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Heavy metal induced physiological and biochemical responses in a temperate legume crop, *Vigna umbellata* T.

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ABSTRACT

It has been revealed in the present study that the rate of germination, root length and shoot length and percentage of normal seedlings of *Vigna umbellata* T. were inversely proportional to the magnitude of heavy metal stress caused by Cd, Pb and Zn. There was a progressive reduction in the level of protein, insoluble carbohydrate, α-amylase and protease activities. However, the studies showed an increase in the levels of soluble carbohydrate and free amino acids in the seed leachate indicating impairment of biosynthetic activities and membrane damage. The cv. Haday of *V. umbellata* was found to be more tolerant to heavy metal stress than the cv. Thangray.

Legumes are one of the richest sources of vegetable protein and form an important component of stable diet all over the world. Rice bean (*Vigna umbellata* T.) is a less known legume and has recently been identified as a suitable addition to the presently existing list of pulses[17]. This plant is used all over the temperate Himalayas as an important crop offering high quality of protein and increasing the input of combined N_2 into the soil. These crops have traditionally been used for rotation with cereal crops in ordet to increase the soil fertility.

Heavy metal toxicity causes multiple direct and indirect effects on plant growth and alters many physiological functions[28]. Heavy metal plays a vital role in the growth and development of plants and may act as cofactors of some enzymes of help in the formation of intermediate metabolites[2]. Rise in the levels of heavy metals in the soil could be attributed to many factors like agricultural practices, soil properties, waste disposal and industrial sewage to agricultural land etc[8]. In view of the present global scenario a complete eradication of heavy metal pollution has become an unrealistic goal. Understanding the responses of plants to their external environment is an attractive targer for improving stress tolerance[21]. Heavy metals cause many deleterious effects to plants such as inhibition of seed germination[23], reduction in plant growth[4] and metabolic disturbances by altering essential biochemical reactions[15].

Zinc is an essential plant nutrient and is involved in a multitude of functions. Zn catalyses diverse chemical reactions affecting

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varied aspects of metabolism[19]. Cadmium is a non essential toxic element that enters the environment through various industrial processes[26]. Cadmium is a wide spread trace pollutant of high toxicity with a long biological half life[10]. Lead, with its increasing concentrations causes reduction in the levels of RNA, DNA and protein with concomitant increase in amino acid content in rice embryo[18].

Systematic studies on the role of heavy metals on physiological and biochemical responses on *Vigna umbellata* under stress is lacking. An understanding of the physiological mechanisms and identification of specific physiological traits conferring heavy metal tolerance could play a major role in development of new varieties for such stress tolerance. Therefore, the present investigation was aimed at understanding the physiological and biochemical responses of two promising rice bean cultivars, *Vigna umbellata* T. cv. Haday and cv. Thangray in order to assess their heavy metal tolerance potentials.

Freshly harvested seeds of *Vigna umbellata* T. cv. *Haday* and cv. *Thangray* were obtained from the Department of Horticulture, Government of Sikkim, Gangtok, Sikkim. The seeds were surface sterilized with 0.1% $HgCl_2$ for 2 minutes and washed for 10 minutes in distilled water (3 times). The washed seeds were allowed to germinate in Petri plates on filter papers soaked separately in the aqueous solution of $PbCl_2$ (Pb 10,100,500 and 1000 μM), $CdCl_2$ (Cd 10,100,500 and 1000 μM) and $ZnCl_2$ (Zn 10,100,500 and 1000 μm). A control set was prepared by soaking the filter paper in distilled water. For each treatment 50 seeds were taken. Each seed lots were irrigated with the pretreating solution after each 36 hrs till the experiment was over. During the entire period of the experiment the environmental conditions were 88 ± 2% relative humidity (RH), 18 ± 2°C temperature and a photoperiod of 10 hrs at an altitude of 2268 M amsl. During the experiment the physiological, biochemical and growth analysis were made after 3,7 and 10 days.

For the analysis of germination data the seed lots (50 each) were allowed to germinate for 7 days as per the international rules of seed testing[12]. Growth behaviours of the two cultivars of rice bean were analyzed in terms of the percentage normal seedlings as well as root length and shoot length was calculated from twenty seedlings of each treatment and the data were recorded from 10 day old uniformly grown seedlings[3].

Samples of protein estimated were take from dehusked seeds. Extraction of protei was made following the method of Kar an Mishra[13] and the estimation was done usir Folin-ciocalteau reagent[16,24].

