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Responses of cadmium exposures on growth, physio-biochemical characteristics and the antioxidative defence system of soybean (*Glycine max* L.)

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Abstract

Soybean (*Glycine max* L.) plants were subjected to different levels of cadmium (Cd) viz. 0.0 (control), 0.05, 0.10, 0.50, 1.00 and 2.00 mM from $CdCl_2$. Exposure to different Cd levels severely inhibited the growth of the plants evaluated in terms of shoot and root lengths, shoot fresh and dry weights. Total chlorophyll content, nitrate reductase activity and total protein content reduced due to various Cd treatments. Non-enzymatic antioxidants such as total ascorbate and glutathione contents increased on occurrence of Cd stress. Cd enhanced lipid peroxidation rate as evident by the TBRAS content. Also, Cd activated the activities of catalase (CAT), peroxidase (APX) and superoxide dismutase (SOD) enzymes in the treated plants. The results suggest that the Cd exposures adversely affected the plant growth, however elicited non-enzymatic as well as enzymatic antioxidants to cope with the harmful consequences of the heavy metal.

Keywords: *Glycine max* (Soybean), Cadmium (Cd^{+2}), Antioxidant, Physiochemical

INTRODUCTION

To date an unprecedented, rapid change in environmental conditions is observed, which is likely to override the adaptive potential of plants, especially the species with their long reproductive cycles. Since plants are sessile organisms and have only limited mechanisms for stress avoidance, they need flexible means for acclimation to changing environmental conditions. Cadmium (Cd) is a widely spread pollutant with no known biological function. It can reach high levels in agricultural soils and is easily assimilated by plants [1]. Moreover, due to neurotoxic, mutagenic and carcinogenic effects, high water solubility and thereby easy entry into human body via food chain render Cd a dangerous environmental pollutant even at a low concentration [2].

When plants are subjected to Cd stress, a variety of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals are generated. These ROS cause oxidative damage in plants [3]. Free radicals are toxic to living organisms unless removed rapidly, destroyed or inactivated by various cellular components. In the absence of effective mechanisms that remove or scavenge free radicals, they can seriously damage plant by lipid per-oxidation, protein degradation, breaking of DNA and cell death [4]. Plants cope with oxidative stress by using antioxidant enzymes such as SOD, CAT, GPX, APX, GR, and non-enzymatic constituents such as ascorbate and glutathione, which are responsible for scavenging excessively accumulated ROS in plants under stress conditions [5, 6, 7]. Soybean (*Glycine max* L.) has been recognized as one of the premier agricultural crops today for various reasons. In brief, soybean is a major source of vegetable oil, protein and animal feed. Soybean, with over 40 percent protein and 20 percent oil, has now been recognized all over the world as a potential supplementary source of edible oil and nutritious food [8]. Soybean is a rich source of edible oil containing no cholesterol and almost none of the saturated fats. Soybean oil surpasses all other oils because it is an ideal food for heart patients and those who wish to avoid heart disease. Besides its nutritive quality, functional properties of soy protein have opened avenues for producing new products and improving the quality of existing standard food products. A chain of soy based industries has emerged in the USA [9]. Oil is extracted for human consumption and industrial uses, and defatted soy meal is converted into various protein rich foods and feed products. In industry, soybean is used in the manufacture of edible lard, margarine, vegetable ghee etc.

Keeping in mind the immense importance of this crop and Cd stress being the deadly threat to its cultivation, experiment was conducted to evaluate the effect of different Cd concentration on growth, physio-biochemical attributed and antioxidant metabolism of soybean.

MATERIAL AND METHODS

Growth conditions and Cd treatments

Field experiment was conducted in the kharif season at the experimental field of Hamdard University, New Delhi. The individual plot size was $6m^2$ (4mX1.5m) having 6 rows with a row to row distance of 15 cm and plant to plant distance of 10 cm. The numbers of plants per m^2 were 15. Before sowing, seeds of soybean were surface sterilized with 95% ethyl alcohol for 5 min and then washed thoroughly with double distilled water. Before seed sowing, Cd was applied at the rates of 0.0 (control), 0.05, 0.10, 0.50, 1.00 and 2.00 mM from $CdCl_2$. The experiment was conducted according to a simple randomized complete block design. Each treatment was replicated five times. The plots were watered as and when required and plants were grown under naturally illuminated environmental

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conditions.

