Egypt. J. Exp. Biol. (Bot.), 13(1): 99 – 110 (2017)

DOI: 10.5455/egyjebb.20170404031852

RESEARCH ARTICLE

Radwan R. Khalil
Mahmoud M.Y. Madany

Stigmasterol relieves the deleterious effects of copper stress in maize

ABSTRACT:

Plant steroids have been implicated to relieve changes induced by heavy metals in plants. Maize *(Zea mays* L.) seeds were primed with stigmasterol (100 ppm) then grown under different levels of copper in the soil (0, 100, 150, or 200 mg kg^{-1} soil) for 40 days. Stigmasterol pretreatment improved the growth of *Zea mays* plants compared with untreated plants under different copper levels. Moreover, stigmasterol pretreatment enhanced membrane stability index, protein and proline content, as well as the activities of nitrate reductase, carbonic anhydrase, peroxidase and catalase. Additionally, grain priming with stigmasterol enhanced the content of photosynthetic pigments in maize plants. Therefore, our results revealed that seed priming with stigmasterol could enhance the tolerance of *Zea mays* plants grown under high levels of copper.

KEY WORDS:

Zea mays; Copper stress; Heavy metal stress; Photosynthetic pigments; Proline; Antioxidant enzymes; Stigmasterol.

CORRESPONDENCE:

Radwan R. Khalil

Department of Botany, Faculty of Science, Benha University, Benha, Egypt.

E-mail: radwan.aboelabbas@fsc.bu.edu.eg

Mahmoud M.Y. Madany

Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza, Egypt, 12613

ARTICLE CODE: 10.02.17

INTRODUCTION:

Maize *(Zea mays* L.) is considered as one of the most important cereal crops worldwide. Biotic and abiotic factors restrict the growth and yield of maize. Copper (Cu) is an essential micronutrient required for plants to maintain normal growth and metabolism. It exists as Cu^{+} and Cu^{2+}, acts as an important structural element in regulatory proteins, and participates in photosynthetic electron transport chain, mitochondrial respiration, oxidative stress response, cell wall metabolism and hormone signaling (Raven *et al.*, 1999). Cu ions also act as cofactors for many enzymes such as Cu/Zn SOD, cytochrome C oxidase, amino oxidase, plastocyanin, and polyphenol oxidase. However, Cu may be potentially toxic above the permissible limit and lead to inhibition of various physiological functions (Thounaojam *et al.*, 2012). To avoid Cu toxicity, plants have developed various strategies like secretion of organic acids, retention of Cu in roots and immobilization in the cell wall (Hu *et al.*, 2007; Wei *et al.*, 2008). Plants have also evolved protective enzymatic mechanisms like peroxidases, superoxide dismutase and catalase reaction to scavenge reactive oxygen species (ROS) (Scandalios, 1993; Teisseire and Guy, 2000). In addition, excess concentrations of Cu may induce toxicity by altering the protein function and activity of enzymes (Hänsch and Mendel, 2009). Toxicity results from the binding of metals to sulfhydryl groups in the protein and cause disruption of the structure and inhibition of protein activity (Morelli and Scarano, 2004). Additionally, excess Cu affects photosynthetic apparatus and disturbs the integrity of thylakoid membranes, resulting in chlorosis and necrosis, stunting, and inhibition of root and shoot growth (Yruela, 2009).

A higher level of antioxidants, namely the enhancement in the activity of antioxidant enzymes in plants exposed to heavy metals is normally associated with improved stress tolerance and assimilation performance (Andrade *et al.*, 2010; Chamseddine *et al.*, 2009). Furthermore, several nitrogenous metabolites, such as amino acids, particularly proline have been shown to accumulate in

plant tissues under Cu stress, indicating a protective action or a regulatory role (Sharma and Dietz, 2006). Proline also serves as a potent nonenzymatic antioxidant which counteracts the inhibitory effects of ROS in plants (Gill and Tuteja, 2010) .

Stigmasterol (StS) plays an essential role in plant growth and development which occurs mostly in free or conjugated form (Hashem *et al.*, 2011) . It is synthesized from β-sitosterol by the cytochrome P450 CYP710A1 via C22 desaturation. Several studies have provided evidence that fluctuation in the StS/sitosterol ratio plays a role in modulating responses of plants to biotic and abiotic stresses (Senthil-Kumar *et al.*, 2013). Griebel and Zeier (2010) found that inoculation of pathogenic microbes on *Arabidopsis thaliana* plants induced an array of metabolic changes that potentially contributed to acquire resistance. They found that accumulation of the StS is a significant process in the *Arabidopsis thaliana*–*Pseudomonas syringae* interaction. However, the role of StS in plants during stress is still poorly understood.

In this context, our work was conducted to examine how StS modulates growth, assimilation and stress defence processes in *maize* plants under different levels of Cu in the soil.

MATERIAL AND METHODS:

Plant materials and growth conditions:

Grains of *Zea mays* L. (single cross 10) were obtained from the Agriculture Research Center (ARC), Giza, Egypt. StS was purchased from MP Biomedicals LLC, Illkirch, France. Maize grains were surface sterilized with 0.1% $HgCl_2$ for 2 min and thoroughly washed with sterile distilled water. The sterilized grains were divided into two groups one of them was soaked in 100 ppm StS solution for 12 h and the other was soaked in distilled water for the same time to provide control.

A pot experiment was conducted in the greenhouse of Department of Botany, Faculty of Science, Benha University. Plastic pots were filled with a mixture of clay-sand (2:1 w/w) soil. Both primed and unprimed seeds were sown 15 cm in depth. Cu (0, 100, 150, or 200 mg kg^{-1} soil) was introduced to the soil in the form of $CuSO_4$. $5H_2O$. All pots were arranged in a randomized complete block design (RCB) with five replicates per treatment under natural conditions (16/8 h light/dark photoperiod and an average temperature of 25 ± 1°C).

The plants were irrigated with half strength nutrient solution until the end of the experimental period, then harvested, and the growth parameters of both shoots and roots were recorded. Some fresh plants from each group were ground immediately under liquid nitrogen and stored at - 20°C until used for biochemical analyses.

Relative water content (RWC) and saturated water deficit (SWD):

The RWC of leaf samples was measured using the method adopted by Barrs and Weatherley (1962) as follows: individual cut leaves were weighed to determine their fresh weight (FW), immersed in water for 24 h at room temperature, re-weighed to determine their water-saturated weight (SW), dried at 80°C, and finally weighed again to give the dry weight (DW). The RWC was then calculated as: RWC = (FW – DW) / (SW – DW) × 100

Measurement of saturated water deficit (SWD) was calculated by the following equation: SWD = 100 - RWC (%).

Electrolyte leakage:

The total inorganic ion leakage from the leaves was measured by the method described by Sullivan and Ross (1979). Twenty leaf discs of 2 cm diameter were placed in a boiling tube containing 10 mL deionized water. The tubes were heated at 45°C (ECa) and 55°C (ECb) for 30 min, each in a water bath and the electrical conductivity (EC) was measured with a conductivity meter (ME977-C, Max Electronics, India). Subsequently, the contents were boiled at 100°C for 10 min and the EC was again recorded (ECc). Electrolyte leakage (EL) was calculated by the following formula:

$$Electrolyte\ leakage\ (\%) = \frac{ECb - ECa}{ECc}\ X\ 100$$

Membrane stability index:

Two hundred mg of leaves were transferred in a falcon tube containing 10 mL double distilled water in two sets. One set was heated at 40°C for 30 min in a water bath and the electrical conductivity (C1) was measured. The second set was boiled at 100°C in a boiling water bath for 10 min and the conductivity (C2) was measured by using a conductivity meter (ME977-C, Max Electronics, India). The MSI was calculated using the formula described by Sairam (1994): MSI = [1 – (C1 / C2)] × 100

Photosynthetic pigments:

The content of chlorophyll *a* and *b* as well as carotenoids from fully expanded fresh leaves was determined (Fadeel, 1962) using 100% acetone, and their contents were calculated using the equation represented by Sestak *et al.* (1971).

