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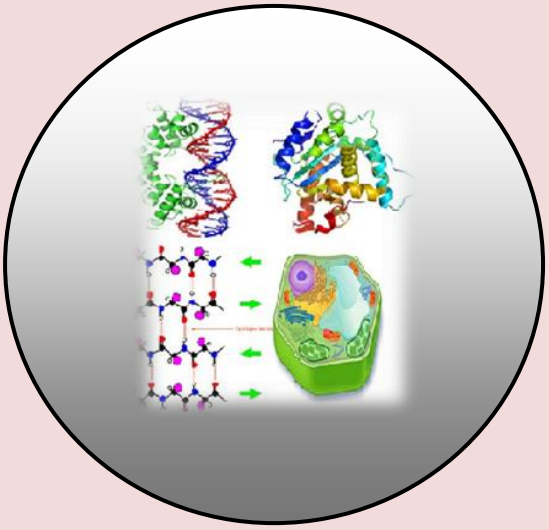

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Expression of Anti-Oxidative activity in Mustard under the Influence of Cadmium Stress

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ABSTRACT

The Contamination of Agricultural soil with Cd is the serious environmental concern all over the world due to anthropogenic activity. It affects the crop productivity and causes oxidative stress in plants. To explore the deleterious effect of oxidative stress of Cd toxicity, a pot experiment was performed to study the effect of different Cd concentrations 1(T1), 5(T2), 10(T3) and 25(T4) mg Cd kg^{-1} in soil on mustard (Brassica juncea L.) hybrid variety (TMMD-2901) under glass house conditions. The results indicated that at higher concentration of Cd (25 mg Cd kg^{-1}) treatment lead to major decrease in growth parameters such as germination percentage, fresh and dry weight (leaf, stem and root) and photosynthesis pigments. Enhance activity of lipid peroxidation (LPO) in the terms of malondialdehyde (MDA) was also observed. Increased activity in antioxidant enzymes such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and ascorbate peroxidase (APX) in mustard variety showed maximum tolerance to heavy metal stress at the 25 mg Cd kg^{-1} in soil. It indicated that anti-oxidative enzymes play an important role in protecting the mustard plant from Cd toxicity.
Key words: Antioxidant Enzymes, Cadmium, Oxidative Stress, Photosynthesis Pigments and Mustard.

INTRODUCTION

Cadmium (Cd) is ecologically hazardous and non essential heavy metal which causes several toxicity problems in plants, animals and humans (Wagner, 1993; Wan and Zhang, 2012). Cd is released in soil, water and atmosphere from different point and non point sources including fertilizer application, sewage, sludge, manure and lime heating systems, metallurgical industries, waste incinerators, urban traffic and cement factories (McGrath et al., 1994; McLaughlin et al., 1996; Di Toppi and Gabbrielli, 1999; Adams et al., 2004; Benavides et al., 2005; Gratao et al., 2005; Kidd et al., 2007). Cd contaminated agricultural soil is the alarming condition for crop productivity and food security on global level, and it has become a serious threat to human health especially in developing countries.

In the environment Cd persists long time due to longer biological half-life (Chien et al., 2002). It is ranked 7th most toxic heavy metal among 20 heavy metals (Morel, 2008). It is toxic to the plants even at low concentration in soil (Das et al., 1997). It enters in the food chain through accumulating in agricultural crops, and reaches highest toxic level in the ecosystem which shows the negative impact on human and animal health (John et al., 2009). It is estimated that the acceptable concentration of Cd in different crops ranges 0.013-0.22, 0.07–0.27, and 0.08–0.28

mg kg^{-1} for cereal, fodder and leguminous plants respectively. But worldwide it is about 0.01to 2 mg kg^{-1} soil with average value of 0.35mg kg^{-1} soil (Kabata-Pendias and Pendias, 2001).

In all the living beings, it is the behaviour of cell that they generate free radicals which cause oxidative stress if they stay longer in the cells without degeneration. The oxidative stress in plants may be caused by Cd toxicity, which shows diverse changes in morphological, physiological and biochemical parameters. Structural symptoms include chlorosis, necrotic lesions, leaf rolling, highly reduced biomass production, growth inhibition, disturbed carbohydrate metabolism and alteration of nutrient homeostasis, and also even the cell death (Azevedo et al., 2005; Santos et al., 2010; Ehsan et al., 2014). In addition, phytotoxicity of Cd also causes changes in photosynthetic efficiency of plants by reducing pigment contents (Somashekarainh et al., 1992; Drazkiewicz et al., 2003; Burzynski and Klobu, 2004; Mobin and Khan, 2007). Cd is also responsible for cytotoxic, mutagenic and genotoxic effects in plant cells (Santos et al., 2010).

