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Involvement of Ca^{2+} in Alleviation of Cd^{2+} Toxicity in Common Bean (*Phaseolas vulgaris* L.) Plants

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Abstract: In common bean plants, Cd caused significant growth retardation in stem length, stem fresh weight, stem dry weight and number of pods per plant by both concentrations (100 and 200 μM) of $CdCl_2$; while, the number of leaves and number of flowers per plant were reduced at high concentration of $CdCl_2$. The root growth parameters were not significantly responding to Cd toxicity. The addition of $CaCl_2$ (100 μM) to Cd-stressed (100 μM $CdCl_2$) plants improved the stem fresh weight, root length, number of flowers and number of pods per plant. The contents of chlorophyll a, chlorophyll b and total chlorophyll were reduced with increasing concentrations of $CdCl_2$; while, carotenoids were higher in Cd-treated plants. The addition of $CaCl_2$ increased the content of chlorophyll a and total chlorophyll, but not chlorophyll b. The total number of protein bands in SDS-PAGE protein profile was reduced in plants treated with $CdCl_2$. However, addition of $CaCl_2$ (100 mM) did not correct the changes in the number of protein bands caused by cd stress. The synthesis of high molecular weight proteins (116 and 85.54 kDa) was completely inhibited by 200 μM $CdCl_2$; while, the synthesis of low molecular weight protein (26.11 kDa) was totally blocked by both $CdCl_2$ treatments. The addition of 100 mM $CaCl_2$ restored the synthesis of a 28.57 kDa protein. The synthesis of a molecular weight polypeptide of 101 kDa was induced by the high concentration of $CdCl_2$. Progressive collapsing; disruption and browning of outer root tissues followed by cell death were noticed after $CdCl_2$ treatments. Addition of $CaCl_2$ relatively corrected the browning and collapsing of tissues caused by $CdCl_2$.

Key words: Cd^{2+} toxicity, Ca^{2+}, common bean, SDS-PAGE proteins

INTRODUCTION

Cadmium ion (Cd^{2+}) a highly toxic element that is widespread in our environment essentially due to human activities such as industrial processes. It is a suspected carcinogen to humans and toxic to living cells even at very low concentrations (Stohs *et al*., 2000).

In plants, a low concentration (5.0 μg) of Cd reduced chlorophyll content and photochemical quantum yield of photosynthesis in *Brassica napus* (Larsson *et al*., 1998; Baryla *et al*., 2001)). Cd also induced the generation of Reactive Oxygen Species (ROS) caused various toxicities in the cells, resulting in inhibition of plant growth and severely suppresses root elongation (Stohs and Bagchi, 1995; Arduini *et al*., 1996).

Studies on metal uptake pointed out that the plant transporter LCAI mediates the uptake of both Ca and Cd in yeast (Clemens *et al*., 1998). Moreover, Cd competes with Ca at both Ca channels (Nelson, 1986) and intracellular Ca-binding proteins (Rivetta *et al*., 1997). Accordingly, the current

investigation was conducted to verify the interacting effect of Ca^{2+} in alleviating the phytotoxicity of Cd^{2+} in common bean plants with a special emphasis on the role of electrophoretic patterns of SDS-PAGE proteins.

MATERIALS AND METHODS

Plant Material

The experimental plant used in this investigation was pure strain of common bean (*Phaseolas vulgaris* L. cv. Nebraska). Seeds were kindly obtained from the Agricultural Research Center in Giza, Egypt.

Experimental Design

For plantation, 25 plastic pots (20 cm) were filled with homogenous pre-sieved garden soil (loamy sand). Surface sterilized common bean seeds were soaked in the pot soil 3.0 cm deep and all pots were watered up to saturation. Seedlings were thinned to three plans per pot, kept in the open garden and irrigated regularly to field capacity until treatments.

Treatments

After two weeks from soaking, the planted pots were randomly subdivided into five equal groups (5 pots each). One group was kept irrigated with pure water and sampled as control. Another two groups were treated with two concentrations (100 and 200 μM) of $CdCl_2$. The last two groups were subjected to two combinations (100 μM + 100 mM and 200 μM + 100 mM) of $CdCl_2$ + $CaCl_2$, respectively.

Sampling and Measurements

After eight weeks from plantation, stem and root lengths, fresh and dry weights, leaf, flower and pod numbers were estimated and recorded.

Statistic

All parameters were statistically analyzed by multiple comparison procedure at $p \leq 0.05$ using t-test and mean separation by Least Significant Difference (LSD) (Steel and Torrie, 1980).

Estimation of Photosynthetic Pigments

The contents (μg g^{-1} dry weight of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids were estimated in the fresh leaves by the method of Lichtenthaler and Wellburn (1983).

