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MUTAGEN EMS TREATMENT OF MICROSPORE-DERIVED EMBRYOS FOR RAPESEED BREEDING (*BRASSICA NAPUS*)

Abstract. Rapeseed embryos obtained from a culture of isolated microspores were treated with various concentrations of ethyl methanesulfonate (EMS) for one hour. As a result, doubled haploid mutants (DHm) of rapeseed were obtained with desirable quantitative traits including improved yield, seed color and fatty acid composition of seed oil in comparison to donor rapeseed cultivars. Analysis of the fatty acid composition of the seeds of obtained DHm M2 showed a significant increase in the percentage of oleic acid in cultivars of to 75.4%, compared with donor cultivars (66.0%). The resulting DHm plants of rapeseed differed from the donor cultivars with high indicators for the weight per plant and the weight of 1000 seeds. At the same time, according to the results of qualitative and quantitative analyses, the best indicators were when processing with mutagen in concentrations of 12 mM EMS.

Key words: Rapeseed, *Brassica napus*, Microspores, EMS, ISSR.

Introduction. Rapeseed is a valuable source of both edible and industrial oil, and can be used as a feed protein. Rapeseed (*Brassica napus olifera Metzg.*) cultivation in Kazakhstan is commercially viable. The breeding process that has aimed to produce new cultivars of rapeseed in Kazakhstan is predominantly performed using traditional methods, however, they are difficult and require a considerable amount of time. Therefore current methods used do not meet modern requirements for the improvement of crop species for commercial purposes. One of the ways in which traditional methods can be improved is through a combination of mutagenesis and haploid biotechnology in the rapeseed breeding process.

The treatment of *Brassica* seeds with mutagens can cause promote the manifestation of traits such as resistance to seed shedding [1], changes in the qualitative composition of oil [2,3] changes in flowering time [4], change in seed coat color from black to yellow [5]. Additionally, haploid biotechnology is widely used in plant breeding practices, primarily for the creation of homozygous lines in one generation. This is especially important in cross-pollinated crops in which self-pollination is difficult or impossible [6].

Mutagenesis *in vitro* has several advantages over traditional breeding methods. For example, processing allows researchers to obtain microspores of homozygous lines with valuable traits, enhance

fatty acid composition and obtain lines that without erucic acid [7]. The advantages of the mutagenesis of haploid cells include: (1) an enhanced ability to avoid chimerism; (2) rapid detection of mutants; (3) the identification of recessive mutants is possible in the first generation; and (4) the timeframe required for producing homozygous mutants is shortened. In addition, use of an increased number of microspores increases the probability of identifying mutants with the more efficient traits. In particular, breeding is already possible at the *in vitro* cultivation stage [8,9]. The effectiveness of mutagenesis using a culture of isolated microspores has previously been demonstrated in several reports [9,10].

Previously, we conducted research that aimed to optimize the cultivation conditions of isolated rapeseed microspores [11]. The study facilitated our continued research on the use of isolated microspore cultures to obtain mutants. EMS is widely used as a mutagen of the *Brassica* family. However, a cultivar of concentrations and processing times have been previously applied. For example, Ferrie et al. (2008) [12] used 2 mM, 4 mM, 8 mM, 10 mM, 12 mM EMS concentrations and the duration of treatment was 1.5 h. Further, He et al. (2004) [13] used 0.05 mM, 0.1 mM and 0.2 mM EMS concentrations for 20 min to produce mutants, and 1 mM, 1.5 mM, 2 mM, 2.5 mM and 3 mM EMS concentrations for durations of 12, 24, and 36 h. Researchers have used a large range of EMS concentrations, and in general, the higher the concentration used, the shorter the duration of treatment should be.

An analysis of literature reveals that obtaining true mutants is possible while using a wide range of exposures to EMS. For our experiments, were treated materials with 4 mM, 8 mM and 12 mM EMS concentrations for one hour. We chose these concentrations and this specific duration of treatment because they are not overly stringent and, in our opinion, should produce mildly mutagenic effects. Microspore-derived embryos were selected as an explant for treatment with EMS in this experiment.

The best, most informative evidence of the occurrence of mutation is demonstrated by assessing changes that occur at the DNA level [14]. Molecular marker analysis is used to study genetic polymorphism and provides results that are more accurate than other methods [15]. Each molecular marker has advantages and disadvantages. The sensitivity of the detection of mutant plants varies depending upon which marker is used. For example, with narcissus mutagenesis, researchers determined that the mutation rates were 8.33% using RAPD (Random Amplified Polymorphic DNA) markers and 15.48% using AFLP (Amplified Fragment Length Polymorphism) markers [16].

In other studies, 330 mutant lily lines were tested simultaneously using ISSR (Inter Simple Sequence Repeat) and RAPD markers. Using ISSR markers, 119 mutant lines were identified at the DNA level. The hereditary variability of various isolated lily mutants, assessed according to their morphological characteristics, reached 36.06% using seven ISSR primers [18]. ISSR markers scan 100 to 1000 bp portions of DNA, and can be used to identify interspecific and intraspecific genetic variation, species, populations, lines. In some cases, ISSR markers can be used to identify individual genotypes, as well as for screening for mutations and detecting known alleles [14]. Tomlekova et al. (2006) [18] reported the successful use of the ISSR method to detect DNA variability in the M1 generation after chemical treatment of cauliflower seeds, *Brassica oleraceae* L, var. *Capitata,* with 0.5, 0.6 and 0.7% EMS.

