МУТАНТНА ЛИНИЯ СЛЪНЧОГЛЕД 103 RM, СЪЗДАДЕНА ЧРЕЗ *ИН ВИТРО* МУТАГЕНЕЗИС НА ГЕНОТИП 249 R
MUTANT SUNFLOWER LINE *103 RM* DEVELOPED BY *IN VITRO* MUTAGENESIS OF GENOTYPE *249 R*

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Abstract

Immature sunflower zygotic embryos of sunflower fertility restorer line *249 R* were ultra sonically treated before planting onto embryo culture medium. Some mutant plants were isolated and thirteen generations were self-pollinated. New sunflower forms with inherited morphological and phytopathological changes were obtained through selection and self-pollination. The genetic alterations which appeared during the mutation procedure included some morphological, biochemical, phytopathological and phenological characters. Line *103 RM*, a subject of this investigation, was characterized by significant changes affecting most of the studied characters. The results showed that mutagenesis in sunflower could be successfully used for developing new lines, useful for heterosis breeding.

Key words: *Helianthus annuus*, immature embryo, embryo culture, ultrasonic, mutant line.

INTRODUCTION

One of the ways for genetic diversity development in cultivated sunflower is inducing mutations by gamma radiation (Encheva et al., 1993, Encheva et al., 2002; Encheva et al., 2003a; Encheva et al., 2003b; Encheva et al*.,* 2003c*;* Encheva, 2009) and chemical mutagenesis (Kostov et al., 2007). The treatment of seeds led to increased variability in many characteristics, such as days to flowering, seed weight, seed husk color, some morphological characters and seed oil content (Giriraj et al., 1990; Jambhulkar and Joshua, 1999; Napipour et al., 2004). Mutagenesis was also used to increase the variation in fatty acid composition of the seeds. (Fernandez-Martinez et al., 1997; Ivanov et al., 1988; Fernandez-Moya et al., 2002; Ebrahimi et al., 2008a).

According to Ahloowalia et al. (2004), gamma radiation was the most commonly used mutagen and suitable for the induction of mutations. Oliveira et al., (2004) obtained resistant to alternaria (*Alternaria helianthi*) sunflower forms, induced by gamma irradiation at a dose of 150 Gy and 165 Gy, and chemical mutagens (EMS). Kostov et al. (2007) increased the resistance of tomatoes to *Orobanche ramosa* L. applying the chemical mutagen EMS.

Alterations due to gamma irradiation of quinoa seeds have been observed for a number of branches, plant height, length of vegetation period, morphology of leaves and flowers of the seed at the two doses of 150 Gy and 250 Gy (Gomez-Pando and de la Varra , 2013). Created mutant forms reduced tendency to lodging and increase yield. Early growing mutants allowed culture to be included in the modern agro-systems.

Some plant organs for generation (seeds, bulbs, cuttings/shoots, etc.) were subjected to extreme physical and chemical agents, which were stress factor for the physiological, biochemical and cytological activity at a cellular level. That caused modifications in the behavior of chromosomes, changes in the growth and development of roots, stems, leaves and flowers. Gamma radiation led to physiological and biochemical mutations caused structural alterations in the chromosomes of plants (Datta et al., 2011).

The combination of *in vitro* culture with induced mutagenesis is the effective way for increasing variation in plants and receiving mutations. The studies of genetic variation in the regenerants of sunflower are significantly less.

Different type of mutations in sunflower after treatment with the chemical mutagen ethyl-methane-sulfonate were reported by Soroka and Lyakh, (2009); Soroka, (2014). According to Soroka, (2014) spectrum of visible mutations at both immature zygotic embryos and mature seeds involved mutations in leaves, stem, inflorescence and physiological mutations. Chlorophyll mutations and mutation of seeds were received at immature seeds, only.

The results of Ebrahimi and Sarrafi, 2012 revealed the effect of gamma radiation at a dose of 75 Gy on the induction of genetic variation in sunflower in relation to the indexes associated to seeds germination. According to the authors, mutant lines M6-39-2-1 and M6-284-1 were better in comparison with the original line AS613 in relation to characteristics percentage of germinated seeds and time for maximum germination under water stress conditions.

