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PRODUCTION MUTANT LINES OF TURNIP RAPE (*BRASSICA RAPA*) AND ITS INTERSPECIFIC HYBRIDS IN THE ISOLATED MICROSPORE CULTURE

This article presents the results of research on the production of mutant doubled haploids of turnip rape (Brassica rapa L.ssp.Oleifera), as well as its interspecies hybrids with rapeseed (Brassica napus). An immature embryo culture was used to increase the productivity of inter-specific hybridization. Studies have obtained interspecific hybrids of turnip rape with rapeseed. The largest number of plants was observed in hybrids where the mother plant was rapeseed. Furthermore, chemical mutagenesis was used to increase the genetic diversity of studied materials. Ethyl methanesulfonate (EMS) has been applied to the treatment of embryos produced in the isolated microspores culture of turnip rape and its interspecies hybrids. Results of embryo regeneration against mutagen on a nutrient medium B5 with gibberellic acid showed that the concentration of 8 mM EMS stimulated the regeneration process in turnip rape cultivars (Zolotistaya – 72% and Yantarnaya – 88%) and showed superior results compared to a low concentration (4 mM). For hybrids compared to high concentration (8mM), a low concentration of 4mM EMS showed superior results. New mutant lines have been obtained as the starting material for selection in the creation of new domestic canola cultivars.

Key words: turnip rape, rapeseed, interspecific hybridization, mutagenesis, isolated microspore culture.

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Оқшауланған микроспора дақылында қышабас (Brassica rapa) және оның түраралық будандарының мутантты линияларын алу

Бұл зерттеуде қышабастың (Brassica rapa L.ssp.Oleifera), және де оның рапс (Brassica napus) өсімдігімен түраралық будандарының екі еселенген мутанттарын алу барысындағы нәтижелер көрсетілген. Түраралық будандардың өнімділігін арттыру үшін оқшауланған жетілмеген ұрық дақылы қолданылды. Зерттеу нәтижесінде қышабас пен рапстың түраралық будандары алынды. Алынған нәтижелер көрсеткендей, аналық форма ретінде рапс өсімдігі болған қиыстыруларда, будандардың саны айтарлықтай көп болды. Сонымен қатар, зерттелілген өсімдік материалдардың генетикалық түрлілігін арттыру мақсатында химиялық мутагенез әдісі қолданылды. Қышабас және оның рапс өсімдігімен түраралық будандарының оқшауланған микроспора дақылында алынған эмбриодтарды өңдеу үшін Этиленметилсульфонат (EMS) мутагені пайдаланылды. Мутагенмен өңделген эмбриодтардың гибберелин қышқылы қосылған B5 қоректік ортасындағы регенерация үрдісі, EMS мутагенінің 8 мМ концентрациясы, қышабас сорттарының регенерациясын (Золотистая – 72% и Янтарная- 88%) арттырып, төмен концентрацияға (4 мМ) қарағанда жоғары нәтиже көрсеткені анықталды. Түраралық будандар үшін, жоғары концентрациямен (8мМ) салыстырғанда, EMS мутагенінің 4мМ төмен концентрациясы жақсы нәтиже көрсеткені анықталды. Каноланың отандық сорттарын жасау мақсатында, селекция үшін бастапқы материал болатын жаңа мутантты линиялары алынған.

Түйін сөздер: қышабас, рапс, алшақ будандастыру, мутагенез, оқшауланған микросппора дақылы.

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Получение мутантных линий сурепицы (Brassica rapa) и ее отдаленных гибридов в культуре изолированных микроспор

