Effect of Excess Iron and Copper on Physiology of Aquatic Plant *Spirodela polyrrhiza* (L.) Schleid

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Received 7 December 2008; revised 15 January 2009; accepted 17 January 2009

ABSTRACT: To elucidate effect of chemical reagents addition on growth of aquatic plants in restoration of aquatic ecosystem, *Spirodela polyrrhiza* (L.) Schleid was used to evaluate its physiological responses to excess iron (Fe^{3+}) and copper (Cu^{2+}) in the study. Results showed that accumulation of iron and copper both reached maximum at 100 mg L^{-1} iron or copper after 24 h short-term stress, but excess iron and copper caused plants necrosis or death and colonies disintegration as well as roots abscission at excess metal concentrations except for 1 mg L^{-1} iron. Significant differences in chlorophyll fluorescence (Fv/Fm) were observed at 1–100 mg L^{-1} iron or copper. The synthesis of chlorophyll and protein as well as carbohydrate and the uptake of phosphate and nitrogen were inhibited seriously by excess iron and copper. Proline content decreased with increasing iron or copper concentration, however, MDA content increased with increasing iron or copper concentration. © 2010 Wiley Periodicals, Inc. Environ Toxicol 25: 103–112, 2010.

Keywords: iron; copper; physiological response; aquatic plant; chemical reagent

INTRODUCTION

Eutrophication is a response in water due to over-enrichment by nutrients (Kumar, 2002; Liu et al., 2006). Cyanobacterial blooms are one of the common consequences of the increasing eutrophication of surface waters. To improve the water quality and to control the formation of cyanobacterial blooms, a lot of physical, chemical and biological measures have been used in the restoration of aquatic ecosystem. However, experimental studies on effects of these measures, especially chemical reagents addition, on growth of aquatic plants are very lacking (Hilt et al., 2006). As is well known, iron and copper are often applied to kill bloom-forming cyanobacteria (Hullebusch et al., 2002) and improve water quality (Haughey et al., 2000), however, this may cause secondary pollution of water environment.

Heavy metal pollution is one of the important topics in plant stress physiology, because of toxicity of metals to plants and potential risk for human health when accumulated in food plants (Kováčik et al., 2008). Though iron and copper are essential trace elements for plants, being involved in many processes of metabolisms (Brown, 1978; Geider and Laroche, 1994; Marschner and Romheld, 1994), excess concentration can result in toxicity, especially in altering chromatin structure, synthesis of chlorophyll and protein, enzyme activity, photosynthesis and respiration, water content and plant biomass yield (Guerinot and Yi, 1994; Mori, 1999; Olaleye et al., 2001; Connolly and Guerinot, 2002; Burzynski and Klobus, 2004).

Only macrophyte-dominated water environment can maintain long-term clear-water state (Søndergaard et al.,

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Contract grant sponsor: National Key Technology R&D program.

Contract grant number: 2006BAC10B02.

Contract grant sponsor: Knowledge Innovation Project of Wuhan Botanical Garden, the Chinese Academy of Sciences.

Contract grant number: 0754531L02.

2007). Therefore, large-scale re-establishment of aquatic plants is of pivotal measure in restoration of aquatic ecosystem. Excess addition of chemical reagents for bloom-forming cyanobacteria killing could aggravate the growth of aquatic plants.

S. polyrrhiza (L.) Schleid is a suitable plant model for toxicity evaluation because of its small size, rapid growth, and ease of culture (Razinger et al., 2007). The main objective of the study is to evaluate the physiological responses of *S. polyrrhiza* (L.) Schleid to excess iron and copper.

MATERIALS AND METHODS

Plant Material and Treatment Conditions

Duckweed plants of *S. polyrrhiza* (L.) Schleid were obtained from an uncontaminated pond at Wuhan Botanical Garden, the Chinese Academy of Sciences, China. The plantlets were rinsed with 0.01 M NaOCl for 30 s to prevent algal growth. Before metal treatment, plants were acclimatized for 5 days in laboratory conditions (160 μmol $m^{-2}s^{-1}$ light with 16 h photoperiod at 25 $\pm$ 1°C) in 10% Hoagland's solution (Hoagland and Arnon, 1950; Srivastava et al., 2006). At the end of this period, uniform and healthy plants were collected and put into 10% Hoagland's solution containing 1, 10, and 100 mg L^{-1} Fe^{3+} or Cu^{2+} (added in the form of $FeCl_3 \cdot 6H_2O$ and $CuSO_4 \cdot 5H_2O$). After 24 h exposure, plants were harvested, washed with double distilled water, blotted and used for the study of various parameters. All chemicals were purchased from Sigma, unless stated otherwise.

