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Cadmium-induced changes in leaf epidermes, photosynthetic rate and pigment concentrations in *Cajanus cajan*

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Abstract

Application of different concentrations of cadmium [5, 10, 15, 25 and 50 μg($CdCl_2$) g^{-1}(soil d.m.)] markedly affected leaves of *Cajanus cajan* (Linn.) Huth. Due to increased Cd content in leaves, stomatal density and size on abaxial epidermis, and the size of stomatal aperture and length and density of trichomes on both leaf epidermes decreased significantly in the treated plants. Net photosynthetic rate and stomatal conductance were reduced significantly at each concentration of cadmium, whereas reduction in intercellular carbon dioxide concentration was significant at 10 μg Cd onwards. The contents of chlorophyll *a*, chlorophyll *b* and carotenoids were relatively low during early stages of plant development under the effect of Cd. Nitrate content, nitrate reductase activity and protein content were also lower in treated plants, compared with control.

Additional key words: carotenoids, chlorophyll, nitrate reductase activity, proteins, stomatal conductance, stomatal density.

Introduction

Cadmium toxicity can disturb metabolic activities and hamper the growth of plants (Ali *et al.* 1998a). It disturbs water balance, supply of nutrients, stomatal function, photosynthesis, nitrogen metabolism and respiration, reduces the size and number of tracheary elements and hence alters the relative proportions of component elements of the conducting tissue (Iqbal and Khudsar 2000). At concentrations above 1 μM Cd, stomatal conductance and water potential are inhibited (Barceló and Poschenrieder 1990, Costa and Morel 1993). The disturbance that starts with water stress leads to alteration in physiological and metabolic processes (Barceló and Poschenrieder 1990) and may eventually cause structural and ultrastructural anomalies (Barceló and Poschenrieder 1999). The present study investigates the responses of leaves of *Cajanus cajan* Linn. (Huth.) to various levels of cadmium stress in terms of morphological, physiological and biochemical variations.

Materials and methods

Healthy seeds of *Cajanus cajan* obtained from IARI, New Delhi were sown in pots each containing 10 kg sterilized soil. After a month of seed germination, five different concentrations of cadmium [5, 10, 15, 25 and 50 μg($CdCl_2$) g^{-1}(soil d.m.)] were added. The untreated plants were considered as control. Sampling was done when plants were 3-month-old (pre-flowering phase), 5-month-old (flowering phase) and 6-month-old (post-flowering phase). Epidermal peels of mature leaves of the control as well as treated plants were obtained in hot nitric acid, following the method of Ghouse and Yunus (1972). The peels, dehydrated in ethanol series and stained with safranin were mounted with DPX mountant on glass slides. The dimensions of stomata and trichomes were measured with an ocular micrometer. Net photosynthetic rate and stomatal conductance were measured using gas-exchange system *LI-COR 6200* (Lincoln, USA). Chlorophyll content was estimated by the method of Hiscox and Israelstam (1979),

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Abbreviations: Car - carotenoids; Chl - chlorophyll, c_i - intercellular CO2 concentration; g_s - stomatal conductance, NR - nitrate reductase; P_N - net photosynthetic rate.

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using 100 mg of fresh leaves and 7 cm^3 of DMSO (dimethyl sulphoxide) taken in vials kept in oven at 65 °C for 2 h. 1 cm^3 aliquot and 3 cm^3 DMSO were taken in a tube and vertexed. Absorbance was read at 663, 645, 510 and 480 nm wavelengths using spectrophotometer (*Beckman DU 640B*, Fullerton, USA). The amounts of chlorophyll *a*, chlorophyll *b* and carotenoids were determined by the formulae of Duxbury and Yentsch (1956) and MacLachlan and Zalik (1963). Activity of nitrate reductase (NR) in fresh leaves was determined by the hydrazine reduction method (Klepper *et al.* 1971). Nitrate content in fresh leaves was estimated following the method of Grover *et al.* (1978), using hydrazine sulphate solution and measuring the absorbance at 540 nm. Concentration of protein was determined by using bovine serum albumin as standard (Bradford 1976). Cd content of leaf tissues was determined by the Atomic Absorption Spectrometer (*Video 11, Thermo Jarrel Ash Corporation*, Franklin, USA). The data were analysed statistically to assess the significance of the variations observed.

