Changes in the antioxidative enzyme activities and lipid peroxidation in wheat seedlings exposed to cadmium and lead stress

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Wheat seedlings were grown in presence of $CdCl_2$ (0-200 µM) and $Pb(NO_3)_2$ (0-2000 µM) separately. The growth of metal-treated seedlings was significantly depressed. The activities of antioxidative enzymes namely superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POX) were altered both in root and shoot tissues of 7-d-old seedlings. Under Cd stress, SOD activity in roots was undetectable even at the lowest Cd concentration (50 µM) whereas in shoots it declined sharply with increasing Cd levels. The activity of CAT declined to a greater extent in roots than in shoots. Even though the POX activity increased nine times in shoots, a decreasing trend was observed in root tissues due to Cd stress. Under Pb stress, the induction in SOD activity and decline in CAT activity were sharper in root tissues than in their shoot counterparts. The POX activity increased both in roots and shoots under Pb stress. Malondialdehyde concentration increased in both roots and shoots of Cd- and Pb-treated seedlings. The results suggest that metal toxicity was associated with oxidative stress. The decline in CAT activity may be the probable reason behind the Cd- and Pb-induced oxidative stress, since the alterations in SOD and POX activities showed different trends under these metal stresses.

Key words: catalase, heavy metals, peroxidase, superoxide dismutase, *Triticum*

Alterações nas atividades de enzimas antioxidantes e peroxidação de lipídeos em plântulas de trigo expostas a cádmio e chumbo: Plântulas de trigo foram cultivadas na presença de $CdCl_2$ (0-200 µM) e $Pb(NO_3)_2$ (0-2000 µM), isoladamente. O crescimento das plântulas tratadas com Cd e Pb foi significativamente reduzido. As atividades de enzimas antioxidantes, dismutase do superóxido (SOD), catalase (CAT) e peroxidase do guaiacol (POX) foram alteradas tanto em raízes como na parte aérea nas plântulas com 7 d de idade. Sob estresse de Cd, não se detectou atividade de SOD nas raízes, mesmo na menor concentração de Cd testada (50 µM), enquanto a atividade da enzima, na parte aérea, decresceu apreciavelmente com o incremento nos níveis de Cd. A atividade da CAT diminuiu, porém mais nas raízes que na parte aérea. A atividade de POX aumentou nove vezes na parte aérea, porém decresceu nas raízes, em resposta ao estresse de Cd. Sob estresse de Pb, a indução da atividade de SOD e o declínio da atividade de CAT foram mais expressivos nas raízes que na parte aérea. A atividade da POX aumentou tanto nas raízes como nas folhas sob estresse de Pb. A concentração de aldeído malônico aumentou tanto nas raízes como na parte aérea das plântulas tratadas com Cd ou Pb. Os resultados sugerem que a toxicidade causada pelos metais foi associada com estresse oxidativo. O declínio da atividade da CAT deve ter tido um papel preponderante nesse contexto, haja vista que as alterações nas atividades da SOD e POX mostraram diferentes padrões sob as condições de estresse causadas por esses dois metais.

Palavras-chave: catalase, dismutase do superóxido, metais pesados, peroxidase, *Triticum*

INTRODUCTION

Nowadays, development has become synonymous with deforestation and progress has become synonymous with pollution. Because of the increased mining and industrial activities in the late 19th and early 20th century, the pollution due to heavy metals has increased considerably worldwide. Among the different heavy metals, cadmium (Cd), lead (Pb) and mercury (Hg) are considered as the most toxic in the environment (Landis and Yu, 1999; Oliveira da Silva et al., 2005). Cadmium and Pb are hazardous heavy metal pollutants that are released to the environment from various sources of modern human activities (Eick et al., 1999; Sanita di Toppi and Gabrielli, 1999). Since most of the activities involving these metals are indispensable for today's life, the environmental contamination and specifically the soil contamination with Cd and Pb is not likely to drop off in the near future. Even though these metals are not essential for plants, because of their water solubility they are readily absorbed by the root systems. The growth and metabolisms of plants are adversely effected by the increasing levels of these metals in the soil environment (Kastori et al., 1992; Balestrasse et al., 2003). Besides that, the metals are also accumulated in different plant parts and thereby enter into the food chain. Therefore, pollution due to heavy metals is significant from nutritional and environmental point of view.

