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CADMIUM INDUCED OXIDATIVE DAMAGE AND ANTIOXIDANT RESPONSES IN ROOTS OF BLACK GRAM [*VIGNA MUNGO* (L.) HEPPER]

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SUMMARY

A study was undertaken to investigate the effect of cadmium (Cd) on seed germination, growth and dry weight accumulation in black gram [*Vigna mungo* (L.) Hepper] cv. T-9, together with an analysis of the changes induced by Cd on the antioxidant enzyme status in the plant roots. Low concentrations of Cd were found to be slightly stimulatory while excess of Cd inhibited root and shoot growth, reduced dry matter accumulation. The activity of antioxidant enzymes varied with the concentration of Cd. A significant increase in malondialdehyde (MDA) content and stimulation of superoxide dismutase (SOD), peroxidase (PER), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) was noted in seedlings exposed to 10, 50 and 100 µM Cd, while catalase (CAT) activity showed significant decrease above 10 µM. Excess of Cd caused lipid peroxidation as well as modified the activity of defense enzymes SOD, PER, CAT, PPO and PAL.

Key words*: Antioxidant enzymes, cadmium, heavy metal, oxidative stress, *Vigna mungo

INTRODUCTION

Anthropogenic activities have resulted in increased contamination of the environment with toxic heavy metals. Among metal pollutants Cadmium (Cd), is regarded as a metal with no known biological function. It is toxic to humans, animals and plants and enters the environment mainly from industrial processes and phosphate fertilizers, and is then transferred to the food chain (Wagner 1993). Environmental pollution by Cd is of increasing scientific interest as Cd is readily taken up by the roots of many plant species and its toxicity is 2-20 times higher than that for other heavy metals (Jagodin *et al*. 1995). Cd has been reported to be strongly phytotoxic causing growth inhibition and even plant death, due to its interaction with photosynthesis, respiration and nitrogen assimilation in plants (Sanità di Toppi and Gabbrielli 1999). It is reported to produce alterations in the functionality of membranes by inducing changes in lipid composition (Ouariti *et al*. 1997).

Cd induced oxidative stress has been documented in plants (Dixit *et al*. 2001, Wu *et al*. 2002). Increased oxidative stress leads to the production of reactive oxygen species (ROS). The activities of antioxidant enzymes are inducible by oxidative stress due to exposure to various types of stresses, and represent a general plant response to adverse conditions (Foyer *et al*. 1994). These enzymes help in scavenging ROS and thus prevent cellular damage. But, the direction and size of the response vary with plant species and tissues analyzed, and the kind and intensity of stress treatment (Schützendübel and Polle 2002). This study focuses on the effect of Cd on seed germination, seedling growth, biomass accumulation, examines the response of antioxidant enzymes, namely superoxide dismutase

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(SOD), peroxidase (PER), catalase (CAT), and also changes in enzymes such as polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) in the roots of black gram (*Vigna mungo*). Malondialdehyde (MDA) concentration, which is an index of lipid peroxidation, was also measured.

MATERIALS AND METHODS

Black gram [*Vigna mungo* (L.) Hepper] cv. T-9 seeds, purchased from the National Seed Cooperation, Thiruvananthapuram, Kerala, were used for the present investigation. Uniform sized seeds were surface sterilized, washed and germinated in, sterilized, covered, disposable petri dishes (15cms diameter) lined with Whatman No.1 filter paper, soaked in varying concentrations (10 µM, 50 µM, 100 µM, 250 µM and 500 µM) of cadmium chloride ($CdCl_2\,2½H_2O$). Distilled water soaked seeds were taken as control. Six replicates of control and the different treatments, each containing 25 seeds per petri dish were used for the experiment. The breaking of the testa was considered as the criterion for germination. The effect of Cd on germination and growth was assessed in terms of percentage (%) of germination after 24 hours (h), and seedling vigor, tolerance index, % phytotoxicity and dry weight after 96 h of germination. Seedling vigor was calculated by multiplying the germination % with total length (cm) of the seedling (Woodstock 1969, Abdul-Baki and Anderson 1973). Tolerence index (TI) calculated according to the formula of Turnur and Marshal (1972).

