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GENOTOXICITY OF LEAD IN LUPIN ROOT CELLS AS EVALUATED BY THE COMET ASSAY

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Abstract: This paper presents the results of a study on the influence of lead (Pb^{2+}) on DNA integrity on plant cells. The study was performed on the root tips of lupin (*Lupinus luteus* cv. Juno) seedlings treated with two selected concentrations of $Pb(NO_3)_2$: 150 and 350 $mg\ l^{-1}$, which were found to inhibit root growth by 50% and 70%, respectively [Rucińska *et al.* Plant Physiol. Biochem. 37 (1999) 37187-37194]. Roots exposed to those external lead concentrations took up about 50 and 70 $mg\ l^{-1}\ Pb^{2+}\ g^{-1}$ fresh weight (FW) over 48 h of incubation. A dose-dependent increase in the degree of root injury was observed in the presence of both tested concentrations.

The genotoxicity of lead in lupin root cells was analysed using a mild alkaline comet assay at pH 12.3, which allows the detection of single strand breaks. The quantity of the DNA fragments migrating away from the nuclear remnant (tail area) increased proportionally to the lead content inside the roots, and was positively correlated with the degree of root injury. At 150 $mg\ l^{-1}\ Pb^{2+}$, a high frequency distribution of nuclei having large values of tail lengths and moments was observed. By contrast, the number of nuclei with minimum values of these parameters increased at 350 $mg\ l^{-1}\ Pb^{2+}$. This data suggests that lead at low concentrations induces the formation of short, rapidly migrating DNA fragments, whereas at higher concentrations, lead probably causes other changes to DNA that result in slower DNA migration in the electric field.

Key Words: Lead, Comet Assay, Lupin

INTRODUCTION

Heavy metals can influence various morphological, physiological and biochemical processes in plants. Lead in particular is known to strongly inhibit plant growth, root elongation, seed germination, seedling development, cell

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division, photosynthesis, transpiration, chlorophyll production, etioplast development and/or lamellar organisation in chloroplasts [1]. However, only limited data is available on the influence of lead on plant genetic material. To evaluate the mutagenicity of heavy metals in plants, the following assays have been proposed: chromosome aberration analysis [2], micronucleus tests [3], DNA fingerprinting analysis [4] and the detection of somatic point mutations and homologous recombination [5].

The alkaline comet assay (single cell gel electrophoresis assay), a sensitive method for detection of DNA damage, was used in order to analyse the genotoxicity of lead in lupin (*Lupinus luteus* cv. Juno) root cells [6]. So far, this method has turned out to be a valuable tool for monitoring DNA damage in the roots of field bean exposed to cadmium [7]. The technique allows the detection of DNA strand breaks within single cells [8]. During electrophoresis, the damaged DNA migrates away from the nucleus to produce a comet shape, which is subsequently visualised by silver or ethidium bromide staining. The distribution of DNA within the comet is estimated using these parameters: tail area, tail length and tail moment. They provide information about the extent of DNA damage within the population of affected nuclei. The method used also gives insight into agent- and dose-specific effects of genotoxic substances [8].

MATERIAL AND METHODS

Plant growth

Seeds of yellow lupin (*Lupinus luteus* cv. Juno) were surface-sterilised with 75% (w/v) ethanol for 5 min, followed by 1% sodium hypochlorite for 10 min. After this treatment, the seeds were thoroughly rinsed with tap water and then soaked for 1 h in distilled water at room temperature. The seeds were germinated in Petri dishes lined with water-moistened filter paper at 22°C in the dark, and left to grow until the roots reached 6-8 mm in length.

Subsequently, the seedlings were treated with $\text{Pb}(\text{NO}_3)_2$ solutions at concentrations of 150 and 350 $\text{mg l}^{-1} \text{Pb}^{2+}$. These concentrations were selected from a wide range of doses (0-350 $\text{mg l}^{-1} \text{Pb}^{2+}$) that cause the inhibition of lupin root growth and the modification of root morphology [9]. At each concentration, 10 seedlings were transferred to a dish containing 5 ml of the test solution and incubated in the dark at 22°C for 48 h. To provide constant immersion of the root tips in the solution, they were covered with moistened filter paper.

Determination of lead content

The samples containing 2 g fresh weight of root tissue were digested by an $\text{HNO}_3/\text{HClO}_4/\text{H}_2\text{SO}_4$ mixture (10/1/1, v/v/v). The amount of lead was determined using a Zeiss-3 model atomic absorption spectroscope.

