Australian Journal of Crop Science AJCS
AJCS 6(3):541-549 (2012) ISSN:1835-2707

The impact of copper ion on growth, thiol compounds and lipid peroxidation in two maize cultivars (*Zea mays* L.) grown *in vitro*

Amina A. Aly*[1] and Amal A. Mohamed[2]

[1]Natural Products Dept., National Centre for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt
[2]Plant Biochemistry Dept., National Research Centre, Dokki, Cairo, Egypt

***Corresponding author: aly_amina@yahoo.co.uk**

Abstract

The aim of the present study was to investigate the effect of increasing copper concentrations (0-100 µM Cu) on the growth, thiol compounds and some other biochemical parameters of maize (*Zea mays* L.) cultivars. For that, plantlets of two different *Zea mays* cultivars (Single Cross 122 and Single Cross 10) characterized by different levels of response to heavy metals stress) were exposed to four Cu levels (0.0, 25.0, 50.0 and 100.0 µM) for 15 days. At seedling stage, significant differences in some parameters were noticed between both cultivars followed by different Cu treatments. For example, copper stress gradually depressed shoots length, roots length and number of roots with increasing Cu levels in MS media. Furthermore, there was an increase in Cu accumulation in the treated shoots compared to the non-treated one, but the accumulation being more pronounced in Single Cross 10 (SC 10) than in Single Cross 122 (SC 122). Chlorophylls and carotenoid pigments were generally decreased by Cu stress, especially under the highest Cu treatment (100 µM) in both cultivars. Proline and total soluble sugars were measured and more pronounced increase was observed in SC 122 compared to SC 10, possibly due to a better capacity to copper stress response. Levels of glutathione, cysteine and lipid peroxidation were increased with increasing of Cu stress. Moreover, the content of total thiols, non protein thiols, protein bound thiols and acid soluble thiols were altered under Cu stress. The basic knowledge of copper stressed responses may further be applied as effective indices for copper tolerance in maize breeding program.

Keywords: Chlorophyll, copper stress, lipid peroxidation, thiols compound, *Zea mays*.
Abbreviations: H_2O_2-hydrogen peroxide; MDA-malondialdehyde; MS- Murashige and Skoog; ROS- reactive oxygen species; TT-total thiols; NPT- non protein thiols; PBT-protein bound thiols; AST- acid soluble thiols.

Introduction

Copper (Cu) is an essential micronutrient for growth and development of plants and plays a significant role in many physiological process. Like most micronutrients, Cu is needed in small amounts by the plant. At cellular level, Cu plays an essential role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron mobilization (Yruela, 2005). Moreover, Cu is required in biological systems as a structural component, and it can be a stress factor causing physiological responses that can inhibit plant growth at higher concentrations in soil (Monni et al., 2000). Excess of Cu concentrations may induce a significant toxic effect by altering the protein function and enzymes activity (Hansch and Mendel, 2009). Toxicity may result from the binding of metals to sulfhydryl groups in the protein, leading to the inhibition of activity or disruption of the structure (Morelli and Scarano, 2004). It was proposed that, Cu interferes with the biosynthesis of the photosynthetic machinery modifying the pigment and protein composition of photosynthetic membranes (Lidon and Henriques, 1991; Maksymiec et al., 1994). Copper can also substitute for Mg in chlorophyll present in both antenna complexes and reaction centers, thus damaging the structure and function of chlorophyll (Tanyolac et al., 2007). In addition, excess Cu concentrations are said to generate oxidative stress due to an increase in the levels of reactive oxygen species (ROS) within subcellular compartments. ROS include the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$), all of which affect mainly lipids, proteins, carbohydrates, and nucleic acids (Brahim and Mohamed, 2011). ROS are also known to damage cell membranes by inducing lipid peroxidation; causing membrane damage, and inactivation of enzymes and alteration of DNA activity (De Vos et al., 1993). Under steady state conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms. The equilibrium between the production and the scavenging of ROS may be perturbed by various abiotic stress factors such as heavy metals. These disturbances in equilibrium lead to sudden increase in intracellular levels of ROS. To overcome this, cells are equipped with enzymatic and non-enzymatic mechanisms to eliminate or reduce their damaging effects. The importance of antioxidant enzymes is their ability to scavenge ROS and thereby prevent oxidative damage. The antioxidant system comprises several enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (PODs) enzymes which play a crucial role to

protect plant from oxidative damage, and it is believed that their amount and activities determine the degree of tolerance in plant (Teisseire and Guy, 2000). The activities of SOD, CAT and PODs can be increased under Cu stress (Srivastava et al., 2006) while in other work, it has been reported that excessive Cu will not increase or even inhibit the activities of SOD, CAT and PODs enzymes in pea seedlings at the applied concentrations and during the treatment period (Chaoui and Ferjani, 2005). In addition to the chain of enzymatic reactions (Asada, 1994), a direct radical scavenging activity performed by non enzymatic chemical species cooperate in the remova1 of the active forms of oxygen (Rama Devi and Prasad, 1998), such as ascorbate (AsA), glutathione (GSH), carotenoids and some low molecular weight compounds containing sulfhydryl groups (Lombardi and Sebastiani, 2005). GSH is an important antioxidant produced by the cell and is responsible for maintenance of antioxidative machinery of the cells integrity under stress (Rennenberg and Brunold, 1994). It is a disulphide reductant that protects thiols of enzymes and react with hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) together with ascorbate (Noctor and Foyer, 1998). The ability of GSH to participate in the redox regulation of cellular processes is presumed to be largely dependent upon its concentration and the ratio between GSH and glutathione disulfide (GSSG). Another mechanism to cope with toxic metals is that, plants activate the sulfur assimilation pathway to provide an enhanced supply of glutathione for the biosynthesis of phytochelatins (PCs), which play a major role in heavy metal sequestration (Ederli et al., 2004). Phytochelatin production induced by copper was accompanied by an increase of total glutathione in maize (Tukendorf and Rauser, 1990).Moreover, plants synthesize and accumulate several organic solutes like sugars, polyols, betaines, proline and sulfur-containing indole derivatives in response to copper stress. Proline plays an important role in osmoregulation, protection of enzymes and stabilization of the machinery of protein synthesis (Choudhary et al., 2007). It also acts as an effective singlet oxygen quencher (Alia and Matysik 2001). Additionally, sugars represent the major accumulating solutes, reserve in the seeds and maintained the osmotic regulation of cells (Bewley and Black, 1994). There are several reports on carbohydrate accumulation during various abiotic stresses in the temperate grasses and cereals from the gramineae family where long term carbohydrate storage occurs during reproductive development (Meier and Reid, 1982). Accumulation of sugars in different parts of plants is enhanced in response to the variety of environmental stresses (Prado et al., 2000). In case of Cu stress (Aalaoui-sossé et al., 2004); adaptation to this stress has been attributed to the stress induced increase in carbohydrate level. Thus the variation that occurs in carbohydrate level, during early developmental stages of seedling under different abiotic stresses is not well understood and information on physiological events involved in this process is scarce. Maize *(Zea mays* L.) is grown throughout the world and is one of the most important cereal and economic oily crop in Egypt. Like other cereal crops, yield of maize is affected by several biotic and abiotic factors. The main objective of the current study was to investigate the effect of copper stress on chlorophylls, proline level, total soluble sugars and reducing sugars together with lipid peroxidation and thiol compounds in two maize cultivars at seedling stage.

