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DNA damage in potato plants induced by cadmium, ethyl methanesulphonate and γ-rays

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Abstract

We have calibrated the alkaline protocol of the plant comet (Single Cell Gel Electrophoresis) assay as a method for detecting the extent of induced DNA damage in potato plants (*Solanum tuberosum* L. cultivar Korela). After 2 and 24 h treatments of the rooted cuttings with the heavy metal cadmium (Cd^{2+}), a dose–response increase in DNA damage was noted versus controls in root nuclei. With a 24 h recovery period, the Cd^{2+}-induced DNA damage in roots increased significantly. No significant increase in DNA damage was demonstrated in leaf nuclei after 24 h Cd^{2+} treatments, but continuous Cd^{2+} treatments for 2 weeks resulted in an increase in leaf DNA damage. This increase may be however associated with necrotic and apoptotic DNA fragmentation, as the affected plants had inhibited growth and distorted yellowish leaves. For comparison, the monofunctional alkylating agent ethyl methanesulphonate, and γ-rays were assessed for induced DNA damage. Analysis of the accumulation of cadmium by inductively coupled plasma optical emission spectrometry demonstrates that roots accumulate almost 9-fold more cadmium than aboveground parts of the rooted potato cuttings. This may explain the absence of Cd^{2+} genotoxicity in leaves after short-term treatments.

Keywords: Comet assay; *Solanum tuberosum* L.

1. Introduction

Potato plants are exposed to various types of environmental agents, either accidentally by compounds present in polluted air, soil or water, or deliberately as in the case of agricultural pesticides and plant growth regulators. For potato plants growing in polluted areas or treated with agricultural chemicals, no genotoxicity assays are available.

The alkaline version of the comet or Single Cell Gel Electrophoresis 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 cross-links (for reviews see Tice et al., 2000; Olive, 2002; Collins, 2004). Although this technique has been primarily applied to animal cells, the incorporation of the comet assay with plant tissues (Koppen and Verschaeve, 1996; Gichner and Plewa, 1998; Menke et al., 2001) significantly extends the utility of plants in basic and applied studies in environmental mutagenesis.

We have calibrated the comet assay procedure (i.e., DNA unwinding and electrophoresis times) for evaluating DNA damage in potato plants and applied the comet assay to rooted potato cuttings treated with (1) the heavy metal cadmium Cd^{2+}; (2) the monofunctional alkylating agent ethyl methanesulphonate; and (3) γ-irradiation. In addition, the accumulation of cadmium in potato roots and aboveground biomass after Cd^{2+} treatments was quantified using inductively coupled plasma optical emission spectrometry.

2. Materials and methods

2.1. Chemicals and media

Cadmium chloride hemi(pentahydrate) (Cd^{2+}, CAS No. 7790-78-5), ethyl methanesulphonate (EMS, CAS No. 62-50-0), the plant growth medium (Phytagel, MS salts), reagents for

Abbreviations: Cd^{2+}, cadmium chloride; EMS, ethyl methanesulphonate

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electrophoresis, and general laboratory reagents were purchased from Sigma Chemical Co., St. Louis, MO. Normal and low melting point agarose were purchased from Roth, Karlsruhe, Germany.

2.2. Plant material

Rooted cuttings of potato (*Solanum tuberosum* L. cultivar Korela) were cultivated and propagated in sterile Magenta containers with a cultivation medium (Opatrná et al., 1997) at 22–26 °C with a 16 h photoperiod each day in a growth room. For the experiments, 5–6 weeks old rooted cuttings were used.

2.3. Cd^{2+} and EMS treatments

For chemical treatments, the potato cuttings were carefully taken out of the Magenta containers, the roots were rinsed in water and immersed in plastic vials containing 22 ml of a defined concentration of Cd^{2+} or EMS dissolved in distilled water. The plants were treated in the dark at 26 °C for 2 and 24 h. For continuous Cd^{2+} treatment schedule of 1 and 2 weeks, the rooted cuttings were grown in Cd^{2+} dissolved in a 50% Hoagland's solution at 22–26 °C with a 16 h photoperiod. For recovery studies, the Cd^{2+} or EMS-treated plants were kept in vials with distilled water for another 24 h at 22–26 °C. For control treatments, the plants were kept for 2 and 24 h in distilled water, or for 1 and 2 weeks in a 50% Hoagland's solution.

