Universitat de Lleida

Departament de Producció Vegetal i Ciència Forestal

Programa de Doctorat
Sistemes Agrícoles, Forestals i Alimentaris

AGRONOMIC STUDY OF TWO ANNUAL *HELIANTHUS* SPECIES NATURALIZED IN ARGENTINA AS POTENTIAL SUNFLOWER CROP GENETIC RESOURCE

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DEDICATORIA

A mis hijos; Francisco José (Fran), Martín Alejandro (Ruso), Lucía Marina (Lucy) y María Magdalena (Magui), que comparten el valor del esfuerzo.

A mis padres; Remo Virginio Cantamutto Comoglio y Mercedes Haydee Sánchez De Filpo, que me enseñaron los valores de la vida.

AGRADECIMIENTOS

Al Dr. Emiliano Sanz Parejo, un colega y amigo, que fue el primero en confiar y abrirme las puertas de la UdL. Ningún otro paso hubiera sido posible sin ese apoyo contundente y desinteresado con que me acompañó desde el principio… *¡Gracias Emiliano!*

A los Dres. Edith Obschatko y Osvaldo Fernández, quienes me halagaron con su manifiesta confianza en apoyo a mi postulación como becario.

Al Rector de la Universidad Nacional del Sur, Dr. José María Fernández, que avaló con firmeza mi presentación como becario.

A la Fundación Carolina, que me dio la enorme oportunidad de poder acceder a una beca de doctorado, sin discriminarme por mi edad.

Al Dr. Juan Antonio Martín Sánchez, por su destacado trato profesional y su inmensa calidad humana, señorial... *¡Gracias Jefe por guiarme con sutileza y por apoyarme siempre!.*

A la Dra. Mónica Poverene (Moni). Colega, amiga y colaboradora. Me trasmitió todo su conocimiento sobre los girasoles silvestres, su deseo de superación y su tesón laboral, que me inspiraron más de una vez. Es mucho lo que me enseñaste y me acompañaste... *¡Gracias Moni!..*

A mis compañeros de trabajo de Bahía Blanca; Ings. Agrs. Marta Miravalles (Pelusa) y Pablo Marinangelli, que en todo momento valoraron mi trabajo y supieron disimular mis ausencias.

Al Ing. Agr. (M.Sc.) Federico Möckel (Fede) que además de lo anterior colaboró en el perfeccionamiento de la Introducción General y Discusión General.

A mi familia. Mis hijos Francisco José (Fran), Martín Alejandro (Ruso), Lucía Marina (Lucy) y María Magdalena (Magui) que siempre compartieron y apoyaron el desafío. Mi esposa Eloísa Gabriela Gaido (Loise) supo acompañar el reto con hidalguía y mantener la familia unida, con mucha paciencia.

A mi hermana María Cristina (Nena) que en forma incondicional me apoyó desde los momentos más difíciles del inicio de la beca. *¡Gracias, Nena!*

Al Ing. Agr. (M.Sc.) Alejandro Presotto (Ale), colaborador incondicional y amable interlocutor. Siempre estuvo apoyándome, compenetrado con el avance de mi tesis, brindando sus ideas y transmitiéndome su fuerza en los momentos difíciles. *Gracias Ale!*

A mis alumnos; Lucas Stanic, Pablo Errazu, Ignacio Sagarzazu (Nacho), Jorge Schaab (Jorgito), Federico Laxague (Fede), Juan Pablo Renzi, Ivana Fernández Moroni (Rusa), Juan Giambelluca (Ruso). Siempre estuvieron incondicionalmente a mi lado.

Al Dr. José Luis Noguera y su cálido grupo humano de Producción Animal del Institut de Recerca e Tecnolgie Agropecuarie (IRTA) de Lleida. Gracias a ellos tuve oficinas, ordenadores, amigos y un espacio inolvidable a la hora del café.

A los amigos latinoamericanos, catalanes y españoles que tuve la fortuna de conocer en Lleida, por ayudarme a sentir y entender mejor la vida; Xiomara Abreu Rosas, Gerardo Hernández Escaldeño y familia, Raquel Quintanilla Aguado, Romi Pena I Subiri, Maite Arbones Ruengo, David Almuzara, Rosa Mestres, Paquita Santiberi.

A Antonia Bosch (Tonyi), que además de lo anterior, escribió el Resum y se ocupó de la impresión de esta tesis.

A mis compañeros de clases en la UdL; Jordi Martí Marsal, Miguel Sánchez y otros, que me introdujeron al manejo del SAS.

Al Departament de Producció Vegetal i Ciència Forestal de la UdL, su extraordinaria gente, autoridades, colegas, personal de apoyo; Tere, Antonio, Dany, Jaume, Vicente, Gustavo, Carlos, Roxana. *Gracias por su calidad humana y profesionalismo!*

A los colegas y amigos de Conreus Extensivos y de la Administración del IRTA; María, Andrea, Teresa, África, Fanny, Betbese, Ezequiel, Ramón, Rosa (Collado), Jordi, Nuria.

A los Ings. Agr. Rubén Miranda y Armando Junquera de la Asociación de Cooperativas Argentinas, quienes hicieron posible el trabajo en contra-estación utilizado para la obtención del Diploma de Estudios Avanzados.

A los miembros del grupo de investigación de girasol silvestre de Bahía Blanca; Dra. Alicia Carrera, Ing. Agr. Soledad Ureta (Sole), Lic. Agustina Gutiérrez (Agus) quienes siempre apoyaron y aceptaron mis propuestas.

Al Departamento de Agronomía de la Universidad Nacional del Sur, por mantener mi lugar de trabajo mientras estudiaba en Lleida.

A los colegas y amigos de la Facultad de Ciencias Agrarias de la Universidad Nacional de Lomas de Zamora, que alentaron mis estudios en Lleida; Ing. Zoot. Fernando Rumiano, Ing. Agr. (M.Sc.) Marcelo Torrecillas, Ing. Agr. (M.Sc.) Luis Bertoia, Ing. Agr. Roberto Castosa (Tito), Ing. Agr. Marcelo Yasky (Ruso), Ing. Zoot. Guillermo Mariategui (Flaco), Ing. Zoot. Jorge Calvo.

A los colegas y amigos que acompañaron las investigaciones que integran esta tesis; Ing. Agr. (M. Sc.) Daniel Alvarez, Dr. Sergio Lenardon, Dr. Lluis Torres, Ing. Agr. (M. Sc.) Raúl Rodriguez (Zurdo).

A Carlita, Jessica, Virginia y demás coautores con quienes compartí la elaboración de los artículos de esta tesis.

El Dr. Gerald Seiler, además de lo anterior revisó cuidadosamente el manuscrito y contribuyó al perfeccionamiento del contenido y expresión de esta tesis. *Thank you very much, Gerald!*

A todos los que me alentaron y facilitaron mis actividades en Lleida.

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ABBREVIATIONS

A	Adventive
AAL	Adolfo Alsina
AAPRESID	Asociación Argentina de Productores de Siembra Directa (Argentina)
AHAS	Hydroxyacetic acid synthetase
ALT	Altitude
ALTU	Plant height
An	Off type plant found between wild *H. annuus*
AN	Annual life cycle
ANEST	Stigma anthocyanin presence
ANFIL	Phyllary width
ANFLIG	Ray flower width
ANHOJ	Leaf width
ANN	*Helianthus annuus* L.
ANNc	Cultivated *H. annuus*
ANNw	Wild *H. annuus*
ANOVA	Analysis of Variance
ANPAL	Pale anthocyanin presence
ANPCyT	Agencia Nacional de Promoción de Ciencia y Tecnología (Argentina)
ANTALL	Anthocyanin in stem and petioles
AOCS	The American Oils Chemist Society (USA)
ARG	Argentina
ARS	Agriculture Research Service (USA)
AS	Asparagine synthetase
ASAGIR	Asociación Argentina de Girasol (Argentina)
AZ	Arizona
BA	Buenos Aires
BAHOJ	Leaf base
BARr	Colonia Barón
BI	Life cycle biannual
br	Sunflower breeding station
Bt	Endotoxin of *Bacillus thuringensis*
CA	California
CAN	Canonical
CAPRIN	Presence of main head
CAT	Catriló
CERZOS	Centro de Recursos Naturales Renovables de la Zona Semiárida (Argentina)
CIC	Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (Argentina)
CL	Clearfield®
CMS	Cytoplasmic male-sterility
CO	Colorado
COCA	Soil calcareous content
CODIS	Disk flower colour
CONABIA	Consejo Nacional de Biotecnología Agropecuaria (Argentina)
CONICET	Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina)
COR	Córdoba
CORD	Instituto Multidisciplinario de Biología Vegetal (Argentina)
CpT1	Trypsin inhibitor
Cry	Bt endotoxins
Cu	Cultivated accession
CUC	*H. cucumerifolius*
CHA	Chaco
CHU	Carhué
DAR	Instituto de Botánica Darwinion (Argentina)
DIA	Diamante
DIAMCAP	Head diameter
DIAMTA	Stem diameter at mid-height
DPLFINFL	Days from 50% flowering to 90% plants without flowers
DRSIDE	Border of dirty roads
DTRFINFL	Days from transplant to 90% plants without flowers
DTRINIFL	Days from transplant to 10% flowering
E	Exotic
EEA	Estación Experimental Agropecuaria
EFSA	European Food Safety Authority
EOLIC	Wind erosion
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
ER	Entre Ríos
EU	European Union
F1-Fn	Filial 1 to n
FAO	Food and Agriculture Organization
FAS	Foreign Agriculture Service (USA)
FORHOJ	Leaf shape

FR	France
FSU	Former Rusia
GM	Genetically modified
GMO	Genetically modified organism
GPS	Geographic Information System
GREP	Frequency of grey pericarp
GRIN	Germplasm Resources Information Network (USA)
GS	Glutamine synthetase
HDOP	Horizontal dilution of precision
Hel	*Helianthus* spp.
HLA	Hilario Lagos
HOJCAP	Leaves on back of head
HRAC	Herbicide Resistance Action Committee
Hwy	Highways
HYDRIC	Water erosion
I	Invasive
IA	Iowa
IANLAR	Width/length index (ratio)
IBPGR	International Board for Plant Genetic Resources
IDIA	Revista de Información sobre Investigación y Desarollo Agropecuario
IL	Illinois
IMI	Imidazolinone
IML	Interactive Matrix Language
In	Isolate off type plant
IN	Indiana
INCLCAP	Head position
INLAM	Leaf blade/petiole index (ratio)
INT	Intermediate
INTA	Instituto Nacional de Tecnología Agropecuaria (Argentina)
IRTA	Institut de Recerca i Tecnología Agroalimentàries de Cataluña (España)
IY	Iodine index
JCE	Juarez Celman
KS	Kansas
L	Leaf length
L/W	Leaf length/leaf width ratio
LAMPEC	LARHOJ/LARPEC
LAPL	Herbario del Museo de La Plata
LARHOJ	Leaf length
LARPEC	Petiole length
LAT	Latitude
LMA	Las Malvinas (Argentina)
LON	Longitude
LOPAL	Pale lobes
LSMEANS	Least-squares means
LP	La Pampa
m.a.s.l	Meters over sea level
M	Mendoza
MAG	Media Agua
MAHOJ	Leaf margin
MAPA	Ministerio de Agricultura, Pesca y Alimentación de España
MER	Villa Mercedes
MG	Oil content
MIX	Population stand with mixture of two species
MOTP	Frequency of motting
MS	Modified soils
MT	Montana
N	Naturalized
nd	Not determinate
Na	Native
ND	North Dakota
NE	Northeast
NE	Nebraska
NEE	Northeast east
NGA	Nueva Galia
NL	The Netherlands
NM	New Mexico
NNW	North northwest
NSA,	National Sunflower Association (USA)
NTA	Non-target Arthropoda
NUCAP	Number of heads
NUFLIG	Number of ray flowers
NUHOJ	Leaf number
NV	Nevada
OECD	Organisation for Economic Co-operation and Development
OK	Oklahoma
OM	Soil organic matter

OSU	Oklahoma State University
OXO	Oxalate oxidase
P	Wild population
PAT	Phosphinothricin acetyltransferase
PC	Principal component
PCA	Principal component analysis
PEBCAP	Disk white hairs
PEL	Pellegrini
PER	Life cycle perennial
PET	*Helianthus petiolaris* Nutt
PET1	Cytoplasmic male-sterility of *H. petiolaris*
PGI	Proyecto de Grupo de Investigación
PH	Soil pH
PI	Passport identification
PICT	Proyecto de Investigación Científica y Tecnológica (Argentina)
PIGFLIG	Ray flower colour
Pn	Off type plant found between *H. petiolaris*
PNCER	Proyecto Nacional de Centros Regionales (Argentina)
POPLSP	Wild *H. annuus* and *H. petiolaris* populations
PPM	Soil available phosphorus
PPT	Annual rainfall
PROV	Province
PRSIDE	Border of paved roads
PUBFIL	Phyllary pubescente
PUBPAL	Pale pubescente
PUBTA	Stem pubescence at maturity
PUFIL	Phyllary tip
RAIN	Average annual rainfall
RAINCO	Rainfall-corrected
RAMI	Branching type
RAN	Rancul
RCU	Río Cuarto
RLARAN	Phyllary Length/Width (ratio)
RR	Glyphosate tolerant
S	South
SA	Salta
SAGPyA	Secretaría de Agricultura, Ganadería, Pesca y Alimentación (Argentina)
SAL	Salliqueló
SALT	Saline areas
SAS	Statistical Analysis Systems
S.D.	Standard Deviation
SD	South Dakota
SE	South east
SES	Santiago del Estero
SHALLOW	Shallow soils
Shape l/w	Leaf/width
SHARP	Sharp landscape due to slope
Sign.	Significance
SL	San Luis
SP	Spain
SMN	Servicio Meterorológico Nacional (Argentina)
SQI	Soil quality index
SJ	San Juan
SPAR	Frequency of sparse pubsescence
SRO	Santa Rosa
SS	Sandy soils
STRP	Frequency of stripes present
SuCMoV	Sunflower Chlorotic Mosaic Virus
SUHOJ	Leaf surface
SUSCEP	Potential wind erosion
SW	Southwest
SWW	Southwest west
TCAL	Mean temperature of the hottest month
TCOL	Mean temperature of the coolest month
THOT	Mean temperature of the hottest month
TRE	Trenque Lauquen
TUB	*Helianthus tuberosus*
TX	Texas
UdL	Universitat de Lleida
UNER	Universidad Nacional de Entre Ríos
UNI	Unión
UNL	Universidad Nacional del Litoral
UNMdP	Universidad Nacional de Mar del Plata
UNS	Universidad Nacional del Sur
UNTu	Universidad Nacional de Tucuman

UPGMA	Unweighted pair group method with arithmetic mean
URI	Uriburu
USA	United States of America
USDA	United States Departament of Agriculture
UT	Utah
VBRC	Valle Bonaerense del Río Colorado (Argentina)
W	Leaf width
WAn	Wild *H. annuus* accession
WASIDE	Side of natural (rivers, streams) or artificial (channels) water courses
We	Weed
WHO	World Health Organization
WIN	Winifreda
WPn	Wild *H. petiolaris* accession
WY	Wyoming
YECODIS	Yellow disk flower

ABSTRACT

The genus *Helianthus* (Asteraceae), native from North America comprises not only the cultivated sunflower *H. annuus* var. *macrocarpus* L., but also some invasive species. The wild *H. annuus* ssp. *annuus* (common sunflower) and *H. petiolaris* Nutt. (prairie sunflower) are two annual species of the genus naturalized in central Argentina. Both species merit interest as genetic resources.

Wild *H. annuus* was probably introduced for forage purposes, but *H. petiolaris* seems to have entered as a seed contaminant. Using multivariate tools, the environment and ecological conditions of the habitats were analysed. A diffusive process from each entry point is suggested, following the terrestrial infrastructure. Invasion took place in a strip of land bordering between the Mollisols and Entisols, the same soil orders as in the centre of origin. Within that strip, each species thrived in different microhabitats transformed by human activity such as fences, fire lines, roadsides, ditches. *Helianthus annuus* showed preference for microhabitats with heavy soils, while *H. petiolaris* preferred sandy soils, as has been reported in North America.

The hypothesis of gene flow between both wild taxa and the crop was tested by the morphological study of the progeny of off type plants and pure populations that flowered in proximity to the other taxa found under natural conditions. Between 0.5 to 18% of hybridization was found. Taking into account the dimensions of the populations found at the points of contact, these frequencies may mean thousands to millions of first generation hybrids each year.

Nine argentine populations of wild *H. annuus* showed enough biodiversity to differentiate among them. The biodiversity contained in this new germplasm was about two thirds of that observed in a sample of wild sunflower from seventeen USA states. The accessions from Argentina showed different combinations of the same traits and a longer life cycle in one accession. The oil content and fatty acid profile did not show values that could merit attention as a source of sunflower oil improvement. Other agronomic traits of interest, such as resistance to SuCMoV or the presence of male-sterility are currently under evaluation.

Seed companies probably lost interest in transgenic sunflower after research demonstrated the existence of wild populations in several regions of the world, the intense gene flow between crop and wild relatives and the probable increase of reproductive capacity due to the acquisition of transgenes. The traits under experimentation in genetically modified (GM) sunflower could improve the performance of the crop but face some market restrictions. The future of GM sunflower depends on the possibility to mitigate the effect of transgenes on the wild and weedy relatives and a change in market place acceptance, which could increase if GM means better quality for the consumer.

RESUMEN

El género *Helianthus* (Asteraceae), que es nativo de América del Norte comprende no solo el girasol cultivado *H. annuus* var. *macrocarpus* L. sino también otras especies que son invasoras. El girasol silvestre *H. annuus* ssp. *annuus* L. y *H. petiolaris* Nutt. son dos especies anuales naturalizadas en la parte central de Argentina. Ambas resultan de interés como recurso fitogenético.

El *H. annuus* silvestre fue probablemente introducido para uso forrajero mientras que pareciera que *H. petiolaris* ingresó como contaminante de semillas. Se estudiaron las condiciones ambientales y ecológicas de los habitats ocupados por las poblaciones mediante análisis multivariante. Se sugiere la existencia de un proceso de difusión desde el punto de ingreso de cada especie, siguiendo la infraestructura de las comunicaciones terrestres. La invasión ocurrió en una banda de territorio en el límite entre Mollisoles y Entisoles, los mismos órdenes de suelos que existen en el centro de origen. Dentro de esa banda, cada especie se ubicó en diferentes microhábitats transformados por actividad antrópica, como alambrados, contrafuegos, banquinas, canales. *Helianthus annuus* mostró preferencia por suelos pesados mientras que *H. petiolaris* prefirió suelos arenosos, en modo análogo a lo informado para América del Norte.

La hipótesis de flujo génico entre los dos taxa silvestres y el cultivo se exploró mediante el estudio de la morfología de la progenie de plantas fuera de tipo y poblaciones puras que habían florecido en proximidad del otro taxa, halladas bajo condiciones naturales. Se encontró entre 0.5 a 18% de hibridización. Considerando las dimensiones de las poblaciones halladas en contacto estas frecuencias pueden significar miles a millones de híbridos de primera generación cada año.

Nueve poblaciones Argentinas de *H. annuus* mostraron suficiente biodiversidad como para diferenciarse entre ellas. La biodiversidad contenida en este nuevo germoplasma fue alrededor de dos tercios de la observada en una muestra de girasoles silvestres de 17 estados de USA. Las accesiones de Argentina mostraron diferentes combinaciones de los mismos caracteres, pero una de ellas presentó un ciclo de mayor duración. El contenido de materia grasa y el perfil de ácidos grasos no presentaron valores que puedan ser de interés para la mejora del aceite de girasol. Se

están investigando actualmente otros rasgos de interés agronómico, como la resistencia al SuCMoV o la presencia de androesterilidad.

Las empresas de semillas perdieron interés en el girasol transgénico luego que las investigaciones revelaran la existencia de poblaciones silvestres en muchas regiones del mundo, intenso flujo génico entre el cultivo y los parientes silvestres y el posible incremento de la capacidad reproductiva por adquisición de transgenes. Los eventos bajo evaluación en girasol genéticamente modificado (GM) podrían mejorar el comportamiento del cultivo pero enfrentan restricciones de mercado. El futuro del girasol GM depende de la posibilidad de mitigar el efecto de los transgenes sobre los parientes silvestres y malezas y a cambios en la aceptación del mercado. Esta podría aumentar si el girasol GM implicara mejor calidad para el consumidor.

RESUM

El gènere *Helianthus (Asteraceae)*, nadiu d'Amèrica del Nord, compren no només el girasol conreuat *H. annuus var. Macrocarpus* L., sinó també d'altres espècies que són invasores. El gira-sol silvestre *H. annuus ssp. Annuus* L. i *H. petiloaris* Nutt. Són dues espècies anuals naturalitzades en la part central d'Argentina. Ambdues resulten d'interès com a recurs fitogenètic.

L'H. annuus silvestre fou probablement introduït per a ús farratger mentre que sembla que l'*H. petiolaris* ingressà com a contaminant de llavors. S'estudiaren les condicions ambientals i ecològiques dels hàbitats ocupats per les poblacions mitjançant anàlisi multivariant. Es suggereix l'existència d'un procés de difusió des del punt d'ingrés de cada espècie, seguint la infraestructura de les comunicacions terrestres. La invasió va ocórrer en una banda de territori en el límit entre Mollisoles i Entisoles, els mateixos ordres de sòls que existeixen en el centre d'origen. Dins d'aquesta banda, cada espècie s'ubicà en diferents microhàbitats transformats per activitat antròpica, com ara tancats de filferro, tallafocs, bancals, canals. *Helianthus annuus* mostrà preferència pels sòls pesants mentres que *H. petiolaris* preferí sòls arenosos, a l'igual que allò constatat per a Amèrica del Nord.

La hipòtesi de flux gènic entre els dos taxa silvestres i el conreu s'explorà mitjançant l'estudi de la morfologia de la progènie de plantes de diferent tipus y poblacions pures que havien florit en proximitat de l'altre taxa, trobades amb condicions naturals. Es trobà entre 0,5 a 18% d'hibridació. Considerant les dimensions de les poblacions trobades en contacte, aquestes freqüències poden significar milers a milions d'híbrids de primera generació cada any.

Nou poblacions argentines d'*H. annuus* mostraren suficient biodiversitat com per diferenciar-se entre elles. La biodiversitat continguda en aquest nou germoplasma fou al voltant de dos terços d'aquella observada en una mostra de gira-sols silvestres de 17 estats d'EUA. Les accessions d'Argentina mostraren diferents combinacions dels mateixos caràcters, però una d'elles presentà un cicle de més llarga durada. El contingut de matèria grassa i el perfil d'àcids grassos no presentaren valors d'interès per a la millora de l'oli de gira-sol. Actualment s'investiguen d'altres trets d'interès agronòmic, com ara la resistència al SuCMoV o la presència d'androesterilitat.

Les empreses de llavors perderen l'interès pel gira-sol transgènic després de les investigacions que revelaren l'existència de poblacions silvestres en moltes regions del món, intens fluxe gènic entre el conreu i els parents silvestres i el possible increment de la capacitat reproductiva per adquisició de transgens. Els esdeveniments en avaluació de gira-sol genèticament modificat (GM) podrien millorar el comportament del conreu però enfronten restriccions de mercat. El futur del gira-sol GM depèn de la possibilitat de mitigar l'efecte dels transgens sobre els parents silvestres i malesa i a canvis en l'acceptació del mercat. Aquesta podria augmentar si el gira-sol GM impliqués una millor qualitat per al consumidor

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Chapter 1 General Introduction

Helianthus could be considered one of the most diverse genus of the Asteraceae family native of the American continent. Due to their morphological and genetic variability, including polyploidy, its systematics is complex and has often been taxonomically revisited (Heiser 1954, 1961, Schilling 2006). It has been considered to comprise from as few as 10 species to more than 200, with 51 species accepted according to Jan and Seiler (2007) with 14 annual and 37 perennial ones. Spontaneous hybridization and introgression are recurrent genetic processes in their native habitat, resulting in morphological intergradations and broad biodiversity in the genus (Heiser 1976). The genus *Helianthus* is an economically and evolutionary important taxon than contains not only one of the world's most important crops, but also a number of wild species that have become models for the study of the genetic adaptation and speciation (Rieseberg et al. 1996, Lexer et al. 2003, Burke et al. 2004, 2005).

North America is the centre of origin of this genus. Many of the *Helianthus* species are wide-ranging geographically and exhibit extensive phenotypic variation, which appears to include hereditable and environmental components (Seiler and Rieseberg 1997). Only a few of the species are rare and restricted in distribution (Rogers et al. 1982). In general the species are widespread and common components of the natural vegetation, showing habitats ranging from disturbed areas to tall grass prairies or climax forest (http://plants.usda.gov). A few species developed adaptation to agricultural systems and are sometimes considered noxious weeds (Whitson et al. 2004).

Together with the perennial *Helianthus tuberosus* L., the annual *H. annuus* was an important food plant domesticated and cultivated by the natives of North America during prehistoric times (Harlan 1992). Cronn et al. (1997), Heiser (1998) and Harter et al. (2004) strongly support that sunflower domestication arose in the central and eastern part of the present territory of USA, around 4000 years ago. There are evidences that the strong directional selection for increased achene size played a central role in sunflower domestication, changing the plant architecture to a monocephalous plant instead of a branched one (Burke et al. 2002).

The cultivated botanical variety of sunflower, taxonomically named *H. annuus* var. *macrocarpus* L. (Heiser 1978), was introduced in Europe in the late 16th century by a Spanish expedition in 1510 (Putt 1978). Sunflower was initially cultivated as an ornamental or rare species at the Madrid botanical garden and from there spread to the other botanical gardens of Europe. By the eighteenth century it was used for consumption during Lent in central Europe since it was not on the list established by the Orthodox Church as forbidden oil species. Initial breeding efforts, started as on-farm selection later followed by successful genetic improvement at the FSU experimental stations of Krasnodar, Saratov, and Rostov in the early 20th century, when the first varieties were produced (Vranceanu 1977).

After the New World was discovered, not only sunflower but also Jerusalem artichoke (*H. tuberosus* L.) was distributed worldwide for decorative or nutritious purposes. The spread of *Helianthus* species included other beautiful ornamentals such as the annuals *H. argophyllus* T.&G., *H. debilis* Nutt. and the perennials *H. tuberosus* L., *H. decapetalus* L., *H. x laetiflorus* Pers., *H. maximiliani* Schrad., *H. x multiflorus* L. and *H. salicifolius* Dietr.

The prairie sunflower *H. petiolaris* Nutt. is another wild annual which probably migrated as a seed contaminant during the commercial trade that is a major route by which non-indigenous organisms are introduced into new habitats (Shimono and Konuma 2007). Outside their native area in North America, some of these species escaped from cultivation, colonized and spread into new environments. Four annual and two perennial *Helianthus* species have been naturalized in at least eight different countries of four continents (Table 1-1).

The most productive Argentine croplands, located in originally grassland plains in the central part of the country, were devoted to agriculture after 1890 (Arriaga 1999). Agriculture started with farmers emigrated from Europe (Taylor 1997) who brought with them several cosmopolitan crop weeds as seed contaminants (Marzocca 1994).

Table 1-1 Countries with naturalized populations of *Helianthus* species, grouped by reproductive habit (Jan, 1997).

Species	Country	Source
A) Annuals, *n* = 17, reproduced by seed		
H. annuus L.	Argentina	Poverene et al. 2002
	Australia	Dry and Burdon 1986
	France, Italy	Faure et al. 2002
	Serbia	Stanković-Kalezić et al. 2007
	Spain	Müller et al. 2006
H. debilis Nutt.	Mozambique	Vischi et al. 2004
H. petiolaris Nutt.	Argentina	Poverene et al. 2002
H. argophyllus L.	Mozambique	Vischi et al. 2004
B) Perennials, *n* = 51, with rhizomes		
H. tuberosus L.	France	Bervillé et al. 2005
	Germany	Kowaric 2005
H. x laetiflorus Pers.	Argentina	Sala et al. 1990

The sunflower crop was started on a small scale at the end of 19th century by Jewish immigrants coming from Eastern Europe who brought improved, highly heterogeneous populations for their own consumption (Kugler and Godoy 1964, Bertero and Vazquez 2003). After one century of cultivation, *Helianthus annuus* L. and *H. petiolaris* Nutt. are considered naturalized components of the flora in this central temperate region of Argentina (Zuloaga and Morrone 1999).

The former Russia Federation, Argentina, China, France, Hungary, India, Romania, Bulgaria, and USA produce up to 83% of the world's sunflower seed production (USDA 2007). Argentina is one of the three largest producers, where it is the fourth most important grain crop, with a cultivated area varying between 2 to 5 million ha (SAGPyA 2008). Argentine farmers show an intense adoption of genetically modified (GM) crops (James 2006), but no sunflower GM varieties are currently available. Wild relatives came to the attention of the National Committee of Agricultural Biotechnology (CONABIA) when seed companies requested the authorization to test and commercialize GM sunflower varieties. The evaluation of risks of transgene escape and eventual environmental impact assessments are required before the releasing of GM varieties in any crop (www.sagpya.mecon.gov.ar/biotecnologia).

Wild *Helianthus* distribution in Argentina was difficult to estimate by herbaria specimens. More than three quaters of about three dozen of *Helianthus* specimens available at the Darwinion Institute, Instituto Multidisciplinario de Biología Vegetal and Herbario del Museo de La Plata are annuals collected in opens spaces of Argentina, but erroneously or incompletely classified (Table 1-2). Seven specimens, misclassified as *H. petiolaris* did not show the characteristic features of hispid leaves - twice as long as broad or more, narrow parallel bracts (≤ 4 mm), white hairs on the tips of disc central pales – which are determinant of the species (Seiler and Rieseberg 1997). These specimens more clearly fit the description of Heiser (1978) of wild or weedy *H. annuus* (Table 1-3)[2]. Non cultivated type of these species (wild or weedy) are expected to be branched, with small heads (disc diameter < 50 mm) and narrower phyllaries (less than 8.5 mm broad), as showed by these specimens. According this feathures, eighteen herbarium specimens could be classified as wild *H. annuus,* and eigth *H. petiolaris* were recognized (Table1-3).

Excluding the garden and experimental field specimens, the wild *H. annuus* were mainly collected outside central Argentina, in most cases in localities whithout actual populations (Table 1-3). Only two of those populations, at Diamante and Río Cuarto, posess collected specimens. In the last locality, the specimen was gathered three decades after Báez and Macola (1954) found a wild population growing there. In the case of *H. petiolaris*, all the examined specimens were collected in the present day area of distribution, but the first specimens were collected nine years after Covas (1966) determined their presecence in Catriló, in a region where it was not present before 1943 (Cabrera 1945).

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[2] In February 2008 Drs. G. Seiler and Ch. Heiser, by observation of digital photographs sent by M. Cantamutto, confirmed the identification of the specimen 17251 as wild *H. annuus* (Table 1-3). By the same procedure, in May 2008 Dr. Heiser also suggested a hybrid origin to the specimen 71246.

Table 1-2 Information data of *Helianthus* specimens deposited in three Argentine herbaria, ordered by year of collection.

COLLECTION DATA					ARCHIVE INFORMATION			
Year	Site	Prov[1]	Habitat information	Collector/s	Herbaria[2]	Code	Spp. [3]	Determined by
1907	Los Cocos	COR	nd	Stukert T.	CORD	17251	PET	Ariza Espinar
1929	Cosquin	COR	nd	Rodrigo A.P.	LAPL	4644	nd	nd
1929	Cosquin	COR	Roadside	Rodrigo A.P.	LAPL	71241	HEL	Cabrera
1929	Elizalde	BA	nd	Cabrera A.L.	LAPL	71246	ANN	Collector
1932	La Plata	BA	Experimental field	Cabrera A.L.	LAPL	72144	ANN	Collector
1940	Villa Angela	CHA	nd	Boffa P.	LAPL	32088	ANNc	Collector
1943	VillaOrtuzar	BA	Experimental field	Duplu M. (seed imported by L. Parodi)	LAPL	57744	HEL	Hunziker A.T.
1945	Alemania	SA	40 cm heigh	Abbiatti D., Claps L	LAPL	55617	nd	nd
1945	Tigre	BA	nd	Lanfrachini A.E.	LAPL	55146	ANNc	Delucchi
1947	Olivos	BA	Domestic garden	Strenstra B.	LAPL	93046	*CUC*	nd
1952	S. Isidro	BA	Experimental field	Burkart A.	DAR	19997	TUB	Collector
1960	Diamante	ER	Sub-spontaneus	Burkart A.	DAR	B 22344	ANN	Collector
1960	Diamante	ER	Sub-spontaneus	Burkart A.	DAR	B 22320	ANN/PET	Collector
1962	La Toma	ER	Sub-spontaneus	Burkart A., N. Troncoso, N. Bacigalupo	DAR	B 23879	ANN	Collector
1962	La Paz	ER	Crop in farm	Burkart A., N. Troncoso, S. Crespo, N. Bacigalupo	DAR	B 23574	ANN	Collector
1963	G.Pedernera	SL	Roadside	Hunziker A., P. Maldonado	CORD	16195	PET	Heiser in litt.
1963	Catrilo	LP	nd	Cano	LAPL	Cano 2808	PET	Delucchi
1965	Diamante	ER	Abundant in gorge	Burkart A., N. Troncoso	DAR	B 26401 b	HEL/PET	Collector
1966	G.Pedernera	SL	Roadside, sandy soil	Anderson D.L.	LAPL	And. 1034	PET	Troiani
1967	S.Fernando	BA	Disturbed land near river	Burkart A.	DAR	26635 a	HEL	Collector
1967	S.Fernando	BA	Disturbed land near river	Burkart A.	DAR	26635 b	HEL	Collector
1969	Toledo	COR	Roadside	Subils R., L. Artico	CORD	992	PET	Ariza Espinar
1972	Mirapampa	BA	nd	Steibel J.	CORD	2206	PET	Collector
1972	Catril-co	LP	Roadisdes, sandy soils	Kiesling R.	LAPL	Kies. 132	PET	Delucchi
1973	Toledo	COR	Abuntant in roadside	Ariza Espinar L.	CORD	LAE2861	PET	Collector
1973	Diamante	ER	In port´s gorge	Burkart A., N. Troncoso, N. Bacigalupo, S. Botta	DAR	B 29548 a	PET	Cabrera
1973	Diamante	ER	In port´s gorge	Burkart A., N. Troncoso, N. Bacigalupo, S. Botta	DAR	B 29548 b	PET	Collector
1976	G.Pedernera	SL	Roadside, sandy soil	Anderson D., H. Molinero, E. Villar, P. Namur	CORD	2992	PET	Collector
1977	Union	SL	nd	Cordo H.	DAR	96041	HEL	Collector
1978	O. de Agua	SE	nd	Hunziker A., R. Subilis, L. Bernardello	CORD	23167	ANN	Ariza Espinar
1979	G.Pedernera	SL	nd	Hunziker A., R. Subilis, L. Bernardello	CORD	23369	PET	Ariza Espinar
1979	G.Pedernera	SL	nd	Hunziker A., R. Subilis, L. Bernardello	CORD	23371	ANN	Collector
1979	S.J.Cachi	SA	nd	Cabrera A., S. Botta, C. Ezcurra, A. Rotman, A. Saenz, N. Troncoso, F. Zuloaga	DAR	30772	HEL	Collector
1979	V.Mercedes	SL	Roadside	Cabrera A.L.	LAPL	CAB 30169	HEL	Collector
1981	R.Cuarto	COR	nd	Hunziker A., R. Subilis	CORD	24154	ANN	Collector
1993	Toledo	COR	Group near railroad	Ariza Espinar L.	CORD	LAE3164	PET	Collector
1999	Isla M.García	BA	Escaped form cultivation	Hurrell J., Ulibarri E., Jankowski, Bonavía A.	LAPL	4147	ANNc	Collector

[1]Prov(Province) code

BA= Buenos Aires
SA= Salta
COR= Cordoba
SE= S. del Estero
ER= Entre Ríos
SL= San Luis
CHA= Chaco

[2]Herbaria identification

DAR = Instituto de Botánica Darwinion
CORD = Inst. Multidic. de Biología Vegetal
LAPL = Herbario Instituto del Museo UNLP
nd = not determined

[3]Spp. Classification

PET= *H. petiolaris*
ANN= *H. annuus*
TUB= *H. tuberosus*
ANNc= Cultivated ANN
INT= Intermediate
ANNw= Wild ANN
CUC= *H. cucumerifolius*
HEL= *Helianthus*

Table 1-3 Adjusted classification according to Heiser (1978) by morphological traits (IBPGR 1985) of the specimens shown in Table 1-2

Identification			Branch	Leaf		Bract		Head			**Classification**	
Herbaria[1]	**Code**	**Year**		Shape	Margin	Wide[2]	Shape	Coulour	Diam[2]	Hears[3]	**Spp.**[1]	**Agreement**[4]
CORD	17251	1907	Yes	Deltoid	Entire	6.5	Rounded	nd	25	No	**ANNw**	No
LAPL	4644	1929	No	Deltoid	Dentate	2	Convergent	nd	17	No	**ANNw**	No
LAPL	71241	1929	Yes	Cordate	Dentate	3.5	Convergent	nd	22	No	**ANNw**	No
LAPL	71246	1929	Yes	Cordate	Undulate	7	Convergent	nd	50	No	**ANNh**	No
LAPL	72144	1932	No	Cordate	Dentate	nd	nd	nd	45	No	**ANN**	Yes
LAPL	32088	1940	No	Deltoid	Undulate	nd	nd	nd	42	No	**ANNc**	Yes
LAPL	57744	1943	Yes	nd	Undulate	5	Rounded	nd	25	No	**ANNw**	No
LAPL	55617	1945	Yes	Deltoid	Dentate	8	Convergent	nd	26	No	**ANNw**	No
LAPL	55146	1945	No	Cordate	Undulate	nd	nd	nd	50	No	**ANNc**	Yes
LAPL	93046	1947	Yes	Cordate	Undulate	3	Convergent	nd	22	No	**ANNw**	Yes
DAR	19997	1952	Yes	Ovate	Crenate	4.5	Convergent	Yellow	25	No	**TUB**	Yes
DAR	B 22344	1960	No	Cordate	Dentate	nd	nd	nd	70	No	**ANNc**	No
DAR	B 22320	1960	Yes	Deltoid	Dentate	4.5	Rounded	nd	30	No	**ANNw**	No
DAR	B 23879	1962	No	Ovate	Dentate	nd	nd	nd	nd	nd	**ANN**	Yes
DAR	B 23574	1962	No	nd	nd	nd	nd	nd	>100	No	**ANNc**	No
CORD	16195	1963	Yes	Lanceolate	Undulate	6	Paralell	nd	30	Yes	**PET**	Yes
LAPL	Cano2808	1963	Yes	Lanceolate	Dentate	2	Paralell	Yellow	20	Yes	**PET**	Yes
DAR	B 26401 b	1965	Yes	Cordate	Dentate	6	Rounded	Yellow	30	No	**ANNw**	No
LAPL	And.1034	1966	Yes	Lanceolate	Dentate	2	Paralell	nd	18	nd	**PET**	Yes
DAR	26635 a	1967	Yes	Deltoid	Undulate	7	Rounded	Yellow	35	No	**ANNw**	No
DAR	26635 b	1967	Yes	Lanceolate	Entire	6	Rounded	Yellow	30	No	**ANNw**	No
CORD	992	1969	Yes	Deltoid	Entire	7	Convergent	nd	22	No	**ANNw**	No
CORD	2206	1972	Yes	Lanceolate	Undulate	nd	nd	nd	25	Yes	**PET**	Yes
LAPL	Kies.132	1972	Yes	Lanceolate	Dentate	2	Paralell	nd	18	Yes	**PET**	Yes
CORD	LAE2861	1973	Yes	Cordate	Dentate	6	Convergent	Purple	35	No	**ANNw**	No
DAR	B 29548 a	1973	Yes	Lanceolate	Dentate	6	Rounded	Yellow	25	No	**ANNw**	No
DAR	B 29548 b	1973	Yes	Deltoid	Dentate	5	Rounded	nd	25	No	**ANNw**	No
CORD	2992	1976	Yes	Lanceolate	Undulate	3	Paralell	nd	23	Yes	**PET**	Yes
DAR	96041	1977	Yes	Deltoid	Dentate	3.5	Convergent	nd	30	Yes	**INT**	Yes
CORD	23167	1978	No	Cordate	Dentate	nd	nd	nd	50	No	**ANNc**	No
CORD	23369	1979	Yes	Lanceolate	Undulate	3	Paralell	nd	22	Yes	**PET**	Yes
CORD	23371	1979	Yes	Deltoid	Dentate	4	Paralell	nd	43	No	**ANNw**	No
DAR	30772	1979	Yes	Cordate	Undulate	nd	Rounded	nd	30	No	**ANNw**	No
LAPL	CAB 30169	1979	Yes	Lanceolate	Dentate	4	Paralell	nd	15	Yes	**PET**	No
CORD	24154	1981	Yes	Deltoid	Crenate	7	Rounded	nd	25	No	**ANNw**	No
CORD	LAE3164	1993	Yes	Cordate	Undulate	6	Rounded	nd	32	No	**ANNw**	No
LAPL	4147	1999	No	Cordate	Undulate	nd	nd	nd	55	No	**ANNc**	Yes

[1] Abreviations as in Table 1-2. **ANNh** = Wild x cultivated *H. annuus*

[2] Bract wide and head disc diameter (diam) in mm

[3] Presence of white hears in the center of the disc

[4] Agreement between herbaria classification and the new determination

A two year exploration was started in 2000 as required by CONABIA to produce a complete assessment of the distribution area of the wild *Helianthus* in Argentina (Poverene et al. 2002). The wild *H. annuus* and *H. petiolaris* were found naturalized and growing extensively in seven Argentine provinces. The area involved, partially coinciding with the central environment of the production area where the sunflower crop (de la Vega and Chapman 2006), is grown between 31° 20' S to 38° 42' S latitude and 60° 38' W to 68° 32' W longitude (Figure 1-1).

The origin and mode of spreading of the wild annual *Helianthus* in central Argentina is unknown. Earliest reports indicate that wild *H. annuus* was intentionally introduced before 1948 in the Cordoba province. Bauer (1991) pointed that the wild genetic resources used at Manfredi Experimental Station by sunflower breeders Báez and Macola (1954) was obtained by a forage experiment conducted in the Rio Cuarto area[3]. Later, *H. petiolaris* was discovered in Catrilo, La Pampa province in 1954 (Covas 1966) probably due to an accidental introduction as a contaminant of forage seeds imported from Texas (M.Sc. A. Luciano, pers. comm.). Another route of entry may have been the introduction of wild species from the USA as germplasm sources intended for breeding programs from 1960 to 1986 (Kinman 1964, Luciano 1964, Seiler and Rieseberg 1997), which escaped and later became naturalized in the central area of the country. There is no evidence that dedomestication can originate wild *H. annuus* populations by endoferality as farmers suspect (Bervillé et al. 2005). Although a volunteer *H. annuus* var. *macrocarpus* was described by Cabrera (1974), all the stable wild populations clearly classified as wild *H. annuus* by several morphological traits (according to Heiser 1978) and no established populations of volunteers were found. However, due to the proximity of weedy relatives to sunflower crop in Argentina; it is entirely possible for wild to crop (volunteers) gene flow to have produced feral populations via exoferality process (Gressel 2005).

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[3] Both Báez and Mácola (1954) as Giordano and Senin Garcia (1967) refer to the wild genetic resource incorporated in the crosses, under the former name of *H. cucumerifolius*. Posterior manuscripts, written by ex-collaborators of the same breeding program, referred to the resource found in Rio Cuarto, Córdoba province, as wild *H. annuus* (Bauer 1992) or *H. annuus* ssp. *annuus* (Bertero and Vazquez 2003).

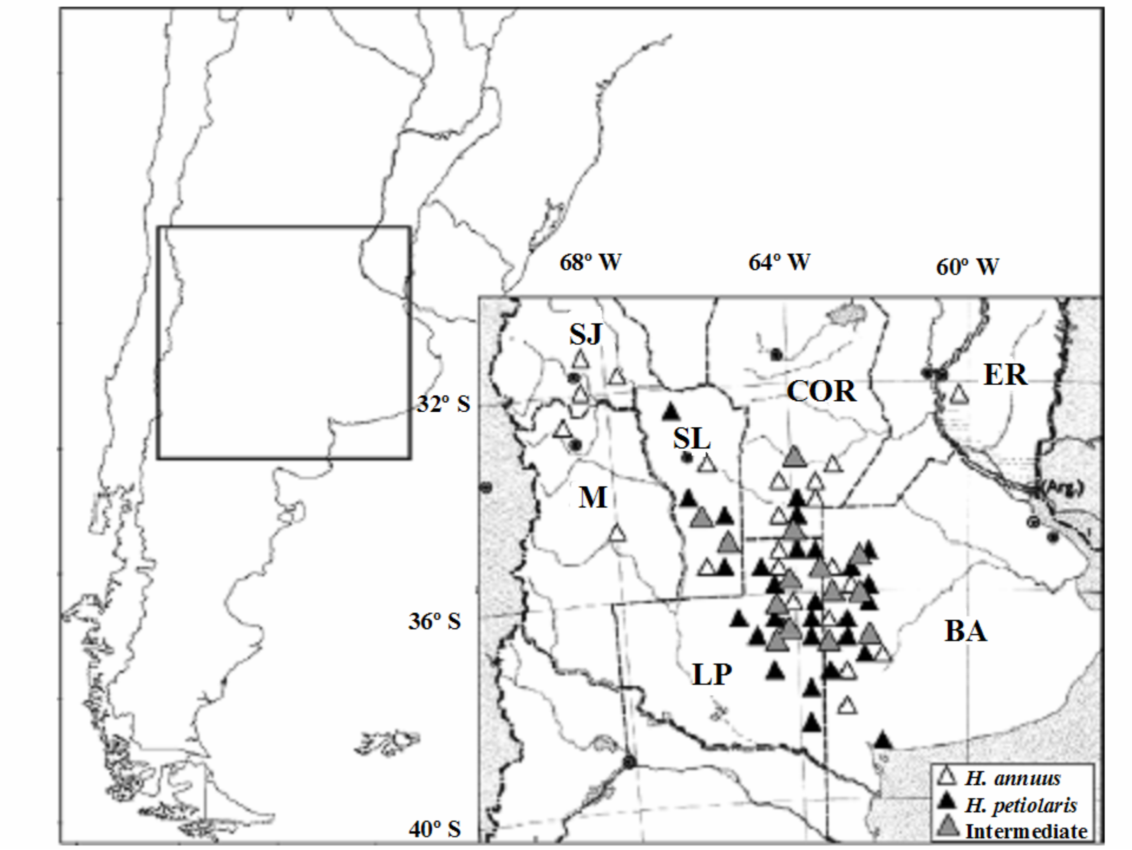

Figure 1-1 Wild sunflowers distribution in central Argentina (From Poverene et al. 2006).

Each triangle indicate a provincial county where the species is present in provinces of Buenos Aires (BA), La Pampa (LP), Córdoba (COR), Entre Ríos (ER), San Luis (SL), Mendoza (M) and San Juan (SJ) .

Volunteers originated from seed that falls to the ground before and after harvest and during grain transportation, are common in all agroecosystems where the sunflower crop is present (Cabrera 1963, Robinson 1978a, Marzocca 1994). These undesirable plants never constitute stable populations and can be controlled easily by cultural and chemical strategies (Robinson 1978b, Lyon et al. 2007). Volunteers are advanced generations of sunflower cultivars, generally top branched, with main head (Faure et al. 2002). These plants differ from the common sunflower and constitute a serious problem in some crop rotations (Anderson 2007).

