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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

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Abstract

Results of a comparative analysis of the frequency and spectrum of cytogenetic anomalies are presented for reproductive (seeds) and vegetative (needles) samples taken from Scotch pine (*Pinus sylvestris, L.*) micropopulations growing at sites with differing levels of radioactive contamination in the Chernobyl NPP 30 km zone, and at the location of a facility for the processing and storage of radioactive wastes (the 'Radon' LWPE, near the town of Sosnovy Bor in the Leningrad Region). The data obtained indicate the presence of genotoxic contaminants in the environment of the tree micropopulations. Chemical toxins make the main contribution to the environmental contamination in the Sosnovy Bor area as compared with the influence of ionising radiation in the Chernobyl 30 km zone. The higher radioresistance of seeds of Scotch pine growing on the area of the 'Radon' LWPE and in the centre of Sosnovy Bor town was revealed with acute γ-radiation.

Keywords: Bioindication; Pinus sylvestris L.; Chromosome aberrations; Chernobyl accident

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1. Introduction

The ‘Radon’ Leningrad regional waste processing enterprise (LWPE) (near Sosnovy Bor in the Leningrad Region) is located at Copor Bay on the coast of the Gulf of Finland (Fig. 1) in the Leningrad NPP sanitary-protective zone. The ‘Radon’ LWPE provides collection, processing and storage facilities for radioactive wastes of low and medium activity from the northwestern region of Russia. It has been recycling metal contaminated with radionuclides, from the Leningrad NPP, at the facility since 1995.

The results from a long-term radiation monitoring programme, carried out by the Regional Ecological Monitoring Laboratory of the “V.G. Khlopin Radium Institute” (Blinova, 1996), have revealed that equipment, and near-surface and surface repositories of radioactive wastes, located within the plant boundary, are the main sources of groundwater pollution. The work carried out in 1992 to seal the waste repositories has not led to noticeable changes in the nature and intensity of the environmental contamination. As a consequence of the industrial activity, there has been an increase in the occurrence of rather high concentrations of radionuclides and other anthropogenic pollutants in surface air, snow, pine tree needles and moss in the vicinity of the ‘Radon’ plant (Blinova, 1996).

The Scotch pine (*Pinus sylvestris, L.*), the dominant tree species in the plant communities of north European and Asian boreal forests, was chosen as a test system for an assessment of the possible effects of the ‘Radon’ enterprise on the environ-

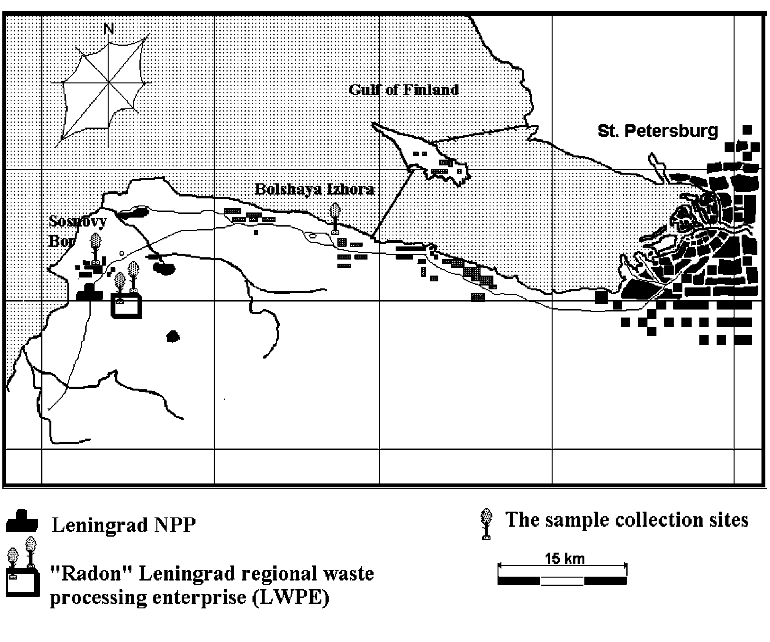

Fig. 1. The locations of the sample collection sites in the area of the Leningrad regional waste processing enterprise (LWPE). “Radon”.

ment. Data on the high radiosensitivity of coniferous tree species were obtained in the early 1960s at the Brookhaven Laboratory (Sparrow and Woodwell, 1962). *Pinus sylvestris, L.* has become one of the primary test objects for ecological and genetic monitoring due to its widespread distribution, the similarity of its radiosensitivity to that of humans, and the utility, reproducability and sensitivity of the available experimental endpoints.

