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# Evaluation of DNA damage in the root cells of *Allium cepa* seeds growing in soil of high background radiation areas of Ramsar – Iran

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ABSTRACT

Plants are unique in their ability to serve as in situ monitors for environmental genotoxins. We have used the alkaline comet assay for detecting induced DNA damage in *Allium cepa* to estimate the impact of high levels of natural radiation in the soils of inhabited zones of Ramsar. The average specific activity of natural radionuclides measured in the soil samples for $^{226}$Ra was 12,766 Bq kg$^{-1}$ whereas in the control soils was in the range of 34–60 Bq kg$^{-1}$. A positive strong significant correlation of the DNA damage in nuclei of the root cells of *A. cepa* seeds germinated in the soil of high background radiation areas with $^{226}$Ra specific activity of the soil samples was observed. The results showed high genotoxicity of radioactively contaminated soils. Also the linear increase in the DNA damage indicates that activation of repair enzymes is not triggered by exposure to radiation in HBRA.



## 1. Introduction

The effects of low doses of ionizing radiation have been a matter of important debate over the last few years. In our natural environment, we are chronically exposed to low doses of ionizing radiation. In some situations, this environmental exposure can reach significant doses, such as in the case of some inhabited areas where the soil displays abnormally high amounts of radionuclides. The use of nuclear energy for civil (power plants) and military (nuclear testing and weapon) purposes led to large radiation exposures in some areas. Hiroshima and Nagasaki (August 1945) and Chernobyl (April 1986) are the most dramatic examples. Therefore, ecosystems require alarms to detect environmental hazards and ensure the safety of the inhabitants as well as the ecosystem. Plants may be used as biosensors of genetic toxicity of environmental pollutants (Grant, 1994; Grant and Salamone, 1994). Plant bioassays, which are most sensitive in detecting genotoxicity of environmental agents, can serve as the first alert for the presence of environmental hazards in water, air, and soil (Kovalchuk et al., 1998).

A number of areas with high background radiation are found throughout the world, such as in Brazil, China, India and Iran. Ramsar, a northern coastal city in Iran, has areas with some of the highest levels of natural radiation measured to date. The hot springs are the main sources for the distribution of natural radionuclides (especially for $^{226}$Ra and its decay products), in turn leading to the creation of high natural radiation areas (HNRA) in the region (Ghiassi-Nejad et al., 2003). $^{226}$Ra is a natural decay product of $^{238}$U and is present in the soil at concentrations of 10–100 Bq kg$^{-1}$ and its half-life is 1620 years. Due to the presence of these natural radionuclides, the inhabitants in some areas of Ramsar receive an annual effective dose that is up to 260 mSv/y, substantially higher than the 20 mSv/y that is permitted for radiation workers (Sohrabi, 1998; Ghiassi-Nejad et al., 2003). The mean effective dose resulting from $^{226}$Ra due to consumption of vegetables from this area is reported to be 12 times greater than the average effective dose resulting from this radionuclide due to foods and drinking water in normal area (Ghiassi-Nejad et al., 2003). Some of studies which have conducted by various molecular methods on HBRAs of Ramsar have shown that the spontaneous level of DNA damage in the lymphocytes of the inhabitants in these regions was significantly higher than control groups (Masoomi et al., 2006; Mohammadi et al., 2006).

Here, by means of the comet assay, we have conducted a preliminary study of plants grown in the soil of high background radiation areas (HBRAs) of Ramsar.

