Microbiota Diversity of *Festuca Sinensis* Seeds in Different Location of Qinghai-Tibet Plateau

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Abstract

Background: The Qinghai-Tibet Plateau is characterized by strong ultraviolet rays, extended sunshine durations, high altitudes, substantial temperature differences between day and night, dry air, and poor soil water and fertilizer preservation ability[54]. Although the environment and climatic conditions of the Qinghai-Tibet Plateau and growth conditions of plants are well known, relatively few studies have been conducted on the effects of environmental factors on seed microbiota diversity on the Qinghai-Tibet Plateau. *Festuca sinensis* is a cool-season perennial grass species suitable for growth on the Qinghai-Tibet Plateau. Therefore, investigating the *Festuca sinensis* seed microflora diversity could play an important role in establishing plant species diversity on the Qinghai-Tibet Plateau.

Results: *Festuca sinensis* seeds were collected from 14 locations on the Qinghai-Tibet Plateau, and their endophyte status and seed microflora were analyzed to determine the effects of endophytes and host growth environment on the microflora of *F. sinensis* seeds. The results showed that the endophyte infection rate of these germplasms ranged from 0% to 80%. Endophyte infection rates were significantly negatively correlated with elevation(P<0.05)and significantly positively correlated with monthly mean temperature (MMT)(P<0.05)and growing monthly mean temperature (GMMT)(P<0.01). Microflora analysis using high-throughput sequencing showed that Proteobacteria, Cyanobacteria, and Bacteroidetes were the most abundant bacteria at the phylum level, and Ascomycota and Basidiomycota were the most abundant fungi at the phylum level in seeds. Regarding the relative abundance of each phylum in different seed lots, significant differences occurred among the 14 ecotypes. Bacterial and fungal diversity indices, including Chao1, Shannon, Simpson, and Adaptive Communication Environment (ACE), showed significant differences among these 14 ecotypes, although they were not consistent among the indices. These diversity indices were correlated with the host growth environment. For example, the Chao richness and ACE indices of bacteria were significantly negatively correlated with monthly mean precipitation, annual mean precipitation, and growing monthly mean precipitation(P<0.05). The Chao richness index of fungi was significantly negatively correlated with MMT, annual mean temperature (AMT), and GMMT (P<0.05). The ACE index of fungi was significantly negatively correlated with MMT, AMT, and GMT(P<0.01). The relative abundance (ACE index) of fungi was significantly positively correlated with elevation. The Chao richness index of fungi was significantly negatively correlated with MMT, AMT, and GMMT.

Conclusions: These results suggest that average precipitation had significant effects on the abundance of bacteria, whereas the endophyte infection rate, elevation, and average temperature significantly affected the abundance of fungi. Temperature and elevation had significant effects on the endophyte infection rate.

Introduction

Festuca sinensis, a native cool-season perennial grass species, is distributed across the cold and semi-arid regions of China. This species, grazed by cattle and sheep, is widely utilized in grassland production on the Qinghai-Tibet Plateau of China[1]. It is also important for grassland establishment, restoration of degraded grasslands, and ecological management. *F. sinensis* is frequently infected by an asexual, symptomless *Epichloë* species[[2, 3]. Many studies of endophyte-grass symbioses have revealed that endophytes can confer a range of benefits to their grass hosts[4]. *Epichloë* endophytes interact mutualistically with their host plant, mainly by enhancing the fitness of the grass host and protecting them from both biotic and abiotic stresses [5]. The enhancing roles of the endophyte on host plant performance may be related to growth hormones[6] or secondary metabolites, such as alkaloids[7]. The *Epichloë* endophyte associated with *F. sinensis* has been isolated and identified by morphology, including colony, texture, conidia, and conidiophores. Recently, a phylogene with a house-keeping gene confirmed that this strain is a new species, *Epichloë sinensis*[8].

The plant microbiome is a key determinant of plant health and productivity and has received substantial attention in recent years[9]. All tissues of a plant can host a microbial community. The biodiversity of microbial communities has important implications for the stability and function of managed and natural ecosystems[10]. The microbiome of plants varies based on the host environment, with each containing core phyla[11]

Seeds are the most basic and extensive plant propagation resources in agricultural production. The quality of seeds directly affects the growth and germination of seeds, and consequently, the growth and development of plants. During seed generation, a new compartment of the microbiome is created. Seeds contain initial reservoirs of endophytes within the endosphere inherited from their parents[12, 13]. Recent omics-based analyses show that plant seeds also contain beneficial, plant genotype-specific microbes, which can be vertically transmitted from one plant generation to the next [14, 15]. Studying the influence of the environment on the microbial diversity of seeds can screen for high-quality germplasm resources in specific regions at high altitudes. Therefore, to ensure excellent planting resources, it is necessary to study the microbial diversity of the seeds.

Although there has been a considerable amount of research dedicated to the effects of *Epichloë* species on pastures, there has been little dedicated to other fungi or bacteria or the wider microbiome. Recent research has also produced conflicting results regarding the pasture microbiome in the context of *Epichloë* fungal endophytes. For example, Nissinen et al. demonstrated that resident *E. coenophiala* as a keystone species, which had divergent impacts on bacterial and fungal communities in the leaf endosphere of *Schedonorus phoenix*, shaping fungal but not bacterial communities[16]. However, Tannenbaum et al. identified the effect of *E. festucae* var. *lolii* on the bacterial microbiomes of pooled young perennial ryegrass seedlings[11]. Hence, it is of substantial importance to study the microflora diversity of different *F. sinensis* seeds from different sites of the Qinghai-Tibet Plateau and their relationships with endophyte status.

The influence of seeds on microbial growth is hypothesized to be caused by nutritional and surface-attachment opportunities. Environmental factors, such as temperature, precipitation, and elevation, could be the main factors affecting microbial diversity. Very few studies have highlighted the effects of environmental factors on seed microbial diversity. Wagner et al. reported that host genotype-by-environment interactions contribute to the complexity of the microbiome assembly on the leaves and roots of *Boechera stricta* in natural environments[17]. Fan et al. showed that the physical properties of soil from different geographic locations affected microbiome community structures[18]. Bryant et al. suggested that bacterial taxon richness and phylogenetic diversity decreased monotonically from the lowest to the highest elevations, and bacterial lineages exhibited significant spatial structure across the gradient[19]. Bulgarelli et al. and Zhang et al. also showed that different biogeographical locations that had vast arrays of climatic conditions, such as precipitation, temperature, pH, and light, influenced the microbiome community structures of barley (*Hordeum vulgare*) and soybeans (*Glycine max*)[20, 21].

Therefore, the goals of this study were to: 1) screen the endophyte status in different lots of *F. sinensis* seeds from 14 locations in the Qinghai-Tibet Plateau; 2) determine the microflora in *F. sinensis* seeds by high-throughput sequencing; and 3) reveal the relationship between endophytes and microflora, and the relationship between the host growth environment and microflora.

Materials And Methods

Seeds materials

Fourteen seed lots of *F. sinensis* were collected from different locations on the Qinghai-Tibet Plateau, as shown in Fig 1 and Table 1. The endophyte status of these seed lots was detected by the aniline blue staining method[22], and 100 seeds of each ecotype were used to determine the endophyte infection rate of the seeds.

Total DNA extraction

Each seed lot had four replicates, with 50 mg of seeds per replication. Genomic DNA of each replication was extracted by the CTAB/SDS method[23], and the purity was determined by 1% agarose gel electrophoresis. DNA was diluted to 1 μg/L with sterile water after the DNA concentration was detected by NanoDrop 2000[24].

High-throughput sequencing

Specific primers with barcodes were synthesized to amplify the bacterial 16s rDNA V4–V5 region and fungal ITS1 or ITS2 region[25]. The primer sequences are listed in Table 2.

