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Breeding Advances in *Passiflora* spp. (Passionflower) Native to Argentina

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ABSTRACT

Passiflora is the largest genus in the *Passifloraceae* family and comprises nearly 500 species. The genus is distributed throughout the tropics and subtropics and the majority of the species are endemic of Central and South America. In Argentina, 19 species grouped in four subgenera are present. *P. alata*, *P. amethystina*, *P. cincinnata*, *P. edulis* f. *edulis* and *P. umbilicata* are the most interesting species for ornamental use due to the size and colour of their flowers. The aim of our breeding program was to obtain new forms for ornamental use. It was also focused in cold tolerance selection. Interspecific crosses have been performed, providing information about the combinatory ability of some species of the genus (*P. alata*, *P. cincinnata*, *P. caerulea*, *P. amethystina*, *P. edulis* f. *edulis* and the hybrid *P.* 'violacea'). *P. alata* and *P. caerulea* were crossed successfully in both directions, while the other combinations showed unilateral incompatibility. Pollen tube growth was arrested in the style in the crosses *P. caerulea* × *P. amethystina* and *P. caerulea* × *P. alata.* The knowledge of the site where incompatibility expresses allowed the design of complementary strategies in order to overcome the barriers to hybridization. The chromosome numbers found in parental species and hybrids was 2n=2x=18. Preliminary results about cold tolerance showed that *P. caerulea* tolerates low temperatures but *P. alata* and *P. amethystina* does not. This tolerance was reflected in their progeny.

Keywords: chromosome number, cold tolerance, interspecific hybridization, pollen tube growth
Abbreviations: DPP, days post-pollination; **RRS**, relative reproductive success

INTRODUCTION

The genus *Passiflora*, which belongs to the *Passifloraceae* family, contains more than 400 species, mostly distributed in Europe and America. The American species are found in the central and southern part of the continent and 19 of them are native to Argentina (Deginani 2001). They belong to 4 of the 23 subgenus described by Killip (1938). The subgenus and Argentine native species are introduced in **Table 1**.

The flowers of *Passiflora* have different petal shapes and colour variability according to the species arousing great interest in the European ornamental plant industry. The crown, typical of *Passifloraceae*, appears in one or several concentric groups; it can be plain or with coloured transversal stripes, providing an exotic appearance and great ornamental value. Apart from this value, some species have edible fruits; others are valued in medicinal uses (Otahola 2000) or represent an entomologic-botanical resource (García and Hoc 1998).

In Argentina, the native genetic resources for the development of ornamental plants have been poorly exploited, but they have been part of some varieties developed by international companies. However, in spite of the huge profits coming from them, Argentina does not receive any benefits.

The Instituto de Floricultura, INTA (Institute of Floriculture, National Institute of Agronomic Technology, Argentina) carried out breeding programs to develop new varieties from native genetic resources.

In the case of *Passiflora*, breeding activities were orientated to obtain varieties with colourful flowers such as *P. alata*, *P. amethystina*, *P. edulis*, *P. cincinnata* and *P.* 'violacea', combined with low temperature tolerance of *P. caerulea*. *P. alata* stands out for its size and the red colour of its petals and also for its abundant and long lasting flowering. *P. amethystina* has fragrant blue flowers whereas *P. cincinnata* and *P. edulis* have purple and white respectively. All of them belong to North Argentina where the climate is warmer and do not tolerate low temperatures. *P.* ´violacea´ is a commercial hybrid from Argentinean *P. caerulea* and *P. kermesina*. *P. caeruela* shows low temperature tolerance and therefore it extends to more southern latitudes (Deginani 2001; Ulmer and MacDougal 2004).

Other breeding programs have been focused on fruit

Table 1 Subgenus and native species of *Passiflora* native to Argentina (Deginani 2001).

Subgenus	Species
Passiflora	*P. alata*
	P. amethystina
	P. caerulea
	P. cincinnata
	P. edulis
	P. elegans
	P. giberti
	P. mooreana
	P. palmatisecta
	P. tenuifolia
	P. tucumanensis
Decaloba	*P. capsularis*
	P. misera
	P. morifolia
	P. suberosa
	P. urnaefolia
Dysosmia	*P. chrysophyla*
	P. foetida
Tacsonioides	*P. umbilicata*

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production, specially using *P. edulis* f. *edulis* and *P. edulis* f. *flavicarpa* (Ulmer and MacDougal 2004). Their improvement aims at increasing disease and pest tolerance, together with cold and extreme warm temperature resistance (Otahola 2000). *P. incarnata* breeding focused on hybrid production with more powerful or highly concentrated active pharmaceutical substances (Ulmer and MacDougal 2004).

Interspecific hybridization is one of the most important sources of genetic variation in breeding ornamental crops (van Tuyl and De Jeu 1997). This strategy was applied to *Passiflora* breeding by Payan and Martín (1975), Ulmer and MacDougal (2004), Pannunzio *et al*. (2006) and Ramírez (2006). When trying to get an interspecific hybrid, crossing barriers can appear and they are the biggest obstacle to overcome (Payán and Martín 1975; van Tuyl and Lim 2003). Some crossings do not produce seeds; some are successful in only one direction (unilateral incongruity) or, are often genotype-dependant (Singh 2003). In *Passiflora*, crossing barriers appeared in interspecific hybridization (Pannunzio *et al*. 2006), some intraspecific (Rego *et al*. 2000) and in self pollination experiments (Rego *et al*. 2000, Suassuna *et al*. 2003). Despite the large number of existent interspecific hybrids of *Passiflora*, combinatory ability of Argentine native species is not known. It is important to mention here that there are self-compatible and self-incompatible species of *Passiflora* (Ulmer and MacDougal 2004).

In *Passiflora*, pollen grains are filtered by incompatibility, which expresses in intraspecific and in self pollinations (Rego *et al*. 2000; Suassuna *et al*. 2003) or by incongruity (Payán and Martín 1975; Nettancourt 1977; Ramírez 2006). Pollen adhesion to the stigma is the first post-pollination event and depends on pollen and stigma surface components. Effective pollen adhesion is followed by pollen hydration with water from the stigma driven by osmotic potential differences. Then, pollen wall proteins are released onto the surface of the stigma and come into contact with components of the stigma pellicle. This is the first chemical interaction between pollen and pistil and may establish the identity of the pollen by the female partner (Shivanna and Sawhney 1997). Souza *et al.* (2006) described the stigma of *P. edulis* f. *flavicarpa* as dry, multiserial, multicellular and papillated. In this type of stigma, pollen adhesion is more critical than in wet-type stigmas, because it depends on the stigmatic pellicle and pollen substances (Shivanna and Sawhney 1997). Rego *et al*. (2000) asserted that, in compatible crosses of *P. edulis* f. *flavicarpa*, pollen tubes showed a uniform layer of callose and callose plugs regularly spaced. They observed two sites of inhibition of pollen tube growth in incompatible crosses: stigma and style. Inhibition in style occurred in the upper and medium third, this is consistent with a gametophytic-sporophytic gene action (Rego *et al.* 2000). In *P. edulis* f. *flavicarpa*, differences in the reciprocal crosses have been observed, which represent a regular pattern in sporophytic systems due to the dominance interaction between pollen and pistil alleles (Rego *et al*. 2000; Suassuna *et al*. 2003). Post-zygotic barriers prevent genetic interchange and can express either an abnormality of the hybrid zygote, low vegetative viability, absence or sterility of one of the plant sexes or infertility at the reproductive stage (Purves *et al*. 2003). Barriers could also be detected when observing the penetration of the pollen tubes in the ovules and the lack of occurrence of fruit set.

