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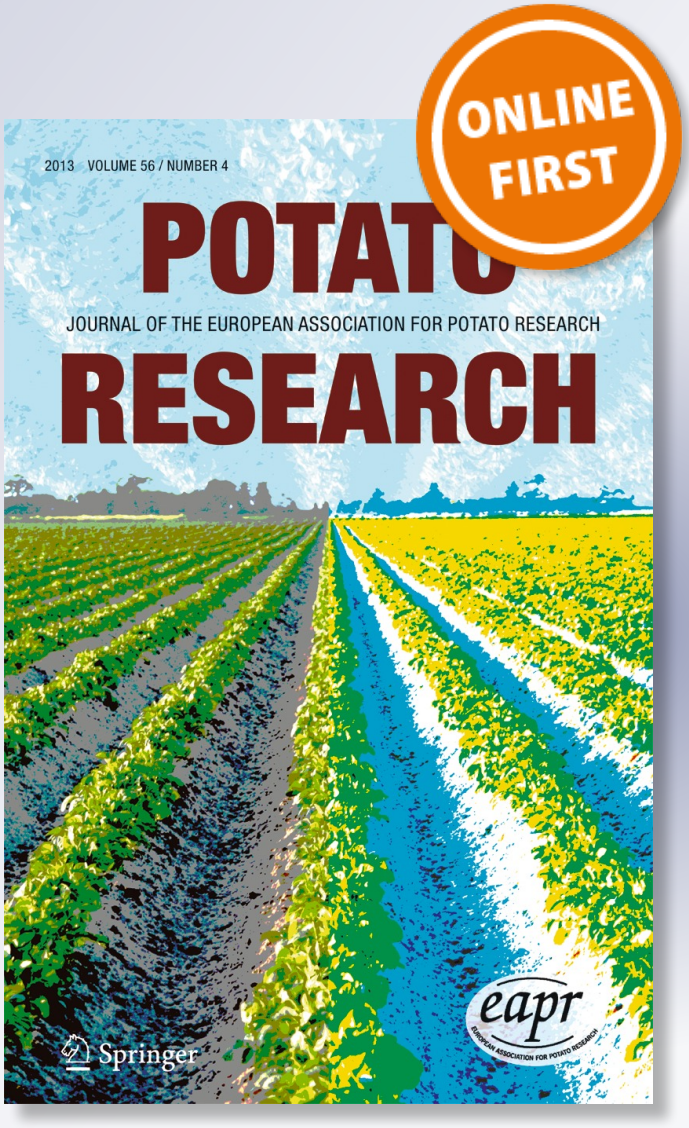

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Abstract Wild and cultivated potatoes form a polyploid series with $2n = 2x$ to $2n = 6x$ ($x = 12$). In nature, they are separated by external and/or internal hybridization barriers that, when incomplete, provide opportunities for gene flow and introgression. Isolation distances estimated in one environment are not necessary extrapolable. As a starting point for pollen-mediated gene flow risk assessment in potatoes, an experiment was set up in the field in one of the major potato growing area in Argentina, with two pollen-pistil compatible tetraploid commercial cultivars with differential molecular marker patterns. The field design consisted of a 10×10 m central square with the pollen donor, surrounded by circles with a male sterile pollen recipient, set every 10 m up to 40 m. The crop was managed as a perennial, and data were recorded over 2 years. Seeded berries were obtained in both years at 30 and 40 m away from the center; all of them contained hybrid seeds as revealed by electrophoretic profiles. We consider that a minimal required isolation distance of 100 m or more would be more suitable for preventing undesirable gene flow in the area.

Keywords Cultivated potato · Gene flow · Hybridization · Isolation distance

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Abbreviations

PCR	Polymerase chain reaction
SSR	Simple sequence repeat
RAPD	Random amplified polymorphic DNA

Introduction

Gene flow is the movement of genes from one population into another. It is an important evolutionary force that can lead to speciation (Mavárez et al. 2006; Mallet 2007) by generation of new gene combinations which can be eventually established in nature if they are favored by selection and become reproductively isolated from other distinct entities. In angiosperms, gene flow can take place by means of pollen, seeds, and/or asexual propagules. There are several examples of hybrid seeds originating from pollen-mediated gene flow between populations of the same species (intraspecific gene flow) (Ellstrand et al. 1989; Jenczewski et al. 1999; Ritala et al. 2002; Abbott et al. 2003; Arnaud et al. 2003; Chen et al. 2004; Van Deynze et al. 2005; among others) and between populations of different species (interspecific gene flow) (Chen et al. 2004; Levin and Kerster 1974; Arriola and Ellstrand 1996; Hansen et al. 2001; Warwick et al. 2003; among others).

