Differential Physiological and Ultrastructural Responses of Cottonseeds under Pb Toxicity

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Abstract

Lead (Pb) is a toxic heavy metal that causes physiological and morphological changes in plants. Our current study was aimed at observing Pb-induced changes in seeds of two upland cotton varieties (TM-1 and Z-747) that were exposed for 24 hours to various Pb concentrations (0, 50, 100, 300, and 500 μM). Seeds of TM-1 showed higher germination percentages, water uptake, and cell viability as compared to Z-747. Changes in nuclei shape, their partial disappearance, and increase in vacuole size were the initial signs of lead toxicity in Z-747, while TM-1 maintained its typical conformations. Cell wall thickening; increase in the number of mitochondria, protein, and lipid bodies; and vacuolation were more prominent in TM-1. In contrast, plasmolytic shrinkage and scattered cell organelles were the key characteristics of Z-747 at higher doses of Pb. Those observations demonstrated that seeds of TM-1 were more tolerant to Pb stress as compared to Z-747 and may be considered a preferred choice for culture on Pb-contaminated soils.

Introduction

Seed is the start of new life and germination studies help to characterize a specific cultivar under stress conditions [1]. Different environmental factors like drought, temperature, salinity, and heavy metal contamination affect seed germination and ultimate growth of plants [2, 3]. Literature reveals that lead (Pb) is a non-essential toxic heavy metal for plants [4, 5]. Its main uptake occurs via roots, where it binds to carboxyl groups of mucilage uronic acid or when it comes in direct contact with polysaccharides of rhizodermal cells. But at the molecular level, the exact mechanism remains a matter of discussion [6]. Pb affects various physiological functions of plants like photosynthesis, water uptake, nutrient balance, and germination [4]. It interferes with carbohy-

drate and protein metabolism in seeds and affects the activities of metabolizing enzymes like amylases, acid invertases, and proteases. Altered activities of these enzymes might negatively affect metabolism of seed reserves needed for radical emergence [7]. Basically, Pb induces biochemical changes that usually trigger some crosstalk between antioxidant enzymes and reactive oxygen species (ROS), including singlet oxygen and hydrogen peroxide [8]. Although ROS plays a significant role in seed development, germination, dormancy, and aging, their overproduction can cause serious effects on seeds. It is under debate whether an increased level of Pb could affect the seed embryo as compared to the endosperm or cotyledons [9].

Besides physiological changes, Pb also causes ultrastructural variations in the cells of different plant parts [10, 11]. From a morphological point of view, chloroplasts and mitochondria represent indispensible components of cellular metal homeostasis in photosynthetic cells [12] and are

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the main targets of abiotic stress [13]. In two species of *Lespedeza*, ultrastructural analyses indicate an increase in the number and volume of starch grains, low density of cell organelles in cytoplasm, some disintegration of mitochondria, dilation of nuclear envelops, and irregular cell wall thickening [14].

Inside cells, plants have developed various mechanisms for detoxifying Pb such as sequestration in vacuole, phytochelatin synthesis, and binding to glutathione and amino acids [15, 16]. The tolerance capacity of plants against Pb is mainly associated with its restrictive localization to their cell walls, synthesis of osmolytes, and activation of the antioxidant enzyme system [17]. So, better understanding of Pb tolerance in cotton seeds requires ultrastructural observations in addition to physiological studies.

Seed coats play a key role in delayed germination, and its structural variations control the diffusion chemistry of seeds when comparing seeds with or without coats in growth medium [18]. To standardize the measure of seed reserves mobilization and to compare the sensitivity of cotton cultivars to Pb stress, seed coats were manually removed in our experimental series. We have focused on seeds of 2 upland cotton germplasms i.e. TM-1 and Z-747 differing in Pb toxicity based on initial screening of seven varieties (data not shown here). Since most research work previously done in cotton is focused on leaves and roots, when studying metal toxicity [19, 20] we tried to explore seed response to elevated levels of Pb. Our main objectives were thus to study physiological behavior as well as ultrastructural changes in seeds of cotton cultivars different in Pb tolerance. To the best of our knowledge, there is no detailed Pb toxicity comparative study yet reported in germinating cotton seeds.

