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Alleviation of Cadmium Toxicity in *Pisum sativum* L. Seedlings by Calcium Chloride

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Abstract

The present investigation was carried out to study the role of calcium chloride in enhancing tolerance and reducing cadmium toxicity in pea seedlings. Some treatment with 1 and 5 mM $CaCl_2$ mitigated cadmium stress by increasing antioxidant enzyme activities: catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO), as well as by elevating contents of ascorbic acid (ASA), tocopherol and carotenoids. On the other hand, total carbohydrate and total soluble proteins decreased with increasing cadmium concentrations in comparison with control plants. However, total phenol, total free amino acids, proline and lipid peroxidation increased with increasing concentrations of cadmium acetate. Electrophoretic studies of protein revealed that cadmium treatments alone or in combination with calcium chloride were associated with the disappearance of some bands or appearance of new bands in pea seedlings. Electrophoretic studies of α-esterase, β-esterase and acid phosphatase isozymes showed wide variations in their intensities and densities.

Keywords: antioxidant enzymes, cadmium toxicity, calcium, non enzymatic antioxidants, oxidative stress, *Pisum sativum*

Introduction

Cadmium (Cd) is a highly toxic trace element that enters the environment mainly from industrial processes and phosphate fertilizers (Liu *et al.,* 2007). It can reach high levels in agricultural soil and is easily accumulated in plants. Cd ions are taken up readily by the plant roots and translocated to the above-ground vegetative parts (Shamsi *et al.,* 2008a). Excessive Cd induces complex changes in plants at genetical, biochemical and physiological levels, leading to phytotoxicity. The most obvious symptoms are (1) depression of plant growth and even resulting in plant death by disturbing the uptake of nutrients (Hassan *et al.,* 2005); (2) destruction of photosynthesis via degradation of chlorophyll (Hassan *et al.,* 2005; Shamsi *et al.,* 2008a) and inactivation of enzymes involved in CO_2 fixation (De Filippis and Ziegler, 1993) or the aggregation of pigment protein complexes of the photosystems (Horvath *et al.,* 1996); (3) interaction with the water balance of the plant and inhibiting stomatal opening (Barcelo and Poschenrieder, 1990); (4) alteration of proline and polyamine contents (Sharma and Dietz, 2006); and (5) accelerating lipid peroxidation and affecting cell membrane fluidity and permeability due to an alteration in the composition of membrane lipids (Molina *et al.,* 2008).

The toxicity of Cd is in part related to oxidative stress, caused by generation of free radicals and reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (El-Beltagi *et al.,* 2008; El-Beltagi, 2011; Gratão *et al.,* 2005; Mohamed *et al.,* 2009; Wahid *et al.,* 2009). ROS are the byproducts of many degenerative reactions in crop plants, which will affect the regular metabolism by damaging the cellular components (Foyer and Noctor, 2002). Plant cells can tolerate ROS by endogenous protective mechanisms involving nonenzymic as well as enzymatic systems (Asada, 1994; Shamsi *et al.,* 2008b). As a consequence, plants evolved cellular adaptive responses like up-regulation of oxidative stress protectors and accumulation of protective solutes (Horling *et al.,* 2003). Antioxidant defense enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR) are the systems designed to minimize the concentrations of superoxide and hydrogen peroxide. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. H_2O_2 is eliminated by catalase and peroxidases, which include both enzymic and non-enzymic H_2O_2 degradation (El-Beltagi, 2001; Peltzer *et al.,* 2002). Catalase dismutates H_2O_2 into water, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Blokhina *et al.,* 2003). The antioxidants such as ascorbate (AsA) and glutathione (GSH) are involved in scavenging ROS primarily via the Halliwell-Asada pathway, which scavenges H_2O_2, while MDAR and GR are involved in the regeneration of ascorbate (Horemans *et al.,* 2000). Stress is also known to affect the metabolism of soluble carbohydrates,

a group of compounds that may act as compatible solutes as well as antioxidants. These compounds usually increase as a result of environmental deficit (Smirnoff and Cumbes, 1989). Another group of compounds, which may be affected by environmental deficit, are total free amino acids. Total free amino acids are often increased in stressed leaves (Pinheiro *et al.,* 2004). Accumulation of protective solutes like proline and total carbohydrates in the leaf is a unique plant response to environmental stresses, specifically to environmental stress (Sakamoto and Murata, 2002). Furthermore, deleterious effects of heavy metal stress in plants may be coupled to other physiological processes via the stimulation of some enzymatic activities that limit cell growth and consequently, accelerate tissue ageing. Several hydrolytic enzymes including glucosidases, esterases, acid and alkaline phosphatases, were pro-posed to be involved in plant morphogenesis, especially in cell wall metabolism that is necessary for its turnover and plasticity (Balen *et al*., 2003). In addition, Kaya (2002) found that, the activity of acid phosphatase (AP) increased in leaves and roots of tomato plants grown at high zinc concentration in the growth media, whereas esterase (EST) is one of most important germination enzymes includes fatty acid esterases that release fatty acids from lipids. Isozymes are an easily obtained class of molecular markers, co- dominant, reliable and can provide valuable information in tracking the transfer of genes between species (Tanksley, 1983). Cd stress can also modulate the expression of antioxidant and hydrolytic isozymes in plant tissue (El-Beltagi *et al.,* 2010; Mohamed *et al.,* 2009).

Calcium is an essential nutrients and a divalent cation that is extremely important in maintaining the strength of plants stems and play a role in regulation of a variety of developmental and photosynthetic processes. This mineral also regulates the absorption of nutrients across plasma cell membranes. Calcium functions in plant cell elongation and division, structure and permeability of cell membranes, nitrogen metabolism and carbohydrate translocation (White, 2000). Calcium regulates the activities of target proteins directly or via calcium-binding proteins, such as calmodulin, which, after binding to Ca^{2+}, activates a number of protein kinases and other proteins in plant cells (Wang and Wang, 2007).

