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Alleviation of Adverse Effects of Copper on *Allium cepa* L. by Exogenous Ascorbic Acid Application

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Abstract
In this study ameliorative effects of ascorbic acid (0.5 mM) on onion (*Allium cepa* L. cv. Red Azarshahr) under copper stress (Cu: 6.5 μM) were studied. Fresh weights of leaves and roots were reduced in seven week old plants under excess copper condition. Cu stress also reduced membrane stability index in leaves. There was a considerable increase in H_2O_2 content and lipid peroxidation in both roots and leaves of plants under excess Cu. Leaves anthocyanin contents, leaves and roots phenolic compounds, soluble proteins and proline contents were increased in response to Cu toxicity. The fresh weights of leaves and roots were improved during addition of ascorbic acid to root medium in stressed and non-stressed plants. Exogenous ascorbic acid significantly increased membrane stability index in Cu stressed plants. We find considerable reduction in anthocyanins, phenolic compounds and proline with ascorbic acid treatments in all plants. Ascorbic acid application also reduced H_2O_2 content and prevented lipid peroxidation in leaves and roots during presence or absence of excess Cu. Treatment of plants with ascorbic acid increased soluble proteins content only in non-stressed plants. The results demonstrate that root applied ascorbic acid ameliorated the copper induced oxidative adverse effects on onion growth.

Keywords: *Allium cepa* L.; Antioxidant; Ascorbic acid; Copper; Membrane

Introduction

Metals are natural elements and many of them are micronutrients essential for plant growth, however, they become phytotoxic at high concentrations (Hall 2002). Stunted growth, leaf penalty and chlorosis are visible symptoms of strong metal toxicity (Cuypers *et al*. 2000). The toxicity is governed by the type of ion and its concentration, and the stage of plant growth (Thapa *et al*. 2012). Copper is essential for photosynthesis, mitochondrial respiration, carbon and nitrogen metabolism, oxidative stress protection, and for cell wall synthesis (Hansch and Mendel 2009). Copper is a redox - active that is a cofactor or a part of a prosthetic group of some proteins. At high concentrations, the redox properties of Cu lead to detrimental toxic conditions and affect plant growth by direct or indirect interference with numerous biochemical processes (Mourato *et al*. 2009). One of the main mechanisms of Cu toxicity is acceleration of oxidative processes by formation of reactive oxygen species (ROS) (Sgherri *et al*. 2003). Plants have different antioxidant defense systems which consist of enzymes and low molecular weight compounds against metal toxicity (Schutzenduble and Polle 2002). These systems are necessary for controlling excessive ROS production during stress and hence, maintaining the correct levels of ROS for growth and signaling (Mittler *et al*. 2004). During stress conditions, the imbalance between the production of ROS and quenching activity of antioxidants lead to injury of normal cell functions by oxidative damage to proteins,

fatty acids, and DNA. Plants with high antioxidative power have been reported to have greater tolerance to oxidative damages. Ascorbic acid (ASA) is a ubiquitous molecule in eukaryotes (Smirnoff and Wheeler 2000) and in plants as an abundant, soluble and stable part of a highly complex and intricate antioxidative system (Foyer and Noctaor 2011). In addition, metabolism is highly sensitive to the ASA pool size because ASA participates in a variety of physiological processes (Foyer and Noctaor 2011). In barley plants under Cu treatment drastic increase in protein carbonyl groups was accompanied by an increase in total ASA and a decrease in reduced ASA reflecting oxidative stress (Demirevska - Kepova *et al*. 2004). ASA reacts with ROS enzymatically, and also reduce non-enzymatically superoxide, singlet oxygen, and hydroxyl radicals (Smirnoff and Wheeler 2000). The ASA deficient mutants of *Arabidopsis thaliana* do not suffer from severe oxidative sensitive stress (Colville and Smirnoff 2008). Therefore, enough endogenous ASA content is necessary to counteract oxidative stress. Exogenous application of ASA by rooting medium can increase the endogenous content of ASA (Athar *et al*. 2008).

The present study was conducted to assess whether exogenous ASA could change and ameliorate the adverse effects of excessive Cu on some biochemical parameters and the results are discussed in relation to the role of ASA in increasing tolerance of onion plants to copper stress.

