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Bio-monitoring the genotoxicity of populations of Scots pine in the vicinity of a radioactive waste storage facility

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Abstract

Results of a long-term (1997–2002) study of the Scots pine populations growing in the vicinity of the radioactive waste storage facility ('Radon' LWPE) are presented. Cytogenetic disturbances in reproductive (seeds) and vegetative (needles) tissues sampled from Scots pine populations were studied to examine whether Scots pine trees have experienced environmental stress in areas with relatively low levels of pollution. The data clearly indicate the presence of mutagenic contaminants in the environment of the pine trees. An increased number of mitotic abnormalities, especially multipolar mitoses was found in the pine tree populations submitted to man-made exposure, which suggests that the cytogenetic damage is mainly caused by chemical contamination. A higher radioresistance of the Scots pine seeds from the impacted populations was shown by use of acute γ-irradiation. During the observation period 1997–2002, pine trees exposed to anthropogenic pollution showed a steady increase of cytogenetic alterations in the root meristem cells.

Keywords: Scots pine tree; Cytogenetic disturbances; Radioactive waste storage; Environmental pollution

1. Introduction

Anthropogenic pollution has become inherent to the modern environment. The global and rapid increase in technogenic stress in the biosphere raises the question about possible consequences for biota, including man, acknowledging that all forms of life are inter-connected and that human health is strongly linked to the ecosystem's health. The ubiquitous presence of genetic variation in resistance to pollutants implies that natural selection for resistance must be occurring in natural populations. However, we still need to learn more about the phenomenon before we can assess its general importance in natural ecosystems. In particular, we need to identify the extent of genetic

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changes occurring when organisms are exposed to chronic, low-level, anthropogenic pollutants.

With this in mind, many efforts have been undertaken to develop effective methods for assessing the quality of the environment. Generally, two approaches are used. The more classical one is to take samples of air, water and soil and analyze them in the laboratory by use of routine physico-chemical techniques. An evaluation of genuine exposure characteristics is complicated, however, because the whole list of mutagens involved should be recognised in advance, since most quantification techniques are able to recognize just a specific compound or its metabolites. Consequently, even exhaustive information on exposures in contaminated sites provides only part of the knowledge necessary to evaluate and assess the harmful potential of pollutants for organisms and communities.

The other approach is to score the genotoxic effects in animals or plants that could be exposed in situ in their natural habitat. In contrast to the specific nature of assessments on exposure, studies of biological effects integrate the impacts of all the harmful agents, including synergistic and antagonistic effects. The use of a direct biomarker for genetically relevant damage in the species of concern as a measurement endpoint could remove much of the uncertainty associated with current ecological risk assessments and provide a meaningful indicator of the biological damage. Genetic changes would initially be less obvious than the direct visible effects of pollutants, but in the long run they could be more significant. Furthermore, if this biological or genetic damage has potential reproductive effects, the biomarker could be an ecologically relevant assessment endpoint as well.

Since the 1970s, higher plant bioassays have been recommended [1] for use in mutation screening and monitoring for the detection of genotoxins in emissions, effluents, or ambient environmental media. In most in situ studies, standard indicator plant species such as *Tradescantia*, *Allium cepa* or *Vicia faba* are transferred to contaminated sites and exposed to genotoxic mixtures under natural conditions. Although very sensitive, this approach has some shortcomings [2,3] and is only partially useful in field conditions. One of the possible ways to overcome these problems is to use naturally growing plant species for monitoring purposes.

The growing quantity of radioactive waste in countries possessing nuclear technologies raises concern about their storage and processing. Facilities for the storage of radioactive waste are potentially one of the main sources [4] of hazard for both the public and the environment. In this paper, we discuss the use of Scots pine (*Pinus sylvestris* L.) populations for the assessment of the genotoxicity arising from a radioactive waste storage facility. Specifically, we used the frequency of cytogenetic disturbances in reproductive (seeds) and vegetative (needles) samples taken from Scots pine populations, in order to examine whether Scots pine trees have experienced environmental stress in areas with relatively low levels of pollution.

2. Materials and methods

2.1. Study sites

Three study sites with different levels of environmental pollutions were chosen in the Leningrad Region of Russia to carry out this study: at the 'Radon' Leningrad regional waste-processing enterprise (LWPE) territory, in the centre of the town of Sosnovy Bor, and near the settlement of Bolshaya Izhora. The latter site is at a distance of 30 km from Sosnovy Bor, outside the area likely to be influenced by the complex of nuclear facilities; it was considered as a reference area. The 'Radon' LWPE is located at Copor Bay on the coast of the Gulf of Finland in the Leningrad NPP sanitary-protective zone near the town of Sosnovy Bor. It includes facilities for collection, processing and storing radioactive waste of low and medium activity from the north-western region of Russia. As a consequence of the industrial activity, there has been an increase in the occurrence of rather high concentrations of radionuclides and other pollutants (especially heavy metals) in surface air, snow, pine tree needles and moss in the vicinity of the 'Radon' plant [5]. Nevertheless, over a long operation period of the nuclear facility (since the early 1970s), doses absorbed by the biota and population from technogenic radionuclides did not exceed the levels officially adopted as permissible. From the radioecological monitoring data [5], the 'Radon' LWPE site was expected to experience the highest environmental stress among the study sites chosen. The external γ-radiation dose rates measured with

the DRG-01T dosimeter at a height of 1 m and averaged over the whole investigation period of 1997–2002 amount to 0.90 pA/kg (12.6 μR/h) in Bolshaya Izhora, 0.92 pA/kg (12.8 μR/h) in Sosnovy Bor, and 1.35 pA/kg (18.8 μR/h) at the 'Radon' LWPE territory.

2.2. Indicator organism and sampling

Scots pine (*Pinus sylvestris* L.), the dominant tree species in North European and Asian boreal forests, was chosen as test species for assessment of the possible effects of the 'Radon' enterprise on the environment. The reproductive organs of the coniferous trees, with their complex organisation and long duration of the reproductive cycle, are the most sensitive to the damaging influence of a wide range of anthropogenic contaminants. Therefore, the occurrence of chromosome aberrations in root-meristem cells of the first mitosis at seed germination is well suited as a biomarker of cumulative environmental exposure. Because coniferous trees generally show a high retention capacity and low turn-over rate for contaminants taken up by aerial biomass from the atmosphere, an assessment of cytogenetic anomalies in the intercalar meristem of young needles also appears to be a promising test system.