The alkaline version of single-cell gel electrophoresis (comet assay), is a powerful genetic assay to quantitatively measure damage to genomic DNA in individual eukaryotic cells, caused by genotoxic agents (Tice et al., 2000). This assay indicates lesions in nucleus DNA

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including single strand breaks, alkali–labile sites and other modifications of the molecules, during the exposure of cells to potent mutagens and ionizing radiation. Because of the simple procedures, high sensitivity, short response time and the requirement of relatively small number of cells and test substances, it has been a powerful tool for determination of genotoxicity (Tice et al., 2000). Higher plants, sensitive to soil, water and air pollutants, have been widely used for the biomonitoring of DNA damage induced by genotoxic agents. The comet assay in the nuclei of various higher plants has been applied before, such as in *Vicia faba* (Koppen and Verschaeve, 1996; Koppen and Angelis, 1998; Koppen et al., 1999), *Allium cepa* (Navarrete et al., 1997; Pincheira et al., 2003), *Nicotiana tabacum* (Koppen et al., 1999; Stavreva et al., 1998; Gichner et al., 2000; Ptacek et al., 2001; Stavreva and Gichner, 2002; Restivo et al., 2002), *Calamagrostis epigejos* (Ptacek et al., 2002), *Impatiens balsamina* (Poli et al., 2003), *Arabidopsis thaliana* (Menke et al., 2001) and barley (Jovtchev et al., 2001). We measured the DNA damage in nuclei of the root cells of *A. cepa* seeds germinated in the soil of high background radiation areas (HBRAs) of Ramsar. This is the first attempt to assess the genotoxicity of radioactively contaminated soils using the *A. cepa* comet assay. In addition, this is the first report on using this bioassay to analyze the genotoxicity in the soil of inhabited HBRA of Ramsar.

The objectives of this study were (1) to generate dose–response curves for DNA migration values from root nuclei of *A. cepa* treated with hydrogen peroxide ($H_2O_2$) and gamma-radiation, (2) to assess the DNA damage in nuclei of the root cells of *A. cepa* seeds germinated in the soil of HBRA in Ramsar, and (3) to examine the differences of radio-sensitivity between the root cells of *A. cepa* seeds germinated in the soils with different activities.

## 2. Materials and methods

### 2.1. Sampling and analyses of natural radionuclides in soils

Soils were sampled from inhabited zones of control and HBRA in Ramsar (Fig. 1) in 2006. About 3 kg of soil was collected from each location. Large stones and other objects were removed. All the samples were air dried, powdered and then sieved through a 1 mm mesh and dried at 105 °C. Samples of about 300 g. were prepared and placed in cylindrical gas-tight containers with the same geometry as the sample container used for efficiency calibration. They were kept for at least 3 weeks in order to reach secular equilibrium between $^{226}$Ra and $^{214}$Bi. Soil samples were assayed for their content of the specific activity of natural radionuclides including $^{226}$Ra, $^{232}$Th and $^{40}$K using the gamma-ray spectrometry system with high purity germanium (HPGe) detector with an energy resolution of 2.0 keV at an energy of 1332 keV, and relative efficiency of 20% in National Radiation Protection Department, Iranian Nuclear Regulatory Authority, Tehran, Iran. The counting time for each sample was 60,000 s except for higher radioactivity samples for which shorter counting times were applied (Ghiassi-Nejad et al., 2003).

### 2.2. Plants

*A. cepa* seeds (Texas Early Grano 502 PRR Holland) were placed on control and contaminated soils in germination boxes under the same conditions inside a greenhouse at Tarbiat Modares University, Tehran, Iran and allowed to germinate in the dark at 25 °C. The roots were harvested after 5 days post-sowing when they were at least 1.5–2.0 cm long. During germination and growth, the plants were irrigated with tap water.

### 2.3. Chemicals

Normal melting point (NMP), low melting point (LMP) agarose and ethidium bromide (EtBr) were obtained from Boehringer Mannheim. All other reagents were purchased from Merck.

### 2.4. $H_2O_2$ treatment and gamma-irradiation

In order to set up the comet assay for plant cells, two independent treatments ($H_2O_2$ and gamma-irradiation) were applied for the germinated seeds in control soil. Roots were immersed in 2 ml plastic microtubes containing 1.5 ml of 0–75 mM $H_2O_2$ dissolved in PBS for 2 h at 25 °C in the dark. Gamma-irradiation of excised *A. cepa* roots was carried out using a $^{60}$Co source of 1858.779 Ci activity and 0.4113501 Gy s$^{-1}$ dose rate (Gamma cell 220, Atomic Energy Canada, Ltd.).

