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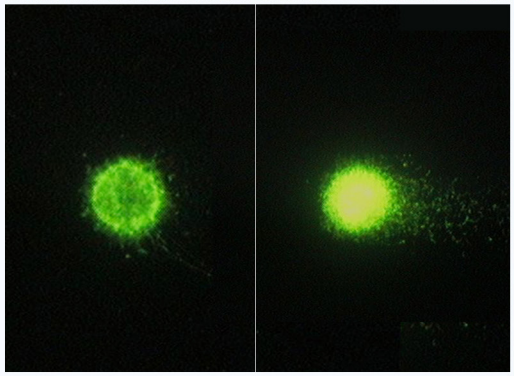

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MORPHOLOGICAL PHENOTYPES, POLLEN VIABILITY AND POLLEN-PISTIL RELATIONS IN THREE NATURAL POPULATIONS OF THE WILD POTATO *Solanum chacoense* BITTER

FENOTIPOS MORFOLÓGICOS, VIABILIDAD DEL POLEN Y RELACIONES POLEN-PISTILO EN TRES POBLACIONES NATURALES DE LA PAPA SILVESTRE *Solanum chacoense* BITTER

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ABSTRACT

Argentina is center of diversity of approximately 35 wild taxonomic potato species that have wide geographical distribution. These species, which form polyploid series with 2n=2x=24 up to 2n=6x=72 (x=12), can reproduce both sexually and asexually. Most of them are diploid obligate outcrossers because they possess a one-locus gametophytic self-incompatibility system. In nature, wild potatoes can present both external and internal reproductive isolation barriers, within and between populations, which can be incomplete. Most accessions in germplasm banks have been assigned specific categories based on morphological phenotypes (taxonomic species concept), without taking into account the natural reproductive behavior of the sampled populations. One of these species, *Solanum chacoense* Bitter (2n=2x=24, 2 EBN [1]), spontaneously grows from the NW provinces to SE Buenos Aires province, in very diverse habitats and overlapping with other taxonomic potato species, with which it can spontaneously hybridize. As preliminary activities of an *in situ* study, individual plants from three populations classified as *S. chacoense* (APEC-1, APEC-2 and APEC-3) that spontaneously grow in Balcarce, Buenos Aires province, were characterized for morphological phenotypes of leaves, flowers and fruits, pollen viability and pollen-pistil relations. The percentage of pollen viability (determined by staining) varied from 34.5 to 98.8% ($\bar{x}$=81.3%) in APEC-1 (n=23), 0 to 98.8% ($\bar{x}$=62.8%) in APEC-2 (n=26) and 63.3 to 95.4% ($\bar{x}$=85%) in APEC-3 (n=21). Variability was also observed for pollen size (<n, 2n, > 2n and 4n, in addition to normal n) and pollen size frequency. Abnormalities were observed at the tetrad stage of meiosis in eight plants of APEC-2 with heterogeneous-size pollen. According to the site of pollen tube arrest, pollen-pistil relationships within APEC-1 and APEC-2 revealed events of cross-incompatibility and/or self-incompatibility, whereas only cross-incompatibility was detected within APEC-3. No relations were found between morphological phenotypes and pollen-pistil reactions, and between corolla shape and pollen viability. The results are indicative of a likely hybrid origin of the studied populations and the need for revising the current taxonomic approaches in wild potatoes.

[1] EBN: Endosperm Balance Number (Johnston *et al.*, 1980)

Key words: pollen viability, pollen-pistil relations, wild potatoes, spontaneous hybrids

RESUMEN

Argentina es centro de diversidad de aproximadamente 35 especies silvestres de papa, que tienen amplia distribución geográfica. Estas especies, que forman series poliploides con 2n=2x=24 hasta 2n=6x=72 (x=12), pueden reproducirse tanto sexual como asexualmente. La mayoría de ellas son diploides, y alógamas obligadas debido a que poseen un sistema de auto-incompatibilidad gametofítica. En la naturaleza, las papas silvestres presentan barreras de aislamiento reproductivo externas e internas, dentro y entre poblaciones, que pueden ser incompletas. A la mayoría de las colecciones de bancos de germoplasma se les ha asignado categoría específica en base a fenotipos morfológicos (concepto de especie taxonómica), sin considerar el comportamiento reproductivo natural de las poblaciones muestreadas. Una de estas especies, *Solanum chacoense* Bitter (2n=2x=24, 2NBE[2]), crece espontáneamente desde las provincias del noroeste hasta el sudeste de la provincia de Buenos Aires, en hábitats diversos y superponiéndose en su distribución con otras especies taxonómicas de papa con las que puede hibridarse espontáneamente. Como actividades preliminares de un estudio *in situ*, se caracterizaron por fenotipos morfológicos de hojas, flores y frutos, viabilidad de polen y relaciones polen-pistilo, tres poblaciones naturales de Balcarce, provincia de Buenos Aires, clasificadas como *S. chacoense* (APEC-1, APEC-2 y APEC-3). El porcentaje de viabilidad del polen (determinado por tinción) varió de 34,5 a 98,8% ($\bar{x}$=81.3%) en APEC-1 (n=23), 0 a 98,8% ($\bar{x}$=62.8%) en APEC-2 (n=26) y 63,3 a 95,4% ($\bar{x}$=85%) en APEC-3 (n=21). Además, se observó variabilidad para tamaño de polen (<n, 2n, >2n y 4n, en adición al polen n normal) y para frecuencia según tamaño. Se observaron anormalidades en el estadio de tétrada en ocho plantas de APEC-2 con polen de tamaño heterogéneo. De acuerdo al sitio de detención del tubo polínico, las relaciones polen-pistilo dentro de APEC-1 y APEC-2 revelaron eventos de incompatibilidad cruzada y/o autoincompatibilidad mientras que en APEC-3 sólo se detectó incompatibilidad cruzada. No se encontraron relaciones entre fenotipos morfológicos y reacciones polen-pistilo, ni entre forma de corola y viabilidad de polen. Los resultados obtenidos son indicativos de un posible origen híbrido de las poblaciones estudiadas y de la necesidad de revisar el enfoque taxonómico en las papas silvestres.

