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Physiological and ultrastructural effects of lead on tobacco

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Abstract

The effects of lead toxicity on leaf gas exchange, chlorophyll content, chlorophyll fluorescence, chloroplast ultrastructure, and opening of stomata were examined in tobacco (*Nicotiana tabacum* L.) plants. Plants were grown in a growth chamber for 7 d in Hoagland nutrient solution supplemented with 0.0 (control), 5, 10, 25, 50, 100, 300 and 500 μM $Pb(NO_3)_2$. Plants treated with 5, 10, and 25 μM $Pb(NO_3)_2$ exhibited no abnormalities. Root and shoot growth, net photosynthetic rate and stomatal conductance were significantly reduced in plants treated with 100, 300 and 500 μM $Pb(NO_3)_2$. In plants treated with 500 μM $Pb(NO_3)_2$, the majority of stomata were closed. The effect of $Pb(NO_3)_2$ on chlorophyll content and chlorophyll fluorescence parameters was negligible. However, in plants exposed to 100, 300, and 500 μM $Pb(NO_3)_2$, the mesophyll cells showed altered chloroplasts with disrupted thylakoid membranes.

Additional key words: chlorophyll content, chlorophyll fluorescence, photosynthetic rate, photosystem II, stomatal conductance, thylakoids, transpiration rate.

Introduction

Lead is one of the hazardous anthropogenic pollutants in both aquatic and terrestrial ecosystems. Pb is not an essential element, so it can be phytotoxic even at low concentrations (Vázquez *et al*. 1992). Exposure to Pb results in a strong reduction of plant growth due to significant alterations in many metabolic pathways. Visible symptoms of toxicity are characterized by smaller leaves and a stunted growth for both shoots and roots. Leaves show chlorosis and necrosis, and roots turn black or dark brown (Burton *et al*. 1984, Ghoshroy *et al*. 1998, Sharma and Dubey 2005). In addition, high Pb concentrations cause a considerable decrease in shoots and roots dry mass (Sharma and Dubey 2005, Boonyapookana *et al*. 2005). Moreover, heavy metals may affect plant physiology resulting in decreased photosynthetic and transpiration rates (Krupa and Baszyński 1995, Kummerová *et al*. 2010). Plants exposed to Pb show decreased photosynthetic and transpiration rates (Burzyński and Klobus 2004, Sharma and Dubey 2005). The effects of heavy metals, including Pb, on photosynthesis are thought to be related to direct and indirect inhibition. Pb directly inhibits the photosynthetic electron transport, the activities of Calvin-Benson cycle enzymes, and net CO_2 influx (Krupa and Baszyński 1995, Sharma and Dubey 2005). Pb can also alter the photosynthetic activity indirectly, distorting the chloroplast ultrastructure or decreasing chlorophyll content of leaves (Påhlsson 1989, Burzyński and Klobus 2004, Sharma and Dubey 2005).

This work is aimed to study the physiological and ultrastructural effects of various concentrations of lead on tobacco.

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Abbreviations: Chl - chlorophyll; E - transpiration rate; F_s - steady state chlorophyll fluorescence; F_m - maximum chlorophyll fluorescence; F_v - variable fluorescence; F_v/F_m - maximum PS II quantum yield; g_s - stomatal conductance; P_N - net photosynthetic rate; PS II - photosystem II.
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Materials and methods

Tobacco (*Nicotiana tabacum* L. cv. Turkish) seeds were germinated and grown in *Promix* for 3 - 4 weeks at 25 ºC. Uniformly sized seedlings were removed from soil and their roots were carefully washed with distilled water. 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 to 300 μmol m^{-2} s^{-1}, day/night temperature 30/23 ºC, humidity 60 ± 5 % and 14-h photoperiod. The nutrient solution was changed every 3 d. Plants were grown for 3 d before they were exposed to 0 (control) 5, 10, 25, 50, 100, 300 and 500 μM $Pb(NO_3)_2$ in Hoagland's solution. The plants were grown for 7 d before root and leaf samples were collected. The experimental design was totally randomized, with three replicates per treatment.

