HELIA, **36**, Nr. 59, p.p. 71-84, (2013) UDC 633.854.78:63.528
DOI: 10.2298/HEL1359071E

APPLICATION OF EMBRYO CULTURE METHOD IN COMBINATION WITH GAMMA IRRADIATION AND ULTRA SOUNDS (Part I)

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Received: October 24, 2013
Accepted: December 05, 2013

SUMMARY

Immature sunflower (*Helianthus annuus* L.) zygotic embryos belonging to R and B sunflower lines were treated with ultra sound and gamma rays before planting in an embryo culture medium. In those circumstances new mutant lines with the altered architecture, increased oil content, 1000-seed weight, number of seed per head, as well as seed yield were developed.

Some mutant lines showed very good combining abilities. Line 12004 R (developed trough Embryo culture method in immature zygotic embryos in combination with gamma irradiation at dose 8 Gy), paternal component of hybrid Goryanin, line 12002 R (developed trough Embryo culture method in immature zygotic embryos in combination with gamma irradiation at dose 8 Gy), paternal component of the commercial hybrid Rada and line 12003 R (developed trough Embryo cluture method in immature zygotic embryos in combination with ultra sound at dose 25.5 W/cm^2 for 1 min) and paternal component of the commercial hybrid Yana were realized. Hybrid Goryanin considerably exceeded the average standard (the Bulgarian commercial hybrids Mercury, Perfect and S-205) by seed yield with 15.2%. Hybrid Rada considerably exceeded the average standard of seed yield by 2.3-7.7% (in relation to Bulgarian commercial hybrid Albena and French commercial hybrid Diabolo). The vegetation period was 115 days. The hybrid Yana considerably exceeded the average standard of seed yield by 7.6%-14.4% (in relation to Bulgarian commercial hybrid Perfect and French commercial hybrid Diabolo). Its vegetation period is 110 days. The hybrids Rada and Yana possess the immunity to the parasite *Orobanche cumana* race G, immunity to *Plasmopara helianhi*-races 300, 330, 700 and 731, immunity to *Phomopsis* and *Macrophomina*, immunity to *Sclerotinia sclerotiorum* (root form) and tolerance to *Phoma.*

Key words: **biochemical, combining ability, commercial hybrids, embryo culture method, gamma rays, *Helianthus annuus* L., mutant lines, morphological, ultra sound**

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INTRODUCTION

The development of variable breeding materials is a primary task of sunflower genetic and breeding programs. New approaches to the tissue culture in combination with induced mutagenesis provide an additional possibility to enrich genetic variability in this crop.

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

Among a few techniques used in modern breeding, the method of mutagenesis is considered to be the most effective one. Mutagenesis, both physical and chemical, proved best for induced mutation 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 agronomical important characters; others can be used as marker traits. Positive results were obtained when induced mutagenesis and tissue cultivation were combined appropriately in tomato (Gavazzi *et al.,* 1987), maize, banana and plantain (Novak *et al*., 1988, 1990), potato (Ahloowalia, 1990), wheat (Cheng *et al.,* 1990), oil crops (Ashri, 1993), rice (Maluszynski *et al.,* 1994) and other crops (Micke *et al*., 1990).

According to Ahloowalia *et al*. (2004) in agriculture more than 2250 cultivars obtained either as direct mutants or derived from their cross-cultures have been obtained worldwide. The author summarized that the economic value of a new variety can lead to the increased yield and enhanced quality of production.

The mature sunflower seeds were subjected to mutagenic treatment more often (Sagadeesan *et al*., 2008). Mutations like yellow leaf veins, fasciation, wrinkled leaf, zigzag stem, zigzag ray florets, stigma emergence and brown patch mutant are new characteristics developed after treating air dry seeds of sunflower variety "Surya" with different doses of gamma rays (Jambhulkar and Joshua, 1999). Lyakh *et al*., 2005 studied the frequency and spectrum of morphological mutations, raised in M2 after sunflower mature and immature seed treatment with ethylmethanesulphonate (EMS). Thirty-three types of mutations were found after treatment of sunflower immature embryos (Soroka and Lyakh, 2009) with ethylmethanesulphonate (EMS).

The aim of this study was to summarize morphological and biochemical mutations in sunflower developed thought Embryo culture method in combination with ultra sonic and gamma irradiation (^{137}Cs) in immature zygotic embryos.

