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Cytogenetic effects of combined radioactive (^{137}Cs) and chemical (Cd, Pb, and 2,4-D herbicide) contamination on spring barley intercalar meristem cells

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Abstract

The frequency of cytogenetic effects in spring barley intercalar meristem cells was studied in the presence of a range of different stressors. There was a non-linear dependence on the concentrations of ^{137}Cs, Cd, Pb, and dichlorophenoxyacetic acid (2,4-D) herbicide contamination in the exposure ranges used. The frequency of cytogenetic effects increased at the lower concentrations of the pollutants more rapidly than at the higher concentrations. Contamination of the soil by lead at a concentration that meets the current standards for permissible content in soil, and by 2,4-D herbicide at the application levels recommended for agricultural use resulted in a significant increase in aberrant cell frequency. In these cases, the extent of the observed cytogenetic effects was comparable with the effect induced by a ^{137}Cs soil contamination of 49.2 kBq/kg, a level that exceeds by 10-fold the maximum level permitted in radionuclide-contaminated areas where people are resident. In most cases, the experimentally observed combined effects of the pollutants studied differed from those expected from an additive hypothesis. When combined with ^{137}Cs contamination, antagonistic effects became increasingly stronger when the second stressor was changed from cadmium to lead, and then to the herbicide, as measured both by tests of the 'frequency of aberrant cells' and the 'aberrations per cell'. Data from this study and previous reported literature suggest that synergistic increases in cytogenetic effects can be induced by the simultaneous influence of several stressors even at low intensities. This indicates that there is a capability for mutual intensification of the effects of environmental factors that actually occur in situations of low-level exposure.
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1. Introduction

An estimation of environmental effects caused by anthropogenic contamination occurring in the biosphere is currently difficult. The relevant mechanisms of a mutagenic and/or poisonous action for most environmental agents have not yet been defined, although some hypotheses have been proposed. The global, rapid increase in man-made stress on the biosphere poses the question about the possible consequences of this contamination for biota as a whole, including man. The highest priority in eco-toxicological research should be given [1] to the contaminants that are known to occur in the field at concentrations high enough to give cause for concern and for which there are reasons to suspect potentiation of effects.

Agricultural ecosystems are of special concern since they are often exposed to mixed pollution, resulting in relatively low, however, not hazard-free, contamination levels. Environmentally released metals and other pollutants are mainly deposited in soil. Excessive transfer of metal ions from contaminated soil to the food chain is controlled by a soil–plant barrier, which sometimes fails to work for certain metals such as lead, which results in the contribution of more than half of the human lead intake through food of plant origin [2]. Therefore, present-day levels of persistent pollutants in agricultural soils may pose a risk both to human health and to biological components of agroecosytems. Although lower than the concentrations normally referred to in evaluations of mutagenicity in laboratory conditions, the widespread presence of these compounds in the environment may have adverse consequences.

Most studies evaluating the genotoxicity of environmental pollutants have been carried out in animal and microbial systems. Studies on putative genotoxic effects in higher plants are relatively few. On the other hand, the mutagenic effects of environmental pollutants in soil cannot be monitored by conventional genotoxicity tests with bacteria [3]. Furthermore, no extraction methods are available for experiments with mammalian cell lines, and results obtained in the commonly used mammalian cell lines with certain heavy metals are highly controversial [4]. Plants are essential component of any ecosystem. Many plant species, particularly crops, are chronically exposed to agricultural chemicals, some of which are mutagenic. Moreover, plants have a high capacity for bio-concentration and bio-conversion. Consequently, an observation of intact plants directly growing in contaminated soil is most suitable for assessing the quality of the environment.

Complex variable processes induced by simultaneous exposure to diverse factors are capable of producing different responses, from antagonistic to synergistic. This is confirmed by numerous examples [5] of non-linear changes in the level and mode of response of biological systems resulting from variations in the order, amounts, and duration of exposure to adverse agents. For low doses or concentrations of a single-factor impact, there has not yet been suggested any reliable theoretical description of the effects of various stressors that would make it possible to predict whether or not the result of a combined action would be additive, synergistic, or antagonistic [6]. Such a theoretical description is a precursor to being able to derive the phenomenology of a biological effect due to combined exposure to different stressors. To achieve this, a well-directed accumulation of knowledge is needed and appropriate experimental data analysis is necessary.

The available database on combined effects is rudimentary and rarely covers exposure ranges large enough to make direct inferences to nowadays low-level exposure situations. The purpose of the present work is to study the cytogenetic consequences for a plant growing under combined radioactive and chemical contamination at levels officially adopted as permissible.

2. Materials and methods

2.1. Cultivation of plants

Spring barley (*Hordeum vulgare* L., variety Zazerskiy 85), one of the most common genetically studied crops [7], was used as a test-species. Barley is a convenient object for studies of induced chromosome aberrations because of its limited number ($2n = 14$) of relatively large (6–8 μm) chromosomes, which are easy to identify. The plants were grown in pots in a greenhouse following the standard procedure [8]. Thirteen plants were grown in each pot filled with 4.5 kg of chernozem leached loamy soil (dry matter). Four replications for each treatment variant including a control

were prepared. Compounds containing heavy metals and ^{137}Cs were mixed in soil. Solution of dichlorophenoxyacetic acid (2,4-D) herbicide was brought to soil surface at the bushing-out phase of barley growth.

