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INDUCED GENETIC VARIABILITY UNDER ETHYL METHANESULFONATE TREATMENT OF IMMATURE EMBRYOS AND MATURE SEEDS

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It was established that the use of immature embryos as the object for mutagenic treatment resulted in the appearance in M_2 of high frequency heritable changes, which amounted to 15.4% for ZL-809 line and varied from 34.1 to 39.9% for ZL-95 line. The frequency of mutations in the case of treating immature embryos was significantly higher than that for mature seeds, where from 8.7 to 9.6% of mutations were observed. Treating mature seeds with ethyl methanesulfonate mutagen produced significantly fewer types of mutations for each line than treating immature embryos

Key words: sunflower, induced mutagenesis, immature embryo, mature seed, M_2 generation, ethyl methanesulfonate, mutation frequency, mutation spectrum.

Introduction. Sunflower is one of the most important commercial crops in the world. In 2013 the acreage under sunflower in the Ukraine only amounted to more than 5 million hectares. However, the available genetic variability of sunflower (*Helianthus annuus* L.) does not currently satisfy the requests of modern breeding. In this regard constant search for ways to increase this variability is conducted [1, 2]. The most commonly used for this purpose are artificial induction of heritable changes or involvement of genetic resources from closely related species.

The method of induced mutagenesis is also essential in this respect, as it has long established itself as a way to produce plants with novel valuable characteristics. As an object for mutagenic treatment mature seeds are commonly used, other objects are treated infrequently. The use of mutagens *in vitro* could be a promising way to provide an extension of genetic variation for different plants. Similar techniques are being currently developed in a number of research laboratories [3-5].

In this context, we proposed a novel approach which consisted in using the method of induced mutagenesis in conjunction with such a biotechnological technique as embryo rescue. Given that gene expression at various stages of plant ontogeny is definitely different, one can expect a different range of variability after treatment of immature embryos, compared with mature seeds. Therefore, a study on the mutagenic effect on immature embryos of sunflower is of both theoretical and practical interest.

The goal of the given paper was to investigate the effectiveness of mutagenic treatment of immature embryos, compared with mature seeds, in the induction of genetic variability in sunflower.

Materials and methods. As the material immature embryos of two lines of cultivated sunflower *Helianthus annuus* L. of Zaporozhye origin – ZL-809 and ZL-95 were used. Some elements of the technology for such mutagenic treatment have been earlier tried on immature seeds [6].

Plants of these lines were grown under greenhouse conditions and isolated before flowering. In 9-11 and 14-16 days after a forced self-pollination of the heads immature achenes with embryos of corresponding age were isolated and treated with 0.02% aqueous solution of ethyl methanesulfonate (EMS) within 16 hours. The

embryos were then plated onto a modified MS nutrient medium and cultured until germination. In controls manipulations were the same, except that distilled water was used for treatment, instead of EMS. Similar treatment with the mutagen was applied to mature seeds. The resulted green seedlings were planted in plastic cups. After emergence of two or three pairs of true leaves the plants were transplanted into the field. Before flowering the plants were individually isolated.

The following year, seeds of the M_1 plants were sown as families in a field for obtaining the M_2 generation. Each M_2 family – M_1 progeny of a single plant. In the field each M_2 family consisted of about 30 plants. During the growing season the plants of M_2 generation were analyzed for visible morphological and physiological characteristics by which experimental plants differed from the control ones.

In general it was analyzed in M_2 generation 145 families of ZL-809 line and 127 families of ZL-95 line. The total number of M_2 plants amounted to over 8000. Before flowering the plants with presumed mutations were isolated. Preliminary frequency of mutations in M_2 (in percent) was determined by the number of families with visible changes in morphological and physiological characteristics related to the total number of examined families. The final frequency of mutations in M_2 was adjusted after confirming inheritance of modified traits.

The electrophoresis of sunflower seed storage proteins was performed in 13% polyacrylamide gel at a current intensity of 100 mA and a voltage of 450 volts for 2.5 hours. Gels were stained during 16-17 hours in a solution of Coomassie brilliant blue R-250. The electrophoretic gels were scanned using an office scanner, and then analyzed with special software (Gel Analyzer 2010 or similar).

The data were statistically processed using MSTAT-C [7] and Statistica [8] software. The essential differences for the frequency of mutations were evaluated using Student's t-test [9].

Results and their discussion. *The spectrum of inheritable changes.* All the inheritable changes were combined into the following seven groups: chlorophyll deficiency, mutations of cotyledon leaves, mutations of true leaves, stem mutations, inflorescence mutations, seed mutations, and mutations of physiological characteristics (Table 1).