2.4. γ-Irradiation

The γ-ray treatment was conducted using a γ-ray device employing a ^{60}Co, Gamma-Cell 220 (Atomic Energy of Canada, Ltd.). The activity of the γ-source was 0.32 Gy min^{-1} (September 2001). The γ-irradiation of plants was done in May 2002 at the Faculty of Nuclear Sciences and Physical Engineering, CTU, Prague. Rooted potato cuttings in Magenta containers with the cultivation medium were irradiated by γ-rays at 20–22 °C. After irradiation, nuclei from the leaves of the irradiated plants were isolated (see below) and the comet assay performed. For recovery studies, the irradiated plants were kept in the Magenta containers for 24 h at 26 °C in the dark.

2.5. Comet assay

After treating roots of cuttings with Cd^{2+} and EMS, or irradiating with γ-rays, excised leaves or roots of the treated plants were placed in a 60 mm petri dish kept on ice and spread with 250 μl of cold 400 mM Tris buffer, pH 7.5. Using a fresh razor blade, the roots or leaves were gently sliced, and the isolated root nuclei collected in the buffer. All operations were conducted under dim or yellow light. The preparation of microscope slides with isolated nuclei was previously described (Gichner, 2003; Gichner et al., 2004). The slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH > 13). If not otherwise stated, the nuclei were incubated for 10 min to allow the DNA to unwind prior to electrophoresis at 0.74 V cm^{-1} (26 V, 300 mA) for 15 min at 4–8 °C. After electrophoresis the slides were rinsed 3 times with 400 mM Tris buffer, pH 7.5, air-dried at room temperature, and stored in boxes.

Air-dried slides were immersed for 5 min in cold water and then stained with 100 μl ethidium bromide (20 μg ml^{-1}) for 5 min, dipped in ice-cold water to remove the excess stain and covered with a coverslip. Mostly 2 × 25 randomly chosen nuclei were analyzed *per* slide using a fluorescence microscope with an excitation filter of BP 536–556 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 percentage of DNA in the tail (% tail DNA) was used as the primary measure of DNA damage (Collins, 2004). Two to three slides were evaluated per treatment and each treatment was repeated at least twice. The averaged median % tail DNA was calculated for each treatment group from the median % tail DNA values of each slide. The % tail DNA values are shown as averaged median means ± S.E. At least 150 nuclei were evaluated for each treatment group.

2.6. Determination of the content of Cd

Rooted potato cuttings were treated for 24 h at 26 °C in the dark with 0–60 μM Cd^{2+}. For each treatment two cuttings were used and each treatment was repeated 3 times. After treatments, the roots were rinsed and the cuttings air-dried. For the determination of the accumulation of Cd, aliquots (1 g) of the dried and powdered roots or aboveground biomass were decomposed in 50 ml quartz-glass beakers at 500 °C for 16 h on a hot plate and in muffle furnace with a stepwise increase of the ashing temperature (Mader et al., 1998). The ash was then dissolved in 20 ml of 1.5% solution of HNO_3. The content of cadmium in the plant digests was determined by inductively coupled plasma optical emission spectrometry with axial plasma configuration (ICP-AES – Varian VistaPro, Australia), equipped by autosampler SPS-5, at spectral line λ = 226.5 nm. Aliquots of the certified reference materials CRM CTA-OTL-1 were mineralized under the same conditions for quality assurance of the analytical data. In this reference material containing Cd = 1.12 mg kg^{-1} dry mass, a total amount of Cd = 1.53 mg kg^{-1} dry mass was detected.

2.7. Statistics

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

3. Results

3.1. Optimization of the comet assay for potato plants

Optimization of the comet assay refers to the most appropriate time for DNA unwinding and electrophoresis. It needs to ensure

