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EFFECT OF CHROMIUM AND H_2O_2 PRETREATMENT ON *CAPSICUM ANNUM* L. AT EARLY GROWTH STAGE

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Abstract

H_2O_2 and distilled water soaked seeds of *Capsicum annum* L. were grown in petri plates followed by treatments with distilled water, 100 and 200 µM Chromium (Cr) solutions. Growth and biochemical parameters were recorded. The seedlings were harvested and analyzed for the germination percentage, rate of germination, seed vigour, chlorophyll content, tolerance index, proline and sugar content. At different concentration of Cr (100 and 200 µM) growth parameters were significantly reduced. Cr toxicity caused a reduction in chlorophyll, tolerance index, accumulation of proline and enhancement of sugar content in shoot and root. While pretreatment of seeds with H_2O_2 had non-significant effect on seed germination and seedling growth stage of *Capsicum annum* L. So it can be concluded that this variety is sensitive to chromium stress and its tolerance cannot be improved by hydrogen peroxide pretreatment.

Key words: Biochemical, Capsicum, Pretreatment, Proline, Vigour.

Introduction

Heavy metals (HM) are one of the major sources of soil pollution (Il'yasova & Schwartz, 2005) and are cause of many environmental issues, linked to industrial and agricultural activities: reduction of microbial activity, soil fertility and crop yield (Yang *et al.*, 2005). Various soils particularly those found in hazardous waste areas are often polluted with heavy metals, for example lead, copper, chromium, and cadmium. Moreover, they may percolate through soils and reach underground waters or may be absorbed by plants (Panda & Choudhury, 2005). Chromium (Cr) is an important element which is found in two states i.e. trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) needed in minute quantities for normal carbohydrate and lipid metabolism. Cr^{6+} is more noxious and mobile as compared to Cr^{3+}, it forms chromate and dichromate, which have high solubility in water and there is no proof of their possible role in plant metabolism (Panda & Patra, 1997). Trivalent is nontoxic and present in living organism while hexavalent is toxic and mostly used in industry. Awareness in chromium toxicity initiated from widespread use in different industrial processes, such as metallurgical and chemical industries. Besides, chromium is utilized on huge scale in industries, for instance paint, pigment, wood preservation, tanneries, paper and pulp production which is resulting in increased Cr quantity in soil and water (Zayed & Terry, 2003). Heavy metals obstruct many physiological attributes causing decrease in the plant growth, photosynthesis and the biomass finally (Jamal *et al*., 2006). Phytotoxicity of Cr. results in decline of seed germination, degradation of photosynthetic pigments and generates oxidative stress in plants (Panda & Patra, 1997 & 2000). Chromium toxicity in plants shows some symptoms like chlorosis of leaf, tissue necrosis, membrane damage, change in soluble protein contents, and germination and growth rate inhibition (Oliveira, 2012).

Capsicum annuum L. commonly called Chilli pepper is domesticated specie of the genus *Capsicum* of solanaceae family (Borges, 2009). Chili pepper is the maximum used spice around the globe (Chukwu, 2006; Nopaintaya & Nye, 1974). In our country Pakistan two species viz. *Capsicum annum* and *Capsicum frutescens* are cultivated on an area of 38.4 thousand hectares and their annual production is 90.4 thousand tones and average yield is 1.7 tons per hectare. Sindh is the major producer of chillies (Ziaf *et al*., 2009; Borges, 2006). Chili pepper also contains some pharmacological characters (Govindarajan & Sathyanarayana, 1991).

Exogenous hydrogen peroxide (H_2O_2) is reported to act as a signal for introducing defense reactions in plants in response to pathogen attack (Alvarez *et al.*, 1998; Levine *et al.*, 1994), oxidative (Morita *et al.*, 1999) and abiotic stresses (Prasad *et al.*, 1994; VanCamp *et al.*, 1998). Pretreatment of seeds with oxidants such as H_2O_2 is known to break seed dormancy (Jann & Amen 1977). Ogawa & Iwabuchi (2001) observed that H_2O_2 promoted germination of *Zinnia elegans* L. seeds which did not germinate because of inhibitors in the pericarp covering the seed within the fruit in a dose-dependent manner. Moreover hydrogen peroxide has also been stated to enhance germination of seeds and growth of sprouts (Narimanov & Korystov, 1997).

