Effects of Zn and Cd accumulation on structural and physiological characteristics of barley plants

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The objectives of this study were to identify the structural changes caused by Zn and Cd accumulation in shoots and roots of barley (*Hordeum vulgare*) plants; and to correlate metal accumulation with anatomical, physiological and morphological changes. Potted plants were exposed to metal treatments of Zn and Cd for 19 and 16 d respectively. Leaves, stems and roots were harvested to identify structural changes and analyze metal accumulation. Barley effectively accumulated Zn (up to 11283 mg kg^{-1}) and Cd (up to 584 mg kg^{-1}) in the shoots. Microscopic structural changes, such as a decrease in intercellular spaces, breakdown of vascular bundles, and shrinkage of palisade and epidermal cells, occurred in leaves, stems and roots of plants treated with high concentrations of Zn. Zinc accumulation also resulted in a significant decrease in water content, fresh weight, dry weight and plant height. Cadmium only caused structural changes in roots at the higher concentrations. Barley plants were able to accumulate significant amounts of Zn and Cd without exhibiting symptoms of phytotoxicity when the metal concentrations were relatively low.
Key words: anatomy, cadmium, *Hordeum vulgare*, microscopy, phytoremediation, zinc

Efeitos do acúmulo de Zn e Cd sobre características estruturais e fisiológicas de plantas de cevada: Os objetivos deste estudo foram identificar as mudanças estruturais causadas pelo acúmulo de Zn e Cd na parte aérea e nas raízes de plantas de cevada (*Hordeum vulgare*), como também correlacionar o acúmulo desses metais com alterações anatômicas, fisiológicas e morfológicas. Plantas cultivadas em vasos foram expostas a tratamentos com Zn e Cd por 19 e 16 d, respectivamente. Folhas, caules e raízes foram coletados para identificarem-se alterações estruturais e o acúmulo desses metais. As plantas efetivamente acumularam Zn (até 11283 mg kg^{-1}) e Cd (até 584 mg kg^{-1}) na parte aérea. Houve alterações estruturais microscópicas em folhas, caules e raízes de plantas tratadas com alta concentração de Zn, tais como decréscimo nos espaços intercelulares, comprometimento dos feixes vasculares e encolhimento das células epidérmicas e do parênquima paliçádico. Acumulação de Zn também resultou em decréscimos significativos no conteúdo de água, na massa fresca, na massa seca e na altura das plantas. Apenas em altas concentrações de Cd observaram-se mudanças estruturais nas raízes. Plantas de cevada acumularam quantidades significativas de Zn e de Cd sem exibir sintomas de fitotoxidade quando a concentração dos metais testados foi relativamente baixa.
Palavras-chave: anatomia, cádmio, fitorremediação, *Hordeum vulgare*, microscopia, zinco

INTRODUCTION

The release of metals and other pollutants in excess into the environment by industrial, mining, agricultural and other operations, poses a considerable threat to the environment. Zinc and cadmium are common environmental pollutants which are widely distributed in soils causing concern at many of the metal contaminated sites in recent years. The main sources of Zn and Cd are

from weathering of minerals and soils, atmospheric deposition from mines, smelters and refineries, iron and steel industries, refuse incineration, industrial and domestic effluents (ATSDR, 1994, 1999; Raskin and Ensley, 2000). Both Zn and Cd bind to soil particles for a long time and accumulate to toxic concentrations in soil resulting in significant risk to the health of natural ecosystems (Brown et al., 1994). Phytoremediation, the use of plants for environmental restoration, has been proposed as a cost effective, environmentally friendly, *in situ* and onsite remediation technology for metal contaminated soils (Raskin and Ensley, 2000; Lasat, 2002; Gratão et al., 2005).

