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The effects of exogenous salicylic acid in the antioxidant defense system in canola plants (*Brassica napus* L.) exposed to copper

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Abstract

With the aim of assaying the possible interactions of copper (Cu) and salicylic acid (SA) in canola, the present research was conducted.Plants were treated withfour concentrationsof $CuSO_4.4H_2O$ (0, 50, 75, and 100 µM), and/or two levels of SA(0 and 250 µM).Proline contents in leaves were significantly increased by Cu treatments, in contrast to SA. Proline contents in roots were reduced by copper treatments, especially the mixed treatments with SA, while the individual application of SA was the most significant effective one. The Cu induced lipid peroxidations were significantly alleviated by the foliar application of SA. SA and/or Cu had significantly increasing effects on H_2O_2 contents in the root and leaf tissues. SA accelerating effects on the activities of peroxidase (POD) in root tissues were more effective than Cu, in contrast to leaf. The promoting impacts of individual applied levels of Cu on the leaf catalase (CAT) activities were declined by the exogenous SA. As opposed to SA, Cu application resulted in reductions in the root CAT activities. The activities of superoxide dismutase (SOD) were significantly elevated by the applied concentrations of Cu in the both root and leaf organs, as opposed to SA induced changes. In conclusion, it seems that the exogenous supplementation of SA may ameliorate the damaging effects of copper stress.

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Introduction

Toxicity of heavy metals is one of the major abiotic stresses and it can lead to serious damages to the health of humans, animals and plants. Nowadays contamination with heavy metals has been considered as one of the main concerns in environment protection (Drzewiecka *et al.,* 2013). Referable to the high reactivity of these metals, they may directly influence the growth, senescence and metabolism.

Copper is a vital micronutrient for plants and its involvements in critical process, including photosynthesis, mitochondrial respiration, superoxide scavenging, ethylene perception, etc (Drzewiecka *et al.,* 2013). However, its presence in excess levels in soil, water, and the atmosphere can cause multiple toxic impacts (Darcansoy Iseri *et al.,* 2011) and has been proposed as a universal environmental issue mainly due to mining, industrial, and agricultural practices (Elguindi *et al.,*2011). The excess Cu ions may result in the accelerated accumulation of active oxygen species (AOS) via Fenton–Haber–Weiss reactions (El-Tayeb and El-Enany, 2007), thereby inducing oxidative stress (Penarrubia *et al.,*2010). Tissue damages under the influence of copper occur when the capacity of antioxidativesystemisless thanthe amount of produced AOS (Kovacik *et al.*, 2008). The deleterious effects of copper on varieties of plants have been recorded (Mocquot *et al.,*1996; Chen *et al.,*2000; Yildiz *et al.,* 2009).

Salicylicacid (SA) has an essentialrolein regulating thegrowthand differentiation as well as, plant response toenvironmentalstresses. It has been well documented that SA triggers plant defense responses against pathogenic agents and establishes systemic acquired resistance (SAR) (An and Mou, 2011;Loutfy *et al.*, 2012**)**. SA, an important signaling compound,maymediatesomeadaptiveresponsestophysicochemicalstressessuch as heavy metal, low temperature, droughtandsalinity (Moussa and El-Gamal, 2010; Kadioglu *et al.,* 2011).

As stress tolerance is a complex process (Krasensky and Jonak, 2012) and partly due to overlaps between defense-related responses to various environmental and biotic stresses; therefore, the exogenous application of SA may affect plant responses to different abiotic stresses. In recent years, there are more interests in mitigating the signs of abiotic stresses by supplementation of signaling compounds like SA. However, the effects of SA depend on the applied concentrations and plant species are varied and the mechanisms triggered by SA are controversial issues. Therefore, more study is required to illustrate different aspects of the interactions between plant, SA and other environmental factors. There are limited researches about physiological changes induced by the interactions of SA and Cu in plants.The objective of this study was to assay the possible physiological changes induced by the interactions of Cu and SA in canola (*Brassica napus* L.) To clarify the underlying mechanisms involved in interactions between SA and copper.