Results

The leaves of *C. cajan* were hypostomatic. Stomatal density and the dimensions (length and width) of stomatal apparatus and stomatal apertures declined with age of the plant. Each of these parameters was significantly reduced on the abaxial epidermis during each developmental stage of the treated plants. The maximum reduction (about 45 % in density, 31 % in length and 32 % in width of stomata) occurred at the highest Cd dose during pre-flowering stage. Reduction in size of stomatal aperture was greatest (about 28 % in length and 33 % in width) during post-flowering stage (Table 1). Length and density of trichomes on both leaf epidermes also decreased with plant age as well as

Table 1. Stomatal density [cm^{-2}], the size of stomata and stomatal apertures [μm], and stomatal conductance [mmol m^{-2} s^{-1}] on abaxial epidermis of hypostomatic leaves at different developmental stages of *Cajanus cajan* plants grown on different Cd concentrations [μg g^{-1}(soil d.m.)]. Means ± SD, $n = 25$. Differences between control and Cd-treated samples are significant at 1 % level.

$CdCl_2$ [μg g^{-1}]	Pre-flowering	Flowering	Post-flowering	Pre-flowering	Flowering	Post-flowering
	Stomatal density			Length of stomata		
0	26.40 ± 6.00	26.88 ± 10.1	31.52 ± 7.96	31.68 ± 1.51	31.95 ± 1.49	32.16 ± 1.84
5	20.64 ± 4.48	20.64 ± 8.92	22.24 ± 6.04	24.72 ± 2.63	25.20 ± 1.50	29.28 ± 1.30
10	19.04 ± 6.24	20.48 ± 9.36	22.08 ± 6.08	23.28 ± 2.49	24.60 ± 1.73	28.08 ± 2.10
15	18.60 ± 11.7	19.04 ± 5.20	20.96 ± 8.68	22.64 ± 4.84	24.60 ± 1.93	24.72 ± 1.56
25	18.40 ± 6.00	18.40 ± 7.72	20.80 ± 8.48	22.08 ± 1.70	22.68 ± 1.74	24.00 ± 2.29
50	17.20 ± 6.80	18.08 ± 6.08	20.48 ± 9.36	21.96 ± 1.67	22.44 ± 1.50	23.12 ± 4.72
	Width of stomata			Length of stomatal aperture		
0	24.56 ± 2.29	22.32 ± 1.51	22.01 ± 1.50	23.28 ± 1.79	22.56 ± 1.52	19.92 ± 1.46
5	19.68 ± 1.51	19.44 ± 1.75	18.00 ± 1.73	19.20 ± 1.50	18.84 ± 2.03	18.24 ± 1.20
10	19.20 ± 1.50	18.72 ± 1.30	17.76 ± 1.92	19.08 ± 1.46	18.60 ± 2.59	17.16 ± 1.37
15	18.60 ± 1.22	17.04 ± 1.42	17.04 ± 1.88	18.72 ± 1.79	18.60 ± 2.12	17.04 ± 1.42
25	17.04 ± 1.42	16.80 ± 1.93	16.56 ± 1.52	18.00 ± 1.93	17.52 ± 2.40	16.94 ± 1.52
50	16.68 ± 1.51	16.68 ± 1.74	16.44 ± 1.52	17.88 ± 1.83	16.20 ± 1.50	15.48 ± 1.21
	Width of stomatal aperture			Stomatal conductance		
0	7.86 ± 1.29	7.38 ± 1.31	6.96 ± 1.21	0.15 ± 0.004	0.10 ± 0.011	0.071 ± 0.050
5	5.58 ± 0.81	5.52 ± 0.94	5.52 ± 0.94	0.08 ± 0.008	0.05 ± 0.004	0.020 ± 0.002
10	5.52 ± 0.95	5.46 ± 0.85	5.28 ± 1.15	0.07 ± 0.010	0.04 ± 0.002	0.010 ± 0.005
15	5.46 ± 0.95	5.34 ± 0.97	4.92 ± 1.46	0.05 ± 0.010	0.03 ± 0.004	0.006 ± 0.001
25	5.34 ± 0.75	5.16 ± 1.15	4.68 ± 1.45	0.04 ± 0.010	0.03 ± 0.001	0.004 ± 0.002
50	5.22 ± 1.07	5.04 ± 1.29	4.62 ± 1.43	0.02 ± 0.008	0.01 ± 0.008	0.003 ± 0.001

