Journal of Plant Physiology and Breeding
2017, 7(1): 41-52 ISSN: 2008-5168

Antioxidative Responses of *Eucalyptus camaldulensis* to Different Concentrations of Copper

Anahita Shariat[1*], Mohammad Hassan Assareh[2] and Abbas Ghamari-Zare[1]

Received: October 29, 2015 Accepted: April 8, 2017
[1]Biotechnology Research Department, Research Institute of Forests and Rangelands, Tehran, Iran
[2]Seed and Plant Certification and Registration Institute, Bolvar Nabovvat, Karaj, Iran
[*]Correspondence author; Email: shariat@rifr-ac.ir

Abstract
Anthropogenic activities have caused important increases in soil Cu levels not only in urban areas but also in croplands. This study was designed to find out the effect of different concentration of copper on physiological and biochemical changes in *Eucalyptus camaldulensis* seedlings. Seeds of *Eucalyptus camaldulensis* were grown in marble chips and irrigated with nutrient solution mixed with copper (control, 5, 10, 20 mM) for 10 months and after this period, leaf, stem and root tissues were harvested. Copper content was determined by ICP-OES and some characters such as proline, pigments, catalase (CAT), peroxidase (POX), superoxid desmotase (SOD) and weight of different tissues were measured. The concentrations of copper in root tissue were higher than leaf and stem tissues and stem concentration was lower than the concentration of leaf. The proline content was raised by increasing metal concentrations, but the content of pigments decreased. The activity of antioxidative enzymes, CAT, POX and SOD positively increased up to 10 mM Cu treatment and then slightly decreased in both leaf and root tissues. These results suggest that eucalypts have efficient mechanism to tolerate Cu excess, as evidenced by accumulating of osmoprotectants and antioxidative enzymes. Also eucalypts under stress can accumulate copper four times more than the control treatment without serious symptoms in growth, therefore it is a feasible plant for hyperaccumulation of copper and declining the environmental pollution.

Keywords: Antioxidative enzymes; Chlorophyll; Cu; *Eucalyptus camaldulensis*; Proline

Introduction

A major environmental concern is the contamination of soil and environment resulting from the dispersal of industrial and urban wastes activities (Ghosh and Singh 2005). Copper is a redox active transition metal and an essential micronutrient (Ducic and Polle 2005). It plays a significant role in a number of physiological processes such as the photosynthetic and respiratory electron transport chains, nitrogen fixation, protein metabolism, antioxidant activity, cell wall metabolism and hormone perception (Flores-Cáceres *et al*. 2015). Copper can also be a potentially toxic element when tissue concentrations exceed only slightly from the optimal demand (Assareh *et al*. 2008; Fariduddin *et al.* 2009; Elobeid and Polle 2010). Copper toxicity can occur from the repeated use of copper-containing fertilizers, fungicides and pesticides to leaf or soil (Sonmez *et al.* 2006). Soil organic matter and pH are main factors governing Cu availability (Fong *et al*. 2015). In addition, Cu is reported to produce reactive oxygen species (ROS) and enhance antioxidant enzyme activity in plants (Merlin *et al*. 2012). A number of different reactive oxygen species, including the superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$) and the hydrogen peroxide (H_2O_2) are produced when plants are under heavy metal stress. Production of

excess ROS in heavy metal stressed plants may be a consequence of the distribution of the balance between their production and the antioxidative enzyme activity, composed of enzymic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) (Lukatkin *et al*. 2014; Liu *et al*. 2015). Excessive amounts of Cu can lead to anatomical, morphological and physiological changes, such as inhibited nutrient uptake, reduced photosynthesis rates and plant growth (Ambrosini *et al*. 2015). It can act strongly on chromatin, seed germination, plant vigor, iron intake and senescence processes (Lequeux *et al*. 2010; Cambrolle *et al*. 2015). Toxicity symptoms also can be observed in various regions of the roots such as root apex and cell walls by increasing, shortening and thickening of lateral roots and reducing the density of the root (Zhang *et al*. 2007). Plants under copper toxicity appear stunted, are usually bluish in color and eventually turn yellow or brown. Growth analysis studies in poplar (*P. tremula* L. x P. *alba* L.) showed that low concentrations of copper had no inhibitory effect on culture quality (i.e., degree of chlorosis and browning) and shoot development (Bojarczuk 2004). The physiological response of *Eucalyptus rostrata* due to exposure to heavy metal air pollutants showed significant increase in proline content and relative peroxidase activity (El-Khatib *et al*. 2004).

The aim of this study was to evaluate antioxidative and osmoprotective pattern against copper stress in *Eucalyptus camaldulensis* and understanding the role of defense system in efficient Cu tolerance and detoxification strategy adopted by the plants as well as the possibility of using such models in contaminated areas.

