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Physiological and Biochemical Responses of Two Rice Bean (*Vigna umbellata* T.) Cultivars to Heavy Metal Stress

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Abstract

The physiological and biochemical responses of two cultivars of *Vigna umbellata* T. viz. Haday and Thangray under the influence of three heavy metals Cd, Pb and Zn have been studied. Heavy metal stress severely jeopardized the vigor of the seeds as evidenced by reduced germ inability, photosynthetic pigments, peroxidase activity and the content of macromolecules. Of the three chemicals used the effect of Cd was found to be most deleterious and the cv Thangray was more susceptible to such effects.

Key words : **Heavy metals, *Vigna umbellata*, Ion toxicity, Dehydrogenase, TTC stainability.**

Heavy metal toxicity causes multiple direct and indirect effects on plant growth and alters many physiological functions (1). 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 (3). 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 target for improving stress tolerance (4). Heavy metals cause many deleterious effects to plants such as inhibition of seed germination (5), reduction in plant growth (6) and metabolic disturbances by altering essential biochemical reactions (7). Zinc in an essential plant nutrient and is involved in a multitude of functions. Zn catalyses diverse chemical reactions affecting varied aspects of metabolism (8). Cadmium is a non-essential toxic element that enters the environment through various industrial processes (9). 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 (11).

Legumes are important as it is not only one of the richest sources of vegetable protein but also increases soil fertility by fixing atmospheric N_2 into the soil. Rice bean (*Vigna umbellata* T.) is a less known legume and has recently been identified as a new addition to the existing list of pulses (12). This plant is used all over the Himalayas as an important crop offering high quality of protein.

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 physiological and biochemical responses of two promising rice bean cultivars, *Vigna umbellata* T. cv *Haday* and cv *Thangray* to assess their heavy metal tolerance potentials.

Methods

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 (three times). The washed seeds were allowed to

germinate in petri plates on filter papers soaked separately in the aqueous solution on $PbCl_2$ (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 lot was irrigated with the pretreating solution after every 36 hours till the experiment was over. During the entire period of the experiment the environmental conditions were relative humidity 88±2% (RH), temperature 18±2 C, and a photoperiod of 10 hours at an altitude of 2168 m amsl. During the experiment the physiological and biochemical analyses were made after 3, 7 and 10 days.

For the analysis of germination data the seed lots (50 each) were allowed to germinate in petridishes and the time for 50% germination of seeds (T_{50}) was determined for each treatment and the control following the method described by Coolbear et al (13). Samples of protein estimation were taken from dehusked seeds. Extraction of protein was made following the method of Kar and Mishra (14) and the estimation was done using Folin-ciocalteau reagent (15) as modified by Peterson (16).

Photosynthetic pigments (chlorophyll-*a*, *b* and carotenoids) were extracted and estimated from the 10-day old seedlings raised from the control and heavy metal treated seeds. For the estimation of photosynthetic pigment 50 mg of leaf tissue was homogenized with 5 ml of 96% ethanol and centrifuged for 10 minutes at 5,000 rpm. The estimate was done by following the method of Lichtenthaler (17).

Extraction of nucleic acid was made from seed kernels following the method described by Cherry (18) and the quantitative estimation of the same was done following the method of Markham (19) as modified by Choudhury and Chatterjee (20).

To analyzed electrical, conductivity 5 g of seed lots of each treatment were immersed in 25 ml of deionized distilled water for 16 hours at room temperature. Leakage of electrolytes was then measured from the pooled seed leachate of the control and treated samples by a direct reading conductivity meter (21).

For the estimation of peroxidase activity 100 mg tissue was homogenized with 5 ml of 300 µm Na-phosphate buffer (pH 6.8) and centrifuged for 10 minutes at 1000 rpm. The supernatant was used as the enzyme source and the enzyme activity was estimated following the method of Kar and Mishra (14). The activity of all the enzymes was determined according to Fick and Qualiset (22).

