Plant Science Research

ISSN 0972-8546

Hexavalent chromium alters the antioxidative efficiency and increases Lipid peroxidation in germinating wheat seeds

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ARTICLE INFO

Article history:
Received : 13 November, 2021
Revised : 7 December, 2021
Accepted : 30 December, 2021

Keywords:

ascorbic acid content,
catalase,
germination percentage,
oxidative stress,
peroxidase,
superoxide dismutase

ABSTRACT

Wheat (*Triticum aestivum* L. cv. Sonalika) seeds were germinated in presence of hexavalent Cr (0.5, 1.0, 2.0, and 4.0 mM) for 24 h and germination percentage, ascorbic acid content, proline content, soluble protein content, activities of antioxidative enzymes like superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and peroxidase (POX; EC 1.11.1.7) along with lipid peroxidation level were determined in embryonic tissues. It was found that the activities of SOD, CAT and POX increased with increase of metal in the medium and it was significant ($p < 0.05$) even at lowest concentration of the metal tested. The level of lipid peroxidation increased significantly ($p < 0.05$) in the embryonic tissues. Ascorbic acid content decreased whereas proline content increased in the embryonic tissues in response to increase in Cr concentration in the medium. The results indicated the imposition of oxidative stress situations during germination stage by Cr (VI) stress which might be one of the probable reasons behind Cr induced toxicity in germinating seeds.

1. Introduction

Chromium (Cr) pollution has now become a serious environmental problem throughout the world (Shahid *et al.,* 2017). This is mainly because of the multiple industrial uses of this metal such as leather tanning, mining and electroplating. Along with its other affects, Cr pollution poses a serious threat to crop growth and human health (Ertani *et al.,* 2017; Sharma *et al.,* 2020). Differently from other heavy metals like cadmium and copper, Cr mainly exists in two valence states i.e. Cr (VI) and Cr (III) (Ashraf *et al.*, 2017). 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 (III) is less mobile, less toxic and is mainly found bound to organic substances in the environment (Becquer *et al.*, 2003). It's worth noting that Cr (VI) has stronger stability, mobility and toxicity than Cr (III) (Choppala *et al.*, 2018). Germination is a phase in plant growth and development during which the radicle and plumule emerge out of the seed upon absorption of water. Since Cr does not have any physiological role in plants and the embryonic tissues are tender during germination, exposure to chromium during this stage is expected to cause toxicity. Studies on the rice (*Oryza sativa* L.) (Sharma *et al.*, 2016) and corn (*Zea mays* L.) (Hou *et al.*, 2014), revealed that the seed germination of plants was inhibited to varying degrees under Cr stress.

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. Heavy metals like Cd and Pb are known to impose oxidative stress situations in plants by altering the natural antioxidative efficiency of the cells (Dey *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),

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hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) beyond the cell's capacity of endogenous antioxidative protective system to scavenge them off. 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 works on detail antioxidative efficiency of germinating seeds exposed to Cr are rare. Even though some studies like germination percentage and lipid peroxidation level in embryonic tissues of germinating wheat seeds exposed to Cr (VI) were reported from this laboratory earlier (Dey *et al.* 2009) but other analyses related to antioxidative efficiency were not done to ascertain a probable mechanism of Cr induced toxicity. Therefore, in this work a detail study on the activities of antioxidative enzymes like superoxide dismutase (SOD, EC 1. 15. 1. 1), catalase (CAT, EC 1. 11. 1. 6) and peroxidase (POX, EC 1. 11. 1. 7) along with lipid peroxidation level have been determined in germinating embryonic tissues of wheat under exposure to chromium. At the same time assessment of some metabolies like soluble protein, ascorbic acid and proline has been done in order to understand their probable role during Cr stress in germinating seeds.

2. Materials and Methods

2.1 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. In different Petri dishes 50 uniform size surface sterilized wheat seeds were spread over filter paper, moistened with 15 ml of 0.5, 1.0, 2.0 and 4.0 mM Cr (VI) as $K_2Cr_2O_7$, prepared with half strength Hoagland's solution. In another Petri dish 15 ml of half strength Hoagland's solution, without Cr, was taken as control. The Petri dishes were covered and kept in an incubator at 30°C for 24 hr. Number of seeds germinated in each Petri dish was counted to determine the percentage of germination. The germinated embryonic tissues, leaving the endosperm portion, were excised from the seeds and taken for different biochemical analyses.

