https://doi.org/10.26651/allelo.j./2018-45-1-1171 0971-4693/94 Euro 20.00
Allelopathy Journal 45 (1): 01-12 (September, 2018) *International Allelopathy Foundation* 2018
Table: 2, Figs : 4

Identification of Allelochemicals responsible for soil degradation in continuously cropped Tea plantations

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(Received in revised form: July 21, 2018)

ABSTRACT

Rhizosphere soils from Tieguanyin tea plantations of 0, 3, 9 and 25 years old were extracted and eluted through 5 resins of different polarity. Tea seedlings were then grown with the 5 eluents and their effects were determined on the photosynthetic indices and leaf quality. Among the 5 resins, soil eluents through ADS-7 resin most strongly inhibited the root length and height of the receptor lettuce, photosynthetic indices and leaf quality of new tea plants. And the inhibitory effects increased with soil age, especially, the ADS-7 resin eluents from 25-year-old soil showed the strongest inhibitory effect. The GC-MS analysis of ADS-7 resin eluents showed the presence of amides, esters, acids and other types, which changed significantly as the tea plant soil age increased, and the content of all acids increased significantly, while the phenolic ketone content of all soil samples remained unchanged.

Key words: Allelochemicals, bioassay, continuously planted soils, GC-MS, leaf quality, photosynthetic indices, resins of different polarities, rhizosphere soil, soil toxicity, Tieguanyin

INTRODUCTION

In China, Anxi county (Quanzhou, Fujian Province, China) is the major tea producing region, (40,000 ha of tea plantations) with annual tea production of 68,000 tons. The Tea plant (*Camellia sinensis* (L.) O. Ktze) is a perennial evergreen woody plant. The cultivation of Tieguanyin tea bushes normally takes 2-3 years, from planting to picking with maximum yield in the 7th year. As the age of cropping increases, changes occur in the soil environment leading to increasing autotoxicity, which is detrimental to its growth (2,3,13,19,25). Wang *et al.* (22) reported that the practice of continuous cropping resulted in deterioration in Tieguanyin tea plants, leading to a significant decrease in the quality of tea leaves. Ye *et al.* (23,24) reported that as the age of cropping increased, rhizosphere soil autotoxicity of Tieguanyin tea plant increased and the quality indexes of tea leaves decreased. Lin *et al*. (14,15) reported that rhizosphere soil from older tea plantations had a lower pH and the functional diversity of the rhizosphere soil microbes varied in tea plants of different ages. Also, rhizosphere soil analysis revealed that the proportion of acidic compounds increased with soil age. We have previously shown (11) that the amount of

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autotoxic allelochemicals including six phenolic acids [namely protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, benzoic acid, syringic acid and cinnamic acid], in the rhizosphere soil from Rougui tea plants increased over time. In the present study, we have analyzed the allelochemicals in the rhizosphere soils from Tieguanyin tea plantations of 0, 3, 9 and 25 years and determined their effects on growth and leaf quality of new tea plants.

MATERIALS AND METHODS

Materials

Rhizosphere soils (3-, 9- and 25-year-old) were collected from Tieguanyin tea plantations, located in Long Juan Township, Quanzhou Anxi County, China**.** Soil which had not been used to grow Tieguanyin tea plants previously, was also collected as control and classified as 0 year soil sample. Samples were collected in May 2016 from different sampling points, located at a longitude of 117°93'E and latitude of 24°97'N, with altitude of 650-800 m. In this region, annual rainfall is 1800 mm, relative humidity is 80%, and annual average temperature is 16-18°C.

Rhizosphere soil sampling was done according to the method of Fujii (5), For this, 100 tea plants of each age were randomly selected. The dead branches and leaves on the surface of the soil were removed. Soil around the tea plants at a radius of 15–25 cm and depth of 25–35 cm were collected as Rhisosphere soil samples. The samples were mixed and the amount of each age soil reached about 15 Kg**.**

The physico-chemical properties of the tea plantation soils and control soil sample were measured as per the *Handbook for Analysis of Soil and Agricultural Chemistry* (12), (Table 1).