Analyses of growth attributes and chlorophyll content

Growth and biochemical attributes were determined at flowering stage i.e.75 days after sowing (DAS). The plants from each treatment were harvested with the roots carefully and shoot and root lengths were recorded. Plants were washed with tap water to remove adhering foreign particles. The plants were dried at 80°C for 48 h, and dry weights were recorded individually.

Total chlorophyll content in fresh leaves was estimated by the method of Lichtenthaler and Buschmann [10]. The fresh tissue from interveinal leaf-area were ground using a mortar and pestle containing 80% acetone. The absorbance of the solution was recorded at 662 and 645 nm for chlorophyll estimation using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan).

Determination of nitrate reductase (NR) activity

Nitrate reductase (E.C. 1.6.6.1) activity in the leaf was determined by the intact tissue assay method of Jaworski [11]. Chopped leaf pieces (200 mg) were incubated for 2 h at 30ºC in a 5.5 mL reaction mixture, which contained 2.5 mL of 0.1 M phosphate buffer, 0.5 mL of 0.2 M potassium nitrate, and 2.5 mL of 5% isopropanol. The nitrite formed subsequently was colorimetrically determined at 540 nm after azocoupling with sulphanilamide and napthylethylene diamine dihydrochloride. The NR activity was expressed as nM NO_2 g^{-1} FW h^{-1}.

Estimation of protein and proline content

Protein content was determined by the method of Bradford [12]. Fresh leaves (0.5 g) were homogenised with a mortar and pestle in 50 mM Tris/HCl (pH 7.5) on ice. Proteins were extracted in 0.1 M sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate and 1 mM EDTA. The slurry was centrifuged for 15 min at 12000×*g*. Protein concentration in the samples was estimated as mg g^{-1} FW.

The proline content was estimated by the method of Bates et al. [13]. The leaf material was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. The supernatant was used to estimate proline content. The reaction mixture, which consisted of 2 ml acid ninhydrin and 2 mL of glacial acetic acid, was boiled at 100°C for 1 h. After terminating the reaction in an ice bath, the reaction mixture was extracted with 4 mL of toluene and absorbance was read at 520 nm.

Lipid peroxidation rate (TBRAS content)

Oxidative damage to leaf lipids was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated by the method of Cakmak and Horst [14] (1991). TBARS were extracted from 0.5 g chopped fresh leaves, ground in 5mL of 0.1% (w/v) trichloroacetic acid (TCA). Following the centrifugation at 12000×*g* for 5 min, an aliquot of 1 mL from the supernatant was added to 4mL of 0.5% (w/v) TBA in 20% (w/v) TCA. Samples were incubated at 90ºC for 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation was performed at 10000×*g* for 5 min, and absorbance of the supernatant was read at 532 nm on a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan) and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. TBARS content was expressed as nmol g^{-1} fresh weight.

Antioxidant enzymes assay

Catalase activity was measured according the methods given by Chandlee and Scandalios [15] with a slight modification. The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.4 mL of 15 mM H_2O_2 and 0.04 mL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Peroxidase was assayed by the method of Kumar and Khan [16]. Assay mixture of APX contained 2 mL of 0.1M phosphate buffer (pH 6.8), 1 mL of 0.01M pyrogallol, 1 mL of 0.005M H_2O_2 and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25 ºC after which the reaction was terminated by adding 1 mL of 2.5N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a reagent blank prepared by adding the extract after the addition of 2.5N H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. One unit of the enzyme activity corresponded to an amount of enzyme that change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

Superoxide dismutase activity was assayed as described by Beauchamp and Fridovich [17]. The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1M methionine, 2×10^{-5}M KCN and 5.6×10^{-5}M nitroblue tetrazolium salt (NBT) dissolved in 3 mL of 0.05 M sodium phosphate buffer (pH 7.8) and 3mL of the reaction medium was added to 1 mL of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Philips 40W fluorescent tubes in a single row. The reaction was initiated at 30 ºC for 1 hour. Identical solutions that were kept under dark served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity was expressed as U mg^{-1} protein. One unit (U) is defined as the amount of change in the absorbance by 0.1 h^{-1} mg^{-1} protein.

