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Rhizosphere fungal community structure succession of Xinjiang continuously cropped cotton

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ABSTRACT

The large-scale long-term plantation of cotton in the Xinjiang region has been accompanied by a regular and wide outbreak of soil-borne fungal diseases such as *verticillium* wilt, which significantly damaged the local cotton industry. High-throughput sequencing data showed that the cotton field cultivation management measures pose a significant influence upon the original ecological soil fungal community structure. During long-term continuous cropping of cotton, a new soil fungal community structure emerges after several repeated adjustments over five years. The number of *verticillium* wilt pathogens in the soil increased rapidly with prolonged continuous cropping time, reaching a maximum at around the 10th y; moreover, the abundance of the *verticillium* wilt pathogen only serves as one of numerous essential factors for disease occurrence. The fungal community structure and the abundance of *verticillium* wilt pathogens in local cotton fields are gradually formed under joint effects of year-long continuous cropping and supporting cultivation management measures.

1. Introduction

Xinjiang is one of the first regions that started to plant cotton in China, and it is also the only plantation base for long-stapled cotton in China. Due to the unique natural environmental conditions of Xinjiang, which make this region suitable for cotton plantation, the plantation area of cotton has been extended since the 1980s. It has ranked at the top in China in terms of indexes such as plantation area, total output, and average yield per mu for 24 consecutive years. Furthermore, because of crop singleness in Xinjiang, especially in the main production area, the plantation area of cotton accounts for more than 95 %; therefore, continuous cropping is quite common in the region. This long-term and wide-spread cotton cultivation in the region has caused an interesting phenomenon where the continuously cropped cotton in newly cultivated land produces a high yield in the first three years with no disease; however, in the following years, soil-borne diseases such as fungal *verticillium* wilt and fusarium wilt start to occur (Lang et al., 2012). Moreover, the occurrence rate and increasing scale with the prolonging of continuous cropping time, causes unstable output or even the complete loss of harvest, thus leading to huge damage to the local cotton industry. That phenomenon is also labeled “cotton cancer” due to the lack of effective measures for prevention and treatment (Han et al., 2010). Relevant research showed the phenomenon to be closely related to the changes of microbial community structure in soil caused by continuous cropping of cotton (Zhang et al., 2013). However, the process of new changes to the fungal community structure and its succession remain to be studied, as well as the abundance changes of the pathogen group that causes soil-borne fungal diseases to cotton, which falls into in the process of fungal community succession. Solving the above two issues has great significance in studying soil fungal community and crop interaction, as has the development of new methods to overcome the obstacles of cotton continuous cropping in Xinjiang.

At the early stage, the DGGE method discovered that the soil microbial community structure transformed from “bacterial” to “fungal” during the continuous cropping of cotton (Zhang et al., 2013). However, methods of molecular ecology such as DGGE cannot soundly dissect the composition of the microbial community structure due to defects such as small number of detected bands, PCR amplification preference to certain bacterial genuses, and little biological information covered by DNA fragments; therefore, these methods are unsuitable for the exploration of the continuous succession of the microbial community structure in

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such complicated ecological systems as soil (Urashima et al., 2012). In recent years, high-throughput sequencing technology has followed rapid development, enabling increased sequencing length, uplifted sequencing throughput, and improved data reliability for analysis. At present, this technology has developed into the most powerful tool for studying microbial ecosystems (Fierer et al., 2013; Xu et al., 2012).

Utilizing high-throughput sequencing technology, this study comparatively analyzed the composition and differences of soil fungal community structures under both conditions of original ecology and more than 30 y of continuous cotton cropping in the arid and semi-arid environment of Xinjiang. Furthermore, it also explored the succession rules of the soil fungal community structure. In addition, the change of *verticillium* wilt pathogens (Verticillium, Moniliaceae, Moniliales, Hyphomycetes, Deuteromycotina) and its enriched number in soil in response to prolonged continuous cropping time was detected using the real-time quantitative PCR method. Combining these two results, this study interpreted the occurrence rules of *verticillium* wilt during the process of long-term continuous cropping as well as the mechanism of rotating cropping in overcoming continuous cropping obstacles.

2. Materials and methods

2.1. Sampling

Samples were collected at the Awati county cotton region (The sampling location was within E 79º45′81″-46′55″, N 39º31′47″-45′50″) on June 5th, 2015 (soil physiochemical properties: pH = 8.52, total organic content 12.78 g/kg, total nitrogen 0.713 g/kg, total phosphorus 0.956 g/kg, total kalium 44.3 g/kg, available nitrogen 37.8 mg/kg, available phosphorus 20.7 mg/kg, and available potassium 213.1 mg/kg). According to the information from the local agricultural department, this was the time when *verticillium* wilt and fusarium wilt started to break out. Soils with the year limit for continuous cropping of 0 (uncultivated soil), 1, 3, 5, 10, 15, 20, 25, 30, and soil with 1 y of rotational cropping of maize after long-term continuous cropping of cotton were sampled and respectively labeled as AW0, AW1, AW3, AW5, AW10, AW15, AW20, AW25, AW30, and AWL. For each period of continuous cotton croping, a 6 cm inner diameter boring stick was used for a vertical drilling with the plants at the center, and the soil in a depth of 1–20 cm was taken. Soils from five plots with the same continuous cotton croping period were mixed in equal amounts form a sample (n = 10). Ten soil samples were obtained in total, and then brought back to laboratory to be stored at −4 °C for use within one week.