Materials and Methods

Seeds of *Eucalyptus camaldulensis* were germinated in sterilized pot filled by marble chips in a controlled temperature glasshouse (20°C day/15°C night). The experimental design was completely randomized with four treatments and five replications. When seedlings reached the two-leaf stage the half-strength Hogland solution was used for irrigation (Moor 1960). After 10 weeks, when the seedlings were grown to 15 centimeters, sufficient $CuSO_4.2H_2O$ was added to the nutrient solution drums to give initial four treatments. Cu concentrations were: Control (nutrient solution), 5, 10 and 20 mM. Samplings were carried out from stamen leaves of different treatments with 10-month interval. The Cu concentration of marble were measured five times (bimonthly) during the experiment. If there were any significant differences after this period, the pots would be watered and, therefore, the concentration of Cu would be adjusted.

Estimation of pigments, soluble sugar, proline and stomata number

Chlorophyll a, b and carotenoid contents were estimated by the method of Wintermans and Motes (1965) and Jason (1978). Leaf samples (0.25 g) were homogenized in 4.5 cm^3 of acetone (80%). Absorbance was recorded at 645, 663 and 470 nm (Spectrophotometer CECIL Model 3000, Cambridge, UK). Free proline content in the

leaves was determined following the method of Bates *et al.* (1973). Total soluble sugar was estimated by anthron reagent (Irigoyen *et al.* 1992). Stomata number per unit leaf area was counted through light microscope.

Estimation of water relations

Leaf water potential (LWP) was measured using the liquid immersion method (Michel 1972). Relative water content (RWC) (%) was calculated using the following equation:

RWC (%) = [(FM - DM)/(TM - DM)] * 100

In the above equation, FM, DM, and TM stand for fresh mass, dry mass and turgid mass, respectively (Boyer 1968).

Protein extraction and quantification

Total soluble proteins were extracted from the leaves and roots for assaying protein by a modification of the method described by Ausubel *et al.* (1987). This consisted of homogenization with a chilled mortar and pestle using a buffer containing ice-cold 50 mM Tris-HCl, pH 7.5, 2 mM EDTA and 0.01% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 11000 rpm for 30 min at 4 °C. Supernatant was recentrifuged at 4000 rpm for 20 min and stored at –20°C for the analysis (Hames and Rickwood 1990). Protein extracts were thawed and their concentration was determined by a colorimetric method, as described by Bradford (1976) using a commercially available reagent (Bio-Rad protein assay dye reagent). In the Bradford assay, protein concentration is determined by quantifying the binding of the dye, Coomassie Brilliant Blue G-250, to the unknown protein solution, as compared to known standards. The tubes containing 100 l aliquots of known concentrations of Bovine Serum Albumin (BSA; 0.156 mg l^{-1} to 10 mg l^{-1} in 0.15 M NaCl) were prepared. Blank tubes containing 100 l of 0.15 M NaCl were also prepared. One ml Coomassie Brilliant Blue solution was added to each tube and stirred on a vortex. The reactions were left at room temperature for 2 min. The absorbance at wavelength of 595 nm was determined against the blank and the standard curve of absorbance versus protein concentration plotted. The reactions containing dilutions of the soluble protein extracts were set up as above and the absorbance at 595 nm was determined. The protein concentration of the extracts was determined from the standard curve using spectrophotometer (CECIL Model 3000, Cambridge, UK).

Analysis of antioxidant enzymes

POX activity was estimated according to the method of Zhang *et al*. (1995). The reaction mixture (3 ml) contained 100 mM potassium phosphate buffer (pH 6.1), 96 mM guaiacol, 12 mM H_2O_2 and enzyme extract. The oxidation of guaiacol was measured by the increase in absorbance at 470 nm. The enzyme activity was calculated using the extinction coefficient 25.5 mM^{-1} cm^{-1} and expressed in units (mg/protein). One unit of enzyme was the amount necessary to decompose 1μmol of substrate per min.

Catalase activity was determined by measuring the decomposition of hydrogen peroxide. About 100 μl of enzyme extract was added into the reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 20 mM H_2O_2. The

decrease of the absorbance at 240 nm was recorded. Activity was calculated using an extinction coefficient of 39.04 mM^{-1} cm^{-1}. One unit of CAT activity was defined as the amount required for decomposing 1µmol of hydrogen peroxide/min/mg protein under assay conditions (Beer and Sizer 1952).

The SOD activity was quantified by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). The reaction mixture (3 ml) contained 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 2.25 mM NBT, 60 µM riboflavin and enzyme extract. After mixing, the contents in the cuvette were illuminated (40 watts light) for 15 minutes. Enzyme extract kept in the dark served as blank, while buffer with no enzyme extract kept in the light served as the control. The absorbance was measured at 560 nm against a blank using a UV visible spectrophotometer. NBT reduction in the light was measured in the presence and absence of enzyme extract. SOD activity was calculated as absorbance of control minus absorbance of sample, giving the total inhibition. One unit of activity was the amount of enzyme required for 50% reduction in color and was expressed in units of the enzyme (mg $protein^{-1}$ h^{-1}) (Giannopolitis and Ries 1977).

