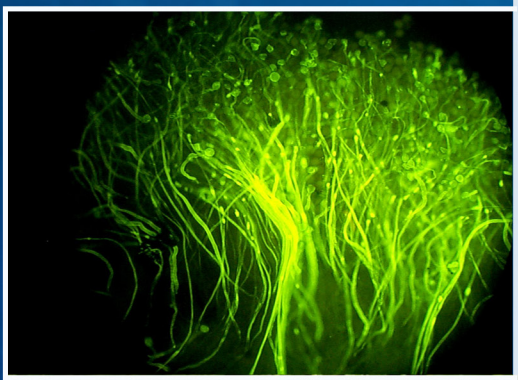
ISSN: 1852-6233

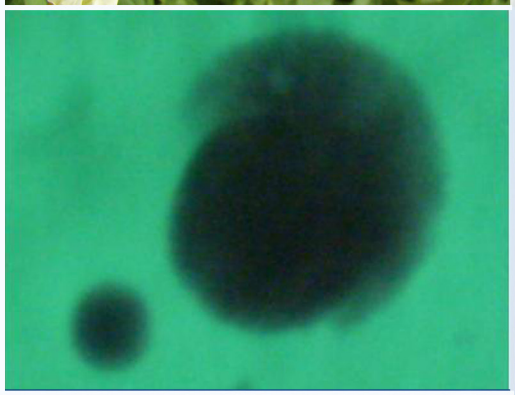
Vol. XXIII Issue 1 2012

Journal of Basic & Applied Genetics

(Formerly MENDELIANA)

JOURNAL OF THE ARGENTINE SOCIETY OF GENETICS
REVISTA DE LA SOCIEDAD ARGENTINA DE GENÉTICA





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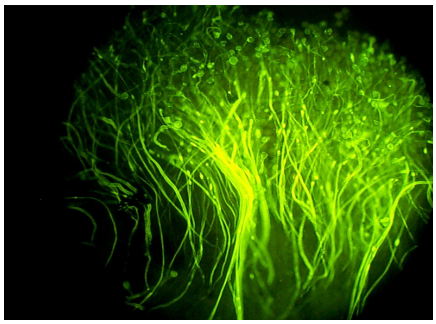
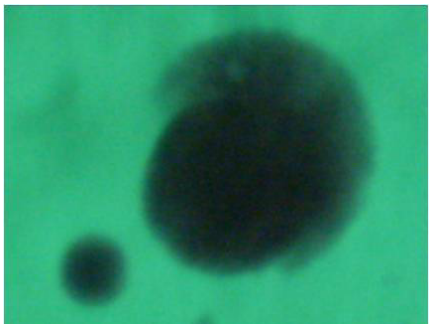


Incompatibilidad polen-pistilo en *S. chacoense Bitter*
Autor: Laboratorio de Genética, Unidad Integrada Balcarce

Solanum chacoense Bitter
Autor: Laboratorio de Genética, Unidad Integrada Balcarce



Micronúcleo observado en sangre periférica de individuo expuesto al consumo de aguas con alto contenido de nitratos.
Autor: M. N. Poli

JOURNAL OF
BASIC AND APPLIED GENETICS

ISSN: 1852-6233

GENOTOXIC EFFECT OF HIGH NITRATE WATER CONSUMPTION IN MEN AND WOMEN FROM NORTHERN MAR DEL PLATA, ARGENTINA

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ABSTRACT

Previous research has shown connections between the exposure to different agents and mutations in germinal and somatic cells. One of such agents, nitrate (NO_3), can be potentially genotoxic for individuals who drink high nitrate well water, and may cause a subsequent impact on their health and on the whole ecosystem. The aim of this research was to conduct genotoxicity assays in somatic cells from people living in the northern area of Mar del Plata city, Argentina. The purpose of these experiments was, in turn, to establish the possible relationships between mutagenic effects and high nitrate water consumption. To this end, different diagnostic tests were carried out to detect potential genetic and/or chromosomal alterations. A non-exposed population equal in age, gender and lifestyle formed the control group. Frequency of Sister Chromatid Exchange (SCE), Micronuclei (MN) and Chromosomic Aberrations (CAs) were determined. Furthermore, Cell Proliferation Kinectis was established through Replication Index (RI). Both a significant increase in MN frequency (Kruskal-Wallis H = 23.79, degree of freedom = 1, $p < 0.001$), and CAs presence (chromosome 9 mosaicism, ring chromosomes, fractures and chromosomal fragments) were observed in the exposed individuals compared with the control group. This genetic damage could be related to the exposure to high nitrate water, thus representing a potential risk to the health of the individuals concerned. However, it does not yet seem to be possible to conclude that this is the only reason that contributes to the mutagenic effects observed.

Key words: mutagenic agents, nitrate, chromosomal aberrations, genotoxicity, micronuclei.

RESUMEN

Se ha demostrado que hay relación entre la exposición a diferentes tipos de agentes y mutaciones en células somáticas o germinales. Uno de estos agentes, el nitrato (NO_3), puede ser potencialmente genotóxico en individuos que, por carecer de servicios de agua de red, consumen agua de pozo con alto contenido de este compuesto, lo cual, a su vez, puede tener impacto en su salud. El objetivo de este trabajo fue realizar ensayos de genotoxicidad en células somáticas de personas que viven en barrios de la zona norte de la ciudad de Mar del Plata, Argentina, con el fin de determinar si existe efecto mutagénico debido al consumo de agua con alto contenido de nitrato. Se realizaron pruebas diagnósticas para detectar posibles alteraciones genéticas y/o cromosómicas. Como control se utilizó una población no expuesta. Se determinó la frecuencia de Intercambio de Cromátides Hermanas (ICH), Micronúcleos (MN), el Índice de Replicación (IR) y la presencia de Aberraciones Cromosómicas (AC). Los resultados mostraron un incremento significativo de la frecuencia de MN (H de Kruskal-Wallis= 23.79, grados de libertad= 1, $p< 0.001$) y la presencia de AC (mosaicismo del cromosoma 9, anillos, fracturas y fragmentos cromosómicos) en las personas expuestas, lo cual evidencia daño del material genético. Este daño genético estaría relacionado con la exposición a aguas con alto contenido de nitratos, constituyendo un riesgo potencial para la salud de las personas expuestas. Sin embargo, no es posible aún inferir que esta sea la única causa que contribuye a los efectos mutagénicos observados.

Palabras clave: agentes mutagénicos, nitrato, aberraciones cromosómicas, genotoxicidad, micronúcleos.

INTRODUCTION

All along their life, organisms are exposed to physical, chemical or biological mutagens. In human beings, it has been observed that both the exposure to different kinds of agents and genetic alterations in germinal or somatic cells are related to congenital malformation development, sterility, autoimmune or degenerative diseases and cancer (Seoane and Dulout, 1999; Carballo *et al.*, 2001).

Nitrate intake above standard levels (45 mg/l or 45 ppm, according to Argentine Food Code, Fan *et al*., 1987) has been related to an increase observed in cases of gastric cancer (Bruning-Fann and Kaneene, 1993) and esophagus and stomach cancer (Anaya Pajuelo *et al*., 1999). The use of fertilizers mainly containing potassium and sodium nitrate produces groundwater aquifer pollution by nitrates. The use of this polluted groundwater as consumption water is therefore the principal means of nitrate exposure. On the other hand, and because nitrate is used as a preservative, it can enter the body through vegetable or meat product intake. Nitrate acts as a nitric oxide (NO) donor, a potentially genotoxic molecule. Chronic use of high nitrate concentrations in water may produce either cytogenetic effects, such as chromosome break (Tsezou *et al*., 1996) or micronucleated lymphocyte frequency increase (Andreassi *et al.*, 2001). Nitrates are quickly absorbed in the body at the gastrointestinal tract level. Microbial action produced both in the environment and in the human body reduces nitrates to nitrites (Eliano *et al*., 1995). Nitrites, in turn, combine with myoglobin to produce methemoglobin or secondary amides, the latter generating nitrosamines. Because methemoglobin cannot carry oxygen to tissues, blood nitrate excess may lead to methemoglobinemia with serious toxic effects or even death in children (Walton, 1951; Sadeq *et al*., 2008). Nitrosamines can methylate DNA and alter its sequence, thus increasing abnormal or neoplasic cell production. Carcinogenic mechanism is more related to the efficacy with which the excision mechanism (elimination) of the nitrogenous bases alkylated by N-nitrous compound works than to the alkylation level itself in a specific location (Loera Gallardo, 1985). Nitrate carcinogenic and teratogenic effect has been observed in animals (Tapia, 2000; Manassaram *et al.*, 2005) whereas in human beings the magnitude of its potential risk has not been fully elucidated to date.

In view of the above, it can be concluded that in the last decades human health has been negatively affected by agro-industrial activity, which is responsible for the exposure to chemical products and genotoxic agents. It thus becomes necessary to carry out genotoxicity assays on a regular basis in order to determine genetic damage level in a given population. In line with this, individuals in risk of suffering alterations that may modify their genetic stability should be monitored (Zalacain *et al*., 2005). The purpose of the present study was therefore to carry out a minimal test set of genotoxicity assays including Sister Chromatid Exchange (SCE), Micronuclei (MN), Replication Index (RI) and presence of Chromosomal Aberrations (CAs) to somatic cells from individuals living in the northern area of Mar del Plata city, Argentina, in order to determine the potential mutagenic effect derived from consumption of high nitrate content water.

MATERIALS AND METHODS

This research was carried out in Alto Camet, Las Dalias and Parque Peña, three neighborhoods located in northern Mar del Plata city, Buenos Aires province, Argentina, which cover a surface of approximately 750 hectares, and with a total population of approximately 15,000 people who consume high nitrate water. Therefore, samples containing up to 137 ppm NO_3 were collected from this water. These samples considerably exceed tolerable standards (45 ppm) (Lasta *et al.*, 2003; Manrique, 2007).

Before sample collection, participants were informed on the steps to follow in the present study as determined by the informed consent approved by the Bioethics Committee of the *Asociación de Genética Humana* (AGHU) in Mar del Plata, and were invited to participate in the study. Samples of peripheral blood from 19 randomly chosen individuals were taken: 7 males and 12 females with an average age of 52.8 ± 12.04 (exposed group). The high nitrate water exposed group was formed of these individuals who proved to keep a record of at least one-year-long residence in the above mentioned neighborhoods. The control group, in turn, was formed of 19 individuals (7 males and 12 females with an average age of 50.4 ± 11.66) who

used water with the allowed level of nitrates. Both groups were gender- and age-matched.

Frequency of SCE, MN and CAs in the exposed and control groups was analyzed in order to study the potential genotoxic damage induced by exposure to polluted water. Peripheral blood samples (4 ml.) were taken using disposable syringes with norheparin (100 U). Fluorescence-plus-Giemsa technique (FPG) was applied for SCE (Perry and Wolf, 1975). This technique consists in differential staining, which, after three *in vitro* cell division cycles, reveals break (damage) and recombination (repair) points. Cells were cultured in a complete medium (RPMI medium 1640 (GIBCO) with 10 mg/ml phytohemagglutinin (PAA) and fetal bovine serum (PAA), supplemented with a 10 mg/ml final concentration bromodeoxyuridine (BrdU) (SIGMA) during 72 hours at 37º C. Cells were then harvested through the addition of colchicines (SIGMA), subsequently incubated in 0.075M KCl hypotonic solution and fixed in Carnoy solution (methanol/ acetic acid 3:1). Chromatid differential staining was performed with Hoechst 33258 (SIGMA) coloring and 2% Giemsa (BIOPUR) staining solution in sheets exposed to UV light for 4 hours. Frequency of SCE and CAs was determined and CPK (cell proliferation kinetics) was established through RI.

A 50 cell-count was performed per individual in the metaphase stage of second mitotic division so as to determine the number of SCE. To study CAs, 100 cells in the metaphase stage of the first mitotic division were analyzed per culture and individual. The amount of AC was estimated for each cell analyzed (Gadano *et al*., 1998). Both the extra chromosomes and the chromosomal fragments found were identified via G-band technique (Seabright, 1971; Barch *et al.* 1997). In order to estimate RI, 100 consecutive cells in the metaphase stage were analyzed per culture and individual. In addition, the proportion corresponding to cells in first (M_1), second (M_2) or third (M_3) mitotic division was counted, RI being determined as RI = ($1M_1$ + $2M_2$ + $3M_3$)/100.

Micronuclei were studied without the addition of cytochalasin B whose reagent was originally used for isolated lymphocytes, and the mayority of laboratories subsequently used it for whole blood cultures (Fenech *et al*., 1999). This is so because cytochalasin improves the sensitivity method by blocking cytokinesis. However, the extent to which mutagen (cytochalasin B) exposure leads to MN formation already *in vivo* or to MN formation *ex vivo* during cell culture as a consequence of persisting DNA damage remains unknown (Speit *et al*., 2011). Cytochalasin B application also has the following drawbacks: 1) it may interfere after MN induction by chemical tests, i.e., it behaves similarly to spindle poison; 2) it may interfere with other cytokinesis inhibitors; and 3) cytochalasin B cytotoxicity varies among cell lines and, sometimes, even among subtypes of the same cell line (Fenech and Morley, 1985a,b; Fenech, 1993; Kirsch-Volders *et al*., 2000). Furthermore, MN already induced *in vivo* can be determined by scoring MN in mononuclear lymphocytes 24 hours after lymphocyte culture initiation (i.e. in lymphocytes not yet divided) (Speit *et al*., 2011). In the present study, the following criteria for MN identification were followed: 1) MN were not refractory; 2) their color was the same or lighter than that of the nucleus from which they had originated; 3) their diameter ranged from 1/16 to 1/3 of the average diameter of the cell nucleus from which they could have originated; 4) they did not overlap with their main nucleus; and 5) they were located at a maximum distance of 3 to 4 nuclear diameters from the cell from which they had originated. Blood samples were cultured in a complete medium, as the one specified above, during 24 h at 37º C in the presence of 5% CO_2. Samples were harvested in the absence of colchicine, incubated in hypotonic solution and subsequently fixed in solution. Staining was made with 3% Giemsa in 0.6M to pH 7 Sorensen´s buffer. In addition, MN percentage in 1,000 mononucleated cells analyzed per individual was determined. Non-parametrical Kruskal-Wallis test was applied for the statistical comparison of the two groups (control vs. exposed) for SCE, RI and MN. The test was performed with SPSS software (10.0 version).

RESULTS AND DISCUSSION

Exposure to high nitrate water in northern Mar del Plata showed evidence of genetic damage in the group exposed to this chemical compound, with a statistically significant increase in MN and CAs

(cell mosaicism, chromosomal fragments and ring chromosomes). The present study revealed that all control individuals yielded standard MN values (from 1 to 3 MN/1,000 mononuclear cells, according to the Human Micronucleus Project, Fenech *et al.*, 1999), whereas 17 out of the 19 exposed individuals showed values above the normal range. The mean number of MN in the exposed individuals (6.84 ± 1.92) increased significantly with respect to that of the control group (2.58 ± 1.17) (Kruskal-Wallis H = 23.79, degree of freedom = 1, p = 0.001) (Fig. 1). The analysis of MN by gender revealed a statistically significant increase in the exposed individuals of both genders, females accounting for 66.26% of such increment (Kruskal-Wallis H = 17.4175, degree of freedom = 1; p=0.0000) and males accounting for 54.20% (Kruskal-Wallis H = 5.2662, degree of freedom = 1; p = 0.0217). These values should be taken into account because they are associated to chromosomal loss or breakup, both having an impact on human health and consequences on the individual and his descendants (teratogenesis to perinatal or infant death, or even neoplasias) (Mudry and Carballo, 2006). In this respect, the use of Fluorescent *in situ* Hybridization (FISH) could be useful to differentiate if the MN defined by the monitored genotoxic action corresponds to a chromosome fragment (clastogenic effect) or to a whole chromosome (aneugenic effect).