$$TI = \frac{\textit{Mean length of the longest root in test solution}}{\textit{Mean length of the longest root in control solution}} \times 100$$

Percent (%) Phytotoxicity was calculated following the standard bioassay technique (Chou *et al.* 1972).

$$\text{Percent phytotoxicity} = \frac{\textit{Radicle length of control - Radicle length of test}}{\textit{Radicle length of control}} \times 100$$

Enzyme extraction and assay

Enzyme extraction and assays on the root samples were carried out after 96 h. Weighed samples (500-1000 mg) were homogenized in known volume of extraction buffer containing sodium phosphate buffer (0.1M, pH 7.5), 1% (v/v) Triton X-100 and 7 mM β mercaptoethanol), centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was preserved in ice. The residue was again extracted with known volume of extraction buffer, centrifuged and the combined supernatant served as enzyme source for SOD, PER, CAT, and PPO.

SOD (*E.C. 1.15.1*), was estimated following the method of Dhindsa *et al.* (1981). Each 3 ml of the assay mixture consisted of 0.1 ml supernatant, 1.5 ml phosphate buffer (0.1 M, pH 7.8), 0.1 ml sodium carbonate (1.5 M), 0.1 ml nitroblueterazolium {NBT (2.25 mM)}, 0.2 ml methionine (200 mM), 0.1 ml ethylenediamine tetra-aceticacid {EDTA (3 mM)}, 0.1 ml riboflavin (60 µM) and 0.8 ml distilled water. The sample tubes were illuminated with 15 W fluorescent lamp for 10 min. The tubes lacking enzyme extract but containing the assay mixture were also illuminated and served as control. A non irradiated complete reaction mixture served as blank. Absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as the quantity of enzyme that reduced the absorbance reading to 50% in comparison with the control.

PER (*E.C.1.11.1.7*), was measured by the modified method of Chance and Machly (1955) and changes in absorbance recorded at 470 nm to the oxidation of guaiacol in the presence of H_2O_2. The assay mixture consisted of 0.1 ml of guiacol (0.02 M), 3.0 ml of sodium phosphate buffer (0.1 M, pH 7) and 0.05 ml of enzyme. The reaction was initiated by the addition of 0.05 ml of H_2O_2. Enzyme activity was expressed as mmols of guaiacol oxidized min^{-1} gm^{-1} Fresh weight (F.wt.) of tissue.

CAT (*E.C.1.11.1.6*), activity was determined by the method of Kar and Mishra (1976). The reaction mixture contained 3 ml of phosphate buffer, 1 ml of 0.1 M hydrogen peroxide (H_2O_2) and 1.0 ml enzyme extract and the mixture was incubated at 25°C for 1 min. The reaction is stopped by the addition of 10 ml of 2% H_2SO_4 and titrated against 0.01 N potassium permanganate ($KMnO_4$) until a faint pink color persisted for 15 seconds. The volume of $KMnO_4$ used was noted. The control has

enzyme activity stopped by addition of 2% H_2SO_4, prior to the addition of enzyme extract. One unit of enzyme activity is defined as that amount of enzyme which breaks down 1µmole of H_2O_2 min^{-1} under the assay conditions described.

PPO (*EC.1.14.18.1*) was determined by the method of Yamaguchi *et al.* (1970). The assay mixture consisted of 1.0 ml catechol (0.01 M), 1.0 ml proline (0.01 M), 2.5 ml sodium phosphate buffer (0.01 M, pH 6.5) and 0.5 ml enzyme extract. The change in absorbance was recorded at 495 nm. The enzyme activity was calculated and expressed mmols of catechol oxidized min^{-1} gm^{-1} F.wt of tissue.

