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TOXICOLOGICAL EFFECTS, OXIDATIVE STRESS AND BIO-ACCUMULATION IN THE TISSUES OF *Phaseolus vulgaris* L. BEAN SEEDLINGS FOLLOWING CADMIUM EXPOSURE

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Abstract

Demirezen Yilmaz D., Uruç-Parlak K., Vural C.: Toxicological effects, oxidative stress and bio-accumulation in the tissues of *Phaseolus vulgaris* L. bean seedlings following cadmium exposure . Ekológia (Bratislava), Vol. 31, No. 1, p. 92–104, 2012.

A hydroponic experiment was carried out in a growth chamber to investigate the impact of cadmium (Cd) levels on the anatomical, morphological, and biochemical characteristics of a bean. The membrane lipid peroxidation (LPO), proline accumulation and the activity of bean seedling catalase following Cd toxicity were investigated. A significant increase in lipid peroxidation (LPO) and a the stimulation of catalase (CAT, 1.11.1.6) activity were recorded in bean seedlings subjected to 2, 4, 8 and 16 ppm Cd. Plant height and chlorophyll content of the seedlings were also significantly affected in a dose-dependent manner by cadmium treatment. In addition a high cadmium concentration caused a significant increase in the number and size of the spongy mesophyll cells. The cortical tissue of a cadmium-exposed shoot is broader and contains large cells. Results also indicated that nodulation was inhibited by increasing metal concentrations and there was a considerable amount of proline accumulation observed in response to Cd treatment. The results also indicated that increasing the activity of the anti-oxidant enzymes was insufficient to protect the cell membrane against Cd toxicity.

Key words: LPO, cadmium, antioxidant enzymes, proline, bean

Introduction

Cadmium (Cd) is one of the globally distributed toxic elements which enters the environment from natural sources such as the weathering of rocks and also anthropogenic impacts and is then is transferred to the food chain (Alia, Matysik, 2001; Bates et al., 1973). It exerts adverse

effects on morphology and growth including chlorosis, reduction in biomass, inhibition of root elongation and photosynthetic processes and finally death. In addition it is the cause of enzyme activitie inhibition water imbalance and alterations in membrane permeability as well as disturbances in mineral nutrition (Bosma et al., 1991; Bradford, 1976). Depending on its concentration and the plant species involved, Cd can either inhibit or stimulate the activity of several anti-oxidative enzymes before any visible symptoms of toxicity appear (Brown, Shrift, 1982).

Phyto-toxicity from heavy metals is closely related to production of reactive oxygen species (ROS) in plants (Mithofer et al., 2004). It has been demonstrated that excessive nickel and cadmium lead to a significant increase in the concentration of hydrogen peroxide (H_2O_2) (Boominathan, Doran, 2002) and also to membrane lipid per-oxidation in a few plant species (Baccouch et al., 2001).

In this study we investigated the effects of Cd toxicity on growth parameters, membrane LPO, proline accumulations and the catalase activity (CAT) in the tissues of a bean cultivar.

Material and methods

Plant growth, sample analysis and metal estimation

Seeds of the Akman bean cultivar, used in this study were obtained from Central Agricultural Research Institute of Ankara in Turkey. The seeds were surface-sterilized with 10% sodium hypochlorite solution for 10 min and then washed and imbibed in distilled water for 1 day. The imbibed 10–15 seeds were planted in plastic cups covered with cheesecloth containing Hoagland's solution and grown for 7 days in a growth chamber at 25 °C with a 16 hour light: 8 hour dark photo-cycle in a light intensity of 40 $mmolm^{-2}$ s^{-1}. Heavy metal treatment was started on the 8th day by applying Hoagland's solution of 2, 4, 8 and 16 ppm cadmium chloride and this treatment was continued for three days. Cadmium concentrations were determined in preliminary experiments. The solutions were interchanged every 24 hours. After 15 days, the leaves were harvested and used directly for analysis or they were frozen in liquid nitrogen and stored at -20 °C for later use.

Dried bean samples were digested in 10 ml of concentrated HNO_3, using a CEM microwave digestion system. The volume of each sample was adjusted to 25 ml using double de-ionized water following this digestion (Demirezen Yılmaz, 2007). Determination of the cadmium concentrations in all samples was conducted by Inductively Coupled Plasma Optical Emission Spectrometry (Varian), with all samples analyzed in triplicate.