Phusion® Hi-Fi PCR Master Mix (New England Biolabs) was used for all PCR reactions. Each PCR mixture contained 5 μL genomic DNA (40–60 ng), 1.5 μL forward primer (10 μM), 1.5 μL reverse primer (10 μM), 1 μL Toyobo, 1 μL KOD FX Neo Buffer (2X), 10 μL dNTP (2mM), and ddH2O was added for a total 50 μL volume. PCR was performed under the following conditions: 1 cycle at 95 °C for 10 min, 15 cycles at 95°C for 1 min, at 50 °C for 1 min, at 72 °C for 1 min, and finally 72 °C for 7 min. The PCR products were qualified with electrophoresis in 2% agarose gel and were purified by magnetic beads using TruSeq® DNA PCR-Free Sample Preparation kits as per the manufacturer's instructions. Then, the purified product was amplified with 30 μL PCR mixture, including MPP-A (10 μM) 1 μL, MPP-B (10 μM), 1 μL of 2× pHusion HF MM 20 μL, and ddH2O 8 μL. The PCR was performed under the following conditions: 1 cycle at 95 °C for 30 s, 10 cycles at 95 °C for 10 s, at 65 °C for 30 s, at 72 °C for 30 s, and finally at 72 °C for 7 min. These PCR products were equivalently mixed after the DNA concentration was quantified using the enzyme labeling method[35]. The PCR mixtures were purified by 2% agarose gel and extracted using kits. The DNA library was constructed after homogenization and quantified by Qubit and q-PCR (AB 9902). After the library was qualified, NovaSeq6000 was used for sequencing[26].

Data analysis

Trim Galore FLASH2 software[27]was used for quality control and filtering the original sequence to obtain optimized sequences with high quality and reliability (clean reads)[28]. The valid data for subsequent analysis were obtained through chimeric filtering based on clean data. The number of operational taxonomic units (OTUs) was classified at a 97% similarity level. Finally, the OTU representative sequence was selected and compared with the database to complete taxonomic annotation of the OTUs.

Alpha and beta diversity values were calculated using standard metrics, such as Chao1, Simpson, Shannon, and ACE (Adaptive Communication Environment), and are available in QIIME [29].Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows (Version 23.0; SPSS, Inc., Chicago, IL). The differences in alpha diversity indices between the groups were analyzed using R software (Version 2.15.3). Statistical significance was determined using a one-way or two-way analysis of variance (ANOVA). When the ANOVA indicated a significant difference, Fisher's least significant difference (LSD) test was applied to conduct multiple pairwise comparisons. Statistical significance was set at $P < 0.05$.

Meteorological data were obtained from the China Meteorological Center (Http://data.cma.cn). According to the longitude, latitude, and altitude of each sampling point, the thin plate smoothing spline algorithm in package Anusplin 4.4 was used to calculate the Kriging difference (Version 4.4, Canberra, Australia; http://fennerschool.

anu.edu.au/files/anusplin44.pdf) to obtain the monthly mean temperature (MMT) and monthly mean precipitation (MMP) at each sampling point from 2006 to 2015. Additionally, the growing season of *F. sinensis* is from April to August, and the average temperature and precipitation from April to August were calculated as the mean temperature and precipitation during the growing season. Microsoft Excel 2016 was used to calculate the MMT, MMP, annual mean temperature (AMT), annual mean precipitation (AMP), and the growing monthly mean temperature (GMMT) and growing monthly mean precipitation (GMMP).

Spearman correlation analysis was used to analyze the correlation between environmental factors, endophyte infection rate, and α-diversity of the most abundant bacterial and fungal phyla. All correlations and tests were considered significant at $p<0.05$. Origin software was used for redundancy analysis (PCA). When an acute angle is presented, it indicates a positive correlation between the two; however, an obtuse angle indicates a negative correlation. PCA diagrams were drawn using R software (Version 2.15.3). PCA analyses were performed using the ade4 package and ggplot2 package of R software (Version 2.15.3).

Results

Principal Component Analysis (PCA) of endophyte infects rate and environmental factors

The endophyte infection rate of these 14 seed lots ranged from 0% to 80%(Table 1). PCA was used to explain the relationship between the endophyte infection rate and elevation, GMMP, MMP, MMT, and GMMT. The PCA suggested that the endophyte infection rate was positively correlated with GMMT and MMT, whereas it was negatively correlated with elevation, GMMP, and MMP(Figure 2). Spearman correlation analyses showed that the endophyte infection rate was significantly positively correlated with MMT($P<0.05$) and GMMT($P<0.01$), whereas it was significantly negatively correlated with elevation ($P<0.05$Table 4).

Sequencing annotation

After 16S rDNA sequencing, an average of 104,769 tags was detected per sample, and 65,201 tags were obtained on average after quality control, with the efficiency of quality control reaching 62.28%. Sequencing was clustered into OTUs with 97% identity, and a total of 3,003 OTUs were obtained. Taxonomic annotations of these OTUs were then conducted using the SILVA138 database. There were 2,862 (95.30%) OTUs that could be annotated in the database.

After fungal ITS sequencing, an average of 103,696 tags was measured per sample. The quantity of effective data under quality control reached 65,156, and the quality control efficiency reached 62.88%. The OTUs were clustered with 97% identity, and 636 OTUs were obtained. Species annotation was performed with the OTU sequence and the Unite databases, and 592 (93.08%) OTUs could be annotated.

For bacterial sequencing, Proteobacteria, Cyanobacteria, and Bacteroidetes were the most abundant phyla. Alphaproteobacteria, Cyanobacteria, and Gammaproteobacteria were the most abundant classes. Sphingomonadales and Rickettsiales were the most abundant orders. Sphingomonadaceae was the most abundant family. For fungal sequencing, Ascomycota and Basidiomycota were the most abundant phyla. Sordariomycetes, Dothideomycetes, and Tremellomycetes were the most abundant classes. Hypocreales, Pleosporales, and Xylariales were the most abundant orders. Nectriaceae and Microdochiaceae were the most abundant families.

Microflora diversity in seeds

Veen plot analysis of bacteria and fungi OTUs in seeds

A total of 1062 bacterial OTUs were obtained by clustering in the Venn diagram(Fig 3a), and 117 OTUs were shared by seed bacterial communities, accounting for 11.02% of the total number of OTUs. Ecotype S99 had the largest number of OTUs (252), followed by ecotypes S116 (248) and S52 (225). Ecotype S057 and S85 had the lowest number of OTUs (170), followed by ecotype S041 (180). A total of 253 fungal OTUs were used in the Venn diagram (Fig 3(b)), and 85 OTUs were shared by fungal communities in the seeds of 14 ecotypes, accounting for 33.60% of the total number of OTUs. Ecotype S52 had the highest number of OTUs (139), followed by ecotype S22 (126). Ecotypes S99 (87), S85 (87), S261 (90), and S116 (90) had the lowest number of OTUs.

α-Diversity of bacterial and fungal communities in seeds

There were significant differences between these ecotypes for all of these α-diversity indices(Table 3). The Chao richness index of bacteria in ecotype S116 was significantly higher than that in ecotypes S124, S66, S85, S261, and S284($P<0.05$), and the Chao richness index of bacteria in the other ecotypes was not significantly different. The Shannon index of bacteria in ecotype S261 was significantly higher than that in ecotype S8($P<0.05$), and the Shannon index of seed bacteria failed to exhibit a significant difference between ecotypes S8, S261, and the others. The Simpson index of bacteria in ecotypes S124, S57, S099, S8, S85, and S261 was significantly higher than that in ecotypes S22 and S66($P<0.05$). The ACE index of

bacteria in ecotypes S116, S22, and S52 was significantly higher than that in ecotypes S124, S57, S66, S85, S261, S3, and S284(P<0.05).

The Chao richness index of fungi in ecotype S52 was significantly higher than that in ecotype S85(P<0.05; Table 3) and was not significantly different from other ecotypes. The Shannon index of fungi in ecotypes S57 and S8 was significantly higher than that in ecotypes S85 and S284(P<0.05) and were not significantly different from that in the other ecotypes. The Simpson index of fungi in ecotypes S32, S124, S57, and S8, was significantly higher than that in seeds of ecotype S284(P<0.05) and was not significantly different from that in other ecotypes. The ACE index of fungi in ecotype S52 was significantly higher than that in ecotype S85(P<0.05) and was not significantly different from other ecotypes.