Materials and methods

Planting and treatment of Plants

Seeds of canola (Brassica napus L. CV. Hyola) were prepared from the reliable center. The seeds disinfected with a 2% (v/v) sodium hypochlorite solutionfor five minutes andthenrinsedseveral times withsteriledistilled water. The germinated seeds (25° C) were planted inpotscontainingperlite. The seedlings were irrigated with Hoaglandsolution (pH of the nutrient solution was adjusted to 6). 21-day-old plants were treated with Hoagland solution containing four concentrationsof $CuSO_4$, 4H2O (0, 50, 75, and 100 μM), and/or two levels of salicylic acid (0 and 250 μM) in 8 different treatment groups. Changing solutions and SA supplementations (foliar application) were done twice a week. Theplants were grown in a controlled growth chamber (the photoperiod: 16h/8h, the light intensity: 190 μmol photon $m^{-2}s^{-1}$ and the relative humidity: 75%). Afterthree weeks, the sampleswere harvested and frozeninliquidnitrogen and stored at -70° Cfor biochemical analyses.

Prolinemeasurement

Prolinecontent was determined based on the method of Bates *et al.*, 1973. The prolinewas extracted from

0.2 g fresh tissueswith10 ml of sulfosalicylicacid (3% w/v). The resultinghomogenatewas centrifuged. 2ml of thesupernatant was mixed with 2 ml of glacial acetic acidand2 mlninhydrin reagentforonehour at100° C. Then it was cooled in icebath. The mixture was vigorously mixed with 4 ml toluene and finally, the absorption of toluene phase was read at 520 nm. The proline concentrations were determined based on the proline standard curve and expressed as µmol g^{-1}FW.

Lipidperoxidation

The lipidperoxidation rate was determined by measuringmalondialdehyde(MDA) according to the method described by Heath and Packer, 1968.0.2gof freshplanttissue was ground in3 ml oftrichloroaceticacid (0.1% w/v). The extract was centrifugedfor 15min. 1ml of thesupernatant was taken and then4 ml of 20% trichloro aceticacid was added to it which contained 0.5% of thiobarbituric acid. The mixturewasheatedfor 30minutesin a water bathat 95°C. Then it wascooledinice and centrifugedfor 5 min. The absorbance was read in 532 nm. The value for the non-specific absorption at 600 nm was subtracted. The extinction coefficient of 155 $M^{-1}cm^{-1}$ was used for calculating the amount of MDA and it was expressed in µmol g^{-1}FW.

Hydrogen peroxide (H_2O_2) measurement

H_2O_2 concentration was measured according to the method previously described by Velikova *et al.*, 2002. Briefly, 0.2g of fresh plant material was ground with 3 ml of trichlroacetic acid (TCA) (0.1% W/v) in the ice bath and then centrifuged. 0.5 ml of the supernatant was added to 0.5 ml of phosphate buffer (10 mM, pH7) and 1 ml of 1M KI. The absorbance was read at 390 nm. The amount of H_2O_2 concentration was determined by the extinction coefficient of 0.28 $\mu M^{-1}cm^{-1}$ and its amount expressed in µmol g^{-1}FW.

Extraction and determinations of activities of antioxidant enzymes

0.5g of fresh plant material was homogenized with 5 ml of phosphate buffer (pH 7.5 and 50 mM) which contained 1 mM Na_2EDTA and 1% polyvinylpyrrolidone (PVP) on ice. The homogenized mixture was centrifuged for 30 minutes at 4°C. Supernatant was used for measuring protein content and activities of enzymes.

Catalase (CAT) activity

Catalase activity was determined according to the absorbance reduction in 240 nm for 1 minute as previously described by Aebi, 1984. The reaction mixture contained 50 mM potassium phosphate buffer (pH7) and 15 mM H_2O_2 and 100 µl of enzyme extract. Enzyme activity was calculated using the extinction coefficient (39.4 $mM^{-1}cm^{-1}$).

Peroxidase (POD) activity

The peroxidase reaction mixture was composed of acetate buffer (0.4 M and pH 5), hydrogen peroxideand benzidine in 50% methanol. The reaction was started by adding 0.2mlof enzymeextract. Then the absorbancechanges were measured at530nm (Abeles and Biles, 1991).

The ascorbate peroxidase (APX) activity

The ascorbate peroxidaseactivity was determined by observing decrease in absorbance at290nm(Nakano and Asada1981). The reaction mixture contained 50 mM potassiumphosphate buffer (pH 7),0.5mMascorbate, 0.1 mM H_2O_2, and 100 µl oftheenzymeextract. The enzymeactivitywas calculatedusing the extinction coefficient (2.8 $mM^{-1}cm^{-1}$).