2.2 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 for overnight. The supernatants were collected for protein estimation using bovine serum albumin as standard, as described by Lowry *et al.* (1951).

2.3 Extraction and estimation of malondialdehyde (MDA):

In this study, the level of lipid peroxidation was measured by estimating MDA 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).

2.4 Extraction and estimation of proline content:

Poline content was estimated following the method of Bates *et al.* (1973). The embryonic tissues were homogenised in 3% aqueous solution of sulphosalicylic acid and centrifused at 5000 rpm for 10 min. Then, 2 ml of supernatant was taken in a test tube to which 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent were added. The contents were mixed and boiled in a water bath at 100°C for 1 hr. The test tubes were cooled and 4.0 ml of toluene was added to each, mixed vigorously and then allowed to separate the phases for few min. The upper chromophore phase was collected carefully and absorbance was measured at 520 nm in a spectrophotometer. The proline content was determined by comparing the absorbance with that of a standard curve drawn with known concentrations of proline.

2.5 Extraction and estimation of ascorbic acid content:

For the estimation of ascirbic acid, the method of Mitsui and Ohta (1961) was followed. The embryonic tissues were collected and washed thoroughly with distilled water and then air-dried. The tissues were homogenized with 6% metaphosphoric acid. The homogenates were centrifuged at 5,000 rpm for 10 min. The supernatants were used for spectrophotometric analysis of ascorbic acid.The assay mixture was composed of 2 ml of 2% sodium molybdate and 2 ml of 0.15 N sulphuric acid. After this, they were mixed and chilled. Then 1 ml of 1.5 mM monobasic sodium phosphate buffer was added. Finally 1 ml of tissue extract or standard ascorbic acid solution was added to it. Then, it was incubated at 60°C in a water bath for 40 min, then cooled and centrifuged. The absorbance was taken at 660 nm in a

spectrophotometer. The ascorbic acid content of the tissue extract was calculated by comparing the absorbance of the samples with the standard curve drawn with 0 to 176 µg per ml of ascorbic acid.

2.6 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 this study. For enzyme extraction, embryonic 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 -4^0 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/ gFW) 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/ gFW) was assayed by measuring the decreasing concentration of H_2O_2 at 240 nm due to CAT activity (Aebi, 1983) and the activity was calculated by using the extinction coefficient of 40.0 mM^{-1} cm^{-1} for H_2O_2 at 240 nm. For assaying POX, guaiacol and H_2O_2 were used as substrates. The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm, as described by Kar and Feierabend (1984) and the peroxidase activity (µ katal/ gFW) was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} due to tetraguaiacol formation under assay conditions.

2.7 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 and standard deviations are indicated. For analyzing the level of significance among the means, ANOVA test using Sigmaplot 11.0 was performed.

3. Results and Discussion

Germination of seed is an important phase in the life of the plants where upon imbibition of water the radicle and plumule emerge out of the seeds rupturing the seed coat. This is a crucial initial stage that ensures the healthy growth and development of plants. Like other phases of plant growth, the germination phase is also affected by different biotic and abiotic factors including heavy metals. In this study, wheat seeds were exposed to Cr (VI) and after 24 h the number of seeds germinated was counted to determine the % of germination and the results are presented in Fig. 1. It has been found that with increase in the Cr in the medium, there was decrease in the germination % of the seeds and it was significant even at the lowest concentration of the metal tested (0.5 mM). The process of seed germination is regulated by the mobilization of reserve food, such as starch, protein, and phytate etc. and the transfer of digested derivatives to the growing embryonic axis. The inhibitory effect of Cr (VI) might have occurred by decreasing the activities of key enzymes involved in carbohydrates, amino acid and peptides metabolism in germinating seeds (Zeid, 2001; Kuriakose and Prasad, 2008; Sethy and Ghosh, 2013). Similar suppression in seed germination due to exposure to Cr (VI) has also been reported in *Vigna radiata* (Samantaray, 2002); *Triticum aestivum* (Dey *et al.,* 2009; Dotaniya *et al.*, 2014); *Beta vulgaris*, *Daucus carota, Solanum melongena,* and *Raphanus sativus* (Lakshmi and Sundermoorthy, 2010); and in tomato (Khan *et al*., 2021). In general, the process of germination require high energy which is taken from rapid oxygen uptake and oxidative phosphorylation (Hourmant and Pradet, 1981). It is a fact that the mobilization of reserve food and oxidative phosphorylation causes the formation of reactive oxygen species (ROS) resulting in oxidative damage and consequent several metabolic alterations (Tommasi *et al*., 2001). Therefore, analysis of antioxidative efficiency in seeds germinating in presence of Cr (VI) is essential in order to understand the mechanism of toxicity and to manipulate the conditions to restore the seed germination in contaminated sites.

