*BioMetals* **17:** 379–387, 2004.



# Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*

Maria Drążkiewicz*, Ewa Skórzyńska-Polit & Zbigniew Krupa
*Department of Plant Physiology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland*
**Author for correspondence (Fax: (48-81) 37 59 01; E-mail: mdrazki@biotop.umcs.lublin.pl)*

Received 30 January 2003; Accepted 31 October 2003; Published online: April 2004

*Key words:* antioxidant enzymes, heavy metal, reactive oxygen species

**Abstract**

Content of reactive oxygen species (ROS): $O_2^{\bullet-}$, $H_2O_2$ and $OH^{\bullet}$ as well as activities of antioxidant enzymes: superoxide dismutase (SOD), guaiacol peroxidase (POX) and catalase (CAT) were studied in leaves of *Arabidopsis thaliana* ecotype Columbia, treated with Cu excess (0, 5, 25, 30, 50, 75, 100, 150 and 300 $\mu$M). After 7 days of Cu action ROS content and the activity of SOD and POX increased, while CAT activity decreased in comparison with control. Activities of SOD, POX and CAT were correlated both with Cu concentration (0–75 $\mu$M) in the growth medium and with $OH^{\bullet}$ content in leaves. Close correlation was also found between $OH^{\bullet}$ content and Cu concentration. Oxidative stress in *A. thaliana* under Cu treatment expressed in elevated content of $O_2^{\bullet-}$, $H_2O_2$ and $OH^{\bullet}$ in leaves. To overcome it very active the dismutase- and peroxidase-related (and not catalase-related, as in other plants) ROS scavenging system operated in *A. thaliana*. Visual symptoms of phytotoxicity: chlorosis, necrosis and violet colouring of leaves as well as a reduction of shoot biomass occurred in plants.

*Abbreviations*: CAT = catalase. – DMSO = dimethyl sulfoxide. – MSA = methane sulfinic acid. – NBT = nitroblue tetrazolium. – POX = peroxidase. – ROS = reactive oxygen species. – SOD = superoxide dismutase

## Introduction

When oxygen comes into contact with metabolic systems it can be transformed into more reactive forms such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) (Smirnoff 1993). These reactive forms of oxygen (ROS) are continuously produced in living cells as a by-product of metabolism (Salin 1988, Bowler *et al.* 1992, Scandalios 1993). High oxygen concentration caused by photosynthetic oxygen evolution, along with photorespiration in $C_3$ plants which produces large amounts of ROS in the form of $H_2O_2$, and efficient absorption of light energy favour increased ROS production (Scandalios 1993, Smirnoff 1993). Chloroplasts are considered potentially the most efficient source of ROS (Bartosz 1997). $O_2^{\bullet-}$ and $^1O_2$ are produced in them by oxygen reduction at PS1 and PS2 (see Smirnoff 1993). Moreover, $^1O_2$ is formed in chloroplasts as a result of excitation energy transfer from chlorophyll to the ground state of oxygen. Other sources of $O_2^{\bullet-}$ are electron transport chain of endoplasmic reticulum and NADH or NADPH utilising electron transport chain of the nuclear envelope (Bartosz 1997 and refs therein). Mitochondria are also important intracellular generators of both $O_2^{\bullet-}$ and $H_2O_2$ (Hernández *et al.* 1993). Microsomal membranes show NAD(P)H-dependent superoxide formation, while peroxisomal and glyoxysomal ones perform it in the presence of NADH. Peroxisomes and glyoxysomes produce $H_2O_2$ apart from $O_2^{\bullet-}$ (Scandalios 1993, Smirnoff 1993). According to Foyer *et al.* (1997), chloroplasts are the principle site of $H_2O_2$ generation. $H_2O_2$ is formed from superoxide anion radicals by action of SOD (Salin 1988). It can also be a product of many other non-enzymatic or enzymatic processes in plants (Foyer *et al.* 1997 and refs therein). Hydroxyl radicals are generated in

Haber-Weiss reaction. Transition metals like Cu and Fe catalyse the formation of $OH^{\bullet}$ in the Fenton and metal-catalyzed Haber-Weiss reactions (Bowler *et al.* 1992, Smirnoff 1993, Bartosz 1997). These radicals can also be formed from $H_2O_2$ and triplet state of chlorophyll (Ramalho *et al.* 1998). ROS attack lipids, proteins and nucleic acids causing lipid peroxidation, protein denaturation and DNA mutation (Salin, 1988, Bowler *et al.* 1992, Scandalios 1993, Alscher *et al.* 1997, Bartosz 1997 and refs therein, Maksymiec 1997 and refs therein).

To prevent such a damage, plant cells are equipped with antioxidant systems. One of them consisting of superoxide dismutase [EC1.15.1.1] (SOD), catalase [EC 1.11.1.6] (CAT), peroxidases [EC 1.11.1.7] (POXs), ascorbate, $\alpha$-tocopherol reacts with ROS. Another regenerates oxidised antioxidants. This system includes glutathione, glutathione reductase, ascorbate, mono- and dehydroascorbate reductases (Smirnoff 1993). Under normal conditions the production and destruction of ROS is regulated well in cell metabolism. However, under environmental stress the balance between prooxidative and antioxidative reactions is shifted in favour of the former. This shift of the balance has been defined as oxidative stress (Alscher *et al.* 1997, Bartosz 1997, Foyer *et al.* 1997). Various environmental conditions can induce oxidative stress in plants (Scandalios 1993, Smirnoff 1993, Alscher *et al.* 1997, Bartosz 1997, Foyer *et al.* 1997, Pell *et al.* 1997). In the midst of them researchers were especially interested in water deficit (Smirnoff 1993, Sgherri *et al.* 1995) and ozone (Pell *et al.* 1997). Oxidative stress induced by Cu was also studied. Attention was paid mainly to the defence system in plants (Chongpraditnum *et al.* 1992, Luna *et al.* 1994, Weckx and Clijsters 1996, Mazhoudi *et al.* 1997, Devi and Prasad 1998, Navari-Izzo *et al.* 1998, Teisseire *et al.* 1998, Mallick and Rai 1999). However, the formation of ROS received less interest (Luna *et al.* 1994). Some authors concluded on ROS generation indirectly, basing on the activity of antioxidant enzymes.