Superoxide dismutase (SOD) activity

SOD activity was measured according to the method of Giannopolitis and Ries, 1977. 3 ml ofreaction mixturecontained50mMpotassiumphosphate (pH 7.8) and 13mM methionine, 75 µM nitro blue tetrazolium (NBT), 20 µM riboflavin, 0.1 mM Na_2EDTA and 100µl ofenzymeextract. The sampleswere exposedto fluorescent lightfor 15minutes. Oneunit ofenzyme activityisthe amountofenzymecausing50% inhibitionofNBTreductionin560nm. The enzyme activity was expressed in unit enzyme per minute permgprotein.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and mean differences between groups were assessed by Duncan's multiple-range test using SPSS software.

Results

In leaf tissues the supplementations of SA and/or Cu, especially the former one, led to decreases in lipid peroxidation rates (Fig. 1). The enhanced lipid peroxidation levels resulted from copper in root tissues, whereas Cu induced lipid peroxidations were significantly alleviated by the foliar application of SA (Fig. 2).

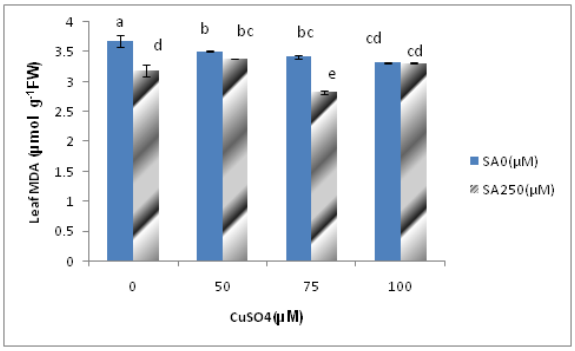

Fig. 1. Effects of Cu in combination with SA on MDA contents in leaves. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

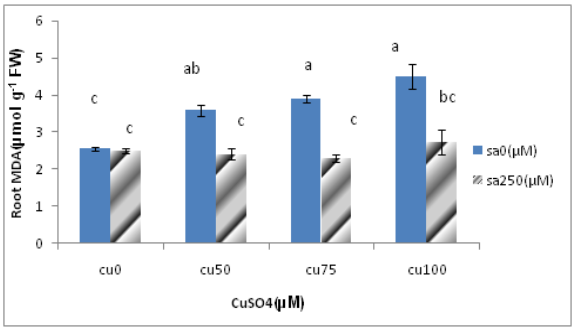

Fig. 2. Effects of Cu in combination with SA on MDA content in the roots. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test

SA and/or Cu, especially the former one, had significantly increasing effects on H_2O_2 contents in the root and leaf tissues, compared to control groups (Fig. 3 and 4).

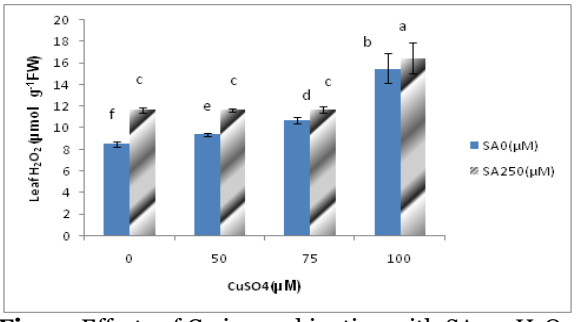

Fig. 3. Effects of Cu in combination with SA on H_2O_2 content in leaves. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

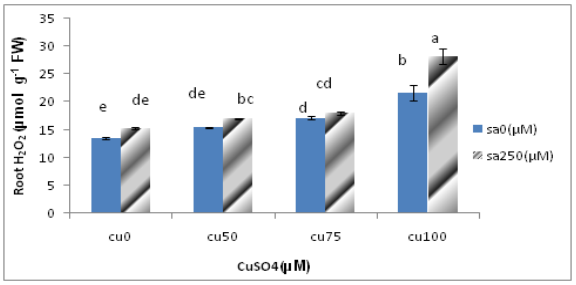

Fig. 4. Effects of Cu in combination with SA on H_2O_2 content in the roots. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

The promoting impacts of individual applied levels of Cu on the leaf CAT activities were significantly declined by exogenous SA (Fig. 5). In contrast to SA, Cu application resulted in significant reductions in the root CAT activities (Fig. 6). In comparison to control samples, the significantly higher amounts of POD activities were observed in SA and/or Cu treated plants (Fig. 7 and 8). SA accelerating effects on the activities of POD in root tissues were more effective than Cu, in contrast to leaf (Fig. 7 and 8). The results shown in figure 9 clearly indicated that the inducing impacts of Cu treatments on leaf APX activities were reduced by SA. The significantly promoted activities of root APX caused by the individual foliar application of SA while Cu and particularly its mixed treatments with SA reduced the activities of the mentioned enzyme, in comparison to the control (Fig. 10). The activities of SOD were significantly elevated by the applied concentrations of Cu in the both root

and leaf organs, as opposed to SA induced changes (Fig. 11 and 12). In comparison to the control, proline contents in leaves were significantly increased by Cu treatments, in contrast to SA (Fig. 13). Proline contents in roots were reduced by copper treatments, especially the mixed treatments with SA, while the individual foliar application of SA was the most significant effective treatment to improve proline contents in root tissues, compared to control (Fig. 14).

