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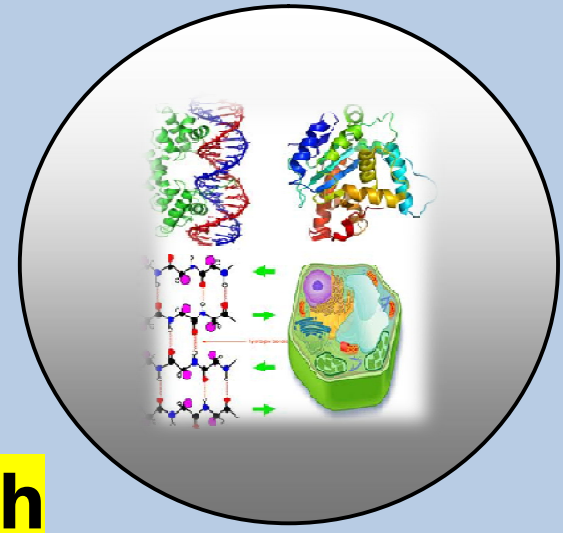

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RESEARCH PAPER

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Effect of Cadmium on Seed Germination, Photosynthesis and Biochemical Aspects of Pea Seedlings

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ABSTRACT

An attempt has been made to assess the response of Pisum sativum L. var. Swati under influence of cadmium chloride ($CdCl_2$) with special reference to growth, morphology and biochemical aspects. Surface sterilized seeds of pea were exposed to various concentrations of $CdCl_2$ ranging (100, 200, 500 µM and control) in petriplates. Results showed that seed germination was highly inhibited by cadmium toxicity. In addition, root and shoot growth showed significant decreases in response to Cd toxicity. Photosynthetic pigments were found to be decreased with increasing cadmium concentration in the medium. The results showed that there was a significant increase in malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and proline contents. Increased activity of peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) were observed in Cd toxic pea plants. This indicated that Cd stress induced an oxidative stress response in pea plants, characterized by an accumulation of MDA, H_2O_2 and increased activities of SOD, POD and CAT. Root and stem Cd concentrations were found to be increased and the highest Cd concentration occurred in roots, followed by the stem.
Keywords: Cadmium, Proline, Lipid Peroxidation and Hydrogen Peroxide.

INTRODUCTION

Cadmium (Cd), a non-essential element, is among the most hazardous environmental pollutants for humans, animals and plants even at low concentrations (Benavides *et al.,* 2005; Mobin and Khan 2007; Wahid and Ghani 2008).

Cadmium with a density of 8.6 g cm-1, is a wide spread heavy metal, released into the environment by power stations, heating systems, metal working industries, waste incinerators, urban traffic, ement factories and a byproduct of phosphate fertilizers (Huang *et al*., 2004; He *et al.,* 2006; Chen and Liu 2006). In areas little affected by human activities, Cd is only released during the weathering of rocks. The largest quantity of Cd enters the soil during the disposal of sewage sludge and waste materials, which contain more Cd than all the other sources put together. Highly polluted soils containing over 100 mg kg^{-1} Cd is reported in China, France and some other countries (Goncalves *et al.,* 2009). The concentrations of Cd in lithuanian soils are not as high, but there exists another problem: in the fields close to the highways, the previous main risk factor lead has now been replaced by cadmium (Antanaitis *et al.,* 2007).

Cadmium can seriously affect plant metabolism in several ways and induce oxidative stress (Vito´ria *et al.,* 2001; Pandey and Singh 2012), although the intensity depends on the species, metal concentration and duration of exposure (Benavides *et al.,* 2005). Cadmium pollution is of increasing scientific interest since Cd is readily taken up by the roots of plants and its toxicity is generally considered to be 2–20 times higher than that of other heavy metals. Previous studies showed that Cd concentration in edible portions of commonly consumed vegetables often exceeded the safe limits of both national and international standards (Sharma *et al.,* 2006, 2007, 2009a; Singh *et al.,* 2010). Since Cd is a fairly immobile element, its accumulation in soils can become dangerous to all kinds of organisms. High to very high Cd concentration have been found to be carcinogenic, mutagenic and teratogenic for a large number of animal species (Degraeve, 1981; Romero-Puertas *et al.,* 2004).