2.2. DNA extraction, PCR amplification, and high-throughput sequencing

The FastDNA®spin kit (MP bio, Santa Ana, USA) was used for the total DNA extraction of soil samples. The operation followed the instructions provided by the manufacturer and for every sample three repetitions were conducted. The total DNA of repeated samples were stored at −20 °C for later use. The ITS3 (GCATCGATGAAGAACGCAGC)/ITS4 (TCCTCCGCTTATTGATATGC) primer was used to amplify the 18S rRNA's ITS region in the total DNA of the samples (Huang et al., 2014). The 30 μL reaction system consisted of: Phusion Master Mix (2×) 15 μL, forward or reverse primer 1.5 μL (2 μM), DNA for every sample (1 ng/μL) 10 μL, double distilled water 2 μL. The reaction procedure for the Bio-rad T100 gradient PCR amplifier followed: 98 °C, 1 min; 98 °C, 10 s; 55 °C, 30 s; 72 °C, 30 s for a total of 30 cycles; 72 °C for 5 min. The amplified segments of the ITS region were quantified using NanoDrop-1000 (USA). After complete mixing of equal density samples and 1×TAE agarose gel electrophoresis at a density of 2 %, the 340–438 bp main bands were recovered using the GeneJET gel recovery kit, obtained from Thermo Scientific. The above samples were used to build a library with NEB Next® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs). The library was built after passing Qubit quantification and library detection and was sequenced on the Illumina HiSeq platform by the Novogene Company (Beijing, China). The accession number of the detected sequence in this study in NCBI SRA database was SRS2004117.

2.3. Bioinformatics and statistical analysis

Novogene edited an already existing script to specifically remove barcode and primer sequences. To differentiate signature sequences, 6 bp signature sequences were used at the front end of the forward primers of every sample. Furthermore, the number of sequences that was available per sample after optimization for accurate analysis reached at least 35,000 (Magoč et al., 2011) The UPARSE pipeline (v7.0.1001) was used to conduct the analysis of OTU-relevant bioinformation for the obtained sample sequences, with the similarity set to 97 %. For species annotation of the representative sequences found from OTUs, both the RDP Classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) and the GreenGene database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) were used (with the threshold set to 0.8–1). Moreover, the community composition of each sample was analyzed from the five classification levels of kingdom, phylum, class, order, family, genus, and species.

The alpha diversity of samples and the indexes of Observed-species were analyzed for full sample similarity, and the Chao1, Shannon, Simpson, ACE, and Goods-coverage were calculated using the Qiime software (Version 1.7.0). R software (Version 2.15.3) was used to obtain the rarefaction curve, rank abundance curve, species cumulative curve, and Venn diagram, and alpha diversity inter-index group differences were analyzed (Caporaso et al., 2010). In the beta diversity analysis, Qiime (Version 1.7.0) was used to calculate weighted and unweighted Unifrac distances to build a UPGMA sample clustering tree. Using the vegdist and hclust functions in the vegan package of the R language, sample similarity clustering, OTU heat map, and β diversity distance calculation were obtained. Furthermore, using the R software package (Version 2.15.3), graphs such as PCA and NMDS were drawn. For PCA analysis, the software packages of ade4 and ggplot2 in the R software were used; for NMDS analysis, the vegan software package in the R software was used (Wang et al., 2007). All data reported in this study represent the average of three replicates. All statistical analyses were performed via SPSS 22.0 software for windows.

2.4. Real-time quantitative PCR measurement of verticillium wilt pathogens within samples

Cotton *verticillium* wilt pathogens originated from the culture preservation room of the Chinese Academy of Agricultural Sciences, and a method proposed by Qin et al. was adopted for strain cultivation and DNA extraction (Qin et al., 2006). The Specific fragments obtained from the amplification with the ITS sequence-based specific primer V1/V2 (5′-CATCAGTCTCTCTGTTTATACCAACG-3`/5′-CGATGCGAGCTGTAACTACTACGCAA-3′) were used to quantitatively detect the number of *Verticillium dahliae* in soil samples (Zhu et al., 1999). The reaction systems for amplifying the specific fragments include: 10×buffer 5 μL, $MgCl_2$ 2 mmol L^{-1}, dNTP 200 μmol L^{-1}, forward or reverse primers (V1/V2) 0.4 μmol L^{-1}, DNA 2 μL, Taq enzyme (TaKaRa) 1.25 U, water was added to reach 50 μL. Reaction conditions were: 94 °C predegeneration for 5 min; 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s for 35 cycles; 72 °C for an extension for

7 min. A standard curve was established with 10-fold serially diluted recombinant *Escherichia coli* pMD19-T plasmid containing the above specific fragments. Real-time fluorescence quantification of the PCR reaction system: 2×SYBR Green Mix (TaKaRa) 5 μL; 10 μmol L^{-1} upstream or downstream primers (V1/V2) 0.2 μL, DNA 1 μL, adding ddH_2O to reach 10 μL. Amplification reaction: 95 °C predegeneration for 2 min; 95 °C for 5 s, 60 °C annealing and extension for 30 s, and amplification for 40 cycles. The quantitative PCR reaction was conducted on a StepOne Real-Time PCR System (ABI) amplifier. The standard curve obtained was $y = -3.8254x + 41.583$ ($R^2 = 0.9927$). The template DNA was extracted with step 1.2, with the LOD being 2 bacteria/g soil.

