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EFFECTS OF *TRICHODERMA* STRAINS ON THE MICRO-ECOLOGY OF THE RHIZOSPHERE SOIL OF *DACTYLIS GLOMERATA* THROUGH HIGH-THROUGHPUT SEQUENCING ANALYSIS

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Abstract

In the study, we used high-throughput sequencing as a tool for testing and comparing the microbes from two soil samples in terms of the microbial quantity, uniformity and diversity, showed that after the exposure to *Trichoderma*, the quantity of rhizosphere bacteria and *Actinomycetes* increased by 29.82%, as well as the relevant increasing of community uniformity and diversity. The quantity of rhizosphere eukaryotic microbes dropped by 9.1% while the species diversity was higher than CK with the comparative prominence of dominant species. On the total quantity of the microbes, T1 with exposure to *Trichoderma* was 4.51% higher than CK. In addition, the exposure to *Trichoderma* can increase the quantity of such communities as *Nitrospirae* which can promote the metabolism of mineral elements. The results showed that the application of *Trichoderma* can increase the number of the rhizosphere bacteria and actinomycetes of *Dactylis glomerata*, reduce the number of harmful fungi, improve the microbial diversity, facilitate the reproduction of some probiotics and improve the soil's micro-ecology.

Key words: High-throughput sequencing; *Trichoderma*; Soil micro-ecology; Diversity; *Dactylis glomerata*.

Introduction

Fungi play an important role in solving global challenges in agriculture (Lange, 2014). The beneficial effects of fungi on plants depend on complex nutrient and chemical signals as well as climatic and soil factors (Ortíz-Castro *et al*., 2009). *Trichoderma* spp. widely distributed in the nature, it can be found all over the world (Jaklitsch and Voglmayr, 2015). At the same time, *Trichoderma* spp. is a class of plant growth promoting fungi (PGPF), the secondary metabolites produced by the *Trichoderma* can protect plants from soil borne fungal diseases in agricultural production, and promote the growth, development and yield of plants (Woo *et al*., 2014), and it has been widely used in biological control of phytopathogens such as *Rhizoctonia solani*, *Fusarium oxysporum*, *Chondrostereum purpureum* and *Armillaria mellea* (Benítez *et al*., 2004; El-Komy *et al*., 2015). *Trichoderma* spp. belongs to the subdivision of Deuteromycotina, Class-Hyphomycetes, Order-Hyphomycetales, Family-Hyphomycetaceae which is an important bio-control fungal to plant diseases, and has a significant role in growth-promoting. A researcher find that *Trichodermu lignorum* have antagonistic effects on several fungi in the soil, and then people gradually recognized the disease control function of *Trichoderma* on the soil (Weinding, 1932). In recent years, *Trichoderma* is generally considered to be the most likely bio-control agent to replace a series of chemical fungicide, as an antagonistic microorganisms *Trichoderma* has abound resources and can promote crop growth in resource-rich, playing an increasingly important role in the development of sustainable agriculture (Liu *et al*., 2016).

High-throughput sequencing technology has become the most commonly used method to investigate the microbial community diversity in micro-ecology. It is capable of depth sequencing of environmental microorganisms, and can sensitively detect the extremely subtle shifts of environmental microorganisms resulting in the changes of external environment, which has an important theoretical and practical significance in the study of the relationship between organisms and the environment, environmental management and the utilization of microbial resources (Amaral-Zettler *et al*., 2009; David *et al*., 2016). Soil microbes affected the absorption and transformation of soil nutrients, the changes of soil microbial quantity directly or indirectly affected the transformation of nutrients in the soil (de Vargas, *et al*., 2015; Li *et al*., 2014). In this study, two soil samples (control CK and *Trichoderma* treatment T1) were treated with high throughput sequencing, Illumina Miseq PE300 were used to study the effects of *Trichoderma* on soil micro-ecology. By high-throughput sequencing, we studied the effect of *Trichoderma* on the rhizophora micro ecology of Orchardgrass, and studied on the promoting mechanism of *Trichoderma* to Orchardgrass from changes of soil microbial quantity and species. Thus settled the foundation for the efficient utilization of *Trichoderma* in the forage production, reduced the application of chemical fertilizers and pesticides, which was conducive to the sustainable development of environment and ecology (Masayuki, *et al*., 2014).