Pine cones were collected in the autumn of the years 1997–2002 from Scots pine trees in three populations located at the study sites. Young shoots, 20–30 mm in length, were sampled at the same three sites and, additionally, near the boundary fence of the 'Radon' LWPE, in the spring of the years 1998–2002. On each plot, samples were taken from 10–15 trees within homogenous stands where pine trees were abundant.

2.3. Sample treatment and cytogenetic analysis

The sampled pine cones were conditioned to ripen up to dehiscing. The resultant seeds were de-winged by hand and germinated on damp filter paper in Petri dishes at a controlled temperature of 24 °C. The seedling roots in the stage of first mitosis (at a length of 8–14 mm) as well as the needle sampled were fixed in acetic acid:alcohol (1:3). Temporary squashed preparations of seedling root apical meristem and intercalar meristem of young needles were made and stained with aceto-orcein. In each preparation, all the ana-telophase cells (2700–16,500 cells at each plot) were scored to determine the fraction of cells with alterations. Chromosomal aberrations in ana-telophases (chromatid (single) and chromosome (double) bridges and fragments) were distinguished from mitotic abnormalities (multipolar mitoses and lagging chromosomes) because of their different origins (the origin of chromosomal aberrations are affected chromosomes, while mitotic abnormalities are due to an injured mitotic apparatus, particularly the mitotic spindle).

2.4. Irradiation of seeds

A portion of the seeds was acutely irradiated at room temperature with γ-rays from ^{60}Co (Lutch Irradiator, Latenergo, Latvia) at a dose of 15 Gy, dose rate of 0.6 Gy min^{-1}. After irradiation, seeds were germinated and, at a root length of ≈8–14 mm, 23–55 seedlings were immediately fixed in acetic acid:alcohol (1:3). One thousand and four hundred to 5000 ana-telophase cells were scored for cytogenetic alterations.

2.5. Statistical analysis

A set of cytogenetic data picked up was optimised by a method of an empirical distribution analysis to provide an estimation of the examined parameters with a certain relative probable error (25%) at the given confidence level (95%) [6]. The values were screened and statistical outliers were excluded from the calculation. Variations in the scored values between trees in all the populations were statistically insignificant, and the data for these trees were, therefore, pooled within every studied population into single sets. To determine the significance of the difference between the sample mean values, the Student's *t*-test for independent variance was applied. To fit the temporal changes of the cytogenetic alteration frequency, a regression analysis was used [7].

To test a hypothesis whether mathematical model 2 fits the experimental data significantly better than model 1, the Hayek criterion was applied [8]:

$$H = \sqrt{\frac{\mu(R_2^2 - R_1^2)}{1 - R_2^2}}, \quad R_2^2 > R_1^2,$$

where R_1^2 and R_2^2 are the multiple correlation coefficients for models 1 and 2, and μ the degrees of freedom of model 2. *H*-statistics follows the Student distribution.

3. Results

3.1. Frequency of aberrant cells

In the present study, attempts were made to understand whether or not any differences existed in responses of the reference Scots pine population and those under low-level stressful influence, measured as frequency of cytogenetic disturbances. Although most angiosperm species have a reproductive cycle lasting for several months, it takes Scots pine seeds at least 18–20 months to mature from micro- and megaspore formation [9]. Such a long maturation period means that significant and observable DNA damage may accumulate in the undifferentiated stem cells, even from exposure to low doses (or dose rates) or low concentrations of anthropogenic contaminants. This latent cumulative damage mainly appears when cells come into the first mitosis during seed germination, and can be registered as chromosome aberrations in the root meristem cells. The yields of aberrant cells in the seedling root apical meristem of the reference samples (from Bolshaya Izhora in 1997–2002) showed no significant differences (Table 1). The frequencies of cytogenetic disturbances in seedling root meristem and needle meristem for samples collected at the 'Radon' LWPE site and near the fence in 1997–2002 were significantly above the corresponding reference values (Table 1). The frequencies of cytogenetic disturbances at the 'Radon' LWPE site were, however, well below those found in our previous study [10] in the Scots pine populations from the 30 km Chernobyl NPP zone where doses of 10–20 Gy were received [11] in 1986 following the Chernobyl accident. While the incidence of cytogenetic damage in the samples from the 30 km Chernobyl NPP zone increased [10] with radiation exposure, the cytogenetic damage found in the seed and needle samples from the 'Radon' LWPE site were much larger than those expected to occur from the very small external doses monitored.

The cytogenetic damage in both the seed and needle samples from the Sosnovy Bor site were

Table 1
Aberrant cell frequency in seedling root apical and needle intercalar meristem of Scots pine (mean ± S.E.)

Year	Plot	Seedlings		Needles	
		Cells scored	Aberrant cells, %	Cells scored	Aberrant cells, %
1997	Bolshaya Izhora	14643	0.60 ± 0.06		
	Sosnovy Bor	12342	1.19 ± 0.10[a]		
	'Radon' LWPE	7927	1.53 ± 0.14[a]		
1998	Bolshaya Izhora	12217	0.53 ± 0.07	10156	0.97 ± 0.10
	Sosnovy Bor	12832	1.30 ± 0.10[a]	12084	1.36 ± 0.11
	'Radon' LWPE fence			11376	1.60 ± 0.12[a]
	'Radon' LWPE	9437	1.73 ± 0.13[a]	5274	2.73 ± 0.22[a]
1999	Bolshaya Izhora	16482	0.57 ± 0.06	5549	0.81 ± 0.12
	Sosnovy Bor	8302	1.36 ± 0.13[a]	3724	1.67 ± 0.21[a]
	'Radon' LWPE fence			4026	1.49 ± 0.19[a]
	'Radon' LWPE	5613	1.73 ± 0.17[a]	3943	2.21 ± 0.23[a]
2000	Bolshaya Izhora	9885	0.66 ± 0.08	11206	0.88 ± 0.09
	Sosnovy Bor	3517	1.73 ± 0.22[a]	5798	1.64 ± 0.17[a]
	'Radon' LWPE fence			5361	1.79 ± 0.18[a]
	'Radon' LWPE	2674	2.28 ± 0.29[a]	4374	2.29 ± 0.23[a]
2001	Bolshaya Izhora	14007	0.69 ± 0.07	6823	0.89 ± 0.11
	Sosnovy Bor	5229	1.84 ± 0.19[a]	3663	1.69 ± 0.21[a]
	'Radon' LWPE	4355	2.18 ± 0.22[a]	3191	2.76 ± 0.29[a]
2002	Bolshaya Izhora	13790	0.70 ± 0.07	10868	0.88 ± 0.09
	Sosnovy Bor	5929	1.75 ± 0.17[a]	5333	1.78 ± 0.18[a]
	'Radon' LWPE	4912	2.16 ± 0.21[a]	5441	2.21 ± 0.20[a]

