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CHANGES IN PHYSIOLOGICAL ATTRIBUTES AND BIOCHEMICAL MARKER RESPONSES IN VEGETABLE CROPS GROWN UNDER CADMIUM STRESS : A COMPARATIVE STUDY

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Abstract

Rapid urban and industrial developments have contributed to the elevated levels of heavy metals in the urban environment. It consequently resulted into elevation in the concentration of metal like cadmium (Cd) in soil and vegetable crops. Cadmium is one of the toxic and hazardous metals and it resulted into degradation in the quality of food. So to find out the level of toxicity, in the present study different vegetable crops *viz*. *Amaranthus caudatus, Solanum lycopersicum, Solanum melongena* and *Trigonella foenum-graecum* were grown in Cd (6 mg kg^{-1} soil) contaminated soil up to the seedling stage. In order to show difference in tolerance behaviour and level of toxicity among different vegetable crops against metal stress particularly Cd: growth, responses of oxidative biomarkers, antioxidative enzymes, pigment content and photosynthetic O_2 evolution/ consumption rate were estimated. All the vegetable crops grown under Cd contaminated soil showed decrease in total chlorophyll content, photosynthetic O_2 yield and consequently in the economic yield of plants. As far as tolerance behaviour is concerned the generation of oxidative biomarkers and responses of antioxidative enzymes were observed. It was found that in all the studied vegetable crops Cd enhanced the oxidative stress markers: $O_2^{\bullet ?}$, H_2O_2 and MDA contents. Among all the vegetable, percent increase in reactive oxygen species was found to be higher in *S. melongena* as compared to others. In response to oxidative stress, antioxidants were increased in all the vegetable crops. The activities of antioxidative enzymes: SOD, CAT, POD, APX, and GST were increased in all the vegetables, but it was the maximum in *A. caudatus* as compared to others, which suggest that this plant is more tolerant against metal stress showing higher yield (297 mg $plant^{-1}$) and lesser toxicity in presence of Cd.

Key words : Cadmium (Cd), naphthylethylene diamine dihydrochloride (NEDD), heavy metal, trichloroacetic acid.

Introduction

Heavy metal contamination issues are common in developing countries including India and elsewhere through various activities such as mining, smelters, coal-burning power plants and excessive use of phosphate fertilizer in agriculture (Groppa *et al*., 2012). Heavy metal accumulation in soil is of concern in agricultural production due to its toxicity in vegetable crops and adverse effects on food safety and marketability. Among all the known heavy metals, cadmium (Cd) is a non essential and toxic element to plants, animals as well as human beings. The principle cause of prolonged presence of Cd in the environment is their non-biodegradable nature, which enters and accumulates in human body through food chain, consequently causes various health hazards. Cadmium is toxic to plant cells, even at low concentrations. Leaf concentrations greater than 5–10 μg Cd g^{-1} dry weight is toxic to the most plants (White and Brown, 2010). The toxic effects of Cd on plant metabolism such as inhibition in photosynthesis: light reactions and Calvin cycle, reduction in stomatal density and CO_2 conductance and also alteration in nutritional status of plants (Sun and Shen, 2007; Durand *et al*., 2010); hence, reduction in the growth and crop productivity were noticed (Gill *et al*., 2011). Cadmium at toxic level in tissues leads to excessive production of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) causing cell death due to membrane lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acid (Gill and Tuteja, 2010). In order to persist through harsh stresses initiated by ROS, plants possess a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to metal stress. The most

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important antioxidant enzymes are: superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and glutathione-*S*-transeferase (GST). The enzyme SOD catalyzes the $O_2^{\bullet-}$ to H_2O_2 and oxygen. However, high concentration of H_2O_2 is also toxic to cells and has to be further detoxified by catalase and peroxidases to water and oxygen.

