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Ultra structural deformation of plant cell wall under heavy metal stress in *Arabidopsis thaliana*

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Abstract
Nowadays, heavy metal pollution has become a serious environmental problem on global scale. The heavy metals are non-biodegradable in nature, which can easily accumulate in the organisms of lower trophic level, and enter to the human body system through food chain. Plants, like all other organisms, have evolved different mechanisms to maintain physiological concentrations of essential metal ions and to minimize exposure to non-essential heavy metals. As a first line of defence, many plants exposed to toxic concentrations of metal ions attempt to prevent or reduce uptake into root cells by restricting metal ions to the apoplast, binding them to the cell wall or to cellular exudates, or by inhibiting long distance transport. From this backdrop, the present experiment highlighted the effect of copper (Cu) heavy metals at 0, 0.5, 1.0, 1.5 and 2 mM of copper salt ($CuSO_4.5H_2O$) stress (mentioned as Cu stress) in the MS0 medium on *Arabidopsis thaliana* in terms of growth physiology, metal uptake, biochemistry, and ultra-structural deformation. Ultra structural damage was recorded highest for Cu vascular system at both 1.5 and 2 mM concentration. However, at 2 mM of Cu was the showed highest deformation in root and leaf was observed. Reactive oxygen species (ROS) levels were also higher in plants under stress. Cell wall measurements showed that heavy metal stress has effect on cell wall thickness and also on biomass accumulation. Thus, the first line of defence i.e. cell wall gets affected in the plants when under heavy metal stress.

Keywords: heavy metal, growth physiology, ultra structural deformation, *Arabidopsis thaliana*, confocal microscopy, scanning microscopy

Introduction
Heavy metals such as Cu and Zn are essential for normal plant growth and development since they are constituents of many enzymes and other proteins. However, elevated concentrations of essential and non-essential heavy metals in the soil can lead to toxicity symptoms and the inhibition of growth of most plants. The toxicity symptoms seen in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular-molecular level. Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element resulting in deficiency effects (Van Assche and Clijsters, 1990) [20]. In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress (Dietz *et al.*, 1999) [21]. Tolerance to heavy metals in plants may be defined as the ability to survive in a soil that is toxic to other plants, and is manifested by an interaction between a genotype and its environment (Macnair *et al.*, 2000) [22]

Nowadays, heavy metal pollution has become a serious environmental problem on global scale. Heavy metal is a member of a loosely defined subset of elements that exhibits metallic properties. It mainly includes the transition metals, some metalloids, lanthanides, and actinides. These metals when present in excess in the environment disturb the balance of the ecosystem. The heavy metals are non-biodegradable, accumulate in the organisms at lower tropic level, and enter to the human body system through food chain resulting in serious threats to human health (Patra *et al.*, 2004) [23]. Heavy metals such as Cu, Zn and Ni play a dual role in plant physiology. They are essential micronutrients for the plant development and growth, but are toxic to them at higher concentrations (Munzuroglu and Geckil, 2002) [26]. Also, plants during their growth are subjected to non-essential heavy metals such as Hg^{+}, Cd^{2+} and Pb^{2+} that are present naturally in the soil or water or as a contaminant from anthropogenic activities (Li *et al.*, 2005) [27]. It is also known that the metal sensitivity and toxicity of the plants is not only influenced by the concentration and the type of the toxicant, but also on the developmental stage of the plant (Liu *et al.*, 2005) [19].

