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Effects of Copper on the Changes of Membrane Lipids, Photosynthetic Pigments and Thiol-containinng Compounds in *Zinnia elegans* Leaves

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ABSTRACT : Five-week-old plants of *Zinnia elegans* were grown in culture medium containing 50 μM $CuSO_4$. Plasma membrane was isolated and purified from both leaves of control and susceptible plants with method of polyethylene glycol/dextrin two-phase partition system. The major fatty acids of plasma membrane were identified as palmitic acid (16 : 0), linoleic acid (18 : 2) and linolenic acid (18 : 3). The ratios of unsaturated fatty acids to saturated fatty acids were reduced in copper-treated plants. The productions of lipid peroxides and nonprotein thiol-containing compound were obviously enhanced in copper-treated plants. Copper also caused the increment in chlorophyll content and total amounts of carotenoids in young leaves.

KEY WORDS : Copper, Membrane lipids, Pigments, Thiol-containing compounds, Toxicity, *Zinnia elegans*

INTRODUCTION

Copper is a micro-element and an activator of enzymes participating in the electron transport of photosynthesis and the redox reactions in plant cells (Shuman, 1994). The net uptake of plant nutrient was also influenced by the increasing copper level (Lidon and Henriques, 1993). However, excess copper in plants interfered with numerous physiological processes including inhibition of chlorophyll synthesis, retardation of photosynthetic electron flow and oxidation of cellular protein thiols (Ouzounidou, 1994; Sandmann and Boger, 1980). Furthermore, excess copper induced formation of free radicals that cause lipid peroxidation and it altered the properties and functions of plasma membrane (de Vos *et al.*, 1991).

Plants protect themselves against heavy metals stress by cell compartmentation, increasing the activities of antioxidant enzymes and producing metal-binding compounds such as phytochelatin and metallothioneins (Steffens, 1990). Plants in response to heavy metals were induced the production of phytochelatin via enzymatic biosynthesis (Grill *et al.*, 1989). The capability of phytochelatin induction for copper is lower than that for cadmium in rice seedlings (Yan *et al.*, 1993). However, excess copper enhancing the cytosolic Cu/Zn superoxide dismutase (SOD) activity in soybean root was reported (Chongpraditnun *et al.*, 1992).

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In the present study, the effects of copper on membrane lipids of plasma membrane, photosynthetic pigments and thiol-containing compounds in plants were examined.

MATERIALS AND METHODS

Plant materials

Seeds of *Zinnia elegans* were surfacedly sterilized in 2 % hypochlorite containing 0.1 % Tween 20 for 20 min. After being thoroughly washed, seeds were placed on the 4 L meshed plastics' containers immersed in Hoagland's solution for germination and hydroponically cultured. The nutrient solutions were changed in every three-days. Five-week-old seedlings exposed to 50 μM $CuSO_4$ for 0 to 7 days. The copper-treated plants were collected for further study.

Quantitation of copper in plants

Roots, stems and leaves of plants were separately collected, dried in oven at 70 ℃ for two days, and cut into pieces. Measured 0.2 g of dried plant material in quartz test tubes were mixed with 10 mL of 14 N nitric acid for one day. The mixture, then, was digested in Prolabo MX-350 Microdigesty until cleaning, diluted properly, filtered and finally detected by a Hitachi Z-6100 Atomic Spectrophometer (Lidon and Henriques, 1993).

