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Interspecific hybridization between sunflower and wild perennial *Helianthus* species via embryo rescue

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Abstract

Interspecific crosses were made between the cultivated sunflower inbred line HA89 and accessions of five wild perennial *Helianthus* species *(H. giganteus* L., *H. laevigatus* T. & G., *H. resinosus* Small, *H. pauciflorus* Nutt. and *H. decapetalus* L.) resistant to broomrape (*Orobanche cernua* Loefl.). Using the genetic male-sterile isogenic version of that line as female, successful reciprocal crosses were also obtained with all these wild species except with *H. decapetalus*. Five-day-old hybrid embryos were excised and cultured *in vitro*. In all cases, few mature plants were obtained from embryos in early developmental stages (early heart and globular) but up to 28% mature plants were obtained from embryos in later stages of development. Very immature embryos were difficult to excise without damage. Hybrid embryos and mature plants were obtained from all five wild species. Interspecific hybrid embryos from different wild species showed distinct developmental potentials, the proportion of hybrid embryos in different developmental stages varying among species. Differences in the proportion of hybrid embryos at the several developmental stages were also obtained for the reciprocal crosses (cultivated × wild), which showed higher proportion of fully developed embryos. Hybrids involving *H. giganteus* and cultivated sunflower were difficult to obtain without the use of embryo culture. Embryo culture proved to be an useful tool to overcome post-zygotic hybrid incompatibility in different *Helianthus* spp., and facilitated interspecific transfer of resistance to *O. cernua*.

Introduction

The narrow genetic base in domesticated germplasm is a major concern of sunflower breeders worldwide. Wild *Helianthus* spp. offer a significant amount of genetic diversity for using in further improvement of cultivated sunflower, including important traits such as disease and insect pest resistance, cytoplasmic male sterility, fertility-restoration, agronomic and seed-oil characteristics, drought tolerance, protein content, and fatty acid composition (Seiler & Rieseberg, 1997). Therefore, the wild perennial *Helianthus* spp. are becoming more important as a genetic resource in broadening the germplasm base of sunflower, and the use of interspecific hybridization is increasing in many sunflower breeding programs (Thompson et al., 1981; Laferrière, 1986; Seiler & Rieseberg, 1997).

Conventional crossing methods have sufficed to produce interspecific hybrids between cultivated sunflower and some of the wild *Helianthus* spp., especially the annual diploids. However, most of the wild perennial diploid species remain untapped as usable germplasm because of difficulties in interspecific hybridization with cultivated sunflower due to the abortion of embryos at an early developmental stage (Seiler & Rieseberg, 1997). In many cases these difficulties can be overcome using embryo rescue. The sunflower embryo culture, first developed by Chandler & Beard (1983), greatly facilitated interspecific hybridization. Culturing requirements for sunflower embryos have been investigated, and further modifi-

cations have been made for rescuing immature interspecific hybrids (Finer, 1987; Jan, 1988; Denat et al., 1991; Espinasse et al., 1991; Kräuter et al., 1991; Jan, 1997). Responses of sunflower embryos to tissue culture have been shown to depend on the composition of media and genotype of the plant. Survival of intraspecific sunflower hybrid embryos *in vitro* is also related to the stage of embryo development (age, size and shape) at the time of culture (Espinasse et al., 1985). Experiments relating embryo developmental stage at the time of transfer to medium and *in vitro* survival for sunflower interspecific hybrids have not been reported before.

A high proportion of wild perennial *Helianthus* spp. are resistant to several races of broomrape (*Orobanche cernua* Loefl.) in Spain (Ruso et al., 1996). A program of interspecific transfer of broomrape resistance from these species into cultivated sunflower was initiated in 1994 (Sukno et al., 1998). Development of sunflower breeding populations resistant to *O. cernua* derived from interspecific crosses has been achieved (Skoric, 1992) but interspecific transfer of resistance genes is frequently restricted by incompatibility problems. In this paper we report the success of interspecific hybridization between five resistant wild perennial species with different ploidy levels, and cultivated sunflower via embryo rescue. The objectives of this study were (1) to evaluate embryo development for the five cross combinations, (2) to investigate the rate of embryo development *in vitro*, and (3) to determine the embryo survival rate with regard to embryo developmental stages.

