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СЕЛЕКЦИЯ НА ТЕХНИЧЕСКИ И ДРУГИ КУЛТУРИ
BREEDING of TECHNICAL CROPS

SUNFLOWER MUTANT LINE, DEVELOPED USING INDUCED MUTAGENESIS

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Резюме

Енчева Ю., 2009. Слънчогледова мутантна линия създадена чрез индуциран мутагенез.

Незрели зиготни зародиши от слънчогледов възстановител на фертилността RHA-857 са третирани с ултразвук преди да бъдат платирани върху хранителна среда за Ембрио култура. Получените растения са изолирани и самоопрашени в продължение на няколко генерации.

Генетичните изменения появили се по време на мутационния процес включват 15 морфологични и биохимични признака. В сравнение с контролната линия RHA-857, намаление в средната стойност на показателите бе регистрирана при 60.0% от общия брой признаци и обратно, доказано повишение бе наблюдавано при 13.3%. Създадена бе мутантна линия възстановител на фертилността с по-високо маслено съдържание, по-ниско стъбло, устойчива на фома, толерантна на фомопсис и стабилно наследявани в следващите генерации. Това е ценна и желана комбинация в селекционната прогрема при слънчогледа. Нашите резултати показват, че индуцирания мутагенез може успешно да се използва за създаване на нови линии слънчоглед, полезни в селекционната програма.

Ключови думи: слънчоглед - *Helianthus annuus* - незрели зиготни зародиши - ултразвук - мутагенез

Abstract

Encheva, J., 2009. Sunflower mutant line, developed using induced mutagenesis

Immature sunflower zygotic embryos of sunflower fertility restorer line RHA-857 were treated with ultrasound before plating to medium for Embryo culture. All plants produced were isolated and self-pollinated for several generations. The genetic changes occurring during the mutation procedure included fifteen morphological and biochemical characters. In comparison to the check line RHA-857, decreasing of the mean value of the indices was registered for 60.0 % of the total number of characters and vise verse, significant increasing was observed for 13.3 %. It was created mutant sunflower restorer line with increased oil content in seed, reduced plant height, resistance to *Phomopsis helianthi* and *Phoma macdonaldii* and stable inheritance in the next generations. This is valuable and desire combination at breeding program of sunflower. Our results showed that induced

mutagenesis can be successfully used to develop new sunflower lines, useful for breeding programme.

Key words: sunflower - *Helianthus annuus* - immature zygotic embryos - ultrasound-mutagenesis

INTRODUCTION

The development of variable breeding material is a primary task of the genetic and breeding programs in sunflower. The new approaches, tissue culture in combination with induced mutagenesis, provide an additional possibility to enrich genetic variability in this crop.

The studies on genetic variation in regenerants from sunflower are not very numerous. Significant changes and molecular evidence for genetic variation in sunflower regenerants obtained through the direct organogenesis method in sunflower genotypes were reported by Encheva *et al*., 2003.

Induced mutagenesis, both physical and chemical, proved favorable for mutation induction in tissue cultures. Encheva *et al*. (1993, 2002, 2003a, b) have reported statistically significant changes in morphological characters of plants regenerated from immature zygotic embryos of sunflower, independently and in combination with gamma irradiation or ultrasound. Positive results were obtained when induced mutagenesis and tissue cultivation were combined appropriately in potato (Ahloowalia, 1990), in oil crops (Ashri, 1993), in tomato (Gavazi *et al*., 1987), in rice (Maluszynski *et al.,* 1994), many crops (Mike *at al*., 1990), in maize, banana and plantain (Novak *et al.,* 1988, 1990) and in wheat (Cheng *et al.,* 1990).

According to Ahloowalia (2001) in agriculture more than 1800 cultivars obtained either as direct mutants or derived from their crosses have been released worldwide in 50 countries.

Black (*Phoma macdonaldi*/*Leptosphaeria lindquistii*) and grey spots caused by *Diaporthe/Phomopsis helianthi* Munt.Cvet.et al. are a serious problem for sunflower production in a number of countries worldwide (Scoric, 1994; Gulya, 1997; Carre, 1993), including Bulgaria (Encheva, V. & P. Shindrova, 1990). The disease has epiphytotic outbreaks in years with hot and moist weather during sunflower vegetation. Breeding of resistant cultivars and hybrids is the most reliable method for disease control.

