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# MUTANT SUNFLOWER LINE R 12003, PRODUCED THROUGH *in vitro* MUTAGENESIS

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SUMMARY

Immature sunflower (*Helianthus annuus* L.) zygotic embryos of sunflower fertility restorer line R 2574 were treated with ultrasound before planting on the embryo culture medium. As a result some chlorophyll, leaf and inflorescence mutations were observed. New sunflower forms with inherited morphological, biochemical and phytopatological changes were obtained through selection and self-pollination. The line R 12003, subjected to investigation in this study, was characterized with significant changes concerning most of the studied characters. The contribution of the present research connected to the investigated mutation was the appearance of resistance to the parasite broomrape. The increased number of seeds per head in line R 12003, increased oil content in seed and 100% resistance to the parasite *Orobanche* are the desired combination in the breeding programme of sunflower.

**Key words:** ***Helianthus annuus*, embryo rescue, ultrasound, mutant line, resistance (*Plasmopara helianthi*, *Phomopsis helianthi*, *Phoma macdonaldii*, *Orobanche cumana*)**

## INTRODUCTION

Improving plasticity and enriching genetic potential of sunflower can be realized through gene mutations and recombinations.

Among the other techniques used in modern breeding, the method of mutagenesis is considered as an effective one. Mutagenesis, both physical and chemical, proved favourable for mutation induction in tissue cultures. It is a technique which allows widening a heritable variability by inducing new traits. Some of them can be of interest as agronomically important characters; others can be used as marker traits.

Another promising technique is embryo culture (Plotnicov, 1983). The method allowed considerable shortening of the breeding process.

The mature seeds were subjected to mutagenic treatment more often (Jambhulkar *et al.*, 1999; Sagadeesan *et al.*, 2008). Lyakh *et al.*, 2005 studied the frequency and spectrum of morphological mutations, raised in $M_2$ after the treatment of mature and immature seed with ethylmethanesulphonate (EMS).

Costov *et al.* (2007) increased resistance of tomato to *Orobanche ramosa* L. by application of EMS. Thirty-three types of chemical mutation at sunflower immature embryos were found, described and classified by Soroka and Lyakh, 2009. Encheva *et al*. (1993, 2002, 2003, 2008 and 2009) 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. Encheva *et al*., 2008 and Encheva, 2009 created sunflower lines resistant to parasite broomrape after treatment of immature zygotic embryos with ultrasound.

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 by the narrow genetic base of commercial sunflower which has to be enlarged by the utilization of wild species, mutagenesis or tissue culture. Therefore development of new lines and hybrids resistant to diseases and to parasite broomrape is very important for sunflower breeding.

Broomrape is widespread in parts of Europe, Spain, the Near East and China (Škorić, 1994). According to Kaya *et al.* (2004), about 80% of the sunflower areas in Turkey (Trakia region) are infested with seeds of the parasite. Virulent races are overcoming the resistance of varieties and hybrids in Turkey, where the new races seem to be more virulent than the races present in other countries.

Broomrape also presents a serious problem to sunflower production in Bulgaria. Although a large number of *Orobanche*-resistant hybrids have been developed during the last years, there are still large areas infected with this parasite (Chindrova, 1994).

Downy mildew is a major disease in the regions with the mass production of sunflower. Currently the races of the pathogen with varying virulence are being observed.

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 (Škorić, 1994; Gulya, 1997; Carre, 1993), including Bulgaria (Encheva, and Shindrova, 1990).

Apart from chemicals and good agronomy practices recommended for control of diseases and broomrape on sunflower, the breeding method is the most efficient and economically most advantageous. The development of resistant lines, hybrids and their introduction in practice will lead to restricted propagation and distribution of the pathogens, and in the course of time, cleaning of the infected fields (Škorić, 1985; Vear and de Labrouhe, 1997; Hahn and Degener, 1999; Roustaee *et al.,* 2000).

The aim of this study was: a) to develop variable initial breeding material from sunflower fertility restorer line R 2574 after treatment of immature zygotic embryos with ultrasound at dose 25.5 w/cm$^2$ for 1 min. before planting it in the nutrition

medium, and b) to evaluate the new line R 12003 morphologically, biochemically and for resistance to phoma, phomopsis, downy mildew and parasite broomrape.

