J. Plant Biotechnology(2004) Vol. 6(4). pp. 259~264

Journal of Plant Biotechnology

Amelioration of Cd^{++} Toxicity by Ca^{++} on Germination, Growth and Changes in Anti-Oxidant and Nitrogen Assimilation Enzymes in Mungbean (*Vigna mungo*) Seedlings

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Abstract

The present study describes the ameliorating effect of Ca^{++} on Cd^{++} toxicity on the germination, early growth of mungbean seedlings, nitrogen assimilation enzyme. s- nitrate reductase (NR), nitrite reductase (NIR), anti-oxidant enzymes (POD, CAT and SOD) and on the accumulation of hydrogen peroxide and sulphydryls. Cd^{++}inhibited seed germination and root and shoot length of seedlings. While NR activity was down- regulated, the activities of NIR, POD and SOD were up- regulated with Cd^{++} treatment. Cd^{++} treatment also increased the accumulation of sulphydryls and peroxides, which is reflective of increased thiol rich proteins and oxidative stress. Ca^{++} reversed the toxic effects of Cd^{++} on germination and on early growth of seedlings as well as on the enzyme activities, which were in turn differentially inhibited with a combined treatment with calcium specific chelator EGTA. The results indicate that the external application of Ca^{++} may increase the tolerance capacity of plants to environmental pollutants by both up and down regulating metabolic activities.

Abbreviations: Cd^{++}= cadmium, Ca^{++} = calcium, NR= nitrate reductase, NIR=nitrite reductase, POD = peroxidse, SOD= superoxide dismutase, CAT= catalase, EGTA= ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid.

Key words: Cadmium, antioxidant and nitrogen assimilation enzymes, calcium, signal transduction, sulphydryls and peroxide accumulation, *Vigna mungo*.

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Received Feb. 22, 2004; Accepted Dec. 13, 2004

Introduction

With increasing air, water and soil pollution, plants are constantly exposed to higher concentrations of gaseous pollutants and heavy metals. As plants are sessile and non-mobile they have to cope up with this situation. Consequently, they have evolved strategies to combat these situations through certain physiological and biochemical mechanisms. The intra-cellular regulation of concentrations of heavy metals and other pollutants is determined by several factors such as energy dependent efflux of metals, changes in oxidative metabolism by the generation of reactive oxygen species and by the synthesis of new proteins and peptides such as phytochelatins (Hernanbez et al. 1993; Mehra and Tripathi 2000).

Cd^{++} is an important environmental pollutant with high toxicity to animals and plants and is released into the environment through many avenues such as traffic, metal-working industries, mining and as a by-product of mineral fertilizers (Nriagu 1990). Sedimentary rock type soils, volcanoes and ocean sprays are some of the natural sources of Cd^{++} (Page and Bingham 1973). Ca^{++} regulates numerous physiological processes in plants as a second messenger including the response to environmental stresses and is now a well-known component of signal transducing pathways in plants and exerts its effects by binding to Ca^{++} receptors or Ca^{++} binding proteins such as calmodulin and Ca^{++} dependent protein kinases (Hepler and Wayne 1985; Ladror and Zielinski 1989; Rengel 1992; Sheen1996; Knight and Knight 1999). However, the role of Ca^{++}+ in heavy metal toxicity is not well documented. The literature on the involvement of Ca^{++} and Ca^{++} binding proteins in the response of plants to heavy metal stresses is scanty and the signal transduction pathways have not been worked out

(Sheen 1996). This paper, therefore, presents data indicating the involvement of Ca^{++} in the homeostasis of cadmium. It is shown that Ca^{++} is able to ameliorate some of the Cd^{++} effects through its involvement in nitrogen assimilation and anti-oxidant enzymes.