Materials and methods

Plant material

Wild perennial species *H. giganteus* L. (2n = 34; PI 503250), *H. laevigatus* T.& G. (2n = 102; PI 468740), *H. resinosus* Small (2n = 102; PI468879), *H. pauciflorus* Nutt. (2n = 102; PI 435869), and *H. decapetalus* L.(2n = 89–99) were grown at Fargo, ND in 1995, together with *H. annuus* L. inbred line HA89 and the nuclear male-sterile isogenic line NMSHA89 (Jan, 1992). Heads of wild species were bagged before anthesis, and when the outer rings of florets started to elongate they were emasculated early in the morning by removing the anthers with forceps, then washed with a spray of water, and pollinated with HA89 pollen in the afternoon. This procedure was repeated in successive days as the flowering progressed toward the center of the head. For reciprocal crosses, bulk pollen of each wild species was applied onto the heads of NMSHA89.

Embryo culture

Single sunflower heads were harvested 5 days after pollination and the number of heads and seeds recorded. Achenes were surface-disinfected by dipping them in a 20% bleach solution (50 g cl L^{-1}) with a trace amount of Tween 20 (polyoxyethylene sorbitanmonolaurate) during 10 min, and then rinsed three or four times with sterile distilled water. Afterwards, achenes were dissected under a stereo microscope, and the embryos, once excised from the embryo sacs, were placed on a growth medium in Petri dishes. Embryo shapes were defined according to Newcomb (1973) as globular (G), early heart (EH), heart (H), and full development or torpedo (FD). Some embryos were cultured in ovules when we were unable to remove them. These explants were recorded as round shape or early-globular stage (O) (Figure 1).

The numbers of developed seeds per head, empty seeds, and damaged embryos, as well as embryo developmental stage were recorded. Hybrid embryos were cultured on the artificial medium following the two-step procedure of Chandler & Beard (1983). Five-day-old hybrid embryos were first cultured on solid growth medium in Petri dishes with Gamborg's B5 macronutrients supplemented with vitamins (nicotinic acid, 1 mg L^{-1}; thiamine × HCl, 10 mg L^{-1}; pyridoxine × HCl, 1 mg L^{-1} and myo-inositol, 4000 mg L^{-1}), amino acids (L-alanine, 1000 mg L^{-1}; L-glutamine, 800 mg L^{-1}; L-serine, 160 mg L^{-1}; L-tryptophane, 50 mg L^{-1} and L-cysteine, 10 mg L^{-1}), 0.05 mg L^{-1} of NAA (alpha naphthalene acetic acid), 120 g L^{-1} of sucrose, and 7 g L^{-1} of agar (Gamborg et al., 1968). After one to two weeks, the enlarged embryos were transferred to a germination medium in test tubes with B5 salts, 10 g L^{-1} sucrose and agar. Some modifications (Jan, 1997), consisting of the addition to the media of morpholino ethasulfonic acid (MES) to maintain a constant pH of 5.5 and a change in the germination medium from liquid to solid with 0.7% agar and increasing sucrose to 20 g L^{-1} were adopted. Three to five weeks later, the plantlets with well developed root systems were transferred to 3.5-cm-diameter Jiffy pellets, and placed in a growth chamber at 23–25 °C for one week. Afterwards, the young seedlings were transplanted into 11-cm-diameter clay pots in the greenhouse, and root-tips were sampled for chromo-

