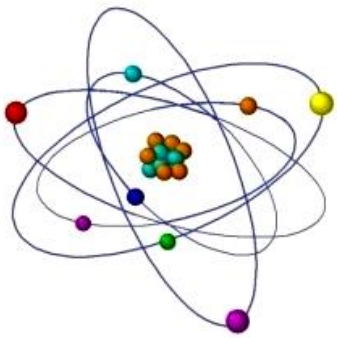
# CONNECTION OF X-RAY INDUCED GENOMIC INSTABILITY AND REMOTE EFFECTS OF ANTIOXIDANT PRODUCTION IN THE CHAMOMILE PLANT



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ABSTACT: *The main purpose of research is verification the hypothesis about the preservation of signs of radiation-induced genomic instability at the flowering stage of the chamomile plant after pre-sowing seed irradiation and the interaction of dose-dependent changes in the level of DNA damage and stimulation of antioxidant production.*

*The study carrying out on two genotypes of chamomile, Perlyna Lisostepu variety and its mutant, using pre-sowing seed radiation exposure at dose levels 5–15 Gy. Studies of the rearrangement of the primary DNA structure of under different doses were studied on plant tissues at the flowering stage using – ISSR and RAPD DNA markers. Dose-dependent changes relative to the control of the amplicons' spectra were analyzed using the Jacquard similar- ity index. Antioxidants such as flavonoids and phenols were isolated from pharmaceutical raw materials (inflorescences) using traditional methods.*

*Preservation of multiple DNA damages at the stage of plant flowering under pre-sowing seed irradiation at low doses confirming. It was found that the largest rearrangements of the primary DNA structure of both genotypes, manifested in reduced similarity with the control spec- tra of amplicons, were observed under irradiation dose levels 5–10 Gy. There was a tendency to approach this indicator to the control under 15 Gy dose, which means increasing efficiency of the reparative processes. The relationship between the polymorphism of the primary structure of DNA by ISSR-RAPD-markers in different genotypes and the nature of its rearrangement under radiation exposure was shown. Dose dependences of changes in the specific content of antioxidants were non-monotonic with a maximum at 5–10 Gy. Comparison of dose dependences of changes in the coefficient of similarity of the spectrum of amplicons between irradiated and control variants with nonmonotonic dose curves in the specific content of antioxidants allowed to suggest that there was the antioxidant protection stimulation under the doses corresponding to low efficiency of repair processes. The decrease in the specific content of antioxidants followed the restoration of the genetic material normal state. The interpretation of the identified phenomenon has been based on both known connection between the effects of genomic instability and the increasing yield of the reactive oxygen species and general principles of antioxidant protection.*

Key words: X-ray, short –term effects, remote effects, induced genomic instability, ntioxidant

## INTRODATUON

The study of the effects of radiation exposure has revealed a variety of metabolic changes in irradiated organisms [3, 7, 9, 21]. Under acute and chronic exposure protective reactions are stimulated, including an increase in the content of radioprotective substances [2,3,9,17,34]. Most plant radioprotectors have antioxidant, antitumor, immunomodulatory, and anti-inflammatory effects and have long time been used in medicine [2, 7, 9, 17, 21, 34]. This enables the biotechnological implementation of radiation exposure to improve the quality of pharmacological raw materials. This approach has shown number of technological advantages. First, irradiation of plants under narrow interval of low doses allows stimulating the production of necessary metabolites without reducing the yield of pharmaceutical raw materials [33]. Second, an increase in the yield of target metabolites in a mature plant has been shown several months after simple procedure jf presowing seed irradiation [33,41]. In addition to their practical significance, these data are theoretically important for understanding the mechanisms of formation of an interactive picture of the long-term and remote aftermath of irradiation. The stimulation of a wide range of anti- oxidants reflects the only one component of the organism's systemic response to radiation, which includes the development of damage, various protective and restorative processes.

The cell genome is the main target of radiation exposure [21]. Recent decades have shown that DNA is vulnerable to both constitutive intracellular factors and external exposure [1, 5, 13,14]. Both diverse protection and reparation mechanisms lead to success in the trans- mission of hereditary information. The study of the effect of low doses on various biological systems revealed the occurrence of multiple genetic damages under direct or indirect radiation exposure. This phenomenon has entered the scien- tific terminology under the name of radiation-induced genome instability (RIGI) [1, 4, 14]. According to modern notions, RIGI is the occurrence of de novo multiple genetic lesions in a significant number (up to 30%) of descendants of irradiated cells, accompanied by activation of mobile elements and other epigenetic rearrangements, increased levels of reactive oxygen species (ROS) [24,36,39]. These features of radiation- induced genomic instability suggest the possibility of maintaining it for several months in a completely developing plant organism and stimulating an increase in the level of antioxidants.

