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Intergeneric hybidization of sunflower (*Helianthus annuus* L.) with spiny plumeless thistle (*Carduus acanthoides* L.)

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Abstract: *Carduus acanthoides* L (spiny plumeless thistle) a biannual wild species with $2n = 22$ chromosomes was crossed with *Helianthus annuus* L. When crossing, pollen from *C. acanthoides* germinated and pollinated the stigmas of sunflower lines HA 89A after 48 h. The crossability rate was low, but seeds and hybrid plants were obtained. The F_1 plants strongly resembled the cultivated sunflower with the most important bio-morphological characters, even though they had an intermediate type of heritability. The hybrid nature was confirmed by RAPD markers. The polymorphism between *H. annuus*, *C. acanthoides*, and their F_1 hybrids was studied using RAPD. The result showed introgression of *C. acanthoides* in the hybrid progeny. It was established that the wild species carried *Rf* genes for the CMS PET-1. After self-pollination and sib-pollination of the F_1 plants and back-crossing with cultivated sunflower, F_2, BC_1 and next generation hybrid progenies were obtained. The investigation encompassed the period 2000–2007 and 2014–2018. Some of the new lines have been included in a heterosis breeding program for developing hybrids for the sunflower market.

Keywords: *Carduus acanthoides*; intergeneric hybridization; morphological characteristic; RAPD; sunflower.

Introduction

The relationships of the genera *Helianthus* and *Viguiera* was discussed by Blake (1918). Chromosome numbers have been reported for four of the 11 species of Tithonia, all $n = 17$, the same as that of diploid species of *Helianthus*. In *Viguiera* there were eight species reported with $n = 17$, two with $n = 34$, two with $n = 8$, and one each with $n = 12$ and $n = 18$. A limited number of intergeneric hybridizations has been attempted between various species of both *Tithonia* and *Viguiera* with

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Helianthus. Probably the progenitors of *Helianthus* were with chromosomal numbers $n = 9$ and $n = 8$ (Heiser 1963, Heiser et al. 1969). Globally, some successful intergeneric crosses with cultivated sunflower have been made. Morozov (1947) was the first, who crossed plants of *Helianthus annuus* L. with *Carthamus tinctorius* L. and *Onopordon acanthium* L. In the first and second hybrid generations, the plants were similar to the maternal parent cultivated sunflower. The author suggested that it was a case of "absorbed" paternal inheritance. Christov and Panajotov (1991) reported the first intergeneric hybrid, obtained between *H. annuus* and *Tithonia rotundifolia*. Successful intergeneric crosses with different species from the *Compositae* family were made by Christov et al. (1994), Vassilevska-Ivanova et al. (1996), Atlagic et al. (1997), Christov (1998, 2013); Vassilevska-Ivanova et al. (1999), Vassilevska-Ivanova and Tcekova (2002), Encheva and Christov (2005), Encheva et al. (2003, 2005), Vassilevska-Ivanova (2005), Reyes-Valdés et al. (2005), Luévanos–Escareño et al. (2010), and Gómez-Martínez and Reyes-Valdés (2016). Christov and Vassilevska-Ivanova (1999) obtained hybrid plants from the cross of cultivated sunflower with *Carduus acanthoides*. The combinations were lines HA 89A (with CMS ARG-1) × *C. acanthoides* and line 3004 (with CMS ARG-3) × *C. acanthoides*. Hristova-Cherbadzhi (2004) analyzed the condition and the functional activity of the photosynthetic apparatus by determining the quantity of chlorophyll fluorescence and pigments content of *H. annuus*, *C. acanthoides* and their F1 hybrid, at the spot where the breach of the photosynthetic apparatus was awaited. The degree of chlorophyll fluorescence was measured in the field at two different temperatures. It was noticed that the results of the different parameters were similar. This showed identity in the response of the studied accessions, regardless of the fact that they were representatives of different genera.

Intergeneric hybridization contributes to the establishment of the relative's intergenerational relationships, for the genetic diversity and for the naturally increased variability, using the potential of the genera from family *Compositae*. The experiment for intergeneric hybridization of cultivated sunflower with *C. acanthoides* aims to find other options that could be an alternative to improve modern sunflower.