В данной статье представлены результаты исследования по получению мутантных удвоенных гаплоидов сурепицы (Brassica rapa L.ssp.Oleifera), а также ее межвидовых гибридов с рапсом (Brassica napus). Для увеличения продуктивности межвидовой гибридизации была использована культура изолированных незрелых зародышей. В результате исследований были получены межвидовые гибриды сурепицы с рапсом. Наибольшее количество полученных растений наблюдалось у гибридов, где материнской формой являлся рапс. Более того с целью увеличения генетического разнообразия изучаемого материала использовался химический мутагенез. Этиленметилсульфонат (EMS) был применен для обработки эмбриодов полученных в культуре изолированных микроспор сурепицы и межвидовых гибридов сурепицы с рапсом. Результаты регенерации эмбриоидов на фоне мутагена на питательной среде В5 с гиббереллловой кислотой показали, что концентрация в 8 мМ EMS стимулировала процесс регенерации у сортов сурепицы (Золотистая – 72% и Янтарная- 88%), и показала лучший результат по сравнению с низкой концентрацией (4 мМ). Для гибридов по сравнению с высокой концентрацией (8мМ), низкая концентрация 4мМ EMS показала лучший результат. Получены новые мутантные линии, как исходный материал для селекции в создании новых отечественных сортов канолы.

Ключевые слова: сурепица, рапс, отдаленная гибридизация, мутагенез, культура изолированных микроспор.

Introduction

Canola is the trade name of rapeseed (*Brassica napus*) and turnip rape (*Brassica rapa L. ssp. Oleifera*) cultivars which seed oil is used for food purposes. In the last decade, a demand on canola oil from rapeseed (*Brassica napus*) and turnip rape (*Brassica rapa L. ssp. Oleifera*) raised dramatically worldwide. In Kazakhstan, where almost all produced rapeseed have been exclusively exported, there is an increasing trend for internal consumption.

Turnip rape has the AA genome where haploid number of chromosomes is 10 (n = 10), unlike rapeseed, which genome AACC (n=19). Turnip rape has shorter growing season and it is more productive in harsh climates and poor soil. We consider that the demand on turnip rape among farmers will rise in nearest future due to climate change, since there are more dry years in Kazakhstan now.

Interest in turnip rape in different countries is definitely linked to its resistance to abiotic environmental stress factors compared to rapeseed [1]. At the same time, turnip rape yield is significantly lower compared to rapeseed in good climatic conditions. As a solution for this issue, hybrids and/or synthetic hybrids may be created. In this case, a significant improvement in yield can be achieved [2]. In order to get the highest productivity (heterosis) in hybrids and synthetic hybrids, homozygous lines are used as parents, since such lines are more convenient for studying their combining ability [3]. Therefore, one of the most important steps in breeding is a quick production of homozygous lines with valuable qualitative and quantitative traits and resistance to stress factors.

However, conventional oilseed crop breeding is a labor-intensive and very long process. In addition, creating homozygous lines also requires long time for self-pollination. The most effective method for that is the method of isolated microspore culture.

Isolated microspore culture has been used for a while to produce doubled haploids in Brassica species. However, despite widespread application of this method in breeding, its application in obtaining doubled haploids requires certain improvements [4], especially for turnip rape, as its embryogenesis depends strongly on plant genotype [5].

In addition to increasing demand on canola oil, special attention is paid to its quality. Canola oil is considered to be one of the healthiest vegetable oils [6]. Particularly, cultivars with high oleic acid content are the most valuable ones [7]. Therefore, developing cultivars with this particular trait is a perspective direction in oilseed crop breeding. One

of the ways to increase its efficacy is expanding genetic diversity of the plant material. This can be achieved using mutagenesis.

The efficacy of mutagenesis can be significantly increased using cell culture. Furthermore, isolated microspore culture increases it even more due to identical chromosome set. As a result, recessive mutations are not hidden by the dominance effect. Various mutagens are used to treat anthers and microspores in the culture. EMS is the most common chemical mutagenesis, however, sodium azide (NaN3) [8] and gamma UV radiation [9] are used instead in many studies. However, EMS is considered the most effective and convenient chemical mutagen [10]. At the same time, chemical mutagenesis in isolated microspore culture may lower embryogenesis rate, thereby causing difficulties in growing plants from embryos. For that reason, researchers [11] used secondary callus embryos, as well as embryo callus to clone embryogenic material obtained in isolated microspore culture.

The aim of this work was to use the culture of isolated microspores to obtain homozygous mutant doubled haploids of turnip rape and its interspecific hybrids.

Materials and Methods

The research materials were cultivars and lines of turnip rape (*Brassica rapa*) and rapeseed (*Brassica napus*).

Method of *Brassica* hybridization.

