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Full Length Research Paper

Effects of different autotoxins on antioxidant enzymes and chemical compounds in tea (*Camellia sinensis* L.) Kuntze

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Continuous cropping obstacle is a serious problem in tea (*Camellia sinensis* L.) planting. In order to understand the mechanism of this phenomenon, SOD, POD, CAT and MDA in the roots of tea tree and chemical compounds in leaf of tea were analyzed. The results showed that the autotoxins had both inhibitory and stimulatory effects on antioxidant activities, stimulatory effects increased with time at low concentration, but the inhibition increased with the increasing of growing years. The antioxidant enzyme activities increased first but decreased then, with the peak value differed in different treatments. The autotoxins also increased the MDA contents, while decreased the contents of Chl-a, Chl-b, carotenoids and water extracts in the fresh tea leaves. Results indicated that the activities of SOD, CAT and POD depended on autotoxins; their time of action and concentration and various physiological processes of tea tree were inhibited due to the contents of Chl-a and Chl-b decreased. These results suggested the adverse effects of autotoxins on the enzymes of antioxidant defense system resulting in lipid peroxidation in roots of tea seedlings and the autotoxins could impact tea yield and quality.

Key words: *Camellia sinensis* L., autotoxicity, autotoxins, peroxidase, catalase, superoxide dismutase, malondialdehyde, chemical compounds.

INTRODUCTION

Autotoxicity exists both in natural and agricultural eco-system, the later have attracted increasingly scientist's attention (Singh et al., 1999; Zhang et al., 2009). It is a process in which a species or its decomposing residues release phytotoxins into the environment to inhibit germination and growth on the same species (Zhang et al., 2010; Saffari et al., 2010). Autotoxicity has been documented in a number of plant species and are believed to be involved in natural and agricultural ecosystem, (Hegab et al., 2008; Singh et al., 1999; Yu et al., 2000). However, it is commonly observed in continuous cropping systems and pure stands of perennial crops and causes the reduction in plant growth and productivity in agriculture and forestry (Batish et al., 2001; Kamal et al., 2008; Umer et al., 2010). Phytotoxins were accumulated in the surface soil layer, which can result in the inhibition of seed germination and growth of surrounding plant (Walker et al., 2003; Sampietro et al., 2006), it has been most frequently identified as cause of degradation and replant failure (Singh et al., 2008). De Candolle suggested that the 'soil sickness' problem in agriculture might be due to exudates of plants (Bertin et al., 2003; Sampietro et al., 2006). Many crops such as rice (*Oryza sativa*) (Chou and Chiou, 1979), tomato (*Lycopersion esculentum*) (Hassan and Ghareib, 2009; Singh et al., 2008), alfalfa (*Medicago sativa*) (Chon et al., 2002), cucumber (*Cucumis sativus*) (Yu et al., 1997) have exhibited autotoxicity.

Tea (*Camellia sinensis* L.) is cultivated extensively in many developing countries in Asia and Africa. It is an important crop in the hilly and mountainous area in southern China. However, the tea tree cannot be cropped continuously due to the rapid tea yield and quality

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Abbreviations: SOD, POD, CAT, MDA etc.

decrease over time after the establishment of tea tree (Anaya et al., 2002; Mondal et al., 2004) and thus, reduction in the economy of tea farmers. Regeneration in old tea garden was very difficult. Therefore, the obstacle in continuous cropping has been a serious problem in tea production and quality.