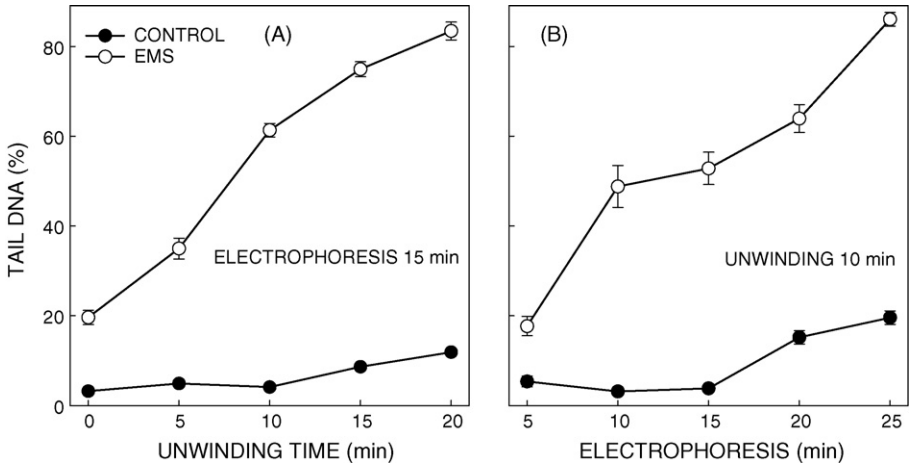

Fig. 1. The average median % tailDNA in leaf nuclei after 24 h ethyl methanesulphonate (6 mM) treatment of rooted potato cuttings as a function of DNA unwinding (A) or electrophoresis (B) time. The error bars represent the standard error of the mean.

the minimal DNA migration in control samples, and at the same time maximum sensitivity of treated samples.

3.1.1. Effect of DNA unwinding time

Treatment at pH >13 before electrophoresis unwinds the double stranded DNA and converts it to single-stranded DNA. The effect of unwinding time (0–20 min) on % tail DNA values was investigated with nuclei isolated from leaves of potato cuttings treated with 6 mM EMS for 24 h and from control plants (Fig. 1A). The electrophoresis time was kept constant at 15 min. A significant increase ($P < 0.05$) in % tail DNA was observed in the EMS-treated plants as a function of increased unwinding at 5 min ($35.0 \pm 2.3\%$) and above compared to the absence of unwinding ($19.6 \pm 1.6\%$). After 20 min unwinding the % tailDNA increased to $83.4 \pm 2.0\%$. In nuclei from control plants, a significant ($P < 0.05$) increase in the % tailDNA values was observed with unwinding times of 15 min ($8.6 \pm 0.5\%$) and above compared to the % tailDNA after 10 min unwinding ($4.1 \pm 0.3\%$). Since, in the control set longer unwinding times induced significant increase in the % tailDNA values, we chose a DNA unwinding time of 10 min for the remaining of the experiments.

3.1.2. Effect of electrophoresis time

We conducted experiments to determine the optimum electrophoresis time that would generate the highest resolution for EMS dose–response curves. The effect of electrophoresis time (0–25 min) on % tailDNA values was investigated with nuclei isolated from leaves of potato plants treated with 6 mM EMS for 24 h and from control plants (Fig. 1B). The unwinding time 10 min was kept constant. In the EMS-treated plants, the % tailDNA increased significantly ($P < 0.05$) from $17.7 \pm 2.1\%$ at 5 min electrophoresis to $86.0 \pm 1.5\%$ at 25 min of electrophoresis. In nuclei from control plants, the % tailDNA significantly increased after 20 min electrophoresis to a value of $15.2 \pm 1.5\%$ compared to $3.8 \pm 0.4\%$ with a 15 min of electrophoresis. Thus, for the remainder of the experiments we have applied a 10 min unwinding and a 15 min electrophoresis time.

3.2. Effect of a 24 h treatment with Cd^{2+} and EMS

Rooted potato cuttings were treated for 24 h with 10–50 μM Cd^{2+}. The nuclei were isolated from leaves and roots and the comet assay was performed (Fig. 2). With increasing concentration of Cd^{2+} the % tailDNA in root nuclei significantly ($P < 0.05$) increased from $10.4 \pm 0.4\%$ (control) to $60.3 \pm 3.3\%$ after a 30 μM Cd^{2+} treatment, followed by a decrease to $52.9 \pm 5.7\%$ with 40 μM Cd^{2+} treatments. However, this decrease is not significant ($P = 0.285$). After treatments with 50 μM Cd^{2+} the number of root nuclei that were suitable for analysis was limited (Fig. 2A). By contrast, analysis of DNA damage in nuclei isolated from the leaves of rooted potato cuttings treated with 10–50 μM Cd^{2+} for a period of 24 h did not result in a significant increase ($P = 0.981$) in the % tailDNA.