Although Cd is not an essential element for plants, it gets easily absorbed and accumulated in different plant parts. Excess Cd causes a number of toxicity symptoms in plants, sure as stunted growth, chlorosis, and blackening of root system. Plants growing in medium with high level of Cd showed deleterious effect in photosynthetic processes, such as chlorophyll content and photosynthesis (Fatoba and Udoh, 2008, Singh and Pandey 2013), upsets mineral nutrition and water balance, changes hormonal status and affects membrane structure and permeability (Sengar et al., 2008, Haouari *et al.,* 2012).

The permissible limit of Cd for drinking water is 5 mg L^{-1} and the maximum permissible discharge level for the effluents is about 2 mg L^{-1}. There have been a number of cases where ill health has been associated with chronic occupational exposure to dust, aerosols and fumes which contain the metal. A condition of chronic Cd poisoning which occurred in the "Jintsu River Basin" in Japan is known as 'Itai-Itai' disease. Excess Cd in the diet has been found to impair kidney function and hence disturb the metabolism of Ca and P and cause bone disease (Tripathi *et al.,* 1997; Singh *et al.,* 2010).

Some studies have suggested that an oxidative stress could be involved in Cd toxicity by either inducing oxygen free radical production or by decreasing enzymatic and non-enzymatic antioxidants (Somashekaraiah, Padmaja & Prasad 1992; Stohs & Bagchi 1995; Shaw 1995). Reactive oxygen species (ROS) are known to cause the oxidative modification of proteins and the generation of reactive aldehydes as by-products of lipid peroxidation.

Proteins can be affected by ROS either directly by oxidation of amino acid side chains, or by secondary reactions with aldehydic products of lipid peroxidation or glycosylation, giving rise to the production of carbonyl-groups in the protein molecule. In plants Cd toxicity can cause reduction in growth (Kopyra and Gwozdz 2003; Singh and Pandey 2013). At cellular level Cd act as a pro-oxidant and induces oxidative stress by generation of reactive oxygen species (ROS) (Kopyra *et al.,* 2006) as observed by enhance lipid peroxidation, hydrogen peroxide generation (Smeets et al., 2005; Pandey and Singh 2012). It also alters the level of antioxidant enzymes as a defense mechanism against Cd induced ROS production (Rodriguez-Serrano *et al.,* 2006; Singh and Pandey 2013).

Pea is a quick growing, an annual herbaceous vine that requires the trellis to support growth. It flourishes well in well-drained, sandy soil supplemented with adequate moisture and cool weather conditions. Green peas are one of the most nutritious leguminous vegetables, rich in health benefiting phyto-nutrients, minerals, vitamins and anti-oxidants. Peas are relatively low in calories on comparison with beans, and cowpeas. 100 g of green peas provide only 81 calories, and no cholesterol. Peas are starchy, but high in fiber, protein, vitamins, minerals, and lutein. Dry weight is about one-quarter protein and one-quarter suga. Pea seed peptide fractions have less ability to scavenge free radicals than glutathione, but greater ability to chelate metals and inhibit linoleic acid oxidation (Aluko *et al.,* 2010).

The aim of this study is to define Cd effects on the accumulation and distribution of Cd in roots and shoots, and growth and development of plant and chlorophyll amounts of leafs in pea. The present study was undertaken to investigate the effect of Cd on growth, and photosynthesis of pea at seedling stage, in order to clarify physiological effect of Cd stress on pea plants.

MATERIAL AND METHODS

The seeds of pea (*Pisum sativum* L var. Swati) were surface-sterilized with 5% (v/v) mercuric chloride solution and washed properly with deionised manesty still water (MSW) before sowing. The composition of nutrient solution excluding cadmium used was a modification of Hoagland nutrient solution which consisted of 4.0 mM $CaNO_3$, 2.0 mM $MgSO_4$, 4.0 mM KNO_3, 0.4 mM $(NH4)_2SO_4$, 2 µM $MnSO_4$, 0.3 µM $CuSO_4$, 0.8 µM $ZnSO_4$, 30 µM NaCl, 0.1 µM Na_2MoO_4, 1.43 mM KH_2PO_4 and 20 µM Fe-Na-EDTA (Hoagland and Arnon 1950). Cadmium was supplied as cadmium chloride with variable concentrations. Ten lots of petridishes containing 50 seeds each were set up for each treatment and seeds raised without cadmium solution was treated as control. The nutrient solutions were changed every alternate day to maintain the desired level of nutrients.