Genomic similarities between parental species are another important issue to consider. The possibility of a cross is an indirect measure of the genomic relationship between parents. Crosses between similar genome species often produce normal fruits, but with different genomes, either embryo abortion or hybrid sterility is usually the rule (Benavente *et al.* 2008).

The objective of this work was to evaluate interspecific compatibility between *P. alata*, *P. amethystina*, *P. caerulea*, *P. cincinnata*, *P.* 'violacea' and *P. edulis* f. *edulis*, in order to provide information about their combinatory ability. We also aimed at ranking the crosses in order to establish the couple partnership for the best reproductive efficiency, finding the sites in which incompatibility is expressed, and establishing the similarities between the chromosomes of the species involved in the crosses. We also focused on searching cold-tolerant genotypes.

Table 2 Codes and sites of origin of the genotypes of *Passiflora* cultivated in the Instituto de Floricultura, INTA (Floriculture Institute, National Institute of Agronomic Technology, Argentina).

Species	Code	Origin
P. alata	20041119A1	Vivero Ferrari, Buenos Aires
P. alata	20050814B1	San Justo, Buenos Aires
P. alata	20100131A1	Puerto Madero, Buenos Aires
P. caerulea	20050318A1	Guayquiraró, Corrientes
P. caerulea	20050608A1	INTA Castelar property, Buenos Aires
P. caerulea	20051202G1	Cerro Corá, Misiones
P. caerulea	20050608A2	INTA Castelar property, Buenos Aires
P. caerulea	20071124A4	Chañar Ladeado, Santa Fe
P. caerulea	05-102	Salta Capital, Salta
P. caerulea	05-101	Valle de Lerma, Salta
P. caerulea	05-43	Castelar, Buenos Aires
P. caerulea	05-42	Ciudad Universitaria property, UBA
P. amethystina	20070707A7	Temaiken Park donation
P. 'violacea'	20050603A1	Commercial
P. cincinnata	05-46 # 1	Particular donation
P. cincinnata	05-46 # 2	Particular donation
P. edulis f. *edulis*	20091009D1	Montecarlo, Misiones

MATERIALS AND METHODS

Plant material

The plant material belongs to the *in vivo* collection of *Passiflora* of the Instituto de Floricultura INTA (Institute of Floriculture INTA) (34º 36' south latitude, 58º 40' west longitude).

Five native species *P. alata*, *P. amethystina*, *P. caerulea*, *P. cincinnata* and *P. edulis* f. *edulis* and one commercial hybrid *P.* 'violacea' (derived from the cross of Argentine native *P. caerulea* and *P. kermesina*) were used for experiments (**Fig. 1**). In **Table 2** species, code and origin of genotypes of *Passiflora* collection used in this work are presented.

Materials were grown in a heated greenhouse with minimum controlled temperature of 10°C, in 10-L pots with a substrate composed of soil, river residue, grinded pine leaves and pine bark (1:1:1:1).

Hybridization

Reciprocal crosses between the mentioned species were performed. Prior to pollination, the mature buds of the female parent were carefully opened and anthers were removed. Pollination was carried out at anthesis. After that, flowers were bagged to prevent further pollination. Pollen grains were checked for viability in crossings by Alexander test (Alexander 1969), analyzing at least 500 pollen grains of each species. Only more than 80% viability pollen grains were used for the experiments. Mature fruits were collected and seeds were counted. The aril was immediately removed from the seeds. Seeds were scarified and washed for 24 h (Ferreira 2005). Then, seeds were sown in bags with vermiculite and kept at room temperature.

The relative reproductive success (RRS = number of fruits obtained/number of pollinated flowers × number of seeds/number of ovules) was calculated (Dafni 1992).

Pollen tube growth

Pistils were removed from the flowers after 1, 2 and 3 days post-pollination (DPP) and fixed in FAA (formaldehyde-acetic acid-ethanol, Merck Argentina) for at least 24 h. They were softened with sodium hydroxide 8N (Merck Argentina) and stained for callose with aniline blue (Schmid Gmbh and Co Germany) 1% in aqueous solution (Martin 1958). Pistils sections (stigma, style and ovary) were displayed on slides by dissection followed by light-

Fig. 1 *Passiflora* species used in interspecific crossings. (**A**) *P. amethystina*, (**B**) *P. caerulea*, (**C**) *P. edulis* f. *edulis*, (**D**) *P.* 'violacea', (**E**) *P. alata*, (**F**) *P. cincinnata*. Scale bars = 1 cm.

squashing under a cover slip. Pistil squashes were viewed on an Olympus BX50 (Olympus Japan). Pollen tubes were identified by the fluorescence of the callose on the walls and pollen tube plugs, and then photographed.

Observations of hybrids

Hybrids phenotypes of flowers and leaves were presented by pictures.

Pollen grain viability of the hybrids was evaluated with Alexander's technique (1969). This technique admits the detection of aborted and non aborted pollen grains. At least 500 pollen grains were noted for each hybrid and a percentage of viability was calculated.

Chromosomal analysis

In order to attain roots in active mitotic division for the observation of mitotic chromosomes, segments of stems of each species with indole-3-butyric acid hormone (1000 mg/L) were placed in a substrate including peat, perlite and vermiculite (2:1:1). The root tips were pre-treated with colchicine (0.025%) and fixed in Farmer solution (ethanol: acetic acid, 3: l). Feulgen staining (Darlington and La Cour 1962) followed the hydrolysis with hydrogen chloride 1N for 10 min at 60°C. To intensify the coloration, acetic-hematoxylin and ferric citrate as mordant were additionally used. Once coloured, roots were treated for 1 h with a buffer solution pH 4.5-4.8 (citric acid-sodium citrate) at 37°C, with an enzyme mixture of pectinase and cellulase. Fifteen metaphasic cells of 4 different genotypes of *P. caerulea*, seven cells of 2 genotypes of *P. alata*, five cells of 1 genotype of *P. amethystina* and four cells of 2 genotypes of *P. edulis* f. *edulis* were analyzed.

