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Bacterial community diversity in rhizosphere of tea plants using16S rDNA amplicon sequencing technique

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ABSTRACT

Illumina-based sequencing approach was used to characterize the changes in the bacterial community of tea plant rhizosphere soils with different ages. More than 6,000 effective tags were obtained for each replication. The community was composed of 10-dominant group at the phylum level and the relative read abundance of different bacterial phyla changed with planting age. Canonical Correlation Analysis (CCA) revealed that the rhizosphere samples of different planting ages differed significantly. Increase in the plantation age had great impact on the soil bacterial community. The bacterial diversity was positively related to the soil pH, polyphenol oxidase and catalase activities, and negatively related to other soil environmental factors, includingtotal N, P, K, available N, P, K, organic matter, polyphenol oxidase, urease, sucrose, acid phosphatise, peroxidise.

Keywords: Bacterial community, *Camellia sinensis*, continuously planted soils, diversity, environmental factors, microbial community, rhizosphere soil, soil factors, soil toxicity, tea plantation, 16S rDNA amplicon sequencing technique.

INTRODUCTION

Tea is one of the most important economic crop in China, which has the world's highest cultivated area (30). The tea plantations in 2014 covered 2.74 million ha, an increase of 32.5% over the acreage in 2009. However, tea production/ha decreased by 24kg in 2014 than 2009. Zheng *et al*. (32) reported that continuous harvesting of tea leaves, pruning and poor soil fertility causes the imbalance in soil ecological system of tea plantations and this decreased the tea yield. Many attempts have been made to improve the low-yield tea plantations using different methods such as soil conditioners and fertilization patterns, but this has little effect (3,4).

The decline in tea yield and quality are related to the development of continuous cropping problem caused by accumulation of tea root exudates, imbalance of soil nutrients, soil acidity and increase in harmful soil microorganisms (11,17,22,23,32). In general, long term cultivation leads to the formation of special microecological system (pH, redox potential and microbial activity) in rhizosphere of tea plants (7,15,20,28). Soil pH and nutrients affects the soil microorganisms and this changes the soil microbial activities, their population and diversity (24,33). Wu *et al*. reported changes in soil fungi and bacteria caused by changing soil pH, available P and other factors (27). Lin *et al.* (18,19) found that with increase in tea soil age, the pH decreased and the soil microbial

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community diversity significantly differed in soils of different ages. The soil fertility of tea plantations had significant correlation with changes in the magnitude of soil fungi (24). Many studies have focused on the improvement and change in the microbial community structure in rhizosphere soil of tea plants, but did not study the relationship between soil environmental factors and microbial community during the ageing process. We collected soil samples from 4-, 14-, 28-, 32-year-old Rougui tea plantations and determined the soil physico-chemical properties and microbial bacterial community structure using 16S rDNA amplicon sequencing technique.

MATERIALS AND METHODS

Soil sampling

The experiment was conducted during April, 2014 in our research field in Xing village, Wuyishan City, Fujian Province [latitude: 27°32′ N, longitude: 117°54′ E, altitude, 450-550 m, annual mean temperature:16-18°C and annual rainfall, 2000 mm]. The tea plantation had Rougui cultivar of different ages. The total area of tea plantation was 65 ha, [17 ha of 4-year-old, 24 ha of 14-year-old, 11 ha of 28-year-old and 2.4 ha of 32-year-old tea plants].

Rhizosphere soil samples were collected as per Fujii *et al*. (9). Ten plants from each plantation lot were selected randomly. Using the five diagonal point sampling method, soil samples around the tea roots in a radius of 15-25 cm and depth of 25-35 cm were collected (31). Soil from uncultivated area was used as the control. The soil samples were air-dried, crushed and sieved through 25-mesh sieve, then sub-sampled as per method of coning and quartering (12) for subsequent analyses.