Materials and Methods

Seed Treatment and Growth Conditions

Two cultivars of upland cotton were used in our experiments i.e. TM-1, a standard genetic line, and Z-747, an extending breeding material suitable for the Xinjiang region of China. Seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 5-6 min, washed thrice with distilled water, and seed coats were removed manually. Eight seeds in each Petri dish were supplied with 10 ml of half strength modified Hoagland solution containing five different levels of $Pb(NO_3)_2$ i.e. 0, 50, 100, 300, and 500 μM. Covered Petri dishes were kept in a growth chamber under dark conditions for 24 hours at 28±2ºC with relative humidity of 60%. After 24 hours, germinated seeds were treated with Na_2-EDTA for 15-20 min to remove unbound Pb, followed by thorough washing with distilled water. Modified Hoagland solution comprised of 500 μM $(NaH_4)_2SO_4$, 500 μM $MgSO_4$, 200 μM K_2SO_4, 1000 μM KNO_3, 600 μM $Ca(NO_3)_2 \cdot 4H_2O$, 200 μM, KH_2PO_4, 10 μM $FeSO_4 \cdot 12H_2O$, 0.5 μM $MnSO_4 \cdot H_2O$, 0.25 μM $ZnSO_4 \cdot 7H_2O$, 0.05 μM $CuSO_4 \cdot 5H_2O$, 100 μM H_3BO_3, and 0.02 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, adjusted to pH

6.5. To avoid precipitation of $Pb(NO_3)$, 50 μM Na_2-EDTA was also added to the nutrient medium.

Physiological Parameters

After treatment, seeds were first properly blotted dry between tissue papers to remove excess water and changes in seed weight and relative weight increase were carefully noted in both varieties. Water content was determined based on fresh and dry weights using the formula:

$$WC (\%) = [(FW - DW)/FW] \times 100$$

For dry weight determination, samples of TM-1 and Z-747 were oven dried at 65ºC for 72 hours.

Cell viability was determined by triphenyl tetrazolium chloride (TTC) reduction method [21]. A standard curve was drawn using a 0.4% TTC solution, where (Y = 0.003X + 0.009), Y represent = OD 485 value, and X = μg TTF (Tetrazolium triphenyl formazan content).

Pb Accumulation in Seeds

For Pb uptake, seeds of TM-1 and Z-747 were dried to constant weight at 70ºC for 72 hours, ashed in a muffle furnace at 250ºC initially for 2 hours, followed by 500ºC heating for another 10 hours. The ash of each sample was dissolved in 5 ml 1:1 HNO_3. Final volume of the extract was 10 ml with distilled water addition, and each sample was filtered twice to get the clear extract. Pb content was determined by an inductively coupled plasma mass spectrometer (ICP-MS 7500a, Agilent). Pneumatic nebulization technique was applied using a peristaltic pump with sample uptake @ 1 ml·min^{-1}. To minimize any memory effects, the system was operated in mass scanning mode, after tuning ion lenses. Each standard, sample, or blank was aspirated for about 2 min before data acquisition. An appropriate interval time (2 min) was maintained between the sample and blank. The data were expressed as μg·g^{-1} dry weight.

Transmission Electron Microscopy

Small sections of radical base (2-3 mm in length) of TM-1 and Z-747 were selected for electron microscopy observations. Sections were first fixed in 2.5% glutaraldehyde (v/v) in 0.1 M BPS (sodium phosphate buffer, pH 7.2) for more than 4 hours and then post-fixed in 1% OsO_4 in phosphate buffer (0.1 M, pH 7.2) for 1.5 hours. Specimens were dehydrated with a graded series of ethanol for 15-20 min i.e. 50%, 70%, 80%, 90%, 95%, and 100%, followed by absolute acetone for 20 min. Then specimens were placed in 1:1 mixture of absolute acetone and final spur resin for 1 hour at room temperature and transferred to mixture of absolute acetone and final spur resin (1:3) for 3 hours. Finally, specimens were put in final resin spur a mixture overnight. Ultra thin sections (90 nm) stained with uranyl acetate and lead citrate, mounted on gold, were examined with a transmission electron microscope (model H-7650 Hitachi, Japan) operating at 60 kV.

