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Plant in Response to Copper Toxicity

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ABSTRACT: Ten-day-old seedlings germinated from seeds of tested plants obtained from commercial sources and Cu-polluted open field were transferred to a Hoagland's solution containing various concentration of copper. Young plants grew better in Hoagland's solution containing 2 μM copper than that of plain one. Among eighteen tested plants, *Miscanthus floridulus* and *Dianthus barbatus* were more tolerant to excess copper. Most Cu-treated plant showed growth retardation in root and shoot, and sometimes leaf chlorosis. Structural changes of cells induced by copper toxicity were found in extracellular matrix and intracellular organelles. Breakage of membrane systems frequently appeared in various tissues of susceptable plants. Chloroplast abnormalities occurred in leaf tissues. Electron-dense granules were found in cell wall, vacuoles, chloroplast and cytoplasm. Acid phosphatase and peroxidase are verified to be the potential markers for the plants in response to copper stress.

KEYWORDS: Copper, toxicity, structure, peroxidase, acid phosphatase, superoxidase, dismutase, isozymes.

INTRODUCTION

Bioassay and biological monitoring of heavy metal pollution have been extensively studied (Martin and Coughtrey, 1982). However, the successness of measurement is based upon the choose of proper plant species and right developmental stage. The susceptiblity of plants to heavy metals in the field varys with plant species, property and composition of soils, and the way of pollution (Woolhouse, 1983; Herstein and Jäger, 1986). Hydroponic culture of seedlings of different plant species could provide a better way for screening test of indicator and tolerant plants (Fernandes and Henriques, 1991).

The excess metals cause the damages of plants in various ways (Woolhouse, 1983). The symptoms of copper toxicity are the retardation of root elongation at first appearance, and the destruction of thylakoid membrane and plasmalemma at microscopic level (Fernandes and Henriques, 1991; Sandmann and Boger, 1983). Peroxidase and acid phosphatase as markers for screening Cd-tolerant plant were examined and only peroxidase markedly changes in the less tolerant plant (Herstein and Jäger, 1986). Plant in responsing to various concentration of copper showed distinct changes in isozyme patterns of superoxide dismutase and peroxidase (Palma et al, 1987). Excess copper induced a cytosolic Cu, Zn-superoxide dismutase isozyme of soybean root had also been reported (Chongpradinun *et al*, 1992).

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In the present study, seedlings of *Zinnia elegans* and *Cosmos sulfureus* in response to copper treatment were examined through their changes in morphology and isozyme patterns of acid phosphatase, peroxidase and superoxidase dismutase.

MATERIALS AND METHODS

1. Plant materials

Seeds of potential plants obtained from both the open field of heavy metal polluted regions and the commercial sources of ornamental plants. After 2-day's incubation at 30°C, five to thirty germinating seeds were transfered to a stainless steel net supported on water surface. Ten-day-old young seedlings were shifted in Hoagland's solution containing different concentrations of copper. After ten day treatment, plants were collected for further studies.

2. Chlorophyll content

For assay of chlorophyll, the method of Mackinney (1941) was adopted. About 0.2 g of leaf blade was homogenated wth 2 ml of 80% acetone and then another 2 ml of 80% acetone was added to make final volume in 5 ml. Spectrophotometric measurement was carried out at OD_{652}. The total chlorophyll can be calculated as follows:

Total chlorophyll (mg) = OD_{652} x 1000/34.5 x 5/(1000 x 0.2) x 25

3. Isozyme analysis

Crude extracts of plant tissues was electrophoretically separated with 7.5 % polyacrylamide gel and 0.38 M glycine buffer (pH 8.3). Gel thickness was 0.75 mm and voltage was set at 120 V. For detection of acid phosphatase, the stainer contained 0.2 M sodium acetate buffer (pH 5.0), 0.1 % fast red TR salt, 5 mM $MgCl_2$ and 0.1 % α-naphthyl phosphate; for peroxidase, the reaction mixture contained 0.1% benzidine, 0.1 % H_2O_2 and 0.2 M phosphate buffer (pH 7.0); and for superoxidase dismutase, the method of Fridovish's group (Clare *et al.*, 1984))was followed.After staining, gels were photographed and dried.

