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Cytogenetic Tests in the Assessment of the Genotoxicity of River Water

Kwasniewska, J.*, Jaskola, R. and Maluszynska J.

Department of Plant Anatomy and Cytology, University of Silesia, Jagiellonska 28, 40-032 Katowice, Poland

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ABSTRACT: The aim of this study was to evaluate and compare the sensitivity of plant bioassays: *Tradescantia*-micronucleus (Trad-MCN) tests using BNL 02 and 4430 clones, and *Crepis capillaris* hairy roots chromosome aberrations test. The evaluation of the sensitivity of the tests was based on the example of the analysis of the genotoxicity of water samples from two natural water reservoirs in Poland: the Rawa River, heavily polluted by industry, and the Goczalkowice - drinking water reservoir. Both tests showed genotoxicity of the Goczalkowice and Rawa waters. The results suggested the stronger genotoxic effect of water samples on *Tradescantia* 4430 clone compared to BNL 02. *Tradescantia* 4430 was a more sensitive bioindicator of genotoxicity than *C. capillaris* hairy roots. However it has been proved that *Crepis capillaris* hairy roots could be used as a convenient system for the environmental monitoring of water samples. Our study is the first example of using the TUNEL test (TdT-mediated dUTP nick end labeling) to determine the genotoxicity of water pollutants.

Key words: Crepis capillaris,Hairy roots,Tradescantia,TUNEL test,Water samples

INTRODUCTION

The increasing pollution of the environment requires the establishment of sensitive systems that provide data on adverse effects to human health. Plant bioassays have been widely used for some time to detect environmental mutagens and carcinogens (Grant 1999, Ma 1999, Monarca *et al.*, 2003, Majer *et al.*, 2005). Although numerous plant species play an important role in this field, *Tradescantia* is especially recommended for environmental biomonitoring (Grant *et al.*, 1992, Grant and Owens, 1998, Rodrigues *et al.*, 1997, Misik *et al.*, 2011). *Tradescantia* clone 4430 is the most often used, but other *Tradescantia* clones are also good alternatives for *in situ* studies (Ichikava *et al.*, 1995, Naumann *et al.*, 1997, Suyama *et al.*, 2002). *Tradescantia* was proposed as a model for monitoring atmospheric pollution, especially of radiation (Ma 1979, Ma *et al.*, 1996, Ruiz *et al.*, 1992). Various studies revealed the usefulness of the *Tradescantia* micronucleus test (Trad-MCN) for *in situ* monitoring of water pollution. The study of the effect of industrial effluents (Chen *et al.*, 1983, Zheng 1985, Lah *et al.*, 2008, Mielli *et al.*, 2009), marine pollution (Chen *et al.*, 1981) and drinking water (Lo, 1985) with the Trad-MCN test proved its utility to detect low-level genotoxicity in water samples. There is no need to know the composition and the concentration of genotoxic agents present in the natural water to evaluate their mutagenicity in *Tradescantia* cells (Fang 1980). The *Tradescantia* micronucleus bioassay is considered to be the most sensitive, as meiotic chromosomes are more susceptible to mutagens than mitotic ones. It is still of importance to improve existing, or to introduce new, sensitive biomarkers and bioindicators of genotoxicity, and the recent developments in molecular biology and biotechnology techniques has made such progress possible. Root tips of seedlings are the most frequently used for assessing chromosome or DNA damage in the mitotic cells of higher plants. The necessity of the large number of seeds required for germination can be omitted by the application of a culture of hairy roots obtained after transformation with *Agrobacterium rhizogenes*. Among the accepted plant bioassays the *Crepis capillaris* chromosome aberrations test is well recognized (Grant and Owens, 1998), *viz* its simple karyotype, fast growth, genetic stability, and a higher sensitivity compared to primary roots make it convenient for the evaluation of chromosome damage. Based on an analysis of the effects of mutagenic treatment, *C. capillaris* hairy roots were proposed as a convenient system for monitoring environmental mutagenic agents, especially for water samples and complex chemical mixtures (Juchimiuk *et al.*, 2005), although studies on the