The aim of this study was: a) to develop variable genetic material from the sunflower line 249 R by treatment with ultra sound of immature zygotic embryos at a dose of 25.5 w/cm^2 for 3 min, and b) to carry out biometric and biochemical investigations of both control genotype 249 R and the mutant line 103 RM.

MATERIALS AND METHODS

The Bulgarian fertility restorer line 249 R, witch is highly homozygotic (more 18 generations), was used as donor material.

Methods

Plants from control line 249 R were grown in the field and self-pollinated manually. The isolated immature zygotic embryos (13-16 days old) were treated with ultra sonic (the seeds were flooded in water and the whole surface well moistened) at dose 25.5 W/cm^2 for 3 min before plating on nutrition medium M for further growing (Azpiroz et al.): 1/2 MS (Murashige, T. and F. Skoog, 1962) macro salts, MS micro salts, B5 vitamins (Gamborg et al., 1968), 20 g/l sucrose, pH-5.7. Immature embryos were aseptically isolated and sterilized under the following conditions: 1) 1 min in 95 % ethanol; 2) 15 min in bleaching solution (2.7 % Cl); 3), followed by several washings with sterile distilled water.

The conditions for cultivation were: 25^o C, 16/8 h photoperiod for one week. The rooted plants were transferred to soil and self-pollinated under greenhouse conditions.

Field experiments

Biometric evaluation of control line 249 R and mutant lines 103 RM

As a result from long-term selfing and individual selection, new sunflower mutant line 103 RM was produced in R13M13 generation. The main criterions for selection of the new line were morphological, biochemical, phytopathological and phenological differences from control line 249 R. In each generation biometric studies of plants were carried out.

The morphological and biochemical investigation, phytopathological evaluation and phonological observation of the new mutant line and the control genotype were made during 2010-2012.

The biometric evaluation of the control genotype 249 R and the new developed mutant line 103 RM was made on 10 plants for each individual year, and included 15 main agronomic traits as plant height, leaf width, leaf length, number of leaves, leaf petiole length, head diameter, number of branches, length of branches, diameter of branch head and stem diameter, 1000 seed weight, oil content in seeds, seed length, seed width and seed thickness**.**

1000 seed weight (g) was determined on three samples of 50 seeds per head each. The control data were collected from plants of the original line 249 R which was grown in field together with the mutant plants.

Biochemical analysis

Nuclear-magnetic resonance (Newport Instruments Ltd., 1972) was used to determine the oil content of air-dried deeds.

Phytopathological evaluation of both control genotype 249 R and the mutant line 103 RM was carried out regarding to the pathogen caused grey spots (*Phomopsis helianthi*) on sunflower. The investigations were carried out in field conditions at Dobrudzha agricultural institute in the period 2010-2012.

Statistical analysis

The control genotype and developed new mutant line were analyzed statistically with regard to the agronomic traits such as plant height, leaf width, leaf length, number of leaves, leaf petiole length, head diameter, number of branches, length of branches, diameter of branch head, stem diameter, seed length, seed width and seed thickness**,** 1000 seed weight and oil content in seeds. Analysis of the experimental data was by the statistical package BIOSTAST 6.0.

RESULTS AND DISCUSSION

Isolated zygotic embryos of homozygous line 249 R were treated with ultra sonic before plated on embryo culture medium. The mutant line 103 RM, an object of this study, was derived from the regenerants, obtained from variants of treatment with ultra sonic at a dose of 25.5 W/cm^2 for 3 min. After individual selection, one of the lines was selected on the base of its morphological, physiological and biochemical differences, compared to the control line 249 R. Statistically proven differences were observed in ten from the all tested indexes (Table 1).