Excessive production of reactive oxygen species (ROS) is the indication of oxidative stress, such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). It can damage the cell components through lipid peroxidation, inactivation of enzymes and deoxyribonucleic acid (DNA), protein and electrolyte leakage (Hossain et al., 2012; Saidi et al., 2013; Howladar, 2014). The degree of Cd toxicity and its accumulation in plants from soil depend on the number of factors including bioavailability of metal, organic matter, pH, growth stage of plant, redox potential, temperature as well as presence of chelating substances and duration of exposure to heavy metal. They influence the Cd toxicity level, its absorption and distribution in plants (Bingham, 1979; Hardiman and Jacoby., 1984; Benavides et al., 2005; Metwally et al. 2005).

To overcome ROS generation in response to various environmental stress, plants have well equipped strategies with antioxidative defense system to mitigate the oxidative stress. It include both enzymatic and non enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), ascorbic acid (AsA), glutathione reductase (GR), α-tocopherols, glutathione and carotenoids etc. In such stress conditions these enzymes and non enzymatic antioxidants are up and down-regulated, and contribute higher tolerance to plants (Ahmad et al., 2010).

In the present study mustard (*Brassica juncea* - TMMD2901) was undertaken as the test plant. In Asian continent especially in India this Indian mustard is one of the fastest growing, high yielding and high rate biomass producing species which can be used in metal polluted soil for phytoremediation. This is oil seed crop plant belonging to Brassicaceae family. Apart from edible oil it is also used for medicine and vegetable purpose. Residual part of seed is used as cattle feed and dry part of plant is used as a fuel in villages of India. The aim of this study was to explore the effect of low and high level of Cd on mustard growth, photosynthetic pigments and certain important oxidative stress responsive enzymes.

MATERIALS AND METHODS

Plant growth conditions and application of Cd treatment

The experiments were carried out during growing rabi season (winter) under glass house conditions in Botany department, Lucknow University, Lucknow (India). The experimental geographical area of Lucknow district lies in between Latitude: 26°50'21" N, Longitude: 80°55'23" E and altitude 126 m. The seeds of mustard (*Brassica juncea* L.) hybrid variety (TMMD 2901) were obtained from an authentic commercial seed supplier company in Lucknow. Before filling soil the inner surface of each pot (diameter 30 cm) was covered with polythene sheet cover. Subsequently, different concentration in the form of cadmium chloride ($CdCl_{2)}$ was dissolved in distilled water, and added into each pot filled with 10 kg soil and compost mixture (Sandy loam) in 3:1 ratio. The pots were left for 3 days before sowing under semi controlled conditions. Seeds were surface sterilized with mercuric chloride (0.1 %, w/v) then washed several times with distilled water and soaked overnight in distilled water at room temperature before sowing.

Fifty mustard seeds were sown in each earthen pot and kept in glass house conditions. The plants were allowed to grow under natural sunlight/dark condition and temperature. The average day/night temperature was 26/16±3˚C and relative humidity was 70±2%. The experimental pots were setup in complete randomized block design and divided into 5 groups, with three replicates in each, Plants were subjected to following treatments-

(I) CTL: Control (without Cd treatment)
(ii) T1: 1 mg $CdCl_2$ kg^{-1} soil
(iii) T2: 5 mg $CdCl_2$ kg^{-1} soil
(iv) T3: 10 mg $CdCl_2$ kg^{-1} soil
(v) T4: 25 mg $CdCl_2$ kg^{-1} soil

To maintain the moisture content of the pot 100 ml of tap water was used to irrigate the plants in each pot every alternate day in the evening.

Phenotypic growth parameters and germination percentage

The total seedlings in each pot were counted on 5^{th} day. Later on the thinning was done and total 3 plants were maintained in each pot. Growth parameters were recorded at the end of the experiment. However, the fresh weight of shoot, root and leaf was taken after proper washing with distilled water. Plant parts were dried at 70˚C for 48 h (2 days) in an oven for dry weight. The germination percentage of seeds was calculated as follows.

$$\text{Germination } \% = \frac{\text{Number of germinated seeds in a pot}}{\text{Total number of seeds sown in a pot}} \times 100$$

Physico-chemical characteristics of soil and DTPA (Diethylenetriamine pentaacetate) extractable metals (Zn, Fe, Mn, Ni and Cd)

Physico-chemical properties of experimental soil were investigated after proper mixing of soil and compost (3:1) before sowing the seeds in earthen pots. 50 gm of dried soil sample (before Cd treatment) was ground in pestle mortar for the analysis. Texture of soil was determined by hydrometer method given by Gee and Bauder (1979). Soil water suspension (1:2.5) was used for pH (LAB MAN, model no. LMPH-10) and electrical conductivity (EC) measurement by using conductivity meter (LAB MAN, model no. LMCM-29) according to Kalra and Maynard (1991). Organic matter and organic carbon were determined by the method of Walkley and Black (1934). For DTPA extractable metals, 10 gm dried soil was taken in conical flask with 20 ml of DTPA extract (0.1M TEA (triethanol amine), 0.01M $CaCl_2$, 0.005M DTPA), and mixture was shaken at 180 rpm for 2 hours (Lindsay and Norvell, 1978). Supernatant was filtered with whatman No.1 (110nm) filter paper. The samples were analyzed for different metals by using Atomic Absorption Spectrophotometer (Thermo Jerrell Ash Video 12 E: AA/AE Chicago, IL).