Chromosomal aberrations were detected in individuals from the exposed group. In three cases, CAs corresponded to mosaicism of chromosome 9. One individual showed 47,XX,+9p(2)/47,XX,+9(4)/46,XX(94) karyotype. The other two exhibited 47,XX,+9(2)/46,XX(98) karyotype and 47,XX,+9(1)/46,XX(99), respectively. The presence of an extra chromosome 9 was determined by G banding technique. Likewise, a fourth individual revealed chromosome rings of different sizes (3 large and 1 small) 47,XY,+r(?)(4)/46,XY(96). The chromosomes to which these rings corresponded could not be determined. In other cases as well as in the individuals exposed, chromosome fractures and fragments were observed. However, no dicentric chromosomes, chromatid breaks or multirradial chromosomes were observed in the exposed individuals analyzed (Table 1). It could thus be inferred that there is a likely relationship between nitrate-contaminated water intake and the above-mentioned anomalies. In addition, such relationship could be related to a possible mutagen activity of nitrites/nitrates. The frequency of chromosome 9 trisomy in pure line (all cells) is very low in human beings who have a low survival rate at birth as a result of the multiple malformations and lethal effects caused by this genetic disorder (Inostroza *et al*., 2002; Rodríguez, 2005). Individuals with chromosome 9 mosaicism in the exposed group had no malformations. The low mosaicism of chromosome 9 observed in the present study could be acquired by the exposure to mutagen agents.

Replication Index decreased in the exposed individuals (1.19 ± 0.3) with respect to the control group (1.27 ± 0.22). However, these differences were not statistically significant (Kruskal-Wallis H = 3.2465, degree of freedom = 1; p = 0.0716) (Fig. 2). The major difference in RI was observed in males (Kruskal-Wallis H = 0.268, degree of freedom = 1; p = 0.605) with respect to females who presented no statistically significant differences (Kruskal-Wallis H = 2.204, degree of freedom = 1; p = 0.138).

Frequency of SCE in the exposed individuals group (4.48 ± 2.98) was higher than that in the individuals belonging to the control group (3.71 ± 1.62), however, SCE frequency showed no significant statistical differences (Kruskal-Wallis H = 3.1921, degree of freedom = 1; p = 0.074) (Fig. 3). The analysis of SCE frequency for both genders revealed that the exposed females outnumbered the non-exposed ones by 30%. As far as males are concerned, the increase was just 7%. Still, the statistical analysis showed no significant differences between SCE frequencies based on gender neither for females (Kruskal-Wallis H = 2.1182, degree of freedom = 1; p = 0.1456) nor for males (Kruskal-Wallis H = 1.0714, degree of freedom = 1; p=0.3006) for this result.

In the present study, the number of SCE by metaphase ranged from 3 to 5 in the control group, and from 5 to 8 in the exposed group. Even though the values are within the basal range and are not statistically different, there is a trend towards a SCE frequency increase in the individuals of the exposed group. Previous research has reported a close relationship between SCE high frequencies and cancer predisposition (Spitz and Bondy, 1993; Cortés Gutiérrez *et al*., 2000). Thus, although this increase is not statistically significant in the SCE

frequencies of exposed individuals, it should not be disregarded and extensive research efforts should be devoted in the near future including a larger number of participants and other variables so as to rule out or identify a possible correlation between high nitrate water exposure and carcinogenesis.

Several factors could affect the parameters analyzed and should be taken into account. It was shown that MN frequency correlated with age in both genders. It was also observed to be affected by dietary factors (Fenech *et al.,* 1999) and occupational exposure (Kirsch-Volders *et al.*, 2000). Further studies have suggested that the average frequency of micronucleus cells does not differ between smokers and non-smokers and between males and females (Sarto *et al.,* 1990). In other words, findings are, in general, contradictory and therefore further studies should be carried out. Moreover, in spite of the fact that age does not play a significant role in cytogenetic manifestations (Pérez-Herrera *et al.*, 1999; Ceballos-Quintal *et al.*, 2002) and although there seem to be no relevant gender-related differences in SCE (Pérez-Herrera *et al*., 1999), an early study contradicted these findings (Verma and Babu, 1995). Other variables related to lifestyle which could be considered genotoxic are tobacco use and alcohol ingestion as they are known to affect chromosomal stability. Alcohol seems not to induce SCE formation although it could be responsible for structural CAs (López *et al*., 2001). In the present study, although individuals from both groups were asked about tobacco use, no obvious conclusions could be drawn.

Results from the present study are consistent with those of previous research reporting that high nitrate levels in water could induce genotoxic effects (Tsezou *et al.,* 1996) and cytotoxicity as well as a slowing down of the cell cycle (Andreassi *et al.*, 2001). A pattern of genetic damage (MN increase and trisomies) similar to the one observed by high nitrate water use was found in a northern Argentine population exposed to arsenic-contaminated water (Dulout *et al*., 1996). On account of this, both compounds are likely to cause similar deleterious effects on human health.

Taken together, results from the present study lead us to infer a relationship between high nitrate water use and genetic damage, the former being therefore a potential threat to the health of those living in Mar del Plata and drinking nitrate water as a part of their daily diet. Still, it cannot yet be concluded that high nitrate concentration in water is the only cause of the mutagenic effects observed although this research tried to establish nitrate concentrations as the only difference between the exposed and control groups.

Furthermore, taking into account the direct action human beings exert on the environment (use of fertilizers and compost, among others), their lifestyles (cured meat consumption and nitrates use as food additive), and the high nitrate levels in water, soil and food, they all contribute to creating a continuous source of undesired contamination. As a result of the increasing number of human beings exposed to nitrate/nitrite polluted water, it becomes necessary to carry out more thorough studies at local level. Such studies will greatly contribute to either establishing or discarding causal relationships among these substances and their effects at a molecular and cellular level, and to determining their impact on individual and population health within Argentine communities. Thanks to the results from the present study, regular tap water supply was secured to one of the affected communities analyzed.

ACKNOWLEDGEMENTS

Authors are grateful to Dr. Clotilde Úbeda and Dr. Andrea Perinetti from the *Departamento de Investigación del Instituto Nacional de Epidemiología "Dr Juan H. Jara"* of Mar del Plata, to Dr. Guillermo Manrique and Dr. Luciano Luppi from the *Universidad Nacional de Mar del Plata*, to people working at the *Programa de Autoproducción de Alimentos de la Oficina del INTA* from Mar del Plata and to Professor Marta D. Mudry Ph. D., Director GIBE- Principal Investigator of CONICET and Associated Professor at *Universidad de Buenos Aires* (UBA), Evolutive Biology Research Group (GIBE). M.N.P. is a fellow of the CONICET and P.F.I. is a researcher of the CONICET.

Individual	Numerical CAs	Chromosome Break	Chromosomal Fragment	Chromosome Rings	Gaps
1	47,XX,+9p(2)/47,XX,+9(4)	-	-	-	-
2	47,XX,+9(1)	-	1 corresponding to chromosome 13	-	-
3	47,XX,+9(2)	1	-	-	-
4	-	-	-	4 (3 small and 1 large)*	-
5	-	-	-	-	2
6	-	-	-	-	3
7	-	-	-	-	1
8	-	-	2	-	-

Table 1. Types and amounts of CAs in individuals exposed to high nitrate water

Number of cells analyzed per individual = 100. No dicentric chromosomes, chromatid breaks or multirradial chromosomes were observed in the exposed individuals analyzed. Individuals 9 to 19 were not included in the table because they evidenced no chromosomal abnormalities.

*The chromosomes to which these rings corresponded could not be determined.

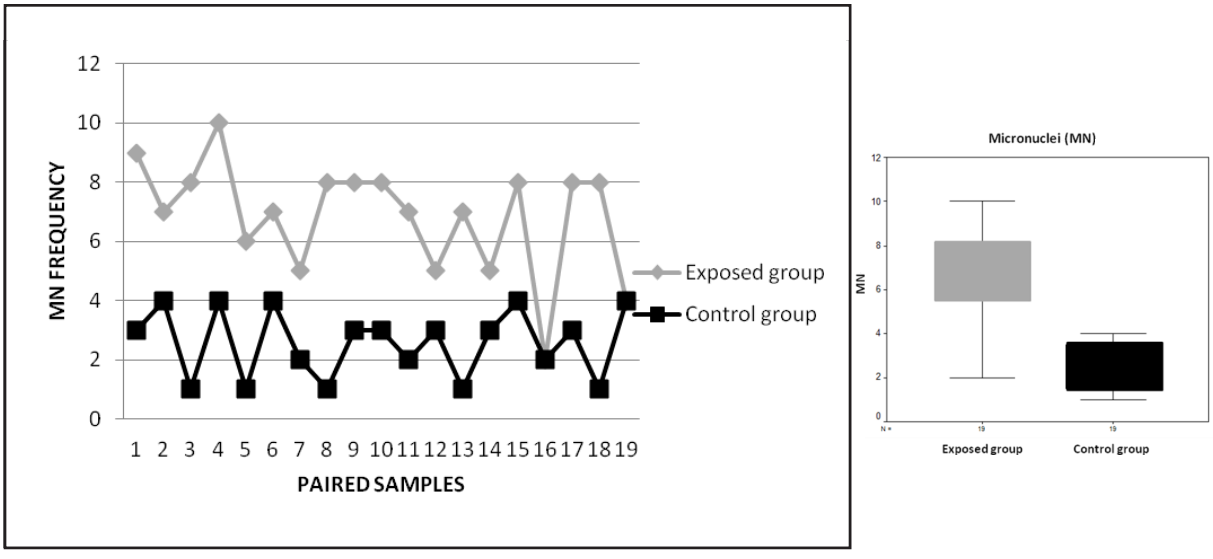

Figure 1. Frequency of MN in males and females: control and exposed groups in relation to high nitrate water consumption in the northern area of Mar del Plata city.

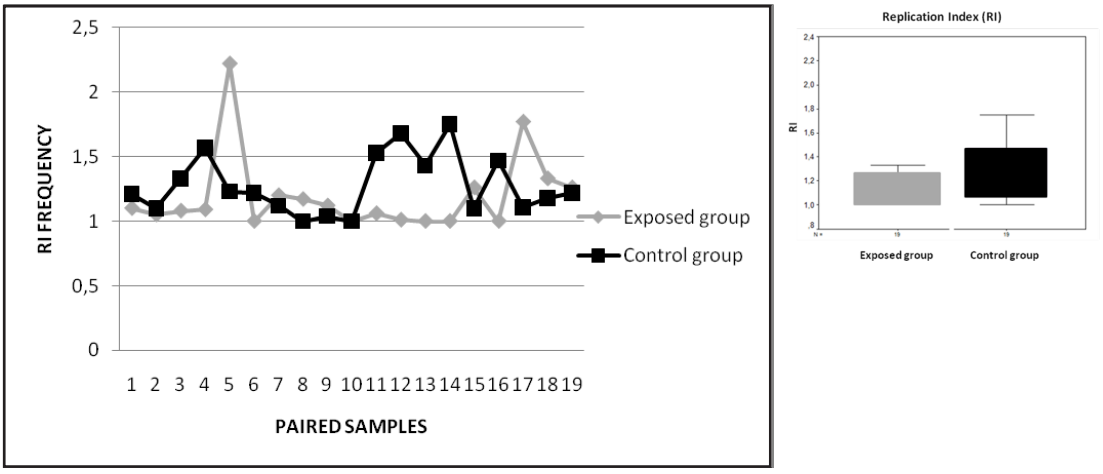

Figure 2. Frequency of RI in males and females: control and exposed groups in relation to high nitrate water consumption in the northern area of Mar del Plata city.

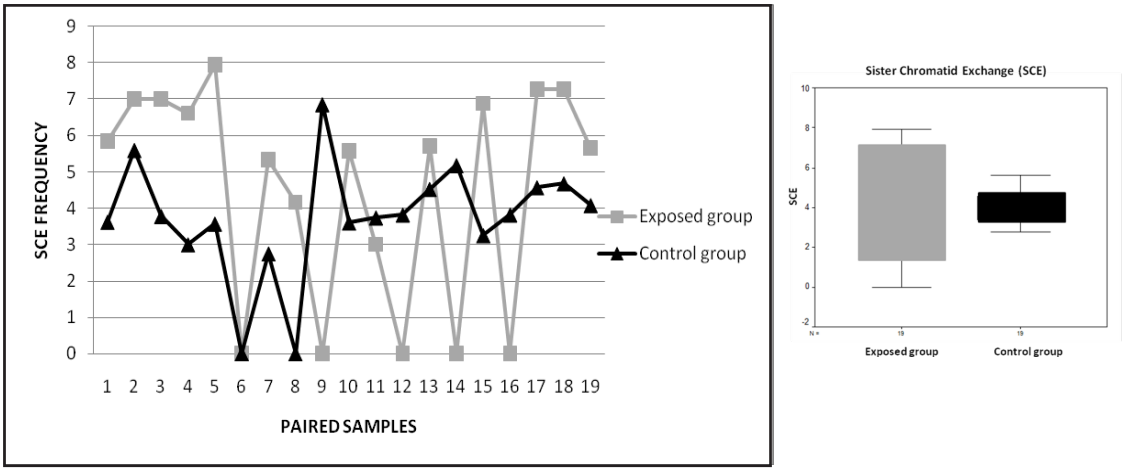

Figure 3. Frequency of SCE in males and females: control and exposed groups in relation to high nitrate water consumption in the northern area of Mar del Plata city.

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JOURNAL OF
BASIC AND APPLIED GENETICS

ISSN: 1852-6233

DISECCIÓN DE LA CORRELACIÓN EN VARIABLES QUE MIDEN LA APTITUD PANADERA DEL TRIGO Y MODELOS PREDICTORES DE LA CALIDAD INDUSTRIAL

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ABSTRACT

Baking quality is one out of four objectives in a breeding program for wheat bread. This is a quantitative variable governed by many genes and strongly influenced by environmental conditions. Several authors have examined this complex variable through different statistical methods. In the present study, path analysis was used to build quality models through the dissection of the correlation between two variables and to compare them in four Argentine wheat sub-regions. The model built with 21 variables yielded high coefficients of determination (over 95%) in all sub-regions, except V South subregion (6.47%). To build Model II, those variables showing either high correlation or scarce contribution to Model I were removed so that the coefficient of determination (R^2) markedly increased in V South sub-region (from 6.47 to 30%). In the remaining sub-regions this value decreased as follows: II North > II South > IV. This wide variation among R^2 values was attributed to the variables that formed each model and to the high environmental influence over them. A high correlation among the same variables was observed in the four sub-regions. As for the contribution of each variable to Model II depending on each sub-region, common variables such as Wet Gluten, Bakery Strength, Index Gluten, Dough Equilibrium and Flour Ash were observed. Other variables were typical of some of the sub-regions. Dissection of each sub-region explained why some variables had low correlations with Loaf Volume while others scarcely contributed to the model. The variables that built Model II in each of the sub-regions could be considered as participants in a breeding index.

