

UDC 575.222.7  
Original scientific paper

## BACKCROSSES IN INTERSPECIFIC HYBRIDIZATION IN SUNFLOWER

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Atlagić J., D. Panković, and A. Pekanović (2003): *Backcrosses in interspecific hybridization in sunflower*. – Genetika, Vol. 35, No. 3, 187-197.

When incorporating desirable traits (resistance to causal agents of various diseases) from the wild relatives into the cultivated sunflower, some undesirable ones are introduced too (branching, small head diameter, low oil content, etc.). To overcome this problem, backcrosses (F<sub>1</sub> interspecific hybrids x cultivated sunflower) are used, although very often desirable traits are lost in the process. Cytological analysis (meiosis and pollen viability) and molecular markers (RAPD) were used to estimate what portion of the parental species genome was present in the interspecific hybrids of the F<sub>1</sub> and BC<sub>1</sub>F<sub>1</sub> generations. The results showed that the percentage of irregularities at meiosis increased from F<sub>1</sub> to BC<sub>1</sub>F<sub>1</sub> gen. They also indicated the presence of aneuploids and sterility in the cross between the hexaploid species *H.rigidus* and cultivated sunflower. The genetic distance between the parents was 83%, that between *H.rigidus* and the F<sub>1</sub> hybrid 54 - 61%, and that between *H.annuus* and F<sub>1</sub> hybrid 70-76%. In the BC<sub>1</sub>F<sub>1</sub> generation, the genetic distance from *H.annuus* decreased to 58-66% and that from *H.rigidus* increased to 69-76%.

*Key words:* Sunflower, interspecies, backcrosses, morphology, cytogenetics and molecular markers

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## INTRODUCTION

Interspecies hybridization is a method that is often used in sunflower breeding. In breeding programs wild relatives are used as donors of desirable traits (for example disease resistance) into the cultivated sunflower. This method is accompanied by several difficulties such as: cross incompatibility, decreased fertility or complete sterility of interspecies hybrids. Not only desirable traits are introduced but undesirable ones as well (branching, small head diameter, low oil content etc.). ATLAGIĆ (1991) has shown that some agronomically important traits in  $F_1$  interspecies hybrids are often inherited in a dominant or partially dominant manner, which indicates that they are difficult to eliminate. Backcrosses, defined by BOROJEVIĆ, 1992) as the method of convergent breeding, are used to introduce gene(s) for one or two traits, or for the fixation of respective genes in the good standard varieties.

*Helianthus rigidus* is a hexaploid sunflower species that is used in breeding programs for increased resistance to diseases (SEILER, 1992) and as a source of high protein content in the seed (71%) (GEORGIEVA-TODOROVA and HRISTOVA, 1975). This species was used a lot in the breeding program in Novi Sad. Interspecies  $F_1$  hybrids (ATLAGIĆ, 1996a) and hybrid combinations of  $BC_1F_1$  generation (ATLAGIĆ, 2000), from the crosses of several *H. rigidus* populations and sunflower lines of cultivated sunflower were obtained. In cited papers, the possibility to use *H. rigidus* in breeding programs was estimated by the appearance of male sterility, and the estimation of genetic distance between *H. rigidus* and cultivated sunflower by cytological methods (difference in number and the structure of chromosomes).

The aim of this paper was to use both cytological methods and DNA molecular markers (RAPD) in order to identify  $F_1$  hybrids (*H. rigidus* x *H. annuus*) and  $BC_1F_1$  hybrids ( $F_1$  x *H. annuus*), and to determine genetic distance between crossed species and progeny. It is particularly important to estimate the partitioning of the genome of parental species in interspecies hybrid  $F_1$  and in  $BC_1F_1$  generation. These results can help breeders to evaluate the number and the type of backcrosses when they apply interspecies hybridization in the breeding of cultivated sunflower.

