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Full Length Research Paper

Response of growth and antioxidant system of heavy metal-contaminated tomato plants to 24-epibrassinolide

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The plants of tomato (*Lycopersicon esculentum* Mill.) were grown in the presence of cadmium or lead (100 to 200 µM Cd^{2+} or Pb^{2+}) and sprayed with 2 µM of 24-epibrassinolide (EBL), and were sampled at 4 weeks after transplanting. The plants exposed to Cd^{2+} or Pb^{2+} exhibited an increase in the activity of antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase) and the level of total ascorbate and reduced glutathione. However, EBL treatment further induced the enzymatic and non-enzymatic system in tomato plants treated with heavy metals. This positive effect was reflected in the improvement of plant growth in the presence of Cd^{2+} or Pb^{2+}. EBL plays an important role in plant responding to heavy metal stress, demonstrating an anti-stress effect on tomato plants contaminated by heavy metals.

Key words: 24-Epibrassinolide, heavy metals, growth, antioxidant system.

INTRODUCTION

Heavy metals are of great concern as soil pollutants because they lead to loss in agricultural productivity and can threaten the health of human beings and animals through the food chain. Several industries and agricultural activities contribute to heavy metal contamination of agricultural lands in peri-urban areas (Rattan et al., 2002). Biochemical adaptations to abiotic stresses among heavy metal stress in plants involve various biochemistry changes of the cell. One of the biochemical changes that occurs when plants are subjected to heavy metals stress is the production of reactive oxygen species (ROS) such as the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). Under normal growth conditions, the production of ROS in cells is very low, while under stress conditions such as drought, salinity, chilling and heavy metals, the production of ROS increases in plant cells. ROS can have a detrimental effect on normal metabolism through oxidative damage to lipids, proteins, and nucleic acids.

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However, many plants that grow in media contaminated with heavy metals have evolved several adaptations. Recent studies have demonstrated that the activities of these antioxidant enzymes and the levels of antioxidant molecules can be increased under various environmental stresses. To control the level of ROS and to protect the cells, plants possess low molecular weight antioxidants (ascorbic acid, glutathione, carotenoids, and tocopherols) and antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) that scavenge ROS. SOD is the major superoxide radical scavenger and its enzymatic action results in H_2O_2 and O_2 formation. The production of SOD activity (H_2O_2) is still toxic and must be eliminated by conversion to H_2O in subsequent reactions. CAT and several classes of peroxidases like APX scavenge the H_2O_2 that produced (Bajguz, 2010). The ascorbate–glutathione cycle seems to be a mechanism of great importance in controlling the cellular redox status, especially after application of heavy metals (Sharma and Dietz, 2009). Ascorbic acid is a primary as well as a secondary antioxidant. As secondary antioxidant, it plays an important role in the regeneration of α-tocopherol. Also, non-protein thiol groups, especially glutathione, exert several important roles in protection of plants from environmental stress factors, especially in the

case of cadmium and lead toxicity (Sharma and Dietz, 2009; Triantaphylidès and Havaux, 2009).

Recently, brassinosteroids (BRs) have shown to be included as one of the plant hormones. Like other plant hormones, BRs were shown to participate at very low concentration to control numerous processes associated with plant growth and development (Friedrichsen and Chory, 2001). BRs encourage plant growth (Hayat et al., 2010; Rady, 2011) via controlling several processes in plants such as urging nucleic acid and protein synthesis (Bajguz, 2000; Khripach et al., 2003), encouraging several enzymatic activities (Hasan et al., 2008; Rady, 2011), photosynthetic efficiency (Hasan et al., 2011), and increase fruit set (Ali et al., 2006). The mechanism by which BRs control these processes has not been completely clear. Heavy metal stress has been shown to be one among several abiotic stresses countered by BRs (Clouse and Sasse, 1998; Hasan et al., 2008, 2011; Bajguz and hayat, 2009; Hayat et al., 2010; Rady, 2011).