The purpose of the investigation was to verify the hypothesis about the preservation of signs of radiation- induced genomic instability at the flowering stage of the plant – chamomile (Matricaria chamomilla L.) after presowing seed irradiation, the interaction of dose-dependent changes in the level of DNA damage and stimulation of antioxidant production.

## MATERIALS AND METHODS

The research in the conditions of vegetation experiment carrying out on two genotypes of chamomile – Perlyna Lisostepu, Ukrainian selection and its mutant. Seed material receiving from the Research Plant of Medicinal Plants of the Institute of Agroecology and Nature Management of NAAS of Ukraine, Lubny, Ukraine. The choice of these genotypes for the purposes of the study basing on previous studies of chamomile genotypes of pharmacy selection

in different European countries that have revealed a significant difference in physiological and biochemical characteristics of these two genotypes [34, 42]. An assessment of intraspecific relationships [31] by ISSR-RAPD polymorphism using UPGMA software (available online at http://genomes.urv.cat/UPGMA/) found that the Jacquard similarity index of these genotypes according to ISSR sequences are 0.47–0.75; RAPD sequences are 0.39–0.55, respectively. To date, the mutant of Perlyna Lisostepu is involved in variety testing: assessment of difference, homogeneity, stability of traits.

Dry seeds were irradiated on an X-ray unit RUM-17 (Russia) at doses of 5, 10, and 15 Gy, dose rate 1.42 × 10—2 Gy/s, voltage — 200 kV, current 10 mA, filter Cu (0.5 mm). There were 10 grown plants per pot with 1.5 kg of loamy soil. For one variant of the experiment, five pots were used, i.e. 50 plants. For molecular analysis, DNA was iso- lated from the vegetative mass of plants in the flowering phase, the material was selected by taking a small amount of tissue from all plants, followed by mixing and averaging immediately before DNA isolation. Conditions for collection, preparation and storage of pharmaceutical raw materials describing previously [33].

There are several approaches for detecting genome instability under different types and doses of radiation exposure. The most common is the method of estimating stable and unstable chromosomal aberrations, which conceding as one of the main markers of radiation damage to humans, animals and plants [20].

Today, molecular genetic approaches have become widespread [24]. One of them is very promising in terms of assessing the vulnerability of certain DNA sequences in PCR with different types of primers. This approach using to study the extent of different DNA sequences under different doses of X-ray exposure using 8 ISSR and 10 RAPD primers. The focus of the RAPD and ISSR study of microsatellite sequences was based on previous data, which revealed the appearance of atypical amplicons in these sequences, that indirectly indicated the ‘rejection’ of more serious DNA disorders at ‘checkpoints’and mitosis [32].

ZymoResearch (Quick-DNA Plant/Seed Miniprep Kit) was used to isolate DNA according to the manufacturer’s protocol. PCR was carried out using a GeneAmp PCR 2400 thermocycler (Applied Biosystems, Foster City, CA). Checking the nativeness of the isolated DNA, as well as sep- arating the products of PCR reactions, was carried out in a 1.7% agarose gel with TBE buffer, visualized in the presence of ethidium bromide on a UV transilluminator, and then photographed. When performing electrophoresis in the ‘pocket’ of the gel, 5 ll of DNA solution was made. GeneRuler 50 bp was used as a molecular weight marker. Two types of primers were used for PCR: OPA and ISSR (Metabion, Planegg, Germany) and a ready mixture of reagents for DNA amplification of PCR MIX 2x-R (Neogene, Ukraine, Kyiv). The nucleotide sequences of the primers are shown in Table 1. The 25 µl PCR reaction mixture contained: 12.5 µl of MIX 2x-R PCR, 1.75 µl of primer, 5.75 µl of deionized water and 5 µl of total genomic DNA. Amplification with ISSR primers included the following steps: initial denaturation for 4 min at 94 ◦C, 40 cycles; denaturation at 94 ◦C — 45 s, annealing — 45 s (temperature differed for each primer, specified by the manufacturer), elongation at 72 ◦C — 45 s; the final elongation lasted 7 min at 72 ◦C. Amplification with OPA primers included the following steps: initial denatur- ation for 5 min at 94 ◦C, 40 cycles; denaturation at 94 ◦C — 40 s, annealing — 40 s (temperature differed for each primer, specified by the manufacturer), elongation at 72 ◦C — 2 min; the final

elongation lasted 10 minutes at 72 ◦C. The nucleotide sequences of the primers are shown in Table 2. The total phenolic content was determined with the Folin–Ciocalteu reagent (FCR) [12].

