



Study of reproductive behaviour in low-chill apples in warmer zones of Argentina



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ARTICLE INFO

Article history:

Received 27 August 2015

Received in revised form 4 December 2015

Accepted 11 December 2015

Available online 2 January 2016

Keywords:

Malus domestica

Apomixis

Self-compatibility

Lack of chilling

Fruit set

Parthenocarpy

Cross-compatibility

ABSTRACT

The domesticated apple (*Malus × domestica* Borkh.) shows abnormalities in its flowering biology when it is growing in mild-winter areas. Hence, the flowering phenology and the mating system (e.g., open-, cross- and self-pollination, apomixis and parthenocarpy) of Caricia, Eva, and Princesa apple cultivars were studied in three consecutive growing seasons. The flowering period ranged between August and October with wide variation between years, especially in relation to the overlapping of the full-bloom periods. Fruit set by selfing was significant, reaching a mean value of 20% with a minimum of 4.5% and a maximum of 43.7%, depending on the growing season and the cultivar. The self-incompatibility index showed significant changes between years in Eva and Princesa but not in Caricia. Fruit and seed set were significantly greater in cross- and open-pollination treatments than by self-pollination, apomixis or parthenocarpy. Those variables were affected by a growing season × treatment interaction. The results indicated that Caricia, Eva, and Princesa apples show partial to full self-incompatibility, depending strongly on the growing season. However, better fruit and seed set rates are obtained by cross-fertilization.

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1. Introduction

World apple production is mainly located in temperate areas in which medium- to high-chill cultivars are widely growing. Low-chill apple cultivars have high adaptation and adequate productivity in warm-winter areas, where traditional cultivars fail to produce a regular annual yield. These cultivars allowed the expansion of apple production into warmer areas (Hauagge and Cummins, 2001). Among low-chill apple cultivars, Eva (IAPAR 75), Caricia (IAPAR 77) and Princesa (NJ56 × Anna) have been successfully grown in Latin America, Africa, and the Middle East (Castro et al., 2015a,b; Pommer and Barbosa, 2009). These varieties, in particular Eva, are grown in very low-chill regions. Less than 350 chill hours are sufficient for growing these cultivars (Castro et al., 2015a,b; Denardi et al., 1988; Hauagge and Tsuneta, 1999; Pommer and Barbosa, 2009).

Typically, *Malus × domestica* cultivars growing in temperate zones show gametophytic self-incompatibility (Golz et al., 1995).

Although self-pollination and apomixis are possible reproductive functions (Baoquin et al., 1995; Campbell et al., 1991; Nyéki, 1996; Qu et al., 2008; Soltesz, 2003), stable fruit production occurs under a cross-pollination system (Hegedus, 2006; Matsumoto, 2014). Furthermore, even though some cultivars show cross-incompatibility, this is a rare event in apple (Hegedus, 2006). However, the apple and other tree species of the high latitudes display some problems related to their flowering biology when they are growing in mild-winter areas. Marginal chilling accumulation may result in a late and erratic bud break, a widely variable flowering period (Dennis, 2001; Erez, 2001), shift to self-fertility (Soltesz, 2003; Verheij, 1990), and abnormalities in floral bud development (Oukabli et al., 2003). Furthermore, in severe cases of under-chilling, flower bud abortion was observed (Lyrene, 2005). These events represent a serious problem for growing apples with high-chill requirements in mild-winter areas. Such symptoms may also be observed in apple cultivars with low-chill requirements growing in mild-winter environments, even in areas with chilling conditions close to their requirements (Lichou and Thiery, 1981; Mohamed, 2008; Zuñiga et al., 1991).

The knowledge of the reproductive behaviour of the low-chill apple cultivars under mild-winter conditions has a great impact on

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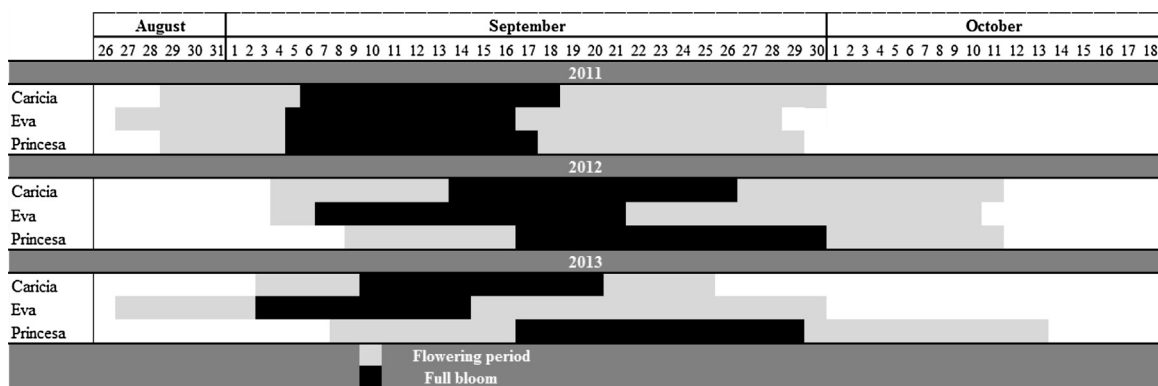


Fig. 1. Flowering period of Caricia, Eva, and Princesa apples during 2011–2013 growing seasons. The chilling accumulation was 400 chilling hours (CH) in 2011, 326 CH in 2012, and 342 CH in 2013.

orchard design and management, mainly in the context of climate change that would reduce the availability of cold more in tropical and subtropical areas than in temperate zones (Luedeling et al., 2011). For example, both the coincidence of bloom periods and the degree of cross-compatibility of a given pair of cultivars impacts on orchard design, fruit quality and yield (Soltesz, 2003). The extent of self-compatibility and its stability along the growing seasons will influence the decision to use solid blocks of a single cultivar or to incorporate pollen donors (Soltesz, 2003; Volz et al., 1996). Each of these decisions will affect the fruit quality and yield.

Thus, the aim of this work was to study the reproductive behaviour of three low-chill apple varieties cultivated in a mild-winter area during three consecutive seasons.