Soil toxicity bioassay

Toxicity of tea soils of different ages was determined by soil extracts bioassay according to Li *et al*. (16) and Ye *et al*. (31). Five germinated seeds of cabbage (*Brassica pekinensis*) were sown in Petri dishes (9 cm dia) and 3 mL soil extracts was added. In control, 3 mL distilled water was used. There were six replications. The Petri dishes were then placed in climate chamber [25^{o}C for12 h (7:00 - 19:00) at light intensity of 2400 Lux]. After 3-days, the root length, plant height, dry weight of cabbage seedlings were measured. Compared to the control group, the relative inhibition (I %) of the soil extracts on the three parameters (31) of the receptor plant was calculated

$$I\ \% = [(\text{Control} - \text{Treatment})/\text{Control}] \times 100\%.$$

Soil Physico-chemical properties: Soil nutrients status was determined according to Lao (14), pH, organic matter, available N, P and K according to Jia *et al.* (13). Various soil enzymes (Catalase, polyphenol oxidase, urease, Acid phosphatase, sucrase, dehydrogenase and peroxidase) activities were determined asperthe methods of Guan (10).

Soil DNA extraction

For soil DNA extraction, 3 replications of soil samples of same age were randomly selected. Total genome DNA of samples was extracted using a Fast DNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA), according to the manufacturer's instructions. DNA concentration and purity was monitored on 1% agarose gels and DNA was diluted to 1ng/μL using sterile water.

Bacterial 16S rDNA amplification

To measure the diversity and components of tea soil bacterial community in each sample, the protocol of Caporaso *et al.* was used (1). PCR amplifications were conducted with the 515f/806r primer set which amplifies theV4 region of the 16S rDNA in bacteria. The 5′ terminus of reverse primer contains a 6-bperror-correcting barcode unique to each DNA sample. Further, DNA was amplified according to the protocol described previously (21).

Illumina sequencing and data analysis

Amplicon sequencing on the Illumina MiSeq platforms and further statistical analysis of data were done by Novogene Bioinformatics Technology Co., Ltd, Beijing, China. The original data obtained by sequencing were spliced and filtered to obtain effective data for data analysis. Using Uparse v7.01001 software, effective data were grouped into a operation unit with 97% consistency (6) and sequences meeting to the Operational Taxonomic Units (OTUs) were selected. Through RDP Classifier (Version2.2) methods (26) and GreenGene database (5), the OTUs sequences were annotated and classified and further species information and abundance distribution of each sample at each level was calculated according to the annotation results. With Qiime software (Version1.7.0), Alpha diversity indexes were calculated to gain the richness and diversity of species in each sample. Tukey test between groups was conducted on the Alpha diversity indexes to analyze the diversity differences among the 5 soils. Using R software (Version2.15.3), the dilution curve was drawn to measure the reasonableness of sequencing data volume. The annotated sequence was classified and analyzed using RDP classifier and then the differences of the top 10 bacterial communities at phylum level among the 5 soils were presented, the results of which were shown with a clustering tree.

Correlation analysis

Canonical Correlation Analysis(CCA) is most commonly used algorithms for mining data association relations. With Canoco 4.5 software, CCA based on OTUs of soil microbial community was done, to distinguish the 15 soil samples. Using Spss 19.0, spearnman correlation coefficients were calculated between environmental factors and OTUs abundance of each sample. The two results were analyzed together and presented in the same figure.

RESULTS AND DISCUSSION

Characteristics of soils of different ages

The physico-chemical properties of rhizosphere soils of different ages is shown in Table 1. Compared with the control soil, the pH of the soils decreased with age, while other parameters showed increasing trend. Variation trend of Soil PH was consistent with previous results (12,13).

Enzyme activities of rhizosphere soils of different ages (Table 2) showed that as the soil planting age increased, acid phosphatase, urease, sucrase and polyphenol oxidase decreased, while, the peroxidase, catalase and dehydrogenase increased (Table 2).

Nutrients are the basis of tea growth. The activities of acid phosphatase, sucrase and urease in soil are positively correlated with P, N and C cycles in soil (8). It was seen that with the increase in soil age, the activity of nutrients-cycling-related enzymes decreased in soil, reflected the nutrients availability in the soil. This factor may be contributing to the decreased yield of tea plants with age.