Wild *H. annuus* shows higher competitive capacity and reproductive plasticity, causing great interference in soybean (Geier et al. 1996), wheat, sorghum (Rosales-Robles et al. 2002, 2005), and corn (Deines et al. 2004) crops. Due to the risk of spreading, the wild, weedy and feral *H. annuus* populations are under observation in other invaded regions of the world (Bervillé et al. 2005, Müller et al. 2006, Vischi et al. 2006, Stanković-Kalezić et al. 2007). Argentina seems to be the only country where *Helianthus petiolaris* is naturalized. Both annual invader *Helianthus* species, with ruderal strategies (Grime 1974, Kolar and Lodge 2001), are not yet considered weeds in the central area of this South American country.

Crop to wild interaction and plant invasions are biological processes of concern under agriculture perspective (Booth et al. 2003, Inderjit 2005). Weed and weedy relatives can exchange genes with the crop and evolve into new forms under the selective forces imposed by environmental, ecological and anthropogenic factors of the agroecosystem (Harlan 1992). Introgression between crop cultivars and their relatives is an ongoing process affecting the genetic diversity of several grain crops as canola (*Brassica napus* L.), maize (*Zea mays* L.), pearl millet (*Pennisetum glaucum* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L. Moench) and their wild relatives (Arriola and Ellstrand 1996, Jarvis and Hodgkin 1999, Ellstrand 2003, Chapman and Burke 2006a, Wolfe and Blair 2007).

Wild-crop hybridization has a potential to influence the evolutionary ecology of related wild and weedy populations and little is known about the persistence or ecological effects of crop genes that enter wild populations via pollen movement. New combinations of genes resulting from hybridization between wild and their crop cultivars, followed by effective introgression, have been important in the evolution of several invasive plants (Ellstrand et al. 1999, Hancock 2005, Schmeller et al. 2005, Campbell et al. 2006, Hall et al. 2006). The range expansion of the *Helianthus* genus into their native distribution area has been in part explained by selection of extreme transgressive phenotypes generated after interspecific hybridization (Rieseberg et al. 2007). The gene flow between wild annual *H. annuus* and *H. petiolaris* and the sunflower crop, under natural selective forces of the central Argentine agroecosystems, could have created new specie variants that would merit attention because of their evolutionary implications.

Cultivated sunflower and its wild progenitor, *H. annuus* show high rates of hybridization, and cultivar genes could persist in the wild populations up to 40 years (Linder et al. 1998). First generation of wild-crop hybrids had lower fitness than wild genotypes, especially when grown under favorable crop conditions, but this do not constitute a real barrier to the introgression of crop genes (Snow et al. 1998). Gene flow with nearby wild *H. annuus* facilitates the evolution and persistence of some weedy populations in USA (Kane and Rieseberg 2008).

Due to the fact that the risk assessment of wild *H. annuus* is of more immediate concern, little consideration has been given to the possibility that *H. petiolaris* hybridize with cultivated sunflower. In sites where these species grow in mixed stands, they produce inter-specific hybrids (Heiser 1947). Both species are diploids with n = 17 chromosomes, but the existence of several translocations causes plants to show low fertility at F1 generation, that could be recovered by backcrosses (Rieseberg et al. 1999). There is evidence that at least three stable annual species have originated from their hybridization (Rieseberg et al. 2003). Sympatric populations of *H. petiolaris* showed a low but detectable rate of introgression, indicating that the genome is permeable to introgression (Rieseberg et al. 1999). In a scenario where both annual *Helianthus* invaders interact with the sunflower crop (Poverene et al. 2004), there are opportunities to evolve through new species variants via gene exchange (Hancok 2005, Campbell et al 2006).

There is a continued need to collect, maintain, evaluate, and enhance wild *Helianthus* germplasm for future utilization in cultivated sunflower (Jan and Seiler 2007). Genetic resources of a crop consist of the total pool of genetic variability that exist in the same species or within species with which the crop plant is sexually compatible (Harlan and de Wet 1971, Maxted et al. 2006). Typically, wild relatives of crop plants usually are genetically much more diverse than cultivated lineages. Genetic diversity is thought to contribute to long term preservation of species by allowing them to adapt quickly to changes in their environment. Diversity in germplasm is also critical to crop breeding programs, but it has not been fully exploited (Harlan 1976).

The genetic diversity of the wild and weedy species can make a significant contribution to sunflower in developing countries by providing genes for resistance (tolerance) to pests and environmental stresses, allowing the crop to become and remain economically viable (Thompson et al. 1981). Wild and weedy species offer opportunities to increase genetic variability in domesticated sunflower through interspecific hybridization (Skoric 1992, 1993, Faure et al. 1999). The wild annuals *H. annuus* and *H. petiolaris* naturalized in central Argentina could constitute a distinct genetic resource with valuable genes for crop improvement.

The wild-weed relative complex of sunflower has demonstrated to be an important genetic resource useful to the crop. Disease resistance (Báez and Mácola 1954, Putt and Sackston 1957), insect resistance (Rogers 1992) and herbicide tolerance (Al-Khatib et al. 1998) are examples of successful transference of traits obtained from the wild ancestor *H. annuus* to the crop. Recently, a natural mutation of the Acetohydroxyacid synthase, detected under repeated herbicide spaying in a weedy population from Kansas, was transferred to create crop cultivars with herbicide tolerance to the iminazolinone family, commercially denominates Clearfield® (CL) (Miller and Al-Khatib 2002, Kolkman et al. 2004). The CL technology, released in the new millennium, has been extensively adopted by farmers from USA and Argentina (www.sunflowernsa.com, www.asagir.org.ar).

The wild relative complex of *Helianthus* genus has supported the modern sunflower hybrid seed industry in the last three decades. A remarkable source of cytoplasmic male-sterility (CMS) was obtained in France by Leclercq (1969) by an interspecific cross involving wild *H. petiolaris* as female and *H. annuus* as pollen donor. Other alternative CMS sources and some restorer genes have been developed by crosses involving the wilds *H. argophyllus* T. & G., *H. maximiliani* Scharader and *H. resinosus* Small, and a dozen of other wild species (Jan and Seiler 2007). In spite of the intense efforts to obtain useful sources, including the use of artificial mutagenesis, the traditional French CMS cytoplasm of wild *H. petiolaris*, codified as PET1 (Serieys 1991), is still the main progenitor of the most adopted commercial hybrids around the world.

Variability for oil concentration also exists in wild species. While oil concentration is lower in the wild species than in cultivated sunflower, backcrossing to cultivated lines quickly raises the oil concentration to an acceptable level. Reduced concentrations of saturated palmitic and stearic acids have been observed in a population of wild *H. annuus* (Seiler 2004). Wild sunflower species have been a valuable source of resistant genes for many of the common pathogens of cultivated sunflower. *H. petiolaris* is a major source of genes for Verticillum wilt (*Verticillium dahliae* Kleb.) resistance (Hoes et al. 1973) and downy mildew (*Plasmopara halstedii* (Farl.) Berl and de Toni) and rust (*Puccinia helianthi* Schwein) in sunflower. Resistance genes for these pathogens occur frequently in the wild annual species (Tan et al. 1992).

The knowledge of a particular habitat and adaptations of wild populations occurring therein can help to identify potential sources of genes for a desired trait. Based on the habitat of a population and its immediate environment, selection of potential accessions for a particular characteristic may become easier, more accurate and more efficient (Jan and Seiler 2007). Heiser (1947) reported that *H. annuus* occurred throughout most of North America on disturbed, mesic, heavy soils that are wet in the spring but that dry out by midsummer, while *H. petiolaris* occupied xeric, sandy soils in western North America. Rogers et al. (1982) described these species sharing a wide sympatric distribution. Geographic, environmental, ecological and anthropogenic forces conditioning the naturalization process of the two wild annual *Helianthus* in the central Argentine landscape are largely unknown.

Biotechnology offers the opportunity to overcome some important constrains or improve the quality of sunflower crop (Paniego et al. 2007). A widely acknowledged risk associated with transgenic crops is the possibility that hybridization with wild relatives will transfer transgenes having the capacity to persist in wild populations (Armstrong et al. 2005). It is imperative to predict the environmental impact if transgenes reach wild or feral relatives, taking as reference point the impact of plants developed by traditional breeding (Conner et al 2003). Wild relatives could represent an obstacle to the release of genetically modified (GM) sunflower varieties in the environment, because of the risk of their genetic modification via gene flow (Snow et al. 1998, 2003, OECD 2004). Introgression by vertical and diagonal gene transfer could result neutral or null genes depending on natural selection pressure (Michaud 2005, Chapman and Burke 2006b). If wild populations acquire transgenes for resistance to diseases, herbivory, environmental stress, or commonly used herbicides, they could become more abundant or invade previously unsuitable habitats. Targeted, statistically sound, rigorously conducted, multi-trophic studies are needed to correctly assess the possible risks of GM crops before release into commerce (Clark 2006).

Objectives:

The objectives of this thesis were to answer the following main questions:

1. Is it possible to identify the existence of natural forces that drove the colonization process of the wilds annuals *H. annuus* and *H. petiolaris* into the landscape of central Argentina?

2. Is it possible to demonstrate the existence of geneflow between the wild annuals *H. annuus* and *H. petiolaris* and sunflower crop in the central Argentine scenario?

3. Is it possible to consider the wild *H. annuus* from Argentina as a new genetic resource with potential interest for the sunflower crop?

4. Is it possible to identify traits of interest for the sunflower crop in the wild *H. annuus* from Argentina?

5. How could biotechnology and GM sunflower varieties impact agroecosystems and the sunflower agro-industrial process?

Scope:

This thesis has been organized in three general and nine specific chapters. Chapters 2, 4, 6 and 10 have been published in referred and indexed journals. Chapter 7 has been accepted by a specialized journal. Chapters 5 and 9 have been published in the proceedings of the 16th and 17th International Sunflower Conferences. Chapters 3 and 8 have been submitted to high impact journals.

Chapter 1 is a general introduction to the theme of the study: the two wild annual *Helianthus* species naturalized in the central area of Argentina: *H. annuus* and *H. petiolaris*.

A chapter 2 and 3 studies the dispersion process of both wild *Helianthus* species under natural conditions.

Chapters 4, 5 and 6 focus on the gene flow between the wild *H. annuus*, *H. petiolaris* and sunflower crop, also under natural conditions.

Chapter 7 described the ecology of the wild annual *Helianthus* naturalized in the central area of Argentina.

Chapter 8 and 9 demonstrates the potential genetic value of the biodiversity contained in the wild *H. annuus* from Argentina by means of comparison to seventeen populations imported from North America.

Chapter 10 analyzed the advances in research and the changes in the intention to release transgenic (GM) sunflower, which significantly decreased after the demonstration of intense gene flow between wild populations and crop, documented in this thesis with data and presented in Chapters 4 to 6.

Chapters 11 and 12 contain a general discussion and conclusions of the research.

The author visited all the sites described in this thesis several times during 2000 to 2008 years, travelling more than 53,000 km in 42 explorations trips to collect the data analysed in Chapters 2, 3 and 7. He located several previously unknown populations of wild *H. annuus* in Buenos Aires and San Juan provinces and detected the misidentification of a population from Diamante. Also the author, alone or with collaborators, annotated the relevant herbaria specimens of Argentina (Chapter 1) and collected all the samples used in the research of this thesis, including the off-type plants (Chapter 4), wild and cultivated plants flowering coincidentally with the others taxa (Chapters 5 and 6). Chapters 8 and 9 are based in research done by students under the author’s supervision. Chapter 10 is based in a manuscript written by the author as a graduate course requirement at UdL.

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Chapter 2 Multi-scale analysis of two annual *Helianthus* species naturalization in Argentina

2008

Agriculture Ecosystem Environment 123:69-74

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Abstract

The localization of two wild annual *Helianthus* species recently established in Argentina *H. annuus* and *H. petiolaris* was found to be connected with climate, soil, and agro-ecosystem variables. The habitats of both species, being mainly roadsides, were strongly related to disturbance, but they were also found in riparian areas and within crops. Microhabitat conditions allowed clear differentiation among the species' preferences; *H. petiolaris* appeared associated with sandy soils with low organic matter content, while *H. annuus* showed preference for more fertile and fine textured soils. In the western region of the country, irrigation strongly modified the environment favouring *H. annuus*. No variables allowed characterization of the three sites where both species grew together.

Key Words: Invasive species, *Helianthus annuus*, *Helianthus petiolaris*, Habitat, Soil properties.

Introduction

Two wild annual species of *Helianthus* (Asteraceae) native to North America have been naturalized in central Argentina in the last sixty years. Cabrera (1963) and Covas (1966) found *H. petiolaris* Nutt. in La Pampa and Buenos Aires provinces at the end of the 1950's. But only recently have stable populations of wild *H. annuus* L. been recognized (Poverene et al. 2002). Wild *H. annuus* causes great interference in soybean (Geier et al. 1996), wheat, sorghum (Rosales-Robles et al. 2002, 2005), and corn (Deines et al. 2004) crops. The wild or wild-derivative *H. annuus* is also an invader in Australia (Dry and Burdon 1986) and Europe (Faure et al. 2002) whereas a related annual species, *H. argophyllus*, has been found naturalized in Africa (Quagliaro et al. 2001).

Heiser (1947) reported both sunflower species as differing in the habitats where they were found in the centre of origin. *H. annuus* occurred throughout most of North America on disturbed, mesic, heavy soils that are wet in spring but dry out by midsummer, while *H. petiolaris* occupied xeric, sandy soil in western North America. In sites where these species grow in mixed stands, they produce inter-specific hybrids (Heiser 1947, Rieseberg et al. 1999). There is evidence that at least three stable annual species have originated from their hybridization (Rieseberg et al. 2003). In Argentina, both annual *Helianthus* invaders interact with the sunflower crop (Poverene et al. 2004).

The annual *Helianthus* species invasive process in Argentina has three merits that help to understand the colonization driving forces. Both wild sunflowers are crop-related species, the invasive process is relatively recent and the Argentine agro-ecosystem is still growing in complexity and scale. The goal of this work was to find relationships between climate, soil, agro-ecosystem, and the localization of the two wild annual sunflower species in Argentina.

Methods

The plant populations of the two annual *Helianthus* species studied fulfilled two main criteria. To be considered, they must had been observed at least for two different years at the same place or within few meters apart, and contain more than two dozen individuals. Wild species were identified as *H. annuus* ssp. *annuus* and *H. petiolaris* ssp. *petiolaris* based on morphological traits as previously described (Poverene et al. 2002). Neither volunteer sunflowers characterized by a main head and top branching (Types 0-3 according to Hockett and Knowles 1970) and ephemeral life, nor wild isolated and hybrid plants were considered.

The study area was comprised of ten Argentine provinces: Buenos Aires, Santa Fe, Entre Ríos, Chaco, Tucumán, San Juan, Mendoza, Córdoba, San Luis, and La Pampa. The survey on wild annual *Helianthus* populations was based on specialized systematic bibliography (Cabrera 1963, Covas 1966, Zuloaga and Morrone 1999) and weed science references (Marzocca 1994). Sunflower breeders and researchers provided valuable information about wild *Helianthus* population localization. Besides, the herbaria of Entre Ríos, Litoral and Tucumán universities were also examined for voucher specimens. The 33 exploration trips accomplished in summer and fall between 2000 and 2006 covered 40,442 km.

Latitude (LAT) and longitude (LON) were determined using a manual GPS system. The nearest locality in the province and county (provincial department) was used as reference to determine the altitude (ALT) variable from de Fina (1992).

The average annual rainfall (RAIN), mean temperature of the hottest (THOT) and coolest month (TCOL) of the nearest locality were also obtained from de Fina (1992). Seven populations from irrigated sites in San Juan and Mendoza provinces were not comparable with other populations, and were excluded when considering annual rainfall with the “rainfall-corrected” (RAINCO) variable.

Plant populations were found in three of the ecological regions described by Burkart et al. (1999): Pampa, Espinal, and Shrubs of Plateau and Plains. In the central temperate area of the country, rainfall decreases from more than 1000 mm in the east to less than 200 mm in the west. From the east, Pampa is a grass steppe without woody species, followed by Espinal, an intermediate savannah, with grasses and scarce xeric trees, mainly *Prosopis* spp. The western Shrubs of Plateau and Plains is an arid steppe with predominance of *Larrea* spp. and tough grasses.

The geographic coordinates of each population site were used to determine the cartographic unit according to the INTA (1990) soil atlas. Order, great group, suborder, and area of each soil type were registered. When sites were situated on complex cartographic units, including more than one soil type, the different soil types of that unit were computed. It included present (PRESENT) or potential (SUSCEP) wind erosion (EOLIC), present or potential water erosion (HYDRIC), lodging (LODGING), Na^+ excess (SODICITY), and sharp landscape due to slope (SHARP) and shallow soil (SHALLOW).

The "habitat" variable was determined by the position of each population site within the landscape. Those populations situated along the border of paved (PRSIDE) or dirt roads (DRSIDE), in roadside ditches, up to fences, and at the first unploughed meters into farm plots, were considered “roadside”. Whereas, when they were localized along the side of natural (rivers, streams) or artificial (channels) water courses disturbed by man or cattle, they were classified as “waterside” (WASIDE). The populations growing on saline areas, near halophytic species (*Atriplex* spp., *Distichis* spp., *Suaeda* spp.) were classified as “salt site” (SALT), but those populations found growing in cultivated lands, within crop, in the same row and/or between them, interfering with cultivated plants were classified as “crop” (CROP). However, if the wild plants seemed to be growing in the same line as the crop, as seed contaminants, they were not taken into account. Other habitats were also considered: rangelands, hills and forests.

Composite surface soil samples (0 - 15 cm) were collected at each site with wild sunflower plant stands. In the laboratory, the soil samples were air dried and sieved to < 2 mm. Standard procedures were used for general soil analyses. The soil pH (PH) was measured using a glass electrode pH-meter (soil : water, 1 : 2.5); organic carbon was determined by wet-chemical oxidation in a H_2SO_4 - $K_2Cr_2O_7$ mixture (Walkley – Black) and transformed into a soil organic matter (OM) value with the conversion factor of 1.72; available phosphorus (PPM) was extracted with 0.03M NH_4F + 0.025M HCl (Bray – Kurtz I) and measured photometrically with molybdenum blue. Texture analysis (% CLAY, SILT, SAND) on HCl and H_2O_2 treated and chemically [0.05M $(NaPO_3)_6$ and 0.15M Na_2CO_3] dispersed samples was carried out by a combination of sieving and pipette method; and calcareous content estimated (COCA) by the reaction which took place in the soil when adding 10% HCl. The soil Quality Index (SQI) was estimated by calculating the ratio between the soil organic matter content and the clay + silt contents (Pieri 1995).

The normality of the 14 defined continuous variables was evaluated using the Shapiro-Wilks test. Due to the lack of normality observed in at least one classification group for all the variables, the comparison of soil, climate, and geographic site conditions between pure *H. petiolaris* (PET), *H. annuus* (ANN) and both species mixed populations (MIX), was performed using Kruskal-Wallis non-parametric analysis. The continuous variables were studied using multivariate discriminant, principal component and cluster analyses. If necessary, variables were transformed by natural log or square root. The categorical variables were analysed using contingence tables and correspondence analysis (Lebart et al. 1984). InfoStat (2002) software was used for these statistical analyses and other tests indicated in each case.

Results

Seventy wild *Helianthus* populations were categorized, comprising 38 *H. petiolaris*, 29 *H. annuus* and 3 mixed stands.

Helianthus petiolaris tended to display a more southern distribution and lower altitude above sea level. However, the range for the latter was wider for *H. annuus* populations. The latitude and altitude of the mixed stands did not differ from both pure populations sites. Considering the mean rainfall (RAIN) and temperature of the hottest (TEHO) and coolest (TECO) months, January and July respectively, no differences were found in the species' localization. Excluding the populations that grew under irrigation in arid lands (RAINCO), *H. annuus* was present at more humid habitats than *H. petiolaris* (Table 2-1). Based on climate variables the ANN and PET population sites were grouped in 6 and 7 clusters respectively, and MIX stands in two groups using mean Euclidean distance. However, discriminant analysis of these 15 representative climates did not show a precise differentiation among them (Figure 2-1).

In drylands of Argentina, the two annual *Helianthus* species grow on a transitional boundary between two defined ecological regions. The median of the distance of both species populations to the boundary between the two eco-regions was less than 10 km, with a range of 48 km to the west for *H. annuus* and 130 km in both directions for *H. petiolaris*. *H. annuus* was more frequently found on Pampas, with sub-humid climate while *H. petiolaris* showed no preference between this eco-region and Espinal, which has semiarid conditions (less than 600 mm rainfall). The mixed stands appeared closer to the limit between both eco-regions. The seven populations found in irrigated areas belonged to the Shrubs of Plateau and Planes region but the environment was extensively modified by irrigation.

Table 2-1 Abiotic habitat characterization of wild sunflowers in Argentina.

The mean and range of stable 38 *Helianthus petiolaris* (PET), 29 wild *Helianthus annuus* (ANN), and mixed stand (MIX) populations in Argentina are showed. In each column, means followed by different letters differ according to Kruskal-Wallis non-parametric test ($p < 0.05$).

GEOGRAPHIC LOCALIZATION

	LAT (°S)	LONG (°W)	ALT (m.o.s.l.)
ANN	33.8b (32.0-37.5)	64.5 (60.6-68.4)	290a (14-609)
MIX	36.5ab (36.2-37.3)	63.7 (62.9-64.2)	142ab (112-169)
PET	36.3a (33.6-37.3)	63.6 (62.4-65.4)	174b (96-515)

CLIMATIC CONDITIONS

	Mean temperature (°C)		Water availability (mm)	
	TEHO	TECO	RAIN	RAINCO
ANN	24.2 (22.4-26.8)	8.5 (6.3-11.2)	605 (109-936)	735a (574-936)
MIX	24.3 (23.8-24.5)	7.5 (7.2-7.7)	580 (535-632)	580b (535-632)
PET	24.0 (22.9-25.0)	7.6 (6.6-8.2)	630 (457-752)	630b (457-752)

SOIL SURFACE CHEMICAL PARAMETERS (0-15 cm)

	PH[1]	PPM	OM (%)
ANN	7.4 (6.3 - 9.0)	32a (5 - 63)	3.2a (1.4 - 8.2)
MIX	7.0 (6.3 - 7.8)	44a (41 - 48)	3.2a (2.3 - 4.6)
PET	7.2 (6.2 - 8.3)	20b (7 - 48)	0.9b (0.2 - 2.9)

SOIL SURFACE PHYSICAL COMPOSITION (0-15 cm)

	CLAY (%)	SILT (%)	SAND (%)
ANN	11.8a (5.0 - 36.0)	28.7a (11.0 - 63.0)	59.5b (15.0 - 83.2)
MIX	8.3a (3.9 - 11.7)	24.1a (11.4 - 34.5)	67.5b (53.8 – 84.7)
PET	3.9b (1.3 - 10.1)	7.1b (1.3 - 17.5)	89.0a (73.7 - 97.0)

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[1] Nomenclature: PH= pH; PPM= available P in ppm; OM= percent of organic matter; LAT= latitude; LONG= longitude; ALT= altitude m over sea level; TEHO= mean temperature of the hottest month; TECO= mean temperature of the coolest month; RAIN= average rainfall; RAINCO= RAIN excluding 7 populations of irrigated areas (see text).

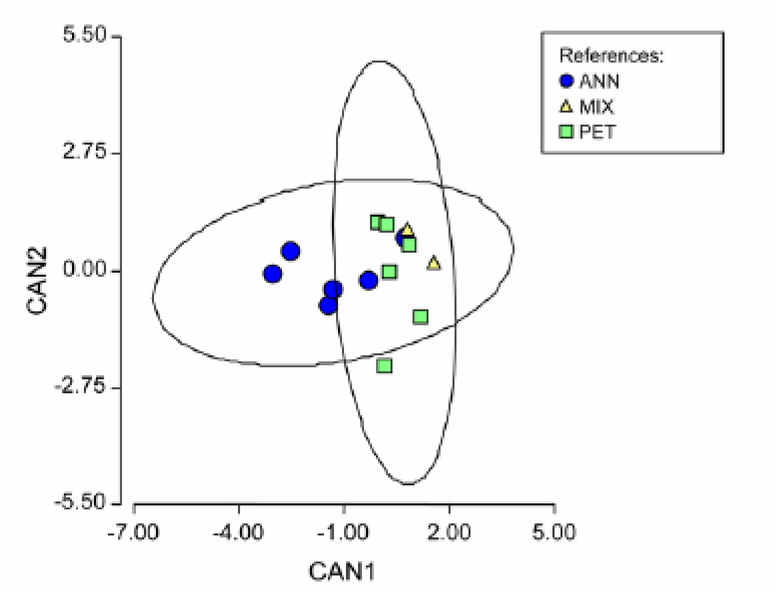

Figure 2-1 Discriminant analysis of 15 representative climates of wild *Helianthus annuus* (ANN), *Helianthus petiolaris* (PET) populations and mixed stands (MIX) in Argentina by means of transformed climatic variables.

Ellipses correspond to 95 % confidence for ANN and PET. First two eingenvalues explain 95 % of variance.

The provincial counties where wild *Helianthus* populations were found comprised 180 cartographic units covering about 30.5 million ha. *H. annuus* was found in 13 units of the Pampean region (11.5% of the area) and in two units of Cuyo region (4%), whereas *H. petiolaris* was found in 17 units of the Pampean region (30.4%). Their distribution was significantly different from the expected random distribution according to Poisson test ($p < 0.01$).

Helianthus petiolaris was located in environments where wind dominated as the natural erosive force in 76% of the sites, whereas wild *H. annuus* was situated in areas where water constituted the main erosive force of nature. There were no clear soil constraints related to the localization of mixed stands, but they were placed in sites with wind erosion susceptibility (Figure 2-2).

Over ten recognized soil orders in Argentina, only Mollisols and Entisols were found associated with both species: 21 *H. petiolaris* populations occurred on Mollisols, 5 on Entisols, and 12 on complex units comprising both orders. Eighteen *H. annuus* populations were found on Mollisols, 10 on Entisols, and one in a complex unit of both orders, whereas mixed stands were found on Mollisols. Chi square test showed highly significant differences in the frequency of both species' association with soil subgroups.

The habitat also defined the species' localization, although not so directly. *H. petiolaris* was frequently found associated with dirt roadsides and fences, whereas *H. annuus* was found on paved or dirt roadsides, riparian, and cultivated sites. Both *H. annuus* and *H. petiolaris* populations were found on fences or roadsides, the former showing a wider range of habitats. It was also found within crops, near water courses and in a few cases, in saline lands. The three mixed stands were found on roadsides and did not differ significantly from both pure populations. Wild *Helianthus* populations were never found in non-disturbed habitats such as forests, range or hills.

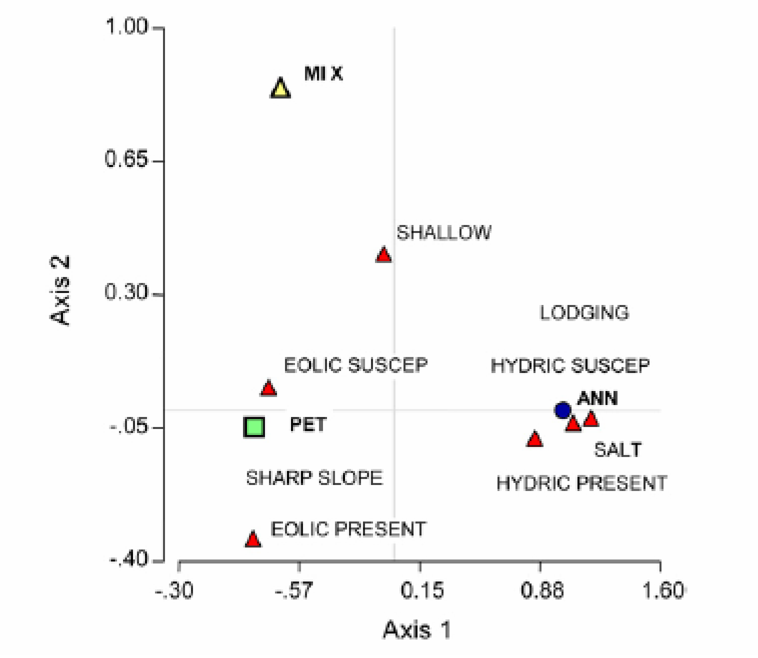

Figure 2-2 Main soil cartographic constraints (triangles, see text) determinant of the macrohabitat (centroids) of *Helianthus petiolaris* (PET), wild *Helianthus annuus* (ANN) and mixed stand (MIX) populations in Argentina according to Multivariate Correspondence analysis (See text). Axis 1 explains 95 % of variance.

Physical and chemical soil properties of the patches (microhabitats) colonized by the two annual wild *Helianthus* species in Argentine showed significant differences in PPM, OM, CLAY, SILT, and SAND content (Table 2-1). *H. annuus* was localized on more fertile, fine textured soils, with high available phosphorus level and OM content. Soil acidity (PH) was similar for both *H. annuus* and *H. petiolaris* sites. Five *H. petiolaris* and three *H. annuus* stands were found in sites with less than 10 ppm of available phosphorus, the lowest value of 5 ppm P for one *H. annuus* population in Buenos Aires province. Eight populations of *H. annuus* grew in soils with pH > 8.0, including an extreme case of pH = 9.0, while only four *H. petiolaris* populations were found in similar situations, with one recorded at pH = 8.3 as extreme value (Table 2-1). In the mixed stands, soil variables did not differ from those corresponding to *H. annuus* distribution (Table 2-1).

Helianthus petiolaris associated with sand, whereas *H. annuus* and mixed populations found better conditions in loamy and clayed textured soils. In fact, *H. petiolaris* populations were found on sandy soils, or sandy combined soils, with less of 2% OM in 95% of the sites. *H. annuus* established on finer textured soils, with less than 75% sand but in a wider range of OM content. More than 50% of the *H. annuus* populations were found on sandy loam soils, where a few *H. petiolaris* populations grew as well. Mixed stands occurred on sandy-loam and loamy-sand textural classes.

The OM content in microhabitats of *H. annuus* was significantly higher than that of the respective macrohabitat ($p < 0.05$), without a strong correlation between them (R^2 = 0.14 *). *H. petiolaris* patches showed a coarser texture in 23/38 populations, with predominance of sandy soils in macrohabitats of loamy and loamy-sand soils (21/23). There were no differences or correlation between OM content in microhabitat and macrohabitat (R^2 = 0.11 n.s.).

Discussion

In Argentina, wild *H. annuus* was used as forage in Cordoba province before 1949 (Bauer 1988) and after that, for sunflower crop breeding (Seiler and Rieseberg 1997; Bertero and Vazquez 2003). Since then, it probably escaped from cultivation. *H. petiolaris*, was probably introduced before 1956 as a contaminant of forage seeds (Covas 1966), most likely in sorghum samples imported from Texas, where the prairie sunflower grows (Dr. A. Luciano pers. comm.).

Wild *H. annuus* and *H. petiolaris* showed strong adaptation between 31° and 38° S latitude, 60.6° and 65.4° W longitude, which was the central temperate mega-environment favourable for sunflower crop (de la Vega and Chapman, 2006). Both species grew in a range of 457 to 936 mm rainfall (excluding irrigated areas), 22.4 to 26.8 °C and 6.3 to 11.2 °C for the hottest and coolest months mean temperatures, respectively.

Wild populations were never found in natural, undisturbed habitats. *H. petiolaris* was found in soil microhabitats where sand predominates, while *H. annuus* patches were associated with silt and clay.

Once introduced into the country, probably in the boundary region, both species spread over the areas which met the appropriate conditions, following a patchily distribution. The wild *H. annuus* could have arrived as seed contaminant or transported along the railways from the east to the irrigated areas of western Argentina. This wild species will probably continue spreading between the two west provinces, because they have connected irrigated areas. On the other hand, *H. petiolaris* has spread over the sandy soils of the central core area.

No variables were found to characterize mixed stands, which could take place at any site into the diffusion areas of both species in Argentina. The co-occurrence in mixed stands created similar conditions for hybridization as in the centre of origin (Rieseberg et al. 1999) that would allow the generation of new ecotypes.

Acknowledgements

We are in debt to Ing. Raúl Horacio Rodriguez (INTA) who kindly guided the first exploration trips. Daniel Alvarez, Carlos Sasovsky and José Gutierrez (INTA), Aurelio Luciano (Private breeder), and botanists from Entre Ríos (UNER), Litoral (UNL), and Tucumán (UNTu) are also very much appreciated for helping. Drs. Alicia Carrera (UNS), Mercedes Echeverria (UNMdP), Ings. Soledad Ureta, Agustina Gutierrez, and Alejandro Presotto (CONICET) helped with the sample collection. Lics. Silvia Luis and Alicia Hernandez (UNS) kindly helped with data treatment. Dr. Nilda Amiotti kindly gave advice for soil taxonomy interpretation.

Thanks to Dr. Daniel Ball (OSU) for critical reading of manuscript.

To the Fundación Carolina, España for a fellowship to the first author MAC.

This research was supported by ANPCyT-PICT 089881 and UNS-PGI 24A106.

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Chapter 3 Migration pattern suggested by terrestrial proximity as possible origin of wild annual *Helianthus* populations in central Argentina.

2008

Submitted to Biological Invasions

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Abstract

Two wild annual *Helianthus* species native to North America have become naturalized in the central agricultural lands of Argentina. This work explores the existence of a migration pattern of *H. annuus* and *H. petiolaris* by means of multivariate techniques and random tests. A minimum connection tree through road distance was created considering dispersal from a unique known entry point for each species. The proposed tree minimized, at the same time, the environmental and the ecological distances calculated by Euclidean and Gower indexes with abiotic and biotic habitat variables. The migration pattern suggests that after their introduction, both wild species moved in successive steps across a biotic and abiotic gradient, aided by human activity across the road connection infrastructure of central Argentina. No evidence of escapes from sunflower breeding stations was found, taking into account population and breeding program locations.

Key Words*:* Plant invasion, ferality, sunflower, weed community, random tests.

Introduction

Only a small proportion of exotic plant species introduced into a new region would have invasiveness and widespread in abundance. After introduction and naturalization, a successful plant invasion will happen if an adequate propagule pressure occurs into a vulnerable habitat (Huston 2004, Martinez-Ghersa and Ghersa, 2006). Usually, as the propagule transportation is promoted by human activity, the plant invader migration patterns present a tendency to follow human transportation routes (Boot et al. 2003). Soil and climate (Mann et al. 1999, de Blois et al. 2002, Sugiyama 2003, Retuerto and Carballeira 2004, Härdtle et al. 2005, Milberg et al. 2006) determine the environmental aptitude for plant invaders and their distribution into a geographic area. Environment and plant community structure could be used to estimate the habitat invasibility for a non native invader (Inderjit et al. 2005).

The Argentina croplands, located in an initially grassland plateau in the central part of the country, were devoted to agriculture after 1890 (Arriaga 1999). Since then, the intense farming activity has promoted the naturalization of several crop-associated weeds, mainly cosmopolitan unintentionally introduced by immigrants coming from Italy and Spain (Taylor 1997). Among others, two annual Asteraceae natives to North America, *Helianthus annuus* L. and *H. petiolaris* Nutt. have naturalized and widespread across the drylands and the western irrigated valleys of Central Argentina (Poverene et al. 2002).

The origin and distribution of the wild *Helianthus* in central Argentina are unknown. The earliest reports indicate that wild *H. annuus* was intentionally introduced before 1948 in Rio Cuarto, Cordoba province, with feeding purposes (Bauer 1991). *H. petiolaris* was found in Catrilo, La Pampa province, in 1954 (Covas 1984) probably introduced accidentally as a contaminant of forage seeds imported from Texas (M. Sc. A. Luciano, pers. comm.). After this, breeding programs in Argentina received wild *Helianthus* species imported from the USA as germplasm sources during the 1960 decade (Kinman 1964, Luciano 1964) and between 1976 and 1986 (Seiler and Rieseberg 1997). According to what farmers suppose, an intense gene flow from the crop to both annual wild *Helianthus* (Poverene et al. 2004, Ureta et al. 2008) made also possible the development of ferality (Gressel 2005). Dedomestication, escapes from the experimental fields or reiterate introductions could be the possible origin of the wild populations in central Argentina.

Wild and weedy sunflowers are ruderal species of which mechanical transportation seems to be the main distribution way. It has been suggested that bison (*Bison bison* Skinner and Kaiser) disseminated the sunflower into the natural distribution area, but road traffic appears to be the modern way for sunflower to spread into new regions (Heiser 2008). In central Argentina the wild populations grow in patches on human-disturbed habitats as firelines, roadsides, and water channels (Cantamutto et al. 2008). Wild, weedy and feral sunflower populations are of concern in invaded regions of the world (Bervillé et al. 2005, Vischi et al. 2006, Müller et al. 2006, Stanković-Kalezić et al. 2007) but no work has been done to understand the forces that drive this invasion process. Other interests concerning those taxa are the potential development of adaptive traits for sunflower breeding (Jan and Seiler 2007) and the risk and consequences of gene exchange with the crop, particularly with transgenic cultivars if they were released (Cantamutto and Poverene, 2007).

Here we explore the existence of migration patterns starting in a unique entry point for each of the two annual wild *Helianthus* invaders in central Argentina using multivariate analysis of environmental and ecological variables of known stable populations. If the habitat connection by ecological and environmental similarities were different from the minimum road connection, the hypothesis of mechanical dispersion from an entry point would be rejected. If the stable wild populations were located nearer the breeding stations where wild resources were used, the second hypothesis of no escapes would also be rejected. The objectives of this work were: 1) To test if the minimum habitat connection tree by road minimizes at the same time the changes occurred during the diffusion process in terms of environmental and ecological variables; 2) To test if the road connection from wild populations to sunflower breeding stations was shorter than from other wild populations.

Methods

The migration pattern of two non-native species in Argentina, *Helianthus annuus* and *H. petiolaris,* was estimated based on the habitat of nine and 13 populations of each species, which are the largest stable populations as well as representative of the diverse agro-ecological distribution in central Argentina (Table 3-1). These are considered the origin of a number of smaller populations and patches (Poverene et al. 2002) developing along roads connecting the habitats. Sometimes plants invade crop fields but always as a continuum of the roadside populations. The study comprises only populations found five or more years in the same place in the 2000-2007 collection trips. Isolated plants or small populations were not considered.

The latitude and longitude of each location were determined with a GPS system (Trimble, horizontal accuracy 1-3 m. differentially corrected, error HDOP $\leq$ 3). Geo-referenced populations were then overlayed on maps of estimated environmental (abiotic) habitat variables. These included altitude, average annual rainfall, and mean temperature of the hottest and coolest month (de Fina 1992). Soil sub-order percent into each soil cartographic unit, average organic matter content, and soil use capacity at every population site were obtained (INTA 1990).

Laboratory analyses of a composite surface sample (0 - 15 cm) of soil collected at each localization was used to estimate microenvironmental habitat variables, as described in Cantamutto et al. (2008).

The agro-ecological characteristics were estimated considering other native and non-native plant community species and the spatially co-occurring crops at wild sunflower habitat. In a survey conducted in February 2007, plant composition and richness were estimated at the location of each wild sunflower population following the method used by Clay and Johnson (2002). At each site, a 100 m by 25 m grid was established. At 10 grid nodes a 2 m^2 circle sample was taken and the relative dominance of each plant species was estimated with an ordinal scale (0 = absent, 5 = dominant). The same scale was used to estimate the dominant landscape representation of crops (wheat, corn, sorghum, soybean, peanut, fruit trees, pasture, and sunflower) in each agroecosystem (neighbouring 10 km of road) associated with the sampled populations.

Table 3-1 Nearest locality and size of representative stable populations used to estimate the diffusion pattern of two annual wild *Helianthus* in Argentina

Locality	Province	Eco-region[1]	Size[2]	Acronym
		Helianthus annuus (ANN)		
Río Cuarto	Córdoba	Espinal	***	RCU
Adolfo Alsina	Bueno Aires	Espinal	***	AAL
Colonia Barón	La Pampa	Pampa	***	BAR
Carhué	Buenos Aires	Pampa	**	CHU
Diamante	Entre Ríos	Espinal	**	DIA
Juarez Celman	Córdoba	Pampa	****	JCE
Las Malvinas	Mendoza	Monte	***	LMA
Media Agua	San Juan	Monte	***	MAG
Rancul	La Pampa	Espinal	*	RAN
		Helianthus petiolaris (PET)		
Catriló	La Pampa	Pampa	***	CAT
Colonia Barón	La Pampa	Pampa	***	BAR
Carhué	Buenos Aires	Pampa	***	CHU
Hilario Lagos	La Pampa	Pampa	*	HLA
Villa Mercedes	San Luis	Espinal	***	MER
Nueva Galia	San Luis	Espinal	***	NGA
Pellegrini	Buenos Aires	Pampa	***	PEL
Salliqueló	Buenos Aires	Pampa	***	SAL
Santa Rosa	La Pampa	Espinal	**	SRO
Trenque Lauquen	Buenos Aires	Pampa	***	TRE
Unión	San Luis	Espinal	*	UNI
Uriburu	La Pampa	Espinal	***	URI
Winifreda	La Pampa	Pampa	*	WIN

[1] Burkart et al. (1999). [2] Population size: *30-300; **301-3000; ***3001-30000; ****>30001

Procedure and statistical analysis

1) Model assumption for each species: If the diffusion occurred through seed transportation starting in an unique entry point, following terrestrial connection that link similar habitats in successive short steps, it would be possible to estimate a migration pattern accomplishing simultaneously three conditions: a) To minimize the total distance covered by road for terrestrial transport; b) To minimize the sum of environmental changes during the migration process. c) To minimize the sum of plant community changes during the migration process.

2) Distance estimation for each one of the three analysis dimensions: Environment and plant community analyses estimated habitat similarities under different dimensions calculated with geographic, environmental and ecological aspects of site variables (Table 3-2) grouped in layers of descriptive information. In each dimension, one habitat was more proximate to the other as the distance between both was shorter. Triangle matrices containing distance information between all pairs of habitats were calculated in the following dimensions: a) Terrestrial transport, estimated through road distances obtained from road maps and dirty roads in Argentina. b) Environmental dissimilarity, calculated through the Euclidean distance (Quinn and Keough 2005) with abiotic variables (Table 3-2) range-transformed and standardized, to avoid scale differences. c) Plant agro-ecosystem dissimilarities, calculated through the complement of Gower index (Gower 1971, Quinn and Keough 2005) considering the complete floristic composition in each habitat. The average dominance or importance of 27 co-occurring species and eight crop or agriculture land uses determined in each site were previously range-transformed and standardized.

3) Connection trees: The minimum connection trees by road distance were calculated using the IML procedure of SAS (2006). Likewise the total distance covered to connect all populations under the three analysis dimensions was calculated and the probability to obtain this value under random was estimated by the procedure described in 4).

4) Connection tree validation: A permutation test was performed in order to verify if the obtained tree was different from random. Fifty thousand paths were simulated through a specially designed macro to connect every habitat with the entry point, without limitation in the number of branches. Each simulation first began with a sorting on the connection order of each habitat with the growing tree. The first population linked to the

entry point, while each of the remainder was sorted to link with any of the populations already connected to the tree. For each simulated pathway the total distance was calculated using the three independent distance matrices. The Gaussian-shaped histograms of distance distribution frequencies were used to test the distance covered by each tree as different from random. In each histogram of path length distribution frequency, the critical values for $p \geq 0.01$ and $p \geq 0.05$ were determined for the least path length.

5) Analysis of the likely escape from experimental fields: The null hypothesis stated that if any population came from wild germplasm escapes in breeding stations, the road distance from the nearest experimental field (which could have used the wild germplasm) would be shorter that the distance to any other wild population. A new triangle matrix was constructed based on the road distance between wild *Helianthus* populations and the breeding stations. Clustering was based on single linkage.

The procedures CORRCAN, DISTANCE, IML, CLUSTER, UNIVARIATE and TREE of SAS (2006) were used to perform the statistical tests.

Results and Discussion

Poverene et al. (2002) showed that wild *Helianthus annuus* displayed a wide distribution across the central area while *H. petiolaris* was confined to a lesser area in central Argentina. In concordance, we found that *H. annuus* populations presented a high variability of abiotic parameters, whereas *H. petiolaris* showed a clear tendency to grow mainly in sandy soils, usually with less organic matter content and constrains for agriculture (Cantamutto et al. 2008). These facts were reflected in the environmental and agro-ecological parameters of the populations considered in the present study (Table 3-2).

For both species the minimum connection tree joining all populations by road distances was highly different from random at the environmental level (Table 3-3). At the agro-ecological level the connection trees for *H. annuus* and for *H. petiolaris* were different from random at $p \leq 0.05$ and $p \leq 0.01$ respectively. Connection trees for *H. annuus* and for *H. petiolaris* are shown in Figure 3-1 and Figure 3-2 respectively.