Owing to considerable evidence of the adverse effects of heavy metals on plant growth, it was hypothesized that 24-epibrassinolide (EBL) can mitigate the injurious effects of heavy metals (that is, cadmium and lead) on growth and antioxidant system in tomato plants. Thus, the primary objective of this work was to examine whether or not EBL could assuage the heavy metal-induced oxidative stress in tomato by regulating the antioxidant defense system involved in stress tolerance.

MATERIALS AND METHODS

Plant materials and growth conditions

The seeds of tomato (*Lycopersicon esculentum* Mill.) cv. Saria were obtained from the Agricultural Research Center (Department of Vegetable Crops, Giza, Egypt). The healthy seeds were surface sterilized with 5% sodium hypochlorite, followed by repeated washing with deionized water. Seeds were sown in seedling trays filled with a nutrient-rich soil mixture. Three weeks after sowing, seedlings were transplanted into plastic pots (40 cm in diameter, 50 cm in deep) filled with sand previously washed using acid then deionized water. Plants were irrigated with ½-strength Hoagland solution every 3 days throughout the duration of the experiment. Seedlings were grown in a greenhouse with temperatures ranging from 22 to 28℃. The average day and night temperatures were 25 ± 3℃ and 18 ± 2℃, respectively. The relative humidity ranged from 35.4 to 71.2%, and day-length from 11 to 12 h. After our preliminary studies, 2 µM concentration of EBL was found to be the most significant in ameliorating heavy metals toxicity of tomato plants (data not shown). The EBL solution was prepared by dissolving EBL in 1 ml ethanol and bringing to the final volume of 1 L with distilled water. The corresponding control was prepared by adding the same concentration of ethanol as in the EBL solution. In addition, 250 µM concentration of cadmium or lead was found to be lethal to plants, while there was no significant change in all plant growth parameters at concentration 50 µM and below (data not shown). Therefore, 100, 150, and 200 µM concentrations of both cadmium and lead were selected for this study and applied along with the nutrient solution. After irrigation, tomato seedlings were sprayed to run-off with 2 µM EBL or distilled water as a control. Sterile solutions of $CdCl_2$ and $PbCl_2$ were used along with the nutrient solution to obtain the required concentration of cadmium (Cd^{2+}) and lead (Pb^{2+}) (100 to 200 µM). Each treatment consisted of 4 replicates and each experiment was carried out twice at different times. After transplanting, four-week old plants were used for various determinations in this study.

Plant growth analysis

Plants were removed from their pots along with the sand and were dipped in a bucket filled with water. The plants were moved smoothly to remove the adhering sand particles and the lengths of root and shoot were measured by using a meter scale. The plants were then placed in an oven run at 80℃ for 24 h. These dried plants were weighed to record plant dry mass. Leaf area was measured manually by using a graph sheet, where squares covered by the leaf were counted to note the leaf area.

Determination of antioxidant levels

Extraction and determination of total ascorbate from tomato leaves was carried out following the method of Kampfenkel et al. (1995). Plant material (1 g) was harvested by filtration and quickly homogenized in liquid N_2 and thereafter extracted with 5% (w/v) TCA. The homogenate was centrifuged for 5 min at 15,600 ×g (4℃). Then the supernatant was transferred to a new reaction vessel and immediately assayed for the ascorbate content in a reaction mixture containing supernatant, 10 mM DTT, 0.2 M phosphate buffer (pH 7.4), 0.5% NEM, 10% TCA, 42% H_3PO_4, 4% 2,2'-dipyridyl and 3% $FeCl_3$.

Determination of glutathione level was essentially as described (De Kok et al., 1986). Briefly, glutathione was extracted from filtered fresh weight in 2 volumes of extracting buffer (2% sulfosalicylic acid, 1 mM Na_2 ethylenediaminetetraacetic acid (EDTA) and 0.15% ascorbate) and homogenized. The homogenate was centrifuged at 12,000 × *g* for 5 min. An aliquot of supernatant was then used for the measurement of the glutathione content in tomato leaf by glutathione assay kit (Sigma Chemical Co., USA).