The aim of the present study was to assess the effect of different concentrations of Cd on physiological and biochemical attributes of seedlings of pea plants and to alleviate the toxic effect of cadmium by addition of calcium chloride.

Materials and methods

Plant material

The experimental plant used in this investigation was pure strain of pea (*Pisum sativum* cv. 'Yasmin') seeds that kindly obtained from the Agricultural Research Center in Giza, Egypt.

Treatments

Seeds were surface sterilized with 0.5% (V/V) sodium hypochlorite for 15 min and rinsed 3 times with distilled water and were placed on the Whatman No. 1 filter paper. One group was moistened with distilled water and sampled as control. Another group was treated with two concentrations of cadmium acetate (25 and 50 μM). The third group was treated with two concentrations of calcium chloride (1 and 5 mM). The last two groups were subjected to two combinations (25 and 50 μM +1 mM) and (25 and 50 μM +5 mM) of cadmium acetate and calcium chloride, respectively. The seeds were allowed to germinate in Petri dishes (15 cm diameter) at 25°C in incubator. After ten days the whole seedlings were collected to determine protein electrophoresis, some isozymes, enzymatic and non enzymatic antioxidants, lipid peroxidation, total phenol, total carbohydrate, proline, total free amino acids and total soluble protein.

Chemical analysis

Assay of total soluble protein

Soluble proteins were measured by the Bio-Rad micro assay modification of the Bradford (1976) procedure using crystalline bovine serum albumin as a reference.

Determination of osmoregulators

Assay of total carbohydrates

Total carbohydrates were determined based on the method of phenol sulfuric acid as described by Dubois *et al.* (1956). Pure glucose was used as standard.

Assay of total phenols

Levels of soluble phenols in cowpea leaves were determined in accordance with Dihazi *et al*. (2003). The absorbance of the developed blue colour was read at 725 nm. Tannic acid was used as standard and the amount of soluble phenols was expressed as mg tannic acid g^{-1} dry weight.

Assay of proline content

The proline content was estimated by the method of Bates *et al.* (1973).

Assay of total free amino acids

Total free amino acids (FAA) content was estimated according to Moore and Stein (1954).

Lipid Peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde content following the method of Heath and Packer (1968). The absorbance of the resulting supernatant was recorded at 532 nm and 600 nm. The non-specific absorbance at 600 nm was subtracted from the 532 nm absorbance. The absorbance coefficient of malondialdehyde was calculated by using the extinction coefficient

of 155mM^{-1} cm^{-1} and the results are expressed as nmol/g F.W.

Assay of non enzymatic antioxidant

Assay of carotenoid contents

Fresh seedlings of pea were used for the estimation of carotenoid contents (Lichtenthaler and Wellburn, 1983).

Assay of ascorbic acid content

Ascorbic acid content was measured according to the method described by Mukherjee and Choudhuri (1983).

Assay of α-Tocopherols content

The α-Tocopherols were determined using the method of Philip *et al*. (1954).

Determination of antioxidant defense enzymes activity

Preparation of enzyme extracts

The pea samples of (1.0 g) were crushed into fine powder using liquid nitrogen. Soluble protein was extracted by homogenizing the powder in 5 ml of 50 mM phosphate buffer (pH 7.8) containing 1mM EDTA and 1% PVP at 4ºC. The homogenate was centrifuged at 15,000 × *g* for 20 min, and the supernatant was used for the following enzyme activity assay.

Assay of catalase (CAT) activity

Catalase activity (CAT; EC 1.11.1.6) was determined by consumption of H_2O_2 using the method of (Aebi, 1983). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer pH 7.0, 15 mM H_2O_2 and 50 µl enzyme extract. The reaction was initiated by adding the H_2O_2. The consumption of H_2O_2 was monitored spectrophotometrically at 240 nm (e = 39.4 mM^{-1} cm^{-1}) for 3 min. Enzyme activity was expressed in µM H_2O_2 g^{-1} h^{-1}.

Assay of peroxidase (POD) activity

Peroxidase (POD) activity was assayed according to the method of Hemeda and Klein (1990). A 100 ml of reaction mixture contained 10 ml of 1% guaiacol (v/v), 10 ml of 0.3% H_2O_2 and 80 ml of 50mM phosphate buffer (pH 6.6). Enzyme extract (75 µl) was added to the reaction mixture in a final volume of 3 ml. The increase in absorbance due to oxidation of guaiacol (extinction coefficient 26.6 mM^{-1} cm^{-1}) was monitored at 470 nm. Enzyme activity was expressed as units g^{-1} h^{-1}.

Assay of polyphenol oxidase (PPO) activity

Polyphenol oxidase activity was assayed by using photochemical method as described by Coseteng and Lee (1987). The reaction mixture contained 50 mM potassium phosphate buffer pH 6.2 and 250 mM catechol and 50 µl enzyme extract. The increasing in absorbance at 420 nm was measured. Enzyme activity was expressed as units g^{-1} h^{-1}.

Electrophoretic analysis of polypeptides and isozymes

Polypeptide pattern was analyzed on 12% SDS polyacrylamide gels according to the method of Laemmli (1970). Isozymes α- and β - esterase and acid phosphatase were analyzed on 12% Polyacrylamide slab gels. Detection of esterase isozymes were carried out by the method described by Scandalios (1964), while acid phosphatase isozymes was detected by method of Wendel and Weeden (1989).

Statistical analysis

Analysis of variance was carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test for significant difference between treatments at $p \leq 0.05$ (Gomez and Gomez, 1984).