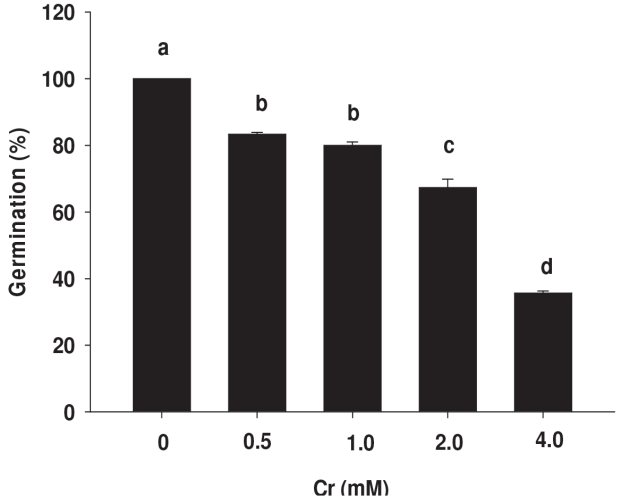

Fig. 1: Effect of Cr (VI) on the germination percentage of wheat seeds after 1 day of incubation. The values are the mean ± SD of three independent experiments. The mean values followed by the same letters are not significantly different ($p<0.05$; ANOVA test).

Proteins are considered to be the building blocks of life and have many functions in living organisms. The soluble protein of a cell mostly represents the total enzymes involved in the metabolic processes. In this study the soluble protein content of the embryonic tissues was estimated and the results are presented in Fig. 2. The results showed that there was increase in the soluble protein content in the tissues with increase in Cr (VI) concentration, except for the concentration of 0.5 mM at which protein content decreased than the control sample. This indicated that probably there was induction in the synthesis of some enzymes in response to Cr exposure as an adaptive cellular strategy to alleviate the stress injury.

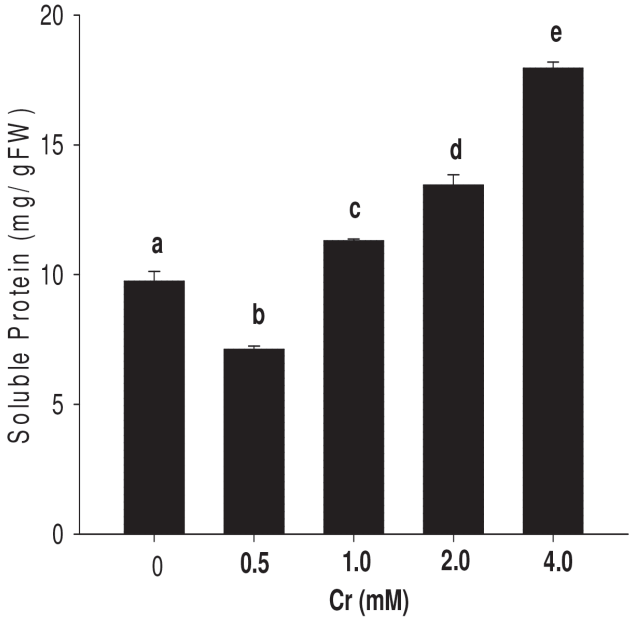

Fig. 2: Changes in the soluble protein content in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