Various samples of mutant plants obtained from the species studied were established using randomly selected primer sequences of tandem repeats to identify mutant plant DNA. As a result of a preliminary screening conducted in the M1 stage, modified plants were selected, and seeds were collected and used to produce plants of the M2 generation. The aim of this work is to create homozygous mutant *B. napus* lines to enhance characters that are useful for breeding.

Materials and Methods.

Materials. The research materials were rapeseed cultivars (*B. napus*) 'Kris' and 'Galant', which were breeds of the Federal V. S. Pustovoit All-Russian Research Institute of Oil Crops (Russia).

Methods. *Microspore culture.* Buds were collected early in the morning hours characterized by intensive pollen division at the single-core microspore stage. The buds were 2–3 mm in size. Buds were pretreated with 10 mg/L silver nitrate solution for 2 d at 4°C temperature. Next, they were sterilized in 50% sodium hypochlorite for 7–10 min, 70% alcohol for 3–5 s, followed by washing three times with distilled water. Then, buds were placed in a cool vortex (10°C) with 30–40 mL of cooled B5 medium [19] without hormones (10–12°C), and homogenized for 7–9 s at high speed. The resulting suspension was filtered using an 80-μm filter. The filtrate was centrifuged at 100 × *g* (Eppendorf, Germany) for 5 min and

the supernatant was removed. The precipitate was re-suspended in 15 mL B5 medium and centrifuged 5 min. The supernatant was discarded and the precipitate was re-suspended in NLN medium with 0.05 mg/L BA (PhytoTechnology Laboratories, US), before being poured into Petri dishes to cultivate microspores. Microspore density in the NLN medium was adjusted to a 35.000 and 50.000 microspores per mL range. The Petri dishes were placed in a thermostat (TSO-1/80-SPU, Russian) with a shaker at 25°C. When torpedo-shaped embryos were formed, Petri dishes were placed under a light at the same temperature [20].

Treatment of somatic embryos with mutagens. When they reached a size of 1.5–2.5 mm, embryos were treated with EMS (Sigma Aldrich, US). Three different concentrations of EMS were added to petri dishes: 4 mM, 8 mM, and 12 mM. The dishes were then placed on a shaker (40–50 rpm) in a thermostat at 25°C for 1 h. After treatment, embryos were dried on sterile paper sheets for 5 s. Dry embryos were transplanted onto solid B5 medium with 0.8% agar and 2% sucrose and incubated for 24 h in a thermostat at 10°C. After incubation, tubes containing embryos were placed under light at 25°C. After two weeks of cultivation, the embryos were transplanted onto fresh B5 medium for regeneration.

After *in vitro* plantlets were obtained, they were cut into three equal pieces and cloned. Two of the three pieces were cloned on B5 medium and one was frozen and stored. Therefore, 1/3 of the plantlets were left for cloning, and 2/3 were transplanted into the soil. Before transplanting into the soil, a ploidy test was performed using the CyFlowPloidyAnalyzer. All haploid plants were treated with 0.05% colchicine solution to double the chromosomal set [21].

Evaluation of agronomic traits in the obtained mutant doubled haploids. To analysis the offspring of the obtained mutants, plants of 'Kris' (4 mM, 8 mM and 12 mM EMS) and 'Galant' (4 mM, 8 mM and 12 mM EMS) cultivars were grown in an experimental field. For this purpose, 100 seeds from each fertile mutant (M1) were selected for sowing. As a result, 50-60 plants for each mutant (M2) were grown. 30 plants from each mutant were selected to analysis agronomic parameters, weight of 1000 seeds (g), seed weight per plant (g).

The determination of fatty acid composition. The fatty acid composition of rapeseed was determined using gas chromatography (GC) [22]. Sample preparation for GC was performed as follows: 0.5 mL oil was extracted from the seeds using a press, 8 μL of the oil was pipetted into a test tube, and 2 mL hexane (Honeywell, Germany) was added to the oil. Afterward, 0.1 mL 5% sodium methylate (Sigma Aldrich, US) was added and the tube was in incubated for 0.5 h with periodic shaking (3 times every 10 min). After incubation, 1 mL distilled water was added, and the tube was shaken and incubated until complete sedimentation was achieved. Then, 1 mL of the upper hexane layer was transferred into a penicillin vial and placed under a fan at room temperature until hexane had completely evaporated. Afterward, 600 μL of chemically pure hexane was added to the penicillin bottle. The GC procedure was performed on Cristal 2000 M, Khromatek, Russia.

