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Fruit Set in Avocado: Pollen Limitation, Pollen Load Size, and Selective Fruit Abortion

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Abstract: Avocado is a woody perennial fruit crop originating in Central America and Mexico domesticated and cultivated in the Americas since pre-Columbian times, currently cultivated in subtropical, tropical, and Mediterranean climates worldwide, with increasing importance in international trade. Avocado shows an exuberant flower production that, however, results in a very low fruit set reflected in a massive abscission of flowers and fruitlets. Several factors are involved in this behavior, and, in this work, we have focused on pollination limitation. The results obtained show that pollen deposition takes place at the female and male stages during the avocado flowering season and that the percentage of flowers with pollen on the stigma varies along the flowering season, probably due to changes in temperature that affect not only the floral behavior but also pollinator activity. However, no embryo or endosperm development took place when pollination occurred during the male flowering phase. Thus, the low number of pollen grains landing on the stigmas of female stage flowers observed under natural pollination conditions might not be enough to ensure a good yield. The production of an excess of flowers and subsequent flower drop of most of the flowers provides the opportunity of a selective fruitlet drop depending on the genotype of the embryo since fruits derived from outcrossing showed higher chances of reaching maturity. Moreover, an important competition for resources occurs among developing fruits and new vegetative growth, conferring importance to the time of flower fertilization for effective fruit set.

Keywords: avocado; fertilization; fruit set; *Persea americana*; pollen load; pollination



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1. Introduction

The avocado (*Persea americana* Mill.) is an evergreen fruit tree native to Central America and Mexico, where it has been domesticated and cultivated since ancient times [1–3]. Avocado belongs to the Lauraceae, a mostly subtropical or tropical family included within the order Laurales that comprises primarily woody perennial species distributed worldwide, mainly in tropical and subtropical regions. One of the main limitations in avocado production worldwide is the massive abscission of flowers and developing fruits, primarily in the first two months following flowering. This results in less than 1% of the fruits remaining on the trees at harvest [4–8]. Several reasons have been proposed to explain this low fruit set in avocado. Among them, the limiting environmental conditions at flowering, lack of sufficient pollinating insects, inefficient use of pollinizer varieties or low flower quality [9–13]. However, general observation in many plant species is that one of the most important factors determining reproductive success in plants is pollination. Pollen limitation, described as the consequence of pistils receiving too few pollen grains to fertilize all the ovules, has been shown to regulate seed and fruit set in different plant species [14]. In many cases [15–21], although the number of pollen grains that arrive on the stigma is usually much higher than the number of available ovules, frequently fertilization and fruit set do not take place. This is often related to the pollen population effect that is linked to a density-dependent pollen phenomenon in which a minimum number of pollen grains on the stigma is necessary to promote appropriate pollen germination and pollen tube

growth. Density-dependent pollen germination has been observed *in vitro* [15] and *in vivo* on the stigmas of a range of species [22–26]. The chemical basis of the pollen population effect on germination has been associated with compounds secreted by the pollen that modify the germination environment, which has a positive effect on pollen performance. Examples include water-soluble growth factors [15], flavonols [27], phytosulphokine-a [19], gibberellins [28], or changes in stigmatic pH causing the inhibition or destruction of pH-sensitive pollen germination inhibitors [29]. In spite of the importance of pollen limitation for seed and fruit production, its physiological and molecular basis are still poorly understood. In avocado, Shoval [30] pointed to the existence of a population effect in pollen germination and tube growth, showing that, although in theory just one pollen tube would be needed for fertilization of the single avocado ovule, the percentage of flowers with a pollen tube reaching the ovary is strongly affected by the number of pollen grains deposited on the stigma. Thus, flowers with fewer than five pollen grains on the stigma resulted in only 4% fertilization, whereas 11% fertilization was observed in flowers with 5–19 grains on the stigma, and about 80% fertilization rate was ensured by the deposition of more than 20 pollen grains. However, under natural pollination conditions, very few avocado flowers are able to accumulate more than 20 pollen grains on their stigmas [31–33]. Furthermore, recent results in this species show that even after adequate pollination, most flowers in avocado will drop, probably due to low flower quality [9,12].

When pollen grains land on the stigma, another factor that plays an important role in fertilization is the genotype of the pollen donor. Evidences are accumulating, suggesting that fertilization is not always a random process [34] and that mate choice occurs in numerous plant species [35–46]. Pollen competition is estimated to be higher in outcrossing than in selfing plants because pollen load in outcrossers is expected to be genetically more diverse [47].

