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BRIEF COMMUNICATION

Zinc induces DNA damage in tobacco roots

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Abstract

We applied the alkaline version of the single-cell gel electrophoresis (comet assay) to seedlings of heterozygous tobacco (*Nicotiana tabacum* L. var. *xanthi*) treated with zinc acetate dihydrate (20 to 80 mM Zn^{2+} for 2 h or 2 to 12 mM Zn^{2+} for 24 h). A dose dependent increase in DNA damage expressed by the tail moment values were observed in nuclei isolated from the roots after 2 and 24 h Zn^{2+} treatments. In contrast, Zn^{2+} did not induce significant DNA damage to leaf nuclei, with the exception of 10 or 12 mM Zn^{2+} for 24 h. Somatic mutations, identified as dark green, yellow, and dark green/yellow double sectors on the pale green tobacco leaves were not detected after any Zn^{2+} treatments. The accumulation of Zn in roots and shoots was determined by inductively coupled plasma optical emission spectrometry and the Zn content in roots was about three times higher than in shoots.

Additional key words: comet assay, ethyl methanesulphonate, *Nicotiana tabacum,* somatic mutations.

The alkaline version of the single cell electrophoresis assay (comet assay) can quantitatively measure DNA damage including single strand breaks, double strand breaks, alkali labile sites (primarily apurinic and apyrimidinic sites), incomplete excision repair sites, and DNA crosslinks (Tice *et al*. 2000). Although this technique has been primarily applied to animal cells, the comet assays with plant tissues (Koppen and Verschaeve 1996, Gichner and Plewa 1998, Gichner *et al*. 2009) significantly extends the basic and applied studies in environmental mutagenesis.

The comet assay detects acute DNA damage in nuclei of leaf and root cells whereas the leaf primordial cells within the apical meristem are the target cells of the somatic mutations (Dulieu and Dalebroux 1975). Mutations are induced in these primordial cells and as the leaf grows, division of the mutant cells develop into individual clones of cells that appear, *e.g*., on the pale green leaves of heterozygous *Nicotiana tabacum* var. *xanthi* as dark green, yellow, and dark green/yellow double sectors. Thus, the somatic mutation frequency can be evaluated first 2 to 3 weeks after the treatment on the newly formed leaves.

The objectives of this study was to determine if excess of zinc, an essential microelement, induces in tobacco plants DNA damage in nuclei of leaf and root cells evaluated by the comet assay and according to occurrence of somatic mutations on leaves.

The well-known mutagen ethyl methanesulphonate (EMS), zinc acetate dihydrate, *Phytagel*, Murashige and Skoog medium, reagents for electrophoresis, and general laboratory reagents were purchased from *Sigma Chemical Co*., St. Louis, MO, USA. Normal (NMP) and low (LMP) melting point agaroses were purchased from *Roth*, Karlsruhe, Germany. Reagents for the Zn content determination and the certified reference material were purchased from *Analytika and Lach-Ner*, Prague, Czech Republic.

The double heterozygous pale green *Nicotiana tabacum* L., var. *xanthi* (a_1^+/a_1; a_2^+/a_2) plants were used (Dulieu and Dalebroux 1975). The seeds were sterilized by an immersion in 70 % (v/v) ethanol for 2 min

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Abbreviations: EMS - ethyl methanesulphonate; TM - tail moment; NMP - normal melting point, LMP - low melting point; ROS - reactive oxygen species.
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followed by immersion in 4.5 cm^3 of distilled water + 0.5 cm^3 of 5.25 % (m/v) sodium hypochlorite + 0.005 cm^3 of 10 % (v/v) *Triton X-100* for 20 min. The seeds were washed five times in sterile distilled water. Each seed was placed in a vented container with 50 cm^3 of sterile solid growth medium (Gichner *et al.* 2009). The plants were grown at temperature of 26 °C, relative humidity of 60 %, a 16-h photoperiod, and irradiance of 200 μmol m^{-2} s^{-1}. At the 4 - 5 true leaf stage, the tobacco plants were carefully taken out of the containers, the roots were rinsed with water and the plants were used for Zn^{2+} and EMS treatments.

