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# Biphasic effect of copper on growth, proline, lipid peroxidation and antioxidant enzyme activities of wheat (*Triticum aestivum* cv. Hasaawi) at early growing stage

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**Abstract**

The biphasic effects of $Cu^{+2}$ on the growth and some biochemical parameters of Hassawi wheat were studied. Hassawi wheat seeds were grown in various copper ($Cu^{+2}$) levels (0, 2, 10, 20, 40, 80 and 100 mM) for 30 days. $Cu^{+2}$ was applied on soil as $Cu_2SO_4.5H_2O$. The results showed that $Cu^{+2}$ concentration at 2 mM level promotes the growth and tested biochemical parameters of Hassawi wheat plants and can be considered as optimal dose. However, the studied parameters did not significantly change when $Cu^{+2}$ applied more than the above mentioned concentration up to 10 mM, and thereafter the growth and biochemical parameters were significantly reduced, compared to untreated control plants. Furthermore, the activities of antioxidant enzymes such as, catalase, peroxidase, ascorbate peroxidase and superoxide dismutase were also increased. The results support the biphasic effect of copper on Hassawi wheat growth. The stimulatory effect of $Cu^{+2}$ on the biosynthesis of free amino acids, proline and antioxidant enzyme activities could serve as important components of antioxidative defense mechanism against $Cu^{2+}$ toxicity.

**Keywords**: Cations uptake, free amino acids, ion leakage, tolerance index.
**Abbreviations**: APX: ascorbate peroxidase; CAT: catalase; POD :peroxidase; SOD: superoxide dismutase.

**Introduction**

Hassawi wheat is one of the most economic important cereal crops and a major source of food, and widely cultivated in Saudi Arabia. World production of wheat is ~607 million tons, making it the third most-produced cereal after maize (784 million tons) and rice (651 million tons) (FAO, 2007). Copper ($Cu^{+2}$) is an essential trace element needed for the normal growth and development of cereal crops. Both absence and excess of copper inhibits the plant growth and impairs important cellular processes. Therefore, optimum copper concentration ensures a normal growth and development of plants (Jain et al., 2009). However at high concentration, copper was shown to inhibit plant growth by hampering important cellular processes such as photosynthesis and respiration (Fariduddin et al., 2009). Higher plants take up copper from the soil solution mainly as $Cu^{2+}$. The range of copper concentrations in soil is generally between 2-250 ppm, and plant healthy tissues uptake 20-30µg $g^{-1}$ dry weight (Khatun et al., 2008). The copper uptake from soil depends on the ability of plants to transfer the metal across the soil–root interface and the total amount of $Cu^{2+}$ present in the soil (Agata and Ernest, 1998). Plants grown in the presence of high level of $Cu^{2+}$ normally show chlorotic symptoms and reduced biomass production (Bernel et al., 2004; Yruela 2005). The accumulation of proline in plants is a general response to some abiotic stress (Jain et al., 2001). Thus, it is reasonable to link the metabolism of proline and heavy metals stress in plants. Free amino acids and proline have been found to accumulate in response to $Cu^{2+}$ exposure (Chen et al., 2004; Fariduddin et al., 2009; Al-Hakimi and Hamada, 2011). Excess copper concentrations generate the oxidative stress due to an increase in the levels of reactive oxygen species (ROS) within subcellular compartments. ROS include the superoxide radical ($O2^{-\bullet}$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical (•OH), all of which affect mainly lipids, proteins, carbohydrates, and nucleic acids (Mittler et al., 2004). To overcome this, cells are equipped with enzymatic mechanisms to eliminate or reduce their damaging effects. The antioxidant enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD) have a vital role to scavenge ROS and thereby prevent oxidative damage. The balance between ROS generation and eradication determines the survival of the system (Khatun et al., 2008; Golshan et al., 2011). $Cu^{+2}$ toxicity is related to disturbances in the uptake of other essential elements (Pätsikkä et al., 2002), and membrane properties by oxidation of membrane lipids. The injurious membrane effect can; however; be estimated from the increase of MDA, one of the lipid peroxidation products (Jouili and El Ferjan, 2003). Copper ion activity does not always accurately predict copper toxicity due to competition effect caused by other cations such as $Ca^{2+}$ and $Mg^{2+}$ (Campbell, 1995). Studies on the response of Hassawi wheat to the exogenous copper would be highly significant for understanding the optimal doses required to grow, and its adaptation to copper toxicity. Therefore, this study was designed to investigate the biphasic effect of different concentrations of copper on growth, proline, lipid peroxidation and antioxidant enzyme activities of Hassawi wheat. This could be help to understand the copper toxicity and the mechanism of Hassawi wheat tolerance to copper. This study may be useful to the farmers of this area to understand the fact that up to which copper level the Hassawi wheat could be grown.