Results and discussion

In plants, ROS are produced continuously as by products of various metabolic pathways that are localized in different cellular compartments (Gratão *et al.,* 2005), but under heavy metal stress conditions, their formation might be in excess of antioxidant scavenging capacity. Generation of ROS by heavy metal caused damage to all biomolecules, especially proteins, due to the higher rate constants of the reaction of the superoxide anion with amino acid side chains. Tab. 1 showed a significant reduction in the level of soluble proteins in pea seedlings with increasing cadmium acetate concentrations. It can be also observed that total soluble proteins content were significantly increased in pea seedlings treated with calcium chloride alone. On the other hand, 1.0 and 5.0 mM of $CaCl_2$ in combination with cadmium concentrations caused non significant effect as compared with the corresponding control. Cadmium treatments caused reduction in protein level in pea seedlings. Similar results obtained by Bhardwaj *et al.* (2009) who reported that treatment with lead and cadmium caused reduction in the soluble protein level in *Phaseolus vulgaris.* The reduction in the level of soluble protein may be due to (i) enhanced protein hydrolysis, (ii) catalytic activity of lead, (iii) enhanced protein degradation process and (v) decreased availability of amino acids and denaturation of enzymes involved in protein synthesis. Also, cadmium and lead may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of reactive oxygen species which led to reduced protein content (El-Beltagi, 2004; El-Beltagi *et al.,* 2010; Ibrahim *et al.,* 2012).

The results presented in Tab. 1 revealed that the concentrations of total free amino acids were increased significantly upon Cd exposure, where the most prominent effect was at 50 µM Cd as being compared with control plants. On using $CaCl_2$ alone or in combination with different concentrations of cadmium, significantly increases in total free amino acids in pea seedlings were observed. In addition, treatment with different concentrations of cadmium acetate (25 and 50 µM) caused significant in-

Tab. 1. Effect of different concentrations of cadmium on the contents of total soluble protein, total free amino acid and proline in pea seedlings in presence or absence of calcium chloride

Treatments	Total soluble protein µg/ g F.W.	Total free amino acid mg/g F.W.	Proline µg/ g F.W.
Control	11.7±1.2^{c}	43.77±2.2^{f}	20.57±1.5^{d}
25 µM Cd^{2+}	9.89±0.9^{d}	91.83±3.7^{ab}	34.17±2.0^{a}
50 µM Cd^{2+}	8.51±1.0^{e}	74.50±2.9^{cd}	35.63±2.1^{a}
1.0 mM Ca^{2+}	13.18±1.5^{a}	61.33±2.5^{e}	22.10±1.7^{cd}
5.0 mM Ca^{2+}	12.96±1.4^{ab}	79.83±2.7^{c}	23.47±1.7^{bcd}
25 µM Cd^{2+}+1.0 mM Ca^{2+}	12.68±1.3^{abc}	68.60±2.5^{de}	28.63±1.8^{abcd}
25 µM Cd^{2+}+5.0 mM Ca^{2+}	11.97±1.2^{bc}	81.30±2.7^{c}	30.73±2.1^{abc}
50 µM Cd^{2+}+1.0 mM Ca^{2+}	12.56±1.3^{abc}	97.10±3.0^{a}	27.07±1.9^{abcd}
50 µM Cd^{2+}+5.0 mM Ca^{2+}	12.47±1.3^{abc}	82.37±3.2^{bc}	32.73±2.4^{ab}
$LSD_{5\%}$	0.918	9.95	9.83

Each value is expressed as mean±SE. Data with different superscript letters were significantly different ($p \leq 0.05$). F.W Fresh weight; D.W Dry weight

creased in proline content in pea seedlings as compared with control plants. In addition, application of 1.0 mM $CaCl_2$ alone or in combination with 25 and 50 µM of cadmium acetate caused non significant effect in the proline content, while 5.0 mM $CaCl_2$ alone caused non significant effect but caused significant increased when combined with and 50 µM of cadmium acetate as compared with control plants (Tab. 1). On using $CaCl_2$ alone or in combination with different concentrations of cadmium, significantly increases in total free amino acids in pea seedlings and significantly increased and non significant effect in proline content were observed (Wu *et al.,* 2004; Zengin and Munzuroglu, 2005). Proline is considered to be one of the most characteristic metabolic consequences of heavy metal stress. The presence of amino acids especially proline may play a role in protection from desiccation and from the harmful effects derived from solute accumulation. It has been often suggested that proline accumulation may contribute to osmotic adjustment at the cellular level and enzyme protection stabilizing the structure of macromolecules and organelles. Proline accumulation may help the plant to survive short periods of heavy metals and recover from stress. Besides, high proline concentration measured in sludge-treated nodules could also contribute to a protective role as scavenger of ROS (El-Beltagi *et al.,* 2011; Türkan and Demiral, 2009). Increase in proline content may be either due to *de novo* synthesis or decreased degradation or both.

Total soluble carbohydrates are the major soluble constituent that helps the plants in osmotic adjustment. Also, carbohydrates provide rapidly growing cells with energy and with the carbon skeletons required to synthesize organic compounds (Taiz and Zeiger, 2010). The total carbohydrate content in pea seedlings was significantly higher in the control plants or those treated with 1.0 mM of calcium chloride alone or in combination with 25 and 50 µM of cadmium acetate, while treatment with 5.0 mM $CaCl_2$ alone caused significant increased in the total carbohydrate content but caused non significant effect when combined with 25 and 50 µM of cadmium as being compared with the corresponding control (Tab. 2). The significant decrease in total carbohydrates content in pea seedlings with increasing cadmium concentrations are in agreement with the result reported by Bhardwaj *et al.* (2009). This may be due to suppression of the activity of enzymes of carbohydrate metabolism under Cd stress (Sanitá di Toppi and Gabbrielli, 1999; Verma and Dubey, 2001). Applying calcium chloride either alone or in combination with cad-

Tab. 2. Effect of different concentrations of cadmium on the contents of total carbohydrate, total phenol and lipid peroxidation in pea seedlings in presence or absence of calcium chloride