Microscopy and morphometry

The methods developed by Panou-Filotheou et al. (2001) were used in the determination of the anatomical and morphological changes in tissues. Microscopic investigations were carried out under OLYMPUS BH-2 Research Light Microscope and photographed with a Nikon Coolpix 4500 photo-microscope.

Chlorophyll determination

The amount of chlorophyll was determined according to the method described by Knudson et al. (1977).

Lipid per-oxidation

The LPO was determined by the method of Teresa et al. (2003) by measuring the amount of TBARS. Approximately 0.3 g leaf tissue from control and treated plants was homogenized in 3 ml of 5% trichloroacetic acid (TCA) using cold mortar and pestle. Homogenates were then transferred into fresh tubes and centrifuged at 12.000 g for 15 minutes at room temperature and 4 ml of 0.5% thiobarbituric acid (TBA) in 20% freshly prepared TCA solution was added to 1 ml of supernatant and then incubated at 96 °C for 30 min. The tubes were then cooled by transfer into an ice bath and centrifuged at 12.000 g for 10 minutes. The absorbance of the supernatant was recorded at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. TBARS content was determined by using its extinction of 155 $mM^{-1}1\ cm^{-1}$.

Proline determination

The amount of proline was determined according to the method described by Bates et al. (1973). Approximately 0.3 g of leaf tissue from control and treated plants was homogenized with the addition of 5 ml of 3% sulphosalicylic acid solution. The leaves were homogenized by using a cold pestle and mortar. Homogenates were then centrifuged at 5000 g for 10 minutes at 4 °C. For each sample, a glass tube containing 2 ml of acid ninhydrin (0.31 g ninhydrin, 7.5 ml of acetic acid, and 5 ml of 6M phosphoric acid), 2 ml of 96% acetic acid and 1 ml of 3% sulpho-salicylic acid were prepared, and 2 ml supernatant from each homogenate was added to the tubes. The tubes were incubated at 96 °C for 1 h in a hot-water bath and 4 ml of toluene was added to each tube, followed by mixing. The absorbance of the pink red upper phase was recorded at 520 nm against the toluene blank, and a standard curve for proline in the range of 0.01 μM−1.5mM was constructed to determine the proline concentration in each sample.

Enzyme extraction and protein determination

Fresh leaf tissue weighing 1 g was homogenized in liquid nitrogen in a pestle and then mortar and suspended in 3.0 ml of 0.1 M pH 7.5 Trise-HCl buffer which contained 0.5 mM of ethylenediaminetetraacetic acid EDTA and 1.0% of polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 15.000 g for 20 minutes and the supernatant was used as an enzyme source in all enzyme analysis. The protein concentration in the leaf extracts was determined according to the Bradford method (1976), and bovine serum albumin was used as the standard.

Determination of catalase (EC 1.11.1.6)

The activity of CAT was measured according to the method described by Aebi (1984). For CAT, the assay medium consisted of 20 mM sodium phosphate buffer (pH 7.5), 6 mM H_2O_2, and crude extract containing 3 $mgml^{-1}$ protein in a final volume of 1 ml. The reaction was initiated by the addition of H_2O_2. The decrease in the absorbance of H_2O_2 was recorded at 240 nm for 3 minutes. The enzyme activity was calculated as the amount of H_2O_2 consumed using the H_2O_2 extinction coefficient of 40 $mM^{-1}\ cm^{-1}$ at 240 nm.

Statistical analysis

The significance of the difference between mean values obtained from at least 3 independent experiments was determined by one-way analysis of variance (ANOVA) at 95% confidence interval by using SPSS for windows (SPSS version 10, 2002).

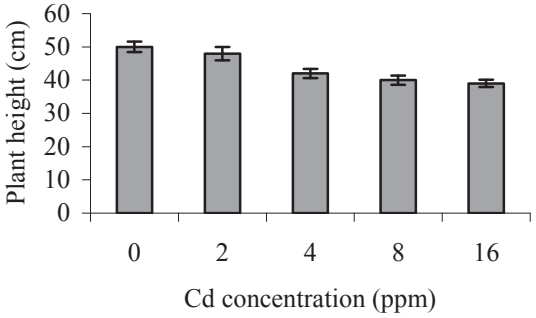

Fig. 1. Plant heights of the bean seedlings subjected to 2, 4, 8, 16 ppm Cd.

