
FULL LENGTH ARTICLE

Alleviation of chromium induced toxicity in *Cicer arietinum* L. seedling by calcium

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The present study intends to assess the role of calcium (Ca^{2+}) in elevating endurance capacity and ameliorating chromium toxicity in chickpea seedlings, as calcium is a potential stress reliever by interacting with signaling pathways. Being a valued protein rich crop of India and susceptible to Cr contamination from irrigated water, toxicity amelioration study of chickpea is justified. Calcium was applied alone (10 mM, 20 mM) and also in combination with Cr (25 μM and 50 μM) to antagonize the damaging effect of Cr and to restore the plant growth in chickpea seedling. The exogenous application of Ca alleviates the detrimental effects of Cr by the upregulation of antioxidative enzymes, such as catalase (CAT), peroxidase (POD), glutathione reductase (GR) as well as non enzymatic antioxidants (proline, phenol, non-protein thiol). Formation of endogenous H_2O_2 was moderately reduced with the supplementation of Ca with increasing chromium concentrations in comparison to control plants. The formation of Malondialdehyde (MDA) which is the indicator of membrane damage due to lipid peroxidation showed significant reduction with Ca co-treatment. Though CAT and GR revealed elevated activity for both concentration of Cr with Ca supplementation, super oxide dismutase (SOD) activity was not upregulated very significantly. Ca dependent toxicity mitigation was more pronounced in 25 μM Cr treatment rather than 50 μM and 20 mM Ca supplementation was found to be more effective. This investigation will help the potential fields of remediation and mitigation of Cr toxicity in near future.

Key words: chromium; calcium; phytotoxic; proline; antioxidant; superoxide dismutase, reactive oxygen species; catalase; glutathione reductase.

INTRODUCTION

Chromium (Cr) is the seventh most abundant element in the earth's crust. It is present in the ecosystem as a result of the weathering of the earth's crust and moreover it is released in the environment due to anthropogenic activities. Untreated effluents from various industries are the prominent sources of Cr in the environment. It can be deposited in fertile agricultural soil and is easily taken up by plants.

According to Agency of Toxic Substances and Disease Registry (2007) Cr occupies 77 th position in the list of most hazardous substances of the world as it is carcinogenic, immune suppressor and causes allergic reaction, ulcers, kidney and liver damage. Trivalent Cr (III) and hexavalent Cr (VI) are the two stable oxidation stages of Cr that is persistent in soil. Both forms differ in terms of solubility, mobility, bioavailability and toxicity. Being more mobile, Cr VI is more phytotoxic in comparison to Cr III. Hexavalent Cr is used in various industries such as, chrome plating, leather tanning, paint and plastic industry. According to Environmental Protection

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Agency (EPA) less than 0.1 ppm concentration of Cr in drinking water is permissible on the basis of health consideration. A maximum acceptable limit of Cr soil is 10-1000 mg/kg (Singh *et al.*, 2013). Cr is toxic for agronomic plants at about 5-100 mg/g in soil (Oliveira, 2012). Non toxic range of Cr concentration in plant is 1 µg/g (Oliveira, 2012). Excessive Cr can be detrimental as it induces physiological, biochemical and genetical alterations in plants cells (Singh *et al.*, 2013; Gomes *et al.*, 2017). The phytotoxic effects of Cr are primarily dependent on the valence (Cr⁶⁺) of the metal, which determines its uptake, translocation and accumulation (Singh *et al.*, 2013). Though Cr is needed in extremely less amount as micronutrient in plants but accumulation of Cr in excess can affects growth, photosynthesis, chlorophyll biosynthesis, enzymatic activities, root hair development, RuBISCO function, photophosphorylation and cause ultrastructural damage to cell membranes (Singh *et al.*, 2013). Cr reduces the size of peripheral part of the antenna complex resulting decrease in total chlorophyll, chlorophyll a/b ratio and carotenoid content (Panda and Chowdhury, 2005). Generation of reactive oxygen species (H₂O₂, OH⁻, O₂⁻) under Cr stress can impart acute oxidative stress leading to the damage of DNA and proteins (Rodriguez *et al.*, 2011; Singh *et al.*, 2013). Cr disturbs redox homeostasis by stimulating the formation of excessive ROS that affect the regular metabolism by damaging the cellular components (Pandey *et al.*, 2009). These toxic effects cause lipid peroxidation, protein oxidation, damage to nucleic acids, and decline in plant growth which even results in plant death. For the mitigation of ROS induced oxidative stress, plants have evolved an array of non-enzymatic and enzymatic antioxidative defense system that consists mainly of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and glutathione reductase (GR) (Maiti *et al.*, 2012; Pourrut *et al.*, 2013). The upregulation of antioxidants in heavy metal stressed plants depends upon the severity of oxidative damages and the tolerance potential of plants. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. H₂O₂ is degraded by catalase and peroxidases into nonreactive water (Shahid *et al.*, 2014). Among non-enzymatic antioxidants ascorbate (AsA), non-protein thiol group, glutathione (GSH)

and phenol are identified as ROS scavenger. Accumulation of free proline as protective osmolyte in heavy metal stressed plant is well documented (Maiti *et al.*, 2012).