Table 1. Physico-Chemical properties of tea plantations soils of different planted years

	planted years			
	0 (control)	3	9	25
pH	5.85	5.64	5.24	4.53
Organic matter* ($g \cdot kg^{-1}$)	9.02	10.68	12.54	13.26
Total N *($g \cdot kg^{-1}$)	1.62	1.36	1.23	1.37
Total P* ($g \cdot kg^{-1}$)	1.05	1.32	1.46	1.27
Total K *($g \cdot kg^{-1}$)	1.76	1.38	1.69	1.48
Available N **($mg \cdot kg^{-1}$)	27.2	25.83	29.38	25.34
Available P** ($mg \cdot kg^{-1}$)	79.5	73.53	76.85	78.26
Available K** ($mg \cdot kg^{-1}$)	305.6	335.18	319.83	289.37

*($g \cdot kg^{-1}$) **($mg \cdot kg^{-1}$)

Bioassay of different polar resin eluents

After drying the rhizosphere soil samples under shade for three days, the different samples were powdered and sieved through a 40 mesh sieve. Five kg of each sieved air dried rhizosphere soil sample was then mixed with 20 L distilled water and the suspension was subjected to ultrasonication at 360 W for 1 h and then was shaken (120 r/min) for 1 h. The suspension was stirred every 10 min during ultrasonication. The resultant suspension was then filtered to separate the soil and filtrate. The residual soil was then subjected again to the ultrasonication and this was repeated 4 times. The 5 filtrates were combined and concentrated in vacuo by rotary evaporation at 45 °C to give a total volume of 5 L, corresponding to a final concentration of 1 g soil per mL. The five soils with different ages were treated similarly as the above method(9).

Resins of different polarity (ADS-7, ADS-21, ADS-F8, ADS-17 and ADS-8) were used for the adsorption of soil allelochemcials, were purchased from Tianjin Nankai Synthetic Technology Co., Ltd. (http://www.tjhecheng.com/Products.aspx?id=112**).** They were presoaked in 100% ethanol for 24 h and then washed with distilled water until no ethanol remained. Each 5 L tea tree rhizosphere soil extracts was then divided into 5 parts (one L each). For allelochemical adsorption on the resin, 200 g of each resin was added to 1 L of extract and shaken at 120 r/min for 24 h. The supernatant was then filtered through filter paper, and the resin was then eluted with 600 mL of methanol at 120 r/min for 12 h. The methanolic eluent was filtered through a 0.45-μm membrane filter and concentrated *in vacuo* to a volume of 200 mL which was used in bioassays and this corresponded to a final concentration of 5 g soil per mL methanol.

For Bioassay, 5 mL methanolic eluents were added to filter papers with a diameter of 9cm placed in sterilised (5h at 120 °C) Petri dishes. Petri dishes containing 5 mL of only methanol were kept as control. The Petri dishes were then placed in a fume hood to enable the methanol to evaporate. Five mL of sterilized distilled water was then added to each dish, and then 10 pre-germinated lettuce seeds were placed on the filter paper. The Petri dishes were placed in an artificial climate chamber [25 °C, light 12 h (7:00-19:00) every day]. The root length and height of the receptor lettuce seedlings was measured after 3 days. The effects of different resin eluents on the receptor were evaluated by determining the percent inhibition relative to control using the formula:

$$PI\ (\%) = (1 - \text{treatment} / \text{control}) \times 100\%$$

All tests were done in triplicate and the PI value is the average of three replicates.

Growth and quality of tea seedlings with different polar resin eluents

For this, 50 mL each of the rhizosphere soils eluents, eluted using different resins (ADS-7, ADS-21, ADS-F8, ADS-17 and ADS-8), were concentrated by rotary evaporation at 45 ℃ to 10 mL, and then diluted to 2 L with sterile distilled water and stored at -20 ℃ till use. Soil that had not been used to grow tea plants was air-dried and passed through 40 mesh sieves and used as the test soil to grow the tea seedlings. Ten kg of powdered soil was placed into pots (25 cm height and 35 cm dia), and 6 one-year-old Tieguanyin tea seedlings were transplanted into each pot. After 30 d, the soil in each pot was loosened and 2L of resin eluent was slowly poured onto the soil in each pot, taking care to ensure that the eluent was evenly distributed in the soil. These tea seedlings were irrigated with 1L of water for each pot every day and allowed to grow for 60 days, with three replicates for each eluent**.**