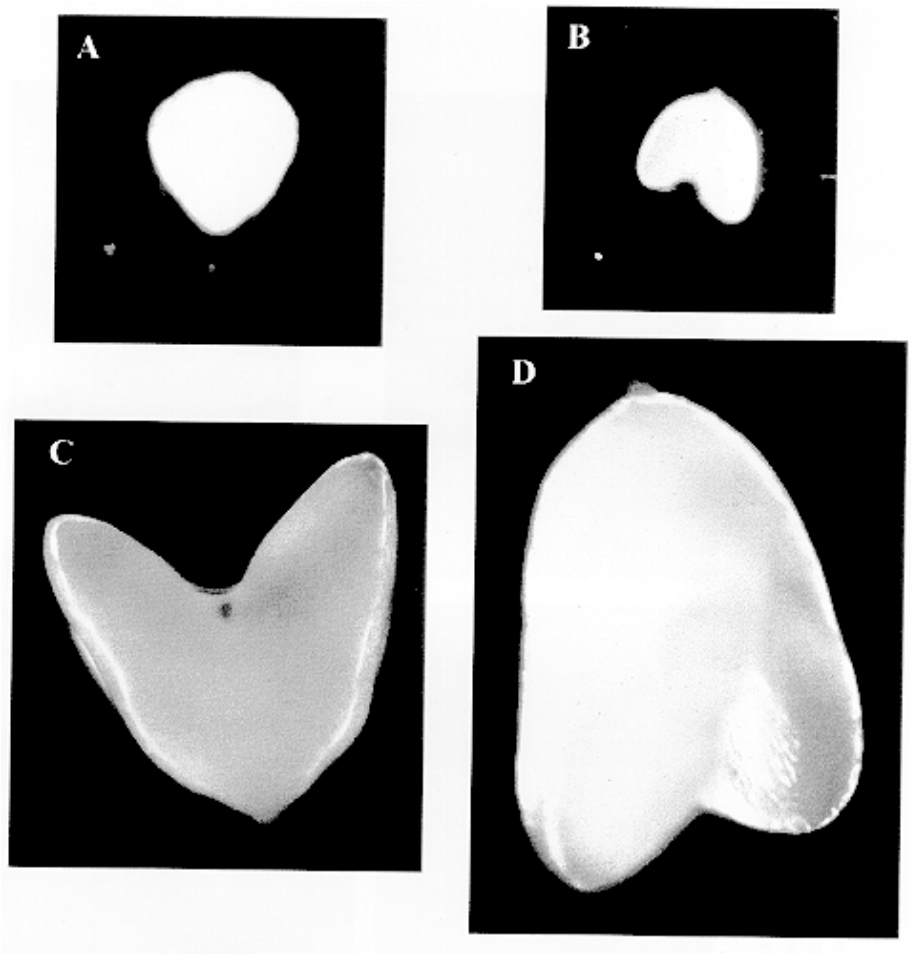

Figure 1. Developmental stages of sunflower embryos: A) globular, B) early heart, C) heart, D) fully developed or torpedo.

some counts. The number of plantlets regenerated and mature plants obtained were recorded. Embryos that either died or developed abnormally into calli were referred to as 'survival failure'. Embryo survival was calculated as the percentage of embryos alive (either in the growth or the germination medium) with regard to the number of plated embryos (Monnier, 1976). The efficiency of the embryo rescue (EER) was calculated by the rate number of plants obtained / number of embryos rescued.

Hybrid identification

The root-tip chromosome number, plant morphology, and pollen stainability were used to identify the hybrid status of plants. Chromosome number of the wild parents and F_1 plants were determined using the Feulgen staining technique according to Jan (1996). Root tips were collected and placed in ice water for 18 h, then fixed in 3:1 ethanol-glacial acetic acid solution for 4 h, hydrolyzed in 1N HCl at 60 °C for 12 min, and then treated with an aqueous solution of 0.2% pectinase and 0.2% cellulase for 20 min before they were dipped in the staining solution. Leaf and flower shape/size of parental and hybrid plants were compared. Pollen stainability of the wild species and F_1 hybrids was determined according to Alexander (1969) and expressed as percentage of total pollen grains. A minimum of 200 pollen grains for each plant were scored.

Table 1. Seeds and embryos obtained from interspecific crosses between five perennial *Helianthus* species and cultivated line HA89 by embryo rescue

Cross	Number of plants[a]	Number of crosses	Developed seeds per cross	Excised embryos plated	Excised embryos empty		Stage of development of the embryos rescued[c] (% of the total excised embryos)				
				No.	No.	%	O	G	EH	H	FD
H. decapetalus × HA89	6	178 (147)[b]	0.48	81	25	23.6	17.3	14.8	28.4	30.9	8.6
H. giganteus × HA89	6	108 (11)	15.12	1081	435	28.7	20.3	50.0	21.2	5.6	2.9
H. laevigatus × HA89	6	126 (51)	7.59	568	164	22.4	11.8	8.3	13.4	48.7	17.8
H. pauciflorus × HA89	6	62 (11)	15.52	389	525	57.4	17.7	17.5	20.6	20.6	23.6
H. resinosus × HA89	2	36 (7)	10.06	312	26	7.7	5.2	11.8	14.7	30.2	38.1
Total	26	510 (227)		2431	1175	32.6	15.9	29.0	18.7	22.0	14.4

[a] = number of wild plants used from each accession.
[b] = unsuccessful crosses are shown between brackets.
[c] = O = preglobular, G = globular, EH = early heart, H = heart, FD = full development or torpedo.