The autotoxicity related soil problems in tea garden were reported, the aqueous extracts of tea leaves, pericarps and stems significantly inhibited the seed germination and root and shoot length of tea seedlings (Cao et al., 1996; Chou et al., 1981). Roots in soil directly comes in close contacts with autotoxic substances released from the plants during their growth, therefore, it is the key organ for the exploring autotoxicity. In previous research, accumulation of phenolic acids originating from dominant plant species of the plant community leads to the inhibition of seed germination, seedling growth and photosynthesis (Blum et al., 1999; Chon et al., 2002; Ezekwesili et al., 2010; Sampietro et al., 2006). These compounds inhibited plant growth by affecting many physiological processes (Blum et al., 1999; Uremis et al., 2009). Recently, the role of the antioxidant system in plant in response to environmental stress has received wide attention (Ahmed et al., 2010; Halliwell et al., 1987; Xu et al., 2010; Prasad et al., 1999; Scebba et al., 1998; Sun et al., 2010; Wang et al.,.2009). The antioxidant defence system in plant cell includes both enzymatic, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) (Zhang et al., 2009; Zheng et al., 2010) and malondialdehyde (MDA) has been considered as a suitable biomarker for lipid peroxidation (Mittler et al., 2002; Mustapha et al., 2009). Altered activities of these antioxidant enzymes and antioxidants have been commonly reported and are used frequently as indicators of oxidative stress in plants (Mittler et al., 2002; Jin et al., 2010; Wang et al., 2010; Zhang et al., 2009). However, few studies have simultaneously investigated the respective responses of root system and shoot system to allelopathic agents and the mechanism of action of autotoxins in tea seedlings is still unknown. To investigate the mechanism between autotoxins and membrane lipid peroxidation, the objective of this study was to determine the effects of root exudates autotoxins on antioxidant enzymes activity and index of oxidative stress (activities of SOD, POD and CAT) and damage degree (MDA content) in root and chemical compounds in the fresh tea leaves were detected.

MATERIALS AND METHODS

Preparation of soil extraction

The dried soils (1000 g, layer 10 to 30 cm) from old tea garden were soaked in distilled water (1000 ml) in a shaker for 24 h. The extracts were filtered by a 0.45 µm hydrophilic membranes and concentrated to 100 ml by using rotary evaporators under 40 to 45℃. The aqueous extracts were kept at 4℃ for further experiments.

Pot culture

The tea seedlings of GSCT17 variety (vegetative seedling by single node cutting) at eight leaves stage were treated by the aquatic extracts of rhizosphere soils of different old years tea garden (15, 30 and 50 years) at the same conditions in the greenhouse at South China Agricultural University (Guangzhou) under natural light at 28/20℃ (D/N) for 30 days, were sprayed with the soil extraction water for three times and the tea tree seedling growth ware analyzed. Each treatment with 30 potted plants was arranged randomly. Fresh tea leaves sample were plucked on 30 days and stored for assays of chlorophylls and carotenoids and the contents of chemical components. All analyses were performed in triplicate.

Preparation of root samples

Tea roots were sampled on 5, 10, 20 and 30 days after trans-planting. Roots were collected immediately after frozen with liquid nitrogen and stored in fridge for further enzyme analysis. Crude antioxidant enzyme solutions were extracted: 0.5 g root was taken and ground in 1 ml 50 mM phosphate buffer (pH 7.8) with liquid nitrogen. After adding 3 ml phosphate buffer, the ground roots was centrifuged (4 000 rpm) at 4℃ for 20 min and the supernatant was used to determine the enzyme activities with Beckman UV/visible light spectrophotometer.

Enzyme analysis

SOD solution was prepared using modified Marklund method. Crude extract was added to 4.5 ml reaction solution containing 100 mM Tris-HCl buffer (pH 8.2), 1 mM EDTA 2Na and 4.5 mM pyrogallol-HCl solution. Then absorbance was measured at 325 nm at start and 1 min later. SOD activity was expressed as µmol/min/ mg protein.

For POD activity measurement, 1 ml crude extract was added in 4 ml reaction medium containing 200 mM phosphate buffer (pH 6.0), 19 ul guaiacol (100%) and 28 ul H_20_2 (30%). The absorbance was measured at 420 nm within 5 min. Activity of POD was expressed as µmol/min/mg protein.

CAT solution was made by adding 0.2 ml crude extract in 3 ml reaction solution including 200 mM phosphate buffer, 100 mM H_2O_2. Absorbance was measured after 4 min H_2O_2 consumption at 240 nm. CAT activity was in µmol/min/mg protein.

MAD analysis

Total 4 ml solution was prepared by adding 1.5 ml crude extract into a mixture containing 20% trichloroacetic acid solution and 0.5% thiobarbituritic acid for MDA testing. The solution was boiled for 30 min and immediately cooled down and then centrifuged for 10 min. The supernatant were used for MDA measurement at 532 and 600 nm. The MDA content was calculated by correcting for compounds derived from MDA-TBA action and was expressed in nmol/g FW (fresh weight) as per following formula:

$$MDA = \frac{(D523-D600)*V}{1.55*10^{-1}*M}$$

Where, $1.55*10^{-1}$ is the extinction coefficient of MAD.

