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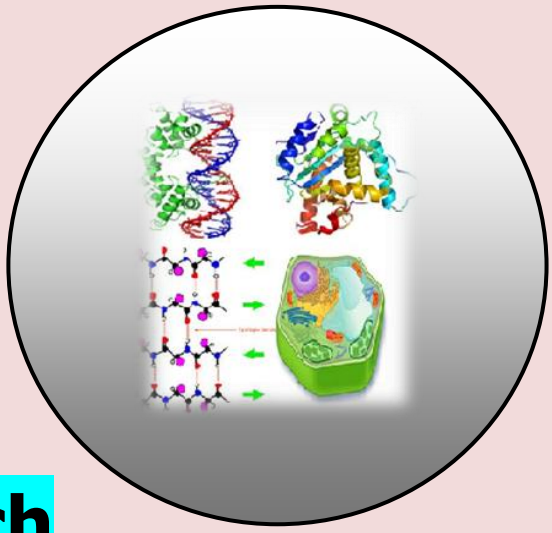

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Alleviation of Cadmium Toxicity in *Solanum lycopersicum* via Regulation of Photosynthetic Pigments and Antioxidative Machinery by Exogenous Zinc Application

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ABSTRACT

We investigated the alleviation of Cadmium toxicity in Tomato (Solanum lycopersicum c.v. S 22) by Zinc application. In this study, the effects of cadmium (Cd), and the combined effect of Cd+Zn (Zinc) on the growth of Solanumlycopersicum were investigated. Different concentrations (0, 200, 500, 1000 mg/kg soil) of Cd alone and also with a fixed concentration of Zn (200 mg/kg soil) were used to grow tomato plants in glasshouse pot culture experiment. Results showed decline in plant growth, germination percentage, Chlorophyll and Carotenoid content with increase in Superoxide dismutase, Lipid peroxidation and Ascorbate peroxidation in Cd-treated tomato plants with comparison to control. When Cd was given along with Zn it was observed that the Zn supply clearly reduced Cd toxicity in tomato. These results suggested that application of Zn to Cd-stressed plants alleviate Cd-induced oxidative stress in Solanum lycopersicum.
Keywords: Ascorbate peroxidation, Cadmium, Lipid peroxidation Solanum lycopersicum and Superoxide dismutase.

INTRODUCTION

Heavy metal contamination of soils is a serious global environmental issue. Cadmium (Cd) is one of the topmost toxins among other 20 toxins and acknowledged as a human carcinogen. It is considered as tenacious and possible environmental contaminant which can cause severe toxicity to all living organisms (Kikuchi, 2007; Gill, 2011; and Bolan, 2013). Exposures to high Cd concentration have been found to be mutagenic and teratogenic in a large number of animal species (Degraeve, 1981). It has been reported that the *in vivo* effect of estrogen and induced uterine hyperplasia or early onset of puberty can be mimic by a low concentration of Cd (5-10µg/kg) in animals (Johnson *et al*., 2003). Cd is a major environmental contaminant and has been classified as an element of 'intermediate toxicity' (Duxbury, 1985). Cd is emitted from a wide spectrum of natural and man-made source such as volcanic eruption, fossil burning, industrial emission, automobile exhausts and sewage disposals. Cd occurs in almost all soils, surface waters, and plants, and it can subsequently allocate in the human food chain. It is a major environmental issue. It is transferred easily to the root of the plant due to its unique property of non-redox water-soluble nature. Through root, it is transported to the aerial parts where it significantly impedes vital cellular processes including respiration and CO_2 fixation. Some morphological indications of Cd toxicity are the deceleration in plant growth, chlorosis, necrosis, epinasty, stunted growth, cell death and disturbance in mineral homeostasis, and inhibition of photosynthesis

