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GENETIC VARIABILITY IN SUNFLOWER AFTER MUTAGEN TREATMENT OF IMMATURE EMBRYOS OF DIFFERENT AGES

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SUMMARY

The frequency and spectrum of morphological and physiological mutations obtained in M_2 and M_3 generations after sunflower immature embryos treatment with ethyl methanesulphonate (EMS) have been studied. Immature 9 - 10 and 14 - 15-day-old embryos of two genotypes were treated with EMS at the concentration of 0.02% for 16 hours. Thirty-three types of mutation were found, described, and classified into the following groups: chlorophyll deficiency (3 types), cotyledon mutation (1), leaf mutations (6), stem mutations (9), inflorescence mutations (11), seed mutation (1) and physiological mutations (2). Differences were observed between genotypes for the spectrum and frequency of mutation. Mutation frequency after immature embryo treatment in the M_2 generation did not exceed the amount of mutations in M_3. At the same time, some mutations such as sterility, leaf venation, and different shapes of leaf lamina were unique for the M_3 generation. Morphological mutations were rarest after mutagen treatment of immature 9 - 10-day-old embryos.

Key words: **sunflower, ethyl methanesulphonate, immature embryos of different age, morphological and physiological mutations, mutation spectrum and frequency**

INTRODUCTION

Sunflower is a widely grown oil crop around the world. In the Ukraine, for example, its acreage in 2008 amounted to 3.9 million ha. Genetic basis of sunflower, however, is not broad enough to satisfy all expectations. Therefore, many sunflower breeding programs started are aimed at the development of new genetic variability suitable to cover growing demands of sunflower growers. As diverse

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source populations are a prerequisite for crop improvement, new approaches should be tried in sunflower breeding.

Among the few techniques used in modern breeding, the method of mutagenesis is considered an effective one. In the work with mutagenesis, however, it happens sometimes that only one induced mutation in a thousand is of importance for a breeder. Even this small value, however, saves considerable funds and contributes to a faster variety development. So far, more than 2500 crop varieties have been developed or improved by induced mutagenesis (Nazarenko, 2007).

Another promising technique is *in vitro* culture. Plant breeders usually rescue valuable but weak or immature embryos to prevent their degeneration and raise mature plants (Sharma, 1996). The method is successfully used in breeding for disease tolerance (Faure *et al.*, 2002), interspecific hybridization (Sukno *et al.*, 1999) and even in conventional breeding process, to produce more generations per year (Soroka, 2000).

Usage of both of these techniques, induced mutagenesis and embryo rescue, could serve for rapid and successful breeding of sunflower as it broadens the available genetic variability, combines several mutations in a single genotype and even isolates new traits, not detected in this crop earlier (Rapoport, 1986).

Ripe seeds are typically treated with a mutagen. At the same time there are scant data on the use of other plant parts as explants for mutagenic treatment (Latado *et al.*, 2004). Immature embryos could also serve as explant as it is supposed that different set of genes is expressed at this stage of development.

MATERIAL AND METHODS

Two sunflower lines, Zl-809 and Zl-95, were used as experimental material. These lines are widely used as source material in development of new sunflower hybrids. Immature 9 - 10 (EMS 2) and 14 - 15-day-old (EMS 1) seeds, isolated from sunflower capita, were treated with 0.02% of ethyl methanesulphonate (EMS) solution or water (control) for 16 hours and thoroughly washed under tap water. After that, both treated and control seeds were surface-sterilized in 70% ethanol and 50% bleach solution, rinsed several times in sterile water and incubated on a modified MS (Murashige, Skoog, 1962) medium until germination at the temperature of 25±3°C and 16/8 h day/night photoperiod.

Germinated plantlets were planted into the soil : sand mixture in plastic pots and allowed to acclimatize and develop several pairs of true leaves. The plants were then transplanted in the field and grown there until ripeness. Before flowering, plants were isolated and then self-pollinated. Next year, seeds of M_1 plants were sown in the field in the row-family design. Each M_2 family consisted of not more than 30 plants. Seed germination, flowering and ripening dates, and a number of morphological traits were analyzed. Before flowering, plants with traits that differed

from the control were isolated. Seeds were collected manually from each individual plant.

