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Effects of phenanthrene on chemical composition and enzyme activity in fresh tea leaves

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ABSTRACT

Phenanthrene pollution around roots of tea (*Camellia Sinensis*) seedlings and its effects on antioxidative enzymes and chemical composition in fresh tea leaves during 7 days of hydroponic culture were investigated. Phenanthrene concentration in the fresh tea leaves gradually increased over the 7 days. The activities of polyphenol oxidase (PPO) and superoxide dismutase (SOD) were promoted at the beginning of phenanthrene contamination and were suppressed from the fifth day, while peroxidase (POD) and catalase (CAT) followed an opposite trend. On the third day, the contents of water extract, amino acid and caffeine had peak values, and the protein content was the lowest. The contents of polyphenols and total sugar were increased on the first day and then began to decline. On the seventh day, all chemical components decreased, indicating that phenanthrene may reduce tea quality. Linear correlation analysis showed that the activities of PPO, POD and CAT and the content of polyphenols in the tea leaves correlated well with the concentration of phenanthrene in the tea leaves.

1. Introduction

Tea is the most popular beverage consumed by over two thirds of the world's population (Yukiaki & Yukihiko, 1999). Global tea production in 2007 was 3.60 million tonnes, of which nearly 30.6% was produced in China. Tea has many functional components such as polyphenol, caffeine and theanine. A number of published studies have provided scientific evidence for the health-promoting properties of tea consumption, including reduction of cholesterol, depression of hypertension, antioxidation and antimicrobial effects, and protection against cardiovascular disease and cancer (Ho, Lin, & Shahidi, 2008; Kurodo & Hara, 1999). However, due to both the chemical treatment of crops and post-harvest manipulation, a number of residues of chemical contaminants have been detected in tea leaves, such as heavy metals, fluoride, and pesticides (Pilar, Natalia, Nerea, Eva, & Manuel, 2007; Xia & Tu, 2008). These contaminants may affect tea yield and quality, and pose a health threat to tea drinkers.

Polycyclic aromatic hydrocarbons (PAHs) are a large class of compounds well-known for their mutagenic, carcinogenic and teratogenic activities (Bohuslav, Jana, & Vladimir, 2002; Silvano et al., 2001). PAHs have two or more fused aromatic rings and are mainly produced during the incomplete pyrolysis or combustion of organic matter and may be transported over long distances before deposition and accumulation in vegetation (Kang et al., 2005; Vania, Larisse, Coretta, & Peter, 2005). PAHs may threaten human health when exposed to human from a wide variety of sources, such as occupation, smoking, diet, drinking water, and outdoor/indoor air particulates (Fismes, Perrin-Ganier, & Empereur-Bissoneet, 2002; Kang et al., 2005; Liu, Luo, Cao, & Jiang, 2002; Vania et al., 2005).

PAHs have been detected in some processed teas and fresh tea leaves around the world, indicating that PAHs could be accumulated from the environment (Lin, Zhu, He, & Tu, 2006; Pilar et al., 2007). The transfer of PAHs from tea leaves into tea infusion has also been evaluated (Lin & Zhu, 2006). However, to date no information is available on the effect of PAHs on the chemical composition and antioxidant activities of fresh tea leaves. The antioxidant machinery is composed of enzymes and non-enzymatic components. The enzymatic component is made of free radical scavengers like superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (Mahaboob & Gurjot, 2007). Some researches also found that polyphenols, catalysed by polyphenol oxidase (PPO), were highly related to the antioxidant activity in tea leaves (Kyung, Choong, Hyungjae, Moon, & Chang, 2008). Tea extracts, mainly composed of soluble polysaccharide, protein, amino acid, caffeine, and polyphenol, determine the quality of processed tea. Environmental stress can affect these components and then

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influence the sensory quality (flavour, taste and colour) of tea infusion (Li, Yu, Li, & Li, 2007).

