

APPLICATION OF SOME BIOTECHNOLOGICAL ACHIEVEMENTS IN SUNFLOWER BREEDING

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SUMMARY

Basic *in vitro* techniques, linked with the increase of genetic variability and acceleration of the breeding process, have been mastered and applied in sunflower breeding programs at Dobroudja Agricultural Institute, Bulgaria.

The method of direct organogenesis has been successfully used for genetic modifications of fertility restorer lines RHA-857, 147 R and Z-8-A and for overcoming the inability for crossing between *H. annuus* (hybrid Albena) and *Helianthus tuberosus*, *Helianthus salicifolius*, *Helianthus maximiliani*, *Helianthus silphoides*, *Verbisina helianthoides* and between *H. mollis* and *H. annuus*.

The method of γ -induced parthenogenesis has been successfully applied to the cultivated sunflower (*H. annuus* L.) for obtaining doubled haploid lines. The parthenogenetic ability of both maternal and paternal parents and the gamma-irradiation effect on parthenogenic embryo development have been studied.

A number of researches have been focused on the genetic potential of wild species from the genus *Helianthus*. Our studies investigated the possibilities of the methods for cultivation of embryos and anthers from interspecific origin. They included the use of the embryo cultivation technique under *in vitro* conditions until the production and testing of finished lines that could directly participate in the breeding process. Embryos were isolated from interspecific hybrids (cultural x wild and wild x cultural) with the participation of the species *H. resinosus* M-046, *H. strumosus* M-059, *H. salicifolius* M-045, *H. ciliaris* M-092, *H. giganteus* M-030, *H. maximiliani* M-087 and *H. glaucophyllus* M-012. The studies included and investigated 51 new lines which originated from embryos of the above interspecific crosses.

A number of lines obtained from embryos of the above interspecific crosses exhibited valuable economic indices and high combining ability. They were included as components in highly productive hybrids.

Key words: sunflower, somaclonal variation, induced parthenogenesis, embryo culture, anther culture, distant hybridization

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INTRODUCTION

A number of *in vitro* and biochemical methods have been developed, which aim to increase the available genetic variability of the initial breeding material or to intensify the breeding process by applying appropriate methods for evaluation and selection of desirable genotypes and unification of the heterozygous progenies culture (Gunda Mix, 1985; Mezzarobba and Jonard, 1986; Gürel *et al.*, 1990; Then-gane *et al.*, 1994; Vasić *et al.*, 2000).

Although remarkable results have been achieved by applying biotechnology in many crops, the studies on the use of various *in vitro* methods for production of forms with valuable characteristics in sunflower have been unsatisfactory. A lack of statistically significant changes after plant regeneration from immature embryos of sunflower was pointed out by Freyssinet and Freyssinet (1988) who used a direct organogenesis system. Somaclonal variation transmitted to the next generation has been described by Pugliesi *et al.* (1991) and Roseland *et al.* (1991). However, sufficient data describing the scope and heritability of the induced somaclonal variation are not available.

By the present review we make an attempt to reveal the recent progress in development and use of *in vitro* techniques in sunflower.

Somaclonal variation in sunflower

The results achieved at the Dobroudja Agricultural Institute are the first systematic investigation on somaclonal variation in sunflower. The studies on lines obtained by the method for induction of direct organogenesis and somatic embryogenesis from immature embryo show that it can be successfully applied for generation of genetic variation that can be included in the program for production of new initial breeding material as shown in Figures 1, 2, 3 and 4 (Encheva *et al.*, 1993; Encheva, 2000). The modifications in comparison to the initial line were confirmed by RAPD analysis, too (Encheva, 2000).

The response of lines with normal cytoplasm, fertility restorers, hybrids and open pollinated varieties to *in vitro* cultivation of immature embryos by the methods of direct organogenesis and somatic embryogenesis was investigated. The results revealed that the group of F₁ hybrids was most responsive to *in vitro* cultivation in all five nutrition media used (85%), while the group of restorers was the least responsive (Table 1) (Encheva *et al.*, 1993).