2. Materials and methods

2.1. Plant material and study area

Three low-chill apple cultivars grafted onto MM111 rootstock were used for this work: Caricia (IAPAR 77), Eva (IAPAR 75), and Princesa (NJ56 × Anna). The cultivars Caricia and Eva were originated in a breeding programme at the Instituto Agrônomo do Paraná (IAPAR) (Hauagge and Tsuneta, 1999) and Princesa was originated in a breeding programme at the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI) (Denardi et al., 1988). Both IAPAR and EPAGRI are government research institutions of Brazil. All trees were grown since 2001 in the Campo Experimental de Cultivos Intensivos y Forestales (CECIF) orchard at the Facultad de Ciencias Agrarias of the Universidad Nacional del Litoral in Argentina (31°24′02.22″ S.; 60°54′01.61″ W.; thirty-two meters above sea level). According to the Thornthwaite climate classification, the study area has a subhumid-humid mesothermal climate with little or no water deficiency (Mosconi et al., 1981).

The natural chilling accumulation was 400 chilling hours (CH) during 2011, 326 CH in 2012 and 342 CH in 2013. The CH accumulation was calculated according to the Crossa-Raynaud model (Crossa-Raynaud, 1956), which is the most suitable for the study area (García et al., 2011). The experimental plot was grown with supplemental drip irrigation. The temperature and relative humidity during the pollination day and the following 17 days are shown in Fig. 3.

2.2. Floral phenology

The length of the blooming period was registered in 10 trees per cultivar. Growth stages ranging from 60 to 69 on the pome fruit BBCH phenological scale (Meier, 2001) were recorded in each tree. Thus, beginning of flowering was considered when 10% of the

flowers were in the anthesis phase (growth stage 61). The full bloom was registered when 50% of the flowers were in anthesis phase and the end of flowering when most of the petals fell in 80% of trees (growth stages 65 and 69).

Pollen availability was qualitatively evaluated by touching the anthers with a brush and observing its presence. Stigmatic receptivity was evaluated with hydrogen peroxide (Osborn et al., 1988).

2.3. Pollination treatments

Different sets of inflorescences of each cultivar were randomly assigned to one of the following treatments in the 2011, 2012, and 2013 growing seasons:

(1) *Open-pollination (control)*: Non-manipulated flowers were exposed to free pollination.

(2) *Induced self-pollination (self-compatibility)*: Flowers were isolated with friseline bags in growth stage 55 (Meier, 2001) and hand-pollinated using pollen from flowers of different trees of the same clone (clonal geitonogamy) in growth stage 59 (Meier, 2001).

(3) *Cross-pollination (xenogamy)*: Emasculated flowers were isolated with friseline bags and subsequently hand-pollinated using pollen from another cultivar. The combination of cultivars is shown in Tables 1–3. The flowers were emasculated by cutting off the anthers in growth stage 57. The flowers were pollinated in growth stage 59.

(4) *Apomixis*: Flowers were bagged at growth stage 55 with friseline bags, emasculated at growth stage 57, and isolated again with no hand-pollination.

(5) *Parthenocarpy*: Fruit set without fertilization was evaluated through the rate of seedless fruits formed from non-manipulated flowers (Nyéki, 1996).

For treatment (5) the experimental unit was a single fruit of a given cultivar. For treatments (1)–(4), the experimental unit was an inflorescence containing 4–5 flowers. To minimize experimental error, non-emasculated small flowers and previously opened flowers were removed from selected inflorescences. Before the pollination treatments the pollen viability was evaluated by a Fluorescein test (Heslop-Harrison and Heslop-Harrison, 1970) and by a germination test according to Dantas et al. (2005).

Fruit set (percentage of fruits formed per flower) and seed set (percentage of viable seeds formed per fruit) were variables used to compare the treatments. Seeds were considered as viable when they were not flattened (Moriya et al., 2005). Fruit set was recorded before “June drop” (about 30–60 days after full bloom, depending on the growing season) and seed set was recorded at harvest. It was not possible to register the seed set for the 2013 season.

The self-incompatibility (SI) index was calculated with the values of seed set obtained in treatments (2) and (3) (Lloyd and Schoen,

