Original article

Microscopic analysis of lead accumulation in tobacco (Nicotiana tabacum var. Turkish) roots and leaves

Rami Alkhatib\(^a,b,*,\) Emad Bsoul\(^c\), Douglas A. Blom\(^d\), Kajal Ghoshroy\(^f\), Rebecca Creamer\(^a\), Soumitra Ghoshroy\(^d,e\)

\(^a\) Electron Microscopy Lab, New Mexico State University, Las Cruces, NM 88003, United States
\(^b\) Department of Biotechnology and Genetic Engineering, Jordan University of Science and Technology, Irbid, Jordan
\(^c\) Department of Biology and Biotechnology, The Hashemite University, Zarqa, Jordan
\(^d\) Electron Microscopy Center, University of South Carolina, Columbia, SC 29208, United States
\(^e\) Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, United States
\(^f\) Division of Science, Mathematics and Engineering, University of South Carolina at Sumter, Sumter, SC 29150, United States

**ARTICLE INFO**

Article history:
Received 22 April 2013
Received in revised form 10 June 2013
Accepted 27 June 2013

Keywords:
Lead nitrate
Hoagland's solution
Invagination
Ferritin
Chloroplast

**ABSTRACT**

A hydroponic experiment was carried out to localize lead accumulation in tobacco roots and leaves. Plants were grown for seven days in Hoagland's solution supplemented with different concentrations of lead nitrate \([\text{Pb(NO}_3\text{)}_2]\); 0.0 (control), 10 \(\mu\text{M}\), 100 \(\mu\text{M}\), and 500 \(\mu\text{M}\). Growth was inhibited in plants treated with 100 \(\mu\text{M}\), and 500 \(\mu\text{M}\). Light and electron microscopic studies showed that lead accumulated mainly in cell walls and vascular tissues of roots of plants exposed to 100 \(\mu\text{M}\), and 500 \(\mu\text{M}\) lead nitrate. In contrast, roots exposed to 10 \(\mu\text{M}\) lead nitrate did not show any detectable accumulation of lead in the roots. The TEM images confirmed the presence of lead outside the epidermis of the roots in the form of electron dense clusters of fine needles. No lead was detected in the leaves. However, plants exposed to 100 \(\mu\text{M}\) lead nitrate were characterized by the presence of ferritin clusters in the chloroplasts. This suggests a protective mechanism by the plant to prevent oxidative damage caused by Pb. Plants exposed to 500 \(\mu\text{M}\) lead nitrate showed mesophyll cells containing altered chloroplasts with disrupted thylakoid systems.

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1. Introduction

According to the Environmental Protection Agency (EPA), lead (Pb) is the most common heavy metal contaminant in the environment [17]. Anthropogenic activities such as mining, smelting, burning of fossil fuel, effluents from storage battery, and the manufacturing of pesticides and fertilizers are some of the primary sources of Pb contamination of the environment [2,11,21,26]. Several studies showed that increased concentrations of Pb cause detrimental effects on plant growth [6]. Visible symptoms of toxicity are characterized by smaller leaves and a stunted growth for both shoots and roots [25]. Leaves show some chlorosis and necrosis and roots turn black or dark brown [15,24,28]. In addition, high concentrations of Pb cause a considerable decrease in dry weights of shoots and roots [5,24,25]. After Pb is absorbed by the plant's root system, it accumulates in different parts of the plant. In general, Pb becomes highly concentrated in the root system as compared to other parts of the plant [24]. Thus, the concentration of Pb in the aerial parts of the plant decreases
as the distance from the root increases [24]. This is due to higher accumulation of Pb in cell walls of the root than other parts of the plant [1,24] and the ability of Pb to bind the carbonyl groups of the carbohydrates galacturonic acid and glucuronic acid in the root cell wall [8].

Numerous studies have shown that Pb is likely to inhibit and interfere with various physiological processes. Plants exposed to Pb ions show decreased photosynthetic and transpiration rates [18,24,25]. These responses are thought to be related to the distortion of the chloroplast ultrastructure, reduced chlorophyll content of the leaves, CO₂ deficiency as a result of stomatal closure, and the inhibited activities of the Calvin cycle enzymes [1,18,24]. Ceratophyllum demersum exposed to Pb(NO₃)₂ showed obvious changes in chloroplast fine structure [10]. Other plants showed a reduction or absence of starch grains when exposed to heavy metals, including Pb [9,14,24]. In general, small quantity of Pb reaches chloroplasts and mitochondria [24].

We hypothesize that Pb will be sequestered and restricted in the root system, mainly in the cell wall. This work aimed to study the localization of accumulated Pb and characterize the method of accumulation in tobacco roots and leaves. We also examined the ultrastructural changes in root and leaf cells treated with various concentrations of Pb.