Estimation of proline content:

Free proline was extracted and determined in fresh leaves (Bates *et al.*, 1973). One gram of fresh leaves was homogenized in 10 mL of 3% aqueous sulfosalicylic acid and filtered by using filter

paper. Two mL of the filtrate was mixed with 2 mL glacial acetic acid and 2 ml of acid ninhydrin reagent and heated for 1 h at 100°C. The reaction mixture was extracted with 4 mL of toluene, mixed vigorously in a test tube for 15 - 20 sec. The chromophore containing toluene was aspired from the aqueous phase and keep at room temperature. The absorbance was read at 520 nm using toluene as a blank. Proline concentration was expressed as µmol g^{-1} FW of leaves.

Protein content:

Extraction of water-soluble proteins was carried out (El-Tayeb *et al.*, 2006). A known dry weight of leaves was boiled in 1 ml distilled water for 2 h, then centrifuged at 6000 g. Determination of soluble proteins is carried out using the modified Folin-Lowry method adopted by Hartree (1972). One ml of the clear protein extract was mixed with 0.9 ml of alkaline sodium carbonate solution, and heated in a water-bath at 50°C for 10 min. After cooling, 0.1 ml copper sulphate-potassium sodium tartrate solution was added to the mixture and allowed to stand for 10 min at room temperature, followed by addition of 3 ml of 10% Folin-phenol reagent with immediate mixing. After 30 min, the absorbance of the blue colour was recorded at 750 nm against water-reagent blank. The concentration of protein was determined using bovine serum albumin standard curve then expressed as mg albumin g^{-1} FW.

Determination of carbonic anhydrase activity:

The activity of CA (EC 4.2.1.1.) in the leaves was measured following the method described by Dwivedi and Randhawa (1974). The leaf samples were cut into small pieces in cysteine hydrochloride solution. These leaf samples were blotted and transferred in a test tube, followed by the addition of phosphate buffer (pH 6.8), 0.2 M $NaHCO_3$, bromothymol blue and, finally, the methyl red as indicator. This reaction was titrated against 0.5 N HCl. The activity of the enzyme was expressed as µmol CO_2 g^{-1} FW min^{-1}.

Determination of nitrate reductase activity:

The activity of NR (EC 1.7.1.1) was determined. The leaf samples were cut into small pieces and transferred to vials containing phosphate buffer (pH 7.5), potassium nitrate and 5% isopropanol (Jaworski, 1971). These vials were incubated for 2 h at 30 ± 2 ºC in dark, then add 0.15 mL of Sulphanilamide solution (1%) and 0.15 mL of NED-HCl (0.02%). The absorbance was recorded at 540 nm and the NR activity was expressed as µmol NO_2 g^{-1} FW h^{-1}.

Assay of antioxidant enzymes:

Half g of leaf tissue was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone. The homogenate was centrifuged at 27,600 × *g* for 10 min at 4°C, and the supernatant was used as source of the enzymes CAT (EC 1.11.1.6), (EC1.11.1.7) and SOD (EC 1.15.1.1). For the estimation of POX activity (Maehly and Chance, 1955), the enzyme extract (0.1 mL) was added to the reaction mixture consisting of pyrogallol, phosphate buffer (pH 6.8) and 1% H_2O_2. The change in the absorbance was read at every 20 secs for 2 min at 420 nm on a spectrophotometer. A control set was prepared by adding double-distilled water instead of enzyme extract. The reaction mixture for CAT consisted of phosphate buffer (pH 6.8), 0.1 M H_2O_2 and enzyme extract (1.0 mL). H_2SO_4 was added to the reaction mixture, after incubating it for 1 min at 25°C, and was titrated against potassium permanganate solution. The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Beauchamp and Fridovich, 1971). The reaction was initiated by adding 50 µL enzyme extract to the reaction mixture (50 mM phosphate buffer; pH 7.8, 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA) and placed under a 15-W fluorescent lamp (Philips Light Company, Lynm, MA, USA). The reaction was started by switching on the light and was allowed to run for 10 min. 50% inhibition by light was considered as one enzyme unit.

Statistical analysis:

The experiment was conducted under completely randomized block design. Mean values were calculated from measurement of three replicates of samples and standard errors of the means were also calculated. All data were statistically analysed by one-way analysis of variance (ANOVA) and Duncan's multiple range test was used to discriminate among means with the level of significance at $p \leq 0.05$. Differences between StS-primed and unprimed maize plants within each Cu level were analysed using Student's *t*-test at probability levels of 0.05, 0.01, or 0.001. To elucidate the interaction between Cu and StS on the measured parameters, a two-way ANOVA was performed. All statistical tests were performed using the computer program PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS:

Growth parameters:

A two-way ANOVA indicated a significant effect of copper and StS interaction on the leaf area, length of both shoot and root as well as fresh and dry weights of shoot and root (Table 1). The effects of StS treatment on shoot and root length, leaf area per plant, as well as fresh and dry weights of shoots and roots of *Zea mays* plants under different concentration of

Cu were investigated (Figs 1 & 2). The different concentrations (0, 100, 150, or 200 mg kg^{-1} soil) of Cu significantly reduced the measured growth characteristics. The reduction was proportionate to the applied concentration of Cu, where maximum reduction was noticed at 200 mg kg^{-1} soil Cu in shoot length (41.3%), root length (74.9%), area of leaves (62.5%), fresh and dry weights of shoots (69.2 % and 77.8%, respectively) fresh and dry weights of roots (71.4% and 29.5%, respectively) as compared with those of untreated control plants. However, application of StS significantly increased shoot length, root length, area of leaves, fresh and dry weights of shoots and root as compared with the corresponding controls (Figs 1 & 2).

Table 1. A two-way ANOVA for the effect of stigmasterol, Cu treatments and their interaction on the different measured parameters in *Zea mays* (numbers represent *F* values; ns = non-significant; *=P < 0.05; **=P < 0.01; *** = P < 0.001).

Parameter	Cu	Stigmasterol	Cu x Stigmasterol
Shoot Length	243.2***	967.6***	3.518*
Root Length	113.8***	138.4***	15.00***
Shoot FW	93.53***	87.61***	4.772*
Root FW	1712***	898.4***	44.07***
Shoot DW	3556***	5485***	22.35***
Root DW	55.73***	334.5***	4.599*
Leaf Area	1559***	1229***	120.5***
EL	1681***	1853***	515.4***
MSI	1227***	278.1***	103.1***
RWC	39.39***	189.5***	2.540ns
SWD	28.69***	182.9***	1.622ns
Chl *a*	121.7***	59.62***	1.595ns
Chl *b*	653.4***	119.2***	64.45***
Carotenoids	53.56***	34.52***	0.7254ns
Total Pigment Content	438.0***	135.3***	22.48***
NR	93.89***	184.1***	32.30***
CA	16.08***	36.72***	7.739**
Total Protein	584.6***	585.9***	57.30***
Proline	283.9***	1937***	33.12***
SOD	296.5***	940.5***	133.0***
POX	6.449**	362.0***	46.49***
CAT	993.2***	1660***	192.7***