3. Results

3.1. High-throughput sequencing of group data

The total number of sequences in the 10 samples, which could be used for accurate analysis was 496,923 with an average length of 302–311 bp. Among the samples, AW3 was the one with the smallest number of 24,246. When the number of effective sequences obtained for samples reached 22,500, the saturation curve of the detected species approached the plateau, while the differences in the number of observable species were large. The sample coverage degree ranged from 0.998 to 0.999 (Table 1). At different levels from kingdom to genus, more than 96 % of the obtained sequences for the samples could be accurately classified, and at the classification level of phylum, it contained four major phyla of the fungal circle, with *Ascomycota* accounting for the largest percentage. Even for the AW3 sample with the lowest content, this also reached more than 95 %. Therefore, the following analysis (related to the classification level) mostly centered on *Ascomycota* (Fig. 1). Then, the data of the top 10 species with the largest abundance in various classification levels were compared. For cotton planting for only one year, the original ecological fungal community structure underwent significant changes from the community structure composition to various component abundances, starting from the level of class; the differences of samples with different years of continuous cropping mainly affected the abundance of various genuses; and the analysis of more detailed classification levels would produce a clearer abundance changing tendency for different genuses. Furthermore, after long-term continuous cropping of cotton, rotating cropping of maize for only one year can also lead to large changes of the soil fungal community structure. Analysis from a low classification level showed that major changes happened to several genuses like *Podospora* (Fig. 1).

3.2. Diversity index and core OTU

Sample AW15 had the lowest Shannon index of 1.582, followed by samples AW1 and AW25. The Shannon index of other samples was much higher than that of AW15, with the highest being 4.317 for AWL, and the Shannon index of various continuous cropping samples showed repeated fluctuations with prolonged time of continuous cropping. The changing rules of Simpson, chao1, and ACE indexes with the prolonged time of continuous cropping were consistent with that of the Shannon index; however, their difference originated in AW20, which was the sample with the highest chao1 and ACE index (Table 1). Sample AW15 contained the least OTU, namely 133, followed by 146 of AW3, and the sample AW20 contained the most, namely 217 (Fig. 2). Via comparison of the OTU differences in samples, the number of OTU shared by all samples was 49; therefore, it was the largest group; however, there were also their own shared OTUs between different samples. With regard to the specific OTU of samples, AW25 was the one with the least, namely two, while AW0 was the one with the most, namely 39, and the number for rotating cropping samples was 20 (Fig. 2).

3.3. Microbial community grouping

Similarity analysis showed that these 10 samples could be clustered into one big cluster. AW0, had the largest difference and was finally clustered (Fig. 3). In unweighted Unifrac clustering, rotating cropping AWL samples firstly clustered with long-year continuous cropping samples of AW10, AW20, AW25, and AW30, followed by short-year continuous cropping samples of AW1, AW3, AW5, and AW15. In weighted Unifrac clustering, rotating cropping sample AWL (most similar to sample AW1) was clustered with long-year and short-year continuous cropping samples alternately with a long clustering distance (Fig. 3). OTU heatmap clustering of the most widely distributed 35 orders for six phyla such as *Ascomycota* and *Basidiomycota* in 10 samples showed that the 10 samples were clustered into one big cluster in terms of overall similarity; however, the manner of clustering combined the clustering order of unweighted Unifrac and weighted Unifrac. Moreover, all 35 classes could be clustered into three major clusters in terms of clustering means. Among these, seven classes such as *Chaetothyriales*, *Mucorales*, and *Agaricales* were clustered into the first, with sample AW1 having the largest differences to others; 10 classes like *Eurotiales*, *Pleosporales*, and *Tremellales* were clustered into the second, with sample AW0 having the largest differences to others; and the other 18 classes were clustered into the third cluster (Fig. 4).

Through beta diversity heapmap analysis of the weighted data, it became clear that at the first year of cotton plantation, fungal communities had seen comparatively strong changes (0.868), and the changes remained at a medium level with the extended time of continuous cropping, which was not changed even by the rotating cropping of maize. Unweighted data also revealed these changes; however, these were much milder (Fig. 5). NMDS analysis showed that sample AW0 had large differences to all other continuous cropping samples, and the long-year continuous cropping samples

Table 1
Diversity of the 10 soil samples in fungus with different succession cropping histories.