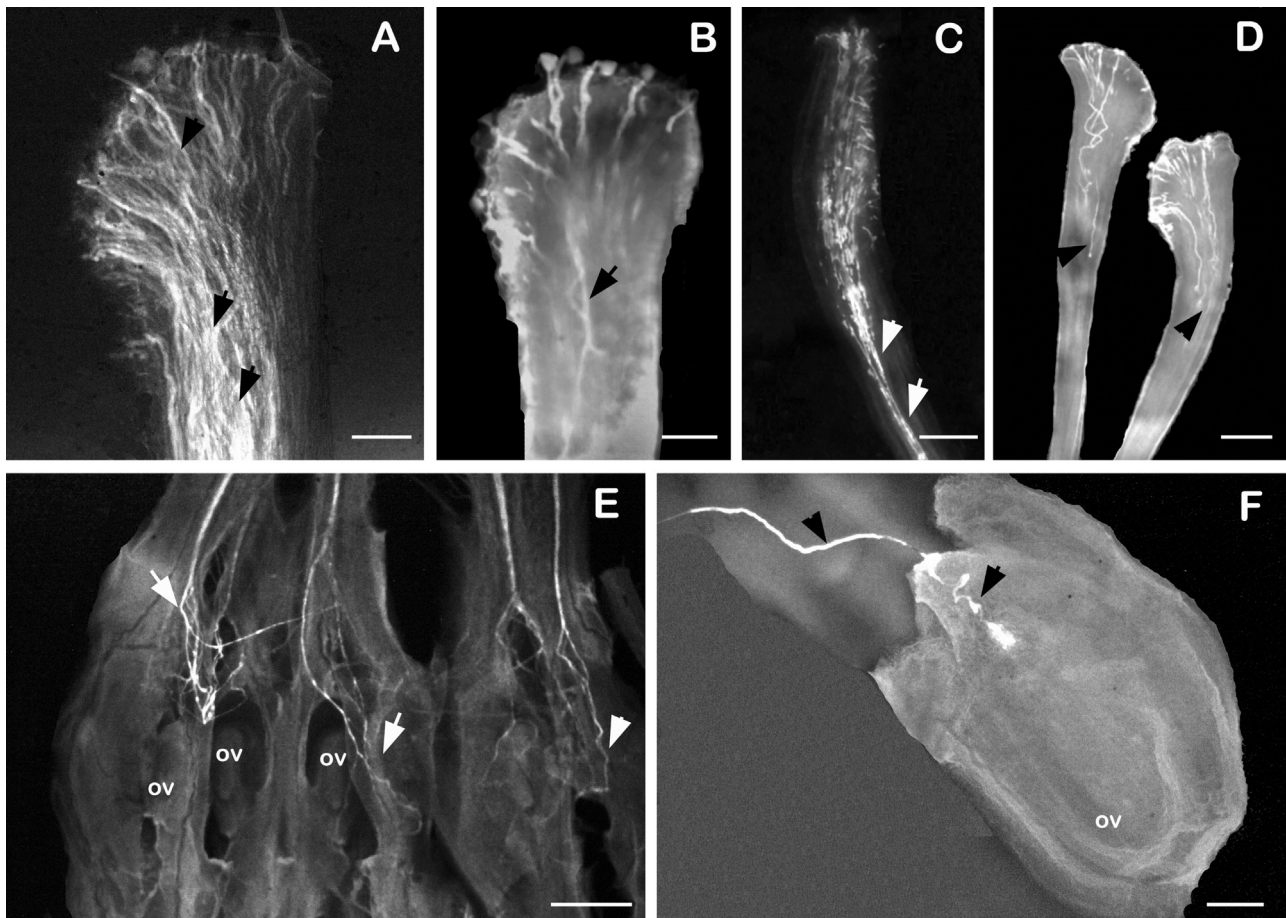


Fig. 2. *In vivo* pollen germination and pollen tube growth across the pistils. (A) Pollen germination in cross-pollinations and self-pollinations when the cultivar behaved as partially self-compatible. (Note the high rate of pollen germination and pollen tube growth. Black arrows indicate pollen tubes growth. Scale = 100 μ .) (B) Pollen germination in self-pollinations when the cultivar behaved as fully self-incompatible. (Note the low rate of pollen germination and pollen tube growth. Black arrow indicates pollen tubes growth. Scale = 100 μ .) (C) Pollen tube growth across the style in cross-pollinations and self-pollinations when the cultivar behaved as partially self-compatible. (Note that the pollen tubes are not arrested in the style, as is shown by white arrows. Scale = 250 μ .) (D) Pollen tube growth across the style in fully self-incompatible pollinations. (Note that the pollen tubes are arrested in the style, as is shown by black arrows. Scale = 250 μ .) (E) Pollen tube growth into the ovaries: the white arrows show pollen tubes (ov: ovules. Scale = 250 μ .) (F) Ovule fertilization: black arrow indicates pollen tube reaching the ovule (ov: ovule. Scale = 100 μ .)

Table 1
Pollination treatments applied to the Caricia apple cultivar during three consecutive growing seasons. (SI index: self-incompatibility index; CH: chilling hours). Different letters in the same column indicate significant difference by DGC test ($p \leq 0.05$). “–” indicates that no data are available. Compatibility status: the lower limit of partial self-compatibility was established at SI index = 0.20. The cross-compatibility status was established according to Maliga (1953) cited by Nyéki (1996).

Growing season	C.H.	Treatment	Female cultivar	Male cultivar	No. of flowers utilized	Initial fruit set (%)	No. of seeds/fruit	SI index	Compatibility status
2011	400	Open pollination	“Caricia”	–	72	52.7b	6.4a	–	
2012	326	Open pollination	“Caricia”	–	72	66.5a	2.1b	–	
2013	342	Open pollination	“Caricia”	–	72	43.0b	–	–	
2011	400	Self-pollination	“Caricia”	“Caricia”	72	43.7b	1.5b	0.49	Partially self-compatible
2012	326	Self-pollination	“Caricia”	“Caricia”	72	13.8c	2.6b	0.40	Partially self-compatible
2013	342	Self-pollination	“Caricia”	“Caricia”	72	13.6c	–	–	
2011	400	Cross-pollination	“Caricia”	“Eva”	72	81.0a	5.9a	–	Cross-compatible to a large extent
2012	326	Cross-pollination	“Caricia”	“Eva”	72	63.2a	6.3a	–	Cross-compatible to a large extent
2013	342	Cross-pollination	“Caricia”	“Eva”	72	73.3a	–	–	Cross-compatible to a large extent
2011	400	Cross-pollination	“Caricia”	“Princesa”	72	87.7a	6.4a	–	Cross-compatible to a large extent
2012	326	Cross-pollination	“Caricia”	“Princesa”	72	54.6b	6.2a	–	Cross-compatible to a large extent
2013	342	Cross-pollination	“Caricia”	“Princesa”	72	45.3b	–	–	Cross-compatible to a large extent
2011	400	Apomixis	“Caricia”	–	72	6.5c	2.6b		
2012	326	Apomixis	“Caricia”	–	72	8.5c	0.3b		
2013	342	Apomixis	“Caricia”	–	72	6.0c	–		
r^2						0.32	0.45		
Treatment effect (T)						0.0001	0.0001		
Growing season effect (GS)						0.0048	0.0001		
T \times GS effect						0.0177	0.0001		

