Flower age and pollenizer could affect fruit set in late-blooming self-compatible almond cultivars under warm climatic conditions

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ABSTRACT

The effect of the pollination time and of the pollen origin was studied in three self-compatible and late-blooming almond genotypes in order to evaluate their effect on fruit set and yield. The full self-compatibility of the three genotypes was clearly assessed as fruit sets after self-pollination were similar to those obtained after cross-pollination with pollen from two different genotypes. Sets reached the level of a commercial production, ranging from 34.02 to 49.98% when the flowers were pollinated at the best pollination time, two days after emasculation. Pollination at later times significantly decreased fruit set, as well as high temperatures, negatively affecting stigma receptivity and, consequently, pollen germination and fruit set. Thus, early pollination is essential for self-compatible almond cultivars, mainly if these cultivars are grown in regions with warm conditions in late winter and early spring.

1. Introduction

The pollination process in fruit trees involves the release, transport, and deposition of pollen from the anthers onto a stigma. Almond [*Prunus amygdalus* Batsch syn. *P. dulcis* (Mill.) D.A. Webb] cultivars are, with few exceptions, self-incompatible (SI), thus making cross-pollination essential for yielding acceptable crops because the commercial part of the fruit is a seed (Socias i Company, 1990). The development of consensus and specific molecular markers linked to the *S*-alleles (Tamura et al., 2000; Channuntapipat et al., 2003), involved in the recognition and inhibition of the pollen tube growth in pistils harbouring the same *S*-genotype, has allowed the establishment of cross-incompatible groups of the most important almond cultivars grown around the world (Kodad and Socias i Company, 2009a). Although this progress has allowed checking the cross-compatibility between cultivars before planting in commercial orchards, the most important problem for efficient pollination is the synchronisation of flowering time of both cultivars in order to maximize the possibilities of pollen interchange. Flowering time is affected by temperatures before bloom (Alonso and Socias i Company, 2009), and the success of the pollination process is additionally affected by other climatic conditions such as rain, wind or fog during bloom, as they distress the activity of the pollen vectors in the orchard. The release of new autogamous almond cultivars (Socias i Company et al., 2009) has been directed to avoid the problems related to pollination, thus allowing the establishment of orchards with a single cultivar and, as a consequence, facilitating their management and solving the frequent situations of a deficient pollination resulting in low yields (Socias i Company, 1990).

However, some self-compatible cultivars have shown setting and production problems (Godini et al., 1994; Socias i Company et al., 2004), raising the question of whether the introduction of adequate insect vectors in the mono-varietal orchards must be maintained to

ensure optimum pollination for increasing fruit set (Godini et al., 1994). Several factors conditioning fruit set, and consequently yield, have been identified and studied, such as bud density and factors determining the floriferous capability of a genotype (Bernad and Socias i Company, 1998; Dicenta et al., 2006); the ability for the flower population to be pollinated and fertilized (Socias i Company et al., 2005), which depends on the genetic control of incompatibility (Dicenta et al., 2002); the proportion of flower sterility (Socias i Company, 1983); the environmental conditions (Socias i Company et al., 2005); and the inbreeding effect (Alonso and Socias i Company, 2005).

Kodad and Socias i Company (2009b) have reported that the effective pollination time could be considered as a determinant factor for fruit set in 'Guara', an autogamous cultivar, and pointed out the importance of the early pollination of flowers. The concept of effective pollination period (EPP) was introduced by Williams (1965) to assess floral receptivity in apple, and was defined as the period during which pollination was effective for producing fruit. This period is determined by the longevity of the ovules minus the time-lag between pollination and fertilization, provided that this resulting value does not exceed the length of stigma receptivity. EPP plays a clear role in controlling fruit set and yield of temperate fruit crops (Sanzol and Herrero, 2001). In almond, yield has been shown to be determined by the number of flowers per tree and the EPP (Griggs and Iwakiri, 1964; DeGrandi-Hoffman et al. 1989; Vezvaei and Jackson, 1994). Several factors related to pollination-fertilization efficiency, such as stigma receptivity (Ortega et al., 2004), pollen tube kinetics (Alonso and Socias i Company, 2005), ovule longevity (Pimienta and Polito, 1982), temperature (Socias i Company et al., 2005), and chemical treatments (Socias i Company and Gómez Aparisi, 2002; Yi et al., 2006), were studied and their importance was underlined in limiting fruit set in almond cultivars.