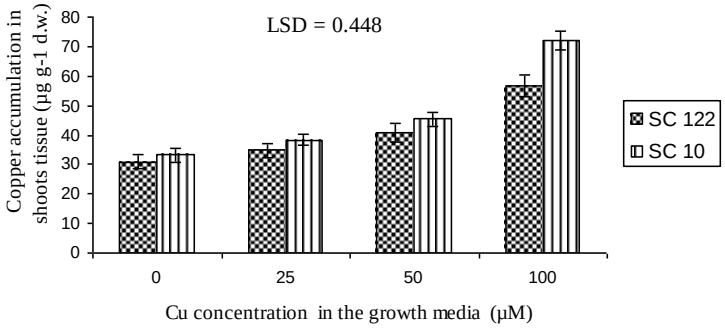

Fig 1. Cu accumulation in shoots of two maize cultivars, (SC 122) and (SC 10) grown on MS media supplemented with different concentrations of Cu for 15 days. LSD; means the interaction between Cu treatment and the maize cultivar at ($p<0.05$ and bars indicate standard division± S.D. (n=3).

Results and discussion

Plant growth

After exposure of plantlets to different concentrations of Cu (0.0, 25.0, 50.0 and 100.0 µM) for 15 days, morphological changes were observed and the growth rate of both cultivars was gradually decreased by increasing Cu concentration. Copper stress treatments significantly ($P\leq 0.05$) decreased shoots length, roots length, and number of roots compared to those plantlets grown under control condition (Table 1). Shoots length was reduced in both cultivars in the presence of Cu in the culture media. SC 122 cultivar displayed 62.63% shoots length reduction over the control (100%) while the reduction percentage was 70.91% in SC 10 after 15 days exposure to 100 µM Cu stress. In SC 122 cultivar, the control treatment (0.0 µM Cu) and the highest Cu treatment (100 µM) had the longest (28.32 cm) and shortest roots length (18.10 cm), respectively. Number of roots per plant showed more pronounced decrease in the SC 10 cultivar (69.83 %) compared to SC 122 cultivar (48.25%) at 100 µM in comparison with control (100 %). On the other hand, percentage of shoots dry matter fluctuated at different Cu levels in both cultivars. In the present study, copper stress retarded the plantlets growth of both maize cultivars by inhibiting shoots length, roots length and number of roots. One of the most rapid responses to toxic Cu levels is inhibition of root growth (Eleftheriou and Karataglis, 1989). Root growth has also been considered as a very sensitive indicator to heavy metal stress (Wilkins, 1978). Heavy metals have been shown to cause many morphological, physiological and biochemical changes in plants, such as growth inhibition (Benavides et al., 2005; Farouk et al., 2011). The inhibitory action of excess copper in shoots length, roots length and dry matter may be due to reduction in cell division, and toxic effect of Cu on photosynthesis. These contributed to the retardation of normal growth (Sonmez et al., 2006; Manivasagaperumal et al., 2011), the authors demonstrated that, higher concentrations of copper decreased the growth, and dry matter production.

Table 1. Shoots length, roots length, number of roots and shoot dry matter percent in two maize cultivars (SC 122) and (SC 10) grown on MS media supplemented with different concentrations of Cu for 15 days.

Maize cultivars / Cu (µM)	Shoots length (cm plant^{-1})			Roots length (cm plant^{-1})			No. of roots plant^{-1}			Shoot dry matter (%)		
	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean
0.0	33.75 ± 1.690	31.35± 1.120	32.55c	28.32± 0.661	27.80± 0.953	28.06d	10.57± 0.430	9.68± 0.11	10.13d	7.28± 0.018	9.23± 0.015	8.26a
25.0	29.11± 1.110	29.73± 1.620	29.42b	24.75± 0.307	24.42± 0.628	24.59c	9.50± 0.500	7.93± 0.070	8.72c	10.97± 0.030	11.28± 0.030	11.13b
50.0	26.85± 1.110	27.70± 1.40	27.27b	20.62± 0.730	22.01± 1.127	21.32b	7.93± 0.070	6.78± 0.050	7.36b	11.08± 0.050	12.13± 0.040	11.61b
100.0	21.14± 1.940	22.23± 2.75	21.68a	18.10± 1.390	19.50± 0.868	18.80a	5.1±0.4 50	6.76± 1.00	5.93a	13.60± 0.260	9.02± 0.515	11.31c
Mean	27.71a	27.75a		22.95a	23.43a		8.28b	7.79a		10.73b	10.41a	
LSD 0.05												
Cultivars	N.S			N.S			0.399			0.191		
Cu	2.21			0.79			0.564			0.271		
Cultivars × Cu	N.S			N.S			N.S			0.383		

Data with different letters were significantly different at P≤ 0.05. The values are mean ± S.D. (n=3). Not significant; N.S

Table 2. Chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TC) and total carotenoids (Cx+c) in the shoots of two maize cultivars (SC 122) and (SC 10) grown on MS media supplemented with different concentrations of Cu for 15 days.

