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SOME PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO COPPER OF DETACHED CUCUMBER (*CUCUMIS SATIVUS* L.) COTYLEDONS PRE-FLOATED IN SALICYLIC ACID

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Abstract

Salicylic acid (SA) is a growth regulator that promotes growth of plants under stress and non-stress conditions. In the present investigation we studied the role of salicylic acid in copper induced physiological and biochemical changes and the possible induction of oxidative stress in detached cucumber cotyledons. Detached cotyledons of young cucumber seedlings were floated in 150 ppm SA. Then, the responses of these cotyledons to different concentrations (0, 10, 20 ve 50 mM) of copper (CuCl2. H2O) were investigated. In detached cucumber cotyledons exposed to increasing Cu concentrations, the fresh weight accumulation and the photosynthetic pigment content were decreased. Furthermore, the levels of some important parameters regarding oxidative stress in the cotyledons, namely lipid peroxidation (MDA), glutathione (GSH) and proline were increased. In the detached cucumber cotyledons pre-floation process with SA alleviated the negative effect of Cu (20 mM and 50 mM Cu) on growth parameters.

Key words: Cucumber, Cotyledon, SA, Copper, Toxic effect.

Introduction

Many changes arising in the process of growth can be observed in detail through the tissue culture. At present, plant production in closed systems where there are various food solutions has gained scientific acceptance (Jung *et al*., 2001). These food solutions sometimes consist of phenolic compounds, which are secondary plant metabolites. Use of phenolic compounds in tissue culture technique is new and thus, the studies in this field are scarce. Salicylic acid (SA) is an endogenous growth regulator of phenolic nature and is reported to take part in the regulation of the physiological process in plants (Sakhabutdinova *et al*., 2003). Phenylpropanoids are increased or it may be synthesized de-novo in response to adverse environmental conditions, plays an important role in regulation of biochemical, physiological and molecular responses in plant (Singh *et al*., 2007). Phenol derivates are reported to affect enzymatic reactions from beginning to end by either modifying some mineral food and various organic substances in the medium or by influencing kinetin and IAA level (Lee & Skoog, 1965). Recently, it was demonstrated that SA significantly reduced the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene in apple discs and pear cell suspension cultures (Romani *et al*., 1989). However, it is not known whether SA inhibits the synthesis of ACC and/or conjugation of ACC. While low concentrations of cytokinin, gibberellin, auxin and brassinosteroid accelerate growth (Jang *et al*., 2000) ethylene, abscisic acid and jasmonic acid retard it (Wilson, 2007). Li *et al*. (1992) established that SA inhibited the activity of ACC synthase enzyme, preventing the formation of ethylene and chlorophyll loss. In this research, we found an increase in the chlorophyll content compared to the control assosiated with treatment of decreasing SA concentrations. When SA is applied into plants separately, depending on the concentration, it creates stimulative and inhibitive effects on physiological responses (Fariduddin *et al*., 2003). Kumar *et al*. (2010) reported that salicylic acid increased contents of chlorophyll and total nitrogen in isolated cucumber cotyledons. However, higher salicylic acid concentrations inhibited above physiological characteristics. Ananiev *et al*. (2004) reported increases in chorophyll biosynthesis in excised cotyledons of *Cucurbita pepo* L. in response to growth regulator. These results support that SA affects physiological process related to growth and development in cucumber plants. Further increased of chlorophyll synthesis, seedling development and dry mass due to reduced concentrations of SA while higher concentrations were observed to be inhibitory. Sakhabutdinova *et al*. (2003) established that treatment of wheat plants with 0.05 M SA increased the level of cell division within the apical meristem of seedlings roots which caused an increase in plant growth. Salicylic acid is used for regulation of oxidative stress in plants subjected to unfavorable environmental conditions. They suggested that salicylic acid (SA) significantly reduced the Chl a/b ratio and the level of lipid peroxidation in Cu-stressed plants. The same study revealed that, exogenous application of SA appeared developed protective reactions to the photosynthetic pigments and a reduction in membrane damage in sunflower (El-Tayeb *et al*., 2006). The protective role of salicylic acid in attenuating heavy metal toxicity has been reported in several plants like rice, pea, maize and sunflower (Guo *et al*., 2009; Popova *et al*., 2009; Krantev *et al*., 2008; Sibgha *et al*., 2009). Copper (Cu) is an essential micronutrient for plants. Copper loading of agricultural soils may originate from the application of sewage sludge or fungicidal sprays (El-Tayeb *et al*., 2006). Higher plants take up copper from the soil solution mainly as Cu^{2+}. For most crop species, the critical level for copper toxicity in leaves is above 20–30 µg g^{-1} dry weight (Robson and Reuter, 1981). Copper at high concentrations can be a stress factor triggering physiological responses (Yruela, 2005). Copper ions are responsible for many alterations of the plant cell. They negatively affect nitrogen and protein metabolism, causing a reduction of chlorophyll contents and inhibit some photosynthetic functions in