## Results and Discussion

### *Growth and copper tolerance index*

The toxicity of copper in soil depends on the available concentration of copper than its total dose in soil. Our results showed that, fresh weight (FW), dry weight (DW), water content and tolerance index (TI%) (Fig 1 A- D, respectively) of $Cu^{+2}$-treated plants, did not change significantly up to 10 mM $Cu^{2+}$, thereafter they were significantly reduced, compared to untreated control plants. However, 2 mM $Cu^{+2}$ tended to increase these parameters. Such biphasic effects of copper were revealed by other investigators using other crop plants (Fageria, 2002; Deef, 2007; Mathad and Pratima, 2009; Verma et al., 2011). They reported that application of copper at lower concentrations increased the plant's dry weight, while excess of copper reduced the biomass production of these plants. The promotion effect of $Cu^{+2}$ at 2 mM on biomass production may be attributed to the beneficial vital role of $Cu^{+2}$ in plant growth and development. Thus, it could be concluded that the role of copper as a fertilizer was more obvious than its toxicity at this level. However, the significant reductions due to excess copper concentration indicated that the higher concentrations of $Cu^{+2}$ have toxic effects on the growth of Hassawi wheat seedlings leading to inhibition of both cell elongation and division (Souguir et al., 2008).

### *Total free amino acids and proline*

The data shows that the accumulations of total free amino acids (Fig 2A) and proline (Fig 2B) were significantly increased at higher copper concentrations of soil. However, low copper concentrations up to 20 mM had non-significant increase on both total free amino acids and proline. The maximum increase in free amino acids and proline content at the highest level of $Cu^{2+}$ was about 148 % and 190% over the control, respectively. In agreement with our results, several studies have been indicated that free amino acids content increases in plant tissues upon $Cu^{2+}$ exposure (Liao et al., 2000; Mazen, 2004; Al-Hakimi and Hamada, 2011). Amino acids are regarded to play a significant role in metal chelation, by which heavy metal detoxification and tolerance in plants take place (Hall, 2002). Thus, it could be suggested that, the positive response of total free amino acids under $Cu^{2+}$ stress in the present work might be a detoxification response of Hassawi wheat to copper exposure, to tolerate the presence of excess $Cu^{2+}$. The accumulation of proline in plants is a general response to some abiotic stress (Jain et al., 2001; Fariduddin et al., 2009; Jaleel and Azooz, 2009). However, these results could be attributed to proline accumulation, which seems to be due to water stress generated by $Cu^{2+}$ stress as evident water content reduction in Hassawi wheat seedlings (Fig 1C). In fact, the proline accumulation was in parallel with the decrease in water content. It is also proposed that, proline acts as a source of carbon and nitrogen for rapid recovery from the stress, and also serves as stabilizer of plasma membrane and some macromolecules and free radical scavenger (Jain et al., 2001). Moreover, energy supply for growth and survival play important role in protecting plants against extreme stress conditions (Sankar et al., 2007; Fariduddin et al., 2009). This is in accordance with the alleviating effect of proline under excess of heavy metals (Mehta and Gaur, 1999; Wang et al., 2009). Generally, the results indicated that $Cu^{2+}$ concentration up to 20 mM did not cause a significant increase in free amino acids and proline content, suggesting that the concentrations of $Cu^{2+}$ up to 20 mM are not toxic for Hassawi wheat seedlings.