Preparations of plasma membrane

Isolation and purification of plasma membrane followed the PEG-dextran two-phase partition method of Widell and Larsson (1981). Samples of thirty-gram fresh leaves were cut into small pieces with scissors, mixed with three volumes of extraction buffer containing 25 mM Tris-HCl, 0.25 M sucrose, 3 mM EDTA and 2.5 mM fresh dithiothretol at pH 7.5, settled for 10 min and homogenized twice in Polytron PT 3000 at 4,000 rpm for 10 sec, filtered with 4 layers of cheesecloth and centrifuged at 12,000 g for 15 min. Collected supernatant was recentrifuged at 80,000 g for 1 h. The pellet was gently suspended with suspension buffer containing 5 mM Tris-maleate, 0.25 M sucrose at pH 7.8 by the help of painting brush and it became microsomal membrane fraction. Microsomal membrane fraction was proportionally mixed with 6.0% PEG-dextran two-phase system and centrifuged in swing bucket rotor at 1,000 g for 5 min. The upper PEG fraction was countercurrently processed with same PEG-dextran two-phase system for three times. Pooled purified plasma membrane fraction was diluted with 4 volumes of extraction buffer and centrifuged at 150,000 g for 1 h. The white pellet was resuspended with extraction buffer for further identification or storage at -80 ℃.

The purity of isolated plasma membrane was identified by using the assay of ATPase activity with the methods of trichloroacetic acid (Gallagher and Leonard, 1982). Taken 0.44 ml of substrate solution containing 0.25 M sucrose, 30 mM Tris-MES (pH 6.5), 50 mM KCl, 3 mM of $MgCl_2$, 0.2 mM of $(NH_4)_6MoO_{24}$ and 3 mM fresh ATP was mixed with 20 μL of 0.25 % Triton X-100, 10 μL of inhibitor (1 mM NaN_3, 50 mM KNO_3, or 0.1 mM Na_3VO_4) and 30 μL of plasma membrane fraction (protein > 3 μg). Reaction mixture was gently

stirred, incubated at 37 ℃ for 30 min. The reaction was terminated with 200 μL of 25 % trichloro-acetic acid and 300 μL of 1 % sodium dodycyl sulfate. Measured 0.8 ml of reaction product combined with 1 mL of color reagent (ferrous-sulfate-ammonium-molybdate) were shaken, reacted for 30 min at room temperature, and detected its absorbance at 660 nm by a spectrophotometer. The activity of ATPase was expressed as μmole/Pi/mg protein/min. Primary potassium phosphate was the standard for calibration.

Analysis of fatty acids in plasma membrane

Lipids in plasma membrane were extracted using the method of Bligh and Dyer (1959). Purified lipids in chloroform were stored at -80 ℃ or directly methylated by the method of Morrison and Smith (1964). Methylated fatty acids in chloroform were further separated and detected by a HP 5890 gas chromatography with Supelco #2330 capillary column (30 m x 0.25 μm) and HP 5971 mass selective detector. The retention time and peak area of methylated tripentadecanion (Sigma T4257) were used as the internal standard for qualitative and quantitative analyses of individual fatty acid.

Quantitation of lipid peroxides :

The detection of lipid peroxides followed the methods of Somashekarariah *et al.* (1992). Measured 0.5 g of leaf blades were mixed with 5 ml of 0.1 % trichloroacetic acid (TCA). The mixtures were homogenized in Polytron at 4, 000 rpm for 10 sec, and centrifuged at 10,000 g for 5 min. The supernatant (1 ml) was completely mixed with 4 ml of 20 % of thiobarbituric acid, heated at 95 ℃ for 30 min, cooled in ice-bathed water and centrifuged at 10,000 g for 15 min. Collected supernatant was measured by a spectrophotometer at absorption of 532 nm.

Pigment analysis with high performance liquid chromatography (HPLC)

Taken 0.2 g of leaf blade were frozen in liquid nitrogen, ground into powder, shakily extracted with 10 ml of 80 % acetone, and centrifuged at 5,000 g for 10 min. Pigment extracted in acetone and purified in ether were further separated with reversed phase-HPLC and monitored at 445 nm (Lu *et al.*, 1995). The running of carrier phases was using 75 to 100 % acetonitrile/methanol (3:1, v/v) for 22 min at a flow rate of 1.5 ml/min and 100 % acetonitrile/methanol (3:1, v/v) for 48 min at a flow rate of 2.5 ml/min.

