Bulgarian Journal of Agricultural Science, 15 (No 2) 2009, 109-118
Agricultural Academy

CREATING SUNFLOWER MUTANT LINES (*HELIANTHUS ANNUUS* L.) USING INDUCED MUTAGENESIS

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Abstract

ENCHEVA, J., 2009. Creating sunflower mutant lines (*Helianthus annuus* L.) using induced mutagenesis. *Bulg. J. Agric. Sci.*, 15: 109-118

Immature sunflower zygotic embryos of sunflower fertility restorer line 374 R were treated with ultrasound and gamma radiation before plating embryos to culture medium. All plants were isolated and self-pollinated for several generations. New sunflower forms with inherited morphological and biochemical changes were obtained. The genetic changes occurring during the mutation procedure included fourteen morphological and biochemical characters.

In comparison to the check line 374 R, decreasing of the mean value of the indexes was registered for 33 % of the total number of characters and vise verse, significant increasing was observed for 60 %.

Mutation for resistance to the local population of *Orobanche cumana* (race A-E) was obtained from the susceptible Bulgarian control line 374 R. Two investigated mutant lines possessed 100 % resistance to *Orobanche* and stable inheritance in the next generations. Our results showed that induced mutagenesis in sunflower can be successfully used to develop new lines useful for heterosis breeding.

Key words: sunflower, *Helianthus annuus*, immature zygotic embryos, gamma radiation -^{137}Cs, ultrasound, mutagenesis, new breeding material, resistance, *Orobanche cumana*

Introduction

Development of new sunflower hybrids possessing high disease resistance and new oil and protein qualities depends in the availability of suitable genetic resources. A main task of the sunflower breeders are to established and recombine the available genetic variability and to create the most suitable recombinants for the specific environment by breeding and evaluation.

Because of the narrowed germplasm of cultivated sunflower the new approaches, tissue culture in combination with induced mutagenesis, provide an additional possibility to enrich genetic variability in this crop. Induced mutagenesis, both physical and chemical, proved favorable for mutation induction in tissue cultures.

Encheva et al. (1993, 2002, 2003) have reported statistically significant changes in morphological and biochemical characters of sunflower. Plants were regenerated from immature zygotic embryos through direct organogenesis method along and in combination with gamma irradiation. Positive results were obtained when induced mutagenesis and tissue cultivation were combined appropriately in tomato (Gavazi et al., 1987), maize (Novak et al., 1988) and 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.

Although sunflower breeding has been very successful throughout the last decades, a number of aims remain to be achieved, e.g. resistance to various diseases and the parasite *Orobanche*. However, these efforts are obviously limited in commercial sunflower which has to be enlarged by the utilization of wild species, mutagenesis or tissue culture. Therefore development of new lines resistant to the parasite broomrape is very important for sunflower breeding.

Broomrape (*Orobanche cumana* Wallr.) is a parasite on the roots of sunflower plants and causes serious damages to sunflower production (Skoric, 1994). The genus is spread on 16 million hectares in the Mediterranean region and South-west Asia., and the most important species, *O. cumana*, has become a limiting factor for the crop in Eastern Europe and in Spain (Casteljon-Munoz et al., 1991). According to Kaya et al. (2004), about 80 % of the sunflower areas in Turkey (Trakia region) are infested with seeds of the parasite. Furthermore, the parasite forms new more virulent races which overcome the resistance of the varieties and hybrids commonly used in production; Pacureanu-Joita et al., 1998; Alonso, 1996; Fernandez-Martinez et al., 2000). This complicates the control of broomrape.

Broomrape presents serious problems to sunflower production in Bulgaria, as well. It is constantly expanding its distribution area, forming new more virulent races (Shindrova, 1994). This leads to considerable losses expressed, on the one hand, in yield decrease, and on the other - in worsened quality of the obtained produce (Shindrova et al., 1998). Because of the narrowed germplasm of cultivated sunflower, induced mutagenesis can be another alternative method of conventional ones for producing plants resistant to the parasite *O. cumana*.

The aim of this study was: a) to develop mutant sunflower restorer lines through induced mutagenesis, and b) to evaluated the new genetic material for resistance to the local population of the parasite *O. cumana* (races A-E) and c) to carry out morphological and biochemical investigations on the new lines.