*Corresponding author E-mail:jolanta.kwasniewska@us.edu.pl

genotoxicity of environmental pollutions using *C. capillaris* hairy root cultures have not previously been carried out. The evaluation of the genotoxicity of different agents can be improved by the application of molecular techniques. One of the methods is the TUNEL (TdT-mediated dUTP nick end labeling) test based on labeling DNA strand breaks. It enables the detection of DNA breaks in a single nucleus (Juchimiuk *et al.*, 2003, Havel *et al.*, 1996).

The aim of this study was to evaluate and compare the sensitivity of plant bioassays: *Tradescantia*-micronucleus (Trad-MCN) tests using BNL 02 and 4430 clones, and *Crepis capillaris* hairy roots chromosome aberrations test. The evaluation of the sensitivity of the tests was based on the examples of the analysis of the genotoxicity of water samples from two natural water reservoirs in Poland: the Rawa River, heavily polluted by industry, and the Goczalkowice - drinking water reservoir. The sensitivity of the TUNEL assay to the detection of DNA fragmentation in the environmental monitoring was analysed.

MATERIALS & METHODS

Water samples were collected from the Rawa River in Katowice and the Goczalkowice Reservoir in Upper Silesia, Poland, in the spring of 2008 and 2009. The Rawa is a minor river which has lost most of its natural river character, and it is now mostly a heavily polluted sewage waterway due to the industrialization of Silesia. It was reported to be crystal clear in the 19th century. The program for improving water and restoring the ecology of the river was introduced few years ago. Currently it is categorized as sewage of 5th purity class. The Goczalkowice Reservoir, built on the Vistula River, is the biggest largest drinking water reservoir in this part of the country. The physico-chemical values classify its water to the 2nd or 3rd purity class, depending on the season. It is slightly polluted by the agricultural activities around it. Nowadays the quality control of water, and especially drinking water, is of major concern. Chemical analyses of water from these reservoirs are routinely performed (data from Provincial Inspectorates for Environmental Protection in Katowice, 20), and the selected chemical parameters of the Rawa and Goczalkowice waters are given in Tab. 1. All water samples were transported to the laboratory after sampling and tested immediately.

Hairy root cultures of *Crepis capillaris* (L.) Waller (2n=6) and *Tradescantia* clones 4430 and BNL 02 were used in the study. The roots were induced by direct infection of the leaves of *C. capillaris* with *Agrobacterium rhizogenes*, strain ATCC15834; and then maintained on a liquid ½ MS medium (Murashige *et al.*, 1962) in the dark at 20°C on a rotary shaker (Juchimiuk *et al.*, 2005). The culture used in this study was already characterized in terms of its chromosome stability, and was about 8 years old. Plants of *Tradescantia* clones 4430 and BNL 02 were cultivated under standardized conditions: 16/8h light/dark, temperature 22ºC.

The time of exposure to the water being tested was 24h, and fresh *Tradescantia* cuttings and the *Crepis* root culture were used for the treatment. The Trad-MCN (*Tradescantia* micronucleus) test was performed according to the standard protocols established by Ma (1981), and for each experiment 25 cuttings were placed in Erlenmeyer flasks containing the water samples. After treatment the cuttings were washed 3 times for 5 min and postincubated for 24h in tap water. After treatment *Tradescantia* inflorescences were fixed in ethanol: acetic acid (3:1 v/v) for 4h at room temperature and then stained in Feulgen's solution. In order to analyze the frequency of micronuclei the inflorescences were dissected under a stereomicroscope, and the anther fragments discarded. For each experiment 25 slides were prepared and 1000 tetrads per group were analyzed in total.