Therefore, in this investigation attempt has been made to assess the effect of H_2O_2 pretreatment in ameliorating toxic effects of chromium on *capsicum annum* L. growth and biochemical parameters: chlorophyll, proline and sugar content, tolerance index, rate of germination and germination percentage. Present study was aimed to find the effects of Cr ion toxicity and H_2O_2 priming on germination and seedling characters of *Capsicum annuum* L.

Materials and Methods

The experiment was conducted in Plant Physiology Laboratory, Department of Botany, Lahore College for Women University Lahore. Seeds of advance line of (suraj mukhi) of chilli (*Capsicum annum* L.) were obtained from Punjab seeds corporation Lahore, Pakistan. The seeds were sown in petriplates. The petriplates were kept in incubator at constant temperature (20±2 ^{0}C). There were seven sets of petriplate, three replicates per treatment. Suggested concentrations (100 and 200µM) of potassium dichromate ($K_2Cr_2O_7$) were applied.

Treatments: 100 and 200 µM solutions of chromium were prepared using $K_2Cr_2O_7$. In the first treatment distilled water was applied and in second treatment seeds were first soaked for 8 hours in distilled water and then distilled water was applied. In the third treatment, seeds were first soaked for 8 hours in H_2O_2 and then distilled water was applied. In the fourth treatment 100µM and in fifth 200µM Cr solutionwas applied to seeds. In the sixth and seventh treatments H_2O_2soaked seeds for 8 hours were applied with 100µM and 200µM Cr respectively. In each set of petri plates 5ml of respective treatment was added after the seeds were placed. To each set of petri plate 2ml of treatments was added daily.

Germination record: Germination was recorded daily. The number of germinated seeds was counted after one week from the start of experiment. Germination percentage was calculated by Close and Wilson (2002) method and seed vigour by Abdul Baki and Anderson (1973).

Tolerance Index: The tolerance index was estimated by the formula given below:

TI = *Mean root length in treatment applied / mean root length in distilled water × 100*

Chlorophyll and carotenoid content determination: Chili leaves were weighed and crushed finally in pestle and mortar. About 4 ml of 80% acetone was added in it. The solution of crushed leaves and acetone was filtered. Now the solution was taken in cuvette of spectrophotometer. The absorbance at different wavelength (i.e. 470nm, 645nm and 663nm) was noted. The amount of chlorophyll was calculated according to standard formulae (Arnon, 1949) and content of carotenoid was calculated according to the formula of Lichtenthaler and Welburn (1983).

Sugar content: Dubois *et al*. (1956) method was utilized for measuring leaf sugar content. For measuring sugar content 0.05gm of root/shoot was weighed and crushed finally with pestle and mortar, 1mL of distilled water was added in it. 5% v/v phenol was prepared by mixing 5mL phenol with 95mL distilled water. In 0.01mL of extract, 1mL of phenol was added, and then it was kept for 1 hour. After 1 hour 5mL conc.H_2SO_4 was added. Now the solution was taken in cuvette of spectrophotometer. The absorbance of sugar at wavelength 420nm was noted. The amount of sugar was calculated by preparing standard curve for known concentrations of glucose.

Proline estimation of leaves and root: Proline was measured from root and leaves of chilli (*Capsicum annum* L) by the method of Bates *et al.* (1973). **Statistical analysis:** Data was analyzed to find out the significance of variance with one-way ANOVA using Costat for Windows. Duncan's multiple range test (Steel & Torrie, 1980) was used to find meaningful differences among treatments at the level of significance P < 0.05.

Results

Various levels of chromium toxicity produced different effects on growth germination percentage, rate of germination, seed vigour, chlorophyll, carotenoid, proline, sugar content and tolerance index of *Capsicum annum* L.