Plants are very often subjected to a variety of environmental stresses that are known to generate reactive oxygen species (ROS) and create oxidative stress situations (Elstner et al., 1988; Baisak et al., 1994; Shah et al., 2001). The pollutant metals are involved in different ROS generating mechanisms within the cells (Stohs and Bagchi, 1995) and thereby induce oxidative stress. Both Cd and Pb can enhance the prooxidant status of the cell by reducing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems and affecting iron-mediated processes (Pinto et al., 2003). These metals are also known to interrupt the photosynthetic electron transport chain which leads to generation of superoxide and singlet oxygen (Asada and Takahashi, 1987). The effects of Cd- and Pb-induced oxidative stress in plants have been reported in the forms of either increase or decrease in the antioxidative enzyme activities and alterations in the levels of antioxidant molecules (Chaoui et al., 1997; Malecka et al., 2001; Schutzendubel et al.,

2001). In most of the studies the antioxidative efficiency under metal stress has been documented either in roots or in shoots. But reports on the changes in the antioxidative efficiency both in roots and shoots due to Cd- and Pb-induced oxidative stress under identical experimental conditions are very scarce. Therefore, in this study attempts have been made to find the possible reason behind Cd- and Pb-induced oxidative stress in wheat seedlings by assessing the activities of antioxidative enzymes like superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and guaiacol peroxidase (POX, EC 1.11.1.7), both in roots and shoots under identical experimental conditions.

MATERIAL AND METHODS

Growth of plant material and imposition of heavy metal stress: Wheat (*Triticum aestivum* L. cv. Sonalika) seeds of uniform size were selected and surface sterilized with freshly prepared filtered 3% solution of commercial bleaching powder (calcium oxychloride) for 30 min, followed by washings for several times with distilled water. The seeds were then germinated on moist filter paper in Petri dishes in the dark and after 24 h, uniform seeds were transferred to nylon nets stretched over small plastic containers containing 150 mL solutions of different concentrations of cadmium chloride ($CdCl_2$) and lead nitrate ($Pb(NO_3)_2$), prepared with distilled water. The metals were studied separately to observe their independent effects on the plants. The concentrations used for Cd were 50, 100, 200, 500, 1000 and 2000 μM and that for Pb were 200, 500, 1000 and 2000 μM. Distilled water, without any metal, was used as control. Twenty germinated seeds were kept on each container to maintain the uniformity of the growth condition and stress imposition. The plants were grown with 8 h light/16 h dark cycle, at $30 \pm 1^{o}C$, under a photosynthetic photon flux density of *ca.* 90 μmol m^{-2} s^{-1} at the surface of the nylon net. Seven-day-old seedlings (6 d of exposure to heavy metals) were sampled from all the treated and control containers and root and shoot tissues were collected separately for biochemical analyses.

Evaluation of seedling vigour: To assess the effects of Cd and Pb on growth, 7-d-old seedlings were collected and root and shoot lengths were measured. The values were compared with those of the control plant.

Extraction and estimation of soluble protein: Root and shoot tissues were homogenized separately with cold 50 mM sodium phosphate buffer under ice-cold conditions, and the resulting homogenates were centrifuged at 0ºC at 17,000 *g* for 10 min. An equal volume of 20% (w/v) trichloroacetic acid (TCA) was added to the supernatants and these were kept overnight in a refrigerator to facilitate complete precipitation of soluble protein. The samples were then centrifuged at 1900 *g* for 15 min and the pellets washed successively with 10% cold TCA, ethyl alcohol, ethyl alcohol: chloroform (3:1, v/v), ethyl alcohol: ether (3:1, v/v) and finally with ether. The pellets were evaporated to dryness and solubilized by resuspending with 0.3 N NaOH for 16 h at 37ºC. The samples were centrifuged and supernatants collected for protein estimation following the method of Lowry et al. (1951), using BSA as standard.