4. Electron and light microscopies

Roots and leaves of control and Cu-treated plants were immersed in 0.1 M cacodydate buffer containing 2.5 % glutaraldehyde and cutted with razor blade into small cubes (<1 mm). The small cubes were soaked in fresh fixation solution for 2 h, washed three times in rinse buffer, and postfixed in 1 % osmium tetraoxide for 4 h. Being washed three times in distilled water, the cubes were dehydrated through ethanol series, infiltrated with series of Spurr's resin and finally embeded in pure resin. Pure resin with tissue segments were polymerized at 70 °C for more than 8 h. Plastic sections in golden color were obtained from ultramicrotomy with glass or diamond knife, doubly stained with uranyl acetate and lead citrate, and observed under Hitachi H-600 at 75 KV.

For light microscopic observation, 1 mm thick sections were collected with glass knife, stained with toludine blue, observed and photographed with Zeiss Photomicroscope III.

RESULTS

Two field trips to Cu-mine at Tsuei-fang in Taipei County gave us the chances to collect the major-existing plant species, such as *Miscanthus floridulum*, *Pteris vitlata* and *Histiopteris incisa*. Most seeds harvested from the field showed high contamination of molds and poor germination rate (<1%).

Seedlings of *Zinnia elegans* and *Cosmos sulfureus* grew better in medium containing 2 μM copper than that of in plain Hoagland's solution (Fig. 1). The critical concentration of copper for *Z. elegans* and *C. sulfureus* were 35 μM and 50 μM, respectively. Root elongation of primary root and development of lateral roots were greatly inhibited in higher copper concentraions (Table 1). However, the chlorophyll content in young leaves of susceptable plants was not significantly disturbed (Table 2), and sometimes, chlorosis happened in the vicinity of small veins.

Table 1. Effect of Cu concnetrations on root growth of three-week-old plants.

Copper (μM)	Root length (cm)	
	Zinnia elegans	*Cosmos sulfureus*
Control	9.32 ± 3.32	7.83 ± 1.75
2	12.41 ± 3.67	12.55 ± 3.57
100	8.25 ± 1.48	5.42 ± 1.49
500	7.25 ± 2.40	5.48 ± 1.52

Table 2. Effect of Cu concentrations on chlorophyll content in leaves of three-week-old plants.

Copper (μM)	Chlorophyll content (mg / g fresh wt)	
	Zinnia elegans	*Cosmos sulfureus*
Control	1.31 ± 0.12	1.13 ± 0.16
2	1.56 ± 0.10	1.66 ± 0.20
100	1.13 ± 0.18	1.89 ± 0.12
500	1.34 ± 0.14	1.92 ± 0.18

Different plants had their distinct isozyme patterns in zymogram of three tested enzymes (Fig. 2). As shown in Figure 2A, zinnia plants embodied four isozymes of acid phosphatase and cosmos plants five. Two minor bands of isozymes did disappear in cosmos plants treated with high copper concentration, whereas one isozyme showed quantitatively changes both at lowest and highest Cu concentrations in zinnia plants. There are five isoperoxidases in zinnia plants and six in cosmos plants (Fig. 2B). Their isozyme patterns did reflect the differences among the treatments of various copper concentrations. In cosmos plants, one isozyme disappeared in the treatment of copper concentrations above

2 μM. Their activities of two minor isozymes in zinnia were enhanced when copper concentrations in media were increased. Both of zinnia and cosmos plants have only two kinds of superoxide dismutase isozymes. Unlike the reulsts of cadmium treatment, isozymes of superoxide dismutase in both plants almost prevailed the similiar isozyme patterns treated with different copper concentrations (Fig. 2C).