Sample name	Observed species	shannon	simpson	chao1	ACE	Goods coverage
AW0	185	3.776	0.862	222.143	217.308	0.998
AW1	121	2.109	0.498	130.731	139.72	0.999
AW3	146	3.543	0.839	187	190.255	0.998
AW5	135	3.137	0.792	166.167	169.364	0.998
AW10	140	3.13	0.743	158.455	167.192	0.999
AW15	97	1.582	0.399	108.55	114.105	0.999
AW20	185	4	0.859	240.25	252.973	0.998
AW25	102	1.87	0.568	123.368	132.091	0.999
AW30	122	3.365	0.819	142.312	145.359	0.999
AWL	185	4.317	0.902	234.038	242.804	0.998

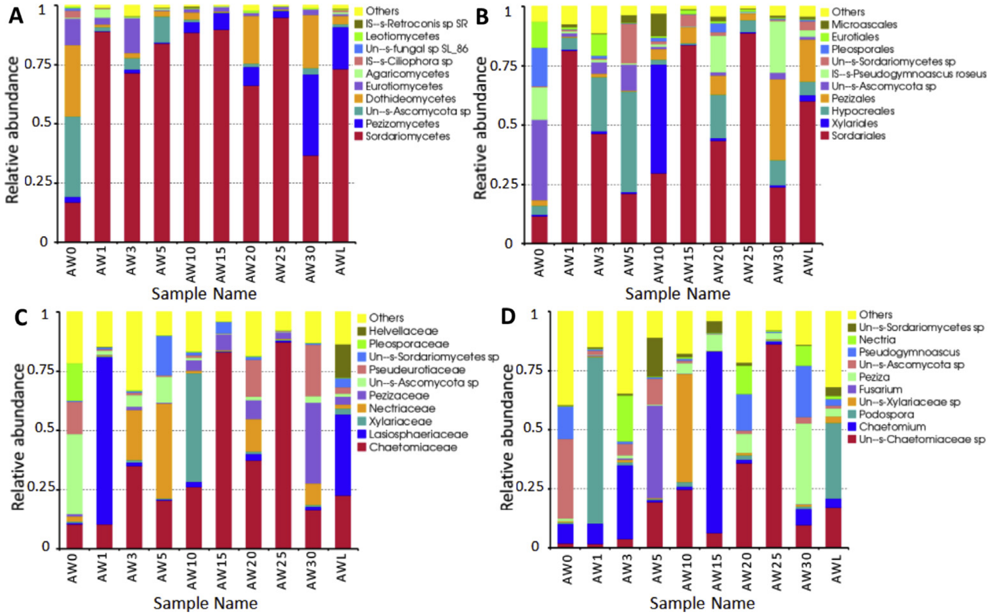

Fig. 1. Abundances of different class/order/family/genus (A/B/C/D) in fungus in all 10 soil samples. The abundance is presented in terms of percentage of total effective fungal sequences per sample.

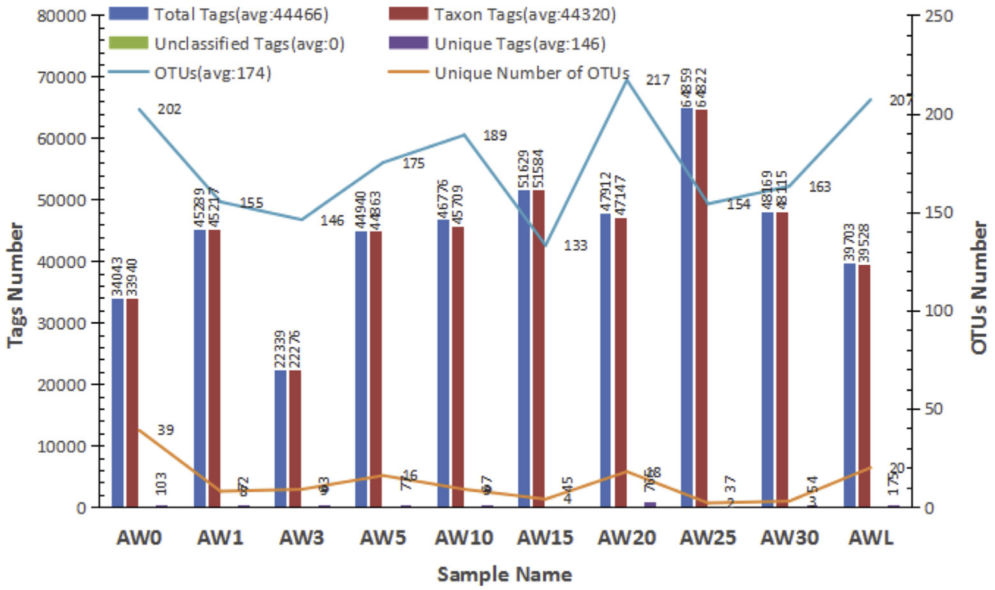

Fig. 2. Number of tags and OTUs in the 10 soil samples. Each bar represents total tags, taxon tags, unclassified tags, unique tags, and each line represents OTUs and unique number of OTUs of the 10 samples.

and the rotating cropping samples AWL were comparatively concentrated (Fig. 6). PCA data showed that AW0 and AW1 were farthest from each other; however, the other samples were relatively concentrated in-between; while among other samples, rotating samples AWL were closest to samples of AW0 and AW1 (Fig. 7).

