

## Brief Report

# Pollen-mediated gene flow from a commercial potato cultivar to the wild relative *S. chacoense* Bitter under experimental field conditions in Argentina

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**Capurro, M. A., Camadro, E. L. and Masuelli, R. W. 2013.** Pollen-mediated gene flow from a commercial potato cultivar to the wild relative *S. chacoense* Bitter under experimental field conditions in Argentina. – *Hereditas* 150: 60–65. Lund, Sweden. eISSN 1601-5223. Received 17 May 2013. Accepted 30 September 2013.

The common potato, *Solanum tuberosum* ssp. *tuberosum* (tbr,  $2n = 4x = 48$ ; 4EBN), has many closely related wild tuber-bearing species. Around 28 to 35 of them spontaneously grow in Argentina overlapping, in some areas, with the crop and/or experimental transgenic potatoes. Although it is well proven that hybridization barriers in potatoes can be incomplete, information on gene flow between cultivated and wild germplasm is scarce. Thus, a gene flow field experiment with a circular array was set up in Balcarce, Argentina, in 2009, and evaluated over two seasons. The tetraploid tbr cultivar Huinkul MAG and one compatible cloned genotype of the related wild potato *S. chacoense* Bitter (chc,  $2n = 2x = 24$ ; 2EBN), which produced  $2n$  eggs, were used, respectively, as pollen donor and receptor. Berries with hybrid seeds – as revealed by ploidy and RAPD profiles – were obtained in one season, at 30 m from the pollen donor. These results reinforce others previously obtained with the same pollen donor and a male sterile tbr cultivar in a similar array, pointing out to the need of increasing isolation distances in areas of overlap between cultivated and wild potato germplasm to prevent or minimize undesirable pollen-mediated gene flow.

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Wild potatoes spontaneously grow in the Americas, from southwestern USA to southern Chile and Argentina (HAWKES and HJERTING 1969). In Argentina, there are around 28 to 35 wild taxonomic species (HAWKES and HJERTING 1969; SPOONER and HILMANS 2001), most of them self-incompatible, insect-pollinated and allogamous, with sexual and asexual reproduction.

Potatoes are extensively cultivated in Latin America, in areas that are centers of origin and/or diversity of related wild species, many of which behave as weeds of the crop (HAWKES and HJERTING 1969; ERAZZÚ et al. 2009). In Argentina, field experiments with transgenic potatoes are carried out in areas of overlap with both, wild species and non-transgenic potato crops. Since 1991, the Secretary of Agriculture has authorized the evaluation of 32 transgenic events in potato – five in the greenhouse and 27 in the field – using an isolation distance of 10 m (CONABIA 2011). Since information on gene flow and risk assessment in the country is scarce or lacking, the isolation distance was established according to regulations in other countries.

Natural potato populations are isolated by external and internal hybridization barriers (CAMADRO et al. 2004). When isolation barriers are incomplete, hybrids can be spontaneously formed; these hybrids can be fertile and able to set seeds upon crossing (either spontaneous or controlled). There is well documented scientific evidence on the extent of interspecific (homoploid and heteroploid) hybridization in potatoes (BEDONNI and CAMADRO 2009; ERAZZÚ et al. 2009; MASUELLI et al. 2009; LARROSA et al. 2012). Interspecific hybridization has also been extensively used in potato breeding around the world (ROSS 1986; JANSKY et al. 2013) and international efforts are being placed on wild potato germplasm conservation and use in pre-breeding (GLOBAL DIVERSITY TRUST 2012).