Determination of the antioxidant enzymes activities

Enzymatic extracts were obtained from 1 g of fresh weight of tomato leaf. The biomass was filtered and homogenized in liquid N_2. Next, the samples were homogenized with 0.05 M phosphate buffer (pH 7.0) containing 0.1 M EDTA and 1% polyvinylpyrrolidone (PVP) at 4℃. The plant biomass : extraction buffer (w/v) proportion was 1:2. The homogenate was centrifuged for 10 min at 15,000 × *g* (4℃) and the supernatant was dialyzed overnight in phosphate buffer. Estimation of the activity of the selected enzymes was performed as follows. The protein concentration in the homogenate was determined according to Lowry et al. (1951).

Superoxide dismutase (SOD; E.C. 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) and the change in absorbance was measured at 560 nm (Beauchamp and Fridovich, 1971). The reaction mixture consisted of 25 mM phosphate buffer (pH 7.8), 65 µM NBT, 2 µM riboflavin, enzyme extract, and TEMED and the reaction mixture was exposed to light of 350 µmol m^{-2} s^{-1} for 15 min.

Catalase (CAT; EC 1.11.1.6) activity was determined following Aeby (1984) method. The rate of H_2O_2 decomposition at 240 nm was measured spectrophotometrically and calculated using a molar extension coefficient ε = 45.2 mM^{-1} cm^{-1}. The reaction mixture consisted of phosphate buffer, 0.1 mM H_2O_2 and supernatant. One unit of catalase activity was assumed as the amount of enzyme that

Table 1. Effect of EBL on the shoot and root lengths, leaf area plant^{-1} and plant dry mass of tomato plants treated with heavy metals (cadmium or lead) (n=4).

Treatment	Parameter			
	Shoot length (cm)	Root length (cm)	Leaf area plant^{-1} (cm^2)	Plant dry mass (g)
0 (control)	48.4	18.4	169.2	16.8
100 µM Cd	40.7	15.9	140.2	13.8
100 µM Cd + 2 µM 24-EBL	45.0	17.1	157.4	17.8
150 µM Cd	37.3	14.8	130.4	12.0
150 µM Cd + 2 µM 24-EBL	41.2	15.7	144.0	13.9
200 µM Cd	33.8	12.8	118.2	10.2
200 µM Cd + 2 µM 24-EBL	37.2	14.0	130.0	12.0
100 µM Pb	34.1	13.1	119.6	13.0
100 µM Pb + 2 µM 24-EBL	36.8	14.0	128.6	14.9
150 µM Pb	26.4	11.0	92.2	10.8
150 µM Pb + 2 µM 24-EBL	30.9	11.9	108.0	12.9
200 µM Pb	22.9	8.8	80.2	8.0
200 µM Pb + 2 µM 24-EBL	26.8	10.3	92.0	10.3
$LSD_{0.05}$	3.6	1.6	11.6	1.3

decomposed 1 nmol of H_2O_2 per mg of soluble protein per minute at 30℃.

Ascorbate peroxidase (APX; EC 1.11.1.11) was determined according to the method described by Nakano and Asada (1981). The reaction mixture consisted of phosphate buffer, 5 mM sodium ascorbate, 0.1 mM of H_2O_2 and supernatant. Total ascorbate peroxidase activity was determined as the decrease in absorbance of ascorbate at 290 nm and calculated using a molar extension coefficient ε = 2.8 mM^{-1} cm^{-1}. The enzyme activity was calculated as the amount of the enzyme that oxidizes 1 nmol of ascorbate consumed per mg of soluble protein per min at 30℃.