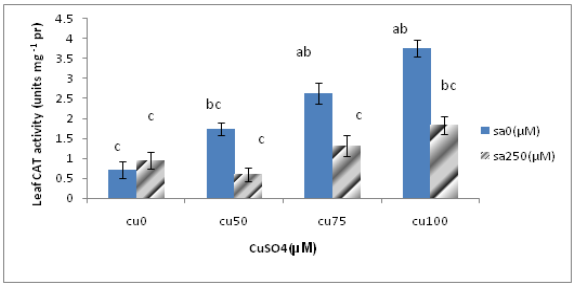

Fig. 5. Effects of Cu in combination with SA on CAT activity in leaves. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

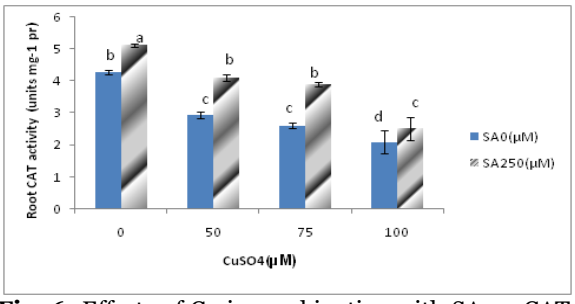

Fig. 6. Effects of Cu in combination with SA on CAT activity in the roots. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

Discussion

The assessment of the applied treatments of SA and/or Cu on lipid peroxidation rates reflected that SA had considerable alleviating impacts on the Cu enhanced lipid peroxidations as it was shown by fewer amounts of produced MDA recorded in Cu-SA combined treatments. It is obvious that heavy metals trigger the accumulations of AOS and oxidative stress. Therefore, these findings might be attributed to the inducing effects of SA on antioxidant system, thereby ameliorating oxidative stress. The alleviating effects of SA on plants exposed to heavy metal stress have been reported in various plants, including maize (Krantev *et al.*, 2008), pea (Popova *et al.*, 2009), wheat (Moussa and El-Gamal, 2010), *Phaseolus aureus* and *Vicia sativa* (Zhang *et al.*, 2011). Exogenous SA-induced declines in lipid peroxidation rates have been recorded in varieties of plants exposed to different stress conditions (Zhang *et al.*, 2011; Horvath *etal.*, 2007; El-Tayeb and El-Enany, 2006; Hayat *et al.*, 2010; Popova *et al.*, 2009; Kerantev *et al.*, 2008; Bin *et al.*, 2010). MDA levels in sunflower leaves were not affected by Copper treatment due to low contents of copper in leaves (Kovacik *et al.*, 2008).

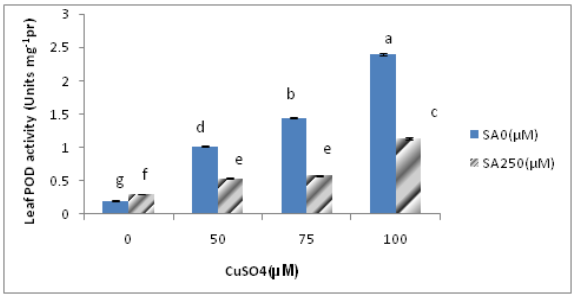

Fig.7. Effects of Cu in combination with SA on POD activity in leaves. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

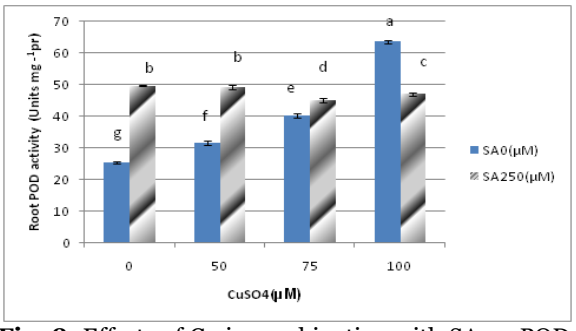

Fig. 8. Effects of Cu in combination with SA on POD activity in the roots. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

