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Rania Samy Hanafy
Abdel Haleem Mohammed Ahmed

Alleviation of copper stress on tomato (*Lycopersicon esculentum* var. Castel rock) plants using ascorbic acid

ABSTRACT:

This study showed the effect of Cu stress on Tomato (*Lycopersicon esculentum* var. Castel rock) plants. Effects of foliar spraying of plants with ascorbic acid (AsA) (200 ppm) under Cu stress (10, 50, and 250 µm) on growth, yield parameters and some chemical constituents of tomato plants were evaluated. Results demonstrated that increasing Cu levels resulted in gradual significant reductions in growth, yield parameters, carbohydrates, proteins and auxin contents. On the other hand, Cu stress caused a significant increase in proline, phenols, anthocyanins and ABA contents. Treatment with 200 ppm ascorbic acid (AsA) as a foliar spray mitigated Cu stress by inducing antioxidant enzymes activities responsible for anti-oxidation, e.g., catalase, peroxidase and polyphenol oxidase in tomato leaves under different concentration of Cu by detoxification, as well as improving all the above recorded parameters. Furthermore, treating the plants with Cu concentrations alone or in combination with AsA leads to variation in the banding pattern of proteins. The accumulated Cu in fruits increased with increasing concentrations of Cu and gradually decreased by using AsA. These results indicate that the adverse effects of Cu toxicity on tomato plant and its accumulation in fruits were partially alleviated by treating tomato plants with ascorbic acid.

KEY WORDS:

Copper, Tomato, Phenols, Proline, Ascorbic acid, Abscisic acid

CORRESPONDENCE:

Rania Samy Hanafy

E-mail: raniaamin91@yahoo.com

Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Cairo, Egypt.

Abdel Haleem Mohammed Ahmed

Biological and Geological Sciences Department, Faculty of Education, Ain Shams University, Cairo, Egypt.

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INTRODUCTION:

Copper (Cu) is considered one of the heavy metals that is a micro-element essential for plant growth and development. It plays important key roles in many physiological and biochemical activities such as the photosynthesis process, electron transport chains, protein, lipid and fatty acid metabolism, cell wall metabolism, nitrogen fixation, ethylene sensing, antioxidant activity and hormone balance. In addition, it is a major compound of plastid protein and plastocyanin and an enzymatic cofactor; amino acid oxidase and cytochrome C oxidase (Solomon and Lowery, 1993). Problem arises when copper is present in higher levels in plant cells. In general, excess heavy metals in the soil can lead to toxicity symptoms and the inhibition of growth of most plants. Toxicity resulting from excessive Cu might be due to damage of biological macro-molecules via redox cycling and glutathione depletion then altered sulfhydryl homeostasis (Fendereski *et al*., 2015).

Excess copper can induce oxidative stress, which occurs because of the production of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, hydrogen peroxide and singlet oxygen, which proved to cause inhibition in plant growth and several damages to photosynthetic pigments, damage of cell membrane, lipids, proteins, carbohydrates, amino acids, nucleic acids and binding to SH

groups via the catalytic action of enzymes (Mei *et al.,* 2015). In addition, excess copper rates reduced soil nutrients which affect plant productivity. Copper can bind strongly to organic matter, clay minerals in the soil that makes them unavailable for plant uptake (Azeez *et al.,* 2015). The plant cell can be protected from ROS by the combined action of enzymatic antioxidant defence like catalase, peroxidase, polyphenol oxidase and non-enzymatic antioxidant defence mechanisms like ascorbate, proline, anthocyanin, phenolic compounds and glutathione for protection against Cu toxicity (Gao *et al.,* 2008).

Tomato is the second most widely popular vegetable crops produced and consumed in the entire world. It belongs to Solanaceae. It is important in maintaining health and vigour. Tomato is a good source of vitamins A, B and C. It has been previously shown that tomato is a Cu sensitive crop (Mami *et al.,* 2011). Ascorbic acid; Ascorbate (vitamin c) as water-soluble vitamins regarded as intrinsic antioxidant compound in plants that accumulates in different parts of plant (especially in leaves) playing a vital role in the ameliorate abiotic stresses through enzymatic and non-enzymatic detoxification in the ascorbate-glutathion pathway, serving as electron donors, stable ascorbyl free radical and directly reduced superoxide, singlet oxygen, hydroxyl radicals and toxic H_2O_2 accumulation that leads to tolerance of the plants to oxidative stresses (Agami, 2014). In the present work gives overview of some important features of copper toxicity and conducted to assess whether exogenous water soluble ascorbic acid could confer and ameliorate the adverse effects of excessive Cu on some morphological and biochemical parameters in tomato plant.

MATERIAL AND METHODS:

Plant material:

Seeds of Tomato -super strain B- (*Lycopersicon esculentum* var. Castel rock) were obtained from the Crop Institute, Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt. The pot experiment was conducted in the greenhouse of the Faculty of Education, Ain Shams University. Homogeneous ten tomato seeds were surface sterilized by 0.01 M $HgCl_2$ solution for 3 minutes, washed thoroughly with distilled water. The seeds were sown in pots (25 cm in diameter and 25 cm in depth) filled with equal amounts of homogeneous loam based garden soil (3 Kg soil/ pot). Soil characteristics were sand 80%; silt 15.5%; clay 4.5%; organic matter 0.45 with % pH 7.8; EC 0.4 dSm^{-1}. After one week from sowing, thinning was done to leave 6 uniform seedlings in each pot for experimentation. The pots were divided into 3 major groups. The pots of the first group (10 pots) were irrigated with tap water (holding capacity is 80%) and were used as a control. The 2nd group was irrigated with tap water until the 3-leaf stage appeared then they were divided into three sets (10 pots/set) and irrigated with an aqueous solution of 10, 50, and 250 µm of Cu $(CH_3COO)_2$ (600ml/ pot). The 3rdgroup subdivided into three sets (10 pots/ set) and irrigated with the equal amount of Cu $(CH3COO)_2$ concentrations (10, 50, and 250 µm) and then at 5-leaf stage, pots were sprayed with 25 ml/plant of ascorbic acid (200 ppm). Spraying treatment was carried out two times with two weeks interval. Growth measurements (shoot and root length, number of lateral roots, number of leaves/ plant, leaf expansion, shoot and root fresh, and dry weights) were carried out 45 days after sowing. Ten replicates of plants for each growth parameters were measured and triplicates of leaves for total carbohydrates, total protein, proline, total phenol, anthocyanin and enzyme activities (catalase, peroxidase, and polyphenol oxidase) were analysed. Leaves from terminal part were taken for auxins, ABA and protein electrophoresis. At harvesting time (120 days after transplanting), the fruits were collected for investigating yield parameters (fruit size, fruit fresh, and dry weight).