2.2. Treatment concentrations of ^{137}Cs, Cd, Pb, and 2,4-D herbicide

^{137}Cs was added to the soil at specific activities of 4.92, 24.6, and 49.2 kBq/kg in the form of nitrate ("Isotope", Russia). Such levels of specific activities at radionuclide-contaminated territories correspond to contamination densities of 1.48, 7.4, and 14.8 MBq/m^2 (40, 200, and 400 Ci/km^2).

According to the current Russian Federation standards [9], maximum permissible concentrations for Cd and Pb in soil with pH 4.0–6.0 are 2 and 32 mg/kg, respectively. Lead and cadmium were each applied to the soil at three concentrations (30, 150, and 300, and 2, 10, and 50 mg heavy metal/(kg soil), respectively) in the forms of nitrates—$Pb(NO_3)_2$ and $Cd(NO_3)_2{\cdot}4H_2O$ ('Reachim', Russia). The lead nitrate is the only form that is readily soluble among the inorganic salts of lead, which was a reason to use nitrate salts of cadmium as well to avoid a possible confounding influence of anions on the study results.

2,4-D herbicide was used in the form of amine salt (50% solution in water; NITIG, Bashkiria) at an application rate of 1 or 2 L/ha per preparation. These are the treatment rates of this compound recommended for agricultural application in Russia for spring crops [10].

To study cytogenetic effects induced by combined exposure to radioactive and chemical contamination, a complete factorial pattern of two-component experiments (^{137}Cs + Cd; ^{137}Cs + Pb; ^{137}Cs + 2,4-D herbicide) was performed. All experimental schemes were undertaken simultaneously with a common control variant.

2.3. Calculation of radiation doses absorbed

The doses of absorbed radiation were estimated for the growing point of a plant from the values of ^{137}Cs specific activities in the soil. It was assumed that the exposure period prior to sampling of the leaf meristem was 23 days, including 5-day exposure after the germs had appeared above ground. The doses for the time interval while the meristem was beneath the soil surface were calculated for a geometry of an infinite homogeneous source for β-radiation, and a geometry of a hemisphere having the same mass of soil in a plant pot, for γ-radiation. The contribution of γ-radiation from other pots to the absorbed dose was taken into account. The dose estimates are given in Table 1. Note that these doses absorbed by the critical point of a plant growing in a pot filled with contaminated soil are approximately 1.64-fold less than would be absorbed if a plant grew in soil with the same specific activity in natural conditions.

Table 1
Estimated absorbed doses to spring barley intercalar meristem cells

Soil specific activity of ^{137}Cs (kBq/kg)	Dose (mGy)		
	D_γ	D_β	$D_{\gamma+\beta}$
4.92	0.20	0.28	0.48
24.6	1.00	1.40	2.40
49.2	2.00	2.80	4.80

2.4. Cytogenetic analysis

As endpoint of genotoxicity, a frequency of ana- and telophase cells with chromosomal aberrations within intercalar meristem was used. Intercalary leaf meristem was sampled at the fifth stage of organogenesis (at 21–25 days of germination) from 13 plants of each of 4 replicas and fixed in acetic acid:alcohol (1:3). Temporal squashed preparations were made and stained with aceto-orcein as described by Constantin and Nilan [7]. All slides were coded and analyzed blind (i.e. an operator did not know the treatment used). From 484 up to 500 ana–telophases were assayed for each treatment variant to determine the frequency of cells with chromosome abnormalities and the number of aberrations per cell. Since there were no significant differences between replicas, the data were pooled together for analysis.

2.5. Statistical analysis

The available set of experimental data of 4 replicas × 484–500 ana–telophase cells scored for each treatment variant was sufficient for a statistically reliable validation of the examined cytogenetical values at a confidence level of 0.95 and the relative probable error, ε = 25–30%. To determine the significance of

the difference between sample mean values, Student's *t*-test was applied.

To test a hypothesis whether mathematical model 2 fits the experimental data significantly better than model 1, the Hayek criterion was applied [11]:

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

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

To measure the deviation of an observed response from an additive one, and to classify the effect produced by a combined impact into additivity, synergism, and antagonism, an interaction coefficient K_w was used, which is determined [12] as a ratio of the increment induced by the combined action of two (or more) agents at levels X_1 and X_2 to the sum of the increments of a measured effect produced by each factor separately:

$$K_w = \frac{I(X_1, X_2)}{I(X_1, 0) + I(0, X_2)},$$
$$I(X_1, X_2) = E(X_1, X_2) - E(0, 0),$$

where $E(X_1, X_2)$ is the value of a biological effect under study and $E(0, 0)$ is the spontaneous level.