### *Antioxidant enzyme activities*

Antioxidative enzyme activities play a vital role in the plant antioxidative defense system. Copper treatments exhibited a non-significant change in the activities of CAT and APX, up to 10 mM $Cu^{2+}$, and up to 20 mM in case of POD, whereas excess $Cu^{2+}$ in the soil led to a highly significant increase in the activities of these enzymes. On the other hand, the activity of SOD did not significantly increase at most $Cu^{2+}$ concentrations, as compared with control (Fig 3A-D). The increase in the activity of CAT (Fig 3A) and POD (Fig 3B) was more obvious than the other enzymes. It has been confirmed in many studies that excess copper could promote and stimulate the generation of ROS leading to increase in the activities of antioxidant enzymes as a defense system (Mittler et al., 2004; Fariduddin et al., 2009; Verma et al., 2011). This is in harmony with our results which showed that growing of Hassawi wheat seedlings in excess $Cu^{2+}$ caused a significant increase in the antioxidant enzyme activities, which can be considered as circumstantial evidence of enhanced ROS production. The higher activity of CAT and POD at the higher concentrations of $Cu^{2+}$ is a sound indication of Hassawi wheat ability to cope with ROS. Therefore, it could be concluded that the increase recorded in the activities of antioxidant enzymes may be attributed to the adaptive defense system of Hassawi wheat seedlings against the toxic effect imposed by $Cu^{2+}$. However, this was insufficient for full protection of Hassawi wheat against ROS, especially at the higher levels of $Cu^{2+}$. On the contrary, the non-significant increase in SOD activity might be due to the increasing rate of ROS scavenging by the other antioxidant enzymes, and the increased $H_2O_2$ due to the inactivation of SOD may function in oxidative stress leading to the induction of peroxidase antioxidant systems (Basu et al., 2010). SOD activity appeared not to be directly responsible from remarkable protection against oxidative injury, whereas CAT, POD and APX might be important for Hassawi wheat plants, which are possibly equipped with mechanisms that only provide protection during $Cu^{2+}$ stress conditions.

### *Lipid peroxidation and ion leakage*

Copper can affect the membrane properties by oxidation of membrane lipids, which can be estimated from the increase of MDA, one of the lipid peroxidation products. The membrane damage is indirectly assessed by the conductivity of ion leakage (EC) from the cells. The results showed that the content of lipid peroxidation as MDA (Fig 4A) and electrolyte leakage (Fig 4B) of Cu-treated plants did not reflect any significant changes up to the level of 10 mM $Cu^{2+}$. Excess $Cu^{2+}$ induced a sharp significant increase in these contents especially in MDA (about 4- fold at the highest $Cu^{2+}$ level) as compared with control plants. Similar findings have been reported in rice and *Arabdopsis thaliana* exposed to copper stress (Chen et al., 2004; Skorzynska-Polit et al., 2010, respectively). The present results and those obtained by Skorzynska-Polit et al. (2010) showed a positive correlation of ion leakage and MDA content. This correlation cleared that the insignificant increase of ion leakage and MDA content at low $Cu^{2+}$ concentrations is important sign of higher oxidative damage limiting capacity under these concentrations of $Cu^{2+}$ stress, while the significant increase at the higher levels of $Cu^{2+}$ was as a results of plasmalemma injury caused by ROS (Yin et al., 2008). It suggests that