Chlorophyll (Chl) and carotenoids (Car) in leaves were extracted in 80% acetone by the method of Lichtenthaler (1987).

Lipid peroxidation was measured in terms of malondialdehyde (MDA) formation (Heath and Parker, 1968). Fresh leaves were homogenized with 0.1% trichloroacetic acid and centrifuged at 10,000 xg for 5 min. The supernatant was treated with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA and the mixture was incubated at 95^{o}C in water bath for 30 min.

Samples were cooled immediately in ice bath and centrifuged at 10,000xg for 10 min. The absorbance was read at 532 nm and adjusted for non-specific absorbance at 600 nm. The concentration of MDA was estimated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide (H_2O_2) was estimated by method of Brennan and Frenkel (1977). Finely chopped leaves were ground in chilled pestle mortar in acetone and centrifuged at 10,000 xg. The pellet was discarded. To the supernatant, titanium tetrachloride was added. The precipitate formed was solubilized in chilled liquid ammonia and centrifuged. Residue was washed with acetone to remove chlorophyll. The residue was dissolved in 2N H_2SO_4 and the color intensity was read at 415 nm. The results have been expressed as µmol H_2O_2 g^{-1} fresh weight.

Ascorbate (Asc) was assayed according to the method of Law *et al*. (1983) by extracting fresh leaf tissue in 10% TCA. After adding NaOH the extracts were centrifuged for 5 min in a microfuge and to the supernatant 150 mM phosphate buffer pH 7.4, was added. The color was developed by adding 10% TCA, 44% orthophosphoric acid, 4% bipyridyl in 70% ethanol and 3% ferric chloride. The samples were incubated at 37°C for 40 min and color developed was read at 525 nm. Total ascorbate was determined in the supernatant by reduction of dehydroascorbate (DHA) to Asc by 10 mM dithiothreitol and 0.5% N-ethylmaleimide which was added after 15 min. Amount of ascorbate was determined by preparing a standard curve with L-ascorbic acid (Sigma).

The activities of enzyme catalase (CAT) and peroxidase (POX) were assayed in the fresh leaf tissue extracts (10%) prepared in glass distilled water. Catalase (EC 1.11.1.6) was assayed by an adaptation of the permanganate titration method described by Pandey and Sharma (2002). Peroxidase (EC 1.11.1.7) was assayed by addition of suitable enzyme extract to a reaction mixture containing 0.1 M phosphate buffer pH 6.0, 0.01% H_2O_2 and 0.5% p-phenylene diamine. Reaction was stopped by adding 4 N H_2SO_4. The colour intensity was read at 485 nm.

For assay of antioxidant enzymes, fresh leaves were homogenized with 150 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA and 2% PVP for assaying SOD. The homogenate was centrifuged at 15,000 g for 10 min. and the supernatant was used as the enzyme preparation. All enzyme preparations were made at 4°C.

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by measuring the ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in a reaction mixture containing 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 0 to 50 µl enzyme extract. Riboflavin was added last and tubes were illuminated for 10 min. Blanks were not illuminated and the above reaction mixture without the enzyme extract developed the maximum color at 560 nm. One unit of SOD represents the amount that inhibits the NBT reduction by 50% (Beauchamp and Fridovich, 1971).

Standard analyses of variance (ANOVA) were used to assess the significance of treatment means. The data are presented as mean values ± standard error (SE, n=3). Differences between treatments means were compared using LSD at the 0.05 probability level.

RESULTS

Tissue concentration and content of cadmium

Tissue concentration of Cd in shoot and roots showed an increase with increase in Cd supply. Maximum accumulation of Cd as compared to control was found in 500 µM Cd supplied seedlings. Cadmium concentration was higher in the roots (695.225, 908.320, 1020.025 µg Cd g^{-1} dry weight) than in shoot (375.250, 440.122, 522.532 µg Cd g^{-1} dry weight).

Photosynthetic pigments

Chlorophyll a, b and carotenoids concentration in leaves of pea seedlings was decreased in 100 to 500 µM Cd supplied seedlings, as compared to the leaves of control.

