



Evaluating an Interspecific *Helianthus annuus* × *Helianthus nuttallii* Line for Use in Sunflower Breeding Program

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ABSTRACT

Interspecific cross was made between the common sunflower inbred line HA89 and an accession of wild *Helianthus nuttallii* ($2n=2x=34$) resistant to the most harmful disease complex and tolerant to drought and high temperature. The recombinant inbred line was a BC₁F₁₅ progeny. The most remarkable feature observed was the vigorous plant habit that manifests itself in measurable morphological characters such as increment plant height, stem diameter and diameter of the head. The line was non-branched (monocephalic) without anthocyanin pigmentation and possessed good agronomic characteristics. Along with the morphological and reproductive traits, some biochemical characteristics related to antioxidant activity were associated to wide hybridization. The overall characteristics of HA-Hnutt line make it a useful plant material for research on interspecific hybridization in *Helianthus* genus.

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Introduction

During the last few years, there has been an increasing interest in the use of wild sunflower relatives in particular breeding programs which aim was providing useful materials to support practical breeding strategies. To produce potential new cultivars, interspecific and intergeneric hybridizations have been performed between common *H. annuus* and its wild relatives (Faure et al., 2002; Breton et al., 2012; Vassilevska-Ivanova et al., 2013; Vassilevska-Ivanova et al., 2014; Vassilevska-Ivanova et al., 2015; Liu et al., 2017; Seiler et al., 2017). *Helianthus* is a diverse genus comprised of 51 species with 14 annual and 37 perennial, all native to North America (Shilling, 2006). The very narrow genetic base of common sunflower has been broadened by the infusion of genes from wild species, which continue to be a valuable source of desirable agronomic traits. *Helianthus nuttallii* T. & G. ($2n=2x=34$) (Nuttal sunflower) was involved in our hybridization program since it appears to display an immune tolerance to *Sclerotinia* in USDA genetic stocks (Feng et al., 2007; Seiler et al., 2017), resistance to the most harmful disease complex (Seiler and Marek, 2011), and also it has shown to have drought tolerance caused by water deficit under field condition (Seiler et al., 2017).

In the course of our study on wide hybridization in sunflower, we obtained a number of recombinant inbred lines distinguishing from both parental species. Notably, some of these lines could be used as a pre-breeding plant material in many modern breeding programs that make substantial use of wild relatives.

Herewith, we report the results of interspecific hybridization *Helianthus annuus* × *Helianthus nuttallii*, in particular, development of a recombinant inbred line produced by conventional cross. The effect on antioxidant capacity as well as the content of the antioxidants such as ascorbate, tocopherols, phenols and flavonoids, and antioxidant enzymes was investigated.

Materials and Methods

Plant Material

The interspecific line *H. annuus* × *H. nuttallii* originated from pollination of cytoplasmic male sterile (cms) line HA89 (female), a public oilseed sunflower inbred maintainer line released by US Department of Agriculture–Agricultural Research Service, with bulked pollen from wild perennial *Helianthus nuttallii* (male). The population of *H. nuttallii* was grown from seeds

originally obtained from the experimental garden located at Bloomington (Indiana), USA. The cross was made using the conventional hybridizing method. Each experiment was carried out on flower head that had been protected from foreign pollen by bagging. First-generation hybrid plants were verified using morphological and cytological methods, and F₁ hybrids were back-crossed to common sunflower to obtain BC₁. Some BC₁ progeny revealed a vigorous phenotype which was considered as valuable for further breeding. These plants were selected for and fixed after the BC₁ to produce an interspecific line (namely HA-Hnutt). Seeds from advanced plant generations were produced after self-pollination under a bag. The plants growth conditions that were employed have already been described (Vassilevska-Ivanova and Naidenova, 2005). The line represented here is F₁₅ progeny of selfed plants; it was raised in the Experimental Field of the Institute of Plant Physiology and Genetics, Sofia, Bulgaria (located at 42°50' N, 23°00' E, 595 m above the sea level) during 2011-2016. Seeds were sown in 5 m rows spaced 0.70 cm apart with three replications. The sowings were performed in late-April. Conventional management practices were used.