After 60 days, the photosynthetic physiological and and quality of tea leaves were determined. Photosynthetic rates, transpiration rates and chlorophyll content of tea leaves of the seedlings were measured using a LI-6400 portable photosynthetic apparatus. A bud and two leaves of each tea plant were selected at random, and kept at 105º C for 60 min and then dried at 80º C to a constant weight. They were then ground and sieved through a 60 mesh sieve. The contents of tea polyphenols, Theanine and Caffeine in tea leaves were determined as per the standards procedures of tea quality, National Standards of People's Republic of China (6,7,8).

GC-MS Analysis of Resin eluent

For this, 20 mL of resin eluent with the strongest inhibitory activity to the receptor and tea seedlings was extracted using a separating funnel with 50 mL of ethyl acetate (thrice) and the volume of the combined extracts was reduced to 5 mL using reduced pressure distillation at 40 ℃. The extract was then lyophilized to a powder and then redissolved in 0.5 mL of high purity ethyl acetate, and then passed through a 0.22 μm filter and used for GC-MS analysis.(9)

GC-MS analysis was done using an Agilent Model 7890A/5975C GC-MS fitted with a DB-5 capillary column (30 m × 0.25 mm). The initial temperature was 50 ℃ for 5 min increasing it to 180 ℃ at a rate of 10 ℃/min; held at 180 ℃ for 4min and increasing to 220 ℃ at a rate of 20 ℃/min; held at 220 ℃ for 5 min; increasing to 280 ℃ at a rate of 5 ℃/min; then held at 280 ℃ for 5min. The inlet temperature was 280 ℃ with helium as carrier gas at a flow rate of 0.8 mL/min. Sample injection volume was 2 μL. The interface temperature was 250 ℃. The ion source temperature was 230 ℃ and the four rod temperatures were 150 ℃. Full scans were carried out using a scanning speed of 0.4s over a scanning range of 15-900(m/z). Mass spectra of compounds were searched and identified by comparison with the standard spectra in the Spectrum Library (NIST 98 & WILEY), ignoring undetectable peaks and peak areas <0.5%, with all compounds then being classified according to their molecular structure and the functional groups.

Statistical Analysis of Data

Variance and significance analysis were carried out using Excel, DPS software.

RESULTS AND DISCUSSION

Effect of different polar resin eluents on lettuce

The bioassay results (Fig 1) showed that the inhibitory effect of eluents on root length and plant height of lettuce seedlings varied with the different resin eluents. The ADS-7 resin eluent of the same age soil was most inhibitory. Further, the inhibitory effects on the receptor by eluents of rhizosphere soils of different ages using the same adsorbing resin was in the order: 25 > 9 > 3> 0 years. It was thus clear that the inhitory effects in the soil increased with the age of the plantations.

Effect of Eluents on photosynthetic indices of tea plants

Fig 2 shows the effects of different eluents on photosynthetic indices of tea plants. The photosynthetic indices of tea plants decreased with the increase of soil age, and the

ADS-7 resin eluent had the strongest effect. After treating with ADS-7 resin eluents of different age soils, the photosynthetic rate of tea tree leaves decreased from 38.25 to 27.53μmol CO_2 m^{-2} s^{-1}, the transpiration rate decreased from 19.79 to 10.52mmol H_2O $m^{-2}s^{-1}$, and the chlorophyll content decreased from 54.21 to 38.28 (SPAD). Similarly, the lowest photosynthetic indices were noticed with plants treated with this ADS-7 resin eluent. Thus, both the age of the soils and the type of resin used influenced the effects on the various indices examined herein.

