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Antioxidative efficiency of *Triticum aestivum* L. exposed to chromium stress

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Abstract: *Wheat (Triticum aestivum L. cv. Sonalika) seedlings were grown in presence of $K_2Cr_2O_7$ (10, 50 and 100 ppm) for 7 days and growth, total chlorophyll, 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) and lipid peroxidation were determined in root and shoot tissues. Growth of the seedlings was significantly ($p \leq 0.05$) depressed and at 100 ppm, root length was reduced by 63% and shoot length by 44% in comparison to the respective controls. Total chlorophyll loss in shoots was about 46% at 10 ppm of $K_2Cr_2O_7$ which further increased to 80% at 100 ppm. Both in root and shoot tissues, activities of SOD and CAT declined with increase of metal in growth medium and it was significant ($p \leq 0.05$) even at lowest concentration of the metal tested. But POX activity showed a different trend. In root tissues it was decreased whereas in shoots, there was many fold increase in the activity (about 370% over control at 100 ppm). Malondialdehyde (MDA) content increased both in root and shoot tissues, but it reached significant ($p \leq 0.05$) level at 50 ppm in roots and at 100 ppm in shoot tissues. Even though antioxidative enzyme activities were not assayed in germinating embryos, inhibition in germination percentage (by 40% at 100 ppm) and increase in lipid peroxidation level (by 71% over control at 100 ppm) were observed in 2-day-old embryos, germinated in presence of $K_2Cr_2O_7$ (10, 50 and 100 ppm). The results indicated the imposition of oxidative stress situations both during germination and early stages of seedling growth by Cr^6 stress, which might be one of the probable reasons behind Cr toxicity in plants.*

Key words: *Antioxidative enzymes, Cr^6, Lipid peroxidation, Wheat*
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Introduction

Environmental release of different compounds of chromium occurs mainly because of the widespread use of this metal in various industries such as refractories, metallurgical and chemical. Chromium compounds are extensively used in leather processing and finishing, drilling muds, electroplating cleaning agents, production of refractory steel, catalytic manufacture and in the production of chromic acid (Shanker *et al*., 2005). Out of the total industrial use of Cr, 40% is being used in leather processing industries which, therefore, are the major cause for high influx of Cr to the environment (Barnhart, 1997). In India, annually about 2,000-32,000 tons of elemental Cr escape into the environment from tanneries only. Since the modern human life is very much dependent on these industrial processes, environmental contamination due to Cr is not likely to reduce in near future. There are various valence states of Cr. But in biological systems, the stable forms of Cr are the trivalent (Cr^3) and the hexavalent (Cr^6). Other valance states are unstable and short lived in biological systems. Hexavalent Cr usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions and is therefore, highly soluble in water. It is highly mobile and considered as the most toxic form of Cr. On the other hand, Cr^3 is less mobile, less toxic and is mainly found bound to organic substances in the environment (Becquer *et al*., 2003).

Because of its complex electronic chemistry, Cr, in contrast to other toxic heavy metals like Cd, Hg, Pb and Al, has received little attention from plant scientists. Even though some crops are not affected by low concentrations of Cr (3.8×10^{-4} µM) (Huffman and Allaway, 1973), the metal is toxic at higher concentrations to most of the higher plants (Davies *et al*., 2002). The phytotoxic effects of Cr includes inhibition of seed germination, loss of photosynthetic pigments, reduction in growth and yield along with several other physiological anomalies (Barcelo and Poschenrieder, 1997; Shanker *et al*., 2005). Recently, specific toxicant "signatures" with respect to Cr and other toxic metals have been detected using genome base technologies such as microarrays which are of helpful in human health risk assessment programs (Yoon *et al*., 2008). Heavy metals like Cd, Pb, Hg *etc.* are known to impose oxidative stress situations in plants by altering the natural antioxidative efficiency of the cells (Verma and Dubey, 2003; Dey *et al*., 2007; John *et al*., 2007; Zhang *et al*., 2007). Oxidative stress situations are generally created when there is generation of higher amounts of reactive oxygen species (ROS) like superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) beyond the cell's capacity of endogenous antioxidative protective system to scavenge them off (Elstner *et al*., 1988; Halliwell and Gutteridge, 1999). Thus oxidative stress is essentially a regulated process and the equilibrium between oxidative and antioxidative capacities determines the fate of plants. Assessment of antioxidative efficiency of plants subjected to heavy metal stress is very vital to understand the toxicity mechanism and perhaps this is why lots of works have been done in this aspect for different heavy metals, but chromium has received little attention in this regard which may be due to its complex electronic chemistry. Therefore, in this study attempts have been made to assess the antioxidative efficiency of wheat seedlings exposed to Cr stress by measuring the activities of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT) and guaicol peroxidase (POX), both in roots and shoots, under the

identical experimental conditions. Some aspect of oxidative metabolism was also evaluated during germination of the wheat seeds.

