

Synergistic Defensive Mechanism of Nitrate Reductase (in-vivo, endo and invitro) and Antioxidative Enzymes in Excised Greening *Phaseolus Vulgaris* Leaf Segments Against Mercury Stress

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Abstract – The present study was designed to investigate the effects of mercury on morphological, enzymes of antioxidant and nitrate reductase activity at five different concentrations (C, 0.001, 0.01, 0.1, 1mM) with the metal. The comparative study is for correlate the accumulation of enzymes with respect to mercury in an effective way. In morphological study, there was decrease in seed germination, shoot and root length and decline in germination period with the increasing concentration of mercury was observed. Sand treatment with mercury shows more pronounced effect in comparison to seed treatment. However in which four hour seed treatment shows more inhibitory morphological signs. In nitrate reductase study, there was decrease in enzyme activity at higher concentration. The activities of antioxidative enzymes i.e Guaiacol peroxidase and catalase followed the same trends as antioxidants first increased up to a concentration of 0.1 mM Hg and then decreased at 1mM concentration. The results of present study, obtained were suggested two important pathways involved in Hg stress and tolerance in *Phaseolus vulgaris* and also allow a deep understanding of the natural defensive mechanisms in plants under heavy metal stress.

Keywords – Mercury, *Phaseolus Vulgaris*, Nitrate Reductase, Antioxidant, Morphological, Peroxidase, Catalase.

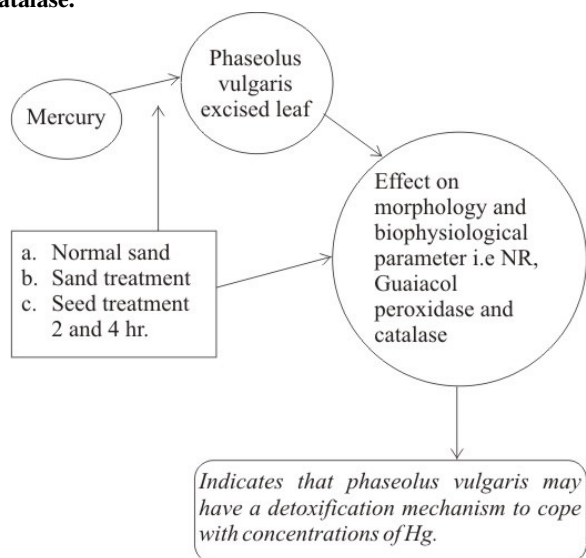


Fig. 1. Graphical abstract

I. INTRODUCTION

Bioaccumulation of heavy metal by higher plants is a serious point of discussion by many researchers.¹

One of the toxic heavy metal is mercury; causes gene-toxicity and phyto-toxicity². Higher plants provide a useful model for determine heavy metal pollution level in soil and monitoring environmental pollutants.³

Nitrogen assimilation is the formation of organic nitrogen compounds like amino acids from inorganic nitrogen compounds present in the environment. The product of nitrate assimilation is ammonia.⁴

Antioxidants are important species which possess the ability of protecting organisms from damage caused by free radical-induced oxidative stress.⁵ The intracellular level of H₂O₂ is regulated by a wide range of enzymes, the most important being catalase and peroxidase. Catalase inactivates H₂O₂ to oxygen and water.⁶

Rajma are exposed to heavy metals in their natural environment as a result of various human activities, including burning of fossil fuel, fertilizers, pesticides and the use of pigments and batteries.⁷ Therefore a more and briefly knowledge of the phyto-toxic effect of mercury is important.

This research gives an overview of the different enzymatic and physiological clues for better detoxification mechanism and its molecular analysis.

II. MATERIALS AND METHOD

2.1 Plant Growth

Seeds of *phaseolus vulgaris* were rinsed in running water for 2 min. Seeds were surface sterilized with 0.1% HgCl₂ for 30 sec. and then washed with three times autoclaved double distill sterilized water.

2.2 Metal Treatment

Treatment with metal at three levels in order to correlate uptake, accumulation and comparison of the same with the following treatment schedules.

a. Excised young leaves from normal acid washed sand were cut into small segments and treated with metals of desired concentrations in continuous light for 24 hrs.

b. Seeds were treated with metals for 2 hrs and 4 hrs followed by thorough wash and subsequently planted on acid washed sand contained in plastic pots. Primary leaves

of the seedling were kept aside for various analytical determinations.

c. Acid washed sand was treated with different concentration of Mercury for 24 hrs contained in plastic pots. Primary leaves of the seedling were kept aside for various analytical determinations.

