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Meiotic abnormalities underlying pollen sterility in wild potato hybrids and spontaneous populations

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ABSTRACT

Wild potato species are widely distributed in the Americas, where they spontaneously grow in very diverse habitats. These species – with low chromosome differentiation – form polyploid series with 2n = 2x, 3x, 4x and 6x (x = 12). They are isolated in nature by external and internal hybridisation barriers that can be incomplete, allowing hybridisation in areas of sympatry. Nevertheless, most accessions in germplasm banks, regardless of genetic background of the sampled spontaneous populations, have been assigned specific categories based on morphological characters. To further investigate the extent of hybridisation in the group and for comparative purposes, pollen viability was estimated in (i) artificial hybrids between a commercial cultivar (Calén INTA) of the common potato (tetraploid *Solanum tuberosum* ssp. *tuberosum*) and the tetraploid cytotype of the related wild species *S. gourlayi*, and (ii) samples of plants (accessions) and inflorescences of natural populations from Argentina, tentatively classified as 'presumed hybrids' (*S. infundibuliforme–S. gourlayi*) and 'species' (*S. infundibuliforme*, *S. gourlayi* and *S. chacoense*). Regardless of origin, 98 out of 103 plants analysed had zero to 70% pollen viability (zero to 40% in eight of them). Pollen grains were of variable size and morphology and, in mostly male sterile plants, the only viable pollen grains were 2n and/or 4n. Furthermore, male sterile plants shared various abnormalities in meiosis I and II (unpaired chromosomes, unequal chromosome distribution, precocious/lagging chromosomes, parallel, tripolar, fused and multiple spindles, unequal size nuclei, dyads, triads and pentads in addition to normal tetrads, among others). These results provide novel evidence to support field observations of early potato botanists on the extent of spontaneous hybridisation in wild Argentinian potato populations, which is not reflected in the current taxonomy and has significant consequences for germplasm conservation and breeding.

INTRODUCTION

The common potato, *Solanum tuberosum* ssp. *tuberosum* L. (tbr, 2n = 4x = 48, 4 EBN), has a large number of related wild species that are widely distributed in the Americas (Hawkes 1990). These species, which grow spontaneously in very diverse habitats, form polyploid series with 2n = 2x, 3x, 4x and 6x (x = 12), with the diploid species being most frequent. Chromosome differentiation in the group is low, allowing pairing and recombination in hybrids (Ramanna & Hermsen 1979; Dvorak 1983; Masuelli & Camadro 1997). In fact, Matsubayashi (1991) recognised a common A genome that, modified structurally to a lesser or greater degree, gave origin to four additional homeologous genomes (B, C, D, E).

Wild potato species (collectively known as 'potatoes') have two alternative modes of reproduction: sexual (by seeds) and asexual (by stolons and tubers). They possess an S-locus gametophytic self-incompatibility system that ensures allogamy in the diploids; however, allogamous polyploids can be selfed if the pollen grains carry two different S-alleles, due to a phenomenon known as 'competition interaction' (see Frankel & Galun 1977).

In nature, potatoes are isolated by external and internal hybridisation barriers. The external barriers are mostly spatial and ecological (Hawkes 1963), whereas the internal barriers can be pre- and post-zygotic (see Camadro *et al.* 2004). Prezygotic barriers act at the pollen–pistil level, preventing pollen germination in the stigma or interfering with normal pollen tube growth in the style and/or ovary (Camadro *et al.* 2004; Bedogni & Camadro 2009; Erazzú *et al.* 2009). Among the post-zygotic barriers, endosperm abortion (and consequent embryo abortion) has been regarded as the most relevant (Johnston *et al.* 1980). However, embryo abortion can precede endosperm abortion due to incongruency between the uniting genomes (Masuelli & Camadro 1997), F_1 hybrids can be highly male sterile (Abdalla & Hermsen 1972a,b; Grun

1979; Santini *et al.* 2000, among others; see Camadro *et al.* 2004) and the F_2 and later segregating populations can break down (Hawkes & Hjerting 1969).

In wild potatoes, the pre-zygotic barriers can be incomplete due to segregation in pollen and pistil of genes involved in the incompatibility reactions (Camadro & Peloquin 1981; Bedogni & Camadro 2009; Erazzú *et al.* 2009). Among the post-zygotic barriers, that posed by the endosperm can be circumvented by the functioning of 2n gametes (Johnston *et al.* 1980), which are gametes or gametophytes with unreduced chromosome numbers formed by inherited cytological alterations in meiosis (Mok & Peloquin 1975; Camadro 1986; Peloquin *et al.* 1999). In F_1 hybrids, male sterility can be the result of genome differentiation in the parents or the action of nuclear and/or cytoplasmic genes (Grun 1979; Iwanaga *et al.* 1991; Ortiz *et al.* 1993; see Camadro *et al.* 2004). In fact, incompatibility, male sterility and endosperm have been regarded as substitutes of genome differentiation in evolution of the group.

Based on morphological field observations in collection expeditions over the years, various researchers (Brücher 1953; Correll 1962; Hawkes 1963; Ugent 1966; Spooner & Van den Berg 1992; among others) reported the occurrence of spontaneous wild potato hybrids in natural habitats. However, researchers have been in general reluctant to recognise their importance in evolution of the group, assigning mainly specific categories on the basis of plant morphology to accessions in germplasm banks, without taking into account either the population structure or the reproductive behaviour of the sampled populations at the collection sites (see Masuelli *et al.* 2009). The approach used to classify the wild germplasm is, however, of utmost importance for *in situ* and *ex situ* conservation of genetic resources, given that both pre- and post-zygotic hybridisation barriers can be acting, thus altering gene frequencies upon sexual reproduction, and also for evaluation of broad introgressive events and for parent selection in breeding programmes in which heterotic responses are to be explored.

To provide further evidence on the extent of spontaneous hybridisation in wild potatoes, we carried out a comparative study of pollen viability in artificial hybrids and samples of spontaneous populations (accessions and inflorescences) that were collected in their natural habitat and classified as 'presumed hybrids' and 'species' for conservation purposes in germplasm banks. The meiotic abnormalities underlying male sterility in these genetic materials, regardless of their classsification as 'species' or artificial/'presumed' hybrids, are also reported and discussed.

