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EFFECT OF HIGH-ENERGY IONIZING RADIATION ON THE DNA CONTENT AND GENETIC VARIATION IN CHRYSANTHEMUM PLANTS REGENERATED FROM IRRADIATED OVARIES

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ABSTRACT

This study aimed to evaluate the range of quantitative and qualitative genetic changes in chrysanthemum plants regenerated in vitro from ovaries irradiated with high-energy photons (5, 10, and 15 Gy) and high-energy electrons (10 Gy). The highest DNA loss (up to 11%) was observed in plants originating from ovaries treated with 10 Gy high energy. AMOVA revealed significant differences between populations of plants representing different mutagenic treatments (18% for RAPD-based and 22% for SCoT-based analysis). The incidence of genetic changes was strongly correlated with the applied type and dose of ionizing radiation. The highest genetic distances to reference plants were observed for plants regenerated from 15 Gy high-energy photon (5.8% with RAPD and 1.7% with SCoT) and 10 Gy high-energy electron-treated explants (6.0% with RAPD and 2.9% with SCoT). Considerable changes in the phenotype of mutants were not necessarily correlated with the extent of genetic alterations. Qualitative and quantitative methods of evaluating post-radiation genetic changes should be combined for reliable detection of variant plants at early developmental stages.

Key words: *Chrysanthemum* × *morifolium* (Ramat.), AMOVA, flow cytometry, mutation breeding, RAPD markers, SCoT markers microscopy

INTRODUCTION

The discovery of ionizing radiation of different natures in the late 19th century started a new era of controlled deployment of ionizing radiation in human service. It soon appeared that the applications of ionizing radiation were numerous, and one of them was the intended induction of alterations in plant genomes [van Harten 1998].

Ionizing radiation in nature comes both from cosmic radiation and the radioisotopes of the chemical elements making up the Earth. For induced mutage-

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nesis in plants, the sources of ionizing radiation are mainly the devices used for clinical purposes, facilities belonging to research institutes or gamma-fields and gamma-rooms used for plant studies in Eastern Asia [Yamaguchi et al. 2010, Ulukapi and Ozmen 2018, Ibrahim et al. 2018].

Currently, various types of ionizing radiation are successfully and routinely applied in mutation breeding programs, leading to the creation of novel cultivars in essential crops, helping to produce plants resistant to diseases, pests, and harsh environments, as well as expressing valuable traits facilitating cultivation or increasing their ornamental value [Datta 2020]. Although modern biotechnology offers targeted mutation methods based on CRISPR/Cas technologies, the induction of random mutations with different mutagenic agents remains one of the essential tools in crop improvement [Holme et al. 2019]. Moreover, it constantly demands improvements in addressing new challenges from technological advances in radiation sources [Schelake et al. 2019, Kokurewicz et al. 2019].

Physical mutagens are used more often than chemical mutagens to induce mutations in plant breeding, mainly since they are less environmentally and staff-hazardous. Among physical mutagens, ionizing radiation is first due to its efficiency in mutant induction combined with human and environmental friendliness during administration. There are two main types of ionizing radiation applied in breeding: electromagnetic waves of a particular wavelength, namely, high-energy photons (X-rays and γ-rays), or beams of particles possessing their own mass and charge (high energy-electrons and ions) [Gudkov et al. 2019]. Modern, technologically advanced facilities used in radiotherapy treatment offer ionizing radiation of high-energy particles up to 25 MeV [Kokurewicz et al. 2019], bringing the opportunity to apply radiation at megavoltages in plant mutation breeding. Previously, mutation breeding with X-rays was based on low-energy devices, in which radiation energy varied from 10 to 400 keV [van Harten 1998]. In recent experiments, high-energy photons and electrons were applied with beam energies of 6 MV and 6 MeV, respectively [Slater 2012, Miler et al. 2021]

High-energy electrons, as well as ions, interact with matter by direct ionization [Gerbi 2006]. Since electrons are charged particles, their mode of action differs from electromagnetic waves (photons and gamma rays). High-energy electrons are less penetrating but produce denser ionization within irradiated tissue [Parsons 2013]. High-energy electrons were used as mutagenic factors in a recent study on chrysanthemums and proved to be an efficient and promising agent in triggering phenotype mutations [Miler et al. 2021].

Electromagnetic waves (photons), particularly X-rays and γ-rays, since they do not carry an electric charge, cannot interact directly, and their efficiency is related to the concentration of water molecules in the absorbent tissue [Gudkov et al. 2019]. To date, numerous studies have deployed photons as mutagenic factors in plant breeding for different ornamental plants (e.g., chrysanthemum, gerbera, rose, bleeding heart, cape primrose, African violet, lily, gladiolus, dahlia), as well as with different target tissues (shoot fragments with or without buds, leaves, calli, scales, seeds, pollen grains) [van Harten 1998, Datta 2014, 2020, Kulus et al. 2022]. The range of absorbed doses of radiation expressed in Gy (1 gray = 1 J kg^{-1}) applied for plant breeding varies from several to hundreds Gy, with 5–25 Gy being the most effective for most soft tissues (such as leaves, shoots, etc.) and 50–700 Gy acting well for seeds [van Harten 1998]. The dose rate value expressed as the dose absorbed within the time unit (Gy min^{-1}) is another measure important for planning mutagenic treatment, and it is directly related to the source of radiation used for breeding. For example, gamma fields based on a cobalt-60 source emit low dose rate radiation ranging from 0.001–0.1 Gy min^{-1} (depending on the distance from the source), while clinical devices operate in the range of 2–4 Gy min–1 within a distance of 1 m [Shigematsu et al. 2012, Slater 2012].

Cultivated chrysanthemums (*Chrysanthemum* × *morifolium* Ramat.) are among the topmost-selling ornamentals worldwide [Anderson 2007, Spaargaren and van Geest 2018]. Numerous new chrysanthemum cultivars are submitted annually to grant plant breeders rights, many obtained with induced mutagenesis [CPVO and IAEA databases 2022]. With its rich genetic background, high heterozygosity, and polyploidy, this species seems to possess an inexhaustible range of possible new variants and mutants concerning inflorescence color, shape, and size, as well as

plant architecture traits, which are essential for floricultural markets [Teixeira da Silva and Kulus 2014]. Both allopolyploidy and centuries of breeding activity on chrysanthemums contributed to the lower natural fertility of this species [Anderson 2007]. On the other hand, polyploid plants possessing several, not only two, copies of crucial genes can be favored in hostile conditions or under mutagenic factor pressure [Sattler 2016]. Combining these features makes chrysanthemums prone to mutation breeding programs, a technique widely used in horticultural breeding practice [Datta 2020].