In aerobic organisms, generation of reactive oxygen species (ROS) takes place as a consequence of oxygen metabolism. These ROS include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). Singlet oxygen ($^1O_2$) is another form of ROS which is formed when ground state molecular oxygen receives some extra energy as a result of which one of its unpaired electron in the outermost orbital gets excited and jumps to a higher state. The superoxide radical and hydroxyl radical are oxygen free radicals whereas hydrogen peroxide and singlet oxygen are non-radicals. These ROS are highly reactive which can oxidatively damage the cellular macromolecules at the sites of their generation causing various toxic effects and even cell death (Halliwell and Gutteridge, 2007; Pourrut *et al*., 2011). However, under normal conditions the toxicity of ROS are not realized due to the neutralizing effects of endogenous antioxidative defense systems which include the antioxidative enzymes and the low molecular weight antioxidative compounds (Elstner, 1982; Elstner *et al.*,1988; Halliwell and Gutteridge, 2007; Caverzan *et al*., 2016; Wu *et al.,* 2017). But under certain developmental stages and when the organisms are exposed to biotic and abiotic stresses the generation of ROS takes place beyond the scavenging capacity of the endogenous antioxidative protective systems and under such situations, the organisms are said to be exposed to oxidative stress (Elstner *et al.*, 1988; Halliwell and Gutteridge, 2007; Yu *et al*., 2019). Thus, there exists a delicate balance between the ROS generation and their scavenging by antioxidative protective systems and hence, the ability of plants to cope with oxidative stress is characterized by the degree of antioxidant activities (Shahzad *et al.,* 2018; Anjum *et al.,* 2015). Therefore, it is highly essential to assess the level of antioxidants under metal stress in order to determine the mechanism of toxicity. In this study the activities of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) have been assayed along with determination of ascorbic acid content in the germinating embryonic tissues and the results are presented in Figures 3, 4, 5 and 6 respectively.

The SOD is responsible for dismutation of superoxide radical to O_2 and H_2O_2 and thereby decreases the chances of buildup of this toxic oxygen species (Halliwell and Gutteridge, 2007; Yu *et al*., 2019). Here in this study, the SOD activity increased in the embryonic tissues with increase in the Cr (VI) in the medium (Fig. 3) which indicated that there was protection against the superoxide radical. But protection against the deleterious effects of H_2O_2 is equally important since it is the product of the SOD catalysed superoxide dismutation reaction along with being formed through other metabolic routes. Catalase and peroxidases are the principal antioxidative enzymes that decompose H_2O_2 and thereby reduce its buildup and further toxicity in the cell (Elstner, 1982; Halliwell and Gutteridge, 2007; Foyer and Noctor, 2009). In this study the activities of both CAT and POX were found to increase with increase in the Cr (VI) in the medium (Fig. 4 and 5). This indicated that protection against H_2O_2 was also maintained in the germinating wheat seeds under Cr stress. This increase in the activities of SOD, CAT and POX might be due to *de novo* synthesis of these enzymes in response to Cr stress which has been corroborated by the increase in soluble protein content in the tissues mentioned earlier (Fig. 2).

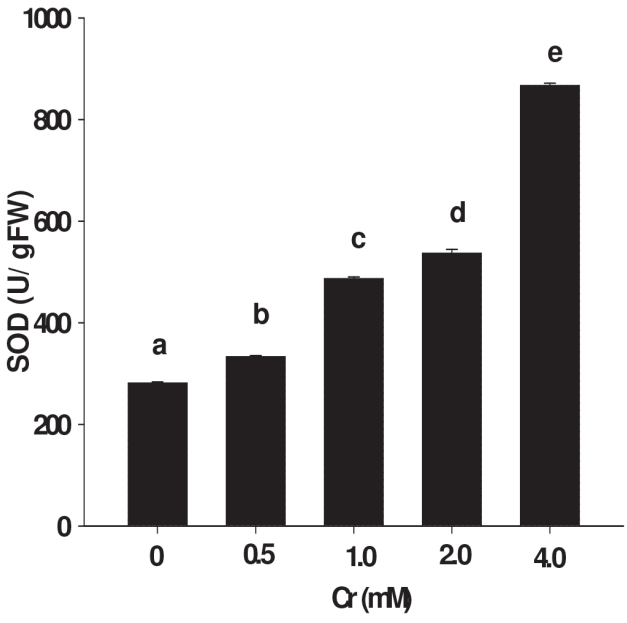

Fig. 3: Changes in the superoxide dismutase (SOD) activity in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