The possible effect of the pollen source on fruit set in self-compatible almond cultivars must be known because in these cultivars fruits are obtained from self-pollination. Consequently, the ability of these cultivars to produce acceptable yields must be assessed in order to recommend them for planting in single-cultivar commercial orchards. Fruit set obtained after hand self- and cross-pollination have been compared (Dicenta et al., 2002; Socias i Company et al., 2005; Ortega et al., 2006; Kodad and Socias i Company, 2008), showing that self-pollination does not negatively affect yield in some genotypes, whereas others showed lower fruit sets when self-pollinated as compared with cross-pollination (Godini et al., 1994; Alonso and Socias i Company, 2005; Socias i Company et al., 2005; Kodad and Socias i Company, 2008).

So far, all studies have utilized a single source of foreign pollen in the cross-pollination treatments, or pollen of unknown origin in the case of open pollination. As a consequence, our objective was to asses the influence of stigmatic receptivity and different pollenizers on fruit set in late-blooming self-compatible almond cultivars.

2. Materials and methods

2.1. Plant material

The experiments were conducted over two consecutive years on three almond genotypes from the almond breeding programme of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), in Zaragoza, Spain, including two released cultivars, ‘Felisia’ (Socias i Company and Felipe, 1999) and ‘Mardía’ (Socias i Company et al., 2008), and one advanced selection (I-2-12). These genotypes are all late-blooming and self-compatible, sharing the S_f allele responsible of self-compatibility in almond (Felisia: S_8S_f;

Mardia: S_6S_f; I-2-12: S_3S_f). The treatments were carried out on three trees of these genotypes grafted in 1998 on the almond × peach hybrid clonal rootstock 'Garnem' (Felipe, 2009) and planted in the orchard in 2000. These plants are maintained according to standard cultural management. Pollenizers included two traditional cultivars, 'Marcona' ($S_{11}S_{12}$) and 'Fournat de Brézenaud' ($S_{24}S_{25}$), grown in the same location. The CITA experimental station is located in Zaragoza, at latitude 41º 38' 50" N and longitude 0º 53' 07" W, at 220 m over sea level.

2.2. Pollen grain germination

Pollen was obtained by desiccating anthers for 48 h at room temperature and storing it at 4ºC in glass vials until pollination. Pollen germination was tested on a solidified culture medium consisting of 0.3 mM sucrose, 0.6 mM calcium nitrate, 1.6 mM boric acid and 0.8% agar in a Petri dish (Hormaza and Herrero, 1996). Petri dishes were incubated at 22°C for 6 hours and pollen germination was observed under light microscope. A pollen grain was considered germinated when the length of the pollen tube exceeded its diameter (Ducon, 1968). The percentage of pollen grain germination was calculated for each sample.

2.3. Effective pollination period

EPP was determined according to Williams (1970) on tree homogenous branches selected at random around the canopy of the three trees of each genotype, including the different directions around the canopy and being of the same order of branching, of an approximate length of 1 m and placed at about 1.5 m above ground. Only flower buds at Stage D (Felipe, 1977) were left on the branches for emasculation as their evolution indicated that they were at one day before anthesis (Kodad and Socias i Company, 2009b). Emasculated flowers (~ 100

flower buds) were hand self-pollinated or cross-pollinated with ‘Marcona’ and ‘Fournat de Brézenaud’ pollen, at 0, 2, 4, 6 or 8 d after emasculation. Intact flowers were left for assessing the anthesis day. Fruit set (i.e., the percentage of pollinated flowers that produced fruit) was recorded in June, approximately three months after bloom.