To assign the response to the groups mentioned above, a statistical testing of K_w on the equality to 1 is necessary, which was realised through examining a null-hypothesis about an absence of differences between mean values of numerator and denominator in the expression for K_w, using Student's *t*-test. If the null-hypothesis cannot be rejected, then $K_w \sim 1$ and the resulting effect of the combined action would be classified as additive; if the null-hypothesis fails, the effect is classified as antagonistic when $K_w < 1$, and as synergistic when $K_w > 1$.

An ANOVA analysis was applied through a STATGRAPHICS software application. According to [13], the sum of squares (SS) that a factor is responsible for in ANOVA shows the contribution this factor makes to the total variance, an assumption that was adopted to estimate the relative contributions of agents and their interaction in an effect induced by combined exposure.

3. Results

3.1. Separate exposure to ^{137}Cs, Pb, Cd, and 2,4-D herbicide

3.1.1. Frequency of aberrant cells

The frequency of aberrant cells increased with the concentration of ^{137}Cs contaminating the soil (Table 2), but this effect becomes statistically significant only at the highest specific activity of 49.2 kBq/kg when a total dose of 4.8 mGy had been delivered to the intercalary meristem cells (Table 1).

The addition of Cd or Pb into soil led to a significant increase in the frequency of cytogenetic effects in all examined treatments (Tables 2 and 3). The soil contamination with cadmium of 2 mg/kg, which is the maximal permissible level for Cd, was the only case where the cytogenetic effect was insignificant (Table 2).

Even minimum dose of 2,4-D produced a significant (almost two-fold) increase in the occurrence of cytogenetic aberrations (Table 4). The cytogenetic effect was weakly dependent on the dose of pesticide—doubling the application dose barely changed the frequency of aberrant cells (Table 4). Our results are consistent with the findings presented in previous studies [14–16] where either no effect or a weak 'dose–effect' dependence was found within a range of effective concentrations of pesticide chemicals. Thus, the application of 2,4-D herbicide at doses recommended for agricultural use resulted in a statistically significant increase in the frequency of aberrant cells. These results are reinforced by the fact that other types of pesticide treatment at doses recommended in regular agricultural practices also lead to statistically significant increase of the genetic aberration frequencies in different higher plant bioassays [16–18], including those with *H. vulgare* L. root-tip cells and pollen-mother cells after seed soaking and spray treatments [19,20].

Both the lead and cadmium concentrations, which are close to the values adopted in Russia as the maximal permissible levels, and also the amount of 2,4-D herbicide advised as the optimum application dose induced cytogenetic damage in barley intercalar meristem at a level comparable with the effect of ^{137}Cs contamination at 49.2 kBq/kg. Such radioactive contamination exceeds by 10-fold the maximum level permitted in

Table 2
Aberrant cell frequency and number of aberrations per aberrant cell for a combined exposure to ^{137}Cs and Cd, and corresponding values of the interaction coefficient K_w

^{137}Cs (kBq/kg)	Cd (mg/kg)						
	0	2	10	50	2	10	50
	Aberrant cell frequency (%)				K_w		
0	10.4 ± 1.4	15.4 ± 1.6	18.2 ± 1.7[a]	20.8 ± 1.8[a]			
4.92	14.0 ± 1.6	26.0 ± 2.0[a]	30.2 ± 2.1[a]	30.8 ± 2.1[a]	1.84[*]	1.74[**]	1.46[**]
24.6	15.4 ± 1.6	18.0 ± 1.7[a]	18.4 ± 1.7[a]	20.4 ± 1.8[a]	0.76[*]	0.63[**]	0.65
49.2	17.6 ± 1.7[a]	14.8 ± 1.6	15.2 ± 1.6	19.2 ± 1.8[a]	0.36[**]	0.32[**]	0.50[*]
	Aberrations per aberrant cell						
0	1.13 ± 0.11	1.26 ± 0.09	1.17 ± 0.04	1.29 ± 0.03[a]			
4.92	1.93 ± 0.10[a]	1.41 ± 0.21	1.90 ± 0.25[a]	1.72 ± 0.21[a]	0.31[**]	0.91	0.61[*]
24.6	1.76 ± 0.10[a]	1.66 ± 0.22[a]	1.80 ± 0.19[a]	1.49 ± 0.06[a]	0.70	0.99	0.45[**]
49.2	2.02 ± 0.13[a]	1.88 ± 0.10[a]	1.76 ± 0.22[a]	1.74 ± 0.08[a]	0.73[*]	0.67[*]	0.58[**]

Interaction coefficient (K_w) differs from 1: $^{*}p < 5\%$; $^{**}p < 1\%$.
[a] Significant difference from the control, $p < 5\%$.

radionuclide-contaminated areas where people are resident [21].

3.1.2. The number of aberrations per aberrant cell

The ratio between the aberrant cell frequency and the number of aberrations per aberrant cell (NAAC) changes during exposure to diverse stressors and these differences can illustrate the underlying biological mechanisms of action of the stressors. Tables 2–4 present data on average NAACs in the spring barley intercalary meristem caused by radioactive and chemical contamination.