3.4. *Change of V. dahliae content in soil*

After optimization of RT-PCR conditions, No V1/V2 specific bands were obtained in the sterile water control group; however, few of them could be obtained in sample AW0. When the specific band content after 35 cycles of the amplification of AW0 sample was regarded as the threshold, the number of cycles that were necessary for amplification to reach the threshold rapidly decreased with prolonged continuous cropping time for the samples of 1–10 y continuous cropping. Among these, the average cycle number for AW10 was minimal, namely 5.3, with the number of cycles for other long-term continuous cropping samples staying at a relatively low level (Fig. 8). With reference to sample AW10 that contained the most *V. dahliae* DNA calculated through standard curve, the content of *V. dahliae* DNA in AW0-3 samples increased stably. The content of *V. dahliae* DNA in AW3-10 samples rapidly increased; while in other long-year continuous cropping samples, it did not constantly increase but rather remained at a relatively high level. The content of *V. dahliae* DNA in AWL with one year of

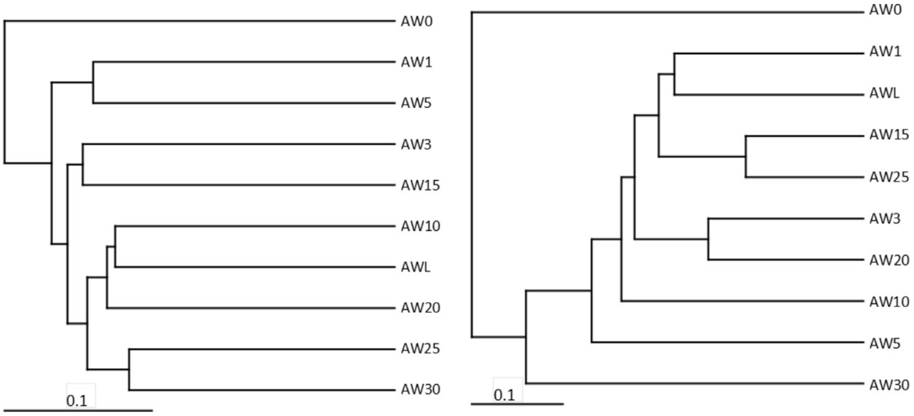

Fig. 3. Analysis of sample similarity in the 10 soil samples (left: unweighted Unifrac, right: weighted Unifrac).

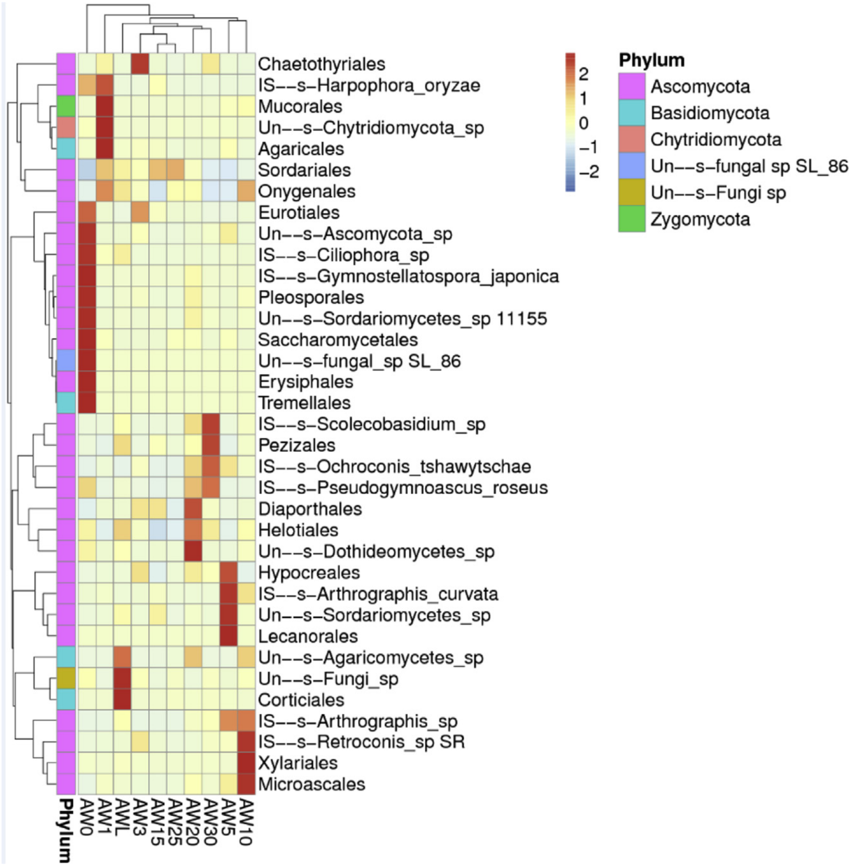

Fig. 4. 35 different orders in fungium the 10 soil samples. Double hierarchical dendrogram shows their distribution. The fungus phylogenetic tree was calculated using the neighbor-joining method and the relationship among samples was determined via Bray–Curtis distance and the complete clustering method. The relative values for fungal orders are indicated with color intensity with the legend indicated at the top right corner.

rotating cropping of maize showed an apparent declining trend (Fig. 8).

