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CADMIUM INDUCED MORPHOLOGICAL AND PHYTOTOXIC EFFECTS ON DIFFERENT PLANT PARTS OF MAIZE (*ZEA MAYS* L.) SEEDLINGS : EVIDENCE FOR ALTERATIONS IN NUTRIENT DYNAMICS AND ENZYME ACTIVITIES

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Abstract

Cadmium (Cd) stress induced changes in morphology, phytotoxic effects of varied maize seedling plant parts were investigated and these changes were correlated with the alterations in nutrient dynamics and enzyme activities. The Cd stress was imposed through Hoagland Nutrient Solution (HNS) and the treatments were 10, 20, 30, 40 and 50 µm. Samples were collected from 14 days old seedlings with four well-unfurled leaves. Changes in morphology and phytotoxicity were analyzed through shoot length, root length, leaf length & width and leaf area. Chlorophyll, relative water content, cell membrane stability and nitrate reductase (NRase) were estimated to understand the functioning of maize under Cd stress. The data were statistically analyzed using IRRISTAT and treatment means were compared by Duncan's Multiple Range Test (DMRT). Lower concentration of Cd did not produce visible toxicity symptoms but concentration beyond 30 µm was fatal. The correlation values for Cd retention capacity of different tissues were estimated by using SPSS. Positive correlation between media Cd and tissue Cd content was realized. Cd at higher concentrations caused root decay and reduced nutrients uptake. Roots were more sensitive than leaves. Plant height, leaf area and chlorophyll b were reduced significantly at higher concentrations. The activity of nitrate reductase (NRase) was significantly reduced in younger leaves. Increased tissue Cd concentration had reduced uptake of nitrogen, potassium, zinc, manganese whilst phosphorus uptake was enhanced. The normal metabolic functioning of maize was altered at Cd stress. The root sensitivity, RWC, CMS, level of chlorophyll b and NRase in leaves under Cd stress could be used as markers to select the Cd stress tolerant maize genotypes that would be used for maize improvement programs targeted to the niche environments.

Key words : Maize, cadmium, morphology, phytotoxicity, nutrient dynamics, enzyme activities.

Introduction

Cadmium (Cd) is a natural trace metal present in non-toxic level as a part of mineral deposits. Cd enters the environment by natural and human processes. Anthropogenic activities such as mining and urbanization processes are significantly contributing approximately 90 percent of the cadmium found in surface waters. Uses of phosphatic fertilizers are also worsening the situation. Cd has no known biological function in living organism but it is easily taken up by crops and accumulated in roots, leaves, stem (s) and grains owing to easy mobility. Consumption of these plant products causes several serious health hazards (Yazihan *et al*., 2008). Cd is strongly phytotoxic, it interferes with the normal metabolic process causing growth inhibition and plant death at supra optimal concentration (Simonetti *et al*., 2016). The visible phytotoxic symptoms are caused either due to alterations in the functionality of cell membranes or in the activity of enzymes of metabolic pathways. Cd may influence plant physiological and biochemical processes through negatively influencing the concentrations and functions of nutrient minerals that otherwise would be performing protective roles against the toxic effects of Cd (Khan *et al*., 2007). The immediate effects of low Cd concentration must be considered to have an insight on the primary site of action of Cd toxicity and its subsequent changes, which will help to identify Cd tolerant genotypes in the screening

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programs. Cd produces alterations in the structural integrity and functionality of membranes through lipid peroxidation (Cagno *et al*., 2001). Photosynthesis is highly sensitive to escalated level of Cd and the green pigment is the prime target (Benavides *et al*., 2005) and reduces the chlorophyll production (Moussa *et al*., 2010). Cd toxicity is having significant inhibitory and stimulatory correlation with uptake and translocation of necessary micronutrients (Nedjimi *et al*., 2009) and results in impaired growth. Cd also inhibits nitrate reductase (NR) activity (Chaffei *et al*., 2004). Therefore, the present experiment aimed to investigate the Cd induced alterations in growth parameters, membrane integrity, essential nutrient contents, thereafter planned to exploit these manifestations to identify Cd tolerant maize genotypes.