Metal Tissue Content

After 24 h exposure to iron and copper, harvested plants were washed thoroughly with demineralized water, blotted and oven dried at 80°C for 3 days. The resulting material was placed in a desiccator and cooled to room temperature before being weighed. A subsample of known weight of dried material was then acid digested in 5 mL of 30% HNO_3 at 90°C for a minimum of 8 h. The concentration of iron and copper in the diluted digests was determined by atomic absorption spectrophotometer (AA-6800, Shimadzu Corporation, Japan) (Batty and Younger, 2003).

Photosynthetic Pigment Assay

Photosynthetic pigments were extracted in 95% ethanol in dark for 24 h. Afterwards the sample was centrifuged for 10 min at 8000 $\times$ *g*. The supernatant was collected and read at 665, 649, and 470 nm. The contents of chlorophylls and carotenoid were calculated using the equations given by Lichtenthaler and Buschmann (2001).

Chlorophyll Fluorescence Measurements

Leaf chlorophyll fluorescence was measured using a Plant Efficiency Analyzer (PEA, Hansatech Instruments Ltd., UK), by which the maximum photochemical efficiency of PSII (Fv/Fm) were obtained. Before measurements, the samples were dark-adapted for 30 min.

Soluble Protein and Carbohydrate Contents in Tissue

Protein content was estimated following the method of Bradford (1976) using serum albumin as standard protein. Carbohydrate content was determined using the phenol-sulfuric acid method (Kochert, 1978).

Phosphate-Phosphorus and Nitrate-Nitrogen Contents in Tissue

Plant material was homogenized with 5 mL of Milli-Q water (18.2 MΩ·cm, Millipore Corp., France). The homogenate was put into tube with screw cap and placed in boiling water bath for 30 min. Then the tubes were taken out and cooled to room temperature. The subsample was centrifuged at 800 $\times$ *g* for 10 min. Then the supernatant was collected and diluted to volume for determination of phosphate-phosphorus and nitrate-nitrogen in tissue (Li, 2000). Phosphate-phosphorus was measured using the ascorbic acid method (American Public Health Association, 1995). Nitrate-nitrogen was determined using the salicylic acid-sulfuric acid method (Li, 2000).

Proline and Lipid Peroxidation

Proline content was measured according to the method described by Bates et al. (1973). Plant material was homogenized with 5 mL of 3% (v/v) sulfosalicylic acid. The homogenate was centrifuged at 800 $\times$ *g* for 15 min. Free proline present in the supernatant was treated with acid-ninhydrin at 80°C for 1 h. The reaction was terminated in an ice bath and the colored complex was extracted in toluene. Its absorbance was recorded at 520 nm. The standard curve for proline was prepared by dissolving proline in 3% (v/v) sulfosalicylic acid covering the concentration range 0.1–5.0 μg mL^{-1}.

Lipid peroxidation was determined by estimation of the malondialdehyde (MDA) content. Plant material was homogenized with 3 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid (w/v). The homogenate was incubated at 100°C for 30 min and reaction was stopped in ice or cooled water. The samples were centrifuged at 10,000 $\times$ *g* for 10 min and absorbance was recorded at 450, 532, and 600 nm. The MDA concentration was determined by the following formula: C_{MDA} (μmol L^{-1}) = $6.45(A_{532}–A_{600})–0.56A_{450}$, from which the absolute concentration (μmol g^{-1} FW) of MDA was calculated (Li, 2000).