Materials and Methods

Studied areas and strategy: The study was conducted in a *Trichoderma*-dominated land and a land without *Trichoderma* spp. located in the experimental farm of Southwestern University, Rong Chang campus (As shown in Fig. 1). Soil sampling: T1 are the soil samples of *Dactylis glomerata* with the inoculation of *Trichoderma* strain M1, CK as control group are the ones without *Trichoderma* in the preliminary experiment. Samples of soils were collected using sterile equipment.

Total DNA extraction and Genomic PCR amplification: Total soil DNA of the two samples was extracted using the E.Z.N.A. ™, DNA Isolation Kit (Omega Bio-tek, Inc. United States) according to the manufacturer's instructions. The purity and concentration of DNA was detected by Nano Drop 2000, and the DNA integrity by 1% agarose gel electrophoresis. Using ABI GeneAmp ® 9700 PCR spectrometer PCR amplification of the genome. The 16S rRNA V3-V4 region of the bacteria was first amplified using 338F5'-ACTCCTACGGGAGGCAGCA-3', 806R5'-GGAC TACHVGGGTWTCTAAT-3' primers. Amplification procedure: a. 1x (95ºC pre-degenerated 3 min); b. 27x (95ºC denaturation 30s; 55ºC annealing 30s; extending 45s); c. 72ºC extension ending in 10 min. The ITS region of fungus was amplified using primer 0817F 5'-TTAGCATGGAATAATRRAATAGGA-3', 1196F 5'-TCTGGACCTGGTGAGTTTCC-3' (Rousk *et al*., 2010). Amplification procedure: a. 1x (95ºC pre-degenerated 3 min); b. 35x (95ºC denaturation 30s; 55ºC annealing 30s; extending 45s); c. 72ºC extension ending in 10 min.

Quantitative measurement of PCR products, Library construction and sequencing: After 3 cycles of amplication for each sample, the PCR product of the same sample was mixed; then 2% agarose gel electrophoresis was used for detection, AxyPrepDNA gel extraction Kit (Axygen firm) for gel extraction of PCR product and Tris-HC1 for eluting. With reference to the preliminary quantitative results of electrophoresis, the blue fluorescence quantitative system of QuantiFluor ™-ST (Promega Corporation) was applied to detect and measure PCR product, then in accordance with the sequencing requirements of each sample, a corresponding mixing was made. Miseq library was constructed and sequenced by Shanghai Meiji pharmaceutical technology, Ltd, and the sequencing platform is Illumina Miseq PE300.

Bio-information analysis process: In order to improve the quality of the analysis results, under the precondition of the rich amount of sequences, the quality of sequences should also be ensured. Therefore, raw data must be filtered before processing to obtain optimized sequences. After the removal of chimeric sequences OTU clustering analysis was conducted for taxonomic analysis of the OTU representative sequences. Such OTU-based clustering analysis can be used for various diversity indices analysis of OTU, as well as the detection of the sequencing depth; based on the taxonomic information, the statistical analysis of community structure can be made at every level of the classification. All the analysis above also lay the foundation for a series of further in-depth statistical and visual analysis of the community structure.

Fig. 1. Two different lands of *Dactylis glomerata*. A presents the soil samples of *Dactylis glomerata* with the inoculation of *Trichoderma* strain M1, B presents the ones without *Trichoderma as* control group, plus one detailed (small square=1m and large square=2m) at the bottom.

Results

Total soil DNA, PCR amplification results of soil bacterial 16S rRNA and fungi ITS: Total soil DNA of sample CK and sample T1 was repectively extracted and purified for testing; the results were: DNA concentration 213.6, 194.4ng·μL$^{-1,}$ OD260/280 : 1.91, 1.92, OD260/230: 0.85, 1.74, respectively. So DNA is qualified for subsequent PCR amplification (Fig. 2).

Figure 3 shows that after the electrophoresis detection of the PCR amplification products from samples, there were around 500bp sequences from PCR products in soil fungi ITS region while around 590bp sequences from PCR products in soil bacterium, showing that the size and concentration were suitable for subsequent tests.

Analysis of microbial diversity in soil samples: When studying the microbial diversity of the community ecology, the diversity of singleton (Alpha diversity) could be used to reflect the abundance and diversity of microbial communities, including a series of statistical analysis index to estimate the species abundance and diversity in environment. If the similarity percentage values among sequences were more than 97, they were clustered into one OTU. In the analysis, the following statistic and analytic index were used: species richness, Ace index, Chao index, Shannon Index, Coverage index, Simpson Index etc.

