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ORIGINAL PAPER

Accumulation, subcellular localization and ecophysiological responses to copper stress in two *Daucus carota* L. populations

Wenshan Ke · Zhiting Xiong · Mingji Xie · Qin Luo

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Abstract Copper accumulation, subcellular localization and ecophysiological responses to excess copper were investigated using pot culture experiments with two *Daucus carota* L. populations, from a copper mine and an uncontaminated field site, respectively. Significant differences of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations and antioxidant enzyme [superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX)] activities of leaves under Cu treatment were observed between the two populations. At high Cu concentrations (400 and 800 mg kg^{-1}), a significant increase in contents of MDA and H_2O_2 but a significant decrease in activities of SOD, CAT and APX were observed in uncontaminated population. Contrarily, the population from copper mine maintained a lower level of MDA and H_2O_2 but higher activities of SOD, CAT and APX. Copper accumulation in roots and shoots increased significantly with the increase of copper concentrations in soils in the two populations. No significant difference of the total Cu in roots and shoots was found between the two populations at same copper treatment. There were also no striking differences of cell wall-bound Cu and protoplasts Cu of leaves between the two populations. The difference was that Cu concentration in vacuoles of leaves was 1.5-fold higher in contaminated site (CS) population than in uncontaminated site population. Hence, more efficient vacuolar sequestration for Cu and maintaining high activities of SOD, CAT and APX in the CS population played an important role in maintaining high Cu tolerance.

Keywords Antioxidant enzymes · Copper subcellular localization · Cu resistance · *Daucus carota* L. · Lipid peroxidation

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Introduction

Heavy metal contamination in soils can be a powerful selective force in plant evolution (Antonovies et al. 1971). Specialized metal-resistant plants can colonize in the metalliferous soils. Plants growing in the metalliferous soils are often genetically distinct from the same species growing in soils with low heavy metal content (Ye et al. 2003).

These plants develop high resistance or tolerance to the heavy metals, forming heavy metal resistant populations (Baker 1978; Ernst 1990). Specific mechanisms may have developed in the resistant or tolerant populations via natural selection in response to heavy metal contamination of soils. There is a variety of metal resistant or tolerant mechanisms in varied plant species. But two basic strategies can be classified in these mechanisms (Tong et al. 2004). One strategy is to detoxify heavy metal ions entering the cytoplasm through inactivation via chelation or conversion of a toxic ion into a less toxic or easier to handle form and/or compartmentalization (Clemens 2001; Hall 2002; Lou et al. 2004; Tong et al. 2004). Plants, accumulator or hyperaccumulators with the strategy can accumulate large amounts of heavy metals in plant tissues even in aerial parts. Conversely, the other strategy is to restrict uptake and accumulation of metals by preventing the metal from being transported across the plasma membrane, either by increased binding of metal ions to the cell wall or by reduced uptake through modified ion channels, or by pumping the metal out of the cell with active efflux pumps (Tong et al. 2004). Most resistant or tolerant plants with the strategy often accumulate less metal in tissues when compared with conspecific non-tolerant individuals (Baker et al. 1983; De Vos et al. 1992; Ouzounidou et al. 1994; Clemens 2001; van Hoof et al. 2001; Llugany et al. 2003).

Cu is an essential micronutrient and a component of several enzymes mainly participating in electron flow and catalyzing the redox reactions (Ouzounidou et al. 1991). However, excess copper can interfere with numerous physiological processes (Fernandes and Henriques 1991). Cu^{2+} may cause oxidative stress by inducing production of reactive oxygen species (ROS), such as $O_2^{\cdot-}$, H_2O_2 and OH•, via Haber–Weiss and Fenton reactions (Aust et al. 1985). Tolerance and protective mechanisms have evolved to scavenge these free radicals. Antioxidant enzymes such as peroxidase, ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) and antioxidant compounds such as glutathione, ascorbate and carotenoids (Zhang and Kirkham 1996) play a key role in controlling the cellular level of these radicals and peroxides. The response of these antioxidant enzymes to metal stress varies with the plant species and the metal species (Mazhoudi et al. 1997). Thus, the knowledge of how plants cope with metal-induced oxidative stress is of considerable importance in understanding the metal tolerance mechanisms evolved by plants.

Daucus carota L. (Umbelliferae) is one of dominant plant species growing in Cu mine at Tonglushan hill in Hubei Province of China. Mining activities at this hill dated back about 3,000 years, which caused considerably elevation of Cu concentration in the local soil. In previous investigation, it was found that *D. carota* was the only with a shoot/root ratio for Cu >1 in several dominant species investigated (Ke et al. 2001). However, little information is available on the Cu accumulation capacity and the Cu-resistance mechanism of *D. carota*. *D. carota* is also found in non-contaminated sites. We hypothesize that this population growing at Cu contaminated sites (CSs) would have adapted itself to the contaminated habitat by forming specialized Cu-resistant mechanisms, and would differ from those from the non-contaminated site in antioxidant response to Cu stress.