PCA of microflora in seed

The PCA diagram based on the number of OTUs at the genus level showed that bacterial diversity was distributed separately at 10.83% and 8.47% on the first two principal components, which explained 19.3% of the variation in bacterial communities from all seeds(Fig 4a). Seed bacterial communities could be divided into three main groups. These three groups included ecotypes S41, S8, S57, and S32, ecotypes S85, S284, S261, S124, and S99, and ecotypes S3, S52, S116, S22, and S66. The grouped ecotypes suggested that the bacterial communities in the seeds in each ecotype had a greater similarity. PCA of fungal diversity was distributed separately at 9.66% and 7.4% on the first two principal components, which explained 17.06% of all seeds' variation in the fungal community(Fig 4b). Seed fungal communities could be divided into two groups. These two groups included ecotypes S32, S124, S116, S57, S99, S41, S8, S85, S261, and S3, and ecotypes S284, S66, S22, and S52.

Relative abundance of the bacterial and fungal phyla

The relative abundance of the top 10 bacterial phyla in seeds were Proteobacteria, Cyanobacteria, Bacteroidetes, Chloroflexi, unidentified bacteria, Actinobacteria, Acidobacteria, Firmicutes,Elusimicrobiota,and Verrucomicrobiota(Fig 5). Proteobacteria, Cyanobacteria, and Bacteroidetes were the most abundant phyla. The microbiomes of each seed lot were different. The relative abundance of Proteobacteria in seeds of ecotypes S124, S99, S41, S52, S66, S8, S85, S261, S3, and S284 was over 50%, which was the most important bacterial phylum in seeds. The relative abundances of the three most abundant phyla in each seed lot are shown in Table 4. The relative abundance of Proteobacteria in ecotype S284 was significantly higher than that in ecotypes S32, S116, S22, S57, S41, S52, S66, S8, and S3(P<0.05), and was not significantly different from that in ecotypes S124, S99, S85, and S261. The relative abundance of Cyanobacteria in ecotype S22 was significantly higher than that in ecotypes S124, S261, and S284(P<0.05) and was not significantly different from that in other ecotypes. The relative abundance of Bacteroidota in seeds of ecotype S261 was the highest, significantly higher than that in the other ecotypes(P<0.05). The relative abundance of Chloroflexi in seeds of ecotype S57 was significantly higher than that in the seeds of other ecotypes(P<0.05).

The relative abundance of fungal phyla is shown in Fig 6. These include Ascomycota, Basidiomycota, Mortierellomycota, Chytridiomycota, Glomeromycota, and Glomeromycota. Ascomycota and Basidiomycota were the most abundant fungi, with a relative abundance of more than 50%. Ecotype S261 had the highest relative abundance of Ascomycota(Table 4), which was significantly higher than that in ecotypes S22 and S66(P<0.05), and the relative abundance of Ascomycota in ecotype S22 was significantly lower than that in ecotypes S41 and S261(P<0.05). The relative abundance of Basidiomycota in ecotypes S116 and S8 was significantly higher than that in ecotypes S22 and S85(P<0.05), and the relative abundance of Basidiomycota in ecotype S85 was significantly lower than that in ecotypes S32, S116, S66, and S8(P<0.05). The relative abundance of mycophyla in ecotype S22 was significantly higher than that in ecotypes S32, S116, S57, S99, S41, S8, S261, S3, and S284(P<0.05).

Correlation analysis of α-diversity with environment factors

Correlation analysis between α-diversity and environmental factors (Table 5) showed that the Chao richness index and ACE index of bacteria were significantly negatively correlated with MMP, AMP, and GMMP($P<0.05$). However, the Simpson index of bacteria was significantly positively correlated with MMP, AMP, and GMMP($P<0.05$). The diversity indices lacked a significant correlation with MMT, AMT, GMMT, GMMP, elevation, and endophyte infection rate($P<0.05$). The Chao richness index of fungi was significantly negatively correlated with the MMT, GMMT, and endophyte infection rates($P<0.05$). The ACE index of fungi was significantly negatively correlated with MMT, AMT, and GMMT($P<0.01$) and significantly positively correlated with elevation($P<0.05$).

Correlation analysis of the most abundant bacteria and fungi with environmental factors

Correlation of the relative abundance of the three most abundant phyla, including Proteobacteria, Cyanobacteria, and Bacteroidetes, with environmental factors, was analyzed(Table 6). The relative abundance of Proteobacteria was significantly positively correlated with MMP, AMP, GMMP, and elevation($P<0.05$). The relative abundance of Cyanobacteria was significantly negatively correlated with MMP, AMP, and GMMP($P<0.01$). The relative abundance of Bacteroidetes was significantly positively correlated with GMMP($P<0.05$). The correlation of fungal diversity of the two most abundant phyla, including Ascomycota and Basidiomycota, with environmental factors, was also analyzed(Table 6). The relative abundance of Ascomycota was significantly positively correlated with MMP, AMP, and GMMP($P<0.01$). The relative abundance of Basidiomycota was significantly negatively correlated with the endophyte infection rate($P<0.05$). PCA analysis was conducted to evaluate the relationship between bacterial and fungal diversity and abundance with environmental conditions. The PCA analysis(Fig 7a) of environmental factors and bacterial diversity indices suggested that the Chao richness index and ACE index were positively correlated with the endophyte infection rate, GMMT, and MMT. In contrast, it had a negative correlation with MMP, GMMP, and elevation. The bacterial Shannon and Simpson indices were positively correlated with the endophyte infection rate, GMMT, MMT, MMP, and GMMP, whereas they were negatively correlated with elevation. The PCA analysis(Fig 7b) of environmental factors and fungal diversity indices suggested that the fungal Chao richness index positively correlated with elevation, whereas it was negatively correlated with GMMT, MMT, endophyte infection rate, GMMP, and GMMP. The ACE index of fungi positively correlated with GMMT, MMT, endophyte infection rate, GMMP, and GMMP, whereas it was negatively correlated with elevation. The Shannon and Simpson indices of fungi were positively correlated with GMMT, MMT, and endophyte infection rate, whereas they were negatively correlated with GMMP, GMMP, and elevation. The relationship between the relative abundance of the most abundant bacterial and fungal phyla and endophyte infection rate, elevation, MMP, GMMP, MMT, and GMMP were also analyzed by PCA (Fig8c and Fig 8d). Proteobacteria was positively correlated with GMMP, GMMP, and elevation but negatively correlated with the GMMT, MMT, and endophyte infection rate. Cyanobacteria positively correlated with the endophyte infection rate, whereas it was negatively correlated with elevation, GMMT, MMT, GMMP, and MMP. Bacteroidota was positively correlated with GMMT, MMT, endophyte infection rate, GMMP, and MMP but negatively correlated with elevation (Fig 8c). Ascomycota was positively correlated with GMMP, MMP, GMMT, MMT, and elevation, whereas it was negatively correlated with the endophyte infection rate. Bacteroidota was positively correlated with elevation, GMMP, and MMP but negatively correlated with the GMMT, MMT, and endophyte infection rate(Fig 8d).

Discussion

There is a rich microbial community comprising a diverse range of bacteria [30] and fungi[31] on the surface and inside plant seeds. Here, we characterized the microbiota of seeds from 14 different locations on the Qinghai-Tibet Plateau at the phylum level; Proteobacteria, Cyanobacteria, and Bacteroidetes were the most abundant bacteria, and Ascomycota and Basidiomycota were the most abundant fungi. The structure and diversity of microbiota in each seed lot were different, and the proportional abundance of each phylum varied with seed ecotype. The bacterial communities of 14 different seed lots only shared 11.02% bacterial OTUs and 33.60% fungal OTUs, suggesting that the microbiome in different F. sinensis seeds had different components.Studies have shown that different environmental conditions affect the propagation, infection, and

transmission of various microbes, and different species carry different types and numbers of microbes. Even the same seed can carry different fungi under different culture conditions[32].