2.4. Stigma receptivity

Stigma receptivity was determined on the same three trees. Flowers were emasculated and hand self-pollinated or cross-pollinated with ‘Marcona’ and ‘Fournat de Brézenaud’ pollen at 0, 2, 4, 6, or 8 d after emasculation. For each pollination treatment, 10-15 flowers were collected 1 and 4 d after pollination, fixed in 1:1:18 (v/v/v) FAA (formaldehyde-acetic acid-70% ethanol), rinsed several times in water, and autoclaved in a 5% solution (w/v) of Na_2SO_3 for 12 min at 1.2 kg cm^{-2}. Samples were maintained at 2-4ºC until examination of pollen germination on the stigmas. The percentage of stigmas with pollen tubes in the upper part of the style were determined using a Leitz Ortholux II (Wetzlar, Germany) microscope with UV illumination via an Osram HBO 200 W/4 mercury lamp after staining with 0.1% (w/v) aniline blue in 0.1M potassium phosphate (Linskens and Esser, 1957). Each stigma was considered receptive when it was able to support pollen hydration, germination, and initial pollen-tube growth into the transmitting tissues of the style (Sanzol et al., 2003). The percentage of pistils with pollen penetrating the stigma 1 d after pollination, out of 25-30 pistils examined, was determined as an index of stigma receptivity.

2.5. Statistical analysis

All statistical analyses were performed using the SAS 2000 programme (SAS Institute, Cary, NC, USA). Analysis of variance used the PROC GLM procedure to distinguish the effects of pollination time and year. Means were separated by Duncan's multiple range test ($P < 0.05$).

2.6 Meteorological data

Climatic parameters during flowering were measured at a station located in an adjacent sprinkler-irrigated grass plot. The daily minimum and maximum temperatures (°C), humidity (%), and wind speed (ms^{-1}) during the flowering period and 8 d after emasculation are shown in Fig. 1 and 2 for the two years of the study.

3. Results and discussion

3.1 In vitro pollen germination

Pollen germination was evaluated for the five almond cultivars included. In 2006, pollen germination of the pollenizers was 82% and 89% for 'Fournat de Brézenaud' and 'Marcona' respectively. For the pollen receivers it was 94%, 92%, and 92% for 'Felisia', 'Mardía', and I-2-12 respectively. In 2007, pollen germination was 90%, 92%, 92%, 90%, and 89% for 'Fournat de Bréznaud', 'Marcona', 'Felisia', 'Mardía', and I-2-12 respectively. These percentages agree with those already reported in almond (Weinbaum et al., 1984; Hill et al., 1985; Martínez-Gómez et al., 2002). Although the pollen of the early blooming varieties 'Fournat de Brézenaud and 'Marcona' had to be stored for 1 to 2 months at 4 °C to be used for pollinating the late blooming genotypes, Martínez-Gómez et al. (2002) reported that this

179 temperature was suitable for almond pollen storage for up to 2 months. The germination
180 percentages obtained were high and considered sufficient to ensure the correct development
181 of pollen tube growth and fertilization (Martínez-Gómez et al., 2002).
182
183 *3.2 Pollination day effect*
184
185 The analysis of variance of the percentage of pistils with germinated pollen and fruit set
186 revealed that the day of pollination and the genotype × day interaction were significant (Table
187 1 and 2). In the same way, the pollen receiver and the year were significant (Table 1 and 2).
188 The present results showed that the day of pollination, the pollen receiver and the year are
189 important factors determining the stigmatic receptivity and fruit set in almond cultivars, as
190 already pointed out by Ortega et al (2004). In our study, selection I-2-12 showed the highest
191 number of pistils with pollen tubes in the upper part of the style in both years, whereas
192 'Felisia' showed the lowest value in 2006 (Table 3). Not all stigmas were receptive at
193 emasculation for all genotypes in both years (Table 3), probably due to immature stigmas as
194 reported in almond cultivars (Ortega et al. 2004; Yi et al., 2006). In the same way, fruit set
195 with pollination time at day 0 was lower than that for days 2 and 4 (Fig. 3), as already
196 observed (Ortega et al., 2004; Kodad and Socias i Company; 2009b). The lowest values of
197 fruit set were obtained with pollination times at days 6 and 8, coinciding with the lowest
198 stigma receptivity (Fig. 3). Acceptable fruit sets were obtained following pollination from day
199 0 to day 4 after emasculation in both years for all cultivars (Fig. 3), coinciding with the
200 duration of EPP in almond, reported to be between 4 and 6 days, depending on the cultivar
201 and the temperature during bloom (Ortega et al., 2004; Kodad and Socias i Company, 2009b).
202 When the statistical analysis was done for each pollination time, the results showed no
203 significant differences between years for the time of 0 and 2 days after emasculation for