## MATERIAL AND METHODS

A part of the experiments were carried out under laboratory conditions, while others were carried out at the experimental field of Dobroudja Agricultural Institute-General Toshevo.

### Development of mutant lines

The Bulgarian fertility restorer line R 2574, which is highly homozygotic, was used as donor material. The 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 R 2574 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 1 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 rinsing in sterile distilled water several times. Immature zygotic embryos were aseptically isolated and placed on nutrition medium M for further growing (Azpiroz *et al.*): 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°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 a greenhouse.

### Biometric evaluation of control line R 2574 and line R 12003

The biometric evaluation and biochemical analysis of the control genotype and the newly 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, plant height, internode length, leaf width, leaf length, number of leaves, petiole length, head diameter, stem diameter, number of branches, length of branches, seed per 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 original line R 2574 which 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 R 2574 and the obtained line R 12003 was performed with regard to the local *Orobanche* population and the diseases phomopsis, phoma and downy mildew at the Sunflower Phytopathology Laboratory and infection fields of DAI - General Toshevo.

The evaluation for resistance to downy mildew and broomrape was done according to standard methodologies (Gulya *et al*., 1991; Panchenko, 1975). The phytopathological evaluation of lines was performed with regard to downy mildew *Plasmopara halstedii* (Farl.) Berlese & de Toni-race 300, 700 and to the local *Orobanche* population (race A-F). In order to characterize the resistance to downy mildew, the method suggested by Gulya *et al*. (1991) was used. The evaluation of 50 plants from a line was carried out using standard methodology: 0%=S (sensitive); 100%=R (resistant).

Broomrape resistance was evaluated under greenhouse conditions according to Panchenko (1975), being slightly modified to the local conditions. 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%.

The evaluation for resistance to attacks of grey spots (*Phomopsis helianthi*) and black spots (*Phoma macdonaldii*) was performed in the filed and in artificial infection plots. The type and severity of the attack were read one week after mass flowering according to the following scales:

Type of grey spot attack:

0 - no symptoms;

1 - a necrotic lesion up to 5 cm in diameter;

2 - a necrotic lesion over 5 cm in diameter;

3 - several merged necrotic lesions on the stem;

4 - a stem broken at the place of infection.

Type of black spot attack:

0 - no symptoms;

1 - a necrotic lesion near the petiole;

2 - several necrotic lesions on the stem;

3 - the entire stem is covered with necrotic lesion or the stem is broken.

Attacking rate: what portion of the plant's stem is covered with lesions of the pathogen (1/3, 2/3 or 3/3) (Encheva and Kiryakov, 2000).

**Statistical analysis**

The check line R 2574 and developed mutant line R 12003 were analyzed statistically with regard to the agronomic traits such as oil in the seed, 1000 seed weight, plant height, internode length, leaf width, leaf length, number of leaves, petiole length, head diameter, stem diameter, number of branches, length of branches, seed per head, seed length, seed thickness and seed width.

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

## RESULTS AND DISCUSION

Immature sunflower (*Helianthus annuus* L.) zygotic embryos of the sunflower fertility restorer line R 2574 were treated with ultrasound before planting on an embryo culture medium. *In vitro* mutagenesis was done with embryo culture method, which allowed isolation of embryos before terminating their development and their planting on nutrition medium to grow *in vitro* seedlings.

Mutation process included chlorophyll mutations (Table 1) as *Xanthovirescens* (Figure 1), *Albovirescens* (Figure 2), *Viridomaculata* and *Xantha* of necrotic type (Figure 3). Most of the *Xanthovirescens* and *Albovirescens* cotyledons were developed to green plants.

*Figure 1: Chlorophyll mutation Xanthovirescens*

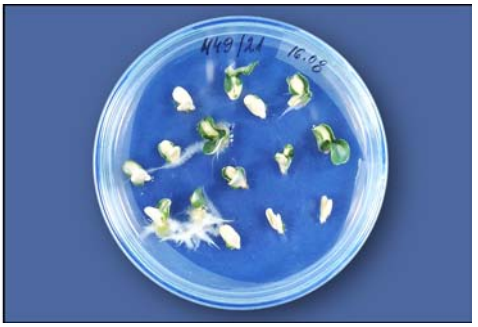

*Figure 2: Numerous chlorophyll mutations-Albovirescens*

*Figure 3: Sunflower mutant plants with chlorophyll types Viridomaculata and Xantha of necrotic type*

*Figure 4: Leaf mutations (malformed leaf)*

Another type of observed mutation was leaves without top or with double tops (Figure 4). We observed dwarf plants, full sterile plants, plants with decrease number of ray flowers, early flowering plants, malformed leaf, split leaf blades, fan-

shaped venation, small and malformed capitulum, absence of ray florets, decreased number of ray florets and replacement of ray leaves with bract leaves. Some of these mutations were published by Soroka and Lyakh (2009) after treatment of immature zygotic embryo with EMS.