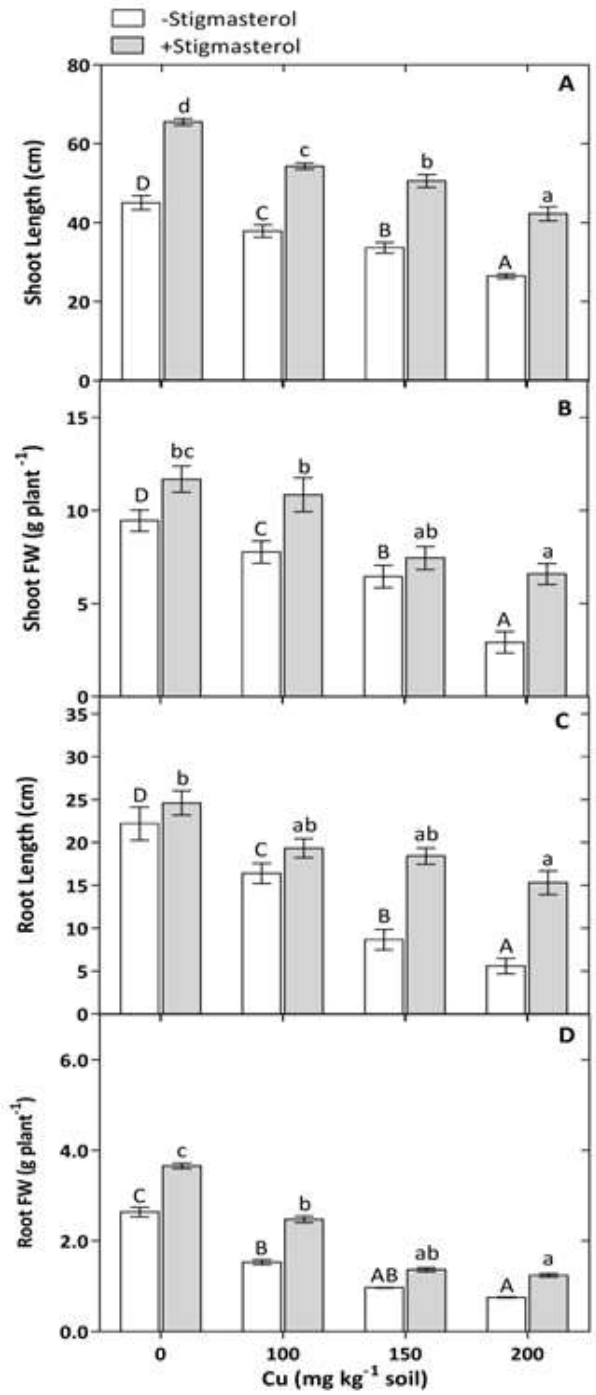


Fig. 1. Effect of different Cu concentrations on the growth of *Zea mays* plants untreated or treated with 100 ppm stigmasterol. *The shoot length* (A), *shoot FW* (B), *root length* (C), and *root FW* (D) were recorded under different levels of Cu in either absence or presence of StS. Each value is the mean of three independent replicates and vertical bars represent the standard error. Same letters indicate no significant difference (P < 0.05) as analysed by *Duncan test* (upper and lower case letters are used for stigmasterol-untreated or treated sets, respectively).

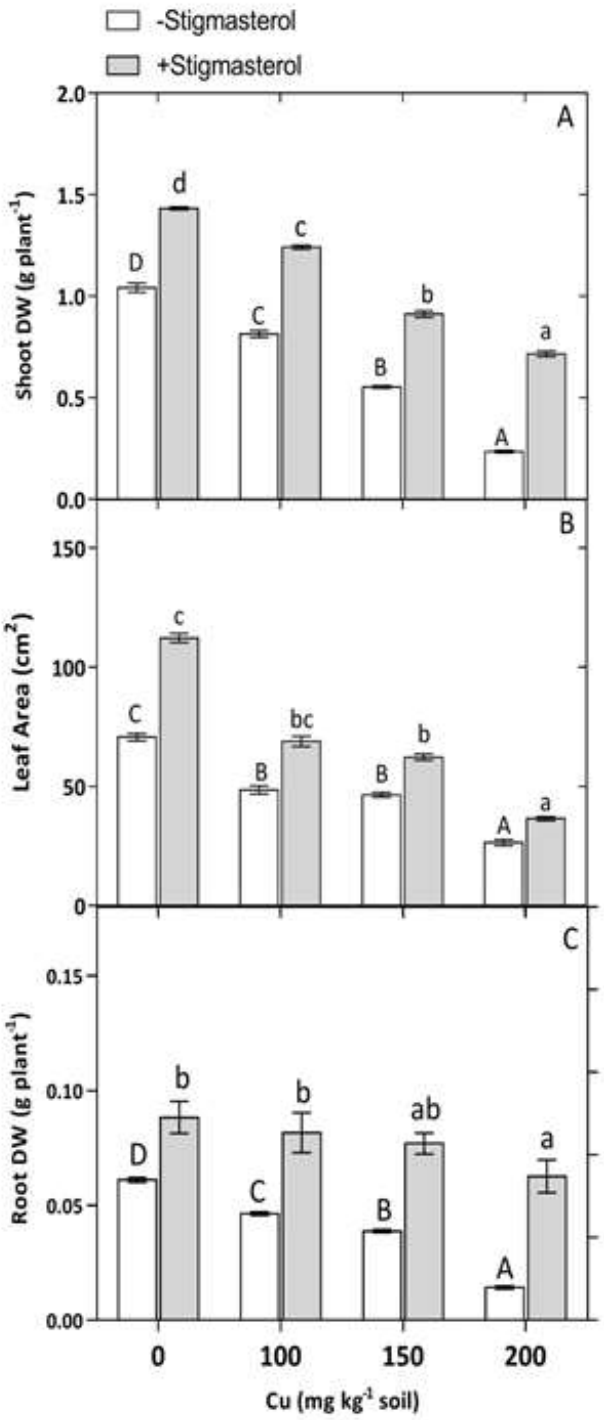


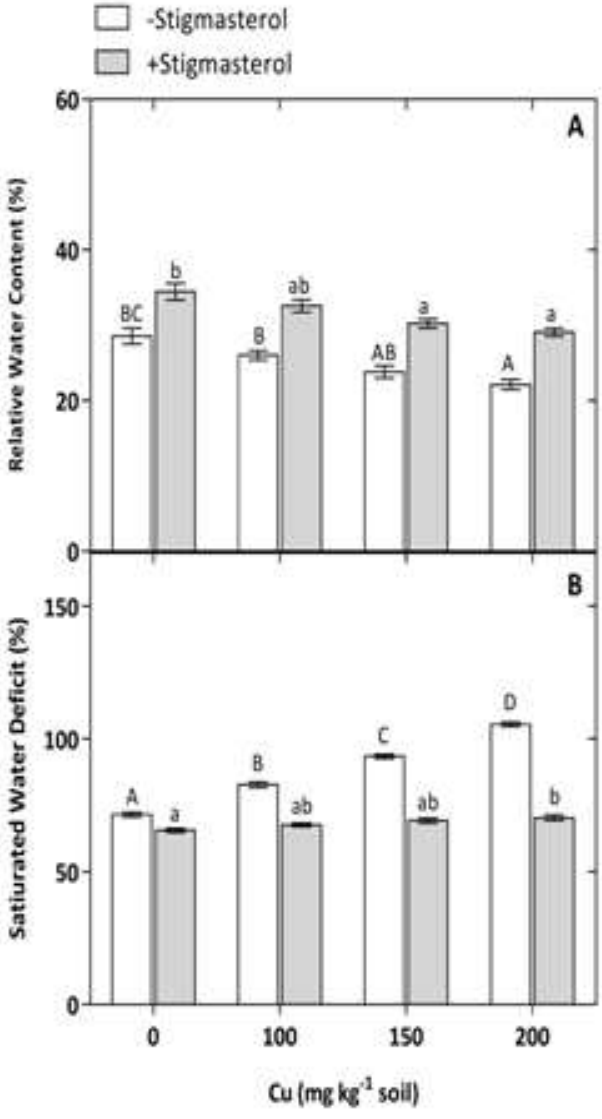
Fig. 2. Effect of different Cu concentrations on the growth of *Zea mays* plants untreated or treated with 100 ppm StS. *The shoot DW* (A), *leaf area* (B), and *root DW* (C) were recorded under different levels of Cu in either absence or presence of stigmasterol. Each value is the mean of three independent replicates and vertical bars represent the standard error. Same letters indicate no significant difference ($P < 0.05$) as analysed by *Duncan test* (upper and lower case letters are used for stigmasterol-untreated or treated sets, respectively).