MATERIAL AND METHODS

Plant material

The genetic material was individual plants of (i) tetraploid hybrid families obtained by controlled reciprocal crosses carried out in our laboratory between the common potato, *S. tuberosum* L. ssp. *tuberosum* (tbr, 2n = 4x = 48; 4 EBN) cv. Calén INTA, and the tetraploid accession OKA 7547 of the wild potato species *S. gourlayi* Hawkes (grl, 2n = 2x = 24, 2 EBN and 2n = 4x = 48, 4 EBN), the latter provided by the Potato and Forage Germplasm Bank, INTA, Balcarce (PFGB), Argentina; (ii) wild potato accessions collected as plants/tubers in a site in NW Argentina, that had been tentatively classified as *S. gourlayi* (grl, ClI 1,707, CLl 1710, ClI 1712), *S. infundibuliforme* Philippi (ifd, Cll 1715, ClI 1717) and *S. infundibuliforme–S. gourlayi* hybrids (ifd–grl, ClI 1716), also provided by the PFGB; and (iii) samples of inflorescences of wild spontaneous diploid populations from NW Argentina, collected and tentatively classified as *S. chacoense* Bitter (chc, E 1 and E 5) by one of the authors (Table 1).

Pollen viability

Except for the chc populations, which grew spontaneously in nature, individual plants (genotypes) of the 'species'/artificial and 'presumed' interspecific (from here on, interspecific hybrids) were grown from either seeds or tubers. Seeds were germinated in Petri dishes and seedlings at the 2–4 leaf stage were transplanted into individual pots with a mixture of sterilised soil and sphagnum moss (3:1, v/v); tubers were planted in individual pots and, along with the seedlings, were

Table 1. Provenance of the genetic material and number of plants used for pollen analyses.

species[a]	population/family[b]	location[c]	geographic coordinates	altitude (m)	no. of plants used for pollen analyses
chc	E 1	San Miguel de Tucumán (T)	27°07′ S-65°19′ W	219	2
	E 5	San Andrés (T)	26°08′ S-65°15′ W	314	4
grl	Cll 1707	Humahuaca (J)	23°00′ S-65°27′ W	3650	6
	Cll 1710	Humahuaca (J)	23°00′ S-65°27′ W	3650	11
	Cll 1712	Humahuaca (J)	23°00′ S-65°27′W	3676	3
ifd	Cll 1715	Humahuaca (J)	22°59′ S-65°27′ W	3659	25
	Cll 1717	Humahuaca (J)	22°59′ S-65°27′ W	3653	2
ifd–grl	Cll 1716	Humahuaca (J)	22°59′ S-65°27′ W	3659	26
tbr × grl	cv. Calén × OKA 7547A	Tumbaya (J)[d]	23°36′ S-65°35′ W[d]	3300[d]	6
grl × tbr	OKA 7547 × cv. Calén	Tumbaya(J)[d]	23°58′ S-65°38′ W[d]	3500[d]	18

[a]chc: *S. chacoense*; grl: *S. gourlayi*; ifd: *S. infundibuliforme*; tbr: *S. tuberosum* ssp. *tuberosum*; tentative classification (Potato Germplasm Bank, EEA Balcarce INTA, and one of the authors).

[b]Collectors: OKA = Okada; Cll = Clausen and Ispizúa; E = Erazzú.

[c]J: Jujuy province; T: Tucumán province.

[d]corresponds to the wild progenitor accession.

grown in a screenhouse in the spring season. Agrochemicals were applied as needed throughout the growing cycle. At flowering, two flowers were removed from each plant to indirectly estimate pollen viability using a light microscope for pollen samples stained with safranine (Essad 1962). For the chc populations, inflorescences with flower buds at various stages were directly sampled in the field during a collection expedition; they were used for both pollen and meiotic analyses. R-software (R Development Core Team 2010), version 2.12.0, was used to represent pollen viability of individual plants within each population/family.

2n pollen production and abnormal pollen features

In 5–8 random microscopic fields (100×), a minimum of 200 pollen grains was scored for staining, shape and presence of abnormal features. Whenever pollen samples of heterogeneous size were observed, the percentage of pollen grains larger or smaller than the average pollen grain of the sample was recorded and taken as an indication of the formation of gametophytes with chromosome numbers differing from the expect n = x in diploids and n = 2x in tetraploids. For each 'species'/interspecific hybrid, and based on the volume of a sphere (roughly the volume of a potato pollen grain), pollen grains with diameters 1.26 times larger than the average of each species were considered to be 2n, those 1.59 times larger were considered to be 4n, and those smaller than the average were considered to be aneuploid or 'micro'-pollen grains. For each population/family, the percentage of pollen grains in each category was calculated by averaging the percentages (for the same category) recorded for individual plants. In this way, each plant makes an equal contribution to the total, regardless of the number of pollen grains that can eventually be screened.

At the end of the growing cycle, tubers of the tbr–grl hybrid plants were harvested for the repeat planting.

Meiotic analyses and mechanisms of 2n pollen formation

In the following season, tubers of the tbr–grl hybrid plants that had exhibited unexpected features in the previous season (*i.e.* pollen of heterogeneous size, 2n pollen and/or 4n pollen in addition to n pollen, pollen sterility, *etc.*) were planted in pots in the screenhouse. At flowering, buds at various developmental stages were fixed in a 96% alcohol:glacial acetic acid solution (3:1, v/v) for 24 h and then transferred to 70% ethanol until use. For microscopic observations, buds were rinsed with 45% glacial acetic acid, placed on a slide, and anthers removed and gently squashed in a drop of acetocarmine with the help of a needle, to release the meiocytes. Next, coverslips were placed on the preparations (one anther/slide, to avoid mixing meiotic stages if anthers differed in maturity), and observed under a light microscope, starting with the more advanced stages and going backwards if abnormalities were detected. The microphotographs were obtained with an Olympus Q-Color 3 digital camera (Olympus America Inc., Center Valley, PA, USA).