Castration is performed on buds of 3-4 mm and an insulator close to the readiness of the pistil for pollination. Then pistil pollinated by paternal form pollen and close the insulator onto which the date of castration and pollination.

Extracting immature germs of *Brassica* hybrids and growing to plants *in vitro*.

Pods obtained at day 15-17 after hybridization are sterilized in 50% sodium hypochlorite solution for 10 min, in 70% alcohol for 3-5 sec and washed in distilled water. After sterilization, the germs are extracted under laminar flow and transferred in MS medium with 1 mg/l of kinetin, IAA 0.1 mg/l, gibberellic acid 1 mg/l, casein hydrolysate 10 mg/l, pH 5.8. 10-15 days later, regenerants derived from the germs are transferred in MS medium with half of the salt content, no hormones and grown until complete formation of roots and shoots.

Isolation of a microspore culture.

Buds (2-3 mm in size) were collected in field conditions early in the morning at the single-nucleus microspore stage during hours of intense pollen division. The pretreatment of buds was carried out in a 10 mg/l silver nitrate solution at + 4°C for 2 days. Subsequently, the buds were sterilised with 5% sodium hypochlorite for 7-10 min and 70% alcohol for 3-5 sec, followed by three washes with distilled water. The buds were then placed in a cool micromixer (10°C) using 30-40 ml of cooled B5 medium [12] without hormones (10-12°C) and homogenised for 7-9 sec at high speed. The resulting suspension was passed through a filter (80 μm), and the filtrate was centrifuged (Eppendorf, Germany) at 100 g for 5 min. The supernatant was decanted, and 15 ml of the B5 medium were added to the precipitate, followed by centrifugation for 5 min. After repeating the previous step, the precipitate was poured into Petri dishes. Subsequently, NLN medium with 0.05 mg/l benzylaminopurine (BA) was added for microspore cultivation. The concentration of microspores in the NLN medium was adjusted to 35,000-50,000 microspores per ml. Petri dishes were placed in a temperature controller with a shaker at 25ºC. As soon as torpedo-like embryoids appeared, the Petri dishes were exposed to light at the same temperature [13]. Embryoids derived from the isolated microspore culture were transplanted onto Gamborg B5 solid nutrient medium supplemented with 1 mg/l gibberellic acid. As regeneration proceeded, the plants were transplanted onto Murashige-Skoog hormone-free medium with half salt composition, one plant per tube, and placed into a room with controlled light and temperature.

Mutagen treatment of somatic embryoids.

Upon reaching 1.5–2.5 mm in size, the embryoids were treated with EMS (Sigma Aldrich, US) mutagen. EMS was added to the Petri dishes in three concentrations: 4 mM, 8 mM, and 12 mM. The dishes were then placed on a shaker (40–50 rpm) in a temperature controller (TSO-1/80-SPU, Russian) at 25°C for 1 h. After the treatment, the embryoids were dried on a sterile paper sheet for 5 seconds. Thereafter, they were transplanted onto solid B5 medium with 0.8% agar and 2% sucrose and incubated for 24 hours in a thermostat at 10°C. After the incubation, the tubes with embryoids were placed under light at 25°C. Following two weeks of cultivation, the embryoids were transplanted onto fresh B5 medium for regeneration.

Colchicine treatment.

After *in vitro* regenerants 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 kept in storage. The clones were grown up to the five-leaf stage and treated with 0.05

% colchicine (AppliChem, Germany) at 4°C for 16 h. Treated plantlets were then washed three times with distilled water. Next, they were planted into soil and grown to obtain seeds of doubled haploid plants [14].

Results and Discussion

Turnip rape is more resistant to abiotic stresses than rapeseed [1]. However, Kazakhstan does not have its own cultivars of turnip rape [15] and grows it on small areas, apparently due to low yields compared to rapeseed. Therefore, there is a need to create cultivars adapted to local soil and climatic conditions. During the period of our previous studies (2018-2021), mutant and hybrid turnip rape lines were obtained, which were distinguished by qualitative and quantitative characteristics [16].