Relative water content (RWC) and saturated water deficit (SWD):

The effect of Cu on RWC was Cu concentration-dependent. The maximum reduction in water loss was triggered by 200 mg kg^{-1} soil. However, exposure of the plants to Cu increased the value of saturated water deficit (SWD) as compared to untreated control plants. Application of StS significantly increased RWC and decreased SWD treated with 100, 150, or 200 mg kg^{-1} soil Cu, respectively (Fig. 3 A & B).

Electrolyte leakage (EL) and membrane stability index (MSI):

Electrolyte leakage (EL) and membrane stability index (MSI) showed a different pattern to each other (Fig. 3 C & D). Treatment with different concentrations of Cu significantly increased the EL with maximum increase by 200 mg kg^{-1} soil Cu. The treatment of StS reduced this increase in EL observed in all Cu-stressed plants. On the other hand, exposure of maize plants to Cu caused a decrease in the MSI. In contrast, StS primed plants showed a significant increase ($p < 0.05$) in the MSI in all Cu levels as compared to the control. A significant effect of Cu and StS interaction was recorded by two-way ANOVA ($P < 0.001$, Table1).



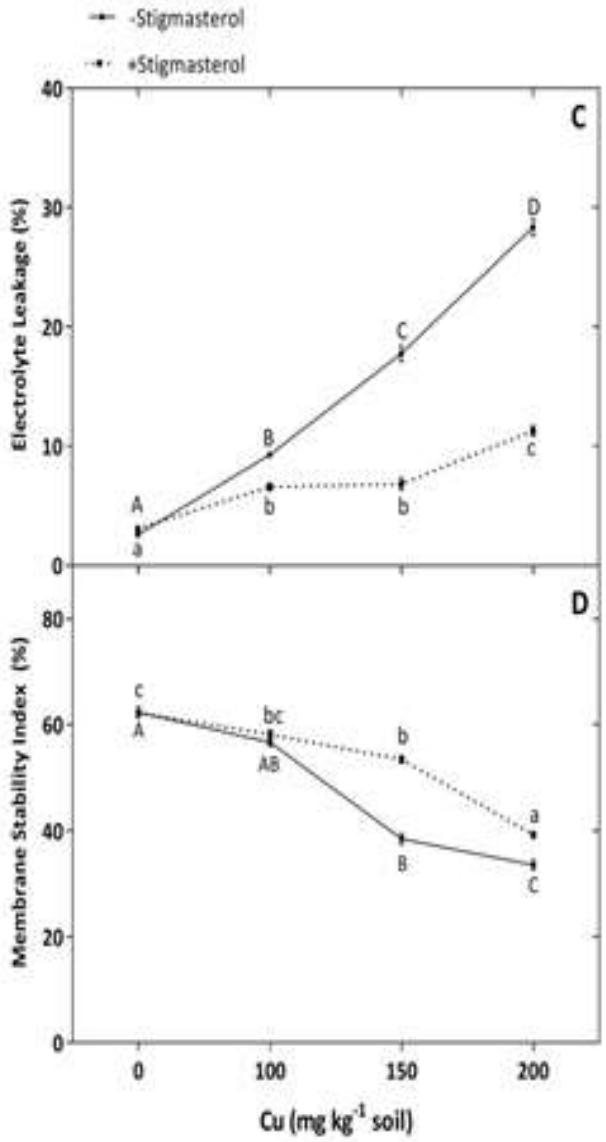


Fig. 3. Effect of different Cu concentrations on *relative water content* (A), *saturated water deficit* (B), *electrolyte leakage* (C), and *membrane stability index* (D) under different levels of Cu in either absence or presence of StS. Each value is the mean of three independent replicates and vertical bars represent the standard error. Same letters indicate no significant difference ($P < 0.05$) as analysed by *Duncan test* (upper and lower case letters are used for stigmasterol-untreated or treated sets, respectively).

Photosynthetic pigments:

The contents of photosynthetic pigments (chl *a*, chl *b*, and carotenoids) were significantly reduced by Cu treatments (Fig. 4 A, B, & D). This reduction is concentration dependent reaching its maximum value at the highest level of Cu in the soil (about 87%) as compared to the control. However, application of StS significantly restored the values and increased the contents of chl *a*, chl *b*, and carotenoids in all levels of Cu with respect to their controls.

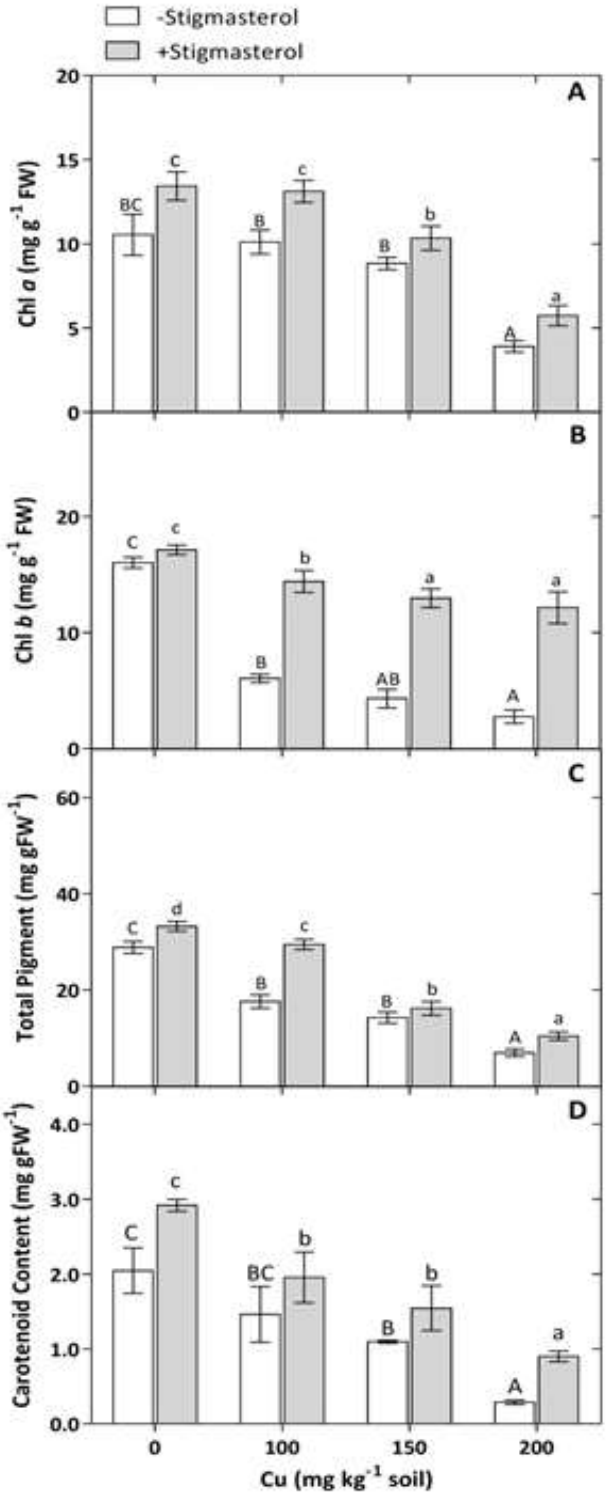


Fig. 4. Effect of different Cu concentrations on *chl a* (A), *chl b* (B), *total pigments* (C), and *carotenoids* (D) under different levels of Cu in either absence or presence of StS. Each value is the mean of three independent replicates and vertical bars represent the standard error. Same letters indicate no significant difference ($P < 0.05$) as analysed by *Duncan test* (upper and lower case letters are used for stigmasterol-untreated or treated sets, respectively).

