Characterization of hybrids from crosses between cultivated *Helianthus annuus* L. and subspecies *rydbergii* (Britton) Long of perennial diploid *Helianthus nuttallii*

Miroslava M. Hristova-Cherbadzi, Michail Christov
Dobroudja Agricultural Institute, General Toshevo 9520, Bulgaria,
E-mail: mirahristova@yahoo.com; michailhristov@yahoo.com

ABSTRACT

The subspecies *rydbergii* (Britton) Long of the perennial diploid species *Helianthus nuttallii* was included in hybridization with the cultivated sunflower *Helianthus annuus* L. The investigation encompassed the period 1999-2007. *H. nuttallii* ssp. *rydbergii* could be crossed with the cultivated sunflower, but hybridization was difficult and the crossability rate was low. Seeds were obtained at both directions of crossing and hybrid plants - from the direct crosses. All F_1 plants showed an annual growth cycle. The heritability in first generation was intermediate but the plants strongly resembled the wild species in their most important biomorphological traits. The polymorphism between *H. annuus*, *H. nuttallii* spp. *rydbergii* and their F_1 hybrids was studied by RAPD. The F_1 plants were also cytologically investigated. From *H. nuttallii* ssp. *rydbergii* were transferred in F_1 genes that controlled such characters as, period of vegetation, plant height, type of branching, size and form of inflorescence and seeds, degree of anthocyanin coloration, seed oil content (60.84%), resistance to *Plasmopara helianthi*, races 300 and 700, *Phomopsis helianthi*, *Phoma macdonaldii* and *Orobanche cumana*. It was established that the subspecies was a source of *Rf* gene for CMS Pet-1 and the control was dominant and monogenic. As a result of self-pollination, sib-pollination of the F_1 plants and back-crossing with cultivated sunflower, F_2, BC_1 were obtained. F_3 - F_8 and next to F_6BC_1 generations were produced. Some of the obtained hybrid forms were included in a program for developing lines for heterosis breeding in sunflower. The hybrids with the perennial *H. nuttallii* ssp. *rydbergii* constitute a real contribution to interspecific hybridization because successful hybridization with this wild species had not yet been reported.

Key words: *Helianthus nuttallii* spp. *rydbergii* – interspecific hybridization – RAPD – sunflower.

INTRODUCTION

H. nuttallii Torrey and Gray. is a perennial diploid (2n = 34) species, belonging to section *Divaricati*, series *Corona Solis* (Schilling and Heiser, 1981). It has three subspecies: *H. nuttallii* ssp. *nuttallii* Torrey and Gray, *H. nuttallii* ssp. *parishii* (Gray) Heiser and *H. nuttallii* ssp. *rydbergii* (Britton) Long (Rogers et al., 1982). The hybridization of cultivated sunflower with diploid perennial species in comparison to diploid annual species *Helianthus* is difficult (Christov, 1991, 1996; Seiler and Rieseberg, 1997). No information regarding the obtaining of successful interspecific hybridization between *H. nuttallii* ssp. *rydbergii* and the cultivated sunflower had hitherto been found.

MATERIALS AND METHODS

The investigation encompassed the period 1999-2007. The subspecies *rydbergii* (GT-M-173) of the perennial species *H. nuttallii* was included in hybridization with cultivated sunflower *H. annuus* L. Hybridization was carried out through reciprocal crosses realized under field conditions. The sterile analogues of lines 2607, 6075, HA89 and HA402 (cytoplasmic male sterile lines in CMS PET-1) were used as female parent of the cultivated sunflower in direct crosses. In the reciprocal crosses, the florets in the inflorescences of the wild species were castrated manually and were pollinated with pollen from line 2607B. To obtain F_2 and BC_1, self-pollination, sib-pollination and back-crossing of F_1 to cultivated sunflower were made. Phenological observations of the F_1 hybrids and the next hybrid generations were conducted during the vegetation period. Biometric parameters and description of the main morphologic characters and biologic peculiarities of all F_1 hybrids were performed. The seed set (the number of inseminated disk florets) was calculated as a ratio between the seeds obtained and total number of disk florets in the inflorescence. 1000 seed weight was calculated by measuring two samples, each of 10, 25 or 50 seeds. Back-crossing with cultivated sunflower as a mother was used with the aim to confirm the presence of fertility restorer genes (*Rf* genes) in F_1 hybrids transferred from species *H. nuttallii* ssp. *rydbergii*. The reactions to diseases were studied using standard methodologies (Acimovič, 1979; Vear and Tourvieille, 1987; Encheva and Kiryakov, 2002). The seed oil content was determined by nuclear

