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Full Length Article

Improvement of Breeding-Valuable Traits of Rapeseed (*Brassica napus)* using Mutagenesis

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Abstract

This study evaluated the agronomic and qualitative characteristics and cold resistance of 46 doubled haploid mutant *B. napus* lines (M2 and M3) that were obtained by treating haploid embryos derived from a culture of isolated microspores with the EMS mutagen (8 and 12 m*M*). The fatty acid composition of seed oil, the agronomic characteristics forming the yield and the resistance to cold temperatures were considered. The analyses showed highly significant differences between the mutants and original cultivars in all the studied characteristics. Mutant lines DHK12-3, DHK12-4, DHK12-8, DHG12-16, DHG12-18 and DHG12-10 combined improved indicators of fatty acid composition with high yield and cold-tolerance. Only mutant lines obtained at a high mutagen concentration (12 m*M*) were more resistant to the cold than the original cultivars. The obtained lines were homozygous since they were doubled haploids, and the stability of agronomic and qualitative traits was observed in two generations. The results obtained confirm the higher productivity of doubled haploid mutant lines, as well as their availability to variable environmental conditions.

Keywords: Rapeseed; Doubled haploid mutant line; EMS; Homozygous; Mutation breeding

Introduction

Rapeseed *(Brassica napus* L.*)* is an important oilseed crop worldwide. The different fatty acid components of certain oils make them more suitable for specific purposes. For example, breeding has produced low erucic acid and glucosinolate (double low or "00") in seeds of the genus *Brassica*, leading to the development of a food grade variety called 'canola' (Stefansson and Kondra 1975). The high oleic acid content and low linolenic acid content of rapeseed oil are very important for long-term storage as polyunsaturated acids (linolenic acid) tend to oxidize and are unstable during frying (Matthäus 2006; Scarth *et al.* 1988). Another important indicator of the quality of edible oil is the ratio of saturated and unsaturated fatty acids.

In this respect, the best genotype is considered to be the one in which the sum of palmitic and stearic saturated fatty acids is much less than the sum of unsaturated acids (Sharafi *et al.* 2015). These properties have been shown to have positive effects on human health by reducing diseases, such as heart disease and several neurological disorders (Chew 2020; Bennouna *et al.* 2021).

In Kazakhstan, the northern regions are the most suitable for rapeseed cultivation. The main influencing factor on the cultivation of rapeseed in these regions is the duration of the cold season, up to 160 days (https://www.kazhydromet.kz/ru/klimat/klimat-kazahstana). The most effective method for obtaining cold-tolerant rapeseed plants is genetic engineering (Peng *et al.* 2018). However, there is a critical attitude towards genetically modified crops. The breeding work on the creation of new cultivars is hindered by the low level of genetic variability in germplasm. The solution to this problem is the use of mutagenesis in rapeseed breeding (Viana *et al.* 2019). The main advantage of mutation breeding is the ability to improve one or more traits without changing the main genome (Oladosu *et al.* 2016). In recent years, induced mutations have been widely used to obtain oilseeds resistant to abiotic factors (Emrani *et al.* 2015; Hussain *et al.* 2017; Channaoui *et al.* 2019).

Mutagenesis is an effective and simple method of obtaining a valuable starting material that can later be used to improve agricultural crops (Shu *et al.* 2012). The widely used chemical mutagen ethyl methanesulfonate (EMS) has been applied to generate important recessive and dominant genomic mutations at a high rate, thereby creating useful genetic variations necessary for plant breeding (Ul-Allah *et al.* 2019). EMS mutagenesis is an efficient approach to create mutations in the genes of allopolyploid species, such as *B. napus*. These mutagens induce non-lethal DNA point mutations that can persist in the genome due to their self-pollination ability (Gilkrist *et al.* 2013). In the studies of

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Shan *et al.* (2020), it was demonstrated that the use of the chemical mutagen EMS can change the level of fatty acids and improve the quality of seed oil. Changes in fatty acid composition after treatment with the EMS mutagen have been observed in winter rapeseed (Spasibionek 2006) for Ethiopian (Velasco *et al.* 1997) and Indian (Prem *et al.* 2012) mustard. In Spasibionek (2006), mutant lines were obtained with an increased level of oleic acid (approximately 76%) and a decrease in linoleic and linolenic acids (8.5 and 7.5%, respectively).

In addition, mutagenesis is widely used in rapeseed breeding to obtain new cultivars with desired agronomic traits, such as early maturity, dwarfism, resistance to abiotic stress, and high yields, which are difficult to obtain with traditional breeding methods (*e.g.*, hybridization) (Parry *et al.* 2009; Ali and Shah 2013; Amosova *et al.* 2019). Traditional breeding is laborious and requires considerable time. In addition, all cultivars and hybrids of the food direction (the low content of erucic acid and glucosinolates) were obtained from the same cultivar "Tawer"; therefore, they have a rather narrow range of variations in traits. Thereby, mutagenesis is used to expand the genetic diversity of the source material for food selection. In Channaoui *et al.* (2019), rapeseed mutants were obtained, which showed the highest 1000-seed weight and also were earlier and characterized by a higher number of pods per plant. Currently, most rapeseed cultivars have a seed color from yellow to black. Studies show that rapeseed mutants with yellow seeds have a thin shell, and a higher oil and protein content than cultivars with black seeds (Facciotti 2003). It is known that the use of mutagenesis in representatives of the genus *Brassica* contributes to the manifestation of such traits as resistance to a certain class of pesticides, and resistance to abiotic stress factors, diseases, and changes in the composition of fatty acids that improve the quality of seed oil (Harloff *et al.* 2012; Yu *et al.* 2019; Guo *et al.* 2020).