Analysis of bacterial community diversity index: The sequenced data of bacteria and *Actinomycetes* in soil samples; a total of 12,823 16S rRNA reads from bacteria and 16647 from the *Actinomycete*s in soil samples were obtained (Table 1), representing the OUT values of 601 species of bacteria and 671 species of *Actinomycetes* respectively. The species abundance in samples reflected in the actual OTU values in the sample. Chao index are estimates of species abundance in the sample, and typically larger than OTU values; the results of Chao index were 613 and 630 respectively, showing the good abundance in the sample. Coverage reflects the real situation of microorganism in the sample; the higher the value, the more possible the sequences were detected in the sample; CK and T1 had approximate coverage, showing that this sequencing work is well finished. The Shannon index and the Simpson index were used to estimate the uniformity and diversity of microorganisms in a sample, Shannon index goes in direct proportion with the community's diversity while Simpson in reverse proportion with the community's diversity. Results showed compared to that of CK, the number of bacteria and *Actinomycetes* of T1 increased by 29.82%, and the uniformity and diversity of bacterial communities in T1 was also better than CK.

Analysis of eukaryotic microbial diversity index: The sequencing data of Eukaryotic microbes in soil samples. As a result, a total of 25255 (CK) and 23148 (T1) fungal 18S sequences (Reads) were obtained, representing 104 and 117 species of fungi (OTU). The results show that Eukaryotic microbes in T1 was decreased by 9.1% in comparison to CK while had higher species abundance than CK, and dominant species stood out even more (Table 2).

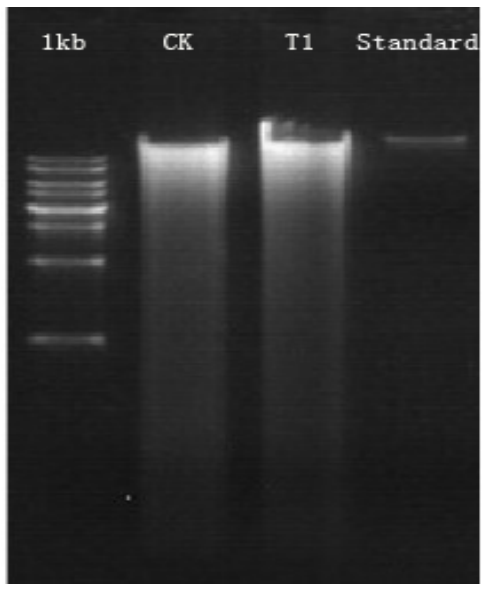

Fig. 2. Agarose gel electrophoresis result of soil DNA.

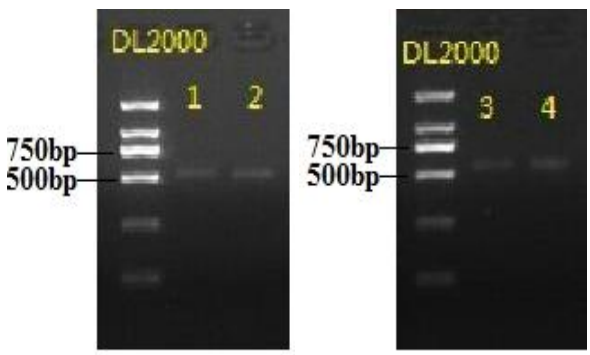

Fig. 3. Agarose gel electrophoresis result of soil fungal and bacteria DNA, DL2000: DL2000^{TM} DNA Marker; 1: CK Fungi ITS; 2: T1 Fungi ITS; 3: CK 16S rRNA; 4: T1 16S rRNA.

Composition of soil microbial community

Composition of soil bacteria and actinomycetes communities: On the phylum levels if the total abundance percentage value was bigger than 2, they were taken as the dominant flora; of the two soil samples the content of Proteobacteria were 47.06% and 53.5% respectively at the highest point. Among them, in sample T1 with the application of *Trichoderma*, *Proteobacteria, Gemmatimonadetes, Nitrospirae, Deferribacteres* had increased in varying degrees in the entire share of microbial communities compared to the control group while *Firmicutes, Bacteroidetes, Chloroflexi, Acidobacteria* and *Actinobacteria* showed varying degrees of decline in proportion of whole microbial communities (Figs. 4-6).