Results

Effect of Cd treatment on plant growth parameters and metal accumulation

Increasing concentrations of cadmium considerably affected the plant height and a significant decrease was observed in response to heavy metal toxicity (Fig. 1). The mean plant height under 16 ppm Cd stress was 39 cm compared to the control plant height of 50 cm (Fig. 1). In this study, the 16 ppm treatment group was most affected, although all other groups, except 2 ppm were also significantly different to the control group with $p < 0.05$).

Bean plants exhibited morphological alterations after exposure to increasing concentrations of cadmium. Results showed that high concentrations of cadmium in the media affected the morphology of *Phaseolus vulgaris* in a number of ways. For example, significant differences in size and, plant height were obtained between control and metal-treated plants. The control group leaf size was larger than those of other groups (data not shown). Furthermore, the results obtained indicated that nodulation decreased linearly with increasing bean cadmium levels (Table 1). For example, nodulation was significantly inhibited by the addition of 16 ppm Cd to 7 ± 0.99 compared with the control with 41 ± 0.11.

T a b l e 1. Effect of cadmium on root nodulation of *Phaseolus vulgaris*. (Means within in the same columns with different letters are statistically significant; $p < 0.05$).

Number of plants treated with Cd	Cd concentrations (ppm)	Number of nodules /bean for Cd
45	0	41 ± 0.11[a]
45	2	27 ± 0.8 [b]
45	4	18 ± 1.2 [b]
45	8	11 ± 0.9 [c]
45	16	7 ± 0.99 [d]

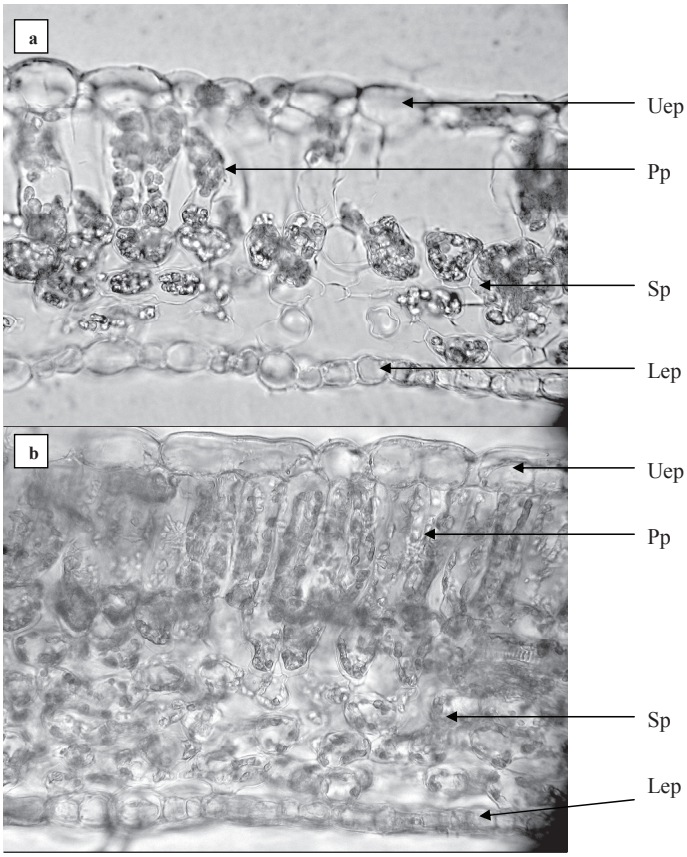

Fig. 2. Comparison of cross sections of leaf in control (a), 16 ppm Cd treated (x400). Uep – upper epidermis, Pp – Palisade parenchyma, Sp – spongy parenchyma, Lep – lower epidermis.