Materials and Methods

Plant material, growth conditions and imposition of Cr stress: Wheat (*Triticum aestivum* L. cv. Sonalika) seeds were selected for uniform size and surface sterilized with freshly prepared filtered 3% solution of commercial bleaching powder (calcium oxychloride) for 30 min, followed by washings with distilled water for several times. The seeds were germinated on moist filter paper in Petri dishes in dark and after 24 hr of germination; uniform seeds were transferred to nylon nets stretched over small plastic containers containing 150 ml solutions of different concentrations (10, 50 and 100 ppm) of $K_2Cr_2O_7$ (for Cr^6), prepared in half strength Hoagland's solution. In another container, Hoagland's solution without $K_2Cr_2O_7$ was taken as control. Twenty germinated seeds were kept on each container to maintain uniformity of stress imposition and growth conditions. The plants were grown with 8 hr light :16 hr dark cycle, at 30 ± 1°C, under a photosynthetic photon flux density of 90 µmol m^{-2} s^{-1} at the surface of the nylon net. Seven-days-old seedlings (6 days of exposure to Cr) were collected from all the treated and control containers and different biochemical analyses were done in root and shoot tissues separately.

To study the effect of Cr on the germination, 30 uniform size surface sterilized (as mentioned above) wheat seeds were spreaded over filter paper (moistened with 10 ml of 10, 50 and 100 ppm $K_2Cr_2O_7$, prepared with half strength Hoagland's solution) in different Petri dishes. 10 ml of Hoagland's solution, without $K_2Cr_2O_7$ was taken in another Petri dish as control. The Petri dishes were covered and kept in an incubator at 30°C for 48 hr. Number of seeds germinated in each Petri dish was counted to determine the percentage of germination.

Evaluation of seedling vigour: To study the effect of Cr on growth, 7-days-old seedlings grown in presence of $K_2Cr_2O_7$ were collected and root and shoot lengths (cm) were measured. The values were compared with those of the control plants.

Extraction and estimation of total chlorophyll: Total chlorophyll content of 7-days-old seedlings was extracted by homogenizing the shoot tissues with 80% acetone. The homogenates were centrifuged at 1900 *g* for 10 min and the absorbance of the supernatant was read at 645 and 663 nm wavelength in a spectrophotometer and total chlorophyll (mg g^{-1} fresh wt.) content was calculated as described by Arnon (1949).

Extraction and estimation of soluble protein: For soluble protein extraction, small volumes of respective enzyme extracts (the preparations of which are given below) utilised for enzyme assays, were used separately. For protein precipitation, an equal volume of 20% (w/v) trichloroacetic acetic acid (TCA) was added to the enzyme supernatants and were kept overnight in a refrigerator. The pellets were then 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 with 0.3 N NaOH. The supernatants were collected for protein estimation using bovine serum albumin as standard, as described by Lowry *et al*. (1951).

Extraction and estimation of malondialdehyde (MDA): In this study, the level of lipid peroxidation was measured by estimating MDA (nmol g^{-1} fresh wt.) which is a decomposition product of peroxidized polyunsaturated fatty acid components of membrane lipid. Thiobarbituric acid (TBA) was used as the reactive material and the extraction and estimation was done following the method of Heath and Packer (1968). Along with the root and shoot tissues of 7-days-old seedlings, MDA was also estimated in 2-days-old germinating embryos (taking whole embryo, leaving endosperm portion).