Total protein and proline contents:

The contents of total protein decreased with increasing Cu level compared with those of untreated plants (Fig. 5A). Soaking maize grains in StS resulted in significant increases in the contents of total protein in leaves of *Zea mays* plants. The maximum increase is 35.8%, over the control was triggered by 200 mg kg^{-1} soil Cu + StS. The plants treated with Cu had higher level of proline content compared with untreated control plants (Fig. 5B). The magnitude of the increase was proportionate to the concentration of Cu. StS induced a significant reduction in proline content by about 54% compared to the reference controls, in plants treated with 100 mg kg^{-1} soil Cu + StS. A highly significant effect of Cu, StS and their interaction on the accumulation of protein and proline in maize plants was detected ($P < 0.001$, Table 1).

Nitrate reductase (NR) activity:

Cu stress (150, 200 mg kg^{-1} soil) caused a significant reduction in the activity of NR in *Zea mays* plants (Fig. 5C). The decrease was proportionate with concentration of Cu. The maximum decrease was triggered by highest rate of Cu (32.19% lower from the control). However, soaking treatment with StS to Cu stressed plants (200 mg kg^{-1} soil Cu) resulted in a significant increase in the activity of NR by about 41%, over the stressed control plants.

Carbonic anhydrase (CA) activity:

Plants grown in the presence of Cu had a significant decrease in the activity of CA (Fig. 5D). However, the soaking treatment of StS significantly increased the activity of the enzyme by about 44%, over the control. A highly significant effect of Cu, StS and their interaction on the activity of CA in maize plants was detected (Table 1).

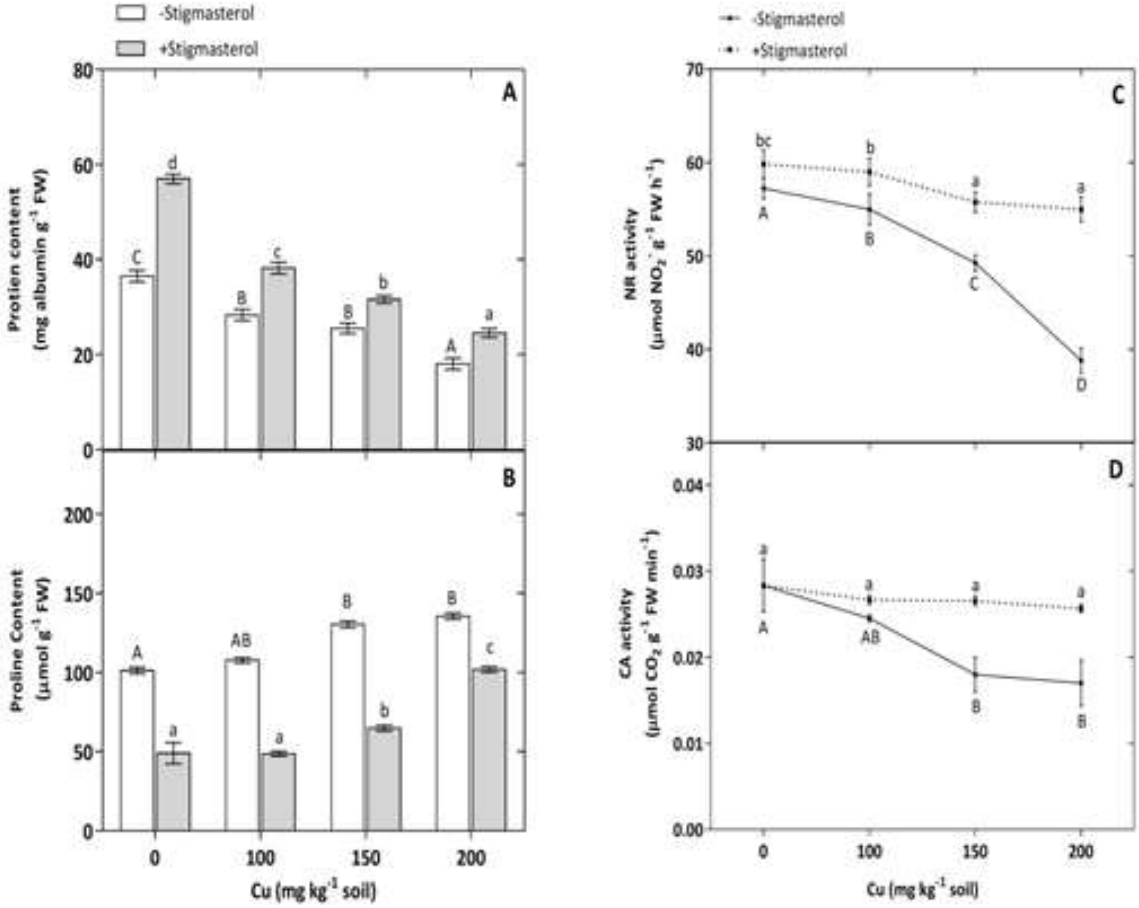


Fig. 5. Effect of different Cu concentrations on *protein content* (A), *proline content* (B), *NR activity* (C), and *CA activity* (D) under different levels of Cu in either absence or presence of StS. Each value is the mean of three independent replicates and vertical bars represent the standard error. Same letters indicate no significant difference ($P < 0.05$) as analysed by *Duncan test* (upper and lower case letters are used for stigmasterol-untreated or treated sets, respectively).

Activities of antioxidant enzymes:

The effect of StS pretreatment and Cu on the activities of POX, CAT, and SOD in maize plants is shown in figure 6 A-C. StS-primed plants showed significant higher activities of POX, CAT and SOD as compared with untreated plants. Moreover, two-way

ANOVA revealed a high significant effect of both Cu and StS, as well as their interaction upon the activities of POX, CAT and SOD (Table 1).