From the Figure 1(a) it is clear that most of the treatments showed non-significant ($p<0.05$) influence on germination percentage but in case of Cr 200µM significant decrease was observed as compared to control but the effect of H_2O_2 application was non-significant under control as well as stressed conditions. Cr treatments showed significant ($p<0.05$) effects on rate of germination in contrast to control treatment whereas influence of distilled water and H_2O_2 seed soaking on rate of germination was found to be nonsignificant ($p<0.05$) in all cases. Figure 1(b) illustrates significant ($p<0.05$) effect of different treatments in case of seed vigour. Minimum seed vigour was observed under Cr 200µM. Under unstressed condition H_2O_2 seed dipping caused a significant reduction in seed vigour whereas under stress condition the effect of H_2O_2 was found to be nonsignificant ($p<0.05$).

A significantly ($p<0.05$) lower tolerance index was observed in all stress treatments and H_2O_2 seed soaking caused a decrease in tolerance index (Figure 1(c)). Tolerance index was found to be maximum when seeds were pre-soaked in distilled water and minimum in H_2O_2 pretreated seeds under 200µM Cr stress.

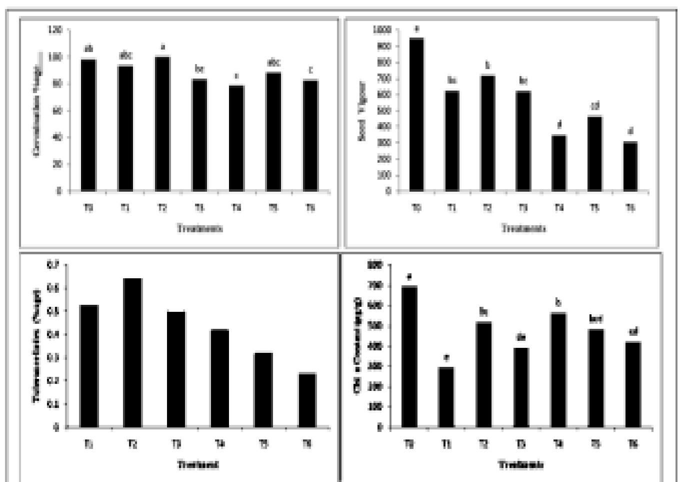

Figure 1: Influence of Chromium toxicity and H_2O_2 seed soaking on (a) germination %age (b) seed vigour (c) tolerance index (%) (d) chlorophyll *a* (µg/g) of *Capsicum annum* L. leaves Where T0= control; T1= Seeds soaked in Distilled water; T2= Seeds soaked in H_2O_2; T3= 100 µM Cr; T4= 200 µM Cr; T5= 100 µM Cr + Seeds soaked in H_2O_2; T6= 200µM Cr+ Seeds soaked in H_2O_2

Significant ($p<0.05$) decrease in chlorophyll *a* content (Figure 1(d)) was noticed in all treatments in comparison to that of control. In distilled water seed priming case, seedlings showed significantly ($p<0.05$) minimum chlorophyll *a* content. H_2O_2 had nonsignificant effect on chlorophyll *a* content under 100µM chromium concentration. Whereas under unstressed condition and 200 µM chromium stress it caused a reduction in chlorophyll *a* content as compared to respective treatments without H_2O_2.

All treatments showed nonsignificant ($p<0.05$) effect on chlorophyll *b* (Figure 2(a)) except distilled water priming which revealed significant ($p<0.05$) decline. Significantly ($p<0.05$) lower total chlorophyll content (Figure 2(b)) was observed as compared all other treatments in case of distilled water priming. All other treatments caused a significant decrease in total chlorophyll content as compared to control. Distilled water treatment resulted in significant lessening of chlorophyll *a*, chlorophyll *b* and total chlorophyll content. No significant influence on chlorophyll *a/b* ratio was found under most of the treatments (Figure 2 (c)). Only Cr 100µM resulted in significant decrease of chlorophyll *a* to chlorophyll *b* ratio and pretreatment of H_2O_2 under 200µM Cr stress significantly decreased this ratio as compared to respective stress alone.