The common potato, *Solanum tuberosum* ssp. *tuberosum* ($2n=4x=48$, 4EBN[1]), is one of the most important world crops, both in cultivated acreage and as source of carbohydrates and other nutrients in the human diet (FAOSTAT 2012). In Argentina, more than 90,000 ha are cultivated with potatoes each year (MinAgri 2012). The species is a tetraploid with tetrasomic inheritance and a narrow genetic base that slows genetic progress in breeding (Camadro 2010). However, it has a large number of wild closely related tuber-bearing species (collectively known as 'potatoes'), which are potential sources of desirable genes for breeding (Ross 1986). Around 35 of these wild species spontaneously grow in both undisturbed and disturbed habitats along the territory, sometimes as weeds of potato and other summer crops (Hawkes and Hjerting 1969; Erazzú et al. 2009).

The extent and probability of gene flow in flowering plants depend, in general, on the action of external and/or internal isolating barriers that can either prevent or hinder hybridization. The external barriers are, for example, separation in space (spatial); flowering phenology (temporal); type of habitat (ecological); and in allogamous species pollinated by insects, birds, or small mammals, floral structure (mechanical). The internal barriers, on the contrary, reside in the tissues of the plant itself; they can be classified as pre- and post-zygotic because they can occur, respectively, at the pollen-pistil level and at the level of embryo, endosperm or both tissues (Hadley and Openshaw 1980; Camadro et al. 2004). In potato, the internal barriers are under genetic control. On the basis of crossing data from various studies carried out over the years with a number of species, it has been hypothesized that the genes involved in both the pre- and the post-zygotic barriers are segregating (Camadro and Peloquin 1980; Johnston et al. 1980; Ehlenfeldt and Hanneman 1988; Camadro and Masuelli 1995; Camadro et al. 2004).

[1] EBN = Endosperm Balance Number

Pollen-mediated gene flow between cultivars under field conditions has been reported for various species, i.e., maize (*Zea mays*) (Luna et al. 2001; Goggi et al. 2007), sunflower (*Helianthus annuus*) (Arias and Rieseberg 1994; Ureta et al. 2008), and rose (*Rosa* sp.) (Debener et al. 2003). In potatoes, gene flow experiments have been carried out in Europe and New Zealand with transgenic cultivars as pollen donors and non-transgenic cultivars as pollen traps. In these experiments, the pollen donors (transgenic cultivars) were Desirée (McPartlan and Dale 1994; Skogsmyr 1994), Iwa (Tynan et al. 1990; Conner and Dale 1996), and Rua and Ilam Hardy (Conner and Dale 1996), and the pollen receptors (non-transgenic cultivars) were Iwa (Tynan et al. 1990), a mixture of breeding lines (Conner and Dale 1996), Desirée (McPartlan and Dale 1994), and Stina (Skogsmyr 1994). Also, gene flow experiments have been carried out but with non-transgenic cultivars (Desirée and British Queen) (Petti et al. 2007). The results were very variable regarding the formation of hybrid seeds in the various field experiments: from a long distance (1,000 m) from the pollen donor with the larger percentage (72%) in the near vicinity (Skogsmyr 1994) to short distances up to 9 m (Tynan et al. 1990), 10 m (Conner and Dale 1996; McPartlan and Dale 1994), and 21 m (Petti et al. 2007), but at very low rates. In diploid potatoes, gene flow up to 80 m away from the pollen source has been reported (Schittenhelm and Hoekstra 1995).