Chemical compounds analysis

Fresh tea leaves sample of 5 g was extracted with 100 ml of 80% acetone in water (v/v) until colorless by grinding with sand and a

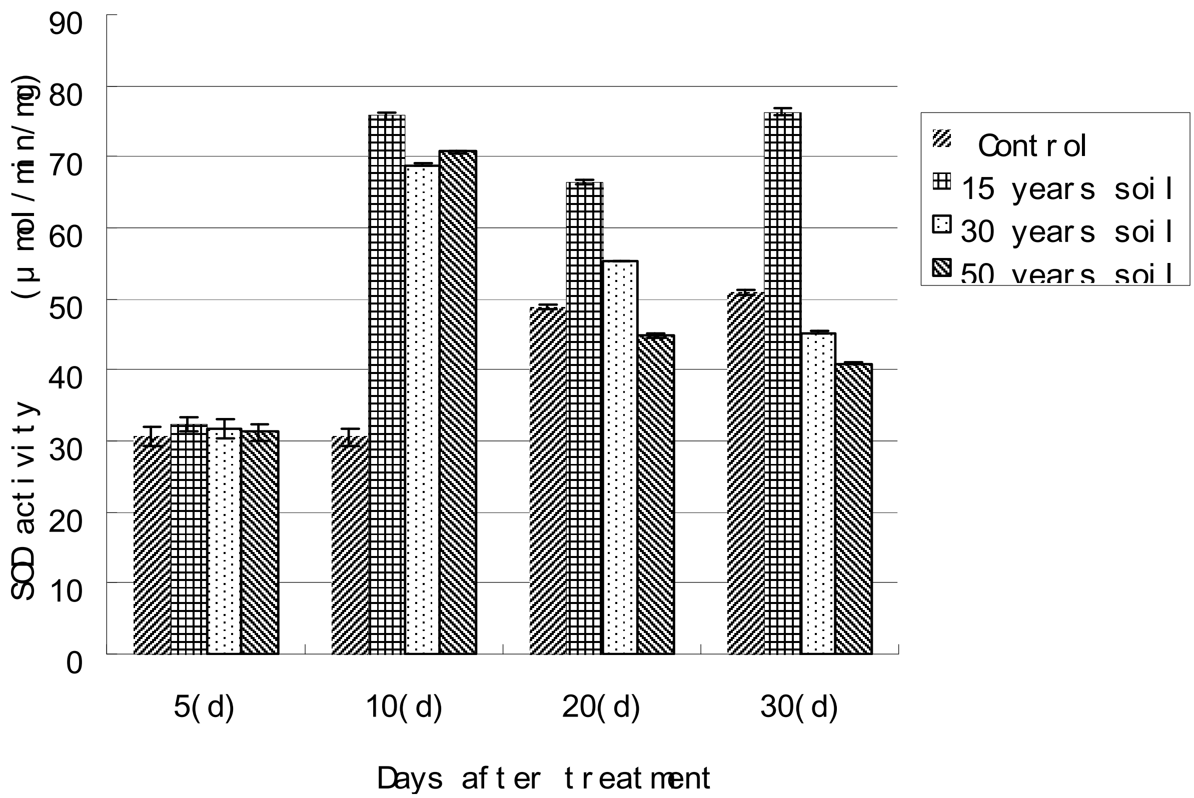

Figure 1. Effects of different soil extractions of tea plants on SOD activity in tea seedling.

small amount of $CaCO_3$ in a mortar using a pestle. For Chl, absorbance of extract after dilution was measured at 663 and 645 nm. For carotenoids, the absorbance was measured at 440 nm. The contents of Chl and carotenoids were calculated with following equations:

Chl-a (mg/g) = (12.7×D663-2.69×D645)/[(1- S)×W]

Chl-b(mg/g) = (2.27×D645-4.68×D663)/[(1- S)×W]

Carotenoids (mg/g) = (4.7×D440-(1.38×D663+5.48×D645))/[(1-S)×W]

Where, S (%) is the moisture content. Tea moisture was measured using avacuum oven based on an international standard method. Water extracts of tea leaf were determined by an international standard method. Briefly, a volume of 50 ml of tea extract prepared as described earlier was evaporated in dish on boiling water bath to rough dry and further dried in an oven at 103°C to complete dryness and then weighted after cooling down to room temperature in a silicagel desiccator.