Structural changes in the cells owing to excess copper treatment could be occurred both in extracellular matrix and intracellular organelles. The leakage of cell components were often observed in root (Fig. 3). The stainablity of cell wall was enhanced and mineral deposit was also found associated with cell wall. In root vascular tissues of Cu-treated plant, protoplasm of parenchyma cells was easily separated from cell during section preparation and plasmolyzed protoplasm showed variations in shape. The breakage of plasmalemma frequently appered in various tissues of susceptable plants. Small leaves of Cu-treated plants, which were resulted from copper toxicity, showed closed stomata , irregularities in structure of mesophyll cells, deposits distinctly discharged in vacuoles and cell wall of epidermis and vascular tissues (Figs. 4,5). In leaf epidermal cells, the outer tangential wall was much thicker than the inner wall. Such irregularity in wall thickness were formed through uneven apposition (Fig. 6).. Chloroplasts in mesophyllous cells of susceptable plant aggregated in group and their abnormalities were in appearance of membrane dilatation in granal thylakoid and chloroplast envelope, and the increasing in numbers of plastoglobuli (Figs, 5, 7). Oil drops in cytoplasm of vascular tissues were also found (Figs. 2, 7, 8). Mineral deposits were found in cell wall as well as in vacuoles, chloroplasts and cytoplasm (Figs.5-8). The occurrence of mineral deposits varied from cell to cell and tissue to tissue.

DISCUSSION

Copper is a trace element for plant grown and development. Both Cu-deficiency and Cu-excess will resulted in plant growth abnormalities (Woolhouse, 1983). In the present studies, most plants grow better in the medium containing 2 μM copper than in plain medium. All plants cultured in medium over this concentration did show the symptoms of copper toxicity. However, the critical concentrations of copper for root growth retardation were much lower than that for shoot stunt and leaf chlorosis. Similiar results have been reported in maize and rice (Hogan and Rauser, 1981; Baszynski et al., 1982).

Symptoms of copper toxicity in field-growing plants were less reported. Soil colloids and organic matters with strong binding of copper in soils often severely restricted the copper mobility, and copper available for plant uptake is low (Bjerre and Schierup, 1985; Fernandes and Henriques, 1990). Copper mobility inside the plants is also restricted by cell wall and xylem fluid (White *et al.*, 1981). A large proportion of the copper absorbed by the plants is withhold in roots, and only small fraction of coppper was translocated to shoots. Therefore, copper levels in root is related to its concentration in the soil, whereas its levels in the shoot did not reflect the copper concentrations in the soil (Jarvis and Whitehead, 1981). Cu-tolerant plants in response to excess copper toxicity could be traced initially from root growth retardation, root browning and bending, shoot curving and finally