Cell wall has a role to play in metal ion uptake and tolerance, binding properties of the cell wall and its role as a mechanism of metal tolerance has been a controversial one. Earlier reports have been reviewed (Ernst *et al.*, 1992) [24] and there have only been a few more papers on this topic. Although the root cell wall is directly in contact with metals in the soil solution, adsorption onto the cell wall must be of limited capacity and thus have a limited effect on metal activity at the surface of the plasma membrane. It is also difficult to explain metal-specific tolerance by such a mechanism (Ernst *et al.*, 1992) [24]. However, Bringezu *et al.* reported that the heavy metal-

tolerant *Silene vulgaris* ssp. *humilis* accumulated a range of metals in the epidermal cell walls, either bound to a protein or as silicates (Bringezu *et al.*, 1999) [5]. There is also evidence that heavy metal stress can cause cell damage in the plants (Mondal *et al.*, 2013) [25]. Keeping in mind the above facts, the objectives of the present experiment are to understand the toxic effect of these metals on plants and their accumulation pattern in different parts of the plant and observation of ultrastructural deformation of xylem and phloem under such heavy metal stress. Numerous works has been done by considering different heavy metals, but use of Cu heavy metal and observe its effect on ultrastuctural deformation is very uncommon.

Materials and Methods

Plant material and stress treatment

Arabidopsis thaliana seeds were surface sterilized, rinsed with sterile water and stratified at 4 °C for two days on half-strength Murashige and Skoog (½ MS; 1962) medium supplemented with 1 % agar, 1 % sucrose. The seedlings were transplanted to the soil mixture of vermiculite: peat moss: perlite (1:1:1) in the greenhouse under a 16 h light and 8 h dark cycle at 20 ±1 °C and light intensity of 60–70 µmol PPFD m^{-2} s^{-1} and irrigated with ½ MS salts, weekly. For stress treatment, 21 days old seedlings were supplemented with desired concentration of 0, 0.5, 1.0, 1.5 and 2 mM of copper salt ($CuSO_4.5H_2O$) stress (mentioned as Cu stress) in the MS0 medium. Three biological replicates were collected from each sample at respective time points after salt stress treatment.

In-situ ROS staining

In situ ROS staining was done in accordance with Beyer and Fridovich (1987) [3], on the basis of the principle of NBT (nitroblue tetrazolium) reduction to blue formazan by $O_2^{-\cdot}$. The intracellular concentration of ROS ($O_2^{-\cdot}$) was directly proportional to the development of intensity of blue colour in the leaves. Briefly, leaf tissue was detached from the wild type and transgenic plants and vacuum infiltrated with 10 mM sodium azide (NaN3) in 10 mM potassium phosphate buffer for 1 min. The infiltrated leaf tissue was incubated in 0.1% NBT (nitroblue tetrazolium) in 10 mM potassium phosphate buffer; pH 7.8 for 30 min. The stained leaf tissue was boiled in acetic acid: glycerol: ethanol (1:1:3) solution to remove other pigments and the stain content was visually documented under Carl-Zeiss Stereo DiscoveryV12 with Axiovision software. This experiment was repeated three times from three biological replicates.

Physiological Parameters

After 15-16 days of treatment, the fresh weight and length of root and shoot of the plants were measured along with other yield parameters. After completion of Arabidopsis lifecycle both under salt and stress conditions, the biomass calculations in terms of rosette diameter, number of leaves, root and shoot biomass was done. Also, yield measurements in terms of number of pods, seeds/pod and total number of seeds/plant were done.

Microscopy

For confocal microscopic analysis, inflorescence stems of WT and transgenic lines were harvested and fixed in formalin, glacial acetic acid and ethyl alcohol (FAA, 1:1:18) at room temperature. Sections of 8–10 µm thickness were cut and stained with 1 % safranin and 4 % fast green. These sections were mounted and examined using Confocal Laser Scanning Microscope (Zeiss LSM510 meta Gmbh, Germany).

For SEM analysis, segments from the apical 1 cm of stem cross-sections were fixed in a mixture of 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4 for 1 h and then with 1 % OsO4 in 0.1 mol/l cacodylate buffer, pH 7.4 for 30 min. After critical point drying, the samples were sputter-coated with gold, and the coated samples were viewed with a Hitachi S-3400N field emission SEM using an accelerating voltage of 30 kV. For TEM study, stem slices were fixed in 4 % (w/v) paraformaldehyde and 1 % (v/v) glutaraldehyde in 0.1 mM phosphate buffer, pH 7.2, for 4 h at room temperature and then postfixed in 1.33 % OsO4 in cacodylate buffer, pH 7.2, and stained with 1.5 % uranyl acetate. All samples were dehydrated in acetone series, followed by propylene oxide. Embedment was in Araldite, Epon, and dodecenyl succinic anhydride in proportions 1:1:3. Polymerization was carried out at 80 °C, and micrographs were taken with a Tecnai G2 TF20 electron microscope (FEI, Netherlands).