In chrysanthemum, various ionizing radiation types (gamma rays, X rays, heavy ion beams) with different dosages were used as physical mutagenic factors for breeding purposes [Yamaguchi et al. 2008, Yamaguchi et al. 2010, Kaul et al. 2011, Kang et al. 2013]. Mutagenic treatment in contemporary experiments is conducted mainly with the application of in vitro techniques, particularly with nonmeristematic explants, due to the higher than in vivo efficiency of regeneration, higher frequency of solid, nonchimeric mutants and, thus, better breeding results expressed as novel mutant cultivars [Datta 2014]. Nonetheless, applying ovaries as mutagenesis target explants has not been the standard practice since, in breeding, they primarily serve as a source of haploid plants in gynogenesis protocols. Consequently, the response of ovaries has not yet been studied in terms of genetic effects resulting from the utilization of high-energy electrons or photons classified as low linear energy transfer (LET) types of radiation. The term "LET" is related to the tissue penetration ability of the radiation applied and the types of interaction with matter: low LET radiation types (gamma rays, photons, electrons) penetrate tissues more deeply, while high LET types (heavy ion beams and fast neutrons) show less tissue penetration with higher tissue damage resulting from energy deposition near the primary particle track [Jo and Kim 2019].

In this study, we investigated the incidence of quantitative and qualitative changes in the DNA of chrysanthemum plants regenerated from ovary explants irradiated with different types and doses of low LET high-energy ionizing radiation: photons and electrons. Moreover, we addressed whether the genetic changes revealed with molecular markers reflect the actual phenotypic variations.

MATERIAL AND METHODS

Plant material, irradiation, and further cultivation

The plants used in this experiment were chrysanthemums (*Chrysanthemum* × *morifolium*/Ramat./) 'Profesor Jerzy' (Polish cultivar, pot type with large -sized, white, whole, flat inflorescence) regenerated in vitro from ovaries excised from inflorescences that were previously subjected to different doses of ionizing radiation. Details concerning the irradiation procedure, regeneration efficiency, management of plants, and phenotype results in regenerated plants are presented in detail by Miler et al. [2021].

The objects (whole inflorescences in full-blossom cut from the stem, put into 350 ml glass jars, immersed in double-distilled water, and loaded with plastic clay to obtain 2-cm-deep immersion) were irradiated at the Oncology Center in Bydgoszcz, Poland. Conventional medical accelerators from Varian Medical Systems (Palo Alto, CA, USA) were used for the experiment. Two types of ionizing radiation were used: high-energy photons and high-energy electrons. For a high-energy photon dose rate of 3.19 [Gy min^{-1}], the total doses delivered were 5, 10, and 15 Gy at nominal accelerating potential (NAP) beam energy at 6 MV (Vitalbeam v. 2.7). For a high-energy electron dose rate of 1.42 [Gy min^{-1}], the total delivered dose was 10 Gy at a beam energy of 6 MeV (Clinac 2300CD Silhouette). Explants were irradiated at 100 cm source-surface distance (SSD) at 2 cm depth (aimed at ovaries gathered in the receptacle of inflorescences immersed in double-distilled water).

For *in vitro* regeneration, 80 ovaries representing each treatment (type and radiation dose) were excised from the irradiated inflorescences (control explants were excised from nonirradiated inflorescences), surface sterilized, and cultivated in vitro. In vitro culture establishment, regeneration from ovaries with a two-step protocol, rooting, and acclimatization were conducted according to Miler and Muszczyk [2015] with modifications by Miler and Jędrzejczyk [2018]. According to this protocol, the regeneration of shoots occurs from somatic tissues of the ovary wall.

Following acclimatization, regenerant plants were cultivated in a greenhouse together with reference plants, which were plants of the same cultivar, 'Profesor Jerzy', multiplied vegetatively with cuttin-

gs without an in vitro phase. Reference plants served as true-to-type templates for evaluating the incidence of genetic changes in regenerants since control plants that emerged from in vitro explants might undergo somaclonal variation.

The impact of ionizing radiation on the plant genome was investigated in terms of quantitative traits, which were 2C DNA contents in regenerants, and qualitative traits, which were intra- and interpopulation variations of regenerants. Moreover, individual plant variants with visually changed inflorescence traits selected after the full-flowering stage were studied in terms of genetic alterations.

Flow cytometric analysis

Changes in the nuclear DNA content in regenerant plants due to irradiation treatments of original explants were studied using flow cytometric analysis. The genome size was estimated in young and fresh leaves of chrysanthemum collected from randomly selected plants: the reference 'Profesor Jerzy' plants (propagated from cuttings in a greenhouse), the control (regenerated in vitro from nonirradiated ovaries) and the regenerants originating from irradiated ovaries, 20 to 46 plants were tested for each treatment. The samples were prepared according to the procedure described by Jędrzejczyk and Śliwińska [2010] using Galbraith's buffer (45 mM MgCl2, 30 mM sodium citrate, 20 mM 3-(N-morpholino)propanesulfonic acid, 0.1% (v/v) Triton X-100, pH 7.0 [Galbraith et al. 1983]) supplemented with propidium iodide (PI 50 µg mL–1), ribonuclease A (RNase A 50 µg mL^{-1}) and 2.0% (w/v) antioxidant polyvinylpyrrolidone (PVP-10). The genome size was measured in 5000–7000 nuclei for each sample using a CyFlow Ploidy Analyzer (Sysmex Partec GmbH, Görlitz, Germany) and linear amplification. The CyFlow Cube program (Sysmex Partec GmbH) analyzed the histograms. The 2C DNA content was calculated using the *Chrysanthemum* × *morifolium*/ V. faba 'Inovec' (2C = 26.90 pg [Dolezel et al. 1992]) 2C peak positions on the histogram of fluorescence intensities. The nuclear DNA contents were transformed into megabase pairs of nucleotides using the conversion 1 pg = 978 Mbp [Dolezel and Bartos 2005]. The results were estimated using a one-way analysis of variance, and the significance of differences between means was verified with Duncan's test ($p \leq 0.05$).

Molecular marker analyses

To evaluate the impact of the types and doses of irradiation on the genetic variation in plants regenerated from irradiated explants, two types of genetic marker systems based on single arbitral primers were applied, namely, randomly amplified polymorphic DNA (RAPD) [Welsh and McClelland 1990, Williams et al. 1990] and start codon targeted (SCoT) [Collard and Mackill 2009]. The sequences of the primers used are shown in Table 1. The selection of primers for the experiment was based on the effective primers used in previous studies for chrysanthemum genetic distance estimations [Miler and Kulus 2018, Feng et al. 2016].

Total genomic DNA was extracted from fresh leaves of greenhouse-grown plants using a ready-to-use Genomic Mini AX Plant Spin column kit (A&A Biotechnology, Poland). The concentrated stocks of DNA were suspended in TE buffer (10 mM TRIS and 1 mM EDTA, pH = 8.0) and stored at –20°C, while the working solutions, with a concentration of 20 ng μL^{-1} DNA, were based on 10 mM TRIS at pH = 8.0. A QuantiFluor dsDNA System (Promega) monitored genomic DNA concentration and purity.

RAPD and SCoT marker systems were applied, each based on 10 primers. Each 25 µL reaction volume contained 0.25 mM dNTP mix, 1 µM single primer, 1.25 U DNA Taq polymerase, 2 mM MgCl2, 20 ng template DNA (0.8 ng μL^{-1}), and deionized water to volume.