4. Analyses and discussion

4.1. *Influence of cultivation mode upon soil fungal community structure*

The interaction between microorganism and plant remains one of the focuses of the research on continuous cropping obstacles; however, few influenced the soil cultivation management measures upon soil microbial community structure (Zhu et al., 1999). Through a comparison of the changes in soil fungal community structure one year before and after cotton cultivation from the classification levels of class, order, family, and genus, this study showed that although cotton was cultivated for only one year, the new soil fungal community structure in its early form had already emerged. I.e., the cultivation management mode used to support cotton cultivation exerted a larger influence upon the original ecological fungal community structure compared to the long-term continuous

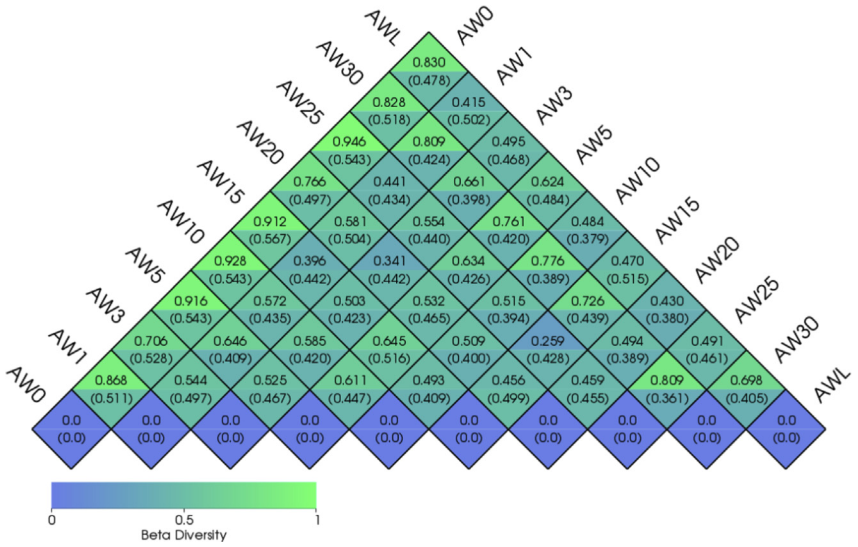

Fig. 5. Beta_diversity heat maps of the 10 soil samples (up: weighted, down: unweighted). The relative values for sample family are indicated by color intensity with the legend at the bottom. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

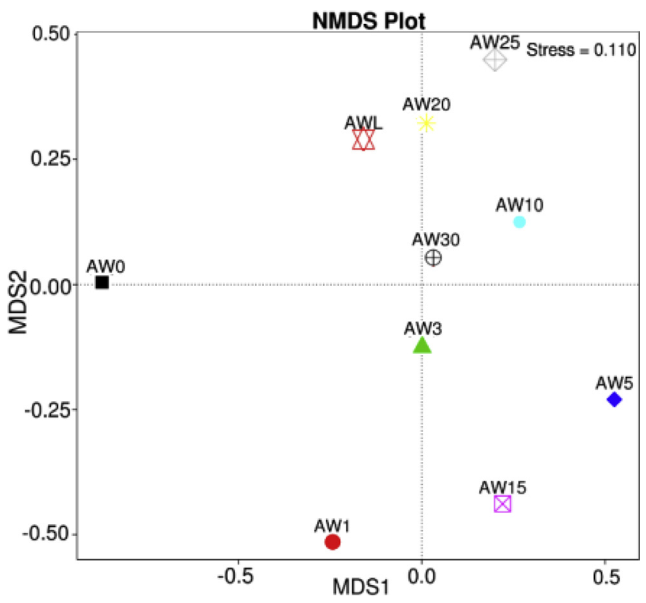

Fig. 6. Nonmetric Multidimensional Scaling (NMDS) analysis of the 10 soil samples.

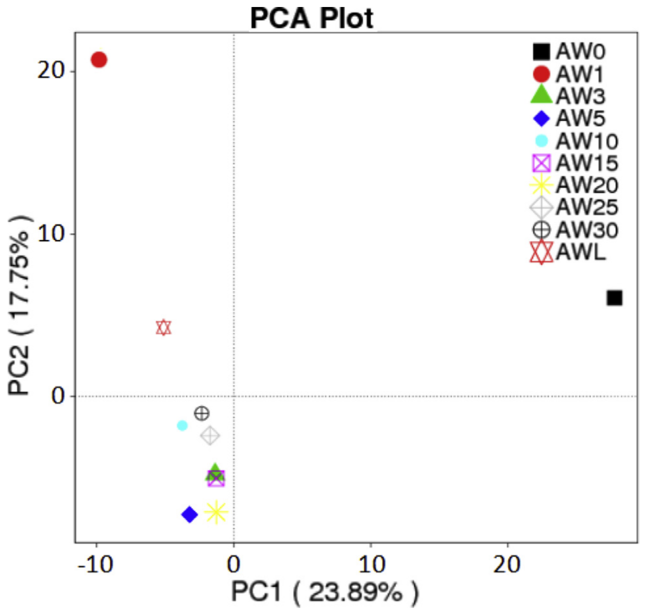

Fig. 7. Principal Component Analysis of 10 soil samples calculated via QIIME. PCA plot analysis based on R software for all samples.