PAL (*E.C. 4.3.1.5*). PAL activity was assayed by the method of Brueske (1980). Weighed samples (500-1000 mg) were crushed in 80% ice cold acetone, filtered through Whatman No. 42 filter paper and residue collected. After evaporation of the acetone content of the residue it was dissolved in 5 ml of 0.2 mM borate buffer pH 8.8 containing 5 mM ß-mercaptoethanol and used as enzyme source. To 0.2 ml of the enzyme extract, 1 ml of (0.1 M) L- phenylalanine and 0.5 ml of 0.2 M borate buffer pH 8.8 was added and incubated in a water bath at 40°C for one hour. In the control, phenylalanine was added after the addition of TCA. The reaction stopped by adding 0.5 ml of 1 M TCA. The absorbance read at 290 nm and the activity was expressed as µg of *trans*-cinnamic acid formed gm^{-1} F.wt. of tissue.

MDA content was determined using thiobarbituric acid (TBA) reaction (Heath and Packer 1968). Weighed tissue (100-500 mg) was homogenized in 5 ml 1% TCA and the homogenate was centrifuged at 10000 rpm for 15 min at room temperature. The supernatant was mixed with equal volume of TBA reagent (0.5% TBA in 20% TCA). The mixture was incubated at 95°C for 30 min, cooled in ice and centrifuged for 10 min at 5000 rpm to clarify the solution. Absorbance was measured at 532 nm and corrected for nonspecific absorbance by subtracting the absorbance at 600 nm. MDA content was calculated using the extinction coefficient of 155 mM cm^{-1}.

Statistical analysis was done using SPSS 7.5 software**.** Difference between the control and treated plants were analyzed by one way ANOVA taking $p<0.05$ as significant according to Duncan's multiple range test. Figures in tables indicate mean values ± standard deviation. Numbers followed by the same letter in the same column are not statistically different.

RESULTS AND DISCUSSION

Cd at high concentrations decreased seedling length and dry matter yield (Table 1). Toxic effect of Cd was more pronounced in the root than shoots. Seedling growth assessed in terms of seedling vigor, tolerance index, % phytotoxicity and seedling vigor was adversely affected by increasing concentrations of Cd (Table 2). Seed germination was less affected by Cd when

Table 1. Effect of cadmium on seedling growth and dry matter yield in *Vigna mungo,* after 96 h of germination.

Treatment $CdCl_2$ (µM)	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Root dry wt. (mg $seedling^{-1}$)	Shoot dry wt. (mg $seedling^{-1}$)
0	6.91 ± 0.12^{d}	5.71 ± 0.54^{c}	12.61 ± 0.85^{d}	3.18 ± 0.29^{d}	9.58 ± 0.51^{d}
10	7.35 ± 0.13^{e}	6.09 ± 0.19^{d}	13.45 ± 0.30^{e}	2.51 ± 0.46^{c}	10.01 ± 0.50^{d}
50	7.41 ± 0.48^{e}	5.56 ± 0.60^{bc}	12.96 ± 0.15^{d}	2.12 ± 0.18^{b}	9.39 ± 0.86^{c}
100	5.34 ± 0.19^{c}	5.26 ± 0.23^{b}	10.57 ± 0.12^{c}	1.97 ± 0.29^{b}	9.29 ± 0.62^{c}
250	2.39 ± 0.13^{b}	4.29 ± 0.91^{a}	6.67 ± 0.19^{b}	1.22 ± 0.16^{a}	7.52 ± 0.79^{b}
500	1.36 ± 0.17^{a}	4.04 ± 0.17^{a}	5.39 ± 0.28^{a}	1.06 ± 0.91^{a}	6.91 ± 0.59^{a}

* mean values ± standard deviations followed by the same letters in the same column are not statistically significant as per Duncan's multiple range test ($p<0.05$)

Table 2. Effect of cadmium on seed germination, tolerance index, phytotoxicity and seedling vigor in *Vigna mungo.*