Treatments	Total Carbohydrate mg/g D.W.	Total Phenol mg tannic acid/g D.W.	Lipid Peroxidation total MDA nmol/ g F.W.
Control	1.38±0.08^{de}	2.20±0.1^{f}	22.47±1.0^{b}
25 µM Cd^{2+}	0.92±0.07^{ef}	3.59±0.2^{b}	31.77±1.3^{a}
50 µM Cd^{2+}	0.77±0.05^{f}	4.89±0.3^{a}	38.83±1.9^{a}
1.0 mM Ca^{2+}	2.56±0.1^{a}	2.84±0.5^{cd}	18.77±1.4^{bc}
5.0 mM Ca^{2+}	2.43±0.2^{ab}	3.15±0.4^{c}	15.70±1.5^{bc}
25 µM Cd^{2+}+1.0 mM Ca^{2+}	2.21±0.2^{abc}	2.34±0.2^{ef}	13.20±1.0^{c}
25 µM Cd^{2+}+5.0 mM Ca^{2+}	1.88±0.3^{cd}	2.60±0.2^{df}	15.67±1.2^{bc}
50 µM Cd^{2+}+1.0 mM Ca^{2+}	2.01±0.2^{bc}	2.42±0.2^{ef}	18.30±1.6^{bc}
50 µM Cd^{2+}+5.0 mM Ca^{2+}	1.72±0.1^{cd}	2.57±0.3^{de}	17.13±1.6^{bc}
$LSD_{5\%}$	0.592	0.328	10.64

Each value is expressed as mean±SE. Data with different superscript letters were significantly different ($p \leq 0.05$). F.W Fresh weight; D.W Dry weight

mium concentrations induced variable recovery in carbohydrate contents in seedlings of pea plants. These results are in agreement with those obtained by Hassanein (1998) and Gaafar *et al.* (2012) who reported that carbohydrate content in white radish and *Triticum aestivum* L. significant decline as the heavy metal (cadmium and lead) concentration increased. While treating the plants with Ca^{2+} induced variable recovery in carbohydrate contents of radish plants.

Also, the results presented in Tab. 2 revealed that the total phenol level was significantly increased in pea seedlings grown at 25 and 50 μM cadmium acetate as compared with those of untreated plants. Application of calcium chloride caused significant increased in the accumulation of phenols in pea seedlings as being compared with the control treatment. On the other hand, treatment of pea seeds with calcium chloride (1.0 and 5.0 mM) in combination with cadmium acetate caused non significant effect in total phenol content except at 50 μM cadmium and 5.0 mM calcium which caused significant increased as compared with control plants.

Considerable variations in the total phenol contents were induced by applying different concentrations of cadmium. Similar data have been reported by Lavid *et al.* (2001). They ascribed the increase of phenolics to the increase in activity of enzymes involved in phenolic metabolism, suggesting *de novo* synthesis of phenolics under heavy metal stress. The involvement of phenolics as intermediates in lignin biosynthesis can reflect the typical anatomical change induced by stressors that increase in cell wall endurance and create physical barriers preventing cells against harmful action of heavy metals. In plant tissues many phenolic compounds are potential antioxidants: flavonoids, tannins and lignin precursors may act as ROS (reactive oxygen species) scavenging compounds. Polyphenols contain an aromatic ring with –OH or OCH_3 substituents which together contribute to their biological activity, including antioxidant action. They have been shown to outperform well-known antioxidants, AsA and α-tocopherol, *in vitro* antioxidant assays because of their strong capacity to donate electrons or hydrogen atoms. Polyphenols can chelate transition metal ions, can directly scavenge molecular species of active oxygen, and can inhibit lipid peroxidation by trapping the lipid alkoxyl radical. They also modify lipid packing order and decrease fluidity of the membranes (Arora *et al.,* 2000). These changes could strictly hinder diffusion of free radicals and restrict peroxidative reactions. Moreover, it has been shown that, especially, flavonoids and phenylpropanoids are oxidized by peroxidase, and act in H_2O_2-scavenging, phenolic/AsA/POD system. There is some evidence of induction of phenolic metabolism in plants as a response to multiple stresses (Michalak, 2006).

The lipid peroxidation contents (MDA) of the cadmium acetate treated samples were significantly higher than that of control (Tab. 2). Results showed that, cadmium acetate (25 and 50 μM) induced intense lipid peroxidation as indicated by increase in the thiobarbituric acid reactive substances (TBARs) product in seedlings of pea plants. Application of calcium chloride either alone or in combination with cadmium caused non significant effect in the level of TBARs in seedlings pea plants except at 25 μM of cadmium in combination with 1.0 mM of calcium as compared with control plants.

The increase in lipid peroxidation of pea seedlings with increasing concentrations of cadmium acetate as shown by malondialdehyde (MDA) concentration, indicates that cadmium toxicity was linked to lipid peroxidation because Cd produces excessive reactive oxygen species including superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) causing cell death due to oxidative stress (Shah *et al.,* 2001). Similar data have been reported for many plant species including bean (Bhardwaj *et al.,* 2009) and *Arabidopsis* (Saffar *et al.,* 2009). Application of calcium chloride either alone or in combination with cadmium caused significantly reduced or non significant effect in the level of MDA in pea plants. These results are in agreement with those observed by Wang and Song (2009) using calcium chloride.