Results and Discussion

The rates of germination (T_{50}) were found to be affected with different heavy metal treatments. The effect was found to be detrimental to the rate of germination and lengthens the period of T_{50} with its increasing concentration. The effect of $CdCl_2$ at 1,000 µM was found to be most detrimental. When the concentrations of these metals were increased from 10 to 500 µM the increasing trend in the time taken for germinations was gradual, however, at 1,000 µM the increase was rather abrupt (Fig. 1). Heavy metals at higher concentration inhibited seed germination (3). Ion toxicity which is implicated in the inhibition of protein activity, changes in cellular permeability

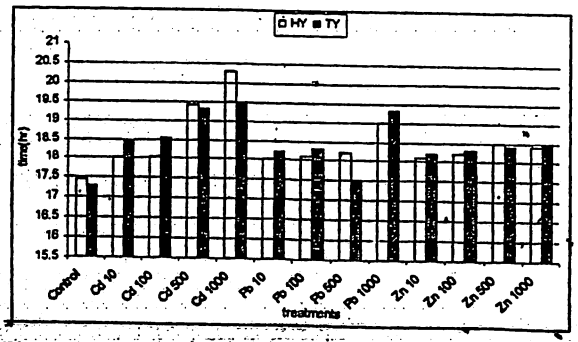

Figure 1. Effect of heavy metal treatment wit $CdCl_2$ (Cd 10, 100, 500 and 1,000 µM), $PbCl_2$ (Pb 10, 100, 500 and 1,000 µM) and $ZnCl_2$ (Zn 10, 100, 500 and 1,000 µM) on time (hour) to 50% seed germination (T_{50}) of *Vigna umbellata* cv *Haday* (HY) and cv *Thangray* (TY) under laboratory conditions.

Table 1. Effect of heavy metal treatment with $CdCl_2$ (Cd 10, 100, 500 and 1,000 µM), $PbCl_2$ (Pb 10, 100, 500 and 1,000 µM) and $ZnCl_2$ (Zn 10, 100, 500 and 1,000 µM) on the protein content (mg/g) of *Vigna umbellata* cv *Haday* and cv *Thangray* seeds under laboratory conditions. Values are mean ± SE of three replicates.

Treatment	*Vigna umbellata* cv *Haday* Days after treatment			*Vigna umbellata* cv *Thangray* Days after treatment		
	3	7	10	3	7	10
Control	80.01±1.80	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	22.31±0.90
Pb10	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	89.7±0.75	90.08±0.20	18.2±0.20
Pb 1000	87.00±3.1	95.5±3.70	17.2±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±0.30	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±0.65	97.2±3.60	99.9±4.10	13.4±0.10

and direct toxicity to the embryo and seedling is most probably responsible for the reduction in the rate of germination (24). However, the toxic effects of Zn were not visible at the concentration used in this experiment except at 1,000 µM where is showed some inhibition (Fig. 1). 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.

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 metals (Table 1). The cultivar *Thangray* seemed more prone to protein depletion over the 10-day period.

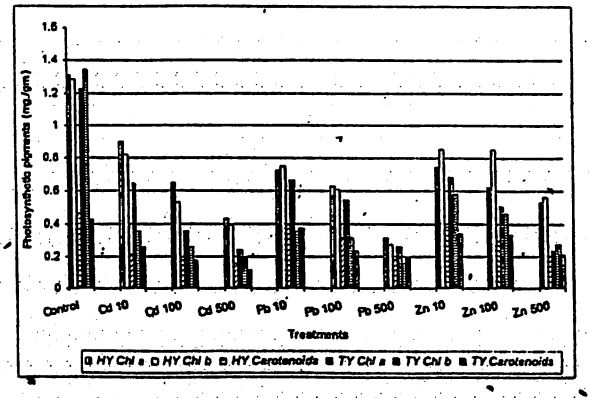

Figure 2. Effect of heavy metal treatment wit $CdCl_2$ (Cd 10, 100, 500 and 1,000 µM), $PbCl_2$ (Pb 10, 100, 500 and 1,000 µM) and $ZnCl_2$ (Zn 10, 100, 500 and 1,000 µM) on the photosynthetic pigments (mg/g of fresh tissue) of *Vigna umbellata* cv *Haday* (HY) and cv *Thangray* (TY) under laboratory conditions. Values are from 10 day old seedlings.