Materials and methods

C. acanthoides is an herbaceous biennial prickly plant ($2n = 22$). The species is of European origin and found in all regions of Bulgaria as part of the wild vegetation in dry and rocky places to 1300 m elevation.

The investigation encompassed the period 2000–2007 and 2014–2018. It included the cultivated sunflower *H. annuus* and the herbaceous biennial species *C. acanthoides*. *H. annuus* was represented by two lines 2607A and HA89A ($2n = 34$) with CMS PET-1.

Hybrid plants were grown and hybridized under field conditions. Regular phenological observations were made during the vegetative period. Biometric measurements and description of the main morphologic characters and biologic peculiarities of the F_1 hybrids were recorded. Similar investigations were carried out with the next generations as well. To obtain F_2 and BC_1 plants, self-pollination, sib-pollination, and back-crossing of F_1 to cultivated sunflower were necessary.

The presence of fertility restorer genes for CMS PET-1 was observed. The female fertility of the plants was determined by the amount of seeds obtained after open pollination, and the weight of 1000 seeds measured in three samples, each of 50 seeds. The disease resistance of the hybrids and the seed oil content were studied. Testing for resistance to *Plasmopara helianthi* was made according to standard methods (Vear and Tourvieille 1987), while resistance to *Sclerotinia sclerotiorum* and *Phomopsis helianthi* was made according to an adapted method of Encheva and Kiryakov (2002). Pollen viability was determined by a standard method (Owczarzak 1952). The germination of pollen grains on stigma of female parent plant was observed with the aid of a fluorescence microscope (Kho and Bäer 1968). The stigmas of *H. annuus*, pollinated with pollen of *C. acanthoides* were fixed by 24 and 48 h. Electrophoresis of seed storage proteins of F_1 hybrids from the crosses *H. annuus* x *C. acanthoides* and its parents was made using a 12.5% SDS-PAGE gel, pH 8.8 (Laemmli 1970) with the addition of five M urea (Vladova et al. 1989). Proteins were stained with Coomassie Brilliant Blue R-250. RAPD analysis was performed in order to determine the hybrid nature of the new F_1 sunflower hybrids. The total DNA was isolated from the youngest sunflower leaves by a modified method of Dellaporta et al. 1983. Kits for PCR analyses (Ready To Go PCR Beads, Amersham Pharmacia Biotech Inc.) and for amplification of random DNA sequence, RAPD decamer primers from Operon Technologies, USA, OPA-01, OPA-02, OPB-01 and OPB-07 were used. Amplified products were separated by electrophoresis and visualized on 2% agarose gel. The PCR programing was: 5 min at the temperature of 95 °C; 45 cycles of 1 min at 95 °C, 1 min at 36 °C and 2 min at 72 °C; 5 min at 72 °C. DNA marker 50 bp (Amersham Biosciences, USA) was used. The oil content of the seeds was quantitatively determined by using a nucleus-magnetic resonance (Newport Instruments Ltd 1972).

Results and discussion

Morphological characterization of *Carduus acanthoidesis* L. ($2n = 22$)

C. acanthoidesis is cross-pollinated. The plant (MB-3) used in the study had the following characteristics. In the first year of development, rosettes were formed (Figure 1B). The stem of the adult plant (second year) was an erect, prickly, with branches mainly in the upper half (Figure 1A). The leaf petiole was lanceolate and deep-pinnate notched and triangular, each with 3–5 lobes, leading to the sharp and hard spines (Figure 1C). Inflorescences were ovate, single or assembled in