Lipid peroxidation

Lipid peroxidation was observed in Cd toxic pea seedlings. This was evident from the accumulation of MDA in the seedlings grown at different levels of Cd. Lipid peroxidation was found to be higher at 100 to 500 µM Cd supplied pea seedlings as compared to control.

Hydrogen peroxide (H_2O_2)

Accumulation of H_2O_2 was observed in pea seedlings exposed to 100 to 500 µM Cd supply as compared to seedlings of control treatment. This accumulation was reported to be 140, 172 and 260% more than that in control.

Ascorbate

Concentration of ascorbate was found to be increased with increasing Cd concentration in growing medium when compared to control treatment. Ascorbate concentration increased with 120, 155, 180% in 100, 200 and 500 µM Cd supplied pea seedlings. Increase in ascorbate concentration was found to be more pronounced in 500 µM Cd supplied pea seedlings.

Proline

As compared to control plants proline was found to be accumulated in cadmium toxic pea seddlings. Accumulation of proline was least in 100 µM Cd supplied pea seedings (132%) and maximum in 500 µM Cd supplied seedlings (220%) as compared to control seedlings.

Antioxidative enzymes

Superoxide dismutase (SOD): Application of cadmium in nutrient solution increased the activity of SOD in shoots of pea seedlings. Application of 100 to 500 µM Cd increased the antioxidative efficiency, and significant increase in SOD activity was observed in leaves of 500 µM Cd as compared to control seedlings.

Catalase (CAT): Activity of CAT was found to be increased in shoots of seedlings receiving 100 to 500 µM cadmium supply as compared to control treatment. Catalase activity was increased with 120, 145 and 160% in 100, 200 and 500 µM Cd treated pea seedlings when compared to the control seedlings.

Peroxidase (POD): As compared to control, in all levels of Cd toxic pea seedlings the activity of POD was found to be increased. Seedlings supplied with 100 to 500 µM Cd showed with 108, 125 and 155% increase in POD activity.

DISCUSSION

Increasing concentration of Cd in the nutrient solution produced a significant reduction in growth and visual symptoms of Cd toxicity in pea plants. Cadmium toxicity caused reduction in leaf size, which was particularly marked in younger leaves. Plants receiving 100, 200 and 500 µM Cd supply developed chlorosis in leaves after three days. Leaves turned chlorotic and their margins turned golden brown and scorched 5 DAT. Interveinal chlorosis followed by necrotic patches appeared in young trifoliate leaves of pea plants supplied with 200 and 500 µM Cd, after five days. The number and length of lateral roots as compared to the control was markedly reduced in cadmium toxic plants. A thickening of lateral roots and browning of root tissues were also observed. The decrease in dry matter production in the roots and shoot increased with increase in Cd concentration and exposure time of the plants to Cd. Root tips and total root length decreased significantly with increasing concentration of cadmium when compared with control treatment, maximum reduction was observed in 500 µM Cd treated pea seedlings.

Cadmium toxic plats showed a greater accumulation of Cd in all plant parts. In comparison to shoot, root accumulates more Cd. It has been suggested that an excess of essential cations such as zinc and calcium has a protective effect against cadmium toxicity (Antonovics *et al.,* 1971; Cosio *et al.,* 2004). This is interpreted as a result of competition for uptake.

Our results suggested a reduction in photosynthesis rates under Cd stress, as indicated by a significant decrease in total chlorophyll content. Reduced amounts of chlorophyll in leaves exposed to Cd stress were also reported in *Phragmites australis* (Pietrini *et al.,* 2003) and black gram, pea and maize (Singh *et al*., 2008, Pandey and Singh 2012; Singh and Pandey 2013). Supply of Cd caused a decrease in concentration of chlorophyll a, b and total chlorophyll. Carotenoid concentration was also suppressed due to the Cd. Carotenoids protect chlorophyll from photooxidative destruction and therefore a reduction in carotenoid could have a serious consequence on chlorophyll pigments. The inhibition of growth seen by the decrease in dry biomass and shoot elongation may be due to reduction in photosynthesis which is caused by reduction in total chlorophyll content (Sandalio et al 2001). The mechanism by which Cd brings about leaf chlorosis is unclear. It was reported that Cd affected chlorophyll biosynthesis and inhibit protochlorophyll reductase and aminolevulinic acid synthesis (Stobart *et al.,* 1985). It has been suggested that Cd could have a direct action on the enzymes of the chlorophyll biosynthesis pathway (Padmaja *et al.,* 1990; Boddi *et al.,* 1995) or on the proper assembly of the pigment protein complexes of the photosystems (Krupa *et al.,* 1987; Krupa, 1988; Horvath *et al.,* 1996).