Materials and methods

Seeds of onion (*Allium cepa* L. cv. Red Azarshahr) were surface-sterilized in sodium hypochlorite solution 1%, rinsed with sterilized water and germinated on moist filter papers in the dark for one week. Seven-day-old seedlings were transferred to plastic pots containing half-strength Hoagland solution, and were placed at 27 ± 2/20 ± 2 ^{o}C day/night, 16 h photoperiod, 250 μmol $m^{-2}s^{-1}$ photon fluence and relative humidity of 40%. After 5 weeks, at the stage of third leaf opening, seedlings were randomly subdivided into two groups. One group was received half-strength Hoagland solution (control) and other group was subjected to half-strength Hoagland solution containing ASA (0.5 mM) for three days. Three days after treatments with ASA, $CuSO_4.5H_2O$ (0 and 6.5 μM) was added to medium for one week. One day after one week treatments, shoots and roots were weighted for recording fresh weights.

For assay of H_2O_2 content, aliquots of fresh tissues were homogenized in potassium phosphate buffer (50 mM, pH = 6.5) and centrifuged. To supernatant, titanium reagent was added and re-centrifuged. The absorbance of supernatant was measured at 410 nm and calculated using the extinction coefficient 0.28 μM^{-1} cm^{-1} and expressed as μmol/g FW (Jana and Chaudhuri 1981).

For assay of thiobarbituric acid reacting substances (TBARS), aliquots of fresh tissues were homogenized in 20% trichloroacetic acid (TCA) containing 1% thiobarbituric acid and centrifuged. The mixture was incubated in water bath for 20 min at 95 ^{o}C and cooled immediately. The absorbance was measured at 532 nm, corrected for 600 nm and calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Heat and Packer 1968). Data expressed as μmol/g FW.

In assay of membrane stability index (MSI), leaf segments were washed with deionized water and incubated at 25 $^{\circ}$C on a rotary shaker for 24 h,

subsequently electrical conductivity of the solution (EC_1) was determined. Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (EC_2) was obtained after equilibration at 25 °C. Leaf cell MSI was determined according to the following formula (Sairam *et al*. 2003): MSI = [1 - (C1/ C2)] × 100

For assay of soluble protein content, aliquots of fresh tissues were homogenized in Tris – HCl (1 M, pH= 6.8) and centrifuged. Protein content was determined in supernatant using bovine serum albumin (BSA) as a standard at 595 nm (Bradford 1976) and expressed as mg/g FW.

For assay of proline content, aliquots of fresh tissues were homogenized in 3% sulphosalicylic and centrifuged. Free proline content was quantified using ninhydrin reagent and expressed as μmol/g FW (Bates *et al*. 1973).

In assay of phenolic compounds content, aliquots of fresh tissues were homogenized in methanol, kept in room temperature for 15 min and centrifuged. Pellet was washed again with methanol and centrifuged again. Two supernatant were mixed. To volumes of supernatant, distilled water and 15% Na_2CO_3 were added and mixed. After three minutes, Folin - Ciocalteau reagent was added and mixed. The solution heated in a 45 ^oC oven for 15 min, after incubation for 60 min at room temperature and dark, the absorbance at 750 nm was measured and calculated from a standard curve for Gallic acid and expressed as mg/g FW (Singleton *et al*. 1965).

For assay of anthocyanin content, aliquots of fresh leaf tissues was homogenized in acidified methanol (99: 1, methanol: HCl), kept in the dark at room temperature for 24 h and after centrifugation, the absorbance was measured at 550 nm. Data expressed as absorbance (ABS)/g FW (Wanger 1979).

Statistical analyses

The experiment was arranged in a completely randomized design with four replications. The data were statistically analyzed by one way analysis of variance and the Least Significant Difference (LSD) test was used to compare the means at $p \leq 0.05$. The results are presented as the average estimations (± standard error) for each treatment.

Results

The onion plants under Cu stress showed a significant reduction in fresh weights of shoots and roots, but exogenous application of ASA improved the shoots and roots fresh weights under non-stress and stress conditions (Figure 1).

Accumulation of H_2O_2 in the leaves and roots was significantly increased due to Cu stress. ASA significantly reduced the accumulation of H_2O_2 in the leaves and roots of the control and stressed plants (Figure 2).

In this study, TBARS content was increased by Cu stress in the leaves and roots, and ASA significantly reduced TBARS content in both the control and Cu stressed plants (Figure 3).

The plants exposed to Cu stress showed a statistically significant decrease in MSI of leaves compared with control plants. Exogenous application of ASA through the rooting medium only increased MSI considerably in the stressed plants (Figure 4).

