



Physiological characteristics of *Ficus tikoua* under antimony stress

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Abstract: A greenhouse culture experiment was used to evaluate the effects of antimony (Sb) stress on *Ficus tikoua* (*F. tikoua*). The results showed that the growth of *F. tikoua* leaves was significantly inhibited when Sb concentration was higher than 30 $\mu\text{mol/L}$, and no significantly inhibitory effect of Sb on the roots and stems of *F. tikoua* was found in all the treatments, implying that leaves were more sensitive to Sb toxicity than roots and stems. Antimony concentration in the roots was higher than that in the stems and leaves. To reduce reactive oxygen species (ROS) level in the *F. tikoua*, the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) increased with Sb treatments, but the SOD and CAT were more early active than POD. Although the decrease of chlorophyll content with high Sb treatments (450 $\mu\text{mol/L}$) was observed at the end of the experiments, the positive impact on chlorophyll content was observed with all the Sb treatments at the early period. No significant difference of the maximum quantum efficiency of PSII and quantum yield of PSII electron transport values with different Sb treatments was observed at the end of this experiment, suggesting that the photosynthesis was not inhibited with Sb concentration below 450 $\mu\text{mol/L}$. The results implied a certain tolerance to Sb stress for *F. tikoua*. This meets the essential condition for utilization in Sb contamination environments.

Key words: antimony; biomass; antioxidant enzyme activity; *F. tikoua*; chlorophyll fluorescence

1 Introduction

Antimony (Sb) is a toxic element with adverse effects on humans and the environment. An increase in Sb mining and smelting processes has resulted in the release of large quantities of Sb, causing serious Sb contamination in local environment [1,2]. Even at low concentration, Sb can cause irreparable damage to the environment [3]. Antimony is known to suppress germination and growth, inhibit photosynthesis, affect membrane structure and permeability, and damage the structure and function of photosystem II (PSII) in plants [4,5]. Research has indicated that the inhibition of photosynthesis is a primary damage mechanism in plants exposed to Sb even at a lower concentration [4,6]. Under Sb stress, the chlorophyll synthesis was inhibited and plastoquinone pool was diminished in plants, with a significant reduction in the total area between the fluorescence induction curves [7]. Also, Sb exposure resulted in more strong inhibition of electron transport both on PSII donor side and acceptor side than the

oxygen-evolving complex and light-harvesting pigment-protein complex II [8].

A typical symptom of Sb toxicity is oxidative damage. In addition, Sb can induce the production of reactive oxygen species (ROS), including superoxide radicals (O^{2-}), hydroxyl radicals ($\bullet\text{OH}$) and hydrogen peroxide (H_2O_2), which react very rapidly with DNA, lipids and proteins to cause cellular damage [9]. The excessive ROS reacts with lipids, proteins and pigments, which results in membrane damage and enzyme inactivation [9]. Antimony toxicity leads to the generation of ROS by inhibiting the electron transport chain in the chloroplast and mitochondria [9,10]. To counter ROS, plants produce antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT).

Ficus tikoua (*F. tikoua*), an endemic species in China belonging to the Moraceae family, has been recognized as a valuable restoration species and a medicinal source [11,12]. *Ficus tikoua* has been found wildly growing in the active and abandoned Sb mining areas. During the past decade, research focused on the

evaluation of biological activities of *F. tikoua* [13]. Few research studies have investigated the photosynthetic response of *F. tikoua* to metal/metalloid stress [14]. Moreover, little is known about the response of *F. tikoua* to Sb treatment. Thus, the objective of this study is to assess the Sb resistance capacity of *F. tikoua* in preparation for eventual application to the re-vegetation of Sb-polluted soils.