RESULTS

Effects of soil extractions of different years of tea plants on SOD activity in tea seedling

The different soil extraction (15, 30 and 50 years) had both inhibitory and stimulatory effects on SOD activities. Positive effect was observed at low concentration (15 years), but with the increase of concentration (30 and 50 years), SOD activities increased on the 10th and 20th day and decreased finally, on the 30th day (Figure 1). The trend in SOD activity found in the present first increase and then decrease trend in 30 and 50 years treatments, in which activity peaks appeared at the 10th day. The results showed that stimulatory effects increased with time at low concentration, but the inhibition increased with the increasing of growing years.

Effects of soil extractions of different years of tea plants on POD activity in tea seedling

The different old years of tea garden soil extractions increased the POD activity over to control at 5, 10 and 20 days after treatment. The trend in POD activity showed fast increase and peak appeared on the 10th day for all treatments, then fast decrease after 30 days (Figure 2). The results showed that POD was more dependent on the time of their action than on concentration.

Effects of soil extractions of different years of tea plants on CAT activity in tea seedling

The different old years of tea garden soil extraction treatments had no significant effects on CAT activities on

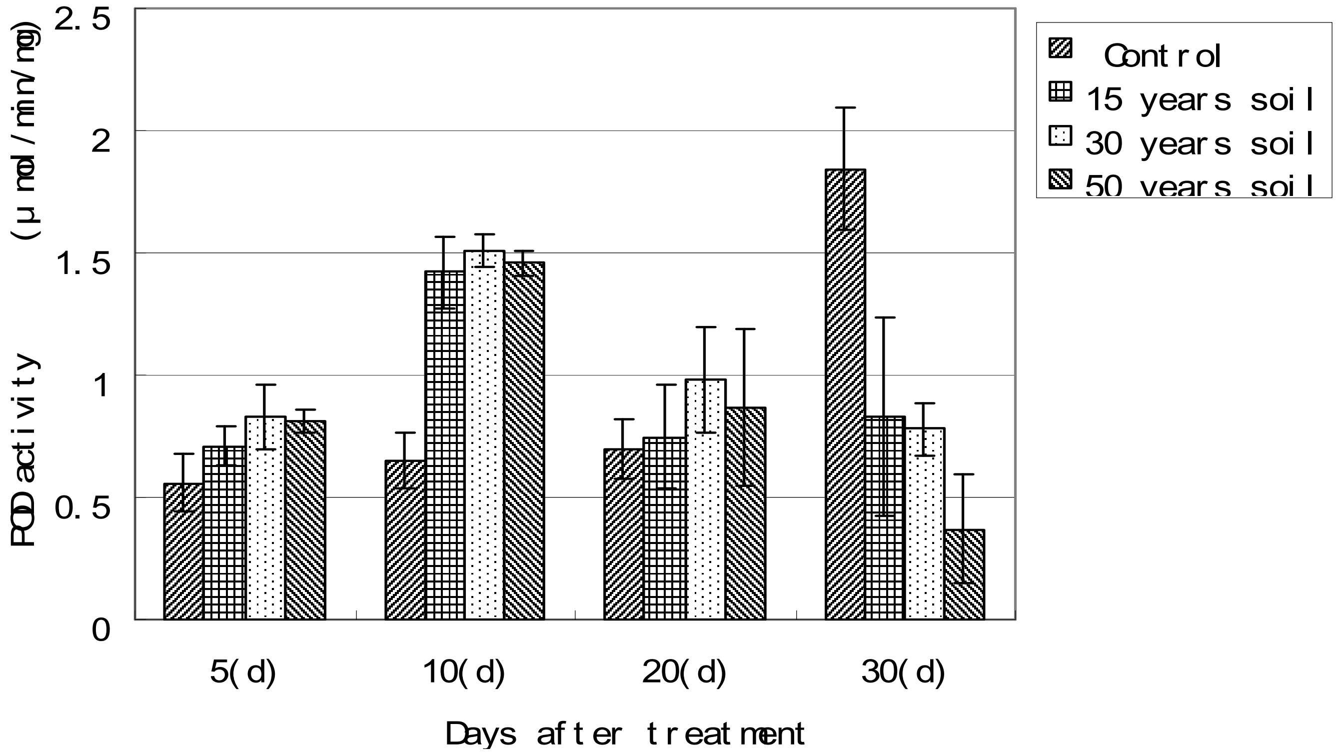

Figure 2. Effects of different soil extractions of tea plants on POD activity in tea seedling.

