Copper Toxicity and Bioaccumulation in Chinese Cabbage (*Brassica pekinensis* Rupr.)

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ABSTRACT: Copper is among the major heavy metal contaminants in the environment with various anthropogenic and natural sources. Human health risk from heavy metal bioaccumulation in vegetables has been a subject of growing concern in recent years. To investigate Cu phytotoxic effects and bioaccumulation in the popular vegetable Chinese cabbage (*Brassica pekinensis* Rupr) as well as the implications for human health due to Cu in the vegetable supply, seed germination and pot culture experiments with this vegetable were carried out. Six levels (0, 0.008, 0.031, 0.125, 0.5, and 2.0 mM/L) and 3 levels (0, 0.2, and 1.0 mM/kg) of Cu treatments were performed for the seed germination and pot culture experiments, respectively. The LC_{50} of Cu for seed germination of Chinese cabbage was 0.348 mM/L. In the pot culture experiments, Cu treatments significantly increased electrolyte leakage and peroxidase activity of shoot tissues, demonstrating Cu phytotoxicity to the plants. On the other hand, Cu treatments significantly stimulated, instead of reduced, chlorophyll content. Cu treatments did not show a significant effect on shoot biomass. Compared to the control, Cu treatments significantly elevated the Cu content of the shoots—9.9, 42.5, and 119.0 mg/kg (DW) of Cu were detected in the 0, 0.2, and 1.0 mM/kg treatments, respectively. These results showed that although the plants accumulated an elevated copper content and suffered damage to some extent under Cu treatment, they looked healthy. It was suggested that Chinese cabbage with an elevated Cu content and without showing visible symptoms of damage possibly could cause a risk to human health from the transfer of the metal in food. © 2005 Wiley Periodicals, Inc. Environ Toxicol 20: 188–194, 2005.

Keywords: copper; *Brassica pekinensis*; electrolyte leakage; peroxidase activity; chlorophyll; biomass; Cu concentration; human health risk

INTRODUCTION

Copper is a major heavy metal contaminant that results from mining, metal processing, fertilizers, fungicides, agricultural and municipal wastes, sewage sludge dispersal, as well as traffic emissions (Lepp and Dickinson, 1994; Wang, 1997; Xiong, 1998; Kabata-Pendias and Pendias, 2001). Although it is an essential micronutrient for normal plant metabolism, playing an important role in a large number of metalloenzymes, photosynthesis-related plastocyanin, and membrane structure, copper has been reported to be among the most toxic of heavy metals (Wong and Bradshaw, 1982; Li and Xiong, 2004). Excess Cu inhibits plant growth and seed germination, induces chlorophyll degradation, and interferes with photosystem activity (Balsberg Pahlsson, 1989; Fernandes and Henriques, 1991; Yruela et al., 1996; Caspi et al., 1999). At the molecular level, Cu ions generate reactive oxygen species such as O_2^-, H_2O_2, and $\cdot OH$ (Van Assche and Clijsters, 1990; Babu et al., 2001). These reactive radicals cause oxidative damage of lipids, proteins, and nucleic acids. Cu ions also are responsible for alterations of membrane integrity in plant cells. Cu-mediated membrane lipid peroxidation causes membrane damage, thus changing membrane permeability and

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leading to electrolyte leakage (Devi and Prasad, 1998; Tarhanen et al., 1999; Quartacci et al., 2001). Plants have evolved several antioxidant defense mechanisms to protect themselves from oxidative damage. Among these mechanisms, peroxidases (POD) play an important role in quenching reactive oxygen species and subsequently alleviating their toxic effects (Van Assche and Clijsters, 1990). As a result, when exposed to metal stress, plants usually show peroxidase induction (Koricheva et al., 1997; Devi and Prasad, 1998; Teisseire and Guy, 2000; Diaz et al., 2001).