The objective of this experiment was to test the hypothesis that Cu excess in a growth medium induces oxidative stress with Cu concentration dependent intensity and variability and activates defence system in *Arabidopsis thaliana*. Induction of oxidative stress was investigated in terms of $O_2^{\bullet-}$, $H_2O_2$, $OH^{\bullet}$ content in leaves of these plants and activation of defence system in terms of SOD, POX and CAT activity. We also hypothesize that there is a relationship between a level of individual ROS and activity of some antioxidant enzymes. Copper is an essential micronutrient for plants, but it can also be a toxic element (for review see Maksymiec 1997) when applied in amounts higher than its optimal level. Copper as a transition metal could particularly catalyse the formation of harmful free radicals.

## Materials and Methods

### *Plant material and growth conditions*

*Arabidopsis thaliana* (L.) Heynh wild type Columbia variety plants were cultivated as described earlier (Drążkiewicz *et al.* 2003) and treated with Cu excess 0, 5, 25, 30, 50, 75, 100, 150 and 300 $\mu$M as $CuSO_4 \cdot 5H_2O$ for 7 days. Plants were grown in a growth chamber 11 h light/13 h dark at a light intensity of 140 $\mu$mol $m^{-2}$ $s^{-1}$, day/night temperature 23/19 °C. At least 5 plants were used for every Cu concentration. After the treatment a shoot biomass was determined and then all leaves of rosette for individual Cu concentration were cut, one-gram-samples were prepared, conserved in liquid nitrogen and analysed. Three independent experiments were carried out.

### *Enzyme and ROS extraction*

Leaves conserved in liquid nitrogen were crushed in a mortar. The powder was homogenised in ice cold 50 mM phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and 1% polyvinylpyrrolidone PVP-40 (w/v) (Milosevic and Slusarenko 1996). The homogenate was centrifuged at 15 000 g at 4 °C for 20 min in Beckman Avanti centrifuge. The supernatant was collected and used for assays of enzyme potential activities and ROS content (except $OH^{\bullet}$). The plant material and isolation buffer were applied at proportions 1:4 and 1:2 to extract enzymes and ROS, respectively.

### *ROS assays*

$O_2^{\bullet-}$ level was determined according to Green and Hill (1984). The reduction of nitroblue tetrazolium (NBT) to formazan by superoxide anion was monitored as an absorbance at 490 nm increase, using the absorbance coefficient 100 $mM^{-1}$ $cm^{-1}$. Measurements were carried out with the reaction mixture containing 40 $\mu$L SOD (1 mg/mL) and without it. Final volume of the

reaction mixture was 1 mL. Results were presented as nmol NBT reduced per gram of leaf fresh weight.

Trapping assay was applied to detection of hydroxyl radicals. $OH^{\bullet}$ content was monitored according to the procedure described by Babbs *et al.* (1989) with modification. In colorimetric determination of $OH^{\bullet}$ content, dimethyl sulfoxide (DMSO) was used as a molecular probe for trapping $OH^{\bullet}$. Upon oxidation by $OH^{\bullet}$, DMSO yields MSA (methane sulfinic acid) – a stable product, which can be determined by colour reaction (Babbs *et al.* 1989). The leaves conserved in liquid nitrogen were homogenized in 5% DMSO (1:2 w/v). The homogenate was centrifuged at 15 000 g at 4 °C for 20 min in Beckman Avanti centrifuge, and the procedure according Babbs *et al.* (1989) was carried out. The absorbance of the samples was measured at 425 nm, the absorbance coefficient being 2088 $M^{-1}$ $cm^{-1}$. The results were presented as $\mu$mol MSA per gram of leaf fresh weight.

$H_2O_2$ content was determined according to Pick (1986) with modifications. The assay was based on horseradish peroxidase-dependent oxidation of phenol red by $H_2O_2$ leading to the formation of a compound, which at alkaline pH exhibited increased absorbance at 600 nm. The reaction mixture (the total volume 460 $\mu$L) consisted of 50 $\mu$L leaf extract, phenol red solution in 0.05 M potassium buffer pH 7.0 (final concentration – 0.52 mM), horseradish peroxidase (final concentration 43.5 unit/mL). The mixture was shaken for 10 min at 25 °C and centrifuged. Then 100 $\mu$L of the supernatant were mixed with 1 mL of 1M NaOH. The absorbance at 600 nm was measured by Shimadzu UV-160A spectrophotometer. An amount of $H_2O_2$ was calculated basing on the absorbance coefficient $19.8 \cdot 10^3$ $M^{-1}$ $cm^{-1}$ and presented as $\mu$mol $H_2O_2$ per gram of leaf fresh weight.