2.3 Morphological Assay

For this study, acid washed sand treated with different concentration of metal and treated seed for two and four hour was used. For After a week seedlings were harvested then root and shoot were separated for measurements. Seed germination was also counted. All values counted in three replicates of experiment^{8,9}

2.4.1 Nitrate reductase assay The activity of nitrate reductase in the treated material will be estimated *in-vivo*¹⁰ or *in-vitro*¹¹ method with slight modification. Endogenous nitrate pool in the leaf segments were estimated according to Aslam (1981)¹². The absorbance was read at 540 nm after 20 min by using UV-spectrophotometer. Briefly, 20 ml incubation buffer containing 0.1 M phosphate buffer and 25% iso-propanol then Incubated for one hour at 30°C. It was mixed with sulfanilamide and NED. After 20-25 min the reading is taken at 540 nm.

2.4.2 Antioxidant enzyme: Peroxidase was estimated according to Maehly, 1954¹³. POD catalyses the transformation of guaiacol to tetraguaiacol (brown product). The oxidation of guaiacol was measured by the increase in absorbance at 436 nm for 1 min. Activities of catalase (EC 1.11.1.6) was assayed in fresh leaf tissue extracts by using a modification of the method of Zhou, 2001¹⁴ and Zhang, 1990¹⁵ as mentioned in Cui and Wang, 2006 with slight modification¹⁶. Catalase activity was expressed as μmol of H_2O_2 decomposed per min per gram of fresh weight ($\mu\text{mol}/\text{min}/\text{g}$ FW).

III. STATISTICAL ANALYSIS

Each experiment was repeated at least thrice and data presented are the average value and standard deviation value of findings. Statistical data collected from one-way ANOVA test software.

IV. RESULT

4.1 Effect on Germination, Shoot, and Root Length

In sand treatment the highest percentage of seed germination was observed in 0.01 with 65% in comparison to control then continuously decreased. In 1 mM there was only 36% germination (Table 1). In seed treated for two hour 48% germination was observed in 0.1 where as in four hour treated seed in 0.01 with 43% highest germination. In seed treated for two hour 1 mM have only 23%, where as in four hour treated seed 28 % in 0.001 least germination followed by 1 mM with no germination was found.

The growth of *phaseolus vulgaris* seedlings was assessed in terms of shoot and root length. It was found in sand treated, shoot length was linearly decreased from control to 1 mM concentrations of Hg or significantly ($P < 0.01$) inhibited at higher concentrations. A 58 % reduction in shoot length was observed at a concentration of

0.001mM, with respect to the control. Root length was continually decreased from control to 1 mM concentration.

In seed treated with two hour, shoot length was more inhibited at 0.1 mM with 78.23% reduction where as in four hour treatment more inhibition in 0.001 mM concentration. Root length was decreased form control to 1mM in two hour seed treated where as in four hour 0.01mM shows more inhibition followed by 0.001 mM. (Table 2 and 3)

Period of seedling germination was found 130% times at 1mM concentration more inhibited than control during sand treatment. In seed treatment with two hour at higher concentration decline in period of seedling germination than control where as in four hour seed treatment there was significantly inhibition ($P < 0.01$) shown in 0.1 mM concentration. (Table 4)

4.2 Effect of Hg on Nitrate Reductase Activity

4.2.1 In-vivo NR: Mercury shows (normal sand) decline in activity form lower to higher concentration and inhibition was more pronounced at 0.1 and 1 mM concentration. Where as in sand treated 1mM shows significant (<0.001) inhibition than followed by 0.01, 0.001 mM. The level of inhibition was different in seed treated two and four hour. In two hour 1 mM concentration shows significant inhibition (<0.001) than followed by 0.01 and 0.1 mM where as in four hour 0.01 mM concentration shows more significantly enzyme inhibition. (Table 5)

4.2.2 Endogenous NR: In normal sand there was, significantly value found in 0.1 and 1 mM conc. Supply of mercury during sand treatment shows significantly decrease in activity at 0.1 mM followed by 1 and 0.001 mM conc. Mercury inhibited endo nr activity in seed treated two hour at 0.1 mM concentration where as in four hour 0.001 mM concentration significantly. (Table 6)

4.2.3 In-vitro NR: Treatment of Hg in normal sand leaf and sand treated Hg shows significantly inhibition in 0.1 mM followed by 1 mM conc. where as in seed treated with two hour shows more pronounced effect on 0.1 mM followed by 1 mM and in four hour 0.1 mM shows more inhibition followed by 0.001 mM concentration. (Table 7)

