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MUTANT SUNFLOWER LINE R 171, PRODUCED THROUGH *IN VITRO* MUTAGENESIS OF IMMATURE EMBRYOS

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Abstract

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Immature sunflower (*Helianthus annuus* L.) zygotic embryos of sunflower fertility restorer line 2571 R were treated with ultrasound before plating in the 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 variations included 16 morphological and biochemical agronomic traits. In our study the plant height, length of branches, leaf petiole length, head diameter and seed yield per head were the most unstable, based on all investigated characters. In comparison to the control line 2571 R, increasing in the mean value of the indices was registered for 68.8 % of the total number of characters. Stability after the number of leaves, number of branches demonstrated induced mutagenesis, seed width and seed thickness. Reduction of mean value was register for 1000 seed weight. i.e. 6.3 % of the indices. Increasing of seed yield per head, oil content in seed and resistance to parasite *Orobanche cumana* of the new mutant line 171 RM is desire combination at breeding program of sunflower.

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

Introduction

Sunflower is one of the most important oil crops in the world. In Bulgaria, for example it occupied area of 690 000 ha. The changed climatic conditions consisted development of new genetic variability suitable to cover increased necessities of sunflower growers. Because of limited genetic base of this crop, new approaches should be applied in sunflower breeding program.

Combination of induced mutagenesis and embryo culture method provides an additional possibility to enrich sunflower genetic variability and acceleration of the breeding process. The method is comparatively easily applicable and has considerable practical value because of the rich genetic variation which they may induce.

Positive results were obtained when induced mutagenesis and tissue cultivation were combined appropriately in tomato (Gavazi et al., 1987), in maize, banana and plantain (Novak et al., 1988, 1990), potato (Ahloowalia, 1990), wheat (Cheng et al*.,* 1990), oil crops (Ashri, 1993), in rice (Maluszynski et al*.,* 1994), other crops (Mike et al., 1990) and in sunflower (Encheva et al., 2002, 2003a, 2003b, 2008, 2009; Soroka and Lyakh, 2009),

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

Broomrape is a parasite on the roots of sunflower plants and causes serious damages to sunflower production (Skoric, 1994). Losses may be severe, near 100 % in parts or even entire fields under extreme circumstances. Broomrape presents serious problems to sunflower production in Bulgaria, as well. This leads to considerable losses expressed, on the one hand, in yield decrease,

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and on the other - in worsened quality of the obtained product (Shindrova et al*.,* 1998). With a view of limiting the parasite's distribution and decreasing the losses it causes, it would be preferable to develop new lines resistant to the broomrape.

The aim of this study was: a) to evaluate the new mutant line 171 RM morphologically, biochemical and for resistance to parasite broomrape.

Material and Methods

A part of the experiments were carried out under laboratory conditions, and another – at the trial field of Dobroudja Agricultural Institute-General Toshevo.

Developing of mutant lines

The Bulgarian fertility restorer line 2571 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. homozygote to the highest possible degree. Therefore, the control line 2571 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 seeds (13-16 days old) were treated with ultrasound at dose 25.5 W/cm^2 for 30 min. Immature seeds 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. Immature zygotic embryos were aseptically isolated and 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. The conditions for cultivation were 25^{o}C, 16/8 h photoperiod for one week. The plants, which formed roots, were transferred to soil and were further grown and self-pollinated in greenhouse.

Biometric evaluation of control line 2571 R and mutant line 171 RM

The biometric evaluation and biochemical analysis of the control genotype and the new developed mutant line were made on 10 plants for each individual year, and included 16 main agronomic traits as oil in the seed, 1000 seed weight, seed yield per head, plant height, leaf width, leaf length, number of leaves, petiole length, head diameter, diameter of branch head, stem diameter, number of branches, length of branches, 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 original line 2571 R that was grown in the field together with mutagenic plants.

Biochemical analysis

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

Phytopatological evaluation

The phytopathological evaluation of the control genotype 2571 R and the obtained mutant line 171 RM was performed with regard to the local *Orobanche* population at the Sunflower Phytopathology Laboratory of DAI - General Toshevo.

Broomrape resistance was evaluated under greenhouse conditions according to Panchenko (1975), slightly modified to the local conditions. The phytopathological evaluation of lines was performed with regard to the local *Orobanche* population (race A-F). Broomrape resistance was calculated as percentage of non-infected plants. The reaction of 50 plants from each line was recorded using the following scale: 0-100 %.