leaves (Fernandes & Henriques, 1991; Foy *et al*., 1978; El-Jaoual & Cox, 1998; Kevresan *et al*., 2001). The decline in chlorophyll content in plants exposed to heavy metals stress such as Cu is believed to be due to: (a) inhibition of enzymes associated with chlorophyll biosynthesis (John *et al*., 2009); (b) inhibition of uptake and transportation of other metal elements such as Mn, Zn and Fe by antagonistic effects (Jayakumar *et al*., 2009; John *et al*., 2009). Cu is extremely toxic and can catalyze the formation of active oxygen species in the cell in haber-Weiss reaction (Kurepa *et al*., 1997). One of the major consequences of Cu toxicity is oxidative stress mediated by increased levels of reactive oxygen species (ROS). It is also known to damage cell membranes by binding to sulphydryl groups of membrane proteins and inducing lipid peroxidation (MDA) (De Vos *et al*., 1992; Liu *et al*., 2004). Other authours (Chen *et al*., 2001; Saha *et al*., 2012) have reported similar increase in lipid peroxidation in plants exposed to Cu. It has been suggested that Cu induced GSH and proline contents in rice (Thounaojam *et al*., 2012; Chen *et al*., 2001). CuSO4 was effective in inducing proline accumulation in detached rice leaves under both light and dark conditions. CuSO4 treatment resulted in an increase in abscisic acid content in detached rice leaves. The possibility that proline accumulation induced by excess Cu is mediated through abscisic acid is discussed (Chen *et al*., 2001). Proline accumulation in plant tissues has been suggested to result from (a) a decrease in proline degradation; (b) an increase in proline biosynthesis; (c) a decrease in protein synthesis or proline utilization; and (d) a increase in protein hydrolysis (Charest & Phan, 1990). Being a redox metal, Cu can interfere with various physiological processes including lipid peroxidation a toxicity indicator for plants exposed to Cu (Baryla *et al*., 2000). The primary site of Cu toxicity lies at the cell membrane level including the photosynthetic membranes (De Vos *et al*., 1992). The objective of this study was to determine the effect of pre-flotation with SA on detached cucumber cotiledons exposed to copper concentrations based an physiological and biochemical.