The success of phytoremediation depends on the selection of plant species that maximize the contaminant removal (Ebbs and Kochian, 1998; Raskin and Ensley, 2000). Hyperaccumulators like *Thlaspi caerulescens* have been shown to be effective in phytoextraction of Zn and Cd (Baker and Brooks, 1989). However, recent evidence suggests that moderate accumulating high biomass species, such as *Brassica juncea* (Kumar et al., 1995; Salt et al., 1995) may be more effective than hyperaccumulators in phytoextraction of Zn and Cd from contaminated soils. Several mechanisms by which the Zn and Cd toxicity is avoided in the plants have been reported (Vazquez et al., 1994; Kupper et al., 1999; Frey et al., 2000; Aravind and Prasad, 2005; Benavides et al., 2005), but the structural changes which refers to the adaptive significance for high levels of these metals have been studied in only *Thlaspi caerulescens* (Vazquez et al., 1992; Vazquez et al., 1994), *Arabidopsis halleri* (Zhao et al., 2000) and *Brassica juncea* (Salt et al., 1995; Maruthi Sridhar et al., 2005) among metal accumulators. In non-hyperaccumulators accumulation of Zn and Cd affects the stomatal function, electron transport, chlorophyll synthesis and relative water content at the plant level and cell disruption, cell wall modification, localized cell collapse and death and degeneration of nuclei at the cellular level in different plants (Denny and Wilkins, 1987; Barceló et al., 1988; Van steveninck et al., 1990; Vazquez et al., 1992).

Barley (*Hordeum vulgare*) has been identified as a plant for efficient uptake and accumulation of Zn and Cd with a phytoremediation potential equal to that of mustard plants (Ebbs and Kochian, 1998). To be an efficient metal accumulator, plants require efficient detoxification and tolerance mechanisms at both the cellular and plant level. Hence it is important to study how the different plant and cellular characteristics are affected when Zn and Cd are accumulated in barley plants. In this study the physiological, morphological and anatomical characteristics of barley plants were evaluated with reference to their Zn and Cd phytoextraction potential. The objectives of our study were to identify the structural changes caused by Zn and Cd accumulation in shoots and roots of barley plants; and to correlate metal accumulation with anatomical, physiological and morphological changes. We also monitored the changes in different plant characteristics of barley plants throughout the metal accumulation process using non-destructive and non-invasive plant spectral reflectance techniques. The results of the spectral study were reported in Maruthi Sridhar et al. (2007).

MATERIAL AND METHODS

Plant culture and phytoremediation experimental design: The barley seeds used were from a commercial variety obtained locally. The soil used for the pot study was Miracle-Gro Potting Mix from Miracle-Gro Lawn Products Inc. (Marysville, OH). The nitrogen, phosphorous and potassium content of the potting mix was 0.18%, 0.1% and 0.1%, respectively. The seeds were sown in plastic pots, each containing approximately 2.0 kg of potting mix. The plants were kept outdoors in an enclosed area except during extreme weather conditions. The seedlings were thinned to four plants per pot at the two-three leaf stage. Modified Hoagland's nutrient solution (Hoagland and Arnon, 1950) was supplied to the plants daily, after the plants attained three to four leaves. The composition of the nutrient solution was 0.5 mM $Ca(NO_3)_2$, 3.1 mM NH_4NO_3, 0.01 mM KH_2PO_4, 50.0 mM KCl, 0.2 mM $CuSO_4$, 12.0 mM H_3BO_4, 0.1 mM $NiSO_4 \cdot 6H_2O$, 2.0 mM $MnSO_4 \cdot H_2O$, 0.5 mM $ZnSO_4 \cdot 7H_2O$, and 0.2 mM $MgSO_4$. To simulate heavy metal contamination in soil, plant groups were supplied with 2 mM (ZnT1), 10 mM (ZnT2), 50 mM (ZnT3) and 150 mM (ZnT4) of Zn solution, supplied as $ZnSO_4 \cdot 7H_2O$; and with 1 mM (CdT1) and 10 mM (CdT2) of Cd solution, supplied as $CdCl_2$. All the treatment groups along with a control or untreated (T0) plant group were arranged in a completely randomized

design with eight replicates in each Zn group and five replicates in each Cd group. The metal treatment with the application of assigned metal solution for each treatment group was started at five weeks after sowing at the rate of 100 mL pot^{-1} d^{-1} for Zn-treated plants and at six weeks after sowing for Cd-treated plants. The metal treatments were applied daily and supplemented with nutrient solution to avoid any water and nutrient deficiencies. The control (T0) group was supplied with only nutrient solution. All the Zn- and the Cd-treated groups were treated groups were respectively harvested 19 d and 16 d after the start of the metal treatment application. This is because the higher Zn- (ZnT3 and ZnT4) and Cd-treatment (CdT2) groups started to show chlorotic symptoms by 19^{th} and 16^{th} day respectively.

