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Impact of chromium toxicity on *in-vitro* growth of *Vigna mungo* (PDM 139)

Abstract

Petri dish culture experiments conducted to study the effect of chromium (Cr^{+6}) on metabolic activities such as pigments, chlorophyll, pheophytin, carotenoid, total protein, catalase, peroxidase and guaiacol peroxidase (GP_x) of black gram (*Vigna mungo* L. PDM139). Seedlings were evaluated on basis of 7 day exposure under different concentration of chromium such as 5,10 & 15mg/L. These concentrations were significantly affected chlorophyll, pheophytin, carotenoid, catalase, peroxidase and guiacol peroxidase activity in seedling of black gram. Study shows significant ($p<0.05$) decrease in total chlorophyll in presence of high level of chromium concentration 5% (1.603 ±0.04), 10% (1.361 ±0.04)& 15% (1.211 ±0.09) as compared to control (1.798 ±0.03). Similar observations also reveal significant ($p<0.05$) decrease in the pheophytin for different Cr^{+6} concentrations 5%(2.290±0.06), 10%(1.963±0.09) & 15%(1.682±0.04). The investigation shows that Catalase, peroxidase & GP_x activity significantly increase in different concentration of chromium as compare to control.Although the level of total pheophytin significantly decrease with reference to elevated dose of chromium concentration.

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Keywords

Chromium, *Vigna mungo*, seedling, pigments

Introduction

Heavy metal pollution is one of the current and most troublesome environmental problems due to mismanagement of effluents coming out from industries that contribute significantly to soil contamination. At elevated levels, these toxic substances may pose considerable loss to human and animal health as well as enormous loss in production. Chromium is one of the important pollutants among other pollutants like cadmium, led, mercury and aluminum. The major cause for the high influx of chromium to the biosphere is the leather industry which accounts for 40% of the total industrial use (Barnhart, 1997), Chromium hexavalent is considered the most toxic form which usually occurs, associated with oxygen as chromate (CrO_4^{-2}) or dichromate ($Cr_2O_7^{-2}$) Chromium trivalent is less toxic, less mobile and is mainly found bound to organic matter in soil and aquatic environments (Bacquer *et al.*, 2003). Until now, methods used for their remediation were not suitable for practical applications due to multiple factors such as high cost, large destruction of soil structure and fertility and low efficiency. Some of these methods were excavation and landfill, thermal treatment, acid leaching and electroclamation. Thus the development of bioremediation strategies for heavy metal contamination is necessary (Cheng *et al.,* 2002).

Environment pollution is a global concern with water pollution as one of the most impactful issue due to its direct use in various purposes. The major sources of water pollution are industrial effluents and the wastewater generated from various industries which is being discharged to the common drainage (or) nearby soil (Lokhande and Vaidya, 2004). Pollution is the greatest threat posed to humanity and even to the whole biosphere. In growing countries like India there is a growing concern for the environmental pollution caused by wastewaters. In most parts of India, the liquid wastes are either discharged into the water courses (or) on to the land causing severe pollution problems. The recycling of wastes can be considered a great boon in combating the pollution problems in the present situation of resource scarcity where important components of wastewater can be recycled in various ways. The use of waste water for irrigation has emerged in the recent past is an important way of utilization of wastewaters taking the advantage of the presence of considerable quantities of calcium, potassium and magnesium along with some other essential elements. The other advantage of wastewater irrigation includes an important aspect of pollutant removal. The pollutants are partly taken up by the plants and transformed in the soil to harmless forms while some is held in the soil without causing any damage. The use of wastewaters for irrigation may be much more beneficial, especially in the arid and semi-arid regions of the world including India. Though, it seems quite promising to use wastewaters for agriculture, it is marred by