### 2.5. Comet assay

After $H_2O$- (control), $H_2O_2$-, and gamma-irradiation treatments, or cultivation of *A. cepa* in polluted soils, 10 excised roots were placed in a 60 mm Petri dish kept on ice and spread with 0.2 ml of cold 400 mM Tris buffer, pH 7.5. Using a fresh razor blade, the roots were gently sliced. The plate was kept tilted in the ice so that the isolated nuclei would collect in the buffer (Gichner et al., 2000). All operations were conducted under dim or yellow light.

Regular microscope slides were dipped into a solution of 1% NMP agarose prepared with phosphate-buffered saline at 50 °C and dried overnight at room temperature. Onto each slide, nuclear suspension (0.05 ml) and 1% LMP agarose (0.05 ml) 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 micropipette tip and a coverslip was placed on the mixture. The slide was placed on ice for 10 min, then the coverslip was removed and a final layer of 0.5% LMP agarose (0.1 ml) was placed on the slide. A coverslip was placed on the LMP agarose and the slide was kept at 4 °C for 10 min. The coverslips were removed and the slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO) to remove most nuclear protein sleaving supercoiled DNA as "nucleoids" to improve the quality of microscope observations. The slides were kept at least 1 h at 4 °C in the lysing solution, then placed in a horizontal gel electrophoresis tank with a freshly prepared cold electrophoresis solution (1 mM $Na_2EDTA$ and 300 mM NaOH, pH > 13), incubated for 15 min to allow the DNA to unwind and to resolve alkali–labile sites. The electrophoresis at 0.65 V cm$^{-1}$ (18 V, 230 mA) at 4 °C to a level about 2.5 mm above the upper agarose surface was done for 20 min (using a power supply LKB 2301 MACRODRIVE 1). They were then rinsed with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min three times, fixed by methanol and stained later with 80 μl ethidium bromide (20 μg ml$^{-1}$) for 5 min, dipped in ice-cold water to remove the excess ethidium bromide. The slides were examined at 200× magnification with a fluorescence microscope (Nikon Eclipse E600) which was fitted with an excitation filter of 510–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm.

The samples were coded and evaluated blind. For each slide, at least 25 random nuclei were scored. For the $H_2O_2$ and gamma-irradiation experiments, two slides were evaluated per treatment and each treatment was repeated twice. For the in situ experiments, three slides were evaluated per sample (total 75 nuclei). A computerized image analysis system (cometscore v1.5) was used to measure the tail moment values (integrated value of tail DNA density multiplied by the migration distance).

### 2.6. Statistical analysis of the data

Data were analyzed using the statistical and graphical functions of software package of SPSS v11.5 and Excel. To correlate between DNA damage and specific activity of $^{226}$Ra in the soil samples, a Pearson Correlation test was conducted. The DNA damage, as expressed by the tail moment (TM) values are given as averaged means ± standard errors (S.E.).

## 3. Results

### 3.1. Gamma spectrometry

Concentrations of natural radionuclides in soil samples from inhabited Ramsar zones are summarized in Table 1. Fig. 1 shows the regions where the soil samplings were performed. The village Talesh Mahalle showed the highest level of natural radiation. For the HBRA, the average specific activity of $^{226}$Ra, $^{232}$Th and $^{40}$K were 12,766, 31 and 611 Bq kg$^{-1}$, respectively. The specific activity of $^{226}$Ra in the control soils was in the range of 34–60 Bq kg$^{-1}$,thus markedly lower than in HBRA, whereas for $^{232}$Th and $^{40}$K approximately the same average specific activity was measured in the soils.