## MATERIALS AND METHODS

Hexaploid species *H. rigidus* (population 72272 obtained by exchange of material with INRA, Montpellier), that was grown in wild species collection of the Institute of Field and Vegetable Crops at Rimski Šančevi, was used in this investigation. Cultivated sunflower, used as the other parental component for interspecies crosses, was line OCMS 74 (B analog). Since  $F_1$  interspecies hybrids between perennial species and cultivated sunflower have the ability of vegetative propagation, most important hybrid combinations were kept and propagated in the wild species collection. Three hybrid combinations of  $F_1$  generation (with *H. rigidus*) were used for backcrosses with cultivated sunflower, and from obtained combinations in  $BC_1F_1$  generation, one was analyzed in detail.

Phenological observations of *H.rigidus*, F<sub>1</sub> (*H.rigidus* x OCMS 74) and BC<sub>1</sub>F<sub>1</sub> (F<sub>1</sub> x OCMS 74) were done in the field, in 1999. Plants were visually screened for the occurrence of male fertility and sampled for cytological and RAPD analysis. Cytological methods involved the analysis of meiosis (acetocarmine method; GEORGIEVA-TODOROVA, 1976) and pollen viability determination (ALEXANDER, 1969).

Genomic DNA was isolated from frozen sunflower leaves (DELLAPORTA *et al.*, 1983). The polymorphism of DNA was examined by the application of RAPD markers (Random Amplified Polymorphic DNA) (WILLIAMS *et al.*, 1990). Nine DNA samples were analyzed: 2 bulk (5-10 plants) parental samples, 3 F<sub>1</sub> plants and 4 BC<sub>1</sub>F<sub>1</sub> plants. Eight decamer primers, previously defined as highly polymorphic for different sunflower inbred lines, were used (PANKOVIĆ *et al.*, 2000). Genetic distances (%) between parental genomes, as well as between parents and their progeny, were calculated from the ratio of polymorphic RAPD fragments over the total number of RAPD fragments (HONGTRAKUL *et al.*, 1997).

## RESULTS

**Morphological characteristics** - F<sub>1</sub> hybrid plants resembled to the “wild” parent in general: they were perennials and branched. But their leaves and inflorescences were bigger, and the flowering time was shorter than in the “wild” parent (Fig.1). The occurrence of male sterile plants ranged from 0 to 87.5%.

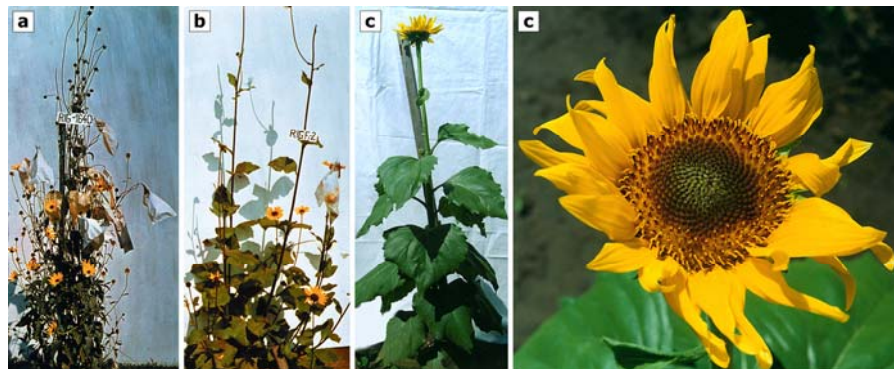


Fig.1. *H. rigidus* species (population 72272) (a), F<sub>1</sub> interspecies hybrid (b), BC<sub>1</sub>F<sub>1</sub> hybrid plant and flower (c)

BC<sub>1</sub>F<sub>1</sub> plants of one hybrid combination differed phenotypically. They had some characteristics of “wild” parent (leaf thickness, the shape of the inflorescence and bracts), but they were not branched, they had bigger heads, large leaves and wooded stem (Fig.1). Male sterility occurrence ranged between 0 and 100%.