*Figure 5: Standard line 2574 R*

*Figure 6: Mutant line 12003 R*

Table 1: Type of morphological mutations in M1 and their descriptions

| N | Type of mutation | Characteristic |
|---|---|---|
| | I Chlorophyll deficiency mutations | |
| 1 | Xanthovirescens | Yellow-green cotyledons |
| 2 | Albovirescens | White-green cotyledons |
| 3 | Viridomaculata | Yellow-green leaves |
| 4 | *Xantha* of necrotic type | Yellow-green spots on the leaves, transforming into necrotic segments |
| | II Leaf mutations | |
| 5 | Malformed leaf | Malformed, split leaf blades |
| 6 | Dichotomous venation | Fan-shaped venation |
| | III Steam mutations | |
| 7 | Low-growing | Plant reduced in height |
| | IV Inflorescence mutations | |
| 8 | Malformed capitulum | Malformed |
| 9 | Absence of ray florets | |
| 10 | Few ray florets | Decrease number of ray florets |
| 11 | Replacement of ray leaves with bract leaves | |
| 12 | Small capitulum | Decreased size of capitulum |
| 13 | Malformed capitulum | Inflorescence consisting of many malformed heads |

In our study some mutant plants were isolated and self-pollinated for several generations. New sunflower forms with inherited morphological, biochemical and phytopathological changes were obtained through selection and self-pollination.

The line R 12003 (Figure 6) was preferred due to its significant differences with the control line R 2574 (Figure 5) resistance to phomopsis, phoma, downy mildew and broomrape and good combining ability.

Table 1 presents data on the significant change of the mean value for the character plant height. Plant height is one of the morphological indices most often investigated in cultural sunflower. It is considered a quantitatively inherited character. Increased plant height is controlled by dominant genetic effects (Putt, 1966), 57% dominant effects and 30% additive effects (Lay and Khan, 1985) and dominance to over dominance (Kovačik and Škaloud, 1990).

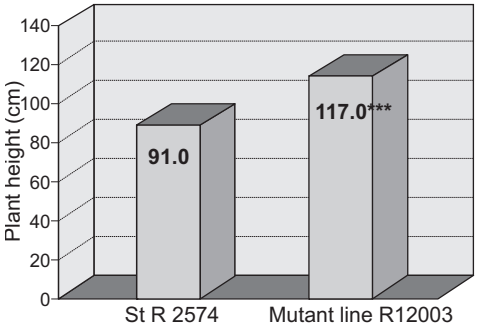


*Figure 7: Plant height (cm) of control line R 2574 and mutant line R 12003 (***-P=0.1%)*

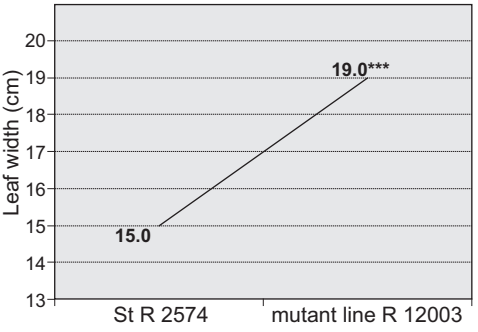


*Figure 8: Leaf width (cm) of standard line R 2574 and mutant line R 12003 (***-P=0.1%)*

The observed difference was considerable when their increase was taken into account. The new mutant line possessed plant height with 26.2 cm more than the control R 2574 (Figure 7). The difference was at the highest degree of significance. Vice versa decrease in plant height has been reported using the direct organogenesis method in combination with gamma irradiation (Encheva *et al.*, 1993, 2002).