Table 1. Germination percentage, inhibitory germination rate, change in weight, and relative percent weight increase (RPWI) of two cotton varieties i.e. TM-1 and Z-747 under Pb stress.

Treatment Pb (μM)	Germination percentage (%)		Change in weight (g)		RPWI (%) TM-1 over Z-747
	TM-1	Z-747	TM-1	Z-747	
0	86.01±7.90a (0)	93.10±13.26a (0)	112.3±5.8a	104.2±0.0b	8
50	93.71±15.07a (8.2)	80.88±15.37ab (-15.1)	173.9±119.2a	98.5±14.3b	77
100	87.26±12.64a (1.4)	87.64±23.65ab (-6.2)	219.4±161.0a	155.9±18.6ab	41
300	83.92±18.93a (-2.5)	63.36±32.56ab (-46.9)	146.9±93.3a	131.5±31.7ab	12
500	71.98±14.56a (-19.5)	48.06±7.25b (-93.7)	198.7±122.9a	170.8±60.9a	16

Values are the mean of three replications ± SD. Variants possessing the same letters are not statistically significant at P<0.05. Parenthesis contain inhibitory germination rate.

Table 2. Seed biomass and water content of two cotton varieties, i.e. TM-1 and Z-747 as affected by elevated levels of Pb.

Treatment Pb (μM)	Fresh Weight (g)		Dry Weight (g)		Water content (g)	
	TM-1	Z-747	TM-1	Z-747	TM-1	Z-747
0	1.09±0.07a	0.77±0.06a	0.35±0.05ab	0.24±0.02a	0.65±0.04b	0.53±0.04a
50	1.03±0.05a	0.72±0.07a	0.34±0.03ab	0.25±0.04a	0.71±0.01ab	0.51±0.06a
100	1.10±0.06a	0.71±0.06a	0.36±0.01a	0.24±0.01a	0.72±0.05a	0.50±0.05a
300	1.03±0.03a	0.79±0.01a	0.38±0.03a	0.23±0.00a	0.67±0.01b	0.52±0.01a
500	0.90±0.01b	0.80±0.02a	0.29±0.04b	0.22±0.01a	0.58±0.04c	0.53±0.00a

Values are the mean of three replications ±SD. Variants possessing the same letters are not statistically significant at P<0.05.

Data obtained from the experiments were subjected to one-way analysis of variance (ANOVA) using a statistical software DPS (data processing system). The significant difference among different treatment means was determined by LSD method.

Results

Effects of Pb on Germination and Quantitative Traits

Radical appearance of 1 mm length was set as a benchmark for germination rate. Data revealed non-significant changes in TM-1 germination rate while Z-747 showed radical inhibition at elevated Pb levels as compared to control (Table 1). Comparative analysis demonstrated 50% more germination in TM-1 vs. Z-747 at 500 μM. Similarly, at the highest Pb level, 19.5% and 93.7% inhibitory rates were observed in TM-1 and Z-747, respectively, over their respective controls.

Various quantitative traits such as change in weight, fresh and dry weight, as well as water content percentage were also studied. Results showed non-significant changes in seeds' weight of TM-1 at all levels of treatment (Table 1). However, TM-1 absorbed 8%, 77%, 41%, 12%, and 16% more water as compared to Z-747 at 0, 50, 100, 300, and 500 μM of Pb, respectively. Although fresh and dry weights of TM-1 and Z-747 were not affected by Pb but values were higher in TM-1 than Z-747, in all treatments (Table 2).

Effects of Pb on Cell Viability and Its uptake

Elevated levels of Pb induced changes in cell viability of TM-1 and Z-747, as TTC reducing capacity (Table 3). A stimulatory effect on cell viability of TM-1 was found at 50 and 100 μM, but a 31% decline was observed at 300 μM Pb over control. However, consecutive decline in viable cells was seen in variety Z-747. A comparison revealed 16%, 63%, 89%, 1%, and 110% more viability in TM-1 as compared to Z-747 at 0, 50, 100, 300, and 500 μM Pb, respectively.