2. Materials and methods

2.1. Plant material

Tobacco (Nicotiana tabacum var. Turkish) seeds were germinated and grown in Promix for 3–4 weeks at 25°C. Uniformly sized seedlings were removed from soil and their roots were carefully washed with distilled water to remove Promix particles. Plants of similar heights and equal number of leaves were placed in Hoagland’s solution (bio-WORLD, Dublin, OH, USA) and grown hydroponically in a growth chamber at a photosynthetic photon flux density (PPFD) of 250–300 μmol m⁻² s⁻¹ under controlled temperature (23–30°C), humidity (60 ± 5%), and photo-period (14 h of light). The nutrient solution was changed every 3 days. Pb was added in the form of Pb(NO₃)₂. Plants were placed in Hoagland’s for 3 days before they were exposed to 10, 100, and 500 μM lead nitrate mixed in Hoagland’s solution. Both 100 μM and 500 μM lead nitrate were toxic to plants; meanwhile 10 μM lead nitrate was non-toxic [25]. Toxicity symptoms caused by excess Pb include stunting, chlorosis and blackening of root system. It also inhibits photosynthesis, upset water balance, and affects membrane structure [25]. Control plants were not exposed to any Pb. The plants were grown for 1 week in Pb solution and root and leaf samples were collected.

2.2. Light and transmission electron microscopy

Root and leaf samples (0.5–1.0 cm) from Pb-treated tobacco plants were fixed in 2.5% glutaraldehyde buffered at pH 7.4 in 0.1 M cacodylate buffer for overnight at 4°C. When fixed, the samples were rinsed with 0.1 M cacodylate for five times, 2 min each. The samples were postfixed in cacodylate-buffered (pH 7.4) 1% osmium tetroxide, dehydrated in a graded series of ethanol (50%, 70%, 80%, 95%, and 100%) for 10–15 min each, and embedded in freshly prepared Spurr’s resin [3].

For light microscopy, thick sections of resin embedded root tissues (0.5 μm) were cut with a histo knife (Diatome, Hatfield, PA, USA), stained with 1% toluidine blue (Epoxy Tissue Stain, EMS, PA, USA) and examined using a Zeiss light microscope (Zeiss, Oberkochen, Germany) equipped with a digital camera (Micromax, Princeton, NJ, USA). For transmission electron microscopy, thin sections of root and leaf samples (70 nm) were cut with a diamond knife, stained with uranyl acetate [16] followed by lead citrate [7], and examined using Hitachi H7650 and H8000. Energy dispersive spectroscopy (EDS) analysis was performed on thin sections of root and leaf tissues using a JEOL 2100F 200 kV field-emission gun TEM equipped with an Oxford Instruments 30 mm solid-state X-ray detector. Spectra were collected with the Oxford INCA system, at several locations in the thin sections of resin blocks.

3. Results

The plants exposed to 10 μM lead nitrate did not show any visible accumulation of Pb in the roots and the overall appearance of the roots was very similar to that of the control plants. The roots exposed to 100 and 500 μM lead nitrate showed severe reduction in overall size and dry weight when compared to that of the control (data not shown). The light microscopic images of Pb-treated plant root sections showed thickening of cell walls in the vascular tissue, enlargement of the intercellular space in the cortex and severe distortion in the shape of the cells as compared to the control root sections and 10 μM Pb-treated root sections (Fig. 1a and b). The roots of 100 and 500 μM Pb-treated plants also showed the presence of Pb as dark precipitates in the vascular tissue and to some extent in the cortical and epidermal cells and cell walls (Fig. 1c and d). The TEM images confirmed the presence of Pb outside the epidermis and also in the form of electron dense clusters of fine needles primarily in the cell walls (Fig. 2c and d). In addition, the cells in the vascular tissue showed the presence of Pb as electron dense deposit in the cell walls (Fig. 3b). The EDS study (Fig. 4) confirmed the clusters as pure Pb (Pb crystals), and no Pb was detected in any other parts of the cells (data not shown). The root epidermal cells in 100 and 500 μM Pb-treated plants also showed severe inward invagination of the cell walls (Fig. 3a). Leaves of plants exposed to 10 μM lead nitrate did not show any visible accumulation of Pb and the overall appearance of the chloroplast was very similar to that of the control plants (Fig. 5a and b). In contrast, localized black spots were observed inside the chloroplast of plants exposed to 100 μM lead nitrate (Fig. 5c). None of these spots were observed in the chloroplasts of the plants treated with 500 μM lead nitrate, however, the ultrastructural organization of chloroplasts was altered and large grains of starch were observed compared to chloroplasts from control plants (Fig. 6d). The EDS study (Fig. 6) confirmed the black spot clusters in the chloroplasts of the 100 μM treated plants were iron. Same black spots
were observed in plants treated with 50 μM lead nitrate (data not shown). Pb was not detected in the chloroplasts of 500 μM Pb-treated plants, and no iron was detected either (Fig. 6). Furthermore, this study demonstrated accumulation of Pb in the form of electron dense crystalline precipitates in various parts of the root tissue. The exposure to high concentrations of Pb also resulted in severe distortion of epidermal cell walls, and overall disruption in the organization of the vascular tissue was clearly evident in the roots.