Determination of photosynthetic pigments

Photosynthetic pigment contents (chlorophyll a, chlorophyll b, total chlorophyll and carotenoid were estimated in plant leaves by the method of Arnon (1949). 100 mg fresh leaves were extracted in 10 ml (80% v/v) chilled acetone. Leaf suspension was centrifuged at 2000 rpm for 10 minutes. The absorbance of supernatant was read with the help of spectrophotometer (LAB UV 300 plus double beam spectrophotometer) at 663, 645, 510, 480 nm, against 80% acetone used as a blank. The concentrations of various pigments were expressed in mg g^{-1} fresh weight of tissue. The carotenoid content was estimated by using formula given by Duxbury and Yenstch (1956).

Lipid peroxidation determination

It was determined in the term of malondialdehyde (MDA) concentration. MDA is the product of lipid peroxidation to assess membrane damage in the plant tissues which was estimated by the content of thiobarbituric acid reaction (Heath and Packer, (1968). 0.5 gm fresh leaves of control and treated plants were homogenized in 5ml of 0.1 % TCA in pestle and mortar, Homogenate was centrifuged at 10,000×g for 5 min. then 2.0 ml supernatant was added to 2.0 ml of 0.5% TBA containing 20% TCA (w/v). Mixture sample was heated on water bath for 30 min at 95^0C and then cooled immediately on ice bath. After cooling it was again centrifuged at 10,000×g for 15 min. Absorbance of clear supernatant was read at 532 and 600 nm using spectrophotometer. The non specific absorbance at 600nm was subtracted from the absorbance at 532 nm. MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol MDA/100mg fresh weight.

Catalase activity

Catalase (CAT, EC. 1.11.16) activity was assayed by the method of Euler and Josephson (1927). The reaction was carried out in 50 ml test tube, in which 10 ml substrate mixture having 10 % H_2O_2 and 0.1 M phosphate buffer (pH 7.0) was added. The Reaction was started by addition of 1ml suitably diluted enzyme extract. Reaction was allowed to proceed for 5 minutes. Thereafter, the reaction was stopped by the addition of 5ml 2N H_2SO_4. The blank was run simultaneously in which 2N $H_2SO_4$was added prior to the addition of enzyme extract. It was then titrated against 0.01N $KMnO_4$. The enzyme activity was expressed as µM H_2O_2 degraded mg^{-1} protein.

Determination of Superoxide dismutase activity

The activity of superoxide dismutase (SOD, EC. 1.15.1.1) was determined in leaves by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). The total (3ml) volume of reaction mixture contained 50 mM phosphate buffer (pH 7.8), 75 µM NBT, 13 mM methionine, 2µM riboflavin, 0.1mM EDTA and 0.5 ml enzyme. The riboflavin was added in the end. The tubes were shaken by hand and illuminated for 10 minute. One unit of SOD activity was considered as the amount of enzyme required to cause 50% inhibition in the reduction of NBT, and absorbance was measured using spectrophotometer at 560nm at 560 nm (LAB UV 300 plus double beam spectrophotometer). Blanks were run in the same way but without illumination. The enzyme activity was expressed as change in optical density (ΔOD) between sample and blank OD mg^{-1} protein.

Determination of Peroxidase activity

Peroxidase (POD, EC. 1.11.1.7) activity was determined in fresh leaves by the method of Luck (1963). The reaction mixture contained 5.0 ml (0.1M) phosphate buffer (pH 6.0), 1ml (0.01%) H_2O_2 and 1ml (0.5%,w/v) p-phenylenediamine. The reaction was started by addition of 1 ml diluted enzyme extract and allowed to proceed at 25^0C for 5 min. The reaction was stopped by addition of 2 ml 5N H_2SO_4. The blanks were also run simultaneously in which 5N H_2SO_4 was added prior to addition of enzyme extract. The reaction mixture was kept in a refrigerator for 20-30 min and then centrifuged at 400g for 10 min at room temperature. The optical density of the supernatant was read at 485 nm on spectrophotometer. The enzyme activity was expressed as change in optical density (ΔOD) between the sample and the blank and expressed as ΔOD mg^{-1} protein.