[2] NBE: Número de Balance del Endosperma (Johnston *et al.*, 1980)

Palabras clave: híbridos espontáneos, papas silvestres, relaciones polen-pistilo, viabilidad de polen

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INTRODUCTION

Wild potatoes (*Solanum* L. sp.) are native to the Americas, where they present a wide geographical distribution. In fact, wild potato populations spontaneously grow in very diverse -and sometimes contrasting- habitats from southwestern USA, through Mexico and Central America, to southern Chile and Argentina, and extending to the east into Brazil, Uruguay and Paraguay (Hawkes and Hjerting, 1969). They form polyploid series with 2n=2x=24 up to 2n=6x=72 (x=12), being diploid the most frequent ploidy level. The number of taxonomic wild potato species varies according to various taxonomic treatments. In this paper, we follow Hawkes and Hjerting´s (1969) treatment because it more adequately accomodates genetic results than more recent treatments.

The successful colonization of diverse environments by wild potatoes is reflected in the variety of morphological phenotypes that they exhibit, from those that are very similar to the common (cultivated) potato, *Solanum tuberosum* L. ssp. *tuberosum*, to others that are quite different. They may have either simple or, more often, compound leaves, with leaflets varying in shape from lanceolate to ovate and elliptic, with or without interjected leaflets. Flowers can be present either arranged in inflorescences or solitary. Corollas can have various shapes, from very stellate to stellate, semi-rotated and rotated, among others, with colors varying from white to cream, blue, and purple (Hawkes and Hjerting, 1969). The current taxonomy in the group makes use of the Taxonomic Species Concept, according to which specific status is assigned to a plant or sample of plants on the basis of morphological phenotypes in comparison with holotypes, which are herbarium specimens -or drawings or pictures of the specimens- that were used for the formal description of a species or lower-rank taxon (Camadro *et al.*, 2012).

Breeding system

Wild potatoes have two alternative modes of reproduction available to them: sexual (by seeds) and asexual (by stolons and tubers), which confer particular advantages to spontaneous populations in terms of genetic flexibility under varying conditions and fitness under stable ones (Camadro *et al.*, 2012). Both, wild and cultivated potatoes possess a gametophytic self-incompatibility system controlled by the multiallelic S-locus, which prevents self-fertilization (and also crossing between closely related individuals) as a result of identity of S-alleles in pollen and style. Such self-incompatibility system, very strict in diploids and one of the most studied intraspecific barriers in higher plants (Takayama and Isogai, 2005), ensures the maintenance of high levels of heterozygosity in the population.

Cytogenetics

Normal meiosis in potatoes consists of a first meiotic division followed (without previous cytokinesis) by a second meiotic division in which the Anaphase II spindles are oriented at about 60º angles, the formation of two simultaneous cleavage furrows, and the final tetrahedron disposition of the four microspores (Mok and Peloquin, 1975). Abnormalities in spindle orientation in Anaphase II ("parallel spindles", "fused spindles" or "tripolar spindles") are rather frequent in potatoes and can lead to the formation of 2n pollen (den Nijs and Peloquin, 1977; Camadro and Peloquin, 1980; Veilleux *et al.*, 1982; Watanabe and Peloquin, 1991; Carputo *et al.*, 2003). In "parallel" and "fused" spindles, the second meiotic division spindles are, respectively, parallel or parallel and fused, and a single cleavage furrows produces a dyad of two 2n microspores. In the tripolar configuration, spindles are fused at one end and cleavage furrow formation results in a triad of one 2n and two n microspores. The 2n microspores are genetically equivalent to those formed by restitution of the first meiotic division (Peloquin *et al.*, 1999). The formation of 4n microspores is a consequence of the absence of the normal simultaneous cytokinesis after the second meiotic division (Larrosa *et al.*, 2012).

Reproductive barriers

Higher plants, in general, are isolated by external and internal hybridization barriers. The external barriers can be spatial (physical separation), ecological (*e.g.* adaptation to different habitats or ecological niches in the same geographical area) and seasonal (*e.g.* differences in flowering period in the same geographical area) (Hadley and Openshaw, 1988), whereas the internal barriers, which reside in the plant tissues themselves, can be either pre- or post-zygotic. In wild potatoes, pre-zygotic barriers act at the pollen-pistil level, either preventing pollen germination on the stigma or interfering with normal pollen tube growth along the style and/or in the ovary (Camadro *et al.*, 2004). Post-zygotic barriers act after fertilization and can result in the

formation of inviable or weak F_1 hybrids, sterile F_1 and/or F_2 hybrids, and weak and/or sterile advanced segregating generations. F_1 inviable hybrid formation may be due to disharmony between the genomes of the parental species in the embryo, abnormal development of the endosperm (or collapse of this tissue that lead to embryo abortion) (Hadley and Openshaw, 1988) or overgrowth of maternal tissue in the ovule (Masuelli and Camadro, 1997; Geerts *et al.*, 2002; among others). But even if F_1 hybrids are formed, they can be highly sterile (most frequently, male sterile) or, if they are vigorous and fertile, they can produce weak or sterile F_2 plants or more advanced segregation progenies (Hadley and Openshaw, 1988).

