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Studies on antioxidative enzymes induced by cadmium in pea plants (*Pisum sativum*)

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Abstract

Pea plants (*Pisum sativum* cv. Swati) exposed to different concentration of cadmium (50, 100, 200 μM Cd) under controlled glass house conditions were quantified for different physiological parameters and antioxidative enzymes. In pea plants, Cd produced a significant inhibition of growth and induced chlorosis, marginal yellowing and necrosis in young leaves, the effect being most pronounced at 200 μM Cd supply. An alteration in the activated oxygen metabolism of pea plants were also detected as evidenced by an increase in concentration of H_2O_2 and TBARS along with decrease in the chlorophyll and carotenoid concentration in leaves. Cadmium toxicity induced an increase in non- protein thiol, ascorbate, proline and cysteine concentration. A significant increment in the activity of SOD, APX and GR, and a decrease in CAT was observed as a result of Cd treatment. The enhanced activity of SOD and inhibition of CAT and POD produces a high build up of H_2O_2 which appears to be the main cause of oxidative stress due to Cd toxicity in pea plants.

Key words

Cadmium, Antioxidative enzymes, Oxidative stress, *Pisum sativum*

Introduction

Among the heavy metals cadmium (Cd) is a highly toxic and persistent environmental contaminant introduced into the soil, through anthropogenic activity, phosphate fertilizers, sewage sludge and atmospheric fallout from industrial and municipal activity (Wagner *et al*., 1993). Although Cd is not essential for plants but because of its mobility in soil and solubility in water it is readily absorbed by plants, where it adversely affects the growth and metabolism and in high concentration it can lead to cell death and destruction of whole plant (Benavides *et al.,* 2005). Cadmium enters into the cells due to its similar chemical and physical characteristics to plant nutrients, using Ca channels or Fe, Mn or Zn transporters and reduces the uptake of iron (Fe), nitrogen (N), phosphorus (P), potassium (K), zinc (Zn), copper (Cu) and sodium (Na) (Wojcik *et al*., 2006). Cadmium is one of the most toxic metals in plants because it is active at concentrations much lower than other heavy metals and it also irreversibly replaces other metal ions in essential metalloenzymes (Jackson *et al*., 1990). Cadmium accumulation in plants causes disturbance in membrane function (Hernandez and Cooke, 1997), enzyme activity (Tamas *et al*., 2006) cell division (Fojtova *et al*., 2002) and cellular redox homeostasis (Romero-Puertas *et al.*, 2004; Ortega-Villasante *et al*., 2005). Cadmium is a non-redox metal but has been found to induce oxidative stress in plant cells (Somashekaraiah *et al*., 1992). In some plants, Cd induced changes in activities of ROS scavenging enzymes including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) have been demonstrated (Dixit *et al*., 2001; Wu *et al*., 2003). The present study aims to study the mechanism of Cd induced oxidative damage and antioxidative enzymes in pea plants at varying concentrations and at different growth stages.

Materials and Methods

Pea plants (*Pisum sativum cv.* Swati*)* were grown under glasshouse conditions in purified silica sand in 5 l polyethylene pots. The nutrient solution containing 4 mM Ca $(NO_3)_2$, 4 mM KNO_3, 2 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 0.33 mM H_3BO_3, 0.1 mM Fe-EDTA, 10 μM $MnSO_4$, 1.0 μM $CuSO_4$, 1.0 μM $ZnSO_4$, 0.1 μM Na_2MoO_4, 0.1 mM NaCl, 0.1 μM $CoSO_4$ and 0.1 μM $NiSO_4$ was supplied for 20 days. Plants were divided into 4 lots. While one lot served as control the other three lots were superimposed with Cd supplied as cadmium chloride ($CdCl_2$) at 50, 100 and 200 μM respectively. The nutrient solution was supplied daily. For each treatment, there were three pots, each containing five plants. A visual record of growth changes and foliar symptoms was maintained after supply of Cd. Six days after treatment (DAT) when leaves of plants displayed initial visual symptoms of toxicity and 12 DAT

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when the symptoms became extremely severe plants were quantified for biomass yield and leaf tissue concentration of Cd along with chlorophyll, carotenoids, hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substances (TBARS), ascorbate (Asc), non-protein thiol, cysteine, proline and activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR).