Cell wall Measurements

Measurements of cell wall thickness and shape were done with a Hitachi S-3400N field emission SEM using an accelerating voltage of 30 kV.

Statistical analysis

All experiments were conducted with at least three independent repetitions in triplicates. All values are shown as the mean ± the standard deviation. The statistical analysis was performed using Statistica software (v.7). The statistical significance between the mean values was assessed by analysis of variance (ANOVA) applying Duncan's Multiple Range Test (DMRT). A probability level of $P \leq 0.05$ was considered significant.

Results and Discussion

Effect of heavy metal stress on plants

Arabidopsis plants were given heavy metal stress (0, 0.5, 1, 1.5 and 2 mM) and changes in the phenotype and morphological changes were recorded (Fig.1). The first response of plants to any metal stress is growth adjustment (Lei *et al.*, 2007) [8]. Under control conditions, plants phenotype was normal, leaves were green and plants were healthy (Fig.1). At 0.5 mM conditions plants, some changes in the plant phenotype were observed but with the increase in stress i.e. 1.5 mM metal stress, wilting and chlorosis was observed which is an indication of stress acclimation in plants (Fig. 1). Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress. Plants exposure to low level heavy metal stress activates an array of processes leading to an improvement of plant stress tolerance. ROS generation

under heavy metal stress (Fig.2) was also observed, and it was seen that under control conditions (0 mM) ROS production was not observed even after 18d period but in those plants where 0.5, 1, 1.5 and 2 mM heavy metal stress was given, ROS accumulation was seen and its accumulation increased with the increase in duration of stress (Fig.2). The degree of toxicity of heavy metals depends on the rate of production of reactive oxygen species like superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). Entry of the heavy metals into food chain is the result of consumption of plant materials grown in contaminated soil (Zou, *et al.*, 2006). Net photosynthesis rate was also observed under control and stress conditions (Fig.3), it was observed that under stress conditions the rate of photosynthesis decreased and at highest level of metal stress (2 mM) the photosynthesis rate dropped quickly. This indicates that plants have shut down the photosynthesis when experiencing heavy metal stress as a feedback tolerance mechanism to escape stress. There is very strong inhibitory effect of lead on chlorophyll a and b content in mustard (Fargašová, 2001) [7] and tomato plants (Beyersmann and Hartwig, 2008) [8]. This change is due to oxidative stress (Azad *et al.*, 2011) [11] and the inhibition of chlorophyll biosynthesis as a result of heavy metal accumulation in the plant tissue because lead also prevents photosynthetic activity of enzymes like δ-aminolevulinic acid dehydratase (Prasad and Prasad, 1987) [11]