Amplification was performed in a C1000 Thermal Touch Cycler (Bio-Rad, USA) under the following conditions for RAPD: one cycle of 4 min at 94°C for initial DNA denaturation; 35 cycles of 1 min at 94°C for denaturation, 1 min at 36°C for annealing, and 2 min at 72°C for DNA extension; the last cycle was followed by a final extension step of 4 min at 72°C; for SCoT: one cycle of 4 min at 94°C for initial DNA denaturation; 35 cycles of 1 min at 94°C for denaturation, 1 min at 50°C for annealing, and 2 min at 72°C for DNA extension; the last cycle was followed by a final extension step of 10 min at 72°C.

The amplified DNA fragments were separated horizontally on a 1.5% (w/v) agarose gel (LE basic, Blirt, Poland) in TBE buffer (90 mM TRIS, 90 mM boric acid, 2 mM EDTA, pH = 8.0) first at 90 V for 10 min and then at 120 V for 110 min (Biometra P25) and detected by staining the gel with 18 µL ethidium bromide at a concentration of 10 mg mL^{-1} in 300 mL of gel.

The separated DNA fragments' sizes were estimated using a 100-5000 bp DNA Ladder (DNA GeneRuler Express DNA Ladder, Thermo Science). The PCR product was visualized in UV light with a GelDoc System (Bio-Rad, USA). Gel images were recorded and analyzed using GelAnalyzer 2010 software. Reliable RAPD and SCoT loci from two PCR repetitions were recorded using a binary system, indicating the presence or absence of bands as 1 and 0, respectively, and binary matrices were created.

Plants treated with different types and doses of irradiation were considered populations, and the overall impact of irradiation treatments on the genetic variation within and between populations (treatments) was evaluated based on binary data for diploid dominant markers using GenAlEx 6.5 [Peakall and Smouse 2006, 2012] and the Statistica 13.3 software package (Tibco, USA).

All analyses were performed separately for RAPD and SCoT genetic markers. The number of genotypes tested was 14 per treatment (population), and six were analyzed (the reference, the control, and the four irradiation treatments). Relationships between different populations of plants were recorded based on the tree-building method and PCoA. Dendrograms were built based on Nei's coefficients of genetic distances. Principal coordinates analyses (PCoA) based on mean population binary genetic distances with data standardization. Analysis of molecular variance (AMOVA) was performed, and the significance of the results was verified at $p \leq 0.001$. Frequencies of monomorphic, polymorphic, and unique bands were recorded, as well as the mean heterozygosity in populations of regenerants. Polymorphism information content (PIC) was calculated to evaluate the individual primer capacity for polymorphism detection.

Additionally, after achieving the full-flowering stage, variants showing inflorescence alternations compared to reference plants were subjected to flow cytometry 2C DNA content measurements and molecular marker analyses.

RESULTS

Quantitative effects

The 2C DNA content in regenerants was affected by the dose and type of irradiation to which the original explant was subjected. The mean 2C DNA content of the studied hexaploid chrysanthemum plants ranged from 16.78 pg for the regenerants originating after treatment with 10 Gy high-energy electrons to 17.77 pg for the control plants, which corresponded to 16 411 and 17 379 Mbp, respectively (Fig. 1). In the reference plants, the genome size range was the narrowest, varying between 17.48 and 18.11 pg/2C. The mean value was similar in the reference and control plants obtained from nonirradiated ovaries (17.74 and 17.77 pg/2C, 17 350 and 17 379 Mbp, respectively).

All the obtained regenerants originating from irradiated explants possessed a lower nuclear DNA content than both the reference and the control (Fig. 1); nonetheless, the differences were not significant for plants regenerated after 5 and 10 Gy photon irradiation. Within the group of plants originating from high-energy photon treatments, the lowest mean genome size (17.33 pg/2C; 16 949 Mbp) was observed in the plants resulting from 15 Gy high-energy photon treatment, and the 2C DNA content ranged from 16.09 pg to 18.17 pg. A slightly higher mean genome size in this group of regenerants was found in plants originating from explants irradiated with 10 Gy photons (17.39 pg/2C; 17 007 Mbp), and the 2C DNA content ranged between 16.38–18.18 pg. For regenerants obtained after 5 Gy irradiation, the genome size was the highest (mean 17.46 pg/2C; 17 076 Mbp) in this group of treatments and ranged from 16.64 pg/2C to 18.05 pg/2C. The lowest mean DNA content of 16.78 pg/2C (16 411 Mbp) was observed in plants irradiated with 10 Gy electrons, and the genome size varied between 15.93 pg/2C and 17.81 pg/2C (Fig. 1). With the increase in the delivered irradiation dose, the genome size ranges within populations of plants representing particular treatments was broader. The difference in genome size between the lowest and highest nuclear DNA content varied from 0.63 pg/2C in the reference plants to 2.08 pg/2C in the regenerants obtained after 15 Gy photon irradiation.

Interestingly, the change in DNA content altered only in one direction –"downward," namely, the higher the dose delivered, the higher the loss of DNA, which points out a higher probability of occurrence of deletions over another type of mutation.

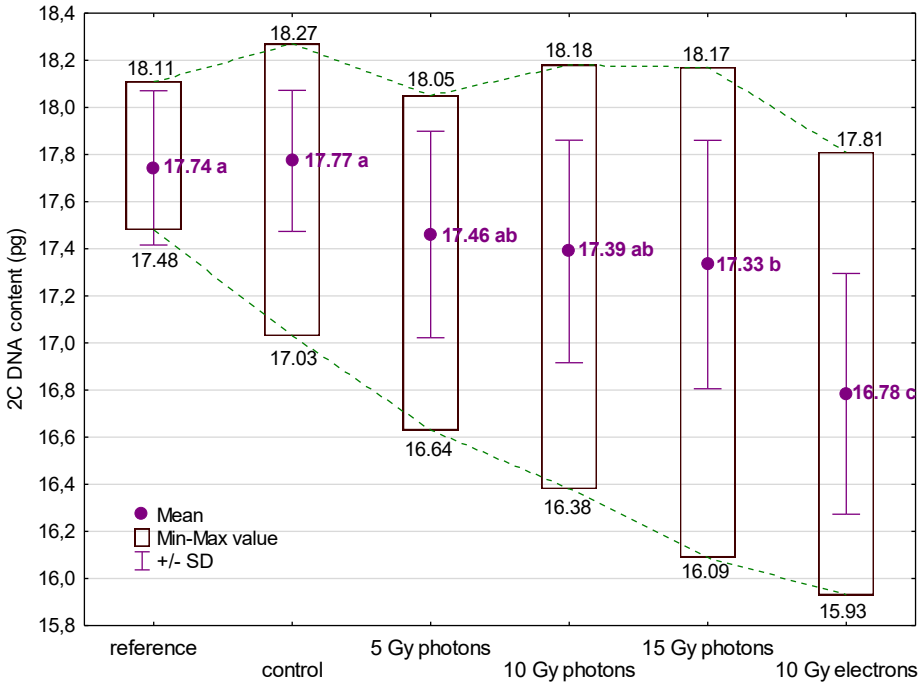

Fig. 1. 2C DNA content (pg) in 'Profesor Jerzy' plants regenerated in vitro from explants irradiated with different doses of high-energy photons and electrons; reference – plants propagated vegetatively with shoot cuttings and cultivated in a greenhouse without in vitro culture phase (true-to-type control), control – regenerants derived from nonirradiated ovaries ex vitro; Bars indicate minimum and maximum values observed within treatments, whiskers indicate mean ± standard deviation (SD). According to Duncan's test, means followed by the same letter do not differ significantly at $p \leq 0.05$

Qualitative effects

From the total number of 20 tested primers, 19 (except for primer RAPD E) gave reliable and replicable results of banding patterns. Primers RAPD A and SCoT 12 produced the highest number of 12 loci (Tab. 1).