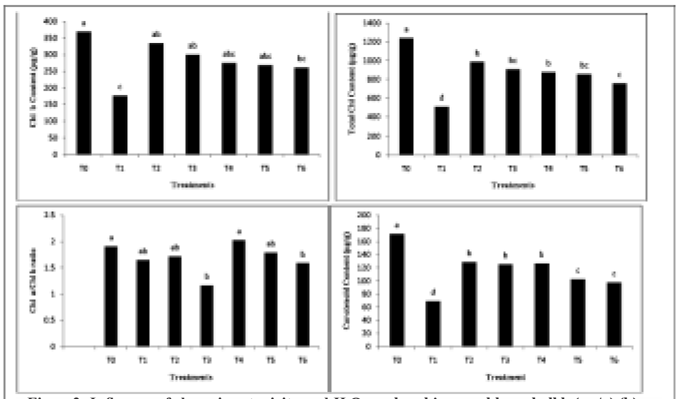

Figure2: Influence of chromium toxicity and H_2O_2 seed soaking on chlorophyll b (µg/g) (b) total chlorophyll (µg/g) of *Capsicum annum* L. leaves (c) chlorophyll *a/b* ratio (d) carotenoid Where T0= control; T1= Seeds soaked in Distilled water; T2= Seeds soaked in H_2O_2; T3= 100 µM Cr; T4= 200 µM Cr; T5= 100 µM Cr + Seeds soaked in H_2O_2; T6= 200µM Cr+ Seeds soaked in H_2O_2

Significantly ($p<0.05$) lower carotenoid content under all the treatments was comparable to control. H_2O_2 pretreatment significantly decreased the carotenoid content under both stressed and unstressed condition (Figure 2(d)).

Root sugar content (Figure 3a) was found to be maximum under Cr 200µM treatment. H_2O_2 under stress condition caused significant reduction in content of root sugar with respect to corresponding stress treatments without H_2O_2 pretreatment. While significant ($p<0.05$) influence of different treatments was observed in case of shoot sugar content (Figure 3b) Maximum shoot sugar content was observed in seedlings growing under 200µM chromium. H_2O_2 had significant negative effect on shoot sugar content under both stressed condition.

Root proline content was found to be higher in H_2O_2 pretreated seedlings under control condition and in stress treatments. 200µM H_2O_2 pretreatment was observed but 200 µM

H_2O_2 pretreatment caused a decrease in proline content. Significantly higher shoot proline content was observed under 100µM chromium stress as compared to seedling under control condition (Figure 3 c & d).

Discussion

It is well established that different biotic and abiotic stresses (Dat *et al*. 1998; Hernandez *et al.*, 1995) result in excess production of reactive oxygen species (ROS) as H_2O_2 in plant tissues. The exogenous supply of which were thought to have some positive effects but these oxidants may also act as stress signals. During present work several of the metabolic and physiological processes of *Capsicum annum* L. seed germination and seedling growth were found to be affected by Cr

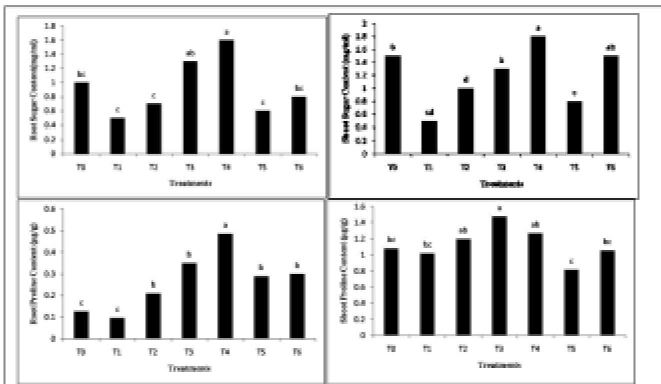

Figure 3 .Effect of chromium toxicity and H_2O_2 seed soaking on (a) root sugar content (mg/ml) (b) on shoot sugar content (mg/ml) (c) root proline content(µg/g) (d) shoot proline content (µg/g) of *Capsicum annum* L. Where T0= control; T1= Seeds soaked in Distilled water; T2= Seeds soaked in H_2O_2; T3= 100 µM Cr; T4= 200 µM Cr; T5= 100 µM Cr + Seeds soaked in H_2O_2; T6= 200µM Cr+ Seeds soaked in H_2O_2