Non-enzymatic antioxidants assay

Total ascorbate content

Total ascorbate content was assayed by the method of Law et al. [18]. A standard curve in the range of 0-100 n moles of ascorbate was used for calibration. Values were corrected for absorbance by eliminating the supernatant in the blank prepared. Values were expressed in n moles g^{-1} FW.

Total glutathione content

Total glutathione was determined by the glutathione recycling method of Anderson [19]. Fresh leaves (0.05 g) were homogenised in 2 mL of 5% sulphosalicylic acid at 4°C. The homogenate was centrifuged at 10,000×*g* for 10 min. To a 0.5 mL of supernatant, 0.6 mL of reaction buffer (0.1 M Na-phosphate, pH 7, 1mM EDTA) and 40 µL of 0.15% 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were added and read at 412 nm after 2 min. To the same, 40 µL of 0.4% NADPH and 2 µL of glutathione reductase (GR; 0.5 enzyme unit) were added and reaction was run for 30 min at 25°C. The samples were again read at 412 nm to determine total glutathione content.

Statistical analysis

Each plot was treated as one replicate and all the treatments were replicated five times. The data was analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Mean values were statistically compared by Duncan's Multiple Range Test (DMRT) at *p<0.05 % level using different letters.

RESULTS

The presence of Cd in the soil medium significantly lowered the values for growth attributes (Shoot and root length, shoot fresh and dry weights). Shoot length noted in 2.00 mM Cd treated plants was lowest compared to control plants showing the reduced growth under

toxic levels of Cd (Table 1). The root length was most severely affected by Cd toxicity compared to other growth attributes. In 2.00 mM Cd treated plants; the root length was 123.5% reduced compared to untreated plants. Shoot and root dry weights were also significantly reduced by different Cd concentrations. At 2.00 mM Cd (the highest applied concentration), the shoot and root dry weights of the plant were reduced maximally (Table 1).

Chlorophyll content was also reduced in Cd stressed plants and the most toxic effect was noted at 2.00 mM concentration of Cd at which the content was 76.6% less than that of untreated plants (Table 1).

Treatments	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	*chl* content (mg g^{-1} FW)
Control	21.70±2.38^{a}	12.52±1.95^{a}	23.43±2.46^{a}	3.23±0.42^{a}	1.48±0.04^{a}
0.05 mM Cd	20.53±2.17^{b}	11.81±1.75^{b}	22.67±2.34^{ab}	3.01±0.38^{ab}	1.37±0.04^{b}
0.10 mM Cd	18.41±2.05^{c}	10.30±1.89^{bc}	21.82±2.28^{b}	2.65±0.33^{b}	1.33±0.03^{c}
0.50 mM Cd	16.20±1.74^{d}	09.57±1.31^{c}	18.26±1.93^{c}	2.16±0.29^{c}	1.19±0.02^{d}
1.00 mM Cd	13.13±1.68^{e}	07.21±1.26^{d}	15.69±1.81^{d}	1.68±0.25^{d}	1.03±0.02^{e}
2.00 mM Cd	08.90±1.25^{f}	05.62±1.19^{e}	12.34±1.70^{e}	1.24±0.22^{e}	0.84±0.02^{f}

Table 1.Effect of different concentrations of Cd^{+2} on growth parameters and total chlorophyll content of *Glycine max* L.
The data shown are means of five replicates ± SE. Means within a column followed by the same letter are not significantly different ($p \leq 0.05$).

The Cd stress decreased the activity of nitrate reductase (NR) in soybean plants. The inhibitory effect of the metal was proportionate to the concentration applied and the highest applied concentration (2.00 mM Cd) decreased the activity of NR by 59.5% compared to control (Table 2).