The aim of this study was: a) to develop variable R lines from sunflower through induced mutagenesis *in vitro* in initial genotype RHA-857, and b) to evaluated the new genetic material for resistance to phomopsis and phoma, and c) to carry out biometric and biochemical investigations on the new line.

MATERIAL AND METHODS

A part of the experiments were carried out under laboratory conditions, and another – at the field trial of Dobroudja Agricultural Institute-General Toshevo. The morphological and biochemical traits of the new mutant line and the control genotype were studied during 2006-2008.

A/ Laboratory experiments

Induced mutagenesis, In vitro cultivation of immature zygotic sunflower embryos and

Developing of mutant line

The American fertility restorer line RHA-857, witch is highly homozygtic, was used as donor material. A main requirement to the initial plant material used according to the methods of embryo culture in combination with ultrasound is to be genetically pure, i.e. ho-

mozygotic to the highest possible degree. Therefore the control line RHA-857 with very good morphological uniformity was chosen as initial material for induced mutagenesis.

Plants were grown in the field and were hand-pollinated. The immature zygotic embryos (11-13 days old) were treated with: 1) ultrasound at dose 25.5 W/cm^2 for 3 min. Immature embryos were 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.

Sterilized embryos were aseptically isolated and plating on nutrition medium M for further growing (Azpiroz *et al*.1988): 1/2 MS (Murashige, T. & F. Skoog, 1962) macro salts, MS micro salts, B5 vitamins (Gamborg *et al*., 1968), 20 g/l sucrose, pH-5.7. Sixty zygotic embryos were plated for each variant. The conditions for cultivation were: 25^o C, 16/8 h photoperiod for one week. R0M0 plants (nomenclature according Novak at al., 1988), which formed roots were transplanted for further grown, self-pollinated and harvested by single plants under greenhouse conditions. The seeds produced (R1M1) were sown in the field.

B/ Field experiments

Biometric evaluation of control line RHA-857 and the mutant line 106 RM

As a result from selfing and individual selection, new sunflower lines were produced in R8M8 generation. The lines were investigated with regard to some main characteristics concerning breeding in sunflower. In each generation biometric studies of plants and biochemical characterization of seeds were carried out. The evaluation of the check line RHA-857 and the new developed mutant line 106 R was made on 10 plants for each individual year, and included 15 main agronomic traits as oil content in seed, 1000 seed weight, plant height, leaf width, leaf length, number of leaves, leaf petiole length, head diameter, stem diameter, number of branches, length of branches, diameter of branch head, seed length, seed thickness and seed width. 1000 seed weight (g) was determined on three samples of 50 seeds per head each.

The control data were collected from plants of the genotype RHA-857 which was grown in field together with the mutant plants.

C/ Biochemical analysis

To determined the oil content of air-dry seeds from the materials included in the study, Nuclear-magnetic resonance (Newport Instruments Ltd., 1972) was used.

D/ The Phytopathological evaluation of the control genotype RHA-857 and seeds from the obtained mutant line 106 RM was performed with regard to the phomopsis (*Phomopsis helianthi*, Munt.-Cvet. et al.) and phoma *(Phoma macdonaldii*, Boerema/*Phoma oleracea* var. *helianthi-tuberosi* Sacc) at the infection fields of DAI, General Toshevo.

The evaluation was carried out according to standard methodologies during the period 2004-2007. According Peres and Regnault, 1986 the natural infection was estimated at the end of September. The plant residues (stems) showing symptoms of grey and black spots were collected from the field. At stage 5-6th leaf of sunflower 3-4 stems per m^2 were scattered between the rows. Each week the plants were irrigated from the beginning of buttoning till mass flowering. The number of sprinklings was determined by the meteorological conditions and varied from 2 to 3 per week. The reaction of the hybrids was registered at full flowering according to the following scale for *phomopsis :* 0 - no symptoms; 1 - small single spot around the leaf petiole; 2 - spots up to 5 cm long; 3 - spots covering two or more internodules; 4 - stem breaking at the place of damage.