Furthermore, high cadmium concentration caused a significant increase in the number and size of spongy mesophyll cells, and the increase in the spongy cell biomass was at the expense of the intercellular spaces (Fig. 2). However, the results also indicated that palisade parenchyma cells did not increase in a similar way. Additionally, a further difference in chloroplasts was observed between the leaf anatomy of the control and heavy metal treated plants. Chloroplasts appeared larger and more numerous in the control leaves than those in cadmium treated leaves of *P. vulgaris*. Furthermore, growth of the main roots was inhibited and the numbers of lateral roots were reduced by the 8 and 16 ppm cadmium treatments (Fig. 3).

The mean values of cadmium in the *P. vulgaris* tissues studied are illustrated in Table 2. The concentrations of cadmium in this plant's tissues are quite variable, including, 0.3–9.43

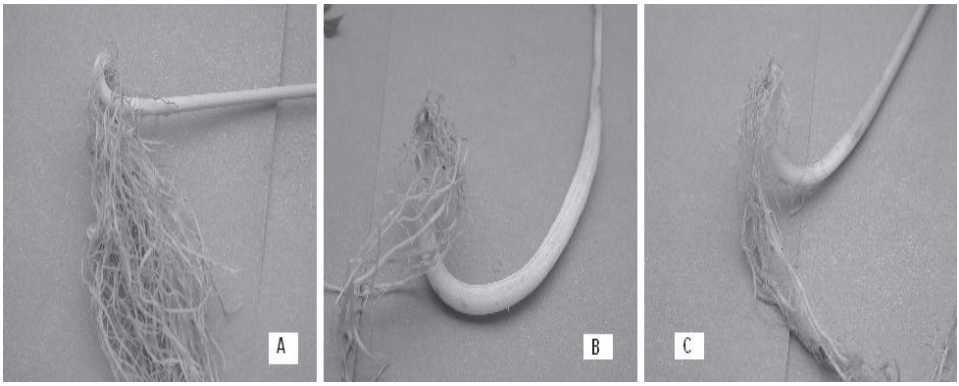

Fig. 3. The differences in size and shape of roots in control (A), 16 ppm Cd treated (B) and 4 ppm Pb treated (C).

ppm for roots and 0.1–0.7 ppm for leaves. The accumulation of cadmium in the roots is higher than in the leaves and shoots.

Effect of Cd treatment on chlorophyll content

A significant decrease was observed in the chlorophyll content at 2, 4, 8, and 16 ppm Cd applications ($p < 0.05$). The most and the least affected groups were those treated with 16 and 2 ppm Cd. There were approximately 1.8 fold differences between the control and 16 ppm Cd application (Fig. 4) and no significant difference was observed between the 8 and 16 ppm Cd levels.

Effect of Cd treatment on LPO

The effect of Cd treatment on LPO was determined by evaluating the tissue contents of TBARS. Cadmium toxicity caused significant increase in TBARS contents in the leaf tissue in a dose dependent manner (Fig. 5). TBARS accumulation was statistically significant above

T a b l e 2. Cadmium concentrations (ppm) in different tissues of *P. vulgaris.* (Means within in the same rows with different letters are statistically significant; $p < 0.05$).

	Cadmium (ppm)				
0	2	4	8	16	
Root	0.3 ± 0.001^{a}	1.2 ± 0.07^{a}	8.5 ± 1.4^{b}	9.4 ± 2.2^{c}	9.43 ± 1.1^{c}
Shoot	0.2 ± 0.001^{a}	0.9 ± 0.01^{b}	2.1 ± 0.3^{b}	2.7 ± 1.1^{b}	3.1 ± 0.9^{c}
Leaves	0.1 ± 0.002^{a}	0.4 ± 0.01^{a}	1.1 ± 0.1^{b}	1 ± 0.05^{b}	0.7 ± 0.04^{a}

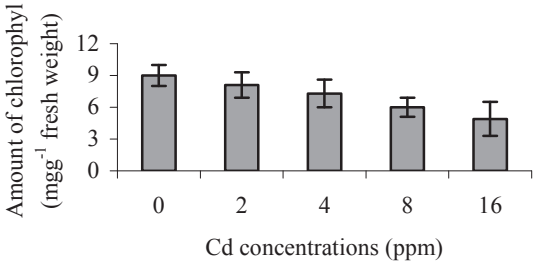

Fig. 4. Amount of chlorophyll in the leaf tissues of the bean seedlings subjected to 2, 4, 8, 16 ppm Cd.