Thus, the working collection of turnip rape was selected according to various useful signs corresponding to the food direction (the content of erucic acid and glucosinolates), high yield and seed quality. The collection consisted of cultivars of Russian (2 cultivars) and German/Germanic selection (5 cultivars), as well as lines of doubled haploids obtained by us from cultivars of Russian selection. The seeds of the Russian and German/German breeding had yellow seeds (Figure 1 A and B). The seeds of the doubled haploids of interspecific hybrids differed in black, brown and light brown colors of seeds (Figure 1 C, D and E). Cultivars with yellow seeds have a number of advantages due to a higher percentage of oil in them, a higher protein content and a low fiber content [16]. There are a number of studies aimed at creating hybrids and mutants of the *Brassica* family with yellow seeds [17].

A) seeds of the Zolotistaya cultivar; B) seeds of mutant DHZ4-1 lines; C) D) E) seeds of doubled haploids
Figure 1 – Seeds of collectible cultivars and lines

The main way to obtain a new source material is hybridization (intervarietal and interspecific). Using methods of interspecific hybridization of the *Brassica* family, high-oleic lines were obtained, as well as lines resistant to environmental stress factors [18;19;20]. In our experiment, a culture of isolated embryos was used to increase the productivity of interspecific hybridization.

The initial material for hybridization was the lines of doubled haploids (DH) of the food direction of the turnip rape (*Brassica campestris*). All DH lines are homozygous and have a low content of erucic acid and glucosinolates corresponding to the nutritional direction, as well as high yield and good seed quality.

The selection of combinations for hybridization was determined by qualitative and quantitative characteristics. In our experiments, 524 immature embryos from ten combinations were introduced into the culture *in vitro*. Not all immature embryos germinated into plants, the largest number of plants obtained was observed in hybrids where rapeseed was the maternal form (Galant x Yantarnaya and Kris x Zolotistaya). In total, we obtained 205 plants *in vitro* from eight hybrid combinations (Table 2). The resulting plants were cloned 3 clones of each line, for further transplantation into the ground (Figure 2). Despite the fact that visually hybrid plants grew and developed normally, not all of them turned out to be fertile.

In our study, chemical mutagenesis was used in the isolated microspores culture in order to expand the genetic variability of the studied genotypes. EMS is a widely used chemical mutagen that is used to generate important recessive and dominant genomic mutations at a high rate. This creates the basis for the selection of useful genetic variations necessary for plant breeding [21]. The use of EMS is widely practiced in the *Brassicaceae* family as a mutagen.

Table 1 – Obtaining plants *in vitro* from interspecific hybrid immature embryos

Name of hybrid combinations	Number of pollinated plants	Number of castrated buds	Number of immature embryos planted	The number of plants obtained *in vitro*
Galant x Yantarnaya	20	100	180	91
Kris x Zolotistaya	20	100	76	38
Zolotistaya x Yantarnaya	20	80	71	33
Yantarnaya x Zolotistaya	20	80	55	26
Galant x CR 307	20	100	45	12
Kris x CR 307	20	100	51	5
Zolotistaya x CR 2186	20	80	21	-
Yantarnaya x CR 2186	20	80	25	-
Total	160	720	524	205

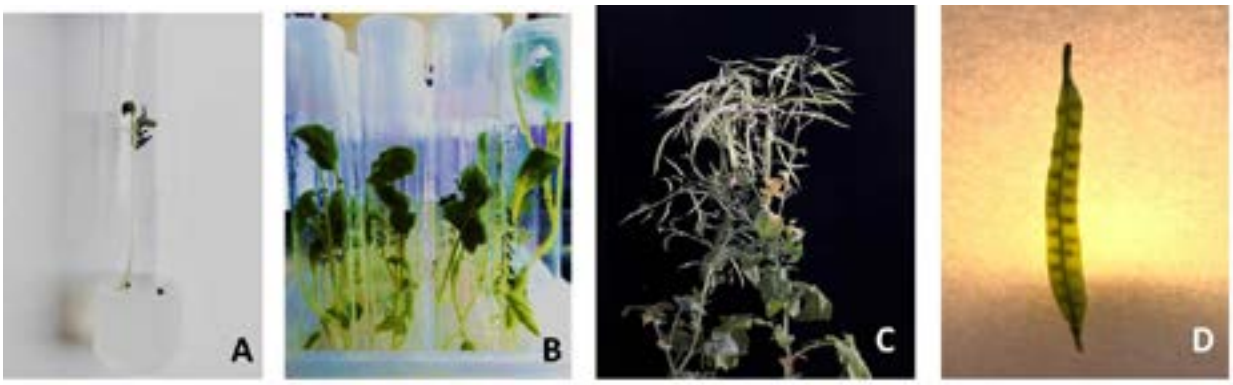

Figure 2 – Sequential cultivation of immature embryos to produce interspecific hybrids of rapeseed with turnip rape. (A) germinated germs; (B) cloned hybrid plantlets; (C) fertile hybrid plant; (D) pod with seeds