magnetic resonance (NMR). Cytological analyses were carried out on the meiosis of pollen mother cells (PMC) according to Georgieva-Todorova (1976). Pollen viability was determined by a standard methodology (Atlagic, 1990). RAPD analyses were performed in order to determine the hybrid nature of the new F_1 forms. Total DNA was isolated from the youngest sunflower leaves by the method of Dellaporta et al. (1983) with some modifications. 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. PCR program was: 5 min. in temperature 95^0C; 45 cycles of 1 min in 95^0C, 1 min in 36^0C and 2 min in 72^0C; 5 min in 72^0C. DNA marker 50 *bp* Amersham Biosciences, USA was used.

RESULTS

The analysis of the results presented in Table 1 showed that the *H. nuttallii* ssp. *rydbergii* could be successfully crossed with *H. annuus*. The percentage of successful crosses (inseminated heads) of the direct crosses was from 72.7 to 80.0 % and of the reciprocal crosses - 58.3 %. In the combination *H. annuus* x *H. nuttallii* ssp. *rydbergii* 15 seeds and 4 F_1 hybrids and in the combination *H. nuttallii* ssp. *rydbergii* x *H. annuus* 28 seeds and any hybrid plant were obtained.

Table 1. Crossability of cultivated sunflower *H. annuus* and wild perennial *H. nuttallii* ssp. *rydbergii*.

Crosses	Pollinated inflorescences			Insemination inflorescence, %	Total number seeds	Hybrid plants	
	total number	with seed					
		number	%			number	%
H. annuus x *H. nuttallii*	24	18	75.00	0.47	113	36	32.99
H. nuttallii x *H. annuus*	12	7	58.33	3.31	28	0	0

The F_1 hybrid (Fig. 1a) had an annual growth habit and a vegetation period similar to that of cultivated sunflower, in contrast to the wild perennials (Fig. 1b, Table 2). All F_1 plants had anthocyanin coloration, especially at cotyledon phase and along the stem. The intensity was similar to that of wild species. In cultivated sunflower anthocyanin coloration was absent. Its presence in hybrids proved the transfer of genetic material from *H. nuttallii* ssp. *rydbergii* to the genotype of the F_1 hybrids and was a suitable morphological marker.

a.

b.

c.

Fig. 1. Plants of: a) *H. nuttallii* ssp. *rydbergii* GT M 173 /№ 18; b) F_1 hybrid: *H. annuus* - HA89A x *H. nuttallii*; c) hybrids №1073 from cross 2607B x F_1 (HA 89A x *H. nuttallii*).

RAPD analyses of F_1 plants and their parents were performed to prove their hybrid nature. The analysis was carried out on those fragments that were well visible (Fig. 2). Out of 4 primers used for amplification of the parents and the hybrid, all showed specific fragments for the wild or cultivated species and the hybrid and fragments represented in the three genotypes. The comparison of the amplification profiles of F_1 plants and the father genotype was based on the presence or absence of the fragments. Primer OPA-01 allowed to amplify two specific fragments for the wild species and the hybrid (size: 400 *bp* and around1300 *bp*), the primer OPA-02 - specific fragment with size over 1300 *bp*, the

Table 2. Characteristics of parents and F_1 hybrids.