**Table 1. Names and the nucleotide sequences of the ISSR DNA markers**

| Primer | Sequences | Primer | Sequences |
|---|---|---|---|
| ISSR 5 | $5^0$-CAC ACA CAC ACA CAC AAC-$3^0$ | ISSR 826 | $5^0$-ACA CAC ACA CAC ACA CC-$3^0$ |
| ISSR 807 | $5^0$-AGA GAG AGA GAG AGA GT-$3^0$ | ISSR 24 | $5^0$-AGA GAG AGA GAG AGA GGC-$3^0$ |
| ISSR 810 | $5^0$-GAG AGA GAG AGA GAG-$3^0$ | ISSR 834 | $5^0$-AGA GAG AGA GAG AGA GCT T-$3^0$ |
| ISSR 825 | $5^0$-ACA CAC ACA CAC ACA CT-$3^0$ | ISSR 842 | $5^0$-GAG AGA GAG AGA GAG ACT G-$3^0$ |

**Table 2. Names and the nucleotide sequences of the RAPD DNA markers**

| Primer | Sequences | Primer | Sequences |
|---|---|---|---|
| OPA-01 | $5^0$-CAG GCC CTT C-$3^0$ | OPA-06 | $5^0$-GGT CCC TGA C-$3^0$ |
| OPA-02 | $5^0$-TGC CGA GCT G-$3^0$ | OPA-07 | $5^0$-GAA ACG GGT G-$3^0$ |
| OPA-03 | $5^0$-AGT CAG CCA C-$3^0$ | OPA-08 | $5^0$-GTG ACG TAG G-$3^0$ |
| OPA-04 | $5^0$-AAT CGG GCT G-$3^0$ | OPA-09 | $5^0$- GGG TAA CGC C-$3^0$ |
| OPA-05 | $5^0$-AGG GGT CTT G-$3^0$ | OPA-10 | $5^0$-GTG ATC GCA G-$3^0$ |

The reaction mixture containing 0.1 ml of sample, 0.5 ml of (1/10 dilution) of FCR, 1 ml of $H_2O$, was mixed, after 1 min, 1.5 ml of 20% sodium carbonate was added, shacked and incubated 2 h in the dark at the room temperature. The absorbance of blue complex was determined at 760 nm using SF-46 (Shimadzu UV-1280, Kyoto, Japan) spectrophotom- eter. The total phenolic contents were calculated in mg of gallic acid equivalent (GAE) per g of dry flower weight by using gallic acid calibration curve.

The flavonoids content in extracts was determined using a method of flavonoid–aluminum complex formation [12]. Reaction mixture containing 0.5 ml of sample, 0.5 ml of 2% of $AlCl_3$ in 50% EtOH solution, 2 ml of 70% EtOH was shacked and incubated 20 min at room temperature. The absorbance of yellow complex was determined at 410 nm using Shimadzu UV-1280 spectrophotometer (Kyoto, Japan). The flavonoids content calclating in mg rutin per g dry flower weight by using rutin calibration curve.

Two statistical approaches and, accordingly, two types of indicators were used to characterize changes in amplicon sets after PCR with ISSR and RAPD DNA markers in both control and exposed variants. The indicator '% of atypical ampli- cons' was calculated as the ratio of the number of amplicons other than the control to their total number; the nonparametric Van der Waerden *X*-test [26] was used to assess the significance of the difference in averages for different var- iants of the experiment. Jacquard's similarity index was used as an indicator of

group, cluster changes in these set of amplicons of different lengths [15]. Calculations were performed using UPGMA software (available online http://genomes.urv.cat/UPGMA/).

Statistical analysis of changes in the content of low molecular weight antioxidants – the mean value (*X*) and standard deviations (Sx) were calculated by traditional methods. Significance of differences between variants of the experiment was evaluated by the parametric Student's *t*-test, confidence interval, *p*¼.95 [26].

## RESULTS AND DISCUSSION

The electrophoregrams (Figures 1 and 2) show significant differences in the number and length of amplicons in the analysis of RAPD and ISSR DNA markers in control variants of the both chamomile genotypes. This indicates the different involvement of these sequences in the organization of the genome of the cultivar Perlyna Lisostepu and its mutant.