[a] Difference from the reference site is significant; $p < 5\%$.

significantly higher (Table 1) than the reference values in all cases, with the exception of the needles sampled in 1998. These results were rather surprising because cytogenetic disturbances in this population were not expected to exceed the control. Moreover, dose rates within this site did not notably exceed the reference values throughout the whole observation period. However, the data strongly suggest the presence of some mutagenic contaminants in the environment of the Scots pine populations, not only at the 'Radon' LWPE site, but also in the town of Sosnovy Bor.

3.2. Temporal changes in the yield of cytogenetic disturbances

The temporal changes of the cytogenetic disturbances in seedling root meristem from 1997 to 2002 are shown in Fig. 1. Fitting the data by linear model showed strong differences in temporal dependence observed in the reference and exposed Scots pine populations. Thus, rates of cytogenetic disturbances increased with time by $0.03 \pm 0.01\%$ per year in the reference, and by 0.14 ± 0.03 and $0.15 \pm 0.04\%$ per year in Sosnovy Bor and the 'Radon' LWPE pine populations, respectively, as estimated from the linear regression coefficients, and differed significantly ($p < 0.05$) between the reference and the exposed populations.

The temporal dependence of the level of cytogenetic disturbances in the intercalar meristem of the needles are clearly different (Fig. 2) from those in the root meristem. Although the percentage of aberrant cells simulated by the linear regression model for the Bolshaya Izhora population lies significantly below ($p < 0.05$) those for the impacted populations, there is no statistically significant tendency for an increase of the percentage of aberrant cells with time.

Although the time dependence of aberrant cell frequencies in seedling root meristem could be satisfactorily fitted with a linear model, the steady growth of cytogenetic damage with time in all the pine populations required more detailed analysis. Table 2 presents results of a non-linear regression of experimental data by different mathematical models in comparison with an approximation quality of the linear model. The findings demonstrate that, in all the pine populations, polynomial models 2–4 fit the data much better than the linear model 1 since they provide lesser squared sums of residuals (SSR) and greater Fisher

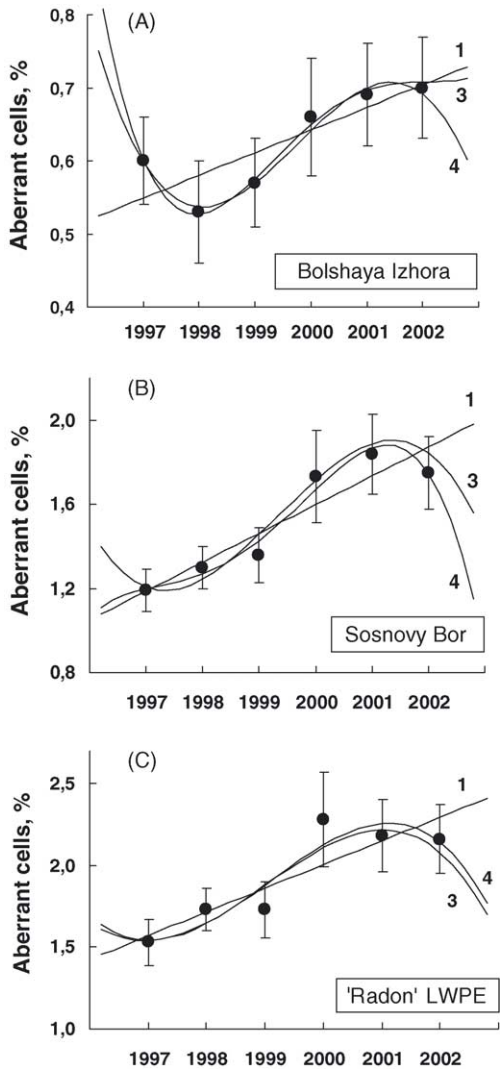

Fig. 1. Aberrant cell percentages in seedling root meristem of Scots pine trees in dependence of year and their approximation by the best models. 1—linear model, 3 and 4—polynomial models of third and fourth degree, respectively (see Table 2).

statistics (F) and multiple correlation coefficient (R^2) values.

In calculating the structural minimization criterion (T) [12], however, a 'penalty' is applied for an approximating model complication. In the reference population of Bolshaya Izhora, T-values show that the benefits of models 3 and 4 are not stipulated by an augmentation of the model pliability through the additional items. And finally, a Hayek criterion testifies against the linear

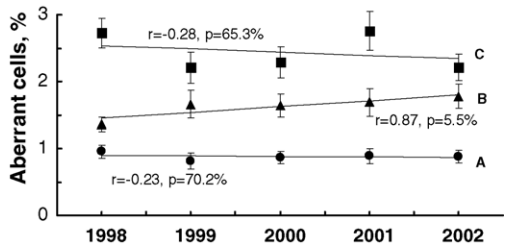

Fig. 2. Linear regressions of the aberrant cells percentage in intercalar meristem of Scots pine trees. Test-sites indication: A—Bolshaya Izhora, B—Sosnovy Bor, C—'Radon' LWPE.

model in favor of model 3. In other words, this could be considered as a proof for cytogenetic parameters to have a tendency of cyclic fluctuations over time at the reference site (Fig. 1A).