To estimated *phoma* ware use four level scale: 0 - no symptoms; 1 - necrotic spot around the leaf petiole; 2 - several merge necrotic spots; 3 - all stem is cover with necrotic spots or stem breaking at the place of damage.

F/ Statistical analysis

The developed new mutant line were analyzed statistically with regard to the agronomic traits such as oil content in seed, 1000 seed weight, 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 thickness and seed width.

The following statistical analysis was performed: a) variance analysis using the following model: $Y_{яjk}=μ+б_я+в_j+(бв)_{яj}+e_{яjk}$ (Everett, B.S., 1984)**.** Analysis of the experimental data was done by the statistical package BIOSTAST 6.0.

Table 1. Dispersion analysis of the studied indices. Harvest years 2006-2008

Indices	A	B	A x B	E
Plant height	1926.67'	7665.00*	781.67**'	21.48
Head diameter	33.75***	9.65***	1.55**	0.31
Leaf length	3.75	100.35**'	13.65*	4.07
Leaf width	1.67	189.82**'	40.82***	3.81
Stem diameter	163.35**	349.55**'	225.95**'	2.61
Number of brunches	79.35***	232.52**'	41.15**	6.03
Length of branches	166.67**	623.22**'	87.32***	8.68
Number of leaves	26.67**	9.22**	30.72***	2.52
Diameter of branch head	0.27	3.15*	3.32**	0.63
Leaf petiole length	273.07**'	41.32***	34.62***	0.81
1000 seed weight	2184.07*	185.62**	63.01	24.19
Oil content in seed	522.15**'	80.00***	92.60***	2.10
Seed length	14.02***	0.02	0.02	0.02
Seed width	14.02***	0.02	0.02	0.05
Seed thickness	0.02	0.02	0.02	0.02
df	1	2	2	54

А-генотип, В-околна среда, статистическа доказаност при -* p=0.05, ** p=0.01 and *** p=0.001
A – genotype, B – environments, statistical significance at - * p=0.05 , ** p=0.01 and *** - p=0.001

Фигура 1. Контролен генотип RHA-857 R (ляво) и мутантна линия 106 RM(дясно)
Figure 1. Control genotype RHA-857 R (left) and mutant line 106 RM (right)

RESULTS AND DISCUSSION

Evaluation according to quantitative traits in mutant line 106 RM

Mutant line 106 RM (Figure 1) originating from the American fertility restorer line RHA-857 (Figure 1) were selected due to their statistically significant and economically important morphological and biochemical changes and resistance to the *Phomopsis helianthi* and *Phoma macdonaldii*.

Differences with the highest level of statistical significance were established in the genetic potential of the indices oil content in seed, 1000 seed weight, plant height, number of leaves, leaf petiole length, head diameter, number of branches, length of branches, stem diameter, seed length and seed width (Table 1).

Factor B (environmental conditions) had a effect on a large part of the characters such as: oil content in seed, 1000 seed weight, plant height, leaf width, leaf length, number of leaves, leaf petiole length, head diameter, stem diameter, number of branches, length of branches and diameter of branch head. It was found out that the char-

acters seed width, seed length and seed thickness were stable and not affected by the changes in the climatic conditions.

The interaction of the two factors (A and B) was significant for the indices oil content in seed, plant height, leaf width, leaf length, number of leaves, leaf petiole length, head diameter, stem diameter, number of branches, length of branches and diameter of branch head (Table 1).

Statistical significance of the investigated factors, as well as genotype x environment (G x E) interaction, was established for the characters oil content in seed, plant height, number of leaves, leaf petiole length, head diameter, stem diameter, number of branches and length of branches (Table 1).

Таблица 2. Морфологични и биохимични характеристики на мутантна линия 106 RM, създадена чрез индуциран мутагенез. Години на изследване 2006-2008, средни данни

Table 2. Morphological and biochemical characteristics of mutant lines 106 RM, developed by induced mutagenesis. Harvest years 2006-2008, average data.