in plants (Sandalio, 2001; Kikuchi, 2007;Naser, 2009; Gill, 2011 and Bolan, 2013). Cd has also been found to provoke oxidative stress either by enhancing superoxide radical production and lipid peroxidation, or by reducing the enzymatic and non-enzymatic antioxidants. The accumulation of reactive oxygen species (ROS) molecule such as H_2O_2 and superoxide radical (O^{2-}) damage the cellular components like DNA, proteins and lipids (Lopez *et al*., 2006). Cd triggers a series of three waves of ROS generation, first with the NADPH oxidase-dependent accumulation of hydrogen peroxidase (H_2O_2), followed by the accumulation of superoxide anions (O^{2-}) in mitochondria, and lastly, fatty acid hydroperoxide, as perceived in tobacco cells (Garnier, 2006). High ROS levels disrupt plant metabolism which can damage lipids, proteins, and DNA. Thus plants have developed several strategies to counteract Cd toxicity, among which Zinc-induced defence is of great importance. That is why plant cell needs to control ROS with the help of Zinc and release the several antioxidative enzymes including superoxide dismutase, ascorbate peroxidase, and catalase (Arvind *et al*., 2005).In more than 300 enzymes Zinc has been specified as a cofactor element because it is vital in protein synthesis and gene expression in plants (Coleman, 1998; Cakmak, 2000 and Broadly, 2007). Zinc is a second most abundant transition metal in living organisms after Fe. Zinc is taken up predominantly as a divalent cation (Zn^{2+}) at high pH. It is presumably also taken up as a monovalent cation ($ZnOH^{+}$). In plants as well as well as in other biological systems Zn exists only as ZnII, and does not take part in redox reactions. Zinc is required for maintenance of the integrity of bio-membranes. It binds to phospholipid and sulfhydryl groups of membrane constituents and forms tetrahedral complexes with cysteine residues of polypeptide chains and thereby protects membrane lipids and proteins against oxidative damage. Cd and Zn have many physical and chemical resemblances. Cadmium and Zinc (IIB transition elements) have similar electronic configuration and valance state, possessing equal affinities for sulphur, nitrogen and oxygen ligands (Nieboar, and Richardson., 1980) and also have similar geochemical and environmental properties (Nan *et al*., 2002)

Cd is a toxic heavy metal and Zn is an essential element which makes this connotation interesting as it raises the possibilities that the toxic effect of Cd may be preventable by Zn. Tomato plant an important vegetable that suffers from Cd toxicity in areas with the heavy metal pollution (Lopez-millan, 2009 and Feng, 2010). This study is focused to evaluate the effect of increasing amount of Cd and its alleviation by Zinc application in tomato (*Solanumlycopersicum*), which is consumed worldwide in the human diet.

MATERIAL AND METHODS

PLANT MATERIAL AND CADMIUM TREATMENT

Tomato (*Solanum lycopersicum*c. v. S 22) seeds were obtained from the authorized agency(Golden Agri Genetic India limited) and sterilized with mercuric chloride (0.1%, w/v) for 2 min. The seeds were then carefully washed and soaked in distilled water for overnight. Thereafter, seeds were transferred in earthen pots (23 cm in diameter) filled with 10 kg of sandy loam soil and compost (3:1) in the glass house of the Department of Botany, Lucknow University, Lucknow, India, under semi-controlled conditions. The inner surfaces of pots were lined with a polythene sheet. The treatments were arranged in a complete randomized triplicate manner. $CdCl_2.H_2O$ and $ZnCl_2$ were used for Cd and Zn supply respectively. All chemicals used were of analytical grade Fisher Scientific. Chemicals were dissolved in double distilled water and added to the soils Cd alone concentrations of Cd 0, 200, 500, and 1000 Cd mg/kg applied Cd alone or in combined Cd + Zn (200mg/kg). The treated soil was packed into pots and equilibrated in a glasshouse for 2 days before being used for planting. Plants were analysed for all parameters after 45 days of sowing except germination percentage which was evaluated after 7 days of sowing:

$$\text{Germination } \% = \frac{number of germinating\ seeds\ in\ pot}{total number of seeds\ sown\ in\ a\ pot} \times 100$$

SOIL SAMPLE

The soil used in the study was sandy loam in texture with pH of 6.5; electrical conductivity (EC) of soil, 1.09 $dSm^{-1}$was measured in the labby a method (Jackson 1973).

GROWTH AND BIOMASS YIELD

Root and shoot lengths together with fresh and dry weights were measured after washing and rinsing tomato plants with tap and distilled water, respectively. For the dry weight of roots and shoots, the plants were oven dried the 70°C for 48 hours.