Materials and Methods

Growing of seedlings

Seeds of mungbean (*Vigna mungo* variety 19 a summer season crop) were obtained from a local seed store. Seeds of uniform size were selected, washed with distilled water and treated with 0.1% mercuric chloride (w/v) for 2 min after which they were thoroughly rinsed with distilled water. The seeds were then sown in petridishes lined with cotton pads moistened with water or solutions of specified concentrations of Cd^{++} singly and in combination with Ca^{++} with or without EGTA a Ca^{++} specific chelator. The experiments were con ducted in a temperature controlled illuminated growth chamber (Heraus-Votsch, temp 25±2℃, 60% RH, 16,000 lux light). Percent germination and early growth of seedlings were recorded every day up to 96 hr. The seedlings were sampled for the extraction of antioxidant, nitrogen assimilation enzymes, total sulphydryls and peroxides at the end of the experiments. The data presents the mean of three independent experiments.

Extraction of enzymes

Seedlings without roots (5.0 g) were extracted in 50 mM phosphate buffer (pH 7.0) containing 1.0% PVP in the ratio of 1:2 (w/v) for anti-oxidant enzymes. For the extraction of NR and NIR phosphate buffer of pH 7.8 was used and the medium also contained 1 mM DTT and EDTA. The seedlings were frozen with liquid nitrogen and homogenised in a pestle and mortar. The extract was filtered through 4 layers of cheese cloth. The homogenate was then centrifuged at 4℃ in a refrigerated centrifuge (Sorvall RC 5 B) at 15,000 xg for 20 min. The supernatant was designated as the crude enzyme and was used after desalting by passing it through a column of Sephadex G 25. Protein in the extracts was determined by the method of Lowry et al. (1951) using BSA as a standard.

Nitrate and nitrite reductase activity

Nitrate reductase activity was determined according to the procedure used earlier (Sane et al. 1987). The assay mixture (2 ml) contained 1.5 ml of 100 mM phosphate buffer (pH 7.5), 0.2 ml of 0.1 M KNO_3, 0.2 ml of NADH (1mg/ml). The activity was measured as the amount of nitrite produced per min after incubation of the reaction mixture for 1 hr. For the determination of nitrite reductase activity, the disappearance of nitrite was measured essentially by the method of Sharma and Sopory (1984).

SOD activity

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride by the method of Dhindsa et al. (1981). The 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 μM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0-100 μl enzyme extract in each case. The riboflavin was added in the end and the tubes were shaken and placed below a light bench fitted with two 15 W fluorescent tubes. The reaction was started by switching on the lights and was allowed to run for 10 min when the lights were switched off and the tubes were covered with a black cloth. The reaction mixture without the enzyme developed the maximum colour and the non-irradiated reaction did not develop any colour and served as the control. The absorbance of the reaction mixture was read at 560 nm in a spectrophotometer (Bausch and Lomb, Spectronic 2000). The amount of enzyme resulting in 50% inhibition of the photochemical reaction was regarded as one unit of enzyme activity and the data is presented as units per mg protein. To ensure that it was the SOD that was measured, a control was run with 3 mM KCN that inhibited the enzyme activity almost completely.

POD activity

POD activity was measured spectrophotometrically as guaiacol peroxidase (Zaharieva et al. 1999) by following the formation of tetraguaiacol at 470 nm (e = 26.6 mM^{-1} cm^{-1}). The reaction mixture (1 ml) consisted of phosphate buffer (50 mM pH 7.0) 2 mM H_2O_2 and 2.7 mM guaiacol. The reaction was started by the addition of 100 ml enzyme extract in each case. One unit of enzyme activity is defined as amount of enzyme required for the consumption of 1 m mol of H_2 O_2 /min. The level of peroxidase activity is reported on mg protein basis.

Catalase activity and accumulation of H_2O_2

CAT activity was determined by the oxidation of hydrogen peroxide at 240 nm spectrophotometrically (ε of H_2O_2 = 46.3 $mM^{-1}cm^{-1}$) by the method of Aebi, 1983. The activity is expressed as change in OD. The accumulation of hydrogen

peroxide in each treatment was also monitored. The H_2O_2 contents were calculated from a standard curve drawn using different concentrations of H_2O_2.

Non-protein sulphydryls

Total non protein sulphydryls contents in the control and treated seedlings was measured. The samples (500 mg fresh wt) were frozen in liquid nitrogen and extracted in sulphosalicyclic acid using the method described by Gupta et al. (1999).

