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EFFECT OF CALCIUM CHLORIDE ON HEAVY METAL INDUCED ALTERATION IN GROWTH AND NITRATE ASSIMILATION OF *SESAMUM INDICUM* SEEDLINGS

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Key Word Index—*Sesamum indicum*; copper; cadmium; heavy metal; lead; nitrate reductase; Pedaliaceae.

Abstract—In the early growth phase of *Sesamum indicum* cv. PB-1, the decrease in fresh and dry mass was higher with 1.0 mM Cd^{2+} than with the same level of Pb^{2+} and Cu^{2+}. Recovery from the metal stress was considerable in the root fresh weight and almost completely in the root dry weight when 10.0 mM (1.9 EC), calcium chloride was supplied to the growing seedlings along with the metal salts in various combinations. Accumulation of Pb^{2+}, Cd^{2+} and Cu^{2+} was differential to the metals and the plant parts when supplied without or with 10.0 mM calcium chloride. The order of endogenous metal accumulation was $Cu^{2+} > Cd^{2+} > Pb^{2+}$ and roots accumulated more metal than the leaves in the absence, as well as in the presence, of calcium chloride. Calcium chloride could recover loss of *in vivo* NRA in roots caused by either of the metal combinations, whereas the salt could recover the loss in leaf NRA caused only by Pb^{2+}–Cd^{2+} (1.0 mM each). Response of root and leaf NRA was on the other hand, different when the enzyme was assayed directly using an *in vitro* assay method, and the salt accelerated the loss in enzyme activity drastically. The organic-N content of root and leaf was, however, increased significantly ($p < 0.001$) with calcium chloride alone and with the metals supplied in various combinations. Our data indicate that instead of a high endogenous accumulation of Cu^{2+}, Cd^{2+} and Pb^{2+} in roots and leaves the metal toxicity is recovered to a great extent in the presence of 10.0 mM calcium chloride in the root environment regarding growth and nitrate reduction of the roots and leaves of young sesame seedlings.

INTRODUCTION

Heavy metals, e.g. Pb^{2+}, Cd^{2+} and Cu^{2+} have specific effects on the biosphere including plants. Usually different toxic metals exist in complex combinations of each other in nature and sometimes they may interact with certain other stresses. These metals are known to affect seed germination [1], seedling growth [2] and plant metabolism [3–6]. In our previous studies a differential response of root and leaves of sesame cultivar PB-1 to Pb^{2+}, Cd^{2+} and Cu^{2+} and an antagonistic effect of NaCl to this differential toxicity have been reported [7]. Calcium chloride recovers the Pb^{2+} induced decrease in biomass accumulation of mungbean seedlings [8] and inhibition of NRA in sesame cultivar HT-I at a concentration of 10.0 mM [6].

It has been observed that Ca^{2+} channels play major roles in the initiation of a large number of signal transduction processes in higher plant cells, including bud formation, polar growth, gas exchange regulation, secretion, movements and light regulated and hormone regulated growth and development [9].

The present work was planned to study whether the effects of heavy metals can be modified by the presence of $CaCl_2$ in the nutritional environment of the plants.

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RESULTS AND DISCUSSION

Seedling growth and endogenous accumulation of lead, cadmium, copper and calcium

The supply of 1.0 mM Pb^{2+}, Cd^{2+} and Cu^{2+} in alternate combinations (1:1) to *Sesamum indicum* cv. PB-1 decreased the fresh weight of root and leaf (significant $p < 0.001$) and dry weight of root (significant $p < 0.05$) (Table 1). The most drastic decrease in fresh weight of the seedling was noticed in Cd^{2+}–Pb^{2+} and Cu^{2+}–Cd^{2+}, which was more pronounced in the roots (88–92%) than in the leaves (37–56%). The leaf dry weight, on the other hand, increased in most of the combinations, but this increase was found statistically

Table 1. Effect of $CaCl_2$ on Pb^{2+}, Cd^{2+} and Cu^{2+} caused reduction in growth of *Sesamum* seedlins