Composition of eukaryotic microbial community in soil sample: If the total abundance percentage value was bigger than 2, they were taken as the dominant flora; of the eukaryotic micro-organisms in the two soil samples the content of *Ascomycota* (SAC fungi) is 70.98% and 78.38% respectively at the highest point. Compared with CK, *Ascomycota, Eukaryota*-unclassified, *Ciliophora, Basidiomycota,* and *Centrohelida* in group T1 all had

varying degrees of increase indicating that *Trichoderma* boosted the growth of these eukaryotic microorganisms in the soil while *Nucleariida, Metazoa, Choanomonada, Zoopagales* all had different degrees of reduction, indicating that *Trichoderma* inhibited the reproduction of these eukaryotic microorganisms in the soil (Figs. 7-9).

Distribution and abundance of soil microbes: The color change of heatmap could reflect the data of a two-dimensional matrix or table which can intuitively display the size of the data value by default color shades (Jami *et al*., 2013). The data were often clustered by species similarity or abundance similarity of samples and then presented in the heatmap chart, which could cluster high abundance species and low abundance species in different blocks, and in which color gradients and similarity levels could reflect the community composition's similarity and difference of multiple samples in different taxonomy. The species abundance heatmap of the bacteria sample on phylum levels, in which the closer a color approaches red, the higher proportion the species account for in the sample. Results show that in soil sample T1 and CK the composition of bacteria had differences but such difference was not significant (Fig. 10). The results of the species abundance heatmap of eukaryotic micro-organisms on phylum levels indicating that there were significant difference of eukaryotic micro-organism composition in sample T1 and CK (Fig. 11).

Table 1. Analysis of bacterial and *actinomycetes* diversity index.

Sample number	OUT value	Chao index	Ace index	Coverage index	Shannon index	Simpson index
CK	601	613	612	0.9976	5.54	0.008
T1	617	630	630	0.9971	5.69	0.006

Table 2. Analysis of nuclear microbial diversity index.

Sample number	OUT value	Chao index	Ace index	Coverage index	Shannon index	Simpson index
CK	104	104	104	0.9999	2.66	0.1140
T1	117	117	118	0.9999	2.43	0.2154

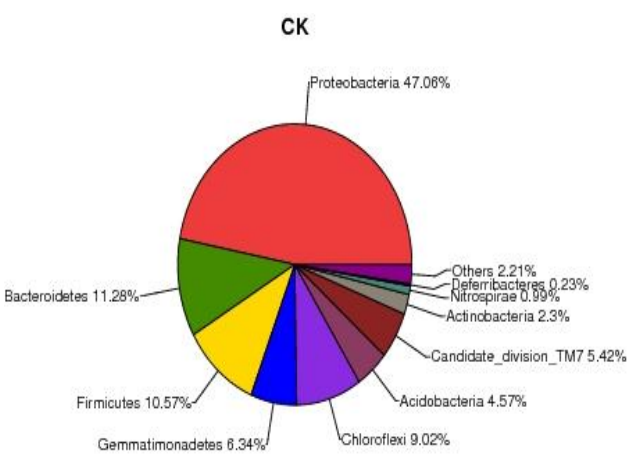

Fig. 4. Bacteria and *actinobacteria* phylum levels of community structure diagram components of CK, these with abundance lower than 1% were merged into the other part; the same below.

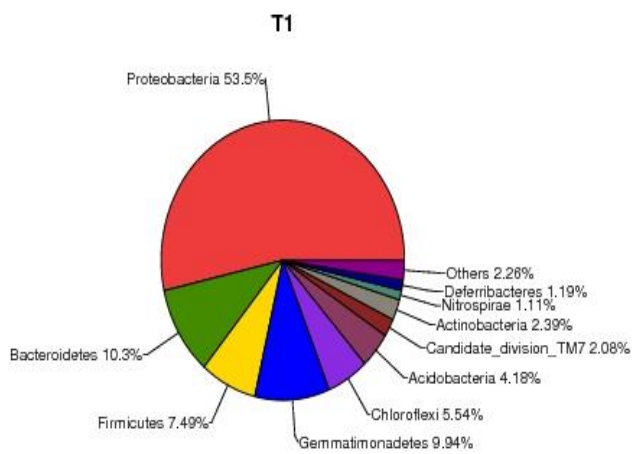

Fig. 5. Bacteria phylum and *actinobacteria* levels of community structure diagram components of T1.