Studies on uptake and accumulation of Cu by plants have been reported recently (Llugany et al. 2003; Liu and Xiong 2005). Knowledge of the subcellular localization and identification of heavy metals can provide essential information on metal toxicity and bioaccumulation mechanisms (Liu et al. 2004). The Cu and other elements localization and heavy mental tolerance mechanisms in plants have been studied by electron dispersive X-ray spectroscopy (EDXS; Kupper et al. 2000; Monni et al. 2002), electron energy loss spectroscopy (EELS; Neumann et al. 1995; Barcelo and Poschenrieder 1999; Liu et al. 2004; Rau et al. 2006), proton induced X-ray analysis (PIXE; Krämer et al. 1997) and Synchrotron radiation X-ray fluorescence microprobe technique (Shi et al. 2004). EDXS and EELS of these techniques were most usually used to reveal subcellular localization of Cu and other metals (MacFarlane and Burchett 2000; Brunner and Frey 2000; Monni et al. 2002; Rau et al. 2006). However, both methods have their advantages and limitations (Stelzer and Lehmann 1993; Bucking et al. 1998; Monni et al. 2002). Careful

attention is needed to ensure that mobile ions are not redistributed during sample preparation and that the ultrastructure of the plant tissues is preserved (Krämer et al. 1997).

A modified protocol of fractionation techniques is also often used in analysis of metal subcellular distribution, especially in analysis of the vacuoles function. For example, Krämer et al. (2000) and Song et al. (2003) demonstrated successfully the function of vacuoles in metal compartmentation and tolerance in *Thlaspi* species and plants *Arabidopsis*, respectively.

Soil pot experiments were conducted in this study. The aims were to reveal: (1) the differences of subcellular distribution of Cu between two *D. carota* populations, (2) and ecophysiological responses to copper stress and the possible mechanisms underlying the difference.

Materials and methods

Plant material and Cu treatment

Two natural populations of *D. carota* L. were selected for study, one population from Cu-contaminated site (CS), the other from uncontaminated site (UCS). The seeds of the CS population were collected from the outcrop of Cu mine at Tonglushan hill in Hubei Province, China, and the seeds of the UCS population were collected from the Yanxi lake nearby in Wuhan city, China.

In each population, great quantities of seeds were taken from a large sample with more than 60 randomly selected plants, and the seeds were finally pooled. The seeds for the pot experiments were randomly taken from the pooled ones. Background values of copper content in the soils of the two sites are listed in Table 1. The soil used for plant culture in this study was collected from the Oil Crop Institute of China Agriculture Science in Wuhan city. Some parameters of the soil were measured as follows: pH 7.1 ± 0.12, cation exchange capacity 5.8 ± 1.4 cmol kg^{-1} dry soil, organic matter content 18.4 ± 2.1 g kg^{-1}, total Cu 58.6 ± 8.74 mg kg^{-1}, available Cu 9.6 ± 1.62 mg kg^{-1}.

The seeds were sterilized in 0.3% hypochlorite for 20 min and washed three times with double distilled water. Then the seeds were transferred into a Pyrex dish and displayed on a filter paper sheet. Germination took place in an incubator with the following condition: photoperiod 16 h light/8 h darkness, day/night temperature 25°C/20°C. After germination, seedlings were evenly planted in 15-cm-diameter and 6-cm-depth round plastic pots filled with 400 g (dry weight, DW) soils, each pot with 15 seedlings. Then the seedlings were grown under natural light and ten seedlings of even growth per replicate were hold. When the seedlings had five or six leaves, Cu treatment was conducted. Cu^{2+} (as $CuSO_4{\cdot}5H_2O$) solution was added to the pots at five levels: 0, 100, 200, 400 and 800 mg kg^{-1} dry soil. Each treatment had three replicates. There were 15 pots (15 independent replicates) each population in this experiment.

In addition, experiments with another three replicates of each treatment at the levels of 0, 100 and 400 mg kg^{-1} Cu treatments were conducted for the analysis of localization of leaves Cu. There were nine pots (nine independent replicates) each population in this experiment.

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 0.4 g leaf fresh weight (FW) obtained at Cu treatment for 7 days according to Heath and Packer (1968). MDA is a product of lipid peroxidation by

Table 1 Copper concentration, organic matter content and pH in the local soils supporting the natural populations of *D. carota* L. (0–10 cm depth)

Sample site	Cu_{total} (mg/kg)	Cu_{NH_4Ac} (mg/kg)	C_{org} (%)	pH (H_2O)
Polluted area	7789.8 ± 542.7	704.9 ± 48.3	1.68 ± 0.06	6.51 ± 0.43
Unpolluted area	32.4 ± 6.4	8.6 ± 1.2	2.46 ± 0.09	6.34 ± 0.40

Results are means ± standard deviation (n = 3)

thiobarbituric acid (TBA) reaction. Fresh leaf tissues were homogenized with 10% TCA and centrifuged at 10,000 *g* for 10 min at 4°C. Two milliliters of supernatant was mixed with 2 mL 0.6% TBA and the mixture was heated at 95°C for 30 min, quickly cooled on ice and then centrifuged at 4,000 *g* for 20 min. The MDA concentration of the resultant supernatant was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Antioxidant enzyme assay

Fresh leaf samples of both populations obtained at Cu treatment for 7 days were used for enzyme analysis. For extraction of enzyme, leaf tissues (0.5 g FW) were homogenized with ice-cold 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1% Triton X–100 and 1% polyvinylpyrrolidone PVP-40 (w/v) in a chilled pestle and mortar. The homogenate were centrifuged at 12,000 *g* for 30 min at 4°C. Supernatant was used for enzyme activity and protein content assays. All assays were done at 4°C. All spectrophotometric analyses were conducted by a Shimadzu (UV-1600) spectrophotometer.

