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Analysis of microbial diversity of tea tree (*Camellia sinensis* (L.) O. Ktze.) sick rhizospheric soil using soil metaproteomic technology

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ABSTRACT

Soil metaproteomics technology was used to analyse and undertand the changes in microbial diversity in rhizospheric soil of Tieguanyin tea trees (*Camellia sinensis* (L.) O. Ktze.**)** of different ages. The results showed We identified 2911 protein spots in the rhizosphere soil of tea tree. These were derived from 388 species of microorganisms [331 species of bacteria (85.31 %) and 57 species of fungi (14.69 %)]. According to the analysis of microbial species in tea tree rhizospheric soil with different ages, the number of microbial species in 0, 4, 9 and 30-years old rhizospheric soils were 346, 350, 345 and 354, respectively. Among them, 310 microbial species co-existed in the rhizospheric soil with different ages tea trees. The analysis of results of microbial species and function showed that, as the tea tree age increased, the pathogenic microorganisms in its rhizospheric soil increased, while the microorganisms, for decomposing harmful substances, probiotics and microorganisms related to carbon cycle and nitrogen cycle decreased. The microbial structure and functions of tea tree rhizospheric soil changed significantly with increase in tea tree age.

Keywords: Age of tree, bacteria, *Camellia sinensis,* fungi, microbial diversity, rhizospheric soil, soil metaproteomics, soil sickness, Tea tree

INTRODUCTION

There are many species and large number of crop soil rhizospheric microorganisms and the selection between roots and soil microorganisms was based on co-evolution, (8,21,22). The unique loop formed by the interactions of plant root system, soil and microorganism changes the physical and chemical properties of soil with time, thereby changing the structure and diversity of rhizosphere microbial community (2,4).

Tea plantation is relatively simple secondary ecosystem. After years of cultivation, the tea garden becomes prone to degradation due to (i). Natural ageing of tea trees, (ii). Soil environment changes after years of tea tree cultivation, (iii). Accumulation of factors not conducive to the growth of tea trees causing "Soil Sickness" and (iv). Soil acidification increases in tea gardens (3,5,19).

The development of crop based "Soil Sickness" is closely related to the changes in the soil ecosystem (22). Recently, many researchers used molecular biology techniques to study the changes of microbial diversity in rhizospheric soil of continuous cropping of peanut (*Arachis hypogaea*) (6), pseudostellariae radix (*Pseudostellariae heterophylla*) (33), cucumber (*Cucumis sativus*) (23), and watermelon (*Citrullus lanatus*) (32). Thus the continuous cultivation of crops could easily lead to the changes in the rhizosphere soil microorganisms and causing the ‘Soil Sickness’ due to soil borne diseases. This decreases the number of beneficial bacteria and increases the number of pathogenic bacteria (16,20,33). In terms of tea trees, Lin *et al.* (17) found that with the increase in age of tea

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trees, the pH value of rhizosphere soil decreased, and the structure of microbial community changed, leading to the decline in tea tree yield and quality. Wang *et al.* (27) reported that with the increase of tea tree age, the amount of soil microbial flora in the rhizosphere of tea tree was adversely affected, and the number of acidophilic microorganisms increased, while the others decreased. Thus age of tea tree, easily lead to differences in the soil microbial structure. Jia *et al.* (13) reported that as the tea tree age increased, the relative abundance of 10-dominant bacterial group at the phylum level changed with planting age and soil environmental factors controls the bacterial diversity. However, current studies focussed on the changes in the microbial flora or their number in the rhizospheric soil of tea tree after continuous planting, becuause there were few reports on specific species of microorganisms and changes in their community structure.