Materials and Methods

Plant material

Seeds of maize (*Zea mays* L.) inbred UMI 665 were washed three times with double distilled water and further surface sterilized in 3% v/v hydrogen peroxide for 20 min. Surface sterilized seeds then rinsed thrice with distilled water, and transferred into large sized petri plates aseptically and kept on the filter paper imbibed with 2 mM $CaSO_4$. Petri plates were covered by thin perforated plastic film and kept in dark chamber for germination for three days. Healthy seedlings were transferred to the plastic jars containing 1.5 L of Hoagland Nutrient Solution (HNS). HNS was well aerated and renewed once in two days. Growth conditions were 25 ± 2^0C temperature, $90\pm$ 2 per cent relative humidity (ISTA, 1999). Cd was added as $CdCl_2$ at the following concentrations 0, 10, 20, 30, 40 and 50 µm. Each treatment was replicated thrice. Plants grown in HNS without Cd were served as control.

Growth parameters

Fourteen days old seedlings with four well-unfurled leaves were harvested and growth parameters *viz*., shoot length, root length, leaf length and width at mid length of each leaf and leaf area were documented. The distance between the basal node of the stem and tip of the youngest leaf was measured and expressed in centimeter (cm) as soot length. Root length was measured from the base of the shoot to the tip of the root by using graph paper line intersection method. Leaf area was calculated using the equation $Y = 0.8077 \times Lf \times Lw + 0.3044$ (Lf – leaf length, Lw – leaf width and Y- leaf area) (Mocquot *et al*., 1996). Each plant was subdivided into root, 3rd leaf (L3), 4th leaf (L4) and the remaining shoots. L3, L4 and roots from each treatment were pooled and cut into small pieces with sterile scissors. One gram of sample from all treatments was frozen in liquid nitrogen for enzyme assay. The remaining samples were oven – dried for determining mineral contents.

Cadmium estimation

Oven dried samples were wet digested with HNO_3, H_2SO_4, $HClO_4$ at 9:2:1 ratio and Cd content was estimated by atomic absorption spectro photometer. The correlation values for Cd retention capacity of different tissues were estimated by using SPSS version 10.0.

Chlorophyll and Relative water content

Chlorophyll contents were measured as suggested by (Arnon, 1994). Relative water content (RWC) was estimated by adopting formula advocated by (Weatherly, 1950) and expressed in percentage.

Cell Membrane stability

Ten seeds were soaked in HNS containing different Cd concentrations for 48 hours at room temperature. The rate of injury to cell membrane was estimated through the measurement of electrolyte leakage from the cells. After the stipulated time, the final conductivity was measured using EC meter. The per cent injury was calculated using the formula of (Blum and Ebercon, 1981).

$$\text{Per cent injury} = 1 - [1-(T1 - T2)/1 - (C1 - C2)] \times 100.$$

T and C refer to mean of treatments and control respectively and the subscripts 1 and 2 are initial and final conductivity.

Nitrate reductase (E.C. 1.6.6.1)

Nitrate reductase (NR) activity was estimated by using the method described by (Hageman *et al*., 1971) and expressed in µ moles NO_2 produced g^{-1} h^{-1} fresh $weight^{-1}$.

Mineral nutrition estimation

Total plant nitrogen content was estimated by micro-kjeldhal method according to (AOAC, 1990) and expressed in percentage on dry weight basis. Total Phosphorus was determined spectrophotometrically by the molybdo-vanadate method according to (Chapman and Pratt, 1961) and expressed in percentage dry weight basis. Potassium uptake was estimated according to Chapman and Pratt (1961) in triple acid extract using flame photometer type-21 and expressed in percentage on dry weight basis.