CHLOROPHYLL AND CAROTENOID CONTENT

Total chlorophyll, chlorophyll a, chlorophyll b contents were measured according to Arnon (1949). For estimation of photosynthetic pigments, plant leaves (300 mg) were extracted in 80% chilled acetone. The resulting suspension was centrifuged for 10 min 10,000× g, and the absorbance of the supernatant was measured spectrophotometrically at 480, 510, 645 nm and 663 nm using spectrophotometer (Toshniwal TSUV 75). Chlorophyll contents were expressed in terms of mg chlorophyll present /g fresh weight of tissue. Carotenoid contents were estimated according to the method of Duxbury and Yentsch (1956).

$$Chlorophyll'a' = 12.7\ (A663) - 2.69\ (A645) \times \frac{V}{1000\ \times W}$$

$$Chlorophyll'b' = 22.9\ (A645) - 4.68\ (A663) \times \frac{V}{1000\ \times W}$$

$$TotalChlorophyll = 20.2\ (A645) + 8.02\ (A663) \times \frac{V}{1000\ \times W}$$

$$Carotenoid = 7.6\ \times (A480) - 1.49\ \times (A510) \times \frac{V}{1000\ \times W}$$

Where

A = absorbance at specific wavelengths
V = final volume of chlorophyll extract
W = fresh weigh of tissue extracted

LIPID PEROXIDATION

Lipid peroxidation in leaves was determined by estimation of MDA content following the method of Heath and Packer, 1968. Five hundred milligrams of material was homogenized in 5 ml of 0.1 % TCA. The homogenate was centrifuged at 10,000 × g for 5 min. For every 2 ml of aliquot, 4 ml of 20% TCA containing 0.5% thiobarbituric acid was added. Mixture was heated at 95 °C for 30 min and then cooled quickly on ice bath. The resulting mixture was centrifuged at 10,000 × g for 15 min and the absorbance of the supernatant was taken at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.Level of malondialdehyde (MDA) measure the membrane damage in test plants.

ANTIOXIDATIVE ENZYMES

Superoxide dismutase (SOD) activity was measured spectrotrophotomertically at 560 nm according to Beauchamp and Fridovich (1971), based on the inhibition of photochemical reduction of nitro blue tetrazolium (NBT). One unit of enzyme activity was defined as the quantity of SOD required for 50% inhibition of NBT reduction. Total Ascorbate peroxidase (APX) activity was determined by measuring the decrease in absorbance at 290 nm (Ɛ = 2.8 mM^{-1} cm^{-1}) due to oxidation of ascorbic acid to dehydroascorbate, according to Nakano and Asada (1981).

PROTEIN

Protein contents were determined according to Lowry (1951) using bovine serum albumin as a calibration standard.

STATISTICAL ANALYSIS

The experiment was conducted in completely randomized design (CRD) with three replications. The data were analysed by one-way ANOVA using software program Sigmastats 4.0. It was followed by a comparison of mean value using Holm Sidak method at $p \leq 0.05$.

RESULTS

PLANT GROWTH, BIOMASS, AND GERMINATION PERCENTAGE

Cadmium exposure significantly affects the normal growth and development of tomato plants. The fresh and dry weight of root, shoot, and leaves of tomato plant decreased in a dose-dependent manner, when treated with different Cd concentration (200, 500 and 1000 mg/kg soil). The maximum toxicity for all the tested parameters was observed at a higher concentration of Cd alone i.e. 1000 mg/kg soil. Supplementation of Zn (200 mg/kg soil) with Cd in tomato plants reverted the toxicity symptoms up to some extent (Table 1).

The effect of Cd toxicity on germination percentage showed that with increasing concentrations of Cd, germination percentage decreased gradually. The germination percentage was observed as 75.56, 64.45 and 22.62% at 200, 500 and 1000 mg/kg Cd, respectively. The results of Cd toxicity with supplemented Zn showed that germination percentage increased to a substantial level as compared to Cd alone treated tomato plants. The sequence of increasing germination percentage was observed 77.78, 75.56 and 64.48% at 200 Cd+200 Zn, 500 Cd+200 Zn and 1000 Cd+200 Zn mg/kg soil, respectively (Table 1).

Table 1. Effect of Cadmium applied alone and in combination with Zinc on the biomass, and germination percentage of tomato leaf extract.