The variability in the reported data calls the attention to the necessity of determining minimum isolation distances in areas in which experimental or commercial fields with transgenic potatoes are grown in overlap with either one or both, non-transgenic potatoes and wild-related species. As an example, the Secretary of Agriculture of Argentina has authorized the evaluation of 32 transgenic events in potato since 1991, five in the greenhouse and 27 in the field, using an isolation distance of 10 m (CONABIA 2011). These authorizations are granted by taking into account, among other considerations, isolation distances used in other countries for transgenic and non-transgenic crops, because gene flow and risk assessment studies have not been carried out locally, except for a recent publication (Bravo-Almonacid et al. 2011), in which an extremely low probability of gene flow was suggested. It is, however, important to collect local data for establishing isolation distances because pollination in potatoes is naturally carried out by insects. The presence, number, and activity of pollinating insects at a given site during flowering are affected, among other factors, by environmental conditions such as temperature, humidity, and wind direction and strength.

In Balcarce, investigations were initiated to determine pollen gene flow between two tetraploid commercial potato cultivars and between one of them and an endemic wild diploid-related species. Since the local data are scarce, and as a starting point, we conducted a field experiment to detect if hybridization was possible over distances up to four times larger than the isolation distance established by regulatory agencies in Latin America. In this paper, we report the results of a cultivar to cultivar field experiment. The final objective of the initiated investigations is to develop—in the near future—gene flow models between cultivars (transgenic and non-transgenic) and between these and wild potato species in both directions of the cross, for risk assessment in both natural and cultivated ecosystems. These models would be of value for countries that are centers of origin or diversity of crop wild relatives and also crop producers.

Materials and Methods

Field Experiment

For the field experiment, 'Huinkul MAG'—a highly male fertile Argentinean cultivar—was used as pollen donor, and 'Spunta'—a highly male sterile Dutch cultivar, compatible with the donor and with differential SSR and RAPD banding patterns—was used as pollen recipient. Both non-transgenic cultivars are commonly grown in the area.

The field experiment was established at the Estación Experimental Agropecuaria (EEA) Balcarce of the Instituto Nacional de Tecnología Agropecuaria (INTA) in Balcarce (37° 45′ 51.04″ S, 58° 17′ 28.41″ W, 125 m altitude), Buenos Aires province, Argentina. The experimental station is located in one of the most important potato producing areas of the country. The pollen donor was placed in the center of the plot (in a 10×10 m^2, in 14 rows with a distance of 70 cm between them and 20 cm between plants) and the pollen recipient in the periphery, in discontinuous circles at 10, 20, 30, and 40 m away from the center (two rows in each circle and the same arrangement as the central square) (Fig. 1). The maximum distance between the plot center and the farthest circle was established taking into account the minimum legislated distance (10 m) and the availability of cultivated land for the experiment. Four alleys determined four quadrants, denominated North, South, East, and West for the purpose of this experiment with the nearest potato field at a distance of 2,000 m away and with soybean and corn crops growing in the vicinity, respectively, in the first and the second year.

Tubers were planted at different times in the mid-Spring months of 2008 to ensure simultaneous flowering of donor and recipient plants. Tubers of the pollen donor were planted on October 22th and 27th, and November 3rd, 7th, and 12th (three 10-m-long

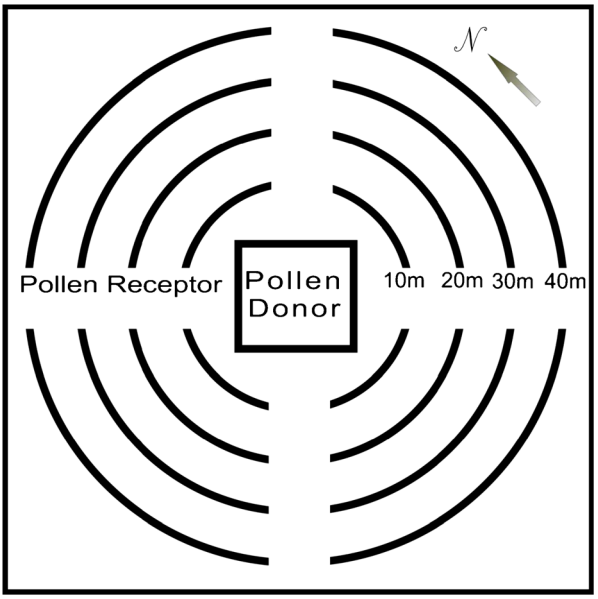

Fig. 1 Field design for estimating gene flow distances. *Arrow* indicates the North