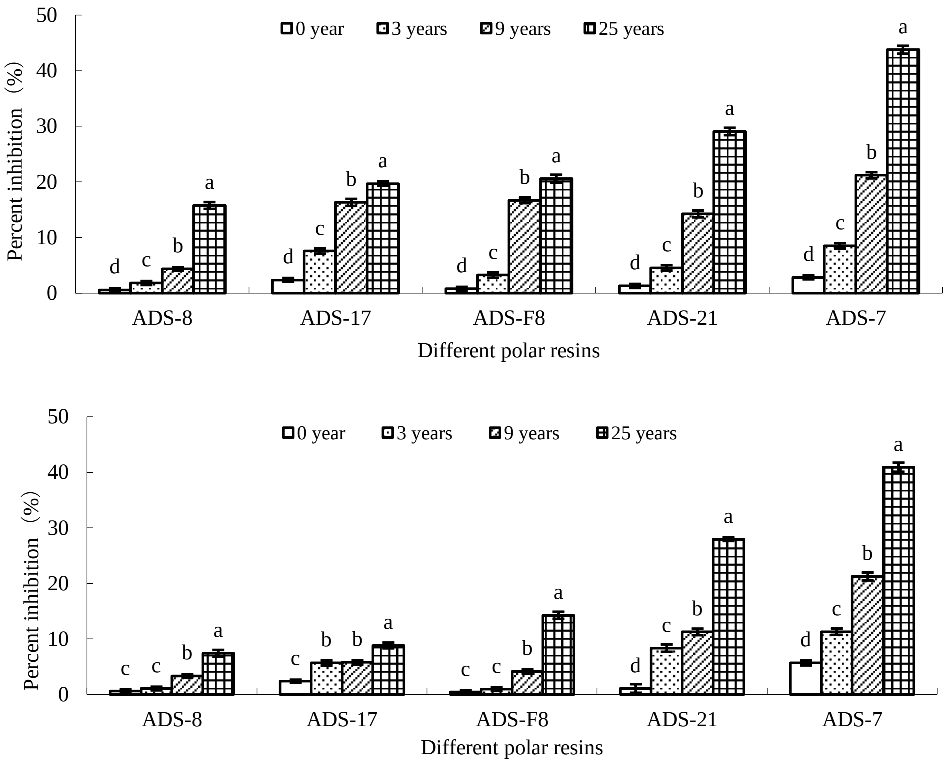

Figure 1. Bioassay of eluents from resins of different polarities
Note: The bars represent standard errors of the mean (n=3). Different letters indicate significant differences at $p < 0.05$.

Effects of different eluents on the quality indices of tea leaves

As seen in Fig 3, eluents from different resins also influenced the quality indices of tea leaves, and the eluent from ADS-7 resin showed the strongest activity. The leaves of plants treated by ADS-7 resin **e**luent had decreased content of polyphenols (282.83 to 235.27 mg/g,) theanine (14.02 to 8.32 mg/g,) and caffeine (28.13 to 18.02 mg/g). Further, the quality indices also changed significantly among different soil ages. As with above, the ADS-7 resin eluent had the strongest effect on the quality indices, especially, from the 25-year-old soil.