**Characteristics of meiosis and pollen viability** - All phases of meiosis in OCMS 74 (♂) proceeded normally. The number of bivalents was 17. Pollen viability was 97.8%. In *H.rigidus*, population 72272, there were 51 bivalents in



In BC<sub>1</sub>F<sub>1</sub> interspecies hybrid plants meiosis was followed with many irregularities. Chromosomes paired irregularly in diakinesis. Bivalents, quadrivalents, trivalents and univalents were detected. The number of bivalents was low (17-24) in comparison to total number of chromosomes (50-60). In the same time the number of univalents was high. Aneuploidy has appeared in BC<sub>1</sub>F<sub>1</sub> hybrid plants.

Chromosomal fragments, that were linked to other chromosome by thin threads, were found in some meiocytes. Chromosomal bridges were detected in anaphase I and telophase II, and dislocated chromosomes were found in metaphase I (Fig.2), anaphase I and telophase II. Pollen viability in male fertile plants was very low and ranged from 1.95- 16.44% (Table 1).

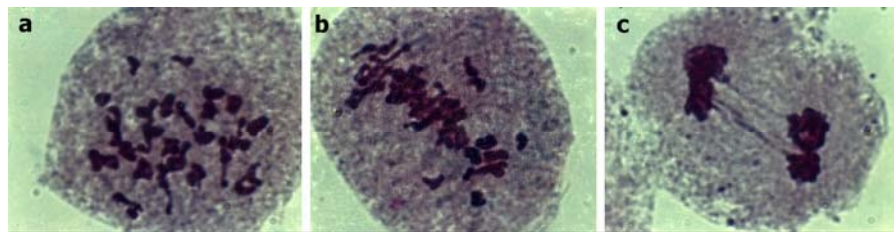


Fig.2. Diakinesis (bivalents, univalents and fragments) (a), Metaphase I (fast chromosomes) (b), Telophase II (chromosome bridges) (c)

**Analysis of polymorphic PCR products** - Eight decamer primers, previously shown to be highly polymorphic for different sunflower inbred lines, have been used (PANKOVIĆ *et al.*, 2000). The average number of synthesized fragments ranged from 3 to 6, and their size varied from 400 to 1600bp. Only few fragments were universal for both parents. There were a lot of fragments that were specific for one of the parents, thus indicating the differences in genome and facilitating the determination of fragments in progeny.

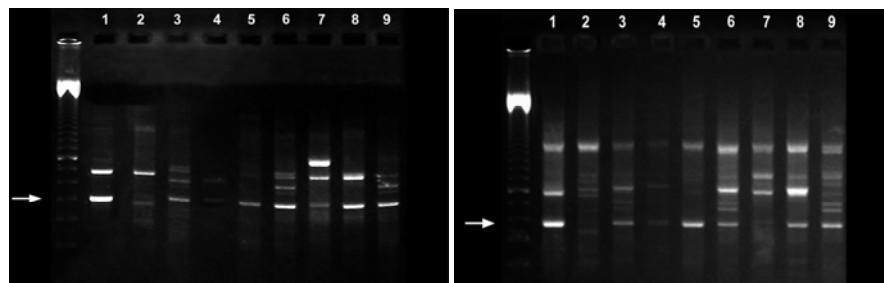


Fig. 3. RAPD fragments amplified with primers UBC-39 (a) and UBC-43 (b)

Nine samples of PCR products amplified on: genomic DNA of father (cultivated sunflower *H.annuus*), genomic DNA of mother (*H.rigidus*), three sam-

ples of genomic DNA of F<sub>1</sub> generation and four samples of genomic DNA of BC<sub>1</sub>F<sub>1</sub> generation, were applied to the gel in the respective order (Fig.3).