Commercial source: Merck Argentina with the exception of hematoxylin, colchicine, cellulase and pectinase that were purchased from Sigma Aldrich, Argentina. Feulgen staining was performed with basic fuchsin (Mallinckrodt Baker Inc, USA).

Flow cytometric analysis

Young leaves of hybrids and parents (*P. alata*, *P. caerulea*, *P. cincinnata*, *P. amethystina*, *P. alata* × *P. caerulea* hybrid, *P. alata* × *P. cincinnata* hybrid and *P. amethystina* × *P. alata* hybrid), were chopped with a sharp razor blade in nuclei extraction buffer and the suspension containing released nuclei was passed through a 50 µm filter. Then, the nuclei in filtrate suspension were stained with a solution containing 4',6-diamidino-2-phenylindole (Sigma Aldrich Argentina) (Otto 1990). After shaking the solution gently, samples were analysed with a flowcytometer (PA Ploidy Analyser,

Table 3 Number of fruits with seed obtained over pollinated flowers, fruit set percentage calculated for the interspecific crosses of *Passiflora* and significant differences among them (Fisher's exact test 0.05).

Interspecific cross	Fruits set /flowers pollinated (nº/nº)	Fruit set (%)	Significant differences (Fisher's exact test)	Reciprocal cross	Fruits set /flowers pollinated (nº/nº)	Fruit set (%)	Significant differences (Fisher's exact test)
P. alata x P. cincinnata	5/11	45.4	a	*P. cincinnata x P. alata*	0/8	0	d
P. alata x P. caerulea	14/51	27.4	abc	*P. caerulea x P. alata*	4/77	5.1	d
P. amethystina x P. caerulea	6/23	26	abc	*P. caerulea x P. amethystina*	0/15	0	d
P. 'violacea' *x P. caerulea*	12/63	19	abcd	*P. caerulea x P.* 'violacea'	0/13	0	d
P. 'violacea' *x P. alata*	7/46	15.2	bcd	*P. alata x P.* 'violacea'	0/18	0	d
P. amethystina x P. alata	2/29	6.8	cd	*P. alata x P. amethystina*	0/8	0	d
P. 'violacea' *x P. edulis* f. *edulis*	1/18	5.5	d	*P. edulis* f. *edulis x P.* 'violacea'	-	-	d

Different letter indicates significant differences.

Table 4 Mean number of seeds per fruit and ovules per ovary and percentages of relative reproductive success, reproductive efficiency and germination for the successful interspecific crosses of *Passiflora.*

Interspecific crosses	Seeds/fruit (mean)	Ovules/ovary (mean)	Relative reproductive success (%)	Germination (%)
P. alata x P. cincinnata	222	269.07	37.5	0.02
	n = 5	n = 14		n = 215
P. alata x P. caerulea	149.43	269.07	15.24	2.39
	n = 5	n = 14		n = 2092
P. amethystina x P. caerulea	59.8	181.83	8.57	17.72
	n = 6	n = 6		n = 299
P. 'violacea' *x P. caerulea*	41	315.16	2.47	8.94
	n = 5	n = 6		n = 123
P. 'violacea' *x P. alata*	12	315.16	0.58	8.33
	n = 4	n = 6		n = 12
P. amethystina x P. alata	21	181.83	0.79	11.9
	n = 2	n = 6		n = 42
P. 'violacea' *x P. edulis* f. *edulis*	4	315.16	0.07	50
	n = 1	n = 6		n = 4
P. caerulea x P. alata	2.46	264.2	0.16	41.6
	n = 13	n = 13		n = 32

Partec). Relative DNA content was estimated according to the prominent peak in each measurement.

Cold tolerance preliminary experiment

For testing hybrids and parental species cold tolerance in suburban (34° 36' south latitude, 58° 40' west longitude) Buenos Aires, Argentina, the plants were prepared in 10 L pots with a substrate composed by soil, river residue, grinded pine leaves and pine bark (1:1:1:1). Ten repetitions for genotype were considered. Plants were placed randomly on tables in field conditions during 2010. Mean temperature of the coldest month of 2010 was 9°C (min. 2°C, max. 16°C), and 16 days with temperatures below 0°C were registered and the absolute minimum temperature was -6°C. Plants were evaluated weekly by visual rank of its leaves damage as 1: no damage, 2: less than 50% damage, 3: more than 50% damage and 4: death. Damage was considered when chlorotic, necrotic or fallen leaves were observed.

Data analysis

The obtained data were analyzed with non-parametric statistical methods. A comparison of two proportions based on Fisher's exact test was used to check significant differences between the fruit set capacity of the interspecific crosses, the pollen tube growth in four crosses, and the hybrids pollen grain viability. A chi-square test was used to evaluate if fruit set capacity depends on species used as female parent. The statistical tests were carried out with InfoStat version 1.2 software. InfoStat is a Statistical Software developed by a team composed of teacher-researchers of Statistics, Biometry and Experimental Design of the National Córdoba University - Argentina.

Tests were considered significant when *P* values were lower than 0.05.

RESULTS

Hybridization

We were able to obtain interspecific hybrids from some species considered in this study. Number of fruits containing seeds, number of pollinated flowers in each cross combination and fructification percentage is presented in **Table 3**. The highest fruit set was obtained for the cross *P. alata* × *P. cincinnata* with 5 viable fruits over 11 flowers pollinated. This cross and *P. alata* × *P. caerulea*, *P. amethystina* × *P. caerulea*, *P.* 'violacea' × *P. caerulea*, *P.* 'violacea' × *P. alata*, *P. amethystina* × *P. alata*, *P.* 'violacea' × *P. edulis* f. *edulis*, *P. caerulea* × *P. alata* were successful, while the crosses *P. cincinnata* × *P. alata*, *P. alata* × *P. amethystina*, *P. caerulea* × *P.* 'violacea', *P. alata* × *P.* 'violacea', *P. caerulea* × *P. amethystina* and *P. alata* × *P. amethystina* did not produce any fruit.

The capacity as female parent measured through a chi-square test ($P < 0.05$; 3 Ld) showed that when female parents were *P. alata* and *P.* 'violacea', the amount of produced fruits (19 and 20, respectively) was more than expected randomly (12.06 and 17.41). In the case of *P. amethystina*, the amount (8) was similar to the expected one (7.13), while when *P. caerulea* was the female parent the amount of fruit produced (4) was less than expected (14.39).