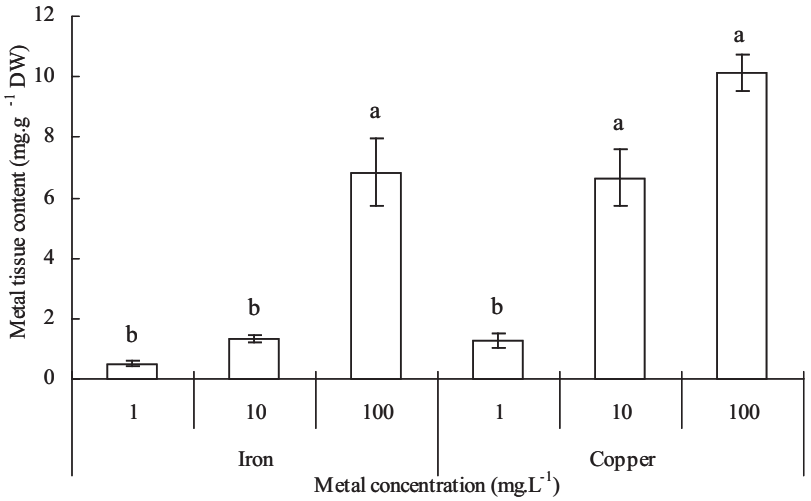

Fig. 1. Bioaccumulation of iron and copper in *S. polyrrhiza* (L.) Schleid after 24 h of treatment. All the values are mean of triplicates ± SD. One-way ANOVA followed by Duncan's multiple range test was used to determine the significant difference between treatments ($P < 0.05$).

Statistical Analysis

Data presented are the mean of triplicates ± SD. One-way ANOVA followed by Duncan's multiple range test was performed to determined the significant difference between treatments. Significance was set for $P < 0.05$.

RESULTS

Metal Tissue Accumulation and Effect on Growth

Plants accumulated high amount of iron and copper in a concentration dependent manner. Accumulation of iron and copper both reached maximum at 100 mg L^{-1} iron or copper after 24 h short-term stress. The maximal values were 6.84 ± 1.09 and 10.14 ± 0.61 mg g^{-1} DW, respectively (Fig. 1).

Table I shows the visual appearance of *S. polyrrhiza* (L.) Schleid and culture medium after 24 h excess iron and copper stress treatments. 10 mg L^{-1} iron as well as 1 and 10 mg L^{-1} copper induced plants to release daughter fronds from the mother frond, resulting in colony disintegration. Furthermore, root abscission occurred in 1 and 10 mg L^{-1} copper treatment groups. Precipitation only occurred in 10 mg L^{-1} iron treatment group, while other culture media were all clear (Table I).

Effect on Photosynthetic Pigment

Photosynthetic pigments, including Chl *a*, Chl *b*, total Chl and carotenoid, exhibited similar response upon short-term

TABLE I. Visual appearance of *S. polyrrhiza* (L.) Schleid and culture medium after 24 h excess iron and copper treatments

Metal Concentration (mg L^{-1})		Symptoms	Culture Medium
Iron (Fe^{3+})	1	No symptoms, fronds were green	Colorless and clear
	10	Colony disintegration, chlorosis occurred, purple in back of fronds was bleached	Slight orange originally; after 24 h treatment, a small amount of precipitation, colorless and clear
	100	Fronds were dark brown and necrotic or dead, but colony integration, fronds and roots weren't separated	Orange but clear, no precipitation
Copper (Cu^{2+})	1	Colony disintegration, purple in back of fronds still existed, root abscission occurred	Colorless and clear
	10	Colony disintegration, chlorosis occurred, purple in back of fronds was bleached, root abscission occurred	Colorless and clear
	100	Leaves were dark brown and necrotic or dead, but colony integration, fronds and roots weren't separated	Colorless and clear