Assays of sulfhydryl groups

HPLC separation of sulfhydryl group was done by using the method of Vogeli-Lange and Wagner (1990) with minor modification. Measured 0.3 g of frozen tissue powder in Eppendorf tube were mixed with 0.6 ml of 3.5 N HCl for 6 h and centrifuged at 13,000 g for 30 min. Acid soluble supernatant was applied to a C18 reverse-phase column of Beckman 126 AA HPLC pre-equilibrated with 0.05 % (v/v) phosphoric and, then, were fractionated with a linear gradient of 0-20 % acetonitrile in 0.05 % phosphoric acid at a flow rate of 0.75 ml/min. Collected fractions with retention time within 20 min were measured their absorbance at 412 nm. Glutathione was also used as a standard peak for calibration.

RESULTS AND DISCUSSION

Copper is a trace element for plant growth and development and both Cu-deficiency and Cu-excess will result in growth abnormalities (Woolhouse, 1983). The previous study showed that most plants grew better in the medium containing 2 μM copper than in plain medium (Tsay *et al.*, 1995). The critical concentrations of copper for root growth retardation were much lower than that of shoot stunt and leaf chlorosis. Similar results have been reported in maize and rice (Hogan and Rauser, 1981; Baszynski *et al.*, 1982). Copper was accumulated in the root tissues and less transported to the shoot regions of plants (Lidon and Henriques, 1993). *Z. elegans* exposed to excess copper had significantly higher copper concentration in roots than that in stems and leaves (Table 1). Symptoms of copper toxicity in early stage are internode shortening, old leaf chlorosis (but young leaf became dark green) and root shortening (Fig. 1). Similar results have been reported in other cereal and ornamental plants (Fernandes and Henriques, 1991).

Table. 1. Distribution of copper in root, stem and leaf tissue of *Zinnia elegans* under control condition and treatment for one week.

	Copper concentration (mg/g dry weight)	
	treatment condition	
plant tissue	control*	50μM $CuSO_4$ #
Root	0.04±0.00**	8.86±0.05
Stem	0.02±0.00	0.30±0.01
Leaf	0.02±0.00	0.06±0.00

*Half- strength Hoagland's solution

#Half- strength Hoagland's solutions containing 50μM $CuSO_4$

**All measurements were in quadruplicate

The plasma membrane is the permeability barrier of plant cells and the first site of the cell to sense changes in outside environment. Plasma membrane obtained from leaves of *Z. elegans* could be further purified with the method of PEG-dextran two-phase partition (Larsson, 1983). Vanadate, an inhibitor of K^+-stimulated Mg^{++}- dependent ATPase of plasma membrane, can be used in combination with azide and nitrate to distinguished plasma membrane-enriched fraction from the rest in two-phase system. As shown in Table 2, ATPase activity in PEG fraction was almost completely inhibited by vanadate (only 1.39 % of activity left). However, its activity was enhanced or only slightly affected by azide (184 % of activity) and nitrate (93.1 % of activity left), specific inhibitors for mitochondrial and tonoplast ATPases, respectively (Gallagher and Leonard, 1982). The ATPases in dextran was also inhibited by vanadate, but with a great relief of inhibition (28.1 % of activity left). Meanwhile, Its activity was also partially inhibited by azide (71.8 % activity left) and nitrate (75.8 % activity left). All the above results indicated that dextran fraction contained intracellular membrane including sources of membrane from endoplasmic reticulum, Golgi, tonoplast, mitochondria, chloroplasts, microbodies, and contaminated plasma membrane. Thus, PEG fraction was rich in a higher purity of plasma membrane and used in the further experiments.