Highest RRS was 37.5% for the cross *P. alata* × *P. cincinnata*. *P. alata* × *P. caerulea*, *P. amethystina* × *P. caerulea* and *P.* 'violacea' × *P. caerulea* values were 15.2%, 8.6% and 2.5%, respectively (**Table 4**). The rest of the successful crosses showed a RRS under 1%.

The highest seed germination percentage was 50% for the cross *P.* 'violacea' × *P. edulis* f. *edulis*, and 41.6% for *P. caerulea* × *P. alata*, while the lowest were 0.02% for *P. alata* × *P. cincinnata*, and 2.4% for *P. alata* × *P. caerulea.* These results are not consistent with the relative reproductive success or fructification percentage for these crosses.

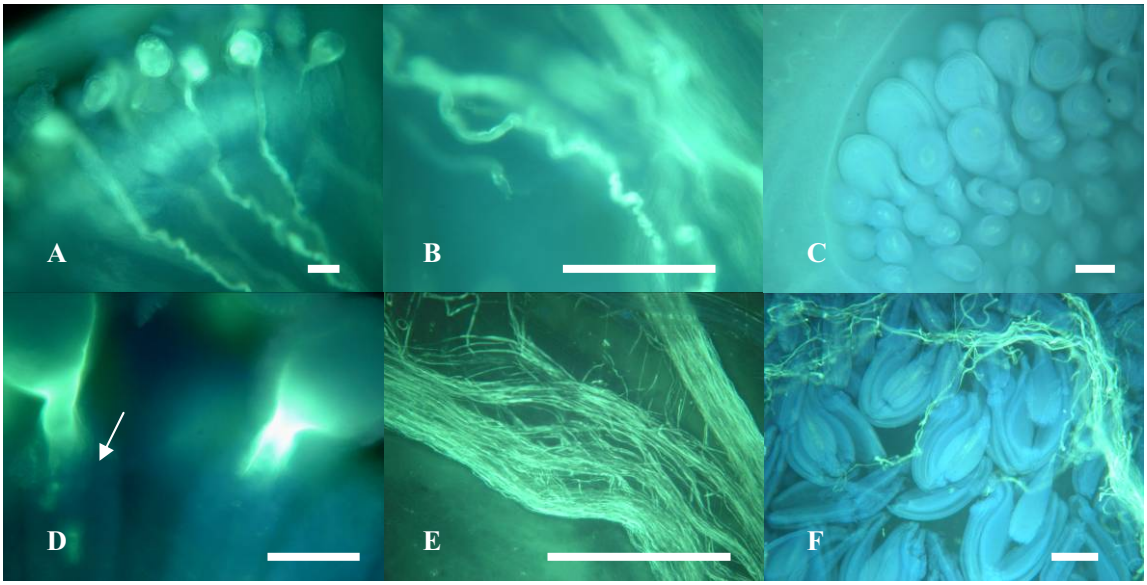

Fig. 2 Pollen tube grow through pistils in incompatible and compatible crosses of *Passiflora*. (**A**, **B**, **C**) stigma, style and ovary in an incompatible cross, respectively. (**D**, **E**, **F**) stigma, style and ovary in a compatible cross, respectively. Scale bars = 50 µm. The arrow indicates the stigmatic papillae.

Fig. 3 *Passiflora* interspecific hybrids: aspect of plants. (**A**) *P.* 'violacea' × *P. edulis* f. *edulis* hybrid, (**B**) *P. amethystina* × *P. caerulea* hybrid, (**C**) *P. amethystina* × *P. alata* hybrid, (**D**) *P.* 'violacea' x *P. caerulea* hybrid.

Fig. 4 *Passiflora* interespecific selected hybrids. (**A**, **B**) *P. amethystina* × *P. caerulea*, (**C**, **D**, **E**) *P. alata* × *P. cincinnata*, (**F**) *P.* 'violacea' × *P. alata*, (**G**, **H**) *P.* 'violacea' × *P. caerulea*, (**I**) *P.* 'violacea' × *P. edulis* f. *edulis*, (**J**) *P. alata* × *P.caerulea*. Scale bars = 1 cm.

Pollen tube growth

The pollen tube growth study revealed conspicuous differences between incompatible and compatible crosses of *Passiflora*. Spiralling of the pollen tubes in the stigma, through the style and the lack of pollen tubes in the ovary indicated that pollen tubes were arrested in the style in incompatible crosses (**Fig. 2A-C**). On the contrary, normal entrance of the pollen tubes through the stigmatic papillae, normal growth through the style and arrival of them to the ovary occurred in compatible crosses (**Fig. 2D-F**).

Daily pollen tube growth in interspecific crosses of *Passiflora* is presented in **Table 5**. Pollen tubes of *P. alata* in *P. amethystina* arrived at the ovary in 28.6% of the cases at 1

Table 5 Pollen tube growth the interspecific crosses of *Passiflora.*

		1 DPP	2 DPP	3 DPP
P. amethystina x *P. alata*	S	71.4	33.3	0
	O	28.6	66.7	100
	n	7	6	7
	F	a	a	a
P. amethystina x P. caerulea	S	100	81.8	66.6
	O	0	18.2	33.4
	n	9	11	9
	F	a	ab	b
P. caerulea x *P. amethystina*	S	100	100	100
	O	0	0	0
	n	7	6	8
	F	a	b	b
P. caerulea x *P. alata*	S	100	100	100
	O	0	0	0
	n	8	6	6
	F	a	b	b

DPP: days post-pollination; S: cases with pollen tubes until style; O: cases with pollen tubes in the ovary; n: number of samples; F: significant differences according to Fisher's exact test. Different letter indicates significant differences.

Table 6 Leaf damage of *Passiflora* selected hybrids and parental species after 2010 Buenos Aires winter period (mode).

Hybrid/parents	1	2	3	4
P. 'violacea' *x P. edulis* f. *edulis*				X
P. 'violacea' *x P. caerulea # 1*		X		
P. 'violacea' *x P. caerulea # 2*			X	
P. alata x P. cincinnata # 1				X
P. alatax P. cincinnata # 2				X
P. alata x P. cincinnata # 3				X
P. amethystina x P. caerulea # 1	X			
P. amethystina x P. caerulea # 2	X			
P. amethystina x P. caerulea # 3	X			
P. amethystina x P. caerulea # 4	X			
P. alata # 1				X
P. alata # 2				X
P. amethystina				X
P. caerulea # 1	X			
P. caerulea # 2	X			
P. edulis f. *edulis*				X

1: no damage, 2: less than 50% damage, 3: more than 50% damage, 4: death
P. cincinnata values are not presented due to any rooting capacity.