The seed leachate obtained by imm sing 5 g of seed lots of each treatment 25ml of deionised distilled water for 16 at room temperature were used for mea ring the levels of soluble carbohydrate free amino acids. For the analysis of solu carbohydrate 1 ml of the seed leachate added to 3 ml of anthrone reagent in condition and kept in hot water batl 5 minutes, cooled down to room tem ture and the estimation was done a McCready *et al.*[20]. For analyzing the amino acid content 2 ml of seed lea

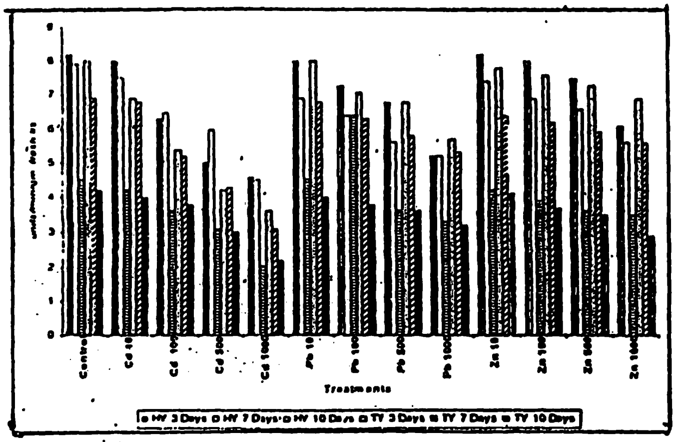

Fig. 1. Effect of heavy metal treatment with $CdCl_2$(Cd 10, 100, 500 and 1000 µM), $PbCl_2$(Pb 10, 100, 500 and 1000 µM) and $ZnCl_2$(Zn 10, 100, 500 and 1000 µM) on the protease activity (units/min./g of fresh tissue) of *Vigna umbellata* cv. Haday (HY) and cv. Thangray (TY) under laboratory conditions.

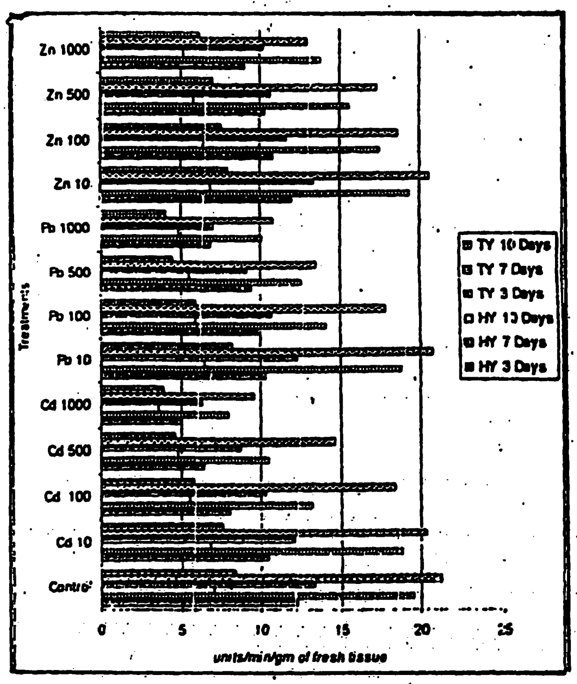

Fig. 2. Effect of heavy metal treatment with $CdCl_2$(Cd 10, 100, 500 and 1000 µM), $PbCl_2$(Pb 10, 100, 500 and 1000 µM) and $ZnCl_2$(Zn 10, 100, 500 and 1000 µM) on the α– amylase activity (units/min./g of fresh tissue) of *Vigna umbellata* cv. Haday (HY) and cv. Thangray (TY) under laboratory conditions.

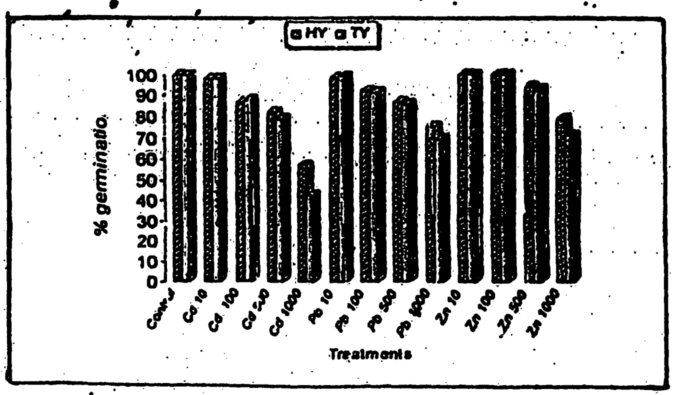

Fig. 3. Effect of heavy metal treatment with $CdCl_2$(Cd 10, 100, 500 and 1000 µM), $PbCl_2$(Pb 10, 100, 500 and 1000 µM) and $ZnCl_2$(Zn 10, 100, 500 and 1000 µM) on the seed germination percentage of *Vigna umbellata* cv. Haday (HY) and cv. Thangray (TY) under laboratory conditions.