with the dose applied. The trichomes were longer but less abundant on the abaxial epidermis than on the adaxial epidermis. The maximum reduction in length was about 23 % on adaxial epidermis and 31 % on abaxial epidermis, while that in density was nearly 41 % and 34 %, respectively, at 50 μg Cd treatment (data not given). Net photosynthetic rate (P_N), stomatal conductance (g_s), and intercellular carbon dioxide concentration (c_i) declined with plant age and were significantly lower in all stages of plant development in the treated plants with the exception that decrease in c_i was non-significant at 5 μg Cd dose. The maximum reduction (83 % in P_N, 96 % in g_s and 59 % in c_i)

was detected at 50 μg Cd during the post-flowering stage (Tables 1, 2). The chlorophyll (Chl) *a* and *b* and carotenoid (Car) contents (Table 2) also decreased with plant age as well as with cadmium stress. In case of Chl *a* and Chl *b*, the decline was non-significant at low doses (5 and 10 μg Cd). The maximum reduction in Chl *a* and Chl *b* (30 % and 65 %, respectively) appeared during pre-flowering stage, while the maximum decline (46 %) in Car content occurred during post-flowering stage at 50 μg Cd dose. Nitrate reductase (NR) activity increased while nitrate content decreased with the growing age of the plant. However, both exhibited a significant decline in treated plants in comparison with control. The maximum reduction (64 %) in NR activity occurred at flowering stage, while that of nitrate content (79 %) in pre-flowering stage at 50 μg Cd dose. The quantity of total soluble proteins in leaves decreased in relation to increasing plant age and cadmium stress, with the maximum decline (about 70 %) occurring in pre-flowering stage at 50 μg Cd treatment (Table 3). Accumulation of Cd ions in leaf tissues at different developmental stages showed a rising trend with the increasing Cd concentration in the soil medium and also with plant age (Table 3).

Table 2. Leaf net photosynthetic rate [μmol (CO_2) m^{-2} s^{-1}], intercellular CO_2 concentration [μmol mol^{-1}], and pigment concentrations [μg g^{-1}(d.m.)] at different developmental stages of *Cajanus cajan* plants grown on different Cd concentrations [(μg g^{-1}(soil d.m.)]. Mean ± SD, $n = 25$, ** = significant at 1 % level, * = significant at 5 % level, NS = non-significant.

$CdCl_2$ [μg g^{-1}]	Pre-flowering	Flowering	Post-flowering	Pre-flowering	Flowering	Post-flowering
	Net photosynthetic rate			Intercellular CO_2 concentration		
0	11.53 ± 0.35	9.50 ± 0.61	6.92 ± 0.76	264.62 ± 2.83	246.02 ± 24.71	223.56 ± 29.56
5	7.08 ± 1.91**	6.80 ± 0.51**	4.09 ± 0.21**	242.08 ± 2.02**	226.02 ± 27.87^{NS}	218.38 ± 27.84^{NS}
10	6.87 ± 1.11**	4.49 ± 0.46**	3.50 ± 0.14**	240.42 ± 6.02**	202.92 ± 23.51*	153.80 ± 28.80**
15	6.16 ± 1.05**	4.02 ± 0.03**	3.23 ± 0.08**	216.35 ± 5.17**	191.47 ± 30.10**	120.12 ± 14.97**
25	5.66 ± 0.72**	3.23 ± 0.20**	2.35 ± 0.08**	195.64 ± 3.00**	176.17 ± 28.49**	109.02 ± 26.08**
50	2.66 ± 0.31**	2.38 ± 0.65**	1.15 ± 0.02**	177.42 ± 6.49**	150.70 ± 21.41**	91.88 ± 10.83**
	Chlorophyll *a*			Chlorophyll *b*		
0	9.96 ± 0.89	4.53 ± 0.36	4.09 ± 0.39	6.68 ± 1.43	2.06 ± 0.13	1.89 ± 0.23
5	9.32 ± 0.59^{NS}	4.29 ± 0.56^{NS}	3.53 ± 0.29*	5.43 ± 0.86*	1.99 ± 0.17^{NS}	1.69 ± 0.26^{NS}
10	9.12 ± 1.76^{NS}	3.96 ± 0.16*	3.39 ± 0.43*	5.39 ± 0.76*	1.86 ± 0.13^{NS}	1.63 ± 0.13*
15	8.79 ± 0.23*	3.89 ± 0.09*	3.13 ± 0.46*	4.83 ± 0.29*	1.73 ± 0.33**	1.56 ± 0.19*
25	8.46 ± 0.33**	3.63 ± 0.39*	3.09 ± 0.06**	4.76 ± 0.19*	1.73 ± 0.13**	1.39 ± 0.09**
50	6.96 ± 0.69**	3.36 ± 0.43**	2.93 ± 0.23**	3.79 ± 0.56**	1.66 ± 0.13**	1.16 ± 0.19**
	Carotenoids					
0	1.49 ± 0.13	1.36 ± 0.03	1.23 ± 0.03			
5	1.23 ± 0.02**	1.19 ± 0.03**	0.99 ± 0.02**			
10	1.16 ± 0.02**	1.09 ± 0.06**	0.99 ± 0.03**			
15	1.09 ± 0.02**	1.03 ± 0.03**	0.93 ± 0.00**			
25	0.99 ± 0.16**	0.93 ± 0.02**	0.86 ± 0.02**			
50	0.83 ± 0.02**	0.76 ± 0.03**	0.66 ± 0.03**			