The total number of loci generated with RAPD and SCoT primers were 51 and 80, respectively. RAPD B (PIC value 0.50) and RAPD A (PIC value 0.45) were the most discriminating primers. Five of the tested RAPD primers revealed no or little polymorphism information, while among SCoT primers, four had very low PIC varying from 0.0–0.06. Although SCoT primers generated an overall higher number of loci than RAPD primers (8.0 and 5.1, respectively), they were less informative, and the banding patterns were more uniform (mean PIC value 0.14 and 0.18 for SCoT and RAPD primers, respectively).

Polymorphic loci analysis showed a two times higher mean number of polymorphic loci recorded with RAPD molecular markers than with SCoT (12.42% and 6.67%, respectively; Tab. 2). Reference plants were uniform in banding pattern, and no polymorphic loci were detected, confirming their high genetic stability. The highest share of polymorphic loci within the population was found in 10 Gy electron regenerants: 35.29% and 16.25% for RAPD and SCoT, respectively. Notably, as detected with both marker systems, a higher share of polymorphic loci was observed in the control plants (from nonirradiated explants) than in the high-energy photon-originated plants.

The total numbers of loci generated with RAPD and SCoT were 51 and 80, respectively. Frequency-based analysis showed that banding patterns within all populations were formed predominantly by bands frequent in more than 95% (Fig. 2). For RAPD, the lowest number of 40 bands was produced in the reference, 10 Gy photon and 15 Gy photon plants, while the most significant number was detected in the 10 Gy electron plants (50 bands). Higher numbers of bands were detected with the SCoT marker system – from 72

Table 1. Name, sequence, number of generated loci, and polymorphism content information (PIC) of primers used in the genetic analysis of chrysanthemum plants regenerated from irradiated ovaries

RAPD primers				SCoT primers			
Name	5′-3′ sequence	Number of loci	PIC	Name	5′-3′ sequence	Number of loci	PIC
A	GGG AAT TCG G	12	0.45	SCoT 3	CAA CAA TGG CTA CCA CCG	12	0.30
B	GAC CGC TTG T	6	0.50	SCoT 4	CAA CAA TGG CTA CCA CCT	11	0.00
C	GGA CTG GAG T	4	0.37	SCoT 8	CAA CAA TGG CTA CCA CGT	8	0.18
D	GCT GCC TCA GG	4	0.00	SCoT 12	ACG ACA TGG CGA CCA ACG	7	0.22
E	TAC CCA GGA GCG	0	0.00	SCoT 13	ACG ACA TGG CGA CCA TCG	6	0.06
F	CAA TCG CCG T	2	0.00	SCoT 25	ACC ATG GCT ACC ACC GGG	10	0.12
G	GGT GAC GCA G	7	0.22	SCoT 26	ACC ATG GCT ACC ACC GTC	6	0.27
H	CCC AGT CAC T	4	0.00	SCoT 27	ACC ATG GCT ACC ACC GTG	9	0.23
I	TGG CGT CCT T	5	0.01	SCoT 28	CCA TGG CTA CCA CCG CCA	7	0.00
J	AGC GTG TCT G	7	0.25	SCoT 33	CCA TGG CTA CCA CCG CAG	4	0.01
	Total:	51			Total:	80	
	Mean:	5.1	0.18		Mean:	8.0	0.14

Table 2. Share (%) of polymorphic loci resulting from RAPD and SCoT genetic marker analyses in populations of 'Profesor Jerzy' reference plants and regenerated *in vitro* from explants irradiated with different doses of high-energy photons and electrons

Share (%) of polymorphic loci	RAPD based	SCoT based
Reference	0.00	0.00
Control	13.73	7.50
5 Gy photons	11.76	5.00
10 Gy photons	5.88	5.00
15 Gy photons	7.84	6.25
10 Gy electrons	35.29	16.25
Mean ±SE	12.42 ±4.98	6.67 ±2.18

in reference plants to 79 in 10 Gy electrons plants. The highest number of eight unique bands was detected in 10 Gy high-energy electron plants with RAPD. No unique bands were recorded for reference plants.

The fluctuations in heterozygosity (He) partly reflects polymorphic loci distribution (Fig. 2, pink curves). The lowest He value was observed in reference plants (0.00) in both marker systems. The heterozygosity curve increased in control plants from nonirradiated ovaries (0.034 for RAPD and 0.021 for SCoT). With the SCoT system, it remained at a similar level for plants derived from ovaries irradiated with photons (0.013, 0.020, and 0.024 for 5, 10, and 15 Gy, respectively), while in the RAPD system, it dropped for 10 and

