### WILD SUNFLOWERS RESEARCH IN ARGENTINA

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#### SUMMARY

Since 2000 a research is in progress on wild sunflowers in Argentina, comprising naturalized H. annuus and H. petiolaris populations, and their relationship with cultivated sunflower. The study includes morphological, phenological, biochemical and molecular aspects, addressed to explain dispersal and adaptive processes and gene flow within sunflower crop. We present here a summary of results obtained during the last five years.

#### Key words: sunflower, wild species, gene flow, hybridization, phenotypic characterization.

#### INTRODUCTION

Two wild annual sunflower species, Helianthus annuus and H. petiolaris, were introduced in Argentina about 50 years ago and they became naturalized. Although the introduction events are unclear there were probably several entries, possibly as gene source for sunflower breeding and as contaminants in forage seed lots (Bauer 1991; Bertero and Vazquez 2003; A. Luciano, personal communication). However, it was not until seed companies became interested in the release of genetically modified (GM) sunflower varieties that wild relatives caught the attention of the National Committee of Agricultural Biotechnology (CONABIA) in Argentina. In fact, information about the risks of transgene escape and eventual environmental impact are required before any GM crops could be released. This study started with the aim of gathering information for CONABIA in January 2000. We present herein a survey of our research in wild sunflowers, as it was presented at the 10<sup>th</sup> FAO Consultation Meeting, Novi Sad, Serbia and Montenegro, in July 2005.

#### MATERIALS AND METHODS

As we survey several aspects of the research, indications of materials and techniques are merged with results. Details can be obtained from the referenced papers or from the authors.

### **RESULTS AND DISCUSSION**

#### Plant exploration for wild Helianthus species

Between 2000 and 2005, 28 field collection trips were successfully completed. The species of interest spread over seven provinces of Argentina. The area extends over the central part of the country, between  $31.3^{\circ}$  and  $38.7^{\circ}$  S latitude,  $60.6^{\circ}$  and  $68.3^{\circ}$  W longitude (Figure 1).

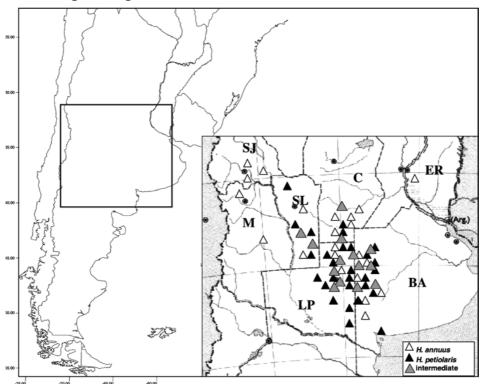


Figure 1: Wild sunflowers distribution in central Argentina, provinces of Buenos Aires (BA), La Pampa (LP), Cordoba (C), Entre Rios (ER), San Luis (SL), Mendoza (M) and San Juan (SJ). Each triangle marks a provincial county where the species is present.

Individuals of both species are mostly encountered in patches along roadsides and disturbed soils (Poverene *et al.* 2002). *Helianthus annuus* and *H. petiolaris* sometimes invade summer crops although not as frequently as in North America. Because of the invasions into crops they may be considered weeds in the core zone of the sunflower crop.

*Helianthus annuus* grows in a wide range of habitats and soils, from eastern Entre Rios to western Mendoza and San Juan provinces, with the largest populations located in Cordoba. *H. petiolaris* is more restricted to sandy soils of central La Pampa and the borders of Buenos Aires and San Luis provinces. Both species grow on Mollisols; *H. annuus* is frequently found on Argiaquolls while *H. petiolaris* populations grow on Haplustolls. Both species show large variation in soil order and suborder preference, mostly on soils subjected to eolic erosion.