### 3.2. $H_2O_2$ treatment

Roots of *A. cepa* were treated with 0–75 mM $H_2O_2$ for 2 h and then nuclei were isolated after treatment. Fig. 2A illustrates the DNA-damaging effects of $H_2O_2$ on excised roots, expressed by mean tail moment values. The TM (±S.E.) values of root nuclei, with increasing concentrations of $H_2O_2$, increased from 4.12 ± 0.62 (negative control) to 115.02 ± 3.63 μm (75 mM $H_2O_2$). The result of regression analysis shows that the dose effect relationship between DNA damage (TM) and the $H_2O_2$ concentration ($D$)

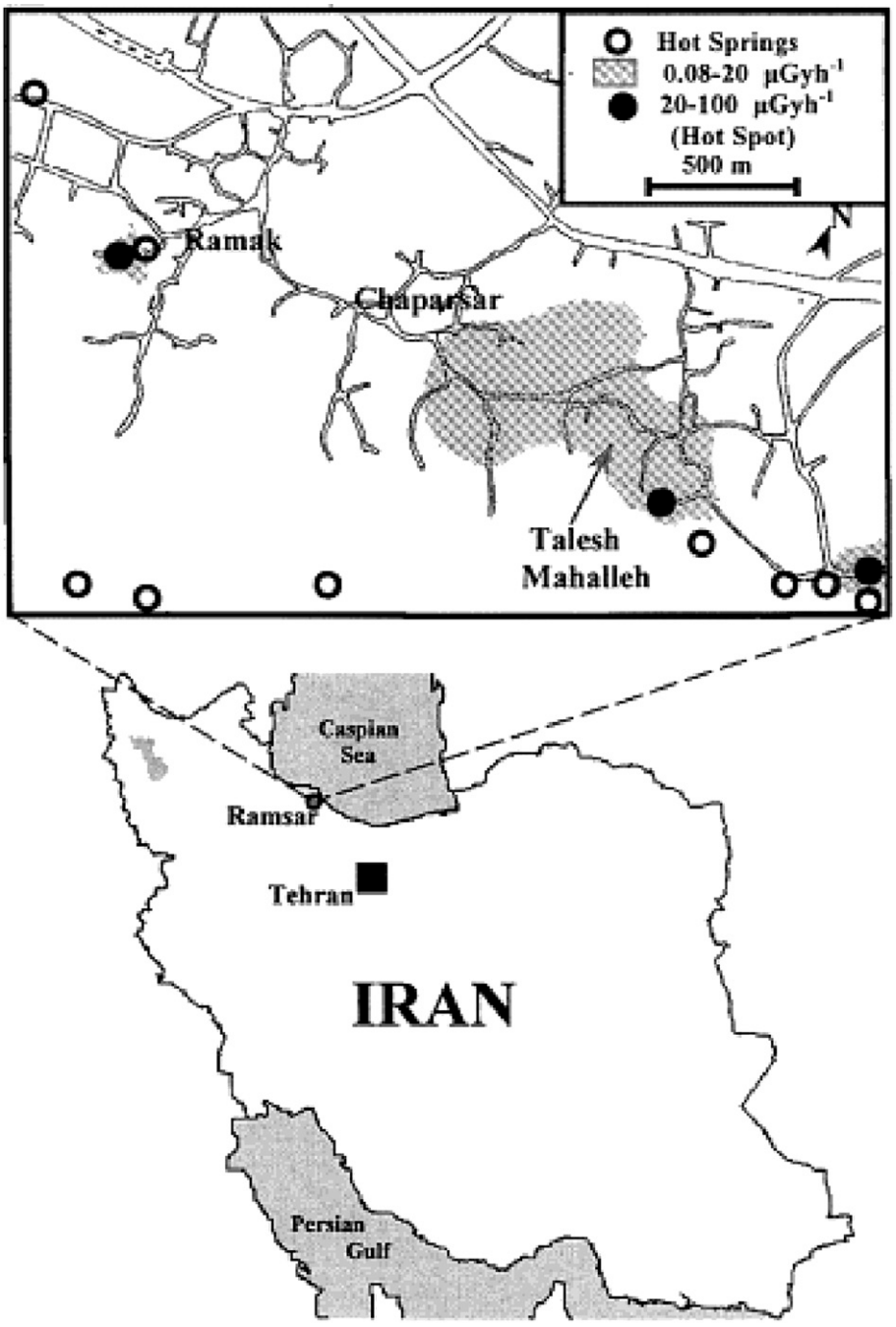


**Fig. 1.** Geographical location of Ramsar and its HBRA.

fits well with the linear equation ($TM = 18.93 + 1.44D$, $R^2 = 0.94$, $P < 0.001$) (Fig. 2A).

### 3.3. Gamma-irradiation

Roots of *A. cepa* were irradiated with 0–50 Gy gamma-radiation and nuclei were isolated immediately after irradiation. Fig. 2B illustrates that with increasing gamma-radiation doses, TM values (±S.E.) of nuclei increased from 3.78 ± 0.51 (control) to 132.72 ± 3.68 μm (50 Gy). The result of regression analysis shows that the dose effect relationship between DNA damage (TM) and the irradiation dose ($D$) fits well with the linear equation ($TM = 16.09 + 2.34452D$, $R^2 = 0.97$, $P < 0.001$) (Fig. 2B).

### 3.4. DNA damage in the roots of A. cepa seeds cultivated on the soil of HBRA

*A. cepa* seeds were germinated on contaminated soil from inhabited HBRA and on soil from clean control areas of Ramsar. The number of germinated seeds was almost the same for all sampled soils. The assessment of DNA damage in the root nuclei of *A. cepa* plants are