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DNA damage measured by the Comet assay in eight agronomic plants

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Abstract

For most crops growing in polluted areas or treated with agricultural chemicals, no genotoxicity assays are available. We have studied the possibility of using the alkaline protocol of the plant-based molecular assay – the Single Cell Gel Electrophoresis (SCGE) assay (also called Comet assay) as a method for detecting induced DNA damage in 8 agronomic important plants (ordered according to the diameter of the nuclei): sugar beet, alfalfa, tobacco, lentil, maize, potato, hard wheat, and bread wheat. The monofunctional alkylating agent ethyl methanesulphonate (EMS) was applied as a model genotoxic agent on young excised leaves of the tested crops for 18 h at 26 °C in the dark. With increasing concentrations of 2 to 10 mM EMS, the DNA damage, expressed by the averaged median tail moment values, significantly increased in nuclei of all crops studied. No correlation between the diameter of nuclei and sensitivity to EMS treatment was observed. The data obtained demonstrate the feasibility of using the Comet assay for detecting induced DNA damage in crops.

Additional key words: *Beta vulgaris*, ethyl methanesulphonate, *Lens esculenta*, *Medicago sativa*, *Nicotiana tabacum*, single cell gel electrophoresis, *Solanum tuberosum*, *Triticum aestivum*, *Triticum durum*, *Zea mays*.

Introduction

For most plant genetic bioassays only specific tester lines can be used and these testers are at present not available for most crops. This limitation hampers or prevents the detection of the genotoxicity of environmental pollutants in crops growing on polluted soil or treated with agrochemical chemicals. To overcome this limitation we used a plant-based molecular assay – the Single Cell Gel Electrophoresis (SCGE) assay, also called the Comet assay, to detect induced DNA damage in 8 selected crops. With the exception of tobacco (*Nicotiana tabacum*), no genotoxicity assays are available at present (Gichner and Plewa 1998). The alkaline version of the 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).

The objectives of this study were: *1*) to define the parameters for SCGE analysis using nuclei isolated from leaves of the crops, *2*) to generate concentration-response curves for DNA migration values from crop leaves treated with the monofunctional alkylating agent ethyl methanesulphonate (EMS), and *3*) to measure the size of the diameter of the nuclei of the tested crops with the aim to find if a correlation exists between the nuclei size and its sensitivity to genotoxic treatment.

Material and methods

Plants: Sugar beet (*Beta vulgaris* L. cv. Elán), alfalfa (*Medicago sativa* L. cv. Jitka), tobacco (*Nicotiana tabacum* L. var *xanthi*), lentil (*Lens esculenta* Moench. cv. Lenka), maize (*Zea mays* L. cv. Dragon), potato (*Solanum tuberosum* L., cv. Korela), tetraploid hard wheat (*Triticum durum* Desf. cv. Soldur), and bread

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Abbreviations: EMS - ethyl methanesulphonate; TM - tail moment; SCGE - single cell gel electrophoresis.
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wheat (*Triticum aestivum* L. cv. Chinese Spring). The crops are ordered according to the diameter of the nuclei.

Chemicals: Ethyl methanesulphonate (EMS, CAS No. 62-50-0), reagents for electrophoresis, normal melting point (NMP) and low melting point (LMP) agarose, and general laboratory reagents were purchased from *Sigma Chemical Co.* (St. Louis, MO, USA).

Mutagenic treatment: For the experiments we used young leaves of crops cultivated in a greenhouse of the Institute of Experimental Botany, Prague, Czech Republic). The lower parts of leaves of the tested crops were immersed in 2 cm^3 plastic microtubes containing 1 cm^3 of 0 to 10 mM EMS dissolved in distilled water for 18 h at 26 °C in the dark.

Isolation of nuclei and preparation of slides: After control (H_2O) or EMS treatments, excised leaves were placed in a 60 mm Petri dish kept on ice and spread with 0.2 cm^3 of cold 400 mM Tris buffer, pH 7.5. Using a fresh razor blade, the part of the leaf not immersed in the treatment solution, was gently sliced. The plate was kept tilted in the ice so that the isolated nuclei would collect in the buffer. All operations were conducted under dim or yellow light.