Protein content was affected by Cu stress and there was a considerable increase in soluble protein content in the leaves and roots of stressed plants relative to control. ASA increased soluble

protein content in the leaves and roots of control plants, but unaffected of stressed ones (Figure 5).

The results showed that proline was accumulated in the leaves and roots of Cu stressed onion plants. Application of ASA induced a reduction of proline content in the leaves and roots of control and stressed plants (Figure 6).

In the present study, excess Cu was found to be associated with an increase in phenolic compounds of the leaves and roots. Rooting medium applied ASA reduced phenolic compounds in the leaves and roots of both control and Cu stressed plants (Figure 7).

The results indicated that Cu treatment increased anthocyanin content in the leaves. ASA considerably reduced anthocyanin content in the leaves of both control and Cu stressed plants (Figure 8).

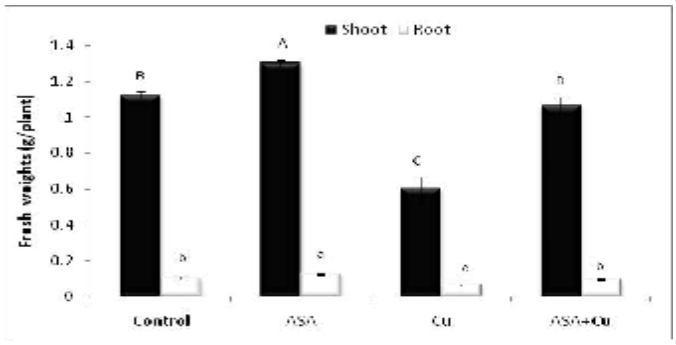

Figure 1. Effects of Cu and ASA on fresh weights of shoots and roots of onion plants. (Results are the means from four replicates ± SE. Means followed by different letters are significantly different at P≤ 0.05.)

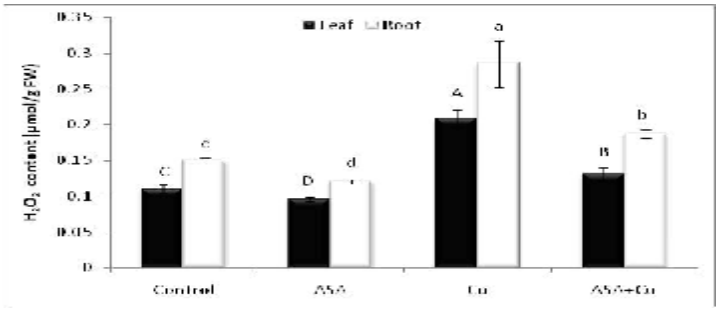

Figure 2. Effects of Cu and ASA on H_2O_2 content in leaves and roots of onion plants. Other details as in Figure 1.

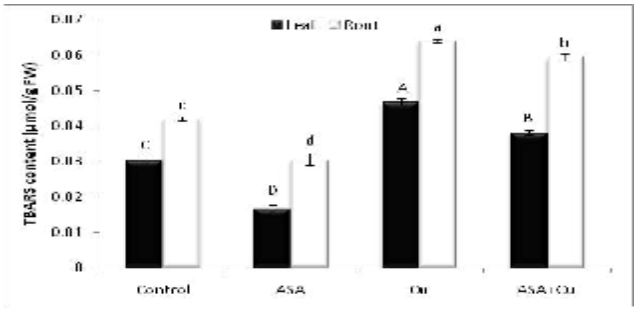

Figure 3. Effects of Cu and ASA on TBARS content in leaves and roots of onion plants. Other details as in Figure 1.

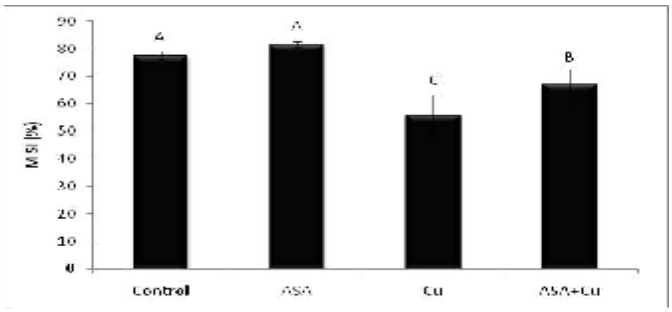

Figure 4. Effects of Cu and ASA on MSI in leaves of onion plants. Other details as in Figure 1.

