samples from the rhizosphere were air-dried and sieved through a 2-mm mesh for soil physicochemical properties analysis. The following soil physicochemical characteristics were analyzed according to the methods described by the Bao et al [38]: The content of organic matter (SOM) was determined by external heating with potassium dichromate. Soil pH (1:2.5= soil: distilled water) was determined using a pH meter. Soil Water content (SWC) was determined by weighing. The total nitrogen (STN) content was determined using the perchloric acid-sulfuric

149 acid digestion method. The total phosphorus (STP) content was determined by acid
150 digestion (molybdenum-antimony colorimetry). The total potassium (STK) content
151 was determined by acid digestion (atomic absorption spectrometry). The total salt
152 (TS) content was determined by atomic absorption spectrometry. Nitrate nitrogen
153 (SNN) and ammonium nitrogen (SAN) contents were analyzed using 0.01 M calcium
154 chloride extraction. The available phosphorus (SAP) content was determined by
155 sodium bicarbonate extraction (molybdenum-antimony colorimetry). The available
156 potassium (SAK) content was determined by ammonium acetate extract method
157 (atomic absorption spectrometry).

158 **2.4 Determination of bioactive compounds in the root of medicinal licorices**

159 The root samples were dried at 60°C for 72 h to constant weight (it has been
160 confirmed that glycyrrhizic acid (GIA) and liquiritin (LI) do not decompose at this
161 temperature [39]). The dried root samples were ground to a powder with a pestle and
162 mortar and passed through a 60 mesh sieve. An aliquot (0.2 g) of powdered root
163 sample was extracted with 71% chromatographic methanol in an ultrasonic bath (250
164 W, 40 kHz) at room temperature. The extract was then centrifuged at 12,000 rpm for
165 10 minutes and the supernatant was filtered (0.22-μm pore size) (Agilent, USA). The
166 GIA and LI contents in the dried root samples (0.2 g) of the medicinal licorices were
167 determined by high-performance liquid chromatography (HPLC, Agilent-1260
168 Infinity, USA) using an Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm)
169 with mobile phase (chromatographic methanol: ultra-pure water: 36% glacial acetic

acid = 71:28:1) and mobile phase (acetonitrile:0.5% glacial acetic acid = 1:4) respectively, and a gradient elution flow rate of 1.0 mL•min-1. GIA and LI were detected at 254 nm and 276 nm, respectively. The injection volume was 5 μL and the column temperature was 30°C. The GIA and LI reference materials (CAS#1405-86-3 and CAS#551-15-5, respectively) were purchased from Solarbio and used for calibration purposes. The total flavonoid content (GTF) in medicinal licorices was determined by ultraviolet spectrophotometry at 334 nm with the liquiritin standard (CAS#551-15-5) as the control.

2.5 DNA extraction and library construction

After immersion in liquid nitrogen, genomic DNA was extracted from the samples using the DNA Quick Plant System kit (Tiangen, China) according to the manufacturer's instructions. The purity and concentration of DNA were detected evaluated using a NanoDrop2000 (Thermo Fisher Scientific, USA). According to the concentration, each DNA sample was diluted a final concentration to 1 ng/μL with sterile distilled water for use as a DNA template.

The ITS (Internal Transcribed Spacer) rDNA genes of the ITS1 region were amplified using specific primers (ITS5-1737F 5'-GGAAGTAAAAGTCGTAACAAGG-3'and ITS2-2043R 5'-GCTGCGTTCTTCATCGATGC-3') with barcodes [40]. PCR analyses were carried out with Phusion ® High-Fidelity PCR Master Mix and GC Buffer (New England Biolabs) to ensure amplification efficiency and accuracy. PCR runs started at 95 °C

for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 5 min.

The PCR product was mixed with the same volume of 1× TAE and then was detected by 2% agarose gel electrophoresis. The PCR product was purified from the target strip using a GeneJET Gel Extraction Kit (Thermo Scientific). The libraries were constructed using a TruSeq ® DNA PCR-Free Sample Preparation Kit (Illumina, USA) according to the manufacturer's instructions, and index codes were added. The library quality was assessed on the Qubit ® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, amplicon sequencing was performed using the Illumina HiSeq2500 platforms at the Beijing Compass Biotechnology Co., Ltd. (Beijing, China).