Mutations of chlorophyll deficiency, in the case of immature embryo treatment, were represented by the following types – *viridis* (1) and *xantha* (2). Those mutations are common to many crops. They have been found earlier by us when treating mature and immature sunflower seeds with a mutagen [6]. In the case of mature seeds mutations of this type have not been found for the studied lines ZL-95 and ZL-809.

One type of cotyledonary leaf mutations, named as “deformed cotyledons” (3) was isolated in this study only when treating immature embryos. Mutant seedlings carried curved, often firmly encased or dissected, cotyledonary leaves.

Changes of true leaves included such types as corrugated leaf (4), tube-shaped leaf (5), malformed leaf (6), and a large leaf (7). In the mutant with a tube-shaped leaf the first pair of true leaves grows together and develops into a tube-shaped formation (Fig.). As plant grows and new leaves emerge the tube ruptures but its presence in the plant can be recognized even by the end of vegetation. Of all the types of leaf mutations, when mature seeds were treated, there were revealed only two – tube-shaped leaf and malformed leaf.

Stem mutations were presented with a branching stem (13) and such changes of habitus as low-growing (8), low-growing with a strong habitus (9) tall with a strong habitus (10), a strong habitus (11), as well as tilted stem (12). Branching mutants

possessed 1-3 side shoots at the basal part of the plant. Mutants with the changed habitus had fewer internodes (low-growing), larger leaves (a strong habitus), larger leaves and a reduced height (low-growing with a strong habitus). The mutation of tilted stem was easily visible at the end of the growing season, when the upper part of the stem with a ripening head bent almost to the ground. Most of stem mutations were found after immature embryo treatments. Only low-growing and low-growing plants with a strong habitus were also found when the mutagen influenced mature seeds.

Fig. The morphotype of «tube-shaped leaf» sunflower mutant

Eleven types of inheritable changes composed the group of inflorescence mutations – few bracts (14), many bracts (15), malformed bracts (16), capitulum fasciation (17), capitulum inclination angle (18), few ray florets (19), many ray florets (20). All of them, except one (few bracts), were found only when processing immature embryos. The mutant plants with few bracts were simultaneously characterized by a reduced number of ray florets. The mutation of malformed bracts caused their significant proliferation. Two mutant types possessed an altered, both decreased and increased, quantity of ray florets.

After mutagenic treatment of immature embryos there was revealed one mutation of seeds. The mutants had reddish-brown color of seed coat, whereas control seeds were black.

Physiological mutations were represented with early- and late-flowering plants. Compared to the control, such mutants had a shortened or extended, by the period of 2-4 days, stage of seedling emergence–flowering. Early flowering plants were found after treatment of both immature embryos and mature seeds.

The spectrum of mutations in M_2 after treatment of immature embryos and mature seeds with the mutagen is illustrated in Table 1. From the given table it is clear that the treatment of immature embryos with EMS was more effective than the treatment of mature seeds. Thus, in ZL-809 line, when immature embryos were used as a processing object, there were obtained 7 types of inheritable changes, whereas in the case of mature seeds – 3 types only. In ZL-95 line after treatment of mature seeds there were also revealed minor mutational changes, unlike after treatment of immature embryos (9 and 14 types of mutations, depending on embryo age).

In the M_2 generation the spectrum of mutations, in treatments with different age of immature embryos, differed. The differences referred to both the number of types of mutations and their matching a specific group of mutations.

In general, the spectrum of mutations in M_2 was quite broad. The total number of mutant types, we have identified when treating embryos of both ages in lines ZL-95 and ZL-809, amounted to 22. Morphological changes were caused by mutations in chlorophyll synthesis, by mutations of cotyledons, true leaves, stem, inflorescence, and seed. Subsequently, genetic control was established for many of the identified in the course of this study morphological mutations [10, 11].

A part of the mutants resulted from the treatment of immature embryos with the chemical mutagen ethyl methanesulfonate was evaluated by electrophoretic spectra of seed storage proteins of (Table 2).