Determination of total carbohydrate:

Total carbohydrate content was measured using the method described by Asgharipour *et al.* (2011). 0.03 mg of the fine dry powder of leaves and 10 ml of 1N sulfuric acid were added. The samples were placed in a boiling water bath for 60 minutes and cooled down and then 0.1 g of barium carbonate was added. The samples were filtered through Whatman filter paper No.1. One ml of the filtrate, 1 ml of 5% phenol and 5 ml of concentrated sulfuric acid were mixed. The absorbance is determined at 490 nm.

Extraction and Estimation of total protein:

Total protein was done by the method of Lowry *et al.* (1951). Alkaline tartrate reagent (20 g sodium carbonate and 0.5 g tartrate) was dissolved in 1000 ml of (0.1N) sodium hydroxide. 10 µl of the protein sample were added to 5 ml of the alkaline copper reagent, and allowed to stand for 15 min. at room temperature. Immediately, the dilution folin reagent (0.5 ml) was then mixed with the mixture and allowed to stand at room temperature for 30 min. The resulting colour of the samples was measured at 750 nm.

Extraction and quantification of total phenols:

Total phenols were determined using the method described by Dihazi *et al*. (2003). One gram of fresh leaf sample was homogenized in 50ml of 80% cold methanol solution for three times at 90ºC. Then combined extract was and filtered through Whatman filter paper No.1. The filtrate is

completed to a known volume of methanol. One ml of extract, deionized water (10 ml), one ml of folin-ciocalteu reagent (10%) and sodium carbonate 20% (2 ml) were mixed. After 30 min. in darkness the absorbance was measured at 750 nm and expressed as mg tannic acid g^{-1} fresh weight.

Determination of proline content:

Leaf proline content was determined using a colorimetric method (Bates *et al*., 1973). Fresh weight of leaves (0.5 g) was blended in 3% sulfosalicylic acid (10 ml) then and centrifuged at 10.000 g for 15 min. The filtrate (2 ml) was mixed with 2 ml of ninhydrin and 2 ml of glacial acetic acid. The reaction mixture was incubated in a water bath at 90ºC for 30 mins. Then the reaction was terminated in an ice bath. The reaction was extracted by using 4 ml of toluene and vortex and the process was done for 15 seconds and lasted for 20 min. in the dark at room temperature for separation of toluene. The absorbance of toluene phase was measured at 520 nm. using proline as a standard.

Determination of anthocyanin:

Anthocyanin content was determined according to the method of Krizek *et al.* (1993). 0.2 g of leaf samples were homogenized in 10 ml of acidified methanol (hydrochloric acid: methanol, 1: 99, v/ v) then centrifuged at 18.000 g for 30 min at 4°C, and the supernatant was filtered through Whatman No.1 then, the filtrate was stored in darkness at 5°C for 24 h. The absorbance was determined at 550 nm. The content was expressed as µmol/ g FW.

Enzymes assays:

Sample preparation was determined by Mukherjee and Choudhuri (1983). 2.5 g of fresh leaf sample was frozen in liquid nitrogen and grounded to the frozen powder. 10 ml of extraction buffer (100 mM phosphate buffer (pH 7.0), 0.1 mM Na_2-EDTA and 0.1 g of PVP) was added to the leaf powder. The homogenate was filtered and centrifuged at 15.000 g for 10 min. Catalase (CAT) (EC 1.11.1.6) activity was measured according to Aebi (1984). The reaction mixture (3 ml) contained phosphate buffer (50 mM, pH 7.0), 1 ml of enzyme extract and 30% (w/ v) H_2O_2 was added. The enzyme activity was assayed by the colorimetric decomposition of H_2O_2 colorimetric at 240 nm. (Havir and Mellate, 1987). One unit of catalase activity was expressed as the amount of the enzyme, which caused catalysed the oxidation of one µmole H_2O_2 per minute (Montavon *et al.,* 2007). CAT activity was expressed as enzyme units per gram fresh weight (U/g Fwt). Peroxidase (POX; EC 1.11.1.7) activity was assayed as described by Kumar and Khan (1982). The mixture consisted of 0.5 ml of enzyme extract, 1 ml of 0.01 M pyrogallol, 2 ml of 0.1 M phosphate buffer (PH 6.8) and 1 ml of 0.005 M H_2O_2. The mixture was incubated at 25°C for 5 minutes and then the reaction was stopped by adding 1 ml of 2.5 N sulphuric acid. The purpurogallin formed was measured at 420 nm. One unit of peroxidase activity was expressed as the amount of the enzyme, which caused 1 µmole purpurogallin formation per minute. Polyphenol oxidase (PPO; EC 1.10.3.1) was detected according to Kumar and Khan (1982). The mixture consists of 0.5 ml of enzyme extract, 2 ml of 0.1 M phosphate buffer (pH 6.0) and 1 ml of 0.1 M catechol was incubated for 5 min. at 25°C. The reaction was terminated by 1 ml of 2.5 N sulphuric acid. The amount of quinone formed was assayed at 495 nm.