*Antioxidant enzymes*

SOD [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) to formazan at 560 nm. The amount of reduced NBT was calculated, using the absorbance coefficient 100 $mM^{-1}$ $cm^{-1}$. One unit of SOD was defined as the enzyme amount causing 50% inhibition reduction of NBT to formazan. The results were expressed in units per mg of protein.

CAT [EC 1.11.1.6] activity was measured following Aebi (1984). The decomposition of $H_2O_2$ was followed by absorbance decrease at 240 nm for 90 sec and was calculated per 60 sec. The results were presented as $\Delta_{240}$ per min per mg of protein.

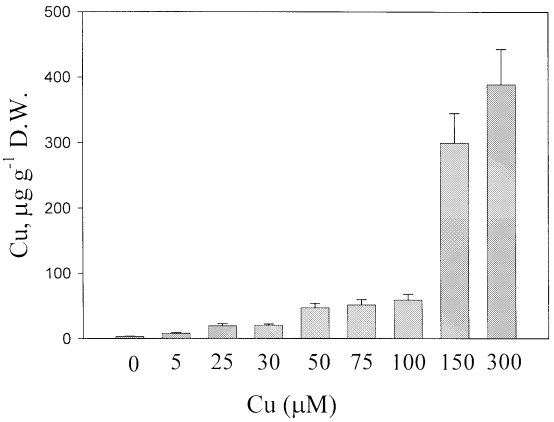


*Fig. 1.* Cu accumulation in leaves of *Arabidopsis thaliana* exposed to copper stress for 7 days. Data are means ± SE.

POX [EC 1.11.1.7] activity was determined as described by Milosevic and Slusarenko (1996). The reaction mixture contained 100 mM phosphate buffer (pH 6.25), 0.012% guaiacol and 0.03% $H_2O_2$ and enzyme extract. Guaiacol oxidation followed at 470 nm for 3 min and the obtained value was calculated per 1 min. The results were presented as $\Delta_{470}$ per min per mg of protein.

Protein was assayed according to the method of Bradford (1976) using bovine serum albumin as standard.

*Copper content*

Copper accumulation in leaves was measured by atomic absorption spectrophotometer in samples mineralized in a mixture containing $HClO_4 : HNO_3 : H_2O$ (1.5 : 1.5 : 10 v/v).

*Statistical analysis*

GraphPad InStat tm Software was applied for calculations. Sample variability is given as the standard error of the mean values. Correlation coefficients were also evaluated. The level of significance was set at $P < 0.05$.

## Results

In *Arabidopsis thaliana* grown under Cu excess its content in leaves increased gradually with Cu concentration in the growth medium up to 100 $\mu$M. Then

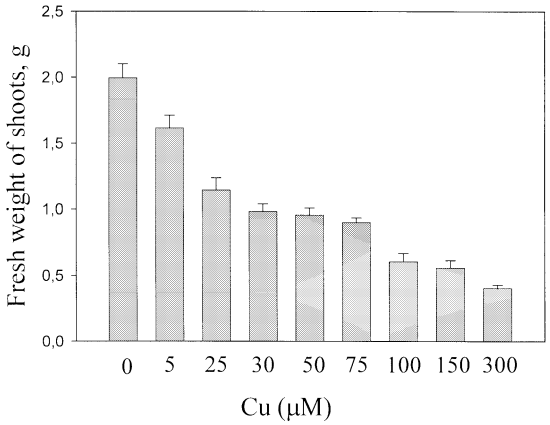


*Fig. 2.* Fresh weight of shoots of *Arabidopsis thaliana* treated with Cu excess for 7 days. Data are means ± SE.

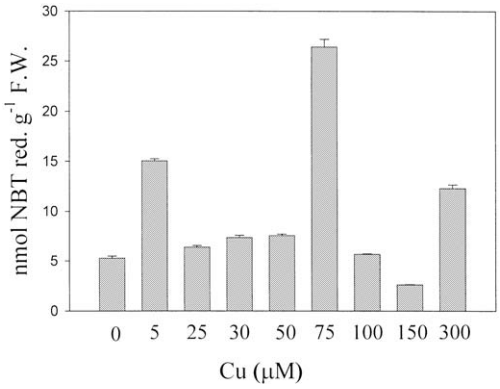


*Fig. 3.* The content of superoxide radical ($O_2^{\bullet-}$) in leaves of *Arabidopsis thaliana* treated with Cu for 7 days. Data are means ± SE.

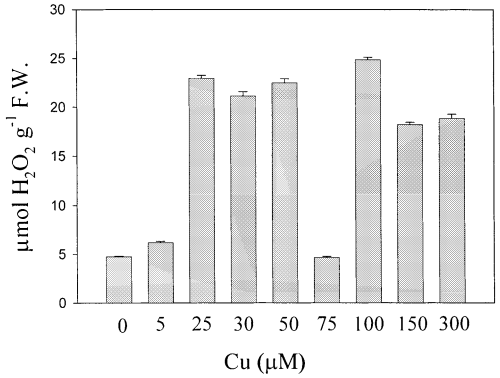


*Fig. 4.* $H_2O_2$ level in leaves of *Arabidopsis thaliana* treated with Cu for 7 days. Data are means ± SE.