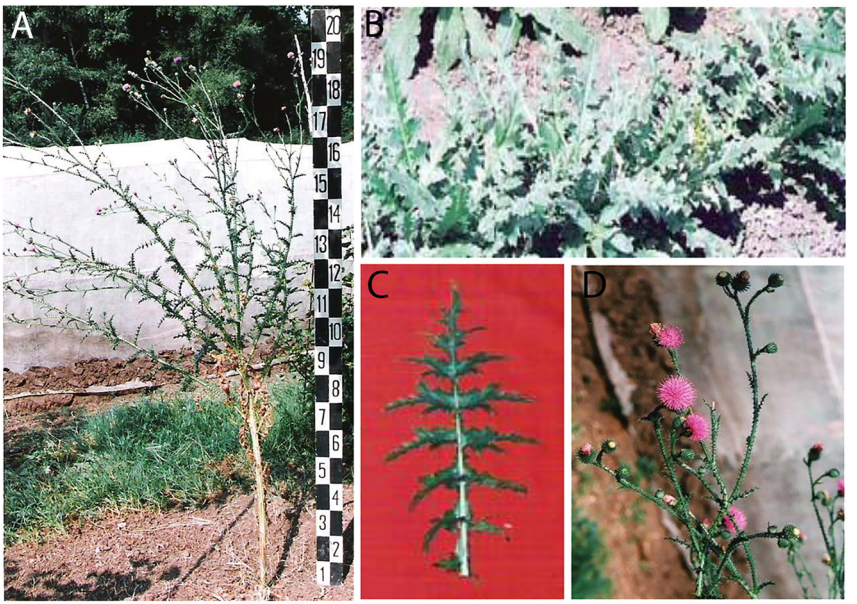

Figure 1: *C. acanthoides*: A. Plant; B. Rosette; C. Leaf; D. Inflorescences.

groups, arranged on branches of different lengths (Figure 1D). The bracts ended in spines and enclosed many filamentous red-purple disk flowers.

The seeds were small, flattened, and with a winglet like a kite. They were light gray in color.

The oil content in the seed of *C. acanthoidesis* ranged from 36.9 to 39.8%. The seeds with highest oil content had the following fatty acid composition: linoleic acid (65.7%); oleic acid (21.2%); stearic acid (2.5%) and palmitic acid (10.6%). As Christov et al. (2004) said in his study plants from *C. acanthoides* can have a resistance/tolerance to pathogens of Phomopsis, downy mildew, and Sclerotinia.

"*Germination of pollen grains from* C. acanthoides *on stigmas of cultivated sunflower*"

Normal germination of pollen grains from *C. acanthoides* on the stigmas of cultivated sunflower is necessary for successful hybridization between the two species. This appears to be completed by 48 h with stigmas from line HA 89A, regardless of the genetic distance of both species (Figure 2).

Table 1 presents results showing the successful crosses of *C. acanthoides* and *H. annuus*.

The crossability rate was low with few seeds obtained. For the *H. annuus* line HA 89A × *C. acanthoides* (MB-3) combination, one hybrid plant was obtained, and for *H. annuus* line 2607A × *C. acanthoides* (MB-3), four hybrid plants were obtained.

Figure 2: Germination of pollen grains on from *C. acanthoides* on stigmas of line HA 89A. Crossability of cultivated sunflower *H. annuus* L. with *Carduus acanthoides*.

Table 1: Crossability between *H. annuus* and *C. acanthoides*.

Crosses	Pollinated inflorescences			Total number of seeds	Hybrid plants	
	Total number	With seeds				
		Number	%		Number	%
H. annuus HA 89A × *C. acanthoides*	12	2	16.67	3	1	33.33
H. annuus 2607A × C. *acanthoides*	14	2	14.29	9	4	44.44

Characterization of the F_1 plants

F1 plants have an annual life cycle. The hybrids have an intermediate type of appearance, but with phenotype closely resembling cultivated sunflower.

The length of the vegetation period of the F1 hybrids for both crosses was similar to a mid-early form of cultivated sunflower (Table 2).

Table 2: Characteristics of F_1 hybrids.

