African Journal of Biotechnology Vol. 11(13), pp. 3173-3178, 14 February, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2364
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

The effect of pre-application of salicylic acid on some physiological and biochemical characteristics of tomato seedling (*Lycopersicon esculentum* L) growing in cadmium containing media

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Accepted 30 January, 2012

In this study, the role of pre-application with salicylic acid on induced physiological and biochemical changes and the possible induction of oxidative stress on tomato grown in a cadmium-containing medium were investigated. This present study examined the effects of salicylic acid pre-application on the responses of twenty-five-day-old tomato (*Lycopersicon esculentum* L.) seedlings to cadmium. For this purpose, the plants were applied 1.5 mM salicylic acid solution for 16 and 24 h and then treated with 0, 10, 25 and 50 µM cadmium solutions for five days. After the treatments, biochemical changes in the seedlings such as malondialdehyde, proline, protein and pigment contents (after 16 and 24 h), seedling and leaf length, seedling fresh weight (after 24 h) were examined. Salicylic acid pre-application was found to significantly differently alleviate the typical harmful effects caused by different cadmium concentrations.

Key words: *Lycopersicon esculentum*, salicylic acid, cadmium, toxicity.

INTRODUCTION

Salicylic acid (SA) as a potent endogenous signal molecule is involved in eliciting specific responses to several biotic and abiotic stresses in plants (Borsani et al., 2001). Several researchers suggested that this substance might be a new plant growth regulator (Hayat and Ahmad, 2007). Pancheva et al. (1996) showed that long-term treatment of barley seedlings with 100 µM and 1 mM SA reduced root and seedlings growth, chlorophyll and protein contents and the rate of CO_2 assimilation. Fariduddin et al. (2003) reported increased chlorophyll (a+b) content, nitrate reductase activity and net photosynthetic rate due to low concentrations of SA, while higher concentrations were observed to be inhibitory to them in *Brassica juncea*. SA is supposed to increase the functional state of the photosynthetic apparatus in plants either by the mobilization of internal tissue nitrate or chlorophyll biosynthesis (Shi et al. 2006).

SA is involved in the modulation of osmotic stress (Singh and Usha, 2003), and salt stress (Gautam and Singh, 2009). It has been shown that SA provides protection in maize (Janda et al., 2008) and winter wheat plants (Tasgin et al., 2003) against low-temperature stress, induces termotolerance in mustard seedlings (Chen et al., 1997; Dat et al., 1998), ozone or UV light in *Arabidopsis thaliana* (Sharma et al., 1996), drought in bean and tomato plants (Senaratna et. al., 2000). Zahra et al. (2010) reported that exogenous SA applications against salinity stress protected tomato seedlings. However, the physiological and biochemical basis for its mechanism of action in abiotic stress tolerance is still not clear. Recently, it has been noted that SA produces favourable effects against oxidative damage resulting from heavy metal pollution. The rapid increase in the amount of organic acid in plants exposed to heavy metal stress suggests that these acids are employed as an element of tolerating oxidative stress by the plant (IIdikó et al., 2005). In higher plants, heavy metal toxicity is generally associated with growth inhibition and reduction

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in biomass production (Woolhouse, 1983). The stress induced by heavy metals leads to a variety of direct and indirect effects on almost all physiological processes in plants. The toxic effects of heavy metals on plant physiology and metabolism are highly complicated and depend on the type of the plant, as well as the nature and concentration of the heavy metal. A correlation was established between heavy metal toxicity and oxidative stress in plant cells.

