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Research Article

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Comparative Biochemical Analysis of the Short Term Responses of *Cicer arietinum* L. to Cd and Pb Toxicity

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ABSTRACT

Heavy metal stress is one of the major abiotic stresses that cause environmental pollution in recent decades. These metals unlike other organic pollutants are not degraded and converted into harmless compounds via biological processes. The study indicates that the treatment of Chickpea by cadmium and lead at different stage of growth resulted in decrease of all the biochemical parameters observed such as chlorophyll 'a' and 'b' content, nitrate reductase activity(E.C.1.6.6.1), proline content, SOD activity(E.C.1.15.1.1) and peroxidase activity(E.C.1.11.1.7).

Key words: Heavy metals, Nitrate reductase, Proline, Peroxidase, Superoxide dismutase.

INTRODUCTION

Heavy metals are naturally occurring elements and are present in varying concentrations in all ecosystems *i.e.*, aquatic or terrestrial. Of all the toxic heavy metals, cadmium ranks the highest in terms of damage to plant growth and human health. The important source of cadmium is non-ferrous metal industry, mining [4] use and disposal of batteries, metal contaminated wastes and sludge disposal, application of pesticides and phosphate fertilizers lead to dispersion of Cd [1,11]. The most important feature that makes it available to the soil and plant too is its solubility in water which is very high. Cadmium is a mobile element, get easily absorbed by root and transported to shoots in competition with that of element get easily absorbed by roots and transported to shoots in competition with that of elements such as K, Ca, Mg, Fe, Mn Cu, Zn and Ni, across the same transmembrane carrier [9]. Moreover, its uptake and accumulation in plants poses a serious health threat to plants and animals via the food chain [10]. Survival under stressful conditions depends on plant's ability to perceive the stimulus, generate and transmit signals and induce biochemical changes that mediate the stress tolerance is an important step towards a better understanding of how plants adjust to an adverse environment. Chickpea (*Cicer aeritinum* L.) is the third most important pulses crop in the world after dry bean and dry peas. The chickpea is an edible legume (English "pulse") of the family Fabaceae, subfamily Faboideae. The name *Cicer* is of Latin origin and is derived from Greek word 'Kikus', meaning force or strength. The centre of origin of Chickpea is eastern Mediterranean and according to De Candolle the fact that gram has a Sanskrit name would indicate that the crop has been under cultivation in India longer than any other country. It is a small, multibranched herbaceous plant. It is cultivated on 12.7 million ha. in 45 countries across the globe producing 8.7 million tons with productivity of 0.84 t/ha. India is the largest producer in the world account for more 66 % of the total world production, it is cultivated over an area of 8.26 million hectares giving 6.2 million tons of production with an average yield of 751 Kg/ha . It is a major source of protein in human diet and animal feed. Protein content of 19-21% and carbohydrate content of 60% from chickpea, making it a valuable

dietary staple for less affluent people[7]. A comparison of amino acid content of various dietary proteins reveals that chickpea protein is comparable to beef or fish. It appears; therefore, when supplemented appropriately, it provides an excellent quality of dietary protein to overcome future food shortages.

EXPERIMENTAL SECTION

DESIGN
DETAILS OF LAYOUT
Experimental design: Completely Randomized design (Table:1)
No. of treatments combination: Five
No. of replications: Five

BIOCHEMICAL PARAMETER

1. Chlorophyll content [1]

The total chlorophyll content was estimated as described by Arnon method [1949]. The leaves from the sample plants were selected and 0.1g weighed. The leaves were measured with 80% acetone in a mortar and pestle and the aliquot was transferred to the test tubes and centrifuged for 10 min. The supernatant was separated and final volume of the sample was made to 10 ml by 80% acetone. After making the volume to 10 ml the absorbance was recorded at 663nm and 645nm using spectrophotometer and 80% acetone was used a blank. From the absorbance value total chlorophyll content was determined by using the formula.

