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Cytogenetic effects of radon activity concentrations in water on meristematic cells of *Allium cepa* L. root

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ABSTRACT

In this study, the results of cytogenetic analysis of the influence of various radon activity concentrations in water on meristematic cells of *Allium cepa* L. are presented. The cytotoxicity and genotoxicity test used in this study was the *A. cepa* test. To reliably consider the biological effects of ionizing radiation coming from radon, it is necessary to modify this test system, adjust the process of exposing the samples in accordance with the migration potential of radon gas. Evaluation of the cytotoxicity and genotoxicity of radon in water, various parameters of *A. cepa* L. were analyzed, such as root number, mitotic and phase indexes (*MI* and *PI*). All applied radon activity concentrations in water, ranging from 100.62 to 1,006.25 Bq/L, caused inhibition of root growth, reduction of mitotic index (*MI*), and a significant increase in the number of aberrant cells, in the treated meristematic cells of *A. cepa* L.

INTRODUCTION

After the discovery of X radiation and natural radioactivity, it became evident that radiation exposure leads to the induction of negative effects for humans. Among the first to warn about harmful effects of the newly discovered radiation were Thomas Edison, William Morton, and Nikola Tesla, pointing to eye irritations, which occurred during experiments with X-rays and fluorescent substances. In June 1896, Nikola Tesla recommended that "experimenters should not get too close to the X-ray tube" (Hrabak et al., 2008). The wider effects of the harmful effects of ionizing radiation came to the fore after the first explosion of atomic bombs in 1945, which were dropped on Hiroshima and Nagasaki. Epidemiological studies of Hiroshima and Nagasaki survivors exposed to the atomic bombing, as well as data from studies on patients exposed to radiotherapy, provided a large amount of information on the effects of ionizing radiation on human health.

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Based on significant experience gained through numerous studies of the health impact of radon in uranium mines, as well as in other mines, radon, and radioactive radon offspring have been identified as causes of lung cancer (UNSCEAR, 2000; ICRP, 1993; Adrovic, 2017; WHO, 2016). Radon is one of the most biologically important radioisotopes in nature because together with its decay products, makes a major contribution to human exposure of natural radiation sources and it has been identified as a public health problem (EPA, 2005).

When cells are exposed to ionizing radiation, standard physical effects between the radiation and atoms or molecules of the cells first appear, followed by possible biological damage (Turner, 2007). Ionizing radiation directly or indirectly disrupts the integrity of biomacro-molecules, resulting in a wide range of structural and functional changes. By the action of ionizing radiation, all types of molecules inside the cell can be damaged, and for the cell the most important damage are in the DNA molecules. Ionizing radiation is a strong mutagen, which leads to changes in the DNA molecule, causes genomic

instability, increases the frequency of mutations above the present normal level of mutations in the cell (Rodrigues et al., 2005). These changes can be at the level of smaller molecules, as well as at the level of proteins, DNA, and RNA, the most important organic molecules. Numerous experiments have shown that the number of mutations caused by radiation is proportional to the exposure dose used (Emery, 1986). Today, it is known that the effects of ionizing radiation depend not only of the size of absorbed dose, but also of the time of radiation exposure. The term dose strength or dose rate should not be confused with the term ionizing radiation intensity (Adrović, 2006). The strength of a dose represents the energy of radiation that remains in the irradiated matter, while the intensity or intensity of radiation represents the energy of that radiation that falls per unit area of the irradiated material per unit time. The problem of radiation protection, as well as other genotoxic agents, is a multidisciplinary problem and occupies a very important place in biological disciplines.