Plants were separated into leaves, stem and roots and total biomass was determined by oven drying (70°C) the samples. The tissue Cd concentration was analyzed in roots and leaves in wet acid digest ($HClO_4$:HNO_3) by atomic absorption spectrophotometer (Perkin Elmer A Analyst 300). Chlorophyll (a+b) and carotenoids were extracted in 80% acetone and measured spectrophotometrically (Perkin Elmer UV/VIS Lambda Bio 20) by method of Lichtenthaler (1987). Lipid peroxides were measured in terms of thiobarbituric acid reactive substances (TBARS). Fresh leaf material was extracted in 1% trichloroacetic acid (TCA). The supernatant was centrifuged and treated with 0.5% TBA dissolved in TCA. The reaction mixture was incubated in boiling water bath for 30 min and TBARS were measured spectrophotometrically at 532 nm after adjusting for non- specific absorbance at 600nm (Heath and Packer, 1968). Proline was estimated by the ninhydrin method of Bates *et al*. (1973). Cysteine content in leaves was measured following the method of Gaitonde (1967). Ascorbate was extracted in 10% TCA and assayed according to Law *et al*. (1983) by following the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid and measuring the color intensity of the Fe^{2+}- α, α-bipyrridyl complex at 525 nm. Non-protein thiols were estimated by the method of Ellman (1959) in the reaction mixture containing 10 mM dithiobis 2-nitrobenzoic acid (DTNB) and 0.1 mM glutathione reduced (GSH). The colour intensity of extract was measured in a spectrophotometer at 412 nm within 15 min. For assay of SOD, APX and GR fresh leaf tissue was ground in potassium phosphate buffer (50 mM, pH 7.0), containing EDTA (1 mM) and PVP (2%). The extracts were centrifuged at 15000 g for 10 min and the supernatant was assayed for the enzyme activities. SOD was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) (Beuchamp and Fridovich, 1971). For determining APX activity as per the method of Nakano and Asada (1981), the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate and 0.1 mM H_2O_2. The enzyme activity was assayed by following the oxidation of ascorbate at 290 nm. Estimation of GR was carried out by following the oxidation of NADPH and monitoring the decrease in absorbance per min at 340 nm as described by Jablonski and Anderson (1978) in a reaction mixture containing100 mM phosphate buffer (pH 7.0), 1.0 mM oxidised glutathione (GSSG), 1.0 mM EDTA, 0.1 mM NADPH and 25 to 50 µl of the enzyme extract. CAT and non specific peroxidases (POD) were extracted by homogenization of the fresh leaf tissue in ice cold glass distilled water (1:10) in a chilled pestle and mortar. CAT was assayed by a modification of the method of Euler and Josephson (1927) and POD according to Luck (1963). Enzyme activities have been expressed on the basis of total soluble proteins in the enzyme preparations, as determined by the dye binding method of Bradford (1976).

All measurement was made on samples drawn in triplicate and the data were statistically analysed (ANOVA) for significance

Table - 1: Effect of cadmium (Cd) treatment on the leaf area, dry matter yield and tissue Cd in pea (*Pisum sativum* var. Swati) plants