Table 1: Productivity of some hybrids and lines obtained after embryo culture from polycross population

Combination	Productivity averaged for three years in % of mean standard (Albena and San Luka)				Mean
	1997	1998	1999	2000	
1041R x 8Gy	130.8	115.6	-	102.3	116.2
147R x 6 Gy	112.3	110.8	-	103.0	108.7

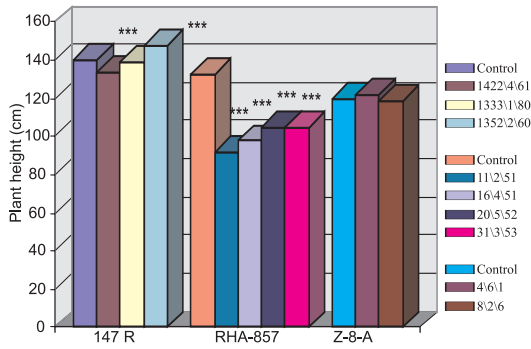


Figure 1: Significant changes of the index plant height in R11-R12 somaclonal families originating from genotypes 147 R, RHA-857 and Z-8-A during the period 1997-1999

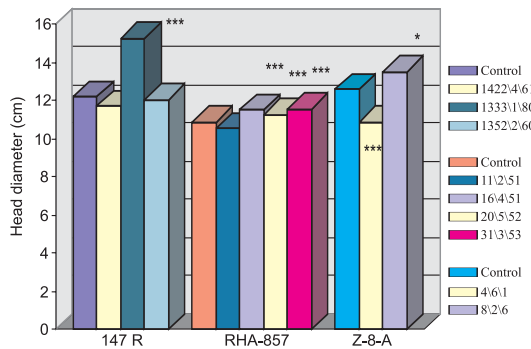


Figure 2: Significant changes of the index head diameter in R11-R12 somaclonal families originating from genotypes 147 R, RHA-857 and Z-8-A during the period 1997-1999

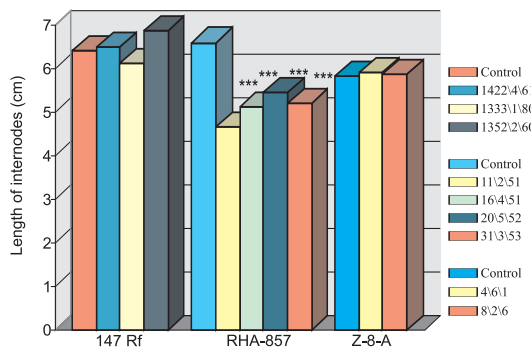


Figure 3: Significant changes of the index length of internodes in R11-R12 somaclonal families originating from genotypes 147 R, RHA-857 and Z-8-A during the period 1997-1999

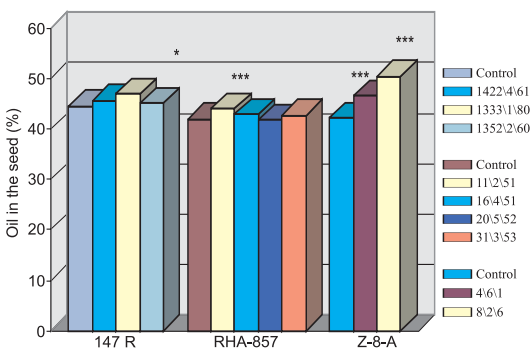


Figure 4: Significant changes of the index oil content in seeds (%) in R11-R12 somaclonal families originating from genotypes 147 R, RHA-857 and Z-8-A during the period 1997-1999

It was established that the medium A1 modified by us and the use of explants produced under greenhouse conditions were very suitable for induction of direct organogenesis from immature embryos, allowing the application of this method all year round. Lines with high regeneration capacity were isolated; these can be successfully used in program for genetic transformation, cell selection and mutagenesis.

An investigation was carried out to compare somaclonal variation to induced mutagenesis with gamma rays in sunflower (Encheva *et al.*, 1993). Data showed that the modifications were identical, but they however differed in intensity with regard to flowering date and some morphological and biochemical characteristics. It can be added that the gamma-ray treatment had a positive effect on the regeneration frequency of the studied genotypes.

As a result from these experiments a number of lines with valuable economic indices and high combining ability were obtained. They were included as components in highly productive hybrids (Encheva, 2000).

A combination of gamma irradiation and *in vitro* culture has been used by several authors in order to expand the scope of somaclonal variation in maize and wheat (Novak *et al.*, 1988; Cheng *et al.*, 1990) and a positive effect of this approach was observed.

