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Soil toxicity and microbial community structure of Wuyi rock tea plantation

X.L. Jia[1], J.H. Ye[1], Q. Zhang, L. Li, Y.L. Hu[1], M.Z. Zheng[1] ,
Y.C. Hong[1], F.Q. Wang[1] and C.Z. Wu[1,2*]
College of Life Sciences, Fujian Agriculture and Forestry University,
Fuzhou, 350002, China.
E-mail: jiaxl2010@126.com

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ABSTRACT

The effects of tea soils of 4-, 14-, 28- and 32-planted years on tea photosynthetic indices and the protective enzymes [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and malondialdehyde (MDA)] activities of replanted tea leaves were examined. It was found that these activities decreased significantly with increase in planted years. The MDA levels increased significantly with increase of soil planted years. The levels of polyphenols, caffeine and free amino acids in tea leaves were lower in tea seedlings grown in 14-, 28- and 32-years soils than in 0- and 4-year soils. In laboratory bioassay, the aqueous soil extracts inhibited the lettuce (*Lactuca sativa* L.) growth significantly with increased number of planted years. The contents of three phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid and cinnamic acid) were significantly higher in 14-, 28- and 32-year soils than that in 0- and 4-year soils. The microbial community of tea soils varied significantly than control. These results confirmed that soil toxicity exists in older tea soils of Wuyi rock tea area. The accumulation of phenolics, deficiency of carbon sources and change in the microbial communities appeared to be the main characteristics of older tea plantation soils.

Key words: Amino acids, *Camellia sinensis*, continuously planted soils, enzymes, *Lactuca sativa*, lettuce, microbial community, phenolic acids, planted years, polyphenols, rhizosphere soil, soil toxicity, tea plantation

INTRODUCTION

Wuyi rock tea is China's most popular tea from the rocky mountain region, Wuyishan County, North Fujian Province, China. Because of great demand for this tea, farmers have reclaimed several tea plantations in this region and even cultivated tea in plains. For quality tea production, the reclaimed plantations are planted with single variety at high density, with heavy fertilization and weeds and pest control management. However, the production and quality of tea decreases with increasing number of planted years.

This is attributed to reduced soil fertility and deterioration of soil quality. Attempts had been made to resolve this problem by supplemental fertilization, by use of bactericides or germicides, or by straw mulching. The effect of these practices was negligible in

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*Corresponding author, [1] Fujian Provincial Key Laboratory of Eco-Industrial Green Technology, Wuyi University, Wuyishan 354300, China. [2] Forestry College, Fujian Agriculture and Forestry University, Fuzhou, 350002, China.

reclaiming the sick soils. It is not known, how the growth and quality of newly planted tree trees is affected, when replanted in old tea plantations. Ye *et al.* (27,28) reported that in Tieguanyin tea plantations, South Fujian Province, China, the continuously cultured tea soils had negative effects on the replanted tea seedlings. In this study, new tea seedlings were planted in soils collected from the plantations of 4, 14, 28 and 32-years, and the changes in photosynthetic indices, protective enzymes [superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and malondialdehyde (MDA)] activity, microbial community status and the quality indices of tea leaves were examined. This study aimed to determine the soil toxicity in older tea plantations of Wuyishan rock tea area, study its effect on the growth and quality of replanted tea tree and changes in the microbial communities.

MATERIALS AND METHODS

The experiment was conducted during April, 2014 at tea plantation with Rou-Gui cultivar (a representative variety of Wuyi rock tea), located in Xing village, Wuyishan County Fujian Province, [latitude 27°32′ N and longitude 117°54′ E, with altitude of 450-550 m, annual mean temperature of 16-18°C and annual rainfall about 2000 mm]. The total area of tea plantation was 65 ha [17 ha of 4 years old tea trees, 24 ha of 14 years old tea trees, 11 ha of 28 years old tea trees and 2.4 ha of 32 years old tea trees, respectively].

Soil samples from around the tea trees from different planted years were collected as per Fujii *et al.* (6). Ten trees from each planted lot were selected randomly. Soil samples around the tea tree roots in radius of 15-25 cm and 25-35 cm depth were collected by the five diagonal point sampling method (27). Soil from the area where no tea was cultivated, was used as control. The soil samples were air-dried, crushed and sieved through 25-mesh sieve. The soil samples were then sub-sampled, as per the procedure by the method of coning and quartering. The soil sub-samples of each planted year were then used for subsequent analyses.