Material and Methods

Cotyledons isolated from seedlings and experimental design: Seeds of cucumber (*Cucumis sativus* L. cv Beit Alpha) were sterilized with 0.01% HgCl2 for about 10 min, washed thoroughly with tap water followed by distilled water. Seeds were placed on moist whatman No.1 filter paper in petri dishes (11cm diameter) for germination in darkness at 25± 2ºC for 48 h. Homogeneous germinated seeds were grown, in culture room (where they were kept under 230 lux, having the photoperiod 16/8, temperature 27± 2, 25± 2ºC and relative humidity 60-70%) for 72 h. Seedlings were grown in sand culture using Hoagland's solution. Cotyledons isolated from 5-d-old cucumber seedlings. Available detached cotyledons of uniform size were transferred to a sterile petri dishes contining SA (150 ppm) aqueous solution and were pre- flotated for a period of 15 min. The pH the solution was adjusted to 6.5 with 1 mol/ L NaOH. After this period, detached cotyledons (after being washed) were transferred to a sterile petri dishes (they contained from filter paper soaked with different test solutions of Cu (0, 10, 20 and 50 mM)) and were treatment for a period of 16 h. We used 10 ml of each test solution. As a result of this treatments, cotyledons were harvested for analysis. At the end, in cotyledons fresh weight changes and chlorophyll a+b, carotenoid, MDA, GSH and proline content were determined.

Changes in fresh weight of cotyledons determination and biochemical analysis: At first changes in fresh weight of cotyledons were determined (Çanakcı, 2003). In each analysis about 0.5 g of fresh cotyledons were used. For the purpose of identifying the amount of photosynthetic pigments, fresh cotyledon tissue was obtained and in 50 ml 80% acetone extracted. The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were measured by the spectrophotometric method by using 80% acetone extraction. After that, the absorbance of these extracts was separately read at 645, 663 and 440 nm wavelengths against a acetone blank. Using the absorbance values, chlorophyll (a+b) and carotenoid amounts were estimated by the equations of Witham *et al*. (1971). Proline analysis was carried out using acid ninhydrin method (Bates *et al*., 1973). Accordingly, fresh cotyledon tissue was homogenized in 10 ml 3% sulphosalicylic acid. After all other, their absorbances were read at 520 nm wavelengths in the spectrometer against a blank. The method of Heath & Packer (1968) was used to determine the amount of lipid peroxidation (MDA) in cotiledons. After all other, their absorbances were read at 532 nm wavelengths in the spectrometer against a blank. Additionally, fresh cotyledon tissue extraction was performed according to Ellman (1959) method and Griffith (1980) method was used to determine the amount of GSH in cotyledons. Their absorbances were read at 412 nm wavelengths in the spectrometer against a blank.

Statistical analysis : Three replicates for each treatment were maintained. Results were analyzed using one-way ANOVA (SPSS 15.0 Evaluation Version Production Mode Facility). The difference between treatments were considered significant at $p<0.05$. Duncan test was performed to compare means.

Results

Growth response to Cu toxicity: The data on effect of SA and Cu on growth parameters are presented in Fig. 1A. Exposure to high Cu concentrations resulted in dramatic decreases in fresh weights as compared with the control for detached cotyledons of cucumber (*Cucumis sativus* L.) (0.086 % and 0.070 % at 20 mM Cu, SA+20 mM Cu and 0.253 % and 0.210 % at 50 mM Cu, SA+50 mM Cu respectively) ($p\leq0.05$). Pre-floated with SA before exposure to 50 mM Cu however, showed 19.72 % alleviation in the decreased fresh weight. SA alone caused a very small increase in the fresh weight as compared with the control (about 0.078 %) ($p\leq0.05$). Pre-floated with SA before exposure to Cu alleviated the loss of fresh weight due to Cu supply and increased the content to pigments.

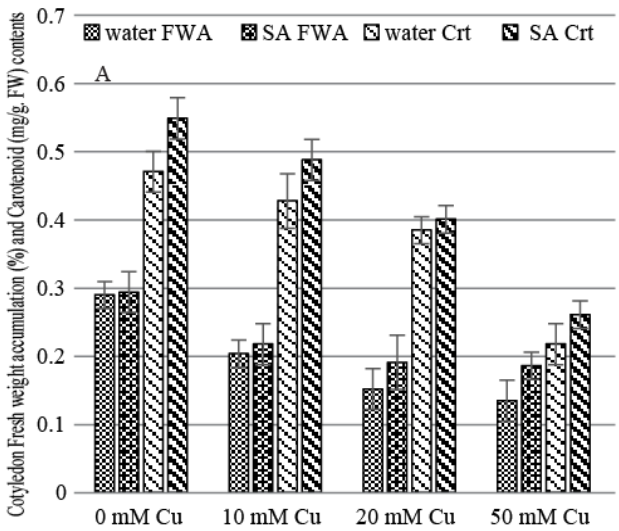

Fig. 1A. Effects of copper (16 h) on the fresh weight accumulation (%) and contents carotenoid in detached cucumber (*Cucumis sativus* L.) cotyledons pre-floated on water (H_2O) or SA (150 ppm) solution for 15 min.