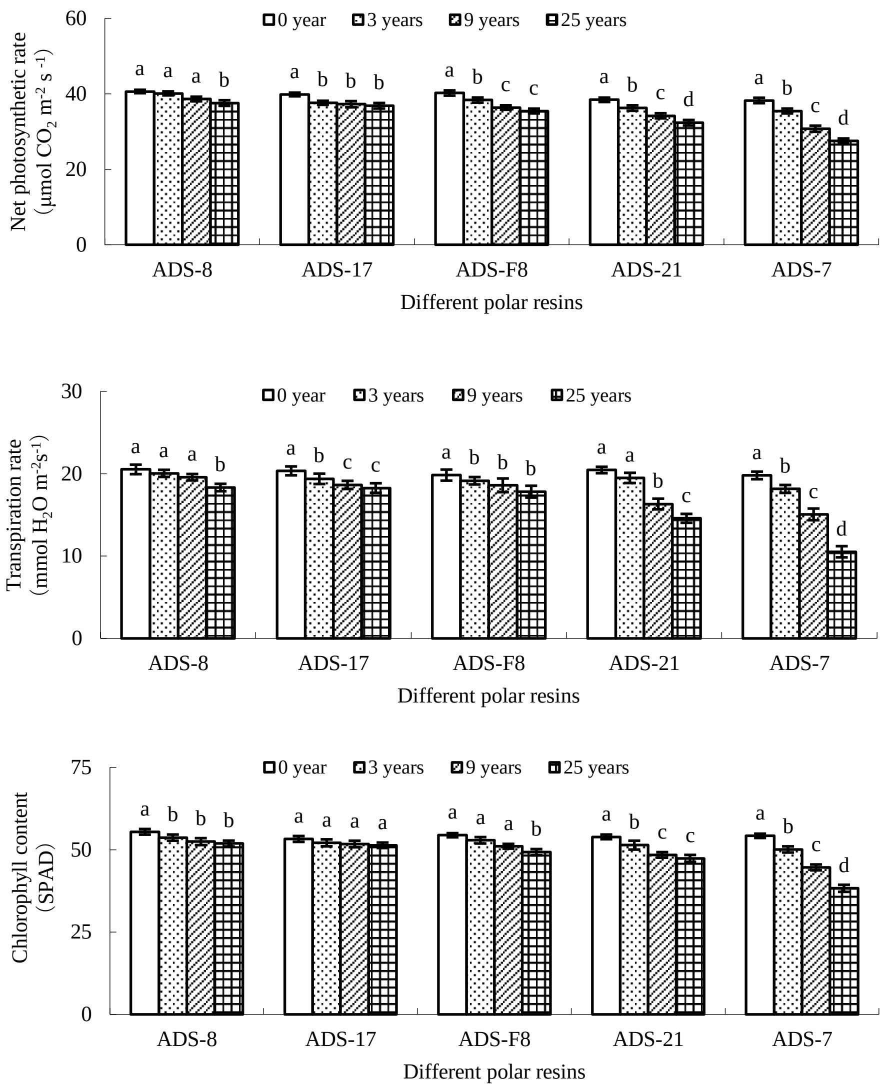

Figure 2. Effects of different resin eluents on the photosynthetic index of tea leaves
Note: The bars represent standard errors of the mean (n=3). Different letters indicate significant differences at $p< 0.05$.

GC-MS analysis of ADS-7 resin eluents

GC-MS analysis of ADS-7 resin eluents (Fig 4, Table 2) showed the presence of 24, 28, 29 and 29 substances in the rhizosphere soil samples from tea plantations of 0,3, 9 and 25 years, respectively. The compounds found were acids, amides, esters, phenolic ketones and others.