2 Experimental

2.1 Plant cultivation and Sb treatment

Cuttings of *F. tikoua* from an antimony mine area in Lengshuijiang city, Hunan province, China (111°29'E, 27°45'N) were grown on water-saturated perlite in a growth chamber in a greenhouse. The cuttings were illuminated with three 25 W fluorescent lamps; the photoperiod was set as 14 h/10 h and the light intensity was 150 $\mu\text{mol}/(\text{m}^2 \cdot \text{s})$ photosynthetically active radiation (PAR). Day/night temperatures of 25 °C/18 °C were applied, and the humidity was 60%–80%. Cuttings were supplied with half-strength Hoagland nutrient solution until all six or seven leaves were completely unfolded. Thereafter, four plants of *F. tikoua* were carefully transferred to plastic pots that were filled with perlites. Four Sb concentrations (0, 30, 150 and 450 $\mu\text{mol}/\text{L}$) were tested in this study. Antimony was supplied as $\text{C}_4\text{H}_4\text{KO}_7\text{Sb} \cdot 1/2\text{H}_2\text{O}$. Each pot was supplied with 50 mL of Sb-containing half-strength Hoagland solution at 5 d intervals. All conditions were the same as those described above.

2.2 Observation of biomass and antimony in plant tissue

After 78 d, the Sb-exposed plants were collected and then rinsed three times with deionized water. The roots, stems and leaves were separately collected, dried at 70 °C and weighted. The plant samples were ground and sieved through a 1 mm sieve and digested with $V(\text{HNO}_3):V(\text{HClO}_4)=4:1$. The Sb concentration in the digested solution was quantified by atomic fluorescence spectrometry (AFS) (Titan AFS–810).

2.3 Assay of antioxidant enzymatic activities and MDA content

Approximately 0.2 g of fresh tissue was homogenized in a pre-cooled mortar with 5 mL precooled sodium phosphate buffer (50 mmol/L, pH 7.8) solution. And then the supernatant was collected for detection of enzyme activity by centrifugation for 20 min at 11000g and 4 °C [10]. The supernatant was used to determine SOD, POD, CAT, MDA and soluble sugar concentration according to the following photo-chemical method.

1) SOD assay was performed according to the method of TANG et al [15], with some modifications. Briefly, 3 mL reaction mixture that contains 0.3 mL of 750 $\mu\text{mol}/\text{L}$ nitroblue tetrazolium (NBT), 0.3 mL of 20 $\mu\text{mol}/\text{L}$ riboflavin, 0.3 mL of 130 mmol/L methionine, 0.3 mL of 100 $\mu\text{mol}/\text{L}$ EDTA- Na_2 , 1.5 mL of sodium phosphate buffer (50 mmol/L pH 7.8), 0.2 mL of deionized water and 0.1 mL of enzyme extract was placed under light with an average photon flux density of 78 $\mu\text{mol}/(\text{s} \cdot \text{m}^2)$ for 20 min at 25 °C, and the absorbance of the reaction mixture was recorded at 560 nm. Reaction solution placed in the dark was used as the control. One unit of enzyme activity was defined as the amount of the enzyme that resulted in 50% inhibition of the rate of NBT reduction.

2) POD activity was determined by measuring the absorbance changes at 470 nm and 25 °C [10]. 0.1 mL of enzyme extract was added into a mixture solution of 1 mL 0.3% H_2O_2 , 0.9 mL 0.2% guaiacol, 1 mL sodium phosphate buffer (50 mmol/L, pH 7.0) to start enzymatic reaction. One unit of POD enzyme activity was defined as the amount of the enzyme that caused an increase in absorbance at 470 nm of 0.1 per minute.

3) CAT activity was measured by monitoring the decrease of H_2O_2 at 240 nm for 1 min at 25 °C [16]. 0.1 mL enzyme extract was added in a mixture solution containing 1 mL 0.3% H_2O_2 and 1.9 mL sodium phosphate buffer (50 mmol/L, pH 7.0) to initiate the reaction. One unit of CAT activity was calculated as the amount of enzyme that caused a reduction in absorbance at 240 nm of 0.1 per minute.