RESULTS

Pollen viability

The percentage of viable pollen, indirectly determined by staining 103 individual plants (genotypes), varied both between and within populations: from zero in one plant of chc E 1 to 99.3% in another plant of ifd ClI 1715, but 46 of these plants had <70% viable pollen (Fig. 1). The two populations tentatively classified as 'chc' (E 1 and E 5) exhibited the lowest average percentage of viable pollen (5.7% and 32.7%, respectively); these percentages were much lower than those exhibited by the tbr–grl artificial hybrid families and the 'presumed grl–ifd hybrid' accessions. Also, ifd ClI 1717, which had been assigned specific category, had a 10% reduction in average pollen viability in comparison with the tbr–grl artificial hybrids. In contrast, ifd ClI 1715, in the same specific category as ClI 1717, had the highest average percentage pollen viability (91.8%). The individual viable pollen percentages within each population/family are presented in Fig. 1, with the population/family median and mean values and outliers.

Pollen size and 'protoplasm'

The results of the analyses of pollen size and pollen 'protoplasm' are presented in Tables 2 and 3, respectively. From

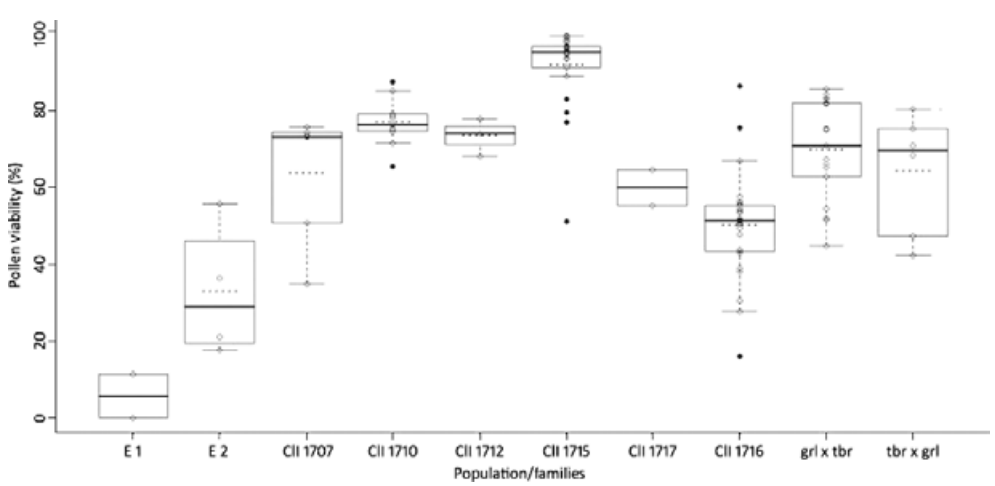

Fig. 1. Individual values (%) of pollen viability in populations/families classified as species and presumed hybrids, and in artificial hybrids. Box = 50% of the central data; highlighted line = median; dotted line = mean; filled points = outliers.

Table 2. Percentage (with minimum and maximum) of pollen grains classified according to size as <n (including micro pollen grains), n and >n. For each population/family, the percentage of pollen grains in each category was calculated by averaging the percentages (for the same category) recorded for individual plants.

	population/family[b]									
	classified as species							natural and artificial hybrids		
	chc		grl			ifd		ifd–grl	tbr × grl	grl × tbr
pollen size[a]	E 1	E 5	Cll 1707	Cll 1710	Cll 1712	Cll 1715	Cll 1717	Cll 1716	Calén × OKA 7547A	OKA 7547 × Calén
<n[c]	0	4.2 (0.6–9.4)	8.5 (0.9–14.9)	4.3 (0–12)	4.8 (2.6–8)	1.5 (0–16.3)	0	15.6 (1.7–45.4)	4.5 (0–9.6)	2.1 (0–14.9)
micro	0	0.7 (0.6–1)	4.5 (0.9–11.2)	1.6 (0–5.3)	0.5 (0–1.7)	0.2 (0–1)	0	8.1 (0–39.3)	4.5 (0–9.6)	2.1 (0–14.9)
n	95.0 (93.9–96.1)	88.0 (78.3–94.4)	86.4 (77.7–95.8)	87.9 (76.3–98)	86.8 (82.4–91.2)	94.0 (68–100)	96.4 (96–96.9)	80.2 (51–97.6)	94.1 (82.5–100)	92.6 (61.2–100)
>n	5.0 (3.9–6.1)	7.9 (5.1–12.4)	5.1 (2.6–7.4)	7.9 (2–18.6)	8.4 (6.2–9.7)	4.5 (0–15.7)	3.6 (3.1–4)	4.2 (0–13.5)	1.4 (0–7.9)	5.3 (0–36.6)
no.[d]	321	340	205	223	188	234	221	244	201	198

[a]approximately expected according to chromosome number.
[b]chc: *S. chacoense*; grl: *S. gourlayi*; ifd: *S. infundibuliforme*; tbr: *S. tuberosum* ssp. *tuberosum*; tentative classification (Potato Germplasm Bank, EEA Balcarce INTA and one of the authors). Collectors: OKA = Okada; Cll = Clausen and Ispizúa; E = Erazzú.
[c]including micro pollen grains.
[d]average number scored per population.

Table 3. Percentage (with minimum and maximum) of pollen grains according to pollen protoplasm[a]. For each population/family, the percentage of pollen grains in each category was calculated by averaging the percentages (for the same category) recorded for individual plants.

	populations/families[b]									
	classified as pure species							natural and artificial hybrids		
	chc		grl			ifd		ifd–grl	tbr × grl	grl × tbr
pollen protoplasm	E 1	E 5	Cll 1707	Cll 1710	Cll 1712	Cll 1715	Cll 1717	Cll 1716	Calén × OKA 7547A	OKA 7547 × Calén
normal	4.0 (0–8)	43.7 (20.3–70.8)	67.4 (34.8–80)	79.5 (73.6–87.5)	76.9 (73.5–80.9)	93.3 (50.9–99.3)	59.7 (55.2–64.3)	57.6 (22.6–86.3)	64.0 (42.3–80.3)	69.9 (44.8–85.5)
shrunken	64.9 (43.2–86.5)	56.1 (29.2–79.4)	3.6 (0.9–8.9)	2.6 (1–4.9)	4.0 (2.7–5.2)	0.7 (0–9.1)	1.0 (0–2)	1.4 (0–3.2)	4.3 (0–11.1)	5.1 (2–17.5)
other type	31.1 (5.6–56.8)	0	3.3 (0–11.1)	4.3 (0.7–10.3)	5.8 (2–9.3)	0.3 (0–2.5)	0.3 (0–0.5)	0.3 (0–2)	2.6 (0–11.1)	3.0 (0–9.6)
lacking	0	0.2 (0–0.6)	25.8 (16.6–45.2)	13.6 (7.2–19.6)	13.3 (7.1–16.3)	5.8 (0.7–37.4)	39.0 (33.2–44.8)	40.7 (12.2–75.9)	29.1 (14.6–52.3)	22.1 (5.7–51.7)
no.[c]	303	306	222	239	208	234	223	248	210	242

