Differential Response of Black Gram towards Heavy Metal Stress

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Abstract. Black gram (*Vigna mungo* L. cv Shekhar-2) was grown in refined sand with complete nutrition (control) and at 0.25 mM each of As, Cd, Co, Cr and Pb. Toxicity of heavy metals on biomass, followed the order: As> Cr> Cd> Co> Pb. Plants treated with As could not survive and wilted within 1 week of supply. Pigments and protein in Cr and Co treated plants were significantly reduced as compared to control. Lipid peroxidation, peroxidase and superoxide dismutase was higher in Cr and Co than Cd and Pb treated plants. Results show that black gram is extremely sensitive to arsenic followed by chromium. Cobalt and cadmium are also toxic for growth of *Vigna mungo*, however, Co mainly disturbs the metabolism of the plant. Lead was least toxic for growth of black gram.

Keywords: Black gram, growth, heavy metals, metabolism

1 Introduction

The word ‘‘heavy metals’’ refers to any metallic element having a relatively high density and is toxic even at low doses (Lenntech Water Treatment and Air Purification 2004). Heavy metals like cadmium, cobalt, chromium, lead etc are environmental pollutants of immense significance and their contamination issues are becoming common day by day. Heavy metal toxicity has significant relevance to plants as when grown in metal polluted sites show altered growth and metabolism. Metal contamination severely affects the physiological and biochemical processes in plants (Nagajyoti et al. 2010). Pollution of agricultural soil by heavy metals is now a critical environmental concern because of its potential adverse ecological impact. Such toxic elements are considered as soil pollutants due to their extensive occurrence and their acute and chronic toxic effect on plants growing in such soils. These heavy metals not only accumulate in plant but also enter into the food chain, thus making heavy metal pollution quite significant both from nutritional as well as environmental point of view.

Plants respond to various types of stresses depending upon their genetic makeup. Heavy metals show unconstructive effects on physiological and metabolic processes of plants like photosynthesis, water relations, protein synthesis, nutrient absorption etc (Wani et al. 2012; Agarwala et al. 1995; Upadhya and Panda 2009). High concentrations of these metals cause toxicity in plants, cause reduction in growth and metabolism, and have been shown to generate oxidative stress which usually accompany an increase of reactive oxygen species like hydrogen peroxide, superoxide and hydroxyl radicals (Stohs and Baghi 1995; Pietrini et al. 2003; Milone et al. 2003). Few heavy metals are also known to interrupt the photosynthetic electron transport chain which leads to generation of superoxide radical and singlet oxygen (Asada and Takahashi 1987) and thus enhance the peroxidant status of the cell by reducing the antioxidant glutathione (GSH) pool, activating calcium dependent systems affecting the iron-mediated processes (Pinto et al. 2003). Well documented studies are there showing effect of different heavy metals on plants but reports on impact of various heavy metals on a single plant are very scarce. So, in the present study, an attempt has been made to evaluate the response of plant black gram towards various heavy metals.

2 Materials and Methods

Black gram (*Vigna mungo* L. cv Shekhar-2) plants were grown in re- fined sand in a glass house at an ambient temperature (25°–30°C). Plants were grown in polyethylene containers of 10 L capacity at 0.25 mM concentrations of different heavy metals (As, Cd, Co, Cr and Pb). Arsenic was supplied as sodium arsenate, Cd as cadmium sulphate, Co as cobalt sulfate, Cr as potassium dichromate and Pb as lead nitrate in nutrient solution. The composition of complete nutrient solution was as follows: 4 mM KNO_3; 4 mM $Ca(NO_3)_2$; 2 mM $MgSO_4$; 1.5 mM NaH_2PO_4; 0.1 mM Fe–EDTA; 0.1 mM NaCl; 30 mM H_3BO_3; 1 mM $CuSO_4$; 1mM $ZnSO_4$; 0.2 mM Na_2MoO_4; 0.1mM $CoSO_4$; and 0.1 mM $NiSO_4$(Agarwala and Sharma, 1976). Initially the seedlings were maintained in complete nutrient solution for 21days. After 21days, plants were separated into six lots. One lot was allowed to grow with full nutrient solution to serve as control. The other five lots received 0.25 mM solutions of different heavy metals. The nutrient solution was supplied daily except on Sundays when each pot was flushed with distilled water to remove adsorbed nutrients in accumulated deleterious substances from routine medium. For each treatment 3 replicates were taken. The pots were arranged in randomized block design.