Phenotypic Observations

The morphological traits were recorded of thirty random plants for hybrid line and its parent. Methods used in this investigation for hybridizing plants, fertility tests and morphological comparisons are the same as those described in previous report (Vassilevska-Ivanova and Naidenova, 2005). The following phenotype traits were recorded: plant height (cm), head diameter (cm), days to flowering (50%), days to maturity (50%), pollen staining using the acetocarmine test, thousand seeds weight (TSW) (g), and the kernel ratio (%) using the equation: Kernel ratio (%) = kernel weight (g)/grain weight (g) × 100. Morphological floral characteristics included the number of ray flowers, the length of the corolla of ray flowers (cm), and the width of the corolla of ray flowers (cm). All floral characteristics were measured at the end of anthesis.

Antioxidant Enzyme Analyses

Enzyme extracts were prepared by homogenizing plants tissue in a pre-chilled mortar in 20 ml chilled extraction buffer (pH 7.8). Extracts were then centrifuged at 12 000 g for 30 min at 5°C. Enzyme assays were conducted immediately following extraction. Superoxide dismutase (SOD) (EC 1.15.1.1) was measured by photochemical method described by Giannopolitis and Ries (1977). Assays were carried out under illumination. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of p-nitro blue tetrazolium chloride reduction at 560 nm. Catalase (CAT) (EC 1.11.1.6) activity was assayed in a method following Beers and Sizer (1952) with minor modifications. Activity was determined by following decomposition of H₂O₂ (extinction coefficient, 39.4 mM⁻¹ cm⁻¹) at 240 nm. The enzyme activity was expressed in catalytic units-mmol H₂O₂ mL⁻¹ min⁻¹ per mg protein. Guaiacol peroxidase (GPO) (EC 1.11.1.7) activity was determined according to Urbanek et al. (1991). The oxidation of guaiacol in the presence of H₂O₂ was

measured as the increase in absorbance recorded at 470 nm. The enzyme activity was expressed as nmol H₂O₂ mg protein⁻¹ min⁻¹ (Plewa et al. 1991). Ascorbate peroxidase (APX) (EC 1.11.1.1) activity was determined measured according to the method of Nakano and Asada (1981). The concentration of oxidized ascorbate was calculated by the decrease in absorbance at 290 nm. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 mM⁻¹ cm⁻¹), and was expressed as moles of ascorbate oxidized per milligram of protein per minute (McKersie and Leshem 1994). Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Antioxidant Capacity

The total antioxidant capacity in fresh leaves was measured from bleaching of the purple coloured methanol solution of free stable radical by the method of Tepe et al. (2006). Antioxidant capacity of the sample was calculated using the equation:

$$\% \text{ Antioxidant activity (I)} = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

Where A_{blank} is the absorbance of the control sample (containing all reagents except the test compound), and A_{sample} is the absorbance of plant extracts.

The concentration of total phenols in the extracts was measured spectrophotometrically by Folin-Ciocalteu method and calculated as caffeic acid equivalents (Pfeffer et al. 1998). The results were expressed in milligrams of caffeic acid per gram of dry weight. The total flavonoids content was measured spectrophotometrically by the method of Zhishen et al. (1999) using a standard curve with catechin as the standard. Total flavonoids content was expressed in milligrams of catechin equivalents per gram of dry weight.

Statistical analysis

Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to Fisher LSD test at the 5% level using a statistical software package (Statgraphics Plus, version 5.1 for Windows).

Results

Phenotype Characteristic and Agronomic Traits

The morphological features of both parental genotypes and the selected line (*H. annuus* × *H. nuttallii*) were assessed (Table 1). In many respects the developing hybrid line revealed intermediacy to the parental species. At maturity, the all plants definitely distinguished in comparison with its parents showing vegetative vigor and increment in the growth in height and in the head diameter (Table 1). The leaves were usually well developed, dark green, mostly alternate of *H. annuus* type with deltoid shape and entire leaf margin. As the whole, line HA-Hnutt had more above ground vegetative biomass and smaller seeds than *H. annuus*. The time to bloom (50 %) was accelerated by 10% in the hybrid plants compared with the cultivated sunflower line. The earlier flowering may be attributed to developmental changes that presumably resulted after interspecific

hybridization, suggesting that it should be possible to breed for the sunflower plants with reduced crop production time (Warner and Walworth, 2010). Furthermore, the line HA-Hnutt has a high degree of pollen stainability (about 93%) which is a measure in hybridization studies as a proxy for true pollen viability; the pollen grains are typically formed and well stained (Table 1).