Copper content analysis

For measuring the elements, plants were harvested 10 months after being exposed to Cu treatments. Plants were rinsed four times in the deionized water. Then, root, stem and leaf tissues were separated. The plant parts were placed in paper bags and dehydrated at 70 °C for 48 hours then digested in %65 nitric acid, 37% hydrochloric acid and 30% hydrogen peroxide (Westerma 1990). Heavy metal analysis was performed using individually coupled plasma-optical emission spectrometry (ICP-OES Integra XL, GBC, Australia). Detection limit for ICP-OES was 10µgL^{-1} for Cu.

Data analysis

Data for each variable were tested for normality and homogeneity of variances and transformations were made when necessary to meet the underlying statistical assumptions of analysis of variance. Duncan's multiple range test at confidence level of 95% was used to separate means using SPSS 13. Standard error of mean (SE) was used to indicate means variability.

Results

Effect of copper supply on physiological characters

Proline, soluble sugars and photosynthetic pigment (Chlorophyll a, b and carotenoids)

There was significant difference among treatments for leaf proline content. The leaf proline concentration of 20 mM Cu treatment was 2.4 times more than the control. However, there was not any significant difference between the 5 mM Cu treatment and the control. Similar to proline content, soluble sugars increased progressively by increasing copper supply. The concentration of soluble sugars in the leaves was more than two times for the 20 mM copper treatment than the control. There was an inverse relationship between increasing the severity of

copper concentration and chlorophyll content. Total chlorophyll content and chlorophyll a significantly decreased when copper concentration rose to 20 mM but no significant change was observed for chlorophyll b and carotenoids under copper stress (Table 1).

Table 1. The effects of toxic concentrations of copper on the growth and physiological characteristics of *Eucalyptus camaldulensis*

Parameters	Control	Cu 5 mM	Cu 10 mM	Cu 20 mM
Proline (µgg^{-1}f.w.)	4.86 ± 0.53^{b}	5.38 ± 0.76^{b}	9.43 ± 1.45^{a}	11.64 ± 0.70^{a}
Soluble sugars (µgg-1d.w.)	669.5 ± 120.9^{b}	790.2 ± 97.8^{b}	876.2 ± 134.9^{b}	1614.0 ± 374.3^{a}
Total chlorophyll (mgg^{-1}f.w.)	3.56 ± 0.29^{a}	3.16 ± 0.17^{a}	3.03 ± 0.12^{a}	2.83 ± 0.15^{b}
Chlorophyll a (mgg^{-1}f.w.)	2.10 ± 0.12^{a}	2.10 ± 0.06^{a}	1.96 ± 0.07^{ab}	1.73 ± 0.03^{b}
Chlorophyll b (mgg^{-1}f.w.)	1.04 ± 0.12^{a}	0.97 ± 0.02^{a}	0.95 ± 0.03^{a}	0.83 ± 0.03^{a}
Carotenoids (mgg^{-1}f.w.)	8.87 ± 0.92^{a}	8.53 ± 0.52^{a}	9.00 ± 0.25^{a}	9.10 ± 0.35^{a}
Leaf weight (gr)	7.81 ± 0.5^{a}	6.94 ± 0.4^{ab}	6.81 ± 0.3^{ab}	5.82 ± 0.3^{b}
Stem weight (gr)	3.52 ± 0.3^{a}	3.15 ± 0.2^{a}	3.05 ± 0.3^{a}	2.14 ± 0.2^{b}
Root weight (gr)	1.43 ± 0.2^{a}	1.23 ± 0.14^{a}	0.91 ± 0.3^{a}	0.82 ± 0.1^{a}
LWP (MPa)	-0.72 ± 0.08^{a}	-0.73 ± 0.04^{a}	-0.84 ± 0.02^{b}	-0.88 ± 0.05^{b}
RWC (%)	86.72 ± 4.2^{a}	85.6 ± 5.1^{a}	84.3 ± 4.5^{a}	76.7 ± 3.7^{b}
Adaxial number	980 ± 87^{a}	1050 ± 173^{a}	950 ± 57^{a}	930 ± 115^{a}
Abaxial number	2300 ± 33^{a}	2333 ± 66^{a}	2250 ± 137^{ab}	2066 ± 88^{b}
Stem Cu (mgkg-1D.W.)	46.4 ± 3.8^{d}	89.6 ± 4.6^{c}	122.6 ± 6.9^{b}	176.6 ± 10.6^{a}
Root Cu (mgkg-1D.W.)	44.7 ± 10.3^{d}	521.6 ± 23.3^{c}	1024.4 ± 59.4^{b}	2459.3 ± 96.9^{a}
Leaf Cu (mgkg-1D.W.)	20.3 ± 3.6^{d}	38.3 ± 3.3^{c}	47.0± 2.91^{b}	84.4± 7.89^{a}
Leaf TSP (mg g-1 F.W.)	11.3 ± 0.3^{c}	11.9 ± 0.2^{c}	13.4 ± 0.4^{b}	17.5 ± 0.4^{a}
Root TSP (mg g-1 F.W.)	2.52 ± 0.2^{d}	3.11 ± 0.1^{c}	4.02 ± 0.2^{b}	5.12 ± 0.1^{a}
Leaf POX (U mg-1 protein)	9.35 ± 0.7^{c}	18.3 ± 1.3^{b}	60.4 ±3.3^{a}	54.2 ± 3.8^{a}
Root POX (U mg-1 protein)	16.6 ± 0.8^{c}	33.2 ± 2.0^{b}	72.3 ± 2.9^{a}	68.5 ± 3.8^{a}
Leaf CAT (U mg-1 protein)	11.4 ± 0.5^{c}	13.6 ± 1.2^{c}	25.6 ± 1.1^{a}	21.2 ± 0.9^{b}
Root CAT (U mg-1 protein)	24.2 ± 0.7^{d}	38.4 ± 1.5^{c}	53.2 ± 2.1^{a}	45.4 ± 1.8^{b}
Leaf SOD (U mg-1 protein)	9.53 ± 0.7^{c}	18.5 ± 1.3^{b}	60.1 ± 3.3^{a}	54.3 ± 3.8^{a}
Root SOD (U mg-1 protein)	16.7 ± 0.8^{c}	33.2 ± 2.0^{b}	72.6 ± 2.9^{a}	68.1 ± 3.8^{a}