Regular microscope slides were dipped into a solution of 1 % NMP agarose prepared with water at 50 °C, dried overnight at room temperature and kept dry in slide boxes until use. Onto each slide, nuclear suspension (0.05 cm^3) and 1 % 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 micropipet tip and a coverslip was placed on the mixture. The slide was placed on ice for a minimum of 5 min. Next, the coverslip was removed and a final layer of 0.5 % LMP agarose (0.1 cm^3) was placed on the slide. A coverslip was placed on the LMP agarose and the slide was kept at 4 °C for 5 min.

Comet assay: In order to analyze the EMS-induced DNA damage, the agarose slides with isolated nuclei from EMS-treated leaves were placed in a horizontal gel electrophoresis tank containing freshly prepared ice-cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH), pH >13). Depending on the crop tested the nuclei were incubated at pH >13 for 5 or 15 min to allow the DNA to unwind prior to electrophoresis at 0.72 V cm^{-1} (26 V; 300 mA) for 15 or 30 min at 4 °C. After electrophoresis the slides were rinsed 3 × with 400 mM Tris, pH 7.5, stained with 0.08 cm^3 ethidium bromide solution (20 μg cm^{-3}) for 5 min, dipped in ice cold water to remove the excess ethidium bromide and covered with a coverslip and analyzed using a fluorescence microscope with an excitation filter of 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. For each slide, 25 randomly chosen nuclei were analyzed. The tail moment (tail DNA density multiplied by the migration distance) was used as the primary measure of DNA damage. Three slides were evaluated per treatment and each treatment was repeated at least twice. From the repeated experiments, the average median tail moment (TM) value was calculated for each treatment group from the median TM value of each slide (Lovell *et al.* 1999).

Measurement of the diameter of the nuclei: Isolated nuclei embedded in agarose, without additional unwinding and electrophoresis, were stained with ethidium bromide as in the Comet assay. The diameter of the nuclei was measured using the Head Extent parameter of the software *Komet version 3.1*. Fifty nuclei were measured for each crop.

Statistical analysis: Data were analyzed using the statistical and graphical functions of *SigmaPlot 4.01* and *SigmaStat 2.03* (*SPSS, Inc.*, Chicago, IL, USA). The median TM values were used in a one-way analysis of variance test. If a significant F-value of $P < 0.05$ was obtained, a Dunnett's multiple comparison versus the control group analysis was conducted.

Results

Comet assay: *Beta vulgaris*: With shorter unwinding (5 min) and electrophoresis times (15 min), the DNA damage induced by EMS and expressed by the TM was comparatively low and reached after 10 mM EMS only 19.9 ± 1.4 μm. Thus the unwinding time was increased to 15 min and the electrophoresis to 30 min (Fig. 1*A*). These conditions led to a significant increase of the TM values from 3.0 ± 0.5 μm (negative control) to 51.9 ± 1.5 μm (10 mM EMS) ($F_{5,35} = 307.0$; $P < 0.001$).

Medicago sativa: After a 18 h treatment period with 2 to 10 mM EMS, nuclei were isolated from leaves. With 5 min unwinding and 15 min electrophoresis, the average median TM ± SE. significantly increased from 0.30 ± 0.1 to 46.7 ± 1.8 μm ($F_{5,35} = 145.1$; $P < 0.001$) (Fig. 1*B*).

Nicotiana tabacum: With unwinding (5 min) and electrophoresis time (15 min), the DNA damage induced by EMS and expressed by the TM value was low and reached after 10 mM EMS only 25.2 ± 0.4 μm (Fig. 2*A*). Electophoretic conditions (15 min unwinding, 30 min electrophoresis) resulted in a significant increase of the

TM value from 0.9 ± 0.1 μm to 68.9 ± 3.5 μm ($F_{5,33}$ = 163.3; $P < 0.001$).

Lens esculenta: Five min unwinding and 15 min electrophoresis resulted in a significant increase of the TM value from 0.9 ± 0.1 μm to 68.9 ± 3.5 μm (Fig. 2*B*) ($F_{5,33}$ = 163.3; $P < 0.001$).

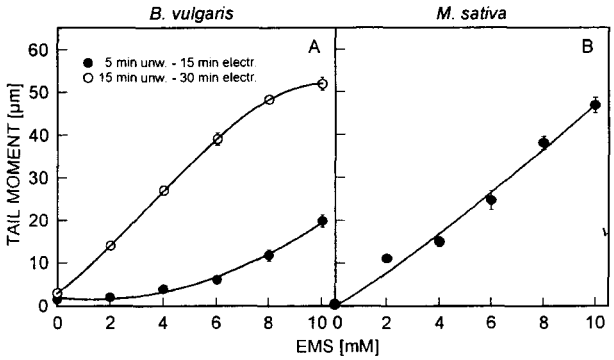

Fig. 1. Dose-response curves of the average medial tail moment values as a function of ethyl methanesulphonate (EMS) treatment of leaves of *Beta vulgaris* (*A*) and *Medicago sativa* (*B*) for 18 h at 26 °C in the dark. The *error bars* represent ± SE of the mean.