A comparison of climate parameters made with 31 Argentinean and 46 US localities representing the span of wild H. annuus populations showed that the geographic range of US populations was almost four times larger than the range for Argentinean populations. Thirty-one stable populations, found in two or more successive years were localized by GPS positioning. Site variables on altitude, annual rainfall, and mean temperature were estimated based on the nearest locality (De Fina 1992). Those were compared with 993 accessions of 39 US states from GRIN-USDA germplasm bank (www.ars-grin.gov2/cgi-bin/npgs/html) grouped in 46 localities for which climate data were taken from www.worldclimate.com (Cantamutto et al. 2005). Highly significant differences were found in latitude and altitude distribution between the two countries (Table 1). Although the mean temperature of the colder month was lower for the US sites compared with the Argentinean sites, there were no significant differences in the mean temperature of the warmest month and the mean annual rainfall. The wider span of the occupied area in the center of origin with respect to Argentina may indicate that the expansion process of exotic wild sunflowers is likely to continue. In fact, successive explorations indicate a continual expansion of the colonized area.

	North America	Argentina <sup>1</sup>
Latitude (°)	$39.7\pm5.4~\text{N}$	34.1 ± 1.6 S **
Altitude (m a. s. l.) <sup>2</sup>	$701\pm478$	266 ± 195 **
Annual rainfall (mm)	$514\pm329$	$614\pm240~\text{ns}$
Warmest month mean temperature (C°)	$\textbf{28.8} \pm \textbf{3.3}$	$24.3\pm0.9~\text{ns}$
Coldest month mean temperature (C°)	$\textbf{-1.8}\pm7.2$	8.5 ± 1.3 **

 Table 1: Site climate variables for *H. annuus annuus* L. populations in North America and Argentina

<sup>1</sup>Significant at the 0.01 level. <sup>2</sup>Meters above sea level.

Gene flow between the two wild species and the cultivated sunflower is evidenced by intermediate forms often found in wild populations and tilled lands. Hybridization and introgression might have shifted genetic variation in Argentinean populations that may now differ from variation at the center of origin. At present, our collection has 135 *H. petiolaris* and 90 *H. annuus* accessions plus samples from over 20 localities where hybrid forms are common. Seed samples of 17 *H. annuus* and 18 *H. petiolaris* accessions were kindly supplied in 2004 by the USDA-

germplasm bank.		
1. Plant characters	S. bataticola	
Anthocyanin in hypocotyl	Drought tolerance	
Stem pubescence	Lodging	
Branching type	5. Flowering characters	
Presence of main head	Leaves at head back	
Plant height	Number of ray flowers	
Stem diameter at medium height	Ray color	
Head position	Ray width	
Head number	Ray length	
2. Leaf characters at medium height	Phyllary number	
Leaf width	Phyllary disposition (oppressed, loose)	
Leaf length	Phyllary tips (acute, acuminate)	
Leaf size (WxL)	Phyllary pubescence	
Petiole length	Phyllary length	
Leaf blade/petiole index	Phyllary basal width	
Leaf base	Phyllary L/W ratio	
Leaf shape	Pappus presence	
Leaf hairs appearance	Pale length (relative to flowers)	
Leaf surface appearance	Pale pubescence	
Leaf hair coverage	Pale lobes	
Leaf margin	Pale anthocyanin presence	
Leaf number	Stigma anthocyanin presence	
Leaf shape variation along stem	Head diameter	
Leaf arrangement (opposite, alter)	Disc flower colour	
Anthocyanins is stem and petioles	Disc white hairs (presence/ absence)	
3. Plant cycle characters	6. Seed yield characters	
Days from transplant to 10% flowering	Seeds from selfing	
Days from 10% to 50% flowering	Seeds from open pollination (OP)	
Days from transplant to end flowering	100 OP-seed weight	
Date of transplant	Hull percentage	
Number of plants after transplant	Achene length	
4. Disease characters	Achene width	
Downy mildew	Achene shape	
Leaf rust	Achene color	
Stem rust	Achene design (striate, maculate)	
Alternaria, leaf Phoma	Achene pubescence	
Verticillium	Mottles in pericarp	
Stem Phoma	Seed set	
Sclerotinia		

 Table 2: Morphological and phenotypic characters used for statistical analysis of wild

 Helianthus annuus. Underlined traits were used as descriptors for the INTA germplasm bank.

ARS North Central Regional Plant Introduction Station, Ames, Iowa, through Dr. Gerald Seiler, covering a broad range of variability. A comparative analysis on morphology and molecular characters is in progress and should provide information about the origin and variability of Argentinean populations. Besides, there are wild entries from the center of origin in North America at germplasm banks, comprising annual and perennial species, at two experiment stations of the National Institute of Agricultural Research of Argentina (INTA).