Maize cultivars / Cu (µM)	Chl a (mg g^{-1} f.w.)			Chl b (mg g^{-1} f.w.)			TC (mg g^{-1} f.w.)			Cx+c (mg g^{-1} f.w.)		
	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean
0.0	0.995± 0.030	0.893± 0.010	0.944d	0.215± 0.200	0.203± 0.070	0.209d	1.21± 0.140	1.096± 0.030	1.150d	0.052± 0.060	0.048± 0.011	0.049d
25.0	0.721± 0.02	0.663± 0.004	0.692c	0.187± 0.010	0.175± 0.010	0.181c	0.908± 0.040	0.838± 0.150	0.873c	0.039± 0.071	0.036± 0.012	0.038c
50.0	0.508± 0.020	0.699± 0.010	0.604b	0.103± 0.310	0.196± 0.030	0.149b	0.611± 0.070	0.895± 0.190	0.753b	0..027± 0.013	0.037± 0.020	0.032b
100.0	0.357± 0.010	0.469± 0.010	0.413c	0.091± 0.140	0.136± 0.220	0.114a	0.448± 0.060	0.612± 0.140	0.532a	0.024± 0.016	0.033 0.011	0.029a
Mean	0.645a	0.681b		0.149a	0.178b		0.794a	0.860b		0.036a	0.039b	
LSD 0.05												
Cultivars	0.001			0.014			0.014			0.0017		
Cu	0.014			0.019			0.020			0.0025		
Cultivars× Cu	0.020			0.030			0.028			0.0034		

Data with different letters were significantly different at P≤ 0.05. The values are mean ± S.D. (n=3).

Copper accumulation

Results indicated significant increase in Cu concentration in shoots tissue with increasing Cu levels in the growth media (Fig. 1). Cu concentration in shoots of SC 122 was significantly lower (65.4 µg g^{-1} d.w.) than that in SC 10 (77.2 µg g^{-1} d.w) at 100 µm Cu treatments. Increasing of copper level in the growth media resulted in the higher accumulation of copper by plants. The lower performance of SC 10 could be linked to a high accumulation (assimilation + adsorption) of Cu in its roots. SC 10 allocated more Cu to the shoots than SC 122, which, at the whole-plant level, may be typical of a less-tolerant species. In this concern, Ouzounidou (1994) made similar observations that, uptake of copper increased with the increased application of copper in *Alyssum montanum* L. and this view is also supported by Cambrolle et al. (2011). Moreover, similar result is obtained in plant stressed by other metal such as Pb, El-baltagi and Mohamed (2010) reported that, the concentration of Pb in plant tissue increased with the increasing of Pb in the growth media of radish plant (*Raphanus sativus* L). From the above mentioned discussion, it could be suggested that, both cultivars showed different response when grown under Cu stress.

Chlorophylls content

Data in Table (2) showed that copper stress caused significant reduction ($P<0.05$) in the content of chlorophyll a, chlorophyll b, total chlorophyll and total carotenoids in both cultivars. In case of control plantlets, total chlorophyll content was lower in SC 10 than in SC 122. However, 100 µM Cu caused a conspicuous decrease in total chlorophyll content in both cultivars and SC 122 exhibited lesser chlorophyll damage (37.02%) than SC 10 (55.83%) compared with control (100%). Although, copper was an essential micronutrient for the growth and development of plants at low levels, it could be a strong inhibitor of photosynthesis when Cu in excess (Frankart et al., 2002). The loss in chlorophyll content could be due to peroxidation of chloroplast membranes or replacing of magnesium in chlorophyll molecule by copper (Mal et al., 2002).

Proline and sugars contents

Table (3) shows a gradual significant increase ($P<0.05$) in the accumulation of proline in both cultivars at all stress regimes. However, at 100 µM Cu, the level of proline content was higher significantly ($P\leq0.05$) in SC 122 (6.18 mg g^{-1} f.w.) than in SC 10 (3.23 mg g^{-1} f.w.) compared to controls (0.540 and 0.360 mg g^{-1} f.w.) respectively. Similar trend was observed for total soluble sugars, however reducing sugars fluctuated at different Cu levels in the growth media. Because proline commonly involved in absorption of OH radical, protect the plant from stress with an appreciable affinity to forming various complexes with cupric ions, which may be partly attributable to the reduction of Cu toxicity (Wu, 1998). Moreover, proline increases the stress tolerance of the plants through such mechanisms as osmoregulation, protection of enzymes against denaturation, and stabilization of protein synthesis (Kuznetsov and Shevyakova, 1997). Accumulation of free proline in response to Cu, Cd and Zn was determined in non tolerant and metal-tolerant *Silene vulgaris* (Moench) Garcke; and the results showed that the constitutive proline concentration in leaves was 5 to 6 times higher in the metal-tolerant ecotype than in the non tolerant ecotype (Schat et al., 1997). Variations in total soluble sugars in both tested maize plantlets as affected by Cu supply are presented in Table (3). It is clear that the increasing levels of Cu generally induced an increase effect on the accumulation of total soluble sugars in both maize cultivars. On the other hand, reducing sugars were lower in Cu-grown plantlets at 100 µM compared with control one. In this concern, Verma and Dubey (2001) studied the effect of cadmium stress (100 µM and 500 µM Cd) on the content of total soluble sugars in two rice cultivars during a 5 to 20 d exposure in the growth medium, an increase in the content of total soluble sugars was observed. The authors indicated that, an increase of soluble sugar accompanied with increased activity of acid invertase and sucrose synthesis. In addition to the role of sugars in osmoregulation, the soluble sugars allow the plants to maximize sufficient carbohydrates storage reserves to support basal metabolism under stressed environment (Hurry et al., 1995; Dubey and Singh, 1999). On the other hand, excess Cu may interfere with the biosynthesis of the photosynthetic machinery and may modify the pigment and protein components of photosynthetic membranes (Maksymiec et al., 1994). This effect may lead to a decrease in the production of carbohydrates (Al-Hakimi and Hamada, 2011).