It is of substantial importance to study seed microbe diversity because microbes in and/or on seeds may affect plant growth and health[33]. Cankar et al. (2005)demonstrated that Picea abiescontain bacteria in seeds had growth-promoting characteristics[34]. Puente et al. (2005) showed that some cactus seed bacteria could migrate into the rhizosphere and facilitate the release of mineral nutrients by pulverizing rocks through the production of organic acids, thereby promoting seedling growth[35]. Fungi associated with seeds can influence seed viability and seed bank structure[36]. The pathogenicity of pathogenic fungi is mainly reflected in the reduction of seed germination rate, inhibition of seedling growth, and decrease in seedling biomass[37]. Previous studies have shown that fungi are responsible for more than 70% of all major crop diseases. Nearly one-quarter of food crops worldwide are affected by mycotoxins, such as aflatoxins, ergot toxins, Fusarium toxins, patulin, and tenuazonic acid. After analyzing the microflora of F. sinensis seeds, the sequences from Fusarium, Epicoccum, Microdochium, and some other fungi were observed, suggesting that these fungi may cause leaf spot disease in the field. Therefore, it is important to study the diversity of seed fungi to prevent seed diseases.

A total of 54 genera and 129 species of specific bacteria were isolated from the surface and interior of more than 30 kinds of crop seeds, among which Proteobacteria was the main group. Firmicutes, Actinobacteria, and Bacteroidetes are the second most common bacteria[38]. This study was consistent with the results of previous studies, and Proteobacteria was the most abundant bacteria, followed by Cyanobacteria and Bacteroidetes. Proteobacteria play a key role in phylogenetic, ecological, and pathogenic processes and participate in energy metabolism, such as oxidation and photosynthesis of organic and inorganic compounds[39]. Bacteroidetes are increasingly regarded as specialists for degrading high molecular weight organic matter, namely proteins and carbohydrates[40]. Ascomycota and Basidiomycota were the most abundant fungi, consistent with previous studies conducted on alpine meadows in Yushu Tibetan Autonomous Prefecture, which showed that most fungi belonged to the phylum Ascomycota[41]. Ascomycota mainly decomposes cellulose and lignin, and its growth may depend on more readily available energy sources, such as soluble carbohydrates [42]. Basidiomycota mainly produces lignin-modifying enzymes to degrade lignin [43]. Ascomycota and Basidiomycota are widely distributed in plants, aquatic ecosystems, and soils in different proportions[44].

The endophyte infection rate of F. sinensis seeds on the Qinghai-Tibet Plateau was systematically investigated, and the endophyte infection rate ranged from 0–80%. Twelve of the 14 seed lots were found to infect Epichloë endophytes, and the endophyte infection rate was high. These high infection rates suggest that the Epichloë endophyte is very important for F. sinensis in grasslands. The asexual Epichloë is extant in the embryos of seeds and is transmitted through seeds[45]. These different seed lots had different endophyte infection rates. Hyphae of the endophyte E. sinensis resident in seeds may have an impact on seed microbiota. The correlation analysis showed that the endophyte infection rate only had a relationship with fungal Chao1 and ACE indices, which suggested that endophytes only affected the fungal microbiome rather than the bacterial microbiome. The relationship between endophyte and host microbiome was consistent with the research on S. phoenix[46], which demonstrated that resident E. coenophiala shapes fungal but not bacterial communities in the leaf endosphere of S. phoenix. The present study provides more evidence that the Epichloë endophyte in the host impacts the host seed microflora.

Environmental factors have different effects on the abundance and diversity of seed microorganisms. High-altitude ecosystems are generally characterized by low temperature, variable precipitation, decreased atmospheric pressure, and soil nutrient stress, which have major impacts on biodiversity[47].Temperature was the dominant driver of the bacterial and fungal diversity gradients. Seeds of different ecotypes carry different abundant bacteria that adapt to different environments, including elevation, temperature, and humidity. Correlation analysis suggested that the Chao richness index and ACE index of bacteria were significantly negatively correlated with MMP, AMP, and GMMP($P < 0.05$). The Chao richness index of fungi in seeds was significantly negatively correlated with MMT, AMT, and GMMT. The ACE index of fungi was significantly negatively correlated with MMT, AMT, and GMT($P < 0.05$). The relative abundance (ACE index) of fungi was significantly positively correlated with elevation($P < 0.05$). The Chao richness index of fungi was significantly negatively

correlated with MMT, AMT, and GMMT($P < 0.05$). The endophyte infection rate was also affected by environmental factors, which were significantly negatively correlated with elevation and positively correlated with temperature. These results suggest that precipitation had significant effects on the abundance of bacteria, whereas the endophyte infection rate, elevation, and temperature significantly affected the abundance of fungi. This study confirmed that environmental factors, such as temperature, precipitation, and elevation, may be the main factors affecting microbial diversity. Latif S et al. showed that the physical properties of soil from different geographic locations affected the microbiome community structure[48]. Bryant et al. suggested that bacterial taxon richness and phylogenetic diversity decreased monotonically from the lowest to highest elevations and bacterial lineages exhibited significant spatial structure across the gradient[49]. Bulgarelli et al. and Zhang et al. also showed that different biogeographical locations influenced the microbiome community structures of barley and soybeans[50, 51].Tannenbaum et al. showed that genotype-by-environment interactions contributed to the complexity of the microbiome assembly in seeds of perennial ryegrass[52]. In this study, F. sinensis seed lots were obtained from different sites on the Qinghai-Tibet Plateau, possibly because the combination of both host genotype and environment interactions led to the variation among the microbiome of these 14 seed lots.

Little attention has been paid to the sensitivity of microbial communities to precipitation. Precipitation seasonality significantly reduced the diversity of the fungal community but not that of the bacterial community[53], which is different from this study. This might possibly be due to the fact that the fungal community was well adapted to precipitation change, but the bacterial community was adapting to changes in precipitation. Therefore, in this study, precipitation seasonality had a significant effect on the most abundant bacteria and the overall observation and analysis of the effects of environmental factors on the diversity of microbes and the most abundant microbial phyla in this experiment. However, the specific mechanism of influence needs requires further research and confirmation.

Conclusion

The most abundant fungi in F. sinensis seeds were Ascomycota and Basidiomycota. The most abundant bacteria were Proteobacteria, Cyanobacteria, and Bacteroidetes. The formation of these microflora in F. sinensis seeds was related to environmental factors. Precipitation had significant effects on the abundance of bacteria, whereas the endophyte infection rate, elevation, and temperature significantly affected the abundance of fungi.

Declarations

Supplementary information

meteorological data

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Authors' contributions

PT designed the experiments, YG did the experiment and analysis. YJC,YQZ provided seeds,YG,PT, YL,YQZ, YJC,and ZBN wrote the manuscript.All anthors reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any

commercial or financial relationships that could be construed as a potential

conflict of interest.

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Tables

Table 1 The information about *F. sinensis* seeds from different locations of Qinghai-Tibet Plateau

Seed ecotype	Elevation/m	Endophyte infected rate	Longitude(E)	Latitude(N)	Location
S3	4542	0%	91 ° 53 '580"	31 ° 20 '039"	Luoma Town,Naqu City,Tibet
S8	5197	0%	91 ° 55 '336"	32 ° 54 '246"	Tanggula Mountains, Golmud City, Qinghai
S22	2912	72%	97 ° 99 '044"	37 ° 17 '138"	Keyukezhen Town,Delingha City Qinghai
S32	2589	64%	101°57 '234"	36 ° 21 '641"	Sanhe Town,Ping'an Country Qinghai
S41	3129	73%	102° 06 '214 "	36 ° 20 '021"	Bazanggou Township, Ping'an Country Qinghai
S52	4617	52%	93 ° 57 '485"	35 ° 32 '876"	Qumahe Township,Qumaleb country Qinghai
S57	2994	80%	102°06 '348 "	36 ° 20 '508"	Bazanggou Township, Ping'an Country Qinghai
S66	4617	20%	93 ° 57 '485"	35 ° 32 '876"	Qumahe Township,Qumaleb country Qinghai
S85	3534	74%	93 ° 07 '325"	29 ° 58 '065"	Gubo'gyamda Country,Nyingchi City,Tibet
S99	3060	56%	101 ° 58 '846"	36 ° 17 '059"	Gucheng Township , Ping'an Country Qinghai
S116	2912	48%	97 ° 99 '044"	37 ° 17 '138"	Keyukezhen Town,Delingha City Qinghai
S124	2741	80%	101 ° 57 '437"	36 ° 20 '047"	Sanhe Town,Ping'an Country Qinghai
S261	4003	32%	91 ° 54 '782"	29 ° 44 '692"	Zhaxigang,Maizhokunggar County,Tibet
S284	4897	54%	91 ° 50 '961"	29 ° 46 '564"	Zaxigang,Maizhokunggar County,Tibet