stigmatic receptivity and fruit set, whereas for 4, 6 and 8 days the differences were significant (data not shown). Thus, the year effect on stigmatic receptivity and fruit set is related to the time of pollination, which in turn is related to the climatic conditions during bloom, but not to the pollen receiver. In fact, the stigmatic receptivity decreased 4 days after emasculation, independently of the pollen receiver and the pollen donor. This decrease has already been described in almond (Griggs and Iwakiri 1975, Ortega et al., 2004), and we have observed differences in the rate of decrease between years, being more drastic in 2006. However, the reduction of fruit set with pollination time was more drastic in 2007 than in 2006 for 'Felisia' and 'Mardía' than for selection I-2-12 (Fig. 3). The year effect on stigma receptivity could be due to different climatic conditions, mainly temperatures during bloom (Ortega et al., 2004). However, fruit set could also be affected by frost damage during bloom and during the first stages of fruit growth (Felipe, 1988). In the present study no abnormal climatic conditions were observed during fruit growth, which could drastically affect fruit set (data not shown). Relative humidity and wind speed also could affect stigmatic receptivity during bloom. In both years of the study, the average humidity during this period was more than 60% (Fig. 2). The average wind speed, however, was higher in 2007 than in 2006 during the blooming time of 'Felisia' and I-2-12 (Fig. 2), although for 'Mardía' it was similar in both years of the study.

However, not all genotypes behaved similarly in both years. 'Mardía' and 'Felisia' showed a drastic decrease of stigma receptivity and fruit set during the first year as compared with selection I-2-12 (Table 3). In 2006, emasculation day was March 13 for I-2-12, March 25 for 'Felisia', and March 28 for 'Mardía' (Fig. 1). At blooming time of I-2-12 temperatures were lower, with maximum temperatures under 20°C, mainly during the first days after emasculation (Fig. 1), whereas for 'Felisia' and 'Mardía' maximum temperatures were higher, between 21-26°C, probably affecting the stigma receptivity and fruit set of these cultivars. In 2007 the maximum temperatures during the blooming period of all genotypes were lower than

in 2006, generally under 20°C (Fig. 1). Under these conditions, the stigmas maintained their receptivity and offered a good support for pollen germination and pollen tube penetration into the style, explaining the high stigma receptivity for all genotypes in 2007. Since the decrease of stigmatic receptivity was more drastic in 2006 than in 2007 (Table 3), it appears that the most important factor affecting stigmatic receptivity under the climatic condition of the present experiment is temperature during bloom, not humidity or wind.

Selection of very late blooming cultivars has been adopted in order to avoid damage by late spring frosts, characteristic of many inland regions where almond growing has expanded. However, not all genotypes react in the same way to high temperatures. Additionally, it was supposed that later blooming, coinciding probably with higher temperatures, would favour pollen transport, germination and growth, but our results show that late-blooming almond selections require a previous evaluation of adaptability to high temperatures because fruit sets may be negatively affected if flowers are not pollinated efficiently during the first days after anthesis.

3.3. Pollen source effect

The statistical analysis showed that the pollination treatment was not significant for stigmatic receptivity and fruit set (Table 1 and 2), clearly showing that fruit set in self-compatible almond cultivars depends primarily on the genotype and the climatic conditions of the year, but not on the pollen source. Fruit sets were similar for all cultivars in the two years after both self- and cross-pollination (Fig. 3). As the main objective of the almond breeding programme was the obtaining of self-compatible and late blooming almond cultivars, the present results assess that this objective was reached. Self-pollination gave a similar or better set than cross-pollination, confirming that self-pollen did not negatively affect fruit set and,