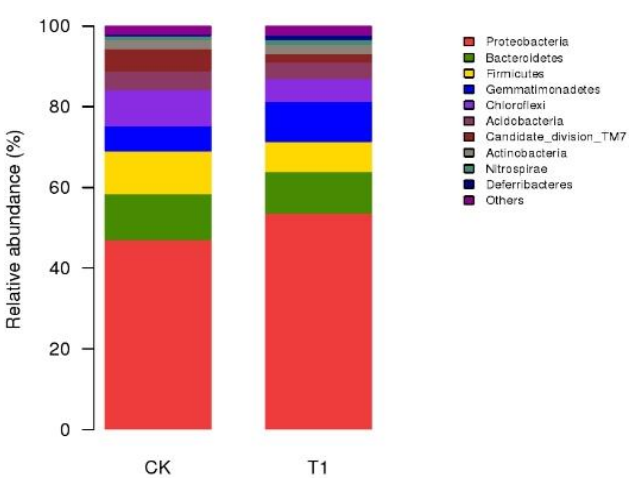

Fig. 6. Bacteria and *actinobacteria* phylum levels of community structure diagram components of soil.

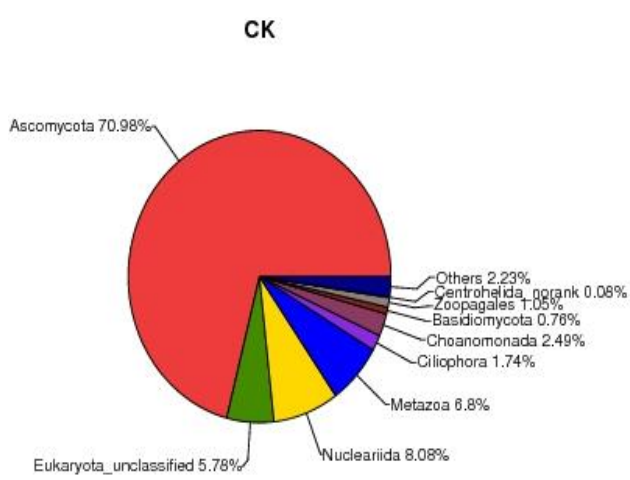

Fig. 7. Nuclear microbial phylum levels of community structure diagram components of CK.

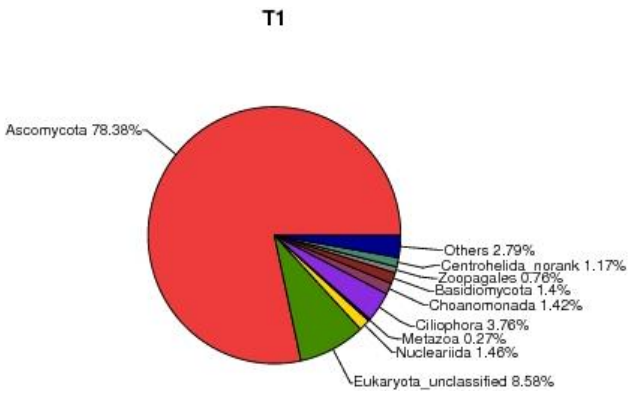

Fig. 8. Nuclear microbial phylum levels of community structure diagram components of T1.

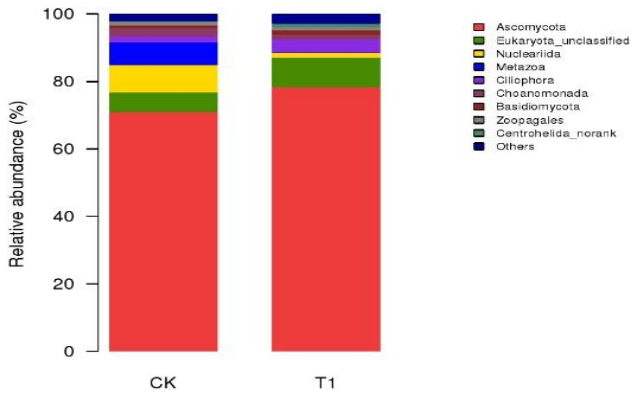

Fig. 9. Nuclear microbial phylum levels of community structure diagram components of soil.