several constraints due to various problems like soil salinity, interaction of chemical constituents of the wastes with the uptake of nutrients and changes in soil property and micro flora. More than fifty percent of the tanneries in the country exist in Tamil Nadu. There are about 25 tanneries operating in Tamil Nadu, India. In India leather industry contributes significantly towards exports, employment generation and occupies an important place in Indian economy. The tanning industry is one of the major consumers of water and most of it is discharged as wastewater, which contains high amounts of heavy metals (Sinha *et al*., 2002). In India, leather-tanning industries play a prominent role as it contributes fifteen percent of the total production capacity of the world. These tanneries specialize in processing hides into heavy leather. The enormous pollution load along with the toxic nature of wastewater makes the tanneries a potential threat to the areas in the vicinity of their location. The tannery wastewater is being contaminated with high levels of metals (Fe, Cu, Zn, Mn, and Cr), which when consumed causes serious health hazards. Thus a detailed scientific study becomes a necessity before any specific waste can be used for irrigation (or) a particular crop with specific soil and climate (Arindam, 2001; Singh and Sinha, 2004; Senthil Kumar and Sekar, 1998).

Bioremediation is the use of biological agents like bacteria, fungi and plants to remove (or) degrade the pollutants from the contaminated soil. This technology has appeared to reduce the enormous costs and environmental disturbance that are associated with current clean up polluted soil on agricultural crops before are used for crop cultivation (Panwar *et al*., 2002). Phytoremediation is an emerging technology which uses plants and their associated microbes to remove, degrade the polluted medium. It has been successfully employed for remediation of different kind of contaminants. Tree species play a major role in reclamation of pollutants. Growing of tree seeding is one of the most promising and potentially effective techniques for the removal of pollutants (An *et al.*, 2006 and Sankar Ganesh, 2008). The toxic compounds are trapped into the trunks of such tree species which will remain for a longer time and will not come to the food chain as well. It is an affordable technology that is most useful when the contaminates are with the root zone of plants. Amendments act as vital remediation measures. Primarily the amendment microorganisms are found to be very effective to degrade, the environmental contaminant into less toxic forms. There are number of previous literature available regarding the bioremediation of industrial polluted soil (BentjenIsteve, 2002; Shimp *et al*., 1993). So the present work deals with bioremediation of tannery effluent polluted soil by growing tree species and amendments mixed polluted soil and its response on black gram, *Vigna mungo* L.

The modern world is facing a major environmental crisis in the name of heavy metal pollution of water. The global heavy metal pollution is increasing in the environment due to increase in number of industries. Heavy metals like cadmium, lead, zinc, cobalt and chromium are major components of industrial wastewaters. Among heavy metals, chromium plays a major role in polluting our water environment. The two oxidation states in which Chromium co-exists in the environment are trivalent chromium and hexavalent chromium respectively. The hexavalent chromium is released from various industries such as electroplating, leather tanning, textile printing, textile preservation and metal finishing. Chromium compounds are strong carcinogens and mutagens that can reach the target organs of human through drinking water. Chromium is often mixed with industrial effluents that are used for irrigation. The uptake of excess concentrations of heavy metals reduced the growth of plants (Abdel-Azeem and El-Nahas, 1996; Salem,2000). The effluent irrigation adversely affects plant growth and development (Panda and Choudhury, 2005; Shanker *et al*., 2005; Yongpisanphop *et al*., 2005). This alternation in plant growth is correlated with the disruption of the physiological and cytological processes in plant cells. By this way the toxic effect of heavy metals greatly affect the processes of respiration, photosynthesis and mitotic activity (Ewais, 1997).Other previous studies showed that the germination, growth and yield of plantswere severely affected by high concentration of chromium (Lakshmi and Sundaramoorthy, 2003; Sankar Ganesh et al., 2006; Purohit *et al*., 2003; Chidambaram *et al*., 2006; Sundaramoorthy and Sankar Ganesh, 2007). Very limited research work has been done on the effect of chromium on germinationof implant cells hence this research attempts to deal with the effect of different concentrations of total chromium on the mitotic index and the biochemical changes in blackgram (*Vignamungo*)root tip cells.

Materials and Methods

Present study was designed to study the effects of various concentrations of Chromium effect on various stages of plant growth and development. Various biochemical parameters; (a) Pigments: chlorophyll, pheophytin and carotenoid (b) Biochemical: amylase, total protein etc. (c) Enzymatic: catalase, peroxidase.