Gene flow in flowering plants can be either minimized or prevented by external and/or internal breeding barriers. Internal barriers – residing in the plant tissues themselves – are classified as pre- and post-zygotic depending on their occurrence, respectively, either at the pollen–pistil or at the embryo, endosperm or other levels (HADLEY and

OPENSHAW 1980; CAMADRO et al. 2004). Internal barriers are under genetic control; it has been proposed that their controlling genes are multiallelic and that they can segregate in meiosis, providing the opportunity for gene flow between otherwise incompatible populations (CAMADRO and PELOQUIN 1980; EHLENFELDT and HANNEMAN 1988; CAMADRO and MASUELLI 1995). A very frequent post-zygotic barrier is abnormal endosperm development. It has been proposed that this barrier is controlled by genetic factors (EBN = Endosperm Balance Numbers) (JOHNSTON et al. 1980). Otherwise pollen–pistil compatible plants can produce viable seeds if they have the same EBN or, if their EBN is different, when the plant with the lower EBN produces  $2n$  gametes (MOK and PELOQUIN 1975). This type of gametes or gametophytes with the sporophytic chromosome number are produced by either pre-meiotic, meiotic or post-meiotic alterations, that can occur occasionally or be under genetic control (CAMADRO 1986).

In the Netherlands, the biological containment of the common potato was assessed by establishing its crossability with two wild related non tuber-bearing species, *S. dulcamara* and *S. nigra*, under various environmental conditions (EIJLANDER and STIEKEMA 1994). It was concluded that gene flow by pollen dispersal from potato to its most common wild relatives in western Europe was highly unlikely. However, as previously stated, wild potato species in the Americas are frequently weeds of the potato crop and all potential pollinators are present in the various environments. It has been demonstrated that viable and fertile hybrids can be formed between wild and cultivated potatoes in partially or fully compatible genotypic combinations (CAMADRO et al. 1998; RAIMONDI and CAMADRO 2003; CELIS et al. 2004; SCURRAH et al. 2008).

For Argentina, there is a recent paper on gene flow assessment between a transgenic cultivated genotype ( $4x$ ) and a diploid wild potato; based on negative results of a field experiment, the authors concluded that the probability of hybridization in the field is extremely low (BRAVO-ALMONACID et al. 2011). But it is necessary to point out that genotypically highly diverse wild potatoes grow spontaneously along the territory, sometimes in sympatry with the crop, and that spontaneous hybrids between wild and cultivated potatoes have been reported (JACKSON and HANNEMAN 1999; SCURRAH et al. 2008). As an example, BRUCHER (1955) observed plants with  $2n = 2x = 24$  and  $2n = 3x = 36$  in the surroundings of fields cultivated with *S. tuberosum* ssp. *andigenum* ( $2n = 4x = 48$ ) in the high Andean region, which were probably the result of hybridization processes that were taking place since remote times.

The knowledge of the dynamics of gene flow in centers of origin or diversity is a crucial instrument to evaluate potential risks of experimental trials and transgenic crop

production in those areas. Thus, the objective of this research was to determine minimum gene flow distances between one commercial cultivar and the closely related wild species *S. chacoense* Bitter under experimental field conditions in Argentina. The results complement those of a previous experiment on gene flow between two potato cultivars under the same experimental conditions (CAPURRO et al. 2010), and provide a starting point for assessment of potential risks due to pollen-mediated gene flow in potatoes, in both natural and cultivated ecosystems.

## MATERIAL AND METHODS

### *Selection of species and genotypes*

For selecting sexually compatible genotypic combinations for establishing the field experiment, controlled hand-pollinations were made in the greenhouse in two growing seasons (2007 / 2008 and 2008 / 2009) using various commercial cultivars of *S. tuberosum* ssp. *tuberosum* (tbr,  $2n = 4x = 48$ ; 4EBN) as males and individual plants of various accessions of seven wild taxonomic species ( $2n = 2x = 24$ ; 2EBN) as females. Plants (genotypes) had to meet three requirements: 1) to be pollen–pistil compatible as females with tbr, 2) to produce  $2n$  eggs to circumvent the EBN barrier in the  $4x \times 2x$  crosses and 3) to exhibit differential molecular patterns. The reference materials are listed in Table 1.