Molecular methods. DNA was isolated from plants using the standard CTAB (cetyl trimethylammonium bromide) method. Leaf tissue (100 mg) as placed in 700 μl of CTAB extraction buffer (100 mM Tris (pH 8.0), 5 M NaCl, 20 mM EDTA (pH 8.0), 0.2%), (p/v) β-mercaptoethanol and 2% (p/v) CTAB), and heated at 60°C for 30 min. The DNA was extracted with one volume of chloroform:isoamyl alcohol (24:1), and precipitated in the presence of isopropanol. The DNA precipitate was washed with 70% ethanol, dried, and dissolved in 30 μl TE (10 mM Tris-HCl (pH 8.0), 1 мМ EDTA, pH 8.0). After adding 1 μl of RNAse (10 mg/mL), DNA concentration were determined using a NanoDrop spectrophotometer.

PCR analysis. DNA amplification was performed using ISSR primers [14] (table 1). For ISSR analysis, the polymerase chain reaction (PCR) was carried out using a reaction volume of 25 μl, which contained 2 μl 10 × Tag buffer, 2 uL of a mixture of 4 dNTPs (2 mM), 2 μL ISSR marker (10 pM), 0.2 μl, Tag DNA polymerase (5U/μL), and 1.0 μL DNA (100 ng/μl). The following thermocycling program was used to perform the randomly amplified DNA polymorphism protocol (ISSR-analysis): pre-denaturation at 94°C for 10 min; followed by 35 cycles that included a 30 s denaturation at 94°C, a 30 s primer annealing step at 48°C and a 1-min elongation step at 72°C.

Table 1 – Names, nucleotide sequences, and annealing temperature of 6 ISSR-primers

Primers	Sequence (5'- 3')	Annealing temperature (°C)
BV-11	CTC TCT CTC TCT CTC TAT	45.4
BV-17	CAC ACA CAC ACA GT	44.7
BV-41	GAG GAG GAG GC	41.0
BV-53	GAG AGA GAG AGA GAG AA	45.7
BV-47	GTG GTG GTG GC	44.0
BV-50	AGA GAG AGA GAG AGA T	47.0
Abbreviation: ISSR, Inter Simple Sequence Repeat.		

Electrophoresis of random-primer amplified products was carried out in agarose (2.5% agarose, TAE-buffer, 5 µl of ethidium bromide), and PAAG gel (8%) using a vertical, and horizontal electrophoresis chamber. Each amplified DNA fragment on the gel was considered an individual descriptor with a specific molecular weight, which was determined using a 100 bp + 1.5 Kb DNA Ladder (Fermentas)molecular weight marker. After electrophoresis, the gel was analyzed in UV-light.

DNA bands traveling at the same rate of movement were equal in size. The bands were scored using a binary in which bands that appeared to be the size of the target were given a score of 1, while the invisible band was given a score of 0. Data were then analyzed with UPGMA (Unweight-Pair Group Method with Arithmetic Means) software based on the Jaccard genetic similarity index within PAST (Paleotological Statistics Software Package for Education and Data Analysis).

Statistical analysis. Significant results were tested using Analysis of Variance (ANOVA) by applying the Duncan's LSD and Tukey HSD test with the program SPSS 22 (IBM). Means with different letters are regarded as statistically significant at $p < 0.05$.

Results and Discussion. The rapeseed embryos obtained via the culture of isolated microspore cultivars, Kris and Galant, were used to obtain mutant lines. Embryos were formed from individual