Table 1. Physico-chemical characteristics of Tea plantations soils of different ages

Parameters	Tea plantation soil age (Years)				
	0 (control)	4	14	28	32
pH	4.97±0.02a	4.86±0.12a	4.45±0.03b	4.2±0.02c	4.17±0.01c
OM($g \cdot kg^{-1}$)	10.24±0.24a	11.87±0.34b	13.37±0.32c	14.15±0.23c	14.32±0.46c
AN ($mg \cdot kg^{-1}$)	113.5±2.63a	126.55±4.10b	125.89±..11b	127.98±4.48b	130.76±5.65b
AP ($mg \cdot kg^{-1}$)	10.38±0.13a	12.22±0.31b	12.07±0.17b	12.79±0.38b	12.58±0.19b
AK ($mg \cdot kg^{-1}$)	95.76±2.44a	103.89±6.12a	108.79±5.02a	112.33±6.50a	111.31±4.76a
TN ($g \cdot kg^{-1}$)	1.45±0.15a	1.59±0.13a	1.63±0.08a	1.78±0.11a	1.71±0.15a
TP ($g \cdot kg^{-1}$)	1.23±0.02a	1.33±0.01b	1.37±0.02b	1.35±0.02b	1.37±0.01b
TK ($g \cdot kg^{-1}$)	10.61±0.05a	12.20±0.09a	12.89±1.33b	13.45±0.17b	13.55±0.31b

OM : Organic matter, AN : Available N, AP : Available P, AK : Available K, TN : Total N, TP : Total P, TK : Total K. Different letters(a,b,c) indicate significant differences at $p < 0.05$.

Table 2. Soil enzyme activities of Tea plantations soils of different ages

Enzyme	Tea plantationssoil age (years)				
	0 (control)	4	14	28	32
ACP[$mg \cdot (g \cdot min)^{-1}$]	0.27±0.01a	0.28±0.01a	0.32±0.02b	0.39±0.01c	0.43±0.01d
URE($mg \cdot g^{-1}$)	5.53±0.34a	6.23±0.13b	6.71±0.16b	7.95±0.21c	8.21±0.34c
SC($mg \cdot g^{-1}$)	21.61±1.24a	21.65±1.65a	26.72±1.28ab	29.86±2.30bc	33.76±2.65c
PPO ($mg \cdot g^{-1}$)	1.92±0.08a	2.38±0.15b	2.91±0.08c	3.30±0.18c	3.28±0.23c
POD ($mg \cdot g^{-1}$)	0.88±0.0.3a	0.83±0.02b	0.77±0.01c	0.73±0.01c	0.66±0.02d
CAT($mL \cdot g^{-1}$)	7.69±0.45a	7.74±0.19a	6.84±0.32ab	6.20±0.19b	5.97±0.28b
DH[$(g \cdot (kg \cdot d)^{-1}$]	0.39±0.02a	0.33±0.01b	0.28±0.03b	0.22±0.01c	0.22±0.02c

ACP : Acid phosphatase, URE: Urease, SC : Sucrose, PPO : Polyphenol oxidase, POD : Peroxidise, CAT : Catalase, DH : Dehydrogenase. Different letters indicate significant differences at $p < 0.05$.

Laboratory bioassay

Plants release secondary metabolites in to the soil during the growth and biomass decay. After continuous cropping, secondary plant metabolites accumulate in the soil, which may have adverse impacts on the same plants (17). In this study, we tested the effects of the soil extracts from soils of different aged tea plants on the cabbage. The results showed that the aqueous extracts of soils significantly inhibited the growth of cabbage seedlings (Fig. 1). As the tea-planting age of soil increased, the root length, plant height and dry weight of cabbage were significantly inhibited. This showed that there were some toxic substances in the aqueous soil extracts, which adversely affected the cabbage growth. These result are consistent with our earlier findings (13). Wu *et al.* (29) also found that the effect of continuously planting soils on receptors plants in bioassay tests, aggravated as soil age increased. Chen *et al.* (2) also reported similar results about the allelopathic potential of soil after continuous cropping of tobacco. In our bioassay, we used cabbage and not tea plants, presuming that the results would be similar. From these results, it was concluded that the autotoxicity potential of tea soil increases with increase in cropping years.