rows/date), whereas tubers of the pollen recipient were planted on October 27th and November 7th (one row per date in each discontinuous circle away from the central plot). Spontaneous weeds, including the endemic diploid wild potato *Solanum chacoense* Bitter, were allowed to grow between circles. Supplementary irrigation was provided once a week and agrochemicals were applied as needed, but taking special care that the frequency and time of applications would not negatively affect the pollinating insects. To control *Epicauta aspersa* ('Bicho Moro,' a blister-beetle), cipermetryne was applied only when the foliage damage became visible; to control *Phytophthora infestans* (late blight), 80% Mancozeb was applied when the climatic conditions favored the development of the pathogen. Observations on the activity of potential pollinating insects were taken during the flowering periods.

The environmental conditions were monitored with a Davis VantagePro 2 weather station situated in the proximities of the field plot. Data were recorded from the beginning to the end of flowering, as the average of every hour.

The cultivars were managed as perennials by not harvesting the tubers at the end of the 2008/2009 growing season. Thus, data were obtained in two seasons (environments): 2008/2009 and 2009/2010.

Pollen Viability

Pollen samples were taken twice a week during the flowering period to estimate viability by staining. Flowers were sampled from at least two plants of cv. 'Spunta' (pollen recipient) per quadrant and per row and five plants of cv. 'Huinkul MAG' (pollen donor); fresh pollen was collected in the laboratory from individual flowers and stained in a drop of acetocarmine (2 g carmine in 45 ml of 45% acetic acid plus 55 ml H_2O) on a glass slide and covered with a cover slip. Around 200 pollen grains were scored in each slide; those plump and fully colored were considered viable.

Berry Identification and Harvest

During flowering, the experimental plot was visited twice a week to visualize recently formed berries on recipient plants; these berries were tagged—recording date, quadrant, and distance from the pollen source—and wrapped with a cotton mesh. Berries were collected 40 days after the last wrapping and seeds (if any) were counted. Following, seeds were surface sterilized with a 5% sodium hypochlorite solution, treated overnight with 1,500 ppm GA_3, and placed to germinate in Petri dishes with humid filter paper in a chamber at 23 °C, with an 18 h light photoperiod. Seedlings were transplanted into pots in a greenhouse, with a 3:1 *v/v* mixture of soil and peat moss. Leaves were collected from the seedlings to obtain DNA samples.

In 2009, we worked on the experimental field plot planted in 2008, following an identical methodology, except for the fact that spontaneous weeds were mechanically controlled (cut at 8–10 cm height to prevent flowering). This control was carried out because a cloned genotype of *S. chacoense* was incorporated in discontinuous circles at various distances away from the central plot to obtain gene flow estimates from the cultivated pollen donor to the wild species using the same experimental plot (Capurro et al. 2013).

DNA Extraction, PCR Analyses and Primer Selection

To detect hybrid progeny, we analyzed nuclear random amplified polymorphic DNA (RAPD) using decameric primers from Biodynamics (Argentina) and microsatellite (SSR) markers: STM 0019, STM 1020, STM 1045, STM 0019, STM 2005, STM 1104, STM 3016, STM 2022, STM 1106, STM 0052, and STM 1049 (Milbourne et al. 1997). The molecular RAPD and SSR analyses were performed in both cultivars and progenies, and a bulked sample of eight spontaneous plants (growing as weeds in the first year) of *S. chacoense* from each one of the four quadrants (32 in total) was also analyzed with RAPD markers to discard unwanted pollen from the wild endemic relative.