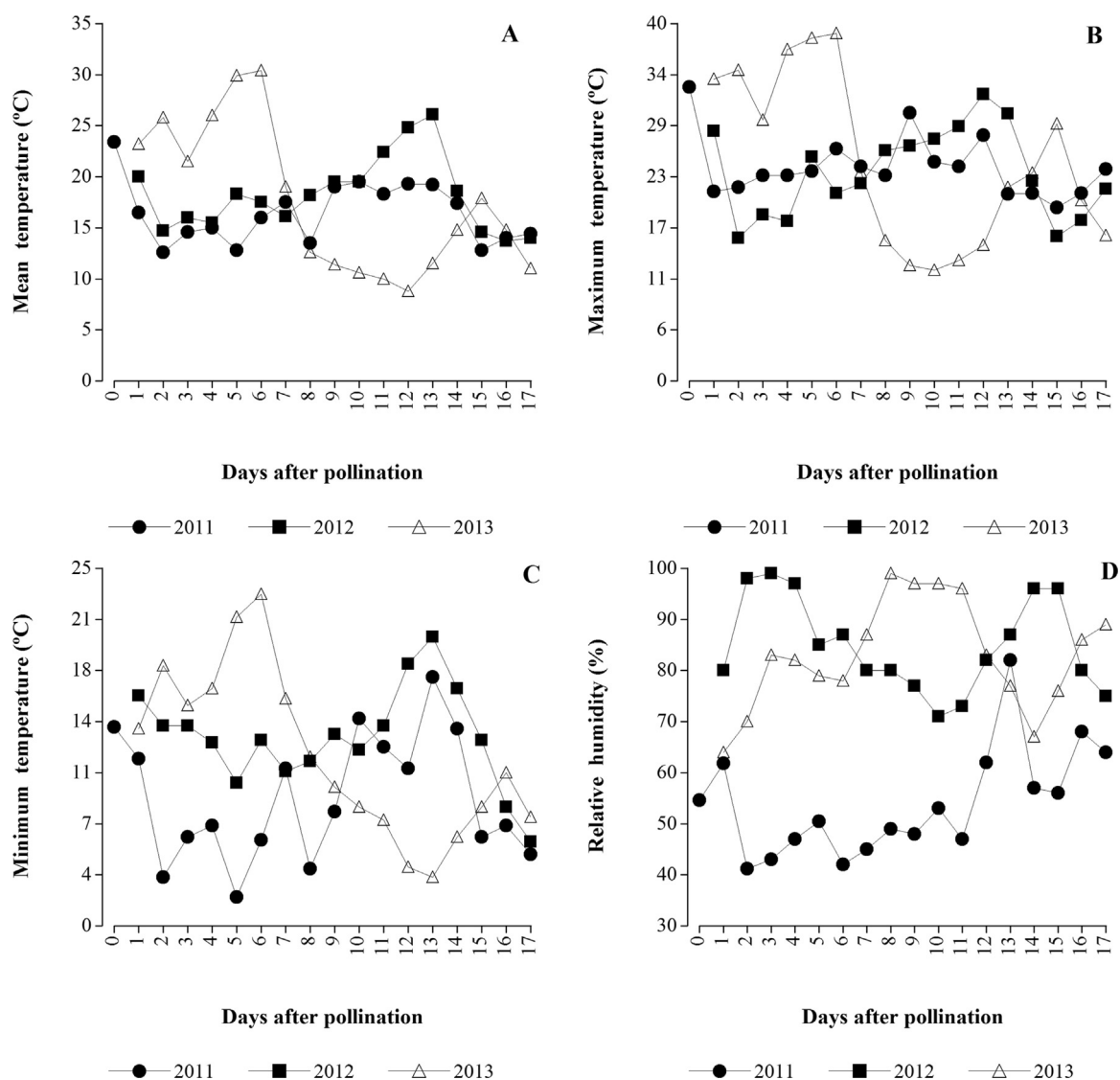


Fig. 3. Meteorological conditions during pollination experiments. (A) Mean temperature. (B) Maximum temperature. (C) Minimum temperature. (D) Relative humidity.

Table 2

Pollination treatments applied to the Eva apple cultivar during three consecutive growing seasons. (SI index: self-incompatibility index; CH: chilling hours). Different letters in the same column indicate significant difference by DGC test ($p \leq 0.05$). “–” indicates that no data are available. Compatibility status: the lower limit of partial self-compatibility was established at SI index = 0.20. The cross-compatibility status was established according to Maliga (1953) cited by Nyéki (1996).

Growing season	C.H.	Treatment	Female cultivar	Male cultivar	No. of flowers utilized	Initial fruit set (%)	No. of seeds/fruit	SI index	Compatibility status
2011	400	Open pollination	“Eva”	–	72	89.1a	2.8a	–	
2012	326	Open pollination	“Eva”	–	72	43.3b	3.0a	–	
2013	342	Open pollination	“Eva”	–	72	68.2a	–	–	
2011	400	Self-pollination	“Eva”	“Eva”	72	6.7c	0.0b	0.00	Fully self-incompatible
2012	326	Self-pollination	“Eva”	“Eva”	72	38.1b	1.4b	0.64	Partially self-compatible
2013	342	Self-pollination	“Eva”	“Eva”	72	4.5c	–	–	
2011	400	Cross-pollination	“Eva”	“Caricia”	72	85.0a	4.5a	–	Cross-compatible to a large extent
2012	326	Cross-pollination	“Eva”	“Caricia”	72	16.3c	4.0a	–	Cross-compatible to a medium extent
2013	342	Cross-pollination	“Eva”	“Caricia”	72	77.7a	–	–	Cross-compatible to a large extent
2011	400	Cross-pollination	“Eva”	“Princesa”	72	72.0a	5.0a	–	Cross-compatible to a large extent
2012	326	Cross-pollination	“Eva”	“Princesa”	72	51.8b	4.2a	–	Cross-compatible to a large extent
2013	342	Cross-pollination	“Eva”	“Princesa”	72	44.5b	–	–	Cross-compatible to a large extent
2011	400	Apomixis	“Eva”	–	72	5.6c	1.3b	–	
2012	326	Apomixis	“Eva”	–	72	3.5c	2.7b	–	
2013	342	Apomixis	“Eva”	–	72	6.0c	–	–	
r^2						0.52	0.19		
Treatment effect (T)						0.0001	0.0041		
Growing season effect (GS)						0.0001	0.9342		
T × GS effect						0.0001	0.7811		

Table 3
Pollination treatments applied to the Princesa apple cultivar during three consecutive growing seasons. (SI index: self-incompatibility index; CH: chilling hours). Different letters in the same column indicate significant difference by DGC test ($p \leq 0.05$). “–” indicates that no data are available. Compatibility status: the lower limit of partial self-compatibility was established at SI index = 0.20. The cross-compatibility status was established according to Maliga (1953) cited by Nyéki (1996).

