Journal of Stress Physiology & Biochemistry, Vol. 9 No. 2 2013, pp. 232-241 ISSN 1997-0838

ORIGINAL ARTICLE

Effect of Ionic and Chelate Assisted Hexavalent Chromium on Mung Bean Seedlings (*Vigna radiata* L. wilczek. var k-851) During Seedling Growth

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Received December 29, 2012

The effect of Cr^{+6} with and without chelating agents were assessed in mung bean seedlings grown hydroponically. It was noted that the growth parameters showed a declining trend with increasing Cr^{+6} concentrations without chelate application. Among the seedlings grown with chelated chromium complexes, Cr^{+6}–DTPA (10µM) showed highest growth rate of roots as well as shoots. At higher concentration of Chromium i.e. Cr^{+6} (100µM), there exhibited high chlorophyll content in mung bean leaves where the seedlings showed stunted growth. The seedlings treated without and with chelated chromium complexes showed increased proline content as compared to control. The enzymatic study showed that, the catalase activity was maximum in shoots as compared to roots and the reverse is true in the case of peroxidase activity i.e. the roots showed higher value than that of the shoots.

Key words: Catalase; Chromium; DTPA; EDTA; EDDHA; Mung bean; Peroxidase

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Heavy metals are integrated components of the Biosphere and occur naturally in soils and plants. Chromium (Cr) is the seventh most abundant heavy element on earth (Katz and Salem, 1994; Mohanty and Patra, 2011) and occurs in several oxidation states ranging from Cr^{-2} to Cr^{+6} with trivalent (Cr^{+3}) and hexavalent (Cr^{+6}) states being the most stable and common in terrestrial environment. Cr^{+3} is essential for animal and human health (Katz and Salem, 1994; Mohanty and Patra, 2011) but unlike Cr^{+3}, Cr^{+6} is a potent, extremely toxic, carcinogenic and causes death to animals ad humans, if ingested in large doses (Zayed and Terry, 2003; Mohanty and Patra, 2011). Ingestion of plant derived chromium containing food materials; primarily pulses provide a major portion of the daily chromium intake. It is therefore essential to ascertain the toxicity effects of free and chelated chromium on toxicological, physiological and biochemical changes during the growth of mung bean plants.

Heavy metal pollution may suppress or even kill a sensitive plant and soil microbial communities and lead to a shift in their functional diversity and structure (Flie Bbach *et al.* 1994; Zayed and Terry, 2003). Many mutagenic, toxic and carcinogenic effects in biological systems caused by chromium compounds have been reported (WHO, 1988; Debetto and Luciani, 1988; Panda and Patra, 1997; Zayed and Terry, 2003; Nayak *et al.* 2004; Mohanty *et al.,* 2005; Mohanty and Patra, 2012). Extensive studies on the toxic effects of chromium and role of chelated chromium compounds in ameliorating these toxic effects have been taken up. This study of induced phytoextraction through the use of such chelators opens a path towards the emerging trends of clean up process as the traditional areas are expensive, difficult, inefficient and sometimes detrimental to soil structure and fertility (Negri and Hinchman, 1996, Mohanty and Patra, 2011). The current phytoremediation techniques are based on the toxic effects of Cr and have been optimized by the use of chelating agents (Huang *et al.* 1997; Mohanty *et al.,* 2005; Mohanty and Patra, 2011; Mohanty and Patra, 2012).

There is notable dearth of information in the literature pertaining to the influence of different chelated chromium compounds like EDTA, DTPA and EDDHA on plant growth, physiological and biochemical changes in mung bean, which requires substantial investigation. There are reports on influence of chelating agents on uptake of chromium by plants (Athalye *et al.* 1995) and the effect of organic acids on mobilization of Cr^{+3} in wheat (Srivastava *et al.* 1999), but all these are not concerned with the role of chelated chromium compounds in ameliorating the toxicity effects of chromium during seedling growth. The present study describes the phytotoxic effects of Cr^{+6} and the ameliorating role of chelated compounds in mung bean seedlings. The attempts have been made to investigate and study the varying degrees of effect at different concentrations of Cr^{+6} and chelated chromium compounds on seedling growth, physiological and biochemical activities of mung bean.