Results

Germination

Table 1 shows that 95-100% seeds germinated in the con trol and the presence of Ca^{++} did not have any appreciable effect on the germination. Cd^{++} (200 μM) inhibited germination by 62-65% (Table 1) and this effect was ameliorated by Ca^{++}. Ca^{++} at a concentration of 200 μM restored 48% of Cd^{++}caused inhibition but the effect of Ca^{++} itself did not differ from the control. EGTA inhibited the germination by 50% (Table 1) and did not restore the Cd^{++} caused inhibition. However, Ca^{++} in combination with EGTA (200 μM each) reversed 48% of EGTA caused inhibition of germination and 58% of the inhibition of germination caused by Cd^{++} and EGTA together. But EGTA itself did not show any additive inhibitory effect on Cd^{++}caused inhibition when given in combination (200 μM each).

Early seedling growth

Both root and shoot lengths were inhibited by Cd^{++}. Root and shoot lengths were inhibited by 80% and 63% respectively with 200 μM Cd^{++} as compared to control. While Ca^{++} itself did not affect either root or shoot lengths, it ameliorated 68% of inhibition of root length and 52% of shoot length caused by Cd^{++} (Table 1). A 20% inhibition of root length and 35% inhibition of shoot length was observed by EGTA treatment (200 μM). This inhibition was completely reversed by Ca^{++} (Table 1). Cd^{++} + EGTA given in combination also inhibited root and shoot lengths, but 56% of this inhibition was overcome by Ca^{++} + when given in combination with Cd^{++}+ EGTA.

NR and NIR activity

Cd^{++} depressed the NR activity of the seedlings by 60% but stimulated the NIR activity by 70% (figure 1). Ca^{++} (200 μM) stimulated both NR and NIR activities by 30 and 20% respectively. Ca^{++} also ameliorated the Cd^{++}caused inhibiton of NR activity by 55% and Cd^{++}caused promotion of NIR by 45%. EGTA (200 μM) lowered the NR activity by 15-20% but had no effect on NIR activity. EGTA also did not have any effect on Cd^{++}caused inhibition of NR activity but reduced the Cd^{++}caused stimulation of NIR by 40%. Ca^{++}in combination with Cd^{++} and EGTA reversed the Cd^{++}inhibition of NR activity by 50% and reduced the Cd^{++}caused stimulation of NIR activity by 33% (Figure 1).

Sulphydryl content

Cd^{++} brought about ten fold increase in sulphydryls as compared to control. Ca^{++} (200 mM) itself had no effect and did not induce any SH accumulation but reduced the Cd^{++} caused accumulation of sulphydryls by 50% when given in combination with Cd^{++}or EGTA (Figure 2). It may be noted that Cd^{++} induced accumulation of SH was reversed by 33%

Table 1. Effect of Cd^{++}, Ca^{++} and Ca^{++} chelator EGTA singly and in combination on the germination and early growth of *Vigna mungo* L seedlings.

Treatment	Germination (%)[a]	Root length (cm)[b]	Shoot length (cm)[b]
Control	97±3.0	5.16±0.46	5.02±0.79
Cadmium (Cd^{++}) 200 μM	36±2.48	1.05±0.42	1.86±0.39
Calcium (Ca^{++}) 200m μM	95±3.9	5.10±0.43	4.75±0.50
Cd^{++} 200 + Ca^{++} 200m μM	69±3.08	3.33±0.69	3.92±0.42
EGTA 200m μM	46±2.96	4.71±0.33	3.23±0.57
Cd^{++} 200 μM + EGTA 200 μM	38±3.09	1.99±0.72	3.37±0.52
Ca^{++} 200 μM + EGTA 200 μM	88±4.06	5.07±0.51	5.10±0.57
Cd^{++} 200 μM + Ca^{++} 200 μM + EGTA 200 μM	90±4.26	4.53±0.47	4.48±0.49

[a]Data taken after 24 hr., [b]Data taken after 96 hr.

when EGTA and Ca^{++} were applied alongwith Cd^{++} but the SH content was still 3-4 times higher than the control (Figure 2).