Growing season	C.H.	Treatment	Female cultivar	Male cultivar	No. of flowers utilized	Initial fruit set (%)	No. of seeds/fruit	SI index	Compatibility status
2011	400	Open pollination	“Princesa”	–	72	35.1b	6.7a	–	
2012	326	Open pollination	“Princesa”	–	72	21.3b	1.2c	–	
2013	342	Open pollination	“Princesa”	–	72	31.9b	–	–	
2011	400	Self-pollination	“Princesa”	“Princesa”	72	20.7b	0.7c	0.11	Fully self-incompatible
2012	326	Self-pollination	“Princesa”	“Princesa”	72	4.6c	1.3c	0.33	Partially self-compatible
2013	342	Self-pollination	“Princesa”	“Princesa”	72	38.3b	–	–	
2011	400	Cross-pollination	“Princesa”	“Caricia”	72	59.1a	5.3b	–	Cross-compatible to a large extent
2012	326	Cross-pollination	“Princesa”	“Caricia”	72	55.2a	4.2b	–	Cross-compatible to a large extent
2013	342	Cross-pollination	“Princesa”	“Caricia”	72	32.5b	–	–	Cross-compatible to a large extent
2011	400	Cross-pollination	“Princesa”	“Eva”	72	70.3a	5.5b	–	Cross-compatible to a large extent
2012	326	Cross-pollination	“Princesa”	“Eva”	72	53.4a	5.4b	–	Cross-compatible to a large extent
2013	342	Cross-pollination	“Princesa”	“Eva”	72	30.4b	–	–	Cross-compatible to a large extent
2011	400	Apomixis	“Princesa”	–	72	2.5c	3.0c	–	
2012	326	Apomixis	“Princesa”	–	72	3.5c	1.0c	–	
2013	342	Apomixis	“Princesa”	–	72	1.6c	–	–	
r^2						0.33	0.58		
Treatment effect (T)						0.0001	0.0001		
Growing season effect (GS)						0.0005	0.0001		
T × GS effect						0.0018	0.0001		

1992; Moriya et al., 2005; Zapata and Arroyo, 1978). The SI index ranges from 0 to 1 (Zapata and Arroyo, 1978), with 0 the value for a complete self-incompatible species and 1 for a fully self-compatible species. According to Lloyd and Schoen (1992), an SI index >0.75 is a clear boundary between a fully self-compatible and a partially self-compatible species or cultivar. However, the boundary between a partially self-compatible and a fully self-incompatible species or cultivar is less clear. In agreement with the data published by Zapata and Arroyo (1978), Moriya et al. (2005) and Lloyd and Schoen (1992), we consider an SI index <0.20 as related to a fully self-incompatible species. Hence, according to SI index, each of the cultivars tested were fully self-compatible (SI index >0.75), partially self-compatible (SI index between 0.20 and 0.75) or fully self-incompatible (SI index <0.20).

On the other hand, the cross-compatibility of a given cultivar combination was established according to Maliga (1953) cited by Nyéki (1996) as follows: (a) cultivars that can fertilize another to a negligible extent (fruit set $\geq 0\%$ and <2%), (b) to a slight extent (fruit set $\geq 2\%$ and <10%), (c) to a medium extent (fruit set $\geq 10\%$ and <20%), (d) to a high extent (fruit set $\geq 20\%$ and <30%) and e) to a large extent (fruit set $\geq 30\%$).

For treatments (2) and (3), pollen from each cultivar was collected from forced flowers on cut branches (Nyéki, 1996). The branches were cut from several trees of each cultivar when inflorescences were at a growth stages 56–57 (Meier, 2001) and forcing at $25 \pm 1^\circ\text{C}$ until dehiscence of anthers. The pollen of each cultivar was collected individually with a different brush, placed in an Eppendorf tube (5 mL), and cold-stored until its utilization. All pollinations were performed using a different brush for the pollen of each cultivar.

2.4. In vivo pollen germination

Pollen tube growth in pistils was evaluated for treatments (2) and (3). Ten flowers were removed 48–96 h after pollination and fixed in FAA (formalin: acetic acid: alcohol, 1:1:3). The pollen tube growth was evaluated using the aniline blue staining protocol (Martin, 1959). The tissues were observed with a fluorescence microscope (Leica DM1000), and pictures were taken with a Canon EOS Rebel T2i (DS126271) camera (Fig. 2). A pollen grain was con-

sidered to be germinated when the pollen tube length was two-fold longer than the pollen grain diameter (Fig. 2).

2.5. Statistical design and data analysis

A factorial arrangement of treatments 4×3 (four treatments, three years of experimentation) in a complete randomized design was used for the data of each cultivar. The experimental unit was a single inflorescence. The data of experiment (5) were analysed separately in a 3×3 factorial (three cultivars and three years of experimentation). Eighteen inflorescences were used for each treatment combination (Tables 1–3) and for treatments (1) and (4). These replicates were selected from six trees of each cultivar at 1.3 m from the ground on both sides of the horizontal-palmette. Hence, 72 flowers were used in each case and every experimental year. Finally, 100 fruits per year and per cultivar were used on treatment (5).

Differences in fruit set and seed set among treatments were analysed by the adjustment of general linear models (GLMs) with the *lme* function of the *nlme* package (Pinheiro et al., 2011) of the R statistical language (R Development Core Team, 2011), using the interface provided by InfoStat (Di Rienzo et al., 2012). Means of statistically significant factors obtained with GLMs were compared using the Di Rienzo, Guzman, and Casanoves (DGC) test (Di Rienzo et al., 2002). Normality and homoscedasticity were tested graphically (Montgomery and Runger, 2003). To avoid variables transformation, variance structure was modelled with the *varexp* function of the *nlme* package of R (Pinheiro et al., 2011) for seed set.

3. Results

3.1. Floral phenology

The flowering period of all cultivars ranged between August and October, with slight differences among them, within each growing season (Fig. 1). Thus, during the 2012 and 2013 seasons, the onset of the flowering period of all cultivars was delayed about six days compared to the 2011 season.

On the other hand, the onset and length of full-bloom periods was related both to the cultivar and the growing season (Fig. 1).