[mg chlorophyll 'a' per gm= [12.7(A663)- 2.64(A645)] x V/(1000 x W)]

2. Nitrate Reductase Activity [8]

For *in vivo* nitrate reductase activity measurement plant samples were incubated at 30 ℃ for 30 min in the dark and then 1 mL of supernatant was used for nitrite estimation by diazo coupling reaction and absorption of pink colour was read at 560nm in spectrophotometer [Elico, SL196]

3. Peroxidase Activity [6]

The homogenates (0.2% w/v) of the leaves were prepared in Tris HCI buffer (0.05M, pH 7.0 with I% B mercaptoethanol) and were centrifuged at 12,000 rpm (7000 Xg) for 20 minute at 4 ℃. The supernatants obtained were used for enzyme assay. The assay mixture comprised 2 ml of phosphate buffer (0.1M, pH 7.0), 1.0 ml of pyrogallol (0.005M), 1.0 ml of H_20_2 solution (0.05M) and 1 ml of supernatant. The mixture was incubated at 25°C for 5 minute and the reaction was stopped by adding 0.5 ml of H_2SO_4 (5% v/v). The amount of purpurogallin formed was determined by reading the optical density at 420 nm. Peroxidase specific activity was expressed as Units/rag protein where unit is [tmol purpurogallin formed/100 mg tissue/minute at 25 °C.

4. Superoxide dismutase activity [5]

Three ml of the reaction mixture containing 0.1 ml of 1.5 M sodium carbonate, 0.2 ml of 200 mM Methionine, 0.1 ml of 2.25 mM NBT, 0.1 ml of 3 ml MDTA, 1.5 ml of 100 mM Potasium phosphate buffer, 1 ml of distilled water and 0.1 ml of enzyme extract were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken in test tubes in duplicate from each enzyme sample. Two tubes without enzyme extract were taken as control. The reaction was started by adding 0.1 ml riboflavin (60µM) and placing the tubes below a light source of two 15W florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes by black cloth. Tubes without enzyme extract developed maximum colour. A non irradiated complete mixture that did not develop colour served as blank. Absorbance was recorded at 560nm in spectrophotometer [Elico, SL196].

Enzyme Unit (EU) = (Enzyme $_{Light}$-(Enzyme $_{light}$-Enzyme $_{dark}$))/(Enzyme $_{light}$/2)

5. Proline content [3]

Leaf sample (0.5 g) was homogenized in 5 ml of sulphosalicylic acid (3%). It was centrifuged at 6000 rpm for 10 min and supernatant was collected. Reduce was again extracted twice with 5 ml, 3% aqueous sulphosalicylic acid. All the supernatant fractions were pooled and final volume was made to 15 ml. The extract (2ml) was taken in the test tube and 2 ml ninhydrin regent and 2 ml glacial acetic acid were added. The reaction mixture was put in boiling water bath for 30 min. After cooling mixture, 5ml toluene was added. Then solution mixture was shaken vigorously

and toluene fraction separated by separated funnel. The absorbance of toluene fraction was read at 520 nm with the help of spectrophotometer against toluene blank. Concentration of proline in the plant sample was estimated by estimated by referring to a standared curve of proline.

Table: 1. Details of the Treatment

T1	Control
T2	$PbNO_3$ 100 mg/kg
T3	$PbNO_3$ 200 mg/kg
T4	$CdNO_3$ 100 mg/kg
T5	$CdNO_3$ 200 mg/kg

Table: 2. Chlorophyll 'a' content

Treatments	30 DAS	45DAS	Mean
T_1	53.00	57.40	55.2
T_2	37.78(28.7)	42.23(26.4)	40.01
T_3	35.75(32.5)	41.48(27.7)	38.62
T_4	30.43(42.5)	34.00(40.7)	32.22
T_5	27.60(47.9)	30.03(47.6)	28.82
Mean	36.91	41.03	
SEM±	2.01	2.09	
C.D. at 5%	6.04	6.27	

Figure in parentheses represent percentage decrease (-) over control. DAS: Days after sowing

Table: 3. Effects of Cadmium and Lead on Nitrate Reductase activity (µ moles NO_2 g^{-1} fresh weigh hr^{-1})

