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Biochemical and Toxicological Effects of Cadmium on *Phaseolus vulgaris* L.

Running title: Cadmium stress in *Phaseolus vulgaris* L.

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Abstract: ***Phaseolus vulgaris L. (kidney bean) is an annual leguminous pulses, rich in antioxidant flavonoids and proteins. This group of plants are being used in eastern Himalaya for different type of ethinic medicinal and edible purpose. On the contrary, Cadmium as a toxic heavy metal has ranked number 7 among top 20 toxins. Manufacturing of paints, plastics, fungicides, fertilizers, Ag-Cd batteries, nickel-cadmium storage batteries, printing ink etc. are common sources of entering Cd(II) into environment. The seeds are collected from seed banks germinated and grown with Hoagland medium under various concentrations of cadmium. Quantum yield of primary photochemistry were estimated using Handy PEA. The biochemical compounds are estimated following standard methods at weekly intervals. The proline content increases with increase in Cd^{2+} concentrations (R^2=0.69). But there was a concentration dependent decrease in other biochemical compounds such as total soluble protein, Chl a, b and yield parameter of photosynthetic electron transport for Cd^{2+} induced toxicity. The root length decrease more significantly (F=8.3; P<0.01) than the shoot length and leaf count with increasing Cd^{2+} induced toxicity on roots. Hence, this may be concluded that 80 ppm Cd^{2+} concentration in Phaseolus vulgaris is detrimental while up to 60 ppm the plant shows better adaptability.***

Keywords: ascorbic acid, cadmium, proline, *P. vulgaris*, quantum yield

1. Introduction

Phaseolus vulgaris L. (kidney bean) is an annual leguminous pulses taken as cooked food for its great nutritional value. It is a widely grown food legume in different parts of India and rich in antioxidant flavonoids and proteins. This group of plants has been identified as one of the dominant legume taxa, which is being used in eastern Himalaya for different type of ethinic medicinal and edible purpose [1]. Its seeds are used as spice and have medicinal values for the treatment of dyspepsia, rheumatism, asthma and constipation [2].

Cadmium is a widespread nonessential toxic heavy metal, which has been identified as a significant pollutant due to its high toxicity and solubility in water. It has ranked number 7 among top 20 toxins[3]. Naturally occurring Cd levels are very low, soluble Cd concentration in non-Contaminated soil varies from 0.01 to 5 $\mu g.kg^{-1}$ [4] whereas metal contaminated soils contain about 600 mg kg^{-1} of Cd [5]. Manufacturing of paints pigments, plastics, fungicides, fertilizers, silver cadmium batteries, nickel-cadmium storage batteries, glass, ceramic alloys, photo-electric cells, photo conductors, rectifiers, printing ink automobile tyres, motor oils etc. are the common sources of entering Cd into environment.

Cadmium is easily taken up by roots from contaminated soil and translocated into leaves of plant species. High concentration of Cd cause toxicity in plants which results decrease in carbon assimilation, generate oxidative stress, induce stomatal closure and disturb plant water status. The toxicity of Cd also inhibits chlorophyll synthesis, damage root tips, reduce nutrient Uptake, impair photo synthesis and inhibit plant growth [6],[7],[8],[9],[10],[11],[12].The effect of Cd^{+2} toxicity on growth and development of plants are mainly based on visual symptoms, such as chlorosis, necrosis, leaf epinasty, Yellowish discoloration, on biomass reduction, stunted growth, decrease and change in mineral composition etc. [13]. Excess Cd induces complex changes in plants at gentical, biochemical and physiological levels leading to phytotoxicity.

The toxic effect of Cd^{+2} on leguminous plant has been discussed. However, investigation of photosynthetic responses coupled with biochemical variations under cadmium stress in *Phaseolus vulgaris* has not been elucidated so far. This study is expected to provide us evidences for accumulation and changes in physiological and biochemical parts in *P. vulgaris* towards Cd^{+2} stress. This novel approach focuses to produce healthy food material as well as a sustainable agriculture.

2. Materials and Methods

2.1 Collection of seed and Experimental Setup

Seeds of *Phaseolus vulgaris* L. (Label No. 301, variety: RONORS-IMPORTED, Lot No.101, Genetic purity (Min): 98%, Physical purity (Min): 98%, Inert matter (max): 2%, moisture (Max): 6%, chemical used: Thiram/Captan) were collected from seed center shop, Rabitalkies square, Bhubaneswar. Fresh and healthy seeds of common bean legume (*P. vulgaris* L.) were surface sterilized with 0.1% $HgCl_2$ for 3 min and continuously washed under running tap followed by distilled water and allowed to germinate in the dark on moistened filter paper at 25^{o}C. Germinated seedlings were then allowed to grow in sterile sand (oven dried at 100 ± 2^{0}C for 24 h) with Hoagland nutrient medium. Different treatment protocols (10ppmCd^{+2} to 150ppmCd^{+2}) with untreated control were set up for 1month experiment with 15 days of exposure.