Vegetable considered as important source of essential micro and macro nutrients, vitamins, antioxidants, metabolites and fibres in food items. As vegetables are one of main component of human diet, hence the reduction in food quality and productivity through metal contamination cannot be underestimated (Singh and Prasad, 2014; Bashri and Prasad, 2015). However, little is known about the toxic effect of Cd on the early establishment of vegetables, and further how the growing plants cope with the toxicity through physiological and biochemical adaptations. Thus, the aim of the present work to study level of toxicity and to find out the tolerance mechanism of different vegetables *viz*. *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum* grown under cadmium stress.

Materials and Methods

Experimental set up, cadmium treatment and growth conditions

Seeds of *Amaranthus caudatus*, *Solanum lycopersicum*, *S. melongena* and *Trigonella foenum-graecum* were sterilized with 2% (v/v) sodium hypochlorite solution for 15 min, then thoroughly washed with distilled water and soaked for 4 h. Thereafter, seeds were wrapped in muslin cloth and placed in dark at 25±2ºC for germination. After 24 h the germinated seeds were sown in plastic pots filled with 150 g sandy loam soil. Cadmium (6 mg Cd Kg^{-1} soil) was applied in the form of $CdCl_2$ and mixed with soil before sowing of seeds. The germinated seedlings were grown in plant growth chamber (CDR model GRW-300 DGe, Athens) under photosynthetically active radiation (PAR) of 150 µmol photons m^{-2} s^{-1} with 16:8 h day-night regime and 65±5% relative humidity at 26±1^{0}C. After 30 days of growth, the treated and untreated seedlings were harvested for analyzing the various parameters.

Growth

The growth was determined by measuring the fresh weight (FW) of seedlings of *A. caudatus*, *S. lycopersicum*, *S. melongena* and *T. foenum graecum* at the time of harvesting (30 days after seed sowing) using single pan electronic balance (Contech, CA 223, India).

Estimation of pigment contents

Fresh leaves (20 mg) from treated and untreated seedlings were cut into small pieces and photosynthetic pigments were extracted in 80% (v/v) acetone. The extracts were centrifuged; the absorbance of the supernatant was recorded at 663.2, 646.5 and 470 nm spectrophotometrically (Shimadzu double beam UV–Visible spectrophotometer-1700). The amount of chlorophylls (Chl *a* and *b*) and carotenoids (Car) were calculated according to the equations given by Lichtenthaler (1987).

Estimation of photosynthesis and respiration rates

Photosynthesis and respiration rates were estimated in terms of oxygen evolution/ consumption from leaf discs in presence and absence of light, respectively, using Clark type oxygen electrode (Digital Oxygen System, Model-10, Rank Brothers, UK) by methods of Kurra-Hotta *et al*. (1987). After removing mid veins fresh leaves (50 mg) were sliced into 1 mm wide strips by keeping them in a Petri dish having 0.5 mM $CaSO_4$ solution. The pieces were transferred in 3 ml 50 mM HEPES-NaOH buffer (pH 7.6) containing 20 mM $NaHCO_3$ and finally placed in air tight vessel of oxygen electrode. Photosynthesis in terms of oxygen evolution was estimated under the saturating illumination of 400 µmol photon m^2 s^{-1}, photosynthetic active radiation while the respiratory activity (O_2 consumption) of each sample was determined under darkness for 3 min. The temperature was maintained at 25°C by water jacket around the vessel. Photosynthetic and respiratory rates were expressed as µmol oxygen evolved/ consumed g^{-1} FW h^{-1}.