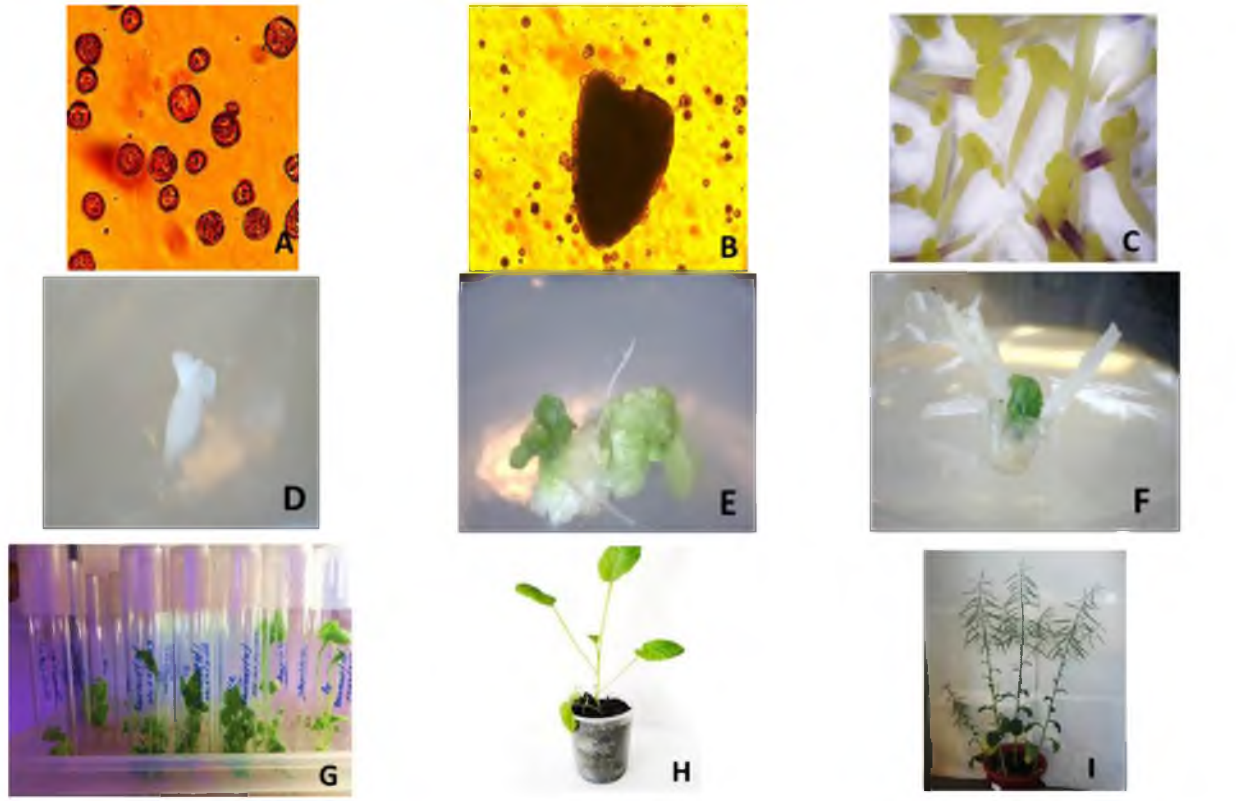


Figure 1 – Obtained doubled haploid rapeseed mutant of rapeseed in cultures of isolated microspores.
(A) Microspores that had been cultivated for one week. (B) heart-shaped embryos are shown. (C) Microspore-derived embryos for EMS are shown and a (D) EMS-treated embryo (1 week after treatment) on agar medium. (E , F) Regenerated plantlets from embryos treated with the EMS are shown. (G) Cloned plantlets and (H) mutant plants in the soil after colchicine treatment and (I) doubled haploid mutant (fertile) plants are shown.

microspores, through several stages of development (figure 1 A, B). Mature embryos with visible bipolar structures were treated (figure 1C).

We assumed that the EMS concentrations used in the experiment, 4mM, 8 mM, 12 mM, after 1 h of treatment did not produce serious lethal mutations, and instead produced only a few changes in the rape genome. Our results showed that rates of embryo survival in rapeseed when treated with EMS were good, even at the 8 mM concentration (figure 2). Regeneration took place at a fairly high frequency. Some stimulation of the regeneration process occurred as a result of the low concentrations of mutagen. Also, negative effects of increased mutagen concentrations (12 mM) on plant regeneration should be noted.

During regeneration, some plants displayed leaf changes due to albinism (figure 1D, E, F). However, as shown (figure 2A), the majority of the plants grew without disturbance. Resulting mutant haploid plantlets were transplanted onto hormone-free MS medium with a half set of macro and micro salts (figure 1G). The cloning of plantlets was carried out using the same medium, and the reproduction rate was 1:3. Next, 1/3 of the plantlets were left for cloning, and 2/3 of were transplanted to soil (figure 1H, I).

Before transplanting to the soil, a ploidy test was performed. All haploid plants were treated with a 0.05% colchicine solution to double the chromosome set (figure 2B).

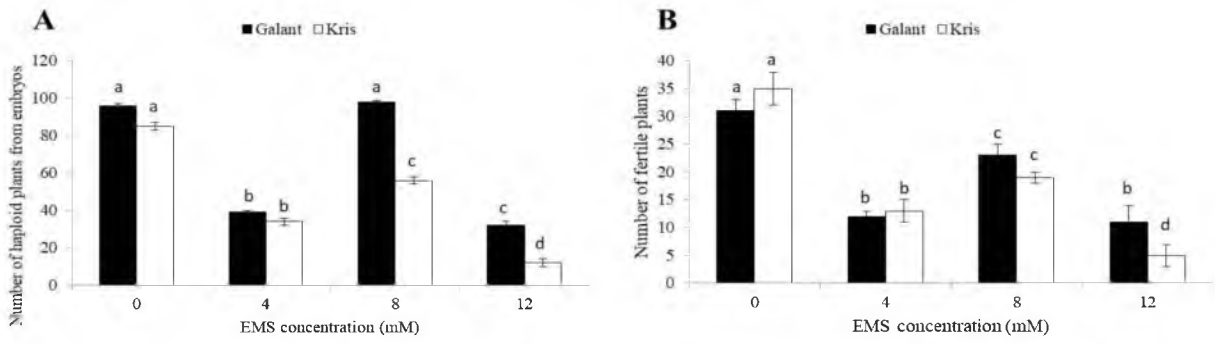


Figure 2 – Plant regeneration from microspore-derived rapeseed embryos (*Brassica napus*) treated with EMS. (A) Number of haploid plants from embryos. (B) Number of fertile plants. Data are means ± SD bars of three experiments (n = 40 embryos; n =30 plantlets per experiment), measured in triplicate. The same letter indicates no significant difference and the different letters indicate a significant difference (Duncan's LSD test, $p < 0.05$)