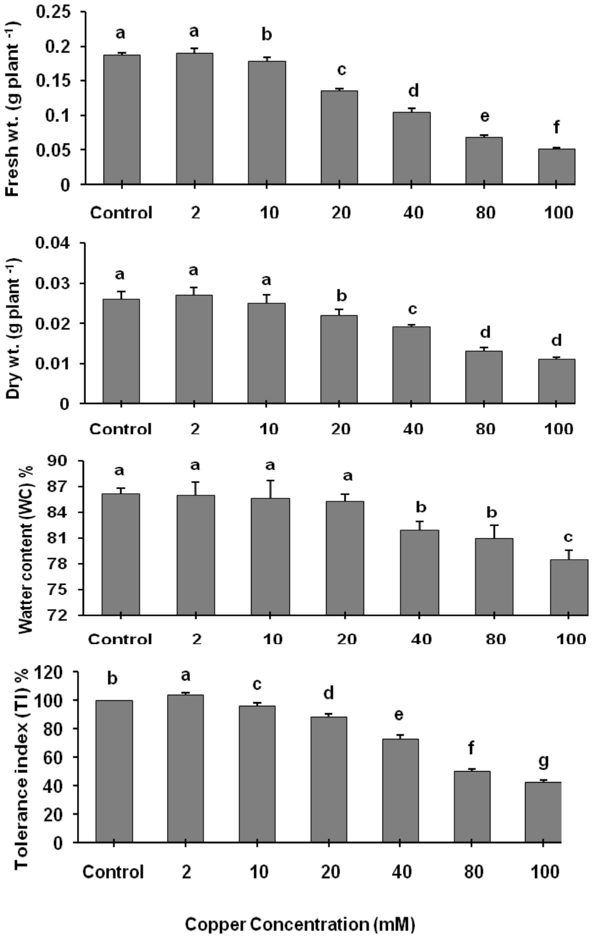


**Fig 1.** Fresh weight (A), dry weight (B) (g plant$^{-1}$), water content (WC%) (C) and tolerance index (TI%) (D) in Hassawi wheat seedlings, exposure to various concentrations of copper ($Cu^{+2}$). Vertical bars represent ± SD of three replicates (*n*=3). Bars carrying different letters are significantly different at P <0.05 between the control and copper treated-plants.

excess $Cu^{2+}$ caused severe oxidative stress, resulting in generation of more MDA. This observation seems to be related with the redox-active nature of $Cu^{2+}$, which catalyses the formation of extremely reactive hydroxyl radicals (Pinto et al., 2003). These results suggest that Hassawi wheat had ability to tolerate $Cu^{2+}$ stress up to 10 mM, in which an efficient antioxidant enzyme is involved. The significant increase of MDA and EC in plants, exposed to excess $Cu^{2+}$, indicated that increase of lipid proxidation in Cu-treated plants led to disorder of plasmatic membranes, resulting in increase of ion leakage due to the increase of $Cu^{2+}$ level in soil medium.

### *Uptake of some cations ($Cu^{2+}$, $Ca^{2+}$ and $Mg^{2+}$)*

Ion regulation is an important essential factor for heavy metals tolerance mechanisms. Application of $Cu^{2+}$ in the soil significantly increased copper concentration (Fig 5A) in Hassawi wheat seedlings. Similarly, Ouzounidou (1994) and Cambrolle et al. (2011) reported that the uptake of copper increased with increasing copper concentrations. $Cu^{2+}$ concentration up to 20 mM had no significant effect on the

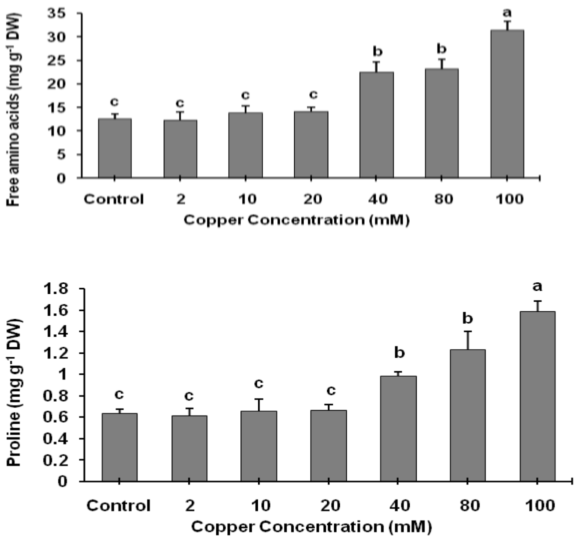


**Fig 2.** The content of free amino acids (A) and proline (B) (mg g$^{-1}$ DW) in Hassawi wheat seedlings, exposure to various concentrations of copper ($Cu^{+2}$). Vertical bars represent ± SD of three replicates (*n*=3). Bars carrying different letters are significantly different at P < 0.05 between the control and copper treated-plants.

uptake of $Ca^{2+}$ (Fig 5B) and $Mg^{2}$ (Fig 5C). However, a significant increase in these contents (Fig 5B and C) was observed with increasing of external $Cu^{2+}$ as reported by Cambrollé et al. (2011). Fageria (2002) found that the copper content can decrease the concentration of calcium and magnesium in rice. On the other hand, the copper content has no effect on these cations in bean plant. Due to this controversy, Ouzounidou et al. (1995) and Mocquot et al. (1996) reported synergistic, antagonistic or no effect of copper on the uptake of macro and micronutrients, depending on crop species and concentration of copper. Therefore, it could be concluded that copper had a synergistic effect rather than antagonistic effect on the uptake of $Ca^{2+}$ and $Mg^{2+}$ in Hassawi wheat plants. The high absorption zone of Hassawi wheat might permit these plants to absorb a sufficient amount of $Ca^{2+}$ and $Mg^{2+}$, which in turn, share in the high affinity of the reactive center of the photosynthetic apparatus.