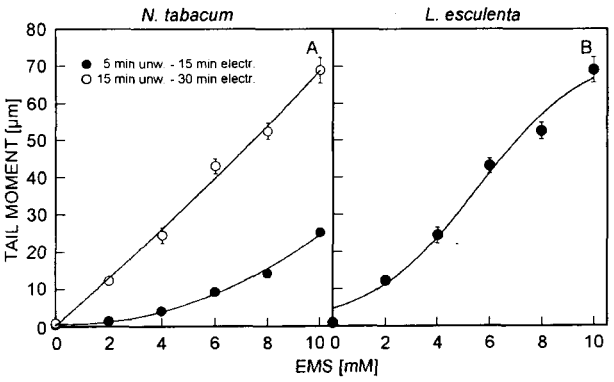

Fig. 2. Dose-response curves for *Nicotiana tabacum* (*A*) and *Lens esculenta* (*B*) (see Fig. 1 for details).

Zea mays: Five min unwinding and 15 min electrophoresis was sufficient to demonstrate a significant increase of EMS-induced DNA damage in nuclei of *Z. mays* (Fig. 3*A*). The TM values increased from 1.2 ± 0.3 μm to 37.0 ± 1.9 μm ($F_{5,35}$ = 55.9; $P < 0.001$). Prolonged 15 min unwinding and 30 min electrophoresis resulted in heavily damaged nuclei.

Solanum tuberosum: With 5 min unwinding and 15 min electrophoresis time, the DNA damage induced by EMS and expressed by the TM was low and reached after 10 mM EMS only 28.1 ± 1.6 μm. (Fig. 3*B*). Electrophoretic conditions extended to 15 min uwinding and 30 min electrophoresis resulted in a significant increase of the TM value from 4.0 ± 0.2 to 75.9 ± 2.9 μm ($F_{5,35}$ = 274.3; $P < 0.001$).

Triticum durum: Five min unwinding and 15 min electrophoresis of the slides with EMS treated nuclei of *T. durum* resulted in a significant increase of the TM values from 0.8 ± 0.07 μm to 66.9 ± 2.3 μm ($F_{5,34}$ = 303; $P < 0.001$) (Fig. 4*A*).

Triticum aestivum: Electrophoretic conditions of 5 min unwinding and 15 min electrophoresis were sufficient to demonstrate a significant increase of EMS-induced DNA damage in nuclei of *T. aestivum*. The TM values increased from 0.8 ± 0.05 μm to 67.6 ± 3.2 μm ($F_{5,31}$ = 214; $P < 0.001$) (Fig. 4*B*).

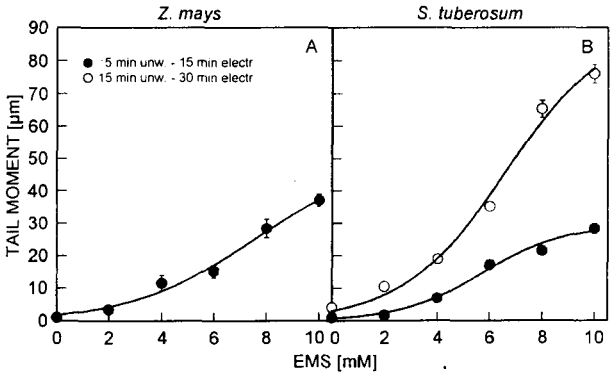

Fig. 3. Dose-response curves for *Zea mays* (*A*) and *Solanum tuberosum* (*B*) (see Fig. 1 for details).

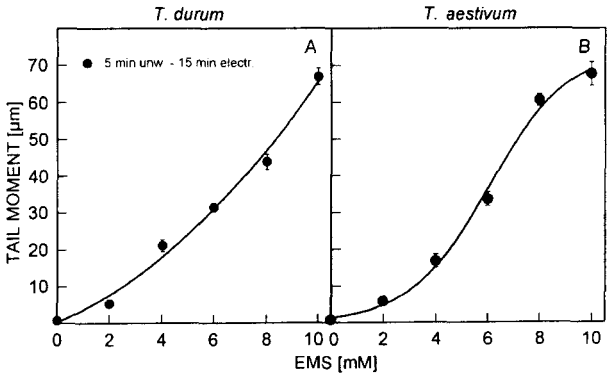

Fig. 4. Dose-response curves for *Triticum durum* (*A*) and *Triticum aestivum* (*B*) (see Fig. 1 for details).