Table 3-2 Selected variables used to estimate the diffusion process of two annual *Helianthus* species in Argentina by multivariate analysis (means ± SD)

	Wild *H. annuus*	*H. petiolaris*
Localization		
Latitude (ºS)	34.6 ± 2.1	35.9 ± 0.9
Longitude (º W)	66.3 ± 2.5	64.0 ± 1.0
Environment		
Altitude (m.a.s.l.)	267 ± 202	199 ± 123
Hottest month temperature (ºC)	24.2 ± 1.1	24.3 ± 0.4
Coolest month temperature (ºC)	8.1 ± 1.5	7.7 ± 0.4
Rain (mm year^{-1})	591 ± 259	601 ± 78
Irrigation (mm year^{-1})	67 ± 123	0.0 ± 0.0
Soil unit organic matter (%)	2.2 ± 1.1	1.2 ± 1.1
Soil unit use capacity (1-7 scale)	4.1 ± 1.2	4.8 ± 1.2
Haplustolls (%)	13.3 ± 33.2	52.3 ± 40.0
Argiustolls (%)	20.3 ± 27.6	3.1 ± 11.1
Hapludolls (%)	7.1 ± 16.0	6.2 ± 22.2
Ustipsaments (%)	0.0 ± 0.0	16.9 ± 20.6
Torripsnaments (%)	22.2 ± 44.1	7.7 ± 27.7
Natraqualfs (%)	2.8 ± 5.5	6.15 ± 17.1
Microenvironment		
Available P (ppm)	30.4 ± 15.8	25.4 ± 13.0
Organic matter (%)	3.6 ± 1.0	1.6 ± 1.1
Clay (%)	13 ± 8	6 ± 3
Loam (%)	30 ± 13	14 ± 12
Sand (%)	57 ± 20	80 ± 15
Plant community		
Co-occurring plants (abundance 0-5 scale)		
Cynodon dactylon	0.89 ± 0.33	0.77 ± 0.44
Chenopodium albus	0.78 ± 0.67	0.92 ± 0.86
Sorghum halepensis	0.78 ± 0.67	0.92 ± 0.49
Melilotus albus	0.89 ± 0.60	0.23 ± 0.60
Centaurea solstitialis	0.67 ± 1.12	0.46 ± 0.97
Salsola kali	0.56 ± 0.73	0.92 ± 0.76
Eragrostis curvula	0.56 ± 0.53	0.69 ± 0.63
Associated crops (importance 0-5 scale)		
Sunflower	2.67 ± 1.41	1.23 ± 0.83
Wheat	2.11 ± 1.45	0.85 ± 0.80
Sorghum	1.56 ± 1.01	1.60 ± 1.60
Soybean	1.67 ± 1.32	1.23 ± 1.01

Table 3-3 Differences from random distribution on the connections between habitats of two wild annual *Helianthus* showed in Figure 3-1 and Figure 3-2, with road (km), environment (Euclidean) and plant community (Gower) distances

		Distance dimension		
		Road	Environment	Community
	Wild *Helianthus annuus*			
Total connection		2021	31.2	2.17
Significance		**	**	*
Upper limit at random	p ≤ 0.05	3195	37.5	2.20
	p ≤ 0.01	2874	35.1	2.07
	Helianthus petiolaris			
Total connection		870	31.3	2.85
Significance		**	**	**
Upper limit at random	p ≤ 0.05	1775	45.4	3.03
	p ≤ 0.01	1609	42.6	2.86

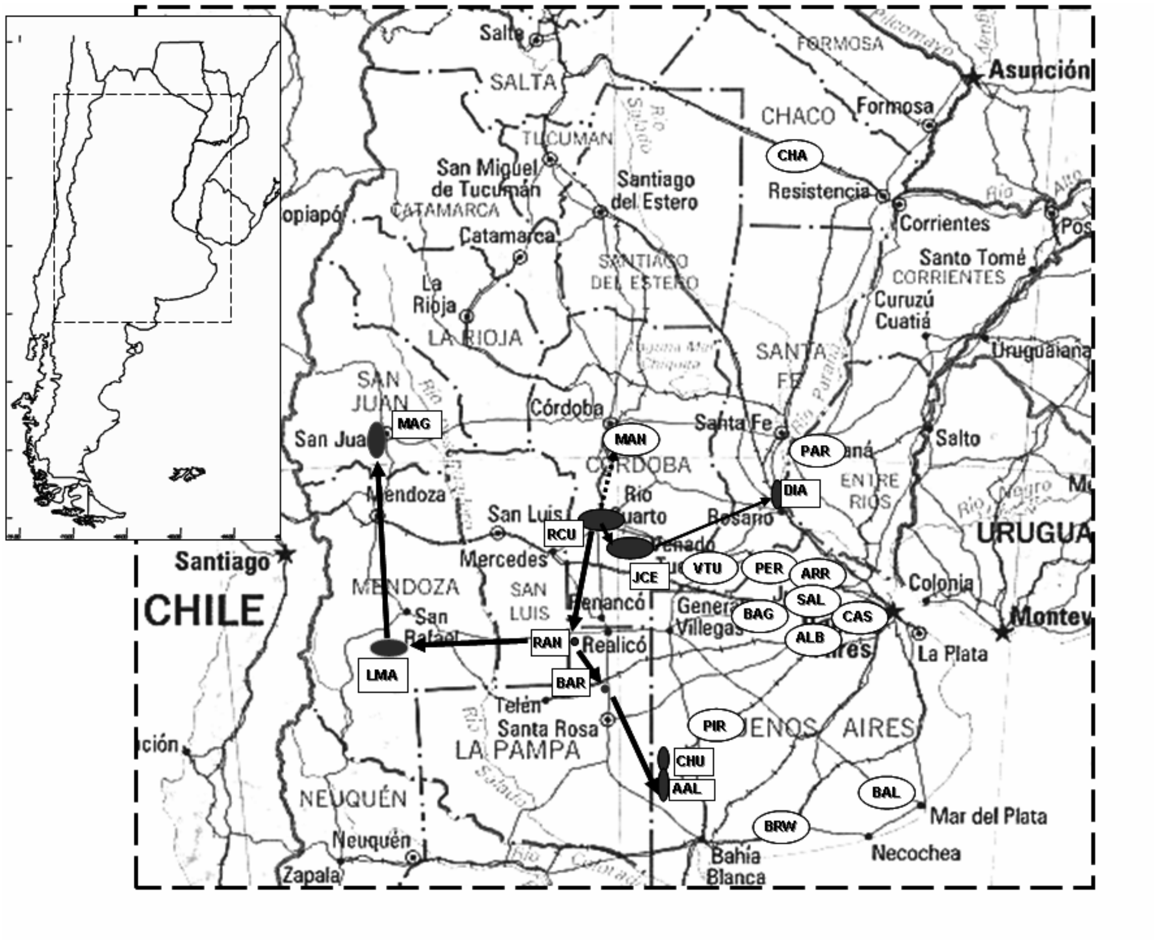

Figure 3-1 Migration pattern suggested by road connection, environmental, and plant community proximities between wild *Helianthus annuus* stable populations.

Population names as in Table 3-1. Symbol shapes indicate the main axis of the population area. The connection between Rio Cuarto and Diamante implies crossing the river (thin arrow). Breeding stations which likely used wild germplasm are located in Alberti (ALB), Arrecifes (ARR), Baigorrita (BAI), Balcarce (BAL), Barrow (BRW), Castelar (CAS), Charata (CHA), Manfredi (MAN), Paraná (PAR), Pergamino (PER), Pirovano (PIR), Salto (SAL), Venado Tuerto (VTU). MAN station used wild sunflower from RCU (dotted arrow).

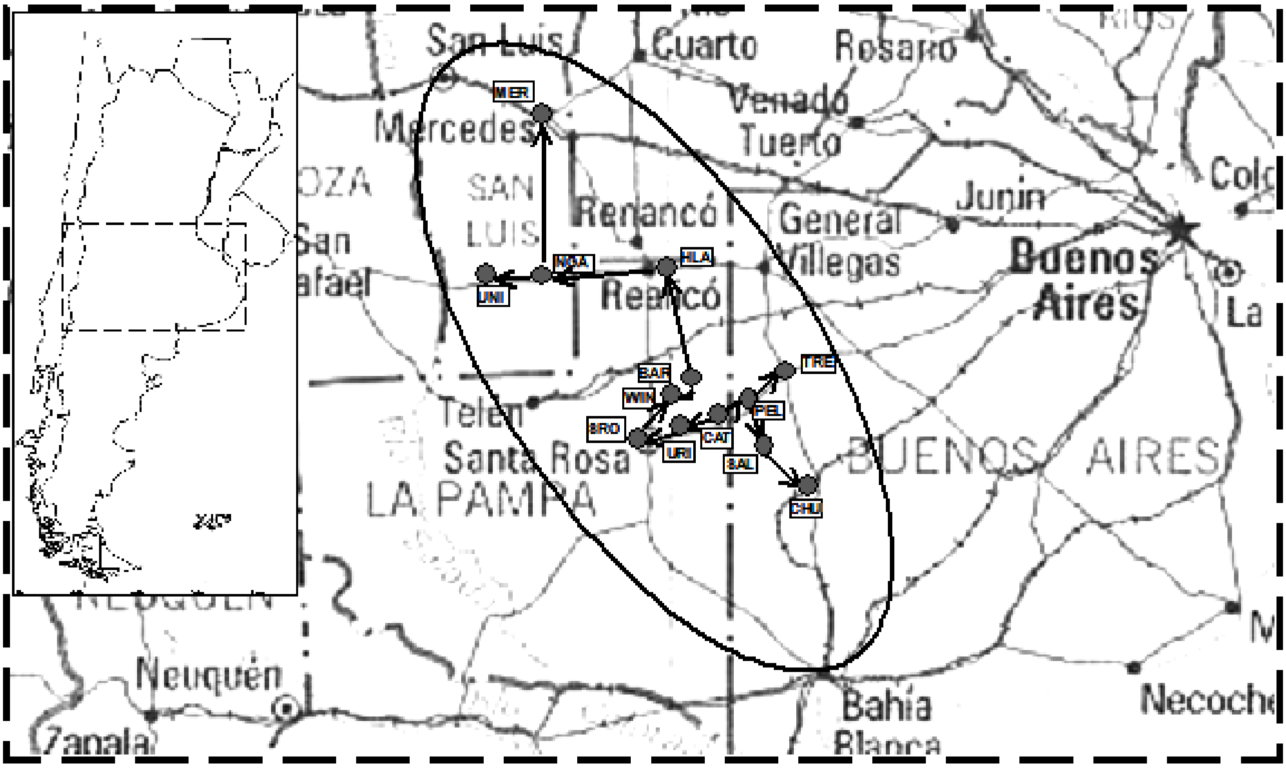

Figure 3-2 Migration pattern suggested by road connectivity, environmental, and plant community proximities between habitats of *Helianthus petiolaris* stable populations.

Population names as in Table 3-1. The ellipse shows the area where isolated plants were found in 2000-2007 surveys.

On the whole the pattern suggests that *H. annuus* migrated from Rio Cuarto towards four main extreme points (Figure 3-3). Diamante (NE), Media Agua (NNW), and Las Malvinas (SWW) represent migration distances of ca. 500 km (Figure 3-3a). Between these extreme points, Diamante means the smallest dissimilitude accounted in terms of community and environmental changes. The other two habitats, both situated in the western irrigated area, entail wider changes in terms of plant community, because populations co-occur with different crops such as fruit trees (Figure 3-3b), but the model estimated a unique entry to the irrigated west area followed by mobilization within this area. Carhue (SE) was the fourth extreme destination reached by *H. annuus*, which means 600 km of migration, being the community composition change greater than the former three points.

Las Malvinas population was first observed by Dra. E. Camadro (pers. comm) in 1999. Differing from Media Agua, sunflower seed production is common in this irrigated area and this activity could be the possible connection to Rio Cuarto and Rancul area. The Rancul-to-Carhue branch showed an equilibrate sequence of steps connecting with Rio Cuarto-Juarez Celman axis. Migration might have been promoted by agriculture activity which is usually accomplished by machinery moving within the area. Migration to Diamante is more difficult to explain because there is evidence of the presence of this species in a voucher specimen at the Darwinion Institute herbarium (Cabrera 1974, http://www.darwin.edu.ar) collected in 1960, before the land connection of 1969, when a tunnel below river Paraná was opened. Even though at environmental level, Diamante showed more proximity to Rio Cuarto than to the other extreme destinations.

The proposed migration pattern of wild *H. annuus* agrees with a previous phenotypic study in an experimental field (Presotto 2007). Las Malvinas population showed some traits of cultivated sunflower (*H. annuus* var. *macrocapus*) which were addressed to seed production activity in this area that could promote gene flow. Juarez Celman, Río Cuarto and Media Agua clustered together based on phenotypic variables. Rancul, Colonia Baron, Adolfo Alsina and Carhué clustered in other group. Diamante differed from the remaining wild populations because of its long life cycle and frequency of yellow disc flowers.

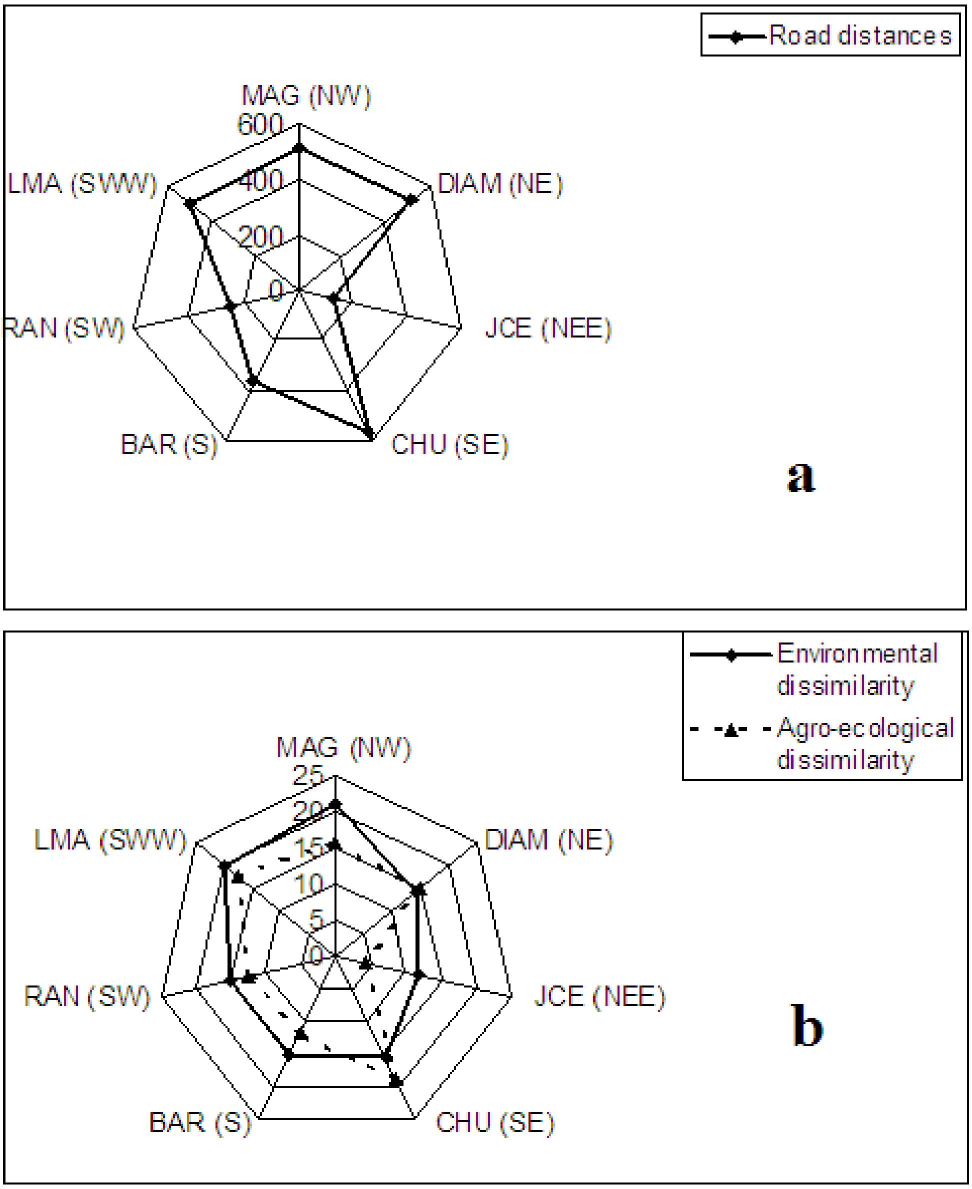

Figure 3-3 Distances from the entry point of wild *Helianthus annuus* (Rio Cuarto, Cordoba province) to the main populations in Argentina.

a) Road distance; b) Environmental and ecological distances expressed as percent of the total distance to connect all populations

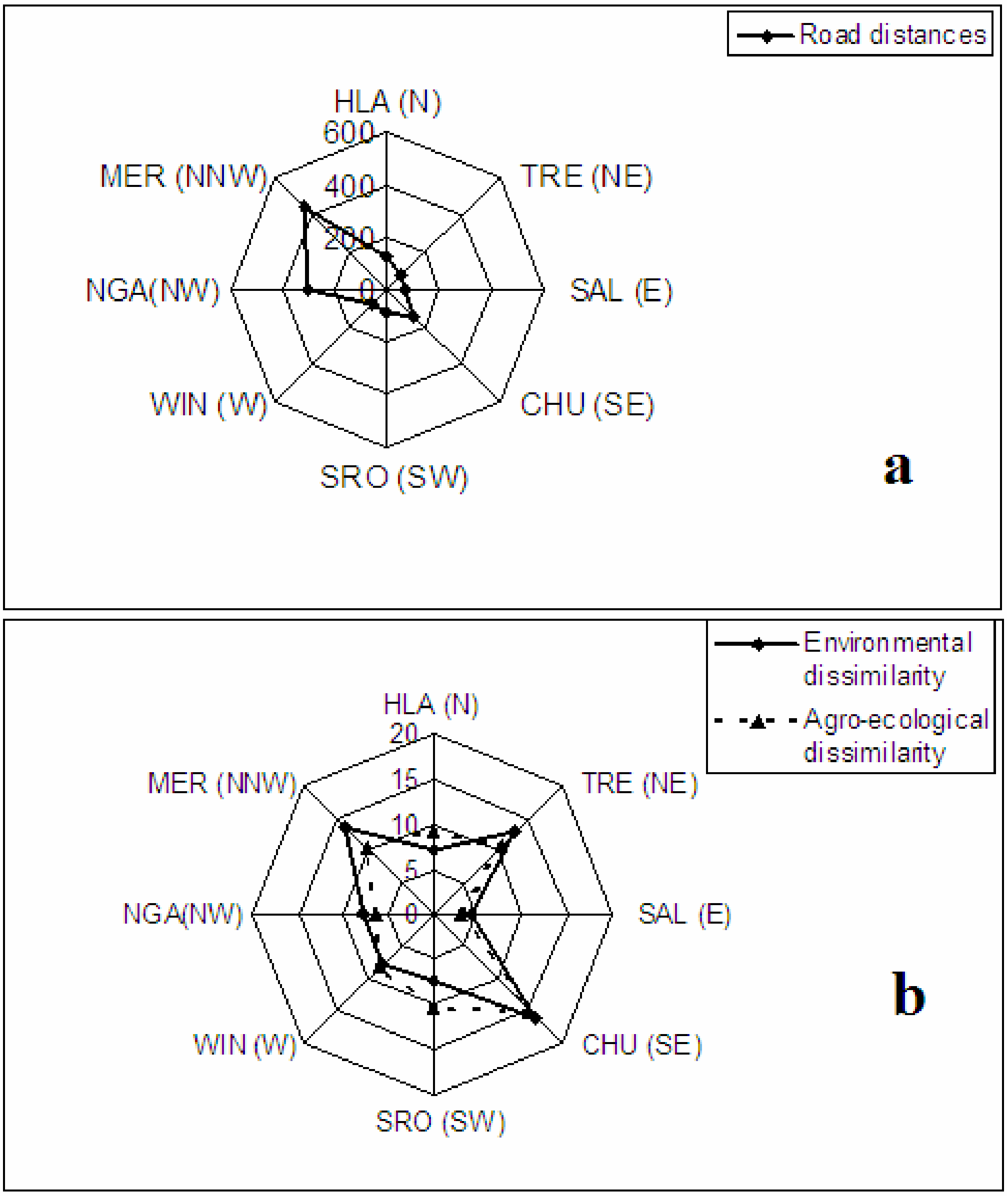

Figure 3-4 Distances from the entry point of *Helianthus petiolaris* (Catriló, La Pampa province) to main populations in Argentina.

Environmental and ecological distances expressed as percent of the total distance to connect all populations

Helianthus petiolaris can be found in an extended area but as very small patches or isolated plants without forming permanent populations (ellipse in Figure 3-2). Stable populations seem to have undergone the more intense environment and plant community changes during the SE migration to Carhue (Figure 3-4b). While the road distance was smaller with respect to the NNW migration, the accomplished environmental and ecological changes accounted to reach this destination were the greatest observed in the species. The migration towards San Luis province in NW and NNW direction implies near 400 km, and was also accompanied with strong environmental changes. A specimen at the Multidisciplinary Institute of Vegetal Biology Herbarium (http://imbiv.unc.edu.ar/cpi/imbiv.htm) gives testimony that this step occurred before 1963 (Table 1-2). To the Northeast, Trenque Lauquen represents a great change in terms of environment and plant community although it is located at a short road distance from the entry point.

Edaphic variables differenced the micro-environment of both wild annual *Helianthus* species (Figure 3-5) but showed overlapping as previously described (Cantamutto et al. 2008). Cluster analysis formed three main groups, with clear predominance of one of each species in two of them but mixed in the remainder. Populations of *H. petiolaris* and *H. annuus* from an overlapping zone clustered together in this third group. These populations corresponded to Colonia Baron, where an intense gene flow between both wild species was apparent (Cantamutto et al. 2007). Edaphic factors of the microhabitat seem to have minor influence over plant community distribution (Cousens et al. 2006) and thus not useful to describe the migration pattern.

Although five wild *H. annuus* stable populations were found near sunflower breeding stations, their origin as escapes can be ruled out because of the closer vicinity of other wild population. The mean distance between each breeding station and the nearest wild *H. annuus* populations was 299 ± 154 km, while the mean distance between each one of the five populations and the nearest one reached 170 ± 212 km. Clustering of road distances between wild *H. annuus* populations including the breeding stations confirm Rio Cuarto as origin (Figure 3-6). Early sunflower breeding programs in Argentina did not use wild genetic resources until wild sunflower species were first included among crossing materials at Manfredi experimental station, in the 1950s (Giordano and Senin Garcia 1967, Bertero and Vazquez 2003). Rio Cuarto population is the closest to Manfredi but it cannot be considered as an escape from this breeding station because it was the source of germplasm for that program (Bauer 1991). The central and southern populations of Rancul, Colonia Baron, Adolfo Alsina, and Carhue are the only populations more proximate to a breeding program than to Rio Cuarto. By road proximity they joint with the historic Pirovano station connected before with Rio Cuarto, but in this breeding program the wild sunflower has never been used. The more direct connection of this group of populations was with Rio Cuarto-Juarez Celman axis through Rancul branch (Figure 3-1). This connection leaves breeding stations at Barrow and Balcarce out of consideration as possible sources for these populations. Las Malvinas and Media Agua were far away from registered breeding programs. Taking into account road distances, Diamante was the only population neither readily connected with Rio Cuarto nor from breeding stations. There were six experimental fields nearer to this population than from Rio Cuarto, but none of them was active before 1960.

Regarding *H. petiolaris*, there are evidences of its usage as disease resistance source in breeding stations of eastern and northern Argentina in the decade of 1960 (Luciano 1964, Bertero and Vazquez 2003) but escapes from there seem unlikely. The road distance from those breeding stations to the nearest populations is of 412 ± 57 km, whereas the populations are placed 94 ± 76 km from each other.

In Argentina it was possible to suggest a migration pattern from an entry point of each two wild annual sunflowers through multivariate analysis of road, environment and plant community proximity between existing representative stable populations. The closer proximity between permanent wild populations with respect to breeding stations turns unlikely the escapes as origin. According to these results the alternative hypothesis of mechanical dispersion from an entry point appears as the most acceptable origin of the actual wild sunflower stable populations in central Argentina.

Acknowledgements

To Profs. Federico Möckel and Raúl Rodríguez for advice about sunflower breeding programs in Argentina. To Drs. Ricardo Rodriguez and Bruce Maxwell for their useful suggestions on the manuscript. To the Fundación Carolina, España for a fellowship to the first author MAC. This research was supported by ANPC y T-PICT 089881 and UNS-PGI 24A106.

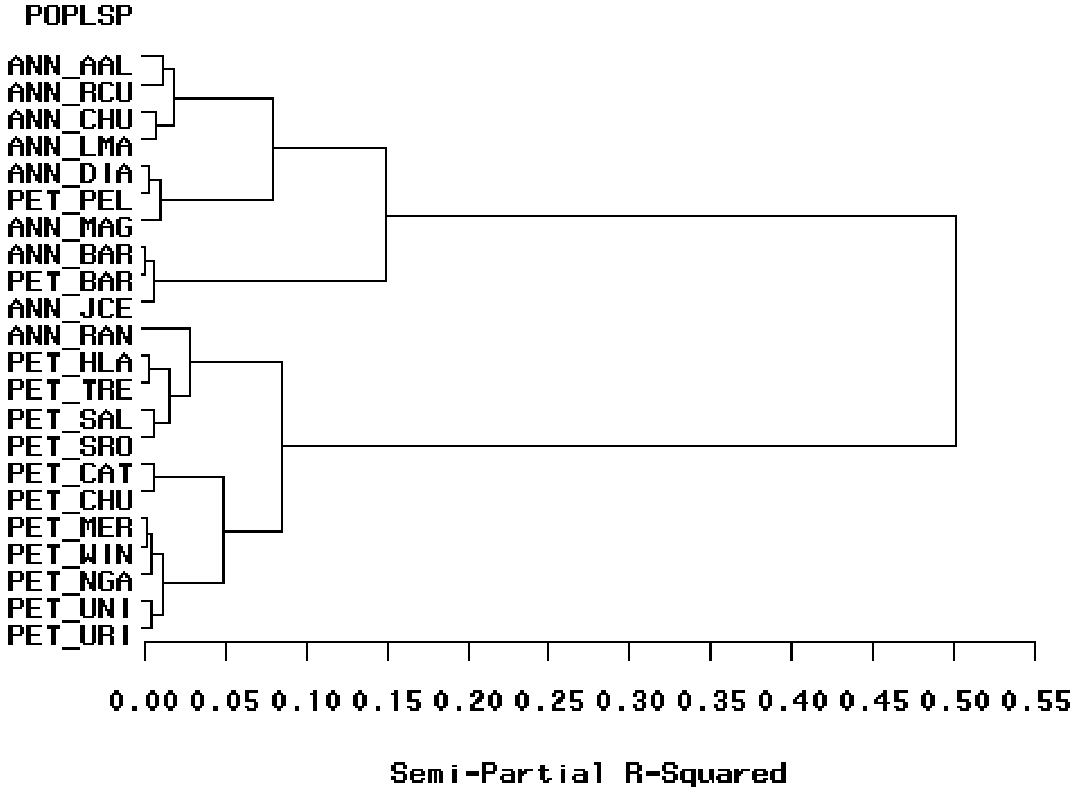

Figure 3-5 Clustering of wild *Helianthus annuus* (ANN) and *H. petiolaris* (PET) populations (POPLSP) in Argentina landscape by micro-environment habitat.

The sites (same nomenclature as Table 3-1) were clustered by Ward minimum variance criteria of Euclidean distance of soil test variables range transformed and standardized. Pseudo T-Squared Statistics indicated the existence of three main clusters

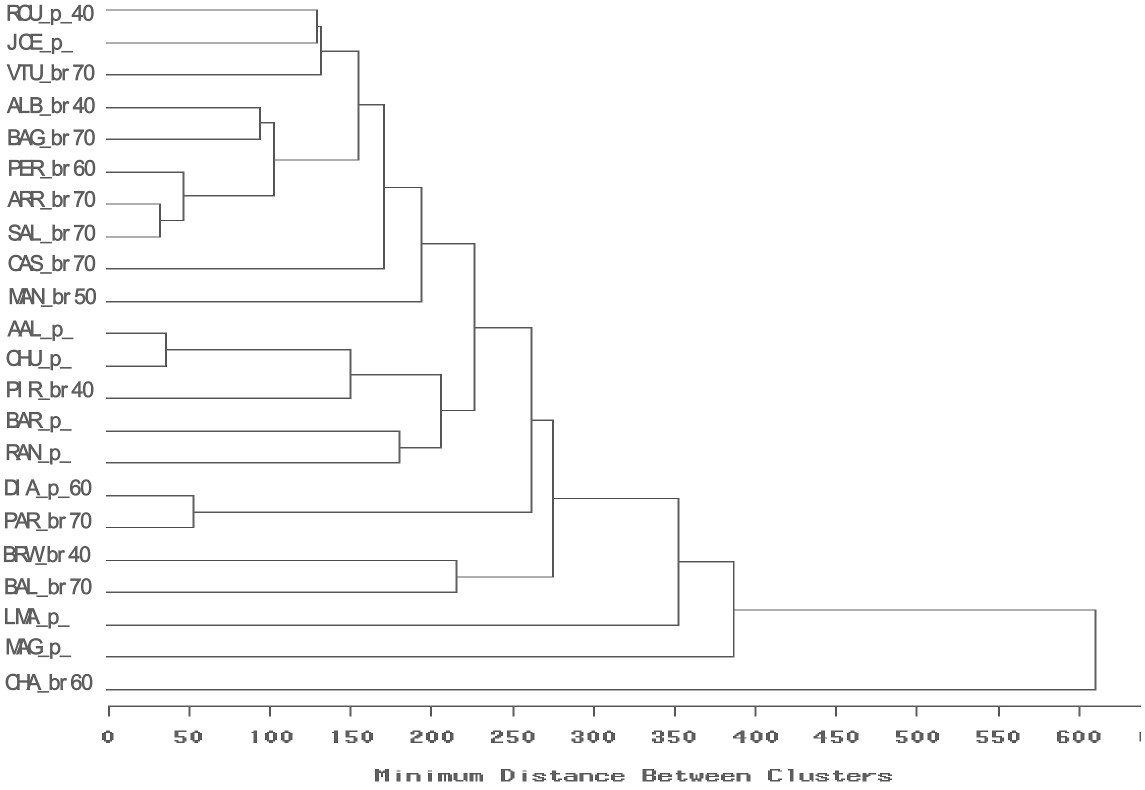

Figure 3-6 Cluster ordination of road distances by hierarchical agglomerative single linkage between Argentine wild *Helianthus annuus* populations (p) and sunflower breeding stations (br).

Names as in Figure 3-1. The two digit numbers indicate the decade of the first register of wild populations (before Poverene et al. 2002) or the possible beginning of wild sunflower cultivation in experimental field.

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Chapter 4 NATURAL HYBRIDS BETWEEN CULTIVATED AND WILD SUNFLOWERS IN ARGENTINA

2008

Genetic Resources and Crop Evolution DOI 10.1007/S10722-008-9326-X

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Abstract

Two introduced wild species *Helianthus annuus* L. and *H. petiolaris* Nutt. have been widespread in central Argentina and overlap the sunflower crop region. Intermediate off-type plants between both wild and cultivated species are often found, what is of concern because of the recent release of imidazolinone resistant varieties and the likely use of genetically modified sunflower cultivars. The progeny of 33 off-type plants obtained in 14 representative sites of the diffusion area were studied to confirm hybrid origin. Germination, survival, morphological traits and days to flowering confirmed hybridization between crop and both wild species, when compared to eight accessions of typical wild plants. Some progenies were presumably crop-wild *H. annuus* hybrids, some originated from the cross of cultivated plants and *H. petiolaris*, and two were the advanced generation of a cultivated hybrid. Hence, morphological traits were a good clue for the identification of spontaneous hybrid plants at field. The results indicate that crop-wild hybridization and introgression occur at different places in central Argentina. This fact may represent a way to herbicide resistance escape and future transgene escape if GM sunflower cultivars are released for commercial use.

Keywords: Wild *Helianthus*, sunflower crop, gene flow, morphology, fertility.

Introduction

Sunflower, *Helianthus annuus* L. var. *macrocarpus*, is a traditional oil crop in Argentina positioned fourth in the world production. In the last years, a remarkable increase of soybean crop displaced sunflower crop towards less adapted southwestern central plains (de la Vega et al. 2007) causing a decline in sunflower production. During the 2006/2007 season, sunflower acreage in Argentina fell to 2.45 million hectares, less than 50% of record area in the last decade. The new crop region greatly overlaps the distribution area of two wild *Helianthus* species which have widespread through the country in the last 60 years (Covas 1966, Poverene et al. 2002). The use of new imidazolinone herbicide tolerant (IMI) varieties and genetically modified (GM) cultivars could place sunflower again as one of the main crops in Argentina. Nevertheless, the release of GM sunflower seems improbable in the next years. GM soybean, maize, and cotton have been commercially released in Argentina, but unlike sunflower, none of them has naturalized wild relatives. Diffusion of varieties carrying novel traits could modify wild *Helianthus* populations via gene flow. Environmental impact depends on the frequency of trait transference and on its ability to enhance growth and fertility by conferring selective advantages to wild plants (Hails and Morley 2005, Hooftman et al. 2005, Mercer at al. 2006).

The genus *Helianthus* (Asteraceae) is native to North America and comprises 51 annual and perennial species, which are diploids, tetraploids and hexaploids, with basic chromosome number of x=17 (Heiser 1978, Seiler and Rieseberg 1997, Jan and Seiler 2007). *H. annuus* L. and *H. petiolaris* Nutt are annual diploid species naturalized over the central part of Argentina (Cantamutto et al. 2008). *H. petiolaris* is more abundant and its botanical description matches subspecies *petiolaris* (Heiser 1961). It grows on sandy soils forming extensive patchy populations. *H. annuus* displays a very variable morphology and corresponds to subspecies *annuus* (Heiser 1954). Both species are sympatric in several localities in the central part of the country and often invade sunflower, maize and soybean crops.

Gene flow among cultivated sunflower and both wild *Helianthus* species has been extensively studied in the centre of origin (Arias and Rieseberg 1995, Whitton et al. 1997, Linder et al. 1998, Snow et al. 1998, Rieseberg et al. 1995, 1999a, Burke et al. 2002). In Argentina, Covas and Vargas Lopez (1970) first described intermediate plants between cultivated sunflower and *H. petiolaris*, but there are no detailed studies about natural occurrence of crop-wild introgression. Plants with intermediate morphological characters are often found in wild populations of both species, along roadsides and in cultivated fields. Those plants could originate from crosses between the cultivated sunflower and the wild species or may represent the advanced generations of a crop cultivar, namely volunteers (Reagon and Snow 2006). Alternatively, they could come from hybridization of both wild species. Morphological characterization of plants constitutes the first step to assess hybrid origin. Trait intermediacy and reduced fertility or fitness in progeny analysis are more reliable indicators of interspecific crosses or wild-crop gene flow. Different classes of hybrids can be classified using morphological characters, if the areas where intermediate plants occur are considered as hybrid zones (Rieseberg and Carney 1998).

The goal of this study was to confirm hybridization processes between wild species and cultivated sunflower through phenotypic analysis of progenies of morphologically intermediate plants found in central Argentina. We hypothesize that if intermediate plants are in fact of hybrid origin, progeny tests would reveal a segregation of parental characters, a reduced fertility and/or fitness.

Materials and Methods

A number of off-type plants with intermediate morphology were found in different counties of four provinces (Figure 4-1, Table 4-1). Thirty three healthy plants were chosen for this study in 14 sampled sites: 31 plants were found in *H. petiolaris* populations or growing in rather isolated conditions, in small patches along roadsides. One plant was collected in a cultivated field and another was found in a wild *H. annuus* population. They were representatives of many others in those populations, showing a phenotype that made them conspicuous among the surrounding plants. Field assays were established with seeds of one to three heads of the 33 off-type plant, a. bulk seed sample of wild *H. annuus* and *H. petiolaris* from eight localities, and a sunflower commercial hybrid (DK H3881). The progeny of each off-type plant is described as a family.

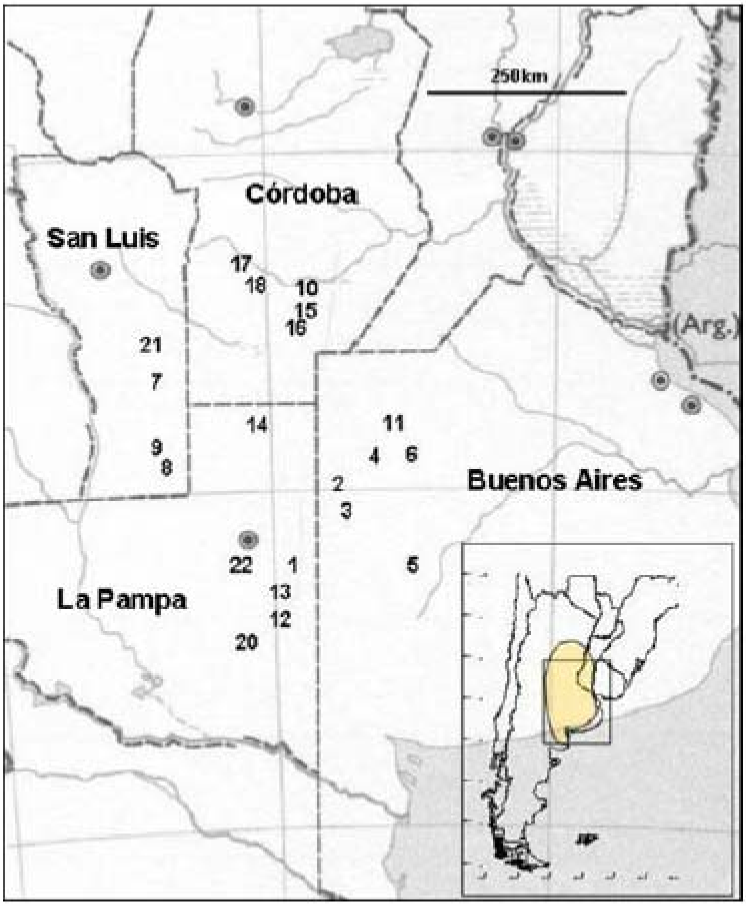

Figure 4-1 Wild *Helianthus* sampling sites in four central provinces of Argentina, related to the sunflower crop region (shaded in detail). Numbers refer to Table 4-1.

Table 4-1 Off type plants and wild *Helianthus* accessions studied by progeny tests.

Population of origin, geographic site, and map reference to Figure 4-1. Off-type plants were identified according to the population where they were found: P for *H. petiolaris*, A for *H. annuus*, C for crop, I for isolate plants. Seed was bulk collected in wild populations, WA for *H. annuus* and WP for *H. petiolaris*.

Mother plant	Population	Province	County	Map #
		Off type plants		
P1, P2	*H. petiolaris*	La Pampa	Atreucó	1
P3, P4	*H. petiolaris*	Buenos Aires	Salliqueló	2
P5, P6	*H. petiolaris*	Buenos Aires	Salliqueló	3
P7	*H. petiolaris*	Buenos Aires	T. Lauquen	4
P8 to P15	*H. petiolaris*	Buenos Aires	Guaminí	5
P16, P17	*H. petiolaris*	Buenos Aires	T. Lauquen	6
P18, P19	*H. petiolaris*	San Luis	G. Dupuy	7
P2O	*H. petiolaris*	San Luis	G. Dupuy	8
P21	*H. petiolaris*	San Luis	G. Dupuy	9
A1	*H. annuus*	Córdoba	J. Celman	10
C1	sunflower crop	Buenos Aires	T. Lauquen	11
I1, I2	isolate[1]	La Pampa	Atreucó	12
I3, I4	isolate[1]	La Pampa	Atreucó	13
I5 to I10	isolate[1]	La Pampa	Realicó	14
		Wild accessions		
WA1	*H. annuus*	Córdoba	J. Celman	15
WA2	*H. annuus*	Córdoba	J. Celman	16
WA3	*H. annuus*	Córdoba	Río Cuarto	17
WA4	*H. annuus*	Córdoba	J.Celman	19
WP1	*H. petiolaris*	Buenos Aires	Tres Lomas	20
WP2	*H. petiolaris*	La Pampa	Utracán	21
WP3	*H. petiolaris*	San Luis	G. Dupuy	22
WP4	*H. petiolaris*	La Pampa	Capital	23

[1] Growing in small patches along roadsides.

Seed of the 33 off-type plants and of eight wild accessions (Table 4-1) were germinated in Petri dishes in 1 mM gibberellic acid to break dormancy (Seiler 1998), then transferred to a sand and peat mix (3:1w/w) in the greenhouse. When the seedlings reached about 10 cm height they were transplanted into 9 m length plots randomly in the field. Each plot comprised a family of 30 plants spaced 0.30 m and distance between plots was 1.4 m. Irrigation and weed control were performed weekly to ensure plant growth.

Germination in Petri dishes was recorded, except for plants P18 and P19, which were sown directly in the field plots, because they were included later in the experiment. After transplanting to the field, the following traits were recorded for every plant in each plot: Seedling survival (%); plant height (m, recorded in intervals); branching (0-4 from no branching to fully branched from the base according to Luczkiewicz (1975), anthocyanin presence in stems and petioles (yes/no); leaf type (*annuus, petiolaris*, intermediate); days from transplant to flowering. Leaf morphology was cordate or subcordate with serrate margins (classified as *annuus*-type), wide or narrow triangular-shaped with cuneate base (classified as *petiolaris*-type) or intermediate forms. The following traits were recorded in three heads per plant: bract (phylaries) width (cm); disc diameter (cm); disc color (yellow, purple); seed color (grey, brown, grey and brown, others); seed design (complete, stripes, mottled, both stripes and mottled); seed pubescence (from 1= glabrous to 4= very pubescent); seed length (mm); pollen viability (%); and seed set (%). Survival was recorded when plants reached the reproductive stage and completed life cycle, being 100% if the 30 plants transplanted per plot survived. Pollen viability was assessed by differential staining (Alexander 1980). Heads at anthesis were shaken over a clean slide to collect fresh pollen, then a staining drop was added and at least 300 pollen grains were counted on each slide. Three slides were fixed per head. Seed set was determined as the proportion of the filled seed per head over the total number of disk florets per head. Traits of continuous variation were analyzed by principal components analysis (PCA) of character x character correlations. Means per plot and standard deviations were graphically represented. A hybrid index was calculated based on the numerical scores of the categorical traits branching type (0 to 4), anthocyanin presence (0-1), disc color (1-2), and leaf type (1 to 3). The index of each plant was the sum of scores for the four traits. The highest score was assigned to the wild taxa and the lowest score corresponded to the cultivated genotype. Hybrid indexes were graphically represented as histograms. A consensus graphic was achieved by Procrustes analysis of all metric and categorical variables (Gower 1975). Multivariate analyses were performed using the InfoStat (2006) program.

Results

Most mother plant heads had a high proportion of aborted seeds. Germination ranged from 2 to 60% among families, with some plants showing a slow development. Families I3, P2, P10 and P15 failed to survive in the field and only one plant survived among progeny of P19. Weakness or premature death was observed in families I2, I4, P1, P8, P9, and P14. Dwarfism or fasciation was observed in families P5, P6, P8, P11, P12, and P13. Many surviving plants among these progenies produced an early head with abnormal ligules and bracts and died before anthesis. Mother plants of all these families were off-type plants found within *H. petiolaris* populations or isolate in fields where this species has established (Table 4-1) and sunflower cropping is widespread, thus presumably interspecific hybrids.

Germination in wild accessions was slow reaching 50-60% in *H. annuus* and 40-50% in *H. petiolaris*. The surviving families showed within-plot segregation of several traits. Healthy plant height varied from less than 0.5 m to almost 3 m. Table 4-2 shows mean values per family in germination, plant survival and plant height.

A lower variation for metric characters as compared with families was observed in the wild accessions (Figure 4-2). Disc diameter, bract width, seed size and days from transplant to flowering in families were almost always intermediate between wild and cultivated sunflower. Families P1, I4, P20 and P21, showed a shorter disc diameter than wild accessions and high sterility. Low pollen viability and seed set indicated reduced fertility in families P1, I1, I3, and P20. Families A1 and P14 had very high fertility, while families I5, I6, I8, I9, and I10 were almost as fertile as wild accessions (Figure 4-3). Mother plants I5 to I10 were found in fields where wild *H. annuus* has established (Table 4-1) so except P14, those plants were presumably wild-crop *H. annuus* intraspecific hybrids. In one plant of each family P16, I5, and I6 male sterility was observed.

Table 4-2 Germination, survival and plant height in 29 off-type families and eight wild *Helianthus* accessions.

Identification numbers as in Table 4-1.

Family	Germination %	Survival %	Plant height m	Family/ Wild accession	Germination %	Survival %	Plant height m
P1	20	3	<0.5	I1	50	23	0.5-1
P3	5	100	1.5-2	I2	45	10	0.5-1
P4	2	92	1.5-2	I4	2	11	0.5-1
P5	10	78	0.5-1	I5	40	86	1-1.5
P6	10	100	0.5-1	I6	50	100	1.5-2
P7	2	97	1-1.5	I7	20	81	0.5-1
P8	5	100	1-2	I8	45	100	0.5-2
P9	2	56	0.5-1	I9	45	95	1-2
P11	30	75	<0.5	I10	55	60	<0.5
P12	10	90	<0.5	A1	40	92	0.5-2
P13	30	90	0.5-1	C1	60	85	1.5-2.5
P14	30	75	0.5-1	WA1	50-60	96	2-3
P16	5	81	1-1.5	WA2	50-60	83	2-3
P17	10	93	0.5-1.5	WA3	50-60	78	2-3
P18	nd[1]	100	1-1.5	WA4	50-60	96	2-3
P19	nd	one plant	1.5-2	WP1	40-50	100	1.5
P20	20	96	0.5-1	WP2	40-50	82	1-1.5
P21	8	100	0.5-1	WP3	40-50	100	1-1.5
				WP4	40-50	100	1-1.5

[1] No data

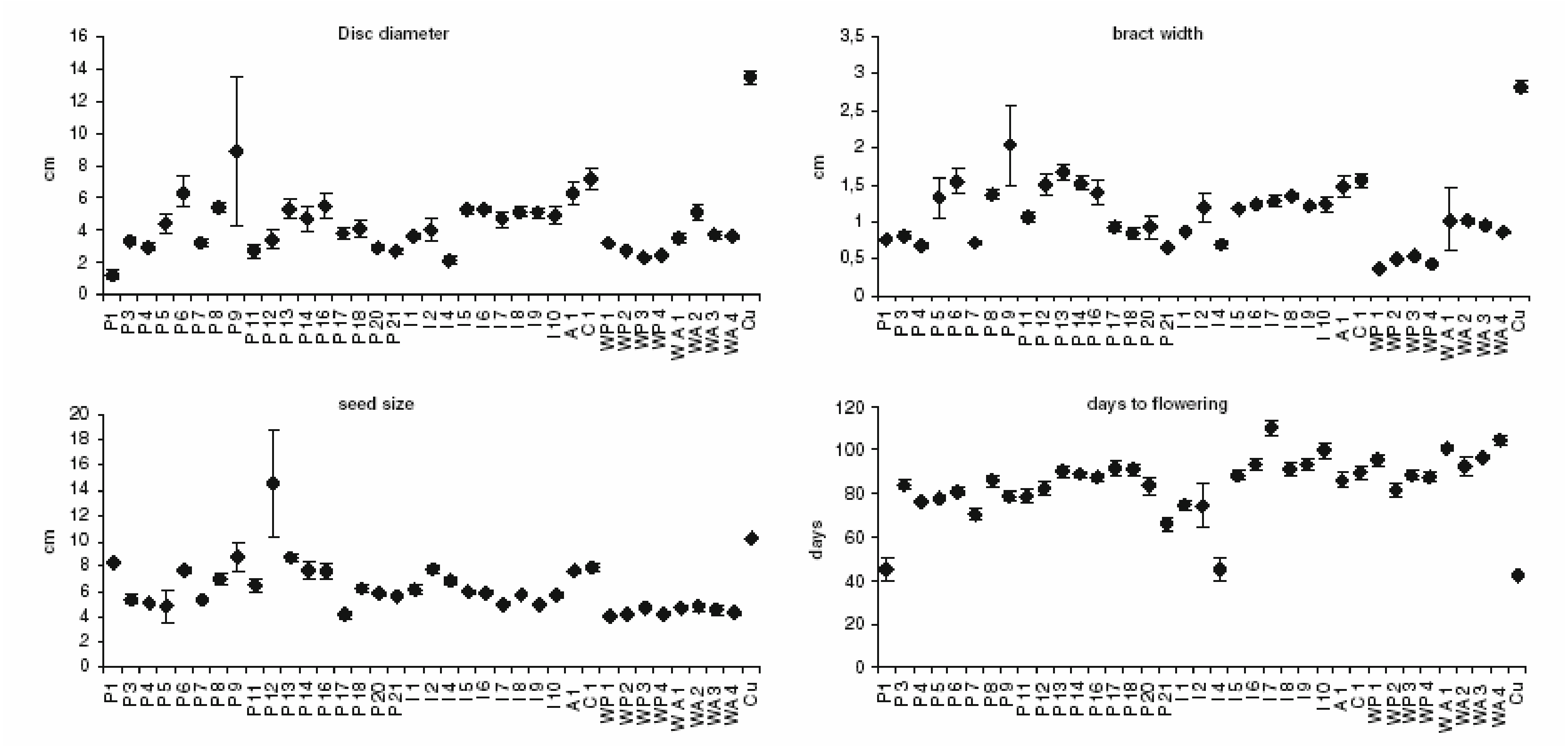

Figure 4-2 Phenotypic metric traits (mean ± SD) in off-type plant families, wild accessions *H. petiolaris, H. annuus,* and a representative sunflower cultivar, DK3881. Identification numbers as in Table 4-1.