Avocado, as many other angiosperms, shows different mechanisms destined to avoid self-fertilization [48,49]. Avocado shows synchronous protogynous dichogamy where each perfect flower opens twice; first functionally as a female flower with a receptive stigma; then the flower closes and, the following day, reopens functionally as a male flower with the stigma no longer receptive and dehisced anthers. Depending on the flowering behavior, avocado genotypes can be classified into two different groups (A and B). In type A cultivars, the flowers open in the morning of the first day of the cycle in the female stage, close at midday, and reopen the afternoon of the following day in the male stage. In type B cultivars, the flowers open in the afternoon of the first day of the cycle in the female stage, close in the evening, and reopen the following morning in the male stage. Under optimum conditions, the floral behavior is predictable. However, the flower opening cycle is highly sensitive to environmental conditions, mainly temperature.

In order to advance in the knowledge of the main limitations for fruit set in avocado, the objectives of this study were to evaluate (a) pollen deposition in avocado flowers at the male and female stages during the flowering season; (b) whether embryo and endosperm development occur when flowers are pollinated during the functionally male stage; (c) the effect of the size of stigmatic pollen loads on fruit set, (d) whether outcrossed fruits show higher survival than selfed fruits, and (e) whether the time of fertilization has an effect on the probability of a flower becoming a fruit.

2. Materials and Methods

The experiments were carried out during three consecutive flowering seasons in an experimental avocado orchard of the ‘Hass’ cultivar, the most widely grown avocado cultivar worldwide, located at the IHSM La Mayora (Málaga, Spain) at latitude 36°45′ N, longitude 4°4′ W and altitude 35 m above sea level. In this orchard, six beehives were placed per hectare at the beginning of the flowering season to guarantee pollination.

2.1. Pollen Load at the Male and Female Stages during the Avocado Flowering Season

Each avocado flower opens twice, first functionally as a female (stamens at a 90-degree angle to the central pistil), closes overnight, and reopens the following day functionally as a male flower (with three stamens close to the central pistil and the other six at an approximately 40-degree angle from the pistil). To evaluate pollen deposition in avocado flowers opening functionally as female and as male and, therefore, to establish the floral stage when the main deposition of pollen grains takes place, flowers at the end of the male and female opening cycles were collected randomly (when the stigma was no longer accessible on closing male and female functional stages, respectively) from several inflorescences located in different trees of the avocado cv Hass. A minimum of fifty flowers at the end of their male and female stages were collected weekly during the approximately four weeks of the flowering season of 'Hass' under our environmental conditions during which the average temperature was 17.5 °C with a mean of the minimum temperatures of 12.2 °C and a mean of the maximum temperatures 22.8 °C. The samples were collected on sunny days during which pollinating insect activity is higher. A total of 243 flowers at the female stage and 203 flowers at the male stage were fixed in FAA (70% ethanol: glacial acetic acid: formalin [18:1:1, v/v/v]) [50]. For microscopical observations, pistils were washed in water three times, 1 h per wash, autoclaved for 10 min at 1 kg/cm² in 5% Na₂SO₃, squashed, and stained with 0.1% aniline blue in 0.1N K₃PO₄. Preparations were examined under a Leica DM LB2 microscope (Leica Microsystems Wetzlar, Germany) with UV epifluorescence using a BP 515–560 exciter filter and an LP 590 barrier filter. Pollen adhesion and germination were evaluated, and the number of pollen tubes was counted at different levels of the pistil. Pollen adhesion was evaluated by counting the number of pollen grains on the stigma after fixation.

Statistical analyses were performed using SPSS 27.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences in pollen deposition among flowers fixed at the male and female stages were evaluated using the Student's *t*-test at the 0.05 significance level. At each stage, pollen deposition was compared during the four weeks of the flowering season with a generalized linear model with pollen adhesion and germination as dependent variables and time as the independent variable.

2.2. Embryo and Endosperm Development in Flowers Pollinated at the Female and Male Stages

One hundred 'Hass' (floral type A) flowers were hand-pollinated daily at the male and female stages using pollen from 'Fuerte' (floral type B) following the procedure described by Alcaraz and Hormaza [11]. Another set of 'Hass' flowers at the female stage was emasculated and bagged to avoid insect pollination. Hand pollination in male stage flowers was carried out in seven trees maintained in a controlled growth chamber under the means of temperature registered during the month of April of the previous year (Min = 11.8 °C, Max = 22.7 °C, and Mean = 16 °C) and three relative humidities (50, 75, and 95%) with a photoperiod of 16 light hours and 8 dark hours.