Roots of tobacco seedlings were immersed in plastic vials in 22 cm^3 of 20 to 80 mM Zn^{2+} for 2 h or in 2 to 12 mM Zn^{2+} for 24 h. For the positive control, treatments with 2 to 10 mM EMS for 2 h or 1 to 4 mM EMS for 24 h were applied. Zn^{2+} or EMS were dissolved in distilled water. The plants were treated in the dark at 26 °C.

For soil experiments, 2- to 3-week-old tobacco seedlings were cultivated in plastic pots (diameter 7 cm, depth 9 cm) filled with 150 cm^3 of soil (Chernozem, pH 7.2). The soil was contaminated with 70 cm^3 of 2.5 to 15 mM zinc acetate dissolved in distilled water. In the soil before contamination, the total zinc content was 88.4 mg kg^{-1} and the mobile-portion was 2.39 mg kg^{-1}. The plants were cultivated at day/night temperatures of 28/22 °C, relative humidity of 60 %, a 16-h photoperiod, and irradiance of 150 μmol m^{-2} s^{-1} for 2 weeks. In soil experiments, only the DNA damage in leaf nuclei was studied. When tobacco seedlings are cultivated in soil for a longer period, it is difficult to use the roots for comet analysis as soil particles cling to the roots and the number of nuclei is very low.

After Zn^{2+} or EMS treatment, a small piece of leaf (approximately 2 × 2 cm) or tufts of roots were placed in a 60 mm Petri dish kept on ice and spread with 0.25 cm^3 of cold 400 mM Tris buffer, pH 7.5. Using a fresh razor blade, the roots or leaves were gently sliced, and the isolated nuclei collected in the buffer. All operations were conducted under dim or yellow light. Microscope slides were dipped into a solution of 1 % (m/v) NMP agarose prepared with water at 50 °C, dried at room temperature overnight, and kept dry in slide boxes until use. Onto each slide, nuclear suspension (0.05 cm^3) and 1 % (m/v) LMP agarose (0.05 cm^3) prepared with phosphate-buffered saline were added at 40 °C. The nuclei and the LMP agarose were gently mixed by repeated pipetting using a cut micropipete tip, and a coverslip was placed on the mixture. The slides were cooled on a steel tray on ice. The coverslips were removed and the slides were placed in a horizontal gel-electrophoresis tank (*Horizon 20.25 GIPCO-BRL*, Gaithersburg, USA) containing a freshly prepared cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH > 13). The nuclei were incubated at 0.74 V cm^{-1} (26 V, 300 mA, *PowerPac 300*, *BioRad*, Hercules, USA) for 15 min to allow DNA to unwind prior to electrophoresis and then at 4 °C 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 and then stained with ethidium bromide (20 μg cm^{-3}) for 5 min, dipped in water to remove the excess stain and covered with a coverslip.

Mostly 50 randomly chosen nuclei *per* slide, and 2 to 3 slides *per* treatment were evaluated. The experiments were repeated twice, so that the number of nuclei evaluated *per* concentration of the tested agent was 150 to 200. A fluorescence microscope (*BX 60*, *Olympus*, Tokyo, Japan) with a band pass excitation filter of 546/10 nm, 546/10 nm, a barrier filter of 590 nm, and a computerized image-analysis system (*Komet v. 3.1*, *Kinetic Imaging*, Liverpool, UK) was used for visualization. The tail moment (TM; integrated value of tail DNA density multiplied by the migration distance) was used as the primary measure of DNA damage. The TM values are shown as the means of medians ± SE.

Eight seedlings at the stage of 4 to 5 leaves were treated with 2 to 8 mM Zn^{2+} or 1 to 4 mM EMS for 24 h in the dark. Then the roots of the seedlings were rinsed, and the seedlings were cultivated in glass vials with 50 % Hoagland's solution in a growth chamber at conditions mentioned above for 2 - 3 weeks. The dark green, yellow, and dark green/yellow double sectors (Dulieu and Dalebroux 1975) were identified on the pale green leaves newly formed after the treatment (on the 1st leaf at day 14 and on the 2nd leaf at day 21). The mutation data are expressed as the mean number of sectors *per* leaf.