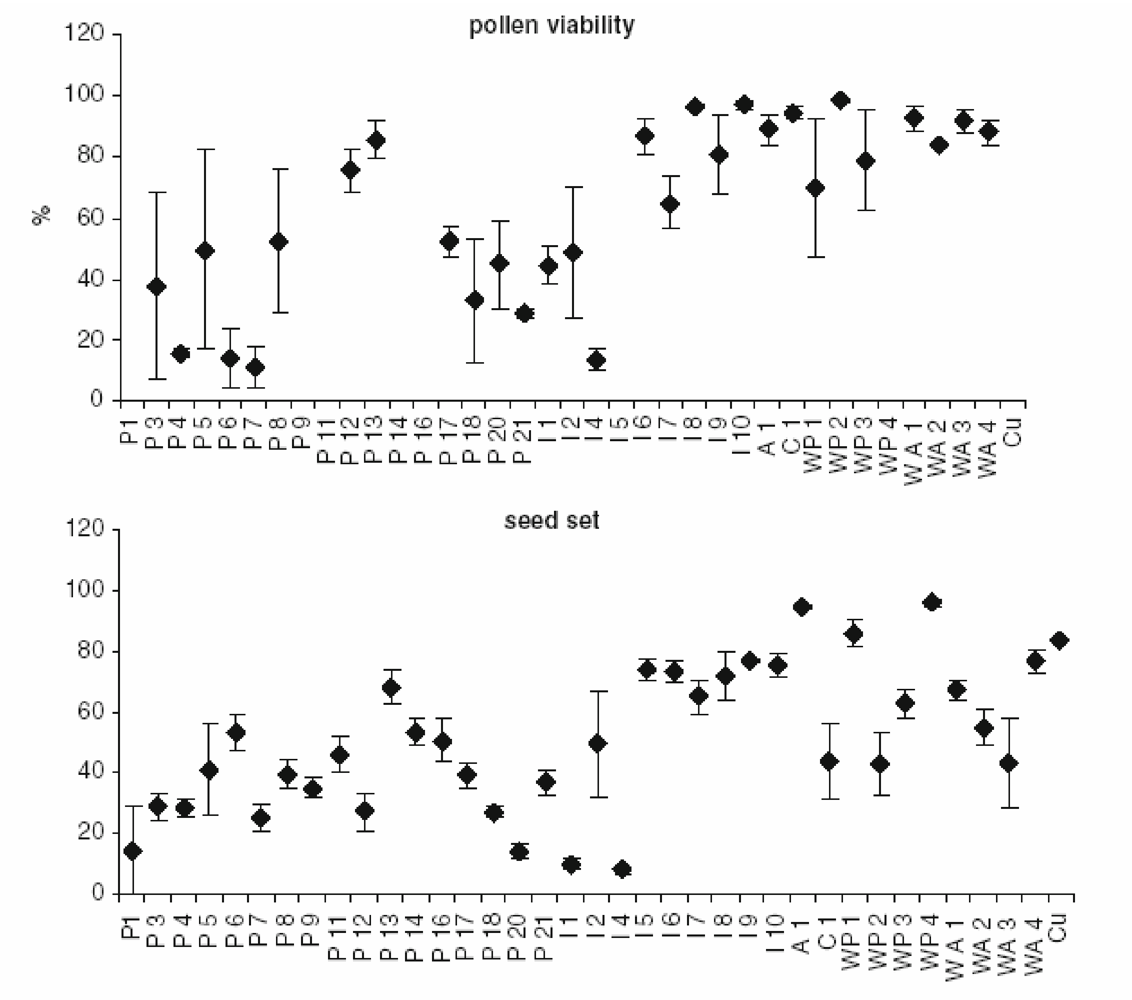

Figure 4-3 Fertility traits in off-type plant families, *H. petiolaris* and *H. annuus* accessions, and the sunflower cultivar DK3881. Identification numbers as in Table 4-1.

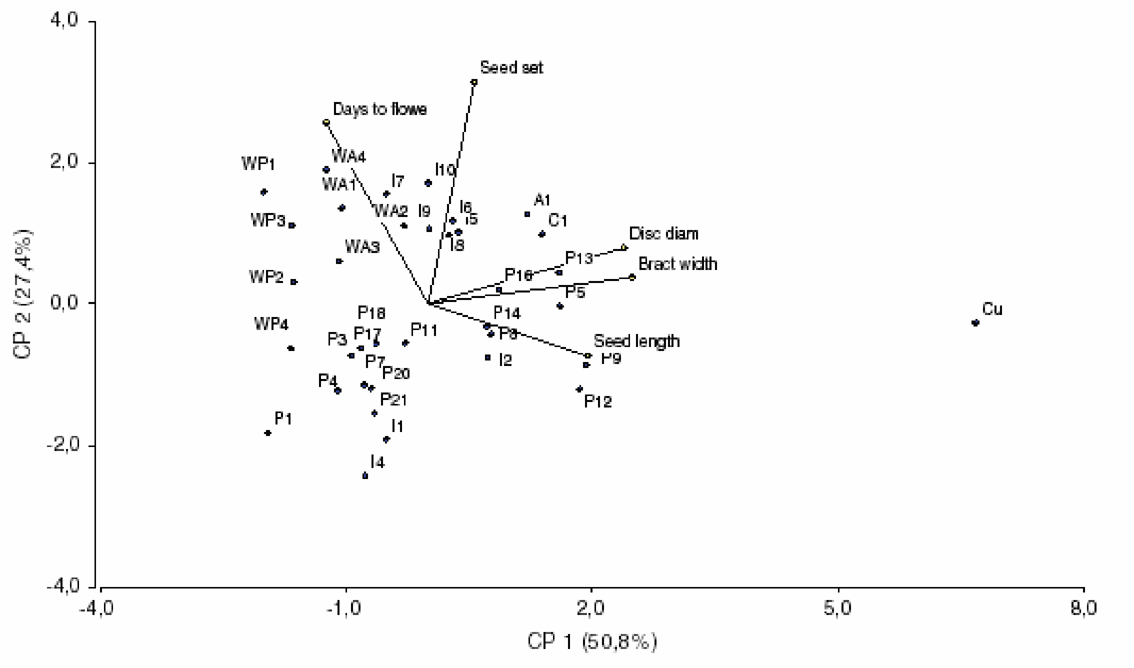

Figure 4-4 Principal component analysis in 29 off-type families, eight wild accessions and a sunflower cultivar.

Identification numbers as in Table 4-1. Two first CP explain over 75% of variability.

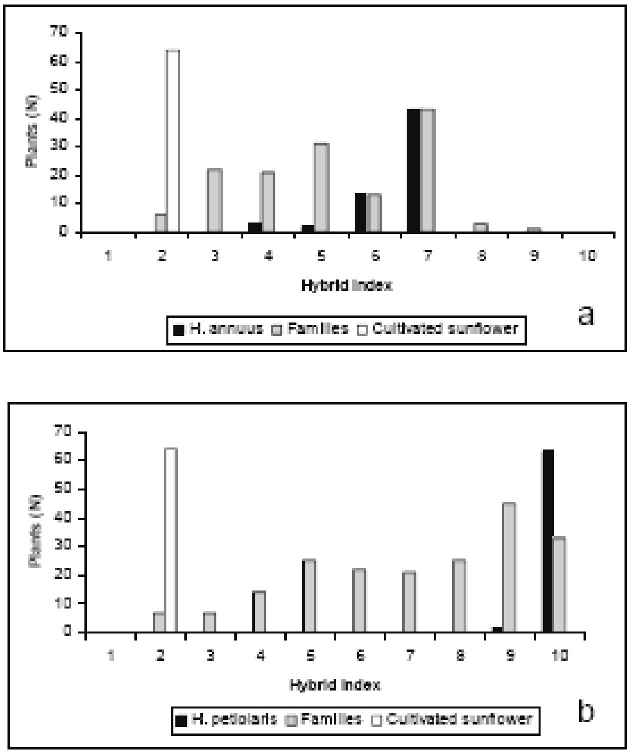

Figure 4-5 Hybrid index based on categorical traits of putative parent species plants and progenies of 29 off-type plants.

a) Wild parent species *H. annuus*. b) Wild parent species *H. petiolaris*. In both figures, white bars are cultivated plants, black bars are typical wild plants, and grey bars are off-type plant progenies.

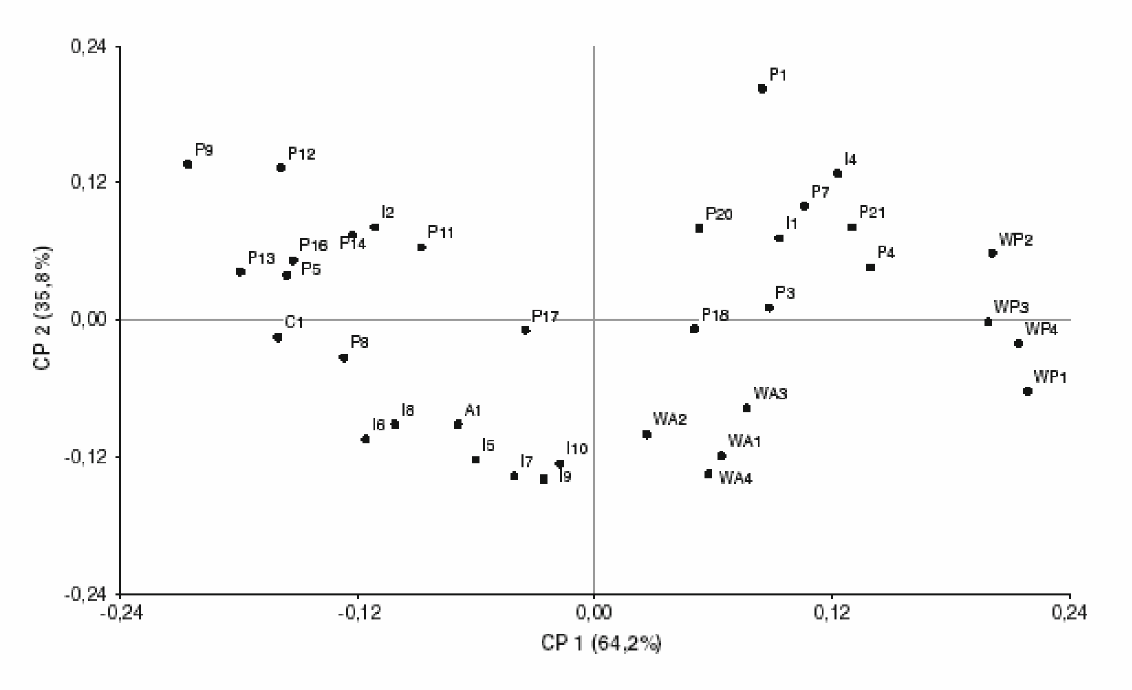

Figure 4-6 Procrustes generalized analysis from metric and categorical data matrix of families and wild sunflower accessions.

Identification numbers as in Table 4-1.

The principal component analysis based on metric traits showed a good separation along the first component. Off-type families showed an intermediate position between the extremes wild *H. annuus* (WA1-4), *H. petiolaris* (WP1-4) and the cultivated strain (Cu). Disc diameter and bract width largely determined this first component. The second component segregated *H. petiolaris* hybrids (P1-P21, I1-I4) from *H. annuus* hybrids (I5-I10, A1, C1) mainly based on seed set and days to flowering (Figure 4-4).

Branching type, leaf shape, anthocyanin presence, disc colour, and seed appearance (colour, design, and pubescence) were different in families than in wild species accessions. Wild accessions presented branches on the whole plant, without a main head, except for some *H. annuus* plants which had upper branching (type 2) or a main head (type 3). Types 0, 2 and 3 are present in cultivated sunflower, whereas 1 and 4 are typical of wild forms. Most families segregated different branching types.

Segregation of leaf types was frequent among progenies within a plot, but one type often predominated. Most wild *H. annuus* and *H. petiolaris* plants showed red-purple stems and petioles. Among families, three presented anthocyanins in every plant, five presented no coloured plants, and the remaining segregated by anthocyanin presence or absence.

H. petiolaris accessions showed an intense purple red colour in disc florets, while wild *H. annuus* also had red discs, with less amount of pigment on the corolla lobes. Yellow discs with no anthocyanin-pigmented florets and black tubular corolla are typical of cultivated sunflower. Among off-type families, seven had yellow discs in every plant; eight had plants with anthocyanin-pigmented lobes and 13 segregated by yellow and red coloured discs. Black colour in the tubular corolla was not observed, the entire corolla being yellow in those yellow-disc plants. Ray flower petals were always yellow in wild accessions and progenies. Anthocyanin pigments in flowers and vegetative parts were associated and both traits segregated in 12 families.

Seed appearance was very variable. In wild accessions, seeds were mostly grey or brown. Off -type families showed black, olive and white seeds as well. Regardless the colour, epidermis was mottled in *H. petiolaris* accessions and presented mottling or stripes in *H. annuus* accessions, whereas families varied from completely coloured, mottled, stripes, or both, and many segregated the various types of epidermis. *H. petiolaris* accessions presented a typical dense pubescence, less dense in wild *H.*

annuus accessions and progenies intergraded from dense pubescence to almost glabrous, like the cultivated sunflower.

Hybrid index based on four categorical traits showed that most progenies of intermediate morphology had intermediate scores between the extreme parental phenotypes. All plants were graphically represented, including wild and cultivated pure species (Figure 4-5).

When metric and categorical traits were combined by Procrustes analysis, off-type families showed an intermediate distribution between wild and cultivated sunflower (Figure 4-6) similar to that observed in hybrid index results. The first two axes explained 81% of the variability contained in all the traits. ANOVA showed 82.4% consensus among metric and categorical traits. Families were clustered mainly according to their collection site.

Discussion

Wild-crop hybridization can influence the evolutionary ecology of related wild or weedy taxa, especially when they co-occur, have overlapping flowering periods~~,~~ and share pollinators (Snow et al. 1998). A very conspicuous phenotypic variation characterized most studied families derived from off-type plants as compared with pure wild *H. annuus* and *H. petiolaris* accessions growing in the same conditions. Segregation of categorical traits was considered as an evidence of the mother plant hybrid origin, although some variation was expected within wild accessions because the two species are self-incompatible outcrossers. Wild-crop hybridization is frequent in Argentina because of the extensive overlapping among wild *Helianthus* distribution and sunflower crop acreage (Poverene et al. 2004, Ureta et al. 2008 and unpublished data).

Poor germination in bulk seeds of wild accessions was ascribed to the typical dormancy of the wild species (Seiler 1998). Most off-type plant seeds germinated earlier, but there was a high failure due to seed inviability, as expected in interspecific hybrid progenies. A reduced dormancy was found in wild-crop hybrids of sunflower by Snow et al. (1998). Many families (i.e. P1-2-8-9-10-14-15, I2-3-4) showed plants with severe delay in development, dwarfism, weakness or premature death which would be a consequence of chromosome or genic imbalance in progenies from interspecific crosses between *H. annuus* and *H. petiolaris* (Rieseberg et al. 1995)

Variability in plant height was within expectable limits, for this trait is a quantitatively inherited character, being tallness from 0.2 m to more than 1 m in *H. petiolaris* and 1-3 m in *H. annuus*. Given that height is very variable among commercial hybrids -the putative cultivated parents- no statistical comparisons were attempted.

Variation in metric traits was also higher within and among off-type families compared to wild species. This was first described by Heiser (1947) in natural and artificial hybrids between *H. annuus* and *H. petiolaris*, and other interspecific hybrids in the same genus (Nikolova et al. 1998). Disc diameter, bract width, and seed size were good indicators of hybridization as stated by Heiser (1947) and Ferreira (1980) who compared natural and artificial hybrids between these two species. Similar results are shown in Figure 4-2.

Progenies of off-type plants may show both parental and intermediate morphological characters (Rieseberg and Carney 1998). The life cycle, represented by days to flowering, was mostly intermediate in families. The two which had a very short cycle were probably early generation hybrids between *H. petiolaris* and cultivated sunflower; while I6 showed a very long cycle and seemed a *H. annuus* wild-crop hybrid. Life cycle was somewhat distorted because wild accessions had a later emergency compared to families. However, data are valid for comparative purposes. The late flowering of I7 family suggests that life cycle could be transgressive in wild-crop hybrids. If hybrid plants flowered earlier than wild ones, they would primarily intercross originating advanced generation hybrids, not backcrossing progenies. Transgressive hybrids would have an opportunity to establish in slightly different habitats or ecological niches (Lexer et al. 2003). Nevertheless, overlapping life cycles of wild and hybrid plants often occur, as in the sites where mother plants were collected.

Very reduced fertility in some families suggests that mother plants were early generation interspecific hybrids. Recovery of fertility occurs within few generations after hybridization (Heiser 1947, Rieseberg et al. 1999b). Families I5, I6, I8, I9, and I10 with comparatively high fertility may represent advanced hybrid generations or backcrosses to the wild parent, *H. petiolaris*. Higher fertility is expected in wild-cultivated *H. annuus* hybrids, where no chromosome barriers to gene flow occur and crop alleles persist in wild populations for many generations after hybridization (Whitton et al. 1997). Male sterility probably came from cultivated x wild crosses, through volunteers (Reagon and Snow 2006).

Branching has a complex inheritance mode (Luczkiewicz 1975, Miller and Fick 1997). Upper branching or a central head in some families and in wild *H. annuus* plants pointed to gene flow from cultivated sunflower. Intermediate leaf shape and anthocyanin presence was frequent within families. Anthocyanin pigment in stems and leaves is usually dominant and very common in wild accessions. Variation and segregation of these traits among families were considered as evidence of crop hybridization and introgression (Bervillé et al. 2005). Variability in seed coat colour and pubescence was also very high. Stripes is a dominant trait characteristic of many old sunflower varieties in Argentina and was present in 25 out of 29 analyzed families.

Wild *H. annuus* populations showed some variation in disc, bracts and seed size, and crop traits were observed in two accessions collected in an area traditionally devoted to sunflower and exposed for many years to crop gene flow. The lower variation observed in *H. petiolaris* accessions, as compared with *H. annuus*, agrees with the species' reports in North America (Heiser 1954, 1961).

Based on seed viability, survival, collection site and morphology, off-type families were assigned to specific wild-crop crosses. There were four family classes according to the variation pattern among plants. The first one was characterized by poor germination, low viability and development abnormalities. In progenies P1, I2, I4, P9, and P14 a high plant mortality was observed, with complete loss of families of P2, I3, P10, and P15. Most of the surviving ones died before anthesis or developed abnormally, showing dwarfism or fasciation in families P5, P11, P12, and P13. As all these families originated from *annuus*-like or intermediate plants growing among typical *H. petiolaris*, the mother plants were probably the first generation of an interspecific cross and the abnormalities arose as a consequence of genetic differences between parental species.

A second class showed germination ranging from 2 to 50%, a high phenotypic variation among plants, and sterility. Families I1, P3, P4, P6, P7, P8, P20, and P21 segregated in *petiolaris*-like and *annuus*-like plants, while P18 and P19 showed an intermediate plant type. Their mother plants were mainly of intermediate type growing in *H. petiolaris* populations and may represent second generation hybrids or backcrosses to *H. petiolaris*.

A third family class showed germination between 45 and 60% (except for one having 20%). Families A1, P17, C1, and I5 to I9 had more restricted variability to *annuus*-like plants, and sterility was not apparent. Mother plants resembled somewhat atypical wild *H. annuus* and were probably originated from crosses between wild and cultivated *H. annuus*. The most common situation is fertilization of wild plants by cultivated pollen donors, but the reciprocal cross is seldom found in seed production fields (Reagon and Snow 2006). As C1 was representative of a number of similar plants grown in the crop rows, it is possible that they were a consequence of wild pollen contamination in the seed production field.

The last class comprised families P16 and I10 They germinated well and gave a rather uniform progeny, showing characteristic domesticated traits, some plants had male sterility and poor seed set. This class may represent progenies of a volunteer sunflower and was not the result of a wild-cultivated cross. Volunteers and their progenies are very common along roadsides and railways, and can easily be misidentified as wild plants (Reagon and Snow 2006).

Consensus analysis of metric and categorical traits confirmed family arrays, grouping each family in a different quadrant, while wild pure species formed two separate clusters. Hybrid index graphically demonstrated that most progenies of the presumable hybrid plants were intermediate between cultivated sunflower and wild species, though this method gives the same hybrid index score to phenotypically different plants (Briggs and Walters, 1997) and does not allow differentiation among families.

Partial germination, survival and seed set in the first and second classes ensure that hybridization and introgression occur between the two species, in spite of chromosomal and genic barriers (Rieseberg et al. 1995, 1999a). Patterns of introgression are similar in natural hybrid zones and in experimental lines, and also in geographically distant zones (Rieseberg et al. 1999b). Natural hybridization between wild *H. annuus* and *H. petiolaris* in North America has originated three homoploid species (Rieseberg et al. 1990; Rieseberg 1991). Environmental variation and natural selection play an important role in this kind of speciation, and geographically isolated populations might speciate in parallel. Species recently introduced in a new habitat, which quickly colonize extensive areas would probably spread on similar soils, occupy similar environments, and hybridize with closely related taxa. However, all sampled off-type plants were found in pure species stands and there were no evidences of both wild species growing together at the same sites. Thus hybridization between wild *Helianthus* species was discarded as a probable origin of studied intermediate plants.

Argentina is the second country following the US in GM crop production (over 18 million hectares) and 84 field trials for GM sunflower varieties have been approved. Our results provide enough evidence that sunflower wild-crop hybridization frequently takes place at several sites within the cultivated area in Argentina. Imidazolinone tolerance and eventually other novel traits will certainly be transmitted to wild *Helianthus* populations via pollen movement in the next years, as reported by Al-Khatib et al. 1998 and Massinga et al. 2003. One concern about crop-wild gene flow is that intermediate plants are often found in western Buenos Aires and La Pampa provinces, especially in those counties of high sunflower production. However, Burke et al. (2002) have pointed that research should focus on the fitness consequences of the particular gene that is transferred to wild populations, rather than on the rate of hybridization.

The results demonstrate that hybridization occurs in all the distribution range of wild *Helianthus* species within the sunflower cultivation area. Both first generation hybrids and advanced generation hybrids, or backcrosses could be found in the same site showing that crop-wild hybridization and introgression are recurrent processes.

Acknowledgments

The authors thank to the National Research Council of Argentina (CONICET) for a fellowship to MSU. Statistical help from S. Luis and A. Hernandez is greatly appreciated. This research was supported by grants ANPCYT- PICT 08-9881 and UNS-PGI 24A106.

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Chapter 5 *Helianthus petiolaris* in Argentina and spontaneous hybridization with cultivated sunflower

2004

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Abstract

Helianthus petiolaris has been naturalized in central Argentina and its geographical distribution overlaps the sunflower crop region. Intermediate forms between both species are often found in wild populations. To study variability and occurrence of natural hybrids, 26 representative populations growing up to 100 m far of sunflower crops in three provinces were sampled and a common garden experiment was conducted. Highly significant differences were found among populations for morphological quantitative traits, and also there was variation for qualitative traits and phenology. Hybrid origin of intermediate plants found in 10 out of 26 populations was assessed through morphology, phenology and fertility. Hybrid plants were rather different and all were at least partially fertile. These results have biological implications concerning to gene flow and introgression, and practical ones regarding the possibility of releasing genetically modified sunflower cultivars.

Introduction

H. petiolaris Nutt. (Asteraceae) is a diploid species (x=17) native to North America and well known as a source of cytoplasmic male-sterility. It was introduced in Argentina about 50 years ago, presumably as contaminants in forage seed lots, and found favourable conditions in the semiarid environments and sandy soils of the central region of the country. At present, it is widespread in La Pampa, San Luis, Western Buenos Aires and Southern Cordoba provinces, between 33º 14’ and 38 48 S (Poverene et al. 2002). Populations are patchily distributed along roadsides, wire fences, and in the external rows of crops, mainly sunflower, but soon disappear when the soil is plowed. Plants grow from November to April. Morphological description matches *H. petiolaris* ssp. *petiolaris*, according to Heiser (1961). However, phenotypic variability is found within and among populations. Genotypic variation was also found through isozyme markers analysis (Poverene et al. 2003).

The extensive overlapping with sunflower crop regions, the coincidence of life cycles and common pollinator insects facilitate interspecific crosses between *H. petiolaris* and sunflower, *H. annuus* var. *macrocarpus*. Although both species differ in chromosome constitution – only seven out of 17 chromosomes are collinear in both species - and there are important barriers to hybridization (Rieseberg et al. 1995), hybrids have been found since many years in Argentina (Covas and Vargas López 1970, Ferreira 1980). Hybridization and introgression between *H. petiolaris* and *H. annuus* have been extensively studied in their centre of origin, where they have originated at least three new homoploid species (Rieseberg et al. 1996, 1999a,b, Rieseberg and Linder 1999, Buerkle and Rieseberg 2001).

We found intermediate forms between *H. petiolaris* and *H. annuus* in several of the 150 sites in Argentina where *H. petiolaris* accessions were collected along three years. According to the site where they were found, it was possible to infer the direction of pollen flow, from cultivated to wild plants o reciprocal (Cantamutto et al. 2003). Seeds of 32 intermediate plants were sown in the experimental field of the Agronomy Department and progeny tests demonstrated segregation of phenotypic characters of both species (Poverene et al. 2003).

The aim of this work was to study *H. petiolaris* distribution and variability in Argentina, occurrence and characterization of natural hybrids from crosses with cultivated sunflower. Gene flow between both species and its consequence is of concern because this may imply a secondary centre of genetic variability for these *Helianthus* species in the Southern hemisphere. Moreover, sunflower is one of the most important oil crops in Argentina and there is an interest in releasing genetically modified cultivars, which are at present under evaluation, and will demand information about potential environmental impact.

Materials and Methods

Between 2000 and 2003 we made a number of trips to study geographical distribution of *H. petiolaris* populations in the country. Collection of plant specimens and seed allowed gathering 150 accessions. In 2003, a common garden experiment in the experimental field of the Agronomy Department comprised 26 accessions from different sites of La Pampa, San Luis and Buenos Aires provinces. These populations were growing up to 100 m far from sunflower crops at the time of collection. Bulked samples of seed were collected from wild heads exposed to pollen flow from the crop in each site[4]. A subset of seeds was grown in the greenhouse and transplanted to field plots, in a completely randomized design with two replications. Plots were of 20 m length, with plants spaced 0.20 m at each side of the drip tape, and distance between rows was 1.5 m. A plot of cultivated sunflower (Dekalb 3900) was sown at the same time.

Phenotypic variation was studied through morphology and phenology. Data on leaf size and shape, head and disc diameter, bract (phyllary) width, days to flowering, life cycle length, were collected on six representative plants of each plot, three leaves and three heads of each plant. Data were subjected to ANOVA and Kruskal-Wallis non parametric analysis (because of deviations from a Gaussian distribution of some traits), Principal component analysis and Cluster analysis based on mean linkage and mean Euclidean distance. Qualitative traits as disc flower and seed color, leaf appearance (undulate/flat) and margin (entire/serrated), plant tallness, branching pattern and pathogen symptoms were also recorded.

The same morphological and phenological data were used to characterize presumably hybrid plants, from pollination by cultivated sunflower. Fertility was estimated through

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[4] For head collection methodology, see Discussion chapter.

pollen viability (stained as Alexander 1969) and seed set. Data from each hybrid were compared with data from three *H. petiolaris* plants of the same accession and with three plants of cultivated sunflower. Mean comparisons (Hochberg test), Principal component analysis and a hybrid index (Grant 1989) for some traits (leaf margin and appearance, disc flower color) were calculated.

Results and Discussion

Phenotypic variation

H. petiolaris populations varied for qualitative traits, in tallness and leaf appearance. Tall ones, more than 1 m height, were more frequent (16/26) than short ones, less than 1 m. Undulate leaves were more frequent (18/26) than flat ones. One population segregated for red and yellow disc flowers, the remaining presented purple red disc flowers only. Seeds varied in color, being reddish brown or gray, sometimes yellowish, but always hairy and maculated. Most populations (22/26) showed more than one seed color. Populations did not show any visual symptoms of disease, although some of them showed powdery mildew at the end of life cycle.

All the quantitative characters showed highly significanant differences among populations with ANOVA ($p<0.0089$) and Kruskal-Wallis tests ($p<0.0045$). Principal component analysis did not reveal any agreement between morphological variation and geographical location of populations. The first component explained 45% of variance and was correlated with phyllary width, leaf length and shape. The third component explained 14% of variance and was correlated with leaf width and head diameter (Figure 5-1).

Lack of concordance with origin was observed in wild population of *H. annuus* in the centre of origin and addressed to their human-dispersed nature (Arias and Rieseberg 1995). The lack of a clinal structure in Argentine *H. petiolaris* populations indicate that random drift has been more important than adaptive processes in differentiation among populations, which are typically patchy and ephemeral. The blurred geographic pattern can also be addressed to multiple events of introduction and dispersion through trucks and trains, which drag heads and seeds along their ways. The results agree with seed isozyme variation, previously assayed in 22 populations (in preparation). Cluster analysis formed four main groups; each one contained some geographically close related accessions, but also no related ones. Two groups included those populations where interspecific hybrids were found, suggesting that some populations share traits, however inconspicuous, that could arise from introgression with *H. annuus*. Field collection trips along three years indicate that *H. petiolaris* is progressively spreading. Variability among and within populations would allow adaptation to different environments.

Interspecific hybridization:

Gene flow from cultivated sunflower to *H. petiolaris* and recurrent hybridization events were confirmed when intermediate plants were found in 10 out of the 26 *H. petiolaris* populations in the field. Frequency of intermediate plants per population ranged from 0.005 to 0.02, with mean of 0.013. Their hybrid origin was conclusively demonstrated through morphology and fertility. Intermediate plants were taller than *H. petiolaris* plants of the same accession, less branched and mostly above, bigger heads and leaves, wider discs and phyllaries. Pollen stainability and seed set were lower than in *H. petiolaris* plants (Table 5-1, Figure 5-2).

The hybrid index showed that leaf margin and appearance, and disc flower colour were intermediate between *H. petiolaris* and cultivated sunflower. Hybrid plants were phenotypically very different, 1.20-1.80 m height, mostly wide cordate leaves but sometimes smaller and lanceolate, mostly long petioles and sometimes very thick. Half of hybrids had very large discs and phyllaries and half resembled more the *petiolaris* type. Variation in leaf, disc and phyllary size was the most informative for CP analysis, which placed hybrids between both parental species (Figure 5-3).

Life cycle of eight hybrids was intermediate (136-205 days) between *H. petiolaris* accessions (146-220d) and the cultivated sunflower (<125d), but six hybrids showed a much longer cycle (>220d) being transgressive. New morphological and phenological traits allow ecological differentiation of hybrids from their paternal species, generating favourable conditions to divergence.

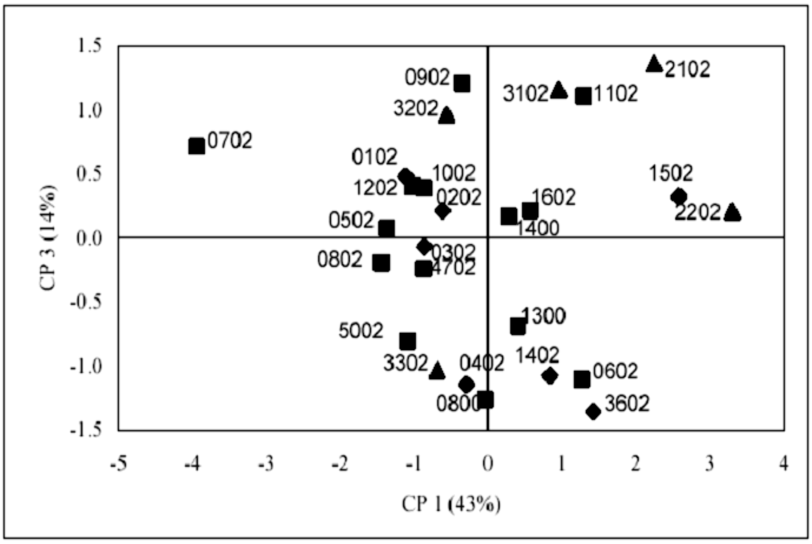

Figure 5-1 Principal component analysis of 26 populations of *H. petiolaris*.

Numbers of Buenos Aires (squares), La Pampa (rhombs) and San Luis (triangles) provinces correspond to accessions in our collection.

Table 5-1 Some characters in sunflower interspecific hybrids and parent species. Mean data and standard deviation, significance and Hochberg mean comparison.

Character	*H. petiolaris*	Intesp. hybrids	Cultivated sunflower	Sign.
Leaf length	8.24 ± 1.51(a)	14.59 ± 2.70(b)	22.83 ± 4.44(c)	**
Leaf width	5.26 ± 0.94(a)	12.99 ± 3.08(b)	21.5 ± 6.58(c)	**
Leaf shape l/w	1.59 ± 0.22(b)	1.14 ± 1.33(a)	1.09 ± 0.13(a)	**
Disc diameter	2.41 ± 0.33(a)	4.09 ± 0.97(b)	13.5 ± 1.14(c)	**
Seed set	71.8 ± 5.26(a)	11.67 ± 24.19(b)	-	**
Pollen stainability	82.46 ± 13.24(a)	30.64 ± 18.61(b)	-	**

Values followed by the same letter within a row are not significant different at P=0.05.

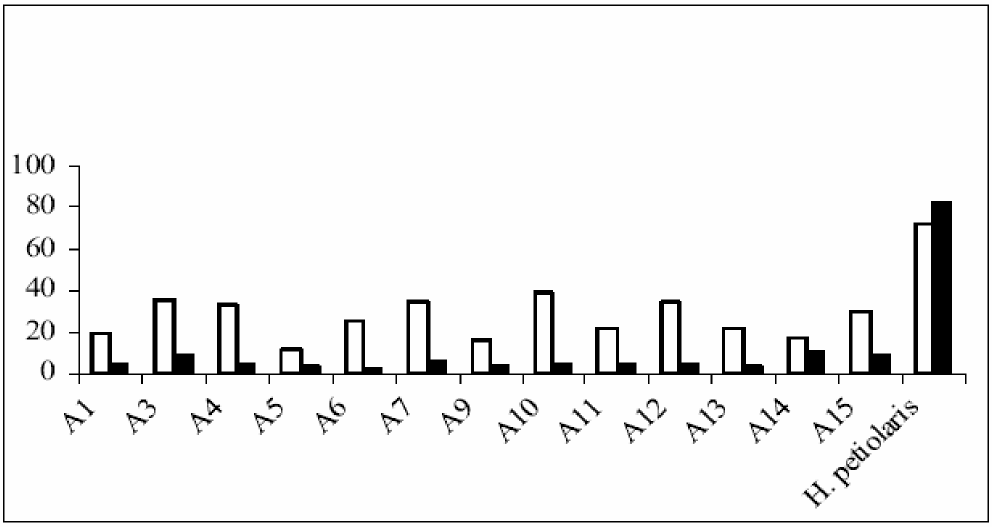

Figure 5-2 Pollen stainability (white bars) and seed set (black bars) percentage in intraspecific hybrids (A1-A15) and *H. petiolaris* (mean data of four populations).

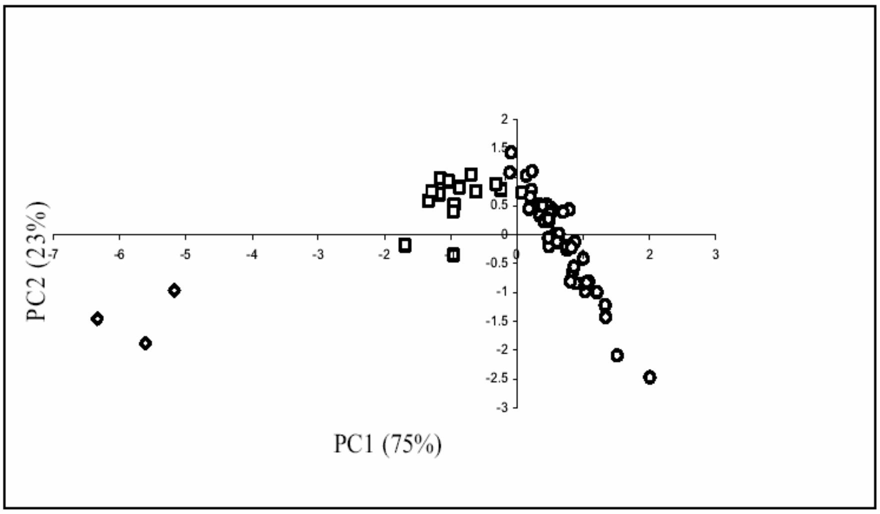

Figure 5-3 PC analysis of morphological traits in *H. petiolaris* (circles), cultivated sunflower (rhombus) and hybrids (squares).

In spite of partial chromosomal and genic barriers between both species (Rieseberg et al. 1995), our results confirm that repetitive hybridization and gene flow are frequent in *H. petiolaris* populations growing close to sunflower crops in Argentina. Given that these species have been in contact during at least 50 years under adaptive conditions, new genetic resources for breeding may be found in the wild. Transgenes would probably spread to feral populations if genetically modified cultivars were released, yet we cannot assert that it would have any environmental impact. In order to study a second generation in the summer of 2003/04, pollen of each hybrid plant was used to pollinate *H. petiolaris* plants of the same population. Seed was collected from these backcrosses and also from the open-pollinated hybrids. Plants from wild populations in Figure 5-1, hybrids, backcrosses and advanced generations are at present under molecular marker analysis.

Acknowledgments

We thank to Lic. S. Luis and A. Hernandez for valuable help with statistics. This work was supported by ANPCYT-PICT 08-9881 and UNS-PGI 24/A106 grants. Also by CIC, Buenos Aires Province through a fellowship to M.S. Ureta.

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Chapter 6 Gene flow among wild and cultivated sunflower, *Helianthus annuus* in Argentina

2008

Agriculture Ecosystem & Environment 123:343-349

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Abstract

Naturalized populations of wild sunflower, *Helianthus annnus*, occur in six provinces of central Argentina, usually near sunflower crops. Plants of intermediate morphology indicate that gene flow might take place in both senses. In order to quantify gene flow between cultivated and wild sunflower, an experimental stand of sunflower was sown surrounded by plots of wild plants at increasing distances. Hybridization rate was estimated using a crop specific isozyme marker, and a mean of 7% progenies were crop-wild hybrids. The nearest wild plants (3m from the cultivar) showed the highest percentage (18%) of gene flow, which was found to decrease with distance, up to 500 m. Pollen flow from wild plants to crop, evaluated through morphological characters in the progenies of cultivated plants from a stand invaded by wild sunflowers, produced 3.75% intermediate plants.

Key words: gene flow, wild *Helianthus*, sunflower, volunteers, hybridization.

Introduction

Agricultural biotechnology was massively adopted by farmers in Argentina in the last 10 years. At present, 40% cotton, 60% corn and almost all soybean production is transgenic over a total acreage of 18 million ha. Argentina is among the main world producers of sunflower, following Russia and Ukraine, and is the first sunflower oil exporter (SAGPyA 2006). About 77 genetically modified (GM) sunflower varieties have been authorized for field trials, but none of them has been released yet. Whereas in Argentina there is no wild species genetically related to the commercially approved GM crops, sunflower has flowering relatives growing synchronously in areas of commercial production. Wild *Helianthus annuus* and *H. petiolaris*, native to North America established about 60 years ago and occur in patches over seven central provinces (Poverene et al. 2002).

In the agro-ecological landscape other potential recipients of pollen from crop are the volunteers, plants spontaneously originated from seed of cultivated sunflower, which are frequently found in roadsides and fallow fields following sunflower cropping. Volunteers and off-type volunteers play an important role in crop-wild gene flow through pollen and seeds. Off-type volunteers arise from wild pollen contamination in sunflower seed production fields (Reagon and Snow 2006). As a pollen source, such feral plants carrying GM traits could contaminate conventional crops and affect the non-GM seeds commercialisation.

Wild *H. annuus* is considered the ancestor of the cultivated sunflower, *H. annuus* var. *macrocarpus*, and despite many phenotypic differences between them, they are interfertile (Heiser 1954, Rieseberg and Seiler 1990, Burke et al. 2002a). Hybridization reaches 42% and alleles from cultivated sunflower persist in frequencies up to 38% in wild sympatric populations (Arias and Rieseberg 1994, Whitton et al. 1997, Linder et al. 1998).

Morphologically intermediate forms were found among wild typical plants in several locations in central Argentina (Poverene et al. 2004). Agro-ecological conditions in Argentina differ from those in North America, and probably determine different relationships between sunflower crop and wild *Helianthus* populations. Therefore, their geographical overlapping (Burke et al. 2002b) was first investigated and an experimental method based on genetic markers (Arias and Rieseberg 1994) was used in order to estimate crop-wild gene flow. Also, wild-crop gene flow was estimated using a morphological characterization approach.

The following questions were posed: Is there overlap between wild populations and sunflower cultivation? Do volunteer sunflowers increase the risk for gene flow? How readily are wild populations pollinated by crop plants? How readily are crop plants pollinated by wild populations?

Materials and Methods

Exploration for wild sunflower populations was carried out in eight provinces in the central part of the country. Heads were collected at random along one or several transects depending on the size of each population and they included dry, senescent heads and mature heads with still green bracts (phyllaries) to avoid achene loss due to shattering. Evidence of crop-wild hybridization was assessed through the observation of a number of phenotypic traits, mainly branching type, head number and size, disc and seed color, anthocyanin presence, leaf, phyllary (bract) and seed size.

Volunteers were surveyed along 30 km of Hwy 51, which connects western Buenos Aires province with Bahia Blanca city port. No wild sunflower plants were found in this area. Volunteers were very abundant, as they came from seeds fallen from trucks. Plants growing on the roadside were directly examined and screened for two traits: male sterility and branching (Miller and Fick 1997). Male parents of commercial hybrids usually carry the dominant male-fertility restorer gene and the recessive gene for branching, while females carry the alternative alleles, so F1 hybrids are heterozygous for both traits. In order to evaluate the genetic constitution of volunteers, chi-square statistics was used in F2 and F3 generation to test the goodness-of-fit of the observed phenotypic classes to expected ratios under single gene hypothesis.

Pollen flow from crop to wild plants

A field experiment comprising cultivated and wild materials was planned to simulate natural flow conditions. We used isozyme data to obtain a more accurate estimation of the gene flow. The isozyme marker was selected based on results from a molecular screening of 13 wild populations of *H. annuus*, four commercial hybrids (DK 3881, 3900, 3915, and 4033) and the inbred line HA89. The study was intended to assess genetic variability of the wild species and to search for specific crop markers (unpublished data). Upon this analysis, we chose for the field experiment hybrid

DK3881 and a wild population collected in Cordoba province which differed in the acid phosphatase enzyme locus *Acp-1*.

The experimental field design was similar to that used by Arias and Rieseberg (1994). A 25 m x 25 m sunflower plot was sown in the centre of a field covering 60 ha, with a commercial planting density. Sowing was done weekly to ensure pollen production during the wild sunflower flowering. About 1000 to 1250 sunflower plants were in anthesis each week. Honeybees from hives in the vicinity ensured pollination.

Around the cultivated stand, 16 plots of 4 m^2, each comprising at least 10 wild *H. annuus* individuals were established along four rays oriented according to cardinal points, at distances of 3 m, 100 m, 300 m and 500 m, plus two more plots located at 1000 m and 1200 m. Wild *H. annuus* seeds were germinated in plastic trays and planted into pots in the greenhouse. Seedlings at 4-6 leaf stage were transplanted to the experimental field. No other wild, volunteers or cultivated sunflowers were present in the surrounding area. After flowering was complete, heads of wild sunflower plants were protected with plastic mesh to prevent bird predation and shattering. Mature heads were harvested and bagged by plot and by plant. For isozyme analysis, 40-45 seeds were taken at random from a seed pool within each wild plot, and evaluated for *Acp-1* marker. Enzymes were extracted from seeds and resolved on horizontal starch gels (Carrera and Poverene 1995). Wild-crop hybrid frequency was calculated by counting heterozygous individuals over the total of individuals analyzed at each distance. The rate of pollen dispersal is defined as the total pollen flow expected at each distance assuming a concentric, circular arrangement of wild plants around crop. Therefore, it was estimated by multiplying hybrid frequencies by *π (pi) d* (diameter). Analysis of variance was carried out to study the effect of ray orientation and distance on hybrid frequency. Hybrid frequency values were arcsine square-root transformed.

Pollen flow from wild plants to crop

A commercial field planting of sunflower located in La Pampa province was found invaded by wild sunflower plants. Heads of cultivated plants were collected at regular intervals and a quarter section of each was sampled. A subset of achenes was sown in the experimental field and the plants obtained were screened for wild-crop intermediate phenotypes. Achenes from the same wild population collected two years before together with a commercial sunflower hybrid (cv. DK 3881) were also included in the experimental field. In order to assess hybridization, 28 morphological traits (18 continuous: plant height, stem diameter, head position and number, leaf width, length, and size (WxL), leaf number, petiole length, blade/petiole index, ray width, length and number, phyllary width, length, and L/W ratio, phyllary number, disc diameter; 10 categorical: branching type, presence of main head, leaf base, margin and surface, leaves at head back, phyllary disposition, disc flower color, pale and stigma anthocyanins) were registered on each plant. Differences among plants were compared through Kruskal-Wallis non-parametric analysis and discriminant analysis of continuous variables, and cluster analysis based on categorical variables using the InfoStat program (2002).

Results

Wild *H. annuus* populations occurred in six central provinces in Argentina, from eastern Entre Rios (32º 03' S, 60º 38’ W) to western Mendoza and San Juan, being the latter the Northwestern border for the species (31º 20’ S, 68º 32’ W). The Southern border was placed in Buenos Aires (37º 36’ S, 62º 53' W). The largest populations were found in Cordoba and Entre Rios. Isolated plants were found in three localities of San Luis. The populations were patchily distributed along roadsides and on disturbed soils. The crop area mainly comprises the western part of Buenos Aires, northeast of La Pampa, and central and southern Cordoba provinces. Besides, there are areas devoted to seed production between 31º 30’ and 35º 20’ S in the same provinces, including Mendoza. During the field exploration and collection, evidence of crop-wild hybridization was apparent in some populations because of intermediary in morphology traits.

Intermediate phenotypes were found in populations of La Pampa, Córdoba, Buenos Aires, and Mendoza provinces, in 21 out of 90 sites where seed was collected. Some off-type individuals were observed within cultivated lots in Buenos Aires and La Pampa, although no large wild population was present in the area. A number of crop-wild hybridization events are detailed in Table 6-1.

Volunteers were present along roadsides in all the sunflower cultivation area. In the studied area, out of 582 screened volunteers, 351 were fertile and unbranched, 102 were fertile and branched, 108 were male-sterile and unbranched, and 21 were male-sterile and branched. This represents a ratio of 77.8% fertile: 22.2% male-sterile and a 78.8% unbranched: 21.1% branched ratio. If volunteers were mostly the first generation progeny of sunflower commercial hybrids, a ratio of 75% fertile (R-) and 25% sterile (rr) would be expected. If all volunteers were F3 plants, a ratio of 83% fertile: 17% sterile would be expected (Table 6.2). Male-fertile: sterile ratios were in agreement with an F2 generation but the presence of some F3 individuals could not be discarded.

Considering the branching trait the 78.8% unbranched to 21.1% branched ratio was in accordance to the recessive character b1 described by Putt (1964). This trait was analyzed under the same assumptions as the fertility trait and the χ^2 goodness-of-fit values were $0.20>P>0.10$ for the F2 ratio expectation and $P<0.001$ for the F3 ratio expectation.

Table 6-1 Morphological evidence and site characteristics of wild-crop hybridization events observed over 4 years in central Argentina.

County and province	Morphological evidence of hybridization	Site characteristics	Year
	Evidence in wild *H. annuus* populations		
J.Celman, Cordoba	Head and leaf size, branching	Roadsides and fallow lands along Hwy 4 and 24	2000 2003
A. Alsina, Buenos Aires	Yellow convex discs, single large head on top	Roadsides of Hwy 60 and lateral dust roads	2002
Rancul, La Pampa	Yellow discs, wide bracts, anthocyanins	Roadsides of Hwy 188	2002
San Rafael, Mendoza	Single head, yellow discs, wide bracts and leaves	Roadsides of Hwy 179 and dust roads, border of irrigation ditches, farm limits	2002
Albardon, Caucete, San Juan	Yellow discs, long bracts, variable branching and cycle	Farm limits and within vineyards	2002
Quemu, Quemu, La Pampa	Single large head on top, striate pericarp	Roadsides of Hwy 10	2002
Diamante, Entre Ríos	Yellow discs, variable color in pericarp	Access to port roads, riverside and city limits	2003
Rio Cuarto, Cordoba	Disc size, single large head on top	Roadsides of Hwy 35 and lateral dust roads	2003
	Evidence in sunflower crops		
Trenque Lauquen, Buenos Aires	Numerous small heads, anthocyanin in disc and stem, pubescent achenes	Commercial field lots	2001
Realico, La Pampa	Tall, numerous heads, wide discs, variable color in pericarp	Isolate plants in fallow lands, city limits	2001

Table 6-2 Expected and observed numbers of fertile and male-sterile plants among volunteers, considering the natural progeny of commercial hybrids (equivalent to F2) and progeny of the previous year's volunteers (F3).