To obtain mutant plants, secondary embryos of interspecific hybrids and cultivars of turnip rape were treated with the EMS mutagen, which were obtained in the isolated microspores culture (Figure 2). The concentration and time of treatment of embryos with the EMS mutagen was established by us in previous studies [22]. A week after the mutagen treatment, changes in color were noticed on the embryos. Such processes were mainly observed during processing with a concentration of 8mM EMS. After 14 days, some embryos acquired a green color and then regenerated. At the same time, in some cases, darkening of embryos was observed, which later died. The results of regeneration of embryos against the background of mutagen on a nutrient medium in 5 with gibberellic acid showed that a concentration of 8 mM EMS stimulated the regeneration process in the cultivars of turnip rape (Zolotistaya – 72% and Yantarnaya- 88%), and showed a better result compared to a low concentration (4 mM) (Figure 4). The results obtained during these studies are consistent with the data M. Tavakoli and m. E. Shariatpanahi [23] and He et al. [24], that the average mutagen concentration promotes the regeneration of embryos and embryonic callus. At the same time, for hybrids, compared with a high concentration (8 mM), a low concentration of 4mM EMS showed the best result (47.2% for isGxY and 64.8% for isKxZ). As is known, mutagenesis in the isolated microspores culture educes embryogenesis and regeneration of plants [25; 26]. Our results confirmed this phenomenon, since at high concentrations; the results of regenerations of secondary embryos in hybrids were lower than in cultivars.

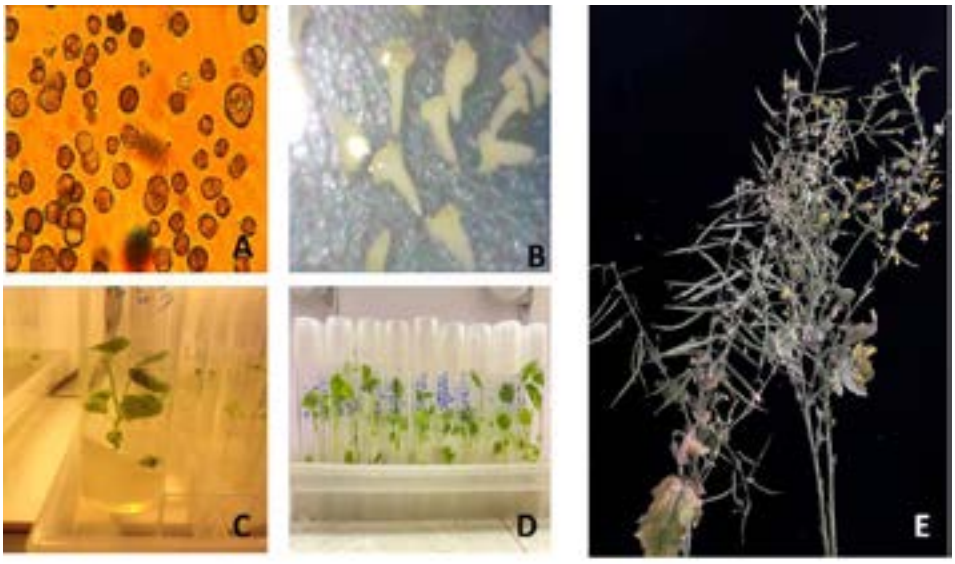

Figure 3 – Production of doubled haploid mutants of B.rapa and their hybrids in isolated microspores culture and regeneration of plants from embryos treated with EMS mutagen. (A) The process of formation of embryos from microspores and (B) mutagen treatment; (C) (D) the resulting mutant plantlets; (E) mutant fertile plant