In common sunflower, *H. annuus*, the number of outer flowers of the head (ray or ligulate flowers) can be considered to be a specific qualitative trait characterizing putative hybrids (Fambrini et al., 2003). In our case, the plants produced inflorescences and flowers with normal structure intermediate in size (smaller than *H. annuus* but larger than *H. nuttallii*); differences in the number of ray flowers and width of corolla of ray flowers became apparent between both parents (*H. annuus* and *H. nuttallii*) and the selected line, thus indicating that the change in the floral characteristics in hybrid plants was restricted to ray flowers (Table 1).

Antioxidant Capacity and Antioxidant Enzymes

Among the all three genotypes tested, the DPPH radical scavenging activity varied from 61.15 to 93.12% in the leaves, and from 78.22 to 93.48%, in the flowers (HA 89 line and hybrid line), respectively (Table 2). FRAP of investigated genotypes varied from 11.060 to 41.883 $\mu\text{mol Fe}^{2+}\text{g}^{-1}$ DW in the leaves (line and *H.*

annuus HA 89), and from 25.928 to 46.779 $\mu\text{mol Fe}^{2+}\text{g}^{-1}$ DW in the flowers, respectively. Both DPPH and FRAP methods together give a good estimate of the antioxidant potential of the studied plants, acting by different mechanisms. In the current study, the total antioxidant activity of leaves and flowers extracts measured by FRAP was found to be much lower than either parental samples. Alternatively, the level of antioxidants as measured by DPPH was higher in the hybrid line compared with the common sunflower line and wild parent *H. nuttallii*.

The amount of total phenolics varied slightly in plant materials and ranged from 6.647 to 8.849 mg of caffeic acid/g dry sample in the leaves, and from 5.996 to 8.144 mg of caffeic acid/g dry sample in the ray flowers, respectively (Table 2). The highest amount was found in HA-Hnutt line, and the lowest in the cultivated sunflower *H. annuus*. The flavonoid contents in the leaves and ray flowers of both parents and hybrid line are given in Table 2. In the leaves, HA-Hnutt line had higher value of 13.843 mg catechin/g dry sample and *H. annuus* had the lowest value of 11.819 mg catechin/g dry sample, respectively. The difference in the content of tocopherols is clearly expressed in the leaves of HA-Hnutt line and between parents as *H. annuus* has markedly low content (Table 2). In hybrid line, total ascorbic acid (vitamin C) concentration in the leaves as well as in the ray flowers was significantly lower than in common sunflower and wild *H. nuttallii* (Table 2).

Table 1 Agronomic characteristics of *H. annuus* HA 89, *H. nuttallii* and interspecific line *H. annuus* \times *H. nuttallii*

Characters	<i>H. nuttallii</i>	Line (hybrid)	<i>H. annuus</i> HA 89
	mean \pm SD**	mean \pm SD	mean \pm SD
Flowering			
Days to bloom (50%)	70	64	71
Self-compatibility (%)	50	40	0
Pollen staining (%)	93	93	pollenless
Plant height at flowering (cm)	245.0 \pm 3.95	185.8 \pm 1.15	153.6 \pm 0.81
lim	235-255	182-190	145-160
Number of branches	highly branched	0	0
lim			
Stem width, cm	1 \pm 0.01	4.6 \pm 0.06	3.2 \pm 0.02
lim	0.9 - 1.1	4.5 - 4.8	3-3.8
Floral morphology			
Flower colour	sunny yellow	sunny yellow	sunny yellow
Disc colour	light	light	light
Number of ray flowers	15.33 \pm 0.61	29.8 \pm 0.6	47.1 \pm 1.23
lim	16-22	27-33	42-54
Length of the corolla of the ray flowers (mm)	1.77 \pm 0.03	5.82 \pm 0.29	9.54 \pm 0.11
lim	1.7-1.9	5.4-6.4	8.9-9.1
Width of the corolla of the ray flower (mm)	0.75 \pm 0.02	1.93 \pm 0.04	2.49 \pm 0.04
lim	0.7 - 0.8	1.0 - 1.2	2.3 - 2.7
Number of leaves		21.4 \pm 0.78	31 \pm 1.2
lim		19-28	25-37
Maturity			
*Head diameter (cm)	1.23 \pm 0.02	30.9 \pm 0.55	24.2 \pm 0.36a
lim	1.2-1.3	5-7	21-26
Days to maturity (50%)	170	139	162
Post harvest			
1000 seed weight (g)	3.02 \pm 0.46a	63.6 \pm 1.07	77.4 \pm 2.38
Kernel ratio (%)	0	40.09	66.92

*-diameter of apical head, **SD=standard deviation

Table 2 Antioxidant capacity of an interspecific line *H. annuus* x *H. Nuttallii**