In the literature, wild potato hybrids have been reported as spontaneously growing in diverse -and often contrasting- natural habitats (Correll, 1962; Hawkes, 1963; Ugent, 1966; Okada and Clausen, 1982; among others). In general, however, researchers have been reluctant to recognize the importance of homoploid hybridization in the evolution of the tuber-bearing *Solanum* (see Masuelli *et al.*, 2009). Specific categories are usually assigned to individual plants or population samples on the basis of morphological phenotypes, not taking into account the reproductive behavior and genetic structure of the sampled populations (Masuelli *et al.*, 2009; Camadro *et al.*, 2012; Larrosa *et al.*, 2012). However, classification of wild germplasm is of great importance for *in situ* and *ex situ* conservation of genetic resources, including the application and/or development of methodologies and strategies for proper sampling of the natural genetic variability and its efficient conservation and use.

Solanum chacoense Bitter

One of the wild taxonomic species with the widest geographical distribution is *S. chacoense* Bitter (2n=2x=24, 2NBE), which spontaneously grow in Brazil, Paraguay, Bolivia, and Argentina, both in the wild and as a weed in cultivated potato fields. In Argentina, its distribution encompasses over 1,500 km in a North-South transect, occupying very diverse habitats, such as forests, open ranges, dry hillsides and river banks and, in modify habitats, ditches, roadsides, railway embarkments and stone fences among others, both in the shade and in full sun, in damp humiferous soils as well as in soils with high content of clay, sand or stones, at sea level and up to 3,000 masl. In its distribution, *S. chacoense* populations overlap with populations of various other wild taxonomic species, and spontaneous hybrids have been described by Correll (1962) and Hawkes and Hjerting (1969). The species is considered of great value for genetic improvement of the cultivated potato, as a source of heritable resistance/tolerance to adverse biotic and abiotic stresses, and of traits of industrial interest (Ross, 1986; Love *et al.*, 1998; Jansky *et al.*, 2013).

In southern Buenos Aires province, spontaneous wild potato populations grow in disturbed habitats and as weeds in commercial fields of cultivated potatoes and other summer crops. According to plant morphology and geographic distribution, these populations have been classified as *S. chacoense*. The objective of this work was to characterize three of these populations which spontaneously grow in the experimental fields of *Estación Experimental Agropecuaria Balcarce*, *INTA*, according to morphological phenotypes of leaves, flowers and fruits, pollen viability and pollen-pistil relations. This preliminary study on the phenotypic variability and reproductive behaviour of *S. chacoense* populations is the first carried out *in situ* -regarding the previously mentioned characteristics- in wild potatoes.

MATERIAL AND METHODS

Plant material

Three spontaneous wild potato populations classified as *S. chacoense* according to morphological phenotypes of leaves, flowers and fruits as described by Hawkes and Hjerting (1969), were sampled in the experimental grounds of *Estación Experimental Agropecuaria Balcarce*, *INTA*, in southeastern Buenos Aires province, and assigned the code APEC. Twenty-six randomly sampled plants (genotypes) of APEC-1, 23 of APEC-2 and 29 of APEC-3 were analyzed (Table 1).

Table 1. Provenance of three spontaneous populations, tentatively classified as *S. chacoense* Bitter, from southeastern Buenos Aires province and number of sampled plants.

Population	Nº of sampled plants	Localities	Geographic Coordinates		Altitude (m)
APEC-1	26	Balcarce	37°45´32´´S,	58°17´45´´W	141
APEC-2	23	Balcarce	37°45´37´´S,	58°18´09´´W	128
APEC-3	29	Balcarce	37°43´30´´S,	58°17´56´´W	134

Morphological characterization

Individual plants of the three populations were morphologically described according to characteristics of leaves (presence/absence of marginal teeth, pubescence of terminal and lateral leaflets, and presence/absence of intercalary leaflets), flowers (corolla shape, colour, and morphology) and fruits (shape and colour), based on the descriptions of Hawkes and Hjerting (1969). Morphological phenotypes were recorded by digital photography. Within each population, variability for one or more morphological traits was visually observed (*e.g.* corolla shape and/or fruit color); plants were then grouped according to the differential trait(s) for the crossing work.

Pollen viability

At bloom, two flowers were removed from each plant. In a few plants, only flower buds could be sampled. Pollen samples were stained with acetocarmine solution (0.2 g carmine, 45 ml 45% glacial acetic acid and 55 ml distilled water) in the laboratory, for observation under an optical microscope. Approximately 150 grains were recorded in at least four randomly taken visual fields. Pollen grains that were fully stained, plump and with well-defined contours were considered as viable, whereas those that were poorly (or not) stained, and/or irregularly shaped were considered as inviable. Pollen viability was then considered in relation to corolla shape of open flowers.

Pollen size

In samples with heterogeneous size pollen, approximately 150 grains were measured in four to six random microscopic fields. Following Larrosa *et al.* (2012) and based on the volume of a sphere, pollen grains with diameters 1.26 times larger than the average pollen diameter of each sample were considered to be 2n, those with diameters 1.59 times larger were considered to be 4n, and those with diameters smaller than the average were considered to be <n. Then, the frequency of pollen grains in the five size categories (<n, n, 2n, >2n, 4n) was calculated for each population, as an average of the percentages obtained for the same category using individual plant values. Microphotographs were obtained with an Olympus Q-Color 3 digital camera (Olympus America Inc., Center Valley, PA, USA).

Sporad analysis

Sporad analysis was performed in eight plants of population APEC-2 from which only flower buds could be sampled and in which unexpected pollen characteristics had been observed, such as heterogeneity in size (<n, 2n,>2n) and/or low viability. Following the standard technique, flower buds were fixed in 3:1 (v/v, absolute alcohol: glacial acetic acid) for 24 h and then transferred to 70% ethanol until use. For microscopic observations, anthers were removed from each bud and gently squashed in a drop of acetocarmine (0.2 g carmine, 45 ml of glacial acetic acid and 55 ml of distilled water) in a slide to release the sporads; after placing coverslips, slides were observed under a light microscope. Microphotographs were obtained with an Olympus Q-Color 3 (Olympus America Inc., Center Valley, PA, USA) digital camera.