Cd is one of the most aggressive heavy metals and may induce oxidative stress in plants (Hegedús et al., 2001). Cd is a strong environmental pollutant and it is toxic to plants. It can reach high levels in agricultural soils and is easily assimilated by plants. It is released from metals, industrial processes, phosphate fertilizers and rock mineralization process. The high mobility of this metal in soil-plant system makes its entrance to the food chain easier (Nogawa et al., 1987). Cd directly affects chlorophyll biosynthesis (Gadallah, 1995). In another study, it was found that Cd treatment of tomato plants decreased photosynthetic pigment content, and fresh and dry weight of plant (Lopez-Millan et al., 2009). Cd growth inhibition might be due to the inhibition of cell division and elongation rate of cells that mainly occurs by an irreversible inhibition of proton pump responsible for the process (Liu et al., 2003). Krantev et al. (2008) reported that shoot-root length growth and shoot fresh weight accumulation, as well as the chlorophyll content were reduced in Cd-treated maize plants. Cd^{+2} ions are known to cause alterations in the functionality of membranes by affecting their lipid composition (Quariti et al., 1997). Cd toxicity leads to accumulation of H_2O_2, oxidative damage (for example, lipid peroxidation), membrane leakage, and finally cell death (Shützendübel and Polle, 2002). Functions of plasma membrane are adversely affected by environmental stresses that can be measured as the level of membrane lipid peroxidation. Malondialdehyde (a product of membrane lipid peroxidation) content could reflect the degree of membrane lipid peroxidation. Proline accumulation may also be part of the stress signal influencing adaptive responses (Maggio et al., 2002). The mechanisms of proline action are not fully understood, but it has been suggested that its increased accumulation permits osmotic adjustment as well as providing protection for some of the enzymes (Sharma et al., 1998). Their data showed that the malondialdehyde (MDA) and proline content in Cd-treated maize plants increased with increasing Cd concentration (Krantev et al., 2008). SA has been reported to be directly involved in the signaling of various antioxidant responses (Larkindale and Knight, 2002). Pre-application of salicylic acid increased NR activity, but decreased MDA and proline content in plants grown under saline stress conditions (Gautam and Singh, 2009). SA pretreatment alleviated Cd toxicity in barley (Metwally et al., 2003), rice (Guo et al., 2009), maize (Krantev et al., 2008), pea (Popova et al., 2009), and *Oryza sativa* (Choudhury and Panda, 2004). SA pretreatment alleviated the negative effect of Cd on growth parameters (Guo et al., 2009; Popova et al., 2009), the rate of photosynthesis and chlorophyll content (Krantev et al., 2008). In addition, in SA pre-applied plants, it reduced the increase in both proline (Popova et al., 2009; Krantev et al., 2008) and MDA (Guo et al., 2009; Popova et al., 2009; Krantev et al., 2008; Metwally et al., 2003) depending on Cd-induced stress. This present study aims to examine how SA pre-application affects the severity of toxic stress caused by cadmium surplus in tomato seedlings.

MATERIALS AND METHODS

Plant materials and experimental design

Tomato (*Lycopersicon esculentum* L. cv.sancon) seeds were submerged in distilled water for 4 h, surface sterilized with 0.5 % (m/v) hypochloride sodium for 30 min, rinsed several times with distilled water and then germinated on moist filter paper placed in sterilized Petri dishes. Then three-day-old 5 germinated seeds were planted in pots containing vermiculite. The pots were kept in a growth chamber under controlled conditions which had 23 – 27°C temperatures; the conditions of the photoperiod chamber were 16 h light/ 8 h dark with natural light and relative humidity between 60% and 70%. Then the pots were irrigated with 0.6 L basic nutrient solution (30%). Nutrient solution: $(Ca(NO_3)_2.4H_2O$ 708.45; $MgSO_4.7H_2O$ 492.94; KH_2PO_4 136.09; K_2SO_4 348.50; $(NH_4)_2SO_4$ 396.39; $CaCl_2.2H_2O$ 441.09; H_3BO_3 2.868; $MnSO_4.H_2O$ 1.545; EDTA-Fe 33.0345; $ZnSO_4.7H_2O$ 0.220; $CuSO_4.5H_2O$ 0.080; $Na_2MoO_4.2H_2O$ 0.0299). Nutrient solution was aerated twice a day, and changed three times in a week. 1.5 mM SA concentration was prepared in deionized water. Five different $CdCl_2$ doses (10, 25 and 50 µM) prepared in nutrient solutions were used as test solutions. Twenty-five-day old seedlings were used in the analysis and the seedlings which showed abnormal growth were eliminated. Before SA treatments, roots of the seedlings were washed with deionized water, and the seedlings were divided into eight groups, each consisting of 10 seedlings. The solutions were applied to the roots of seedlings. Four groups were placed into jars containing deionized water and four into jars containing 1.5 mM SA solution. SA pre-application to the seedlings was performed in two different ways for 16 and 24 h. At the end of this period of time, the roots of the seedlings were washed, and the seedlings were transferred into jars containing 0, 10, 25 and 50 µM cadmium solutions. After being kept in cadmium concentrations for 5 days, the seedlings were taken and their seedling and leaf length, seedling fresh weight, malondialdehyde, proline, protein and photosynthetic pigment amounts were measured.