In the affected populations not only polynomial but also other models ($\sqrt{t}$, $\ln t$) show some advantage over the linear one (Table 2). Although there is a similar tendency to temporal fluctuations of aberrant cell frequencies (Fig. 1B and C), however, there is no model with confident preference over the linear approximation (Table 2).

3.3. Analysis of the cytogenetic disturbances spectrum

Table 3 presents the number of alterations of different types and frequencies of the chromosomal (double and single) and mitotic (chromosome laggings and multipolar mitoses) disturbances observed.

Table 2
Quality of approximation of time dependence of the percentage of aberrant cells in Scots pine seedlings root meristem by various models

Model	n	SSR, $\times 10^{-3}$	F	R^2, %	T, $\times 10^{-3}$	H	p
Bolshaya Izhora							
1. $a_0 + a_1 t$	2	7.3	9.2^{**}	69.6	3.6	–	–
2. $a_0 + a_1 t + a_2 t^2$	3	**5.5**	$\mathbf{10.1}^{**}$	**77.0**	5.5	0.7	0.51
3. $a_0 + a_1 t + a_2 t^2 + a_3 t^3$	4	**0.4**	$\mathbf{122.4}^{***}$	**98.4**	**0.8**	**4.2**	**0.05**
4. $a_0 + a_1 t + \cdots + a_4 t^4$	5	**0.1**	$\mathbf{201.9}^{***}$	**99.5**	**0.6**	5.5	0.11
5. $a_0 + a_1 \sqrt{t}$	2	9.3	6.3^{*}	61.2	4.6		
6. $a_0 + a_1 \ln(t)$	2	12.0	4.0	50.1	6.0		
7. $a_0 + a_1 e^t$	2	10.0	5.5^{*}	57.7	5.0		
8. $a_0 + a_1/t$	2	17.0	1.5	27.4	8.5		
Sosnovy Bor							
1. $a_0 + a_1 t$	2	53.0	24.7^{***}	86.1	26.5	–	–
2. $a_0 + a_1 t + a_2 t^2$	3	**46.0**	22.0^{***}	**88.0**	46.0	0.5	0.64
3. $a_0 + a_1 t + a_2 t^2 + a_3 t^3$	4	**13.0**	$\mathbf{55.9}^{***}$	**96.5**	**26.0**	1.8	0.22
4. $a_0 + a_1 t + \cdots + a_4 t^4$	5	**10.0**	$\mathbf{35.4}^{***}$	**97.3**	50.0	1.4	0.39
5. $a_0 + a_1 \sqrt{t}$	2	**51.0**	$\mathbf{25.8}^{***}$	**86.6**	**25.5**	0.3	0.79
6. $a_0 + a_1 \ln(t)$	2	63.0	20.1^{**}	83.4	31.5		
7. $a_0 + a_1 e^t$	2	216.0	3.1	43.3	108.0		
8. $a_0 + a_1/t$	2	119.0	8.9^{**}	68.9	59.5		
'Radon' LWPE							
1. $a_0 + a_1 t$	2	115.0	12.7^{**}	76.1	57.5	–	–
2. $a_0 + a_1 t + a_2 t^2$	3	**88.0**	$\mathbf{13.5}^{**}$	**81.9**	88.0	0.7	0.52
3. $a_0 + a_1 t + a_2 t^2 + a_3 t^3$	4	**60.0**	$\mathbf{14.1}^{**}$	**87.6**	120.0	1.0	0.43
4. $a_0 + a_1 t + \cdots + a_4 t^4$	5	**60.0**	7.0^{*}	**87.6**	300.0	0.7	0.61
5. $a_0 + a_1 \sqrt{t}$	2	**102.0**	$\mathbf{15.0}^{**}$	**70.0**	**51.0**	0.6	0.61
6. $a_0 + a_1 \ln(t)$	2	**103.0**	$\mathbf{14.7}^{**}$	**78.6**	**51.5**	0.5	0.63
7. $a_0 + a_1 e^t$	2	322.0	2.0	33.3	161.0		
8. $a_0 + a_1/t$	2	149.0	9.0^{**}	69.2	74.5		

a_0, a_1, . . ., a_4—regression coefficients; t—time (year of observation); n—number of parameters in a model; SSR—squared sum of residuals; F—Fisher statistics; R^2—multiple correlation coefficient; T—structural identification criteria [12]; H—Hayek statistics; p—significance of H-statistics. The regression with a given model is significant at: *—$p < 10\%$, **—$p < 5\%$, ***—$p < 1\%$. Values showing that the given model exceeds the linear model in approximation quality are marked in bold.

Table 3
Alteration spectrum and frequencies of different types of disturbance in root meristem cells of Scots pine seedlings