In contrast to above parameters, the proline content increased by various Cd treatments. The highest content was noted when the plants were supplied with 2.00 mM of Cd through soil medium (Table 2). Protein content decreased on increasing Cd concentrations; the lowest protein content was noted at 2.00 mM Cd applied (Table 2).

The level of total ascorbate and glutathione was measured in order to determine the non-enzymatic antioxidants by soil applied Cd in plants. Both the previous contents were found higher in the treated plants, as compared to control (Table 2).

Treatments	NR activity (n mol NO_2^- g^{-1} FW h^{-1})	Proline content (mg g^{-1} W)	Total protein content (mg g^{-1} FW)	Total ascorbate content (n mol g^{-1} FW)	Total glutathione content (n mol g^{-1} FW)
Control	284.53±5.14^{a}	5.65±0.55^{e}	30.38±1.56^{a}	26.76±1.27^{f}	50.41±1.52^{f}
0.05 mM Cd	280.82±5.01^{ab}	5.82±0.64^{de}	29.39±1.44^{b}	28.22±1.66^{e}	53.32±1.71^{e}
0.10 mM Cd	268.71±4.87^{b}	6.30±0.69^{d}	28.64±1.38^{c}	32.47±1.87^{d}	59.24±2.01^{d}
0.50 mM Cd	236.52±4.65^{c}	8.95±0.81^{c}	26.23±1.23^{d}	37.47±1.87^{c}	67.69±1.88^{c}
1.00 mM Cd	213.28±4.49^{d}	10.31±0.89^{b}	24.69±1.11^{e}	42.81±2.15^{b}	75.32±1.71^{b}
2.00 mM Cd	178.36±3.74^{e}	13.62±0.96^{a}	20.62±1.02^{f}	48.23±2.21^{a}	84.41±1.52^{a}

Table 2. Effect of different concentrations of Cd^{+2} on NR activity, protein and proline contents, and total ascorbate and glutathione contents of *Glycine max* L.
Means within a column followed by the same letter are not significantly different ($p \leq 0.05$). The data shown are means of five replicates ± SE.

The values of TBRAS content and antioxidant enzymes significantly enhanced in the plants subjected to Cd stress. The TBRAS content was measured as an indicator of oxidative stress / membrane damage. In the leaves of the plants treated with different concentrations of Cd, TBRAS content was increased progressively. The highest content was noted when the plants were supplied with 2.00 mM of Cd through soil medium (Fig.1). There was a high CAT activity noted in the plants receiving different Cd treatments having a most pronounced effect on 2.00 mM concentration of Cd (Fig.1). Compared to control, the activity of APX and SOD were also significantly increased in the Cd treated plants and the application of 2.00 mM Cd to the soybean plants showed highest activity of APX and SOD (Fig.1).

DISCUSSION

The ill effects generated by Cd toxicity impaired the growth of the plants as evident by the shoot and root lengths, shoot fresh and dry weights. A gradual decrease in root and shoot elongation rate was observed with the increase in Cd concentrations. The reduction in root and shoot elongation with increasing concentrations of Cd has also been observed by many workers in soybean [20, 21, 22]. Among growth parameters, the most degenerative effects, of the different Cd concentrations applied, were noted on the root growth as the roots are the easily affected region of Cd toxicity. Cd toxicity block the mechanism of cell division and as a result of this root become shunted and brittle, root hair development is poor and root apices become swollen and damage. Detailed spatial studies have indicated that within the root, the root apex, and more specifically the distal part of the transition zone within the apex, is the primary target of Cd toxicity [23]. The promptness of the root growth inhibition upon exposure to Cd indicates that Cd quickly disrupts root cell expansion and elongation, prior to inhibiting cell division [21]. Increasing concentrations of Cd reduced the height of the plant and also fresh and dry weights per plant. The observed lower values for fresh and dry weights of the plant upon Cd treatments are in agreement with many researchers investigating the response of various plant species to Cd stress [20, 24].

