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Full Length Research Paper

Effects of copper stress on antioxidative enzymes, chlorophyll and protein content in *Atriplex halimus*

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Our study showed the effect of Cu on *Atriplex halimus* grown in hydroponics conditions. The aim of this work was to investigate some enzymatic systems response of this plant to copper stress. Analysis was carried on enzymatic profiles, protein tenor and chlorophyll content of *A. halimus* leaves. Two months after sowing, plants were subjected to different concentrations of $CuSO_4$ (50, 500, 1000 and 2000 µM) and samples were analyse after 6, 24 and 48 h. Results demonstrate that chlorophyll content declined progressively with increasing concentrations of copper. In contrast, protein content decreased after 6 h to 38% at 2000 µM $CuSO_4$, and then increased after 48 h to 155% at 500 µM $CuSO_4$. Non-denaturing polyacrlamide gel electrophoresis (PAGE) revealed three catalase (CAT) isoformes, three superoxide dismutase (SOD) isoformes and five peroxidase (POX) isoformes. One new SOD isoforme and two new CAT isoformes were found as response to high concentration of Cu. The bands density of these enzymes increased with increase of Cu-dose. These results indicate that stress of Cu induced changes in *A. halimus* metabolism with stimulation of new gene expressions involved in the mechanism of abiotic plant defence.

Key words: *Atriplex halimus*, copper (Cu), oxidative stress, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), isoenzyme.

INTRODUCTION

Heavy metal (HM) contamination is one the most serious environmental problems that limits plant productivity and threatens human health. Cu toxicity appears on productive cropland treated repeatedly with Cu-containing pesticides and fertilizers (Demirevska-Kepova et al., 2004).

The sensitivity of plants to heavy metals depends on an interrelated network of physiological and molecular mechanisms such as: (i) uptake and accumulation of metals through binding to extracellular exudates and cell wall constituents; (ii) efflux of heavy metals from cytoplasm to extranuclear compartments including vacuoles; (iii) complexation of heavy metal ions inside the cell by various substances, for example, organic acids, amino acids, phytochelatins and metallothioneins; (iv) accumulation of osmolytes and osmoprotectants and induction of antioxidative enzymes; (v) activation or modification of plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged cell structures (Kabata-Pendias and Pendias, 2001; Cho et al., 2003; Tremel-Schaub and Feix, 2005).

Cupric ions are responsible for many alterations of the plant cell. They negatively affect nitrogen and protein metabolism, cause a reduction of chlorophyll contents and inhibit some photosynthetic functions in leaves (Foy et al., 1978; El-Jaoual and Cox, 1998; Kevresan et al., 2001). One of the major consequences of Cu toxicity is oxidative stress mediated by increased levels of reactive oxygen species (ROS); Cu^{2+} can catalyze the formation of ROS and particularly, the highly reactive hydroxyl radical, via Fenton-type reactions or Haber–Weiss reactions. ROS includes the superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), all of which affect mainly lipids, proteins, carbohydrates and nucleic acids

Plants possess homeostatic mechanisms to maintain the correct concentration of essential metals like Cu in different cell compartments. A regulated network of metal transport, chelation, trafficking and sequestration activeties functions and provides the uptake, distribution and detoxification of excess metal ions. Distinct homeostatic

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mechanisms are reported for Cu due to their different chemical characteristics. Cu mobility inside the plant is restricted. A large proportion of Cu absorbed by the plants is retained in the roots. In the cells, Cu is bound to various ligands (Cu chaperones, metallothioneins and phytochelatins). Excess Cu is sequestered in vacuoles. Heavy metal toxicity symptoms are revealed after disturbance of homeostatic mechanisms (Demirevska-Kepova et al., 2004).

To cope with ROS and alleviate their toxic effects, plants possess several antioxidative systems. The antioxidant system comprises several enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX). SODs are considered to be the first line of defence against ROS, which is responsible for the dismutation of $^{\bullet}O_2^-$ generating H_2O_2 and O_2. The bulk of H_2O_2 is removed by CATs, localized in peroxisome, and POXs localized in vacuoles, cell walls and the cytosol (Mittler, 2002; Demirevska-Kepova et al., 2004; Ghamsari et al., 2007).