Key words: path analysis, bread wheat, industrial quality

RESUMEN

La calidad panadera es uno de los cuatro objetivos en un plan de mejoramiento de trigo pan. Al ser una variable cuantitativa está regida por muchos genes y afectada fuertemente por las condiciones ambientales. Estudios previos han analizado esta compleja variable a través de diferentes métodos estadísticos. En el presente trabajo se aplicó el análisis del coeficiente de sendero para construir modelos de calidad a través de la disección de la correlación entre dos variables y comparar los mismos en cuatro subregiones trigueras argentinas. El Modelo I planteado con 21 variables dio valores altos en los coeficientes de determinación (superiores a 95%) en todas las subregiones, excepto la subregión V Sur (6,47%). Al plantear el Modelo II, eliminando las variables de alta correlación y de poco aporte al Modelo I, el coeficiente de determinación (R^2) aumentó considerablemente en la subregión V Sur (de 6,47 a 30%). En tanto que en las restantes subregiones este valor disminuyó de la siguiente manera: II Norte > II Sur > IV. La amplia variación encontrada entre los valores de R^2 se atribuye a las variables que conforman cada modelo y a la elevada influencia ambiental sobre las variables. Ha sido coincidente con esto la alta correlación en las mismas variables en las cuatro subregiones. Con respecto al aporte de cada variable que conformó el Modelo II según la subregión considerada, se observaron variables en común tales como Gluten Húmedo, Fuerza Panadera, Gluten Index, Equilibrio de la Masa y Ceniza en Harina. Otras variables fueron típicas de alguna de las subregiones. La disección de cada subregión explicó por qué algunas variables tenían baja correlación con Volumen de Pan y por qué otras contribuyeron muy poco a la variable dependiente. Las variables que conformaron el Modelo II en cada una de las subregiones podrían tomarse como partícipes en la formación de un índice de selección.

Palabras clave: coeficiente de sendero, subregiones trigueras, índice de calidad

INTRODUCCIÓN

Los principales objetivos en la obtención de nuevas variedades de trigo pan, *Triticum aestivum* L, se centran en altos rendimientos en grano, óptima calidad panadera, buena sanidad y adaptación a varios ambientes (Evans, 1998). La calidad panadera es uno de los aspectos de mayor interés tanto para el fitomejorador como para los diferentes integrantes de la cadena comercial. Esta es una variable compleja, de tipo cuantitativa, influida por el ambiente y dependiente del genotipo y de la interacción entre ambas (Poehlman y Sleper, 2003). Varios autores han analizado la calidad panadera tomando diferentes parámetros que la definen y a través de diversos métodos analíticos determinaron la aptitud industrial de los cultivares de trigo (Miranda y Salomón, 2001; Salomón y Miranda, 2008; Molfese y Seghezzo, 2010; Cuniberti *et al.*, 2011).

Diferentes parámetros agronómicos y químicos, tales como la proteína en grano, la fuerza panadera, la estabilidad farinográfica, el tiempo de desarrollo de la masa, el aflojamiento de la masa y el volumen de pan influyen directamente o indirectamente sobre la calidad panadera (Wrigley y Bietz, 1988). En la mejora de la calidad es importante entender claramente las relaciones entre cada uno de estos parámetros y la calidad. Debido a que son muchos los factores involucrados, es difícil para el mejorador elegir por cuál de ellos seleccionar los materiales.

Un programa de mejoramiento de la calidad panadera en trigo implica el uso de ciertas estrategias, entre las que se destaca el estudio de sus componentes (Poehlman y Sleper, 1996), es decir, de las variables agronómicas y químicas cuya interacción manifestarán la calidad final requerida. Estas interacciones pueden dar valores no significativos del verdadero efecto individual de cada componente sobre el carácter en estudio (calidad). Los principales responsables de las variaciones en los valores de correlación responden tanto a efectos genéticos como a la interacción entre variables.

Un parámetro que calcula la relación entre variables es el coeficiente de correlación, el cual es útil al momento de cuantificar la magnitud y la dirección de las variables a pesar de que no indica el efecto directo e indirecto que ejerce cada variable sobre la calidad *per se*. Para solucionar este problema, se aplicó el análisis de sendero (*path analysis*, Wright, 1934; Li, 1956; Dewey y Lu, 1959; Cruz y Regazzi, 1997), el cual permite descomponer la correlación entre un componente (X) y el producto final (Y) en un efecto "directo" (P) de X sobre Y y en efectos "indirectos" de X sobre Y, los que se hacen efectivos por vía de la relación de X con otros componentes de Y (X_1, X_2, etc.). El efecto "directo" puede interpretarse como una correlación parcial de X_1 e Y, una vez excluidos los efectos de X_2 y X_3. La estimación de los efectos directos e indirectos de los componentes sobre el producto final se efectúa a partir de la resolución de un sistema de ecuaciones que tendrá tantas incógnitas como componentes investigados.

El objetivo del análisis de sendero (Niles, 1922) es brindar posibles explicaciones causales de las correlaciones observadas entre una variable respuesta (dependiente) y una serie de variables predictoras (independientes). En el análisis de sendero se pretende construir modelos de causa-efecto entre las variables a través de la disección de la correlación entre dos variables como la suma de dos tipos de efectos; estos son efectos directos de una variable sobre otra (senderos simples) y efectos indirectos de una variable sobre otra, a través de una o más variables independientes (senderos compuestos) (Balzarini *et al.*, 2008). En nuestro país varios autores han aplicado esta metodología de análisis en otras especies con otras variables cuantitativas, por ejemplo, tricepiro y triticale (Paccapelo *et al*., 2004), cebadilla criolla (Abbott *et al*., 2007), agropiro alargado (Abbott *et al*., 2009), trigo doble propósito (Morant *et al.*, 2009).

El área de producción triguera argentina está dividida en siete sub-regiones, división que se ha basado en variables agrobioecológicas (Zarrilli, 1997). El comportamiento varietal es diferencial tanto en rendimiento (Calzolari *et al*, 2012) como en calidad (Calzolari y Polidoro, 2004).

El objetivo de este trabajo fue encontrar un modelo que contenga las variables que modifican la aptitud panadera en trigo pan con la finalidad de predecir de esta manera los efectos directos e indirectos de cada una de ellas en la calidad panadera y comprobar si las mismas se mantienen en diferentes subregiones trigueras del país. Las variables que formen el modelo serán las que conformen un índice

Subreg.	GH	GI	TD	ESTAB	AFLOJ	L	W	P/L	CNIZH	RTOH	PMIL	PROTGR	CNIZGR	PH
Sur	0,428	0,381		0,347			0,338	0,229	-0,471	0,061		0,548		-0,09
Norte	0,405	0,314	0,261		-0,238		0,521	0,102	0,041	-0,21	0,254	0,547		
	0,441	0,112	0,04	0,00023			0,251	-0,3			0,034	0,535	-0,053	
Sur	0,24	0,1	0,27	0,18	-0,27	0,06	0,29	0,03	0,06	-0,05	-0,26	0,31	0,01	

Tabla 1. Coeficientes de Correlación entre Volumen de Pan y las variables correspondientes al Modelo II en cada una de las subregiones analizadas. Significancia al 5% (en negrita) y al 1%: (subrayada)

Gluten Húmedo (GH), Gluten Index (GI), Tiempo de Desarrollo (TD), Estabilidad Farinográfica (ESTAB), Aflojamiento de la Masa (AFLOJ), Extensibilidad (L), Fuerza Panadera (W), Relación de Equilibrio P/L (P/L), Cenizas en Harina (CNIZH), Rendimiento en Harina (RTOH), Peso de Mil Granos (PMIL), Ceniza en Grano (CNIZGR), Peso Hectolítrico (PH), Volumen de Pan (VP).

Subreg.	GH	GI	TD	ESTAB	AFLOJ	L	W	P/L	CNIZH	RTOH	PMIL	PROTGR	CNIZGR	PH
II Sur	29,16	30,25		12,04			1,00	0,09	17,64	0,01		0,002		0,81
II Norte	22,18	16,89	0,48		0,56		8,29	0,49	11,22	1,64	0,000003	0,21		
IV	2,25	5,29	0,36	0,01			0,01	3,24			3,24	18,49	0,01	
V Sur	17,64	2,56	2,56	1,21	6,25	20,25	9,00	13,69	0,64	1,21	1,00	0,001	1	

Tabla 2. Efectos directos del Modelo II para cada subregión analizada. Contribución de cada variable al modelo (Mariotti, 1986)

Gluten Húmedo (GH), Gluten Index (GI), Tiempo de Desarrollo (TD), Estabilidad Farinográfica (ESTAB), Aflojamiento de la Masa (AFLOJ), Extensibilidad (L), Fuerza Panadera (W), Relación de Equilibrio P/L (P/L), Cenizas en Harina (CNIZH), Rendimiento en Harina (RTOH), Proteína en Grano (PROTGR), Peso de Mil Granos (PMIL), Ceniza en Grano (CNIZGR), Peso Hectolítrico (PH).

PROTGR	Efecto Directo	-0,0044
Efecto Indirecto	**Vía**	
	RTOH	0,0024
	GH	0,35
	GI	0,12
	ESTAB	-0,0022
	W	0,06
	P/L	-0,0022
	CNIZH	0,01
	PH	0,01
r con VP		0,548

GI	Efecto Directo	0,55
Efecto Indirecto	**Vía**	
	PROTGR	-0,00097
	RTOH	0,00035
	GH	-0,20
	ESTAB	-0,01
	W	0,05
	P/L	-0,01
	CNIZH	0,01
	PH	-0,003
r con VP		0,380

Tabla 3. Disección de los efectos directos del componente Proteína en Grano y Gluten Index en la subregión II Sur

Proteína en Grano (PROTGR), Gluten Húmedo (GH), Gluten Index (GI), Estabilidad Farinográfica (ESTAB), Fuerza Panadera (W), Relación de Equilibrio P/L (P/L), Cenizas en Harina (CNIZH), Rendimiento en Harina (RTOH), Volumen de Pan (VP). r Coeficiente de Correlación.

de selección a aplicar en materiales selectos de un programa de mejoramiento genético para calificar genotipos de trigo por calidad industrial según su comportamiento en diferentes ambientes.

MATERIALES Y MÉTODOS

Los datos analizados fueron extraídos de la base proporcionada por la Red de Ensayos Territoriales de Trigo de la campaña 2008 correspondientes a las subregiones II Sur (Chacabuco, Bellocq, Plá, 9 de Julio, General Villegas), II Norte (Maciel, Marcos Juárez, Pergamino, Rafaela), IV (La Dulce, Tandil, Miramar, Barrow, Balcarce) y V Sur (Anguil, Bordenave). Cada subregión incluyó localidades representativas de la producción de trigo.

La siembra de las parcelas experimentales se realizó en bloques completos al azar, con tres repeticiones, aplicándose el mismo diseño estadístico en todas las localidades. El análisis se realizó por subregión triguera, formando una muestra compuesta de cada localidad previa conjunción de las tres repeticiones del ensayo. Se conformaron así 71 muestras para la subregión V Sur, 69 para la subregión IV, 89 en la II Norte y 43 en la II Sur, en las mismas no estaban incluidos todos los genotipos y las valoraciones de calidad individual eran diferentes[1].

La variable Volumen de Pan (cm^3) (VP) fue tomada como dependiente. Se eligió esta variable debido a que es la que representa la prueba directa en pequeña escala de la aptitud de las harinas y reúne todas las propiedades del trigo pan apto para la industrialización (Trigo Argentino, 2012).

Las variables independientes analizadas fueron: Peso de Mil Granos (PMIL, g), Proteína en Harina (PROTH, %), Gluten Index (GI), Tiempo de Desarrollo (TD, min), Tenacidad (P, mm), Fuerza Panadera (W, Joule x 10^{-4}), Ceniza en Grano (CNIZGR), Peso Hectolítrico (PH, g/hl),

[1] Los lectores pueden consultar la lista completa de materiales evaluados en la página *web* de INASE

Gluten Húmedo (GH, %), *Falling Number* (FN, cm), Estabilidad Farinográfica (ESTAB, min), Extensibilidad (L, mm), Relación P/L, Cenizas en Harina (CNIZH), Proteína en Grano (PROTGR, %), Gluten Seco (GS, %), Humedad de la Harina (HDD), Aflojamiento de la Masa (AFLOJ, min), Índice de Hinchamiento (G), Rendimiento en Harina (RTOH) y Volumen Específico (VESP). Las correlaciones fenotípicas simples entre las variables y entre estas y la variable dependiente se calcularon utilizando el coeficiente de correlación de Pearson (r) como medida de la magnitud de la asociación lineal entre dos variables que no depende de las unidades de medida de las variables originales. El coeficiente de determinación (R^2) de la variable VP y el análisis de coeficiente de sendero (*path analysis*) y el resto de los cálculos se realizaron con el programa estadístico InfoStat (Di Rienzo *et al.*, 2011).

RESULTADOS

Subregión II Sur

Se planteó un Modelo I completo con todas las variables medidas. El coeficiente de determinación del mismo fue de 98,75%. El análisis de correlación simple mostró que las variables L vs G, GH vs GS, PROTGR vs PROTH, P/L vs P, L y G y VP vs VESP estaban altamente correlacionadas ($p<0,01$). Por lo tanto, las variables citadas en segundo término en la dupla se eliminaron de este modelo. En la correlación P/L vs P, L y G se decidió tomar la variable P/L ya que aporta más información que P y L por separado. El análisis de coeficiente de sendero planteado con 21 variables mostró que FN, TD, AFLOJ, CNIZGR y PMIL contribuyeron poco al modelo (0,0001 y 0,001%, resultados no mostrados).

Teniendo en cuenta este análisis, se planteó el Modelo II extrayendo las variables antes enumeradas, que incluyó solo 10 variables. Las relaciones causales con VP se pueden ver en la Tabla 1. Se observó una correlación altamente significativa y positiva entre VP y los componentes PROTGR, GH y GI, en tanto que fue negativa con CNIZH. Hubo correlación significativa y positiva con W y ESTAB. La Tabla 2 muestra los efectos directos de cada variable independiente con VP y el aporte de cada variable al Modelo II. Las variables que más aportaron fueron GH, GI, ESTAB y CNIZH.

En esta subregión, la disección de la correlación se aplicó a la variable PROTGR por su alta correlación con VP y falta de aporte al modelo elegido, y a la variable GI por ser la de máximo aporte al modelo. La alta asociación de VP con PRTOGR estuvo dada por un fuerte efecto de GH y GI por vía indirecta y en menor medida por W (Tabla 3). Este Modelo II tuvo un coeficiente de determinación del 68,08%.