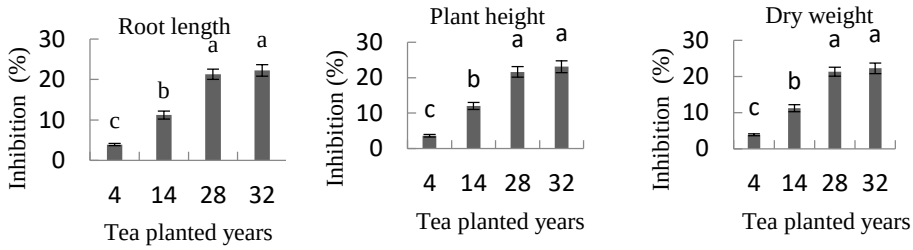

Figure 1. Inhibitory effects of soil extracts on cabbage growth.
The bars represent standard errors of the mean (n=6). Different letters indicate significant differences at $p < 0.05$

Annotated results of OTUs from bacteria in soil samples

As seen in Table 3, the 15 DNA samples sequenced by Illumina HighSeq produced a total of 1072921 effective tags (73450, 73890, 70017, 69962 and 70321, respectively), which were assigned to the 5-soils. The length of effective tags of the 15 samples were in the range of 253-258 and their average length was 254.These were clustered into OTUs at 97% similarity level using Uparse software (Uparse v7.01001). RDP Classifier method and GreenGene database were then used to annotate species for the representative sequences of each OTU and this showed about 67071, 69126, 64629, 65392 and 64934 effective Tags from the 5 soils obtained OTUs notes and further analysis gained 2916, 3391, 3069, 2198 and 2014 OTUs of each soil (Tab. 3). As seen in Table 4, the number of annotated sequences declined with the decrease in the annotation level, most of which could be annotated to the genus level. However, the sequences annotated to the species level are far less than that at the genus level.

Table 3. Sequencing data and OTUs notes

Sample Name	Raw PE(#)	Raw Tags	Clean Tags	Effective Tags	AvgLen (nt)	Taxon Tag	OTU_num
CK1	79,479	77,939	76,783	74,770	253	62396	2896
CK2	80,362	78,318	76,860	75,368	255	69953	2937
CK3	86,049	71,974	70,857	70,213	256	68864	2915
RA1.1	85,624	83,454	82,166	80,477	254	74548	3644
RA1.2	77,259	75,123	73,841	72,019	255	67477	3216
RA1.3	74,232	72,073	70,489	69,174	256	65354	3314
RA2.1	83,165	72,544	70,420	69,529	258	65770	3298
RA2.2	77,091	75,465	74,361	72,722	254	68627	3066
RA2.3	72,871	71,387	70,312	67,801	253	59490	2844
RA3.1	71,036	69,864	68,841	67,415	253	62756	2053
RA3.2	86,213	77,635	75,454	74,284	257	71510	2288
RA3.3	72,692	71,575	70,588	68,187	253	61912	2255
RA4.1	77,456	76,112	75,057	73,660	254	69477	2161
RA4.2	78,120	76,916	75,835	73,111	253	64123	1933
RA4.3	88,308	67,952	65,485	64,191	254	61203	1948

Raw PE: The original down machine PE reads; Raw Tags: Tags sequences obtained after splicing; Clean Tags: A sequence of filtered low quality and short length; Effective Tags: The final step in the subsequent analysis of the Tags sequence after filtering the chimera; Avglen: The average length of Effective Tags; Taxon Tag: To build OTUs and get the notes Tag; OTUs number: The number of OTUs obtained per sample

Table 4. Number of sequences of each sample in each classification after OTUs annotation

Sample Name	Kingdom	Phylum	Class	Order	Family	Genus	Species
CK1	62396	61008	59857	55150	30220	13051	2129
CK2	69953	68463	67193	61599	36113	16990	1863
CK3	77736	76855	76104	71401	43431	18897	2413
RA1.1	74548	72849	70969	64169	45650	17738	2457
RA1.2	67477	65936	64060	56499	42827	17204	2220
RA1.3	65354	64049	62353	56059	41931	15855	2058
RA2.1	65770	64208	62542	56201	40341	15101	2001
RA2.2	68627	67344	65908	58709	43012	17200	2533
RA2.3	59490	57663	56065	51536	37176	16172	2712
RA3.1	62756	61966	60833	57364	35176	13975	2147
RA3.2	71510	70498	69201	64567	44641	14569	2339
RA3.3	61912	60947	59892	56939	40455	17558	2799
RA4.1	69477	67811	66362	52658	34533	12773	1598
RA4.2	64123	62301	60790	49535	34802	12626	1519
RA4.3	61203	59721	58269	50627	35025	10187	1487

Microbial richness and diversity in tea soils of different ages

Rarefaction curves drawn using R software (Version 2.15.3) showed that when the number of sequencing reads reached10000, the curve presented a smooth and upward status and the OTUs number closed to saturation (Fig. 2**),** which directly reflected the rationality of sequencing data volume and met the needs of subsequent analysis. As seen from table 5, good coverage of all 15 samples was above 0.98, indicating that the sequencing depth basically covered all species in the samples and further data analysis can be done.