Parents and hybrid	FRAP ($\mu\text{mol Fe}^{2+} \text{g}^{-1} \text{DW}^{-1}$)		DPPH (%)	
	Leaves	Ray flowers	Leaves	Ray flowers
<i>H. annuus</i>	41.883a	46.779bc	61.15a	78.22a
<i>H. nuttallii</i>	33.996b	41.702b	83.00b	83.09a
Hybrid	11.060a	25.928a	93.12bc	93.48ab
	Phenols (mg/g DW)		Flavonoids (mg/g DW)	
	Leaves	Ray flowers	Leaves	Ray flowers
<i>H. annuus</i>	6.647a	5.996a	11.819a	8.309a
<i>H. nuttallii</i>	7.200ab	7.981b	12.505b	13.534a
Hybrid	8.849b	8.144b	13.843c	9.601a
	WS-AOC ($\mu \text{mol g}^{-1} \text{DW}^{-1}$)		LS-AOC ($\mu \text{mol g}^{-1} \text{DW}^{-1}$)	
	Leaves	Ray flowers	Leaves	Ray flowers
<i>H. annuus</i>	248.471b	495.059b	0.650a	0.800a
<i>H. nuttallii</i>	473.412c	611.765c	1.150b	0.950a
Hybrid	183.529a	103.059a	1.300c	0.950a

*Data were reported as means \pm standard error. Standard errors were represented as vertical bars which were the mean of three values (n=3); letters in common indicate no significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA.

Table 3. Antioxidant enzymes of an interspecific line *H. annuus* x *H. Nuttallii**

Parents and hybrid	CAT (units $\text{mg pr}^{-1} \text{min}^{-1}$)		GPO (units $\text{mg pr}^{-1} \text{min}^{-1}$)	
	Leaves	Ray flowers	Leaves	Ray flowers
<i>H. annuus</i>	16.250b	13.140b	92.498a	31.010a
<i>H. nuttallii</i>	5.691a	3.513c	476.095c	200.526b
Hybrid	15.286a	4.651a	294.057b	23.217a
	APX ($\mu\text{mol ASC mg pr}^{-1} \text{min}^{-1}$)		SOD (units $\text{mg pr}^{-1} \text{min}^{-1}$)	
	Leaves	Ray flowers	Leaves	Ray flowers
<i>H. annuus</i>	0.720b	0.400c	56.422b	36.154b
<i>H. nuttallii</i>	0.109a	0.158b	13.253a	57.385c
Hybrid	0.068a	0.080a	13.527a	9.803a

*Data were reported as means \pm standard error. Standard errors were represented as vertical bars which were the mean of three values (n=3); letters in common indicate no significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA.

Determination of the Level of CAT, SOD, GPO, and APX in the Three Sunflower Genotypes

Analysis of CAT activity revealed differences between three genotypes, which were evident both in the leaves and ray flowers (Table 3). The most striking of these differences was the activity of CAT in perennial *H. nuttallii*. GPO activities in the leaves for three selected genotypes were higher than that these in the ray flowers, despite of the almost equal phenolic content in both plant organs (Table 3). The highest value was established in the leaves of *H. nuttallii*. However, the GPO activity of hybrid line was intermediate of two parental genotypes. In common sunflower *H. annuus*, SOD-activity was statistically higher than activities recorded in wild sunflower and HA-Hnutt line (Table 3). There was no substantial difference in the level of SOD in the leaves sample of *H. nuttallii* and hybrid line. However, in ray-flowers of hybrid plants, the level of SOD was strongly reduced compared with both parental genotypes. Common sunflower showed the highest APX activity in the leaves when compared with *H. nuttallii* and hybrid line (Table 3). Similar trend revealed the APX activity in the ray flowers. Hybrid, however, revealed the lowest APX activity in the leaves than either parent.