Atriplex halimus (Chenopodiaceae) is a xerohalophyte which is perennial and native in arid and semi arid Mediterranean regions. This species tolerate well, harsh conditions such as salinity (Wong and Jager, 1978; Bajji et al., 1998), light stress (Streb et al., 1997), drought (Martinez et al., 2004) and cold (Salahas et al., 2002; Walker et al., 2008), moreover, recent researches demonstrated its ability to tolerate high concentration of heavy metal (Lutts et al., 2004; Nedjimi and Daoud, 2008; Manousaki and Kalogerakis, 2009).

The present experiment was undertaken to investigate a change in antioxidant enzymes, total protein and pigment content in *A. halimus* treated with $CuSO_4$ in order to contribute to an understanding of *A. halimus* adaptation to environmental stress.

MATERIALS AND METHODS

Plant material and growth conditions

A. halimus is considered as a plant model for stress studies due to its tolerance to many drastic conditions. Fruits of *A. halimus* were collected during December, 2008 from wild plants in Mascara, west of Algeria (0.3561110 longitudes, 35.3194440 latitudes). Seeds were removed from the bracts by hand. They were then placed to germinate in plastic jars filled with sand demineralised as support of plant; the jars were irrigated with distilled water and put at 25°C for 48 h in an incubator. Then, they were transferred to a controlled-environment chamber with a 14 h light-10 h dark cycle, respectively, and air temperatures of 25 ± 2°C. The relative humidity was 70% (day) and 80% (night) and photosynthetically active radiation (PAR) was 350 µmol m^{-2} s^{-1}, provided by a combination of fluorescent tubes (Philips TLD 18W/83, Germany, and MAXIMA FL20T8D/18). Irrigation was ensured by a nutritive solution containing: 5 mM KNO_3, 2 mM $MgSO_4 \cdot 7H_2O$, 5.5 mM Ca $(NO_3)2,4H_2O$, 1 mM KH_2PO_4, 25 µM KCl, 3 µM $MnSO_4 \cdot 4H_2O$, 10 µM H_3BO_3, 1 µM $ZnSO_4 \cdot 7H_2O$, 0.3 µM $CuSO_4 \cdot 5H_2O$, 1 µM $Na_2MoO_4 \cdot 2H_2O$, 7.5 µM $FeSO_4$ and 7.5 µM ethylenediaminetetraacetic acid (EDTA) (Lutts et al., 2004). The pH of the solution was adjusted to 6.1. Each treatment was replicated three times and each replicate included ten plants (that is, 30 plants per treatment).

The solution was renewed each week. Two months after sowing, plants were subjected for 48 h to different concentrations of Cu (as $CuSO_4$, 50, 500, 1000 and 2000 µM) diluted in distilled H_2O; the metal solution was made once. Controls contained 0.3 µM Cu in the nutrient medium.

Extraction and analysis of antioxidant enzymes

One gram of fresh leave was homogenised on ice using a mortar and pestle in 1.5 ml of 0,1 M Tris-HCl buffer (pH 8) containing 70 mM β-mercaptoethanol, 26 mM sodium metabisulfite, 11 mM ascorbic acid and 4% polyvinylpyrrolidon (PVP) (Kaplan et al., 2002). The homogenate was centrifuged at 15 000 g for 20 min at 4°C, and the supernatant (enzyme extract) was stored at -20°C for later enzyme electrophoretic separation.

Polyacrylamide gel electrophoresis (PAGE) and antioxidant enzymes activity staining

Electrophoresis buffers and gels were prepared as described by Laemmli (1970) except that sodium-dodecyl-sulphate (SDS) was excluded. Electrophoresis was carried out under non-denaturing condition in 10% polyacrylamide gels for POX and SOD and 8% for CAT activity staining. A constant current of 10 mA per gel was applied for 24 h at 4 °C. Equal amounts of protein were loaded onto each lane.

SOD activity staining

After electrophoretic separation, SOD activity was determined as described by Brewer (1967). Briefly, the gel was rinsed in distilled water and incubated in daylight in a reaction mixture containing 80 ml of 50 mM Tris HCl buffer (pH 8.5), 10 mg MTT, 6 mg PMS and 15 mg $MgCl_2 \cdot 6H_2O$. The enzyme bands are seen as pale zones on a dark blue background.