Before each experiment all the glasswares viz. petridishes, pipettes, flasks, bottles, test tubes, funnels and beakers etc. were all detergent cleaned first, washed thoroughly with tap water followed with distilled water, sterilized with 70% alcohol and dried well. Seeds of uniform size, weight and colour of aforementioned plants were selected, and surface sterilized with 0.1% $HgCl_2$ for the prevention of surface fungal/bacterial contamination. Twenty seeds were grown on filter paper in each petridish. In each petridish 10ml solution was supplied as described below and the experiment was performed in triplicate. The fresh solutions were applied every alternate day for the prevention of contaminants and to maintain the concentration.

Petri-culture experiments: Petri-dish culture experiments were conducted to study the effect of different levels of Chromium (VI) on seedling growth in black gram. Chromium was given as $K_2Cr_2O_7$ at concentration of 5, 10, and 15 mg/L, and distilled water serve as control. 3 ml of test solution was applied every day for maintain of concentration and prevention of contamination. The nutrient solution was supplied at 3rd day. The seedling growth was carried out at in natural environment the physiology and bio-chemical analysis related to seedling growth and developments were carried out 7th day. The pigments, protein, catalase, peroxidase and GPx activity observed in each sample at the end of experiment respectively.

Nutrient Solution: The composition of nutrient solution for petri-dish experiment was same as given by Hewitt (1966). Macronutrient and micronutrient stock solutions were prepared from analytical

reagents grade salts (AR) except Fe-EDTA solution which was prepared by the method of Jacobson (1951) as described by Hewitt (1966) using ethylene diamine tetra acetic acid, ferrous sulphate and potassium hydroxide. The composition of nutrient solution for growing plants as described by Hewitt (1966) and modified for Indian conditions by Agarwala and Sharma (1976) was as follows:
Composition of nutrient solution:

Nutrient elements	Forms in which supplied	Levels of Supply (mM)
A) Macronutrient		
Potassium	KNO_3	4
Calcium	$Ca(NO_3)_2$	4
Nitrogen	Nitrate of K and Ca	12
Phosphorus	$NaH_2PO_4.24H_2O$	15
Magnesium	$MgSO_4.7H_2O$	2
Sulphur	Sulphate of Mg	2
B) Micronutrients		
Iron	K_2-Fe-EDTA	100
Manganese	$MnSO_4.H_2O$	10
Boron	H_2BO_3	13
Copper	$CuSO_4.5H_2O$	1
Zinc	$ZnSO_4.7H_2O$	1
Sodium and Chloride	Na-Cl	10
Nickel	$NiSO_4.7H_2O$	0.1
Cobalt	$CoSO_4.7H_2O$	0.1

Nutrient solution was supplied to all the growing seedling at every third day and no other treatment was given when nutrient solution was supplied.

Pigments' estimation was done by using the method of Arnon (1949) amended by Lichtenthalce (1987). For the estimation of chlorophyll 100 mg fresh mass in 10 ml acetone (80 %) was homogenized, centrifuged at 5000 rpm at 4ºC for 10 minutes. In one test-tube pure 80% acetone solution was also taken for the reading of blank. The supernatant was observed in spectrophotometer (Chemito UV 2000) at the wavelength 645 nm, 652 nm, 663 nm, 655nm, 510nm and 480nm for estimation of total chlorophyll, chlorophyll a, chlorophyll b, total pheophytin, pheophytin a, pheophytin b and carotenoid respectively. The pigment contents were expressed as mg/gm fresh weight of tissue

chl_a = 12.7(O.D.663) - 2.69(O.D.645) x V/1000 x 1/wt
chl_b = 22.9 (O.D.645) - 4.68(O.D.663)x V/1000 x 1/wt
chl (Total) = 34.5 (O.D. 652) x V/1000 x 1/wt
$pheophytin_a$ = 20.5xO.D 666 - 5.87xO.D655 xV/1000 x1/wt
$pheophytin_b$=31.96XO.D 655 - 13.40xO.D666xV/1000 x1/wt
pheophytin (Total) = 6.75xO.D666+26.03xO.D655 xV/1000 x1/wt
carotenoid (Total) = 7.6XO.D480-1.49xO.D510 xV/1000 x1/wt
V = volume, Wt = weight in gm