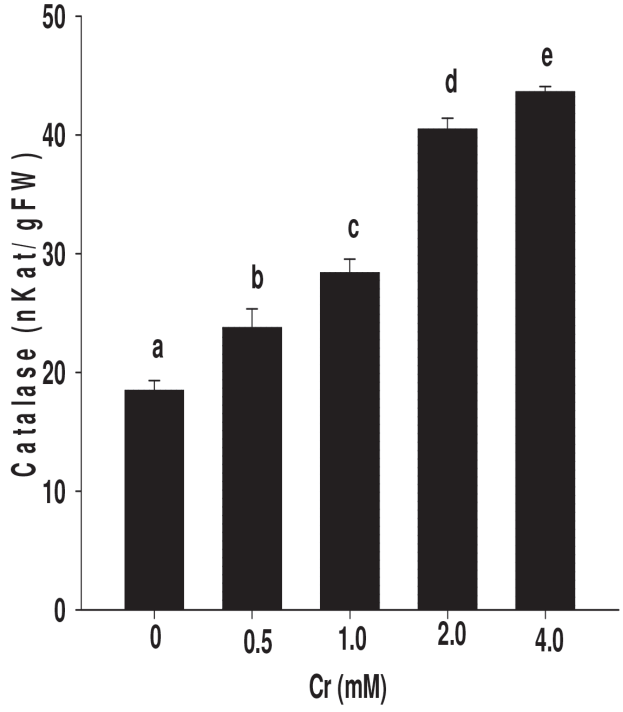

Fig. 4: Changes in the catalase (CAT) activity in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

Alterations in the activities of SOD, CAT and POX have been reported in various systems under exposure to different heavy metals (Verma and Dubey, 2003; Dey *et al*., 2007; Dey *et al*., 2009; Pati *et al*., 2014) and the metal induced toxicity have been ascribed to the imposition of oxidative stress in respective cases. Even though the increase in SOD and CAT activities in the embryonic tissues under Cr stress, as reported in this study, may be attributed to enhanced efficiency of the cells to protect against superoxide radical and H_2O_2, augmentation in POX activity may be correlated to some other reason. The enhanced POX activity may be due to increased release of cell wall bound peroxidases in the cells and such enhancement in POX activity has been found in plants exposed to different metals (Verma and Dubey, 2003; Srivastava *et al*., 2006; Dey *et al*., 2007; Dey *et al*., 2009; Pati *et al*., 2014). However, decrease in POX activity has also been observed when the metal concentration was high (Srivastava *et al*., 2006; Pati *et al*., 2014). According to Zhang *et al*. (2007) enhancement in POX activity is a biomarker of heavy metal stress and hence imposition of stress due to Cr (VI) can also be presumed in this study. In this situation the elucidation of the role of ascorbic acid, the low molecular weight antioxidant, in the seeds germinating in presence of Cr seems very logical.

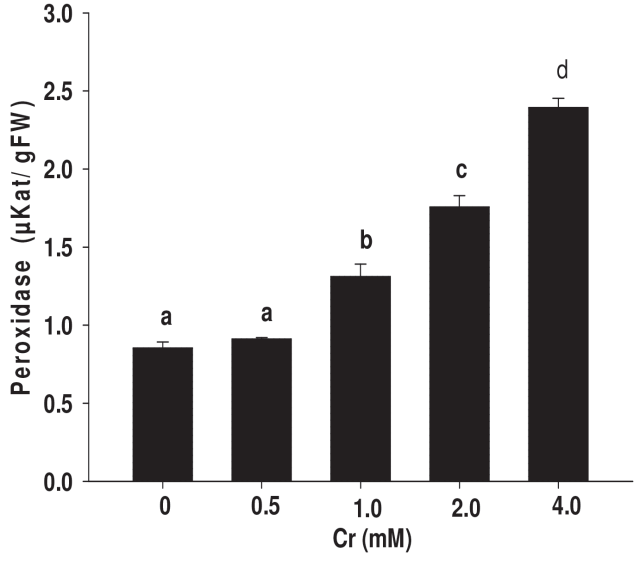

Fig. 5: Changes in the peroxidase (POX) activity in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the same letters are not statistically significant ($p<0.05$; ANOVA test).