Gamma-induced parthenogenesis in sunflower

There were several studies on the use of *in vitro* techniques for production of haploid and doubled haploid plants in cultivated sunflower, such as anther culture (Gunda Mix, 1985; Mezzarobba and Jonard, 1986; Gürel *et al.*, 1990; Thengane *et al.*, 1994, Vasić *et al.*, 2000), pollen culture (Gürel *et al.*, 1991; Todorova *et al.*, 1993) and ovule culture (Gelebart and San, 1987). However, none of these techniques can be successfully applied to breeding programs of sunflower due to its low regeneration frequency.

In 1993, investigations on the production of doubled haploid lines of sunflower by completely different means, based on γ -induced parthenogenesis, started at the Dobroudja Agricultural Institute (Todorova *et al.*, 1994).

As a result, conditions for reproducible regeneration of doubled haploid lines in *Helianthus annuus* L. were estimated (Todorova *et al.*, 1997).

It was found that the efficiency of the method was affected by the interaction between the genotype of the pollen source, the strength of γ -radiation effect and the genotype of the initial forms for parthenogenic induction (Figures 5, 6 and 7). By using mixed pollen from the best pollen sources studied, the genotype specificity of the pollen donor was partially overcome (Todorova and Ivanov, 1999). In a series of consecutive studies, radiation doses which suppress the fertilization ability of the pollen, but preserve its stimulating effect on the embryo sac of sunflower, were determined (Todorova and Ivanov, 2000).

A total of 762 new lines were already produced by this method; these were included in the breeding programs of the Dobroudja Agricultural Institute.

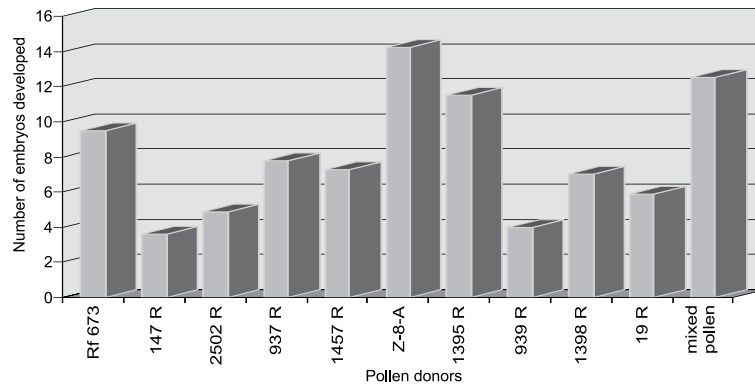


Figure 5: Effect of pollen donor genotype on the efficiency of γ -induced parthenogenesis in sunflower (*Helianthus annuus L.*)

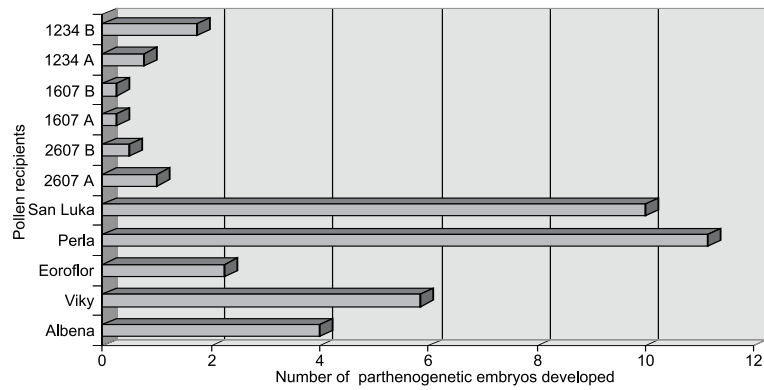


Figure 6: Parthenogenetic embryo development of some sunflower lines and hybrids after pollination with gamma-irradiated pollen

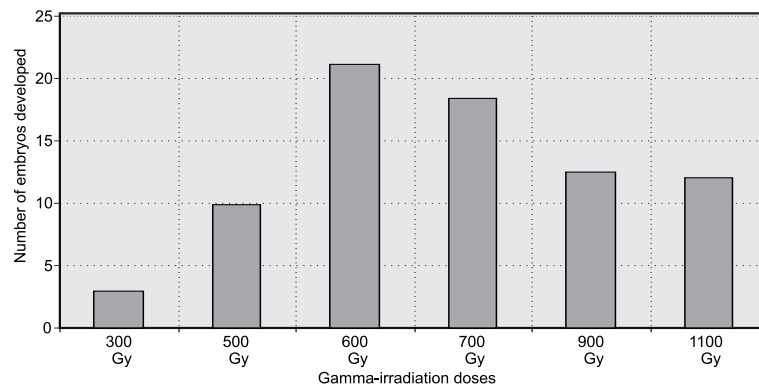


Figure 7: Effect of gamma-irradiation on parthenogenetic embryo development in sunflower (*Helianthus annuus L.*)