Cd stress (mg/kg)	Control	Cd 200	Cd 500	Cd 1000	Cd 200 + Zn 200	Cd 500 + Zn 200	Cd 1000+ Zn 200
Germination percentage (%)	91.12± 5.87	75.56±8.01	64.45±4.45	22.62± 8.0*	77.78± 4.45	75.56± 8.89	64.48± 5.87
Stem fresh weight (g)	49.26± 0.40	45.68±0.81	44.26±0.81	31.12± 2.65*	43.32± 0.25	37.65± 0.65	27.5± 1.63*
Root fresh weight (g)	6.05± 0.20*	3.51±0.30*	3.01± 0.08*	2.06± 0.07*	4.30± 0.07*	2.31± 0.20*	1.40± 0.10*
Leaf fresh weight (g)	19.49± 1.04*	16.44±1.33*	15.10± 0.01*	11.11± 2.09*	14.07± 2.58*	13.20± 3.62*	11.16± 0.81*
Stem dry weight (g)	24.91± 0.38*	15.20±0.15*	12.23± 0.66*	10.31± 0.33*	21.46± 0.66*	12.27± 0.54*	10.31± 0.74*
Root dry weight (g)	2.41± 0.16*	1.43±0.09*	1.29± 0.10*	0.80± 0.09*	1.62± 0.08*	1.08± 0.09*	0.56± 0.10*
Leaf dry weight (g)	3.49± 0.71*	3.42±0.69*	5.60± 1.14*	4.33± 0.88*	6.20± 1.26*	2.7± 0.55*	4.9± 1.00*

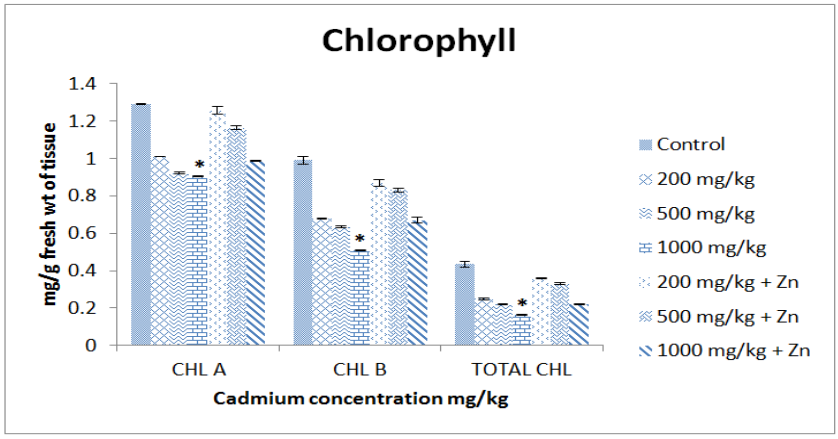
The values are mean of 3 replicates ± S.E. *Data significant at $p<0.05$. Multiple comparisons *vs.* control group (Holm Sidak method). Overall significant level = 0.05 (Zn = 200mg/kg $ZnCl_2$).

CHLOROPHYLL PIGMENTS AND CAROTENOID

The photosynthetic pigments showed a drastic reduction (34, 52, 62 and 28% of Chl a, Chl b, total Chl, and Carotenoids, respectively) in 1000 mg/kg Cd-treated plants incomparison to the control (Fig. 1 and 2). The addition of Zn (Cd+200 Zn, 500 Cd+200 Zn, and 1000 Cd+200 Zn mg/kg soil) to the medium with Cd restored the photosynthetic pigments levels. The sequence of increasing photosynthetic parameter (Chl a, Chl b, total Chl, and Carotenoids) was observed 29, 32, 51 and 9% at 1000 Cd+200 Zn mg/kg soil, respectively (Fig. 1 and 2). These results suggest that Zinc modify the toxicity of Cadmium in treated tomato plants.

LIPID PEROXIDATION

The effect of Cd toxicity on lipid peroxidation was measured by evaluating the TBARS levels in Cd-treated tissues. Compared to control, plants exposed to Cd showed an increase in TBARS level (Fig. 3). Results showed that tomato plants treated with different Cd dose (200, 500, 1000 Cd mg/kg) resulted into marked increase in the MDA content as25, 80 and 109% respectively compared to control. When treated with Zinc application the MDA content decreased by 21, 60, and 89% respectively (Fig. 3) compared with Cd alone treated tomato plant. Thus results indicated that application of Zinc might play a crucial role in scavenging radicals, thus preventing lipid peroxidation by excess active oxygen produced under Cd stress.