DNA was extracted according to Haymes (1996), and its concentration and quality were estimated using a spectrophotometer (BioRad Smart Spec 3000). The amplification reaction was carried out in a volume of 25 μl consisting of 1X reaction buffer (Promega); 2 mM ClMg; 100 mM of each dNTPs; 200 nM of the corresponding RAPD primer; 0.5 U of GoTaq Polimerase (Promega); and 40–60 ng of DNA. The thermocycling profiles were 3 min incubation at 94 °C, followed by 45 cycles at 94 °C for 30 s, 40 °C for 1 min, and 72 °C for 2 min and a final elongation at 72 °C for 5 min. Following the amplification, 12 μl from each PCR reaction was run in a 1.5% *m/v* agarose gel at 80 V for 55 min; the markers used were a 1 kb and a 100 bp ladder (1:10). The RAPD reaction was adjusted to obtain clear and defined bands in 1.5 agarose gels. The SSR products were run in 6% acrylamide gel in a Gibco S2 sequencing gel electrophoresis apparatus. Electrophoresis was carried out at 85 W for 2.5 h using a 100 bp molecular marker and silver-stained.

Results

Flowering and Pollen Viability

The overlapping flowering period of pollen donor and recipient extended from December 12th to January 1st in the 2008/2009 season and from November 30th to December 25th in the 2009/2010 season. Plants in the circle closer to the center of the plot flowered very poorly in both years. Pollen viability along the flowering period in the pollen donor varied from 77 to 95% in 2008/2009 and from 85 to 96% in 2009/2010; on the contrary, almost no pollen production was detected in the recipient plants in both years and the few pollen grains that were observed were, apparently, inviable.

Berry and Seed Harvest

In summer 2009, 115 berries were collected in the pollen recipient plants, at every distance from the pollen source and in each quadrant. Only three of these berries had seeds (18, 21, and 22 seeds/berry, respectively): One of them was collected in a circle situated 30 m away from the center of the plot, in the North quadrant, and two were collected in circles at 40 m from that center, in the North and South quadrants, respectively. In summer 2010, a total of 36 berries were formed on the recipient plants.

Only four of them—three collected in the East quadrant at 30 m from the pollen source and one in South quadrant at 40 m from that source—had seeds (36, 41, 48, and 70 seeds/berry, respectively).

DNA Analyses

For the SSR analysis, DNA electrophoresis was carried out in a sample of three seedlings from each of the seven berries harvested over the two growing seasons. The selected primer was STM 2022, because it generated differential bands in cv. 'Huinkul MAG' (pollen donor) and cv. 'Spunta' (pollen recipient). In the pollen donor, five bands were generated at the 185, 190, 195, 242, and 259 bp positions, whereas two bands (at 190 and 195 bp) were generated in the recipient. The banding patterns of the pollen donor, pollen recipient, and resulting progeny are presented in Fig. 2.

For RAPD analysis, DNA electrophoresis was carried out in bulked samples of eight seedlings from each one of the seven berries that were harvested over the two growing seasons. Primers 1 and 6 were selected because they generated reproducible differential bands in cv. 'Huinkul MAG' (pollen donor) and cv. 'Spunta' (pollen recipient). In the pollen donor, five bands were generated with primer 1 (380, 400, 550, 700, and 910 bp positions) and six with primer 6 (300, 350, 410, 500, 550, and 750 bp positions), respectively. In the recipient, three bands were generated at the 380, 550, and 650 bp positions with primer 1 and at the 300, 400, and 550 bp positions with primer 6. The banding patterns of the pollen donor, pollen recipient, and resulting progeny, as well as those of the 32 plants of *S. chacoense* from the field are presented in Fig. 3. All plants derived from the harvested seeds had at least one paternal band.

Environmental Conditions

The 2008/2009 growing season was extremely dry due to the effects of La Niña current. While the average rainfall had been 418 mm for the last 20 years, the rainfall in the 2008/2009 season was 156 mm. The rainfall for the 2009/2010 growing season was 317 mm, slightly lower than the average. The supplementary irrigation was insufficient to revert the water deficit. As a consequence, flowering was scarce in both seasons, but mainly near the center of the plot where the automatic irrigation system was placed and water was not homogeneously delivered. During flowering, winds blew predominantly from the NE, N-NW, and N-NE directions in the first season and from the N-NW, N and N-NE directions in the second.