SOD activity

Cd^{++} (200 μM) treatment increased the SOD activity 2 fold over control. Ca^{++} reversed this effect by 41% (Figure 3). However, both Ca^{++} and EGTA when used alone also increased SOD activity by 70% and 25% respectively. EGTA reversed the Ca^{++} effect completely but was unable to ameliorate the Cd^{++} caused stimulation of SOD activity to any appreciable extent (Figure 3).

POD activity

Cd^{++} increased POD activity over control by 2-3 fold (Figure 4). While Ca^{++} (200 mM) also increased POD activity by 50% but reduced the Cd^{++} caused stimulation by of POD by 35-40%. EGTA although itself had no effect on POD activity but reduced the Cd^{++} caused stimulation by 40%. A similar ameliorating effect was observed when EGTA, Ca^{++} and Cd^{++} were given in combination (Figure 4).

CAT activity

The CAT activity was only marginally increased by Cd^{++} (200 mM) treatment. Ca^{++} and EGTA (200 μM) each alone inhibited the CAT activity by 50% over control. This inhibition was reduced when Ca^{++}or EGTA were given in combination with Cd^{++}(Figure 5).

Peroxide accumulation

A three-fold enhancement of hydrogen peroxide accumulation was observed by Cd^{++} treatment over control. Ca^{++} treatment was ineffective in this respect but reduced the Cd^{++} - induced peroxide accumulation by 40% (Figure 6).

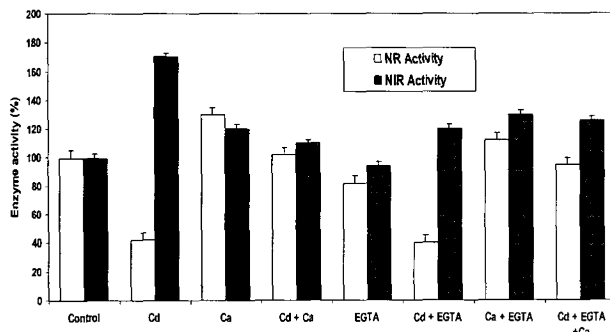

Figure 1. Nitrate and nitrite reductase (NR and NIR) activities (%) in the mungbean seedlings treated with 200 μM each of Ca^{++}, Cd^{++} and EGTA (Ca^{++} specific chelator) given singly and in combination (values are mean $\pm$ S.E., n=3).

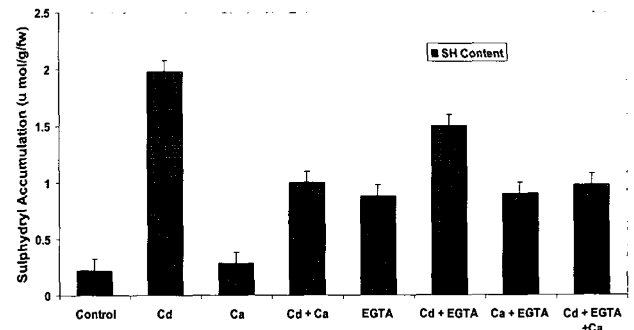

Figure 2. Total non-protein sulphhydryls in the mungbean seedlings treated with 200 μM each of Ca^{++}, Cd^{++} and EGTA (Ca^{++} specific chelator) given singly and in combination (values are mean $\pm$ S.E., n=3).

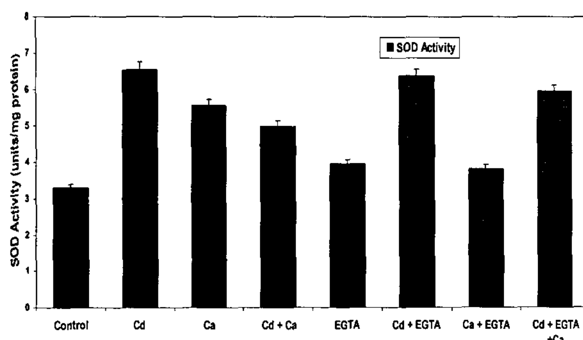

Figure 3. Superoxide dismutase (SOD) activity (units/mg protein) in the mungbean seedlings treated with 200 μM each of Ca^{++}, Cd^{++} and EGTA (Ca^{++} specific chelator) given singly and in combination (values are mean $\pm$ S.E., n=3).