Extraction and estimation of antioxidative enzymes: The activities of three antioxidative enzymes, *viz*, superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POX) were assayed in the root and shoot tissues of 7-days-old wheat seedlings. For enzyme extraction, root and shoot tissues were homogenized under ice-cold conditions in extraction buffers containing 10% (w/v) insoluble polyvinylpolypyrrolidone. 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. Homogenates were centrifuged at 17, 000 *g* for 10 min at 0°C and the resulting supernatants were desalted by passing through gel filtration columns, packed with presoaked Sephadex G-25 (fine). The eluted fractions were tested for protein and the fractions responding to protein test were collected and used for the assay of the enzyme. The activity of SOD (U mg^{-1} protein) was assayed by measuring the inhibition of superoxide driven nitrite formation from hydroxylamine hydrochloride, following the method of Das *et al*. (2000). SOD activity was calculated using the formula Vo/V-1, where Vo is the absorbance at 543 nm of the control (without enzyme) and V is the absorbance of sample (with enzyme) at the same wavelength. Catalase activity (nkatal mg^{-1} protein) was assayed by measuring the decreasing concentration of H_2O_2 at 240 nm due to CAT (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. Guaiacol and H_2O_2 were used as substrates to assay POX activity. The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm, as described by Kar and Feierabend (1984). Peroxidase activity (µ katal mg^{-1} protein) was calculated using the extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ due to tetraguaiacol formation.

Statistical analysis: All the experiments were performed at least for three times with three replicates in each time. The mean values are presented in Figures with SD. For analyzing the level of significance among the means (of either root or shoot tissues for a particular parameter, not between root and shoot tissues), LSD test (Gomez and Gomez, 1984) was performed.

Results and Discussion

Germination of seeds is usually considered as the first visible indication of growth which is regulated by several physical and

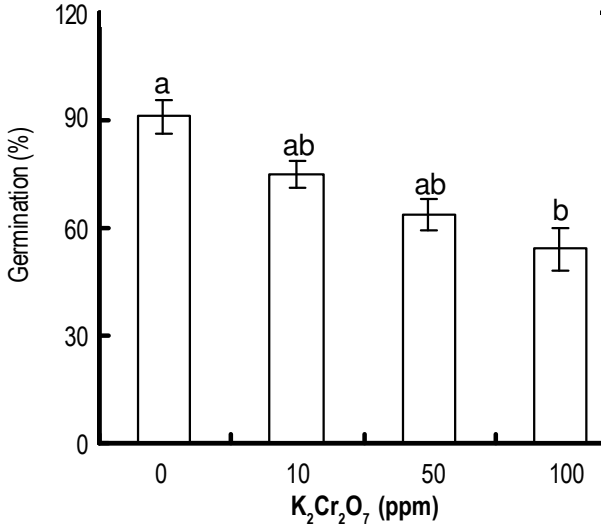

Fig. 1: Effect of $K_2Cr_2O_7$ on the germination percentage of wheat seeds after 2 days of incubation. The values are the mean ± SD of three independent experiments. The mean values followed by the same letter are not significantly different ($p \leq 0.05$; LSD test)

physiological conditions. In this study, even though the reduction in germination percentage (as recorded after two days of incubation) was concentration dependent, it reached significant level ($p \leq 0.05$) only at 100 ppm of $K_2Cr_2O_7$ (Fig. 1). Like Cr, other heavy metals (Cd, Pb and Hg) are also known to inhibit seed germination in different plant species (Al-Yemeni, 2001; Pandey *et al*., 2007). Although the reason behind Cr induced germination inhibition was not investigated in this study, decrease in both α and β-amylase activities under Cr stress was reported by Zeid (2001). The amylase activity hydrolyses starch and supply sugar to developing embryos. The decrease in amylase activity under Cr treatment thus decreases sugar availability to developing embryo which might be one of the reasons behind germination inhibition. As reported herein, the inhibition in germination by Cr has also been reported in many studies (Corradi *et al*., 1993; Nayari *et al*., 1997), but studies on the mechanisms behind such inhibition are rare, excepting few (Zeid, 2001). Therefore, detailed studies on the physiological changes during germination under Cr treatment would help in understanding mechanisms behind Cr induced germination inhibition. Such studies will also help in manipulating the conditions in Cr polluted sites to restore germination.