Glutathione reductase (GR; EC 1.6.4.2) was determined according to Jablonski and Anderson (1978). The reaction mixture consisted of 10 mM GSSG, 1 mM Na_2EDTA, 200 mM phosphate buffer, and supernatant was pre-incubated at 25℃ for 5 min. The reaction was initiated by an addition of 1 mM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and the rate of oxidation of NADPH was monitored at 340 nm. The enzyme activity has been expressed as micromole NADPH oxidized mg^{-1} protein min^{-1}.

Statistical analysis

The values for the parameters were subjected to statistical analysis, following the standard procedure described by Gomez and Gomez (1984). The 'F' test was applied to assess the significance of the treatment, at 5% level of probability.

RESULTS

The effects of EBL on the growth (expressed as shoot and root lengths, plant leaf area and plant dry mass) of tomato plants grown under heavy metals stress are presented in Table 1. The presence of Cd^{2+} or Pb^{2+} in the growing medium inhibited the growth of tomato plants. All measured growth parameters decreased proportionally with increasing heavy metal concentration. Heavy metal at the highest dose 200 µM caused significant decrease in all growth parameters. At heavy metal concentrations of 100 to 200 µM, a combination with EBL appeared to have a stronger stimulatory effect on all growth parameters compared with a single heavy metal (which displayed stronger inhibitory effect). The inhibitory effect on growth of tomato plants treated with EBL and both heavy metals was on behalf of lead as compared with cadmium. The data provided indicate that under the influence of EBL combined with heavy metals, an increase in the growth parameters of tomato plants occurs. EBL has an anti-stress effect on tomato plants contaminated by heavy metals (Cd^{2+} or Pb^{2+}).

Table 2 presents the response of various antioxidant enzymes (SOD, CAT, APX, and GR) and non-enzymatic antioxidants (ascorbate and GSH) in tomato plants treated with heavy metals (cadmium or lead) and EBL. Control treatment possessed the minimum value of antioxidant enzymes activities. An increase in activities of heavy metals (Cd^{2+} > Pb^{2+}) was evident. The activities of enzymes demonstrated a positive correlation with an increase in the concentration of heavy metal. In the case of plants treated with heavy metals and 2 µM EBL stimulation of antioxidants activities was also observed, especially at the highest concentration (200 µM) of heavy metals. EBL enhanced the activity of antioxidant enzymes with respect to the plants treated with Cd^{2+} or Pb^{2+} alone. Changes in antioxidant enzymes were accompanied by significant changes in non-enzymatic antioxidants. Increase in the activities of APX and GR in tomato plants is indicative of activation of ascorbate-glutathione cycle in that plant. Total ascorbate and reduced glutathione content increased with increasing supply of heavy metals. EBL also enhanced the content of these compounds in tomato plants treated with Cd^{2+} or Pb^{2+}.

Table 2. Effect of EBL on the activity of enzymatic antioxidants and the contents of non-enzymatic antioxidants in the leaf of tomato plants treated with heavy metals (cadmium or lead) (n=4).

Treatment	Parameter					
	SOD	CAT	APX	GR	Ascorbate	GSH
0 (control)	98.4	27.7	61.2	26.9	401.4	25.8
100 µM Cd	138.5	55.6	110.1	65.0	487.3	38.6
100 µM Cd + 2 µM 24-EBL	164.3	65.7	136.4	74.3	646.9	41.0
150 µM Cd	157.8	84.3	134.9	89.7	589.4	42.7
150 µM Cd + 2 µM 24-EBL	185.2	109.5	156.6	111.4	684.3	53.7
200 µM Cd	165.8	112.9	165.9	123.8	633.9	50.6
200 µM Cd + 2 µM 24-EBL	222.4	136.5	200.0	168.6	714.5	93.1
100 µM Pb	178.1	71.5	141.5	83.5	626.6	49.6
100 µM Pb + 2 µM 24-EBL	231.3	89.5	175.4	95.5	831.8	52.7
150 µM Pb	202.9	108.3	173.4	115.4	757.9	54.9
150 µM Pb + 2 µM 24-EBL	258.2	140.9	201.3	143.2	879.9	69.1
200 µM Pb	213.3	145.2	213.3	159.1	815.2	65.1
200 µM Pb + 2 µM 24-EBL	295.8	175.5	257.2	216.8	918.7	94.7
$LSD_{0.05}$	13.8	9.2	13.2	9.5	56.9	4.4