Discussion

The researchers has separately adopted normal PCR and reverse transcription PCR with combination of DGGE to analyze the bacteria community's structure and composition of chrysanthemum in different growth and development stages in the rhizosphere soil and root tip, indicating that the dominant bacteria are mainly *Pseudomonas*, *Comamonas*, *Variovorax* SP., *Acetobacter*, *Bacillus* and *Arthrobacter* (Duineveld *et al*., 2001). Most microbes of *Actinobacteria* and *Bacteroidetes* mainly degrade cellulose, and even many hard-to-degraded aromatic compounds, playing an important role in the mineralization of the soil (Nie *et al*., 2009). Researchers studied the effects of *Trichoderma* chlamydospores preparation on soil microbial quantity through experiment, and the results showed that after applying *Trichoderma* preparation the number of bacteria in rhizosphere soil slowly increased while fungi significantly decreased in comparison with control group (Regragui and Lahlou, 2005). The experiment also indicated that the adding of *Trichoderma* can increase the diversity of soil bacteria (Hermosa *et al*., 2004). According to studied the effects of *Trichoderma harzianum* on regulating the soil microbial communities and soil enzyme activities of rice seedling bed through experiment which showed that compared with control group the number of bacteria in rhizosphere soil of the inoculated rice seedling increased by 50.7% while the number of fungi reduced by 16.15%, indicating *Trichoderma harzianum* could efficiently regulate the rhizosphere microbial community composition of the rice seedling; thus the soil micro-ecosystem environment was improved (Tewari and Singh, 2012).

Soil microorganisms affect the absorption and transformation of soil nutrients, and the quantity changes of soil microbes directly or indirectly affect the conversion of nutrients in soil. Based on the analysis results of the changes of soil microbial diversity, this experiment showed that *Proteobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Deferribacteres* all have varying degrees of increases while *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Acidobacteria* and *Actinobacteria* all have varying degrees of decline in proportion of whole microbial communities. This is consistent with the above research results. *Nitrospira* participates in nitrogen's fixation, form- transformation in soil, and can secrete large amounts of organic acid to activate mineral elements in soil, so it is crucial to maintaining soil productivity (Azcon & Barea, 1975; Nelson & Mele, 2006). In the soil, *Actinomycete* participates in the cycling of organic matter, forming soil structure, and secreting antibiotics (Joergensen & Wichern, 2008). In this study, the percentage of *Actinobacteria* in T1 with *Trichoderma* treatment had somehow declined a little but the total amount of *Actinobacteria* still increased in compassion to control group.

The diversity and community structure and composition of soil fungi may have a profound impact on the ecosystem, as well as balance of the ecosystem. Fungi are the main components of soil biomass and organic matter decomposition, playing an important role in agricultural eco-system (Ding *et al*., 2007). The research into eukaryotic microbial diversity in soil showed that in the sample soil with *Trichoderma* treatment *Ascomycota*, *Eukaryota-unclassified*, *Ciliophora*, *Basidiomycota*, *Centrohelida* displayed varying degrees of increase, while *Nucleariida*, *Metazoa*, *Choanomonada*, *Zoopagales* had different degrees of reduction, which is consistent with other researches (Qian *et al*., 2015; Hannula *et al*., 2013).

Diversity index is one of the most effective ways to evaluate the diversity of soil microbial community, high diversity index indicates higher microbial community diversity. The Shannon index indicates the species quantity in species community the bigger the value is, the more diversified the species are in the community, ACE and Zhao index reflect the species abundance, which are commonly used to estimate the total categories of species, the bigger the value is, the more the categories; the bigger the value of Simpson index, the more prominent the dominant species in biological community (Pielou, 1974; Li *et al*., 2015). Results of this study show that in the soil sample T1 with the implantation of *Trichoderma* the volume of bacteria increased by 29.82% compared to CK, and species abundance, diversity, and homogenies were all higher than CK. On the Eukaryotic microbes, the volume of Eukaryotic microbes in T1 declined by 9.1% compared to CK while species abundance was higher than CK; dominant species stood out. On the total amount of microorganisms, T1 with *Trichoderma* treatment increased by 4.51% compared to CK. Soil bacteria can change soil's physical and chemical nature, and play a leading role on effective absorption of soil nutrients and promotion of plant growing (Kagohashi *et al*., 2013). Many fungi are pathogenic fungus of crops and the implantation of *Trichoderma* reduced the number of harmful fungi through such means as space competition, nutrition competition or hyperparasitism, enriched the groups of soil microbes, changed community structure of soil microorganisms, promoted the reproduction of beneficial microorganisms and improved soil micro-ecological environment.