Table 2 The primer sequences utilized in this study

Types of sequencing	Primer name	Primers sequence
16sDNA	515F	(5'-ACTCCTACGGGAGCAGCA-3')
	806R	(5'-GGACTACHVGGGTWTCTAAT-3')
ITS	ITS5-1737F	(5'-CTTGGTCATTTAGAGGAAGTAA-3')
	ITS2-2043R	(5'-GCTGCGTTCTTCATCGATGC-3')

Table 3 α-diversity of bacteria and fungi communities in seeds

Seed ecotype	bacteria				fungi			
	Chao1	Shannon	Simpson	ACE	Chao1	Shannon	Simpson	ACE
S3	471.769abcd	3.639 ab	0.792abcd	478.857bc	176.519ab	3.553abc	0.845ab	179.835 ab
S8	551.169abcd	4.112 b	0.837ab	563.386abc	185.746ab	3.78a	0.869 a	185.448 ab
S22	571.910abcd	2.791 ab	0.673cd	589.278ab	181.22ab	3.303abc	0.823 ab	179.552 ab
S32	505.643 abcd	3.885 ab	0.773abcd	513.608abc	157.982ab	3.707ab	0.875 a	160.059 ab
S41	529.829abcd	3.962 ab	0.805abc	542.505abc	153.964ab	3.41abc	0.828 ab	156.961 ab
S52	604.213abc	3.295ab	0.73bcd	614.796ab	205.715a	3.356abc	0.829 ab	210.739 a
S57	489.317abcd	4.595 ab	0.826ab	499.292bc	172.3ab	3.766a	0.886 a	171.290 ab
S66	421.225 cd	2.498 ab	0.662d	431.058bc	178.480ab	3.406abc	0.846 ab	181.712 ab
S85	389.623d	4.042 ab	0.828ab	392.482c	129.748b	3.099bc	0.832 ab	126.345 b
S99	655.146ab	4.315 ab	0.826ab	581.811abc	155.979ab	3.527abc	0.860 ab	158.928 ab
S116	680.762a	4.074 ab	0.807abc	692.481a	158.973ab	3.449abc	0.843 ab	163.983ab
S124	454.200 bcd	4.421 ab	0.831ab	459.145bc	160.959ab	3.592ab	0.868 a	163.321 ab
S261	416.486cd	5.122 a	0.873a	422.716bc	156.875ab	3.535abc	0.848 ab	160.459 ab
S284	430.691cd	4.153 ab	0.8abc	435.502bc	158.767ab	2.943c	0.746 b	161.060ab

Note: Different lowercase letters in the table indicate significant differences in the same index (P=0.05).

Table 4 The relative abundance of the most abundant bacteria and fungi

Seed ecotypes	The most abundant bacteria			The most abundant fungi	
	(Proteobacteria)	(Cyanobacteria)	(Bacteroidota)	(Ascomyota)	(basidiomycota)
S3	59.37%bcd	29.17%abc	4.16%bc	59.46%abc	13.93%abc
S8	56.21%bcd	28.80%abc	6.65%bc	49.80%abc	18.95%a
S22	49.95%cd	46%a	1.44%bc	32.63%c	8.99%bc
S32	47.97%cd	38.47%ab	7.26%bc	46.44%abc	16.73%ab
S41	60.44%bc	27.60%abc	6.80%bc	65.23%ab	13.69%abc
S52	52.57%cd	42.61%ab	2.96%bc	43.57%abc	10.60%abc
S57	39.55%d	30.37%abc	6.58%bc	52.96%abc	13.7%abc
S66	54.74%bcd	42.83%ab	0.58%c	36.46%bc	17.22%ab
S85	67.21%abc	18.65%abcd	4.49%bc	51.00%abc	5.9%c
S99	63.68%abc	19.94%abcd	8.22%bc	49.25%abc	12.28%abc
S116	47.99%cd	37.74%ab	7.80%bc	48.19%abc	19.61%a
S124	66.15%abc	16.23%bcd	10.79%b	46.54%abc	11.62%abc
S261	74.35%ab	0.95%d	19.18%a	67.15%a	12.51%abc
S284	81.73%a	3.54%cd	6.80%bc	61.22%abc	16.61%abc

Note: The values in the table are relative abundances, and different lower case letters indicate significance between different ecotype.

Table 5 correlation coefficient between seed microbialα-diversity and environmental factors

Environmental factors	bacteria				fungi				Endophyte infected rate
	Chao1	shannon	simpson	ACE	Chao1	shannon	simpson	ACE	
MMT(℃)	0.263	0.480	0.208	0.303	-0.639*	0.226	0.254	-0.684**	0.619*
MMP(mm)	-0.620*	0.483	0.565*	-0.642*	-0.461	0.128	0.108	-0.437	0.022
AMT(℃)	-0.263	0.480	0.208	-0.303	-0.639*	0.226	0.254	-0.684**	0.652*
AMP(mm)	-0.620*	0.483	0.565*	-0.642*	-0.461	0.128	0.108	-0.437	0.022
GMMT(℃)	-0.165	0.470	0.212	-0.223	-0.642*	0.278	0.324	-0.697**	0.674**
GMMP(mm)	-0.620*	0.483	0.565*	-0.642	-0.461	0.128	0.108	-0.437	0.022
Elevation	-0.093	-0.063	-0.016	-0.090	0.288	-0.125	-0.153	0.310*	-0.636*
Endophyte infected rate	-0.085	0.090	0.029	-0.094	-0.313*	-0.088	0.067	-0.354*	x

Note: "*"indicates a significant correlation at P<0.05; ""indicates a very significant correlation at P<0.01.**

Table 6 correlation coefficient between the relative abundace of most abundance bateria and fungi and environmental conditions

Environmental factors	The most abundance bacteria			The most abundance fungi	
	(Proteobacteria)	(Cyanobacteria)	(Bacteroidota)	(Ascomyota)	(Basidiomycota)
MMT(℃)	0.086	-0.403	0.523	0.449	-0.097
MMP(mm)	0.547*	-0.712**	0.306	0.708**	-0.035
AMT(℃)	0.086	-0.403	0.523	0.449	-0.097
AMP(mm)	0.547*	-0.712**	0.306	0.708**	-0.035
GMMT(℃)	0.044	-0.364	0.535*	0.359	-0.141
GMMP(mm)	0.547*	-0.712**	0.306	0.708**	-0.035
Endophyte infected rate	-0.163	-0.199	-0.177	0.165	0.168
Elevation	0.374*	0.025	0.108	-0.049	-0.418*

Note: within a confidence interval of P=0.05,"*"indicates a significant correlation，""indicates a very significant correlation**