## Materials and methods

### *Plant growth and treatments*

Grains of Hassawi wheat (*Triticum aestivum* cv. Hassawi) were surface sterilized with mercuric chloride (0.1 %) for 5 minutes, and then they were rinsed 3 times with distilled water. Homogenous Hassawi wheat grains are sown in plastic pots, lined with polyethylene bags containing 2 kg of dried soil. The grains were irrigated with half Hogland solution supplemented with various $Cu^{+2}$ concentrations [0.0 (control), 2, 10, 20, 40, 80 and 100 mM], which were added as $Cu_2SO_4.5H_2O$ dissolved in deionized water and poured into the Hassawi soil at field capacity (without drainage) at the time of sowing. Plants were grown in a growth chamber maintained at 25/19 °C day/night, 60% relative humidity, 14/10 light /dark. The pots were irrigated with normal water

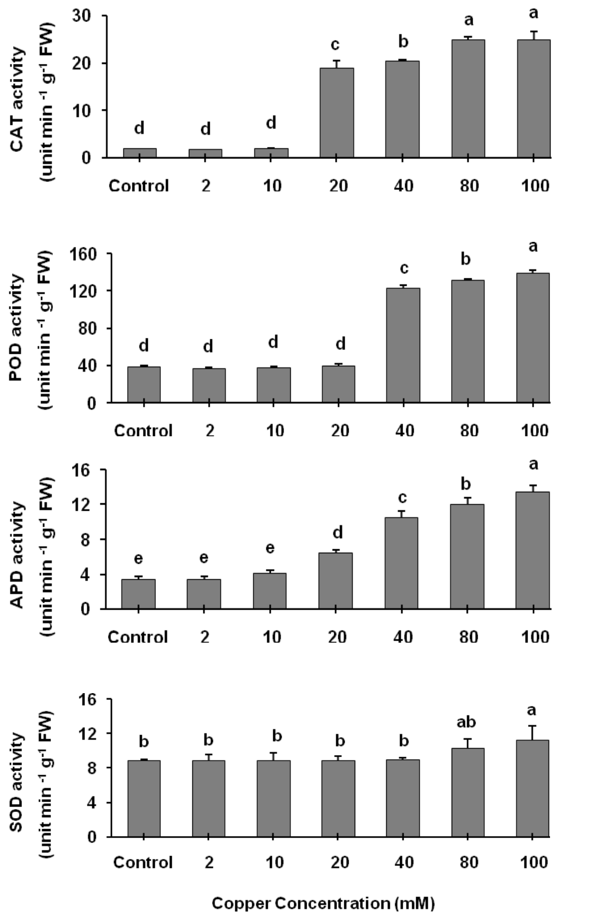


**Fig 3.** The activity of antioxidant enzymes (unit min $^{-1}$ g$^{-1}$ FW) of catalase (CAT) (A), peroxidase (POD) (B), ascorbate peroxidase (APX) (C) and superoxiddesmutase (SOD) (D) in Hassawi wheat leaves, exposure to various concentrations of copper ($Cu^{+2}$). Vertical bars represent ± SD of three replicates (*n*=3). Bars carrying different letters are significantly different at $P < 0.05$ between the control and copper treated-plants.