4) The Sb-induced oxidative damage (membrane liquid peroxidation) was estimated by measuring the MDA concentrations [17]. The MDA content was assayed using a solution containing 3 mL of 20% (w/V) trichloroacetic acid, which included 0.5% (w/V) thiobarbituric acid and 1 mL enzyme extract. The solution was kept in boiling water bath for 20 min and then quickly cooled. After refrigeration, the homogenate was centrifuged at 5000g and 25 °C for 10 min. The absorbance of the supernatant was recorded at 450, 532 and 600 nm, respectively. The content of MDA (C_1) ($\mu\text{mol}/\text{L}$) was calculated from the following formula: $C_1=[6.45(A_{532}-A_{600})-0.56A_{450}]$, where A_{450} , A_{532} , A_{600} represent the absorbance values at 532, 600 and 450 nm, respectively. The content of soluble sugar (C_2) (mmol/L) was calculated by the following formula [18]: $C_2=11.71 \times A_{450}$.

2.4 Chlorophyll estimation and chlorophyll a fluorescence measurement

To determine chlorophyll and carotenoid contents in leaves, the homogenate of fresh leaves (0.2 g) was extracted for 24 h in 25 mL of 80% acetone in darkness.

The contents of chlorophyll a, chlorophyll b and carotenoids in the extracts were measured at 646, 663 and 470 nm with a spectrophotometer (UV-4100, Hitachi), respectively [19].

Chl fluorescence of the fully expanded, fourth leaf from top was measured. All fluorescence measurements were carried out in darkness for 40 min to obtain equilibrium of the photosystem II (PSII) redox state. The induction curve (slow kinetics) for PSII was measured using the MINI-version of the Imaging-PAM fluorometer (Walz, Effeltrich, Germany). For fluorescence measurements, the actinic light was set at $135 \mu\text{mol}/(\text{m}^2 \cdot \text{s})$ PAR. The irradiance step was 20 s, and the length of the program was 5 min. The maximum quantum efficiency of PSII (F_v/F_m) and quantum yield of PSII electron transport ($Y(\text{II})$) were calculated [20].

2.5 Statistics

Analysis of variance (ANOVA) was performed using SPSS statistical software. A p -value of <0.05 denoted significance. The Tukey test and subsequent pairwise comparisons were employed to compare significant differences ($p<0.05$) between the means for the treatments. All data presented are mean values of three replicate experiments.

3 Result and discussion

3.1 Growth of plants

The effects of Sb on the growth of *F. tikoua* were evaluated by measuring the dry mass of root, stem and leaf (Fig. 1). The dry mass of the *F. tikoua* leaves treated with Sb concentration higher than $30 \mu\text{mol/L}$ was significantly ($p<0.05$) lower than that of the untreated controls (without Sb application). But the dry mass of stem and root at all Sb concentrations was not statistically different. When heavy metal transported from matrix to roots and further moved to different parts in the shoots, the toxicity occurred, affecting various plant processes [4]. Plant organs have different sensitivities to Sb exposure because of their different biomass allocation patterns when plants were exposed to Sb stress [21]. In this study, the leaf of *F. tikoua* was more sensitive than root and stem under the Sb treatments.

3.2 Sb concentrations in *F. tikoua*

F. tikoua showed the ability to limit Sb transfer from the underground parts to the aboveground parts since Sb concentrations in the roots of *F. tikoua* were significantly ($p<0.05$) higher than that in stems and leaves for all Sb treatments (except for control) (Table 1). Sb concentration in the plants increased with increasing Sb concentration in solution. The maximum Sb

concentrations in the root, stem and leaf were 690.81, 350.12 and 71.41 mg/kg respectively when the plants were exposed to $450 \mu\text{mol/L}$ Sb. According to the research of FENG et al [22] and TSCHAN et al [23], Sb accumulation in different species varies greatly [22,23]. In the present study, Sb concentration in the roots of *F. tikoua* was one order of magnitude higher than those in the stems and leaves, suggesting that the *F. tikoua* would be a plant species to tolerate Sb.