Year	Plot	Number of alterations						Frequency of alterations		
		f″	m″	f′	m′	g	mp	f″ + m″	f′ + m′	g + mp
1997	B. Izhora	16	7	4	44	19	0	0.16 ± 0.04	0.32 ± 0.05	0.18 ± 0.05
	Sosn. Bor	38[a]	33[a]	9	38	28	5[a]	0.74 ± 0.14[b]	0.36 ± 0.06	0.25 ± 0.05
	LWPE	35[a]	18	17[a]	28	24	2	0.62 ± 0.10[b]	0.57 ± 0.10	0.29 ± 0.07
1998	B. Izhora	31	4	4	12	16	0	0.35 ± 0.07	0.12 ± 0.03	0.12 ± 0.04
	Sosn. Bor	44	31[a]	5	28	85[a]	9[a]	0.70 ± 0.10[b]	0.22 ± 0.04	0.63 ± 0.10[b]
	LWPE	56	28[a]	7	25	55[a]	8[a]	0.86 ± 0.13[b]	0.29 ± 0.07	0.70 ± 0.15[b]
1999	B. Izhora	13	23	0	36	22	0	0.22 ± 0.04	0.22 ± 0.03	0.15 ± 0.03
	Sosn. Bor	14	32[a]	0	27	42[a]	9[a]	0.54 ± 0.10[b]	0.32 ± 0.07	0.61 ± 0.09[b]
	LWPE	9	31[a]	0	37[a]	23[a]	10[a]	0.65 ± 0.16[b]	0.62 ± 0.10[b]	0.58 ± 0.10[b]
2000	B. Izhora	12	20	2	20	12	0	0.28 ± 0.04	0.21 ± 0.04	0.10 ± 0.03
	Sosn. Bor	19[a]	12	0	11	23[a]	0	0.93 ± 0.14[b]	0.44 ± 0.12	0.60 ± 0.13[b]
	LWPE	13	9	0	26[a]	14[a]	3	0.80 ± 0.18[b]	0.88 ± 0.15[b]	0.82 ± 0.18[b]
2001	B. Izhora	10	41	1	23	27	0	0.29 ± 0.04	0.15 ± 0.03	0.16 ± 0.03
	Sosn. Bor	10	28	0	32[a]	27	7[a]	0.66 ± 0.11[b]	0.76 ± 0.16[b]	0.58 ± 0.12[b]
	LWPE	9	16	0	29[a]	35[a]	11[a]	0.57 ± 0.12[b]	0.61 ± 0.12[b]	1.15 ± 0.15[b]
2002	B. Izhora	14	57	0	8	27	0	0.43 ± 0.05	0.06 ± 0.02	0.16 ± 0.04
	Sosn. Bor	5	62[a]	1	11	38[a]	2	0.91 ± 0.15[b]	0.18 ± 0.06	0.53 ± 0.09[b]
	LWPE	11	62[a]	0	8	45[a]	1	1.24 ± 0.19[b]	0.13 ± 0.06	0.80 ± 0.14[b]

f″, m″—chromosome fragments and bridges; f′, m′—chromatid fragments and bridges; g—chromosome laggings; mp—multipolar mitoses.
[a] Difference of the disturbance frequency from the reference one is significant, $p < 5\%$.
[b] Difference from the reference site is significant; $p < 5\%$.

The occurrence of mitotic anomalies in the Scots pine populations of Sosnovy Bor and the 'Radon' LWPE site exceed corresponding reference levels, and this excess is significant in every year of study except for 1997 (Table 3). These observations are in agreement with the outcome of a study [13] where the highest number of mitotic abnormalities, especially multipolar spindles, was found at two smelting plant locations. In our present (Table 3) and previous [10] studies, multipolar mitoses in seedling root meristem cells were also found only in the samples from both the 'Radon' LWPE and Sosnovy Bor sites, but not from either the reference sites or those within the 30 km Chernobyl NPP zone. There were some differences in the multipolar mitoses in reproductive (seeds) and vegetative (needles) tissues. Whereas multipolar mitoses were found in seedling root meristem cells of the exposed pine populations in each year of the study except in 2000 in Sosnovy Bor—and their frequency was often well above zero (Table 3)—in needle intercalar meristem only three cells with multipolar mitoses were recognised overall (Sosnovy Bor, 1999 and 2000; 'Radon' LWPE, 2002), and the corresponding frequencies were not different from zero.

In this study, chromosome aberrations (double fragments and bridges) made an unusually high contribution to the total disturbances in root meristem. In the reference population they constituted 25–67% of the total number of alterations. Since similar data were obtained in our studies of Scots pine populations in other regions of Russia considered non-contaminated (Obninsk, 1995 [10]; Bryansk region, 2003, unpublished data), such a phenomenon seems to be peculiar to the study species. Chromosome aberration frequencies in the affected populations showed a steady increase over the reference level throughout the period 1997–2002.

3.4. Acute radiation exposure of the seeds

A portion of the seeds collected in 1999–2001 was subjected to a subsequent acute γ-ray exposure. The

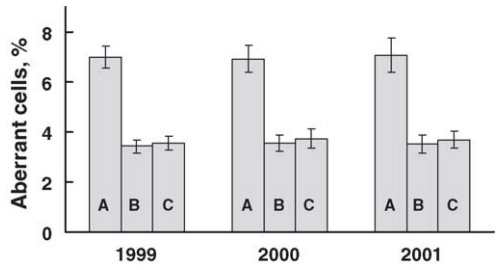

Fig. 3. Aberrant cell frequency in root meristem of Scots pine seedlings grown from seeds exposed to an acute γ-ray dose of 15 Gy. Test sites indication is the same as in Fig. 2.

seeds from the Scots pine populations that are experiencing anthropogenic exposure and are characterized by an enhanced level of cytogenetic disturbances, showed higher resistance to the acute γ-ray exposure than those from the reference population (Fig. 3). As a corollary, a sharp difference in the increment of cytogenetic disturbances between the reference and exposed Scots pine populations induced by acute radiation was observed. The acute γ-ray dose of 15 Gy increased the cytogenetic disturbance level 11.0 times (averaged value from 1999 to 2001) in the reference population, while exposure of the seeds from Sosnovy Bor and the 'Radon' LWPE site to the same radiation dose changed the corresponding values only 2.2 and 1.8 times, respectively. Kalchenko and Fedotov [11] found similar results in studies of the acute γ-radiation resistance of Scots pine seeds collected in the 30 km Chernobyl NPP zone in 1997.

4. Discussion

A highly significant increase in cytogenetic disturbances in both vegetative (needles) and reproductive (seeds) organs of in situ Scots pine trees have been observed in the vicinity of a radioactive waste storage facility. This strongly suggests the presence of genotoxic factor(s) in the environment near the nuclear facilities.

Levels of cytogenetic damage in needles sampled both from the reference and non-reference sites were higher than in the seedling root meristem. Most likely, this originates from the relative differences in sensitivity of the intercalar meristem cells and the root apical meristem cells, and agrees with our earlier data obtained for agricultural plants [14]. The detection of cytogenetic disturbances in our experiment might only be tip of the iceberg, reflecting global structural and functional genome rearrangements induced by external exposure. In this connection, it is important to distinguish between the genetic changes in the vegetative and reproductive organs. The former have effects on adult trees, and thereby on the current population. The latter are less numerous [14,15] but could affect future generations. Both would affect the genetic composition of populations but in rather different ways.