Results

Crosses and embryo culture of interspecific hybrids

The number of plants and heads crossed, seeds developed per head, and the developmental stages of excised hybrid embryos are shown in Table 1. Only 283 (55%) of the 510 crossed heads produced culturable embryos. Hybrid seed formation varied among the species. *H. giganteus* had 89.9% of heads with seeds, in contrast to only 17.5% from *H. decapetalus*. Because of the high proportion of unsuccessful crosses, the number of plated embryos of *H. decapetalus* × HA89 was drastically reduced. The average number of developed seeds per head varied from 0.48 for *H. decapetalus* × HA89 to 15.52 for the cross *H. pauciflorus* × HA89. All plants, except one *of H. pauciflorus*, produced embryos after repeated crosses. Out of the 3606 achenes excised, 2431 (67.4%) embryos were plated. The remaining 1175 achenes (32.6%) were discarded as they were empty. The largest and smallest percentages of empty achenes resulted from crosses involving *H. pauciflorus* and *H. resinosus*, respectively. The percentages of excised embryos at each developmental stage varied between the perennial species used in the crosses. Half of the embryos from *H. giganteus* × HA89 had globular shape, whereas a similar number of the embryos of *H. laevigatus* × HA89 were heart shaped. Stages H and EH predominated in crosses of *H. decapetalus* × HA89, whereas the most frequent stages for crosses *H. resinosus* × HA89 were FD and H. Similar frequencies (17.5–23.6%) were observed for all the stages of development of the excised embryos of crosses *H. pauciflorus* × HA89 (Table 1).

The number of embryos that survived the embryo culture, grew in Jiffy pellets, and reached maturity are shown in Table 2. The percentage of survival and further development of embryos transferred to the germination medium varied among different interspecific hybrids. Embryos from *H. giganteus* × HA89 had a lower survival rate than those of *H. laevigatus* × HA89, *H. pauciflorus* × HA89, *H. resinosus* × HA89 and *H. decapetalus* × HA89. Overall, about 67% of embryos failed to progress through the growth medium, embryo survival being higher for EH-H-FD stages than for O-G developmental stages (Figure 2A).

Plantlets that survived in the germination medium and developed roots were transferred to Jiffy pellets. The percentage of plantlets surviving through the growth in Jiffy pellets (the number of plantlets obtained / total number of plants transplanted to pellets) were higher for crosses involving *H. decapetalus* and *H. resinosus* than for those of *H. pauciflorus* × HA89 and *H. giganteus* × HA89 (Table 2). The percentage of rescued embryos that developed into plants increased as the developmental stage progressed from G to FD. There was a highly significant ($P \leq 0.001$) correlation ($r = 0.6$) between the survival of embryos and the stage of development. Embryo survival was lower for O-G than for EH-H-FD stages. The early-globular stage had, on the average, a slightly higher rate of plantlet formation than the globular stage. Maximum rates of plantlet development were observed for FD embryos, except for *H. giganteus* × HA89 (Figure 2B). More