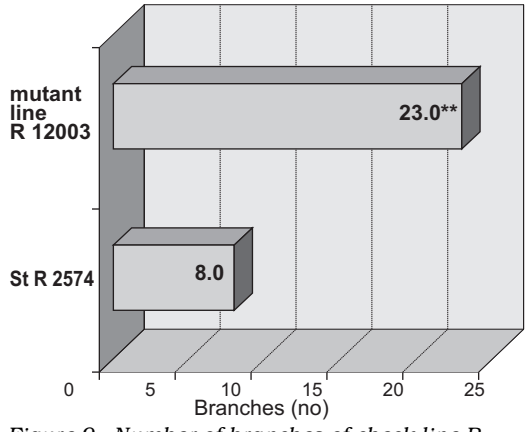


*Figure 9: Number of branches of check line R 2574 and mutant line R 12003. (**-P=1%)*

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 at doses 150 Gy and 200 Gy.

Larger photosynthetic surface at mutant line was possessed through considerable and statistically significant increase in leaf width with 4 cm (Figure 8), leaf length with 2.5 cm (Table 2) and larger number of leaves (Table 2).

A significant increase of number of branches with 15 (Figure 9), plant height, leaves size and number of leaves leads to changes in plant architecture of mutant line R 12003.

Oil content in seed is one of the most important agronomic indices (Figure 10). The results from dispersion analysis demonstrated a significant increase with 2.0% at mutant line R 12003.

Number of seeds per head is one of the characters forming seed yield (Figure 11). In our study we observed the increase of mean value of seeds with 190 numbers in comparison to the check line. The increased oil content and seed yield of the mutant restorer line produced were valuable changes with significant importance for the sunflower breeding programme.

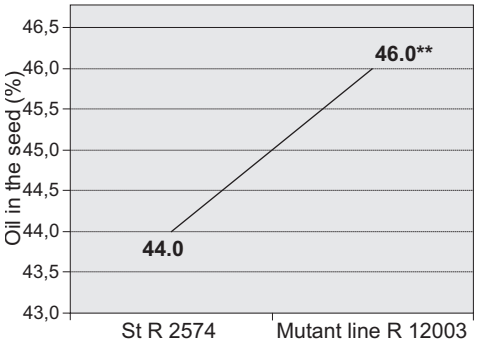


*Figure 10:Oil in the seed (%) of control line R 2574 and mutant line R 12003 (**-P=1%)*

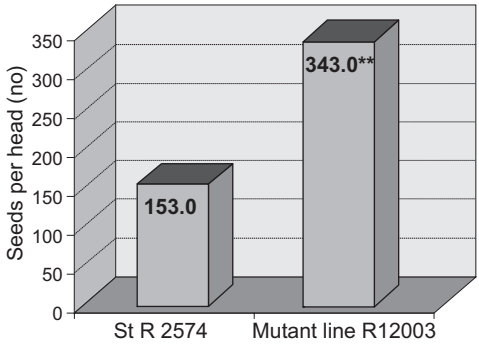


*Figure 11:Seeds per head (no) of control line R 2574 and mutant line R 12003 (**-P=1%)*

Significant positive changes were noted for the characters internode length, stem diameter and head diameter. A maximum value of differences was 1.2 cm, 1.8 cm, and 1.3 cm, respectively.

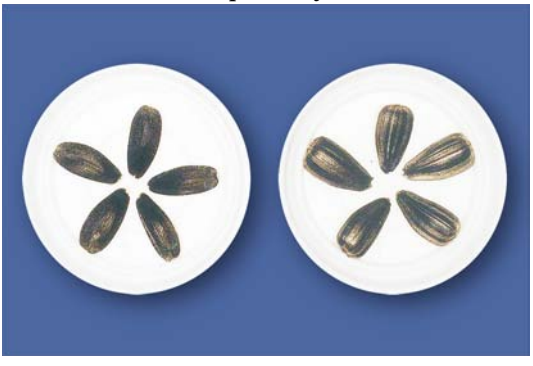

*Figure 12:Seed size of control line R 2574 (left) and mutant line R 12003 (right)*

The increase of 1.1 mm of the character seed length and the decrease of 0.5 mm (Table 2) at seed thickness leads to the change of seeds size of mutant line R 12003 (Figure 12).