Discussion

In *Helianthus* crop breeding programs, wild relatives have been evaluated as genetic resources to develop new cultivars with potential traits that could lead to

improvements (Davey et al, 2010; Breton et al, 2012; Zhang et al., 2017). Wide hybridization has been performed between common sunflower, *H. annuus* and its wild relatives to produce enhanced sunflower germplasms with high yield, high oil content, and desirable fatty acid concentration, as well as novel resistance genes for diseases and insects (Seiler and Marek, 2011; Kaya, 2014; Zhang et al., 2017). In this view, hybridization may play a creative role in providing the genetic "raw material" required to support practical breeding strategies, and also to study the genetic relationships between species in the genus *Helianthus*. Herewith, we examined the late-generation fitness consequences of hybridization between the common sunflower, *H. annuus* and wild perennial diploid *H. nuttallii*. According to Johansen-Morris and Latta (2006), the fitness of later generation recombinant inbred lines (RILs) derived from the cross reflects both the loss of early generation heterozygosity as well as disruption of any collapsed gene complexes present in the parents. Herewith, we have presented a recombinant inbred line (RIL) easily distinguished from both common sunflower and wild perennial parents that will likely be used in sunflower breeding.

As in other studies (Faure et al., 2002; Vassilevska-Ivanova et al., 2003; Breton et al., 2012), the interspecific hybridization affects a wide range of vegetative and reproductive traits. In terms of phenotype, the common sunflower, *H. annuus* appeared to be dominant since hybrid *H. annuus* \times *H. nuttallii* matched closely the female parent for some *H. annuus*-line morphological

traits. The most remarkable feature observed was the vigorous plant habit that manifests itself in measurable morphological characters such as increment plant height, stem diameter and diameter of the head, thus, suggesting the pleiotropic effect of hybridization. Further, the growth habit and flower features had a clearly intermediate character, thus, indicating that characters from both parents are being expressed. Morphological data are consistent with several studies in that the morphological intermediacy of the hybrids between the common sunflower, *H. annuus* and several of its congeners, including *H. mollis* (Faure et al., 2002; Vassilevska-Ivanova et al., 2005; Breton et al., 2012), *H. argophyllus* (Vassilevska-Ivanova et al., 1998), and wild *H. annuus*, *H. petiolaris*, *H. bolanderi* (Vassilevska-Ivanova et al., 2001) has been documented.

Along with the morphological and reproductive traits, some biochemical characteristics related to antioxidant activity were associated to wide hybridization. The comparison of hybrid line and both parental species revealed that antioxidant capacity and the level of antioxidant enzymes such as CAT, SOD, GPO, and APX were altered by the interspecific hybridization to a different extent. It was observed that, among the analyzed biochemical parameters FRAP, ascorbic acid, the amount of total tocopherols, CAT, and GPO content were mostly intermediate. Among these parameters, the total content of tocopherols seems to be of practical interest considering its high antioxidant potential. It was established that the sunflower oil is the richest in α -tocopherol which is the main compound among the tocopherol homologs with the highest biological activities (Seiler and Marek, 2017). This fact could contribute to reinforcing the idea that interspecific hybridization can be a potential tool to improve vitamin E status of the sunflower oils rich in monounsaturated fatty acids. However, further studies are needed to characterize the composition of hybrid oil and to assess its potential as a source of health-promoting bioactive compounds.

The present study indicated significant differences in antioxidant enzymes activity among three investigated genotypes. It was reported that very few studies have attempted to determine the mechanisms behind the patterns of chemical variation, thus reducing ability to predict which hybrids will be more or less variable (Orians, 2000). Also, genetic studies to ascertain whether the pattern of chemical variation arose as a direct by-product of hybridization or whether they evolved *via* other mechanisms, for example, the type of divergence is necessary to be performed. In the leaf samples of HA-Hnutt line, the total phenolic and flavonoids content altered to a different extent (Table 2); the total phenolic content and the flavonoids were higher than these in both parents. In the current study, the antioxidant level of phenols and flavonoids is an indication that both characteristics might be hardly useful as biochemical diagnostic markers for preliminary evaluation of the hybridization events. So, in relation to the sunflowers (*H. annuus* and its wild relatives), the identification of specific biochemical markers that verified the hybrid origin of the plants is needed to be investigated. Thus, we can come closer to mechanisms of wide hybridization and

generalizations of methods for testing of experimental hybrids in the selection studies.

In conclusion, it is possible to produce fertile hybrid plants from crosses between common sunflower, *H. annuus* and wild perennial diploid species *H. nuttallii*. Morphological and biochemical characters provide substantial evidence that *H. nuttallii* is involved in hybridization process. Outcomes of hybridization between both *Helianthus* species show the formation of a stable single headed line with extreme plant habit. Our results suggest that the genetic diversity between two species is not a strong barrier to the hybrid formation. Nevertheless, more research is needed to elucidate the real potential of wild sunflower germplasms and their introduction into breeding programs to develop high performance cultivars.

Conflict of Interest

The authors declare no conflict of interest.

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