DPP and 100% at 3 DPP. The cross between *P. amethystina* × *P. caerulea* did not show any pollen tube beyond the style at 1 DPP and the amount of cases increased between 2 and 3 DPP (18.2 to 33.4%). In the crosses *P. caerulea* × *P. amethystina* and *P. caerulea* × *P. alata*, the pollen tubes were arrested in the style. The crosses *P. caerulea* × *P. amethystina* and *P. caerulea* × *P. alata* did never showed pollen tube arrival at the ovary.

Observations of hybrids

It was possible to obtain vigorous hybrid plants from some interspecific crosses: *P. amethystina* × *P. caerulea*, *P. alata* × *P. cincinnata*, *P.* 'violacea' × *P. alata*, *P.* 'violacea' × *P. caerulea* and *P.* 'violacea' × *P. edulis* f. *edulis* (**Fig. 3**). Nevertheless, the cross *P. amethystina* × *P. alata* produced a hybrid with low vegetative viability and after two years of germination, it did not blossom and showed a poor vegetative growth (**Fig. 3C**).

The floral phenotypes of some selected hybrids are presented in **Fig. 4**. As expected, flowers showed intermediate characteristics compared with the parent species. As regards to flower colour, hybrids showed an intermediate tone between both parents with variations in the size and colour of the pieces of the floral crown. The morphology of the leaves was also different from parents in the hybrid plants (**Fig. 5**).

Fig. 6 shows a picture composition showing flowers, anthers and pollen grains viability of some selected hybrids. The obtained hybrids from the cross *P. amethystina* × *P. caerulea* showed the highest pollen viability. Hybrids from *P.* 'violacea' × *P. caerulea*, *P. alata* × *P. caerulea* and *P.* 'violacea' × *P. edulis* f. *edulis* crosses showed the lowest percentage of pollen viability. When pollen viability was very low, the thecas were closed or semi-closed and the pollen grains inside were sticky while in the anthers with high pollen viability the pollen grains were dusty and separate (**Fig. 6H, 6I**).

Flow cytometric analysis

The relative fluorescence peaks of the parental plants and the hybrid in three combination crosses are presented in **Fig. 7**. Hybrids peaks between the parental species showed that the amount of DNA of the hybrid was intermediate of its parents. Due to this situation, it was possible to make an early detection of the progeny of *P. alata* × *P. caerulea*, *P. alata* × *P. cincinnata* and *P. alata* × *P. amethystina*.

Chromosomal analysis

P. caerulea, *P. alata*, *P. amethystina* and *P. edulis* f. *edulis* showed a karyotype with 2n=2x=18 (**Fig. 8**). The chromosomes were predominantly submetacentric and metacentric. In *P. alata* and *P. caerulea* two pairs of chromosomes with satellites were observed (**Fig. 8A,** 8**F**).

Mean size of chromosomes of parental species was estimated: the mean size of the chromosomes for *P. alata* was 5.12 µm, for *P. amethystina* it was 2.61 µm, and for *P. caerulea* and *P. edulis* f. *edulis* it was 3.68 and 2.66 µm, respectively.

The hybrids from *P. alata* × *P. caerulea* (two hybrids) and *P. amethystina* × *P. caerulea* (one hybrid), had a chromosomal formula of 2n=2x=18, as in their parents. In their mitotic cells, neither aneuploidies nor even another type of abnormalities was detected (**Fig. 8E**).

Cold tolerance preliminary experiment

Preliminary results about leaf damage after the 2010 winter period in some selected hybrids and parental species are presented in **Table 6**. *P. caerulea* tolerated low temperatures as their hybrids using *P. amethystina* as mother plant. *P.* 'violacea' × *P. caerulea* hybrids showed approximately 50% leaf damage.

P. alata, *P. amethystina* and *P. edulis* f. *edulis* did not survive the 2010 winter conditions of Buenos Aires. **Fig. 9** shows the damage found in hybrids and parental species and plant disposition at the experiment.

DISCUSSION

It was possible to obtain hybrid plants through controlled pollinations among the *Passiflora* species considered in this study (*P. alata*, *P. amethystina*, *P. caerulea*, *P. edulis* f. *edulis*, *P. cincinnata* and *P.* 'violacea') providing information about the combinatory capacity of species native to Argentina. The species under study showed that they are able to produce offspring; however, they did not behave in the same way when they were tested as female parent. This differential capability of each species to act as female partner could arise from different mechanisms such as sporophytic incompatibility, traditionally described for self-incompatibility systems. The mechanism occurs due to the recognition between pollen and stigma proteins and, as a result of that, pollen grains do not germinate or produce a short pollen tube. According to Dickinson (1995), this kind of self-incompatibility is associated to trinucleate pollen and dry papillae as found in *Passiflora* (Souza *et al*. 2006). This incompatibility is generated through pollen-pistil interaction between the *S*-alleles that form an allelic series. If the maternal *S*-allele is dominant over the paternal, then the cross is incompatible; if the maternal *S*-allele is recessive to the paternal, the cross is compatible (Brennan and