MATERIAL AND METHODS

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

Development of mutant lines

Bulgarian fertility restorer lines and the line with normal cytoplasm, which is highly homozygotic, were used as the donor material. The main requirement to the initial plant material used according to the methods of Embryo culture in combination with ultra sonic and gamma irradiation is to be genetically pure, *i.e.* homozygotic to the highest possible degree. Therefore the control lines with very good morphological uniformity were chosen as the initial material for induced mutagenesis.

Plants were grown in the field and were hand-pollinated. The immature seeds (13-16 days old) were treated: 1) with the ultra sound at the dose of 25.5 W/cm^2 for 1, 2, 3, 5, 7, 9, 10, 11, 13 and 30 min and 2) with the ionizing radiation such as gamma rays (^{137}Cs) at the dose of 8 Gy and 50 Gy (the power of the dose being 0,338 krad/min=3.38 Gy/min). Immature seeds were sterilized under the following conditions: 1) 1 min in 95% ethanol; 2) 15 min in bleaching solution (2.7% of active Cl); 3) followed by several washings in sterile distilled water. Immature zygotic embryos were aseptically isolated and planted on the 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°C, 16/8 h photoperiod for one week. R0M0 plants (nomenclature according to Novak *et al.*, 1988) which formed roots were transplanted and further grown, self-pollinated and harvested by single plants under greenhouse conditions. The seeds produced (R1M1) and were sown in the field.

Biometric evaluation of control genotypes and mutant R and B lines

Biometric evaluation and biochemical analysis of the control genotype and new developed mutant lines were made on 10 plants for each individual year and included the main 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 yield per head (g), 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 lines which were 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.

Hybridization

To determine the combining abilities of newly developed sunflower mutant lines 12001 R, 12003 R and 12004 R sterile analogues of Bulgarian selfed line 2607 A

and 4558 A were used. The standards for comparison of the new hybrid Goryanin were Bulgarian commercial hybrid Mercury, Perfect and S-205. The standards for comparison of the new hybrid Rada were Bulgarian commercial hybrid Albena and French hybrid Diabolo. The standards for comparison of the new hybrid Yana were Bulgarian commercial hybrid Perfect and French hybrid Diabolo.

The obtained hybrid Goryanin was tested for three years (2003, 2004 and 2005) in the trial field of Dobroudja Agricultural Institute-General Toshevo. The obtained hybrid Rada was tested for two years (2002 and 2004), while hybrid Yana was tested for three years (2004, 2006 and 2007) in the breeding fields of State Variety Testing Commission in six locations according to the block-design method and in three replications, the area of each replication being 25 m^2.

RESULTS AND DISCUSION

Immature sunflower (*Helianthus annuus* L.) zygotic embryos of sunflower R and B lines were treated with ultra sonic and gamma rays before planting on the Embryo culture medium. *In vitro* mutagenesis was combined with Embryo culture method, which allowed isolation of embryos before terminating their development and their planting in the nutrition medium to grow *in vitro* seedlings.

As a results of continuous and individual selection new mutant R and B lines were developed (Table 1). Mutant lines were selected due to their statistically significant morphological and biochemical changes.

Figure 1: Genotype RHA-857 R (left) and mutant line 106 R (right)

Plant height is one of the morphological indices most often investigated in cultural sunflower. It is considered to be a quantitatively inherited trait. Significant changes of the mean value for the trait "plant height" were registered as well. New restorer lines 114 R, 115 R and 116 R with the altered architecture were developed. They originated from the genotype 2574 R treated with gamma irradiation at the dose of 8 Gy (line 114 R) and ultra sounds at the dose of 25.5 W/cm^2 for 1 min (lines 115 R and 116 R). Lines were characterized with the increased plant height within 14.1 cm to 26.2 cm, increased number of branches (8 to 14) and e. ts. (Encheva *et al.*, 2003).

Mutant lines 116 R, 117 R, 118 R, 119 R and 120 R were developed by

Table 1: Morphological and Biochemical mutations in sunflower, developed thought embryo culture method in combination with ultra sonic and gamma irradiation (Cs^{137})