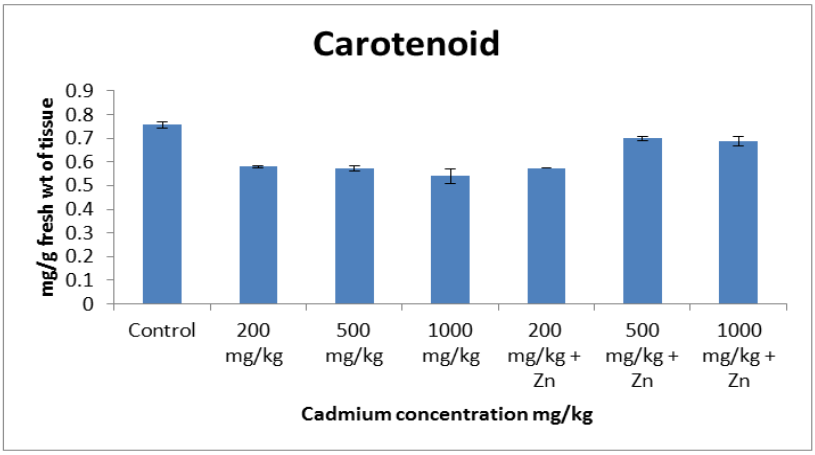


Fig.1 and 2. Effect of Cadmium applied alone and in combination with Zinc on the photosynthetic pigments [Chl = chlorophyll (mg g^{-1} fresh weight), Chl a= Chlorophyll a (mg g^{-1} fresh weight); Chl b= Chlorophyll b (mg g^{-1} fresh weight); Total Chl = Total Chlorophyll (mg g^{-1} fresh weight) and Carotenoid] of tomato leaf extract. The values are mean of 3 replicates ± S.E. *Data significant at p<0.05. Multiple comparisons *vs*. Control group (Holm Sidak method). Overall significant level = 0.05 (Zn = 200mg/kg $ZnCl_2$).

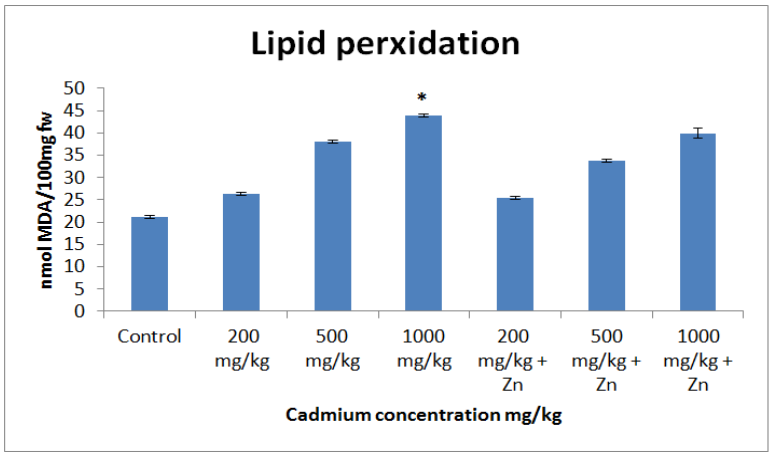


Fig. 3. Effect of Cadmium applied alone and in combination with Zinc on the lipid peroxidation of tomato leaf extract. The values are mean of 3 replicates ± S.E. *Data significant at p<0.05. Multiple comparisons *vs.* Control group (Holm Sidak method). Overall significant level = 0.05 (Zn = 200mg/kg $ZnCl_2$).

SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD) activity was found to beincreased with Cd concentrations (200, 500, and 1000 mg/kg). Itshowed increased 54, 138, and 293% respectively, whencompared with control. Adding of Zinc concentration (200 mg/kg) along with the same concentration of Cd showed adecrease in SOD activity observed which was 21, 60, and 89% respectively (Fig. 4) compared with Cd alone treated plants. However, the results suggested that Cd along with Zn treatments apparently decrease the Cd toxicity.