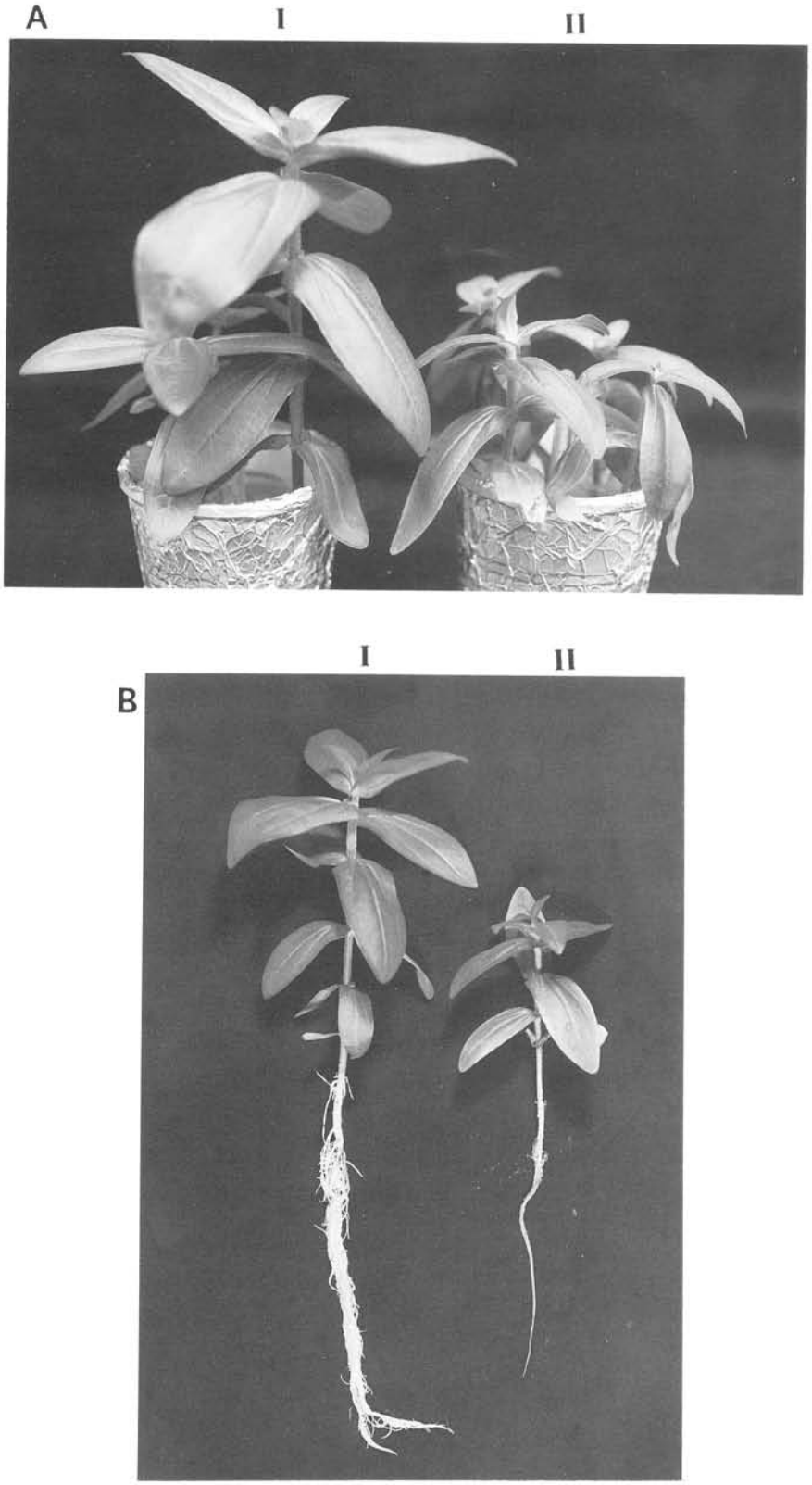

Fig. 1. The general symptoms of copper toxicity in *Zinnia elegans* were internode shortening and old leaf chlorosis. Young leaf became dark green (AI). Root tissue exposed to exccess copper became brown and root growth was inhibited (BII). I: control; II: excess copper-treated.

Table. 2. Effect of various inhibitors on ATPase specific activity in the plasma membrane fraction and intracellular membrane fraction obtained by using 6.0 % two-phase partition system from *Z. elegans* leaves.