Fig. 2B illustrates the DNA-damaging effect of EMS applied for 24 h to rooted potato cuttings. In root nuclei the % tailDNA significantly ($P < 0.05$) increased from $8.1 \pm 0.84\%$ (control) to $71.5 \pm 3.0\%$ with 6 mM EMS. After treatment at higher doses of EMS, the number of nuclei that were suitable for analysis was limited. In nuclei isolated from the leaves of rooted potato cuttings subjected to the same EMS treatment, the % tailDNA significantly ($P < 0.05$) increased from $5.0 \pm 0.34\%$ (control) to $61.8 \pm 4.9\%$ after 8 mM EMS treatment. The DNA damage in roots is mostly higher than that in leaves; in roots 6 mM EMS induced significantly ($P < 0.05$) higher values of % tailDNA ($71.5 \pm 3.0\%$) compared to that induced in the leaves ($50.6 \pm 3.1\%$).

3.3. Effect of a 2 h treatment of roots with Cd^{2+} and EMS followed by a 24 h recovery period

Roots of potato cuttings were treated with 0.4–2 mM of Cd^{2+} for 2 h and nuclei were isolated from the roots after the termination of treatment (Fig. 3A). The % tailDNA values of root nuclei increased significantly ($P < 0.05$) from $10.3 \pm 0.9\%$ (control) to $69.2 \pm 2.5\%$ (2 mM Cd^{2+}). When rooted cuttings were allowed to recover for 24 h in water, the DNA damage in root nuclei

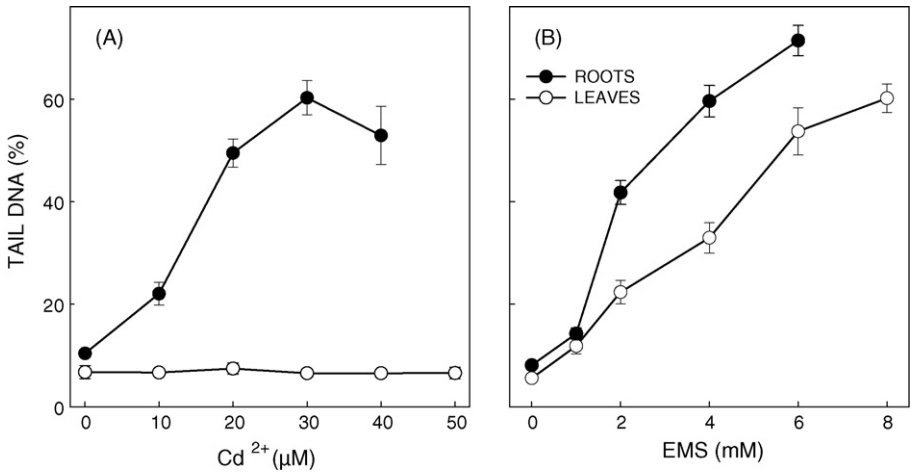

Fig. 2. The average median % tailDNA in nuclei of potato roots and leaves after 24 h cadmium chloride (A) or ethyl methanesulphonate (B) treatment. The error bars represent the standard error of the mean.

increased. For example, with 0.8 mM Cd^{2+} the % tailDNA values increased from 28.5 ± 3.4% (no recovery) to 71.9 ± 1.9% (24 h recovery period).

Similar data were obtained following EMS treatment (Fig. 3B). Rooted potato cuttings were treated for 2 h with 10–50 mM EMS and the nuclei were isolated from roots either immediately after the EMS treatment or after a 24 h recovery period. With increasing concentration of EMS the % tailDNA increased significantly ($P < 0.05$) from 9.2 ± 0.7% (control) to 68.7 ± 2.2% (50 mM EMS). After a 24 h recovery of the EMS-treated cuttings in distilled water, the level of induced DNA damage increased further. The value of % tailDNA after 30 mM EMS treatment at 0 h recovery period was 46.8 ± 1.9% and after a 24 h recovery period was 88.4 ± 0.6%.