Treatments	mg fresh wt (± S.E.)		mg dry wt (± S.E.)	
	Root	Leaf	Root	Leaf
Distilled water	7.46 ± 1.63 (100)	10.08 ± 0.53 (100)	0.45 ± 0.01 (100)	0.58 ± 0.05 (100)
Pb^{2+}–Cu^{2+} (1:1)	5.74 ± 1.62 (77)	9.28 ± 1.01 (92)	0.27* ± 0.07 (60)	0.77 ± 0.08 (133)
Pb^{2+}–Cd^{2+} (1:1)	0.63** ± 0.14 (08)	6.36** ± 0.76 (63)	0.37 ± 0.08 (82)	0.96* ± 0.14 (165)
Cu^{2+}–Cd^{2+} (1:1)	0.92** ± 0.19 (12)	4.40*** ± 0.71 (44)	0.35 ± 0.06 (78)	0.69 ± 0.10 (119)
$CaCl_2$ (10.0 mM; 1.9 EC)	9.01 ± 0.28 (121)	10.37 ± 0.72 (103)	1.18 ± 0.20 (262)	1.50*** ± 0.10 (259)
Pb^{2+}–Cu^{2+} + $CaCl_2$	5.84 ± 0.11 (78)	7.32* ± 0.76 (73)	0.45 ± 0.05 (100)	0.62 ± 0.06 (107)
Pb^{2+}–Cd^{2+} + $CaCl_2$	4.70 ± 0.33 (63)	6.88*** ± 0.31 (68)	0.50 ± 0.04 (111)	0.86* ± 0.10 (148)
Cu^{2+}–Cd^{2+} + $CaCl_2$	5.14 ± 0.47 (69)	6.13*** ± 0.63 (61)	0.47 ± 0.06 (104)	0.87* ± 0.12 (150)

Sesame seedlings were raised for five days as described in Experimental. Roots and leaves were taken for the measurements. Data shown are mean ± S.E. ($n = 6$). Differences marked by asterisks are significant at $p < 0.05$*; $p < 0.01$**; $p < 0.001$***. Values relative to control are given in parentheses. Metal concentrations are 1.0 mM each.

Table 2. Accumulated Level of Pb^{2+}, Cd^{2+}, Cu^{2+} and Ca^{2+} in *Sesamum indicum* roots and leaves exogenously supplied with the metal salts and $CaCl_2$

Treatments	Dry wt ($\mu g\ g^{-1}$)							
	Root				Leaf			
	Pb^{2+}	Cd^{2+}	Cu^{2+}	Ca^{2+}	Pb^{2+}	Cd^{2+}	Cu^{2+}	Ca^{2+}
Distilled water	—	—	—	—	—	—	—	—
Pb^{2+}–Cu^{2+}	96	6	1860	—	8	—	229	—
Pb^{2+}–Cd^{2+}	95	733	—	—	23	558	—	—
Cu^{2+}–Cd^{2+}	—	725	1215	—	—	558	388	—
$CaCl_2$	—	—	—	350	—	—	—	1829
Pb^{2+}–Cu^{2+}–$CaCl_2$	199	—	2148	220	13	—	271	316
Pb^{2+}–Cd^{2+}–$CaCl_2$	114	710	—	216	12	546	—	202
Cu^{2+}–Cd^{2+}–$CaCl_2$	—	713	1251	108	—	597	383	242

Sesame seeds were germinated for five days; roots and leaves were detached and metal content was estimated by atomic absorption spectrophotometric analysis.

significant ($p < 0.05$) only in the case of Pb^{2+}–Cd^{2+} (1:1).

The supply of $CaCl_2$ (10.0 mM; 1.9 EC) to *Sesamum* seedlings during early growth phase increased the root fresh weight and root and leaf dry weight (Table 1). The toxic effect of the Cd^{2+} in various combinations was partially (55–57%) recovered with $CaCl_2$ supply in root fresh weight, but leaf fresh weight seemed to be affected more drastically by the metal toxicity even in the presence of $CaCl_2$, if Pb^{2+}–Cu^{2+} (1.0 mM each) was supplied (Table 1). A partial (17%) recovery in the metal toxicity caused by Cu^{2+}–Cd^{2+} was, however, noticed in the presence of 10.0 mM $CaCl_2$.

An analysis of the metal accumulation in the roots and leaves of sesame seedlings in the presence of 1.0 mM Pb^{2+}, Cd^{2+} and Cu^{2+} salts in alternate combinations with each other as well as with $CaCl_2$ (10.0 mM) showed that the order of metal accumulation in the roots was $Cu^{2+} > Cd^{2+} > Pb^{2+}$ (Table 2). The accumulation of Pb^{2+} and Cu^{2+} was considerably higher in roots than in the leaves of the seedlings, which showed a slow and steady translocation of these metals towards the leaves. The endogenous Cd^{2+} level in roots and leaves of sesame also showed a similar pattern, but the magnitude of differences were less (Table 2). It appears, therefore, that translocation of Cd^{2+} in this cultivar of sesame is more rapid to the aerial parts than that of Pb^{2+} and Cu^{2+}.