The highest level of NAAC occurred in the presence of ^{137}Cs. The smallest among the tested levels of soil contamination with ^{137}Cs, i.e. 4.92 kBq/kg, caused a significant increase in the NAAC so that the spontaneous level was exceeded by a factor of 1.71. Further increase in the ^{137}Cs contamination did not

Table 3
Aberrant cell frequency and number of aberrations per aberrant cell for a combined exposure to ^{137}Cs and Pb, and corresponding values of the interaction coefficient K_w

^{137}Cs (kBq/kg)	Pb (mg/kg)						
	0	30	150	300	30	150	300
	Aberrant cell frequency (%)				K_w		
0	10.4 ± 1.4	16.8 ± 1.7[a]	20.6 ± 1.8[a]	23.8 ± 1.9[a]			
4.92	14.0 ± 1.6	18.6 ± 1.7[a]	21.0 ± 1.8[a]	23.2 ± 1.9[a]	0.82	0.74	0.75
24.6	15.4 ± 1.6	17.4 ± 1.7[a]	18.6 ± 1.7[a]	20.2 ± 1.8[a]	0.61	0.54[*]	0.53[*]
49.2	17.6 ± 1.7[a]	17.4 ± 1.7[a]	19.8 ± 1.8[a]	23.0 ± 1.9[a]	0.52[*]	0.54[**]	0.61[*]
	Aberrations per aberrant cell						
0	1.13 ± 0.11	1.15 ± 0.04	1.23 ± 0.07	1.33 ± 0.13			
4.92	1.93 ± 0.10[a]	1.82 ± 0.08[a]	1.43 ± 0.09[a]	1.74 ± 0.14[a]	0.84	0.33[**]	0.61[*]
24.6	1.76 ± 0.10[a]	1.76 ± 0.15[a]	1.73 ± 0.15[a]	1.54 ± 0.13[a]	0.95	0.82	0.49[**]
49.2	2.02 ± 0.13[a]	1.69 ± 0.08[a]	1.60 ± 0.09[a]	1.67 ± 0.11[a]	0.61[**]	0.48[**]	0.50[**]

Interaction coefficient (K_w) differs from 1: $^{*}p < 5\%$; $^{**}p < 1\%$.
[a] Significantly different from the control, $p < 5\%$.

Table 4
Aberrant cell frequency and number of aberrations per aberrant cell for a combined exposure to ^{137}Cs and 2,4-D herbicide, and corresponding values of the interaction coefficient K_w

^{137}Cs (kBq/kg)	2,4-D herbicide (kg/ha)				
	0	1	2	1	2
	Aberrant cell frequency (%)			K_w	
0	10.4 ± 1.4	19.8 ± 1.8[a]	22.2 ± 1.9[a]		
4.92	14.0 ± 1.6	14.2 ± 1.6	15.6 ± 1.6	0.29**	0.34**
24.6	15.4 ± 1.6	15.4 ± 1.6	16.2 ± 1.6	0.35**	0.35**
49.2	17.6 ± 1.7[a]	16.6 ± 1.7[a]	15.8 ± 1.6	0.37**	0.28**
	Aberrations per aberrant cell				
0	1.13 ± 0.11	1.21 ± 0.12	1.17 ± 0.09		
4.92	1.93 ± 0.10[a]	1.41 ± 0.08[a]	1.40 ± 0.15[a]	0.32**	0.32**
24.6	1.76 ± 0.10[a]	1.48 ± 0.29	1.39 ± 0.11	0.49*	0.39**
49.2	2.02 ± 0.13[a]	1.26 ± 0.05	1.27 ± 0.10	0.14**	0.16**

Interaction coefficient (K_w) differs from 1: *$p < 5\%$; **$p < 1\%$.
[a] Significantly different from the control, $p < 5\%$.

change the NAAC significantly. This can be related to some of the features of ^{137}Cs uptake from the soil to the plant [22]: in particular, the higher the ^{137}Cs contamination density, the lower the relative contribution of incorporated radionuclides to the absorbed dose.

For contamination with heavy metals, the NAAC increased with increasing concentrations of both cadmium and lead in the soil (Tables 2 and 3). However, a statistically significant increase was registered only at the highest (50 mg/kg) concentration of cadmium. The herbicide 2,4-D had no actual influence on the NAAC (Table 4) for the treatment regimes studied.

3.1.3. Analysis of dose (concentration)–effect relationships

The relationships between cytogenetic effects and the concentrations of the stressors obtained can be satisfactorily fitted with a linear model (Table 5). Nevertheless, a number of different regression models were examined with respect to 'goodness-of-fit' of the experimental data. It was shown that the power and logarithmic models: $y = a_p + \exp(-b_p x)$, $b_p < 1$, and $y = a_l + \log(b_l x)$, accordingly, provide the best results at approximating the empirical dependences observed.