Distant hybridization and *in vitro* techniques

The *in vitro* technologies developed are widely used for solving the problems of distant hybridization. We have performed experiments mainly with anther culture from various species and interspecific hybrids, embryo culture of hybrid embryos and direct organogenesis.

The androgenic response was more clearly expressed in the species from the genus *Helianthus* than in the cultivated sunflower, but it was still unsatisfactory. We tested 60 accessions of species with various ploid levels, which were representatives of the groups with annual and perennial cycles of development. Regeneration was achieved in four perennial diploid species: *H. mollis* (M-012), *H. salicifolius* (M-045), *H. decapetalus* (M-043) and *H. strumosus* (M-126). Differences between the species regarding anther culture could be observed and the ploidy level of the regenerants was determined by flow cytometry. Good results were achieved in the 60 interspecific hybrid combinations investigated, obtaining regeneration in 15 of them (Nenova *et al.*, 1992; Todorova *et al.*, 1995; Nenova *et al.*, 1998; Nenova *et al.*, 2000).

Embryo culture significantly increases the efficiency of distant hybridization (Nenova *et al.*, 1990). As a result from its application we obtained a large number of lines with normal cytoplasm from combinations with different species such as *H. resinosus*, *H. salicifolius*, *H. ciliaris*, *H. giganteus*, *H. maximiliani*, *H. glaucophyllus*, etc. Combinations of 128 Rf lines and 219 B lines were tested in competition trials. Table 2 shows the results from a four-year testing of some hybrids which exceeded the standard by the index seed yield per dekar. In 1998, the hybrids 2607 x 445/p, 2607 x 447/p and 2607 x 489/H were tested in France where they also exceeded the standards by 5.4%, 13.9% and 17.7%, respectively.

The method of direct organogenesis in interspecific and intergeneric hybridization was used for the first time in our laboratory (Encheva 1992; Encheva, 2000). A large number of hybrid plants were produced from the following combinations:

<i>H. annuus</i> (hybrid Albena)	x	<i>H. salicifolius</i>
<i>H. annuus</i>	x	<i>H. maximiliani</i>
<i>H. annuus</i>	x	<i>H. silphoides</i>
<i>H. annuus</i>	x	<i>H. tuberosus</i>
<i>H. mollis</i>	x	<i>H. annuus</i>
<i>H. annuus</i> (hybrid Albena)	x	<i>Verbisina helianthoides</i>

This approach seems to be most promising, at least due to the fact that a large number of plants were produced from a single hybrid embryo, while in embryo culture one plant only was obtained.

The hybrid nature of the genotypes stabilized through the combinations mentioned above was also confirmed by RAPD analysis (Encheva, 2000).

Table 2: Hybrids produced with lines originating from interspecific hybridization through embryo cultivation which exceed the standard by seed yield after a 4-year testing, (in %)

Hybrid combination	1997	1998	1999	2000	Mean
2607 x 445/p	115	108	120	109	113
2607 x 447/p	109	105	116	107	109
2607 x 489/H	110	115	126	111	115
2607 x 518/p	117	112	124	108	115
2607 x 524/p	102	110	127	108	112

CONCLUSIONS

The methods available for *in vitro* cultivation of sunflower allow to plan various experiments for production of new initial breeding material, its fast unification and inclusion in breeding programs.

Advantages of these methods can be summed up as follows:

1. Production of suitable hybrid populations of Rf lines, embryo culture and obtaining of dihaploid lines.
2. Creation of suitable hybrid populations of B lines, embryo culture and production of dihaploid lines.
3. Hybridization of outstanding commercial hybrids with B lines, embryo culture and production of dihaploid lines.
4. Production of dihaploid Rf lines from outstanding commercial hybrids.
5. Production of analogues of well established lines by insertion of different characters (disease resistance, quality of oil and protein, etc.), creation of CMS analogues and others.