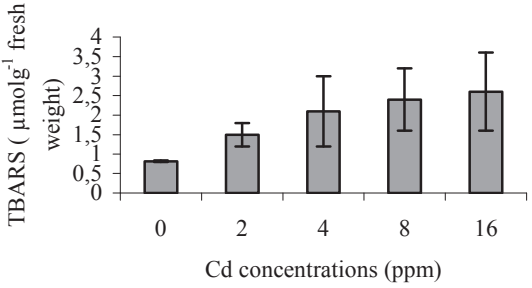

Fig. 5. TBARS levels of leaf tissues in the bean seedlings subjected to 2, 4, 8,16 ppm Cd.

the 2 ppm Cd level, with a 3.2 fold increases, at 16 ppm Cd. The mean TBARS accumulation values between the control and 8–16 ppm, between 2 and 8 ppm and 2–16 ppm Cd levels were statistically significant at $p < 0.05$ (Fig. 5).

Effect of Cd treatment on proline accumulation

The mean differences in proline content between the control and all cadmium treated groups were found to be statistically significant and the mean proline content differences between 2, 4, 8 and 16 ppm Cd treatment were also statistically significant (all at $p < 0.05$). There was approximately a 5.3 fold difference between the control and the 16 ppm Cd treatment (Fig. 6).

Effect of Cd treatment on catalase activity

There was significantly different CAT activity for all Cd treatment levels at the $p < 0.05$ level and a 3.5 fold difference between the control and the 16 ppm Cd treatment (Fig. 7).

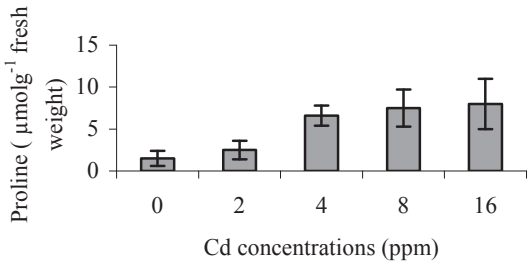

Fig. 6. Proline accumulations in the leaf tissues of the bean seedlings subjected to 2, 4, 8, 16 ppm Cd.

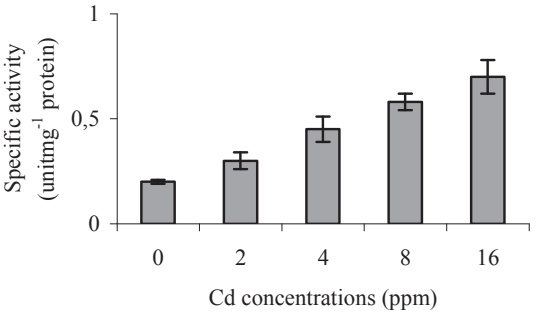

Fig. 7. CAT activity in the leaf tissues of the bean seedlings subjected to 2, 4, 8, 16 ppm Cd.

Discussion

Plants possess a remarkable but differential ability to take up and accumulate various essential and non-essential elements including heavy metals from their external environment. Cadmium is a non-essential element in anti-oxidation reactions in plant cell growth (Zhang et al., 2007). Plants are generally not considered to require cadmium and their tolerance to it is quite low. Plant toxicity at higher concentrations of Cd has been reported by several researchers (Amani, 2008; Teresa et al., 2003).

In this study, *P. vulgaris* was grown in hydroponic culture in the presence of increasing Cd concentrations, so that the plant's uptake and growth and defence mechanisms could be evaluated. The results indicated that Cd is toxic for *P. vulgaris*. The higher accumulation of Cd in *P. vulgaris* found in our study could reflect different cellular mechanisms for the bio-concentration of essential and non-essential trace metals within plants (Richards, 1954; Erdei, Hegedüs, 2002).

Effect of Cd on plant growth parameters and metal accumulation

The critical level of Cd above which a significant reduction in dry-matter yield occurred was found to be different for different plants. An increase in Cd content in plant tissues resulted in a considerable decrease in the biomass for several plants. During this investigation an obvious reduction in plant growth was observed at Cd treatments above 4 ppm in hydroponic cultures (Fig. 1). Decreased seedling vigoour in bean plants due to excessive cadmium could possibly be attributed to the interference of Cd in the metabolic and biochemical processes, such as protein and chlorophyll synthesis. In accordance with the results from this study, Teresa et al. (2003) reported a significant reduction in root and shoot yield in wheat plants after the application of more than 2.2 μM Cd.