2.6 Bioinformatics analysis and statistical analysis

Single-end reads was assigned to samples using Cutadapt [41] software based on their unique barcode and truncated by cutting off the barcode and primer sequence. To avoid the influence of non-microbiota sequences (such as, chloroplast and mitochondrial sequences), the raw sequences were further filtered by Cutadapt software to remove non-microbiota taxa before subsequent analysis. Then raw tags were subjected to a strict quality controlled process using Cutadapt software to obtain high-quality clean reads. Clean reads were obtained by comparison with the reference database (Unite database) [42] using UCHIME algorithm to detect and remove chimeric sequences.

UPARSE software [43] (UPARSE v7.0.1001) was used to cluster the effective tags of all samples into the same operational taxonomic units (OTUs) with ≥97% identity, and taking the sequence with the highest frequency as the representative sequence of each OTU. The taxonomic information for each representative sequence was annotated using the Unite database, and multiple sequence alignment was performed using MUSCLE (Version 3.8.31) software to study the phylogenetic relationship of the representative sequences of OTUs among the 27 root samples. OTU abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences (54,262 reads for sample D.2.1). Subsequent analysis of alpha diversity and beta diversity were performed based on this output normalized data. The raw sequence reads have been deposited in the NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA664554.

Using the FunGuild database based on the species information obtained from amplicon analysis, the ecological functions of existing species in the environment can be inquired.

Alpha Diversity analysis was used to study the complexity of species diversity in a sample through six indices (observed-species, Shannon, Simpson, Chao1, ACE, and Good-coverage) [44]. All indices in the samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

Beta diversity analysis was used to evaluate differences in sample species complexity, which based on weighted Unifrac was calculated by QIIME software. The Un-weighted Pair-group Method with Arithmetic Mean (UPGMA) clustering analysis

was conducted by QIIME software (Version 1.7.0). In addition, R software (Version 2.15.3) was also used to rarefaction curve generation, Wilcoxon rank sum test, Metastat statistical test, Spearman correlation analysis of heat maps and Distance-based Redundancy Analysis (db-RDA). Pearson correlation analysis was run among the bioactive compounds and the soil physicochemical properties. Two-way ANOVA was performed with SPSS 19.0 (IBM Inc., Armonk, USA), and displayed with GraphPad Prism 5.

3. Results

3.1. Sequencing Results

In the root of the three medicinal licorices (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, and *Glycyrrhiza inflata*), a total of 2,118,633 effective sequences were obtained after filtering out low-quality and short sequence reads. The sequencing results for each sample are listed in Table S1. The effective sequences were clustered into OTUs with 97% identity, and a total of 1,063 OTUs were obtained, among them, 91.53% of the effective sequences were assigned to the Kingdom level, 59.27% to the phylum level, 54.37% to the class level, 53.72% to the order level, 46.19% to the family level, 38.01% to the genus level, and 23.52% to the species level by the Illumina HiSeq (Figure 1a). The rarefaction curves showed that the number of OTU in each sample increased gradually with quantity of sequence, thus confirming that the amount of sequencing data was adequate (Figure 1b).

3.2 Alpha diversity

The alpha diversity index of each group was shown in Table 2. Some indexes (Shannon and Chao1) respectively reflected the diversity and richness of microbial communities in samples, the greater the index, the higher the species diversity, the richer the distribution. The Shannon index of the W1 (4.910) sample was the highest. In contrast, that of the D1 (3.393) sample was the lowest. Moreover, we found that D1 had the lowest Chao1 (238.678) and ACE (253.105), while the D3 sample had the highest Chao1 (356.317) and ACE (355.694), respectively. Meanwhile, the results based on Wilcoxon rank sum test showed that the Shannon index was significantly different distribution between *Glycyrrhiza uralensis* and *Glycyrrhiza inflata*, especially 0-20cm at the root depth (Figure 2a). Specifically, the Shannon index in W1 sample was significantly higher than D1 sample ($p < 0.05$). Furthermore, the Chao1 index in *Glycyrrhiza inflata* increased gradually with the downward movement of root depths, and based on Wilcoxon rank sum test showed that the Chao1 index in *Glycyrrhiza inflata* was significantly affected by root depth (Figure 2b). Specifically, the Chao1 index in D3 sample was significantly higher than D1 sample ($p < 0.01$); D2 sample was significantly higher than D1 sample ($p < 0.05$).