CAT activity staining

CAT activity in native PAGE gels was determined according to Harris and Hopkinson (1976). Gel was incubated in 3% H_2O_2 for 15 min and transferred in a 1:1 mixture of solutions of 2% ferric chloride and 2% potassium ferricyanide. Yellow bands of CAT activity appeared on a blue-green background.

POX activity staining

POX activity in native PAGE gel was determined as described by Graham et al. (1964). The gel was incubated in staining solution containing 100 ml of 50 mM sodium acetate buffer (pH 5.0), 50 mg of 3-amino-9-ethyl-carbazole (dissolved in a few drops of acetone) and 0.75 ml of 3% H_2O_2 (freshly prepared) at 4°C until red-brown bands appeared.

Determination of protein concentration

Protein concentration for all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Chlorophyll concentration analysis

For chlorophyll concentration analysis, 0.25 g of leaves were homo-

Table 1. Effects of $CuSO_4$ on chlorophyll content of *A. halimus* grown in hydroponic conditions.

$CuSO_4$ (µM)	Chlorophyll (mg g^{-1}FW)	
	Chl. a	Chl. b
0	1.89 ± 0.089^{A}	1.36 ± 0.043^{A}
50	1.81 ± 0.114^{A,B}	1.32 ± 0.018^{B}
500	1.71 ± 0.028^{B}	1.14 ± 0.008^{C}
1000	1.46 ± 0.046^{C}	0.98 ± 0.022^{D}
2000	1.03 ± 0.068^{D}	0.68 ± 0.026^{E}

Different letters in the same column indicate significant difference at the 5% level according to the Newman-Keuls test. Values represent means ± standard error (n = 3).

genized in 5 ml acetone (80%) and incubated at 4°C in the dark until the leaves were colorless. Chlorophyll a and b contents were measured as a function of absorbance at 663 and 645 nm, respectively (Spectrophotometer JENWAY 6305 UV/Vis), and calculated using the equation described by Arnon (1949).

Statistical analysis

The experiment was set up as a completely randomised design, with three replications of each treatment. Data were analysed statistically, using the Statistical Package for the Social Sciences (SPSS) 7.5 software package, by analysis of variance (ANOVA) and Tukey's multiple range test, to determine differences between means

RESULTS

Typical symptoms of Cu toxicity were developed 2 days after the beginning of the treatment. Yellowing and drying were seen on the leaves of plants treated with 1000 and 1500 µM Cu. The quantity of both chlorophyll a and b was diminished significantly with elevated copper-dose (Table 1) confirming that copper is damaging to the photosynthetic apparatus. A reduction in leaf total soluble protein was observed after 6 h (38% at 2000 µM) (Table 2), then a recovery in their biosynthesis appeared after 24 h, which becomes more significant after 48 h (155% at 500 µM).

Variations in the antioxidant enzyme profiles

Superoxide dismutase isoenzymes

SOD activity staining on a gel after native PAGE revealed the existence of three isoenzymes in leaves (bands 1, 2 and 3) (Figure 1). SOD-2 appeared at 500 µM and according to the densitometric analysis, its band intensity increased at 1000 µM Cu then decreased at 1500 µM Cu. Densitometric analysis also showed a decrease in SOD-3 band intensity with increasing copper-dose.

Peroxidase isoenzymes

Five isoenzymes of POX are revealed after native PAGE in leaves extract of *A. halimus* (Figure 2). Densitometric analysis showed that an increase in 2, 3 and 4 band intensity correlated with copper-dose.

Catalase isoenzymes

Three isoenzymes of CAT were revealed after non-denatured electrophoresis (Figure 3). CAT-1 is very slow and present in all treatments. CAT-2 and CAT-3 bands appeared at 50 µM Cu and its intensity increased with copper amount.

DISCUSSION

When a plant is subjected to any biotic or abiotic stress factor, the first observed response is a decrease in its normal metabolic activities, with a consequent reduction of growth. In this "alarm phase", protein synthesis is one of the most negatively affected anabolic processes together with photosynthesis, transport of metabolites, and uptake and translocation of ions (Bonjoch and Tamayo, 2003).