Results obtained here in this study demonstrated that cadmium concentrations ranging from 4 to 16 ppm inhibited plant growth and the bean plants exhibited morphological alterations on exposure to increasing concentrations of cadmium. Cadmium treatments caused shortening in stem size and a reduction in the root volume and number of nodules in bean plants. Das et al., (1997) stated that plants with numerous thin roots accumulate more metals than those with few broad roots. The greater surface area of thin and long roots compared to that of broad and short roots accounts for the higher Cd absorption by the plant's roots and high levels of cadmium almost completely inhibit nodulation (Table 1). These results agree with the observations described by Chen et al. (2003) who found that cadmium stress has significant deleterious effects on root nodulation.

The results indicated that high cadmium concentration caused a significant increase in the number and size of spongy mesophyll cells. Furthermore chloroplasts in the control leaves appeared larger and more numerous than in the cadmium treated leaves of *P. vulgaris*. Similar observations were obtained from leaves of *Oregano* sp. treated with copper (Panou -Filotheou et al., 2001) and also in *Brassica juncea* by (Epstein et al., 1999) and in *Phaseolus vulgaris* (Geebelen et al., 2002).

Results indicated an increase in Cd accumulation in leaves and roots with increasing Cd concentration in the bean plant's growth medium (Table 2). Cd accumulated primarily in the roots, and small amounts were also transferred to the leaves. Cd distribution in plant tissues indicates a change in chemical form resulting from complexes formed with plant-produced ligands. Most of Cd transported into plants is chelated by chemical compounds such as organic acids and phytochelatins. Only traces of Cd can be detected as a free ion, and Cd toxicity is related to the free Cd contamination. Therefore, in plants which are not hyper-accumulators of Cd, previously been shown, to be the major site of phytochelatin synthesis and hence of Cd accumulation (Di Cagno et al., 1999; Zhu et al., 1999). Results here have also shown that after its application, Cd accumulated in roots at a faster rate and to a much higher concentration than in the leaves (Table 2). Similar results were also found in soybean where approximately 98% of the accumulated Cd was retained in the roots (Becher, Hofner, 1994).

Effect of Cd on chlorophyll content

The chlorophyll content in leaf tissues was significantly lower when Cd levels were above 4 ppm. This inhibition of the chlorophyll biosynthesis caused a subsequent reduced chlorophyll accumulation in leaves which was proportional to the increased Cd level in the hydroponic culture. Similarly, severe Cd toxicity is reported to decrease the total chlorophyll content has been result in chlorosis in maize (Ekmekçi et al., 2008).

Leaf chlorosis is one of the most commonly observed effects of Cd toxicity (Wu et al., 2003). A decrease in the photosynthetic activity may be partly due to the decreased chlorophyll content.

LPO

The TBARS contents of shoot tissues were slightly increased at the 4 ppm Cd level and were more prominent at the higher concentrations of 8–16 ppm (Fig. 5). The level at 16 ppm was 3.2 fold higher than that of the control. The effect of Cd on plant LPO was dependent on cadmium dosage. At higher concentrations, the antioxidative function of Cd seemed to be decreased or reversed with LPO being higher than the low Cd level. Dong et al. (2006) described a similar tendency in Cd-stressed tomato seedlings, where they noticed a significant increase in the MDA content compared to that of the control at different Cd concentration.