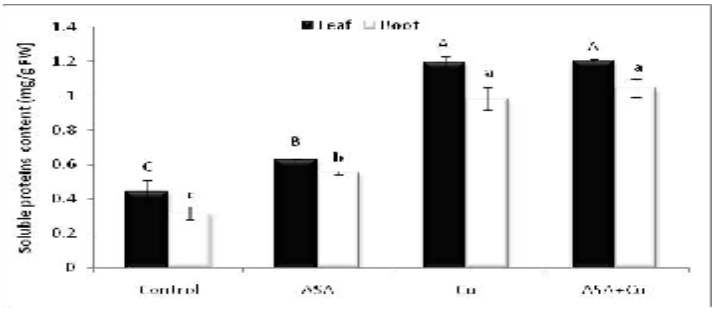

Figure 5. Effects of Cu and ASA on soluble proteins content in leaves and roots of onion plants. Other details as in Figure 1.

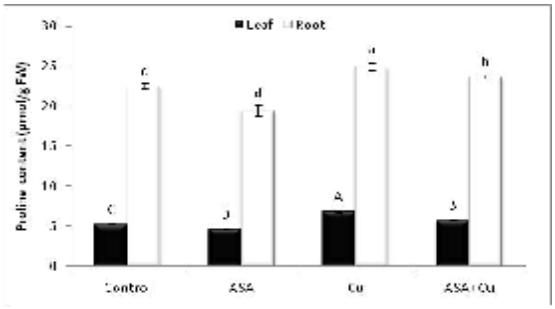

Figure 6. Effects of Cu and ASA on proline content in leaves and roots of onion plants. Other details as in Figure 1.

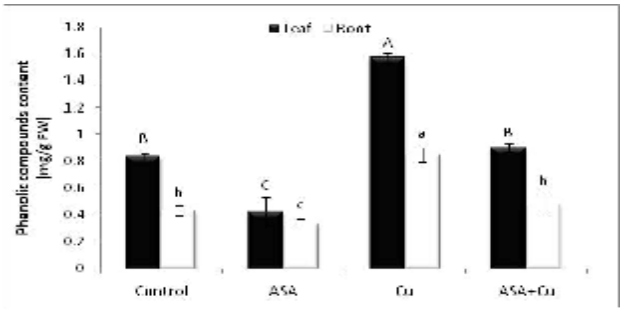

Figure 7. Effects of Cu and ASA on phenolic compounds content in leaves and roots of onion plants. Other details as in Figure 1.

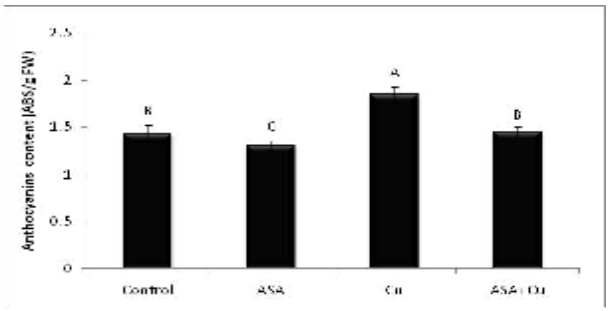

Figure 8. Effects of Cu and ASA on anthocyanins content in leaves of onion plants. Other details as in Figure 1.

Discussion

The growth rate of plants was decreased by Cu stress (Figure 1). The arrest in growth is a general response of plants to Cu and other heavy metals (Mourato *et al*. 2009; Cui *et al*. 2010). The growth inhibition in the presence of heavy metals may be due to some disturbances, such as the cellular water status, mitosis, cell cycle (El-Tayeb *et al*. 2006), hormonal balance (Groppa *et al.* 2007), nitrogen metabolism (Xiong *et al.* 2006) and stiffening of cell walls (El-Tayeb *et al*. 2006). Recently an study indicated that excess Cu inhibition of primary root elongation mediated by auxin redistribution (Yuan *et al*. 2013). The inhibition of growth observed in Cu treated onion plants might partly coincide with the accumulation of free phenolic compounds. An increased level of soluble phenolic acids was observed in carrot plants treated by Cu (Gorecka *et al*. 2007). Exogenous ASA application to Cu treated plants improved growth against the Cu stress (Figure 1). There are some reports which provide evidence that ASA accelerates cell division and enlargement in plants such as wheat (Athar *et al*. 2008) and broad bean (Younis *et al*. 2010). ASA play a role in synthesis of the cell wall hydroxyproline rich extension proteins and detoxification of H_2O_2 (Davey *et al*. 2000). ASA acts as an important cofactor in the biosynthesis of many plant hormones, including ethylene, gibberellic acid and abscisic acid; it also participates in nitrogen fixation (Azzedine *et al*. 2011). ASA induced increase in growth of onion plants under non-stress and Cu-stress conditions may have been due to increase in cell division or cell enlargement, via reduction of H_2O_2 level (Figure 2), by restoring the hormone equilibrium and increase of nitrogen content.