Ascorbate (AsA) is the most abundant, low molecular weight antioxidant that has a key role in defense against oxidative stress caused by enhanced level of ROS. Data presented in Tab. 3 showed that ascorbic acid contents (AsA) were higher in pea seedlings treated with 50 μM of cadmium acetate as being compared with control plants. Application of 1.0 mM calcium chloride in combination with 25 μM cadmium acetate caused significantly increased in ascorbic acid content in seedlings of pea plants as compared with control plants, while the other treatments caused non significant effect. Treatment with different concentrations of cadmium acetate (25 and 50 μM) showed significant increased in α- tocopherol contents in seedlings of pea plants. In addition, using 5.0 mM calcium chloride alone or in combination with 25 and 50 μM of cadmium acetate caused significantly increased in the same contents as compared with control plants (Tab. 3). The results presented in Tab. 3 showed that cadmium concentrations (25 and 50 μM) alone or in combination with 1.0 and 5.0 mM calcium caused significantly increase in carotenoids content in pea seedlings as compared with control plants. Accumulation of α- tocopherol, ascorbic acid and carotenoids levels in cadmium treated pea seedlings as being compared with control indicate their protective role against oxidative damage. Similar to the present results, other investigations have shown increased in α-tocopherol and ascorbic acids levels for different plant species exposed to cadmium (Liu *et al.,* 2007; Molina *et al.,* 2008; Zengin and Munzuroglu, 2005). Calcium chloride application either alone or in combination with cadmium acetate increased carotenoids content as compared with those of control plants. AsA is considered powerful antioxidant because of its ability to donate electrons in a

Tab. 3. Effect of different concentrations of non enzymatic antioxidants in pea seedlings in presence or absence of calcium chloride

Treatments	Ascorbic acid µg/ g F.W.	Tocopherol µg/ g F.W.	Carotenoid mg/g F.W.
Control	2.36±0.03^{c}	5.37±0.4^{f}	4.00±0.4^{f}
25 µM Cd^{2+}	3.33±0.04^{bc}	13.38±1.1^{a}	7.57±0.9 ^{a}
50 µM Cd^{2+}	4.56±0.06^{ab}	11.10±1.0 ^{b}	7.96±0.8 ^{a}
1.0 mM Ca^{2+}	2.93±0.02^{c}	6.91±0.6^{de}	4.07±0.5^{ef}
5.0 mM Ca^{2+}	2.67±0.02 ^{c}	6.22±0.7^{ef}	4.67±0.5 ^{e}
25 µM Cd^{2+}+1.0 mM Ca^{2+}	5.30±0.07^{a}	6.20±0.7^{ef}	5.66±0.7^{c}
25 µM Cd^{2+}+5.0 mM Ca^{2+}	2.76±0.02^{c}	9.63±0.9^{bc}	6.59±0.8 ^{b}
50 µM Cd^{2+}+1.0 mM Ca^{2+}	3.06±0.05^{bc}	5.69±0.5^{ef}	4.44±0.7 ^{e}
50 µM Cd^{2+}+5.0 mM Ca^{2+}	2.74±0.03^{c}	8.44±0.8^{cd}	4.99±0.5 ^{d}
$LSD_{5\%}$	1.57	1.54	0.406

Each value is expressed as mean±SE. Data with different superscript letters were significantly different ($p \leq 0.05$). F.W Fresh weight; D.W Dry weight

number of enzymatic and nonenzymatic reactions. AsA has been shown to play important role in several physiological processes in plants, including growth, differentiation, and metabolism. It provides membrane protection by directly reacting with $O_2^{\cdot -}$, H_2O_2 and regenerating *α*-tocopherol from tocopheroxyl radical and preserves the activities of the enzymes that contain prosthetic transition metal ions (Noctor and Foyer, 1998). AsA has a key role in removal of H_2O_2 *via* AsAGSH cycle (Shida *et al.,* 1997). Oxidation of AsA occurs in two sequential steps, first producing monodehydroascorbate (MDHA) and subsequently dehydroascorbate (DHA). In the AsA-GSH cycle, two molecules of AsA are utilized by APX to reduce H_2O_2 to water with concomitant generation of MDHA. MDHA is a radical with a short life time and can spontaneously dismutate into DHA and AsA or is reduced to AsA by NADP(H) dependent enzyme MDHAR (Miyake and Asada, 1994). While, Tocopherols represent a group of lipophilic antioxidants involved in scavenging of oxygen free radicals, lipid peroxy radicals, and $^1O_2$ (Diplock *et al.,* 1989). Tocopherols are known to protect lipids and other membrane components by physically quenching and chemically reacting with O_2 in chloroplasts, thus protecting the structure and function of PSII (Ivanov and Khorobrykh, 2003). Tocopherols prevent the chain propagation step in lipid autooxidation which makes it an effective free radical trap. Carotenoids also belong to the group of lipophilic antioxidants and are able to detoxify various forms of ROS (Young, 1991). As an antioxidant, they scavenge $^1O_2$ to inhibit oxidative damage and quench triplet sensitizer (3Chl*) and excited chlorophyll (Chl*) molecule to prevent the formation of $^1O_2$ to protect the photosynthetic apparatus. Carotenoids also serve as precursors to signaling molecules that influence plant development and biotic/abiotic stress responses (Li *et al.,* 2008).

The activities of the antioxidant enzymes viz. POD, PPO and CAT in pea seedlings were increased significantly at all cadmium acetate concentrations (25 and 50 µM) as compared with control plants (Tab. 4). On the other hand, treatment with the two concentrations of calcium chloride (1.0 and 5.0 mM) caused significantly decreased in the antioxidant activities as being compared with control plants. Treatment with calcium chloride and cadmium acetate caused significant increased in antioxidant enzymes as compared with control plants except at concentration of 1.0 mM calcium chloride in combination with 50 µM cadmium.

