Bulgarian Journal of Agricultural Science, 19 (No 3) 2013, 578-583
Agricultural Academy

SUNFLOWER MUTATIONS, PRODUCED BY ULTRASONIC TREATMENT OF IMMATURE EMBRYOS OF CULTIVATED GENOTYPE 147 R

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Abstract

ENCHEVA, J., D. VALKOVA and P. SHINDROVA, 2012. Sunflower mutations produced by ultrasonic treatment of immature embryos of cultivated genotype 147 R. *Bulg. J. Agric. Sci.,* 19: 578-583

Immature sunflower zygotic embryos of sunflower fertility restorer line 147 R were treated with ultrasonic before plating to embryo culture medium. Some mutant plants were isolated and self-pollinated for several generations. New sunflower forms with inherited morphological and biochemical changes were obtained through selection and self-pollination. The genetic changes occurring during the regeneration procedure included sixteen morphological and biochemical characters. As a result of our investigation, reducing of the mean values negative were registered for the most of the indices i. e. 68.8 % of the total number of studied characters. The opposite positive changes of the follow indices leaf width, leaf length, petiole length and length of branches were observed, i.e 25 % of traits. Mutation for resistance to the local population of *Orobanche cumana* Wallr. (race A-E) was obtained from the susceptible Bulgarian control line 147 R. Two investigated mutant restorer lines possessed 100 % resistance to *Orobanche* and stable inheritance in the next generations. Our results showed that mutagenesis in sunflower can be successfully used to develop new lines useful for heterosis breeding.

Key words: *Helianthus annuus*, embryo rescue, ultrasonic, mutant line, resistance (*Orobanche cumana* Wallr.)

Introduction

Broomrape is a major problem in sunflower in all southeastern European, Middle East, Mediterranean 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. According to these authors, every 20 years there is epiphytotic occurrence of broomrape in the region. Furthermore, the parasite forms new more virulent races which overcome the resistance of the varieties and hybrids commonly used in production (Alonso, 1996; Pacureanu-Joita et al., 1998; Fernandez-Martinez et al., 2000; Kaya et al., 2004). 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, 2006). This leads to considerable losses expressed, on the one hand, in yield decrease, and on the other - in worsened quality of the obtained production (Shindrova et al., 1998).

Several methods have been used for the control of broomrape such as Mechanical, Chemical and Biological. The expensive and harmful for environments herbicides limited their utilization.

Resistant crops are one of the most effective control methods of broomrape. With a view of limiting the parasite's distribution and decreasing the losses it causes, it would be preferable to develop new sunflower lines and hybrids resistant to the broomrape.

Instead of wild species, induced mutagenesis of ear dry seeds or immature zygotic embryos can be another source for producing plants resistant to the parasite *Orobanche*. Mutant forms resistant to broomrape were produced after treatment of ear dry seeds of open pollinated varieties VNIMMK and Start with gamma rays at dozes 150 Gy and 200 Gy (Christov et al., 1998).

Mutagenesis, both physical and chemical, proved favorable for mutation induction in tissue cultures. Mutation for resistance to *Verticillium* was obtained when induced mutagenesis and tissue cultivation were combined appropriately in tomato (Gavazzi et al., 1987). Mutant sunflower lines resistant to *Orobanche cumana* or *Plasmopara halstedii* were developed by physical mutagenesis in sunflower immature embryos (Encheva et al., 2008; Encheva, 2009; Encheva and Shindrova, 2011).

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The aim of this study was: a) to develop variable R lines from sunflower through induced mutagenesis *in vitro* in initial genotype 147 R, and b) to evaluated the new genetic material for resistance to broomrape and c) to carry out biometric and biochemical investigations on the new lines.

Material and Methods

A part of the experiments was carried out under laboratory conditions, and another one at the trial field of Dobroudja Agricultural Institute-General Toshevo. The morphological and biochemical traits of the new mutant lines and the control genotype were studied during 2009-2011.

A/ Developing of mutant lines

The Bulgarian fertility restorer line 147 R, witch is highly homozygotic, 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. homozygotic to the highest possible degree. Therefore the control line 147 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 ultrasonic (during treatment the seeds were flooded in water, the whole surface well moistened) at dose 25.5 W/cm^2 for 5 min and 7 min, respectively before plating on nutrition medium M for further growing (Azpiroz et al., 0000): 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. 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. Sixty zygotic embryos were plated for each variant. The conditions for cultivation were: 25^{o}C, 16/8 h photoperiod for one week. The rooted plants were transferred to soil and were further grown and self-pollinated under greenhouse conditions.

