

## INTERGENERIC HYBRIDS BETWEEN CULTIVATED SUNFLOWER (*Helianthus annuus* L.) AND *Verbesina helianthoides* (GENUS *Verbesina*) - RAPD ANALYSIS

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### SUMMARY

The method of direct organogenesis has been successfully used for overcoming the inability for crossing between *Helianthus annuus* (cv. Albena) and *Verbesina helianthoides* (genus *Verbesina*). As a result of long-term individual selection in the hybrid materials, fertility restorer lines were produced in the R 10 generation. The applied molecular method (RAPD) confirmed the hybrid nature of the obtained breeding material and indicated an introgression of *Verbesina helianthoides* DNA into some of the hybrid progenies produced. Using the similarity coefficient of Nei and Li (1979), a dendrogram was generated through UPGMA analysis with the aim to determine the genetic distance of the studied genotypes. We were able to demonstrate that RAPD could be used for characterization of intergeneric hybrid progenies in sunflower at a late stage of selection (F<sub>9</sub>) in which an increased genetic variation was discovered.

**Key words:** *Helianthus annuus*, *Verbesina helianthoides* (genus *Verbesina*), direct organogenesis, RAPD analysis

### INTRODUCTION

The wild *Helianthus* species are potential sources of genes for resistance to diseases and pests. They possess considerable variability for most agronomic traits and qualities of seed (Thomson *et al.*, 1981) and could be included in interspecific and intergeneric crosses for increase of genetic variability in cultivated sunflower (Seiler, 1992, 1997; Škorić and Rajčan, 1992; Škorić *et al.*, 1995; Köhler *et al.*, 1997; Thomson *et al.*, 1981).

There are just a few publications on the use of molecular markers for identification of F<sub>1</sub> interspecific and intergeneric hybrids and their progenies. While Kräuter *et al.* (1991) and Faure *et al.* (1998) have used RFLP markers, Köhler *et al.* (1999) have applied the AP-PCR technique. The studies of the majority of authors include hybrid materials at an early stage of breeding. We investigated possibilities of using the RAPD method for confirming the hybrid nature of the progenies from the inter-

generic cross *H. annuus* (cv. Albena) × *Verbesina helianthoides* (genus *Verbesina*) at a late stage of breeding (the F<sub>9</sub> generation).

The aim of the present study was to confirm the hybrid nature of the obtained materials with the help of RAPD analysis.

## MATERIAL AND METHODS

### Plant material

Cultivated sunflower (cv. Albena 2n=34) and the wild species *Verbesina helianthoides* accession V1 (2n=34) (Figure 1) were grown under field conditions at DAI, General Toshevo. The hybrid embryos were obtained after sterilization of maternal pollen with gibberellic acid (GA<sub>3</sub> - 0.045 g/l) and hand pollination with pollen of paternal form.

To determine the hybrid nature of the lines obtained through the direct organogenesis method from the cross *Helianthus annuus* (cv. Albena) × *Verbesina helianthoides*, the RAPD method was used. Twenty one (10 base) primers with random sequences were used to amplify DNA sequences of the lines R 131, R 138, R 140, R 143, R 144 and R 146 obtained from the intergeneric cross.

### Methods

Direct somatic buds and plants from the intergeneric cross *Helianthus annuus* (cv. Albena) × *Verbesina helianthoides* were induced on nutrition media I, II, and III (Encheva *et al.*, 1992). As a result of long-term individual selection in the hybrid materials, fertility restorer lines were produced in the R<sub>10</sub> generation. In the crosses made, the female form had sterile cytoplasm of *Helianthus petiolaris* from Leclerq (1969) and therefore only the fertile forms were considered in this research work, *i.e.*, the ones possessing a gene for restoration. We have not proved the origin of this gene. It may originate both from the female form (the hybrid Albena) and from the wild father parent (*Verbesina helianthoides*); there is evidence that it carries genes for restoration of this cytoplasm (Christov and Vassilevska, 1999). This means that the newly self-fertile plants may possess Rf genes from both parents. The fertility restorer lines were investigated by the RAPD analysis.