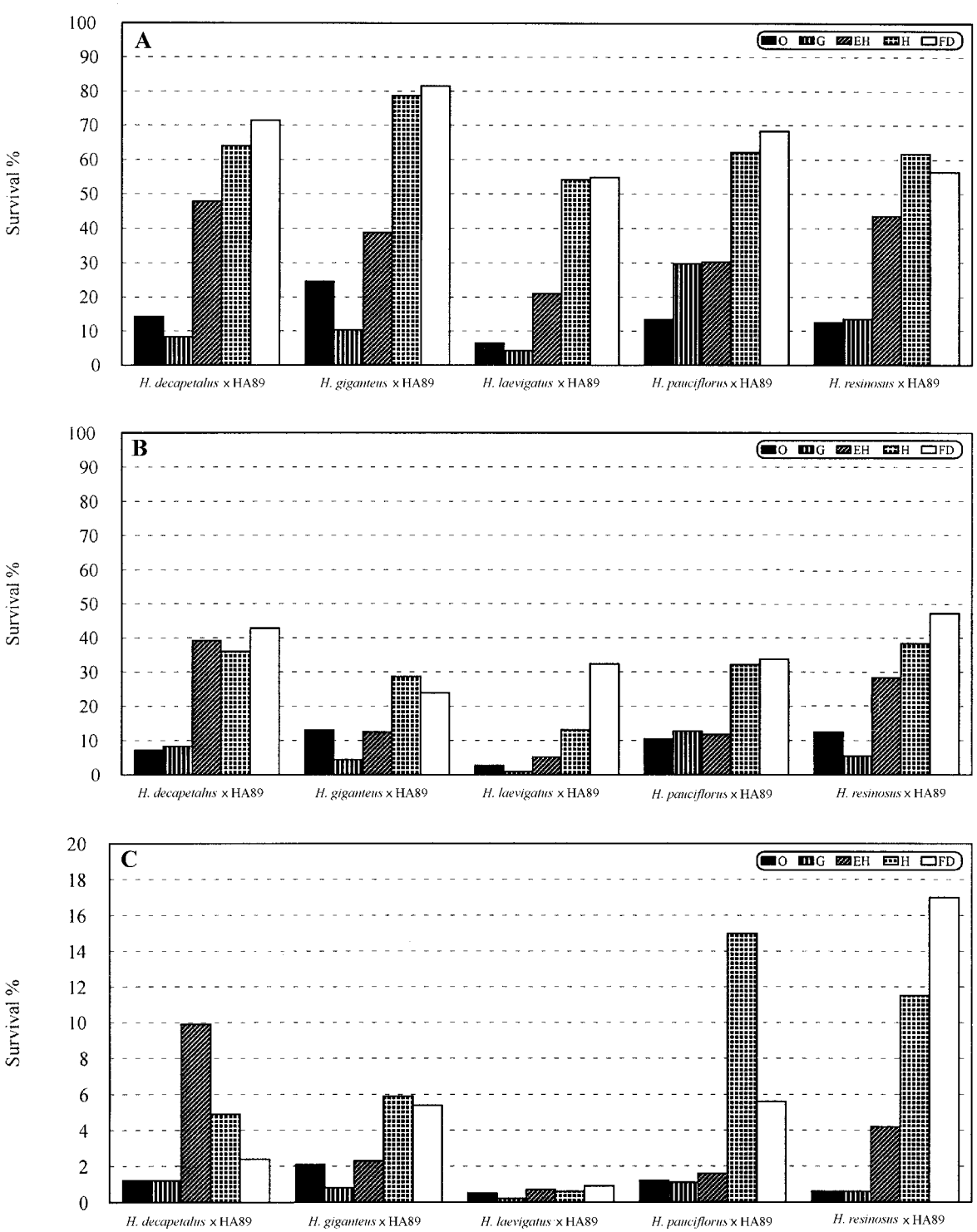

Figure 2. Effect of embryo developmental stages on the survival rates of (A) % of embryos developed in growth medium for further transfer, (B) % of plantlets developed in germination medium for transfer to Jiffy pellets, and (C) % of mature plants established in greenhouse pots.

Table 2. Survival of excised embryos from interspecific crosses between five perennial *Helianthus* species and cultivated line HA89

	In vitro			*Ex vitro*				
		Plantlets transferred to the germination medium		Plants transferred to Jiffy pellets			Mature plants obtained	
Cross	Embryos plated No.	No.	%	No.	%	EER[a]	No.	%
H. decapetalus × HA89	81	35	43.2	23	65.7	28.4	16	69.5
H. giganteus × HA89	1081	135	12.5	41	30.4	3.8	31	75.6
H. laevigatus × HA89	568	287	50.5	145	50.5	25.5	139	95.9
H. pauciflorus × HA89	389	193	49.6	67	34.7	17.2	64	95.5
H. resinosus × HA89	312	152	48.7	109	71.7	34.9	106	97.2
Total	2431	802	33.0	385	48.0	15.83	356	94.8

[a] EER: efficiency in embryo rescue: number of plants obtained / number of total embryos rescued.

than half of the plants of crosses involving *H. decapetalus*, *H. laevigatus*, and *H. resinosus* transferred to the germination medium developed into plantlets as compared to only one third in the case of *H. giganteus* and *H. pauciflorus*. A higher number of embryos died in the growth medium than in the germination medium, particularly for *H. decapetalus*, *H. giganteus* and *H. resinosus* (Table 2).

The efficiency of embryo rescue, EER (number of plantlets obtained / number of embryos cultured) varied with the wild *Helianthus* spp. in the cross, being lowermost for *H. giganteus* × HA89 and highest for *H. resinosus* × HA89 (Table 2). EER was lower for O and G stages than for the other stages, and higher for FD than for EH stage (Figure 2A, B).