Table 2. RAPD markers obtained with eight respective primers (the screening of fragments in parents and their progeny)

RAPD marker	OCMS 74	RIG 72272	F <sub>1</sub> /1	F <sub>1</sub> /2	F <sub>1</sub> /3	BC <sub>1</sub> F <sub>1</sub> /1	BC <sub>1</sub> F <sub>1</sub> /2	BC <sub>1</sub> F <sub>1</sub> /3	BC <sub>1</sub> F <sub>1</sub> /4
	+	-	+		+	-	-	-	-
	+	-	+		+	+	+	+	+
	+	-	-		-	+	+	+	+
	+	-	-		-	-	-	-	-
	+	-	-		-	+	-	+	-
	+	-	-		-	-	-	-	-
UBC 18	-	+	-		+	-	-	-	-
	+	-	-		-	-	-	-	-
	+	-	-		-	+	-	+	+
	-	+	-		-	+	+	-	-
	-	+	+		+	-	+	-	+
	+	-	-		-	-	-	-	+
	+	-	+		+	+	-	-	-
	-	+	-	+	-	-	-	-	-
UBC 39	+	-	+	-	+	+	-	-	-
	-	-	+	+	+	+	+	+	+
	+	-	-	-	-	+	+	+	+
	-	+	-	-	-	+	-	+	+
	-	+	+	+	-	+	-	+	+
UBC 43	+	-	-	-	-	-	+	-	-
	-	+	-	-	-	-	-	-	+
	+	-	+	+	+	+	-	+	+
	-	+	-	-	-	-	-	-	-
	-	+	+		+	+	+	+	+
UBC 211	+	-	-	-	-	-	-	-	-
	+	-	+		-	+	-	+	-
	+	-	-		-	+	+	+	+
	+	-	-		-	-	+	+	-
	+	-	-	-	-	-	-	-	-
UBC 222	+	-	-	-	-	+	+	+	-
	-	+	+	+	+	+	+	+	-
	-	+	-	-	-	-	-	+	+
	-	+	+	+	+	+	+	-	-
	+	-	-	-	-	-	+	+	-

Table 2. continued on next page ...

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RAPD marker	OCMS 74	RIG 72272	F <sub>1</sub> /1	F <sub>1</sub> /2	F <sub>1</sub> /3	BC <sub>1</sub> F <sub>1</sub> /1	BC <sub>1</sub> F <sub>1</sub> /2	BC <sub>1</sub> F <sub>1</sub> /3	BC <sub>1</sub> F <sub>1</sub> /4
	-	+	-		-	-	-	-	-
	-	+	-		-	-	-	-	-
	-	+	-		-	+	-	+	+
UBC 226	+	-	+		+	+	+	-	+
	+	-	-		-	+	-	+	+
	-	+	+		+	-	+	+	-
	+	-	+		-	+	-	+	-
	-	+	-	-	+	-	-	-	-
	+	-	-	-	-	+	+	-	+
	-	+	-	-	-	-	-	-	-
UBC 318	+	-	-	-	-	+	+	-	-
	+	-	-	-	-	+	+	-	+
	+	-	-	-	-	-	-	-	-
	+	-	-	-	-	-	-	-	-
	+	-	+	+	-	+	-	+	+
	+	-	-		-	+	-	-	+
UBC 331	+	-	-		-	+	-	-	-
	-	+	-		-	+	-	+	-
	-	+	+		+	+	-	-	-
	+	-	-		-	-	-	-	-

The criteria for RAPD marker definition was the presence of band in one of the parental lines. Polymorphic products obtained with primers UBC-39 and UBC-43 are shown on figure 3. Arrows are indicating fragments-markers, that were present in both DNA of the father and progeny of F<sub>1</sub> and BC<sub>1</sub>F<sub>1</sub> generation, and absent in mother. Results obtained with both primers indicate the presence of variability between both F<sub>1</sub> and BC<sub>1</sub>F<sub>1</sub> plants. In some individuals extra bands, that were not detected in parental DNA, were found. In total 56 polymorphic RAPD markers were analyzed, and the results are shown in Table 2.