#### Phenotypic characterization of wild sunflower species

Both naturalized species display phenotypic diversity and differences in phenology, though not representing the extreme variability found in North America. Argentinean materials correspond to the taxa H. annuus ssp. annuus and H. petiolaris ssp. petiolaris, as described by Heiser (1954, 1961). Both species showed significant differences among populations for a number of morphological traits when analyzed by ANOVA and Kruskal-Wallis comparisons. Overall phenotypic similarity among populations was assessed by principal component analysis (PCA). No definite clusters were found for *H. petiolaris*. In addition, no correlation with geographic distribution was apparent in this species although the amount of variation was higher in the populations found in regions of traditional sunflower crop production (Poverene et al. 2004a). Plant cycle was correlated with geographic position (latitude and longitude) and bract (phyllary) width, one of the traits that are frequently influenced by gene flow from the crop. A founder effect was not evident. Although we were not able to find hints about the species' spread over the country, data indicate that several simultaneous introduction events had occurred (Poverene et al. 2004b). In addition, seed dispersion may also be influenced by truck and train movement during sunflower harvest and transport.

On the other hand, multivariate analysis of *H. annuus* did reveal population clusters that allow us to make inferences about the dispersion processes. Traits like plant height and branching, plant cycle, leaf size and shape, and seed set were highly variable among accessions.

Five out of the largest Argentinean wild *H* annuus populations were compared with 17 North American populations from Kansas, Illinois, Utah, California, New México, South Dakota, North Dakota, Indiana, Iowa, Colorado, Montana, Wyoming, Arizona, Oklahoma, Texas, Nevada and Nebraska, on the grounds of morphology, phenology and disease symptoms. The aim of this study was to assess processes of introduction and diffusion of this species in Argentina. A common garden experiment was performed to gather data on 71 characters (Table 2) (Presotto *et al.* 2005). Principal component analysis showed that Argentinean populations comprise only a part of the North American variability, but also differentiation processes have taken place during naturalization (Figure 2).

In the summers of 2003 and 2004, nine accessions of wild *H. annuus* were grown in the experiment field at our Agronomy Department. Seeds regenerated

from pollination among 30 heads were incorporated into INTA Manfredi Experiment Station active germplasm bank. These constituted the first Argentinean wild materials officially preserved. Descriptors used for population characterization are included in Table 2.

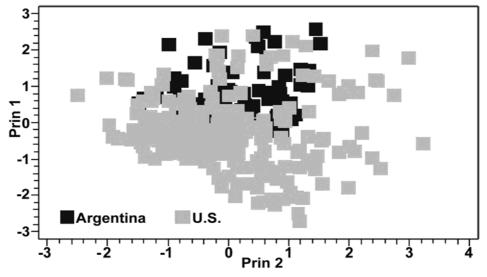


Figure 2: Principal component analysis of wild Helianthus annuus accessions from US and Argentina, based on 71 morphological and phenological traits of Table 2.

Naturalized wild *H. annuus* populations display some promising traits in view of breeding new sunflower cultivars. Accessions grown in the common garden experiment showed differences in susceptibility to Verticillium wilt (*Verticillium dahliae*), downy mildew (*Plasmopara halstedii*) and rust (*Puccinia helianthi*). At this time we are investigating other characters, such as variation in fatty acid composition and life cycle, resistance to diseases, presence of male sterility and tolerance to imazapyr herbicides. In particular, the recent release of imazapyr-tolerant sunflower cultivars in Argentina might have a high impact on wild populations, through hybridization and gene flow.

*H. petiolaris* populations display high resistance to most fungus diseases. Additionally, in the western Buenos Aires and eastern La Pampa provinces, a region of intensive sunflower production, we have observed tolerance or escape of some *H. petiolaris* individuals to the parasitic plant *Cuscuta indecora* var. *longisepala* (*Cuscutaceae*), which causes severe damage to different crops. This fact may deserve attention, because resistance genes might be implicated.