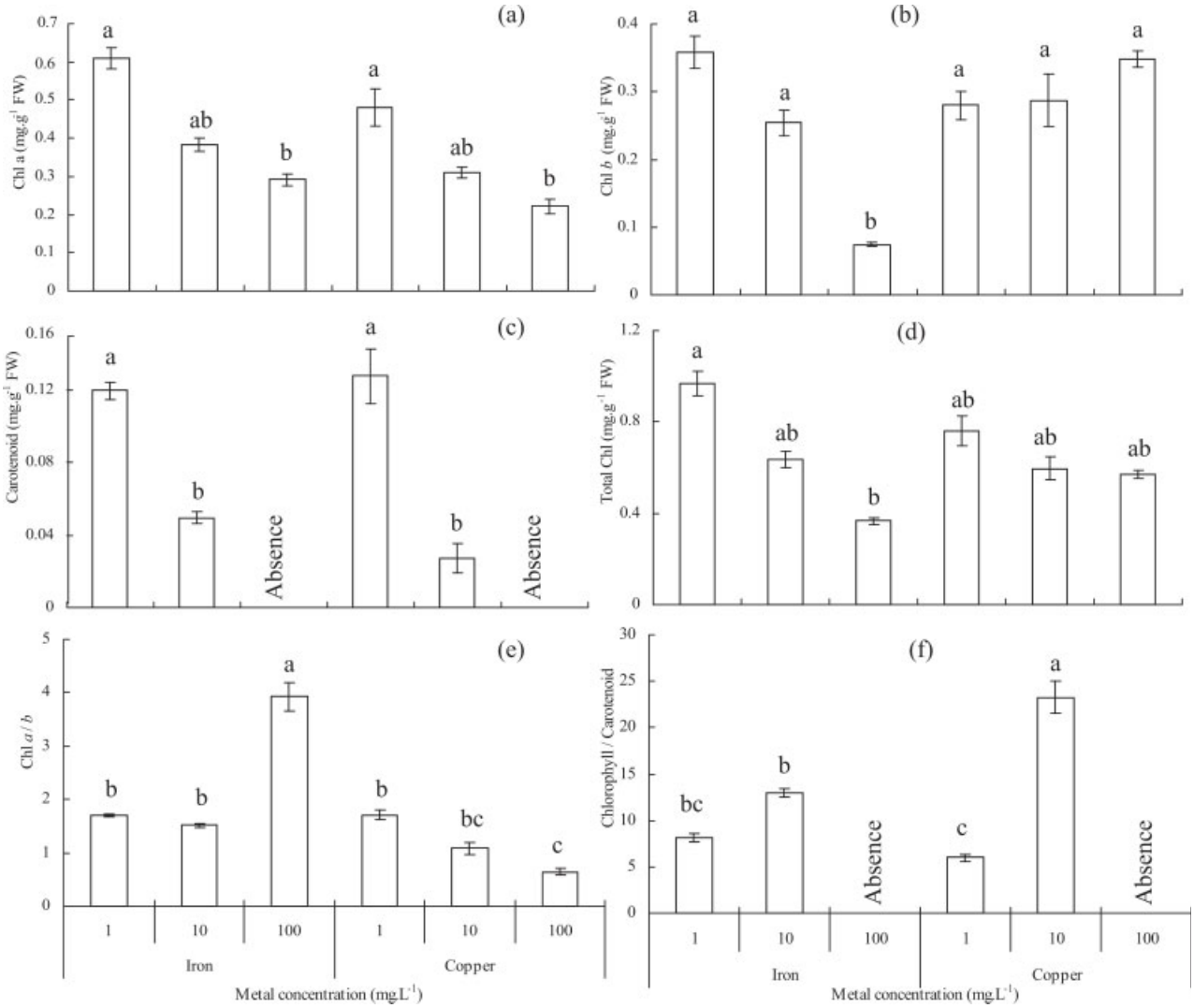

Fig. 2. Effect of excess iron and copper on Chl *a* (a), Chl *b* (b), Car. (c) and total Chl (d) contents, and Chl *a*/*b* (e) and Chl/Car (f) ratios in *S. polyrrhiza* (L.) Schleid after 24 h of treatment. All the values are mean of triplicates ± SD. One-way ANOVA followed by Duncan's multiple range test was used to determine the significant difference between treatments ($P < 0.05$).

excess iron or copper exposure, except that Chl *b* had a slight increase with increasing copper concentration (Fig. 2). The content of pigment in higher metal concentrations accounted for 25–50% of 1 mg L^{-1} metal treatment groups except Chl *b*. The maximal value of Chl *a*/*b* was found at 100 mg L^{-1} iron and 1 mg L^{-1} copper, respectively, while the maximal value of Chl/car occurred in 10 mg L^{-1} iron or copper treated plants.

Effect on Chlorophyll Fluorescence

Significant effect on chlorophyll fluorescence was observed at 1–100 mg L^{-1} iron and copper. Particularly, maximum photosynthetic efficiency (Fv/Fm) was absent in 100 mg L^{-1} iron or copper treatment group (Fig. 3).