Soil nutrients status was determined as per Lao (8). The pH and nutrients contents of soils of different planted years and the control soil met the requirements for tea culture (Table 1), as per the Environmental Requirements for Tea-growing Areas (NY/T 853-2004) by the Chinese Ministry of Agriculture (13).

Pot culture

The effect of soils on newly planted tea seedlings was studied in pot culture. Soil samples (32 kg) were placed in pots (50 cm height and 40 cm dia) and 3 tea seedlings of Rou-Gui cultivar (1-year-old about 35 cm tall) were transplanted in each pot. The treatments were replicated thrice in completely randomized design. These pots were placed in original tea plantation under the natural conditions and managed in the customary manner. One year after transplanting, the physiological and photosynthetic parameters and quality indices of tea tree leaves were measured as described below.

Table 1. pH and nutrients status of tea plantations soils of different ages

Index	Soil planted years					Grade
	0 (control)	4	14	28	32	
pH	4.98±0.03	4.79±0.01	4.45±0.03	4.20±0.02	4.17±0.01	
Organic matter ($g\cdot kg^{-1}$)	10.24±0.21	11.87±0.34	13.37±0.28	14.15±0.16	14.32±0.35	II
Available N ($mg\cdot kg^{-1}$)	113.53±2.60	126.50±3.65	125.89±2.78	127.97±3.86	130.75±4.70	I
Available P ($mg\cdot kg^{-1}$)	10.38±0.13	12.22±0.24	12.06±0.15	12.79±0.32	12.58±0.14	I
Available K ($mg\cdot kg^{-1}$)	95.75±2.50	103.84±5.37	108.78±4.97	112.33±5.40	111.31±4.01	II

Means standard error (SE) from three replications for each determination.
I and II - meet grade I standards and grade II standards, respectively, according to Environmental Requirements for Tea-growing Areas (NY/T 853-2004) by the Chinese Ministry of Agriculture (MOA, 2005)

Photosynthetic indices and protective enzymes in tea seedling leaves

The second internode leaves of tea trees were chosen to determine photosynthetic indices *in situ.* The net photosynthetic rate, transpiration rate, intercellular CO_2 concentration and stomatal conductance were measured by a LI-6400XT Portable Photosynthesis System (Li-Cor, Lincoln, NE, USA). Chlorophyll content was measured by a SPAD-502 PLUS (Konica Minolta Camera Co. Ltd., Japan). These indices were measured at 9 - 11 a.m. on a sunny day and 6 leaves in each pot were used to measure the indices. Measurement of photosynthetic indices by a LI-6400XT Portable Photosynthesis System were done *in situ* without detaching tea leaves (27).

Three top leaves from each tree representing the shoots for processing high quality tea were selected. The second internode leaves were used to determine the protective enzymes activities. Fresh leaves 0.3 g, were added to pre-chilled extraction buffer (50 mM/L phosphate buffer with 1% PVP, pH 7.0), homogenized by grinding on ice and centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was used to determine the activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and malondialdehyde (MDA) content, as per the manual (26). Enzyme activities in plant leaves are expressed as specific activities as per Wang (26)

Quality indices of tea seedling leaves

The rest of the fresh leaves from above, were kept at 105 ^{o}C for 60 min and then dried at 80 ^{o}C to a constant weight. They were then ground and sieved through a 60 mesh sieve. Then the contents of polyphenols, caffeine and free amino acid in tea leaves were determined as per the standards procedures of tea quality, National Standards of People’s Republic of China (14,15,16,17).

Preparation of soil aqueous extracts

The soil aqueous extracts were obtained according to Li *et al*. and Ye *et al*. (9,27). In brief, 50 g of each soil samples were added to 150 mL distilled water, shaken for 30 min and then centrifuged at 1600 x *g* for 10 min. The supernatant was filtered with double filter papers and then with a sterile 0.22-μm membrane filter. The filtrates were used for following experiments.

Soil toxicity bioassay

The toxicity of the continuously planted tea soils was determined by the soil extracts bioassay as per Li *et al*. (9) and Ye *et al*. (27). The soil extracts obtained above were used as test solutions. Lettuce was used as the test plant. Five germinated seeds of lettuce (*Lactuca sativa* L.) were placed on a petri dish (9 cm dia) and 3 mL soil extracts was added. In control, 3 mL distilled water was used. There were six replicates for each test. The petri dishes were then placed in climate chamber [25 ^{o}C, with 12 h (7:00 - 19:00) light of 2400 Lux light intensity]. After 3 days, the root length and plant height of lettuce were measured. All plants were then collected and oven-dried at 120 °C for 30 min and then at 80 °C for 48 h to obtain the plant dry weight. The relative inhibition rate (IR) of the soil extracts on growth of the receptor, compared to the control, was calculated as under:

IR (%) = [(control - treatment)/control] × 100%.