The various defense response of plant against heavy metal stress is the manifestation of intricate signaling network coordinated by Calcium. This divalent cation is an extremely important micronutrient with an immense role in developmental and physiological processes including absorption of ions, growth, signal transmission and protective response to environmental stress (Song *et al.*, 2008). The central role played by Ca in heavy metal detoxification is the upregulation of antioxidant enzyme activities, lowering the lipid peroxidation of biological membranes, upgradation of physiological and biochemical tolerant parameters (Siddiqui *et al.*, 2012; Huang *et al.*, 2017). Calcium ion acts as second messenger that controls the functions of target proteins either directly or by calmodulin dependent pathway, which regulates a number of protein kinases responsible for the onset of physio-biochemical processes attributed to successful development of heavy metals tolerance to plants (Huang *et al.*, 2017).

Chickpea (*Cicer arietinum* L.) belongs to family Fabaceae is well known as Bengal gram or chana. It is a major grain legume, ranking third in global production after bean and pea. Chickpea is a nutrient dense food, with high protein content and good source of dietary fibre and minerals like iron, phosphorous. Chickpea is a valued crop for expanding population of India and is cultivated in the states of Maharastra, Karnataka, Andhra Pradesh, Utter Pradesh and Rajasthan. Irrigation water used in these agricultural fields is highly contaminated (80%) with adjacent industrial effluents containing several heavy metals that is responsible for reduction of dry weight and 5% yield of chickpea annually (Naz *et al.*, 2015). Regarding the bioaccumulation of Cr by Indian pulses, previous study has shown that, chickpea is better accumulator of Cr (4 mg/g) compared to Mung bean (0.98 mg/g) (Behera *et al.*, 2018).

The aim of the present study is to assay the physiological and biochemical changes associated with chromium stress on *Cicer arietinum* seedlings

grown under laboratory condition and to assess the role of calcium in ameliorating the adverse effects of Cr stress by the enhancement of antioxidative defense in chickpea plant. This study only deals with the phytotoxicity of Cr in plant, but assessment of human health risks of Cr contaminated chickpea consumption is not considered here.

MATERIALS AND METHODS

Plant material and treatment conditions

Healthy seeds of chickpea (*Cicer arietinum* L.) were surface sterilized with 5% sodium hypochlorite solution for 15 min and rinsed with distilled water, followed by soaking the seeds overnight in dark. The seeds were germinated in dark for 3 days on moist cotton bed (10 seeds / cotton bed), then transferred to autoclaved beakers containing 2 ml 1x Hoagland's solution- pH 5.5 (1993) [1mM KH_2PO_4 , 5 mM KNO_3 , $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 11.8 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.7 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.32 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.16 μM $(\text{NH}_4)_6\text{MO}_7\text{O}_{24} \cdot \text{H}_2\text{O}$, 46.3 μM H_3BO_3 , 5 μM FeCl_3] and kept in sunlight till day 7, with temperature of $27^\circ\text{C} \pm 2^\circ\text{C}$. On 7 th day different sets of plant samples was treated with potassium dichromate (hexavalent Cr source) and calcium chloride (source of Ca^{2+}) solely and in combination. Total 9 experimental sets were prepared by transferring 5 germinated seeds from cotton bed to each beaker. Treatments are followings: C(control): Cr-0 Ca-0; T1 (25 μM Cr + 0 Ca); T2 (50 μM + 0 Ca); T3 (0 Cr + 10 mM Ca); T4 (0 Cr + 20 mM Ca); T5 (25 μM Cr + 10 mM Ca); T6 (25 μM Cr + 20 mM Ca); T7 (50 μM Cr + 10 mM Ca); T8 (50 μM Cr + 20 mM Ca). All sets of plant were allowed to grow for next 7 days and then analysis of different parameters such as, enzymatic and non-enzymatic antioxidants was carried out. To confirm whether Ca itself imposes any toxicity or not, T3 and T4 experimental sets were prepared and examined also. All sets were grown in triplicate.

METHODS

Growth parameters: Shoot and root length of control as well as treated plants were measured.

Stress related parameters

Assay of endogenous hydrogen peroxide content: Leaf tissue (0.5 g) was crushed with 3ml of 1% TCA. The homogenate was centrifuged at 10000 rpm for 10 minutes and the supernatant of 0.5ml was added with 0.75 ml of 50 mM potassium phosphate buffer (pH 7) and 1.5 ml of 1M potassium iodide (KI) and the absorbance was measured at 390 nm. H_2O_2 content was expressed as $\mu\text{mol H}_2\text{O}_2 \text{ g}^{-1}$ of fresh weight tissue (Jessup *et al.*, 1994).

Assay of Lipid peroxidation: Using thiobarbituric acid (TBA), the amount of malondialdehyde (MDA) content is determined to estimate lipid peroxidation (Heath and Packer, 1968). 0.5g leaf tissue were crushed in 5 ml of 0.1% TCA and centrifuged for 5 min at 1000 rpm. For each 1 ml of aliquot, 4 ml of 20% TCA containing 0.5% thiobarbituric acid was added, followed by heating at 95°C water bath for half an hour and then instantly cooled on an ice bath. This final mixture was spun at 10000 rpm for 15 min and the absorbance was taken at 532 nm and 600 nm. The non specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of MDA was calculated by using the extinction coefficient of 155/mM/cm.