consequently, yield. The strategy of obtaining self-compatible cultivars to avoid the problems related to pollination and management of orchards with multiple cultivars has been successful (Socias i Company, 1990), as confirmed by other results when pollination was done at day 0 or 2 after emasculation (Dicenta et al., 2002; Martínez-García et al., 2011). However, in other cases fruit set after cross-pollination has been higher than after self-pollination (Socias i Company et al., 2004; Martín and Rovira, 2009), stressing the need for a correct evaluation of self-compatibility during the selection process (Socias i Company et al., 2010), as other factors may affect fruit set. These different results are probably not contradictory, but consequence of the effect of inbreeding depression.

The most important criterion to evaluate the degree of self-compatibility for any genotype is its ability to produce a high number of fruit when self-pollinated (Socias i Company et al., 2010), a feature mostly depending on the intrinsic characteristics of the genotype (Socias i Company et al., 2005; Kodad and Socias i Company, 2008). ‘Tuono’ has been a self-compatible almond cultivar repeatedly utilized in most breeding programmes as a source of self-compatibility (Socias i Company, 2002), having given rise to many self-compatible cultivars released in the last years. ‘Tuono’ has been reported to show a clear inbreeding effect (Socias i Company, 2002; Martínez-García et al., 2012), and several inbred genotypes have been identified and described in its progeny (Grasselly and Olivier, 1988; Alonso and Socias i Company, 2005). Inbreeding affords the expression of lethal and deleterious genes, which could cause disruption of pollen tube growth and embryo sac development (Alonso and Socias i Company, 2005; Martínez-García et al., 2012), leading to lack of fertilization and low or nil fruit set (Martínez-García et al., 2012). The level of inbreeding expression may depend on the number of altered genes inherited by each genotype (Lynch and Walsh, 1988). Thus, the effect of self-pollination on fruit set will depend on the presence and number of these deleterious alleles in each genotype. As a consequence, the negative effect of self-pollen on

fruit set of a given genotype is probably due to the level of inbreeding depression manifested in that genotype. Since no differences were found between self- and cross-pollination in these genotypes, they do not show any kind of depression and could be advised to be planted in single-cultivar orchards.

4. Conclusion

The present results confirm the effect of the year, the genotype, the time of pollination, and the warm temperatures during flowering on fruit set. The effective pollination period in almond appears to be variable among genotypes, conditioned by high temperature during blooming, ranging generally between 0 and 6 days after emasculation. It appears that self-pollination does not negatively affect fruit set in these late-flowering self-compatible genotypes, and that the most important factor determining fruit set in these genotypes is pollination time. The efficiency of self-pollination during the first few days (4 days) after emasculation appears to be crucial to ensure high fruit set, and consequently yield, in self-compatible almond cultivars, mainly under warm climatic conditions during bloom. The ability of self-pollination or autogamy depends on the reciprocal position of the stigma and the anthers, because the closer they are the greater the possibility of self-pollination. Thus, the selection of autogamous cultivars is crucial in any almond breeding programme, mainly if these cultivars are planted in regions with warm conditions during late winter and early spring.

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Table 1

Analysis of variance for the number of pistils with pollen tubes in the upper part of the style of the three almond genotypes studied.

Source of variation	df	Mean square[†]		*F-value*	P (>F)
Genotype	2	3084.90	***	15.79	<.0001
Treatment	2	71.74	ns	0.37	0.6931
Genotype × Treatment	4	92.99	ns	0.48	0.7532
Year	1	6809.45	***	34.86	<.0001
Genotype × Year	2	35.15	ns	0.18	0.8354
Year × Treatment	1	65.71	ns	0.34	0.5626
Genotype × Year × Treatment	2	12.65	ns	0.06	0.9373
Day of pollination	4	25209.8	***	129.07	<.0001
Genotype × Day of pollination	8	681.12	**	3.49	0.0009
Year × Day of pollination	4	55.17	ns	0.28	0.8891
Genotype × Year × Day of pollination	8	117.49	ns	0.60	0.7759
Treatment × Day of pollination	8	127.85	ns	0.65	0.7309
Genotype × Treatment × Day of pollination	16	51.24	ns	0.26	0.9983
Year × Treatment × Day of pollination	4	33.77	ns	0.17	0.9521
Genotype × Year × Treatment × Day of pollination	8	29.57	ns	0.15	0.9964
Error	195	195.31			

[†]Significance of the mean squares at $P < 0.001$(**), $P < 0.0001$(***) or non-significant (ns) by Student's *t*-test.