4.3 Effect on Antioxidant Enzymes

4.3.1 Peroxidase: A concentration dependent activity of peroxidase was observed from 0.001 to 0.1 mM of Hg (table 8). The activity slightly inhibited in 0.01 and 0.1 mM in both normal sand and sand treated with Hg. During seed treatment with two hour only in 1mM conc shows significantly increase in activity where as in four hour 71 times more fold inhibition in 0.1mM followed by 0.001mM conc. or least inhibition in 0.01 mM conc.

4.3.2 Catalase: The catalase activity shows a little bit same pattern as peroxidase. During normal sand leaf treatment shows slightly decrease in 0.001 where as in 0.1 and 1 mM shows equal inhibition as in control. Supply of mercury in sand treatment shows more inhibition in 0.001 mM than followed by 0.1 in compare to control. During seed treatment with two hour only in 0.01 and 0.1 mM conc shows more inhibition and significantly activity in 1 mM where as in four hour 0.001mM and 0.01 shows inhibition. (Table 9).

V. DISCUSSION

The seed germination of *phaseolus vulgaris* seedlings was decreased with increased in mercury concentration which was significantly ($P < 0.01$) affected at higher concentrations of Hg (table 1 and 2). Reduction *phaseolus* in biomass at high levels of Hg may be correlated to high Hg accumulation by seedlings. In that case, cells might have to produce extra energy to cope with the high Hg concentration in the tissues.¹⁷ The growth pattern of *phaseolus* seedlings in the presence of Hg was different from pea and spear mint¹⁸, ryegrass¹⁹, tomato²⁰, *Sesbania grandiflora*²¹, *Mentha arvensis*²²

In present study, when mercury was supplied in sand as the mercury concentration increased there was observed decreased in growth parameter. Further when Hg exposed to seed directly as the treatment time period was raised two to four hour there was also decrease in growth parameter. Furthermore no growth in 1 mM concentration was found in four hour treated seed.

Nitrate is currently one of the most hazardous pollutant.²³ The supply of $HgCl_2$ (0.01-1.00) exhibited substantially *in vivo* as well as *in vitro* the total NRA and endogenous nitrate pool in excised bean leaf segments. The toxic effect of Hg was overcome completely by the supply GSH, cystein and sucrose and partially by molybdenic acid and NADH.²⁴

The major factor inducing changes in nitrogenous activity is the concentration of free oxygen inside nodules. Oxygen availability in the infected zone of nodule is limited, among others, by the gas diffusion resistance in nodule cortex. The presence of nitrate may cause changes in the resistance to O_2 diffusion²⁵. In this study, NR (*in vivo*, endogenous, *in vitro*) activity was increased from 0.001 mM to 0.01 then slightly drop in 0.1 and 1 mM in normal sand. During sand and seed treatment 0.01 and 0.1 mM concentration were shown satisfactory inhibition. Further in two hr. seed treatment 1mM also showed inhibitory effect during *in vivo* NR.

The phyto-toxic effect and its mechanism are explored only in few processes in *phaseolus* under mercury stress. The effect of mercury on enzyme activity was previously reported²⁶, which explore the phytochelatin induction due to mercury stress. Hg treatment to *phaseolus* seedlings were noticed by Mor *et al.* (2002)²⁷ on hypocotyl elongation and cell wall loosening. The relatively strong affinities of heavy metal ions for side chain ligands of proteins indicate that enzyme activities and other functional proteins are one of the primary targets of metal toxicity²⁸ (Vallee and Ulmer 1972).

The mercury stress induces an antioxidant enzyme response which involved in hydrogen peroxidase detoxification mechanism of *phaseolus vulgaris* seedlings. Peroxidase is extensively distributed in the plant group and is one of the principal enzymes involved in elimination of active oxygen species (AOS). We found as the mercury stress increase the enzyme activity also increase but slightly decrease in (1mM concentration) at higher concentration. Sand treatment shows more profound significantly values than followed by four hour

treatment. Similarly catalase is one of the most efficient antioxidant enzymes and it plays an important role in maintaining the redox homeostasis of the cell²⁹. Catalase also shows little similar pattern as peroxidase. The increased activities of peroxidase and catalase in *phaseolus* seedlings under three phases Hg treatment may be considered as experimental evidence for tolerance mechanisms developed by this plant under lab condition. Hence sand treatment shown better inhibition we were taken to further step for mercury accumulation or uptake by icp-ms. Compared to the previous reports on Hg accumulation *phaseolus* accumulates and tolerate high concentration of Hg when supplied to sand. At present still plenty of unknown aspects regarding Hg's genotoxicity, namely, the mechanistic, target, and extent of its effects in plants. Plants play a major role in ecology hence, there is need to find process about uptake mechanism of Hg and protect our valuable ecosystem.