The presence of Cd in the soil significantly decreased the level of chlorophyll in the leaves. The decrease in chlorophyll might be mediated through the reduced uptake of Mg, that Cd toxicity reduced the uptake of Mg, which is the integral part of the chlorophyll molecule [25]. Among various enzymes, nitrate reductase (NR) is a key enzyme in the conversion of nitrate to nitrite and its sustained activity is crucial to N assimilation. It was also found that Cd exposure elicited the changes in activity of the nitrate reductase. With increasing Cd concentrations, the activity of nitrate reductase in

leaves was reduced (Table 2). The nitrate reduction response was concentration dependent and at 2.00 mM of Cd caused a 59.5% decrease in the enzyme activity. Reductions in NR activity, reduced nitrogen fixation and ammonia assimilation in nodules have been reported in legumes with application of Cd [26]. The presence of Cd in the soil affected the assimilation of NO_3 in *Cicer arietinum* L. [27, 28].

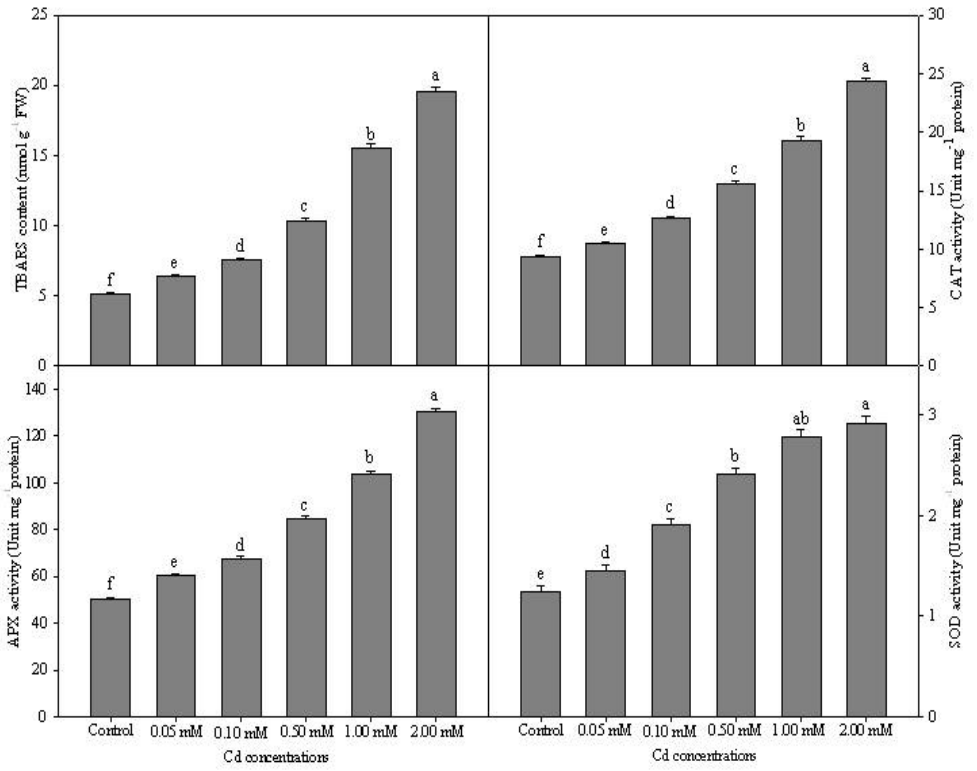

Fig. 1. Effect of different concentrations of Cd^{+2} on TBRAS content, CAT, APX and SOD activities in leaves of *Glycine max* L. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars (┬) show SE.

The Cd stress also resulted in an increase in the level of proline in the treated plants. The accumulation of free proline in response to heavy metal exposure is widespread among plants [2]. The accumulation of proline is a gene-regulated process which is the consequence of the over expression of the genes involved in its biosynthesis and depression of those responsible for its degradation, in the plants under stress. The increase in the level of proline in the present study seems to be mediated through water stress or a physiological drought generated by Cd [29]. However, Kastori et al. [30] suggested that the accumulation of proline in metal-exposed plants is directly due to metal uptake, rather than to water deficit stress. It is possible that the functional significance of proline accumulation under heavy metal stress might include water balance maintenance, scavenging of hydroxyl radicals or metal chelation.