Procedure for microscopic study: Leaf, stem and root samples from 24 barley plants consisting of control (T0) plants and plants treated with ZnT1 to ZnT4 and CdT1 and CdT2 were collected and prepared for light microscopy (LM) and scanning electron microscopy (SEM). Stem and root samples of 5 mm length were excised from 2 cm above and 2 cm below the stem–root intersection, respectively. Leaf samples of 5 mm length were excised from the middle portion of the second leaf from the base of the plant. The samples were immediately fixed in formaldehyde acetic acid for LM and in glutaraldehyde for SEM studies.

For the LM study, leaf and root samples of plants were alcohol dehydrated, paraffin embedded, ultramicrotomed and subjected to safranin (0.1%) - fastgreen (0.2%) staining for further observation (Sass, 1958). For the SEM study, leaf and stem samples were immediately fixed in 2.5% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.1) for 8 h (Johansen, 1940; Sass, 1958), and then dehydrated in ethanol series. The samples were then sealed in parafilm, frozen in liquid nitrogen and fractured transversely using a pre-cooled knife. The cryofractured specimens were critical point dried through carbon dioxide mounted on stubs, and coated with gold-palladium. All materials were observed with a LEO SEM (Carl Zeiss SMT, Cambridge, UK).

Chemical analysis: The plants were cut about 2 cm above the soil level, shoots and roots were harvested, and the roots were washed with deionized water and the samples were dried at 80ºC in an oven for 48 h for chemical analysis. Dry shoots and roots were then ground and weighed. Plant samples (*ca.* 0.5 g) were digested with concentrated HNO_3 and H_2O_2 (Jackson, 1958; Han et al., 2004). The digested solution was filtered and then analyzed for Zn and Cd concentration using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Han and Banin, 1997).

Plant measurements and statistical analysis: The plant heights from the root-stem intersection to the growing tip of the stem were measured at the end of the experiment. The fresh weights and dry weights of the shoots were obtained using an electronic balance. The water content (WC) of the plants was obtained using the formula [(Fresh weight – Dry weight)/ Fresh weight]. Statistical analysis was conducted with SAS statistical software (SAS Institute Inc., Cary, NC, USA). The GLM procedure was used for the analysis of different metal treatments, with means separated by the Duncan multiple range test at $P < 0.05$. The CORR procedure was used for correlation analysis with means separated at $P < 0.05$. Least significant difference was used for comparisons between the treatment means.

RESULTS

Effect of Zn and Cd accumulation on plant growth and physiology: Barley plants grew steadily in all of the Zn- and Cd-treated groups and chlorosis was not visually observed during the treatment process except in higher Zn- (ZnT3 and ZnT4) and Cd-treatment (CdT2) groups towards the end of the experiment. Metal accumulation in plant shoots and roots, metal concentration in the soil and plant growth characteristics including: fresh weight, dry weight, WC and plant heights are summarized in Table 1 for Zn- and in Table 2 for Cd-treated plants. Metal accumulation in plant shoots and roots increased significantly ($P < 0.05$) in both Zn- (Table 1) and Cd- (Table 2) treated groups with increase in applied metal solution concentration. The fresh weight, dry weight, WC and plant height decreased significantly ($P < 0.05$) in both ZnT3- and ZnT4-treated plants when compared to the control (T0) plants. In the case of Cd-treated groups WC showed significant changes in CdT2-treated plants compared to control (T0) plants.

Table 1. Accumulation of Zn in shoots, roots and soil, fresh weight and dry weight of shoots, water content (WC), and height of plants treated with Zn at the end of the experiment. Values are means ± SE of eight replicates. Within each column, means followed by a distinct letter differ significantly from each other at the 0.05 probability level. T0 = control plants; ZnT1, ZnT2, ZnT3, and ZnT4 designate respectively plants treated with 2, 10, 50 and 150 mM of Zn solutions.

Treatment	Shoot concentration (mg kg^{-1} DW)	Root concentration (mg kg^{-1} DW)	Soil concentration (mg kg^{-1} DW)	Fresh weight (g)	Dry weight (g)	WC (%)	Plant Height (cm)
T0	97 ± 11 d	141 ± 51 d	41 ± 3 d	53 ± 1. 7 a	10.7 ± 0.6 a	80 ± 0.8 a	29 ± 0.5 a
ZnT1	686 ± 26 c	1865 ± 193 c	825 ± 215 c	58 ± 7 a	10.8 ± 1.3 a	81 ± 0.2 a	29 ± 1 a
ZnT2	893 ± 93 c	1920 ± 158 c	962 ± 191 c	49 ± 4.7 a	9.1 ± 0.9 b	82 ± 0.3 a	29 ± 1 a
ZnT3	4955 ± 298 b	7820 ± 838 b	5984 ± 1054 b	42 ± 2.5 b	9.2 ± 0.5 b	78 ± 0.4 b	23 ± 1.2 b
ZnT4	11283 ± 667 a	18638 ± 591 a	21062 ± 5191 a	31 ± 3.5 c	7.8 ± 0.8 c	74 ± 0.5 c	21 ± 1.2 c