Determination of the degree of root injury

Seedlings were incubated for 15 min with 0.05% w/v Evans blue solution, and then rinsed with four changes of distilled water to remove the excess of unbound

dye. Six hundred mg of root tips (5 mm) were excised on ice and dye bound to dead cells, then solubilized in a solution (1.2 ml) of 50% v/v methanol and 1% w/v SDS for 30 min at 50°C and quantified by absorbance measurement at 600 nm [10].

Isolation of nuclei

The nuclei of the root cells were isolated as described by Pfosser *et al.* [11]. The roots were cut 5 mm from their ends and about 20 mg of root tips (from 30 seedlings) were immediately chopped with a razor blade in 1 ml of ice-cold 0.2M Tris pH 7.5 containing 4 mM MgCl₂·6H₂O and 0.5% w/v Triton X-100 (Tris-MgCl₂ buffer). The nuclei were filtered through a 52 µm nylon net, and sedimented by centrifugation at 200 g for 5 min (4°C) in a swinging bucket rotor. The pellet was then resuspended in 200 µl of Tris-MgCl₂ buffer. The integrity of the nuclei was checked under a microscope (150 × magnification) after staining with ethidium bromide (10 µg ml⁻¹) using an excitation filter of 515-560 nm (data not presented).

Comet assay

The degree of DNA damage was determined using the alkaline comet assay, according to the method described in a review paper [6]. Each microscope slide was precoated with a layer of 1% normal melting point agarose and thoroughly dried at room temperature. Next, 100 µl of 0.8% low melting point agarose at 37°C was mixed with 20 µl of the nuclear suspension and dropped on top of the first layer. The slides were allowed to solidify for 2 min on an ice-cooled tray and were then immersed in ice-cold lysing solution (1 M NaCl; 30 mM NaOH, 0.5% w/v SDS, pH 12.3) for 1 h. After lysis, the slides were placed in a horizontal gel electrophoresis chamber and the DNA was allowed to unwind for 1 h in the electrophoretic buffer, which contained 30 mM NaOH and 1.5 mM EDTA at pH 12.3 [6]. Electrophoresis was conducted for 20 min at 25 V (1 V cm⁻¹) in a chamber cooled on ice. Then the slides were washed twice with water, dried for 1 h at room temperature, and silver stained according to [12]. The gels were fixed for 10 min in a solution containing 15% w/v trichloroacetic acid, 5% w/v zinc sulphate, and 5% v/v glycerol. Then the slides were washed three times in deionized water and dried overnight at room temperature. Before silver staining, the gels were re-hydrated for 5 min in deionized water. The staining solutions were freshly prepared before use. 34 ml of Solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% tungstosilicic acid, 0.15% v/v formaldehyde, 5% sodium carbonate) was gently added to 66 ml of solution A (5% sodium carbonate). The slides were soaked in this staining solution for 20 min, rinsed three times in deionized water, and air-dried.

The comets were observed using 150 x magnification, and the extent of DNA migration was determined using the ScionImage image analysis system [13] and macro Comet_Scoring_Macro, which optimise the measurements.

Three parameters were analysed: tail area (length x width), tail length (measured from the right edge of comet head) and tail moment (the integrated value of tail density multiplied by the migration distance). Because the measurement system was not calibrated, all the parameters were expressed in arbitrary units. The tail area is proportional to the amount of total the DNA which migrated from an individual nucleus, while tail length and tail moment are positively correlated with the level of DNA breakage and/or alkali-labile sites, and negatively correlated with the level of DNA cross-links [14].

Data analysis

Generally, 300 comets were scored per dose (100 comets were randomly selected from three replicated slides). The data from three experiments was pooled and the mean values (\pm Std. Dev. and \pm Std. Error) were calculated. The Least Significant Difference (LSD) test was used to determine statistical differences between the control values and the values of each concentration. The distribution of the total number of comets among the various classes was presented graphically in the form of histograms, separately for tail area, length and moment [15]. The calculations and graphs were done using the computer program Statistica (StatSoft, Inc.).