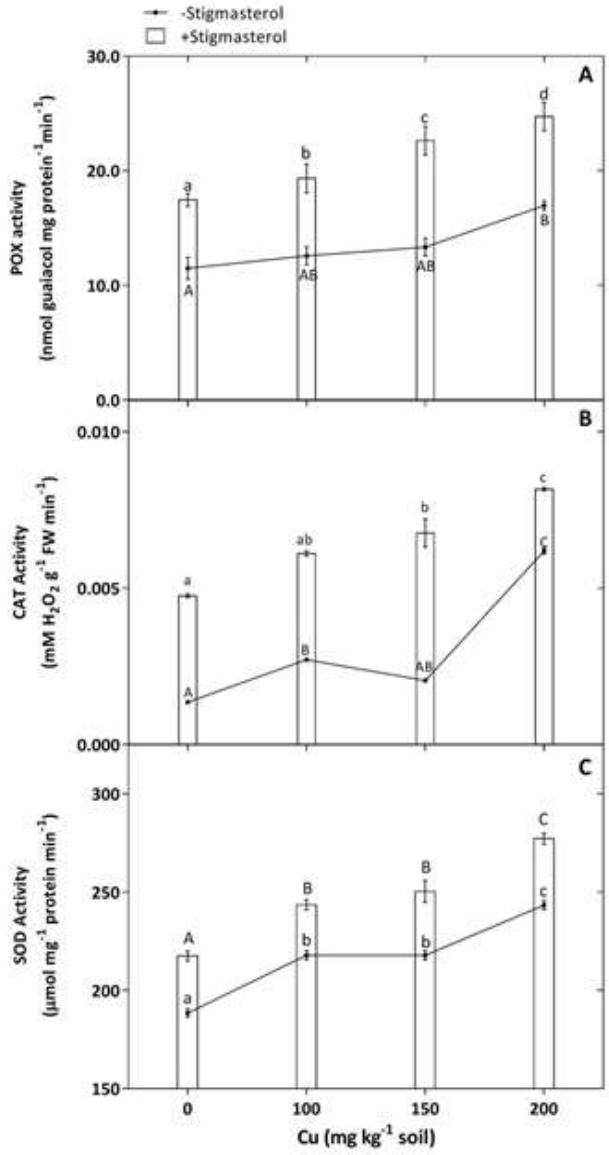


Fig. 6. Effect of different Cu concentrations on the activities of *POX* (A), *CAT* (B), and *SOD* (C) under different levels of Cu in either absence or presence of StS. Each value is the mean of three independent replicates and vertical bars represent the standard error. Same letters indicate no significant difference ($P < 0.05$) as analysed by *Duncan test* (upper and lower case letters are used for stigmasterol-untreated or treated sets, respectively).

DISCUSSION:

Heavy metals have been shown to cause many morphological, physiological and biochemical changes in plants, such as growth inhibition (Farouk *et al.*, 2011). It has been found that, Cu stress retarded the plant growth of maize cultivar by inhibiting shoot and root length, area of leaves, fresh and dry weights of shoot and roots. The inhibitory action of excess Cu in shoot length, root length and dry matter may be due to reduction in cell division, and toxic effect of Cu on photosynthesis that in turn contributed to the retardation of normal growth (Manivasagaperumal *et al.*, 2011; Sonmez *et al.*, 2006) . Plant biomass reduction is a common response observed in plants exposed to high levels of Cu (Andrade *et al.*, 2010; Thounaojam *et al.*, 2012). A significant decrease in shoot and root fresh weight was detected as Cu availability increased, suggesting that Cu-induced toxicity at higher concentrations.

Typical sterols (sitosterol and StS) play a regulatory function in plant development like those of brassinosteroids (BRs) (He *et al.*, 2003). Application of StS enhanced the overall growth of *Zea mays* plants and improved the values of shoot length, area of leaves per plant, fresh and dry weights of shoots and roots. This is, probably, by increasing the efficiency of water uptake and utilization, enhancing cell division and/or cell enlargement, resulting in longer shoots and roots and increasing leaf area which, results in larger surface area available for anabolic activities. This is culminated with higher fresh and dry matter of root and shoots. Similar results were obtained by Abd El-Wahed *et al.* (2001) .

In the present work, the high level of Cu concentration induced an increase in water deficit and decrease in relative water content. The significant decrease in relative water content might be due to the Cu toxicity causing wilting and plasmolysis in plant cells. These results are further investigated in wheat under chromium toxicity (Panda and Patra, 2000). Loss of water could be due to the production of reactive oxygen species (ROS) which damage membranes and leakage of cell saps through lipid peroxidation (Gill and Tuteja, 2010). Membrane damage can be evaluated indirectly by measuring solute leakage (electrolyte leakage) from cells (Ekmekçi *et al.*, 2008) and the MSI (Ali *et al.*, 2008). Increasing the Cu level in the present study caused a marked increase in electrolyte leakage and decreased MSI of maize plants. Application of StS corrected the stress-mediated damage to the plasma membrane, as was evident from the significant increase in membrane stability and the significant decrease in membrane leakage of Cu stressed *Zea mays* plants. Similar results were obtained by Hamada (1986) who found that brassinolide modifies membrane structure/stability under stress conditions.

The decline in chlorophyll content in *Zea mays* plant exposed to Cu is believed to be due to: (a) inhibition of enzymes associated with chlorophyll biosynthesis; (b) inhibition of uptake and transportation of other metal elements such as Mn, Zn, and Fe by antagonistic effects (John *et al.*, 2012).

The loss in chlorophyll content could be due to peroxidation of chloroplast membranes or replacement of magnesium in chlorophyll molecule by Cu (Mal *et al.*, 2002). Similar decrease in chlorophyll content under Cu stress was reported in *Atriplex halimus* (Brahim and Mohamed, 2011) and mangrove seedlings (Zhang *et al.*, 2007) . It has also been reported that epibrassinolide (EBL) alone and as a follow-up treatment to the stressed plants enhanced the SPAD value of chlorophyll.

Beside membrane damage, the accumulation of ROS also influences the key processes of assimilation. CA catalyses the inter-conversion of CO_2 to HCO_3^-, and its activity is regulated by the photon flux density, CO_2 concentration, availability of Zn (Tiwari *et al.*, 2005) and the expression of genes encoding CA protein (Kim *et al.*, 1994). At the level of the leaf, excess Cu decreased the content of chlorophyll and altered the structure of the chloroplast and thylakoid membrane (Yruela, 2005). Therefore, it might be due to interference of Cu modifying the structure and fluidity of the plasma membrane, thereby reducing the uptake of Zn which is responsible for the expression of genes encoding CA protein. It was proposed that Cu interferes with the biosynthesis of the photosynthetic machinery by decreasing the intracellular CO_2 concentration leading to a modification in the pigment and protein composition of thylakoid membranes (Azmat and Riaz, 2012). Our results indicated a decline in NR activity in plants exposed to Cu which could be due to inhibition and / or metabolic dysfunction of the enzyme protein. Furthermore, heavy metals also affect the proper functioning of plasma membrane bound proton pump and the fluidity of the membrane (Obata *et al.*, 1996). Low rate of nitrate uptake under Cu stress could be well explained by the study of Lazof *et al.* (1994) who demonstrated that disruption of nitrate uptake may be due to internal binding of Cu to membrane channel protein or other components of nitrate transport system that could be a possible reason for the decrease in the NR activity. However, the toxic effect generated by Cu could be overcome partially by a follow up treatment with StS and the improved activity of NR might be due to the involvement of BRs in the process of transcription and / or translation (Khripach *et al.*, 2003).

Our results indicated that recovery of chlorophyll contents by StS, played a role in the enhancement of photosynthesis and transpiration in *Zea mays* plants, which might be responsible for increase in Cu tolerance. In agreement with these results, Kalinich *et al.* (1985) stated that the application of StS enhanced the photosynthetic efficiency and enzyme activity in beans. In addition, Abd El-Wahed (2001) found that the contents of the photosynthetic pigments chl *a*, chl *b*, and carotene were increased in maize as sitosterol concentration increased. Treatment with EBL triggered an increase in contents of chl *a*, chl *b*, and carotenoids in the rape leaves under cold treatment at 2°C (Janeczko *et al.*, 2007). Moreover, EBL reduces the toxic effect of cadmium on photochemical processes by diminishing the damage of photochemical active reaction centres and the activity of O_2 evolving centres, as well as maintaining efficient photosynthetic electron transport (Janeczko *et al.*, 2005).