Table 3. Genetic distance between parents and their progeny (%)

	♂	♀	F <sub>1</sub> /1	F <sub>1</sub> /2	F <sub>1</sub> /3	BC <sub>1</sub> F <sub>1</sub> /1	BC <sub>1</sub> F <sub>1</sub> /2	BC <sub>1</sub> F <sub>1</sub> /3	BC <sub>1</sub> F <sub>1</sub> /4
♂	0	83	70	76	76	60	65	58	66
♀	83	0	54	77	61	69	69	74	76

Genetic distance between parents and progeny of F<sub>1</sub> and BC<sub>1</sub>F<sub>1</sub> generation was calculated from the ratio of polymorphic RAPD fragments over total number of RAPD fragments, that were presented in the Table 2. The genetic distance between parents: *H.rigidus* and *H. annuus* was high (83%) (Table 3). Genomic DNA of F<sub>1</sub> hybrids were more similar to the mother (*H.rigidus*) (GD 54-77%) than to the father (OCMS 74) (GD 70-76%). In BC<sub>1</sub>F<sub>1</sub> plants genetic distance from the father

lowered (OCMS 74) (58-66%), whereas genetic distance from the mother (*H.rigidus*) increased (69-76%) (Table 3).

#### DISCUSSION

Morphological traits of  $F_1$  and  $BC_1F_1$  hybrids indicate that the transfer of parental traits to progeny develops gradually. Variability of  $F_1$  hybrids from one hybrid combination was low, whereas this was not the case with  $BC_1F_1$  interspecies hybrids. It was shown earlier that the identification of successful crosses can be estimated by the use of morphological traits (ATLAGIĆ, 1996a, 2000; GEORGIEVA-TODOROVA, 1990; CHRISTOV, 1991).

Results on high percentage of male sterility occurrence in  $F_1$  and  $BC_1F_1$  plants as well as decreased pollen viability in male fertile plants indicate that there are genetic differences between *H.rigidus* and *H.annuus*.

Small number of bivalents in regard to total number of chromosomes, the occurrence of multivalents and univalents, as well as the high percentage of irregularities in phases of meiosis in  $F_1$  and especially  $BC_1F_1$  hybrids, confirms problems in crossing these two species. The cause is not only different number of chromosomes in crossed species but also different structure of chromosomes of translocation type (the occurrence of quadrivalents) and of inversion type (chromosome bridges). Similar results were observed by ATLAGIĆ (1996b) when analysing the meiosis of  $F_1$  and  $BC_1F_1$  interspecies hybrids of following crosses: *H.nuttallii* x *H.annuus*, and *H.laevigatus* x *H.annuus* (ATLAGIĆ and ŠKORIĆ, 1999). It was expected that the back crosses with cultivated sunflower will contribute to decreased irregularities in meiosis, as suggested by WHELAN (1979) and WHELAN and DORRELL (1980). These authors have found that back crosses significantly decrease meiotic aberrations detected in  $F_1$  generation. They even claim that meiosis develops normally in  $BC_3$  and  $BC_4$  generation.

The high number of univalents was determined in  $BC_1F_1$  hybrids (*H.rigidus* x *H.annuus*) x *H.annuus*, and small number of bivalents in comparison to total number of chromosomes, indicate that chromosome pairing is often regular. The smaller number of bivalents is the result of wild parent's chromosomes autosyndesis, whereas the multivalents are the result of nonhomologous chromosome pairing of crossed species. Univalents are unpaired chromosomes. As the results aneuploidy plants appear in  $BC_1F_1$  generation (ATLAGIĆ i ŠKORIĆ 1999; ATLAGIĆ, 2000; GEORGIEVA-TODOROVA, 1984).

The results obtained with molecular markers confirm the conclusions obtained by analysis of morphology and cytogenetics: the interspecies hybrid was successfully obtained, and there is high variability among  $BC_1F_1$  interspecies hybrids. In contrast to applied classical methods, RAPD markers revealed variability among  $F_1$  hybrids as well. SOSSEY-ALAOUI *et al.* (1998) have shown that cloned RAPD fragments resemble repetitive sequences, which are very efficient in detecting genomic differences.