Pb accumulation in both varieties followed a gradual increase as its concentration increased in growth medium, except in Z-747 at 500 μM Pb (Table 3). Maximum Pb uptake in TM-1 (4.3 μg·g^{-1} Fw) and Z-747 (3.7 μg·g^{-1} Fw) were observed at 500 and 300 μM Pb, respectively. Taken together, TM-1 absorbed 3-fold more Pb than Z-747 at the highest concentration of Pb.

Ultrastructural Observations

Microscopic observations of controls in both varieties revealed well-developed cell architecture. Intact cell membrane and cell wall, number of mitochondria, large vacuole, well-shaped nuclei and nuclear membranes, and one or

Table 3. Cell viability as TTF (µg·g⁻¹ Fw) and Pb content (µg·g⁻¹ DW) of two cotton varieties, i.e. TM-1 and Z-747, as affected by different levels of Pb.

Treatment Pb (µM)	Cell viability		Pb content	
	TM-1	Z-747	TM-1	Z-747
0	117.30±27.80abc	101.43±42.91a	0.292±0.03d	0.403±0.21e
50	137.89±7.75ab	83.72±2.96ab	0.719±0.13d	0.685±0.21d
100	143.00±25.52a	75.19±9.12abc	1.408±0.04c	3.134±0.20b
300	89.25±16.61c	63.05±17.23bc	3.238±0.20b	3.703±0.12a
500	97.03±15.57bc	45.42±10.66c	4.305±0.70a	1.425±0.09c

Values are the mean of three replication ±SD. Variants possessing the same letters are not statistically significant at P<0.05.

more nucleoli were found in microscopic images. Smooth and rough endoplasmic reticulum with attached ribosomes, protein and lipid bodies were also seen throughout the cytoplasm (Figs. 1 A-B). However, cytoplasm appeared denser in TM-1 as compared to Z-747, probably due to lipid and protein bodies' assembly (Fig. 1 A). Vacuolization was more pronounced in Z-747, with the presence of small and larger size vacuoles surrounded by tonoplast membranes. Membrane-bound peroxisomes could also be seen in Z-747 control cells (Fig. 1 B).