Ascorbate peroxidase

The activity of ascorbate peroxidase (APX, EC. 1.11.1.11) was determined in leaf extract by estimating the rate of ascorbate peroxidation by the method described by Nakano and Asada (1981). Total 3.0 ml reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 0.5mM sodium ascorbate, 0.1mM EDTA, 0.1 mM H_2O_2 and 0.1ml enzyme extract. The reaction was initiated by adding H_2O_2 in reaction mixture. Decrease in absorbance was recorded at each 30 second interval for 3 min at 290 nm. The quantity of ascorbate oxidized was calculated using extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ and expressed in µmol of ascorbate oxidized min^{-1} mg^{-1} protein.

Estimation of protein content

Protein contents were estimated according to Lowry et al (1951) by using bovine serum albumin (BSA) as a standard.

RESULTS

Soil analysis

The results obtained in soil analysis of pre sowing are shown in table 1. The soil was sandy loam in texture, slightly alkaline pH 7.8, EC 0.269 dsm^{-1}, organic matter 1.14% and organic carbon 0.66%. Diethylenetriamine pentaacetate (DTPA) extractable metals Zn, Fe, Mn, Ni and Cd in soil were 6.91, 11.78, 2.43, 1.64, 0.00 mg kg^{-1} respectively.

Table 1. Soil analysis (at pre-sowing before Cd treatment).

Parameters	Obtained value
Texture	Sandy loam
pH	7.8
EC (dsm^{-1})	0.269
Organic carbon (%)	0.66
Organic matter (%)	1.14
Zn ($mgkg^{-1}$soil)	6.91
Fe ($mgkg^{-1}$soil)	11.78
Mn ($mgkg^{-1}$soil)	2.43
Ni ($mgkg^{-1}$soil)	1.64
Cd ($mgkg^{-1}$ soil)	0.00

Effect of Cd on seed germination and growth attributes

The effects of Cd on seed germination, plant height, fresh and dry weight of plant (leaves, stem and root) are shown in table 2. Seed germination percentage was observed decline from T1 to T4 treatment by 86%, 80%, 73% and 66% in comparison to control. As increase in Cd concentration, increased inhibition trend was observed in germination percentage. It was reduced by 3.70%, 11.11%, 18.51%, and 25.92% at concentration of 1, 5, 10, and 25 mg Cd kg^{-1} soil respectively (T1toT4) as compared to control. The plant height was gradually reduced from control to higher Cd concentrations treatment. It was observed to be reduced by 15.54%, 26.47%, 35.29% and 49.57% (T1 to T4) as compared to control. Both fresh and dry weight of leaves, stem and root were found to be highly reduced in the treatment of T4 (25mg Cd kg^{-1} soil) by 70.75%, 69.36%, 78.27 % and 71.19%, 65.40%, 70.24%, respectively, in comparison to control.

Table 2. Effect of Cd on seed germination, growth and biomass yield in mustard (*Brassica juncea*).

Treatments	Germination %		Fresh weight (gm)			Dry weight (gm)		
		Plant height (cm)	Leaf	Stem	Root	Leaf	Stem	Root
Control	90.00 ± 0.577	39.66 ± 0.333	7.066 ± 0.735	24.00 ± 0.577	2.47 ± 0.315	1.226 ± 0.150	4.663 ± 0.087	0.683 ± 0.060
T1	86.66 ± 0.333 (3.70%)	33.5 ± 0.763* (15.54%)	5.506 ± 0.173 (22.07%)	16.66 ± 0.670* (30.58%)	1.646 ± 0.176* (33.33%)	0.906 ± 0.065 *(26.08%)	3.466 ± 0.202 *(25.66%)	0.483 ± 0.043 *(29.26%)
T2	80.00 ± 0.577 (11.11%)	29.16 ± 0.726* (26.47%)	3.726 ± 0.126 (47.26%)	12.423 ± 0.288* (48.23%)	1.373 ± 0.121* (44.39%)	0.646 ± 0.086* (47.28%)	2.703 ± 0.089 *(42.03%)	0.373 ± 0.020 *(45.36%)
T3	73.33 ± 0.333 (18.51%)	25.66 ± 0.333* (35.29%)	2.503 ± 0.051 (64.57%)	9.843 ± 0.078* (58.98%)	1.186 ± 0.043* (51.95%)	0.493 ± 0.053 *(59.78%)	2.173 ± 0.043 *(53.39%)	0.303 ± 0.031 *(55.60%)
T4	66.66 ± 0.666 (25.92%)	20 ± 0.577* (49.57%)	2.066 ± 0.536* (70.75%)	7.353 ± 0.376* (69.36%)	0.536 ± 0.021* (78.27%)	0.353 ± 0.027 *(71.19%)	1.613 ± 0.057 *(65.40%)	0.203 ± 0.026 *(70.24%)

Each value represents mean of 3 replicates ± SE. *Mean data were significant at $p<0.05$. Multiple comparisons Vs control group (Holm Sidak method). Overall significant level = 0.05. Control- 0, T1- 1, T2- 5, T3- 10, T4- 25 mg Cd kg^{-1} soil respectively. Percentage decrease value in comparison to control is given in parenthesis.

