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BEATA KOŚMIDER[1*], REGINA OSIECKA[1] & JAN T. SICIŃSKI[2]
[1*] Department of Cytogenetics and Plant Molecular Biology, University of Łódź, 12/16 Banacha Str., 90–237 Łódź, Poland
[2] Department of Botany, University of Łódź, 12/16 Banacha Str., 90–237 Łódź, Poland
[*] e-mail: beatakosmider@interia.pl

APPLICATION OF MICRONUCLEUS ASSAY IN PLANTS TO MONITOR ENVIRONMENTAL POLLUTION *IN SITU*

Abstract: Samples of 5 vascular plant species: *Achillea millefolium* L., *Artemisia vulgaris* L., *Festuca gigantea* (L.) VILL., *Ranunculus acris* L. s. str. and *Vicia angustifolia* L. were collected near Fuel Storage Station, Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki and Sewage Treatment Plant in Sulejów. Genotoxicity of environmental pollution was analysed using micronucleus assays in pollen mother and root tip cells in comparison with plant material collected in the Tatra and Babia Góra National Parks. Abnormalities in morphology of plants growing in polluted areas were also found. The cytogenetical analyses revealed positive correlation between the percentage of micronuclei and the presence of heavy metals in soil. Pollen mother cells (germ line) were found more susceptible to DNA damage as compared with root tip cells (somatic line).

Key words: monitoring *in situ*, environmental pollution, micronuclei, pollen mother cells, root tip cells.

1. INTRODUCTION

The negative effects caused by anthropogenic factors e.g. industrial pollution, expansion of urban-industrial agglomerations, use of chemical compounds in agriculture, deposition of dangerous refuses, exhaustion of natural resources, pollution of ecosystems and even radioactive contamination of natural environment are considered one of the major problems of the present time. This situation disturbs homeostasis of ecosystems and exerts influence on all organisms which are connected with one another by food chains.

Monitoring system is one of the elements of the interdisciplinary program used to control and prevent further contamination of human environment. It registers simultaneously emission of pollution and its consequences for ecosystems. Physico-chemical methods are used to define concentrations of various factors in natural environment as well as biological research is conducted to reveal their influence on living organisms and determine pollution effects (CIEPAŁ 1992). Methods of monitoring *in situ* are used among others to analyze morphological and anatomical changes in plants and an impact of various pollutants on the structure and organization of plant genomes. DNA damage can be detected by including genotoxicity assays to methods of environmental pollution monitoring *in situ*.

The micronucleus assay is useful in biomonitoring and ecotoxicology as a sensitive method to detect chromosomal abnormalities induced by mutagens both *in vivo* and *in vitro* (STOPPER, MÜLLER 1997). Micronuclei are formed through loss of whole chromosomes or their fragments during cell division. The micronucleus assay is simple and easy to perform and belongs to the standard cytogenetics assays recommended in different monitoring systems and ecotoxicology (MA, GRANT 1982).

The aim of this research was to analyze frequency of micronuclei occurrence in pollen mother and root tip cells of plant species collected from polluted areas and from the National Parks. These experiments were carried out in order to evaluate the usefulness of micronucleus assay to monitor environmental pollution *in situ*.

2. MATERIALS AND METHODS

The plant species *Achillea millefolium* L., *Artemisia vulgaris* L., *Festuca gigantea* (L.) VILL., *Ranunculus acris* L. s. str. and *Vicia angustifolia* L. were collected in the polluted areas (Fuel Storage Station, Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki and Sewage Treatment Plant in Sulejów) and from Tatra and Babia Góra National Parks in 1998. Whole inflorescences and root tips were cut for cytogenetic analyses. Moreover, plants for herbarium material were brought.

Approximately 50 individual plants were observed in each studied area. Stereomicroscope and botanical key were used to identify plant species (SZAFER et. al. 1986). Additionally, abnormalities in their morphological structures were also observed. For cytogenetical analyses the root tips and flower buds (MA 1982) were fixed in Carnoy solution (1 : 3 icy acetic acid : 96% ethanol) and stored in 70% ethanol at 4°C (GRANT 1982). Various staining methods (Feulgen method, aceto-carmine, Mayer's hematoxylin) were used. The dry-ice method was applied. Four preparations were made from pollen mother cells and root tips of each species. The percentage of the micronuclei was determined in 1000 root tip cells per slide and in 100 pollen mother cells. The standard deviations and Student test were also applied.