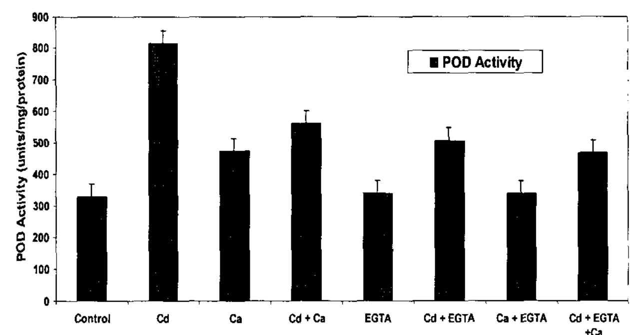

Figure 4. Peroxidase (POD) activity (units/mg protein) in the mungbean seedlings treated with 200 μM each of Ca^{++}, Cd^{++} and EGTA (Ca^{++} specific chelator) given singly and in combination (values are mean $\pm$ S.E., n=3).

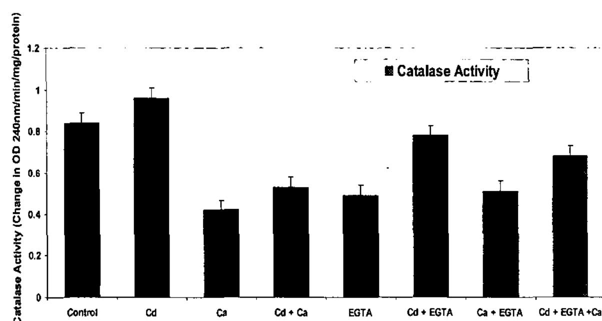

Figure 5. Catalase (CAT) activity (change in OD 240 nm/mg protein) in the mungbean seedlings treated with 200 μM each of Ca^{++}, Cd^{++} and EGTA (Ca^{++} specific chelator) given singly and in combination (values are mean ± S.E., n=3).

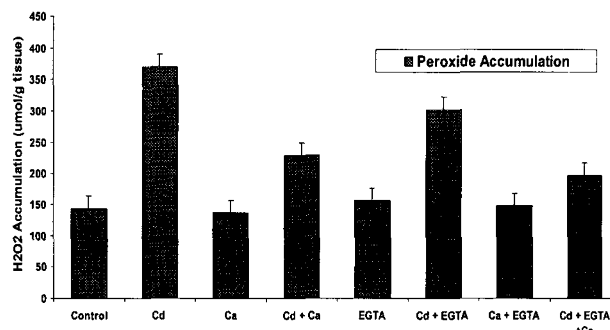

Figure 6. Accumulation of H_2O_2 (OD 240 nm) in the extracts of mungbean seedlings treated with 200 μM each of Ca^{++}, $Cd^{++}$and EGTA (Ca^{++} specific chelator) given singly and in combination (values are mean ± S.E., n=3).

Similarly EGTA itself had no effect but reduced the Cd^{++} - induced enhancement by 25%. However, EGTA in combination with Ca^{++} reduced the Cd^{++} - induced peroxide accumulation by 53% (Figure 6).