Copper is an essential micronutrient for the growth and development of plants since it is a structural and catalytic component of many proteins and enzymes involved in a variety of metabolic pathways (Teisseire and Guy, 2000). In plants that possess only low or no stress tolerance mechanisms, acute damage and senescence will occur rapidly. Besides loss of chlorophyll, ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO) and other chloroplast proteins are hydrolysed and exported via phloem, followed by hydrolysis of mitochondrial proteins and vascular tissues. Therefore, low protein concentrations should be interpreted as a clear symptom of stress damage in plants (Bonjoch and Tamayo, 2003).

However, in response to unfavourable conditions, most plants will activate their stress coping mechanisms such as acclimation of metabolic fluxes, activation of repair processes and long-term metabolic and morphological adaptations, which conform the named general adaptation syndrome (Lichtenthaler, 1996). Such mechanisms include *de novo* synthesis of proteins with specific adaptive functions, osmotic adjustment, antioxidative defence, among others.

The decrease in protein levels observed in *A. halimus* leaves during six hours after application of Cu, must be the result of the excess copper that generates free radicals that cause cellular damage at the DNA level and organelles such as mitochondria or lysosomes as described by Lee and Wei (2001). But increase observed after 24 and 48 h is the result of the accumulation of free amino acids as histidine, proline and cysteine in tissues,

Table 2. Effects of $CuSO_4$ on protein content of *A. halimus* grown in hydroponic conditions.

CuSO4 (µM)	Protein content (mg g^{-1}FW)		
	After 6 h	After 24 h	After 48 h
0	24.68 ± 01.95	26.06 ± 01.81	33.00 ± 03.85
50	23.81 ± 03.41 (-3.53%)	30.84 ± 03.28 (+18.34%)	95.85 ± 14.77 (+154.88)
500	19.30 ± 0.40 (-21.78%)	29.65 ± 04.09 (+13.75)	95.99 ± 31.62 (+155.25)
1000	18.89 ± 02.49 (-23.46%)	29.46 ± 0.74 (+13.05)	92.82 ± 20.88 (146.82)
2000	15.17 ± 02.06 (-38.54%)	28.54 ± 01.38 (9.52)	73.74 ± 08.67 (96.08)

Percentage of control values are given in parentheses.

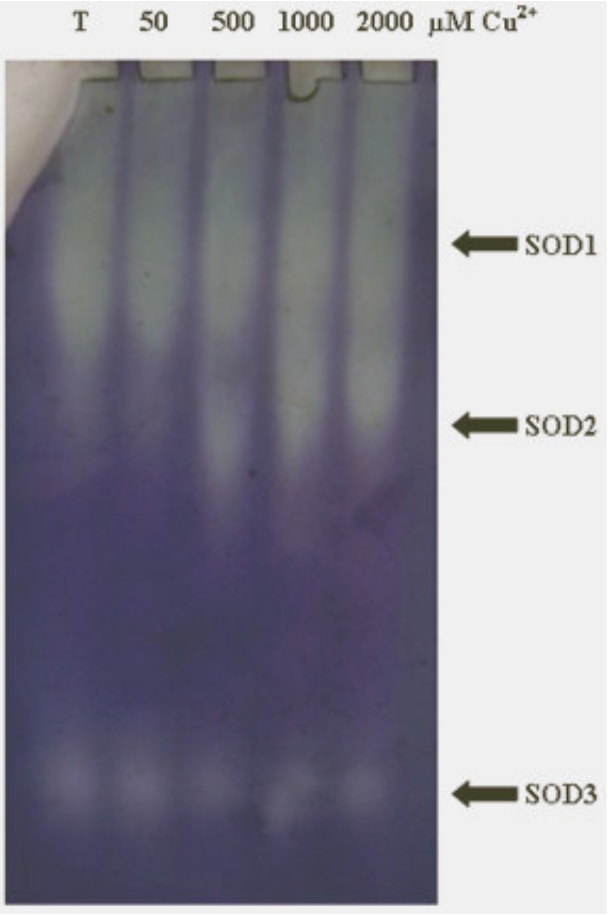

Figure 1. Isoenzyme patterns of SOD in *A. halimus* leaves after 48 h treatment with Cu. Different isoforms are numbered from cathode to anode.