The visible symptoms of heavy metals toxicity on black gram were recorded periodically. Plants were sampled and separated into various parts for dry matter yield and also tissue analysis for iron content at 10, 20 and 30 days of metal supply. The oven dried plant parts were digested in 10:1 nitric:perchloric acids (Piper, 1942) and in clear digests the concentration of iron was estimated by Atomic Absorption Spectrophotometer. In leaves of black gram, the concentration of chlorophylls (a and b), carotenoids, protein, lipid peroxidation, and activities of anti oxidative enzymes were measured at 15 days of heavy metal supply. Total chlorophyll, chlorophyll a and chlorophyll b contents were measured according to Arnon (1949). Chlorophyll contents were expressed in terms of mg chlorophyll present / g fresh weight of tissue. Carotenoid contents were estimated according to method of Duxbury and Yentsch (1956). Lipid peroxidation in terms of malondialdehyde (MDA) was determined to access the membrane damage in black gram plants. For the measurement of lipid peroxidation, TBA (Thiobarbituric acid) test was used to measure MDA level as an end product of lipid peroxidation (Heath and Packer, 1968). Activity of catalase (CAT) enzyme was estimated according to Euler and Josephson (1927). Peroxidase (POD) activity was estimated according to Luck (1965). Superoxide dismutase (SOD) activity was assayed by the method of Beauchamp and Fridovich (1971) by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazoliun (NBT). Protein contents were determined according to Lowry et al. (1951) using bovine serum albumin as a calibration standard. The experiment was conducted in a completely randomized design (CRD) with 3 replications. The data were analyzed by One Way ANOVA using software program Sigmastats 3.5. It was followed by comparison of mean values using Holm Sidak method at $p \leqslant 0.05$.

3 Results

3.1 Visible Symptoms and Growth

The symptoms of arsenic toxicity appeared earliest in arsenic treated black gram with plants showing complete wilting within 2 days of metal supply and plants treated with As failed to survive beyond 9-10 days and collapsed. Visible symptoms of chromium toxicity also appeared very early within 2-3 days as wilting of older leaves. Growth of black gram was almost stopped and old leaves showed induction of senescence. The entire lamina turned yellow together with leaf petiole and stem. Plants could not survive beyond 20-21 days and eventually collapsed. The effect of cadmium supply appeared after 5 days of metal supply as chlorosis of young leaves. Cadmium treated plants managed to survive upto 23-24 days of metal supply after which they wilted.

Black gram didn't show very significant visible symptoms of cobalt and lead toxicity. Plants survived till the end with control. Cobalt treated plants only showed very mild chlorosis of young leaves. Lead treated plants didn't show any visible symptoms. However, very marginal growth reduction was observed at later stages of growth in Pb and Co treated plants as compared to control.

3.2 Biomass

Reduction in total biomass among heavy metal treated plants followed the order: As> Cr> Cd> Co> Pb, where Pb showed least and As showed maximum reduction (table 1).

Table 1: Effect of different heavy metals on biomass of black gram at different stages of growth.

Total dry weight/ plant	Control (C)	Arsenic (As)	Cadmium (Cd)	Cobalt (Co)	Chromium (Cr)	Lead (Pb)
10 DAS	0.41±0.003	0.28±0.002* (–30.2%)	0.44±0.02* (+9.5%)	0.49±0.03* (+22.4%)	0.385±0.026* (–5.2%)	0.57±0.02 (+40.2%)
20 DAS	0.88±0.047	----	0.4±0.115* (– 54.1%)	0.694±0.021* (– 19.9%)	0.27±0.0073* (– 68.79%)	0.93±0.047* (+ 7.8%)
30 DAS	1.525±0.03	----	----	0.97±0.01* (– 36.3%)	----	1.36±0.002* (– 11.11%)

Values are means of 3 replications ± SEM. Values in bracket ( ) show percent increment/decrease over control. *statistically significant difference (P ⩽ 0.001). Multiple comparison Vs control group- (Holm Sidak method) overall significance level 0.05. DAS= Days after metal supply.