Growth parameters of seedlings and biochemical analyses

Determination of the seedling growth rate

Growth parameters were examined in seedlings at the end of 24-h pre-applications with SA. In order to determine seedling growth rate, the seedling length (cm/plant), leaf length (cm) and fresh weight (g/plant) of the seedlings were measured.

Determination of lipid peroxidation and proline level

Seedlings which were pre-applied with SA for 16 and 24 h were

Table 1. Toxic effects of cadmium on tomato (*Lycopersicon esculentum* L.cv) seedlings which were pre-applied 1.5 mM SA (24 h) and those which were SA-free. Growth parameters: Seedling length, Leaf length and Seedling fresh weight.

Cadmium-5d	Seedling length (cm/plant)		Leaf length (cm)		Seedling fresh weight (g/plant)	
	SA pre-applied 24 h					
	0 mM	1.5 mM	0 mM	1.5 mM	0 mM	1.5 mM
0 µM	26.02 ± 2.06	27.02 ± 2.09	6.90 ± 0.72	6.72 ± 0.77	0.775 ± 0.02	0.793 ± 0.03
10 µM	24.03 ± 1.35	25.50 ± 1.69	6.20 ± 0.97	6.33 ± 0.82	0.718 ± 0.04	0.756 ± 0.03
25 µM	16.70 ± 1.75	21.01 ± 1.84*	4.50 ± 0.53	4.83 ± 0.45	0.612 ± 0.03	0.688 ± 0.03*
50 µM	13.80 ± 1.31	16.80 ± 1.30*	3.81 ± 0.68	4.16 ± 0.38	0.499 ± 0.04	0.596± 0.04*

*Compared to the associated group; $p < 0.05$ probability levels, respectively. Data are means ±SE (n:10).

used in the analyses. Lipid peroxidation was measured using TBARS method (Heath and Packer, 1968). For this purpose, 0.5 g foliar tissue was homogenized in 10 ml 0.1% TCA. The homogenate was centrifuged at 10000 g for 15 min. Then 1 ml of the surface phase was collected and added to 0.5% TBARS prepared in 4 ml 20% TCA and 0.04 ml butylated hydroxyl toluene (4% solution prepared in BHT-ethanol). The mixture was heated at 95°C for 30 min and cooled down in an ice bath. The samples were centrifuged at 10000 g for 15 min. The surface phases were taken and added 3 ml butanol. The mixture was centrifuged again, and the phases were separated. The surface phase was put into a spectra tube and its absorbance was recorded at 532 nm in a blind manner. Then the amount of malondialdehyde in the sample was determined as nmol.g^{-1} FW. Proline analysis was carried out using acid ninhydrin method (Bates et al., 1973). Accordingly, 0.5 g leaf tissue was homogenized in 10 ml 3% sulphosalicylic acid. The homogenized mixture was filtered through Whatman No 2 filter paper. 2 ml of the filtrate was put into a test tube together with 2 ml ninhydrin and 2 ml glacial acetic acid; the mixture was left to react at 100°C for 1 h and then rapidly cooled. Four ml toluene was added on this mixture and vortexed for 15 to 20 s; and the toluene phase was aspirated and taken into spectra tubes. After it cooled down to room temperature, its absorbance was measured at 520 nm in a blind fashion. Then the amount of proline in the sample was determined as µmol. g^{-1} FW.