The plant cell membranes are generally considered as the primary site of injury and destabilization of membrane is attributed to lipid peroxidation. The increased concentration of O_2 and H_2O_2 lead to lipid peroxidation, causing membrane damage (Sairam and Srivastava, 2000, Pandey *et al.,* 2009; Anjum *et al.,* 2011; Namdjoyan *et al.,* 2011). It is well documented that MDA is a product of cell membrane lipid peroxidation (Shah *et al.,* 2001) and its content *in vivo* can indicate the extent of oxidative stress in plants and cell membrane homeostasis.

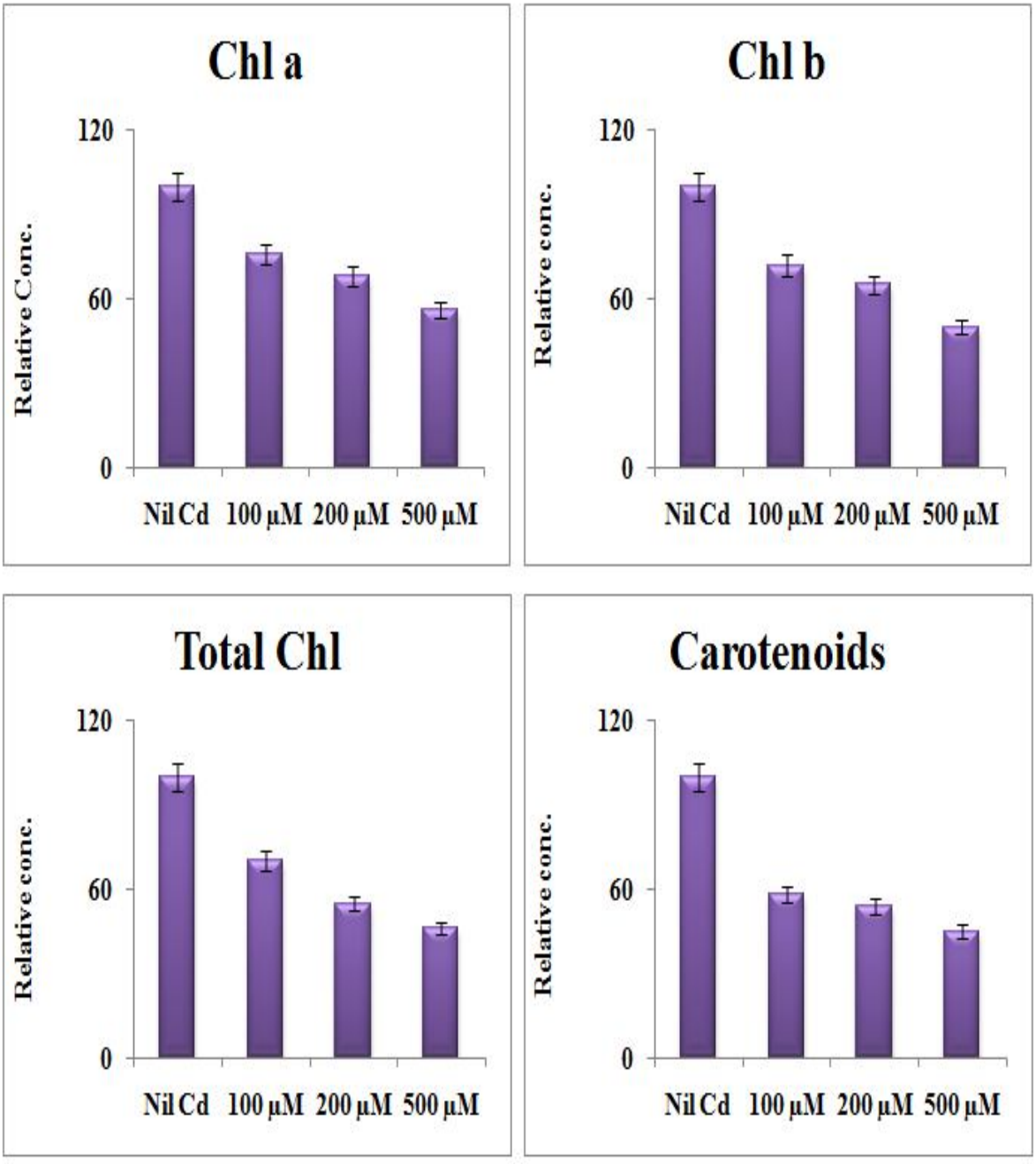

Fig. 1 Effect of cadmium toxicity on relative concentration of photosynthetic pigments in pea (*Pisum sativum* L. var. Swati) seedlings in solution culture.