Table 2: Significant morphological changes in the indices of line R 12003, developed from the control line R 2574. Averaged data

| Genotype 2574 R | Number of leaves | Leaf length | Petiole length | Seed length (mm) | Seed thickness (mm) | Seed width (mm) |
|---|---|---|---|---|---|---|
| | Mean (no) | Mean (cm) | Mean (cm) | Mean (mm) | Mean (mm) | Mean (mm) |
| Control R 2574 | 21.00 | 16.23 | 10.73 | 9.98 | 3.62 | 4.86 |
| line R 12003- us | 24.00 +c | 18.77 +c | 12.13 +b | 11.10+b | 3.14-a | 4.92 |

a, b and c = significant of differences at the level of 0.05, 0.01 and 0.001, respectively

Based on all 16 agronomic characters investigated, it can be determined that the positive significant differences were registered for plant height, number of leaves, leaf width, leaf length, petiole length, internode length, number of branches, stem diameter, head diameter, number of seeds per head, oil content in seed and seed length. This account for 75% of all characters studied. Vice versa, the negative change was registered for seed thickness, *i.e.*, 6.3% of the total number of traits. Stability after treatment of immature zygotic embryos with ultrasound was demonstrated by the character length of branches, seed width and 1000 seed weight.

**Evaluation of line R 12003 for resistance to some economically important diseases and parasite on sunflower**

Resistance of the mutant line to the local population of the broomrape races A-F distributed in Bulgaria was established. The control line R 2574 was susceptible to this parasite.

In Bulgaria where sunflower is grown commercially, the successful production is endangered by many fungal pathogens and parasites. Losses may be severe, near 100% in parts or even entire fields under extreme circumstances. Because of the narrowed germplasm of cultivated sunflower, mutagenesis is applied as an alternative method of conventional ones.

In the nature the polyploid perennial species are considered as sources of resistance. Among them, *H. tuberosus*, *H. scaberimus*, *H. divaricatus* and *H. rigidus* have been most frequently used as a source of 100% resistance to Orobanche (Pustovoit, 1976). Studies of Christov, 1996 confirmed *H. tuberosus* as a source for Orobanche resistance, too. In Spain, Ruso *et al*. (1996) and Fernandez-Martinez *et al*. (2000) found resistance to several virulent races, including race F, in 29 perennial wild species. Fernandez-Martinez *et al*., 2008 reported the resistance to broomrape found in race F sunflower lines developed after cross-breeding with perennial wild species *H. grosseserratus*, *H. maximiliani* and H. divaricatus.

Burlov and Kostiuk (1976) and Pogorleckii and Gesele (1976) discovered that broomrape resistance was controlled by a dominant gene which was designated as *Or*. Many studies have showed a monogenic control by a single dominant gene over

sunflower resistance against races A-E (Ish-Shalom *et al.*, 1993; Sukno *et al.*, 1999), although two dominant genes (Dominguez, 1996b) and one recessive gene (Ramaiah, 1987). 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 line to the local *Orobanche* population-race F can also be obtained through induced mutagenesis of cultured sunflower, by treatment of immature zygotic embryo with ultrasound, in particular. The same mutation, resistance to the parasite broomrape, was obtained in all variants of treatment of the initial genotype R 2574. Genetically, this regularity could be explained with existence of similarity of quantity of mutable genes in close relative organisms. The results allow us to presume that the resistance of the mutant sunflower line 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 also obtained after EMS treatment of pollen of tomato (Gavazi *et al.*, 1987).

The three years evaluation of line R 12003 showed immunity to *Phoma macdonaldii*, resistance to *Plasmopara halstedii*–race 300 and 700 and middle resistance to *Phomopsis helianthi*.

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 line.

## CONCLUSION

As a result of treatment of immature zygotic embryos of the sunflower genotype R 2574 with ultra sound some chlorophyll, leaf and inflorescence mutations were observed. Owing to continuous self-pollination and selection, the line 12003 was obtained, which was distinguished by its morphological, biochemical and phytopathological differences.

The contribution of the present research connected to the investigated mutation was the appearance of resistance to the parasite broomrape. The studied line showed very good combining ability and it was included in the sunflower breeding program.

Having combined induced mutagenesis with embryo culture method, it was concluded that the new variability was exclusively due to the effect of the mutagen. This conclusion 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 a short period of *in vitro* cultivation of immature zygotic embryos.

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