Hairy root cultures were washed 3 times for 5 min with distilled water after treatment, and post-incubated for 24h in a ½ MS medium. In order to determine the number of chromosomal aberrations in root-tip meristems the roots were fixed, as describe above. Cytogenetic slides were prepared using the Feulgen's squash technique. For each treatment group chromosomal aberrations (bridges, fragments and laggard chromosomes) were counted in 200 cells during anaphase and early telophase. Ten slides, each made from 10 roots, were analyzed.

MH (maleic acid hydrazide, Sigma, CAS 123-3301) was used as a positive control of the experiment. It was dissolved in tap water (*Tradescantia)* or distilled water (*C. capillaris*) immediately before use. The MH treatment conditions for *Tradescantia* – 0.26 mM (6h) proved to be clastogenic, both in our studies (data not presented) and those of Ma *et al.* (1984). The treatment of *C. capillaris* hairy roots with 0.5 mM MH for 4h was used according to our earlier experiments (Juchimiuk *et al.* 2005). Clean tap water was used as a negative control for *Tradescantia* and distilled water for *C. capillaris*. Each experiment was replicated twice. The differences between the two groups were statistically evaluated by Student's *t*- test.

The TUNEL (TdT-mediated dUTP nick end labeling) test was performed in order to analyze the frequency of nuclei with DNA fragmentation in the cells of *Crepis* hairy roots. After the MH treatment of the samples the roots were postincubated 24h and then

the test was performed, and the frequency of TUNEL-positive nuclei was also estimated. One cm long *Crepis* root tips were fixed with freshly prepared 4% paraformaldehyde (Fluka) for 1h at room temperature. The TUNEL reaction using an *In situ* Cell Death Detection Kit, Fluorescein (Roche) was performed according to the procedure described earlier by Juchimiuk and Maluszynska (2003). The positive control was a 50 µl DNAse solution (1U) applied to one slide of the control sample. For a negative control of the TUNEL reaction a mixture without terminal transferase was used. Five root tips were used to make 1 slide, and the slides were stained with 2 µg/mL DAPI (4',6-Diamidino-2-phenylindole), air dried and mounted in Citifluor.

The slides were evaluated with a fluorescence microscope using an FITC filter (with an excitation filter of 495 nm and a barrier filter of 525 nm) and a DAPI filter (with an excitation filter of 355 nm and a barrier filter of 450 nm). Labeled nuclei were counted and the total number analyzed was calculated. The frequency of labeled cells was done on the basis of 2000 cells analyzed on two slides for each treatment. The differences between the two groups were statistically evaluated using the Student's *t*- test.

RESULTS & DISCUSSION

The results of the Trad-MCN are presented in Fig. 1. The treatment with water from the Rawa and Goczalkowice caused a significant increase in the frequencies of tetrads with micronuclei. After treatment with samples from the Goczalkowice reservoir the increase in *Tradescantia* 4430 clones was about 4.5 times, and only about 15% for BNL 02. The use of the Rawa samples on *Tradescantia* cuttings also led to an increase in the frequency of tetrads with micronuclei,- x5 times in the 4430 clone, and nearly x2 in BNL 02. No significant differences in the frequencies of tetrads with micronuclei between seasons, and repetitions of the experiments were observed (summarized data for all experiments are presented in Fig. 1. However, it must be noted that the frequencies of tetrads with micronuclei of 4430 and BNL 02 clones, which occurred spontaneously, were different: only 2.7% for *Tradescantia* 4430 and 7.7% for BNL 02. The lower frequency of tetrads with micronuclei after exposure to tap water rather than to the Goczalkowice samples is probably due to the effective water treatment processes. The frequencies of tetrads with spontaneously occurring micronuclei in *Tradescantia* 4430 plants were previously reported by Ma *et al.* (1987) – 4.8% and Gong *et al.* (2003) – 4.5-5.2%. In our experiments 0.26 mM MH was used in the Trad-MCN test using 4430 and BNL 02 clones. The frequencies of tetrads with micronuclei in both clones after MH treatment were very similar – 28.9% for 4430 clone and 30.2% for BNL 02. The number of micronuclei per cell was analyzed, as well as the total frequencies of the tetrads with micronuclei. The number of micronuclei in the tetrads after treatment with natural water was 1 (Fig. 5a), whereas after treatment with MH as many as four micronuclei could be seen (Fig. 5b).