Таблица 1. Биометрични показатели на контролния генотип и слънчогледовата мутантна линия 103 RM, създадена чрез третиране с ултразвук на незрели зиготни зародиши на линия 249 R
Table 1. Biometric characteristics of control genotype and the sunflower mutant line 103 RM developed through ultra sonic treatment of immature zygotic embryos of line 249 R

Показатели Characteristics	Контрола 249 R Control 249 R	Мутантна линия 103 RM Line 103 RM	LSD
Височина на растение (cm) Plant height (cm)	98,50	**102,7+b**	Gd 5%=2,81
Брой листа (no) Number of leaves (no)	28,00	28,00	Gd 5%=1,31
Ширина на лист (cm) Leaf widt (cm)	12,17	**13,30+c**	Gd 5%=0,42
Дължина на лист (cm) Leaf length (cm)	13,80	14.43	Gd 5%=0,76
Дължина на листна дръжка (cm) Leaf petiole (cm)	9,49	9,57	Gd 5%=0,65
Диаметър на стъбло (mm) Stem diameter (mm)	18,46	**17,60-a**	Gd 5%=0,82
Диаметър на пита (cm) Head diameter (cm)	14,20	**12,20-c**	Gd 5%=0,69
Брой разклонения (no) Number of brunches (no)	8,00	9,00	Gd 5%=1,23
Дължина на разклонения (cm) Length of branches (cm)	15,36	**11,33-c**	Gd 5%=2,02
Диаметър на пита на разклонения (cm) Diameter of branch head (cm)	5,23	5,03	Gd 5%=0,61
Дължина на семе (mm) Seed length (mm)	0,95	**0,92-b**	Gd 5%=2,46
Ширина на семе (mm) Seed width (mm)	0,35	**0,49+c**	Gd 5%=2,53
Дебелина на семе (mm) Seed thickness (mm)	0,21	**0,32+c**	Gd 5%=2,57
Масленост, % Oil content in seed, %	44,08	**48,31+c**	Gd 5%=1,84
Маса на 1000 семена (g) 1000 seed weight (g)	23,93	**27,60+b**	Gd 5%=1,90

a, b, c = доказани различия при нива 0.05, 0.01 и 0.001, съответно.
a, b and c = significant differences at levels 0.05, 0.01 and 0.001, respectively.

Фиг. 1. *Контролна линия 249 R*
Fig. 1. *Control line 249 R*

Фиг. 2. *Мутантна линия 103 RM*
Fig. 2. *Mutant line 103 RM*

The height of plants is one of the most frequently studied characters in sunflower. It was considered as a quantitative heritable trait. In our experiment, the mutant line 103 RM was distinguished with increasing of height with 4.2 cm compare to the control line. The increased index value was with high degree of statistical significance.

A change in the mean value was registered in the mutant line 103 RM. The trait leaf width was increased with 1.5 cm and the character leaf length - 0.63 cm. The difference is statistically significant at the first index only.

Statistical decreased of head diameter with 2.0 cm and with 0.85 cm of stem diameter was observed at mutant line 103 RM.

Changes with high statistical significance were observed with respect to the length of the branches. While for the control line 249 R, the mean value of the trait was 15.36 cm, but for the mutant line 103 RM was detected a decrease with 4.1 cm.

One of the most important traits of sunflower is oil content in seeds. Increasing in the oil content with 4.23% of the mutant line 103 RM was with the higher degree of statistical confidence. Such mutation is detected by Soroka, 2014 in mutant F2 plants, obtained after treatment of immature and mature sunflower seeds with ethyl methane sulfonate (EMS).

The 1000 seeds weight is another important character determined the yield of sunflower. In our study the increasing of the value with 4.27 g was observed.

The data in Table 1 provided information on three traits concerning the morphology of the seed. Changes have occurred in seed length, width and thickness (Figure 3). An increase in the arithmetic mean with a high degree of significance is registered at the three indexes, as the most significant is that of the seed width with a 0.14 mm, followed by a seed thickness with 0.11 mm.

Based on all 15 studied characteristics it can be concluded that the decrease in average value of the traits compared to the control 249 R is recorded for head diameter, stem diameter, length of branches and seed length (4 of 15) i.e. 26.7% of the total number of characters studied.

Vase verse statistically significant increase in arithmetic mean is registered for the height of plants, leaf width, oil content in seed-%, 1000 seeds weight, seed width and seed thickness (6 of 15) i.e. 40% of the total number of traits studied.

Фиг. 3. *Семена от контролната линия 249 R (ляво) и мутантната линия 103 RM (дясно)*
Fig. 3. *Seeds of control line 249 R (left) and mutant line 103 RM (right)*

Such mutations with high stem, large leaves and higher oil content in seeds (%) were obtained from Christov and Nikolova, (1996) after treatment of air-dry seed of the sunflower lines L-2969, L-3004 and variety Peredovik with ultra sonic and EMS. The authors also found an increase in the size of plants and increase oil content in the seeds (%) in line L-3004 after treatment of air dry sunflower seeds with gamma rays Co^{60}.