The analysis of the results of studies carried out on *Pinus sylvestris* in the Chernobyl NPP accident control zone, at the East Urals radioactive trail, and in the vicinity of the Semipalatinsk nuclear test site (Kalchenko and Spirin, 1989; Sidorov, 1994; Kozubov and Taskaev, 1994; Kalchenko et al., 1995; Shevchenko et al., 1996; Kalchenko and Fedotov, 2001) has identified a number of test systems suitable for indicating the effects of low-level radiation. The reproductive organs of the coniferous trees, with their complex organisation and the long duration of the generative cycle, are the tissues most sensitive to the damaging influence of a wide range of anthropogenic contaminants. Although most angiosperm species have a reproductive cycle lasting for several months, it takes Scotch pine seeds at least 18–20 months to mature from micro- and megaspore formation (Kozubov and Taskaev, 1994; Shevchenko et al., 1996). Such a long maturation period means that significant and observable DNA damage may be accumulated in the undifferentiated stem cells even at low doses (or dose rates) from, or exposure to low concentrations of, anthropogenic contaminants. Because coniferous plants generally show a high retention capacity and low turnover rate for contaminants taken up by the 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. In either case, the damage to the DNA mainly appears as chromosome aberrations at the first mitosis.

2. Materials and methods

Pine cones were collected in the autumns of 1997–2000 from *Pinus sylvestris, L.* trees in three micropopulations located at: the ‘Radon’LWPE site; the town of Sosnovy Bor; and, a distance of 30 km from the town (the settlement of Bolshaya Izhora) and outside the likely area of influence of the complex of nuclear facilities (Fig. 1.). Young shoots, 20–30 mm in length, were collected at the same sites, and from the immediate vicinity of the boundary fence of the ‘Radon’ LWPE site, in the springs of 1998–2000. On each plot, samples were taken from 10–15 trees and fixed with ethanol–acetic acid (3:1). The variation in the values of the measured parameters, between trees in a given micropopulation, was not statistically significant, and all the data for these trees were, therefore, pooled into a single set. The results of a previous study of the variation of cytogenetic anomalies in pine seeds sampled from two areas of differing degrees of radionuclide contamination in the Chernobyl 30 km zone, and a control area, were available for comparison. The pine seeds for this study were obtained from cones, sampled in 1995, from three micropopulations of trees located at: the asphalt–concrete plant (ACP) (a zone of sub-lethal lesions where a dose of 10–20 Gy was received in 1986 following the accident (Kalchenko and

Fedotov, 2001); the village of Cherevach (a relatively "clean" area within the 30 km zone); and, the town of Obninsk in the Kaluga Region (a remote, and ecologically safe control area).

The samples of pine cones were allowed to ripen up to dehiscing. The resultant seeds were allowed to germinate on damp filter paper in Petri dishes at a controlled temperature of 24 °C. As with most other wild plant species, the pine has extremely variable, time-dependent, rates of seed germination and seedling growth. The root length necessary for the fixation of the first mitosis was estimated by taking account of the fact that the initial elongation of the root at germination arises from the differentiation and extension of germ cells that have been formed at embryogenesis, together with a study to determine the number of ana-telophases in seedling roots of increasing length. The resulting data (Table 1) indicate that the peak incidence of first mitoses appears to be at a root length of about 10 mm, and that second mitoses occur at lengths of 20 mm and more. Schevchenko et al. (1996), using a slightly different technique for seed germination, fixed *Pinus sylvestris, L.* seedlings with a root length of 7–8 mm to observe cells in first mitosis.

The seedling roots in the stage of first mitosis (8–14 mm in length) were fixed in ethanol–acetic acid (3:1). Temporary squash preparations of seedling root apical meristem and the intercalar meristem of young needles were made and stained with aceto–orcein. In each preparation, all ana-telophase cells were scored for chromosome aberrations and the proportion of anomalous cells was determined. The spectrum of aberrations identified and scored included chromatid (single) and chromosome (double) bridges and fragments, multipolar mitoses, and lagging chromosomes (genome mutations). Note that this method of anaphase analysis reveals aberrations in the cells of the root apical meristem that had been induced during the period from gametogenesis through to the maturation and harvesting of the seed; chromosome alterations which had arisen in the vegetative stages prior to flowering and gameto-

Table 1
Dependence of cell number at the stage of first mitosis on the root length of *Pinus sylvestris, L.* seedlings

Root length, mm	Cells scored	Dividing cells	
		Number	Mean±SE, %
2	1789	0	–
4	4097	0	–
6	2310	18	0.78±0.18
8	2640	72	2.73±0.32
10	5155	159	3.08±0.24
12	2987	53	1.77±0.24
14	2531	31	1.22±0.22
16	4194	30	0.72±0.13
18	4505	14	0.31±0.08
20	2916	43	1.47±0.22

genesis would have been eliminated in meiosis except for the symmetrical translocations and inversions that are not detected by this method.