Statistical analysis

The check line 2571 R and developed mutant line 171 RM were analyzed statistically with regard to the agronomic traits such as oil in the seed, 1000 seed weight, seed yield per head, plant height, leaf width, leaf length, number of leaves, petiole length, head diameter, diameter of branch head, stem diameter, number of branches, length of branches, seed length, seed thickness and seed width.

The following statistical analysis was performed: a) variance analysis using the following model: $Y_{ijk}=\mu+\alpha_i+\beta_j+(\alpha\beta)_{ij}+\varepsilon_{ijk}$ (Everett, 1984)**.** Analysis of the experimental data was done by the statistical package BIOSTAST 6.0.

Results and Discussion

Mutant line 171 RM (Figure 1) originating from the Bulgarian fertility restorer line 2571 R (Figure 1) were selected due to their statistically significant morphological and biochemical changes and resistance to parasite *Orobanche cumana* (races A-F).

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

Fig. 1. Control line 2571 R (left) and mutant restorer line 171 RM (right)

Factor B (environmental conditions) had a significant effect on a large part of the traits such as: head diameter, leaf width, leaf length, stem diameter, number of brunches, length of branches, number of leaves, diameter of branch head, petiole length, seed length, seed yield per head, 1000 seed weight and oil content in seed (Table 1). It was found out that the characters plant height, seed width and seed thickness were stable and were not affected by the changes in the climatic conditions.

The interaction of the two investigated factors (A x B) was significant for the indices plant height, stem diameter, number of leaves, seed width, seed thickness, 1000 seed weight and oil content in seed (Table 1).

Plant height is one of the morphological indices most often investigated in cultural sunflower; it is consider a quantitatively inherited character. The statistically significant variation at the character plant height was towards increase of the mean value with 19.7 cm according to the control 2571 R (Figure 2). Vice versa, decrease in plant height of sunflower has been reported in using the direct organogenesis method independently

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

Indices	A	B	A X B	E
Plant height	5801.67***	0.42	130.12*	31.67
Head diameter	176.82***	34.2***	2.07	0.81
Leaf length	35.27***	15.80**	2.47	2.44
Leaf width	22.82**	47.40***	0.87	2.93
Stem diameter	91.27***	210.82***	34.52***	2.84
Number of brunches	2.82	55.05***	3.32	2.76
Length of branches	792.07***	59.15**	20.82	11.79
Number of leaves	40.02***	154.12***	12.62**	1.69
Diameter of branch head	6.02*	13.27***	0.87	1.12
Leaf petiole length	432.02***	18.87**	4.47	2.41
Seed length	10.05***	0.53**	0.02	0.08
Seed width	0.00	0.00	0.17*	0.05
Seed thickness	0.00	0.12	0.20*	0.04
1000 seed weight	67.50*	116.63***	97.30**	10.58
Oil content in seed	44.43**	98.85**	14.63*	4.10
seed yield per head	369.95***	98.31***	7.32	6.40

A – genotype, B – environmental conditions, A X B - interaction, * - statistical significance by p=0.05, ** - p=0.01, *** - p=0.001

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

Significant changes in leaf size was registered in studded line 171 RM. Considerable increase of the mean value of both indices was observed (18.3 cm leaf width and 18.9 leaf length in comparison to 17.0 cm and 17.4, respectively, in the check variant).

Breeding to improve stem strength is a major objective of researchers of sunflower. Increasing stem diameter of line 171 RM may lead to improve standability of the plants.

Regarding length of branches, the observed statistical difference was only in direction towards increase. The statistical changes in comparison to the control were to 7.3 cm.

Oil content in seed is one of the most important agronomic indices (Table 2). A significant increase of 2.4 % was observed at mutant line 171 RM. One of the aims of our study was to develop variable R lines from sunflower with higher oil content through induced mutagenesis in initial genotype 2571 R. The increased oil content of the mutant restorer line developed is a valuable change with significant practical importance for the sunflower breeding program. The data presented at this study confirmed the conclusions made previously that ultrasound in R lines (Encheva at al., 2003a, b) and in B lines (Encheva at al., 2004) leads to genetically increasing of oil content in seed.