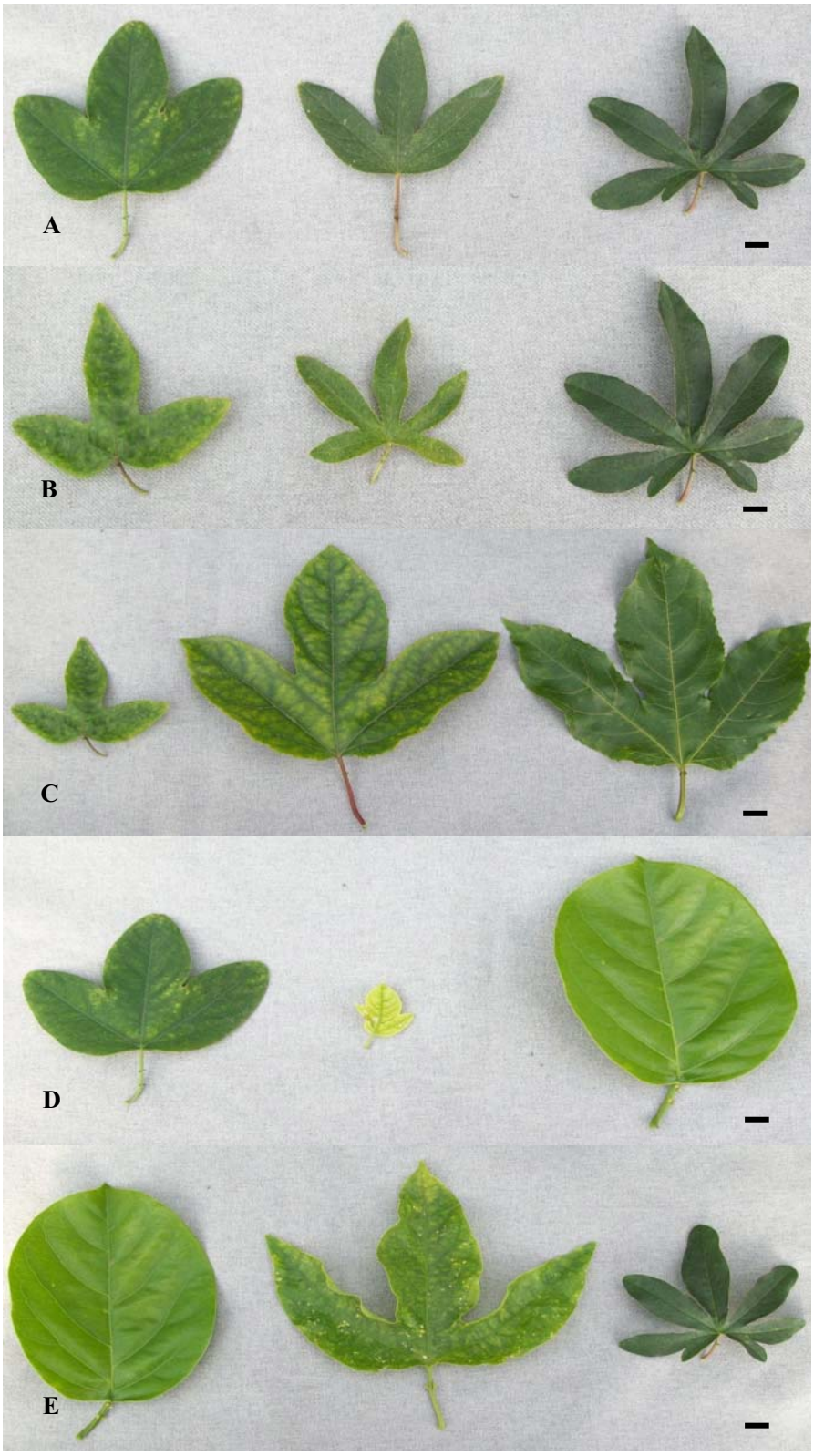

Fig. 5 Leaf morphology of hybrids (middle) and parents (female at left, male at right) of *Passiflora*. (**A**) *P. amethystina* × *P. caerulea*, (**B**) *P.* 'violacea' × *P. caerulea*, (**C**) *P.* 'violacea' × *P. edulis* f. *edulis*; (**D**) *P. amethystina* × *P. alata*, (**E**) *P. alata* × *P. caerulea*. Scale bars = 1 cm.

Hiscock 2010). S-alleles exist in several self-incompatible species and it could be possible that they act inhibiting not only self-pollination, but also interspecific hybridization (Nettancourt 1977; Pandey 1977; McClure *et al*. 2000). This kind of sporophytic incongruity could explain the success of one cross and the failure of the reciprocal as occurred in *Passiflora* interspecific hybridization (unilateral incompatibility). Pandey (1973) and Nettancourt (2001) described unilateral incompatibity in *Nicotiana* and they also asserted that the expression of this incompatibility occurs in the style. We hypothesize that *P. alata* and *P.* 'violacea' would own an *S*-allele of the most recessive of the allelic series and this could put them in a "better mother situation".

Gametophytic incompatibility acts when the pollen and the style lead one or two same alleles, which would avoid fertilization in both directions. Rego *et al*. (2000) and Suassuna *et al*. (2003) arrived at this conclusion for *P. edulis*. In almost all cross combinations considered in this work, seeds were produced at least in one cross direction so unilateral incompatibility was confirmed. Because of this, we deduced that crossings did not respond to the gametophytic

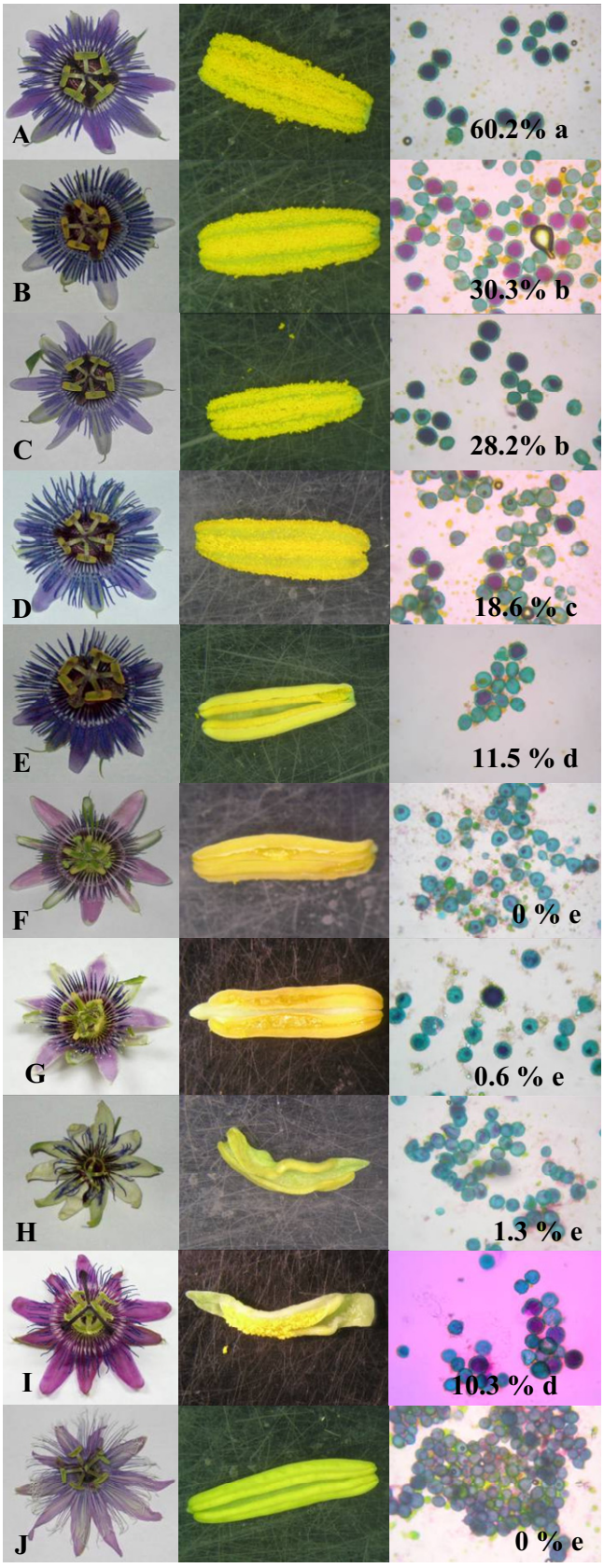

Fig. 6 Flowers, anthers and pollen viability test (Alexander, 1969) of *Passiflora* interspecific hybrids. (**A**, **B**, **C**, **D**, **E**). *P. amethystina* × *P. caerulea* hybrids, (**F**, **G**) *P. alata* × *P. caerulea* hybrids, (**H**, **I**). *P.* 'violacea' × *P. caerulea* hybrids, (**J**) *P.* 'violacea' × *P. edulis* hybrid. Different letter indicates significant differences.

determination of incompatibility.