Results and Discussion

Visual symptoms of toxicity

No visible toxicity symptoms were observed in HNS containing Cd concentration ranging from 10 µM to 30 µM. But, beyond 30 µM, it caused several toxicity

symptoms. Production of toxicity symptoms after 40 µM were also reported in *Palisada flagellifera* (Simonetti *et al*., 2016). Plants grown with higher Cd concentration produced smaller sized leaves and exhibited stunted growth than control. Cd caused delayed unfolding of primary leaves, yellow coloration along the length of the leaves (chlorosis) and leaf tip drying. These effects were caused by alterations in the content of other elements Table 1. Cd inhibited metabolism of Mg, Fe, Mn and Zn (Gussarson *et al*., 1995). The concomitant increase in root Cd caused deficiency of these elements, leaf chlorosis and necrosis. At higher concentration, roots possessed very few decayed secondary roots and root hairs. Browning and decay of root tips were also witnessed. Browned roots were less flexible and broke off easily than control plants. Darker roots are perhaps due to root death. Pronounced effect of Cd on root was also reported in barley (Lachman *et al*., 2015).

Tissue cadmium level

Cd concentration both in roots and leaves increased markedly when plants were exposed to Cd-containing HNS, compared to the control (fig. 1). The tissue Cd level was postitvely correlated with growth media concentration. Similar findings of positive correlation was also reported (Roy *et al*., 2016) Cd concentration in roots, L3 and L4 ranged from 90 to 320, 72 to 139 and 49 to 89 µM kg^{-1} DW, respectively. Cd accumulation in roots and older leaves were higher. Roots had three and two fold higher Cd concentration at 50 µM than the younger leaves (L4). The roots retained much of the observed Cd. The linear line was best fitted using SPSS Version 10.0, the r^2 values were estimated and they were 0.9832, 0.8853, and 0.8514 for roots, L3 and L4, respectively. This would be explained by the facts that a larger pool of chelated metal facilitates metal uptake by replacing ions uptake or it is possible that Cd would be taken up directly; root browning and decay explained the later. In addition, immobilization of Cd by cell wall and extra cellular carbohydrates might also made roots to accumulate more Cd. At the highest Cd concentration, the leveling off Cd in the leaf tissues may represent a "plateau" effect, which has been observed in plants grown in environments with increasing concentration of metal pollutants. Akin results of higher root Cd content were reported in *Sesuvium portulacastrum* and *Mesembryanthemum crystallinum* (Ghnaya *et al*., 2005).

Morphological parameters

An orderly decrease in morphological parameters *viz*., shoot length, root length and leaf area have been observed with increased Cd concentration. Gradual decrease in above said growth parameters was observed but effects became evident at 40 µM of Cd (figs. 2 & 3). The statistically significant reduction were observed, when tissue Cd concentration reached to 126 µg Kg^{-1} DM in L4, 76 µg Kg^{-1} in L3 and 286 g Kg^{-1} DM in roots. The root growth was most affected than shoot growth. The percentage of reduction in root growth between different Cd concentrations was higher than shoot length. At higher concentrations the root growth was almost arrested. These results are in agreement with (Simonetti *et al*., 2016). While higher leaf Cd concentration was reported in sorghum (Roy *et al*., 2016). Cd induced adverse effects on the successful growth and development of plant was earlier reported (Da-lin *et al*., 2013).This is attributed by (i) increased cross linking of pectins in the middle lamellae cause increased cell adhesion and binds to cell wall and thus showed resistance to growth (ii) metal induced increase in cell wall bound peroxidase activity enhance phenolic cross linking that could inhibit cell wall expansion, (iii) larger parenchyma cells, increased resistance to radial flow of water and mineral nutrients, (iv) Cd binds to cell wall, increases the cortex thickness, causing impairment in cell expansion rate and leads to reduced root growth (Poschenrider *et al*., 1989). The abnormalities of mitotic division and chromosomal aberrations were attributed to elongation (Siddiqui *et al*., 2009).