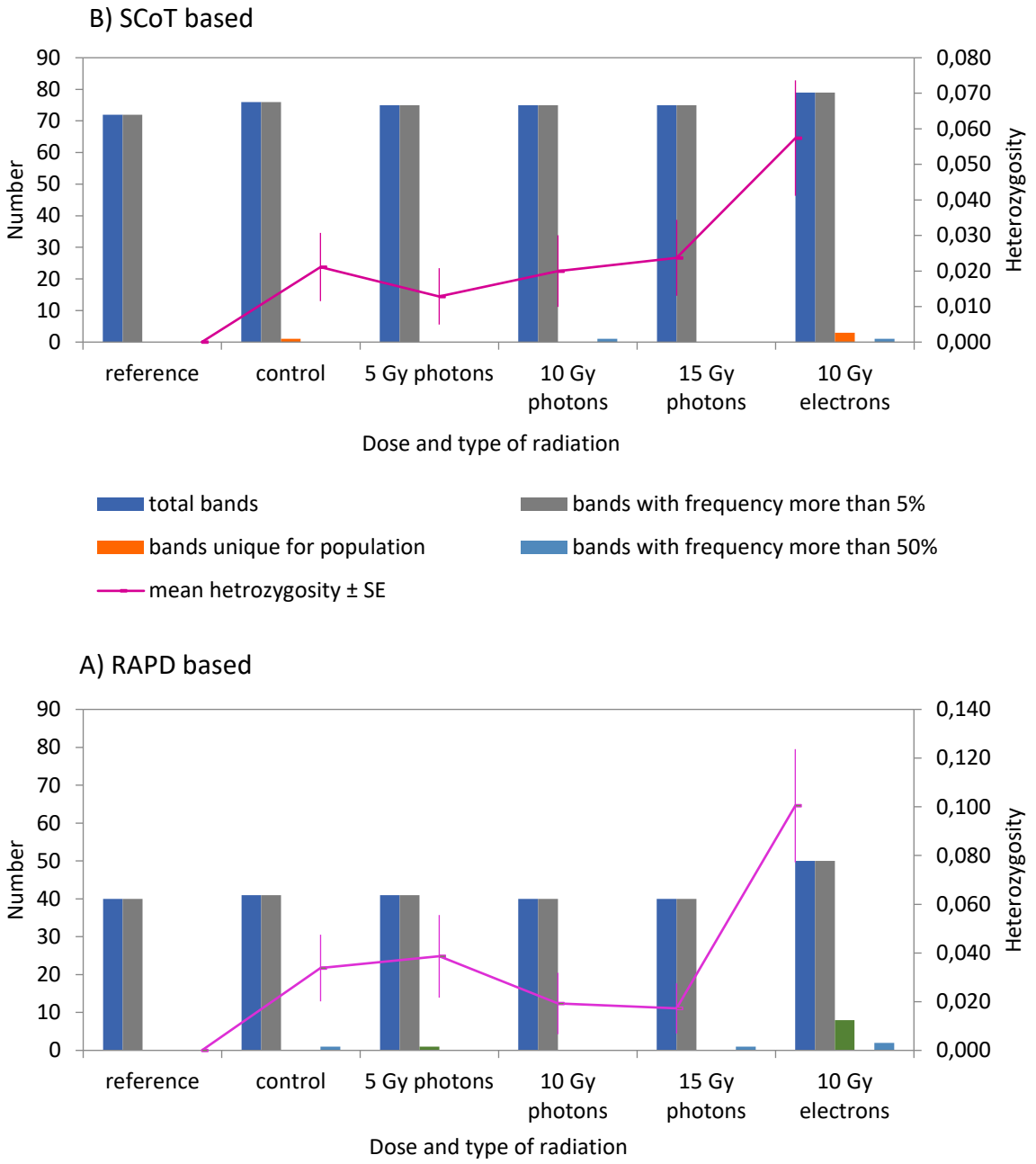

Fig. 2. Analyses of frequencies of bands and heterozygosity obtained with RAPD (A) and SCoT (B) genetic markers in populations of 'Profesor Jerzy' reference plants and regenerated in vitro from explants irradiated with different doses of high-energy photons and electrons

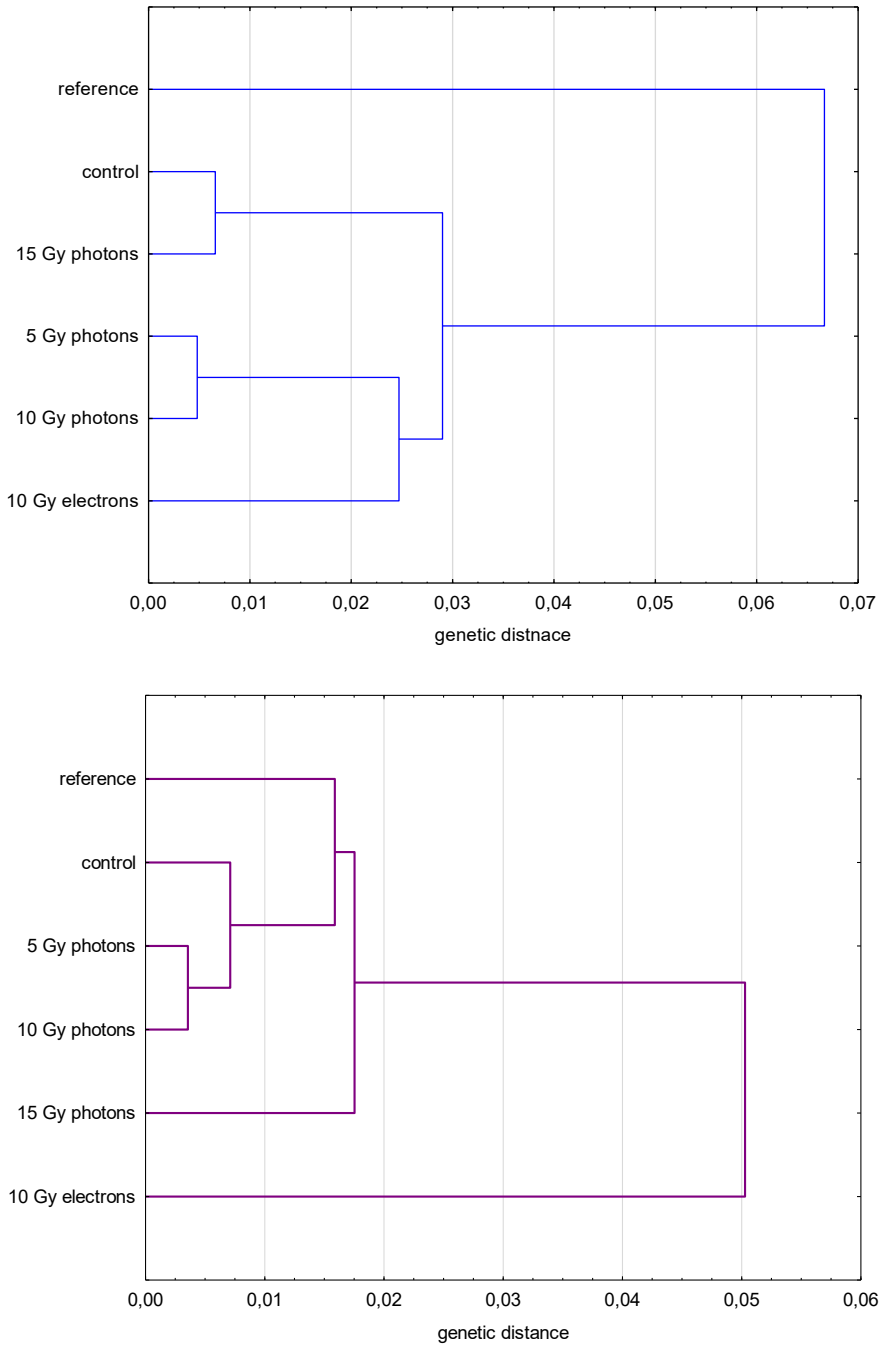

Fig. 3. Dendrogram based on Nei's genetic distance matrix, calculated from RAPD (A) and SCoT (B) marker analyses for populations of 'Profesor Jerzy' chrysanthemums regenerated in vitro from explants treated with high-energy photons and electrons. References are the plants of the original cultivar propagated vegetatively with shoot cuttings and cultivated in a greenhouse without an in vitro culture phase

