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Evaluation of DNA damage and mutagenicity induced by lead in tobacco plants

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ABSTRACT

Tobacco (*Nicotiana tabacum* L. var. *xanthi*) seedlings were treated with aqueous solutions of lead nitrate (Pb^{2+}) at concentrations ranging from 0.4 mM to 2.4 mM for 24 h and from 25 μM to 200 μM for 7 days. The DNA damage measured by the comet assay was high in the root nuclei, but in the leaf nuclei a slight but significant increase in DNA damage could be demonstrated only after a 7-day treatment with 200 μM Pb^{2+}. In tobacco plants growing for 6 weeks in soil polluted with Pb^{2+} severe toxic effects, expressed by the decrease in leaf area, and a slight but significant increase in DNA damage were observed. The tobacco plants with increased levels of DNA damage were severely injured and showed stunted growth, distorted leaves and brown root tips. The frequency of somatic mutations in tobacco plants growing in the Pb^{2+}-polluted soil did not significantly increase. Analytical studies by inductively coupled plasma optical emission spectrometry demonstrate that after a 24-h treatment of tobacco with 2.4 mM Pb^{2+}, the accumulation of the heavy metal is 40-fold higher in the roots than in the above-ground biomass. Low Pb^{2+} accumulation in the above-ground parts may explain the lower levels or the absence of Pb^{2+}-induced DNA damage in leaves.

1. Introduction

Because of many industrial activities, lead (Pb) is a ubiquitous pollutant in the environment [1]. Accumulation of lead in the atmosphere and in soil can become dangerous to all kinds of organism, including plants. Positive as well as negative results have been reported on genotoxic properties of lead in plants [2–5]. The pathway(s) of lead-induced genotoxicity are still unknown, but may involve the interaction of Pb with DNA, either directly or indirectly *via* oxidative stress [6].

In a recent paper [7] we have reported that a cultivation of tobacco seedlings in soil from a polluted locality in the Czech Republic resulted in a low but significant increase in DNA damage in leaf nuclei measured by the comet assay. As the polluted soil contained a very high content of lead, we studied the effect of this metal on tobacco plants in detail.

In the work presented here, we have treated tobacco plants with lead nitrate (Pb^{2+}) and (1) evaluated Pb^{2+}-induced DNA damage in root and leaf nuclei by the comet assay, (2) assessed the toxic effects of Pb^{2+} by measuring the changes in leaf area, and (3) scored the frequency of somatic mutations in leaves. In addition, Pb content in the soil in which the tobacco plants were cultivated, and the content of Pb in the roots and leaves of the tested plants were measured by inductively coupled plasma optical emission spectrometry.

2. Materials and methods

2.1. Chemicals and media

Ethyl methanesulfonate (EMS, CAS No. 62-50-0), lead nitrate (Pb^{2+}, CAS No. 10099-74-8), maleic hydrazide (MH, CAS No. 123-33-1), the plant growth medium (Phytagel, MS salts), reagents for electrophoresis, and general laboratory reagents were purchased from Sigma Chemical Co., St. Louis, MO. Normal (NMP) and low melting point (LMP) agarose were purchased from Roth, Karlsruhe, Germany. Reagents for the Pb content determination and the certified reference material were purchased from Analytika and Lach-Ner Ltd., Czech Republic.

2.2. Tobacco growth and Pb^{2+} treatment conditions

Double heterozygous light green *Nicotiana tabacum* L. var. *xanthi* ($a_1{}^+/a_1$; $a_2{}^+/a_2$) plants were used [8]. A detailed description of tobacco cultivation was published previously [9].

(1) For 24-h or 7-day treatments, the roots of tobacco seedlings were immersed in plastic vials containing 22 ml of a solution of Pb^{2+} dissolved at a defined concentration in distilled water. For the control, the seedlings were immersed in distilled water. The plants were treated for 24 h in the dark at 26 °C or for 7 days at 22–26 °C in a plant growth room with a 16-h photoperiod.

Abbreviations: Pb^{2+}, lead nitrate; EMS, ethyl methanesulfonate; MH, maleic hydrazide.