Days after treatment	Plant part	Cd (µM) supply				LSD (P=0.05)
		0	50	100	200	
				Area (cm^2)		
6	Leaves	48.64±2.78	38.22±1.66	25.30±0.97	22.72±1.41	2.452
12		43.93±2.63	30.40±0.57	28.79±1.25	21.39±0.99	4.353
				Dry matter yield (g $plant^{-1}$)		
6	Leaves	0.691±0.028	0.617±0.021	0.556±0.031	0.491±0.023	0.062
	Stem	0.402±0.030	0.351±0.021	0.348±0.028	0.316±0.035	0.034
	Root	0.065±0.011	0.040±0.005	0.036±0.003	0.031±0.002	0.006
	Whole plant	1.158±0.058	1.008±0.062	0.940±0.045	0.838±0.058	0.132
12	Leaves	1.180±0.063	0.088±0.047	0.749±0.051	0.575±0.048	0.092
	Stem	0.643±0.058	0.565±0.050	0.450±0.045	0.391±0.028	0.124
	Root	0.095±0.007	0.078±0.005	0.070±0.006	0.064±0.003	0.003
	Whole plant	1.918±0.071	1.531±0.055	1.269±0.068	1.030±0.035	0.026
				Tissue Cd (µg g^{-1}d.wt.)		
6	Leaves	0.50±0.019	30.16±1.412	51.25±2.59	76.21±4.85	2.34
	Root	3.29±0.091	187.10±5.35	247.75±8.33	460.59±9.23	5.45
12	Leaves	0.62±0.020	14.18±0.089	15.52±0.991	99.30±4.056	2.13
	Root	2.83±0.085	117.88±4.62	160.53±9.76	669.50±12.52	8.96

Values are mean of three replicates + SE

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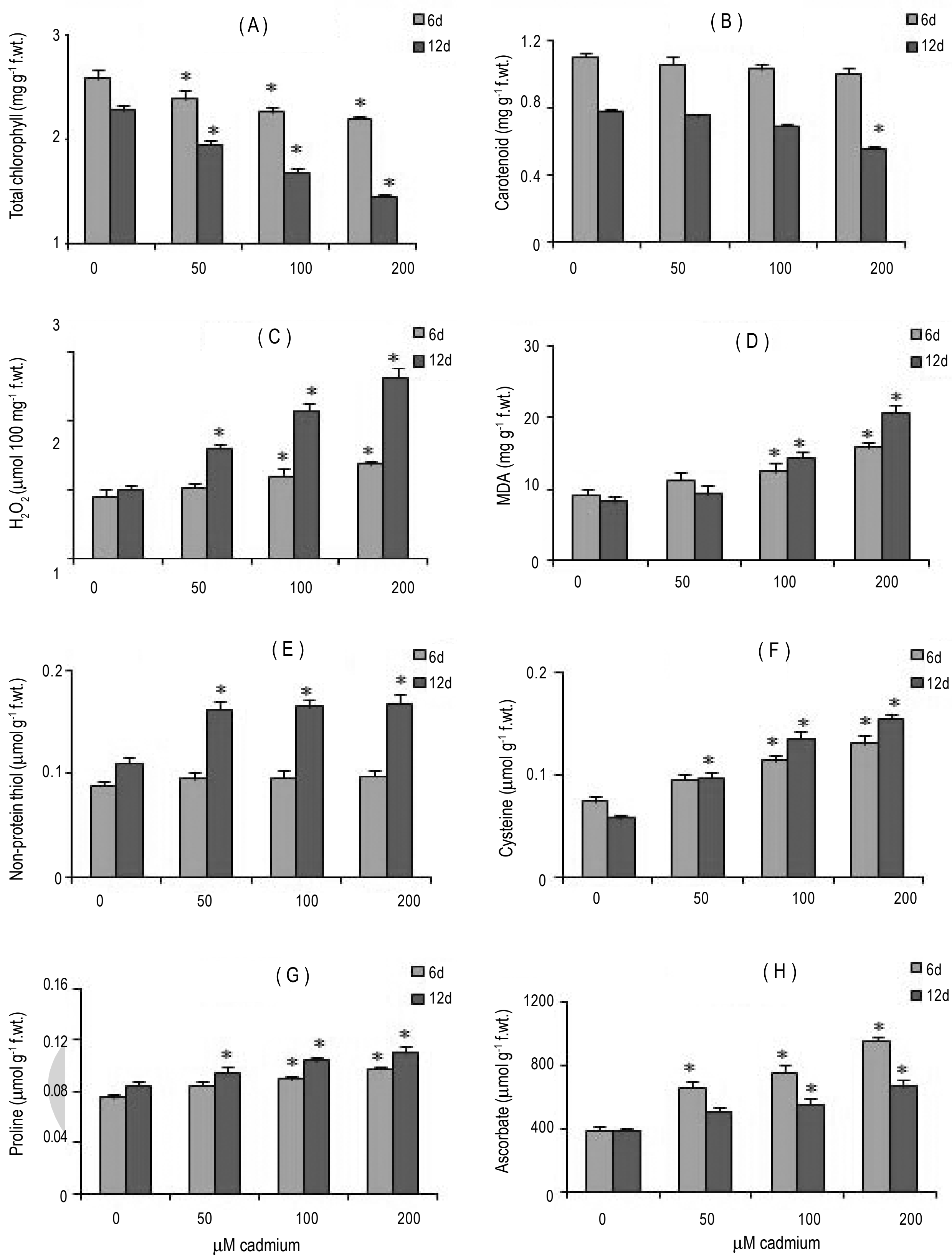