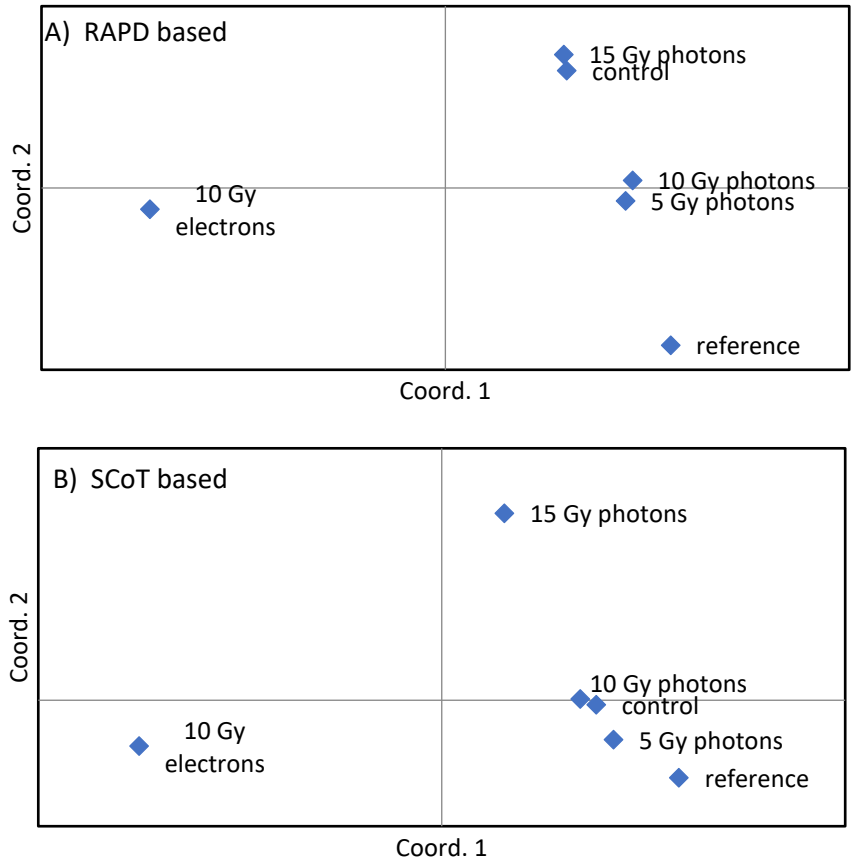

Fig. 4. Principal coordinates analyses (PCoA) based on mean population binary genetic distance matrix with standardized data for populations of chrysanthemums regenerated in vitro from explants treated with high-energy photons and electrons. Plots were created based on RAPD (A) and SCoT (B) marker analyses. References are the plants of the original cultivar propagated vegetatively with shoot cuttings and cultivated in a greenhouse without an *in vitro* culture phase

15 Gy photon-derived plants (0.039, 0.019 and 0.017 for 5, 10 and 15 Gy photons, respectively). The highest He value was observed in 10 Gy electron-treated plants (0.100 and 0.057 for RAPD- and SCoT-based analysis, respectively).

Analysis of molecular variance (AMOVA) confirmed that genetic changes in plants belonging to different groups of treatments were significant; thus, the populations varied significantly at $p \leq 0.001$ ($\Phi PT = 0.179$ and 0.223 for RAPD-based and SCoT-based analysis, respectively). Regarding molecular variance, the irradiation dose and type were the discriminating factors. Both molecular marker systems applied in the experiment indicated similar molecular variances between populations (among treatments), 18% and 22% of the total detected genetic variation for RAPD- and SCoT-based analysis, respectively. Consequently, AMOVA showed high genetic variance within treatments (82% and 78% for RAPD- and SCoT-based analysis, respectively), suggesting a relatively high number of individual variants within populations. Although the populations were relatively uniform in terms of banding pattern, the frequency of unique bands was high enough to differentiate populations of plants, as shown with AMOVA.

Two methods of population clustering were applied (tree building and PCoA), which resulted in slightly different distributions of populations into clusters depending upon the molecular marker system used.

A dendrogram based on Nei's genetic distance (Figure 3), constructed on the base of RAPD markers, presented a considerable distance between reference

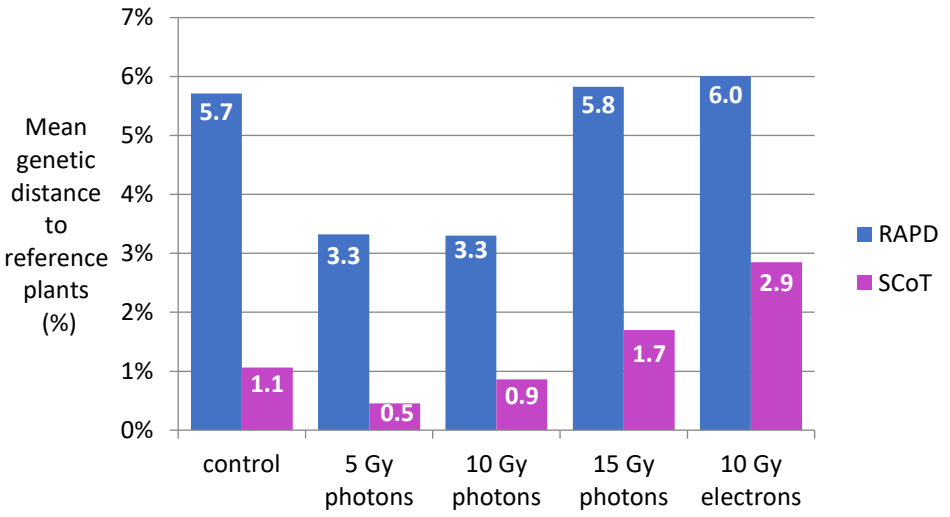

Fig. 5. Mean genetic distance (%) of populations of 'Profesor Jerzy' plants regenerated *in vitro* from explants irradiated with different doses of high-energy photons and electrons to plants propagated vegetatively with shoot cuttings and cultivated in a greenhouse without an *in vitro* culture phase. Genetic distance was calculated according to Nei's coefficient

plants and all regenerants gathered in two minor clusters (first cluster: control and 15 Gy photon plants; second cluster: 5 Gy and 10 Gy photons, plus 10 Gy electron plants). The SCoT-based tree showed different distributions of populations into clusters: the first cluster formed with reference, control, 5 and 10 Gy photon plants close together (genetic distance less than 2%), the second cluster formed with 15 Gy photon plants, and the third cluster formed with plants regenerated from 10 Gy high-energy electron-irradiated ovaries. In the case of both trees, the highest distances were detected between the reference and 10 Gy high-energy electron plants.

Principal coordinates analyses (PCoA), based on the Euclidean distance matrices, showed generally similar clustering compared to the tree-building method (Fig. 5). For RAPD-based PCoA, there were two clusters consisting of two groups of plants: 1) control and 15 Gy photons and 2) 10 Gy and 5 Gy photons; reference plants and 10 Gy electrons plants were located separately. For SCoT-based PCoA, there was one cluster consisting of plants representing reference, control, 5 and 10 Gy photons, while the 10 Gy electrons and 15 Gy photons plants were highly distanced from the main cluster and each other.

Values of Nei's genetic distances of regenerants to reference plants were considerably two- to six-fold higher in RAPD than in SCoT analyses (Fig. 6). Notably, for 5 Gy photon-originated plants, the genetic distance to reference plants was lower than for control plants (approx. two times lower in both marker systems). For SCoT, the highest genetic distance to reference plants was indicated for 10 Gy high-energy electron regenerants (2.9%), while for the RAPD control, 15 Gy photon- and 10 Gy electron-originated plants showed similarly high genetic distances from 5.7 to 6%. In general, all the parameters describing the level of genetic variation were considerably higher, as detected with the RAPD molecular marker system, than with SCoT.