It is interesting to note that a higher accumulation of Ca^{2+} was estimated in the leaves than in the roots (Table 2). Further Ca^{2+} accumulation was reduced to a significantly lower level if the metal salts were also supplied to the seedlings, but the endogenous accumulation of the toxic metals was not restricted in the presence of $CaCl_2$; rather, Pb^{2+} accumulation was increased in the presence of $CaCl_2$ (Table 2).

It has been proposed that the effects of plant hormone on growth and development are mediated by hormone induced shifts in cytoplasmic Ca^{2+} levels with Ca^{2+} acting as an intracellular messenger conveying information about the nature of a particular stimulus or stress impinging on the cell to target proteins that guide the cellular response [10]. Thus, Ca^{2+} plays a pivotal role in signal transduction by communicating signal perception at a localized receptor to other parts of the cell, where the effectors of the cellular responses are located. The transient rise in cytosolic Ca^{2+} concentration is detected by calcium response elements—cell calcium receptor protein, e.g. calmodulin [11]—calcium dependent protein-kinase, phosphatases [12] and Ca^{2+} stimulated phospholipases [13] which activate a number of enzymes in plant parts.

Our data show that calcium influx in the roots and leaves of sesame seedlings do not increase in the presence of the metals, whereas the endogenous metal accumulations are increased in most cases if an abundant amount of $CaCl_2$ is present in the root environment (Table 2). In spite of low calcium and higher metal accumulation the root and leaf growth recovered very significantly, especially in the case of cadmium-caused toxicity. How plants manage this high level of endogenous metal in the presence of $CaCl_2$ is an important question raised by this study.

It is presumed that the presence of the heavy metals around the plants in the environment induces the synthesis of chelatin, a peptide which binds the free metal entering into the plant system via absorption through the roots. Studies on the biosynthesis and activity of phytochelatins (which seem to be specific for the three metals in the presence of $CaCl_2$) may be a possible answer, but these could not be carried out due to the lack of appropriate facilities. Our earlier reports with this cultivar of sesame indicate that it is more sensitive to cadmium than to lead [6, 7, 14], which is possibly related to a differential accumulation of the metals (Table 2).

The accumulation of more lead, cadmium and copper in the roots than in the leaves of the seedlings may be due to direct exposure of the organ to the metals and it is in accordance with other sutdies [6, 15, 16]. Calcium, however, seems to accumulate more in the leaves than in the roots (Table 2), indicating a different sink system for these ions in the roots and leaves in sesame.

The presence of Ca^{2+} along with the heavy metal may induce the signal to the plant, which is first received by the root to induce phytochelatin synthesis to abolish/antagonize the toxicity to some extent. Second, Ca^{2+} increases the permeability of the plasma membrane, leading to a greater accumulation of toxic metals and other ions in aerial plant parts. However, the heavy metals are not drastically toxic in the presence of $CaCl_2$ possibly due to the activation of some stress tolerance mechanism in the tissue. The response of fresh weight of sesame primary roots and leaves to Ca^{2+} is different from that of the dry weights as it has no significant effect on the root and leaf fresh weight and a pronounced increase in root and leaf dry weight (Table 1). It is also evident from measurements on other organs in which Ca^{2+} as well as the heavy metal causes an increase in dry weight, especially in the leaf (Table 1). A differential metal effect on the biomass of roots and leaves of sesame had been discussed in our previous papers [7, 14].

Nitrate reductase activity

Alternate combinations of 1.0 mM Pb^{2+}, Cd^{2+} and Cu^{2+} inhibited *in vivo* NRA of five day old roots and leaves of sesame significantly ($p < 0.001$) (Table 3). Among these metals Cd^{2+} and Cu^{2+} appeared to be more toxic to the root NRA in either of the combinations possibly because roots accumulate high amount of Cu^{2+} and Cd^{2+} in comparison with Pb^{2+} (Table 2).

The negative effect of these tissues was, however, not observed in an *in vitro* assay of NRA (Table 3). Further, exogenously supplied $CaCl_2$ (1.9 EC; 10.0 mM) decreased (65%) *in vivo* NRA of root and *in vitro* enzyme activity significantly ($p < 0.001$), both in root (71%) and leaf (85%) of five day old sesame seedlings (Table 3). The *in vivo* NRA of leaf, however, was hardly affected by $CaCl_2$. It is interesting to note that $CaCl_2$ and the toxic heavy metals, i.e. Cu^{2+}, Cd^{2+} and Pb^{2+} inhibited *in vivo*

Table 3. Effect of $CaCl_2$ in the presence of Pb^{2+}, Cd^{2+} and Cu^{2+} on *in vivo* and *in vitro* nitrate reductase activity of *Sesamum* seedlings