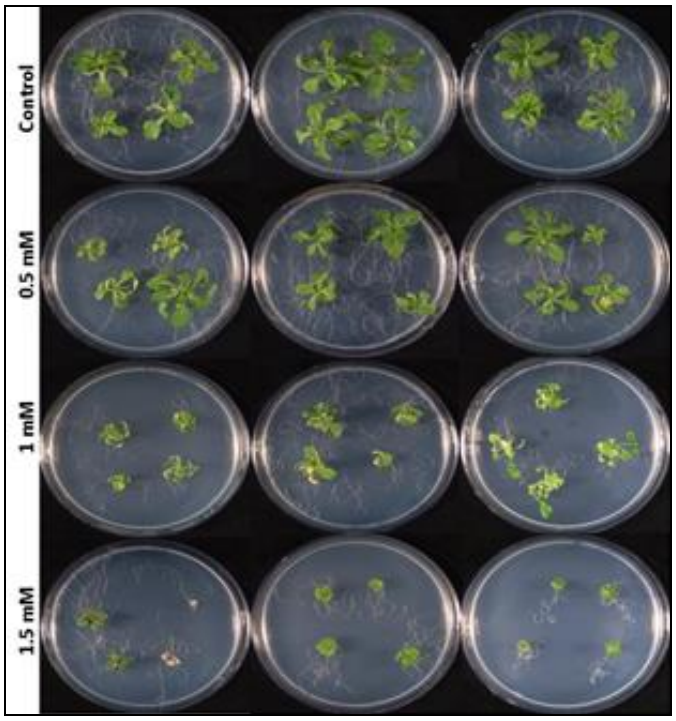

Fig 1: Growth of Arabidopsis under control (0 mM) and Cu stress (0.5, 1, 1.5 mM).

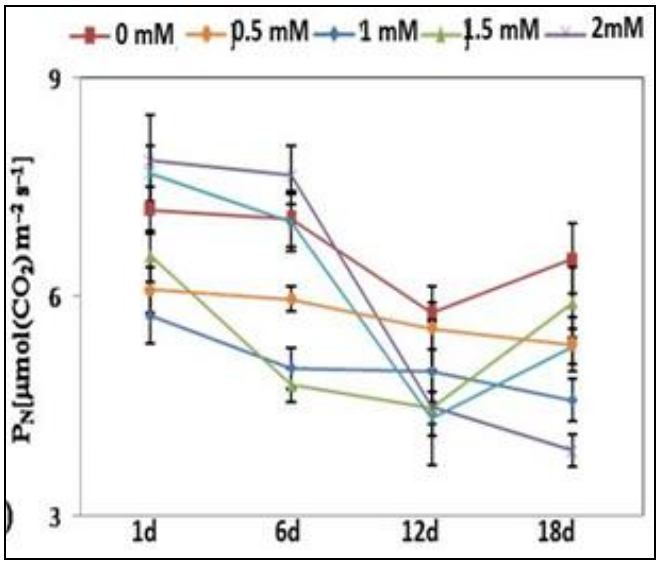

Fig 2: Comparative net rate of photosynthesis (P_N) of Arabidopsis under control (0 mM) and Cu stress (0.5, 1, 1.5, 2 mM) after 1d, 6d, 12d and 18d of stress.