Extraction and quantification of auxins and ABA:

Frozen samples were grounded in 80% cold methanol followed by multiple extractions with methanol at 0ºC for 2 hours. The extracts of auxins and abscisic acid (ABA) were carried out according to Wasfy and Orrin (1975). The quantification was made utilizing Gas Liquid Chromatography (GLC) (Varien Vesta, 6000) as described by Vogel (1975).

Protein Electrophoresis:

The electrophoretic protein profile of tomato leaves was analysed according to (SDS-PAGE) technique as described by (Laemmli, 1970). It was performed by using 12% running gel and 3% stacking gel. Running gel consist of 30% (wt/vol) acrylamide, 0.8% (wt/vol) methyl bis acrylamide, 1.5 M Tris-HCl (PH 8.3), 10% (wt/vol) SDS, 9.3 ml H_2O, 23 ml of tetramethylethylenediamine and 10% (wt/vol) ammonium persulfate solution. Stacking gel contained 30% (wt/vol) acrylamide, 0.8% (wt/vol) methyl bis-acrylamide, 0.5 M Tris-HCl (PH 6.8), 10% (wt/vol) SDS, 10.10 ml of H_2O, 23 ml of tetramethylethylenediamine and 10% (wt/vol) ammonium persulfate. Running buffer consist of 0.125 M Tris-HCl (PH 8.5), 0.96 M glycine and 0.5% (wt/vol) SDS. The sample buffer was used with an equal volume of protein extraction supernatant. Gel was stained with Coomassie blue (0.5 g/l) and distained with 5% MeOH/ acetic acid mixture. De-stained gel was photographed and protein banding pattern was analysed using gel protein analyser version 3 (MEDIA- CEBRNE TICE, USA).

Determination of cu in fruit:

Copper content in fruit tissue was determined by atomic absorption spectrometry as described by Chapman and Pratt (1978).

Statistical analysis:

The results were statistically analysed by one-way analysis of variance and the Least Significant Difference (LSD) test was

used to compare the means at p ≤ 0.05 according to SAS (2006).

RESULTS:

Data presented in table 1 show that the effect of Cu stress on tomato plants varied according to Cu level. Treatment of plants with the lowest concentration of copper (10 µm) caused significant increases in all growth parameters (shoot and root lengths, number of roots and leaves, leaf expansion, and root fresh and dry weights) as compared with the control plants. However, increasing the Cu concentrations (50 and 250 µm) caused significant reductions in the above-mentioned parameters. Treatment of plants with concentrations 10, 50 µm of Cu showed non-significant changes in shoot fresh and dry weights while significant decrease was obtained with high concentration (250 µm) of Cu in the same parameters as compared with control plants. The results also showed that, spraying tomato plants with ascorbic acid (AsA) (200 ppm) resulted in visible increases in all the above-mentioned growth parameters as compared with untreated and treated plants with the corresponding Cu levels. These increments were much more pronounced at the concentrations of Cu level (10 and 50 µm). Significant decreases were observed in case of plants sprayed with ascorbic acid and grown under the highest concentration of Cu stress (250 µm) (Table 1).

Irrigating tomato plants with the low concentration of Cu (10 µm) caused significant increase in yield parameters (fruit size, fruit fresh and dry weights) as compared with control plants, while the concentrations of Cu (50 and 250 µm) caused an opposite pattern of change (Table 1). Foliar spraying of tomato plants with ascorbic acid caused significant increase in yield parameters as compared with control and the corresponding Cu levels. Significant decreases were observed in case of plants sprayed with ascorbic acid and grown under the highest concentration of Cu stress (250 µm) (Table 1).

Table 1. Effect of foliar spraying with ascorbic acid on growth and yield parameters of tomato plants grown under different Cu concentrations. Different letters are significantly different at P ≤ 0.05 according Least Significant Difference (L.S.D) test.

Samples	Shoot Length (cm)	Root Length (cm)	No. of lateral roots	No. of leaves/ plant	Leaf area (cm^2)	Shoot fresh wt. (g)	Shoot dry wt. (g)	Root fresh wt. (g)	Root dry wt. (g)	Fruit size (cm^3)	Fruit fresh wt. (g)	Fruit dry wt. (g)
Control	35.2	13.5	31	6.4	51.3	14.47	1.291	2.071	0.258	21.61	24.03	1.514
10 µm Cu	42.1[a]	16.2[a]	35 [a]	7.6[a]	58.7[a]	16.89[c]	1.343[c]	2.617[a]	0.362[a]	28.33[a]	31.82 [a]	1.803[a]
50 µm Cu	30.4[b]	11.4 [b]	27[b]	5.5[b]	42.3[a]	12.07[c]	1.069[c]	1.750[b]	0.330[a]	17.91[b]	21.05[b]	1.212[b]
250 µm Cu	26.9[b]	8.3[b]	22[b]	4.3[b]	30.8[b]	8.21[b]	0.890[b]	1.23[b]	0.206[b]	14.01[b]	13.89[b]	1.040[b]
10 µm Cu+ AsA	43.4[a]	19.6[a]	37[a]	8.8[a]	61.5[a]	18.59[a]	1.730[a]	3.816[a]	0.424[a]	51.26[a]	34.67 [a]	1.851[a]
50 µm Cu+ AsA	41.6[a]	16.6[a]	35[a]	8.4[a]	59.6[a]	17.62[a]	1.572[a]	3.571[a]	0.346[a]	29.03[a]	29.17 [a]	1.613[c]
250 µm Cu+AsA	29.2[b]	11.2[b]	26[b]	7.2[b]	38.7[b]	11.37[b]	1.034[b]	1.593[b]	0.211[b]	16.87[b]	18.16[b]	1.317[b]
L.S.D. at 5%	4.804	1.508	3.505	0.774	5.475	3.152	0.228	0.224	0.034	2.442	1.793	0.173