The increase in H_2O_2 due to Cu treatment (Figure 2) is in accordance with previous studies on other plants (Panda 2008; Cui *et al*. 2010). Cu^{2+} ions at toxic concentration inhibit photosynthetic electron transport, promoting the formation of $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ (Yruela 2009). The decrease of free radicals scavengers caused by heavy metals may change balance of free radical metabolism toward H_2O_2 accumulation. Although H_2O_2 accumulation leads to oxidative stress, it has been reported that generation of H_2O_2 during abiotic stress could be as part of signaling cascade leading to protection from stress. In ASA treated plants, the H_2O_2 content showed a

significant reduction (Figure 2). There have been reported that ASA decreases the H_2O_2 content in wheat under salt stress (Azzedine *et al*. 2011) and in mung bean under heat stress (Kumar *et al*. 2011). ASA reacts directly with H_2O_2 and converts that to water via ascorbate peroxidase reaction in ascorbate–glutathione cycle (Foyer and Noctaor 2011).

Figure 3 illustrates large increase in the contents of TBARS during Cu stress. In agreement, MSI showed reduction in the Cu stressed plants (Figure 4). Similar results were reported by Sanchez-Viveros *et al*. (2010) and Tamas *et al*. (2006). The plant plasma membrane may be regarded as the first living structure that is a target for Cu toxicity. Heavy metals induce severe lipid peroxidation leading to the formation of lipid radicals. Lipid molecules in general and unsaturated ones in particular are sensitive to ROS presence under stress conditions. The elevated levels of lipid peroxides are generally accepted as a marker of oxidative stress. The induction of oxidative stress in plants by heavy metals is well documented (Cuypers *et al*. 2000; El-Tayeb *et al*. 2006). Cu as one of the redox active metals which catalyzes the Fenton reaction can accelerate the generation of highly damaging $OH^{\bullet}$ radical from $O_2^{\bullet-}$ and H_2O_2 (Avery 2001). Cu stress leads to increase activity of lipoxygenase, catalyzing lipid peroxidation (Yurekli and Porgali 2006). By a cascade of cyclical reactions, the lipid peroxidation leads to repetitive formation of short chain alkanes and lipid acid aldehydes. The net result of these reactions is changing the composition and fluidity of membrane lipids, oxidation and cross linking of protein thiols, ion leakage and inhibition of key membrane proteins such as the H^+ - ATPase (Hall 2002). The plants treated with ASA showed significant reduction in TBARS content and membrane damage (Figures 3 and 4) in accordance with the observations on onion plants under salinity conditions (Salama 2009). Similar inhibitory effects of exogenous ASA on lipid peroxidation were reported in salt stressed canola (Dolatabadian *et al*. 2008) and water stressed maize (Dolatabadian *et al*. 2009) seedlings. The results obtained by using the transgenic plants and mutants, confirmed the role of ASA in oxidative stress or scavenging free oxy-radicals (Khan *et al*. 2011). ASA acts as a primary substrate in the cyclic pathway for enzymatic detoxification of H_2O_2. In addition it acts directly to neutralize $O_2^{\bullet-}$ radicals, singlet oxygen and as a secondary antioxidant during reductive recycling of the oxidized form of α–tocopherol, another lipophilic membrane associated antioxidant molecule (Shalata and Neumann 2001). Possibly the protective effect of ASA is more related to scavenge of ROS, and its binding to the membrane lipids may stabilize the membrane structure, and so maintains its functions but the actual mechanisms are not yet clear.