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(2) For soil experiments, the tobacco seedlings were cultivated for 2–6 weeks in plastic pots ($2r = 7$ cm) filled with 150 ml of garden soil. The soil was previously "polluted" with 50 ml (Experiment I) or 70 ml (Experiment II) of a defined concentration of Pb^{2+} dissolved in distilled water. For the control, seedlings were cultivated in un-polluted garden soil. Eight seedlings were cultivated for each concentration of Pb^{2+}. The plants were treated at 22–28 °C in a plant growth room with a 16-h photoperiod.

2.3. Comet assay

After Pb^{2+} treatment, excised leaves or roots of the treated plants were placed in a petri dish kept on ice and spread out with cold 400 mM Tris buffer, pH 7.5. Using a fresh razor blade, the leaves or roots were gently sliced, and the isolated nuclei collected in the buffer. The preparation of microscope slides with agarose, and a detailed procedure of the comet assay using nuclei isolated from tobacco plants was previously described [9]. In our recent experiments we found out that the third layer of 0.5% agarose is not necessary, so we omitted this step. Slides were incubated in alkaline electrophoresis buffer for 15 min prior to electrophoresis at 0.74 V cm^{-1} (26 V, 300 mA) for 25 min. After electrophoresis, the slides were rinsed three times with 400 mM Tris buffer, pH 7.5, air-dried at room temperature, and stored in boxes. Air-dried slides were immersed in water for 10 min and then stained with 100 μl ethidium bromide (20 μg ml^{-1}) for 5 min, dipped in water to remove the excess stain and covered with a coverslip. In most cases 2×25 randomly chosen nuclei were analyzed *per* slide using a fluorescence microscope with an excitation filter BP 546/10 nm and a barrier filter of 590 nm. A computerized image-analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was employed. The tail moment (TM; integrated value of tail DNA density multiplied by the migration distance) was used as the primary measure of DNA damage [10]. Two to three slides were evaluated per treatment and each treatment was repeated at least twice. The TM values are shown as the means of medians ± S.E. At least 150 nuclei were evaluated for each treatment group.

2.4. Measurement of the leaf area and the somatic mutations assay

The leaf area was measured and somatic mutations were scored in plants after they had grown in polluted soil for 2, 4 and 6 weeks. Only the leaves newly formed after treatments were used. The leaf area of one leaf per plant, thus eight leaves per tested soil was measured by a planimeter and expressed in cm^2. The mean ± S.E. of measurements was calculated.

Somatic mutations on one leaf per plant, thus on eight leaves per tested soil were scored using a stereomicroscope. The frequency of somatic mutations was expressed as mean ± S.E. Three main types of mutagenic events were scored: (1) dark green, (2) yellow and (3) green/yellow twin sectors [8].

2.5. Pb content analysis

2.5.1. Soil sample collection

Soil samples were taken 24 h after lead nitrate was added to the soil, at the time when tobacco seedlings were planted into the pots, and after 6 weeks of tobacco cultivation. The particle size of the garden soil used as a control was less than 5 mm and contained Cu = 55.9, Cd = 0.79, Zn = 254 and Pb = 35.2 mg kg^{-1} soil. The soil samples were air-dried at 20 °C, ground in a mortar and passed through a 2-mm plastic sieve.

2.5.2. Total Pb content in the soil

Aliquots (0.5 g) of air-dried soil samples were decomposed in a digestion vessel with a mixture of 8 ml concentrated nitric acid, 5 ml hydrochloric acid, and 2 ml concentrated hydrofluoric acid. The mixture was heated in an Ethos 1 (MLS GmbH, Germany) microwave-assisted wet digestion system for 33 min at 210 °C. After cooling, the digest was quantitatively transferred into a 50-ml Teflon® vessel and evaporated to dryness at 160 °C. The digest was then dissolved in 3 ml of a mixture of nitric and hydrochloric acid (1:3), transferred to a 25-ml glass tube, filled-up with de-ionized water, and kept at laboratory temperature until measurement.

Aliquots of the certified reference material RM 7003 Silty clay loam were mineralized under the same conditions for quality assurance of the analytical data. In the reference material containing Pb = 76.2 ± 6.4 mg kg^{-1}, a total amount of Pb = 79.6 ± 3.8 mg kg^{-1} soil was detected.

2.5.3. Content of exchangeable Pb fraction

The exchangeable fraction represents Pb bound on a soil sorption complex. This fraction can easily be released into soil solution and subsequently become available to plants.