[a]protoplasm refers to protoplasm *sensu stricto* plus the plasmatic membrane.
[b]chc: *S. chacoense*; grl: *S. gourlayi*; ifd: *S. infundibuliforme*; tbr: *S. tuberosum*; tentative classification (Potato Germplasm Bank, EEA Balcarce INTA); Collectors: OKA = Okada; Cll = Clausen and Ispizúa; E = Erazzú.
[c]average pollen grain number scored per population.

herein on, we will refer to the protoplasm *sensu stricto* plus the plasmatic membrane (the plant cell without considering the cell wall) as 'protoplasm'. All populations/families produced pollen of heterogeneous size. Pollen grain diameters varied from smaller than the expected for normal n up to the expected for 4n grains (Table 2, Fig. 2a–c), except two populations (chc E 1 and ifd ClI 1717), which did not have pollen in the smaller class. Moreover, chc E 5 was the only population/family that produced 4n pollen grains (0.4%), which were included in the >n class (Fig. 2b).

Of the two populations with the highest numbers of analyzed plants – ifd–grl ClI 1716 and ifd ClI 1715 – the first exhibited the highest and the lowest average percentages of pollen grains, respectively, in the <n (15.6%) and n (80.2%) categories, whereas the second had almost the lowest and the highest average percentages of pollen grains, respectively, in the <n (1.5%) and n (94.0%) categories. However, both populations exhibited similar percentages of pollen grains in the >n category. At the individual plant level, the highest percentage of pollen grains in the <n category was 45.4% in ifd–grl ClI 1716 whereas the highest percentages of pollen grains in the >n category varied from 12.4% to 36.6% in plants of chc E 5, grl ClI 1710, ifd ClI 1715 and ifd–grl ClI 1716 and in the tbr–grl artificial hybrid families. The highest average percentages of pollen grains were in the n category (Fig. 2d). Although n size pollen grains were the most frequent in all populations/families, various abnormalities were observed in their protoplasm (Table 3, Fig. 2e–k).

According to protoplasm, pollen grains were classified as 'normal' (Fig. 2d), 'shrunken', 'other type' and 'lacking' (Table 3). chc E 1 had the lowest average percentage of pollen grains in the 'normal' (4.0%) and 'lacking' (0%) categories and the highest values in the 'shrunken' (64.9%) and 'other' type (31.1%) categories. On the other hand, the highest average percentage of pollen grains with 'normal' protoplasm (93.3%) and the lowest percentage of pollen with 'shrunken' protoplasm (0.7%) were observed in ifd ClI 1715, which also had one of the highest percentage of n pollen grains, as previously mentioned; this population had a very low average percentage of pollen with 'other type' protoplasm (0.3%), although the lowest value was observed in chc E 5 (0%). The spontaneous 'presumed' ifd–grl hybrid, ClI 1716, had the highest average percentage of pollen in the 'lacking' protoplasm category (40.7%).

The protoplasm in the Shrunken category (Fig. 2h–j) took several forms, with the protoplasm occupying most of the inner space (Fig. 2h) or less than half of it (Fig. 2j); the most extreme forms (Fig. 2j) were observed in the chc populations (E 1 and E 5), but the less extreme forms (Fig. 2h) were most frequent. Due to the many forms that were observed, and for the sake of simplicity, pollen grains with (i) normal shape and abnormal protoplasm, with lobes resembling dyads (dyad-like), triads (triad-like) (Fig. 2g) and tetrads (tetrad-like), and (ii) abnormal shape and odd protoplasm (Fig. 2e and f), were included in the Other type category (see Table 3). For this last category, all populations/families (except chc E 5) showed at least one of the previously mentioned forms. Pollen grains with dyad-like protoplasm were absent only in chc E 1and ifd ClI 1717, whereas those with triad-like protoplasm were present in all populations/families; the tetrad-like form was absent in grl ClI 1712, ifd ClI 1715 and ifd ClI 1717 (data not shown); chc E 1 was the only population that presented another odd morphology (star-like) of protoplasm (Fig. 2e). Empty pollen grains were classified into the Lacking (protoplasm) category (Fig. 2k).

Most plants within all accessions/families with at least six plants analysed (60%) exhibited high homogeneity in the percentage of pollen abnormalities in the various categories (data not shown), except ifd ClI 1715, with an average percentage of <n pollen grains of 1.52, had 16 out of 25 plants with zero and three plants with 9% to 16% pollen grains in this category.

Meiotic analyses and mechanisms of 2n pollen formation

Cytological analyses were carried out in a sample of seven male sterile plants of tbr–grl artificial hybrids and accessions of ifd, grl and presumed ifd–grl hybrids. Various abnormalities were observed in Meiosis I and Meiosis II such as lagging chromosomes in Anaphase I and II, chromosome bridges in Anaphase I, parallel, tripolar and pentapolar Anaphase II spindle orientation, and monads, dyads and triads, in addition to normal tetrads, at the tetrad stage (data not pre-