2.2 Study of Growth Parameter

Freshly collected plants were designed in a chart paper from control to 150ppm sequentially and then a photograph was taken. Number of leaves of each plant was counted and noted. The root length and shoot length of each plants was measured in a scale and note down.

2.3 Estimation of photosynthetic quantum yield

The quantum yield of leaves of each plant was measured through the instrument Plant efficiency analyzer (Handy PEA; Hansatech instrument Ltd., Norfolk, UK). Prior to measurement, a leaf clip (4 mm dia measuring area) with closed lid was inserted into the leaf and dark adoption was made for 10-15 min after which fluorescence rise was induced on application of continuous actinic red light (685 nm) Saturation pulse at an irradiance of 3000 μmol photon/m^2s [14]. The F_v/F_m reading appeared on the handy PEA was noted down.

2.4 Extraction and estimation of total soluble protein

Fresh leaf sample (100 mg) were homogenized with 5 ml of 10% ice cold TCA by a pre-chilled mortar and pestle, incubated overnight at 4^{o}C and centrifuged at 10,000g for 10min. For removal of pigments, the pellet was washed with 100% acetone and subsequently washed with 80% ethanol, ethanol/chloroform (3:1 v/v) and diethyl ether to remove phenolic compound. Washed pellets were then suspended in 5ml of 0.1 N NaOH followed by the method of Lowery et al. [15] for estimation of total soluble protein.

2.5 Estimation of Proline

Freshly collected leaf sample (100 mg) were homogenized with 5 ml of 3% aqueous sulfo-salicylic acid and centrifuged at 12,000g for 10min. Two milliliters of supernatant was taken and 2ml of glacial acetic acid and 2 ml of freshly prepared acid Ninhydrin reagent (Ninhydrin + Glacial acetic acid + 6M H_3PO_4) were added and proline estimation was completed following methods of Bate et al.[16].

2.6 Estimation of Ascorbic Acid

One hundred milligrams of fresh leaves were homogenized with 5ml of 6% meta-phosphoric acid and centrifuged at 7,000g for 10 min. Two milliliter of 2% sodium molybdate and 2 ml of 0.1N sulphuric acid were mixed and chilled and 1 ml of monobasic sodium phosphate and 1 ml of supernatant were added. The mixture was then subjected to estimate ascorbic acid following methods of Mitsui and Ohta[17].

2.7 Estimation of chlorophyll

One hundred milligrams of freshly collected of leaves were homogenized with 80% acetone followed by centrifugation at 6,000 rpm for 5 min. Then the absorbance was taken at 663 nm and 647nm using UV-Vis spectrophotometer (λ 25, Perkin-Elmer, U.S.A.). The Chlorophyll contents of leaf sample were estimated and expressed in mg/g fresh weight by Porra *et al.*[18].

3. Result and Discussion

In the present investigation the seeds of *P. vulgaris* L. were selected to estimate alternations in growth, physiological and biochemical parameters under different treatments of cadmium. For each treatment there were five replicas of the plants taken and the experiment was carried out thrice for better results. Though 10 to 150 ppm cadmium ($CdCl_2$) were taken for experiment against a control with no cadmium chloride, this experiment have >90 degrees of freedom which resembles a statistically viable outcomes.

3.1 Cadmium stress altering Biochemical responses in P. vulgaris L.

The experimental study reveals a steady increase in proline content i: e 61% from 10ppm to 150 ppm and 54% from 10 to 60ppm after 15days and 30days of Cd^{+2} exposures to plants. (Table 1) Throughout experiment the control plant sustains same proline level as it had no Cd^{+2} content. Due to stress effect the plants above 60ppm died to 30 days exposure. On the other hand the Protein level of *P. vulgaris* L. demonstrated a rapid declination (70%) from 10 to 150ppm as well as 10 to 60 ppm (48%) after 15days and 30days of study respectively (Table 1). Plants from 80 to 150 ppm couldn't sustain to 30day exposure due to more stress of cadmium. Like Proline level the Ascorbic acid content showed a gradual increment in Cd^{+2} stress both in 15days and 30days of study. It was calculated 141% increase from 10 to 150 ppm after 15days study and 78% from 10 to 60ppm after 30days (Table 1). In control plant no significant alternation in Ascorbic acid was noticed throughout experiment.