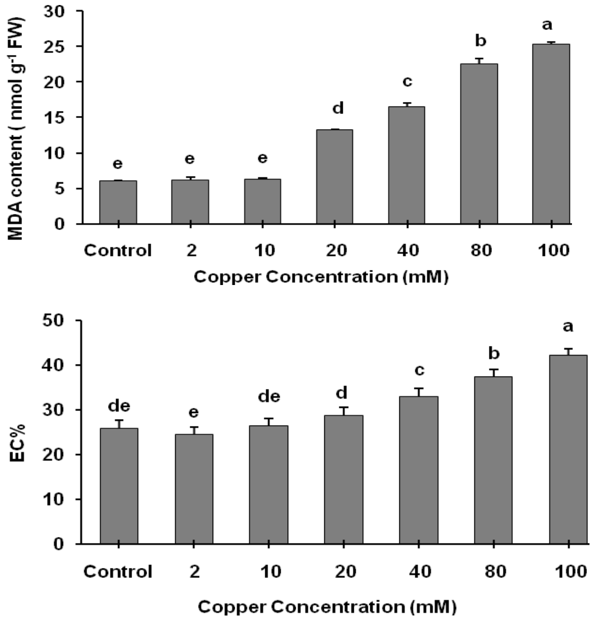


**Fig 4.** The content (nmol g$^{-1}$ FW) of malondialdehyde (MDA) (A) and ion leakage (EC%) (B) in Hassawi wheat leaves, exposure to various concentrations of copper ($Cu^{+2}$). Vertical bars represent ± SD of three replicates (*n*=3). Bars carrying different letters are significantly different at $P < 0.05$ between the control and copper treated-plants.

at field capacity through the whole experimental period (30 days). Three replicates of each treatment were prepared, and each treatment had 10 plants. Antioxidant enzyme activities and MDA in leaves were measured 15 days after sowing (DAS). At the end of the experiment (on day 30), the Hassawi wheat seedlings were harvested.

***Growth parameters***

To determine fresh weight, the harvested plants were rinsed with deionized water, and blotted on paper towels before being weighed. Dry matter yields of the seedlings were determined after drying the washed freshly seedlings in an aerated oven at 80 ºC to constant weight. The dry tissues were grinded into fine powder and stored in sealed glasses at room temperature for various analytical experiment.

***Copper tolerance index***

Copper tolerance index (TI) was calculated as the quotient of the dry weight of plants grown under copper treated and control conditions (Bálint et al., 2002) according to this formula:

$$TI\ (\%) = \frac{\text{Dry weight of copper-treated plants} \times 100}{\text{Dry weight of copper-untreated plants (control)}}$$

***Electric conductivity***

Ion leakage was measured as electrical conductivity (EC%) according to Yan et al. (1996). The percentage of electrolyte leakage was calculated according to this formula: EC (%) = (C1/C2) × 100. Where C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

***Determination of free amino acids and proline***

Free amino acids were determined according to the method of Lee and Takahashi (1966). Proline content was determined by ninhydrin method (Bates et al., 1973).

***Assays of some antioxidant enzyme activities***

***Enzyme extraction***

The samples were prepared as described by Mukherjee and Choudhuri (1983). A leaf sample (0.5 g) was frozen in liquid nitrogen and ground using a porcelain mortar and pestle. The frozen powder was added to 10 ml of 100 mM phosphate buffer ($KH_2PO_4/K_2HPO_4$) pH 7.0, containing 0.1 mM $Na_2EDTA$ and 0.1 g of polyvinylpyrrolidone.