cropping of cotton (Gómez et al., 2009). Moreover, after long-term continuous cropping of cotton, rotating cropping of maize for just one year changed the soil fungal community structure in the same manner (Orgiazzi et al., 2012). There were huge differences to the above two crop cultivation management modes; however, both of them have proven that under the local environment and climate conditions, the cultivation management mode exerts a significant influence upon the soil fungal community structure (Denis et al., 2015). The reasons may be due to the following aspects: Firstly, the sampling location was at the center of the Eurasian continent far from the ocean; therefore, the weather was dry and lacked rain, the soil was dry and alkaline with few organic components. These environmental features equipped the soil in the region with a relatively simple yet special original ecological fungal community structure, which was comparatively more sensitive to the changes in external environment and ultimately underwent significant adaptive adjustments (Eleni et al., 2012). Secondly, during the cultivation of cotton, manual management measures such as regular irrigation, fertilization (mainly nitrogen), and use of mulching film significantly changed the physiochemical features of soil such as permeability, fluctuation range of moisture, and ground temperature (Jerbi et al., 2015). Compared to the influence of cotton growing upon soil microorganisms, these factors contributed more to the formation of new soil fungal community structures (Wu et al., 2011). At present, many reports exist on the causes of continuous cropping obstacles (Xiong et al., 2015). Furthermore, with the rapid development of molecular ecology technology, many studies have started to pay attention to the close relationship between the changing rules of the soil microbial community structure and continuous cropping obstacles (Hilton et al., 2013; Wu et al., 2013); however, few reports show a relatively fixed cultivation of

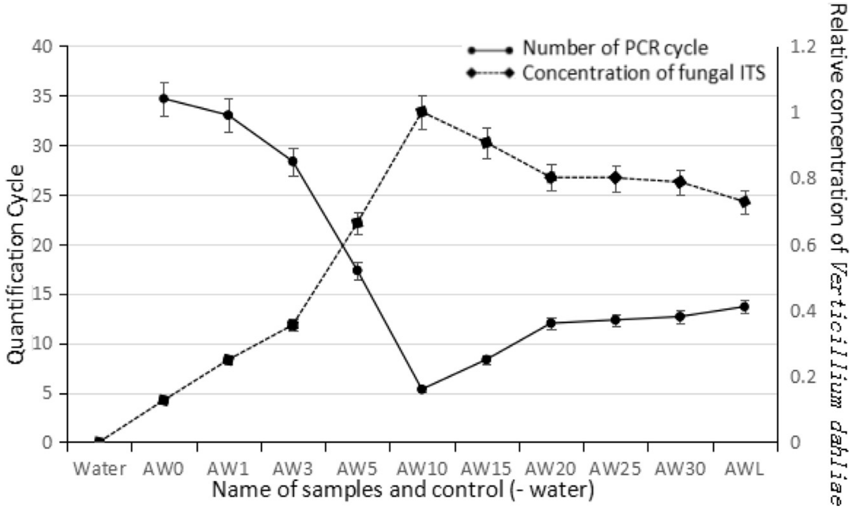

Fig. 8. Results of the 10 soil samples with *V. dahliae* via Real time RT-PCR.

certain crop to be the major reason for soil microbial community structure changes (Chen et al., 2014). In addition, reports involving its influence means and depth are also rare (Sun et al., 2016). This study chose the soil microbial ecological structure of Xinjiang as research object, with the goal to unraveling the influencing degree and means of cultivation management mode upon the soil microbial community structure. The results are expected to inhibit or relieve the agricultural damage caused by continuous cropping obstacles of cotton through a more in-depth research on the relationship between specific crop cultivation management modes and soil-borne fungal OTU groups that cause cotton diseases.

4.2. *Change rules of soil fungal community structure under long-term continuous cropping of cotton*

During the 0–30 y of continuous cropping of cotton in this region, the influences of cotton cultivation management measures featured consistent artificial intervention and continuous cropping of cotton upon soil fungal community structure. In addition, within a small region of the same town and the same soil conditions, cotton fields with 0–30 y of continuous cropping history of cotton co-existed; hence, facilitating the study on the changing rules of soil fungal community structure under long-term continuous cropping. The soil sampling time was June, during which cultivation measures entered the normal stage and both the local climate and soil temperature were suitable for fungal growth and fungal soil-borne diseases spreading. Therefore, the reliability of the research results will not be influenced by the existence of a large amount of fungal hypha. The α diversity changing rules in the results revealed the following conclusions: Firstly, soil fungal diversity during continuous cropping of cotton was not rapidly and continuously adjusted to the diverse structure compositions under the threat of a new environment; however, it approached stability through repeated adjustments with the fluctuation range moving from large to small. It was predicted that the plantation mode of both half-year cultivation and half-year retirement determined by local climate conditions will lead to the soil fungal community structure being influenced by cotton cultivation and undergoing restorative changes under the influence of local natural climate and environment during the period of retirement (Zhang et al., 2013). Therefore, the original soil fungal community structure showed a strong self-restoring capacity (Kozlova et al., 2008). Moreover, the new soil fungal community structure came into being after 10–15 y of continuous cotton cropping, yet the time for the new bacterial community structure was only 5–10 y (Zhang et al., 2013). The reason could be due to bacteria having a stronger adaptive ability to external environment than fungi. In particular, the temperature and moisture in the local natural climate conditions underwent sharp changes during one year, which had a larger influence upon fungi than bacteria (Kennedy et al., 2015).