Glutathione, cysteine and lipid peroxidation

Glutathione (GSH) content in shoots of stressed plantlets tended to increase gradually with increasing of copper levels in the growth media of both cultivars (Table 4). However, at 100 µM, Cu caused an increase in GSH content in both cultivars; moreover SC 122 exhibited higher GSH (393.2 %) than SC 10 (241.6%) compared to control (100%). The results showed significant differences between treatments and interaction of cultivars and treatments ($P\leq0.05$) for GSH level. In the same table, cysteine content tended to decrease gradually with increasing of copper levels in the growth media of both cultivars. In control plantlets, cysteine content was lower in SC 10 (0.307 µmol g^{-1} f.w.) than in SC 122 (0.648 µmol g^{-1} f.w.). Meanwhile, 100 µM Cu caused a conspicuous decrease in cysteine content in both cultivars and SC 122 exhibited higher cysteine (52.31%) than SC 10 (48.34%) compared to control (100%). On the other hand, copper stress caused a significant increase in the levels of malondialdehyde (MDA) in both cultivars compared to control treatment (Table 4). MDA values reached to the maximum level at 100 µM Cu treatment in both cultivars, but the rate of increment was higher in SC 10 (40.17 nmol MDA g^{-1} f.w.) than the SC 122 (37.63 nmol MDA g^{-1} f.w.). Heavy metal toxicity is considered to induce the production of reactive oxygen species and may resulted in significant damage to cellular constituents. Glutathione plays a prominent role in scavenging free oxy-radicals (Zhang and Kirkham, 1994; Galli et al., 1996). Copper treatment increases the total glutathione contents in the shoots of the two maize cultivars (Table 4). It is widely accepted that detoxification of metal ions within plant tissues usually depends on chelation by appropriate ligands. Antioxidants like cysteine, proline, ascorbic acid and glutathione play an important role in detoxification of toxic metal ions (Singh and Sinha, 2005). Lipid peroxidation is a biochemical marker for the free radical mediated injury. The present results showed an increase in the level of lipid peroxides with increasing concentrations of Cu, indicating that Cu induces oxidative stress in maize plants. Similar to our observations, some report showed that MDA content increased significantly with increasing copper concentra-

Table 3. Proline, total soluble sugars and reducing sugars contents in the shoots of two maize cultivars (SC 122) and (SC 10) grown on MS media supplemented with different concentrations of Cu for 15 days.

Maize cultivars / Cu (µM)	Proline (mg g^{-1} f.w.)			Total soluble sugars (mg g^{-1} f.w.)			Reducing sugars (mg g^{-1} f.w.)		
	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean
0.0	0.54± 0.020	0.360± 0.040	0.450a	75.78± 0.12	72.66± 0.15	74.22a	36.89± 0. 17	39.07± 0. 12	38.98b
25.0	0.867± 0.03	0.92± 0.100	0.894b	80.08± 0.04	79.51± 0. 18	79.79b	35.84± 0. 21	41.54± 0. 17	38.69b
50.0	2.75± 0.020	2.97± 0.018	2.860c	85.46± 0.03	84.29± 0. 14	84.88c	48.69± 0. 16	44.24± 0. 11	46.46c
100.0	6.18± 0.010	3.23± 0.030	4.700d	93.44± 0.09	90.29± 0. 28	91.87d	31.52± 0. 14	36.97± 0. 15	34.24a
Mean	2.584b	1.87a		83.69b	81.64a		38.24a	40.46b	
LSD 0.05									
Cultivars	0.126			0.541			1.207		
Cu	0.178			0.765			1.710		
Cultivars × Cu	0.253			1.080			2.410		

Data with different letters were significantly different at P≤ 0.05. The values are mean ± S.D. (n=3).

Table 4. Glutathione (GSH), cysteine and lipid peroxidation (MDA) contents in the shoots of two maize cultivars (SC 122) and (SC 10) grown on MS media supplemented with different concentrations of Cu for 15 days.

Maize cultivars / Cu (µM)	Glutathione (µmol g^{-1} f.w.)			Cysteine (µmol g^{-1} f.w.)			Lipid peroxidation (nmol MDA g^{-1} f.w.)		
	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean
0.0	0.177± 0.010	0.160± 0.070	0.168a	0.648± 0.110	0.307± 0.010	0.410d	18.63± 0.190	17.73± 0.140	18.17a
25.0	0.294± 0.030	0.214± 0.050	0.254b	0.444± 0.100	0.260± 0.080	0.349c	23.45± 0.050	22.70± 0.140	23.07b
50.0	0.496± 0.040	0.251± 0.070	0.318c	0.359± 0.010	0.184± 0.010	0.274b	28.98± 0.070	31.18± 0.140	30.07c
100.0	0.696± 0.070	0.389± 0.060	0.528d	0.259± 0.012	0.136± 0.080	0.206a	37.63± 0.040	40.17± 0.180	38.89d
Mean	0.415b	0.253a		0.408a	0.211b		27.17a	27.94a	
LSD 0.05									
Cultivars	0.0377			0.042			1.038		
Cu	0.053			0.06			N.S		
Cultivars × Cu	1.377			2.075			NS		

Data with different letters were significantly different at P≤0.05. The values are mean ± S.D.(n=3). N.S; Not significant.

tions in germinating rice seeds exposed to 0.2 to 1.5 mM Cu (Ahsan et al., 2007) and in roots of *Brassica junicea* treated with 8 µM Cu (Wang et al., 2004). Increasing concentrations of MDA, which is a product of lipid peroxidation, is an indicator of oxidative stress after heavy metal dosing; the increase correlates with the increase of metal concentrations.