Means±standard error; Means with different letters in a row are significantly different ($p \leq 0.05$) according to the Duncan's multiple range test; LWP: leaf water potential; RWC: relative water content; TSP: total soluble protein; POX: peroxidase; CAT: catalase; SOD: superoxide dismutase

Growth characters, leaf water potential, relative water content and stomatal number

Under the 20 mM copper concentration, older leaves were red; a trend that progressed up the plant with time and eventually all leaves became red. The seedlings showed minimal growth at the maximum concentration of Cu (20 mM). Stem weight decreased from 3.5 to 2.1 g fresh weight

i.e., nearly 40% lower as compared to the control, at the highest copper concentration. Root weight was not affected significantly by the Cu concentration. LWP and RWC decreased when copper supply increased. It demonstrated that osmotic adjustment is a common response to copper excess in eucalypts. Copper supply did not affect adaxial stomata frequency significantly difference but abaxial stomata decreased by the Cu treatment.

Protein and antioxidative enzyme activity

Leaf total soluble proteins showed an increasing trend with the increase in copper level. However, this increase was more pronounced for the 20 mM Cu concentration as compared to the control. Total soluble proteins increased from 11.3 to 17.5 mg g^{-1} F.W. in shoots and 2.5 to 5.1 mg g^{-1}F.W. in roots at the maximum copper concentration, i.e., nearly 54% and 100% higher, respectively as compared to the control. The relationship between POX, CAT and SOD and the Cu concentration in the growth medium was depicted in Figure 1. POX and CAT activity were increased significantly in the leaf and root tissues up to 10 mM Cu concentration (600% and 425% increment in the leaf and root tissues, respectively for POX) (227% and 220% increment in leaf and root tissues, respectively for CAT) (Figure 1 a,b). Thereafter, it slightly decreased at 20 mM (Figure 1b), however, the difference with the control treatment was significant. In general, the CAT and POX activity was significantly increased at increasing concentrations of Cu treatment. SOD activity increased significantly up to 10 mM Cu in both leaf and root tissues (Figure 1c). Maximum SOD activity was 660% and 450% higher in the leaf and root tissues, respectively for 10 mM Cu compared to the control. The SOD activity slightly decreased at 20 mM Cu concentration.

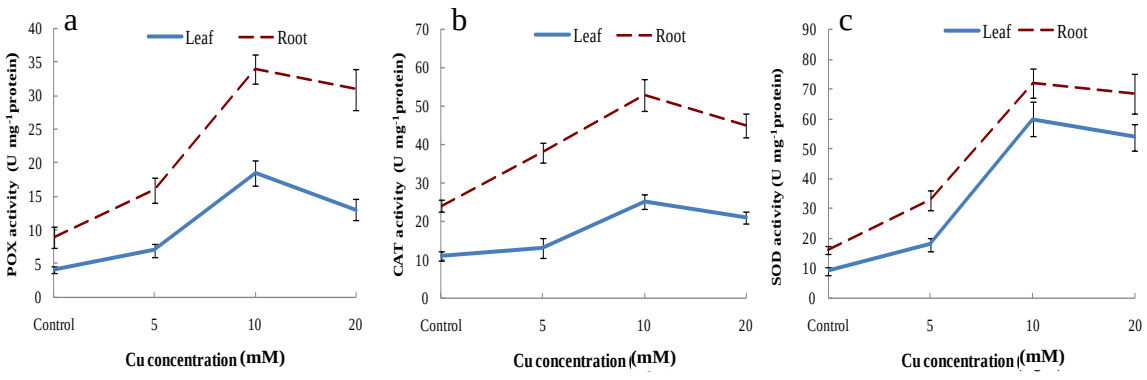

Figure 1. Effects of copper heavy metal stress on POX (a), CAT (b) and SOD (c) antioxidative enzyme activities in leaf and root tissues of *E. camaldulensis*. The error bars indicate mean ± SE (n= 5). POX: peroxidase; CAT: catalase; SOD: superoxide dismutase.