Effect on Soluble Protein and Carbohydrate in Tissue

The content of soluble protein in tissue reached maximal value at 1 mg L^{-1} iron or copper, beyond which the content of protein was decreased. The decreases were significant in 10 and 100 mg L^{-1} iron or copper treatment groups (Fig. 4).

The maximal content of carbohydrate in tissue occurred at 1 mg L^{-1} iron and copper. The content of soluble carbohydrate was decreased when iron concentration was higher than 1 mg L^{-1}. In addition, no significant difference of

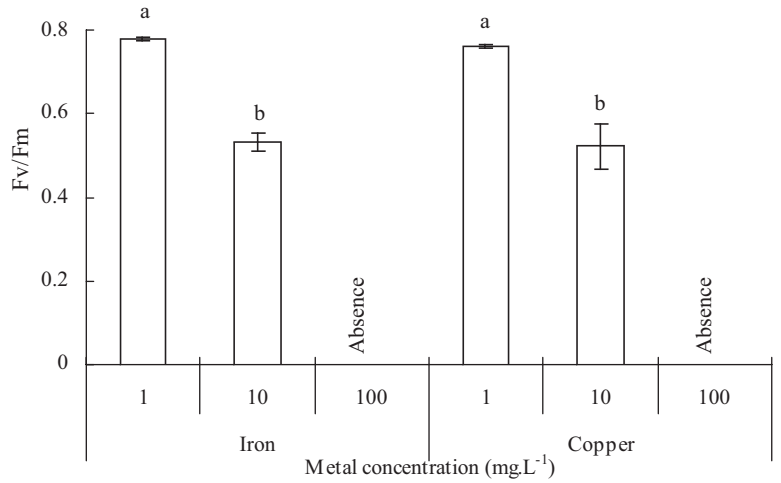

Fig. 3. Effect of excess iron and copper on photochemical efficiency (Fv/Fm) in *S. polyrrhiza* (L.) Schleid after 24 h of treatment. All the values are mean of triplicates ± SD. One-way ANOVA followed by Duncan's multiple range test was used to determine the significant difference between treatments ($P < 0.05$).

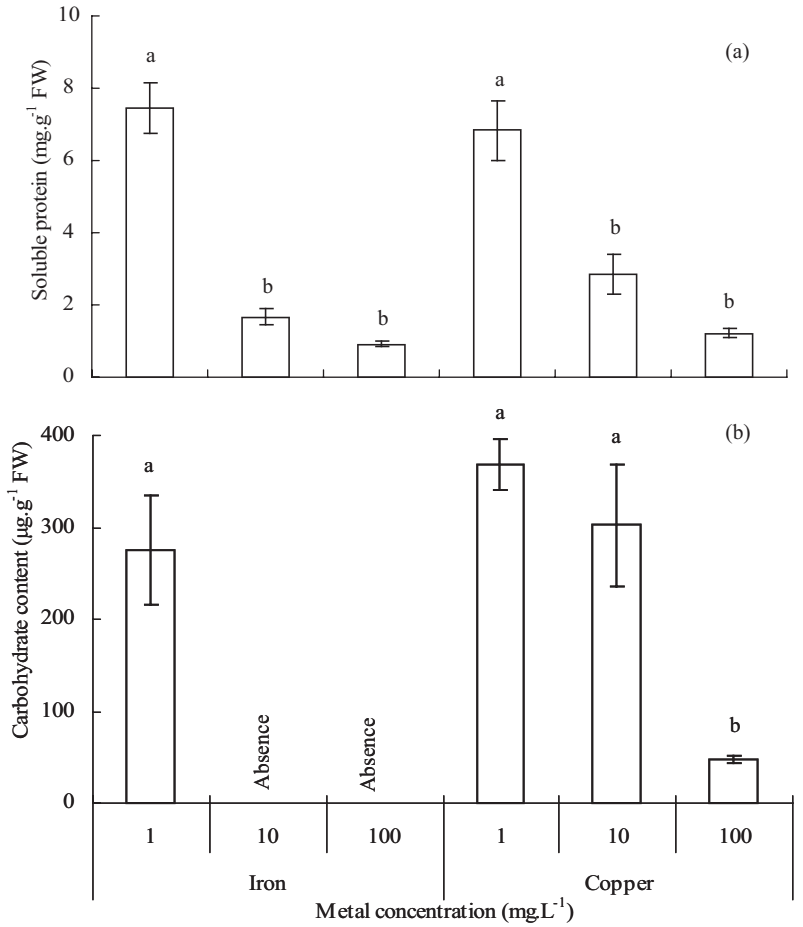

Fig. 4. Effect of excess iron and copper on soluble protein (a) and carbohydrate (b) contents in *S. polyrrhiza* (L.) Schleid after 24 h of treatment. All the values are mean of triplicates ± SD. One-way ANOVA followed by Duncan's multiple range test was used to determine the significant difference between treatments ($P < 0.05$).