In comparison with the control plants, the growth of the treated plants was significantly reduced as observed in the form of decrease in root and shoot lengths under all the concentrations of metal tested (Fig. 2). Decrease in root and shoot lengths started even at 10 ppm of $K_2Cr_2O_7$ (lowest concentration of the metal tested) and at 100 ppm, the root length was reduced by 63% and shoot length was reduced by 44%. This indicated a greater toxic effect of Cr on roots than on shoots. Decrease in root and shoot lengths are a well documented effect due to different heavy metal toxicity in plants (Rout *et al*., 1997; Al-Yemeni, 2001; Dey *et al*., 2007). As

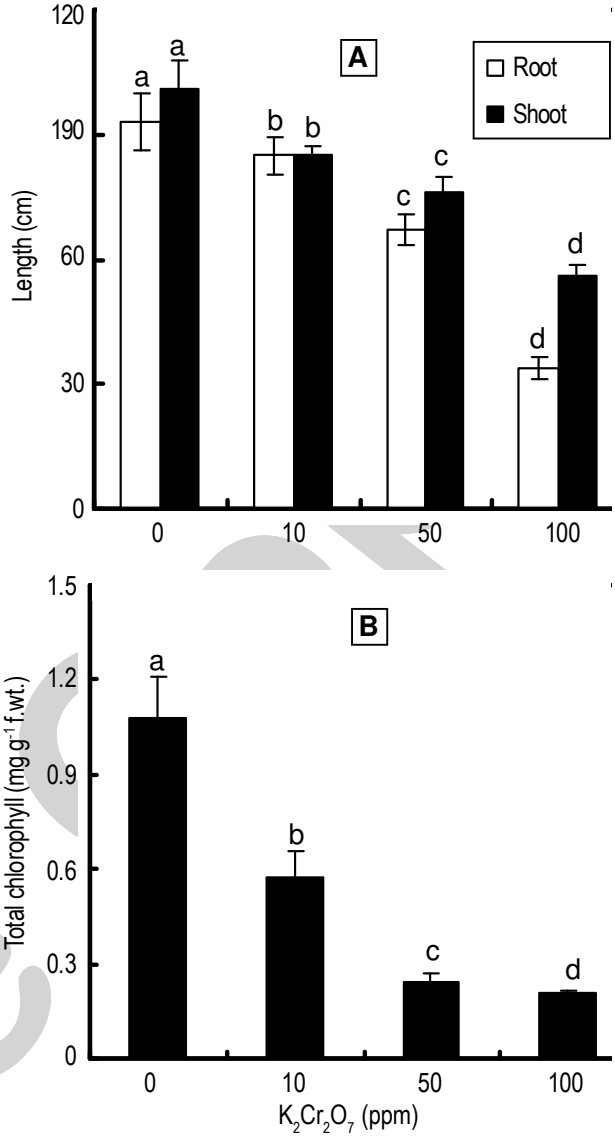

Fig. 2: Changes in root and shoot length (A) and total chlorophyll content (B) of 7-days-old wheat seedlings grown in presence of different concentrations of $K_2Cr_2O_7$. The values are the mean ± SD of three independent experiments each with three replicates. The mean values of a particular tissue type (either root or shoot), followed by the same letter are not significantly different ($p \leq 0.05$; LSD test)

reported by Prasad *et al*. (2001), root growth is more sensitive to Cr than other heavy metals like Cd and Pb. The decreased root growth in presence of Cr could be due to inhibition of root cell division and/or elongation which might have occurred as a result of tissue collapse and subsequent inability of the roots to absorb water and nutrients from the medium (Barcelo *et al*., 1986). The altered root growth also affected the shoot growth since there was limited nutrient supply to shoot tissues. Besides, the Cr transported to the aerial parts could have also affected the physiological processes contributing to the reduction in the plant height. In addition to the growth reduction, there was also decline in total chlorophyll content of shoot tissues due to Cr^6, present in the growth medium (Fig. 2).