In many cases treatment with gamma-rays, ultrasound, etc., may be added to these schemes.

Table 3: Comparison of hybrids of line 2607 CMS against some DHL with pedigree including line 147Rf x collective pollen

Variant	Number	Num. of lines exceeding mean standard	% of yield exceeding the standard			
			100-105	105-110	110-115	>115
2607 CMS x DHL (K)	14	7	-	2	1	4
2607 CMS x DHL(US)	7	7	2	-	1	4
2607 CMS x DHL(GR)	8	6	-	2	3	1
Sum	29	20	2	4	5	9

K - DHL produced from non-treated embryos

US - DHL produced from ultrasonic treated embryos

GR - DHL produced from gamma-ray treated embryos

By using these methods and schemes, a significant number of new lines have already been produced, their combining ability was investigated, highly productive

hybrids were released, and one of those hybrids was registered with the Bulgaraen State Varietal Commission in 2001 (Tables 3 and 4).

Table 4: Productivity of some hybrids resistant to downy mildew and broomrape

Combination	Yield (averaged for three years) in % of the mean standard (Albena and San Luka)			
	1998	1999	2000	Mean
2607 x BT 29 PI K	105.8	112.6	103.1	107.2
2607 x BT 84 PI GR	124.4	109.3	108.0	113.9
2607 x BT 182 PI GR	116.7	103.3	113.6	111.2
2607 x BT 205 PI K	127.9	108.0	102.7	112.9
2607 x BT 226 PI US	120.2	103.3	104.4	109.3
2607 x BT 227 PI US	133.7	101.0	103.7	112.8

Molecular analysis techniques are now being successfully adopted. RAPD analysis has already become a routine procedure. The hybrid nature of 70 lines produced from crosses of species from genus *Helianthus* and back-crossed several times with line HA2607 was confirmed with the help of this analysis.

Obviously, it is possible to conclude that the *in vitro* technologies already adopted and applied allow to realize various experiments leading to creation of rich initial breeding material. Valuable forms can be selected from this material, which can be fast unified, studied and included in breeding programs.

REFERENCES

- Encheva, J., 2000. Production and study of initial sunflower breeding material by using *in vitro* techniques. Doctoral thesis, Sofia.
- Encheva, J., M. Christov, P. Ivanov, 1992. Use of direct organogenesis *in vitro* from immature embryos of interspecific and intergeneric hybrids of *Helianthus annuus*. Proc. 13th International Sunflower Conference, Pisa, Italy, 2: 1455-1460.
- Encheva, J., P. Ivanov, F. Tsvetkova, V. Nikolova, 1993. Development of a new initial breeding material in sunflower (*Helianthus annuus* L.) using direct organogenesis and somatic embryogenesis. Euphytica 68: 181-185.
- Encheva, J., P. Ivanov, G. Liu, 1993. Genotype responsiveness of several sunflower lines, hybrids and open pollinated varieties to *in vitro* manipulation of immature embryos. Biotechnology and Biotechnol. Eq. 4: 78-82
- Freyssinet, M. and G. Freyssinet, 1988. Fertile plant regeneration from sunflower (*H. annuus* L.) immature embryos. Plant Science 56: 177-181.
- Gelebart, P. and San, Lh., 1987. Obtention de plantes haploides par culture *in vitro* d'ovaires et d'ovules non fécondes de Tournesol (*Helianthus annuus* L.). Agronomie 7: 81-86.
- Gürel, A., Nichterlein, K. and Friedt, W., 1991. Shoot regeneration from anther culture of sunflower (*Helianthus annuus* L.) and some interspecific hybrids as affected by genotype and culture procedure. Plant Breeding 106: 68-76.
- Gürel, A., Kontowski, S., Nichterlein, K. and Friedt, W., 1991. Embriogenesis in microspore culture of sunflower (*Helianthus annuus* L.). Helia 14: 123-128.
- Mezzarobba, A. and Jonard, R., 1986. Effets du stade de prelevement et des pretraitements sur le developpement *in vitro* d'antheres prelevees sur le tournesol (*Helianthus annuus* L.). Physiologie vegetale, C. R. Acad. Sc. Paris t. 303. Serie III, 5: 181-186.
- Mix, G., 1985. Antheren- und Ovarienkultur von Sonnenblumen (*Helianthus annuus* L.). Landbauforschung Voelkenrode 35: 153-156.
- Nenova, N., M. Christov, P. Ivanov, 1990. Embryo culture experiments of some *Helianthus* hybrids. International Youth Conference on Genetics, Albena, pp. 202-205.