Seeds of M_2 plants were sown the subsequent year to produce the M_3 generation. Each M_3 family was a progeny of a single plant, selected from the M_2 family. About 30 seeds were sown for each M_3 family (one row per family) to produce about 10-20 plants per row. The M_3 plants were analyzed for the traits selected for in M_2. Simultaneously, all M_3 families were visually analyzed for possible morphological or physiological mutations, which were not selected for in M_2 or earlier. Before flowering, the observed possible mutant plants were artificially isolated, and self-pollinated at flowering time. Seeds were collected manually, individually from each plant. Two controls were used: immature 9 - 10 and 14 - 15-day-old embryos treated with distilled water.

Table 1: Survival rate of M_1 sunflower plants after treatment of immature embryos of different age with ethyl methanesulphonate

Treatment (variant)	Embryos treated with EMS	Plants transplanted to field	Plants in the field that produced seeds	Survival rate (plants with seeds), %
ZL-809				
Control 1	75	59	51	68.0±5.39
EMS 1	238	169	115	48.3±3.24**
Control 2	73	70	8	11.0±3.66###
EMS 2	224	0	0	0**. ###
ZL-95				
Control 1	67	36	34	50.7±6.11
EMS 1	243	77	65	26.7±2.84***
Control 2	123	61	41	33.3±4.25##
EMS 2	165	34	32	19.4±3.08**

, * -differences from the control are significant at $P<0.01$ and $P<0.001$, respectively;
##, ### -differences between the embryos of different age are significant at $P<0.01$ and $P<0.001$, respectively.

RESULTS AND DISCUSSION

Survival rate of M_1 plants

Survival rate of sunflower plants after EMS and control treatments of immature embryos were first analyzed. As shown in Table 1, after EMS treatment, most embryos perished even before normal plantlets were developed and transplanted to the field. Some of them, however, wilted after planting in the field or did not produce viable seeds at ripeness. The main reason for plantlet death was dying-off of the embryo root. On the whole, the survival rate of the M_1 plants after treatment of immature embryos, both 9 - 10 and 14 - 15-day-old, decreased greatly in comparison with the control. However, the survival rate of plants raised from younger embryos was even lower. For example, all plantlets of line ZL-809 from 9 - 10-day-old embryos perished before being transplanted to the field.

Table 2: Types of morphological and physiological mutations in M_2 and M_3 and their brief description

N	Type of mutation	Characteristic
I Chlorophyll deficiency mutations		
1	*Viridis*	Light green seedling and plant
2	*Xantha*	Yellow green leaf bracts and bottom of the capitulum
3	*Xantha* of necrotic type	Yellow-green spots on the leaves, transforming into necrotic segments at the end of growing season
II Cotyledon mutations		
4	Malformed cotyledons	Curved, often fused or split, cotyledons
III Leaf mutations		
5	Goffered leaf	Undulated leaf edges
6	Tube-shaped leaf	First pair of true leaves accreted as a tube
7	Malformed leaf	Malformed, curved, often fused or split leaf blades, accreted or dissected petioles
8	Big leaf	Leaf of a large size
9	Dichotomous venation	Fan-shaped venation, corrugated leaf, small petiole-stem angle
10	Erect leaf	Reduced petiole-stem angle
IV Stem mutations		
11	Low-growing	Plant reduced in height
12	High-growing	Plant enlarged in height
13	Strong low habit	Low-growing plants with strong stem and large leaves
14	Strong high habit	High-growing plants with strong stem and large leaves
15	Strong habit	Plants with strong stem and large leaves
16	Tilted stem	Plant top with the head curving very close to the ground at the end of vegetation period
17	Tobacco-like plant	Plant with shortened internodes, oval leaves, wide and oval cotyledons, decreased number of short ray florets
18	Stem fasciation	Flattened stem
19	Branching	One-three lateral shoots at the basal part of stem
V Inflorescence mutations		
20	Few bracts	Decreased number of bracts
21	Many bracts	Increased number of bracts
22	Malformed bracts	Considerable overgrowth of bracts
23	Malformed capitulum	Inflorescence consisting of many malformed heads
24	Capitulum inclination angle	Platform capitulum
25	Partial capitulum sterility	Underdeveloped generative tissues at head center
26	Full sterility	Male and female sterility, defective development of male and female generative structures, absence of ray florets
27	Few ray florets	Decreased number of ray florets
28	Many ray florets	Increased number of ray florets
29	Short ray florets	Reduced length of ray florets
30	Goffered ray florets	Undulated and curved ray florets
VI Seed mutations		
31	Seed color	Brown-red color of seed coat
VII Physiological mutations		
32	Early flowering	Emergence-flowering period shortened by 2-4 days
33	Late flowering	Emergence-flowering period prolonged by 2-4 days