Characters	*H. annuus* line HA89 A	F_1: *H. annuus* - HA89A x *H. nuttallii* ssp. *rydbergii*	*H. nuttallii* ssp. *rydbergii* M 173
Physiological development			
Life cycle	annual	annual	perennial
Period of vegetation, day	114	132	195
Flowering central head, day	9 - 10	6 - 8	6 - 7
Flowering full plant, day		36	59
Morphological characters			
Hypocotyl			
Anthocyanin coloration	absent	medium	medium
Stem			
Plant height, *cm*	105 - 110	90 - 150	70 - 140
Branched	absent	in top half-past	in top half-past
Position of lateral heads		below	below
Number of branches	0	6 - 12	9 - 37
Coloration	green	dark green with anthocyan	dark green with anthocyan
Intensity of hairiness	weak	medium	medium
Leaf			
Number of leaves	24 - 28	32 - 39	74 - 81
Length of leaves, *cm*	25 - 27	13 - 18	11 - 12
Width of leaves, *cm*	24 - 25	6 - 9	4 - 5
Shape	cordate	oblong-cordate	lanceolate wide to oval
Coloration	green	green	dark green
Serration of entire	medium	medium	fine
Length of leaf petiole, *cm*	12 - 15	2 - 4	1
Anthocyanin coloration	absent	medium	medium
Inflorescence			
Attitude of the central head	half-turned down	half-turned down	half-turned down
Shape of grain side	convex	convex	convex
Head diameter, *cm*	22 - 25	7 - 12	1.6 - 1.9
Number of bracts	52	16 - 19	29
Coloration	green	dark green	dark green
Number of ray flowers	36	11 - 19	21
Coloration	yellow	orange-yellow	orange-yellow
Shape	ovate	ovate	ovate
Number of disk flowers	1211 - 1465	91 - 152	86 - 97
Disk flowers color	yellow	purple	purple
Anthocyan of stigma	absent	medium	medium
Coloration of pollen	yellow	orange	orange
Seed			
Friability	absent	at full ripeness	at full ripeness
Length/width/thickness, *cm*	1.1 / 0.7 / 0.4	0.9 / 0.4 / 0.3	0.5 / 0.2 / 0.1
Size	medium	small	small
Shape	ovate wide	ovate wide	ovate
Trichomes	very weak	weak	medium
Main color	gray-black	gray-brown / black	gray-brown
Technological characters			
Seeds after self-pollination	0	3 - 6	0 - 1
Insemination:			
- self-pollination, %	0	2.48 - 4.80	0 - 1.09
- free pollination, %	71.5	0.83 - 15.70	38.5
1000 seed weight, *g*	57.6	x	5.9
Oil, %	49.8	x	31.2

Fig. 2. Electrophoresis spectra of amplification profiles of the three genotypes with OPA-01, OPA-02, OPB-01 and OPB-07 primers.

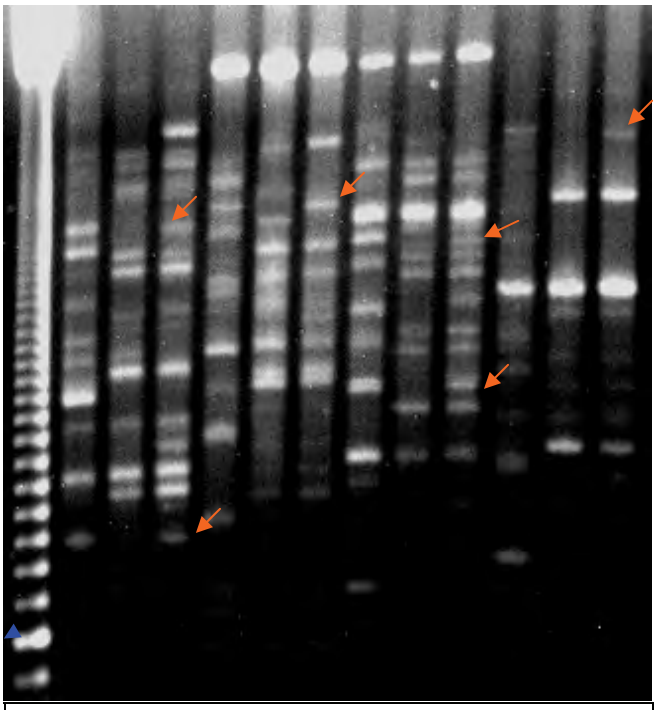
1250 *bp*

1. *H. nuttallii* ssp. *rydbergii* (M 173)

2. *H. annuus* - line 6075

3. F_1 *H. annuus* - line 6075 x *H. nuttallii* ssp. *rydbergii*

250 *bp*



primer OPB-01 – two specific fragments with size 750 *bp* and 1300 *bp* and the primer OPB-07 - one specific fragment with size over 1300 *bp*. The results confirmed that there was a polymorphism in the amplification PCR profiles of *H. annuus*, *H. nuttallii* and *H. annuus* x *H. nuttallii ssp. rydbergii*, i.e. the RAPD analysis confirmed the hybrid nature of the F_1 material obtained from crosses between the line 6075 and the wild species.