The objective of the study was to elucidate the effects of phenanthrene (one important PAH, Fig. 1) on enzyme activity and chemical composition in fresh tea leaves. Variations of the activities of SOD, POD, CAT and PPO and the contents of main chemical components in fresh tea leaves under the pollution of phenanthrene around the roots of tea seedlings were detected, and the probable mechanisms discussed.

2. Materials and methods

2.1. Hydroponic experiment of tea seedlings

Two-year-old tea (*Camellia Sinensis*) seedlings of Wuniuzao, one of the most important economic tea varieties in China, were granted by the Tea Research Institute of Chinese Academy of Agricultural Sciences from its tea plantation in Hangzhou City of Zhejiang Province in China. After collected and thoroughly washed with water, these seedlings were starved in water for one week, and then grew in the nutrient solution for another week. Six liters nutrient solutions were added in each 10 l plastic bucket. The components of nutrient solution (Lin, Tu, & Zhu, 2005) were as follows: 20 ppm $(NH_4)_2SO_4$, 10 ppm NH_4NO_3, 3.1 ppm NaH_2PO_4, 40 ppm K_2SO_4, 15 ppm $CaCl_2 \cdot 2H_2O$, 0.35 ppm EDTA FeNa $\cdot 3H_2O$, 25 ppm $MgSO_4 \cdot 3H_2O$, 20 ppm $Al_2(SO_4)_3 \cdot 18H_2O$, 0.1 ppm $ZnSO_4 \cdot 7H_2O$, 0.1 ppm H_3BO_3, 0.025 ppm $CuSO_4 \cdot 5H_2O$, 1 ppm $MnSO_4 \cdot H2O$, 0.05 ppm $Na_2MoO_4 \cdot 2H_2O$. All of these components were of analytical grade with a purity of >98%. Uniform tea seedlings, about 60 cm in height with about 15 leaves, were selected and every six of them were bundled together with sponge in the lower part of the stems and fixed through a drilled hole in a board which covered the bucket to ensure that the seedlings were upright and their roots were immersed just below the surface of nutrient solution.

The experiment was designed into two groups. Group 1 was set as a blank control without phenanthrene. In group 2, six milliliters acetone solution with phenanthrene (1 mg/ml) was added into the 6 l nutrient solution in each bucket. Phenanthrene was purchased from ACROS ORGANICS (Geel, Belgium) with a purity of >90%. Tea seedlings were cultured in a greenhouse for 7 days at a temperature of 25–30 °C in the daytime and 15–20 °C in the nighttime. Each sample had three replicates, and all treatments were located randomly. During the hydroponic period, the nutrient solution was stirred once a day to increase dissolved oxygen. Acetone in group 1 and phenanthrene solution in group 2 were supplemented every day. Tea leaves were plucked on the first, third, fifth, and seventh days and stored for assays of antioxidant enzyme activities and the contents of phenanthrene and chemical components.

2.2. Determination of enzyme activities

One gram fresh tea leaves, 0.6 g polyvinylpolypyrolidone (PVPP) and 20 ml cold buffer solution were homogenized (by a Kultra Turrax Ika T18 Basic Blender) at 14,000 rpm. The mixture was extracted at 4 °C for 4 h, and then centrifuged at 1776*x*g and 4 °C for 15 min (by a Beckman J2-HS centrifuge). The supernatant was then used for the assays of the activities of PPO, POD, CAT and SOD (Huang, 1997).

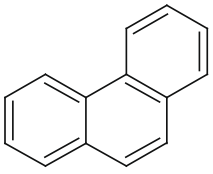

Fig. 1. Molecular structure of phenanthrene.

PPO activity reaction system, containing 1 ml of enzyme extract, 2 ml of citrate and phosphate buffer solution (pH 5.6, 0.1 M), 0.4 ml of proline (10 mg/ml), and 1 ml of catechol (1 mg/ml), was kept in 37 °C water for 10 min. PPO activity was determined by spectra photometric method using HP 8453 UV–visible G1103A Hewlett–Packard at 460 nm. One unit of PPO activity was defined as 0.1 change of absorbance per minute.