Subregión II Norte

En esta subregión, al plantearse el Modelo I se encontraron las mismas asociaciones entre las variables, con el mismo grado de significancia ($p<0,001$) que en la anterior subregión en el modelo completo con 21 variables. El coeficiente de determinación del mismo fue de 97,94%. Las variables que evidenciaron poca contribución al Modelo I (menores o iguales a 0,01%, resultados no mostrados) fueron FN, ESTAB, PH y CENIZGR. En base a estos resultados, se construyó nuevamente el Modelo II, en esta oportunidad con 11 variables cuyas correlaciones se muestran en la Tabla 1.

Se observó una correlación altamente significativa y positiva entre VP y los componentes PROTGR, GH, GI y W. Solo se encontró una sola correlación significativa positiva entre VP, RTOH y TD y dos negativas, con AFLOJ y con PMIL. La Tabla 2 muestra los efectos directos de cada variable independiente con VP y el aporte de cada variable al Modelo II. Las variables que más aportaron en este modelo fueron GH, GI, W y CNIZH.

En esta subregión la disección de la correlación se aplicó a las variables PROTGR y W por su alta correlación con VP y falta de aporte al modelo elegido, y a la variable GI por ser la de máximo efecto real a VP (Tabla 4). La alta asociación de VP con PRTOGR estuvo dada por un fuerte efecto por vía indirecta de GH, RTOH y GI, en menor medida por W, en tanto que la asociación con W estuvo dada por vía indirecta por las variables GI, GH y CNIZH, en ese orden de importancia. En lo que respecta a GI, esta variable tuvo efectos indirectos positivos sobre VP a través de GH y W. Este Modelo II tuvo un R^2 de 95,89%.

PROTGR	**Efecto Directo**	**0,046**
Efecto Indirecto	**Vía**	
	RTOH	0,062
	GH	0,365
	GI	0,04
	TD	-0,03
	AFLOJ	-0,022
	W	0,159
	P/L	0,011
	CNIZH	-0,084
	PMIL	-0,000099
r con VP		0,547

W	**Efecto Directo**	**0,288**
Efecto Indirecto	**Vía**	
	PROTGR	0,026
	RTOH	0,034
	GH	0,147
	GI	0,207
	TD	-0,028
	AFLOJ	-0,035
	P/L	-0,012
	CNIZH	-0,107
	PMIL	-0,000068
r con VP		0,521

GI	**Efecto Directo**	**0,411**
Efecto Indirecto	**Vía**	
	PROTGR	0,005
	RTOH	-0,007
	GH	-0,15
	TD	-0,031
	AFLOJ	-0,04
	W	0,145
	P/L	-0,031
	CNIZH	0,013
	PMIL	0,00001
r con VP		0,314

Tabla 4. Disección de los efectos directos de los componentes Proteína en Grano, Peso de Mil y Gluten Index en la subregión II Norte

Proteína en Grano (PROTGR), Gluten Húmedo (GH), Gluten Index (GI), Tiempo de Desarrollo (TD), Fuerza Panadera (W), Relación de Equilibrio P/L (P/L), Cenizas en Harina (CNIZH), Rendimiento en Harina (RTOH), Peso de Mil Granos (PMIL), Volumen de Pan (VP). r Coeficiente de Correlación.

Subregión IV

En esta subregión, al plantearse el Modelo I se encontraron las mismas asociaciones entre las variables, con el mismo grado de significancia ($p<0,001$) que en las subregiones anteriores para el modelo completo con 21 variables. El R^2 de dicho modelo fue de 95,72%. Hubo variables que aportaron muy poco al Modelo I pero estos aportes, a diferencia de las otras subregiones, fueron en general más altos, extrayéndose del modelo las variables con porcentajes de aportes menores o iguales a 0,50% (resultados no mostrados). En base a estos resultados se construyó, como en los casos anteriores, el Modelo II. En esta oportunidad fue con 10 variables, cuyas correlaciones se muestran en la Tabla 1. Se observó una correlación altamente significativa y positiva entre VP y los componentes PROTGR y GH. Solo se observó una correlación significativa positiva entre VP y P/L y negativa entre W y P/L. La Tabla 2 muestra los efectos directos de cada variable independiente con VP y el aporte de cada variable al Modelo II. Las variables que más aportaron en este modelo fueron PROTGR, GI, P/L, PMIL y GH, en orden descendente, teniendo P/L y PMIL el mismo valor de contribución. En esta subregión la disección de la correlación se aplicó a las variables GH por su alta correlación con VP y falta de aporte al modelo elegido (Tabla 5). La alta asociación de VP con GH estuvo dada por un fuerte efecto por vía indirecta de PROTGR y P/L. Este Modelo II tuvo un coeficiente de determinación del 36,94%.

GH	**Efecto Directo**	**0,15**
Efecto Indirecto	**Vía**	
	PROTGR	0,35
	GI	-0,08
	TD	-0,01
	ESTAB	0,0018
	W	0,0023
	P/L	0,09
	CNIZGR	0,00099
	PMIL	-0,07
r con VP		0,44

Tabla 5. Disección de los efectos directos de componente Gluten Húmedo en la subregión IV

Proteína en Grano (PROTGR), Gluten Húmedo (GH), Gluten Index (GI), Tiempo de Desarrollo (TD), Estabilidad Farinográfica (ESTAB), Fuerza Panadera (W), Relación de Equilibrio P/L (P/L), Peso de Mil Granos (PMIL), Ceniza en Grano (CNIZGR), Volumen de Pan (VP). r Coeficiente de Correlación.

Subregión V Sur

Se planteó el Modelo I con las 21 variables independientes obteniendo un coeficiente de determinación de 6,47%. El análisis de correlación simple mostró que las variables L vs G, GH vs GS, PROTGR vs PROTH y VP vs VESP estaban altamente correlacionadas ($p<0,01$). Por lo tanto, se eliminaron de este modelo las variables citadas en segundo término en la dupla. El análisis de coeficiente de sendero mostró que algunas variables contribuyeron poco al modelo planteado (0,0001 y 0,001%, resultados no mostrados). Ellas fueron P y VESP, aunque a esta última ya se había considerado eliminarla por su alta correlación con VP en el análisis anterior (0,0001%). También se sacaron del modelo las variables FN y PH dado que su efecto real sobre VP fue muy bajo, menor que el 0,001%. De esta manera, quedaron en el análisis 14 variables, las cuales conformaron el Modelo II. Las relaciones causales con VP se presentan en la Tabla 1. Se observó una correlación significativa y positiva entre VP y los componentes GH, TD y W, en tanto fueron negativas con AFLOJ y PMIL. El único componente que mostró correlación altamente significativa y positiva fue PROTGR.

La Tabla 2 muestra los efectos directos de cada variable independiente con VP y el aporte de cada variable al Modelo II. Las variables que más efecto real tuvieron sobre la variable dependiente fueron L, GH, P/L, W y AFLOJ. La Tabla 6 muestra la disección de los efectos indirectos del componente PROTGR que tuvo una correlación positiva y significativa con VP, aunque en el Modelo II no se manifestó su contribución. Los componentes que mayor efecto real tuvieron en forma indirecta a la correlación positiva y significativa fueron GH, W, P/L y PMIL. El resto de los componentes lo hicieron en forma negativa: GI, ESTAB, L y CNIZGR.

Otro componente analizado fue L, el cual no

mostró correlación con VP pero tuvo el efecto más alto en el modelo. La partición del coeficiente de correlación mostró que indirectamente GH y P/L fueron los componentes de mayor influencia sobre la variable L. (Tabla 3). Este Modelo II tuvo un coeficiente de determinación del 30%.

PROTGR	**Efecto Directo**	**-0,0031**
Efecto Indirecto	**Vía**	
	GH	0,3
	GI	-0,04
	TD	0,01
	ESTAB	-0,0026
	AFLOJ	0,03
	L	-0,13
	W	0,05
	P/L	0,05
	CNIZH	0,0021
	RTOH	0,02
	PMIL	0,05
	CNIZGR	-0,03
r con VP		0,31

L	**Efecto Directo**	**-0,45**
Efecto Indirecto	**Vía**	
	GH	0,27
	GI	-0,06
	TD	-0,06
	ESTAB	0,02
	AFLOJ	-0,03
	W	0,02
	P/L	0,31
	CNIZH	-0,01
	RTIH	0,02
	PMIL	0,03
	PROTGR	-0,00088
	CNIZGR	-0,01
r con VP		0,06

Tabla 6. Disección de los efectos directos del componente Proteína en Grano y Extensibilidad en la subregión V Sur

Proteína en Grano (PROTGR), Gluten Húmedo (GH), Gluten Index (GI), Tiempo de Desarrollo (TD), Estabilidad Farinográfica 0(ESTAB), Aflojamiento de la Masa (AFLOJ), Extensibilidad (L), Fuerza Panadera (W), Relación de Equilibrio P/L (P/L), Cenizas en Harina (CNIZH), Rendimiento en Harina (RTOH), Peso de Mil Granos (PMIL), Ceniza en Grano (CNIZGR), Volumen de Pan (VP). r Coeficiente de Correlación.

DISCUSIÓN Y CONCLUSIONES

El Modelo I planteado con 21 variables dio valores altos en los coeficientes de determinación (superiores a 95%) en todas las subregiones, excepto la V Sur (6,47%). Esto sugiere que existen muchas más variables no consideradas en este estudio que podrían incorporarse en dicha subregión, lo que posiblemente haría aumentar su coeficiente. De acuerdo con los hallazgos del presente trabajo, algunas variables de esta subregión contribuyeron poco en la explicación de la variable dependiente VP tomada como respuesta en el Modelo, existiendo, además, correlaciones parciales negativas entre variables dependientes y la independiente. Al plantear el Modelo II, se eliminaron las variables de alta correlación (por existir colinealidad entre ellas) y las de escasa contribución al mismo, de modo que R^2 aumentó considerablemente en la subregión V Sur (de 6,47 a 30%). Esto se

atribuye a que este segundo modelo no contenía las variables que por vía indirecta hacían disminuir el coeficiente en esta subregión, lo cual indica que dichas variables influyeron negativamente. En las restantes subregiones ese valor disminuyó en orden decreciente de la siguiente manera: II Norte > II Sur > IV al eliminar las variables de poca contribución. Esta amplia variación encontrada entre los valores de R^2 se atribuye a las variables que conforman cada modelo, a la elevada influencia ambiental sobre ellas y a los diferentes genotipos incluidos en cada subregión. Ha sido coincidente la alta correlación en las mismas variables (1 o cercana a 1) en las cuatro subregiones, entre las cuales se citan GH vs GS, PROTGR vs PROTH, VP vs VPESP, L vs G, P/L vs L, P/L vs P y P/L vs G.

En cuanto al aporte de cada variable que conformó el Modelo II según la subregión considerada, hubo variables en común tales como GH, W, GI, P/L y CNIZH. Algunas variables fueron propias de algunas subregiones, tales como ESTAB en la II Sur, PMIL en la IV, L y AFLOJ en la V Sur. La disección de cada subregión explicó por qué algunas variables tenían bajas correlaciones con VP y otras aportaron muy poco al modelo elegido.

Las variables que conformaron el Modelo II en cada una de las subregiones podrían tomarse como participantes en la formación de un índice de selección. La Tabla 7 muestra las variables que conformarían dicho índice según la subregión considerada. El actual Índice de Calidad (IC) que utiliza el Comité de Cereales de Invierno para la calificación de los nuevos cultivares inscriptos en grupos de calidad industrial incluye solamente cuatro de las variables encontradas en el presente trabajo: GH, ESTAB, W y PROTGR (Miranda y Salomón, 2001). Este IC ha sido aplicado en 10 campañas trigueras pertenecientes a la Red de Ensayos Territoriales. Se propone analizar esos datos, o parte de ellos, con el nuevo índice obtenido en el presente trabajo diferenciando los datos por subregión. Este tipo de análisis resulta de interés ya que tiene en cuenta relaciones funcionales y causales directas e indirectas entre los componentes de la calidad que poseen alta influencia ambiental. De esta manera, se podría mejorar la eficiencia de la selección en trigo pan.

Subregión	**Variables del Índice**					
	Alto aporte a la variable VP en el Modelo II					Correlación altamente significativa con VP
II Sur	GH	GI	ESTAB	CNIZH		PROTGR
II Norte	GH	GI	W	CNIZH		PROTGR
IV	GH	GI	PROTGR	PMIL	P/L	
V Sur	GH	AFLOJ	W	L	P/L	PROTGR

Tabla 7. Variables que conformarían un índice de calidad según la subregión analizada

Proteína en Grano (PROTGR), Gluten Húmedo (GH), Gluten Index (GI), Estabilidad Farinográfica (ESTAB), Aflojamiento de la Masa (AFLOJ), Extensibilidad (L), Fuerza Panadera (W), Relación de Equilibrio P/L (P/L), Cenizas en Harina (CNIZH), Peso de Mil Granos (PMIL), Volumen de Pan (VP).

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JOURNAL OF
BASIC AND APPLIED GENETICS
ISSN: 1852-6233

INTROGRESSION OF CULTIVATED SUNFLOWER IN EXOTIC *Helianthus petiolaris* POPULATIONS

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ABSTRACT

Cultivated sunflower *Helianthus annuus* and the wild exotic *H. petiolaris* are sympatric species in an extensive region of central Argentina. Both species are sexually compatible, they overlap their flowering time and share pollinators in the region. Although there are important barriers to introgression between them, interspecific hybrids are occasionally found in contact zones. We present evidences of crop introgression in *H. petiolaris* populations under natural conditions and controlled and semi-controlled crosses in the experimental field using morphological traits. If crop genes persist in subsequent generations after hybridization, it could then be possible to find crop morphological traits and molecular markers in hybrid offsprings. Plants of *H. petiolaris*, sunflower and plants of intermediate morphology (IMP) were characterized in their natural habitats, and progenies of IMPs were grown in a common garden and compared to progenies of interspecific hybrids obtained by open pollination and backcrosses to *H. petiolaris*. Variability of IMPs in the wild was comparable to variability under experimental conditions, thus confirming introgression from sunflower in *H. petiolaris* populations. Molecular markers confirmed introgression. This has implications on the usage of herbicide tolerant sunflower varieties and the likely release of genetic modified varieties in agro-ecological regions invaded by *H. petiolaris* populations.

Key words: crop-wild, intermediate morphology, interspecific hybridization, RAPD.

RESUMEN

El girasol cultivado *Helianthus annuus* y la especie silvestre exótica *H. petiolaris* son simpátricos en una extensa región central de Argentina. Ambas especies son sexualmente compatibles, superponen su floración y comparten polinizadores en las condiciones locales. Aunque hay importantes barreras a la introgresión entre ellas, en las zonas de contacto se encuentran ocasionalmente híbridos interespecíficos. En este estudio presentamos evidencias de la introgresión del cultivo en las poblaciones naturales de *H. petiolaris* y cruzamientos controlados y semi-controlados producidos en el campo experimental, utilizando caracteres morfológicos. Si los genes del cultivo persisten en las generaciones posteriores a la hibridación, sería posible encontrar rasgos morfológicos y marcadores moleculares del cultivo en la descendencia de los híbridos. Se caracterizaron plantas de *H. petiolaris*, girasol y plantas de morfología intermedia (IMP) en sus sitios naturales. Se criaron las progenies de las IMPs en un jardín común y se compararon con progenies de híbridos interespecíficos y sus retrocruzas con *H. petiolaris* producidas bajo condiciones controladas. La variabilidad fenotípica de las IMPs en el hábitat natural fue similar a la observada en la progenie de cruzas interespecíficas criadas bajo condiciones experimentales, lo cual confirma la introgresión del girasol en *H. petiolaris*. Los marcadores moleculares también confirmaron la introgresión. Este hallazgo tiene implicancias sobre el uso de variedades de girasol tolerantes a herbicida y la posible liberación de variedades genéticamente modificadas en regiones agro-ecológicas invadidas por poblaciones de *H. petiolaris*.