Extraction and estimation of malondialdehyde (MDA): The level of lipid peroxidation was measured by estimating MDA, a decomposition product of peroxidized polyunsaturated fatty acid component of membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968). The tissues were homogenized with 5% (w/v) TCA and 1 mL of homogenate was mixed with 4 mL of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixtures were heated at 95ºC for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 1900 *g* for 10 min. The absorbance of the coloured supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm and for absorbance at 532 nm of the correction blank. For the reference blank 1 mL of 5% (w/v) TCA was mixed with 4 mL TBA reagent and for the correction blank 1 mL of tissue homogenate was mixed with 4 mL of TBA reagent 20% (w/v) TCA. Concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} for MDA at 532 nm.

Enzyme extraction and assay: Root and shoot tissues were homogenized under ice-cold conditions in extraction buffers containing 10% (w/v) insoluble PVPP. The buffers used were: 50 mM sodium phosphate buffer, pH 7.4 for SOD, and 50 mM sodium phosphate buffer, pH 7.5 for CAT and POX. The homogenates were centrifuged

at 17,000 *g* for 10 min at 0ºC. The resulting supernatants were desalted by passing through gel filtration columns, packed with pre-soaked Sephadex G-25 (fine grade). The eluted fractions were tested for protein and the fractions responding to the test were pooled and used directly for the assay of the enzyme. Superoxide dismutase was assayed by measuring the inhibition of superoxide-driven nitrite formation from hydroxylamine hydrochloride, as formulated by Das et al. (2000). The enzyme activity was calculated from the value of V_o/V -1, where V_o is the absorbance (read at 543 nm) of the control (without enzyme) and V is the absorbance of the sample (with enzyme). Catalase was assayed by measuring the decrease in the H_2O_2 concentration at 240 nm (Aebi, 1974) and the activity was calculated by using the extinction coefficient of 40.0 mM^{-1} cm^{-1} for H_2O_2 at 240 nm. Peroxidase was assayed using guaiacol and H_2O_2 as the substrates. The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm as described by Kar and Feierabend (1984); the activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} due to tetraguaiacol formation.

Presentation of data and statistical analysis: One unit of SOD was defined as the amount that inhibits the superoxide-driven nitrite formation from hydroxylamine hydrochloride by 50% under the assay conditions. Activities of CAT and POX were expressed in katals, i.e., one mole of substrate consumed or product formed per second. All the experiments were performed at least for three times with three replicates each time. The mean values are presented with the SD. The significance of difference between means (of either root or shoot tissues for a particular parameter, not between root and shoot tissues) was computed following the LSD test (Gomez and Gomez, 1984).

RESULTS

Relative to control plants, growth of the wheat seedlings was drastically reduced due to Cd and Pb at all tested concentrations (Figure 1). For Cd, there was an almost complete inhibition of growth from 200 µM onwards; even at 50 µM Cd, growth was inhibited (~60%) in both roots and shoots. Therefore, all the analytical studies were restricted to treatments in the range of 0-200

μM of Cd. For Pb, growth of roots and shoots was significantly reduced, even at the lowest concentration (200 μM). At 2000 μM of Pb, lengths of both shoot and root were very small and growth was almost fully inhibited.