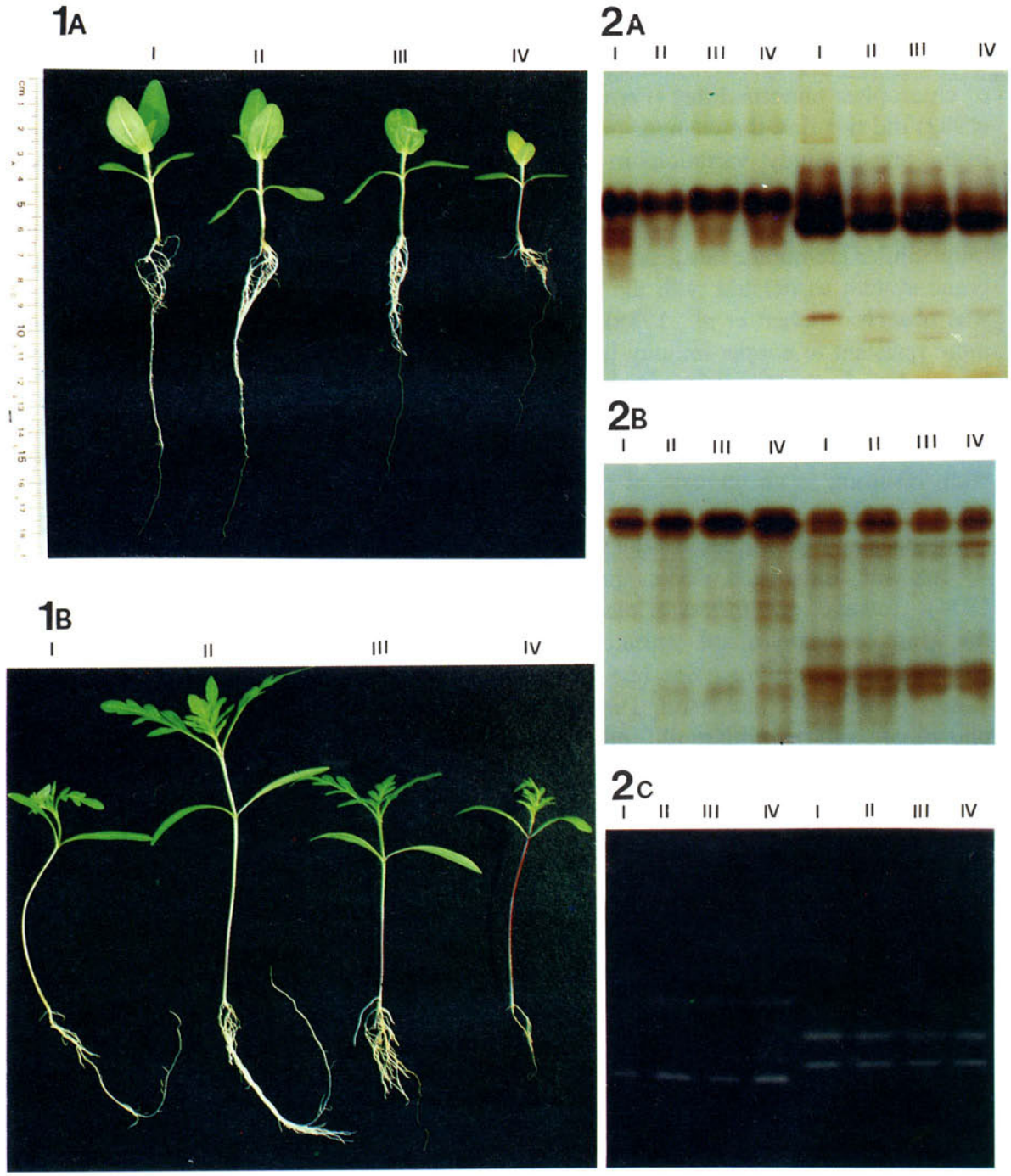


Fig. 1. External features of plants grown in different copper concentrations . A, yellow cosmos. B, zinnia. The copper concentrations in treatments I, II, III, and IV were control (copper in Hoagland's solution), 2, 100 and 500 μM respectively.

Fig. 2. Zymograms of acid phosphatase, peroxidase and superoxide dismutase (SOD) from zinnia (lanes 1-4 from left) and cosmos (lanes 5-8 from left) plants growing at various copper concentrations. A, acid phosphatase. B, peroxidase. C, SOD. Symbols I, II, III, and IV denote copper concentrations for control, 2, 100 and 500 μM, respectively.

chlorosis in leaf blade. Similiar results were found in acer, pinus and many other plants (Foy *et al.*, 1978). However, Cu-sensitive plants often showed early leaf yellowing. Ultrastructural study did reveal that all chlorotic leaves are associated with different kinds of chloroplast abnormalities (Fernandes and Henriques, 1990) .However, Baszynski *et al* (1982) did not find the changes in photosynthetic apparatus of spinach exposed to copper. In the present study, variations in chloroplast structure and shape were found in different green and yellowing leaves.

Cu-induced peroxidation of membrane lipid resulting in the extensive degradation of intracellular membrane has been suggested (Matto et al., 1983). The disintegration of membrane system associated with the accumulation of oil drop in cytoplasm and chloroplast was observed (Chen *et a*l., 1988). However, membrane systems in mitochondria seened more resistant to copper toxicity than that of chloroplast (Fernandes and Henriques, 1991). It was also observed in this study.

In various kinds of plants, copper deposits had been found in organelles of cells in different tissues or organs(Baszynski *et al.*, 1982). The deposits was associated with cell wall, resulting in an increase of the electron dense in cell wall. Electron dense granules often found in vacuoles of Cu-treated algae and higher plants were also reported (Fernandes and Henriques, 1991).

Isozymes have been used as the biochemical markers of plant development (Scandalios, 1974). However, the fitness of plant expression are quitely depended on plant species and the kind of heavy-metal stress. Acid phosphatase in roots of Cu-tolerant clones of *Deschampsia caespitosa* was less sensitive to copper inhibition than that of non-tolerant clones (Cox and Hutchinson, 1980). In the present study, the isozyme patterns of acid phosphtase and peroxidase did reflect the changes of copper concentration. Similar results were reported in pea (Palma et al., 1987). Isozyme patterns for screening Cu-tolerant need further study of intraspecific level.

Legends to Figures:

Fig. 3. Cross section of root vascular bundle in Cu-treated plants and its vincity showing the deposit of copper in cells (arrowheads) and leakages in collapsed cells.