it raised very rapidly, reaching about 390 $\mu$g $g^{-1}$ of dry weight at 300 $\mu$M Cu (Figure 1). In the plants a reduction of shoot biomass ocurred. It decreased to 81%, 45% and 20 % of control at 5 $\mu$M, 75 $\mu$M and 300 $\mu$M Cu, respectively (Figure 2). Chlorosis was a visible symptom of Cu toxicity (Photo 1A-C). It appeared already in rosette of plants exposed to 5 $\mu$M Cu in a top of the oldest leaves. At 25 $\mu$M Cu it expanded in the oldest leaves, and in plants treated with 30 $\mu$M Cu first necrotic changes in the top of the oldest leaves were observed. They enlarged gradually at 50 $\mu$M, 75 $\mu$M and 100 $\mu$M Cu. In leaves of *A. thaliana* exposed to 100 $\mu$M Cu chlorosis started also in younger leaves and, moreover, a violet pigmentation occurred along the veins. Total necrosis of some older leaves was found in plants treated with 150 $\mu$M Cu, in addition to violet colouring along veins. However, exposure of plants to 300 $\mu$M Cu resulted in intensification of chlorosis, particularly near veins of younger leaves, and also in intensification of necrosis in older leaves, as well as in intensification of violet pigmentation along veins of both older and younger leaves.

In leaves of *Arabidopsis thaliana* exposed to Cu excess elevated $O_2^{\bullet-}$ content was found at a low metal concentration (5 $\mu$M), where it reached 285% of control (Figure 3). The plants grown at 75 $\mu$M Cu showed an extremely high $O_2^{\bullet-}$ level (500% of control). It was also relatively high in plants exposed to 300 $\mu$M Cu (230% of control).

The increase of $H_2O_2$ content in Cu treated *A. thaliana* was particularly high when the metal concentration in the growth medium was 25–50 $\mu$M and 100–300 $\mu$M (Figure 4). These plants contained $H_2O_2$ in amounts 440–480% of control and 380–520% of control, respectively. A course of changes both in $O_2^{\bullet-}$, $H_2O_2$ content and activities of POX and CAT (Figures 3, 4, 7, 8) in leaves of *A. thaliana* treated with lower (0–75 $\mu$M) and with higher Cu concentrations suggest that interrelationship between ROS levels, enzyme activities and Cu concentration can vary in significance. Therefore correlation coefficients between these parameters were calculated in the range 0–75 $\mu$M and 0–300 $\mu$M Cu (Table 1). Not quite significant correlation ($0.05 < P < 0.1$) occurred between $H_2O_2$ and $O_2^{\bullet-}$ content in leaves of *A. thaliana* exposed to the Cu concentration range 0–300 $\mu$M, but it was not found in the range 0–75 $\mu$M Cu (Table 1).

The level of $OH^{\bullet}$ in *A. thaliana* leaves was positively correlated with Cu concentration in the growth medium in the both Cu concentration ranges (Table 1).

*Table 1.* Interrelationships between ROS level, antioxidant enzyme activity in leaves of *A. thaliana* and Cu concentration in the growth medium.

| Parameters | Cu concentration, $\mu$M | | | |
|---|---|---|---|---|
| | 0–75 | | 0–300 | |
| | R | P | R | P |
| Cu-CAT | −0.9633 | <0.01 | −0.6426 | <0.1 |
| Cu-SOD | 0.9242 | <0.01 | 0.8968 | <0.001 |
| Cu-POX | 0.9493 | <0.01 | 0.6243 | <0.1 |
| Cu-OH$^{bullet}$ | 0.9690 | <0.001 | 0.7790 | <0.01 |
| OH$^{\bullet}$-SOD | 0.8529 | <0.05 | 0.7130 | <0.05 |
| OH$^{\bullet}$-POX | 0.9896 | <0.001 | 0.9275 | <0.001 |
| OH$^{\bullet}$-CAT | −0.9079 | <0.01 | −0.7945 | <0.01 |
| $O_2^{\bullet-}$-POX | 0.7867 | <0.1 | 0.6290 | <0.1 |
| $O_2^{\bullet-}$-$H_2O_2$ | −0.5750 | >0.1 | −0.5879 | <0.1 |

The correlation was closer in the range of the 0–75 $\mu$M metal concentrations. OH$^{\bullet}$ content in leaves of the plants grown at 5 $\mu$M Cu reached 180% of control, at 75 $\mu$M Cu it was equal 490% of control, while at 300 $\mu$M Cu – 600% of control (Figure 3).

SOD activity raised gradually from 120% of control in the plants exposed to 5 $\mu$M Cu to 400% of control in those treated with 300 $\mu$M Cu (Figure 6). Activity of this enzyme was significantly correlated with concentration of the metal and the correlation coefficients were similar in the Cu concentration ranges 0–75 $\mu$M and 0–300 $\mu$M (Table 1).

Very large increase of POX activity was observed in leaves of *A. thaliana* under Cu treatment (Figure 7). It was 240% of control at 5 $\mu$M Cu, 990% at 75 $\mu$M Cu, and 810% at 300 $\mu$M Cu. POX activity was correlated with Cu concentration, particularly closely in the range 0–75 $\mu$M Cu.

CAT activity decreased gradually, when metal concentration increased and reached about 40% of control at 75 $\mu$M Cu (Figure 8). Significant correlation occurred between these parameters in the range of 0–75 $\mu$M Cu (Table 1).

Activities of SOD, POX and CAT were significantly correlated with OH$^{\bullet}$ content in leaves of *A. thaliana* (Table 1). In the case of POX and CAT activities correlation coefficients had higher values in plants exposed to 0–75 $\mu$M Cu than to 0–300 $\mu$M Cu. Moreover, not quite significant correlation was found between POX activity and $O_2^{\bullet-}$ level in leaves of *A. thaliana* (Table 1).