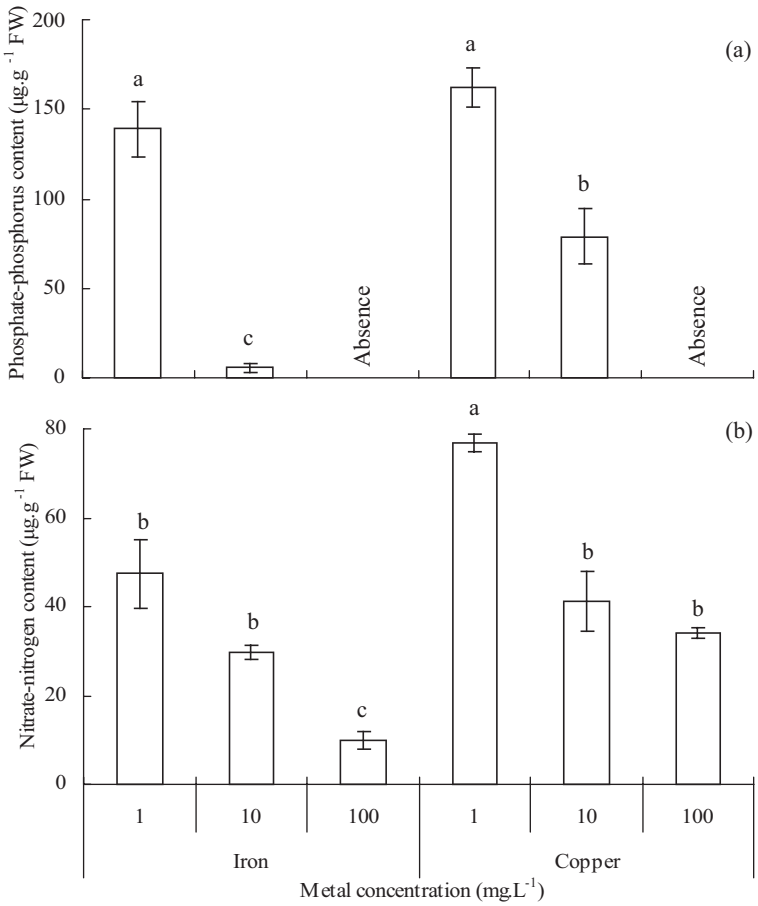

Fig. 5. Effect of excess iron and copper on phosphate-phosphorus (a) and nitrate-nitrogen (b) contents in *S. polyrrhiza* (L.) Schleid after 24 h of treatment. All the values are mean of triplicates ± SD. One-way ANOVA followed by Duncan's multiple range test was used to determine the significant difference between treatments ($P < 0.05$).

soluble carbohydrate level was found between 1 and 10 mg L^{-1} copper (Fig. 4).

Effect on Phosphate-Phosphorus and Nitrate-Nitrogen Contents in Tissue

Figure 5(a) shows that iron had significant influences on absorption and accumulation of phosphate-phosphorus compared with copper. The content of phosphate-phosphorus in tissue was absent at 100 mg L^{-1} iron and copper.

Nitrate-nitrogen was significantly influenced by high iron or copper dose leading to decrease by 20 and 50% in 100 mg L^{-1} iron and copper compared with 1 mg L^{-1} iron or copper, respectively. No significant differences were found between 1 and 10 mg L^{-1} iron as well as 10 and 100 mg L^{-1} copper [Fig. 5(b)].

Effect on Proline and MDA Contents in Tissue

Proline content decreased with increasing iron or copper concentration, however, MDA content increased with increasing iron or copper concentration. The maximal value of proline in tissue occurred at 1 mg L^{-1} iron or copper, on the contrary, the maximal content MDA in tissue was found at 100 mg L^{-1} iron or copper (Fig. 6).