Results indicated significant increase in soluble protein content in the tissues with increasing Cu levels in the growth media (Figure 5). The accumulation of proteins in plant organs due to heavy metals is well known (Demirevska-Kepova *et al*. 2004). In addition, mechanism of heavy metals detoxification and tolerance in plants involves the chelating of metals by organic

acids, amino acids or peptides and metalloproteins (Suresh and Ravishankar 2004). Phytochelatins and metalloproteins play an important role in transport, translocation and detoxification of heavy metals (Cobbett and Gldsbrough 2002). Cu chaperones, proteins containing a Cu-binding domain(s), play an essential role in Cu homeostasis in conferring tolerance to excess Cu (Shin *et al*. 2012). Also heavy metal stress can lead to expression of defense genes that encode heat shock protein that have a main role in detoxification of degraded proteins (Hall 2002). The soluble protein content increased with application of ASA (Figure 5). These findings are in agreement with those reported by Dolatabadian *et al*. (2008). Increase in protein content by ASA may be due to de novo synthesis of new proteins (Azooz and Al-Fredan 2009). Bassuony *et al*. (2008) have shown that ASA treatments induced a significant alteration in the enzymes of protein metabolism and that ASA might act as activators of protein synthesis. New proteins may play an inductive role in triggering a special system helping onion plants to tolerate copper stress.

Accumulation of free proline was observed in response to excess Cu (Figure 6). Proline functions as an osmolyte, receptor of nitrogen, radical scavenger (especially $OH^{\bullet}$), electron sink, chelator of metal ions, stabilizer of macromolecules and a cell wall component (Sharma and Dietz 2006). Some investigators have reported the accumulation of proline in response to Cu toxicity (Lombardi and Sebastiani 2005). Chen *et al*. (2001) found excess Cu-induced proline accumulation in detached rice leaves to be related to proteolysis and increased activities of D1-pyrroline-5-carboxylate reductase or ornithine-d-amino-transferase. Proline accumulation may be realized by increased synthesis, release from macromolecules or decreased degradation. Rooting medium applied ASA caused reduction in proline content (Figure 6). In agreement, Alqurainy (2007) reported that application of ASA reduced the accumulation of proline in salt- stressed bean plants. ASA is cofactor of proline hydroxylase and causes the conversion of proline into hydroxyproline (Davey *et al*. 2000); also it has antioxidant property, and thereby ASA application can reduce proline content.

Phenolic compounds and anthocyanin contents in Cu stressed plans tended to increase with applying of Cu in the growth media (Figures 7 and 8). An increase in the level of phenolic compounds in plants treated with excess Cu was reported in earlier studies (Sgherri *et al*. 2003). Phenolics compounds have various functions in plants. An enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions. Phenolics compounds possess hydroxyl and carboxyl groups, able to bind particularly iron and copper; also phenolic compounds especially flavonoids and phenylopropanoids, are oxidized by peroxidase, and can directly scavenge ROS (Michalak 2006). These results concluded that during heavy metal stress phenolic compounds can act as metal chelators due to their high tendency to chelate metals. An increase of phenolic compounds may be due to the increase in activity of enzymes involved in phenolic

compounds metabolism and de novo synthesis of them under heavy metal stress. The induction of foliar anthocyanins has been implicated in the acquisition of tolerance to environmental stresses. Anthocyanins, acting as antioxidant or as osmoregulators in plant cells, are associated with enhanced resistance to the effects of chilling and freezing, heavy metal contamination, desiccation and wounding (Gould 2004). In the present study, ASA treatment resulted in a considerable reduction of phenolic compounds and anthocyanin contents in the onion plants (Figures 7 and 8). This is in agreement with results obtained by Hariri *et al*. (2010). But some investigators have reported the increase of phenolic compounds in presence of ASA (Eid *et al*. 2011; El-Lethy *et al*. 2011). Phenoxyl radicals are toxic to living systems because of their ability to initiate free radical chain reactions in the membrane and their propensity to cross link with a variety of molecules. Phenolic compounds can be regenerated from phenoxyl radicals by non-enzymatic reaction with ASA inhibiting the formation of degraded products (Michalak 2006). Decrease of phenolic compounds (and flavonoids) by ASA may be due to its oxidation by the antioxidant enzymes which use phenols as their substrate and may also due to the decline in its biosynthesis.

In conclusion, the results of present work revealed that root applied exogenous ascorbic acid can remarkably increase the tolerance of onion plants for copper stress and improve growth. The increase in plant tolerance for copper stress was associated with the antioxidant activity of ascorbic acid and a partial inhibition of copper induced increases in membrane lipid peroxidation by active oxygen species. In onion plants levels of H_2O_2, TBARS and proline in roots were higher than those of leaves.

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