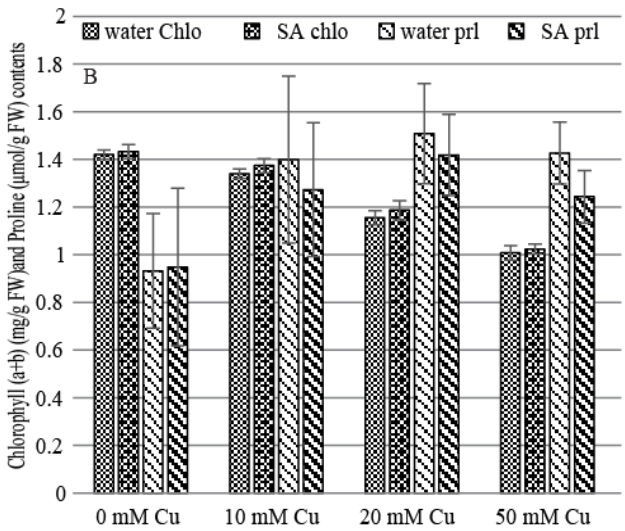

Fig. 1B. Effects of copper (16 h) on the chlorophyll (a+b) and proline contents in detached cucumber (*Cucumis sativus* L.) cotyledons pre-floated on water (H_2O) or SA (150 ppm) solution for 15 min.

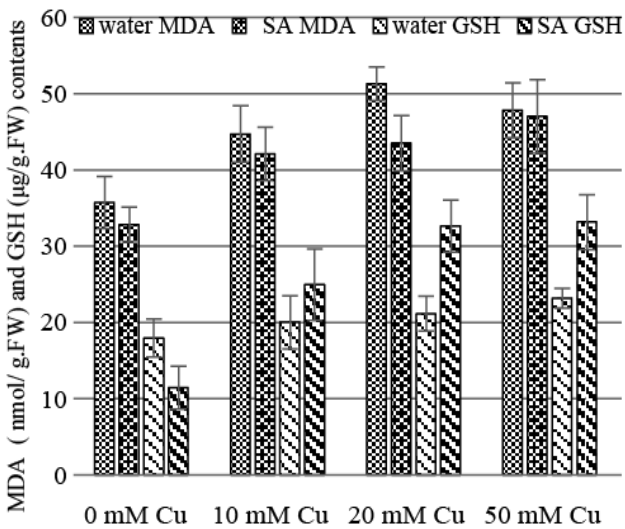

Fig. 2. Effects of copper (16 h) on the MDA and GSH contents in detached cucumber (*Cucumis sativus* L.) cotyledons pre-floated on water (H_2O) or SA (150 ppm) solution for 15 min.

Chlorophyll (a+b) content showed 5.64%, 18.67%, 16.41%, 29.02% and 27.89% reduction in 10 mM Cu, 20 mM Cu, SA+20 mM Cu, 50 mM Cu and SA+50 mM Cu treated cotyledons respectively ($p \leq 0.05$). Carotenoid content showed 29.66%, 24.83%, 45.59%, 34.14%, 53.44% and 35.87% reduction in 10 mM Cu, SA+10 mM Cu, 20 mM Cu, SA+20 mM Cu, 50 mM Cu and SA+50 mM Cu treated cotyledons, respectively. Pre-floated with SA before exposure to 50 mM Cu however, showed 37.77% increase of the carotenoid levels (Fig. 1B) ($p \leq 0.05$).