SOD, superoxide dimutase (nmol NO_2 mg protein^{-1} min^{-1}); CAT, catalase (nmol H_2O_2 mg protein^{-1} min^{-1}); APX, ascorbate peroxidase (nmol ascorbate oxidized mg protein^{-1} min^{-1}); GR, gluthatione reductase (nmol NADPH oxidized mg protein^{-1} min^{-1}); ascorbate (ng ascorbate mg protein^{-1}); GSH, reduced glutathione (nmol GSH mg protein^{-1}).

DISCUSSION

Overproduction of ROS is a common consequence of different stress factors, including heavy metals. To maintain metabolic functions under stress conditions, the balance between generation and degradation of ROS is required, otherwise oxidative injuries may occur. The level of ROS in plant tissues is controlled by an antioxidant system that consists of antioxidant enzymes (SOD, CAT, APX and GR) and non-enzymatic low molecular weight antioxidants (ascorbate and glutathione) (Schutzendubel and Polle, 2002). Therefore, it was expected that the exposure of tomato plants to heavy metal stress (cadmium or lead) could elevate the level of antioxidant enzymes as well as that of ascorbate and glutathione. However, the interesting thing that emerged in the present study was that the treatment of plants with EBL both in absence and presence of stress enhanced the activities of antioxidant enzymes as well as the level of ascorbate and glutathione (Table 2). Cao et al. (2005) demonstrated, based on molecular, physiological and genetic approaches, that the elevation in antioxidant enzymes was the consequence of enhanced expression of de-etiolated-2 (DET2) gene, which enhanced the resistance to oxidative stress in Arabidopsis.

Heavy metals are non-degradable and can accumulate and concentrate as they move up the food chain. The toxic effect of heavy metals on plant growth and development is commonly known, through inhibition of photosynthesis and respiration. Inhibited biosynthesis of chlorophyll and carotenoids, and reduced phosphorylation are most frequently observed symptoms of metal toxicity (Prasad, 2004). Despite these problems, plants are able to grow on sites contaminated with heavy metals as a result of several mechanisms, which reduce the toxicity of metal ions. These include the immobilization of heavy metals within cell wall, reduction of metal uptake at plasmamembranes, sequestration of metals in vacuoles, and complexation of metal ions within cytoplasm by phytochelatins (Bajguz, 2002; Cobbett and Goldsbrough, 2002).

When plants are subjected to heavy metal stress, a variety of ROS are generated. Plant hormones are integrated in the regulation of stress response and plant development (Zhang et al., 2006). It was shown that application of BRs modified antioxidant enzymes such as SOD, CAT, APX and GR, and non-enzymatic antioxidants, such as ascorbic acid, tocopherols, carotenoids, glutathione, etc. in plants under stress conditions. Vitamins C, E, and glutathione react directly or via enzyme catalysis with $OH^{\bullet}$, H_2O_2 or $O^{\bullet}_2$, while carotenes directly operate as effective quenchers of reactive intermediary forms of oxygen (Gapper and Dolan, 2006; Bajguz and Hayat, 2009). The stimulation of ascorbate in response to heavy metals suggests its role in ROS detoxification generated by stress. Ascorbate is known to operate as an antioxidant either in direct chemical interaction with free oxyradicals or during the reaction catalyzed by ascorbate peroxidase (Nakano and Asada, 1981). Moreover, ascorbate oxidation affects the redox balance of other metabolites such as glutathione which being themselves involved in the perception of cellular redox unbalance (Kampfenkel et al., 1995). The