Protein Electrophoresis

Extraction of Total Protein

Total protein extracts were prepared by extracting appropriate weight from the frozen plant material with 0.125 M Tris/borate, pH 8.9. All the obtained extracts were kept at 4°C for 24 h and centrifuged at 10000 rpm for 20 min. The supernatants were used for electrophoresis.

Gel Electrophoresis

SDS-PAGE was carried out with gel slabs according to the method of Laemmili (1970). Protein subunit bands were stained with Coomassie blue R-250 by standard techniques. The gel was scanned using Gel pro- Analyzer ver. 3.3 (Media Cypermetics 93-97).

Histological Examination

Eight-week-old common bean plant roots were used. Plants were cultivated under similar conditions of Cd toxicity and Ca effect assay. Root specimens were fixed in formalin: acetic acid: ethanol (FAE). Tissues were dehydrated in n-butyl alcohol, infiltrated and embedded in pure paraffin wax (m.p. = 56-58°C) as described by Johansen (1940). A rotary microtome was used to prepare serial sections (10 μ), which were then stained with safranin and light green or hematoxilin. Stained sections were examined and photographed with Zeiss Microscope.

RESULTS

Growth Parameters

Cd Phytotoxicity caused noticeable growth retardation in most of the growth parameters of common bean (*Phaseolas vulgaris* L.) plants. These growth parameters were significantly reduced by both 100 and 200 μM $CdCl_2$ (Table 1). The stem length, stem fresh weight, stem dry weight and number of pods per plant were significantly reduced by both concentrations (100 and 200 μM) of $CdCl_2$; while, the number of leaves and number of flowers per plant were reduced only by the high concentration (200 μM) of $CdCl_2$. The root growth parameters were not significantly responding to Cd toxicity. The addition of $CaCl_2$ (100 μM) to Cd-stressed (100 μM $CdCl_2$) common bean plants improved the stem fresh weight, root length, number of flowers and number of pods per plant.

Photosynthetic Pigments

As shown in Table 3, the contents of chlorophyll a, chlorophyll b and total chlorophyll were reduced in foliage leaves of common bean plants in response to the applied concentrations (100 and 200 μM) of $CdCl_2$; while, carotenoids were higher in Cd-treated plants. The addition of $CaCl_2$ increased the content of chlorophyll a and total chlorophyll, but not chlorophyll b.

Protein Electrophoresis

Cd stress induced both qualitative and quantitative changes in SDS-PAGE protein profile of common bean plants (Fig. 1, Table 2). The total number of protein band was reduced from 27 in untreated (controlled) plants to 25 and 23 bands in plants treated with 100 and 200 μM $CdCl_2$, respectively. However, addition of $CaCl_2$ (100 mM) did not correct the changes in the number of protein bands caused by Cd stress. The synthesis of high molecular weight proteins (116 and 85.54 kDa) was completely inhibited by 200 μM $CdCl_2$; while, the synthesis of low molecular weight protein (26.11 kDa) was totally blocked by both $CdCl_2$ treatments. The addition of 100 mM $CaCl_2$ restored the synthesis of a 28.57 kDa protein. The synthesis of a molecular weight polypeptide of 101 kDa was induced by the high concentration of $CdCl_2$.

Table 1: Mean vegetative and reproductive growth parameters of common bean (*Phaseolas vulgaris* L. cv. Nebraska) plants treated with $CdCl_2$ (100 and 200 μM) and $CdCl_2$ + $CaCl_2$ (100 or 200 μM + 100 mM), respectively

Treatment	Stem length (cm)	Stem fresh weight (g)	Stem dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	No. of leaves/plant	No. of flowers/plant	No. of pods/plant
C	73.00	21.80	6.70	11.00	9.20	1.30	14.00	11.66	9.30
T_1	68.00	18.10	4.70	10.30	11.60	2.50	11.60	10.00	7.00
T_2	67.00	15.20	3.81	10.00	7.16	1.60	8.30	7.30	7.30
T_3	70.00	21.00	4.86	12.40	11.50	2.80	12.00	12.33	8.60
T_4	68.00	16.33	4.00	14.50	8.16	1.53	10.30	9.30	8.00
LSD (p = 0.05)	2.432	2.268	1.622	2.011	2.136	1.232	2.193	2.093	1.435

C: Controlled plants, T_1, T_2: Plants treated with 100 and 200 UM $CdCl_2$, respectively, T_3: Plants treated with 100 uM $CdCl_2$+100 mM $CaCl_2$, T_4 = Plants treated with 200 uM $CdCl_2$+100 mM $CaCl_2$

Table 2: Comparative analysis of relative concentration, molecular weight (M.wt.) and rate of mobility (R_m) of SDS-PAE protein profile of common bean (*Phaseolas vulgaris* L. cv. Nebraska) plants treated with $CdCl_2$ or $CdCl_2$ + $CaCl_2$