Proteins are the expression of life functions. Using soil metaproteomics technology, the overall proteome of the microbial community in the environment can be directly analyzed, and then the microorganisms in the environment can be determined (12,14). Soil microbial diversity was very rich and the protein abundance in most soils was low, due to the loss of some low-abundance proteins in the protein extraction process (7,26). However, the application of soil proteomics to study the change in soil microbial diversity reflects the change in the soil microbial community structure (1,25). Therefore, this study used rhizosphere soil of Tieguanyin tea tree of different ages and applied the soil metaproteomics technology to analyze the soil microbial diversity and functional changes. This study aimed to (i). analyze the soil microbial diversity using the soil metaproteomics, (ii). the effects of tea garden degradation on microbial species amd (iii). provide a theoretical basis for the restoration of degraded tea garden.

MATERIALS AND METHODS

The rhizosphere soil of Tieguanyin (Asexual tea tree) tea tree, cultivated for 4, 9 and 30 years, was collected from the Longjuan Town, (N24°57 '53.89 ", E117°40' 8.74 ") Fujian province, place of its origin. The soil without tea plant (0 years) was used as control. All soil samples with different aged trees (0, 4, 9, 30 years) were used to extract proteins. The sampling method of rhizosphere soil of tea trees was as under (9): 5 tea trees (Normal growth, free from diseases and insect pests) of different ages were selected respectively, deciduous leaves were removed from the surface of tea trees, tea trees were dug out, and the soil about 1 cm from the root of tea trees was shaken off. Each sample was of 200 g (collected from 3- spots, mixed and homogenized). The control soil sample (200 g) was collected as under: after removing the surface vegetation and litter, the soil upto 20 cm depth was collected from 3-spots (Each 10 m apart). The collected soil was sifted through 40 mesh sieve and stored at -80 ℃ for subsequent extraction of soil proteins. The basic physical and chemical indexes of the rhizosphere soil of tea trees with different ages were shown in Table 1.

Table 1. Fertility status of Tea Tree soil after different years of conitnuous cropping

Tree age (Years)	Nitrogen, Phosphorus, Potassium contents					
	Nitrogen		Phosphorus		Potassium	
	Total (g/kg)	Available (mg/kg)	Total (g/kg)	Available (mg/kg)	Total (g/kg)	Available (mg/kg)
0	2.63	27.2	1.37	79.3	1.72	305.2
4	2.58	29.3	1.64	87.4	1.29	312.1
9	2.47	28.1	1.21	88.5	1.71	320.2
30	2.49	28.7	1.24	89.2	1.69	324.5

(i). Soil protein extraction

The rhizosphere soil proteins of tea trees with different ages were extracted as per the method of Wang *et al* (28) briefly described below. 5 mL of citrate buffer (0.25 M, pH 8.0) and SDS buffer (1.25% w/v SDS, 0.1 M Tris-HCl, pH 6.8, 20 mM DTT) were added into 2.0 g fresh soil, respectively. These mixtures were shaken at 2800 rpm for 3 h (Citrate buffer), then 1 h (SDS buffer) and centrifuged at 15,000 rpm, 4 ^{o}C for 15 min. Supernatants were filtered through 0.45 μm millipore to separate citrate and SDS buffer extractions. Further, the sediment were mixed together, added 5 mL extracting solution (Citrate buffer 2.5 mL and SDS buffer 2.5 mL), shaken at 2800 rpm for 1.5 h and centrifuged at 15,000 rpm, 4 ^{o}C for 15 min, and then supernatants were filtered through 0.45 μm Millipore membrane.

The supernatants of SDS extraction, citrate extraction and SDS+citrate mixed extraction were mixed, then 6 mL buffered phenol (pH 8.0) was added into the filtered liquor mixture being shaking at 2800 rpm for 30 min. After being centrifuged at 15,000 rpm, 4 ^{o}C for 15 min, the phenol phase was collected and mixed and added 6-volumes of cold methanol plus 0.1 M ammonium acetate at -20 ^{o}C, 12 h. Proteins were recovered by centrifugation at 15,000 rpm, 4 ^{o}C for 25 min, and washed with cold methanol first and then thrice with cold acetone. Thereafter, air-dried naturally in sterile super clean bench and stored at -80 ^{o}C for further use.