POD activity reaction system, containing 0.05 ml of enzyme extract, 2 ml of water, 1 ml of guaiacol (3 μl/ml) as donor, and 1 ml of H_2O_2 (3 μl/ml) as substrate, was kept in 35 °C water for 5 min. POD activity was determined by spectra photometric method at 470 nm. One unit of POD activity was defined as 0.1 change of absorbance per minute.

CAT activity reaction system, containing 0.1 ml of enzyme extract, 1.9 ml of water, and 1 ml of H_2O_2 (2 μl/ml) as substrate, was kept in 35 °C water for 5 min. CAT activity was determined by spectra photometric method at 240 nm. One unit of CAT activity was defined as 0.01 change of absorbance per minute.

SOD activity reaction system, containing 0.2 ml of enzyme extract, 2 ml of SOD I solution, and 1 ml of SOD II solution, was illuminated at 4000 Lx for 15 min. SOD I solution is the 0.05 M phosphate buffer (pH 7.8) containing 3 mg/ml methionine, 0.1 mg/ml NBT (nitroblue tetrazolium), 0.05 mg/ml EDTA; SOD II solution is the 0.05 M phosphate buffer (pH 7.8) containing 2.26 μg/ml riboflavin. SOD activity was determined by spectra photometric method at 560 nm. One unit of SOD activity was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions.

2.3. Extraction and determination of phenanthrene and chemical components in fresh tea leaves

The extract and analysis of phenanthrene was described elsewhere (Lin et al., 2006).

Three gram fresh tea leaves were pulverized and extracted with 100 ml boiling water at 100 °C for 30 min. The infusion was filtered through "Double-ring" No. 102 filter paper (Xinhua Paper Industry Co. Ltd., Hangzhou, China) for chemical composition determinations. Fifty milliliters of the tea extract were evaporated and dried at 103 °C for 3 h to a steady weight. The final dried solid was weighed. Water extract content was defined as the ratio of the solid weight to the dry weight of tea leaves in the 50 ml tea extract. The contents of polyphenol, amino acid, protein and total sugar were determined by a spectrophotometer using the following methods: polyphenol by ferrum tartrate dying method, amino acid by ninhydrin dying method, protein by coomassie brilliant blue dying method, and total sugar by anthrone reagent dying method (Lin et al., 2006; Zhong, 1989).

The contents of catechins and caffeine were analyzed using a high-performance liquid chromatography (HPLC) system (SHIMADZU LC-2010A, Kyoto, Japan) (Tu, Xia, & Watanabe, 2005). Ten microlitres of the tea extract sample were injected for HPLC analysis under the following conditions: The column used was VP-ODS (250L × 4.6). The mobile phase A was acetic acid/acetonitrile/water (0.5:3:96.5, v), and B acetic acid/methanol/water (0.5:30:69.5, v). At a flow rate of 1.0 ml/min at 35 °C column temperature using the Shimadzu SPD ultraviolet detector, peaks were observed at a wavelength of 280 nm. Catechins standard sample was from Sigma Company. All organic solvents used for sample preparation and analysis were HPLC grade.

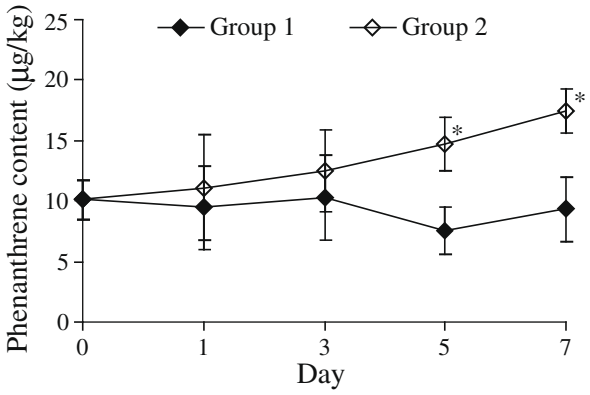

Fig. 2. Change of phenanthrene content in the fresh tea leaves during the experiment. * represents significant difference between the group 1 and the group 2 ($p < 0.05$).