Today, test organisms are used with great success as indicators of radioactive contamination of the environment. Radioisotopes are potent genotoxic stressors, which can be cytologically detected by cellular inhibition, metaphase disturbance, induction of chromosomal aberrations, numerical and structural, ranging from chromosomal fragmentation to disruption of the mitotic spindle, and thus all subsequent dependent mitotic phases (Tedesco and Laughinghouse, 2012). The test organism *A. cepa* L. has been adopted by the International Plant Biological Research Program (IPPB) for monitoring or testing environmental pollutants (Nefic et al., 2013; Matsumoto et al., 2006; Ma, 1999). This test organism meets most of the essential requirements required in biomonitoring (Leme and Marin - Morales, 2009). The test organism *A. cepa* L. allows the assessment of a wide range of effects, such as chromosomal aberrations and disorders in the mitotic cycle, which can predict DNA damage in human chromosomes when exposed to similar agents.

MATERIALS AND METHODS

Complementary physical and biological dosimetry methods were applied in this study to reliably consider the biological effects of radon activity concentration, as the dominant natural source of ionizing radiation in the environment. In this study, were used the most modern research methods and measuring systems for the detection of ionizing radiation. In physical dosimetry were used sensitive, multiparameter measurement systems to monitor the effects of small doses of ionizing radiation. The biological effect of radiation dose in biodosimetry is based on cytogenetic analysis of biomarkers, which reflect the damage caused by ionizing radiation. The higher plants have been recognized as excellent genetic models for detecting mutagens in the environment, and today test organisms are necessary for monitoring cytogenetic studies (Ernst, 2003). In this study, the *A. cepa* test was used to determine the genotoxic and cytotoxic effects of radon activity concentration in water.

Methods and instrumentalization for radon detection

Alpha GUARD PQ 2000 PRO was used to detect radon activity levels in all ambient of environments. This system used integrated sensors, in addition to radon concentration, simultaneously measures air temperature, atmospheric pressure, and relative humidity, those meteorological parameters that are deeply correlated with the distribution of radon. Reliable detection of radon in water requires a complex and sensitive experimental procedure, which due to the migratory properties of radon gas must be carried out according to strict rules. In addition to the Alpha GUARD PQ 2000 PRO measuring system, the equipment includes Aqua KIT and Alpha PUMP. Aqua KIT is a special set of glass vessels for degassed radon from water and a safety vessel for optimizing the operation of the apparatus (Aqua KIT, 1997). The glass containers of the Aqua KIT apparatus provide a hermetically sealed space of radon emitted from the water sample, as well as the fast exchange of samples, which prevents incorrect measurements as a result of radon leakage from the equipment. In the closed gas circulation of Aqua KIT glass vessels, radon was expelled from the water sample using the Alpha PUMP system (2001).

This pump contains electronic pump speed adjustment (0.03 L/min, 0.05 L/min, 1 L/min). The connecting pipes route the radon gas path between degassing vessel, safety vessel, Alpha PUMP, and activated carbon filter box, to the central Alpha GUARD system. The box of activated carbon filter was used for the separation of exhaled radon from the tested water samples. With this measurement system, the difficulties associated with the detection of radon in water are minimized. This system is supported by a professional Alpha EXPERT software package for multiparameter analysis as well as graphical visualization of the obtained data and their archiving.

Preparation of water samples with different radon activity concentrations

For cytogenetic analysis of the influence of radon activity concentrations on meristematic cells of onion tissue, samples of distilled water with the same volume (0.4 L) were prepared, in which certain amounts of standard ^{226}Ra were added as a direct source of radon isotope ^{222}Rn. For sample preparation, the reference standard of the Czech Metrology Institute, Inspectorate for Ionizing Radiation was used, which in 500 ml of solution contains 550 ng ^{226}Ra, specific activity of 40.2 $Bq \cdot g^{-1}$ and activity of 20.13 kBq, on