Aliquots (1 g) of air-dried soil samples were extracted with 0.11 M CH_3COOH in a ratio 1:20 (w/v) for 16 h at 20 °C [11]. The soil extracts were centrifuged (Hettich Universal 30 RF) at 3000 rpm ($460 \times g$) for 10 min and the supernatants were kept at 6 °C until measurement.

2.5.4. Pb content in tobacco plants

Aliquots (1 g) of air-dried powdered tobacco roots and above-ground biomass, or whole seedlings, were decomposed in a borosilicate glass test-tube in a mixture of oxidizing gases ($O_2 + O_3 + NO_x$) at 400 °C for 10 h using a Dry Mode Mineralizer Apion (Tessek, Czech Republic). The ash was dissolved in a 20 ml of 1.5% HNO_3 (electronic grade purity) and kept in glass tubes until measurement [12].

Aliquots of the certified reference material RM NCS DC 733350 Poplar leaves were mineralized under the same conditions for quality assurance of the analytical data. In the reference material containing Pb = 1.5 ± 0.3 mg kg^{-1}, a total amount of Pb = 1.3 ± 0.1 mg kg^{-1} dry mass was detected.

2.5.5. Pb content measurement

Pb content in the soil and in the plants was measured by inductively coupled plasma optical emission spectrometry with axial plasma configuration (ICP-AES–Varian VistaPro, Australia), equipped with an autosampler SPS-5, at spectral line λ = 220.4 nm. Two to three samples were analyzed per variant.

2.6. Statistics

Data were analyzed using the statistical and graphical functions of SigmaPlot 8.0 and SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). When a significant *F*-value of $P < 0.05$ was obtained in a one-way analysis of variance test, a Dunnett's multiple-comparison test between the treated and control groups was conducted. For all statistical tests the significance level was set at $P < 0.05$.

3. Results

3.1. Effect of a 24-h treatment with Pb^{2+}

After a 24-h treatment with Pb^{2+}, nuclei were isolated from roots and leaves, and the comet assay was conducted (Fig. 1). With increasing concentrations of Pb^{2+} in the range of 0.8–1.6 mM, the average median tail moment in root nuclei increased significantly from 2.3 ± 0.3 μm (control) to 51.3 ± 2.3 μm after treatment with 1.6 mM. However, treatments with higher Pb^{2+} concentrations (2 mM and 2.4 mM) resulted in a significant decrease of DNA damage compared with the treatment with 1.6 mM Pb^{2+}.

By contrast, in leaf nuclei the same treatment did not result in a significant increase of DNA damage. For example, the control TM (2.6 ± 0.5 μm) and the TM after treatment with 2.4 mM Pb^{2+} (4.5 ± 0.5 μm) were not significantly different.

3.2. Effect of a 7-day treatment with Pb^{2+}

Tobacco seedlings were treated with 25–200 μM Pb^{2+} for 7 days. After treatment, nuclei were isolated from leaves and roots, and the comet assay was performed (Fig. 2).

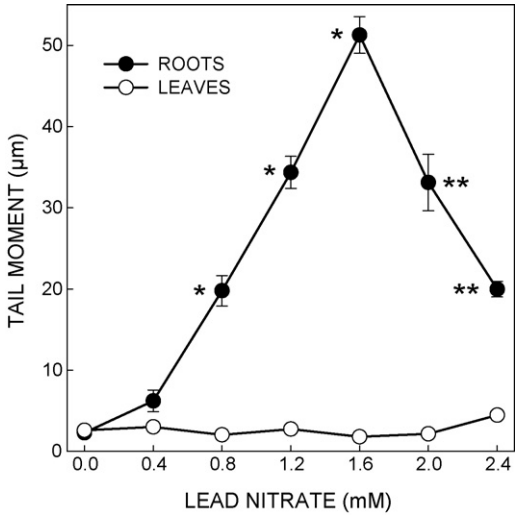

Fig. 1. The average median tail moments (TMs) in root and leaf nuclei after a 24-h treatment of tobacco (*Nicotiana tabacum* var. *xanthi*) seedlings with lead nitrate. The error bars represent the standard error of the mean. *Significantly ($P < 0.05$) different from the control. **Significantly ($P < 0.05$) lower than the TM after treatment with 1.6 mM lead nitrate.