Palabras clave: cultivo–silvestre, morfología intermedia, hibridación interespecífica, RAPD

INTRODUCTION

Sunflower, *Helianthus annuus* var. *macrocarpus* L. (crop, domesticated sunflower) and the wild exotic *H. petiolaris* Nutt. are sympatric species in an extensive region of central Argentina. Both species overlap their flowering time and share pollinators in the invaded landscape (Poverene *et al*., 2004). Because reproductive barriers between both species are incomplete (Rieseberg *et al*., 1999a) interspecific hybrids with intermediate morphological traits are occasionally found in contact zones (Heiser, 1947; Rieseberg *et al.,* 1999b; Ureta *et al*., 2008). In Argentina plants with intermediate morphology (IMP) have been found for several consecutive years in wild *H. petiolaris* populations near sunflower fields in districts of Buenos Aires and La Pampa provinces (Poverene *et al.,* 2006). This could be indicative of the natural occurrence of mating events between both species in the region (Poverene *et al*., 2008).

An increasing number of studies as of 2000 have documented gene flow from crops to wild relatives, thus indicating the potential risk of gene or transgene escape via hybridization (Jenczewski *et al.,* 2003; Hails and Morley, 2005). However, the impact of allele release depends on the likelihood of its persistence in wild populations, which, in turn, depends on the fitness of plants bearing the trait, which is difficult to prove beyond the first hybrid generation (Vacher *et al.,* 2004). Most studies on crop-wild hybridization have documented first generation hybrids but little progress has been made on generations after hybridization (Kirkpatrick and Wilson, 1988; Langevin *et al.,* 1990; Robert *et al.,* 1991; Klinger *et al.,* 1991; Santoni and Bervillé, 1992; Arias and Rieseberg, 1994; Brubaker and Wendel, 1994; Arriola and Ellstrand, 1996). The bare observation of first generation hybrids does not prove gene flow from the crop into wild or weedy populations on account of the fact that interspecific hybrids might be almost completely sterile. Demonstration of crop gene introgression into wild populations is essential since the persistence of either genes or transgenes to confer adaptive advantages in wild populations is an ecological risk (Linder *et al.,* 1998). For example, plants could acquire herbicide tolerance through crop pollen flow, rendering wild or weedy populations more difficult to control in agricultural lands. The herbicide-resistant sunflower varieties and the experimental assays with genetically modified varieties (MAGYP, 2012) that could be released in the future are of concern, sunflower being a species of high-risk category in terms of gene escape probability after crop-wild hybridization according to Ahl Goy and Duesing (1996). On the other hand, as wild populations are more tolerant to adverse conditions than crops, a transgene conferring resistance to a pest or an herbicide may not necessarily represent the same advantage as for the crop. On the contrary, it could have a fitness cost due to pleiotropic effects and the impact of a transgene could result in both beneficial and cost effects. This might represent an adaptive cost in a new environment (Vila-Aiub *et al.,* 2009).

In view of the above, the purpose of the present study was to account for interspecific introgression of sunflower in *H. petiolaris* invasive populations under natural conditions. Particular attention was paid to whether or not morphological variability in *H. petiolaris* populations at different sites was a consequence of crop introgression following natural hybridization. Two *H. petiolaris* populations located in the sunflower cropping area of Argentina were studied focusing on the following queries: 1) do controlled crosses between hybrid plants and wild species in the experimental field induce morphological variability similar to that observed under natural conditions?, and 2) can this comparison help explain hybridization under natural conditions? In an attempt to answer these queries, IMPs, which were suspected to have arisen from interspecific hybridization and were found under natural conditions, and IMPs obtained in the experimental field through controlled and semi-controlled crosses, were studied. Different plant materials were analyzed in three experiments, namely i) plants of sunflower crop, *H. petiolaris*, and IMPs under natural conditions; ii) progenies of the latter from a common garden; and iii) progenies of interspecific hybrids obtained in a common garden. Plants were characterized taking into account several morphological traits for their comparison among the different experiments. The underlying hypothesis was that if crop genes persist in subsequent generations after hybridization, it seems likely that there will be crop morphological traits or molecular markers in the offspring of hybrids or their backcrosses to the wild species.

MATERIALS AND METHODS

Experiment #1

We studied IMP individuals in two regions of Argentina where *H. petiolaris* (HP) has naturalized in the sunflower cropping area. Both species overlap in flowering time and the presence of IMPs is frequent. Off-type plants showing atypical records of morphological traits within *H. petiolaris* populations were considered as IMPs. Large *H. petiolaris* populations grow along roadsides and among sown fields, in a patchy distribution. A total of 10 IMPs were analyzed in Trenque Lauquen (approximately S35˚49.5', W62˚49.5'), and 10 IMPs in Catriló (approximately S36˚30.1',W63˚44.7'). Data were collected from 49 *H. petiolaris* plants from both locations and 15 sunflower plants from a crop lot in the former site were used as controls.

Experiment #2

Progenies of another set of 10 IMPs collected from Trenque Lauquen, following the same criteria as in experiment #1, were analyzed in a common garden (IMPp-TL). Seeds were placed in plastic trays on wet paper and kept at 4ºC for one week to break dormancy. They were sown in the greenhouse and they grew with appropriate watering and temperature until seedlings had 4-6 leaves. At this stage, they were transplanted to the experimental field in a completely randomized design, each plot representing the progeny of a single plant. Plants from six HP populations and one sunflower hybrid cultivar (cv. Dekalb 3881) were included in adjacent plots. A total of 91 plants were analyzed.

Experiment #3

Progenies of eight interspecific *H. petiolaris* – sunflower crop natural hybrids from seven different HP populations from La Pampa and Buenos Aires provinces, already described in Gutierrez *et al.* (2010) were analyzed. Hybrid offsprings were obtained in the experimental field either by open-pollination (OP) or backcrosses (BC) to the wild parent. Male parents of OP were not identified, being comprised of the pollen community of the experimental field, which consisted in *H. petiolaris*, cultivated sunflower and products of interspecific crosses. Backcrosses (BC) were obtained from previously bagged *H. petiolaris* heads, applying pollen from crop-wild hybrids with a cotton brush. The experiment included OP and BC plants derived from natural hybrids and pure *H. petiolaris* plants from seven locations in La Pampa, Buenos Aires and San Luis provinces (Table 1). Seeds were placed in plastic trays on wet paper and kept at 4ºC for one week to break dormancy. They were grown in the greenhouse as described above and transplanted to the experimental field in a completely randomized design. A sunflower hybrid cultivar (cv. Dekalb 3881) was included in adjacent rows. A total of 44 HP, 49 BC, 83 OP, and 10 sunflower plants were analyzed.

Morphological characterization and data analysis

The quantitative and qualitative morphological traits analyzed in each experiment are shown in Table 2. In experiment #2 (IMPp in a common garden) four metric traits difficult to measure in natural populations because of wild fruit shattering, namely filled seed number (FS), empty seed number (ES), fertility (F) measured as viable seeds (FS/FS+ES), and seed length (SL), were added. Metric data were subjected to Principal Component Analysis (PCA, bi-plot) from a correlation matrix. Means were compared using the non-parametric Kruskal-Wallis test. A hybrid index for categorical traits was calculated using a scale for each variable (Table 3). The highest and lowest values in the scale correspond to pure parental species and intermediate values correspond to plants showing intermediate morphology (Briggs and Walters, 1997). By means of discriminant analysis of morphological data, IMPs from experiment #1 were classified into one of the groups including the two stabilized species (HA and HP) and the progeny of their interspecific hybrids (BC or OP) characterized in experiment #3. Data analyses were performed with InfoStat (2006).

Molecular characterization

Molecular analysis was carried out in progenies of some crosses in the common garden to evaluate the frequency of crop specific markers in advanced generations of interspecific hybrids. The selected biotypes were descendants of previously confirmed crop-wild hybrids, in which crop had been the pollinating parent (Gutierrez *et al*., 2010). They included three crop-wild interspecific hybrids (HI0802, HI0902, and HI1002), 21 open-pollinated

descendants (seven individuals per interspecific hybrid, OP0802, OP0902, OP1002), 21 backcrosses to the wild parent (seven individual per interspecific hybrid BC0802, BC0902, BC1002) and sunflower inbred lines previously studied by Gutierrez *et al*. (2010), namely HA89, HA369, HAR2, HAR3, HAR5, and RHA274. Young leaves were lyophilized and DNA was isolated with CTAB method (Hoisington *et al*., 1994). RAPD markers were amplified using two primers (A2 and B5, Operon Technologies) showing three crop-specific markers found in the crop-wild interspecific hybrids analyzed (Gutierrez *et al*., 2010). Amplifications were carried out in a total volume of 25µl with 50 ng of purified DNA as template, 30 ng of primer, 1U of Taq DNA polymerase, and a final concentration of 2 mM MgCl2, 10X buffer, and 50 mM of dNTP. Reactions were placed in a PTC-100 MJ Research Thermal Cycler programmed for a 6-min cycle at 94ºC, 40 15-sec cycles at 94ºC, a 45-sec cycle at 40ºC, a 1-min cycle at 72ºC, and a 7-min final extension at 72ºC. Amplification products were separated by electrophoresis in 1.5% TAE agarose gels and detected by staining with ethidium bromide.

RESULTS

Morphological characterization

Experiment #1

PCA (bi-plot) based on 11 metric morphological traits collected in the field explained 87.5% of the variability recorded in the first two axes. It showed two different groups: one consisting of cultivated sunflower plants and the other consisting of *H. petiolaris* and IMPs. The latter scattered between both parental species (Fig. 1). Most IMPs from Trenque Lauquen (IMPs-TL) were observed to have an intermediate position among stabilized species whereas several of them from Catriló (IMPs-C) were closer to *H. petiolaris* plants. Variability was observed to be higher in the plants from Trenque Lauquen than in those from Catriló.

Metric morphological traits analyzed by Kruskal-Wallis test showed differences between *H. petiolaris* and IMPs-C for five out of 11 of the traits analyzed. Highly significant differences were found between IMPs-TL and *H. petiolaris* for every trait, except bract number (Table 4). The hybrid index showed that IMPs-C and IMPs-TL were intermediate between *H. petiolaris* and sunflower although some of them showed no differences with respect to pure wild plants (Fig. 2).

Experiment #2

Many seeds from IMP individuals were inviable or seedlings died before the reproductive stage. The PCA (bi-plot) of morphological traits of 103 viable plants (including HP and sunflower plants) explained 73% of variation in the first two axes and showed a clear separation of the pure species along PC1. Progenies of IMP-TL (IMPp-TL) were more related to *H. petiolaris* plants (Fig. 3). Highly significant differences in Kruskal Wallis test were found between IMPp and the pure species, *H. petiolaris* and sunflower (Table 4). The hybrid index showed that IMPp were intermediate between *H. petiolaris* and sunflower although some of them showed no differences with respect to pure wild plants (Fig. 4).

The PCA (bi-plot) based on 12 metric morphological traits measured in a common garden explained 75% of variability in the first two axes (Fig. 5). The OP plants were found scattered between both parental species although some plants were closer to the wild species. Many, though not all, BC plants were similar to the female parent, *H. petiolaris*. A higher variability was observed among OP compared with BC.

Kruskal-Wallis test showed that eight metric morphological traits differentiated BC and OP individuals from *H. petiolaris* and sunflower. Highly significant differences were found between OP and BC progeny of the first generation hybrids and cultivated sunflower except for bract (phyllary) number. No differences in ligule length and width were found between *H. petiolaris* and BC (Table 5). Furthermore, the hybrid index showed that BC and OP plants were intermediate between *H. petiolaris* and sunflower although some of them showed no differences with respect to pure wild plants (Fig. 6).

Descriptive discriminant analysis of materials from experiments #1 and #3 confirmed, in general, PCA results in each experiment (Fig. 7). Prediction ellipses showed the probability of finding a plant within a given group with 95% confidence level. Cross-classification table showed group membership prediction of 20 IMP plants (Table 6). Five and

seven IMPs from Catriló and Trenque Lauquen were classified as interspecific hybrid descendants (OP or BC). Some HP plants from Trenque Lauquen site (not identified in Table 6) were found and classified as BC and OP.

Molecular characterization

Two out of three crop-specific markers (A2720, A2820 and B5680) were found in the progeny of interspecific hybrids in experiment #3. Band B5680 was not found in any of the plants analyzed. Four out of 21 (19%) 0802 OP plants derived from one interspecific hybrid plant showed specific markers of cultivated sunflower. Two of them were found to have band A2720 and two were observed to have band A2820. Two out of 21 (9.5%) backcrosses of the same population (0802 BC) showed the presence of crop-specific marker A2720.

H. petiolaris	*OP progenies*	*BC progenies*	*District*	*Province*
HP 0102	1	1	Guaminí	Buenos Aires
HP 0502	1	1	Catriló	La Pampa
HP 0802	2	1	Atreucó	La Pampa
HP 0902	1	1	Capital	La Pampa
HP 1002	1	1	Capital	La Pampa
HP 3202	1	1	G. Pedernera	San Luis
HP 4702	1	1	Realicó	La Pampa

Table 1. Materials studied of *H. petiolaris* (HP), open-pollinated crop-wild hybrid progenies (OP) and backcrosses to the wild parent (BC). Numbers indicate the sites from where seeds were collected. Two hybrids from 0802 population yielded one OP progeny each.

Metric variable and symbol		Categorical variable and symbol	
plant height	PH	branching type	BT
stem diameter	SD	main head	MH
leaf width	LeW	leaf base	LB
leaf length	LeL	leaf shape	LS
petiole length	PL	leaf area	LA
ligule number	LiN	leaf margin	LM
ligule width	LiW	bract tip	BT
ligule length	LiL	palea anthocyanin	PA
bract number	BN	stigma anthocyanin	SA
bract length	BL	disk flower color	CD
bract width	BW	disk chaff hair	HD
disk diameter	DD		
filled seed number	FSN		
empty seed number	ESN		
fertility (FS/FS+ES)	F		
seed length	SL		

Table 2. Morphological traits taken into account for plant description following Rieseberg and Morefield (1995). Variable units are in cm except for head, bract, and seed number.

Variable	Category		
	1	2	3
Branching type	Total branching	No branches	-
Main head	No	Yes	-
Leaf base	Cuneate	Cordate	-
Leaf shape	Lanceolate	Cordate	-
Leaf surface	Flat	Waxy	Curled
Leaf margin	Curled	Serrate	Deeply serrate
Bract tip	Acute	Acuminate	-
Palea Anthocyanin	Present	Absent	-
Stigma anthocyanin	Present	Absent	-
Disk flower color	Red	Yellow	-
Disk chaff hair	Present	Absent	-

Table 3. Categories assigned to morphological traits for hybrid index analysis.