Lack of statistically proved changes was observed for traits number of leaves, length of leaf, length of leaf petiole, number of branches and diameter of branch head. Our results confirm the conclusion of Skirvin, 1978 that the physical or chemical mutagenesis is suitable for induction of mutations in tissue culture.

In support of our study may be cited and the conclusion of Atanassov, 1988 that the ability of experimental mutagenesis in immature zygotic embryos at an early stage of their development are larger compared to air dried seed.

Characteristics of the mutant line 103 RM according the International Classification of UPOV

Table 2 presented the qualitative differences between the mutant line 103 RM and a control line 249 R, according to 16 characteristics from the International classification of UPOV. The leaves of the mutant line RM 103 were medium and dark green to small and green for the control line. The shape of the cross section of the leaves is flat in the control and weak convex to the new line. Height of flat blade compared to the base of petiole at the stem was higher for the mutant line 103 RM, and lower for the control line 249 R. Ray flowers were densely arranged in line 103 RM, in comparison to the control line, which ray flowers were smaller and less. Leaf of the base of the head of mutant line was round and enveloped it slightly, whereas in the control line it was elongated and did not cover it. The position of the head at maturity of control line was half-turned down with vertical stem, and in line RM 103 it was half-turned down with inclined stem. At the phenological observation a delay of the beginning of flowering with 4 days was established for the mutant line.

Type of branching of plants is a characteristic that differed substantially for both lines. Whereas the branches in the control line were on the whole stem, in the mutant line 103 they were mostly on the top.

The most significant difference was observed in terms of the shape, size and color of seeds. In the mutant line 103 RM seeds were medium large, ovoid wide and without stripes in the middle. In the control line 249 R seeds were small, elongated and with strong express in the Middle.

Evaluation of mutant line and control genotype for resistance to *Septoria helianthi Ellis et Kellerman*

Leaf spot blight on sunflower appeared in early stages of plant development. The disease favored of average temperatures and frequent rainfall during the vegetation. The first symptoms appeared on the leaf lamina of the lower floors and later attacked those from upper floors. Brown spots are irregularly shaped and yellow halo.

Таблица 2. Различия в някои качествени характеристики на слънчогледовата мутантна линия 103 RM и контролната линия 249 R според международната класификация на UPOV
Table 2. Differences in some quality characteristics of sunflower mutant line 103 RM and control line 249 R according to international classification UPOV

Характеристика Characteristics	Контролна линия 249 R Control line 249 R	Мутантна линия 103 RM Mutant line 103 RM
Лист: размер Leaf: size	малък small	среден medium
Лист: цвят Leaf: color	зелен green	тъмнозелен dark green
Лист: форма на напречно сечение Leaf: shape of cross section	плоска flat	слабо изпъкнала weak convex
Лист: форма на връхната част Leaf: size of the tip	тяснотриъгълна narrow triangular	широкотриъгълна wide triangular
Лист: уши Leaves: wax	малки small	няма или много малки absent or very weak
Височина на върха на петурата в сравнение с основата на дръжката при стъблото Height of flat blade compared to the base of petiole at the stem	ниска short	висока high
Време на цъфтеж Time of flowering	рано early	средно рано medium
Гъстота на езичести цветове Density of ray flowers	редки sparse	гъсти much
Листче в основата на питата Leaf of the base of the head	удължена elongated	кръгла rounded
Листче в основата на питата/положение в сравнение с питата Leaf of the base of the head/attitude in comparison to the head	не я обхваща not enveloped	обхваща я слабо enveloped slightly
Пита: положение Head: attitude	полуобърната, с право стъбло half-turned down with vertical stem	полуобърната, с извито стъбло half-turned down with inclined stem
Тип на разклоняване на растенията Type of branching	по цялото стабло full branching	предимно на върха predominantly in the top
Семка: размер Seed: size	малка small	средна medium
Семка: форма Seed: shape	удължена elongated	яйцевидно закръглена ovoid wide
Семка: дебелина спрямо ширина Deed: thickness in comparison to the width	тънка thin	средна medium
Семка: ивици по средата Seed: lateral stripes	силно изразени strong expressed	липсват absent

At severe attacks, tearing lamina defoliation was observed. From diseased plants receive lower seed yield and smaller seed oil content were obtained from damaged plants.