Under radiation exposure, the appearance of atypical amplicons of both longer and shorter length is observed in comparison with the amplicons in the control. Irradiation causes direct and indirect damage to DNA, a variety of endogenous chemical transformations associated with the action of ROS [7,12,29,31], the formation of single and double breaks. Double breaks and their misrepair are basic trigger mecha- nisms of chromatin reorganization [20]. The possible results of such a reorganization could be the formation of intercalary and terminal deletions, inversions, duplication, translocation [20]. These types of chromosome reorganizations lead to changes in the length and primary sequence of DNA. An additional important mechanism of chromatin reorganization is also the hypervariability, the formation of new microsatellites as replication and repair errors through microsatellite DNA sequences [5,8,13,18, 27].

Thus, the interaction of two mechanisms – radiation- induced chromatin reorganization and the phenomenon of hypervariability of microsatellite sequences might cause a change in the length and primary structure of the DNA molecule, and manifest itself as different from the control, increased or decreased in length amplicons.

Figure 3(A,B) shows the trend of the average frequency of atypical amplicons depending on the dose during ISSR and RAPD DNA markers' analysis.

There is a noticeable difference in the average frequency of atypical amplicons depending on the dose in ISSR and RAPD DNA markers' analysis. However, estimates for the non-parametric Van der Waerden *X*-test only indicate a close to significant 95% confidence interval difference between the percentage of atypical ISSR-PCR amplicons of Perlyna Lisostepu genetic material and its mutant at 5 Gy and between mutant variants exposed with 10–15 Gy. Indeed, this indicator does not focus on the changes in total, cluster changes in the structure of their spectra. This allows us to consider the rate of '% of atypical amplicons' is not informative enough to characterize the effect of radi- ation on DNA stability, at least for the selected set of markers and dose range. The data shown in Figures 1 and 2 in terms of changes in the spectra of amplicons are obtained under the exposure with the involvement of the Jacquard similarity index. There are two groups of indicators necessary for the analysis: similarity between spectra of amplicons of different genotypes at the same radiation dose (Table 3) and similarity of spectra of amplicons under different doses within one genotype (Tables 4 and 5).