Variable	HP	IMP-C	IMP-TL	HA
Stem diameter	0.9 ± 0.2 a	1 ± 0.3 a	1.1 ± 0.2 a	2.1 ± 0.5 b
Leaf width	5.8 ± 1.2 a	6.5 ± 2.3 a	10.3 ± 2.1 b	23.3 ± 5.1 c
Leaf length	8.2 ± 1.5 a	8.9 ± 3.3 a	13.4 ± 3.7 b	23.4 ± 3.5 c
Petiole length	8.1 ± 1.4 a	9.9 ± 2.1 b	13.6 ± 3 c	14.2 ± 2.8 c
Ligule number	21.3 ± 3 a	23.9 ± 3.3 b	27.4 ± 2.1 c	42.6 ± 8.7 d
Ligule width	1.1 ± 0.2 a	1.2 ± 0.1 a	1.5 ± 0.3 b	1.9 ± 0.2 c
Ligule length	2.9 ± 0.4 a	3.1 ± 0.8 a	4.1 ± 1.2 b	8.8 ± 0.7 c
Bract number	31.4 ± 4.1 a	32.1 ± 2.1 ab	33.7 ± 4.1 ab	35.1 ± 3.4 b
Bract length	1.4 ± 0.2 a	2.4 ± 1 b	3.3 ± 0.7 b	7 ± 1 c
Bract width	0.4 ± 0.05 a	1.2 ± 0.1 b	0.9 ± 0.3 b	3.6 ± 0.4 c
Disk diameter	2.3 ± 0.3 a	2.7 ± 0.4 a	4.1 ± 1.1 b	17.6 ± 3.6 c

Table 4. Morphological traits (means ± SD) of *H. petiolaris* (HP), domesticated sunflower (HA), intermediate plants of Catrilo (IMP-C) and Trenque Lauquen (IMP-TL) in field conditions. Variable units are cm, except for ligule and bract number. Means followed by different letters indicate significant differences in Kruskal–Wallis test (P<0.05) N=84 plants.

Variables	HP	BC	OP	HA
Stem diameter	0.9 ± 0.3 a	1.1 ± 0.3 b	1.6 ± 0.6 c	2.3 ± 0.5 d
Leaf width	5.5 ± 1.1 a	7.1 ± 2.7 b	11.9 ± 5.4 c	25.6 ± 4.6 d
Leaf length	8.2 ± 1.3 a	9.8 ± 2.7 b	13.7 ± 5.0 c	25.0 ± 2.8 d
Petiole length	8.3 ± 1.5 a	9.8 ± 2.6 b	11.7 ± 3.8 c	15.1 ± 3.3 d
Ligule number	20.8 ± 3.4 a	23.1 ± 4.2 b	25.5 ± 5.0 c	41.2 ± 8.5 d
Ligule width	1.1 ± 0.3 a	1.1 ± 0.3 a	1.4 ± 0.3 b	1.9 ± 0.1 c
Ligule length	2.8 ± 0.5 a	3.0 ± 0.7 a	4.0 ± 0.6 b	8.7 ± 0.7 c
Bract number	30.2 ± 3.7 a	32.6 ± 4.5 b	33.4 ± 6.1 b	34.7 ± 3.8 b
Bract length	1.3 ± 0.2 a	1.6 ± 0.3 b	2.0 ± 0.5 c	7.1 ± 1.0 d
Bract width	0.4 ± 0.1 a	0.5 ± 0.1 b	0.6 ± 0.2 c	3.6 ± 0.5 d
Disk diameter	2.3 ± 0.2 a	2.7 ± 0.5 b	3.3 ± 0.7 c	19.0 ± 3.7 d

Table 5. Morphological traits (means ± SD) of *H. petiolaris* (HP), sunflower (HA), and progeny of their interspecific hybrids produced by controlled backcrosses to the wild parent (BC) or under open-pollination in the experimental field (OP). Variable units are in cm except for ligule and bract number. Means followed by different letters indicate significant differences in Kruskal–Wallis test (P<0.05).

Biotypes	HA	HP	OP	BC	Total	Error (%)
HA	25	0	0	0	25	0.00
HP	0	96	3	13	112	14.29
OP	0	5	58	20	83	30.12
BC	0	22	14	13	49	73.47
C-IMP	0	5	2	3	10	
TL- IMP	0	3	4	3	10	
C+TL- IMP	0	8	6	6	20	

Table 6. Cross-classification of *Helianthus petiolaris* (HP), domestic sunflower, *H. annuus* (HA), backcrosses (BC), open-pollinated plants (OP), and 20 Catrilo (C) and Trenque Lauquen (TL) IMP considered as unknown individuals.

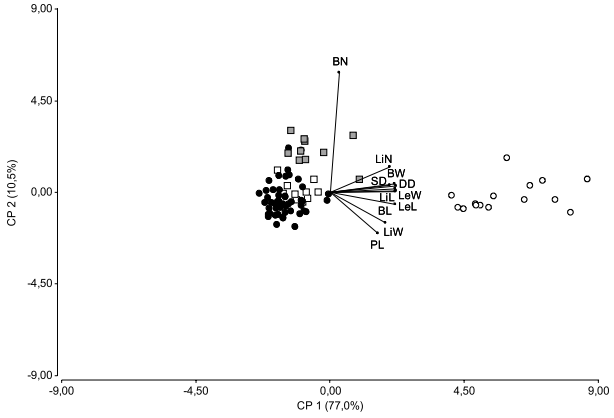

Figure 1. Principal component analysis (bi-plot) based on field data of 11 metric morphological traits. Each point represents an individual of *H. petiolaris* (black circles), domesticated sunflower (white circles), Catrilo IMP (white squares) and Trenque Lauquen IMP (gray squares) in field conditions. For bi-plot references see Table 2. N=84 plants.

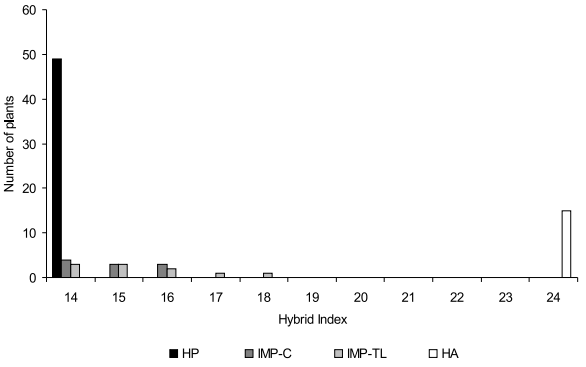

Figure 2. Hybrid index based on 11 categorical morphology traits of *H. petiolaris* (HP), cultivated sunflower (HA), intermediate morphology plants of Catrilo (IMP-C) and Trenque Lauquen (IMP-TL) in field conditions. N=84.

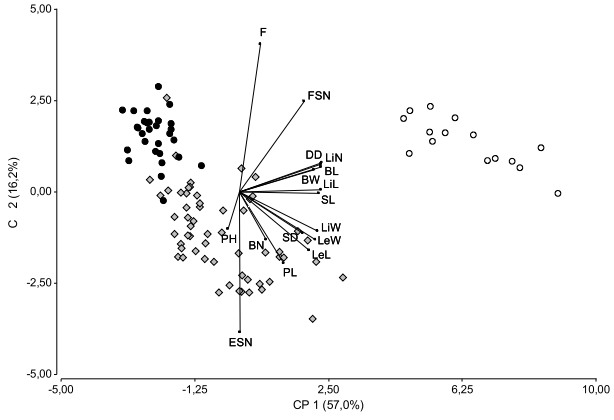

Figure 3. Principal component analysis (bi-plot) based on 16 metric morphological traits. Each point represents a descendant of a single HP (black circles), cultivated sunflower (open circles) and IMPp (gray diamonds) grown in common garden conditions. For bi-plot references see Table 2. N = 103 plants.

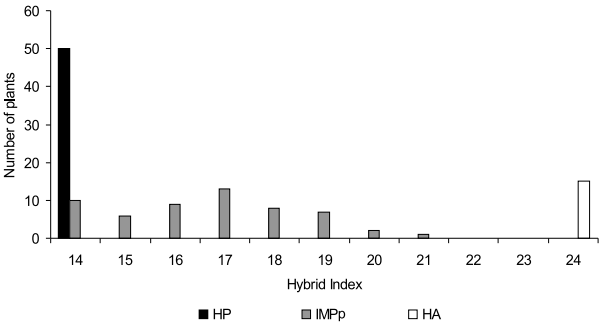

Figure 4. Hybrid index based on 11 categorical morphology traits of *H. petiolaris* (HP), cultivated sunflower (HA) and progenies of intermediate morphology plants (IMPp). N=103.

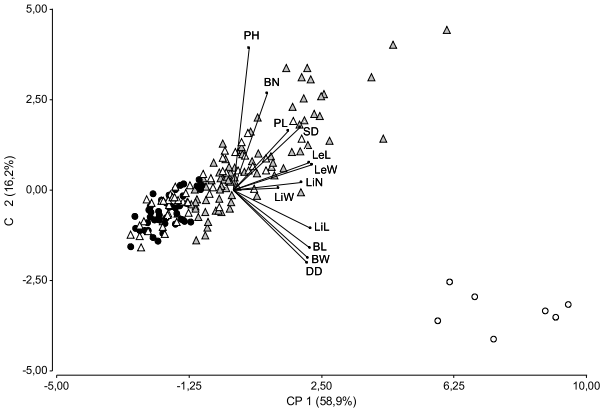

Figure 5. Principal component analysis (bi-plot) based on 12 metric morphological traits of *Helianthus annuus* (HA), *H. petiolaris* (HP), their advanced generations from open-pollinated hybrids (OP) and backcrosses (BC) obtained in the experimental field. Each point represents an individual of HP (black circles), BC (open triangles), OP (gray triangles) and sunflower, HA (open circles) biotypes. For bi-plot references see Table 2. N=186 plants.

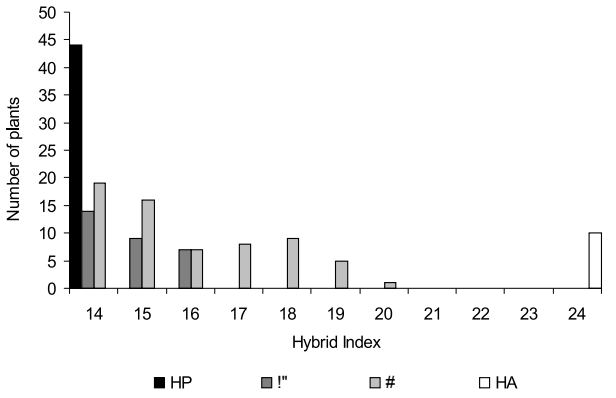

Figure 6. Hybrid index based on 11 categorical morphology traits of *Helianthus petiolaris* (HP), domestic sunflower, *H. annuus* (HA), backcrosses (BC), and open- pollinated progeny (OP) in the experimental field. N=186.

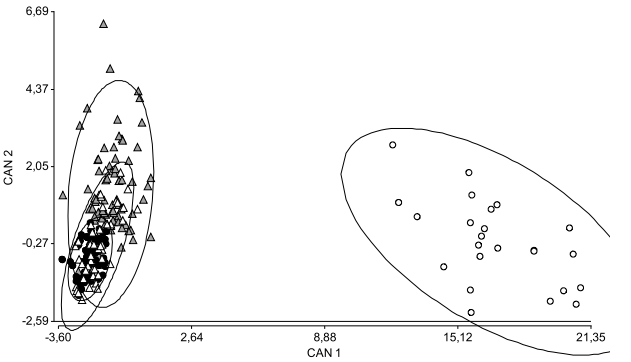

Figure 7. Discriminant análisis based on metric morphological traits of *Helianthus annuus* (HA, open circles), *H. petiolaris* (HP, black circles), their advanced generations from open-pollinated hybrids (OP, gray triangles), and backcrosses (BC, open triangles). Each point represents an individual. Prediction ellipses correspond to 95% confidence level.

DISCUSSION

This is a comparative analysis through which experimental data are compared with data collected under natural conditions, this being the procedure recommended to to assess gene flow (Ellstrand, 1995; Kareiva *et al.*, 1996). In the three experiments conducted, PCA, mean differences and hybrid indexes showed morphological differences between the parental species sunflower *H. annuus* and the wild exotic *H. petiolaris*. Polygenic (metric) traits tended to be intermediate in hybrids as well as in many cases of F1 and advanced-generation interspecific hybrids in *Helianthus* (Rieseberg and Ellstrand, 1993). In the present study, advanced generations from open-pollinated hybrids between these two species were intermediate and similar to *H. petiolaris* when hybrids backcrossed to wild plants. The intermediate morphology of the IMPs collected in Catriló and Trenque Lauquen showed similar variability, thus indicating that crop introgression is likely to occur naturally in agro-ecosystems where both species are sympatric. Data from progenies of controlled crosses in the common garden confirmed this although variability was not as high as in progenies of natural crosses. This was attributed to a higher variability under natural conditions, which led to carry out experiment #2 in order to standardize experimental conditions and highlight genotypic differences. Segregation within plots also confirmed the hybrid origin of intermediate plants. On the other hand, experiment #2 showed a straight relationship between *H. petiolaris* populations and fertility and between cultivated sunflower plants and filled seeds. Both are characteristics of stabilized species, in contrast to the association between intermediate morphology and empty seeds as a consequence of low gamete viability in interspecific hybrids (Rieseberg *et al.,* 1998). This confirms that while barriers to hybridization between *H. annuus* and *H. petiolaris* are not complete (Rieseberg *et al*., 1999a), there is a chromosomal incompatibility causing a low production of fertile seeds in hybrid plants. Chromosomal differences consist of at least 11 rearrangements affecting 10 out of 17 pairs of chromosomes in both *Helianthus* species (Burke *et al*., 2004). This could greatly affect introgression because recombination rates are reduced in non-collinear portions of the genome (Rieseberg *et al.,* 1995). However, fecundity parameters recover fast in advanced-generation hybrids in agreement with previous field experiments (Gutierrez *et al.,* 2011).

Discriminant analysis classified 60% of the IMPs as progenies of interspecific hybrids between *H. petiolaris* (as female parent) and sunflower (as male parent) and no IMP seemed to relate with the sunflower group. We therefore hypothesized that introgression was different in each site. In Trenque Lauquen hybridization and introgression occur repetitively due to extensive sunflower cultivation every year, determining a stable frequency of IMP individuals. In contrast, in Catriló hybridization occurs occasionally and advanced-generation hybrids resemble more HP plants. In addition,

cross-classification table predicting membership of 20 unknown plants also showed some HP plants collected in Trenque Lauquen classified as BC or OP. This reveals introgression traits in such HP plants although these traits were not observed during data collection. The BC and OP plants inaccurately classified in Table 5 show that these groups are phenotypically heterogeneous due to the recombination and segregation of interspecific traits.