Photosynthetic pigment content under Cd stress

Photosynthetic pigments such as chl a, chl b, total chlorophyll and carotenoids were adversely affected by Cd treatment (fig.1). It was observed that the amount of chlorophyll b was decreased to a greater extent as compare to chlorophyll a, with increasing Cd concentration. However, this decline was more in T4 (25mgkg^{-1} Cd) than T1, T2 and T3 treatment. Chlorophyll a and b were found to be reduced by 5.82%, 1051%, 15.21%, 26.51% and 15.06%, 20.03%, 28.86%, 45.29% respectively, as compared to control. Therefore, Chlorophyll a:b ratio was higher at maximum Cd concentration and showed increasing trend by 2.08, 2.10, 2.24, 2.53 with 1.88 in control. Remarkable decrease in the level of total chlorophyll and carotenoid content was observed with cadmium concentration increased (T1 to T4) by 9.16%, 13.90%, 20.17%, 33.21% and 4.32%, 11.82%, 18.91%, 34.06% as compared to control (fig.1).

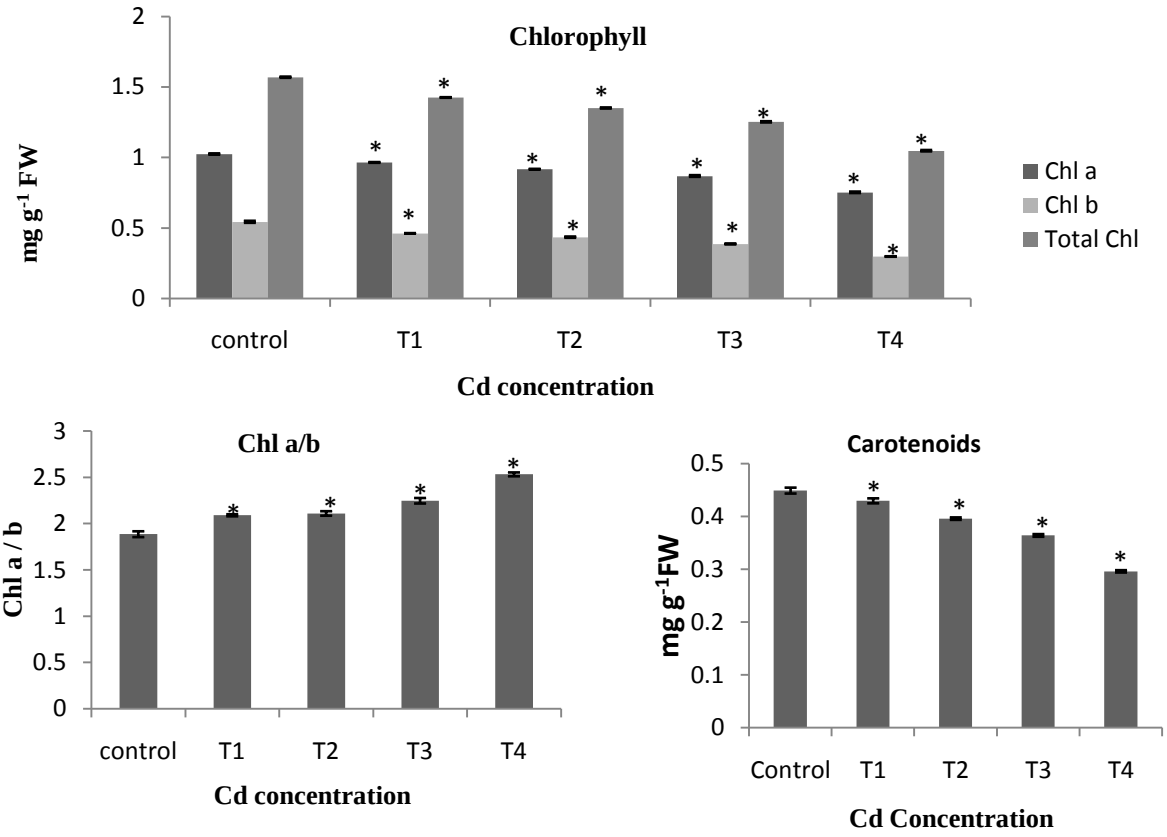

Figure 1. Effect of Cd on chlorophyll Chl a, Chl b, total Chl, Chl (a/b) ratio and Carotenoid content in a leaves of *Brassica juncea* L. Each value represents mean of 3 replicates ± SE. *Mean data were significant at p<0.05. Multiple comparisons Vs control group (Holm Sidak method). Overall significant level = 0.05. Control- 0, T1- 1, T2- 5, T3- 10, T4- 25 mg Cd kg^{-1} soil.