To make a choice between the linear, power, and exponential models, the Hayek criterion was applied, of which the results are presented in Table 5. The only case when the linear model proved superior over the other two models was a fitting of the NAAC induced by lead nitrate, and this follows from the Hayek criterion ($p < 0.1\%$). In all other cases, the linear model even failed to show the highest values of multiple correlation coefficient, R^2. The dependencies of aberrant cells induction on soil contamination were best fitted by the power model and this was true for all the pollutants studied. In approximating the NAAC, the linear model was inferior to the other models for soil contamination with ^{137}Cs, but a choice between the power and logarithmic models could not be made using the Hayek criterion. For contamination with Cd and 2,4-D pesticide, it was impossible to select one superior model for the NAAC among the three (Table 5), but the values of R^2 were higher for the non-linear models.

3.2. Combined exposure to ^{137}Cs and Cd

3.2.1. Aberrant cells frequency

The maximum frequency of aberrant cells was found (Table 2) for a combination of cadmium with the lowest ^{137}Cs specific activity, i.e. 4.92 kBq/kg. When the ^{137}Cs contamination in soil was studied alone, it did

Table 5
Comparison of approximations of experimental data by three regression models

Substance	Model	Aberrant cell frequency			Aberrations per aberrant cell		
		R^2	H	p	R^2	H	p
^{137}Cs	Linear	78.04	6.00	<0.01	42.58	4.21	<0.05
	Logarithmic	87.21	4.44	<0.05	91.67	[a]	[a]
	Power	93.31	[a]	[a]	91.36	0.34	[b]
Cd	Linear	60.73	21.6	<0.001	44.70	1.01	[b]
	Logarithmic	94.32	8.06	<0.01	58.63	0.04	[b]
	Power	99.75	[a]	[a]	58.66	[a]	[a]
Pb	Linear	78.40	18.03	<0.001	99.87	[a]	[a]
	Logarithmic	87.67	13.91	<0.001	50.04	33.91	<0.001
	Power	99.81	[a]	[a]	83.28	19.57	<0.001
2,4-D	Linear	87.52	49.94	<0.001	34.41	1.91	[b]
	Logarithmic	98.77	15.61	<0.01	76.77	[a]	[a]
	Power	99.99	[a]	[a]	76.60	0.12	[b]

[a] The best regression model with the highest value of R^2.
[b] No significant difference is found by the Hayek criterion between the given and the best regression models.

not result in a significant increase in aberrant cell frequency. These data are noteworthy in view of the ^{137}Cs activity that occurs in the territories contaminated by the Chernobyl accident [21]. In the presence of cadmium, an increase in the ^{137}Cs specific activity over 4.92 kBq/kg resulted in a reduction in aberrant cell frequency of up to two-fold (Table 2). The highest examined ^{137}Cs contamination of 49.2 kBq/kg by itself produced a significant increase in aberrant cell frequency. However, in combination with the low cadmium concentrations of 2 and 10 mg/kg, there is no increase in aberrant cell frequency, which is actually lower than that induced by the separate exposures to each agent. The effect did not strongly depend on the cadmium concentration (Table 2).

In Table 2, the values of K_w and the results of statistical testing on the variation of K_w to 1 are presented for all combinations of cadmium and ^{137}Cs concentrations. There was a significant synergistic effect for combinations of the lowest ^{137}Cs specific activity of 4.92 kBq/kg with all the cadmium concentrations tested. When the ^{137}Cs contamination of the soil was increased at constant cadmium concentration, the synergistic effect changed to an antagonistic effect. Thus, the effects observed experimentally for combined exposures essentially differ from the total of the contaminants' individual effects. Therefore, information about the separate effects of Cd and ^{137}Cs may not be adequate for assessing and predicting the consequences of their combined exposure.

3.2.2. The number of aberrations per aberrant cell

As in the case of separate exposures, the NAAC was mainly dependent on the presence of ^{137}Cs; however, it did not show any dependence on the level of radioactive contamination density (Table 2). The increase in the NAAC under the combined exposure in comparison with soil contamination by cadmium alone was statistically significant, except for the treatment with the lowest concentrations of cadmium and ^{137}Cs. Thus, the additional application of ^{137}Cs in cadmium-treated soil consistently produced an increase in the NAAC. There was a similar role pertaining to cadmium in the test of aberrant cell induction above.

3.2.3. Relative contribution of ^{137}Cs and cadmium to induction of cytogenetic effects

According to ANOVA, ^{137}Cs contamination is responsible for 53 and 87% of SS in the total variance of the aberrant cells frequency and NAAC, while Cd takes only 38 and 0.06%, respectively. Thus, the relative contribution of caesium to the cytogenetic effect is more important than that of cadmium. Interaction effects are responsible for less than 1% of the discrepancy.

