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Efficient interspecific hybridization in the genus *Helianthus* via "embryo rescue" and characterization of the hybrids

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Summary. With the aid of the "embryo rescue" technique, interspecific hybrids in the genus *Helianthus* could be raised with a recovery rate of 41%. Altogether, 33 different hybrid combinations were realized using the cultivated form, both as a female and male parent. The hybrids obtained have been identified by different methods, i.e., by comparison of leaf morphology, pollen stainability, chromosome number and by RFLP analysis. The former three methods are useful to obtain global information, while the RFLP analysis allows a rapid and safe characterization in early developmental stages of the hybrids.

Key words: *Helianthus* sp. – Interspecific hybridization – "Embryo rescue" – RFLP analysis

Introduction

The growing interest in alternative crop production has led to an expansion of sunflower and other oil crops in recent years. However, this expansion revealed several problems closely linked with the narrow genetic basis of the present cultivars (Arnaud 1986), which are almost exclusively hybrids. Most of these varieties are very sensitive to fungal diseases. Therefore, resistance against such pathogens needs substantial improvement. For example, varieties resistant to the most dangerous diseases, *Sclerotinia sclerotiorum* and *Botrytis cinerea,* are necessary for an economically successful cultivation in northern regions.

Furthermore, commercial sunflower hybrid seed production is based on one single source of cytoplasmic male sterility (cms), first reported by Leclerq (1969). Therefore, all hybrids thus far available worldwide are closely related with regard to their identical cytoplasm (Arnaud 1986). In order to broaden the basis of cytoplasmic sterility, interspecific hybridizations have already been attempted (Whelan 1980, 1981; Anaschenko 1981; Heiser 1982; H. Serieys, unpublished manuscript). Furthermore, with the aid of such wide crosses, genes for resistance can be transferred from wild species to cultivated varieties. Therefore, the nuclear as well as the cytoplasmic genetic basis of sunflower can be broadend via interspecific hybridization. However, reports on successful interspecific crosses with perennial species are comparatively rare (Carter 1978; Whelan 1978). Nevertheless, with the help of embryo culture *in vitro* the recovery rate of hybrid plants can be increased (Bohorova 1982; Chandler and Beard 1983; Georgieva-Todorova 1984). So far the success rate has been too low to apply this technique in a breeding program (Georgieva-Todorova 1984; Bohorova et al. 1985). It was our aim, therefore, to increase the regeneration capacity of interspecific hybrids in order to recover sufficient numbers of offspring for further selection and screening for characteristics mentioned above. In addition, we attempted to establish a fast and early applicable method for characterization of the hybrids.

Materials and methods

Plant material

Sunflower (*Helianthus annuus* L.) cytoplasmic male-sterile lines, HA89 and Baso, and their maintainer lines have been consecutively cultivated under field conditions at the experimental station of Rauischholzhausen (University of Giessen) in order to extend the flowering period. A collection of 30 different

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Table 1. Media composition for embryo culture

B5 (per liter): 150 mg $CaCl_2 \times 2H_2O$, 150 mg $NaH_2PO_4 \times H_2O$, 2.5 g KNO_3, 134 mg $(NH_4)_2\ SO_4$, 250 mg $MgSO_4 \times 7H_2O$, 37.25 mg Na_2-EDTA, 27.9 mg $FeSO_4 \times 7H_2O$, 8.5 mg $MnSO_4 \times 4H_2O$, 4.3 mg $ZnSO_4 \times 7H_2O$, 3.1 mg H_3BO_4, 0.41 mg KJ, 0.0123 mg $CuSO_4 \times 5H_2O$, 0.125 mg $Na_2MoO_4 \times H_2O$, 100 mg myo-inositol, 10 mg thiamine × HCl, 1 mg nicotinic acid, 1 mg pyridoxine × HCl, 90 g sucrose, pH 5.6, 7 g agar-agar