Table 1

The spectrum of visible mutations in M_2 after ethyl methanesulfonate treatment of immature embryos and mature seeds of sunflower
(2006-2010)

№	Type of mutation	Line, treated material				
		ZL-95			ZL-809	
		Embryos of 14-16 days	Embryos of 9-11 days	Mature seeds	Embryos of 14-16 days	Mature seeds
Mutations with impaired synthesis of chlorophyll in seedlings and adult plants						
1	*Viridis*	+				
2	*Xantha*				+	
Mutations of cotyledonary leaves						
3	Malformed cotyledons	+	+			
Mutations of true leaves						
4	Goffered leaf	+				
5	Tube-shaped leaf	+		+		
6	Malformed leaf			+		
7	Big leaf	+	+			
Mutations of stem						
8	Low-growing	+	+	+		
9	Low-growing with strong habitus	+	+		+	+
10	High-growing with strong habitus	+				
11	Strong habitus		+			
12	Tilted stem	+	+			
13	Branching	+	+			
Mutations of inflorescence						
14	Few bracts				+	
15	Many bracts	+				
16	Malformed bracts		+			
17	Malformed capitulum	+				
18	Capitulum inclination angle	+			+	
19	Few ray florets	+				+
20	Many ray florets				+	
Mutations of seed						
21	Seed color		+			
Physiological mutations						
22	Early flowering				+	+
23	Late flowering				+	
Total		14	9	3	7	3

As can be seen from the Table 2, the presence of only the first component in the two-component HEL 6 locus was characteristic for the electrophoretic spectrum of seed storage proteins of the reference line ZL-95. Similar spectrum in the given locus was typical for a mutant sample with a reddish-brown color of seeds. However, electrophoretic spectrum in the HEL 6 locus of *xantha* line was represented by the second component of this locus, and of the line with tilted stem – by both components.

A distinctive feature of the electrophoretic spectrum for a number of mutants of ZL-95 line was also another component composition and a higher degree in the intensity of individual protein components in the HEL 4 locus, compared with the reference line. Those differences from the source line were characteristic for the *xantha* and tilted stem mutants.

Table 2

Mobility of polypeptides in sunflower mutant samples and the source line ZL-95 at HEL 4 and HEL 6 loci, Rf (2013)

Polypeptide zone	**Source line**	**Mutant sample**		
	ZL-95	*Xantha*	Tilted stem	Reddish-brown color of seed coat
HEL 4				
1	0,70	0,70	0,69	0,69
2		0,75	0,75	
3		0,77	0,77	
4	0,79			0,78
5	0,81	0,81	0,81	0,81
6	0,84	0,84	0,83	0,83
HEL 6				
1	0,94		0,94	0,93
2		0,98	0,97	

Some mutant types, such as *viridis* and several others, have been identified in our previous study after treatment of mature and immature sunflower seeds with ethyl methanesulfonate [6].

In addition to morphological and physiological changes, a number of samples identified in M_2 after treatment of immature embryos and mature seeds were also analyzed for changes in such an important biochemical characteristic as the total oil content of the seeds. We have detected plants with both low and high oil content of the seeds.

The frequency of inheritable changes. The data on the frequency of visible inheritable changes in M_2 confirmed significant influence of the mutagen on the immature embryos, which was reflected in the appearance of a significant number of mutations in this case. For ZL-809 line they amounted to about 15%, and for ZL-95 line – almost 3 times more. As can be seen from the Table 3, the frequency of mutations when treating embryos of both ages with the mutagen was significantly greater than when treating mature seeds of both sunflower lines. The difference in the frequency was observed depending on the line only, which was obviously due to the significant differences in the genetic background of the two samples studied. For the line ZL-809 an increase in this index when processing immature embryos (as

compared to mature seeds) was almost 180%, whereas the differences for the line ZL-95 were even greater (3-4 times).

Table 3

The frequency of mutations in M_2 after ethyl methanesulfonate treatment of immature embryos and mature seeds of sunflower, %

(2006-2010)

Type of mutation	Line, treated material				
	ZL-95			ZL-809	
	Embryos of 14-16 days	Embryos of 9-11 days	Mature seeds	Embryos of 14-16 days	Mature seeds
1	1,6				
2				0,9	
3	4,8	3,1			
4	1,6				
5	3,2		2,4		
6			4,8		
7	1,6	3,1			
8	8,0	3,1	2,4		
9	6,3	3,1		0,9	2,9
10	1,6				
11		3,1			
12	1,6	3,1			
13	1,6	6,2			
14				1,8	
15	1,6				
16		6,2			
17	1,6				
18	1,6			0,9	
19	3,2				2,9
20				1,8	
21		3,1			
22				1,8	2,9
23				7,3	
Total frequency	39,9±6,17	34,1±8,51	9,6±4,60	15,4±3,46	8,7±4,83
Total in the control	6,67±6,44	0	0	0	0