Effect of Cd on Lipid peroxidation content

The results obtained are shown in terms of malondialdehyde (MDA), a product of lipid peroxidation. Which is accumulate in the tissue of leaves, and showed the degree of membrane damage. It was found to increase under the influence of Cd treatments as compared to the control (fig 2A). The lipid peroxidation enzyme activity significantly increased in all treatment from T1 to T4 by 36.58, 41.85, 46.55 and 54.80 nmol MDA 100 mg^{-1} FW as compared to control (32.73). Treatment with T4 (25mgkg^{-1} Cd) was observed to cause maximum enhancement of lipid peroxidation. It was found that the percentage of MDA content increased by 11.75%, 27.86%, 42.23%, and 67.42% from T1 toT4 treatment respectively, as compared to control.

Activity of Antioxidant enzymes in response to Cd stress

In leaves of mustard plant some antioxidants enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) were examined after 80 days of Cd treatment. These enzymes were activated when different concentration of Cd (T1, T2, T3, and T4) was applied in soil.

In the present investigation, the SOD activity significantly increased by 6.80, 8.84, 11.17 and 14.11 mg^{-1} protein in reference to control(6.07). It was observed that SOD activity was maximum at T4 (25 mg Cd kg^{-1}) and minimum at T1 (1mg Cd kg^{-1}), and showed the increasing trend by 12.01%, 45.64%, 83.85%, and 132.33 % in T1 to T4 under the influence of Cd concentration as compared to control (fig. 2B).