SA and/or Cu, especially the former one, had significantly increasing effects on H_2O_2 contents in the root and leaf tissues. H_2O_2 may act as a signal

metabolite and influence defense related genes, thereby improving tolerance to stress conditions. Thus, slightly and temporally raised H_2O_2 may play critical roles for acclimatizing to the changing environment by influencing the pattern of gene expression. Increased H_2O_2 accumulation usually associated with changes in the cellular redox status resists the plant cell against environmental stresses (Maksymiec, 2007). Stimulated H_2O_2 contents under heavy metal treatment have been reported in different plant species (Guo *et al.*, 2007; Drazkiewicz *et al.*, 2004; Moussa and El-Gamal, 2010; Zhang *et al.*, 2011; Jing *etal.*, 2007; Maksymiec, 2007). Raising the amount of H_2O_2 under the influence of SA has been observed in various studies (Horvath *et al.* 2007; Jing *et al.* 2007; Hayat *et al.* 2010; Panda and Patra, 2007). H_2O_2 concentration induced by SA is related to inhibition of APX and CAT activity (Kadioglu *et al.*, 2011).

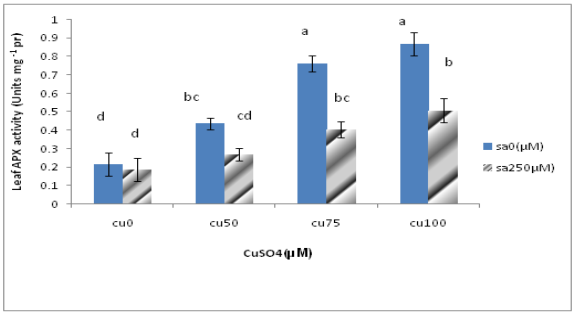

Fig. 9. Effects of Cu in combination with SA on APX activity in leaves. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

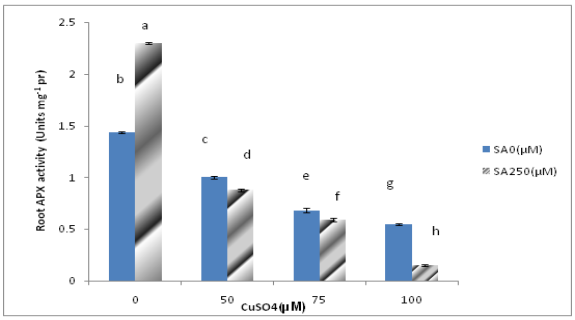

Fig. 10. Effects of Cu in combination with SA on APX activity in the roots. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

The obtained results reflected that the antioxidant systems in leaf and root tissues were significantly influenced by SA and/or Cu, as it was indicated by the modified activities of SOD, CAT and POD. The foliar application of SA led to the induced systemic defense responses, long distance responses, in root tissues. The adapted activities of H_2O_2-metabolizing enzymes, CAT, POD and SOD, in combination with the influenced concentrations of essential nutrients by Cu and/or SA may be responsible for the changed metabolism observed in the treated plants. It is clear that plants possess enzymatic and non enzymatic mechanisms to protect cellular structures under stress conditions. The accelerated activities of SOD observed in root tissues due to the simultaneous usage of Cu and SA may play an important role in repelling additional $\dot{O}_2^-$ radicals and ameliorating the resistance against Cu stress.

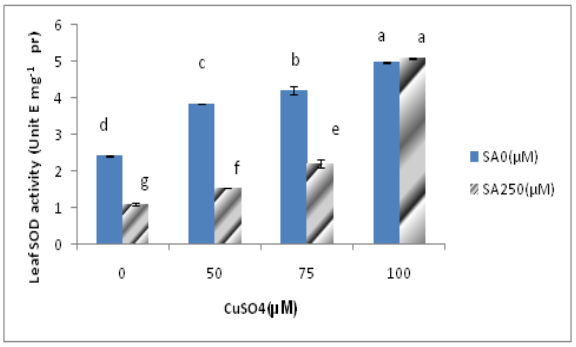

Fig. 11. Effects of Cu in combination with SA on SOD activity in leaves. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