Total thiols, non-protein thiols, protein bound thiols and acid soluble thiols

Copper stress treatments resulted in a significant gradual increase of total thiols (TT) of both cultivars (Table 5). The content of total thiols increased significantly by 133.14, 152.44 and 184.50%, respectively in 25.0, 50.0 and 100.0 µM Cu treatments in the cultivar SC 122 compared with control (100%). The same trend was observed in cultivar SC 10, but the increasing level was more pronounced in cultivar SC 122 than in cultivar SC 10. Level of non-protein thiols (NPT) decreased to the minimum level (72.99% lower than control) at 100.0 µM Cu concentration for the cultivar SC 122. The level of protein bound thiols (PBT) was significantly (P≤0.05) increased by increasing Cu level in the growth media of both cultivars. A significant decline (P≤0.05) in acid soluble thiols (AST) level was observed in both cultivars and this decline was increased by increasing Cu concentration in the growth media which was maximum at 100.0 µM Cu (54.16% and 57.32% lower than control 100%) for SC 122 and SC 10, respectively. Metals stress has been reported to enhance the sulfur reduction pathway by affecting not only the sulfur uptake and transport but also by inducing the enzymes of the pathway (Rausch and Wachter, 2005; Herbette et al., 2006). Such change in levels of sulfhydral compounds may be indicative of transient disturbance of metabolism as in maize plant, glutathione depletion was attributed to copper induced phytochelatin synthesis (De Vos et al., 1993). The latter investigator observed that, copper in some plants caused a 50% decline in total glutathione, thus suggesting that copper induced the synthesis of some non-protein SH compounds other than glutathione (Ernst et al., 2000).

Table 5. Total thiols (TT), non-protein thiols (NPT), protein bound thiols (PBT), and acid soluble thiols (AST) in shoots of two maize cultivars (SC 122) and (SC 10) grown on MS media supplemented with different concentrations of Cu for 15 days.

Maize cultivars / Cu (µM)	TT (µmol g^{-1} f.w.)			NPT (µmol g^{-1} f.w.)			PBT= (NPT-TT) (µmol g^{-1} f.w.)			AST (µmol g^{-1} f.w.)		
	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean	SC 122	SC 10	Mean
0.0	0.513± 0.014	0.441± 0.04	0.477a	0.274± 0.04	0.312± 0.044	0.298c	0.239± 0.023	0.129± 0.068	0.148a	0.637± 0.043	0.485± 0.063	0.561d
25.0	0.683± 0.020	0.586± 0.080	0.635b	0.267± 0.023	0.295± 0.019	0.281c	0.416± 0.054	0.291± 0.047	0.253b	0.487± 0.039	0.340± 0.063	0.414c
50.0	0.782± 0.050	0.642± 0.041	0.712c	0.247± 0.042	0.268± 0.073	0.258b	0.508± 0.023	0.374± 0.046	0.441c	0.434± 0.028	0.326± 0.051	0.380b
100.0	0.949± 0.040	0.799± 0.060	0.874d	0.200± 0.030	0.228± 0.060	0.214a	0.749± 0.068	0.571± 0.045	0.660d	0.345± 0.071	0.278± 0.033	0.311a
Mean	0.732b	0.617a		0.247a	0.366b		0.428b	0.341a		0.476b	0.357a	
LSD 0.05												
Cultivars	0.017			0.008			0.015			0.018		
Cu	0.024			0.113			0.021			0.025		
Cultivars× Cu	0.034			0.016			0.029			0.036		

Data with different superscript letters were significantly different at P≤0.05. The values are mean ± S.D.(n=3)

To sum it up, the present data have shown that metabolic constituent such as (Cu accumulation, chlorophylls, proline, total soluble sugars, glutathione, cysteine, lipid peroxidation and thiol compounds) response to Cu stress activated markedly in shoots of SC 122 more than SC 10 after 15 days stress period to protect the plant from oxidative damage. Conclusively, SC 122 cultivar can be successfully grown in Cu-rich areas.

Materials and methods

Plant materials and copper treatments

The present research work was conducted at the National Centre for Radiation Research and Technology, Atomic Energy Authority, Cairo-Egypt. Two maize (*Zea mays* L.) cultivars named; Single Cross 122 (SC 122) and Single Cross 10 (SC 10) (which were characterized by their different levels of response to heavy metals stress) were obtained from Field Crops Research Institute, Agriculture Research Centre, Giza-Egypt. Seeds of both maize cultivars were surface sterilized for 1 min in ethanol 70% (v/v), 20 min in sodium hypochlorite 5% (v/v) and rinsed five times with sterile water. Surface sterilized seeds were half strength of Murashige and Skoog (MS/2) medium (Murashige and Skoog, 1962) supplemented with 2.5% (w/v) sucrose, 0.8% (w/v) plant agar. Different copper concentrations (0.0, 25.0, 50.0 and 100.0 µM) as $CuSO_4.5H_2O$ were added to the medium before autoclaving. The experiment was arranged as randomized complete block design (RCBD) with 10 replicates and five seeds per replicate. Cultures were maintained in growth chamber under 16/8 hrs light/dark photoperiod with 50 µmol $m^{-2}s^{-1}$ illumination at 25±1ºC. After 15 days seedlings were harvested, washed with double distilled water, plotted and used for the following measurements.

Growth parameters

Ten plantlets from each treatment were taken at random; the shoots length (cm), roots length (cm) and number of roots per plant were measured. The shoots were then dried in a forced oven at 70°C for 72 hrs, and the dry matter was recorded (AOAC, 2000).

Copper concentration

To detect copper concentration, shoots sample were ashed, dissolved in 10 % HCl with bidistilled water. After ashing, copper was detected as described in AOAC (2000), by using Atomic Absorption Spectrophotometer (Perkin Elmer-100) and the result was expressed as µg g^{-1} d.w.