Determination of reactive oxygen species and index of oxidative damage

Superoxide radical (SOR) was estimated by the method of Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of superoxide radical ($O_2^{\bullet-}$) in the supernatant obtained from leaf homogenate of plants grown in treated and non-treated soils. This assay is based on the formation of NO_2^- from hydroxylamine in the presence of $O_2^{\bullet-}$. Each sample (150 mg) was homogenized in 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 10,000 g for 10 min at 4°C. The reaction mixture consisted of 65 mM potassium phosphate buffer (pH 7.8), 10 mM hydroxylamine hydrochloride and extract (1 ml) was incubated for 20 min at 25°C. After this, 17 mM sulfanilamide and 7 mM naphthylethylene diamine dihydrochloride (NEDD) were mixed to the incubated reaction mixture. After 15 min of incubation, diethyl ether was mixed to the same reaction mixture gently and

centrifuged at 2000 g for 5 min. The absorbance of the colored aqueous phase was recorded at 530 nm. Standard curve prepared with NO_2^- was used for SOR calculation. For the determination of H_2O_2, fresh samples (150 mg) of each set were homogenized in 0.1 percent (w/v) trichloroacetic acid (Velikova *et al*., 2000). The reaction mixture (2 ml) contained tissue extract (500 ml), 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI solution. Absorbance of reaction mixture was read at 390 nm. Hydrogen peroxide concentration was calculated using a standard curve prepared with H_2O_2. Index of oxidative damage to test sample was determined by estimating thiobarbituric acid reactive malondialdehyde (MDA) a product of lipid peroxidation following the method of Heath and Packer (1968).

Measurement of antioxidative enzymes

For the extraction of antioxidant enzyme superoxide dismutase (SOD, EC 1.15.1.1) fresh leaves (100 mg) from each set was homogenized in an ice cold 100 mM EDTA-phosphate buffer (pH 7.8). The homogenate was centrifuged for 20 min at 10,000 g at 4 °C and supernatant was used as the enzyme extract. The activity was determined by the photochemical NBT reduction method as described by Giannopolitis and Reis (1977). The 3 ml reaction mixture contained 1.3 µM riboflavin, 13 mM L-methionine, 0.05 M Na_2CO_3 (pH 10.2), 63 µM p-nitroblue tetrazolium chloride (NBT) and 100 µl enzyme extract. Reaction was carried out for 15 min in similar test tubes at 25°C under an illumination of 75 µmol photons m^{-2} s^{-1}. The initial rate of reaction as measured by the difference in increase in absorbance at 560 nm in the presence and absence of extract was proportional to the amount of enzyme. One unit of SOD activity is defined as the amount of enzyme required to cause 50 per cent inhibition in the reduction of NBT.

Catalase (CAT; EC 1.11.1.6) activity was determined by the method given by Aebi (1984). Leaves (100 mg) of each sample was homogenized with 3 ml 50 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0) and centrifuged at 6000 g for 15 min. The supernatant obtained was used as enzyme. The reaction mixture contained 0.5 ml 60 mM H_2O_2, 1.3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 200 µl enzyme extract. The decrease in absorbance of the solution was recorded at 240 nm for 1 min. The concentration of H_2O_2 in each sample was calculated using the extinction coefficient 39.4 mM^{-1} cm^{-1}. One unit of CAT activity is the amount of enzyme dissociating 1 nmol H_2O_2 min^{-1}.

Peroxidase (POD, EC 1.11.1.7) activity in the leaves of each set was determined according to the method of Zhang (1992). Fresh leaves (100 mg) of each sample was homogenized in 2 ml 50 mM phosphate buffer (pH 6.1). The homogenate was centrifuged at 10,000g and the supernatant was used as enzyme extract. Peroxidase activity was measured with guaiacol as the substrate in a total volume of 3 ml. The reaction mixture (3 ml) consisted of 50 mM potassium phosphate buffer (pH 6.1), 1 percent guaiacol, 0.4 percent H_2O_2 and 200 µl enzyme extract. Increase in the absorbance due to the oxidation of guaiacol (extinction coefficient 25.5 mM^{-1} cm^{-1}) was measured at 470 nm. One unit of POD activity is the amount of enzyme oxidizing 1 nmol guaiacol min^{-1}.