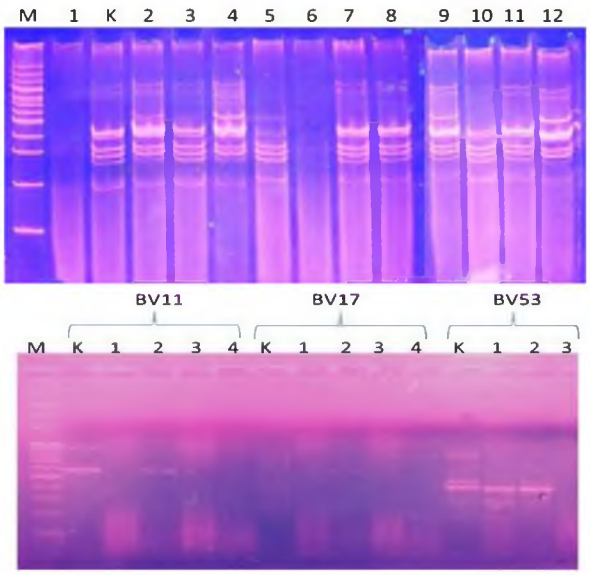
Seeds from mutant plants of *B.napus* cultivars (M1) were sown in an experimental field. During flowering, each plant was covered with an insulator. As a result, M2 seeds were obtained by growing mutant doubled haploids of rapeseed (table 2).

Table 2 – Seed mass of mutant doubled haploids (M2) obtained during treatment of embryos with the EMS mutagen at a concentration of 12 mM

Genotype	Seed yield per plant (g)	Weight of 1000 seeds (g)
Galant (control)	5.2 ± 1.4	3.1 ± 0.1
DH2G12	6.5 ± 2.3	3.5 ± 0.2
Kris (control)	5.9 ± 1.5	3.0 ± 0.4
DH2K12	6.8 ± 1.9	3.6 ± 0.1
Values tabulated are mean ± SD at three replications.		

To further verify and identify promising lines, an analysis was carried out using molecular ISSR markers of M2-generation rapeseed. ISSR markers have previously been used to screen for genetic diversity [23]. To assess the level of genetic diversity in rapeseed mutant and parental forms, we selected 6 ISSR primers.

The electrophoregram (figure 3) using BV11, BV17, BV53, BV47 primers revealed visible differences between mutant plants and starting material. Mutant plants possessed 550 bp, 250 bp, fragments that were absent in the original plants. As well as mutant lines DH2G12-6, DH2K12-3 and DH2K12-4 were distinguished by the absence of loci/bends, which showed that mutation and genetic changes occurred.



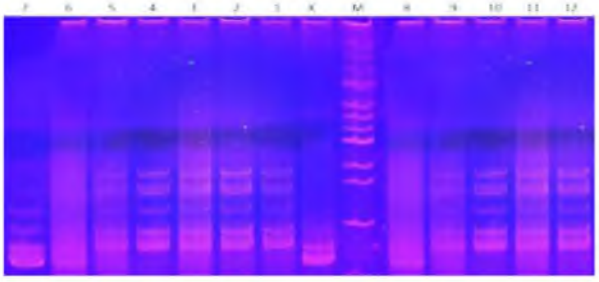


Figure 3 – The ISSR profiles of the 12 mutant Galant lines using BV11 (A), BV53 (B) (K) Control (Galant); (1) DH2G12-1; (2) DH2G12-2; (3) DH2G12-3; (4) DH2G12-4; (5) DH2G12-5; (6) DH2G12-6; (7) DH2G12-7; (8) DH2G12-8; (9) DH2G12-9; (10) DH2G12-10; (11) DH2G12-11; (12) DH2G12-12 and 4 mutant Kris lines using BV11, BV17 and BV53 primers (C) (K) control (Kris); (1) DH2K12-1-1; (2) DH2K12-2; (3) DH2K12-3; (4) DH2K12-4 are shown. Lane numbers indicate the following: (M) (100–3000 b.p. ladder)

The presence of individual loci or the absence of one of the genotypes indicates that genetic changes in plants had occurred as a result of *in vitro* mutagenesis [24]. Changes observed during molecular analyses were caused by point mutations, displacement, a nucleotides that was removed from the sequence [25]. This molecular analysis performed can be used to facilitate the breeding of mutant lines of rapeseed cultivars. In the future, we analyzed only those mutant lines that had the prospect of practically useful for breeding. As a result, we determined that the best mutants were plants that were obtained via exposure to EMS at a concentration of 12 mM.

According to quantitative characteristics of the data, mutant plants were relatively higher indicators of the mass of plants compared with the control and the mass of 1,000 seeds was determined (table 2). In addition, mutant seeds of two plants of the Galant cultivar (DH2G12-5, DH2G12-6) had seeds that were different in color – from light brown to black (figure 4), while the donor cultivar Galant had black seeds. Changing the color of the seed coat from black to yellow is an important positive trait for cultivars of rapeseed (canola).

Figure 4 – Mutant seeds (EMS 12 mM) of the Galant cultivar are shown as follows: (A) control; (B) mutant seeds DH2G12-5; (C) mutant seeds DH2G12-6

A key indicator of the value of rapeseed breeding was determined principally by assessing the fatty acid composition of seed oil [26]. Another indicator of the nutritional composition of rapeseed was the absence of erucic acid. In our experiments, erucic acid was not found in either donor or mutant lines. An important indicator of the quality of edible oil is the ratio of saturated to unsaturated fatty acids. In this regard, the best genotypes are considered to be ones in which the sum of palmitic and stearic saturated fatty acids is smallest [12]. In addition, rapeseed genotypes, which have high oleic acid and low linolenic acid content are ideal.