It was observed that the full-bloom period of all cultivars in the 2011 growing season was entirely overlapped. However, in 2012 there was an overlay of <50% between the full-bloom periods of the Eva and Princesa cultivars. Furthermore, in 2013 no overlay was observed between the full-bloom periods of these cultivars. In all the three-year period of the study the overlap of the entire bloom period between cultivars was greater than 50% (Fig. 1). It is worth mentioning that the progression of flowering was not uniform on any cultivar observed; on the contrary, at least two “waves” of flowering were observed on each cultivar during the blooming period (data not shown). These waves were related to the position of the flowers: the flowers on the terminal position (both on spurs or shoots) opened before the flowers on the lateral position on long shoots (more than 20 cm long).

Flowers exhibited protogyny in all the cultivars studied. Stigmatic receptivity started in growth stage 59 and pollen shed started between growth stages 63 and 65. Thus, the anther dehiscence of the first set of stamens took place in growth stage 63. However, the second set of stamens continued elongating until its own dehiscence, which occurred at growth stage 65.

3.2. Pollination treatments

3.2.1. Fruit set

In Caricia, the fruit set varied as an effect of a growing season \times treatment interaction ($p=0.017$; $r^2=0.32$). The fruit set by cross- and open-pollination was higher than by self-pollination, within each growing season, except in 2011 (Table 1). In cross-pollinations, Eva proved to be the best pollen donor in both the 2012 and 2013 growing seasons, as fruit set was higher than using Princesa as pollen donor. Furthermore, using Eva as the pollen donor, the fruit set was equal or greater than by open-pollination in all the growing seasons. Using Princesa as the pollen donor, the fruit set was higher than by open-pollination only in the 2011 growing season; in 2013 it was equal and in 2012 it was lower. With either Princesa or Eva as pollen donors the fruit set surpassed 30%.

The fruit set by self-pollination was higher than 10% in all the growing seasons. The fruit set in 2011 was higher than it was in both 2012 and 2013.

In Eva, the fruit set was also affected by a growing season \times treatment interaction ($p=0.0001$; $r^2=0.52$). The fruit set by cross- and open-pollination was higher than by self-pollination, except in 2012 (Table 2). With regard to cross-pollination, there was no clear evidence of the superiority of either pollen donor (Caricia or Princesa). In the 2011 growing season there was no difference in fruit set attributable to either pollen donor, but in 2012 the highest fruit set was reached with pollen of Princesa, while in 2013 this was observed using pollen of Caricia. This pattern was also observed comparing the performance of either pollen donor against the performance of open-pollination in those growing seasons. As occurred by using Caricia as the female, the fruit set by cross-pollination was higher than 30%.

In contrast, the fruit set by self-pollination was only higher than 10% in the 2012 growing season, which was the highest of the three-year study.

In Princesa, as in previous cultivars, the fruit set was affected by a growing season \times treatment interaction ($p=0.0018$; $r^2=0.33$). Unlike the observed fruit set of the previous cultivars, the fruit set by cross-pollination was higher than by both self- and open-pollination in each growing season excluding 2013 (Table 3). As there was no difference in the rate of fruit set using either Caricia or Eva as pollen donors, both of them could be utilized as pollinators. Furthermore, the fruit set by cross-pollination was higher than 30% on all the growing seasons. However, the fruit set by open- and cross-pollination was lower in 2013 compared with the previous growing seasons. With regard to fruit set by self-pollination, it

was observed that it was higher than 10% on most of the growing seasons.

The rate of fruit set by apomixis was 7% in Caricia, 5% in Eva, and 2.5% in Princesa apples without significant differences between them (Tables 1–3). The fruit set by parthenocarpy was only affected by the cultivar ($p=0.003$, data not shown). Therefore, the mean fruit set of parthenocarpic fruits was 12.5%, 5.5%, and 15.5% in Caricia, Eva, and Princesa, respectively.

3.2.2. Seed set

In Caricia, the seed set was affected by a growing season \times treatment interaction ($p=0.0001$; $r^2=0.45$) (Table 1). The seed set by cross-pollination was higher than by self-pollination in both years (2011 and 2012). Furthermore, the interaction effect was observed when analysing the seed set of flowers subjected to open-pollination. In 2011 the seed set of this treatment was equal to the cross-pollinations, but in 2012 the seed set fell to a value equivalent to that obtained by self-pollination.

In Eva, the seed set varied just as an effect of the treatment ($p=0.0041$; $r^2=0.19$) (Table 2). The seed set was higher by both cross- and open-pollination than by self-pollination, irrespective of the growing season. However, the seed set was lower than what observed on Caricia or Princesa.

In Princesa, the seed set was affected by growing season \times treatment interaction effect ($p=0.0001$; $r^2=0.58$) (Table 3). The seed set was higher by cross-pollination than by self-pollination in both growing seasons. The seed set by open-pollination varied widely among growing seasons: in 2011 it was higher than by cross-pollination, but in 2012 it was equivalent to self-pollination.

The seed set by apomixis was low and was mainly affected by the cultivar (Tables 1–3). As a result, the seed set by apomixis was 1.5 seeds per fruit in Caricia, and 2.0 seeds per fruit in Eva and Princesa. Thus, the rate of seed set by apomixis was similar to that reached by self-pollination.

3.2.3. Self-incompatibility (SI) index

The SI index in all cultivars was lower than 0.75. In Eva and Princesa there was a clear effect of the growing season on the SI index (Tables 2 and 3, respectively). Thus, the SI index accounted for self-incompatibility in 2011 (400 CH) and partial self-compatibility in 2012 (326 CH) in those cultivars. On the contrary, the SI index showed no variability related to growing season in Caricia (Table 1). As a result, the SI index in this cultivar indicated partial self-compatibility behaviour in both 2011 and 2012.

3.2.4. In vivo pollen germination

Observations *in vivo* of pollen germination and pollen tube growth showed pollen tubes growing through the style and reaching the ovary in more than 60% of observed cross-pollinated flowers (Table 4 and Fig. 2). The rate of pollen tubes that reached the ovary was lower only in the Princesa \times Caricia treatment during the 2012 growing season. Thus, as a mean value, the pollen tubes reached the ovary in 83% of the cross-pollinated flowers, regardless of the cultivar. In hand-self-pollinated flowers there were differences in pollen tube growth through the style between cultivars and growing seasons (Table 4). In Caricia, between 8% and 13% of the flowers exhibited pollen tubes reaching the ovary in 2011 and 2013, respectively. However, during 2012 pollen tube growth was arrested in the style, and consequently no pollen tubes reached the ovary. Similarly, in Eva 43% of the flowers showed pollen tubes reaching the ovary in 2012 whereas the pollen tube growth was arrested in the style during both 2011 and 2013. Finally, in Princesa there was no substantial difference between pollen tube growths during the study (Table 4).