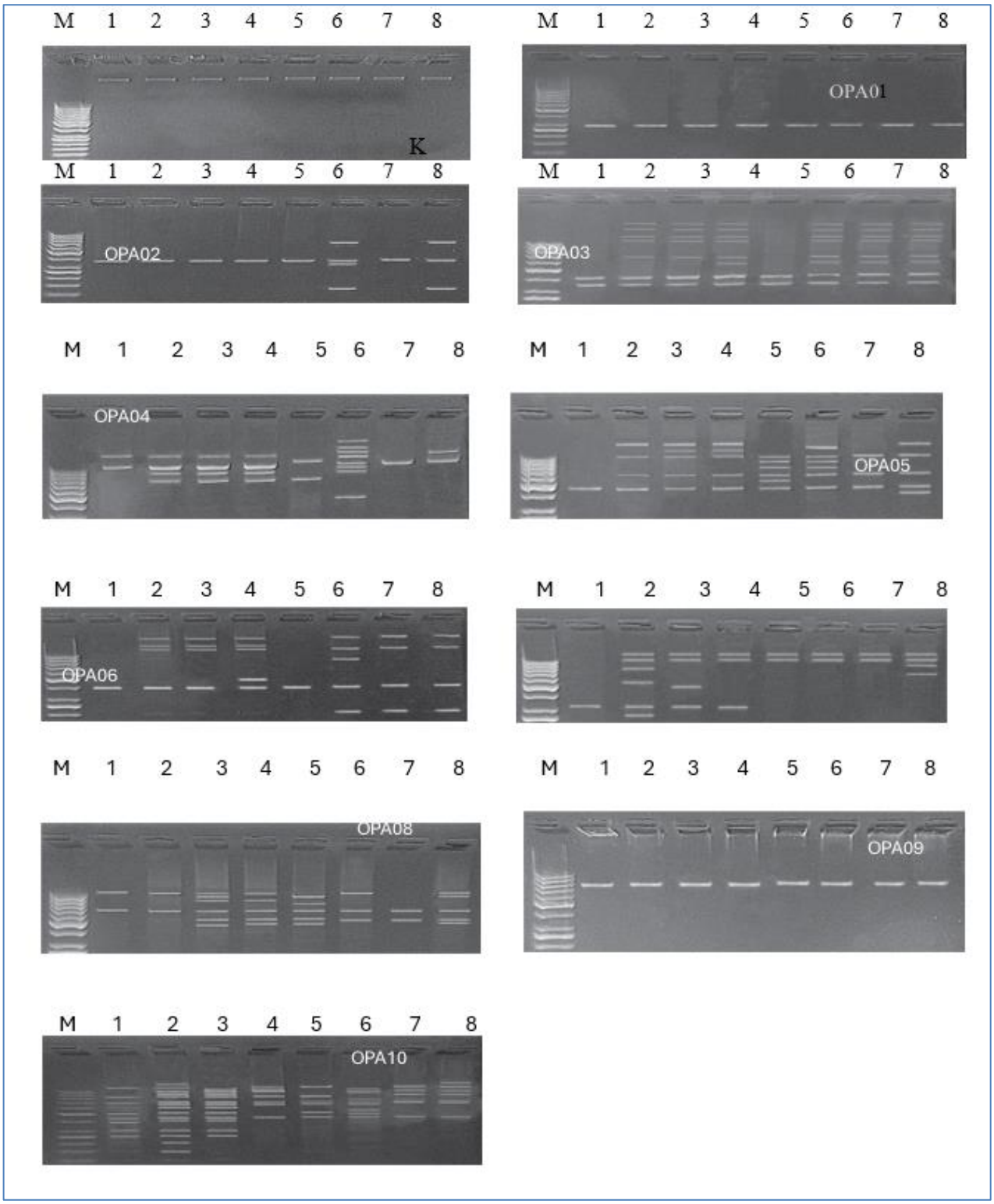


**Figure 1. Electrophoregram of RAPD-PCR products.**

K - control of DNA nativeness; M - molecular weight marker GeneRuler 50 bp; 1 - Perlyna Lisostepu, control; 2 - Perlyna Lisostepu 5 Gy; 3 - Perlyna Lisostepu, 10 Gy; 4 - Perlyna Lisostepu, 15 Gy; 5 - mutant, control; 6 - mutant, 5 Gr; 7 - mutant, 10 Gr; 8 - mutant, 15Gy.