Superoxide dismutase (EC 1.15.1.1) activity was assayed according to the method of Beauchamp and Fridovich (1971) in terms of its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.8), 75 μM NBT, 13 mM L-methionine, 0.1 mM EDTA and 0.002 mM riboflavin. Reactions were carried out at 25°C, under light intensity of about 300 $mmol^{-1}$ m^{-1} s^{-1} through 10 min. One unit of SOD was defined as the enzyme amount causing 50% inhibition reduction of NBT and the enzyme activity was expressed in units per milligram protein.

Catalase (EC 1.11.1.6) activity was determined by measuring the disappearance of H_2O_2 according to Aebi (1984). The decomposition of H_2O_2 was followed by absorbance decrease at 240 nm for 90 s and was calculated per 60 s. The reaction mixture contained 50 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA and 15 mM H_2O_2. Addition of H_2O_2 started the reaction and μmol mL^{-1} H_2O_2 destroyed per minute was defined as one unit of CAT.

Ascorbate peroxidase (EC 1.11.1.11) activity was measured following Nakano and Asada (1981). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA Na_2 and 250 mM H_2O_2. The oxidation of ascorbic acid was measured by the decrease in absorbance at 290 nm, and the enzyme activity was calculated by using the absorbance coefficient (2.8 mM^{-1} cm^{-1}) for ascorbic acid. One enzyme unit was defined as μmol mL^{-1} oxidized ascorbate per minute.

Total soluble protein content in enzyme extracts was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Hydrogen peroxide

The hydrogen peroxide (H_2O_2) content of the samples was colorimetrically measured as described by Jana and Choudhuri (1981). Leaf tissues (0.4 g FW) were homogenated with 1.5 mL of 50 mM potassium phosphate buffer, pH 6.5. The homogenate was centrifuged at 6,000 *g* for 20 min at 4°C. To determine H_2O_2 content, 3 mL of extracted solution was mixed with 1 mL of 0.1% titanium sulfate in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6,000 *g* for 15 min at 4°C. The resultant supernatant was read at 410 nm. H_2O_2 content was calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}

Chlorophyll determinations

Chlorophyll concentrations in leaf extracts, protoplasts and vacuole-enriched fractions were estimated according to the method of Alan (1994).

Isolations of cell walls, protoplasts and vacuoles

Leaf tissues after 100 mg kg^{-1} and 400 mg kg^{-1} Cu treatments (including the control) for 7 days were experimented with isolations of cell walls

and protoplasts, separately. Cell walls were done according to the method described by Fritioff and Greger (2006). The leaf tissues were homogenized in liquid nitrogen immediately and then extracted in extraction buffer comprising 500 mM sucrose, 1.0 mM dithiothreit (DTT), 5.0 mM ascorbic acid, 50 mM Hepes (4-(2-hydroxyethyl)–1-piperazineethanesulfonic acid) and 1.0% (w/v) polyvinyl polypyrrolidone, adjusted to pH 7.5 with NaOH. The homogenate was sieved through a nylon cloth (100 μm mesh size) and washed with extraction buffer; this residue, together with the pellet retained after centrifugation of the filtrate at 100 *g* for 5 min, constituted the cell wall-bound metals.

The isolation of protoplasts was carried out according to a modified protocol based on Krämer et al. (2000). Leaves were placed in Petri dishes (1 g of leaf material per dish) containing 10 mL of washing buffer supplemented with 0.05% (w/v) BSA, 0.5 mM DTT, 2% (w/v) Cellulysin and 0.1% (w/v) pectolyase Y-23. Digestion of cell walls was carried out for 8 h in the light (1,600 lux) at 25°C with gentle shaking at 30 rpm. Ten milliliters of digest were filtered through a nylon mesh (114 μm) and rinsed twice with 5 mL of washing buffer. For protoplast purification approximately 3.5 mL of filtrate was layered onto 1.5 mL of washing buffer containing 20% (w/v) sucrose in a glass centrifuge tube. The gradient was centrifuged in a swinging bucket rotor at approximately 100 *g* for 20 min. The protoplasts were collected from the interface with a pipette and mixed gently with 6 mL of CPW20S solution (containing 20% sucrose), and the suspension was centrifuged at 100 *g* for 15 min. Protoplasts were collected at the sucrose interface, suspended gently in 10 mL CPW10M (10% mannitol) to obtain a protoplast suspension. The supernatant was then removed, and protoplasts were suspended in washing buffer to obtain a protoplast suspension of 1×10^6 to 1.5×10^6 protoplasts per milliliter, as determined by quadruplicate counting of aliquots on a hemacytometer. Aliquots of this suspension were used for the preparation of vacuoles and for the quantification of Cu.