Mutant R and B lines	Type of mutations	Publications
Lines: 114 R; 115 R; 116 R	Morphological changes; increased oil content in seeds (%); increased 1000 seed weight; increased number per head	Encheva, J., Christov, M., Nenov, N., Tsvetkova, F., Ivanov, P., Shindrova, P. and Encheva, V., 2003. Developing genetic variability in sunflower (*Helianthus annuus* L.) by combined use of hybridization with gamma radiation or ultrasound. Helia 26(38): 99-108.
Lines: 116 R; 117 R; 118 R; 119 R; 120 R	Morphological changes; mutation for seeds size and seed coat color	Encheva, J., Shindrova, P and Penchev, E., 2008. Developing mutant sunflower lines (*Helianthus annuus* L.) through induced mutagenesis. Helia 31(48): 61-72.
Lines: 193 R; 194 R	Morphological changes; increased oil content in seeds (%); mutation for seeds size and seed coat color	Encheva, J., 2009a. Creating sunflower (*Helianthus annuus* L.) mutant lines using induced mutagenesis. B.J.A.S. 15(2): 109-118.
Line: 106 R	Morphological changes; increased oil content in seeds (%); mutation for seeds size and seed coat color	Encheva, J., 2009b. Sunflower (*Helianthus annuus* L.) mutant line, developed using induced mutagenesis. In: Field Crop Studies, Breeding and technical crops. Vol. V-1: 109-117.
Lines: 74 B; 78 B; 85 B; 88 B	Morphological changes; Increased 1000 seed weight; mutation for seeds size	Encheva J., Petrov, P. and Shindrova, P., 2010. Developing mutant B lines in sunflower (*Helianthus annuus* L.) trough induced mutagenesis. International Scientific conference, 14-17 October, Plovdiv, 5-12.
Lines: 97 R; 98 R; 99 R; 100 R; 101 R	Morphological changes; increased oil content in seeds (%)	Encheva, J. and Shindrova, P., 2011. Developing mutant sunflower lines (*Helianthus annuus* L.) through induced mutagenesis and study their combining ability. Helia 34(54): 107-122.
Line: 171 R	Morphological changes; increased oil content in seeds (%); increase seed yield per head (g)	Encheva, J., Valkova, D. and Shindrova, P., 2012a. Mutant sunflower line 171 R, produced through *in vitro* mutagenesis of immature embryos. B.J.A.S. 18(3): 342-347.
Line: 12003 R	Morphological changes; increased oil content in seeds (%); increase seed yield per head (g); mutation for seeds size and seed coat color	Encheva, J., Shindrova, P., Valkova, D. and Encheva, V., 2012b. Mutant line 12003 R, produced through by *in vitro* mutagenesis. Helia 35(56): 19-30.
Lines: 143 R; 145 R	Morphological changes; increased seed yield per head (g)	Encheva, J., 2013. Mutant sunflower lines, developed through ultra sonic treatment of immature embryos of genotype 377 R. B.J.A.S. - in press

treating the genotype 147 R with ultra sounds at the dose of 25.5 W/cm^2 for 5, 7, 9, 11 and 13 min, respectively. Specific for them were decreased plant height (13.8 to 22.4 cm) and decreased length of branches (8.4 to 9.5 cm) (Encheva *et al.*, 2008). Plant height reduction after treating the immature zygotic embryos of maize with 5 Gy was reported by Novak *et al.* (1988).

After treating the genotype RHA-857 with ultra sounds at the dose of 25.5 W/cm^2 for 3 min line 106 R (Figure 1) was produced with the decreased mean value of many morphological characters (Encheva, 2009b).

Lines 97 R, 99 R (Figure 2), 100 R (Figure 2) and 101 R, developed after treating the control line 381 R with ultra sounds at the dose of 25.5 W/cm^2 for 1 min and line 98 R for 3 min showed increased plant height (6.5 cm to 23.3 cm), decreased length of branches (12.5 cm), decreased leaf size, increased number of branches with 11 and e. ts. (Encheva and Shindrova, 2011).

Figure 2: Mutant lines 99 R and 100 R

Figure 3: Genotype 2571 R (left) and mutant line 171 R (right)

After treating the line 2571 R with ultra sounds at the dose of 25.5 W/cm^2 for 30 min, the mutant line 171 R (Figure 3) was produced. It was characterised by the increased plant height (19.7 cm), increased length of branches (7.3 cm), increased mean value of other morphological characters (Encheva *et al.*, 2012a).

Line 12003 R, produced after treating the genotype 2574 R with ultra sounds at the dose of 25.5 W/cm^2 for 1 min was characterized by the increased plant height

(26.2 cm), increased length of branches (15 cm), increased size of leaves, as well as the increased mean value of other morphological characteristics (Encheva *et al.*, 2012b).