For the extraction of ascorbate peroxidase (APX; EC 1.11.1.11) 100 mg fresh leaves of each sample was homogenized in 2 ml of chilled 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM ascorbic acid and centrifuged at 15,000 g for 20 min at 4°C and supernatant was used as enzyme. Reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and 200 µl enzyme extract. The decrease in absorbance was measured at 290 nm for 1 min. The enzyme activity was calculated by an extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada, 1981). One unit of enzyme activity is defined as 1 nmol ascorbate oxidized min^{-1}.

Glutathione-*S*-transferase (GST, EC 2.5.1.18) activity was determined following the method of Habig *et al*. (1974). Fresh leaves (100 mg) from each sample were homogenized in 100 mM potassium phosphate buffer (pH 6.25) and after centrifugation the supernatant was used as enzyme extract. Enzyme assay was carried out in 2 ml reaction mixture containing 100 mM potassium phosphate buffer (pH 6.25), 0.75 mM CDNB (1-chloro-2, 4-dinitrobenzene), 30 mM GSH (reduced glutathione) and 0.2 ml enzyme extract. The increase in absorbance due to the formation of conjugates between GSH and CDNB was monitored at 340 nm. Enzyme activity was calculated by using an extinction coefficient 9.6 mM^{-1} cm^{-1}. One unit (U) of enzyme activity is defined as 1 nmol of CDNB-conjugates formed min^{-1}.

Statistical analysis

Student 't' test of significance was performed to show the significant difference for the data of yield and photosynthetic pigment contents, photosynthesis and respiration rate of experimental vegetables crops grown under control and Cd treated soil. Results were also statistically analyzed by analysis of variance. Duncan's multiple range test was applied for mean separation for

Table 1 : Effect of cadmium on photosynthetic pigment contents (mg g^{-1} FW), photosynthesis (µmol oxygen evolved g^{-1} FW h^{-1}) and respiration (µmol oxygen consumed g^{-1} FW h^{-1}) rate of *A. caudatus*, *S. lycopersicum*, *S. melongena* and *T. foenum-graecum*.

Parameters	*A. caudatus*		*S. lycopersicum*		*S. melongena*		*T. foenum-graecum*	
	Control	Cd-6 mg Kg^{-1}	Control	Cd-6 mg Kg^{-1}	Control	Cd-6 mg Kg^{-1}	Control	Cd-6 mg Kg^{-1}
Chlorophyll *a*	1.50±0.04	1.43±0.02*	1.55±0.02	1.49±0.02*	1.52±0.03	1.38±0.02**	1.53±0.02	1.44±0.02*
Chlorophyll *b*	0.37±0.01	0.31±0.01*	0.37±0.01	0.31±0.01*	0.40±0.01	0.28±0.01**	0.48±0.01	0.39±0.01*
Carotenoids	0.61±0.01	0.55±0.01*	0.60±0.009	0.54±0.009*	0.39±0.006	0.35±0.005*	0.35±0.006	0.32±0.005*
Photosynthesis	67.67±1.17	57.00±0.98*	45.62±0.79	38.00±0.65*	39.90±0.69	32.70±0.56*	55.25±0.95	46.20±0.80*
Respiration	16.00±0.27	18.00±0.31*	11.21±0.19	12.47±0.21*	10.50±0.18	13.13±0.22*	12.30±0.21	13.73±0.23*

Values with '*' and '**'shows significant differences at $P < 0.05$ and $P < 0.01$ level, respectively between plants grown in control and Cd treated soil according to the student 't' test.

significant differences among treatments at $P<0.05$ significance level (SPSS 16). The results presented are the means ± standard error of three replicates.

