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Novel Plant Bioassays for Monitoring the Genotoxicity of Drinking Water from the Inhabited Areas of the Ukraine Affected by the Chernobyl Accident

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The worst nuclear accident in history happened on the 26th of April 1986 in Ukraine causing the prolonged release of radioactive material into the environment and contamination of huge territories in Ukraine, Russia and Belarus. About 20% of the ^{137}Cs released during the accident was deposited in Ukraine (Izrael et al., 1997). In Ukraine the affected areas were classified into 4 zones in accordance with the Ukrainian State law: zone 1 - "exclusion zone" where the ^{137}Cs contamination exceeded 40 Ci/km^2, from which the population was evacuated in 1986; zone 2 - "obligatory resettlement zone" – where ^{137}Cs contamination constituted 15-40 Ci/km^2; zone 3 "of guaranteed voluntary resettlement of population" (contamination ranged between 5-15 Ci/km^2) and zone 4 "of enhanced radiological control" (1-5 Ci/km^2 of ^{137}Cs). Zone 3 and 4 as well as a part of zone 2 are still inhabited. Numerous studies have been conducted to evaluate the long-term genetic consequences of the accident on living organisms. Monitoring of the genotoxicity of drinking water in the areas affected by the Chernobyl accident is one of the key elements in the disaster management program.

To analyze the genetic effects of radioactive contamination on living organisms a choice of test system is critical. The ideal system has to be eukatyotic, provide a large number of offspring, be easy to handle and provide results on the molecular level. Higher plants were recently proven to be very suitable and reliable systems for biomonitoring. We made use of the well established *Allium cepa* test alongside a new transgenic *Arabidopsis thaliana* plant-based bioassay to study the genotoxicity of the drinking water from the inhabited areas of Ukraine contaminated after the Chernobyl accident. The *Allium cepa* chromosome aberration test provided a rapid screen for toxic and genotoxic effects of chemicals and metal ions (Grant 1994; Fiskesjo 1985; Fiskesjo1993 a). This test has been extensively used for the wastewater monitoring (Fiskesjo 1993b; Nielsen and Rank 1994; Rank and Nielsen 1994; Smaka-Kincl et al., 1996). Moreover, recent studies have proved the *Allium cepa* test as a reliable method for detection of cyto- and genotoxicity of chronic irradiation stemming from the radioactively polluted soil (Kovalchuk O., 1998; Kovalchuk I., 1998).

The *Allium cepa* system described above is sensitive and useful, yet the changes it measures are not understood at the molecular level. The new assay we previously introduced for the study of genotoxicity of radioactively polluted soils, was based on a well-established system which allows the detection of homologous recombination (HR) events in whole living plants (Swoboda et al., 1994). *Arabidopsis thaliana* plants were transformed with two overlapping, non-functional truncated versions of a chimeric β-glucuronidase (*uidA*) marker gene as a recombination substrate (Swoboda et al., 1994).

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In cells in which events of HR at this transgenic locus have occurred the *uidA* gene was restored. Upon histochemical staining, cells expressing β-glucuronidase and their progeny could be precisely localized as blue sectors on white plants, representing recombination events that restored the disrupted gene, and could be easily scored.

Ionizing radiation is a potent mutagene producing double-strand breaks (DSBs) in DNA. DSBs are in part repaired by homologous recombination (Puchta and Hohn, 1996). The DSBs occurring in the repeated part of the transgene are subject to repair. Thus, repair of DSBs via homologous recombination pathway leads to the restoration of β-glucuronidase activity. These events consequently represent a measure for the level of DNA strand breaks in the studied marker gene and, by presumption, of the whole plant genome (Puchta et al., 1995; Kovalchuk I. et al., 1998; Kovalchuk O. et al., 1999). Consequently, we planned and conducted large scale monitoring of the genotoxicity of drinking water using both *A. cepa* and transgenic *A. thaliana*-based tests in parallel.