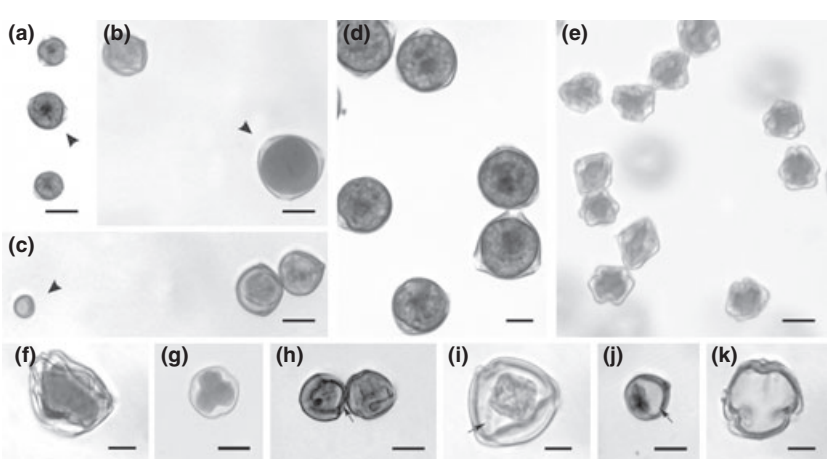

Fig. 2. Pollen larger or smaller (arrow) than expected n – a: 2n, b: 4n and c: micro. Pollen shape and protoplasm* – d: both normal; e, f: both abnormal. Pollen with normal shape and with protoplasm – g: tri-lobulated (triad-like); h–j: shrunken, from least extreme (h) to most extreme form (j) (arrow: empty cellular space); and k: absent (most frequent male sterility form). *protoplasm refers to protoplasm *sensu stricto* plus the plasmatic membrane; Bars = 20 μm (a, e); 10 μm (b, c, g, h, j); 5 μm (d, f, i, k).

sented). Herein, we report a detailed analysis (with frequencies) of meiotic abnormalities performed in the chc samples that had the highest percentages of male sterility, starting at the latest stages and working backwards (Tables 4 and 5). At the tetrad stage, the four plants analysed presented from 54.5% tetrads in chc E 5.25 to 80.7% in chc E 5.2; however, abnormal tetrads were from approximately two (in chc E 5.2) to seven (in chc E 1.3) times more frequent than normal tetrads (Table 4). In the four plants analysed, triads exhibited the expected one large (2n) and two small, similar size (n) cells, but also unexpected forms and sizes; therefore, they were classified as either normal or abnormal. The percentage of triads varied from 4.9% in chc E 5.25 to 25.9% in chc E 1.3, with abnormal triads being almost three (in chc E 5.6) to six (in chc E 1.3) times more frequent than normal triads. chc E 5.25 exhibited, in addition, 39.8% monads (in chc E 5.6 the percentage was much smaller, 7.4%) and it was the only genotype in which a pentad was observed (Table 4). Besides the number of cell per meiocyte, the abnormalities observed at the tetrad stage varied from one to all cells in a triad/tetrad with rough borders (instead of the normal smooth borders), to cells with unexpected sizes and shapes (Fig. 3). In chc E 5.2, the four cells in a tetrad resembled (after staining) mature pollen grains, as they were covered by a refractive layer (Fig. 3i).

Table 4. Percentage of monads, triads, tetrads and pentads at tetrad stage of meiosis of four genotypes from two populations tentatively classified as *S. chacoense*.

S. chacoense genotype[a]	monad	triad[b]		tetrad[b]		pentad	no.[c]
		N	A	N	A		
E 5.2	0	3.5	15.8	26.3	54.4	0	57
E 5.6	7.4	4.4	11.8	22.1	54.4	0	68
E 5.25	39.8	0.8	4.1	11.4	43.1	0.8	123
E 1.3	0	3.7	22.2	9.3	64.8	0	54

[a]E = Erazzú (collector).
[b]N: normal; A: abnormal (tetrads with cells of different size and/or non-tetrahedron disposition, cells in tetrads/triads with non-spherical shape and/or rough borders, *etc.*).
[c]meiocytes analysed per genotype.

The number of meiocytes with abnormalities in Meiosis I and II in four genotypes of the two populations tentatively classified as chc is presented in Table 5. From a total of 489 analyzed meiocytes, 310 (63.4%) presented at least one abnormality, the abnormalities being more frequent in Meiosis II (73% of 204 meiocytes) than in Meiosis I (56.5% of 285 meiocytes). Among them, (i) more than 12 chromatine bodies (Fig. 4a) in chc E 5.3 and chc E 5.2; (ii) precocious chromosome migration and abnormal chromosome disposition (out of the equatorial plate) in Metaphase I (Fig. 4b) in chc E 5.2 and chc E 1.3; (iii) asynchronous chromosome migration, lagging chromosomes and chromosome bridges in Anaphase I in chc E 5.2 (Fig. 4e and f); (iv) an asynchronous cell, with a group of metaphasic chromosomes at the equatorial plate and the other group already migrating (Fig. 4d) in chc E 5.6; (v) abnormal chromosome disposition in the equatorial plate and also precocious chromosome migration in Metaphase II (Fig. 4c) in chc E 5.6 and chc E 5.25; (vi) asynchronous chromosome migration, lagging chromosomes and fused, parallel, tripolar and other abnormal dispositions of spindles (Fig. 4h–k) in Anaphase II in chc E 5.6 and chc E 5.25; (vii) chromosome bridges in Anaphase II (Fig. 4g) in chc E 5.6; and (viii) lagging chromosomes in Telophase I (Fig. 4l) in chc E 5.2 and Telophase II (Fig. 4m) in chc E 5.6 and chc E 5.25.

DISCUSSION

In nature, tuberous *Solanum* species are reproductively isolated mainly through external barriers. In areas of overlap these barriers are reinforced by internal barriers that reside in the plant tissues themselves (Hawkes & Hjerting 1969; Camadro *et al.* 2004). These internal barriers, as proposed by Summers & Grun (1981), probably arose as subproducts of adaptive divergence rather than positive selection of specific mechanisms. In the absence of external barriers, the internal barriers allow coexistence of more than one species in a given area while maintaining their specific limits.

Potatoes can reproduce sexually and/or asexually; however, and until now, the prevalent mode of reproduction in nature has not been ascertained. Notwithstanding, there is morphological, genetic and molecular evidence that supports the assertion that sexual reproduction is important in the wild. In fact, when population samples (accessions of germplasm

Table 5. Number of meiocytes with abnormalities in Meiosis I and Meiosis II in four genotypes of two populations tentatively classified as *S. chacoense*.