To obtain new mutant rapeseed cultivars with improved quality characteristics and resistance to abiotic stress factors, mutagenesis is widely used in the culture of isolated microspores (Kott 1995; McClinchey and Kott 2008). The main advantage of induced mutagenesis is the expansion of genetic diversity for the desired traits. At the same time, the advantage of obtaining doubled haploids is the rapid production of homozygous lines. The various homozygous lines obtained in this way are a valuable source for breeding new cultivars.

Chemical mutagenesis is considered as an effective mean in improving the yield and quality trait of crop plants (Kong *et al.* 2020). Moreover, EMS is one of the most powerful chemical mutagens used to induce mutation in crop plants (Sharamo *et al.* 2021). The present study was carried out to investigate the effect of mutation induced by EMS on qualitative and quantitative characters of doubled mutant haploid rapeseed lines in M2 and M3 mutant lines and to identify and select the most interesting ones for their use as a valuable germplasm in the rapeseed breeding program.

Materials and Methods

Plant materials

The objects of research were previously obtained 46 doubled mutant haploid lines of rapeseed cultivars "Galant" and "Kris". All mutant lines were derived by treatment with EMS mutagen of embryos obtained in the culture of isolated microspores (Fig. 1). Haploid embryos were treated with EMS aqueous solution at concentrations of 8 and 12 m*M* for 1 h (Daurova *et al.* 2020). The mutagen concentration and treatment time were optimized in our previous studies (Zhambakin *et al.* 2015). As the haploid plantlets were formed, they were treated with 0.05% aqueous colchicine solution to double the chromosome set. Further, they were transplanted into the ground under controlled conditions and mutant seeds of doubled haploids were obtained.

The originator of the cultivars (Kris and Galant) is the V.S. Pustovoit All-Russian Research Institute of Oil Crops.

Determination of fatty acid composition

The fatty acid composition of rapeseed was determined using gas chromatography (GC). Sample preparation for GC was performed as follows: 0.5 mL oil was extracted from the seeds using a press and 8 μL of the oil was pipetted into a test tube, to which 2 mL of hexane (Honeywell, Germany) was added. Subsequently, 0.1 mL 5% sodium methylate (Sigma Aldrich, US) was added and the tube was 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 the hexane had completely evaporated. Subsequently, 600 μL of chemically pure hexane was added to the penicillin bottle. The GC procedure was performed on a Cristal 2000 M (Khromatek, Russia; GOST R 51483-99 1999).

Characteristics of vegetative growth

For the analysis of offspring, mutant plants of the cultivars "Galant" (8 and 12 m*M* EMS) and "Kris" (8 and 12 m*M* EMS), as well as the original cultivars, were grown in the experimental field. Morphological parameters were collected at the harvest stage and measured using 30 randomly selected mutant plants and 20 plants of each parent genotype. At maturity plant height (cm) and number of pods per plant were determined. After harvest number of seeds per pod was counted in laboratory whilst 1000-seed weight (g) and the weight of seeds per plant (g) was determined by a precision balance.

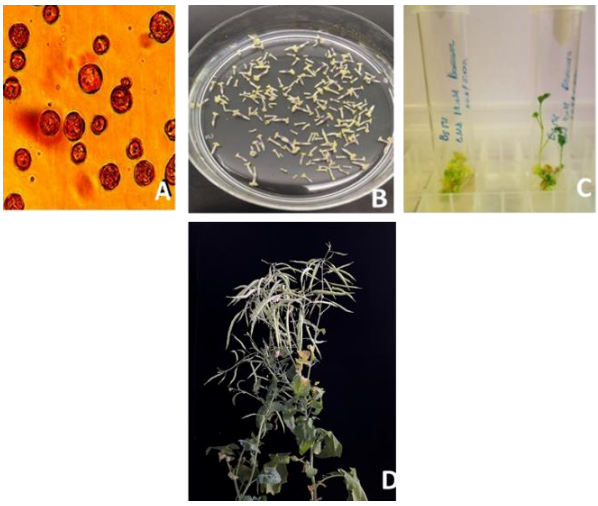

Fig. 1: Produced rapeseed doubled haploid mutants in cultures of isolated microspores. (A) Microspores that had been cultivated for one week. (**B**) Microspore-derived embryos and EMS-treated (**C**) Regenerated plantlets from embryos treated with the EMS are shown. (**D**) doubled haploid mutant (fertile) plants are shown

Determination of cold resistance

Forty-six mutant plants and 2 original materials (Galant and Kris) were grown to the stages of 3–4 leaves at a temperature of 25°C and then were placed in a programmable low-temperature thermostat for 2 h at temperatures of 4°C, 0°C, and – 4°C. For each temperature, 12 plants were treated with three repetitions. After low-temperature treatment, the plants were placed in controlled conditions for recovery at a temperature of 22–25°C for 3 weeks (16 h day/8 h night in 5000 lux light mode) and the survival degree was assessed (Yan *et al.* 2018).