Results and Discussion

Growth of vegetable crops

Heavy metal pollution has developed as an ecological problem, which threatens primary and secondary consumers and finally human beings. This problem also influences plants growth. Since seedlings stage in the plant life cycle is a vulnerable stage so, the present study is concerned with the effects of cadmium contamination at seedling stage of *Amaranthus caudatus*, *Solanum lycopersicum*, *Solanum melongena* and *Trigonella foenum-graecum*. In the present study, Cd showed its toxic effect by decreasing the fresh weight of all studied vegetable crops *A. caudatus*, *S. lycopersicum*, *S. melongena* and *T. foenum-graecum* at particular concentration of Cd (6 mg Kg^{-1} soil) (fig. 1). Similar to this Gill *et al.* (2011) have also reported the decrease in growth of *Brassica juncea* under Cd stress. The reduction in growth of all vegetables under Cd stress might be due to (i) decreased pigment content (table 1), (ii) decreased rate of photosynthesis (table 1) and (iii) enhanced generation of ROS (fig. 2). As compared to control, reduction in growth was maximum in *S. melongena* and minimum in *A. caudatus* (fig. 1).

Photosynthetic pigment content and photosynthetic O_2 evolution/consumption rate

Photosynthetic pigment (Chl *a,* Chl *b* and Car) contents and photosynthetic O_2 evolution rate showed significant decline under Cd stress and similar to growth maximum decline was observed in *S. melongena* and minimum in *A. caudatus.* The results were in consistent with earlier report where Cd inhibited the biosynthesis of chlorophyll (Qian *et al*., 2009). Cd can also inhibit the rate of CO_2 fixation by reducing the activity of enzymes of Calvin cycle including Rubisco and also induces alteration in the redox cycling of oxygen-evolving center (Popova *et al*., 2009). As a result of this Cd may declined the rate of photosynthesis (Popova *et al*., 2009), hence decreased the growth of plants as found in present study (fig. 1). Data presented in table 1 shows that the dark respiratory O_2 uptake rate was significantly increased with Cd, which could be correlated with supply of ATP needed to carry on basic metabolism of plants (Prasad and Zeeshan, 2005). Furthermore, Cd is known to induce uncoupling effect, hence mitochondrial respiration (measured as oxygen consumption) might have increased to balance the ATP production (Tiwari *et al.,* 2002; Prasad and Zeeshan, 2005).

Oxidative stress biomarkers and antioxidative response

One of the common outcomes after Cd exposure is the increase in production of reactive oxygen species (ROS), which is potentially harmful for the cell components of all the vegetable crops. A significant increase in the extent of oxidative stress was noticeable in test plants with Cd stress applied (vs. control). The contents of H_2O_2 and SOR displayed significant increase in comparison with the control; the maxima occurred in *S. melongena* seedlings (fig. 2). Exposure to Cd resulted in an accumulation of lipid peroxidation products in leaves of all tested seedlings (fig. 2b). Malonidealdehyde (MDA) content is the most common indictor of oxidative damage, which is product of membrane lipid peroxidation. Among all the vegetables, percent increase in MDA content was found to be higher in *S. melongena*, whereas other species showed lower reduction that may be due to tolerant behavior of these species (fig. 2). The Cd load in

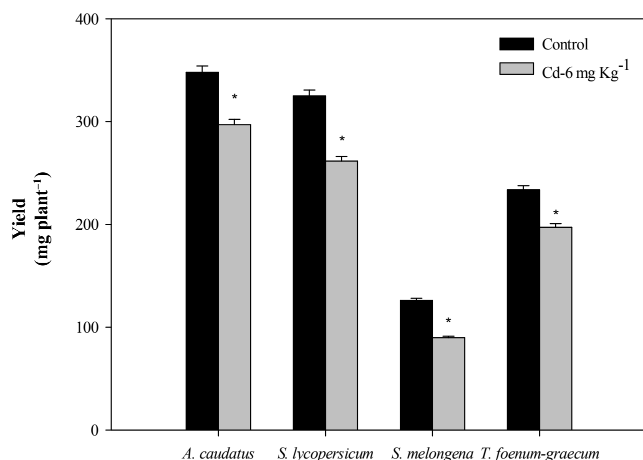

Fig. 1 : Effect of cadmium on yield of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*. Values with '*' shows significant differences at $P < 0.05$ significance level between plants grown in control and Cd treated soil according to the student 't' test.