3.4. Continuous Cd^{2+} treatments

Rooted potato cuttings were treated for 1 or 2 weeks with 2.5–12.5 μM Cd^{2+} dissolved in a 50% Hoagland's solution and the comet assay was performed on the nuclei isolated from the leaves. As demonstrated in Fig. 4, after 1 week of continuous treatment, a slight but significant ($P < 0.05$) increase in the % tailDNA was observed at 7.5 μM Cd^{2+} and above. However, these treatments were associated with damaged leaves and a strong inhibition of root growth. Similarly, a 2-week continuous treatment with concentrations from 5 to 12.5 μM Cd^{2+} resulted in an increase of % tailDNA from 18.0 ± 2.1% to 49.4 ± 3.5%, compared to the control of 7.3 ± 0.6%. In all cases the increase of % tailDNA was associated with strongly distorted and yellowish leaves and with an inhibition of root growth.

3.5. γ-Irradiation

Rooted potato cuttings in plastic Magenta containers were exposed to 5–30 Gy and nuclei for the comet assay analysis were isolated from the leaves immediately or after a 24 h recovery period (Fig. 5). With no recovery time, a significant ($P < 0.05$) dose-dependent increase in the % tailDNA values from 4.1 ± 0.3% (control) to 67.4 ± 2.4% (30 Gy) was observed. After a 24 h recovery period, the % tailDNA values in the leaf nuclei of the irradiated plants significantly ($P < 0.05$) decreased, e.g., from the value of 67.4 ± 2.4% (30 Gy) to 10.7 ± 1.2%.

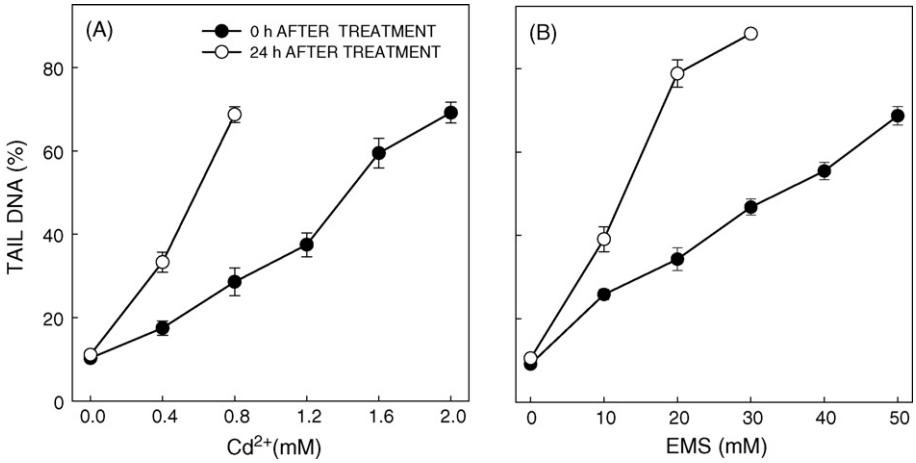

Fig. 3. The average median % tailDNA in nuclei of potato roots after 2 h cadmium chloride (A) or ethyl methanesulphonate treatment (B) followed by a 24 h recovery period. The error bars represent the standard error of the mean.

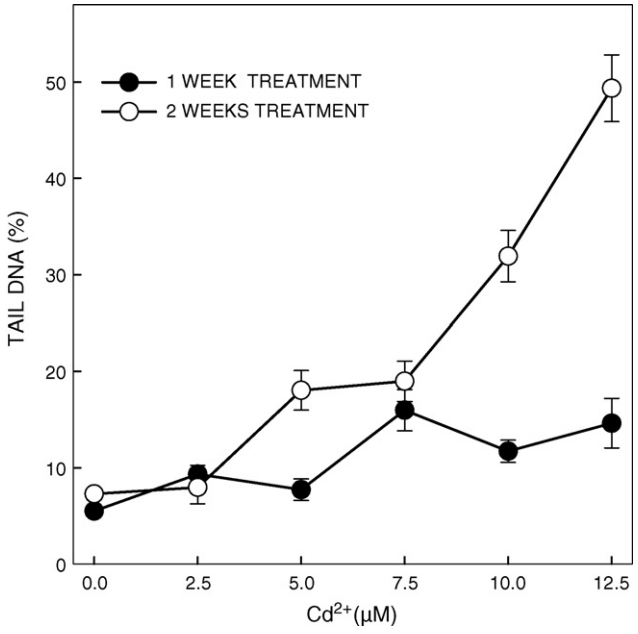

Fig. 4. The average median % tailDNA of leaf nuclei after 1 and 2 weeks treatment of rooted potato cuttings with cadmium chloride. The error bars represent the standard error of the mean.