2.1. Characteristics of studied area

2.1.1. Fuel Storage Station

Although the high concentrations of heavy metals and ether-extractable substances (Tables 1, 2) indicating organic-laden water in this station and its surrounding were known to be well above-average, the actual content of organic substances has not been determined there (unpublished data). Aromatic hydrocarbons, lead compounds, mineral oils and wastes from alkaline fuel purification which are released to soil and water from the Fuel Storage Station were found on the list of dangerous wastes (Dz.U. 2001; Dz.U. 2002).

Table 1: Mean values of chemical analyses of sewage from Fuel Storage Station and Sewage Treatment Plant in Sulejów (unpublished data from these stations). The highest threshold limit values of pollution in sewage inflow to water and into soil (Dz.U. 1991). **BOD** – biochemical oxygen demand; **ChOD** – chemical oxygen demand; **nt** – not tested. Values exceeding limits are given in bold

Specification	Fuel Storage Station	Sewage Treatment Plant in Sulejów			Limit value	Unit
		inflow	sewage outflow	sludge		
BOD_5	33.7	32	12	nt	50	mg/l
Cadmium	nt	nt	nt	3.51	0.2	mg/l
Chloride	9.16	30	42	nt	1 000	mg/l
ChOD	133.6	90	39	nt	250	mg/l
Cooper	nt	nt	nt	**124.45**	0.5	mg/l
Lead	nt	nt	nt	**90.78**	0.5	mg/l
Reaction	6.86	7.3	7.9	6.9	6.5–8.5	pH
Sulphates	nt	41.8	36.2	nt	500	mg/l
Total nitrogen	4.06	nt	nt	**50.35**	30	mg/l
Total suspension	7.6	51	13	nt	35	mg/l
Zinc	nt	nt	nt	**2 192.15**	2	mg/l

Table 2: Soil contamination near and in Fuel Storage Station (**1** – tankage facilities, **2** – tank cleaning department, **3** and **4** – station boundaries, **5** – 50 m away from station; unpublished data from Fuel Storage Station). The highest threshold limit values of pollution in sewage inflow to water and into soil (Dz.U. 1991).

Indicator	1	2	3	4	5	Limit values	Unit
Ether-extractable substances	**117**	**5 220**	**165**	**106**	**118**	50	mg/l
Lead	<6	**15.5**	<6	<6	<6	0.5	mg/l
Reaction	7.37	7.32	7.69	**4.87**	**4.7**	6.5–9.0	pH

2.1.2. Sewage Treatment Plant in Sulejów

Domestic and municipal wastes inflow to this sewage treatment plant (Table 1). Localization of sewage treatment plants is important because substances harmful for water environment are usually present there. These include wastes containing among others: cadmium, lead, mercury, phosphorus and compounds of chromium, zinc and copper (Dz.U. 2001).

2.1.3. Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki

Until 1991 industrial wastes were disposed directly on this dumping site, which meant with probability of landfill leachate occurrence, but after modernisation of the waste site wastes were deposited in a rational way (SADURSKI, LISZKOWSKA 1993). However, in spite of the fibre production stoppage this waste site is still dangerous for the environment (Table 3). Its negative environmental impact is not exactly known but soil degradation was detected within 200-m radius (DANIELAK et al. 1993). There are still stable substances which have negative impact on oxygen balance in water (e.g. nitrites), copper compounds and cyanides (Dz.U. 2001; Dz.U. 2002).