Table 2. Concentrations of Cd in shoots, roots and soil, fresh weight and dry weight of shoots, water content (WC), and height of plants treated with Cd at the end of the experiment. Values are means ± SE of five replicates. Within each column, means followed by a distinct letter differ significantly from each other at the 0.05 probability level. T0 = control plants; CdT1 and CdT2 designate respectively plants treated with 1 and 10 mM of Cd solutions.

Treatment	Shoot concentration (mg kg^{-1} DW)	Root concentration (mg kg^{-1} DW)	Soil concentration (mg kg^{-1} DW)	Fresh weight (g)	Dry weight (g)	WC (%)	Plant Height (cm)
T0	45 ± 14	187 ± 44 b	223 ± 67 b	31.5 ± 5 a	7.1 ± 1 a	77 ± 0.8 a	29 ± 1.3 a
CdT1	51 ± 4 b	351 ± 13 b	235 ± 27 b	28.8 ± 2.3 a	7.2 ± 0.5 a	75 ± 1.1 a	28 ± 0.8 a
CdT2	584 ± 22 a	3676 ± 505 a	2668 ± 773 a	25.9 ± 2.5 a	7.5 ± 0.6 a	71 ± 0.8 b	29 ± 0.7 a

Pearson's correlation coefficients were calculated to identify the effect of metal accumulation on the physiological and morphological characters of the plant. Correlations between Zn and Cd accumulation in shoots and roots to dry weight, WC and plant height are given in Table 3. There were statistically significant ($P < 0.01$; $P < 0.001$) negative correlations between Zn accumulation in shoots and roots to WC, plant dry weight and plant height (Table 3). In Cd-treated plants, statistically significant ($P < 0.01$) negative correlation was seen only between Cd accumulation in shoots and roots to WC (Table 3).

Table 3. Correlation coefficients between Zn ($n = 8$) and Cd ($n = 5$) concentrations in roots and shoots and plant characteristics: water content (WC), dry weight and plant height. *ns*, non significant ($P > 0.05$); *Statistically significant ($P < 0.01$); ** Statistically significant ($P < 0.001$).

Metal concentration (mg kg^{-1} DW)	WC (%)	Dry weight (g)	Plant height (cm)
Zn Root	-0.86**	-0.40*	-0.73**
Zn Shoot	-0.89**	-0.34*	-0.77**
Cd Root	-0.80*	0.01 *ns*	0.11 *ns*
Cd Shoot	-0.70*	-0.1 *ns*	0.06 *ns*

Effect of Zn and Cd accumulation on plant internal structure: The ZnT3- and ZnT4-treated plants showed gradual changes in leaf structure with an increase in metal concentration compared to the control group (T0). The SEM micrographs showed more compact epidermal and mesophyll cells with thickened cell walls in the leaves of both ZnT3- (Figure 1B) and ZnT4- (Figure 1C) treated plants compared to the T0 group (Figure 1A). The decrease in cellular size and intercellular spaces with an increase in metal concentration affected the thickness of the leaf in Zn-treated plants. The leaf thickness and cell sizes were calculated from the LM cross sections. The average ($n = 5$) leaf thickness decreased significantly ($P < 0.05$) from 157 μm in control (T0) plants to 151 μm in ZnT3- and 148 μm in ZnT4-treated plants. Similarly, the average ($n = 5$) epidermal cell size decreased significantly ($P < 0.05$) from 270 μm^2 in control plants to 241 μm^2 in ZnT3- and 217 μm^2 in ZnT4-treated plants. In both the cases, the light microscopic results were confirmed by detailed SEM