Aliquots (1 g) of both air-dried powdered tobacco roots and shoots 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*, Prague, Czech Republic). The ash was dissolved in 20 cm^3 of 1.5 % (m/v) HNO_3 (electronic grade purity) and kept in glass tubes until measurement (Miholová *et al.* 1993). The Zn content in the plants was measured by inductively coupled plasma optical emission spectrometry with axial plasma configuration (*ICP-AES-Varian VistaPro*, Mulgrave, Australia), equipped with auto-sampler *SPS-5*, at spectral line λ = 206.2 nm. Each measurement was repeated 3 times. The experimental conditions were as follows: power of 1.2 kW, plasma flow of 15.0 dm^3 min^{-1}, axillary flow of 0.75 dm^3 min^{-1}, and nebulizer flow of 0.9 dm^3 min^{-1}. The certified reference material, oriental tobacco leaves (*CTA-OTL-1*), contained 49.9 mg(Zn^{2+}) kg^{-1}(d.m.). Aliquots of the reference material were mineralized under the same conditions as described above for quality assurance of the analytical data. A total amount of 49.5 mg(Zn^{2+}) kg^{-1}(d.m.) was detected.

Data were analyzed using the statistical and graphical functions of *SigmaPlot 8.0* and *SigmaStat 3.0* (*SPSS Inc*., Chicago, IL, USA). If in a one-way *ANOVA* a significant *F*-value ($P < 0.05$) was obtained, the Dunnett's multiple comparison test between the treated and control group was conducted. Differences between two groups were statistically evaluated by the paired *t*-test.

With increasing concentrations of Zn^{2+} in the range of 20 - 80 mM, the average median tail moment for root nuclei increased significantly from 2.8 ± 0.6 µm (control) to 19.7 ± 2.1 µm after 60 mM Zn^{2+} treatment (Fig. 1*A*). For the comet assay using leaf nuclei, the 2 h Zn^{2+} treatment was not sufficient to induce any effect. The seedlings must be immersed in the test agent for at least 18 h to enable the agent to be evenly distributed in all leaves (Gichner and Plewa 1998).

The 24 h treatment of the roots with 8 mM Zn^{2+} resulted in a significant increase of the TM values from 2.0 ± 0.2 µm (control) to 31.2 ± 0.4 µm but at higher Zn^{2+} concen-tration (10 mM), a decrease of DNA damage (TM = 16.4. ± 2.4 µm) was noted (Fig. 1*B*). In contrast, in leaf nuclei, the 24-h treatment did not result in a significant increase of DNA damage within the concentration range from 2 to 8 mM Zn^{2+}. At higher Zn^{2+} concentrations of 10 and 12 mM, the TM values increased to 4.7 ± 0.7 and 8.6 ± 0.5 µm, respectively. The tobacco seedlings showed wilting leaves, so that this increase of TM values may be associated with necrotic DNA fragmentation manifested by the formation of comet images (Gichner *et al*. 2009).

As a positive control, the alkylating agent EMS (2 - 10 mM) was applied to tobacco roots for 2 h and to tobacco roots and leaves for 24 h. The TM values increased significantly with increasing EMS concentrations after 2 h (Fig. 2*A*). Similarly, the significant increase of DNA damage was observed in leaf and root nuclei after 24 h treatment (Fig. 2*B*). In contrast to Zn^{2+} treatment, EMS induced high levels of DNA damage not only in the root nuclei, but also in the leaf nuclei.

The DNA damage in leaf nuclei from the soil grown seedlings increased significantly after application of 12.5 mM Zn^{2+} for 2 weeks (TM = 4.6 ± 0.2 µm) compared to control leaf nuclei (2.7 ± 0.2 µm). This increase in DNA damage was accompanied with severe wilting of leaves. Application of 15 mM Zn^{2+} resulted in high toxic effects to the seedlings so that the comet assay could not be applied. EMS (3 mM) increased a TM value to 18.3 ± 2.6 µm (Table 1).