Assay of free proline: Free proline amount was determined ($\mu\text{g/g}$ tissue) from a previously prepared standard curve following a standard protocol (Bates *et al.*, 1973). 500mg leaves were crushed in 5ml 0.1M sulfosalicylic acid and was centrifuged at 5000 rpm for 30 minutes. Then 5 ml glacial acetic acid, 5ml ninhydrin solutions were added to the 2 ml supernatant, and heated at boiling water bath for 60 mins. Then the resulting mixture was extracted with toluene in separating funnel and absorbance was measured at 520 nm.

Assay of total phenols: 0.25 g of leaf was added to 2.5 ml ethanol and centrifuged at 2°C for 10 min. The supernatant was taken and mixed with 2.5 ml of 80 % ethanol and was allowed to centrifuge. Then the supernatant was evaporated to dryness, followed by addition of 3 ml water, 0.5 ml folin phenol reagent and

2 ml of sodium carbonate (20%). The reaction mixture was boiled in water bath for 1 min and OD was taken at 650nm (Mallik and Singh, 1980). Catechol was used to prepare standard curve.

Assay of non protein thiol content (NPS): 0.5g fresh samples were crushed in 5 ml of 5% meta-phosphoric acid and centrifuged at 12000 rpm. Reaction mixture was made with 0.5 ml plant extract, 2.5 ml of 150 mM phosphate buffer (pH 7.4), 5 mM EDTA, 0.5 ml 6 mM 2-nitro benzoic acid. This mixture was allowed to incubate at room temp for 5 min, followed by the measurement of OD at 412 nm (Cakmak *et al.*, 1992). Calculation was done from the standard curve of reduced GSH.

Assay of antioxidative enzymes

Catalase (CAT) activity (CAT, EC 1.11.1.6) : 0.5 g of fresh leaf was crushed in 0.067M phosphate buffer (pH 7) for estimation of catalase activity (Woodburry *et al.*, 1971). The homogenate was centrifuged at 12000 rpm for 30 minutes at 4°C and this supernatant was the source of enzyme. In 1ml of the reaction mixture containing potassium phosphate buffer (pH 7), 250 µl of supernatant and 60 mM H₂O₂ was used to start the reaction. OD was measured for 3 minutes at an interval of 10 seconds at 240nm in a spectrophotometer. Molar extinction coefficient, = 39.4 mM⁻¹cm⁻¹ to be used for enzyme activity calculation.

Guaiacol Peroxidase (EC: 1.11.1.x) : Reaction mixture (3ml) containing 50 mM phosphate buffer (pH 5.8), 1.6 µl H₂O₂, 1.5 µl guaiacol and 0.2 ml enzyme extract. The GPX activity was measured at 770 nm and then calculated using the extinction coefficient 26 mM⁻¹cm⁻¹ for tetraguaiacol and was expressed in mol tetraguaiacol/min/mg tissue (Fang and Kao, 2000).

Superoxide dismutase (SOD EC 1.15.1.1) : The activity of SOD was expressed as unit per milligram protein (Bauchamp and Fridovich, 1971). One unit activity of SOD is the amount of protein needed to stop 50% reduction of NBT under light. 3 ml reaction mixture was prepared with 50 mM potassium

phosphate buffer (pH 7.8). 13 mM methionine, 75 µM nitroblue tetrazolium, 2 µM riboflavin, 0.1 mM EDTA and 0.8 ml of enzyme extract. This final reaction mixture was kept in test tubes under 30 cm below light source of 15W lamp for half an hour followed by the measurement of OD at 560 nm.

Glutathione reductase (EC 1.8.1.7) : 50 mM Tris HCl buffer (pH 7.6) was used to homogenize 200 mg leaf and centrifuged at 14000 rpm for 20 min. The final reaction mixture of 1 ml was prepared by adding 50 mM Tris-HCl buffer pH 7.6, 0.15 mM NADPH, 1mM GSSG (oxidized glutathione), 3mM MgCl₂ and 200 µl enzyme extract. Reduction of absorbance of NADPH at 340 nm was recorded carefully by measuring OD of reaction mixture. The enzyme activity was represented as µmol NADPH oxidized / min / mg protein (Schadle and Bassham, 1977).

Statistical analysis

All experiments were performed in random repetition of triplicates. All datasets obtained from the experiments were analyzed with analysis of variance (ANOVA) followed by Tukey's Multiple Range Test (TMRT) using SPSS 11.0 statistical package. Significance level was compared at $p < 0.05$. All data were represented as means, with $\pm$ SD.