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 lines and the control genotype were studied during 2005-2008.

A/ Laboratory experiments

Induced Mutagenesis, in vitro Cultivation of Immature Zygotic Sunflower Embryos and Developing of Mutant Lines

The Bulgarian fertility restorer line 374 R, 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 and gamma radiation is to be genetically pure, i.e. homozygotic to the highest possible degree. Therefore the control line 374 R with very good morphological uniformity was chosen as initial material for induced mutagenesis.

Plants were grown in the field and were hand-pollinated. The isolated immature zygotic embryos (11-13 days old) were treated with: 1) ultrasound at dose 25.5 W/cm^2 for 10 min and 2) ionizing radiation such as gamma rays (^{137}Cs) at dose of 50 Gy (the power of the dose being 0.338 krad/min=3.38 Gy/min). 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.

Sterilized embryos were plating on nutrition medium M for further growing (Azpiroz et al.,1988): 1/2 MS (Murashige and 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: 25ºC, 16/8 h photoperiod for one week. The M1 plants which formed roots were transplanted to soil and were further grown and harvested by single plants under greenhouse conditions. The M2 seed were grown in the field.

B/ Field experiments

Biometric evaluation of control line 374 R and the mutant lines 193 RM and 194 RM

As a result from selfing and individual selection, new sunflower lines were produced in R5M5 generation. The main criterion for selection was resistance to *Orobanche cumana.* The lines were investigated with regard to some main characteristics concerning breeding in sunflower, also. In each generation biometric studies of plants and biochemical characterization of seeds were carried out. The evaluation of the check line 374 R and the new developed mutant lines 193 RM and 194 RM 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 374 R 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 374 R and F3 seeds from the obtained mutant lines 193 RM and 194 RM was performed with regard to the local *Orobanche* population (race A-E) at the Sunflower Phytopathology Laboratory during 2005-2007. Broomrape seeds were collected from several regions in Bulgaria. Broomrape resistance was evaluated under greenhouse conditions according to Panchenko (1975), slightly modified to local conditions. A mixture of soil and sand (2:1) was prepared, and 0,2 mg broomrape seeds were added to each kilogram of the mixture. Sunflower was grown in this substrate in the following order: 50 plants + 10 plants (standard-AD-66) in every container. They were placed in a greenhouse under controlled conditions with irrigation. Forty-five days after planting, the roots of all sunflower plants were cleaned and checked for the existence of the parasite. Broomrape resistance was calculated as percentage of non-infected plants. The reaction of 50 plants from each genotype was recorded using the following scale: 0 % = S (sensitive); 100 % = R (resistant**).**

E/ Statistical analysis

The developed new mutant lines 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: à) variance analysis using the following model: $Y_{ijk}=\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$ (Everett, 1984); b) cluster analysis by Euclidean linkage distances (Elliott et al., 1982). Analysis of the experimental data was done by the statistical package BIOSTAST 6.0.

Results and Discussion

Evaluation according to quantitative traits in mutant lines

The study included economically important morphological and biochemical traits. Mutant lines (Figure 1) originating from the fertility restorer line 374 R were selected due to their resistance to the parasite *Orobanche cumana* and statistically significant morphological and biochemical changes.

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

Table 1
ANOVA (MS-mean square) of the studied indices

Indices	A	B	A x B	E
Plant height	111.51***	2585.54*****	284.41***	14.75
Head diameter	110.53***	21.23***	0.77	1.42
Leaf length	54.68***	214.04***	16.76***	2.99
Leaf width	89.54***	183.88***	14.08***	1.93
Stem diameter	365.48***	173.51***	16.99***	2.74
Number of brunches	41.68***	114.54***	11.21***	1.97
Length of branches	779.48***	2576.68***	56.64	29.25
Number of leaves	20.63***	344.03***	64.67***	1.05
Diameter of branch head	13.14***	15.88***	0.06	0.93
Leaf petiole length	52.14***	12.21***	1.48	0.96
1000 seed weight	1275.11***	35.85*	27.50*	8.65
Seed length 24.58	24.58***	0.28	0.93***	0.16
Seed width	0.011	0.011	0.03	0.13
Seed thickness	3.24***	0.58*	0.11	0.15
Oil in seed	173.79***	6.01	34.08***	3.11
df	2	2	10	81

A – genotype, B – environments, *,-statistical significance by - p=0.05, **p=0.01 and *** - p=0.001

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 and seed thickness.