Table 3: Mean values of chemical analyses of water extracts from dumping site of Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki (SIUTA et al. 1991) and the highest threshold limit values of pollution in sewage inflow to water and into soil (Dz.U. 1991). Values exceeding limits are given in bold

Indicator	Content in waste			Limit values	Unit
	fibres	plastics	viscoses		
Cooper	<0.1	<0.1	<0.1	0.5	mg/l
Nitrates	<0.1	<0.1	<0.1	30	mg/l
Reaction	**1.54**	**1.34**	**13.8**	6.5–8.5	pH
Sulphates	**12 000**	**550**	40	500	mg/l
Total nitrogen	<1	<1	<1	30	mg/l
Zinc	**117**	**4**	**5**	2	mg/l

2.1.4. Tatra and Babia Góra National Parks

They are located far from industrial centres and should be potentially unpolluted. However, increased level of sulphur dioxide, nitric oxides and heavy metals were detected here. Precipitation that increases with the height above sea-level also increases pollution inflow. Strong winds cause an air-mass mixing and facilitate dust settlement in a moist soil zone. Lasting low temperature increases gases solubility, causes plant mechanical damages and facilitates pollution infiltration (GRODZIŃSKA, SZAREK 1996).

3. RESULTS

3.1. Abnormalities in morphology of plants collected in the studied area

3.1.1. Fuel Storage Station

Achillea millefolium L.: single leaves at the stem base while in the upper part they were abnormally numerous and growing in whorled arrangement; lingual flowers were not developed.
Artemisia vulgaris L.: excessive thickness of roots.
Festuca gigantea (L.) VILL.: above-average growth; leaf blade thickened considerably; larger sizes of leaves.
Vicia angustifolia L.: some leaves abnormal bigger; variability in their sizes; leaf development disturbed e.g. splitting of leaf tips.

3.1.2. Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki

Achillea millefolium L.: abnormally numerous leaves in nods; inflorescences untypical growing from the middle part of a steam; scanty flowers.
Vicia angustifolia L.: deformation of a whole plant; untypical variability in sizes of leaves which are very narrow and small; abnormal distribution of flowers in leaf corners; S-shaped pods; disturbances in leaf developments e.g. splitting of leaf tips.

3.1.3. Sewage Treatment Plant in Sulejów

Ranunculus acris L. s. str.: fasciated stems; leaves of abnormal shapes and sizes; deformed inflorescences.

3.2. Micronuclei analysis

3.2.1. Pollen mother cells

Figure 1 shows examples of micronuclei in pollen mother cells observed in plant species collected in the studied area. The percentage of the micronuclei in pollen mother cells of *Ranunculus acris* L. s. str. in relation to the area of its growing were presented in Figure 2. Their lowest number was noticed in the material from Tatra (2%) and Babia Góra National Parks (4.3%). On this ground a suggestion could be drawn concerning the degree of contamination of the studied area. Greater number of these structures (10%) in pollen mother cells of plants growing on drying beds in Sewage Treatment Plant in Sulejów as compared with its adjacent (5.8%) was also observed which can be connected with the high concentration of heavy metals in the former place (Table 1). The high percentage of micronuclei was observed both in *Achillea millefolium* L. (47.3%) growing on the dumping site of Chemical Fibre Factory "Wistom" and in *Artemisia vulgaris* L. (15.5%) from Fuel Storage Station (Fig. 3).

Fig. 1: Micronuclei in pollen mother cells: ⟨1⟩ – *Ranunculus acris* L. s. str. ⟨2⟩ – *Achillea millefolium* L.

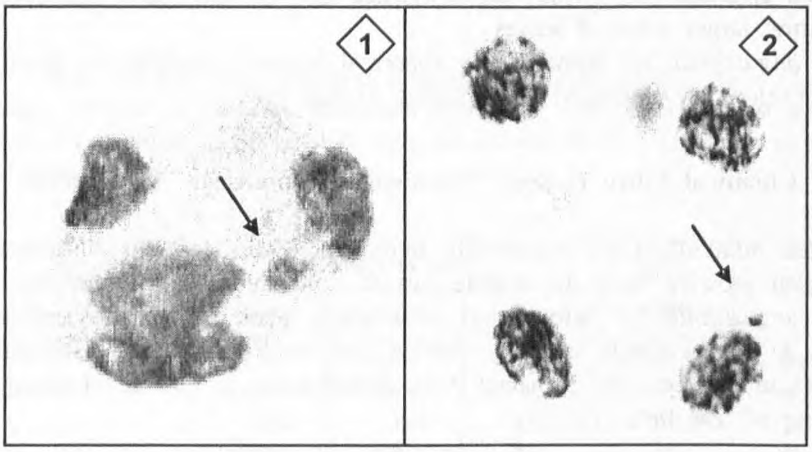


Fig. 2: The percentage of micronuclei in pollen mother cells of *Ranunculus acris* L. s. str. collected in the studied area: **1** – Tatra National Park; **2** – Babia Góra National Park; **3** – area of Sewage Treatment Plant in Sulejów; **4** – drying beds in Sewage Treatment Plant in Sulejów. (*) statistical significance (Student test; $p<0.05$).