The results clearly showed the stronger effects of water samples on *Tradescantia* 4430, and thus confirm the higher sensitivity of this clone compared to BNL

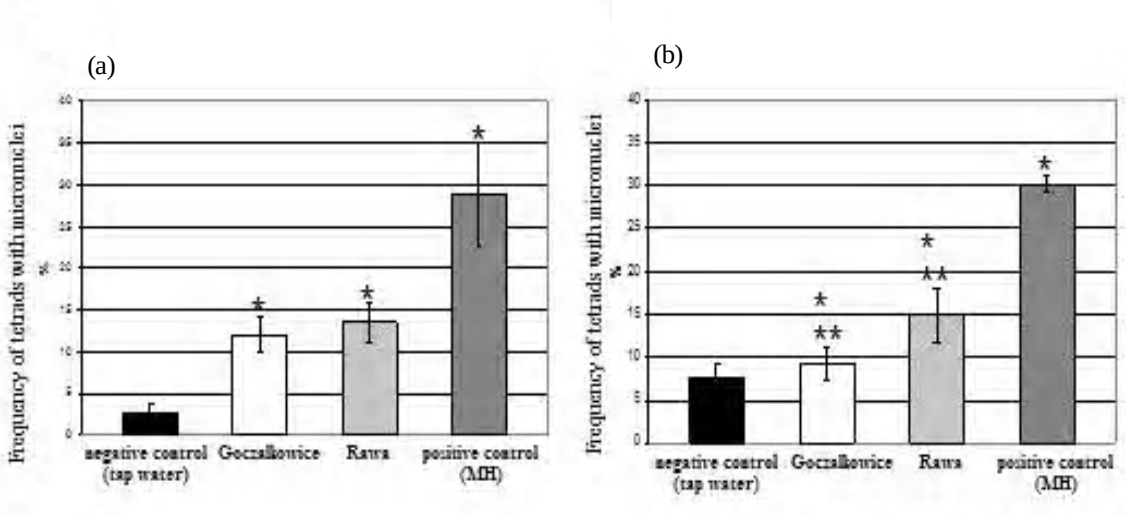

Fig. 1. Frequency of tetrads with micronuclei in *Tradescantia* clones: 4430 (a) and BNL 02 (b) after treatment with tested samples

Statistical analysis was performed according to the Student's *t*-test. Statistically significant in the compared group at $p < 0.05$: *between negative control and treatment groups, ** between Rawa and Goczalkowice treatment groups

02. However, the frequencies of tetrads with micronuclei after treatment were still about half as much as after MH treatment. No comparison of the sensitivities of *Tradescantia* clones to environmental factors is previously known. Naumann *et al.* (1997) have only shown the stronger effects of chemical mutagens treatment in 4430 clones as opposed to the BNL02 clone. However, the *Tradescantia* 4430 clone is used as the most sensitive among other clones and it is classically employed in detecting environmental genotoxic pollutants (Guimares *et al.*, 2000). It has been shown previously that *C. capillaris* hairy root cultures proved to be a more sensitive system for the evaluation of the cytogenetic effects of test agents (MH, X-ray) than primary roots (Juchimiuk *et al.*, 2005). The effect of treatment with the tested water samples was observed as changes in the mitotic activity of hairy roots cells (Fig. 2). Control hairy roots cells were characterized by a relatively high value of their mitotic index – 14.7%, although it should be taken into consideration that growing conditions on ½ MS medium are standardized. A decrease in the frequency of dividing hairy root cells after treatment was observed, and the mitotic index of the Rawa sample was 4.7-fold lower than that observed in the control hairy roots, and also after similar treatment with MH. Only a 2.5-fold decrease in the mitotic index was caused by treatment with the Goczalkowice sample. Surprisingly, the hairy roots transferred to distilled water were characterized by a lower mitotic activity (5.7%) than roots from the ½ MS. According to the expectations, the mitotic index in hairy roots cells exposed to tap water was higher than after exposure to distilled water – 8.3%. The drastic decrease of mitotic activity in hairy roots cells treated with distilled water is probably due to a lack of nutrients and mineral salts. The presence of mineral salts in tap water caused mitotic activity to be higher than after exposure to distilled water. However, the decrease of mitotic activity after treatment with Rawa, and similarly for MH, indicates the effects of these water samples. The mitotic activity of the hairy roots cells is not a good indicator of the genotoxicity of water samples tested because even in hairy roots placed in distilled water a significant decrease of the frequency of dividing cells was observed.