The experimental data were interpreted using a method for statistical analysis of empirical distributions (Geras'kin et al., 1994). This technique allows the determination of the optimum sample size required to derive estimates of the study parameters with a fixed probable error at a given confidence level. Student's test and the method of confidence intervals were used to determine statistical significance.

3. Results and discussion

In the early years after the Chernobyl accident, the quality of the seed produced by spruce and pine trees depended on the absorbed dose (Kozubov and Taskaev, 1994; Shevchenko et al., 1996), i.e., the weight of 1000 seeds, and their germination rate, decreased, while the proportion of immature seeds showed a regular increase with absorbed dose. By 1995, however, the level of radioactive contamination in the Chernobyl NPP 30 km zone had ceased to produce a significant effect on the variation in these attributes. In the micropopulations of the Sosnovy Bor region, the morphometric indices are also within the normal range of spontaneous variation, though there is some tendency towards increases in both the proportion of immature seeds, and the number of abortive seed buds.

The use of cytogenetic criteria offers the possibility of an improved sensitivity for the detection of biological effects arising from anthropogenic sources of contamination. The data provided in Table 2 show that cytogenetic damage in both vegetative (needles) and reproductive (seeds) organs of Scotch pine is significantly higher in the samples from the experimental plots as compared with the controls. Note that the aberration rate in needles is higher than in the seedling root apical meristem for both the experimental and control samples. This is in agreement with our earlier data obtained for agricultural plants concerning the relative differences in cytogenetic sensitivity of the intercalar, and root apical, meristem cells (Geras'kin et al., 1996; Geras'kin et al., 1998).

The yields of cytogenetic anomalies in the seedling root apical meristem of the control samples (from Obninsk in 1995, and from Bolshaya Izhora in 1997–2000) show no significant differences within the range of spontaneous variation (Table 2), and the data may be pooled for analysis. The frequencies of cytogenetic anomalies in seedling root apical meristem and needle meristem for samples collected at the 'Radon' LWPE site in 1997–2000 are significantly above the corresponding control values (Table 2). The frequencies of cytogenetic anomalies at the 'Radon' LWPE site are, however, lower than those found in the Scotch pine micropopulations in the sub-lethal zone (the ACP site in the Chernobyl NPP 30 km zone). It should be noted that, while the incidence of cytogenetic damage in the samples from the Chernobyl NPP 30 km zone increases with the radiation exposure, the cytogenetic damage found in the seed and the needle samples from the 'Radon' LWPE site cannot be attributed to the radiation exposure alone.

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

Table 2
Aberrant cell frequency (%) in seedling root apical meristem and in needle intercalar meristem of Scotch pine (*Pinus sylvestris, L.*)

Year	Plot	Dose rate, $\mu R \cdot h^{-1}$	Cells scored	Aberrant cells	
				Number	Mean±SE, %
		Seedling root apical meristem			
1995	Obninsk, Kaluga Region	12.6±0.3	1994	12	0.60±0.17
	Cherevach (30 km Chernobyl NPP zone)	250±10	2011	41	2.04±0.32[a]
	Asphalt-concrete plant (30 km Chernobyl NPP zone)	2690±85	2049	89	4.34±0.45[a]
1997	Bolshay Izhora	13.3±0.8	14643	88	0.60±0.06
	Sosnovy Bor	13.3±0.6	12342	147	1.19±0.10[a]
	'Radon' LWPE	24.0±0.7	7927	121	1.53±0.14[a]
1998	Bolshay Izhora	13.3±0.8	12217	65	0.53±0.07
	Sosnovy Bor	13.3±0.6	12832	167	1.30±0.10[a]
	'Radon' LWPE	24.0±0.7	9437	163	1.73±0.13[a]
1999	Bolshay Izhora	13.3±0.8	16482	94	0.57±0.06
	Sosnovy Bor	13.3±0.6	8302	113	1.36±0.13[a]
	'Radon' LWPE	24.0±0.7	5613	97	1.73±0.17[a]
2000	Bolshay Izhora	13.3±0.8	9885	65	0.66±0.08
	Sosnovy Bor	13.3±0.6	3517	61	1.73±0.22[a]
	'Radon' LWPE	16.0±0.7	2674	61	2.28±0.29[a]
		Needle intercalar meristem			
1998	Bolshay Izhora	13.3±0.8	10156	98	0.97±0.10
	Sosnovy Bor	13.3±0.6	12084	164	1.36±0.11
	'Radon' LWPE fence	12.2±0.7	11376	182	1.60±0.12[a]
	'Radon' LWPE	24.0±0.7	5274	144	2.73±0.22[a]
1999	Bolshay Izhora	13.3±0.8	5549	45	0.81±0.12
	Sosnovy Bor	13.3±0.6	3724	62	1.67±0.21[a]
	'Radon' LWPE fence	12.2±0.7	4026	60	1.49±0.19[a]
	'Radon' LWPE	24.0±0.7	3943	87	2.21±0.23[a]
2000	Bolshay Izhora	13.3±0.8	11206	98	0.88±0.09
	Sosnovy Bor	13.3±0.6	5798	95	1.64±0.17[a]
	'Radon' LWPE fence	12.2±0.7	5361	96	1.79±0.18[a]
	'Radon' LWPE	16.0±0.7	4374	100	2.29±0.23[a]