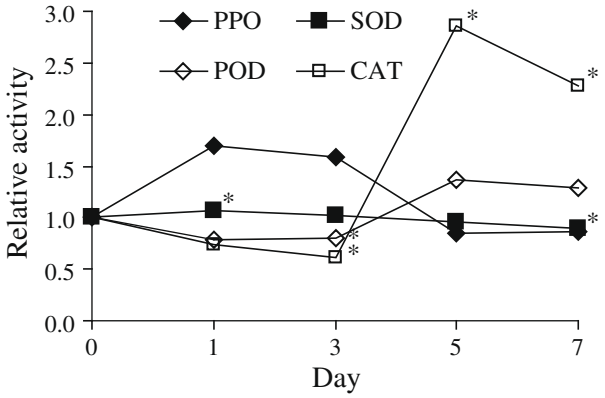

Fig. 3. Relative enzyme activities in fresh tea leaves subjected to phenanthrene stress. Relative values were defined as the ratio of group 2 to group 1, and the values in group 1 were 1. * represents significant difference between the group 1 and the group 2 ($p < 0.05$).

2.4. Statistical analysis

All experiments were performed in triplicate. Significance of the difference between group 1 and group 2 was evaluated by analysis of variance (ANOVA) followed by Tukey's studentized range test carried out on the SAS system for windows V8. Probability value of $p < 0.05$ was used as the criteria for significant differences. Dynamic changes of enzymes and chemical components during the hydroponic period (from the first day to the seventh day) were depicted by their relative values defined as the ratio of group 2 to group 1. Linear regressive analysis between the relative values of phenanthrene content and enzyme activities and chemical component contents were also carried out.

3. Results and discussion

3.1. Phenanthrene content in tea leaf

Phenanthrene content of the tea leaves in group 2 gradually increased in the 7 days (Fig. 2), with a range of 17.7–95.0% higher than that in group 1. There were significant differences in the phenanthrene contents between group 1 and group 2 on the fifth and seventh days when the phenanthrene contents were 14.7 and 17.5 μg/kg in group 2, respectively, which were 95.0% and 87.5% higher than that in group 1, respectively. Our previous research showed that phenanthrene can be greatly accumulated by tea plant roots from water, but only about 4.98% of the root accumulated phenanthrene could be translocated to leaves (Lin et al., 2006). Despite the little increase of phenanthrene, an obvious aging process could be observed in the leaves of group 2. The leaves in group 2 became more yellow and crisp than in group 1, suggesting that phenanthrene could affect the metabolism and growth of tea plant.

3.2. Effects of phenanthrene on enzyme activity

The activity changes of SOD, POD, CAT and PPO in the tea leaves during the hydroponic period were shown in Fig. 3. The activities of PPO and SOD in the samples treated with phenanthrene were both promoted on the first day, and then declined, with relative activities of 1.69–0.85 and 1.06–0.90, respectively. There were significant differences between group 1 and group 2 on the first and seventh days in SOD activity. The responses of POD and CAT to phenanthrene were contrary to the trends in the activities of SOD and POD. They decreased during the first three days, and then were activated from the fifth day. The relative activities of POD ranged from 0.79 on the first day to 1.36 on the fifth day, and CAT from 0.61 on the third day to 2.86 on the fifth day. There were significant differences between group 1 and group 2 on the third day in POD activity, and on the third, fifth and seventh days in CAT activity.