The number of plants of each cross that reached maturity from embryo culture is shown in Table 2. All crosses of *H. laevigatus* and *H. resinosus* with HA89 produced embryos that developed into putative hybrid plants. Four plants of *H. decapetalus*, one of *H. giganteus*, and one of *H. pauciflorus*, produced only a few embryos when crossed to HA89, and these did not reach the mature stage (data not shown). Less than 5% of the F_1 plants of *H. laevigatus* × HA89, *H. pauciflorus* × HA89, and *H. resinosus* × HA89 died after transferring them to clay pots in the greenhouse. Plant death increased to 24.4% and 30.5%, respectively, for the F_1 of *H. giganteus* × HA89 and *H. decapetalus* × HA89 (Table 2). In all cases, the number of mature plants produced from embryos in early developmental stages remained low, but it greatly increased when embryos in late developmental stages were used (Figure 2C).

Except for *H. decapetalus*, one or several crosses were made between NMSHA89 and the other four perennial *Helianthus* accessions, in order to study the survival of cultured embryos in comparison to the reciprocal crosses. The number of embryos from each stage of development and *ex vitro* plants, as well as the number of excised embryos and the percentage plant survival are shown in Table 3. The average size of embryos developed from those crosses were much larger than that of embryos obtained when the wild species were used as the maternal parents. Good rates of recovery were obtained when the cultivated NMSHA89 line was used as parental female, except for NMSHA89 × *H. giganteus* for which no interspecific embryos were formed. As for the reciprocal crosses, differences in the percentage of embryos rescued from each developmental stage were also found. The percentage of FD embryos rescued from NMSHA89 × *H. laevigatus*, NMSHA89 × *H. pauciflorus*, and NMSHA89 × *H. resinosus*, was much higher than for the reciprocal crosses, but it was lower for O, G and EH stages (Tables 1 and 3). When the cultivated line was used as female parent, EER was much higher than that of the reciprocal crosses (Tables 2 and 3). This rate was more than double in interspecific embryos of NMSHA89 × *H. laevigatus* and NMSHA89 × *H. pauciflorus* as compared to their reciprocals.

Characterization of hybrids

F_1 hybrid plants were closer to the wild parent than to the cultivated one in growth habit (branching and perennials) but they flowered earlier than their wild

Table 3. Effect of embryo developmental stages of interspecific crosses between NMSHA89 and three perennial *Helianthus* species, and the efficiency of embryo rescue

Cross	Embryos plated	Embryos rescued from each stage of development (%)[a]					
		O	G	EH	H	FD	EER[b]
NMSHA89 × *H. laevigatus*	50			8.0	46.0	46.0	64.0
NMSHA89 × *H. pauciflorus*	30	10.0	6.7	6.7	10.0	66.6	36.7
NMSHA89 × *H. resinosus*	49		8.2	6.1	16.3	69.4	49.0
Total	129	2.3	4.6	7.0	26.4	59.7	51.9

[a] O = preglobular, G = globular, EH = early heart, H = heart, FD = full development ot torpedo.
[b] EER: efficiency in embryo rescue: number of plants obtained/number of total embryos rescued.

Table 4. Chromosome number, pollen stainability, and percent of hybrid plants of wild *Helianthus* species and their interspecific hybrids with HA89

Pedigree	Chromosome number	Pollen stainability (%)		Hybrid plants %	Physiological type
		range	mean		
H. decapetalus	89–99	52–97	83		Perennial
H. giganteus	34	63–99	91		Perennial
H. laevigatus	102	95–99	98		Perennial
H. pauciflorus	102	95–99	97		Perennial
H. resinosus	102	94–99	98		Perennial
HA89	34	–	99		Annual
H. decapetalus × HA89, F_1	61–68	3–94	30	93.7	Perennial
H. giganteus × HA89, F_1	34	0–30	7	72.4	Perennial
H. laevigatus × HA89, F_1	68	32–72	53	92.6	Perennial
H. pauciflorus × HA89, F_1	68	15–69	42	100	Perennial
H. resinosus × HA89, F_1	68	32–58	46	48.0	Perennial