RESULTS AND DISCUSSION

The effect of lead nitrate on the DNA integrity of lupin roots was tested at two concentrations of the metal: 150 and 350 mg l⁻¹, at which root growth was inhibited by 50% and 70%, respectively [9]. It was previously shown that these concentrations cause a defined effect on the growth of seedlings [9], mitotic index [16], accumulation of PR-10 proteins (pathogenesis related) [17], level of reactive oxygen species and activity of antioxidant enzymes [9]. The degree of root growth inhibition was correlated with the internal metal content within the cells (Fig. 1A) and the level of root cells injury (Fig. 1B). Roots exposed to 150 mg l⁻¹ Pb²⁺ took up about 50 mg l⁻¹ Pb²⁺ g⁻¹ FW (fresh weight) after 48 h of incubation. The amount of lead inside the roots increased to 70 mg l⁻¹ Pb²⁺ g⁻¹ FW at an external concentration of 350 mg l⁻¹. This increase was not directly related to the higher lead concentration in the medium, and this may suggest that seedlings are capable, at least partially, of limiting the uptake of Pb²⁺. Yang *et al.* [18] indicated that compounds secreted from the roots, such as oxalate, may precipitate lead on the root surfaces and thereby reduce the bioavailability of this metal. The entry of lead into the protoplast might be reduced by the binding of metal ions to the cell wall or by the suppression of transmembrane pumps and channels [19]. These protective mechanisms were revealed not to be sufficient to sustain the integrity of the plasma membranes of lupin roots. Using Evans blue uptake, a dose-dependent increase of root injury was observed in the presence of both tested concentrations (Fig. 1B).

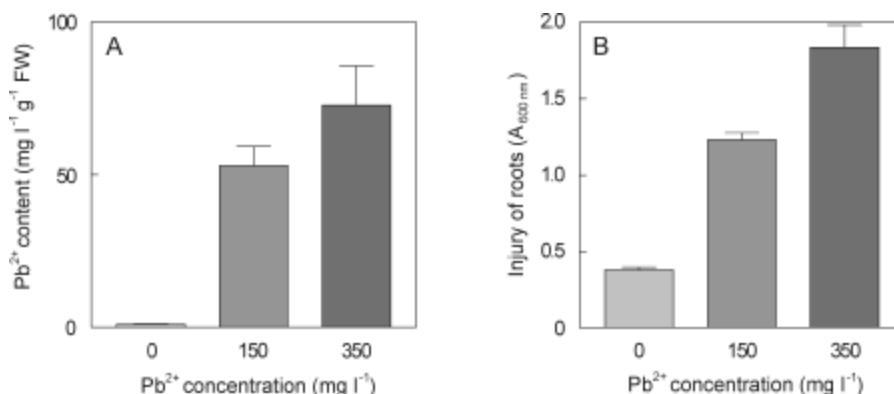


Fig. 1. Lead uptake and its effect on root injury. A. The lead content in the roots of lupin seedlings after 48 h incubation in control conditions or exposure to 150 and 350 mg l⁻¹ Pb(NO₃)₂. The Pb²⁺ content was determined by a Zess-3 model atomic absorption spectroscope. Values are the means ± S.D. of three independent experiments (n=3). B. The degree of root injury after 48 h incubation in control conditions or exposure to 150 and 350 mg l⁻¹ Pb(NO₃)₂. The injury was determined by Evans blue staining and quantified by measuring absorbance at 600 nm. Values are the means ± S.D. of three independent experiments (n=3).

DNA damage in control and lead-treated roots was estimated using the alkaline comet assay followed by silver staining. Fig. 2 shows an individual isolated nucleus comprising a head and a tail, forming a comet image. The head corresponds to the amount of DNA that still remains in the region of the nuclear matrix, whereas the tail visualises the fragments of DNA migrating from the nuclei [8]. Within experimental variants (control, 150 and 350 mg l⁻¹ Pb²⁺), the high degree of heterogeneity among the values of tail area, length and moment were scored (Fig. 3). At both concentrations, lead induced a significant increase ($P < 0.001$) in the tail area (Fig. 3A). The areas of individual tails were measured and divided into classes. The frequency of comets of classes 0-0.6 in the control was higher than for lead-treated roots (Fig. 4A). By contrast, the number of comets in classes 0.6-1.1 was higher at both metal doses: 150 mg l⁻¹ and 350 mg l⁻¹ of Pb²⁺ (Fig. 4B, 4C). Moreover, lead at the higher concentration caused an increase in the fraction of comets with larger areas (1.1-2.0) than that observed for lead at the lower concentration (Fig. 4B, 4C). This data indicated that the amount of DNA liberated from the nuclei of lupin roots is correlated with the external metal concentration.

The various plant genotoxic tests demonstrated that lead strongly induce DNA damage in a concentration-dependent manner [2, 3, 5]. The results obtained for lupin roots with the application of the alkaline comet assay and based on tail area measurements are consistent with previous observations.

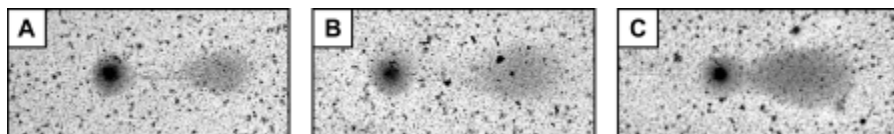


Fig. 2. Comet images after alkaline single-cell gel electrophoresis followed by silver staining. Nuclei were isolated from lupin roots of control seedlings (A) or those exposed to 150 (B) and 350 (C) $\text{mg l}^{-1} \text{Pb}(\text{NO}_3)_2$ for 48 h.