Factor B (environmental conditions) had a effect on a large part of the characters such as: 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 and seed thickness. It was found out that the characters seed width, seed length and oil content were stable and not affected by the changes in the climatic conditions (Table 1).

Fig. 1. Control Bulgarian genotype 374 R (left), mutant lines 193 RM and 194 RM (right)

The interaction of the two factors (A and B) was significant for the indixes 1000 seed weight, plant height, leaf width, leaf length, number of leaves, number of branches, stem diameter, seed length and oil in seed.

Statistical significance of the investigated factors, as well as genotype x environment (G x E) interaction, was established for the characters 1000 seed weight, plant height, leaf width, leaf length, number of leaves, number of branches, length of branches and

Table 2
Morphological and biochemical characteristics of mutant lines 193 RM and 194 RM, developed by induced mutagenesis. Harvest years 2005-2008, average data

Traits	Control line 374 R	Line 139 RM 50 Gy	Line 194 RM us	LSD
Plant height, cm	73.43	74.23	77.10 +b	Gd 5%= 2.88
Number of leaves, no	20	18.00 -b	19	Gd 5% = 1.20
Leaf width, cm	18	15.77 -c	14.60 -c	Gd 5% = 0.93
Leaf length, cm	16.03	15.23	13.40 -c	Gd 5% = 1.07
Petiole length, cm	9.67	7.40 -c	7.37 -c	Gd 5% = 0.33
Stem diameter, mm	13.57	20.37 +b	18.33 +c	Gd 5% = 1.00
Head diameter, cm	9.47	12.53 +c	13.00 +c	Gd 5% = 0.35
Number of branches, no	5	7.00 +c	5	Gd 5% = 0.74
Length of branches, cm	28.17	20.60 -c	18.47 -c	Gd 5% = 2.89
Diameter of branched Head, cm	4.89	5.73 +c	6.17 +c	Gd 5% = 0.54
Seed width, mm	3.07	3.1	3.07	Gd 5% = 0.18
Seed length, mm	9.4	10.93 +c	11.00 +c	Gd 5% = 0.23
Seed thickness, mm	4.36	4.93 +c	4.87 +a	Gd 5% = 0.20
Oil content in seed, %	38.44	42.74 +c	42.47 +c	Gd 5% = 1.10
1000 seed weight, g	31.76	42.78 +c	43.30 +c	Gd 5% = 1.68

a,b and c = significant differences at levels 0.05, 0.01 and 0.001, respectively.

stem diameter (Table 1).

Plant height is one of the morphological indices most often investigated in cultural sunflower (Table 2). Significant changes for line 194 RM was towards increasing of the mean with 3.67 cm. according to the control line 374 R. Instead slightly increasing of plant height the two mutant lines were characterized with increasing of stem diameter with 4.8 and 6.8 mm, respectively (Table 2). Leaf length, leaf width and petiole length were characterized with maximum decreasing of mean in comparison to the check line 374 R with 3.40 cm, 2.63 cm and 2.30 cm, respectively (Table 2). With acceptation of leaf length of line 193 RM all data for other traits were with the highest level of statistical significance. Decreasing of the size of leaves is negative change concerning photosynthetic surface**.**

Head diameter showed increasing of mean in the two mutant lines with 3.06 and 3.53 cm, respectively. The similar tendency was observed for the diameter of branches head (with 0.84 cm and 1.28 cm.). Head diameter showed increasing of mean value in the two mutant lines with 3.06 and 3.53 cm, respectively. The similar tendency was observed for the diameter of branches head (with 0.84 cm and 1.28 cm). The differences at the two indexes were at the highest degree of significance.

In lines 193 RM and 194 RM a decrease of the length of branches (with 7.6 and 9.7 cm) was registered.