Copper concentration in plants

Cu concentration in the root tissue was higher than the shoot tissue. The shoot Cu concentration increased as the relative dry weight decreased. Root Cu concentration also increased as the shoot dry weight decreased (leaf<stem<root). There was more differentiation between root Cu concentrations as dry weight decreased. Absorption of Cu increased from 46.4 to 176 in stems, from 44 to 2459 in roots and from 20 to 84 $mgkg^{-1}$D.W. in leaves; nearly 3.7 times more in stems, 56 times more in roots and four times more in leaves by the copper treatment as compared to the control.

Discussion

This study showed that the Cu excess inhibited the plant growth and induced oxidative stress. There were strong morphological differences between copper treated and control plants. The absence of visual damage to the seedlings suggests that *Eucalyptus camaldulensis* plants have efficient mechanisms to tolerate Cu excess under the present experimental conditions. Our result clearly showed that compatible solutes such as soluble sugars and proline accumulated under Cu excess. Osmotic adjustment by accumulation of compatible solutes, which include sugars, glycerol, amino acids, sugar alcohols and other low molecular weight metabolites, is one of the mechanisms evolved by plants to overcome stress (White *et al.* 2000, Verslues *et al.* 2006). Moreover, Filippou *et al*. (2014) showed that proline accumulated in tissues of the stressed plants due to the increased rate of its synthesis by pyrroline 5 carboxylate synthetase and the decreased rate of its degradation by proline oxidase enzyme. Similar response to copper treatment was previously noticed in various plants (El-Khatib *et al*. 2004; Elobeid and Polle 2010). In this study *E. camaldulensis* showed photosynthetic susceptibility to high doses of Cu as total chlorophyll and chlorophyll a significantly decreased. A lower content of chlorophyll and alterations of chloroplast structure and thylakoid membrane composition have been found in leaves of barley (Demirevska-Kepova *et al*. 2004); tea (Camellia sinensis) (Dey *et al*. 2014) and alfalfa (Flores-Cáceresa *et al*. 2015). It has been proposed that Cu interferes with the biosynthesis of the photosynthetic machinery modifying the pigment and protein composition of photosynthetic membranes (Fariduddin *et al*. 2009). Excess copper can induce alterations in the photosynthetic and respiratory processes, enzyme activity, DNA and membrane integrity, all of which could lead to growth inhibition (Fariduddin *et al*. 2009; Flores-Cáceres *et al*. 2015). Decreased plant growth might be associated with the inhibition of mitotic index noticed with heavy metal treatment (Lequeux *et al*. 2010; Malar *et al*. 2014). Plant tolerance to heavy metal stress is estimated based on their root and/or shoot growth inhibition by the metal present in a nutrient solution (Flores-Cáceresa *et al*. 2015; Ambrosini *et al*. 2015).

Decreasing of LWP and RWC demonstrated that osmotic adjustment is a common response to copper excess in eucalypts. Relative water content change has been suggested as an indicator of

phytotoxicity after heavy metal stress (Zn and Cr) in Chinese brake and Indian mustard fern (Su *et al*. 2005). Relative water content in the leaves was slightly higher in copper treated plants than in the control at the end of the Cu treatment. It is most likely that metal stress induced stomatal closure, triggered over the course of the experiment due to the atmospheric carbon fixing activities that were compromised as a consequence (Brunet *et al*. 2008).

No significant difference was obtained between Cu treatments and the control in terms of adaxial stomata frequency but abaxial stomata decreased by the Cu concentration. Significant decrease of LWP, RWC and abaxial stomata must be related to osmotic adjustment. This may reflect the fact that abaxial guard cells are usually more sensitive to environmental signals such as changes in light intensity or quality, soil water status, ambient humidity and CO_2 concentration (Lu 1988; Wang *et al*. 1998). Plant species vary in their capacity for Cu accumulation. For example Gharbi *et al.* (2008) reported that lettuce had a relatively higher potential for Cu uptake and translocation than spinach. Cu accumulation also differed among plant organs (Gharbi *et al.* 2008). In *Brassica pekinensis* Rupr Cu treatments significantly elevated the Cu content of the shoots (Xiong and Wang 2005). Lombardi and Sebastian (2005) reported that rootstocks of fruit trees, commonly used to regulate growth, precocity, fertility and yield, can also be used to solve problems associated with soil pollution such as copper excess. They demonstrated that *Prunus cerasifera* is quite tolerant to copper and mobilizes catalase and superoxide dismutase in order to mitigate copper stress damages.