The isolation of vacuoles was carried out according to a modified protocol based on Krämer et al. (2000). To achieve the release of intact vacuoles, a protocol was used in which protoplasts were subjected to a gentle osmotic shock in combination with a pH increase in the medium and low concentrations of a mild detergent (Wagner and Siegelman 1975). Protoplast suspension, 1.5 mL, was added to 6 mL of ice-cold lysis medium containing 344 mM mannitol, 0.75 mM CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonicacid), 25 mM HEPES–Tris, pH 8.0 and 1.25 mM EGTA. The suspension was gently stirred with a toothpick for 10 min. Aliquots of 1.25 mL of the lysed protoplast suspension were dispensed into 15-mL disposable glass centrifuge tubes and mixed gently with 2.1 mL of a solution containing 500 mM mannitol, 1 mM EGTA, 0.5 mM CHAPS, 20 mM HEPES–Tris, pH 8.0 and 20% (w/v) sucrose. Ten microliters of a 0.3% (w/v) solution of neutral red in water was added to one of the tubes to monitor the position of the vacuoles in the density gradient. The mixture was carefully overlaid first with 1.25 mL of a solution containing 475 mM mannitol, 0.2 mM CHAPS, 20 mM HEPES–Tris, pH 8.0, and 10% (w/v) sucrose and then with 1.25 mL of a solution containing 475 mM mannitol, 0.2 mM CHAPS and 20 mM HEPES–Tris. The gradient was centrifuged for 30 min at 650 *g*. Vacuoles were collected at the 0%/10% sucrose interface and suspended in 5 mL solution containing 475 mM mannitol, 0.2 mM CHAPS, 20 mM HEPES–Tris, pH 8.0 and 10% sucrose. Quadruplicate aliquots were counted immediately using a hemacytometer after addition of neutral red. Aliquots were taken from all phases of the gradient and analyzed for Cu.

The yield of intact vacuoles was determined by staining with neutral red to improve visibility and quadruplicate visual counting of structures under the light microscope using a hemacytometer. The red color of the vacuoles after neutral red staining indicated that they were able to maintain a low inside pH at a pH of 8.0 in the medium, suggesting that the vacuolar membranes were sealed against net proton leakage. The total number of protoplasts lysed and vacuoles recovered were calculated by taking into account the volume of protoplast suspension lysed and the

volume of vacuole suspension recovered, respectively. Based on these values, vacuolar yield and contamination were determined as shown in Table 2, assuming that one vacuole was released per protoplast. All steps were carried out on ice or at 4°C to minimize any carrier-mediated efflux from vacuoles. After lysis, the osmotic potential of the medium was increased to stabilize the vacuoles, and vacuoles were purified by centrifugation in a density gradient optimized for purity and recovery of intact vacuoles. Recovery of vacuoles was approximately 24% and contamination of the vacuole-enriched fraction with chlorophyll was low (Table 2).

Measurement of biomass and Cu concentration

Four weeks after Cu treatment, the plants were harvested for determining of biomass and plant total Cu. Plants were washed with double distilled water for three times, then separated into roots and shoots (leaves), and dried in an oven at 70°C for 48 h. Biomass (DW) was determined and the dried plant tissues (powder samples) were digested with a mixture of HNO_3 and $HClO_4$ [3:1 (v/v)], heated on an oven. After cooling, the extracts were diluted and made up to 25 mL with 1 M HNO_3. Leaf samples, cell walls, protoplast suspensions and vacuole suspensions above were also dried, weighed and then wet digested separately. Copper concentrations of the extracts were determined by flame atomic absorption spectrophotometer (model WF-5).

Statistical analysis

The results were analyzed by using two-way analysis of variance (ANOVA) to test the effect of Cu treatment on plant biomass, Cu concentration, H_2O_2 and MDA contents and SOD, CAT and APX activities. If the *F*-value showed significant differences ($P < 0.05$), the means were compared by Tuckey HSD test. Calculation was conducted in statistic 6.0.

Results

Biomass

A significant difference in plant biomasses was observed between the two *D. carota* populations (Table 3). At control and low copper treatments (100 mg kg^{-1}), the shoot biomass and the root biomass were higher in UCS population than in

Table 2 Chlorophyll content (total), vacuolar yield and purity of vacuole-enriched fraction from leaves of two *D. carota* populations

One week of Cu exposure (mg kg^{-1})	Chlorophyll of whole leaf (mg g^{-1} FW)	Chlorophyll of protoplast (μg per 10^6 protoplasts)	Number of counted vacuoles[a] (% of lysed protoplasts)	Chlorophyll of counted vacuoles (% of lysed protoplasts)
CS				
100	1.81 ± 0.31 a	69.3 ± 4.1 a	24.1 ± 3.5 a	1.32 ± 0.44 a
400	1.64 ± 0.62 b	64.5 ± 5.2 a	23.7 ± 2.4 a	1.43 ± 0.62 a
UCS				
100	1.58 ± 0.32 b	56.7 ± 4.8 b	23.8 ± 4.6 a	1.36 ± 0.61 a
400	1.41 ± 0.24 c	42.6 ± 3.4 c	24.3 ± 3.8 a	1.24 ± 0.52 a
ANOVA				
$F_{population}$	7.143*	7.152*	3.377 ns	1.842 ns
F_{Cu}	6.954*	8.415*	4.158 ns	2.413 ns
$F_{population \times Cu}$	7.84*	4.21 ns	2.841 ns	2.648 ns

Different letters in the same column are significant at $P < 0.05$ according to Tuckey HSD test

ns non-significant $P > 0.05$

* $P < 0.05$

[a] Number of vacuoles and total chlorophyll content of counted vacuoles were determined in the vacuole-enriched fraction, and these values are expressed as a percentage of the respective values measured in the total volume of protoplast suspension lysed to obtain the vacuoles. Values are means of two experiments (three replications of each experiment) ± SE