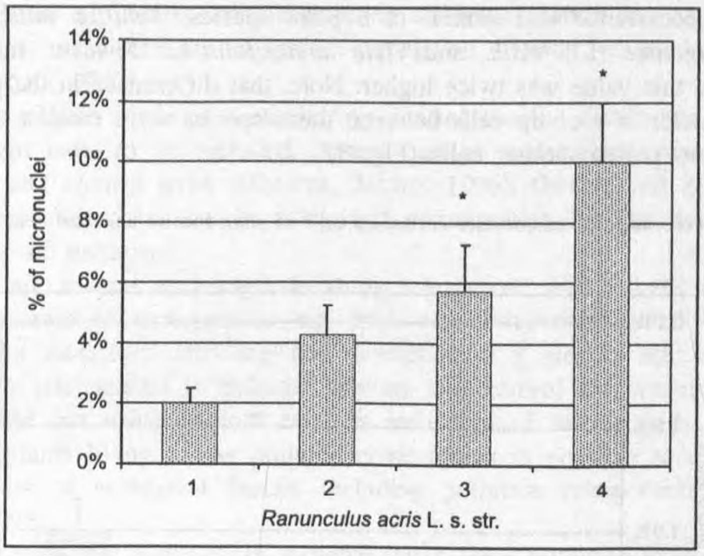
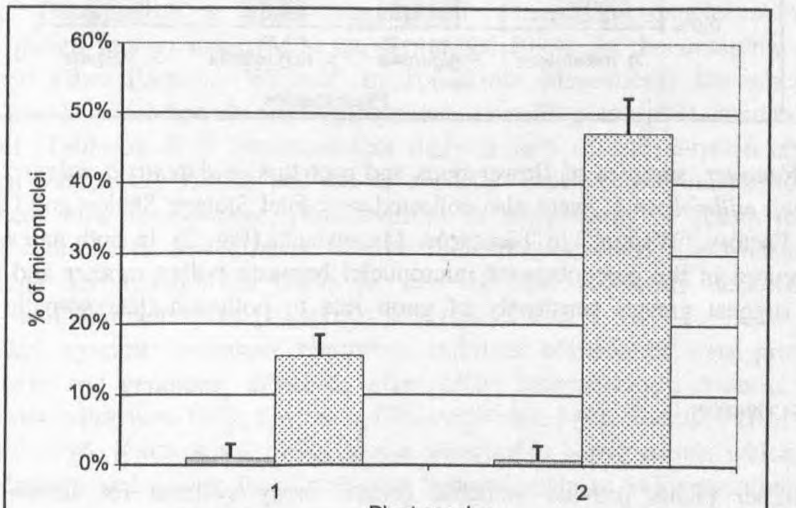