Treatment of *A. thaliana* plants with 30 $\mu$M and 100 $\mu$M Cu resulted in the rise of $H_2O_2$ level (440% and 520% of control, respectively) higher than other ROS (Figures 3–5). It was accompanied by very similar increase of both POX and SOD activities (230% and 200% of control at 30 $\mu$M Cu and 340% of control at 100 $\mu$M Cu, respectively) (Figures 6, 7). However, the greatest increase both of $H_2O_2$ content and POX activity was characteristic of the plants treated with 25, 50 and 150 $\mu$M Cu, while at 75 $\mu$M Cu the highest rise of POX activity and $O_2^{\bullet-}$ level occurred.

## Discussion

In leaves of *Arabidopsis thaliana* exposed to Cu for 7 days extremely high accumulation of the metal occurred at 150 $\mu$M and 300 $\mu$M Cu (Figure 1). It was accompanied by particularly strong reduction of shoot biomass (Figure 2). Considerably greater copper accumulation in roots than in shoots, decrease of the root length and changes in chloroplast ultrastructure were observed in *A. thaliana* cultivated in the medium with copper excess for 14 days (Wójcik and Tukiendorf 2003). Cu concentration depending decrease of shoot biomass in *A. thaliana* plants exposed to Cu for 7 days was accompanied by also Cu concentration depending chlorotic and necrotic changes in leaves and their violet colouring (Photo 1–3). Oxidative stress occurred in these plants and it was expressed as the content of $O_2^{\bullet-}$, $H_2O_2$ and OH$^{\bullet}$ considerably greater than in control plants (Figures 3–5), in which ROS are continuously produced as a by-product of metabolism (Salin 1988, Bowler *et al.* 1992, Scandalios 1993). Earlier studies on oxidative stress induced by heavy metals showed that the roots of plants exposed to lead formed enhanced total pool of free radicals, when the metal was applied at a sublethal concentration (Rucińska *et al.* 1999). However, in *Arabidopsis thaliana* plants even slight Cu excess (5 $\mu$M) in the growth medium induced oxidative stress in leaves (Figures 3–5). The $O_2^{\bullet-}$ level was especially high in these plants (Figure 3) and it could result from the slight increase in SOD activity at 5 $\mu$M Cu (Figure 6).

OH$^{\bullet}$ content in leaves of *A. thaliana* depended significantly on Cu concentration in the growth medium. Cu as the transition metal was favourable both for Fenton reaction and metal-catalyzed Haber-Weiss reaction (Bartosz 1997), which led to high OH$^{\bullet}$ content in leaves (Figure 5). $O_2^{\bullet-}$ and $H_2O_2$ levels were more mutually related, than Cu concentration depend-

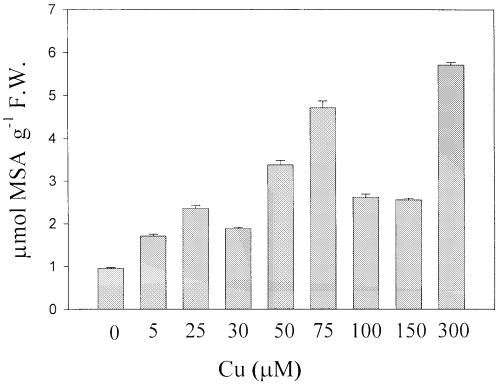


*Fig. 5.* Hydroxyl radical ($OH^{\bullet}$) content in leaves of A*rabidopsis thaliana* treated with Cu for 7 days. Data are means ± SE.

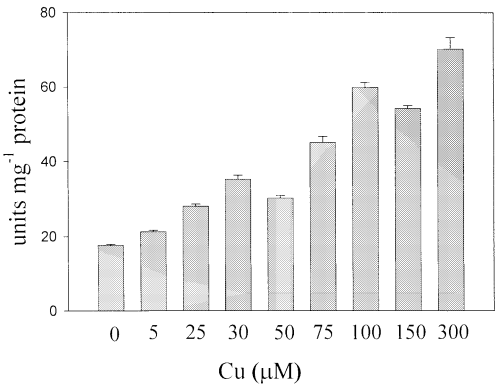


*Fig. 6.* Superoxide dismutase (SOD) activity in leaves of *Arabidopsis thaliana* treated with Cu for 7 days. Data are means ± SE.

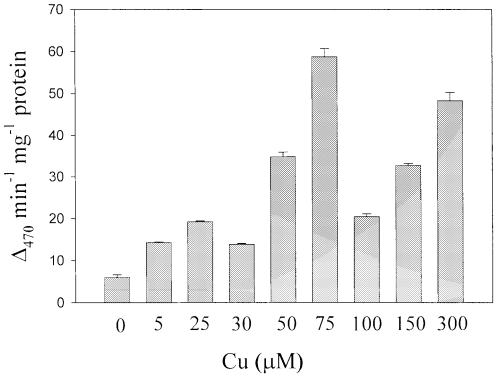


*Fig. 7.* Guaiacol peroxidase (POX) activity in leaves of *Arabidopsis thaliana* treated with Cu for 7 days. Data are means ± SE.