The Shannon index, Simpson diversity index, Chao index and ACE index were calculated with Qiime software (Version 1.7.0) (25). Differences in the significance analysis of 4 indices from the 15 samples by Student-Newman Keuls test showed that Chao1 and ACE indexes were not significant between 28 and 32 years. In 0, 4 and 14 years, the richness of soil microbial communities in the same group was basically same**.** Meanwhile, analysis of Shannon and Simpson indices also showed that differences in 28 and 32-year-old soils were smaller and very smaller among the 0, 4 and 14-year-old soils, indicating that the diversity of soil microbial communities in each group had certain similarity.

Microbial community in rhizosphere soils

The 10 dominant phyla or groups were determined by the mother programme (25). The overall bacterial composition of different samples was similar, while the distribution of each phylum varied (Fig.3). In all samples, Acidobacteria, Proteobacteria, Chloroflexi and Actinobacteria were the most dominant phyla, accounting for >75% of the reads. Compared with other samples, control soil had significantly higher percentage of Nitrospirae, Thermotogae and Gemmatimonadetes and low percentage of Chloroflexi and WD272. Weighted UniFrac analyses showed that distance is 0.025, 15 samples were divided into 5-groups on the basis of relative read abundance of different bacterial phyla, which is in accordance with their planted ages.

Table 5. Diversity analysis of OTUs in Tea soils

Sample name	Shannon index	Simpson index	Chao1 index	ACE_ index	Goods_ coverage
CK1	8.9	0.992	2882.405	2932.605	0.99
CK2	8.707	0.992	2867.133	3056.282	0.987
CK3	8.705	0.992	3320.609	3093.755	0.989
RA1.1	9.369	0.996	3964.562	3911.078	0.982
RA1.2	9	0.994	3131.46	3293.365	0.986
RA1.3	9.087	0.994	3248.803	3394.666	0.986
RA2.1	9.108	0.994	3270.153	3412.918	0.986
RA2.2	8.823	0.994	2913.526	3066.49	0.987
RA2.3	8.912	0.994	2811.658	2838.962	0.988
RA3.1	8.189	0.99	2000.483	2044.019	0.993
RA3.2	8.183	0.987	2163.664	2273.423	0.992
RA3.3	8.59	0.993	2205.728	2256.73	0.992
RA4.1	8.412	0.992	2074.789	2178.964	0.992
RA4.2	8.384	0.993	1852.984	1930.371	0.993
RA4.3	8.267	0.992	1898.247	1947.576	0.993

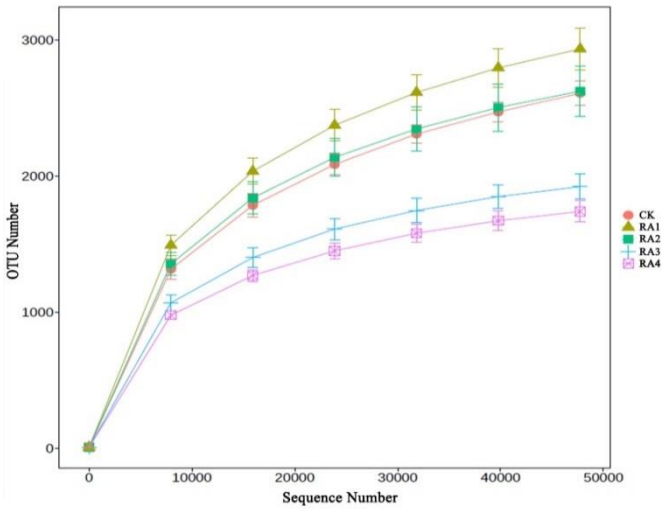

Figure 2. Rare faction curves of 15 samples

Correlation analysis between the soil microorganisms and environmental factors

Using Canoco and Spss software, correlation analysis (CCA) between soil microorganisms and environmental factors was calculated. The CCA showed that the first and second CCA components were able to explain 42.5% of the total bacterial variation