[a] Difference from the control is significant; $p<5\%$

Bor site was elevated above the control values, significantly so in all cases except for the needles sampled in 1998. Thus, the data show the presence of mutagenic contaminants in the environments of the Scotch pine micropopulations not only at the the 'Radon' LWPE site, but also in the town of Sosnovy Bor. Although this result was unexpected, it was supported by the statistical analysis of the data.

Additional information on the possible factors affecting the pine plants may be obtained from an analysis of the spectrum of structural mutations (Table 3). In particular, it should be noted that tripolar mitoses, rather rare anomalies, were found in the preparations from seeds sampled at both the LWPE 'Radon' site and Sosnovy Bor, but not in the samples from either the control sites or those within the Chernobyl NPP 30 km zone. The appearance of tripolar mitoses is possibly linked to spindle damage (Alieva and Vorobiev, 1989). It has been reported by Bessonova (1992) that a significant increase in the incidence of tripolar mitoses (none was found in the controls) in *Syringa vulgaris L.* and *Armeniaca vulgaris Lam.* was associated with the contamination of the local soils with heavy metals. From this, together with the dosimetric data presented in Table 2, it may be concluded that there is likely to be significant contamination of the Sosnovy Bor region with genotoxic chemicals.

In the current situation, when the possible effects of human activities on the biota have become a particular concern, it is pertinent to consider the adaptive potentials of natural populations. One consequence of chronic irradiation in natural populations is an apparent increase in the mean radioresistance—the so-called "radio-adaptation phenomenon"; this has been observed in studies in the East Urals trail region (Cherezhanova and Alexakhin, 1971; Shevchenko et al., 1992) and in the results of the responses of seeds to an additional, acute γ-radiation exposure. Experimental studies of repair inhibitors, dose- effect relationships for low and high LET radiations, measures of unscheduled DNA synthesis and an effective restoration of single strand breaks (Shevchenko et al., 1992; Sergeeva et al., 1985) allows the conclusion to be drawn that the divergence of populations in terms of radioresistance is connected with selection for changes in the effectiveness of repair systems and is not accompanied by visible morphologic alterations (Shevchenko et al., 1992).

A proportion of the seeds collected in the Sosnovy Bor region in 1998 was subjected to an acute γ-ray exposure of 15 Gy (at a dose rate of 0.6 Gy/minute). The seeds from the Scotch pine populations growing in the town of Sosnovy Bor and at the 'Radon' LWPE site appear to be significantly more resistant to acute radiation exposure than the controls (Table 4). Kalchenko and Fedotov (2001) have found similar results in studies of the acute γ-radiation resistance of *Pinus sylvestris, L.* seeds collected in Chernobyl NPP 30 km zone in 1997. Note that the number of generations of pine trees during the existence of the 'Radon' LWPE, and following the Chernobyl accident, is obviously insufficient to allow for natural selection, in the classical sense, on the basis of the efficacy of repair systems. At the same time, a somatic adaptive response can hardly account for the observed changes because it is the seeds whose response to acute irradiation was studied (the seeds were kept for six months under controlled conditions that precluded the operation of additional influences). One possible explanation relates to selection on the basis of cellular radiosensitivity, i.e, the selective elimination of sensitive cells and their apparent replacement with more radioresistant cells (Kalchenko and Fedotov, 2001). An alternative explanation concerns the possible existence of an induced epigentic change in the activity of functional genes that is heritable over a number of cell generations (Mikheev et al., 1999). The possible molecular mechanisms underlying such a change have been discussed previously (Geras'kin, 1995; Geras'kin and Sarapul'tzev, 1995).