In long-term experiments carried out around five nuclear power plants in Japan [16], significantly increased mutation frequencies in the stamen hairs of *Tradescantia* have been correlated to the operation periods of the nuclear facilities and the predominant wind direction, but not to the accumulated external radiation exposure of the inflorescences. The increases of pink mutations detected in the *Tradescantia* stamen hairs were much larger than those expected to occur with the very small external doses monitored. Nevertheless, in that study, ionizing radiation was suggested [16] to play a decisive role in the increasing mutation incidence. In our study the external dose is also too small to explain the observed effect. However, unlike the hypothesis suggested in [16], it does not seem that ionising radiation is a determining factor in the enhanced cytogenetic response in the vicinity of the 'Radon' LWPE.

Additional information on the possible factors affecting the trees may be obtained from analyzing the spectrum of cytogenetic disturbances. This is a very important, but also a most neglected topic. The specificity of an impact from pollutants of a certain type implies [17,18] that spectrum of cytogenetic disturbances can in principle be applied to deduce the agent responsible for an increase in the level of abnormalities.

Though Scots pine is very often used in biological monitoring programs [9–11,13], publications on the spectrum of cytogenetic abnormalities in Scots pine, both in control conditions and under environmental stress, are scarce in the literature. Thus, total frequencies of chromosomal aberrations and mitotic abnormalities are given in [13] for two species of the genus *Pinus* growing in the vicinity of two smelting plants in Slovakia. Detailed information on aberration spectra obtained from our preliminary observations of Scots pine populations growing in the vicinity of the 'Radon' LWPE as well as in the Chernobyl Exclusion Zone were presented in [10]. Other authors

either have used non-cytogenetic test-systems or contented themselves with total rates of cytogenetical disturbances without subdividing them into groups. Thus, to our knowledge, Table 3 presents the most exhaustive data on the cytogenetic disturbance spectrum in reproductive (seeds) and vegetative (needles) tissues of Scots pine induced spontaneously and under conditions of environmental stress.

Concerning the high contribution of chromosome aberrations to the spectrum, this phenomenon does not seem to refer to exposure to radiation or any radio-mimetic agent, but biological features of the test species. An explanation could possibly be the long maturation period when regular spontaneous disorders might be processed in such a way that they appear as chromosome aberrations at the stage of first mitosis in germinated roots. Despite this feature of Scots pine, an indication of environmental stress with chromosome aberrations in seedling root meristem shows stable and consistent results, since the aberration frequencies in Scots pine populations of Sosnovy Bor and the 'Radon' LWPE test sites were significantly increased compared with the reference population throughout the 6 years of observation (Table 3).

From Table 3, the occurrence of mitotic anomalies in the exposed populations is above the reference values in all cases. Furthermore, multipolar mitoses, rather rare anomalies, were found only in the samples from the 'Radon' LWPE and Sosnovy Bor sites, but not in the samples from either the reference sites of Bolshaya Izhora and Obninsk [10], or the radioactively contaminated sites within the 30 km Chernobyl NPP zone [10]. An appearance of multipolar mitoses is possibly linked to spindle damage [19,20]. A significant increase in multipolar mitoses was found in *Syringa vulgaris* L. and *Armeniaca vulgaris* Lam. (none was found in the controls) [21]; authors associated them with heavy-metal soil contamination. This is in line with the results obtained in the study of *Pinus sylvestris* L. and *Pinus nigra* Arn. growing in the vicinity of two smelting plants in Slovakia [13]. Indeed, heavy metals are typical aneugenic agents that preferentially act on DNA indirectly [22,23], but can efficiently induce spindle damage [20,24]. Thus, the analysis of the spectrum of cytogenetic disturbances taken together with the dosimetry data suggest that the main contribution to induction of cytogenetic effects registered in the Sosnovy Bor region is most likely caused by the environmental contamination with genotoxic chemicals. Similar results were obtained in our previous work [18] on genotoxicity and toxicity of water samples from a radium production industry storage-cell area (Komi Republic, Russia) by means of the *Allium*-test, and in a study of genetic variability and reproductive potential in *Gambusia affinis* populations in radioactively contaminated reservoirs in Oak Ridge, USA [25].

The observations of the Scots pine populations over several years made it possible to trace temporal changes in cytogenetic damage. If registered variations were not stochastic but causal, ignoring them could lead to an unsuitable or even wrong forecast of the further development. The clear and steady increase of cytogenetic alterations was revealed in the root meristem cells. A reason for this is not easy to find out. Man-made influences of nearby facilities would seem to be an apparent cause of this stable temporal increase in the frequency of aberrant cells in the exposed populations. On the other hand, the slight increase of cytogenetic disturbances in the control population may reflect global environmental deterioration. It is generally accepted that regular fluctuations with time are inherent in many of natural processes. It is, therefore, not surprising that a more thorough consideration of time dependence revealed (Table 2) that cytogenetic parameters at the reference site show a trend to cyclic fluctuation over time, whereas in the technogenically affected populations these peculiarities could not be revealed with confidence. Thus, the technogenic impact in this region is strong enough to destroy natural regularities. This very interesting finding needs further investigation.

The level of cytogenetic disturbances in root meristem of seedlings and intercalar meristem of needles differed in a qualitative manner. The most plausible reason for this is a difference in the biological features of the test systems used. For the seedling root meristem analysis, aberrations are registered at the stage of first mitosis, when most of the primary damages appear that have accumulated over a long period. Therefore, this test-system reflects the cumulative exposure of the plants. When analyzing the cytogenetic disturbances in the intercalar meristem of the needles, we are dealing with a non-synchronized cell population. The frequency of cytogenetic disturbances in this case reflects a balance between the continuous induction caused by ongoing exposure and the elimination of cells bearing aberrations from the cell population. Consequently,

this endpoint provides a snapshot picture of the current exposure rates. The essential differences of these test-systems in their uptake and exposure time of man-caused pollutants cannot be ruled out.