Figures

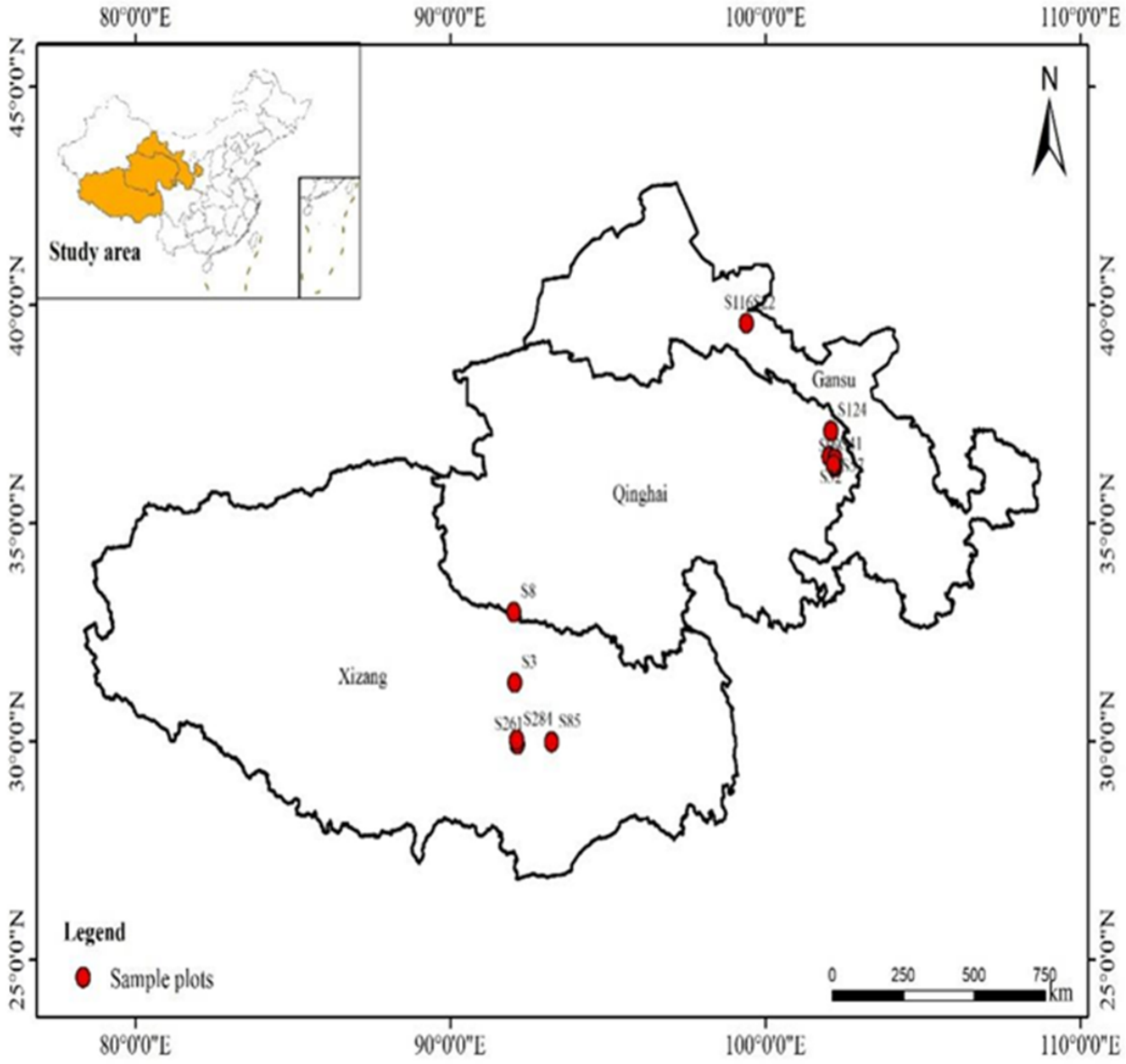

Figure 1

Figure 1

Approximate geographical location of each Festuca sinensis ecotype seeds Note: the red solid circle on the map stand for sample collection location

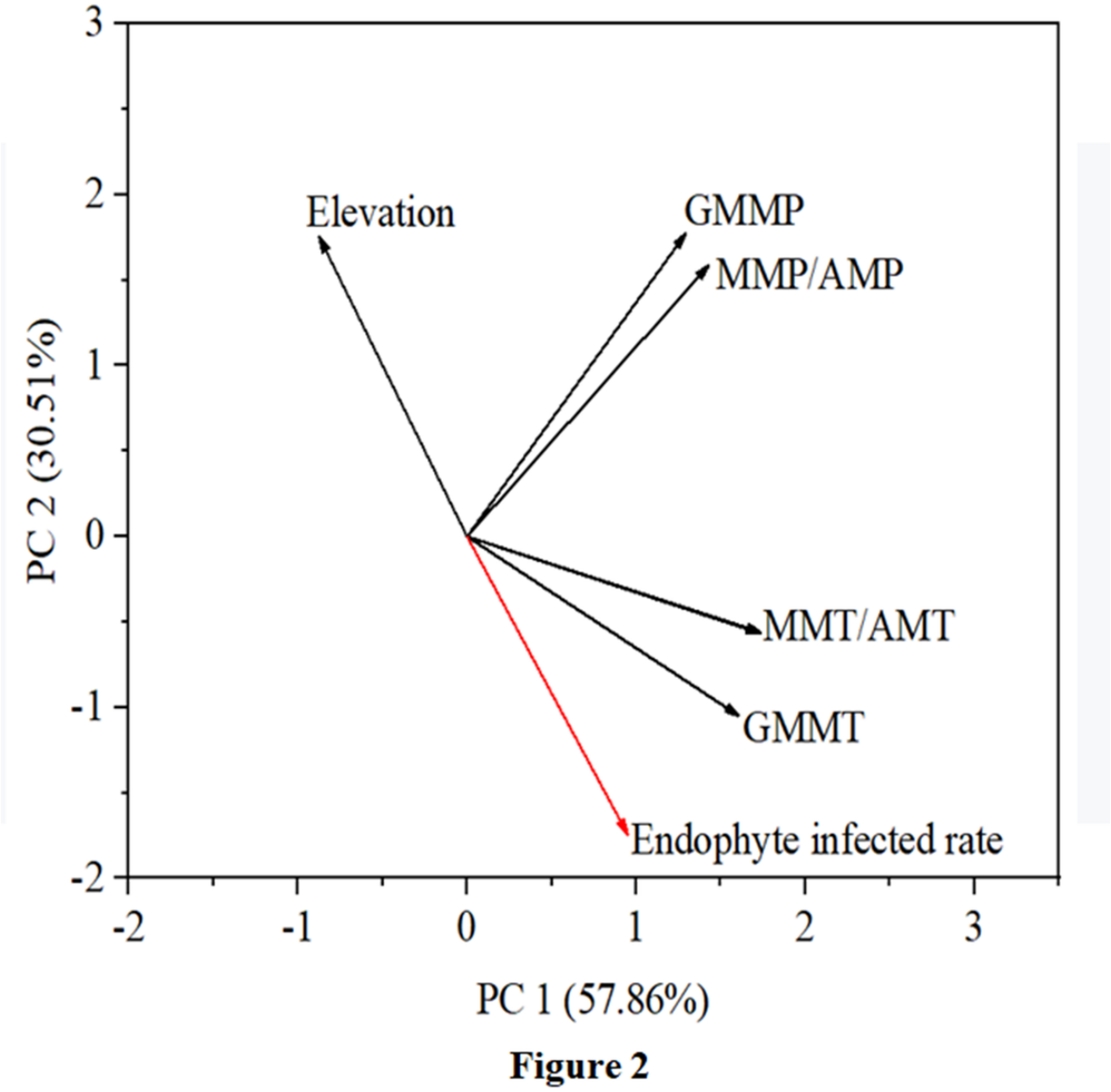

Figure 2

Figure 2

Effect of environmental factors including elevation, GMMP, MMP, MMT and GMMT on endophyte infects rate (PC1=55.16%; PC2=33.14%). Note: MMT meaning monthly mean temperature, MMP meaning monthly mean precipitation, AMT meaning annual mean temperature, AMP meaning annual mean precipitation, GMMT meaning the growing monthly mean temperature and GMMP meaning growing monthly mean precipitation.