We showed that the high level of copper caused an oxidative stress and resulted in a fast and high production of antioxidant components, such as CAT, SOD and POX. The SOD activity was slightly decreased at the higher concentrations of Cu (>10 mM), probably because of the harmful effects of over production of H_2O_2 or its poisonous ROS derivatives. Enzymatic activity is induced by ROS production through indirect mechanisms, such as lipid peroxidation or disruption of the electron transport chain in chloroplast and mitochondria (Sharma and Dietz 2008). Similar results were reported by Zhang *et al*. (2007) and Fengtao *et al*. (2013). Enhanced level of SOD activity may be attributed to the production of more active oxygen species (AOS) or over expression of genes encoding SOD. A slight decrease in SOD activity noticed at the higher Cu dose may be due to the inhibition of enzyme activity by excess H_2O_2 content that is a product in various cellular compartments. The effects of high concentration of Cu have been reported previously in citrus, radish and *Arabidopsis thaliana* (Merlin *et al*. 2012; Lukatkin *et al*. 2014; Liu *et al*. 2015). Cu toxicity causes an oxidative burst with rapid H_2O_2 production in *Arabidopsis* and release into the apoplast of plants (Liu *et al*. 2015). Cu can affect membrane integrity in citrus that would lead a reduction of root water content, affecting pressure potential and growth (Merlin *et al*. 2012). The same effect was observed on raddish (*Raphanus sativus* L.) by Lukatkina *et al*. (2014)

and on *Lens culinaris* Medik. by Janas *et al*. (2010), where a high Cu amount was accumulated in the roots, inducing oxidative stress and excessive ROS production, resulting in the rapid deterioration of membrane lipids. Increased POX activity is considered a reliable indicator of heavy metal impact (Malar *et al*. 2014). POX in turn catalyzes H_2O_2-dependent oxidation of substrates (Passardi *et al*. 2005). Previous studies in other plants have also shown changes in POX activity in response to heavy metal exposure (Zhang *et al*. 2007; Kim *et al*. 2010). Zhang *et al*. (2007) showed that the metal tolerant mangrove species, *Kandelia candel*, was able to maintain higher levels of POX activity than another mangrove species *Bruguiera gymnorhiza*, at higher concentrations of heavy metals, and that there was a significant difference in POX activity between these two mangrove plants. These results suggest that enhanced POX activity correlates with heavy metal exposure, and that POX has a protective role against oxidative stress and the accumulation of ROS in response to heavy metal exposure. It is generally known that heavy metal stress is related to physiological and biochemical process alterations owing to generated oxidative stress in plants (Sharma and Dietz 2008). The activity of antioxidant enzymes can prevent ROS overproduction and oxidative destruction by heavy metal conditions, which may lead to fatal damage at cellular and whole plant level (Noctor and Foyer 1998). CAT eliminates H_2O_2 by breaking it down directly to form water and oxygen. However, it operates alone and has low substrate affinity for H_2O_2 per cycle. POXs have a much higher affinity for H_2O_2 and require a reductant (Cosio and Dunand 2009). Therefore, the highest SOD activity suggests that SOD can serve as a better intrinsic defense tool to resist heavy metal induced oxidative stress in plant.

Conclusions

Eucalyptus camaldulensis is one of fast growing and evergreen of eucalypts that is used for extending silviculture and leads to study of declining pollution and impressing in uptaking heavy metals. This study confirms several aspects of the physiology of *Eucalyptus camaldulensis* with respect to copper supply. In this study, *Eucalyptus camaldulensis* adapted to higher doses of Cu treatment and the level of antioxidative enzymes was enhanced significantly. Results strongly suggest that *Eucalyptus camaldulensis* is not affected by oxidative stress, in spite of the presence of higher dose of Cu in the irrigation water, as would be anticipated for a species that has efficiently survived in a highly polluted environment. Increased SOD, CAT and POX activity appear to play key roles in the antioxidant defense response of *Eucalyptus camaldulensis* seedlings when exposed to Cu heavy metal toxicity. Enhancing the antioxidant enzymes could help to overcome metal toxicity from ROS detoxification. Despite the fact that research efforts have produced an enormous amount of information, we are far from understanding the complete circuits of stress reactions. Only a few components of many pathways have been the subject of investigations. Future priorities should be aimed at the identification of molecules connecting pathways and of key components in each pathway. It is proposed that our

understanding of plant stress tolerance can be greatly refined by thorough characterization of individual genes and assessing their contribution to the stress tolerance. The results showed that eucalypts can accumulate copper four time more than the control treatment without serious symptoms in growth, therefore it can be used for declining of environmental pollution. This result is important in the planning ecologically and economically advantageous *Eucalyptus* planting. Further research should be conducted on the tolerance of other species.