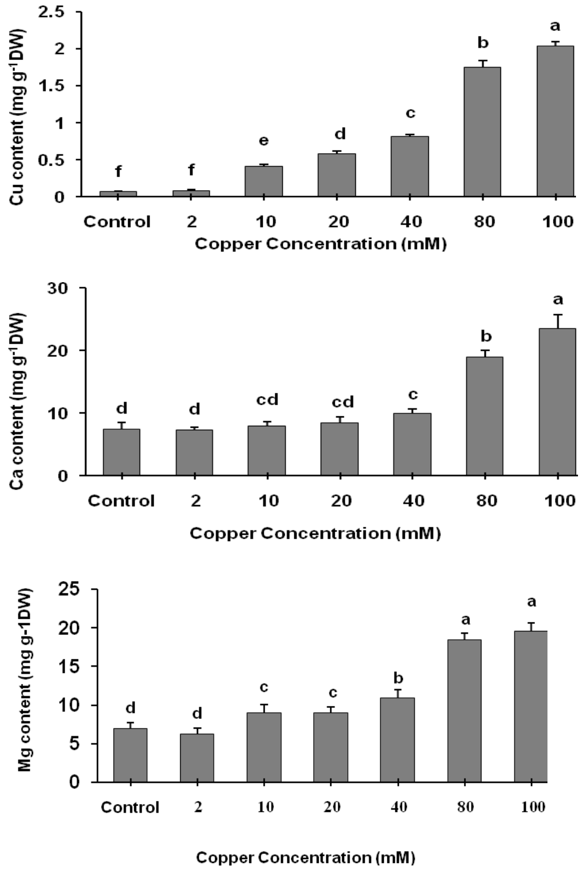


**Fig 5.** The content (mg $g^{-1}$DW) of copper ($Cu^{2+}$) (A), calcium ($Ca^{2+}$) (B) and magnesium ($Mg^{2+}$) (C) in Hassawi wheat seedlings, exposure to various concentrations of copper ($Cu^{+2}$). Vertical bars represent ± SD of three replicates ($n$=3). Bars carrying different letters are significantly different at P < 0.05 between the control and copper treated-plants.

The homogenate was centrifuged at x 15,000 *g* for 10 min at 4°C., and the resulted supernatant was collected and stored at 4°C for CAT, POD, APX and SOD assays.

### *Assay of catalase activity*

Catalase (EC 1. 11. 1. 6) activity was assayed according to Aebi (1984). The activity of catalase was estimated by the decrease of absorbance at 240 nm for 1 min as a consequence of $H_2O_2$ consumption (Havir and McHale, 1987).

### *Assay of peroxidase activity*

Peroxidase (EC 1. 11. 1. 7) activity was determined according to Maehly and Chance (1954) by the oxidation of guaiacol in the presence of $H_2O_2$. The increase in absorbance due to formation of tetraguaiacol was recorded at 470 nm (Klapheck et al., 1990).

### *Assay of ascorbate peroxidase activity*

The activity of ascorbate peroxidase (EC 1. 11. 1. 11) was assayed using the method of Chen and Asada (1992), by measuring the decrease in absorbance at 290 nm for 1 min caused by ascorbic acid oxidation.

### *Assay of superoxide dismutase activity*

Superoxide dismutase (EC 1. 15. 1. 1) activity was measured as described by Dhindsa et al. (1981). Absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT.

### *Lipid peroxidation*

It was measured as the content of malonyldialdehyde (MDA) using the thiobarbioturic method (Zhao et al., 1994), and expressed as nmol of MDA formed using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$ as nmol (MDA) $g^{-1}$ FW.

### *Determination of cations*

0.5 g of dried powder samples were transferred to 50 $cm^3$ digestion flasks, and supplemented by 2 ml perchloric acid 80% and 10 ml of concentrated $H_2SO_4$ and the flasks heated gently over a hot plate until the solution become colorless. Digested material diluted by double distilled water to 100 ml. Copper ($Cu^{2+}$), calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) were estimated in the extract solution by atomic absorption spectrophotometer model Perkin Elmer 3110 USA.

### *Statistical analysis*

All of data were subjecdted to one-way analysis of variance (ANOVA) using SPSS 12.0 software. Vertical bars in the figures indicate the mean values ± SD based on independent three determinations ($n$ =3). Least significant difference (L.S.D) test was used to assess the differences between the mean values of control and copper-treated plants; $p < 0.05$ was considered statistically significant.

## Conclusion

In conclusion, our results revealed that the optimal $Cu^{+2}$ level (2 mM) had a favorable effect on the growth of wheat. Hasaawi wheat tolerated $Cu^{+2}$ up to 10 mM, and survival up to 100 mM. Toxic effects of $Cu^{+2}$ were reflected by reduction in growth parameters of Hassawi wheat seedlings, while the contents of MDA and ion leakage are increased. The stimulatory effect of $Cu^{+2}$ on the biosynthesis of free amino acids and proline as well as antioxidant enzyme activities may play an important role in protecting Hassawi wheat plants against $Cu^{+2}$ toxicity at lower concentrations. Therefore, it could be suggested that the elevated levels of these parameters and antioxidant enzyme activities, at least in part, were responsible for the development of resistance against copper stress in Hassawi wheat. Further, these plants have the ability to grow in $Cu^{2+}$ polluted areas by altering their various physiological processes.


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