From the characteristics concerning the size of seeds, increasing was observed in seed length and seed thickness of the two mutant lines. A maximum value of differences was 1.6 mm and 1.57 mm, respectively.

Positive changes of 1000 seed weight mean value was registered in the two investigated lines. The increasing was from 11.0 to 11.5 g. and was statisti-

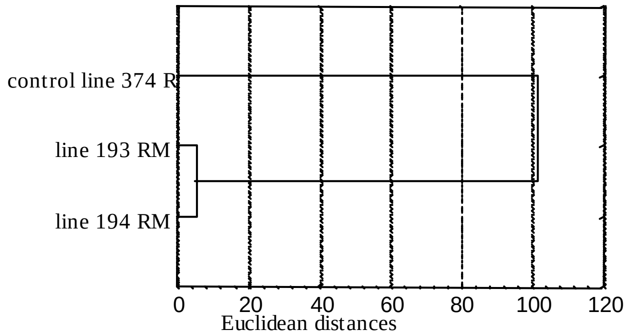

Fig. 2. Degree of similarity between control line 374 R and mutant lines 193 RM and 194 RM

cally proved (Table 2). Oil content in seed is one of the most important agronomic indices of sunflower. Significant increase of 4.03 and 4.3 % respectively was observed at lines 193 RM and 194 RM.

It can be concluded that the mutant lines have higher stem, larger head, larger seeds and higher 1000 kernel weight. These are positive changes leading to increased of yield per head. Another positive character is the thicker stem related to increased lodging resistance. An especially valuable change is the increased oil content in seed. All these differences from the initial check variety are very valuable and significant for the sunflower breeding program.

Leaf width, leaf length, petiole length, stem diameter, head diameter, length of branches, diameter of branched head, seed length, seed thickness, 1000 seed weight and oil content were the most unstable, based on all investigated characters. In the two mutant lines the changes indexes was the same number.

Based on all 15 agronomic characters investigated, it can be determined that the reduction in the means in comparison to the check line 374 R was observed for number of leaves, leaf width, leaf length, petiole length and length of branches i.e. 33 % of the total number of traits. Vise verse, significant differences in direction to increasing mean value was registered for plant height, stem diameter; head diameter, steam diameter, diameter of branched head, seed length, seed thickness, 1000 seed weight and oil content i.e. 60 % of the total number of characters. The index seed width remains stable for the two investigated lines and not affected by the mutagenic treatment.

At the variant with ultrasound it was observed larger changes at the mean value of 8 characters, while at the variant with gamma irradiation it was 5 out of 15.

It can be summarized that observed morphological and biochemical changes in the mutant lines 193 RM and 194 RM, developed with two different mutagen are deviations in the values of the most important indexes, but new characters in sunflower were not observed.

Cluster analysis for resistance to broomrape, agronomic and morphological traits

Investigation on the Euclidean distance between control line 374 R and mutant lines 193 RM and 194 RM

In order to characterize the investigated lines as fully as possible and to determine the relations between them and the similarity in their response to various conditions, cluster analysis was applied. Grouping was based on *Orobanche cumana* resistance and on the calculated mean arithmetic values from 15 characters during a 3-year period of investigation and their variation under year conditions.

Cluster analysis was carried out calculating the Euclidean distances between the investigated lines. The dendrogram of phytopathological, morphological and biochemical classification resulted in the differentiation of the control genotype and the new mutant lines into two main clusters. The first cluster included control line 374 R, the second-lines 193 RM and 194 RM. The dendrogram shows the big Euclidean distance between the new developed lines and the check line.

The big distance of mutant lines and control line 374 R was due to the fact that they differ mainly with resistance to the parasite *Orobanche cumana.* The new lines have higher and thickness steam, larger central head and head of branches, larger seeds, increased oil content and higher 1000 seed weight. On the other hand the lines possess lesser length of petioles and branches.

Despite a big difference between the investigated lines and check line 374 R, the dendrogram shows a lower distance between themselves. That may be explained by similar resistance to the parasite broomrape, morphological traits and reaction to the factors of environment.

Evaluation of the sunflower mutant lines for resistance to local Broomrape population

In most countries where sunflowers grow commercially successful production is endangered by many fungal pathogens and parasites. Losses may be severe, near 100 % parts or even entire fields under extreme circumstances (Skoric, 1994).