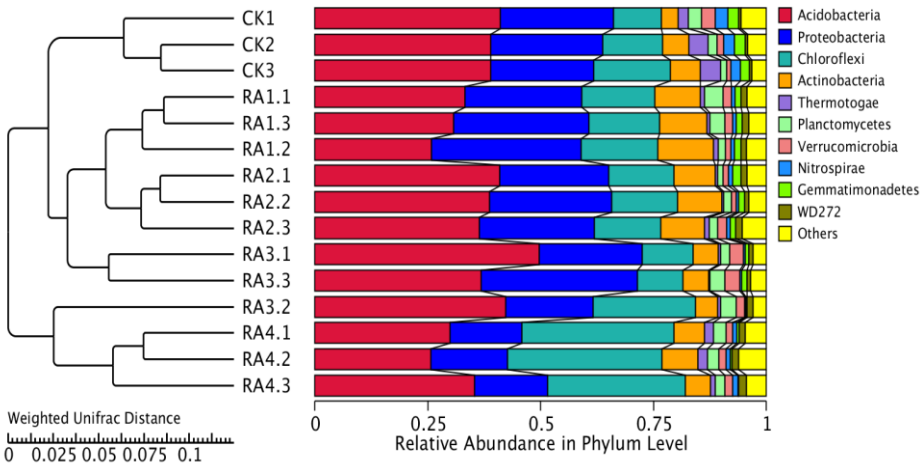

Figure 3. Comparison of the bacterial communities at the phylum level.
Relative read abundance of different bacterial phyla within the different communities. Sequences that could not be classified into any known group were labeled ‘‘Other’’.

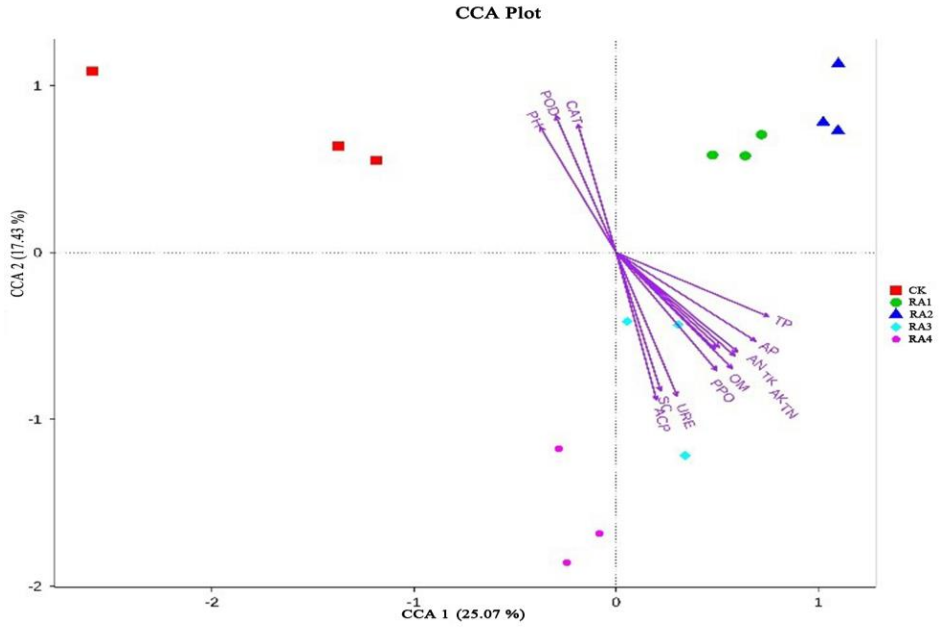

Figure 4. CCA analysis (CCA) between soil physico-chemical indices and microorganism based on OTUs

CONCLUSIONS

The practice of long term cultivation of tea plants in tea plantations, contributes to the soil degradation, during which many harmful substances accumulate to cause imbalance in environmental factors. Thereby, the diversity and composition of soil microbial community also changes and these changes could have harmful impact on the growth of tea plants, which became more serious with the increasing tea planted soil age. This study revealed the role of environmental factors in rhizosphere soil bacterial community development process. This may be useful in restoring the soil micro-ecological system and reduce the impact of ageing on tea plant growth.

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