In control seedlings, SOD activity was lower in roots than in shoots (Figure 2). With increasing Cd concentration, there was a sharp decline in SOD activity
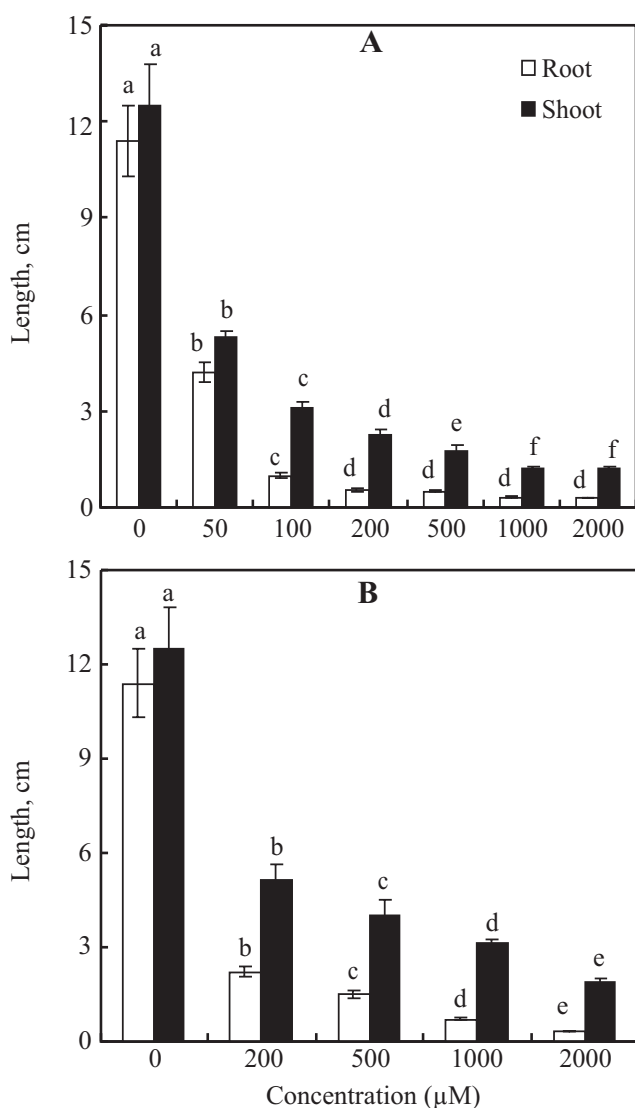
Figure 1. Changes in the root and shoot length of 7-d-old wheat seedlings grown in presence of incresing concentrations of CdCl$_2$ (**A**) and Pb(NO$_3$)$_2$ (**B**), separately. The values are the mean ± SD of three independent experiments each with three replicates. The mean values, of a particular tissue type (either root or shoot), followed by the same letter are not significantly different (*P* ≤ 0.05; LSD test).

in shoot tissues, reaching significance even at the lowest Cd concentration tested (50 μM), whereas in root tissues there was a complete loss of SOD activity starting from 50 μM onwards. The activity of CAT nearly halved in roots at 50 μM of Cd, and thereafter small changes were noted at higher Cd concentrations (Figure 2). By contrast, in shoot tissues the decline in CAT activity was not so sharp; e.g., it was 32% at 200 μM of Cd. The trends in POX activity were different in root and shoot tissues. Roots of control plants had 58 times more POX activity than shoot tissues. With the imposition of Cd stress, POX activity declined in roots but ultimately increased nine-fold in shoots, in comparison with control plants (Figure 2).

There were alterations in the activities of all three enzymes in wheat plants grown under Pb stress (Figure 3). The SOD activity increased both in root and shoot tissues with increasing Pb concentrations. However it should be noted that although roots of control plants had lower SOD activity than that of shoots, with the imposition of metal stress the increase in SOD activity in roots was remarkably sharper than in shoots; at 2000 μM of Pb, enzyme activity in root tissues was about three times higher than that in shoot tissues. The CAT activity declined significantly with Pb stress, but to a greater extent in roots than in shoots. In contrast, POX activity showed an opposite trend, i.e. it increased both in root and shoot tissues as the concentrations of Pb increased in the growth medium. In general, at all the concentrations, roots had a much higher POX activity than that of shoots.

Malondialdehyde concentration was enhanced both in root and shoot tissues with the severity of metal stress (Figure 4). However, Cd-induced increased MDA accumulation reached statistical significance only at 200 μM of Cd. For Pb, MDA was significantly larger in root tissues at 1000 and 2000 μM than in control plants whereas in shoots such difference started occurring at 200 μM Pb, but with no appreciable change at higher concentrations.