3.6. Accumulation of total Cd in roots and aboveground biomass

Treatment of rooted potato cuttings with 60 μM Cd^{2+} for 24 h resulted in about a 170-fold increase in the total Cd content in the aboveground biomass (67.7 ± 15.3) compared to the control (0.4 ± 0.1 mg kg^{-1} dry mass) (Table 1). By contrast, after the same treatment, the roots accumulated almost 9-fold more cadmium than the aboveground biomass and showed nearly a 400-fold increase in Cd contents (594 ± 87.3) compared to the control (1.5 ± 1.2 mg kg^{-1} dry mass).

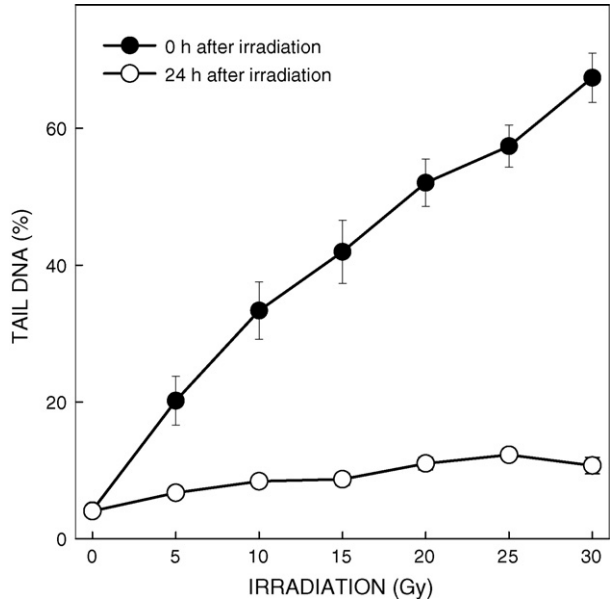

Fig. 5. The average median % tailDNA in leaf nuclei as a function of γ-irradiation of rooted potato cuttings evaluated immediately after irradiation or after a 24 h recovery period. The error bars represent the standard error of the mean.

Table 1
Comparison of total cadmium accumulation in aboveground biomass and roots of potatoes (*Solanum tuberosum* L. cultivar Korela) after 24 h treatment of rooted cuttings with Cd^{2+}

Cd^{2+} (μM) 24 h treatment	Cd in aboveground biomass (mg kg^{-1} dry mass)	Cd in roots (mg kg^{-1} dry mass)
0	0.4 ± 0.1	1.5 ± 1.2
20	23.9 ± 9.1	206 ± 35.3
40	52.0 ± 5.2	431 ± 80.5
60	67.7 ± 15.3	594 ± 87.3

The results represent means of three repetitions ± S.E.

4. Discussion

The comet assay protocols for plants and animals differ due to the presence of the plant cell wall. Plant nuclei cannot be isolated like nuclei from human and animal cells by standard lysing, but they are isolated mechanically by slicing the plant tissue with a razor blade (see Section 2.5); the lysing step can be avoided.

The use of the comet assay for potato plants required a calibration of the unwinding and electrophoresis conditions, as these conditions differ for each plant species. In potato plants, we have determined that, to obtain low DNA migration in the control treatments and a dose–response increase in DNA migration after treatment with the model alkylating agent EMS, the best conditions were a 10 min unwinding time followed by a 15 min of electrophoresis at 0.74 V cm^{-1} and 300 mA (Fig. 1). Similar studies showed that for performing the comet assay in tobacco (*Nicotiana tabacum* L.) plants, a 15 min unwinding and a 25 min electrophoresis provides the best results (Gichner et al., 2006).

Three genotoxic agents were applied in this study: (1) cadmium chloride; (2) ethyl methanesulphonate; and (3) γ-rays.