3.3 Beta diversity

Beta diversity analysis was used to evaluate differences in microbial community composition among the samples. The Unweighted Pair-Group Method with Arithmetic (UPGMA) cluster analysis was performed to study similarity in the

composition of endophytic fungal community among different samples, and the clustering results were integrated with species relative abundance at phyla taxon level in each group. As shown in Figure 3a, the results of UPGMA cluster tree based on Weighted Unifrac distances showed that samples from G3, G2, G1 and W1 were clustered together, and samples from W3, D2, W2 and D1 were clustered together (Figure 3a). Meanwhile, for the difference analysis between the beta diversity, a Wilcoxon rank sum test based on Weighted Unifrac distances was constructed (Figure 3b), and the results showed that there were significant differences in beta diversity between *Glycyrrhiza uralensis* and *Glycyrrhiza inflata*, which was consistent with UPGMA cluster tree. Specifically, there were significant differences in beta diversity between D1 and D2 samples ($P < 0.05$), D3 and W3 samples ($P < 0.05$), and D1 and W1 samples ($P < 0.01$) (Figure 3b), which indicated there were significant differences in endophytic fungal community composition in roots of medicinal licorices between different species and different root depth.

3.4 Composition of fungal community in the root of medicinal liquorices

According to the OTUs sequence and Unite database, 8 phyla, 23 classes, 53 orders, 102 families, 140 genera and 141 species were annotated. The endophytic fungal phyla with the greatest abundance from nine groups were enumerated in Figure 4a. Ascomycota dominated the observed sequences at the phylum level, representing 91.821%, 60.558%, 39.956%, 79.651%, 62.305%, 54.241%, 82.176%, 81.928% and 80.290% of the total number of species in D1, D2, D3, G1, G2, G3, W1, W2 and W3,

296 respectively. In addition, Basidiomycota occupied a large part of the relative
297 abundance in D2 (21.348%), D3 (28.440%), G2 (10.631%), G3 (12.523%), W2
298 (6.749%) and W3 (5.110%), respectively. Meanwhile, our results showed that the
299 relative abundance of Ascomycota gradually decreased with the downward movement
300 of root depths. For the difference analysis at the Phylum classification level, a
301 MetaStat statistical test based on species abundance was constructed, and the results
302 showed that the relative abundance of Ascomycota in *Glycyrrhiza inflata* significant
303 difference distribution at different root depth (Figure 4b). Specifically speaking, the
304 relative abundance of Ascomycota at D1 sample (91.821%) was significantly higher
305 than D3 sample (39.956%) (Figure 4b).

306 In terms of genus, we listed the top 10 dominant fungal genera in each group in
307 Figure 4c: *Fusarium* was found to be the predominant genus in D1 (27.907%), G1
308 (23.944%), G2 (31.071%), G3 (25.381%), W1 (19.253%) and W3 (18.215%).
309 Meanwhile, the abundance of *Paraphoma* was high in the D1, D3 and W3 samples,
310 accounting for 27.738%, 23.937% and 13.980%, respectively. *Helminthosporium*
311 occupied a large part of the relative abundance in D1 (26.567%), G1 (25.124%), W1
312 (8.224%) and W2 (17.408%), respectively. *Sarocladium* occupied a large part of the
313 relative abundance in D2 (3.326%), G1 (16.547%), G2 (17.243%), G3 (21.897%) and
314 W1 (4.218%), respectively, the abundance of *Cladosporium* was high in D2 (6.446%),
315 D3 (2.721%) and W3 (15.174%). *Cadophora* (13.200%) and *Psathyrella* (10.917%)
316 were found to be the most dominant in D2 sample. *Tomentella* (14.472%) was found
317 to be the most dominant in D3 sample. *Conocybe* (12.068%) was found to be the most

dominant in G3 sample (Figure 4c).