The injurious effect of Cu may be alleviated by enzymatic and non-enzymatic antioxidants. Proline is a proteinogenic amino acid that accumulates in *Zea mays* plant in response to Cu stress. Although, proline has long been considered an important compatible osmolyte yet it has multiple functions in stress adaptation, recovery, and signaling (Gill and Tuteja, 2010). Nowadays, it is considered as a potent non-enzymatic antioxidant. The greater accumulation of proline in leaves exposed to the highest Cu level (200 mg kg^{-1} soil) indicated the importance of proline accumulation in Cu tolerance. In fact, higher proline production has been correlated with Cu tolerance in lichen photobiont *Trebouxia erici* (Bačkor *et al.*, 2003), in jack bean (Andrade *et al.*, 2010), in rice plants (Thounaojam *et al.*, 2012), in chickpea (Singh *et al.*, 2010), and rice-detached leaves (Chen *et al.*, 2001). In the present study, also the level of proline increased in *Zea mays* plants treated with Cu and decreased with StS treatment. This finding might be explained by the fact that StS enhances the biosynthesis of other amino acids and their incorporation into proteins (Raksha *et al.*, 2015).

The activity of antioxidant enzymes have been shown to change in some plants when subjected to environmental stress conditions (Gomes-Junior *et al.*, 2006). In this study, under Cu stress, superoxide dismutase and peroxidase antioxidant enzymes increased and CAT activity decreased, which might have led more accumulation of H_2O_2 to a toxic level. In this regard, Feierabend *et al.* (1992) showed that under stress conditions, inactivation of CAT is linked to H_2O_2 accumulation. A significant increase in endogenous H_2O_2 content and a marked decline in CAT activity is reported during induced thermotolerance in mustard (Dat *et al.*, 1998). CAT deactivation by salt stress may be a result of prevention of new enzyme synthesis (Feierabend and Dehne, 1996). Application of StS ameliorated the effect of Cu stress, reduced the activity of SOD and increased the activity of POX and CAT in *Zea mays* plants. High activity of CAT in StS treated plants under Cu stress suggests that the treated plants possess a better

scavenging ability. The elevation in antioxidant enzymes by BRs was the consequence of enhanced expression of *det2* gene, which enhanced the tolerance to oxidative stress in *Arabidopsis* (Cao *et al.*, 2005). Data reported by Chamseddine *et al.* (2009) in *S. lycopersicon* showed a decline in SOD, CAT, and APX activities after prolonged Cu exposure, indicating that the scavenging function of these enzymes is impaired with prolonged Cu treatment (Chamseddine *et al.*, 2009).

The present study indicated that application of StS increased Cu tolerance of *Zea mays* plants by (1) increasing chlorophyll content and photosynthesis, (2) increasing membrane stability index and decreasing in electrolyte leakage, and (3) improving antioxidant enzyme activities and protecting against Cu induced oxidative stress. These results could be exploited for repairing soil contaminated with heavy metal and improve (yield) production of *Zea mays*.

ACKNOWLEDGMENTS:

The authors would like to thank the Botany Department, Faculty of Science, Benha University for funding this study and for permitting us to carry out the experiment using their laboratory facilities. The authors would like to thank DR. Qazi Fariduddin, associate professor of plant physiology and biochemistry, Botany Department, Faculty of Science, Aligarh Muslim University, India for his comments that help improve the manuscript.

REFERENCES:

Abd El-Wahed MS, Ali ZA, Abdel Hady MS, Rashad SM. 2001. Physiological and anatomical changes on wheat cultivars as affected by sitosterol. J. Agr. Sci. Mans. Univ, 26(9): 4823–4839.

Ali B, Hasan SA, Hayat S, Hayat Q, Yadav S, Fariduddin Q, Ahmad A. 2008. A role for brassinosteroids in the amelioration of aluminium stress through antioxidant system in mung bean (*Vigna radiata* L. Wilczek). Environ. Exp. Bot. 62(2): 153–159.

Andrade SAL, Gratao PL, Azevedo RA, Silveira APD, Schiavinato MA, Mazzafera P. 2010. Biochemical and physiological changes in jack bean under mycorrhizal symbiosis growing in soil with increasing Cu concentrations. Environ. Exp. Bot, 68(2): 198–207.

Azmat R, Riaz S. 2012. The inhibition of polymerization of glucose in carbohydrate under Cu stress in *Vigna radiata*. Pak. J. Bot, 44(1): 95–98.

Bačkor M, Fahselt D, Davidson, R.D, Wu, CT. 2003. Effects of copper on wild and tolerant strains of the lichen photobiont *Trebouxia erici* (Chlorophyta) and possible tolerance mechanisms. Arch. Environ. Contam. Toxicol. 45(2): 159–167.

Barrs HD, Weatherley PE. 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. Aust. J. Biol. Sci., 15(3): 413–428.

Bates LS, Waldern RP, Teare ID. 1973. Rapid determination of free proline for water-stress studies. Plant Soil, 39(1): 205–207.

Beauchamp C, Fridovich I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem., 44(1): 276–287.

Brahim L, Mohamed M. 2011. Effects of copper stress on antioxidative enzymes, chlorophyll and protein content in *Atriplex halimus*. Afr. J. Biotechnol., 10(50): 10143–10148.

Cao S, Xu Q, Cao Y, Qian K, An K, Zhu Y, Binzeng H, Zhao H, Kuai B. 2005. Loss-of-function mutations in DET2 gene lead to an enhanced resistance to oxidative stress in Arabidopsis. Physiol. Plantarum, 123(1): 57–66.

Chamseddine M, Wided BA, Guy H, Marie-Edith C, Fatma J. 2009. Cadmium and copper induction of oxidative stress and antioxidative response in tomato (*Solanum lycopersicon*) leaves. Plant Growth Regul., 57: 89-99.

Maehly AC, Chance B. 1954. The assay of catalases and peroxidases. Methods Biochem. Anal., 1: 357-424.

Chen CT, Chen L, Lin CC, Kao CH. 2001. Regulation of proline accumulation in detached rice leaves exposed to excess copper. Plant Sci., 160(2): 283–290.

Dat JF, Lopez-Delgado H, Foyer CH, Scott IM. 1998. Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiol., 116(4): 1351–1357.

Dwivedi RS, Randhawa NS. 1974. Evaluation of a rapid test for the hidden hunger of zinc in plants. Plant Soil, 40(2): 445–451.

Ekmekçi Y, Tanyolac D, Ayhan B. 2008. Effects of cadmium on antioxidant enzyme and photosynthetic activities in leaves of two maize cultivars. J. Plant Physiol., 165(6): 600–611.

El-Tayeb MA, El-Enany AE, Ahmed NL. 2006. Salicylic acid-induced adaptive response to copper stress in sunflower (*Helianthus annuus* L.). Int. J. Bot., 2(4): 372-379.

Fadeel AA. 1962. Location and properties of chloroplasts and pigment determination in roots. Physiol. Plantarum, 15(1): 130–146.

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 Physiol. Biochem., 7(2): 99-116.

Feierabend J, Dehne S. 1996. Fate of the porphyrin cofactors during the light-dependent turnover of catalase and of the photosystem II reaction-center protein D1 in mature rye leaves. Planta, 198(3): 413–422.

Feierabend J, Schaan C, Hertwig B. 1992. Photoinactivation of catalase occurs under both high-and low-temperature stress conditions and accompanies photoinhibition of photosystem II. Plant Physiol., 100(3): 1554–1561.

Gill SS, Tuteja N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem., 48(12): 909–930.

Gomes-Junior RA, Moldes CA, Delite FS, Gratão PL, Mazzafera P, Lea PJ, Azevedo RA. 2006. Nickel elicits a fast antioxidant response in *Coffea arabica* cells. Plant Physiol. Biochem., 44, 420–429.

Griebel T, Zeier J. 2010. A role for β-sitosterol to stigmasterol conversion in plant-pathogen interactions. plant J., 63(2): 254–268.

Hamada K. 1986. Brassinolide in crop cultivation. In: "Plant Growth Regulators in Agriculture. (McGregor P. Ed.)". Taiewan FFTC, vol. 1, pp. 190–196.

Hänsch R, Mendel RR. 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Curr. Opin. Plant Biol., 12(3): 259–266.