#### **Oilseed** quality

Fatty acid composition was studied in the largest wild *H. annuus* populations in Argentina. Seed was collected at nine localities and grown in a common garden in

Table 3: Oil composition of the largest wild <i>H. annuus</i> populations in Argentina, showing differences among populations and pollination mode (open or sibbed in a common garden). Province references as in Figure 1.	ition of the or sibbed i	largest wild n a commor	H. annuus 1 garden). P	population: rovince refe	a of the largest wild <i>H. annuus</i> populations in Argentina, show sibbed in a common garden). Province references as in Figure	1a, showing Figure 1.	differences a	among popul	lations and	pollination
	lio			ш	Fatty acids(g kg <sup>-1</sup> of seed oil content) <sup>1</sup>	g kg <sup>-1</sup> of seed	l oil content) <sup>1</sup>			
Population number and province	content <sup>1</sup> (%)	Palmític 16:0	Estearic 18:0	Oleic 18:1	Linoleic 18:2	Linolenic 18:3	Arach. 20:0	Eikos. 20:1	Behe. 22:0	Ligno. 24:0
1403 (C)	27.0d	48.45ab	31.04a	238.17bc	668.37ab	0.85	2.29a	1.94bc	6.33	1.71a
1802 (LP)	21.3a	53.84de	36.63b	252.38c	641.43a	0.79	2.84bc	2.02cd	7.34	2.04b
2003 (ER)	23.6b	65.69f	42.10c	162.86a	714.97c	0.89	2.88c	1.54a	6.20	1.86ab
2402 (M)	23.7b	48.14a	30.98a	260.21 c	644.82a	0.66	2.46abc	2.09d	7.34	2.13b
2702 (SJ)	23.3ab	55.20e	33.48ab	218.29b	678.07b	0.82	2.50abc	1.88b	6.84	2.13b
4102(C)	24.5bc	50.37bc	31.11a	239.20bc	664.59ab	0.75	2.44abc	1.98bcd	6.67	2.07b
4902 (LP)	23.6b	50.27b	32.33ab	239.50bc	663.36ab	0.75	2.42abc	1.98bcd	6.37	2.04b
6202 (BA)	27.9d	50.37bc	31.53a	247.66bc	656.55ab	0.61	2.35a	1.91bc	6.09	1.92ab
6402 (BA)	26.4cd	52.31cd	31.20a	235.09bc	667.32ab	0.81	2.40ab	1.92bc	6.26	1.98ab
F population	**	**	**	**	**	N.S.	*	**	N.S	*
F pollination	N.S.	N.S.	N.S.	**	**	N.S.	N.S.	N.S.	N.S.	N.S.
Mean values with the same letter are not significantly different (Tukey, P=0,05). F values marked with * and ** are significant at 0.05 and 0.01 levels, respectively.	same letter a	re not signific	cantly differe	nt (Tukey, P	=0,05). F valı	ues marked w	vith * and ** a	are significant	: at 0.05 and	0.01 levels,

HELIA, 29, Nr. 44, p.p. 65-76, (2006)

the experiment field. Seed samples from those localities were sown in a randomized design with two replicates. Both open pollinated seed and seed from sib mating were collected from the plants. In this way we were able to compare seeds coming from three sources: each population site in the wild, open pollinated and sibbed in a common garden. We compared oilseed content and fatty acid composition among accessions and also between pollination modes. A 30 g sample of seed of each accession under the three conditions was analyzed by HPLC. Results were compared with oilseed content and composition of 85 US populations assessed by NMR and gas chromatography, obtained from GRIN-USDA http://www.ars-grin.gov2/cgibin/npgs/html. One-way ANOVA and Tukey test were performed using the SAS statistical package. Highly significant differences among populations were detected for oilseed content, palmitic and stearic acid content and also in minor fractions (Table 3). We found that oilseed content was higher in plants growing in the common garden conditions compared with those in the wild (P < 0.05). However, no differences in total oil content were found between open pollinated and sibbed seeds collected in the common garden. Populations showed highly significant differences in most fatty acid fractions, though no differences were found between pollination modes, except for oleic and linoleic acids. Differences in oilseed content may be associated with differences in growing conditions, especially those related to soil water availability. Oilseed variability in local populations was lower than in US populations (Seiler and Brothers 2000). Nonetheless, variability seems to be sufficient to consider local wild resources of interest for breeding purposes.