Phenolic acids in tea soils

Phenolic acids in the soil extracts were quantified by SPE-HPLC method (29). The six phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, benzoic acid, syringic acid and cinnamic acid, purchased from Aldrich Chemical Co., USA) were used as standards for the calibration curves. A mixture of 6 phenolic acids was made by diluting the stock standards in methanol. The concentrations of the mixture were set at 5 gradients based on the sensitivity and the detection limits of each phenolic acid, that is: 1.00, 2.00, 5.00, 8.00 and 10.0 μg/mL of protocatechuic, *p*-hydroxybenzoic, vanillic, benzoic and syringic acid and 0.50, 1.00, 2.00, 4.00 and 6.00 μg/mL of cinnamic acid. The 100 mL of soil extracts obtained as described above were loaded on to Cleanert PEP solid phase extraction (SPE) cartridge (Agela, China). The cartridge was first washed with water (5 × 4 mL) and then eluted with methanol (3 × 4 mL). The methanol fraction was concentrated under nitrogen to 500 μL. Then the samples and the standards of phenolic acid mixtures were analyzed using an Agilent 1206 HPLC (Agilent Technologies, USA) equipped with a C18 reverse column (ZORBAX SB-C18, 150 mm × 4.6 mm, 5 μm). The mobile phase was a mixture of methanol (A) and 1% phosphoric acid (B) with the following gradient elution program: A:B = 27:73 (9 min), A:B = 30:70 (2 min) and A:B = 50:50 (4 min). The flow rate of the mobile phase was 1.6 mL/min and fractions were monitored at 280 nm. The injection volume was 5 μL. The concentrations of single phenolic acids in rhizosphere soil extracts of tea tree were calculated directly from calibration curve.

Soil microbial community

The 50 g of each soil samples were added to 150 mL distilled water and shaken for 30 min. The supernatants were used as test solutions to determine the soil microbial community by Biolog EcoPlate method (5,21) In brief, the EcoPlates were pre-heated to

25 ^{o}C, then 150 μL of aqueous soil extract (prepared as above) was added into each well. The plates were incubated at 28 ^{o}C in an incubator. There were three replications for each sample. The absorbance at 590 nm was measured by ELISA reaction plate reader after 7 days. The microbial populations represented the average well color development (AWCD), were calculated as under:

$$AWCD=[\sum(C-R)]/N,$$

Where C: Absorbance from 31 carbon source wells and R: Absorbance value of control well and N: Number of carbon source (25).

Statistical analysis of data

All experimental data were subjected to one-way analysis of variance (ANOVA) followed by least significant difference (LSD) analysis at $p < 0.05$, carried out with IBM SPSS 20.0 program. Principal component analysis (PCA) and correlative analysis were all conducted with SPSS 20.0 software.

RESULTS AND DISCUSSION

Effect of soils on photosynthetic indices, enzymes activities and quality indices of replanted tea seedlings

There were no significant differences in photosynthetic indices (except net photosynthetic index) of tea seedlings leaves between the control (0 year) and 4 years soil (Fig. 1). However, the net photosynthetic rate, stomatal conductivity, intercellular CO_2 concentration, transpiration rate and chlorophyll content decreased significantly with increase in the soil planted years. Activity of the protective enzymes (SOD, POD and CAT) in tea seedling leaves decreased significantly with increase of planted years, but did not differ significantly between control (0 year) and 4 years (except POD) (Fig. 2). The MDA levels increased significantly with increase in soil planted years but did not differ significantly between control (0 year) and 4 years soil.

The quality indices of tea leaves were not significantly different between the control (0 year) and 4 years soil (Fig. 3). However, the contents of polyphenols, caffeine and free amino acids in leaves of tea trees cultured in 14-, 28- and 32-year soils were significantly lower, than in 0- and 4-year soils.

This suggests that when tea seedlings are replanted on the continuously planted soils, photosynthesis, protective enzymes activity and the major quality indices of tea seedlings decrease with the increase in the number of years of plantation. These results are reflection of the number of years rather than the pH or nutrients contents of soils. Earlier we reported that the continuously cultured tea soils significantly affects the growth and quality of Tieguanyin tea, another famous tea from Anxi County, located in the south of Fujian Province, China (27,28). The results reported here are similar and confirmed that soil sickness of tea plantation is common in Fujian Province of China and soil sickness increased with the number of years of plantation.