Показатели Indices	Контролна линия RHA-857 Control line RHA-857	Линия 106 RM ултра звук Line 106 RM ultrasound	LSD
Plant height (cm)	109.67	**98.33-c**	Gd 5% = 2.14
Number of leaves (no)	28.10	**29.43+b**	Gd 5% = 0.78
Leaf width (cm)	16.07	16.40	Gd 5% = 0.73
Leaf length (cm)	16.90	16.40	Gd 5% = 0.90
Petiole length (cm)	12.27	**8.00-c**	Gd 5% = 3.99
Stem diameter (mm)	18.90	**15.60-c**	Gd 5% = 0.77
Head diameter (cm)	10.80	**9.30-c**	Gd 5% = 0.27
Number of branches (no)	11.53	**9.23-c**	Gd 5% = 1.18
Length of branches (cm)	16.50	**13.17-c**	Gd 5% = 1.42
Diameter of branched head (cm)	5.17	5.03	Gd 5% = 0.39
Seed width (mm)	9.97	**9.00-c**	Gd 5% = 0.07
Seed length (mm)	3.97	**3.00-c**	Gd 5% = 0.11
Seed thickness (mm)	2.00	2.00	Gd 5% = 0.12
Oil content in seed (%)	40.00	**45.90+c**	Gd 5% = 0.79
1000 seed weight (g)	44.10	**32.03-c**	Gd 5% = 2.59

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

Фигура 2. Лист на мутантна линия 106 RM (ляво) и контрола RHA-857 R (дясно)
Figure 2. Leaf of mutant line 106 RM(left) and control RHA-857 R (right)

Plant height is one of the morphological indices most often investigated in cultural sunflower, it is consider a quantitatively inherited character. Breeding to improve stem strength is a major objective of the researchers of sunflower. In our study the significant change in line 106 RM (Figure 2) was towards decrease of the mean index value of plant height with 11.3 cm according to the control RHA-857 (Table 2).

Stem breakage due to adverse growing conditions can reduce yields significant in some years. Reducing plant height may lead to increase in yields by increasing standability of sunflower.

Decrease in plant height has been reported in somaclonal lines (Encheva *et al*., 1993, 2002,

2003) and in using the direct organogenesis method in combination with gamma irradiation (Encheva *et al*., 1993, 2002). Novak *et al*. (1988) reported plant height reduction after treatment of immature zygotic embryos of maize with 5 Gy.

Decrease in plant height of sunflower plants has also been observed by Hristov, 1996, after treatment of air dry seeds with gamma rays.

In this study reduced plant height with similar number of leaves showed that the internodes length was reduced in mutant line. According Miller & Hammond, 1991 the additive component of genetic effects controlling reduced height with similar number of leaves in sunflower ranged from 48 to 71 %, while the dominant component ranged from 3 to 16 %.

Instead decreasing of plant height the mutant line was characterized with decreasing of stem diameter with 3.3 mm. (Table 2).

The number of branches, length of branches and leave petiole length (Figure 2) were characterized with decreasing of mean in comparison to the check line RHA-857 with 1.86 cm., 3.33 cm., and 4.27 respectively (Table 2). The differences at the three indices were at the highest degree of significance.

Differences were observed in relation to the indices seed width and seed length. The reduction at the new line was with 0,97 и 0,97 mm, respectively. Instead those two traits, the seeds of mutant lines 106 RM were differ with their black color, while at the control, seeds were black with green stripes in both marginal and lateral (Figure 3).

Фигура 3. Семена на контрола RHA-857 R (ляво) и мутантна линия 106 RM (дясно)
Figure 3. Seeds of control RHA-857 R (left) and mutant line 106 RM (right)

Oil content in seed is the most important agronomic index of sunflower. A significant increase of 5.9 % was observed at the mutant line 106 RM. One of the aims of our study was to develop variable R lines from sunflower with higher oil content through induced mutagenesis. The increased oil content of the mutant restorer line produced is a valuable change with significant practical importance for the sunflower breeding programme. The data presented at this study confirmed the conclusions made previously that ultrasound in R lines (Encheva *at al*., 2003) and in B lines (Encheva *at al*., 2004) leads to genetically increasing of oil content in seed.