One fully-expanded mature leaf 5 - 10 cm from the top of each plant was selected to measure gas exchange. Net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), vapor pressure deficit (VPD) and leaf temperature (T_L) were measured on 6 cm^2 leaf area using an infrared gas analyzer-based photosynthesis system (*LI-6400*, *Li-Cor*., Lincoln, NE, USA). All measurements were taken at PPFD of 1000 μmol m^{-2} s^{-1}, using a built-in LED light source, flow rate of 400 μmol s^{-1} and CO_2 concentration of 400 μmol mol^{-1}.

Chl fluorescence was measured with a pulse amplitude *OS5-FL* modulated chlorophyll fluorometer (*Opti-Sciences*, Tyngsboro, MA, USA). Prior to the measurements, the leaves were dark-adapted for 30 min. After the dark period, all centers of PS II were open. After reading (F_0), the maximum fluorescence (F_m) was induced by 1-s pulse of saturating "white light" (10 000 μmol m^{-2} s^{-1}) (Burzyński and Klobus 2004). Variable to maximum fluorescence ratio (F_v/F_m) was calculated as $(F_m - F_0)/F_m$.

Five leaf discs (0.6 cm diameter) were taken from three mature leaves avoiding any mid-rib and stored at -20 ºC for subsequent HPLC analysis. Leaf pigments and antioxidants were assayed according to Ratnayaka *et al*. (2003) with modifications. Frozen leaf discs were weighed and homogenized in liquid nitrogen under dim light and incubated with 0.35 cm^3 acetone in the dark at 4 ºC for 30 min. Homogenate was centrifuged at 7000 *g* for 5 min at 4 ºC, the supernatant was filtered through 0.2 μm nylon membrane filter (*Millex®-GN*, *Millipore*, Bedford, MA, USA), and injected onto the HPLC (*Agilent 1100* automated with *Chemstation A.08.03*, *Agilent Technologies*, Palo Alto, CA, USA). Chromatographic separation was performed on a *Spherisorb® ODS-1* reversed phase column (5 μm particle size; 250 mm × 4.6 mm i.d.) protected by a *Altima*™ C-18 guard column (5 μm; 7.5 × 3.0 mm, *Grace Davison Discovery Science*, Deerfield, IL). The mobile phase consisted of two components: A - acetonitrile:methanol: Tris-HCl (0.1 M, pH 7) (72:8:3, v:v:v) and B - methanol: ethyl acetate (68:32, v:v). An isocratic elution of 100 % A for the first 2 min was followed by a linear gradient from 100 % A to 100 % B from 2 to 16 min. A 2 min linear gradient from 100 % B to 100 % A, and an isocratic elution with 100 % A for the next 4 min were used to re-equilibrate the column before the next injection. Flow rate was 0.8 cm^3 min^{-1} and injection volume was 0.025 cm^3 with the autosampler at 4 ºC. Chlorophylls and carotenoids were detected at 440 nm using a photodiode array detector, and α-tocopherol was detected using a fluorescence detector with excitation at 295 nm and emission at 340 nm. Xanthophyll cycle pigments deepoxidation was computed as (Z+A)/(Z+A+V) where Z, A and V are zeaxanthin, antheraxanthin and violaxanthin, respectively. Antheraxanthin and violaxanthin standards were purchased from *DHI Water and Environment*, Horsholm, Denmark. Chlorophyll *a* and *b*, β-carotene, zeaxanthin, lutein and α-tocopherol standards were purchased from *Sigma-Aldrich*, St Louis, MO, USA.

Root and leaf samples (0.5 - 1.0 cm) from control and Pb-treated tobacco plants were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight at 4 ºC. When fixed, the samples were rinsed with 0.1 M cacodylate five times for 2 min each. The samples were postfixed in 1 % osmium tetroxide in cacodylate-buffered (pH 7.4), dehydrated in a graded series of ethanol (50, 70, 80, 95, and 100 %) for 10 to 15 min each, and embedded in freshly prepared Spurr's resin (Spurr 1969). Thin sections (70 nm) of leaf samples were cut with a diamond knife, stained with uranyl acetate (Epstein and Holt 1963) followed by lead citrate (Reynolds 1963), and examined using *Hitachi H7650* (USA) transmission electron microscope. For scanning electron microscopy, dehydrated samples were dried in liquid CO_2 using critical point dryer (*LADD*™, Willingston, USA) and later sputter coated with palladium using a *Desk IV* (*Denton vacuum,* USA) sputter coater. The leaf samples were examined using a *Hitachi TM-1000* tabletop scanning electron microscope. Widths of the stomatal openings were measured on 6 random micrographs per treatment.