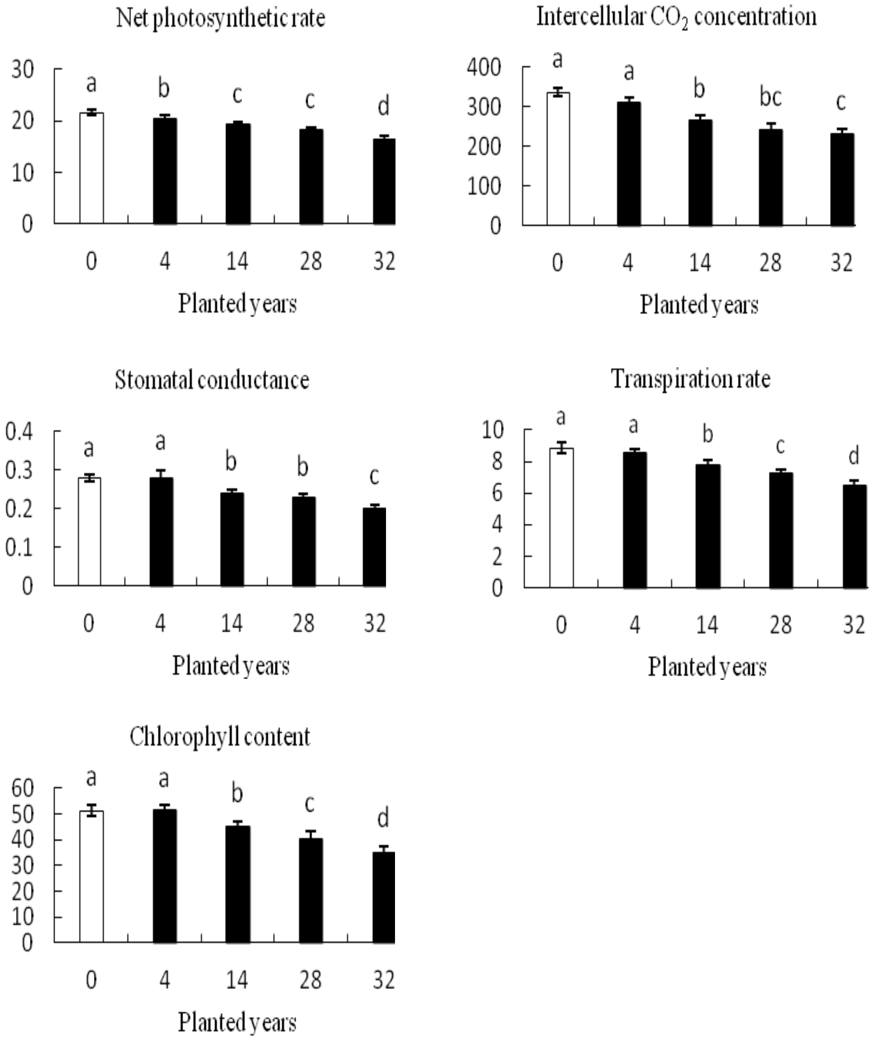

Figure 1. The photosynthetic indices of tea seedling leaves from soils of different planted years.
The bars represent standard errors of the mean (n=6). Different letters indicate significant differences at $p < 0.05$.
Net photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), Stomatal conductivity (mol H_2O m^{-2} s^{-1}), Intercellolar CO_2 concentration (μmol CO_2 mol^{-1}), Transpiration rate (mmol $H_2Om^{-2}s^{-1}$), Chlorophyll content (SPAD value). SPAD is a read data from the LI-6400XT Portable Photosynthesis System, which is used directly to represent the Chlorophyll content of plant leaves.