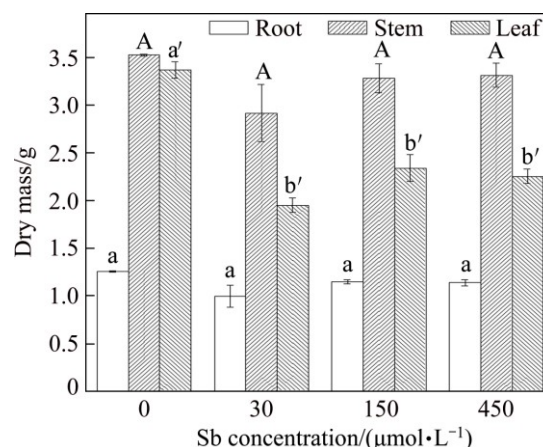


Table 1 Sb content in different parts of *F. tikoua*

Sb concentration/ ($\mu\text{mol} \cdot \text{L}^{-1}$)	w(Root)/ ($\text{mg} \cdot \text{kg}^{-1}$)	w(Stem)/ ($\text{mg} \cdot \text{kg}^{-1}$)	w(Leaf)/ ($\text{mg} \cdot \text{kg}^{-1}$)
0	$0.42 \pm 0.02^{\text{AB}}$	$0.50 \pm 0.06^{\text{A}}$	$0.25 \pm 0.03^{\text{B}}$
30	$134.77 \pm 1.11^{\text{A}}$	$12.23 \pm 0.70^{\text{B}}$	$2.09 \pm 0.32^{\text{B}}$
150	$459.44 \pm 23.95^{\text{A}}$	$94.19 \pm 3.93^{\text{B}}$	$23.44 \pm 1.79^{\text{B}}$
450	$690.81 \pm 22.99^{\text{A}}$	$350.12 \pm 28.79^{\text{B}}$	$71.41 \pm 0.68^{\text{C}}$

Note: Values followed by same uppercase letters are not significantly different at $p=0.05$, for same Sb treatments; values followed by same lowercase letters are not significantly different at $p=0.05$, for different Sb treatments.

3.3 Effect of Sb on antioxidant enzyme activity

The activities of POD, SOD, and CAT responded to Sb stress with different concentrations in the different exposure periods are shown in Fig. 2. The SOD activity increased with increasing Sb concentration in matrix (Fig. 2(a)). The SOD activity in all Sb treatments is higher than that in control treatment. Especially, $450 \mu\text{mol/L}$ Sb treatment significantly resulted in increase in SOD activity during the whole experiment period as compared with control treatment. The CAT activity increased with increasing Sb concentration in matrix (Fig. 2(b)) and CAT activity in *F. tikoua* was improved after exposure to $150 \mu\text{mol/L}$ Sb for 7 d. All Sb treatments resulted in higher CAT activity than the