The wild diploids were crossed as females with tbr, after emasculation. When seeded fruits were formed, chromosome numbers of the derived progenies were determined under a light microscope by following standard procedures of pre-treatment with 8-hydroxyquinolein, fixation in 3:1 (v/v, 98% ethanol: glacial acetic acid), hydrolysis in 1N HCl at 60°C, staining with basic fuchsin and squashing on a slide. Plant morphological phenotypes of parents and progeny, if any, were observed. DNA was extracted from individual plants according to HAYMES (1996). RAPD (random amplified polymorphic DNA; Biodynamics A series) and SSR (microsatellites; MILBOURNE et al. 1997) markers were assayed to further select one wild genotype, compatible with one tbr cultivar,  $2n$  eggs producer and with differential expression. The genotype that met the three requirements was in vitro cloned to obtain enough plants for establishing the field experiment.

### *Field experiment*

The field experiment was established in Balcarce, Buenos Aires province, Argentina ( $37^{\circ}45'51.04''S$ ,  $58^{\circ}17'28.41''W$ , 125 m altitude). The selected pollen donor (cv. Huinkul MAG) was placed in the center of the plot (in a  $10 \times 10$  m square, in 14 rows with a distance of 70 cm between rows and 20 cm between plants) and the

Table 1. Number of wild diploid species accessions, genotypic combinations with tetraploid cultivars and pollinated flowers, and number of fruits and seeds obtained with each pollinator in  $2x \times 4x$  crosses.

Wild species*♀	No. accessions	No. pollinated flowers	No. genotypic combinations	No. fruits and seeds obtained with each ♂ parent (4x, 4 EBN)			
				Huinkul MAG	Pampeana INTA	Innovator	Calén INTA
<i>S. chacoense</i>	14	901	339	2 (3, 5)**	9	1	12
<i>S. gourlayi</i>	10	335	139	1	0	0	0
<i>S. infundibuliforme</i>	6	282	103	0	0	0	0
<i>S. microdontum</i>	3	203	87	0	0	0	—
<i>S. tarijense</i>	1	101	32	0	0	***	—
<i>S. venturii</i>	1	96	38	0	0	—	—
<i>S. acaule</i>	1	64	27	0	0	—	—

\*All  $2x$ , 2EBN, except *S. acaule* (4x, 2EBN)

\*\*seeds in parenthesis

\*\*\*cross not performed.

selected pollen receptor (one cloned genotype of *S. chacoense*) was transplanted in the periphery, in patches of  $2 \times 2$  m at 10, 20, 30 and 40 m away from the center, at a density of 16 plants  $m^{-2}$ . Four alleys determined four quadrants: North, South, East and West, for the purpose of this experiment (Fig. 1). There were no potato fields at least at 2000 m away from the experiment; only soybeans were grown in the near vicinity in the first year and corn in the second.

No spontaneous weeds were allowed to grow in the experimental field and spontaneous *S. chacoense* plants were kept at a minimum of 70 m away from the farthest experimental distance. The activity of potential pollinat-

ing insects was observed during flowering. The cultivar was managed as a perennial by not harvesting the tubers at the end of the first growing season.

Pollen samples were taken twice a week during flowering to indirectly estimate viability by staining with 1% acetocarmine. Flowers were sampled from at least two plants/patch of the pollen receptor and five plants of the pollen donor; pollen was collected in the laboratory, stained and observed under a light microscope. Twice a week, along the flowering period, recently formed berries in the receptor plants were wrapped with cotton mesh. Forty days after the last wrapping, berries were collected and seeds were harvested in the laboratory.

Seeds were washed and disinfected with a 20% common bleach solution. After treatment overnight with 1500 ppm  $GA_3$ , seeds were sown in petri dishes. Derived seedlings were transplanted into pots in the greenhouse. In seedlings that closely resembled the artificial hybrids obtained during the initial selection process, chromosome counts were carried out to identify tetraploid progeny, if any. Molecular analyses were performed, as previously described, in the identified individual tetraploid plants.