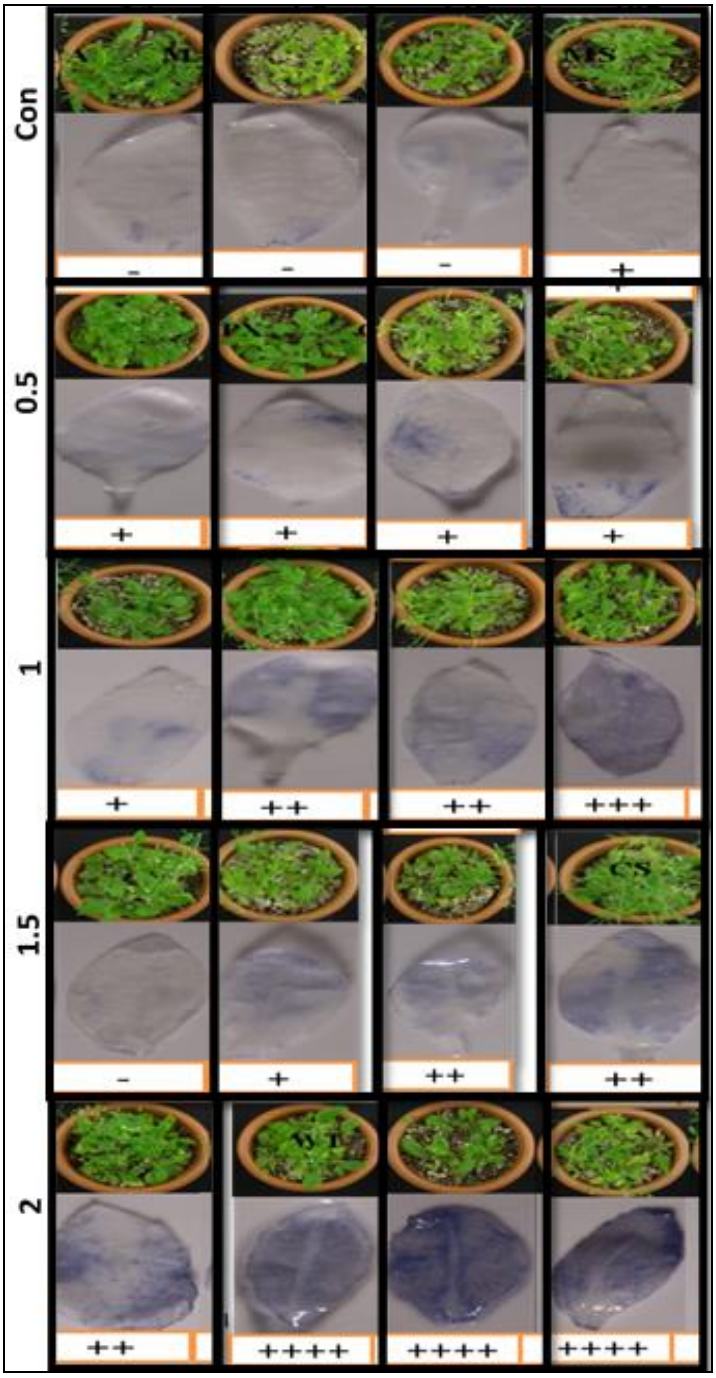

Fig 3: Growth of Arabidopsis under control (0 mM) and Cu stress (0.5, 1, 1.5, 2 mM) after 1d, 6d, 12d and 18d of stress.

Morphological and Developmental Phenotypes under Heavy metal stress

Heavy metals such as Cu and Zn are essential for normal plant growth, since they are essential component of many enzymes and other proteins. However, elevated concentration of both essential and non-essential metals can result in growth retardation and cellular toxicity. The internal structure of the stems was characterized in Arabidopsis under control and stress conditions, which revealed that vascular bundles were well connected with interfascicular fibers forming a continuous cylinder of lignified tissue around the pith. Under heavy metal stress, thinner stems in plants had smaller vascular bundles and less developed fibers in the interfascicular tissue under stress conditions (Fig. 4). This indicates that plant vascular system and the cell wall is experiencing the heavy stress. Thus in the present study, anatomical investigation of vascular structures using confocal and electron microscopy clearly showed that disruption and distortion in the morphology of plants with heavy metal stress (Fig. 4). The wealth of information gained from the Arabidopsis genome sequence (Arabidopsis Genome Initiative 2000), coupled with the powerful tools available to Arabidopsis researchers (Seki *et al.*, 2002; Rhee *et al.*, 2003) [13, 12], has facilitated much progress within the cell-wall research community in identification of genes encoding enzymes involved in cell-wall biosynthesis.