RESULTS

Ca promotes plant growth under Cr stress

Root and shoot length of Cr treated plants reduced considerably compared to untreated plants (Fig. 1). Maximum reduction of both root (46%) and shoot (46.8%) was found in 50 µM Cr treated set (T2). Though co-treatment of 10 mM Ca (T5) has withdrawn Cr induced root (20.6%) and shoot (24.7%) growth inhibition, but better Cr toxicity alleviation was recorded with 20 mM Ca dosage in (T8) root (36%) and 10 mM Ca dosage (T7) in shoot (34%).

Cr alters membrane integrity and osmoregulation

Highest endogenous H₂O₂ production (83%) was found (Fig. 2) in 50 µM Cr treated plant (T2) which was 33% reduced by simultaneous application of 20

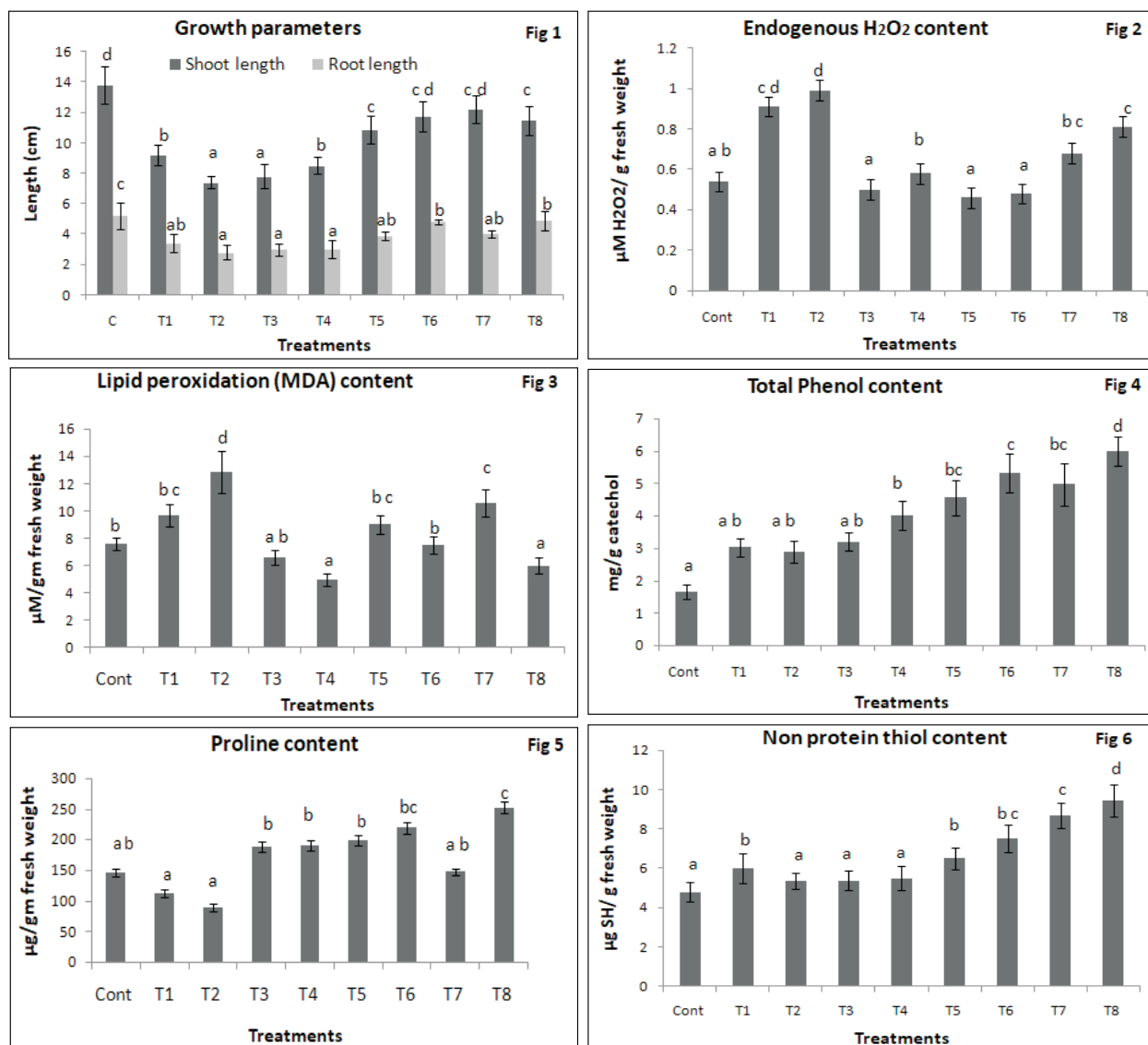


Fig. 1. Growth parameters; **2.** Estimation of endogenous H₂O₂ content; **3.** MDA content; **4.** total phenol content; **5.** proline content; **6.** non protein thiol content in *Cicer arietinum* under various treatment [C(control): Cr-0 Ca-0; T1(25 μM Cr + 0 Ca); T2(50 μM + 0 Ca); T3(0 Cr + 10 mM Ca); T4(0 Cr + 20 mM Ca); T5(25 μM Cr + 10 mM Ca); T6(25 μM Cr + 20 mM Ca); T7(50 μM Cr + 10 mM Ca); T8(50 μM Cr + 20 mM Ca)]

mM Ca (T8). When Ca was applied alone (T3) prominent decline in H₂O₂ accumulation was recorded. One of the adverse effects of heavy metals exposure is lipid peroxidation, which is quantitatively measured by malondialdehyde (MDA) formation. Highest lipid peroxidation (69%) was observed (Fig. 3) in maximum Cr concentration (T2). But 20 mM Ca in combination with 50 μM Cr (T8) was found to be more effective in lowering membrane damage (48%).