the 5th and 10th day after treatment (Figure 3). The activity of CAT increased after the 20th day in all treatments, but was lower than the control; this increasing trend of enzyme activity was very slight. The trend in CAT activity showed peak on the 20th day for all treatments, then decrease after 30 days. The CAT activity in all treatments was sharply decreased simultaneously over control, but its difference in all treatments was little.

Effects of soil extractions of different years of tea plants on MDA contents in tea seedling

The oxidative effects of autotoxins in the lipid degradation were indicated by MDA contents. The results showed that the autotoxins could increase the MDA contents. The MDA contents increased with the increase of growing years (Figure 4). Significant stimulatory effects of autotoxins on MDA contents were observed when compared with the control, in which case the MDA was accumulated with prolonged duration and increase in concentration.

Effects of different soil extractions on chemical components of tea leaves

The change in trend of chemical components of tea leaves were the same for all treatments and the contents of Chl-a, Chl-b, carotenoids and water extracts in fresh tea leaves were decreased (Table 1). Results indicated that the autotoxins could inhibit physiological synthetic processes of Chl-a, Chl-b, carotenoids and other chemical components of tea leaves, which suggested that autotoxins could impact tea quality; the results showed that photosynthesis of tea tree were inhibited by autotoxins, the inhibition increased with the increasing of growing years.

DISCUSSION

The plant growth and germination rate under allelechemicals stresses have been observed in tea seedlings in previous studies (Anaya et al., 2002; Cao et al., 1994). Autotoxins considerably increased the production of peroxides and active oxygen species (AOS) in allelochemicals treated root tips (Zuo et al., 2010; Yu et al., 1997) and forced the plant to over synthesize O_2 and H_2O_2 followed by the perturbations of antioxidant enzymes system. The same antioxidant enzymes and MDA content did not change over to the control on the 5th day. It indicated that the defensive system of the plant prevailed due to slight overloading, and free radicals were alleviated by scavengers due to their relative short endurance.

Furthermore (on the 10th day), the synthesis of free radicals predominated over the defensive quality of the system, while the antioxidant enzyme system was