#### Molecular diversity

Molecular analysis revealed high levels of polymorphism in both wild species. Genetic differentiation for RAPD markers among *H. petiolaris* populations assessed by ANOVA showed significant variation both among and within populations. This would indicate a low gene exchange among populations, possibly related to their patchy distribution. As it could be expected, the populations showing higher morphological variation also showed higher molecular diversity. Cluster analysis performed on molecular data based on Dice index grouped *H. petiolaris* populations in two clusters. Notoriously, populations included in one of the clusters had previously evidenced crop hybridization, since a number of plants showing intermediate morphology had been found within them the year before. Thus, the molecular pattern proved useful to demonstrate gene flow and introgression from the crop.

Table 4: Diversity parameters in *H. annuus* populations in the center of origin, wild and cultivated sunflower in Argentina: polymorphic loci (P), mean number of alleles per locus (A) and mean expected heterozygosity (H)

Populations	Р	А	н
Wild <i>H. annuus</i> in the US <sup>1</sup>	0.56	1.65	0.21
Wild H. annuus in Argentina	0.43	1.60	0.17
Cultivated inbred lines and hybrids <sup>2</sup>	0.42	1.47	0.15

<sup>1</sup>From Cronn et al. 1997 ; <sup>2</sup> From Carrera et al. 2002.

Isozymes and SSR analysis revealed high genetic variability in local populations of wild *H. annuus*. Table 4 shows isozyme variability parameters for wild and cultivated sunflower. Measures of genetic variation were slightly lower than those reported for the center of origin (Cronn *et al.* 1997) showing that populations have not been subjected to strong diversity reduction during the introduction process. The presence of different unique alleles over the whole range of distribution does not agree with the single origin hypothesis of wild *H. annuus* in Argentina and it could rather suggest multiple introductions events.

Allelic composition for 29 microsatellite (SSR) loci of wild and cultivated sunflower was similar. Even so, cultivated sunflower has a subset of alleles. These results are in agreement with a wild ancestor-crop derivative relationship. The high value of average allele number points to the wild populations as important reservoir of genetic variability. Allele frequencies varied between populations, allopatric and sympatric with sunflower crop. Difference in number of plants harboring alleles from the crop was highly significant (P < 0,005) (Figure 3).

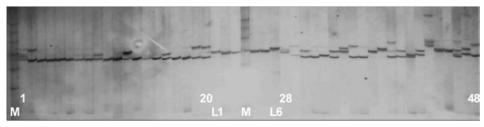


Figure 3: SSR analysis in wild H. annuus populations, allopatric (lanes 1-20) and sympatric (lanes 28-48) with sunflower crop. Central lanes show ladder (M) and sunflower inbred lines (L1 to L6).

We have not been able to find specific SSR markers for cultivated sunflower yet. It is possible that the isozyme and microsatellite alleles shared by wild and crop plants might represent common genome zones for *Helianthus annuus*. Alternatively, it could be suggested that the wild populations might have been under crop genetic flow in their native land, before their introduction to Argentina.

#### Interspecific hybridization and gene flow

Plants of intermediate morphology are often found in wild populations of both *H. annuus* and *H. petiolaris* (Poverene *et al.* 2004a). Head diameter, bract (phyllaries) width, leaf size and branching make hybrid plants conspicuous enough among wild type ones to indicate a likely  $F_1$  hybrid origin. Additionally, these characters point out the cultivated sunflower as the putative parent. One of the characters also notorious in hybrid plants was seed size. We performed an analysis of a sample of plants with the above mentioned characteristics and their families in a common garden study and we were able to confirm the suspected hybrid status. Backcrosses or advanced hybrid generations are not as easily recognized based on morphological traits. Therefore, molecular marker analysis would be necessary to reliably esti-

mate hybridization. Gene flow from the crop to the two wild species was also estimated by sampling mature heads of wild plants which had flowered at the same time of a close sunflower crop. The progenies were analyzed both for morphological traits and molecular markers. Moreover, gene flow from the wild species to the crop was also confirmed.

Hybrid plants were identified based both on morphology and molecular markers in *H. petiolaris* populations. RAPD markers showed unique bands for crop and wild plants (Figure 4). The hybrid individuals showed a combination of parental bands, although a petiolaris-like molecular phenotype was also frequent among the hybrids. The unique bands observed in cultivated sunflower constitute potential useful markers to estimate gene flow.