Statistical analyses

In this research, at least three biological replications ($n \geq 3$) were designed for each experiment. All values are means ± standard deviation (SD). The measurement of the mean value, the standard deviation and the coefficients of variation (CV%) of each trait were calculated using Excel 2010. Data were processed using one-way ANOVA, and mean separation was done by a Duncan's multiple range test. Statistical analyses were performed using the computer program SPSS 22 (IBM).

Results

Differences in fatty acid composition between original cultivars and doubled haploid (DH) mutant lines

When evaluating 46 mutant lines (2 and M3) and lines with a low total content of saturated acids a low content of linolenic acid and a high content of oleic acid were distinguished. Based on the total content of saturated fatty acids, all mutants showed a reduced total concentration of saturated fatty acids and an increased concentration of oleic acid in comparison with the original cultivars. In general, mutant lines showed a significant difference compared with the original plants (Table 1). In DH mutant lines, DH2K12-4, DH2K12-5, DH2K12-12, DH2K8-6, DH2K8-7, DH2G12-13, DH2G12-18, DH2G12-19, DH2G8-2 and DH2G8-3, the oleic acid content varied from 74.2 to 74.6%, respectively and was 6.6–8.6% higher than that in the original cultivars. Meanwhile, in the M3 mutant plants, a slight decrease in the content of oleic acid was observed in comparison with the M2 mutant lines (1.5%). However, in relation to the control sample, they were significantly higher (5.0–6.5%). High oleic acid mutants in M2 (DH2K12-4, DH2K12-5, DH2K12-12, DH2K8-6, DH2K8-7, DH2G12-13, DH2G12-18, DH2G12-19, DH2G8-2 and DH2G8-3) showed the highest stability in the composition of fatty acids in M3. The content of oleic acid in these mutant plants ranged from 71.5 ± 1.9% to 73.2 ± 1.6%, while in the control cultivars it was 66 ± 1.5%. As shown in Table 1, the M3 mutant lines of Kris had significant reliability in the percentage ratio of oleic and linolenic acids. Moreover, DH3K12-4 had high significance for two fatty acids, an increased concentration of oleic acid (73.3 ± 1.6%) and a low level of linolenic acid (3.5 ± 0.3%). All of the selected M3 mutant lines of the «Galant» cultivar had high authenticity based on the results of oleic and linolenic acid. The oleic acid level in 8 «Galant» mutant lines varied from 71.5 ± 1.2% to 73.3 ± 0.7%. The content of linolenic acid was significantly lower than the control sample, *i.e*., from 2.9 ± 0.1% to 5.3 ± 0.7%. The increase/decrease in oleic acid content was accompanied by a concomitant decrease/increase in the content of linoleic and linolenic acids.

Change in quantitative characteristics

According to the results of the analysis of variance, EMS mutagen exposure significantly affected the variability of all studied quantitative traits in the M2 and M3 generations (Table 2). Higher variability was observed in the M2 mutants for traits, such as the number of seeds per pod, the number of pods per plant, and the seed yield per plant. In the M3 mutants, indicators such as the number of pods and the weight of 1000 seeds were the most stable. A higher degree of variability was observed in mutants obtained by treatment with 12 *mM* EMS.

Variation in the parameters of yield attributes (1000-seed weight and the weight of seeds per plant) of mutant doubled haploids is shown in Table 3. Treatment with the mutagen led to significant ($P \leq 0.05$) differences between original cultivars and doubled haploid mutants in the weight of seeds per plant and the weight of 1000 seeds.

In the original parents, the weight of seeds per plant was 5.3g (Galant) and 5.8 g (Kris) and the weight of 1000

Table 1: Fatty acid composition of seeds in *B. napus* cv.. Kris, Galant and DH mutants (M2 and M3)