(ii). Soil protein data collection

The tea tree rhizosphere soil protein (about 100 μg) of different ages trees was taken, respectively, and enzymatic hydrolysis was done to identify the soil proteins. The methods were as under. The enzymatic hydrolysis of soil protein was used as per FASP method (29). The specific steps were: 100 μg soil protein was dissolved in 8 M Urea, 10 mM DTT, total volume made to 200 μL at 37 ^{o}C, 2.5 h, added 50 mM IAA, reaction occurred in dark room for 40 min. After centrifugation at 12,000 g for 10 min, the sediment was added to 400 μL 8 M Urea, then centrifuged at 12,000 g for 10 min. The sediment was added in to 400 μL 50 mM NH_4HCO_3 solution, and centrifuged at 12,000 g, 4 ^{o}C for 10 min, and then filtrate was discarded. This step was repeated twice. After that, the sediment was added into 200 μL 50 mM NH_4HCO_3 solution and trypsin (Soil protein: Trypsin= 50 : 1, mass ratio), and enzymolysised for 16 h at 37 ^{o}C, and centrifuged at 12000 g, 4 ^{o}C, finally, filtrate was collected and freeze-dried.

High performance liquid chromatography (HPLC) was used to separate the soil proteins, with specific parameters as under: HPLC (EasyNano LC1200), chromatographic column (C18, 3µm, 100Å, 75µm × 15 cm), sample (9 μL), mobile phase (A: 0.1% Formic acid in water; B: 0.1% Formic acid, 80% Acetonitrile in water), time (90 min), chromatographic gradient parameters were shown in Table 2.

Table 2. The chromatographic gradient parameters for 90 min

Time (min)	Mobile phase A	Mobile phase B	Flow velocity (nL/min)
0	95%	5%	600
16	90%	10%	600
66	70%	30%	600
82	55%	45%	600
83	5%	95%	600
90	5%	95%	600

The LC-MS/MS was used to indentify the soil proteins, the mass spectrum parameters were: mass spectrometer (Thermo Scientific Q Exactive HF), spray voltage (2.2 kV)，capillary temperature (320 °C)，S-lens RF Level (60)，resolution setting (Level 1, 120,000@m/z 200; Level 2, 30,000@m/z 200), parent ion scan range (m/z 400-1200), product ion scan range (start from m/z 100), MS1 AGC (3e6), ion implantation time (20 ms), MS2 AGC (1e6), ion implantation time (50 ms), ion screening window (2.0 m/z), fracture mode (HCD, energy NCE 27), Data-dependent MS/MS (Top 20), dynamic elimination time (20 s).

Ten μL samples were taken and collected by DDA (Data Dependent Acquisition), and the collected results were used to build the database of microbial species.

Data analysis

Firstly, Proteome Discoverer 2.1, international mainstream proteome analysis software, was used to search and compare the protein data collected by DDA. The retrieval database was performed using Fungus and Bacteria databases downloaded from the uniprot web site. Fungus was used for Swissprot and Trembl full library, and Bacteria as Swissprot library; the search engine uses Sequest HT. At the same time, to remove false positive results, the positive and negative library retrieval strategy was adopted for data retrieval, and the screening condition of FDR ≤ 1% was adopted for both the peptide level and the protein level of the retrieval results. The database retrieval parameters are shown in Table 3. The rest data were analyzed by EXCEL.

Table 3. The retrieval parameters of database

Item	Value
Enzyme	Trypsin
Miss Cleavages	2
Variable modifications	Oxidation (M), Deamidated(N,Q)
Fixed modifications	Carbamidomethyl(C)
Peptide Mass Tolerance	± 10 ppm
Fragment Mass Tolerance	± 0.02 Da
Peptide FDR	Less than 1%
Protein FDR	Less than 1%

RESULTS AND DISCUSSION

Construction of rhizosphere soil microorganism database of tea tree

Soil microorganisms are rich in species and diverse in functions. These microorganisms perform different functions in the soil, including beneficial and harmful aspects (21). The results of this study identified 2911 protein spots in the rhizosphere soil of tea tree, which were derived from 388 species of microorganisms, including 331 species of bacteria (85.31 %) and 57 species of fungi (14.69 %).