S. chacoense Genotype[a]	meiotic stage[b]												
	Metaphase I		Anaphase I		Telophase I		Metaphase II		Anaphase II		Telophase II		
	N	A	N	A	N	A	N	A	N	A	N	A	no.[c]
E5.2	45	55	55	34	15	2	–	–	–	–	–	–	204
E5.6	–	–	–	–	–	–	13	36	20	52	9	16	146
E5.25	–	–	–	–	–	–	6	27	2	12	5	6	58
E1.3	9	70	–	–	–	–	–	–	–	–	–	–	79

[a]E = Erazzú (collector).
[b]N: normal; A: abnormal (chromosome disposition out of equatorial plate, precocious, laggards and asynchronous chromosome migration, chromosome bridges, fused, parallel and tripolar spindles disposition, *etc.*).
[c]meiotic cells analysed per genotype.

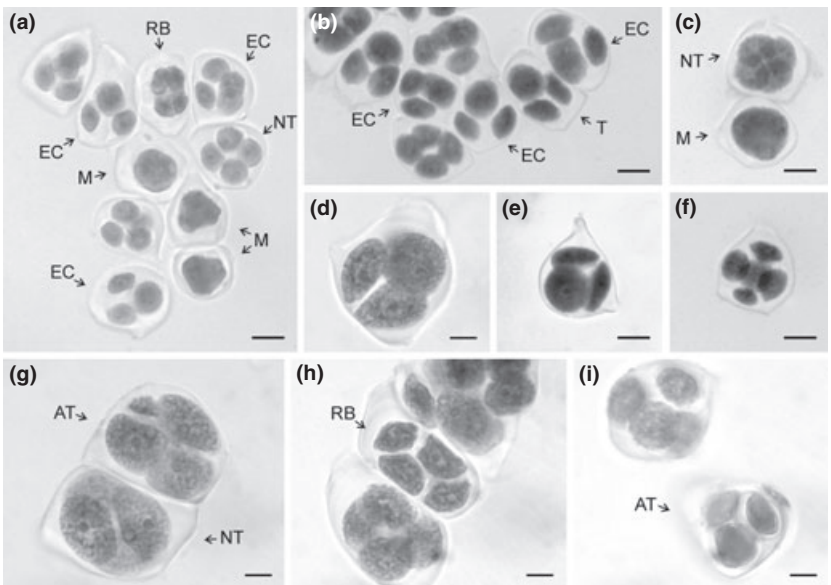

Fig. 3. Abnormalities at the tetrad stage. a, b: General view with normal tetrads (NT), tetrads with rough-bordered (RB) and elliptical cells (EC), triads (T) and monads (M); c: normal tetrad (NT) and monad (M); d, e: triads varying in size and shape of smaller cells; f: pentad; g: tetrads with normal (NT) and abnormal size and shape of cells (AT); h: tetrad with rough-bordered cells (RB); i: abnormal tetrad with cells having apparent exine deposition (AT) like pollen grains. Bars = 10 μm (a, b, c, e, f); 5 μm (d, g, h, i).

banks, classified as 'species') have been characterised using more than one type of molecular marker, plants within accessions have been found to be more variable than plants of different accessions of the same species (Bedogni & Camadro 2009; Erazzú *et al.* 2009).

Both pre- and post-zygotic barriers are common in potatoes, both in intra- and inter-specific combinations (according to current taxonomy) and within and among accessions of a given species (see Camadro *et al.* 2004). Hybridisation is successful only in partially or completely compatible genotypic combinations at the three levels (pollen–pistil, embryo and endosperm). In this regard, morphological and molecular evidence has been provided on the hybrid origin of *S x rechei* (Clausen & Spooner 1998), and *S x ruiz-lealli* (Raimondi *et al.* 2005). Over the years and in succesive generations, the F_1 hybrids and their segregating progeny can give rise to hybrid complexes with intricate genetic relations (Ugent 1966, 1970; Hawkes & Hjerting 1969).

Twentieth century potato botanists have recognised the widespread presence of interspecific hybrids in nature (Brücher 1953; Correll 1962; Hawkes & Hjerting 1969; Spooner & Van den Berg 1992), as well as the extensive overlap of character states and the weak morphological and geographical separation in some complexes as, *e.g.* Brevicaule, which contains the putative ancestors of the common potato (Van den Berg *et al.* 1998). Moreover, the molecular data, like the morphological data, fail to support the taxa with species-specific electrophoretic bands (Miller & Spooner 1999). Notwithstanding there has been a reluctance to recognise the importance of interspecific hybridisation in nature and, effectively, potato botanists have assigned mostly specific categories to new collections. But Masuelli *et al.* (2009) have provided strong evidences on the role of homoploid hybridisation as an evolutionary force in the group.

In species that are compatible at the pollen–pistil, embryo and endosperm levels, one of the most important post-zygotic barriers is male sterility of the F_1 hybrid. Male sterility could be the result of genome differentiation in the parental species, which can negatively affect chromosome pairing and distribution, resulting in unbalanced (and/or non-viable) gametes or gametophytes, the presence of either nuclear or cytoplasmic genes or the interaction between nuclear genes from the male gamete of one species and cytoplasmic genes from the female gamete of the other species (see Frankel & Galun 1977; Iwanaga *et al.* 1991; Ortiz *et al.* 1993). The latter phenomenon, known as gene-cytoplasmic male sterility, was extensively studied in potatoes by Grun (1979). Many other authors have reported similar phenomena in F_1 hybrids derived from matings involving various wild and cultivated species (see Camadro *et al.* 2004). Among these authors, Buck (1960) and Abdalla & Hermsen (1972b) reported the interaction of *S. verrucosum* cytoplasmic genes and nuclear genes of *S. phureja*, *S. chacoense* and *S. tuberosum* (all of which are diploid wild species, except the last, which is a cultivated tetraploid).

In the generation of hybrids using controlled crosses for basic or applied studies, strong incompatibilities can be encountered, thus hindering the process; therefore, the number of genotypic combinations that can be attempted in a given work is limited by the number of viable seeds (genotypes) of each accession that a germplasm bank can provide to the researcher. The generation of the 4x tbr–grl hybrids was very time-consuming, thus, spontaneous 'presumed' ifd–grl hybrids (which had one parental species in common with the artificial hybrids) were obtained from a germplasm bank to facilitate the work, because the study of these hybrids also allows valid comparison with the other genetic materials that have been classified as species for conservation purposes.