Chlorophyll

Cd strongly inhibited chlorophyll production as witnessed by the yellowing and reduced chlorophyll content in L3 and L4 (table 2). Prominent yellowing of L4 was observed beyond 20 µM, implies that chlorophyll reduction was significant in L4 than L3. The difference in chlorophyll 'b' content between 50 µM and control were 0.74 and 0.80 in L3 and L4 and they were 0.33 and 0.39 for chlorophyll 'a'. Chlorosis is the first Cd induced visible phytotoxic symptom and is due to decreased synthesis of green pigment. Declined production of chlorophyll b was also reported by Fargasova (2001) and total chlorophyll by Miyadate *et al*. (2001). Altered chlorophyll biosynthesis is due to inhibition of protochlorophyllide reductase and water splitting enzyme located at the oxidizing site of photo-system II as it is evidenced by reduced amount of Mn (table 3). Cd stress induced reduction in the activity of photo-system II was earlier reported in poplar plants. These alterations are affecting the normal functioning of the photosynthetic machinery through the reduction in the activity of carbonic anhydrase enzyme (Roy *et al*., 2016), which alter photosystem components and consequently accelerates the deregulation of CO_2 homeostasis in leaves thereby

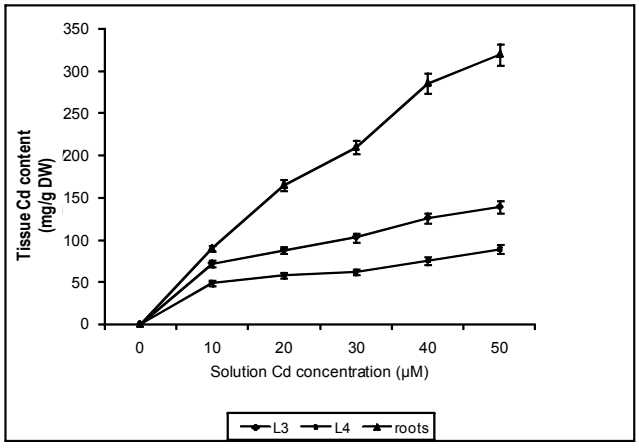

Fig. 1 : Effect of cadmium treatment on tissue cadmium content of different plant tissues of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at ≤0.01.

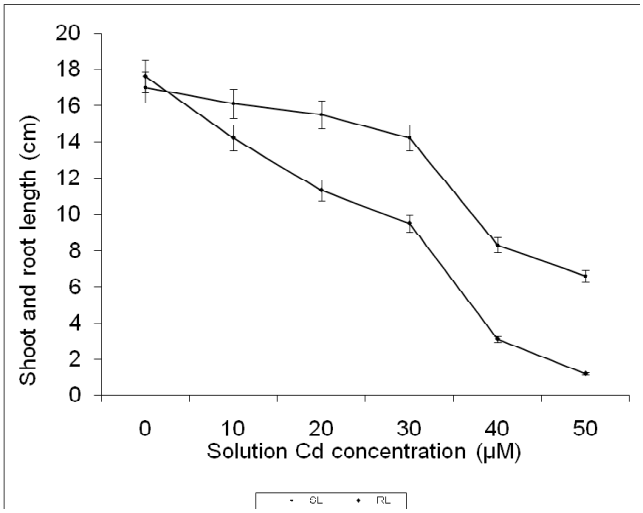

Fig. 2 : Effect of cadmium treatment on shoot length (SL) and root length (RL) of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at ≤0.01.

photosynthesis (Alessandro *et al*., 2013). These deregulations in turn affect the normal growth of the plants under Cd stress.