## RESULTS AND DISCUSSION

### Species and genotype selection

Over both seasons (2007 / 2008 and 2008 / 2009), 765 wild species  $\times$  tbr genotypic combinations (1982 pollinated flowers) were made to detect sexual compatibility. Average pollen viability of the male parents was 86%. Six of combinations involving *S. gourlayi* and *S. chacoense* produced tetraploid hybrid seeds. One *S. chacoense* genotype was selected as female parent for the field experiment because it had been collected locally

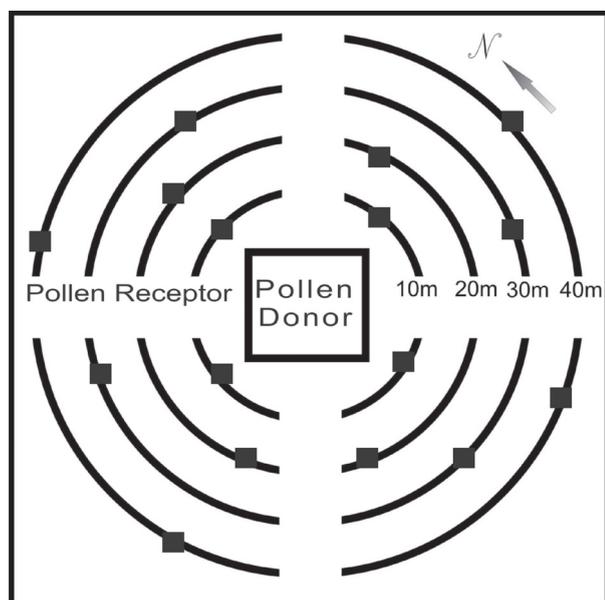


Fig. 1. Field design; boxes identified the position of the *S. chacoense* patches.

(and, thus, abundant flowering was expected), was compatible with cv. Huinkul MAG (already planted as the pollen donor in a previous cultivar to cultivar field experiment) and exhibited differential molecular profile.

Cloned plants obtained by micropropagation of the selected *S. chacoense* genotype were transplanted into the field in November after rustication in a greenhouse.

Genotypes of pollen receptor, *S. chacoense*, and pollen donor were chosen because they were pollen–pistil compatible. Moreover, the pollen receptor was selected for 2n eggs production for circumventing the post-zygotic endosperm barrier in the  $2x \times 4x$  cross, if there were no embryo barriers. The gametophytic self-incompatibility system of *S. chacoense* prevented selfing and crossing among the in vitro cloned plants.

#### Field experiment data

Flowering period of the pollen donor extended from 30 November to 25 December in the first season and from 1 December to 2 January in the second. Pollen viability along that period in the pollen donor varied from 85 to 96% in the first season and from 82 to 95% in the second; for the receptor the flowering period extended from 9 December to 13 February in the first season, and from 13 December to 22 February in the second season.

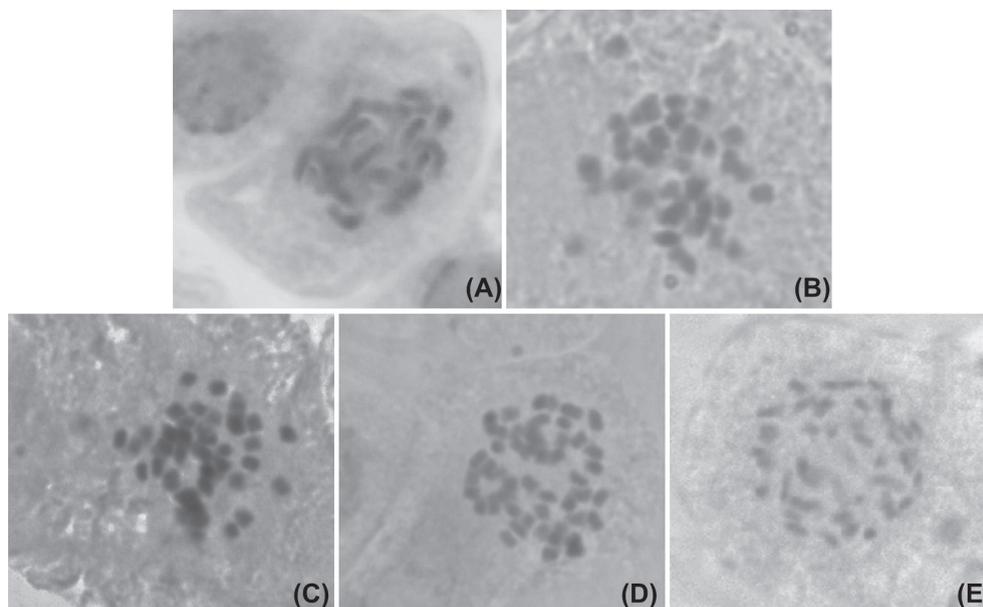
In summer 2009 / 2010 and summer 2010 / 2011, 479 and 798 berries were collected, respectively, in the pollen receptor plants, in each quadrant and at every distance from the pollen source. The total number of berries harvested in both seasons were, respectively, 116 and 172 at