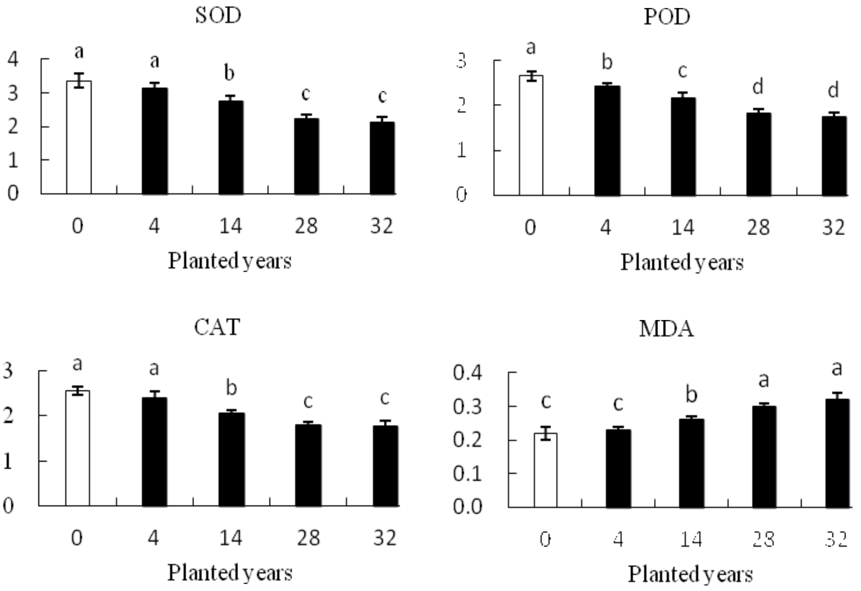

Figure 2. The protective enzymes of tea seedling leaves from plants in soils of different planted years. The bars represent standard errors of the mean (n=3). Different letters indicate significant differences at $p < 0.05$. SOD (unit·mg^{-1} protein); POD (△OD470 min^{-1}·mg^{-1} protein); CAT (mg (H_2O_2)·min^{-1}·mg^{-1} protein); MDA (μmol·mg^{-1}·protein)

Effect of soil extracts on receiver plants in laboratory bioassay

In laboratory bioassay, the aqueous extracts of the soils significantly inhibited the growth of lettuce (Fig. 4). The extent of inhibition of the root length, plant height and plant dry weight significantly decreased with increase of planted years of the plantation. This suggested the presence of some toxic substances in the aqueous soil extracts which are harmful for the growth of lettuce.

Quantification of phenolic acids in tea tree soil extracts

The contents of three phenolic acids (protocatechuic acid, *p*-hydroxybenzoic acid and cinnamic acid) were significantly higher in 14-, 28- and 32-year soils than that in 0- and 4-year soils (Fig. 5). This showed that older soils have higher contents of these phenolics, which are harmful to the growth of the replanted tea plants.

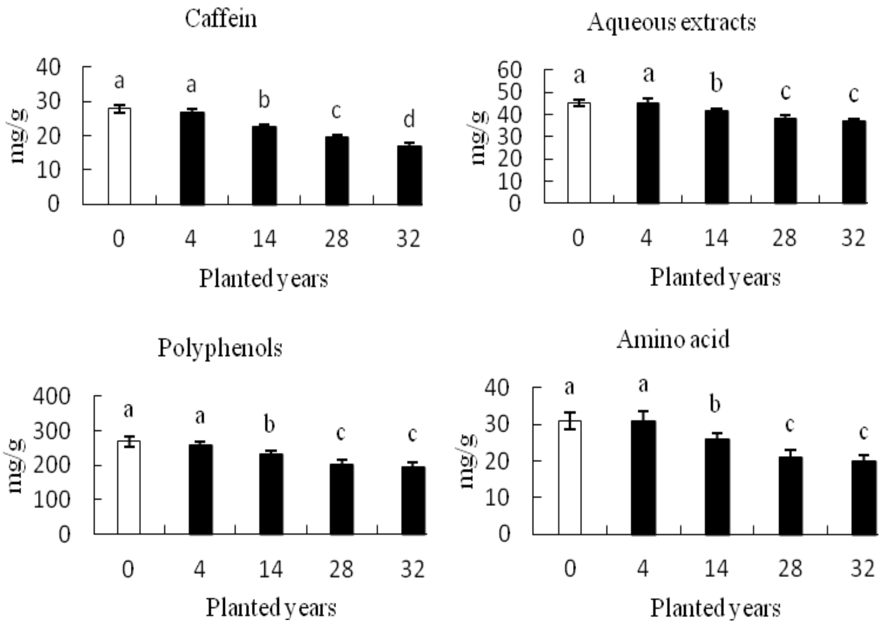

Figure 3. The quality indices of tea seedling leaves from soils of different planted years. The bars represent standard errors of the mean (n=3). Different letters indicate significant differences at $p < 0.05$