It is generally accepted that heavy metal toxicity to plants is positively correlated to the concentration of the metals in plant tissues; higher metal concentrations in the tissues usually induce stronger damage to the plants. On the other hand, the damage caused by heavy metals is involved with tolerance of the plants (Liu and Xiong, 2004). Some species or populations are sensitive to heavy metal stress, whereas others are tolerant, showing little inhibition or damage, even if they grow in a severely polluted environment and accumulate high concentrations of heavy metals in their tissues. Heavy metal-tolerant species of higher plants have been reported in many families such as Cruciferae, Caryophyllaceae, Gramineae, Leguminosae, and Asteraceae (Kruckeberg and Kruckeberg, 1990; Brooks, 1998). The tolerance of plants to elevated concentration of heavy metals in growth media and in tissues raises a health risk to humans and animals because of the bioaccumulation of a high concentrations of the metals in plants (Kabata-Pendias and Pendias, 2001). Heavy metals that are tolerated by plants and are toxic to humans and animals are easily transferred to consumers through food supply and consequently cause health problems. This is a special problem in vegetables with roots and leaves that are edible by human beings because heavy metals are usually accumulated in these parts.

Brassica pekinensis Rupr (belonging to Cruciferae) is a popular leafy vegetable in China. This vegetable has a large number of varieties and can be cultured throughout the year depending on the varieties. It was found that this species could accumulate high concentrations of heavy metals under conditions of nutrient deficiency (Xiong et al., 2002) and high concentrations of NH_4Cl and $FeCl_3$ in the growth medium (Xiong and Feng, 2001). In recent years a relatively large area of farmland has been contaminated by heavy metals because of industrialization and urbanization in China. This vegetable often is grown on farmland near highways, in mining areas where soils are usually contaminated by heavy metals, and in soils amended with heavy metals contained in sewage sludge. This suggests that the plant can tolerate some extent of heavy metal contamination and that as a food supply it could create a health risk to consumers. The aim of the present study was to investigate the phytotoxic effects and bioaccumulation of Cu in the plant *Brassica pekinensis* Rupr. The results of this investigation have implications for heavy metal ecotoxicology involved with the vegetable supply for food.

MATERIALS AND METHODS

Plant Material and Seed Germination

The cultivar Xiayangbai of Chinese cabbage (*Brassica pekinensis* Rupr.) was used for the experiments. Seeds of this cultivar were purchased from a local seed market in Wuhan. The seeds were sterilized in 3% formalin (CH_2O) for 5 min to prevent fungal growth, washed with distilled water for several changes, and soaked in water overnight. The soaked seeds were placed in plastic dishes 10 cm in diameter with a layer of filter paper on the bottom. In each dish 15 seeds were evenly placed on the paper, and 10 mL of the Cu solution with the required Cu^{2+} [as $Cu(NO_3)_2$] concentration was added. The Cu^{2+} concentrations in the solution were 0, 0.008, 0.031, 0.125, 0.5, and 2.0 mM/L. Each treatment had two replicates (dishes). Exposure was maintained for 72 h under dark condition at 25°C. Seeds with a root longer than 1 mm were regarded as germinated, and the germination rate and root and shoot lengths were recorded.

Plant Preculture and Cu Treatment

Pot experiments were conducted to test Cu phytotoxicity and Cu accumulation in the plants. The soil for the plant culture was a natural loamy soil, collected from the campus of Wuhan University. Some of the soil parameters were: pH 6.2, cation exchange capacity 31.9 cM/kg (+) dry soil, total Cu 13.6 mg/kg dry soil, and organic matter content 3.1%. The air-dried and mixed soil was screened to pass through a 1-mm sieve, and water content was determined. The screened soil was placed in 14-cm-diameter round plastic pods, and each pod was filled with 400 g (DW) of soil. Seed sterilization, soak, and germination were the same as described above. The germinated seeds were evenly sown in the soil-filled pots, each pot containing 20 seeds. The pots were placed in a plastic greenhouse, watered daily, and fertilized occasionally with Hoagland's solution depending on the growth status of the plants. When seedlings had developed 5 or 6 leaves, they were thinned to retain 15 uniform plants, and the pods were translocated to a cultural facility with supplementary lighting (14-h photoperiod, 4000 lux) and a temperature of 15°C–25°C. Then Cu^{2+} [as $Cu(NO_3)_2$] solution was added to the pods at three levels: 0, 0.2, and 1.0 mM/kg dry soil.