3.3. Combined exposure to ^{137}Cs and Pb

3.3.1. Frequency of aberrant cells

The aberrant cell frequency under the combined soil contamination of ^{137}Cs and lead differs little from that caused by lead alone (Table 3). Similar to the case of cadmium + ^{137}Cs, the effect is strong for the different concentrations of Pb when combined with the lowest (4.92 kBq/kg) ^{137}Cs specific activity. As the lead concentration rises, the aberrant cell frequency also increases. However, statistically significant excesses of aberrant cell frequency over the level induced by separate exposure to ^{137}Cs occur (Table 3) for combinations of the lowest ^{137}Cs soil contamination with lead concentrations of 150 and 300 mg/kg of soil only.

In contrast to the ^{137}Cs + Cd combined exposure, the interaction in the ^{137}Cs–Pb combination is either additive or antagonistic (Table 3). Consistent antagonistic effects are seen when the contaminants are applied at high concentrations. Thus, a prediction of cytogenetic consequences of the lead nitrate + ^{137}Cs soil contamination based on the additive model would be incorrect for the concentration ranges examined here.

3.3.2. The number of aberrations per aberrant cell

As for the separate introduction of the pollutants into the soil, the presence of lead did not significantly change the NAAC, but ^{137}Cs contamination raised this value considerably. The NAAC in the combined soil contamination with lead nitrate and ^{137}Cs is higher compared with both the control level (Table 3) and the level induced by lead alone. At the same time, lead shows some protective effect since an increase in lead concentration in ^{137}Cs-contaminated soil reduces NAAC. This antagonistic effect is statistically significant for nearly all the examined combinations of ^{137}Cs and Pb (Table 3).

3.3.3. Relative contribution of ^{137}Cs and Pb to the cytogenetic disturbances induction

The results of the variance analysis indicate that the value of the aberrant cell frequency mainly depends on the extent of Pb exposure (89% of the total variance), while the NAAC is determined by the extent of ^{137}Cs exposure (83%). In a previous study [12], in which there was no radionuclide introduced into the soil but an acute external radiation exposure, the resulting aberrant cell frequency depended on the γ-radiation dose and the soil contamination with lead nitrate to the same degree, whereas the lead exposure was decisive in determining the NAAC.

3.4. Combined exposure to ^{137}Cs and 2,4-D herbicide

3.4.1. Frequency of aberrant cells

For the combined ^{137}Cs + 2,4-D herbicide treatments, a decrease in cytogenetic effects occurred in comparison with the separate exposures in almost all cases (Table 4). The maximum aberrant cell frequency was observed for the combination of 1 kg/ha of 2,4-D herbicide and the maximum ^{137}Cs specific activity, i.e. 49.2 kBq/kg. Interaction coefficient values indicated statistically significant antagonistic effects for all the combinations of contaminants (Table 4). Thus, for the whole range of concentrations examined, the experimental effect differed from the predictions of the additive model.

3.4.2. The number of aberrations per aberrant cell

The NAAC values for ^{137}Cs + 2,4-D exposure (Table 4) were higher than in the control. The NAAC was below that observed in the separate exposures to ^{137}Cs in all cases, i.e. the additional treatment with 2,4-D pesticide led to a decrease in the NAAC. This decrease was statistically significant except for the combination of 24.6 kBq/kg ^{137}Cs + 1 kg/ha of 2,4-D herbicide. Analysis of the K_w values (Table 4) indicates antagonistic interactions for all combinations of ^{137}Cs and 2,4-D herbicide studied. The heavy metals demonstrate a similar but less pronounced effect as shown above. The observed antagonisms may in part be due to the ability of the 2,4-D herbicide to inhibit ^{137}Cs ion uptake from the soil to a plant [23].

3.4.3. Relative contribution of ^{137}Cs and 2,4-D pesticide to induction of cytogenetic effects

The herbicide makes a much greater contribution (40% of the total variance) to the aberrant cell frequency than does ^{137}Cs (19%) for their combined application, but ^{137}Cs is more effective with respect to aberrant cell damage (31%) than the herbicide (12%). The interaction effects (antagonisms) are very notable in this case since the SS values for the interaction of

these two factors are 41% for the aberrant cell frequency and 57% for the NAAC.

4. Discussion

To our knowledge, this is the first published information on the assessment of genotoxic effects due to separate and combined exposure to heavy metals, herbicide, and radionuclides in the intercalar meristem of spring barley. An analysis was carried out to demonstrate the qualitative differences in induction of cytogenetic effects in spring barley intercalar meristem by various stressors. The herbicide 2,4-D produced an increase in aberrant cell frequency, but had little effect on the NAAC. Soil contamination with ^{137}Cs, in contrast, influenced the NAAC more strongly than the aberrant cell frequency. The heavy metals changed both parameters, although they affected the aberrant cell frequency to a greater extent. The observed distinctions in these empirical dependences are caused by differences in uptake mechanisms from the soil and the biological effects on the cells.