### DNA extraction for RAPD analysis

DNA extraction for RAPD analysis was carried out using genomic DNA extracted by a modified method of Doyle and Doyle (1990). Sunflower leaf tissue was ground to a fine powder in liquid nitrogen. The frozen powder (2.5 g) was transferred to 15 ml hot hexadecyltrimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris HCl (pH 8.0), 1.4 mM NaCl, 20 mM EDTA, 1% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 0.2% β-mercaptoethanol) and incubated at 65°C for 30 min. with occasional shaking. An equal volume of chloroform: isoamylalcohol (24:1 v/v ) was added and mixed by inver-

sion, then centrifuged at 6000 g and 4°C for 10 min. The aqueous phase was transferred to a fresh tube and re-extracted with an equal volume of chloroform: isoamylalcohol (24:1 v/v) and centrifuged at 5000 g and 4°C for 10 min. The aqueous phase was removed and transferred to a fresh tube again and precipitated in 1.0 ml ammonium acetate (10 M) and 1.0 ml sodium acetate (3 M, pH 5.5) and 2/3 VT 2-propanol (4°C). Finally, the precipitated DNA was dried and resuspended in TE buffer (10 mM Tris HCl (pH 8.0) and 1 mM EDTA, pH 8.0). After treatment with Rnase, the DNA concentration was measured by using a flourometer (Model TKO 100, Hoefer Scientific Instruments, Serva, Germany).

#### **DNA amplification**

Twenty-one short primers (10 bp), OPA-12, OPA-16, OPAE-01, OPAE-03, OPAH-15, OPAJ-19, OPAJ-20, OPAK-05, OPAK-08, OPAO-14, OPAO-18, OPAO-20, OPAP-04, OPAP-05, OPAS-12, OPAT-12, OPAV-10, OPAW-17, OPAW-18, OPAW-19 and OPW-04, with random sequences were applied to characterize DNA sequences of the lines produced (Table 1).

**Primer selection** was based on the information content, clarity and reproducibility of banding patterns. Amplifications were carried out in a 20 µl volume containing 1.5 units polymerase Stoffel fragment "Goldstar" (Eurogentec); 1 × reaction buffer; 6 mM MgCl<sub>2</sub>; 0.4 mM each of dATP, dCTP, dGTP and dTTP; 25 ng of template DNA; add 20 µl H<sub>2</sub>O and 0.3 µM of primer. The amplifications were performed using a Thermal Cycler 9600 (Perkin Elmer Cetus, Norwalk, USA). The thermal cycle was programmed for a first denaturation step of 4 min. at 94°C followed by 45 cycles of 2 min. at 94°C, 1 min. at annealing temperature 36°C and 2 min. extension step at 72°C.

**Amplification products** were resolved by gel electrophoresis in 2% agarose in 0.5 TBE (89 mM Tris pH 8.0, 89 mM boric acid and 0.5 M EDTA) stained with ethidium bromide and visualized on an UV screen.

**Molecular size** of the amplification products was estimated using a 100 bp DNA ladder (Gibco BRL, Life Technologies).

#### **Analysis of amplification profiles**

**Amplification profiles** of *Helianthus* genotypes were compared with each other and bands of DNA fragments were scored as present (1) or absent (0). The computer program "RFLPscan" (Scanalytics, Billerica, MA, USA) was used to estimate the data for all the 21 primers. The similarity was then analyzed on the basis of the number of shared amplification products according to Nei and Li (1979). A dendrogram based on similarity coefficients was generated with the program 'NTSys' (Version 1.80, Exeter Software, Setauket, NY, USA) by using the unweighted pair group method of arithmetic means (UPGMA).

## RESULTS AND DISCUSSION

**Characterization of intergeneric hybrids in cultivated sunflower (*Helianthus annuus* L.) by the molecular method (RAPD)**

To confirm the hybrid nature of the sunflower lines produced by the direct organogenesis method from the intergeneric cross *Helianthus annuus* (cv. Albena) × *Verbesina helianthoides*, the RAPD method was applied.

Twenty one (10 base) primers with random sequences were applied to characterize DNA sequences of the lines R 131, R 138, R 140, R 143, R 144 and R 146 obtained from the intergeneric cross. The number of polymorphic bands obtained varied from 396 (primer OPAO-18) to 2556 (primer OPAK-08). Primers OPAK-08, OPAK-05, OPAW-18 and OPW-04 amplified the largest numbers of DNA products (Table 1).

Table 1: Total number and size of RAPD patterns generated by 21 operon primers

Primer	Max (bp)	Min (bp)	Bands (no)
OPA-12	1753	425	861
OPA-16	2073	350	956
OPAE-01	1326	284	486
OPAE-03	2533	289	1431
OPAH-15	2051	306	1035
OPAJ-19	1490	192	1081
OPAJ-20	1697	301	1225
OPAK-05	2052	326	2346
OPAK-08	1379	130	2556
OPAO-14	1985	230	1176
OPAO-18	1945	377	396
OPAO-20	1819	206	1431
OPAP-04	2052	254	946
OPAP-05	2036	297	1830
OPAS-12	2238	417	1275
OPAT-12	1669	273	1485
OPAV-10	1848	360	1711
OPAW-17	2199	279	946
OPAW-18	2035	255	2145
OPAW-19	1840	291	820
OPW-04	1846	214	2016

Some bands were common for all six hybrid progenies. Band (1000 bp) was specific for lines 146 R, 131 R, 138 R and 143 R.