toxicity and H_2O_2 seed soaking.

As seed germination is the first phase of plant life affected by any stress the ability of a plant seed to germinate under Cr stress would be an indication of its tolerance to this metal (Peralta *et al*., 2001). In the present study, it was noted that there was a significant correlation between level of chromium toxicity, germination percentage (Fig.1) and rate of germination. As reported by Rout *et al.,* (2000) germination of *Echinochloa colona* seeds was reduced to 25% with Cr. (200 µM) concentrations. Parr and Taylor (1982) also observed that 500ppm of Cr concentration in soil caused a reduction of germination up to 48% in *Phaseolus vulgaris.* It was also observed that H_2O_2 did not affect the rate of germination and germination percentage, contrasting to the reports made in recent years by different workers (Tamas *et al.*, 2004; Schopfer *et al*., 2001) who reported that H_2O_2 and other oxidants can also be used in a positive manner in plant tissues, e.g. in a seed. H_2O_2 exogenously given to the seeds might played a role in mobilization of lipids by providing the stores of carbon and energy which are needed for the production of enzyme hydrolase and its secretion from the aleurone layer (Palma & Kermode, 2003). It means that dose of H_2O_2 given exogenously during present study did not influence any of these processes.

Seed vigour is reported as a key seed quality indicator which is required to be analyzed to complement germination and viability checks to get understanding about the seed performance of a lot in the field or under storage conditions. A significant ($p<0.05$) effect of all the treatments was observed in seed vigour index *Capsicum annum* L. (Fig. 1). Sharma *et al*. (2005) performed a study during which spinach growth was assessed

under different concentrations of chromium (0, 1.25, 2.5, 5, 10, 20, 40, 80, 160, and 320 mg Cr kg^{-1} soil). Zero germination was recorded under 320 mg Cr kg^{-1} rate so it was clear that increasing concentration of chromium decrease seed vigour index.

A significant ($p<0.05$) decrease of tolerance index was observed in *Capsicum annum* L. with the increase in chromium concentrations during the present study, like the study made by Prodgers and Inskeep (1981) that too much of toxic metals generally reduces plant growth. Our results were found to be similar to the findings of Kabir *et al.* (2008) who reported that the tolerance limit of *Thespesia populnea* L. progressively declined with enhanced levels of heavy metals.

The amounts of chlrophyll *a*, chlorophyll b and consequently total chlorophyll were lower under stress conditions. Total chlorophyll and chlorophyll b content declined more at higher levels of Cr (VI). An excess of chromium might have prevented the incorporation of iron into the protoporphyrin, which resulted in the decrease of chlorophyll pigment. Our results corroborates with previous reports made by Bera *et al*. (1999). Heavy metal-induced decrease in chlorophyll had been observed in indian mustard and mung bean (Simonova *et al*., 2007), cape gooseberry, pepino, pepper, tobacco and tomato (Thiebeauld *et al*., 2005), *Phaseolus vulgaris* (Zengin and Munzuroglu, 2005) and *Alternanthera philoxeroides* (Deng *et al*., 2007). As the chlorophyll content was minimum in distilled water treatment, according to Al-Ansari (2002) research control seedlings which were irrigated with distilled water were totally dependent on their original seeds reserves of minerals. These seeds may suffer deficiencies in some essential or trace elements leading to reduced rates of anabolic reactions and a below normal concentrations of proteins, carbohydrates and photosynthetic pigments as compared with seedlings irrigated with stresstreatments.