4. Discussion

Stress caused by the presence of high Pb levels in the medium contributed to the disruption of the plant growth and development mainly in the roots. Regardless the
concentrations of lead nitrate in the Hoagland’s medium, roots with symptoms were the main accumulation site for Pb. It appears that the inhibition of root growth under high levels of lead is caused by the inhibition of cell division in root tips [29]. A reduction in root growth, mitotic irregularities and chromosome stickiness were observed, when the effect of different concentrations of lead nitrate was studied on root growth, cell division, chromosome morphology and the nucleolus of root tips of onion (Allium cepa) [22]. In this study, Pb taken up by the plant roots primarily localized in the vascular tissue and cell walls, however, proximity of epidermal cells to lead nitrate induced severe damage in their cell walls. The movement of Pb in the root is primarily via the apoplast, however, higher concentrations of Pb may disrupt the casparian strips of the endodermis allowing Pb ions to move into the vascular tissue of the plant. This suggests that endodermis act as a partial barrier since some of the Pb ions move through the vascular tissues [27]. Also, the strong binding of Pb to carboxyl groups of carbohydrates in cell walls of roots slows down its movement via apoplast [8]. Within the cell, the majority of the Pb is sequestered in the vacuole [24]. In Stigeoclonium the formation of such vacuoles is important for the sequestration of excess metal ions [4]. Inward invagination of the cell walls of 100 μM and 500 μM treated plants suggests a mechanism of Pb detoxification, to protect the cell contents from its effects, by forming pinocytotic vacuoles.

Fig. 3. TEM images showing Pb in outside epidermal cells and inward invagination of cell wall (a), and in vascular tissue cell (VTC) (b) of 500 μM Pb-treated N. tabacum roots.

Fig. 4. EDS data obtained from the analytical TEM show a strong Pb peak (arrows) in the lead crystals in the 100 μM and 500 μM Pb-treated plants (c and d). The 10 μM Pb-treated plants did not show any Pb peak (b) as well as control (a).
Fig. 5. TEM images showing chloroplasts of *N. tabacum* for control (a), 10 µM (b), 100 µM (c), and 500 µM (d) Pb-treated plants. Cell wall (CW), starch (S), and grana (G). Ferritin clusters (arrow) were detected in the 100 µM Pb-treated plants chloroplast. Scale bar = 500 nm.

In general, heavy metals decrease the concentration of chlorophyll in leaves either by inhibiting its synthesis or by inducing its degradation [13]. In Pb-treated plants, the enhancement of chlorophyll degradation occurs due to increased chlorophyllase activity [20].

In plants treated with 100 µM lead nitrate, the ultrastructural organization of the chloroplast (Fig. 5c) was similar to those of control plants; however, accumulation of iron was observed in these chloroplasts in the form of small black spots (Fig. 5c). This suggests the formation of some specific defense protein such as ferritin, which is known to be involved in iron solubility with a high capacity of storage of iron atoms. Ferritin accumulation in the chloroplast is often related to cell protection against damage due to free radicals enhanced by oxidative stress [12]. Previous studies have indicated that transgenic plants accumulating alfalfa ferritin exhibit tolerance to iron excess and necrotic damage caused by viral and fungal infections [19]. Wuystwinkel et al. [23] reported that tobacco plants overexpressing soybean ferritin exhibit paraquat-mediated oxidative stress tolerance. One of the phytotoxic effects of Pb in plants appears to be the induction of oxidative stress due to enhanced production of reactive oxygen species (ROS) [24]. The production of these ROS in larger amounts can pose a severe threat to the plant. Chloroplasts of plants treated with 500 µM lead nitrate exhibited a reduction in grana stacks and usually were deformed and shrunken. This may be related to the disturbances in transport of electrons in the photochemical phase of photosynthesis [13]. Also, chloroplasts were characterized by the presence of large

Fig. 6. EDS data obtained from the analytical TEM show a strong iron (Fe) peak (arrow) in the lead crystals in the 100 µM Pb-treated plants (a) and no Pb was detected in the 500 µM Pb-treated plants chloroplast (b).
starch grains. This suggests both an increase of photosynthetic activity and the inhibition of carbohydrate transport from leaves to non-photosynthetic organs [14] may have occurred.

5. Conclusion

This study has shown that if Pb is available to a tobacco plant, the root system will take up Pb and restricts its movement to the upper parts of the plant. Plants treated with 10 μM lead nitrate did not show any abnormalities or ferritin clusters formation and plants were similar to control plants. This suggests that 10 μM lead nitrate is not toxic or lethal to plants. Despite the fact that Pb was not detected in the leaves 500 μM lead treated plants, Pb adversely affected tobacco leaves. The ultrastructure of the chloroplasts was altered with disrupted thylakoid systems (reduction of their number, swelling, and condensation). Iron can be scavenged from abundant iron-containing proteins (such as plant ferredoxins), mainly in a situation of iron deficiency; ferritin serves only as a last resort source of iron. The absence of ferritin in the chloroplasts of 500 μM Pb-treated plants suggests a phytotoxic effect of Pb at this concentration; preventing plants from producing ferritin.

Conflict of interest

The authors declare that they have no conflict of interest. We confirm that this manuscript does not infringe any other person’s copyright or property rights, all authors have contributed substantially to the manuscript, and all authors have agreed to publication of the work.

Acknowledgment

The authors gratefully acknowledge the work of Nour Abdo for her assistance in experiment preparation.

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