MATERIALS AND METHODS

Water was sampled from the private wells in the villages from the inhabited 'Chernobyl' and uncontaminated control areas. Chemical and radiological studies of the water samples were conducted. The pH of the water samples ranged between 6.95 and 8.10. For chemical analysis water samples were collected in clean bottles and acidified by the addition of 3 ml of aqueous solution of nitric acid (1:1, V/V) per litre of water. All samples were stored at 4°C before analysis. Chemical analysis included assaying water samples for presence of ammonium, nitrates, nitrites, chlorides and for the content of Cu, Zn, Pb and Cd. Water from the clean and radioactively contaminated territories was also assayed for ^{137}Cs and ^{90}Sr activity, using the β- and γ- spectrometry devices RUB – 91 and RUG - 91M, respectively (Kovalchuk et al., 1998; Moiseev and Ivanov, 1991).

The *Allium cepa* seeds were germinated in the Petri dishes with the water from control and contaminated areas in the dark at 22 °C (10mL/plate). Germination rates were recorded and roots were harvested during the second mitotic cycle, when they were 1.5-2.0 cm long. The harvested roots were fixed in 3:1 (70% ethanol : acetic acid) for 24 hours and then stained with 2% orcein stain (Pausheva, 1980). Root tips were squashed in 45% acetic and examined for mitotic index and cells with chromosomal aberrations. The mitotic index was determined by examination of 400 cells per slide, 5 slides per sample. Chromosomal aberrations were determined by the examination of 500 normal anaphase and early telophase cells (100 cells per slide). The total number of aberrations was taken as 100% and the contribution of each aberration type was expressed as a percentage.

Transgenic *A. thaliana* plants were sown in the Perti dishes on the medium prepared using control or contaminated water and grown under 16 hours light and 8 hours dark at 24°C. The histochemical staining was done on the plants at the full rosette stage (30 days after germination) as described (Swoboda et al., 1994; Kovalchuk I. et al, 1998).). Homologous recombination frequencies (HRF) were obtained by counting recombination events (sectors) in each plant separately, summing them up and relating these data to the number of plants in the population as previously described (Kovalchuk I. et al., 1998). About 200 seeds (±10%) were sown and analysed for each experimental condition. The experiments were repeated 3 times.

Most statistical procedures were described by Sokal and Rohlf, 1995. For each group of samples the mean values were calculated. For the determination of the significance of the difference between the means, the Student's *t*-test for independent variance was used. Correlation analysis was carried out and the significance of the correlation coefficients obtained was tested using Fisher's Z-transformation (Sokal and Rohlf, 1995) as previously reported (Kovalchuk I. et al., 1998). Statistical analysis and plotting were performed using Excel 7.0 for Windows 98 programs.

RESULTS AND DISCUSSION

The radiological characteristics of the clean and radioactively contaminated areas of Ukraine are summarized in Table 1.

Table 1. Radiological characteristics of the soil and water from the clean and radioactively contaminated villages in Ukraine, provided by Regional Sanitary Service and measured as described in Kovalchuk I. et al., 1998 and Moiseev and Ivanov, 1991.

Zone	Village	Soil surface contamination, (C		Specific activity of th water samples, Bq/l
			^{137}Cs	^{90}Sr
Control	Pidlissya	0.1	<3.7	<30
Enhanced radiological control (zone 4)	Pidvysoke	1.5	<3.7	<30
	Potichok	1.8	<3.7	<30
	Stetseva	3.1	<3.7	<30
	Rusiv	3.3	<3.7	<30
Obligatory resettlement zone (zone 2)	Loznitsa	15.0	<3.7	<30
	Narodichi	20.0	<3.7	<30
	Velyki Klischi	40.0	<3.7	<30

The villages Velyki Klischi and Loznitsa and the town Narodichi, all from the obligatory resettlement zone, showed the highest levels of soil radioactive contamination. Notably, the specific activity of the water samples from these villages were below the detection levels for both ^{137}Cs and ^{90}Sr - 3.7 and 30 Bq/kg, respectively (Table 1). The results of the chemical analysis of the water from clean and contaminated territories are shown in Table 2.