illustrated in Fig. 3. The activities of $^{226}Ra$ in the range from 1040 to 59,100 Bq kg$^{-1}$ in the soil of HBRA, induced TM values (±S.E.) that ranged from 29.35 ± 1.58 to 165.70 ± 6.62 μm. Thus, the DNA damage of the roots of *A. cepa* was much higher in the soil of HBRA than in the control area. The result of regression analysis shows that the dose effect relationship between DNA damage (TM) and the specific activity

**Table 1**
Specific activity (mean ± error) of natural radionuclides in the soil samples from different high background radiation and control areas of Ramsar

| Sampling place | Activity of natural radionuclides in dried soil (Bq kg$^{-1}$) | | |
|---|---|---|---|
| | $^{226}$Ra | $^{232}$Th | $^{40}$K |
| Sang bone | 34.1 ± 1.99 | 25.6 ± 2.4 | 589 ± 16.2 |
| Kash | 44.4 ± 2.79 | 10.6 ± 2.3 | 670 ± 53.2 |
| Vazir garma | 59.5 ± 3.21 | 43.8 ± 3.8 | 530 ± 34.8 |
| Talesh Mahalle (station 1) | 1040 ± 37.5 | 43.5 ± 1.6 | 715 ± 44.3 |
| Khak sefid (station 1) | 1400 ± 38.8 | 32.2 ± 8.9 | 634 ± 38.6 |
| Talesh Mahalle (station 2) | 6870 ± 100 | 26.7 ± 11.7 | 495 ± 38.4 |
| Talesh Mahalle (station 3) | 7550 ± 91.6 | 34.8 ± 3.2 | 582 ± 47.6 |
| Khak sefid (station 2) | 7590 ± 168 | 25.2 ± 1.8 | 812 ± 51.3 |
| Talesh Mahalle (station 4) | 8190 ± 96.3 | 37.2 ± 3.4 | 567 ± 32.8 |
| Khak sefid (station 3) | 9830 ± 124 | 22.8 ± 4.1 | 654 ± 49.1 |
| Ramak (station 1) | 9920 ± 119 | 29.6 ± 1.6 | 668 ± 47.4 |
| Ramak (station 2) | 9940 ± 181 | 22.8 ± 4.1 | 709 ± 53.7 |
| Talesh Mahalle (station 5) | 19,000 ± 191 | 30.4 ± 3.5 | 485 ± 27.4 |
| Talesh Mahalle (station 6) | 59,100 ± 555 | 36.1 ± 4.4 | 402 ± 29.3 |