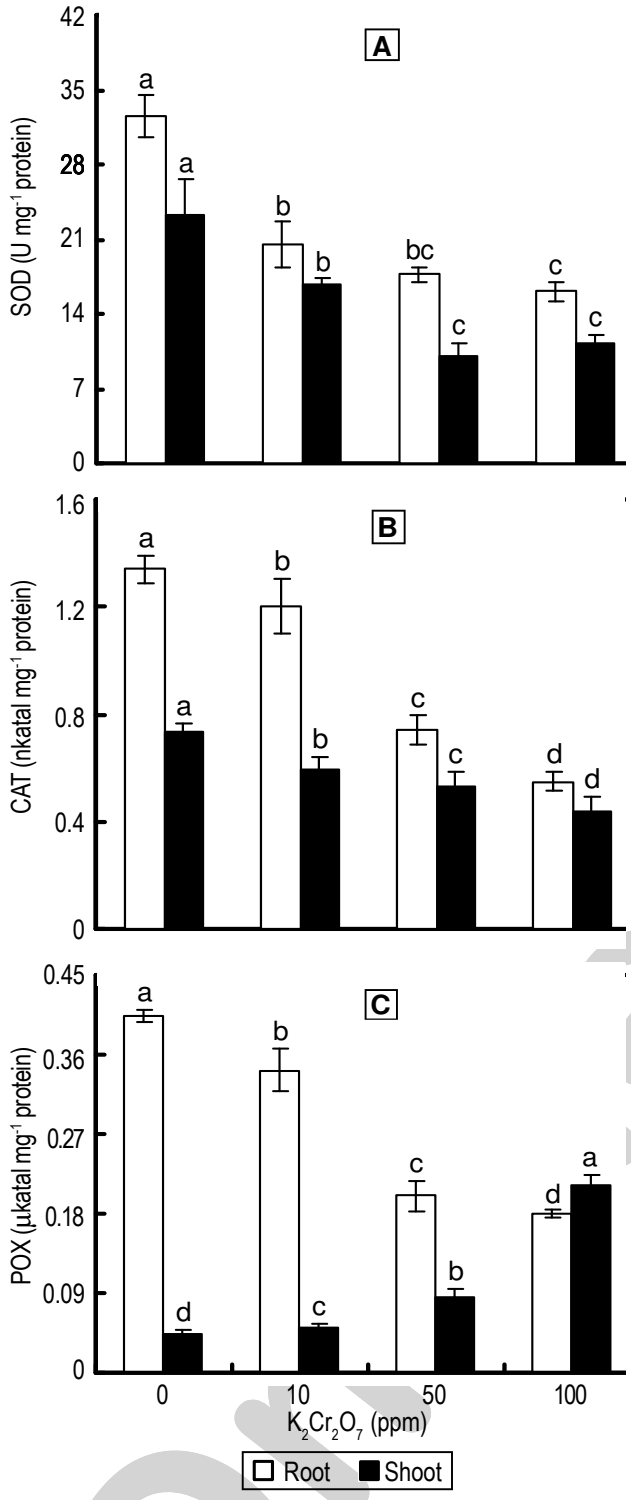

Fig. 3: Changes in the activities of superoxide dismutase (SOD) (A), catalase (CAT) (B) and guaiacol peroxidase (POX) (C) in root and shoot tissues of 7-days-old wheat seedlings grown in presence of increasing concentrations of $K_2Cr_2O_7$. The values are the mean ± SD of three independent experiments 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 ($p \leq 0.05$; LSD test)

There was about 46% of pigment loss at 10 ppm of $K_2Cr_2O_7$ which increased to 80% at 100 ppm. The loss of pigments could have a negative impact on photosynthetic process. Since the loss of chlorophyll is also an indication of prevalence of oxidative stress situation in plants (Shaw, 1995), imposition of oxidative stress due to Cr toxicity can be presumed in this study.