Name of doubled haploid line	Fatty acid						Name of doubled haploid line	Fatty acid					
	16:0	18:0	16:0 + 18:0	18:1	18:2	18:3		16:0	18:0	16:0+18:0	18:1	18:2	18:3
Kris	3.7 ± 0.6^{ab}	2.0 ± 0.4^{b}	5.7 ± 1.0^{bcde}	67.6 ± 1.0^{f}	17.3 ± 0.7^{c}	5.9± 0.2^{c}	Galant	3.6± 0.3^{abcd}	2.0 ± 0.2^{a}	5.6 ± 0.5^{ab}	66.0 ± 2.0^{k}	18.0 ± 0.2^{de}	7.4 ± 0.2^{a}
M2 mutants													
DH2K12-3	3.5 ± 0.4^{ab}	2.0 ± 0.3^{b}	5.5 ± 0.6^{cde}	71.8 ± 1.0^{bc}	18.1± 1.2^{b}	3.9± 1.2^{a}	DH2G12-13	3.0± 0.5^{abcd}	2.0 ± 0.2^{a}	5.0 ± 0.5^{ab}	74.5 ± 1.2^{a}	16.6 ± 0.2^{gh}	4.6 ± 0.4^{cd}
DH2K12-4	3.7 ± 0.2^{ab}	2.2 ± 0.2^{b}	5.9± 0.7^{abcde}	74.2 ± 0.8^{a}	15.0 ± 1.1^{a}	2.1 ± 0.9^{j}	DH2G12-16	3.2 ± 0.4^{ef}	1.8 ± 0.5^{a}	5.0 ± 0.9^{b}	73.5 ± 1.1^{c}	17.1 ± 0.1^{fg}	2.8 ± 0.1^{fgh}
DH2K12-5	3.6 ± 0.3^{ab}	1.8 ± 0.2^{b}	5.4 ± 0.5^{e}	74.3 ± 0.9^{a}	17.4± 0.6^{bc}	4.0± 0.8^{c}	DH2G12-18	3.8 ± 0.2^{ab}	1.9 ± 0.3^{a}	5.7 ± 0.5^{ab}	74.6 ± 1.2^{a}	22.3 ± 0.2^{a}	2.7 ± 0.1^{fgh}
DH2K12-12	3.8 ± 0.4^{ab}	2.5± 0.3^{ab}	6.3 ± 0.7^{abcd}	72.4 ± 0.9^{bc}	15.7±0.6^{de}	4.6± 0.4^{e}	DH2G12-19	3.4±0.4^{cdef}	2.4 ± 0.5^{a}	5.8 ± 0.9^{ab}	74.1 ± 0.9^{ab}	19.6 ± 0.1^{c}	2.5± 0.1^{fgh}
DH2K12-13	3.6 ± 0.3^{ab}	2.3 ± 0.2^{b}	5.9 ± .5^{abcde}	71.4 ± 0.8^{bc}	15.6± 0.3^{de}	4.6± 0.2^{e}	DH2G12-10	3.2 ± 0.3^{ef}	1.8 ± 0.2^{a}	5.0 ± 0.5^{b}	73.5 ± 0.9^{c}	17.1 ± 0.6^{fg}	2.8 ± 0.2^{fgh}
DH2K12-14	4.3 ± 0.4^{a}	2.4± 0.4^{ab}	6.7 ± 0.8^{abc}	71.3 ± 0.6^{cd}	13.9 ± 0.8^{f}	3.6± .4^{hi}	DH2G8-3	3.8 ± 0.2^{ab}	1.9 ± 0.4^{a}	5.7 ± 0.6^{ab}	74.6 ± 0.9^{ab}	22.3 ± 0.1^{a}	2.7 ± 0.1^{fgh}
DH2K8-6	3.2 ± 0.2^{b}	2.3 ± 0.2^{b}	5.5 ± 0.4^{cde}	73.6 ± 1.1^{ab}	14.6 ± 0.9^{f}	3.3 ± 0.5^{i}	DH2G8-1	3.3 ± 0.3^{def}	2.2 ± 0.4^{a}	5.5 ± 0.7^{ab}	73.4 ± 0.5^{c}	21.3 ± 0.3^{b}	2.1 ± 0.4^{gh}
DH2K8-7	3.5 ± 0.3^{ab}	2.0 ± 0.4^{b}	5.5 ± 0.7^{de}	74.2 ± 0.9^{a}	14.3 ± 1.0^{f}	4.1± 0.3^{g}	DH2G8-2	3.6± 0.4^{abcd}	2.4 ± 0.3^{a}	6.0 ± 0.7^{ab}	74.3 ± 0.6^{ab}	21.2 ± 0.1^{b}	2.3 ± 0.1^{def}
							M3 mutants						
DH3K12-3	3.4 ± 0.3^{ab}	2.3 ± 0.3^{b}	5.8 ± 0.6^{cd}	70.8 ± 0.9^{bc}	19.3 ± 1.5^{b}	5.9± 1.1^{a}	DH3G12-13	3.1 ± 0.4^{abc}	2.0 ± 0.2^{a}	5.1 ± 0.6^{b}	73.2 ± 0.9^{ab}	17.6 ± 0.7^{i}	5.3 ± 0.7^{cd}
DH3K12-4	3.8 ± 0.2^{ab}	2.4 ± 0.2^{b}	6.2 ± 0.4^{bcde}	72.8 ± 1.2^{ab}	16.6 ± 1.0^{a}	3.5 ± 0.3^{j}	DH3G12-16	3.2 ± 0.3^{4def}	1.6 ± 1.2^{a}	4.8 ± 0.^{5ab}	71.5 ± 1.2^{cd}	19.0 ± 0.8^{g}	3.5 ± 1.4^{efg}
DH3K12-5	3.7 ± 0.6^{ab}	2.0 ± 0.2^{b}	5.7 ± 0.8^{e}	73.2 ± 1.6^{a}	18.2± 0.9^{bc}	5.2± 0.6^{c}	DH3G12-18	3.6 ± 0.3^{ab}	2.0 ± 0.3^{a}	5.6 ± 0.6^{b}	73.3 ± 0.7^{ab}	21.0 ± 0.7^{a}	3.6 ± 0.7 ^{de}
DH3K12-12	3.5 ± 0.3^{ab}	2.6± 0.3^{ab}	6.1 ± 0.6^{abc}	71.5 ± 1.9^{bc}	17.7± 0.8^{de}	5.6± 0.3^{e}	DH3G12-19	3.6 ± 0.8^{ef}	2.1 ± 0.5^{a}	5.7 ± 1.3^{ab}	72.8 ± 0.7^{a}	19.8 ± 0.9^{de}	2.9 ± 0.1^{ef}
DH3K12-13	3.5 ± 0.3^{ab}	2.3 ± 0.2^{b}	5.8 ± 0.5^{bcde}	70.8 ± 1.8^{bc}	19.3± 0.9^{de}	4.5± 0.7^{e}	DH3G12-10	3.1 ± 0.3^{3cef}	1.6 ± 0.7^{a}	4.7 ± 1.0^{ab}	72.1 ±0.6 ^{bc}	18.2 ± 0.7^{f}	4.2± 0.7^{efgh}
DH3K12-14	3.9 ± 0.2^{a}	2.7± 0.4^{ab}	6.6 ± 0.6^{ab}	70.3 ± 1.0^{cd}	15.2 ± 1.1^{f}	4.6± .9^{hi}	DH3G8-3	3.5 ± 0.1^{ab}	2.0 ± 0.2^{a}	5.5 ± 0.3^{a}	73.1± 0.9^{abc}	20.3±0.02^{hi}	3.4±1.1^{efgh}
DH3K8-6	3.3 ± 0.2^{b}	2.1 ± 0.1^{b}	5.4 ± 0.3^{cde}	72.1 ± 1.1^{ab}	16.7 ± 0.6^{f}	4.7 ± 0.6^{i}	DH3G8-1	3.5 ± 0.7^{a}	2.0 ± 0.7^{a}	5.5 ± 1.4^{a}	72.0±0.2^{abc}	22.3 ± 0.7^{bc}	4.0 ± 0.9^{fgh}
DH3K8-7	3.7 ± 0.3^{ab}	2.7 ± 0.7^{b}	5.7 ± 1.0^{cde}	72.8 ± 1.7^{a}	15.2 ± 0.8^{f}	5.4± 0.7^{g}	DH3G8-2	2.9 ± 0.5^{ab}	2.0 ± 0.4^{a}	4.9 ± 0.9^{b}	73.1± 1.0b^{c}	21.5 ± 0.9^{de}	3.5 ± 0.4^{fgh}