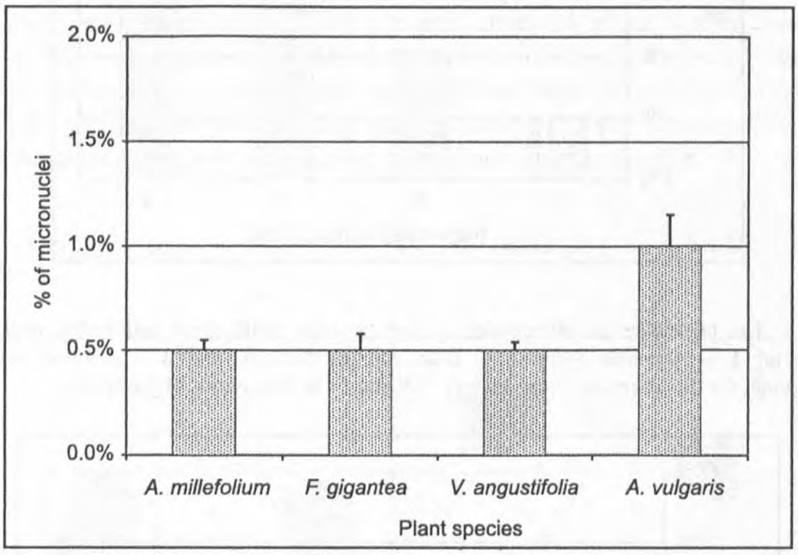
Fig. 3: The percentage of micronuclei in root tip cells (solid grey) and pollen mother cells (dotted) in: **1** – *Artemisia vulgaris* L. (Fuel Storage Station), and **2** – *Achillea millefolium* L. (dumping site of Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki).



3.2.2. Micronuclei analysis in root tip cells

The number of micronuclei in root tip cells in 4 plant species collected near Fuel Storage Station was compared (Fig. 4). The percentage of these structures occurrence was similar in 3 plant species: *Achillea millefolium* L., *Festuca gigantea* (L.) VILL. and *Vicia angustifolia* L. However, in *Artemisia vulgaris* L. this value was twice higher. Note, that differences in the percentage of micronuclei in root tip cells between these species were smaller (Fig. 4) as compared to pollen mother cells (Fig. 3).

Fig. 4: The percentage of micronuclei in root tip cells of plant species collected near Fuel Storage Station



Moreover, samples of flower buds and root tips of *Artemisia vulgaris* L. and *Achillea millefolium* L. were also collected near Fuel Storage Station and Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki (Fig. 3). In both cases distinct differences in the percentage of micronuclei between pollen mother and root tip cells suggest greater sensitivity of germ line to pollution than somatic one.

4. DISCUSSION

Higher plants provide valuable genetic assay systems for screening and monitoring environmental pollutants (GRANT 1994). Natural environment is characterised by different biotic and abiotic conditions that are connected with particular phytocoenosis. Because of that finding the same plant species in different places in area poses some problems. However, plants collected from different areas were also analysed for *in situ* studies (DANIEL et. al. 1997). Moreover, pollution can affect the distribution of plants. It was reported that the abundance of plants strongly depends on actual conditions e.g. increased soil acidity causes decrease in species population (DANIEL et. al. 1997; BANÁSOWÁ, ŠUCHA 1998).

In the system of *in situ* monitoring control species distributed in natural environment have to be included. Assays can be undertaken employing flora from test and control areas (MICIETA, MURIN 1996). GRANT and ZURA (1982) also recommended the use in situ weed communities for the detection and monitoring of mutagens.

There are several advantages in using microspore mother cells and tetrads as bioindicators of mutagenicity e.g. high sensitivity conditioned by haploid state, when mutations effecting the development of pollen are immediately evident. *In situ* studies in polluted regions and control (in botanical garden) were carried out using haploid cells as indicators of mutagenicity. Therefore by using plants living in the polluted environment is possible to evaluate the effectiveness of ecological factors including pollution components (MICIETA, MURIN 1996).

In the Tatra and Babia Góra National Parks some pollution is also observed but it is mainly a result of common gaseous emission. In *Ranunculus acris* L. s. str. collected in the Tatra National Park micronuclei number (2%) in pollen mother cells was lower than from the Babia Góra National Park (4.3%; Fig. 2). However, these results are comparable to those obtained for pollen mother cells of *Tradescantia* in which spontaneously formed micronuclei number is 5% in pollen mother cells (MÜLLER, STREFFER 1994). In the dumping site of Chemical Fibre Factory "Wistom" in Tomaszów Mazowiecki the amount of heavy metals and other chemical compounds exceeding accepted standards was detected (Table 3). It is important that there is lack of soil sorption complex that can bind heavy metals and act as a buffer which causes inflow of toxic substances into plant tissues. Abnormalities in morphology of plants growing on soil with excess concentration of toxic mineral compounds could result from their high accumulation in plants as well as from secondary deficiency of macro- and microelements (FABISZEWSKI et al. 1983).