The parasitic phanerogame *Orobanche cumana* grow on sunflower roots, resulting in weak and dwindled plants, with thin steam. The parasite enhances the transpiration of damaged plants, which in drought conditions are withering, even if attacked by a small number of parasitic plants (Iliescu et al., 1998). Broomrape presents serious problems to sunflower production in Bulgaria, as well (Shindrova, 1994).

Hence, there is a need for greater variability providing additional sources of resistance to diseases, and insects, and seed quality characteristics among modern cultivated sunflower (Seiler, 1992). At present cultivated hybrid sunflower utilizes an extremely narrow genetic base, mainly the male sterile sytoplasm derived from the wild species *H. petiolaris* (Leclercq, 1969). Genetic variability, as resistance to parasite *Orobanche cumana* in particular may be increase using induced mutagenesis (Encheva et al., 2008).

In our study except the established morphological and biometrical changes a mutation was observed in the reaction of the genotypes towards *Orobanche cumana* parasite. The initial genotype 374 R was susceptible to broomrape. The mutant lines 193 RM and 194 RM showed 100 % resistance to the local broomrape population. These results were confirmed during three years of evaluation. On the basis of these data the conclusion was drawn that the resistance of the new lines was due to the mutagenic treatment with ultrasound and gamma radiation, respectively**.** This result confirmed our previous investigation with ultrasound (Encheva et al., 2008a, 2008b).

The results allow us to presume that the resistance of the mutant sunflower lines to *Orobanche cumana* occurred as a result from a single gene dominant mutation. Similar conclusion has been made by Christov et al. (1996), analyzing the type of resistance to broomrape of mutant sunflower forms obtained through irradiation of air dry seeds with gamma rays. The authors found out that it was controlled by a single dominant gene. The dominant resistant gene mutants were obtained after EMS treatment of pollen of tomato (Gavazi et al., 1987).

Many studies at classical breeding programme at sunflower show a monogenic control by a single dominant gene over sunflower resistance against races A to E (Ish-Shalom-Gordon et al., 1993; Sukno et al., 1999) and although two dominant genes (Dominguez, 1996b).

In contrast to the resistance derives from cultivated sources, the race F resistant population BR4, derived from wild species, was found to be under the control of a single dominant gene designated *Or6* (Perez-Vich et al., 2002). Pacureanu et al. (2004) reported also a single dominant gene controlling the resistance to race F in Romania.

In our experiment we prove that 100 % stable resistance of the sunflower mutant lines to the local *Orobanche* population can also be obtained through induced mutagenesis. The same mutation as resistance to the parasite broomrape, was obtained in variants of treatment with ultrasound and gamma irradiation. This suggests that the same mutable regions were affected in sunflower genome during *in vitro* treatment with mutagens.

Combining induced mutagenesis in immature zygotic embryo 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 ultrasonic. 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 et al., 2008a, 2008b).

Although induced mutagenesis is a random and unpredictable process, it is an invaluable fact that the occurred mutation of resistance to the parasite broomrape is of stable inheritance in the progenies of the fertility restorer lines.

Our results confirmed the conclusion of Skirvin (1978), that mutagenesis, physical or chemical, is favorable for induction of mutations in tissue cultures. 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, 1988).

Conclusion

Following the main problems of sunflower breeding at DAI, morphological, biochemical and phytopathological variability was developed by treatment with ultrasonic and gamma radiation.

In comparison to the check line 374 R, decreasing of the mean value of the indexes was registered for number of leaves, leaf width, leaf length, petiole length and length of branches i.e. 33 % of the total number of investigated characters.

Vise verse, significant increasing was observed for plant height, stem diameter, head diameter, steam diameter, diameter of branched head, seed length, seed thickness, 1000 seed weight and oil content i.e. 60 %.

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 lines with positive changes as resistance to parasite *Orobanche cumana*, increased oil content, 1000 seed weight, head diameter and stem diameter. This is very important and desire combination at breeding program of sunflower.

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

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Received October, 12, 2008; accepted for printing January, 23, 2009.