Better result was noted in 20 mM Ca co treated with 25 μM Cr (T6) which showed 57% decrease in membrane damage with respect to sole Cr treatment (T1). For detoxification of ROS plants use various phenolic compounds as potential antioxidants. Fig. 4 revealed that sole 20 mM Ca treated plants (T4) had 2.4 folds increased phenolic content compared to control set. But co treatment of 20 mM Ca with Cr (T8) was found to be most effective as it showed 2.47 folds increase in

phenolics with respect to 50 μ M solo Cr stressed plants (T2).

Proline is an osmolyte that functions as a signalling molecule (Maiti *et al.*, 2012). Though solo application of Ca (T3) revealed increase in proline accumulation (23.6%) compared to control plant (Fig. 5) but more significant increase in proline production (27.4%) was observed in 20 mM Ca co treated plants (T8) in comparison to only Cr treated set (T2).

Ca modifies anti-oxidative defense response under Cr stress

To cope with environmental stress, plant produces non enzymatic and enzymatic antioxidants, which scavenge Reactive Oxygen Species (ROS), thus preventing oxidative stress. The tripeptide glutathione is sulphur containing major non protein thiol (NPSH)

and is involved in sequestration of heavy metals and detoxification of xenobiotics (Kao *et al.*, 2015). Elevation of NPSH content (12.5%) was observed in sole Ca treatment (T3/T4) compared to control set (Fig. 6), but simultaneous application of 20 mM Ca with 50 μ M Cr (T8) revealed better production (84%) with respect to sole Cr treated experimental set (T2).

In this study Cr toxicity has led to significant changes in antioxidative enzymes status. Significantly, 3 folds higher level of catalase activity (Fig. 7) was recorded in 20 mM Ca co treated plants (T8) in compared to 50 μ M Cr treated set (T2), while application of Cr alone showed 4.5% decline in compared to control set. With comparison to control set, Guaiacol peroxidase activity (Fig. 8) showed 16.5% decline with higher Cr toxicity (T2), but this decreased activity was compensated (54%) better with Cr and 20 mM Ca combined treatment (T8). GR activity (Fig. 9) showed