parents. Size and shape of leaves and inflorescences of hybrids were intermediate between those of parents. Chromosome number and pollen stainability are shown in Table 4. Hybrid status for crosses between polyploid perennials and HA89 was verified by chromosome numbers in root-tip cells. All plants were hybrids for the cross *H. pauciflorus* × HA89. Among all the putative F_1 plants of *H. decapetalus* × HA89, *H. giganteus* × HA89, and *H. laevigatus* × HA89, more than 70% were confirmed as hybrids, whereas less than 50% of F_1 plants could be confirmed as hybrids for *H. resinosus* × HA89. Embryos from self-pollination were all FD, except for one from EH stage in *H. decapetalus* × HA89. The F_1 hybrids did not show variability in chromosome number, except for *H. decapetalus* × HA89 which chromosome number varied from 61 to 68, in correspondence with the frequent aneuploidy (2n = 89–99) in the parent accession of *H. decapetalus*. The pollen stainability was very high for all wild species and cultivated lines studied, with the exception of some aneuploid plants of *H. decapetalus* which had a minimum value of 52% and some *H. giganteus* plants (Table 4). The pollen stainability of F_1 hybrids was lower than that of their parent plants, and varied among hybrid combinations. The pollen stainability of diploid (2n = 34) hybrid *H. giganteus* × HA89 was very low (averaging 7%). In contrast, the three tetraploid (2n = 68) F_1 hybrids, resulting from crosses between wild hexaploid *Helianthus* spp. and cultivated sunflower had an average pollen stainability between 42% and 53%. Pollen stainability averaged 30% in the hybrid *H. decapetalus* × HA89,

although variability between plants was very high and one plant reached 94% (Table 4).

Discussion

Espinasse et al. (1985) defined the developmental stages of sunflower intraspecific embryos according to size and shape rather than age, since embryos of the same age could differ in size and shape depending on environmental conditions after crossing. Our results show that the proportion of the different-shaped embryos of interspecific hybrids was species dependent, age not being a reliable character to define embryo developmental stage because, for the same age, this stage varied with the species under study. The variation of the F_1 embryo developmental stage at the excision time indicated interspecific variability for crosses with cultivated lines. A variation of *in vitro* growth requirements of different species has been emphasized for interspecific crosses within legume species (Phillips & Collins, 1979; Stafford & Davis, 1979).

Similar to results of Espinasse et al. (1985), *in vitro* behaviour of interspecific hybrids and viability of sunflower plantlets were also influenced by size and developmental stage of the embryo within the same species. We observed that underdeveloped embryos had a much lower survival rate than embryos rescued at heart or fully developed stages (Figure 2A and B). In spite of that, some interspecific embryos cultured *in vitro* were able to develop fully through all the stages of embryogenesis and formed mature plants not only from the well formed embryos but also from early globular and globular embryos, which contrasts with results reported by Espinasse et al. (1985). Genetic differences in the ability of different hybrids of cultivated × perennial wild species to progress towards seed production was suggested as well as the need of embryo culture to secure hybrids. Raghavan (1976) reported a low rate of survival for very immature embryos, and indicated that culturing globular to heart shaped embryos was difficult as they may have more complex nutritional and physiological requirements. Monnier (1976), working with interspecific hybrids of a crucifer plant, suggested the culture of ovules instead of very small embryos to avoid dissecting damages. Our results support his observations, since early-globular-stage embryos of sunflower cultured in ovules showed a slightly higher rate of plantlet formation as compared to that of dissected globular embryos.

Chandler & Beard (1983) developed a two-step procedure for culturing immature interspecific *Helianthus* embryos, and successfully produced 53 interspecific hybrids, of which 21 had not been reported previously. However, the number of hybrid plants produced was low and further modifications were proposed by different authors (Jan, 1988; Denat et al., 1991; Espinasse et al., 1991). Interspecific hybrids of *H. annuus* with *H. decapetalus*, *H. resinosus* and *H. pauciflorus* were produced using embryo culture by Georgieva-Todorova (1984), Jan (1988), and Kräuter et al. (1991). Iuoras (1987) obtained four (3.9%) and two (1.2%) plants from 101 and 169 cultured embryos, respectively, of *H. pauciflorus* × HA89, whereas our recovery rate for this species and the other species crossed was much higher. The lowest recovery rate was observed in *H. decapetalus* × HA89. This species was expected to have $2n = 6x = 102$, but almost all our plants were found to be aneuploids with $2n = 89$ to 99. Our results indicated either that fertilization in this species did not occur or that most of the hybrid embryos aborted within five days after crossing. A total of 23 hybrid plants were obtained but 30% of them died before reaching maturity (Table 2). Espinasse et al. (1991) concluded that recovery of interspecific embryos was strongly dependent on the genotype of the cultivated sunflower female parent used. Kräuter et al. (1991) successfully cultured immature embryos ranging from globular to full development stages, obtaining plants from 33 sunflower interspecific hybrid combinations. They regenerated 481 (41%) of the 1178 embryos cultured, but this could be slightly reduced if they followed their regenerates through maturity. Their success could be due in part to the use of cultivated line as female in 21 of the 33 interspecific crosses. In our study, the efficiency of embryo rescue, EER, of interspecific hybrids varied from 3.8% for *H. giganteus* × HA89 to 34.9% for *H. resinosus* × HA89 when the cultivated line was used as male parent. Pooling data from all crosses led to a regeneration rate of 14.6% when wild species were used as females, whereas it averaged 51.9% for the reciprocal crosses (Tables 2 and 3). These values are comparable to those reported by Kräuter et al. (1991). The size of the embryos appears to be much influenced by the maternal parent, the larger embryos on cultivated lines developing better than their reciprocal crosses, which resulted in higher culturing success. The reciprocal differences within the same cross combination was observed not only in the number of hybrid plants obtained but also in the percentage of rescued embryos