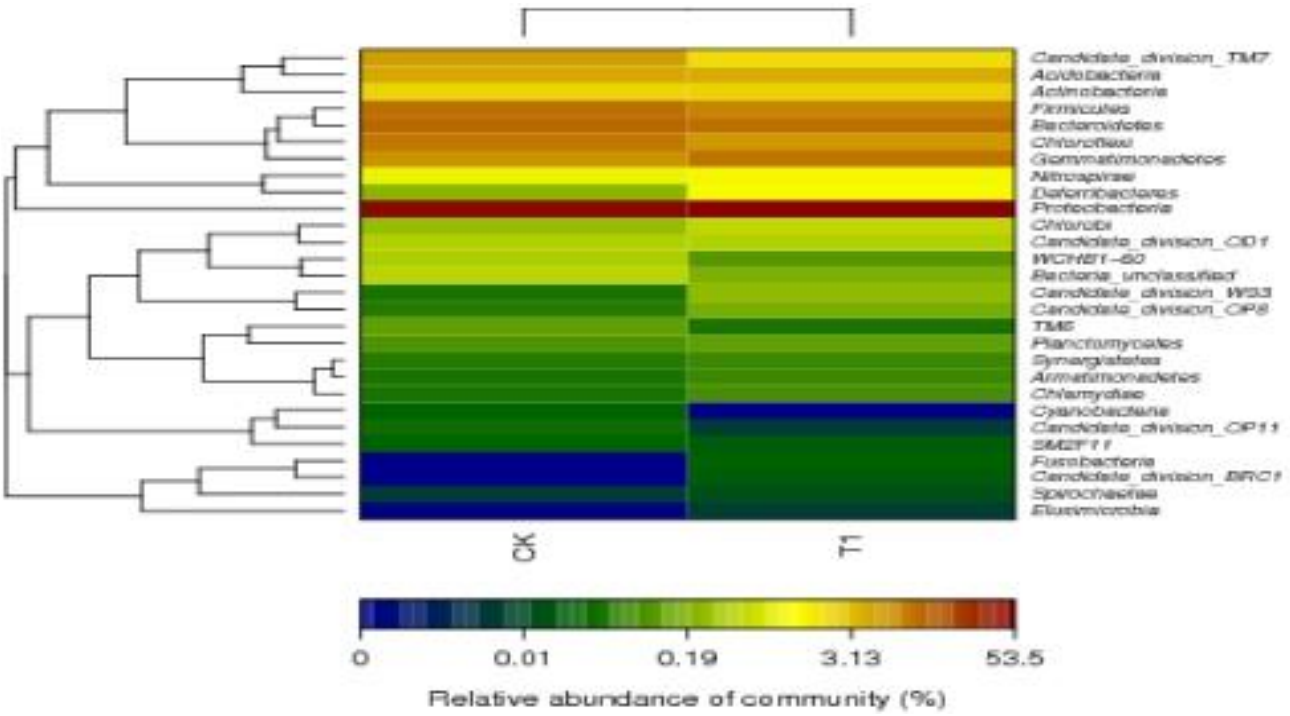

Fig. 10. Samples phylum levels of bacteria species abundance heatmap.