Mutant lines 143 R and 145 R were characterized by the increased plant height (35.7 cm), increased length of branches (9.9 cm), increased head diameter (8.9 cm) and increased leaf size. Mutant lines were developed after treating the line 377 R with ultra sounds at the dose 25.5 W/cm^2 for 1 min and 2 min respectively (Encheva, 2013).

The application of method Embryo culture in combination with ultra sounds at the dose of 25.5 W/cm^2 for 1, 3 and 5 min was used to developed new mutant lines with normal cytoplasm (B), also. The new mutant lines 74 B (1 min), 78 B (3 min), 85 B (3 min) and 88 B (5 min) were developed by treating the genotype 197 B. Specific for them was the alteration of plant height in two directions (Encheva *et al.*, 2010). However, in many cases the changed traits had a synergistic effect on the cultivation of the crop, agronomic inputs, crop rotation and utilization (Ahloowalia *et al.*, 2004). For example, sunflower hybrids developed with short height mutant lines have contributed significantly to the increased grain yield because of their resistance to lodging and high planting density.

The increased oil content of the mutant restorer lines produced is a valuable change with significant practical importance for the sunflower breeding programme. In the line 116 R, generated from genotype 2574 R increased mean value of oil in seed by 2% was observed (Encheva *et al.,* 2003).

The lines 193 R and 194 R developed from line 374 R showed significant increase of oil in seed by 4.1% and 4.3%, respectively (Encheva, 2009a). Significant increase of oil in seed by 5.9% in the line 106 R was registered. The origin of line was the genotype RHA-857 (Encheva, 2009b). Lines 97 R, 98 R, 99 R, 100 R and 101 R with the origin from line 381 R showed the increase of oil in seed in (by 2.5% to 7.0%) (Encheva and Shindrova, 2011). Line 171 R produced after treating the line 2571 R was characterized by 2.4% increase of oil in seed (Encheva *et al.*, 2012a). Line 12003 R differed from the genotype 2574 R by 2% increase of oil in seed (Encheva *et al.*, 2012b).

1000-seed weigh is an index has a direct relation to sunflower yield. Considerable increase of 11.0 g and 11.5 g was registered in mutant lines 193 R and 194 R, originated from the genotype 374 R (Encheva, 2009a). Such mutation was observed in mutant lines with normal cytoplasm 74 B, 78 B, 85 B and 88 B. They exceeded the mean value of genotype 197 B with 5.4 g to 6.7 g (Encheva *et al.*, 2010)

The seed number per head is another index having direct relation to sunflower yield. Positive changes in this trait were observed in lines 114 R, 115 R and 116 R, generated from genotype 2574 R. Exceeding was in diapason of 48 to 232 seeds per head (Encheva *et al.* 2003).

Line 12003 R produced after treating the line 2574 R demonstrated the increase of trait seed yield per head by 190 numbers (Encheva *et al.*, 2012b).

Mutations were also registered in the indices seed size and seed coat color. Reduction of the indices seed width and seed length of mutant line 106 R was

observed as well (Figure 4). Seeds coat of new line were different by their black color, while at the control RHA-857 seeds coat were black with gray stripes in both marginal and lateral (Encheva, 2009b).

Figure 4: Seed size and seed coat color of line RHA-857 R (left) and mutant line 106 R (right)

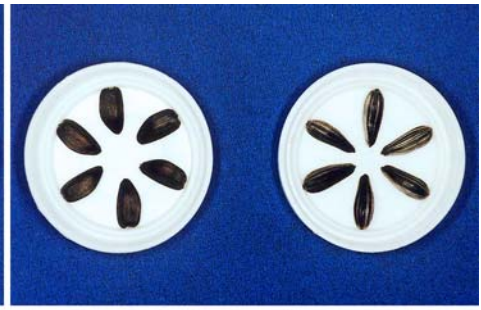

Figure 5: Seed size and seed coat color of line 2571 R (left) and mutant line 171 R (right)

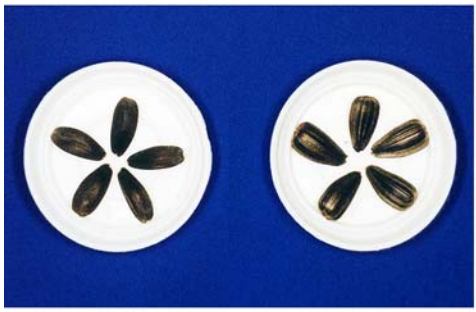

Figure 6: Seed size and seed coat color of line 2574 R (left) and mutant line 12003 R (right)