Table 2

Analysis of variance for fruit set in the three almond genotypes studied.

Source of variation	df	Mean square[†]		*F-value*	P (>F)
Genotype	2	1004.69	***	12.63	<.0001
Treatment	2	204.93	ns	2.58	0.0589
Genotype × Treatment	4	49.99	ns	0.63	0.6429
Year	1	7128.98	***	89.60	<.0001
Genotype × Year	2	778.14	***	9.78	<.0001
Year × Treatment	2	34.17	ns	0.43	0.6515
Genotype × Year × Treatment	4	53.99	ns	0.68	0.6076
Day of pollination	4	10305.80	***	129.54	<.0001
Genotype × Day of pollination	8	326.11	**	4.10	0.0002
Year × Day of pollination	4	159.72	ns	2.01	0.0553
Genotype × Year × Day of pollination	8	128.71	ns	1.62	0.1224
Treatment × Day of pollination	8	65.55	ns	0.82	0.5825
Genotype × Treatment × Day of pollination	16	38.34	ns	0.48	0.9535
Year × Treatment × Day of pollination	8	76.16	ns	0.96	0.4710
Genotype × Year × Treatment × Day of pollination	16	65.13	ns	0.82	0.6634
Error	180	79.5			

[†]Significance of the mean squares at $P < 0.05$(*), $P < 0.001$(**), $P < 0.0001$(***) or non-significant (ns) by Student's *t*-test.

Table 3

Mean values of number of pistils with pollen tubes in the upper part of the style 24 hours after pollination for the three almond genotypes studied after different pollination treatments and pollination times.

Genotype	Treatment[z]	Day of pollination after emasculation				
		0	2	4	6	8
		2006				
Felisia	⊗	89.41	91.48	59.72	32.76	18.92
	×F	90.74	92.36	62.18	26.19	14.17
	×M	86.67	88.33	49.44	28.69	16.19
Mardía	⊗	88.89	89.63	64.60	30.28	17.41
	×F	91.11	90.00	62.22	38.15	20.74
	×M	90.28	88.89	52.98	39.49	15.74
I-2-12	⊗	92.32	89.03	51.67	37.41	30.26
	×F	90.86	89.63	62.96	44.95	24.66
	×M	91.90	90.32	64.81	44.07	31.11
		2007				
Felisia	⊗	94.71	95.12	70.83	55.45	31.72
	×F	90.74	94.21	66.13	53.17	29.17
	×M	91.90	93.89	71.11	52.98	29.84
Mardía	⊗	92.96	92.96	70.79	48.98	34.44
	×F	91.11	92.96	70.00	51.85	30.74
	×M	90.28	91.11	67.98	49.15	30.56
I-2-12	⊗	90.46	89.03	73.33	57.08	37.78
	×F	93.64	92.96	73.70	57.88	35.03

×M	92.80	95.08	67.78	51.48	34.44

[z] ⊗: self-pollination; ×F; cross-pollination with ‘Fournat de Brézenaud’ pollen; ×M: cross-pollination with ‘Marcona’ pollen.

Figure legends

Fig. 1. Maximum, minimum and mean daily air temperatures during the blooming period in 2006 (A) and 2007 (B) at the experimental site.

Fig. 2. Average relative humidity and wind speed during the blooming period in 2006 (A) and 2007 (B) at the experimental site.

Fig. 3. Mean values of fruit set for I-2-12 (A), ‘Felisia’ (B) and ‘Mardia’ almond genotypes after different pollination treatment and pollination times during the two years of study.

⊗: self-pollination; ×F; cross-pollination with ‘Fournat de Brézenaud’ pollen; ×M: cross-pollination with ‘Marcona’ pollen.