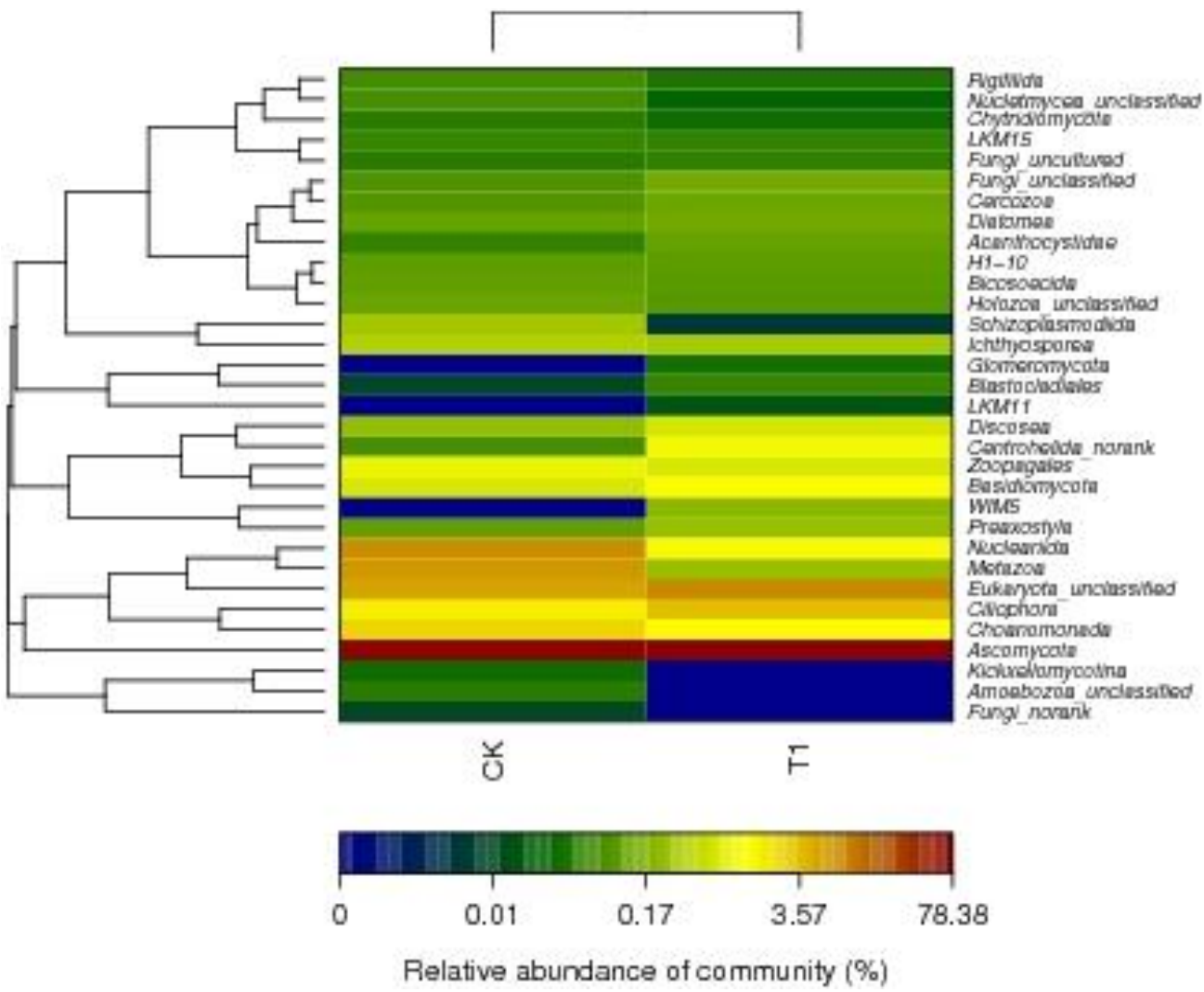

Fig. 11. Samples phylum levels of nuclear microbial species abundance heatmap.

Conclusion

By high-throughput sequencing of microorganisms in two soil samples (control CK and T1 with *Trichoderma* treatment), we obtained 12,823 and 16,647 bacterial 16S rRNA sequences respectively from the two soil samples representing 601 and 617 species of bacteria, respectively. We also obtained 25,255 (CK) and 23,148 (T1) fungal 18S sequences, representing 104 and 117 species of respectively in two soil samples. In terms of microbial number, homogeneity and diversity, compared to the control group, the number of rhizosphere bacteria and the *actinomycetes* after the treatment of *Trichoderma* rose by 29.82%, community, homogeneity and diversity also increased. The number of eukaryotic microorganisms in rhizosphere declined by 9.1%, but species abundance is higher than CK, the dominant species were prominent. From total amount of microbes, the total amount of microbes in T1 with *Trichoderma* treatment was higher than control CK by 4.51%. In addition, the *Trichoderma* treatment can increase such communities' quantity as *Nitrospirae* which could promote metabolism of mineral elements. The above data proves that the implantation of *Trichoderma* could increase the number of rhizosphere bacteria and *actinomycetes* of *Dactylis glomerata*, reduce the number of harmful fungi, raise the microbial diversity, promote the microbial reproduction and improve soil micro-ecological environment.

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