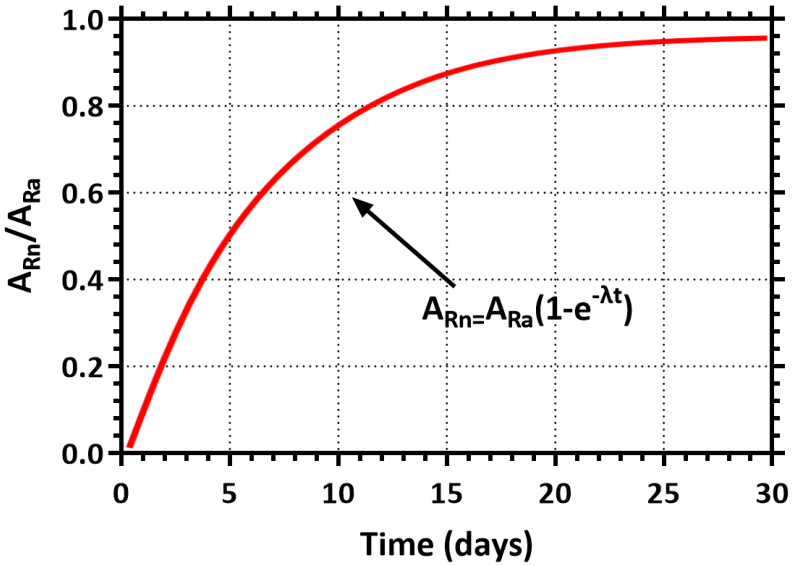

Figure 1. Emanation rate ^{222}Rn expressed via ^{226}Ra activity.

12 May, 2020 year. Ten water samples were prepared with a range of radon activity concentrations of 100.62; 201.25; 301.87; 402.50; 503.12; 603.75; 704.37; 805.00; 905.62; and 1,006.25 Bq/L. Distilled water was used as a negative control (NC). This range of radon activity concentrations in water is realistic in nature, and biological systems can potentially be exposed to them. The prepared water samples were placed in glass containers, hermetically sealed in desiccators of identical volumes. The original procedure applied in the study aimed to optimize the influence of a given radon activity concentration on the treated onion bulb samples. Like as, all other physico-chemical parameters in the prepared water samples were identical, so radon activity concentration was the only variable. The prepared water samples were left for 30 days in well-sealed desiccators, in order to establish an equilibrium between the concentrations of ^{226}Ra and ^{222}Rn isotope activity (Figure 1).

Because of the current scientific problem-dilemma, related to perception of the effect of low radiation doses on biological systems, samples of *A. cepa* L. bulbs were targeted with such broad values of radon activity concentration in water. In this way, were optimized the assumptions for detecting the potentially possible hermetic response of the *Allium* cells. In the available scientific literature, no research has been identified that is similar to this research, and which refers to the effects of radon activity concentration in the water on test organisms. The experiment was adjusted to the migration potential of radon gas, its physico-chemical characteristics so that the exaltation of radon would be reduced to a minimum value (Figure 2).

After a period of 30 days, the onion sets (10 bulbs) were exposed for 48 h to radon activity concentrations. To prevent radon agitation from the water in the desiccators and imbalance to radioactive equilibrium, the bulbs were placed as carefully as possible in contact with the surfaces of water samples of different concentrations. As the descendants of radon ^{222}Rn are the main sources of gamma radiation dose, which comes from the dominant natural radioactive uranium ^{238}U series, along with measuring of radon activity concentration, measurements of gamma radiation dose rate were performed continuously throughout the experimental study. Measurements were performed by using the Gamma Tracer system, measuring a range from 10 nSv/h - 10 mSv/h (Genrich, 1996).

Allium cepa test

A. cepa test is widely used for determination of the genotoxic and cytotoxic substances found in water. The *A. cepa* test is a short-term test with many advantages: low cost, ease of handling, onion cell chromosomes are relatively large chromosomes, which enables easy detection of possible chromosomal damage.