Treatment	30DAS	45DAS	Mean
T_1	0.31	0.37	0.34
T_2	0.28(24.3)	0.23(25.8)	0.26
T_3	0.18(51.3)	0.17(45.1)	0.18
T_4	0.23(37.8)	0.22(29.0)	0.23
T_5	0.07(54.0)	0.13(58.0)	0.10
Mean	0.214	0.224	
SEM±	0.02	0.02	
C.D. at 5%	0.05	0.05	

Figure in parentheses represent percentage decrease (-) over control. DAS: Days after sowing

Table: 4. Effect of Cadmium and Lead on Proline content (µg g^{-1} fresh weight)

Treatment	30DAS	45DAS	Mean
T_1	20.50	22.68	21.59
T_2	24.25(18.29)	28.00(23.4)	26.13
T_3	29.38(43.31)	31.75(39.9)	30.57
T_4	28.50(39.0)	30.25(33.3)	29.38
T_5	35.00(70.7)	34.75(53.2)	34.88
Mean	27.53	29.49	
SEM±	0.66	1.86	
C.D. at 5%	1.98	4.57	

Figure in parentheses represent percentage Increases (+) over control. DAS: Days after sowing

Table: 5. Effect of Cadmium and Lead on Peroxidase activity (U g^{-1} fresh weight min^{-1})

Treatment	30 DAS	45DAS	Mean
T1	13.8	14.6	14.2
T2	14.3(3.6)	16.0(9.5)	15.2
T3	16.5(19.5)	17.5(19.8)	17.0
T4	17.8(28.90)	19.5(33.50)	18.7
T5	19.3(39.8)	21.5(47.2)	20.4
Mean	16.3	17.8	
SEM	0.22	0.33	
C.D. at 5%	0.67	1.15	

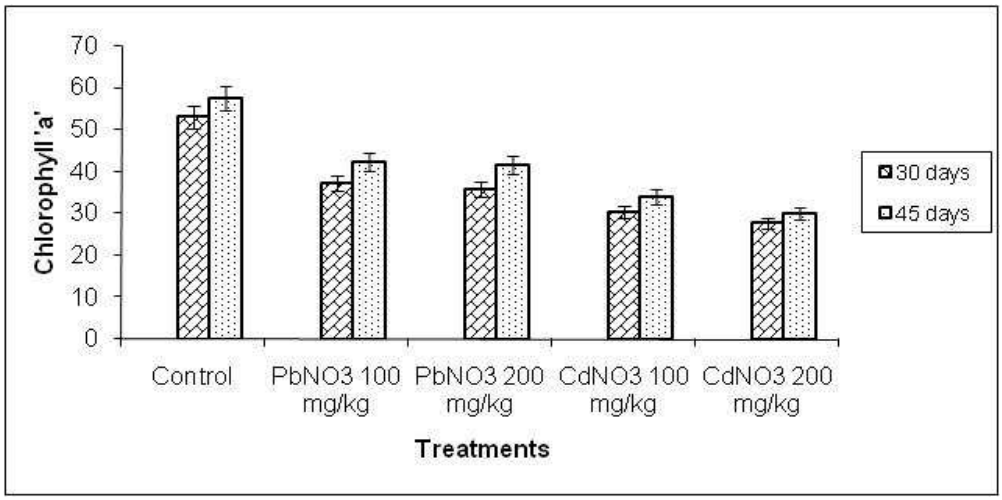
Figure in parentheses represent percentage Increases (+) over control. DAS: Days after sowing

Table: 6. Effect of Cadmium and Lead on SOD (U g^{-1} fresh weight)

Treatment	30 DAS	45DAS	Mean
T1	253.50	305.00	279.25
T2	343.75(35.6)	392.00(28.5)	367.88
T3	358.50(41.4)	396.75(30.0)	377.63
T4	307.50(21.3)	332.75(9.0)	320.13
T5	455.50(79.6)	509.75(67.1)	482.63
Mean	343.75	387.25	
SEM±	15.25	20.89	
C.D. at 5%	45.72	62.63	