The detrimental effects of Pb started at 50 µM Pb, when conformational changes appeared in the shape of nuclei, accompanied by the disappearance of nucleoli and an increase in the size of vacuoles of Z-747 (Figs. 1 C-D).
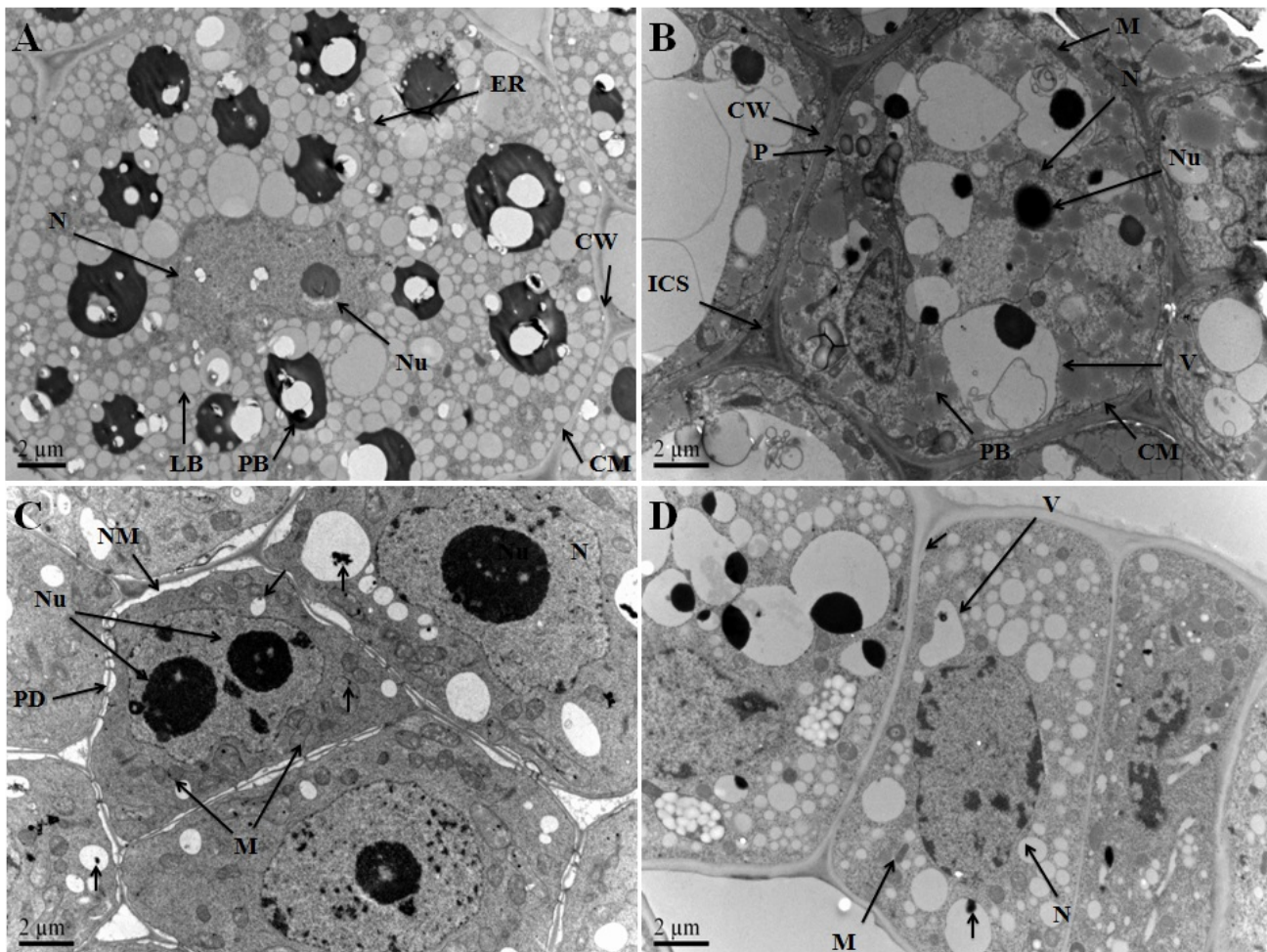

Fig. 1. (A, B) TM-1 and Z-747 at 0 µM Pb (C, D) TM-1 and Z-747 at 50 µM Pb. Electron micrographs of radical base cells of two upland cotton TM-1 and Z-747 showing nucleus (N), nucleolus (Nu), nuclear membrane (NM), mitochondria (M), endoplasmic reticulum (ER), cell wall (CW), vacuole (V), protein bodies (PB), lipid bodies (LB), peroxisomes (P), intracellular spaces (ICS), plasmadesmeta (PD), and cellular membrane (CM) in the control cells (A and B), and 50 µM Pb (C and D) level of TM-1 and Z-747, respectively. Small arrows (→) represent Pb particles.

But TM-1 maintained a typical nucleus round shape structure and well-defined nucleoli (Fig. 1 C). Plasmolysis, increase in the number of mitochondria and intercellular spaces; accumulation of Pb in vacuoles and alongside cell walls was similar in TM-1 and Z-747. The plasma membrane seems more intact in micrographs of TM-1 as compared to Z-747. At 100 µM, there was greater increase in intercellular spaces, cell wall size, mitochondria, and vacuoles of TM-1 (Figs. 2 E-F). Endoplasmic reticulum (ER) lost its integrity and was found scattered throughout the cytoplasm along with ribosomes. Cell membrane could be seen still attached to cell walls in TM-1 at 100 µM Pb. Most of the cells contained more than one vacuole, while others showed just one larger vacuole. On the other side, in Z-747, cell membrane disappearance, deformation of cell wall, disturbance in cell organelles, and disintegration of mitochondria and peroxisomes was found at 100 µM (Fig. 2 F). The nuclear membrane lost its round structure and nucleoli disappeared or disintegrated and were scattered in the nucleoplasm.