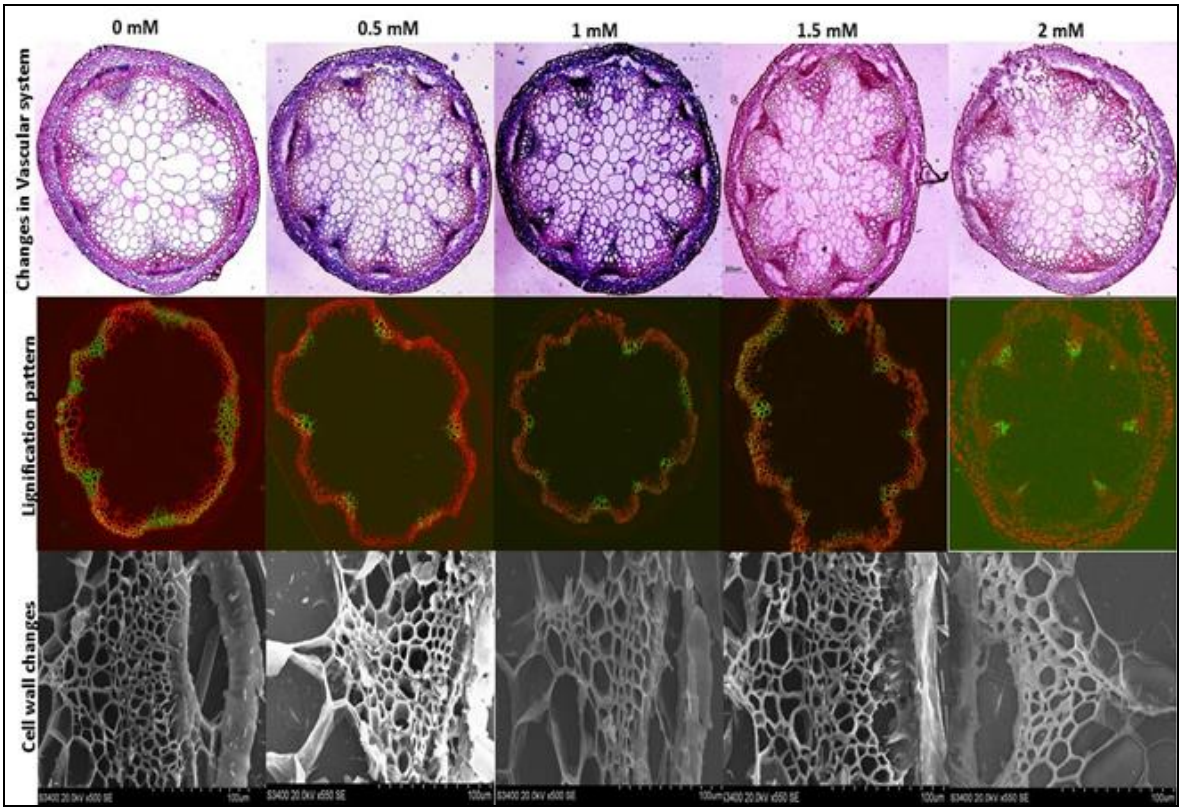

Fig 4: Stem sections of Arabidopsis stem observed with bright field, confocal and scanning microscope under control (0 mM) and Cu stress conditions (0.5, 1, 1.5 and 2 mM).

Effect of drought stress on yield and Biomass

The Arabidopsis showed better performance under control conditions in terms of growth parameters such as, plant height, root length, rosette area and number of leaves (Table 1) as compared to stress conditions. Several yield parameters such as size of pod, number of pods and total seed yield were calculated (Table 1). It was observed that under stress conditions yield and biomass accumulation decreased, which indicates that plants are experiencing extensive drought stress. Decrease in plant biomass under chromium stress was reported earlier on different plants (Dixit *et al.*, 2002; Sharma & Sharma, 1996) [6, 18]. This can be explained by the fact that under heavy metal stress, availability of water became restricted to all the parts of the plants or due to disturbed carbohydrate and nitrogen metabolisms and reduction in protein synthesis (Sharma & Sharma, 1996) [18].

Table 1: Biomass and yield attributes of Arabidopsis under control (0 mM) and heavy metal stress (0.5, 1, 1.5 and 2 mM) conditions

S. No	Attributes	0 mM	0.5 mM	1 mM	1.5 mM	2 mM
1	Plant Height (cm)	28.3	26.2	24.4	23.4	20
2	Root Length (mm)	8.4	6	4.5	4.3	3.2
3	Rosette Area (cm^2)	5	4.3	3.5	2	2
4	No. of Leaves	15	11	8	5	5
5	No. of Pods	44	39	37	35	30
6	Pod size (cm)	1.7	1.3	1.2	0.94	0.80
7	Total seeds	984	782	759	624	539