DISCUSSION

In the present study, the most prominent symptom of Cd (as well as Pb) toxicity was found to be the stunted growth of 7-d-old seedlings. Like other stresses, Cd stress is also expected to alter the activities of
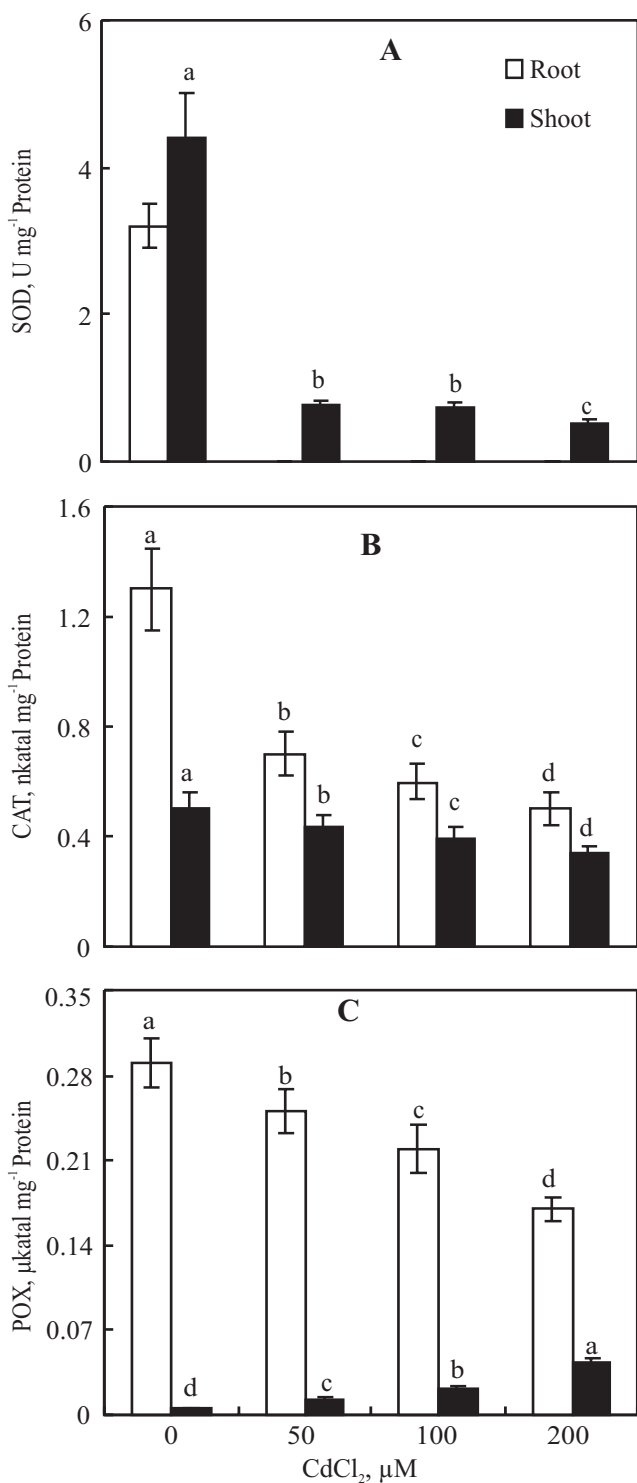
Figure 2. Changes in the specific activities of superoxide dismutase (SOD) (**A**), catalase (CAT) (**B**) and guaiacol peroxidase (POX) (**C**) in root and shoot tissues of 7-d-old wheat seedlings grown in presence of incresing concentrations of $CdCl_2$. See legend to Figure 1 for details.
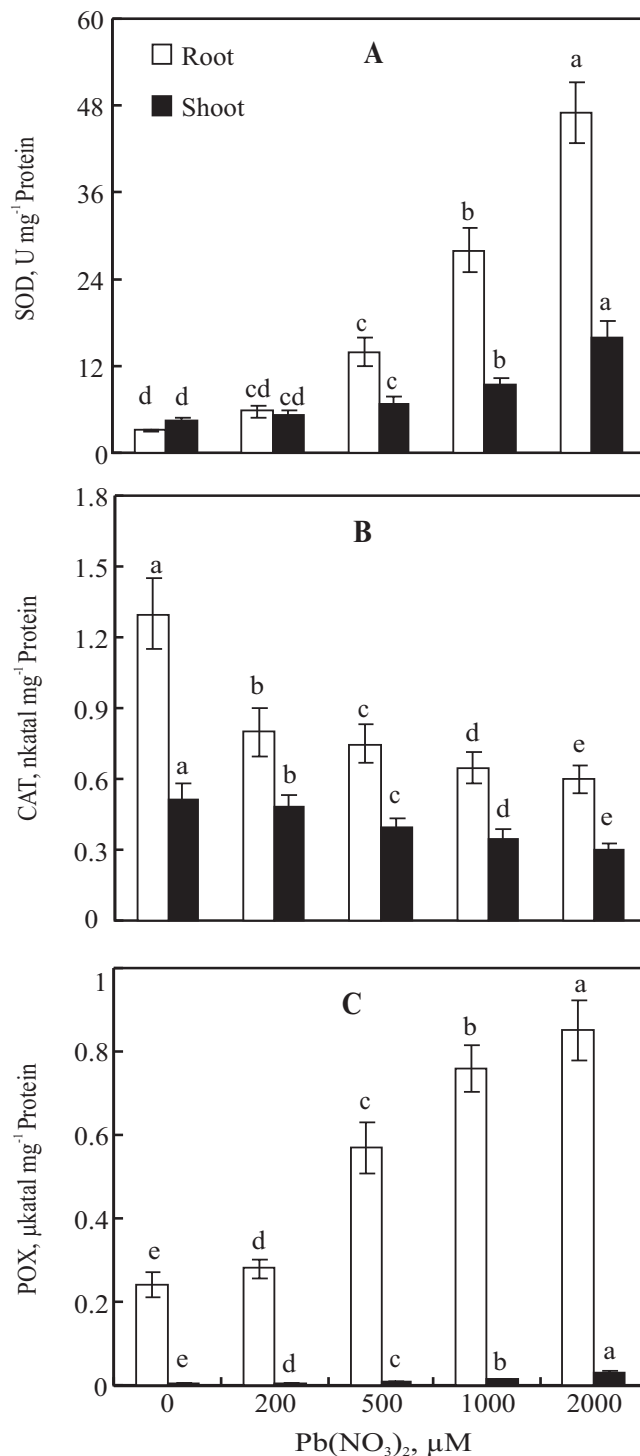