DISCUSSION

Duckweed plants of *S. polyrrhiza* (L.) Schleid exhibited high accumulation of iron or copper at 100 mg L^{-1} iron or copper, respectively. High accumulation properties of duckweed for metal species have already been documented in previous studies (Bassi and Sharma, 1993; Li and Xiong, 2004a,b; Montvydiene and Marčiulioniene, 2004; Mukherjee et al., 2004; Drost et al., 2007; Horvat et al., 2007; Hou et al., 2007; Razinger et al., 2007, 2008). Compared with *Lemna minor* Linn., *S. polyrrhiza* (L.) Schleid had much more capacity of metal accumulation in this study (Fig. 1). The accumulation of metals in aquatic plants is often accompanied by a variety of morphological and physiological changes, some of which directly contribute to tolerance

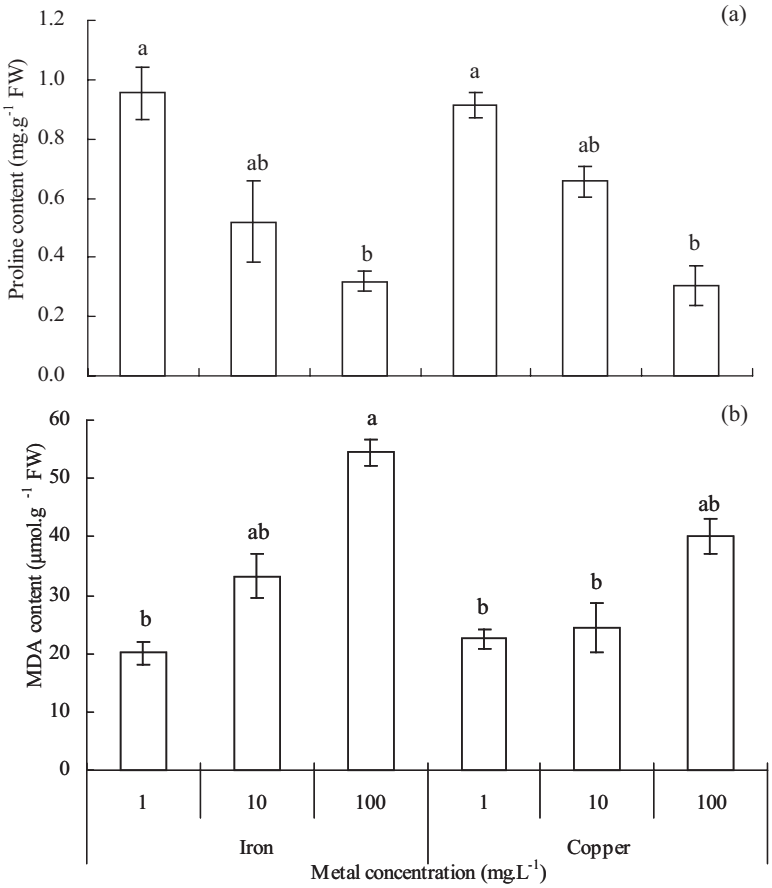

Fig. 6. Effect of excess iron and copper on proline (a) and MDA (b) contents in *S. polyrrhiza* (L.) Schleid after 24 h of treatment. All the values are mean of triplicates ± SD. One-way ANOVA followed by Duncan's multiple range test was used to determine the significant difference between treatments ($P < 0.05$).

capacity of the plants (Prasad et al., 2001; Ding et al., 2007).

Morphological toxicity symptoms were observed at designed metal concentrations except for 1 mg L^{-1} Iron (Table I). Excess iron and copper caused plants necrosis or death and colonies disintegration as well as roots breakup. Our results were in line with the findings of Li and Xiong (2004a,b).