The only way for disease control was using of resistant lines, varieties and hybrids of sunflower.

In this study besides statistically proved morphological changes, mutation for resistance to the disease *Septoria helianthi* Ellis et Kellerman was also established. The initial genotype showed susceptibility to septoriosis on infection field. Through years of testing under the same climatic conditions, the genotype 249 R was valuated as fully susceptible to the disease, whereas the mutant line 103 RM was resistant.

The conclusion is that this mutation was resulted from the influence of mutagen ultra sonic. This was because the nutritional medium M, used for further development of immature zygotic embryos, did not contain any mutagens. Similar mutation connected to the resistance to septoriosis was received after the treatment of immature zygotic embryos of other genotype 377 R with ultra sonic at a dose of 25.5 W/cm^2 for 1 min and 2 min. Sustainability in both obtained mutant lines 143 RM and 145 RM was steadily inherited in the next generations.

Although induced mutagenesis could not be predicted or programmed, in this research a mutation for resistance to *Septoria* disease, have obtained, besides morphological mutations. This mutation was inherited steadily by the progeny of the mutant line.

The mutation is valuable, because the new line will be included in heterosis breeding for developing of disease-resistant hybrids.

CONCLUSIONS

1. Statistically significant changes in most of the studied morphological characteristics were received. The increase in plant height, reduction in the length of the branches, a change in the location of the branches, the increase in the size of the leaves generates a mutant line 103 RM with altered architectonic.

2. Rreceiving of mutations both in terms of higher oil content and higher 1000 seeds weight at line 103 RM were valuable combinations suitable for heterosis breeding of sunflower.

3. Differences at 10 morphological and biochemical parameters and more than 16 indexes according to classification of UPOV and the phenological change later flowering gives us the reason to believe that we have obtained new mutant line 103 RM characterized by distinctness, uniformity and stability from the control line 249 R.

4. Mutation for resistance to the disease *Septoria helianthi* Ellis et Kellerman was obtained. The rise mutations were due to the influence of mutagen ultra sonic, because the medium M used for further development of the young plants did not consist mutagenic factors and duration of cultivation of immature zygotic embryos was short.

5. The changes in the mutant line were variations in the values of important agronomic indexes, but the emergence of new signs were not observed.

6. The induction of mutations was unpredictable process, but in our study was obtained morphological, biochemical, phytopatological and phenological changes with stable inheritance in progeny of the new mutant line.

REFERENCES

Encheva, Yu., 2013. Mutantni linii slanchogled, sazadeni chrez tretirane s ultrazvuk na nezreli zarodishi ot clanchogled na genotip 377 R. Selskostopanska nauka 46 (2): 34–40.

Atanassov, A., 1988. Biotechnology of agriculture, Sofia: pp. 278.

Azpiroz, I.S., P. Vincourt, H. Serieys, A. Gallais, 1988. La culture *in vitro* des embryous immatures dans l'acceleration du cycle de selection des lignees de tournesol et ses effects morphovegetatifs. Helia 10: 35–38.

Ahloowalia, B.S., M. Maluszynsky, K. Nichterlein, 2004. Global impact of mutation-derived varieties. Euphytica: 187–204.

Christov, M., V. Nikolova, 1996. Increasing of the sunflower genetic diversity by mutagenesis. In: Proceeding of 14th ISC Beijing/Shenyang, China, pp. 19–30.

Datta, S.K., D. Chakrabarty, A.K. Verma, B.K. Banerji, 2011. Gamma ray induced chromosomal aberration and enzyme related defense mechanism in *Alium cepa* L. Caryologia 64(4): 388–397.

Jambhulkar, S.J., D.C. Joshua, 1999. Induction of plant injury, chimera, chlorophyll and morphological mutations in sunflower using gamma rays. Helia 2: 63–73.

Ebrahimi, A., P. Maury, M. Berger, F. Shariati, P. Grieu, A. Sarrafi, 2008b. Genetic improvement of oil quality in sunflower mutants under water stressed conditions (ed), The 17th International sunflower conference, (pp. 2: 509–512) Cordoba, Spain.