	Treatment and Band (%)						
Band No.	1	2	3	4	5	R_m	Mol. wt. (KDa)
1	0.71	0.42	--	0.55	--	0.07	116.00
2	--	--	0.16	--	--	0.10	101.01
3	0.32	0.21	--	0.26	--	0.18	85.54
4	1.21	2.37	0.95	1.88	1.77	0.22	79.28
5	0.07	0.76	0.46	0.57	0.44	0.25	76.81
6	0.04	--	0.42	0.19	--	0.27	72.33
7	1.03	2.06	0.81	1.77	2.34	0.33	66.22
8	0.30	1.21	0.97	0.58	0.80	0.36	48.15
9	0.48	0.24	--	0.22	0.46	0.40	36.75
10	0.82	0.86	0.45	0.63	0.97	0.43	35.39
11	1.53	2.52	1.02	1.37	2.70	0.47	33.06
12	0.29	0.38	0.44	--	0.57	0.50	31.68
13	0.20	0.36	0.33	0.22	0.10	0.52	30.97
14	0.42	0.59	0.39	0.33	0.21	0.54	30.04
15	0.21	--	--	0.30	0.14	0.58	28.57
16	1.19	1.89	0.65	1.19	1.95	0.61	27.20
17	0.28	---	--	--	--	0.64	26.11
18	2.89	4.09	1.04	2.33	3.29	0.67	24.65
19	15.20	14.00	29.00	21.40	21.00	0.71	23.21
20	17.80	32.40	21.20	20.80	28.90	0.76	21.68
21	8.40	5.61	7.59	6.37	5.81	0.79	20.68
22	3.05	4.31	3.29	3.63	2.24	0.82	19.70
23	3.43	1.87	1.22	1.06	2.27	0.84	19.22
24	3.19	0.73	0.92	1.47	0.55	0.86	18.65
25	2.33	3.35	1.63	1.31	1.88	0.89	17.75
26	1.93	1.98	0.72	0.27	0.47	0.91	17.26
27	1.54	1.82	0.45	1.25	0.90	0.94	16.34
28	3.07	2.38	1.19	2.41	1.07	0.98	15.51
Bands/lane	27	25	23	25	23		

T_1: Untreated (control) plants, T_2 and T_3 Plants treated with 100 and 200 μM $CdCl_2$, T_4 and T_5 Plants treated with 100 $CdCl_2$+100 mM $CaCl_2$ and 200 μM $CdCl_2$ + 100 mM $CaCl_2$; respectively

Table 3: Photosynthetic pigments (μg g^{-1}) in foliage leaves of common bean (*Phaseolas vulgaris* L.) C, untreated (Controlled) plants treated with $CdCl_2$ or $CdCl_2$ + $CaCl_2$

	Chlorophyll (μg g^{-1})			
Treatment	Chlorophyll (a)	Chlorophyll (b)	Carotenoids	Total chlorophyll
C	28.82	18.96	0.87	47.77
T_1	22.24	9.07	5.96	31.33
T_2	15.48	6.96	3.36	22.44
T_3	25.05	5.76	6.87	31.81
T_4	18.42	6.43	4.33	24.85

C: Untreated (control) plants, T_1 and T_2: Plants treated with 100 and 200 μM $CdCl_2$, T_3 = Plants treated with 100 μM $CdCl_2$+ 100 mM $CaCl_2$, T_4: Plants treated with 200 μM $CdCl_2$ +100 mM $CaCl_2$

Histological Staining

Thin sections from common bean root (Fig. 2) indicated progressive collapsing and disruption of the epidermal and cortical tissue layers of the root tissues and browning of tissues followed by root cell death caused by $CdCl_2$ treatments (Fig. 2B, C) compared to the root of untreated plants (Fig. 2A). The addition of $CaCl_2$ relatively prevented the brown coloration and collapsing of tissues caused by the low and high concentrations of $CdCl_2$ (Fig. 2D, E).

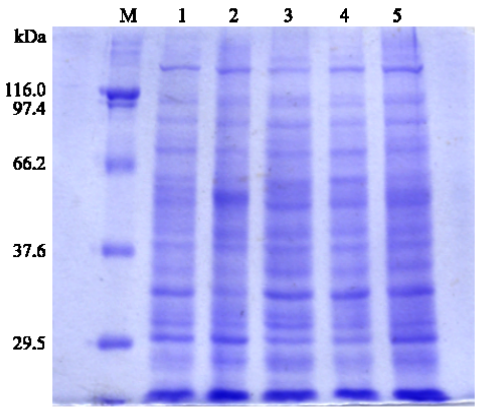

Fig. 1: Electrophotograph of SDS-PAGE of total proteins of common bean (*Phaseolas vulgaris* L. cv. Nebraska) plants. 1, untreated (controlled) plants (track 1); 2 and 3, plants treated with 100 and 200 μM $CdCl_2$ (tracks 2 and 3); 4 and 5, plants treated with 100 and 200 μM $CdCl_2$ + 100 mM $CaCl_2$ (tracks 4 and 5); M, molecular weight markers used on polyacrylamide gel