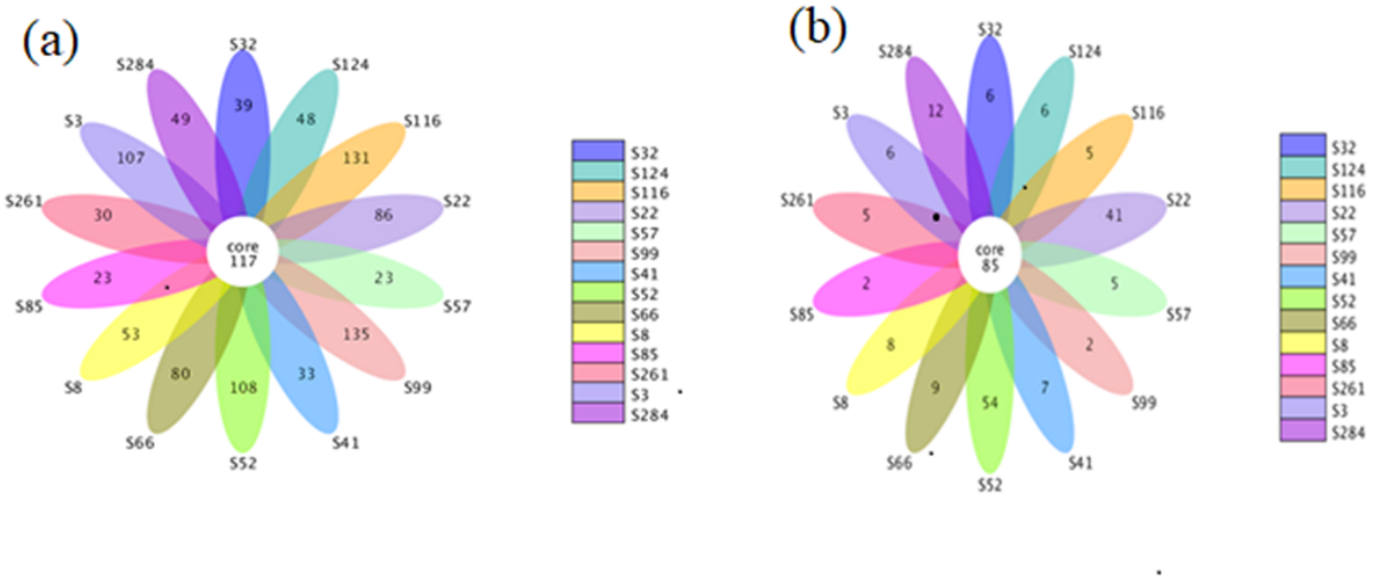

Figure 3

Figure 3

Veen petals of bacteria (a) and fungi (b) at the OTUS level showing the number of commonly and uniquely expressed genes in different seed ecotypes.

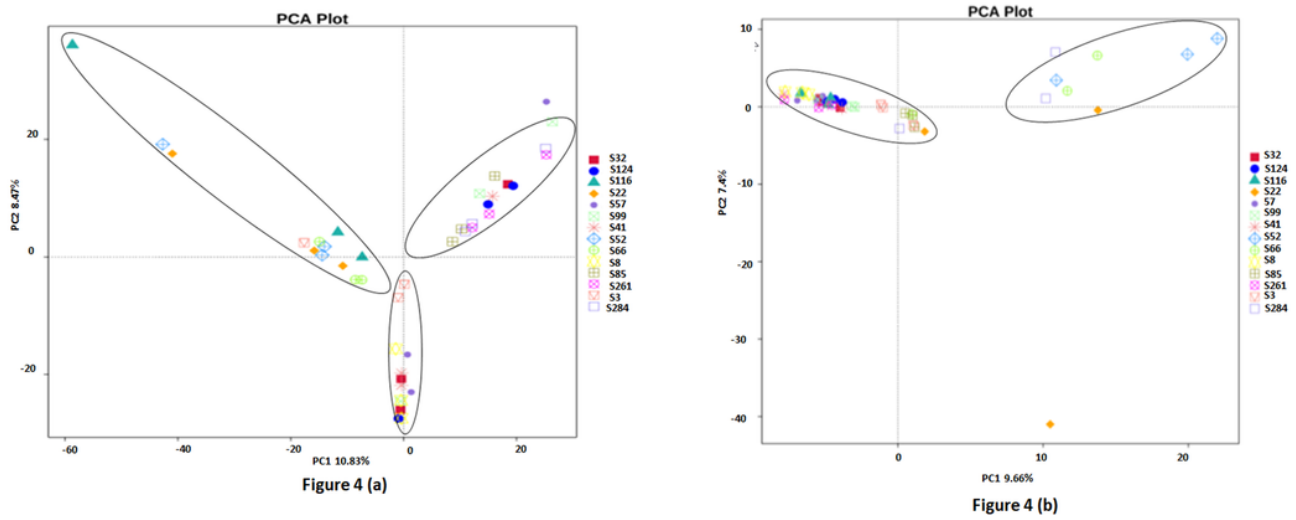

Figure 4

principal component analysis (PCA) of seed bacterial community (a) and seed fungi community (b) based on number of OTUs at genus level (a: PC1=10.83%; PC2=8.4%; b: PC1=9.66%; PC2=7.4%).

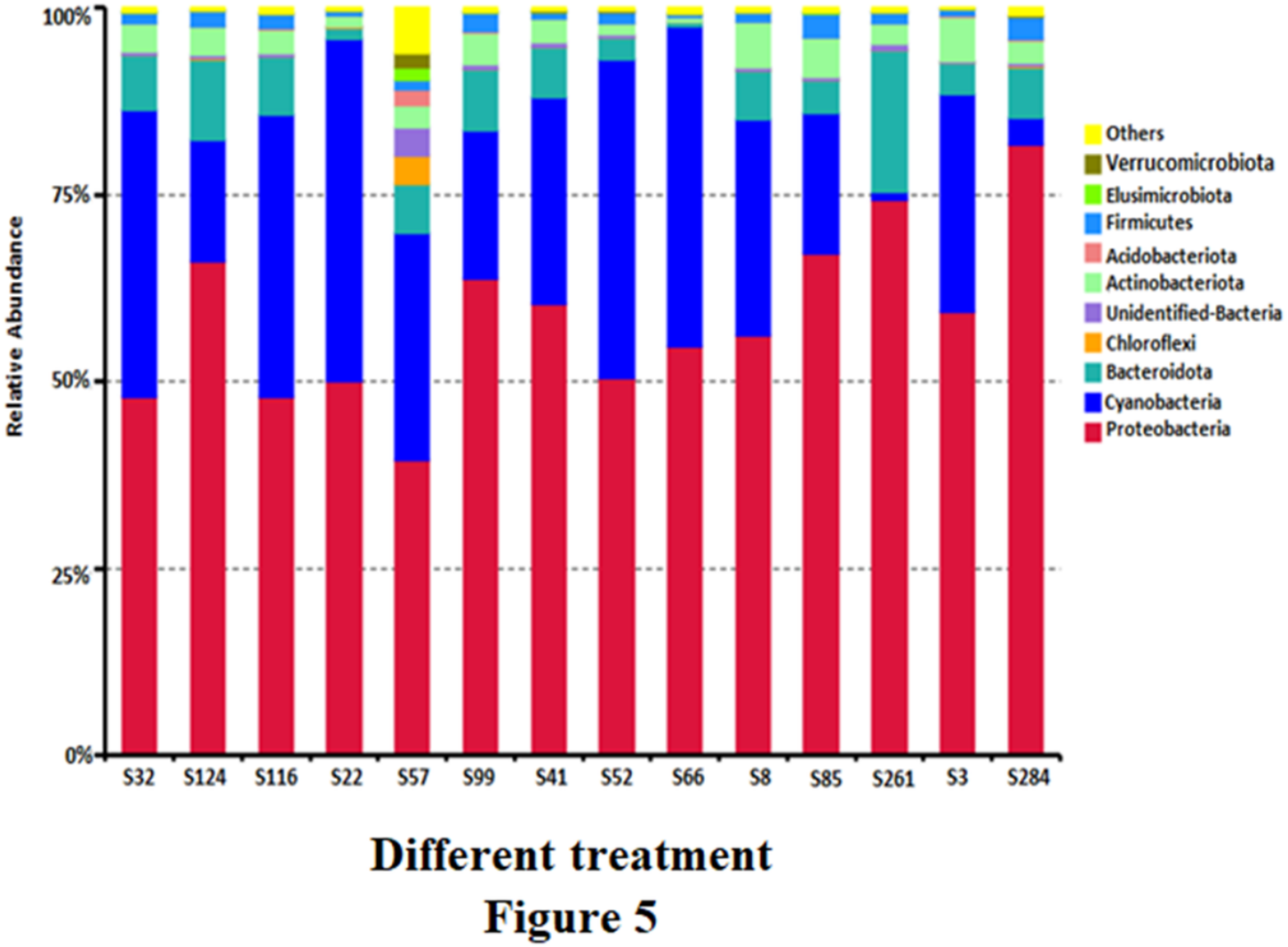

Figure 5

Figure 5

The relative abundance of the top 10 phylum of bacteria in each seed lot through 16s ribosomal RNA sequencing