Discussion

The results presented in this paper indicate that Ca^{++} can ameliorate the toxic effects of Cd^{++} in germination and early seedling growth of mungbean seedlings and the reversal is manifested through its effect on nitrogen assimilatory enzymes (NR and NIR) and anti-oxidant enzymes (SOD, POD and CAT). While NR activity was down regulated by Cd^{++} treatment, the effect increased with the increasing concentration (data not given), the activities of NIR, SOD, POD and CAT were up-regulated. The Ca^{++} treatments in combination with Cd^{++} reversed these effects completely or partially. While the role of Ca^{++} in ameliorating Al toxicity is known (Rengel 1992) but its effect in amelioration of Cd^{++} effects is not well documented. A stimulatory effect of Ca^{++} on *in vitro* NR activity was reported by us earlier (Sane et al. 1987) and an inhibition of NR activity by lead and other heavy metals in hydrophytes was reported by Rai et al. 1995. The effect of Cd^{++} on NIR activity observed here is in contrast to that of NR. The NIR activity was promoted by Cd^{++} treatment. But similar to NR the stimulatory effect of Cd^{++} on NIR activity was also reversed by Ca^{++}. EGTA itself only slightly reduced NR activity but reversed the Cd^{++} induced NIR promotion. Ca^{++} and EGTA together reduced the inhibitory effect of EGTA alone and the three together behaved in similar way. The effects of heavy metal stresses on NIR and their interaction with Ca^{++} are not well known. While the role of Ca^{++} and nitrogen oxide (NO) in the signalling pathways of pathogen defence are known in literature but that of other species of nitrogen such as nitrogen dioxide and nitrite in relation to heavy metal effects is still unknown. Therefore, it is possible that nitrite along with Ca^{++} may be involved as a signal in the detoxification of heavy metal effects. The increased activities of SOD, POD and CAT and H_2O_2 accumulation are indicative of oxygen burst as a result of Cd^{++} treatment. SOD catalyses the removal of super oxide anions by yielding hydrogen peroxide and molecular oxygen and thus, protects the cells from the deleterious effects of superoxide free radicals (O_2^-). Heavy metals are known to generate oxygen free radical species as active oxygen species especially in the fresh water ecosystems (Frostner and Prasi 1979). But there are hardly any reports on the effect of Ca^{++} in scavenging these active oxygen species. It is interesting, therefore, to note that while Ca^{++} itself had only a slight effect on POD, CAT and SOD activities but reversed the Cd^{++} induced high activities of these enzymes (Figure 3, 4 and 5). There was a marked increase in H_2O_2 accumulation in the Cd^{++} treated seedlings and Ca^{++} reduced this accumulation significantly (Figure 6). In the presence of EGTA, the higher POD and CAT activity induced by 200 μM Cd^{++} is reduced by Ca^{++} as well as by EGTA and also by a combination of Cd^{++} + Ca^{++}+ EGTA. The role of Ca^{++} in ameliorating the Cd^{++} toxicity through scavenging this oxygen burst through the anti oxidant enzymes is an interesting and novel finding of these investigations. Price et al (1994) in deed reported an increase in cytosolic Ca^{++} due to oxidative stress in tobacco seedlings as a result of chilling injury. But in case of SOD activity, neither Ca^{++} nor EGTA is able to reverse the effect appreciably and even when the three- Cd^{++}, Ca^{++} and EGTA were given together the SOD activity still remained high.

The effects of calcium on the accumulation of sulphydryls (a common phenomenon in heavy metal toxicity) are also interesting. There was an accumulation of sulphydryls as a result of Cd^{++} treatment but Ca^{++} had little effect (Figure 2).

The levels of sulphydryls still remained high with combinations of Cd^{++} and Ca^{++}. The levels of sulphydryls remained higher even with EGTA and Cd^{++}+EGTA. None of Ca^{++} or EGTA alone or together could bring the levels of sulphydryls to the control levels (Figure 2). The accumulation of more sulphydryls as a result of Cd^{++} application is also indicative of the synthesis of sulphydryl- rich peptides (phytochelatins) consequent upon Cd^{++} application. It has been reported that sulphydryls as GSH may be involved in signalling pathways during acclimation of plants to chilling and cold (Kocsy et al 2001). From these studies it is apparent that external application of Ca^{++} may increase the tolerance capacity of plants to the environmental pollutants such as Cd^{++} both by up- and down-regulating metabolic activities. Work is now in progress to pin point the exact role of Ca^{++} in the amelioration of Cd^{++} toxicity by measuring the Cd^{++} contents of seedlings treated with Ca^{++} and Cd^{++} together. The preliminary results (unpublished) are suggestive of a disturbance of the Cd^{++}uptake by Ca^{++}.

Acknowledgements

The authors are thankful to Dr. P. Pushpangadan, Director National Botanical Research Institute Lucknow for providing the lab facilities. Technical help provided by Miss Aquila Bano is also acknowledged.

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