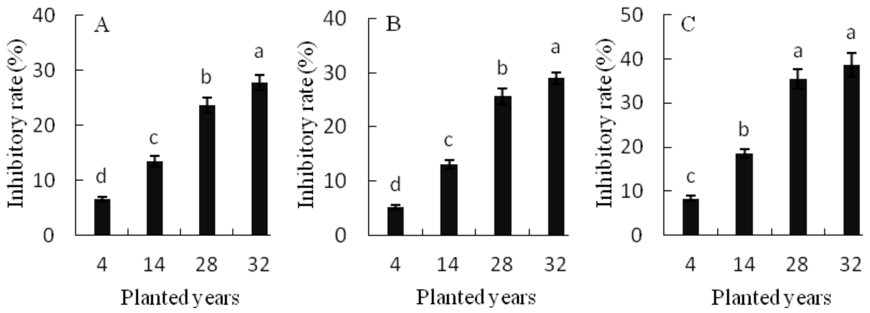

Figure 4. Inhibitory effect of soil extracts on lettuce growth.
A - root length, B - plant height, C - plant dry weight.
The bars represent standard errors of the mean (n=6). Different letters indicate significant differences at $p < 0.05$

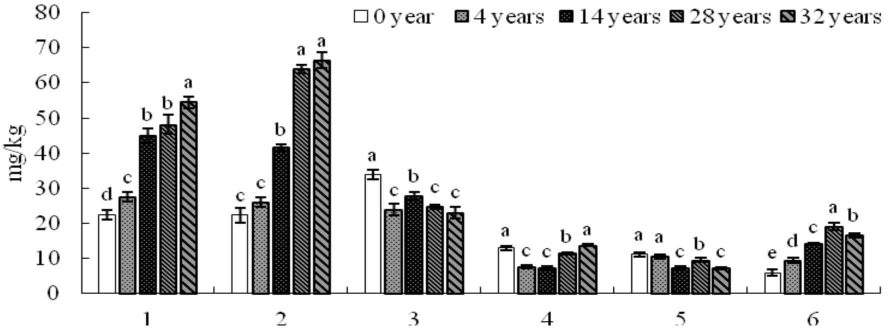

Figure 5. Changes in the phenolic acid content of soils of different ages.
1:protocatechuic acid; 2:*p*-hydroxybenzoic acid; 3:vanillic acid; 4:benzoic acid; 5:syringic acid; 6:cinnamic acid. Phenolic acids in soil expressed on air dry basis.

Soil microbial community structure

The AWCD values showed that carbon source utilization by soil microbes did not differ significantly among most of the tested soils (except the 32-year soil). The Carbon source use by soil microbes to amino acid utilization was not significantly different between 14-, 18 and 32-year tea soils. Carbon source use by soil microbes to amide also was also not significantly different among the tested soils. However, carbon source utilization by soil microbes to fatty acid and carboxyl derivatives utilization was significantly higher in tested soils, compared to the control. Carbon source utilization of soil microbes to phenolic acids utilization was irregular and significantly different among tested soils. The highest value was of 14-year soil (Fig. 6).

Principal component analysis (PCA) showed that utilization of 31 carbon sources by soil microbes could be separated into four principal components (Fig. 7), including PC1 (49.149%), PC2 (23.588%), PC3 (0%) and PC4 (11.894%), respectively. The PC1 of control soil (0 year) was located at the negative side and other tested soils were located at the positive side, indicating PC1 could differentiate the blank soil from the planted soils. The PC2 of 4- and 14-years soils was located on the negative side, while 28- and 32-years soils was located at the positive side, indicating there were significant differences between these soils in their soil microbial community. Similarly, PC4 indicated significant difference in soil microbial community between 4-, 14-years soils and 28-, 32-years soils (Fig. 7).

The utilization of 18 out of 31 carbon sources was significantly correlated with the PC1, PC2 and PC4 (not in PC3) (Table 2). The 11 components were positively correlated including 3 carbohydrates, 3 carboxyl derivatives, 2 amino acids, 2 amides and 1 fatty acid. The 7 components were negatively correlated including 6 carbohydrates and 1 amino acid. These results indicated that the accumulation of acidic compounds and deficiency of