Proline accumulation

Proline has been shown to play an important role in recovering from environmental stresses including heavy metal stress in plants and microorganisms. Drought, salinity, and heavy metal toxicity induce proline accumulation in algae and higher plants (Stadtman, 1990). However, the function of proline accumulation in response to metal stress is still not very clear. Significant proline accumulation was observed in the leaves of Cd-treated bean seedlings in a dose dependent manner (Fig. 6). Proline accumulation may be induced as a result of ROS accumulation. The mechanisms by which proline reduces the oxidative damage include the physical quenching of singlet oxygen and chemical reaction with hydroxyl radicals (Alia, Matysik, 2001). Due to its chelating ability, proline may also be a defence mechanism for the survival of stressed plants by binding with metal ions. Increase in both proline and TBARS content with increasing metal ion concentration is indicative of a correlation between free radical generation and proline accumulation. Although, it has been suggested that proline can protect plants from heavy metal toxicity (Farago, Mullen, 1979), many researchers believe that the proline accumulation is simply an indicator of various stresses, and that it is not involved in protecting plants against metal toxicity. Evidence that proline accumulation did not occur until plant tissues had already been damaged demonstrated that heavy metal-induced proline accumulation obviously did not prevent metal toxicity (Lutts et al., 1996; Schat et al. (1997). Similarly, despite substantial proline accumulation, the membrane

damage exhibited by LPO did not decrease over the course of this study. This therefore also suggests that proline accumulation is not involved in protection against cadmium induced oxidative damage.

Catalase

Plants have evolved various protective mechanisms to eliminate or reduce ROS. One of them is the enzymatic antioxidant system, including SOD, APX, CAT, GR, GST and Glutathione peroxidase (GSH-Px). Each of these enzymes has a physiological function under non-stressed conditions, but their activity or quantity is increased under oxidative damage. Many biotic and abiotic stresses including drought, extreme temperature, soil salinity and heavy metals are recognized as causing oxidative damage to plants either directly or indirectly by triggering an increase in ROS. In resisting oxidation damage, these antioxidant enzymes and certain other metabolites play an important role in the adaptation and ultimate survival of plants during stressful periods (Mittler, 2002). This study suggests that Cd toxicity leads to production of lipid peroxides and induces some of the crucial enzymes in the antioxidant defence system in bean seedlings. However, since the role that cell antioxidant systems play in Cd detoxification is still not clear. Cd detoxification mechanisms at the cellular level and the methods plants use to defend themselves against Cd still require investigation. Our results demonstrated that Cd toxicity-mediated changes in the catalase activity are dose dependent. Significantly higher activity was observed for CAT which scavengers of H_2O_2 and this increased activity may result in the reduced formation of superoxide anion radicals. As depicted in Fig. 7, increased LPO levels and decreased chlorophyll contents were observed when a remarkable in CAT activity occurs. The antioxidant responses of this bean to Cd treatment were found to be dose dependent.

These results indicate that the increase in the activity of antioxidant enzymes (CAT) was not adequate to protect the bean cells from the Cd-induced toxic damage. In accordance with this finding, Cavalcanti et al. (2004) reported that SOD, CAT, and POX do not confer protection from oxidative damage in the salt-stressed leaves of cowpea. Similar results were obtained when *Allium sativum* was subjected to varying levels of Cd. Although treatments ranging from 1 to 10 mM caused a clear increase in CAT activity, they also caused an increase in the LPO level (Zhang et al., 2005). CAT is a universal oxido-reductase which decomposes H_2O_2 to water and molecular oxygen and it is thus one of the major ROS scavenging mechanisms involved in the removal of toxic peroxides (Mittler, 2002).

In summary, our results strongly suggest that higher cadmium levels causes oxidative stress in bean cells they are capable of causing membrane damage through production of ROS and they interfere with chlorophyll metabolism. These higher Cd levels also induce CAT activity. Furthermore, although antioxidant enzymes significantly induced they are at insufficient levels to protect bean cells from Cd toxicity. Consequently, it is most likely that antioxidant enzyme activities are not involved in the mechanism(s) of Cd stress tolerance. Although the physiological mechanisms responsible for cadmium's effect on antioxidant enzymes cannot be explained by results obtained during this current investigation, it is

note- worthy that higher Cd concentrations did trigger the bean's antioxidant responses by activation of oxido-reductases. Nevertheless, Cd-induced oxidative stress occurred at high concentrations, exhibiting its role as a pro-oxidant, and this is supported by previous reports (Cartes et al., 2005; Hartikainen et al., 2000; Nowak et al., 2004). These results suggest that there must be alternative ways in which Cd-tolerant plants detoxify the effects of Cd. It is further suggested that detection and analysis of induced proteins in stressed Cd-tolerant plants can help increase our understanding of the mechanism of Cd tolerance in plants.

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