Inhibitor	ATPase specific activity* (μmole Pi/mg protein/ min) Plasma membrane (PEG)	Intracellular membrane (Dextran)
Control	0.555(100)	0.139(100)
$NaVO_4$(0.5mM)	0.077(1.39)	0.039(28.1)
NaN_3(1mM)	0.517(93.1)	0.099(71.4)
KNO_3(50mM)	1.021(184)	0.105(75.8)

*ATPase activity was assayed in the presence of 0.25 M sucrose, 30 mM Tris-MES (pH 6.5), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM $(NH_4)_6Mo_7O_{24}$, 3 mM ATP and various inhibitors as indicated. Number in parentheses indicates percent of control.

Plant cell membranes are dynamic in behavior with lipid composition that is changed with variation in the external environment. Moreover, the ratios of unsaturated/saturated fatty acids in plasma membrane not only influence membrane stability, but also directly or indirectly affect the intrinsic enzyme activities and ion pumps (Carruthers and Melchior, 1986). An increase in the degree of fatty acid saturation was observed in wheat under salt stress. This changes correlated with the more rigid plasma membrane has been suggested (Mansour *et al.*, 1994). In the present study, palmitic acid (16 : 0), linoleic acid (18 : 2) and linolenic acid (18 : 3) were identified as the three major fatty acids in plasma membrane of *Z. elegans* (Table 3). Copper-treated plants showing a decrease in their unsaturated/saturated fatty acid ratios. Such changes in composition of membrane lipid may reduce membrane fluidity, selectivity of membrane transport and intrinsic enzyme activity.

Table. 3. Effect of copper on fatty acid composition of plasma membrane purified from th leaves of *Z. elegans*.

Fatty acid	Fatty acid composition (μg/mg protein) Control			50 μM $CuSO_4$ #		
	0 day	2 days	4 days	0 day	2 days	4 days
14:0	0.03±0.00*	0.03±0.0	0.04±0.00	0.04±0.00	0.05±0.04	0.07±0.00
16:1	0.11±0.02	0.14±0.01	0.11±0.01	0.21±0.00	0.24±0.01	0.29±0.01
16:0	0.87±0.02	1.01±0.05	0.83±0.01	0.55±0.01	0.62±0.02	0.84±0.03
18:2	0.88±0.03	0.97±0.06	0.81±0.08	0.34±0.01	0.41±0.01	0.44±0.02
18:3	0.64±0.02	0.82±0.05	0.64±0.02	0.39±0.01	0.41±0.02	0.51±0.03
18:1	ND**	ND	ND	0.12±0.01	0.13±0.01	0.12±0.01
18:0	0.12±0.01	0.14±0.01	0.15±0.0	0.10±0.00	0.13±0.01	0.16±0.01
Others	0.05±0.00	0.13±0.02	ND	ND	ND	ND
C16/C18+	0.59	0.60	0.59	0.8	0.8	0.9
Unsat/Sat++	1.63	1.68	1.6	1.64	1.56	1.38

Plants pretreated with 50 μM $CuSO_4$ for one week
* All experiments were in quadruplicate
** ND: No detect
+ C16 fatty acids/ C18 fatty acids ratio
++ Unsaturated fatty acids/ saturated fatty acids ratio

Excess copper blocked photosynthetic electron flow chain in the chloroplast to cause a decrease in photochemical efficiency and form reactive destructive oxygen radicals (Woolhouse, 1983). Cu-mediated oxidative stress due to free radicals was evidenced by the accumulation of lipid peroxides (Fig. 2). Plants confronted with copper-affected damages were induced the formation of glutathione and/or activation of antioxidant enzyme such as SOD and peroxidase that played the major roles in the scavenger of free radicals (Chongpraditnun *et al.*, 1992; de Vos *et al.*, 1992).