Plants of hybrid origin are difficult to identify in wild populations after the second generation following hybridization. This is clearly shown by hybrid index and PCAs, through which it could be observed that BC and IMP-C, and OP and IMP-TL individuals (though in lower proportion) overlap with the wild species. Furthermore, progeny test carried out on 10 IMPs-TL in the common garden showed segregation, the latter being characteristic of a hybrid progeny. In both cases, IMPs were interspecific hybrids of different generations. Furthermore, Trenque Lauquen IMP individuals could be interpreted as corresponding to advanced back-cross generations receiving pollen from both *H. petiolaris* and sunflower. Catriló IMP individuals could be interpreted as corresponding mostly to backcrosses to *H. petiolaris*. Trenque Lauquen is a district with the largest area of sunflower crop production in Buenos Aires province, having, in fact, reached a 50% higher of the total production in the province in the last five years (MAGPyA, 2012). Although IMPs have been found in every season for the last 10 years, *H. petiolaris* plants are similar to those of the centre of origin (Poverene *et al.,* 2008) and no "intermediate biotypes" have been established as populations in those sites. Hybrid biotype stabilization is likely to require ecological divergence either to co-exist with parent species or to occupy a different ecological niche, as occurred in North America where three homoploid annual species were found to have arisen from hybridization between *H. annuus* and *H. petiolaris* (Rieseberg, 1997; Rieseberg *et al.,* 2003, Karrenberg *et al.,* 2007). These species exhibit several transgressive traits (significantly exceeding parental trait values) while results from the present study showed no transgressive traits in advanced-generation hybrids, thus not accounting for ecological divergence. In contrast, IMPs were found to evidence gene flow preventing reproductive isolation from occurring. In addition, no discrete or different ecological niches associated to the sampled sites were found, both of which are located in the Pampean ecological region exhibiting similar micro-habitat parameters (Cantamutto *et al.,* 2008).

In the cases in which intermediate morphological characters are not evident, it is necessary to use additional techniques such as molecular markers. In *H. petiolaris* populations growing adjacent to cultivated sunflower plots in North America, plants were observed to show no morphological evidence of hybridization but revealed crop gene introgression when surveyed with molecular markers (Rieseberg *et al.,* 1999b). Also, crop alleles were found to persist in wild *H. annuus* populations for five generations at moderate frequencies (Whitton *et al.,* 1997). In line with this, and based on the specific markers of sunflower inbred lines found in progenies of interspecific hybrids, results from the present study demonstrate that sunflower crop alleles may also persist in *H. petiolaris* populations after hybridization.

The morphological traits analyzed in the present study as well as the evidence of gene flow after the first generation of hybridization confirm introgression from sunflower into *H. petiolaris* (Heiser, 1947; Rogers *et al*., 1982; Rieseberg *et al*., 1999b). Gene spread to related wild populations cannot be prevented from occurring because crop pollen can reach long distances by insect and other pollinator transportation (Arias and Rieseberg, 1994; Ureta *et al*., 2008). Furthermore, although sunflower crop introgression reduces hybrid plant fitness, the fast recovery of fecundity parameters in the generations following hybridization allows predicting that traits conferring an ecological advantage, such as herbicide tolerance or disease resistance, are likely to diffuse into wild populations (Gutierrez *et al.,* 2011). Herbicide tolerance introgression in wild annual *Helianthus* was confirmed (Massinga *et al.,* 2005; Presotto *et al.,* 2012). This suggests that sunflower gene or eventually transgene escape to wild relative populations in Argentina could not be discarded.

ACKNOWLEDGMENTS

Authors thank the National Research Council of Argentina (CONICET) for a fellowship granted to GA. This research was supported by grants ANPCYT PICT 2286 and PAE-PICT-2007-00020.

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JOURNAL OF
BASIC AND APPLIED GENETICS
ISSN: 1852-6233

OROBANCHE CUMANA WALLR. RESISTANCE OF COMMERCIAL SUNFLOWER CULTIVARS GROWN IN ARGENTINA

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ABSTRACT

The parasitic weed *Orobanche cumana* Wallr. (broomrape) is one of the major limiting factors in worldwide sunflower production. However, it is absent in the centre of origin and in the sunflower crop areas of América. It has not yet been elucidated if *O. cumana* naturalization in sunflower habitats in Argentina is restricted as a result of either abiotic constraints or resistance in grown commercial cultivars. The aim of the present study was to assess the degree of resistance of commercial sunflower cultivars grown in Argentina to *O. cumana*. More than 95% of the tested sunflower cultivars were, in general, susceptible to broomrape attack. Although three cultivars were found to evidence an acceptable response to broomrape attack, only one of them showed complete resistance to *O. cumana*. This disregards genetic resistance of grown cultivars as being the reason for the absence of broomrape in Argentina. In view of this, future studies should focus on other biotic and abiotic factors affecting broomrape growth and development which could be potentially responsible for the absence of *O. cumana* in Argentina.

Key words: broomrape, incidence, severity.

RESUMEN

La planta parásita *Orobanche cumana* Wallr. (jopo) es una de las mayores limitantes de la producción de girasol en los principales países productores del mundo. Sin embargo, está ausente en el centro de origen y en las áreas de cultivo de girasol en América. No ha quedado claro aún si la naturalización de *O. cumana* en los hábitats de girasol de Argentina está limitada por restricciones abióticas o por la resistencia de los cultivares comerciales. El objetivo de este estudio fue investigar la resistencia a *O. cumana* de los cultivares comerciales de girasol en Argentina. En general, más del 95% de los cultivares probados fue susceptible al ataque de jopo; solamente uno de ellos mostró completa resistencia a *O. cumana*. Esto descarta la posibilidad de considerar a la resistencia genética de los cultivares utilizados como la causa de la ausencia de jopo en Argentina. Por lo tanto, los estudios futuros deberían focalizarse en otros factores bióticos y abióticos que, por afectar el crecimiento y desarrollo del jopo, podrían ser responsables potenciales de la ausencia de este parásito en Argentina.

Palabras clave: jopo, incidencia, severidad.

INTRODUCTION

The parasitic weed *Orobanche cumana* Wallr. (broomrape) is one of the major limiting factors in sunflower production among the principal sunflower-producing countries all over the world. As of the earliest studies initiated in Russia by Dr. V.S. Pustovoit at the beginning of the last century, genetic resistance combined with herbicides has been the best way to control this weed (Eizenberg *et al.*, 2006; Rubiales *et al.*, 2009; Fernández-Martínez *et al.*, 2010). Nonetheless, in spite of the worldwide use of these control strategies, infested areas keep on revealing a significant increase. This parasitic weed, which is native of Russia, is widely distributed in the whole Eurasia continent, including the Black Sea region (Antonova *et al.*, 2009), Serbia (Masirevic and Medic-Pap, 2009), Romania (Pricop *et al.*, 2011), Turkey (Demirci and Kaya, 2009), Spain (Fernández Martínez *et al.*, 2009), Israel (Eizenberg *et al.*, 2003) and Southern areas of France. Broomrape has also been recently detected in Africa (Amri *et al.*, 2012).

Broomrape is absent in the centre of origin of sunflower and in the sunflower crop areas of América. As a result of an intense sunflower seed exchange among regions worldwide and due to the very small size of *Orobanche* seeds, absence of accidental introductions seems to be almost impossible. It has not yet been confirmed if *O. cumana* naturalization in Argentinean sunflower habitats is limited due to abiotic constraints (Miladinovic *et al.*, 2012). The habitats suitable for broomrape invasion in Argentina have been observed to be different from invaded habitats in Serbia particularly in terms of mean temperature during the coolest months. Also, *O. cumana* has been found in some Spanish habitats where winter temperatures are similar to those in Argentina (Cantamutto *et al.*, 2012). Both natural and broad genetic resistance in Argentina sunflowers could be potentially responsible for broomrape absence in Argentina. In view of the above, the purpose of the present study was to assess resistance of Argentine commercial sunflower cultivars to *O. cumana*.

MATERIALS AND METHODS

A representative sample of commercial Argentine sunflower germplasm including a hundred of commercial hybrids from 22 private seed companies and one old open pollinated variety (Impira INTA) from a national institution (breeders) was analyzed. Sunflower cultivars were grouped as i) traditional (TRAD; n=68), ii) imidazolinone resistant or Clearfield® (CL; n=23), and iii) high oleic (SQ; n=10) varieties. Ninety-six of the sampled hybrids were included in the sunflower network of experimental analyses carried out by the *Instituto Nacional de Tecnología Agropecuaria* (INTA) during the 2010-2011 growing season.

Samples of sunflower cultivars grown in Argentina were carefully cleaned and treated with 1.05 mg/g seed of metalaxyl-m. Treated seeds were sent to the Institute of Field and Vegetable Crops, in Novi Sad, Serbia, following phytosanitary regulations from Argentina and Serbia.

Broomrape resistance screening was performed as described by Terzic *et al.* (2010). Ten-litre pots were filled with a mixture of sand: perlite: peat in a 1:1:1 ratio and 70 mg of *O. cumana* seed, race E. Ten sunflower plants per pot were grown in the greenhouse at 25°C - 16:8 h photoperiod during six weeks. After seven weeks, the plants were completely cleaned and broomrape attack incidence and severity were determined. Incidence (INC) was calculated as the ratio attacked plants: total plants (n=10); Severity was calculated as broomrape attachment number per sunflower plant (TSPL).

For severity values, ANOVA was done using InfoStat (2008) where each sunflower plant was treated as one replication (n=10) in a completely randomized design, factorial arrangement with three factors: cultivar, breeder and group. *O. cumana* incidence was determined considering cultivars as replicates (n=68, 23, 10) and two factors: breeder and group. Each cultivar was identified by a letter for the breeder and a number corresponding to the variety. Due to commercial implications, identification of the cultivars analyzed is restricted but available to each breeder upon request.

RESULTS AND DISCUSSION

In general, sunflower cultivars grown in Argentina were found to show a high incidence of *O. cumana* attack, with no differences among the above-mentioned three groups (Table 1). Mean attack severity corresponded to susceptible cultivars (Terzic *et al.*, 2010) and no effect of breeder either on incidence or on attack severity was observed (Data not shown). In the most susceptible subgroup among traditional cultivars, severity reached more than eight broomrape attachments per plant and complete incidence (Fig. 1). The open variety Impira INTA (coded as L3) was included in this category. On the other hand, in four cultivars from this group broomrape emergence was lower than two attachments per plant. Furthermore, two traditional cultivars showed a good resistance level as no emergence of broomrape was observed. However, broomrape nodules were found on their roots. The I3 cultivar was observed to have only one infested plant with seven broomrape nodules. Due to the hybrid nature of I3 cultivar, this unusual response to inoculation could be attributed to an unintentional seed contamination.

On the other hand, high severity of broomrape attack was observed in all Clearfield cultivars, with two or more broomrape tassels per plant and complete parasitic incidence (Fig. 2). The high susceptibility of Clearfield cultivars to broomrape could be the consequence of a breeding process on account of the fact that as in Clearfield cultivars less attention is paid to genetic resistance to this parasite since broomrape could be chemically controlled in Clearfield crops (Kaya *et al.*, 2012). Among high oleic sunflowers, W15 cultivar showed complete resistance to broomrape attack, with no root attachments (Fig. 3). The remaining nine high oleic cultivars showed complete incidence, with more than two broomrape attachments per plant.

In general, more than 95% of the tested sunflower cultivars grown in Argentina were susceptible to broomrape attack. Although three cultivars showed an acceptable response to broomrape attack, only one of them showed complete resistance to *O. cumana*. This is indicative of a general vulnerability to *O. cumana* diffusion in Argentina.

Findings from the present study reveal that the majority of the commercial sunflower cultivars grown in Argentina are susceptible to broomrape. The possibility of considering genetic resistance of grown cultivars to *O. cumana* as the reason for broomrape absence in Argentina is therefore discarded. Future studies should therefore be conducted on other biotic and abiotic factors affecting broomrape growth and development which could be potentially responsible for the absence of this parasite in Argentina.

Group	TSPL	INC (%)
Traditional	4.5 ± 0.3	97.2 ± 1.7
Clearfield	4.8 ± 0.3	100.0 ± 0.0
High Oleic	6.2 ± 1.0	90.0 ± 10.0
ANOVA	ns	ns

Table 1. Severity (TSPL= *Orobanche* attachments per sunflower plant) and Incidence (INC=attacked plants/total inoculated plants) mean ± SE for the three groups of Argentine sunflower cultivars inoculated with *O. cumana*, race E.

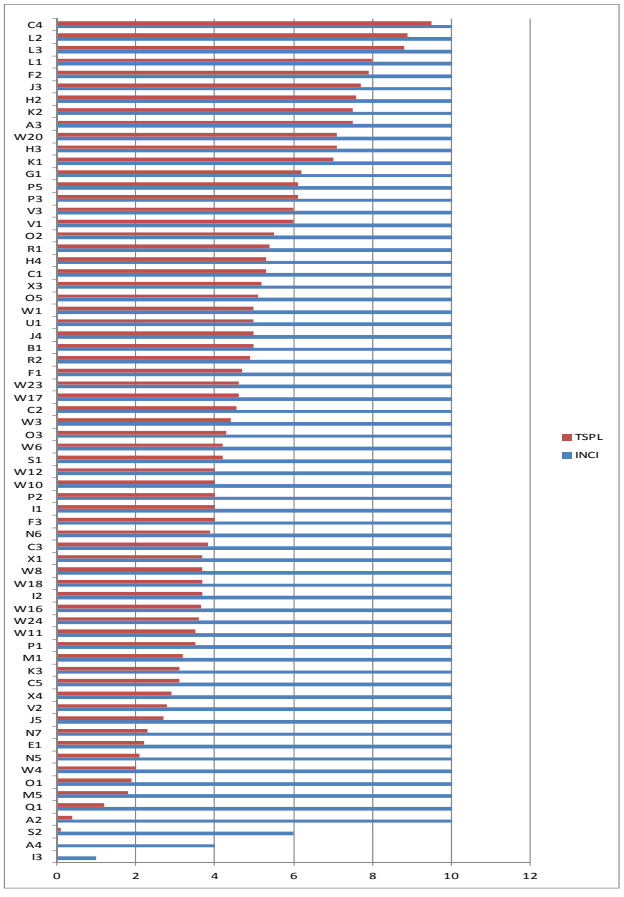

Figure 1. Broomrape (*O. cumana* Race E) severity (as broomrape attachments per plant = TSPL, LSD = 2.5) and incidence (attacked plants per ten plants = INC) of traditional Argentine sunflower cultivars.

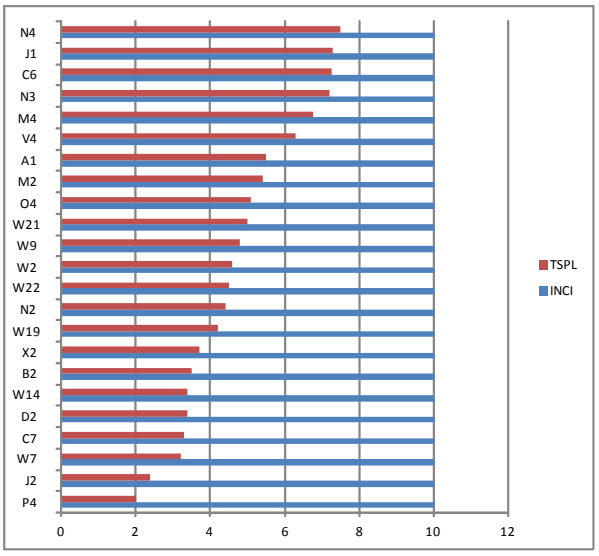

Figure 2. Broomrape (*O. cumana* Race E) severity (as broomrape attachments per plant = TSPL. LSD = 2.6) and incidence (attacked plants per ten plants = INC) of Argentine Clearfield (CL) sunflower cultivars.