a: significant increase; b: significant decrease; c: Non-significant

The obtained results showed that, total carbohydrate and total protein contents decreased significantly as compared to control plants in response to irrigation with different Cu levels (Table 2). They decreased gradually with increasing Cu levels. The same effect appears clearly in tomato plants treated with AsA under Cu levels, with additional increases in total carbohydrates and total protein. Meanwhile, data in the same table showed that, proline, total phenol and anthocyanin contents of tomato increased significantly with increasing Cu levels as compared with control plants. The similar effect appeared clearly in tomato plants treated with AsA under different Cu levels, but with lower magnitude as compared with Cu treated plants with additional increases with increasing the Cu concentrations. Changes in the activity of antioxidant enzymes activities are the consequence of oxidative stress. Changes in enzymatic activities of catalase, peroxidase and polyphenol oxidase in tomato leaves under different concentration of Cu either without or with AsA treatment was assayed are shown in figure 1. Results indicates that when plants are exposed to lower level of Cu (10 µm) caused significant decreases in the activities of all enzyme mentioned above, while the concentrations 50 and 250 µm caused significant increases in the activity of the same enzymes in Cu treated plants alone or combined with AsA.

Table 2. Effect of foliar spraying with ascorbic acid on total carbohydrate, total protein, proline, total phenol content and anthocyanin content of tomato leaves grown under different Cu concentrations. Different letters indicate a significant difference at P≤0.05 according to Least Significant Difference (L.S.D) test.

Samples	Total carbohydrate mg/g DW	Total protein mg/g FW	Proline µg/g FW	Total phenol mg tannic acid/ g FW	Anthocyanin µmol/g FW
Control	8.6	8.99	10.37	1.637	0.616
10 µm Cu	3.4[b]	7.58[b]	19.04[a]	2.770[a]	0.710[a]
50 µm Cu	1.9[b]	5.05[b]	20.87[a]	3.012[a]	0.887[a]
250 µm Cu	1.2[b]	3.10[b]	28.59[a]	6.415[a]	1.232[a]
10 µm Cu + AsA	6.1[b]	8.04[b]	10.71[a]	2.125[a]	0.746[a]
50 µm Cu + AsA	2.7[b]	6.57[b]	12.92[a]	2.207[a]	0.761[a]
250 µm Cu + AsA	1.8[b]	3.99[b]	18.10[a]	3.138[a]	0.992 [a]
L.S.D. at 5%	0.215	0.615	1.273	0.155	0.075

a: significant increase; b: significant decrease; c: Non-significant

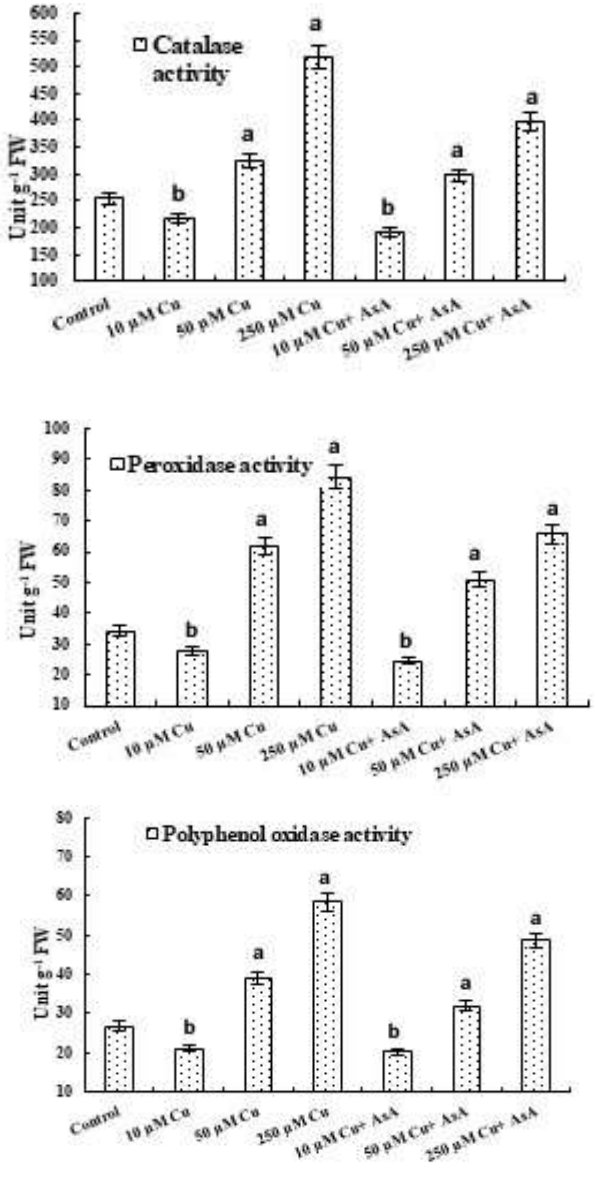

Fig. 1. Effect of foliar treatment of ASA on catalase, peroxidase, and polyphenol oxidase activities of tomato plants grown under control and different concentrations of Cu. Different letters are significantly different at P ≤ 0.05 according Least Significant Difference (L.S.D) test

The obtained results showed that the auxins content of leaves was significantly decreased by Cu levels. There was a considerable decrease in auxins accumulation with increasing Cu levels as compared with control plants. Irrigation of plants with the lower concentration of Cu (10 µm) caused significant increase in auxins content as compared with control plants. While with the increasing of Cu concentrations 50 and 250 µm caused an opposite pattern of change. The values of auxins in AsA- treated plants were in the result with more pronounced increase than those of Cu treated plants. The obtained results showed that different concentrations of Cu caused gradual significant increases in ABA content of the leaves as compared with control plants (Fig. 2).