This test does not require prior preparation of the tested samples, as well as the addition of an exogenous metabolic system. A special feature of this test is applicable to a large number of cell types. Very well elucidated cytogenetic properties of *A. cepa* L., a small number of chromosomes in the diploid chromosome set, are additional advantages of the *A. cepa* test, which proved to be a reliable model system for measuring the influence of cytogenetic potential of pollutants in the

Figure 2. The original experimental setup preparation of samples with different radon activity concentrations.

environment. Metaphase chromosomal abnormalities, which are detected in the *A. cepa* test procedure, are not excluded from occurring in human chromosomes when exposed to similar agents. There is a large number of worldwide scientific reports for an excellent correlation of this plant system with the mammalian system (Fiskesjo, 1988). For monitoring or testing of environmental pollutants, the *A. cepa* test has been adopted by the International Plant Biological Processes Program (Ma, 1999).

The *A. cepa* test uses a mitotic index and certain nuclear abnormalities to evaluate cytotoxicity and micronucleus analysis, to check for mutagenicity of various agents. Mitotic and replication indexes are used as indicators of adequate cell proliferation (Gadano et al., 2002), which can be measured using this plant test system. Mitotic index is defined as the ratio of the number of cells in mitosis and the total number of analyzed cells. We have long considered it a very important criterion for tissue growth and multiplication. Assuming that mitosis occurs at random intervals, mitotic index and measured mitosis duration were used to calculate the interphase length, which value shows how radon activity concentration in water affected cell division, whether it was proliferative, inhibitory, or did not affect the division process at all. At different stages of mitosis, mitotic index, phase index, as well as the total percentage of abnormalities at different stages are calculated using the following formulas:

$$MI(\%) = \frac{TDC \times 100}{TC} \quad (1)$$

$$PI(\%) = \frac{TC \times 100}{TDC} \quad (2)$$

Where: *MI* - mitotic index; *PI* - phase index; *TDC* - total number of dividing cells; *TC* - total number of dividing and non-dividing cells.

Interpretation of mitotic index results was performed in relation to the mitotic index of *A. cepa* L. negative control. Special emphasis should be placed on the values of knowledge from plant cytogenetic techniques, which enable further development and increase the reliability of the *A. cepa* test. Sensitivity and correlation studies of the *A. cepa* test system and other test systems are essential for reliable risk assessment, as well as the extrapolation of data to other organisms, primarily humans, which was the aim of this study.

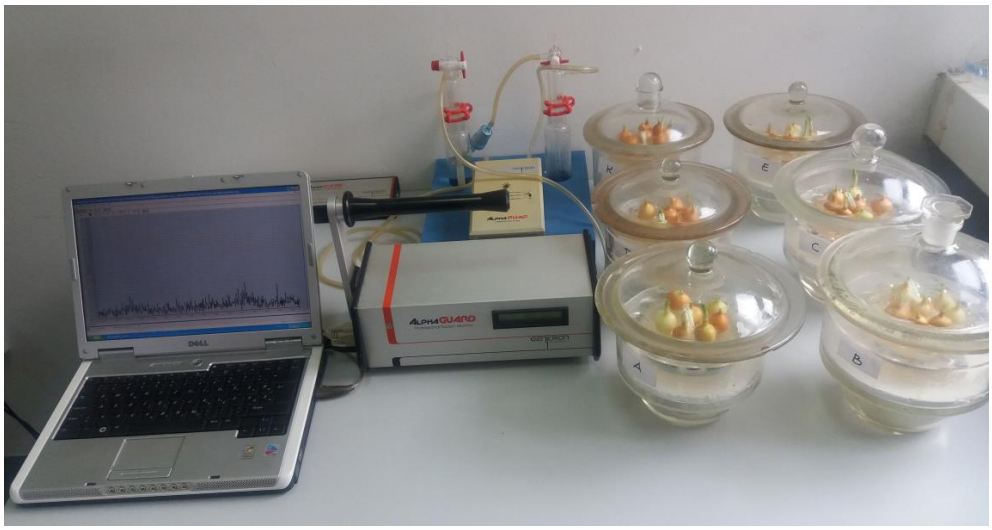

Figure 3. Onion bulbs on germination at selected radon activity concentrations in water.