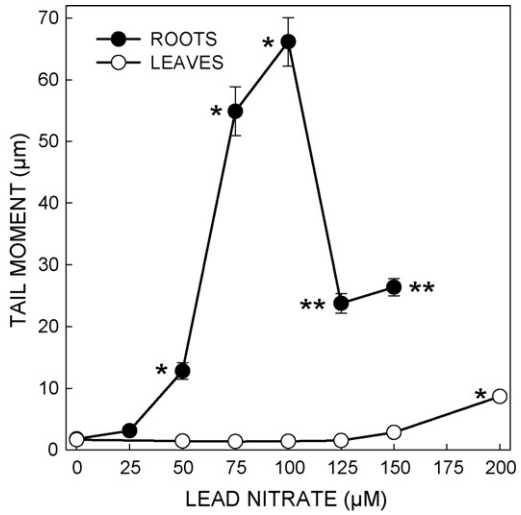

Fig. 2. The average median tail moments (TMs) in root and leaf nuclei after a 7-day treatment of tobacco (*Nicotiana tabacum* var. *xanthi*) seedlings with lead nitrate. The error bars represent the standard error of the mean. *Significantly ($P < 0.05$) different from the control. **Significantly ($P < 0.05$) lower than the TM after treatment with 100 μM lead nitrate.

With increasing concentrations of Pb^{2+} in the range of 25–100 μM, the average median TM in root nuclei increased significantly from 1.8 ± 0.2 μm (control) to 66.2 ± 3.9 μm (100 μM). However, treatments with 125 μM and 150 μM Pb^{2+} resulted in significantly lower TM (23.8 ± 1.6 μm and 26.5 ± 1.4 μm, respectively) compared with the treatment with 100 μM Pb^{2+}. Still higher concentrations of Pb^{2+} could not be applied as the number of nuclei was very low. At concentrations of 75 μM Pb^{2+} and above the root tips became brownish.

In leaf nuclei, treatment with 50–150 μM Pb^{2+} did not result in a significant increase of DNA damage. There was a slight, but significant increase in TM (8.7 ± 0.7 μm) in treatment with 200 μM Pb^{2+} compared with the control (1.6 ± 0.3 μm). The treatment with 200 μM Pb^{2+} resulted in an inhibition of the apical meristem, so no new leaves were formed.

3.3. Toxic, mutagenic, and DNA-damaging effects in tobacco plants cultivated in Pb^{2+}-polluted soil

Two independent experiments were conducted. As the volume of Pb^{2+} solutions applied to the soil differed in both experiments (50 ml in Experiment I and 70 ml in Experiment II), the data could not be summed.

3.3.1. Experiment I

Results of 2-, 4- and 6-week cultivations of tobacco plants in Pb^{2+}-polluted soil are presented in Table 1. The average leaf area of plants cultivated for 6 weeks in soil with 120 mM Pb^{2+} was about 10 times lower (6.7 ± 0.8 cm^2) than the leaf area of plants cultivated in control soil (69.3 ± 2.6 cm^2).

The increased frequency of somatic mutations would be the evidence of mutagenic effects of the Pb^{2+}-polluted soil. However, no difference in the frequency of somatic mutations between polluted and control soil was observed (Table 1).

DNA damage (expressed as tail moment) in the leaves of the plants cultivated for 6 weeks increased significantly from 3.4 ± 0.3 μm (control) to 11.7 ± 2.1 μm (120 mM Pb^{2+}).

Maleic hydrazide, a herbicide and plant growth regulator, was used as a positive control for the somatic mutation assay. MH induced 37.8 ± 3.2 mutant sectors *per* leaf. The alkylating agent EMS was the positive control for the comet assay. EMS induced DNA damage, expressed by TM = 45.2 ± 2.6 μm.

3.3.2. Experiment II

The results of 2-, 4- and 6-week cultivations of tobacco plants in Pb^{2+}-polluted soil are presented in Table 2. The average leaf area decreased with increasing Pb^{2+} concentration. For example,

Table 1
Leaf area, frequency of somatic mutations (mutant sectors *per* leaf), and DNA damage (tail moment) in the leaves of tobacco plants (*Nicotiana tabacum* var. *xanthi*) cultivated for 2–6 weeks in soil polluted with lead nitrate (Experiment I)