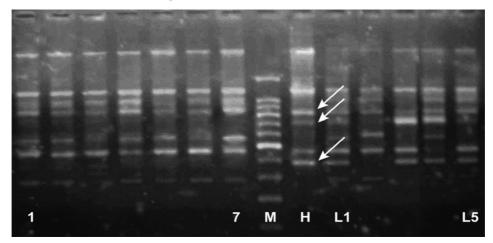


Figure 4: RAPD analysis of H. petiolaris plants (lanes 1-7), a natural wild x crop hybrid (H) and sunflower inbred lines used as testers (L1-L5). Arrows show markers bands from the crop (Operon primer A2, M is a 100 bp ladder).

#### **Ongoing work**

**Gene flow from IMI sunflowers to wild populations.** Screening for imidazolinone resistance in wild populations and pollination of wild *H. annuus* plants with an IMI (Clearfield®) sunflower cultivar are currently under way. The aim of this project is to study the influence of this trait in wild sunflowers, searching for molecular markers and changes in population composition and fitness, through survival and fecundity parameters.

Male sterility in *Helianthus annuus* ssp annuus local populations. Male sterile plants found in the 2003-2004 experiment field were sibbed with normal plants on the same plot. The  $S_1$  plants grown in 2004-2005 showed complete recovery of fertility. Heads of those plants were either self-fertilized or subjected to diallelic crosses. We will evaluate fertility in the next generation and compare it with other male-sterility sources. The restoration of PET-1 source will be evaluated in the crosses with MS HA89 and A10 registered lines.

**Characterization and fitness of sunflower crop** - *H. petiolaris* hybrids. Crop genes beyond the  $F_1$  generation following hybridization and the establishment of crop genes in wild populations are being investigated through molecular markers. Crop-wild hybrids fitness is compared with wild plants fitness as a component of determining the risk of transgene escape. Advanced generation hybrids and back-crosses to wild plants are under study in a number of populations where natural  $F_1$  hybrids have been found.

**Molecular comparison of US and Argentinean materials.** We are currently investigating other molecular markers in *H. annuus*, RAPD and ISSR (DNA polymorphisms based on amplified fragments primed by microsatellite sequences). We hope that they will render more informative markers to estimate crop-wild hybridization. We have already identified some useful markers to study gene flow from the crop to *H. petiolaris* populations, but we need to screen a higher number of populations in order to confirm the validity of this approach.

**Combining ability of local** *Helianthus annuus* **ssp.** *annuus* **populations**. During the summer 2004-2005 we crossed two male-sterile lines (A89 and A10) with plants from five Argentine populations of *H. annuus* ssp *annuus*. Scant seed was obtained, so that it was necessary to repeat the crossing the following summer. The  $F_1$  seed will be planted in regional evaluations of the sunflower crop area. Agronomic traits such as life cycle, vigor, plant architecture, disease resistance and yield will be evaluated in different crop conditions in order to assess potential value for breeding.

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# INVESTIGACION SOBRE GIRASOLES SILVESTRES EN ARGENTINA

#### RESUMEN

Desde 2000 se lleva a cabo una investigación sobre girasoles silvestres en Argentina, que comprende las poblaciones naturalizadas de *H. annuus* y *H. petiolaris* y su relación con el cultivo de girasol. El estudio abarca aspectos morfológicos, fenológicos, bioquímicos y moleculares, tendientes a explicar los procesos de dispersión, adaptación y flujo génico con el girasol cultivado. Se presenta aquí un resumen de los resultados obtenidos en los últimos cinco años.

# RECHERCHE SUR LE TOURNESOL SAUVAGE EN ARGENTINE

#### RÉSUMÉ

Depuis 2000 des recherches sur le tournesol sont en cours en Argentine. Elles incluent les populations naturalisées *H. annuus* et *H. Petiolaris* et leur rapport envers le tournesol cultivé. Les observations comprennent les aspects morphologiques, phénologiques, biochimiques et moléculaires et elles ont pour but d'expliquer les processus de dispersion et d'adaptation ainsi que l'écoulement de gène dans la culture du tournesol. Nous présentons un résumé des résultats obtenus au cours des cinq dernières années.