Pollen tube growth study confirmed the presence of prezygotic barriers in the crosses *P. caerulea* × *P. amethystina* and *P. caerulea* × *P. alata.* In these cases, pollen tubes exceeded post-pollination stages in the stigma (adhesion,

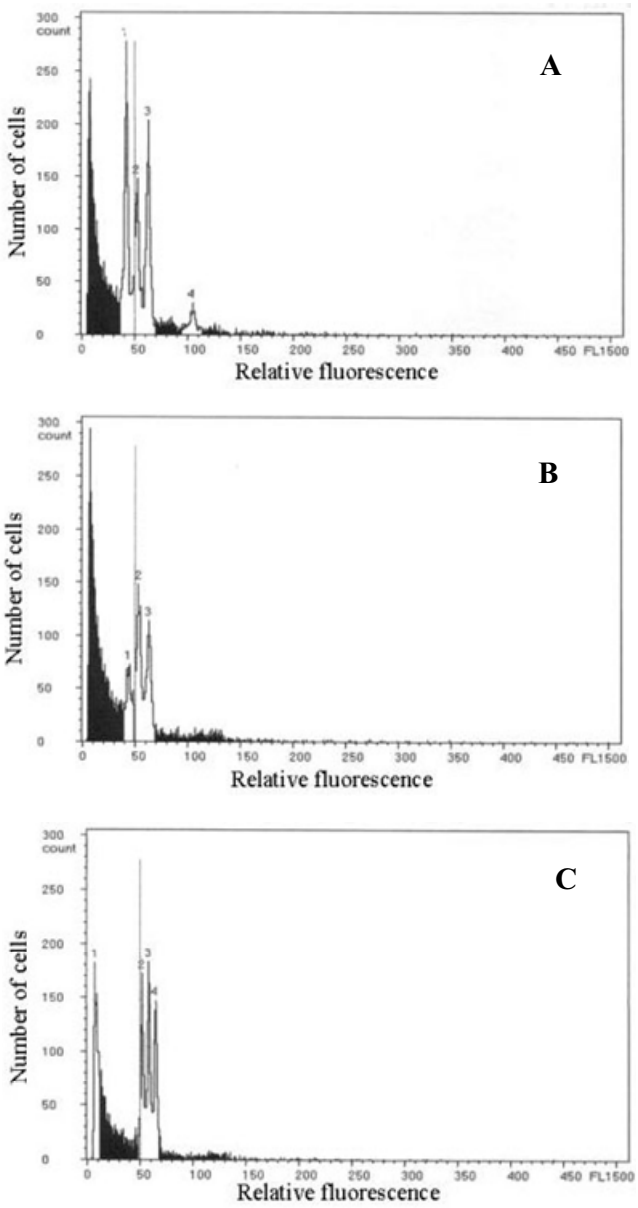

Fig. 7 Flow densitometry histograms of parental species and hybrids of *Passiflora*. (**A**) *P. alata* (right), *P. alata* × *P. caerulea* hybrid (middle), *P. caerulea* (left); (**B**) *P. alata* (right), *P. alata* × *P. cincinnata* hybrid (middle), *P. cincinnata* (left); (**C**) *P. alata* (right), *P. alata* × *P. amethystina* hybrid (middle), *P. amethystina* (left).

hydration, germination and entry of pollen tubes through stigmatic papillae). This suggests that, in these species, stigmatic barriers or any mechanism in this post-pollination instance did not exist. This situation differed from Rego *et al.* (2000) for *P. edulis*, where they found pollen tube growth inhibition at the stigma because of terminal callose heavy plugs in the pollen tubes.

In the interspecific crossings considered, none of the cases showed fruit production when *P. amethystina* acted as a male parent. This fact could be due to pollen-pistil interactions in the styles of the female partners. In the other crosses with poor fruit set, *P. caerulea* × *P. amethystina and P. caerulea* × *P. alata*, pollen tubes stopped growing in the style. Nevertheless, the last cross produced fruits but fruit set was lower among the crosses that produce any fruit. This could indicate a sporadic situation due to an environmental factor that favours fertilization. Azcón-Bieto and Talón (2000) asserted that climatic factors can induce sterility while others as high temperatures could inactivate incompatibility proteins (van Tuyl 1997).

Through the reproductive success values we can come to the conclusion that a barrier to hybridization occurs due

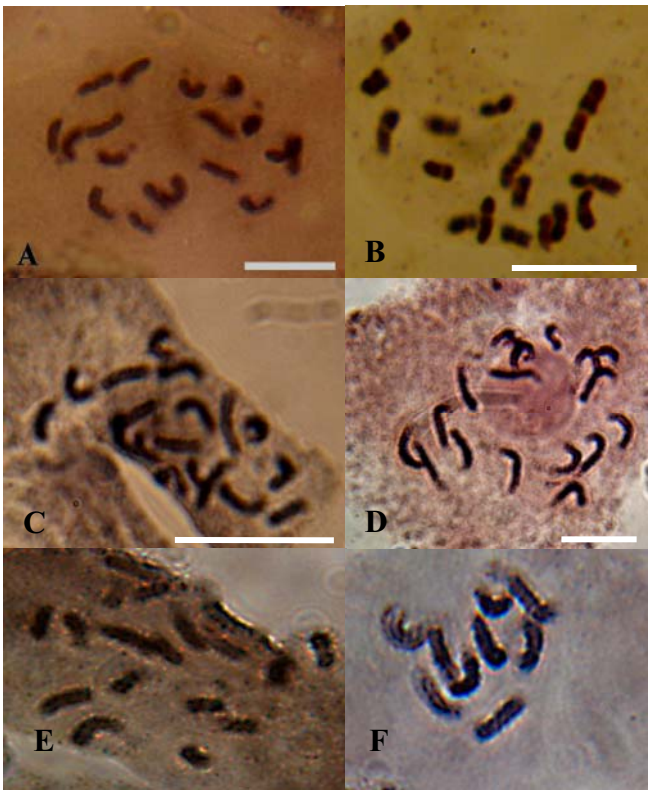

Fig. 8 Chromosomes of parental species and hybrids of *Passiflora*. (**A**) Metaphase of *P. caerulea*, (**B**) Metaphase of *P. edulis*, (**C**) Metaphase of *P. amethystina*; (**D**) Prometaphase of *P. alata*, (**E**) Metaphase of *P. alata* × *P. caerulea* hybrid; (**F**) Monoploid cell in *P. alata*. Scale bars = 10 μm.

to the lack of fruit set in some crosses and problems in seed germination. The causes of germination failure could be due to an error in the embryo development, in the endosperm development, in the embryo-endosperm compatibility or seed dormancy (Lester and Kang 1998; Kinoshita 2007).