Catalase (EC 1.11.1.6) activity was determined by using the method of Euller and Josephson (1927) with recent modifications. The 5 % extract of seedling was used for the estimation of catalase activity in terms of ml H_2O_2 degraded/gm. fresh weight of tissue. For each sample two test tubes were taken, one for catalase activity, second for its blank. In each test tube added 2 ml buffer (citrate phosphate buffer pH 7.0), 2 ml 0.5 % H_2O_2, added 2 ml distilled water and 2 ml enzyme extract incubated for 10 minutes. After that 2 ml 4N H_2SO_4 was added to stop the reaction, but in case of blanks, H_2SO_4 was added before H_2O_2. After ten minutes final volume was titrated against 0.01N $KMnO_4$.

Peroxidase (EC 1.11.1) activity was measured by using the method of Luck (1963). The 2.5 % extract was used for the estimation of peroxidase activity in the terms of ml OD / gm fresh weight of tissue. Using 2 ml citrate phosphate buffer pH 6.0, 1 ml extract and 1 ml 0.5% H_2O_2, incubation for 10 minutes, 2 ml 4N H_2SO_4 was added to stop the reaction, but in case of blanks, H_2SO_4 was added before adding H_2O_2, centrifuged at 40C at 5000 RPM for 10 minute and optical density measured at 485 nm wavelength, with the help of spectrophotometer.

Guaiacol peroxidase (GPx) (EC 1.11.1.7) activity was assayed according to the method of Hemeda and Klein (1990). A 100ml of reaction mixture was prepared by adding 10ml of 1% guaiacol (v/v), 10ml of 0.3% $H_2O_2$and 80ml of 50mM phosphate buffer (pH 6.6). Enzymes extract (75µl) was added tomreaction mixture in a final volume of 3 ml. the increase in absorbance due to oxidation of guaiacol (extinction coefficient 26.6 mM-1 cm-1) was monitored at 470nm. Enzyme activity is expressed as units mg-1 protein (1 unit=µmoles of guaiacol oxidized min-1.

Total Protein contents were estimated by using the method of Lowry et al. (1951). The 2.5% extract of plant tissue was used for protein estimation in terms of mg protein/gm fresh weight of tissue. 100 µl of substrate was taken. Then one ml of reagent 'C' (A mixture of 50 ml 25% Na_2CO_3 in 0.1 NaOH and 1 ml of 0.5% $CuSO_4$ in 1% sodium citrate) was added and incubated for 10 minutes. After that 0.1ml folinciocalteu reagent was added and incubated at room temperature for 30 minutes, and optical density was measured at 750 nm wavelengths, with the help of spectrophotometer.

Protein = O.D. x 158.43mg/gm fresh weight of tissue

Stastistical Analysis: The data observed in the experiments were averaged from three replicates and statistically analyzed for the calculation of standard error (S.E.) and student't' test was administered for testing the hypothesis with the help of computer software sigma stat 2.0. The statistically analyzed graphs were prepared with the help of computer software sigma plot 2001. The data shown are the averages of three replicates ± S.E. and significant (*) at <0.05 level.

Results and Discussion

To explore the early seedling growth some Petri dish culture experiment were conducted using pulse (black gram) to find out the sensitivity of chromium hexavalent.

Pigment concentration: Noteworthy decline in pigment with increased chromium exposure was evident in chlorophyll content (a, b, total) however, carotenoid content were found increased exposed to chromium treatment as compared to control (Table-1).