Cross	Fertile progeny	Male-sterile progeny
F2		
Rr x Rr	3/4R-	1/4rr
Observed	453	129
Expected	436.5	145.5
χ^2 goodness-of-fit	0.20>P>0.10	
F3		
1/12 RR x RR	1/12RR	
4/12 RR x Rr	4/12R-	
4/12 Rr x Rr	3/12R-	1/12rr
1/12 rr x RR	1/12R-	
2/12 rr x Rr	1/12R-	1/12rr
Total	10/12R-	2/12 rr
Observed	453	129
Expected	485	97
χ^2 goodness-of-fit	P<0.001	

Gene flow

It was not possible to identify crop-specific alleles to assess gene flow within the whole collection. However, DK3881 showed homozygosis for the slowest acid phosphatase allele *Acp-1-e*, which was rare in wild populations and completely absent in the analyzed accession of Cordoba province. Hybrid progeny was easily identified by the presence of bands which were absent in maternal plants, displaying a typical heterozygous three-banded pattern attributed to a dimeric structure of the active enzyme. Fifty three out of 760 analyzed seeds (7%) were crop-wild hybrids. Frequency values per stand ranged from 0 to 0.275. Crop-wild hybrids were found up to 500 m from the cultivated pollen source. Mean values and standard deviation for each distance are shown in Figure 6-1. Significant differences in hybrid frequency were observed between distances ($P<0.009$). No significant differences in pollen flow were detected for the same distance among the four rays. No evidence of gene flow was detected at the 1000 m and 1200 m sites. The maximum pollen dispersal rate was found at 300 m, being the distance at which pollen was transported with the highest relative amount (Figure 6-2). This means that the relative amount of pollen required to produce a given hybrid frequency was greater at 300 m than at 3 m.

Regarding gene flow from wild plants to crop, three morphologically intermediate plants out of 80 were found (3.7%), obtained from the heads collected in a field invaded with wild sunflowers. The intermediate plants differed from cultivated sunflower in eight morphological characters but none from the wild plants. Stem diameter had an intermediate value between wild plants and crop (Table 6-3).

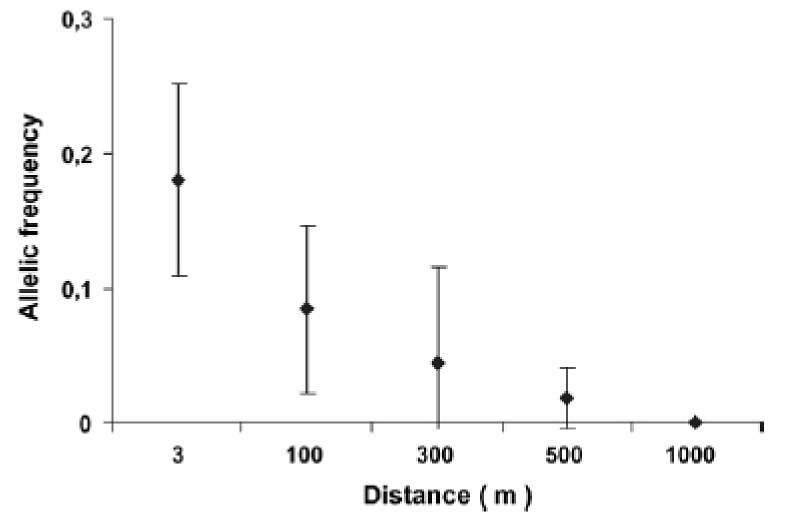

Figure 6-1 Frequency of sunflower cultivar marker among progeny of wild plants. Values represent the mean and standard deviation at each distance (n=40-45).

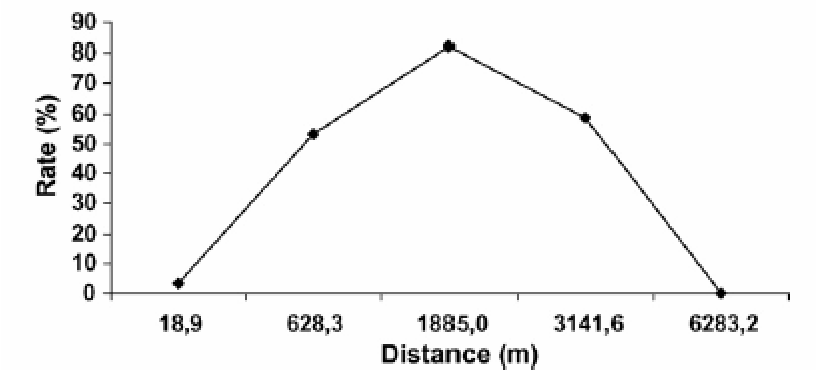

Figure 6-2 Crop pollen dispersal rate estimation into wild plants situated at five distances from a central sunflower pollen source.

Table 6-3 Morphological traits of hybrid plants from sunflower crop pollinated by wild sunflower and representative parental phenotypes.

Means are significantly different at P<0.01 (**) or P<0.001(***).

Character	Wild *H. annuus* (♂ phenotype)	Intermediate plants (hybrids)	Sunflower (♀ phenotype)	Significance
Plant height (cm)	157.80a	134.33b	108.80c	***
Stem diameter (cm)	1.69b	1.93ab	2.36a	***
Head number (n)	75.60a	33.67b	1.00c	**
Leaf width (cm)	18.00b	19.03b	25.60a	**
Ray length (cm)	4.13b	4.73b	8.72a	***
Ray flower number (n)	24.60b	22.67b	41.20a	***
Bract number (n)	27.67b	26.90b	33.70a	***
Bract L/W ratio	2.96a	2.95a	2.02b	***
Disc diameter (cm)	3.77b	4.23b	19.00a	***

Values followed by the same letter within a column are not significantly different at P=0.05.

Discriminant analysis based on 18 continuous traits confirmed that the three intermediate plants grown from seeds harvested on crop heads displayed a different morphology compared to wild or cultivated sunflower The variables which most contributed to discriminate between cultivated and wild sunflower (canonical axis 1 in Figure 6-3) were ray and phyllary width and head number, whereas those which best explained differences among intermediate and true type plants (canonical axis 2) were phyllary length, ray width, and head number. Cluster analysis based on 10 categorical traits showed that intermediate plants were more similar to wild than to cultivated plants (Figure 6-4).

Discussion

Overlap between wild populations and sunflower crop

Wild *Helianthus annuus* were found forming extensive clumps from the warm riverside in Entre Rios, to the rather xeric environments of San Juan. Populations varied in size and had a patchily distribution. Based on acreage of provincial counties, overlapping between wild sunflower distribution and cropped area, which was 1.96 million ha in 2005 (SAGPyA 2006) was estimated at 37%. This included the area devoted to seed production where gene flow could affect hybrid seed purity. Observed off-type individuals within cultivated lots in Buenos Aires and La Pampa, probably originated from wild pollen flow in the seed production fields. A similar situation is of concern also in the USA and France (Faure et al. 2002, Reagon and Snow 2006).

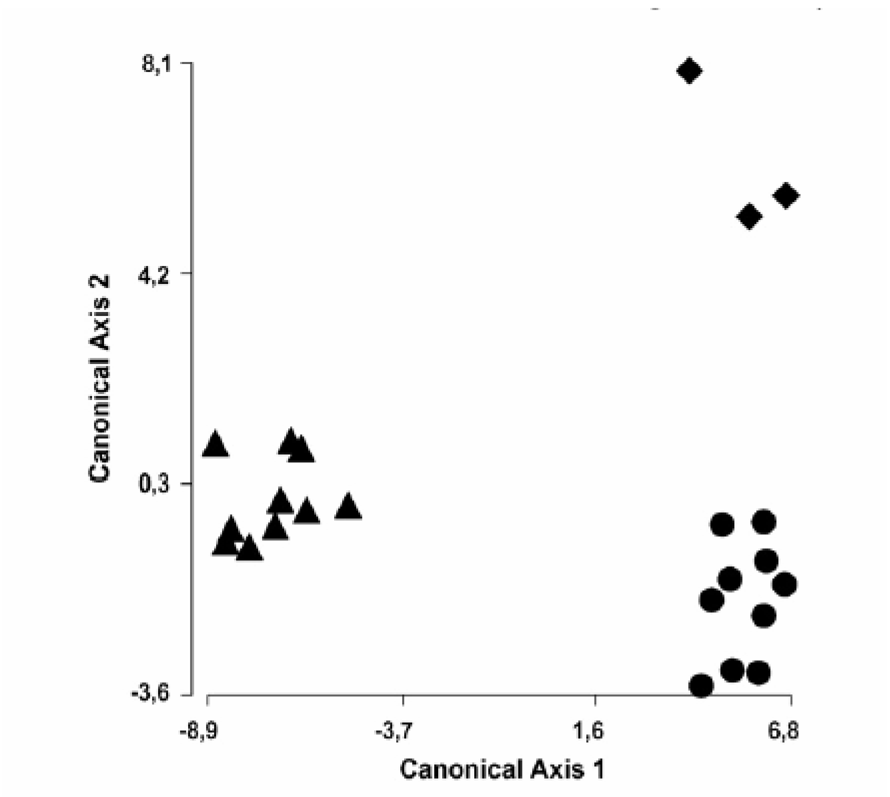

Figure 6-3 Discriminant analysis of progenies from heads harvested in a crop field invaded by wild sunflowers, based on 18 metric morphological traits.

Each point represents the score for an individual. Crop-like plants (triangles) and intermediate plants (diamonds) were found among progenies; wild plants (circles) were included as controls.

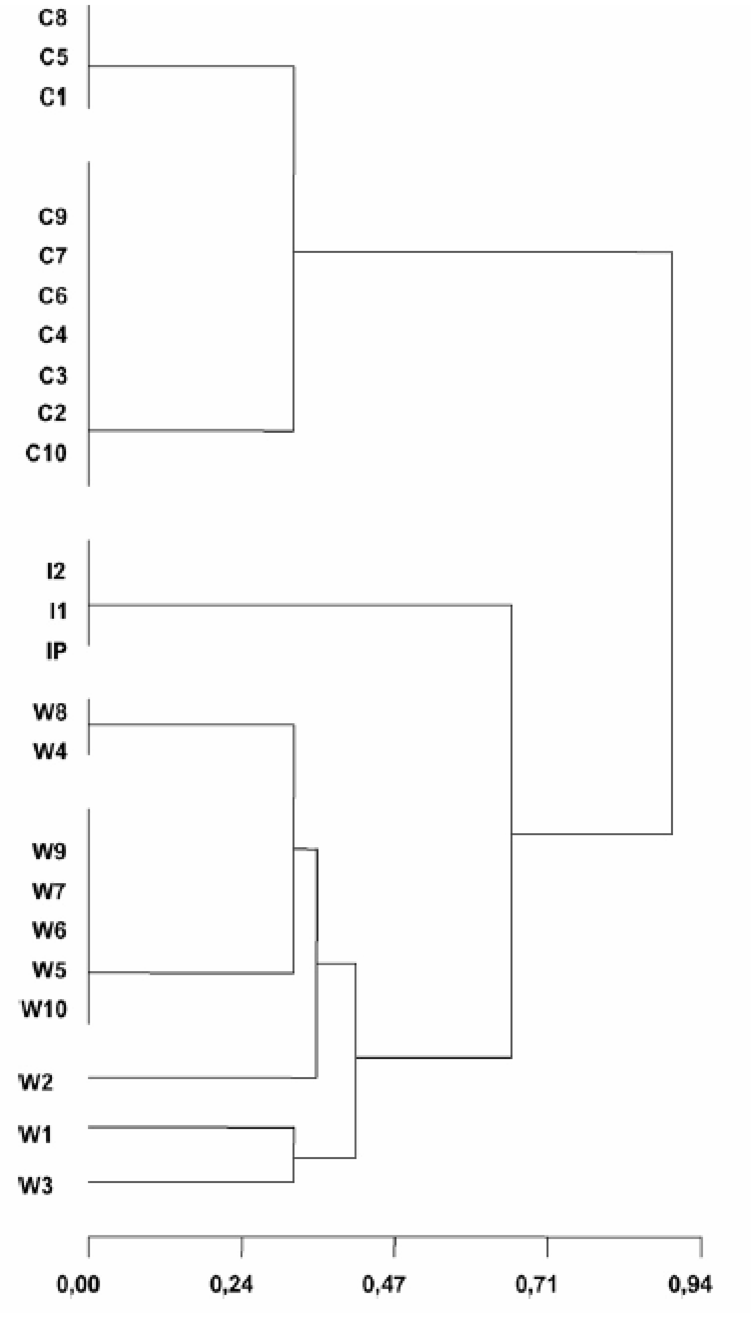

Figure 6-4 Cluster of crop-like (C), wild *H. annuus* (W), and intermediate (I) plants based on categorical morphological traits (standardized variables).

UPGMA clustering on the basis of Gower's distance matrix (Cophenetic correlation 0.992)

Plants with intermediate morphology were found in 23% of the collecting sites, so hybridization and gene flow between wild and cultivated sunflower seems a frequent event, as observed in six provinces. Overlapping flowering period between cultivated and wild sunflower occurs from December to February, being honey bees, wild bees and butterflies the main pollinating insects (Poverene et al. 2004). Thus, gene flow is expected to occur in the whole range of sunflower cultivation in the central part of the country. Wild *H. annuus* has also established in Spain and Italy and interferes with crop (Faure et al. 2002).

Volunteer sunflower increase the risk for gene flow

Volunteers were found as feral plants growing in the whole crop area, as in the USA (Reagon and Snow 2006) and in France (Faure et al. 2002). The phenotypic ratios found among volunteers confirmed their origin from seeds of commercial hybrids (F1) lost during transport. It cannot be discarded that at least some of these plants came from seeds of volunteers grown the year before (F2:F3). One out of five individuals was male-sterile or branched, becoming an extremely vulnerable plant susceptible to gene flow, thus increasing the chance for novel traits dissemination. Volunteers could act as a genetic bridge through which transgenes would spread to wild or cultivated plants (Reagon and Snow 2006). If GM sunflowers were released in Argentina, this would certainly take place over the whole cropping area.

Wild populations pollinated by crop plants

The occurrence of crop-wild hybridization was confirmed using isozyme markers, a reliable approach for the detection of such events. The overall mean frequency of hybrids (7%) was comparable to that obtained by Arias and Rieseberg (1994) in Mexico (10%). The region where the gene flow experiment was conducted displays warmer temperatures and lower humidity compared with the core sunflower production zone where higher hybridization rates could be expected. Arias and Rieseberg (1994) also found significant differences in gene flow rates between localities.

It was possible to track the distance and direction of pollen movement from source plants. No significant differences between cardinal rays mean that there was no effect of wind direction on pollinator activity. A leptokurtic distribution of pollen dispersal was observed, similar to that observed in North America. Average hybrid frequency half-declined from one distance to the next, but no hybrids were found at 1000 m. This finding and the maximum pollen dispersal rate sustain the isolation distance of 3000 m recommended by The National Committee for Agricultural Biotechnology in Argentina for GM sunflowers management. Wild sunflower carrying the transgene that confers white mould resistance did not show a significant increase in fitness measured by seed output (Burke and Rieseberg 2003) while plants with Bt transgene produced more seeds than non-transgenic individuals (Snow et al. 2003). In Argentina, traits under field experimentation in sunflower include tolerance to herbicides (glyphosate, glufosinate), insect tolerance (Lepidoptera, coleopteran), fungal resistance, and other fitness-enhancing traits such as increased nitrogen assimilation (Cantamutto and Poverene 2007). These transgenes are expected to be neutral or beneficial in wild sunflower populations and may spread quickly, but this does not mean that they will result in more invasive weeds (Snow et al. 2005).

Crop plants pollinated by wild populations

Gene flow also occurs from the wild species to the cultivated sunflower. This was evidenced by intermediate morphological traits in plants grown from seeds of sunflower heads collected in a field invaded by wild *H. annuus.* Frequency of intermediate plants was of 3.75 %, in a random sample of such seeds. The plants resulted morphologically more similar to the wild parent and this was considered enough evidence of gene flow from wild plants to crop, because a molecular screening would have been impractical.

One caveat regarding this experiment is the low number of plants examined, even though the finding of three intermediate-type plants out of 80 was enough evidence of wild-crop gene flow. Furthermore, this pollen flow is expected to be lower than crop-wild pollen flow for several reasons. First, in the Argentine landscape the number of cultivated plants is several times higher than wild plants, which usually occur in small patches. In the 2005/06 season, sunflower acreage in the invaded provinces was of 1.7 million hectares. Considering about 40,000 plants per ha, this means over 70 billion cultivated plants. Second, big sunflower heads attract more insects because of larger nectaries and anthers than the small wild heads, therefore crop pollen could represent the majority of the pollen load in the insects. Finally, during crop flowering time (7-10 days) the amount of available wild pollen is much lower, because heads are small, with 3-4 cm discs, even though wild plants flower for a longer time (20-40 days).

Gene flow in Argentina was demonstrated by the extent of overlapping areas of wild and cultivated sunflower, hybridizing frequencies, and recurrence of wild-crop hybrids. Sunflower can be considered a crop of high probability of transgene and herbicide tolerance transference.

Acknowledgements

To National Research Council of Argentina (CONICET) for a fellowship to MSU. Also we thank technical assistance from A. Garayalde and A. Gutierrez, and greatly appreciate statistical help from S. Luis and A. Hernandez. This research was supported by grants ANPCYT- PICT 08-9881 and UNS-PGI 24A106.

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Chapter 7 Ecological characterization of wild *Helianthus annuus* and *H. petiolaris* germplasm in Argentina

2008

Accepted in Plant Genetic Resources: Characterization and Utilization

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Abstract

Helianthus annuus and *H. petiolaris* (Asteraceae) are wild sunflowers native to North America but have become naturalized in central Argentina covering an area of about five million hectares. Wild *H. annuus* has been recognized as invader species in several countries, but no research has been done to study the ecological determinants of their distribution. In a survey covering seven provinces between 31º58'-38º S and 60º33'-69º W, we described the ecology of the main wild populations. Wild *Helianthus* populations were located in three of the 18 ecological regions of Argentina, on five Mollisol and seven Entisol soil groups. The associated plant communities were comprised of 60 species belonging to 16 families, all being frequent components of the native flora. Disease symptoms were seldom observed in wild populations, with *Alternaria helianthi* being the most commonly observed pathogen. Population size varied from less than 100 to more than 100,000 plants, covering from 100 to more than 60,000 m^2 with densities most frequently up to 3 plants per m^2, but reaching 80 plants per m^2 at certain sites. Intermediate plant phenotypes between wild species and cultivated sunflower were found in one-third of the populations providing evidence of intense gene-flow. Hybrid swarms were found at three localities with a population sizes between 100 and 10,000 individuals.

Key Words: Community, density, diseases, habitat, sunflower, populations.

Introduction

Helianthus annuus L. and *H. petiolaris* Nutt. (Asteraceae) are annual, diploid species native to North America where the former has a wide distribution and the latter is restricted to the central region (Heiser et al. 1969, Rogers et al. 1982). Wild or common *H. annuus* tends to be weedy, always located in habitats that have been disturbed. The prairie sunflower, *H. petiolaris*, usually grows in sandy soils, but it is also found as an adventive weed elsewhere (Seiler and Rieseberg 1997). Both species have several botanical forms and are systematically complex (Heiser 1954, 1961, Seiler and Rieseberg 1997, Jan and Seiler 2007). *Helianthus annuus* is the ancestral species of cultivated sunflower (Heiser 1978, Burke et al. 2002).

Both species are valuable germplasm resources with traits that have been transferred into cultivated sunflower, i.e. cytoplasmic male-sterility (CMS) from *H. petiolaris* (Leclercq 1969), but Rieseberg and Seiler (1990) provided evidence that CMS may have been derived from *H. annuus*, disease and pest resistance, oil quality and other traits for crop breeding (Seiler 1992). These species are also crop weeds in North America (Geier et al. 1996, Rosales Robles et al. 2002, Deines et al. 2004) and are beginning to invade summer crops in Argentina.

Sixty years after the first introduction of *H. annuus* and *H. petiolaris* they have become naturalized in the central area of Argentina (Covas 1966, Bauer 1991, Poverene et al. 2002). At present, their distribution significantly overlaps that of the sunflower crop. As in the Northern hemisphere, flowering time of both wild species and the cultivated sunflower coincide and they share pollinators, mainly honeybees, bumblebees, and other wild bees, favoring gene flow (Burke et al. 2002, Poverene et al. 2004) and natural hybridization processes (Rieseberg et al. 1998, 1999b).

Wild *H. annuus* has been recognized as an invader species in several countries (Berville et al. 2005), but at present *H. petiolaris* has been naturalized only in Argentina. The study of the invasive process of these annual species could help to understand and prevent analogous processes in other regions of the world. Cantamutto et al. (2008) studied the environmental conditions of these invader species distributions, but there is no available information about the ecology of these wild sunflower species. The objective of this research was to describe the eco-geographic distribution of *H. annuus* and *H. petiolaris* in Argentina and to characterize the populations in their natural habitats.

Materials and Methods

Agro-ecological descriptions of populations were made during a collection trip across seven central provinces of Argentina, during February, 2007 (Figure 7-1). Thirty previous explorations carried out between 2000 and 2006 provided data on population locations, habitat, and soil type. Collected information included botanical name, collection site (province, district, location, latitude, longitude, and altitude), environmental conditions (habitat) and community (dominance of co-occurring plant species), estimated population size, plant density, plant size, and morphological variation. Also, the occurrence of prevalent sunflower diseases was recorded: downy mildew (*Plasmopara halstedii*), rust (*Puccinia helianthi*), white rust (*Albugo tragopogonis*), *Alternaria helianthi*, *Verticillium dahliae*, *Phoma macdonaldii*, and *Sclerotinia sclerotiorum* wilt.

The geographic coordinates were used to determine the agro-ecological regions (Burkart et al. 1999) and soil taxa (INTA 1990) corresponding to each population. The order, great group, suborder, area, and predominant texture of each soil type were used to describe the habitat environment of both species (Bouma 2003).

Data recorded for each population included occupied area, density, and total number of individuals. The total area was the sum of one to five quadrants measured at each site which contained all the individuals. The density was estimated by 10 samples within the quadrants, taken at regular intervals along the main transect across each population, with a 0.25 m^2 circle. The total number of plants was then calculated as a product of area x mean density. In the case of populations growing in continuous patches, the limit was established as the point where the distance between two patches was greater than the longest side of the quadrant.

Plant community density was recorded for each quadrant following a semi-quantitative method (Clay and Johnson 2002). At each collection site, data were collected from 10 points on a uniformly spaced grid coordinate system. At each grid point (a 2 m^2 circle) abundance was qualified as following: 0= absent; 1= less than 5 plants m^{-2}; 2= 6-10 plants m^{-2}; and 3= more than 10 plants m^{-2}. The 20 most frequent species were characterized by life cycle, origin, and status.

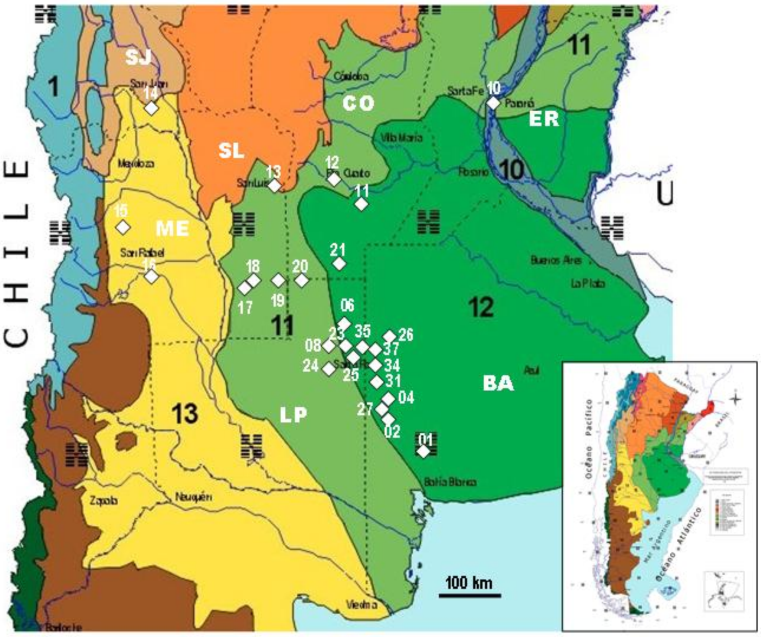

Figure 7-1 - Wild *Helianthus* populations (white numbers) sampled in three ecological regions (black numbers) of central Argentina: 11 Espinal, 12 Pampa, 13 Shrubs of Plateau and Plains. Soils in the Pampa region are mainly Mollisols whereas Entisols predominate in the other two regions. Provinces are Buenos Aires (BA), Cordoba (COR), Entre Rios (ER), La Pampa (LP), Mendoza (ME), San Juan (SJ), San Luis (SL) (Map from Burkart et al. 1999; scale 1:15,000,000).

To estimate the number of plants potentially exposed to gene flow, a mean was obtained for population size based on the ranges observed in more than 50% of the populations. Cultivated plant number was computed taking into account the minimum acreage per cultivated field (30-60 ha) and the minimum number of plants per hectare (40,000) usually sown. Frequencies of gene flow between the three taxa were obtained from our previous research (Poverene et al. 2004, Cantamutto et al. 2007, Ureta at al. 2008).

Results and Discussion

Collection site data characterization

Helianthus annuus populations were widespread ranging from 31º58' to 37º31'S, and 60º33' and 68º14'W, at an altitude of 128 to 600 m.a.s.l. (Table 7-1). Plants grew in patches in disturbed habitats such as roadsides, ditches, fence rows, and field margins in the sunflower production areas (Buenos Aires, La Pampa and Cordoba provinces). They were also patchily distributed along irrigation channels in the western provinces (Mendoza and San Juan) and also growing along crags for several kilometers of the coastal rivers in eastern Entre Rios province. Population size varied from a few dozen individuals to more than 100,000 plants, with mean densities varying between 0.25 and 6 plants m^{-2}. However, some populations reached 72 and 80 plants m^{-2} in Mendoza and Cordoba provinces respectively. Most plants were very robust with heights over 2.80 m. Regarding the number of plants, wild populations in the center of the country were only one magnitude of order lower than crop populations that are usually between 1-2.5 million plants (Ureta at al. 2008).

Table 7-1 Frequency of selected populations and habitat characteristics of wild *Helianthus annuus* and *H. petiolaris* collected in Argentina.

Ecogeographic data	*Helianthus annuus* (%)	*Helianthus petiolaris* (%)	Both (mixed stands) (%)
Altitude (m.a.s.l.)			
<300	55	60	25
>300	18	40	75
Not recorded	27	0	0
Population size (Nº plants)			
<100	9	0	0
101-1000	0	30	50
1001-5000	55	30	25
5001-10000	18	20	25
10001-50000	9	20	0
>50000	9	0	0
Surface area (m^2)			
100-1000	27	20	25
1001-10000	27	70	75
10001-50000	18	0	0
>50000	27	10	0
Mean plant density ($pl.m^{-2}$)			
<1	27	40	50
1-3	64	50	50
>3	9	10	0
Maximum plant density ($pl.m^{-2}$)			
16-25	22	40	0
11-15	23	10	50
5-10	33	20	0
<5	22	30	50
Plant height (cm)			
>280	22	0	0
200-280	45	10	50
<200	33	90	50

Table 7-1.Continuation

Soil texture			
Loam	9	0	0
Loamy sand	18	40	25
Sand	0	60	0
Sandy loam	64	0	75
Silt loam	9	0	0
Habitat			
Roadside, intersection	55	80	75
riverside	9	0	0
field margin	18	10	25
within crop	0	10	0
ditch	18	0	0
Volunteers			
present	46	10	25
absent	55	90	75
Intermediate plants			
present	36	30	100
absent	64	70	0

Helianthus petiolaris was the most frequent species, but was geographically more restricted ranging from 35º08' to 38º08'S and 62º16' and 65º56'W, and up to 455 m in altitude (Table 7-1). Populations were very numerous in the eastern La Pampa and western Buenos Aires provinces, reaching 18,000 plants with a mean density of 0.25 to 6 plants m^{-2}, and up to 40 plants m^{-2} in the former. Sparser populations were found in southern San Luis. In Cordoba, this species seemed to be confined to the southern extreme of the province. *Helianthus petiolaris* often grows in field margins and seldom invades sunflower, corn, or pasture crops. Most populations were found in roadsides and road intersections on disturbed sandy soils. Compared to previous collection trips, the species seemed to be more widespread, although population size and density are strongly dependent on climatic conditions, particularly moisture. Wild *Helianthus* populations were found in the agricultural regions where soybean, maize, sunflower, and wheat are the predominate crops.

Two perennial *Helianthus* populations were found in Mendoza, probably *H. tuberosus* or *H. x laetiflorus*, but were difficult to identify because they were just beginning to flower. These kinds of feral populations also occur in the Buenos Aires province where they are usually established by rhizomes discarded from gardens (Sala et al. 1990).

Gene flow

Volunteer plants from the cultivated sunflower crop were found among wild ones and many plants showed intermediate morphological traits, indicating a frequent crop-wild gene exchange in Buenos Aires and La Pampa provinces, where there is a large sunflower crop acreage, and Mendoza where there are areas devoted to sunflower seed production. Volunteers can considerably enhance sunflower crop-wild hybridization acting as a bridge for genetic transfer of crop traits into wild populations (Reagon and Snow 2006). Persistent cultivar gene flow determines high levels of introgression and the replacement of wild populations by advanced generation hybrids (Linder et al. 1998). Morphologically intermediate plants indicated that also crop-*H. petiolaris* hybridization occurs when they come into contact. Variation was observed for leaf size, presence of anthocyanin in stems and petioles, ray color, and white pubescent disc flowers in the center of the head.

The magnitude of crop-wild gene flow in Argentina has been estimated through field observations and previous experiments (Poverene et al. 2004, Ureta et al. 2008 and unpublished data). Although the hybridization frequency was similar to that observed in

North America (Arias and Rieseberg 1994, Rieseberg et al. 1999a), plants are so numerous that even at a low frequency of interspecific crosses, thousands of F1 hybrids are likely produced every year (Figure 7-2).

Sunflower crop genes persist for several generations in wild populations (Whitton et al. 1997, Linder at al. 1998) and can modify wild populations depending on their fitness (Alexander et al. 2001, Cummings et al. 2002) and on the environments where they grow (Mercer et al. 2007). The high number of morphologically intermediate crop-wild plants observed in two-thirds of the populations in central Argentina could be assessed to gene flow. The consequences of the frequent hybridization process have yet to be fully evaluated

Three hybrid swarms of both wild species were found, one in Buenos Aires and two in La Pampa. These swarms included wild type plants, intermediate, and a number of volunteers from crop plants. In the largest swarm from La Pampa, the northern half was comprised of 2,600 plants with about 15% being annuus-like and a plant density of 1.34 plants m^{-2}. The southern part was comprised of about 8,000 plants, with 50% being annuus-like and the rest petiolaris-like. Many intermediate plants were observed in the central zone. When these species come into contact in North America, they can often form hybrid zones that have given rise to three other species via homoploid speciation (Rieseberg et al. 1990, 1991; Rieseberg 1991). Although both species have become established in Argentina rather recently, hybridization and introgression processes are taking place in this new environment.

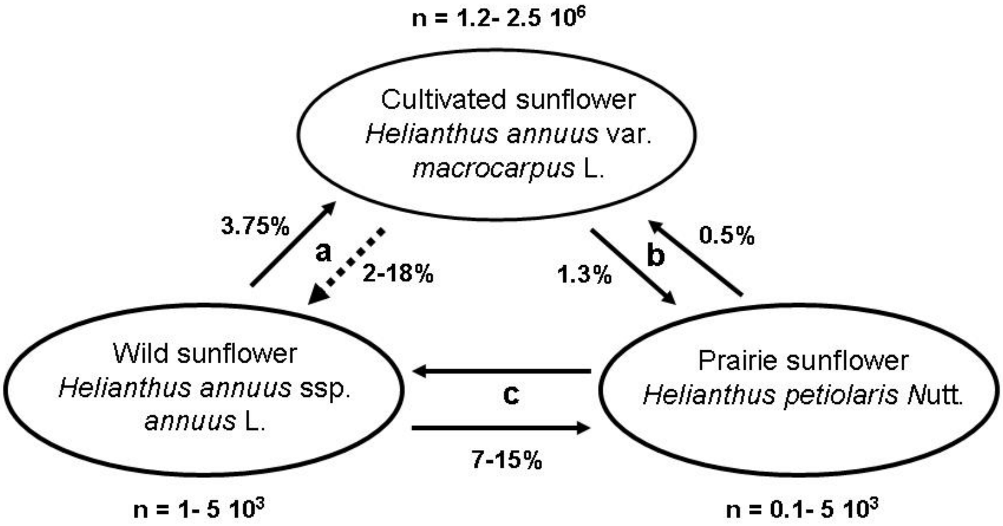

Figure 7-2 Gene flow frequencies among cultivated and wild sunflowers in Argentina and number (n) of plants estimated as a range from data in Table 7-1. a: from Ureta et al. (2008); b: from Poverene et al (2004); c: from Cantamutto et al. (2007). Gene flow values were estimated in natural conditions, except one which came from a planned field experiment (dotted arrow).

Ecology

Wild species populations were found in three of the 18 ecological regions described in Argentina by Burkart et al. (1999). These include the Pampa, Espinal, and Shrubs of Plateau and Plains (Figure 7-1). From the east, Pampa is a grass steppe without woody species, followed by Espinal, an intermediate savannah, with grasses and scarce xeric trees. The western Shrubs of Plateau and Plains is an arid steppe with the predominance of shrubs and tough grasses. Both wild sunflower species extend along a SE-NW boundary which coincides with the limit between Pampa and Espinal regions. The subhumid region called Pampa is a cultivated area which corresponds to grasslands ploughed within last 140 years, while the semiarid region, called Espinal comprises savannahs with xerophytic shrubs, where agriculture is much more recent.

Soil taxonomy as an indicator of ecosystem processes can predict potential plant species suitable habitats (Mann et al. 1999). Mollisols, Alfisols, and Entisols orders cover only 18% of the world's temperate areas, but predominate in the centre of origin of the genus *Helianthus*, where together they cover 48% of the USA surface. The soils of the Central Great Plains of North America, the common distribution area for the two annual species *H. annuus* and *H. petiolaris* (Rogers et al. 1982) belong to these orders (USDA 1999). In Argentina, *H. petiolaris* and *H. annuus* populations were found on five Mollisol and seven Entisol groups (Table 7-2). Species distributions were significantly associated with soil subgroups according to Pearson Chi-square test (Pearson χ^2 <0.001, highly significant). The 14 soil subgroups where the *H. annuus* populations were found cover 9.9 million ha, while the 11 subgroups associated with *H. petiolaris* cover 13.1 million ha (INTA 1990). In this central area, where the sunflower crop has moved to in the least 10 years, there is a high probability of observing new wild sunflower populations because of the favourable macro-habitat conditions.

The plant communities associated with the wild *Helianthus* species were comprised of 60 species belonging to 16 families. Of these, 32 were found associated with both wild species. Most frequent species were *Sorghum halepensis*, *Cynodon dactylon*, *Eragrostis curvula* (Poaceae), *Chenopodium album*, *Salsola kali* (Chenopodiaceae), and *Centaurea solstitialis* (Asteraceae). Except for *E. curvula*, these species are weeds and related to disturbed soils (Marzocca 1994). *Eragrostis curvula* or "weeping lovegrass" has become established in sandy soils subjected to wind erosion. Nineteen other species were found only in *H. annuus* communities, with *Melilotus albus*

(Fabaceae) being the most common. This forage species is salt tolerant and has become established in humid soils with medium to high salt levels (Marzocca 1994). Nine other species were only found in *H. petiolaris* communities, where *Cenchrus pauciflorus* (Poaceae) was the most frequent. This is a noxious weed, very common in sandy soils of the western central region (Marzocca 1994). Differences for the latter two species' association with wild sunflowers were significant ($p<0.05$). Table 7-3 presents life cycle, origin, and status of the 20 species most frequently found associated with wild sunflowers in the explored provinces, considered as the dominant community species. Among these, six species are considered noxious weeds of agriculture in Argentina. All the cited species are frequent components of the flora in central Argentina and none of them was indicative of a specific ecosystem. This reinforces the hypothesis that abiotic factors, particularly disturbance determine the wild *Helianthus* colonization (Cantamutto et al 2008) and that the two wild sunflowers will expand their distribution when the habitat and opportunity arises.

Table 7-2 Frequency of stable wild *H. petiolaris* (PET), *H. annuus* (ANN) populations and mixed stands (MIXED) associated with 16 of the 65 soil taxa defined by INTA (1990) for the colonized provincial counties.

Soil			Population frequency (%)		
Order	Group	Subgroup	ANN	MIXED	PET
Mollisols	Argiaquolls	typic	4.65		
"	Argiudolls	aquic	6.98		
"	"	typic	4.65		
"	Argiustolls	typic	6.98	25.0	2.70
"	Hapludolls	various	4.66		
"	"	entic	18.60		12.16
"	"	thapto-argidic			4.06
"	"	typic	6.98		4.06
"	Haplustolls	entic	9.30	50.00	31.08
"	"	litic	4.65	25.00	2.70
"	"	various			12.16
"	"	udortentic	6.98		
Entisols	Torrisfluvents	typic	13.95		
"	Torripsamments	various	6.98		1.35
"	Udipsamments	typic	2.32		1.35
"	Ustisfluvents	typic	2.32		
"	Ustipsamments	typic			20.27
"	Ustorthentst	typic			8.11

Table 7-3 The 20 dominant community species most frequently associated with wild sunflower populations in central Argentina.

Species	Family	Cycle[1]	Origin	Status	Provinces
Chenopodium album	Chenopodiaceae	AN	E	I, MS	BA,COR, LP,SL,M,SJ
Sorghum halepense	Poaceae	PE	E, A	We*	all
Cynodon dactylon	Poaceae	PE	E, A	We*	all
Centaurea solstitialis	Asteraceae	AN	E, A	We, SS	BA,COR,ER,LP,SL
Salsola kali	Chenopodiaceae	AN	E, A	We*	BA,LP,M,SL
Eragrostis curvula	Poaceae	PE	E, N	SS	BA,COR,LP,SL,M
Melilotus albus	Fabaceae	AN	E, A	SS	all
Portulaca oleracea	Portulacaceae	AN	E, N	MS	all
Tagetes minuta	Asteraceae	AN	Na	SS	all
Setaria verticillata	Poaceae	AN	E, A	MS	all
Eleusine indica	Poaceae	PE	Na	SS	BA,COR,ER,LP
Diplotaxis tenuifolia	Cruciferae	PE	E, A	We*	BA,COR,LP,SL,M,SJ
Amaranthus quitensis	Amarantaceae	AN	Na	We*	BA,ER,M,SJ
Chenopodium multifidum	Chenopodiaceae	PE	Na	MS	all
Medicago sativa	Fabaceae	PE	E, A	MS	BA,COR,ER,LP,M,SJ
Cenchrus pauciflorus	Poaceae	AN	Na	SS	BA,COR,ER,LP,SL,M
Heterotheca latifolia	Asteraceae	AN	E, A	We, SS	COR,LP,SL
Onopordon acanthium	Asteraceae	BI	E, N	We*	BA,LP
Polygonum aviculare	Polygonaceae	AN	E, A	MS	all
Solanum elaeagnifolium	Solanaceae	PE	Na	MS	all

*Agricultural epidemic

[1] **Life cycle:** AN= annual, PE= perennial, BI= biannual. **Origin:** Na=native, E= exotic, A= adventive, N= naturalized. **Status:** We= weed, I= invasive, MS= modified soils, SS= sandy soils. **Provinces:** BA= Buenos Aires, LP=La Pampa, SL= San Luis, COR= Cordoba, M= Mendoza, SJ= San Juan, ER= Entre Rios, all= all the 7 explored provinces.

Disease symptoms were observed in only 25% of the wild populations. *Alternaria* lesions on leaves were the most frequent, with *A. helianthi* being the most likely pathogen. *Puccinia helianthi* was often found on volunteer plants, but never on wild plants. Table 7-4 presents the observed diseases and the frequency of affected plants. Most populations were free from diseases and confirmed that wild sunflower species are potential gene reservoirs for fungus and virus resistance.

Wild *H. annuus* and *H. petiolaris* form large populations distributed over an area of about five million hectares in central Argentina. Since their establishment 60 years ago they have continuously increased their area, behaving as an invasive species providing evidence that they will continue spreading. These species offer opportunities for research covering various scopes. First, both wild *Helianthus* constitute germplasm reservoirs of biotic and abiotic gene resistance for crop improvement. Second, wild populations subjected to gene flow may acquire crop traits (i.e. herbicide tolerance) that modify their fitness enhancing invasiveness or weediness, changing ecological relationships in their environment. Lastly, hybrid zones allow comparative studies with the center of origin regarding processes of parallel adaptation and speciation.

Acknowledgements

This research was supported by grant UNS-PGI 24A106 and special funds from Postgraduate Studies Department of the Universidad Nacional del Sur for the visit of Dr. Gerald J. Seiler.

Table 7-4 Observed diseases on wild *Helianthus* populations from central Argentina.

Population		Disease	Frequency of infected plants (%)
1007	*H. annuus*	Alternaria	70
1107	*H. annuus*	Alternaria	90
		Phoma black stem	90
1207	*H. annuus*	Alternaria	10
1307	*H. petiolaris*	Alternaria	10
1407	*H. annuus*	Virus (SuCMoV)[1]	40
		Powdery mildew	10
1607	*H. annuus*	Alternaria	20
2107	*H. petiolaris*	Alternaria	40
2507	*H. annuus*	Alternaria	30
2607	*H. petiolaris*	Alternaria	20
	crop-wild hybrids	Alternaria	50
		Phoma black stem	20
3507	*H. petiolaris*		
	crop-wild hybrid	Phoma black stem	10

[1] Field identification by leaf lesions.

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Chapter 8 Novel biodiversity in wild *Helianthus annuus*

2008

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Abstract

Wild *Helianthus annuus* is a non-native invader naturalized in central Argentina during the last 60 years. It was intentionally introduced for forage purposes and probably escaped from cultivation. Argentine phenotypic biodiversity of nine populations from different geographic regions in central Argentina were compared using multivariate techniques with 17 populations from the USA grown in a common garden (S 38º 41’, W 62º 14’). Wild populations from Argentina reflected about two-thirds of the phenotypic variability of those from the center of origin, but showed adaptation to local conditions which allowed differentiation between populations from each hemisphere. Most Argentine populations showed phenotypic similarity with some North American populations, mainly those from Illinois and Indiana. The presence of traits corresponding to domesticated sunflower was present in wild populations from both continents. Bract width over 0.8 cm differentiated Argentine populations as an evidence of crop introgression, but this trait was also present in wild populations from the USA. The great phenotypic biodiversity found in wild *H. annuus* from Argentina did not reflect founder effects and could represent a novel biodiversity in the newly colonized environment enhanced by the intense gene flow in the Argentine landscape. The lack of canonical correlation between climate and phenotype in the Argentina provinces provides evidence suggesting that the adaptation process is still ongoing.

Key Words Ferality - Morphology - Non-native invader - Phenology - Sunflower

Introduction

Being a relatively new culture in the Old World, sunflower and its wild relative complex have received considerable interest in the history of crop domestication and agriculture (Harlan1992). *Helianthus annuus* L. (Asteraceae) is native to North America and has spread as an oil crop and as an ornamental (Heiser 1954, Seiler and Rieseberg 1997, Schilling 2006). The domesticated sunflower, *H. annuus* var. *macrocarpus* has become a well adapted crop in Eastern Europe and Argentina, but their wild relative *H. annuus* spp. *annuus* have been found as a non-native invader in Australia (Dry and Burdon 1986) and Southern Europe (Bervillé et al. 2005), sometimes being considered a weed or a crop contaminant.

The modern sunflower crop, domesticated in America more than 4000 years before present (Harter et al. 2004) was significantly improved as an oil crop by Russian breeders during the 19º century. This traditional crop was introduced into Argentina through varieties brought by European immigrants in the 1930s that successfully became adapted to local agro-ecosystem conditions (Bertero and Vázquez 2003). One decade latter, wild *H. annuus* was intentionally introduced as a forage crop in the central part of the country (Bauer 1991). It is unclear how in the following 60 years the species spread throughout central Argentina (Poverene et al. 2002) colonizing well defined habitats at the environmental level (Cantamutto et al. 2008).

In the Argentine scenario, de-domestication via exo-ferality was considered feasible due the proximity of sunflower and its weedy relative's complex (Gressel 2005). This possible origin could be enhanced by the intense gene-flow under local agro-ecological conditions (Poverene et al 2004), where sunflower is an extensively grown crop (Ureta et al. 2008). Actually, the wild populations are distributed west of the more-adapted area of the sunflower crop (de la Vega and Chapman 2006).

In their naturalized habitat (Poverene et al. 2002) the wild *H. annuus* populations show considerable morphological differences that could be attributed to phenotypic plasticity (Richards et al. 2006). A common garden study minimizes environmental effects (Bender et al. 2002, Sugiyama 2003) allowing for the measurement of genetic variability. Phenotypic traits could be considered suitable for this purpose (Rawashdeh et al. 2007) without incongruence sometimes accounted for by molecular techniques (Soleimani et al. 2007).

We hypothesize that the naturalized *Helianthus annuus* in Argentina consist of variants of the true wild germplasm, containing a proportion of the biodiversity from the centre of origin. Since these populations originated from another country and have become naturalized, their potential value as a unique genetic resource could justify their preservation..

Materials and Methods

Germplasm samples from Argentina were collected from nine wild *Helianthus annuus* populations from different geographic regions previously described (Cantamutto et al. 2008). The provinces of Córdoba, La Pampa and Buenos Aires contributed two populations each; Rio Cuarto (RCU) (S 33º 09', W 64º 20'), Juarez Celman (JCE) (S 33º 40', W 63º 28'), Colonia Barón (BAR) (S 36º 10', W 63º 53'), Rancul (RAN) (S 35º 04', W64º 46'), Adolfo Alsina (AAL) (S 37º 16', W 62º 59`) and Carhué (CHU) (S 37º 16', W 62º 55'), respectively. Entre Ríos, San Juan and Mendoza provinces has one populations each; Diamante (DIA) (S 32º 03', W 60º 38'), Media Agua (MAG) (S 31º 57', W 68º 27') and Las Malvinas (LMA) (S 34º 47', W 68º 15'), respectively.

Wild germplasm from North America was represented by 17 populations. States of origin and passport numbers were: Arizona (AZ) PI 468571, California (CA) PI 468580, Colorado (CO) PI 468621, Illinois (IL) PI 435540, Indiana (IN) PI 468633, Iowa (IA) PI 597901, Kansas (KS) PI 586851, Montana (MT) PI 586821, Nebraska (NE) PI 586867 Nevada (NV) PI 468596, New Mexico (NM) PI 468537, North Dakota (ND) PI 586807, Oklahoma (OK) PI 468483, South Dakota (SD) PI 586835, Texas (TX) PI 468504, Utah (UT) PI 468607, and Wyoming (WY) PI 586824 (for more information see www.ars-grin.gov/cgi-in/npgs/acc/display.pl?1080516).

Seedlings were grown for 30 days in the greenhouse at 20-25ºC in 28 x 54 cm 200-cell plastic trays and then transplanted in rows 2.10 m long with 0.25 m between plants for an equivalent population of 19.047 plants ha^{-1}. Plots at the Agronomy Department, Universidad Nacional del Sur, Bahia Blanca, Argentina (S 38º41’, W 62º14’) were drip irrigated for maximum plant growth. Data were collected from 238 and 248 individuals from Argentina and USA respectively, during 2004 to 2006 summer seasons, including 9 to 24 populations each year. Observations were made on more than 20 individuals per population, except for four USA accessions with poor germination which had only

14 to 18 individuals. Original seed was used for the first year, while re-generated seed was used in subsequent years. Controlled pollinations for seed regeneration were made by hand on heads of more than 20 sibbed individuals and then covered with polyamide bags until the end of flowering.