Values tabulated are mean ± SD at three replications.
Values with different alphabetical superscripts are significantly different ($P \leq 0.05$) according to DMRT.
16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; DHnNn- doubled haploids, number of generation, name of cultivar, number of plant

Table 2: Vegetative parameters in *B. napus* cv. Kris, Galant and DH mutants (M2 and M3)

Traits	Parental plant (Kris)		After mutagen treatment (Kris)				Parental plant(Galant)		After mutagen-treated (Galant)			
	$\bar{X}$ ±S	CV	8 мМ EMS		12 мМ EMS		$\bar{X}$ ±S	CV	8 мМ EMS		12 мМ EMS	
			$\bar{X}$±S	CV	$\bar{X}$ ±S	CV			$\bar{X}$ ±S	CV	$\bar{X}$ ±S	CV
M2 mutants												
Plant height (cm)	136.4 ± 9.6	7%	116.2 ± 23.6	20%	96.8 ± 26.4	27%	132.5 ± 5.3	4%	117.0 ± 23.7	20%	94.7 ± 12.6	13%
Number of pods	114.4 ± 21.3	18%	160.0 ± 75.0	47%	184.6 ± 131.7	70%	137.5 ± 15.6	9%	167.8 ± 33.8	33%	201.3 ± 123.6	35%
Number of seeds in pods	15.0 ± 2.8	18%	18.0 ± 5.2	29%	21.0 ± 7.2	34%	17.2 ± 3.2	19%	17.9 ± 6.2	30%	19.8 ± 5.9	35%
1000-seed weight (g)	3.1 ± 0.9	29%	3.6 ± 0.8	22%	3.8 ± 1.3	34%	3.4 ± 0.6	18%	3.7 ± 1.6	39%	3.8 ± 1.5	43%
weight of seeds per plant (g)	6.5 ± 1.6	25%	7.1 ± 2.8	37%	11.6 ± 4.3	39%	6.3 ± 1.3	21%	6.8 ± 3.3	49%	10.3 ± 5.6	58%
M3 mutants												
Plant height (cm)	134.0 ± 13.0	10%	115.2 ± 4.2	4%	120.4 ± 12.3	10%	126.0 ± 3.3	3%	124.6 ± 4.4	12%	119.3 ± 12.1	10%
Number of pods	125.0 ± 30.6	24%	165.0 ± 38.4	23%	166.9 ± 32.9	20%	125.0 ± 10.3	8%	152.1 ± 31.9	21%	190.0 ± 36.9	19%
Number of seeds in pods	16.0 ± 3.2	20%	15.3 ± 6.3	40%	17.4 ± 6.4	41%	14.5 ± 2.1	14%	12.0 ± 4.5	37%	14.6 ± 8.6	58%
1000-seed weight (g)	3.1 ± 0.9	29%	3.7 ± 1.3	25%	3.9 ± 0.5	35%	3.3 ± 0.8	24%	3.6 ± 0.6	16%	3.8 ± 0.5	13%
weight of seeds per plant (g)	5.9 ± 1.2	20%	6.8 ± 2.3	34%	9.2 ± 4.8	52%	6.3 ± 1.7	27%	4.2 ± 2.3	37%	9.0 ± 3.3	54%

Each meaning represents the mean value ± standard deviation; CV - Coefficient of variation

seeds was 3.3 (Galant) and 3.1 g (Kris). In the M2 mutants of «Kris», the seed weight per plant varied from 5.3 to 17.4 g, and the weight of 1000 seeds varied from 3.7 to 4.7 g. The weight of seeds per plant in the M2 mutants of «Galant» ranged from 5.4 to 20 g and the weight of 1000 seeds ranged from 3.8 to 4.4 g. According to the characteristics of the weight of seeds per plant and the weight of 1000 seeds, three mutants of the Galant cultivar (DH2G12-1, DH2G12-3, DH2G12-10) and five mutant lines of the Kris cultivar (DH2K12-3, DH2K12-7, DH2K12-8, DH2K8-2, DH2K8-5) were distinguished. The results of morphological traits showed that the M3 mutants retained all the quantitative traits that M2 had.

