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DEVELOPING MUTANT SUNFLOWER LINE (*HELIANTHUS ANNUUS* L.) BY COMBINED USED OF CLASSICAL METHOD WITH INDUCED MUTAGENESIS AND EMBRYO CULTURE METHOD

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Abstract

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The isolated immature zygotic F1 embryos, produced with pollycross method were treated with ultrasound before plating on nutrition medium *in vitro*. Some mutant plants were isolated and self-pollinated for several generations. New sunflower form with inherited morphological, biochemical and phytopatological changes were obtained through selection and self-pollination. The genetic changes included 15 morphological and biochemical agronomic traits.

In comparison to the mother line in the cross 2568 R, decreasing in the mean value of the indexes was registered for 73.3 %. of the total number of characters and vice verse, significant increasing for oil content in seed, seed length, seed width and seed thickness i.e. 26.7 %.

Increasing oil content in seed, very good combining ability, resistance to *Plasmopara halstedii* (Farl.) and to the parasite *Orobanche cumana* of the new mutant line is desire combination at breeding program of sunflower. Hybrid No 61 developed with the participation of line 35 RM considerably exceeded the standard (commercial hybrids San Luka) by seed and oil yield. Polycross method in combination with induced mutagenesis in sunflower can be successfully used to develop new mutant line useful for heterosis breeding.

Key words: Helianthus annuus, immature zygotic embryos, ultrasound, mutagenesis, new breeding material, resistance, *Plasmopara halstedii* (Farl.), *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 new approach as combination of polycross method with induced mutagenesis of immature zygotic embryos, provide an additional possibility to enrich genetic variability in this crop and acceleration of selection process. They are comparatively easily applicable and have considerable practical value because of the rich genetic variation which they may induce.

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 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 wheat (Cheng et al., 1990), in oil crops (Ashri, 1993) and in rice (Maluszynski et al., 1994).

Although sunflower breeding has been very successful throughout the last decades, a number of aims remain to be achieved, e.g. resistance to downy mildew and to the parasite broomrape. However, these efforts are obviously limited by the narrow genetic base of commercial sunflower which has to be enlarged by the utilization of wild species, mutagenesis or tissue culture.

Downy mildew, caused by *Plasmopara halstedii* (Farl.) is one of the main diseases in most of the sunflower growing areas of the world. In recent years a number of authors have reported the occurrence of new, more virulent races of the pathogen which demonstrate resistance to the fungicides used up to now (Molinero- Ruiz et al., 2002; Baldini et al., 2006).

The facts mentioned above show the necessity to making systematic studies on the resistance to downy mildew end breeding resistant lines and hybrids.

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, and on the other - in worsened quality of the obtained produce (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 and hybrids resistant to the broomrape.

The aim of this study was: a) to develop restorer line from sunflower with higher oil content through polycross method in combination with induced mutagenesis, and b) to evaluated the new genetic material for resistance to *Plasmopara halstedii* (Farl)-race 330 and to the local population of the parasite *Orobanche cumana* (races A-E) and c) to carry out biometric investigations on the new line 35 RM, and d) to study combining ability of the new restorer line produced.

Material and Methods

To carry out investigation, line 2568 R as female parent and seven self-pollinated R lines as pollen donor were selected from the collection of the Dobroudja Agricultural Institute. The pollination was done by mixed pollen of lines 2568 R, Z-8-A, 2571R, 763 R, 808 R, 7011 R and 683 R (Table 1). This is so cold polycross method, used in breeding of cross pollinated crops (Stoyanova et al., 1977).

A part of the experiments were carried out at the trial field of Dobroudja Agricultural Institute-General Toshevo, and another under laboratory conditions. The morphological and biochemical traits of the new mutant line and the mother genotype in the interlinear hybridization were studied during 2005-2007.

Polycross hybridization

The fertility restorer lines included in the polycross were of known quality composition, possessed good agronomic indexes and good combining ability. The cross was realized under field conditions at DAI-General Toshevo. Hybrid embryos were obtained by ster-

Table 1
Fertility restorer lines, used as pollen donors in the pollycross procedure

R lines	*Orobanche cumana*	*Plasmopara halstedii*	*Phomopsis helianthi*
2568 R	100	100	2
Z-8-A	0	100	2
2571 R	100	100	2
763 R	0	100	0
808 R	0	100	2
7011 R	0	100	0
683 R	100	100	2

ilizing pollen from the mother plants by gybberelic acid (GA_3-0.045 g/l) and hand-pollinated with mixed pollen from the pollycross population. To avoid foreign pollination, the necessary number of plants, both from the sterile form and the pollen sources, were isolated immediately before beginning of flowering.