$Pb(NO_3)_2$ (mM)	Cultivation (weeks)	Leaf area (cm^2 ± S.E.)	Mutant sectors *per* leaf (±S.E.)	Tail moment (μm ± S.E.)
0	2	38.6 ± 2.9	0.4 ± 0.2	2.9 ± 0.4
20	2	34.3 ± 3.0	0.4 ± 0.2	3.5 ± 0.4
40	2	29.9 ± 0.4	0.4 ± 0.2	4.0 ± 0.4
60	2	18.0 ± 1.1*	0.2 ± 0.2	3.7 ± 0.6
80	2	13.2 ± 1.2*	0.4 ± 0.2	2.9 ± 0.5
100	2	14.7 ± 1.7*	0.2 ± 0.2	2.7 ± 0.2
120	2	8.3 ± 0.6*	0.4 ± 0.2	2.0 ± 0.1
0	4	61.1 ± 2.5	0.9 ± 0.2	3.1 ± 0.3
20	4	62.3 ± 4.7	0.7 ± 0.2	4.0 ± 0.6
40	4	43.5 ± 2.2*	0.7 ± 0.2	3.7 ± 0.7
60	4	16. 7 ± 1.8*	0.5 ± 0.2	3.6 ± 0.4
80	4	15.2 ± 2.1*	0.2 ± 0.2*	4.1 ± 0.6
100	4	12.0 ± 1.7*	0.4 ± 0.2	5.6 ± 0.6
120	4	7.0 ± 0.9*	0.2 ± 0.2*	11.6 ± 2.0*
0	6	69.3 ± 2.6	0.6 ± 0.3	3.4 ± 0.3
20	6	58.3 ± 2.9	0.9 ± 0.3	3.1 ± 0.6
40	6	44.9 ± 3.6	0.6 ± 0.3	2.6 ± 0.2
60	6	23.2 ± 3.3*	0.4 ± 0.2	3.2 ± 0.3
80	6	15.0 ± 2.5*	0.4 ± 0.2	2.9 ± 0.5
100	6	20.4 ± 3.7*	0.5 ± 0.2	6.6 ± 0.9*
120	6	6.7 ± 0.8*	0.4 ± 0.2	11.7 ± 2.1*
MH[1]	4	36.7 ± 2.1*	37.8 ± 3.2*	
EMS[2]	2			45.2 ± 2.6*

* Significantly ($P < 0.05$) different from the control.
MH[1]: tobacco plants treated with 0.05 mM maleic hydrazide.
EMS[2]: tobacco plants treated with 0.04 mM ethyl methanesulfonate.

Table 2
Leaf area, frequency of somatic mutations (mutant sectors *per* leaf), and DNA damage (tail moment) in the leaves of tobacco plants (*Nicotiana tabacum* var. *xanthi*) cultivated for 2, 4 and 6 weeks in soil polluted with lead nitrate or 240 mM KNO_3 (Experiment II)

$Pb(NO_3)_2$ (mM)	Cultivation (weeks)	Leaf area ($cm^2 \pm$S.E.)	Mutant sectors *per* leaf (±S.E.)	Tail moment ($\mu m \pm$S.E.)
0	2	46.2 ± 5.0	1.0 ± 0.3	2.4 ± 0.6
40	2	24.4 ± 1.5*	1.5 ± 0.4	2.6 ± 0.2
60	2	21.0 ± 2.7*	1.0 ± 0.3	2.4 ± 0.3
80	2	10.6 ± 1.4*	0.5 ± 0.3	2.1 ± 0.7
100	2	8.3 ± 1.2*	0.6 ± 0.2	1.2 ± 0.1
120	2	6.6 ± 1.2*	0.4 ± 0.2	2.0 ± 0.5
KNO_3	2	41.2 ± 2.5	1.1 ± 0.5	3.1 ± 0.2
0	4	59.0 ± 3.5	1.0 ± 0.3	2.6 ± 0.6
40	4	23.5 ± 2.5*	0.6 ± 0.3	2.8 ± 0.4
60	4	16.9 ± 1.9*	0.5 ± 0.2	1.2 ± 0.2
80	4	11.7 ± 1.7*	0.5 ± 0.2	2.0 ± 0.3
100	4	6.1 ± 1.0*	0.6 ± 0.3	5.3 ± 0.5
120	4	5.9 ± 0.9*	0.2 ± 0.2	5.3 ± 0.5
KNO_3	4	54.6 ± 1.9	0.7 ± 0.3	3.1 ± 0.3
0	6	80.5 ± 6.3	1.0 ± 0.3	1.7 ± 0.2
40	6	24.4 ± 1.5*	1.0 ± 0.2	2.9 ± 0.2
60	6	21.7 ± 3.3*	0.4 ± 0.3	3.5 ± 0.6
80	6	12.3 ± 1.5*	0.4 ± 0.2	4.2 ± 0.3
100	6	6.3 ± 0.8*	0.2 ± 0.2	9.7 ± 1.6*
120	6	3.7 ± 0.5*	0.4 ± 0.2	11.1 ± 1.1*
KNO_3	6	75.6 ± 3.8	0.9 ± 0.2	2.7 ± 0.3