Pollen-pistil relations

Controlled pollinations using individual plants (genotypes) were performed -following an incomplete diallel mating design- within and between morphological groups in each population, and according to the availability of flower buds. Flower buds were emasculated one or two days before anthesis and a minimum of two flowers were

pollinated for each combination of genotypes. Forty-eight hours after pollination (HAP), and following Martin´s (1958) technique, pollinated pistils were fixed in FAA (1:8:1, v/v/v, acetic acid, 80 ethanol, 40% formaldehyde) for at least one day. For pollen-pistil compatibility studies, fixed styles were stained with aniline blue and observed under a fluorescent microscope. Reactions were classified as (1) compatible (pollen tubes reached the ovules) or (2) incompatible at: (a) stigma (pollen grains did not germinate or, if they did, pollen tube growth was shortly arrested) and (b) style (pollen tube growth was arrested in either the first, second or last third). Pollen-pistil reactions were then considered in relation to morphological phenotypes.

RESULTS

Morphological characterization

In the three populations, variability for morphological phenotypes was observed (Figure 1), although all of them fell into the formal description of Hawkes and Hjerting (1969). All plants presented compound leaves, varying in shape from lanceolate to ovate and elliptic in APEC-1, and from lanceolate to elliptic in APEC-2 and APEC-3. In APEC-1 and APEC-2, interjected leaflets were either present or absent, whereas in APEC-3, they were always present. In the three populations, white flowers with five petals were arranged in inflorescences. Variability for corolla shape of open flowers (according to Hawkes and Hjerting, 1969) was observed in APEC-1, from very stellate to stellate and semi-rotated, and in APEC-2, from very stellate to stellate; the latter population also presented some flowers with complex morphologies, *e.g.* corollas were either partly stellate and partly semi-rotated or very stellate and stellate. In APEC-3, flowers were very stellate to stellate and, less frequently, actinomorphous instead of zygomorphous. In the three populations, fruits were ovate and green with rough white spots, but in APEC-2, smooth green fruits were also observed.

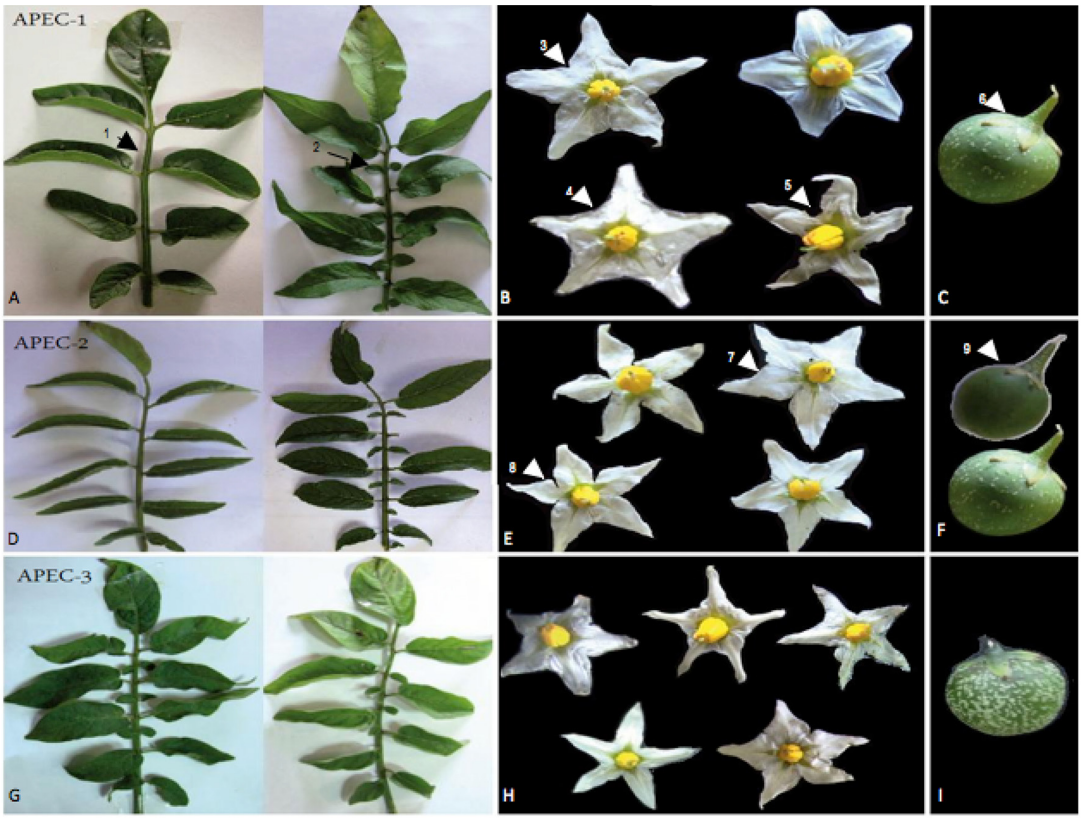

Figure 1. Morphological phenotypes in three spontaneous *S. chacoense* populations from southeastern Buenos Aires province. APEC-1 population (A-C), APEC-2 population (D-F), APEC-3 population (G-I). Examples of : leaf (1) compound without interjected leaflet , (2) compound with interjected leaflet; corolla shape (3) stellate, (4) semi-rotated, (5) very stellate, (7) partly stellate and partly semi- rotated, (8) partly stellate and partly very stellate; fruit color (6) green with rough white spots, (9) green.