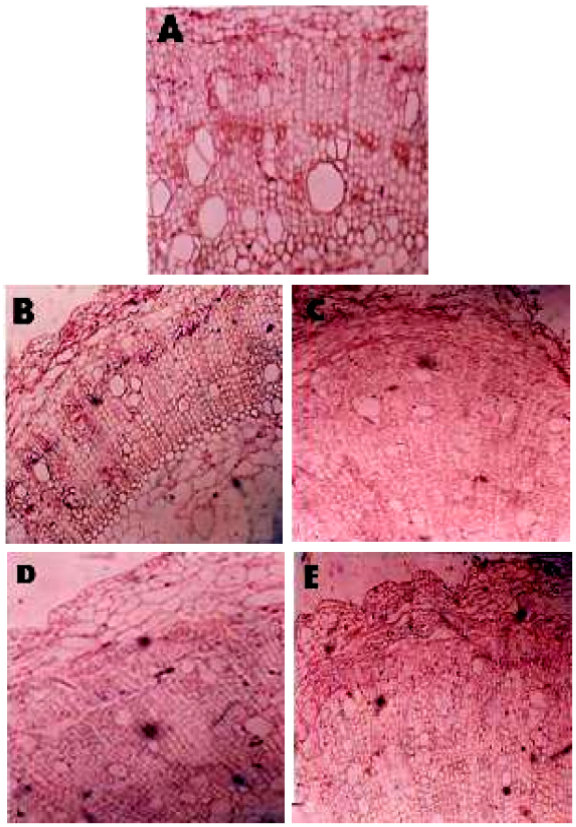

Fig. 2: Effect of $CdCl_2$ on the epidermal and cortical tissues of common bean (*Phaseolas vulgaris* L.) plant roots. (A) untreated plants, (B and C) plants treated with 100 and 200 μM $CdCl_2$, respectively, (D) plants treated with 100 μM $CdCl_2$ + 100 mM $CaCl_2$ and (E) Plants treated with 200 μM $CdCl_2$ + 100 mM $CaCl_2$, respectively

DISCUSSION

In common bean plants, the results of this investigation reported on a variety of phytotoxic effects of Cd to plant growth, chlorophylls, protein synthesis and root tissue damages as well. Consistent findings reported that free Cd and other heavy metal ions often reduce cellular activities for a variety of reasons; for example, by generating oxidative stress and inhibition of enzyme reactions. Suzuki (2005) reported on plants incubated for two weeks in a sublethal level (200 μM) of Cd, root cells survived but with irregular thickening of cell walls and enlarged and unusually formed cells. Although it is not clear which mechanism caused those growth changes in root cells, Cd may have altered some metabolic pathways i.e., cell division block through the lack of Glutathione (GSH) integrity in plant roots (Vernoux *et al*., 2000), or Cd affected directly GSH metabolism (Xiang and Oliver, 1998).

In this report, it was showed that Ca reduced the toxic effects of Cd in common bean plants. Relevant results reported that Ca reduced the uptake, accumulation and toxic effects of Cd in *Arabidopsis* (Suzuki, 2005) and radish (Rivetta *et al*., 1997). Cd tolerance increased in tobacco in the presence of Ca, that probably due to the active exclusion of toxic Cd by the formation and excretion of Cd/Ca containing crystals through the head cells of trichomes (Choi *et al*., 2001). It was also previously reported that the accumulation of Cd on seed germination of radish is reduced by high concentration of Ca (Rivetta *et al*., 1997).

Several mechanisms for Ca alleviation of mineral toxicity have been adopted. A proposed mechanism is the displacement of cell-surface toxic cations by Ca. Since plasma membrane surfaces are negatively charged, high level of Ca^{+2} would reduce cell-surface negativity and alleviate the harmfulness of cationic toxicants (Kinraide, 1898). The other proposed mechanism is the uptake of Cd through calcium channels blockers, diltiazem, verapmil, nifedipine and nitrendipine (Blazka and Shaikh, 1991). It is evident that a large number of carrier proteins are involved in the transport of Cd (Maser *et al*., 2001). As a major defense mechanism, the inactivation of these metal ions could be accomplished by complexing them with phytochelatins such as metalothionin and cysteine-rich proteins (Di Toppi and Gabbrielli, 1999; Song *et al*., 2004).

In conclusion, Ca ion could be applied in alleviating Cd Phytotoxicity in common bean plants in moderately Cd-contaminated soils. Such treatments would be worked out if the Cd concentration outside could be kept below a critical threshold level (Haag-kerwer *et al*., 1999). Further investigations would focus on elucidating the precise role of Ca in decreased Cd influx into plants.

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