Upon exposure to pollutants, organisms usually attempt to metabolize and depurate directly, minimizing any cellular damages. Protective mechanisms generally appear to alter such enzyme activities, which can scavenge the free radicals originated from the biotransformation of pollutants (Cheung, Zheng, Li, Richardson, & Lam, 2001). If the free radicals are not eradicated efficiently, oxidative stress would occur and cause a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses, and then lead to the oxidation of biomolecules (i.e. lipids, proteins, and nucleic acids) or even cell death (Boscolo, Menossi, & Jorge, 2003; Cai, Ma, Gao, & Yang, 2005; Wang et al, 2008). So these enzymes can be regarded as biomarkers of exposure or effects (Cheung et al., 2001). SOD is responsible for catalyzing the dismutation of superoxide radicals ($O_2^{\cdot -}$) to O_2 and H_2O_2 (Boscolo et al., 2003), which constitutes the first line of defence against ROS (Dhiraj & Sanjay, 2005). In addition, when plant cells are injured or upon aging, separated PPO and polyphenols contact and react to produce quinine to lighten the harm (Alfred & Eitan, 1979). This was also proven in this test that during the first three days, the PPO activities of the treatments were increased more than 50%.

However, the raising phenanthrene concentration would reduce the enzyme activities, which might be due to enzyme protein damage by excessive H_2O_2 (Xiao, Lei, Ning, & Hong, 2008). H_2O_2 is toxic to cells and has to be further detoxified. Otherwise it will cause Haber–weiss reaction to produce ·OH which is a strong oxidant (Cai et al., 2005). The oxidation of proteins to form carbonyls occurs via the hydroxyl radical, since neither H_2O_2 nor $O_2^{\cdot -}$ is reactive enough to provoke oxidation (Boscolo et al., 2003; Cai et al., 2005). CAT and POD can both cut off the Haber–weiss reaction by detoxifying hydrogen peroxide to H_2O and O_2. POD activity is responsible for the degradation of hydrogen peroxide. H_2O_2 would lead to oxidative stress without a parallel increase in the POD activity in the cell (Boscolo et al., 2003). In addition, CAT removes the bulk of H_2O_2 (Faezeh, Akio, & Hiromi, 2005). On the fifth day, hydrogen peroxide was elevated probably due to the increasing phenanthrene content, and the activities of POD and CAT were stimulated. However, H_2O_2 is a major substance for biosynthesis and accumulation of phenolic compounds and lignification in plants (Faezeh et al., 2005). Usually, the POD activity is high in

Table 1
Changes of relative chemical contents[a] in the fresh tea leaves subjected to phenanthrene stress.

Day	Water extract	Polyphenol	Amino acid	Protein	Total sugar	Caffeine
1	0.98	1.07	0.90*[b]	1.13	1.33*	0.81
3	1.03	1.04	1.02	0.83	1.26*	1.34
5	0.97	0.94	0.84*	1.42*	1.09*	0.87
7	0.96	0.92	0.96	0.86	0.91*	0.71

[a] Relative values were defined as the ratio of group 2 to group 1, and the values in group 1 were 1.
[b] * represents significant difference between group 1 and group 2 ($p < 0.05$).

Table 2
Linear correlation coefficient between the relative phenanthrene content and relative enzyme activities and relative chemical contents[a] during the 7 days' hydroponic culture.

	PPO	SOD	POD	CAT	Water extract	Polyphenol	Amino acid	Protein	Total sugar	Caffeine
Phenanthrene	−1.09*[b]	−0.15	0.74*	2.67*	−0.48	−0.18*	−0.09	0.25	−0.39	−0.36

[a] Relative values were defined as the ratio of group 2 to group 1, and the values in group 1 were 1.
[b] * represents significant linear correlation ($p < 0.05$).

aged organizations, and low in young organizations. A lower activity of POD will be more beneficial for the growth. Because POD not only can participate in the breakdown of H_2O_2, but also can biosynthesize some carbohydrate to lignin in the presence of H_2O_2, which will stiffen the cell walls and cause the aging of organization (Faezeh et al., 2005). That could account for the observation in our experiment that the appearance of tea leave becoming crisp and yellow, which coincided with the increased POD activities in the group 2 on the fifth and seventh days.