* Significantly ($P < 0.05$) different from the negative control (tap water).

after 6 weeks of cultivation there was a significant decrease from 80.5 ± 6.3 cm^2 (control) to 3.7 ± 0.5 cm^2 (120 mM Pb^{2+}). In contrast, for the same plants, there were no significant differences in the frequency of somatic mutations. In fact, the frequency of somatic mutations was slightly higher in the controls (1.0 ± 0.3 for control and 0.4 ± 0.2 for 120 mM Pb^{2+}). This may be explained by the very small leaf size of plants growing in polluted soil, which prevents the detection of small mutant sectors.

After 6 weeks of cultivation, the DNA damage in tobacco plants growing in the soil polluted with 100 mM and 120 mM Pb^{2+} was low (TM = 9.7 ± 1.6 μm and 11.1 ± 1.1 μm, respectively), but significantly higher than in the control (1.7 ± 0.2 μm).

To find out whether high nitrate concentration affects the plant growth and DNA damage (as demonstrated in Experiment I for high Pb^{2+} concentrations), we used also 240 mM KNO_3 as a negative control. This concentration of KNO_3 corresponds to the concentration of nitrate present in the highest Pb^{2+} solution (i.e. 120 mM). No significant decrease in average leaf area was observed after 6 weeks of cultivation (75.6 ± 3.8 cm^2 for 240 mM KNO_3 and 80.5 ± 6.3 cm^2 for control). Similarly, there were no significant effects of nitrate on the frequency of somatic mutations and DNA damage. These results demonstrate that the toxic and DNA-damaging effects of 120 mM $Pb(NO_3)_2$ are not associated with the presence of nitrate.

3.4. Accumulation of Pb in roots and above-ground biomass after 24-h treatments with Pb^{2+}

Treatment of 6-week-old tobacco seedlings with 2.4 mM lead nitrate for 24 h resulted in about a 180-fold increase in the above-ground total Pb content (1460 mg kg^{-1} dry mass) compared with the control (8 mg kg^{-1} dry mass) (Table 3). In contrast, after the same treatment the roots accumulated almost 40-fold more Pb than the above-ground biomass and showed nearly a 7000-fold increase in Pb content (57749 mg kg^{-1} dry mass) compared with the control (8 mg kg^{-1} dry mass).

3.5. Pb content in the soil and in the plants after a 6-week treatment with Pb^{2+}

Following a single addition of 50 ml 120 mM lead nitrate to the soil, total Pb content and exchangeable Pb fraction were 13 509 mg kg^{-1} and 459 mg kg^{-1}, respectively, whereas in control soil total Pb content and exchangeable Pb fraction were 35 mg kg^{-1} and 0.9 mg kg^{-1} of soil (Table 4). After 6 weeks, the total Pb content had decreased to 7003 mg kg^{-1} and the exchangeable Pb fraction had decreased to 145 mg kg^{-1} soil. This decrease is caused partly by the uptake of Pb by the tobacco plants and also by the dilution of Pb in the soil by water used for watering the plants.

Table 3
Comparison of total Pb accumulation in above-ground biomass and roots of tobacco (*Nicotiana tabacum* var. *xanthi*) after a 24-h treatment of seedlings with $Pb(NO_3)_2$

$Pb(NO_3)_2$ (mM)	Pb in above-ground biomass (mg kg^{-1} dry mass)	Pb in roots (mg kg^{-1} dry mass)
0	8	8
0.8	453	38099
1.2	622	51972
1.6	1368	51168
2.0	1129	53454
2.4	1460	57749

The results are means of three analyses (above-ground biomass) or means of two analyses (roots).