Pollen viability

The percentage of viable pollen, indirectly estimated by staining in 78 individual plants (using open flowers and fixed buds), varied between and within populations: from 0 in seven plants of APEC-2 to 98.8% in one plant of APEC-1 and in another of APEC-2 (Figure 2). APEC-2 had the lowest average percentage of pollen viability (62.8%; range= 0%-98.8%) in comparison to APEC-1 (81.3%; range= 34.5%-98.8%) and APEC-3 (85%; range= 63.3% -95.4%).

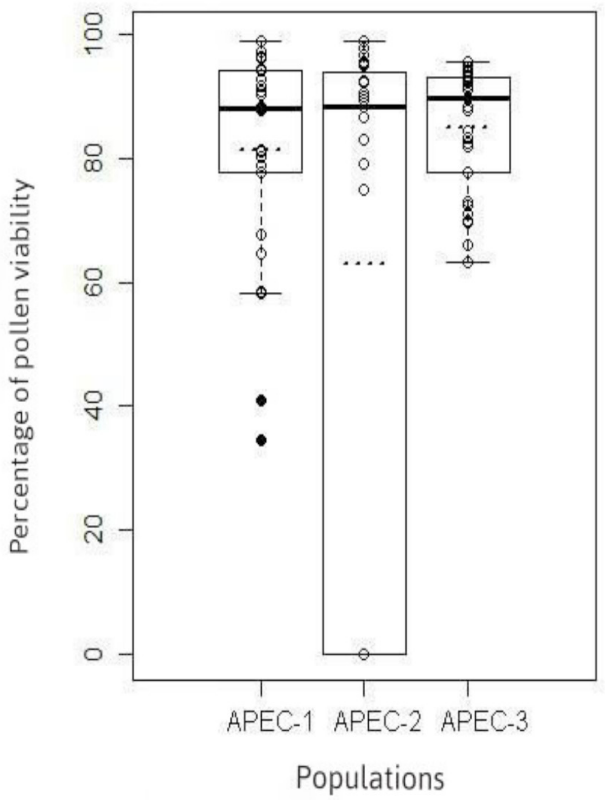

Figure 2. Percentage of pollen viability in three spontaneous *S. chacoense* populations (APEC-1, APEC-2 and APEC-3) from southeastern Buenos Aires province. Boxes: 50% of the central data; highlighted line: median; dotted line: mean; filled circles: outliers.

Pollen Size

Variability for pollen size and frequency of <n, 2n,>2n and 4n pollen grains, in addition to the expected n, was detected in the three populations (Table 2, Figure 3). Individual values for each category varied widely within each population. In relation to the average values, APEC-1 had the lowest percentage of <n pollen grains (0.04% *vs.* 0.46% in APEC-2 and 0.60% in APEC-3). APEC-1 also had the lowest percentage of >2n pollen grains (0.06% *vs.* 0.20% in APEC-2 and 0.23% in APEC-3). In turn, APEC-1 was the only population in which 4n pollen grains (0.03%) were detected. APEC-3 also presented the highest percentage of 2n pollen grains (0.67% *vs.* 0.22% in APEC-1 and 0.30% in APEC-2). The highest average percentage of pollen according to size corresponded to the expected n (99.7%), and was observed in APEC-1. No relation was found between pollen viability and corolla shape of open flowers in any of the populations (Table 3).

Sporad analysis

In the eight plants of APEC-2 that exhibited unexpected pollen characteristics, such as heterogeneity in size (<n, 2n,>2n, in addition to normal n) and low fertility, various abnormalities were observed at the tetrad stage (Table 4, Figure 4).

From 72 to 240 sporads were analyzed. The percentage of tetrads varied from 76% in plant 1 to 100% in plant 16, being the number of abnormal *vs.* normal tetrads approximately three times higher in the former and 70 times higher in the latter. The percentage of triads ranged from 0.9% in plant 2 to 6.9% in plant 9. Plant 1 also produced 3% dyads, one order of magnitude higher than the observed in plant 4 (0.4%). Monads were observed only in plant 1 (1%) and plant 2 (0.9%). The percentage of pentads varied from 0.8% in plant 9 and plant 4 to 15% in plant 1. Only plant 1 exhibited hexads (4%) and only plant 5 produced heptads (0.7%). Relations between sporad abnormalities and corolla shape could not be explored in the eight plants, because fully opened flowers were not available at the time of bud sampling and later on in the season.

Pollen-pistil relations

Twenty-six crosses (combinations of genotypes) were performed in APEC-1 (Table 5). The reactions observed were: 1) compatible, 30.8%, 2) incompatible, 69.2%, with inhibition of pollen tube growth in: a) stigma (26.9%), b) first third of style (19.2%), c) second third of style (3.9%), d) last third of style (11.5%), and e) stigma + first third of style (7.7%).

In APEC-2, 24 crosses were performed (Table 6). The reactions observed were: 1) compatible, 54.2%, 2)

incompatible, 45.8% with inhibition of pollen tube growth in: a) stigma (12.5%), b) first third of style (4.2%), c) last third of style (8.3%), d) stigma + first third of style (4.2%) e) stigma + last third of style (12.5%) and f) first third of style + second third of style (4.1%).

In APEC-3, 12 crosses were performed (Table 7). The reactions observed were all incompatible, with inhibition of pollen tube growth in: a) stigma (91.7%) and b) last third of style (8.3%).

No relation was found between morphological within-group phenotypic variation and pollen-pistil reactions (data not shown).

Table 2. Percentage (with minimum and maximum range) of pollen grains classified according to size as <n, n, 2n, >2n y 4n (viable and non-viable) in three spontaneous *S. chacoense* populations from southeastern Buenos Aires province.