Table 1. Effect of heavy metal treatment with $CdCl_2$ (Cd 10, 100, 500 and 1000 μM), $PbCl_2$ (Pb 10, 100, 500 and 1000 μM) and $ZnCl_2$ (Zn 10, 100, 500 and 1000 μM) on the protein (mg/g) of *Vigna umbellata* cv. *Haday* and cv. *Thangray* seeds under laboratory conditions.

Treatments	*Vigna umbellata* cv. *Haday* Days after treatment			*Vigna umbellata* cv. *Thangray* Days after treatment		
	3	7	10	3	7	10
Control	80.01±1.8	69.2±1.50	44.5±0.15	84.0±1.20	78.2±1.80	49.0±0.20
Cd 10	80.0±0.20	99.0±2.70	26.55±0.05	87.9±0.85	98.4±2.40	15.2±0.15
Cd 100	89.25±0.75	98.92±0.60	25.55±0.25	95.7±2.70	98.0±0.29	19.05±0.10
Cd 500	72.5±0.20	89.4±0.90	23.6±0.50	92.6±3.20	99.05±0.89	27.21±1.05
Cd 1000	80.01±2.70	83.7±2.10	16.8±0.18	94.2±1.90	96.3±0.30	2.31±0.90
Pb 10	77.0±0.20	99.05±0.55	21.0±0.10	99.0±0.01	97.0±0.13	18.8±0.80
Pb 100	77.5±2.30	96.00±5.10	19.8±0.80	86.2±0.60	98.0±0.12	16.5±0.25
Pb 500	95.28±2.70	99.05±0.55	18.1±0.10	89.07±0.75	90.08±0.20	18.2±0.20
Pb 1000	87.00±3.1	95.5±3.70	18.2±0.20	91.5±0.50	92.0±0.60	17.1±0.29
Zn 10	82.2±0.60	93.9±0.50	21.8±0.70	97.6±0.4	99.0±0.20	21.2±0.30
Zn 100	95.11±5.10	98.0±2.10	20.7±0.40	92.9±0.5	98.0±2.10	18.2±0.30
Zn 500	76.2±0.60	99.26±2.50	17.3±0.15	89.4±3.00	99.9±3.70	17.2±0.05
Zn 1000	77.4±0.60	99.92±0.80	20.5±0.45	97.2±3.60	99.9±4.10	13.4±0.10

was added with 2 ml of 1% ninhydrin solution and boiled for 20 minutes in a water bath placing glass marbles at the top of the test tubes and the absorbance was measured at 570 nm[22].

The extraction for protease activity was done by homogenizing 200mg of tissue in 5ml of chilled 0.1M Na-phosphate buffer (pH 6.5) and centrifugation of the homogenate for 10 minutes at 10000 rpm. The supernatant was used as the enzyme source. The assay mixture contained 1 ml of the enzyme extract, 0.1 ml of $MgSO_4 . 7H_2O$ (0.1 M) and 1 ml of BSA (0.5 mg/ml)[25]. To estimate the α- amylase activity 300 mg of seed tissue was homogenized with 10 ml. of pre-chilled Na-phosphate buffer (0.1 M). The supernatant was taken as the enzyme source after centrifugation at 5000 rpm for 10 minutes. The assay mixture consisted of 0.5 ml of enzyme extract and 0.1 ml starch solution and the reaction was stopped by adding 3 ml of I_2-HCl solution (60 mg KI and 16 mg I_2 dissolved in 0.5 N HCl) after 10 minutes of incubation at 37°C. The colour developed was measured at 620 nm[14]. The activity of these enzymes was determined according to Fick and Qualset[7].

Table 2. Effect of heavy metal treatment with $CdCl_2$ (Cd 10, 100, 500 and 1000 μM), $PbCl_2$ (Pb 10, 100, 500 and 1000 μM) and $ZnCl_2$ (Zn 10, 100, 500 and 1000 μN) on the leaching of soluble carbohydrate (mg/g/25 ml) of *Vigna umbellata* cv. *Haday* and cv. *Thangray* seeds under laboratory conditions.