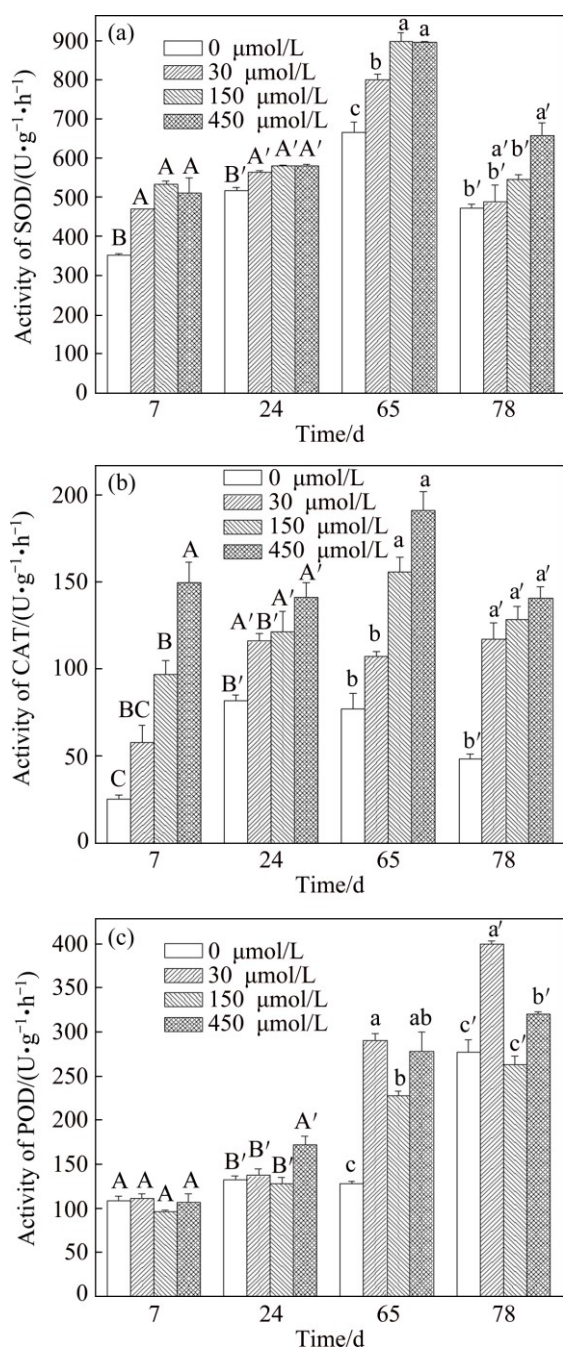


Fig. 2 Activities of antioxidant enzymes in *F. tikoua* under Sb treatments: (a) SOD; (b) CAT; (c) POD

control. Especially, the significant increase of CAT activity during the all exposure period was observed with 150 and 450 $\mu\text{mol/L}$ Sb treatments. However, POD activity in *F. tikoua* has different change trends in the different exposure periods (Fig. 2(c)). In the early exposure period (24 d), POD activity significantly increased only with 450 $\mu\text{mol/L}$ Sb treatment. With prolonging exposure time till 65–78 d, POD activity significantly increased with low Sb concentration treatment, such as 30 $\mu\text{mol/L}$ Sb. Heavy metals exert toxic effects through the production of ROS, which have a variety of harmful effects in plant cells including lipid

peroxidation [24]. To cope with ROS or alleviate their damaging effect, plants have evolved enzymatic and nonenzymatic antioxidant mechanisms. Generally, improvement of stress tolerance is related to an increase in the activity of antioxidant enzymes, and plants may experience oxidative damage due to the inability of the antioxidative enzymes to tolerate severe stress [25,26]. In the present study, to avoid oxidative damage, the complex antioxidant defense systems of plant regulated the contents or activities of antioxidants (SOD, POD and CAT) in response to Sb stress. The results indicate that Sb could significantly change the enzyme activity of *F. tikoua* even the concentration is as low as 30 $\mu\text{mol/L}$. Antimony could either inhibit or stimulate the activity of several antioxidant enzymes before any visible symptoms of toxicity appear [9,10]. A more efficient antioxidative system likely resulted in lower oxidative stress and reduced membrane damage.

3.4 Lipid peroxidation and soluble sugar content

Figure 3(a) shows MDA content of *F. tikoua* in all Sb treatments. In the early exposure period (7 and 24 d) the higher Sb concentration showed higher MDA content of *F. tikoua* than the lower Sb concentration. The highest MDA content was observed with 450 $\mu\text{mol/L}$ Sb treatment at 24 d. At the end of the experiment (65 and 78 d), there was no significant difference of MDA content with 150–450 $\mu\text{mol/L}$ Sb treatments and control treatment, while the highest MDA content was observed with the 30 $\mu\text{mol/L}$ Sb treatment. Usually, the high level of MDA was expected because the metalloids contents were very high [25]. These suggested that *F. tikoua* could alleviate lipid peroxidation under different Sb levels over time. Besides, the low Sb concentration (30 $\mu\text{mol/L}$ Sb) stress on *F. tikoua* worked later than the high one (Fig. 3). The soluble sugar was considered as the important matter to adapt the heavy metal stress [27]. In this work, soluble sugar content, except for 30 and 150 $\mu\text{mol/L}$ Sb treatments at 24 d, significantly increased with increasing Sb concentration in matrix (Fig. 3(b)). The results suggest that the increase soluble sugar in the *F. tikoua* adapted to Sb stress was very important. These findings are in agreement with previous results reported for other heavy metals [28].