Primer OPAJ-19 produced some fragments (300 bp) in the hybrid intergeneric progenies which had been detected in the wild species *Verbesina helianthoides* (Figure 1). These DNA fragments indicate the introgression of *Verbesina helianthoides* DNA into some of the hybrids progenies produced. Band (300 bp) was spe-

cific for lines 140 R, 144 R and 143 R. This is a demonstration that DNA polymorphism could be detected between the amplified products of the 6 inbred lines.

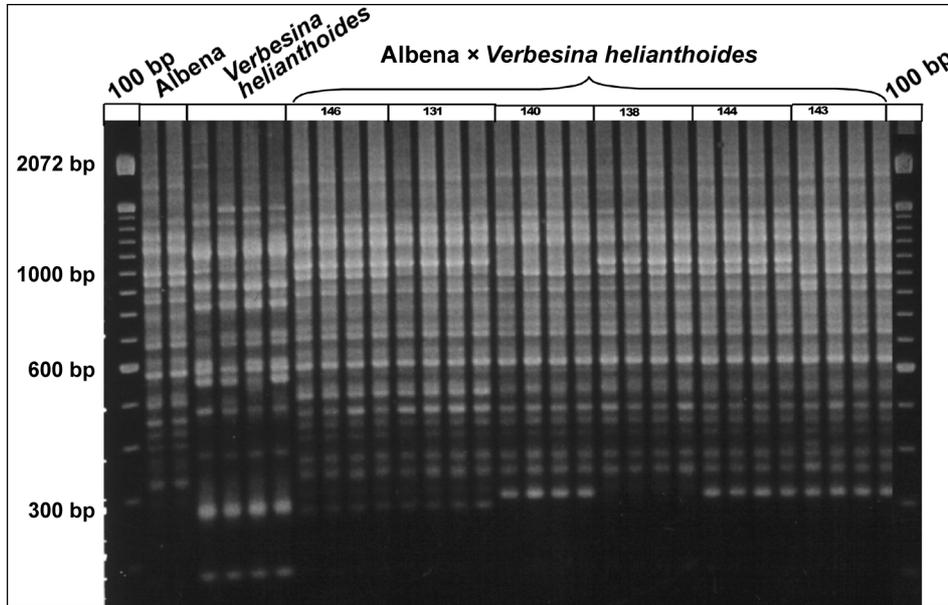


Figure 1: Genetic profile of intergeneric sunflower hybrids progenies (*Helianthus annuus* × *Verbescina helianthoides* based on primer OPAJ-19. Lines 2 and 3: female parent (cv. Albena). Line 4-7: male parent (*Verbescina helianthoides*); lines 8-31: intergeneric hybrid progenies: R 146, R 131, R 140, R 138; R 144, R 143; lines 1 & 32: 100 bp ladder. (Gibco BRL, Life Science Technology)

Using the similarity coefficient of Nei and Li, 1979, a dendrogram was generated through UPGMA analysis with the aim of determining the genetic distance of the studied genotypes. In the constructed scheme (Figure 2) three main groups can be recognized (*Verbescina helianthoides*, the hybrid Albena and the hybrid progenies).

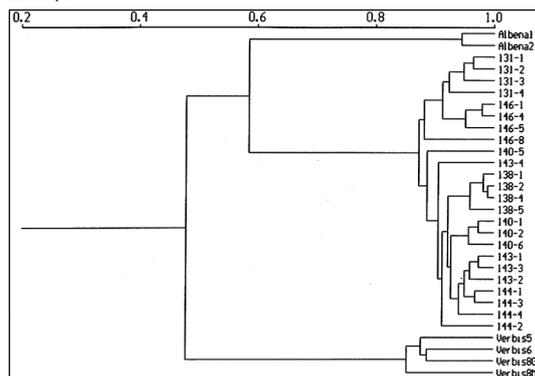


Figure 2: Constructed scheme of UPGMA analysis of genetic distance of the studied genotypes

The group of hybrid progenies was significantly closer to cultivated sunflower than to *Verbesina helianthoides*. This result was expected due to the fact that the lines were studied after 9 generations of selfing. The data indicate that *Verbesina helianthoides* has a significant genetic distance from the other genetic plasma studied. This result is evident in the dendrogram because *Verbesina helianthoides* formed a cluster neither with the cultivated sunflower (the hybrid Albena) nor with the group of intergeneric progenies. The cluster analysis based on Nei and Li matrix showed a basic group which included intergeneric hybrid progenies. Lines R 131, R 138, R 146 and R 144 were more homogenous in this group than lines R 140 and R 143.