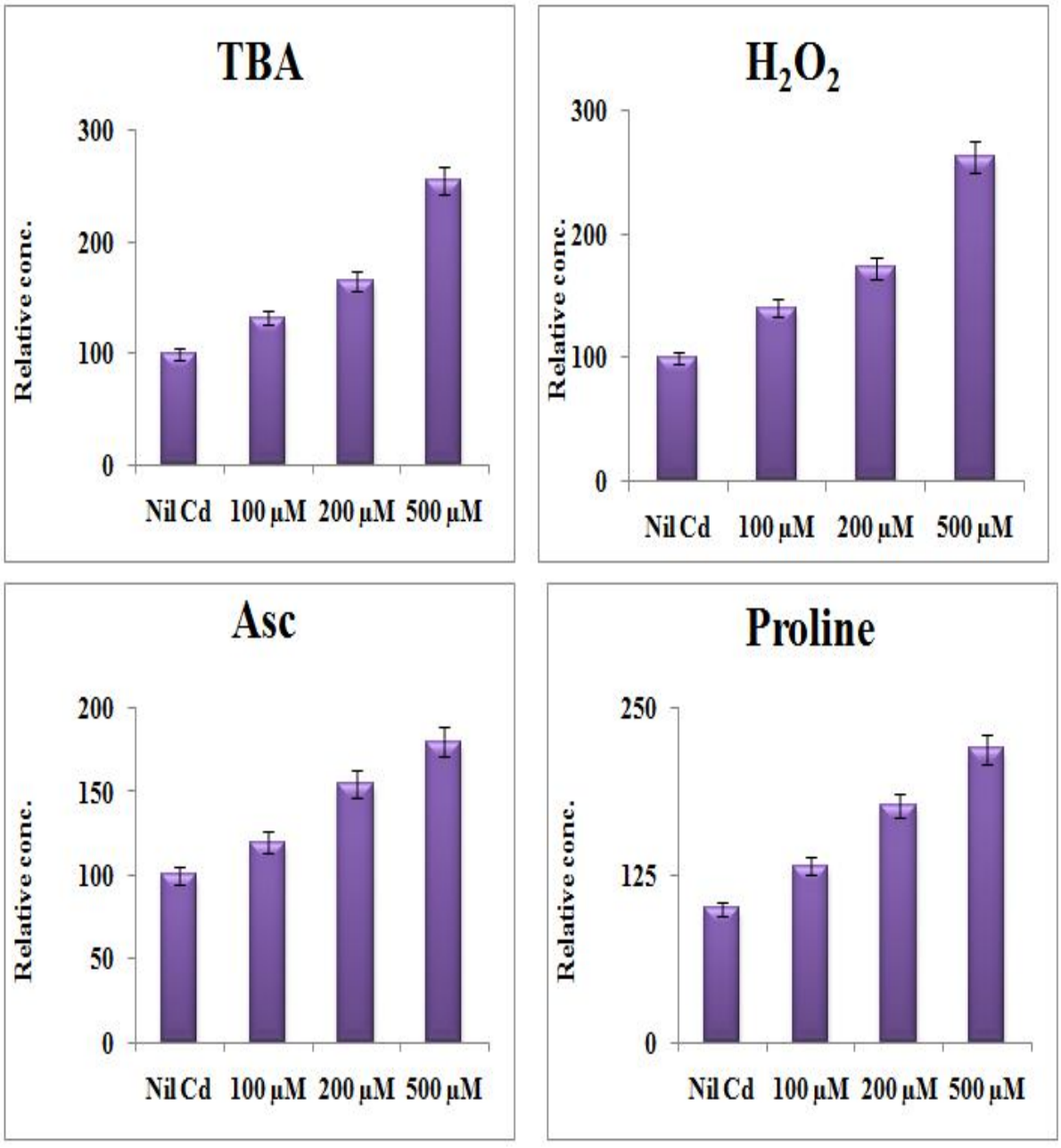

Fig. 2. Effect of cadmium treatment on relative concentration of Lipid peroxidation (LPO), Hydrogen peroxide (H_2O_2), Ascorbate and Proline in shoots of *Pisum sativum* L. var. Swati) seedlings in solution culture.