Genetic analyses of individual variants

Detailed analysis of the selected inflorescence variants revealed no simple correlation between considerable changes in the phenotype of plants and the qualitative or quantitative DNA characteristics (Tab. 3). For example, in two variants, v2, and v5, there were considerable differences in DNA content and genetic distance to reference plants, which was in line with the significantly different color of their inflorescences (dark yellow compared to white in reference). In contrast, variant v7 showed high phenotypic variation, while its genetic distance to reference plants observed with RAPD and SCoT markers was low (0.0 and 1.2%, respectively), and its DNA content remained similar to

Table 3. Characteristics of chrysanthemum variants obtained from ovaries compared to reference plants. Traits different from those of the reference plants are indicated exclusively; the reference inflorescence phenotype was white, full, flat shape; the mean 2C DNA content of the reference was 17.74 pg, and it was treated as 100%

Variant origin (symbol)	Inflorescence phenotype description*	Stability in vegetative propagation	Nei's genetic distance (%) to reference based on molecular markers		2C DNA content	
			RAPD	SCoT	absolute (pg)	relative to reference (%)
Control (v1)	tubular	yes	6.1	1.2	17.69	99.7
5 Gy photons (v2)	dark yellow	yes	4.0	3.8	16.64	93.8
5 Gy photons (v3)	dark yellow	yes	0.0	1.2	16.83	94.9
10 Gy photons (v4)	pinkish	yes	4.0	1.2	16.94	95.5
10 Gy photons (v5)	dark yellow, tubular	yes	4.0	1.2	16.54	93.2
10 Gy photons (v6)	light yellow	no	2.0	2.5	17.25	97.2
10 Gy photons (v7)	dark yellow, semi-full, tubular	yes	0.0	1.2	17.69	99.7
15 Gy photons (v8)	dark yellow, incurved	yes	6.1	3.8	16.68	94.0
15 Gy photons (v9)	light yellow	no	2.0	1.2	16.74	94.4
10 Gy electrons (v10)	pinkish	yes	32.1	0.0	17.13	96.6

that of the reference. Moreover, the genetic variation differed in some variants regarding the system used: variant v3 showed no genetic distance with RAPD and 1.2% with SCoT, while variant v10 originated from high-energy electron-treated explants and showed extremely high genetic distance with RAPD markers (32.1%) and none with SCoT markers. Nonetheless, phenotypic variants differed in at least one genetic characteristic compared to the reference plant: the DNA quality expressed by genetic distance based on RAPD, SCoT markers, or both, or the DNA quantity expressed as 2C DNA content.

DISCUSSION

Induced mutagenesis is not only a standard tool enriching the variation for efficient selection of desired traits in breeding programs but also a valuable approach for numerous investigations aimed at plant physiology and development, as well as genome function and structure [Holme et al. 2019, Shelake et al. 2019]. The application of whole-genome sequencing in mutant plants representing model plant species revealed that single nucleotide polymorphisms and small deletions were the predominant mutation types induced with various physical mutagens. In contrast, larger deletions and insertions rarely occurred [Jo and Kim 2019]. In contrast, our study observed such a significant loss of DNA in plants regenerated after mutagenic treatments that it could result from more remarkable genome changes, most likely deletions or even whole chromosome eradication. An extremely low DNA content (15.93 pg) was recorded for the plant that emerged from the 10 Gy high-energy electron treatment, which equaled 89.8% of the reference DNA content. Since the chrysanthemum possesses 54 chromosomes, we can assume that the mean chromosome weight is approximately 1.81 pg, 1.85% of the reference genome. Thus, we can estimate the loss equivalent of almost six chromosomes in this particular variant plant. Indeed, the deletions might be scattered within the whole genome, and the actual number of chromosomes could only be recorded with

the cytological study. However, these estimations help us to understand the extent of the influence of ionizing radiation on the genome size in chrysanthemum plant cells.

Yamaguchi et al. [Yamaguchi et al. 2008, 2010] treated explants with different types (heavy ions and γ-rays) and doses (up to 60 Gy) of ionizing radiation, which resulted in the highest 8% loss of DNA content after ion beam irradiation and the highest 3.8% loss after γ-ray treatment. Similar to our research, they also noted a correlation between the dose and the decrease in DNA content. X-rays were also more effective in inducing deletions in mutant cultivars than γ-rays [Miler et al. 2020]. Our study observed the most significant change in nuclear DNA content in plants that emerged from ovaries treated with high-energy electrons and high-energy photons (X-rays) at the highest dose (15 Gy).

It must always be taken in mind that the dose, dose rate, or type of irradiation and the kind of tissues subjected to irradiation can affect the outcome of mutagenic treatment. This phenomenon is related to the explant's regeneration capacity [Miler and Zalewska 2014]. However, it could also be determined by the water content in absorbing tissue, which strongly affects radiation performance and the ability to ionize directly or indirectly [Turner 2004]. In our research, we observed that the plants regenerated from explants exposed to a dose of 10 Gy with a 6 MeV high-energy electron beam (direct action) responded more effectively than the explants exposed to high-energy photons (X-rays; indirect action), both in terms of DNA content and genetic diversity.

Notably, in our experiment, we observed considerable differences between the characteristics of the plants originating from the same dose of 10 Gy but different irradiation types: photons and electrons. They vary regarding the share of polymorphic loci indicated with RAPD (5.88% for 10 G photons and 35.29% for 10 Gy electrons) and SCoT markers (5.00% and 16.25%, respectively). Moreover, the 2C DNA content in plants obtained from 10 Gy electron-irradiated ovaries was 0.61 pg lower than that in 10 Gy photon-irradiated ovaries. Similarly, the heterozygosity indices were three to five times higher (based on RAPD or SCoT markers, respectively) for 10 Gy electron plants than for 10 Gy photon plants. The quality factors of high-energy electrons and high-energy photons are the same for human tissues, although their modes of action are different [Winiecki 2020]. High-energy electrons, negatively charged particles possessing low mass, have lower matter penetration ability and produce higher ionization on their pathway within irradiated objects [Gerbi 2006, Parsons 2013]. Therefore, the question arises as to whether the variation between characteristics obtained with the same doses but different types of radiation in plants could result from different structures of plant tissues, i.e., higher hydration (over 90%) compared to human tissue (less than 70%). The higher concentration of reactive oxygen species (ROS) produced with direct ionization (high energy-electron action) may interfere more intensively with the structure and function of DNA [Reisz et al. 2014]. In this case, the threshold dose of high-energy electrons for plant tissue should be much lower than for human tissue. The quality factors for electron and photon beams in plant tissue differ, unlike for human tissue.