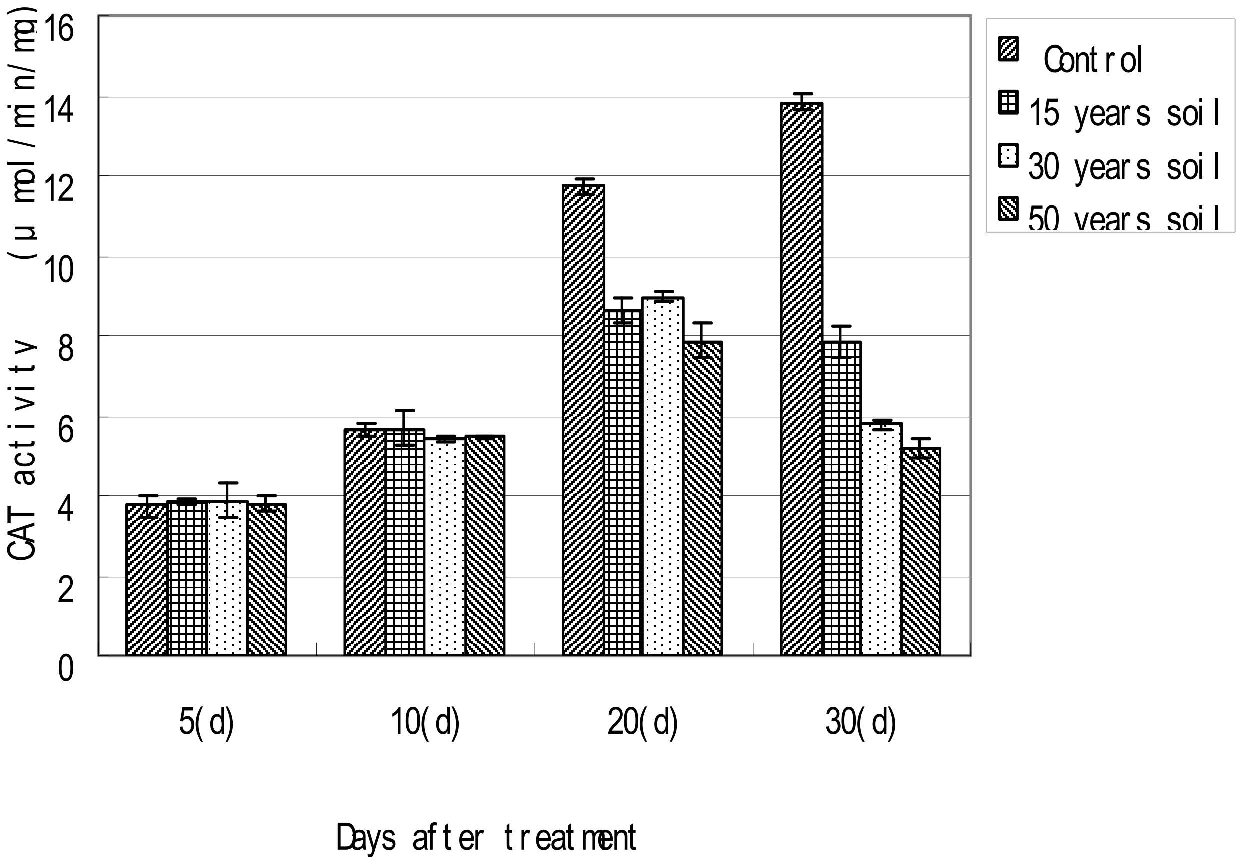

Figure 3. Effects of different soil extractions of tea plants on CAT activity in tea seedling.

activated and the activity increased. Similar findings have been reported (Wang et al., 2009). The SOD activity was stimulated due to decrease in the content of O_2; when the plant is exposed to autotoxins, a low concentration and the harmful damage is lessened (Prasad et al., 1999). The same results were also obtained with other allelochemicals (Hegab et al., 2008). POD activity greatly increased due to H_2O_2 accumulation. Therefore, the membrane lipid peroxidation MDA contents increased in all treatments. The MDA increases, when a plant is subjected to various stress conditions (Baziramakenga et al., 1995). By the end of the treatment (on the 30^{th} day), the allelochemicals caused deep oxidative stress in target tissues and degraded the antioxidant mechanisms (Yasar et al., 2010). The activity of POD, SOD and CAT in 30 and 50 years were drastically reduced during this period leading to more MDA. Our results indicated that autotoxins had both significant inhibitory and stimulatory effects on antioxidant activities. Positive effect was observed at low concentration and the inhibition trend at higher concentration. Plants release the allelochemicals as mixture rather than a single compound (Makoi and Ndakidemi, 2007). The effects of individual constituents are often different from mixtures, in which the synergic and antagonistic effects occur (Rizvi et al., 1999).

Large amount of polyphenols and coffeine forms in the leaves of tea plant, have been described as allelchemicals (Cao et al., 1994, 1997) and a large portion of these easily soluble and degradable compounds penetrated into the soil with rain, dew and fog and accumulate in the surface soil layer that may inhibit the seedling growth. The phenolic acids were one of the main types of autotoxin compounds in root exudation and accumulated in the surface soil layer in which the seedlings growth was inhibited (Baziramakenga et al., 1995; Yu et al., 1997). There was significant difference on activities of antioxidant enzymes in different soil extracts. The possible reasons are difference in autotoxin compounds and relative contents in different soil extracts, the autotoxins in the leaves of tea into the soil by rain, dew or fog and decomposition of tree residues and the stay time of various autotoxins in the soil were different, some easily soluble and degradable compounds were lost with time.