Vazquez *et al.*, 1987 studied that few of the heavy metals, for example, chromium in toxic amount may cause chlorosis, which is resulted by change in the amount of essential minerals; it may also reduce photosynthesis due to stomatal closure and also reduction in intercellular spaces and change within chloroplast. It was also concluded by Chatterjee and Chatterjee, (2000) that higher concentrations of heavy metals cause negative effect on biomass, iron concentration, chlorophyl "a" and "b", in cauliflower. However according to Saha *et al*., (2010), pretreatment of seeds with H_2O_2 caused decrease in chlorophyll in comparison to that of non-treated plants. It could have acted as an added stress signal. Nonsignificant effect of all treatments appeared in chlorophyll a to chlorophyll b ratio (Fig.2) whereas previously Zou *et al*. (2009) studied that at 100µM Cr (IV) treatment, chlorophyll a decreased and chlorophyll decreased versus the control.

Carotenoid Contents: Carotenoids can act as antioxidants which reduce Reactive Oxygen Species (ROS) produced due to Cr toxicity. But contrasting to this a significant ($p<0.05$) decrease of carotenoid content was observed under increased chromium concentration (Fig.2). Decrease in plants carotenoids had been already reported under Cr stress (Choudhury & Panda, 2004; Panda & Choudhury, 2004; Panda, 2003; Panda *et al*., 2003; Tripati and Smith, 2000;, 2000; McGrath, 1982). While opposite result was obtained in some aquatic plants, a rise in carotenoids content was noticed under Cr stress (Ripati & Smith, 2000; Vajpayee *et al*., 2001).

Sugar Content: Sugar is an important energy constituent needed for all living organisms. Plants manufacture this organic substance during photosynthesis and respiration. The concentration of soluble sugar indicates the physiological activity of plant body. The sugar content in plants grown under laboratory conditions and field environment varied due to different doses of Cr application, while maximum was observed in Cr 200µM treatment. Sugar content was found to be more in shoot than in root and work of Hall, (2000); Kochian, (1995); Rellen-alvarez *et al.*, (2006) supported this statement by explaining that the rise in leaf sugar content under various Cr. treatment of Cr might be a strategy to overcome the Cr toxicity on plants by increasing the production of carbohydrates.

Proline Content: Proline, is the amino acid which acts not only as compatible solute but also work as a source of carbon and nitrogen for after stress recovery and growth, as a protector of membranes and machinery of protein synthesis as a free radicals scavenger, regulate redox potential and also protect the proteins from denaturation (Saha *et al*., 2010). Under stress treatment proline accumulated more in roots (Fig. 3) but not in leaves with the increase of chromium there was significant ($p<0.05$) increase in proline content these findings were in accordance to Pandey and Sharma (2002) who demonstrated that heavy metals caused increase in proline content in the leaves of cabbage, and proposed a correlation with the altered water status of the stressed plants. Schat and coworkers (1997) recognized accumulation of proline in the leaves of Cd-stressed *Silene vulgaris*. This increased proline content might also be a reason of increased tolerance of this variety of *capsicum annum* as proline act as protective compound under different stressful conditions as Matysik *et al*. (2002) supported this hypothesis by saying that proline is also involved in antioxidant defense.

Conclusion

The results of this study showed that increase in the concentrations of Cr, caused changes in various growth attributes of *Capsicum annum* L. at seed germination and seedling growth stages. At 100µM Cr studied parameters were not much affected by chromium toxicity but as the concentration increases (200µM) seedlings showed remarkable reduction in various physiological and biochemical parameters. Moreover pretreatment with H_2O_2 was not helpful in increasing Cr tolerance. So it can be concluded that this variety is sensitive to higher level of Cr stress at germination and seedling growth stages. More over this dose of H_2O_2 might have acted as an additional stress signal so it cannot be used to increase tolerance to this level of Cr toxicity in this particular variety of capsicum. So other priming treatments can be tested for increasing tolerance to Cr stress in *Capsicum annum* L.

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