The recently developed molecular techniques are highly specific tools for differentiation and classification of genotypes. These modern tools complement the more classical methods based mainly on morphological, biochemical and physiological traits, allowing us to determine the degree of genetic similarity within populations of a species and between species and related genera. Among these methods RAPD has turned out to be an especially efficient tool due to its simplicity, low quantity of target DNA necessary for genetic analysis, high automation and no need for radioactivity.

## CONCLUSIONS

The intergeneric cross *Helianthus annuus* × *Verbesina helianthoides* was obtained by the method of direct organogenesis. The available literature does not report of previous development of this hybrid combination. Our results are also the first attempt to apply molecular techniques in proving the hybrid nature of inbred lines obtained at a late stage of the breeding process. The cluster analysis confirmed the hybrid nature of the intergeneric progenies. Cultivated sunflower, *Verbesina helianthoides* and their hybrid progenies were clearly distinguishable. Our data confirmed the conclusion drawn by Köhler and Friedt (1999) that the lines which originate from the same interspecific or intergeneric cross form their specific cluster, *i.e.*, the related lines could be grouped into a separate gene pool. RAPD markers indicated also an introgression of wild genome portions into some of the obtained inbred lines of the cross during the selfing procedure. We were able to demonstrate that RAPD could be used for characterization of intergeneric hybrid progenies in sunflower at a late stage of selection (F9) in which an increased genetic variation was discovered. This genetic variation could be a valuable source of resistance to diseases, of increased quantity and quality of oil in seed and improvement of other agronomic indices. The intergeneric hybrids provide valuable initial material for increasing genetic variation in sunflower and genetic markers could help breeders to characterize this new material.

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**HÍBRIDOS INTERGENÉRICOS ENTRE EL GIRASOL CULTIVADO (*Helianthus annuus* L.) Y *Verbesina helianthoides* (GENUS *Verbesina*) - ANÁLISIS RAPD**

## RESUMEN

El método del organogénesis directo, fue aplicado con éxito para la superación de la barrera para el cruzamiento entre *Helianthus annuus* (cv. Albena) y *Verbesina helianthoides* (género *Verbesina*). Como resultado de una selección individual de varios años del material híbrido, las líneas restauradores de fertilidad fueron creadas en la generación R<sub>10</sub>. El método molecular RAPD confirmó la naturaleza híbrida del material de selección obtenido, e indicó la introducción de DNA de la especie *Verbesina helianthoides* en algunas de las descendencias híbridas creadas. Utilizando el coeficiente de similitud (Nei y Li, 1979), se ha construido el dendrograma, mediante el análisis UPGMA, con el fin de determinar la distancia genética entre los genotipos investigados. Hemos

logrado demostrar que RAPD puede utilizarse para la caracterización de la descendencia de los híbridos de girasol intergenéricos en la fase tardía de selección (F<sub>9</sub>). Por el método aplicado se ha determinado la existencia de la variación genética aumentada.

**HYBRIDES INTER GÉNÉRIQUES ENTRE LE TOURNESOL  
DE CULTURE (*Helianthus annuus* L.) ET *Verbesina  
helianthoides* (GENUS *Verbesina*) - ANALYSE RAPD**

RÉSUMÉ

La méthode d'organogénèse directe a été utilisée avec succès pour surmonter l'impossibilité de croiser *Helianthus annuus* (cv. Albena) et *Verbesina helianthoides* (genus *Verbesina*). Le résultat d'une sélection individuelle à long terme du matériel hybride, les lignées restauratrices de fertilité ont été produites dans la génération R 10. La méthode moléculaire RAPD a confirmé la nature hybride du matériel de culture obtenu et indiqué un apport d'ADN de *Verbesina helianthoides* dans certaines progénitures hybrides obtenues. En utilisant le coefficient de similarité de Nei et Li, 1979, un dendrogramme a été composée par l'analyse UPGMA dans le but de déterminer la distance génétique des génotypes étudiés. Nous avons pu démontrer que l'analyse RAPD peut être utilisée dans la caractérisation de progénitures hybrides inter génériques du tournesol à un stade tardif de sélection (F<sub>9</sub>). L'existence d'une variation génétique accrue a été confirmée par l'utilisation de la méthode.