Among chromosome aberrations in anaphase and telophase chromosome fragments, laggards and bridges were observed (Fig. 5 c, d), with usually more than one chromosome aberration per cell. The frequencies of chromosomal aberrations are presented in Fig. 3. The frequency of spontaneous chromosomal aberrations in hairy root cells growing on MS medium was about 2%, and this level did not change when the culture was transferred to distilled water. There was a significant difference in the frequencies of chromosome aberrations in hairy roots treated with the Goczalkowice and Rawa samples; a 6.6% frequency for Goczalkowice water (and similar if tap water was used), whereas in roots treated with Rawa water were 2 times higher, at 12.8%. The strongest cytogenetic effect was observed in hairy roots treated with the positive control of the experiment, 0.5 mM MH – 42.2%. As for the Trad-MCN test no significant differences in the frequencies of chromosome aberrations between replicates were observed, and thus the mean data are presented.

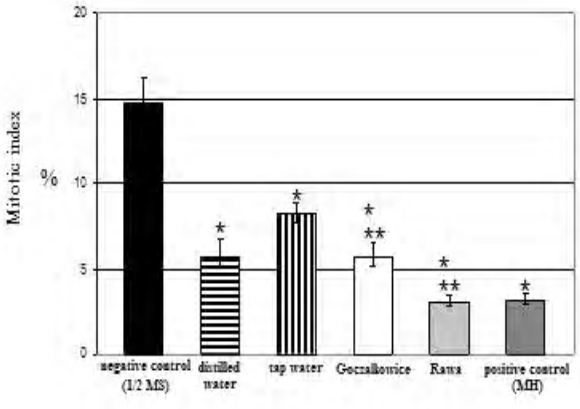

Fig. 2. Mitotic index (%) of *C. capillaris* hairy roots after treatment with analyzed samples

Statistical analysis was performed according to the Student's *t*-test. Statistically significant in the compared group at $p < 0.05$: *between negative control and treatment groups, ** between Rawa and Goczalkowice treatment groups

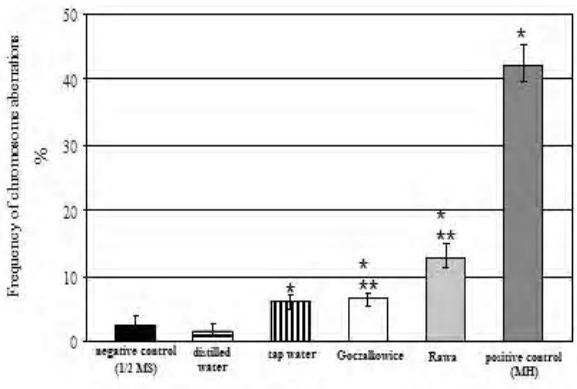

Fig. 3. Frequency of chromosome aberrations in *C. capillaris* hairy roots after treatment with tested samples

Statistical analysis was performed according to the Student's *t*-test. Statistically significant in the compared group at $p < 0.05$: *between negative control and treatment groups, ** between Rawa and Goczalkowice treatment groups

A significant increase in the frequency of chromosome aberrations after treatment with Goczalkowice water, and especially Rawa water, indicates their potential genotoxicity. Results of the analysis of the chromosome aberrations in hairy roots after treatment with Goczalkowice and Rawa waters suggest that this system has high utility for the genotoxicity testing of complex natural samples.