B/ Field experiments

Biometric evaluation of control line 147 R and mutant lines 158 RM and 159 RM

As a result from long-term selfing and individual selection, new sunflower mutant lines were produced in R10M10 generation. The main criterion for selection was different morphology and resistance to broomrape. The lines were investigated with regard to some main characteristics concerning breeding in sunflower, also. In each generation biometric studies of plants were carried out.

The biometric evaluation of the control genotype and the newly developed mutant lines was made on 10 plants for each individual year, and included 16 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, number of branches, length of branches, diameter of branch head and stem diameter, seeds per head, 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 147 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.

C/ Phytopatological evaluation

The phytopathological evaluation of the control genotype 147 R and the obtained mutant lines was performed with regard to the local *Orobanche* population (race A-E) at the Sunflower Phytopathology Laboratory during 2003-2005.

Broomrape resistance was evaluated under greenhouse conditions according to Panchenko (1975), slightly modified to local conditions. Broomrape resistance was calculated as percentage of non-infected plants. The reaction of 50 plants from each genotype was recorded using the following scale: 0-100 %.

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 and stem diameter, seeds per head, seed length, seed width and seed thickness

The following statistical analysis was performed: a) Student's T-test, b) Cluster analysis by Euclidean linkage distances (Elliott at al., 1982). Analysis of the experimental data was by the statistical package BIOSTAST 6.0.

Results and Discussion

The isolated sunflower immature zygotic embryos of highly homozygotic Bulgarian fertility restorer line 147 R (Figure 1) were treated with ultrasonic before plating on nutrition medium for further growing. The new lines 158 RM (Figure 1) and 159 RM (Figure 2) object of this study arose from two different regenerants. They were selected due to their statistically significant morphological and biochemical changes and resistance to parasite *Orobanche cumana* (races A-F).

One of most investigated indices in cultural sunflower is plant height. It is consider a quantitatively inherited character. Variation was towards decrease of the mean value with 25.8 cm. and 22.7 cm. according to the control line 147 R (Table 1). The difference was with the highest statistical significance. Similar mutation with reduced plant height independently or in combination with gamma irradiation or ultra sound was register by Encheva et al. (1993, 2002, 2003). Novak et al. (1988) reported plant height reduction after treatment of immature zygotic embryos of maize with 5 Gy.

Leaf size of the new lines were smaller than check 147 R. Increasing of the mean value of leaf width was observed (16.8 cm for both mutant lines with comparison to 15.7 cm in the check variant). Increasing of leaf length with 0.76 cm was registered for line 159 R, only. Statistical increase of petiole length with 2.0 cm and 1.9 cm was demonstrated at two studied lines. Mutations as high stem and large leaves were obtained by Christov and Nikolova (1996) after ultrasonic treatment of dry seed of sunflower lines L-2969, L-3004 and cultivar Peredovik.

Decreased plant height, head diameter, number of branches, lead to the development of line 158 RM and 159 RM with changed architecture. Oil content at the two lines was statistically decreased with 3.9 % and 1.9 %. Another important trait as 1000 seed weight showed decreasing with 10.1 g and 7.1 g, also.

In our study, decreasing of head diameter is in correlation with number of seeds per head. Considerable reduction of seeds with 148 and 150 was observed at mutant line 158 RM and 159 RM.

In our study the number of leaves was the most stable, based on all investigated characters. Decreasing of mean value of seed length, seed width and seed thickness showed different size of the mutant lines. Besides the change size of seeds, the new mutant lines differ in the color. The control

Fig. 1. Mutant line 158 RM (left) and control restorer line 147 R (right)

Fig. 2. Mutant restorer line 159 RM

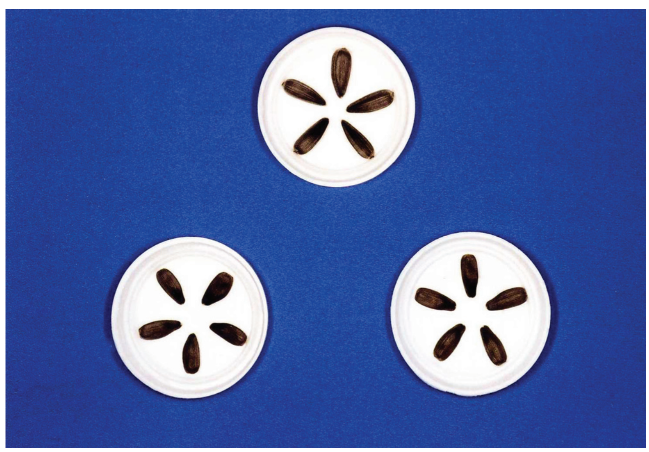

Fig. 3. Seed size of control line 147 R (above) and mutant lines 158 RM (left) and 159 RM (right)

line 147 R has black color with marginal and lateral grey stripes, while new lines have black color (Figure 3).