Onion seeds were germinated on the water from the clean and contaminated areas. Although the activity of water samples was below the detection level of the spectrometry devices, we observed a slight decrease of the germination rates from 89.4±4.2 in control to 80.0±5.5 in Velyki Klischi ($P<0.05$), accompanied with a decrease of mitotic activity from 38.9±4.2 in control to27.9 ± 2.8 in Velyki Klischi (Figure 1). To study the chromosome aberrations, the root tips were analysed for the presence of cells with bridges, fragments, vagrant and sticky chromosomes, c-mitosis and multipolar anaphases. Bridges and fragments are clastogenic effects, both resulting from chromosome- and chromatid-breaks; vagrants and multipolar anaphases are consequences of weak c-mitosis and can lead to aneuploidy. Chromosome stickiness is usually an irreversible effect, generally leading to cell death (Fiskesjo,1993a). We noted an increase in the fraction of aberrant cells in the root tips of the *Allium cepa* seeds germinated in the water samples from the villages Rusiv and Stetseva of zone 4 and from the villages of zone 2. This was mainly due to the increase of the fraction of cells with bridges and fragments (Figure 2).

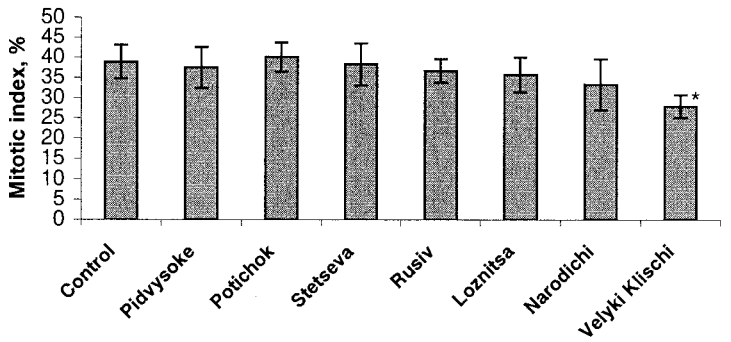

Figure 1. Mitotic index in the root tip cells of *Allium cepa*. *P<0.05.

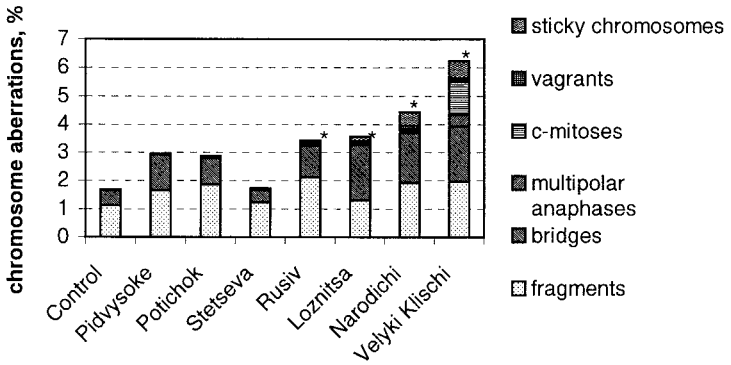

Figure 2. Distribution of the chromosomal aberrations observed in the root tip cells of *Allium cepa*. *P<0.05.

Correlation analysis did not reveal any significant correlation of the chromosomal aberrations with any of the chemical parameters of the water samples. In contrast, the changes observed were found to be strongly and significantly correlated with the soil surface contamination in the studied villages (r>0.85, P<0.05).

Transgenic *A. thaliana* plants were grown on the medium prepared using water from the studied areas. We noted a slight increase of the homologous recombination (HR) frequency in the plants grown on the water samples from the villages of the obligatory resettlement zone (zone 2). No significant increase was noted in the villages of the zone of enhanced radiological control (Figure 3). Similarly with the *A. cepa* test, we did not find any correlation between HR in plants and the chemical parameters of the studied water samples, but, the increase of HRF was found to be strongly correlated with the soil surface contamination in the villages studied (r>0.9, P<0.05). This statistically significant relationship was of particular interest because the correlation of the recombination frequency in the tested *A. thaliana* plant populations with the soil surface contamination in the villages studied was most closely approximated by an exponential growth regression model. In accordance with this model recombination frequencies in the exposed plant populations were slowly rising with the

increase of the soil surface contamination observed in the villages. We noted that the increase of homologous recombination in transgenic *A. thaliana* plants was paralleled by an increased contribution of the fraction of cells with bridges and fragments, indicating a high rate of DNA breakage and non-precise recombination events, to the total aberration rate monitored in *Allium cepa* ($r>0.9$, $P<0.05$).