Tab. 4. Effect of different concentrations of cadmium on the activities of antioxidants enzymes in pea seedlings in presence or absence of calcium chloride

Treatments	POD Unit / g F.W./ h	PPO Unit/ g F.W. / h	CAT µmole H_2O_2 /g F.W. /h
Control	5.78±0.6^{ef}	3.39±0.2^{f}	19.07±1.7^{ef}
25 µM Cd^{2+}	10.09±1.0^{b}	4.45±0.3^{b}	24.80±2.0^{b}
50 µM Cd^{2+}	13.37±1.2^{a}	5.25±0.6^{a}	26.77±2.2^{a}
1.0 mM Ca^{2+}	5.07±0.7^{ef}	2.87±0.2^{g}	17.57±1.5^{fg}
5.0 mM Ca^{2+}	4.56±0.6^{f}	2.83±0.4^{g}	16.57±1.5^{g}
25 µM Cd^{2+}+1.0 mM Ca^{2+}	7.11±0.8^{cde}	3.68±0.5^{de}	21.43±2.3^{cd}
25 µM Cd^{2+}+5.0 mM Ca^{2+}	8.82±0.9^{bc}	3.93±0.3^{c}	22.13±2.1^{c}
50 µM Cd^{2+}+1.0 mM Ca^{2+}	6.08±0.8^{def}	3.44±0.3^{ef}	20.57±1.0^{de}
50 µM Cd^{2+}+5.0 mM Ca^{2+}	8.11±0.9^{bcd}	3.89±0.4^{cd}	19.73±1.9^{e}
$LSD_{5\%}$	2.32	0.245	1.53

Each value is expressed as mean±SE. Data with different superscript letters were significantly different ($p \leq 0.05$). F.W Fresh weight; D.W Dry weight

It is clearly shown that enzymatic antioxidants (peroxidase, polyphenyl oxidase and catalase) activities in pea seedlings were gradually increased by increasing the concentrations of cadmium. These results are in agreement with that reported by Radeva *et al.* (2010). Peroxidase which participates in lignin biosynthesis might build up a physical barrier against toxic heavy metals. It is supported by the results obtained by Saffar *et al.* (2009) peroxidase and polyphenyl oxidase activities increased in *Arabidopsis thaliana* seedlings with increasing cadmium concentrations when compared to the untreated plants. Moreover, the increase in CAT activity as found herein was also observed by Gomes-Júnior *et al.* (2006) in *Coffea arabica* under Cd-stress conditions. This increase suggests a compensatory mechanism of defense against oxidative stress caused by toxic metal concentrations and can be explained by increase in its substrate to maintain the level of H_2O_2 as an adaptive mechanism of the plants. The activities of antioxidative enzymes in plants treated with calcium chloride in combination with cadmium acetate were significantly increased or non significant effect as being compared with control plants. This indicated that Ca^{2+} is able to increase the biosynthesis of antioxidative enzymes under metal toxicity. These results are in agreement with the observation obtained by Wang and Song (2009) who found that Ca^{2+} application in *Trifolium repens* L. seedlings treated with Cd^{2+} promote the activity of antioxidative enzymes.

Variation of SDS-PAGE banding patterns of protein extracted from pea seedlings in response to cadmium acetate and calcium chloride treatments are shown in Tab. 5 and Fig. 1. Cadmium treatments alone or in combination with calcium chloride were associated with the disappearance of some bands or appearance of new bands. It is clearly shown that, the total number of protein bands in treated and untreated pea plants ranged between 11 and 16 bands. The untreated plant contained 13 polypeptide bands of molecular weights ranged between 170 and 11.9 kDa. The protein banding patterns of seedlings in all treatments comprise four major bands. These bands were recorded at the molecular weights 107, 73, 56 and 15 kDa.

Concerning Cd^{2+} treatments alone or in combination with $CaCl_2$, they induced *de novo* synthesis of nine stress proteins in seedlings having molecular weights ranged between 225 and 8.8 kDa. These inducible proteins might be metal binding polypeptides whose function is to sequester and detoxify excess metal ions which known as phytochelatins. The inducible protein which has molecular weight 8.8 kDa might be phytochelatins. Moreover, the appearance of a polypeptide with a molecular weight 18 kDa in seedlings of treated pea may be calcium binding protein

Tab. 5. Effect of different concentrations of cadmium acetate on the protein patterns separated by SDS- PAGE in pea seedlings in presence or absence of calcium chloride

NO.	M.Wt kDa	Treatments								
		Control	1.0 mM Ca^{2+}	5.0 mM Ca^{2+}	25 µM Cd^{2+}	50 µM Cd^{2+}	25 µM Cd^{2+}+1.0 mM Ca^{2+}	25 µM Cd^{2+}+5.0 mM Ca^{2+}	50 µM Cd^{2+}+1.0 mM Ca^{2+}	50 µM Cd^{2+}+5.0 mM Ca^{2+}
1	225	-	-	-	-	+	+	-	+	+
2	187	-	-	-	+	-	-	-	+	-
3	170	+	-	+	+	+	-	-	+	-
4	155	+	+	+	+	-	+	-	-	+
5	136	+	+	+	+	+	-	-	+	-
6	107	+	+	+	+	+	+	+	+	+
7	73	+	+	+	+	+	+	+	+	+
8	56	+	+	+	+	+	+	+	+	+
9	48	-	+	-	+	-	-	-	-	-
10	46	+	+	+	-	+	+	+	+	+
11	39	+	+	+	-	+	+	+	+	+
12	34	-	+	+	+	+	+	+	+	-
13	29	+	+	-	-	-	-	-	-	+
14	20	+	-	+	+	-	+	+	+	-
15	18	-	+	+	+	+	+	-	-	+
16	15	+	+	+	+	+	+	+	+	+
17	13.5	+	+	+	-	+	-	+	+	+
18	13.1	-	-	-	+	+	+	-	-	-
19	12.7	-	+	+	-	-	-	-	+	+
20	11.9	+	-	-	-	-	-	+	-	-
21	10.4	-	+	+	-	+	-	-	+	+
22	8.8	-	-	-	+	+	+	+	+	-
Total No. of bands		13	15	15	14	15	13	11	16	13