Comparison of the cytogenetic effects of the Goczalkowice water sample in meiotic (*Tradescantia* 4430) and mitotic cells (*C. capillaris* hairy roots) confirmed the findings of Sax (1938), that meiotic chromosomes are more susceptible to genetic damage than mitotic ones (even from different species). The frequency of *Tradescantia* 4430 cells with aberrations increased 4.5 fold, whereas for *C. capillaris* hairy roots cells it was only 2.6%. In contrast, treatment with the more polluted Rawa samples resulted in a similar 5-fold increased in the frequency of cells with aberrations, both in meiotic and mitotic cells. Therefore, the hairy roots system proved to be highly sensitive to DNA damage, since the detection of the cytogenetic effect of natural samples was possible. Other studies comparing the sensitivity of the Trad-MCN test using 4430 clone and the *Vicia faba* micronucleus test for ionizing radiation (Minouflet *et al.*, 2005), and water from Lake Superior, United States (Grant *et al.*, 1992), showed stronger genotoxic effects in meiotic cells, but it needs to be remembered that seedlings, not isolated root cultures, were used. In terms of our previous study (Juchimiuk *et al.*, 2005) it is probable that primary roots, routinely used in genotoxicity studies, might not be sensitive enough for monitoring natural samples with low contamination e.g. the Goczalkowice sample. Such studies on the sensitivity of different testing systems are crucial for a detailed evaluation of the environmental impact on living cells.

The TUNEL test, previously used in apoptosis studies, was adapted for the detection of DNA damage in plant mutagenesis (Juchimiuk and Maluszynska, 2003). The test was used to visualize DNA breakage in hairy roots cells, which occurred spontaneously or was induced by tested water samples (Fig. 5 e, e‘-f, f‘). The differences in the frequency of TUNEL-positive nuclei between replicated experiments were not statistically significant, so the mean frequencies are presented. Nuclei with TUNEL-specific signals were sporadically observed in untreated hairy roots *ca* 4% (Fig. 4). All tested water samples caused DNA fragmentation in the nuclei of hairy roots, and the TUNEL test revealed the highest increase of the frequency of labeled nuclei after treatment with the Rawa sample, about 55%. Treatment with the Goczalkowice sample caused DNA fragmentation in almost 45% of nuclei. Treatment of the hairy roots with distilled water also caused DNA fragmentation, with a five-fold higher frequency than in hairy root cells from the ½ MS medium. This might be due to the stress conditions (e.g. lack of mineral salts) correlated with transferring the culture from the growing medium. Negative and positive controls of the TUNEL reaction were used in the experiments to prove that this idea is correct. TUNEL-specific fluorescence was not seen in the negative control (no terminal transferase used), whereas about 70% of nuclei were labeled in the positive control (DNAase solution was used prior to the TUNEL reaction). The 0.5 mM MH, used as positive control of the treatment experiments, caused DNA fragmentation in 55% of nuclei.

The conclusions following from the TUNEL test confirmed those from analysis of the chromosomal aberrations in *C. capillaris* cells. Both Goczalkowice and Rawa water samples induced DNA changes. Interestingly, the frequencies of TUNEL-positive nuclei were a few times higher than the frequencies of cells with chromosome aberrations – 6.8 and 4.2 for Goczalkowice and Rawa, respectively. It is probable that an effective DNA break repair occurred in the hairy root cells and not all DNA breaks led to chromosome aberrations. It is also probable that DNA single strand breaks occurred predominantly, and that they are preferentially restored. One exception is if MH was used, where a high frequency of chromosome aberrations (42.2%) is correlated with a high frequency of TUNEL-positive nuclei (55%). Our studies proved that the TUNEL test is an effective method to monitor