The ascorbic acid content of the embryonic tissues has been found to be significantly affected due to Cr (VI) stress. The results presented in Fig. 6 show that there was decline in ascorbic acid content with increase in the Cr in the medium. Ascorbic acid is an important low molecular weight antioxidant of the cell which is known to scavenge the ROS like superoxide radical, hydroxyl radical and singlet oxygen (Bodannes and Chan, 1979). Among the different low molecular weight antioxidants ascorbate plays an important role in chloroplasts. In plant cells about 30-40% of the total ascorbate is localized in chloroplasts and its concentration in the stroma is about 50 mM (Foyer and Noctor, 2005). High levels of reduced ascorbic acid and glutathione are necessary in order to remove H_2O_2 permanently. Therefore, several enzymes involved in the ascorbate-glutathione pathway operate together in the chloroplasts to ensure the neutralization of H_2O_2. The pathway includes interrelated redox reactions involving ascorbate, glutathione, and NADPH (Asada, 1999).

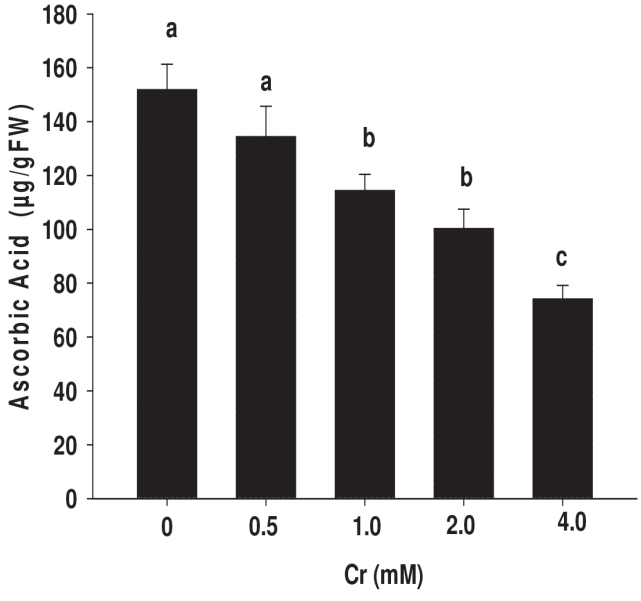

Fig. 6: Changes in the ascorbic acid content in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the same letters are not statistically significant ($p<0.05$; ANOVA test).

Even though neither the glutathione content nor the activities of enzymes of ascorbate-glutathione pathway have been analysed in this study, the decrease in the ascorbate content in the tissues indicated the low level of antioxidant status which might favour the pro-oxidation process. Therefore, measurement of lipid peroxidation level was highly essential since the ROS are a well-known cause of damage to membranes through lipid peroxidation process (Su *et al.*, 2019). Malondialdehyde (MDA), a decomposition product of peroxidised polyunsaturated fatty acid component of membrane lipid taking thiobarbituric acid as the reactive substance, content was measured to determine the level of lipid peroxidation. The results presented in Fig. 7 show that lipid peroxidation level increased with increase in the Cr (VI) in the medium.

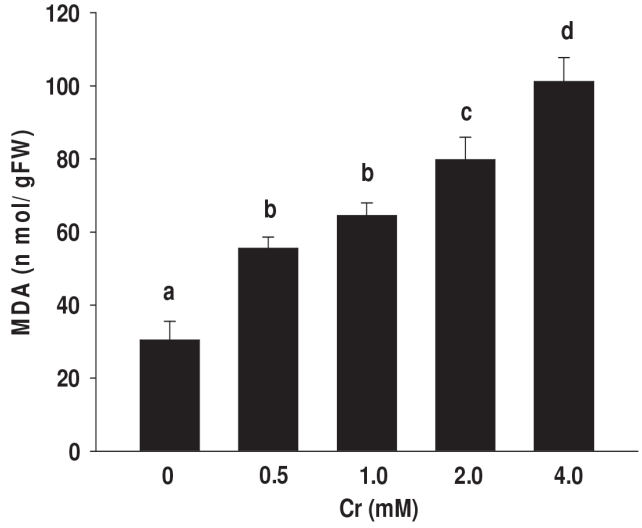

Fig. 7: Changes in the lipid peroxidation level in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the same letters are not statistically significant ($p<0.05$; ANOVA test).

The hydroxyl radical is known to be the most potentially toxic species in aerobic cells. The polyunsaturated fatty acid components of membrane lipids are highly susceptible to hydroxyl radical attack which 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). The increase in lipid peroxidation level (Fig. 7) indicated that there was imposition of oxidative stress due to Cr (VI) in the embryonic tissues of germinating wheat seeds. The increase in the SOD and CAT activities indicated that the defense against superoxide radical and H_2O_2 was high in the seeds during germination but probably it was not enough to maintain the required antioxidant pool in the tissues. The decrease in the ascorbic acid content due to Cr stress (Fig. 6) further corroborates this assumption. Under these circumstances, further studies involving the determination of glutathione content and the activities of enzymes involved in the ascorbate-glutathione pathway are essential to elucidate the mechanisms of Cr -induced oxidative stress in germinating wheat seeds.