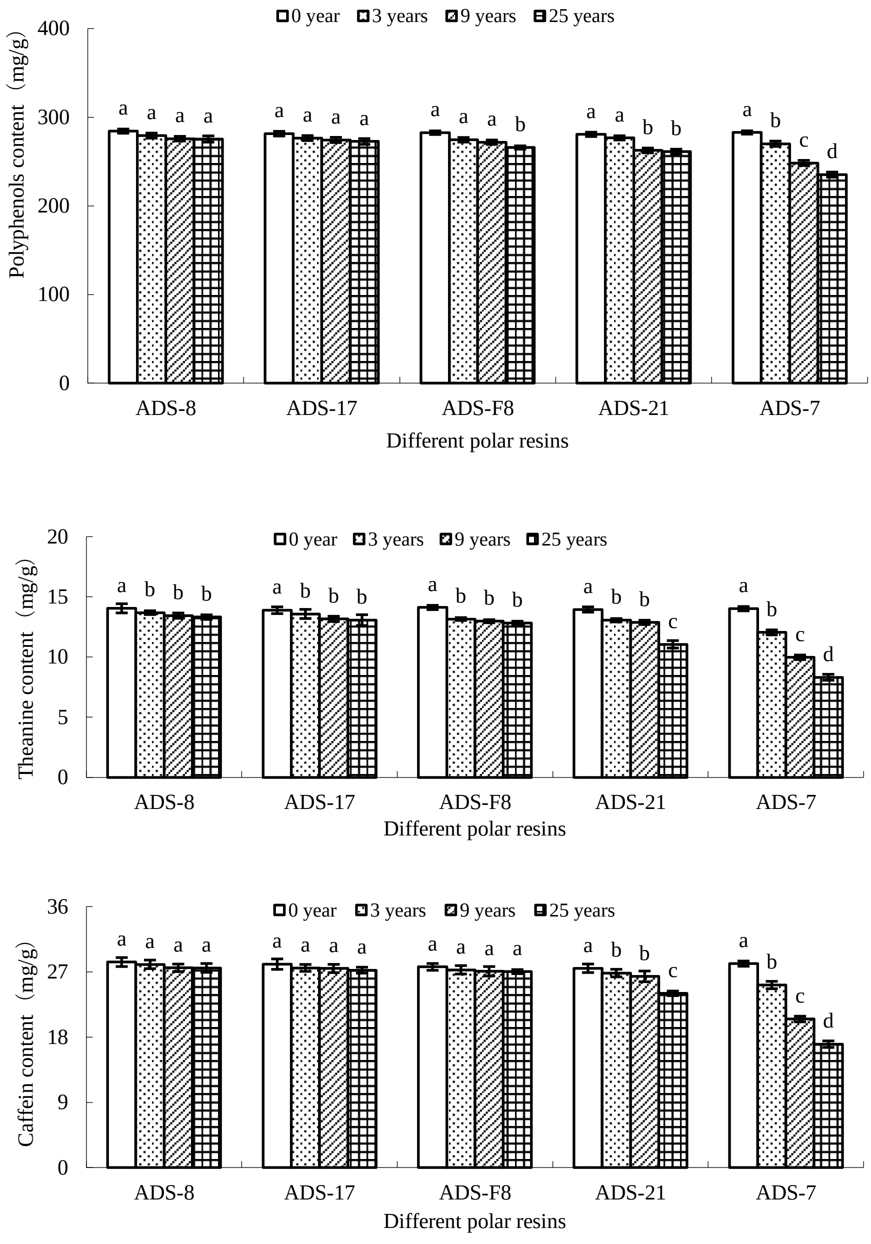

Figure 3. Effects of different resin eluents on the quality of tea leaves
Note: The bars represent standard errors of the mean (n=3). Different letters indicate significant differences at $p< 0.05$.

As the planting age increased, the relative content of amides, esters and other types showed a downward trend, while acids showed the increasing trend. There were little differences in the relative content of the phenolic ketones. These results indicate that acidic compounds accumulated over time in the rhizosphere soils of tea trees that lead to a decrease in soil pH from 5.85 (Control soil) to 4.53 (25 year old soil), which may be responsible for the degradation of tea plantation soils. These findings are consistent with research reported previously (11), which also described the accumulation of acidic compounds in the rhizosphere soil of Rougui tea plants in Wuyishan tea plantation. In the previous study (11), the content of 6 phenolic acids in soils were determined quantitatively by using SPE-HPLC method (11,27). The trend in accumulation of the phenolic acids in the tea plantations appears to be similar and is likely dependent on the tea plantation *per se*, irrespective of the location of the tea plantations.

GC-MS analysis of the soil eluents showed the presence of 9 different acids in the ADS-7 resin eluents of rhizosphere soil, which increased significantly with the increase of the soil age. Among the 3 amides, the amount of hexadecanamide decreased, while dodecanamide increased with soil age. Of the 6-esters present, the levels of triacontyl acetate, BIS(2-Ethylhexyl)phthalateand dibutyl phathalate showed significant downward trend, while 1-Hexadecanol, acetate significantly increased. Of the 7-phenolic ketones present, the amount of vanillin and p-Hydroxybiphenyl increased significantly, while, the amount of 1,1'-bis-(2,4,6-trihydroxy-1, 3-phenylene) ketone showed significant downward trend. Among others, 2,3-dimethylnaphthalene and 2-naphthyl methyl ketone also showed significant downward trend.

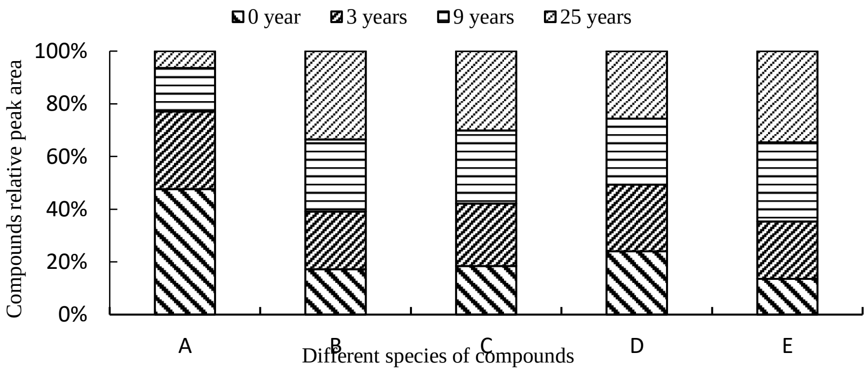

Figure 4. Composition of ADS-7 resin eluent from tea rhizosphere soils of different ages
A：Acids；B：Amides；C：Esters；D：Phenolic and Ketones；E：Other。