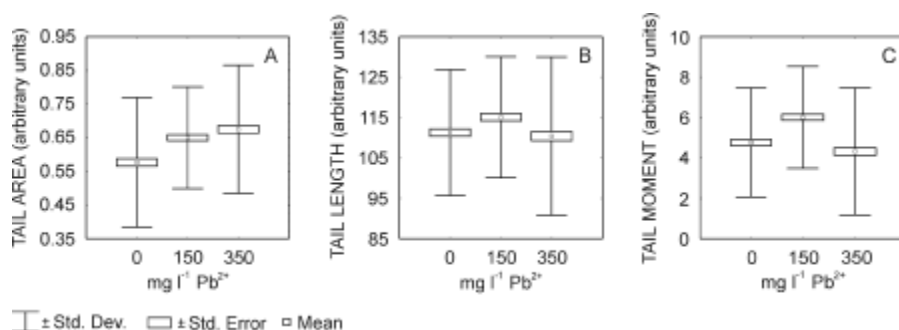


Fig. 3. The tail area (A), length (B) and moment (C) of comets obtained from lupin root nuclei of control seedlings or those exposed to 150 and 350 $\text{mg l}^{-1} \text{Pb}(\text{NO}_3)_2$ for 48 h. Results for 300 comets scored per dose were pooled and the mean values (\pm Std. Dev. and \pm Std. Error) were calculated.

A marked increase in tail length ($P < 0.05$) and moment ($P < 0.001$) was seen only after incubation with the lower lead concentration of 150 mg l^{-1} (Fig. 3B, 3C). The distribution of comets with the respective representative tail lengths from the control and low and high concentration lead-treated roots is shown in Figs. 4D, E and F. The highest frequency of comets with tails between 100-110 was detected for the control roots (Fig. 4D), while for the roots treated with 150 and 350 $\text{mg l}^{-1} \text{Pb}^{2+}$, the comet distributions were symmetrical (Fig. 4E, 4F). The tail moment had a more significant statistic distribution. In the case of the control plants, the most abundant fraction of the comets had a tail moment between 2-6 (Fig. 4G), while at 150 mg l^{-1} of Pb^{2+} , the maximum lay between 4-8 (Fig. 4H). The most pronounced effect was seen between 6-12, where the number of comets was almost twice as high as that for the control. When 350 mg l^{-1} of lead was used, the maximum returned to values between 0-6 (Fig. 4I). At this lead concentration, the 0-2 value comet frequency was 3-fold higher than for the control.

The analyses of comet images in Fig. 2 and histograms in Fig. 4 provide insight into the dose-specific differences in the types of DNA damage caused by lead. In general: long, thin tails are induced at the lower concentration of 150 $\text{mg l}^{-1} \text{Pb}^{2+}$ (Fig. 2B, 4E, 4H), while short, thick tails with DNA fragments in the vicinity of the comet head are visible at the higher concentration (Fig. 2C, 4F, 4I).