Characteristics	*H. annuus* 2607A	F_1*	*Carduus acanthoides*	F_1**	*H. annuus* HA89A
Physiological development					
Period of vegetation (days)	118	124–131	135–150	133	114
Morphological characteristics					
Plant height (cm)	130–135	145–170	2.00	150	105–110
Number of branches (*n*)	0	11–23	9–27	8	0
Length of leaf branches (cm)	0	40–125	70–105	30–70	0
Length of leaves (cm)	24–27	17–19	12–15	21	27
Width of leaves (cm)	22–26	11–13	3–5	15	25
Length of leaf petiole (cm)	11–13	8–10	0	10	12–15
Head diameter (cm)	17–19	6–9	0.8–1.3	11	22–25
Technological characteristics					
1000 seeds weight (gm)	61.4	x	2.9	x	57.5
Oil (%)	43.0	x	36.9–39.8	x	49.8

F1* – *H. annuus* line 2607A × *C. acanthoides*; F1** – *H. annuus* line HA89A × *C. acanthoides*.

The stem of plants from both types of crosses is erect and branched. Branches exceed the height of the central stem and are tertiary. The stem color ranges from green to dark green with varying degrees of anthocyanin coloration. The leaves are green. Their shape is similar to the leaves of cultivated sunflower. The leaf margins of the plant from the combination *H. annuus* line HA89A × *C. acanthoides* are shown in Figure 3 with finely serrated rarely placed single spines, while the leaves from combination *H. annuus* line 2607A × *C. acanthoides* have serrated margins, like the cultivated sunflower.

The corolla and stigma are yellow with traces of light purple edges and the ray flowers and pollen are orange. The central head diameter is 6 to 11 cm.

All F_1 plants were male-fertile indicating that the genome of *C. acanthoides* has restorer genes (*Rf*) for CMS PET-1. Pollen viability of F_1 plants *H. annuus* × *C. acanthoides* ranged from 12.8 to 42.8%, while *C. acanthoides* was from 93.9 to 95.4%.

Christov (1992, 2000, 2001a) reported the transfer of *Rf* genes for CMS PET-1 from *T. rotundifolia* and other species from the Compositae family into the cultivated sunflower. He also developed new *R*-lines (Christov 1999, 2001b).

Figure 3: F_1 *H. annuus* – HA89A × *C. acanthoides*.

The presence of branching and anthocyanin pigmentation in the first generation of crossing of cultivated sunflower (lines HA 89A and 2607A) × *C. acanthoides*, the traits characteristic for thistle, the resulting deformed plants and the

male fertile F_1 plants in CMS PET-1 are evidence for successful hybridization and an indicator for transfer of genetic material into the genome of the hybrids from the species *C. acanthoides*.

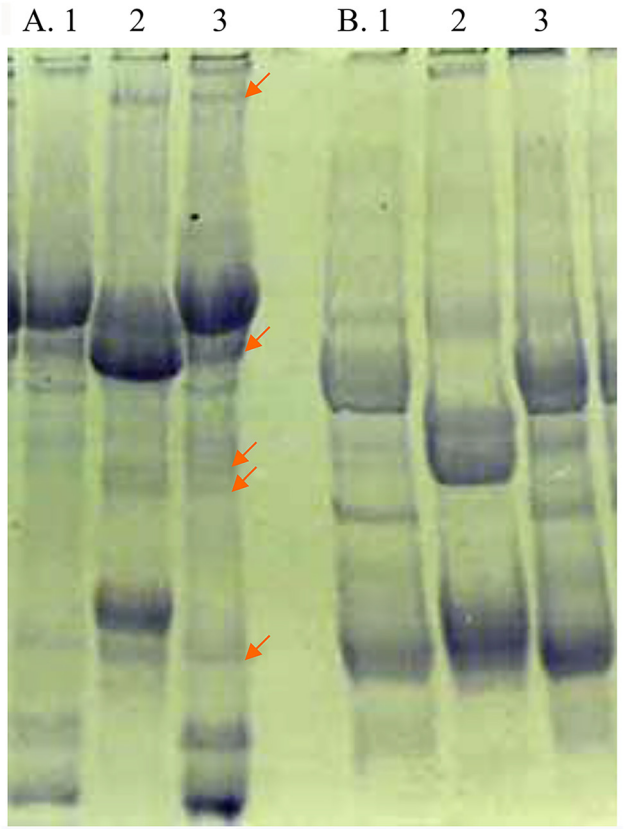

Figure 4: Electrophoregram of storage proteins from parent forms and F1 plants from cross *H. annuus* line 2607A × *C. acanthoides*. 1. *H. annuus* – l. 2607A, 2. *C. acanthoides*, 3. F_1: *H. annuus* × *C. acanthoides*.