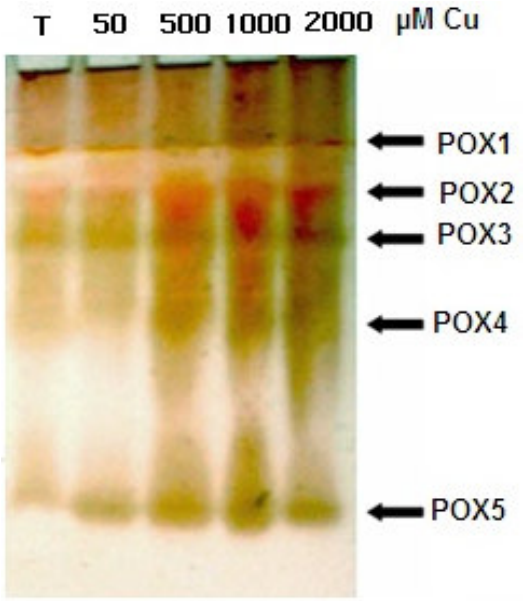

Figure 2. Isoenzyme patterns of POX in *A. halimus* leaves after 48 h treatment with copper. Different isoforms are numbered from cathode to anode.

this has been observed in stress metal such as Cd (Nedjim and Daoud, 2009).

The decline in chlorophyll content in plants exposed to heavy metals stress such us Cu is believed to be due to: (a) inhibition of enzymes associated with chlorophyll biosynthesis (John et al., 2009); (b) inhibition of uptake and transportation of other metal elements such as Mn, Zn and Fe by antagonistic effects (Jayakumar et al., 2009; John et al., 2009). Similar decrease in chlorophyll content under heavy metal stress was reported earlier in cyanobacteria, unicellular chlorophytes (*Chlorella*), gymnosperms such as *Picea abies* and angiosperms such as *Zea mays*, *Quercus palustrus* and *Acer rubrum* (Siedlecka and Krupa, 1996). The decrease in chlorophyll content was also reported in sunflower (Zengin and Munzuroglu, 2006) and in almond (Elloumi et al., 2007).

Results concerning SOD, CAT and POX activities in this study are based on gel electrophoresis analysis by densitometer. These results showed a strategy of defence of *A. halimus* against oxidative stress induced by Cu, which results in a change in the expression of the antioxydant enzymes. Similar results are obtained in plants stressed by the same metal, Cu (Demirevska-Kepova et al., 2004), or other metals such us Mn, Pb, Ni and Cd (Kopyra and Gwozdz, 2003; Demirevska-Kepova et al., 2004; Sobkowiak et al., 2004; Gomes-Junior et al., 2006).

Stress that disrupts the cellular homeostasis, including

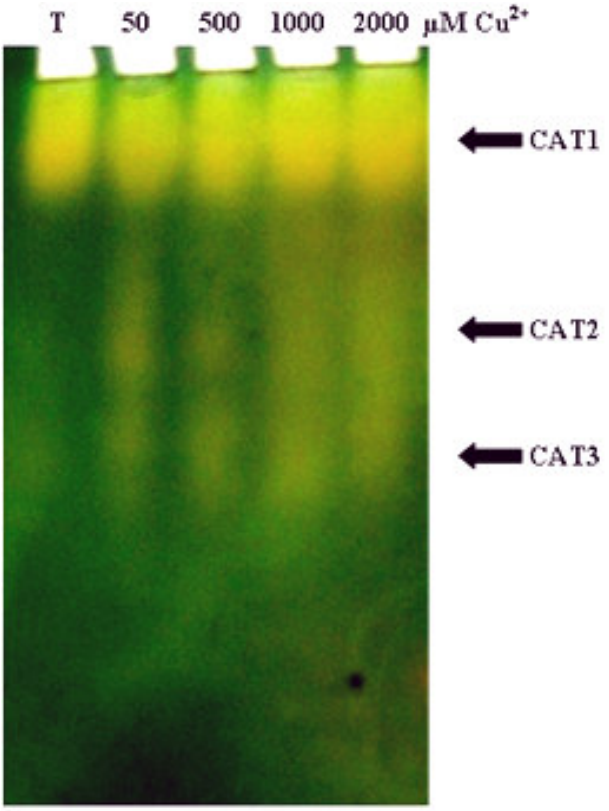

Figure 3. Isoenzyme patterns of catalase in *A. halimus* leaves after 48 h treatment with copper. Different isoforms are numbered from cathode to anode.