Table 3 Biomass (g DW pot^{-1}) in two populations of *D. carota* after 4 weeks of culture in copper amended soil

Cu treatment (mg kg^{-1} soil)	Root		Shoot	
		% of control		% of control
UCS				
0	0.316 ± 0.026 ab	100	0.339 ± 0.007 (100) b	100
100	0.336 ± 0.026 a	106.3	0.406 ± 0.019 (119.8) a	119.8
200	0.278 ± 0.006 b	87.98	0.34 ± 0.012 (100.3) b	100.3
400	0.216 ± 0.013 c	68.4	0.303 ± 0.017 (89.4) c	89.4
800	0.191 ± 0.006 cd	60.4	0.243 ± 0.002 (71.7) d	71.7
CS				
0	0.236 ± 0.014 cd	100	0.268 ± 0.032 (100) c	100
100	0.251 ± 0.043 bc	106.4	0.336 ± 0.065 (125.3) b	125.3
200	0.256 ± 0.008 bc	108.5	0.340 ± 0.045 (127.1) b	127.1
400	0.243 ± 0.002 cd	102.9	0.278 ± 0.025 (103.7) c	103.7
800	0.222 ± 0.012 cd	94.1	0.260 ± 0.015 (97.1) cd	97.1
ANOVA				
$F_{population}$	17.223***		7.552***	
F_{Cu}	9.936**		9.915**	
$F_{population \times Cu}$	12.095***		2.374 ns	

Results are means ± standard deviation ($n = 3$). Different letters in the same column are significant at $P < 0.05$ according to Tuckey HSD test

ns non-significant $P > 0.05$

** $P < 0.01$; *** $P < 0.001$

CS population. At higher copper treatments (≥400 mg kg^{-1}), however, the shoot biomass and the root biomass were lower in UCS population than in CS one ($P < 0.05$). The tendency of change in biomasses was also different between the two populations. In CS population, no significant change was observed in biomasses of roots and shoots except that the shoot biomasses increased significantly at 100 mg kg^{-1} and 200 mg kg^{-1} Cu treatments. In UCS population, a significant reduction of biomasses in roots and shoots was observed at ≥200 mg kg^{-1} Cu treatment.

Lipid peroxidation

Lipid peroxidation level in leaves of the two *D. carota* populations was measured as the content of MDA. Marked difference of MDA concentration was observed between the two populations (Fig. 1). MDA concentration of UCS population was significantly lower than that of CS population in control but significantly higher than that of CS population in higher copper treatment (≥400 mg kg^{-1}) ($P < 0.05$). The trend in change of MDA content was also different between the two populations. In UCS population, the MDA content increased significantly with the increase of Cu concentration in soil. In contrast to UCS population, no significant change in MDA content was observed in CS population except for the control.

Effect of copper toxicity on H_2O_2 contents

Significant difference in H_2O_2 contents of leaves was found between the two populations (Fig. 2). In control, H_2O_2 contents were higher in CS population than in UCS population. Under Cu treatment, however, H_2O_2 contents were significantly lower ($P < 0.05$) in CS population than in UCS population. In high Cu treatment (400 and 800 mg kg^{-1}), a significant increase (29.4 and 56.9%, respectively) of H_2O_2 contents was observed in UCS population compared to their controls, whereas no significant change in CS population.

Antioxidant enzymes

The change trend of SOD activity was similar in the two populations after 7 days of Cu treatment

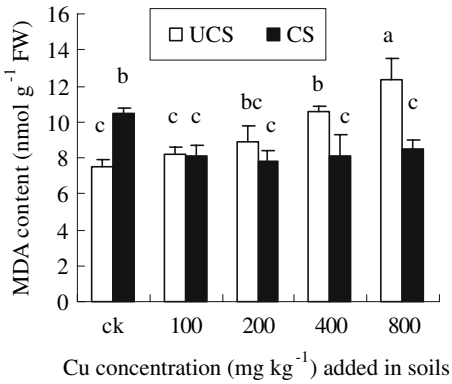

Fig. 1 Malondialdehyde content of two populations of *D. carota* subjected to 7 days of Cu treatment

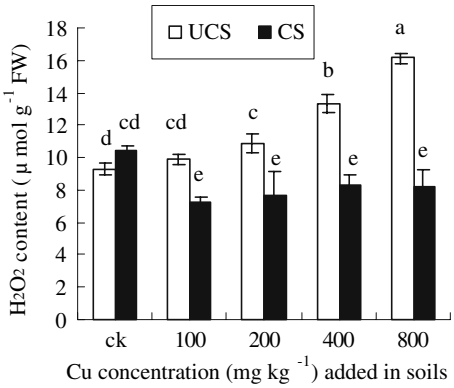

Fig. 2 Hydrogen peroxide contents in leaves of two populations of *D. carota* subjected to 7 days of Cu treatment

(Fig. 3). At 100 mg kg^{-1} Cu treatment, the SOD activity increased in the two populations and then, decreased gradually with the increase of Cu concentration in the soil. But the decrease of SOD activity in UCS population was more pronounced than in CS population. In control, the SOD activity of UCS population was higher that of CS population. At ≥200 mg kg^{-1} Cu treatments, however, the SOD activity of the CS population was significantly higher than that of UCS population ($P < 0.05$).