Relative water content

Leaves and roots have showed gradual reduction in RWC and pronounced reduction was observed in roots (fig. 4). Roots showed its inability to retain water at incubation and thus failed to maintain necessary metabolic activities as evidenced by the root browning and membrane damage. It approached to 55.6 per cent at 50 µm form 98 per cent at control followed by L3 and L4. Similar results were also observed by Poschenrider *et al*. (1989). While L3, sustain the stress by possessing more water content and the reduction percentage in L3 and L4 was though

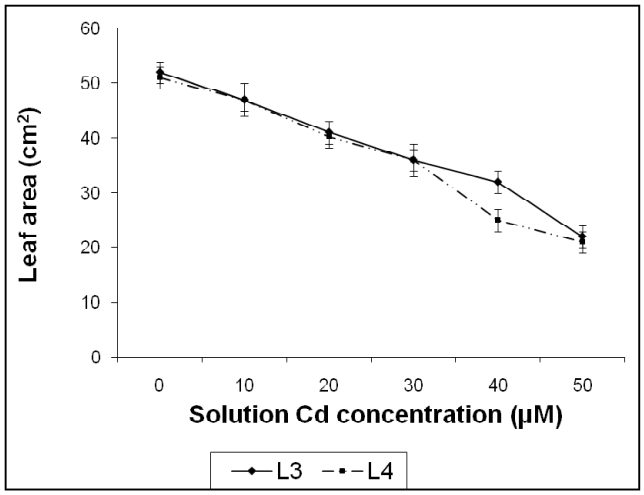

Fig. 3 : Effect of cadmium stress on leaf area of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at ≤0.01.

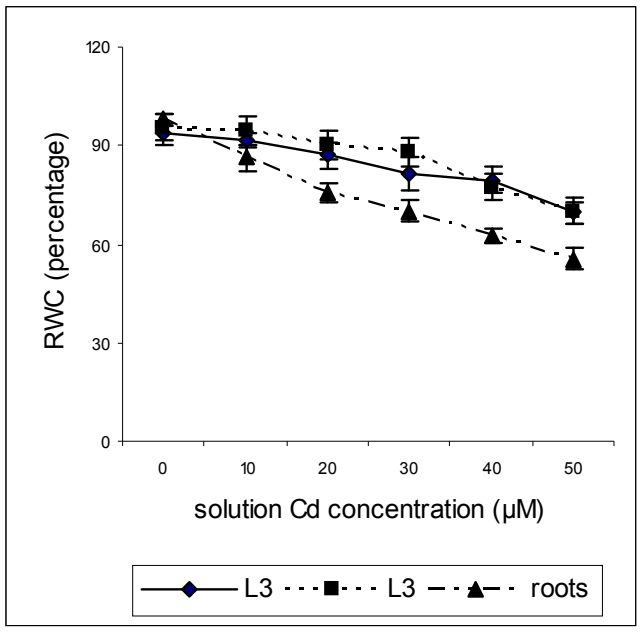

Fig. 4 : Effect of cadmium stress on relative water content in different plant tissues of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at ≤0.01.

gradual it was insignificant between them. It is generally accepted that the metal stressed plants produce less root hair surface and lowers the capacity of plants to explore the soil for nutrients and water. Reduced RWC in roots is also credited by increased resistance to water flow into and within roots, root browning, structural changes in hydro dermis, endodermis and plasmolysis of cortical cells (Barcelo *et al*., 1988). Inhibition of cell division (in pro cambium and cambium) and cell elongation during secondary wall thickening are causing decreased vessel number and radius (Barcelo *et al*., 1988). Reduced RWC under Cd stress was earlier reported by Roy *et al*. (2016).

Table 1 : Effect of cadmium stress on major and micronutrients contents of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at P≤0.01. Different letters indicate significantly different values at a particular time.