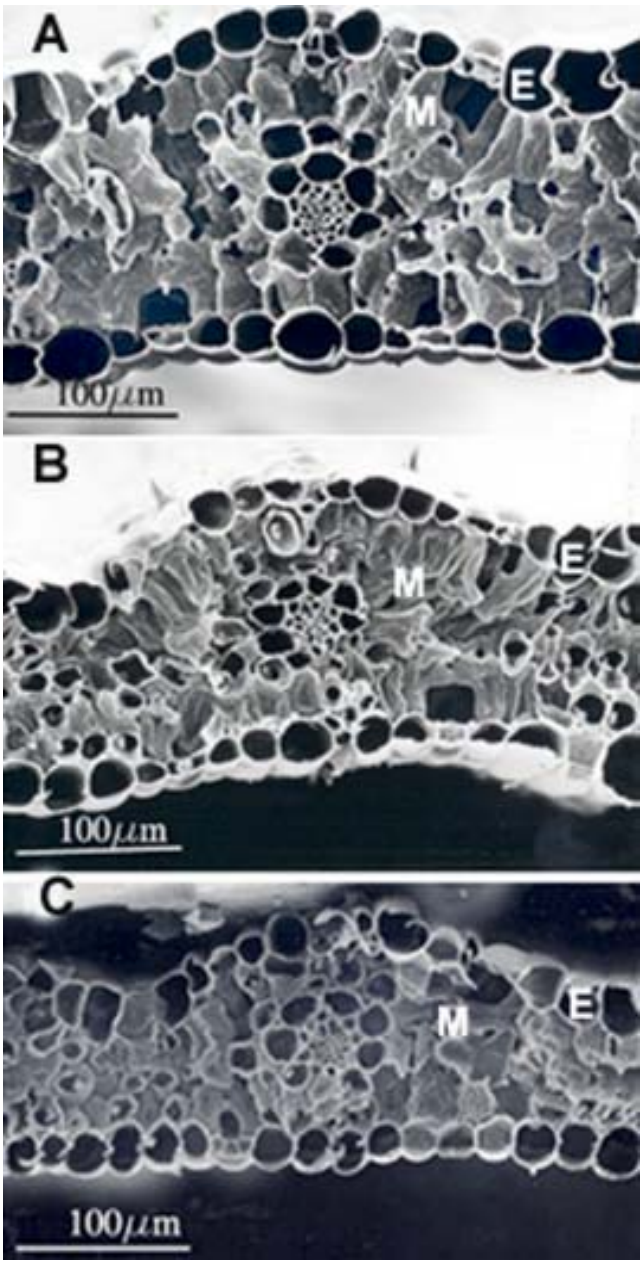

Figure 1. Micrographs (SEM) showing the transverse section of lower leaves of control (T0) (**A**), ZnT3- (50 mM Zn) (**B**) and ZnT4- (150 mM Zn) (**C**) treated plants. The leaves of ZnT3- and ZnT4-treated plants show a decrease in intercellular spaces and reduction in epidermal and palisade cell size compared to the control plants (**A**) by 19th day of metal treatment. E = Epidermal cells, M = Palisade mesophyll cells.

studies. The changes in chloroplasts and cellular components in ZnT1- and ZnT2- treated plants were not distinct from the control group. The magnitude of structural changes increased with an increase in metal accumulation among the Zn-treated groups. In CdT2-treated plants there were no significant changes in the internal structure of leaves compared to the control group (data not shown).

The SEM micrographs of stems of ZnT4- (Figure 2B) treated plants showed breakdown of the cell walls of vascular bundles compared to control plants (Figure 2A). Also the xylem vessels were dilated and lost their shape in the stems of ZnT4-treated plants (Figure 2B). The light micrographs of ZnT4- and CdT2- treated plant roots showed a decrease in their areas of cross section with an increase in the respective metal concentrations compared to the control group. The roots showed breakdown of