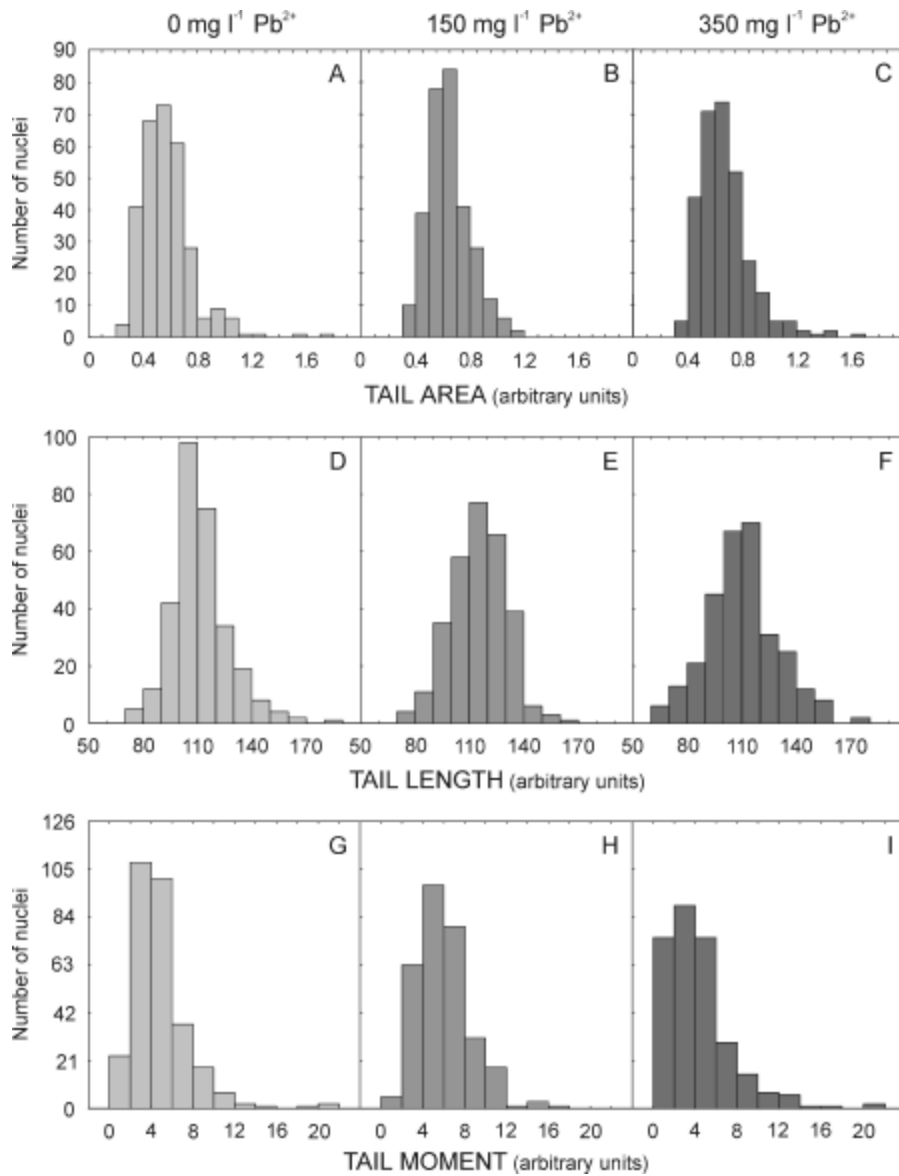


Fig. 4. A histogram of the distribution of comet tail areas (A-C), lengths (D-F) and moments (G-I) in the alkaline comet assay. Nuclei were isolated from lupin roots of control seedlings (A, D, G) or treated with 150 (B, E, H) and 350 (C, F, I) mg l⁻¹ Pb(NO₃)₂ for 48 h. The number of scored comets in each treatment was 300 (100 comets were randomly selected from three replicated slides).

Similar symptoms of lead genotoxicity were observed in isolated human lymphocytes [20]. At concentrations of 0.2 mg l⁻¹ and 2 mg l⁻¹ Pb²⁺, an increase in the tail length was observed, whereas at 207 mg l⁻¹, a decrease was seen. The authors suggested that the former effect of lead could be due to the induction of DNA strand breaks and/or alkali-labile site formation. The latter arise from the formation of DNA-DNA and/or DNA/protein cross-links. A slow migration of DNA has also been reported for field bean nuclei treated with a higher dose (112 mg l⁻¹) of other heavy metals such as cadmium [7]. This phenomenon has been explained by the formation of chromosome bridges and the stickiness of chromosomes observed in barley treated with cadmium [21]. The data presented by Kovalchuk *et al.* [5] suggests that double-stranded breaks induced by lead may be in part repaired by homologous recombination. This process could also occur in our experimental system.

In our earlier studies, the response of antioxidant enzymes to various concentrations of Pb²⁺ (0-350 mg l⁻¹) was examined in lupin seedlings [9]. A dose-dependent increase in the activities of superoxide dismutase and peroxidase from the root tip was observed, whereas catalase and ascorbate peroxidase activities decreased at the higher lead concentrations. Using electron paramagnetic resonance analysis, it was found that at the lead concentration of 350 mg l⁻¹, the level of quinone radicals markedly increased. These results suggest that at higher lead concentrations, the formation of both free radicals and reactive oxygen species is beyond the capacity of the antioxidant system, which in turn may contribute to oxidative DNA modifications. According to Cunningham [22], metal-mediated production of reactive oxygen species in the DNA vicinity generates the premutagenic adduct 8-OHdG (7,8-dihydro-8-oxoguanine), which could mispair with adenine in the absence of DNA repair, resulting in G to T transversion mutations. It has been reported that lead caused an accumulation of a small amount of 8-OHdG in calf thymus DNA [23]. However, the possible participation of reactive oxygen species in the DNA-damaging potential of lead nitrate in lupin roots requires further experimental confirmation.

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