At the same time, details of the composition of the top 10 dominant fungi at other classification levels (Class, Older, Family and Species) were listed in Table S2. Specifically speaking, Sordariomycetes, Dothideomycetes and Agaricomycetes were dominate at the class taxonomic level; the dominant species at the order taxonomic level are Hypocreales, Pleosporales, Thelephorales; the dominant species at the family taxonomic level are Nectriaceae, Phaeosphaeriaceae, Massarinaceae; the dominant species at the species taxonomic level are *Fusarium-solani*, *Paraphoma-radicina*, *Sarocladium-kiliense*.

Based on the ITS amplicons analysis, we obtained the classification and abundance information of endophytic fungal community in root of medicinal licorices; we also pay attention to what role these species play in the ecosystem. The top 25 main ecological function of fungal species based on FunGuild analysis was shown in figure 5. Plant_ Pathogen-Soil_ Saprotroph-Wood_ Saprotroph (32.072%) was found to be the most dominant in G2 sample; Fungal_ Parasite-Plant_ Pathogen (26.567%) was found to be the most dominant in D1 sample; the abundance of Undefined_ Saprotroph was high in the G1, G2 and G3 samples, accounting for 21.271%, 28.330% and 35.555%, respectively. Ectomycorrhizal (21.187%) and Endophyte (13.208%) were found to be the most dominant in D2 sample. However, a much higher proportion of unassigned ecological function existed in all groups.

3.5 The relationship between endophytic fungal communities and the bioactive compounds and soil physicochemical properties

The results of two-way ANOVA showed that the content of the bioactive compounds (glycyrrhizic acid (GIA), liquiritin (LI) and total flavonoid (GTF)) were not significantly affected by the interaction effect between root depth (0-20cm, 20-40cm, 40-60cm) and plant species (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra*) ($P > 0.05$) (Table3). However, the content of LI was significantly affected by the main effect plant species ($P < 0.05$) (Table 3 and Figure 6). As shown in the Figure 6, the contents of LI in root of *Glycyrrhiza uralensis* were significantly higher than those in *Glycyrrhiza inflata* ($P < 0.05$), and the contents of LI in root of *Glycyrrhiza uralensis* were significantly higher than those in *Glycyrrhiza glabra* ($P < 0.05$) (Figure 6a).

In addition, Pearson correlation analysis showed that the content of bioactive compounds was significantly correlated with soil physicochemical properties (Table S3). GIA content in root had a very significant positive correlation with available potassium (SAK) and soil water content (SWC) ($r > 0$; $P < 0.05$), but LI content in root had a very significant negative correlation with SAK and total salt (TS) content ($r < 0$; $P < 0.05$).

Furthermore, spearman correlation analysis showed that the content of LI was significantly positive correlated with alpha diversity index ($r > 0$, $P < 0.05$) (Figure 7). As shown in Figure 7, the content of LI had a very significant positive correlation with Shannon index, Simpson index and Chao1 index ($P < 0.05$), which indicated that

360 the content of LI was accountable for the differences in the diversity of endophytic
361 fungal community in this study.

362 Meanwhile, spearman correlation analysis showed that there was a significant
363 relationship between dominant fungi phylum and bioactive compounds and soil
364 physicochemical properties (Table 4). Specifically, Ascomycota showed a very
365 significant negative correlation with RWC ($r < 0$, $P < 0.01$); Basidiomycota showed a
366 very significant positive correlation with RWC ($r > 0$; $P < 0.01$); Olpidiomycota
367 showed a significant positive correlation with GIA ($r > 0$; $P < 0.05$);
368 Mortierellomycota showed a significant positive correlation with STK, SWC and
369 SAK ($r > 0$; $P < 0.05$); Mucoromycota showed a very significant positive correlation
370 with SOM ($r > 0$; $P < 0.01$), but a significant negative correlation with STP ($r < 0$, $P <$
371 0.01); Rozellomycota showed a significant positive correlation with SOM, STK and
372 RWC ($r > 0$; $P < 0.05$).