No difference was observed in the frequency of somatic mutations in the control treatments compared to the highest Zn^{2+} dose applied (1.6 versus 1.4 mutation sectors *per* leaf). Higher concentrations of Zn^{2+} could not be applied as they inhibited the growth of the apical meristem. The frequency of mutation sectors increased up to 31.2 mutation sectors *per* leaf following 4 mM EMS treatment for 24 h (Table 2).

At each concentration of Zn^{2+} applied, there was about 3-fold higher content of Zn^{2+} in the roots than in the shoots (Table 3). However, the treatment with 10 mM

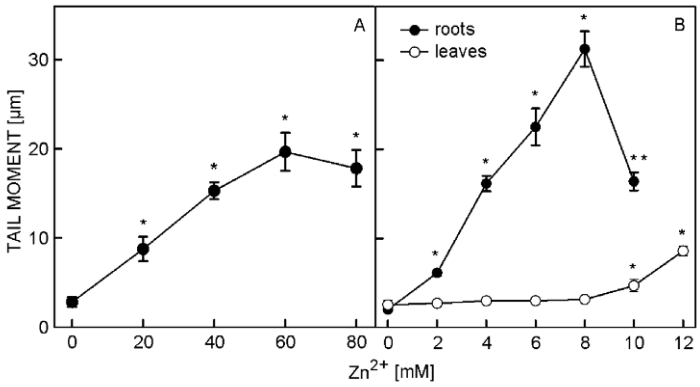

Fig. 1. Tail moment values in tobacco roots and leaves as a function of Zn^{2+} concentration. Treatments lasted 2 h (*A*) and 24 h (*B*). The *error bars* represent SE of the averaged medians; * - significantly different ($P < 0.05$) from the control, ** - significantly lower ($P < 0.05$) than after treatment with 8 mM Zn^{2+}.

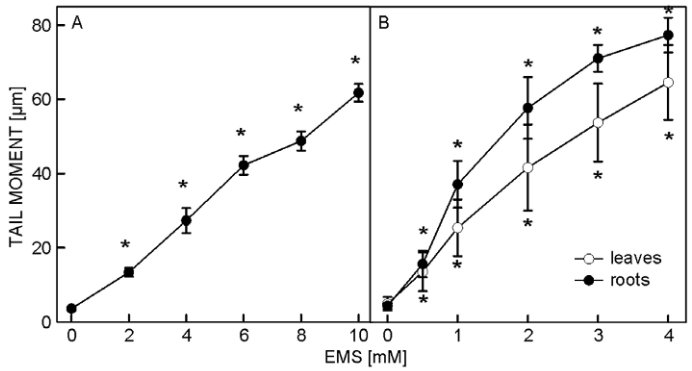

Fig. 2. DNA damage in nuclei of tobacco roots and leaves as a function of EMS concentration. Treatments lasted 2 h (*A*) and 24 h (*B*). The *error bars* represent SE of the averaged medians; * - significantly different ($P < 0.05$) from the control.

Zn^{2+} for 24 h resulted in about a 44-fold increase in the total Zn^{2+} content in the shoot biomass and nearly a 29-fold increase in the Zn^{2+} content in the root biomass.

Table 1. DNA damage expressed by the tail moment (TM) values in nuclei of leaves of *N. tabacum*. var. *xanthi* cultivated for 14 d in soil contaminated with Zn^{2+}. EMS (3 mM) was applied as a positive control. Averaged medians ± SE, n = 150 - 200, * - significantly different ($P < 0.05$) from the control, ++ - very few nuclei.

Zn^{2+} [mM]	TM [μm]
0	2.8 ± 0.3
2.5	3.1 ± 0.4
5.0	3.7 ± 0.09
7.5	3.0 ± 0.4
10.0	2.7 ± 0.3
12.5	4.6 ± 0.3*
15.0	++
EMS	18.3 ± 0.4*

Table 2. Number of somatic mutations (SM) *per* leaf of tobacco seedlings after treatment with Zn^{2+} or EMS for 24 h. Means ± SE, n = 16, * - significantly different ($P < 0.05$) from the control.