for each stage of development. In general, when wild species were used as female, the percentage of embryos in early-developmental-stages was higher than that in the reciprocal crosses, probably because of faster embryo development when cultivated lines were used as female. Feng et al. (1996) observed similar results with interspecific hybrids of *Arachis* spp. and concluded that this might be due to differences among species in storage and utilization of nutrients.

Similarly to our results with hexaploid *H. laevigatus*, *H. pauciflorus* and *H. resinosus*, interspecific hybrids between cultivated sunflower and the perennial species *H. resinosus*, *H. rigidus*, and *H. decapetalus* were also obtained by conventional methods by Georgieva-Todorova (1984) and Atlagic (1996). The use of embryo culture technique is not critical for interspecific crosses in these species but it could accelerate the hybrid plant production and avoid seed dormancy problems when using F_1 seeds. A very limited number of hybrid plants of the cross *H. giganteus* × cultivated *H. annuus* was produced before (Chandler & Beard, 1983; Christov, 1991; Jan, 1997). In our study, the recovery rate for this species was ten times higher than previously reported, but we did not obtain any hybrid plant when embryo culture was not used, indicating the need of embryo culture for the very difficult interspecific hybrids. Our results indicated that the most difficult step to recover interspecific embryos of *H. giganteus* was the survival in the growth medium, since this species had similar recovery rates in the germination medium than some of the hexaploid species.

In agreement with our results (Table 4), a reduction of pollen stainability in F_1 interspecific hybrids with regard to their wild parents was previously reported (Heiser et al., 1962; Georgieva-Todorova, 1984; Jan, 1997). This might be explained by differences in the number and structure of chromosomes in different species (Chandler et al., 1986). Since four of the five species in this study were hexaploid and cultivated sunflower is diploid, chromosome counts easily determined whether the plants were hybrids. For the hybrids between the diploid *H. giganteus* and cultivated sunflower, leaf morphology and pollen stainability were critical for hybrid verification. Pollen stainability of hexaploid *H. decapetalus* was lower than in the other wild species. The low fertility that we found in hybrids with *H. decapetalus* could result from the small sample size used and their aneuploidy, although high tolerance of sunflower to aneuploidy was observed by Jan et al. (1988). In our study, plants with 2n = 99–100 were able to produce interspecific embryos and reached maturity, whereas those with 2n = 89 to 97 produced a small number of embryos that died at very early stages of development. This indicates that it is important not only to use a larger sample size for interspecific crosses, but also to check the number of chromosomes in the wild parents, especially when they are polyploid.

Our preliminary results indicated that the use of combinations of embryo culture and amphiploid production were effective in overcoming cross incompatibility and facilitated interspecific transfer of genes for resistance to *O. cernua* from perennial species into diploid cultivated background (Sukno et al., 1998). Although a number of successful hybridizations involving embryo culture have been reported in sunflower (Kräuter et al., 1991), to our knowledge, detailed studies relating embryo developmental stage at the time of transferring them to growing medium and survival of interspecific hybrids *in vitro* have not been published. Differences in frequency of each stage of development obtained by reciprocal crosses is also reported for the first time. The viability of the interspecific hybrid embryos between wild and cultivated sunflower seems to be related to the embryo developmental stages. We have shown that embryo shape in interspecific hybrids was related to the species. Since some species produce a high rate of globular stage embryos, in order to extend the spectrum of successful interspecific hybridization to the many sources that still remain to be utilized, it will be necessary to adjust the optimal day after pollination for excision, or to culture smaller and less differentiated hybrid embryos. This will require modifications in the culture media and manipulation procedures. Based on the efficiency of regeneration rate in reciprocal crosses of each species, we will be able to decide if the greater efforts required to obtain these crosses are justified by the results obtained.

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