Marked differences in activities of CAT (Fig. 4) and APX (Fig. 5) were found between the two populations. A significant increase of CAT activities was observed in two populations under lower Cu treatments (100 and 200 mg kg^{-1}). But the increase of CAT activity in CS population (238.98 and 387.50% of the control at 100 and 200 mg kg^{-1} Cu treatments, respectively) was more pronounced than that in UCS one (204.45 and 195.80% of the control, respectively). Under higher Cu treatments (400 and 800 mg kg^{-1}), a decrease of the CAT activities was observed in the two populations. But the decrease of CAT activities in CS population was significantly less than that in UCS one. For APX, no significant change was observed in CS population in any Cu treatment when compared to their controls while, a significant inhibition in UCS population (Fig. 5).

Copper concentration in tissues

The copper contents in plant tissues of the two populations depended on Cu concentration in soils and increased significantly ($P < 0.05$) with the increase of copper concentration in the soil

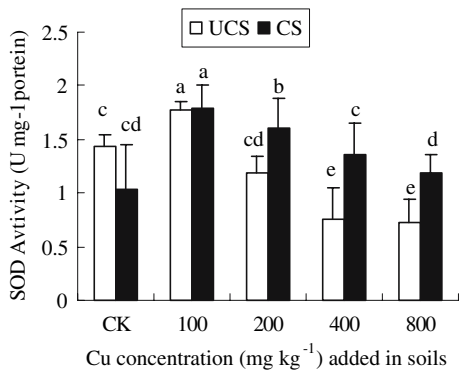

Fig. 3 The effect of Cu treatment on SOD activities in leaves of two *D. carota* populations

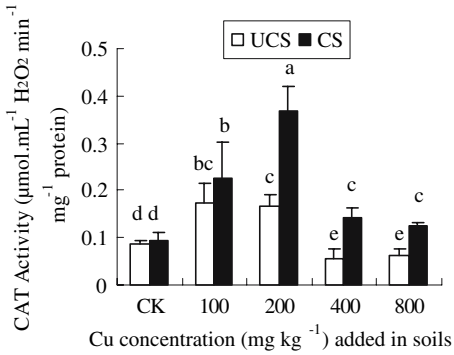

Fig. 4 Catalase activity in two *D. carota* populations subjected to 7 days of Cu treatment

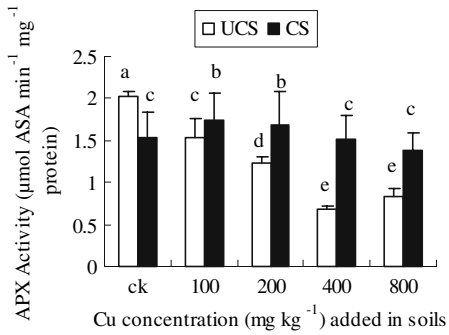

Fig. 5 Ascorbate peroxidase activity in two *D. carota* populations subjected to 7 days of Cu treatment

(Fig. 6). However, no significant difference of Cu concentration in the roots and shoots was observed between the two populations at the same Cu treatment (Fig. 6). Furthermore, it was also found that the copper contents in shoots of two *D. carota* populations were always higher than that in roots (shoots:root > 1) at all treatments, indicating a better transport mechanism from root to shoots in this plant.

Localization of Cu in leaves

In this experiment, the cell wall-bound fractions of Cu, protoplast Cu and vacuolar Cu in the two populations increased at the higher concentration of Cu in the medium (Table 4). The amount of Cu in whole leaves, in protoplasts and in cell walls was approximately equivalent in the two populations (Table 4) at 100 and 400 mg kg^{-1} Cu concentrations in the medium. More than 50% of whole leaf Cu was distributed in cell walls. Protoplast Cu, 44.7 and 50.8%, were localized in the vacuoles in the CS population under 100 and 400 mg kg^{-1} Cu treatments, respectively, whereas a significantly smaller proportion, 29.3 and 33.2% of the protoplast Cu was in the vacuoles in the UCS population ($P < 0.05$). The Cu content of the vacuoles was about 1.5-fold higher in CS population than in UCS population (Table 4).

Discussion

In this study, an increase or no significant change of biomasses in roots and shoots was found in CS population of *D. carota*, whereas a significant decrease of biomasses was observed in UCS population (Table 3). The result indicated that the CS population of *D. carota* might have developed to be a Cu-resistant population.