Further analysis of the function of microorganisms showed (Fig. 1) that the obtained fungi could be divided into 5-categories according to their biological functions viz., (i). Pathogenic fungi (36.84 %), (ii). Microorganisms inhibitory to bacteria or fungi (12.28 %), (iii). Probiotics (5.26 %), (iv). microorganisms for decomposition of harmful substances (3.51 %), and (v). carbon cycle (42.11 %).

While bacteria could be divided into 8-categories viz., (i). Pathogen (47.43 %), (ii). microorganisms inhibit bacteria or fungi (7.25 %), (iii). probiotics (7.5 5%), (iv). microorganisms for decomposition of harmful substances (8.76 %) and (v). nitrogen cycle (9.97 %), (vi). phosphorus cycle (1.51 %), (vii). sulfur cycle (2.42 %) and (viii). carbon cycle (15.11 %), etc. It could be seen that in the rhizospheric soil, microbial species and biological functions were relatively rich, which laid foundation for the analysis of soil microbial changes at the later stage.

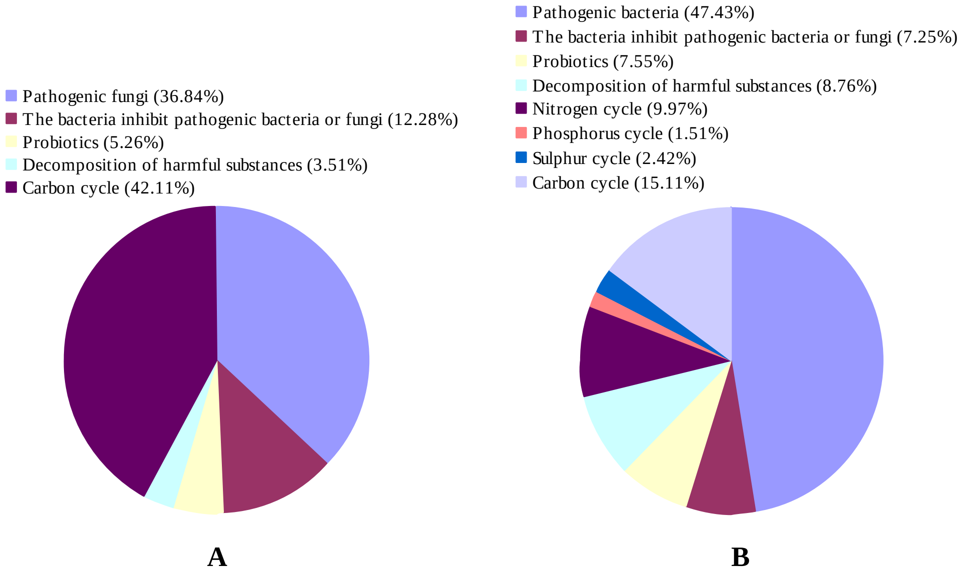

Figure 1. Analysis of microbial species source of soil protein and microbial function.
A: Analysis of fungal function corresponding to soil protein; B: Analysis of bacterial function corresponding to soil protein;

Microbial species analysis of tea rhizosphere soil

In the process of crop planting, with the change of crop planting years, the microorganisms in the soil also changed accordingly. It was reported that during the planting process the *Pseudostellariae heterophylla* and *Rehmanniae*, the number of rhizosphere soil microorganisms and their species also decreased with the increasing planting years (30,33). The results of this study showed that the number of microbial species in the rhizosphere soil of tea tree at 0, 4, 9 and 30 years were 346, 350, 345 and 354, respectively, among which 310 microbial species were present simultaneously in the rhizosphere soil of tea tree at different ages. Further analysis showed that in the rhizosphere soil, the microbial species similarity was 89.60 %, 88.57 %, 89.86 % and 87.57 % in 0, 4, 9 and 30 years old tea trees respectively (Fig. 2). It could be seen that the changes of soil microbial species in the tea rhizosphere soil with different ages are not obvious, which is manifested in their high similarity.