MS-modified (per liter): 165 mg NH_4NO_3, 190 mg KNO_3, 440 mg $CaCl \times 2H_2O$, 370 mg $MgSO_4 \times 7H_2O$, 170 mg KH_2PO_4, 16.9 mg $MnSO_4 \times 4H_2O$, 8.6 mg $ZnSO_4 \times 4H_2O$, 6.2 mg H_3BO_4, 0.83 mg KJ, 0.025 mg $CuSO_4 \times 5H_2O$, 0.25 mg $NaMoO_4 \times 2H_2O$, 27.9 mg $FeSO_4 \times 7H_2O$, 37.25 mg Na_2-EDTA, 0.4 mg thiamine × 3HCl, 100 mg myo-inositol, 10 g sucrose, pH 5.4–5.6, 9 g agar-agar

Helianthus wild species has been established (sources: USDA, Institute of Soil and Crop Management, Unit Sunflower, Bushland, USA, and Institute of Field and Vegetable Crops, Novi-Sad, Yugoslavia). The different, mostly perennial, species have mainly been crossed with HA89, using the wild species as pollinator for the most part, but also as a female parent.

Embryo culture ("embryo rescue")

Five to 10 days after fertilization – depending on the growing conditions – the fertilized seeds weere harvested, disinfected for 10 min in a NaOCl solution (2%), and washed twice. The immature embryos were isolated by opening the seed coat, the integument, and the enclosed embryo sack under a dissecting microscope (Zeiss). The isolated embryos were placed on different media depending on their size. For small embryos (0.2–1.5 mm) either a modified B5 medium (Table 1) according to Gamborg et al. (1968) with a sucrose concentration reported by Espinasse et al. (1985) was used, or embryos (>1.5 mm) were cultured on a modified MS medium (Table 1) according to Murashige and Skoog (1962), revised by B. Foroughi-Wehr (personal communication), in small petri dishes (∅ 5 mm). The embryos were cultured in a growth chamber at 25 °C under permanent light at approximately 136 W/m² (Philips, TLM 40 W).

After the small embryos had reached a size of 2–3 mm, they were transferred onto MS medium. Shoots developed 1–2 weeks after transfer to this medium, after which they were transferred to the greenhouse where they developed into intact plants.

Characterization of the hybrids

Leaf morphology. The leaf shape and petiole rudiments of parental and hybrid plants were compared.

Pollen viability. To determine pollen viability, mature pollen grains were stained in 4% acetocarmine solution according to Alexander (1969). Frequencies of stained and unstained pollen were determined with a light microscope.

Chromosome number. The chromosome number of hybrid plants was determined according to J. Georgieva-Todorova (personal communication). Root tips 1–2 cm in length were immersed into a 0.05% colchicine solution at 25 °C for 30 min. The tissue was subsequently hydrolyzed in 1 *N* HCl at 60 °C for 10 min. The root tips were then transferred to "Schiff's Reagent" ("Feulgen stain") until they reached a dark red color. Afterwards they were squeezed in a drop of acetocarmine (4%) and chromosomes were counted in mitotic metaphase.

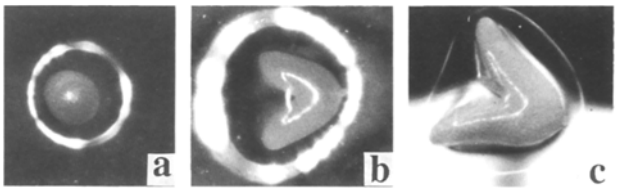

Fig. 1 a–c. Different stages of embryo development. **a** Globular stage, **b** heart-shaped stage, **c** differentiated stage

Table 2. Regeneration of interspecific hybrids (F_1) via embryo rescue (Kräuter 1990)

Cross combinations	278
– without success	155 (56%)
– successful	123 (44%)
In vitro culture of embryos	1,178
– globular stage	141 (12%)
– young heart-shaped stage	495 (42%)
– differentiated stage	542 (46%)
Regenerated plants (rate)	481 (41%)

RFLP analysis. Total leaf DNA of parental and hybrid plants was isolated according to Dellaporta et al. (1983) with an additional purification step on CsCl gradients. DNA was digested with the restriction enzyme *Hin*dIII according to the recommendation of the supplier. DNA fragments were separated electrophoretically on 0.7% agarose gels and transferred to nitrocellulose membranes (Southern 1975). For hybridization, a DNA probe containing part of the 23S rRNA gene (1.1 kbp) from *Helianthus annuus* (obtained from Ph. Heizmann, Lyon) was radioactively labeled by random priming according to Feinberg and Vogelstein (1983). Hybridizations were performed in 50% formamide, 2 × SSC at 37 °C for 12 h. Filters were washed in 2 × SSC and autoradiographed using Fuji RX films.