Hartree EF. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem., 48(2): 422–427.

Hashem HA, Bassuony FM, Hassanein RA, Baraka DM, Khalil RR. 2011. Stigmasterol seed treatment alleviates the drastic effect of NaCl and improves quality and yield in flax plants. Aust. J. Crop Sci., 5(13): 1858-1867.

He JX, Fujioka S, Li TC, Kang SG, Seto H, Takatsuto S, Yoshida S, Jang JC. 2003. Sterols regulate development and gene expression in Arabidopsis. Plant Physiol., 131(3): 1258–1269.

Hu KD, Hu LY, Li YH, Zhang FQ, Zhang H. 2007. Protective roles of nitric oxide on germination and antioxidant metabolism in wheat seeds under copper stress. Plant Growth Regul., 53(3): 173–183.

Janeczko, A, Gullner, G, Skoczowski, A, Dubert, F, Barna, B, 2007. Effects of brassinosteroid infiltration prior to cold treatment on ion leakage and pigment contents in rape leaves. Biol. Plantarum, 51(2): 355–358.

Janeczko A, Koscielniak J, Pilipowicz M, Szarek-Lukaszewska G, Skoczowski A. 2005. Protection of winter rape photosystem 2 by 24-epibrassinolide under cadmium stress. Photosynthetica, 43(2): 293–298.

Jaworski EG. 1971. Nitrate reductase assay in intact plant tissues. Biochem. Biophys. Res. Commun., 43(6): 1274–1279.

John, R, Ahmad, P, Gadgil, K, Sharma, S, 2012. Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation by *Brassica juncea* L. Int. J. Plant Prod., 3(3): 65–76.

Kalinich JF, Mandava NB, Todhunter JA. 1985. Relationship of nucleic acid metabolism to brassinolide-induced responses in beans. J. Plant Physiol., 120(3): 207–214.

Khripach VA, Zhabinskii VN, Khripach NB. 2003. New practical aspects of brassinosteroids and results of their ten-year agricultural use in Russia and Belarus, in: Brassinosteroids. Springer, pp. 189–230.

Kim HJ, Bracey MH, Bartlett SG. 1994. Nucleotide sequence of a gene encoding carbonic anhydrase in *Arabidopsis thaliana*. Plant Physiol., 105(1): 449.

Lazof DB, Rincón M, Rufty TW, Mackown CT, Carter TE. 1994. Aluminum accumulation and associated effects on $^{15}NO_3$ - influx in roots of two soybean genotypes differing in Al tolerance. Plant Soil, 164(2): 291–297.

Mal TK, Adorjan P, Corbett AL. 2002. Effect of copper on growth of an aquatic macrophyte, *Elodea canadensis*. Environ. Pollut., 120(2): 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. Phytol., 3(3): 53-62.

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.

Obata H, Inoue N, Umebayashi M. 1996. Effect of Cd on plasma membrane ATPase from plant roots differing in tolerance to Cd. Soil Sci. Plant Nutr., 42(2): 361–366.

Panda SK, Patra HK. 2000. Nitrate and ammonium ions effect on the chromium toxicity in developing wheat seedlings. P. Natl. Acad. Sci. India, B. Biol. Sci., 70(1): 75–80.

Raksha B, Priya R, Siva R, Babu S. 2015. Comparative computational analysis of stigmasterol biosynthetic genes and proteins in plants. Plant Omics J., 8(1): 1-8.

Raven JA, Evans MCW, Korb RE. 1999. The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. Photosynth. Res., 60(2): 111–150.

Sairam RK. 1994. Effects of homobrassinolide application on plant metabolism and grain yield under irrigated and moisture-stress conditions of two wheat varieties. Plant Growth Regul., 14(2): 173–181.

Scandalios JG. 1993. Oxygen stress and superoxide dismutases. Plant Physiol., 101(1): 7-12.

Senthil-Kumar M, Wang K, Mysore KS. 2013. AtCYP710A1 gene-mediated stigmasterol production plays a role in imparting temperature stress tolerance in *Arabidopsis thaliana*. Plant Signal. Behav., 8(2): e23142.

Sestak Z, Catsky J, Jarvis PG. 1971. Determination of chlorophyll *a* and *b*. In: "Plant Photosynthetic Production. Manual of Methods. (Sestak Z, Catsky J, Jarvis PG. Eds.)". The Hague, Netherlands, Dr. W. Junk NV, pp. 672–697.

Sharma SS, Dietz KJ. 2006. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J. Exp. Bot., 57(4): 711–726.

Singh V, Bhatt I, Aggarwal A, Tripathi BN, Munjal AK, Sharma V. 2010. Proline improves copper tolerance in chickpea (*Cicer arietinum*). Protoplasma, 245(1-4): 173–181.

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

Sullivan C, Ross W. 1979. Selection for drought and heat tolerance in grain sorghum, In: "Stress

Physiology in Crop Plants. (Hussel HSR. Ed.)". Wiley Interscience, New York, pp. 263–281.

Teisseire H, Guy V. 2000. Copper-induced changes in antioxidant enzymes activities in fronds of duckweed (*Lemna minor*). Plant Sci., 153(1): 65–72.

Thounaojam TC, Panda P, Mazumdar P, Kumar D, Sharma GD, Sahoo L, Sanjib P. 2012. Excess copper induced oxidative stress and response of antioxidants in rice. Plant Physiol. Biochem., 53: 33–39.

Tiwari A, Kumar P, Singh S, Ansari SA. 2005. Carbonic anhydrase in relation to higher plants. Photosynthetica, 43(1): 1–11.

Wei L, Luo C, Li X, Shen Z. 2008. Copper Accumulation and Tolerance in *Chrysanthemum coronarium* L. and *Sorghum sudanense* L. Arch. Environ. Contam. Toxicol., 55(2): 238–246.

Yruela I. 2005. Copper in plants. Braz. J. Plant Physiol., 17(1): 145–156.

Yruela I. 2009. Copper in plants: acquisition, transport and interactions. Funct. Plant Biol., 36(5): 409–430.

Zhang FQ, Wang YS, Lou ZP, Dong JD. 2007. Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (Kandelia candel and Bruguiera gymnorrhiza). Chemosphere, 67(1): 44–50.

ستيجماستيرول يخفف من الأثار الضارة لإجهاد النحاس في الذرة

رضوان خليل*، محمود مدني**

* قسم النبات، كلية العلوم، جامعة بنها، بنها، مصر

** قسم النبات والميكروبيولوجي، كلية العلوم، جامعة القاهرة، الجيزة، مصر

الاستيرولات النباتية تساعد في تخفيف التغيرات الناجمة عن المعادن الثقيلة في النباتات. تم زراعة حبوب الذرة المعاملة بستيجماستيرول (100 جزء في المليون) ونمت تحت مستويات مختلفة من النحاس في التربة (0، 100، 150، أو 200 ملجرام/كيلو جرام تربة) لمدة 40 يوما . أدت المعالجة بستيجماستيرول بتحسين نمو نباتات الذرة مقارنة مع النباتات غير المعالجة تحت مستويات النحاس المختلفة. وعلاوة على ذلك، المعاملة بالاستيجماستيرول أدت الى زيادة معامل ثبات الأغشية، والبروتين ومحتوى البرولين، وكذلك أنشطة انزيم النيتريت ريداكتيز، أنهيدريز الكربونى، البيرأوكسيديز والكتاليز. بالإضافة إلى ذلك، أدت معاملة الحبوب بالاستيجماستيرول الى زيادة محتوى الأصباغ في نباتات الذرة .لذلك، كشفت نتائجنا أن البذور المعاملة بالاستيجماستيرول يمكن أن تعزز من مستوى تحمل نباتات الذرة النامية تحت مستويات عالية من النحاس.