Effect of SA on proline, GSH and MDA level to Cu toxicity: Concentrations of the stress metabolite proline increased upon Cu exposure. The most prominent effect was at 20 mM Cu, SA+20 mM Cu and 50 mM Cu, SA+50 mM Cu a nearly 61.90%, 52.23% and 53.10%, 44.13% rise a compared with the control. SA pre-floated countracted the Cu-induced increase in proline levels. Pre-floated with SA before exposure to 50 mM Cu however, showed 6.22% alleviation of the proline level ($p \leq 0.05$) (Fig. 1B). The low concentration of Cu (10 mM Cu) caused a slight increase of the proline and the GSH, but the fresh weight was decreased. No major changes were observed in GSH level in Cu-treated cotyledons as compared with the control. SA alone decreased the content of GSH (about 36.16%). GSH level showed 82.19%, 29.35% and 85.04% increase in SA+20 mM Cu, 50 mM Cu and SA+50 mM Cu-treated cotyledons, respectively ($p \leq 0.05$). Pre-floated with SA before exposure to 20 mM Cu and 50 mM Cu however showed 63.08% and 56.56% increase of the GSH level ($p \leq 0.05$) (Fig. 2). Because higher concentrations of Cu are known to induce oxidative stress, we investigated the damage to membranes by monitoring MDA content. Relative to control, Cu-treated cucumber cotyledons exhibited a higher rate of lipid peroxidation. Pre-floated of cotyledons with SA before application of Cu decreased the level of MDA, the effect was more pronounced in higher Cu concentrations. Pre-floated with SA before exposure to 20mM Cu however, showed 15.20 % reduction of the MDA content. Pre-floated with SA alleviated the effect of Cu on the values of this parameter. MDA was also affected by Cu (the increase was approximately at 10 mM Cu, 20 mM Cu, SA+20 mM Cu, 50 mM Cu and SA+50 mM Cu a nearly 25.21%, 43.69%, 21.84%, 33.89% and 1.63% rise a compared with the control) (Fig. 2).

Discussion

Numerous studies exist in literature on different stress factors in detached leaves. However, no reports are available on Cu and SA. Therefore the present study was conducted to analyze the mechanisms of the beneficial effect of SA on cucumber detached cotyledons exposed to toxic concentration of Cu. We demonstrated that Cu produced a concentration-dependant reduction in the growth for detached cotyledons of cucumber (*Cucumis sativus* L. Beit alpha) measured as fresh weight of cotyledons (Yruela, 2005; Foy *et al*., 1978; El-Jaoual &

Cox, 1998; Kevresan *et al*., 2001). The effort of alleviating the inhibitive effect of higher Cu concentration on growth by SA pre-floated was found to be significant. Growth inhibition in plants in response to heavy metal toxicity has been reported earlier in pea seedlings (Popova *et al*., 2009). Similarly, the growth promoting effect of SA against Cd toxicity has been observed in maize, pea, corn-soybean and *Brassica juncea* plants (Krantev *et al*., 2008; Popova *et al*., 2009; Fariduddin *et al*., 2003). The increase in photosynthetic pigment destruction is a typical consequence of metal toxicity in plants (Pätsikkä *et al*., 2002). Mariann *et al*. (2005) suggested that the low-concentration of some stress-inducing heavy metals slowed down the loss of Chl in detached barley leaves. The decrease in the chlorophyll content of plant tissues treated with a metal may be caused by a problem in the synthesis (John *et al*., 2009) or by an increase in the destruction of this pigment (Foy *et al*., 1978; El-Jaoual & Cox, 1998; Kevresan *et al*., 2001). The data showed that the chlorophyll (a+b) and carotenoid content were reduced in Cu treated cotyledons (De Vos *et al*., 1992) Similarly, high SA concentrations were found to spur chlorophyll (a+b) destruction in radish leaf discs and barley leaf segments (Çanakcı and Munzuroğlu, 2008). In our study, treatment with SA alone had no significant effect on chlorophyll (a+b), carotenoid, MDA and proline content of detached cotyledons.