Cadmium concentration (µm)	Major nutrients (percentage DW)									Micronutrients (ppm)					
	Nitrogen			Phosphorus			Potassium			Manganese			Zinc		
	L3	L4	Roots	L3	L4	Roots	L3	L4	Roots	L3	L4	Roots	L3	L4	Roots
0	0.80^{b}	0.82^{a}	0.74^{c}	1.00^{h}	0.80^{i}	1.04^{h}	0.598^{c}	0.612^{a}	0.492^{f}	52^{o}	268^{a}	82^{k}	62^{b}	59^{c}	65^{a}
10	0.62^{f}	0.74^{c}	0.59^{g}	1.20^{f}	1.00^{h}	1.12^{g}	0.514^{e}	0.605^{b}	0.471^{h}	64^{m}	259^{b}	97^{h}	57^{d}	42^{g}	61^{b}
20	0.59^{g}	0.72^{d}	0.47^{jk}	1.36^{cd}	0.76^{i}	1.03^{h}	0.489^{g}	0.576^{d}	0.465^{i}	88^{j}	240^{c}	112^{f}	54^{e}	41^{g}	59^{c}
30	0.51^{i}	0.68^{e}	0.46^{k}	1.55^{b}	1.30^{e}	1.32^{de}	0.425^{j}	0.513^{e}	0.398^{k}	74^{l}	226^{d}	123^{e}	48^{f}	38^{h}	53^{e}
40	0.48^{j}	0.56^{h}	0.39^{m}	1.60^{ab}	1.36^{cd}	1.36^{cd}	0.391^{m}	0.489^{g}	0.356^{n}	32^{q}	110^{g}	56^{n}	31^{k}	34^{j}	36^{i}
50	0.41^{l}	0.41^{l}	0.35^{n}	1.64^{a}	1.40^{c}	1.40^{c}	0.356^{n}	0.394^{l}	0.315^{o}	29^{r}	91^{i}	37^{p}	28^{l}	21^{m}	33^{j}
	CD=0.02			CD=0.055			CD=1.65			CD=1.53			CD=1.74		

Table 2 : Effect of cadmium stress on photosynthetic pigments of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at P≤ 0.01. Different letters indicate significantly different values at a particular time.

Cadmium concentration (µm)	L 3		L 4	
	Chlorophyll a (mg g^{-1} fresh weight)	Chlorophyll b (mg g^{-1} fresh weight)	Chlorophyll a (mg g^{-1} fresh weight)	Chlorophyll b (mg g^{-1} fresh weight)
0	0.84^{e}	1.26^{a}	0.72^{h}	1.02^{c}
10	0.78^{g}	1.05^{b}	0.68^{i}	0.98^{d}
20	0.65^{j}	0.32^{f}	0.42^{q}	0.62^{k}
30	0.59^{l}	0.68^{i}	0.39^{r}	0.51^{o}
40	0.56^{m}	0.61^{k}	0.35^{s}	0.44^{p}
50	0.42^{q}	0.52^{n}	0.32t	0.32^{t}
	CD = 0.059	CD = 0.064	CD = 0.046	CD = 0.048

Table 3 : Effect of cadmium stress on photosynthetic pigments of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at P≤ 0.01. Different letters indicate significantly different values at a particular time.

Cadmium concentration (µm)	L 3		L 4	
	Chlorophyll a (mg g^{-1} fresh weight)	Chlorophyll b (mg g^{-1} fresh weight)	Chlorophyll a (mg g^{-1} fresh weight)	Chlorophyll b (mg g^{-1} fresh weight)
0	0.84^{e}	1.26^{a}	0.72^{h}	1.02^{c}
10	0.78^{g}	1.05^{b}	0.68^{i}	0.98^{d}
20	0.65^{j}	0.32^{f}	0.42^{q}	0.62^{k}
30	0.59^{l}	0.68^{i}	0.39^{r}	0.51^{o}
40	0.56^{m}	0.61^{k}	0.35^{s}	0.44^{p}
50	0.42^{q}	0.52^{n}	0.32t	0.32^{t}
	CD = 0.059	CD = 0.064	CD = 0.046	CD = 0.048