One of the consequences of chronic irradiation in natural populations has been observed in the East Ural trail region [26,27], i.e. an apparent increase in the mean radioresistance of the seeds to an additional acute γ-radiation exposure—the so-called "radio-adaptation phenomenon". Experimental studies of repair inhibitors, dose–effect relationships for low- and high-LET radiations, measurements of unscheduled DNA synthesis and an efficacy of the recovery of single-strand breaks [26,28] allow the conclusion that the divergence of populations in terms of radioresistance is connected with a selection for changes in the effectiveness of the repair systems. Consequently, the appearance of factors (either of natural origin or man-made) in the plants' environment activates genetic mechanisms, changing a population's resistance to this exposure.

In [29,30] a distinction in genetic structure was found between *Pinus sylvestris* L. populations in areas with different levels of man-made pollution. This supports the assumption that the long-term impact of the nuclear industry facilities on Scots pine populations in the vicinity of the town of Sosnovy Bor, diagnosed reliably by means of the cytogenetic tests, can change their genetic make-up. The natural selection among the available variants that fit in new conditions results in the change of mean values of the quantitative traits. Consequently, an increase of the variability in a population is a reflection of the induced adaptive processes, and a difference of mean values is a quantitative measure of the shifts that occurred under a natural selection pressure. Actually, the data presented in Table 1 indicate that Scots pine populations experiencing anthropogenic exposure have a significantly increased level of cytogenetic variability as a whole. One of the aspects of the adaptive process that occurred in the Scots pine populations is a much higher radioresistance of the seeds, as detected in our experiments with the acute γ-ray exposure (Fig. 3).

Most investigations in which actual evolution of resistance has been confirmed [26,27,31,32] involved plants with relatively short life cycles. In combination with an intense selection, a short life cycle can result in a rapid genetic change in a population. But in most natural ecosystems, the dominant plants are often long-lived trees and other perennials. In combination with lower selection pressures, this probably limits the rate at which natural populations might become significantly more resistant to pollutants. However, the only conclusion that can be drawn from our findings is that the evolution of resistance to pollutants may be extremely rapid in this case also.

The number of generations of pine trees during the existence of the 'Radon' LWPE is obviously insufficient for natural selection, in the classical sense, on the basis of the efficacy of repair systems. Even if virtually all mutations are favourable, it is difficult to imagine that this would lead to a sufficient and rapid adaptation of plant populations within several years, even with the increased mutation rates shown in this study. One possible explanation could be a selection on the basis of cellular radiosensitivity, i.e. a selective elimination of sensitive cells and their apparent replacement with more radioresistant cells [11]. An alternative explanation concerns the possible existence of an induced epigenetic change in the activity of functional genes that is heritable over a number of cell generations. This hypothesis has found support recently [33] when genomic DNA of pine trees exposed during the Chernobyl disaster was shown to be considerably hyper-methylated in a dose-dependent manner. Methylation patterns are believed to be inherited through generations [34]; their changing may lead to alterations in expression of various stress-related and housekeeping genes. Further studies are clearly needed to analyze an involvement of epigenetic mechanisms in the adaptive response of wild plant populations to chronic environmental exposure.

The health status of coniferous forests is most often evaluated using needle coloration, crown transparency and other descriptive features [35]. Therefore, there is a need for a feasible and impartial approach to health assessment in conifers (and other species) at a premonitory stage when environmental stress has not yet affected plant growth or vigour. Our findings demonstrate that analysis of the frequency of cytogenetic disturbances in both the reproductive (seeds) and vegetative (needles) structures of Scots pine may be used to quantify pollution-induced stress not only in areas with prominent damage to the conifers, but also in forests with slight or no visible symptoms of a pollution impact.

Overall, the results described here clearly indicate that the bio-indicator approach based on analysis of the frequency of cytogenetic disturbances in the reproductive and vegetative structures of Scots pine not only provides tools for an efficient diagnosis of man-made contaminations of different types, but also enables us to draw plausible conjectures about their nature, as well as explore the dynamics and trends in adaptive processes in plant populations. Therefore, the use of Scots pine trees for the in situ bio-monitoring of the genotoxicity of the environmental factors seems to be promising.