- Nenova, N., M. Christov, P. Ivanov, 1992. Anther culture regeneration of F1 hybrids of *Helianthus annuus* x *Helianthus smitii* and *Helianthus annuus* x *Helianthus eggertii*. XIII International Sunflower Conference, Piza, Italy, pp. 1509-1514.
- Nenova, N., P. Ivanov, M. Christov, 1998. Anther culture regeneration from *Helianthus mollis* (M-020) and its hybrid combinations. *Biotechnology & Biotechnol. Eq.* 2: 58-60.
- Nenova, N., Cristov, M., Ivanov, P., 2000. Anther culture regeneration from some wild *Helianthus* species. *Helia* 23(32): 65-72.
- Novak, F.J., S. Daskalov, H. Brunner, M. Nestincky, R. Afza, M. Dolezelova, S. Lucretti, A. Herichova and T. Hermelin, 1988. Somatic embryogenesis in maize and comparison of genetic variability induced by gamma radiation and tissue culture techniques. *Plant Breeding* 101: 66-79.
- Pugliesi, C., F. Cecconi, A. Mandolfo and S. Baroncelli, 1991. Plant regeneration and genetic-variability from tissue cultures of sunflower (*Helianthus annuus* L.). *Plant Breeding* 106: 114-121.
- Roseland, C.R., A. Espinasse and T.J. Grosz, 1991. Somaclonal variants of sunflower with modified coumarin expression under stress. *Euphytica* 54: 183-190.
- Thengane, S.R., Joshi, M.S., Khuspe, S.S. and Mascarenhas, A.F., 1994. Anther culture in *Helianthus annuus* L., influence of genotype and culture conditions on embryo induction and plant regeneration. *Plant Cell Reports* 13: 222-226.
- Todorova, M., Dahlhoff, M. and Friedt, W., 1993. Microspore culture in sunflower (*H. annuus* L.). *Biotechnol. and Biotechnol. Eq.* 4B: 83-90.
- Todorova, M., Ivanov, P. and N. Nenova, 1994. Induced parthenogenesis in sunflower (*H. annuus* L.) using irradiated pollen. *EUCARPIA Symp.*, Sept., 1994, Albena, Bulgaria.
- Todorova, M., Ivanov, P., Shindrova, P., Christov, M. and I. Ivanova, 1997. Dihaploid plant production of sunflower (*Helianthus annuus* L.) through irradiated pollen-induced parthenogenesis. *Euphytica* 97: 249-254.
- Todorova, M., Nenova, N., Ivanov, P. and M. Christov, 1997. Plant regeneration through anther culture and induced parthenogenesis in genus *Helianthus*. *Biotechnol. & Biotechnol. Eq.* 4: 27-30.
- Todorova, M. and P. Ivanov, 1999. Induced parthenogenesis in sunflower: effect of pollen donor. *Helia* 22(31): 49-56.
- Todorova, M. and P. Ivanov, 2000. Induced parthenogenesis in sunflower: Effect of gamma irradiation doses. *Proc. of the 15th International Sunflower Conference*, Toulouse, France, 12.06.-15.06.2000.
- Cheng, X.Y., M.W. Gao, Z.Q. Liang and K.Z., Lin, 1990. Effect of mutagenesis treatments on somaclonal variation in wheat (*Triticum aestivum* L.). *Plant Breeding* 105: 47-52.
- Vasić, D., D. Škorić, S. Jocić, 2000. Anther culture of sunflower cultivars. *Proc. 15th Internat. Sunfl. Conf.*, Toulouse, France 12-15. June, L51-L55.

APLICACIÓN DE CIERTOS LOGROS BIOTECNOLÓGICOS EN EL MEJORAMIENTO GENÉTICO DE GIRASOL

RESUMEN

En los programas de mejoramiento genético de girasol en el Instituto Agrícola de Dobroudja (Bulgaria) se han creado y aplicado las técnicas básicas *in vitro*, relacionado con el aumento de variabilidad genética e intensificación del proceso de mejoramiento genético.