of $^{226}$Ra of soil samples ($D$) fits well with the linear equation ($TM = 13.62 + 0.00295D$, $R^2 = 0.83$, $P < 0.001$) (Fig. 3). In addition, several roots were disrupted and immersed in PBS for 24 h and then irradiated with 30 Gy gamma-radiation from $^{60}$Co source. The comet assay was processed immediately after irradiation. Fig. 3 illustrates the DNA damage in cells of these roots. There were no significant differences for induced DNA damages in the samples of various areas showing the occurrence of the same radio-sensitivity. For two places in which the $^{226}$Ra were 1040 and 1400 Bq kg$^{-1}$, probably a technical error has been occurred.

## 4. Discussion

Assessment of the ecological and genetic impact of nuclear pollution on plant populations is of great importance as plants are important commercial products and are consumed by people. Moreover, plants may be used as biosensors of genetic toxicity of environmental pollutants. In this research by the comet assay, the potential for DNA damage in the *A. cepa* as a biomarker of genetic toxicity was considered.

The sensitivity of the single-cell gel electrophoresis (SCGE) or comet assay allows rapid prediction of genotoxic potential of compounds and has been shown to be useful for in vivo and in vitro biomonitoring of environmental pollutants (Angelis et al., 1999; Silva et al., 2000; Lee and Steinert, 2003; Szeto, 2007). On the other hand, the *A. cepa* was among the plant bioassays reviewed by the US Environmental Protection Agency (EPA) GeneTox Program in 1980 (Bernhard et al., 2005; Grant, 1982). This plant has often been used for the determination of cytotoxic and genotoxic effects of various substances (Ma, 1999; Grant, 1999; Bernhard et al., 2005).

In the present study, the *A. cepa* seeds growing on the soil of HBRA were subjected to chronic exposures of ionizing radiation. The assessments of DNA damage showed that this plant has a great sensitivity to $^{226}$Ra concentration in the soil, and can be use as environmental monitoring agent. Similar behavior was observed when the seeds were subjected to acute gamma-ray irradiation. It has been previously shown that chronic irradiation is less effective in inducing mutations in *Nicotiana alata* than acute irradiation (van Gastel and de Nettancourt, 1974). However, in other study in the soils of inhabited areas in the Ukraine contaminated by the Chernobyl accident, a dose-dependent increase in the fraction of aberrant mitoses from control values was found in the *A. cepa*. A strong, significant correlation of $^{137}$Cs activity of soil samples with the percentage of chromosomal abnormalities was observed (Kovalchuk et al., 1998). Also in this work a significant correlation of $^{226}$Ra specific activity of soil samples with the DNA damage in the roots of *A. cepa* was observed. The linear increase in the DNA damage indicates that activation of repair enzymes is not triggered by exposure to radiation in HBRA. It is noteworthy that in the previous studies in this HBRA, conducted for human lymphocytes, a very similar high DNA damage was also observed (Mohammadi et al., 2006; Masoomi et al., 2006). But in the human cases more efficient repair was observed which interpreted to radio-adaptation.

Most available data in studying the effect of radiation have been obtained under acute high external dose. Only very few studies deal with the effect of low-chronic external exposure on the plant (Kovalchuk et al., 1998; Ptacek et al., 2002; Zaka et al., 2002a,b; van Gastel and de Nettancourt, 1974). Previous studies have demonstrated that in *N. tabacum* leaves, chemically induced DNA damage persists over a 72 h recovery period (Stavreva et al., 1998). By contrast, in *N. tabacum*, induced DNA damage by 30 Gy gamma-radiation is completely repaired within 24 h (Ptacek et al., 2001). A very efficient repair of X-ray-induced DNA breaks was previously reported for *V. faba* (Koppen and Angelis, 1998). These data indicate that DNA strand breaks induced by ionizing radiations are rapidly repaired.