Analysis of microbial functions in tea rhizosphere soil

Many researchers believed that long-term planting of crops could easily lead to the simplification of rhizosphere soil microorganisms, leading to the ‘Soil Sickness’ and the reduction in microbials number (18,31). The decrease in number of rhizosphere microorganisms in crops could be beneficial and harmful. For example, after long-term continuous cropping of *Achyranthis bidentata*, the microorganisms related to nutrients cycling in rhizosphere soil increased, and the growth, yield and quality of *A. bidentata* improved (11). However, after continuous cropping of *Pseudostellariae heterophylla*, the number of pathogenic bacteria in rhizosphere soil increased significantly, the number of probiotics decreased, and the yield and quality of *Pseudostellariae heterophylla* decreased

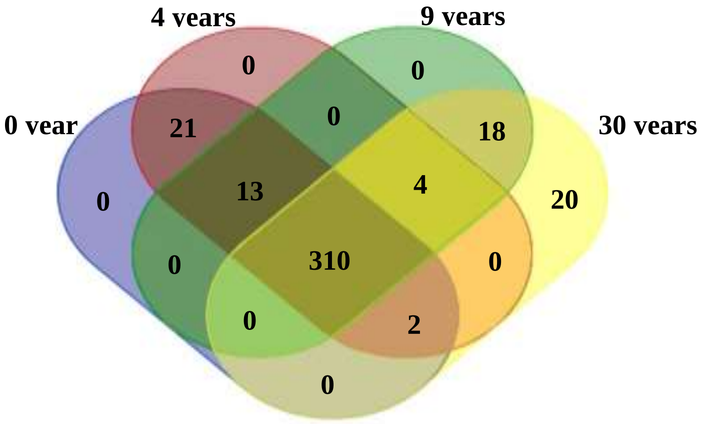

Figure 2. Analysis of the microbial species from different years of tea tree rhizosphere soil

(33). Thus the change in pattern of rhizosphere soil microbial community structure after continuous cropping played an important role in identifying the reasons for the development of continuous cropping soil sickness problem. Soil supprts the tea tree growth, however, after long-term planting of this tree, the microbial function of the rhizosphere soil of tea tree changed?

The results of this study (Table 4) showed that 12 species (0 years), 15 species (4 years), 18 species (9 years) and 29 species (30 years) of pathogenic bacteria were present in rhizosphere soil of tea trees of different ages. The bacteria that inhibited the pathogenic bacteria or fungi, decomposition of harmful substances, probiotics, their spp were 14 species (0 years), 15 species (4 years), 9 species (9 years) and 8 species (30 years) respectively. The results of microbes related to soil carbon cycle, nitrogen cycle, and phosphorus cycle showed that their spp were 11species (0 years), 11 species (4 years), 8 species (9 years) and 7 species (30 years) respectively. It could be seen that the species and number of pathogenic bacteria increased substantially as the age increased, while the probiotics for the nutrients cycle decreased.