Analysis of the fatty acid composition of the seeds of individual M2 mutants was carried out only in individual plants tested for the presence of mutations using molecular markers. Table 3 presents data that revealed that the fatty acid compositions of doubled haploids were different from controls.

Table 3 – Indicators of the percentage ratio of the fatty acid composition of seeds in mutant M2 doubled haploids of rapeseed, obtained when processing androgen embryos treated with 12 mM EMS

Name of doubled haploid line	Fatty acid					
	P (C16:0) %	S (C18:0) %	O (C18:1) %	L (C18:2) %	Ln (C18:3) %	Er (C22:1) %
Galant (control)	3.6 ± 0.1^{a}	2.0 ± 1^{a}	66.0 ± 2^{d}	18.0 ± 2^{a}	7.4 ± 0.2^{a}	< 0.05
DH2G12-1	3.6 ± 0.3^{a}	2.5 ± 0.2^{a}	**72.5** ± 1.5^{abc}	13.8 ± 0.2^{cdef}	4.5 ± 0.3^{b}	< 0.05
DH2G12-2	3.3 ± 0.1^{a}	2.4 ± 0.2^{a}	**75.4** ±0.9 ^{a}	12.2 ± 2.1^{f}	3.0 ± 0^{d}	< 0.05
DH2G12-3	3.8 ± 0.4^{a}	2.9 ± 0.2^{a}	**74.3** ± 1.1^{ab}	12.3 ± 0.3^{f}	3.4 ± 0.2^{cd}	< 0.05
DH2G12-4	3.2 ± 0.2^{a}	2.6 ± 0.2^{a}	71.3 ± 0.2^{c}	15.5 ± 0.6^{bc}	4.4 ± 0.4^{b}	< 0.05
DH2G12-5	3.6 ± 0.5^{a}	2.9 ± 0.3^{a}	70.3 ± 1.5^{c}	16.6 ± 0.4^{ab}	3.5 ± 0.2^{cd}	< 0.05
DH2G12-6	3.6 ± 0.4^{a}	2.9 ± 0.2^{a}	70.2 ± 0.8^{c}	18.1 ± 0.4^{a}	1.6 ± 0.4^{e}	< 0.05
DH2G12-7	3.9 ± 0.2^{a}	2.3 ± 0.3^{a}	71.7 ± 0.9^{bc}	14.7 ± 0.4^{bcd}	4.1 ± 0.2^{bc}	< 0.05
DH2G12-8	3.8 ± 5.4^{a}	2.4 ± 0.3^{a}	72.9 ± 0.4^{abc}	13.4 ± 0.7^{def}	4.1 ± 0.2^{bc}	< 0.05
DH2G12-9	3.8 ± 0.4^{a}	2.6 ± 0.3^{a}	**74.3** ± 0.5^{ab}	12.2 ± 0.3^{f}	3.5 ± 0.2^{cd}	< 0.05
DH2G12-10	3.6 ± 0.4^{a}	2.1 ± 0.3^{a}	70.7 ± 0.3^{c}	12.7 ± 0.3^{ef}	3.1 ± 0.2^{d}	< 0.05
DH2G12-11	3.1 ± 0.1^{a}	2.7 ± 0.1^{a}	72.0 ± 0.2^{bc}	14.4 ± 0.2^{cde}	3.6 ± 0.2^{cd}	< 0.05
DH2G12-12	3.5 ± 0.3^{a}	2.6 ± 0.3^{a}	73.1 ± 0.4^{abc}	15.2 ± 0.1^{bcd}	3.5 ± 0.3^{cd}	< 0.05
Kris (control)	3.7 ± 0.3^{ab}	2.0 ± 0.2^{b}	67.6 ± 0.1^{c}	17.3 ± 0.4^{a}	5.9 ± 0.1^{a}	< 0.05
DH2K12-1	4.1 ± 0.2^{a}	2.8 ± 0.2^{a}	**72.8** ± 0.3^{b}	12.3 ± 0.4^{e}	4.0 ± 0.2^{b}	< 0.05
DH2K12-2	3.7 ± 0.3^{ab}	2.3 ± 0.2^{ab}	**73.5** ± 0.9^{ab}	12.3 ± 0.3^{e}	4.0 ± 0^{b}	< 0.05
DH2K12-3	3.3 ± 0.2^{b}	2.2 ± 0.2^{b}	**73.1** ± 0.4^{ab}	13.3 ± 0.2^{b}	3.3 ± 0.2^{c}	< 0.05
DH2K12-4	3.5 ± 0.4^{ab}	2.4 ± 0.2^{ab}	**74.1** ± 0.2^{a}	12.3 ± 0.2^{e}	3.1 ± 0.2^{c}	< 0.05

Values tabulated are mean ± SD at three replications. Means followed by same letters in the column are not different from one another by Tukey test at the 5% probability level.