Measurement of the diameter of the nuclei: The diameter of nuclei isolated from the 8 selected crops (Table 1) demonstrate great differences in the size of the nuclei, for example in sugar beet (11.4 ± 0.2 μm) it was half of that in bread wheat (24.0 ± 0.5 μm). We have compared the correlation between the nuclei diameter and the DNA damage induced by 10 mM EMS in nuclei of leaves isolated from the studied crops. The TM values presented were obtained after 5 min unwinding and 15 min electrophoresis, as these Comet assay conditions were applied for all crops. The Pearson product moment correlation (r) was only = 0.64. This analysis indicated a very low correspondence between the diameter of the nuclei and their sensitivity to EMS.

The results given in this paper demonstrate that the

Table 1. Diameter of isolated nuclei (means ± SE) harboured in agarose on microscopical slides, and DNA damage as expressed by the average median tail moment value ± SE (TM) in nuclei isolated from crop leaves treated for 18 h with 10 mM EMS, and followed by 5 min unwinding and 15 min electrophoresis in alkaline buffer (pH >13). Ordered according to the diameter of the nuclei.

	Nuclei [μm]	TM [μm]		Nuclei [μm]	TM [μm]
Beta vulgaris	11.4 ± 0.2	19.9 ± 1.4	*Zea mays*	14.3 ± 0.2	37.0 ± 1.9
Medicago sativa	12.8 ± 0.2	46.7 ± 1.8	*Solanum tuberosum*	15.3 ± 0.2	28.1 ± 1.6
Nicotiana tabacum	13.9 ± 0.2	25.2 ± 0.4	*Triticum durum*	18.2 ± 0.4	66.9 ± 2.3
Lens esculenta	14.2 ± 0.3	68.9 ± 3.5	*Triticum aestivum*	24.0 ± 0.5	67.6 ± 3.2

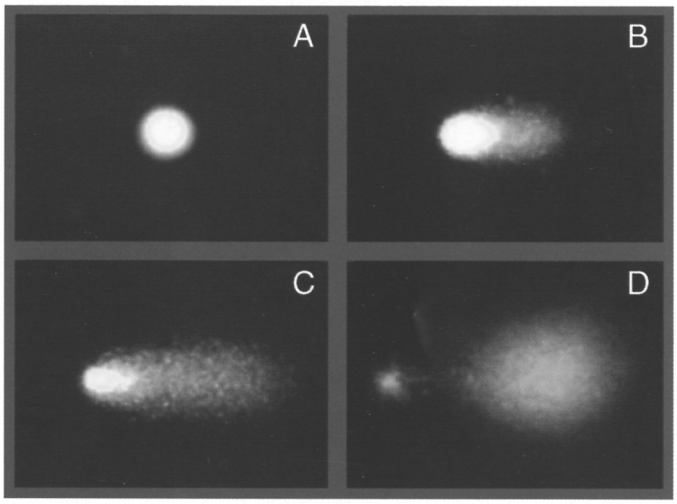

Fig. 5. Images illustrating the induction of DNA damage as expressed in the Comet assay. Control nucleus (*A*) and nuclei with different levels of DNA damage (*B. C. D*).

DNA damage after the lowest applied concentration of EMS (2 mM) was significantly higher ($P < 0.05$) than the DNA damage of the negative control in all the crops tested

DNA damage induced by γ-rays, measured by the Comet assay, is readily repaired within 24 h (Ptáček *et al.* 2001). Thus the Comet assay is not suitable for monitoring the late effects of acute ionizing radiations. The data regarding DNA damage in leaf nuclei of tobacco seedlings treated by alkylating agents were strikingly different (Gichner *et al.* 1999, 2000). Using the Comet assay, DNA damage induced by EMS persisted over 72 h after treatment without significant reduction. Even after 4 weeks the amount of DNA damage in nuclei isolated from mature leaves were significantly higher than compared to the controls. These data indicate that the Comet assay may detect DNA damage inflicted by some types of chemical genotoxic agents long periods after the initial exposure.

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