Seed storage proteins

A study of proteins was performed on F_1 hybrids derived from hybridization of *H. annuus* line 2607A with species *C. acanthoides* and its parents.

The electrophoregram is divided into two parts. Part "A" refers to non-reducing protein fractions, and "B" to reducing fractions (Figure 4).

Fragments specific to one of the parental genotypes and fragments characteristic of both parental forms and hybrid genotypes were observed. The electrophoretic protein spectrum of non-reducing fractions differs from that of the reducing fractions. It shows that protein fractions from the initial forms are present in hybrid genotypes in varying degrees as the intensity of some fractions changed.

Existence of distinct specific fragments for *C. acanthoides* and hybrids was observed only in non-reducing protein fractions.

Characterization of the second generation

Pollen from fertile F_1 plants was put on sterile plants of lines HA 89A and 2607A with CMS PET-1. Fifty-two seeds from four inflorescences were obtained and sown. The total number of BC_1 plants was 47, 25 were fertile, and 22 male-sterile or about a 1:1 ratio. There was diversity of the degree of anthocyanin coloration in the stems, branches, and leaf petioles. All BC_1 plants were branched. The leaves were large, almost identical with serrated margins. The head diameter was 13–18 cm. Eleven plants were selected and self-pollinated to obtain the next generation. Seed set was from 2.15 to 4.83 % with the number of seeds varying from 30 to 67. The seeds did not shatter from the inflorescence before full maturity. They were light gray, gray-black and black in color. The oil content in the seeds was 42.4–43.6%.

All F1 plants were self-pollinated. Seeds were obtained from all 23 F_2 plants with more than four seeds from one inflorescence. The seeds were gray-blue to gray-black in color. All 23 F_2 plants were branched. The shape of the leaves of 19 plants was prolonged, heart-shaped, with the remaining four oval-elongated. The margins of leaves were serrated. In seven plants the leaves had a glossy sheen. In three plants, the top-leaves and inflorescences were underdeveloped. There was different degree of anthocyanin coloration on stems, branches and leaf petioles. Pollen and ray flowers were orange. The head diameter was 10–15 cm. The seeds were of different sizes and gray-white, gray-blue and gray-black in color. The oil content in the seeds ranged from 40.0 to 41.9%.

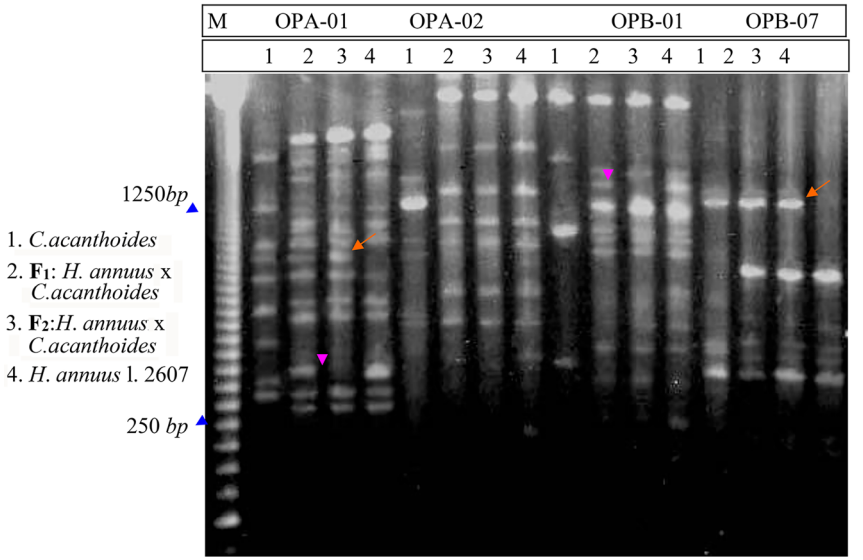

Figure 5: Electrophoregram of amplification profiles of the three genotypes with OPA-01, OPA-02, OPB-01 and OPB-07 primers. 1. *C. acanthoides*, 2. F_1: *H. annuus* × *C. acanthoides*, 3. F_2: *H. annuus* × *C. acanthoides*, 4. *H. annuus* l. 2607.