Proline is a compatible solute help in cellular osmotic adjustments whose accumulation increases when plants are in metal stress condition[19]. Proline acts as a molecular chaperon stabilizing the structure of Protein and to maintain a balance of cell redox potential and also it helps to vacuolar water potential [20],[21]. The present study reveals that Cadmium application had dose-dependent effect on proline content of bean plant. The increase in Proline content shows positive response towards metal stress.

Proteins are essential structural components of cell membrane, cytoplasm, organells etc. [22]. Protein function as receptor molecules which bind and transport specific informational molecules like hormones and auxins and regular activities as well as reducing energy in the form of electrons such as role of Plastocyanin, a small molecular weight protein which transport electron from Plastoquinon to PS I. The later one is an important activity in net photosynthetic quantum yield[23]. The quantum yield data also shows a decreasing trend along with the total soluble protein concentration. This finding corroborates with *R. mucronata* under salinity stress response by earlier report [24]. Proteins that accumulate in plants under metal stress may provide a storage form of Nitrogen and play a role in osmotic adjustment [22]. It is evident from the result that the protein content was decreased for all concentration in *Phaseolus vulgaris* L..The decrease in protein content is due to the oxidative stress induced denaturation or degradation of proteins [25].

Ascorbic acid is an antioxidant molecule and a cofactor for hydroxylase enzymes (e.g. prolyl hydroxylase and violaxanthin de-epoxidase). Ascorbate can act as a carrier of electron transport for photosynthetic activity [26]. The result of the present study reveals the fact that ascorbic acid level increases with increase in time of exposure and cadmium stress indicating antioxidant defense against metal induced oxidative stress. This indicates the plant resistance to metal stress and free radical scavenging activity. Hence Ascorbic acid helps the plant to protect against Oxidative damage and maintains membrane integrity.

3.2 Cadmium induced change concentration of different photosynthetic pigments in P. vulgaris L.

The chlorophyll content of plant under Cd^{+2} stresses denotes a gradual decreasing trend with increase in concentration throughout experimental period. The control plant has little remarkable changes in compared 15^{th} day to 30^{th} day treatment. After 15 days of treatment, the chlorophyll *a* from 10 to 150 ppm decreased 50% where as chlorophyll *b* decreased 84% significantly (Table 2). Likewise after 30 days of exposure the reduction level of chl *a* was 30% and chl *b* was 38% from 10 to 60 ppm plants. The plants of 80, 100,150 ppm died after 30day treatment due to thresh hold limit of stress tolerance.

The Chl *a* fluorescence is an unique tool to asses Oxygenic plant under abiotic stress like light, chilling, heat, salinity, metal, drought etc. The present study reveals the fact the chlorophyll content in the leaves of *P. vulgaris* L. decreases in correspondence with increase in concentration .Reduction of chlorophyll content at higher concentration plants may be due to the interference of metals [18]. cadmium interfered with both PS II and PS I. PS II was highly sensitive to deleterious effect of cadmium and its functioning was inhibited to a much greater extent than that of PS I.

3.3 Effect of cadmium on quantum yield of P. vulgaris L.

The photosynthetic quantum yield (F_v/F_m) rate of *P. vulgaris* L. demonstrated a decline trend with ascending concentration during experimental period. It was accounted 5% reduction after 15days from 10 to 150ppm and 16% from 10 to 60ppm after 30days study (Figure 1). The control plant shows no alternation due to absence of Cd^{+2}.

The derived fluorescence parameter revels many fact about Photosystem II (PS II) integrity and PS II to PS I electron transport efficiency. Out of which F_v/F_m, the quantum yield of electron transport revels the overall efficiency to fix photon of energy converted to reduction energy as $NADPH_2$. This fluorescence parameter ranges between 0.80 to 0.85 for plants from different ecosystems and climatic conditions.

Hence the decrease in F_v/F_m value in *P. vulgaris* L. under Cadmium(II) stress indicates poor photosynthetic performance in terms of quantum yield of electron transport where as the control plant shows normal Photosynthetic activity having an average F_v/F_m value of 0.79.

3.4 Effect of Cadmium stress on growth parameter of P. vulgaris L.

Leaf Count

The experiment reveals that the leaf content of plants abase towards higher concentration both after 15day and 30 day study but the number increase at 30day compare to 15day i.e. 5 to 11 for control plants and maximum 9.33 for treated plants (Table 3). The plants from 80 ppm to 150 ppm died due to high toxic level.