References

Ambrosini VG, Rosa DJ, Corredor Prado JP, Borghezan M, Bastos de Melo GW, Fonsêca de Sousa Soares CR and Brunetto G, 2015. Reduction of copper phytotoxicity by liming: a study of the root anatomy of young vines (*Vitis labrusca* L.). Plant Physiology and Biochemistry 96: 270–280.

Assareh MH and Shariat A, 2008. Seedling response of three Eucalyptus species to copper and zinc toxic concentrations. Caspian Journal of Environmental Sciences 6: 97–103.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (Eds), 1987. Current Protocols in Molecular Biology. John Wiley and Sons, Inc., New York.

Bates IS, Waldern RP and Teare ID, 1973. Rapid determination of free proline for water stress studies. Plant and Soil 39: 205-207.

Beauchamp C and Fridovich I, 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry 44: 276–287.

Beers RR and Sizer IW, 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. Journal of Biological Chemistry 195: 133-140.

Bojarczuk K, 2004. Effect of toxic metals on the development of poplar (*Populus tremula* L. x *P. alba* L.) cultured in vitro. Polish Journal of Environmental Studies 13 (2): 115 – 120.

Boyer JS, 1968. Measurement of the water status of plants. Annual Review of Plant Physiology 9: 351-363.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254.

Brunet J, Repellin A, Varrault G, Terrync N and Zuily-Fodil Y, 2008. Lead accumulation in the roots of grass pea (*Lathyrus sativus* L.): a novel plant for phytoremediation systems? Comptes Rendus Biologies 331: 859–864.

Cambrolle J, García JL, Figueroa ME and Cantos M, 2015. Evaluating wild grapevine tolerance to copper toxicity. Chemosphere 120: 171-178.

Cosio C and Dunand C, 2009. Specific functions of individual class III peroxidase genes. Journal of Experimental Botany 60: 391–408.

Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Hölzer R and Feller U, 2004. Biochemical changes in barley plants after excessive supply of copper and manganese. Environmental and Experimental Botany 52: 253–266.

Dey S, Mazumder PB and Paul SB, 2014. Effect of copper on growth and chlorophyll content in tea plants (*Camellia sinensis* (L.) O. Kuntze). IMPACT: International Journal of Research in Applied, Natural and Social Sciences 2: 223–230.

Ducic Tand Polle A, 2005. Transport and detoxification of manganese and copper in plants. Brazilian Journal of Plant Physiology 17: 103–112.

El-Khatib AA, Faheed FA and Azooz MM, 2015. Physiological response of *Eucalyptus rostorata* to heavy metal air pollution. El-Minia Science Bulletin 15: 429–451.

Elobeid M and Polle A, 2010. Response of grey poplar (Populus x canescens) to copper stress. Plant Stress 4 (special issue 1): 82–86.

Fariduddin Q, Yusuf M, Hayat S and Ahmad A, 2009. Effect of 28-homobrassinolide on antioxidant capacity and photosynthesis in *Brassica juncea* plants exposed to different levels of copper. Environmental and Experimental Botany 66: 418–424.

Fengtao LI, Jianmin QI, Gaoyang Z, Lihui L, Pingping F, Fen TA and Jiantang XU, 2013. Effect of cadmium stress on the growth antioxidative enzymes and lipid peroxidation in two kenaf (*Hibiscus cannabinus* L.) plant seedlings. Journal of Integrative Agriculture 12: 610–620.

Filippou P, Bouchagier P, Skotti E and Fotopoulos V, 2014. Proline and reactive oxygen/nitrogen species metabolism is involved in the tolerant response of the invasive plant species *Ailanthus altissima* to drought and salinity. Environmental and Experimental Botany 97: 1–10.

Flores-Cáceres ML, Hattab S, Hattab S, Boussetta H, Banni M and Hernández LE, 2015. Specific mechanisms of tolerance to copper and cadmium are compromised by a limited concentration of glutathione in alfalfa plants. Plant Science 233: 165–173.

Fong JDM, Masunaga T and Sato K, 2015. Control of micronutrients availability in soil and concentration in rice grain through field water management. Journal of Agricultural Science 7: 163–174.

Gharbi F, Rejeb S, Ghorbal MH and Morel JL, 2008. Plant response to copper toxicity as affected by plant species and soil type. Journal of Plant Nutrition 28: 379-382.

Ghosh M and Singh SP, 2005. A review on phytoremediation of heavy metals and utilization of it's by products. Asian Journal on Energy and Environment 6: 214–231.

Giannopolitis CN and Ries SK, 1977. Superoxide dismutases. Plant Physiology 59: 309–314.

Hames, BD and Richwood M, 1990. Gel Electrophoresis of Proteins, a Practical Approach. Oxford University Press, UK.

Irigoyen JJ, Einerich DW and Sanchez-Diaz M, 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum 84 (1): 58-60.