All statistical analyses for physiological parameters were conducted with the use of *PC SAS* (*v. 9.1.3*; *SAS Institute*, Cary, NC, USA). *ANOVA* and Dunnett's tests were used for all parameters analyses.

Results

The plants exposed to 5 and 10 μM $Pb(NO_3)_2$ did not show any visible reduction in their shoot height and root length, and the overall appearance of shoots and roots was very similar to the control plants (Fig. 1). In contrast, the shoots and roots of plants exposed to 100, 300 and 500 μM lead nitrate showed severe growth reduction

(Table 1). The root system length of plants exposed to 500 μM $Pb(NO_3)_2$ decreased up to 65 %, as compared to control plants. The dry mass of shoots and especially roots was also significantly reduced when compared to control plants (Table 1).

Table 1. Shoot height [cm], root length [cm], shoot dry mass [g plant^{-1}], and root dry mass [g plant^{-1}] of control and Pb-treated tobacco plants. Means of four experiments ± SE. * denote significant differences at 0.05 % level between the Pb-treated and control plants.

Pb^{2+} [μM]	Shoot height	Root length	Shoot dry mass	Root dry mass
0	12.0 ± 0.45	8.3 ± 0.29	0.50 ± 0.018	0.09 ± 0.008
5	10.2 ± 0.70*	7.6 ± 1.03	0.35 ± 0.024*	0.05 ± 0.005*
10	11.4 ± 0.64	8.3 ± 0.58	0.41 ± 0.028*	0.07 ± 0.005*
25	9.4 ± 0.71*	7.7 ± 0.90	0.36 ± 0.026*	0.06 ± 0.005*
50	8.3 ± 0.45*	5.9 ± 0.52*	0.35 ± 0.025*	0.05 ± 0.003*
100	6.9 ± 0.37*	4.7 ± 0.62*	0.31 ± 0.027*	0.05 ± 0.006*
300	6.5 ± 0.44*	3.2 ± 0.23*	0.26 ± 0.017*	0.03 ± 0.003*
500	4.9 ± 0.24*	2.9 ± 0.31*	0.23 ± 0.020*	0.03 ± 0.004*

The photosynthetic rate (P_N) for control plants was 10.9 μmol(CO_2) m^{-2} s^{-1}. P_N drastically decreased at concentrations of $Pb(NO_3)_2$ higher than 10 μM (Table 2). When the plants were exposed to 500 μM $Pb(NO_3)_2$, P_N decreased by 61 %. In addition, transpiration rate (E) and internal CO_2 concentration (c_i) of leaves exposed to 100, 300, and 500 μM $Pb(NO_3)_2$ decreased significantly (Table 2). Stomatal conductance (g_s) was also strongly decreased under Pb stress. In plants exposed to 300 and 500 μM $Pb(NO_3)_2$, g_s decreased by 86 and 92 %, respectively. The width of the stomatal pore in plants treated with 100 μM and 500 μM $Pb(NO_3)_2$ was 9.61 ± 0.69 and 7.28 ± 0.58 μm, respectively (Fig. 2*C*,*D*). However, in plants exposed to 500 μM $Pb(NO_3)_2$ some of the stomatal pores were completely closed. In contrast, control plants and plants treated with 10 μM $Pb(NO_3)_2$ were open and width of the stomatal pore was 16.21 ± 1.06 and 12.59 ± 1.36 μm, respectively (Fig. 2*A*,*B*). Further, plants treated with 500 μM $Pb(NO_3)_2$ showed slightly elongated cells of irregular shape (Fig. 2*D*). The differences in the parameters of chlorophyll fluorescence were negligible (Table 2).