3.3 Lipid Peroxidation (LPO)

MDA (malondialdehyde) content was significantly higher in cobalt and chromium treated plants as compared to control. Increase in LPO was in the order: Co> Cr> Pb> Cd as compared to control (fig. 1).

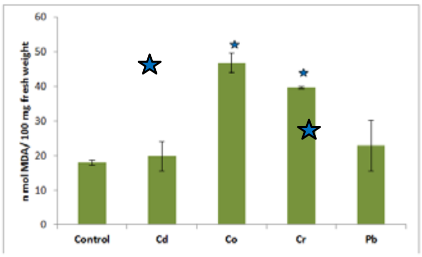

Figure 1. Effect of various heavy metals on status of lipid peroxidation in black gram leaves. Values are means of 3 replications ± SEM. * statistically significant at P ⩽ 0.001 (Holm Sidak method, Comparisons Vs Control group)

3.4 Protein Content

Protein content when analyzed didn't show any drop in Cd treated plants and showed 11.03% increase in Pb treated plants. Reduction of 16.9% and 2.1% was observed in Co and Cr treated plants respectively (fig. 2).

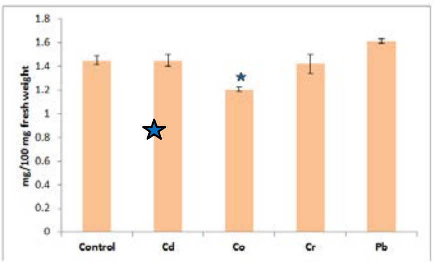

Figure 2. Effect of various heavy metals on protein content of black gram leaves. Values are means of 3 replications ± SEM. * statistically significant at P ⩽ 0.001 (Holm Sidak method, Comparisons Vs Control group)

3.5 Photosynthetic Pigments

Pigment contents showed reduction in mostly all heavy metal treated plants except in Pb treated plants. Drop in total chlorophyll was maximum in Co treated plants (66.43%). Chlorophyll a showed maximum reduction in Cr treated black gram. Chl b was reduced to 70 % in Co treated plants as compared to control. There was 295.2% increase in chl b content in Pb treatment. Carotenoids' content decreased in all but Cd where it showed an increase of 12.77% (table 2).

Table 2: Impact of different heavy metals on photosynthetic pigments of black gram.

Pigments	Control (C)	Cadmium (Cd)	Cobalt (Co)	Chromium (Cr)	Lead (Pb)
Total chlorophyll: mg/g fresh weight	1.233±0.0351	1.133±0.0507 (–8.12%)	0.414±0.0431* (–66.43%)	0.564±0.0176* (–54.26%)	2.955±0.00257* (+139.65%)
Chlorophyll a: mg/g fresh weight	0.627±0.0216	0.548±0.0204 (–12.6%)	0.2305±0.0143* (–63.24%)	0.188±0.0279* (–70.016%)	0.374±0.04* (–40.35%)
Chlorophyll b: mg/g fresh weight	0.608±0.0128	0.585±0.0304 (–3.7%)	0.183±0.0293* (–70%)	0.378±0.0103* (–37.78%)	2.401±0.0615* (+295.2%)
Carotenoids: mg/g fresh weight	0.501±0.00661	0.565±0.0343 (+12.77%)	0.202±0.018* (–59.62%)	0.379±0.0204* (–24.35%)	0.492±0.0291 (–1.796%)
Chlorophyll a : b ratio	1.032±0.000577	0.937±0.000176* (–9.2%)	1.26±0.00577 (+22.1%)	0.497±0.00016* (–51.84%)	0.156±0.000577* (–84.88%)

Values are means of 3 replications ± SEM. Values in bracket ( ) show percent increment/decrease over control. *statistically significant difference ($P \leqslant 0.001$). Multiple comparison Vs control group- (Holm Sidak method) overall significance level 0.05.

3.6 Anti Oxidative Enzymes

The specific activities of anti oxidant enzymes like SOD and POD increased at 0.25 mM each of Cd, Co and Cr. However, Pb treated plants showed reduction in SOD and POD activities. The increase in SOD and POD was maximum in Co treated plants. Catalase decreased in all heavy metal treated black gram plants and the reduction was maximum in Cr treatment (table 3).