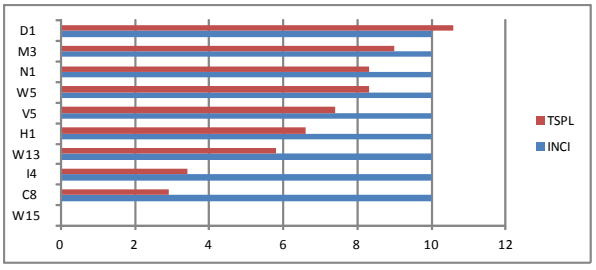

Figure 3. Broomrape (*O. cumana* Race E) severity (as broomrape attachments per plant = TSPL, LSD = 3.3) and incidence (attacked plants per ten plants = INC) of Argentine high oleic sunflower cultivars.

ACKNOWLEDGEMENTS

Authors are grateful to student Federico Laxague for his help in seed preparation. This study was supported with PAE PICT 020 and INTA PNOLE 031031 grants as well as with TR30125 grant from the Ministry of Education, Science and Technological Development of R. Serbia.

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JOURNAL OF
BASIC AND APPLIED GENETICS
ISSN: 1852-6233

IN VIVO SELF-INCOMPATIBILITY RESPONSE IN THE WILD POTATO *SOLANUM CHACOENSE* BITTER

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ABSTRACT

In tuber-bearing *Solanum* species (potatoes), *S*-locus mediated self-incompatibility is under gametophytic control. In this system, self-fertilization is avoided when the same *S*-allele is expressed in both pistil and pollen. Because the ultrastructural details of the self-incompatibility response in this group of species is unknown, the aim of this study was to identify the cellular events involved in this response by using the diploid species *S. chacoense* Bitter as a model. To this end, pollinations were carried out in two genotypic combinations previously identified as (1) compatible and (2) self-incompatible (self-pollination) for the S-locus. Pollinated pistils were fixed on an hourly basis and observed under a transmission electron microscope to detect ultrastructural changes. In the self-incompatible genotypic combination, pollen grains germinated normally but loss of electron density in the mitochondrial matrix of pollen tubes was observed at 1 hour after pollination (HAP), mitochondrial swelling started at 2 HAP and, finally, mitochondrial content was lost at 7 HAP, with the concomitant cessation of pollen tube growth in the upper third of the style. The gametophytic self-incompatibility response in potato shares similarities with a type of programmed cell death.

Key words: pollen tube mitochondria, programmed cell death, self-incompatibility, tuber-bearing *Solanum*

RESUMEN

En las especies tuberosas de *Solanum* (papas), la auto-incompatibilidad mediada por el locus *S* tiene control gametofítico. En este sistema se evita la autofecundación cuando el mismo alelo *S* se expresa en ambos pistilo y polen. Dado que se desconocen los cambios ultraestructurales de la respuesta auto-incompatible en este grupo de especies, el propósito del presente trabajo fue identificar los eventos celulares involucrados en dicha respuesta usando la especie diploide *S. chacoense* Bitter como modelo. Para tal fin, se realizaron polinizaciones controladas en dos combinaciones genotípicas previamente identificadas, respectivamente, como compatible y auto-incompatible (auto-fecundación) para el locus S. Los pistilos polinizados se fijaron en base horaria y se observaron en microscopio electrónico de transmisión para detectar cambios ultraestructurales. En la combinación auto-incompatible, los granos de polen germinaron normalmente pero se observó pérdida de densidad en la matriz de las mitocondrias de los tubos polínicos a 1 hora después de la polinización (HAP), agrandamiento de las mitocondrias a las 2 HAP y, finalmente, pérdida del contenido de las mitocondrias a las 7 HAP, con el cese concomitante del crecimiento de los tubos polínicos en el tercio superior del estilo. La respuesta de auto-incompatibilidad gametofítica en papa comparte similitudes con un tipo de muerte celular programada.

Palabras clave: mitocondrias del tubo polínico, muerte celular programada, auto-incompatibilidad, *Solanum* tuberosos

INTRODUCTION

In self-incompatible plants, self-fertilization or crossing between closely related individuals is prevented by the action of a self-incompatibility locus (or loci) under either gametophytic or sporophytic control (Frankel and Galun, 1977). This phenomenon is widely present in economically important food, feed and ornamental plants or closely related species of the same families. In sporophytic self-incompatibility, pollen phenotype for the *S*-locus is determined by the somatic genotype of the parental plant and, in incompatible pollinations, pollen tube growth stops on the stigmatic surface (e.g., *Brassicaceae*). In gametophytic self-incompatibility, pollen tube growth can stop either on the stigmatic surface (*Papaveraceae*) or in the upper third of the style (e.g., *Solanaceae*, *Rosaceae*, *Scrophulareaceae*) (de Nettancourt, 1977).

The gametophytic self-incompatibility system of *Solanaceae* and *Rosaceae* has an S-RNase-based control in which a single polymorphic *S*-locus is involved (Wang *et al*., 2003). In this system, pollen grain phenotype is gametophytically determined and fertilization is prevented when the same *S*-allele is expressed in both pollen grain and pistil. Wild and cultivated potatoes (*Solanum* spp.) belong to the *Solanaceae* family, which also includes tomatoes and eggplants. Species in this family share the same gametophytic self-incompatibility system.

There are a few reports on the ultrastructural changes underlying pollen-pistil incompatible reactions. In *Lycopersicum peruvian* Mill. (*Solanaceae*) (de Nettancourt *et al.*, 1973) the self-incompatibility phenomenon involves the destruction of the cell wall and the appearance of vesicles. Alterations in mitochondria have been described for *Papaver* spp. (*Papaveraceae*) (Geitmann *et al*., 2004), *Pyrus pyrifolia* (*Rosaceae*) (Wang *et al*., 2009), *Turnera joelii* and *T. scabra* (*Turneraceae*) (Safavian and Shore, 2010), and *Olea europaea* (*Olaceae*) (Serrano *et al.*, 2010). In these studies, self-incompatibility involved no passive reaction but a reaction closely resembling programmed cell death.

Results from Wang *et al*. (2009) in *Pyrus pyrifolia* (*Rosaceae*), using an *in vitro* test, are an important starting point to further explain incompatible reactions under natural conditions, in which not only the S-RNase is present. Nonetheless, it is necessary to confirm this explanation by carrying out assays under natural *in vivo* conditions. Furthermore, no data on the ultrastructural details of the self-incompatible reaction in tuber-bearing *Solanum* species have been published to date. Thus, the aim of the present study was to identify changes at the ultrastructural level in one *S*-locus self-incompatible genotypic combination (selfing) in the diploid wild potato species *S. chacoense* Bitter used as a model.

MATERIALS AND METHODS

Selection of genotypic combinations

Local genotypes of *S. chacoense* were grown in a glasshouse in Balcarce, Buenos Aires province, Argentina (37º45´41˝S; 58º18´41˝W). A full diallel crossing scheme was followed to identify *S*-locus compatible and incompatible genotypic combinations. To this end, flower emasculation and hand pollination were carried out on individual plants (genotypes). Pollinated pistils were removed 48 hs after pollination (HAP), fixed in FAA (40% fomaldehyde: 80º ethanol: glacial acetic acid, 1:8:1 v/v/v) and processed according to Martin (1958). Processed pistils were squashed on a glass slide and examined under an optical microscope with UV light.

Time of occurrence of incompatible reactions during the progamic phase

To determine the time at which self-incompatible pollen tube growth was arrested during the progamic phase, 36 pistils of one plant (genotype) were selfed. Three of these pistils were subsequently fixed per hour, from 1 to 12 HAP, and processed as previously described. Comparison was then made between pollen tube length at each fixation time and at 48 HAP, when fertilization in *S*-locus compatible pollinations already occurred.

Ultrastructural studies

Three pistils from each, one S-locus fully compatible genotypic combination and the selfed male parent of that combination, were prepared for observations with a transmission electron microscope (TEM). To this end, standard procedures with modifications were used (Medina *et al*., 2003). Ultrathin sections (70-90 nm) were obtained with

an ultracut microtome (Reichert, Milton Keynes, UK) using a diamond knife. Sections were then routinely mounted for staining on Formvar© coated 200 mesh copper grids. All sections were analyzed under a Zeiss-910 TEM.

Mitochondrial area was determined in at least 200 mitochondria recorded in three pistils per slide. An Analysis of Variance (ANOVA) was carried out and the Tukey test was used to detect highly significant differences (HSD) between means at the 5% level (R project 2006).

RESULTS AND DISCUSSION

Selection of genotypic combinations

Light microscopy showed differences in pollen tube length among genotypic combinations and selfings at 48 HAP. In some genotypic combinations, pollen tubes were observed to have reached the end of the style at 48 HAP. In contrast, in other combinations and invariably in selfings pollen tube growth was found to be arrested in the upper third of the style. The first group of genotypic combinations was considered to be fully compatible for the *S*-locus whereas the second group was considered to be self-incompatible for this locus because of the characteristic site of reaction. In the incompatible genotypic combinations and selfings, arrest of pollen tube growth occurred at the 7th HAP. One of the compatible genotypic combinations and the male parent of that combination were chosen for the TEM study.

Ultrastructural studies

Since pollen tube arrest in the self-pollination occurred at the 7th HAP, TEM observations were made from the 1st to the 7th HAP. Normal features of active cells were observed in compatible pollen tubes (Fig. 1a-c). In contrast, the following mitochondrial alterations were observed in self-incompatible pollen tubes from the 1st HAP onwards: i) loss of electron density in the mitochondrial matrix at 1 HAP (Fig. 1d), ii) mitochondrial swelling at 2 HAP (Fig. 1e), iii) mitochondrial content degeneration at 7 HAP (Fig. 1f). Average mitochondrial areas per fixation time are shown in Table 1.

Changes in self incompatible pollen tubes in *S. lycopersicum* were observed by de Nettancourt *et al*. (1973). In the present study, both rapid alterations in the mitochondrial area and degeneration of mitochondrial contents after pollination were observed in an incompatible genotypic combination (selfing of one genotype) of the tuber-bearing species *S. chacoense*, in comparison with one compatible genotypic combination in which the same genotype was used as the pollen donor. These changes are consistent with those observed in apoptotic programmed cell death (Häcker, 2000). Similar changes in ultrastructure and time of occurrence of the incompatible reaction were previously reported for an S-RNase system in *P. pyrifolia* by Wang *et al*. (2009); however, their study was carried out in a growth medium (*in vitro*). These changes led Wang *et al.* (2009) to conclude that the only factor affecting pollen tube growth was the S-RNase. *In vivo* assays carried out in *Turnera joelii* and *T. scabra* (Safavian and Shore, 2010) and in *Olea europaea* (Serrano *et al*., 2010; Serrano *et al*., 2012), similarly to the observed by Wang *et al.* (2009) and in the present study, showed conspicuous changes in mitochondria. Even more, apparently and independently of the self-incompatibility system, the incompatible reaction leads to a certain type of cellular programmed death. Findings from the present study complement those derived from the above-mentioned studies and provide the first *in vivo* lines of evidence of ultrastructural changes triggered by a self-incompatible reaction in a potato species.

	Pollen-pistil relationships		
	Self-Incompatible	Compatible	
Fixation time (HAP[a])	area[b] mean-sq µm- (SD)	area[b] mean-sq µm- (SD)	P[c]
1	9.19 (1.12)B	9.52 (0.99)A	0.5472
2	12.86 (2.79)A	8.91 (0.91)A	0.0004
7	11.97 (2.20)A	9.56 (0.99)A	0.005

Table 1. Mitochondrial area in self-compatible and self-incompatible genotypic combinations at various fixation times after pollination.

[a]HAP: hours after pollination; [b]over 200 mitochondria measured; [c]significance level. (SD) standard deviation. Means followed by the same letter within a column are not significantly different at 5% level according to Tukey.

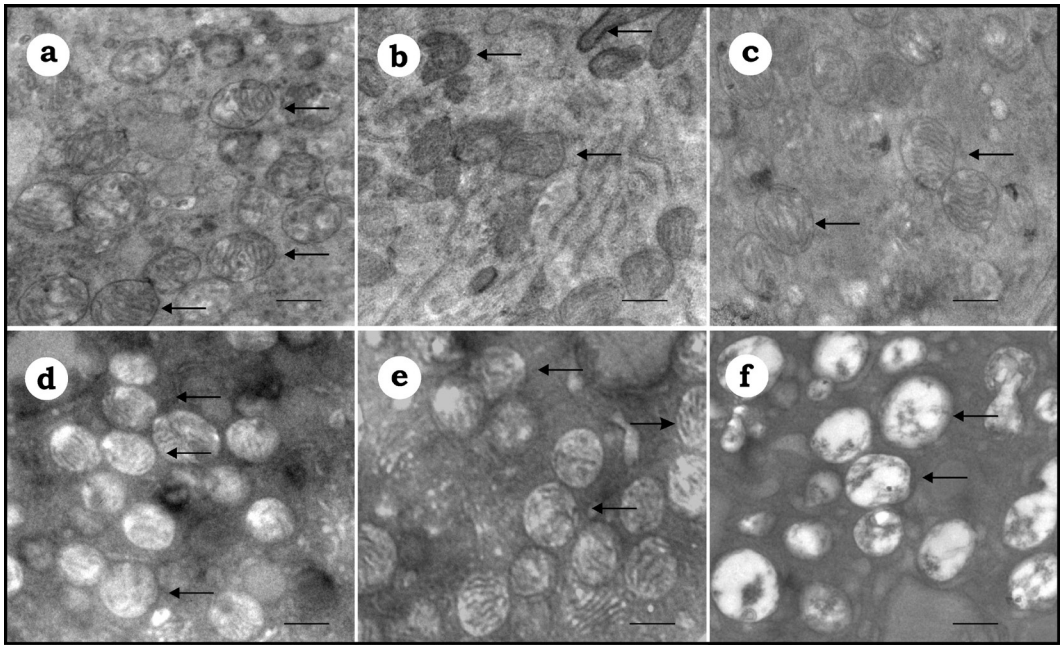

Figure 1. Mitochondrial alterations in gametophytic self-incompatible genotypic combinations (compatible=CC and incompatible= IC). At 1h after pollination (AP): (a) CC and (d) IC, presence of low electron dense mitochondrial content; at 2h AP: (b) CC and (e) IC, swelling and presence of low electron dense mitochondrial content; at 7h AP: (c) CC and (f) IC, mitochondrial content with irregular internal structure. Arrows indicate mitochondria. Bar: 0.28µm.

ACKNOWLEDGMENTS

Authors are indebted to the *Programa Pablo Neruda of the Organización de Estados Iberoamericanos (OEI)*, for the scholarship awarded to CMA to carry out short-term academic activities at the Universidad de Lleida, Spain. This study is part of CMA´s Ph. D. Thesis.

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