In all treatments, Pb did not significantly affect Chl *a* and Chl *b* content and Chl *a*/*b* ratio (data not shown). In addition, the content of other leaf pigments (*e.g.* β-carotene and lutein) were similar to those of the control plants (data not shown). In the mesophyll cells of control plants, chloroplasts showed a regular arrangement of thylakoid membranes (Fig. 3*A*). Chloroplasts of plants treated with 10 μM $Pb(NO_3)_2$ did not exhibit any structural abnormalities (Fig. 3*B*). However, chloroplasts of plants treated with 500 μM $Pb(NO_3)_2$ exhibited large starch grains and a shortening of the intergranal thylakoids (Fig. 3*C*).

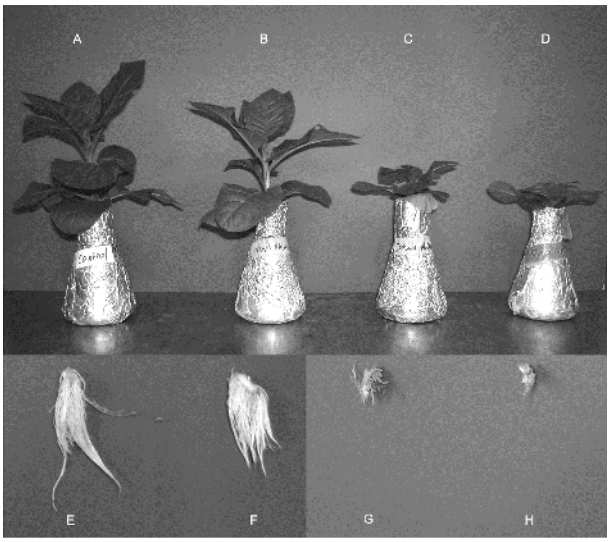

Fig. 1. Effects of lead on tobacco shoot and root growth. *A* - shoot of a control plant. *B* - shoot of 10 μM Pb-treated plant. *C* - shoot of 300 μM Pb-treated plant. *D* - shoot 500 μM Pb-treated plant. *E* - root of a control plant. *F* - root of 10 μM Pb-treated plant. *G* - root of 300 μM Pb-treated plant. *H* - root of 500 μM Pb-treated plant.

Table 2. Photosynthetic rate [μmol(CO_2) m^{-2} s^{-1}], stomatal conductance [mol m^{-2} s^{-1}], transpiration rate [mmol(H_2O) m^{-2} s^{-1}], internal CO_2 concentration [μmol mol^{-1}] and the variable to maximum chlorophyll fluorescence ratio (F_v/F_m) in control and Pb-treated tobacco plants. Means of four experiments ± SE. * - significant differences at 0.05 % level between the Pb-treated and control plants.

$Pb(NO_3)_2$ [μM]	Photosynthetic rate	Stomatal conductance	Internal CO_2 conc.	Transpiration rate	F_v/F_m
0	10.9 ± 0.84	0.38 ± 0.116	262.6 ± 17.8	5.17 ± 0.81	0.855 ± 0.005
5	8.9 ± 0.71	0.26 ± 0.102*	235.0 ± 22.1	4.32 ± 0.94	0.854 ± 0.006
10	10.0 ± 0.99	0.26 ± 0.086*	242.6 ± 17.6	4.36 ± 0.87	0.852 ± 0.005
25	8.5 ± 0.78*	0.16 ± 0.056*	223.7 ± 16.0	3.45 ± 0.71*	0.850 ± 0.006
50	7.6 ± 1.11*	0.12 ± 0.033*	227.0 ± 17.9	2.83 ± 0.58*	0.855 ± 0.004
100	6.2 ± 1.03*	0.07 ± 0.017*	209.9 ± 13.5*	2.14 ± 0.42*	0.855 ± 0.007
300	5.7 ± 0.52*	0.05 ± 0.006*	172.1 ± 12.5*	1.72 ± 0.22*	0.850 ± 0.007
500	4.2 ± 0.61*	0.03 ± 0.004*	164.7 ± 9.7*	1.10 ± 0.17*	0.847 ± 0.007