Abbreviation: P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Ln, lenolenic acid; Er, erucic acid.

Chromatographic analysis showed that the oleic acid content within seed oil of mutant plants of the Galant cultivar was 9.4% higher (from 70.2 % to 75.4%) compared to the control (66.0%). Also, the sum of unsaturated fatty acids was superior in mutant plants of the Galant cultivar to the oil from the seeds of mutant plants of the Kris cultivar. Mutant plants of Kris cultivar (DH2K12-4) had 6.5% (74.1) increased oleic acid content. It should be noted that the mutant plants, DH2G12-5, DH2G12-6, isolated by the lighter color of the seeds, and had a good fatty acid compositions (table 3).

A cluster analysis of obtained data was used to determine genetic distances between eight mutant rapeseed plants (lines) and plants of the control cultivars (Galant (G control)) and Kris (K control)) and a dendrogram was constructed (figure 5). Genetic distances between the studied lines ranged from 0.3 to 1.0. The most genetically similar mutants were DH2G12-2 (isolated by its fatty acid composition in which oleic acid content was 75.4%) and DH2G12-3 (selected as a result of its fatty acid composition, in which oleic acid content was 74.3%). The greatest degree of genetic difference was between DH2G12-1 (isolated by quantitative characteristics) and Galant (control) plants, which demonstrated a high degree of genetic difference and the presence of mutations.

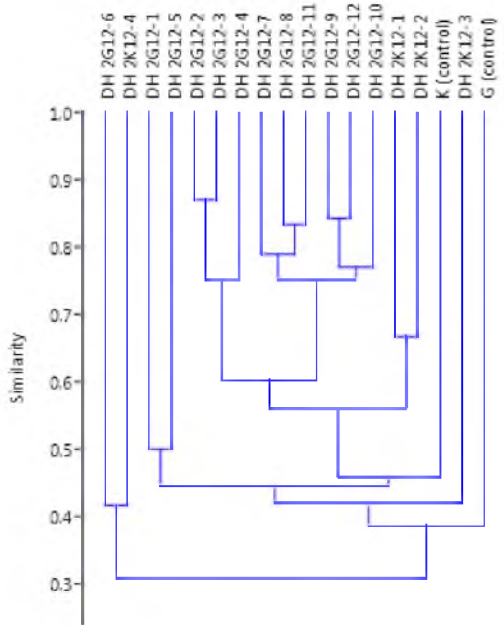


Figure 5 – Phylogenetic tree of mutant rapeseed lines

The creation of clusters of initial plants and their mutants reveal genetic features, and studying the location of genes in different clusters in the dendrogram. Mutant plants DH2G12-1 (isolated by quantitative characteristics and fatty acid composition), DH2G12-2, DH2G12-3, DH2G12-4, DH2G12-5 (identified by seed color) and DH2K12-4 (identified by its quantitative characteristics and fatty acid composition) demonstrate a high degree of genetic difference from their parent plants, since they are located much further from the control.

In our experiment, we used embryos obtained from microspore cultures to perform EMS. The use of chemical mutagenesis directly within cultures of isolated microspores can reduce embryogenesis, and create difficulties throughout the mass production of plants from embryos. Secondary embryos induced from calli have been previously used to solve such difficulties [27] either calli were induced from embryos. Work of Liu et al. (2005) [28] produced doubled haploid mutant rapeseed plants resistant to *Sclerotinia sclerotiorum*.

Conclusion. In our experiments, the processing of mutagenic EMS embryos obtained for the culture of isolated microspores was shown. Mutant doubled haploids of the rapeseed cultivars Galant and Kris, were obtained by both quantitative and qualitative characterization. Researchers determined that the best outcomes of mutagenesis were obtained by processing embryos obtained for the culture of isolated microspores in an EMS concentration of 12 mM.

In the future, the authors plan to use other markers, in particular SNP (Single Nucleotide Polymorphisms) markers, for the analysis of rapeseed mutagenesis. This would provide a more in-depth analysis of mutations that arose as a result of EMS treatment of on embryos obtained in the isolated microspore.

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РАПС СЕЛЕКЦИЯСЫ ҮШІН ОҚШАУЛАНҒАН МИКРОСПОР ДАҚЫЛЫНАН АЛЫНҒАН ЭМБРИОИДТАРДЫ EMS МУТАҒЕНІМЕН ӨҢДЕУ (*BRASSICA NAPUS*)

Аннотация. Микроспор дықылындағы мутагенез рапстың жаңа сорттарын шығаруда селекциялық тәжірибе үшін кеңінен қолданылады. Мутагенездің майдың майлы қышқыл құрамының өзгерісіне оң әсер ететіні белгілі.

Рапс мутагенезі үшін эксперименттерімізде оқшауланған микроспоралар дақылынан алынған эмбриоидтар пайдаланылды. Эксперимент нысаны ретінде рапс – Крис және Галанттың эруксіз сорттары қызмет етті. Экспериментімізде мутагенмен өңдеу ЭМS-4 мМ, 8 мМ және 12 мМ химиялық мутаген концентрациясы кезінде 1,5 - 2 мм өлшемдегі эмбриоидтарда 1 сағат бойы жүргізілді. Мутагенмен өңдеуден кейін эмбриоидтар өсімдіктерді одан әрі қалпына келтіру үшін Гамборгтің жаңа қоректік ортасына ауыстырылды. Бұл әдіс гаплоидты мутантты өсімдіктерді жаппай алуға мүмкіндік береді.