Protein content may be considered as important indicators to assess growth performance of plants under stress conditions. Significant decrease in total protein content was noted due to different Cd treatments compared to control (Table 2). Decrease in protein content could be a consequence of increased protein degradation and/or a decrease in protein synthesis. Balestrasse et al. [20] have shown that under cadmium stress, decrease in protein content was related with increased protease activity in soybean.

The ascorbate cycle plays a crucial role in removing ROS and maintaining the cellular redox status in the different cell compartments [31, 32]. In our study, a dose-dependent response was observed. Low Cd doses produced minimal alteration in this cycle in which only a slight increase in total ascorbate and glutathione was observed, thus allowing the other cycle parameters to remain unchanged. On the other side, medium and high Cd doses produced a dramatic increase in both the contents. Consequently, increased ascorbate and glutathione was likely used to scavenge the over-production of ROS [33].

Lipid peroxidation is an important symptom of heavy metal toxicity, and has been reported in several species. Lipid peroxidation has been reported to be induced by Al treatment in soybean cultivar [14] and in *Artemisia annua* L. [34]. Yamamoto et al. [35] found that there was an increase in lipid peroxidation in pea plants after 4 h of Al treatment. Therefore, the target of oxidative stress varies depending on the plant species under Cd toxicity. In this study, it was noticed that lipid peroxidation rate in Cd exposed plants increased in a dose dependent manner with a maximum damage to the plants treated with 2.00 mM of Cd. Most plant genes so far known to be induced by Cd are either known oxidative stress genes or are induced by a range of conditions that are likely to involve oxidative stress. In addition to these commonalities one of the suggested mechanisms of Cd toxicity is that it causes lipid peroxidation [33, 36].

There is increasing evidence suggesting that oxidative stress is a key damaging factor in plants exposed to a variety of stressful conditions including metal toxicity and that plant resist oxidative stress by inducing activities of antioxidant enzymes [37, 38].

Environmental stresses increase the formation of ROS that oxidize membrane lipids, protein and nucleic acids [39]. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage [40]. All the treatments in which Cd was applied had raised the activities of antioxidant enzymes. With increasing amounts of Cd in the soil medium, a concomitant increase in the activities of CAT, APX and SOD were observed in the exposed plants. Similar to our results Ferreira et al. [36] Mobin and Khan [41] and Liu et al. [33] also reported increase in the activities of antioxidant enzymes under Cd toxicity in different plants. CAT and APX appear to play an essential protective role in scavenging process of ROS when coordinated with SOD [42]. SOD initiates detoxification of singlet oxygen by forming H_2O_2, which is also toxic and must be eliminated by conversion to H_2O in subsequent reactions. In plants, a number of enzymes regulate intracellular H_2O_2 levels, but CAT and APX are considered the most important [43]. Superoxide radicals are toxic byproducts of oxidative metabolism and can interact with H_2O_2 to form highly reactive hydroxyl radicals, which are thought to be primarily responsible for oxygen toxicity in the cell [44]. The dismutation of superoxide radicals into H_2O_2 and oxygen is an important step in protecting the cell and is catalyzed by SOD. The present observations of increased activity of antioxidant enzymes are in agreement with the fact that they remain active for scavenging of ROS.

CONCLUSIONS

From the work presented here, it is quite clear that Cd toxicity resulted in poor growth and yield of soybean plants. The data confirm the inhibitory effect of heavy metal on chlorophyll content and NR activity, which are subsequently slowed down by Cd treatments. The decrease in protein and increase in proline contents of were proportional to the increased doses of Cd, and the most phytotoxic influence was observed at the highest tested concentration (2.00 mM). Treatment with Cd also caused oxidative damage as evidenced by increased lipid peroxide. However, to cope with heavy-metal toxicity, soybean plants were able to promote the activities of antioxidant enzymes as well as non-enzymatic antioxidants.

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