Figure 3. Changes in the specific activities of superoxide dismutase (SOD) (**A**), catalase (CAT) (**B**) and guaiacol peroxidase (POX) (**C**) in root and shoot tissues of 7-d-old wheat seedlings grown in presence of increasing concentrations of $Pb(NO_3)_2$. See legend to Figure 1 for details.
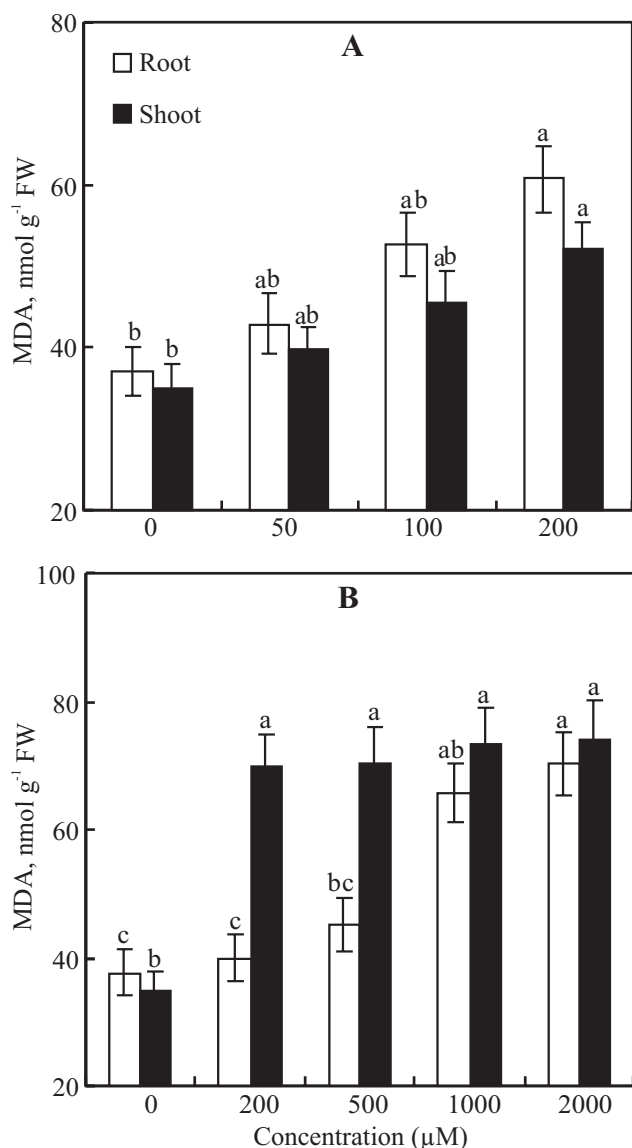
Figure 4. Changes in lipid peroxidation in root and shoot tissues of 7-d-old wheat seedlings grown in presence of different concentrations of CdCl$_2$ (**A**) and Pb(NO$_3$)$_2$ (**B**), separately. See legend to Figure 1 for details.