POD (EC 1. 11. 1. 7) Activity Assay

The POD activity assay was carried out 7 days after the addition of Cu. The fresh leaves (0.5 g) for each replicate were homogenized using a chilled pestle and mortar in 5 mL of 20 mM potassium phosphate. The homogenate was centrifuged at 10 000 rpm for 10 min, and the resulting supernatant was used for POD assay. POD activity was determined in a 4-mL reaction mixture containing 1 mL of

TABLE I. Seed germination rate (%) and root and shoot lengths (mm) of young seedlings of *Brassica pekinensis* subjected to 72 h of Cu solution treatment in culture dishes

Cu treatment (mM/L)	Seed Germination (%)	Root Length (mm)	Shoot Length (mm)
0	100 ± 0 a	8.0 ± 7.3 a	3.9 ± 1.6 b
0.008	96.7 ± 4.7 a	7.8 ± 5.3 a	4.9 ± 2.0 a
0.031	100 ± 0 a	4.6 ± 2.5 b	4.0 ± 1.4 b
0.125	100 ± 0 a	3.3 ± 2.0 b	4.0 ± 1.3 b
0.5	43.3 ± 14.1 b	0.5 ± 0.5 c	0.9 ± 1.1 c
2.0	0 ± 0 c	0 ± 0 c	0 ± 0 d
F	96.72	23.54	62.67
P	< 0.001	< 0.001	< 0.001
$LSD_{0.05}$	14.892	1.975	0.697

Results are means ± SD ($n = 3$). Means with different letters are significantly different from one another ($P < 0.05$) according to *LSD*.

supernatant, 3.35 mM H_2O_2, 0.05% (v/v) guaiacol, and 100 mM sodium phosphate buffer (pH 6.0). How much the supernatant was diluted depended on the enzyme concentration. The rate of increase in absorbance was measured at 470 nm using a UV/vis spectrophotometer (model UV-9100, Beijing Ruili) within 2 min. A unit of POD activity (ΔA_{470}) was defined as the change in absorbance per minute and per gram fresh weight (FW) of the tissue.

Electrolyte Leakage Determination

Plant electrolyte leakage was determined 12 days after the addition of Cu by measuring the conductivity of leaf leachate (IPPAS, 1999). For each replicate, two samples (each 0.5 g, FW) of leaf disk (14 mm in diameter) were prepared with a cork borer symmetrically from either side of the main vein of the leaf blades. One sample was placed in a test tube containing 20 mL of deionized water. The tubes were placed in a vacuum vessel for 30 min at 25°C and then were returned to normal atmospheric pressure for 1 h. Next, the conductivity of the solution was measured with an electrical conductivity meter (model DDS-11A, Shanghai Dapu). The other sample also was placed in a test tube containing 20 mL of deionized water, and the tube was covered. The covered tubes were then immersed in boiling bath for 10 min. After the tubes cooled, solution conductivity was measured with an electrical conductivity meter. Relative conductivity (RC) was calculated according to the formula $RC = Cv/Cb \times 100$, where Cv is the conductivity of leachate from the vacuum-treated samples and Cb is the conductivity of leachate from the heat-killed samples.

Chlorophyll Estimation

The chlorophyll content of the plants was determined 13 days after the addition of Cu. Fresh leaves (0.5 g) were homogenized using a pestle and mortar in 3 mL of 80% acetone with a small amount of quartz sand. The homogenate was filtered through ashless filter paper and made up to 10 mL with 80% acetone. The filtered solution was used for chlorophyll estimation. Absorbance of the solution was measured at 645 and 663 nm using a UV/vis spectrophotometer (model UV-9100; Beijing Ruili). Chlorophyll a, chlorophyll b, and total content were calculated according to the following formulas: $C_a = 12.72\ A_{663} - 2.59\ A_{645}$, $C_b = 22.88\ A_{645} - 4.67\ A_{663}$, $C_T = 20.29\ A_{645} + 8.05\ A_{663}$.

Plant Biomass and Cu Measurement

The shoots were harvested 17 days after the addition of Cu and dried in an oven at 60°C for 24 h. Then the biomass (dry weight) was determined with an electronic balance. The dried samples were ashed in a muffle furnace at 500°C for 15 h. The ash was dissolved in a mixture of concentrated $HNO_3/HClO_4$ [2:1 (v/v)] and heated at a platen heater. After cooling the extract was diluted and made up to 10 mL with 1 M HNO_3. The Cu concentration of the extracts was determined with a flame atomic absorption spectrophotometer (model WF-5; Guizhou Xintian).