10 m, 150 and 272 at 20 m, 69 and 98 at 30 m, and 144 and 256 at 40 m, being 127 and 141 the average numbers of seeds/berry. All harvested seeds were sown in petri dishes and then transplanted into pots in the greenhouse. At the four-leaf stage, the derived plantlets were screened according to morphological phenotypes. Three of the plantlets obtained from one berry that had been harvested in the northern quadrant and at 30 m from the center in the 2009/2010 season, were morphologically similar to the artificial hybrids (obtained by hand pollination) and were  $2n = 4x = 48$  (Fig. 2). A sample of plantlets that differed morphologically from the artificial hybrids were used as checks; all of them were  $2n = 2x = 24$ . In the 2010/2011 season, no tetraploid progenies were identified.

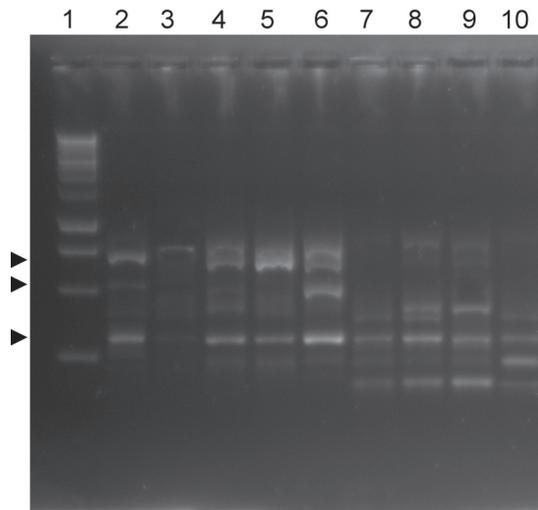
#### DNA analysis

The SSR electrophoretic profiles were inconsistent in *S. chacoense*. Thus, RAPD markers were used for the analysis of the three tetraploid plants, the pollen donor and the pollen receptor.

Primer 2 was selected because the differential banding was reproducible in both, pollen donor and receptor. In the pollen donor, three differential bands were generated at 115, 205 and 290 bp positions, using a 100 bp ladder (1:10), that allowed hybrid characterization. Banding patterns of pollen donor, pollen receptor and resulting progeny, and a bulked sample of eight *S. chacoense* plants per quadrant, are presented in Fig. 3. All  $4x$  plants derived from seeds harvested on the pollen receptor had at least one band from the pollen donor (Fig. 3). The most frequent



**Fig. 2A–E.** (A) mother (*S. chacoense*;  $2n = 2x = 24$ , 2EBN); (B) father (*S. tuberosum* spp. *tuberosum*;  $2n = 4x = 48$ , 4EBN); (C), (D) and (E): tetraploid interspecific hybrids ( $2n = 4x = 48$ ; 4EBN).



**Fig. 3.** RAPD banding pattern for primer 2: 100 bp ladder (1); pollen donor (2), pollen receptor; (3) tetraploid progenies (4–6); bulked DNA from eight *S. chacoense* plants per quadrat (7–10). Arrow heads indicate differential bands (125 bp, 210 bp and 275 bp).

pollinating insect was the large size bumble bee (*Bombus* spp.), which was very active during both season.