Peterson (1983) proposed that the resistance of plants to heavy metal stress was associated with their uptake processes. Studies on the uptake of copper indicated that tolerant plants accumulated more copper in the roots than non-tolerant plants did (Wu and Antonovics 1975; Lolkema and Vooijs 1986; Liu et al. 2004). In this way, less copper arrived at the shoots in tolerant plants than that in non-tolerant plants (Landberg and Greger 1996; Liu et al. 2004; Liu and Xiong 2005). In this experiment, however, no distinguishable difference was detected in the total Cu concentration of roots and leaves between the two *D. carota* populations. Thus, Cu (total Cu) uptake difference cannot explain the resistance difference in this species. But interestingly, we found in

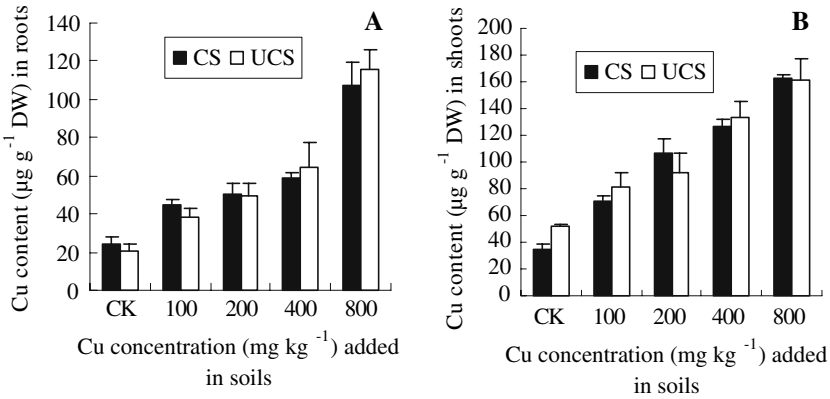

Fig. 6 Cu concentration in roots (**a**) and shoots (**b**) of two *D. carota* populations grown in soils with and without Cu addition

Table 4 Localization of Cu in leaves of two *D. carota* populations

Cu treatment (mg kg^{-1})	Total Cu	Cell walls		Protoplast Cu		Vacuolar Cu	
	(μg g^{-1} DW)	(μg g^{-1} DW)	(%)	(μg g^{-1} DW)	(%)	(μg g^{-1} DW)	(%)
CS							
CK	39.3 ± 3.8 c	16.6 ± 3.6 c	42.6 ± 8.1	17.1 ± 1.8 c	43.5 ± 6.4	3.2 ± 0.6 d	8.1 ± 2.6
100	74.7 ± 3.7 b	40.8 ± 7.2 b	54.6 ± 8.6	22.8 ± 6.4 b	30.5 ± 4.2	10.2 ± 2.1 b	13.7 ± 3.6
400	127.4 ± 5.1 a	68.6 ± 6.2 a	53.8 ± 9.8	35.6 ± 8.4 a	27.9 ± 4.6	18.1 ± 3.3 a	14.2 ± 5.4
UCS							
CK	45.6 ± 4.9 c	18.9 ± 4.1 c	41.5 ± 6.7	19.2 ± 4.8 c	42.1 ± 6.2	3.7 ± 0.9 d	7.9 ± 3.4
100	81.5 ± 10.9 b	42.1 ± 6.4 b	51.7 ± 8.1	23.7 ± 4.3 b	29.1 ± 4.7	6.8 ± 1.8 c	8.4 ± 2.7
400	133.9 ± 11.6 a	70.8 ± 8.4 a	52.9 ± 7.9	36.8 ± 7.1 a	27.5 ± 3.8	12.2 ± 3.1 b	9.1 ± 4.3
ANOVA							
$F_{population}$	1.043 ns	0.976 ns		1.487 ns		1.045 ns	
F_{Cu}	78.518***	57.217***		65.325***		57.217***	
$F_{population \times Cu}$	3.16*	1.241 ns		1.713 ns		3.871 *	

Cytoplasm Cu = protoplasm Cu - vacuole Cu; data did not show in this table

Results are means ± standard deviation ($n = 3$). Different letters in the same column are significant at $P < 0.05$ according to Tuckey HSD test. ''%'' indicates percent of the leaf total Cu

ns non-significant $P > 0.05$

* $P < 0.05$; *** $P < 0.001$

this experiment that most of the Cu was transported from roots to shoots in *D. carota*, which was different from that of *Lotus purshianus* (Wu and Lin 1990). As to our knowledge, it is the first to report the phenomenon under controlled lab conditions. In previous field investigation in Cu mine area, it was also found that *D. carota* was the only with a shoot/root ratio for Cu >1 in several dominant species investigated (Ke et al. 2001). The result indicated that a better transport mechanism from root to shoot might exist in *D. carota*.

Subcellular localization of heavy metals provides further understanding of heavy metal tolerance mechanism. Cell wall is believed to play a role in metal tolerance (Neumann et al. 1997). MacFarlane and Burchett (2000) reported that accumulation of Cu was predominantly in the cell walls. Lou et al. (2004) reported that 68% of Cu in root cell of *Elsholtzia haichowensis* was bound in cell walls. Contrarily, Liu and Kottke (2004) reported that only trace amounts of Cu was detected in the cell walls, while most of the Cu was accumulated in the vacuoles of cortical parenchyma cells in *Allium sativum*. The conflicting results may be due to differences between the plant species and their capacities to accumulate and sequester toxic metals. In present study, more than 50% Cu was bound in cell walls of *D. carota* leaves. However, no significant difference of Cu concentrations in leaf cell walls was found between the two populations. There was also no significant difference in protoplasm Cu concentrations between them. But Cu concentration of vacuoles was significant difference between the two populations (Table 4). In the experiment of isolating vacuoles by sucrose gradient centrifugation, aliquots were taken from all phases of the gradient and analyzed for Cu. There was no significant difference in Cu concentration (averaging 7.1% of Cu in vacuole-enriched fraction) between the 0% sucrose phases of gradients for CS and UCS populations ($P = 0.46$ and 0.38, respectively). This suggests that any difference in vacuolar Cu concentrations between the two populations was not due to differential leakage of vacuolar contents.