Table 4. Analysis of different Microbial Species, source of soil protein and microbial function of tea rhizosphere soil in different planting years

Microbial species	Tea Plantations Age (Years)			
	0	4	9	30
Pathogenic bacteria				
Bacillus cytotoxicus, Baumannia cicadellinicola subsp., Clavibacter michiganensis subsp. , Pasteurella multocida, Polynucleobacter asymbioticus, Salmonella enteritidis, Staphylococcus epidermidis, Staphylococcus saprophyticus subsp	*	*		
Shigella sonnei, Staphylococcus aureus	*	*	*	
Salmonella arizonae, Klebsiella pneumonia	*	*		*
Candidatus Glomeribacter, Erwinia tasmaniensis, Mycoplasma capricolum subsp.		*	*	*
Acinetobacter baumannii, Bordetella pertussis, Burkholderia cenocepacia, Chlamydia trachomatis serovar, Moniliophthora roreri, Pseudomonas syringae pv., Bacteroides fragilis, Campylobacter curvus, Chromobacterium violaceum, Escherichia fergusonii, Pectobacterium atrosepticum, Shimwellia blattae, Sodalis glossinidius			*	*

Alternaria alternata, Botryosphaeria parva, Dichelobacter nodosus, Laribacter hongkongensis, Neisseria meningitidis serogroup, Ralstonia solanacearum, Salmonella newport, Shigella boydii serotype, Shigella flexneri, Vibrio vulnificus, Yersinia pestis				*
Bacteria inhibitory to pathogenic bacteria or fungi				
Collimonas pratensis, Serratia proteamaculans	*	*		
Burkholderia ambifaria, Bacillus subtilis	*	*	*	
Bacillus thuringiensis			*	*
Bacillus licheniformis, Bacillus velezensis				*
Decomposition of harmful substances				
Anoxybacillus flavithermus, Bacillus weihenstephanensis	*	*		
Cupriavidus taiwanensis		*	*	*
Burkholderia lata			*	*
Probiotics				
Cupriavidus necator, Lactococcus lactis subsp., Streptococcus thermophilus, Streptococcus thermophilus, Pseudomonas fluorescens	*	*		
Photobacterium profundum, Actinobacillus succinogenes, Lactobacillus delbrueckii subsp.	*	*	*	
Oceanobacillus iheyensis			*	*
Photorhabdus luminescens subsp., Serratia odorifera				*
Carbon cycle				
Alteromonas mediterranea, Halorhodospira halophila, Tolumonas auensis, Trabulsiella odontotermitis	*	*		
Pantoea ananatis, Thermotoga petrophila, Wigglesworthia glossinidia brevipalpis	*	*	*	
Clostridium acetobutylicum, Synechococcus elongates			*	*
Geobacillus kaustophilus, Phanerochaete chrysosporium, Pseudomonas mendocina				*
Nitrogen cycle				
Pseudomonas entomophila	*	*		
Azotobacter vinelandii, Geobacillus thermodenitrificans	*	*	*	
Paraburkholderia phymatum				*
Phosphorus cycle				
Bacillus halodurans	*	*	*	
Burkholderia multivorans				*

* Microbes detected

The level of soil bacterial community diversity played a decisive role in maintaining the stability of the ecosystem and restoring the system functions after disturbance (10). Latz *et al.* (15) found that the increased diversity of vegetation communities was conducive to the accumulation and growth of beneficial antagonistic bacteria in the underground soil, which inhibited the spread of soil-borne diseases. Therefore, the decrease in bacterial community diversity may result in the structural imbalance of soil microecosystem and the large increase in soil-borne pathogens, may decrease the growth and development of perennial crops. Thus after long-term continuous planting of tea trees, the diversity and structure of soil bacterial community get out of balance, which further affects the growth and development of tea trees.

CONCLUSIONS

Soil suppots the vegetation including the tea tree. This research explored the soil microbial diversity and functions, changes in tea rhizosphere soils with different ages. The results showed that with the increase of tea tree age, rhizosphere soil microbial diversity and functions show significant changes and the soil probiotic species decreased, while the pathogenic bacteria increased. Furthermore, the microbial species that contribute to soil texture improvement and nutrients cycling decreased, which further imbalanced the soil ecosystem environment and the development of soil deterioration leading to the soil sickness, which adversely affected the growth of tea trees.

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