One mutant line from «Kris», DH2K8-7, combined the best indicators of the yield with reduced linolenic acid content.

Table 3: Yield attributes and seed color of *B. napus* cv. Kris and Galant and DH mutants (M2 and M3)

Name of doubled haploid line	weight of seeds from the 1st plant	1000-seed weight	Color of seeds	Name of doubled haploid line	weight of seeds from the 1st plant	1000-seed weight	Color of seeds
Kris	5.8 ± 1.2^{i}	3.1 ± 0.4^{g}	b	Galant	5.3 ± 0.9^{m}	3.3 ± 0.3^{defg}	b
M2 mutants							
DH2K12-3	17.4 ± 1.2^{a}	4.0 ± 0.3^{bcdef}	b	DH2G12-1	20.0 ± 1.5^{a}	4.3 ± 0.2^{bc}	b
DH2K12-4	8.4 ± 0.9^{de}	4.4 ± 0.5^{abcd}	br	DH2G12-3	9.2 ± 0.6^{ef}	4.4 ± 0.5^{bc}	br
DH2K12-5	5.3 ± 0.8^{ij}	3.8 ± 0.2^{cdefg}	y.br	DH2G12-7	6.3 ± 0.9^{ijk}	3.9 ± 0.5^{bcde}	br
DH2K12-6	13.3 ± 1.1^{b}	3.8 ± 0.3^{cdefg}	b	DH2G12-8	6.1 ± 0.8^{jkl}	3.8 ± 0.5^{bcdef}	br
DH2K12-7	9.1 ± 1.2^{d}	4.5 ± 0.4^{abc}	b	DH2G12-9	12.1 ± 1.2^{c}	3.8 ± 0.4^{bcdef}	br
DH2K12-8	11.1 ± 1.2^{c}	4.2 ± 0.3^{abcd}	br	DH2G12-10	17.4 ± 1.3^{b}	4.4 ± 0.1^{b}	b
DH2K12-13	11.0 ± 1.1^{c}	3.7 ± 0.2^{defg}	b	DH2G12-15	9.5 ± 0.9^{e}	3.5 ± 0.5^{defg}	y.br
DH2K12-14	6.2 ± 0.9^{hi}	4.9 ± 0.2^{a}	b	DH2G12-16	10.4 ± 0.7^{d}	3.8 ± 0.6^{bcdef}	b
DH2K8-1	8.3 ± 0.3^{def}	4.3 ± 0.4^{abcd}	br	DH2G12-18	5.4 ± 0.7^{lm}	5.4 ± 0.3^{a}	y.br
DH2K8-2	13.4 ± 0.7^{b}	4.7 ± 0.2^{ab}	b	DH2G8-3	5.9 ± 0.7^{ijk}	4.0 ± 1.0^{bcd}	br
DH2K8-5	13.1 ± 0.8^{b}	4.0 ± 0.2^{bcdef}	br	DH2G8-2	10.3 ± 1.1^{d}	3.9 ± 0.3^{bcde}	br
DH2K8-7	7.0 ± 0.7^{efg}	4.4 ± 0.2^{abcd}	b	DH2G8-4	5.5 ± 0.8^{klm}	3.4 ± 0.6^{defg}	y.br
M3 mutants							
DH3K12-3	15.2 ± 0.9^{a}	3.8 ± 0.3^{cdef}	b	DH3G12-1	18.9 ± 1.6^{a}	3.9 ± 0.7^{bc}	b
DH3K12-4	8.6 ± 0.3^{de}	3.9 ± 0.7^{abc}	br	DH3G12-3	10.4 ± 0.9^{ef}	3.9 ± 0.8^{bc}	br
DH3K12-5	7.3 ± 0.5^{ij}	3.9 ± 0.1^{cde}	y.br	DH3G12-7	7.8 ± 0.9^{ijk}	4.1 ± 0.6^{bcde}	br
DH3K12-6	10.1 ± 0.5^{b}	3.6 ± 0.7^{def}	b	DH3G12-8	6.7 ± 0.8^{jkl}	3.8 ± 0.7^{bcdef}	br
DH3K12-7	7.5 ± 1.1^{d}	4.1 ± 0.7^{ab}	b	DH3G12-9	10.8 ± 1.0^{c}	3.7 ± 0.9^{bcde}	br
DH3K12-8	9.9 ± 1.0^{c}	4.0 ± 0.8^{bcd}	br	DH3G12-10	16.5 ± 0.9^{b}	4.1 ± 0.9^{b}	b
DH3K12-13	10.7 ± 0.5^{c}	3.9 ± 0.9^{def}	b	DH3G12-15	8.6 ± 0.9^{e}	3.7 ± 0.8^{defg}	y.br
DH3K12-14	7.6 ± 0.3^{hi}	4.1 ± 0.8^{ab}	b	DH3G12-16	9.9 ± 0.8^{d}	3.6 ± 0.8^{def}	b
DH3K8-1	8.2 ± 0.7^{def}	3.6 ± 0.7^{abc}	br	DH3G12-18	7.2 ± 1.0^{lm}	4.3 ± 0.7^{a}	y.br
DH3K8-2	11.7 ± 1.3^{b}	4.6 ± 0.7^{abc}	b	DH3G8-3	6.0 ± 0.9^{ijk}	4.0 ± 0.3^{bcd}	br
DH3K8-5	9.7 ± 0.6^{b}	4.1 ± 0.7^{bcdef}	br.	DH3G8-2	7.8 ± 0.7^{d}	3.8 ± 0.4^{bcd}	br
DH3K8-7	8.2 ± 0.6^{efg}	3.8 ± 0.7^{bc}	b	DH3G8-4	7.6 ± 0.7^{klm}	3.7 ± 0.9^{de}	y.br