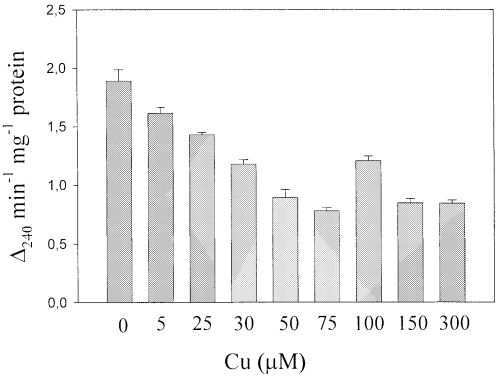


*Fig. 8.* Catalase (CAT) activity in leaves of *Arabidopsis thaliana* treated with Cu for 7 days. Data are means ± SE.

ent (Table 1). Similarly to Cu treated *A. thaliana*, a significant increase of $H_2O_2$ content was observed in Hg stressed tomato seedlings in comparison with control (Cho and Park 2000). However, the changes in $O_2^{\bullet-}$ and $OH^{\bullet}$ content in *A. thaliana* leaves caused by Cu (Figures 3, 5) differed from those in the chloroplasts of Mn-treated rice (Lidon and Teixeira 2000).

The significant correlation between Cu concentration in the range 0–75 $\mu$M in the growth medium and the activity of antioxidant enzymes in leaves of *A. thaliana* (Table 1) indicates that enzymes engaged in antioxidant defence: SOD and POX were activated by Cu, while CAT activity was inhibited. In the plants grown at 75 $\mu$M Cu, where the lowest CAT activity (40% of control) (Figure 8) was accompanied by the highest $O_2^{\bullet-}$ level (500% of control) (Figure 3) this enzyme was additionally inhibited by $O_2^{\bullet-}$. The above relationship supports the opinion of Salin (1988) that inactivation of catalase is among a few effects directly attributed to $O_2^{\bullet-}$. Contrary to *A. thaliana,* Cu treatment of *Ceratophyllum demersum* resulted in a considerable rise of CAT activity (Devi and Prasad 1998). The authors suggested that enhanced $H_2O_2$ production occurred in Cu treated plants displaying increased activity of CAT. However, in leaves of *A. thaliana* the rise of $H_2O_2$ level was found (Figure 4) when CAT activity dropped under Cu treatment (Figure 8). CAT was considered as a key enzyme to decompose $H_2O_2$ produced during photorespiration (Bowler *et al.* 1992, Niewiadomska *et al.* 1999). Diminution of the enzyme activity in *A. thaliana* by Cu resulted in the high level of $H_2O_2$ despite a considerably enhanced POX activity. These suggest that $H_2O_2$ could be a product of photorespiration. Moreover, POX, which can exhibit oxidase activity, mediating the reduction of $O_2$ to $O_2^{\bullet-}$ and $H_2O_2$ (Chen and Schopfer 1999), could also affect $H_2O_2$ level (Baker *et al.* 2000). At some Cu concentrations elevated $H_2O_2$ level in *A. thaliana* could result from diminished ascorbate peroxidase activity as was shown by Drążkiewicz *et al.* (2003). The oxidase activity of peroxidase (Chen and Schopfer 1999)

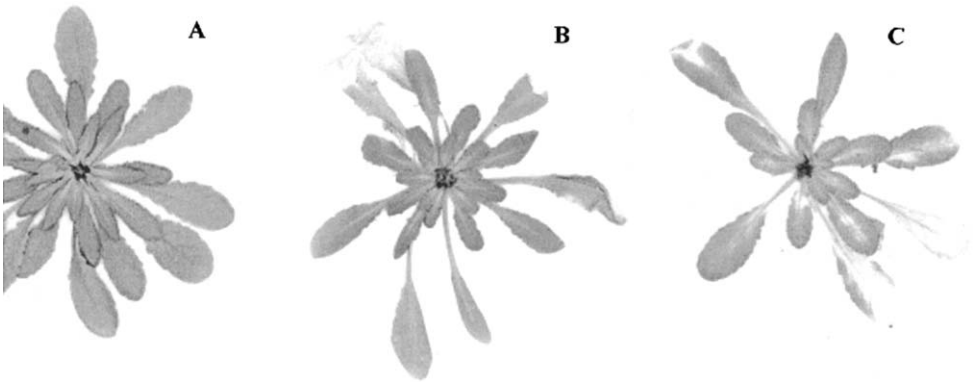


*Photo 1.* Chlorosis and necrosis in leaves of *Arabidopsis thaliana* treated with Cu for 7 days. A – control plant, B – plant exposed to 75 $\mu$M Cu, C – plant exposed to 300 $\mu$M Cu for 7 days.

could be responsible for the high $O_2^{\bullet-}$ level in leaves of *A. thaliana* exposed to 300 $\mu$M Cu (Figure 3), accompanied by the high $H_2O_2$ content (Figure 4) despite their utilisation for $OH^{\bullet}$ formation. However, NADH oxidation by peroxidase, where $H_2O_2$ is utilised (Askerlund *et al.* 1987) could lead to extremely high $O_2^{\bullet-}$ concentration in leaves of *A. thaliana* treated with 75 $\mu$M Cu, accompanied by extremely low $H_2O_2$ level and exceptionally high POX activity.