Treatment $CdCl_2$ (μM)	Germination (%)	Tolerance index (96 h.)	% Phytotoxicity (96h.)	Seedling vigor (96h.)
0	99.13 ± 1.46 [c]	100.00 ± 0.00 [d]	0.00 ± 0.00 [b]	1246.14 ± 22.72 [d]
10	99.00 ± 0.93 [c]	106.50 ± 1.64 [e]	-6.50 ± 1.64 [a]	1332.76 ± 30.24 [e]
50	99.00 ± 0.92 [c]	107.33 ± 4.07 [e]	-7.31 ± 4.07 [a]	1283.32 ± 13.62 [d]
100	94.63 ± 3.44 [b]	77.39 ± 2.79 [c]	22.61 ± 2.79 [c]	988.49 ± 42.33 [c]
250	92.13 ± 5.36 [b]	34.63 ± 1.62 [b]	65.37 ± 1.62 [d]	584.33 ± 79.80 [b]
500	84.12 ± 6.81 [a]	19.68 ± 2.70 [a]	80.31 ± 2.70 [e]	438.47 ± 30.55 [a]

*mean values ± standard deviations followed by the same letters in the same column are not statistically significant as per Duncan's multiple range test ($p<0.05$)

compared to seedling length and biomass; significant differences were observed only at concentrations above 50 μM Cd. In the present work, the plants exposed to 10 μM Cd showed marginal increases in seedling length and vigor. Stimulatory effect of low concentrations of Cd has been reported earlier (Wu and Zhang 2002). The reduction in biomass accumulation may represent the cumulative effects of damaged or inhibited physiological functions. Root elongation was severely affected and root tip turned brown and brittle with increasing levels of Cd. The toxic effect of Cd on the roots affects the entire plant as it is the main pathway for entry of water and nutrients. Excess of Cd caused lipid peroxidation as well as modified the activity of antioxidant defense enzymes SOD, PER, CAT, PPO and PAL (Table 3). SOD is an essential component of the antioxidant defense (Boscolo *et al.* 2003), and is present in all aerobic organisms and in most sub cellular compartments that generate oxidative stress. Analysis of SOD activity in the present study showed an increase in the activity at low concentrations of Cd with a maximum increase of 18% at 100 ìm in comparison with the control. Thereafter, SOD activity declined by 14% and 25% on exposure to 250 and 500 μM Cd respectively, indicating severe damage to the root tissue development and function. PER and PPO activities followed a similar pattern as SOD with Cd treatment (Table 3). The activity of PER and PPO gradually increased with Cd

Table 3. Effect of cadmium on the activities of enzymes and on MDA content in the roots of *Vigna mungo* seedlings after 96 h of germination.

Treatment $CdCl_2$ (μM)	Peroxidase mmols of guaiacol oxidized min^{-1} g^{-1} fw	Catalase units g^{-1} fw	Superoxide dismutase units g^{-1} fw	Phenylalanine ammonia lyase μg t-cinnamic acid $h^{-1}g^{-1}$ fw	Polyphenoloxidase mmols of catechol oxidized min^{-1} g^{-1} fw	Lipid peroxidation MDA μmol g^{-1} fw
0	138.40 ± 8.94 [c]	720.16 ± 23.64 [e]	78.26 ± 3.78 [c]	82.51 ± 4.98 [a]	18.49 ± 1.88 [a]	10.43 ± 1.24 [a]
10	144.78 ± 3.48 [cd]	748.33 ± 14.23 [e]	80.06 ± 7.98 [c]	132.12 ± 6.34 [b]	24.24 ± 1.48 [b]	12.89 ± 1.00 [b]
50	150.51 ± 8.15 [d]	659 ± 19.39 [d]	83.22 ± 3.01 [c]	147.07 ± 6.25 [c]	31.40 ± 1.70 [d]	14.50 ± 1.64 [bc]
100	243.34 ± 6.85 [e]	613.33 ± 17.59 [c]	93.16 ± 3.62 [d]	173.06 ± 7.54 [e]	36.40 ± 2.37 [e]	15.86 ± 1.29 [c]
250	123.35 ± 5.69 [b]	504 ± 21.56 [b]	67.40 ± 3.78 [b]	182.06 ± 3.17 [f]	28.49 ± 2.70 [c]	18.71 ± 1.71 [d]
500	111.16 ± 10.36 [a]	413.33 ± 39.93 [a]	59.16 ± 3.47 [a]	163.55 ± 5.77 [d]	22.15 ± 1.96 [b]	21.86 ± 2.03 [e]