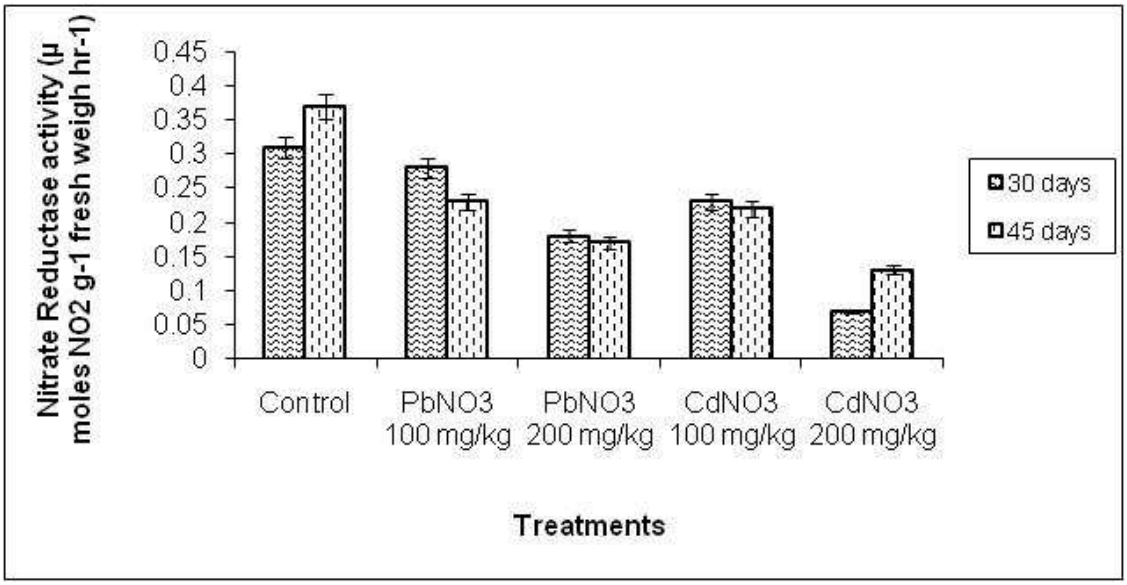
Figure in parentheses represent percentage Increases (+) over control. DAS: Days after sowing

Figure A. Effects of $CdNO_3$ and $PbNO_3$ on amount of chlorophyll 'a' after 30 and 45 days of treatment.



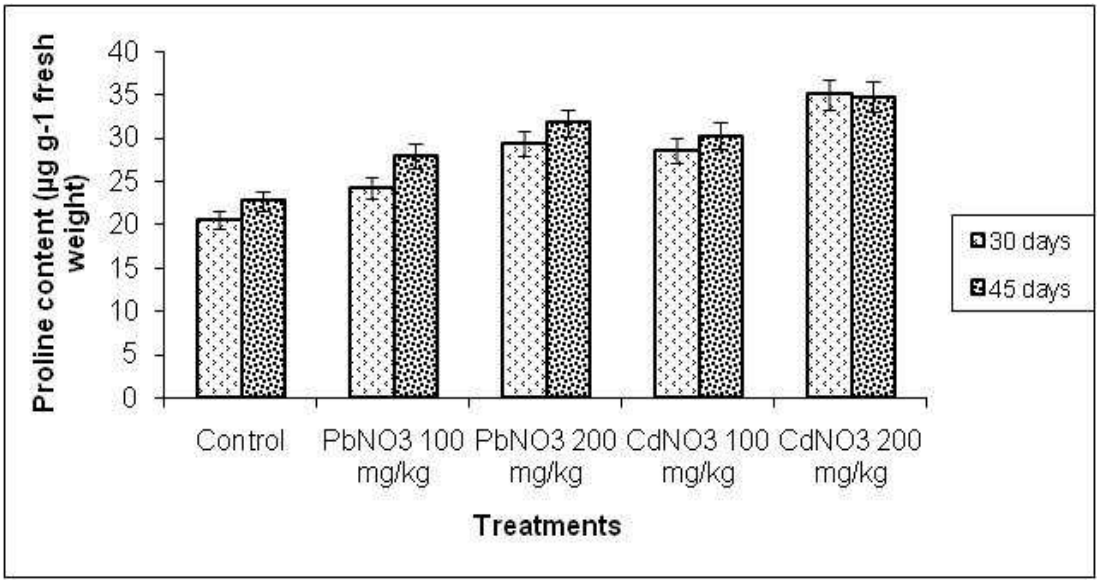
LSD (P= 0.05)

Figure B. Effects of $CdNO_3$ and $PbNO_3$ on Nitrate Reductase activity after 30 and 45 days of treatment.