accumulation of ascorbate and glutathione led to enhancement of plant tolerance toward heavy metals stress (Artetxe et al., 2002; Rucin´ ska-Sobkowiak and Pukacki, 2006; Masood et al., 2012). Tomato plants adopted to grow in the present of heavy metals by increment of ascorbate and glutathione levels, together with increased activities of antioxidant enzymes, such as, CAT, APX, and GR. The increased glutathione level has been shown to correlate with the plant adaptation to heavy metal stress and the reduced glutathione level shows marked alterations in response to this stress (Xiang and Oliver, 1998; Jin et al., 2008; Masood et al., 2012). Moreover, glutathione is also a precursor of phytochelatins produced by plants to immobilize toxic heavy metals. On the other hand, CAT participates in the main defense system against accumulation and toxicity of ROS, and plays a key role in controlling H_2O_2 level in plant cells (Singh et al., 2006; Jin et al., 2008).

In the present study, heavy metals' toxicity resulted in reduction in tomato growth, and their inhibitory effects on the growth parameters were ameliorated by the application of EBL (Table 1). EBL improved growth in terms of shoot and root length, leaf area and plant dry mass. EBL at a concentration of 2 µM caused a considerable increase in growth parameters even under stress and restored the growth to the level of unstressed control. It was shown that EBL treatment during aluminium-related stress stimulated growth in *Phaseolus aureus* (Bilkisu et al., 2003). The application of BRs also improved the performance of mustard (Hayat et al., 2007), radish (Anuradha and Rao, 2007), chickpea (Hasan et al., 2008) subjected to cadmium stress and also of mungbean (Ali et al., 2008b) and mustard (Alam et al., 2007) to aluminium and nickel, respectively. Other studies demonstrated that the application of BRs improved the shoot and root length, fresh and dry mass of *Brassica juncea* under copper stress (Sharma and Bhardwaj, 2007; Fariduddin et al., 2009) and enhanced those of *Phaseolus vulgaris* under cadmium stress (Rady, 2011). The present study demonstrates the ability of EBL to counter the toxic effects of cadmium or lead on growth of tomato plants.

BRs enhanced activity of the antioxidant enzymes (CAT, POD, and SOD) and proline content in chickpea (*Cicer arietinum*) and common bean under cadmium stress (Hasan et al., 2008; Rady, 2011). A significant correlation with the degree of improvements was found, which was measured in terms of nodulation, nitrogen fixation, pigment composition, carbonic anhydrase and nitrate reductase activities. A similar pattern of response together with an elevation in the photosynthesis was observed in the plants of mustard exposed to cadmium through nutrient medium (Hayat et al., 2007). Ali et al. (2008a) also reported that EBL enhanced the level of antioxidant system (SOD, CAT, POD, GR, and proline) under both stress and stress-free conditions. The influence of EBL on the antioxidant system was more pronounced under stress situation, suggesting that the elevated level of antioxidant system, at least in part, increased the tolerance of mustard plants to saline and/or nickel stress, and thus protected the photosynthetic machinery and the plant growth.

Conclusion

Heavy metals are important pollutants in the agricultural environment. The growth of tomato plants was inhibited in growing medium treated with heavy metals (cadmium or lead). The present study revealed that the EBL-treated plants grown in the presence of Cd^{2+} or Pb^{2+} had significantly enhanced the level of antioxidative enzymes (SOD, CAT, APX and GR), the level of ascorbate and glutathione, compared with the untreated plants (receiving cadmium or lead alone) as well as with the control. The results obtained in this study clearly indicated the ameliorative influence of EBL on the inhibitory effect of heavy metals phytotoxicity. The increased resistance due to the application of EBL was reflected in the improvement of plant growth in the presence of heavy metals (Cd^{2+} or Pb^{2+}).

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