Figure 7: Seed size of line 197 B (above) and mutant lines 74 B, 78 B and 88 B (down)

Seeds of mutant line 171 R were black with gray stripes, both marginal and lateral (Figure 5), while that of the donor line 2571 R were black. The new line differed by the increased width and length (Encheva *et al.*, 2012a). The mutant line 12003 R had black seeds with gray stripes, both marginal and lateral (Figure 6), while the donor line 147 R had black color (Encheva *et al.*, 2012b). Line 116 R, 118 R and 120 R had black seed color, and lines 117 R and 119 R - brownish seed color. The genotype 147 R had seeds with black color and gray stripes, both marginal and lateral. The mutant lines did not have stripes (Encheva *et al.*, 2008).

Lines 74 B, 78 B and 88 B (Figure 7) with increased thickness and width of the seeds were developed from the donor line 197 B (Encheva *et al.*, 2010).

Some of the mutations isolated in our studies with immature zygotic embryos were observed by Hristov and Nikolova (1996) after treatment of air dry seeds with gamma rays (^{60}Co) at doses 150 Gy and 200 Gy, EMS and ultra sonic and Jambhulkar and Joshua (1999) after treatment of air dry seeds of sunflower using gamma rays.

Possibility for practical use of hybrids Goryanin, Rada and Yana, produced with participation of mutant lines 12002 R, 12003 R and 12004 R

After long individual selection mutant lines 12002 R, 12003 R and 12004 R were developed and tested for their combining abilities. The results from three-year testing of lines 12002 R, 12003 R and 12004 R revealed very good combining abilities in hybridization. The line 2607 A and 4558 A (sterile analogues of the Bulgarian inbred lines) were used as a tester.

Figure 8: Hybrid "Goryanin" developed from the crossing 4558 A × 12004 R

Hybrid Goryanin (Figure 8) is a simple cross hybrid, developed by crossing of 4558 A × 12004 R. The line 12004 R was developed by Embryo culture method of immature zygotic embryos in combination with gamma irradiation at the doze of 8 Gy at genotype 763 R. Two-factor dispersion analysis was done according to the characteristic seed yield t/ha. The hybrid Goryanin considerably exceeded the average standard of seed yield (Bulgarian commercial hybrids Mercury, Perfect and S-205) by 15.2%. Under the conditions present in Dobroudja Agricultural Institute the average seed yield was 3832.0 t/ha, in comparison to the average standard 3328.0 t/ha. The hybrid possessed the immunity to the parasite *Orobanche cumana* race F, immunity to *Plasmopara helianhi* - races 300, 330 and 700 and tolerance to *Phomopsis* and *Phoma.*

Hybrid Rada (Figure 9) is a simple cross hybrid, developed by crossing the lines 2607 A × 12002 R. The line 12002 R was developed by Embryo culture method of immature zygotic embryos in combination with gamma irradiation at the dose of 8 Gy. (Encheva *et al.*, 2003). The hybrid was approved at the meeting of Expert Commission on oil crop of Executive Agency of Variety Testing, Approbation and Seed Control held on January 13, 2005. The Certificate for new variety is № 10695.

Figure 9: Hybrid "Rada" developed from the crossing 2607 A × 12002 R

Hybrid Rada considerably exceeded the average standard of seed yield (the Bulgarian commercial hybrid Albena and French commercial hybrid Diabolo) by 2.3-7.2%.

In State Variety Commission the middle seed yield was 3430.0 t/ha in comparison to the average standard 3225.0 t/ha. Vegetation period was 115 days. The hybrid possessed the immunity to the parasite *Orobanche cumana* race G, immunity to *Plasmopara helianhi*-races 300, 330, 700 and 731, resistance to *Phomopsis* and *Macrophomina,* immunity to *Sclerotinia sclerotiorum* (root form) and tolerance to *Phoma.*

Hybrid Rada was tested in 2000 at the territory of South France by the company Rustica Prograin Genetique. The hybrid considerably exceeded the average standard of seed yield (French commercial hybrids Prodisol, Allstar and Melody) by 3%.