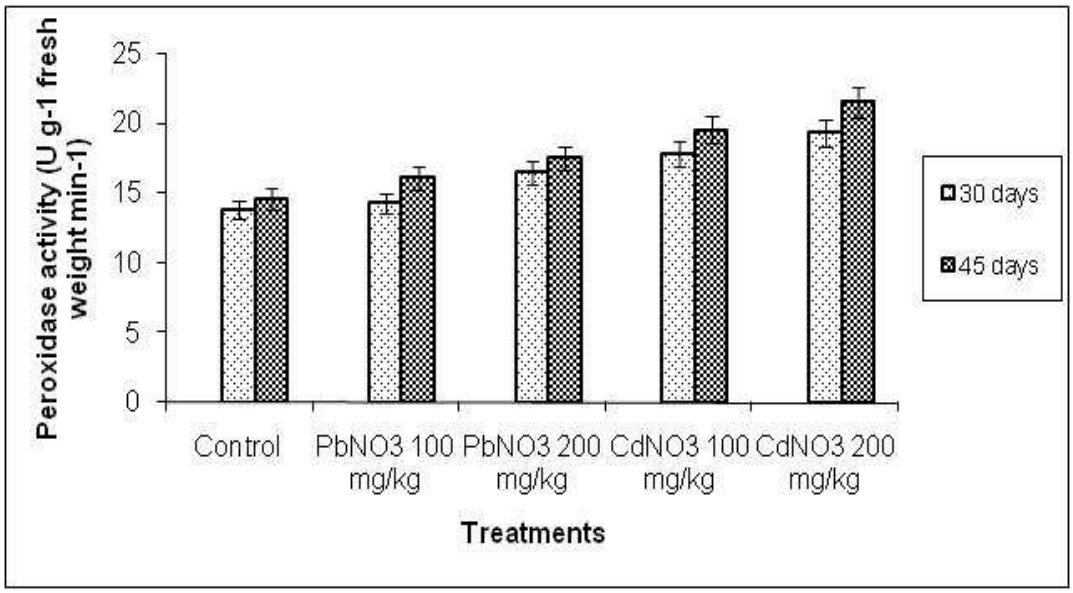
LSD (P= 0.05)

Figure C. Effects of $CdNO_3$ and $PbNO_3$ on Proline content after 30 and 45 days of treatment.



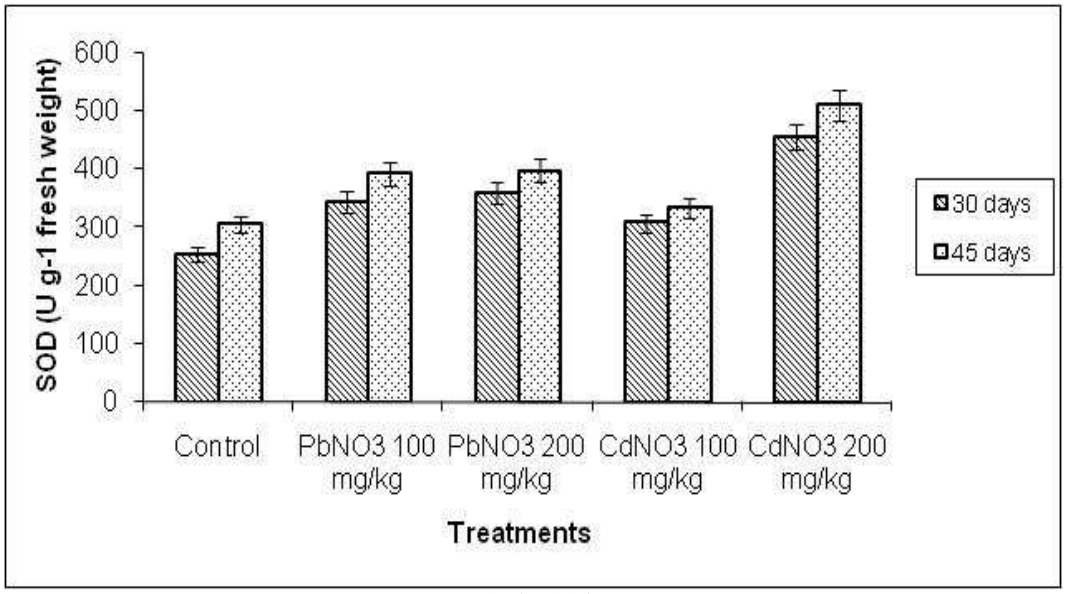
LSD (P= 0.05)