Zn^{2+} [mM]	SM [leaf^{-1}]	EMS [mM]	SM [leaf^{-1}]
0	1.6 ± 0.4	0	1.6 ± 0.3
2	1.5 ± 0.3	1	2.9 ± 0.3
4	1.5 ± 0.2	2	7.7 ± 0.7*
6	1.9 ± 0.4	3	16.0 ± 0.6*
8	1.4 ± 0.4	4	31.2 ± 3.2*

Table 3. Comparison of total zinc content in tobacco shoots and roots after Zn^{2+} treatments for 24 h. Means ± SE, n = 3, * - significantly ($P < 0.05$) higher Zn^{2+} content in roots than in shoots at the same applied concentration.

Zn^{2+} [mM]	Zn^{2+} in shoots [mg kg^{-1}(d.m.)]	Zn^{2+} in roots [mg kg^{-1}(d.m.)]
0	129 ± 15	595 ± 34*
2	718 ± 50	1121 ± 59*
4	1866 ± 265	13223 ± 800*
6	4019 ± 462	13631 ± 960*
8	3352 ± 173	15666 ± 446*
10	5693 ± 666	17108 ± 896*

Zinc is an essential microelement that is indispensable for normal plant growth as it acts as a cofactor of more than 300 enzymes and proteins involved in cell division, nucleic acid metabolism, and protein synthesis (Hacisalihoglu *et al.* 2003). Zn^{2+} added to soil often causes an increase of plant biomass production, stomatal conductance, and the quantum yield of photosystem II (Wang *et al*. 2009). Zn^{2+} does not cause stress at low concentrations but Zn^{2+} free medium is damaging (Giampaoli *et al*. 2012). However, at higher concentrations, Zn^{2+} is toxic to plants like other heavy metals and represents a major environmental concern (Dudka *et al*. 1996). High Zn^{2+} concentrations affects biological molecules, for example, when Zn^{2+} binds to the SH group, it blocks the active site of enzymes, disrupts the cellular homeostasis, and cause oxidative damage by generating reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, and hydroxyl radicals (Tripathi and Gaur 2004). The ROS accumulation causes oxidative damage to cell compounds including DNA (Achary *et al*. 2012) resulting in toxic and genotoxic effects (Steinkellner *et al.* 1998, Kovalchuk *et al*. 2001). However, Zn^{2+} effects do not depend only on its concentration but also on the tolerance of particular plant species (Mýtinová *et al*. 2010). The roots are a primary site for Zn toxicity (Balsberg Pahlsson 1989).

Compared to roots, leaf cells are better equipped with antioxidant defense systems that might protect the nuclear DNA in leaf cells from Zn-induced oxidative stress. In previous studies, it was demonstrated that the activity of catalase, the principal H_2O_2 scavenging enzyme, is about 30 times higher in tobacco leaves than in roots (Gichner 2003). Consequently, the high content of catalase and other enzymes inactivating ROS in leaves prevents DNA damage, as measured by the comet assay, and also somatic mutations.

EMS is an alkylating mutagen that directly reacts with DNA and its DNA damaging effect is not affected by catalase (Gichner 2003). This could explain why EMS induces DNA damage and somatic mutations in tobacco leaves in contrast to Zn^{2+}. The absence of Zn^{2+}-induced DNA damage in leaf nuclei and of somatic mutations may be thus caused by the lower Zn^{2+} accumulation in leaves and high activity of ROS scavenging enzymes.

On the other hand, data in Fig. 1*B* demonstrate a concentration dependent increase of DNA damage in root nuclei induced by Zn^{2+}. However, at the highest Zn^{2+} concentrations, a significant decrease of DNA damage was observed. A similar phenomenon was described following cadmium (Gichner *et al.* 2004) and lead (Gichner *et al*. 2008) treatments of tobacco plants. It was suggested that the formation of DNA-DNA and DNA-protein cross-links at high Pb^{2+} and Cd^{2+} concentrations inhibits DNA migration during electrophoresis. The same explanation may be provided for the data presented in Fig. 1 for Zn^{2+} treatments.

It can be concluded that Zn^{2+} induced DNA damage in nuclei of roots but not in leaves of tobacco seedlings with the exception of treatments associated with high toxic effects on the plants. Zn^{2+} also did not bring about somatic mutations in leaves.

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