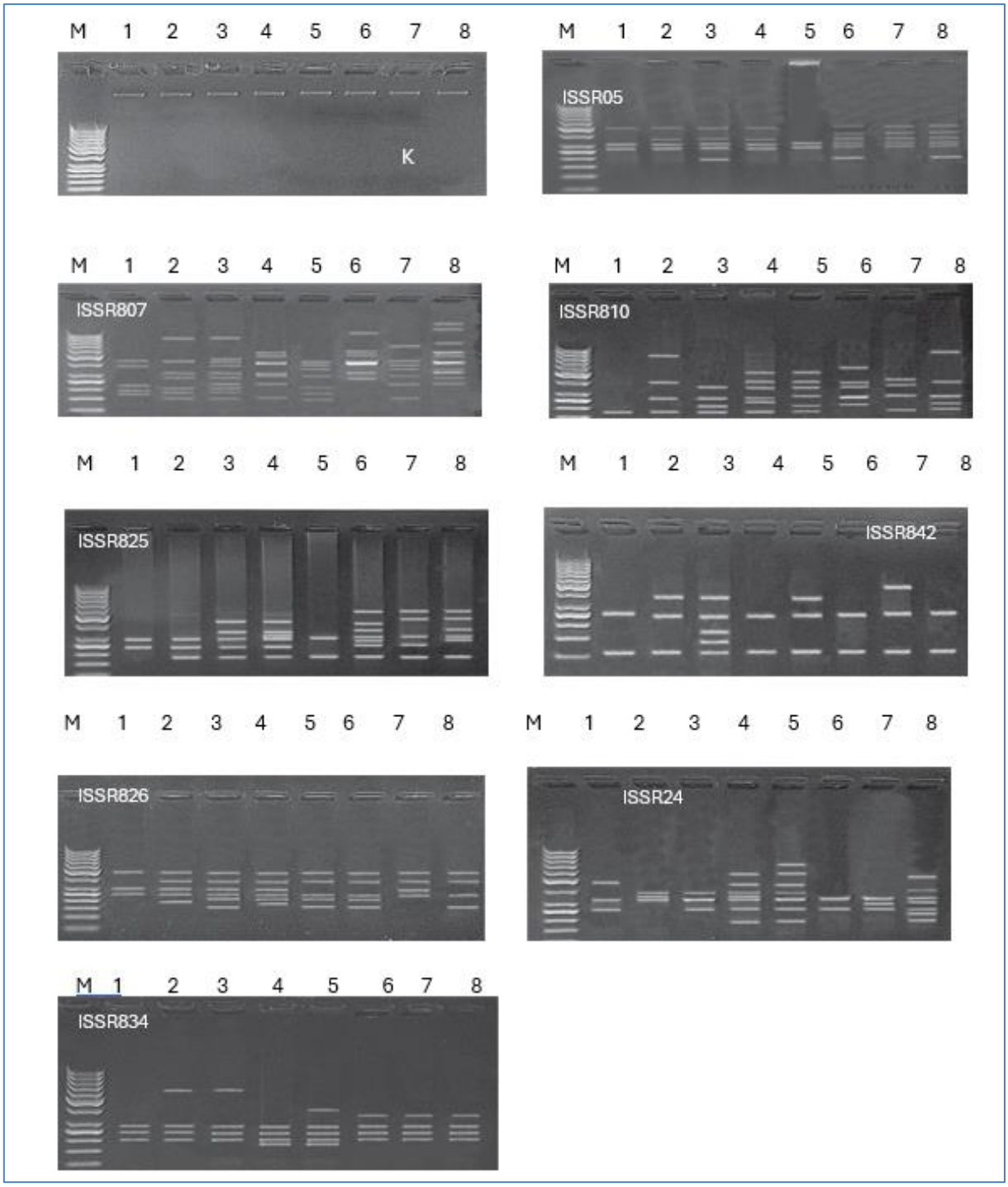


**Figure 2. Electrophoregram of ISSR-PCR products.**

K-control of DNA nativeness. M - molecular weight marker GeneRuler 50 bp; 1 - Perlyna Lisostepu, control; 2 - Perlyna Lisostepu, 5 Gy; 3 - Perlyna Lisostepu, 10 Gy; 4 - Perlyna Lisostepu, 15 Gy; 5 - mutant, control; 6 - mutant, 5 Gy; 7 - mutant, 10 Gy; 8 - mutant, 15 Gy.

There are differences in the similarity of the spectra of amplicons of both control and exposed variants (Table 3). Common in the behavior of this indicator is the most sig- nificant decrease in the similarity spectra of amplicons in Perlyna Lisostepu and its mutant under 5 Gy-dose and the increase in similarity under both 10 and 15 Gy as in ISSR- RAPD – PLR analysis.

Jacquard similarity index for the spectrum of amplicons obtained by ISSR DNA marker for control and exposed var- iants is shown in Table 4 for each of the genotypes.

There is a noticeable difference in the behavior of the similarity index in the selected genotypes. Thus, under Perlyna Lisostepu exposure at 5 and 10 Gy-doses, there is a decrease in similarity with the control variant and an increase in this indicator when exposed with 15 Gy. When the mutant is irradiated, there is a gradual decrease in the similarity of the spectra of the amplicons of the irradiated variants with the control variant. The effects of genome instability at two consecutive doses in different genotypes are also manifested differently. In the Perlyna Lisostepu var- iety, the similarity of amplicon spectra under both 5 and 10 Gy dose levels is replaced by a decrease in similarity between amplicon spectra under both 10 and 15 Gy, while the similarity between amplicon spectra between successive doses in the mutant is gradually reduced.

The indices of similarity between control and irradiated variants behave in a similar way when performing RAPD DNA marker PCR with DNA of both genotypes (Table 5). The greatest decrease in similarity with the control variant is observed under 5 Gy-dose with a slight increase in the index yield under both 10 and 15 Gy. For both genotypes, there is also an increase in similarity of the amplicon spectra of var- iants exposed between two consecutive doses.

Thus, there are some main results of this stage of the study. First, the effects of DNA damage identification in the flowering plants under pre-sowing radiation exposure of dry seeds. Second, the identification of the relationship between the polymorphism of the primary DNA structure in differ- ent genotypes and the nature of its rearrangement under radiation exposure. Third, the finding that the greatest DNA damage of both genotypes is observed under 5–10 Gy and there is a tendency to recover under 15 Gy.

Changes in the content of phenols and flavonoids show differences in the dose dependences of these characteris- tics for both Perlyna Lisostepu and its mutant. The dose dependence of the specific content of flavonoids for the first genotype (Figure 4(A)) has a maximum correspond- ing to a dose of 10 Gy, for the second one to 5 Gy-dose. A significant increase in the specific content of phenols is observed only for the mutant under 5 Gy dose (Figure 4).