3.3. Effects of phenanthrene on chemical composition contents

Sensory quality and healthy effects of tea are related to its chemical composition. Treated with phenanthrene for three days, water extract, amino acid and caffeine had a peak value (2.6%, 2.0% and 34.0% higher than the control level, respectively), and protein had the least content (16.7% lower). The contents of polyphenols and total sugar were both raised to the maximum on the first day (7.3% and 32.9% higher than the control level, respectively), and then declined. On the seventh day, the contents of all chemical components were less than the control group. Water extract, amino acid, caffeine, protein, polyphenols and total sugar were 4.0%, 4.2%, 28.9%, 13.8%, 7.9% and 8.9% lower than the control level, respectively (Table 1). The results of significance analysis were as follows: amino acid was significantly decreased on the first and fifth days; protein was significantly increased on the fifth day; total sugar was significantly higher within the first 5 days and lower on the seventh day. Total sugar can also be accumulated at the beginning of drought stress (Javadi, Arzani, & Ebrahimzadeh, 2008). Sugars are important signals in the regulation of plant metabolism and their accumulation can also induce leaf senescence (Wingler & Roitsch, 2008). This study showed that after three days of PAH pollution, the activities of SOD and PPO decreased, the fresh tea leaves suffered heavier stress and getting old, and the contents of chemical compositions decreased, which suggested that phenanthrene pollution could impact tea quality.

3.4. Linear regressive analysis

Table 2 shows that the activities of PPO, POD and CAT were significantly correlated with the increased concentration of phenanthrene in the tea leaves. Thus, these enzymes may be used as biomarkers for the occurrence of phenanthrene in tea. Polyphenols are a class of important secondary metabolite in vacuole. They have the capacity to quench lipid peroxidation and prevent DNA oxidative damage by neutralizing and detoxifying the radicals produced in the process of exposure to oxidative stress (Yukiaki and Yukihiko, 1999; Kyung, Choong, Hyungjae, Moon, & Chang, 2008). Total phenolics and flavonoids showed a high correlation with antioxidant activity (Kyung et al., 2008). Polyphenol content significantly correlated with the phenanthrene content (Table 2), which may be another circumstantial evidence for the occurrence of oxidative stress in the tea leaves. These correlations suggest that SOD, PPO and polyphenols may build the front line of defense against oxidation resulted from PAH pollution. The promotion of SOD and PPO activities and polyphenol content indicates that the exposure to phenanthrene would induce the formation of superoxide radicals in a quantity greater than the capacity that the pre-existent antioxidant defensive system could remove (Boscolo et al., 2003).

4. Conclusions

PAHs can accelerate senescence process, influence antioxidant enzyme activity, and finally reduce tea quality in the 7 days. The content of phenanthrene in the tea leaves increased gradually during the hydroponic period. Upon exposure to phenanthrene, tea leaves became yellow and crisp. The activities of PPO and SOD were promoted on the first day and suppressed since the fifth day, while the responses of POD and CAT were on the contrary. On the third day, the contents of water extract, amino acid and caffeine reached peak values, while protein content decreased to the lowest. The contents of polyphenols and total sugar were both raised on the first day and then kept reducing. On the seventh day, all chemical components decreased. Linear correlation analysis showed that phenanthrene content in the tea leaves was significantly correlated to the activities of PPO, POD and CAT and the content of polyphenols, suggesting that PPO, POD, CAT and polyphenols were more sensitive to phenanthrene than other items determined in this experiment. The outcome of this work may improve our understanding of the ecotoxicity of PAHs and developing strategies for reducing the risks of PAHs to tea quality and tea drinkers.

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