Table 2. Effect of heavy metal treatment with $CdCl_2$ (Cd 100 and 1,000 µM), $PbCl_2$ (Pb 100 and 1,000 µM) and $ZnCl_2$ (Zn 100 and 1,000 µM) on the DNA and RNA contents (mg/g) of *Vigna umbellata* cv *Haday* and cv *Thangray* seeds under laboratory conditions Values are mean ± SE of three replicates measured fron 10 day old seedlings.

Treatment	*Vigna umbellata* cv *Haday* DNA	RNA	*Vigna umbellata* cv *Thangray* DNA	RNA
Control	0.45±0.15	1.74±0.37	0.41±0.02	0.83±0.01
Cd 100	0.38±0.02	1.05±0.08	0.34±0.11	0.63±0.01
Cd 1000	0.130±0.10	0.88±0.21	0.15±0.10	0.42±0.04
Pb 100	0.37±0.75	1.19±0.008	0.31±0.02	0.71±0.19
Pb 1000	0.25±0.05	0.71±0.40	0.22±0.002	0.53±0.12
Zn 100	0.37±0.05	0.72±0.23	0.41±0.005	0.60±0.02
Zn 1000	0.32±0.02	0.59±0.08	0.39±0.04	0.42±0.12

Table 3. Effect of heavy metal treatment with $CdCl_2$ (Cd 10, 100, 500 and 1,000 µM), $PbCl_2$ (Pb 10, 100, 500 and 1,000 µM) and $ZnCl_2$ (Zn 10, 100, 500 and 1,000 µM) on the peroxidase activity (units/min/g of fresh tissue) of *Vigna umbellata* cv *Haday* and cv *Thangray* seeds under laboratory conditions. Values are mean ± SE of three replicates.

Treatment	*Vigna umbellata* cv *Haday* Days after treatment			*Vigna umbellata* cv *Thangray* Days after treatment		
	3	7	10	3	7	10
Control	28.95±0.004	19.2±0.05	14.25±0.04	27.0±0.03	18.5±0.10	13.7±0.02
Cd 10	27.3±0.02	14.3±0.05	12.99±0.05	25.5±0.23	18.35±0.02	13.25±0.000
Cd 100	24.1±0.009	12.35±0.05	10.47±0.08	23.25±0.05	15.8±0.10	9.74±0.001
Cd 500	20.14±0.03	11.55±0.05	8.40±0.001	20.9±0.09	13.32±0.09	5.75±0.008
Cd 1000	17.1±0.14	8.43±0.06	6.81±0.02	16.15±0.05	8.33±0.03	4.35±0.09
Pb10	28.4±0.1	17.1±0.001	12.75±0.03	24.8±0.13	17.85±0.03	12.9±0.10
Pb 100	28.3±0.21	16.8±0.004	10.5±0.03	22.6±0.07	17.23±0.11	11.65±0.10
Pb 500	24.7±0.08	15.2±0.03	8.53±0.08	23.25±0.07	15.5±0.03	9.23±0.07
Pb 1000	22.8±0.05	12.33±0.05	8.33±0.06	19.6±0.18	11.6±0.02	7.87±0.09
Zn 10	27.93±0.18	18.0±0.06	14.35±0.03	26.5±0.000	12.25±0.04	13.65±0.04
Zn 100	27.78±0.10	16.8±0.05	12.2±0.003	24.6±0.03	17.9±0.02	12.55±0.03
Zn 500	24.15±0.13	15.2±0.48	11.58±0.08	21.5±0.05	16.8±0.05	10.85±0.03
Zn 1000	23.05±0.11	13.55±0.11	10.65±0.006	18.65±0.07	15.45±0.01	8.88±0.15

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 (25). 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 (26) or due to reduced incorporation of free amino acids into protein (27).