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References

[1] W.F. Grant, Higher plant assays for the detection of chromosomal aberrations and gene mutations—a brief historical background on their use for screening and monitoring environmental chemicals, Mutat. Res. 426 (1999) 107–112.
[2] J.R. Lazutka, A. Stapulionyte, D.K. Bjerketvedt, A. Odland, Seasonal variation in the frequency of abnormal anaphases and mitotic index values in wild populations of herb-Paris (*Paris quqdrifolia* L., Trilliaceae): implications for genetic monitoring, Mutat. Res. 534 (2003) 113–122.
[3] R. Laskowski, Why short-term bioassays are not meaningful—effects of a pesticide (imidacloprid) and a metal (cadmium) on pea aphids (*Acyrthosiphon pisum* Harris), Ecotoxicology 10 (2001) 177–183.
[4] I.I. Kryshev, E.P. Ryasantzev, Ecological Safety of Nuclear Fuel Cycle Industry in Russia, IzdAT, Moscow, 2000.
[5] L.D. Blinova, Littoral ecosystem radioecological monitoring at the region of atomic energy enterprises, in: B. Amiro, R. Avadhanula, G. Johansson, C.-M. Larsson, M. Lüning (Eds.), Protection of the Natural Environment. Proceedings of the International Symposium on Ionizing Radiation, vol. 2, Stockholm, Sweden, 1996, pp. 497–503.
[6] S.A. Geras'kin, S.V. Fesenko, L.G. Chernyaeva, N.I. Sanzharova, Statistical method of empirical distribution analysis of coefficient of radionuclides' accumulation in plant, Agric. Biol. 1 (1994) 130–137.
[7] N.R. Draper, H. Smith, Applied Regression Analysis, Wiley, New York, 1981.
[8] J. Gofman, Radiation-induced Cancer from Low Dose Exposure: An Independent Analysis, CNR Book Division, San Francisco, 1990.
[9] G.M. Kozubov, A.I. Taskaev, Radiobiological and Radioecological Studies of Woody Plants, Nauka, St Petersburg, 1994.
[10] S.A. Geras'kin, L.M. Zimina, V.G. Dikarev, N.S. Dikareva, V.L. Zimin, D.V. Vasiliyev, A.A. Oudalova, L.D. Blinova, R.M. Alexakhin, Bioindication of the anthropogenic effects on micropopulations of *Pinus sylvestris*, L. in the vicinity of a plant for the storage and processing of radioactive waste and in the Chernobyl NPP zone, J. Environ. Radioactivity 66 (2003) 171–180.
[11] V.A. Kalchenko, I.S. Fedotov, Genetics effects of acute and chronic ionizing radiation on *Pinus sylvestris* L. inhabiting the Chernobyl meltdown area, Russ. J. Genet. 37 (2001) 427–447.
[12] S.A. Geras'kin, B.I. Sarapul'tzev, Automatic classification of biological objects on the level of their radioresistance, Automat. Rem. Contr. 54 (1993) 182–189.
[13] K. Micieta, G. Murin, Three species of genus *Pinus* suitable as bioindicators of polluted environment, Water Air Soil Pollut. 104 (1998) 413–422.
[14] S.A. Geras'kin, V.G. Dikarev, Ye.Ya. Zyablitskaya, A.A. Oudalova, Ye.V. Spirin, R.M. Alexakhin, Genetic consequences of radioactive contamination by the Chernobyl fallout to agricultural crops, J. Environ. Radioactivity 66 (2003) 155–169.
[15] R.B. Cervantes, J.R. Stringer, C. Shao, J.A. Tischfield, P.J. Stambrook, Embryonic stem cells and somatic cells differ in mutation frequency and type, Proc. Natl. Acad. Sci. USA 99 (2002) 3586–3590.
[16] S. Ichikawa, In situ monitoring with *Tradescantia* around nuclear power plants, Environ. Health Persp. 37 (1981) 145–164.
[17] V.G. Tsytsugina, G.G. Polikarpov, Radiological effects on populations of Oligochaeta in the Chernobyl contaminated zone, J. Environ. Radioactivity 66 (2003) 141–154.
[18] T.I. Evseeva, S.A. Geras'kin, I.I. Shuktomova, Genotoxicity and toxicity assay of water sampled from a radium production industry storage cell territory by means of *Allium*-test, J. Environ. Radioactivity 68 (2003) 235–248.
[19] I.B. Alieva, I.A. Vorobiev, Cells behavior and centrioles at multipolar mitosis induced by nocodasolum, Cytology 31 (1989) 633–641.
[20] A.I. Dovgalyuk, T.B. Kalinyak, Ya.B. Blum, Cytogenetic effects of toxic metal salts on apical meristem cells of *Allium cepa* L. seeding roots, Cytol. Genet. 35 (2001) 3–10.
[21] V.P. Bessonova, The state of pollen as an indicator of the environmental pollution with heavy metals, Russ. J. Ecol. 23 (1992) 45–50.
[22] M. Valverde, C. Trejo, E. Rojas, Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA–metal interaction? Mutagenesis 16 (2001) 265–270.
[23] T. Gichner, Z. Patkova, J. Szakova, K. Demnerova, Cadmium induces DNA damage in tobacco roots, but no DNA damage, somatic mutations or homologous recombination in tobacco leaves, Mutat. Res. 559 (2004) 49–57.
[24] A.I. Seoane, F.N. Dulout, Genotoxic ability of cadmium, chromium and nickel salts studied by kinetochore staining in

the cytokinesis-blocked micronucleus assay, Mutat. Res. 490 (2001) 99–106.
[25] C.W. Theodorakis, B.G. Blaylock, L.R. Shugart, Genetic ecotoxicology I: DNA integrity and reproduction in mosquitofish exposed *in situ* to radionuclides, Ecotoxicology 6 (1997) 205–218.
[26] V.A. Shevchenko, V.L. Pechkurenkov, V.I. Abramov, Radiation Genetics of Natural Populations: Genetic Consequences of the Kyshtym Accident, Nauka, Moscow, 1992.
[27] L.V. Cherezhanova, R.M. Alexakhin, On a biological effect of an increased ionising radiation background and the processes of radioadaptation in populations of herbaceous plants, Russ. J. Gen. Biol. 36 (1971) 303–311.
[28] S.A. Sergeeva, A.B. Semov, V.I. Abramov, V.A. Shevchenko, Repair of radiation-induced damages to plants growing under conditions of chronic exposure to β-radiation, Radiobiology 25 (1985) 774–777.
[29] W. Prus-Glowacki, A. Wojnicka-Poltorak, J. Oleksyn, P.B. Reich, Industrial pollutants tend to increase genetic diversity: evidence from field-grown European Scots pine populations, Water Air Soil Pollut. 116 (1999) 395–402.
[30] V.A. Ducharev, I.I. Korshikov, S.M. Ryabokon, A.A. Kotova, Genetic differentiation of *Pinus sylvestris* L. subpopulations under technogenious pollution, Cytol. Genet. 26 (1992) 7–11.
[31] L.F. Pitelka, Evolutionary responses of plants to antropogenic pollutants, Trends Ecol. Evol. 3 (1988) 233–236.
[32] M.R. Macnair, Heavy metal tolerance in plants: a model evolutionary system, Trends Ecol. Evol. 2 (1987) 354–359.
[33] O. Kovalchuk, P. Burke, A. Arkhipov, N. Kuchma, S.J. James, I. Kovalchuk, I. Pogribny, Genome hypermethylation in *Pinus sylvestris* of Chernobyl—a mechanism for radiation adaptation? Mutat. Res. 529 (2003) 13–20.
[34] O. Mittelsten-Scheid, K. Afsar, J. Paszkowski, Release of epigenetic gene silencing by trans-acting mutations in *Arabidopsis*, Proc. Natl. Acad. Sci. USA 95 (1998) 632–637.
[35] M.V. Kozlov, P. Niemela, J. Junttila, Needle fluctuating asymmetry is sensitive indicator of pollution impact on Scots pine (*Pinus sylvestris*), Ecol. Indicators 1 (2002) 271–277.