Fig. 1: Effect of cadmium treatment on concentration of (A) Total chlorophyll, (B) carotenoids, (C) hydrogen peroxide (H_2O_2), (D) malondialdehyde (MDA), (E) non-protein thiols, (F) cysteine, (G) proline and (H) ascorbate in the leaves of pea plants; Bars indicates ± S.E. of three independent values; * indicates significant differences compared to control at P=0.05

(LSD at P= 0.05). The data in figures are presented as bar diagram of mean values ± standard error (SE, n=3).

Results and Discussion

Increasing concentration of Cd in the nutrient solution produced a significant reduction in growth and visual symptoms of Cd toxicity. Cadmium toxicity caused reduction in leaf size, which was particularly marked in younger leaves. Plants receiving 50 and 100 µM Cd supply developed chlorosis in leaves after 8 days. Leaves turned chlorotic and their margins turned golden brown and scorched 10 DAT. Interveinal chlorosis followed by necrotic patches appeared in young trifoliate leaves of pea plants supplied with 200 µM Cd, after 5 days. The number and length of lateral roots as compared to the control was markedly reduced in Cd toxic plants. A thickening of lateral roots and browning of root tissues were also observed. The decrease in dry matter production in the roots, stem and leaves increased with increase in Cd concentration and exposure time of the plants to Cd. However, decrease in root dry weight was more significant than leaf dry weight (Table 1). According to Zhang *et al.* (2002) the reason for high root sensitivity to Cd (as also observed in the present study) might be related to the fact that roots are the first organ to be in contact with Cd and the accumulated Cd in root is at much higher amounts than the shoots. As compared to leaves (99.30 µg Cd g^{-1} d. wt.) at 12 DAT roots of pea plants receiving 200 µM Cd showed 669.50 µg Cd g^{-1} d. wt. (Table 1).

Supply of Cd caused a decrease in concentration of chlorophyll a, b and total chlorophyll. Carotenoid concentration was also suppressed due to the Cd (Fig. 1). Carotenoids protect chlorophyll from photooxidative destruction and therefore a reduction in carotenoid could have a serious consequence on chlorophyll pigments. In some plant species, the degradation of chlorophyll or the inhibition of its biosynthesis has been proposed as being responsible for the poor photosynthesis and growth reduction produced by this metal (Somashekaraiah *et al*., 1992). In this study, an increase in concentration of H_2O_2 and enhanced level of lipid peroxidation with increasing Cd concentration were found (Fig.1). In aerobic cells, the hydroxyl radicals are known to be formed from H_2O_2 in presence of transition metal ions (Halliwell and Gutteridge, 1999). The unsaturated fatty acid component of membrane lipids is highly susceptible to these $^{\cdot}OH$ radicals and are peroxidised in its presence. In the present study, increased level of H_2O_2 could account for the formation of $^{\cdot}OH$ radicals leading to lipid peroxidation and accumulation of TBARS. Non-protein thiols are involved in heavy metal detoxification and play a role in gene activation and in the protection from oxidative stress (Noctor and Foyer, 1998). Glutathione is a major non-protein thiol and possesses strong antioxidative properties. Glutathione is substrate for phytochelatin which consists of the three amino acids; cysteine, glycine and glutamic acid. In response to Cd, pea plants showed accumulation of non-protein thiols and cysteine. This is possible since both are involved in heavy metal detoxification as substrate for phytochelatins.