Descriptors from USDA (2007) GRIN (Germplasm Resources Information Network) database were selected to assess variability between populations. Metric traits measured on individual plants included: final plant height (ALTU), stem diameter at mid-height (DIAMTA), head position-angle (INCLCAP), leaf number (NUHOJ) and total head number (NUCAP). Leaf width (ANHOJ) and length (LARHOJ), petiole length (LARPEC) and their relationship (ILAMPEC = LARHOJ/LARPEC) were determined on first order leaves at the flowering stage. Ray flower number (NUFLIG), ray width (ANFLIG), ray length (LARFLIG), bract number (NUFIL), length (LARFIL), width (ANFIL) and head diameter (DIAMCAP) were determined on first order heads. The presence of main head (CAPRIN), cuneate leaf base (BAHOJ), cordate leaf shape (FORHOJ), flat leaf surface (SUHOJ), serrate leaf margin (MAHOJ), stem anthocyanin (ANTALL), leaves on back of head (HOJCAP), pale anthocyanin (ANPAL), stigma anthocyanin (ANEST), and yellow disk flower (CODIS) were recorded by individual, and reported as frequencies. Life cycle of each population was computed as days from transplant to beginning (DTRINFL), mid and end (DTRFINFL) of flowering and their sub-periods (DPLFINFL = DTRFINFL - DTRINFL).

The mean of each quantitative trait was estimate by LSMEANS using the GLM procedure of SAS (2002) through the lineal combination of model effects, considering years as blocks and individuals as replicates. Populations were compared by multivariate analysis of LSMEANS for metric traits and frequencies for categorical traits. Principal component analysis (PCA) using correlation matrix to avoid scale effect, revealed the variables which mainly contributed to population differentiation (InfoStat 2002). For cluster analysis, a matrix based on Mahalanobis distance among groups was computed, and agglomeration was performed by Ward's minimum-variance linkage, considering the F pseudo statistic to define the group number (SAS 2002). The eigenvalues of PCA containing more than 80% of the variance were selected to force the formation of two groups through K-means non-hierarchical agglomerative technique, and the probability of population clustering according to their origin was evaluated by the Chi-square test.

Canonical correlations among the abiotic conditions of the population sites and the phenotypic traits were calculated using SAS (2002). Geographic coordinates and altitude of collection site were obtained from USDA (2007) and the Argentine passport data. Mean temperature of the hottest and coolest month and the average annual rainfall of the nearest locality, were obtained from www.worldclimate.com for USA populations and de Fina (1992) for Argentina. Canonical correlations among abiotic and phenotypic variables were performed with the more significant eigenvalues from PCA. Climate parameters, latitude, altitude, rainfall, and temperature of 32 stable populations (Cantamutto et al. 2008) and 46 collection sites representatives of 39 USA states were compared using the Kruskal-Wallis test.

Hybridization with cultivated sunflower *H. annuus* var. *macrocarpus* was estimated through frequency analysis of individuals showing the traits indicative of domestication: presence of main head, yellow disks, phyllary (bract) width over 0.8 cm, and disk diameter over 4.5 cm (Heiser 1978). Populations identified as “wild” in the GRIN database (USDA 2007), the remaining populations from the centre of origin, and Argentine populations were compared using the Kruskal-Wallis test.

Results and Discussion

The Argentine and North American wild population biodiversity was represented by phenotypic traits PCA (Figure 8-1). The Argentine populations were spread over the positive range of PC1, determined by plant height, leaf number and size, and head shape and diameter, though not completely separated from North American populations, which showed a greater variability (Figure 8-1a). Three populations from Diamante (Argentina), Arizona and California (USA) differed from the others because of their longer life cycle by PC3 (Figure 8-1b). The first three components explained 65% of variance, in agreement with that found in wild French populations (Serieys et al. 1997).

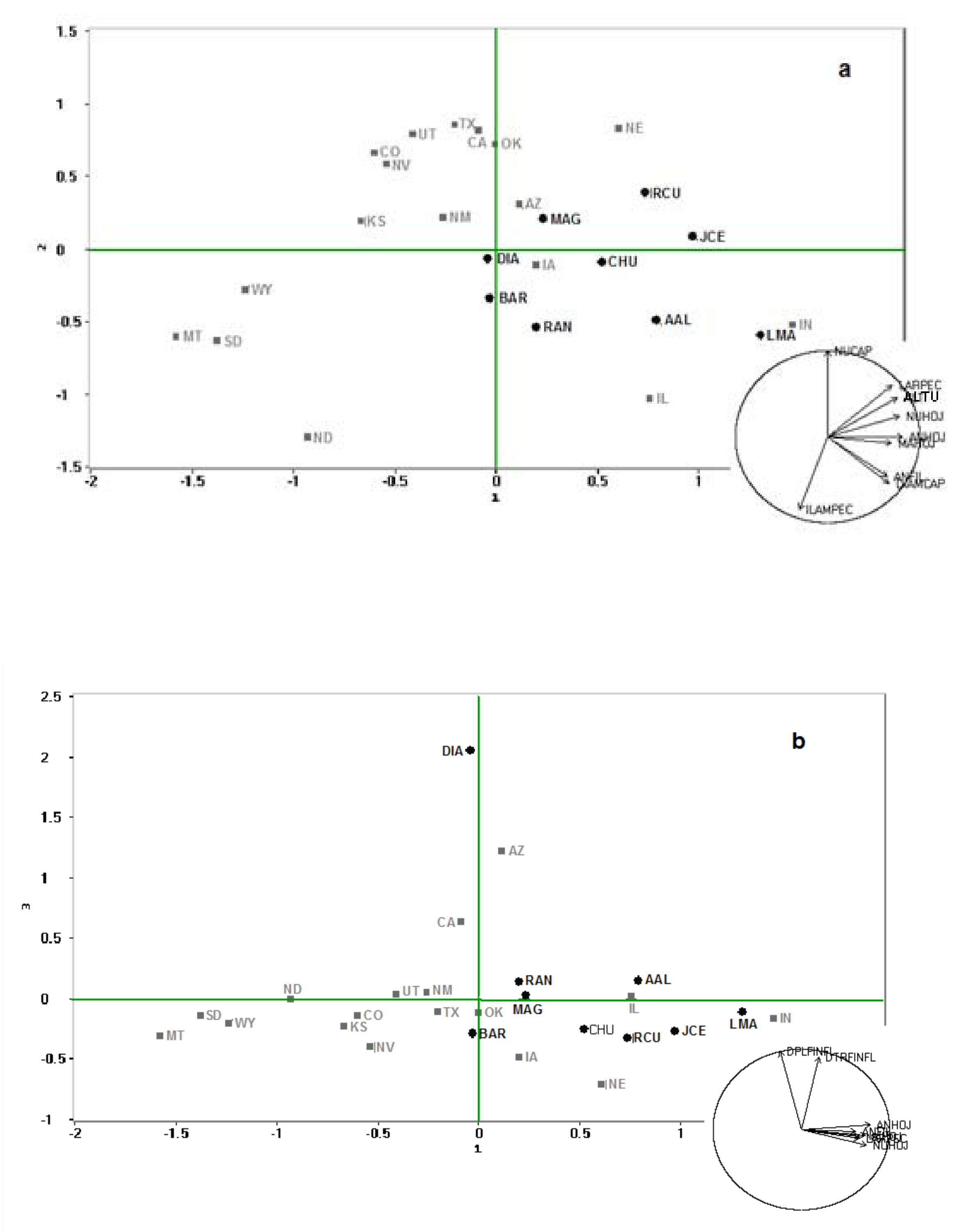

Figure 8-1 Differentiation among 17 North American (two letters) and nine Argentine (three letters, see text) populations of wild *Helianthus annuus* using principal component analysis for 33 normalized characters (for nomenclature see text). The circle below the PCA shows the traits that contributed to separation in each direction.

Ward's minimum-variance hierarchical clustering based on Mahalanobis distance, grouped the populations into three main clusters (Figure 8-2). Five of the nine Argentine populations were grouped with seven North American populations in a single cluster, showing a high similarity between them. The second cluster included four Argentine populations and three North American populations. The last cluster included just seven North American populations and was distantly related to the other two. Most Argentine populations showed phenotypic similarity with some North American populations, a fact that would assume a closer genetic relationship, mainly with the Illinois and Indiana populations. Considering this consistent similitude, wild populations from Illinois and Indiana could possibly be the progenitors of the wild Argentine populations.

The similitude between both country origins was also demonstrated when forcing the formation of two groups by the non-hierarchical K-means clustering method considering the first seven eigenvalues of the phenotype PCA, containing 85.5% of the variance. This method mixed 56% of Argentine populations and 77% of North American populations in the same group, a distribution which did not differ from random (χ^2 p = 0.15).

Canonical correlation analysis between the first two eingenvalues of the PCA retained >82% of the variability of the original environmental and the phenotype in a common garden showing a close relationship for North America populations (Wilks' Lambda = 0.0009 **), but was not the case for Argentine germplasm (Wilks' Lambda = 0.5559 ns). The latitude of the wild species distributed in the Northern Hemisphere ranged from 29.4º to 50.9º N (USDA 2007), while the Argentine distribution in the Southern Hemisphere was significantly narrower ranging from 31.6º to 37.2º S (Table 8-1), even though nearly all the potential habitats in Argentina had similar conditions to USA wild sunflower habitats. The hottest mean monthly temperature varied between 27 to 14º C, while the coolest month varied from 15 to 1º C. Rainfall exceeded 100 mm in all the Argentine territories with only part of the two eastern provinces having more than 1000 mm (www.cima.fcen.uba.ar/Egonzalez/sclima/index.htm). The lack of canonical correlation between the common garden phenotype and environmental variables at the collection site shows that the adaptive process in the Argentine environment is still ongoing.

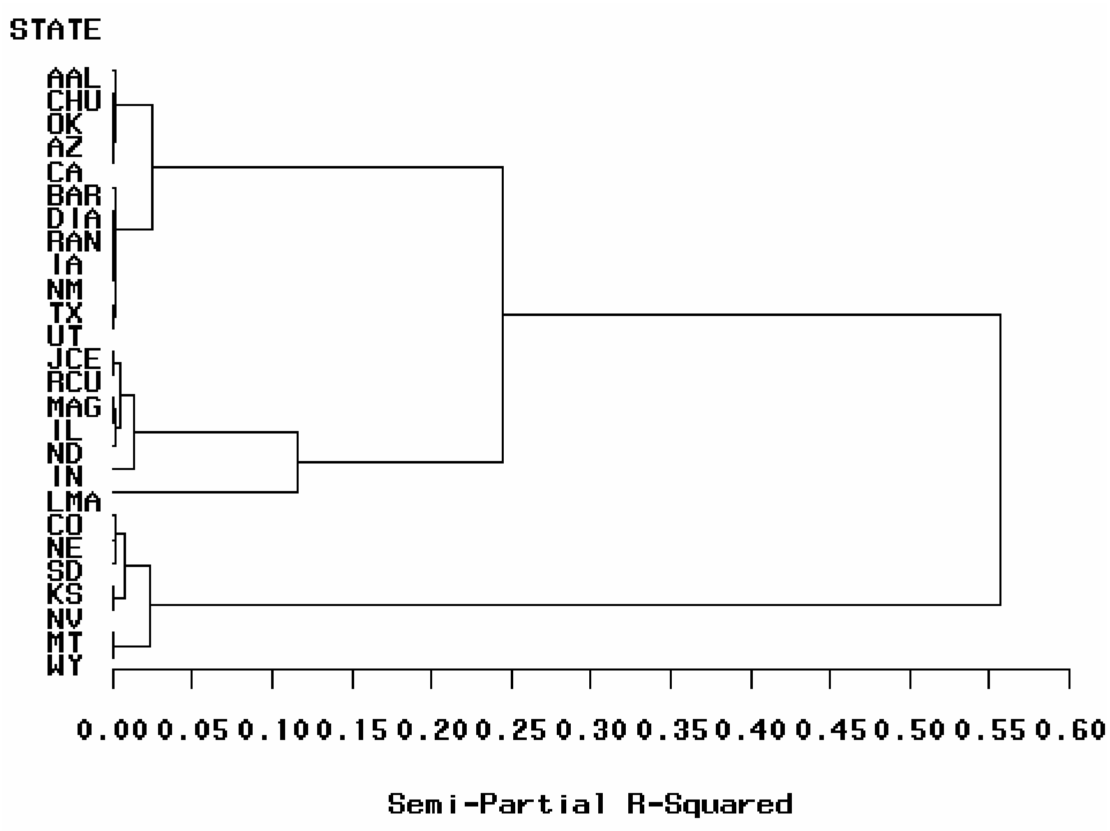

Figure 8-2 Clustering of Argentine and North American *Helianthus annuus* populations, using Ward's minimum-variance linkage hierarchical method based on Mahalanobis distance (nomenclature as in Figure 8-1).

Table 8-1 Geographic and climatic variables (mean ± S.D.) of the habitat from 32 wild stable argentine populations and 46 collection sites of wild sunflower in 39 USA states of North America.

Variable	North America	Argentina [a]
Latitude (°)	39.7 ± 5.4 N	34.1 ± 1.6 S **
Altitude (m.o.s.l.)	701 ± 478	266 ± 195 **
Annual rainfall (mm)	514 ± 329	614 ± 240 ns
Mean temperature of the hottest month (°C)	28.8 ± 3.3	24.3 ± 0.9 ns
Mean temperature of the coolest month (°C)	-1.8 ± 7.2	8.5 ± 1.3 **

[a] Kruskal-Wallis test.

The frequency of plants bearing a main head, yellow disks, or head diameter over 4.5 cm was not different in the North American populations classified as wild, Argentine populations, and the remaining populations from the center of origin (Table 8-2). However, the frequency of plants with phyllaries over 0.8 cm in width was significantly higher among Argentine populations compared to the two North American groups, which did not differ.

In two North American populations from Indiana and Illinois, the frequency of involucral bracts over 0.8 cm in width was higher than 70%, the same as in Argentine populations from Media Agua, Adolfo Alsina, and Las Malvinas. North American populations from Nebraska, Iowa, North Dakota, Kansas, and Arizona and the remaining Argentine populations showed 20 to 50% of plants with phyllaries over 0.8 cm in width.

Introgressive hybridization between crops and their related species has been the origin of some invasive plants (Ellstrand et al. 1999, Hancock 2005; Schmeller et al. 2005, Campbell et al. 2006, Hall et al. 2006). The Argentine agro-ecosystem is a favorable environment for gene flow (Poverene et al. 2004) since the naturalized wild annual sunflower populations are sympatric with sunflower crop over an extensive area (Ureta et al. 2008) and with *H. petiolaris* populations (Cantamutto et al. 2008).

Helianthus annuus populations naturalized in Argentina showed a number of different traits compared to cultivated forms. All populations were completely branched (type 4) and none of them included plants having a single head (type 0), nor plants with basal branching (type 1) or top branching (type 2) according to Hockett and Knowles (1970). However, there were plants with a predominant head (type 3) in populations found in Las Malvinas, A. Alsina, J. Celman, Rancul, and C. Baron, though this trait was associated with profuse branching, with more than 32 heads per plant. Individuals with the same type of branching were also observed in North American populations from Indiana, Illinois, Iowa and North Dakota, although the latter two were identified as wild populations from the passport data for the populations.

Table 8-2 Frequency (mean ± S.D.) of characters associated with a wild type of plant in North American and Argentine *H. annuus* populations evaluated in a common garden.

North American populations have been split in two groups according to the passport data from the original collections.

Characters [b]	North America		Argentina	P [c]
	Wild [a] (n = 7)	Other (n = 10)	(n = 9)	
CAPRIN	0.02 ± 0.04	0.07 ± 0.17	0.13 ± 0.13	ns
YECODIS	0.02 ± 0.04	0.05 ± 0.09	0.05 ± 0.08	ns
ANFIL>0.8 cm	0.19 ± 0.17 a [d]	0.23 ± 0.32 a	0.48 ± 0.23 b	*
DIAMCAP>4.5 cm	0.12 ± 0.19	0.22 ± 0.40	0.23 ± 0.28	ns

[a] Classified as wild type in USDA (2007)

[b] CAPRIN = presence of main head; YECODIS = yellow disk flower; ANFIL = phyllary width; and DIAMCAP = head diameter.

[c] Kruskal Wallis test.

[d] Means followed by the same letter do not differ for $p < 0.05$.

Phyllary (bract) width provides the strongest evidence of introgression with cultivated sunflower in wild populations established in Argentina. Mean phyllary width in Las Malvinas, A. Alsina, and Media Agua populations exceeded 0.8 cm, whereas in the remaining populations some individuals also had bracts exceeding 0.8 cm. Among the North American populations, mean phyllary width was over 0.8 cm in populations from Indiana and Illinois, while Nebraska, Iowa, North Dakota and Kansas populations were classified as wilds according to the passport data in the GRIN (www.ars-grin.gov2/cgi-bin/npgs/html). Nevertheless, those populations also included individuals suspected to have hybridized with cultivated sunflower, as all the Argentine populations cultivated in the common garden. In that case, populations from Argentina would not have introgressed characters from cultivated sunflower to the extent and duration of those from the centre of origin in the USA. It seems that the extreme variability in this species discourages the use of different Latin names for botanical forms (Seiler and Rieseberg 1997) but also makes difficult the assignment of wild populations to a well-defined taxonomical group.

Helianthus annuus populations established in Argentina showed high enough phenotypic variability to differentiate among them. At present, it is accepted that invasive plant populations in Argentina are not different from native populations. The Argentine populations' introgressed with cultivated sunflower traits could not be clearly differentiated from the USA ones. Some traits of Argentine populations were absent in the North American populations, such as life cycle length in the Diamante population with over 180 days, the longest of any population studied. The Diamante location can be considered an extreme habitat for wild sunflowers in Argentina, given the local climatic and soil conditions (Cantamutto et al. 2008). It seems that founder effects did not limit wild *H. annuus* biodiversity in the newly colonized environment of Argentina, because 60 years after their introduction nearly two-thirds of the USA wild germplasm phenotypic variability is still present.

Acknowledgements

Authors thank to the Fundación Carolina, Spain for a fellowship to MC, and to National Research Council of Argentina (CONICET) for a fellowship to AP. Also are grateful to Drs. Ignacio Romagosa and Lluis Torres from Centro UdL-IRTA, Lleida, Spain for assistance with statistical data analyses. This research was supported by grants ANPCYT-PICT 08-9881 and UNS-PGI 24A106.

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Chapter 9 Seed morphology and oil composition of wild *Helianthus annuus* from Argentina

2008

17th. International Sunflower Conference, Córdoba, Spain 8-12 June 2008.

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Abstract

Wild *Helianthus annuus* naturalized in Argentina constitutes a potential genetic resource for use in sunflower crop breeding. Seed morphology and oil content of nine stable Argentine wild populations were characterized and compared to 17 wild accessions from the USA. The achenes were harvested from an experimental field at Bahia Blanca (S 38º 41', W 62º 14') during February, in three successive summers. Seed dimensions of Argentine accessions were within the range of USA accessions, but showed less variability. The lower mottling and higher frequency of stripes in Argentinean populations would be an indication of crop introgression. The oil content, fatty acid composition and iodine value did not differentiate the wild species origins. None of Argentine populations showed a fatty acid composition similar or better than the improved mutant lines reported by other authors. All measured seeds traits showed significant differences, pointing to the existence of high variability in this new wild germplasm from Argentina.

Key words: Achene – Fatty acid – Ferality – Genetic resources – Oil quality – Sunflower

Introduction

Sunflower oil quality which contributes about 80% of the total value of the crop has received considerable breeding efforts in the last 30 years (Fick and Miller 1997). The main use of sunflower oil is as a salad and cooking oil, being a major ingredient in some vegetal butter and shortening products, but it also could be used for industrial purposes in paints, varnishes, plastics, soap, and detergent (Seiler 2007). Sunflower oil has a high potential as a source for biodiesel production to satisfy the demand for a renewable energy (Vannozzi 2006).

Oil physical and chemical properties determine its end-use, with the fatty acid composition and iodine value indicative of the oil characteristics. Traditionally sunflower has been considered as polyunsaturated oil because of its high content of linoleic acid, but breeding selection sometimes helped by chemical mutations, has produced several lines with altered fatty acid composition (Fernandez-Martinez et al. 2006). Low saturated fatty acid content is chosen for edible oil, high oleic mono-unsaturated acid is selected for high temperature processes (as frying or bio-lubricants), whereas high saturated acids are preferentially used for margarine production, because it reduces the need for hydrogenation (Jan and Seiler 2007).

The wild *Helianthus annuus* naturalized in Argentina grows as extended populations in a wide area across the boundary between humid and sub-humid regions (Poverene et al. 2002). Wild and weedy relatives of crops are genetically much more diverse than cultivated lineages and constitute a genetic resource that has not been fully exploited (Maxted et al. 2006). Wild *Helianthus* species provide a resource for improving oil quality in cultivated sunflower (Thompson et al. 1981) and a potential source of altered fatty acid composition (Seiler 2004, 2007). The potential of wild sunflower naturalized in Argentina as genetic resource for oil improvement is unknown.

The objective of this work was to characterize wild *Helianthus annuus* from Argentina as a potential source for sunflower crop oil composition improvement.

Materials and methods

The wild germplasm was represented by nine stable populations from the diverse agro-ecological conditions where it grows in Argentina (Cantamutto et al. 2008). The accessions were from Rio Cuarto (RCU) S 33º 09', W 64º 20', Juarez Celman (JCE) S 33º 40', W 63º 28', Colonia Barón (BAR) S 36º 10', W 63º 53', Rancul (RAN) S 35º 04', W 64º 46', Adolfo Alsina (AAL) S 37º 16', W 62º 59`, Carhué (CHU) S 37º 16', W 62º 55', Diamante (DIA) S 32º 03', W 60º 38', Media Agua (MAG) S 31º 57', W 68º 27', and Las Malvinas (LMA) S 34º 47', W 68º 15'. The accessions were collected by M. Poverene and M. Cantamutto in 2002-2003 during exploration trips, and regenerated in the experimental field in Bahía Blanca (S 38º 41', W 62º 14'), during the summer of 2004 and stored in the Sunflower Germplasm Active Bank at INTA Manfredi Experimental Station (Córdoba, Argentina) as code numbers 832 to 840.

Wild germplasm from North America (USA) represented by 17 populations provided by the USDA-ARS GRIN germplasm system was studied for comparison. States of origin and passport numbers were: Arizona PI 468571, California PI 468580, Colorado PI 468621, Illinois PI 435540, Indiana PI 468633, Iowa PI 597901, Kansas PI 586851, Montana PI 586821, Nebraska PI 586867, Nevada PI 468596, New Mexico PI 468537, North Dakota PI 586807, Oklahoma PI 468483, South Dakota PI 586835, Texas PI 468504, Utah PI 468607, and Wyoming PI 586824 (for more information see www.ars-grin.gov/cgi-in/npgs/acc/display.pl?1080516).

Seedlings were grown in a greenhouse for one month and then transplanted by accessions in the experimental field at 1.9 plants/m^2 density during three successive summers (2003-2006). Drip-irrigation was applied to satisfy plant water demands. To regenerate the populations, heads of 20-30 individuals of each accession were bagged prior to open and hand-pollinated during flowering. Bulk seed of mature heads were collected before achene shattering during the last week of February for sibbed and open pollination heads to minimized flowering date effects (Seiler 1983).

A sample of 30 completely developed achenes from both pollination systems was used for seed description. Seed length, width, and thickness were measured using 10X magnification. The individual seed fresh weight was estimated by the total mass of the achenes. Qualitative traits, shape, pubescence, stripe presence, pericarp colour and mottling were individually determined and computed as frequencies. Argentine qualitative traits were determined using the original seed. Oil composition, fatty acid content and iodine value were evaluated at the EEA INTA Manfredi laboratory by AOCS (2007) approved methods (Ai 3-75, Ce 1-62 and Tg 1a-64) on a 10 g sample of seeds harvested from the experimental field under two pollination systems. Methyl esters of fatty acids were analyzed by Gas Chromatograph Hewlett Packard 6890 with a fire lionization detector and a capillary column HP-INNOWax (Crosslinked Polyethylene Glycol), of 0.32 mm x 30 m x 0.5 mm thick film. Each population was grown for at least two years.

To compare all the accessions, the ANOVA considered country, populations nested in countries, and year as variation sources. For seed qualitative traits of Argentine wild accessions, population and year were considered as sources of variability for the ANOVA. The oil content and fatty acid composition of Argentine accessions were analyzed for open-pollinated and sib-pollinated seed and the pollination system was considered as a source of variability for the ANOVA. LSMEANS were calculated for each parameter and pair-compared using a linear combination of the model using the GLM procedure of SAS (2002). The linear regression between metric parameters was calculated and compared using an ANOVA (Quinn and Keough 2005). Box-plot graphics were obtained with the InfoStat package (InfoStat 2002).

Results and discussion

Argentine seed dimensions possessed about a half of the variability observed in the sample of USA wild sunflowers, with no differences in the relationships between width, length, thick and weight, and were within the extreme values observed in the USA populations (Figure 9-1). Achene weight, length and width of accessions from both hemispheres corresponded to the expected values for wild and weedy populations (Heiser 1978, Seiler 1997).

The frequency of sparse pubescence and grey pericarp was not able to discriminate the between the groups, but stripes and mottling frequency differentiated both wild species origins (Figure 9-2). The ranges of all qualitative traits overlapped for the Argentine and USA wild origins (Figure 9-2). A possible crop introgression in Argentine populations was suggested by their lower mottling (Figure 9-2.b) and higher stripes frequency (Figure 9-2.d) compared to the USA accessions.

Though not included for botanical classification by Heiser (1978), mottling could be considered a wild trait. Stripes are typical of confectionary sunflower (Jan and Seiler 2007) and characterized the first Argentine varieties (Bertero and Vazquez 2003). If introgression happened during the colonization process, a strong selection pressure for small seed size would be expected (Alexander et al. 2001) but not for pericarp traits, that seem to be neutral. This could explain the absence of complete separation using seed dimensions, being larger in Argentine wild accessions but within the range of acceptable sizes for wild sunflower (Heiser 1978). Hybridization with cultivated sunflower, also suggested by a phenotypic study of a number of plant traits (unpublished data), likely took place during the invasive process as a result of the intense gene flow documented in Argentina landscape (Ureta et al. 2008). The introgression process was probably followed by a strong selection for small seed.

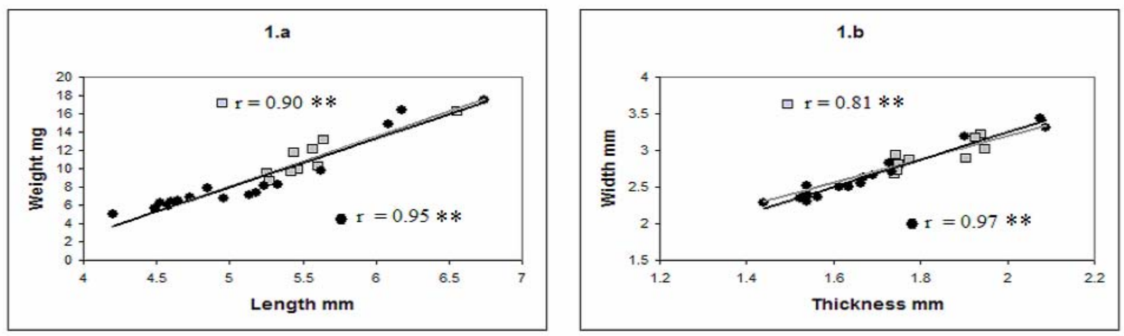
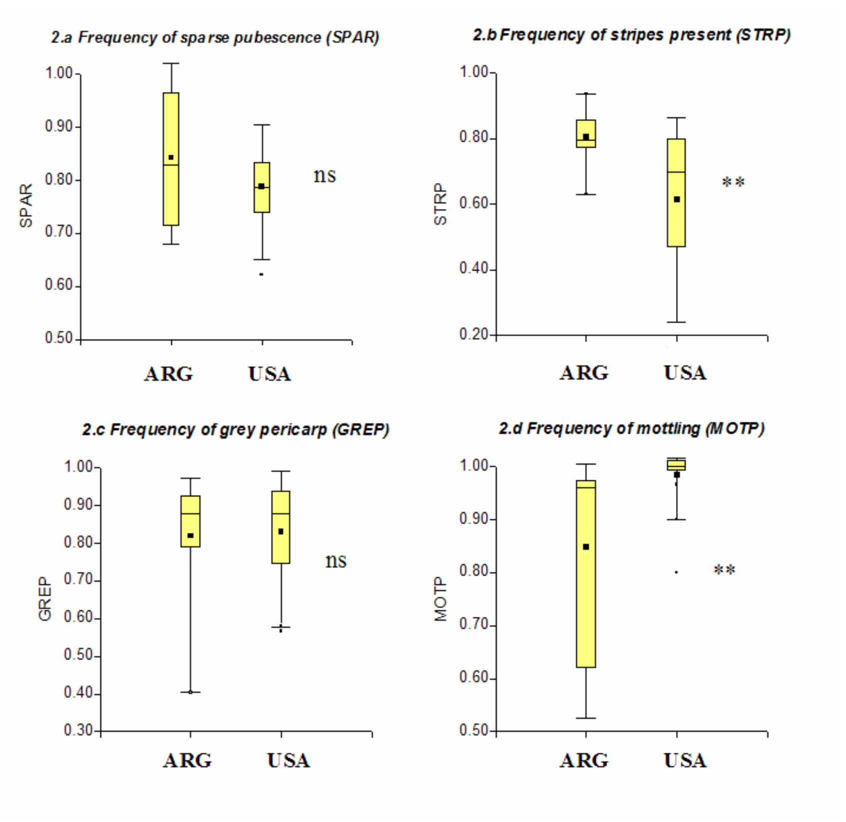


Figure 9-1 Morphological relationships in wild *Helianthus annuus* seeds grown for three years in an experimental field.

Argentine (grey squares) and North American (black circles) populations showed no differences in linear correlation between parameters.



2.a Frequency of sparse pubescence (SPAR)

2.b Frequency of stripes present (STRP)

2.c Frequency of grey pericarp (GREP)

2.d Frequency of mottling (MOTP)

Figure 9-2 Seed morphological descriptors of 26 wild sunflower populations from Argentina and the United States of America (USA) grown for three years in an experimental field.

Box-plots show the LSMEANS distribution, ANOVA differences between both sources are indicated in each case. Year effect was not significant for all traits

Within the Argentine accessions, an ANOVA showed that populations differed for all the analyzed morphological traits (Table 9-1). Year effect was evident only in seed weight and length, probably due to differences in climatic conditions during grain filling. The significant effect of year on pericarp colour could be due to differences in achene size making it difficult to clearly visualize this trait in small seeds.

The Argentine accession, CHU had the smallest seed dimensions significantly different from LMA and MAG, which had the largest achenes (Table 9-1). The CHU accession also had a higher ovoid shape and grey pericarp frequencies. RCU, RAN, and JCE showed mottling in all seeds, significantly different from LMA, MAG and AAL with low mottled seed frequency. Considering all the traits together, RCU, BAR and CHU seemed to be a pure wild strains as opposed to LMA, AAL and MAG which showed introgressed crop-related traits (big seeds, presence of stripes, low mottling). These findings agree with the hypothesis that Rio Cuarto was as an entry point of wild *Helianthus annuus* before 1950s (Bauer 1991) from where the invasive process progressed (Chapter 3).

The oil content, fatty acid composition and iodine value did not show differences between the wild species origins (Figure 9-3) but showed a year effect in fatty acid composition and iodine value. A higher palmitic acid concentration was found (Figure 9-3.b) and a lower oleic acid concentration (Figure 9-3.d) in Argentine accessions, with the other chemical parameters within the ranges observed for the USA wild populations.

Argentine populations showed differences between the accessions for all the chemical parameters (Table 9-2). Oil content between 21.4 to 28.2 % was typical of wild seeds and was only affected by population variability. The year had a significant effect on palmitic acid and highly significant effects on oleic, linoleic, linolenic concentration, oleic:linoleic ratio and iodine value. Even though the grain filling of all analyzed achenes correspond to the same month, a variation between 35.4 to 40.5ºC of maximum temperature registered during this period could explain the year effect since they are influenced by temperature (Harris et al. 1978). Slight variations in nitrogen availability (Steer and Seiler 1990), water regime (Flagella et al. 2002) and night minimum temperature (Izquierdo et al. 2006) can have an effect on oleic and linoleic sunflower concentrations and maybe responsible for the observed year effect.

Table 9-1 Morphological seed traits of nine wild *Helianthus annuus* from Argentina.

Wild	Seed dimensions[2]				Seed traits frequency[3]				
population[1]	Weight mg	Length mm	Width mm	Thickness mm	Ovoid shape	Sparse pubescence	Stripes	Grey pericarp	Mottling
AAL	11.7 bc[4]	5.6 bc	2.9 bd	1.9 a	0.92 a	0.67 c	0.93 a	0.94 ab	0.64 b
BAR	9.3 d	5.5 bc	2.9 bd	1.7 b	0.98 a	0.74 bc	0.77 b	0.99 a	0.98 a
CHU	8.8 d	5.2 c	2.6 e	1.7 b	0.96 a	0.66 c	0.89 a	0.97 a	0.98 a
DIA	10.2 c	5.5 bc	2.8 ce	1.8 b	0.92 a	1.00 a	0.85 a	0.81 bc	0.97 a
JCE	9.4 d	5.4 bc	2.8 ce	1.8 b	0.90 a	0.69 bc	0.62 c	0.95 ab	1.00 a
LMA	17.4 a	6.7 a	3.3 a	2.0 a	0.90 a	0.96 a	0.79 ab	0.41 c	0.53 b
MAG	13.2 d	5.7 b	3.0 ac	2.0 a	0.87 a	1.00 a	0.85 a	0.75 c	0.64 b
RAN	11.4 bc	5.4 c	3.1 ab	1.9 a	0.63 b	0.92 a	0.71 b	0.83 ac	1.00 a
RCU	9.0 d	5.3 c	2.7 de	1.8 b	0.95 a	0.84 b	0.80 a	0.90 ac	1.00 a
					ANOVA				
Population	**	**	**	*	*	**	**	**	**
Year	**	**	ns	ns	ns	ns	ns	*	ns

[1] See text for population codes. [2] Achenes harvested during three years in the experimental field. [3] Original seed accessions and achenes harvest in the experimental field. [4] LSMEANS with different letters showed differences at $p < 0.05$

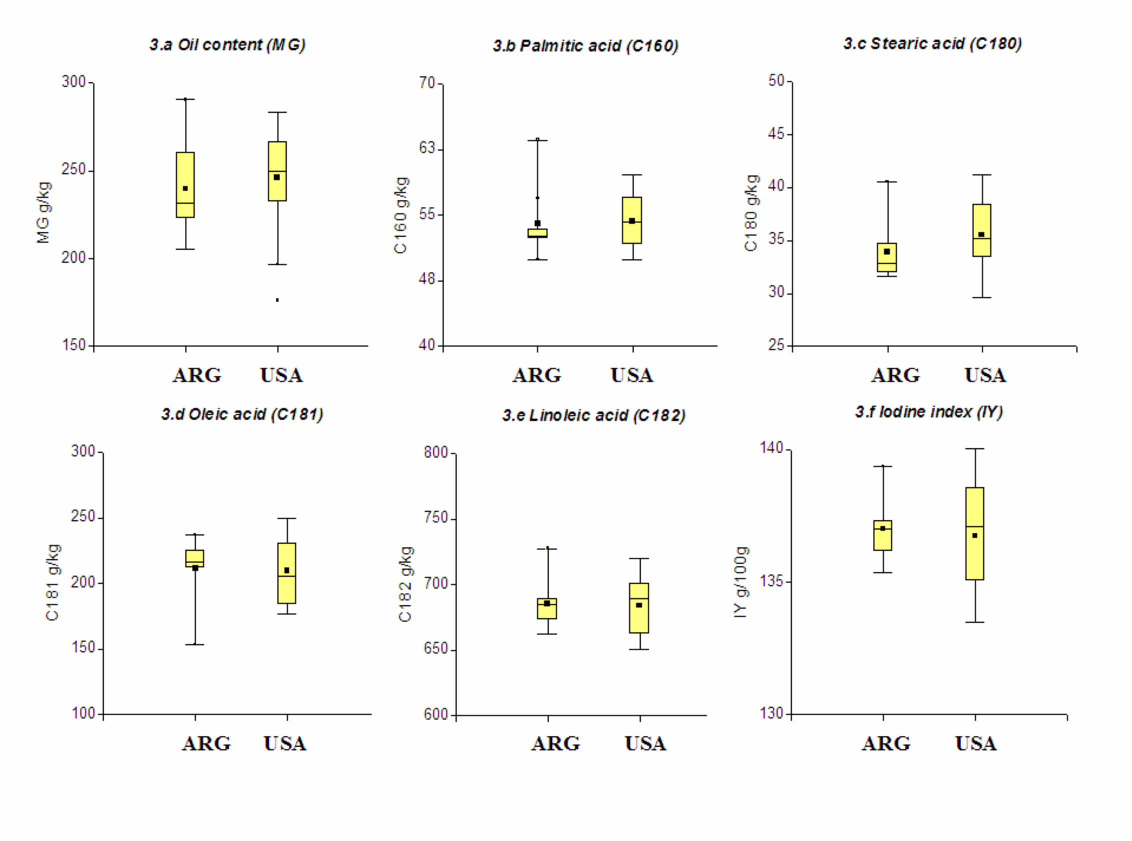


Figure 9-3 Oil composition of wild *Helianthus annuus* open pollinated populations from Argentina (ARG) and North America (USA) grown in Bahia Blanca, Argentina over three years.

No differences were observed between both groups. Box-plots show the LSMEANS distribution of 26 wild populations.

Table 9-2 Oil content and chemical composition of nine wild *Helianthus annuus* populations from Argentina.

Achenes correspond to grain-filling in February with sibbed and open pollination systems. Data are LSMEANS of three years.

Wild `population[1]	Oil content	Fatty acid composition						Iodine value
		palmitic 16:0	stearic 18:0	oleic 18:1	linoleic 18:2	linolenic 18.3	oleic: linoleic	
	g/kg DM			g/kg				g/100g
AAL	282 a[2]	52 cd	32 cd	218 a	684 c	0.71 d	0.32 a	137 cd
BAR	238 bd	52 cd	33 cd	205 ab	696 bc	0.77 cd	0.29 ab	138 bd
CHU	261 ac	54 bc	31 d	199 ab	701 bc	0.86 ac	0.28 ab	139 ac
DIA	217 d	65 a	42 a	135 c	743 a	1.01 a	0.18 c	141 a
JCE	236 bd	52 cd	32 cd	201 ab	700 bc	0.83 bd	0.29 ab	139 ac
LMA	226 cd	51 cd	31 cd	211 a	690 bc	0.76 cd	0.31 a	138 bd
MAG	228 cd	57 b	34 c	181 b	713 b	0.89 ac	0.25 b	139 ac
RAN	214 d	54 bc	37 b	211 a	681 c	0.87 ac	0.31 a	136 d
RCU	270 ab	50 d	31 d	194 ab	711 b	0.92 ab	0.27 ab	140 ab
Pollination								
Sibbed	242	54	33	180	718	0.84	0.25	140
Open	241	54	34	210	686	0.86	0.31	137
			ANOVA					
Population	*	**	**	**	**	*	**	*
Year	ns	*	ns	**	**	**	**	**
Pollination	ns	ns	ns	**	**	ns	**	**
Population x pollination	ns	ns	ns	ns	ns	ns	ns	ns

[1] See text for population code. [2] LSMEANS with different letters showed differences at $p < 0.05$

There was a high significant effect of the pollination system on oleic and linoleic concentration, their relationship and the iodine value (Table 9-2) as expected considering both parent influence. Given the general inverse relationship, sibbed seeds produced lower oleic acid and higher linoleic acid concentration than open pollinated seeds. The 15% gain for oleic acid from open pollination was insufficient to reach the maximum value observed in AAL. The cause of the increased in average oleic content from open pollination could be addressed in future studies.

In general, the fatty acid composition did not show values of interest with respect to those reported for improved mutant lines with altered fatty acid composition (Fernandez-Martinez et al. 2006). None of the Argentine accessions showed less than 39 and 26 g/kg of palmitic and stearic acid content, nor more than 300 g/kg of palmitic acid to be considered low or high in saturated acid content. None of the Argentine accessions showed oleic acid over 860 g/kg or linoleic concentration over 780 g/kg, similar to values of improved mutant lines.

The AAL accession had the highest oleic concentration, but was only different from MAG, RAN and DIA. Among Argentine germplasm, DIA showed the most variability in fatty acid composition, with higher palmitic, stearic, linoleic, linolenic, and iodine values and the lower oleic acid content. This population from Diamante represented a life cycle that is significantly longer than the other North America and Argentine accessions (Chapter 8) and could constitute a unique germplasm of potential value.

Acknowledgements

Drs. Juan Antonio Martín Sanchez and Lluis Torres (IRTA-Spain) made valuable comments. MAC is a fellow of Fundación Carolina (Spain). This research was supported by grants INTA-PNCER 1339, ANPCYT-PICT 08-9881 and UNS-PGI 24A106.

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Chapter 10 Genetically Modified Sunflower Release: Opportunities and Risks

2007

Field Crops Research 101:133-134

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Abstract

Sunflower (*Helianthus annuus* L.) is a crop native to North America for which there are no genetically modified commercial varieties. Some of the transgenic traits incorporated in other crops have already been subjected to research and experimentation in sunflower. Several new traits have also been noted, with the most relevant of these being the increased latex production. GM sunflower release would modify crop management through improved mineral nutrition, weed control, insect and disease resistance, and product quality. In this research, the traits investigated were reviewed and analyzed in connection with main crop constraints. These characters could potentially influence agro-ecosystem components and produce a significant environmental impact. In regions where sunflower coexists with wild relatives this situation could affect germplasm resources, with this being especially important at the centre of origin and where *Helianthus* populations established in Africa, Asia, and Europe.

Key words: sunflower - biotechnology - GM crops - environmental impact - herbicide tolerance - pest control.

Introduction

Sunflower is the fifth most important source of edible oil after soybean, rapeseed, cotton, and peanut. The total world production of 25.8 million tons of sunflower seed goes almost exclusively to oil extraction, providing 8.2% of total world volume, estimated at around 107 million tons. The sunflower crop is important in several Eastern European countries and also in Argentina, which provides more than 10% of world production. Sunflower is considered good quality oil, but does not command the high prices of other edible oils, for which there is greater demand in the most select markets. The mean price of sunflower on the Rotterdam market over the last decade was US$663 per ton, exceeding those of soybean, palm and coconut oils (FAS 2005).

Biotechnology can speed up plant breeding, with many of the techniques complementing rather than substituting conventional methods (FAO 2005a) and some biotechnological products have had a strong impact upon production systems because they have also facilitated crop management. This has led to a major increase in the total area devoted to genetically modified (GM) maize, soybean, cotton and rapeseed production, which now exceeds 90 million ha, most of which are distributed amongst the 14 countries in which these crops have been authorized (James, 2005).

GM soybean constitutes a particularly significant case. Since its release as a commercial crop in Argentina 10 years ago, there have been increases in acreage, yield, and total production of 12.5%, 10.6%, and 25% respectively (SAGPyA 2002). This trend is still continuing and may, at least in part, be associated with RR soybean tolerance to glyphosate herbicide (Monsanto 2002). This simplifies its cultivation under no-till systems immediately after wheat harvest, and greatly facilitates weed control. Moreover, it helps to reduce production costs, making the crop profitable in otherwise marginal areas of Paraguay, Brazil, and Bolivia. These facts help to explain the major increase in production observed in South America during the last 10 years (FAS 2005).

Sunflower and peanut are the only major vegetable oil yielding crops that have no GM varieties authorized for commercial use. This does not imply that versions of these products are not available through research, but just the opposite. In the case of sunflower, an open-pollinated crop native to North America (Heiser et al. 1969, Harter et al. 2004) which has wild relatives throughout the world's crop regions, Europe (Faure et al. 2002), Australia (Dry and Burdon 1986), Africa (Quagliaro et al. 2001, Ribeiro et al. 2001), and Argentina (Poverene et al. 2002). Diffusion to these areas can be addressed to human activity. Crop-wild gene exchange allows transgene escape via gene flow in sunflowers (Whitton et al. 1997, Linder et al. 1998, Rieseberg et al. 1999, Burke et al. 2002). Transgenes from the crop could potentially disperse into wild or weedy populations enhancing their fitness and modifying their ecological interactions (Burke and Rieseberg 2003, Snow et al. 2003). Conversely, wild or weedy sunflowers and volunteers can invade and interfere crop and may modify traits, such as oil composition, via pollen flow (Faure et al. 2002, Bervillé et al. 2004). Furthermore, the impact of GMO release on edible oil marketing could be negative, because of the well known consumer resistance to GM products. So far, these two circumstances have delayed the development of GM sunflower for commercial uses. The goal of this work was therefore to analyze the likely impact of the use of transgenic sunflower on agronomic crop management and to consider the possible consequences of authorization being granted for the commercialization of such products.

Registered GM crops

The level of adoption of GM crops in the USA is the highest in the world, with 49.8 million ha (James 2005). Their diffusion was preceded by intense research and development activity. At present, more than a hundred different GM products have been authorized for commercialization: 13 of these are crops, including maize, soybean, rapeseed, flax, and rice (Table 10-1). The products authorized for farming and industrial uses mainly facilitate weed, pests and/or virus control, and seek to improve quality and facilitate hybrid seed production. These commercial products represent the successful end products from just a few of more than 20,000 authorized trials undertaken with several dozen species.

The GM products available to farmers in Argentina have traits which facilitate crop management, such as herbicide tolerance and insect resistance. Only the soybean, maize and cotton harbouring modifications of these traits have been authorized for commercial use (Table 10-1). From the beginning of the biotechnological registrations in 1991, more than 700 cases have been authorized for research, but with only about 10% of these cases involving sunflower. Each case is an event, a crop line genetically engineered to express a particular trait. In Argentina, the studied events include a smaller number of traits than in the USA, but in addition to the events available in the USA, there have also been modifications aimed at increasing the capacity for nitrogen fixation. Even so, insect resistance and herbicide tolerance accounted for more than 80% of the release permits submitted in 2004.

Australia, a country also actively involved in biotechnology, adds other traits to its GM products (Table 10-1). It has authorized the commercialization of transgenic maize, soybean, potato and sugar beet, and an improved quality GM product, the high oleic soybean, is also now available. Authorized events for controlled research include metabolic transformation in photosynthesis, resistance to salinity, synthesis of new products (alkaloids) and modified quality in grapes, wheat, sugar cane, cotton, and flowers (carnation).

In spite of resistance to the use and consumption of GM products in Europe, a number of crops have already been authorized, and/or are currently under evaluation (Table 10-1). At present, commercial authorizations have been granted for the production and/or consumption of GM maize, rapeseed, endive, soybean, and flowers. The genetic modifications confer upon them similar traits to those previously detailed for the USA, Australia, and Argentina. Among products pending approval there is a variety of potato with modified starch content for industrial use. Amongst European states, Spain stands out as a major producer of transgenic crops, with over 100,000 ha of transgenic maize (James 2005).

Although transgenic sunflower varieties have already been obtained, they remain the subject of ongoing research in both the USA and Argentina. Figure 10-1 shows that the interest in GM sunflower research has decreased in the 21st century, probably because official control bureaus have imposed restrictions in the face of ecological concerns. Although it is impossible to accurately assess the present extent of private research, public registrations on this crop include the traits detailed in Table 10-2. The main impacts upon crop management can be analyzed as follows.

Table 10-1 Traits on released GM plants for cultivation and/or consumption in four selected areas of the word with intense use of GMO.