	NRA μmol NO_2^- $h^{-1}g^{-1}$ fr. wt ($\pm$ S.E.)			
	In vivo		*In vitro*	
Treatments	Root	Leaf	Root	Leaf
Distilled water	0.31 ± 0.03 (100)	0.30 ± 0.01 (100)	0.76 ± 0.06 (100)	0.75 ± 0.05 (100)
Pb^{2+}–Cu^{2+} (1:1)	0.15*** ± 0.01 (48)	0.19*** ± 0.01 (63)	0.87 ± 0.03 (114)	1.03* ± 0.09 (137)
Pb^{2+}–Cd^{2+} (1:1)	0.23* ± 0.01 (74)	0.14*** ± 0.01 (47)	0.72 ± 0.09 (95)	0.70 ± 0.05 (93)
Cu^{2+}–Cd^{2+} (1:1)	0.08*** ± 0.01 (26)	0.12*** ± 0.02 (40)	0.93 ± 0.05 (122)	0.86 ± 0.04 (115)
$CaCl_2$ (1.9 EC)	0.11*** ± 0.01 (35)	0.24 ± 0.02 (80)	0.22*** ± 0.01 (29)	0.11*** ± 0.01 (15)
Pb^{2+}–Cu^{2+}–$CaCl_2$	0.33 ± 0.02 (106)	0.20* ± 0.02 (67)	0.35*** ± 0.01 (46)	0.26*** ± 0.01 (35)
Pb^{2+}–Cd^{2+}–$CaCl_2$	0.32 ± 0.01 (103)	0.42* ± 0.03 (140)	0.34*** ± 0.01 (45)	0.11*** ± 0.02 (15)
Cu^{2+}–Cd^{2+}–$CaCl_2$	0.20* ± 0.02 (64)	0.14*** ± 0.01 (47)	0.26*** ± 0.00 (34)	0.20*** ± 0.01 (27)

Sesame seedlings were raised for five days as described in Experimental. Roots and leaves were detached and organic nitrogen content was estimated as described in Experimental. Data shown are mean ± S.E. ($n = 6$) and are significant: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***. Values relative to control are given in parentheses. Metal concentration in each case is 1.0 mM.

NRA of root when supplied separately, but they produced an antagonistic effect in most cases if supplied in a combination of metals in the presence of $CaCl_2$ (Table 3). Leaf *in vivo* NRA was also reduced in this condition, but there was a profound increase in the presence of Pb^{2+}–Cd^{2+} (1.0 mM each) with $CaCl_2$ (10.0 mM). A similar antagonistic effect of NaCl at 2 and 10 EC salt level in this cultivar of sesame was also noticed when NaCl was supplied alone and along with the metal in various combinations [14]. The data from *in vitro* enzyme assays, however, showed a consistent decrease in the enzyme activity (54–85%) with supply of the metal combinations along with $CaCl_2$ as observed for NaCl [14] (Table 3).

In an earlier study, Kumar *et al.* [6] have reported that exogenously supplied $CaCl_2$ counteracts the inhibition of root NRA in sesame var. HT-I caused by lead acetate supplied exogenously. Inhibition of NRA by Pb^{2+} and Cd^{2+} has been a general observation in several plants when supplied in isolation [5, 6, 15–17]. This inhibition in NRA may be due to a reduced supply of NADH [18], disorganization of chloroplast [19], water stress created by the metal [20], decreased NO_3^- supply to the site of the enzyme synthesis [20, 21], and a direct effect of the metal on the enzyme protein synthesis/activity as it has a strong affinity for any functional SH group of the enzyme [22]. Reduction in the *in vivo* production of NADH due to reduced rates of photosynthesis [23] and respiration [24, 25] in the presence of Cd^{2+} has been reported. Further, the phytotoxic response to Pb^{2+} and Cd^{2+} is more pronounced in the roots than in the leaves, which may be attributed to a restricted translocation of the metal to the site of enzyme action in the leaves and induced synthesis of metal chelating peptides in the leaves as observed for Cd^{2+} [26]. Inhibition of NRA by Pb^{2+} in *Helianthus* and *Sorghum* [27], cucumber cotyledon and root [23], *Zostera marina* root [28], *Zea* and *Pisum* leaves [17], roots and leaves of sesame HT-I [6] and PB-1 [15] has also been observed. The differential action of lead on NRA during *in vivo* and in *in vitro* assays was also found in pea leaves [17] and sesame roots and leaves [7, 14, 15]. Calcium is considered as an important nutrient ion and is known to increase the membrane permeability and, thus, greater availability of other ions to the site of the enzyme action is possible, which may cause an indirect regulation of the enzyme activity in the presence of $CaCl_2$ in addition to its role as secondary messenger for signal transduction [29].