MATERIALS AND METHODS

Plant material and experimental design

Graded dry seeds of green-gram (*Vigna radiata* L. Wilczek. Var K-851) were obtained from "National Seed Corporation", Bhubaneswar. The seeds were stored in dark and cool place for experimental use. Uniform seeds were selected and surface sterilized by soaking in 0.1% mercuric chloride ($HgCl_2$) for about five minutes and then were washed several times with tap water followed by distilled water. The surface sterilized seeds were placed in sterilized petriplates over saturated cotton pads for germination. The seeds were germinated at 25°C in darkness for two days. Different chelated chromium compounds, i.e., Cr^{+6}-EDTA (10µM), Cr^{+6}-DTPA (10µM), Cr^{+6}-EDDHA (10µM) were prepared each in equimolar ratio.

After two days of germination, the healthy seeds were transferred to well-aerated half strength Hoagland nutrient solution (without chromium) in glass culture vessels. Treatment with different concentrations of free chromium and chelated chromium compounds in other culture vessels were also run side by side. The seedlings were grown in growth chamber with 12 hr light period. The white light was provided by filtered cool white fluorescence tubes (36W Philips TLD) with a photon flux density of 52µE m^{-2} s^{-1} (PAR) (Mohanty and Patra, 2012). The nutrient culture solution of the growing mung bean seedlings were changed every

day with freshly prepared solution.

Growth parameter study

The growth parameters like root length, shoot length, fresh matter and dry matter of 5 days old mung bean seedlings were used for study. Different Cr^{+6} concentrations (10µM, 100µM) without and with chelating agents such as EDTA, DTPA and EDDHA were used during growth parameter study. Five days old-seedlings were harvested for measurement of root and shoot length. Fresh and dry matter content of both control and treated samples were measured using electronic balance (Shimadzu Corporation). The dry matter of the seedlings were taken by keeping the seedlings in oven at 80°C for a period of three days or more till constant dry weights were determined.

Analysis of chlorophyll content

The extraction and estimation of leaf chlorophyll content using cold alkaline acetone were done following the method of Nadler *et al.* (1972) with slight modification (Porra, 2002) by the formula of Arnon (1949) using UV-Vis spectrophotometer (Perkin Elmer).

Analysis of proline content

The extraction and estimation procedures for proline were followed by the method of Bates *et al.* (1973).

Extraction and assay of enzymes

Extraction and assay of enzymes (catalase and peroxidase) were followed by the method of Patra *et al.* (1978) and Patra and Mishra (1979). Chilled tissues (leaves/roots) were cut into small pieces and homogenized with cold 0.1M phosphate buffer, pH 6.8 (100 mg tissue : 5ml buffer) in a pre-chilled glass mortar and pestle. The extract was centrifuged at 4°C for 15 min. at 17, 000 rpm in a refrigerated centrifuge. The clear supernatant was used for assay of the catalase enzyme after proper dilution. Catalase activity was estimated by titration with 0.01N potassium permanganate. One unit of catalase activity is defined as that amount of enzyme, which breaks down one micro (µ) mol of H_2O_2/min. under the assay condition (Patra *et al.* 1978.).

For peroxidase, pre-chilled tissues (leaves/roots) were cut into small pieces and homogenized with cold 0.1 M phosphate buffer of pH 6.8 (100 mg fresh tissues: 2ml buffer) in a pre-chilled glass mortar and pestle. Then, the extract was centrifuged at 4°C for 15 min. at 17,000 r.p.m. in a refrigerated centrifuge and the final volume of the clear supernatant was made up to 20ml using phosphate buffer (pH 6.8). The enzyme assay procedure was followed as done previously (Patra and Mishra, 1979). Peroxidase activity was determined from the absorbency at 420nm. One unit of peroxidase activity was expressed in terms of purpurogallin formed, which increased the absorbency by 0.1 O.D. per minute under the assay condition.

Staistical analysis and presentation of data

All the experiments were conducted in completely randomized design and repeated thrice. The data presented in the figures and tables are mean ± SEM of three replicates.