Table 3
Aberrations of different types in the seedling root apical meristem and needle intercalar meristem of Scotch pine (*Pinus sylvestris, L.*)

Year	Plot	Number of aberrations of different types[a.]						Relative proportions of different aberrations, %			
		f ‘	f ‘‘	m’	m’’	g	3p	f ‘+m’	f ‘‘+m’’	g	3p
Seedling root apical meristem											
1995	Obninsk, Kaluga Region	1	1	2	4	6	0	21.4	35.7	42.9	0.0
	Cherevach (30 km Chernobyl NPP zone)	0	7	8	25	10	0	16.0	64.0	20.0	0.0
	Asphalt-concrete plant (30 km Chernobyl zone)	4	6	17	46	21	0	22.4	55.3	22.3	0.0
1997	Bolshay Izhora	4	16	44	7	19	0	53.3	25.6	21.1	0.0
	Sosnovy Bor	9	38	38	33	28	5	31.1	47.0	18.5	3.3
	‘Radon’ LWPE	17	35	28	18	24	2	36.3	42.7	19.4	1.6
1998	Bolshay Izhora	4	31	12	4	16	0	23.9	52.2	23.9	0.0
	Sosnovy Bor	5	44	28	31	85	9	16.3	37.1	42.1	4.5
	‘Radon’ LWPE	7	56	25	28	55	8	17.9	46.9	30.7	4.5
1999	Bolshay Izhora	0	13	36	23	22	0	38.3	38.3	23.4	0.0
	Sosnovy Bor	0	14	27	32	42	9	21.8	37.1	33.9	7.2
	‘Radon’ LWPE	0	9	37	31	23	10	33.6	36.4	20.9	9.1
2000	Bolshay Izhora	2	12	20	20	12	0	33.3	48.5	18.2	0.0
	Sosnovy Bor	0	19	11	12	23	0	16.9	47.7	35.4	0.0
	‘Radon’ LWPE	0	13	26	9	14	3	40.0	33.9	21.5	4.6
Needle intercalar meristem											
1998	Bolshay Izhora	1	6	58	40	0	0	56.2	43.8	0.0	0.0
	Sosnovy Bor	3	13	90	51	14	0	54.4	37.4	8.2	0.0
	‘Radon’ LWPE fence	2	46	59	61	28	0	31.1	54.6	14.3	0.0
	‘Radon’ LWPE	0	17	62	67	13	0	39.0	52.8	8.2	0.0
1999	Bolshay Izhora	0	9	11	7	20	0	23.4	34.0	42.6	0.0
	Sosnovy Bor	0	27	5	20	13	1	7.6	71.2	19.7	1.5
	‘Radon’ LWPE fence	0	17	1	28	14	0	1.7	75.0	23.3	0.0
	‘Radon’ LWPE	1	18	21	32	22	0	23.4	53.2	23.4	0.0
2000	Bolshay Izhora	9	20	2	45	22	0	11.2	66.3	22.5	0.0
	Sosnovy Bor	4	23	6	46	20	1	10.0	69.0	20.0	1.0
	‘Radon’ LWPE fence	1	37	3	41	20	0	3.9	76.5	19.6	0.0
	‘Radon’ LWPE	4	19	2	60	24	0	5.5	72.5	22.0	0.0

[a.] f ‘, m’ - chromatid (single) fragments and bridges; f ‘‘, m’’ - chromosome (double) fragments and bridges; 3p - multipolar mitoses; g- lagging chromosomes.

Table 4
Aberrant cell frequency in the root apical meristem of Scotch pine (*Pinus sylvestris, L.*) seedlings grown from seeds sampled in the region of the town of Sosnovy Bor in 1998 and exposed to acute γ-ray dose of 15 Gy

Plot	Cells scored	Aberrant cells	
		Number	Mean±SE, %
Bolshay Izhora	5505	546	9.92±0.40
Sosnovy Bor	4695	305	6.50±0.36[a.]
'Radon' LWPE	4715	311	6.60±0.36[a.]

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

4. Conclusions

The results of a comparative analysis of the frequencies and spectra of cytogenetic anomalies in the reproductive and vegetative organs sampled from micropopulations of Scotch pines growing at two sites within the Chernobyl NPP 30 km zone, on the 'Radon' LWPE site, and in the town of Sosnovy Bor indicate the presence of genotoxic contaminants. Moreover, in the Sosnovy Bor region, in contrast to the Chernobyl NPP 30 km zone, it appears that chemical toxins make a considerable contribution to the environmental contamination. The treatment of the seeds from the Scotch pine populations growing in Sosnovy Bor and at the 'Radon' LWPE site with additional acute γ-irradiation indicates their greater radioresistance.

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