Previous studies reported that excess metals could modify a number of physiological processes and particularly chlorophyll degradation (Ding et al., 2007). In present study, excess iron and copper caused significant loss of pigments (Fig. 2). Moreover, copper had significant negative correlations with Chl a ($r = 0.9364$, $P < 0.05$), carotenoid ($r = 0.9780$, $P < 0.05$) and Chl a/Carotenoid ($r = 0.9783$, $P < 0.05$), indicating Chl a is more sensitive to adversity stress than Chl b under metal stress. The result was completely contrary to previous studies (Ding et al., 2007; Li et al., 2008). Furthermore, excess iron and copper induced oxidative stress in chloroplasts (Prasad and Strzalka, 1999). When the levels of ROS formed exceed the ROS-quenching ability of plants, peroxidation of chloroplast membranes would occur, which caused the further loss in pigment concentrations (Baszynski et al., 1988).

Carotenoids not only aid in broadening the spectrum of PAR but also protect the light-harvesting pigments in the antenna complexes against photochemical damage caused by excited triplet states and other ROS (Pinto et al., 2003). The reason for the decrease of carotenoids perhaps lies on metal concentration. Pinto et al. (2003) reported that at high or acute levels of metal pollutants, damage to cells occurs because ROS levels exceed the capacity of the cell to cope, however, at lower or chronic, damage to cells occurs because accumulation of heavy metals and subsequent metal toxicity.

The reduced photosynthetic activity is an effect commonly noticed in plants exposed to heavy metal (Frankart et al., 2002; Hanikenne, 2003; Hou et al., 2007; Hu et al., 2007; Küpper et al., 2007; Li et al., 2008). In our experiment, significant correlation was obtained between Fv/Fm and excess iron and copper ($r = -0.9742$, $r = -0.9790$, $P < 0.05$), suggesting ROS caused by excess metal

concentration damaged photosynthetic apparatuses severely. Previous studies indicated that heavy metals primarily disturbed the integrity of thylakoid membranes and changed their fatty acid composition (De Vos et al., 1991), interfered with the biosynthesis of photosynthetic machinery and decreased net photosynthetic rate (Cook et al., 1997; Yruela, 2005).

Aside from the sugars and starches that meet this vital nutritional role, carbohydrates also serve as a structural material (cellulose), a component of the energy transport compound ATP, recognition sites on cell surfaces, and one of three essential components of DNA and RNA (Duffus and Duffus, 1984). In addition, soluble carbohydrate, that is sugar, directly reflects bioavailable carbohydrate content and energy level in plants. In this study, excess metals modified the carbohydrate accumulation and distribution in plants. The low content of soluble carbohydrate suggested that plants consumed a large amount of soluble sugar to maintain the basic physiological functions, such as photosynthesis and respiration. Our results were in agreement with the report of Samarakoon and Rauser (1979) that converse changes of carbohydrate and photosynthesis in leaves of *Phaseolus vulgaris* under excess Ni stress.

Both nitrogen and phosphorus are related to cell division and growth. Elevated concentration of iron and copper resulted in metal toxicity within the plants which impeded the uptake of nitrogen and phosphorus. The result supported the finding of Batty and Younger (2003) that the reason of growth inhibition was not nutrient deficiency but metal toxicity. In addition, nutrient content in plants also affected the synthesis of chlorophyll and protein as well as carbohydrate.

Malondialdehyde (MDA) is a product of lipid peroxidation and an indicator of free radical production and consequent tissue damage (Ohkawa et al., 1979). Our results showed that with the increasing metal concentration, the MDA content increased, which is similar to the effect of heavy metals on other higher plants (Sinha and Saxena, 2006; Singh et al., 2006; Zhang et al., 2007). Proline accumulates in a variety of plant species in response to stresses such as drought, salinity and extreme temperatures. Under stress conditions, proline may act as an osmotic adjustment mediator, a subcellular structures stabilizer, a free radical scavenger and a redox potential buffer and is an important component of cell wall proteins (Molinari et al., 2007). Although its role in plant osmotolerance remains controversial, proline is thought to contribute to osmotic adjustment, detoxification of reactive oxygen species and protection of membrane integrity. Excess metal induced the decrease in intracellular proline content in the study, suggesting osmosis of membranes was affected by stress caused by excess iron and copper, which agreed with the reports on other plants (Alia and Pardha, 1991; Bassi and Sharma, 1993; Sinha and Saxena, 2006; Sinha et al., 2009).

In conclusion, excess iron and copper caused obvious morphological and physiological changes of *S. polyrrhiza* (L.) Schleid. Dose and side effects must be considered seriously in practical application of chemical reagents.

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