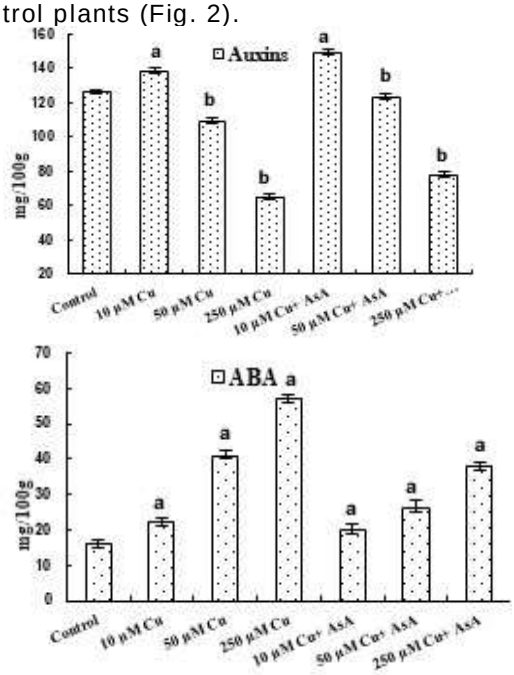

Fig. 2. Effect of foliar treatment of ASA on hormonal changes (Auxins and Ascorbic acid) (mg/100 g) of tomato plants grown under different concentrations of Cu. Different letters are significantly different at P ≤ 0.05 according Least Significant Difference (L.S.D) test.

Ascorbic application decreased the ABA level as compared with plants under Cu stress. Variation in the SDS-PAGE banding pattern of proteins (Table 3 & Fig. 3) extracted from leaves of tomato (*Lycopersicon esculentum* var. Castel rock) in response to Cu stress, showed the disappearance of certain bands and appearance of new bands. Fifteen protein bands with molecular weights ranging between 252.35 and 8.55 kDa were observed in tomato leaves (control). Total number of protein bands was decreased from 15 bands in the control plants to 15, 14 and 12 bands in plants irrigated with 10, 50, and 250 µm Cu, respectively. The leaves extract of tomato plant was characterized by the presence of 4 monomorphic protein bands; their molecular weights were 169.14, 128.61, 63.73, and 51.2 kDa. Therefore, in the present study, Cu stress in general induced synthesis of a new set of protein bands (4 bands) at molecular weights 190.43, 185.59, 107.09, and 13.85 kDa at all Cu stress levels, and at molecular mass 330.86 and 11.35 kDa at the highest level of Cu stress (250 µm) only as compared with control plants. The obtained results also showed the disappearance of protein bands at molecular weights 252.35, 83.8, and 33.85 kDa at all Cu stress levels. These results indicate that the plants irrigated with different Cu levels characterized by appearance of certain new bands and the disappearance of other ones as compared with that of the untreated plants (Table 3). Foliar spraying of tomato plants with ascorbic acid in combination with different levels of Cu induced the appearance of two de novo synthesized protein bands at molecular weights 209.67 and 96.8 kDa in tomato leaves treated with ascorbic acid in combination with different levels of Cu. These bands disappeared in both control plants and plants exposed to Cu stress only. Also, ascorbic acid in combination with Cu level (250 µm) induced synthesis of a new set of protein band at molecular weight 335.8 kDa.

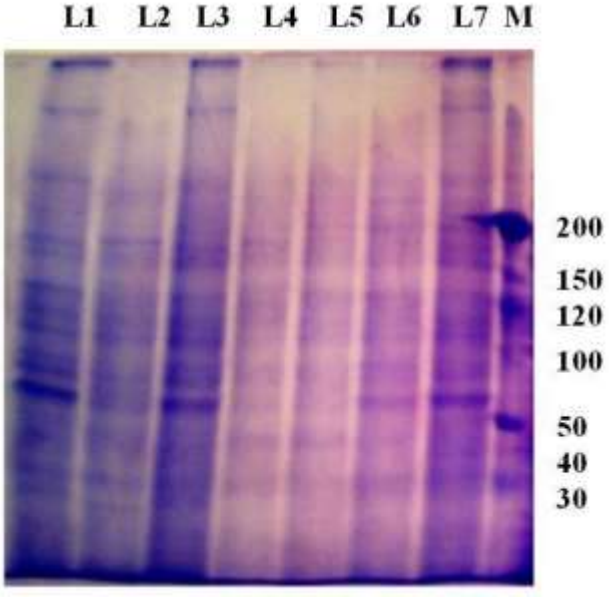

Fig. 3. Protein banding profiles on SDS-PAGE of tomato leaves influenced by foliar spraying with and ascorbic acid and grown under different concentrations. (1) = Control; (2) =10 µm Cu; (3) = 50 µm Cu; (4) = 250 µm Cu; (5) = 10 µm Cu + ASA; (6) = 50 µm Cu + ASA; (7) = 250 µm Cu+ ASA.

Table 3. Molecular weights and electrophoretic proteins banding patterns extracted from leaves of tomato leaves treated with different concentrations of Cu and ASA (200 ppm).

Marker (mol. wt.) (kDa)	Control	Cu (µm)			Cu + ASA (200 ppm)		
		10	50	250	10	50	250
335.8	--	--	--	--	--	--	+
330.86	--	--	--	+	--	--	--
252.35	+	--	--	--	--	+	+
231.48	+	+	--	--	+	+	--
209.67	--	--	--	--	+	+	+
190.43	--	+	+	+	--	--	--
185.59	--	+	+	+	--	--	--
169.14	+	+	+	+	+	+	+
128.61	+	+	+	+	+	+	+
114.23	+	+	+	+	+	--	+
107.09	--	+	+	+	--	--	--
96.8	--	--	--	--	+	+	+
89.5	+	--	+	--	+	+	--
83.8	+	--	--	--	--	--	--
77.4	+	+	--	--	+	+	+
63.73	+	+	+	+	+	+	+
51.2	+	+	+	+	+	+	+
42.51	+	+	+	--	+	+	+
33.85	+	--	--	--	--	--	--
28.51	+	+	+	--	+	+	+
20.77	+	+	+	--	+	+	--
13.85	--	+	+	+	+	+	--
11.35	--	--	--	+	--	--	--
8.55	+	+	+	+	+	--	--
No. of bands	15	15	14	12	16	14	13

Results in present study indicated that treatment of tomato plants with 10 µm Cu induced a slight increase in accumulated Cu in fruits (1.3 and 4.3 µg/g) as compared with control, and gradually increased at 50 and 250 µm of Cu treatments (9.6 and 17.7 µg/g) causing inhibition in fruit parameters. Foliar spraying of tomato plants with ascorbic acid caused lesser accumulation of Cu in tomato fruits (3.0, 7.4, and 12.8 µg/g), respectively with increasing the Cu levels used as compared with treated plants (Fig. 4).