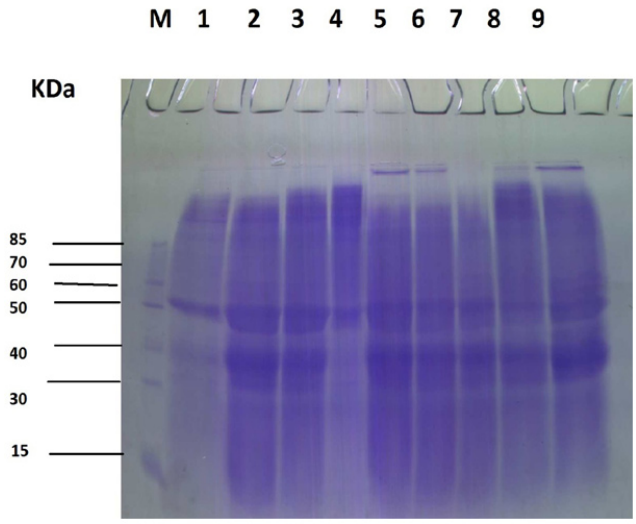

Fig.1. Electrophoretic banding patterns of pea seedlings in response to treatment with different concentrations of cadmium acetate in the presence or absence of calcium chloride

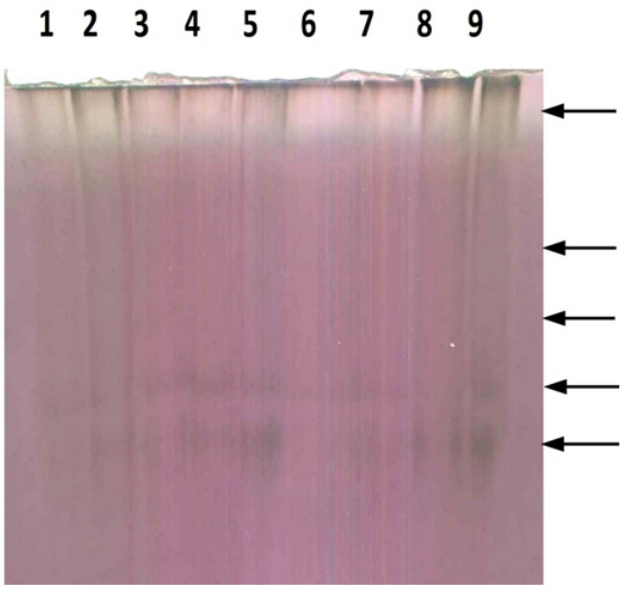

Fig. 2. α- esterase isoenzyme of pea seedlings in response to treatment with different concentrations of cadmium acetate in the presence or absence of calcium chloride

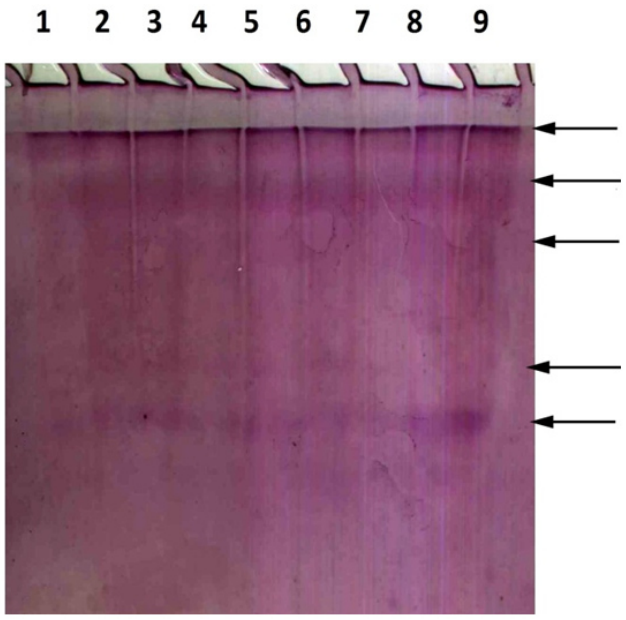

Fig. 3. β- esterase isoenzyme of pea seedlings in response to treatment with different concentrations of cadmium acetate in the presence or absence of calcium chloride

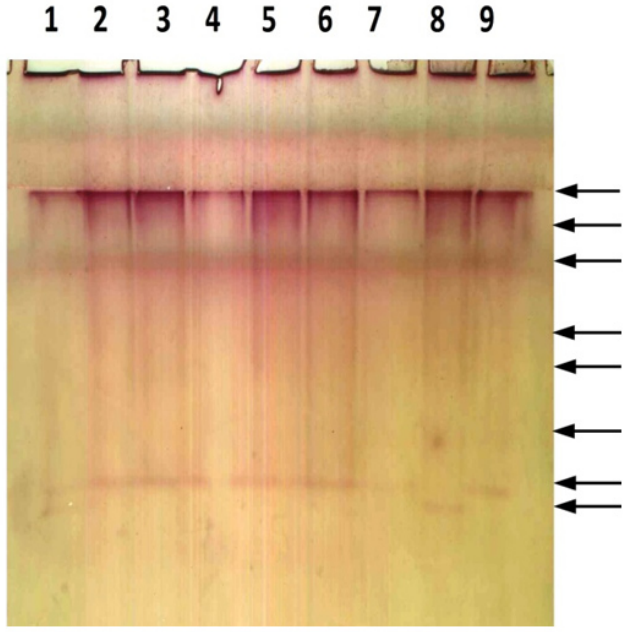

Fig. 4. Acid phosphatase isoenzyme of pea seedlings in response to treatment with different concentrations of cadmium acetate in the presence or absence of calcium chloride

Lanes: 1- Control; 2- 1.0 mM Ca^{2+}; 3- 5.0 mM Ca^{2+}; 4- 25 μM Cd^{2+}; 5- 50 μM Cd^{2+}; 6- 25 μM Cd^{2+}+1.0 mM Ca^{2+}; 7- 25 μM Cd^{2+}+5.0 mM Ca^{2+}; 8- 50 μM Cd^{2+}+1.0 mM Ca^{2+}; 9- 50 μM Cd^{2+}+5.0 mM Ca^{2+}

calmodulin. In this respect, Marme (1989) stated that plants contain several calcium binding proteins, which are considered as ubiquitous protein having molecular mass 17 to 19 kDa. The protein calmodulin is an essential regulatory subunit for some protein kinases. However, calmodulin cannot bind to and activate the enzyme unless it first binds four calcium ions (calcium calmodulin complex). These results are in accordance with Mohamed (2009) who found that treatment with different concentrations of cadmium acetate caused the appearance of a polypeptide with a molecular weight 17 kDa.