Our results of the rise of SOD activity by Cu in *A. thaliana* are convergent with those obtained for soybean (Chongpraditnum *et al.* 1992), wheat (Navari-Izzo *et al.* 1998), cyanobacterium (Devi and Prasad 1998) and oat (Luna *et al.* 1994). However, in *Phaseolus vulgaris* SOD activity showed a significant reduction under Cu treatment (Weckx *et al.* 1996). As was postulated by Chongpraditnum *et al.* (1992), increase of SOD activity under Cu treatment appears to be connected with enhanced synthesis of the enzyme. The induction of SOD may be the result either of a direct effect of copper on the gene for SOD (Navari-Izzo *et al.* 1998) or indirect *via* an increased $O_2^{\bullet-}$ levels (Ramalho *et al.* 1998). The close positive correlation between Cu concentration in the growth medium and SOD activity in *A. thaliana* leaves points to direct Cu effect on this activity. From the significant positive correlation between SOD activity and $OH^{\bullet}$ content in leaves of *A. thaliana* (Table 1) it appeares that very active SOD catalysed $O_2^{\bullet-}$ dismutation leading to $H_2O_2$ formation, which is an indispensable substrate in Fenton and Haber-Weiss reactions producing $OH^{\bullet}$.

POX was shown to be able catalyse a reaction, which results in $OH^{\bullet}$ production (Chen and Schopfer 1999). This is iron-catalyzed Haber-Weiss reaction in which the ferric/perferryl peroxidase couple constitutes an effective biochemical catalyst for the production of $OH^{\bullet}$ from $O_2^{\bullet-}$ and $H_2O_2$, where $H_2O_2$ is a rate-limiting substrate in this reaction. In the presence of $O_2^{\bullet-}$ POX is switched into perferryl form and catalyses the reduction of $H_2O_2$ to $OH^{\bullet}$ (Chen and Schopfer 1999). Both the high $H_2O_2$ level (Figure 4) and the correlation between the $O_2^{\bullet-}$ content and POX activity in leaves of *A. thaliana* (though not quite significant – $0.05<P<0.1$) and in particular, extremely significant correlation POX activity with $OH^{\bullet}$ content (Table 1) point out to possibility of functioning of such a mechanism in these plants. Increased POX activity in plants exposed to Cu stress could be due to *de novo* POX biosynthesis (Mazhoudi *et al.* 1997, Fang and Kao 2000). In *A. thaliana* POX activity was stimulated by water deficiency, UV-B radiation, ozone and sulphur dioxide (Kubo *et al.* 1999) and Cd (Skórzyńska-Polit *et al.* 2003/4). Thus, elevated POX activity is a response of *A. thaliana* common to different stress factors.

According to Scandalios *et al.* (1993) SOD and CAT are considered as the most effective antioxidant enzymes in averting cellular damage. However, in *A. thaliana* the increase of POX activity, greater than other enzymes, was the response of *A. thaliana* to Cu excess. Increase in POX activity accompanied by decrease in that of CAT indicates that $H_2O_2$ was primarily consumed in oxidation processes. SOD also took part in defence reactions, but it was stimulated to a lesser or, at some Cu concentrations, to very similar degree than POX. However, CAT was not involved in the defence system, especially at higher Cu concentrations. Thus, Cu induced POX- and SOD-related ROS scavenging systems in *A. thaliana.*

Using a wide range of Cu concentration and following the content of individual ROS instead of their total pool, as it was done in earlier studies, we showed how various can be the response of the plant to the same stress factor of different intensity. $O_2^{\bullet-}$, $H_2O_2$ or $OH^{\bullet}$ was predominant ROS, depending on Cu concentration applied, except 75 $\mu$M Cu, where increase of $O_2^{\bullet-}$ and $OH^{\bullet}$ content were almost the same. The correlation between Cu concentration in the range 0–75 $\mu$M and: $OH^{\bullet}$ content, POX and CAT activities indicates that, at the lower concentrations of the metal its effect was specific, while at higher ones, it could be both specific and indirect (from secondary action). However, Cu effect on SOD activity seems to be specific at all Cu concentration applied. Contrary to data presented in literature, in *A. thaliana* the relationship between the level of some ROS and activity of individual antioxidant enzymes was shown (Table 1).

## Acknowledgements

This work was supported by the State Committee for Scientific Research (KBN), grant 6 P04C 064 15

## References

Aebi H. 1984 Catalase *in vitro*. *Meth. Enzymol.* **105**, 121–126

Alscher RG, Donahue JL, Cramer CL. 1997 Reactive oxygen species and antioxidants: Relationships in green cells. *Physiol. Plant.* **100**, 224–233

Askerlund P, Larson Ch, Widell S, Møller IM. 1987 NAD(P)H oxidase and peroxidase activities in purified plasma membranes from cauliflower inflorescens. *Physiol. Plant* **71**, 9–19

Babbs ChF, Pham, JA, Coolbaugh RC. 1989 Lethal hydroxyl radical production in paraquat-treated plants. *Plant Physiol.* **90**, 1267–1270

Baker CJ, Deahl K, Domek J, Orlandi EW. 2000 Scavenging of $H_2O_2$ and production of oxygen by horseradish peroxidase. *Arch. Biochem. Biophys.* **382**, 232–237

Bartosz G. 1997 Oxidative stress in plants. *Acta Physiol. Plant.* **19**, 47–64

Beauchamp Ch, Fridovich I. 1971 Superoxide dismutase: Improved asssays and assay applicable to acrylamide gels. *Anal. Biochem.* **44**, 276–287

Bradford M. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254

Bowler Ch, Van Montagu M, Inzé D. 1992 Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 83–116

Chen S, Schopfer P. 1999 Hydroxyl-radical production in physiological reactions. *Eur. J. Biochem.* **260**, 726–735

Cho U-H, Park J-O. 2000 Mercury–induced oxidative stress in tomato seedlings. *Plant Sci.* **156**, 1–9