Intended effect (Involved agent)	USA[1]	Argentina[2]	Australia[3]	EU[4]
	Cultivation and consumption (food and feed)			Consumption
Tolerance to glyphosate (EPSPS, EPSPS + GOX)	alfalfa (only feed), corn, cotton, creeping bentgrass (only feed), rapeseed, soybean, sugar beet, wheat	corn, cotton, soybean	corn, rapeseed, soybean, sugar beet	Corn[5], cotton, rapeseed, soybean
Tolerance to glufosinate ammonium (PAT)	corn, cotton, radicchio, rapeseed, rice, sugar beet	corn	corn, rapeseed, soybean	corn, rapeseed
Tolerance to bromoxynil (Nitrilase)	cotton, rapeseed		cotton, rapeseed	
Tolerance to sulfonylurea	cotton, flax			
Resistance to Lepidoptera (Cry1F, Cry1Ac, VIP3A, Cry2ab, Cry1ac, Cry9C, Cry1Ab)	corn, cotton, tomato	corn, cotton	corn , cotton	corn , cotton
Resistance to Coleoptera (Cry3Bb1, Cry34Ab1, Cry35Ab1, CryIIIA)	corn, potato		corn, potato	corn
Coat virus protein, virus replicase	papaya, potato, squash		potato	
Male sterility (Barnase); fertility restorer (Barstar)	radicchio, rapeseed		rapeseed	rapeseed
Male sterility (DAM)	corn			
Phytase degradation	rapeseed (only feed)			
Increase lysine level (cDHDPS)	corn			
High oleic oil content (GmFad2-1)	soybean		soybean	
High laurate oil content	rapeseed			
Delayed ripening, two traits	tomato			
Delayed ripening (*E.coli*)	cantaloupe, tomato			

1http://www.cfsan.fda.gov/~lrd/biocon.html (Access June 2, 2006).

2http://www.sagpya.gov.ar/biotecnologia/conabia/eventos comerciales (Access June 3, 2006);

3http://www.ogtr.gov.au/rtf/gmorec/gmfoodprod2.rtf (Access June 3, 2006).

4http://ec.europa.eu/food/food/biotechnology/ authorisation/index_en.htm (Access June 3, 2006); http://ec.europa.eu/food/dyna/gm_register/index_en.cfm (Access June 3, 2006). 5By far, Bt Corn is the most widely GMO planted in EU at Spain, France, Portugal and Germany (James, 2005)

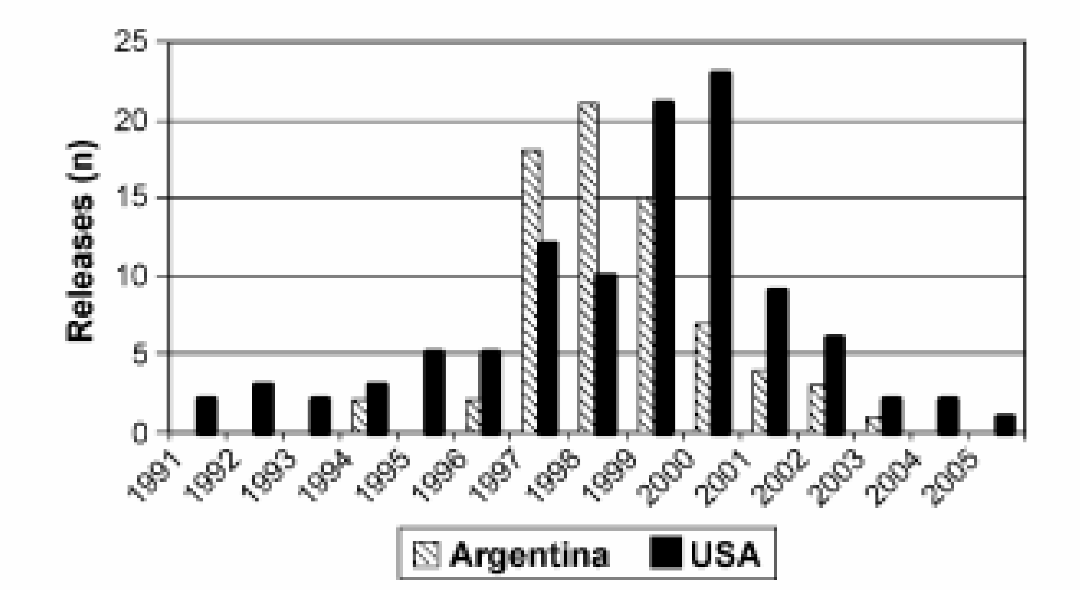

Figure 10-1 GM sunflower requests for testing in the United States of America (USA) and Argentina since 1991.

Table 10-2 Deliberate traits under field experimentation in sunflower[1]

Intended effect	Responsible (ARG= Argentina, USA= United States, FR= France, NL= The Netherlands, SP= Spain)	Brief description
Tolerance to glyphosate	MONSANTO (ARG, USA) INTA (ARG)	5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) synthesis by expression of *Agrobacterium tumefaciens* genes
Tolerance to glufosinate ammonium	ZENECA (ARG) VAN DER HAVE (NL)	Phosphinothricin acetyltransferase (PAT) synthesis by expression of *Streptomyces hygroscopicus* or *S. viridochromogenes* genes
Increased nitrogen assimilation	ZENECA (ARG) VAN DER HAVE (USA, NL)	Ammonium incorporation by Asparagine synthetase (AS) or increased N assimilation by nitrate reductase or nitrite reductase synthesis
Resistance to Lepidoptera	MYCOYEN (ARG) PIONEER (ARG) VAN DER HAVE (AR, NL) INTA (ARG) DOW (ARG)	Bt-derived insect resistance mediated by synthesis of endotoxins (Cry 1F) from *Bacillus thuringensis*
Resistance to Coleoptera	VAN DER HAVE (USA, NL) ZENECA (AGG)	Cowpea (*Vigna unguiculata*) trypsin inhibitor synthesis (CpT1) plus Snowdrop lectin (Nptll(SM))
Fungal resistance	PIONEER (FR, AR, USA) SINGENTA (USA) INTA (AR) ZENECA (ARG) ADVANTA (AR) VAN DER HAVEN (NL)	Oxalate oxidase (OXO) synthesis by expression of wheat or barley genes conferring resistance to *Sclerotinia sclerotiorum*
Rubber yield increased	COLORADO STATE UNIVERSITY (USA)	Enhanced quantity and quality of rubber production by expression of the synthesis complex of *Parthenium argentatum* (Guayule)
Enhanced protein quality	PIONEER (USA) VAN DER HAVE (USA)	Storage protein from *Bertholletia excelsa* (Brazil nut) with high methyonine content.
Modified stearate content	RUSTICA PROGRAIN GÉNETIQUE (FR)	High stearate content. Reduction of stearic acid content
Others	VAN DER HAVE (NL, FR, SP, ARG)	Albumin, asparagine, chalcone, chitinase, fructosyltransferasa, glucanase or levan sucrase synthesis. Chlorsulphuron tolerance, fungal resistance, male sterility/fertility restoration, drought tolerance, marker system, MAC promoter.
Broomrape control	PIONEER (SP)	No available information

[1] Sources: http://biotech.jrc.it/doc/snifs.rtf , http://biotech.jrc.it/deliberate/dbplants.asp; http://www.aphis.usda.gov/brs/status/notday.html; http://www.sagpya.gov.ar/biotecnologia/conabia (access June 1, 2006).

Managing transgenic sunflower crops

Mineral nutrition

Sunflower is a highly nitrogen-dependant crop which, unlike soybean, does not perform nitrogen fixation. This limits its growth and development in poor soils and under no-till situations, where it is necessary to add nitrogen fertilizers (Diaz Zorita et al. 2003). Biotechnology for GM sunflower has been put forward as a possible way to improve nitrogen absorption.

In plants, ammonium absorption, which is an alternative pathway to the nitrogen cycle, is performed through the glutamine synthetase (GS) enzyme. However, in darkness and with a low available C:N ratio, some variants of asparagine synthetase (AS) enzyme, coded by HAS1 and HAS1.1 genes provisionally store N as asparagine, thereby preventing ammonium intoxication (Herrera Rodriguez et al. 2004). In GM plants, AS can substitute GS under conditions that limit its activity (such as in *Medicago truncatula*, Carvalho et al. 2000) and act as an alternative N-storing metabolic pathway (as in *Nicotiana tabacum*, Ferrario-Méry et al. 2002). AS expression in GM sunflower might therefore improve N metabolism and contribute to a more efficient use of this element.

Production system

Sunflower has similar crop requirements to maize and soybean. It cannot be defined as highly tolerant to drought, but its ability to explore the soil profile helps it to survive under drought conditions better than many other species, if there is water available deep in the soil profile. It can be cultivated under conventional tilling, with reduced tilling or under no-till systems, but systems that compact soil should be avoided, because they limit plant growth (Blamey et al. 1997). The use of no-till in rotations including sunflower is highly recommended as it helps to maintain the soil structure due to the rapid decomposition of crop residue once it has been buried (Bowman et al. 2000).

In Argentina, no-till has been adopted by farmers on a large scale; in over 50% of the area devoted to grain production. Soybean is by far the main crop subject to this soil conservation system, being followed by maize and wheat. In contrast, sunflower accounts for less than 3% of the no-till area (AAPRESID 2006). Difficulties associated with the use of postemergence herbicides to control weeds affecting sunflower could explain why no-till have not been adopted by many farmers cultivating this crop.

Compared to the glyphosate tolerant (RR) soybean, weed control under no-till for sunflower is more complex and not always very effective. Weed control under no-till could be improved by allowing sufficient time for preplant herbicide to take effect and by applying granular formulations (NSA 2006). However, granular herbicides are expensive and farmers tend to resist their early application, usually preferring postemergence products. Many herbicides from that group are effective in controlling grass weeds but controlling latifoliate can only be achieved to a certain extent and through the application of a limited range of herbicides (ASAGIR 2006, MAPA 2006). These include aclonifen, which can only be used in early crop stages and which persists in the upper layers of the soil profile (Vischetti et al. 2002).

Although still not widely disseminated, the GM technology that has been developed for sunflower includes tolerance to glyphosate and glufosinate-ammonium herbicides. Both of these herbicides are systemic and neither has residual effects upon the soil. Glyphosate is used on a very large scale and is relatively inexpensive, but reiterated use can promote weed resistance.

The need for RR sunflower to facilitate crop management in no-till systems seemed to disappear with the discovery of genes capable of conferring resistance to herbicides that belong to the imidazolinone (IMI) and sulfonylurea groups and which were found - in wild sunflower populations in Kansas, under field conditions – to inhibit the hydroxyacetic acid synthetase (AHAS) enzyme (Baumgartner et al. 1999, Kolkman et al. 2004). By transferring these mutations to crop germplasm in the USA and Argentina, seed companies created non-GM sunflowers, under the commercial name of Clearfield, that were tolerant to both imazapyr and imazamox (Zollinger 2003). Tolerance gene expression in these new varieties allows herbicide application at advanced stages of crop development, thus controlling the majority of weeds.

The hemiparasitic weed Jopo (*Orobanche* spp.), which constitutes an important crop limitation in the Mediterranean region could be effectively controlled in sunflower if herbicide resistant varieties were availabe. This strategy has proven useful in others crops (Nadula 1998) and coukld be improved if herbicide where brought with the seed, because broomrape affects the roots before emergence. At present, control strategies tend to use a specific gene mechanism which is also obtained in wild species (Fernandez Martinez et al. 2000, Labrousse et al. 2004). However, the continuous appearance of new races of the weed means that a process of constant renewal of resistance sources is required to maintain these control strategies.

Some herbicides that are members of the imidazolinone and sulfonylurea families including imazethapyr (Gressel et al. 1996) inhibit AHAS (Group B) and are therefore useful for controlling *O. cernua* (Alonso et al. 1999). Some other groups have also proven effective against this weed, including glufosinate-ammonium (Valkov et al. 1998) and glyphosate (Collin 1999). This may also be possible with GM sunflower because tolerance to these herbicides is currently under investigation.

There are many cases in the world of weed populations displaying resistance to herbicides that inhibit AHAS (95 cases in 63 genera, including *Helianthus*) and also to other herbicides; this points to the need to keep on searching for new control strategies. Table 10-3 shows selected cases of weed resistance to the chemical group of herbicides which could be used in sunflower, under different management strategies, including two GM varieties at present under research. Given the absence of glufosinate-ammonium resistance among weeds, a good long term strategy could involve incorporating this tolerance through GM sunflower. Moreover, two homologous “bar” and “pat” genes that codify the PAT enzyme have been shown to be safe for this purpose as they do not cause allergy and are rapidly degraded in the gut (Hérouet et al. 2005).

However, research and development should focus on more than simply obtaining broad spectrum herbicide-resistant sunflower. Science and technology policies should also outline and evaluate other integrated management strategies, which are rarely pursued by commercial companies which do not regard them as “retrieving technologies”. Without a doubt there is no single safe way in which to avoid potential problems associated with herbicide-resistant weed development: in agriculture, weed control should be a long term strategy and involve the application of a number of different management techniques (Matthews 1994).

Insect control

Crop insects present a different type of problem. At the centre of origin of sunflower, in North America, there are almost 50 species belonging to genus *Helianthus* (Heiser et al. 1969). Almost 40% of at least 25 different insect species that constitute plagues for this crop are restricted to this genus. On the other hand, in Europe and South America most of the insects that affect sunflower are unspecific (Charlet et al. 1997). Of 16 pests reported during the last five years, three are restricted to the genus *Helianthus*, being found only in the centre of origin. The others are polyphagus and have a number of unspecific controllers, with the main cosmopolitan one being *Helicoverpa armigera* (Table 10-4).

One of the most generalized sunflower constraints caused by Arthopoda is stand establishment failure due to soil larvae: mainly of Coleoptera, Elateridae and Lepidoptera. These plagues which feed on seedling stems and roots at different levels all correspond to polyphagus species. Insects that eat the aerial parts of plants, including some aphids and white flies, can be particularly important during early stages of crop development. A small number of these predators are exclusive to sunflower and are only found at the centre of origin (Charlet et al. 1997, Lopez Bellido 2002).

The relative importance of crop plagues constitutes a dynamic situation that technological developments can do much to change. This does not only relate to improved control methods but also to general changes in the ecosystem. With the increase in no-till surfaces, two previously unnoticed snails of genus *Deroceras* have recently become limiting factors for sunflower crops (Carmona 2001).

Classical sunflower breeding techniques have succeeded in achieving resistance to the European moth (*Homoeosoma nebulella*) which was once the main constraint on the diffusion of this crop in Europe. The source of resistance was found in wild sunflower populations in North America. On the contrary, the domestication of sunflower has reduced the biological control of its American relative, *H. electellum,* in a clear example of a tritrophic relationship. Adult females easily lay eggs in big sunflower flowers, while parasitic Hymenopteran *Dolichogenidea homoeosomae* females find it difficult to do the same and prefer the smaller wild *Helianthus* flowers (Chen and Welter 2003).

Table 10-3 Sunflower postemergence weed control strategies and documented cases of resistance to the chemical group of the corresponding herbicide[1]

Technology (availability)	Herbicides	Chemical group	HRAC Group	Mode of Action	Resistant weeds: total number of cases and selected representative genera
Conventional sunflower (in use)	Aclonifen	Diphenylether	F3	Bleaching: Inhibition of carotenoid biosynthesis	4: *Agrostris, Lolium, Poa, Poligonum*
Sunflower IMI Clearfield ® (recently released in the USA and Argentina)	Imazapyr Imazethapyr Imazamox	Imidazolinones	B	Inhibition of acetolactate synthase or acetohydroxyacid synthase (AHAS)	95: *Amaranthus, Ambrosia, Anthemis, Avena, Bidens,Brassica, Bromas, Chenopodium, Conyza, Cuscuta, Cyperus, Digitaria, Diplotaxis, Echinochloa, Eleusine, Euphorbia, Kochia, Lactuca, Lolium, Papaver, Parthenium, Phalaris, Raphanus, Sagittaria, Salsola, Setaria, Sinapsis, Sysimbrium, Sonchus, Sorghum, Stellaria, Xanthium*
Sunflower RR® (under research)	Glyphosate	Glycines	G	Inhibition of EPSP synthase	8: *Amaranthus, Conyza, Ambrosia, Eleusin, Lolium, Plantago*
Sunflower LL® (under research)	Glufosinate-ammonium	Phosphinic acids	H	Inhibition of glutamine synthetase	Unknown

[1] Source: http://www.weedscience.org/summary/MOASummary.asp (access May 31, 2006)

Table 10-4 Host range, geographic area, and controllers of sunflower pests reported for the last five years[1].

The general information of each pest was taken from Charlet et al. (1997)

Common name	Species	Taxa	Host range	Geographic pest area		Natural controllers	[1] Number and selected references
				Origin	Presence		
Caterpillar pest American bollworm	*Helicoverpa armigera*	Lepidoptera: Noctuidae	Wide range, including sunflower.	Old world	Worldwide	*Chrysoperla carnea, Trichogramma chilonis*, beetles, spiders *Trichogramma* spp.	6, including Sanehdeep, Brar 2003, Ballal, Singh 2003
Sunflower Beetle	*Zygogramma exclamationis*	Coleoptera: Chrysomelidae	Restricted to *Helianthus* spp.	North America	North America	Coccinellidos, Carabidos, Tachinidos. *Myiopharus macellus*	3, including Brewer, Charlet 2004
Caterpillar pest	*Spodoptera litura*	Lepidoptera: Noctuidae	Polyphagous	Old world	Worldwide	Several parasities	2, including Reddy et al 2005
Sunflower Midge	*Contarinia schulzi*	Diptera: Cecidomyiidae	Restricted to *Helianthus* spp.	North America	North America	No record	Hodgson et al 2004
Red Sunflower Seed Weevil	*Smicronyx fluvus*	Coleoptera: Curculionidae	*Helianthus* spp., *Veronica* sp., *Heliopsis* sp.	North America	North America	*Bracon* sp., *Nealiolus* sp., *Trimeromicrus* spp., *Torymus* sp., *Thereva* sp., *Rucifera* sp. *Triaspis aequoris*	Charlet 2002
Banded Sunflower Moth	*Cochylis arthur* and *C. hospes*)	Lepidoptera: Cochylidae	Restricted to *Helianthus* spp.	North America	North America	*Orius* sp., *Glypta* sp., *Chelonus* sp. *Beauveria bassiana* and *Metarhizium anisopliae*	Foster et al 2003
Long-horned Sunflower Stem Girdler	*Dectes texanus*	Coleoptera: Cerambycidae	*Ambrosia* sp., *Xanthium* sp. sunflower, soybean	North America	North America	Seven species of Hymenoptera	Michaud, Grant 2005
Western Corn Rootworm	*Diabrotica virgifera virgifera*	Coleoptera: Crysomelidea	Corn, sunflower	America	America, Europe	*Beauveria bassiana*	Mulock, Chandler 2001 Horvath, Attila 2003
Stem borer	*Mordellistena parvula*	Coleoptera: Mordellidae	Wide range	Eastern Europe	Central and Eastern Europe	No record	Yakutkin 2003
Thrips	*Thrips palmi*	Thysanoptera: Thripidae	Wide range	Southeast Asia	India	Unspecific predators	Satish et al 2004
Bihar Hairy Caterpillar	*Spilosoma obliqua*	Lepidoptera : Arctiidae	Wide range. Very destructive in jute Corchorus spp.	Unknown	India	Wasp, including 4 *Apanteles* spp.	Arora et al 2003
Wireworms Click Beetles	*Agriotes* sp.	Coleoptera: Elateridae	Polyphagous	Depends on species	Worldwide	Predated by Carabidae and birds	Trasca et al 2004
Bug	*Nysius natalensis*	Hemiptera: Orsillidae	Wheat, grasslands, onion, sunflower, alfalfa	Hawaii	South Africa	No record	Plessis et al 2005
Tenebroid beetle	*Opatrum sabulosum*	Coleoptera: Tenebronideae	Wide range	Unknown	East Europe	Ants, beetles	Trotus 2003
Percevejo	*Xyonysius major*	Heteroptera: Lygaeidae	Sunflower	Brazil	Brazil	No record	Aguiar et al 2002
Weevil	*Hypurus* sp.	Coleoptera: Curculionidae	Beans, sunflower, others	Mediterranean area	India	No record	Kumar 2001

The most frequent methods used for insect control in sunflower involve the use of pesticides. A number of chemical products are recommended to control insects that reduce crop stand. Biotechnology could improve this control by helping to develop insect- resistant GM sunflower. However, sustainable management calls for a complete knowledge of the biology of the target pest and its relationship with other components of the agro-ecosystem.

New control options offered by genetic engineering include GM crops that express gene fragments from insecticide proteins of *Bacillus thuringensis* (Bt endotoxins called Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, Cry1Fa, cry3Aa, and others), the *Vigna unguiculata* trypsin inhibitor (CpT1), lectins, and other metabolic inhibitors. The most widespread Bt proteins show strong activity against Lepidoptera, although some bacterial variants have also proven effective also against Diptera (*B. th.* var. *israeliensis*) and Coleoptera (*B. th.* var. *tenebrionis*). There is strong specificity in the action and expression Bt endotoxin. Not all the genes that codify Bt proteins are expressed in the different plant species. Similarly, nor all the Lepidoptera found in a crop are controlled by the same event.

On the other hand, the CpT1 agent is very active against Coleoptera and Orthoptera (Boulter et al. 1989) and is already available in GM crops. Modern biotechnological strategies incorporate the expression of a carrier to improve the toxin penetration and its influx into the insect's haemolymph (Flitches et al. 2004). The ideal GM technology should be environmentally friendly, with a wide spectrum of activity with respect to the target insects, but with few if any effects on beneficial insects (Hilder and Boulter 1999).

The GM sunflowers released into the environment and authorized for research include two groups of events for insect control. The reported Lepidoptera-resistant varieties express the Bt insecticide protein, which is codified by the Cry1F gene. If expressed in the early stages of crop development, this could be a valuable tool for controlling polyphagous moth larvae of genera *Agrotis* and *Euoxa*, which are present in the main sunflower growing regions (Charlet et al. 1997). For *Suleima helianthana*, which bores sunflower roots and stems in North America, control through the use of GM varieties is difficult to justify because the damage caused is seldom significant (Charlet and Brewer 2001).

Bt proteins could also offer excellent possibilities for controlling insect damage to aerial tissues. Lepidoptera that cause important crop damage include *Heliothis* spp *Helicoverpa* spp, *Diabrotica* spp, *Spilosoma* spp, *Colias lesbia*, *Rachiplusia nu*, and *Vanessa cardui.* These species could be controlled through GM technology based on Cry1 variants of the Bt gene. As these species are highly polyphagous, refuges to prevent the selection pressure for insect resistance would not be indispensable, except in cases where all the crops in a given region were GM varieties with the same expression of Bt proteins.

On the other hand, CpT1 could improve stand establishment in cases in which failure is due to Coleoptera of genera *Agriotes* sp., *Melolontha* sp., *Anoxia* sp., and Orthoptera of *Calolampra* spp. and *Teleogryllus* spp. Larvae of these species exhibit subterranean habits and eat plant roots at different stages of crop development, causing the death of seedlings in early attacks (Charlet et al. 1997, Lopez Bellido 2002). To achieve the required impact at crop establishment, the expression of CpT1 toxin should take place early in crop development and involve concentrations that are lethal for the plague. Seedlings are very sensitive to the loss of certain of their parts, so it is therefore important to stop damage as early as possible at the begining of the attack.

Two beetles cause economically important damage in North America (Charlet and Brewer 2001). *Cylindrocopturus adspersus* mainly causes crop damage by lodging in weakened plants whose stems have been bored; this also facilitates the development of fungi. This pest can be controlled through the application of insecticides, though it would also be interesting to explore the genetic resistance of many wild sunflower species. A similar situation occurs with another Coleoptera, *Smicronyx fluvus*, whose larvae develop inside seeds. This is an oligophagous species, which is adapted to only a few hosts and can be controlled with insecticides, sometimes in combination with crop traps. Some parasitic Hymenoptera and Diptera act as controllers, and genetic resistance could therefore be achieved. Females consume head bracts and pollen before oviposition, so the expression of the CpT1 gene in these tissues would help to reduce adult populations. However, the probability of transgene escape points to the need for management strategies that limit the induction of insect resistance and the acquisition of transgenes by other wild host plants. This would provide durable resistance without environmental impact.

At present, control of insects that affect crop establishment is achieved through systemic insecticides that are preventively applied to the seed. Such products offer protection through rejection. One of the most popular of these products is imidacloprid, which has proven utility for the control of soil Elaterids (Pons and Albajes 2002). However, it was withdrawn from the market in France because it was associated with bee mortality, following the consumption of pollen from treated sunflower crops.

The *Sclerotinia* problem

Conventional plant breeding combined with simple management techniques offers a successful way to control most forms of disease affecting sunflower. However, stalk rot and head rot (white rot) which are caused by *Sclerotinia sclerotiorum*, one of the most important diseases affecting the crop worldwide, have yet to be effectively controlled in this way. Chemical control is only recommended to prevent disease from spreading through seeds. Biological control has yet to be successfully applied, but appears to offer some promise (Elad 2000). An algorithm using climate data can be used to determine the risk threshold for *Sclerotinia* in oilseed rape (Makowski et al. 2005) but there is so far nothing similar available for sunflower.

The causal agent responsible for white rot is a polyphagous fungus which attacks many plants, including soybean. The sclerotia, a fungus-resistant tissue, remain viable in the soil for up to five years. Under favourable environmental conditions attacks begin in the roots and stalk or in the head, depending on the stage of crop development. Although there is no the evidence of complete resistance among commercial hybrids (Pedraza et al. 2004) there have been continuous efforts to develop methods enabling early selection (Vuong et al. 2004) and lines with combining ability that help to obtain it (Becelaere and Miller 2004).

A biochemical disease study provided hints as to how to control this problem using biotechnological techniques. Research involving the heads of infected plants has shown that tolerance to white rot is related to the accumulation of phenolic compounds (Prats et al. 2003) and to the absence of the phytotoxic effect of oxalic acid (Baldini et al. 2002). The concentration of oxalic acid increases when tissues are damaged and this can be used as an indirect method for selecting on the basis of tolerance to disease (Vasic et al. 2002).

Biotechnology offers a number of strategies for the control of white rot (Schnabl et al. 2002), including defence activation, fungus inhibition, and detoxification (Lu 2003). GM sunflower might present resistance to damage caused by *Sclerotinia* through over-expression of the oxalate oxidase (OXO) enzyme which degrades oxalic acid to carbon dioxide and hydrogen peroxide as a hypersensitivity mechanism. The first strategy outlined for sunflower by Lu et al. (1998) was also successful in other host plants. Donaldson et al (2001) demonstrated that wheat gene expression of the OXO enzyme in soybean cells walls close to the site of pathogen attacks reduced disease progression.

The OXO effect in sunflower seems to be more than a hypersensitivity mechanism. Hu et al. (2003) demonstrated that fungus-related damage promotes defence gene activation that is independent of cell death in GM plants that express the wheat OXO gene. The OXO expression may also reduce the herbivory action of certain insects, as demonstrated in maize under field conditions (Ramputh et al. 2002).

Product quality

Biotechnology offers other potential improvements in the quality of sunflower products and by-products. The fatty acid composition of some sunflower varieties has been modified through conventional plant breeding and mutagenesis. Although biotechnology could overcome some of the restrictions in this area and pave the way for further advances (Lacombe and Bervillé 2000), its acceptance by the consumer market must also be carefully considered. The high price of sunflower oil is due to it being perceived as a healthy, high quality product. Given that consumers in many countries are opposed to GM food, diffusion of GM varieties would probably affect its price and make sunflower products less popular than soybean alternatives.

After the oil extraction process, the residual sunflower meal has a low value as feed due to the limited level of methionin, an amino acid that is also scarce in other plant products. The Brazil nut (*Bertholletia excelsa*) is an exception to this general rule, providing high concentrations of this amino acid. Its genome has been biotechnologically manipulated in order to improve its amino acid content (Marcellino et al. 1996) and enable it to be transferred to other species. Unfortunately, Brazil nut albumen causes allergy in the natural product and also in GM soybean expressing its traits (Lack 2002). Given that the *Codex Alimentarius* (ftp://ftp.fao.org/es/esn/food/guide_plants) strongly recommends avoiding the transference of genes that cause allergies, interest in this kind of product for food purposes has declined. The situation for sunflower might however be different because the main destination of its meal is animal feed.

Interest in procuring alternative sources of latex has led to a search for increased biosynthesis in sunflower. The goal of this project, which has been exclusively sponsored by a governmental organization, is to commercially produce substitutes for USA imports. The guayule (*Parthenium argentatum*) is a desert shrub that produces a variant of rubber which does not cause allergy and which therefore has a high economic value. Progress in understanding the regulation of rubber biosynthesis in guayule has made it possible to obtain GM plants that offer profitable yields (Cornish and Scott 2005, Veatch et al. 2005). The proposal for GM sunflower aims to achieve expression of the latex biosynthesis complex in sunflower (McMahan 2006) because such an annual crop should facilitate extensive management.

The environmental impact of transgenes

The impact of gene flow from a GM crop depends on the expression of the transgene in the recipient population and how that modifies its fate (Darmency 1994, Jorgensen et al. 1999). In each case, analysis is very complex due to the event in question and the environmental conditions at the location where the release will take place. At present there is a generally perceived need for in-depth, case by case research that takes into account the worst case scenario as a previous step to GMO release into the environment.

A consult to experts (FAO 2005b) concluded that it was necessary to adapt the evaluation methodology for assessing the environmental impact of transgenic crops to the specific conditions of each agricultural system. In the EU the proposed guidelines for evaluating GM plants include a description of related species, the environment in which they occur, and the potential for interaction with other organisms within the agro-ecosystem (EFSA 2004). The USA government control agencies are currently developing a cooperative study to standardize the initial trials for GM ecological risk evaluation (Hellmich et al. 2005).

Gene flow in sunflower can reach 1000 m due to insect pollination (Arias and Rieseberg 1994) and crop genes persist in wild populations for a long time (Linder et al. 1998). In the USA hybridization between GM sunflower and wild populations would be likely because more than half of the crops flower at the same time as wild *Helianthus annuus* ssp. *annuus* populations. At locations with similar flowering times, 10-33% of hybridization has been recorded, with the risk depending on the fitness consequences of the transgene (Burke et al. 2002). The crop can also hybridize with other species of its genus providing at least a few fertile progeny (Rogers et al. 1982). *H. petiolaris* is an example of how crop gene flow can have an impact on wild relative populations (Rieseberg et al. 1999).

Transgene acquisition could have a reproductive cost, but this is not always negative. The Cry1Ac gene expression of Bt in wild *Helianthus* plants reduced damage due to Lepidoptera, thus increasing fecundity (Snow et al. 2003). This would increase seed production in wild populations expressing the Bt gene in environments in which target herbivores are the limiting factor. Furthermore, although they did not exhibit consistent resistance to disease, wild plants backcrossed to GM sunflowers expressing the OXO gene with conferred resistance to white rot did not have modified seed production in the presence of *Sclerotinia* (Burke and Rieseberg 2003). This finding indicates that this transgene would not represent a reproductive cost for wild plants but would confer an adaptive advantage in environments in which disease was present.

No negative effects relating to insecticide protein expression have been reported on non-target Arthropoda (NTA); there are therefore no grounds on which to question the application of this biotechnology. Even so, a careful selection process should be conducted with species which could be affected by GM sunflower release expressing endotoxines. Scholte and Dicke (2005) proposed selecting and testing 4-6 NTA from the most relevant items in food-webs, on the basis of a number of pre-determined characters. The probable effects of endotoxines on soil fauna through root exudates should also be monitored (Saxena et al. 2004).

At the centre of origin, crop-wild hybridization constitutes a primary risk in the use of GM sunflower varieties in view of the modification of wild species of the genus that are useful as a natural germplasm reserve for breeding. A number of *Helianthus* species established in other continents merit other considerations. In Europe there are feral populations of *H. tuberosus* and *H. annuus* that could probably spread to the natural environment (Faure et al. 2002). The former is a hexaploid species which crosses with diploid cultivate sunflower, producing highly sterile F1 derivatives that exhibit numerous meiotic abnormalities. Crop progenies are volunteers which do not pose any risk “per se”, because they do not establish as durable populations. They are only found in fields in which sunflower has been seeded during the previous year and near road and rail transport routes. However, in areas where sunflower coexists with wild relatives, volunteers can potentially act as transgene reservoirs and as a bridge for exchanges between cultivated and wild *Helianthus* (Reagon and Snow 2006). Appropriate control may be necessary to prevent escapes. The recent discovery of established *H. annuus* ssp. *annuus* populations in some sunflower crop regions in southern Spain (Bervillé et al. 2005) might change this situation in Europe.

The east coast of Africa seems to be a relatively new site for the recombination of genus *Helianthus* under field conditions, with intense gene flow. Established populations of *H. argophyllus* and *H. debilis* in Mozambique show a high frequency of hybrids (Vischi et al. 2004). In a similar way, in Argentina naturalized populations of *H. annuus* ssp. *annuus* and *H. petiolaris* hybridize with cultivated sunflower (Poverene et al. 2004a, b). Natural selection pressure outside their centre of origin possibly reduces interspecific reproductive barriers. The increased likelihood of hybridization could generate new biotypes. Populations that easily hybridize with the crop would be potential receptors of these transgenes which could give them some adaptive advantages. To prevent transgene escape, it is necessary to design appropriate strategies for each particular scenario. The best way to prevent gene flow between transgenic rubber producing sunflower and wild relatives would be to use male-sterile plants, which are currently under experimentation (McMahan 2006).

Germplasm banks are invaluable tools for mitigating the consequences of crop gene flow on wild sunflower resources. In addition to the North Central Regional Plant Introduction Station at Ames, Iowa, USA several other countries including Serbia and Montenegro, Bulgaria, Ukraine, and India also preserve wild sunflower collections.

Conclusions

Most of the available transgenes and modulators that have been engineered could be expressed in different crops. However, in sunflower the traits that are being studied for environment release are limited and mainly consist of insect resistance, herbicide tolerance, and special compound synthesis. Leaving aside product marketing considerations, sunflower crops would greatly benefit from the introduction of GM varieties.

GM sunflower release at its centre of origin would have a major impact on wild populations of the genus among which hybridization is a distinct possibility. The marked oligophagy of some Arthropoda that affect the crop contributes to a high risk scenario with respect to insecticide proteins of GM sunflower potentially becoming expressed in wild sunflowers. The probable acquisition of herbicide-tolerant genes could be considered in a different way. A suitable management strategy, involving other herbicides than those associated with the GM event to control wild species, should reduce the risk of resistance developing in these populations. In this sense, tolerance to glufosinate-ammonium in GM sunflower would be promising if it were alternating with the glyphosate-tolerant RR soybean.

The scenario is different in certain areas in Europe, where there are no established wild populations and the risk of environmental impact deriving from insect resistance or herbicide tolerance would be minimal. Even so, as sunflower is a target crop for bees, GM events should ensure that there is no transgene expression in pollen or nectar in order to avoid contaminating honey.

In regions where naturalized *Helianthus* spp. populations coexist with the crop, the risk of transgene escape is very high. In these conditions, experiments should be designed to predict the potential consequences of transgene acquisition and their environmental impact. In non-native environments of the genus, the specificity of Arthropoda controllers and their natural enemies – which are not yet known – may not be as complex as at the centre of origin; this should facilitate the study of GM expressed insecticide proteins. As far as weed control is concerned, the general situation and management considerations should be similar to those mentioned above.

Transgenic events related with sunflower products quality do not suppose any competitive advantage with respect to GM plants and would probably have a low environmental impact. Under present market conditions the only viable event would seem to be hypoallergenic guayule latex. Acquisition of these transgenes by wild *Helianthus* species would be unlikely to destabilise the ecosystem. Even so, it should be remembered that sunflower is a species with a high exposure to gene flow and which continuously generates variability. In view of this, it is necessary to ensure strict environmental monitoring in order to prevent any potentially undesirable outcomes.

Acknowledgements

We would like to thank the Universitat de Lleida and the Institut de Recerca i Tecnologia Agroalimentàries (Centre UdL-IRTA) Lleida, Catalonia, Spain, where this paper was researched and written and to the Fundación Carolina for the fellowships awarded to both of the authors.

Dr. Xavier Pons suggested at MAC the theme of this review as a requisite for the course Producció Extensiva de Conreus Herbacis in the UdL.

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Chapter 11 General Discussion

The two annual wild *Helianthus* species naturalized in Argentina (Chapter 1) are diploid (n = 17) self-incompatible natives from North America. The genus *Helianthus* (Asteraceae) is composed of 51 annual and perennial species including the cultivated sunflower (Heiser et al. 1969). It is a comparatively novel crop among the world's major crops and it encompasses different uses, including as an ornamental (Jan and Seiler 2007). Cultivated sunflower *H. annuus* L. var. *macrocarpus* derives from the species domestication by North American Indians before the discovery of America, followed by Russian farmer's selection and genetic breeding done at the FSU experimental stations in the early 20th century (Vranceanu 1977).

The high variability within *H. annuus* allowed Russian breeders to develop a wide range of agricultural varieties starting from a few strains introduced to Europe for ornamental purposes (Heiser 1951). This biodiversity also comprises a high degree of *endoferality* which would result in volunteer plants able to interfere with the following crops, and *exoferality*, to give rise to stable populations through crosses with native or naturalized wild plants (Gressel 2005, Reagon and Snow 2006). This seems to have happened in Spain and France (Müller et al 2006), in Serbia (Stanković-Kalezić et al. 2007), in Italy (Vischi et al. 2006) and other European countries, where there are populations most likely resulting from seed contaminants (wild or crop-wild seed) imported from the USA (Bervillé et al. 2005), to Australia where wild sunflower likely had been introduced as forage crop (Downes and Tonnet 1982, Dry and Burdon 1986). The ability to develop feral populations also extends to other annual species as *H. argophyllus* and *H. debilis* (Quagliaro et al. 2001, Vischi et al 2004, Ribeiro et al. 2005) and the perennial *H. tuberosus* (Faure et al 2002, Kowarik 2005).

According Heiser (1954, 1961) classifications, the two annual members of the genus naturalized in Argentina (Chapter 1), are the *H. annuus* ssp. *annuus* (common sunflower) and *H. petiolaris* ssp. *petiolaris* (prairie sunflower). Both wild taxa were probably introduced from the USA, naturalized and spread across the central part of the Argentina (Chapter 3).

Forces that drove the colonization process of the wild annuals *H. annuus* and *H. petiolaris* in the landscape of central Argentina.

The scientific information about the environmental and ecological determinants of wild sunflowers invasions is of agronomic interest, but information is scare. Only recently has attention been focused on the distribution of wild sunflower in the Southern part of its native range because of the supposed discovery of domesticated sunflower in early archaeological deposits in Mexico (Heiser 2008, Lentz et al. 2008). In general, it is accepted that distribution of native and non-native plants are strongly determined by climate and soil physicochemical properties (Milberg et al. 1999, Dodd et al. 2002, Peterson et al. 2003, Li et al. 2006, Ursino 2005). The Central Argentina scenario could be considered as a model of study of the invasive processes of wild annual *Helianthus* and a source of information to understand similar process in other countries and to perform ecological forecasts as suggested by Clark et al. (2001).

The field and herbaria surveys accomplished by the author during the summers of years 2000 to 2008, covering over 53,000 km in 42 exploration trips, demonstrated the existence of naturalized wild sunflower populations in three eco-regions of central Argentina (Chapters 2 and 7). The distribution of wild populations overlaps with the present crop area (SAGPYA 2008), into the *central mega-environment* for sunflower, where the crop encounters more stable growing conditions (de la Vega and Chapman 2006). Besides, there are also specialized areas for seed production in western Mendoza, near wild populations, and another in southern Buenos Aires province, both under artificial irrigation (INASE 2008).

The possible origin of the first herbaria specimen of wild *H. annuus*, collected by Dr. T. Stuckert in Los Cocos remains questionable (Figure 11-1). The locality is extremely mountainous, without open spaces for agriculture. The area is unconnected with the open prairie of the Pampas of central Argentina and corresponds to a region colonized three hundred years before initiation of extensive agriculture in Argentina. These lands were initially dedicated to grazing cattle, but at the end of the eighteenth century it became popular as a resort area, specially dedicated to the treatment of certain illnesses. In the beginning of the 20th century, cultural meetings of famous people coming from Europe were frequent (Agüero 1998). In an exploration in February of

2008, we did not find any isolated plants or populations of annual wild *Helianthus* species in the area around Los Cocos and Cosquín.

In eastern central Argentina, the Pampas are a grass steppe strongly transformed by agriculture. The central Espinal is an intermediate savannah, with scarce xeric trees, mainly *Prosopis* spp. The western Monte is an arid steppe with predominance of shrubs as *Larrea* spp. and tough grasses (Burkart et al. 1999). The climate is temperate, and rainfall decreases from near 1000 mm in the east to less than 200 mm in the west (SMN 2008). Wild *H. annuus* grows on the Monte eco-region, but only in habitats under irrigation or near them. In drylands, both wild annual *Helianthus* grow close to the limited zone between the Pampa and Espinal regions (Figure 11-1).

Mollisols, Alfisols and Entisols soil orders cover only 18% of the world temperate areas, but covers 48% of the USA land surface, where the centre of origin of the genus *Helianthus* is located (USDA 1999). More precisely, the soils of the Central Great Plains of North America, the common distribution area for the two annual species, *H. annuus* and *H. petiolaris*, belong to these orders (Rogers et al. 1982). In Argentina all annual *Helianthus* populations are established on Mollisols and Entisols (Chapters 2 and 7).

Soil taxonomy is an indicator of the prevalent ecosystem processes and could be used to estimate the habitat adaptability for given species (Mann et al. 1999, Bouma 2003). Given the similarities among the soils orders preferred by the two annual *Helianthus* in North America and Argentina, it seems that macrohabitat components have contributed to the naturalization process (Chapter 3). The 14 soil subgroups of *H. annuus* habitats reach 9,9 million ha, while the 11 soil taxa associated to *H. petiolaris* cover 13,1 million ha (INTA 1990). In these soils, there is a high probability of observing new populations due to the existence of favourable macrohabitat conditions.

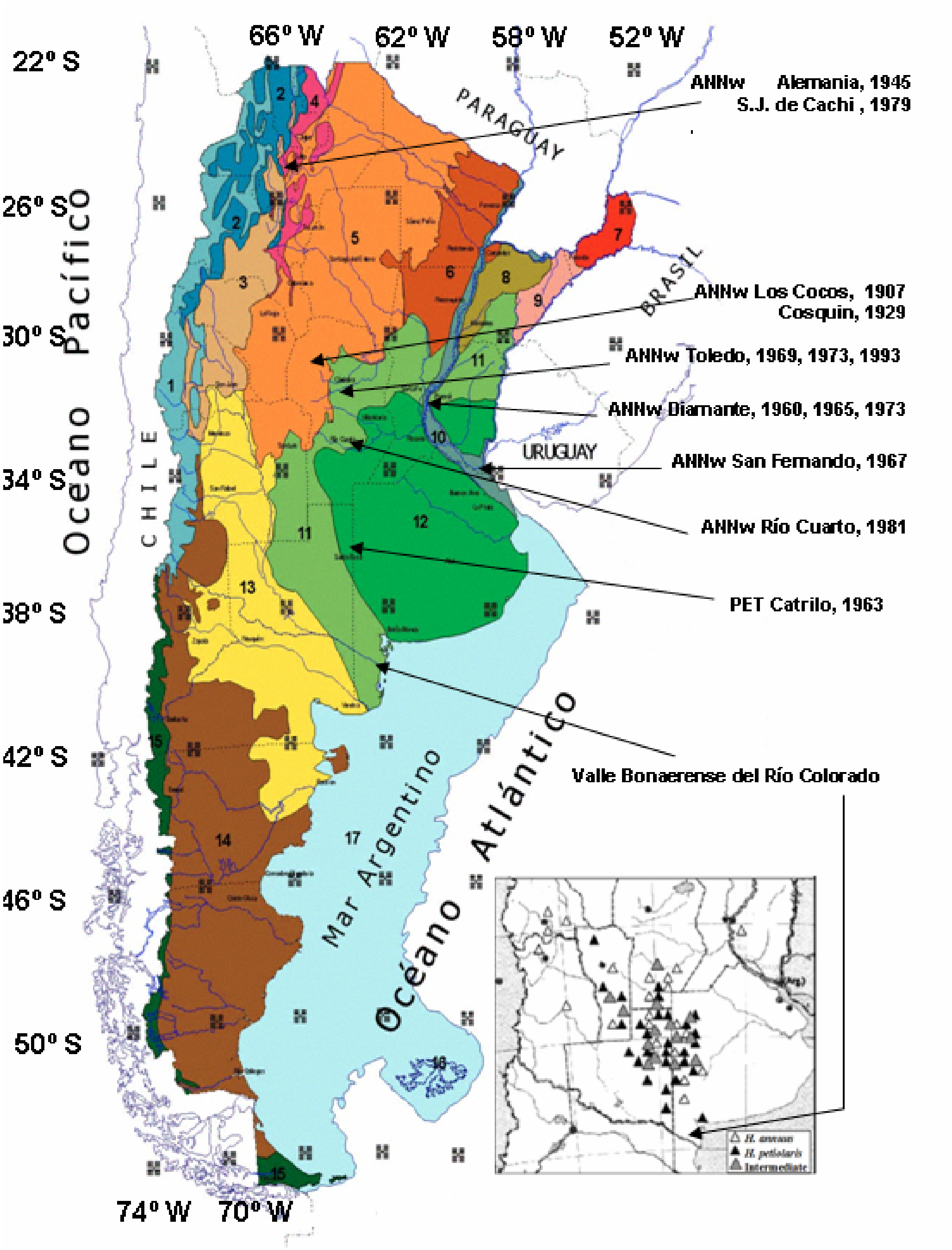

Figure 11-1 Locality information for relevant herbaria specimens of wild *Helianthus annuus* (ANNw) and *H. petiolaris* (PET) specimens (from Table 1-2) and the main sunflower seed production area of Argentina (INASE 2008).

Eco-regions (numbers) according Burkart (1999), actual populations (triangles) from Poverene et al. (2002)

Both non-indigenous invasive species demonstrated their ruderal strategy with high preference for disturbed microhabitats, typically vulnerable to invasions (Grime 1974, Kolar and Lodge 2001, Hierro et al. 2005, Stohlgren and Schnase 2006). Due to the disturbance, the physicochemical soil properties of the wild *Helianthus* patches did not always match those of the predominant soil parameters of the macrohabitat, estimated by the cartographic soil unit (Chapter 2). Both *H. annuus* and *H. petiolaris* microhabitats were preferentially fences, firelines or roadsides. The wild *H. annuus* was also found within crops, at the edge of water courses and in a few cases, in saline areas near irrigated plots, always in places where excess water has collected (Poverene et al. 2002, Chapter 2). Wild *Helianthus* populations were never found in non-disturbed habitats such as forests or rangelands. In suitable macrohabitats, the populations established mainly in microhabitats strongly modified by human activities.

In the USA, wild or common sunflower *Helianthus annuus* is usually found in clay-based mesic soils, always located in habitats that have been disturbed by man or animal (http://plants.usda.gov). *H. petiolaris* usually grows on drier, sandy soils (Seiler and Rieseberg 1997). In concordance, in Argentina *Helianthus annuus* becomes established on soils with less than 75% sand, but in a wider range of OM content (Chapter 2). More than 50% of the *H. annuus* populations were found on sandy loam soils, while only a few *H. petiolaris* populations grew in this soil type. *Helianthus petiolaris* microhabitats had sandy soils, with less than 2% OM in 95% of the sites.