DNA

RAPD analysis of the parent forms, line 2607A, *C. acanthoides* and their F_1 and F_2 generations from the combination *H. annuus* × *C. acanthoides* was carried out. Figure 5 of the PCR profiles showed the results when four primers were utilized. Polymorphic bands for the two parents were observed.

The comparison of the amplification profiles was based on the presence or absence of the fragments in the spectrum of the F_1 material, typical of the male genotype.

Primer OPA-01 allowed the amplification of a specific fragment with size 1050 bp common for the genotypes of *C. acanthoides* and the hybrids from the two generations, and the primer OPB-07 amplified a specific fragment with a size over 1300 bp. Primers OPA-02 and OPB-01 did not show specific fragments for *C. acanthoides* and the hybrids from the two generations.

The results reveal that there is polymorphism in the amplification PCR profiles of the parental forms *H. annuus* and *C. acanthoides*. Introgression was established from the species *C. acanthoides* into F_1 *H. annuus* × *C. acanthoides*. The RAPD analysis data confirmed the hybrid nature of the first generation.

Characterization of the next generations

All next generations were produced by self-pollination. The next hybrid generation showed a continued segregation in the characteristics of vegetation period, anthocyanin coloration, plant height, branching, size and shape of leaves and others.

All F_3 plants were branched. The shape of the leaves was elongated-triangular (Figure 6A). Two of the 61 total plants were male sterile. Two plants, originating from a combination *H. annuus* line 2607A × *C. acanthoides*, were artificially inoculated to test for resistance to Sclerotinia. They overcame the infection and were rated highly resistant. The head diameter was 13–16 cm. The seeds were gray-blue, gray-black and black. The oil content in the seeds ranged from 42.6 to 44.2 %.

The first unbranched plant was obtained in the F_1BC_1 generation. The leaf shape was triangular to heart-shaped and elongated-triangular. From a total of 77 plants, 67 were fertile and 10 male sterile. The central head diameter of the branched plants was from 17 to 22 cm, and 24 cm for unbranched plant. The seeds were gray-blue, gray-black and black. The oil content in the seeds was from 42.8 to 45.5%.

Plant height of F_4 plants ranged from 110 to 140 cm. The head diameter was 15–16 cm. All plants were without anthocyan in coloration and had gray-blue, gray-black and black seeds. The seeds did not shatter from the inflorescence before full

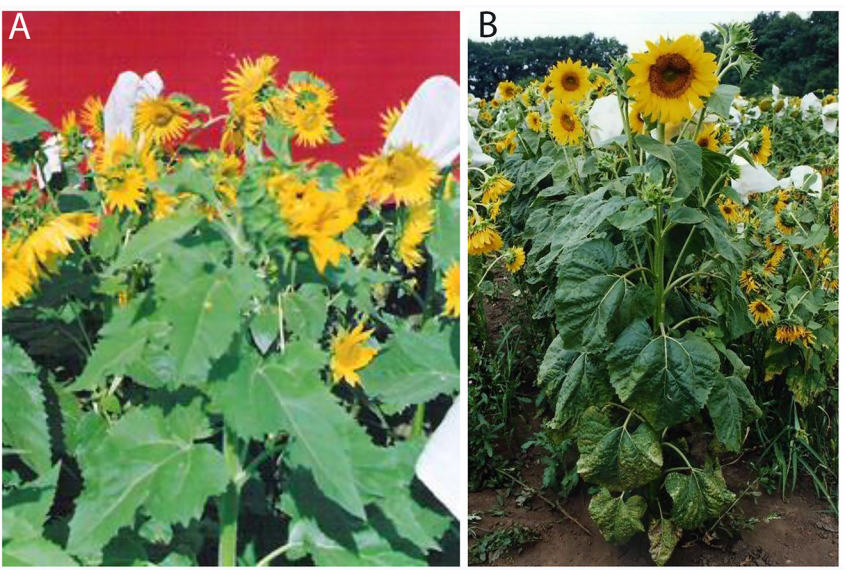

Figure 6: A. F_3 *H. annuus* × *C. acanthoides*, and B. F_7: №2793 (*H. annuus* × *C. acanthoides*).

maturity. Plants of the two F_4 accessions were also rated highly resistant to Sclerotinia.