Chlorophyll a, chlorophyll b and total carotenoid

Shoots (0.5 g) were homogenized in chilled 80 % acetone in a mortar and pestle in the dark at 4°C and the homogenates were centrifuged at 8800 g for 10 min. The supernatants were collected and the absorbance of the acetone extracts was measured at 663, 646 and 470 nm using a UV–visible spectrophotometer. The Chl a, Chl b, total chlorophylls and total carotenoid contents were calculated following the equations of Lichtenthaler (1987). The concentration of Chl a, Chl b, total chlorophylls and total carotenoid were expressed as mg g^{-1} f.w.

Proline content

Proline content was estimated following the method of Bates et al. (1973). Shoots (0.5 g) were homogenized in 3.0% (w/v) sulphosalycylic acid and the homogenate was filtrated. The resulting solution was treated with glacial acetic acid and acid ninhydrin. The mixture was boiled for 60 min in water bath, and then the reaction was stopped placing in an ice bath. The mixture was extracted with toluene, and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm. Proline concentration was calculated from a standard curve ranging from 0.0 to 100 µg proline. Proline content was expressed as mg g^{-1} f.w.

Total soluble sugars

Total soluble sugars were determined based on the method of phenol-sulfuric acid (Dubois et al., 1956). Fresh shoots (0.5 g) of samples were extracted in hot ethanol 80% (v/v) for one hr. The content of total soluble sugars was expressed as mg g^{-1} f.w. using glucose as standard curve (0-100 µg).

Reducing sugars

Reducing sugars were determined by using the method of Miller (1972). Approximately, 0.5 g of fresh shoots were extracted in hot ethanol 80 % (v/v). Then, 3.0 ml of the ethanolic extract was added to 3.0 ml of DNSA (3, 5-dinitro-salicylic acid) reagent. Absorbance was recorded using spectrophotometer at 515 nm. The quantity of reducing sugars was expressed as mg g^{-1} f.w. using glucose as standard curve (0.0-50 µg).

Reduced glutathione (GSH)

GSH was estimated following the method of Anderson (1985). Fresh shoots (0.5 g) were homogenized in 2.0 ml of 5% (w/v) sulfosalicylic acid under cold conditions. The homogenate was centrifuged at 1000 g for 10 min. To 0.5 ml of the supernatant, 0.6 ml of 100 mM phosphate buffer (pH 7.0) and 40 µl of 5-5'- dithiobis-2-nitrobenzoic acid (DTNB) were added. After 2 min, the absorbance was taken at 412 nm. GSH level was expressed as µmol g^{-1} f.w. using GSH as standard.

Cysteine content

The content of cysteine was determined spectrophotometrically, followed the method of Giatonde (1967). Fresh shoots (0.5 g) were homogenized in 5% (w/v) ice-cold perchloric acid. The homogenate was centrifuged at 2800 g for 1 hr at 5°C, the supernatant was filtered and the filtrate (1.0 ml) was treated with acid ninhydrin reagent. The absorbance was read at 580 nm. The amount of cysteine was calculated using pure cysteine as standard curve (0.0-10 µg) and the result was expressed as µmol g^{-1} f.w.

Lipid peroxidation (TBA test)

The level of lipid peroxidation in the shoots tissue was assayed using 2- thiobarbituric acid (TBA) reagent according to Buege and Aust (1978). The absorbance of the end product of lipid peroxidation (mainly malondialdehyde, MDA) was measured at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The level of lipid peroxidation products was expressed as nmol MDA g^{-1} f.w. by using the molar extinction coefficient of 155.10^5 $mM^{-1}cm^{-1}$.

Total thiols (TT)

Total thiols were estimated as described by Nagalakshmi and Prasad (2001). Fresh shoots (0.5 g) were homogenized in 20 mM ascorbate buffer contains 20 mM EDTA, and the homogenates were centrifuged at 12000 g for 20 min at 4°C. Aliquots (0.5 ml) of the supernatants were mixed with 2.4 ml of 200 mM Tris–HCl buffer (pH 8.2) and 0.1 ml of 10 mM DTNB. The color was allowed to develop for 15 min. The absorbance was measured at 412 nm. Total thiols (TT) were calculated using the molar extinction coefficient (ε=13,100 $M^{-1}cm^{-1}$). The result was expressed as µmol g^{-1} f.w.

Non-protein thiols (NPT)

Fresh shoots (0.5 g) were homogenized in a mortar with 5% sulphosalicylic acid (1:10 w/v) and the homogenate was centrifuged at 14000 g for 40 min at 4°C. The assay mixture (1.65 ml) consisted of the obtained supernatant, 0.1M sodium phosphate buffer (pH 7.0), 0.5 mM EDTA and 0.25 mM DTNB were allowed to stand for 10 min at room temperature (Israr et al., 2006). The absorbance was measured at 412 nm and non-protein thiols were calculated using the molar extinction coefficient (ε=13,100 $M^{-1}cm^{-1}$). The result was expressed as µmol g^{-1} f.w.

Protein bound thiols (PBT)

The protein bound thiols were calculated by subtracting the non-protein thiols (NPT) from total thiols (TT) as described by Nagalakshmi and Prasad (2001). The result was expressed as µmol g^{-1} f.w.

Acid soluble thiols (AST)

Acid soluble thiols were determined following the method of Jocelyn (1987). Fresh shoots (0.2 g) were extracted with 0.8 ml of NaOH (0.1M) + sodium borohydride (25 mg ml^{-1}) + 0.4 ml of distilled water, and then centrifuged at 11000 g for 5 min. The resulting supernatant (0.5 ml) was diluted with 0.2 ml HCl (35%) and centrifuged at 11000 g for 5 min. Then, 0.5 ml of DTNB (300 mM) in phosphate buffer (0.5M, pH 7.5) was added to 0.5 ml of supernatant and heated at 30°C for 2 min. Absorbance was determined at 412 nm. Acid soluble thiols level was calculated by using the molar extinction coefficient (ε=13,100 $M^{-1}cm^{-1}$) and the result was expressed as µmol g^{-1} f.w.