Pollen grain size	Population		
	APEC-1 (n=23)	APEC-2 (n=26)	APEC-3 (n=29)
<n	0.04* (0-0.61)	0.5 (0.2-1.6)	0.6 (0.3-4.0)
n	99.7 (96.9-100)	99.0 (96.6-100)	98.5 (94.4-100)
2n	0.2 (0.4-1.2)	0.3 (0.4-0.8)	0.7 (0.4-1.5)
>2n	0.1 (0.2-0.6)	0.2 (0.5-1.7)	0.2 (0.3-1.4)
4n	0.03 (0.2-0.6)	0.0	0.0

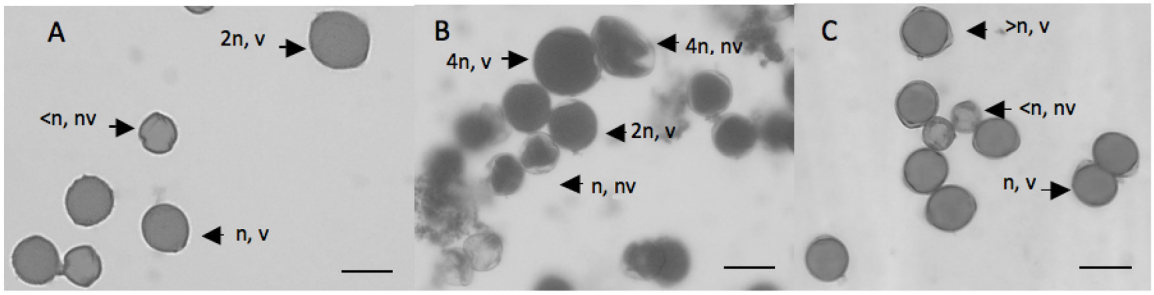

Figure 3. Variability in pollen size and viability in three spontaneous *S. chacoense* populations (A: APEC-1, B: APEC-2 and C: APEC-3) from southeastern Buenos Aires province. v: pollen grains viable, nv: pollen grains non-viable, n: pollen grains with normal size, expected for a gametophytic chromosome number n= x= 12. Bars=10 µm.

Table 3. Corolla shape and percentage of pollen viability in three spontaneous *S. chacoense* populations (APEC-1, APEC-2 and APEC-3) from southeastern Buenos Aires province.

Population	# plants	Corolla shape	% Pollen viability $\bar{x}$ (rank)
APEC-1	3	semi-rotated	71.0 (58.3-90.2)
	9	stellate	90.6 (78.9-97.2)
	13	very stellate	75.9 (41.0-96.4)
	1	partly semi-rotated y partly stellate	98.8
APEC-2	5	semi-rotated	93.9 (86.5-98.8)
	2	stellate	87.6 (83.0-92.1)
	8	partly semi-rotated y partly stellate	92.6 (88.3-96.5)
	1	partly semi-rotated y partly very stellate	97.8
	1	partly stellate y partly very stellate	92.1
	1	very stellate	95.3
APEC-3	8	stellate	81.4 (63.3-93.2)
	18	very stellate	80.2 (66.0-95.4)
	2	partly semi-rotated y partly stellate	76.5 (71.0-81.9)
	2	partly stellate y partly very stellate	93.4 (92.5-94.2)

Table 4. Meiotic abnormalities at the tetrad stage in *S. chacoense* population APEC-2.

Plant	Nº meiocytes analyzed	% of meiotic abnormalities							
		Monad	Dyad	Triad	Tetrad		Pentad	Hexad	Heptad
					Normal	Abnormal			
1	100	1.0	3.0	1.0	20.0	56.0	15.0	4.0	-
2	110	0.9	-	0.9	22.2	75.1	0.9	-	-
4	240	-	0.4	2.9	18.7	77.2	0.8	-	-
5	148	-	-	-	19.0	80.3	-	-	0.7
8	154	-	-	4.5	11.3	81.6	2.6	-	-
9	129	-	-	6.9	5.4	86.9	0.8	-	-
13	90	-	-	4.5	7.8	87.7	-	-	-
16	72	-	-	-	1.4	98.6	-	-	-

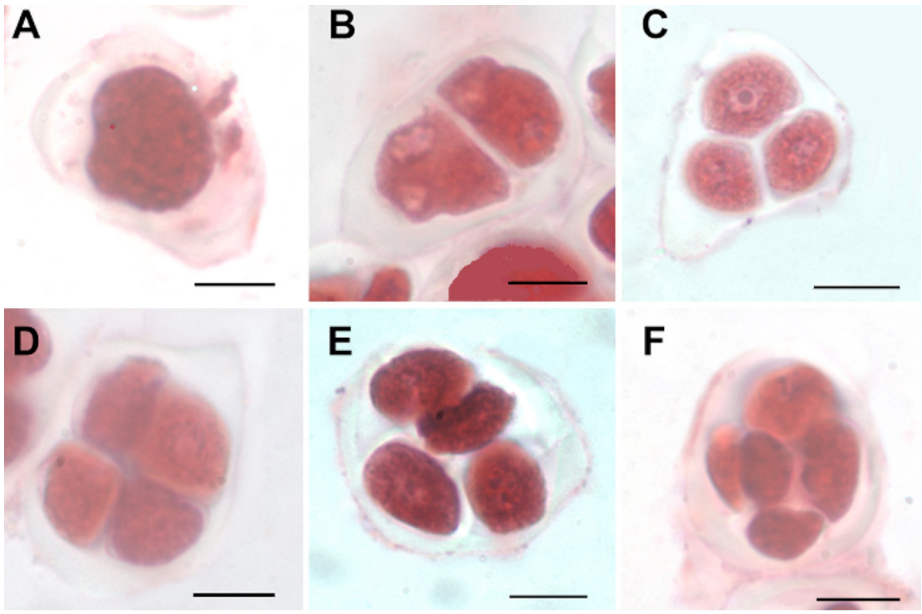

Figure 4. Abnormalities at the tetrad stage of meiosis in *S. chacoense* population APEC-2 from southeastern Buenos Aires province. Monad (A), dyad (B), triad (C), normal tetrad (D), abnormal tetrad (E), pentad (F). Bars=10 µm.