Induced mutagenesis and In vitro cultivation of immature zygotic sunflower embryos

The isolated immature zygotic F1 embryos (11-13 days old) were treated with ultrasound at dose 25.5 W/cm^2 for 1 min. before 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. 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 plants which formed roots were transferred to soil and were further grown and self-pollinated under greenhouse conditions.

Field experiments

Biometric evaluation of mother line 2568 R and mutant line 35 RM

As a result from long-term selfing and individual selection, new sunflower mutant lines were produced in R16M16 generation. The main criterion for selection was height oil content in seed and resistance to downy mildew and 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 mother genotype 2568 R and the new developed mutant line 35 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 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 mother line 2568 R which was grown in field together with the mutant plants.

Biochemical analysis

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

The phytopathological evaluation of the mother genotype 2568 R in the cross, the lines for pollen donors, the obtained mutant line 35 RM and hybrid No 61 was performed with regard to downy mildew *Plasmopara halstedii* (Farl.) Berlese & de Toni-race 330, phomopsis (*Phomopsis helianthi*, Munt-Cvet. et al.) and to the local *Orobanche* population (race A-E) at the Sunflower Phytopathology Laboratory. With a view to characterizing the resistance to downy mildew were used the method suggesting by Gulya at al. (1991). The evaluation of 50 plants from each lines and hybrid was carried out according standard methodologies: 0 % = S (sensitive); 100 % = R (resistant).

The estimation for resistance to phomopsis was performed 20 days after inoculation of plants in budding stage (Tourvieelle et al., 1988). It was using four degree scale: from 0 = R (resistant) to 4=S (sensitive).

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 %=S (sensitive); 100 %=R (resistant).

Hybridization

To determine the combining ability of the new developed sunflower line 35 RM the sterile analogue of the Bulgarian selfed line 4515A was used. The standard for comparing the new hybrid No 61 developed was the Bulgarian commercial hybrid San Luka. The obtained hybrid combination was tested during 2007 at the breeding fields of DAI according to the block-design method, in three replications, the area of each replication being 10 m^2 (Barov and Shanin, 1965).

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 width and seed thickness.

The following statistical analysis was performed: a) variance analysis using the following model: Yijk = X. + Yi + Yj + (YY)ij + eijk (Everett, 1984); b) Student's T-test,

Analysis of the experimental data was by the statistical package BIOSTAST 6.0.

Results and Discussion

Evaluation according to quantitative and qualitative traits in mutant line 35 RM

The embryo culture method was applied on F1 hybrid zygotic embryos, obtained as a result from the cross of fertility restorer line 2568 R and mixed pollen from the pollycoss population of known genetic composition. The method allows isolation of embryos before terminating their development and their plating onto nutrition medium to grow *in vitro* seedlings (Plotnicov, 1983).

Fig. 1. Mutant sunflower line 35 RM (left) and mother line 2568 R (right)

The aim of this study was to investigate some agronomic traits of newly produced sunflower mutant line. Line 35 RM (Figure 1) originating from polycross method in combination with induced mutagenesis was selected due to their statistically significant morphological and biochemical changes and good combining ability.

Table 1 present data on mutant line 35 RM, showing deviation from the respective mother line 2568 R with regard to the mean arithmetic value of 15 morphological and biochemical traits.

Differences with the highest level of statistical significance were established in the genetic potential of the indices head diameter, leaf width, leaf length, stem diameter, number of brunches, length of branches, diameter of branch head, leaf petiole length, oil content in seed, seed length and seed width. (Table 2).

The considerable and statistically significant decrease in plant height with 11.3 cm., leaf width (with 2.6 cm), leaf length (with 4.1 cm.), number of branches (with 5) and length of branches (with 10.8 cm) leads to changes in steam architecture of mutant line 35 RM.

Oil content in seed is the most important agronomic indices of sunflower. A significant increase of 11.2 % was observed at the mutant line 35 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 et al., 2003) and in B lines (Encheva et al., 2004) leads to genetically increasing of oil content in seed.