In our study the plant height, 1000-seed weight and oil content in seed were the most unstable, based on all investigated characters.

The changes indexes (11 from 15) were observed in line 106 RM i.e. 73.3 % of the total number of characters**.**

Based on all 15 agronomic traits investigated, it can be determined that the reduction in the mean value (9 from 15) in comparison to the control RHA-857 R was observed for 1000 seed weight, plant height, leaf petiole length, head diameter, number of branches, length of branches, stem diameter, seed length and seed width, i.e. 60.0 %. of the total number of characters**.**

Vice verse, positive significant differences were registered manly for number of leaves and oil content in seed i.e. 13 % of the total number of indices. Stability after induced mutagenesis of immature zygotic embryos was demonstrated by the characters leaf width, leaf length, diameter of branched head and seed thickness. The line 106 RM was haracterised with light green color of leaves, while in control genotype the color was dark green.

The line 106 RM possess a value of the indices “beginning of flowering” and “mass flowering with four days later than that of the check line**.**

Phomopsis is the most destructive pathogen of sunflower. In extreme cases, it can

compromit sunflower production and cause significant yield losses (Skoric, 1985). After three years phytopatological evaluation the new line 106 RM showed resistance to *Phoma macdonaldii* in comparison to the check line RHA-857 which posses middle resistance. During this study mutant line showed resistance to *phoma,* as a control line.

Combining induced mutagenesis with the embryo culture method, it can be assumed that the new variability obtained is due only to the effect of the mutagen. This assumption is confirmed by the fact that the embryo culture method alone does not generate variation due to the lack of mutagen factors in the nutrition medium and the short period of *in vitro* cultivation of the immature zygotic embryos.

The available literature on sunflower does not provide data on treatment of immature zygotic embryos with ultrasound. In this respect the approach is especially valuable due to the fact that immature sunflower zygotic embryos are treated at an early stage of development, i.e. this is functional tissue (Encheva *at al*., 2008a, b). It was established that the possibilities of experimental mutagenesis in using embryos at an early stage of their development are greater, as compared to air dry seeds (Atanassov, A., 1988).

Although induced mutagenesis is a random and unpredictable process, it is an invaluable fact that the occurred morphological and biochemical mutations were of stable inheritance in the progenies of the fertility restorer line 106 RM.

Our results confirmed the conclusion of Skirvin, 1978, that mutagenesis, physical or chemical, is favorable for induction of mutations in tissue cultures.

CONCLUSION

Following the main problems of sunflower breeding at DAI, morphological, biochemical and phytopathological variability was developed by induced mutagenesis.

Embryo culture method was used because does not generate variation. Our assumption is that the new variability obtained is due only to the effect of the mutagen i.e. ultrasound. In other hand embryo culture method allowed considerable shortening of the breeding process.

Based on all 15 agronomic characters investigated, it can be determined that the reduction in the mean value (9 from 15) in comparison to the control RHA-857 R was observed for 1000 seed weight, plant height, leaf petiole length, head diameter, number of branches, length of branches, stem diameter, seed length and seed width, i.e. 60.0 %. of the total number of traits**.**

Vice verse, positive significant differences was registered manly for number of leaves and oil content in seed i.e. 13 % of the total number of characters.

Stability after induced mutagenesis of immature zygotic embryos was demonstrated by the characters leaf width, leaf length, diameter of branched head and seed thickness.

The line 106 RM was haracterised with different color of leaves and seeds in comparison to the check line RHA-857.

The observed morphological changes were alterations in the ratio between the most important traits, but the synthesis of unusual characteristics were not observed.

We succeed to create mutant sunflower restorer line with increased oil content in seed, reduced plant height, resistance to *Phoma macdonaldii* and *Phomopsis helianthi*. This is very important and desire combination. Induced mutagenesis in sunflower immature embryos is suitable to use in breeding program for production of new breeding material.

Further evaluation is needed to achieve a more complete description of the new line, produced in terms of fertility restoration and general combining ability.

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