PCR-based molecular markers are often applied to detect plant genetic variation induced by external factors or natural processes [Kang et al. 2013, Nybom et al. 2014]. They may be used in population analyses [Bhattacharyya et al. 2013, Feng et al. 2016] and for individual variants [Kaul et al. 2011, Eeckhaut et al. 2020]. The idea behind RAPD markers is that they are generated with short primers (decamers) attached to complementary sites along the genome [Welsh and McClelland 1990, Williams et al. 1990]. In contrast, SCoT primers are more extended (usually 18-mers), and they contain an ATG sequence–start codon, which anchors the primers in complementary sites where functional genes start [Collard and Mackill 2009]. Since SCoT markers are related to coding regions of DNA, they reveal changes in essential regions of the genome; thus, they can be more reliable while searching for functional mutations. Ionizing radiation influences the DNA by single- or double-strand breaks and chemical modifications of the nucleotides [Turner 2004], which may lead to the sequence change in a target site for a particular primer. Consequently, the hybridization of a primer in an altered location can be hampered, resulting in polymorphic loci production, which affects the genetic distance and variation parameters. Moreover, due to the loss of part of the DNA due to mutagenic treatment, some hybridization sites can be lost, leading to polymorphism. The markers used in this study are classified as dominant

types, as they do not discriminate between homozygotes or heterozygotes [Nybom et al. 2014]. Since chrysanthemums are hexaploids, mutations leading to the loss of one of the hybridization sites for a primer, while some homologous sites left intact within other chromosomes, would not be detected. Although RAPD markers in our study revealed higher genetic variance based on DNA polymorphism analyses, the overall patterns of variation detected with SCoT and RAPD markers were similar.

The genetic distance to reference plants in our study was the highest in the plants obtained after treatment with 10 Gy high-energy electrons and 15 Gy photons and, surprisingly, in control plants, while in plants produced after 5 and 10 Gy photon treatment, the genetic distances to reference plants were lower.

The unexpectedly high genetic variation in control plants was confirmed by other measures of variation, such as heterozygosity, polymorphic loci content within populations, and cluster analyses. The genetic variation observed in control plants might most likely be of somaclonal origin. Somaclonal variation often occurs under in vitro conditions, particularly in the case of de novo regeneration [Eeckhaut et al. 2020]. In our study, we used ovaries excised from irradiated inflorescences of chrysanthemum, and nonirradiated ovaries served as the source of control plants. The ovaries performed indirect (via callus) in vitro regeneration of shoots [Miler et al. 2021]. The process of deploying adventitious regeneration of shoots, particularly in the callus phase, is highly prone to genetic instability and often leads to the formation of somaclonal variants [Karp 1995]. Similar to our results, as revealed with AFLP markers [Kang et al. 2013], chrysanthemum control plants showed higher genetic diversity than plants obtained after 50, 70, or even 100 Gy treatments. In another study, genetic distance in variants obtained from ovaries compared to mother plants of chrysanthemum 'Capitola' ranged from 0.93 to 7.69%, which was concluded to be an effect of somaclonal variation [Miler and Jędrzejczyk 2018]. Moreover, in chrysanthemum plants regenerated in vitro from untreated leaf explants, the genetic distance to the mother cultivar ranged from 0.0 to 2.69%, as revealed with RAPD markers [Miler and Kulus 2018]. Our results indicate the high probability of genetic variation with a somaclonal background in plants regenerated from ovaries, which may even exceed the low-dose ionizing radiation effects at the DNA level. Nonetheless, the appearance of effective variants with notably changed phenotypic traits (such as changes in inflorescence color) is undoubtedly triggered by ionizing radiation, even at low doses [Miler et al. 2021]. It can be assumed that regardless of the high genetic variation observed in control regenerant plants, the most significant phenotypic changes are observed in plants formed from irradiated tissues.

Interestingly, in our study, the genetic distance in plants obtained from low-dose (5 and 10 Gy photons) -treated explants was lower than that in control plants derived from nonirradiated ovaries. Living plant cells perform several responses to protect their genetic resources from genotoxic agents such as ionizing radiation [Kim et al. 2019]. Cellular mechanisms (cycle arrest, endoreduplication), as well as DNA repair mechanisms (homologous recombination, nonhomologous end-joining, base excision repair, nucleotide excision repair, mismatch repair), lead to error eradication and prevent mutations [Kim et al. 2019]. Moreover, low doses of ionizing radiation stimulate many physiological processes in plants and DNA damage repair mechanisms [Gudkov et al. 2019]. Ionizing radiation effects in living organisms are nonlinear; e.g., there is no simple correlation between increasing dose and increasing effect; the curve of an effect at low doses goes downward and then rises for higher doses. This phenomenon is called hormesis, which comes from Greek "to excite" [Cuttler and Pollycove 2009] and can be understood as the excitement of cellular repair mechanisms in response to low doses of genotoxic agents. According to our results, we attribute the decrease in genetic variation in plants regenerated from low-dose treated explants to the stimulatory effect of ionizing radiation on DNA repair mechanisms [Kim et al. 2019, Ludovici et al. 2020].

AMOVA in our research showed that approximately 20% of molecular variation resulted from treatment, which means that populations representing plants regenerated from irradiated explants differed significantly and that mutagenic treatment was the differentiating factor. As revealed in other studies based on vegetatively propagated chrysanthemums subjected to various treatments, microwave irradiation-induced 11% inter-treatment molecular variation [Miler and Kulus 2018], while silver nanoparticle application led to 32% molecular variance, depending on the cultivar and marker

system used for polymorphism detection [Tymoszuk and Kulus 2022]. In contrast, for sexually propagated plants, the values of molecular variance are considerably higher: the molecular variation between 13 populations of date palm growing in their natural locations in Iran was estimated with four SCoT primers as 40% [Saboori et al. 2020], while for wild Dendrobium, interpopulation molecular variation was 43.37% [Bhattacharyya et al. 2013].

From the analysis of individual variants in terms of their genetic-based traits compared to morphological traits, we can conclude that regarding the selection of individual mutants, particularly at the vegetative stage of plant development when not all traits are yet visible, it is beneficial to use more than one molecular method to detect potential variation. Arbitral molecular markers systems such as RAPD or SCoT and the value of 2C DNA content applied alone may fail in mutant detection since their resolutions are too low and do not necessarily reflect the fundamental phenotypic changes. Nonetheless, all three methods can serve as good indicators of overall population variation and reveal the probability of occurrence of phenotypic alterations. Implementing combined genome quantity (2C DNA content evaluation) and quality (RAPD and/or SCoT)-based marker systems proved to be a more effective tool in detecting actual variations in contemporarily bred cultivars than using these methods separately. Our results are in line with recommendations for breeders applying for cultivar property rights [Jamali et al. 2019].

CONCLUSIONS

Applying chrysanthemum ovaries as explants subjected to mutagenic treatment leads to profound genetic changes in regenerated plants. The incidence of genetic changes, both quantitative and qualitative, is strongly correlated with the type and dose of ionizing radiation applied: high-energy electrons (10 Gy) act more efficiently in terms of induction of genetic variation than high energy-photons at the same dose, and similarly to a higher dose (15 Gy) of high-energy photons. Ionizing radiation can decrease the DNA content in plants regenerated from irradiated ovaries: up to 11% of genetic material can be lost, equivalent to six Chrysanthemum × morifolium chromosomes. RAPD and SCoT molecular markers revealed that the range of polymorphism induced with high-energy photons and electrons in plants regenerated from irradiated ovaries is dose-dependent. Moreover, high genetic variation can be detected in plants regenerated from nonirradiated ovaries, contributing to the somaclonal variation. Moreover, after irradiation, the hormesis phenomenon can be observed, as the low doses of high-energy photons (5 and 10 Gy) resulted in lower genetic variation than in control plants regenerated from nonirradiated ovaries. Nonetheless, the appearance of effective color chrysanthemum variants from ovaries is triggered by ionizing radiation.

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