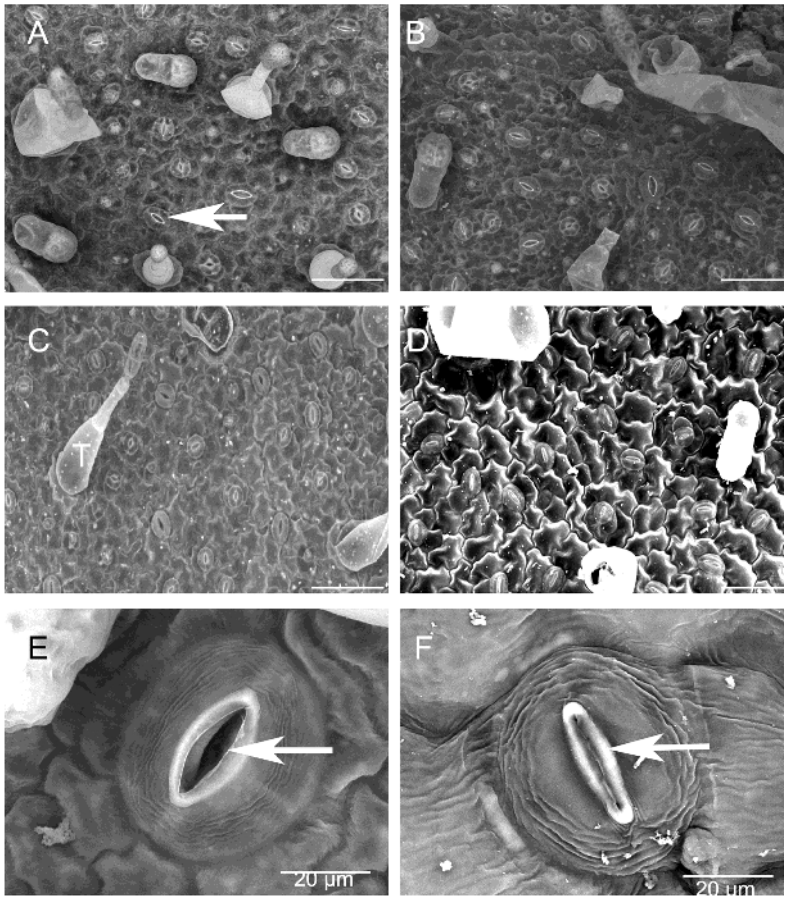

Fig. 2. SEM micrographs of the surface of a tobacco leaf epidermis after 7 d of Pb-treatment. *A* - control. *B* - 10 µM Pb-treated plant. *C* - 100 µM Pb-treated plant. *D* - 500 µM Pb-treated plant. *Scale bar* = 200 µm. *E* - fully opened stomata in 10 µM Pb-treated plant. *F* - fully closed stomata in 500 µM Pb-treated plant. *Scale bar* = 20 µm.