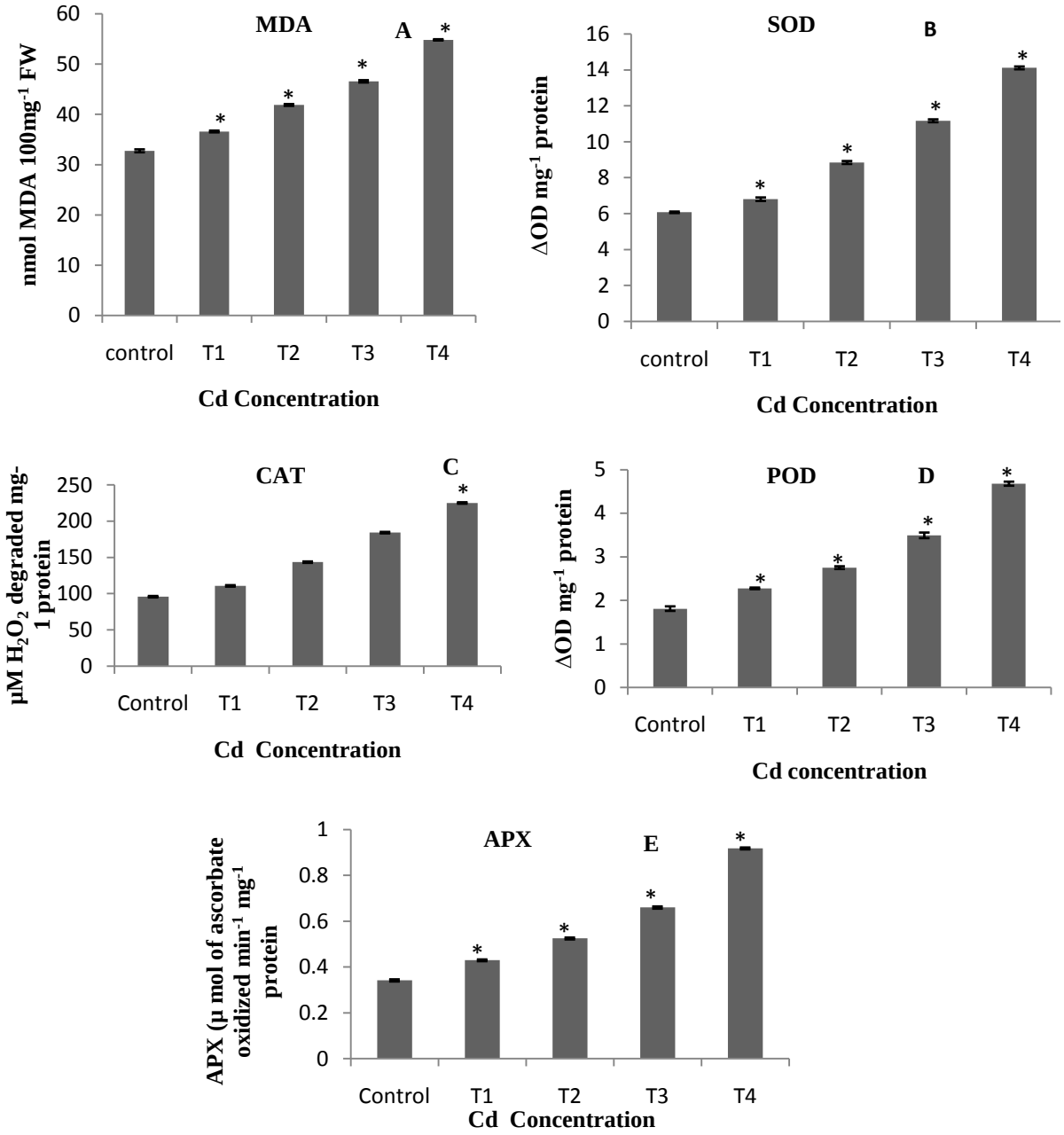

Figure 2. Effect of Cd on the activity of MDA, SOD, CAT, POD and APX in the leaves of mustard plants. Each value represents mean of 3 replicates ± SE. *Mean data were significant at p<0.05. Multiple comparisons Vs control group (Holm Sidak method). Overall significant level = 0.05. Control- 0, T1- 1, T2- 5, T3- 10, T4- 25 mg Cd kg^{-1} soil respectively.

Catalase data are represents in fig. 2C. It was found that the CAT activity in response to Cd toxicity was increased by 110.70, 143.43, 184.18 and 225.06 mg^{-1} protein. But it was significantly increased in T4 treatment only. In terms of percentage it increases by 15.57%, 49.74%, 92.29% and 134.97% respectively, from T1 to T4 treatment over the control.

The level of POD activity under the influence of Cd toxicity was enhanced significantly by 2.27, 2.274, 3.49, and 4.67 in comparison to control (1.80) mg^{-1} protein (fig. 2D). But this enhanced activity was observed to be more in T4 treatment as percentage POD increase was observed to be 25.74%, 52.08%, 93.12%, and 158.78% from T1 to T4 treatment as reference to control.

Similar trend was also observed for APX activity which was found to enhance by 0.42, 0.52, 0.65 and 0.91 mg^{-1} protein (fig. 2E). For control the APX value was 0.34 mg^{-1} protein. It was also noted to increase by 25.65%, 53.51%, 93.02%, and 168.46%, when compared to control.

DISCUSSION

The growth attributes of mustard plant such as plant height, germination percentage, fresh and dry biomass of root, stem and leaves were adversely affected by increasing Cd concentration (table 2). Reduction in growth parameters as affected by Cd has also been reported previously by different workers (Bauddh and Singh, 2012; Zouari et al., 2016; Ahmad et al., 2015) in various plants.

Decline in chlorophyll content in leaves of mustard plant on exposure to Cd even at low concentration (1mg kg^{-1}) could be due to inhibition of important enzyme such as δ-aminolevulinic acid dehydratase and protochlorophyllide reductase etc. which are involved in chlorophyll biosynthesis. It is very well established that the supply of Mg and Zn is required for the synthesis of chlorophyll (Parmar et al., 2013; Elloumi et al., 2014). Previously, Weigel (1985) has also reported that Cd has ability to interfere/inhibit the various steps of the Calvin cycle. However, the substitution of Mg^{++} ions in chlorophyll by toxic heavy metals such as Cd, Cu and Hg was an important cause of chlorophyll damage. This substitution can result in breakdown of photosynthesis by preventing the capture of photosynthetic light in Cd contaminated environment (Küpper et al., 1996; Jamers et al., 2009). The similar decreasing trend of Chl a, Chl b and total chlorophyll on exposure of Cd was observed by several worker (Gosawami and Das, 2015; Silva et al., 2018; Ahmed et al., 2015; Mobin and Khan, 2007; Bauddh and Singh, 2011) in different plants.

Cd act as a potent inhibitor for photosynthesis via non-stomatal limitation, Chl reduction, PSII damage which are more during plant growth inhibition (Liu et al., 2014). Loggini et al. (1999) reported that the increase in Chl a/b ratio is linked with the changes in the light harvesting chlorophyll proteins (LHCPs). The similar results were also reported by Mobin and Khan (2007) in different variety of *Brassica juncea*.

Carotenoids play an important role in photo-protection of chlorophylls in reference to photo-oxidative damage by quenching reactive oxygen species (ROS) in terms of singlet oxygen (Küpper et al., 1996). Due to this efficient unstable free radical scavenger, a reduction in carotenoid is observed due to Cd stress. This may result in an excessive production of ROS that subsequently interferes the plant growth through causing oxidative damage to RNA, DNA and proteins (Choudhary et al., 2012; Mishra et al., 2006). Carotenoid contents decrease with increasing Cd concentration in present experiment. Several authors have observed similar effect in *Brassica juncea, Olea europaea* and *Miscanthus* species (Gosawami and Das, 2015; Zouari et al., 2016; Guo et al., 2016).

