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Sensitivity of *Allium* and *Nicotiana* in cellular and acellular comet assays to assess differential genotoxicity of direct and indirect acting mutagens

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ABSTRACT

We have evaluated the extent of DNA damage induced by direct and indirect mutagens by cellular and acellular comet assays in two plant systems, *Nicotiana tabacum* (wild type tobacco) and *Allium cepa* (common onion). The objectives of this study were: (1) to generate dose–response curves for DNA migration values from root and shoot nuclei of *A. cepa* and *N. tabacum* treated with the direct acting mutagens, ethyl methanesulphonate (EMS), hydrogen peroxide (H_2O_2) and the indirect acting mutagen, cadmium chloride ($CdCl_2$), (2) to assess the differential response between isolated nuclei and nuclei of root and shoot and of both plants and (3) to examine the differences of sensitivity between direct and indirect acting mutagens by cellular and acellular comet assays. Similar sensitivities were evident in both plant systems to direct and indirect acting mutagens. The combination of cellular and acellular comet assays provided valuable insight to the mode of action of the genotoxicants used. The data obtained demonstrated the estimable capacity of the two plant systems to evaluate genotoxicity under different stress conditions and suggests *Allium* is a more desirable test system for rapid monitoring of genotoxicity.

1. Introduction

Plants as biomonitors have a long history (Kovalchuk and Kovalchuk, 2008). For decades they are used as a part of ecological risk assessment of agricultural (Mishra and Srivastava, 2009) and industrial chemicals, solid wastes (Chakraborty et al., 2009) food additives (Giri and Roychoudhury, 1989), and chemically and radioactively polluted soil (Gichner et al., 2007; Saghirzadeh et al., 2008) and water (Matsumoto et al., 2006; Leme and Marin-Morales, 2008). The comet assay or single-cell gel electrophoresis (SCGE) is one of the versatile methods for assessing DNA damage, with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair. SCGE attracts adherents by its simplicity, sensitivity, versatility, speed, and economy, and has been widely accepted as a reliable biomarker for DNA damage (Collins, 2004). 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 (Gichner et al., 2008).

DNA damage induced by genotoxic agents depends on their transport across cellular/nuclear membranes, activating and deactivating intracellular enzymatic processes, levels of radical scavengers and the repair competency of the target cell population. To determine the effect of these cellular processes on the amount of induced DNA damage, the acellular (sub-cellular, cell-free) comet assay was developed (Kasamatsu et al., 1996; Vasquez and Tice, 1997; Thomas et al., 1998; Tice et al., 2000; Szeto et al., 2002). Additionally, the response of intact cells and isolated nuclei can be evaluated, indicating the role of various cellular components in mutagenesis processes (Kasamatsu et al., 1996; Jovtchev et al., 2001; Gichner, 2003a,b; Juchimiuk et al., 2006).

Nicotiana tabaccum var *Xanthi* (tobacco) has been found to serve as an indicator of mutagenicity. With the exception of *Nicotiana* no genotoxicity assays were available (Gichner and Plewa, 1998) for a very long time. However the germination period (approximately 2 months) may appear disadvantageous in case of rapid monitoring.

Due to its sensitivity, the *Allium cepa* (common onion) test was the first of nine plant assay systems evaluated by the Gene-Tox Program of the US Environmental Protection Agency (Grant, 1994). It is now frequently used for environmental monitoring (Leme and Marin-Morales, 2009). *Allium* has several advantages with regard to its shorter germination time, availability and also minimum facility requirement.

In the work presented here, we have compared the DNA-damaging effect of three genotoxic agents in both leaves and roots of tobacco and onion as measured by the cellular and acellular comet assays: (1) ethyl

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methanesulphonate (EMS), a monofunctional alkylating agent, (2) hydrogen peroxide (H_2O_2), a direct acting genotoxic agent with DNA-damaging activity dependent on the level of cell catalase and (3) the widespread heavy metal cadmium (Cd^{2+}) applied in the form of cadmium chloride ($CdCl_2$). By comparing the data obtained in the cellular and acellular comet assays, the role of the cell wall and the cytosol on the level of DNA damage induced by the genotoxic agents tested can be determined.

Moreover, the data obtained could demonstrate the utility of the two plant systems used for their ability to evaluate genotoxicity under different stress conditions and may suggest which system is more appropriate for rapid monitoring of genotoxicity.

2. Materials and methods

2.1. Chemicals

Ethyl methanesulphonate (EMS: CAS No. 62-50-0), Cadmium chloride, hemi pentahydrate, (Cd^{2+}, CAS No. 7790-78-5) reagents for electrophoresis, normal and low melting point agarose (NMP and LMP) were purchased from Sigma Aldrich Chemical Co., India. Hydrogen peroxide (H_2O_2, CAS No. 7722-84) was purchased from Merck (India). The mutagens were dissolved in distilled water.

2.2. Plant material

(1) Equal sized bulbs were chosen from population of a local market variety of the common onion *Allium cepa* L. ($2n=16$). Before use the loose outer scales were removed and dry bases were scraped to expose the root primordial. The onions were positioned for germination directly on autoclaved sand taken in earthen pots—a method, which mimics natural condition (Panda et al., 1990). When the roots were 2–3 cm long they were considered ready for experiment (Mukherjee and Gichner, 2009), shoots (fleshy leaves) from the same bulb were obtained which were 2 cm in length.
(2) Tobacco seeds of commercial variety (*Nicotiana tabaccum* var *Xanthi*) were grown in pots with normal garden soil. Seedlings at 4–5-leaf stage were carefully removed and their roots were rinsed with water.