In comparison to the control line 147 R, increasing in the mean value of the indices were registered manly for leaf width, leaf length, petiole length, length of branches and i.e 25 %. Reduction of mean value was register for plant height, stem diameter, head diameter, number of branches, diameter of branched head, oil in the seed, 1000 seed weight, seeds per head, seed length, seed width and seed thickness 68.8 % i. e. of the total number of characters studied

In comparison to Gavazzi et al. (1987), which results in tomato indicate changing the spectrum and frequency of the mutants at our study observed morphological changes were alterations in the ratio between the most important traits, but the synthesis of unusual characteristics were not observed.

Cluster analysis for agronomic and phytopatological traits

Cluster analysis was carried out calculating the Euclidean distances between the investigated mutant lines and control restorer line 147 R. 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. Figure 4 presents the genetic relation between mutant lines and the control genotype 147 R with regard to *Orobanche cumana* resistance and on the calculated mean arithmetic values from 16 characters during a 3-year period of investigation and their variation under year conditions. In the constructed scheme, two main clusters can be recognized first-control line 147 R, second - lines 158 RM and 159 RM. The dendrogram shows the big Euclidean distance between the newly developed lines and the check line. The big distance of mutant lines and control line 147 R was because they differ mainly with resistance to the parasite *Orobanche cumana.* The new mutant lines have shorter

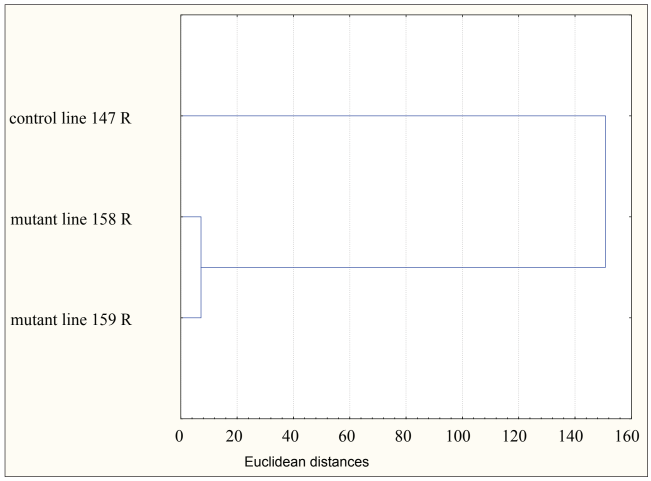

Fig. 4. Degree of similarity between control restorer line 147 R and mutant lines 158 RM and 159 RM

Table 1
Morphological, Biochemical and Phytopatological characteristics of mutant lines 158 RM and 159 RM, developed by treatment of immature zygotic embryos of control restorer line 147 R with ultra sonic. Harvest years 2009-2011, average data

Traits	Control line 157 R	Line 158 RM us	Line 159 RM us	LSD
Plant height, cm	121.33	95.50-c	98.67-c	Gd5%=2.21
Number of leaves, no	23.00	24.00+a	22.00-b	Gd5%=0.63
Leaf width, cm	15.67	16.80+a	16.80+a	Gd5%=0.99
Leaf length, cm	17.47	17.50	18.23+a	Gd5%=0.76
Petiole length, cm	11.53	13.57+c	13.40+c	Gd5%=0.59
Stem diameter, mm	18.47	17.27-b	16.93-c	Gd5%=0.84
Head diameter, cm	16.13	12.50-c	13.07-c	Gd5%=0.31
Number of branches, no	16.00	11.00-c	14.00-b	Gd5%=1.42
Length of branches, cm	20.03	23.30+c	20.07	Gd5%=1.78
Diameter of branched head, cm	5.97	5.70-c	6.07	Gd5%=0.15
Oil content in seed, %	48.00	44.13-c	46.07-c	Gd5%=1.00
1000 seed weight, g	37.89	27.80-c	30.83-c	Gd5%=3.64
Seeds per head, no	237.00	89.00-b	87.00-b	Gd5%=90.58
Seed length, mm	0.82	0.79-b	0.79-b	Gd5%=2.10
Seed width, mm	0.29	0.23-c	0.24-c	Gd5%=0.01
Seed thickness, mm	0.16	0.13-c	0.13-c	Gd5%=1.13
Reaction to broomrape, %	0	100	100	

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

plant height, less value of some characters as seed oil content, seeds per head, size of the seeds, 1000 seed weight, head diameter, stem diameter and number of branches. On the other hand, the mutant lines possess increase arithmetic value for leaf width, leaf length, petiole length and length of branches.