Determination of protein and chlorophyll amounts

In order to establish the amount of protein, 10 seedlings per group were employed. Protein extraction was performed in accordance with Larson and Beevers (1965). The extracts were put into separate experiment tubes. Total protein amount was measured by Lowry method (1951). The extracts were subjected to the procedures described in this method, and the absorbance of the extracts was measured in a spectrophotometer at 725 nm in a blind fashion. Then, the amount of protein in the sample was determined as mg. g^{-1} FW. For pigment analysis, 1 g leave tissue was extracted in 100 ml 80% acetone and absorbance of these extracts was read against blind at 645 and 663 nm wavelengths. To determine absorbance, quartz tubes with a volume of 1 cm^3 were utilized. Using the absorbance values, chlorophyll a+b amounts were calculated as mg. g^{-1} FW (Witham, et al., 1971).

Statistical analysis

All the experiments were repeated at least three times and the presented data were the mean of three separate experiments. Data were analyzed with SPSS 15 for windows software. Significance of difference was tested with one-way anova/Duncan.

RESULTS

In this present study, we examined the responses of growth parameters of tomato seedlings, which were pre-applied SA for 24 h, before cadmium treatment. It was found that high concentrations (25 and 50 µM) of cadmium treatment for 5 days resulted in 20.51% and 17.85% delays in seedling length growth, and 11.04 and 16.27% decreases in seedling fresh weight increase, respectively when compared to seedlings which were pre-applied SA (at $p < 0.05$) (Table1). However, it did not affect leaf length growth when compared to seedlings which were pre-applied SA (at $p \geq 0.05$) (Table1). In our study, pre-application of SA for 16 h, compared to pre-application of SA for 24 h, did not induce significant differences on growth parameters of seedlings. Because of this, only data of 24 h SA was given for the seedling growth parameters. The amount of MDA, which is an indicator of lipid peroxidation in the leaves of SA-free seedlings treated with 25 and 50 µM cadmium concentrations, was measured to increase by 21.29 and 24.78%, 19.70 and 28.40% respectively in comparison to tomato seedlings pre-applied SA for 16 and 24 h (at $p < 0.05$) (Tables 2 and 3). Similarly, it was found that the amount of proline, which is an osmo-protectant serving to tolerate the stress associated with heavy metal in seedlings, increased in the leaves of SA-free seedlings treated with 10 µM cadmium at rates of 11.84 and 0%, with 25 µM cadmium at rates of 15.38 and 10.02%, with 50 µM cadmium at rates of 13.25 and 9.86%, respectively when compared to seedlings pre-applied SA for 16 and 24 h (at $p < 0.05$) (Tables 2 and 3). On account of amounts of soluble protein, any significant difference could not be found between SA-free and SA pre- applied (at $p \geq 0.05$) seedlings treated with cadmium (Tables 4 and 5). Photosynthetic pigment destruction, a typical outcome of vegetative heavy metal toxicity was analyzed with regard to chlorophyll a+b. The pigment amounts in the leaves of SA-free tomato seedlings treated with 10, 25 and 50 µM cadmium were measured to decrease by 4.14 to 5.94%, by 7.84 to 10.38% and 7.85 to 0%, respectively when compared to seedlings pre-applied SA for 16 and 24 h (at $p < 0.05$) (Tables 4 and 5). SA alone caused a small increase of 6.43 to 3.84% in chlorophyll

Table 2. Effects of cadmium toxicity on the MDA and proline contents in the leaves of tomato (*Lycopersicon esculentum* L. cv) seedlings which were pre-applied 1.5 mM SA (16 h) and those which were SA-free.