One of the key observations in TM-1 at 300 µM Pb was the presence of nucleoli and nuclear membranes and greater increase in vacuole size, pushing cells organelles toward the cell wall. Pb signals were scattered throughout the cytoplasm (Fig. 2 G). Nuclear membranes vanished in Z-747 and nucleoli, if seen, floated in the cytoplasm. However, the cell wall was still intact and identification of different cell organelles became difficult in Z-747 at this stage (Fig. 2 H). An increase in the intracellular spaces was a common characteristic found in both varieties. At the highest level of treatment, micrographs of TM-1 cells could be seen with or without clear nucleus, small and large vacuoles filled with cellular debris, and Pb particles (Fig. 3 I). The variety Z-747 exhibited cell wall deformation at 500 µM Pb (Fig. 3 J). Cell membranes disappeared in many cells and identification of other cell organelles was difficult.

Discussion

This study reveals the differential response by seeds of two upland cotton varieties under elevated levels of Pb. A decline in germination percentage under Pb toxicity has been reported in many studies [9, 22] mainly due to decline in water content. We observed that seeds of TM-1 absorbed more water than Z-747, hence more germination at all lev-
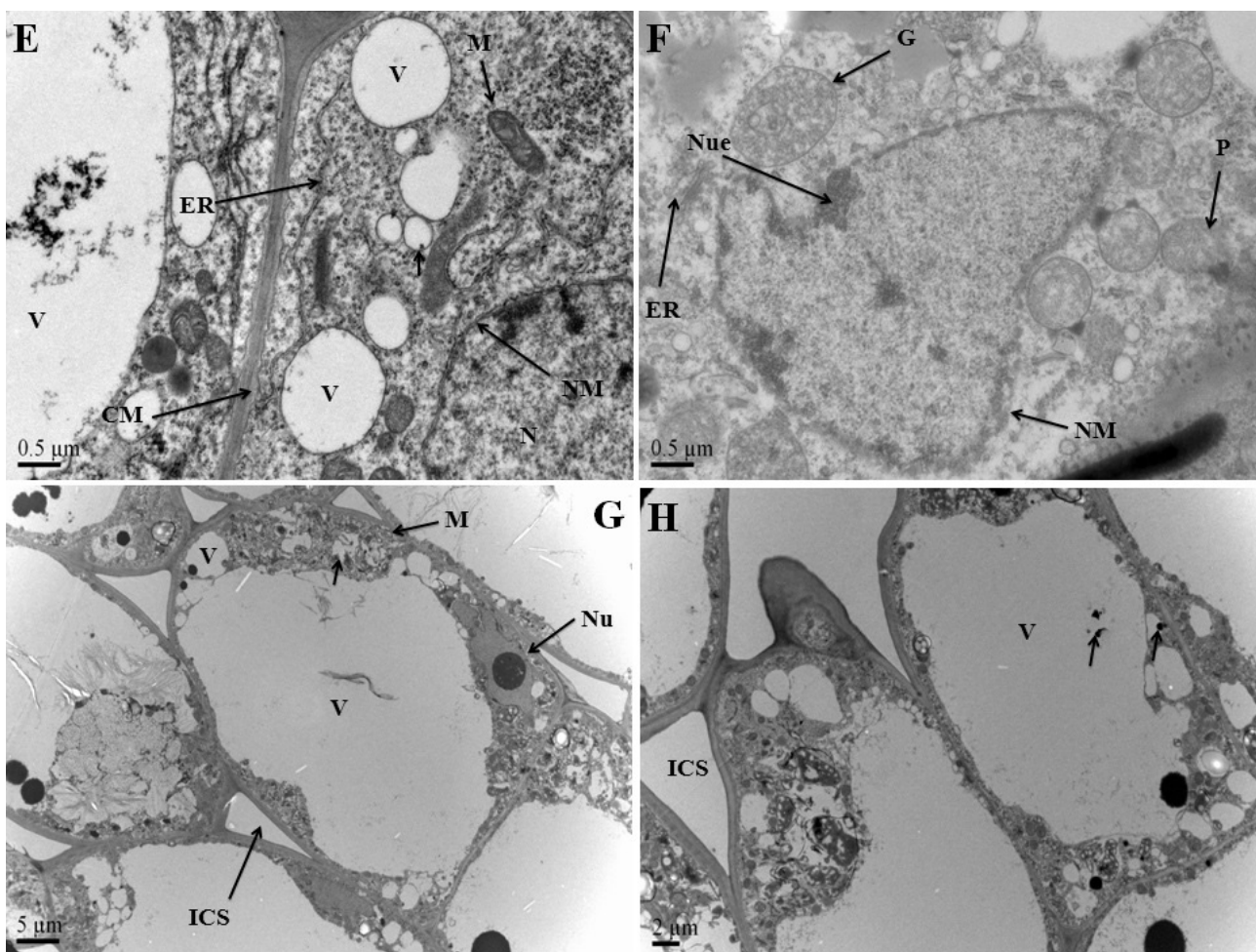