Considering the environmental and ecological conditions of wild annual *Helianthus* habitats, the existence of a migration process cannot be rejected (Chapter 3). The migration pattern suggests that after their introduction at an entry point both wild species moved in successive steps across a biotic and abiotic gradient, aided by human activity through the road connection infrastructure of central Argentina. Wild and weedy sunflowers are ruderal species for which mechanical transportation seems to be the main way of distribution (Humston et al. 2005). It has been suggested that buffalo (*Bison bison* Skinner and Kaiser) disseminated sunflower into the natural distribution area; however, road traffic seems to be the modern way for sunflower spread into new areas, as in Mexico (Heiser 2008).

On the whole, the distribution pattern in central Argentina suggests that *H. annuus* migrated from the entry point at Río Cuarto towards four extreme points up to six hundred km away, moving along the road infrastructure (Chapter 3). The migration to one of these remote destinations is more difficult to explain. Even though the connection with Diamante represents small changes at the community and environmental level, it was not interconnected by land with Río Cuarto in 1960, when the first specimen of this species was collected (Chapter 1).

Considering the achene traits (Chapter 9), Río Cuarto, together with Colonia Barón and Carhué accessions seemed to be pure wild populations as opposed to those from Las Malvinas, Adolfo Alsina and Media Agua, which showed introgressed crop-related traits (large seeds, presence of stripes, low mottling). These findings agree with the hypothesis that Río Cuarto was the entry point of wild *Helianthus annuus* before 1950s (Bauer 1991) from where the invasive process expanded into central Argentina (Chapter 3).

Helianthus petiolaris seems to have traveled shorter distances from their entry point at Catriló, with a maximum of four hundred km up to Villa Mercedes, where it arrived before 1963 (Chapter 1). This locality, together with Carhué and Trenque Lauquen, exhibits the greatest environmental dissimilarities overcome in the invasive process (Chapter 3).

The morphological evaluation of *H. petiolaris* populations planted in our experimental field did not show any agreement between phenotype and the geographical origin (Chapter 5). Another experiment conducted in our experimental field during the summer 2007/08 demonstrated the similarity of all wild *H. petiolaris* accessions from Argentina with one accession from Texas (unpublished data), in agreement with the hypothesis of a unique and accidental entry from this USA state (Chapter 3).

Helianthus petiolaris populations are located far away from breeding programs that could have used this species as a disease resistance source (Luciano 1964, Bertero and Vazquez 2003). Five wild and stable populations of *H. annuus* found near sunflower breeding stations cannot be considered escapes due to the presence of other wild populations in the vicinity, established before the breeding program introduced wild resources (Chapter 3).

The first *H. annuus* population record from Toledo was collected fifteen years after the inclusion of wild sunflower resources in crosses made by the breeders Báez and Mácola (1954) (Chapter 1). Toledo is 38 km away from Manfredi, located on the main railroad and road to Cordoba (Figure 11-1). The specimens collected twice in the successive thirty years at this locality could correspond to segregation from interspecific crosses originated by pollen flow or seed escaped from experimental fields. The specimens showed different phenotypes in the different years of collection as one might expect of an interspecific cross (see Table 1-3). In experimental fields, the natural emergence of intermediate individuals after crosses involving wild *Helianthus* has been reported (Berville et al. 2005). In the summer of 2008, we found only two wild *H. annuus* individuals in Toledo, an insufficient number to be considered a population, but enough to maintain it as genetic resource due the documented presence for 38 years.

As a consequence of the previous analysis (Chapter 3), we found that the area where the 85% of the sunflower seed of Argentina is produced (INASE 2008), is vulnerable to an invasion of wild annual *Helianthus* (Presotto et al. 2007) since the environmental and ecological variables of the habitat are similar to the areas where they are already present in the USA and Argentina (Cantamutto et al. 2008, Figure 11-2). The naturalization of the two annual *Helianthus* in the Valle Bonaerense del Río Colorado (VBRC) in southern Buenos Aires province would place a severe constraint on sunflower seed production and a risk for other regions in the country not invaded yet due to contaminated sunflower hybrid seed usage which could give rise to new feral populations (Faure et al. 2002, Berville et al. 2005).

In fact, we issued an active alert to the VBRC, including an exhaustive cleaning of machinery coming from other regions, rouging of off-type plants from seed production fields, avoiding cultivation of annual *Helianthus* species for ornamental purposes, and removal of all feral forms within the protected region (Cantamutto et al. 2007b).

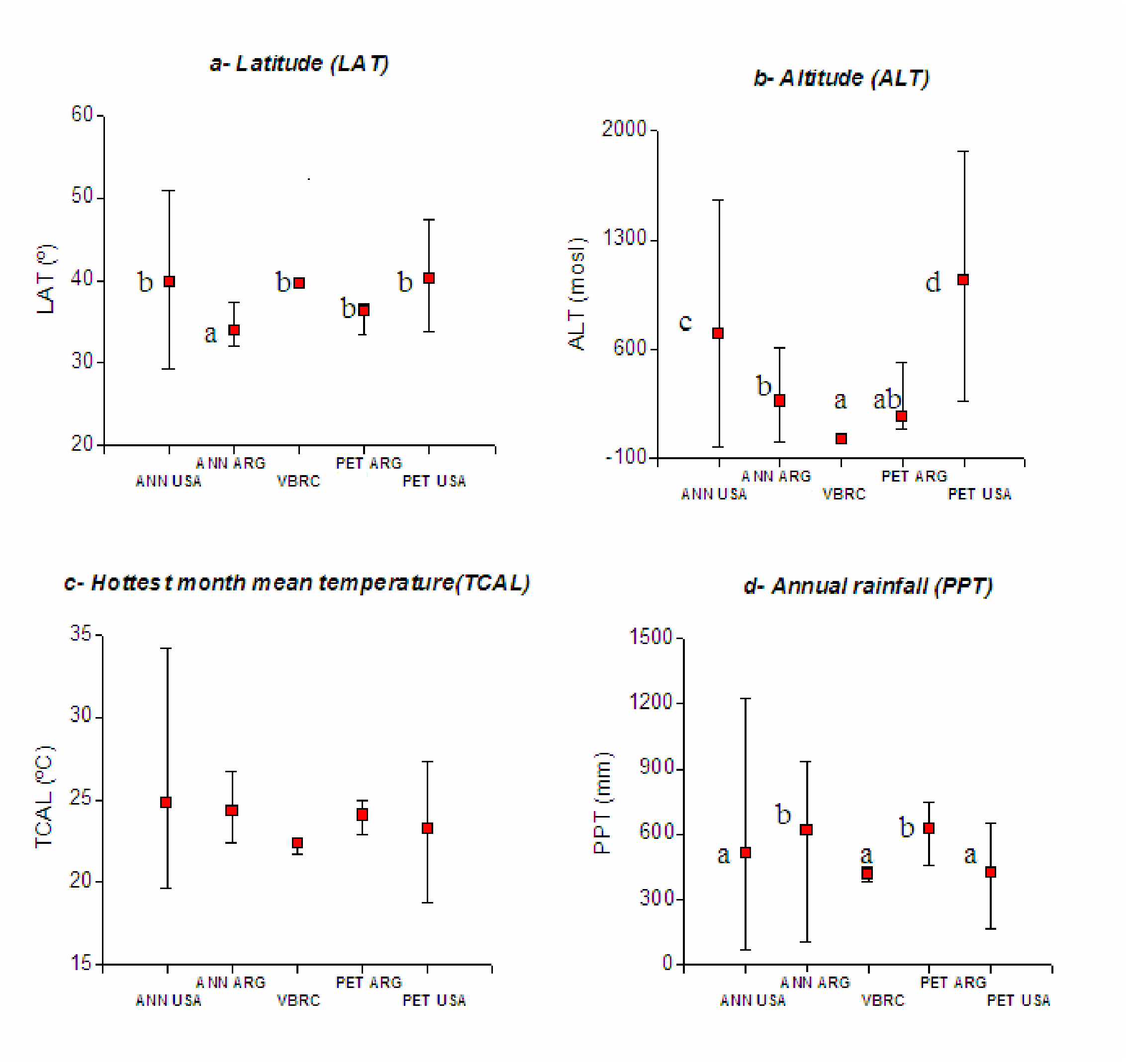

Figure 11-2 Geographical and climatic distribution (mean, range) of *Helianthus annuus* (ANN) and *H. petiolaris* (PET) populations in North America (USA) and Argentina (ARG) compared to that observed in River Colorado Valley in Buenos Aires province (VBRC).

Means significantly different are followed by different letters. Source: Cantamutto et al. (2008).

Gene flow between the wild annuals *H. annuus* and *H. petiolaris* and sunflower crop in the central Argentine scenario

Gene flow between domesticated plants and their wild relatives has evolutionary important consequences. Weed species could evolve towards more efficient competitors via gene exchange and mimicry (Harlan 1992, Hancock 2005, Campbell et al 2006). In the USA, hybridization between *H. annuus* and *H. petiolaris* occurs when the habitats are juxtaposed, which is frequent in areas with human disturbance (Schemske 2000). Generally both species retain their integrity because of the synergistic action of several reproductive barriers (Rieseberg et al. 1995, 1999a, Schwarzbach et al. 2001), but their hybridization have originated at least three new homoploid species (Rieseberg et al. 1996, 1999a, 1999b, Rieseberg and Linder 1999, Buerkle and Rieseberg 2001).

Gene flow between wild and weedy sunflowers has been recognized as a possible mechanism of evolution of weedy populations (Kane and Rieseberg 2008). The introgressed genes must have some ecological implication, for example it may suppress a controlling element (Hails and Morley 2005) or enhance fecundity in the receptive population (Lee and Natesan 2006). Introgression could also result in neutral or null effects, depending on natural selection pressure (Chapman and Burke 2006).

The hypothesis of intense gene flow between the sunflower crop and the wild annual taxa emerged as a result of the numerous morphological and site evidences of hybridization that we observed in the landscape of central Argentina (Cantamutto et al. 2003, Table 6-1). The progenies of 33 off-type plants collected from 14 representative sites of the diffusion area confirmed the existence of an intense interspecific gene flow (Chapter 4). The phenotypic study demonstrated that some progenies were presumably crop-wild *H. annuus* hybrids, some originated from the cross of cultivated sunflower and *H. petiolaris* and other were advanced generations of cultivated hybrids.

The intensity of the gene flow in central Argentina could be influenced by the relative dimension of the populations of wild and cultivated sunflowers. We observed the prairie sunflower developing in clumps of fewer plants up to over 20,000 individuals (Chapter 7), sometimes in close proximity to sunflower crops, exposed it to gene flow. For example, during the 1998/99 season, the sunflower production area in the provinces with naturalized *H. petiolaris* reached 3,6 million hectares, meaning that more than 1,8 10^{11} crop plants potentially releasing pollen (SAGPyA 2008). In our explorations, we frequently observed isolated *H. petiolaris* plants growing among sunflower volunteers or the near crop. The overlapping with sunflower crop zones, the coincidence of life cycles and the existence of common pollinator insects facilitate interspecific crosses between *H. petiolaris* and sunflower (Heiser 1947). Although both species differ in chromosome constitution – only seven out of 17 chromosomes are collinear in both species - and important barriers to hybridization exist, hybrids have been found to exist for many years in Argentina (Covas and Vargas López 1970, Ferreira 1980).

An estimation of crop-to-wild gene flow frequency emerged from the study of the progenies of selected samples of pure wild species that overlap in flowering with the sunflower crop (Chapter 5). To collect only heads that had been exposed to pollen flow from the crop, the author previously compared the phenology of both species. *Helianthus petiolaris* possess indeterminate growth habits and each plant can be flowering for more than a month. The flowering of each head of prairie sunflower extended during 7-10 days. The complete achene development takes place in the following 10-15 days and after then, the head dries and shatters (unpublished data).

In 26 different sites of the provinces of La Pampa, San Luis and Buenos Aires we collected bulked samples of seed from wild populations growing up to 100 m from the sunflower crop at the R6-R7 stages (Schneiter and Miller 1981) just before shattering. From this sample, ten out of 26 sampled populations produced hybrid descendants, which were recognized by their intermediate morphological traits and reduced fertility (Chapter 5).

Although in the USA both annual species overlap in flowering time and pollinators, fertilization by intraspecific pollen is selectively favored, limiting the formation of hybrids (Rieseberg et al. 1995). In spite of this constraint, overall hybridization estimated in this random sample of co-existence in central Argentina reached up to 1.3% (Chapter 5) differing from the parallel situations in North America, where none of the 159 individuals collected in *H. petiolaris* populations growing adjacent to cultivated sunflower fields showed any such morphological indications of hybridization. In this sample, crop introgression was revealed only by molecular markers (Rieseberg et al. 1999a). This could be due to successive natural backcrossing after interspecific hybridization, which recovered the fertility without selection over the neutral genes of cultivated sunflower, thus persisting in wild populations (Whelan 1979, Rieseberg et al. 1998).

Although *H. petiolaris* is not considered a noxious weed in Argentina, in the last few years we often found it invading summer crops in the eastern part of the La Pampa province and the western part of the Buenos Aires province. The expected gene transfer from sunflower crop to *H. petiolaris* populations is of concern regarding the recent commercialisation of imidazolinone tolerant (Clearfield®) hybrids. Herbicide tolerance was transferred with a high frequency (79%) to wild plants (Massinga et al. 2003). A similar situation of risk could occur if genetically modified (GM) sunflower varieties were released (Chapter 10).

Wild *Helianthus annuus* grows in a wide range of habitats in North America (Seiler and Rieseberg 1997) and Argentina (Chapter 2). We observed that the species has a patchy distribution over central Argentina; some populations are extensive with more than 100,000 individuals, while others have only a few plants (Chapter 7). The sunflower production area in the provinces with naturalized *H. annuus* reached a record of 3.7 million hectares in 1998/99 campaign (SAGPyA 2008). At present, it can be estimated that one third of the sunflower crop overlaps with the distribution of wild sunflower (Chapters 2 and 7).

Wild sunflowers are of concern because they can invade and exert crop interference (Faure et al. 2002, Bervillé et al. 2005). However, wild *H. annuus* from Argentina do not seem to be a great hazard to sunflower yield. In our experimental field plots, a wild accession from Colonia Barón showed low interference capacity to the sunflower crop (Errazu et al. 2007). The use of the available space and resources demonstrated by this wild accession was not different from that showed by any crop plant. The distinguished trait observed in the wild accession was great capacity to continue growing and producing seeds after the crop ended flowering (R6). Associated to early shattering and post-dispersal disturbance, this could be a mechanism to promote soil seed bank formation (Moody-Weis and Alexander 2007).

In the centre of origin, wild *H. annuus* ssp. *annuus* often hybridizes with cultivated sunflower, *H. annuus* var. *macrocarpus* (Arias and Rieseberg 1994, Whitton et al. 1997, Linder et al. 1998). Both taxa are genetically close enough to be cross compatible, grow sympatrically, overlapping phenology, and share the same pollinators (Arias and Rieseberg 1994, Burke et al. 2002, 2004). In Argentina, we observed that the overlapping flowering period between cultivated and wild sunflower occurs from December to February, though wild plants bloom from December to April (unpublished data). Pollinating insects are mainly honey bees, wild bees, and butterflies. Thus gene flow is expected to occur over the whole range of cultivated sunflower. Hybridization frequencies up to 15% have been found in natural wild hybrid zones with *H. annuus* as the maternal parent (Rieseberg et al. 1998).

Our experiment performed with a similar design to that used by Arias and Rieseberg (1994) confirmed the high probability of gene exchange between wild *H. annuus* and the sunflower crop in Argentina (Chapter 6). In this experiment, gene flow from cultivated sunflower to the wild relative extended up to 500 m, decreasing from 18% (at 3 m) to 2% of hybrid frequency. No significant differences between cardinal rays were found, meaning that there was no effect of wind direction on pollinator activity. Under those experimental conditions, evidence of pollen flow at 1000 m was not detected, suggesting the necessity of at least this distance to prevent crossing.

In the Argentine landscape, gene flow also occurs from both wild species, which donate pollen to the cultivated sunflower as a female. This was evidenced by intermediate morphological traits in plants grown from seeds of sunflower heads collected by the author in a field invaded by wild *H. annuus* in the province of La Pampa (Chapter 6). Frequency of intermediate plants was of 3.75% in a random sample of seeds sown in our experimental field. Based on 28 morphological traits, those intermediate plants were more similar to the wild parent, which is a clear evidence of gene flow from wild plants to crop plants.

Morphological intermediate plants were also obtained from seeds of cultivated plants sampled by the author and collaborators in nine sunflower crop fields invaded by *H. petiolaris* in the provinces of Buenos Aires and La Pampa (Gutierrez et al. 2006). The heads were collected at R8 stage in fields where the wild sunflower heads were between the active flowering and the shattering phase. Four off-type individuals were found among 851 crop progenies (0.5%). Off-type plants displayed total branching and lacked a main head. Disc flowers were always red, and the disc diameter and phyllary width were intermediate between *H. petiolaris* and cultivated sunflower.

Volunteers are common near sunflower crop fields in the USA and in other countries (Faure et al. 2002, Reagon and Snow 2006). The phenotypic ratios found among volunteers confirmed that they constitute advanced generations (F_2-F_3) of seeds of commercial hybrids growing along the roadsides from grain lost during transport (Chapter 6). Given the usual constitution of commercial hybrids, volunteers segregated as expected in terms of branching character and male-sterile trait from their male and female progenitors. In the analyzed samples more than 20% of volunteers were male-sterile and branched. Both traits increase the potential number of flowers susceptible to pollen flow from crop and wild sunflowers. The progeny of isolated volunteers growing with the prairie sunflowers, collected near Catrilo by Cialzeta and Antonelli (1971), segregated with intermediate plants evidencing an interspecific cross with *H. petiolaris*.

Volunteers are of concern because they could serve as a genetic bridge by which genes from the crop spread to wild or cultivated plants (Reagon and Snow 2006). Sunflower wild-crop hybrids display some fecundity constraints in comparison to true wild-type, such as a smaller number of flower heads and reduced number of seeds per plant (Snow et al. 1998) but this would not prevent crop gene dispersal. Environmental, wild population source and competitive conditions affecting the crop could increase the reproductive capacity of crop-wild hybrids (Mercer et al. 2006). The final rate of gene spread from the crop will be mainly governed by their persistence in the wild population after introgression, and therefore it will become gene-dependent (Burke and Rieseberg 2003, Snow et al. 2003).

The finding of three mixed stands of both wild species growing together enable an intense gene flow between both (Chapter 2), resembling similar situations in the centre of origin that would create favourable conditions for the formation of new ecotypes or new speciation processes (Rieseberg et al. 1996, 1999a,b). We observed in three wild mixed stands from two provinces of central Argentina, with populations sizes between 560 to 10,600 individuals, showing 7 to 15% of plants with intermediate phenotypes among both pure species *H. annuus* and *H. petiolaris* (Cantamutto et al. 2007c). Introgression of biotic resistance traits by hybridization with wild relatives and selection of transgressive phenotypes has been important in the adaptation of *H. annuus* to central and southern Texas (Whitney et al. 2006). Also, new plant species may be formed through hybridization if hybrids escape the homogenizing effects of gene flow from parental species and reach reproductive isolation (Buerkle et al. 2000, Rieseberg et al. 2006). Analogous processes of adaptation and speciation could be ongoing in central Argentina.

Given the agro-ecological conditions in central Argentina, the highest rate of hybridization observed amounted to 18% in the controlled experiment designed to evaluate the pollen flow from the sunflower crop to wild *H. annuus* (Chapter 6). In the worst case scenario, this frequency could mean almost thousand of first generation hybrids each year (Figure 7-2). Prairie sunflower populations subject to crop pollen pressure could produce hundreds of interspecific hybrids at each encounter point (Chapter 5, Gutierrez et al. 2007). The observed hybrid frequencies between sunflower crop and *H. petiolaris* or wild *H. annuus* (<1% to 3.75%) suggest that hybridization crop x wild could represent millions of first generation interspecific hybrids per year (Chapter 6). If the seeds formed by these crosses were assigned to industrial processing, a minor change in the oil quality could be expected, but if the seed falling during grain transportation germinates on roadsides or other disturbed areas, the consequences could be of agro-ecological risk (Berville et al. 2005).

Wild *H. annuus* from Argentina: A new genetic resource of potential interest for the sunflower crop?

During recent years, there has been increasing awareness of the importance of a holistic view of biodiversity, including agriculture biodiversity, conservation for sustainable utilization and development (Ramanatha Rao and Hodgkin 2002). Aggressive collection of wild sunflower germplasm for preservation in seed banks is critical so that germplasm may be made easily available to the sunflower genetics and breeding community (Seiler et al. 2006, Gulya et al. 2007). To preserve the genetic variability of the genus, there are several well maintained *ex-situ* collections of wild sunflowers around the world, for example the Novi Sad collection (Altagić et al. 2006).

Early explorations for rust resistance were undertaken in USA by Dr. Murray Kinman and Dr. Aurelio Luciano in Texas and Oklahoma in 1963 (Seiler and Rieseberg 1997). In Argentina, the explorations for wild *H. petiolaris* were initiated by Cialzeta and Antonelli (1971), who travelled across the provinces of La Pampa, Buenos Aires and Córdoba searching for *Puccinia helianti* resistance. In the following decade, the wild populations from Juarez Celman in Argentina also received attention as germplasm source from breeders of seed companies (Monge Navarro 1987).

The value of the wild *H. annuus* from Argentina as a unique genetic resource was estimated by comparing nine populations from different geographic regions of Argentina and 17 populations from the USA (Chapter 8). Twenty-three quantitative traits showed a continuous range with most of the extreme values in populations from North America (unpublished data). The populations that showed similarities for one group of descriptors differed for other traits, revealing the existence of different phenotypes. *Helianthus annuus* populations established in Argentina could be considered a unique genetic resource, containing new combinations and traits absent in North American populations.

Phyllary (bract) width provides the strongest evidence of introgression with cultivated sunflower in wild populations established in Argentina (Chapter 8). Cultivated sunflower is characterized by bracts over 0.8 cm width, while all the wild or weedy subspecies have bracts less than 1.0 cm wide (Heiser 1954). Mean phyllary width in Las Malvinas, Adolfo Alsina, and Media Agua populations exceeded 0.8 cm, whereas in the remaining populations some individuals also had bracts exceeding 0.8 cm.

Among the North American populations, mean phyllary width was over 0.8 cm in populations from Indiana and Illinois (Chapter 8). The populations from Nebraska, Iowa, North Dakota and Kansas also included individuals suspected to have hybridized with cultivated sunflower, as all the Argentine populations cultivated in the experimental field. In that case, populations from Argentina would not have introgressed characters from cultivated sunflower to the same extent and duration as those from the centre of origin in the USA. It seems that the extreme variability in these species discourages the use of different Latin names for botanical forms (Seiler and Rieseberg 1997) but also hampers the assignment of wild populations to a well-defined taxonomical group.

The achenes of the Argentine accession from Carhué had the smallest seed dimensions, significantly different from those found in Las Malvinas and Media Agua, which had the largest achenes (Chapter 9). The accession from Carhué also had a higher frequency of ovoid shaped seeds and a grey pericarp. The Río Cuarto, Rancul and Juarez Celman accessions showed mottling in all seeds, significantly different from those form Las Malvinas, Media Agua and Adolfo Alsina which had a low mottled seed frequency.

Helianthus annuus populations established in Argentina showed a high enough phenotypic variability to differentiate among them (Chapter 8). It can be accepted that invasive plant populations in Argentina are not different from native populations. Some traits of Argentine populations were absent in the North American populations, such as life cycle length in the Diamante population with over 180 days, the longest population studied so far. The Diamante location can be considered an extreme habitat for wild sunflowers in Argentina, given the local climatic and soil conditions, this phenotypic trait could mean an adaptation to this environment (Chapter 3).

The biodiversity present in the wild populations from Argentina represented nearly two-thirds of that observed in wild populations from the USA (Chapter 8). It seems that founder effects did not limit wild *H. annuus* biodiversity in the newly colonized environment of Argentina, because 60 years after their introduction the high variability of the USA wild germplasm phenotype is still present. Also, the observed biodiversity could have originated from the intense gene flow with the sunflower crop (Chapter 7) or the introgression with *H. petiolaris* (Cantamutto et al. 2007c, Gutierrez et al. 2007).

Traits of interest for sunflower crop from wild *H. annuus* populations from Argentina

The possible existence of adaptation to extreme soil conditions has been observed in wild sunflowers. Three *H. annuus* populations grew in soils under the limit of available phosphorus, below which an economic response to fertilizer can be expected (Diaz Zorita el al. 2003), with a lowest value of 5 ppm P for one *H. annuus* population in Buenos Aires province (Chapter 2). Even though sunflower is not highly sensitive to soil pH (Robinson 1978), eight *H. annuus* microhabitats in Argentina were over the suggested pH limit listed for the USA (http://plants.usda.gov). These findings could indicate the existence of novel traits of interest for breeding.

The Argentine accessions of wild *H. annuus* could contain desirable genes useful for sunflower breeding to improve oil quality (Chapter 9). Sunflower oil quality is of interest because this product contributes about 80% of the total value of the crop (Fick and Miller 1997). Since each end-use of sunflower oil requires a certain fatty acid composition, considerable breeding efforts have been made in the last 30 years, searching for genes useful to obtain specific characteristics of oil composition (Seiler 2004, 2007). The fatty acid composition of some sunflower varieties has been modified through conventional plant breeding and mutagenesis (Garcés et al. 1989, Osorio et al. 1995).

Taken as a group, the oil content, fatty acid composition and iodine value of Argentine accessions of *H. annuus* did not show differences from the wild populations from North America (Chapter 9). The oil content of the Argentine populations cultivated in an experimental field varied between 214 to 282 g/kg, typical values of wild seeds and was only affected by the population as a source of variability.

In general, the fatty acid composition did not show values of interest with respect to those reported for improved mutant lines with altered fatty acid composition (Fernandez-Martinez et al. 2006). None of the Argentine accessions showed less than 39 and 26 g/kg of palmitic and stearic acid content or more than 300 g/kg of palmitic acid to be considered low or high in saturated acid content (Chapter 9). None of the Argentine accessions showed oleic acid over 860 g/kg or linoleic concentration over 780 g/kg, similar to values of improved mutant lines.

The wild *H. annuus* populations from Argentina showed a negative relationship between palmitic (16:0) fatty acid content and oil content (unpublished data). The lack of such a relationship was found in the descendants of crosses with an improved mutant line CAS-3, suggesting the feasibility of simultaneous selection for both traits (Velasco et al. 2007). There was also a negative relationship between stearic (18:0) fatty acid content and oil content in the wild accessions from Argentina similar to Velasco et al. (2007).

Among Argentine germplasm, the Diamante population showed the most extreme values in fatty acid composition, with higher palmitic, stearic, linoleic, linolenic, and iodine values and the lower oleic acid content (Chapter 9). As in phenotypic traits, this population from Diamante could constitute a unique germplasm of potential value. The other chemical parameters of oil quality of accessions from Argentina were within the ranges observed for the USA wild populations.

Prairie sunflower (*H. petiolaris*) from Argentina has also deserved attention as a possible source for industrial processes (Perez et al. 2007). The four samples collected under natural conditions in three Argentine provinces yielded oil content between 27 and 30%, and a higher concentration of unsaturated fatty acids than cultivated sunflower (Perez et al. 2004). Although these results could be influenced by the different environmental conditions during grain filling (Seiler 1983), as in common sunflower (Chapter 9), none of the accessions showed values of interest compared to the improved lines of cultivated sunflower. The potential use of prairie sunflower as a meal for animal feed has been suggested by some authors.

We found other promising traits of agronomic interest in the wild *H. annuus* accessions from Argentina. The first cytoplasmic male-sterility (CMS) source reported was derived from *H. petiolaris* Nutt. (Leclercq 1969) and has been designated as CMS PET1 according to FAO code (Serieys 1991). This CMS system is a very efficient tool in world-wide commercial production of hybrid seeds, and currently most of the commercial hybrids are produced using this system (Serieys 2002). Independent of their origin, other existing CMS sources seem to be similar to CMS PET1 (Horn and Friedt 1999). The use of a single CMS mechanism implies a potential risk as a result of the vulnerability of such a narrow genetic basis (Havekes et al. 1991).

In the summer of 2005, we found two male-sterile plants within Las Malvinas population in the Bahía Blanca experimental field. Fertility was not completely recovered in crosses with the restorer lines from CMS PET1; R432, R307 and R09 (Cantamutto et al. 2007a). This accession could constitute a potential new source of male-sterility[5].

Sunflower chlorotic mottle virus (SuCMoV), a member of the *Potyvirus* genus is one of the most widely distributed potyviruses in cultivated and wild sunflowers from Argentina (Lenardon et al. 2001). A wild *H. annuus* accession collected by the author in Colonia Baron (La Pampa province) artificially inoculated with SuCMoV showed more than 45% of individuals without any visible symptoms (Cantamutto et al. 2007a). The healthy plants showed a virus resistance superior to the source found for Lenardon et al. (2005) in the privately owned line L33, which exhibited isolated chlorotic pinpoints, resembling a hypersensitive mechanism of reaction. We crossed the resistant plants of the accession from Colonia Barón with the pure lines of cultivated sunflower HA89, A09 and A10, in order to obtain resistant lines. The search for other useful genes from Argentine *H. annuus*, such as tolerance to low temperatures, is currently under experimentation in our experimental field

The prairie sunflower from Argentina has also gained attention as a disease resistance source (Cialzeta and Antonelli 1971). A first experimental trial performed by Cáceres et

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[5] The study of this male sterility source has been recently included as doctoral thesis research of Lic. Antonio Garayalde (CONICET) under the supervision of Dr. Alicia Carrera (UNS).

al. (2006) suggested the possibility of detecting some *H. petiolaris* populations with higher levels of resistance to *S. sclerotiorum* than others.

Based on their potential value, nine more diverse wild *H. annuus* populations from Argentina, collected by M. Poverene and M. Cantamutto during 2002-2003 exploration trips were regenerated in the experimental field in Bahía Blanca (S 38º 41', W 62º 12') during 2003-2004 summers. Passport information was completed by Presotto (2004) under the supervision of the author, and the harvest seeds were deposited in the Sunflower Germplasm Active Bank at Manfredi Experimental Station of the Instituto Nacional de Tecnología Agropecuaria (INTA) in Córdoba, Argentina, as code numbers 832 to 840.

Impact of GM sunflower varieties on the agroecosystems and the agro-industrial processes.

Hybrid sunflower seed rapidly diffused to all the world's crop regions thanks to reasonable cost of hybrid seed production using the CMS breeding system (Leclercq 1969). During most of the second half of the 20th century, sunflower breeding techniques were as complex as for other major crops, including interspecific crosses, induced mutation, marker-assisted selection, and other advanced tools (Jan and Seiler 2007). Thus, the crop became a profitable and competitive option for some countries of the former Russia, China, France, Hungary, India, Romania, Bulgaria, USA and Argentina, which comprises 83% of world production estimated at more than 30 million metric tonnes for 2007-2008 (USDA 2007).

Towards the end of the first biotech decade, after the eruption of genetically modified (GM) crops, sunflower still remains a traditional non-GM crop (Chapter 10). This fact makes it clearly different from other crops such as corn (*Zea mays* L.) and soybean (*Glycine max* L.), which rely on the adoption of biotechnologically improved cultivars (Brookes and Barfoot 2006, James 2006).

The majority of transgenic traits incorporated in other crops have already been subjected to research and experimentation in sunflower (Chapter 10). Biotechnology could help sunflower to overcome some crop constraints (Paniego et al. 2007). GM sunflower release would improve the mineral nutrition, weed control, and insect and disease resistance of the crop (Chapter 10).

Gene flow studies became popular when large-scale cultivation of genetically modified (GM) crops became a reality by the end of the 20th century (James 2005). Crop genes can spread through pollen and seed dispersal to populations of related crops, weeds, and wild relatives (Harlan 1992, Ellstrand 2003). For GM crops, case studies, monitoring, and regulations are needed to minimize the negative ecological effects of the release of genetically engineered organisms (Snow et al. 2005).

The horizontal gene flow to other Asteraceae naturalized in Argentina like *Tithonia* spp. or *Verbesina* spp. (Zuloaga and Morrone 1999), seems highly unlikely because their hybridization is only possible using artificial techniques (Sossey-Alaoui et al. 1998, Encheva and Christov 2005). With less reproductive limitations, the implications of gene flow with other sexually compatible *Helianthus* species pose constraints to transgenic sunflower release because of the risk of *vertical* or *diagonal* gene flow (Gressel and Al-Ahmad 2005).

Botanical files would indicate a high ecological risk for GM sunflower release because of the difficulty to keep transgenes restricted within the crop (Conner et al 2003). Sunflower seeds can be dispersed throughout wide distances by trucks and machinery and create ruderal populations (Robinson 1978, Reagon and Snow 2006, Chapters 3, 6). In addition, being an outcrossing, insect-pollinated crop, safe isolation demands distances over to 1 km (Anfinrud 1997, OECD 2004, Chapter 6).

Genetic changes in wild populations constitute a primary risk to GM crops, although non-GM crops can modify them in a similar way. Many weeds have originated from this kind of contact (Snow and Morán Palma 1997, Ellstrand et al. 1999). In view of this, it is worthy to evaluate the rate at which hybridization occurs and the persistence of hybrids that could facilitate introgression and modification of wild populations (Hails and Morley 2005). Gene flow from genetically engineered crops can transfer gene coding for traits such as tolerance to herbicides, insect herbivores, diseases, and environmental stress into wild plants. Some crop-traits can confer advantages to wild populations, enhancing their weediness (Keeler 1989, Vacher et al. 2004). The wild annual *Helianthus* are non native plant invaders (Chapters 1 and 3) and the introgression of crop genes is possible (Chapters 4 and 8).

From 1991 up to 2000, there was a continuous growth in the number of environmental controlled field experiments with GM sunflower in Argentina and USA (Chapter 10). Coincidental to the dissemination of our work showing evidence of crop-wild gene flow (Cantamutto et al. 2003, Chapter 6) and the expected increase of fecundity in wild populations by transgene acquisition (Burke and Rieseberg 2003, Snow et al. 2003), the number of GM sunflower release permits in Argentina and USA have declined. After these findings, the public release of transgenic sunflower began to be viewed as *improbable* by the Argentine Sunflower Association (ASAGIR) (Fonseca et al. 2004, Ingaramo 2006). A similar situation took place in 2004 in the USA, where the National Sunflower Association began to stress the non-transgenic nature of the sunflower crop promoting the consumption of its oil as an alternate to currently used GM oils (www.sunflowernsa.com).

Contrary to this, interest in GM corn and soybean remained high, as evidenced by the interest of seed companies in the development of GM varieties for these crops (Figure 11-3). Differing to Mexico (Ortiz-García et al. 2005), in the USA and Argentina, neither of these crops has naturalized relatives that could be exposed to modifications by means of gene flow from GM varieties.

In Argentina we could not investigate the effect of gene flow from GM sunflower to naturalized wild annual *Helianthus* populations. We try to investigate this point, but we not successful in gaining funding from the national association of sunflower (ASAGIR) for a project in 2003. We were not able to obtain a reason why they were not interested in funding the research. Research from our experimental fields demonstrated that the crop tolerance to herbicides of imidazolinona family (Tan et al. 2005) can be easily transferred to wild *H. annuus* populations (Ureta et al. 2007). A similar situation would be expected for transgenes incorporated into the crop.

There are strategies to minimize environmental risks of GM crops (Gressel and Al-Ahmad 2005). Transgenes could be contained in male-sterile varieties (McMahan 2006) or inserted in chloroplasts (Haygood et al. 2004). Also, it could be possible to mitigate gene flow by using transgenic varieties carrying chromosome translocations and inversions (Bervillé et al. 2005). An alternative would consist of transgene linkage to traits of low persistence in the wild, such as no branching (Snow et al. 1998, Alexander et al. 2001, Claessen et al. 2005). However, as the genus has one of the highest recombination rates among plants (Burke et al. 2004) this strategy would not be reliable enough because the linkage could be broken.

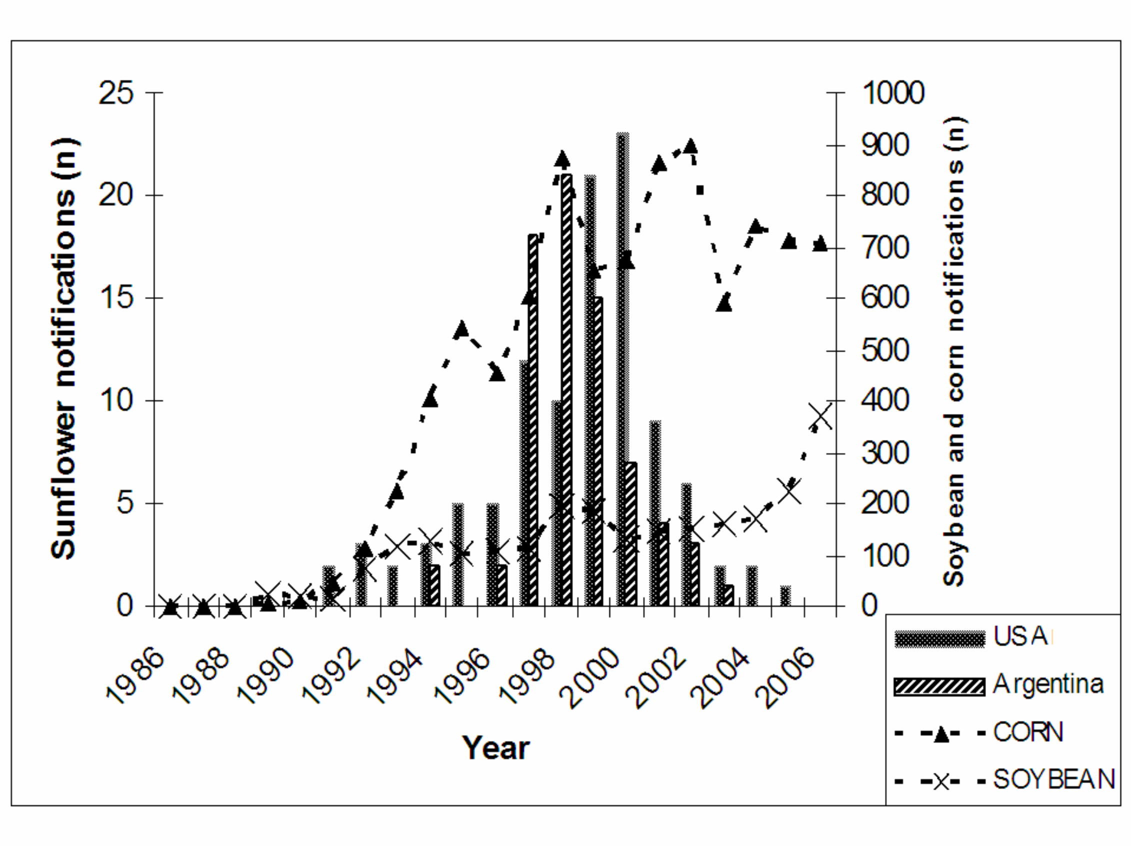

Figure 11-3 Number of GM sunflower notifications[6] compared to GM corn and GM soybean notifications in the USA and Argentina, since 1986. Redraw from Chapter 10.

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[6] Environment controlled experiments authorized by the correspondig corresponding governmental in Argentina (CONABIA) and USA.

In high quality edible oil markets, sunflower presents advantages that make it a very competitive crop and should be used to increase its value. Mid-oleic hybrids obtained from chemical mutation (NuSun) has a fatty acid composition of saturated, mono- and poly-unsaturated acids close to those recommended by WHO (FAO 1994), making sunflower oil superior to olive, in which mono-unsaturated oleic acid predominates. That makes sunflower oil very healthy for cardiovascular care (Jan and Seiler 2007). Sunflower oil is also rich in tocopherol (vitamin E) with anti-oxidant effects (Fernández Martínez et al. 2004). The high price of sunflower oil is due to it being perceived as a healthy, high quality product (Obschatko et al. 2006, NSA 2007). Given that consumers in many countries are opposed to GM food, development of GM sunflower hybrids would probably affect its price and make its products less popular than soybean alternatives. The question still remains if existing markets would accept edible oil coming from a transgenic crop?

If environmental constraints were to be overcome through regulatory flexibility or by obtaining varieties harboring containment or mitigation mechanisms, transgenic sunflower acceptance would be strongly conditioned by consumers' attitude. At present one could expect a complete acceptance in the increasing biofuels market (Table 11-1). However, this competition seems to leave sunflower behind because other suitable crops like soybean and rapeseed already having available transgenic varieties.

The future of transgenic sunflower will be defined by the potential for industrial use and changes in consumer perception. Environmental risk mainly related to difficult-to-control novel sunflower feral forms can be diminished, but may not be eliminated. Nevertheless, risk does not involve merely transgenic varieties, but extends to every new germplasm obtained through classical breeding. Consumer perception would change dramatically if transgenic varieties meant an outstanding improvement of life quality, including environment and health. Until then, advances in other new transgenic crop developments will postpone the usage of sunflower transgenic hybrids.

Table 11-1 Expected acceptance of sunflower transgenic varieties under present market perception.

(Based on Fernández Martínez et al. 2004, Fonseca et al. 2004, and Vannozzi 2006).

Destination	Attributes	GM acceptance	Competitor crop	Available GM varieties
Biodiesel	high oleic acid	total	palm	no
			canola	yes
			soybean	yes
Bio-lubricants	low linoleic acid antioxidants	total	flax	no
			canola	yes
Edible oil Fried products	saturated fatty acids	parcial	canola	yes
Edible oil Salads	flavour, unsaturated fatty acids	low	olive	no
			corn	yes
Confectionary	big and healthy achenes	none	peanut	no
			pistachio	no

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Chapter 12 GENERAL CONCLUSIONS

1. It was possible to identify natural forces that drove the naturalization process of the wild annuals *Helianthus annuus* L. and *H. petiolaris* Nutt. in the landscape of central Argentina.

1.1. The localization of both wild *Helianthus* were found to be interrelated with climate, soil and agroecosystem variables.

1.2. The wild species were naturalized across the boundary between Mollisols and Entisols, the same soil taxa predominant in the centre of origin in North America.

1.3. The habitats of both species were strongly related to disturbance, but they were also found in riparian areas and within crops.

1.4. Microhabitat conditions allowed clear differentiation among the specie's preferences; *H. petiolaris* appeared associated with sandy soils with low organic matter content, while *H. annuus* showed preference for more fertile and fine textured soils.

1.5. The wild *H. annuus* was also found in irrigated areas of the western part of the country.

1.6. No environmental variables allowed for the characterization of three sites where both wild species grew together.

1.7. It was possible to create a connection tree through road links and environmental and plant community similarities from an unique entry point for each species, minimizing the covered distances measured with three independent matrices, based on road distances, environment, and ecological variables.

1.8. It was possible to suggest a migration pattern showing that both wild species moved in successive steps across a biotic and abiotic gradient, aided by anthropic activities.

1.9. No evidence of escapes of wild sunflowers from breeding programs was found.

2. An intense gene-flow between the sunflower crop and both wild taxa was demonstrated in both controlled and natural situations.

2.1. The morphology, phenology, and fertility of the progeny of 33 off-type *Helianthus* plants found isolated or between pure populations in 14 representative points in central Argentina, confirmed the existence of hybridization between the sunflower crop and both wild taxa under natural conditions.

2.2. Hybrid plants between *H. petiolaris* as female and *H. annuus* var. *macrocarpus* as male progenitor were found in the progenies of 10 out of 26 representative pure populations growing up to 100 m away from the sunflower crop in three different provinces of Argentina.

2.3. The hybridization between wild *H. annuus* as female and *H. annuus* var. *macrocarpus* as male reached up to 18% in the wild plants located at 3 m of the crop and diminished to 0 at 1000 m from the pollen source.

2.4. Hybridization from pollen flow from wild *H. annuus* to the crop in a stand invaded by wild sunflowers produced 3.75% intermediate type plants.

2.5. The levels of gene flow under natural conditions could represent up to millions of first generation interspecific hybrids each year.

3. The wild *H. annuus* naturalized in Argentina could be considered a new and unique genetic resource of potential interest for the sunflower crop.

3.1. Nine wild *H. annuus* populations from Argentina reflected about two-third of the phenotypic variability observed in a sample of 17 populations from the centre of origin.

3.2. Nine wild *H. annuus* populations from Argentina reflected enough phenotypic variability to be considered as differentiated populations..

3.3. The Indiana and Illinois populations from North American showed the most phenotypic similarity with the wild *H. annuus* from Argentine.

3.4. The presence of traits corresponding to domesticated sunflower was present in wild populations from Argentina and USA.

3.5. The great phenotypic biodiversity found in wild *H. annuus* from Argentina did not reflect founder effects.

3.6. The lack of canonical correlation between climate and phenotype in the Argentine populations provides evidence suggesting that the adaptation process is still ongoing.

3.7. All accessions from Argentina were different from those found in the USA and could represent new combinations of the same traits present in native populations.

4. It was impossible to conclude about the existence of desirable new traits useful for sunflower improvement in the wild *H. annuus*.

4.1. The oil content and the fatty acid composition of nine populations from Argentina did not show values beyond the range measured in the populations from the USA.

4.2. The oil content and the fatty acid composition of nine populations from Argentina did not reach the extreme values reported for the improved mutant lines.

4.3. Some wild *H. annuus* accessions demonstrated traits of agronomic interest such as virus resistance and male-sterile plants.

5. The currently available GM traits for sunflower could be useful to overcome crop limitations, but they have ecological and market consequences.

5.1. The GM sunflower traits currently available could be successful used to overcome some of the crop restrictions such as weeds, diseases and pest control.

5.2. The main agro-ecological concern of GM sunflowers is the existence of gene flow with wild relatives, mainly, the capacity to develop invasive populations.

5.3 There are biotechnological proposals to minimize the possible impact of the gene flow, such as transgene insertion in chloroplasts; however, none are absolutely safe.

5.4. The main restriction to the continuity of the development of GM sunflower is market acceptance. In the most selective markets, where sunflower oil is valued for its healthy properties, low acceptance is expected. Transgenic sunflower oil would be accepted by crushers; but, in this industry, the crop competes with others that have a more agro-ecological adaptation.

5.5. National associations from the USA and Argentina do not currently support the release of GM sunflower, thus sunflower will probably continue to be identified as a non GM product.

Photo 1: Dr. Gerald Seiler examines a specimen of *Helianthus petiolaris* Nutt. in a roadside of La Pampa province. February of 2007.

Photo 2: Large population of wild *Helianthus annuus* located at Juarez Celman, Cordoba province. February of 2007.

Photo 3: Typical *Helianthus petiolaris* population in the roadside of an unpaved road. Puán, Buenos Aires province, February of 2007.

Photo 4: Isolated plant of *Helianthus petiolaris* found near Villalonga, in Southern of Buenos Aires province. March of 2007.

Photo 5: Dr. Gerlad Seiler and the wild sunflower research group in the experimental field at Bahía Blanca. February of 2007.

From left to right: Lic. Agustina Gutierrez, Dra. Monica Poverene, Ing. Agr. Soledad Ureta, Dr. Seiler, the author and Ms. Sc. Alejandro Presotto.

Photo 6: Dra. Monica Poverene shows an intermediate specimen of annual *Helianthus* found in a sunflower crop near Catriló, La Pampa province. February of 2007.

Photo 7: Dr. Juan Antonio Martín Sanchez and the author with an *Helianthus annuus* plant with wild characteristics, found in the experimental field at Gimenells, Lleida province. May of 2007.

Photo 8: Herbaria specimen of wild *Helianthus annuus* collected in 1907 in Los Cocos, Córdoba province.

Photo 9: The author observes the head morphology of a wild *Helianthus annuus* plant in an extended population at Rio Cuarto, Cordoba province. February of 2006.

Photo 10: Volunteer sunflowers growing in the roadside of National Highway 51, near Cabildo at Buenos Aires Province. February of 2006.

Photo 11: Male-sterile volunteers plants found in the roadside of National Highway 51 near Cabildo, Buenos Aires province. February of 2006.

Photo 12: Student Alejandro Presotto and the author collecting heads of a small wild *Helianthus annuus* population at Rancul, La Pampa province. March of 2004.