The interplay between antioxidant defense system and reactive oxygen species (ROS) is a key metabolic pathway for plant growth, development and acclimatizationto environmental stressors (Hassan *et al*., 2017). Free radicals increasing with oxidative stress, increase lipid peroxidation in plant cells (Quariti *et al*., 1997). In cucumber detached cotyledons pre-floated with SA before Cu application decreased MDA, the effect being more pronounced in 20 mM Cu-treated cotyledons. In addition, it was demonstrated that SA pretreatment decreased MDA accumulation caused by Cu (Saha *et al*., 2012; Baryla *et al*., 2000). Similar results have been reported earlier in maize, barley, pea and rice plants (Krantev *et al*., 2008; Popova *et al*., 2009; Guo *et al*., 2009). In order to repair the damage initiated by ROS plants evolve complex antioxidant metabolism. In our study, it was determined that pre-floated with SA decrease GSH destruction for 20 mM and 50 mM Cu. In the study conducted on rice it was found that GSH content increased under low Cd concentrations and decreased at higher concentrations, as compared to roots of seedling pretreated with SA (Choudhury & Panda, 2004). GSH content generally increased when compared to the control (Thounaojam *et al*., 2012). This increase in GSH levels is suggestive of SA playing a particular role in glutathione biosynthesis (Freeman *et al*., 2005). Therefore, differences in glutathione amounts can be placed on an enzymatic basis (Yoshida *et al*., 2009). Being able to logically discuss the increase of GSH amounts despite the toxic damage of metal requires studies on all antioxidant enzymes. Due to its membrane transmittance regulating and membrane integrity protecting characteristics in cells (El-Tayeb, 2005), SA may have reduce the toxic damage of Cu in cucumber seedlings. Generation of proline is also one of the vital responses of plant under Cu toxicity which is possibly associated with the protection of plant cells against oxidative damage and with signal transduction (Choudhary *et al*., 2007). SA pre-application increased GSH amount in roots and shoots against the toxic effect of Cu and alleviated oxidative damage due to low level of H_2O_2 and MDA in rice (Guo *et al*., 2009). Proline might protect plants from metal toxicity by chelating heavy metals in the cytoplasm or as a hydroxyl radical scavenger (Smirnoff & Cumbes, 1989). The most prominent effect was observed at 20 mM Cu (a nearly half-fold rise compared to the control) (Chen *et al*., 2001). In detached rice leaves, it has been shown that proline is accumulated by excess copper (Chen *et al*., 2001). Proline has been shown to be accumulated in plants subjected to water stress and exposed to excess Cu (Bassi & Sharma, 1993). Kastori *et al*. (1992), also observed proline accumulation in Cu-exposed leaf discs. Proline accumulation appeared also to be a suitable indicator for toxic metal stress. SA pre-floated counteracted the Cu-induced increase in proline levels (Krantev *et al*., 2008; Popova *et al*., 2009; Guo *et al*., 2009). The proline content is also increased with rising Cu concentrations. This result is in agreement with those of other investigators using different plants as experimental materials (Chen *et al*., 2001; Thounaojam *et al*., 2012; Bassi & Sharma, 1993). The elevation of MDA and proline contents indicates that Cu treated cotyledons are exposed to more stress, relative to their control cotyledons. The beneficial effect of SA was seen with all parameters and was shown to be statistically significant except for chlorophyll (a+b). Also in this experiment, fresh weight of cotyledon, and GSH content increase were stimulated in the presence of SA in control cotyledons. The results of the present study suggest that pre-application of SA during the growth period may help the plant tissues to avoid cumulative damage upon Cu exposure. SA may activate Cu tolerance mechanisms different from Cu distribution and antioxidant defence or would enhance repair process.

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