The two Beta diversity methods of NMDS and PCA were adopted to compare the sample results, through which some composition evolution rules of fungal community diversity structure were revealed in response to prolonged continuous cropping time. Firstly, the distance between AW0 and continuous cropping samples was large, indicating that cotton cultivation caused significant changes to the original ecological soil fugal community structure (Tian et al., 2011), which was consistent with the α diversity analysis results. Furthermore, the distance between AW1 and other continuous cropping samples was also large, with AW3 at the middle and others surrounding it. This showed that the new soil fungal community structure was formed during the 3rd year of continuous cropping of cotton; and although the soil fungal community structure remained fluctuating after continuous cropping, it was adjusted around the AW3 basic composition structure, indicating that the interaction between crops and fungal community was a long-term process (Xue et al., 2017). Thirdly, although rotating cropping was only for one year, AWL was adjusted to the level closest to AW0. The soil fungal community structure adapted very fast to influences of cultivation management mode and crops (Raaijmakers et al., 2009). Furthermore, from the OTU heatmap at the classification level of the order, the changes with which OTU genus caused the above three phenomena could be further analyzed. Firstly, the huge changes of original ecological soil fungal community structure that were caused during the 1st year of cotton plantation were realized through the increased abundance of six orders, such as *Mucorales* etc, and the decreased abundance of 10 orders, such as *Pleosporales* etc (Xu et al., 2017). Cotton plantation did neither promote nor hinder all fungi. Moreover, in continuous cropping of cotton for more than three years, some species continued to change; however, the number and changing amplitude of fungi lowered strongly. It can be seen that the slight changes of influencing factors in continuous cotton cropping for different years will also trigger rapid or slow adaptive adjustments of individual orders' abundance. Thirdly, planting maize after cotton cropping for one year caused relatively significant changes to the abundance of several orders, such as *Corticiales* etc. This suggests that changing the type of crops under the local environment could only cause large changes to the fungal abundance of few genera, yet the influence of planting itself upon soil fungal community structure was significant (Buée et al., 2009). Fourthly, for fungal orders with few changes in abundance of cotton continuous cropping showed large changes, if analyzed from lower classification levels of family, genus, and species. In addition, the primers used in this study only facilitated the analysis of *Ascomycota*, and excluded the in-depth research on other phyla of fungi (Gardes et al., 1993; White et al., 1990). Therefore, we hold that for imperfect fungi phylum soil-borne fungal pathogens causing significant influence to cotton plantation, the related abundance changes in soil should be studied from the species level. Only in this way, can the overall process of cotton disease occurrence, development, and outbreak be more accurately explored, thus suggesting effective solutions for its fundamental prevention and treatment.

4.3. *Relationship between* verticillium *wilt pathogenic fungi abundance change and disease occurrence relationship in cotton continuous cropping*

One phenomenon exists in particular in the long-term plantation of local cotton that, although the occurrence rate and scale of fungal *verticillium* wilt and fusarium wilt, kept increasing after years of cotton continuous cropping. Agricultural damage can be controlled due to the effective prevention and treatment measures or dry weather against the occurrence of fungal diseases, so that the plantation year limit could exceed 30. Of course, if diseases will not be properly controlled, their large-scale outbreak will lead to a lack of harvest. An analysis of the research results showed that the phenomenon was closely related to the number of pathogens in soil and the dry weather with little rainfall in the region during the cotton growth period (Zhang et al., 2013). Firstly, at the early stage of cotton cropping, few or no pathogens were present in soil; therefore, disease will not break out, although environmental factors were suitable for their growth. Secondly, with prolonged time of continuous cropping, small-scale disease outbreaks will accumulate pathogens in the soil. Combined with the fact that the pathogens can exist in soil for a long time and local irrigation means promote its spreading, preconditions are provided for a large-scale outbreak of *verticillium* wilt. Thirdly, *verticillium* wilt pathogens will not continue to increase once reaching a certain threshold, which may be related to the survival time of *verticillium* wilt pathogens' hyphae in soil (Niem et al., 2013). Fourthly, when the number of pathogens reaches the minimal required value for outbreak, there is no phenomenon of year-on-year outbreak of disease, which may be related to factors such as outbreak conditions, climate, soil physiochemical features, and disease-resistant varieties (Pullman et al., 1981). For example, the local weather is dry and lacks rain. During summer, it weather conditions of low temperature (25 °C) and high moisture (80 %) suitable for *verticillium* wilt pathogens are rare to occur; therefore, some cotton fields can be used for long-term plantation for more than 30 y. Moreover, the relative content of pathogens in soils of continuous cropping for different years was used in this study. This was because the gene fragments for marking *verticillium* wilt pathogens were multi-copy, and the hyphae of pathogens in soil and pathogens themselves were polynucleate; therefore, relative content can more directly compare the differences between samples. Furthermore, the local agricultural production experience showed that to restore the productivity of cotton fields, constant rotating plantation of other crops that are not infected with *verticillium* wilt for more than five years was the only solution. The number of *verticillium* wilt pathogens in soil will drop after rotating cropping until reaching the minimal degree that will not any longer cause cotton diseases, hence fundamentally preventing the possibility of cotton diseases (Ikeda et al., 2015).

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