(1) Cadmium is a widespread heavy metal, released into the environment from power stations, heating systems, waste incinerators, metal-working industries and from many other sources. Accumulation of cadmium in soil can become dangerous to all kinds of organisms, including plants. Even though the toxic effects of cadmium compounds in plants have been studied over many years, inconsistent results have been obtained about their genotoxic properties (Koppen and Verschaeve, 1996; Steinkellner et al., 1998; Sanità di Toppi and Gabbrielli, 1999; Panda and Panda, 2002). The possible pathway(s) of cadmium induced genotoxicity are still unknown, but may involve the interaction of this metal with DNA, either directly or indirectly *via* the induction of oxidative stress (Valverde et al., 2001). Cadmium chloride induced DNA damage as evaluated by the comet assay in broad bean (*Vicia faba* L.) and tobacco roots, but not in tobacco leaves after 2–72 h treatments (Koppen and Verschaeve, 1996; Gichner et al., 2004).
(2) The monofunctional alkylating agent ethyl methanesulphonate (EMS) generates alkylated DNA bases that may

be sites of DNA excision repair. Incomplete excision repair sites are a source of DNA strand breaks that are detected in the comet assay. Alkylated bases are also substrates for DNA glycosylases which lead to apurinic or apyrimidinic sites (AP sites). Under the conditions of the alkaline comet assay, these AP sites are converted into single-strand DNA breaks via β-elimination (Friedberg et al., 1995). EMS is known to induce DNA damage in plants measurable by the comet assay (Gichner and Plewa, 1998; Koppen and Verschaeve, 1996).

(3) γ-Rays induce DNA damage directly (as a result of deposition of energy in cells) or indirectly (as a result of free radical formations and oxidative damage). The main lesions produced by the physico-chemical interaction between ionizing radiation and DNA are single and double strand breaks, DNA–DNA and DNA–protein cross-links, alkali labile sites and damage to purine and pyrimidine bases (Téoule, 1987; Cadet et al., 1997). All these types of DNA damage can be detected by the alkaline comet assay (Collins, 2004; Olive, 2002; Tice et al., 2000).

Fig. 2A illustrates that a 24 h Cd^{2+} treatment induced a very high level of DNA damage in potato roots, whereas in the leaves the level of DNA damage was on the level of the control till the sub-lethal doses. Analytical studies by inductively coupled plasma optical emission spectrometry demonstrate (Table 1) that after 24 h treatment of rooted potato cuttings with 60 μM Cd^{2+}, the accumulation of the heavy metal is 9-fold higher in the roots than in the leaves. This lower accumulation may explain the absence of Cd^{2+} genotoxicity in leaves. The role of thiol-rich phytochelatins in binding Cd and accumulating it within vacuolar compartments of root cells is well known (Sanità di Toppi and Gabbrielli, 1999) and perhaps responsible for the high Cd content in roots. Cd^{2+} generates various reactive oxygen species (ROS) in plant cells, including H_2O_2 (Olmos et al., 2003). Compared to roots, leaf cells are better equipped with antioxidant defense system that might protect the nuclear DNA in leaf cells from Cd-induced oxidative stress. In previous studies (Gichner et al., 2004) it was demonstrated that the activity of catalases, the principal H_2O_2 scavenging enzymes, is about 30 times higher in tobacco leaves than in roots. Consequently, the high content of catalases, and other enzymes inactivating ROS in leaves, prevents the reaction of ROS with leaf nuclear DNA.

By contrast, the direct acting monofunctional alkylating agent EMS induced a dose–response increase in DNA damage both in potato leaves and roots (Fig. 2B).

Data presented in Fig. 3A and B demonstrate that Cd^{2+}- and EMS-induced DNA damage in roots increases after a 24 h recovery period in water. By contrast, as demonstrated in Fig. 5, most of the γ-ray-induced DNA damage is repaired within 24 h. The data indicate that the standard alkaline protocol of the comet assay may not be suitable for biomonitoring of increased levels of acute radiations, as the induced DNA damage is rapidly repaired. By contrast, the comet assay may be more suitable for monitoring genotoxic effects of environmental chemical pollutants, where the induced DNA damage may persists for a longer period.

Continuous exposures for 1 and 2 weeks to Cd^{2+} resulted in an increase of DNA damage in leaves of rooted potato cuttings (Fig. 4), but this increase was associated with toxic effects: leaves were distorted and yellowish, no new leaves were formed, and the growth of the roots was strongly inhibited. Many of the comet images from the distorted leaves were of the "hedgehog" type (large fan-like tail and small heads). This may be associated with necrotic or apoptotic DNA fragmentation (Gichner et al., 2006).

5. Conclusion

The alkaline version of the comet assay may be used for monitoring the DNA-damaging effects of environmental pollutants in potato plants.

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