All F_1 hybrids had erect and branched stems, colored from dark green to dark anthocyanin and were covered with prickly hairs (Table 2). The branches were situated in the center and the top of the stem, with the exception of 3 out of 4 plants from cross *H. annuus* (HA89A) x *H. nuttallii* ssp. *rydbergii*, with branches and in lower half-stem (Fig. 1a). The size of the inflorescence in F_1 was intermediate and the number of disk florets - similar to those of *H. nuttallii* ssp. *rydbergii*. The disk florets and the stigmas were colored dark purple, and pollen and ray florets were orange. These characters were typical of the wild species. The number of bracts in F_1 was smaller than that in the two parents. The bracts were colored dark green like the male parent form. All F_1 plants with four different sterile analogues were male fertile. The mean percentage of pollen viability of F_1 hybrids was low (31.62%: from 26.38 to 34.96%) and three times lower than the value of *H. nuttallii* ssp. *rydbergii* (92.73%: from 87.62 to 97.32%).

The meiosis in *H. annuus* - line HA89B was normal, and in *H. nuttallii* ssp. *rydbergii* with few aberrances. In the greater part of the cells in diakinesis the chromosomes conjugated completely with 17 bivalents: open from 2 to 7 and closed from 10 to 15 were recorded. Cells with 1 to 3 “X” bivalents were also recorded. Single cells were 15-16 bivalents and 2 univalents or 1 quadrivalent. The cells in metaphase I with aberrances - lagging chromosome were 0.96%. In anaphase I 1.67% there were cells with chromosome bridges and lagging chromosome. The tetrads were observed during telophase II. Reduction division of PMC in the F_1 plants occurred with deviations. Cells with 17 bivalents, cells with 14-16 bivalents and 2-6 univalents and cells with 11-13 bivalents and 2-3 quadrivalents were recorded in diakinesis. The meiotic analyses showed that the mean frequency of closed bivalents per cell in F_1 hybrids was 1.88 and was much lower than the value for *H. annuus* (6.20) and for the wild species (12.30). The highest mean frequency of chiasmata per cell was registered for *H. nuttallii* ssp. *rydbergii* (28.80). It was lower for cultivated sunflower (23.20) and lowest (19.21) for the hybrid. The mean frequency of chiasmata per bivalent for hybrids was lower than that of the parents. The values were 1.13 for F_1, 1.36 for *H. annuus* and 1.69 for wild species. In metaphase I there were cells with 2 to 5 fast chromosomes, which were located on one or two sides of the lamella. In the F_1 hybrid there were cells with non-included chromosomes during anaphase I. The number of lagging chromosomes was up to 8. Cells with 2

chromosome bridges and fragments were recorded. Cells with non-regular distribution during telophase I were observed (19:15), and during telophase II - tetrads, tetrads with up to 6 lagging chromosomes, and tetrads with one micronucleus. It is interesting to note that the different phases of meiosis occurred simultaneously in the same preparation, in the same field of vision. This fact showed the non-synchronous occurrence of the meiosis.