373 At the same time, as shown in Figure 8, there was a significant relationship
374 between the dominant fungi genus and bioactive compounds and soil physicochemical
375 properties. Specifically, *Fusarium* showed a significant positive correlation with LI
376 content ($P < 0.05$); *Paraphoma* showed a significant positive correlation with SAN (P
377 < 0.05), but a significant negative correlation with SAK, TS and SWC ($P < 0.05$);
378 *Helminthosporium* showed a significant positive correlation with PH ($P < 0.05$);
379 *Sarocladium* showed a significant negative correlation with SOM, STN and SNN ($P <$
380 0.05); *Conocybe* showed a significant positive correlation with SWC, but a significant
381 negative correlation with SAN ($P < 0.05$).

382 Distance-based redundancy analysis (db-RDA) based on the Bray–Curtis
383 distance showed that the bioactive compounds and soil physicochemical had
384 significant effects on the differences of endophytic fungal community (Figure 9). The
385 differential distribution of endophytic fungal community was mainly restricted in the
386 first and second ordination axes, and the first ordination axis, the second ordination
387 axis were explained 16.23%, 13.89% of the total variability, respectively (Figure 9).
388 Specifically, among the soil environment factors, SAK content was identified as the
389 factor that most significantly affects the differences of endophytic fungal community
390 ($r^2 = 0.329$, $P < 0.01$), followed by SAN ($P < 0.05$). Among the root factors, the RWC
391 was explained the difference of endophytic fungal communities in roots to the greatest
392 extent ($r^2 = 0.247$, $P < 0.05$), followed by LI content ($P < 0.05$) (Figure 9, Table 5).
393 According to results of the db-RDA analysis, the SAN, SAK, RWC, and LI content
394 were the major factors contributing to the variations in the overall structure of
395 endophytic fungal community in this study.

396 ## 4. Discussion

397 In this study, we investigated the composition and diversity of endophytic fungal
398 communities in different root depth (0-20cm, 20-40cm and 40-60cm) of three
399 medicinal licorices (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, and *Glycyrrhiza*
400 *inflata*) using high-throughput sequencing technology, which provides a large amount
401 of data with more accuracy than that obtained in previous studies using traditional

technology [45-47]. We obtained the composition of endophytic fungal communities at different taxonomic levels (phylum, class, older, family, genus and species) by high-throughput sequencing (Figure 4a, Figure 4c and Table S2). The results showed that there was a specific microbiome in 27 samples of tree medicinal licorices, and the relative abundance of endophytic fungi was correlated with the host plant species and root depth. For example, Ascomycota was the dominant phylum in all samples, followed by Basidiomycota, which result consistent with previous studies [48, 49]. The phylum Ascomycota, as the largest phylum of fungi, has diverse populations and plays an important role in genetics [50], ecology [51] and phylogeny [52]. Such as, the Ascomycota produce large numbers of spores through both asexual and sexual reproduction. Asci can act as small water cannon, spraying spores into the air. Dispersal process of ascospores, spores is important for dissemination of many fungal plant diseases and for the dispersal of many saprophytic fungi [53].

Moreover, our results showed that the relative abundance of Ascomycota gradually decreased with the downward movement of root depths (Figure 4b), which was consistent with the results of Ko, Daegeun et al [54]. On this basis, we found that the relative abundance of Ascomycota in *Glycyrrhiza inflata* had a significant difference at different root depth, but *Glycyrrhiza uralensis* and *Glycyrrhiza glabra* were not significant difference, indicating that some endophytes may preferentially proliferate in a certain ecological region and play different ecological roles from other endophytes. Overall, in addition to soil depth, the relative abundance of endophytes was also related to the genotype of the host plant species. This was consistent with the

results of host genotype and soil conditions on ectomycorrhizal community of poplar clones by Karliński, Leszek et al. [55].