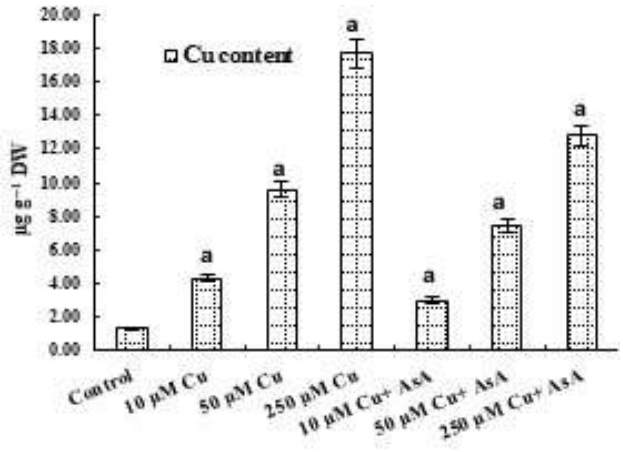

Fig. 4. Effect of foliar treatment of ASA on Cu concentration (µg g-1 DW) in tomato fruits grown under control and different concentrations of Cu treatment. Different letters are significantly different at P ≤ 0.05 according Least Significant Difference (L.S.D) test.

DISCUSSION:

The excess Cu level in soil plays a cytotoxic role and induces stress which caused injury to plants. The obtained results showed that the growth aspects and yield parameters (fruit size, fruit fresh, and dry weights) of tomato plants were decreased with increasing the concentrations of copper. Such reduction in plant might be due to interfering of excess toxicity with important cellular processes such as photosynthesis and respiration which generates oxidative stress that causes disturbance of metabolic pathways like the cellular water status, mitosis division, nitrogen metabolism, hormonal balance and damages macromolecules in plants (Chaparzadeh and Chagharlou, 2013). In addition, excess copper in soil decreases the amount of micronutrient and macronutrients such as zinc, manganese, iron and phosphorus due to their antagonistic relationship (Azeez *et al.,* 2015) which caused nutrients imbalance by reducing nutrients uptake and therefore affects the growth and yield productivity. This result is in harmony with the findings of Mei *et al.* (2015) who demonstrated that treatment of Cu caused a significant reduction in growth parameters in three cotton genotypes. The results also showed that spraying tomato plants with AsA (200 ppm) alleviate the harmful effect of copper and resulted in obvious enhancement in growth parameters under stress conditions by causing a pronounced increase in growth and yield parameters as compared with those of untreated plants. In support of these results, Mei *et al.* (2015) found out that pretreatment of the three genotype cotton seeds with AsA was significantly prevented Cu-growth inhibition and thereby the yield production. The exogenous application of AsA improved the growth and yield production under stress conditions could be explained on the basis that AsA might increase cell division and cell enlargement via detoxification of H_2O_2 via restoring the hormones (ethylene, gibberellic acid and abscisic acid) equilibrium and participates in nitrogen fixation (Azzedine *et al*., 2011). Increasing Cu levels resulted in gradual significant reductions of carbohydrate contents in tomato plants. Limitation in the amount of carbohydrates observed in our study is one of the various effects of Cu stress in plants, the reduced carbohydrates in stressed plants agrees with the results obtained by Al-Hakimi and Hamada (2011) who showed that Cu treatment has an inhibitory effect of carbohydrate accumulation in wheat plants. This effect of excess Cu may be attributed to its effects on the enzymes involved in the cycles of carbohydrate metabolism (Singh *et al.,* 2007). Cu stress might interfere with the biosynthesis of the photosynthetic process leads to degradation of pigment and protein components in photosynthetic membranes (Maksymiec *et al.,* 1994) and, therefore, resulting in reduction of the carbohydrates production. On the other hand, the increasing in carbohydrate levels observed in our study due to treating the stressed plants by ascorbic acid which could be explained on the basis that ascorbate might stimulate the enzymes responsible of sugar hydrolysis (Gul *et al.,* 2015).

The obtained results in table 2 showed that irrigation of tomato plants with various Cu levels decreasing significantly the total protein contents compared to control plants. These results are in line with those obtained by Al-Hakimi and Hamada (2011) who recorded a decrease in total protein of wheat plants with increasing Cu concentrations (0, 5, 10, 20, and 40 mg/l). The reduction in the protein contents under Cu stress could be related to their ability to binding thiol groups of enzymes, which might cause disturbance in protein metabolism or the generation of ROS that cause damaging of proteins, nucleic acids, lipids and leading to cell death (Bai *et al.,* 2003). On the other hand, foliar spraying of tomato plants with AsA exhibited a stimulatory effect on the accumulation of total proteins in tomato leaves; this is similar results are confirmed with those obtained by Gul *et al*. (2015) who indicated that ascorbate was one of the most effective compounds that induce total amino acid and protein synthesis of Guar (*Cymopsis Tetragonoloba*) plants under normal or stressed conditions via inducing the uptake and translocation of N to be involved in various metabolic processes resulting in the production of protein. Also, ascorbic acid induced alterations in the enzymes of protein metabolism and acts as bio activators of

protein synthesis (Bassuony *et al.,* 2008). Increasing protein contents might play an inductive role in triggering a special system that helping tomato plants to tolerate copper stress.