Previous study by Suzuki *et al* (20) had shown that tea trees release amides including caffeine and theobromine into the soil, which together inhibit the growth of tea seedlings. Cao *et al* (1) reported 9-phenolic compounds [Ferulic, cinnamic, vanillic, p-coumaric, benzoic, p-hydroxybenzoic, caffeic, chlorogenic and gallic acid], in the soil extracts from tea plantations of South China agricultural university, Guangdong Province, China, which increased with age of the plantations. Our results also clearly show that components of Tieguanyin soil systems changed significantly with the age of the tea plantation. While the 13 compounds accumulated significantly, the 7 compounds were significantly reduced (Table. 2).

The release and accumulation of exudates by tea roots over a period of years can potentially lead to autotoxicity that can inhibit the tea plant growth and the quality of the tea leaves (11,17). Our findings confirm that as the soils age increased, they accumulate allelochemicals which are toxic to Tea (autotoxic) as well to other plants such as lettuce.

Table 2. Compounds of ADS-7 resin adsorption eluent from tea rhizosphere soil of different ages

Compounds	Chemical	The compounds relative peak area of different years soil (%)				Correlation coefficient
		0 year	3 years	9 years	25 years	
Acids	Benzoic acid	1.24±0.12 d	2.14±0.08 c	2.84±0.05 b	4.25±0.21 a	0.97**
	9,12,15-Octadccatricnoic acid	0.98±0.08 d	1.63±0.14 c	1.95±0.04 b	2.49±0.07 a	0.92*
	Tetradccanoic acid	0 d	1.68±0.09 c	2.88±0.13 b	4.94±0.15 a	0.96*
	Hexadecanoic acid	0 d	1.59±0.15 c	3.87±0.18 b	5.59±0.24 a	0.94*
	2-Butenoic acid	1.45±0.13 d	2.93±0.26 c	4.92±0.23 b	6.47±0.16 a	0.94*
	alpha-Acetamidocinnamic acid	0 c	0 c	1.25±0.25 b	6.64±0.19 a	0.98**
	Cyclohexanecarboxylic acid, 2-(1,1-dimethylethyl)-, trans-	0 d	1.07±0.05 c	2.42±0.12 b	3.85±0.15 a	0.96*
	n-Decanoic acid	1.26±0.15 d	2.38±0.13 c	3.59±0.18 b	5.21±0.15 a	0.97**
	Pentadecanoic acid	0.84±0.06 d	1.51±0.14 c	2.95±0.17 b	3.65±0.13 a	0.92*
Amides	Hexadecanamide	8.92±0.27 a	6.15±0.31 b	3.52±0.19 c	1.09±0.24 d	-0.93*
	9-Octadecenamide, (Z)-	2.38±0.19 a	2.26±0.13 a	2.47±0.12 a	2.34±0.18 a	0.03
	Dodecanamide	0.74±0.05 d	1.42±0.08 c	1.89±0.09 b	2.75±0.12 a	0.96**
Esters	3-Acetoxytridecane	2.33±0.15 d	4.68±0.22 c	5.84±0.21 b	6.65±0.13 a	0.83
	Triacontyl acetate	5.83±0.34 a	3.85±0.18 b	1.89±0.13 c	0.54±0.08 d	-0.91*
	1-Hexadecanol, acetate	1.59±0.12 d	2.16±0.06 c	2.98±0.03 b	4.04±0.12 a	0.97**
	Di-n-octyl phthalate	4.68±0.32 a	4.52±0.27 a	4.84±0.28 a	4.59±0.26 a	-0.1
	BIS(2-Ethylhexyl)phthalate	9.35±0.36 a	8.12±0.18 b	5.43±0.14 c	1.29±0.13 d	-0.99**
	Dibutyl phathalate	6.42±0.28 a	4.46±0.15 b	2.87±0.13 c	1.38±0.06 d	-0.92*
Phenolic and Ketones	2,2-Methylencbis(6-tert-bulyl-4-methylphenol)	4.36±0.22 a	4.34±0.29 a	4.54±0.19 a	4.42±0.18 a	0.34
	Vanillin	0.83±0.04 d	2.42±0.16 c	4.89±0.14 b	6.54±0.12 a	0.93*
	Phenol,3,5-bis(1,1-dimethylethyl)	1.52±0.09 a	1.55±0.18 a	1.49±0.26 a	1.53±0.19 a	-0.03
	p-Hydroxybiphenyl	0 d	0.12±0.05 c	0.36±0.03 b	0.49±0.03 a	0.92*
	Ethanone, 1-(3,4,5-trimethoxyphenyl)-	1.84±0.26 a	1.88±0.15 a	1.89±0.16 a	1.83±0.15 a	-0.43
	Ethanone, 1,1'-(2,4,6-trihydroxy-1,3-phenylene)bis	8.21±0.23 a	6.19±0.17 b	3.24±0.15 c	0.63±0.09 d	-0.95*
	3,5-di-tert-Butyl-4-hydroxyben zyl alcohol	5.58±0.19 a	5.21±0.24 a	5.69±0.27 a	5.45±0.31 a	0.03
Other	Naphthalene, 2,3-dimethyl-	9.87±0.31 a	7.82±0.16 b	4.64±0.12 c	1.28±0.13 d	-0.96**
	2-Naphthyl methyl ketone	10.36±0.27 a	8.57±0.12 b	4.83±0.48 c	1.56±0.12 d	-0.96**
	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-et hylidene-	2.49±0.12 a	2.53±0.19 a	2.52±0.17 a	2.47±0.16 a	-0.62
	2-Hydroxy-2,4-dimethyl-hept-6-en-3-one	5.82±0.24 a	5.87±0.36 a	5.94±0.32 a	5.85±0.26 a	0.08