3.5 Effect of Sb on chlorophyll fluorescence

A positive effect of Sb on Chl a in *F. tikoua* leaves was observed at the early exposure period (7 and 24 d), and even the significant increase in Chl a content was observed with 150 and 450 $\mu\text{mol/L}$ Sb treatments as compared with the control treatment (Fig. 4(a)). However, at the end of the experiment (65 and 78 d), Chl a content was recovered, and Chl a content was not significantly different among most Sb treatments except

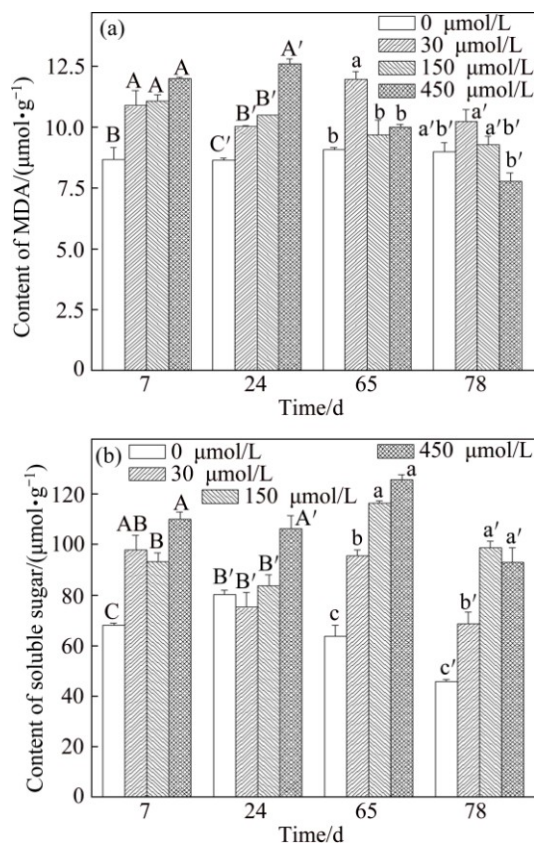


Fig. 3 Contents of MDA and soluble sugar in *F. tikoua* with Sb treatments

for 450 $\mu\text{mol/L}$ at 78 d. The positive effect of Sb on Chl b content was observed at 7 d when Sb concentration was in the range of 30–150 $\mu\text{mol/L}$ (Fig. 4(b)). But there was no significant difference of Chl b content among the most Sb treatments at 24 and 65 d, except for 30 $\mu\text{mol/L}$ Sb at 24 d. At the end of the experiment (78 d), Sb stress at 450 $\mu\text{mol/L}$ Sb treatment significantly decreased Chl b content as compared with control treatment. In the early exposure period (7 and 24 d), carotenoid of *F. tikoua* significantly increased in the 150 and 450 $\mu\text{mol/L}$ Sb treatments as compared with control treatment (Fig. 4(c)). At 65 d, no significant difference of carotenoid content was observed among control, 30, 150 and 450 $\mu\text{mol/L}$ Sb treatments. However, 450 $\mu\text{mol/L}$ Sb treatment slightly decreased carotenoid content of *F. tikoua* at 78 d. Chlorophyll content is a measure of the ability of a plant to convert photosynthetic energy into biomass, or a measurement of the efficiency of a plant to produce biomass. Indeed, Chl a, Chl b, and carotenoid contents are the indication of plant as affected by stress [29]. Reduced chlorophyll content and disturbed chlorophyll synthesis were suggested to be the primary causes of diminished photosynthetic activity. Also, it was reported that the beneficial effects on plant growth in the low dose of heavy metal presence [9,30]. In this work, Chl a, Chl b and carotenoid content varied at the

different Sb exposure time. For the early exposure period, Sb has a positive effect on the Chl a, Chl b and carotenoid contents, but the positive effect was not obviously improved over all the period. At the end of experiment, a significant decrease in contents of Chl a and Chl b was observed at 450 $\mu\text{mol/L}$ Sb treatment. The results suggest that the change of Chl content in the *F. tikoua* under Sb treatment depends on the Sb concentration and the exposure time.