Treatments	*Vigna umbellata* cv. *Haday* Days after treatment		*Vigna umbellata* cv. *Thangray* Days after treatment	
	7	10	7	10
Control	2.7±0.3	8.08±0.4	4.6±0.3	9.7±1.00
Cd 10	4.15±0.15	10.95±0.15	5.35±0.05	11.0±0.1
Cd 100	7.25±0.15	14.95±0.75	7.6±1.00	14.025±0.075
Cd 500	8.05±0.05	17.7±0.5	9.6±0.1	15.2±0.4
Cd 1000	12.3±0.1	20.55±0.5	13.35±1.05	19.55±0.65
Pb 10	3.35±0.05	9.1±0.4	5.45±0.05	10.3±0.3
Pb 100	4.25±0.15	11.5±1.1	8.475±0.025	14.7±0.4
Pb 500	6.7±0.15	13.35±0.45	11.65±0 05	18.3±1.8
Pb 1000	9.4±0.7	14.35±0.95	12.55±0.45	22.0±0.2
Zn 10	2.9±0.1	9.25±0.85	4.9±.1	9.9±0.1
Zn 100	4.6±0.1	10.4±1.00	6.45±0.35	12.95±0.25
Zn 500	6.65±.25	12.6±0.4	7.00±0.1	14.6±0.6
Zn 1000	8.05±0.15	13.2±0.1	10.5±0.45	16.3±0.2

Of the three chemicals $PbCl_2$ and $CdCl_2$ caused gradual reduction in the percentage of germination with its increasing concentration. The rate of decline was particularly higher in $CdCl_2$ treated seeds than those in $PbCl_2$. When the concentration of these two metals was increased from 10 μM to 500 μM the decreasing trend in the rate of germinations was gradual, however, at 1000 μM the decline was rather abrupt (Fig. 3). Heavy metals at higher concentration inhibited seed germination[1]. Ion toxicity which is implicated in the inhibition of protein activity, changes in cellular permeability and direct toxicity to the embryo and seedling is most probably responsible for the reduction in the rate of germination[6]. However, the toxic effects of Zn were not visible at the concentrations used in this experiment except at 1000 μM where it showed some inhibition (Fig. 3.). May be Zn being an essential micronutrient, the deleterious effects are not pronounced at the relatively lower concentrations. Of the two cultivars of the rice bean the cv. Thangray is more vulnerable to heavy metal toxicity. Seedlings abnormality occurred as a result of treatments with the heavy metals. The abnormality

Table 3. Effect of heavy metal treatment with $CdCl_2$ (Cd 10, 100, 500 and 1000 μM), $PbCl_2$ (Pb 10, 100, 500 and 1000 μM) and $ZnCl_2$ (Zn 10, 100, 500 and 1000 μM) on the leaching of free amino acids (mg/g/25 ml) of *Vigna umbellata* cv. *Thangray* seeds under laboratory conditions. (Values are mean ± SE of 3 replicates).

Treatments	*Vigna umbellata* cv. *Haday* Days after treatment		*Vigna umbellata* cv. *Thangray* Days after treatment	
	7	10	7	10
Control	3.0±0.4	8.4±0.90	5.15±0.85	8.25±1.05
Cd 10	3.15±0.05	10.35±1.35	11.7±0.10	19.25±0.75
Cd 100	6.15±1.75	11.25±0.45	12.65±0.15	21.96±0.915
Cd 500	11.25±.85	16.85±0.65	14.1±0.40	28.9±1.25
Cd 1000	14.75±0.25	23.4±1.50	18.05±0.75	37.25±0.75
Pb 10	9.3±0.1	14.3±1.30	10.1±0.20	16.4±1.50
Pb 100	10.05±0.15	16.2±2.75	12.2±0.81	17.0±1.00
Pb 500	14.8±1.20	24.4±0.30	15.25±0.45	19.75±2.05
Pb 1000	15.4±0.30	26.2±1.50	17.1±1.70	26.5±0.90
Zn 10	8.85±0.15	11.9±2.90	7.9±0.10	15.9±0.90
Zn 100	9.6±0.30	15.8±0.70	8.5±0.70	16.2±0.09
Zn 500	11.7±0.30	16.2±0.95	10.85±0.15	17.2±0.21
Zn 1000	12.7±0.10	20.25±1.25	14.75±0.05	24.21±2.25

was most pronounced in the Pb treated seeds than with those treated with Cd and Zn (Table 4).