Fig. 4. Cross section of leaf blade from Cu-treated plants showing collapsed guard cells (arrowheads) and dennsely stained cells in epidermis, mesophyll and vascular bundles .

Fig. 5. Mesophyllous cells of Cu-treated plants showing the aggregated starch grains in abnormal chloroplasts and dense granules (arrowhead) in vacuoles.

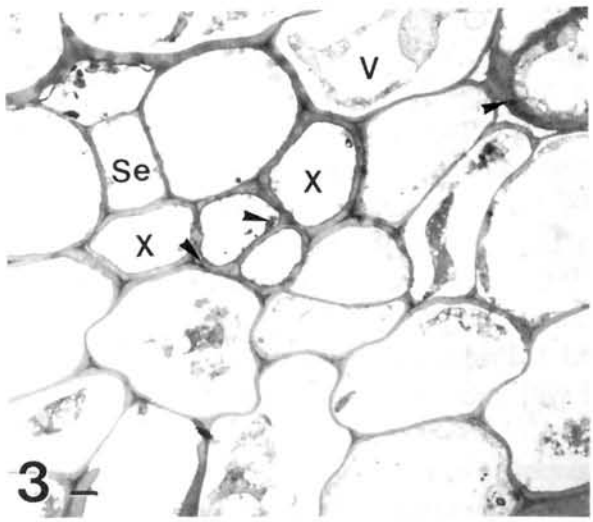
Fig. 6. Epidermal cells in leaf blade of Cu-treated plants with uneven wall-thickening and copper deposits (arrowheads) in the vacuole, chloroplast and cell wall.

Fig. 7. Chloroplasts in mesophyllous cells of Cu-treated plants showing the occurrence of plastoglubili and the abnormality of thylakoid membrane.

Fig. 8. Mesophyllous cells in Cu-treated plants showing their copper deposits (arrowheads) in cytoplasm, chloroplast and vacuole .

All bars in Figures 3-8 are in 1 mm except Figure 4 (10 μm).

Abbreviations: C, Chloroplast; Eu, Upper epidermis; El, Lower epidermis; Pa, Palisade tissues; Pb, Plastoglobuli S, Starch grain; Se, Sieve element; Sp, Sponge tissue; V, Vacuole; Vb, Vascular bundle; X, xylem.



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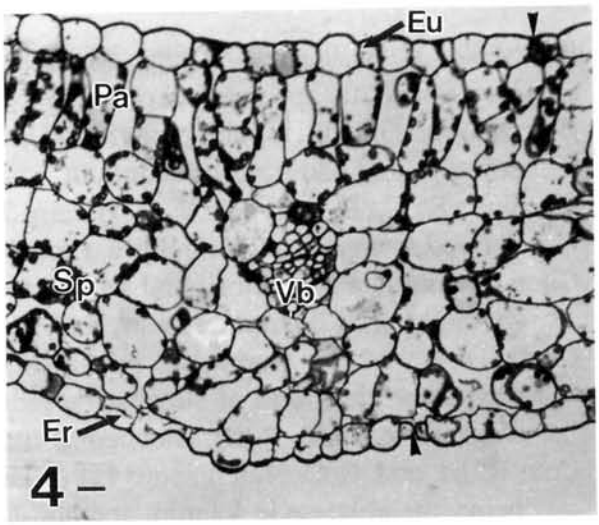

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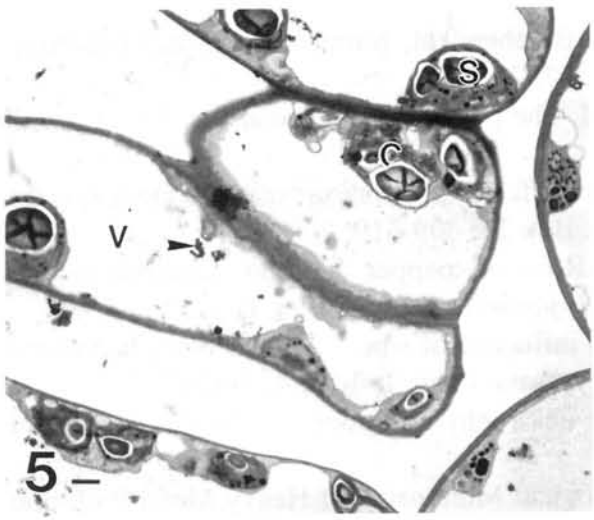