antioxidative enzymes and thereby creating an oxidative stress situation. Superoxide dismutase, CAT and POX are important antioxidative enzymes that function in the cells to prevent the build-up of ROS (Elstner, 1982; Halliwell and Gutteridge, 1999). Superoxide dismutase destroys superoxide, forming H$_2$O$_2$ which in turn may be detoxified by CAT and POX. As a result, the formation of the hydroxyl radical is prevented since it is produced by the interaction of superoxide and H$_2$O$_2$, being catalyzed by

transition metal ions (Elstner, 1982). A very sharp decline in SOD activity in shoot tissues and its complete loss in root tissues, as well as the decrease in CAT activity (Figure 2), indicate the weakening of superoxide and H$_2$O$_2$ scavenging systems due to Cd stress. In addition, the decreased POX activity in roots may also be responsible for the poor efficiency to decompose H$_2$O$_2$ in Cd-treated roots. Even in shoots where POX increased remarkably under Cd stress, its activity may not be linked to efficient H$_2$O$_2$ scavenging. This is because guaiacol peroxidases are localized in cytosol, cell wall, vacuole and in extracellular spaces and induction of activity under Cd stress might be due to increased release of cell-wall-bound peroxidases, as has been observed earlier under various stress situations (Mittal and Dubey, 1991; Zhang and Kirkham, 1994; Dey and Kar 1995). In any case, the observed decreases in SOD and CAT activities may be because of enzyme inhibition, since Cd is known to be a potential enzyme inhibitor (Das et al., 1997; Benavides et al., 2005). Our results support the findings of Schutzendubel et al. (2001) who reported a severe suppression of SOD and CAT, and an almost complete loss of ascorbate peroxidase activity in pine roots after 48 h of exposure to 50 μM Cd. However, contrasting results such as either fluctuation or increase in the activities of these enzymes under Cd stress have also been found (Dixit et al., 2001; Zhang et al., 2007). Although the increase in these enzyme activities has also been linked to the metal tolerance of plants (Zhang et al., 2007), the Cd stress in the present study was accompanied by a weakening of ROS detoxification systems, thus increasing the chances of their accumulation in plant tissues.