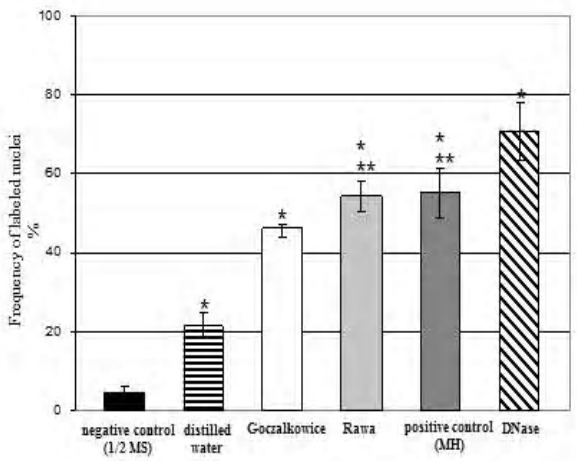

Fig. 4. Frequency of labeled nuclei in *C. capillaris* hairy roots *in situ* detection of DNA fragmentation using the TUNEL test after treatment with MH and water samples used in the study

*Statistically significant between untreated and treated groups at $p < 0.05$ (t-test)

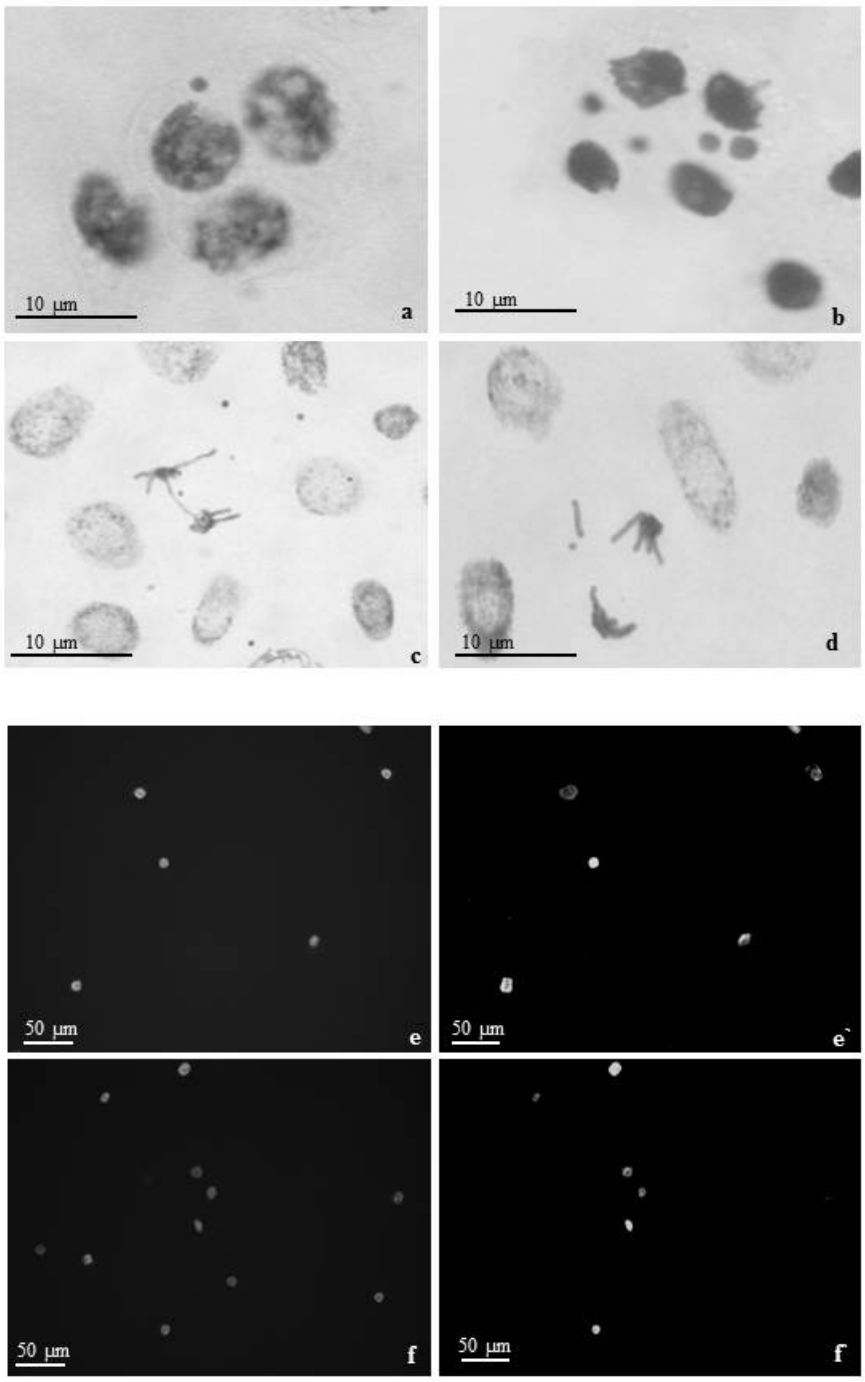

Fig. 5. Results of chromosome aberrations/micronuclei assays and the TUNEL test after treatment with tested water samples

a, b *Tradescantia* 4430 cells in the tetrad state with a different number of micronuclei: one- after treatment with Rawa water sample (a), four - after MH treatment (b)

c, d *C. capillaris* hairy root cells with chromosome aberrations after treatment with Rawa water samples: anaphase bridge, and micronuclei (c), anaphase fragments (d). The micronuclei are also seen in (d).

e, e‘-f, f‘ *In situ* detection of DNA fragmentation in the TUNEL test in *C. capillaris* hairy roots.

e, e‘ after treatment with water samples from the Rawa, all nuclei with DNA breaks; f, f‘ after treatment with water samples from Goczalkowice, approximately half of the nuclei show fluorescence

e,f: DAPI, all nuclei stained; e’,f‘: fluorescein, positive results of the TUNEL test

the DNA damage caused by environmental agents, especially by water samples, even with a low pollution level. The application of the TUNEL test enables the consequences of water sample treatment on the DNA level to be observed immediately after treatment. The TUNEL test applied after a post-incubation time, corresponding to a few cell cycles, and compared with an analysis of the frequency of chromosome aberrations might demonstrate the effectiveness of the repair processes after genotoxic environmental treatment.