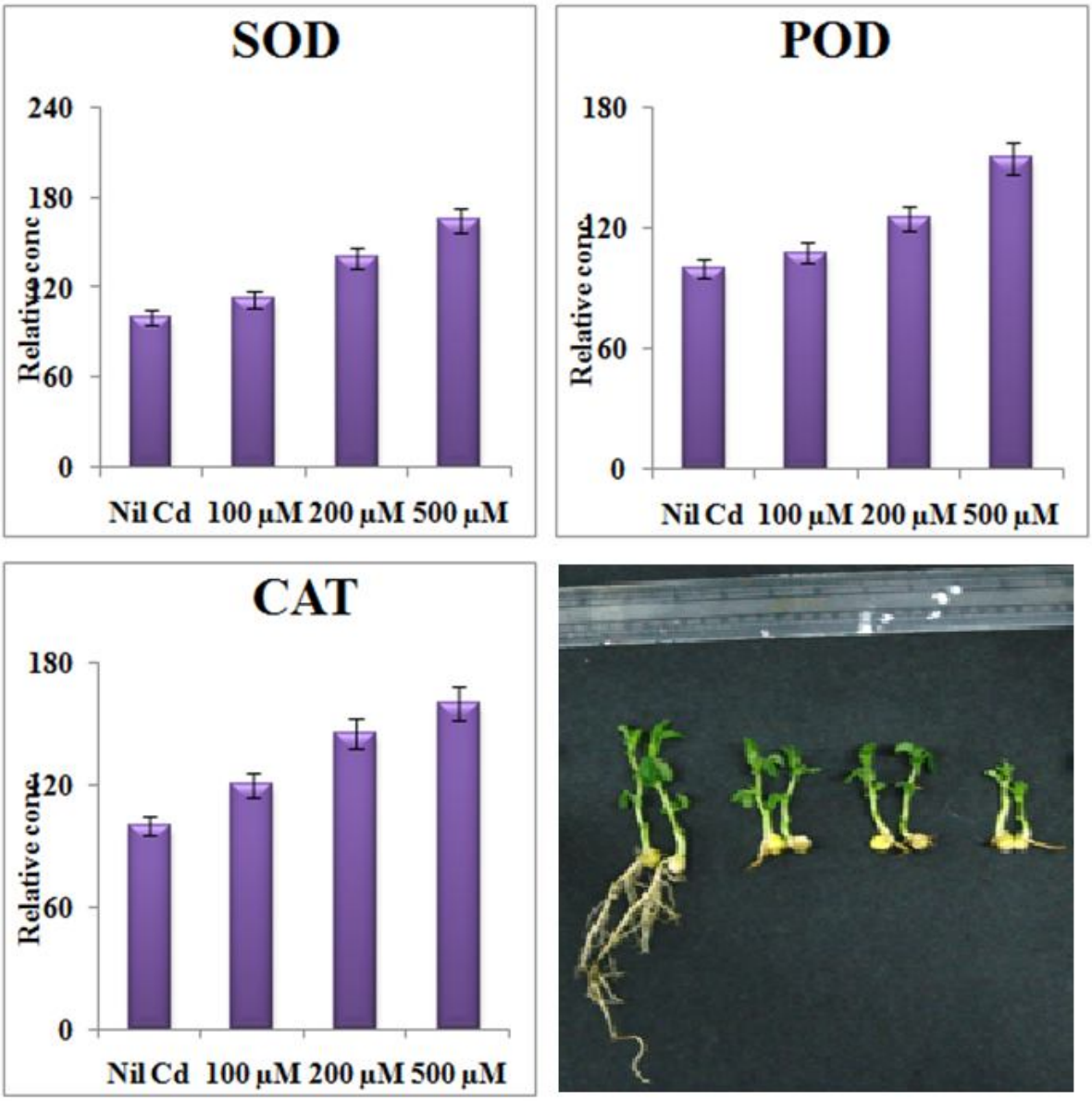

Fig. 3. Effect of cadmium treatment on relative concentration of Superoxide dismutase (SOD), Peroxidase (POD),and Catalase (CAT) in shoots of *Pisum sativum* L. Var Swati) seedlings in solution culture.