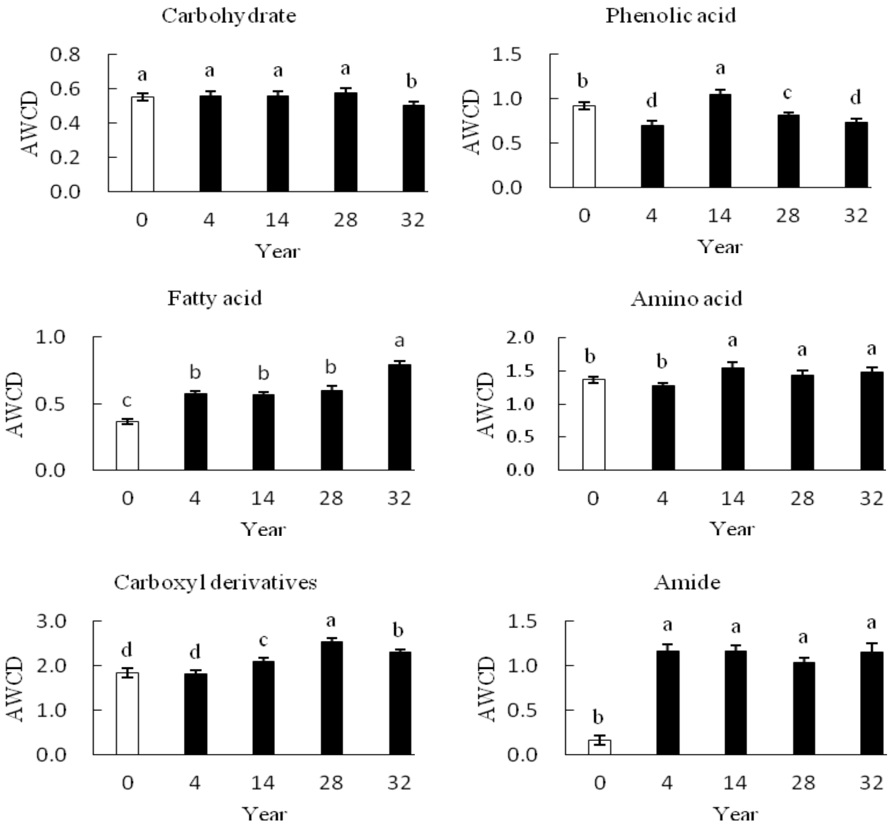

Figure 6. AWCD in Biolog EcoPlates with different carbon sources and soil extacts of different planted years.
The bars represent standard errors of the mean (n=3). Different letters indicate significant difference at $P<0.05$.

some carbohydrate carbon sources, were perhaps the main characteristic in the older tea plantation soils. Suzuki and Waller (22) reported that tea trees release caffeine and theobromine into tea soils and inhibited the growth of shoots and roots of tea seedlings. Cao *et al*. (1) reported that there were 9 phenolic compounds identified in soils extracts of old tea gardens and the total contents increased with the increasing age of tea trees. Lin *et al*. (11,12) reported that the decrease in microbial diversity and an increase in microbes of low metabolic activity in tea soils, may result in the imbalance in microbial community structure resulting in the decline of soil quality and fertility. Li *et al.* (10) reported that the content of the labile organic carbon in tea plantation decreased with the increasing age of tea plants. Based on our results, we suggest that using appropriate agronomic practices to reduce the acidic components and to increase carbohydrates in the soils may alleviate the soil toxicity.

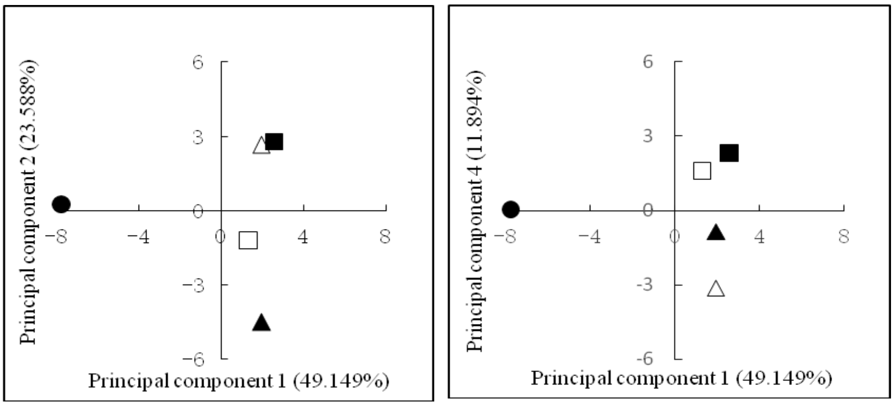

Figure 7. Loadings for principal component analysis (PCA) of carbon source utilization of soil microbes.