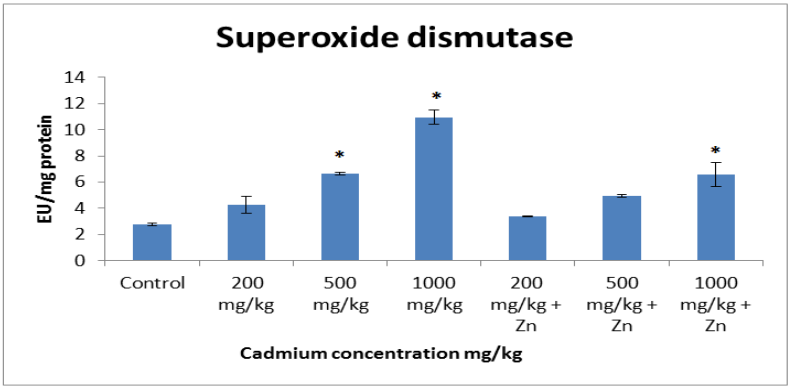


Fig. 4.Effect of Cadmium applied alone and in combination with Zinc on the Superoxide dismutase of tomato leaf extract. The values are mean of 3 replicates ± S.E. *Data significant at p<0.05. Multiple comparisons *vs.* Control group (Holm Sidak method). Overall significant level = 0.05 (Zn = 200mg/kg $ZnCl_2$).

ASCORBATE PEROXIDASE

The Ascorbate peroxidase (APX) activity was found to be increased with Cd concentrations (200, 500, and 1000 Cd mg/kg). It showed increase of 151, 330, and 466% respectively, compared with control. However addition of Zinc concentration (200 mg/kg) along with same concentration of Cd led to decrease in APX activity was observed 71, 253 and 280% respectively (Fig 5.), compared to Cd alone treated plants.

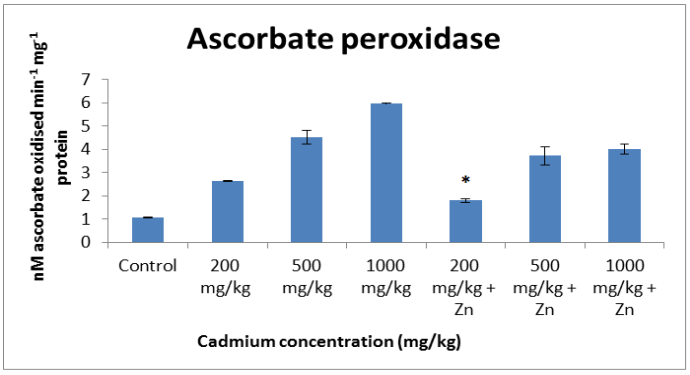


Fig. 5. Effect of Cadmium applied alone and in combination with Zinc on the Ascorbate peroxidation of tomato leaf extract. The values are mean of 3 replicates ± S.E. *Data significant at p<0.05. Multiple comparisons *vs*. control group (Holm Sidak method). Overall significant level = 0.05 (Zn = 200mg/kg $ZnCl_2$).

DISCUSSION

Cadmium is a non-essential and mobile metallic element that negatively affects plant physiological and biochemical processes (Gallego *et al*., 2012). Its contamination of agriculture land is serious environmental problem. It causes the reduction of yield and effect severely the quality of the agricultural field. In this study, we have focused on a vegetable which is commonly known as a tomato. It is largely consumed by people all around the world. If it is Cd contaminated it causes serious problems to the human and further consequences are negative effect on the human health. That's why there is urgent need to reduce Cd accumulation in agriculture lands. Cadmium is non-essential and toxic whereas Zinc is an essential nutrient, and also an important component for many vital enzymes and proteins.

Therefore, application of Zn fertilizer is an ideal method for sustainable development of agriculture. Zn supplementation proved to be beneficial for the system in combating Cd toxicity in *Ceratophyllum demersum* (Aravind *et al*., 2009) and *Solanumlyco persicum* (Cherif *et al.*, 2010). Thus our results showing the deleterious effects of Cd toxicity, could be mitigated by Zn addition in tomato plant (Table 1).The photosynthetic main component is Chlorophyll, which affects the plant growth (Mani *et al*., 2015). Several studies have shown that Cd inhibits chlorophyll biosynthesis in plant leaves (Ali *et al*., 2017). It was observed that the growth of tomato plant is adversely affected by the cadmium and at high dose of cadmium the plant growth was declined and chlorosis as well as necrosis of leaf tips was also observed. The inhibitory effect of Cadmium on plant biomass is in accordance with earlier reports in other plant species (Zhang *et al*., 2005; and Bauddh *et al*., 2016). For plants grown without Zinc addition, there was a significant decrease in biomass production. However, it was less severe in case of Cd+Zn treatments. These findings are similar to the previous reports for tomato (Cherif., 2011), and *Ceratophyllum demersum* (Prasad *et al*.,2005).