Alpha Diversity and Beta Diversity analysis of endophytic fungal community showed significant differences in root depths (0-20cm, 20-40cm and 40-60cm) between *Glycyrrhiza uralensis* and *Glycyrrhiza inflata* (Figure 2 and Figure 3), which indicated that both genotype and ecological region of host plants contributed to the differences of endophytic fungal community. Meanwhile, numerous studies [56] have shown that the adaptation of endophytic fungal community largely depends on the adaptation of host plants to the ecological environment, which indicated that host plants largely determine the colonization and distribution of endophytic fungal community. The relationship between fungus and host plant were also often considered as a flexible interaction, with orientations determined by subtle differences in the expression of fungal genes in response to the host, or conversely, by the host's recognition and response to the fungus. Thus, slight genetic differences in the two genomes control the symbiosis [57].

Furthermore, our results showed that the root depth had a significant effect on the richness and composition of endophytic fungal community (Figure 2b, Figure 3a and Figure 3b), which indicated that different ecological types of endophytic fungi may represent certain ecological regions (different root depth), these should become an important consideration factor that endophytic fungi inoculation. We speculated that this is related to root respiration and soil C content. On the one hand, root respiration, accounts for 60% of total soil respiration, can regulates the metabolism of

446 roots and related microorganisms, and is an important part of terrestrial carbon budget
447 [58]; on the other hand, the content of C in unstable soil varies greatly between
448 different soil depths [59]. Moreover, Noah Fierer et al. [60] demonstrated that the
449 vertical distribution of the specific microbial species was largely related to the
450 decrease in carbon availability with soil depth.

451 However, one weakness in this study was that the samples of three *glycyrrhiza*
452 species were collected from areas which differed in geographical environment. Since
453 it is rare to find three *glycyrrhiza* species in the same habitat, to a certain extent, the
454 soil physicochemical properties can represent the environmental factors in which
455 three *glycyrrhiza* species were growing. Therefore, in this study, in addition to root
456 factor, also included the effects of the soil factors.

457 Numerous studies [61, 62] showed the accumulation of bioactive compounds in
458 medicinal licorice roots is affected by various factors. In this study, the content of LI
459 were more affected by main effect plant species than main effect root depth (Table 3),
460 among them, the content of LI in root of *Glycyrrhiza uralensis* were significantly
461 higher than those in *Glycyrrhiza inflata* and *Glycyrrhiza glabra* (Figure 6a), which is
462 consistent with the results of Zhang et al. [63]. We speculate that this is related to the
463 expressions of some functional genes that are closely associated with the content of
464 bioactive compounds including glycyrrhizic acid and liquiritin in root of licorice
465 species. Some studies [64-66] have shown that key functional genes, such as chalcone
466 synthase gene, 3-Hydroxy-3-methylglutary CoA reductase (HMGR) and squalene
467 synthase (SQS), are involved in transcriptional level regulation process in glycyrrhizic

acid and liquiritin biosynthesis. Although further studies are required to characterize the expression of functional genes of bioactive compounds, this study provides a theoretical basis for the development of strategies to expand the *Glycyrrhiza uralensis* cultivation. On the other hand, the content of bioactive compounds is the result of the interaction between plants and their growing environment, therefore, the accumulation of bioactive compounds in root is influenced by the ecological environment of its. In this study, GIA, GTF and LI content had a positive correlation with soil total nitrogen (STN) ($r > 0$), indicating that soil nutrients can promote the accumulation of bioactive compounds, but not all soil nutrients, such as soil total potassium (STK), have such a function. Although potassium can be involved in many enzyme activation systems in plants and improve plant stress resistance [67], the content of GIA, GTF and LI were negatively correlated with STK ($r < 0$) in this study, which is consistent with the results of Liu et al [68]. In addition, soil available potassium (SAK) had a significant positive correlation with GIA, but had a significant negative correlation with LI (Table S3), indicating that the utilization mechanism of soil nutrients by bioactive compounds is completely different. Although the mechanism by which available potassium regulate bioactive compounds is still unclear, this discovery may form the basis of further in-depth research. In general, these soil factors exhibit habitat specific characteristics are related to the regulation of bioactive compounds in root of licorice.