Cadmium -5d	Malondialdehide (nmol.g^{-1} FW)		Proline (µmol.g^{-1} FW)	
	SA pre-applied 16 h			
	0 mM	1.5 mM	0 mM	1.5 mM
0 µM	4.03 ± 0.26	4.14 ± 0.27	1.97 ± 0.07	1.82 ± 0.08
10 µM	5.44 ± 0.32	4.96 ± 0.38	2.36 ± 0.08	2.11 ± 0.11*
25 µM	7.52 ± 0.41	6.20 ± 0.29*	3.15 ± 0.12	2.73 ± 0.13*
50 µM	8.81 ± 0.55	7.06 ± 0.63*	3.76 ± 0.09	3.32 ± 0.07*

*Compared to the associated group; $p < 0.05$ probability levels, respectively. Data are means ±SE (n:3).

Table 3. Effects of cadmium toxicity on the MDA and proline contents in the leaves of tomato (*Lycopersicon esculentum* L. cv) seedlings which were pre-applied 1.5 mM SA (24 h) and those which were SA-free.

Cadmium -5d	Malondialdehide (nmol.g^{-1} FW)		Proline (µmol.g^{-1} FW)	
	SA pre-applied 24 h			
	0 mM	1.5 mM	0 mM	1.5 mM
0 µM	3.88 ± 0.26	3.71 ± 0.25	2.04 ± 0.09	1.83 ± 0.13
10 µM	5.21 ± 0.32	4.86 ± 0.43	2.84 ± 0.14	2.58 ± 0.12
25 µM	7.35 ± 0.57	6.14 ± 0.61*	3.73 ± 0.11	3.39 ± 0.09*
50 µM	7.64 ± 0.69	5.95 ± 0.58*	4.01 ± 0.08	3.65 ± 0.06*

*Compared to the associated group; $p < 0.05$ probability levels, respectively. Data are means ±SE (n:3).

Table 4. Effects of cadmium toxicity on chlorophyll a + b contents in leaves and protein content in the tomato (*Lycopersicon esculentum* L. cv) seedlings which were pre-applied 1.5 mM SA (16 h) and those which were SA-free.

Cadmium -5d	Soluble protein (mg.g^{-1} FW)		Chlorophyll a+b (mg.g^{-1} FW)	
	SA pre-applied 16 h			
	0 mM	1.5 mM	0 mM	1.5 mM
0 µM	0.979 ± 0.03	1.080 ± 0.06	1.802 ± 0.03	1.926 ± 0.08
10 µM	0.783 ± 0.04	0.850 ± 0.06	1.710 ± 0.03	1.784 ± 0.04*
25 µM	0.564 ± 0.06	0.691 ± 0.08	1.397 ± 0.05	1.516 ± 0.06*
50 µM	0.530 ± 0.03	0.590 ± 0.03	1.103 ± 0.05	1.197 ± 0.04*

*****Compared to the associated group; $p < 0.05$ probability levels, respectively. Data are means ±SE (n:3).

(a+b) content (16 and 24 h), and of 9.35% in protein content (16 h), respectively when compared to the control seedlings (at $p < 0.05$) (Tables 4 and 5).

DISCUSSION

This present study revealed the regulatory effect of SA on the toxicity induced by cadmium in tomato seedlings. Although there are numerous studies examining the effects of cadmium on tomato seedlings, we have not found any literature study addressing it together with SA. Cadmium is a strong environmental pollutant which causes toxicity in plants. The threshold dose of the toxic effect varies with species, and even with varieties within species. In this present study, it was found that in SA-free seedlings grown in cadmium-containing media, rooting decreased and chlorosis occurred in association with pigment destruction, and also necrotic stains occurred as a result of cell destruction by macroscopic. These results agree with those established by many researchers for different plant species (Guo et al., 2009; Lopez-Millan et al., 2009; Krantev et al., 2006/2008; Popova et al., 2009).