RESULTS

The data of growth parameter study were presented in Table 1. Root length and shoot length decreased markedly with increase in free Cr^{+6} concentrations. Among all different types of treatments, the seedlings treated with Cr^{+6}– DTPA (10µM) had the maximum length of roots and shoots. A marked decrease in root length was observed in the seedlings treated with chelated Cr^{+6}

–EDTA (10µM) as compared to the seedlings treated with other chelating agents. The fresh weight and dry weight of the seedlings grown under Cr^{+6}–DTPA (10µM) treatment was maximum. The seedlings fresh matter and dry matter gradually decreased with free Cr^{+6} concentrations. Among the seedlings treated with chelating agents, Cr^{+6}–EDDHA (10µM) showed the minimum root fresh weight and dry weight and Cr^{+6}–EDTA (10µM) had minium shoot fresh weight and dry weight. The results showed a substantial increase in proline content in different free and chelated Cr^{+6} forms.

The total chlorophyll content was found less in all Cr treated seedlings (without and with chelate state) as compared to control. Total leaf chlorophyll content among the seedlings treatment with chelating agent was in the following order; (Table 2) Cr^{+6}–DTPA (10µM) < Cr^{+6}–EDDHA (10µM) < Cr^{+6}EDTA (10µM).

The chlorophyll content was found high in free Cr^{+6} (100µM) treatments due to stunted growth of seedlings.

Among six types of treatment, the seedlings treated with and without chelated chromium complexes showed increased proline content as compared to control (Table 2).

The catalase activity was found maximum in shoots as compared to roots. The free form of Cr^{+6} treatments showed higher catalase activity in the shoots than the chelated ones (Fig. 1). The peroxidase activity in roots, on the other hand, showed higher value as compared to shoots (Fig. 2). At higher free Cr^{+6} concentrations (100µM), both root and shoot peroxidase activities showed higher value. Free chromium supply i.e., Cr^{+6} (10µM), and Cr^{+6} (100µM) stimulated the catalase activity in shoots of mung bean seedlings. However, Cr^{+6} DTPA chelated complex showed a higher catalase value than other chelated complexes. The data showed highest root and shoot peroxides activity in seedlings treated with Cr^{+6} (100µM). Cr^{+6} -EDDHA (10µM) had the least root and shoot peroxidase activity but chromium chelated with EDTA and DTPA at 10µM concentration has moderate values of root peroxidase activity.

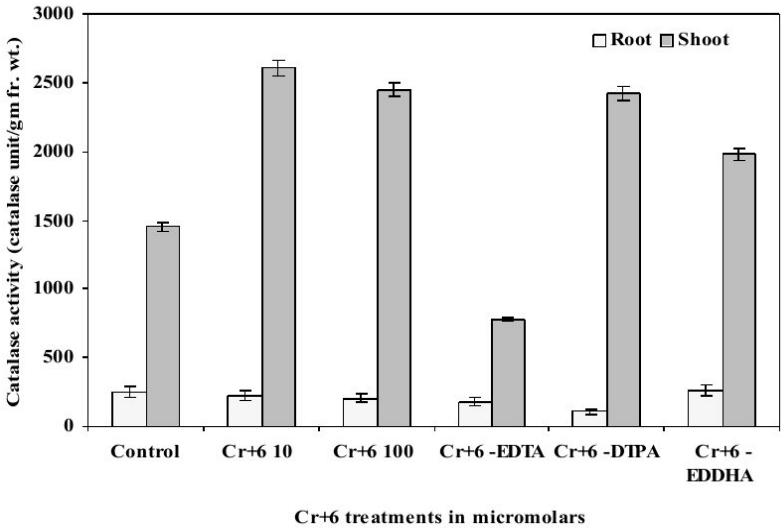

Figure 1 Effect of Cr^{+6} and chelating agents on catalase activity (root and shoot) of 5-days old mung bean seedlings