In the genetic materials analysed in our study, we observed three of the male sterility types described by Abdalla & Hermsen (1972a) in *S. verrucosum*: ordinary sterility (Lacking), partly stained sterility and striped vacuolar sterility (Other type), but also a fourth type (Shrunken), which were the most frequent (Table 2, Fig. 2e–k). We did not observe the tetrad sterility type reported by the previously cited authors in *S. verrucosum*, nor those observed by Santini *et al.* (2000) in haploid tbr × wild species (2x grl, *S. spegazzinii* and chc) diploid hybrids. In fact, the results of Santini *et al.*

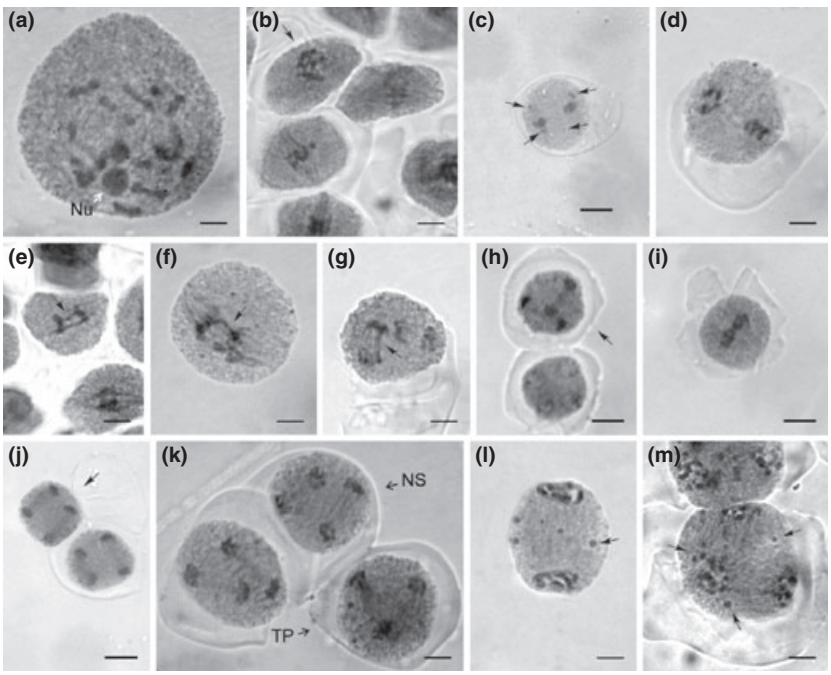

Fig. 4. Abnormalities in Meiosis I and Meiosis II. a: Prophase I cell with more than 12 chromatin bodies (nucleolus= Nu); b: Metaphase I with chromosomes out of equatorial plate (arrow); c: Metaphase II with precocious chromosome migration (arrow); d: asynchronous cell in Meiosis II with a group of metaphasic chromosomes at the equatorial plate (right) and a second group in migration (left); e: Anaphase I with lagging chromosomes (arrow); f: Anaphase I and g: Anaphase II with chromosome bridges (arrow); h–k: dispositions of Anaphase II spindles – h: T form (arrow), i: fused, j: parallel (arrow); k: normal orientation (NS) and tripolar (TP); l, m: lagging chromosomes (arrow) in (l) Telophase I and (m) Telophase II. Bars = 10 μm (c, h, i, j); 5 μm (a, b, d, e, f, g, k, l, m).

(2000) and unpublished data of our group do not agree with those of Hermunstad & Peloquin (1985), who suggested that male sterility of tbr haploids could be reverted to fertility upon crossing with wild species. In addition, all populations/families (except ifd ClI 1715) had pollen grains with abnormal morphology, with average percentages similar to those of the tbr–grl artificial hybrids. The average pollen viability of the presumed ifd–grl hybrid (ClI 1716) was low *per se* but was also approximately 20% lower than the average pollen viability of the tetraploid tbr–grl artificial hybrids (expected to have meiotic disturbances due to hybridisation and tetrasomic inheritance). In contrast, ifd ClI 1715 (collected at the same site) had very high average pollen viability. Similarly, Erazzú *et al.* (1999), working with nine accessions of diploid *S. spegazzinii* from NW Argentina, grouped on the basis of morphological phenotypes, reported variability for pollen fertility within most of them (*i.e.* from 61 to 94% in OKA 4065 and from 65 to 95% in OKA 5662). These results further support to our hypothesis on the extent of hybridisation in natural population and the inadequacy of referring to intra- and inter-specific crosses when working with accessions not genetically or cytogenetically characterised. Thus, pollen viability can be used as a first screen to differentiate hybrid populations or complexes from species populations, although it cannot be discarded that the latter could also contain hybrid plants in areas of sympatry with other populations/species. The low average pollen viability observed in chc E 1 (7.3%) and chc E 5 (32.7%) allow us to speculate that they are actually hybrid entities with chc germplasm.

The cytological observations in ifd–grl ClI 1716 provide additional evidence on its putative hybrid origin, although the occurrence of intra- and inter-specific polyploids cannot be discarded since plants of this accession and of other accessions collected at the same site produced >n pollen, as well as the expected n pollen. Clausen *et al.* (2006) reported variations in plant morphology, chromosome number (in ifd ClI 1715), pollen viability and 2n pollen production in 12 sampled sympatric populations, which is indicative of the presence of hybrid complexes. The accessions classified as grl (ClI 1707) and ifd (ClI 1717) had relatively low average pollen viability (63.4% and 59.7%, respectively); thus, and similar to the hypothesis for the presumed ifd–grl hybrid accession, both could be either hybrids or polysomic polyploids with irregular chromosome distribution in meiosis. Moreover, and through analysis of pollen viability, it would be possible to identify individual plants in otherwise homogeneous populations that could be hybrids and/or polyploids and, therefore, not good representatives of the populations sampled for conservation purposes.