Statistical Analysis

One-way ANOVA was performed to test the effects of Cu on seed germination, root and shoot length, biomass, chlorophyll content, electrolyte leakage, POD activity, and shoot Cu concentration. If the F value showed a significant difference ($P < 0.05$), means were compared with the least-significant-difference (LSD) test. The LC_{50} of Cu for seed germination was calculated according to the linear regression method.

RESULTS

Seed Germination and Shoot Biomass

Seed germination was significantly influenced by Cu ($P < 0.001$; Table I). The 0.5 mM/L Cu treatment remarkably reduced the germination rate, and the LC_{50} (median lethal concentration), calculated as the lethal effect on seed

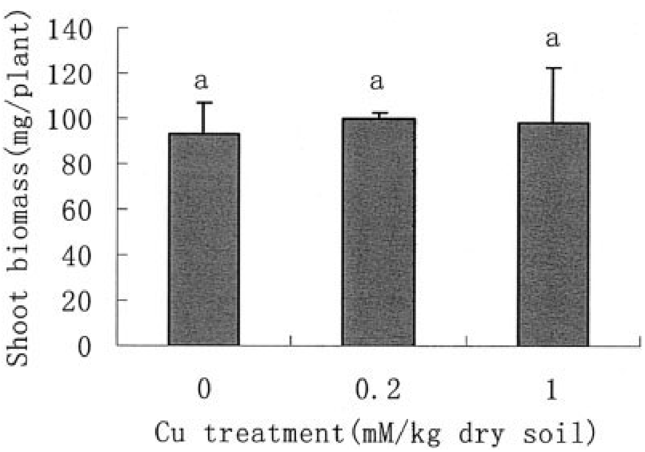

Fig. 1. Shoot biomass (dry weight) of *Brassica pekinensis* subjected to Cu treatments (0, 0.2, and 1.0 mM/kg dry soil). Bars are standard deviation ($n = 3$). Means with the same letter are not significantly different from one another ($P > 0.05$) according to *LSD*.

germination, was 0.348 mM/L. Root and shoot length of the young seedlings also was inhibited by Cu (Table I), but stimulatory elongation of the shoots occurred with the 0.008 mM/L treatment.

Shoot biomass was not significantly influenced by Cu treatment in the soil ($P = 0.844$, $F = 0.174$; Fig. 1).

Chlorophyll Content

Copper treatment led to significant effect on total chlorophyll (a + b) content ($P = 0.028$, $F = 6.831$; Fig. 2). Surprisingly, chlorophyll content increased rather than decreased with an increase in Cu concentration in the soil.

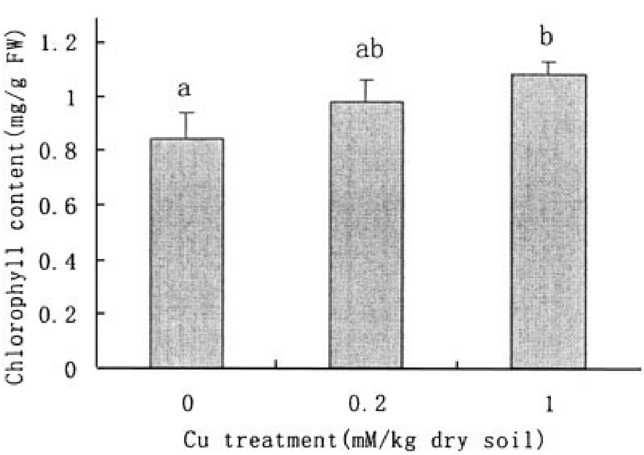

Fig. 2. Chlorophyll (a+b) content of *Brassica pekinensis* subjected to Cu treatments (0, 0.2, and 1.0 mM/kg dry soil). Bars are standard deviation ($n = 3$). Means with different letters are significantly different from one another ($P < 0.05$) according to *LSD*.