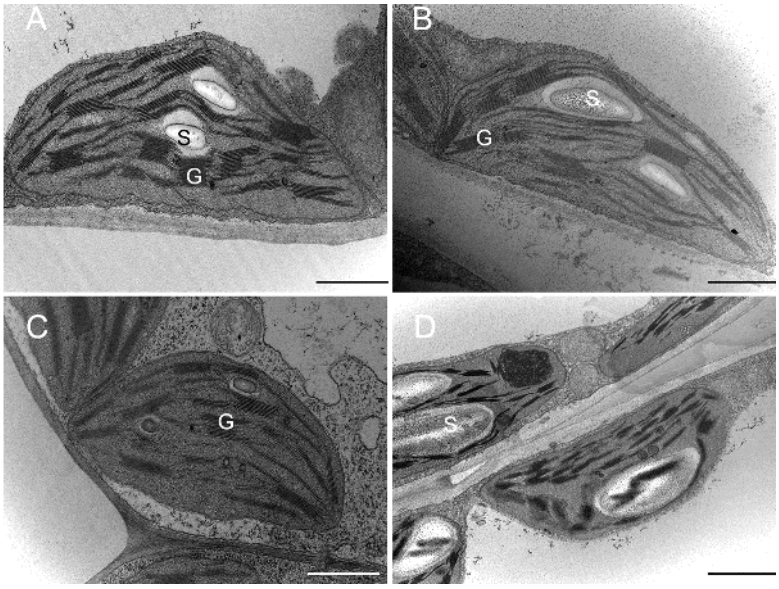

Fig. 3. TEM micrographs of chloroplast of tobacco leaves. *A* - control leaf showing regular arrangement of the grana (G) formed from several thylakoid membranes with regular starch grains (S). *B* - chloroplast of 10 µM Pb-treated plants similar to control plant. *C* - chloroplast of 100 µM Pb-treated plants with normal grana (G). *D* - chloroplast of 500 µM Pb-treated plant exhibiting compact grana with large starch grains (S). *Scale bar* = 500 nm.

Discussion

In this study we showed that stress caused by the presence of high Pb concentration in the growth medium contributed to the reduction of plant growth. Plants treated with 100, 300, and 500 μM $Pb(NO_3)_2$ showed much smaller root system, compared to control plants and plants treated with 5 and 10 μM $Pb(NO_3)_2$. The inhibition of root growth may be due to a decrease in Ca in the root tips, leading to the inhibition of cell division (Haussling *et al.* 1988, Eun *et al*. 2000). In onion treated with different concentrations of $Pb(NO_3)_2$, Wierzbicka (1994) reported a reduction in root growth, mitotic irregularities and chromosome stickiness. In our study, the shoot heights of plants treated with 25, 50, 100, 300 and 500 μM lead nitrate decreased significantly as compared to the control plants (Table 1). This could be due to the decline in P_N (Table 2). In general, plants grown in heavy metals contaminated soils or media show a reduction of plant growth, especially their dry mass (Påhlsson 1989, Ghoshroy *et al*. 1998, Sharma and Dubey 2005, Moussa and El-Gamal 2010). A significant decrease in dry mass of *Plantago major* plant parts was also observed under Pb treatment (Kosobrukhov 2004). However, in maize seedlings treated with Pb, an increase in dry mass of some plant parts was reported, which was due to an increase in the synthesis of cell wall polysaccharides (Wierzbicka 1998).

During exposure to heavy metals, the water status of leaves might be distorted, affecting transpiration rate and water potential of the plants (Poschenrieder and Barcelo 1999). However, water potential of all Pb-treated plants was not significantly affected (data not shown). This suggests that Pb can affect stomatal conductance and consequently transpiration rate also by other mechanism than water potential-induced stomatal closure.

Several biochemical processes are affected by excess Pb, especially photosynthesis and nitrogen metabolism (Påhlsson 1989). In this study, P_N was adversely affected by Pb, however, the PS II efficiency was not affected (Table 2). In general, the content of chlorophyll is lowered in the leaves treated with heavy metals including Pb either by inhibiting its synthesis or by inducing its degradation (Molas 1997). In Pb-treated plants, the enhancement of chlorophyll degradation occurs due to increased chlorophyllase activity (Drazkiewicz 1994), or by lowering the transport rate of Fe to leaves (Burzyński and Klobus 2004). However, Stiborova *et al*. (1986) and Sarvari *et al.* (2002) reported increased chlorophyll content in maize and cucumber at low Pb concentrations. Our results showed that contents of chlorophyll, lutein, β-carotene, and xanthophyll cycle pigments were not affected by lead exposure. This may suggest that the photosynthetic apparatus is rather tolerant to Pb stress and the decrease of P_N is most likely due to the stomatal closure. However, the low Fe content in leaves may affect the structure of the chloroplasts and indirectly P_N (Siedlecka and Krupa 1999). In plants treated with 10 μM $Pb(NO_3)_2$, the ultrastructural organization of the chloroplasts (Fig. 3*B*) was similar to those of control plants, however, chloroplasts of plants treated with 300 and 500 μM $Pb(NO_3)_2$ exhibited a reduction in grana stacking and deformed and shrunken thylakoids. In addition, chloroplasts were characterized by the presence of large starch grains (Fig. 3*D*). Nevertheless, changes of all parameters depend on the severity and duration of metal stress and plant species (Burzyński and Klobus 2004, Sharma and Dubey 2005).

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