VI. CONCLUSION

As it explored, in this study the mercury stress causes morphology and physio-biochemical effect in *phaseolus vulgaris*. In conclusion, the present study revealed that the upper limit of Hg tolerance in *phaseolus vulgaris* seedlings is 1mM. Seedlings bio-physiological responses significantly decreased with increasing concentrations of Hg in the three phase comparative study, suggesting its phyto-toxic effects. A coordinated decrease in enzymatic NR activity and increase in up to 0.01 mM conc. antioxidants (peroxidase and catalase) was noted with an increase in Hg concentrations in the tissues. Methyl mercury can be biologically synthesized in the environment from virtually any mercurial compound; mercury in any form must be considered a potentially-dangerous environmental pollutant. This indicates that *phaseolus vulgaris* may have a detoxification mechanism to cope with such a high concentrations of Hg. Thus, the direction of the plant response depends on the metal concentration and the intensity of the stress.

Table 1. Effect of $HgCl_2$ on germination of *P. vulgaris* seed

Concentration of mercury (mM)	Sand treated with mercury concentrations Percentage %	Seed treated with mercury concentration Percentage %	
		Two hour	Four hour
	24 hour sand treated		
Control (without treatment)	76	53	61
0.001	56	40	28
0.01	65	41	43
0.1	53	48	33
1	36	23	No growth

Table 2. Effect of HgCl₂ on shoot lengths

Concentration of mercury (mM)	Sand treated with mercury concentrations cm Mean± SD	Seed treated with mercury concentration cm Mean± SD	
		Two hour	Four hour
	24 hour sand treated		
Control (without treatment)	211.5± 59.94	129.33± 13.28	109±45.03
0.001	155± 34.87	98±20.78	56.67±1.15
0.01	154 ±13.08	122±45.03	102.33±14.43
0.1	97.67± 4.93	99±10.39	66.33±17.9
1	64.67±6.81*	35.33± 17.9	No growth

Table 3. Effect of HgCl₂ on root lengths

Concentration of mercury (mM)	Sand treated with mercury concentrations cm Mean± SD	Seed treated with mercury concentration cm Mean± SD	
		Two hour	Four hour
	24 hour sand treated		
Control (without treatment)	81.67±2.52	69.33±32.33	45.33±5.77
0.001	61±27.62	57±24.25	20.33±4.04
0.01	83 ±27.84	59.33±25.4	39.67±0.59
0.1	45±20.81	51±13.86	32.67±20.21
1	20.67±3.21	15.67±4.62	No growth

Table 4. Decline in periods of seed germination

Concentration of mercury (mM)	Sand treated with mercury concentrations Growth in days Mean± SD	Seed treated with mercury concentration Growth in days Mean± SD	
		Two hour	Four hour
	24 hour sand treated		
Control (without treatment)	9±2.65	12±2	9.67±2.52
0.001	9.33±2.31	12±2	11.33±1.15
0.01	8.33±1.53	9.67±2.52	13.67±1.53
0.1	10.67±2.52	10.33±1.53	19.33±1.15*
1	10±3.46	10.67±2.31	No growth

Table 5. Effect of mercury on in vivo NR activity in excised bean leaf segments

**p < 0.001 and *p < 0.01.

Concentration of mercury (mM)	Normal sand (µ mol NO ₂ /hr/g) fresh weight Mean± SD	Sand treated with mercury concentrations (µ mol NO ₂ /hr/g) fresh weight Mean± SD	Seed treated with mercury concentration (µ mol NO ₂ /hr/g) fresh weight Mean± SD	
			Two hour	Four hour
		24 hour sand treated		
Control (without treatment)	6.99±1.83	68.56±13.84	60.62±11.39	69.66±18.94
0.001	5.86±2.90	12.85±15.66**	74.54±13.05	47.51±15.30
0.01	6.22±2.81	64.50±35.59*	24.55±4.18**	29.36±3.51**
0.1	6.30±2.34	35.26±11.47	11.46±14.67*	63.93±11.69
1	6.87±2.37	11.19±4.38**	30.73±3.81**	No growth