The meiotic abnormalities observed in the chc populations provide a basis for explaining the formation of heterogeneous pollen size in these and, by extension, in the other populations/families studied. Prophase I cells with more than 12 chromatin bodies are an indication of synaptic problems that can impair the co-orientation of homologous centromeres in the equatorial plate; consequently, chromosome disjunction to opposite cell poles can also be impaired, resulting in laggard and precocious chromosomes, as observed in Metaphase I and II and in Anaphase I and II, as well as the laggards observed in Telophase I and II. In this way, a number of chromosomes larger or smaller than the expected n can be included in Telophase II nuclei, giving origin to tetrads with unequal size cells and, consequently, pollen grains of various sizes. In conclusion, the incomplete synapsis, asynchronous chromosome movement in Meiosis I and II and simultaneous occurrence of meiocytes of the same anther at very different meiotic phases (data not shown) are indications of genetic or genomic conflicts at the nuclear and/or nuclear–cytoplasmic level.

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 60°, the formation of two simultaneous cleavage furrows, and the final tetrahedron disposition of microspores in the tetrad (Mok & Peloquin 1975). Abnormalities in spindle orientation (tripolar, parallel, fused) are rather frequent in potatoes and can lead to the formation of 2n microspores (Mok & Peloquin 1975; Ramanna 1979; Masuelli *et al.* 1992; Peloquin *et al.* 1999; Camadro *et al.* 2008). Moreover, Genualdo *et al.* (1998) reported that 2n pollen formation in a somatic 2x *S. commersonii* × 2x haploid *S. tuberosum* hybrid ocurred through deviations in spatial configurations of both components of the cytoskeleton (microtubulin and microactin) at Meiosis II and in cytokinesis. Thus, in the chc populations, the formation of 2n pollen can be explained by the parallel, fused and tripolar Anaphase II spindle dispositions, whereas the formation of 4n pollen is a consequence of the absence of cytokinesis after the second meiotic division. Although dyads were not observed in the chc plants analysed, their occurrence in the populations cannot be discarded.

In accessions of the wild diploid potato *S. okadae* from NW Argentina and Bolivia, with marked differences in vegetative and reproductive traits and difficult to reproduce by seeds, Camadro *et al.* (2008) 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. In these genetic materials, Bottini *et al.* (2008) observed univalents in Metaphase I, precocious chomosome migration in Anaphase I, precocious and asynchronous chromosome migration in Metaphase II and Anaphase II, and micronuclei at Telophase I and II. Very similar meiotic abnormalities were reported for one accession of *S. chacoense*, also from Argentina (Bottini *et al.* 2009). Irregular meiosis and micronuclei have also been observed in *S. ruiz-lealli*, an Argentinian species of hybrid origin, with very low pollen viability and also difficult to reproduce from seeds (Raimondi 2002; Marfil *et al.* 2009) and in *S. stoloniferum* × *S. tuberosum* artificial hybrids (Panahandeh *et al.* 2008).

In Argentina, *S. chacoense* has a very wide distribution, from the NW region where it grows at very high altitudes (up to 2000 m) to the NE and Central regions, where it grows at low altitudes (below 1000 m), in contrasting environments. This species, which extensively overlaps with other morphological species, exhibits important morphological variation along its distribution (Hawkes & Hjerting 1969). Some accessions from the NW (where the two populations used in the present study were sampled) have been cited as likely hybrids with 2x *S. spegazzinii* (Hawkes & Hjerting 1969; Erazzú *et al.* 1999), introgressed forms with 2x *S. microdontum* (Hawkes 1962), and hybrids with 2x *S. vernei* and 2x *S. venturi* (Hawkes & Hjerting 1969), among others. Also, using artificial controlled crosses, the compatibility of genotypic combinations with other diploid morphological species from the W-NW region, *S. kurtzianum* and *S. ruiz-lealli*, has been described (Raimondi *et al.* 2003).

However, current potato taxonomy makes use of the morphological species concept (Hawkes 1990; Spooner *et al.* 2009), not taking into account the genetic structure of the populations and the breeding relationships within populations and with neighbouring populations growing at a distance such that gene flow and introgression is feasible. In fact, there are no reports in the potato literature on such an approximation to the species problem as has been done for species of *Iris* (Hodges *et al.* 1996; Arnold *et al.* 1999), for example, which share many similarities in their reproductive system with potato species (Grant 1981). Hodges *et al.* (1996), working under field conditions with *Iris* species in North America, demonstrated that the rate of advanced generation hybrid formation can be higher than the rate of initial F_1 hybrid formation. These authors concluded that despite the difficulties encountered in their establishment, the initial F_1 hybrids could be a bridge for the rapid establishment of hybrid zones. Moreover, Arnold *et al.* (1999) provided evidences that, in angiosperms, the extremely low fertility or viability of early generation hybrids (F_1, F_2, BC_1) does not necessarily prevent extensive gene flow and the establishment of new evolutionary lineages in nature, since some hybrid genotypes could have equivalent or higher fitness than their parental genotypes in certain habitats.

To our knowledge, and for wild potatoes, this is the first report in which a comparison has been carried out on male sterility and the occurrence of various meiotic abnormalities in high frequencies in plants of artificial and natural presumed hybrids, accessions from germplasm banks with specific categories and natural populations of an apparently morphological species. The similarities of the results obtained in the various genetic materials studied using this approach, similar meiotic observations reported by other authors for individual genotypes of accessions and specific categories, and field observations of spontaneous hybrids (according to plant morphology) of early potato taxonomists, provide solid evidence on the extent of spontaneous hybridisation in wild potato populations and establish the need to revise the species concept in this group on the basis of population genetic studies.

For germplasm conservation, our results point to the need for complementing morphological characterisation of the sampled populations with genetic and/or cytogenetic studies before assigning a specific category for their incorporation as accessions in germplasm banks. The estimation of male fertility through staining is a low cost, simple, rapid and effective technique that can be complemented with meiotic studies. These pollen and meiotic studies will provide information to establish botanical seed regeneration strategies, and thus avoid genetic drift and the consequent loss of potentially valuable genes through the sexual process (*i.e.* by increasing the number of plants/accession used as progenitors), and to make decisions on what to keep when sympatric populations are sampled.

For breeding, knowledge and better understanding of the reproductive biology of potato, the genetic variability that can be present in accessions of germplasm banks (even in those classified as the same species) and the fact that some of these accessions may actually be hybrids or introgressed forms with reduced fertility, would allow better selection of parents and better application of breeding strategies, thus increasing the efficiency and reducing the cost of developing a commercial cultivar, which currently can take more than 10 years.

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