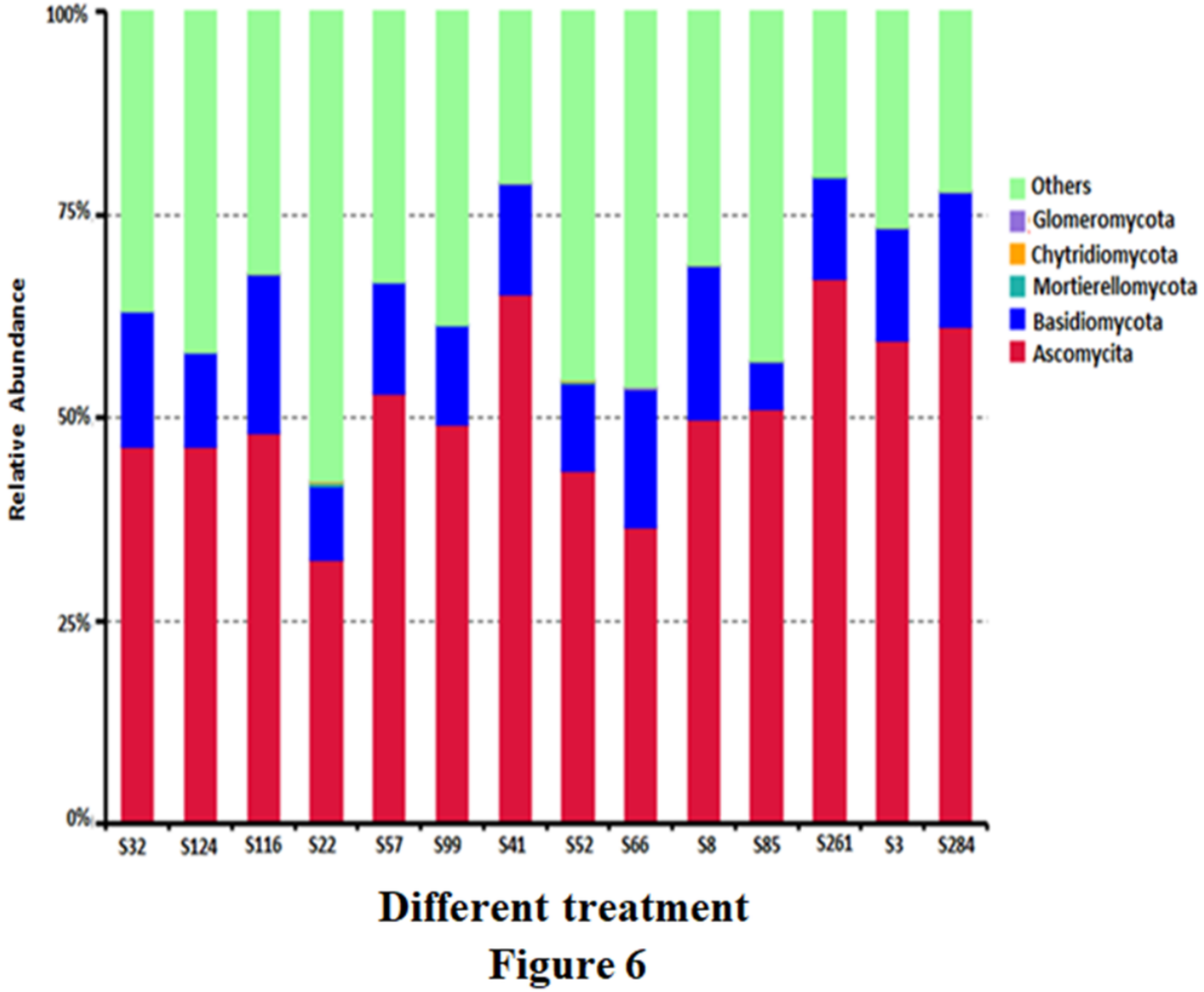

Figure 6

The relative abundance of the top 5 phylum of fungi in each seed lot through ITS sequencing

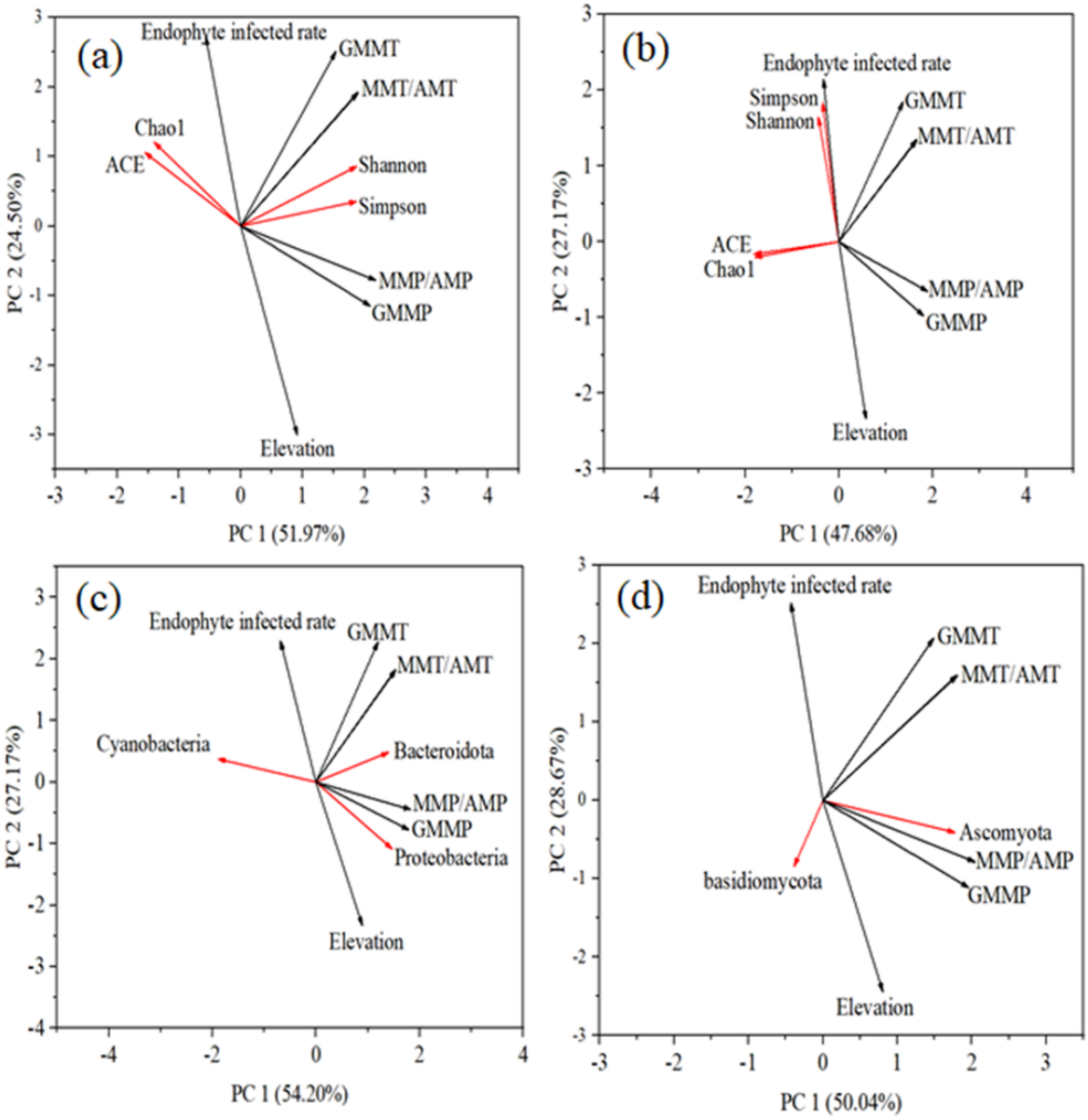

Figure 7

Figure 7

Effect of environmental factors on α-diversity of bacteria (a) and fungi (b), the relative abundance of most abundant bacteria (a) and fungi (b). (a) analysis of α-diversity of bacteria and environmental factors (PC1=47.35%; PC2=25.28%); (b) analysis of α- diversity of fungi and environmental factors (PC1=46.43%; PC2=24.75%); (c) analysis of the abundance of Proteobacteria, Bacteroidota, Cyanobacteria and environmental factors (PC1=49.24%; PC2=28.74%); (d) analysis of the abundance of Ascomyota, Basidiomycota and environmental factors(PC1=45.57%; PC2=29.24%).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformatin.xlsx