Janas KM, Zielińska-Tomaszewska J, Rybaczek D, Maszewski J, Posmyk MM, Amarowicz R and Kosińska A, 2010. The impact of copper ions on growth, lipid peroxidation, and phenolic compound accumulation and localization in lentil (*Lens culinaris* Medic.) seedlings. Journal of Plant Physiology 167: 270–276.

Jenssen A, 1978. Chlorophyll and carotenoids. In: Hellebust JA and Craigie JS (Eds). Handbook of Phycological Methods. Physiological & Biochemical Methods. Pp. 59-65. Cambridge University Press, Cambridge, New York.

Kim YH, Lee HS and Kwak SS, 2010. Differential responses of sweet potato peroxidases to heavy metals. Chemosphere 81: 79–85.

Lequeux H, Hermans C, Lutts S and Verbruggen N, 2010. Response to copper excess in *Arabidopsis thaliana*: impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. Plant Physiology and Biochemistry 48: 673-682.

Liu J, Shi X, Qian M, Zheng L, Lian C, Xia Y and Shen Z, 2015. Copper-induced hydrogen peroxide upregulation of a metallothionein gene, OsMT2c, from *Oryza sativa* L. confers copper tolerance in *Arabidopsis thaliana*. Journal of Hazardous Materials 294: 99–108.

Lombardi L and Sebastian L, 2005. Copper toxicity in *Prunus cerasifera*: growth and antioxidant enzymes responses of in vitro grown plants. Plant Science 168: 797–802.

Lu Z, 1988. The sensitivity of adaxial and abaxial stomatal resistance in wheat leaf to soil water stress. Acta Phytophysiol Sinica 14: 223–227.

Lukatkin A, Egorova I, Michailova I, Malec P and Strzałka K, 2014. Effect of copper on pro- and antioxidative reactions in radish (*Raphanus sativus* L.) in vitro and in vivo. Journal of Trace Elements in Medicine and Biology 28 (1): 80–86.

Malar S, Shivendra-Vikram S, JC-Favas P and Perumal V, 2014. Lead heavy metal toxicity induced changes on growth and antioxidative enzymes level in water hyacinths [*Eichhornia crassipes* (Mart.)]. Botanical Studies 55: 1–11.

Merlin TPA, Lima GPP, Leonel S and Vianello F, 2012. Peroxidase activity and total phenol content in citrus cuttings treated with different copper sources. South African Journal of Botany 83*:* 159–164.

Michel BE, 1972. Solute potentials of sucrose solutions. Plant Physiology 50: 196–198.

Moor RH, 1960. Laboratory Guide for Elementary Plant Physiology. Burgess Pub, Minneapolis.

Noctor G and Foyer CH, 1998. Ascorbate and glutathione: keeping active oxygen under control. Annual Review of Plant Physiology and Plant Molecular Biology 49: 249–279.

Passardi F, Cosio C, Penel C and Dunand C, 2005. Peroxidases have more functions than a Swiss army knife. Plant Cell Reports 24: 255–265.

Sharma SS and Dietz KJ, 2008. The relationship between metal toxicity and cellular redox imbalance. Trends in Plant Sciences 14: 43–50.

Sonmez S, Kaplan M, Sonmez NK, Kaya H and Uz I, 2006. High level of copper application to soil and leaves reduce the growth and yield of tomato plants. Scientia Agricola 63(3): 213–218.

Su Y, Han FX, Sridhar BBM and Monts DL, 2005. Phytotoxicity and phytoaccumulation of trivalent and hexavalent chromium in brake fern. Environmental Toxicology and Chemistry 24: 2019–2026.

Verslues PE, Agrawal M, Katiyar-Agrawal S, Zhu JH and Zhu JK, 2006. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant Journal 45: 523-539.

Wang XQ, Wu WH and Assmann SM, 1998. Differential responses of abaxial and adaxial guard cells of broad bean to abscisic acid and calcium. Plant Physiology 118:1421–1429.

Westerma REL, 1990. Soil Testing and Plant Analysis. SSSA, Madison Wisconsin, USA. 571 pages.

White DA, Turner NC and Galbraith JH, 2000. Leaf water relations and stomatal behavior of four allopatric *Eucalyptus* species planted in Mediterranean southwestern Australia. Tree Physiology 20: 1157-1165.

Wintermans JFGM and Motes AD, 1965 Spectrophotometric characteristics of chlorophyll a and b and their pheophitin in ethanol. Biochimica et Biophysica Acta 109: 440-452.

Xiong ZT and Wang H, 2005. Copper Toxicity and Bioaccumulation in Chinese cabbage (*Brassica pekinensis* Rupr.). Environmental Toxicology 20 (2): 188-194.

Zhang C, Zhou W and Zhu D, 2007. Physiological responses induced by copper bioaccumulation in *Eichhornia crassipes* (Mart.). Hydrobiologica 579: 211–218.