Results presented in table 2 illustrate that proline content of tomato plants significantly increased gradually by increasing the Cu levels as compared with control plants. These obtained results demonstrated that, the physiological role of proline which accumulated in tomato under Cu stress acts as osmolyte, protectant agent and has other roles related to stresses (Ashraf and Foolad, 2007). Several investigators indicated that proline was increased with increasing Cu concentrations. Zengin and Kirbağ (2007) indicated that, proline induced in sunflower (*Helianthus annuus* L.) seedlings as a response to Cu stress. Proline is considered one of the first metabolic responses to stress, which acts as osmoregulation stabilizer in the protein synthesis process, a metal chelator and a hydroxyl radical scavenger and a storage component of carbon and nitrogen for stress recovery and it improves cytoplasmic and mitochondrial enzymes stability and induces expression of stress responsive genes. Moreover, exogenous application of proline is known to improve survival rate of plants under stress conditions Bybordi (2012). In addition, it was observed that tomato plants that were subjected to foliar spraying with different levels of AsA generally have greater proline contents as compared to untreated with gradually decrease as compared with stressed plants. Similar results are in accordance with Małkiewicz *et al.* (2015) who revealed that the exogenous application of ascorbic acid leading to elevate proline contents of tomato seedling under Cu condition. Proline accumulation may be attributed to the increase in its synthesis or inhibition of its degradation and/ or may be a mechanism for stress tolerance.

As results obtained in table 2, increasing Cu levels significantly affected the total phenolic and anthocyanin contents of tomato plants when compared with control. Irrigation of plants to all Cu levels (10, 50, and 250 µm) caused a significant increase in total phenols and anthocyanin contents of the produced plants as compared to control. The magnitude of induction was much more pronounced at 250 µm. Phenol compounds have various functions in plants. The enhancement of phenylopropanoid metabolism and the number of phenolic compounds are observed under different environmental stress conditions Du *et al.* (2010). Our results also support the results of Chaparzadeh and Chagharlou (2013) who found that phenolic and anthocyanin contents in plants were increased with increasing Cu level. Phenol compounds (especially flavonoids) are responsible for the antioxidant induction in the plant. They are oxidized by peroxidase, and can directly scavenge ROS (Michalak, 2006). Also, Phenol compounds possess hydroxyl and carboxyl groups, able to bind particularly copper. So, during copper stress phenolic compounds can act as metal chelators due to their high tendency to chelate metals. The increase of phenolic contents might be due to the increase in activity of enzymes, which involved in phenolic compound metabolism under copper stress Chaparzadeh and Chagharlou (2013). Anthocyanins are considered as an antioxidant component which act as osmo-regulators in plant cells, prevent membrane injury, protein degradation, enzyme inactivation and the disruption of DNA and photo-inhibition that are associated with enhanced resistance to the effects of stress (Gould, 2004). Ascorbate treatment resulted in a considerable increase of phenolic compounds and anthocyanin contents as compared with control plants and gradually decreased as compared with stressed plants. These results agree with results obtained by El-Lethy *et al.* (2011). The protective mechanisms adapted by plants to scavenge ROS were included several anti-oxidative enzymes, which enhanced in plants when exposed to stress conditions. In the present work, it has been observed that, in tomato plants under Cu stress (especially in 50 and 250 µm) CAT, POD and PPO activities were elevated over the controls (Fig. 1). Therefore, it is assumed that the excess Cu inducing defines genes responsible for antioxidant enzymes which contributed to the removal of ROS and protected biomolecules from its attack. The induction of CAT and POD activities in plants by Cu stress plays several roles in heavy metals tolerance, such as scavenging free radicals, trapping heavy metals in polymers and reduces toxic molecules (Wang *et al.,* 2004). This is in harmony with our results which showed a significant increase in the anti-oxidative enzymes (CAT, POD, and PPO) under Cu stress, this increase in these enzymes may be attributed to the adaptive defence system in tomato against the toxic effects imposed by Cu. Exogenous application of AsA significantly increased the specific activity of antioxidant enzymes in stressed plants which decrease the injurious effects of Cu. Padh (1990) reported that ascorbic acid plays an important role in preserving enzymes activities that contain prosthetic transition metal ions. In addition, ascorbic acid (AsA) acts as a primary substrate for enzyme detoxification of hydrogen peroxide (Shalata and Neumann, 2001).

Under heavy metal stress, plants integrate signalling pathways that produce

hormones that have an important role in the defence mechanism. Auxins, cytokinins and abscisic acid have vital roles in stress tolerance of heavy metal stress (Vázquez *et al.,* 2013). In the present study, it was estimated that auxin was significantly increased under low Cu concentration and significantly decreased with increasing Cu concentration. The decreased synthesis of auxins under Cu metal stress could be correlated with a reduction in shoot, root growth and fresh weight of plant. These results agree with Choudhary *et al.* (2010) who showed a reduction in auxins and induction of ABA in radish seedlings under Cu^{2+} and Cr^{6+} metal stress. Foliar spraying of ascorbic acid can reduced the rate of ROS generation, improve the activity of phytohormones (IAA, GA_3, and cytokinins), decreased the level of inhibitor (ABA) and enhance stress tolerance of plants (Ozaki *et al.,* 2003), These results are in line with those obtained by Sakr *et al*. (2010) who reported that when exogenous ascorbic acid were applied to two rice cultivars (Sakha 101 and Giza 178), the promoters (IAA, GA_3 and cytokinin) were increased but the inhibitor (ABA) was decreased. Ascorbic acid is strictly required by some enzymes that are involved in the promoter hormones biosynthesis (Arrigoni and De Tullio, 2002).