As a result of self-pollination and sib-pollination of the central inflorescences of 3 F_1 plants from the cross *H. annuus* line HA 89A x *H. nuttallii* ssp. *Rydbergii*, a total of 21 seeds were obtained, and from the crosses of lines HA 402A, 6075A and 2607A with *H. nuttallii* ssp. *Rydbergii*, a total of 73 seeds after self-pollination. Seed color of F_1 was from gray-brown to black. The seed shape was similar to that of cultivated sunflower, and its size to that of wild species. This is a negative character typical of the wild sunflower species. The number of obtained F_2 plants was 81.13 F_2, all originating from cross HA 89A x *H. nuttallii*, 17 from cross 6075A x *H. nuttallii*, 32 from HA 402A x *H. nuttallii* and 19 from 2607A x *H. nuttallii*. The observed diversity among the F_2 plants was a result of the segregation of the following characters: vegetation period, plant height, type of branching, size of branches, size and shape of leaves, inflorescence and seeds, degree of anthocyanin coloration, oil content in seed (Table 3). Twenty-six out of all 81 F_2 plants were branched along the entire stem, 36 plants had branches in the middle and at the upper half of the stem, and 19 plants had branches near the top. Of all the F_2 plants, 21 were male sterile - 3 F_2 from cross HA 89A x *H. nuttallii*, 9 from HA 402A x *H. nuttallii*, 4 from 6075A x *H. nuttallii* and 5 from 2607A x *H. nuttallii*. The segregation of the characters fertility/sterility was in a correlation close to 3:1 fertile:sterile (Table 4), and in the above groups of crosses the ratio was 10:3; 23:9; 13:4 and 14:5, respectively. The inflorescence of all male fertile F_2 plants (a total of 60) was self-pollinated. Seed color was from gray-brown to anthocyanin-black.

Table 3. Characteristics of F_2, BC_1, F_3, F_1, BC_1 and F_4 hybrids.

Characters	*H. annuus* - HA 89A x *H. nuttallii* ssp. *rydbergii*				
	F_2	BC_1	F_3	F_1BC_1	F_4
Physiological development					
Period of vegetation, day	139	131	118 - 124	113 – 116	115 - 117
Morphological characteristics					
Plant height, *cm*	150 - 180	160 - 220	140 - 180	140 – 160	130 - 150
Number of branches	4 - 9	4 - 8			
Head diameter, *cm*	16	18	15 - 16	16 – 20	15 - 16
Technological characteristics					
1000 seeds weight, *g*	42.2	43.4	43.1	43.6	42.8
Oil %	39.8 - 44.5	48.2 - 52.9	46.5 - 53.0	47.1 - 50.4	49.9 - 53.6

Table 4. χ^2 for F_1, F_2 and BC_1 generations.

Generation	Number of plants		Expected ratio	χ^2*
	fertile	sterile		
F_1	36	0	- (Rfrf)	0
F_2	60	21	3:1 (Rf- : rfrf)	0.037
BC_1	81	76	1:1 (Rfrf : rfrf)	0.159

* χ^2 at level of significance 0.05, 0.01 and 0.001 was 3.841, 6.635 and 10,827.

The total number of BC_1 plants was 234 and all were branched (Table 3). Seed color of BC_1 plants was from dark brown to black. All 77 BC_1 plants from the combination 2607B x F_1 (HA 89A x *H. nuttallii*) were with normal male fertility. About 50 % of BC_1 plants were with normal cytoplasm and the recessive gene *rf* and about 50% with normal cytoplasm and the dominant gene *Rf*. B lines with important characters transferred from *H. nuttallii* ssp. *rydbergii* can be produced from the hybrids with normal cytoplasm and a recessive gene. The hybrid forms with dominant genes were suitable for developing R lines. These BC_1 plants were morphologically similar to the plants from the combination 2607A x F_1 (HA 89A x *H. nuttallii*); from the latter cross 45 fertile and 40 male sterile plants were produced. For cross combinations 2607A x F_1 (6075A x *H. nuttallii*) and 2607A x F_1 (HA 402A x *H. nuttallii*), the number of fertile and male sterile plants was 15:13 and 21:23, respectively. The ratio fertile/sterile BC_1 plants was almost 1:1 (81:76, Table 4).