Figure D. Effects of $CdNO_3$ and $PbNO_3$ on Peroxidase activity after 30 and 45 days of treatment.



LSD (P= 0.05)

Figure E. Effects of $CdNO_3$ and $PbNO_3$ on SOD activity after 30 and 45 days of treatment.



LSD (P= 0.05)

RESULTS AND DISCUSSION

BIOCHEMICAL OBSERVATIONS

1. CHLOROPHYLL 'a' CONTENT (mg g^{-1} fresh weight)

The leaf chlorophyll 'a'content decreased with the increase level of cadmium and lead [Table 2, Figure A]. There was significant reduction in chlorophyll 'a'content at both the growth stage. Maximum reduction in chlorophyll 'a' reduction was observed in T_5 at 30 DAS, whereas, least reduction was observed in T_2 (5.6%) as compared to control. Chlorophyll 'b' content showed similar trend as to chlorophyll 'a'.

2. EFFECT OF CADMIUM AND LEAD ON NITRATE REDUCTASE ACTIVITY

Significant and greater reduction in Nitrate Reductase activity was observed at higher dosed of cadmium and lead [Table 3, Figure B]. Cadmium showed greater depression in Nitrate Reductase activity as compared to lead. Treatment T_5 recorded the least Nitrate Reductase activity and significantly differed with all other treatments. The presence of Cd and Pb significantly inhibited the activity of the enzyme Nitrate Reductase and the degree was dependent on the concentration of the metal used.

3. PROLINE CONTENT

Proline content increases from 30 DAS to 45 DAS in all the treatment except T_5. Highest significant increase in proline content was observed in T_5 (70.7%) as compared to control [Table: 4, Figure C] Greater increase in proline content was observed at 30 DAS as compared to 45 DAS. Lead and cadmium at higher doses resulted in higher production of proline in plant. The accumulation of free proline in response to heavy metal is widespread among plant. The accumulation of proline is a gene regulated process which is the consequence of the overexpression of the gene involved in its biosynthesis and depression of those involved in its degradation, in the plants under stress. The functional significance of proline accumulation under heavy metal stress might include water balance maintenance, scavenging of hydroxyl radical or metal chelates.

4. EFFECTS ON PEROXIDASE ACTIVITY

There was significant increase in peroxidase activity observed with increasing doses of cadmium and lead [Table: 5, Figure D]. Mean peroxidase activity recorded at both growing stages was least in control and highest in T_5 among treatment.

5. EFFECTS ON SUPEROXIDE DISMUTASE ACTIVITY

Changes in superoxide dismutase (SOD) activity are presented in table 5. SOD activity increased significantly with increasing doses of cadmium and lead at both growth stages. Cadmium greater increases in SOD activity as compared to lead [Table 6, Figure E]

CONCLUSION

The proline content, peroxidase and superoxide dismutase activity increase with increasing of heavy metal indicating the induction of antioxidant defence mechanism for self-defence, although they may not cope fully with the production of ROS during heavy metal stress. The highest concentration of Cd and Pb was found to be more injurious for plant.

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