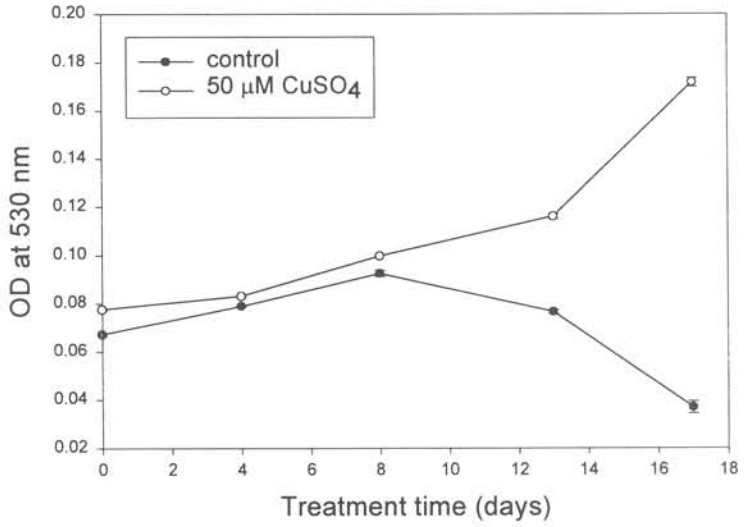

Fig. 2. Effect of copper on the change of lipid peroxides level in *Z. elegans* leaves. Malonaldehyde as lipid peroxidation decomposition product extracted in 20 % tricholoroacetic acid was processed with the TBA reaction for 30 min at 95℃ and determined at 532 nm. Measurements were all in quadruplicate and standard errors were shown as bars indicate.

The age of leaves in *Z. elegans* obviously affected their sensitivity to Cu-treatment. While Cu-treated plants produced young leaves with much higher content of pigment, its old leaves accumulated much less (Fig. 3). Carotenoids were thought to be accessory pigments protect chloroplast against photooxidation (Young and Britton, 1990). Higher level of pigments in young leaf and less level in old leaf might imply that feeding high level of copper. *Z. elegans* not only produced higher content of phytochelatins for formation of Cu-chelating complex (Fig. 4), but also sacrifice old leaf to support a normal metabolism in young leaf.

In response to an excess of heavy metal, plants produce a class of metal-binding ligands including nonprotein sulfhydryl peptides called phytochelatins and gene-encoded proteins called metallothioneins. Their common characteristics are metal-induced, low molecular weight and cystein-rich (Steffens, 1990). Phytochelatins were drastically synthesized in copper-sensitive *Silene cucubalus* within six hours of exposure to copper (de Vos *et al.*, 1992). In *Z. elegans* nonprotein SH-containing peptides were markedly induced by copper treatment in the first 12 h (Fig. 4). These metal-binding compounds have an important role in metal detoxification and reduce the availability of diffusible metal ions within cells.