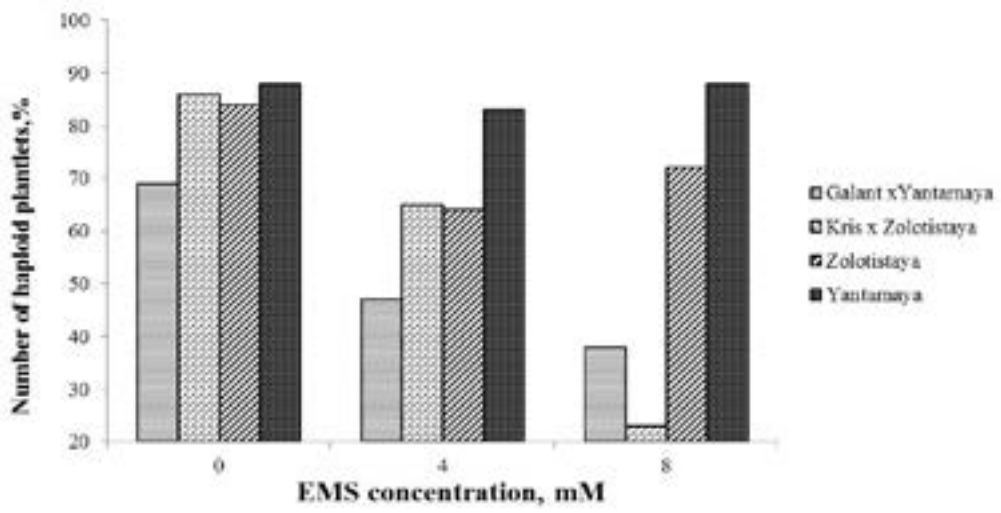

Figure 4 – Number of haploid plantlets from embryos

The production of fertile plants occurred due to the treatment of haploid plantlets with 0.05 % aqueous solution of colchicine. As a result, M1 seeds of turnip rape cultivars and their interspecific hybrids were obtained under controlled conditions (Table 2). Table 2 shows the lines with the highest seed productivity. Chemical mutagen treatment has a certain effect on plant fertility [27]. As a result of our research, some mutant turnip rape lines and their interspecific hybrids turned out to be sterile when treated with the EMS mutagen at a concentration of 8 mM EMS, while at 4 mM EMS all plants were fertile.

Table 2 – The obtained seed material of mutant lines (M1) from the culture of androgen embryos against the background of mutagen

Mutant lines of turnip rape and interspecific hybrids		EMS Mutagen Concentration (mM)	Weight of seeds from one plant, g.
Galant x Yantarnaya	DHGY	0	5,1±0.2
	MDHGY-2	4	4,3±0.8
	MDHGY-2	8	2,5±1.0
Kris x Zolotistaya	DHKZ	0	4,8±0.3
	MDHKZ-1	4	4,0±0.9
	MDHKZ-2	8	3,1±0.8
Yantarnaya	DHY	0	3,8±0.3
	MDHY-1	4	3.1±0.4
	MDHY-1	8	3,9±0.3
Zolotistaya (сурепица)	DHZ	0	3,5±0.3
	MDHZ-2	4	3,6±0,2
	MDHZ-1	8	4,2±0.4

Note: DHNC- n , doubled haploid name of cultivars – number of line; MDHNC- n, mutant doubled haploid name of cultivars – number of line.

Conclusion

The conducted studies have shown that the use of culture of isolated embryos significantly increases the efficiency of obtaining interspecific hybrids of turnip rape and rapeseed. The use of *in vitro* mutagenesis at the level of androgen embryoids is very convenient for the creation of new lines in the *Brassicaceae* family. It has been shown that the ethylmethanesulfonate (EMS) mutagen during the treatment of androgenic embryoids for one hour significantly affects the regeneration of the embryoids of the turnip rape and its hybrids with rapeseed. Studies have shown that the chemical mutagen EMS can be effectively used to produce fertile lines. The obtained new mutant lines will be further studied by qualitative and quantitative characteristics to determine their breeding value.

Conflict of interest

The author declares no conflicts of interest.

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