plant organs can impair important biochemical processes by inducing oxidative stress (fig. 2) due to imbalance between generation and metabolism of ROS in these organs (Anjum *et al*., 2012). Although, Cd is a non-redox active metal, its phytotoxicity as a result of Cd-accrued induction of oxidative stress is extensively reported (Cuypers *et al*., 2012). Thus, a higher oxidative stress (in terms of H_2O_2 and SOR) and its consequences (such as MDA) in *A. caudatus*, *S. lycopersicum*, *S. melongena* and *T. foenum-graecum* are a natural outcome of high Cd burdens.

To evaluate the plant defense against the potential oxidative stress imposed by Cd, the activities of several antioxidative enzymes SOD, CAT, POD, APX, and GST were studied (fig. 3). Reactive oxygen species $O_2^{\bullet-}$ and H_2O_2 have been considered as the central components of signal transduction which triggers the defense genes responsible for antioxidant enzymes, such as SOD (Arrora *et al*., 2002). It is the first line of defense against oxidative stress and our results showed an increase in SOD enzyme activity under Cd toxicity (maximum in *A. caudatus* and minimum in *S. melongena*). This shows that the increase in its activity was probably related to a protective mechanism against oxidative stress. Conversely, the increased enzyme activity contributes to the removal of $O_2^{\bullet-}$ (Gill and Tuteja, 2010). The increase in SOD activity may be the consequence of de novo synthesis of enzymatic proteins or the changes in gene expression (Qadir *et al*., 2004). Enhancement of SOD activity has also been reported in mustard (Ahmad *et al*., 2011) and *B. juncea* (Qadir *et al.,* 2004) under metal stress.