heavy metal toxicity, can enhance the production of ROS and increase the steady-state level of H_2O_2 up to 30-fold (Mittler, 2002). Autoxidation of "free" Cu^+ results in ($^{\bullet}O_2^-$) formation and subsequently in H_2O_2 and $OH^{\bullet}$ production via Fenton-type reactions (Teisseire and Guy, 2000; Polle and Schützendübel, 2004). Although we have not measured H_2O_2 or other ROS in this study, increases are likely to have occurred based on the responses of the antioxidant enzymes that were measured (Gratao et al., 2008).

As already stated in the introduction, the enzymes evaluated in this study are important in the antioxidant responses of plants to stress. Such responses to Cu and other heavy metals have received a great deal of attention in recent years and have been shown to vary considerably among plant species, organs and tissues and are dependent on the Cu concentration and duration of exposure (Sobkowiak et al., 2004; Demirevska-Kepova et al., 2004; Gratao et al., 2008). The activity and expression of genes encoding antioxidant enzymes have been shown to change in some plants when subjected to several environmental conditions and the responses of antioxidants to heavy metal induced oxidative stress have provided variable and controversial results (Gomes-Junior et al., 2006). In the present study, the change in SOD activity in PAGE at all concentrations of Cu^{2+} tested suggested that Cu^{2+} toxicity induces superoxide radicals ($^{\bullet}O_2^-$) in *A. halimus* leaves. This has been found in rape leaves and in *Ottelia alismoides (L.)* pers. plant (Xu et al., 2001, 2003), and in animals (Lin and Lan, 2001). SOD activity increased in PAGE with increase Cu^{2+} concentration, suggesting that SOD was stimulated by scavenging ($^{\bullet}O_2^-$) to protect *A. halimus* leaves from Cu^{2+} toxicity (Gomes-Junior et al., 2006). We found three SOD isoenzymes in *A. halimus* leaves, but the number of SOD isoenzymes varies greatly from plant to another (Gomes-Junior et al., 2006). SOD isoenzymes are found in various compartments of plant cells and can contain Cu and Zn, Fe, or Mn, as cofactors and they are the major ($^{\bullet}O_2^-$) scavenger and their enzymatic action results in H_2O_2 and O_2 formation (Allen, 1995). The new bands found suggest that stress of copper, stimulate the expression of a novel genes of SOD involved in plant defense.

The rapid elevation in CAT and POX activity in gel may be another major mechanism of Cu induced ROS protection and may be associated with the elevation of H_2O_2 level by the higher SOD activity, or another Cu-induced oxidative stress source (Gomes-Junior et al., 2006). The level of H_2O_2 in plant cells is under the control of CAT and POX, which can lower concentration if it is produced in excess. CAT eliminates H_2O_2 by breaking it down directly to form water and oxygen. It is less efficient than POD in H_2O_2 scavenging because of its low substrate affinity. In flowering plants, there are three genes that encode CAT isoforms. External factors stimulate the transcription of these genes in a different way (Słomka et al., 2008). We observed only three isoforms of CAT in leaves and five isoformes of POX. POX is widely distributed in the plant kingdom and it catalyses H_2O_2-dependent oxidation of substrate. Moreover, POD participating in lignin biosynthesis can build up a physical barrier against toxic heavy metals (Zhang et al., 2007).

Conclusion

In conclusion, appearance of new forms of CAT and SOD, and the fast increase in antioxidant enzymes activities in correlation with the amount of copper, suggest on one hand, that copper induced the free radicals in the leave of *A. halimus*, and on the other hand, that these enzymes are mobilized quickly to limit the cellular damage and increase the antioxidant capacity of *A. halimus* to cope with copper stress.

The increase in protein content may be another strategy of *A. halimus* to cope with the excess of copper by the synthesis of proteins with specific adaptive functions which remain to be identified.

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