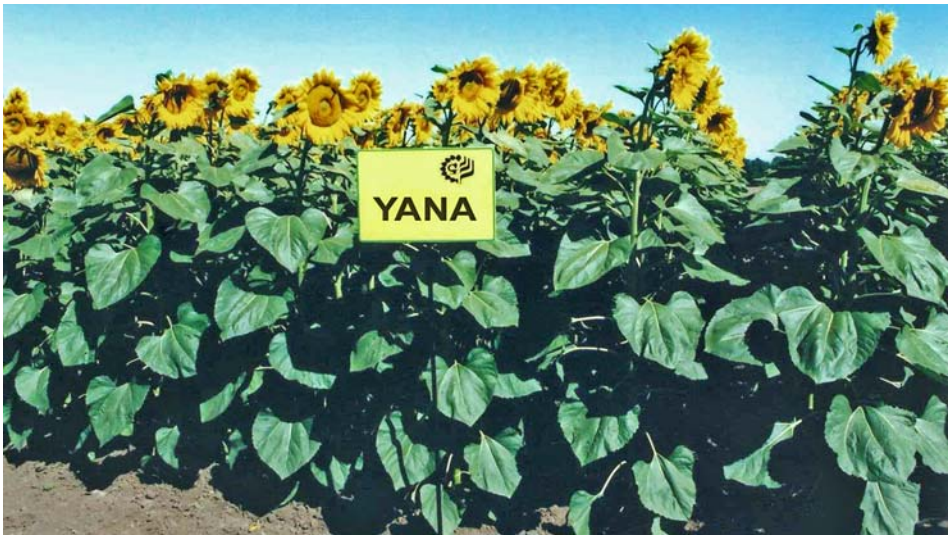

Figure 10:Hybrid "Yana" developed from the crossing 2607 A × 12003 R

Hybrid Yana (Figure 10) is a simple cross hybrid, developed by crossing the lines 2607 A × 12003 R. (Encheva *et al.,* 2012c). The line 12003 R was developed by Embryo culture method of immature zygotic embryos in combination with ultra sounds at the dose of 25.5 W/cm^2 for 1 min. Hybrid Yana considerably exceeded the average standard of seed yield (the Bulgarian commercial hybrid Perfect and French commercial hybrid Diabolo) by 10.0%. In State Variety Commission the average seed yield was 3493.0 t/ha in comparison to the average standard 3167.0 t/ha. Maximum seed yield was in 2004 - 4200.0 t/ha. Its vegetation period was 110 days. Hybrid Yana was approved at the meeting of Expert Commission on oil crop of Executive Agency of Variety Testing, Approbation and Seed Control held on April 9, 2008. The certificate for new variety is № 10819. The hybrid possessed the immunity to parasite *Orobanche cumana* race G, immunity to *Plasmopara helianthi,* races 300, 330, 700 and 731, resistance to *Phomopsis*, immunity to *Macrophomina,* immunity to *Sclerotinia sclerotiorum* (root form) and tolerance to *Phoma.*

CONCLUSION

We succeed to create mutant sunflower lines with increased oil content in seed, increased seed yield per head, increased 1000-seed weight and increased number of seed per head, change architecture and improve combining abilities. This is a desirable combination during the sunflower breeding program.

The available literature on sunflower does not provide the data on treatment of immature sunflower zygotic embryos with ultra sounds. 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 and their cells were at different phases of mitotic cycle. *i.e.* this is a functional tissue. The most sensitive is the interphase during which the "S" stage was realized (in which synthesis of DNA occurred). In this phase of mitosis the influence of mutagen factors is the strongest and it can cause translocations in nucleus apparatus. This is expected to increase the frequency of mutations to a higher rate in comparison to the usual approach of treating air dry seeds.

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 advantage in this case is that this allows isolation of embryos before terminating their development and their planting onto the nutrition medium to grow *in vitro* seedlings.

We concluded that similar changes occurred in several immature embryos of the same control genotype. This allows us to assume that there are mutable locations in the cultural sunflower genome resulting from induced mutagenesis.

The use of ultra sonic or gamma irradiation for occurrence of single mutants controlled by one or several genes, while preserving other positive characters of well-adapted genotype, is one of the most useful application of induced mutagenesis. These include characters such as plant height, 1000-seed weight, seed per head, maturity and disease resistance which contribute to the increased yield and quality traits, *e.g.* modified oil content, and size and quality of seeds.

Although induced mutagenesis is a random and unpredictable process, it is an invaluable fact that the occurrence of morphological and biochemical mutations is of stable inheritance in the progenesis of R and B lines.

Studied mutant lines showed very good combining abilities in hybridization. Commercial hybrids Rada and Yana were produced with mutant lines 12002 R and 12003 R, respectively. Hybrid Yana was included in the variety list of the European Union in 2009. German-Rumanian Company Saaten-Union started seed production and distribution of hybrid Yana in Europe and Asia.

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