Hybrid seed inviability, seedlings inviability and vegetative lethality occur commonly in distant intergenomic crosses (Singh 2003). The possibility of cross is an indirect measure of the degree of genomic relationship between the parental species. The result of the crosses suggested that *P. caerulea* has more genomic affinity with *P. amethystina*, because their crosses result in hybrids with viable pollen grains. The phylogeny of the genus *Passiflora* developed by Muschner *et al*. (2003) also permitted to establish their closeness while *P. alata* was placed far from them.

Choromosome number was confirmed as 2n=2x=18. This information is in agreement with the observations performed by Deginani (2001), Deginani and Escobar (2002) and De Melo *et al*. (2001). In spite of this, the mean sizes of the chromosomes of the parental species studied were different. The different sizes of the chromosomes in *P. alata* and *P. amethystina* could explain the conflict of their hybrid's genome which showed a poor vegetative growth. Hansen *et al.* (2006) stated that the monophyletic origin of the genus is strongly determined by the basic chromosome number. In four species of *Passiflora* x=9 was found so we can conclude that a small genetic distance exists among them.

The common origin would also make possible that the species could own identical self-incompatibility genes preserved in the evolution of the different species in such a way that they interact, at present, among species. In this way, the rejection between two genotypes could easily occur at a same or higher frequency inside the same species and between different species (Nettancourt 1977). Another hypothesis that could also relate to the mentioned phenomena, suggests that self-incompatibility genes are binded to other incongruity genes in the *S*-locus forming an *S*-complex (Pandey 1973; Fritz and Hanneman 1989; Nettancourt 2001).

Considering the obtained results, we are able to suggest treatments to overcome interspecific barriers among the native species of *Passiflora*. *P. amethystina* × *P. caerulea* was the only cross that produced hybrid plants capable of producing fertile pollen grains, enough for future crossings. Hybrid plants from the crosses *P. alata* × *P. caerulea*, *P.* 'violacea' × *P. caerulea*, *P. alata* × *P. caerulea* and *P.* 'violacea' × *P. edulis* f. *edulis* which arrived to a reproductive state, turned out to present hybrid's pollen inviability. Hybrid fertility could be restored by polyploidization allowing the pairing of homologous chromosome in the allopolyploid hybrid (van Tuyl 1997). *P. amethystina* × *P. alata* hybrid showed low vegetative viability, poor growth and lack of flowering. For this cross, duplication of ploidy level for the reversion of sterility is also suggested. This reversion was registered for other species through this strategy (Hermsen *et al*. 1981; van Tuyl *et al*. 1997).

In the cases of pollen tubes which were arrested in the style (crosses between *P. caerulea* × *P. amethystina* and *P. caerulea* × *P. alata*), many diverse techniques have been developed to overcome these barriers. Techniques such as style graft, cut style pollination, mentor pollen pollination, growth regulators application, bud pollination and ovary or ovule pollination were suggested (van Tuyl 1997). Payán and Martin (1975) proved the effectiveness of gibberellic acid and α-naphthalene-acetamide application in 1% concentrations to the base of the ovary of different species of *Passiflora*. Van Tuyl (1997) suggested that, besides gibberellins, application of auxins or cytokinins to pedicels or ovaries improve fruit set and seed set in *Lilium* and *Tulipa*. Sometimes, it was possible to observe the arrival of pollen tubes at the ovary without fruit set. In these cases, *in vitro* culture could offer the possibility to overcome some of the problems between the arrival of the pollen tubes to the ovary and the abortion of the fruit (Litz 1993, van Tuyl 1997). Seed of some crosses *P. alata* × *P. cincinnata*, *P. alata* × *P. caerulea*, *P.* 'violacea' × *P. alata*, *P.* 'violacea' × *P. caerulea* and *P. amethystina* × *P. alata*) did not germinate, these barriers could be overcome by the *in vitro* culture of the embryos in the seeds. Embryo culture could avoid their death due to nutrition problems that occurs because of the endosperm development failure or embryo-endosperm compatibility (Litz 1993).

As expected, hybrids phenotypes were intermediate between parents. Some interesting materials could be selected for floriculture industry. We could also advance on cold tolerance selection and confirm the presence of this characteristic in *P. caerulea* as suggested by Deginani (2001) and Ulmer and MacDougal (2004). A more comprehensive research is necessary to get consistent characterization of cold tolerance. This trait is difficult to select in field conditions due to unpredictability of cold temperature occurrence and its coincidence with critical stages of plant development. Despite this, cold tolerance of *P. caerulea* and some of their hybrids was evident since no effect was detected even in flower buds after two winter periods.

CONCLUSIONS

Interspecific hybrids which achieved a reproductive state were obtained in the following crosses: *P. alata* × *P. cincinnata*, *P. alata* × *P. caerulea*, *P. amethystina* × *P. caerulea*, *P.* 'violacea' × *P. caerulea*, *P.* 'violacea' × *P. alata*, *P. amethystina* × *P. alata*, *P.* 'violacea' × *P. edulis* f. *edulis* and *P. caerulea* × *P. alata.* In another cross (*P. alata* × *P. amethystina)* post-zygotic barriers were detected. The existence of pre-zygotic barriers in the crosses *P. caerulea* × *P. amethystina* and *P. caerulea* × *P. alata* was established. *P. alata* outstood as maternal parent while *P. caerulea* did so as male parent. The chromosome number 2n=2x=18 was confirmed for *P. alata*, *P. caerulea*, *P. edulis* and *P. amethystina*. Preliminary results on the selection of cold tolerant hybrids encouraged us to continue with efforts on this topic.

The future of *Passiflora* breeding will depend on the

Fig. 9 Behavior of parental species and hybrids of *Passiflora* before the first cold period. (**A**) *P. caerulea*, (**B**) *P. amethystina*, (**C**) *P. alata*, (**D**) *P. amethystina* × *P. caerulea* hybrid, (**E**) *P.* 'violacea' × *P. caerulea* hybrid, (**F**) *P. cincinnata* × *P. alata* hybrid; (**G**) View of experimental plants.

effective and integrate application of the techniques to overcome incongruity barriers. The detection of hybrids with high viability pollen grains allowed us to continue crossing and obtaining of a segregated progeny. The inclusion of variability from other species not considered in this work could also be interesting.

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