Proline accumulated in leaves of Cd toxic plants and accumulation increased with increase in Cd concentration and duration of supply (12 DAT). The accumulation of free proline in the tissues of several plant species is regarded as a general response to stress (Pesci and Reggiani, 1992). Cadmium ions are known to affect integrity of membranes altering their permeability that can cause water stress like condition resulting in accumulation of proline, as observed by us in the present study (Fig. 1). Ascorbate is a key antioxidant that react with OH radicals, $O_2^{.-}$ and $^1O_2$ (Noctor and Foyer, 1998). Ascorbate reacts directly with the ROS in photosynthetic tissues where it recycles á tocopherol, protects enzymes with prosthetic metal ions, and is utilized as a substrate for ascorbate peroxidase which catalyses H_2O_2 detoxification. The ascorbate concentration increased significantly in the Cd treated pea plants with increasing concentration of Cd at both stages. However, at later stage the level of the antioxidants was reduced indicating enhanced oxidative stress (Fig. 1).

Cadmium toxicity decreased the antioxidative enzyme activity of CAT and POD. The SOD activity showed an enhancement under Cd treatment contradictory to reports by Sandalio *et al*. (2001) who observed a decrease. The SOD, CAT and POD are important antioxidative enzymes that function in the cells to prevent the buildup of ROS (Elstner, 1982; Halliwell and Gutteridge, 1999). Superoxide dismutase is a key enzyme in protecting cells against oxidative stress. This enzyme catalyzes the dismutation of $O_2^{\cdot}$ to H_2O_2 and O_2^{-} which in turn may be detoxified by CAT and POD. An increase in SOD activity as well as the decrease in CAT activity as observed in the present study (Fig. 2) indicates the weakening of H_2O_2 scavenging system due to Cd stress as observed earlier by Pandey *et al*. (2009). The observed decrease in CAT and POD activity may be because of enzyme inhibition, since Cd is known to be a potential enzyme inhibitor (Das *et al*., 1997). Our results support the findings of Schutzendubel *et al*. (2001) who reported a suppression of CAT and POD in pine roots after 48 hr of exposure to 50 µM Cd. Contrasting results such as fluctuation in the activities of these enzymes under Cd stress have also been found (Dixit *et al*., 2001; Zhang *et al*., 2007) accompanied by a weakening of ROS detoxification systems. The APX and GR activity increased with increasing concentration of Cd and the later was more than double over control in leaves of pea plants treated with 200 µM Cd at 12 DAT (Fig. 2). According to Sandalio *et al.* (2001) the GR activity did not show significant change by Cd, while our studies showed an increment in GR activity at both stages. The APX is a main enzyme of glutathione-ascorbate pathway and eliminates peroxides by converting ascorbic acid to dehydroascorbate (Asada, 1992; Foyer and Noctor, 2005). Glutathione reductase activates glutathione-ascorbate cycle and converts GSSG to reduced glutathione. The increase observed in APX and GR activity suggest an induction of the ascorbate-glutathione cycle and this could have a relevant role in protecting cells against Cd toxicity at the early stage of Cd toxicity as also observed by Pandey *et al.* (2009) in spinach. In Cd exposed pea plants, the increase in GR activity at both stages maintained the glutathione turnover. However,