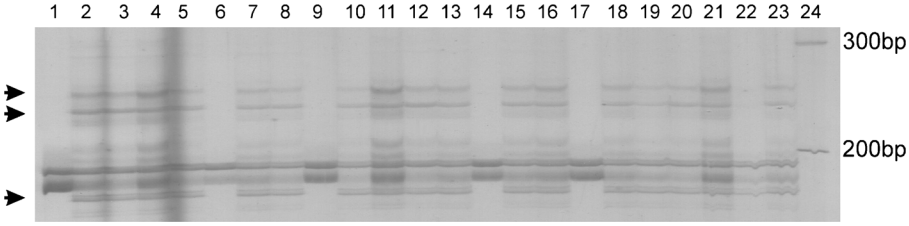

Fig. 2 Banding patterns for STM 2022 of pollen recipient (*1*), pollen donor (*2*), and samples of progenies obtained in the 2009/2010 (3–14) and 2008/2009 (15–23) seasons; ladder (24). *Arrows* indicate the differential paternal bands

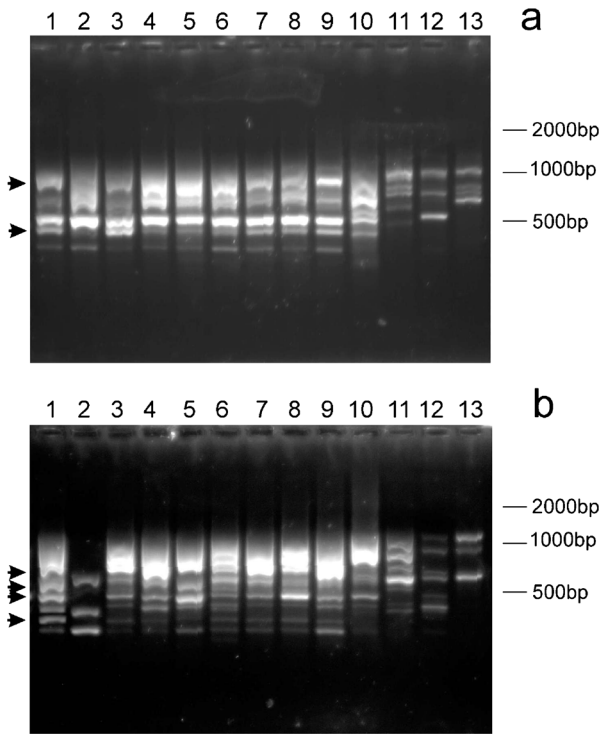

Fig. 3 RAPD banding patterns: (**a**) primer 1 and (**b**) primer 6 of the A series (Biodynamics, Argentina). Pollen donor (*1*), polen recipient (*2*), bulked DNA of eight seedlings of each of seven berries (3–9), bulked DNA of eight plants of *S. chacoense* per each quadrant (10–13)

In the first season, an important activity of pollinating insects was observed, and they seemed attracted by weeds, which grew abundantly between circles and topped the crop at flowering. In the second season, this problem was overcome by trimming weeds at a low height. The most frequent insect visitor was a large size bumble bee (unidentified species of *Bombus*).

Discussion

Data were obtained in two growing seasons that were unusually dry. Particularly in 2008/2009, the supplementary irrigation was insufficient to revert the water deficit. Consequently, the crop and the surrounding weeds suffered from drought stress, and the activity of the pollinating insects was negatively affected. Notwithstanding, hybrid seeds were obtained at various distances from the center (pollen source), including in the circle 40 m away. No relation was found between the direction of the predominant winds and berry formation.

In potato cultivars, it is very difficult to estimate with certain precision—as has been done in maize and sunflower (Goggi et al. 2007; Arias and Rieseberg 1994)—the percentage of flowers that were cross-pollinated and produced hybrid seeds in relation to the number of available flowers. The reason is that potato plants have multiple stems

derived from the vegetative buds of the unit of propagation (one tuber or tuber cut with usually more than one vegetative bud); thus, inflorescences are not formed simultaneously and, at a given time, one plant can have vegetative stems and inflorescences at different physiological stages. The flowering period in the geographical location of the experimental plot usually lasts from 20 to 30 days.