Based on all 15 agronomic characters investigated, it can be determined that the reduction in the mean value (11 from 15) in comparison to the mother line in the cross 2568 R was observed for plant height, leaf width, leaf length, leaf petiole length, stem diameter, head diameter, number of leaves, number of branches,

Table 2
Mean of square of the studied indices

Indices	MSA	MSB	MSA x B	MSE
Plant height	1926.67***	9093.60***	1883.47***	21.13
Head diameter	72.60***	5.02**	1.55	0.69
Leaf length	248.07***	84.65***	12.82***	1.18
Leaf width	104.01***	38.15***	5.52	2.44
Stem diameter	45.07***	1022.87***	0.07	4.2
Number of brunches	370.02***	970.87***	628.07***	5.54
Length of branches	1749.60***	1045.32***	269.15***	6.73
Number of leaves	13.07	113.15***	15.32*	3.83
Diameter of branch head	3.75***	5.22 ***	1.05	0.37
Leaf petiole length	5.22 ***	74.62 ***	0.15	0.94
Seed length	23.44***	0.01	0.01	0.33
Seed width	1.07*	0	0	0.09
Seed thickness	1.07	0.03	0.03	0.16
1000 seed weight	10.42	54.87**	64.87***	8.2
Oil content in seed	1859.71***	113.68***	90.33***	7.08
df	1	2	2	54

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

length of branches, 1000 seed weight, diameter of branch head, i.e. 73.3 % of the total number of traits. Vice verse, positive significant differences were registered for oil content in seed, seed length, seed width and seed thickness i.e. 26.7 % of the total number of characters. Besides the above morphological peculiarities the new line also differed in one quality trait: The ray flower shape of line 35 RM was elongated, while in the mother line was ovoid. It can be summarized that the observed changes in the mutant line are deviations in the values of the most important agronomic indexes, but new characters in sunflower were not observed.

Factor B (environmental conditions) had a significant effect on a large part of the traits such as: plant height, head diameter, leaf width, leaf length, leaf petiole length, stem diameter, number of brunches, length of branches, number of leaves, diameter of branch head, 1000 seed weight and oil content in seed (Table 2).

The interaction of the two investigated factors (A and B) was highly significant for the indices plant height, number of leaves, leaf length, number of branches, length of branches, 1000 seed weight and oil content in seed (Table 2). The lack of statistical significance of the genotype x environment (G x E) interaction, was established for the index head diameter, stem diameter, leaf width, leaf petiole length, diameter of branch head, seed length, seed width and seed thickness.

Possibility for practically use of the hybrid No 61, produced with the participation of line 35 RM

The one year testing of line 35 RM showed 100 % restoration ability and very good combining ability. The sterile analogue of the Bulgarian self-pollinated line 4515 was used as a tester of the hybrids No 61 (Figure 2). A one-factor dispersion analysis of hybrids was carried out with regard to the indices seed yield and oil yield per dekar.

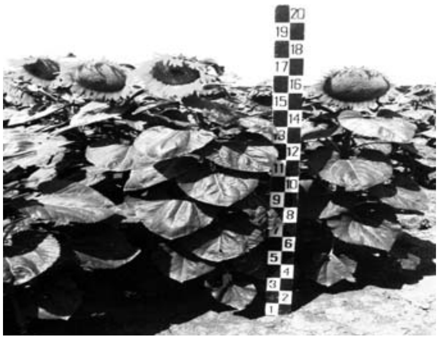

Fig. 2. Hybrid No 61 (4515 A x 35 RM)

The seed yield (Figure 3) of hybrid No 61 produced during the one year period of testing mutagenic line 35 RM exceeded the standard (commercial hybrid San Luka) by 86.1 kg/da or 80.8 % with height level of statistical significance.

Oil yield is another important index. Figure 4 presents data on the investigated hybrid. The results from dispersion analysis of oil yield demonstrated that the significant difference according to the standard was with 43.1 kg/da or 92.4 %.

The newly developed hybrid No 61 were characterized by increased seed and oil yield, resistance to downy mildew race 330 and to the parasite broomrape, which is a desirable combination in the sunflower breeding programs.

Evaluation of the sunflower mutant line 35 RM and hybrid No 61 for resistance to downy mildew and local Broomrape population

Downy mildew of sunflower (*Helianthus annuus* L.) is caused by the obligate parasite *Plasmopara halstedii*. According to the last nomenclature system there are 10 downy mildew races currently existing in the world, as follows: 100, 300, 310, 330, 700, 703, 710, 711, 730, and 770 (Tourvieille at al., 2000). According Miller (1992) and Vear at al. (2000) resistance to downy mildew is controlled by single dominant genes noted Pl and has been found for all known races. The new race 330 was established in North-East part of Bulgaria since 2005 year (Shindrova, 2006). The facts mentioned above show the necessity of breeding resistant lines and hybrids. During this study line 35 RM and hybrid No 61 showed 100 % resistance to race 330 of downy mildew.