An analysis of the frequencies of *Tradescantia* tetrads with micronuclei, chromosome aberrations in hairy root cells and the results of the TUNEL test showed genotoxicity of both the Goczalkowice and Rawa water. Although the genotoxicity of Rawa samples was predictable, the results of the bioassay regarding the Goczalkowice samples were surprising. The presence of a high concentration of nitrites can lead to a genotoxic effect of Goczalkowice water. The genotoxic effect of nitrites in plants (*Tradescantia*) and animals (*Mus musculus*) in Lake Superior was reported earlier (Grant *et al.,* 1992). The very strong genotoxic cytogenetic effects of the Rawa water probably correlate with the occurrence of nitrites, nitrates and other nitrogen compounds, which are well known as potential mutagens (Minouflet *et al.,* 2005).

CONCLUSION

In conclusion, this study proved the genotoxic effect of the Rawa, and surprisingly, Goczalkowice water samples. *Tradescantia* 4430 is a more sensitive bioindicator of genotoxicity than *C. capillaris* hairy roots. However, the *C. capillaris* hairy roots test has also been shown to be excellent and sensitive in the evaluation of the mutagenic effect of water samples, even those only slightly polluted. The TUNEL assay applied to the *in situ* detection of DNA fragmentation seems to be more sensitive in the evaluation of the genotoxicity of abiotic agents, and proved its usefulness in environmental monitoring.

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