We consider that the field design used in this experiment—with the pollen recipient planted in concentric circles around the pollen donor in the center of the field plot—allows better estimations of gene flow in the field than other designs reported in the literature (Arias and Rieseberg 1994; Ureta et al. 2008; Skogsmyr 1994; Bravo-Almonacid et al. 2011). In our design, all wind directions were covered (wind direction and strength can affect foraging of pollinating insects) and also data were obtained in 2 years with and without the presence, respectively, of weeds that could distract the activity of the pollinating agents on the cultivars. Hybrid seed production in the recipient plants by 2n pollen functioning from the weedy *S. chacoense* in the first year cannot be discarded. However, we consider that the probability of such event is low, since 2n pollen producing plants have to be also pollen-pistil compatible with the pollen donor. In this regard, we screened 14 accessions of *S. chacoense* as female parents for compatibility with cv. Huinkul MAG in another gene flow study and obtained only two fruits, with three and five seeds, respectively, from 901 pollinated flowers in 339 genotypic combinations (Capurro et al. 2013).

The pollen recipient, cv. 'Spunta', is male sterile and, thus, progeny screening was facilitated because selfed and intra-cultivar seed production was prevented. A possible drawback of using a male sterile recipient is that pollinating insects are attracted by the bright yellow color of fertile anthers; thus, they are not expected to be as well attracted to pale yellow anthers, such as those of cv. 'Spunta'. Brown (1993) reported that the number of seeds per berry in potatoes was negatively correlated with the rate of outcrossing and, also, that the population dynamics of 'buzz' bees is one of the variables that can affect outcrossing rates between locations and years. In the Brown's (1993) experiment, less than 90 seeds/berry were obtained in the male sterile clones; whereas in our experiment, the number was lower in the first year but approached the number reported in the second year. These differences between years could be the result of the very dry environmental conditions particularly in the first year that affected flowering and the activity of the pollinating insects as previously mentioned.

Pollen flow between potato populations in nature depends mainly on bumble bee pollination, because this insect can typically forage even at high altitudes (over 1,500 m) (Walther-Hellwig and Frankl 2000). The presence of weeds between circles apparently did not have an important effect on pollen dispersal by the pollinating insects—which can fly over long distances without stopping on intermediate plants along their way—because the number of berries with seeds formed in each year was rather similar. The pollen donor and recipient had been previously selected for differential electrophoretic banding patterns. Only a few seedlings in the samples from both seasons did not exhibit the paternal RAPD banding pattern; this could be due to either (1) segregation, if the pollen recipient was not quadruplex for the marker, or (2) intra-cultivar pollinations, if a few pollen grains from Spunta were actually viable.

The distance between plants or populations in the field is an important factor to prevent or reduce undesirable pollen gene flow, but it must be complemented with other precautionary measurements. For transgenic crops, in particular, male sterility appears

to be the most adequate precautionary measure, but it has to be taken into account that gene flow may take place in either one or both directions of a cross and, also, through the movement of seeds and asexual propagules in the landscape.

This report constitutes a first step in the understanding of gene flow and the dynamics of hybridization and eventual introgression in potatoes in Argentina, which is the center of origin or diversity of a large number of related wild species, some of which behave as weeds of the crop. Since only cultivated genetic materials were used in our experiment, it is necessary to carry out additional field studies to assess the associated risks and benefits of using transgenic technology in areas of overlapping transgenic and non-transgenic potatoes and their related wild species. The results of a field experiment under the same environmental conditions, with a non-transgenic commercial cultivar and the wild diploid potato *S. chacoense* (which presents incomplete hybridization barriers with the common potato) has been recently reported in another paper (Capurro et al. 2013).

The suggested isolation distance of 20 m (Conner and Dale 1996) for the environmental conditions of New Zealand appears to be inadequate for Argentina. Functional pollen dispersal over 40 m has been proven to occur in our experiment. Pollination in potatoes is carried out mainly by bumble bees, which can forage around 100 m from their nests (Walther-Hellwig and Frankl 2000), and gene flow in diploid potatoes has been shown to occur up to 80 m from the pollen source (Schittenhelm and Hoekstra 1995). Thus, we consider that minimal required isolation distances of 100 m or more would be more suitable to prevent undesirable gene flow.

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