Changes in shape and size of Cell wall under heavy metal stress

The detailed changes that occur in wall thickness during heavy metal stress were investigated in Arabidopsis. Using scanning electron microscopy (SEM), cell wall thickness was measured in stem prepared by rapid plunge-freezing in liquid nitrogen slush and then freeze-fracturing perpendicular to their long axes (Table 2). Thickening, thinning, and maintenance of wall thickness were observed within this growth conditions, and heavy metal stress. It was concluded that wall thicknesses are regulated by heavy metal stress, are dependent upon the carbon resources available to the cell (Table 2). Recently, it was shown that lignin (Shafi *et al.*, 2014; 2015a, b; 2017) [14, 15, 16] serves as a major component of the casparian strip in *Arabidopsis thaliana* roots and also prevents ion diffusion in the root endodermis (Naseer *et al.* 2012) [10]. Bringezu *et al.* reported that the heavy metal-tolerant *Silene vulgaris* ssp. *humilis* accumulated a range of metals in the epidermal cell walls, either bound to a protein or as silicates (Bringezu *et al.*, 1999) [5].

Table 2: Wall thickness of vessels and fibers in stems of the WT and transgenic lines under heavy metal stress

S. No	Sample	Interfascicular fibers (µm)	Vessels (µm)	Xylary fibers (µm)
1	0 mM	2.411 ± 0.09	0.918 ± 0.02	0.261 ± 0.02
2	0.5 mM	2.139 ± 0.14	0.813 ± 0.03	0.218 ± 0.03
3	1 mM	2.019 ± 0.18	0.655 ± 0.02	0.17 ± 0.02
4	1.5 mM	1.648 ± 0.14	0.475 ± 0.05	0.155 ± 0.02
5	2 mM	1.358 ± 0.14	0.415 ± 0.05	0.132 ± 0.02

Wall thickness was measured from electron micrographs of fibers and vessels. Data are mean (µm) ± SE from cells. Means were compared using ANOVA.

Conclusion

This paper has focused on recent evidence that identifies potential cellular-molecular mechanisms that may be involved in the resistance and tolerance of plants to excess concentrations of heavy metals in the environment. An improved knowledge in these crucial areas will help to further elucidate the molecular mechanism that lie beyond plant metal effect at cell wall level.