The enzymes usually involved in quenching of H_2O_2

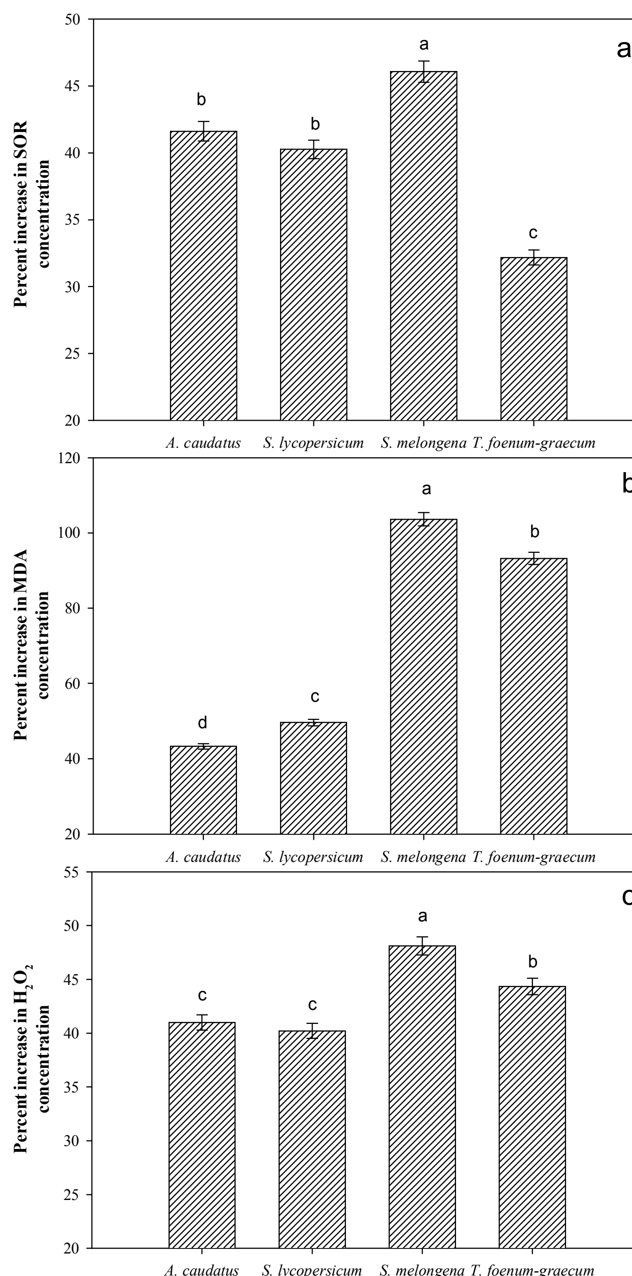

Fig. 2 : Effect of cadmium on percent changes in (a) H_2O_2 (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 266.67±4.13, 494.10±6.28, 191.9±3.65 and 713.70±7.58 nmol g^{-1} FW, respectively), (b) MDA (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 40.13±0.98, 25.28±1.03, 10.30±0.65 and 10.40±0.69 nmol g^{-1} FW, respectively) and (c) SOR (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 195.68±3.65, 73.08±1.33, 238.67±3.85 and 214.50±3.87 nmol g^{-1} FW, respectively) content of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*. Data are means ± standard error of three replicates. Values with different letters show significant differences at $P<0.05$ significance level among treatments according to the Duncan's multiple range test.