Root Length

Under Cd^{+2} stresses, the root length of leguminous plant *P. vulgaris* L. showed a decreasing trend with increase in concentration. In one month experiment, the root length of plant decreases slowly towards higher concentration. In control plant a significant length of 0.45cm increases from 15 day to 30 day study. For concentrated plants the root length varied from 4.67cm at 10ppm to 1.8cm at 150ppm (Table 3).

Shoot Length

It was noticed a remarkable increment in shoot length at 30day plant compare to 15days but the length increased towards higher concentration. The control plant shows a noticeable increase in length i:e 19.37at 15 day to 44.17 at 30 day. The shoot length decreases 18.43cm to 4.3cm from 10 to 150ppm and attained a maximum shoot length of 43.3cm at 10ppm after 30 day study (Table 3).

It is evident from the result of growth parameter that during one month exposure to *P. vulgaris* L. to Cd^{+2} shows a reduction rate in root length and an increment in leaf number and shoot length. The content of Cd^{+2} are higher in roots than in the aerial parts, indicating that the roots act as barrier for translocation and protect the edible parts from toxic contamination [27]. Poor translocation of Cd^{+2} to the shoots and leaves could be due to the sequestration of most of the Cd^{+2} in the vacuoles of the root cells to render it non- toxic, which may be a natural protective response of this plant [2]. It must be noted that Cd^{+2} is a toxic and non-essential element to plants and hence the plant may not possess any specific mechanism to transport the Cd^{+2} [28].

4. Conclusion

The present study reveals the fact that the proline content increases with increase in Cd^{+2} concentration up to a certain level (60 ppm) and then decreases. But other biochemical compounds such as total soluble protein, Chl *a*, *b* and yield parameter of photosynthetic electron transport decreases due to Cd^{+2} induced toxicity. Surprisingly, ascorbic acid level increases for all treatments showing plants innate defense mechanism. The growth parameters such as root length decreases where as shoot length and leaf count increases indicating maximum translocation of Cd^{+2} in roots than shoots there by imposing toxicity on roots. Cd^{+2} may interferes in cell division and hinders in cell elongation in roots tissues as these are the most exposed part of the toxicant.

Hence this may be concluded that 80 ppm Cd^{+2} concentration in *Phaseolus vulgaris* L. is detrimental while

up to 60 ppm the plant shows better adaptability. Again the exogenous supply of Proline may reduces Cd^{+2} toxicity in plants. It is also a fact that Chlorophyll *a* fluorescence tool is a rapid and nondestructive sampling method to investigate metal stress effect in plants. Further studies may require selecting particular factors influencing biochemical and physiological changes in *P. vulgaris* L. under even more Cd^{+2} stress.

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Table 1: Analysis of biochemical components in mg/g fr. wt. of leaf tissue of P. vulgaris L. under Cd stress

Cd^{2+} (ppm)	after 15 day			after 30 day		
	Proline	Protein	Ascorbic acid	Proline	Protein	Ascorbic acid
0	1.16 ± 0.01	0.174 ± 0.133	238.166 ± 1.42	01.18 ± 0.11	0.175 ± 0.029	238.118 ± 7.56
10	1.52 ± 0.02	0.226 ± 0.003	162.593 ± 9.34	11.31 ± 1.86	1.239 ± 0.043	132.061 ± 0.55
20	1.55 ± 0.03	0.275 ± 0.004	155.538 ± 9.75	14.19 ± 0.06	1.469 ± 0.002	128.548 ± 0.73
40	1.74 ± 0.01	0.342 ± 0.002	123.312 ± 7.83	16.30 ± 0.81	1.853 ± 0.007	125.351± 2.74
60	1.76 ± 0.12	0.384 ± 0.003	112.065 ± 9.68	17.51 ± 0.15	2.212 ± 0.093	68.840 ± 2.93
80	1.97 ± 0.04	0.449 ± 0.012	102.642 ± 9.81	18.23± 0.51	2.103± 0.054	59.248± 3.52
100	1.89 ± 0.03	0.495 ± 0.010	99.346 ± 11.48	18.64± 0.44	1.992± 0.025	58.112± 0.57
150	2.45 ± 0.03	0.546 ± 0.002	47.442 ± 2.87	-	-	-

Note. '-' sign indicates no data available.

Table 2: Chlorophyll contents of P. vulgaris L. under Cd stress.