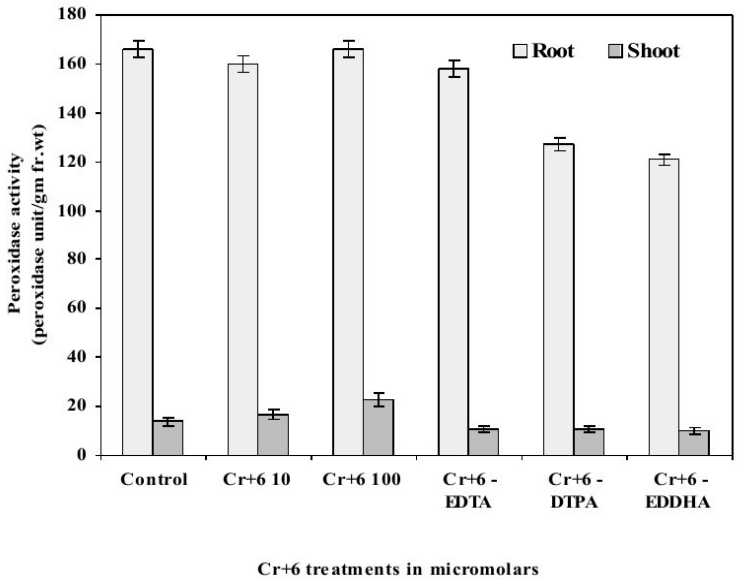

Figure 2. Effect of Cr^{+6} and chelating agents on peroxidase (root & shoot) activity of 5-days old mung bean seedlings

Table 1. Effect of Cr^{+6} and chelated–Cr^{+6} complexes on shoot length, root length, fresh weight and dry weight of 5-days old mung bean seedlings grown in hydroponics condition (values are mean ± SEM of three replicates).

Growth Parameters	Control	Cr^{+6} Treatments (µM)		Chelated Cromium Complex (10µM)		
		10	100	Cr:EDTA (1:1)	Cr:DTPA (1:1)	Cr:EDDHA (1:1)
Root Length (cm)	11.84±0.62	6.38±0.28	2.35±0.20	6.64±0.05	9.54±1.07	9.51±1.20
Shoot Length (cm)	22.96±0.15	17.81±0.41	10.69±0.52	18.92±0.60	19.91±0.59	19.69±0.40
Root Fresh Weight (mg)	47.05±2.51	37.75±0.68	31.05±0.87	40.8±2.75	46.5±2.29	40.65±1.47
Shoot Fresh Weight (mg)	302.6±10.53	208.55±15.2	132.25±1.59	241.3±13.97	260.85±7.0	249.65±4.25
Seedling Fresh Weight (mg)	174.82±6.52	123.15±7.95	81.65±1.23	141.05±8.36	153.67±4.64	145.15±2.86
Root Dry Weight (mg)	2.3±0.09	1.6±0.06	1.35±0.05	1.92±0.16	2.2±0.06	1.9±0.01
Shoot Dry Weight (mg)	18.15±0.43	14.3±0.54	11.1±0.06	16.15±0.74	15.95±0.27	15.45±0.26
Seedling Dry Weight (mg)	10.22±0.26	7.95±0.3	6.22±0.05	9.02±0.45	9.07±0.16	8.67±0.13

Table 2. Effect of Cr^{+6} and chelated–Cr^{+6} complexes on chlorophyll (mg/gm fresh weight of leaf tissue) and proline (µg/gm fresh weight of leaf tissue) contents of 5-days old mung bean seedlings grown in hydroponics condition (values are mean ± SEM of three replicates).

Biochemical Parameters	Cr^{+6} Treatments (µM)			Chelated Cromium Complex (10µM)		
	Control	10	100	Cr:EDTA (1:1)	Cr:DTPA (1:1)	Cr:EDDHA (1:1)
Proline	12.0±0.91	16.0±1.30	36.0±2.11	52.0±0.84	43.0±1.96	49.0±1.48
Total Chlorophyll	0.947±0.012	0.486±0.013	0.905±0.024	0.544±0.008	0.209±0.005	0.256±0.010

DISCUSSION

In natural environment, the plants are exposed to various types of stresses. Now-a-days heavy metal stress is considered as one of the emerging type of stresses. This is caused due to various kinds of natural as well as man-made activities (Zayed *et al.* 1998; Zayed and Terry, 2003; Mohanty and Patra, 2011). The metal toxicity caused either by natural soil properties or by other means like agricultural practices, manufacturing, mixing and waste disposal practices creates several environmental hazards (Zayed *et al.* 1998; Zayed and Terry, 2003; Mohanty and Patra, 2011). Toxicity of heavy metals has received considerable attention partly due to its occurrence in nature and by mining activities. The phytotoxic effect of chromium was reported long time back by Koenig (1910) and subsequently by others (Koening, 1911; WHO, 1988; Debatto and Luciani, 1988; Panda and Patra, 1997; Zayed and Terry, 2003; Mohanty *et al.* 2005; Mohanty *et al.* 2008; Mohanty and Patra, 2012). Application of chromium to plants through *in vitro* studies resulted in reduced rate of growth, damage to cell wall and membranes and changes in metabolic status of plants (Mc. Grath, 1982; Barcelo *et al.* 1985; Panda and Patra, 1997; Panda and Patra, 2000; Cervantes *et al.* 2003; Nayak *et al.* 2004, Mohanty *et al.* 2008 Mohanty and Patra, 2012).