Further studies are clearly needed to analyze the adaptation ability of natural plant populations grown in Chernobyl and to understand the extent of epigenetic control in the genome of these

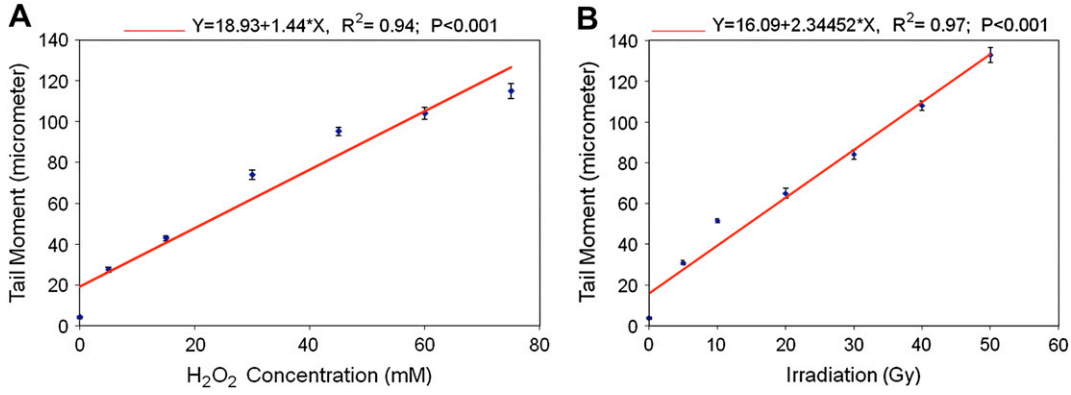


**Fig. 2.** Dose–response curve of the average mean tail moment (TM) values as a function of (A) $H_2O_2$ treatment of roots of *A. cepa* for 2 h at 25 °C in the dark. (B) Gamma-radiation of roots of *A. cepa*, immediately after irradiation. The error bars represent the S.E. of the mean.

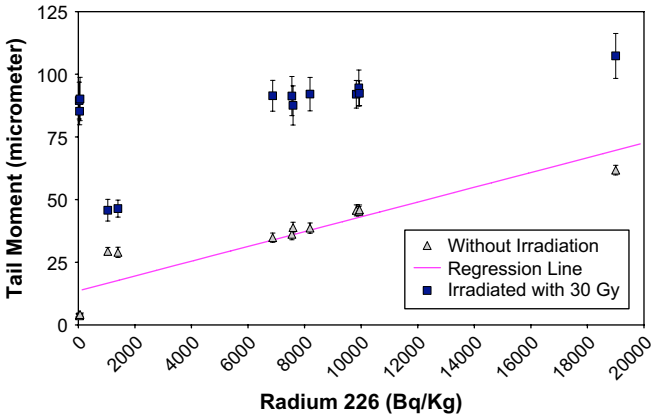


**Fig. 3.** Dose–response curves of the mean tail moment (TM) values of roots of *A. cepa* as a function of $^{226}$Ra specific activity of soil samples (△, without external irradiation and ■, irradiated with 30 Gy gamma-radiation). The error bars represent the S.E. of the mean.

plants. It could be interesting to follow up the radio-sensitivity and repair kinetics of this plant after few generations in the radio-activated soils.

## 5. Conclusion

The present study confirms the *A. cepa* comet assay as a reliable method for detection of genotoxicity of chronic irradiation. In addition, soils from inhabited and contaminated areas were indeed proved to be highly genotoxic. The *A. cepa* bioassay as a fast, inexpensive, and easy sampling may be a useful alternative to analysis the genotoxicity of contaminated soils with $^{226}$Ra.

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