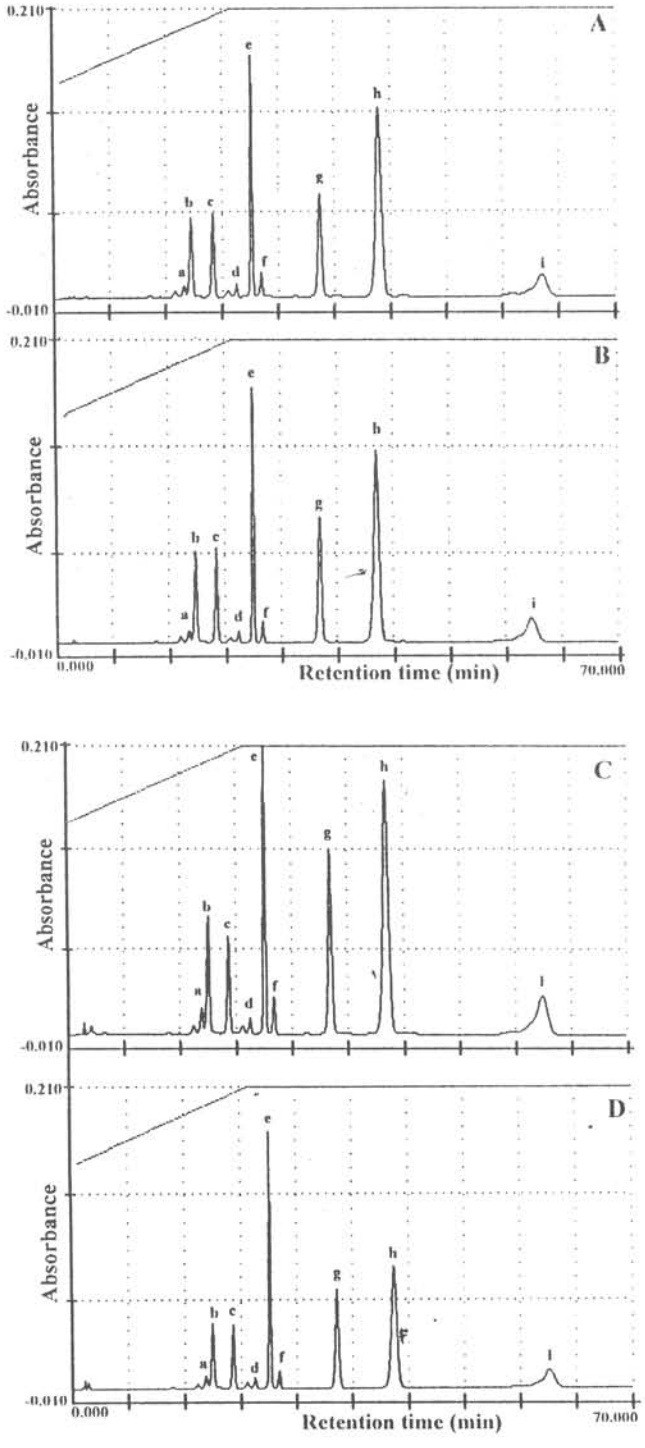

Fig. 3. HPLC profiles of photosynthetic pigments in control (A, B) and Cu-treated (C, D) *Z. elegans* plants. Pigments extracted in acetone and purified in either were assayed by HPLC using 75 to 100 % acetonitrile/methanol (3:1, v/v) for 22 min at 1.5 ml/min, 100 % acetonitrile/methanl (3:1, v/v) in 48 min at 2.5 ml/min on the reverse phase column and monitored at 445 nm. The identified peaks are: a: neoxanthin; b: violaxanthin; c: taraxanthin; d: antheraxanthin; e: lutein; f: zeaxanthin; g: chlorophyll b; h: chlorophyll a; I: β-carotene. (A, C: young leaf; B, D: old leaf).