Superoxide dismutase, CAT and POX are important antioxidant enzymes that prevent the build up of reactive oxygen species like $O_2^{\cdot-}$ (superoxide radical), H_2O_2 (hydrogen peroxide) and subsequently $^{\cdot}OH$ (hydroxyl radical) in the aerobic cells (Elstner, 1982; Halliwell and Gutteridge, 1999). SOD dismutates superoxide radical to O_2 and H_2O_2 and this H_2O_2 is again detoxified to O_2 and H_2O by the enzyme CAT. POXs are also H_2O_2 scavenging enzymes, similar to CAT, but they do so by co-oxidation of a variety of inorganic and organic reduced co-substrates. In this study, the SOD activity was found to be declining both in roots and shoots in a concentration dependent manner (Fig. 3) and the activity was always higher in roots than in shoots. Even at 10 ppm of $K_2Cr_2O_7$, the decline in SOD activity reached significant level in comparison to that of control seedlings. The loss of SOD activity in roots was 37% at 10 ppm which further increased to 50% at 100 ppm. In shoot tissues, SOD activity declined by about 28% at 10 ppm which further increased to 52% at 100 ppm of $K_2Cr_2O_7$. Lower concentrations of Cr^6 is reported to elevate SOD activity in root mitochondria of pea (Dixit *et al*., 2002), but in the present study all the concentrations of $K_2Cr_2O_7$ were found to decrease the SOD activity both in roots and in shoots. Like SOD, CAT activity was also decreased both in roots and shoots (Fig. 3). The decline in CAT activity reached significant level even at 10 ppm of $K_2Cr_2O_7$ and at 100 ppm, there was 59% (in roots) and 40% (in shoots) decline in CAT activity in comparison to the control seedlings. Our results corroborate the findings of Jain *et al*. (2000) where CAT activity was also reported to decline with increase in Cr concentration from 20 to 80 ppm. The decline in SOD and CAT activities in presence of $K_2Cr_2O_7$ indicated the declining efficacy of the tissues to scavenge superoxide radical and H_2O_2 respectively, and thereby increasing the chances of their accumulation. Unlike SOD and CAT, the POX activity showed a reverse trend. It was increased in shoots but decreased in roots with increasing concentration of metal in the growth medium (Fig. 3). The decreased POX activity in roots may additionally be responsible for poor efficacy in decomposing H_2O_2 in Cr treated seedlings. Even in shoot tissues, where there was remarkable increase in POX activity under Cr stress, this may not be linked to efficient H_2O_2 scavenging. This is because guaiacol peroxidases are localized in cell wall, cytosol, vacuole and in extracellular spaces and induction in activity in shoot tissues under Cr stress might be due to increased release of cell-wall bound peroxidases, as have been observed under different stress situations (Mittal and Dubey, 1991; Dey and Kar, 1995; Dey *et al*., 2007). Thus under Cr stress, as found in this study, accumulation of ROS like superoxide radical and H_2O_2 can not be avoided which was mainly due to the alterations in the activities of enzymes like SOD, CAT and POX.

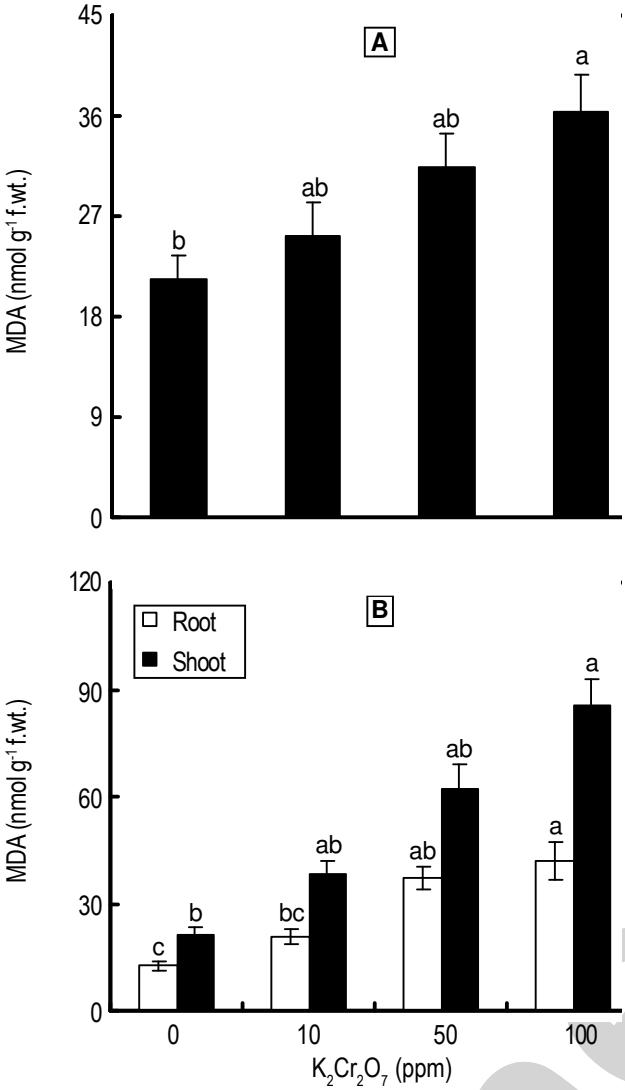

Fig. 4: Changes in lipid peroxidation in germinating wheat embryos (A), and in root and shoot tissues of 7-days-old wheat seedlings (B) in presence of increasing concentrations of $K_2Cr_2O_7$. The values are the mean ± SD of three independent experiments each with three replicates. The mean values of a particular tissue type (either root or shoot), followed by the same letter are not significantly different ($p \leq 0.05$; LSD test)