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 is peroxidised in its presence.

In the present study increased level of H_2O_2 could account for the formation of ·OH radicals leading to lipid peroxidation and accumulation of TBARS.

Cadmium toxicity increased the activity of proline in pea seedlings. Proline could be involved in metal chelation in the cytoplasm. Moreover, proline is a poor inducer of phytochelator synthesis (Farago and Mullen, 1979). The plants exposed to heavy metals seem to induce accumulation of free proline (Bassi and Sharma, 1993; Costa and Morel, 1994).

Ascorbate is a key antioxidant that react with OH radicals, $O_2^{\cdot -}$ and $^1O_2$ (Noctor and Foyer, 1998). Ascorbate reacts directly with the ROS in photosynthetic tissues where it recycles α-tocopherol, protects enzymes with prosthetic metal ions, and is utilized as a substrate for ascorbate peroxidase which catalyses H_2O_2 detoxification. An increase of ascorbate and proline under heavy metal stress has been reported by others (Schat *et al.,* 2006; Choudhary *et al.,* 2007; Pandey and Singh 2012).

Plants possess a number of antioxidant systems that protect them from oxidative damage (Smeets et al., 2005). These defense systems are composed of metabolites such as ascorbate, glutathione, proline etc., and enzymatic scavengers of activated oxygen such as POD, CAT and SOD (**Noctor** and Fayer, 1998; Asada,1999; Sandalio et al., 2001; Mandhania et al., 2006; Pandey and Singh 2012). Each of these enzymes has physiological function under non-stressed conditions, but their activity is increased or decreased under oxidative stress. Superoxide dismutase (SOD) is the first enzyme in the detoxifying process that converts O^{2-} radicals to H_2O_2 at a very rapid rate (Polle and Rennenberg, 1994). Cadmium was found to result in oxidative stress (Hendy *et al.*, 1992; Somashekaraiah *et al.,* 1992) by either inducing oxygen free radical production (Balaknina *et al.,* 2005; Demirevska-Kepava *et al.,* 2006) or by decreasing concentrations of enzymatic and non-enzymatic antioxidants (Somashekaraiah *et al.*, 1992; Stohs and Bagchi, 1995; Sandalio *et al.,* 2001; Cho and Seo, 2004; Mohan and Hosetti, 2006). Peroxidase induction is a general response of higher plants after uptake of toxic quantities of metals (Van Assche and Clijsters, 1990). The SOD activity showed an enhancement under Cd treatment contradictory to reports by Sandalio *et al*., (2001) who observed a decrease. An increase in SOD activity as well as the increase in CAT and POD activity as observed in the present study indicates the weakening of H_2O_2 scavenging system due to Cd stress as observed earlier by Pandey *et al*., (2009). The observed increase 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). The results support the findings of Sandalio *et al*., (2001) who reported a enhancement of SOD, CAT and POD in Cd toxic plants. 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 increase in the H_2O_2 concentration in Cd toxic plants seems to be well correlated with the increase in CAT, POD and SOD activity. In the present study, Cd toxicity was evaluated by the increase in MDA and H_2O_2 content in pea seedlings.

CONCLUSION

The results of the present study have shown that Cd treatment was inhibitory to seed germination, plant growth and biochemical constituents of pea seedlings, when compared to control seedlings. The loss of these may be due to inhibition of cell division, impairment of PSII activity, directly or indirectly inhibits physiological processes such as photosynthesis, plant–water relationships, loss of cellular turgor, inhibiting the activity of the cell and its enlargement, resulting in poor growth and low biomass. The decreased chlorophyll contents of pea might be due to the active involvement of Cd in iron uptake and chlorophyll biosynthesis. So there was a consequents reduction in the growth of root and shoot length, fresh and dry weight, leaf area, chlorophyll and carotinoids of plants. The shoot length of Cd treated pea plants was higher than the root length. Cadmium showed adverse effects on plant growth and development at all concentrations, but maximum inhibition occurred at 500 µM Cd.

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