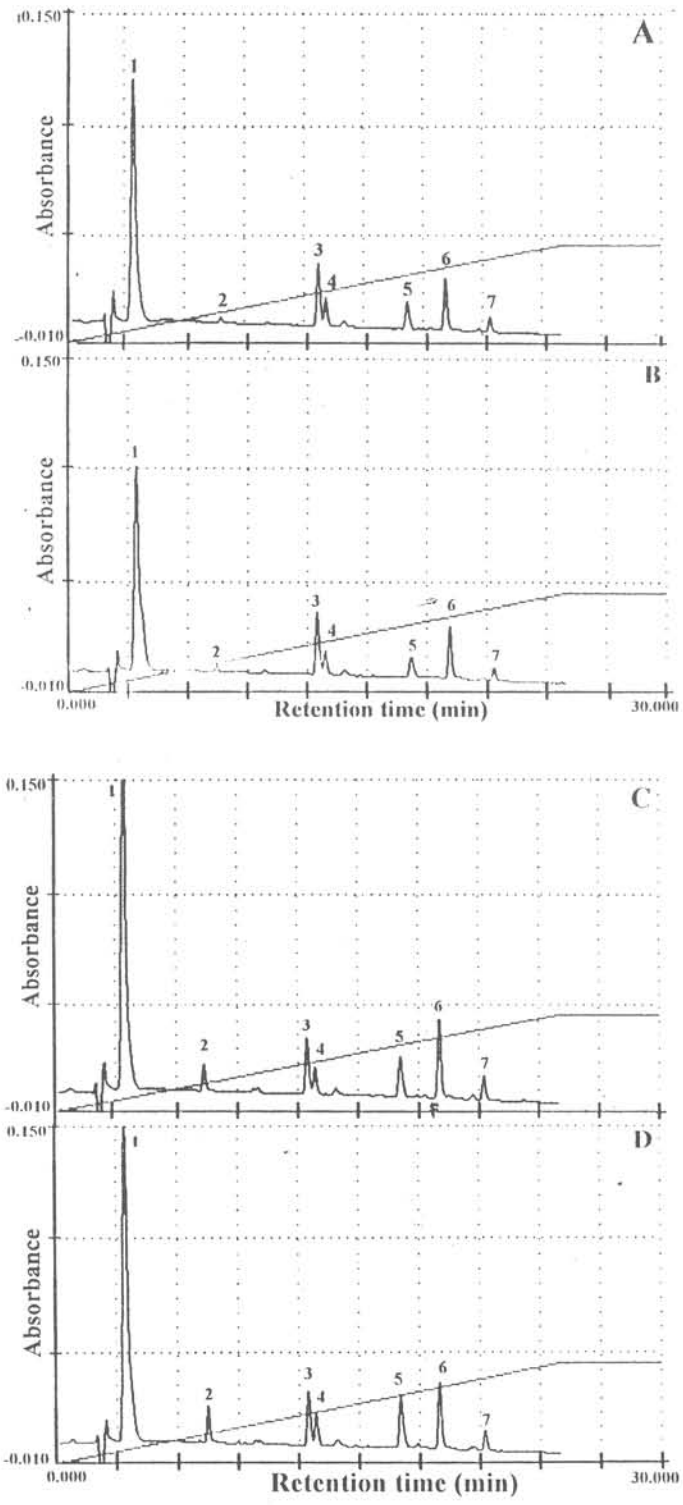

Fig. 4. HPLC profiles of nonprotein thiol-containing compounds from acidic leaf extracts of control and Cu-treated *Z. elegans* plants for A-0 h, B-6 h, C- 12 h and D-24 h. Analysis by HPLC using 0 to 30 % acetonitrile in 0.05 % phosphoric acid over 25 min on C18 reverse phase column. After postcolumn derivatization with Ellman's reagent, thiol-containing compounds were determinted at 412 nm.

As a result of long-term adaptation to heavy metal-polluted environment, plants have evolved a variety of resistant mechanisms (Woolhouse, 1983). Therefore, many researchers attempt to find the physiological characteristics of metal tolerance. In *Silene cucubalus*, restriction of copper uptake was required for copper tolerance, but phytochelatin synthesis was not absolutely related to metal tolerance in whole plant system (de Vos *et al.*, 1991). Plants may simultaneously induce many defensive mechanisms in the face of heavy metal stress. Then, they must regulate the physiological processes to maintain the normal functions in cell (Woolhouse, 1983). In the present studies, changes in plasma membrane properties, accumulation of lipid peroxides, changes in the level of photosynthetic pigments and a newly formed nonprotein SH-peptides have shown under copper treatment. In the further study, we will directly measure plasma membrane fluidity and ion pump efficiency and investigate the relationship between various physiological changes and metal tolerance in plants.

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銅對百日草葉中原生質膜的脂質、光合作用色素及硫化物含量的影響

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摘　　要

五週大的百日菊幼苗以含或不含 50 μM 硫酸銅的營養液培養後，以 PEG-Dextran 雙相分配系純化原生質膜的方法，收集取樣分離與測試。經分析顯示 Palmitic acid (16:0)、Linoleic acid (18 : 2) 及 Linolenic acid (18 : 3) 為三種主要的脂肪酸，不飽和脂肪酸與飽含脂肪酸的比例因銅處理而減低。銅處理也促進脂質過氧化物的生成和非蛋白質性的含硫化物的合成，同時造成百日草幼葉中葉綠素及總類胡蘿蔔素的增加。

關鍵字：銅、膜脂、色素、含硫化合物、毒害、百日草。

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