The seed yield per head is an index having direct relation to sunflower yield. In our study considerable

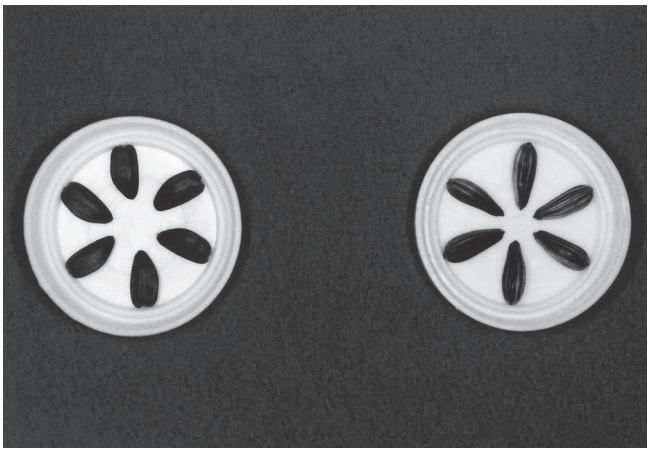

Fig. 2. Seed size of control line 2571 R (left) and mutant line 171 RM (right)

Table 2
Morphological and biochemical characteristics of mutant line 171 RM, developed by induced mutagenesis at immature embryos. Harvest years 2006-2008, average data

Traits	Control line 2571 R	Line 171 RM us	LSD
Plant height (cm)	87.83	107.50+c	Gd 5% = 3.38
Number of leaves (no)	23.00	25.00	Gd 5% = 0.83
Leaf width (cm)	17.03	18.27+c	Gd 5% = 0.72
Leaf length (cm)	17.43	18.97+c	Gd 5% = 0.83
Petiole length (cm)	8.30	13.67+c	Gd 5% = 0.55
Stem diameter (mm)	14.43	16.90+c	Gd 5% = 1.18
Head diameter (cm)	12.23	15.67+c	Gd 5% = 0.44
Number of branches (no)	9.00	8.00	Gd 5% = 0.71
Length of branches (cm)	14.77	22.03+c	Gd 5% = 1.71
Diameter of branched head (cm)	5.87	6.43+a	Gd 5% = 0.55
Seed width (mm)	3.94	3.91	Gd 5% = 0.17
Seed length (mm)	9.03	10.08+c	Gd 5% = 0.20
Seed thickness (mm)	2.54	2.55	Gd 5% = 0.27
Oil content in seed (%)	48.33	50.76+c	Gd 5% = 1.49
1000 seed weight (g)	30.73	27.73-a	Gd 5% = 0.26
Seed yield per head	7.46	15.48+c	Gd 5% = 2.05

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

statistical increase (with of 8.0 g) was register at mutant line 171 RM.

Increasing of head diameter with 3.4 cm and petiole length with 5.4 cm was statistically proved at mutant line 171 RM.

The increasing of plant height, length of brunches, as well as longer petiole length, lead to the development of line 171 RM with changed architecture.

In our study the plant height, length of branches, leaf petiole length, head diameter and seed yield per head were the most unstable, based on all investigated characters. In comparison to the control line 2571 R, increasing in the mean value of the indices were registered manly for oil in the seed, seed yield per head, plant height, leaf width, leaf length, petiole length, head diameter, stem diameter, diameter of branched head, length of branches and seed length., i.e. 68.8 % of the total number of characters studied.

Stability after the characters number of leaves, number of branches demonstrated induced mutagenesis, seed width and seed thickness. i.e. 25 %. Reduction of mean value was register for 1000 seed weight. i. e. 6.3 %.

Instead developed morphological and biochemical changes at line 171 RM we succeed to preserve one very important feature of the control line 2571 R-resistance to broomrape. The mutant line showed 100% resistance to the local broomrape population-race A-F. These results were confirmed during several years of evaluation.

Broomrape presents serious problems to sunflower production in Bulgaria (Shindrova, 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). It can be summarized that the observed changes in the mutant line are deviations in the values of the most important agronomic indices, but new characters in sunflower were not observed.

Conclusions

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

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.

We succeed to create mutant sunflower line with increased oil content in seed, seed yield per head and in addition to preserve one very important feature of the control line 2571 R-resistance to broomrape. The used of ultrasound for occurrence of single mutants controlled by one or several genes while preserving other positive characters of a control line is one of the most useful application of this technique.

Although induced mutagenesis is random and unpredictable process, it is result in genetically heritable variation in sunflower that is suitable to use in breeding program for production of new breeding material.

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