●: control soil (0 year); ▲: 4-year soil; □: 14-year soil; △: 28-year soil; ■: 32-year soil.

Table 2. The main substrates significantly correlated in PCA of 31 carbon source utilized by microbes

Substrates	r	Substrates	r
Principal component 1			
Putrescine	0.99**	D-Xylose	-1.00**
L-Phenylalanin	0.96**	N-Acetyl-D-glucosamine	-0.95**
L-Arginine	0.98**	Glucose-1-Phosphate	-0.99**
L-Serine	0.95**	Glycyl-L-Glutamic acid	-0.96**
D-Mannitol	0.92*	D-Glucuronic acid	-0.85*
α-Ketobutyric acid	0.83*		
Pyruvic acid methyl ester	0.85*		
Principal component 2			
α-Cyclodextrin	0.94**	D-Glucosaminic acid	-0.84*
Tween 40	0.94**		
Tween 80	0.88*		
Glycogen	0.85*		
Principal component 4			
		D-Cellobiose	-0.87*

* significant correlation at 0.05 level, ** significant correlation at 0.01 level. Principal component 3 was not significantly correlated

The correlation analysis showed that microbes utilizing carboxyl derivatives were significantly and positively correlated with the inhibitory levels of aqueous soil extracts on root length and plant dry weight of lettuce (Table 3). The AWCD values of carboxyl derivatives were significantly different among 0, 4 and 14, 28, 32 year soils (Fig. 6) and all 3 carboxyl derivatives of 31 carbon sources in Biolog EcoPlates were significantly and positively correlated with PCA (Table 2). Cao *et al*. (1) reported that in old tea plantations, accumulation of phenolic acids in tea soils resulted in autotoxicity. Acidic compounds are the degradation products of carboxyl derivatives in soil biochemical processes and Microbial community led to accumulation of acidic compounds in tea soils, which may be cause of soil toxicity in the long period planted tea plantations.

Table 3. Correlations among carbon source utilization by soil microbes and soil toxicity

Carbon sources	Inhibitory rate on lettuce		
	Root length	Plant height	Plant dry weight
Carbohydrate	-0.46	-0.51	-0.44
Phenolic acid	-0.10	-0.11	-0.09
Fatty acid	0.73	0.76	0.71
Amino acid	0.51	0.51	0.51
Carboxyl derivatives	0.90*	0.87	0.91*
Amide	-0.48	-0.44	-0.51

*Significant correlation at the 0.05 level.

Soil sickness has been widely seen in continuously cropped agricultural systems, such as corn (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), sunflower (*Helianthus annuus*), cucumber (*Cucumis* spp.), tobacco (*Nicotiana tabacum*), as well as in orchards, coffee and tea plantations (3,7,20,24). The aqueous extracts from stems, leaves, roots and fruit peels of tea trees inhibits the tea seedling growth (2) and also inhibited the germination and seedlings growth of garden cress (*Lepidium sativum* L.), lettuce (*Lactuca sativa* L.), redroot pigweed (*Amaranthus retroflexus* L.), golden foxtail (*Setaria glauca* (L.) P. Beauv.) and *Vicia* sp. (4,18). However, there are only few reports about the effects of soil on replanted tea trees. Our results showed that the soil from old tea plantations had significant negative effects on the photosynthetic indices and protective enzymes in the leaves as well as the quality indices of tea leaves of the replanted tea trees (Fig. 1, 2 and 3). The pH and the nutrients levels of soils examined met the requirements of tea plants growth (Table 1). Obviously, therefore, these consequences seen are not the result of pH or nutrients deficiency in the tested soils. Our results confirmed that soil sickness exists in the long planted plantations of Wuyi rock tea, confirmed that soil sickness in tea plantations is a common phenomenon in China (27,28).

There were significant differences in the microbial community structure between the control soil and the long planted soils, especially in carbon source utilization of acidic compounds and carbohydrates. Further research is needed to know the causes and mechanisms of soil sickness in tea plantations, to develop integrated strategies to protect replanted tea seedlings from soil sickness and to maintain the yields and quality of tea.

CONCLUSIONS

The tea soils from the old tea gardens have negative effects on the growth and quality of the replanted seedlings. The microbial community profile of these soils was also significantly different from the control soils. Soil toxicity exists in old tea plantations of Wuyi rock tea area. The changes in the microbial community may be a cause of soil toxicity and an indicator of soil degradation in old tea plantations.

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