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). The phytopathological evaluation of the mother component in the cross 2568 R and pollen donor line 2571 R and line 683 R and obtained mutant line 35 RM performed with regard to the local *Orobanche* population (race A-E) shown

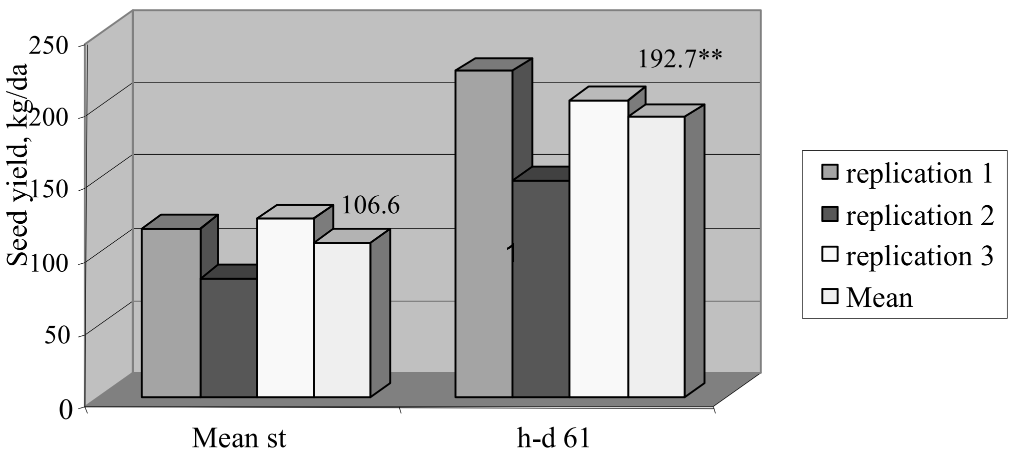

Fig. 3. Comparison of seed yield (kg/da) from hybrid No 61 and standard (commercial hybrid San Luka) during 2007 (- P = 0.1 %)**

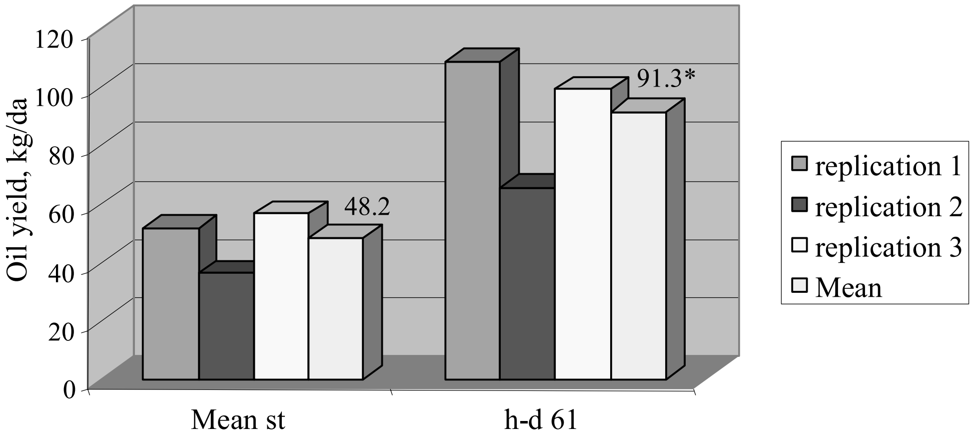

Fig. 4. Comparison of oil yield (kg/da) from hybrid No 61 and standard (commercial hybrid San Luka) during 2007 (* - P = 1 %)

100 % resistance. Resistance to the parasite broomrape is of stable inheritance in the progenies. The phytopathological evaluation of the hybrid No 61 produced with the participation of mutant line 35 RM shows 100 % resistance to broomrape.

Conclusion

Following the main problems of sunflower breeding at DAI, morphological, biochemical and phytopathological variability was developed by combination of polycross method and induced mutagenesis. Combining induced mutagenesis in immature zygotic embryo with embryo culture method, allows isolation of embryos before terminating their development and their plating onto nutrition medium to grow *in vitro* seedlings. Embryo culture method allowed considerably shortens of the breeding process obtaining 5 generations within a single year. We succeed to create mutant sunflower line with increased oil content in seed, resistance to *Plasmopara halstedii*-race 330 and to parasite broomrape and possessing very good combining ability. This is very important and desire combination at breeding program of sunflower.

Polycross method in combination with induced mutagenesis is suitable to use in breeding program for production of new breeding material and highly productive hybrids.

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