The uptake of Pb by the tobacco plants after a 6-week cultivation in Pb^{2+}-polluted soil is summarized in the last column of Table 4. The Pb content in plants growing in soil with 120 mM $Pb(NO_3)_2$ was significantly higher (240 mg kg^{-1}) than in the control (5 mg kg^{-1} dry mass).

4. Discussion

The DNA-damaging effects of lead were assessed by the comet assay in many human and animal systems [6,13,14] and in root tips of lupin (*Lupinus luteus* L.) [5].

In the present study, data in Figs. 1 and 2 demonstrate a concentration-dependent increase of DNA damage in root nuclei. However, at higher Pb^{2+} concentrations a significant decrease of DNA damage was observed. A similar phenomenon was described for lead nitrate treatment of lupin plants [5] and for lead acetate treatment of human lymphocytes [14]. It was suggested that the formation of DNA–DNA and DNA–protein cross-links at high Pb^{2+} concentrations inhibits DNA migration during electrophoresis.

Table 4
The total and exchangeable Pb fraction in soil and the total Pb content in tobacco plants (*Nicotiana tabacum* var. *xanthi*) at the start and at the end of a 6-weeks cultivation in $Pb(NO_3)_2$-polluted soil

Concentration of $Pb(NO_3)_2$ added to soil (mM)	Total Pb content (mg kg^{-1} soil)		Exchangeable Pb fraction (mg kg^{-1} soil)		Total Pb content in plants after a 6-weeks cultivation (mg kg^{-1} dry mass)
	At the start of cultivation	After 6 weeks cultivation	At the start of cultivation	After 6 weeks cultivation	
0	35	66	0.9	0.8	5
20	536	773	12	21	17
40	941	1253	21	53	24
60	8654	4056	453	75	181
80	14495	3452	716	107	186
100	15245	4870	880	130	216
120	13509	7003	459	145	240

The results are means of two analyses.

In the leaves, the extent of Pb^{2+}-induced DNA damage was not significantly increased with the exception of that after treatment with 200 μM Pb^{2+} for 7 days (Fig. 2). Analytical studies by inductively coupled plasma optical emission spectrometry demonstrate (Table 3) that after a 24-h treatment of tobacco with 2.4 mM Pb^{2+}, the accumulation of the heavy metal is 40-fold higher in the roots than in the above-ground biomass. Low Pb^{2+} accumulation in the above-ground parts may explain the absence or lower extent of Pb^{2+}-induced DNA damage in leaves. Our data on Pb content fit well with a general conclusion of many experiments, which showed that Pb primarily accumulates in the roots of several plant species and it is poorly translocated to other plant parts [15,16].

As expected, plants treated with higher Pb^{2+} concentrations were more phenotypically damaged than those exposed to lower Pb^{2+} concentrations, and they did not survive 1 week cultivation in water. Even Pb^{2+} concentrations that induced only a low extent of DNA damage were lethal for the plants within 14 days after the treatment, or had inhibited the cell divisions in apical meristem, so that no new leaves could be formed.

By contrast, EMS induced a high extent of DNA damage (TM up to 70 μm), but did not prevent the formation of new leaves [17]. These results may lead to an assumption that Pb^{2+}-induced DNA damage may be connected with necrotic and/or apoptotic DNA fragmentation and not with DNA damage resulting in genetic alteration. This assumption is supported by the absence of a significant increase in the frequency of somatic mutations, which represent genetic alterations (Tables 1 and 2).

The question has been raised whether the comet assay is a useful method for monitoring genotoxic effects of environmental pollutants in plants growing *in situ*. As demonstrated in Tables 1 and 2, a slight but significant increase in DNA damage was associated with high toxic effects (inhibition of leaf size, brownish and distorted leaves). Similar results were obtained when monitoring the toxic and genotoxic effects in tobacco and potato (*Solanum tuberosum* L.) plants growing on soil heavily polluted with heavy metals [7] or polychlorinated biphenyls [18]. Root nuclei could be more sensitive to environmental pollutants. However, the number of root nuclei that can be evaluated for DNA migration in plants growing for a longer period in polluted soil is very low, soil parts cling to the roots, and so it is difficult to perform the comet assay. Thus the comet assay does probably not represent a suitable method for monitoring genotoxicity of environmental pollutants using plants growing *in situ*.

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