Because of the weakening in superoxide radical and H_2O_2 scavenging systems in the seedlings exposed to Cr stress, as reported herein, elevation in the steady state levels of these ROS in the tissues might have favoured. These two ROS are known to interact in presence of transition metal ions and produce the hydroxyl radical (Elstner, 1982). Hydroxyl radicals are also formed from H_2O_2 in presence of transition metal ions (Halliwell and Gutteridge, 1999). Thus the possibility of generation of higher amount of hydroxyl radicals in the tissues could not be ruled out. In aerobic cells, hydroxyl radical is the most potentially toxic oxygen species that attack the unsaturated fatty acid components of membrane lipids. As a result, membrane lipids are peroxidized and membrane architecture is lost. Therefore, lipid peroxidation is the consequence of free radical mediated reactions and is considered as an indicator of prevalence of oxidative stress situation in aerobic cells (Kappus, 1985). In this study, even though activities of enzymes like SOD, CAT and POX and total peroxide level were not determined in wheat embryos germinated in presence of $K_2Cr_2O_7$, the increased levels of MDA with increasing Cr concentration (Fig. 4 (A) indicated higher rate of lipid peroxidation. Increased lipid peroxidation reached its significant level only at 100 ppm of $K_2Cr_2O_7$, but there was always a rising trend in MDA level in the tissues. This suggests that Cr^6 stress imposes oxidative stress situation during the germination phase of plant growth. This might be one of the reasons behind Cr induced germination inhibition, in addition to the decreased activities of both α and β- amylases, as reported by Zeid (2001). In 7-days-old seedlings, there was also increase in the level of lipid peroxidation, both in roots and in shoots, as concentration of $K_2Cr_2O_7$ increased in growth medium Fig. 4(B). In root tissues the elevation in MDA level was significant at 50 ppm whereas in shoot tissues the increased level of MDA was significant at 100 ppm of $K_2Cr_2O_7$ in the growth medium. There was about 232% (in roots) and 306% (in shoots) increase in the level of lipid peroxidation in the seedlings due to Cr stress. This increased level of lipid peroxidation indicates the prevalence of oxidative stress situation in the seedlings. Such increase in lipid peroxidation has also been observed in several systems under exposure to Pb (Verma and Dubey, 2003), Cd and Pb (Dey *et al.,* 2007) and drought (Nair *et al.,* 2008) stress situations.

Presently, Cr^6 is considered as an important controlled environmental contaminant in many countries round the world (Zayed and Terry, 2003). Hexavalent Cr is actively taken up by plants by a metabolic driven process and the toxic properties of this species originate from the action of this form itself, usually as an oxidizing agent. In this study, the toxic effect of Cr^6 was observed both during germination and seedling growth phases of plant development. The loss of total chlorophyll, alteration in the activities of SOD, CAT and POX and the increase in MDA content in 7-days-old seedlings subjected to Cr stress suggest the possibility of prevalence of oxidative stress situation in the plant. Even though activities of antioxidative enzymes were not analyzed in germinating embryos, the increased lipid peroxidation level indicates the onset of oxidative stress during germination phase. Thus, imposition of oxidative stress might be one of the probable reasons behind Cr^6 induced toxicity in plants. However measurement of steady-state levels of different ROS generated in the tissues subjected to Cr stress would be vital to establish such presumption. At the same time, the roles of enzymes involved in ascorbate-glutathione cycle can not be undervalued. Therefore, analysis of these enzyme activities, estimation of low molecular antioxidants like ascorbate, glutathione *etc.* and also the measurement of Cr absorbed and accumulated by the plants would help in drawing any conclusive decision. Thus, the findings of this study will help in revealing Cr toxicity mechanism which subsequently will facilitate to manipulate the conditions for Cr detoxification in plants growing in Cr contaminated environment.

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