Table 4
Pollen tube penetration on Caricia, Eva, and Princesa apple pistils after 48–96 h of pollination treatments during three consecutive years. (CH: chilling hours; PTG: pollen tubes growing; † indicates that it was not possible to observe pollen tubes in the ovaries).

Pollen tube penetration									
Growing season	CH	Treatment	Female cultivar	Male cultivar	No. of flowers observed	Pistils with PTG (%)	Stigmas with PTG (%)	Styles with PTG (%)	Ovaries with PTG (%)
2011	400	Self-pollination	“Caricia”	“Caricia”	5	90.0	75.0	70.0	8.0
2012	326	Self-pollination	“Caricia”	“Caricia”	25	100.0	75.0	75.0	0.0
2013	342	Self-pollination	“Caricia”	“Caricia”	30	78.0	73.0	63.0	13.0
2011	400	Cross-pollination	“Caricia”	“Eva”	5	100.0	86.0	100.0	90.0
2012	326	Cross-pollination	“Caricia”	“Eva”	30	100.0	67.0	100.0	100.0
2013	342	Cross-pollination	“Caricia”	“Eva”	15	100.0	100.0	100.0	100.0
2011	400	Cross-pollination	“Caricia”	“Princesa”	5	100.0	100.0	95.0	90.0
2012	326	Cross-pollination	“Caricia”	“Princesa”	30	100.0	100.0	100.0	100.0
2013	342	Cross-pollination	“Caricia”	“Princesa”	10	100.0	100.0	100.0	†
2011	400	Self-pollination	“Eva”	“Eva”	5	60.0	50.0	35.0	2.0
2012	326	Self-pollination	“Eva”	“Eva”	23	100.0	83.0	78.0	43.0
2013	342	Self-pollination	“Eva”	“Eva”	15	42.0	8.0	8.0	0.0
2011	400	Cross-pollination	“Eva”	“Caricia”	5	100.0	90.0	90.0	90.0
2012	326	Cross-pollination	“Eva”	“Caricia”	30	100.0	100.0	100.0	100.0
2013	342	Cross-pollination	“Eva”	“Caricia”	25	100.0	100.0	100.0	100.0
2011	400	Cross-pollination	“Eva”	“Princesa”	5	95.0	85.0	80.0	80.0
2012	326	Cross-pollination	“Eva”	“Princesa”	22	100.0	83.0	83.0	83.0
2013	342	Cross-pollination	“Eva”	“Princesa”	15	92.0	85.0	77.0	77.0
2011	400	Self-pollination	“Princesa”	“Princesa”	5	85.0	80.0	80.0	18.0
2012	326	Self-pollination	“Princesa”	“Princesa”	15	66.7	69.0	69.0	15.0
2013	342	Self-pollination	“Princesa”	“Princesa”	30	100.0	90.0	90.0	24.0
2011	400	Cross-pollination	“Princesa”	“Caricia”	5	80.0	80.0	80.0	70.0
2012	326	Cross-pollination	“Princesa”	“Caricia”	24	33.0	21.0	21.0	17.0
2013	342	Cross-pollination	“Princesa”	“Caricia”	10	100.0	100.0	100.0	†
2011	400	Cross-pollination	“Princesa”	“Eva”	5	100.0	93.0	80.0	80.0
2012	326	Cross-pollination	“Princesa”	“Eva”	20	100.0	60.0	60.0	60.0
2013	342	Cross-pollination	“Princesa”	“Eva”	10	100.0	100.0	100.0	†

4. Discussion

The results showed that low-chill cultivars Caricia, Eva, and Princesa produced moderate to high rates of fruit set by selfing under mild-winter conditions, so autogamy is a relevant function under our climatic conditions. Furthermore, apomictic and parthenocarpic fruit set was also observed. However, fruit and seed set were improved by cross-pollinations suggesting that those cultivars are mainly allogamous and dependent on the presence of cross-compatible cultivars to reach high and stable fruit yield in every growing season.

Natural fruit and seed set by open-pollination were relatively high but varied between years in the three studied cultivars. The data of seed set by open-pollination clearly suggest that in 2012 there were poor pollination conditions for Caricia and Princesa. In Eva the pollination conditions were deficient both in 2011 and 2012. In mild-winter areas, chilling accumulation and the temperature during the blooming period are considered the main factors that affect (directly or indirectly) the performance of pollination and fruit set (Dennis, 2001; Soltesz, 2003; Verheij, 1990). The greatest impact of the marginal cold accumulation is observed on the phenology and reproductive and vegetative behaviour of deciduous fruit trees, including apple (Dennis, 2001; Erez, 2001). In our study it was observed that in years of low chilling accumulation the full-bloom period of some of the cultivars tested was delayed and the seed set was reduced. As the flowers exhibited protogyny, the delay or partial overlap in full-bloom periods may cause poor cross-fertilization in the earlier blooming cultivar, given the low availability of cross-compatible pollen. Thus, the poor seed set shown in 2012 may be in part a result of a lower overlap than 50% between the full-bloom periods. The marginal chilling accumulation has been related to low flower bud quality (Oukabli et al., 2003) and to low quantity and quality of pollen grains (Hauagge and Cummins, 2001), so these may have affected our results. Furthermore, the variation of temperature and relative humidity during

bloom observed between growing seasons in our trials (Fig. 3) may have affected the effective pollination period, thus modifying the fruit and seed set by open-pollination between years (Sanzol and Herrero, 2001; Soltesz, 2003; Wani et al., 2010). In addition, factors such as pollen viability during a given growing season (Hauagge and Cummins, 2001), pollen mixture on the stigma (Visser and Marcucci, 1984), and pollen–style interactions (Kron and Husband, 2006) may play a key role in the fruit and seed set by open-pollination.