*mean values ± standard deviations followed by the same letters in the same column are not statistically significant as per Duncan's multiple range test ($p<0.05$)

levels reaching a maximum at 100 µM Cd (75% and 97% increase respectively), and declined thereafter. No significant change in the activities of SOD, PER and CAT was observed in plants subjected to 10 µm Cd when compared with controls. PPOs are responsible for the oxidation of phenolic substrates to quinones and have also been reported to show increased activities in response to various types of stresses (Rivero *et al*. 2001). In this study activities of PPOs showed a trend similar to that of SOD and PER, hence it is suggested that they may have a role in reducing oxidative stress caused by Cd. PAL activity increased significantly with Cd levels reaching a maximum at 250 µM Cd (120% increase). PAL is the entry point enzyme in the phenyl propanoid biosynthetic pathway, and increase in PAL activity in response to various types of biotic and abiotic stresses, has been reported in different species of plants (Hammerschmidt 1999, Ruiz *et al*. 1998, 1999). In *Arabidopsis* suspension cultures, H_2O_2 has been found to induce the expression of glutathione S- transferase and PAL (Desikan *et al*. 1998)

Oxidative stress arises from an imbalance in the generation and metabolisms of ROS, with more of ROS such as H_2O_2 being produced, than that is metabolized. Cd has been reported to cause the production of ROS, in both plants and animal cells (Olmos *et al*. 2003, Szuster-Ciesielska *et al*. 2000). The oxidative enzymes are known to deplete the pool of ROS from accumulating to toxic levels, and thus play an important role in adaptation and survival of plants during stress. The cellular antioxidant enzymes are believed to protect the cell, to some extent, from the damages arising from Cd-induced ROS production. SOD catalyses the conversion of superoxide ion to H_2O_2, whereas CAT scavenges H_2O_2, and PER uses H_2O_2 for oxidation of various inorganic and organic substrates. Increased levels of these enzymes observed in this study, therefore, may reflect a common strategy to overcome the stress due to Cd. The decreased SOD, CAT and PER activities observed at 250 and 500 µM Cd, may be due to the harmful effect of overproduction of H_2O_2, other ROS derivatives and Cd-induced inhibition of protein synthesis which overrides the cellular detoxification mechanism of the plant. The reduced activities of the antioxidant enzymes in the presence of high Cd concentrations represent an indication of the severity of Cd-induced oxidative stress.

MDA levels are generally used as an index of lipid peroxidation under stress conditions. In the present study, MDA concentration increased exponentially with increasing Cd levels indicating, the increased presence of free radicals in the tissue, and consequent increase in lipid peroxidation, and oxidative stress in black gram (Table 3). Protonation of O^{2-} can produce the hydroperoxyl radical (*OH, H_2O_2), which can convert fatty acids to toxic lipid peroxides, destroying biological membranes. Enhanced levels of lipid peroxidation have been reported in both root and leaves of pea plants exposed to Cd (Dixit *et al*. 2001).

In the present study, Cd-stress modified the levels of SOD, PER, PPO, PAL and CAT, the main components of enzymatic defense system in *Vigna mungo*, and the effect varied with the intensity Cd exposure. The pattern of enzyme activities suggests that the tolerance mechanism involves a system that removes free radicals and ROS, preventing their accumulation, which in turn increases the need for the antioxidant/ defense enzymes such as SOD, PER, PPO, CAT and PAL. The increase in the activity of the above enzymes in plants exposed to Cd may be due to increased expression of the genes controlling the biosynthesis of these enzymes and / or increased activation of existing enzyme pools, while reduction in the activity of the enzymes at high concentrations of Cd, may be attributed to decreased synthesis or dysfunctional conformational changes which leads to loss of enzyme activity.

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