Fig. 2. (E, F) TM-1 and Z-747 at 100 µM Pb (G, H) TM-1 and Z-747 at 300 µM Pb. Electron micrographs of radical base cells of TM-1 and Z-747 cell organelles, i.e. nucleus (N), nucleolus (Nu), nuclear membrane (NM), cytoplasm (C), mitochondria (M), endoplasmic reticulum (ER), cell wall (CW), protein bodies (PB), intracellular spaces (ICS), and lipid bodies (LB). Small arrows (→) indicate Pb.
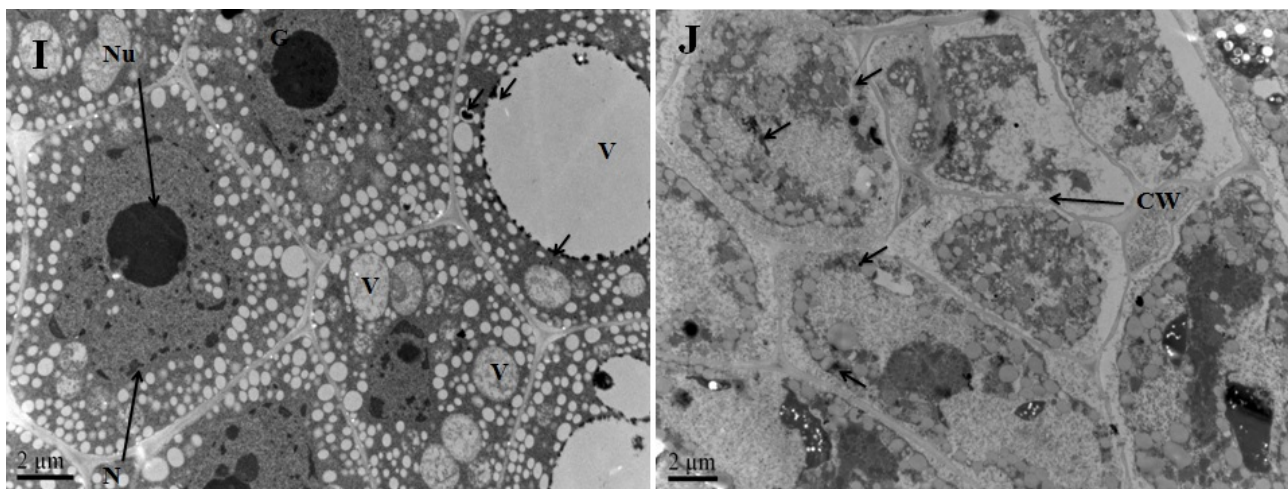
Fig. 3. (I, J) TM-1 and Z-747 at 500 µM Pb. Electron micrographs of radical base cells of TM-1and Z-747 showing different cell organelles, i.e. nucleus (N), nucleolus (Nu), nuclear membrane (NM), intercellular spaces (ICS), mitochondria (M), endoplasmic reticulum (ER), ribosomes (R) cell wall (CW), protein bodies (PB), and lipid bodies (LP) at 500 µM Pb. Small arrows (→) indicate Pb particles.

els of Pb. Importantly, seeds of TM-1 were bigger in size showing better germination. Several studies have shown a direct relationship between seed size and germination [23, 24]. It is known that Pb causes alterations in the activities of key metabolizing enzymes like amylases, acid invertases, and proteases, thus playing a negative role in exploiting seed reserves for radical emergence and delaying or inhibiting germination [7]. The relative better performance of TM-1 seeds may also be attributed to phytochelatin synthesis, activation of antioxidant enzymes [25], metallothioneins and heat shock proteins [16], and higher flavonoid content [26]. Similarly, phytic acid is also considered a major component of protection against ROS as reported in maize seeds [27]. A stimulatory effect on germination in TM-1 at 50 µM Pb is in comparison to earlier experiments that showed that low concentrations of heavy metals are sensitive to germination and detrimental effects appear only at high concentrations [28].