Mutation range in M_2 and M_3 generations

Treating embryos with ethyl methanesulphonate caused a wide range of morphological and physiological mutations in the M_2 and M_3 generations. Their brief description is given in Table 2. Twenty-three types of heritable changes were found in the M_2 generation. These mutations were divided into the following groups: chlorophyll deficiency – 2, cotyledon – 1, leaf – 4, stem – 6, inflorescence – 7, seed – 1, physiological mutations – 2 types. The same number of mutation types was isolated in the M_3 generation. Those were: chlorophyll deficiency – 3, cotyledon – 1, leaf – 4, stem – 5, inflorescence – 7, seed – 1 type and physiological mutations – 2 types. Mutations after 9 - 10 and 14 - 15-day-old immature embryo treatment with water (control) were not found.

Three types of chlorophyll deficiency mutations were noted in the present study, namely *viridis* (Figure 1), *xantha*, and *xantha* of the necrotic type. The first two mutations are common for many agricultural crops. Besides these mutation types, we also isolated plants that showed yellow-green spots on the leaves, which transformed into necrotic segments at the end of the growing season (Figures 2a,b). This mutation reduced neither plant height nor plant productivity.

One type of cotyledon mutations was found in the present study – malformed cotyledons. Such mutant seedlings had curved cotyledons, and often fused or split cotyledons.

Changes of leaves included such types as folded leaf, big leaf, malformed leaf, rolled leaf, dichotomous venation and reduced petiole-stem angle. The rolled leaf mutant had the first pair of true leaves fused into a tube (Figure 3). The mutation of leaf dichotomous venation touched a number of traits such as venation, leaf shape and petiole-stem angle (Figure 4). The mutation to erect leaf showed itself as a small petiole-stem angle only and was not accompanied by modification of the other traits.

The stem mutations were represented as fasciation, branching, and habit changes such as low-growing, high-growing, strong low habit, strong high habit, strong habit, tilted stem, as well as tobacco-like plant. The fasciated mutants included plants with a flattened stem and branched mutants had 1-3 lateral shoots at the basal part of a plant. The mutants with changed habit were caused by a smaller number of internodes (low-growing), larger number of internodes (high-growing), bigger leaves (strong habit), bigger leaves but reduced height (strong low habit), bigger leaves and height (strong high habit). The mutation of curved stem distinctly demonstrated itself at the end of vegetation period when stem top with a head very close to the ground. Tobacco-like plants had obvious differences from the normal plants starting from the seedling stage. They were characterized by several modified traits such as wide and oval cotyledons, shortened internodes, oval leaves and decreased number of short ray flowers (Figures 5a,b).