Allium cepa test methodology

For the needs of experimental research of the study, were selected young, healthy bulbs (*A. cepa* L.), free of loose outer scales, uniform size, and weight from 5.00 to 5.99 g. The bulbs were procured from an organic food producer, who did not use herbicides or fungicides for their cultivation. The bulbs were placed in distilled water to germinate for 48 h. After that, over the next 48 h, the bulbs were fixed and exposed in the original designed glass packaging in exicators with uniform volume and selected radon activity concentrations in water, for further germination (Figure 3).

After the treatments, the roots were cut off and placed in Carnoy's fixative (3:1) ethanol and glacial acetic acid. Then the roots with fixative were left at a temperature of 4°C for 24 h. The next step was hydrolysis in 1 N (normal) HCl for five minutes at 60°C. The HCl solution hydrolyzes the cell wall and roots become softer, which facilitates the preparation of microscope slides. The roots were then placed in distilled water and prepared for preparation of the permanent cytogenetic slides. The preparation of root tip chromosome slides took place in the following order: 2-3 roots are placed on the slide and approximately 2-3 mm of the top part (meristematic cells) is cut off.

A drop of paint (Lacto-Propionic Orcein) is placed on them and they are macerated with the flat end of a metal rod (taper). A cover glass is placed over the macerated tops of roots and pressed with filter paper to absorb the excess color. The final step is to add Canada balsam to slide, which is covered with a cover glass, a smaller and thinner sheet of glass that is placed over the specimen. After that, the slides were ready for microscopic observation.

The microscopic analysis of meristematic cells of A. cepa L., a total of 5,000 cells per treatment, was performed on modern high-resolution biological microscopes the BestScope and Kern & Sohn (models: BS-2063T, OBE-104C825) and imaged using high-resolution cameras (BUC2D-500C, resolution 5 MP, manufacturer: Sony Japan, and camera ODC 832, resolution 5.1 MP, manufacturer: KERN Optics Germany). The data of root number, root length, mitotic, and phase indexes were subjected to statistical analysis.

One-way analysis of variance (ANOVA) was used for statistical analysis. One-way ANOVA and multivariate data analysis were performed using the GraphPad Prism 9 software. The results that differed at the level of $p < 0.05$ were considered statistically significant. Also, the Pearson correlation coefficient was calculated for the observed parameters. The research was conducted in the Laboratory for Detection, Dosimetry, and Radiation Protection, and the Laboratory for Genetics, Faculty of Science, University of Tuzla.

RESULTS AND DISCUSSION

The first results in our study were macroscopic parameters, the root length and number of onion roots. The results were obtained after exposure to different radon activity concentrations for 48 h. The results were obtained from a group of 10 bulbs from each sample, where 5 bulbs of *A. cepa* L. were analyzed. Table 1 shows the growth and number of onion roots in each group of bulbs, expressed as mean value of the variable lengths of the calculation of individual roots, which were exposed to selected radon activity concentrations in water. The obtained results of