Table 3: Status of antioxidative enzymes in black gram in response to different heavy metals.

Anti-oxidative enzymes	Control (C)	Cadmium (Cd)	Cobalt (Co)	Chromium (Cr)	Lead (Pb)
Superoxide dismutase: Enzyme unit(EU)	1.345±0.0779	1.583±0.0549 (+17.5%)	1.6±0.808 (+18.96%)	1.45±0.11 (+7.8%)	1.26±0.0289 (–6.32%)
Peroxidase: ΔOD/mg protein	0.349±0.0159	0.3505±0.017 (+0.43%)	0.400±0.0057* (+14.61%)	0.351±0.000333 (+0.57%)	0.296±0.00404* (–15.2%)
Catalase: μM H_2O_2 /mg protein	496.45±20.756	454.4±26.241 (–8.45%)	407±6.813* (–18.02%)	199.1±11.316* (–59.9%)	415.05±5.193* (–16.4%)

Values are mean of 3 replications ± SEM. Values in bracket ( ) show percent increment/decrease over control. *statistically significant difference (P $\leqslant$ 0.001). Multiple comparison Vs control group- (Holm Sidak method) overall significance level 0.05.

3.7 Iron Content and Its Correlation with Dry Weight

The iron content when estimated in roots, stem, young and old leaves of black gram showed reduction on heavy metal treatment. Fe content and dry weight of plant was found to be positively correlated (fig. 3,4).

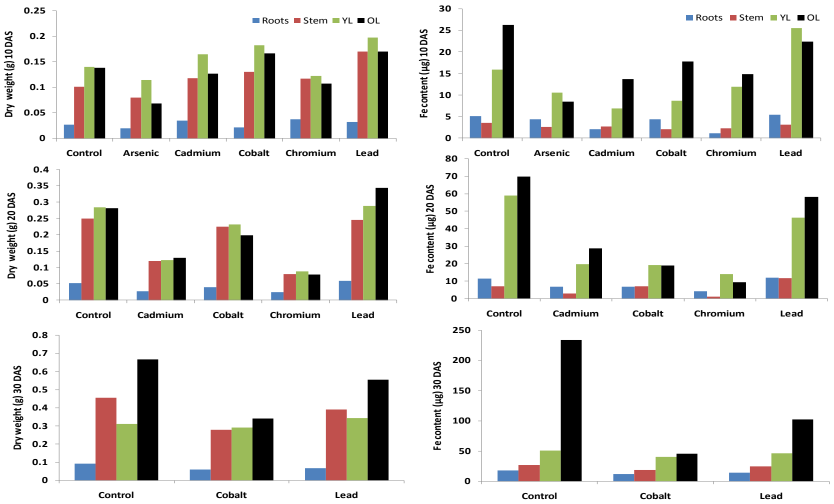

Figure 3. Effect of heavy metals on iron content and dry weight in black gram at different stages of growth.

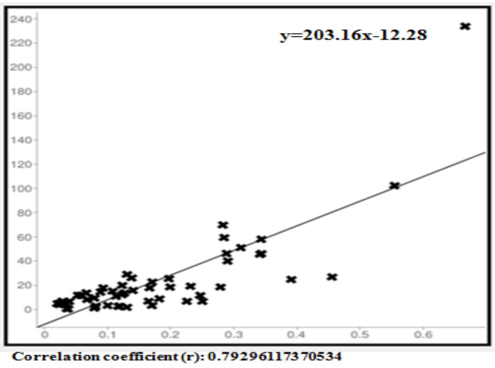

Figure 4. Graph showing correlation between dry weight and iron content in black gram.

4 Discussion

In the present study, growth and metabolism of black gram were affected variably by impact of heavy metals such as As, Cd, Co, Cr and Pb. The metals under study resulted in growth inhibition, and produced characteristic visible effects similar to those described by other workers (Gopal and Khurana 2011; Tewari et al. 2002; Zhou and Qiu 2005; Srivastava and Sharma 2013a,b).