The results of Chl a, Chl b and Total Chl content (Fig. 1) showed severe decrease due to Cd. Similar results were also observed in the carotenoid content of plants. This decline in Chlorophyll and Carotenoid due to Cd has been proposed to be responsible for the reduction in photosynthesis and growth due to this metal (Maksymiec *et al*., 2007). On the other hand, when Cd-treated (200, 500, 1000 mg/kg Cd) plants were supplemented with Zn (200 mg/kg) there was maximum protection and restoration of the chlorophyll levels. Zinc maintains chlorophyll synthesis through –SH group protection of the oxidation-prone δ-Aminolevulinic acid dehydrogenase (ALA dehydrogenase) and protochlorophyllide reductase (Baszyński et al., 1980; Myśliwa-Kurdziel *et al*., 2004). Since ALA dehydrogenase catalysing the conversion of ALA to porphobilinogen requires Mg^{2-} or Zn^{2-} for its effective functioning (Beale, 1999) Zinc plays a role in activating this enzyme, and hence porphobilinogen to chlorophyll moiety. Results clearly indicate that Zn is involved not only in protection of photosynthetic pigments but it also increases the level of Chl a, Chl b, Total Chl and carotenoid as compared to Cd alone treated tomato plants (Fig. 1 and 2). These findings are similar as results reported previously by several workers (Arnon., 1949 and Kirk *et al*., 1965). MDA formation is used as the general indicator of the extent of lipid peroxidation resulting from oxidative stress. In the present study increased lipid peroxidation in response to Cdtoxicity demonstrates the increased generation of reactive oxygen species (ROS) which cause increase the TBARS content. Lipid peroxidation can also be due to a Cd-mediated increase in lipoxygenases (LOXs) activity (Smeets *et al*., 2008). Results demonstrate that the excessive Cd dose (200,500 and 1000 mg/kg) cause oxidative stress that can damage membrane and consequently increase MDA. The results are in accordance with several researchers who also found the same phenomena with MDA content (Tkalec *et al*., 2008; Gratao *et al*., 2008; Balen *et al*., 2011 and Shan *et al*., 2012).Combined treatment of Cd+Zn caused a significantly reduced MDA content in comparison to individual Cd (Fig. 3). These results indicate that Zn decreases the toxicity of Cd and maintain the membrane integrity of treated tomato plants. Superoxide dismutase (SOD) is considered as the major superoxide ($O_2^{\cdot-}$) scavenger and also provides the first line of defence against cellular injury due to environmental stress (Gratão *et al*., 2005).The highly reactive O^{2-} is converted to H_2O_2 by SOD and O_2 and play a key role in quenching ROS (Choudhury *et al*., 2011). Results of experiments showed a significant increase in the SOD activity of Cd-treated tomato plants (Fig.4). This enrichment in activity may be due to over synthesis of this enzyme. When Zn added in combination with Cd there was a very high production of SOD activity, much higher than control. Zn may also protect plants from Cd toxicity by enhancing antioxidative enzymes. It stabilizes superoxide dismutase and hence a higher Zn supplement is able to enhance dismutation of Cd-increased $O_2^{\cdot-}$radical, facilitating its detoxification in the subsequent steps utilizing CAT and APX (Cakmak, 2000). Results show an increase in the activity of APX in response to Cadmium treatments. Combined treatment of Cd and Zn decrease the APX as compared to Cd alone treatment (Arvind *et al*., 2003).

CONCLUSION

The detrimental effect of toxic Cadmium in tomato plant was clearly evident in the form of stunted growth and reduced biomass. This is further supported by elevated activity of oxidative stress markers. The results clearly indicate that tomato plant is quite sensitive towards Cadmium toxicity. Therefore, it could be concluded that an exogenous Zinc supply mitigates the oxidative stress by the improvement of antioxidative enzymes activity in test plant.

Thus, Zn protects several vital cell components such as chlorophyll, membrane lipid from oxidation etc. Such behaviour makes it possible to recommend the plants under Cd toxicity with Zinc application. This experiment is useful for the farmers as they can apply Zinc in agricultural fields to overcome the cadmium toxicity and improve the yields, biomass, and quality of crops if Cadmium contamination is there either in soil or irrigation water.

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