Our previous study [12] showed that acute γ-radiation did not produce any significant increase in the NAAC. In the present work, the significant increase that was observed for this parameter may have been caused by heterogeneity in the dose field resulting from the combined effect of the external γ-irradiation and the internal exposure to ^{137}Cs incorporated in the meristem plant tissues. A much higher frequency of homologous recombination has been reported in plants grown in ^{137}Cs-contaminated soil when compared to acutely irradiated plants [24]. Incorporated radionuclides are carried by passive transport and concentrate in meristem tissues [22] where active cell division takes place. There is heterogeneity of ^{137}Cs distribution in tissues after uptake from soil, so actual doses to meristem cells are an order of magnitude higher than those calculated from an assumedly homogeneous internal distribution [25]. This provides for an enhanced level of cell damage in meristem tissue. Incorporated radionuclides have been shown to be more effective at creating genetic damage than γ-radiation in other recent studies [26,27].

Establishing the nature of the relationship between the biological effect and the stressor concentration in various components of the environment is important to achieve compliance with permissible levels of anthropogenic contamination and to obtain some reasonable estimates of its consequences for biota. Moreover, knowledge of dose–effect dependence gives additional information about the underlying mechanisms of the cytogenetic effects. It is interesting to compare our findings with those of previous studies [28,29], where genotoxicity of soils contaminated with heavy metals was studied with three plant bioassays (micronucleus tests of *Tradescantia* pollen-mother cells and meristematic root-tip cells of *Allium cepa* and *Vicia faba*). Significant genotoxicity was indicated for six heavy metals (As^{3+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, Cr^{6+}, and Ni^{2+}) among the nine studied; linearity was significant in all cases except for Cr^{6+}.

In the present work, similar results are presented for a different plant bioassay. Pb^{2+} and Cd^{2+} demonstrated significant genotoxicity, and the relationship between the cytogenetic effects and the level of soil contamination by these heavy metals can satisfactorily be described with a linear function. However, the non-linear models are shown to fit the experimental data much better than the linear model (Table 5). A non-linear dose–cytogenetic effect relationship was found in meiosis and first mitosis in meristematic root-tip cells of *Hordeum distichon* L. under conditions of aerial and root uptake of ^{90}Sr [27]. The same results have been obtained recently with a *Pisum sativum* root-tip meristem bioassay [30] in that the relationship between aberrant cell frequency and dose was supra-linear within a low dose-range and the biological effect was notably higher than expected.

Most of the empirical dependences obtained here are supra-linear, i.e. the cytogenetic effect yield per unit of dose of the agent examined is higher at low dose than at higher dose. This is in accordance with the results obtained recently in a transgenic *Arabidopsis thaliana* plant-based assay [31], where lead and nickel had a very strong effect on recombination frequencies at the lowest tested concentration of 0.5 mg/l, but did not influence recombination significantly at higher concentrations. This may, in part, be due to the fact [31,32] that the uptake of essential compounds increased linearly, whereas the non-essential heavy metal ions, lead and cadmium, were taken up more efficiently at low than at higher concentrations. The non-linear character of the empirical dependences shown here emphasizes the importance of taking greater care in choosing maximum permissible levels of soil contamination with

chemicals: in the low concentration range, even a small excess over the current standards can result in a disproportionately high increment of cytogenetic aberrations in plants cells.

In the present study, interactions of a low level of ^{137}Cs contamination with any level of cadmium soil contamination were shown to act synergistically on the frequency of aberrant cells. The frequency of aberrant cells does not depend on the cadmium contamination, but is reduced with increasing ^{137}Cs specific activity (Table 2), and the synergistic effects change to additive and antagonistic. This response trend was different from that reported by Shima and Ichikawa [33] who studied the mutagenic interaction between *N*-ethyl-*N*-nitrosourea and X-rays in the stamen hairs of *Tradescantia* clone BNL 4430. The authors found that the difference between the observed and expected somatic mutation frequencies tended to be larger after combined treatments with higher doses.

The phenomenon of damage interaction induced in a cell by different mutagens is one of the mysteries of mutation theory. Experiments on the genetic effects of combined treatments of chemical agents and irradiation on higher plant seeds [34] have shown that the mutagen interaction occurs at the primary damage level. Therefore, during prolonged existence in a cell of the primary damage induced by one of the factors, the process of its transformation into a chromosome aberration may be modulated by the other acting factor. The synergistic and antagonistic effects observed may be caused by the following: (1) a long-term existence and possible modification of the primary damage; (2) possible changes in the efficiency of the repair systems; (3) an interaction between DNA and membrane damages; (4) a mitotic activity stimulation (or repression) by one of the factors and the consequent reduction (or increase) of the time for repairing the DNA damage. No doubt, a deeper understanding of these events would be very useful in widening our knowledge about the mechanisms leading to non-linear effects.