Eleven heritable types of changes fell into the group of inflorescence mutations: few bracts, many bracts, malformed bracts, few ray flowers, many ray florets, short ray flowers, corrugated ray flowers, malformed capitulum, capitulum inclination

Table 3: Spectrum of visible mutations in M_2 and M_3 after EMS treatment of immature sunflower embryos

N	Type of mutation	Line, treatment, generation					
		ZL-95, EMS 1		ZL-95, EMS 2		ZL-809, EMS 1	
		M_2	M_3	M_2	M_3	M_2	M_3
I Chlorophyll deficiency mutations							
1	*Viridis*	+	+		+		
2	*Xantha*					+	+
3	*Xantha* of the necrotic type				+		
II Cotyledon mutations							
4	Malformed cotyledons	+	+	+			
III Leaf mutations							
5	Corrugated leaf	+					
6	Tube-shaped leaf	+	+				
7	Malformed leaf	+	+	+	+		
8	Big leaf	+		+			
9	Dichotomous venation				+		
10	Erect leaf				+		
IV Stem mutations							
11	Low-growing	+	+	+	+		
12	High-growing		+				
13	Strong low habit	+		+		+	
14	Strong high habit	+					
15	Strong habit		+	+	+		+
16	Curved stem	+		+			
17	Tobacco-shaped plant				+		
18	Stem fasciation				+		
19	Branching	+		+			
V Inflorescence mutations							
20	Few bracts					+	
21	Many bracts	+					
22	Malformed bracts			+	+		
23	Capitulum fasciation	+	+		+		
24	Capitulum inclination angle	+	+		+	+	+
25	Partial capitulum sterility						+
26	Full sterility		+				
27	Few ray florets	+					
28	Many ray florets					+	
29	Short ray florets				+		
30	Corrugated ray flowers				+		
VI Seed mutations							
31	Seed color		+	+			
VII Physiological mutations							
32	Early flowering		+			+	
33	Late flowering		+			+	
Total		15	13	10	14	7	4

Figure 1: A light green mutant of viridis type (right) and a normal green plant (left)

Figure 2a: A mutant with yellow-green spots on the leaves

Figure 3: A mutant showing first pair of true leaves fused into a tube

Figure 2b:A mutant showing transformation of yellow-green spots on the leaves into necrotic segments at the end of growing season

Figure 4: A mutant with leaf dichotomous venation, corrugated leaf, and small petiole-stem angle

Figure 5a: A tobacco-like plant with shortened internodes, oval leaves and decreased number of short ray flowers

Figure 5b: A mutant showing oval cotyledons (right) and a normal plant (left)

angle, partial capitulum sterility, and full sterility. The first two types of mutations had changed the number of bracts. The mutant plants with few bracts were simultaneously characterized by a decreased number of ray florets (Figure 6).

Figure 6: A few bracts mutant (left) and a normal plant (right)

The mutation called malformed bracts stood for excessive bract growth. Few ray flowers and many ray flowers mutations were characterized by changed number of ray flowers. Two types of capitulum sterility were found in the present study. One of them was characterized by underdeveloped generative tissues at the head center (partial capitulum sterility). The other mutant had a complete abnormality in the development of the male and female generative structures (Figures 7a,b).

Figure 7a: A mutant showing defective development of male and female generative structures as well as absence of ray florets

Figure 7b: Another mutant with defective development of male and female generative structures

This mutation was also accompanied by the absence of ray florets. The rare mutation type of corrugated ray flowers was found. Mutant plants had undulated and curved ray florets (Figure 8).

One type of seed mutations was isolated after EMS treatment. The mutant line possessed seeds of brown-red color of seed coat while in the control the seeds were black.

Figure 8: A mutant with undulated and curved ray florets

Physiological mutations were represented as early flowering and late flowering plants. These mutants had emergence-flowering period shortened or prolonged by 2-4 days in comparison with the control.

The mutation range in both M_2 and in M_3 generations was rather wide. Morphological variations dealt with mutations of chlorophyll synthesis, cotyledons, leaf blade, stem, inflorescence, and seeds. However, the genotypes studied differed essentially in mutation spectrum – in line ZL-95 it was considerably wider than in line ZL-809 (Table 3). Thus, in ZL-809 line we did not observe some mutations as chlorophyll deficiency, fasciation, leaf blade and cotyledon deformation, etc. At the same time, specific mutations of ray florets and head bracts, which were found in line ZL-809, were not found in line ZL-95. Other types of mutations, such as capitulum inclination angle, strong habit and strong low habit were common for both lines.