The obtained results indicated that tomato plants irrigated with different Cu levels characterized by the appearance and disappearance of bands as compared with that of control plants. In this respect Rout *et al.* (2013) founded that stressed plants developed significant mechanisms of protection against Cu stress via the induction of de novo synthesis of new set proteins. In the present study Cu induced synthesis of a set of new protein bands (4 bands) at molecular weights 190.43, 185.59, 107.09, and 13.85 (Table 3) at all Cu levels. Under Cu stress, the increase of the new synthesized proteins. It is well known that plants respond to heavy metals stresses by the synthesis of related peptide and related proteins that helped for encountering of its inhibitory effects (Didierjean *et al.,* 1996). Furthermore, excess heavy metals are responsible for generating oxidative stress, which increases the levels of reactive oxygen species (ROS) that affect the production of amino acids, protein and nucleic acids (Brahim and Mohamed, 2011). It was also revealed that heavy metal stress altered the patterns of protein synthesis, and this might due to the synthesis of several osmo-responsive genes that may be involved in adaptation to stress. One of the most important responses of plants to abiotic stresses is the activation of some genes involved in stress response (Rasul *et al.,* 2017). Rout *et al.* (2013) revealed that Cu stress could enhance changes in modulating gene expression to adapt to this stress condition. Foliar spraying of tomato plants with ascorbic acid and grown under Cu stress is induced the appearance of two inducible protein bands at molecular weights 209.67 and 96.8 kDa. Moreover, increase in number of protein bands as compared with plants under Cu stress reflect that application of ascorbic modify the expression of Cu-stress inducible proteins and may be due to de novo synthesis of new protein, which are expected to play an important role in plant tolerance (Azooz, 2004). Bassuony *et al*. (2008) has shown that vitamins treatments caused a significant alteration in the enzymes related to protein metabolism so it might act as activators of protein synthesis. The new protein bands and the increase in the intensity indicate that ascorbic acid has stimulatory effect on the protein component, which might be linked with the improvement of plant growth.

Copper is essential for biochemical and physiological metabolism and necessary for maintaining health, but if this metal has an excessive concentration above the recommended limit as established by the FAO/WHO (1999) due to the application of fertilizers and copper based fungicides may sometimes increase it to the alarming levels that may cause diseases (Mohamed *et al.,* 2003). In this study, it was detected the level of copper in untreated tomato fruits (1.3 µg/g); this concentration is found within the FAO/WHO values. This concentration of Cu in tomato fruit was agree or less with the results of Mohamed *et al.* (2003); 4.47 µg/g, Radwan and Salama (2006); 1.83 µg/g, Elbagermi *et al.* (2012); 2.245 µg/g, Brhane and Shiferaw (2014); 0.879- 3.428 µg/g in the edible tomato fruits that are accepted by FAO/WHO guideline value. On the other hand, accumulation of copper increased (4.3, 9.6, and 17.7 µg/ g) with increasing the concentrations of Cu applied to soil which exceeded the FAO/WHO value. So, the excessive Cu in the nutrient solution caused an increase of Cu in tomato fruits and associated symptoms of toxicity; therefore, there is need of remedial action. Application of AsA as a foliar spraying exhibited a partial inhibition effect on the accumulation of copper in tomato fruits treated with copper (3.0, 7.4, and 12.8 µg/g). These results agree with those obtained by Shabana *et al*. (2012) who found that application of vitamin C decreased significantly the concentration of Cu in tomato fruits as compared to the control and polluted plants in the two seasons. In conclusion, the results of present work revealed that the increase in plant tolerance for copper stress was associated with the antioxidant activity of ascorbic acid and it could partially alleviate the inhibitory effects of Cu stress on tomato plants and its accumulation in fruits.

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التخفيف من إجهاد النحاس على نباتات الطماطم (ليكوبيرسيكون إسكولنتوم كاستيل روك) باستخدام حامض الاسكوربيك

رانيا سامي حنفي، عبد الحليم محمد أحمد

قسم العلوم البيولوجية والجيولوجية، كلية التربية، جامعة عين شمس، القاهرة، مصر.

أظهرت الدراسة الحالية تأثير إجهاد النحاس على نباتات الطماطم (ليكوبرزيكون إسكولنتوم كاستيل روك). تم تقييم تأثيرات الرش الورقي للنباتات بحامض الاسكوربيك (200 جزء بالمليون) تحت ضغط النحاس (10، 50 و 250 ميكرومول) على النمو وصفات المحصول وبعض المكونات الكيميائية لنباتات الطماطم. وتظهر النتائج أن زيادة مستويات النحاس أدى إلى انخفاض كبير تدريجي في النمو، صفات المحصول، والكربوهيدرات والبروتينات ومحتويات الأوكسين. من ناحية أخرى، تسبب إجهاد النحاس زيادة كبيرة في البرولين، الفينولات، الأنثوسيانين ومحتويات الايه بى ايه. أدت المعاملة باستخدام 200 جزء في المليون من حمض الاسكوربيك بالرش الورقي إلى تخفيف إجهاد النحاس عن طريق حفز نشاط الانزيمات المضادة للأكسدة، على سبيل المثال، الكاتالاز، البيروكسيديز والبوليفينول اكسيديز في أوراق الطماطم تحت التركيزات المختلفة من النحاس عن طريق إزالة السموم وكذلك تحسين جميع المظاهر المذكورة أعلاه. وعلاوة على ذلك، علاج النباتات بتركيزات النحاس وحدها أو متحدة مع حامض الاسكوربيك يؤدى إلى الاختلاف في نمط حزم البروتينات. تراكم النحاس في الفواكه زاد مع زيادة تركيزات النحاس وانخفض تدريجيا باستخدام حامض الاسكوربيك. وتشير هذه النتائج إلى أن التأثيرات الضارة لسمية النحاس على نبات الطماطم وتراكمها في الفواكه قد تم تخفيفها جزئيا بمعالجة نباتات الطماطم مع حمض الاسكوربيك.