In recent years, a growing number of studies [69-71] have demonstrated that the dynamics of the microflora is driven to a large extent by environmental factors

including soil characteristics (pH, nitrogen, phosphorus and potassium) and climate condition (rainfall and temperature). Consistent with these reports, our results showed that LI, RWC, SAN and SAK content were the major factors contributing to the variations in the overall structure of endophytic fungal community (Figure 9 and Table 5). In addition, we found that the content of LI in root had a very significant positive correlation with diversity of endophytic fungal community (Shannon and Simpson index) ($P < 0.05$) (Figure 7). Liquiritin (LI), the main bioactive compounds of flavonoids, is one of the material basis for clinical efficacy and an important index of the quality of medicinal licorices. Flavonoids can be specifically induced by symbiotic fungus to respond to purified signaling molecules from these organisms when the fungus colonizes. Chen et al. [72] demonstrated that with inoculation of fungi *Glomus mosseae*, *Glycyrrhiza uralensis* plants significantly increased stem and root biomass and liquiritin content in the main root.

Meanwhile, our results showed that soil physicochemical and bioactive compounds had a significant effect on composition of endophytic fungal communities (such as phylum and genus) (Figure 8 and Table 4), which showed that there is an interaction among endophytic fungal community, root and soil factor. This suggests that we may be able to alter the fungal composition by altering soil factors [73], thereby promoting the accumulation of bioactive compounds in plants [74]. In the case of medicinal licorices, Wei Xie et al. [75] shown that P addition and arbuscular mycorrhizal (AM) inoculation could improve plant growth and facilitated glycyrrhizic acid and liquiritin accumulation in *Glycyrrhiza uralensis*. Meanwhile, Y. Orujei et al.

[76] also shown that two species of arbuscular mycorrhizal fungi (AMF) were successful inoculation, the increase in the growth rate and the accumulation of bioactive compounds in licorice roots (*Glycyrrhiza glabra*) were observed compared to control. In general, this study provided useful an information for the development of strategies to improve the production and quality of medicinal licorices, although further studies are required to characterize the functions of these endophytic fungi.

5. Conclusions

In this study, numerous endophytic fungal communities were detected in roots of medicinal licorices based on high-throughput sequencing. Furthermore, we identified significant differences in the relative abundance of Ascomycota among root depth. Furthermore, the alpha diversity analysis and beta diversity analysis showed that the endophytic fungal community structure and composition differed among the species and root depth in medicinal licorices. Moreover, the SAN, SAK, RWC, and LI content were the major factors contributing to the variations in the overall structure of endophytic fungal community in this study. This study clarified the ecological role of non-biological factor (soil and root) in the endophytic fungal community of medicinal licorices, which may provide theoretical basis for the synthesis of bioactive compounds and rational utilization of medicinal plants in production practice.

Data Accessibility Statement

All data generated or analysed during this study are included in this published article. The raw sequence reads of amplicon sequencing have been deposited in the NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA664554 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA664554?reviewer=mk27e1r5vjo257l0n599u2e0en).

Author Contributions

Hanli Dang: Conceptualization; Formal Analysis; Investigation; Writing – original draft; **Tao Zhang:** Investigation; Methodology; Software; **Zhongke Wang:** Investigation; Methodology; Software; **Guifang Li:** Investigation; Methodology; **Wenqin Zhao:** Investigation; Methodology; **Xinhua Lv:** Investigation; Methodology; **Li Zhuang:** Investigation; Supervision; Writing – review & editing.