Nickel, cyanide, cadmium, chromium and lead compounds were proved to have toxic and genotoxic effect on plant cells (SOMASHEKAR, AREKAL 1983; VILLALOBOS-PIETRINI 1987; GABARA, GOŁASZEWSKA 1992; GABARA et al. 1992; GWÓŹDŹ 1996; KACPERSKA 1998). It was shown that heavy metals which cause DNA damage and cancer in animals and humans such as cadmium, chromium, nickel, arsenic were highly genotoxic in *Tradescantia* and *Vicia* micronucleus assays (MA 1982; ENNEVER et al. 1988; MA et al. 1994; KNASMULLER et al.

1998), whereas metals which pose no or minor hazards to man e.g. antimony and copper caused no significant dose-effects in this system (KNASMULLER et al. 1998). Moreover, STEINKELLER et al. (1998) ordered the heavy metals Pb>Cd>Zn>Cu according to decreasing potential genotoxicity in the micronucleus assay in pollen mother cells in *Tradescantia* and in root tip cells in *Allium cepa* and *Vicia faba*.

Cadmium was found to reduce the DNA content in the meristematic zone of *Pisum sativum* and its action in meristem could be attributed to the reduction in the transcriptional activity (WOJTYŁA-KUCHTA, GABARA 1991). Note, that in pollen mother cells of *Ranunculus acris* L. s. str. collected on drying beds in Sulejów, where the excess of lead, zinc and cadmium was noticed (Table 1), the highest percentage of micronuclei (10%) was also observed (Fig. 2). SOMASHEKAR and AREKAL (1983) showed toxic influence of pollutants with high nickel and cyanide contents on *Allium cepa* root tip cells. They observed chromosome breaks, micronuclei, chromatid bridges, dicentric chromosomes and binuclear cells induction, disintegration and fragmentation of nuclei, disturbances in mitotic spindle organization and function.

SMARDAKIEWICZ and WOŹNY (1995) reported that heavy metal absorption in monocotyledonous species was lower than in dicotyledonous. This is in agreement with our observations in *Festuca gigantea* (Fig. 4). *Achillea millefolium* and 70% of grasses are polyploidal plants (MIZIANTY 1995). Polyploidization can be advantageous in occupation of new ecological niches as well as it can facilitate of adaptation to new environmental conditions (KONONOWICZ 1989).

In disturbed habitats microevolution processes resulting in the development of plants resistant to heavy metals present in soil can be observed. FALIŃSKA (1996) reported that sometimes these reactions have an adaptive character and then resistant ecotypes appear. It was demonstrated that species growing in soil naturally rich in metallic elements inherited resistance mechanisms. It was also observed that plant tolerance can be induced and stabilized by gradual introduction of species to polluted areas but attention must be paid not to exceed their ecological resistance.

Existence of plants in polluted environments can be connected with e.g the presence of enzymes able to function despite high levels of heavy metals (SCHNEIDER 1995; GWÓŹDŹ 1996).

The harmful effects of aromatic hydrocarbons in petroleum derivative products, e.g. toluene, n-hexane and n-heptane on plants were also confirmed. They are soluble in lipids and can damage lisosome membranes and DNA can be degraded by DN-ase. In *Vicia faba* root tip cells these compounds caused induction of micronuclei, C-mitosis, chromosomal aberrations and chromosomes with inactive centromers. It was proved that centromere was inactivated already after 1 h incubation with these substances. This caused an increase in chromosome number with inactive or damaged centromeres that did not participate in anaphase and appeared as micronuclei (GOMEZ-ARROYO et al. 1986). Low percentage of micronuclei number in root tip cells (Fig. 4) suggests the occurrence of detoxification mechanism and tolerance.

In conclusion the correlation between the highest number of micronuclei and the presence of heavy metals in soil was noted. Moreover, we found higher number of these structures in germ line in comparison with somatic one which suggests higher sensitivity of pollen mother cells than root tip cells to pollution. This tendency was also reported by KNASMULLER et al. (1998) and STEINKELLER et al. (1998). The presented results confirm usefulness of micronucleus assay in plants to monitor environmental pollution *in situ*.

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