Here, in this study, As treated plants showed greatest depression in growth among various heavy metals tested. Plants could not survive even 10 -11 days after metal supply. Arsenate (As), an analog of phosphate (P), competes for same uptake carriers in the root plasmalemma of plants (Meharg and Macnair 1992) and this is the reason why As is toxic for plant. It replaces P in cells which causes disruption of energy cells in plant. Also, inside the cell, As(V) can be readily converted to As(III), the more toxic of two forms. There are numerous reports, where arsenic has severely inhibited plant growth and reproductive capacity through losses in fertility yield, and fruit production (Garg and Singla 2011). At sufficiently high concentration, As interferes with critical metabolic processes, which can lead to death.

Regarding heavy metal, Cd, it is a known fact that plants grown in high levels of Cd contaminated soil show visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips and death (Sanita di Toppi and Gabbrielli 1999; Wojcik and Tukiendorf 2004; Mohanpuria et al. 2007; Guo et al. 2008). Here also, Cd showed similar results. Cd treated plants showed decrease in their pigment content. Cd is known to produce alterations in the functionality of membranes by inducing lipid peroxidation (Fodor et al. 1995) and disturbances in chloroplast metabolism by inhibiting chlorophyll biosynthesis and reducing the activity of enzymes involved in CO_2 fixation (De Filippis and Ziegler 1993). It is said that, inhibition of root Fe(III) reductase induced by Cd leads to Fe(II) deficiency, here in the present study, Cd treatment resulted in reduction in iron content of the plant. This iron deficiency affects photosynthesis (Alcantara et al. 1994).

As far as cobalt is concerned, plants survived till the end at 0.25 mM Co treatment, probably because this level of Co is not very toxic for growth of black gram. However, at later stages of growth, it restricted the growth of black gram, chlorophyll, protein and catalase activity. This is in agreement with reports on cauliflower (Chatterjee and Chatterjee 2000).

Chromium (Cr) compounds especially $Cr^{6+}$are highly toxic to plants and are detrimental to their growth and development. Chromium stress is one of the important factors that affect photosynthesis in terms of CO_2 fixation, electron transport, photophosphorylation and enzyme activities (Clijsters and Van Assche 1985). Here in the present study, Cr severely affected growth and biomass of black gram. Anti-oxidative enzymes like catalase decreased while SOD and POD increased. There was a marked reduction in chl a than in chl b in Cr treated plants. The more pronounced effect of Cr(VI) on PS I than on PS II activity in isolated chloroplasts has been reported by Bishnoi et al. (1993a, b) in peas. Induction and activation of superoxide dismutase (SOD) and of antioxidant catalase are some of the major metal detoxification mechanisms in plants (Shanker et al. 2003). Gwozdz et al. (1997) found that at lower heavy metal concentrations, activity of antioxidant enzymes increased, whereas at higher concentrations, the SOD activity did not increase further and catalase activity decreased.

Lead (Pb) is one of the most abundant toxic elements in the soil. However, in black gram, it didn't show much detrimental effects. This is in accordance with reports on alfa alfa where there were no visual symptoms of lead toxicity in plants exposed to 100 mg/mL Pb (Porter and Cheridan 1981). Here, in the study, Pb treated plants showed increase in their protein content as compared to control. Also, biomass of plants was higher except at later stages of growth. Chlorophyll a showed decrease in Pb treated plants whereas chlorophyll b showed tremendous increment. This may be due to as PSI is more sensitive towards Pb toxicity. Also, anti oxidative enzymes were not expressed very much in Pb treated plants. All this may be contributed to the fact that Pb precipitates in roots of plants and is not translocated to upper parts in shoots. Thus, plants doesn't experience the toxicity of Pb to a great extent and thus, do not show any visible signs of injury.

5 Conclusion

Among the heavy metals tested, arsenic was most detrimental for growth and metabolism of black gram. Pb didn't show any appreciable damage to plants. Among Co, Cr, and Cd, Cr mainly affected growth of plants whereas Co and Cd made significant damage to the metabolism of *Vigna mungo*. Overall, toxicity impact of heavy metals in black gram followed the order: As> Cr> Cd> Co> Pb, Pb being least toxic for black gram.

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