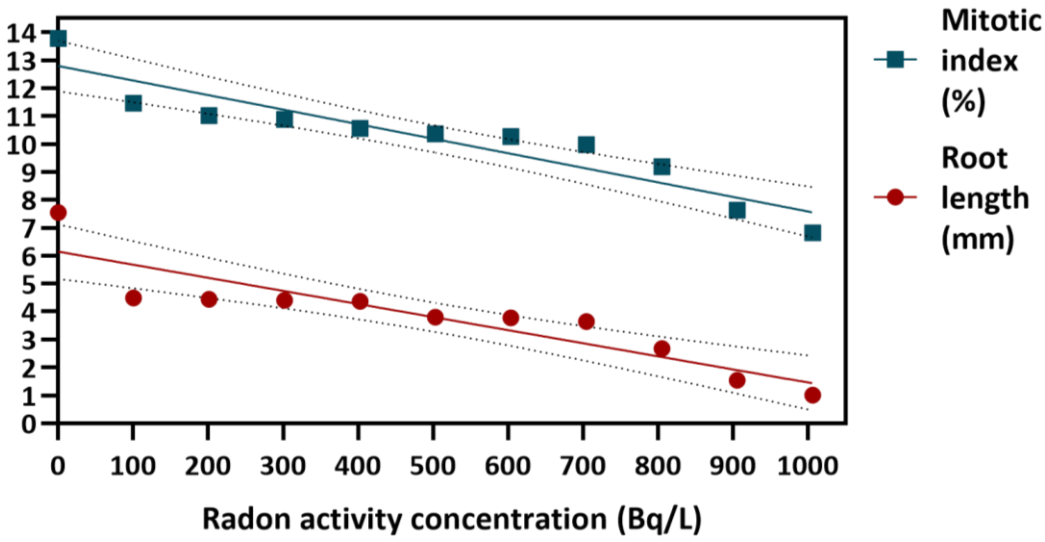

Figure 4. Comparative presentation of the dependence of onion root length and mitotic index induced by different radon activity concentrations in water.

Table 1. Mean root length of *A. cepa* L. and cytological effects induced by different radon activity concentrations in water.

Radon activity concentration (Bq/L)	The mean value of length roots (mm)	Overall number of onion roots	% root growth relative to control
100.62	4.49	168	59.55
201.25	4.44	141	58.90
301.87	4.41	161	58.49
402.50	4.37	149	58.00
503.12	3.80	136	50.40
603.75	3.77	137	50.00
704.37	3.64	135	48.28
805.00	2.68	136	35.54
905.62	1.54	133	20.42
1,006.25	1.01	140	13.40
0 (NC)	7.54	208	100

measuring the length of root growth, show the inhibition of root growth at all applied radon activity concentrations in water. As the radon activity concentration in water increased, the inhibition of root growth also increased. Table 1 shows that the maximum root growth was recorded on the negative control group of onion bulbs, 7.54 mm, the smallest, 1.01 mm, on the bulbs treated at the highest concentration of work activity of 1,006.25 Bq/L. Smaller average length roots on the treated bulbs in relation to control series of bulbs are undoubtedly a reflection of the toxic effect of radon activity concentration, as the dominant variable in this experiment.

The cytotoxicity of radon activity concentration in water was also confirmed by analysis of mitotic index (*MI*). Onion root growth and MI were correlated with radon activity concentrations in water, using linear Pearson correlation coefficients. In the first case, Figure 4, was obtained a strong negative correlation (r = - 0.78) of root growth and levels of radon activity concentrations in water. Also in the second case, was obtained a strong negative correlation (r = - 0.80) of the mitotic index and different of radon activity concentrations in water.

The mitotic index decreased, in relation to negative control, indicate changes caused by chemical action during the growth and development of onion roots (Leme and Marin - Morales, 2009), which causes cell lag in

Table 2. Presentation of mitotic and phases indexes of *A. cepa* L. cells, after exposure to different radon activity concentrations in water.