Table 5. Pollen-pistil relations within and between morphological groups (G) in *S. chacoense* population APEC-1. Inhibition of pollen tube growth in stigma (S) and style (St). Inhibition sites: 1/3, 2/3, 3/3. C: compatible.

	♂	G1			G2					G3		G4	G5	G6		G7			G8
♀		1	2	3	5	7	10	11	13	14	15	17	19	21	22	23	24	25	26
G1	1					S; St 1/3													
	2									C									
	3		C																
G2	5									S; St 1/3									
	7											St 1/3							S; St 1/3
	10			S; St 1/3															
	11													C		S; St 1/3			
	13																		
G3	14					C					St 3/3								S; St 1/3
	15	C								S				c					
G4	17										St 3/3	St 3/3	St 1/3						S; St 1/3
G5	19																		
G6	21											St 2/3							
	22										C	C				St 1/3			
G7	23																		
	24	St 1/3																	S; St 1/3
	25											St 1/3							
G8	26																		

Table 6. Pollen-pistil relations within and between morphological groups (G) in *S. chacoense* population APEC-2. Inhibition of pollen tube growth in stigma (S) and style (St). Inhibition sites: 1/3, 2/3, 3/3. C: compatible.

	♂	G1				G2		G3	G5				G6	G7	
♀		1	2	3	4	8	10	13	15	16	17	18	20	21	22
G1	1														
	2												C		
	3				S										
	4													C	C
G2	8	S		St 1/3, 2/3							St 1/3	St 3/3			
	10														C
G3	13													C	
G5	15											C			C
	16													C	
	17			C											C
	18			S										St 3/3	
G6	20														
G7	21				S; St 1/3	C							S; St 3/3		C
	22	S; St 3/3		C										S; S 3/3	

Table 7. Pollen-pistil relations within and between morphological groups (G) in *S. chacoense* population APEC-3. Inhibition of pollen tube growth in stigma (S) and last third of the style (3/3 St).

	♂	G1				G2			G3		G4		G5		G6		G7	
♀		1	2	3	4	8	9	10	11	14	17	19	20	22	23	24	26	28
G1	1			S														
	2											S						
	3																	
	4																	
G2	8												S					
	9																	S
	10															S		
G3	11																	
	14								S									
G4	17	S								S					S			
	19																	
G5	20																	
	22													S			S	
G6	23																	
	24																	
G7	26				St 3/3													
	28																	

DISCUSSION

In Argentina, *S. chacoense* has a wide distribution, from the northwestern region, where it grows at high altitudes (up to 2000 m) to the northeastern and central regions, where it grows at low altitudes (below 1000 m, including sea level) in various and, sometimes, contrasting environments. This taxonomic species exhibits significant morphological variation throughout its range, in which natural populations largely overlap with other wild and cultivated potatoes (Hawkes and Hjerting, 1969; Erazzú *et al.*, 2009; Okada 1979, pers. comm.). This morphological variation could be the result of adaptation to various macro and micro-environments (possibly underlied by physiological and/or genetic causes) and/or the occurrence of hybridization and introgression processes. The occurrence of the latter phenomena was demonstrated by Bedonni and Camadro (2009) in their morphological, genetic and molecular study with accessions of *S. kurtzianum* Bitt. *et* Wittm. and of *S. chacoense* and other wild diploids that overlap in their distribution in the west-central region of Argentina, and by Larrosa *et al.* (2012), who reported that populations classified as *S. chacoense* from the northwestern and southeastern regions of Argentina had low pollen fertility, underlied by abnormalities at the tetrad stage of meiosis, that closely resembled the observed in artificial and natural presumed hybrids (according to the intermediacy of morphological phenotypes), as will be discussed shortly.

The southeastern region of Buenos Aires province is climatically subhumid-humid. The annual rainfall average is 800 mm, with maximum values from January to March and minimum from June to August. Minimum and maximum daily temperatures, as well as thermal amplitudes, show seasonal changes, with lower values registered between June and August, and higher values between December

and March. The annual average temperature reaches around 13.3º C. The only other wild potato reported for the area is *S. commersonii* Dun. (2n=2x=24; 1EBN) which spontaneously grow in hillsides and grasslands, although "escapes" of other wild potato species from experimental investigations -carried out by other research groups more than 30 years ago in the grounds of the Experimental Station- cannot be discarded.

In the three studied populations, flower colour and flower arrangement in inflorescences were consistent over populations. However, morphological variation was observed for leaf characters, corolla shape and fruit colour.

As described by Hawkes and Hjerting (1969), the taxonomic species *S. chacoense* has leaves arranged in semi-rosettes at the base of the stem, with 4-6 pairs of lateral leaflets and one terminal leaflet, and 0-3 pairs of interjected leaflets, with leaf shape being either ovate, lanceolate or elliptic. Inflorescences consist of many (on average 3-5) flowers, with white and stellate corollas. Fruit shape can be either spherical or ovate, varying in colour from smooth green to green with rough white spots or smooth purple spots.