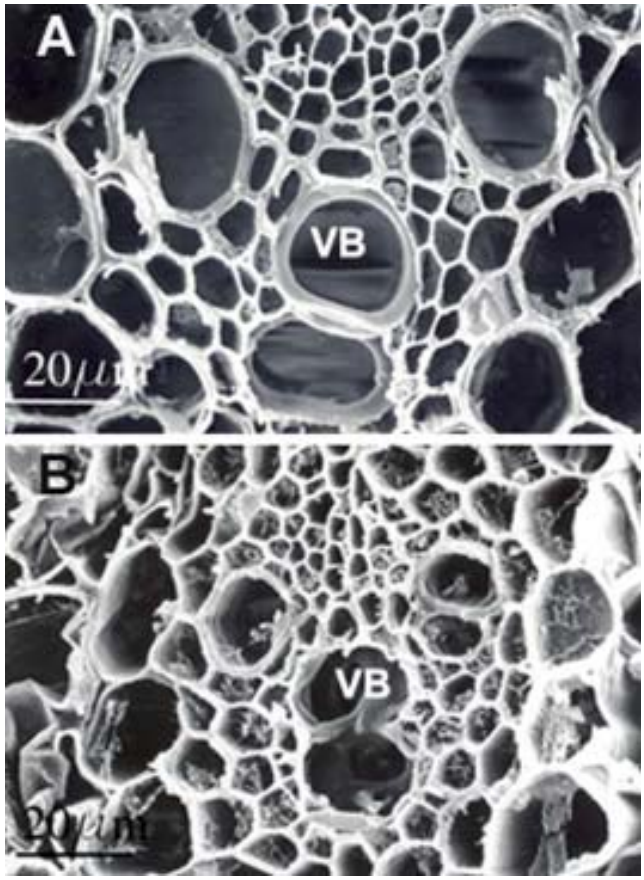

Figure 2. Micrographs (SEM) of transverse section of stems of control (T0) plants (**A**) and ZnT4- (150 mM Zn) (**B**). Note the depositions and breakdown of vascular bundles in stems of ZnT4- (**B**) treated plants compared to control (**A**) group. The plants were sampled on the last day of metal treatment. VB = Vascular bundles. The marked arrows (**B**) show the depositions.

cells in the cortex region in both ZnT4- (Figure 3B) and CdT2- (Figure 3C) treated groups and reduced cell size compared to control (Figure 3A) group. The changes in root, stem and leaf of ZnT1- and ZnT2- treated groups were not significant from the control group.

DISCUSSION

Our study demonstrates significant correlations between Zn accumulation in plant shoots and roots with different plant characteristics. The leaves of Zn-treated plants showed structural changes, such as decrease in intercellular spaces, breakdown of vascular bundles, and shrinkage of palisade and epidermal cells. In the case of Cd-treated plants, no significant structural changes in leaves were observed even though Cd accumulation in plant shoots was confirmed by the analytical results (Table 2). Also no significant correlation was identified between Cd concentrations in shoots and roots with plant dry weight and heights in Cd treated plants.

Our study shows that Zn-treatments at different concentrations increase linearly in metal accumulation in roots and shoots of barley plants. Since Zn acts as a growth promoting micronutrient at lower concentrations, the ZnT1- and ZnT2-treated groups showed no significant changes in dry weight, WC, and increase in plant height compared to the control (T0) group, suggesting that barley has a high tolerance and uptake ability for Zn. It was suggested that 100 mg L^{-1} of Zn concentration was not phytotoxic to *Brassica juncea* (Kumar et al., 1995), when added to a soil mixture. However at higher concentrations (ZnT3 and ZnT4), the plants showed significant changes in physiological and morphological characters, such as reduction in WC, dry weight and plant height (Table 1).

Apart from these physiological and morphological changes, Zn accumulation also results in structural changes in leaves, stems and roots. Structural changes in leaves include shrinkage of epidermal, palisade and spongy parenchyma cells. The Zn accumulation in stems of ZnT4-treated plants was seen as depositions in SEM micrographs (Figure 2B). Several studies verified these depositions as compounds of Zn through X-ray probe microanalysis (Denny and Wilkins, 1987; Van Steveninck et al., 1987, 1990; Vazquez et al., 1992, 1994; Kupper et al., 1999). These depositions were found to be either simple