2.3. Mutagenic treatment

For chemical treatments, the roots of *Allium* and *Nicotiana* were rinsed in water and immersed in glass vials containing 22 ml of defined concentration of EMS (0, 2, 4, 6 and 8 mM), hydrogen peroxide (0, 1, 2, 3 and 4 mM, and 0, 0.2, 0.4, 0.6, 0.8, 0.1 and 1.2 mM in acellular system), and cadmium chloride (0, 0.4, 0.8, 1.2 and 1.6 mM) in distilled water. The plants were treated for 2 h in the dark at 26 °C. After treatment the roots were rinsed in water and kept in vials with 50% Hoagland's solution for another 24 h in dark at 26 °C for recovery. For control treatments, the plants were kept for 2 h and 24 h in 50% Hoagland's solution (Gichner and Plewa, 1998).

2.4. Cellular and acellular comet assay

After chemical treatment of the plants, excised roots/leaves of *Allium/Nicotiana* were placed for 2 min on ice to keep them turgid (Navarrete et al., 1997). For isolation of nuclei, root/leaf tissues, treated or untreated as appropriate (Chakraborty et al., 2009), were placed in a 60 mm Petri dish kept on ice and in cold 400 mM Tris buffer, pH 7.5. Using a fresh razor blade, the roots/leaves were gently sliced. The plate was kept tilted in the ice so that the isolated nuclei would collect in the buffer. 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 (150 μl) and 1% LMP agarose (150 μl) were added at 40 °C. The nuclei and the LMP agarose were gently mixed by repeated pipetting using a cut micropipette tip and a coverslip was placed on the mixture. The slide were placed on ice for a minimum of 5 min, the coverslip was then gently removed. 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). The nuclei were incubated for 15 min to allow the DNA to unwind prior to electrophoresis at 0.72 V cm^{-1} (26 V, 300 mA) for 20 min at 4 °C. After electrophoresis, the slides were rinsed three times with 400 mM Tris, pH 7.5, stained with 80 μl ethidium bromide (20 μg ml^{-1}) for 5 min, dipped in ice-cold water to remove the excess ethidium bromide and covered with a coverslip. All operations were performed under dim or yellow light. For each slide, 25 randomly chosen nuclei were 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 5.5, Kinetic Imaging Ltd., Andor Technology, Nottingham, UK) was employed. The percentage of tail DNA was used as the primary measure of DNA damage. Three slides were evaluated per treatment and each treatment was repeated twice. From the repeated experiments, the averaged median percentage of tail DNA were calculated for each treatment group from the median percentage of tail DNA of each slide (Lovell et al., 1999).

In the acellular comet assay, nuclei from untreated *Allium* and *Nicotiana* root and leaf cells were prepared as outlined above and the slides were immersed in solutions containing different concentrations of EMS (0–8 mM), hydrogen peroxide (0.2–1.2 mM) and $CdCl_2$ (0–1.6 mM) for 2 h at 26 °C in 400 mM Tris–HCl buffer (pH 7.5).

After the treatment period, the slides were rinsed 3 times for 5 min by immersion in cold distilled water, followed by unwinding and electrophoresis as described earlier.

2.5. Statistical analysis

For all statistical analyses, the level of significance was established at $\alpha=0.05$. Data were analyzed using the statistical functions of Sigma Stats.3 software (SPSS, Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) test was conducted.

3. Results

3.1. Concentration–response SCGE analysis of the direct acting mutagens, ethyl methanesulphonate and hydrogen peroxide

After 2 h treatment of tobacco with EMS, nuclei were isolated and a concentration–response analysis in the cellular comet assay was conducted (Fig. 1A). In roots of tobacco, with increased concentrations

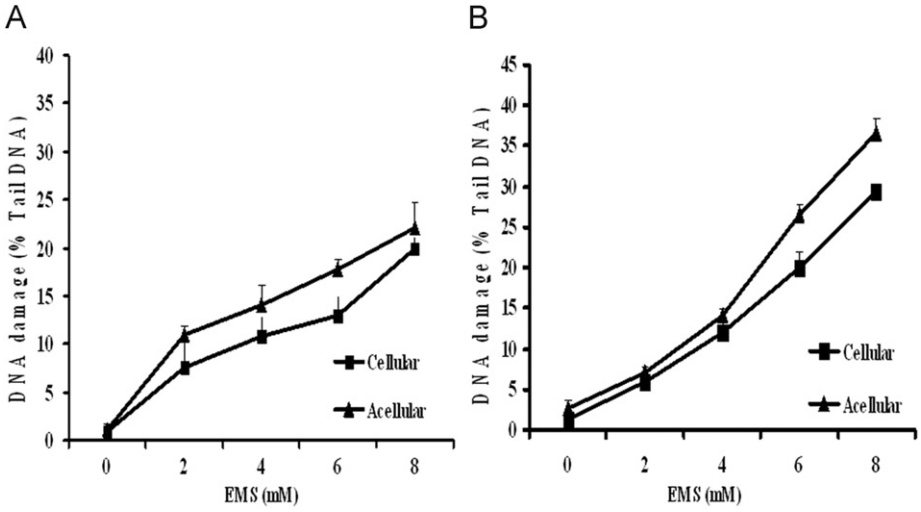

Fig. 1. Concentration-dependent DNA damage induced by EMS in roots of *Nicotiana* (A) and *Allium* (B) measured by cellular and acellular comet assays. The error bars represent the standard deviation.