The pollination experiments showed that Eva, Caricia, and Princesa were capable of producing fruits and seeds after cross- and self-pollination. Based on both fruit set and SI index, these cultivars would be placed in the range of partial self-compatible to fully self-incompatible. Since fruit set depends on numerous environmental and physiological factors, the SI index is more reliable to determine the status of self-compatibility. This index corrects the success of self-pollination for variability in seed production caused by different plant vigour, physiological factors affecting seed production, or different pollination conditions between growing seasons (Lloyd and Schoen, 1992). According to the SI index, self-compatibility was stable through the growing seasons in Caricia but not in Eva or Princesa. In contrast to that which is observed in apple cultivars growing in areas with adequate availability of cold, self-compatibility was a significant function in our research, especially in growing seasons with less chilling accumulation. The rate of autogamy is strongly affected by the environmental conditions such as geographical location (mainly related to the climate), the meteorological particularities of each growing season (Dennis, 2001; Moriya et al., 2005; Soltesz, 2003; Verheij, 1990), the genetic potential of the cultivar (Campbell et al., 1991; Moriya et al., 2005), or their interaction (Moriya et al., 2005; Soltesz, 2003). For example, it was observed that Rome Beauty (a high-chill apple genotype), when cultivated under mild-winter conditions, produced a high parthenocarpic fruit set on emasculated flowers and a high fruit set by selfing (Verheij, 1990). This fact was also observed in Anna

(a low-chill apple genotype): it was self-compatible in Reunion Island (Lichou and Thiery, 1981) but self-incompatible in Costa Rica (Zuñiga et al., 1991)—both mild-winter areas for apple cultivation. Moreover, the European pear Précoce was self-compatible or self-incompatible depending on the growing season in the same location (Moriya et al., 2005). These results are consistent with those observed in our experiment in relation to the wide variation in selfing and SI index shown by Princesa and Eva apples and suggest that the gametophytic self-incompatibility (GSI) system failed to prevent selfing under mild-winter conditions. Internal factors like mutations that silence the S-RNase activity, the duplication of S-locus, the lack of S-proteins in the pollen grain, or defective S-proteins may break down the GSI system (Igic et al., 2007; Stone et al., 2006; Stone and Pierce, 2005; Tsukamoto et al., 2003, 1999). Also, in mild-winter areas the maturity of ovules precedes the beginning of the functionality of the GSI system (Verheij, 1990). Thus, the fruit set by selfing in our research may be in part a result of a hand-self-pollination made when the GSI system was still inactive.

Regarding the hand cross-pollination between Eva, Caricia, and Princesa, the fruit set was higher than 30% in most cases, with the highest seed set, irrespective of the pollen donor. According to Soltesz (2003) a fruit set higher than 10% as a result of cross-pollination is a criterion to consider those cultivars as compatible. Furthermore, when fruit set by hand-cross-pollination is over 30%, there is a large degree of cross-compatibility between the cultivars involved (Maliga, 1953, cited by Nyéki, 1996). Hence, Caricia, Eva, and Princesa must be considered cross-compatible to a large extent in most of the growing season. However, the little variations in fruit set observed in relation to the pollen donor could be associated to differential pollen performance (paternal effect) and physiological factors related to a female recipient, as well as pollen–pistil interactions (Kron and Husband, 2006; Albuquerque et al., 2010). The data of *in vivo* pollen germination allow us to speculate that Caricia, Eva, and Princesa apples do not share any “S” alleles between themselves, as the pollen tubes are not arrested in the style like in fully cross-incompatible combinations (Golz et al., 1995). However, cross-pollination by hand between known partial-cross-compatible apple cultivars produced high fruit set similar to fully cross-compatible cultivars (Schneider et al., 2005). This effect was attributed by the authors to an excess of pollen deposited on the stigma of pollinated flowers. Because our research did not evaluate the presence of S-RNase alleles, we cannot affirm that Caricia, Eva, and Princesa are fully compatible among themselves from a genetic point of view.

With regard to asexual reproduction, unlike what happens in *Malus* (Baoquin et al., 1995; Campbell et al., 1991; Qu et al., 2008; Soltesz, 2003), apomixis (including parthenocarpy) is not a significant event in the reproduction of the studied low-chill apple cultivars, in terms of rate of fruit set.

According to Nyéki (1996), a fruit set from self-pollination ranging from 3% to 10% is considered sufficient for a good apple yield. Based on our results, Eva, Caricia, and Princesa would have an adequate fruit yield if planted in solid blocks as the fruit set by selfing ranged between 4.5% and 43.7%. However, while the fruit set by selfing was significant, it was quite variable between growing seasons. The commercial quality (size, shape, firmness, soluble solid content) of such fruits may be lower than fruits coming from cross-pollination (Soltesz, 2003). Added to this, poor fertilization in the early stages of flowering (as occurred in our self-pollinations) reduces calcium concentration in fruit at harvest, which in turn can affect their post-harvest life (Volz et al., 1996).

To sum up, our results demonstrated that allogamous pollination produced significantly higher and more stable fruit set than selfing in most of the growing seasons. Thus, cross-pollination appears to be a low-risk system that can achieve high fruit set and

better fruit quality. On the other hand, the fact that full-bloom periods of a given pair of cultivars do not overlap by more than 50% in all the growing seasons reinforces the need to use two pollen donors, and/or the need to use dormancy-breaking treatments. Finally, the present study demonstrates that in the low-chill apple cultivars tested, the rate of self-compatibility depends strongly on the growing season. These results have a great impact on apple orchard design and management under mild-winter conditions.

5. Conclusions

The low-chill apple cultivars Caricia, Eva, and Princesa were prone to selfing under mild-winter conditions, but this was quite variable and strongly affected by the growing season. On the other hand, the fruit set by cross-pollination was high and stable through the growing seasons. Our results suggest that the cultivars tested were largely cross-compatible.

Acknowledgements

The authors thanks for reviews' comments and suggestions.

Funding for this research was provided by the Universidad Nacional del Litoral through the CAI+D programme, by Agencia Nacional de Promoción Científica y Tecnológica through the PICT 00477 project and by the Secretaría de Estado de Ciencia, Tecnología e Innovación de la provincia de Santa Fe through project 2010-031-11.

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