Considerable attention has been paid to cell viability and programmed cell death (PCD) issues, having an important role in plant growth and development under different environmental stresses [29]. Formazan, an artificial chromogenic dye, allowed assaying viability via reduction of tetrazolium salts (like TTC) by mitochondrial dehydrogenases [30, 31]. Incubation time with dehydrogenases to produce pink color is the indicator of viable cells [32]. Plasma membrane is impermeable to macromolecules like TTC, which can penetrate only at higher Pb concentrations (i.e. 50 and 100 µM), thus disturbing membrane homeostasis. Similar results were found in Avicennia germinas when exposed to heavy metals [33]. A study conducted on germinating wheat seeds indicated a direct relationship between seed vigor (as observed in TM-1) and cell viability [34]. Besides other factors, higher cell viability in TM-1 may be attributed to glutathione (GSH), which provides a stance against ROS by signal transduction and maintaining active protein function [35].

Seed coat permeability defines the influx of the heavy metal ions to cellular matrix [22]. When exposed, Pb uptake is rapid and accumulation occurs within hours as reported in different species of legumes [36]. Our results showed an increase in Pb uptake in TM-1 and Z-747 at all levels of treatment over control. Similar results were reported in *Pluchea sagitallis* [37]. Pb accumulation and sequestration may be attributed to phytochelatins in TM-1 [38].

Ultrastructural observations are valuable tools to study cell organelles and formation of biological molecules [39]. Similarly, deleterious effects of Pb can be easily evaluated through cell micrographs of desired plant part [10]. From an ultrastructural point of view, our current work is one of very few studies of this kind, at least when it comes to cotton seeds. Typical high resolution morphology of radical base cells could identify differences in both cotton varieties. Micrographs showed cellular change in both varieties but were more prominent in Z-747, even at lowest level of Pb treatment (50 µM). Previous studies report Pb-induced changes in human lymphocytes at very low levels such as 1 µM [40] and in *Lemna minor* root cells at 15 µM [41]. In fact plasma membrane permeability defines Pb accumulation and subsequent changes. Metal ions get attached to thiol groups of proteins and the hydroxyl part of phospholipids to cause membrane permeability [42]. Pb also causes chromosomal anomalies as reported in *Trigonella foenumgraecum* [5] and cell wall modifications due to excessive H_2O_2 production [43].

Tolerant behavior by TM-1 may be due to several factors. Mitochondria, a prominent source of ATP, are the signature for cell protection and resistance capabilities at mild stressful conditions [13]. Tolerance in TM-1 may be attributed to mitochondrial organic acids that possibly reduce oxidative damage [44] and phytochelatin formation as reported in aquatic fern [45]. Comparable ultrastructural changes in roots of mined (tolerant) and non-mined (sensitive) ecotypes of *Elsholtzia argyi* also were reported [11].

Conclusions

The following conclusions can been drawn from the present study:

- Pb adversely affects germination, water content, cell viability, and morphology of cotton seeds.
- The variety Z-747 showed greater decline in germination percentage, cell viability, and ultrastructural variations due to reduction in water content, imbalance in nutrient uptake, plasma membrane permeability, and higher Pb accumulation.
- The variety TM-1 proved more tolerant to Pb stress due to larger seed size, greater water uptake, and controlled permeability to heavy metal ions.
- Sequestration of Pb in vacuoles and cell walls, phytochelatin synthesis, antioxidant enzymes, and defensive genes expression may have played a significant role in Pb tolerance of TM-1.
- TM-1 is more tolerant of Pb stress as compared to Z-747 and may be considered a potential variety for cultivation on Pb-contaminated soils.

Acknowledgements

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