As an osmolyte and compatible solute, proline protects the plants from various stresses and helps the plants to recover from stress more rapidly. It accumulates in many plants in response to environmental stresses which has been positively correlated with the stress tolerance (Serraj and Sinclair, 2002; Ashraf and Foolad, 2007; Hayat *et al*., 2012). Besides acting as an osmolyte, proline plays three important roles in plants during stress situations, i.e., as a metal chelator, a signaling molecule and an antioxidative defense molecule (Smirnoff and Cumbes 1989; Bohnert *et al*., 1995). The proline content of the germinating embryonic tissues was also determined in this study and the results are presented in Fig. 8.

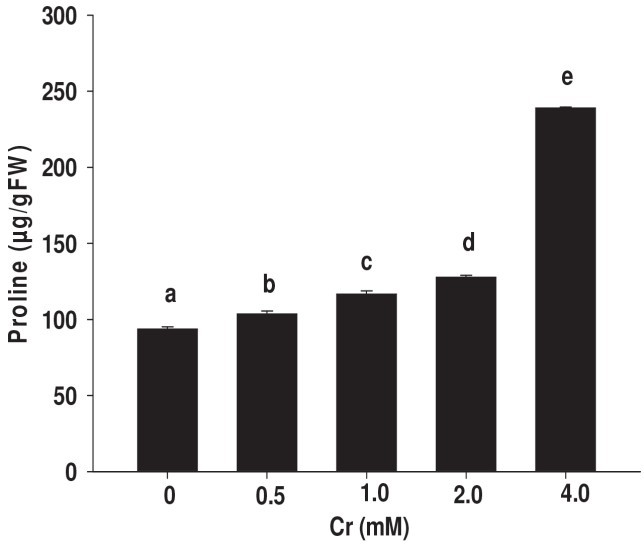

Fig. 8: Changes in the proline content in the embryonic tissues of germinating wheat seeds after 1 day of exposure to Cr (VI). The values are the mean ± SD of three independent experiments each with three replicates. The mean values followed by the different letters are statistically significant ($p<0.05$; ANOVA test).

The increase in the proline content was initially slow up to 2.0 mM of Cr (VI) in the medium which increased rapidly further at 4.0 mM. The accumulation of free proline in plants in response to heavy metal stress has been attributed as a consequence, rather than as a cause of metal tolerance (Schat *et al*., 1997). In this study the increased proline content in the tissues can be viewed as an indicator of stress imposition rather than emphasizing its protective roles, as mentioned above, which is because of the fact that despite enhancement in proline content toxic effects of Cr (VI) on the germinating wheat seeds have been observed in the forms of decrease in germination percentage as well as increase in the lipid peroxidation level.

4. Conclusion

Thus, the findings of this study indicate that Cr (VI) is toxic to the germinating wheat seeds which was observed initially in the form of decrease in germination percentage. In order to elucidate the mechanism of toxicity the activities of antioxidative enzymes like SOD, CAT and POX were analysed. Even though the activities of these enzymes were found to increase, it was perhaps not enough in giving antioxidative protection since pro-oxidative effect was noticed in the form of increase in lipid peroxidation level. The increase in proline content indicated the stress imposition due to Cr (VI). The decrease in the ascorbic acid content can be assumed to be the cause behind the Cr induced toxicity in germinating wheat seeds and for this, further studies involving the determination of glutathione content and enzymes involved in the ascorbate-glutathione pathway are highly essential to validate such proposition and to understand the mechanism of toxicity. The toxicity imposed in the embryonic tissues during seed germination in the form of oxidative injury may persist which may affect the plant during its further growth and development. It is also a fact that maintaining metabolic functions under stress conditions is crucial for plants to survive. Therefore, remediation of soil contaminated with hexavalent chromium should be done before sowing seeds.

Acknowledgements

The authors are thankful to the Head, Department of Environmental Science, Fakir Mohan University, Vyasa Vihar, Balasore-756089 for providing the facilities to carry out this work.

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