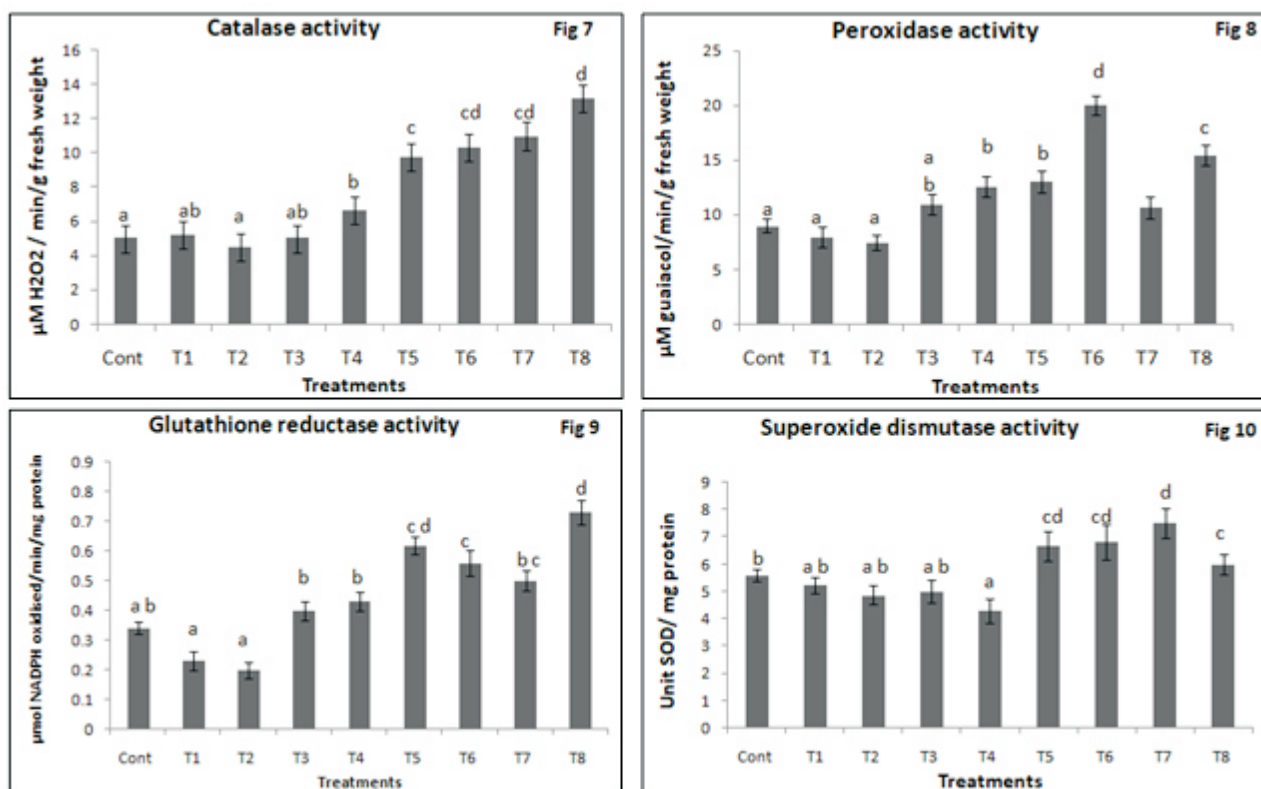


Fig. 7. Estimation of Catalase activity; **8.** Peroxidase activity; **9.** Glutathione reductase activity, **10.** Superoxide dismutase activity in *Cicer arietinum* under various treatment [C(control): Cr-0 Ca-0; T1(25 μ M Cr + 0 Ca); T2(50 μ M + 0 Ca); T3(0 Cr + 10 mM Ca); T4(0 Cr + 20 mM Ca); T5(25 μ M Cr + 10 mM Ca); T6(25 μ M Cr + 20 mM Ca); T7(50 μ M Cr + 10 mM Ca); T8(50 μ M Cr + 20 mM Ca)]

41% decline under 50 μM Cr toxicity (T2) in compared to control. This reduced activity was best alleviated (70%) by 20 mM Ca supplementation (T8). Maximum SOD activity (Fig. 10) was recorded from plants with combined treatment of 50 μM & 10mM Ca (T7) which was 28% higher than solo treatment of 50 μM Cr (T2). But 20mM Ca co treatment (T8) was found less effective here in ameliorating Cr toxicity.

DISCUSSION

The present study reveals that, growth parameters including root (46%) and shoot length (40%) were reduced under Cr toxicity (T1, T2) compared to control (Fig 1). Since roots are the first organ to come in contact with the Cr, their growth is affected largely. The direct exposure of roots to Cr containing media, results in retardation of various cellular activities and cell elongation in root apex (Gomes *et al.*, 2017). Along root inhibition, shoot growth was also impeded by Cr toxicity. Similar type of result was reported earlier, under Cr stress in maize (Mallick *et al.*, 2010) and Cd in Brassica juncea (Ahmad *et al.*, 2015). Sundaramoorthy *et al.* (2010) observed that Cr (VI) caused an extension in cell cycle leading to inhibition of cell division, and thus root growth. The decreased root growth directly affects the water and nutrient absorption and their transportation to aerial plant parts, thereby inhibiting shoot growth. Application of calcium along with chromium in the growth medium, root and shoot length were significantly increased likened to chromium stressed plants. In our study, addition of calcium chloride in the absence of chromium showed no significant increase in both root and shoot length compared to control plants. When Ca was applied alone (T4) shoot growth inhibition was withdrawn by 4% in the treated set, which proves that Ca was not imposing any prominent toxicity. On the other hand, simultaneous application of 20 mM Ca and Cr (T8) causes 30.81% increase in shoot length compared to only Cr treated plants while root growth inhibition was reduced by 12%. Our finding is in accordance with the report of Mukta *et al.* (2019) where Ca was found to induce shoot growth under Cr toxicity in rice seedling.

In plants, ROS are generated spontaneously as by

products of different metabolic activities, but under heavy metal stress, their formation is very acute and detrimental (Maiti *et al.*, 2012). Production of reactive oxygen species is a biomarker of heavy metal poisoning. Formation of endogenous H_2O_2 is directly related to oxidative degradation of biological membranes. Cr in growth solution (T2) increased (83%) the formation of endogenous H_2O_2 in leaf compared to the control (Fig. 2). Maximum Cr sensitivity was recorded in the chickpea seedling treated with 50 μM Cr evident by highest production of H_2O_2 in comparison to the seedling grown with simultaneous treatment of 50 μM Cr and 10 mM (T7) or 20 mM (T8) calcium (Fig. 2). It is quite obvious that presence of Ca ameliorates the excessive formation of H_2O_2 under Cr toxicity as 33% reduction of ROS was noted. The more effective dosage of Ca was 10 mM (T7) as this treatment showed significant reduction (54%) with respect to only Cr stressed plants (T2) in controlling ROS production. Ca induced restriction of H_2O_2 formation is prominently established here. Our observation is in good accordance with few workers (Singh and Prasad 2019) where external application of Ca and S significantly reduced Cr (VI) induced free radical accumulation. But in contrast, 20 mM Ca treatment of 50 μM Cr stressed seedling (T8) showed slight elevated production of H_2O_2 than 10 mM Ca (T7). It can be explained by the facts that, despite the harmful effects of H_2O_2 , it can participate in signalling mechanisms in response to stress (Seth *et al.*, 2011; Maiti *et al.*, 2012), and has been established as a potential elicitor of signal transduction regarding plant metal tolerance (Seth *et al.*, 2011; Maiti *et al.*, 2012).