SA pre-application was established to significantly relieve the retarding effect of high cadmium concentrations (Krantev et al., 2008; Popova et al., 2009) had on length growth and fresh weight increases in seedlings (Metwally et al., 2003; Guo et al., 2009). The growth-inhibiting effect of cadmium might result from its inhibiting cell division and cell elongation rate, as is the case with

Table 5. Effects of cadmium toxicity on chlorophyll a+b contents in leaves and protein content in the tomato (*Lycopersicon esculentum* L. cv) seedlings which were pre-applied 1.5 mM SA (24 h) and those which were SA-free.

Cadmium -5d	Soluble protein (mg.g^{-1} FW)		Chlorophyll a+b (mg.g^{-1} FW)	
	SA pre-applied 24 h			
	0 mM	1.5 mM	0 mM	1.5 mM
0 µM	1.047 ± 0.05	1.168 ± 0.08	2.050 ± 0.04	2.132 ± 0.04
10 µM	0.914 ± 0.06	1.055 ± 0.09	1.836 ± 0.05	1.952 ± 0.05*
25 µM	0.758 ± 0.09	0.894 ± 0.07	1.527 ± 0.06	1.704 ± 0.07*
50 µM	0.597 ± 0.03	0.604 ± 0.05	1.201 ± 0.05	1.239 ± 0.04

*Compared to the associated group; $p < 0.05$ probability levels, respectively. Data are means ±SE (n:3).

other heavy metals (Liu et al., 2003,). Heavy metal toxicity in tall plants is generally accompanied by inhibition of growth and decreased biomass production (Woolhouse, 1983). The increase in amounts of MDA (Metwally et al., 2003; Guo et al., 2009; Krantev et al., 2006) and proline (Popova et al., 2009; Krantev et al., 2006) due to oxidative stress caused by high cadmium concentrations in SA-free seedlings is relieved significantly in seedlings which were pre-applied SA. Similar data have been reported for many plant species (Popova et al., 2009; Krantev et al., 2006). A typical outcome of lipid peroxidation in stressed plants is the elevation of malondialdehyde (MDA) quantity. This condition indicates that membrane integrity, and therefore, membrane structure has been impaired. Proline is a metabolite that is very commonly produced in vegetative tissues suffering from stress conditions. The decline in water content in plants is accompanied by a rapid accumulation of free proline. In other words, the increase in proline amount induced by heavy metals in the foliar tissues is associated with water damage. However, physiological significance of the accumulation of this amino acid in plants inflicted by metal stress has not been fully understood. The amount of soluble protein was found low in cadmium-treated SA-free seedlings, when compared to seedlings which were pre-applied SA, but the difference was not significant (Krantev et al., 2008). As we have noted before, the increase in photosynthetic pigment destruction, which is a typical consequence of heavy metal toxicity in plants (Lopez-Millan et al.,2009), was offset by SA pre-application (Krantev et al., 2006/2008; Popova et al., 2009). The elevation of both MDA and proline amounts indicates that these seedlings are exposed to more stress, relative to their control seedlings. SA application was found to relatively alleviate the retarding and reductive effect brought about by cadmium.

In other words, SA pre-application was observed to prevent the photosynthetic pigment destruction in the leaves of seedlings pre-applied SA, relative to SA-free seedlings. In general, the results showed that pre-application of SA for 16 h is more effective against toxic concentrations of cadmium, when compared to pre-application of SA for 24 h.

Conclusion

The results we have obtained in this study suggest that salicylic acid pre-application reduces the toxic effect of cadmium in tomato seedlings, and particularly in their leaves. However, further studies to be conducted with tomato seedlings are needed to gain deeper insights into this topic.

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