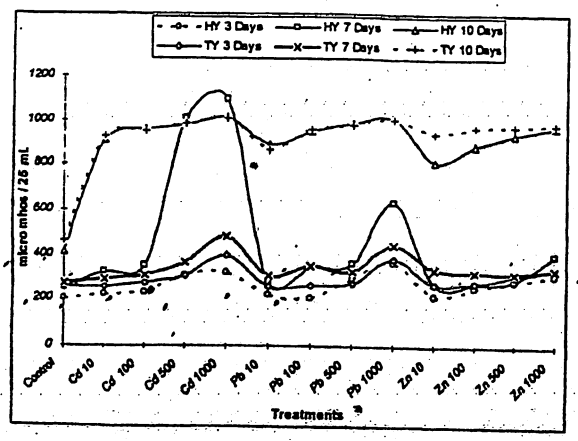

Figure 3. Effect of heavy metal treatment with $CdCl_2$ (Cd 10, 100, 500 and 1,000 µM), $PbCl_2$ (Pb 10, 100, 500 and 1,000 µM) and $ZnCl_2$ (Zn 10, 100, 500 and 1,000 µM) on electrical conductivity (µ mhos/ 25 ml.) on the seed leachates of *Vigna umbellata* cv *Haday* (HY) and cv *Thangray* (TY) under laboratory conditions.

Photosynthetic pigments like chlorophyll-*a*, chlorophyll-*b* and carotenoids in the leaves of 10-day old seedlings showed a declining trend with the increasing concentration of Cd, Pb and Zn. The effect of Cd was most significant in the reduction of the pigment contents. Of the two cultivars of *Vigna umbellata* the cultivar *Thangray* was more susceptible to the effects of heavy metals for decreasing the levels of photosynthetic pigments (Fig. 2). Impaired chlorophyll development by heavy metals may be due to interference with the synthesis of proteins, the structural components of chloroplasts (28).

The quantity of RNA after 10 days of treatment with heavy metals showed a steady decline as compared to the control (Table 2). Growth rate of the germinating seeds is known to be controlled by the RNA content (11). Under the influence of heavy metals the quantity of RNA increased initially up to a period of 24 hours. The amount decreased with

prolonged duration of the treatment (29). As expected the level of RNA showed a sharp decline with increasing dose of heavy metal exposure. The DNA content also showed a steady decline with Cd being responsible for the largest decline and the cv *Thangray* the most vulnerable to heavy metal induced loss of DNA content (Table 2).

A large increase in electrical conductivity was noticed in all the heavy metals treated and untreated seeds. There was proportional increase in tissue permeability with the increase in magnitude of stress. Most remarkable changes in the membrane permeability were observed in the concentration range of 100–1,000 µM Pb and Cd treatment (30). However, the magnitude leakiness was exceeded by two times in the treated seeds over a period of 10 days. It should be mentioned here that the exposure to low concentration Pb and Zn for a short duration checked the electrical conductivity to some extent. Thus we can assume that heavy metals are responsible for the loss of membrane integrity however, exposure for a short time in a low dose is not that deleterious, more so, specially in the cv *Haday* where the membrane integrity was maintained to a considerable extent (Fig. 3).

Declining trend in peroxidase activity was observed in all treatments. However, the decline was not that prominent in the treatment with Zn (Table 3). The decreased activity of peroxidase might be one of the reasons for the degradation of macromolecules causing membrane damage via lipid peroxidation (31).

Data presented in this paper revealed that the heavy metal treatment with Cd, Zn and Pb continuously for a considerable length of time cause severe impairment of physiological and biochemical activities of the *Vigna umbellata* cv *Haday* and cv *Thangray*. The disrupted cellular activities may be due to retardation of biosynthetic activities caused mainly by jeopardizing the action of specific enzymes. All the damages were proportional to the magnitude of the stress. The results demonstrated that the two cultivars of the rice bean *Haday* and *Thangray* slightly differed in their sensitivity to heavy metal stress. The cultivar *Thangray* was more susceptible to damage caused by heavy metals as compared to the cultivar *Haday* and Cd at a concentration of 100 µM and above showed the most damaging effect.

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