Results

Efficient regeneration capacity of interspecific hybrids with the help of embryo rescue

Sexual crosses of wild species, mainly with sunflower female line HA89, and subsequent embryo rescue resulted in the recovery of 481 hybrid plants out of 1,178 embryos isolated (Table 2, Fig. 1). This result corresponds to an average regeneration frequency of 41%. Altogether, hybrids from 33 different cross combinations could be raised (Table 3). Fertility of the plants was identified and backcrosses and/or selfings were made wherever possible.

Characterization of hybrids by comparison of the leaf morphology of parents and hybrids

An identification of all hybrids was not possible on this basis alone. The phenotype of the hybrid HA89 × *H. strumosus* in Fig. 2a is intermediate between the parents, but the hybrids shown in Figs. 2b and 2c could not be

Table 3. Viable hybrids obtained (F_1)

Mother	Father	Fertility	Physiological type	
			annual	perennial
(1)	HA89 × *H. decapetalus*	−		+
(2)	HA89 × *H. decapetalus*/Dijon	+	+	
(3)	HA89 × X *H. originalis*	+		+
(4)	HA89 × X *H. angustifolius*	+		+
(5)	HA89 × *H. resinosus*	+		+
(6)	HA89 × *H. nuttallii*/239	−	+	
(7)	HA89 × *H. nuttallii*/329	−	+	
(8)	HA89 × *H. nuttallii*/103	+		+
(9)	HA89 × X *H. laetiflorus*/HUNG	+	+	
(10)	HA89 × X *H. laetiflorus*/558	+	+	
(11)	HA89 × *H. argophyllus*	+	+	
(12)	HA89 × *H. tuberosus*	+	+	
(13)	HA89 × *H. bolanderi*	−	+	
(14)	HA89 × *H. debilis*	−	+	
(15)	HA89 × *H. strumosus*	+	+	
(16)	HA89 × *H. giganteus*	−		+
(17)	HA89 × *H. grosseserratus*	*		
(18)	HA89 × *H. mollis*	+	+	
(19)	HA89 × *H. rigidus*	−	+	
(20)	HA89 × X *H. laetiflorus*	+/−	+	
(21)	HA89 × *H. maximiliani*	+	+	
(22)	Baso × *H. mollis*	+	+	
(23)	Baso × *H. debilis*	+	+	
(24)	*H. nuttallii*/239 × HA89	+		+
(25)	*H. nuttallii*/329 × HA89	+		+
(26)	X *H. laetiflorus*/558 × HA89	+		+
(27)	*H. argophyllus* × HA89	+	+	
(28)	*H. debilis* × HA89	+	+	
(29)	*H. divaricatus* × HA89	*		
(30)	*H. debilis* × sf-135	+,− **	+	
(31)	*H. decapetalus*/HUNG × sf-135	+,− **		+
(32)	*H. ann. lenticularis* × sf-135	+	+	
(33)	*H. niveus canescens* × sf-135	+	+	