Statistical analysis

The effect of Cu addition to nutrient solution was evaluated by two-way ANOVA and the mean differences were compared by Tukey's test at the 0.05 significance level. The experiment was arranged as randomized complete block design (RCBD). Data shown in tables and figure are the means ± SD of at least three independent replicates. The probability level of 0.05 was used as the criterion for significance in all procedures according to Little and Hills (1992).

Conclusions

From the results obtained in the present investigation, it could be concluded that, SC 122 showed better responses to copper stress than SC 10 at seedling stage. In addition SC 122 showed higher shoots and roots length, chlorophylls, proline, total soluble sugars, glutathione, cysteine, and thiol compounds. On the other hand, contents of reducing sugar and lipid peroxidation were lower in SC 122 than in SC 10 under different copper stress levels. All the above mentioned biochemical parameters might be played an important role in the response of plant to copper stress. Additionally, these parameters may serve as important biochemical markers for copper tolerance trait in plants. Given that such stress impose considerable constraints on crop production. This preliminary study may pave way for further investigation to improve our understanding of the effect of copper stress on some biochemical parameters during seedling stage.

References

Aalaoui-sossé B, Ggenet P, Vvinit-dunand F, Ttoussaint ML, Epron D, Badot PM (2004) Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. Plant Sci 166: 1213-1218.

Ahsan N, Lee DG, Lee SH, Kang KY, Lee JJ, Kim PJ, Yoon HS, Kim JS, Lee BH (2007). Excess copper induced physiological and proteomic changes in germinating rice seeds. Chemosphere 67: 1182-1193.

Al-Hakimi AM, Hamada AM (2011) Ascorbic acid, thiamine or salicylic acid induced changes in some physiological parameters in wheat grown under copper stress. Plant Protect Sci 47: 92-108.

Alia MP, Matysik J (2001) Effect of proline on the production of singlet oxygen. Amino Acid 21: 195-200.

Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. In: Meister A, editor. Methods in Enzymology. New York: Academic press. 113: 548-551.

AOAC (2000) (Association of Official Analytical Chemists). Official Methods of Analysis. 17th ed., Arlington Virginia, USA.

Asada K (1994) Production and action of active oxygen species in photosynthetic tissues. In: Foyer CH, Mullineaux PM [eds.], Causes of Photooxidative Stress and Amelioration Defense Systems in Plants 77-104. CRC Press, Boca Raton, FL.

Bates LS, Waldren RP, Tears ID (1973) Rapid determination of free proline for water-stress studies. Plant and Soil 39: 205-207.

Benavides MP, Gallego SM, Tomaro ML (2005) Cadmium toxicity in plants. Braz J Plant Physiol 17: 21-34.

Bewlay JD, Black M (1994) Seeds. Physiology of developmental and germination. 2nd edition, Plenum press, New York.

Brahim L, Mohamed M (2011) Effects of copper stress on antioxidative enzymes, chlorophyll and protein content in *Atriplex halimus*. Afr J Biotech 10: 10143-10148.

Buege JA, Aust SD (1978) Microsomal lipid peroxidation. Methods Enzymology 52: 302-310.

Cambrolle J, Mateos-Naranjo E, Redondo-Gomez S, Luque T,Figueroa, ME (2011) Growth, reproductive and photosynthetic responses to copper in the yellow-horned poppy, *Glaucium flavum* Crantz. Environ Exp Bot 71: 57-64.

Chaoui A, Ferjani EE (2005) Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings. C R Biologies 328: 23-31.

Choudhary M, Jetley UK, Khan MA, Zutshi S, Fatma T (2007) Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. Ecotoxicol Environ Safety 66: 204-209.

De Vos CHR, Ten Bookum WM, Vooijs R, Schat H, De Kok LJ (1993) Effect of copper on fatty acid composition and peroxidation of lipids in roots of copper tolerant and sensitive *Silene cucubalus*. Plant Physiol Biochem 31: 151-158.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 38: 350-356.

Dubey RS, Singh AK (1999) Salinity induces accumulation of soluble sugars and alters the activity of sugar metabolizing enzymes in rice plants. Biol Plant 42: 233-239.

Ederli L, Reale L, Ferranti F, Pasqualini S (2004) Responses induced by high concentration of cadmium in *Phragmites australis* roots. Physiol Plant 121: 66-74.

El-Beltagi HS, Mohamed AA (2010) Changes in non protein thiols, some antioxidant enzymes activity and ultrastructural alteration in radish plant (*Raphanus sativus* L.) grown under lead toxicity. Not Bot Hort Agrobot Cluj 38:76-85.

Eleftheriou EP, Karataglis S (1989) Ultrastructural and morphological characteristics of cultivated wheat growing on copper-polluted fields. Bot Acta 102: 134-140.

Ernst WHO, Nelissen HJM, Bookum WMT (2000) Combination toxicology of metal enriched soils: Physiological responses of Zn- and Cd-resistant ecotype of *Silene vulgaris* on polymetallic soils. Environ Exp Bot 43: 55-71.

Farouk S, Mosa AA, Taha AA, Ibrahim HM, EL-Gahmery AM (2011) Protective effect of humic acid and chitosan on radish (*Raphanus sativus*, L. var. sativus) plants subjected to cadmium stress. J Stress Physio Biochem 7: 99-116.

Frankart C, Eullaffroy P, Vernet G (2002) Photosynthetic responses of *Lemna minor* exposed to xenobiotics, copper and their combinations. Ecotoxicol Environ Safety 53: 439-445.

Galli U, Schiiepp H, Brunold C (1996) Thiols in cadmium and copper-treated maize (*Zea mays* L.). Planta 198: 139-143.

Giatonde MK (1967) A spectrophotometric method for direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem J 104: 627-633.

Hansch R, Mendel RR (2009) Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Curr Opin Plant Biol 12: 259-266.