Radon activity concentration (Bq/L)	Counted cells	Dividing cells	Aberrant Cells (%)	Deformed non-dividing cells (%)	*MI* (%)	*PI* (%)			
						Pro	Meta	Ana	Telo
100.62	4,885	560	17.86	6.16	11.46±0.69**	38.95	26.32	13.68	21.05
201.25	4,860	535	30.84	7.82	11.01±0.98**	36.26	26.37	16.48	20.89
301.87	4,825	525	40.00	9.00	10.88±0.71**	38.64	21.59	18.18	21.59
402.50	4,730	499	46.70	11.21	10.55±0.73**	30.09	29.13	17.48	23.30
503.12	4,835	501	57.88	15.00	10.36±0.53***	33.23	24.26	20.41	22.10
603.75	4,770	490	71.02	18.10	10.27±0.45***	36.63	20.13	12.12	31.12
704.37	4,925	491	79.43	18.58	9.97±0.62***	34.67	23.44	14.42	26.18
805.00	4,855	446	84.10	20.80	9.19±0.43***	39.43	20.22	15.24	25.11
905.62	4,875	372	87.36	23.90	7.63±0.50****	28.75	22.33	18.99	29.93
1,006.25	4,675	319	92.48	27.00	6.82±0.45****	27.26	19.85	22.31	30.58
Control	4,970	685	8.03	1.21	13.78±0.53	31.39	28.47	16.79	23.35

MI, mitotic index; *PI*, phase index; Pro, prophase; Meta, metaphase; Ana, anaphase; Telo, telophase. NC, negative control.
Significant difference compared to the control group, *ρ<0.05; **ρ<0.01; ***ρ<0.001; ****ρ<0.0001. Analyzed per 5,000 cells for each experimental treatment and negative control.

certain phases of the cell cycle. In the negative control, the value of mitotic index (*MI*) was 13.78%. As the radon activity concentration in water increases, the mitotic index constantly decreases, ranging from 11.46 to 6.82%, according radon activity concentrations from 100.62 to 1,006.25 Bq/L, respectively. The decrease in the *MI* could be due to inhibition of DNA synthesis (Schneiderman et al., 1971; Sudhakar et al., 2001) or blocking in the G_2 phase of the cell cycle, preventing the cell from entering mitosis (Van't Hof, 1968). The MI is an acceptable measure of cytotoxicity in living organisms (Smaka-Kinel et al., 1996), which was confirmed by our study. The effects of radon activity concentration on the MI and frequency of mitotic phases are given in Table 2. The lowest percentage (8.03%) of aberrant cells was observed in negative control and the highest in samples of *A. cepa* L. cells (92.48%) that were exposed to radon activity concentration of 1,006.25 Bq/L. The percentage of aberrant cells began to increase sharply with increasing radon activity concentration. At radon activity concentration of 402.50 Bq/L, the percentage of aberrant cells was 46.70%. It can be seen from Table 2 that prophase is the most frequently recorded phase of the cell cycle. De facto, prophase index is dominant at all radon activity concentrations. The values of the prophase index ranged from 27.26 to 39.43%, metaphase index from 19.85 to 29.13%, anaphase index from 12.12 to 22.31%, and telophase from 20.89 to 31.12%. Based on the values of phase index, it can be concluded that the largest number of cells present in prophase, which is evidence of good proliferative activity.

On the other hand, the existence of all phases of mitosis indicates that the cells regularly complete division, and eliminates the possibility of an extreme prophase index, which would be a consequence of abnormal cell proliferation.

Conclusion

All applied radon activity concentrations, from 100.62 to 1,006.25 Bq/L, caused growth inhibition and changes in the mitotic index of *A. cepa* L. cells. Decreased root growth, as well as a significant increase in aberrant cells, are an indicator of the toxic effects of all applied radon activity concentrations.

There was a significant decrease in root growth and mitotic index of meristematic cells of *A. cepa* L., occurred in bulbs treated with radon activity concentrations higher than 704.37 Bq/L, which is close to the value of 1000 Bq/L taken by the European Union when it is necessary to establish measures to protect public health.

The percentage of chromosomal abnormalities in the different mitotic phases of all treated samples was significantly higher than the percentage of the negative control samples. The obtained results represent a good basis for a more thorough study of the effect of ionizing radiation on biological systems, which are caused by radon activity concentrations, and based on the results of this study, was indicated that the *A. cepa* test, is a sensitive and reliable bioassay, for determining the impact of radioisotopes as contaminants, causing legitimate concern for the state of the environment, where these tests are applied.

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