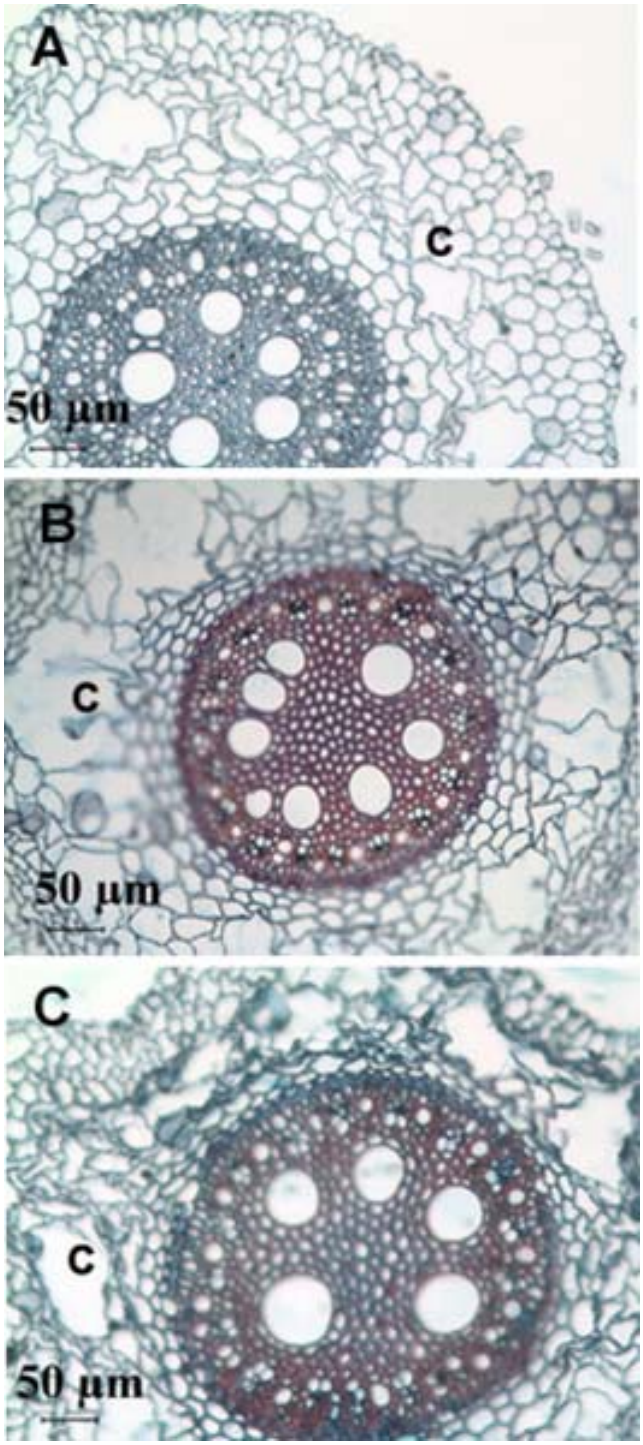

Figure 3. Light micrographs of transverse section of roots of control-T0 (**A**), ZnT4- (150 mM Zn) (**B**) and CdT2- (10 mM Cd) (**C**) treated plants. The micrographs show a breakdown of cells in the cortex region in ZnT4- (**B**) and CdT2- (**C**) treated plants compared to control (**A**) plants. The plants were sampled on the last day of metal treatment. C = Cortex and VB = Vascular bundles. The marked arrows show the breakdown of cortex cells.

salts of Zn or large organic molecules such as protein and carbohydrate complexes with Zn. Most of these studies did not correlate the structural changes to plant characteristics such as physiological and morphological changes and phytoremediation potential of plants.

In our study, we found that higher Zn concentrations (ZnT3 and ZnT4) resulted in a decrease in WC of stems and leaves of barley plants (Table 1), as also judged from the high negative correlation of WC with Zn accumulation in the shoots (r = -0.89) and roots of Zn-treated plants (r = -0.86). In our previous study (Maruthi Sridhar et al. 2005), significant structural changes in leaves, stems and roots of *Brassica juncea* were observed when treated with Zn concentrations of 100 mM to soil mixture for a 12 d treatment period. In this study, structural changes were observed in barley plants treated with Zn concentrations of 150 mM in the plant samples collected on the 19th day of metal treatment period.

In the case of Cd-treated plants no significant changes in WC, dry weight, and plant height at lower concentrations of Cd-treatment groups (CdT1) suggest that the applied concentrations of Cd were within the tolerance limit. This is consistent with barley being a Cd accumulator (Ebbs and Kochian, 1998). However due to significant metal accumulation in the CdT2-treated plants, the cross sections of roots showed break down of cortex cells in light micrographs of roots (Figure 3C).

Barley plants can accumulate significant amounts of Zn and Cd without showing phytotoxicity or reduction in plant growth when the environmental metal concentrations are relatively low. However higher Zn concentrations will result in structural changes in roots, stems and leaves and altered physiological and morphological characteristics. Our study presented here documented some of the physiological implications of structural alterations caused by Zn and Cd due to metal uptake, translocation and accumulation. The Cd and Zn uptake and accumulation patterns and their effect on different plant characteristics can be better understood by our research.

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