Reactive Oxygen Species induced membrane damage of stressed plants is indicated by the production of malondialdehyde (MDA). Estimation of malondialdehyde which is a cytotoxic product of lipid peroxidation acts as vital parameter of oxidative stress due to heavy metal toxicity. The enhanced MDA production (Fig. 3) in chickpea seedlings with increasing concentrations of Cr is supported by the literature that proline accumulation attributed to excessive reactive oxygen species generation due to oxidative damage under Cd, Cr stress (Farooq *et al.*, 2016). 50 μM Cr in growth medium (T2) elevated the formation of MDA in plant with respect to the control

almost 1.7 folds. But the simultaneous application of calcium with Cr significantly decreased the accumulation of MDA in both treated (T5, T6, T8) plant (Fig. 3). The sole application of 20 mM calcium (T4) also reduced MDA accumulation (30%) in comparison to non-treated control set. This indicates that 20 mM concentration of Ca is nontoxic for plants and effective against oxidative damage of membrane. In addition, treatment of 20 mM Ca (T8) was found to be more effective in the reduction (48%) of lipid peroxidation in comparison to (T7) 10mM in 50 μ M Cr (34.5%) stressed seedlings. This result is in agreement with the recent finding of few workers (Mukta *et al.*, 2019) where it has been reported that Ca successfully reduces lipid peroxidation in Cr stressed rice plant. Same observations have been recorded for Cd toxicity in few plants such as *P. vulgaris* (Bhardwaj *et al.*, 2009) and *A. thaliana* (Saffar *et al.*, 2009).

It is well established that polyphenols contribute antioxidative protection as it acts as chelator of metal ions, and can reduce lipid peroxidation by scavenging the lipid alkoxyl radical (Lavid *et al.*, 2001). In this present study prominent changes in the total phenol contents were recorded in different sets of Cr treated plants (Fig. 4). 20 mM concentration of Ca co-treated with 25 μ M Cr (T6) and 50 μ M Cr (T8) showed significant overproduction of phenol almost 1.75 folds and 2.47 folds respectively in compared to sole Cr treated plant (T1, T2) with significant variation.

It is well documented that free proline not only contributes to osmotic adjustment at the cellular level but also very helpful to withstand heavy metals stress (Tripathi, 2013). Proline is well known as a molecular chaperone and maintains the structural integrity of proteins to improvise enzyme activity (Szabados and Savoure, 2010). Our study revealed that on using Ca alone or in combination with different concentrations of Cr, free proline content significantly increases in chickpea seedlings (Fig. 5), where the most prominent effect was at 50 μ M Cr in combination with 20 mM Ca (T8) in comparison with control and sole Cr stressed plants (T2). Though 10 mM Ca alone (T3) or in combination with 25 μ M of Cr (T5) showed insignificant effect in the enhancement of proline content, but 20 mM Ca caused significant increase (2.8 folds) when combined with 50 μ M of Cr (T8) with

respect only 50 μ M Cr treated set (T2). This can be correlated with better growth parameters (Fig. 1) of Cr stressed plant under 20 mM Ca supplementation. Literature revealed that proline induced ROS scavenging is mainly by detoxification of hydroxyl radicals and scavenging of singlet oxygen (Mourato *et al.*, 2012) that helps plant to grow under abiotic stress. Cr toxicity ameliorating capacity of 20 mM Ca co treatment by overproducing proline (Fig. 5) can be further correlated with the lesser lipid peroxidation (Fig. 3) in experimental set T8 also. Our study is in pretty good accordance with various documentations (Beltagi and Mohamed, 2013) where Cd stressed *Pisum* seedlings were remediated with various concentration of CdCl₂. Several reports revealed that Rice seedlings treated with Cr+6 (100 μ mol L⁻¹) and *Cucumis sativus* L. treated with increasing Cr6+ concentrations showed significant elevation in free proline accumulation (Mohanty and Patra, 2011).

The tripeptide glutathione is sulphur containing major non protein thiol (NPSH) and is involved in sequestration of heavy metals and detoxification of xenobiotics (Kao, 2015). Excess formation of glutathione is the prerequisite for the synthesis of heavy metals scavenger peptide phytochelatin (Yu *et al.*, 2018). 84% enhancement of NPSH content was recorded in 20 mM Ca treated 50 μ M (T8) Cr stresses plant (Fig. 6) in compared to sole Cr stressed plant (T2). This is responsible for its better ability to resist cellular metal load, which might be due to the promotion of phytochelatin biosynthesis. Our finding is in good accordance with the study of few researchers (Leao and Oliveira, 2014) that revealed concentration dependent increase of non protein thiol in Lemma against As toxicity. Literature revealed immense importance of phytochelatin, a well known nonprotein thiols, in the mitigation of As and Cd toxicity (Noctor *et al.*, 2012). But recently various workers have reported that under Cr stress in rice, phytochelatin synthesis increase for metal sequestration in plant vacuole resulting Cr tolerance (Yu *et al.*, 2018).