Organic nitrogen content

The supply of various combinations of Pb^{2+}, Cd^{2+} and Cu^{2+} increased significantly the organic nitrogen content of the plant parts with a predominent increase in the root tissue ($p < 0.001$) (Table 4). The supply of $CaCl_2$ (1.9 E.C.; 10.0 mM) alone or with the metal combinations also showed a higher accumulation of organic-N content that was again more pronounced in roots (Table 4). This increase is not always correlated with NRA of the organs during the early seedling growth phase [6, 7, 14]. A slight increase in protein and nitrogen of *Zea* leaves due to Pb^{2+} supply [17] and a concentration dependent increase in soluble protein and organic N of root and shoot of sesame HT-I [6] have also been reported. It is concluded that the metals and in a more general way $CaCl_2$ also increased the translocation of reserve N from the cotyledons to the growing roots and shoots during the early growth phase as it has been correlated with a decreased organic-N content of the cotyledons in sesame under a Pb^{2+} environment [6]. It may also be attributed to the increased synthesis of stress induced peptides and proteins during the given environmental conditions.

EXPERIMENTAL

Seeds of *S. indicum* L. cv. PB-1 were purchased from the National Seed Corp., New Delhi, and sterilized with 0.1% $HgCl_2$ for 2.5 min. They were washed thoroughly

Table 4. Organic nitrogen content of different plant parts as affected by Ca^{2+} in the presence of the heavy metals

	mg nitrogen g^{-1} dry wt ($\pm$ S.E.)			
Treatments	Root		Leaf	
Distilled water	12.75	$\pm$ 0.43 (100)	33.46	$\pm$ 1.68 (100)
Pb^{2+}–Cu^{2+} (1:1)	20.09***	$\pm$ 0.89 (158)	39.60	$\pm$ 1.63 (118)
Pb^{2+}–Cd^{2+} (1:1)	22.15***	$\pm$ 0.56 (174)	27.93	$\pm$ 0.56 (83)
Cu^{2+}–Cd^{2+} (1:1)	28.55***	$\pm$ 0.79 (224)	32.54	$\pm$ 0.91 (97)
$CaCl_2$ (1.9 EC)	26.64	$\pm$ 0.33 (209)	34.69	$\pm$ 0.56 (104)
Pb^{2+}–Cu^{2+}–$CaCl_2$	22.38	$\pm$ 0.36 (175)	42.51*	$\pm$ 3.31 (127)
Pb^{2+}–Cd^{2+}–$CaCl_2$	25.84	$\pm$ 0.49 (203)	42.36***	$\pm$ 0.67 (127)
Cu^{2+}–Cd^{2+}–$CaCl_2$	24.00	$\pm$ 0.17 (188)	40.64**	$\pm$ 1.42 (121)

Sesame seedlings were raised for five days as described in Experimental. Roots and leaves were detached and organic nitrogen content was estimated as described in Experimental. Data shown are mean $\pm$ S.E. ($n = 6$) and are significant: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***. Values relative to control are given in parentheses. Metal concentration in each case is 1.0 mM.

with H_2O before planting, and seedlings were raised in petri dishes containing sterilized filter paper with desired soln at $30 \pm 2°$ in a growth chamber under alternate periods of light (5 klux) and dark (9:15 hr) for 5 days. Seedlings were watered daily with H_2O containing 1.0 mM each of $Pb(OAc)_2$, $Cd(OAc)_2$ and $CuSO_4$ in combination or with $CaCl_2$ (10.0 mM; 1.9 EC). Seedlings were raised without supply of any nutritional soln to avoid interference from other salts including NO_3^- as described earlier [7, 14, 15]. The growth and metabolism of the seedlings during this early growth phase is dependent on reserve metabolites translocated from the germinating sesame seeds. Roots and leaves of 5-day-old seedlings were dissected out and weighed for fr. wt measurements. The tissues were then oven dried at 60° until the dry wt became constant. Metal content was estimated in the oven dried samples of roots and leaves using an atomic absorption spectrophotometer as described earlier [6].

Nitrate reductase activity in the freshly harvested leaves and roots was measured by *in vivo* [30] and *in vitro* [31] methods. Total organic-N content of plant parts was estimated by the micro-Kjeldahl method [32], after digesting the tissue with conc. H_2SO_4; $(NH_4)_2SO_4$ was used as standard. The data presented in this paper are averages of duplicates of three independent experiments. Data were analysed for variance and CD values using the 'F' test to determine the statistical significance of the differences observed.

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