Cd^{+2} (ppm)	Chl *a* (mg/g fr. wt. Leaf)		Chl *b* (mg/g fr. wt. leaf)		Chl *a*/*b* ratio	
	After 15 d	After 30 d	After 15 d	After 30 d	After 15 d	After 30 d
0	0.666± 0.006	0.689± 0.000	0.351± 0.003	0.365± 0.001	1.896	1.886
10	0.585± 0.002	0.602± 0.004	0.337± 0.001	0.349± 0.002	1.737	1.727
20	0.525± 0.002	0.584± 0.004	0.317± 0.001	0.328± 0.002	1.653	1.781
40	0.489± 0.001	0.572± 0.001	0.282± 0.001	0.272± 0.003	1.735	2.106
60	0.441± 0.001	0.422± 0.000	0.246± 0.001	0.215± 0.001	1.795	1.959
80	0.433± 0.003	0.352± 0.002	0.208± 0.002	0.204± 0.002	2.079	1.725
100	0.404± 0.002	0.294± 0.013	0.155± 0.007	0.199± 0.004	2.612	1.477
150	0.289± 0.001	-	0.055± 0.006	-	5.238	-

Table 3: Growth parameters of P. vulgaris L

Cd^{2+} (ppm)	Leaf Count (Numbers)		Root Length (cm)		Shoot Length (cm)	
	15 Day	30 Day	15 Day	30 Day	15 Day	30 Day
0	5.33± 1.52	11.00± 1.00	7.40± 0.10	7.85±0.10	19.37± 0.25	44.17± 0.15
10	5.33± 0.38	9.33± 0.57	4.67±0.21	4.8±0.12	18.43 ±0.40	43.3± 0.10
20	4.33± 0.57	9.00± 1.00	4.20± 0.20	4.6±0.15	16.2 ±0.26	42.23± 0.21
40	4.00± 1.00	8.33± 0.36	3.2 ± 0.20	4.4±0.17	14.6± 0.10	40.43± 0.32
60	3.66± 0.52	7.33± 0.57	2.73±0.15	3.43±0.15	11.5± 0.20	37.37± 0.49
80	3.66± 0.57	4.5± 0.0.24	2.43± 0.06	2.7± 0.31	6.67± 0.25	14.00± 0.58
100	3.33± 0.25	2.00± 0.00	2.2±0.10	1.3± 0.15	5.5± 0.36	7.00± 0.42
150	2.33± 0.46	-	1.8±0.10	-	4.3± 0.30	-

Note. '-' sign indicates no data available.

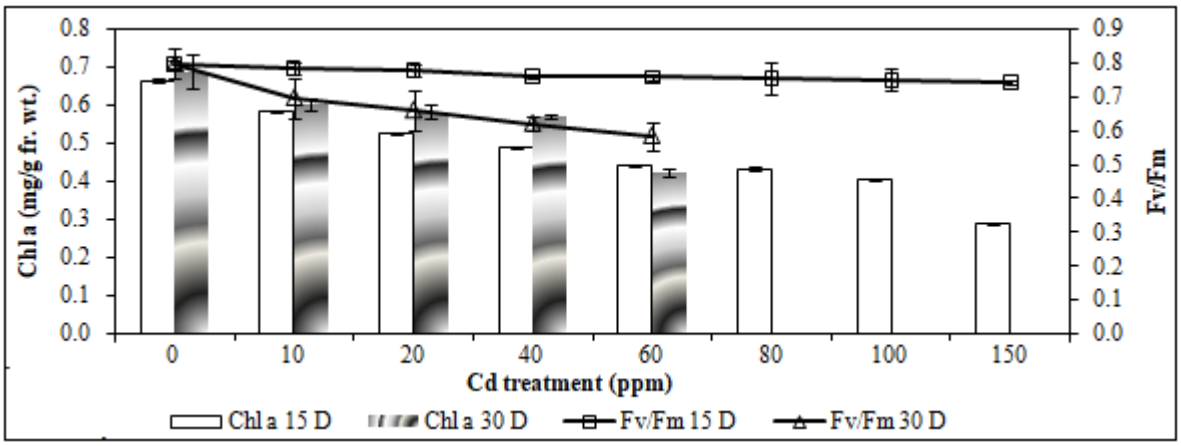

Figure 1: Comparative analysis of Chl *a* and quantum yield parameter *P. vulgaris* L. under Cd stress

Figure 2: Cadmium stress induced retardation in growth in *P. vulgaris* L.

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