Experimental evidences indicate the positive role of Ca supplementation under Cr toxicity in restoration of various growth parameters, membrane integrity and antioxidative status. These findings are in accordance

with the early research works in mustard (Ahmad *et al.*, 2015), almond (Elloumi *et al.*, 2014) and sesame plants under cadmium stress (Abd_Allah *et al.*, 2017). The oxidative damage of plant cell imposed by heavy metal toxicity is ameliorated by enhanced activities of various antioxidative isozymes (SOD, GR, CAT). Superoxide dismutase (SOD EC 1.15.1.1) plays a crucial role in the primary defense against ROS, decreasing the oxidative stress by converting two superoxides radical into H_2O and O_2 . Catalase (CAT, EC 1.11.1.6) further degrades H_2O_2 into H_2O and O_2 . Glutathione reductase (GR, EC 1.8.1.7) is an important member of ascorbate-glutathione cycle that acts as antioxidative shield in any type of oxidative damage. For uninterrupted supply of phytochelatin, GSH pool must be maintained continuously which is done by GR. Glutathione reductase catalyzes the NADPH dependent reduction of glutathione disulfide (GSSG) of cell to glutathione (GSH), which in turn acts as precursor of metal chelator proteins (Ding *et al.*, 2009). Over expression of GR or upregulation of GR activity (Melchiorre, 2009) is directly associated with metal detoxification potential of plants. The activities of the antioxidant enzymes: CAT (Fig. 7), POD (Fig. 8) and GR (Fig. 9) in chickpea seedlings were both increased and decreased among different treatments. Our study revealed that 50 μM Cr application (T2) caused insignificant decrease in CAT activity which can be overcome by 10 mM (T7) and 20 mM (T8) Ca supplementation upto 3 fold. It is pretty correlated with decrease in H_2O_2 accumulation in T6 and T7 treatments. This is due to Ca^{+2} antagonisms with Cr, suggesting that Ca^{+2} controls ROS signalling for mitigating Cr-induced oxidative injury in chickpea seedling. It has already been proven by previous researchers that the first attempt of Ca^{+2} induced metal tolerance is the lowering of H_2O_2 level by modifying ROS signal pathway (Thounaojam *et al.*, 2012). Supplementation of 20 mM Ca^{2+} with Cr (T6,T8) resulted remarkable enhancement in GPOD (4.35 fold) activity, though T6 showed better activity due to lesser Cr conc (25 μM) than T8 (50 μM) (Maiti *et al.*, 2012). Application of 20 mM Ca in combination with Cr (T8) GR showed 3.17 fold increased activity with respect 50 μM Cr stressed plants (T2), which can be correlated with considerably high amount of NPSH accumulation in T8 treatment (Fig. 6). In contrast, 20

mM Ca was proven to be less effective in combination with 50 μM (T8) Cr to alleviate SOD activity compared to 10 mM Ca dosage (T6). Enhanced SOD activity (Fig 10) indicates excess accumulation of superoxide leading to stress (Hakeem *et al.*, 2019). Initially SOD activity declined under Cr stress, but 20 mM Ca supplementation regain its activity by 21% in T8 set. This increase recommends Calcium as a potential antagonist to Cr induced oxidative stress. Our observation of Ca dependent enhancement of antioxidative enzymes activity under metal toxicity are in agreement with the records obtained by early researchers (Wang and Song, 2009) who found that *Trifolium repens* L. seedlings co- treated with Ca^{2+} and Cd^{2+} showed enhanced activity of antioxidative enzymes. Supplementation of calcium with chromium increased CAT, POD, SOD and GR activities with respect to chromium stressed plants is pretty well established in our investigation. Our result can be corroborated with the reports of other workers where Cd-induced oxidative injury was mitigated by alteration of enzymatic antioxidant system in sesame due to Ca (Abd_Allah *et al.*, 2017) application and in mustard by citric acid supplementation (Mahmud *et al.*, 2018). But in the case of SOD activity, less significant change was observed within various concentrations. In both the treatment of Cr, 10 mM Ca^{2+} supplementation was found to be more effective for elicitation of SOD activity (Fig. 10) than 20 mM. This might be explained as higher concentration of Cr produces so huge amount of superoxides (Kabir, 2016) that could not be ameliorated by 20 mM Ca supplementation.

CONCLUSION

In this study, Ca^{+2} was added solely as well as simultaneously with Cr to antagonize the damaging effect of Cr in chickpea seedling as Calcium protects plants from detrimental effect of stress by participating in signaling pathways. From our investigation, it is clear that, the co-treatment of Ca with Cr, helps to overcome the metal induced injury in chickpea seedlings. This amelioration is due to upregulation of antioxidant defense by modulating CAT, POD, GR activity as well as non enzymatic antioxidants. Though 20 mM concentration of Ca

supplementation was not unanimously confirmed as most suitable for Cr toxicity amelioration but can be suggested as optimum dosage for mitigation of micromolar Cr contamination of soil. Outcomes of this investigation not only boost our knowledge about chromium toxicity in *Cicer sp.* but also enlighten the potential fields of remediation and mitigation of Cr toxicity.

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