Values tabulated are mean ± SD at three replications.
Values with different alphabetical superscripts are significantly different ($P \leq 0.05$) according to DMRT.
DHnNn- doubled haploids, number of generations, name of cultivar, number of plants
b-black, br – brown, y.br –yellow brown

Fig. 2: Seeds of original *B. napus* cvs. Kris and Galant and their mutant progeny. Seeds of *B. napus* cvs. Kris (**a1**) and Galant (**b1**), mutant seeds of DH2K12-4 (**a2**) and DH2K12-4 (**a3**); mutant seeds of DH2G12-8 (**b2**) and DH2G12-15 (**b3**)

In addition, a change in seed color was observed in the mutant lines — from yellow brown to black (Fig. 2 and Table 3) — which was maintained over two generations (M2 and M3). The original cultivars had black seeds.

Cold-tolerance screening

DH mutants and the original plants were moved to low-temperature conditions (4, 0 and -4°C) for 12 h (Fig. 3). All of the tested DH mutants and original plants had no obvious morphological changes at a temperature of 4°C. However, the parental plants began to die when treatment occurred under 0°C and -4°C. The DH mutant plants under the same conditions showed wilting leaves, which recovered within 21 days. With a decrease in temperature, the damage to the plants was higher. At a temperature of 0°C, the dehydration of leaves and stems was observed and the edges of the leaves were twisted. The survival rate of original

Fig. 3: Morphological changes in parental cultivars and DH mutants of rapeseed seedlings (Kris and Galant) after 21 days at 22°C and cold stress. **A**, **D**, **G**, **J** – parental plants after 21 days at 22°C (CK), for 6 hours of treatment at 4°C, 0 and -4°C, respectively; **B**, **E**, **H**, **K** and **C**, **F**, **I**, **L** mutant lines DH3G12-10 and DH3K12-8, after 21 days at 22°C (CK), for 6 hours of treatment at 4° C, 0 and -4°C, respectively; B, E, H, K and C, F, I, L mutant lines DH3G12-10 and DH3K12-8, after 21 days at 22°C (CK), 2 h of treatment at 4°C, 0 and -4°C, respectively. Bar, 5 cm

plants at 0°C was 7 and 5% (Galant and Kris), while the viability of mutants was 45 and 43% (Galant and Kris). Under the influence of a temperature of -4°C, the leaves and stems of the original plants were severely dehydrated and showed zero survival, while the mutants that were obtained by treatment with EMS mutagen at a concentration of 12 *mM* showed 49% viability at low temperatures (Fig. 4). Of the 46 analyzed mutant lines, which had different levels of survival, higher viability was shown in the mutant lines DHG12-10, DHK12-8, DHK12-3, DHK12-4, DHG12-16 and DHG12-18, which were derived from treatment with the EMS mutagen at a concentration of 12 *mM* (Fig. 4). As shown in Fig. 3, all of the mutant lines showed high significance compared to the original plants ($P \leq 0.05$) at low temperatures (4, 0 and -4).

Discussion

EMS mutagenesis is an effective approach to create mutations in the genes of polyploidy species such as *B. napus*. Moreover, EMS is the most common chemical mutagen that is used in the culture of isolated microspores

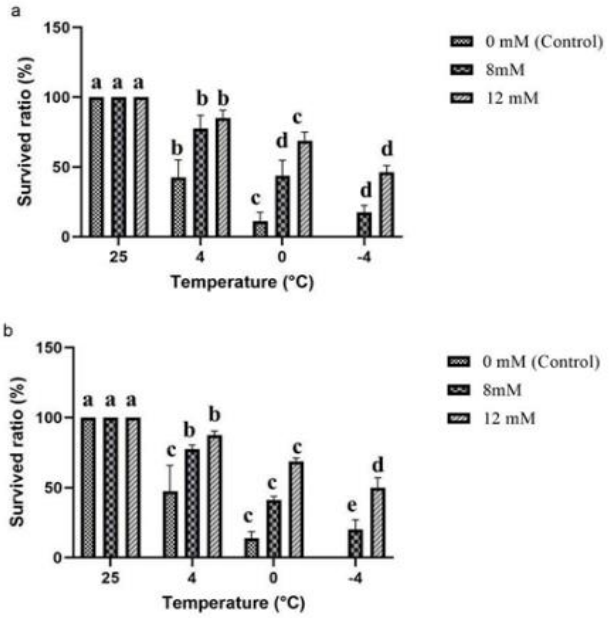

Fig. 4: Effect of the EMS mutagen concentration on survival of DH mutant lines in cold-stress. (**a**) DH mutant lines of *B. napus* cv. Kris and (**b**) DH mutant lines of *B. napus* cv. Galant
Values with different letters are significantly different ($P \leq 0.05$) according to DMR

(Lu *et al.* 2016). In this study, we studied mutants that were derived earlier by us during the treatment of secondary embryos obtained in the culture of isolated microspores (Daurova *et al.* 2020). Secondary embryos or embryonic calluses have been successfully used to produce mutant doubled haploid rapeseed plants resistant to *Sclerotinia sclerotiorum* (Liu *et al.* 2005) and *Leptosphaeria maculans* (Desm.) (Newsholme *et al.* 1989).