The decreased in contents of Chl-a and Chl-b indicated that tea tree photosynthesis was inhibited, then the physiological synthetic processes of chemical components of tea leaves were inhibited, thereby the contents of Chl-a, Chl-b, carotenoids and water extracts in fresh tea leaves decreased in all treatments, which suggested that autotoxins could impact tea yield and quality. The water extracts and carotenoids in fresh tea

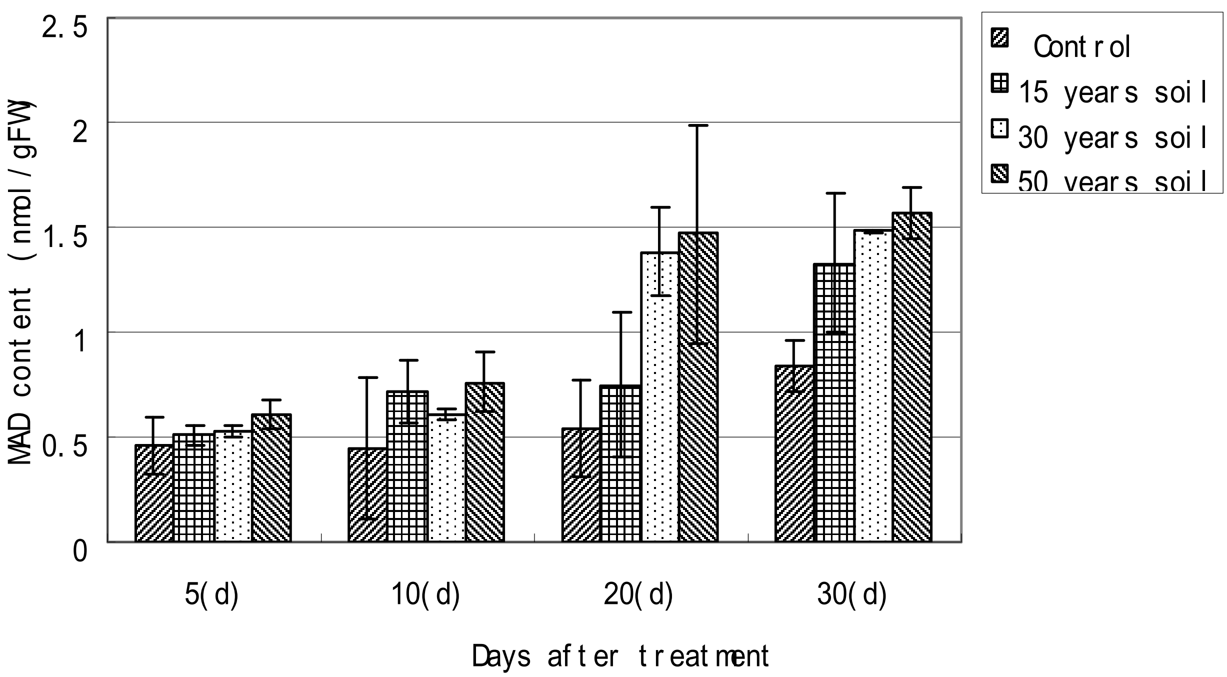

Figure 4. Effects of different soil extractions of tea plants on MAD content in tea seedling.

Table 1. Effects of soil extractions of different years of tea plants on chemical components of tea leaves.

Treatment (year soil)	Chl-a (mg/g)	Chl-b (mg/g)	Carotenoids (mg/g)	Water extract (%)
Control	1.16±3.47[a]	0.70±0.23[a]	0.45±1.15[a]	28.4±0.23[a]
15	1.11±3.08[b]	0.65±0.41[b]	0.43±0.45[b]	25.83±0.41[b]
30	1.03±1.73[c]	0.58±0.11[bc]	0.41±0.07[c]	23.78±0.11[bc]
50	0.81±1.09[cd]	0.47±0.22[c]	0.36±0.04[c]	20.37±0.22[d]

Numbers with different letters within a column refer to significant difference at the 0.05 level according to Duncan's multiple-range test. Control = without Camellia sinensis.

leaves were important constituents of green tea taste and colour quality. During tea processing, carotenoids degrade to form a number of volatile compounds (Liang et al., 2003), highlighting their contribution to aromatic quality of tea. The water extracts are important elicitor of the taste of bitterness and astringency in tea (Liang et al., 2003); there were significantly correlations between perceived taste scores and concentrations of water extract.

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