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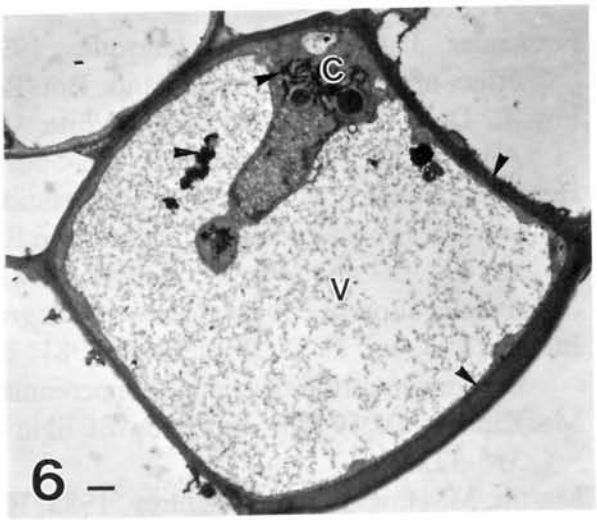

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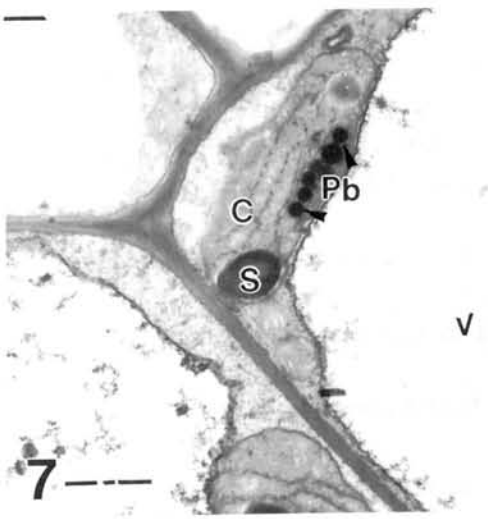

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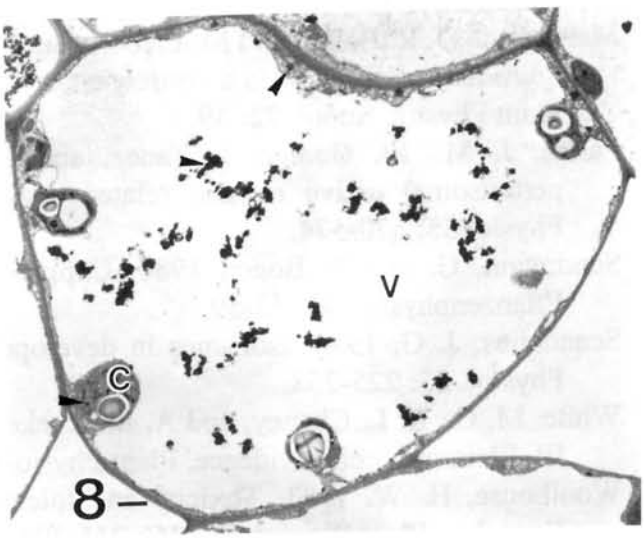

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植物對銅毒害的反應

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摘　　要

污染現場採集所得及買自種子店的種子萌發兩週後植株，經移栽在含不同銅溶液的培養液中兩週後，觀察植物的外表形態及進行比較。長在 2 μM銅濃度的植株比長在其他各種濃度者爲佳，甚至比對照組好。而在檢驗的十八種植物中以五節芒 (*Miscanthus floridulus*)及西洋石竹 (*Dianthus barbatus*) 抗性較高。一般銅的毒害造成根與莖部的生長受抑制，時間一長也會產生葉子黃化。致害在構造上而言，造成細胞外質質深、胞器的變異及膜系滲漏。電子密度高的顆粒或沈澱往往出現在 細胞壁、細胞質、葉綠體及液胞上。酸性磷酸酵素及過氧化酵素在銅毒害的情況下變異顯著，可當生化指標。

關鍵詞：銅，毒害，構造，過氧化酵素，酸性磷酸酵素，同功酵素。

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