The largest number of mutation types was isolated in the M_2 generation after treatment of immature 14 - 15-day-old embryos of ZL-95. Some specific changes were found in the M_3 generation, however. For example, after treatment of younger embryos of line ZL-95, rare mutations such as dichotomous venation, tobacco-like plant, short ray florets, corrugated ray flowers and *xantha* of the necrotic type were isolated. An interesting result of the treatment immature of 14 - 15-day-old embryos of line ZL-95 was the occurrence of full sterility mutation in the M_3 generation. Those mutants were characterized by abnormal development of male and female generative structures and heterozygosity. In general, the spectrum of morphological

Table 4: Mutation frequency in M_2 and M_3 after EMS treatment of immature sunflower embryos, %

Type of mutation	Line, treatment, generation					
	ZL-95, EMS 1		ZL-95, EMS 2		ZL-809, EMS 1	
	M_2	M_3	M_2	M_3	M_2	M_3
1	1.6	1.6		6.2		
2					0.9	0.9
3				3.1		
4	4.8	1.6	3.1			
5	1.6					
6	3.2	3.2				
7	4.8	1.6	15.5	3.1		
8	1.6		3.1			
9				3.1		
10				3.1		
11	8.0	3.2	3.1	3.1		
12		3.2				
13	6.3		3.1		0.9	
14	1.6					
15		4.8	3.1	3.1		0.9
16	1.6		3.1			
17				3.1		
18				6.2		
19	1.6		6.2			
20					1.8	
21	1.6					
22			6.2	6.2		
23	1.6	3.2		6.2		
24	1.6	1.6		6.2	0.9	4.6
25						0.9
26		6.3				
27	3.2					
28					1.8	
29				3.1		
30				3.1		
31		1.6	3.1			
32		1.6			1.8	
33		1.6			7.3	
Total, types	15	13	10	14	7	4
Total, frequency	43.7±6.15	35.1±8.57	46.7±6.19	58.9±8.84	15.4±3.46	7.3±2.49

variations in the M_3 generation resembled that in the M_2 generation, however, some traits appeared to be unique for the M_3 generation, as far as they were not observed in M_2.

Some similar mutant types, like *viridis*, dichotomous venation and others, were isolated in our previous study after treating mature and immature sunflower seed with EMS (Lyakh *et al.*, 2005). At the same time, a different mutation range was found by Jambhulkar and Joshua (1999) when using gamma rays to treat mature sunflower seeds.

Mutation frequency in the M_2 and M_3 generations

The data on the frequency of M_2 and M_3 heritable changes testified to essential influence of the mutagen on the immature embryos, which had been expected to induce a significant number of mutations.

The frequency of morphological and physiological mutations essentially depended on genotype. As evident in Table 4, variations sprang up with higher frequency in line ZL-95 than in ZL-809, both in the M_2 and the M_3 generation. It could be explained by significant differences in the genetic origin of these two samples. At the same time, the treatment of young embryos was characterized by a tendency to increase the frequency of visible heritable changes.

Mutations of malformed cotyledons and leaves as well as habit mutations – low-growing and strong low habit – were most often observed after 14 - 15-day-old embryo treatment in line ZL-95. When young embryos of line ZL-95 were treated with the mutagen, the mutations of malformed leaf, malformed bracts and branching were found with highest frequency. More frequent heritable changes in line ZL-809 were those associated with late flowering.

In the M_3 generation as compared with M_2, the total frequency of heritable changes was at the same level for both lines. The former generation had a high frequency of such visible variations as *viridis*, malformed bracts, stem fasciation and malformed capitulum, as well as head inclination angle after 9 - 10-day-old embryo treatment of line ZL-95. Rare mutations of the *xantha* necrotic type, dichotomous venation, tobacco-like plant and some other mutations were noted in this treatment. EMS treatment of 10 - 14-day-old embryos of line ZL-95 resulted in the highest rate of full sterility. Plants of this mutant type were found in four families and were not noted anywhere else in the present study. Only the mutation of capitulum inclination angle was found with sufficiently high frequency in line ZL-809 (Table 4).