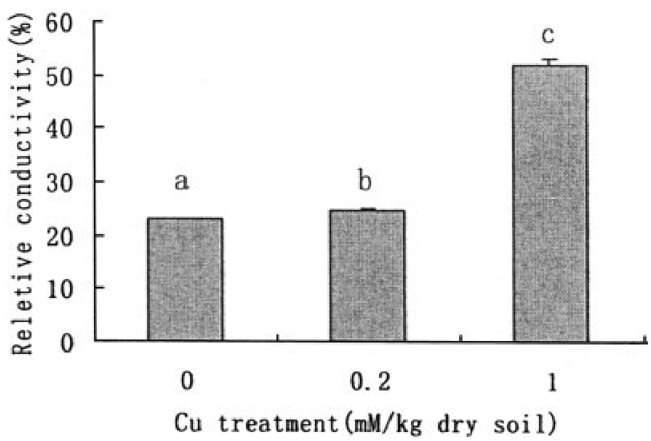

Fig. 3. Relative conductivity of leaf leachate of *Brassica pekinensis* subjected to Cu treatments (0, 0.2, and 1.0 mM/kg dry soil). Bars are standard deviation ($n = 3$). Means with different letters are significantly different from one another ($P < 0.05$) according to *LSD*.

The chlorophyll content with the 1 mM/kg treatment was 27.4% higher than that of the control. These facts indicate that when the Chinese cabbage (cultivar Xiayangbai) plants ware exposed to certain levels of Cu pollution, Cu had a stimulatory effect on chlorophyll content. Cu was found not to have a significant effect on the ratio of chlorophyll a to chlorophyll b (data not shown).

Electrolyte Leakage

The relative conductivity of leaf leachate was strongly affected by Cu ($P < 0.001$, $F = 1622.085$; Fig. 3). Conductivity increased with Cu concentration in the soil. With the 1.0 mM/kg Cu treatment, conductivity showed a 2.25-fold increase over the control. These data indicated that Cu could induce electrolyte leakage of leaf cells when Cu was taken up and translocated to the shoots.

POD Induction

POD activity was significantly induced by Cu treatment ($P = 0.006$, $F = 13.844$; Fig. 4). Activity increased with an increase in the concentration of Cu in the soil. With the 1.0 mM/kg treatment, POD activity was significantly higher than both the control and the 0.2 mM/kg treatment according to the LSD, whereas POD activity with the 0.2 mM/kg treatment was not significantly higher than that of the control.

Cu Concentration in the Shoots

Cu concentration in the shoots was significantly influenced by Cu treatment ($P < 0.001$, $F = 183.916$; Fig. 5). Cu concentration increased markedly with an increase in the soil Cu concentration. With a background level of Cu (the

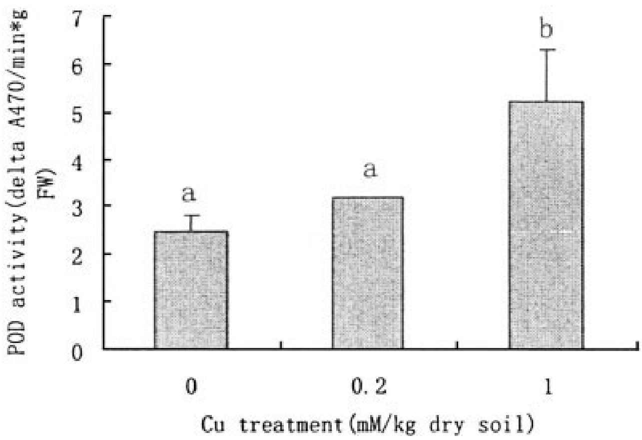

Fig. 4. Peroxidase activity in leaves of *Brassica pekinensis* subjected to Cu treatments (0, 0.2, and 1.0 mM/kg dry soil). Bars are standard deviation ($n = 3$). Means with different letters are significantly different from one another ($P < 0.05$) according to *LSD*.

control, 13.6 mg Cu/kg dry soil), Cu concentration in the shoots was 9.9 mg/kg. With the 0.2 mM/kg treatment, shoot Cu concentration rose to 42.5 mg/kg. With the 1.0 mM/kg treatment, shoot Cu concentration was 119.0 mg/kg (1.9 mM/kg). According to the LSD test, shoot Cu concentration in both treatments was significantly higher than that in the control. These facts showed that when Chinese cabbage (cultivar Xiayangbai) plants were exposed to certain levels of Cu pollution, the shoots could accumulate a relatively high amount of Cu.