* Extinct before flowering
** Reduced fertility

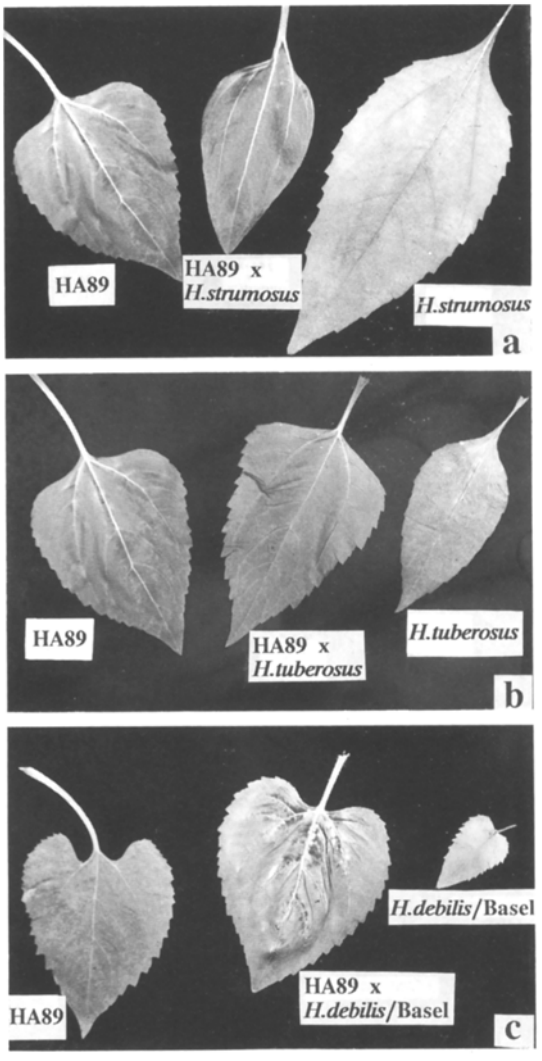

Fig. 2a–c. Comparison of leaf morphology of parents and hybrids (female HA89 cms): **a** cross combination HA89 × *H. strumosus*, **b** cross combination HA89 × *H. tuberosus*, **c** cross combination HA89 × *H. debilis*/Basel

accurately classified on the basis of leaf morphology alone.

Pollen viability and chromosome numbers

Interspecific hybrids are often characterized by reduced fertility. However, the results given in Table 4 were not always sufficient for a full characterization of the hybrids. For example, hybrid HA89 × *H. strumosus* could be easily identified as a hybrid on a morphological basis (Fig. 2a), although the pollen viability (fertility) of the plant reached 92%. For some hybrid combinations, an expected reduced stainability could be confirmed, e.g., HA89 × *H. originalis*, *H. debilis* × HA89, and HA89 × *H. resinosus*.

The same was true for the determination of chromosome numbers: the hybrids investigated so far did not show any additional or missing chromosomes (Fig. 3). Therefore, a characterization of the hybrids on the basis of either fertility or chromosome number does not lead to satisfactory results in most cases.

RFLP analysis, a method to identify the parentage of hybrids

Analysis of nuclear DNA using the 23S ribosomal DNA probe revealed single bands in the *Hin*dIII pattern of both the *H. annuus* line HA89 and the species *H. resinosus*, suggesting that the parents are homozygous with respect to the rDNA gene. A polymorphism in the *Hin*dIII restriction pattern (Fig. 4, lanes 1 and 3) between the two parental species is revealed by the different electrophoretic mobilities of these bands. This polymor-

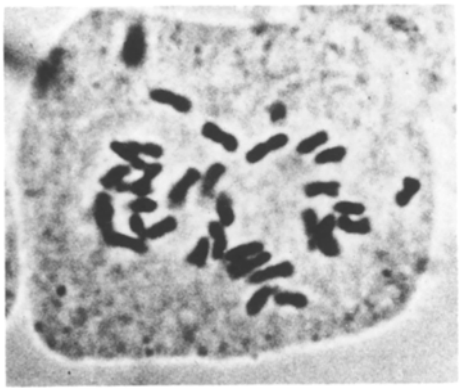

Fig. 3. Chromosome number of the interspecific hybrid HA89 × *H. nuttallii*/102 in mitotic metaphase (2n = 2x = 34); diploid chromosome numbers were also found in HA89 × *H. decapetalus*/Dijon and HA89 × *H. strumosus*, whereas higher ploidy levels were observed in other crosses (e.g., 6x in HA89 × *H. resinosus*)

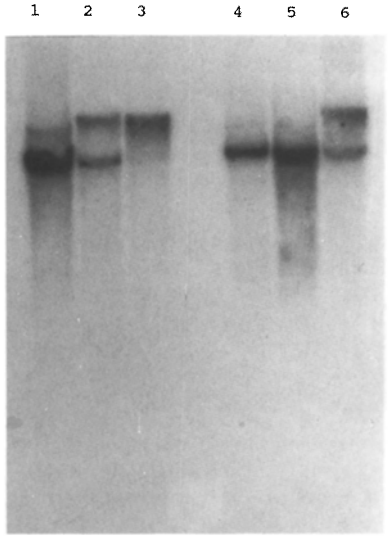

Fig. 4. Results of hybridization with rDNA probe. *1* HA89, *2* HA89 × *H. resinosus* (F_1), *3 H. resinosus*, *4* HA89, *5* HA89 × *H. strumosus* (BC1), *6 H. strumosus*