Lead-induced increase in SOD activity suggests that due to imposition of Pb stress, *de novo* synthesis of enzymatic protein may have occurred which would be more pronounced in root tissues. Similar elevation in SOD activity was also reported in other systems under Pb stress (e.g., Lozano et al., 1996; Verma and Dubey, 2003). A decreased superoxide concentration is thus to be expected, but in parallel with an increased production of H$_2$O$_2$. Peroxidases should play a more significant role than CAT in detoxifying the produced H$_2$O$_2$ since the activity of POX increased, in contrast to that of CAT. That augmentation might be due to increased release of

peroxidases localized in cell walls as has been reported in rice under Pb stress (Verma and Dubey, 2003) and also under other stress situations (Mittal and Dubey, 1991; Zhang and Kirkham, 1994; Dey and Kar 1995). Increase in POX activity is now used as a biomarker of heavy metal stress (Zhang et al., 2007).

In the case of Cd stress, the protection against superoxide and H_2O_2 becomes weakened due to the decline in activities of SOD and catalase which may favour the elevated steady state levels of H_2O_2 and superoxide. These two ROS can react in presence of transition metal ions to produce the hydroxyl radical (Elstner, 1982). In the case of Pb, even though there was some protection against superoxide, the H_2O_2 detoxification mechanism was poor. In aerobic cells, hydroxyl radicals are known to be formed from H_2O_2 in presence of transition metal ions (Halliwell and Gutteridge, 1999). In aerobic cells, the hydroxyl radical is known to be the most potentially toxic species. The unsaturated fatty acid components of membrane lipids are highly susceptible to hydroxyl radical attack and are peroxidized in its presence. Therefore, lipid peroxidation is the consequence of free radical mediated reactions in aerobic cells and is a good indicator of prevalence of oxidative stress (Kappus, 1985). In this study, even though total peroxide level was not determined, increased levels of MDA with increasing Cd and Pb concentrations were found. In the case of Cd stress, there was a continuous increase in the MDA level both in roots and shoots with increasing metal concentration, but in Pb stress, the MDA level peaked at 200 µM of Pb in shoots with no appreciable changes at higher concentrations. This is probably because peroxidizable fatty acid content becomes limiting. Further studies involving membrane lipid analyses and measurement of lipophilic antioxidant levels in the shoot tissues subjected to Pb stress will clarify this point in the future. Thus the increased MDA indicates the prevalence of oxidative stress and perhaps this may be one of the possible mechanisms by which toxicity due to Cd and Pb stress could be manifested in plant tissues. Probably this oxidative stress situation might have occurred due to the alterations in the activities of the antioxidative enzymes. However, prevalence of oxidative stress situation can be conclusively substantiated by measuring the steady state levels of ROS in the tissues.

The sharp decline in CAT activity in roots than in shoots both in presence of Cd and Pb, the complete loss of SOD activity in roots of Cd-treated seedlings and the sharp induction in SOD activity in Pb-treated roots may be due to higher accumulation of these metals in roots, as has been detected in rice for Cd (Shah et al., 2001) and Pb (Verma and Dubey, 2003). Whereas CAT was found to be declining in root as well as in shoot tissues, both under Cd and Pb stress, the other two enzymes showed different trends in two different metal stress, except for POX activity in shoots under Pb stress. Thus we presume that the decline in CAT activity was the key behind the Cd- and Pb-induced oxidative stress. However, the roles of enzymes involved in ascorbate-glutathione cycle cannot be underestimated. Therefore, analysis of these enzyme activities and determination of the contents of low molecular weight antioxidants like ascorbate and glutathione will be helpful in drawing any such conclusion. Since no nutrient solution was supplemented for growing the seedlings for 7 d, the results of this study can be taken as reference for analyzing any antagonistic or synergistic effects of nutrients with Cd or Pb toxicity. At the same time the single effects of these metals on antioxidative enzyme activities, as herein reported, will also be helpful in co-contamination studies, since Cd and Pb seldom occur in isolation in the polluted soil environment.

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