The data on growth parameter study showed that, with the increase in Cr concentration, the growth rate decreased progressively. However, in chelated forms of chromium (Cr^{+6}–EDTA, Cr^{+6}–DTPA and Cr^{+6}–EDDHA at 10µM concentrations) an observation of increased growth rate was found. The growth parameters like root length, shoot length, fresh weight and dry weight decreased markedly at higher free Cr^{+6} concentrations (i. e. at 100µM). The deleterious effects of Cr on seedlings growth are closely related to its concentration in the medium, the higher the concentration; the greater the effectiveness. Similar findings were reported earlier by Bonet *et al.* (1991) studied the inhibitory effect of higher chromium concentration on bush bean (*Phaseolus vulgaris* L.) plants which was also confirmed by others (Cervantes *et al.* 2003; Nayak *et al.* 2004, Mohanty *et al.* 2008 Mohanty and Patra, 2012). Hauschild (1993) reported that hexavalent chromium (Cr^{+6}) was more toxic than trivalent (Cr^{+3}) and thereby causing severe disturbances in the biological systems. The problems of Cr contamination and toxicity need further clarification with respect to its effect on growth and physiological changes.

Accumulation of chromium in food crops represents potential health hazards to animal and humans, if it is accumulated and supplied in hexavalent form at higher concentrations (Zayed *et al.* 1998). Higher concentrations of chromium induce toxicity symptoms and suppress plant growth (Barcelo and Poschenrieder *et al.* 1985; Dubey and Pessarakli, 1995; Cervantes *et al.* 2003; Nayak *et al.* 2004, Mohanty *et al.* 2008; Mohanty and Patra, 2012). The toxicity effects on plant growth was visualized with respect to stunted seedling growth, reduced length of roots and shoots at higher Cr concentrations (i.e, at 100 µM). In the present study it has been observed that, chelating agents reduces the toxicity effect of Cr^{+6} to a greater extent. Among the three types of chelated chromium compounds, Cr^{+6} – DTPA (10µM) showed highest growth rate. Further study Cr–bioavailability in roots and shoots using chelated Cr^{+6} compounds may provide some ideas concerning the detoxification problems.

The study was further extended to chlorophyll

and proline analysis. From all the six types of experiments, the control had the maximum chlorophyll content. But exceptionally at higher concentration of Cr^{+6} supply i.e. at Cr^{+6} (100µM), the chlorophyll content of leaves was significantly high. This corroborates the findings of Bonet *et al.* (1991) on Fe-deficient plants of bush bean and where a significant positive correlation was obtained between the Cr concentration supplied and the chlorophyll concentration in primary and first trifoliate leaves of Fe-deficient plants. Chelate-based nutrition was found to ameliorate the toxicity stress by decreasing the catalase and peroxidase activity at lower concentrations. Related findings were observed by other researchers in different plants (Mohanty *et al.* 2008; Mohanty and Patra, 2012). The increased proline biosynthesis in the Cr^{+6} treated seedlings signifies a stress regulating factor that gives protection in plants under heavy metal toxicity environment.

The overall study showed that chelated chromium compounds have effective role in stimulating growth and ameliorating the toxicity effect of chromium. It has also been demonstrated that among all six types of treatment, control showed high chlorophyll content and unusually at higher concentration of Cr (i.e. at 100µM), chlorophyll concentration was also high. This is due to the fact that, the seedlings exhibited stunted growth for which unusual metabolism was noticed. The amelioration of chromium toxicity has been proved from enzymatic study as well. Hence, chelate-based nutrition is of utmost importance in regulating toxicological, physiological and biochemical changes during seedling growth.

AKNOWLEDGEMENTS

The authors are grateful to DST-PURSE support for providing the necessary financial assistance to the Post Graduate Department of Botany, Utkal University, Bhubaneswar.

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