Алынған гаплоидты мутантты өсімдіктердің бір бөлігі 5 жапырақ фазасына жеткенде 0,05% колхицинмен хромосомдық жиынтықты екі еселеу үшін өңдеді және топыраққа, бақыланатын жағдайға ауыстырды. Нәтижесінде оқшауланған микроспор дақылдарынан алынған эмбриондарды мутагенмен өңдеуде рапстың мутантты екі еселенген гаплоидты өсімдіктері мен оның тұқымы (М1, М2) алынды. Рапстың мутантты және аталық және аналық формаларында генетикалық әртүрлілік деңгейін бағалау үшін 6 праймер ISSR таңдалды. Мутантты линиялар мутация мен генетикалық өзгерістердің болғанын көрсетті және аталық және аналық формаларынан ерекшеленді.

Алынған мутантты екі еселенген гаплоидтардың майлы-қышқылдық құрамын талдау рапс сорттарында олеин қышқылының пайыздық арақатынасының донорлық сорттармен (66,0%) салыстырғанда 75,4%-га дейін айтарлықтай ұлғайғанын көрсетті. Алынған мутантты екі еселенген рапстың гаплоидты өсімдіктері өсімдіктің салмағы мен 1000 тұқымының массасы бойынша жоғары көрсеткіштермен донорлық сорттардан ерекшеленді. Сонымен қатар, сапалық және сандық талдау нәтижелері бойынша жоғары көрсеткіштер 12 mM EMS концентрациясында мутагенмен өңдеу барысында айқындалды.

Зерттеу нәтижелері көрсеткендей, оқшауланған микроспора дақылынан эмбриондардың мутагенезі рапстың сапалы белгілерін жақсартуда үлкен әлеуетке ие. Бұл әдіс мутантты және сонымен қатар қажетті белгілері бар гомозиготалық линияларды жасауға мүмкіндік береді және селекциялық үдеріс тиімділігін айтарлықтай арттыруы мүмкін.

Түйін сөздер: рапс, *Brassica napus,* микроспоралар, EMS, ISSR.

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ОБРАБОТКА МУТАГЕНОМ EMS ЭМБРИОИДОВ, ПОЛУЧЕННЫХ В КУЛЬТУРЕ ИЗОЛИРОВАННЫХ МИКРОСПОР ДЛЯ СЕЛЕКЦИИ РАПСА (*BRASSICA NAPUS*)

Мутагенез в культуре микроспор широко применяется для селекционной практики при выведении новых сортов рапса. Известно положительное влияние мутагенеза на изменение жирнокислотного состава масла.

В наших экспериментах для мутагенеза рапса использовали эмбриоиды, полученные в культуре изолированных микроспор. Объектами для эксперимента служили безэруковые сорта рапса – Крис и Галант. В отличии от других исследовательских работ по обработке мутагеном в культуре изолированных микроспор в нашем эксперименте обработка мутагеном проводилось на эмбриоидах размером 1,5-2 мм при концентрациях химического мутагена EMS - 4 мМ, 8 мМ и 12 мМ в течение 1 часа. После обработки мутагеном эмбриоиды были перенесены на свежую питательную среду Гамборга В 5 для дальнейшей регенерации растений. Данный метод позволяет массово получать гаплоидные мутантные растения.

Часть полученных гаплоидных мутантных растений по достижению фазы 5 листочков обрабатывали 0,05% колхицином для удвоения хромосомного набора и пересаживали в грунт, в контролируемые условия. В результате, при обработке мутагеном эмбриоидов, полученных из культуры изолированных микроспор,

были получены мутантные удвоенные гаплоидные растения рапса, а также их семена (M1, M2). Для оценки уровня генетического разнообразия у мутантных и родительских форм рапса было отобрано 6 праймеров ISSR. Все мутантные линии отличались от родительских форм, показывая, что мутация и генетические изменения произошли.

Анализ жирно-кислотного состава семян, полученных мутантных удвоенных гаплоидов M2, показал значительное увеличение процентного соотношения олеиновой кислоты у сортов рапса до 75,4%, по сравнению с донорными сортами (66,0%). Полученные мутантные удвоенные гаплоидные растения рапса отличались от донорных сортов с высокими показателями по массе с растения и массе 1000 семян. При этом, по результатам качественных и количественных анализов, наилучшие показатели были при обработке мутагеном в концентрации 12 mM EMS.

Наши результаты показали, что мутагенез эмбриоидов из культуры изолированных микроспор имеет большой потенциал для улучшения качественных признаков рапса. Данный метод позволяет создать мутантные и в то же время гомозиготные линии с желаемыми признаками, что может значительно повысить эффективность селекционного процесса.

Ключевые слова: рапс, *Brassica napus,* микроспоры, EMS, ISSR.

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