Acknowledgement

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References

1. Arabidopsis Genome Initiative. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana* Nature, 2000; 408:796-815.
2. Azad HN, Shiva AH, Malekpour R. Toxic effects of lead on growth and some biochemical and ionic parameters of sunflower (Helianthus annuus L. seedlings Current Research Journal of Biological Science, 2011; 3:398-403.
3. Beyer WF, Fridovich I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions Annals Biochemistry, 1987; 161:559-566.
4. Beyersmann D, Hartwig A. Carcinogenic metal compounds: Recent insight into molecular and cellular mechanisms Archives of Toxicology, 2008; 82:493-512.
5. Bringezu K, Lichtenberger O, Leopold I, Neumann D. Heavy metal tolerance of Silene vulgaris Journal of Plant Physiology, 1999; 154:536-546.
6. Dixit V, Pandey V, Shyam R. Chromium ions inactivate electron transport and enhance superoxide generation in vivo in pea Pisum sativum L. cv. Azad root mitochondria Plant Cell Environment, 2002; 25:687-693.
7. Fargašová A. Phytoxic effects of Cd, Zn, Pb, Cu and Fe on Sinapsis alba L. seedlings and their accumulations in roots and shoots. Biologia Plantarum, 2001; 44:471-473.
8. Lei Y, Korpelainen H, Li C. Physiological and biochemical responses to high Mn concentrations in two contrasting Populus cathayana populations. Chemosphere, 2007; 68:686-694.
9. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 1962; 15:473-497.
10. Naseer S, Lee Y, Lapierre CR, Franke C, Nawrath N, Geldner. Casparian strip diffusion barrier in Arabidopsis is made of a lignin polymer without suberin. Proc Natl Acad Sci. USA, 2012; 109:10101-10106.
11. Prasad DDK, Prasad ARK. Altered δ- aminolevulinic acid metabolism by lead and mercury in germinating seedlings of Bajra Pennisetum typhoideum. Journal of Plant Physiology, 1987; 127:241-249.
12. Rhee SY, Beavis W, Berardini TZ *et al.* The Arabidopsis Information Resource TAIR: a model organism database providing a centralized, curated gateway to Arabidopsis biology, research materials and community. Nucleic Acids Research, 2003; 31:224-228.
13. Seki M, Narusaka M, Kamiya A *et al.* Functional annotation of a full length Arabidopsis cDNA collection. Science, 2002; 296:141-145.
14. Shafi A, Chauhan R, Gill T, Swarnkar MK, Sreenivasulu Y, Kumar S *et al.* Expression of SOD and APX genes positively regulates secondary cell wall biosynthesis and promotes plant growth and yield in Arabidopsis under salt stress. Plant Molecular Biology, 2015b; 87:615-631.
15. Shafi A, Dogra V, Gill T, Ahuja PS, Sreenivasulu Y. Simultaneous over-expression of PaSOD and RaAPX in transgenic *Arabidopsis thaliana* confers cold stress tolerance through increase in vascular lignifications. PLoS One, 2014; 9:e110302.
16. Shafi A, Gill T, Sreenivasulu Y, Kumar S, Ahuja PS, Singh AK *et al.* Improved callus induction, shoot regeneration, and salt stress tolerance in Arabidopsis overexpressing superoxide dismutase from Potentilla atrosanguinea. Protoplasma, 2015a ; 252:41-51.
17. Shafi A, Pal AK, Sharma V, Kalia S, Kumar S, Ahuja PS *et al.* Transgenic Potato Plants Overexpressing SOD and APX Exhibit Enhanced Lignification and Starch Biosynthesis with Improved Salt Stress Tolerance. Plant Molecular Biology Reportor, 2017; 35:504-518.
18. Sharma DC, Sharma CP. Chromium uptake and toxicity effects on growth and metabolic activities in wheat, Triticum aestivum L. cv. UP 2003. Indian Journal of Experimental Biology, 1996; 34:689-691.
19. Zou J, Wang M, Jiang W, Liu D. Chromium accumulation and its effects on other mineral Elements in Amaranthus viridis. Acta Biologica Cracoviensia Series Botanica, 2006; 48:7-12.
20. Van Assche F, Clijsters H. Effects of metals on enzyme activity in plants. Plant, Cell and Environment, 1990; 13:195-206.
21. Dietz K-J, Baier M, Kra¨mer U. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In: Prasad MNV, Hagemeyer J, eds. Heavy metal stress in plants: from molecules to ecosystems. Berlin: Springer-Verlag, 1999, 73-97.
22. Macnair MR, Tilstone GH, Smith SE. The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Banuelos G, eds. Phytoremediation of

contaminated soil and water. CRC Press LLC, 2000, 235-250.
23. Patra M, Bhowmik N, Bandopadhyay B, Sharma A. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. Environmental and Experimental Botany, 2004; 52:199-223.
24. Ernst WHO, Verkleij JAC, Schat H. Metal tolerance in plants. Acta Botanica Neerlandica, 1992; 41:229-248.
25. Mondal NK, Das C, Roy S, Datta JK, Banerjee A. Effect of varying cadmium Stress on chickpea Cicer arietinum l seedlings: An Ultrastructural study. Annals of Environmental Science, 2013; 7:59-70.
26. Munzuroglu Ö, Geckil H. Effects of Metals on Seed Germination, Root Elongation and Coleoptile and Hypocotyl Growth in Triticumaestivum and Cucumissativus. Ach. Environ. Contam. Toxicol, 2002; 43:203-213.
27. Li Y, Dhankher O, Carreira L, Balish R, Meagher R. Engineered overexpression of γ-glutamylcysteine synthetase in plants confers high level arsenic and mercury tolerance Environmental Toxicology and Chemistry, 2005; 24:1376-1386.