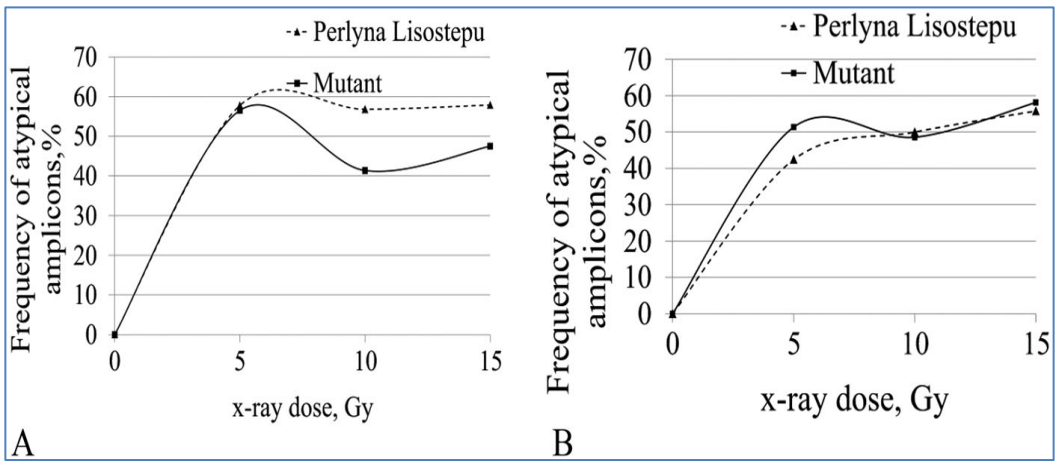


**Figure 3. The trend of changes in the average frequency of atypical amplicons depending on the dose indicated with RAPD (A) and ISSR (B) PCR analysis.**

**Table 3. Jacquard similarity index of the amplicon spectra corresponding to one dose of different genotypes obtained when performing RAPD and ISSR DNA marker PCR.**

| RAPD-PCR variants | M** control | M 5 Gy | M Gy | M 15 Gy | ISSR-PCR variants | M control | M 5 Gy | M 10 Gy | M 15 Gy |
|---|---|---|---|---|---|---|---|---|---|
| PL* control | 0.67 | – | – | – | PL control | 0.75 | – | – | – |
| PL 5 Gy | – | 0.65 | – | – | PL 5 Gy | – | 0.63 | – | – |
| PL 10 Gy | – | – | 0.73 | – | PL 10 Gy | – | – | 0.68 | – |
| PL 15 Gy | – | – | – | 0.81 | PL 15 Gy | – | – | – | 0.71 |

**Table 4. Dose dependence of the Jacquard similarity index of the amplicon spectra through each genotype indicated when performing ISSR DNA marker PCR.**

| ISSR-PCR variants | PL control | PL 5 Gy | PL 10 Gy | PL 15 Gy | ISSR-PCR variants | M control | M 5 Gy | M 10 Gy | M 15 Gy |
|---|---|---|---|---|---|---|---|---|---|
| PL control | 1 | 0.69 | 0.65 | 0.80 | M control | 1 | 0.78 | 0.72 | 0.67 |
| PL 5 Gy | | 1 | 0.82 | 0.77 | M 5 Gy | | 1 | 0.72 | 0.67 |
| PL 10 Gy | | | 1 | 0.72 | M 10 Gy | | | 1 | 0.62 |
| PL 15 Gy | | | | 1 | M 15 Gy | | | | 1 |

**Table 5. Dose dependence of the Jacquard similarity index of the amplicon spectra through each genotype indicated when performing RAPD DNA marker PCR**

| RAPD-PCR variants | PL control | PL 5 Gy | PL 10 Gy | PL 15 Gy | RAPD-PCR variants | M control | M 5 Gy | M 10 Gy | M 15 Gy |
|---|---|---|---|---|---|---|---|---|---|
| PL control | 1 | 0.62 | 0,68 | 0.67 | M control | 1 | 0.70 | 0.71 | 0.77 |
| PL 5 Gy | | 1 | 0,82 | 0.73 | M 5 Gy | | 1 | 0.71 | 0.83 |
| PL 10 Gy | | | 1 | 0.89 | M 10 Gy | | | 1 | 0.78 |
| PL 15 Gy | | | | 1 | M 15 Gy | | | | 1 |