Cell membrane stability

Cd induced alterations in the integrity of cell membranes were studied through membrane leakage. There was no cell membrane damage in control plants but inclusion of Cd in HNS caused damage. Tissue Cd level determined the degree of damage. Cd, by forming disulfide bridges with N and S ligands distorting membrane channels and thus causes ion leakage. At higher Cd concentration more membrane damage was noticed in roots. The percent injury were 84.2 (roots), 79.5 (L3) and 53.7 (L4), respectively (fig. 5). Direct contact of roots to the bioavailable Cd in medium and high tissue Cd could have been attributed to more membrane damage. A positive correlation between accumulated metal in tissue and ion leakage was reported by Mishra *et al*. (2006). More damage to the membranes posed problems to cell functionality and ultimately death. The peroxidation of cell membranes caused impairment in normal function,

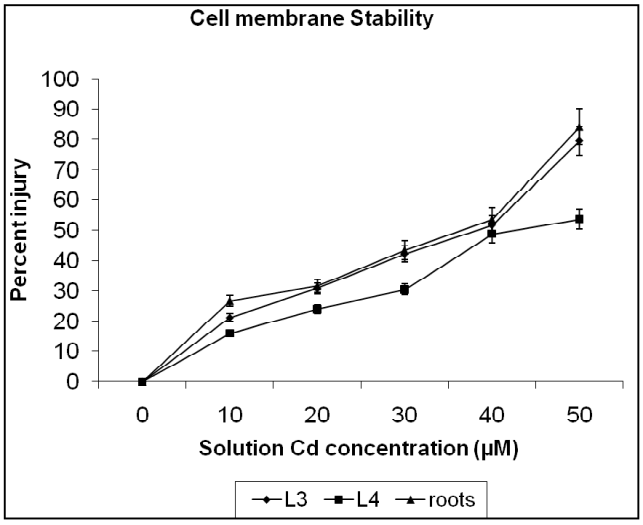

Fig. 5 : Effect of cadmium stress on cell wall stability of different plant tissues of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at ≤0.01.

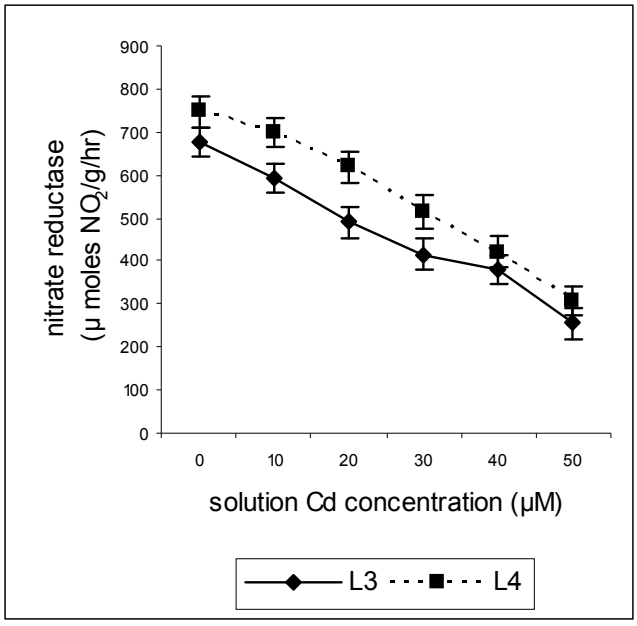

Fig. 6 : Effect of cadmium stress on NR activity of leaves of *Zea mays* L. All the values are mean of triplicates ± S.D. ANOVA significant at ≤0.01.

disturbances to structural integrity and produce irreversible damage to the cell function as a whole. Prominent effect of Cd on electrolyte leakage earlier reported in barley by Lachman *et al*. (2015).