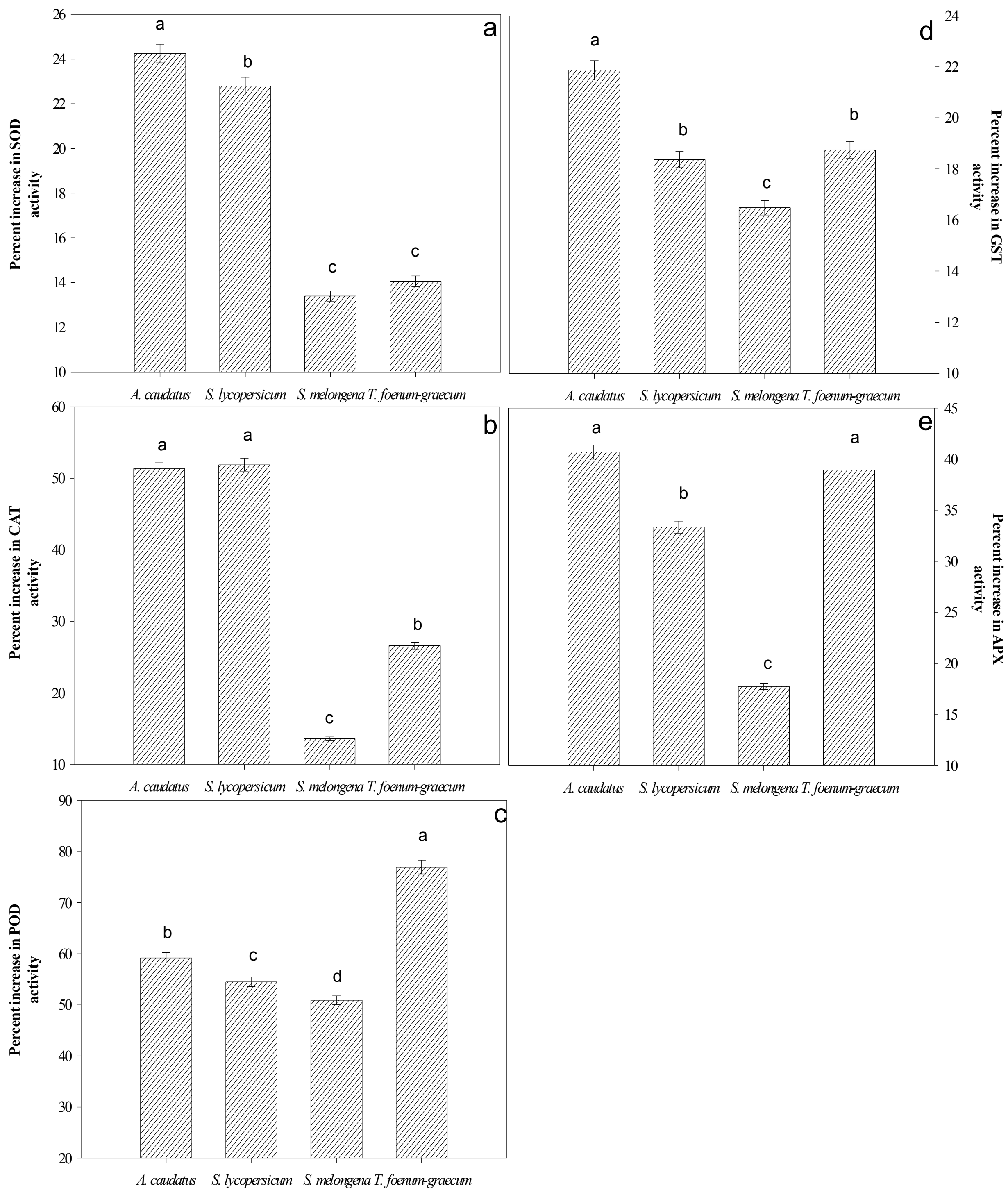

Fig. 3 : Effect of cadmium on percent changes in (a) SOD (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 22.00±0.87, 55.65±1.52, 42.69±0.89 and 29.7±0.78 U g^{-1} FW), (b) CAT (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 98.89±1.55, 535.99±9.54, 1354.73±21.25 and 718.00±12.85 U g^{-1} FW, (c) POD (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 80.39±0.85, 41.53±0.76, 73.00±2.58 and 9662.13±23.75 U g^{-1} FW, (d) APX (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 966.63±14.36, 4714.28±24.63, 3000.00±19.56 and 6325.27±28.45 U g^{-1} FW and (e) GST (control value of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*: 263.00±5.36, 260.41±4.86, 290.16±3.75 and 289.87±3.95 U g^{-1} FW of *A. caudatus, S. lycopersicum, S. melongena* and *T. foenum-graecum*, respectively) activities. Data are means ± standard error of three replicates. Values with different letters show significant differences at $P<0.05$ significance level between treatments according to the Duncan's multiple range test.

are catalase and peroxidase. Our results showed that CAT, POD and APX activity increased with Cd (fig. 3). CAT can directly catalyze the reaction of H_2O_2 to H_2O without any electron donor, and POD needs the help of certain phenolic substrate to complete the process, while APX can convert H_2O_2 to H_2O mainly through the ascorbate/glutathione cycle. CAT activity in all the species was increased with Cd applications compared to the controls. In fact, the role of POD and CAT in plants subjected to excess Cd seems to be highly dependent on plant species (Li *et al.,* 2013). The plants adopted the strategy of accelerating such enzymes as POD and GST for efficiently metabolizing high Cd-mediated H_2O_2 levels and controlling the H_2O_2 accrued consequences like lipid peroxidation. Cd treatment caused a significant increase in GST activity (fig. 3e). GST binds to the heavy metals, so the Cd-induced increase in GST activity in the present study as a result of detoxification response of vegetables. The induction of GST provides additional defense against metal toxicity and keeps the metabolic activities of plants functional.

Conclusion

Cadmium declined the growth of vegetables by declining its pigment contents, rate of photosynthesis and also by enhanced generation of ROS. The defense system in the form of antioxidant plant counteract with ROS under Cd stress. Based upon the result it was concluded that among all the studied vegetables (*Amaranthus caudtus*, *Solanum lycopersicum*, *Solanum melongena* and *Trigonella foenum-graecum*), *Amaranthus caudatus* is found to be the most tolerant crop due to better antioxidant defense system and lesser reduction in growth under Cd stress.

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