Table 6. Effect of mercury on Endo NR activity in excised bean leaf segments

**p < 0.001 and *p < 0.01.

Concentration of mercury (mM)	Normal sand (µ mol NO ₂ /hr/g) fresh weight Mean± SD	Sand treated with mercury concentrations (µ mol NO ₂ /hr/g) fresh weight Mean± SD	Seed treated with mercury concentration (µ mol NO ₂ /hr/g) fresh weight Mean± SD	
			Two hour	Four hour
		24 hour sand treated		
Control (without treatment)	12.78±2.3	16.18±1.54	18.58±0.56	9.79±2.37
0.001	9.43±3.16	10.86±1.73**	7.99±0.79**	19.62±2.30**
0.01	7.43±2.87	15.59±1.33	4.73±0.60*	6.85±2.32
0.1	6.95±3.10*	22.06±0.63**	28.57±0.22**	8.14±2.31**
1	3.99±3.56**	10.72±1.20**	0.84±0.36*	No growth

Table 7. Effect of mercury on in-vitro NR activity in excised bean leaf segments

**p < 0.001 and *p < 0.01.

Concentration of mercury (mM)	Normal sand (µ mol NO ₂ /hr/g) fresh weight Mean± SD	Sand treated with mercury concentrations (µ mol NO ₂ /hr/g) fresh weight Mean± SD	Seed treated with mercury concentration (µ mol NO ₂ /hr/g) fresh weight Mean± SD	
			Two hour	Four hour
		24 hour sand treated		
Control (without treatment)	6.94± 1.27	10.88 ±3.28	27.44±3.00	13.01±0.48
0.001	8.32 ±1.45	5.53 ±2.15	21.99±5.47	16.73±0.25**
0.01	6.79 ±1.80	8.14± 2.76	10.64±5.82**	13.20±0.28
0.1	2.65± 1.34**	3.15± 1.55**	5.99±0.92**	27.36±0.20**
1	1.46± 1.38**	4.28± 1.59**	5.28±1.38**	No growth

Table 8. Effect of mercury on peroxidase activity in excised bean leaf segments

**p < 0.001 and *p < 0.01.

Concentration of mercury (mM)	Normal sand (mg enz./ml original sol /protein) fresh weight Mean± SD	Sand treated with mercury concentrations (mg enz./ml original sol /protein) fresh weight Mean± SD	Seed treated with mercury concentration (mg enz./ml original sol /protein) fresh weight Mean± SD	
			Two hour	Four hour
		24 hour sand treated		
Control (without treatment)	2.58± 0.01	2.02± 0.18	0.21± 0.01	1.58± 0.35
0.001	2.15 ±0.03**	1.03± 0.18**	0.21± 0.01	0.48± 0.15**
0.01	2.46 ±0.02**	1.66 ±0.07**	0.21±0.01	1.19± 0.3
0.1	2.46 ±0.02**	1.44± 0.15**	0.21± 0.01	0.64± 0.25**
1	0.84 ±0.01**	0.99 ±0.06**	0.09± 0.05**	No growth

Table 9. Effect of mercury on catalase activity in excised bean leaf segments

** $p < 0.001$ and * $p < 0.01$.

Concentration of mercury (mM)	Normal sand (μ mol/min/gm fresh weight) Mean \pm SD	Sand treated with mercury concentration (μ mol/min/gm fresh weight) Mean \pm SD	Seed treated with mercury concentration (μ mol/min/gm fresh weight) Mean \pm SD	
			24 hour sand treated	Two hour
Control (without treatment)	36.61 \pm 7.42	49.68 \pm 4.56	38.88 \pm 3.33	45.91 \pm 1.08
0.001	18.6 \pm 4.86**	41.66 \pm 18.81	35.27 \pm 5.46	47.39 \pm 1.19
0.01	25.86 \pm 5.98	49.34 \pm 4.42	32.81 \pm 6.12	47.76 \pm 1.01
0.1	32.84 \pm 6.11	47.62 \pm 4.65	31.68 \pm 6.8	3.22 \pm 0.4**
1	32.98 \pm 6.92	45.13 \pm 4.49	16.95 \pm 0.52**	No growth

VII. ACKNOWLEDGEMENT

Sincerely obliged to Dr. Jot Sharma and Dr. Archana Shrivastav for encouragement and providing necessary administrative and research facility

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