Discussion

Dimensions of stomata and stomatal pore (length and width) in abaxial epidermis of the *C. cajan* leaves were significantly reduced under cadmium stress. A greater inhibition of root growth than of shoot growth (Khudsar *et al.* 2000) might reduce water supply to shoot and lead to stomatal closure (Iqbal and Khudsar 2000). However, a direct effect of Cd on stomatal regulation cannot be ruled out (Sheoran *et al.* 1990a,b). Cd-caused effects on stomatal behaviour could be via abscisic acid (ABA) accumulation (Poschenrieder *et al.* 1989). Reduced size of stomatal aperture and/or the stomatal closure under pollution stress may be an avoidance mechanism or a protective measure (Gupta and Ghouse 1987, Iqbal *et al.* 1996). The decline of stomatal density in *C. cajan* may also be a similar response. The length and density of trichomes on leaf epidermes of the treated *C. cajan* plants remained comparatively reduced throughout.

In the present investigation, P_N, g_s and c_i were reduced significantly at each of the Cd concentrations, thus endorsing several earlier findings (Vassilev *et al.* 1998, Mehindirata *et al.* 1999). The reduction in P_N was maximum in post-flowering stage. P_N in Cd^{2+}-treated plant

was inhibited mainly as a result of low Chl content and the stomatal closure. However, disturbed PS 2 activity and inhibition of various enzymes of PCR-cycle can not be ruled out under Cd stress (for review Iqbal and Khudsar 2000). Cd inhibited activities of alcohol dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase in germinating pea seeds. The effect was concentration-dependent in the range of 0.25 to 1.0 mM $CdCl_2$ (Chugh and Sawhney 1999). Application of Zn as well as Cu can reduce the effect of Cd toxicity (Ali *et al.* 1998a,b).

Table 3. Nitrate reductase activity [μmol(NO_2) g^{-1}(d.m.) h^{-1}], nitrate content [mg g^{-1}(d.m.)], total soluble proteins [mg g^{-1}(d.m.)], and Cd content [μmol mol^{-1}] at different developmental stages of *Cajanus cajan* plants grown on different Cd concentrations [(μg g^{-1}(soil d.m.)]. Means ± SD, $n = 25$, differences between control and Cd-treated samples are significant at 1 % level.