When *F. tikoua* was exposed to Sb treatments, the change of functional photosynthetic state was investigated by using the PAM-methodological approach and the results are shown in Fig. 5. In this study, only a considerable decline of F_v/F_m was observed with 150 and 450 $\mu\text{mol/L}$ Sb treatments at 24 d, implying that *F. tikoua*

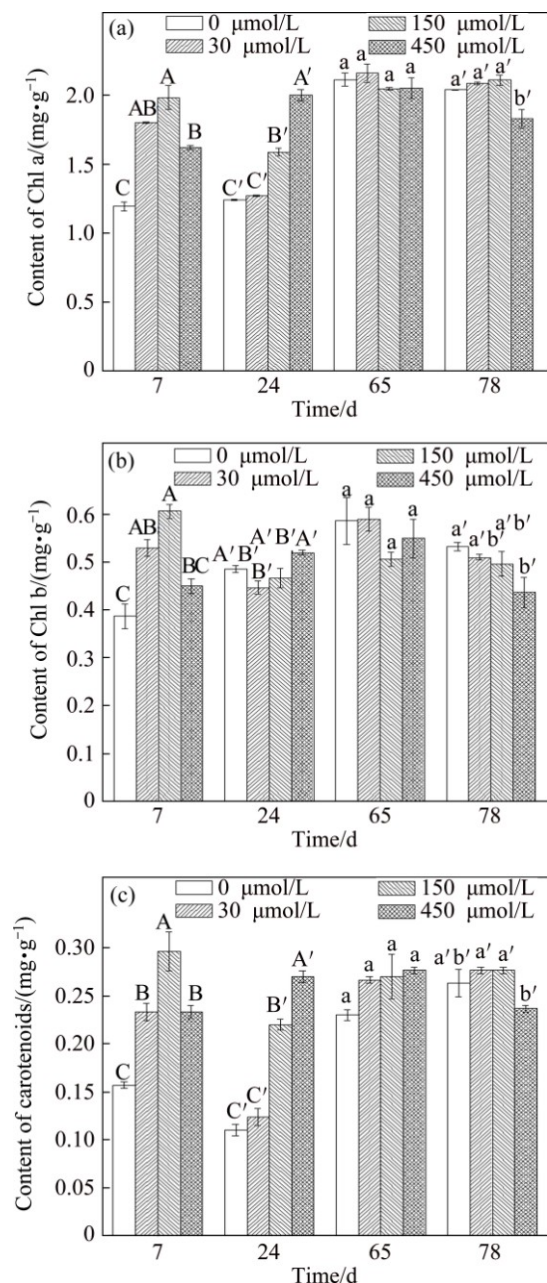


Fig. 4 Chlorophyll content of *F. tikoua* under Sb treatments

was adapted to Sb stress on the reaction center of photosystems II (Fig. 5(a)). $Y(II)$ provides a more realistic impression of the overall leaf photosynthetic condition when the plant is under Sb stress. In the early exposure period (7 d), $Y(II)$ value of *F. tikoua* leaves in 450 $\mu\text{mol/L}$ Sb treatment was higher than that with control treatment. At 24 d, a considerable increase in $Y(II)$ was observed in 30, 150 and 450 $\mu\text{mol/L}$ Sb as compared with control treatment, indicating that photosynthesis was active under Sb stress at early exposure period (Fig. 5(b)). With prolonging exposure time to 65 and 78 d, the effects of Sb stress on $Y(II)$ were not observed because there was no significant difference of $Y(II)$ at all treatments, indicating that photosynthesis of *F. tikoua* was retrieved.