DISCUSSION

Studies have shown that plants grown in Cu-contaminated soil usually accumulate an elevated Cu content in their tissue (Bargagli, 1998). As a result, a series of physiological and toxicological responses take place in plants depending on the Cu concentration in the tissue. It was suggested that 12 ppm be a global value for Cu concentration in plant biomass in unpolluted conditions and that values higher than that lead to toxic responses in plants (Balsberg Pahlsson, 1989; Fernandes and Henriques, 1991). In the present study, a remarkable positive correlation between soil and shoot Cu concentrations was observed, and the Cu concentration reached 43 and 119 mg/kg (DW) with the 0.2 and 1.0 mM/kg Cu treatments, respectively (Fig. 5). These values are several times higher than the suggested normal Cu concentration and thus would be expected to be toxic to the plants. The increased electrolyte leakage (Fig. 3) and POD activity (Fig. 4) clearly illuminated the toxic effects of Cu on the plants. Similar results have been reported in the literature. Membrane leakage caused by Cu, generally regarded as a result of cell membrane damage, was found in *Silene cucubatus* (De Vos et al., 1989), *Ceratophyllum demersum* (Devi and Prasad, 1998), wheat (Quartacci et al., 2001), *Rumex dentatus* (Liu and Xiong, 2004), and lichen (Tarhanen et al., 1999). Also, Cu exposure usually gave rise to POD induction (Koricheva et al., 1997; Devi and Prasad, 1998; Teisseire and Guy, 2000; Diaz et al., 2001; Liu and Xiong, 2004). Both these responses could be explained by Cu-mediated generation of reactive oxygen species (e.g., H_2O_2, O_2^-, or $\cdot OH$). These radicals could cause peroxidation of membrane lipids, thus altering membrane integrity and inducing electrolyte leakage. To protect themselves from oxidative damage, plant cells could scavenge the radicals by induction of POD and other antioxidant enzymes (Van Assche and Clijsters, 1990; Fernandes and Henriques, 1991).

On the other hand, in the experiments we did not observe any visible symptoms of Cu toxicity such as chlorotic and brown points on leaves and leaf margins. Cu treatment increased rather than decreased chlorophyll content (Fig. 2). Chlorophyll content increased with an increase in Cu concentration in the soil and in the plant tissue. A similar result was observed in maize, in which chlorophyll content significantly increased in seedlings cultured in Cu-containing solutions (see Balsberg Pahlson, 1989), whereas in other studies, chlorophyll content was found to be reduced by Cu treatment (Ouzounidou, 1996; Devi and Prasad, 1998; Ralph and Burchet, 1998). The reason for this difference in chlorophyll response is unknown but might be related to tolerance of the plants to the metal. Tolerant plants would increase chlorophyll content presumably because of certain compensatory mechanisms for Cu stress. Also, shoot biomass was not significantly influenced by Cu treatment (Fig. 1). Even if Cu concentration in the shoot reached 119 mg/kg with the 1 mM/kg Cu treatment, the biomass was not reduced compared to the control. According

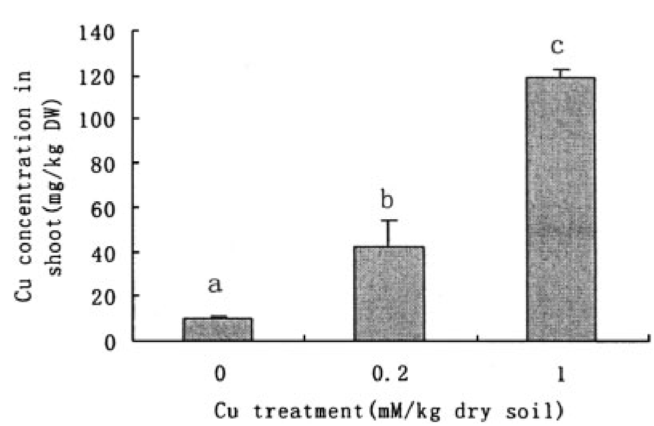

Fig. 5. Cu concentration in dry shoot of *Brassica pekinensis* subjected to Cu treatments (0, 0.2, and 1.0 mM/kg dry soil). Bars are standard deviation ($n = 3$). Means with different letters are significantly different from one another ($P < 0.05$) according to *LSD*.