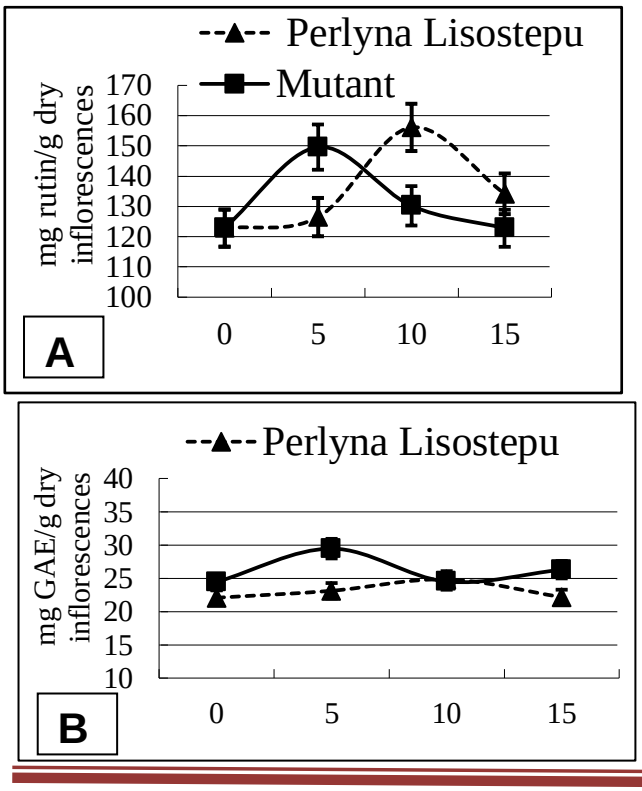


**Figure 4. Dose dependence of total flavonoid (A) and phenolic (B) content in the flower weight of *M. chamomilla* variety Perlyna Lisostepu and its mutant. Confidence interval, P = 0.95.**

4(B). There is a decrease in the content of both antioxidants under 15 Gy.

Maximum content of flavonoids and phenols was indicated under 5 and 10 Gy doses for different genotypes. It corresponded to the greatest loss of similarity with the control vari- ant of amplicon spectra indicated with RAPD

DNA markers for both genotypes and with ISSR DNA markers for Perlyna Lisostepu following increasing these values under 15 Gy.

Comparison of the response to the pre-sowing exposure of both genotypes identifies common trends and shows some differences. Thus, when conducting ISSR-PCR analysis, in contrast to the Perlyna Lisostepu, its mutant does not show major similarity of amplicon spectra with increasing radiation dose (Table 4), and the maximum accumulation of both anti-oxidants corresponding exposure with 5 Gy instead of 10 Gy. A comparison of these two facts suggests the existence of cer- tain differences in the reparative processes in these two genotypes. The existence of polymorphisms in ISSR and RAPD sequences causes some differences in chromatin compaction, which, according to the modern ideas [8,36,37] affects the availability of DNA to transcription and reparation factors, the formation of so-called radiation-induced foci [16], providing the effectiveness of DNA repair.

Thus, all three markers have indicated a nonlinear and non- monotonic dependence of the radiation effects for the low-dose exposure. The most common view is related to the superposition of dose curves of several radiation- induced processes [6,22,39].

Currently, the overall picture consists of several components. First, it is a direct damage of DNA and formation of products of DNA interaction with ROS. The second one is a stimulation of various repara- tive processes. Third, the antioxidant protection increasing by enzymatic and non-enzymatic antioxidants. The fourth mechanism that reduces the appearance of atypical amplicons is the stimulation of processes through checkpoints that block the transition to divide cells with significant DNA damage. Each of the four known radi- ation-induced processes has its own initiation threshold and dose–effect dependence.

However, the identification of individual components of the response to radiation stress leaves the issue opened concerning the structure of their interaction, in this case – interaction of dose-dependent changes in the level of DNA damage and changes in the level of antioxidant protection. Consider a hypothetical interactive picture of radiation- induced processes based on the obtained data. The lowest values of similarity index between control and exposed variants under 5–10 Gy-doses indicated the highest level of DNA damage and low efficiency of repair processes for the field of low doses, which was proven by several studies [16]. The repair of the DNA native structure is associated with the decreased antioxidants' yield. The phe- nomenon could be explained in two ways. First, there is a direct link between the appearance of multiple DNA damage and increasing antioxidant protection. Second, there appears an increasing ROS yield that accompanies the genomic instability. In the second case, it should be assumed that increasing ROS yield is associated not only with the primary oxidative stress developed immediately after the irradiation, but also with the subsequent maintenance as a protective reaction to non-native 'foreign' DNA [31]. While repairing the native DNA structure, the level of ROS is decreased that also leads to decreased yield of the antioxidant protection.

The obtained data confirm the hypothesis about the preservation of signs of genomic instability in the plant at the flowering stage several months after presowing irradiation at low doses. It showed the decrease in the similarity of the set of amplicons with the control after the PCR analysis with both ISSS and RAPD DNA markers, i.e. the loss of DNA nativeness is more significant at lower radiation doses. Up- to-date data allow us to explain this by the low efficiency of repair processes in the range of low doses. There is a narrow range of subthreshold doses to stimulate/induce DNA repair, which stimulates antioxidants production. DNA nativity repair leads to decreasing specific yield of low molecular weight antioxidants.

Thus, antioxidant production stimulation is an example of the long-term consequences of irradiation and the result of interactions between the development of DNA damage and repair processes, recognition of the erroneous DNA primary structure and the corresponding chromatin conformation.

At the same time, obtained data are the beginning of the transition from a purely empirical selection of effective doses, which stimulate the development of certain metabolites to identify the mechanisms that cause the metabolic changes.

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