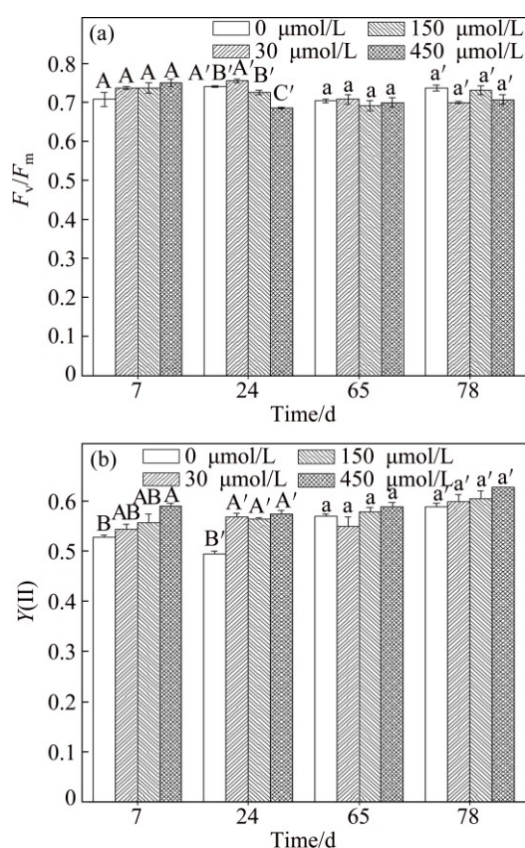


Fig. 5 Effects of Sb on fluorescence quenching parameters

Changes in PSII efficiency due to heavy metal excess were observed in different plant species and were dependent upon the time of exposure to metals and their contents in leaf tissue [30,31]. Generally, F_v/F_m is the most frequently applied Chl fluorescence ratio because it is easy and fast to determine. When plants are exposed to metal/metalloid stresses, decline in F_v/F_m indicates a disturbance or damage to the photosynthetic apparatus [30]. The present results showed that F_v/F_m and $Y(II)$ were active under Sb stress in the early exposure period (7 d). However, there was no significant difference of F_v/F_m and $Y(II)$ at all Sb treatments at the end of this

experiment (65 and 78 d), indicating that the efficiency of the photosynthetic apparatus and the size and number of active photosynthetic centers were not inhibited or decreased in Sb concentration range of 30–450 $\mu\text{mol/L}$.

4 Conclusions

1) *F. tikoua* showed a certain ability to resist and tolerate toward Sb stress. The highest Sb concentration in *F. tikoua* tissues was found in root, followed by stem and leaf.

2) The leaf of *F. tikoua* was more sensitive than root and stem under Sb stress. The biomass of leaf significantly decreased in Sb treatment, but effects of Sb treatment on the biomass of root and stem were not significantly different.

3) Under Sb stress, a negative effect of ROS in *F. tikoua* was eliminated and alleviated by improving SOD, CAT and POD activities and increasing soluble sugar content.

4) The photosynthesis of *F. tikoua* was not inhibited by Sb concentration below 450 $\mu\text{mol/L}$. In the early exposure period, the photosynthesis of reaction centers of photosystems II was active under Sb stress. However, the photosynthesis of *F. tikoua* was retrieved in the prolonging exposure time.

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锑胁迫下地枇杷的生理变化特性

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摘 要: 采用温室培养试验研究锑对地枇杷生理变化的影响。结果表明, 当锑浓度高于 30 μmol/L 时, 地枇杷叶片的生长明显受到抑制, 而地枇杷的根和茎在所有的锑浓度下都没有明显的变化, 表明地枇杷的叶片比根和茎对锑的毒性更敏感, 且在根中锑浓度要高于茎和叶中的。地枇杷通过增加超氧化物歧化酶(SOD)、过氧化物酶(POD)和过氧化氢酶(CAT)活性来减少锑胁迫产生的活性氧(ROS)水平, 但在锑胁迫早期, SOD 和 CAT 比 POD 的作用更明显。在锑胁迫早期, 不同浓度的锑能够在一定程度上增加地枇杷叶绿素的含量, 但在锑胁迫后期(实验结束时), 叶绿素含量在高浓度锑(450 μmol/L)胁迫下显著降低。在整个培养阶段中, 地枇杷最大光量子产量和实际光量子产量值没有显著差异, 表明地枇杷在锑浓度为 450 μmol/L 以下时, 光合作用未受明显抑制。地枇杷具有一定的锑耐受能力, 可用于锑污染土壤修复。

关键词: 锑; 生物量; 抗氧化酶活性; 地枇杷; 叶绿素荧光

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