The results described in [35,36] demonstrate that the inhibition of the DNA repair processes is a common mechanism in metal-induced genotoxicity, but each metal exerts a unique mechanism of repair inhibition. The ability of some agents to modify the efficiency of the repair systems leads to an increase in mutation frequency, due to the spontaneous and induced damages in the DNA molecule that are normally repaired. This explanation for synergistic effects is supported by experiments with different species (*E. coli*, *D. radiodurans*, *S. cerevisiae*, mammalian cells), showing that in the strains mutated in repair, the synergistic effect was much less pronounced than in the wild-type strains [36,37]. Moreover, for isogenic strains of yeast, the synergistic effects of combined treatment were most clearly pronounced in diploid cells of the wild-type, fewer appear in the haploid strains with lowered recovery capacity, and synergistic effects were practically absent in repair-defective mutants [37]. The same results were obtained in the presence of cadmium [38]. The synergistic effects are more typical for the interaction of low-LET radiation with different agents [37] than for high-LET radiation that forms severe non-reparable damages, in combination with the same agents.

The appearance of non-linear effects also depends on the concentration and dose rate of the stressors applied. In response to low-level exposures, the extent of non-linear effects does not drop, but it usually increases. This is because the shape of the dose–effect curve at low dose is mainly determined not by the exposure level, but by the specific features of the biological system, and it is often highly non-linear [39]. Therefore, the combined exposure to different factors more often leads to a synergistic response at low doses and concentrations than at high exposure levels, when the adaptation capacities of the system are exhausted and its response lags behind the increase in intensity of the impact.

The results obtained in this study support the relevance of the mechanisms described above in the formation of synergistic effects. Indeed, ionizing radiation is effective at inducing DNA single- and double-strand breaks leading to chromosome aberrations, and cadmium preferentially acts on DNA indirectly [40,41] by means of interfering with DNA transcription [42] and repair processes [35,36]. Therefore, these factors taken together can act synergistically. In studies on the combined exposure of low concentrations of ^{232}Th and ^{3}H with Cd nitrates on *A. cepa* root-tip cells, a synergistic increase in the frequency of aberrant cells was also revealed [41,43]. Cd is a typical biologically non-essential metal that, by penetrating a cell at high concentrations, can produce a strong toxic effect [32]. This can be the cause of the consistent antagonistic effects observed in this study for the com-

bined exposures of high concentrations of ^{137}Cs and Cd (Table 2).

An implementation of primary damage induced by one damaging agent has been reported to be conditioned by impact intensity, concentration or time dependence [34]. In the case of the combined treatment, the functional state of the biological system becomes very important; this was shown in our experiments on *Tradescantia* and *A. cepa* [44]. Therefore, this may explain why, irrespective of the test-species (barley, onion, and spiderwort) and the experimental conditions used (soil or aqua cultures), there is a mitigation of genotoxic effects for combined exposure of Cd with other stressors (external acute and chronic γ-radiation, incorporated ^{137}Cs and ^{232}Th radionuclides, etc.) up to clear antagonism, when the intensity of the impact (concentration or time of exposure) increases [12,43,44]. These clear antagonistic effects of combined exposure to cadmium at high concentrations and γ-radiation or other metals have been found in plants [45,46] and animals [47].

From the data presented here, the addition of lead and cadmium compounds to the soil together with ^{137}Cs contamination results in an increase of the aberrant cell frequency and a decrease in the NAAC. However, there are some differences in the effects of these heavy metals. Firstly, cadmium is capable of synergistically increasing the mutagenic effect of low doses chronic exposure. Secondly, cadmium makes a considerably smaller contribution to changes in the aberrant cell frequency. Thirdly, while the synergism for the ^{137}Cs + Cd combination found at low levels of contamination changes into antagonism as the ^{137}Cs specific activity increases (Table 2), the ^{137}Cs + Pb combination tends to show an antagonistic interaction (Table 3); this tendency is maximally displayed for the ^{137}Cs + 2,4-D combination (Table 4); and this is similar for both tests of the 'frequency of aberrant cells' and the NAAC.

5. Conclusion

The results of the study indicate that the aberrant cell frequency assay with spring barley intercalar meristem detects genotoxic effects of low levels of radioactive or chemical contamination and can be applied in the bio-monitoring of man-made contamination. The relationship between the extent of cytogenetic disturbances in spring barley intercalar meristem cells and the extent of soil contamination with ^{137}Cs, Cd, Pb, and 2,4-D herbicide was non-linear in the concentration ranges investigated. At low exposures, the cytogenetic effect frequency increased at a greater rate than at higher exposure levels. A combined ^{137}Cs and chemical (heavy metals and herbicide) contamination can induce statistically significant synergetic and antagonistic effects in spring barley intercalar meristem. The experimentally observed response in most cases differed from the additive effect anticipated from the data on the separate effects of the stressors. Considering our data and other literature, the conclusion can be made that the synergistic intensification of cytogenetic effects induced by various factors can generally be observed at low exposure levels. At higher concentrations (doses) or prolonged exposures, additive interactions or antagonistic effects occur more often. The results presented here support the principle that it is possible for environmental factors to mutually intensify their biological effects in situations of low-level contamination of the biosphere. In environmental exposure levels, the safety margins in linear risk estimates might be small and not able [6] to cover an extra risk due to synergistic action. This emphasizes the necessity of taking into account the possible effects of synergetic and antagonistic interactions induced by the combined exposure of different stressors in estimating the consequences of man-made influences on natural and agricultural ecosystems.

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