In present observation, Cd induced increase in MDA content in leaves was noted (fig. 2A). Similar results was also observed in *Ricinus communis*, *Brassica juncea* and *Olea europaea* at different concentration of Cd in soil which increased the level of lipid perixidation (Baudh and Singh, 2012; Zouari et al., 2016; Gill et al., 2011; Ahmed et al, 2015). Exposure to Cd stress in soil increased MDA (in mustard plant caused oxidative damage to cell membrane lipid and protein modification) has also been shown by (Yamauchi et al., 2008., Sharma et al., 2012) due to excessive generation of ROS (Mobin and Khan, 2007) by Cd stress. Increased lipid peroxidation was also reported in various Cd treated plants including *Pisum sativum* (Chaoui et al., 1997; Metwally et al., 2005), rice (Chien et al., 2001), sunflower seedlings (Gallego et al., 1996), Tobacco seedlings (Tkalec et al., 2014) and *Miscanthus* species (Guo et al., 2016).

The accelerated production of ROS in various plant species in response to Cd stress is very well established (Ci et al., 2009; Chen et al., 2014). Both of these antioxidant enzymes (CAT and POD) could catalyze H_2O_2 into H_2O and O_2 but POD has more affinity to alleviating the oxidative damage caused by H_2O_2 in response to Cd stress in leaf of mustard plant (Noctor and Foyer, (1998). In our results, compared with control, the activity of CAT and POD were significantly increased on application of Cd (fig. 2C and 2D).

These results are in agreement with several studies in which the enhanced activity of CAT and POD in maize (*Zea mays*) and *Miscanthus* species on Cd treatment (Anjum et al. 2015; Guo et al. 2016) has been observed. Similar results were also obtained in Cr contaminated maize plant by Anjum et al., (2017).

Catalase has a very high turnover rate but lower affinity than APX to scavenging H_2O_2 in peroxisome (Gill and Tuteja., 2010; Ahmad et al., 2015) in stress conditions. Due to this reason removal of H_2O_2 through catalase may be overcome by the increased activity of APX in leaves of mustard plant shown in fig.2C and 2D. APX is involved in playing important role when maximum amount of H_2O_2 is to be eliminated (Ahmad et al., 2010; Sharma et al., 2012) in the leaves of mustard plant to reduce the oxidative damage. Scavenger property of APX is also very important in water-water and ascorbate-glutathione cycle by utilizing AsA as the electron donor to eliminate/detoxify H_2O_2 (Kangasjärvi et al., 2008). The enhanced activity of APX enzyme is also reported in several previous studies (Gill et al., 2011; Ahmad et al., 2015; Guo et al., 2016 Anjum et al., 2015) in *Brassica juncea*, *Miscanthus* species and maize (*Zea mays*), respectively.

In addition to heavy metal stress, ROS are generated in both unstressed and stressed cells at various locations in plants such as chloroplasts, mitochondria, peroxisomes, apoplast, plasma membranes, endoplasmic reticulum and cell wall (Sharma et al., 2012). On exposure to Cd stress various plants protecting antioxidant enzymes such as SOD, CAT, POD and APX are synthesized more which play important role in reducing the heavy metal effect in mustard plant. SOD is considered as metalloenzyme which provides the first line of defense against ROS which is involved in the disproportionation of $O_2^{\bullet-}$ to H_2O_2 and O_2. It reduces the risk of $OH^{\bullet}$ formation by eliminating the $O_2^{\bullet-}$ through the use of metal-catalyzed Haber-Weiss-type reaction (Alscher et al., 2002; Srivalli et al., 2003; Gopavajhula et al., 2013).

In our study, the activity of SOD was increased from T1 to T4 treatment as shown in (Fig. 2B). This study is supported by different experiments which were done in various plants by Anjum et al., 2008; Ahmad et al., 2015; Gill et al., 2011; Guo et al., 2016. Enhanced activity of SOD, APX, POD, CAT and lipid peroxidation (MDA) indicated that mustard plant has capacity to survive in maximum concentration of Cd at 25 mg kg^{-1}soil. As reference to the control seed germination, plant height and biomass are also drastically reduced on 25 mg Cd kg^{-1}soil treatment.

CONCLUSION

Based on the present study it was revealed that the different levels of exogenous Cd concentration involved in mustard plant treatment. This was showing maximum reduction in seed germination%, and fresh and dry biomass production at T-4 (25 mg Cd kg^{-1} soil) treatment. The data of present study showing more tolerable stress in T-4 Cd concentration (TMMD 2901) as compare to T1, T2, and T3 concentration in reference to increased lipid peroxidation (MDA) as well as activity of antioxidant enzymes such as CAT, POD, SOD, and APX. Which minimize oxidative stress significantly under higher concentration of Cd toxicity and improve the protection of metabolic mechanism.

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