Differences in the overall frequency of mutations between treatments with different embryo age were not found, although mutagenic treatment of the younger embryos resulted in a tendency to increase the frequency of morphological and physiological changes. At the same time there were differences between those treatments in the frequency of individual types of mutations. So, after mutagenic treatment of 14-16 day-old embryos of ZL-95 line the highest frequencies of malformed cotyledons as well as mutations of habitus – low-growing and low-growing with a strong habitus – were observed. The frequency for the last two types of inheritable changes accounted for almost 15% of mutations. When treating the younger (9-11 day-old) embryos of this line with ethyl methanesulfonate, most frequently detected mutations were those of bracts, as well as of branching.

It should be noted that for the most of the controls, both for immature embryos and for mature seeds, no mutations were detected. In one case only – in the control of mutagenic treatment of 14-16 day-old embryos of ZL-95 line, – there was identified mutation of *xantha* type (2). The overall frequency of mutations totaled here 6.67%.

The mutations isolated by us in M_2 generations after the treatment of both immature embryos and mature seeds can be used in sunflower breeding programs as donors of marker traits in hybrid seed production, or to create a starting material as a source of low- and high-growing habitus, elongated and shortened vegetation period, and some other traits of economic importance.

During the development of the described method for the expansion of genetic variability in sunflower using embryo rescue, it was studied the inheritance of a number of revealed morphological mutations and found that they were under simple genetic control [10, 11]. This greatly facilitates the involvement of sunflower samples, carrying those mutant traits, into the breeding process and demonstrates the effectiveness of biotechnological approaches for practical breeding.

Conclusions

It was established that immature embryos of sunflower could serve as an effective object for mutagenic treatment. When they were treated with a chemical mutagen ethyl methanesulfonate the frequency and spectrum of mutations were significantly higher than with mature seeds.

The age of immature embryos influenced the spectrum of inheritable changes but had no substantial effect on the frequency of mutations.

The frequency and spectrum of induced mutations depended significantly on the genetic background of the treated material.

Some of the mutant lines, as compared with the source line, were characterized by the differences in the electrophoretic spectrum of seed storage proteins, which was manifested in the presence / absence of the protein components or their varying intensity at Hel 4 and Hel 6 loci.

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ИНДУЦИРОВАННАЯ ГЕНЕТИЧЕСКАЯ ИЗМЕНЧИВОСТЬ ПРИ ДЕЙСТВИИ ЭТИЛМЕТАНСУЛЬФОНАТА НА НЕЗРЕЛЫЕ ЗАРОДЫШИ И ЗРЕЛЫЕ СЕМЕНА

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Ключевые слова: подсолнечник, индуцированный мутагенез, незрелый зародыш, зрелые семена, поколение M_2, этилметансульфонат, частота мутаций, спектр мутаций.

Резюме. Установлено, что использование в качестве объекта мутагенного воздействия незрелых зародышей приводит к появлению в M_2 высокой частоты наследуемых изменений, которая составляла 15,4% у линии ЗЛ-809 и варьировала от 34,1 до 39,9% – у линии ЗЛ-95. При этом частота мутаций в случае использования незрелых зародышей была значительно выше чем при использовании зрелых семян, где наблюдали от 8,7 до 9,6% мутаций. При обработке зрелых семян у каждой линии выявляли значительно меньше типов мутаций чем в случае обработки мутагеном незрелых зародышей.

ІНДУКОВАНА ГЕНЕТИЧНА МІНЛИВІСТЬ ПРИ ДІЇ ЕТИЛМЕТАНСУЛЬФОНАТУ НА НЕЗРІЛІ ЗАРОДКИ І ЗРІЛЕ НАСІННЯ

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Ключові слова: соняшник, індукований мутагенез, незрілий зародок, зріле насіння, покоління M_2, етилметансульфонат, частота мутацій, спектр мутацій.

Резюме. Встановлено, що використання в якості об'єкта мутагенного впливу незрілих зародків призводить до появи в M_2 високої частоти успадковуваних змін, яка становила 15,4% для лінії ЗЛ-809 і варіювала від 34,1 до 39,9% - для лінії ЗЛ-95. При цьому частота мутацій у разі використання незрілих зародків була значно вищою, ніж при використанні зрілого насіння, де спостерігали від 8,7 до 9,6% мутацій. При обробці зрілого насіння у кожної лінії виявляли значно менше типів мутацій ніж у випадку обробки мутагеном незрілих зародків.

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