Considerable increase in the leaching of soluble carbohydrate was seen in all the seed lots. Following the trend of the electrical conductivity a short exposure to low doses of heavy metals checked the leaching of these compounds to a large extent. Only the data after 7 and 10 days of exposure to heavy metal treatment is presented in this study since in a shorter exposure the variation of data was insignificant to that of the control (Table 2). Data presented in this study revealed that cotinuous treatment with heavy metal caused significant membrane damage as evidenced by increaced amount of free amino acid (Table 3) and soluble carbohydrate in both the cultivars of rice beans. However, each cultivar showed a different sensitivity towards the heavy metals used. Membrane deterioration under the influences of different abiotic stresses is often related to membrane lipid peroxidation caused by free radicals[27]. The membrane deterioration was directly proportional to the extent of heavy metal stress imposed in terms of both increasing concentration of

Table 4. Effect of heavy metal treatment with $CdCl_2$ (Cd 10, 100, 500 and 1000 μM), $PbCl_2$ (Pb 10, 100, 500 and 1000 μM) and $ZnCl_2$ (Zn 10, 100, 500 and 1000 μM) on the root length, shoot length and percentage of normal seedlings of *Vigna umbellata* cv. *Haday* and cv. *Thangray* under laboratory conditions.
(Values are mean of 10 seedlings after 10 days of germination)

Treatments	*Vigna umbellata* cv. *Haday*			*Vigna umbellata* cv. *Thangray*		
	Root length	Shoot length	% Normal seedlings	Root length	Shoot length	% Normal seedlings
Control	29.7	31.7	100	25.7	31.3	100
Cd 10	24.1	24.5	90	21.5	28.6	90
Cd 100	11.3	20.8	80	11.8	24.3	90
Cd 500	5.9	4.4	40	7.9	7.6	70
Cd 1000	3.0	1.2	40	4.1	2.4	60
Pb 10	27.2	27.9	80	24.8	30.4	100
Pb 100	26.2	26.6	80	21.4	25.7	90
Pb 500	20.8	21.8	80	19.8	19.0	90
Pb 1000	6.0	5.8	50	6.6	4.6	30
Zn 10	29.3	30.6	90	25.1	28.2	100
Zn 100	25.2	24.4	90	24.5	24.0	90
Zn 500	24.0	21.1	90	22.5	22.8	80
Zn 1000	20.6	19.8	90	18.8	17.7	70

the heavy metals and increased duration of treatment.

There was a slight increase in the total protein content during the first 7 days of treatment. Subsequently the protein level declined drastically in the seeds treated for 10 days with heavy metal (Table 1). The cultiver *Thangray* seemed more prone to protein depletion over the 10 day period. At first the increase in the level of protein may be explained by assuming the fact that the protein levels rose initially because the tissue produced various types of protein to fight against the odds of stress condition[5]. Subsequently, when the heavy metal toxicity crossed the threshold the protein level was brought down. It might be due to the break down of protein synthesis mechanism at toxic concentrations of heavy metals due to some protein degrading mechanism[9] or due to reduced incorporation of free amino acids into protein[11].

The activity of protease steadily decreased with the concentration and duration of heavy metal treatment. Though it may be expected that the activity of protease will be higher for degrading the protein, however, at an increasing magnitude of heavy metal stress most possibly the disruption of the enzyme nature of protease takes place causing the decline in its activity. The rate of protease decline was found to be slower in cv. *Haday* than the cv. *Thangray*. (Fig. 1). The activity of α-amylase showed a gradual increase at the initial phases. Up to 7 days of the heavy metal treatment the increasing activity was consistent which was probably responsible for increase in the soluble sugar level. However, beyond 7 days the enzyme activity showed a sharp decline as the toxicity disrupted its activity (Fig. 2).

The growth pattern of heavy metal treated seedlings showed a reduction in the root length and shoot length. The growth of the root and shoot length was inversely proportional to the concentration of the heavy metals used. A concentration of 1000 μM solution of heavy metals had the most devastating effects on the growth of the legume. Cd was found to be the most inhibitory and Zn the least inhibitory to the growth of the rice bean *Vigna umbellata* plants (Table 4).

The study presented here showed that heavy metal treatment of *Vigna umbellata* seeds rapidly caused the disruption of the physiological and biochemical activities even in a short spell of 10 days. The results indicated that the chemicals caused damages in the membrane structure and consequently resulted in the disruption of all physico-chemical activities probably by the toxic effects to the functional proteins. The data presented also revealed that the rise in the contents of soluble carbohydrates and free amino acids was caused by the disruption of biological membrane. Similarly, the reduction in the rate of α-amylase and protease activities proved that the enzyme nature was lost by the toxic effects of the heavy metals Cd, Pb and Zn. Of these Cd had the most drastic and Zn had the least damaging effects to the biological parameters studied. Both the cultivars *Haday* and *Thangray* showed similar type of responses, however, it differed in magnitude with cv. *Haday* being more tolerant to the heavy metal stress.

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