Among amplified fragment in progeny, the occurrence of new fragments, not present in parents, was observed. This phenomenon was already described



(AYLIFFE *et al.*, 1994) and is considered to be the result of the amplification of allelic sequences of different size. Many authors have discovered unexpected changes in the DNA organization of interspecies hybrids. For example NATALI *et al.* (1998) have found specific changes of DNA-binding proteins in interspecies hybrids. It is possible that similar changes have happened in F<sub>1</sub> and BC<sub>1</sub>F<sub>1</sub> hybrids investigated in this paper.

The percentage of genetic distance, calculated on the bases of RAPD markers, enables the determination of partitioning of parental genomes in interspecies F<sub>1</sub> and BC<sub>1</sub>F<sub>1</sub> hybrids. The lower genetic distance between F<sub>1</sub> individuals and *H.rigidus* than *H.annuus*, indicates the higher partitioning of wild species genome. To the contrary, in BC<sub>1</sub>F<sub>1</sub> generation the genetic distance to *H.annuus* lowered from 70-76% to 58-66%, and to *H.rigidus* increased from 54-61% to 69-76%. Thus after first back cross, the partitioning of cultivated sunflower genome increased. Further investigations will be improved by the use of more RAPD markers and/or the use markers with higher reproducibility (SSR). Beside that the work should be focused on the investigation of the inheritance of desirable genes (traits) by the use of specific primers and gene expression.

### CONCLUSION

Morphological characteristics, results of cytological analysis and molecular marker's data on parental species, F<sub>1</sub> and BC<sub>1</sub>F<sub>1</sub> hybrids, indicate that the introduction of wild species genome into the genome of cultivated sunflower is gradual and uneven. Therefore the backcross method in interspecies hybridization should be followed with the mentioned analyses in order to determine the point when the desirable genes from wild species have been introduced, and in the same time, the appropriate traits from the cultivated sunflower preserved.

Received October 1<sup>st</sup>, 2003  
Accepted December 29<sup>th</sup>, 2003

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## POVRATNA UKRŠTANJA U INTERSPECIES HIBRIDIZACIJI SUNCOKRETA

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### Izvod

Pri unošenju "poželjnih" svojstava (otpornost na prouzrokovače bolesti) iz divljih srodnika u gajeni suncokret unose se i "nepoželjna" (grananje, mali prečnik glave, nizak sadržaj ulja i dr.). Da bi se prevazišao ovaj problem koriste se povratna ukrštanja ( $F_1$  interspecies hibridi x gajeni suncokret). S druge strane u povratnim ukrštanjima se vrlo često gube "poželjna" svojstva. Primenom citoloških analiza (mejoza i vitalnost polena) i metoda molekularnih markera (RAPD) izvršena je procena koji je udeo genoma roditeljskih vrsta prisutan kod interspecies hibrida  $F_1$  i  $BC_1F_1$  generacije. Rezultati pokazuju da se procenat nepravilnosti u mejozi povećava od  $F_1$  do  $BC_1F_1$  generacije, kao i pojava aneuploida i sterilnosti kod ukrštanja heksaploidne vrste *H.rigidus* i gajenog suncokreta. Genetička udaljenost, izračunata na osnovu polimorfnih RAPD markera, između roditelja je iznosila 83%, između *H.rigidus* i  $F_1$  hibrida od 54 do 61%, a između *H.annuus* i  $F_1$  hibrida od 70 do 76%. Genetička udaljenost  $BC_1F_1$  generacije od *H.annuus* se smanjila i iznosila od 58 do 66%, a od *H.rigidus* se povećala i kretala u opsegu od 69 do 76%.

Primljeno 1. X 2003.  
Odobreno 29. XII 2003.