All F_1 plants *H. annuus* x *H. nuttallii* with four different sterile analogues were male fertile. The presence of fertile F_1 plants indicated that in the genotype of subspecies *rydbergii* there were genes that

controlled restoration of male fertility for CMS Pet-1. Restored genes were transferred from *H. nuttallii* to the hybrid material. In wild species the *Rf* genes were probably homozygous because all F_1 plants were fertile in the formula: S *rfrf* (male sterile) x N *RfRf* (fertile) = S *Rfrf* (fertile), where in the nucleus of the plants from the sterile line the genes controlling the restoration of male fertility were only recessive - *rfrf*, and in *H. nuttallii* ssp. *rydbergii* - only dominant, *RfRf*. The F_1 progeny was fertile and heterozygous. The segregation of the characters fertility/sterility in the F_2 plants was at a ratio close to 3:1 fertile:sterile, and in the BC_1 plants - close to 1:1 (Table 4). Back-crossing with cultivated sunflower as a mother was used with the aim of confirming the presence of *Rf* genes in F_1 hybrids, transferred from species *H. nuttallii* ssp. *rydbergii*. B lines can be developed from the new forms obtained. The value of χ^2 in F_2 and BC_1 generations was lower than the level of significance 5 % (3.841). That determined the accidental nature of the differences between the observed and expected value. This result showed that *Rf* genes from *H. nuttallii* ssp. *rydbergii* were transferred in *H. annuus* and the control for recovery of male fertility at CMS PET-1 was dominant and monogenic.

F_3 - F_8 and next to F_6BC_1 generations were produced. As a result of the selection, new forms suitable for R and B lines were developed. Genes were transferred from *H. nuttallii* ssp. *rydbergii* that controlled such characters as: 100 % resistance to *Plasmopara helianthi*, races 300 and 700 (№ 990, №2505, №2558 and №2559), *Phomopsis helianthi* (№990, №1073, №2497, №2505 and №2558), *Phoma macdonaldii* (№2505) and *Orobanche cumana* (№955, №1066 and №1073), *Rf* gene for CMS Pet-1, suitable type of branching for R lines, high oil content (as in №991 - 53.43 % and №2505 - 60.84 %) and high combining ability.

DISCUSSION

H. nuttallii ssp. *rydbergii* could be crossed with the cultivated sunflower, but hybridization was difficult. The hybrids with this perennial diploid species were a real contribution to interspecific hybridization because successful hybridization with this wild species had not been reported up to now.

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REFERENCES

Acimovič, M. 1979. Evaluation procedures for the intensity of disease occurrence in sunflower. Helia 2:55-57.

Atlagic, J. 1990. Pollen fertility in some *Helianthus* species and their F_1 hybrids with the cultivated sunflower. Helia 13:47-54.

Christov, M. 1991. Possibilities and problems in the hybridization of cultivated sunflower with species of the genus *Helianthus* L. Helia 14(15):35-40.

Christov, M. 1996. Hybridization of cultivated sunflower and wild *Helianthus* species. 2:603-615. In: P.D.S. Caligari & D.J.N. Hind (Eds). *Compositae*: Biology and Utilization. Proc. International Compositae Conference, Royal Botanic Gardens, Kew, UK,

Dellaporta, S.L., J. Wood, and J.B. Hick. 1983. A plant DNA minipreparation: Version II. Plant. Mol. Biol. Rep. 1:19-21.

Encheva, V., and I. Kiryakov. 2002. Method for evaluation of sunflower resistance for *Diaporthe* (*Phomopsis*) *helianthi* Munt. Cnet. et al. Bulgarian J. Agric. Sci. 8:219-222.

Georgieva-Todorova, J. 1976. Mezduvidovi otnoshenia v roda *Helianthus Helianthus* L. Sofia, Bulgaria.

Rogers, C.E., T.E. Thompson, and G.J. Seiler. 1982. Sunflower species of the United States. National Sunflower Association, Bismark, ND.

Schilling, E.E. and C.B. Heiser. 1981. Intrageneric classification of *Helianthus* (*Compositae*). Taxon 30:393-403.

Seiler, G.J., and L.H. Rieseberg. 1997. Systematics, Origin, and Germplasm Resources of the Wild and Domesticated Sunflower. p. 21-66. In: A.A. Schneiter (ed.), Sunflower technology and production. Agronomy Monograph 35. ASA, CSSA and SSSA, Madison, WI, USA.

Vear, F., and D. Tourvieille. 1987. Test de resistance au Mildiou chez le tournesol. CETIOM, Information techniques 98:19-20.