Note: Different letters indicate significant differences at $p < 0.05$.

Among the different resins employed for adsorption of the soil chemicals, the inhibitory effect of the eluent from the ADS-7 resin was the greatest (Fig. 1-3). GC-MS analysis of the ADS-7 resin eluent revealed that soil from older plantations contain higher amounts of acidic compounds (Fig. 4). ADS-7 resin has strong polar surface, which absorbs mostly the polar compounds in the soil.

CONCLUSIONS

The practice of monoculture in tea plantations can result in the composition of rhizosphere soil becoming imbalanced, resulting in increasing concentration of acidic allelochemicals that can lead to strong autotoxic effects. Our studies herein and earlier (11) have shown this phenomenon present in two different tea plantations in China. These problems will increase as the length of time the land is used to continuously grow tea trees increases, eventually leading to significant decreases in the quality of the tea. Therefore, maintenance of the quality and balance of chemicals in soil is important, if changes in soil content caused by continuous tea cropping are to be managed effectively and the quality of the tea leaves is not decreased. This being a common phenomenon, it is necessary to develop technology to eliminate the autotoxic chemcials sothat the quality of the tea from these plantations is maintained.

ACKNOWLEDGEMENTS

H.B. Wang equally contributed to this research work. This work was supported by the National Key Research and Development Program of China (2016YFD0200900)； National 948 Project (2014-Z36) of China; the Project of the Collaborative Innovation Centre of Chinese Oolong Tea Industry, Wuyi University (201310); China Postdoctoral Science Foundation（2016M600493）； Natural Science Foundation of Fujian Province (2017J05057，2017J01649) ；the Open Project of Key Laboratory of Agroecological Processing and Safety Monitoring (FAFU), Fujian Province (NYST-2017-01)；the Project of Scientific Research of Young and Middle-aged teachers, Fujian Province (JAT170573，JAT160504)； Science & Technology Project of Longyan City (2017LY71), Fujian Province of China；the Talent-Recruiting Program of Longyan University (LB2015001).

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