Despite a big difference between the investigated mutant lines and check line 147 R, the dendrogram shows a lower distance between themselves. Similar resistance to the parasite broomrape, similar morphological traits and reaction to the factors of environment may explain that.

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

Broomrape is an obligate, holoparasitic angiosperm that lives to the roots of sunflower depriving them of nutrients and water. From an agriculture point of view, parasitic angiosperm *Orobanche cumana* Wallr. is the most important as it cause serious damage and yield losses in sunflower (Shindrova, 2006).

Christov et al. (2009) reported 13 perennial and 3 annual *Helianthus* species resistant to parasite broomrape, race E. Resistance to the race F broomrape populations was found in germplasm of both cultivated and wild sunflower (Akhtouch et al., 2002; Fernandez-Martinez et al., 2010). The resistance to broomrape, race G established in annual *H. argophylus* was successfully transferred to cultivated sunflower (Valkova et al., 2009).

Instead wild species source of resistance to *Orobanche cumana* can be induced thought mutagenesis. It is an alternative method to conventional ones. In our study except statistically significant morphological and biochemical changes, a mutation was observed at two mutant lines 158 RM and 159 RM towards *Orobanche* parasite, race F. The initial genotype 147 R was susceptible to broomrape (Table 1). The mutant lines developed from immature zygotic embryos by ultrasound treatment demonstrated 100 % resistance to the local broomrape population (A-F) during three years of study. Comparing with check line 147 R, the conclusion was drown that the mutation for resistance of the new lines was due to the ultrasonic treatment. The same mutation, resistance to the parasite broomrape, was obtained in all variants of treatment of the initial genotype 147 R. This allows us to assume that there are mutable locations in the cultural sunflower genome resulting from induced mutagenesis. 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. The result allows us to assume that the resistance of the new lines occurred because of mutation of a single dominant gene. Similar conclusion was 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. Resistant mutants to *Verticillium* with dominant genes were obtained after EMS treatment of pollen of tomato (Gavazzi et al., 1987). Kostov et al. (2007) reported mutant tomato plants resistant to *Orobanche ramosa*, produced by EMS treatment of seeds. Monogenic and dominant inheritance to sunflower broomrape was confirmed by genetic studies of other authors (Pogorlezkii and Geshele, 1976; Burlov and Kostiuk, 1976; Burlov and Arteminko, 1983; Tolmachev, 1984; Sukno et al., 1999), although two dominant genes (Dominguez, 1996b). Vranceanu et al. (1980) announced about five dominant resistant genes Or1 through Or5, which provide cumulative resistance to the five successive races of *Orobanche*. According to Pacureanu-Joita et al. (1998), the gene *Or6* provides resistance to all races - from A to F.

In this study by ultrasonic treatment of immature zygotic embryos it is possible to receive not only morphological changes but also phytopatological, like *Orobanche* resistance.

Conclusion

Ultrasonic treatment of immature zygotic embryos of sunflower allows developing economically important character as resistance to *Orobanche cumana*. Combining induced mutagenesis and embryo culture method it can be assume that the new trait was exclusively due to the effect of mutagen. Embryo culture method alone does not generate variation, due to the lack of mutagen factors in the nutrition medium and a short period of *in vitro* cultivation of immature zygotic embryos. Mutations to resistance to broomrape were obtained in our previous studies with different initial genotype of sunflower. Explanation of such results was the fact that we treated immature zygotic embryos at early stage of their development, i.e. this is already a functional tissue.

Based on all 16 agronomic characters investigated, it can be determined that the reduction in the mean value (11 from 16) in comparison to the control 147 R was observed for plant height, stem diameter, head diameter, number of branches, diameter of branched head, oil in the seed, 1000 seed weight, seeds per head, seed length, seed width and seed thickness i. e. 68.8 % of the total number of characters studied

Vice verse, positive significant differences was registered manly for leaf width, leaf length, petiole length and length of branches i.e. 25 % of traits. 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, 30, 2012; accepted for printing May, 2, 2013.