Herbette S, Taconnat L, Hugouvieux V, Piette L, Magniette ML, Cuine S, Auroy P, Richaud P, Forestier C, Bourguignon J, Renou JP, Vavasseur A, Leonhardt N (2006) Genome-wide transcriptome profiling of the early cadmium response of Arabidopsis roots and shoots. Biochimie 88: 1751-1765.

Hurry VM, Strand Å, Tobiæson M, Gardeström P, Őquist G (1995) Cold hardening of spring and winter-wheat and rape results in differential effects on growth, carbon metabolism, and carbohydrate content. Plant Physiol 109: 697-706.

Israr M, Sahi S, Datta R, Sarkar D (2006) Bioaccumulation and physiological effects of mercury in *Sesbania drummondii*. Chemosphere 65: 591-598.

Jocelyn PC (1987) Spectrophotometric assay of thiols. Methods Enzymol. 143: 44-67.

Kuznetsov W, Shevyakova NL (1997) Stress responses two tobacco cells to high temperature and salinity, proline accumulation and phosphorylation of polypeptids. Physiol Plant 101: 477-482.

Lichtenthaler HK (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymology 148: 350-382.

Lidon FC, Henriques FS (1991) Limiting step in photosynthesis of rice plants treated with varying copper levels. J Plant Physiol 138: 115-118.

Little J M, Hills FJ (1992) In: Statistical methods in agriculture research. Agric Extens Univ Calif. Davis,USA.

Lombardi L, Sebastiani L (2005) Copper toxicity in *Prunus cerasifera*: growth and antioxidant enzymes responses of *in vitro* grown plants. Plant Sci 168:797-802.

Maksymiec W, Russa R, Urbanic-Sypniewska T, Baszyñski T (1994) Effect of excess Cu on the photosynthetic apparatus of runner bean leaves treated at two different growth stages. Physiol Plant 91:715-721.

Mal TK, Adorjan P, Gorbett AL (2002) Effect of copper on growth of aquatic macrophytes *Elodea Canadensiscanadensis*. Environ Pollut 120: 307-311.

Manivasagaperumal R, Vijayarengan P, Balamurugan S, Thiyagarajan G (2011) Effect of copper on growth, dry matter yield and nutrient content of *Vigna radiata* (L.) wilczek. J phytology 3: 53-62.

Meier H, Reid JS (1982) Reserve polysaccharides other than starch in higher plants. In: Encyclopedia of Plant Physiol, New series. Loewus FA., Tanner W (Eds.). Springer-Verlag, Berlin, 13a: 418-471.

Miller GL (1972) Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 31: 426-428.

Monni S, Salemaa M, Millar N (2000) The tolerance of *Empetrum nigrum* to copper and nickel. Environ Pollu 109: 221-229.

Morelli E, Scarano G (2004) Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga *Phaeodactylum tricornutum*. Plant Sci 167: 289-296.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Nagalakshmi N, Prasad MN (2001) Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. Plant Sci 160: 291-299.

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49: 249-279.

Ouzounidou G (1994) Copper-induced changes on growth, metal content and photosynthetic function of *Alyssum montanum* L. plants. Environ Exp Bot 34: 165-172.

Prado FE, Boero C, Gallarodo M, Gonzalez JA (2000) Effect of NaCl on germination, growth and soluble sugar content in *Chenopodium quinoa* willd seeds. Bot Bull Acad Sin 41: 27-34.

Rama Devi S, 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.

Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci 10: 503-509.

Rennenberg H, Brunold C (1994) Significance of glutathione metabolism in plants under stress. Prog Bot 55: 142-156.

Schat H, Sharma SS, Vooijs R (1997) Heavy metal-induced accumulation of free proline in a metal-tolerant and nontolerant ecotype of *Silene vulgaris*. Physiol Plant 101: 477-482.

Singh S, Sinha S (2005) Accumulation of metals and its effects in *Brassica juncea* (L.) Czern. (cv. Rohini) grown on various amendments of tannery waste. Ecotoxicol Environ Safety 62: 118-127.

Sonmez S, Kaplan M, Sonmez NK, Kaya H, Ilker UZ (2006) High level of copper application to soil and leaves reduce the growth and yield of tomato plants. Sci Agric (Piracicaba, Braz.) 63: 213-218.

Srivastava S, Mishra S, Tripathi DR, Dwivedi S, Gupta KD (2006) Copper-induced oxidative stress and responses of antioxidants and phytochelatins in *Hydrilla verticillata* (L.f.) Royle. Aquat Toxicol 80: 405-415.

Tanyolaç D, Ekmekçi Y, Ûnalan A (2007) Changes in photochemical and antioxidant emzyme activities in maize (*Zea mays* L.) leaves exposed to excess copper. Chemosphere 67: 89-98.

Teisseire H, Guy V (2000) Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (*Lemna minor*). Plant Sci 153: 65-72.

Tukendorf A, Rauser WE (1990) Changes in glutathione and phytochelatins in roots of maize seedlings exposed to cadmium. Plant Sci 70: 155-166

Verma S, Dubey RS (2001) Effect of cadmium on soluble sugars and enzymes of their metabolism in rice. Biolo Plant 44: 117-123.

Wang SH, Yang ZM, Yang H, Lu B, Li SQ, Lu YP (2004) Copper induced stress and antioxidative responses in roots of *Brassica junicea* L. Bot Bull Acad Sin 45: 203-212.

Wilkins DA (1978) The measurement of tolerance to edaphic factors by means of root growth. New Phytol 80: 623-33

Wu JT (1998) Role of proline accumulation in response to toxic copper in *Chlorella* sp., (chlorophyceae) cells. J Phycol 34: 113-117.

Yruela I (2005) Copper in plants. Braz J Plant Physiol 17: 145-156.

Zhang J, Kirkham MB (1994) Drought stress induced changes in activities of superoxide dismutase, catalase and peroxide in wheat species. Plant Cell Physiol 35: 785-791.