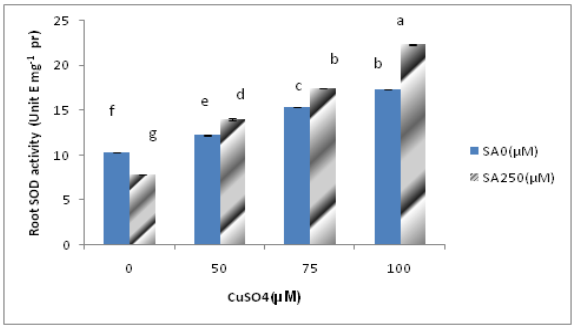

Fig. 12. Effects of Cu in combination with SA on SOD activity in the roots. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

The improved resistance to heavy metal stress in SA-supplemented plants had been attributed to SA-promoted antioxidant system (Zhang *et al.*, 2011). The stimulated antioxidant system caused by the exogenous SA-triggered signaling have been described in various studies (Kovacik *et al.*, 2009; Horvath *et al.*, 2007; Jing *et al.*, 2007; Hayat *et al.*, 2010). SA may directly inhibit the activity of certain H_2O_2-scavenging enzymes such as CAT and APX (Dat *et al.*, 2000; Ganesan and Thomas, 2001). It has been stated that when CAT activity was reduced; other AOS- scavenging enzymes like POD and SOD were increased as a compensation mechanism (Mourato *et al.*, 2009).The response rate of POD enzyme in resistant plants is very high to promote the resistance against stress, in contrast to the sensitive ones (Li and Xiong, 2005). The activities of CAT, POD and SOD have been suggested as a suitable index of defensive system (Drazkiewicz *et al.*, 2004). The presented results indicated that proline contents in leaves and roots were affected by the applied treatments of Cu and/or SA. Free proline accumulation may regard as a suitable index in heavy metal stress. Besides being nitrogen storage, proline accumulations may lead to decreases in growth rate and protection of biomolecules like proteins against heavy metals. These results are consistent with findings of El-Tayeb and El-Enany, 2006. Proline is known as an osmoprotectant, in addition, it can act like an antioxidant in protecting cells against radical damages (Moussa and El-Gamal, 2010). Rises in some amino acids like proline may relieve the damaging effects of excess heavy metal (Mehta and Gaur, 1999; Backor *et al.*, 2004).

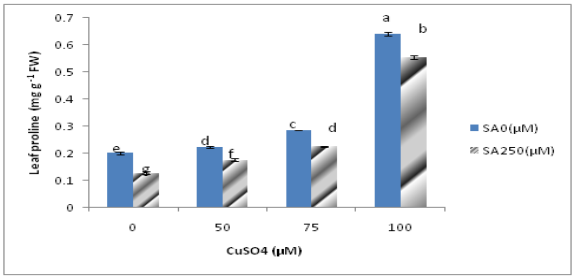

Fig. 13. Effects of Cu in combination with SA on proline content in leaves. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test.

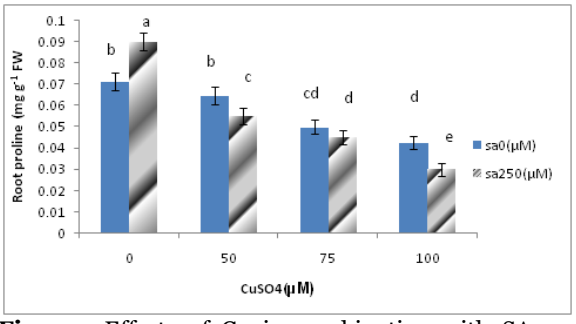

Fig. 14. Effects of Cu in combination with SA on proline content in the root. The data represent means±standard errors. Different letters indicate significant differences between treatments at $p<0.05$ according to Duncan´s test

Cu-induced levels of proline caused by the increase in nitric oxide (NO) concentrations in *Chlamydomonas reinhardtii*(Zhang *et al.*, 2008). Hall (2002) reported that amino acids are strong ligands for heavy metals and has a major role in detoxifying and metal resistance, mainly via creating proline-metal compounds (El-Tayeb and El-Enany, 2006). The foliar usage of SA in wheat plants resulted in the enhanced proline content (Hayat *et al.*, 2010). The pretreatment of tomato seeds improved resistance to salt stress by promoting proline contents (Tari *et al.* 2002).

In conclusion, it seems that the exogenous supplementation of SA may ameliorate the damaging effects of copper stress by inducing systemic defense responses, modifying the activities of antioxidant enzymes, reducing lipid peroxidation and increasing proline content, especially in organs directly exposed to stress.

Acknowledgements

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