Ebrahimi, A., A. Sarrafi, 2012. Genetic variability and identification of markers associated with germination parameters under water stress condition. Iranian journal of genetics and plant breeding 1(2): 1–8.

Encheva, J., P. Ivanov, F. Tsvetkova, V. Nikolova, 1993. Development of a new initial breeding material in sunflower (*Helianthus annuus* L.) using direct organogenesis and somatic embryogenesis. Euphytica 68: 181–185.

Encheva, J., F. Tsvetkova, P. Ivanov, 2002. Creating genetic variability in sunflower through the direct organogenesis method, independently and in combination with gamma irradiation. Helia 25(37): 85–92.

Encheva, J., F. Tsvetkova, P. Ivanov, 2003a. Comparison between somaclonal variation and induced mutagenesis in sunflower (*Helianthus annuus* L.). Helia 26(38): 91–98.

Encheva, J., F. Tsvetkova, P. Ivanov,, 2003b. Comparison between somaclonal variation and induced mutagenesis in tissue culture of sunflower line RHA-857 (*Helianthus annuus* L.). Bulgarian Journal of Agricultural Science 9(2): 179–184.

Encheva, J., M. Christov N. Nenov, F. Tsvetkova, P. Pvanov, P. Shindrova, V. Encheva, 2003c. Developing genetic variability in sunflower (*Helianthus annuus* L.) by combined use of hybridization with gamma radiation or ultrasound. Helia 26(38): 99–108.

Encheva, J., 2009. Creating sunflower (*H. annuus* L.) mutant lines using induced mutagenesis. Bulgarian Journal of Agricultural Science 15(2): 109–118.

Fernandez-Martinez, J.M., J. Osorio, M. Mancha, R. Garces, 1997. Isolation of high palmitic mutants on high oleic background. Euphytica 97: 113–116.

Fernandez-Moya, V., E. Martinez-Force, R. Garces, 2002. Temperature effect on a high stearic acid sunflower mutant. Phytochemical 59: 33–38.

*Ivanov, P., D. Petakov, V. Nikolova, E. Pench*ev, 1988. Sunflower breeding for high palmitic acid content in the oil. 12th International sunflower conference, Novi Sad, Yogoslavia, Int. Sunflower association (pp. 463–465) Toowoomba, Australia.

Gamborg, O.L., R.A. Miller, K. Ojima, 1968. Nutriments requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158.

Giriraj, K., S.R. Hiremath, A. Seetharam, 1990. Induced variability for flowering, seed weight and oil content in parental lines of sun flower hybrid BSH-1. Indian journal of genetics and plant breeding, 50: 1–7.

Gomez-Pando, L.R., A. de la Varra, 2013. Developing genetic variability of quinoa (*Chenopodium quinoa* Willd.) wilh gamma radiation for use in breeding programs. American journal of plant science 4: 349–355.

Kostov, K., R. Batchvarova, S. Slavov, 2007. Application of chemical mutagenesis to increase the resistance of tomato to *Orobanche ramosa* L. Bulgarian Journal of Agricultural Science 13: 505–513.

Murashige, T., F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissues cultures. Plan. Physiol. 15: 473–497.

Nibipour, A.I., B. Yazdi-Samadi, A. Sarrafi, 2004. Genetic control of some morphological mutant in sunflower. Journal of genetic and breeding 58: 157–162.

Oliveira, M.F., A.T. Neto, R.M.V.B.C. Leite, V.B.R. Castiglioni, C.A.A. Arias, 2004. Mutation breeding in sunflower breeding in sunflower for resistance to alternaria leaf spot. Helia 27(41): 41–50.

Soroka, A., V. Lyakh, 2009. Genetic variability in sunflower after mutagen treatment of immature embryos of different ages. Helia 32(51): 33–46.

Soroka, A.I., 2014. Induced genetic variability under ethyl methanesulfonate treatment of immature embryos and mature embryos. Naukovo-tehnicheskiy byuletin institutu oliynih kultur NAAN, № 20: 12–20.

Skirvin, R.M., 1978. Natural and induced variation in tissue culture. Euphytica 27: 241–266.