#### Pollen-mediated gene flow

Pre-zygotic breeding barriers in potato can be either complete, incomplete or absent, both within and between taxonomic species, which have been defined on the basis of morphological phenotypes in relation to holotypes, and not on breeding relations. As previously described, the experiment involved two steps: 1) selection of pollen donor and pollen receptor genotypes, which was based on results of controlled pollinations carried out in a greenhouse, and 2) establishment of the two selected genotypes in the experimental field, in which natural pollination took place. In the first step, different genotypes (that is, different plants belonging to accessions of different taxonomic species) were used as female parents with various male parents, with the objective of identifying pollen–pistil compatible genotypic combinations. The number of pistils that were hand-pollinated per plant was set at the beginning; furthermore, only pistils that were receptive (according to the stage of flower bud development) were pollinated. Then, female parents that were pollen–pistil compatible with a 4x pollinator were selected for 2n egg production under greenhouse conditions to circumvent the EBN barrier, which was expected to occur in the 2x 2EBN × 4x 4EBN cross. A relation could then be established between number of pollinated pistils and number of fruits, and between number of fruits and number of seeds (total number of seeds and number of seeds per fruit).

In the field experiment, pollinations were naturally carried out by insects, whose presence and activity is affected, among other factors, by environmental conditions during flowering (i.e. temperature, humidity, wind direction and strength). Moreover, only pollen grains deposited on receptive stigmas can undergo the progamic phase leading to double fertilization.

In our field experiment, the frequency of berries with hybrid seeds was low (0.2%), and they were obtained in only one of the two evaluated season. Notwithstanding, their occurrence give support to the hypothesis that pollen–pistil compatible 2x and 4x potato genotypes can hybridize in the field if post-zygotic barriers are either absent or can be circumvented.

The results of the field experiment regarding the efficiency of pollination cannot be compared to those of the selection step because, in the former, only one compatible genotypic combination was established, the numbers of receptive flowers per patch and receptive flowers that were actually insect-pollinated cannot be estimated, and the environments were different. Likewise, they cannot be compared to others reported in the literature due to similar considerations, that make the results obtained in a given environment not necessarily extrapolable to other(s).

As an example, in Balcarce and in one season, BRAVO-ALMONACID et al. (2011) assessed gene flow between a transgenic potato derived from cv. Spunta (donor) and genotypes of *S. chacoense* (receptors). The field design consisted of a central plot with the pollen donor, surrounded by rows of the receptor, at distances of 0.2 to 50 m. They obtained negative results after screening thousands of seedlings. It is to be noticed that 1) the pollen donor, cv. Spunta, is known for its very low pollen viability, 2) pollen receptor genotypes had been selected neither for pollen–pistil compatibility nor 2n eggs production, as was the genotype used in our investigation, and 3) the total number of genotypes (individual plants derived from seeds) set in the experimental field was 280; this is, at most, 280 genotypic combinations, a number much lower than the one we tested for selecting the genetic materials for the field experiment.

In Perú, SCURRAH et al. (2008) worked with various Andean cultivated potatoes in farmers' fields, in which native wild potatoes had been included. They demonstrated that hybridization between wild and cultivated germplasm actually occurred under natural conditions and that hybrid progenies can be successful in nature. However, their field design did not allow the estimation of gene flow distances.

The suggested isolation distance of 20 m of CONNER and DALE (1996) for the environmental conditions of New Zealand appears to be inadequate for Argentina, since functional pollen dispersal over at least 30 m has been proven in our experiment. Also, BROWN (1993) has

proposed an isolation distance of 80 m for diploid potatoes. Since pollination in potatoes is carried out mainly by bumble bees, potato pollen transfer over larger distances is possible (SCHITTENHELM and HOEKSTRA 1995). Therefore, a minimal required isolation distances of 100 m or more would be more suitable for the environmental conditions of our country. Notwithstanding, isolation distances could be insufficient to prevent gene flow and thus, complementation with more stringent prevention measures such as the incorporation of male sterility genes along with the transgene(s) of interest should be considered.

*Acknowledgements* – This paper is part of the first author's doctoral thesis. This work was financed by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Plurianual Research Project 112 20080100116) and Universidad Nacional de Mar del Plata (UNMDP, project AGR 283/09). The infrastructure and experimental field were provided by Instituto Nacional de Tecnología Agropecuaria (INTA).

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