The observed F_2BC_1 plants were mostly branched, only four were unbranched. Plant height was 130–160 cm. The central head diameter of branched plants ranged from 15 to 17 cm, and the size for unbranched plants was from 19 to 24 cm. All plants were without anthocyanin coloration and had gray-white, gray-blue, gray-black and black seeds.

Two groups of plants were differentiated in the fourth generation, branched and unbranched. Breeding in the group of unbranched plants aimed to maintain the dense pubescent hairs on the stem, the leaves from the top portion of the stem and the back of the inflorescence. This type of plants is suitable for growing in areas with dry soils in arid regions. Breeding in the branched plant group focused on the creation of plants which are normally developed and equally branched, have similar shaped leaves, and equal-sized inflorescences with relatively high oil content in their seeds. In the fifth, sixth, and seventh generations, the plants appeared identical. The plants showed a specific green color of the whole plant, the stem shape and location of branches, leaves and inflorescences. Seeds in accessions were gray-white to gray-blue and gray-black in color. The seeds from unbranched plants were densely covered with soft white hairs.

The hybridization between *H. annuus* and *C. acanthoides* has led to selection of plants in different generations that show potential valuable for breeding sunflower forms. Forms with full resistance to economically important diseases such as downy mildew, Sclerotinia, and Phomopsis as well as with relatively high seed oil content of 45.2–49.3% were obtained. In all branched and unbranched plants the *Rf* genes was transferred. This makes them suitable for creating the *R* lines (Figure 6B) that can be used in the development of new sunflower hybrids.

Characterization of the new lines

After a six-year lapse, an attempt was made to recover the latest generation of materials. Thus, in 2014, single plants of three F_7 generation (out of eight), and three in BC_1F_5 (out of six) were successfully grown. All plants from the F_7 generation were branched, and two BC_1F_5 were unbranched. The seeds did not shatter from the inflorescence before full maturity. The seeds were gray-white to gray-blue, gray-black and black.

As a result of the selection in the four years of self-pollination, branched and unbranched plants were obtained which were resistant to the diseases downy mildew, Sclerotinia, Phomopsis, and had relatively high seed oil content. They are

Figure 7: F_1 *H. annuus* decorative forms × *C. acanthoides*.

suitable as starting material for developing parental *R* lines, for new sunflower hybrids.

The branched form obtained from the *H. annuus* HA 89A × *C. acanthoides* combination with the highest oil content 48.2% was line Sc 55. This line is characterized by complete resistance – full (100%) to downy mildew and Phomopsis and highly resistantce to Sclerotinia.

The unbranched form obtained from the *H. annuus* 2607A × /*H. annuus* HA 89A × *C. acanthoides* (MB-3) was named line 2667 and had the highest seed oil content of 47.5%. This line is resistant to downy mildew and Phomopsis.

A cross between sterile decorative forms and *C. acanthoides* was also made. F_1 plants were obtained that were fertile (see Figure 7).

Conclusions

Intergeneric hybridization of the cultivated sunflower *H. annuus* with *C. acanthoides* was established, but the degree of crossability was low. A small number of seeds and hybrid plants was obtained. These results indicate that there is a degree of embryonic and post embryonic incompatibility. All F_1 and next generation plants were annual. Inheritance in the first generation had an intermediate phenotype, with more pronounced characteristics of the cultivated sunflower. *C. acanthoides* was a source of *Rf* genes for CMS PET1 with genes that control resistance to economically important diseases such as downy mildew, Sclerotinia and Phomopsis and genes that control the morphological characteristics such as type of branching, density of pubescence on the stems, leaves, and inflorescence.

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