Table 4. Pollen viability of different interspecific hybrids

Female Male parent	Pollen stainability (%)
(1) HA89 × X *H. originalis*	3
(2) HA89 × *H. resinosus*	57
(3) HA89 × *H. laetiflorus*/558	96
(4) HA89 × *H. decapetalus*/Dijon	86
(5) HA89 × *H. strumosus*	92
(6) *H. debilis* × HA89	12

phism has been used to demonstrate the hybrid nature of the F_1 hybrid resulting from the cross HA89 × *H. resinosus* [compare the two bands of equal intensities in Fig. 4, lane 2, one of maternal (lower band), the other of paternal origin (upper band)].

The DNA analysis of the cross combination HA89 and *H. strumosus* did not demonstrate the hybrid nature of the backcross (BC_1) progeny with the DNA probe used here.

Discussion

Embryo culture

A two-step culture procedure as proposed by Chandler and Beard (1983) is essential for the successful cultivation of embryos in young stages, i.e., globular and heart-shaped stages (Fig. 1 a and 1 b). Embryos in differentiated stages (Fig. 1 c) should be placed on regeneration medium for shoot development only, and do not need a medium for initial growth. On high-sucrose-containing medium, embryos in a differentiated stage actually stopped development and started to turn brown. It seems that different osmotic gradients are needed in various stages of development; although sucrose has importance as a nutrient too.

For initial culture, the medium recommended by Espinasse et al. (1985) was used in a slightly modified form, as recommended for *H. annuus* embryos. Chandler and Beard (1983) added phytohormones and amino acids to the initial medium, which we did not find to be necessary. For the second cultural step, a modified MS medium was used with a tenfold reduction of the NO_3^- concentration (B. Foroughi-Wehr, personal communication). In contrast Espinasse et al. (1985) and Chandler and Beard (1983) applied the revised medium composition of Gamborg et al. (1968).

The reported regeneration rate of 41% demonstrates that interspecific hybrids can be raised with the help of the applied technique in an efficient way. Successful crossing or regeneration potential of embryos in the genus *Helianthus* by embryo rescue was also reported by Georgieva-Todorova (1984) and Bohorova et al. (1985). The former author obtained a yield of 0.6–32.5% of mature seeds from the cross *H. annuus* × *H. resinosus*, while Bohorova et al. (1985) gained 15% plant regeneration from the hybrid *H. annuus* × *H. hirsutus* and 70% from the cross combination *H. scaberimus* × *H. annuus*. All these results were obtained with differentiated embryo stages exclusively.

Characterization of the hybrids

Before any further breeding activities are initiated, the hybrid nature of the plants must be confirmed. This is

especially true for cases where wild species have been used as female parent. For technical reasons, a manual castration of the wild species is all but impossible; therefore, self fertilization cannot be excluded. For the reciprocal cross combinations (*H. annuus* × wild species), the male-sterile line HA89 has been used as a female parent, i.e., the hybrid nature of progeny should be certain in these cases. Several attempts at hybrid characterization have been made. The morphological comparison of hybrids and parental leaves and the test of pollen stainability were already applied by Whelan (1978) and Georgieva-Todorova (1984). Our results obtained with these tests, given in Fig. 2 and in Table 4, can only be considered as supplementary. This is also true for the chromosome number determination (Fig. 3). The interpretation of cytological results is especially difficult in cases where the parents have identical chromosome numbers; in this case meiotic investigations would be required in addition.

With the molecular comparison of hybrid and parental DNA, a clear difference could be demonstrated in one cross combination thus far. For a clear identification of other species combinations, the applicability of different DNA probes in combination with different restriction enzymes must be analyzed further. In contrast to the sunflower genome, the maize genome is characterized by many restriction fragment length polymorphisms (e.g., Helentjaris et al. 1985; Beckmann and Soller 1986; Godshalk et al. 1990; Smith et al. 1990). However, the RFLP technique is also applicable for sunflower interspecific hybrid characterization, as demonstrated by the hybrid *H. annuus* HA89 × *H. resinosus*. The applied technique is very useful for investigations even in early stages of development, since DNA can be isolated in any developmental stage even from mummified and dried tissue (Rogers and Bendich 1985).

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