Table 2. Chemical parameters of the water samples from the clean and radioactively contaminated villages in Ukraine. Permissive levels according to WHO, 1993, 1996.

Village	Chemical parameters (mg/L) ±10%							
	pH	Nitrites	Nitrates	Chlorides	Cu	Pb	Cd	Zn
Pidlissya, control	6.95	-	57.2	120	2.4	0.019	0.0012	0.056
Pidvysoke	7.52	0.4	28.5	123	7.4	0.005	0.0018	0.021
Potichok	7.20	-	85.6	87	4.1	0.011	0.0008	0.310
Stetseva	6.90	-	28.5	77	3.2	0.010	-	0.420
Rusiv	6.98	-	85.6	109	1.6	0.014	-	0.340
Loznitsa	7.60	-	57.2	115	1.7	0.002	0.0015	0.010
Narodichi	7.05	-	85.3	86	2.4	0.013	0.0010	0.250
Velyki Klischi	6.80	-	28.5	110	5.4	0.009	0.0005	0.025
Permissive level	N/A	3.	50.0	250	2.0	0.010	0.0030	n/s

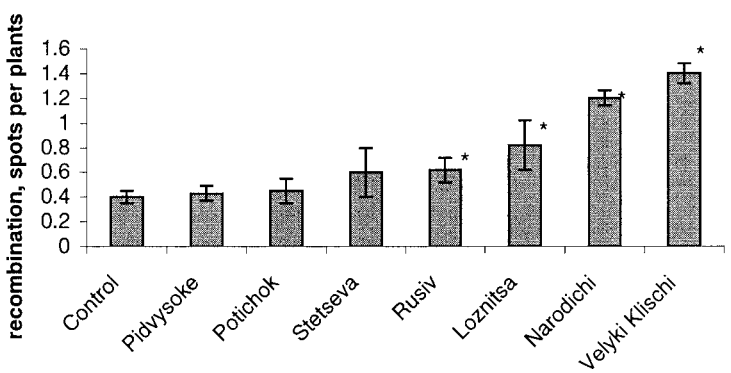

Figure 3. Homologous recombination in the *A. thaliana* plants grown on the water from the radioactively contaminated areas,* $P<0.05$.

Our analyses revealed that the drinking water from the private wells in the village of narodichi and Velyki Klischi belonging to the obligatory resettlement zone contaminated after the Chernobyl NPP accident was genotoxic. We did not find any strong correlation of the microscopic parameters in the *Allium cepa* test and HRF in transgenic plants and the chemical composition of the water from radioactively contaminated areas.

Radiological analyses of the water samples did not reveal any ^{137}Cs or ^{90}Sr activity because the concentration of these nuclides fell below the range that can be detected with the standard equipment. We found that changes in mitotic activity and chromosome aberration rates in *Allium cepa* and the increase of the HRF in *A. thaliana* cultivated on the water from

contaminated regions of Ukraine were correlated with the soil surface radioactive contamination. In contrast to common radiation measuring systems, which did not detect traces of radioactivity in the water, our biomonitoring systems proved to be reliable and sensitive and detected even minute levels of radionuclides. Correlation analysis strongly supports this observation.

Transgenic bioindicator plants and the *Allium cepa* test are powerful bioindicators that can detect the genotoxicity of drinking water despite very low contamination levelsand where dosimetric analysis did not reveal any danger. Previous research indicates that plant bioindicator systems are often more sensitive to certain types of environmental contaminants than animals (Smith, 1991; Wang and Freemark, 1995; Kovalchuk I. et al., 1999). Our plant-based bioassay for monitoring the genotoxicity of drinking water proved to be an effective "alarm system", providing a warning about the possible hazard before economically significant damage has occurred. Our transgenic plants, as sensitive bioindicators at the base of the food chain, may experience the effects of toxicants sooner than higher trophic organisms, thereby reducing the lag period between exposure and significant impact (Lovett Doust et al., 1994). This findings open new horizons for the development of plant-based systems for water monitoring.

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