The three populations fall into the formal description for the evaluated leaf and fruit characters. However, they varied extensively within and between them in corolla shape, which was very stellate to stellate and semi-rotated, actinomorphous in two of them and frequently zygomorphous in the third (APEC 3). In this regard, Hawkes and Hjerting (1969) considered that *S. chacoense* (included in the Commersoniana taxonomic series along with *S. commersonnii* on the basis of corolla stellate) is closely related to species in series Tuberosa, which includes the cultivated and various wild diploid and tetraploid potatoes with which it can form hybrids. In fact, they considered that *S. chacoense* is not so closely related to *S. commersonii* as revealed by the difficulties involved in their hybridization, both in nature and in field experiments. A similar observation on the evolutive divergence between the two taxonomic species was made by Summers and Grun (1981) based on the results of controlled interspecific crosses. However, hybridization between *S. chacoense* and *S. commersonii* may occur in nature, giving origin to 3x hybrids by functioning of 2n gametes from the latter, that would allow the circumvention of the post-zygotic endosperm barrier in the inter-EBN cross. Moreover, Raimondi *et al.* (2003) obtained hybrid seeds in interspecific crosses among accessions of *S. chacoense*, *S. kurtzianum* and other wild potatoes from southern Mendoza (tentatively classified as *S. ruiz-lealii* Brücher), despite the fact that hybridization barriers were detected in some genotypic combinations.

In the present study, the percentage of pollen viability varied both within and between populations, similarly to the reported by Erazzú *et al.* (1999) in nine accessions of diploid *S. spegazzinii* Bitter from northwestern Argentina, which had been previously assigned to three morphological groups by Ispizúa (1994). Similarly, Clausen *et al.* (2006) reported variations in plant morphology, chromosome number, pollen fertility and 2n pollen production in 12 accessions classified as *S. infundibuliforme* Philippi from northwestern Argentina, which is indicative of the presence of hybrids. More recently, Larrosa *et al.* (2012) carried out studies of pollen viability in artificial hybrids between wild diploid species and the common potato (*S. gourlayi* Hawkes x *S. tuberosum* ssp. *tuberosum*), and accessions and samples of spontaneous wild populations from northwestern Argentina classified as "species" (*S. chacoense* or "presumed hybrids" (*S. gourlayi* x *S. infundibuliforme*). They reported high variability for pollen fertility and sterility in all of them, regardless of their origin and taxonomic status.

We detected variability for pollen size (<n, n, 2n, >2n and 4n) and frequency, within and between populations, with the highest frequencies being for the 2n class in two of them and the <n class in the third. The unexpected products at the tetrad stage of meiosis in individual plants of APEC-2 suggest abnormalities in spindle orientation (tripolar, parallel, fused) in Anaphase II that would explain the formation of heterogeneous size pollen. In the two remaining populations, APEC-1 and APEC-3, meiosis was not analyzed because only open flowers were present at sampling time, although it is possible that similar abnormalities occurred in them as well. Similar observations were reported by Camadro *et al.* (2008) in accessions of the wild diploid taxonomic potato species *S. okadae* Hawkes and Hjerting from northwestern Argentina and Bolivia. These authors detected plants with heterogeneous size pollen and dyads and triads, as well as normal tetrads, at the tetrad stage as a result of abnormal Anaphase II spindle orientation. Moreover, Larrosa *et al.* (2012) observed 2n and 4n pollen in addition to normal (n) pollen, high male sterility and various abnormalities at the tetrad stage of meiosis in the hybrids and populations previously described.

In nature, wild potato populations are isolated by external and/or internal hybridization barriers (Camadro

et al., 2004). However, as Camadro *et al.* (2012) have discussed, the internal barriers can be incomplete, providing opportunities for hybridization and introgression within and between populations and ploidy levels in areas of overlap.

The results of the controlled crosses within *S. chacoense* populations carried out in the present study provide evidence of the functioning of reproductive isolation barriers at the pollen-stigma/style level, at various sites, in the three populations. However, fully compatible genotypic combinations were also detected in APEC-1 and APEC-2.

It may be speculated that in those genotypic combinations in which pollen tube growth is inhibited in the 1/3 of the style, as in some of the genotypic combinations analyzed within APEC-1 and APEC-2, both gametophytic self-incompatibility and crossed incompatibility could be occurring. On the contrary, in APEC-3 population, only cross incompatibility occurred since the inhibition sites were the stigma and the last third of the style. Similar results were obtained by Erazzú *et al.* (1999) in the accessions of diploid *S. spegazzinii* previously mentioned. Different sites of pollen-pistil/style incompatibility were observed in controlled crosses between and within morphological groups. These authors speculated that both variability for morphological phenotypes and site of expression of the incompatibility reaction could be the result of spontaneous interspecific crosses in nature.

The variability for morphological phenotypes of leaves, flowers and fruits, pollen viability, pollen size (n, 2n,>2n, 4n) and frequency, and abnormalities at the tetrad stage of meiosis observed in this study are indicative of a possible hybrid origin of the three populations. Since it was first described by Hassler (1911) under the name of *Solanum guaraniticum* and assigned the actual name *S. chacoense* by Bitter in 1912 (in Hawkes and Hjerting 1969), population samples were collected from many parts of its distribution range, but mainly in Buenos Aires and Tucumán provinces (Central and NW Argentina, respectively) and referred to a number of "very unrelated species". Hawkes and Hjerting (1969) considered that eleven microspecies and one botanical variety described by various other authors had to be grouped under the name of *S. chacoense*. In our research group, and working with spontaneous populations that fall into the formal description of the species, we have observed neither normal meiosis nor high pollen fertility (see Larrosa *et al.*, 2012) as it would be expected for a "pure" species with sexual reproduction. Thus, we have proposed that *S. chacoense* and other spontaneous wild potato populations are at various stages of evolutive divergence (see Camadro *et al.*, 2012).

The results of the present study, similar to those previously obtained in the Laboratory of Genetics (EEA Balcarce, INTA) with artificial and spontaneous hybrids, and accessions with specific categories from germoplasm banks, as has been previously discussed, provide further evidence on the role of spontaneous hybridization in wild potatoes populations. They also raised the concern, and poise the need to investigate, other natural populations from a wide geographic area in Argentina which have been classified as *S. chacoense*, given the genetic consequences of the classification approach in germplasm conservation and breeding.

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