CONCLUSIONS

In general, survival rate of M_1 sunflower plants after treatment of immature embryos with ethyl methanesulphonate, both 9 - 10 and 14 - 15-day-old, decreased greatly in comparison with the control. Significant genetic variability in the M_2 and M_3 generations after treating immature sunflower embryos with the mutagen was

revealed. The spectrum of visible heritable changes in the M_3 generation resembled that in the M_2 generation. However, such mutations as sterility, leaf venation, and several shapes of leaf blade appeared to be unique for the M_3 generation, as they were not observed in M_2. A considerable difference in the mutability of the two studied lines was noted. The frequency of morphological and physiological changes did not significantly differ in the M_3 generation from M_2. When treating young embryos, a tendency was noted of increased mutation rate.

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VARIABILIDAD GENETICA EN GIRASOL DESPUES DEL TRATAMIENTO CON MUTAGENO DE LOS EMBRIONES INMADUROS DE LA EDAD DEFERENTE

RESUMEN

La frecuencia y el spectro de mutaciones morfologicas y ficiologicas separadas en M_2 y M_3 despues del tratamiento de los embriones inmaduros de girasol con etilometanosulfonato (EMS) fueron estudiados. Embriones a la edad de 9-10 y 14-15 dias de dos lineas ZL-95 y ZL-809 fueron tratados con EMS en la concentracion de 0,02% durante 16 horas. Fueron obtenidos y descritos 33 tipos de mutaciones que fueron combinados en los grupos siguientes: insuficiencia clorofilica (3 tipos), mutaciones de cotiledon (1), mutaciones de hoja (6), mutaciones de tallo (9), mutaciones de semilla (1), mutaciones de inflorescencia (11), mutaciones fisiologicas (2). Diferencias genotipicas fueran reveladas para el spectro y frecuencia de mutaciones. La frecuencia mutacionica despues del tratamiento de los embriones inmaduros en M_2 generacion no superada considerablemente la cantidad de mutaciones en M_3. Al mismo tiempo algunos mutaciones asi como esterilidad, nervadura

de hoja, formas diferentes de hoja fueran unicas para la M_3 generacion. Despues del tratamiento de los embriones a la edad de 9-10 dias mutaciones morfologicas mas raras fueran separadas.

VARIABILITÉ GÉNÉTIQUE CHEZ LE TOURNESOL APRÈS TRAITEMENT MUTAGÈNE D'EMBRYONS IMMATURES DE DIFFÉRENTS ÂGES

RÉSUMÉ

La présente étude porte sur le traitement d'embryons immatures de tournesol par l'ethylmethanesulfonate (EMS), l'observation des fréquences et du spectre des mutations morphologiques et physiologiques obtenues aux générations M_2 et M_3.

Des embryons immatures de 9-10 et 14-15 jours de deux génotypes ont été traités avec l'EMS à la concentration de 0,02 % pendant 16 heures.

33 mutations différentes ont été identifiées et décrites, regroupées dans les catégories suivantes: déficience chlorophyllienne (3 cas), mutations de cotylédon (1), de feuille (6), de tige (9), d'inflorescence (11), de graine (1) et mutations physiologiques (2). Des différences entre les génotypes pour le spectre et la fréquence des mutations ont été mises en évidence.

La fréquence de mutation après traitement d'embryons immatures en M_2 n'a pas dépassé la quantité de mutations en M_3. Parallèlement, certaines mutations telles que la stérilité, le type de nervure des feuilles, la morphologie foliaire étaient uniques pour la génération M_3. Après traitement mutagène d'embryons immatures de 9-10 jours, les mutations morphologiques les plus rares ont été trouvées.