$CdCl_2$ [μg g^{-1}]	Pre-flowering	Flowering	Post-flowering	Pre-flowering	Flowering	Post-flowering
	Nitrate reductase activity			Nitrate content		
0	9.75 ± 0.06	14.28 ± 0.13	21.57 ± 0.02	11.62 ± 0.09	7.23 ± 0.09	6.46 ± 0.30
5	7.16 ± 0.06	9.16 ± 0.03	17.85 ± 0.03	9.19 ± 0.23	6.23 ± 0.16	5.73 ± 0.13
10	6.43 ± 0.09	7.52 ± 0.20	17.32 ± 0.16	6.63 ± 0.26	5.50 ± 0.13	5.30 ± 0.23
15	5.23 ± 0.09	7.36 ± 0.20	11.85 ± 0.03	5.09 ± 0.16	4.40 ± 0.03	3.46 ± 0.26
25	4.83 ± 0.03	6.83 ± 0.16	11.42 ± 0.16	3.50 ± 0.16	2.50 ± 0.06	2.20 ± 0.09
50	4.33 ± 0.03	5.13 ± 0.03	11.91 ± 0.06	2.50 ± 0.20	2.26 ± 0.09	2.16 ± 0.23
	Total soluble proteins			Cd content		
0	22.91 ± 0.05	16.01 ± 0.04	4.86 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	22.50 ± 0.02	9.22 ± 0.05	4.26 ± 0.02	0.02 ± 0.005	0.03 ± 0.001	0.04 ± 0.005
10	17.52 ± 0.03	8.36 ± 0.06	4.16 ± 0.01	0.04 ± 0.001	0.05 ± 0.010	0.06 ± 0.010
15	16.94 ± 0.02	6.52 ± 0.03	3.89 ± 0.02	0.10 ± 0.005	0.13 ± 0.020	0.16 ± 0.020
25	12.59 ± 0.01	6.02 ± 0.04	3.77 ± 0.01	0.18 ± 0.020	0.20 ± 0.010	0.23 ± 0.020
50	6.93 ± 0.01	5.14 ± 0.06	3.70 ± 0.02	0.26 ± 0.010	0.33 ± 0.020	0.40 ± 0.005

The amounts of Chl *a*, Chl *b*, and Car in *C. cajan* were relatively low under Cd stress. This decline could involve interference with pigment metabolism, as heavy metals are known to inhibit Chl biosynthesis at the level of protochlorophyllide reductase (Stobart *et al.* 1985). Cd^{2+} causes symptoms of iron deficiency in various crops including wheat, rice, soybean, sugar beet, lettuce and maize (Iqbal and Khudsar 2000). Thus, chlorophyll content might fall due to decrease in iron uptake by the root which could lead to retarded chloroplast formation through inhibition of protein synthesis. These effects could be due to impairment in the Mg^{2+} and Fe^{2+} supply to the leaves (Greger and Lindberg 1987).

Nitrate concentration was low in the Cd-treated *C. cajan* plants. Cd toxicity seems to reduce mobilization of the endogenous nitrate. Nitrate has been shown to prevent *in situ* inactivation of enzymes in several systems (Pleg and Aspinal 1981). NR activity was also significantly low in leaves of the treated *C. cajan* plants. *In vivo* production of NADH can be inhibited due to reduced rates of photosynthesis (Bazzaz and Govindjee 1974) and respiration (Reese and Roberts 1985) in the presence of Cd^{2+}. Moreover, Cd^{2+} may stimulate NADH oxidation (Bittel *et al.* 1974) and the subsequent reduction in NADH-pool available to the enzyme. Binding of heavy metals to enzymes, consequently altering the enzyme function, has also been demonstrated (Eicchan *et al.* 1969).

Likewise, the protein content of leaves remained consistently low in the treated lot of *C. cajan.* Protein content of *Lupinus albus* root and shoot has been reported to be affected by Cd^{2+} concentration above 1 μM Cd^{2+} (Costa and Spitz 1997). It was significantly low in Cd^{2+}-treated roots of *Phaseolus vulgaris* (Chaoui *et al.* 1997). A decrease in protein content may be a consequence of decrease in NR activity, as the enzyme is believed to be rate-limiting in the overall assimilation of nitrate (Beevers and Hageman 1969). Cd^{2+} also reduces protein content by affecting protein synthesis as well as the activity of hydrolytic enzymes (Sheoran *et al.* 1990a, Dua and Sawhney 1991).

Accumulation of Cd^{2+} in leaves of *C. cajan* in different stages of development showed a positive correlation with Cd content of the soil. In general, Cd^{2+} accumulates more in roots than in shoots (Lozano-Rodriguez *et al.* 1997, Hernandez *et al.* 1998, Khudsar 1999, Kovačević *et al.* 1999), and causes greater biomass reductions, compared with Ni and Pb (Kovačević *et al.* 1999).

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