Enzymatic activities: The significantlymarked increased catalase, peroxidase and GP_X activities was found in seedling on black gram exposure to all concentration of chromium and above that production

Table-1: Effect of Cr (VI) on chlorophyll (a, b & Total), Pheophytin (a, b &Total) and carotenoids on 7th day in green gram (*Vigna mungo* PDM 139)

Treatments	Chlorophyll (mg/gmf.wt.)			Pheophytin			Carotenoids
	a	B	Total	A	B	Total	(mg/gmf.wt.)
Control	1.179±0.01	0.51±0.01	1.798±0.03	1.454±0.12	0.902±0.17	2.426±0.05	0.982±0.00
5 mg/L	1.031±0.01	0.497±0.04	1.603±0.04	1.358 ±0.07	0.746±0.12	2.29±0.06	0.771±0.00
10 mg/L	0.855±0.02	0.453±0.00	1.361±0.04	1.242±0.01	0.596±0.12	1.963±0.09	0.702 ±0.01
15 mg/L	0.802±0.03	0.357±0.07	1.211±0.09	1.097±0.13	0.538±0.05	1.682±0.04	0.672±0.02

Values are mean of three replicates ± SE and (*) statically significant at P <0.05 level

Table-2: Effect of Cr (VI) on antioxidative enzymes and protein content in greengram (*Vignamungo* PDM 139) on7th day.

Treatments (mg/L)	Catalase (ml H_2O_2hydrolysed/gmf.wt.)	Peroxidase ΔO.D/gmf.wt.	GPx (unit mg^{-1} protein)	Protein (µg/mg f.wt.)
Control	54.667 ±3.52	8.487 ±0.37	28.932 ±1.16	170.312 ±6.64
5 mg/L	82.000 ±5.29	9.940 ±0.98	40.080 ±1.61	162.654 ±5.53
10mg/L	122.000 ±13.01	9.773 ±0.61	52.069 ±0.19	146.547 ±2.99
15mg/L	141.333 ±11.85	10.463 ±0.32	55.367 ±3.05	109.316 ±1.64

Values are mean of three replicates ± SE and (*) statically significant at P <0.05 level

of noticeably higher amount of catalase, peroxidase and GP_X levels was observed compared to control (Table-2).

Protein content: A chromium concentration was increased and significant decline was evident it content of total protein in each treatments in seedlings on black gram seedling (Table-2).

In India, many villages use polluted water as a major source for irrigation of crop plants. The present study was carried out to find out the effects of heavy metal-Chromium on seedling growth of green gram (*Vignamungo*). The germination study was conducted in the laboratory to find out the effect of Chromium- a heavy metal on seedling growth, seed germination, fresh weight and dry weight of black gram seedlings.

In the screening experiment, the seeds of green gram (PDM-139) are allowed to germiniate in glass plates lined with filter paper. They were irrigated with different concentrations (C, 5, 10 and 15 mg/L) of heavy metal chromium. The control was irrigated with distilled water. The black gram seeds were screened for tolerance and susceptible varieties for heavy metal chromium. The germination percentage, seedling growth, and dry weight were taken into consideration for screening experiment. The plant basis of data obtained from germination studies, the variety (PDM-139) showed poor performance under heavy metal- chromium treatment. In similar type of varietal screening experiment was carried out in Black gram cultivars under the influence of heavy metal-Lead by (MumtazHussain, 2006), in Black gram under the influence of Zinc and Copper (Dhankar, 2010), in geen gram under the influence of Cobalt (Jayakumar, 2008), in tomato under the influence of mercury (Chandra Sekar, 2011). In the present study of seed germination was found to be high in control (95%) and gradually decreased on the concentration increased. It was high in control of all varieties, and was very low at concentration (40%) the same trend was observed in another study conducted in tomatoes under the influence of heavy metal-mercury which showed 95% of germination in control while it was 20% of germination in high concentration (Chandra Sekar, 2011), chromium- heavy metal treatment on Green gram (Abbasi-2012). Tannery effluent irrigation in Maize also showed seedling growth more in control than the treated plants. The seed germination was significantly reduced due to the presence of heavy metal - chromium in the rooting medium; it might be because of the decreased photosynthetic pigment chromium hexavalent concentration even causes of chromosomal aberratons in roots of black gram (Sundramoorthy, 2009). The reason for reduced seedling growth under heavy metal treatment could be the reductioni in meristamatic cells present in the cotyledons and endosperms, during seedling groowth hydrolysis of food reserves takes place which is carried out by hydrolytic enzymes. So the activities of hydrollytic enzymes might be affected and the food did not reach to the radical and plumule leading to the reduction in seedling growth.

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