Electrophoretic pattern of α-esterase isozyme are illustrated in Tab. 6 and Fig. 2 exhibited a maximum of five bands. Three monomorphic bands at R_f 0.10, 0.56 and

Tab. 6. Effect of different concentrations of cadmium acetate on α- esterase, β- esterase and acid phosphatase isozymes profiles in seedlings of pea plant in presence or absence of calcium chloride

Treatments										
α- esterase										
NO.	Rf	Control	1.0 mM Ca^{2+}	5.0 mM Ca^{2+}	25 µM Cd^{2+}	50 µM Cd^{2+}	25 µM Cd^{2+}+1.0 mM Ca^{2+}	25 µM Cd^{2+}+5.0 mM Ca^{2+}	50 µM Cd^{2+}+1.0 mM Ca^{2+}	50 µM Cd^{2+}+5.0 mM Ca^{2+}
1	0.10	+	+	+	+	+	+	+	+	+
2	0.38	-	+	-	-	-	-	-	+	+
3	0.46	-	+	-	-	-	-	-	+	+
4	0.56	+	+	+	+	+	+	+	+	+
5	0.64	+	+	+	+	+	+	+	+	+
Total No. of bands		3	5	3	3	3	3	3	5	5
β- esterase										
1	0.036	+	+	+	+	+	+	+	+	+
2	0.16	+	+	+	+	+	+	+	+	+
3	0.28	-	-	-	-	-	-	-	+	+
4	0.41	-	-	-	-	-	-	-	+	+
5	0.53	-	-	+	+	+	+	+	-	-
6	0.63	+	+	+	+	+	+	+	+	+
Total No. of bands		3	3	4	4	4	4	4	5	5
acid phosphatase										
1	0.11	+	+	+	+	+	+	+	+	+
2	0.16	-	+	+	-	+	-	-	+	-
3	0.22	+	+	+	+	+	+	+	+	+
4	0.32	-	-	-	-	+	+	-	+	+
5	0.41	-	+	+	-	-	-	-	+	-
6	0.50	-	+	+	-	+	+	-	-	+
7	0.54	-	-	-	-	+	+	-	-	-
8	0.58	+	+	+	+	+	+	+	+	+
9	0.62	-	-	-	-	-	-	-	-	-
Total No. of bands		4	6	6	3	7	7	3	7	6

0.64 were produced in pea seedlings under control and cadmium treatment alone or in combination with calcium chloride and could be considered as common bands. Two polymorphic bands were recognized. The electrophoretic bands showed a wide variation in their intensities and densities among different profiles.

β-esterase electrophoretic patterns are illustrated in Tab. 6 and Fig. 3. A total of five bands were observed among the profiles at all treatments. These five bands were present in some treatments and absent in the others (polymorphic band) which substantial differences in their intensities and densities. Three bands at R_f 0.04, 0.16 and 0.63 were present in all treatments and could be considered as common bands.

Acid phosphatase electrophoretic pattern is shown in Tab. 6 and Fig. 4. A total number of seven bands were observed among the profile at all treatments. Three bands were found in all treatments and have R_f 0.11, 0.22 and 0.58.

The electrophoretic bands of α-, β-esterase and acid phosphatase showed a wide variation in their intensities and densities among different profiles. These results are in agreement with those reported by Bhardwaj *et al*. (2009) who showed that acid phosphatase, peoxidase and esterase isozyme activities were increased with increasing concentration of heavy metals (cadmium and lead) in *Phaseolus vulgaris* L. plants and Mohamed (2009; 2011) who found that treatment of flax and cowpea plants with different concentrations of cadmium acetate and lead acetate showed a wide variation in their intensities and densities of α-, β-esterase and acid phosphatase isozyme. The intensity of bands in the high concentrations of cadmium acetate (50 µM) alone or in combination with calcium chloride was induced compared to control treatments. This stimulation is known to be caused by a metal induced *de novo* synthesis of the enzyme protein (Pereira *et al.,* 2002). Generally, increasing the activity of hydrolytic enzymes (acid phosphatase) after plants treatment with heavy metals reflects a general senescence response induced by heavy metal ions (Lee *et al.,* 1976). Also, the appearance or the disappearance of the fast bands may be due to that heavy metals acts at the nucleic acid level in which transcriptional, post-transcriptional, translational, or other inhibition processes may be involved.

Involvement of calcium in the alleviation of cadmium toxicity by reducing the uptake, accumulation and toxic effects of Cd was reported in *Arabidopsis* and common bean (Ismail, 2008; Suzuki, 2005). Since plasma membrane surface are usually negatively charged, high level Ca^{2+} would reduce cell-surface negativity and alleviate the harmfulness of cadmium toxicity. There is a report that the uptake of Cd is inhibited by the Ca^{+2} channel blockers (Ismail, 2008). As a major defense mechanism, the inactivation of these metal ions could be accomplished by complexing them with phytochelatins such as metalothionin and cysteine-rich proteins.

In conclusion, Calcium can used to alleviate the harmful effect of cadmium. Calcium regulates the activities of target proteins directly or via calcium-binding proteins, such as calmodulin.

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