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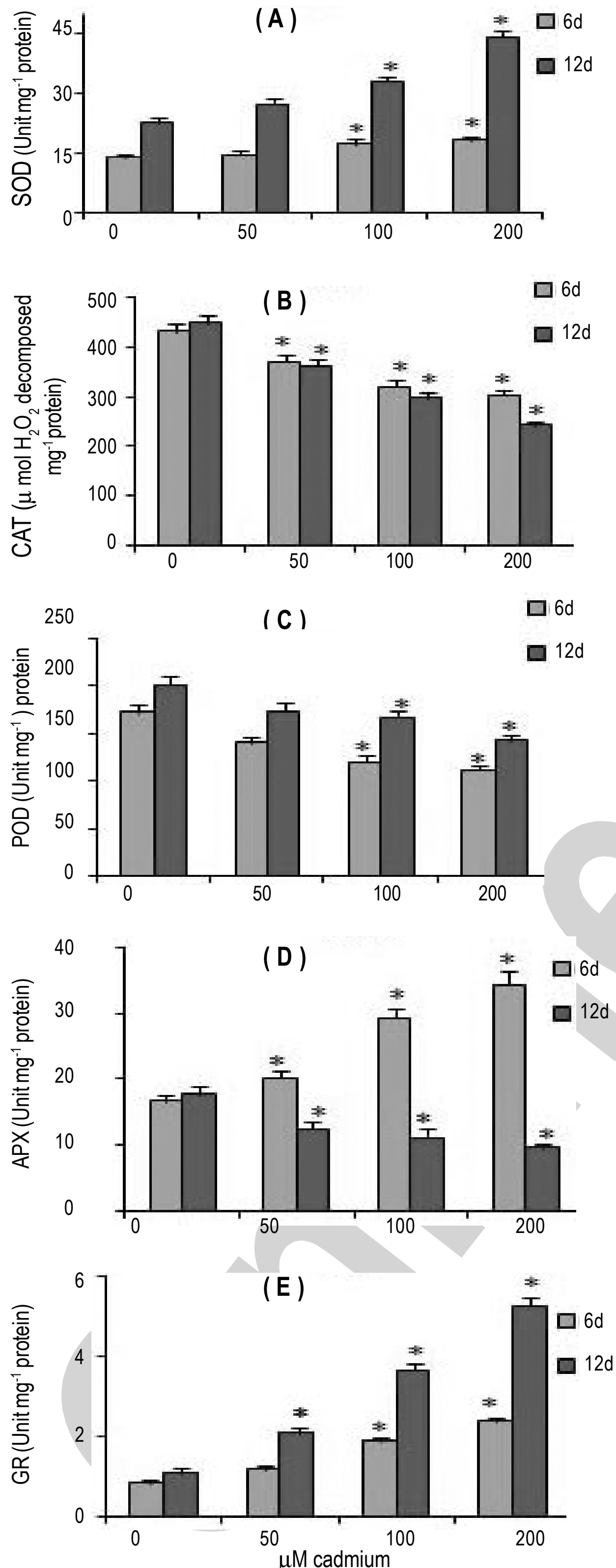

Fig. 2: Effect of cadmium treatment on activity of (A) SOD (superoxide dismutase), (B) CAT (catalase), (C) POD (peroxidase), (D) APX (ascorbate peroxidase) and (E) GR (glutathione reductase) in leaves of pea plants. Bars indicates ± S.E. of three independent values. * indicates significant differences compared to control at P=0.05.

significant decrease in APX at second stage resulted in greater accumulation of H_2O_2 and an inhibitory effect on ascorbate-glutathione cycle.

In conclusion, Cd toxicity causes oxidative damage in pea plants due to accumulation of H_2O_2 as a result of enhanced activity of SOD and low activity of CAT, POD and APX, which are important H_2O_2 scavenging enzymes.

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