Chongpraditnum P, Mori S, Chino M. 1992 Excess copper induces a cytosolic Cu,Zn-superoxide dismutase in soybean root. *Plant Cell Physiol.* **33**, 239–244

Devi SR, Prasad MNV. 1998 Copper toxicity in *Ceratophyllum demersum* L. (Coontail), a free floating macrophyte: Response of antioxidant enzymes and antioxidants. *Plant Sci.* **138**, 157–165

Drążkiewicz M, Skórzyńska-Polit E, Krupa Z. 2003 Response of the ascorbate-glutathione cycle to excess copper in *Arabidopsis thaliana* (L.). *Plant Sci.* **164**, 195–202

Fang W-Ch, Kao ChH. 2000 Enhanced peroxidase activity in rice leaves in response to excess iron, copper and zinc. *Plant Sci.* **158**, 71–76

Foyer ChH Lopez-Delgado H, Dat JF, Scott IM. 1997 Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiol. Plant.* **100**, 241–254

Green MJ, Hill MAO. 1984 Chemistry of dioxygen. *Meth. Enzymol.* **105**, 3–22

Hernández JA, Corpas FJ, Gómez M, del Rio LA, Sevilla F. 1993 Salt-induced oxidative stress mediated by activated oxygen species in pea leaf mitochondria. *Plant Physiol.* **89**, 103–110

Kubo A, Aono M, Nakajima N, Saji H, Tanaka K, Kondo N. 1999 Differential responses in activity of antioxidant enzymes to different environmental stresses in *Arabidopsis thaliana*. *J. Plant Res.* **112**, 279–290

Lidon FC, Teixeira MG. 2000 Oxy radicals production and control in the chloroplast of Mn-treated rice. *Plant Sci.* **152**, 7–15

Luna CM, Gonzales CA, Trippi VS. 1994 Oxidative damage caused by an excess of copper in oat leaves. *Plant Cell Physiol.* **35**, 11–15

Maksymiec W. 1997 Effect of copper on cellular processes in higher plants. *Photosynthetica* **34**, 321–342

Mallick N, Rai LC. 1999 Response of the antioxidant systems of the nitrogen fixing cyanobacterium *Anabena doliolum* to copper. *J. Plant Physiol.* **155**, 146–149

Mazhoudi S, Chaoui A, Ghorbal MH, Ferjani EE. 1997 Response of antioxidant enzymes to excess copper in tomato (*Lycopersicon esculentum,* Mill). *Plant Sci.* **127**, 129–137

Milosevic N, Slusarenko AJ. 1996 Active oxygen metabolism and lignification in the hypersensitive response in bean. *Physiol. Molec. Plant Pathol.* **49**, 148–158

Navari-Izzo F, Quartacci MF, Pinzino C, Vecchia FD, Sgherri CLM. 1998 Thylakoid-bound and stromal antioxidative enzymes in wheat treated with excess copper. *Physiol. Plant.* **104**, 630–638

Niewiadomska E, Gaucher-Veilleux C, Chevrier N, Mauffette Y, Dizengremel P. 1999 Elevated $CO_2$ does not provide protection against ozone considering the activity of several antioxidant enzymes in the leaves of sugar map. *J. Plant Physiol.* **155**, 70–77

Pell EJ, Schlagnhaufer CD, Arteca RN. (1997) Ozone-induced oxidative stress: Mechanism of action and reaction. *Physiol. Plant.* **100**, 264–273

Pick E. 1986 Microassays for superoxide and hydrogen peroxide production and nitroblue tetrazolium reduction using an enzyme immunoassay microplate reader. *Meth. Enzymol.* **132**, 407–421

Ramalho JC, Campos PS, Teixeira M, Nunes MA. 1998 Nitrogen dependent changes in antioxidant system and in fatty acid composition of chloroplast membranes from *Coffea arabica* L. plants submitted to high irradiance. *Plant Sci.* **135**, 115–124

Rucińska R, Waplak S, Gwóźdź EA. 1999 Free radical formation and activity of antioxidant enzymes in lupin roots exposed to lead. *Plant Physiol. Biochem.* **37**, 187–194

Salin ML. 1988 Toxic oxygen species and protective systems of the chloroplast. *Physiol. Plant.* **72**, 681–689

Scandalios JG. 1993 Oxygen stress and superoxide dismutases. *Plant Physiol.* **101**, 7–12

Sgherri CLM, Navari-Izzo F. 1995 Sunflower seedlings subjected to increasing water deficit stress: Oxidative stress and defence mechanisms. *Physiol. Plant.* **93**, 25–30

Skórzyńska-Polit E, Drążkiewicz M, Krupa Z. 2003/4 The activity of the antioxidative system in cadmium-treated *Arabidopsis thaliana*. *Biol. Plant.* **47**: 71–78.

Smirnoff N. 1993 The role of active oxygen in the response of plants to water deficit and dessication. *New Phytol.* **125**, 27–58

Teisseire H, Couderchet M, Vernet G. 1998 Toxic responses and catalase activity of *Lemna minor* L. exposed to folpet, copper, and their combination. *Ecotoxicol. Environ. Safety (sec. B)* **40**, 194–200

Weckx JEJ, Clijsters HMM. 1996 Oxidative damage and defence mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. *Physiol. Plant.* **96**, 506–512

Wójcik M, Tukiendorf A. 2003 Response of wild type of *Arabidopsis thaliana* to copper stress. *Biol. Plant.* **46**, 79–84