El método de la órgano-génesis directa, se ha utilizado con éxito para las modificaciones genéticas de las líneas restaurador RHA-857, 147 R y Z-8-A y para la superación de imposibilidad de cruzamiento entre la especie *H. annuus* (híbrido Albena), por un lado, y *Helianthus tuberosus*, *Helianthus salicifolius*, *Helianthus maximiliani*, *Helianthus silphiodes* y *Verbisina helianthodes*, por otro lado, tanto como entre las especies *H. annuus* y *H. mollis*.

El método de la partenogénesis á-inducida se ha aplicado con éxito en la cultivación de girasol (*H. annuus* L.) para la obtención de las líneas haploides dobles. Se ha estudiado la habilidad partenogénica de las componentes

parentales, femeninas, tanto como masculinas, y también la influencia de la radiación gama en el desarrollo partenogenético del embrión.

Hasta el momento se ha efectuado el gran número de investigaciones, orientadas hacia la investigación del potencial genético de las especies salvajes del género *Helianthus*. Nuestras investigaciones han sido orientadas hacia las posibilidades del método de cultivación del embrión y de antera del origen interspecies, incluyendo la utilización de la técnica de cultivación del embrión en las condiciones *in vitro*, hasta la producción y el ensayo de las líneas que directamente estén incluidas en el proceso del mejoramiento genético. Se han aislado los embriones de los híbridos interspecies (cultivados x salvajes y salvajes x cultivados) con la participación de las especies *H. resinosus* M-046, *H. strumosus* M-059, *H. salicifolius* M-045, *H. ciliaris* M-092, *H. giganteus* M-030, *H. maximiliani* M-087 y *H. glaucophyllus* M-012. En dichas investigaciones han sido incluidas e investigadas 51 líneas nuevas, originadas del embrión de los cruzamientos interspecies mencionados.

Se ha obtenido mayor número de líneas originadas del embrión de las arriba mencionadas interspecies de cruzamiento, con valiosos índices económicos y alta habilidad de combinación, que, como componentes, están incluidos en los híbridos de alto rendimiento.

APPLICATION DE CERTAINES RÉALISATIONS BIOTECHNOLOGIQUES À LA CULTURE DU TOURNESOL

RÉSUMÉ

Des techniques *in vitro* pour augmenter la variabilité génétique et intensifier le processus de culture ont été développées et appliquées dans les programmes de culture du tournesol à l'institut agricole Dobroudja en Bulgarie.

La méthode d'organogenèse directe a été utilisée avec succès pour les modifications génétiques des lignes *restorer* RHA-857, 147 R et Z-8-A et pour surmonter l'impossibilité de croisement entre les espèces *H. annuus* (hybride Albena) d'un côté et *Helianthus tuberosus*, *Helianthus salicifolius*, *Helianthus maximiliani*, *Helianthus silphoides* et *Verbisina helianthodes* de l'autre ainsi qu'entre les espèces *H. annuus* et *H. mollis*.

La méthode de parthénogenèse induite *y* a été appliquée avec succès sur le tournesol de culture (*H. annuus* L.) pour obtenir des lignes doubles haploïdes. L'aptitude parthénogénétique des composants génétiques mâles et femelles et l'effet des rayons gamma sur le développement parthénogénétique des embryons ont été observés.

Plusieurs recherches orientées vers l'étude du potentiel génétique de l'espèce sauvage du genre *Helianthus* ont été faites. Nos études examinaient les possibilités des méthodes de culture d'embryons et d'anthères d'origine interspecies. Elles comprenaient l'utilisation de la technique de culture des embryons dans des conditions *in vitro* jusqu'à la production et au test des lignes directement impliquées dans le processus de culture. Des embryons des hybrides interspecies ont été isolés (cultivés x sauvages et sauvages x cultivés) avec la participation des espèces *H. resinosus* M-046, *H. strumosus* M-059, *H. salicifolius* M-045, *H. ciliaris* M-092, *H. giganteus* M-030, *H. maximiliani* M-087 et *H. glaucophyllus* M-012. Les recherches portaient sur 51 nouvelles lignes provenant des embryons des croisements interspecies déjà mentionnés.

Plusieurs lignes provenant des embryons de ces croisements interspecies ont été obtenues qui avaient un index économique avantageux et une grande aptitude à être combinés. Ils ont été classés comme hybrides très productifs.