Nitrate reductase

Cd reduced the activity of NRase. The degree of reduction was related to HNS Cd concentration (fig. 6). The reduction was more prominent in young leaves (L4). NRase activity was reduced from 746.3 in control to 308.1 at 50 µm Cd. Major reduction between two consecutive stages was witnessed between 10 µm and 20 µm. It was reduced from 592.8 to 490.1 in L3 and from 699.4 to 618.2 in L4. A remarkable decline was noticed at 20 µM. Reduced NRase activity under Cd stress in *Lycopersicon esculantum* L was reported by Simonetti *et al*. (2016). The affinity of metals to sulphydryll groups involved in the catalytic action or structural integrity of enzymes is said to be one of the main mechanisms of enzyme inhibition. In general the molar ratio in which nitrogen and sulfur occur is 25:1 in proteins. To circumvent the Cd induced stress plants tend to synthesis the Cd binding PCs possessing an N: C ratio of 2.5:1. Plants producing substantial amounts of PCs instead of proteins that would have inhibited the NRase activity and increased activity of enzymes of sulfate reduction cycle (Tkalec *et al.*, 2014).

Plant nutrients

Excessive Cd accumulation affects the rate of uptake and distribution of other nutrients and consequently responsible for mineral deficiencies/imbalance and causing impaired growth. In the present study, Cd induced alterations in absorption of other nutrients observed Table 1. At lower concentration (10 µm) there was an increase in P content in leaves and roots. However, at 20 µm there was a reduction in P content. Tissues regain their starved 'P' level on further exposure to escalated level of Cd in HNS and the increase was steady. With Cd accumulation, P content increased both in leaves and roots. Toxic Cd levels inhibited the uptake of P (Vitoria *et al.*, 2003). In the present study, a parallel reduction was observed in N and K content with increasing Cd concentration and the maximum reduction was observed in roots. At 50 µm, L3 and L4 had 0.41g/plant of N but root had 0.35 g/plant. Comparatively the inhibitory effect of Cd on K and Mn absorption was low in young leaves. The root K content at 50 µm was 0.315 g/plant followed by L3 (0.356 g/plant) and L4 (0.394 g/plant). Cd stress reduced uptake of Zn and the reduction was linear. It is clear that though roots can absorb more of Mn and Zn from HNS but its translocation was prevented. This decreased transport is either might be due to production of PCs in the roots or seizing the activity of transporters in translocation pathway. Metal movement to above ground parts comprises of its radical movement in the root and loading into the xylem vessels (Vazquez *et al*., 1987). The reduced influx of Zn in *Sinapsis alba* L was reported (Fargasova, 2001). Reduced uptake of Zn and Fe under Cd stress is ascribed by changes in H^+ ATPase activity and modifications in the selectivity of IRT 1, a Zn and Mn transporter (Korshunova *et al*., 1999) and thus affect the strength of polyvalent cations. Reduced uptake of Zn in presence of Cd was reported in tobacco (Tkalec *et al*.,

2014; Lachman *et al*., 2015).

Statistical analysis

The data were statistically analyzed (Gomez *et al*., 1984) and treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute Biometric unit, the Philippines.

Conclusion

In the present study, tissue Cd content was positively correlated with HNS Cd concentration. Cd at higher concentrations induced phytotoxicity symptoms like leaf yellowing, drying, root browning and decay. Root growth was worst affected. Reduced root volume resulted in low RWC and thus caused hindrance to normal metabolic functions. Cd stress has significantly modified the uptake of major and minor mineral nutrients. Cd stress caused abridged cholorphyll 'b' synthesis and NRase activity. Roots accumulate more of observed Cd than leaves. Increased oxidative stress was realized by enhanced electrolyte leakage. These changes could be used as biomarkers to identify Cd stress tolerant maize lines. However, detailed estimation of phytochelatin sulfhydril synthesis and antioxidant enzymes under Cd stress are to be investigated to confirm and utilizing these findings in future crop improvement programs.

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