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Conflicts of Interest

None declared

Ethics statement

None required

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Figure Legends

Figure 1 . Distribution of the number of tags on each classification level (Kingdom, Phylum, Class, Order, Family, Genus and Species) (a); Rarefaction curves of fungal community composition (b)

Description: Sample Name: D, G and W: *Glycyrrhiza inflata*, *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*; 1, 2 and 3: root depth 0-20cm, 20-40cm, and 40-60cm, respectively; the third number representing the replicate number. The rarefaction curves different colors represent different samples.

Figure 2 . The significance test of the differences of Alpha Diversity

Description: Ordinates are Shannon index (a) and Chao1 index (b), respectively. Abscissa is the group name: D, G and W: *Glycyrrhiza inflata*, *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*; 1, 2 and 3: root depth 0-20cm, 20-40cm, and 40-60cm, respectively. The mark * is significance test $p < 0.05$.

Figure 3. Unweighted Pair-Group Method with Arithmetic (UPGMA) clustering tree base on the weighted unifrac distance (a); and the significance test of the differences of Beta Diversity (b).

Description: a: The left is the UPGMA cluster tree structure, and the right is the distribution of relative abundance of each sample at the phylum level; b: Ordinate is the Beta diversity; Abscissa is the group name: D, G and W: *Glycyrrhiza inflata*, *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*; 1, 2 and 3: root depth 0-20cm, 20-

804 40cm, and 40-60cm, respectively. The mark * is significance test $p < 0.05$.

805 **Figure 4 .** Histograms of relative abundance of the top 10 endophytic fungi at the

806 phyla (**a**) and genera (**c**) level of taxonomy; difference analysis at the Phylum

807 classification level (**b**).

808 Description: Ordinate is the relative abundance; others refers to are sequences with

809 less or not be annotated. Abscissa is the group name: D, G and W: *Glycyrrhiza inflata*,

810 *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*; 1, 2 and 3: root depth 0-20cm, 20-

811 40cm, and 40-60cm, respectively. ** means $P<0.01$.

812 **Figure 5 .** Histograms of relative abundance of the top 25 main ecological function

813 Description: Ordinate is the relative abundance; others refers to are sequences with

814 less or not be annotated. Abscissa is the group name: D, G and W: *Glycyrrhiza inflata*,

815 *Glycyrrhiza glabra* and *Glycyrrhiza uralensis*; 1, 2 and 3: root depth 0-20cm, 20-

816 40cm, and 40-60cm, respectively.

817 **Figure 6 .** Effect of main effect plant species on the bioactive compounds of licorice

818 roots

819 Description: Ordinate is the content of LI (a), GIA (b) and GTF (c); abscissa is the

820 group name: D, G and W: *Glycyrrhiza inflata*, *Glycyrrhiza glabra* and *Glycyrrhiza*

821 *uralensis*, and the mark * is significance test ($p < 0.05$).

822 **Figure 7** . Heatmaps of Spearman correlation analysis

823 Description: Ordinate is the information of environmental factors, and abscissa is the

information of alpha diversity indexes. The correlation coefficient r of Spearman is between -1 and 1, $r < 0$ is negative correlation, $r > 0$ is positive correlation, and the mark * is significance test ($p < 0.05$).

Figure 8．Heatmaps of Spearman correlation analysis

Description: Ordinate is the information of environmental factors, and abscissa is the information of species at the genera level of taxonomy. The correlation coefficient r of Spearman is between -1 and 1, $r < 0$ is negative correlation, $r > 0$ is positive correlation, and the mark * is significance test ($p < 0.05$).

Figure 9．Distance-based redundancy analysis (db-RDA) for all groups

Description: Environmental factors are generally represented by arrows. The length of the arrow line represents the degree of correlation between a certain environmental factor and community and species distribution, and the longer the arrow, the greater the correlation. When the angle between the environmental factors is acute, it means that there is a positive correlation between the two environmental factors, while when the angle is obtuse, there is a negative correlation.

Supplementary Materials

Table S1 Sequencing results of each sample. Table S2 Composition of dominant fungi at each classification level. Table S3 Pearson correlation coefficient of the content of bioactive compounds with soil physicochemical properties.