to Kabata-Pendias and Pendias (2001), growth depression of sensitive plants was observed with a Cu concentration in tissues of 15–20 ppm (DW), and a 10% decrease in yield was most likely with a Cu concentration of 10–30 ppm (DW). These values are much lower than 119 mg/kg with the 1 mM/kg Cu treatment in the present study. These facts suggest that to some extent, Chinese cabbage is a heavy-metal-tolerant vegetable. The reason for this is not clear, and further research on heavy-metal-tolerant mechanisms is needed.

Therefore, the results generated from our study show that although Cu treatment induces electrolyte leakage and POD activity, thus exerting some extent of phytotoxic effects on the plants, it cannot damage plant performance and reduce plant growth and productivity at the level of Cu pollution used in this study, as was shown by the chlorophyll content and biomass. Unfortunately, from the point of view of the food chain, vegetables with an elevated Cu content that do not have visible toxic symptoms (e.g., chlorosis, stunt and yield reduction) would pose a risk to human health. There is the possibility that people will eating healthy-looking vegetables that actually have a high heavy metal content. Copper, as a essential micronutrient for human beings, is a constituent of some metalloenzymes and is required in hemoglobin synthesis and in the catalysis of metabolic oxidation (Strausak et al., 2001). However, the intake of excess Cu is deleterious to health, causing disorders including liver cirrhosis, dermatitis, and neurological disorders (Somer, 1974; Tanner, 1998; Eife et al., 1999). The amount of metals ingested by humans is directly related to their content in foodstuffs. For an adult the estimated safe and adequate daily dietary intake of Cu is 1500–3000 μg (Voutsa and Sarnara, 1998) or 2–5 mg (Xu and Yang, 1995). The FAO (1985) recommended that the daily allowance of dietary Cu be 2.5 mg. According to WHO (1996), the daily amount of needed Cu is 0.7 mg/day for a man and 0.8 mg/day for a woman, and the recommended dietary allowance is 0.9 mg per day for adults. The common values of dietary Cu intake reported by many countries are in the range of 0.7–5.0 mg/day (Onianwa et al., 2001). In this study, the Cu concentration in the fresh weight of the shoots of Chinese cabbage was 0.935, 4.038, and 11.302 mg/kg for Cu treatments of 0, 0.2, and 1.0 mM/kg, respectively. If an adult consumes 0.5 kg daily, a common average value among the Chinese, of healthy-looking Chinese cabbage grown in 1.0 mM/kg Cu–contaminated soil in this study, the adult will have a Cu intake of 5.651 mg. This intake alone is higher than the estimated safe and adequate daily dietary intake of Cu. Plus other sources of Cu intake, such as cereals (Cu FW content in the range of 0.3–13.0 ppm), fruits (0.3–4.0 ppm), nuts (0.2–23.8 ppm), (Kabata-Pendias and Pendias, 2001), beans (11.3–30 ppm DW; Liu, 2001), and eggs (0.9–2.6 ppm; Sun et al., 2003), mean Cu daily intake will be much higher than the estimated safe and adequate daily dietary intake for an adult.

In conclusion, our results have demonstrated Cu phytotoxicity in *Brassica pekinensis*. Cu treatments increased electrolyte leakage and POD activity, showing a significant correlation between Cu concentration in shoots with electrolyte leakage and POD activity. Phytotoxicity also was shown by the decline in seed germination and the inhibition of young seedling growth. However, at the Cu treatment levels used in this study, copper did not induce visible toxic symptoms. Cu treatments stimulated chlorophyll content and did not reduce biomass. The addition of Cu in the soil resulted in Cu bioaccumulation in the edible parts of the vegetable. Cu content in the shoots was significantly positively correlated with Cu concentration in the soil. Given the transfer of heavy metals through the food chain, vegetables with elevated Cu content but that do not have visible symptoms of damage has the possibility of being a health risk in humans.

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