Most researchers mutagenized seeds to obtain rapeseed mutants with improved qualitative and quantitative traits (Shan *et al.* 2020). However, it may take a long time to assess and confirm the changes made in quantitative and qualitative traits (up to 4–5 years), while homozygous doubled haploid mutants allow the selection of plants with the desired traits at the M2 generation (Huang *et al.* 2016). EMS mutagenesis can induce genetic changes in plants and modify the levels of fatty acids in seed oil (Amosova *et al.* 2019). In this study results confirmed that the mutagenesis of haploid embryos leads to mutant homozygous lines with improved fatty acid composition. In particular, vegetable oils with a high content of oleic acid are relevant for a healthy diet (Bowen *et al.* 2019). The fatty acid composition of the doubled haploid seeds obtained by us had a high content of unsaturated (oleic, linoleic, linolenic) and a decrease in the amount of low saturated fatty acids (palmitic and stearic) was observed, in addition, the content of erucic acid was not higher than 0.05%.

In previously published studies, high concentrations of the EMS mutagen were reported to affect quantitative traits, such as plant height (Kumar and Yadav 2010), 1000-

seed weight, and seed weight per plant (Ali and Shah 2013). In our study, EMS mutagenesis induced morphological and agronomic changes among DH mutants of M2 and M3 generations. As a result, it was possible to successfully select mutants showing distinct differences in morphological and agronomic characteristics. Moreover, the results showed that some mutant lines differed in the color of the seeds, which had brown and light-brown shades. *B. napus* with yellow seeds has a number of advantages: high oil content, higher protein content, and low fiber content. Although rapeseed with yellow seeds is not found in nature, there are a number of studies aimed at creating hybrids and mutants of rapeseed with yellow seeds (Facciotti 2003; Rahman *et al.* 2019). In addition, the following undersized lines stood out: DHK12-3, DHK12-4, DHK12-5, DHG12-7 and DHG12-16. Compared with the parent cultivars, these mutants had plant heights ranging from 93 cm to 115 cm, while the control had 129 cm. Stunting of plants (dwarfism) is accompanied by an increase in yield due to a decrease in lodging and an increase in the yield index (More and Malode 2016). In our study, the stunted rapeseed mutants showed high yields compared to the parental plants. Previously, it was found that the best results of mutagenesis were observed when the embryos obtained in the culture of isolated microspores were treated with high concentrations (Rahman *et al.* 2013). The results of our studies confirmed that at a high concentration of the mutagen (12 *mM*), an increase in the weight of 1000 seeds occurs, which was also shown in another work (Channaoui *et al.* 2019).

Low temperature is serious stress that negatively affects the growth and development of plants, reducing yields (Xin and Browse 2000). Cold resistance is an important characteristic of rapeseed, which is sown in the northern regions. Based on previous studies (Fiebelkorn and Rahman 2016), we evaluated 46 mutant doubled haploid rapeseed lines for cold resistance, in comparison with the original varieties at temperatures of 4°C, 0°C and -4°C. Similar work was carried out in the study of McClinchey and Kot (2008), where it was reported that mutant doubled haploids showed increased resistance to cold at -6°C, without noticeable phenotypic changes. In our study, we used the viability index under low-temperature stress as a criterion for identifying cold-resistant mutants. Our data are consistent with the results of previous studies, where the viability of seedlings was evaluated at chilling (4 and 2°C) and freezing (-2 and -4°C) (Lei *et al.* 2019).

Our results demonstrate that EMS treatment of microspores is an efficient procedure to generate mutations resulting in highly diverse phenotypes of rapeseed. Microspore mutagenesis is a rapid approach for creating the homozygous mutants, which can be screened in M_1 generation and can accelerate the creation of new cultivars. Moreover, this approach identifies mutant lines that combine improved quantitative and qualitative traits and cold resistance.

Conclusion

High efficiency of mutagenesis in the culture of embryos obtained from isolated microspores in the selection of spring rapeseed. Mutant lines were identified that combined improved quantitative and qualitative traits and resistance to cold (DHK12-3, DHK12-4, DHK12-8, DHG12-16, DHG12-18, and DHG12-10). In addition, mutant-line DHK12-4 had brown seeds, whereas DHG12-18 was yellow-brown. Overall mutagenesis in the isolated microspore culture expands the genetic diversity of the initial material and is a good tool for the practical breeding of rapeseed. The promising lines obtained as a result of the experiments will be disseminated to breeders for breeding domestic cultivars of spring rapeseed (canola) adapted for cultivation in the northern regions of Kazakhstan.

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Author Contributions

Kabyl Zhambakin and Malika Shamekova: Conceptualization; Kuanysh Zhapar, Zagipa Sapakhova and Dias Daurov: Methodology, Software; Ainash Daurova and Kabyl Zhambakin: Data curation, Writing- Original draft preparation; Kabyl Zhambakin: Supervision; Ainash Daurova, and Kabyl Zhambakin: Writing- Reviewing and Editing.

Conflict of Interest

The authors declare that this work was carried out without any commitments that could result in a potential conflict of interest.

Data Availability

All new research results were presented in this article.

Ethics Approval

Not applicable.

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