In *Thlaspi goesingense*, it was found that the vacuolar Ni concentrations of Ni-tolerant ecotype were significantly higher than that of non-tolerant ecotypes (Krämer et al. 2000). Song et al. (2003) also showed that vacuoles of YCF1-transgenic plants had a fourfold higher uptake of Cd-GS2 that those of wild-type plants *Arabidopsis*. In CS population, more of protoplasm Cu was transported to vacuoles (Table 4). Thus, Cu

concentrations in cytoplasm were less in CS population than in UCS population (cytoplasm Cu = protoplasm Cu - vacuole Cu; data did not show in Table 4). Storage of Cu in vacuoles may suggest that cells reduce free Cu inside the cytosol by metal-binding polypeptides or organic acids, leading to maintenance of low levels of Cu in the cytoplasm of cells (Liu et al. 2004). Therefore, more efficient vacuolar sequestration mechanism for Cu might have developed in the Cu-tolerant ecotype (CS population) of *D. carota*.

Many environmental factors can induce oxidative stress in the cell by generation of ROS. Cu^{2+} as one of these factors participates in the well-known Haber–Weiss cycle, producing ROS, such as $O_2^-\bullet$, H_2O_2 and OH• (Winterbourn 1982). Protonation of $O_2^-\bullet$ can produce the hydroperoxyl radical (OH•, H_2O_2), which converts fatty acids to toxic lipid peroxides, and destroys biological membranes (Weckx and Clijsters 1996; Wu et al. 2003). In this study, higher Cu treatment induced significantly an increase of MDA (Fig. 1) and H_2O_2 (Fig. 2) contents in UCS population ($P < 0.05$). The result indicated that excess Cu caused oxidative stress in UCS population. By contraries, a significant lower MDA and H_2O_2 was observed in CS population, which indicated a better protective mechanism might exist in CS population.

The protective mechanisms adapted by plants to scavenge free radicals and peroxides include several antioxidant enzymes and antioxidant substances. The activity of one or more of antioxidant enzymes is generally increased in plants when exposed to stressful conditions and this elevated activity is correlated to increased stress tolerance (Mazhoudi et al. 1997). It has been previously reported that Cu increases the activities of SOD, APX and CAT (Chongpraditnun et al. 1992; Rama Devi and Prasad 1998; Weckx and Clijsters 1996). However, the response of antioxidant enzymes to copper, and in general to metals, remains controversial and can vary among plant species and among different tissues (Mazhoudi et al. 1997). In our study activities of the antioxidant enzymes were affected differently in two populations. In UCS population, there was an increase in activities of SOD and CAT at low Cu concentrations but significant reduction at higher Cu concentration when compared to the controls (Fig. 3, 4). Similar results were also observed in sensitive *Rumex dentatus* population (Liu et al. 2004), *E. haichowensis* (Liu and Xiong 2005), rice (Maribel and Satoshi 1998) and the maize (*Zea mays* L.) cultivar 3233 (Tanyolac et al. 2006), in which the sensitive variety has lower SOD activity and higher metal ion content in the leaves. Rama Devi and Prasad (1998) also reported that in *Ceratophyllum demersum* L. (Coontail) CAT activity at higher (4 μM) Cu was lower than 2 μM, which indicated that CAT activity was inhibited partly by more Cu accumulation. Lower activities of these enzymes in UCS population indicated that at higher Cu concentrations, antioxidant enzymes may not sufficiently protect the UCS population from oxidative damage. In CS population, exposure to excess Cu concentrations resulted in significant rises in the activities of SOD and CAT when compared with the controls (Fig. 3, 4). The activity of APX also increased at lower Cu concentration (Fig. 5). But the extent of increase in APX activity was lower than that of CAT activity. Thus, CAT might be more effective than APX in eliminating H_2O_2 in the plants. Some reports showed that activities of antioxidant enzymes under different stress conditions were relatively higher in tolerant species than in the sensitive ones (Bor et al. 2003; Shalata et al. 2001). Different response to Cu between two populations is suggested that it could be correlated to the difference of copper content in tissues between the two populations (Liu et al. 2004; Tanyolac et al. 2006). The higher copper content in tissues of sensitive population would be likely exerted a severe toxicity to these enzymes. Higher activities of SOD, CAT and APX in CS population indicated that the Cu-resistant population had stronger capacity in scavenging free radicals, and thus could avoid oxidative damage caused by these free radicals.

In conclusion, the results in this study showed that there were substantial differences in copper-induced oxidative stress, antioxidant enzyme responses to Cu stress and Cu concentration of vacuolar between the two populations of

D. carota. Under Cu stress, the CS population of *D. carota* showed lower H_2O_2 and MDA accumulation in leaves but higher biomasses and higher antioxidant enzyme activities than those of the UCS population. The CS population also showed higher Cu accumulation in vacuoles of leaves, which suggested more efficient vacuolar sequestration for Cu in the CS population, than that of the UCS population. The more efficient Cu sequestration in vacuoles and higher SOD, CAT and APX activities suggested the tolerance capacity of this CS population to protect the plant from oxidative damage. Conclusively, the CS population of *D. carota* can be successfully grown in Cu-enriched areas.

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