Ten pistils hand-pollinated in the male and female stages were collected daily from the day after pollination to 25 days after pollination and fixed in 2.5% glutaraldehyde 0.03 M phosphate buffer [51], dehydrated in a graded ethanol series, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) resin. Resin-embedded material fixed in glutaraldehyde was sectioned at 2 µm and stained with periodic acid-Schiff's reagent (PAS) followed by 0.2% toluidine blue in water [52]. Preparations were observed under a Leica DM LB2 epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Effect of Pollen Load on Fruit Set

To determine whether a minimum number of pollen grains on the avocado stigmas is required for pollen germination and pollen tube growth and to establish a possible relationship between pollen load on the stigma and fruit set, 61 inflorescences were selected in 15 'Hass' trees, and a minimum of 250 flowers were hand-pollinated daily using pollen from 'Fuerte'. The following day, when the flower reopens functionally as male and pollen

tubes had reached the ovary [12], the style of each flower was sliced and fixed individually in FAA, and the fate of the ovaries that remained in the trees was monitored until fruit maturity. Since no effect of cutting the styles one day after pollination on fruit set has been found [12], this method allows establishing a correlation between pollen load on the stigma and the final fate of the ovary of the same flower remaining on the tree. Three different groups of flowers were established based on flower fate; the first group was formed by flowers/fruitlets that dropped early after the flowering season; the second by fruitlets that remained on the trees for at least two months, and the last group was formed by fruitlets that finally remained on the trees until harvest time. Styles were prepared for microscope observation as described in Section 2.1. Pollen grains adhered and germinated on the stigma, and pollen tubes in the upper and lower parts of the style were counted under the microscope.

Data were analyzed with a Multivariate GLM, with pollen adhesion, pollen germination, and number of pollen tubes at the upper and lower parts of the style being the dependent variables, and abscission time the explanatory variable. If the Multivariate GLM were significant, each of the dependent variables was subsequently analyzed with a GLM separately. Post-hoc analyses were done using the Tukey HSD. Correlation among different parameters in each group was established. Germination percentages were arcsine transformed to meet the assumption of normality and homogeneity of variances. Logistic regression was used to determine the effect of pollen load size on fruit set. The number of pollen grains adhered and germinated on the stigma was used as the independent variables. Fruit set was used as the response variable with the following levels: (1) flowers that successfully developed into fruits reaching the maturity stage, (2) flowers that initiated fruit development but aborted, and (3) flowers that did not develop into fruits.

2.4. Fate of the Fruits Developed after Self- and Cross-Pollination

To evaluate whether fruits resulting from outcrossing showed more probability to survive than fruits resulting from self-pollination, 30 inflorescences distributed in 6 'Hass' trees were selected. Since avocado shows protogynous dichogamy, pollen storage is necessary to perform self-pollinations. Since short-term pollen storage at 4 °C has no effect on the pollen capacity to fertilize the avocado ovule [11], male-stage flowers of 'Hass' were collected when all the anthers were dehisced and stored at 4 °C, whereas male-stage flowers from 'Fuerte' (a type B cultivar of green skin fruits used as pollen donor for 'Hass' in different avocado growing regions) were collected at midday, just before performing hand pollinations. Ten flowers of 'Hass' were hand-pollinated daily in each inflorescence by direct contact of the stigma with the dehisced anthers; 5 of them were cross-pollinated with pollen from 'Fuerte' and 5 with pollen from 'Hass'. A total of 1314 'Hass' flowers were hand-pollinated with each pollen-donor genotype.

Since self- and cross-pollinated flowers were competing for resources during all the blooming season with the rest of the flowers within the inflorescence, all the non-pollinated flowers in the inflorescence were removed. To estimate fruit set at maturity, flower and fruit drop was monitored every two weeks until harvest time. To confirm the paternity of the fruits, at harvest time, DNA was extracted from the embryo of the fruits following the method described by Hormaza [53], and the analysis of paternity was carried out using one microsatellite marker, AVAG21, published by Sharon et al. [54], for which no alleles were shared between 'Hass' and 'Fuerte' [10]. A Chi-square test for 2 × 2 contingency tables, with Yate's correction for continuity, was used to determine whether preferential abortion of self-pollinated vs. outcrossed fruits took place in avocado.

2.5. Fruit Set and Pollination Date

To study the possible effect of pollination date on fruit set, the results of fruit set after hand pollination were used, taking into account the pollination date. Since flowering is not synchronous in all the inflorescences, the time of mid flowering in each inflorescence was considered when 50% of the total flowers of the inflorescence were hand-pollinated.

Observations of flower/fruit drop were made weekly during the two months following flowering and every two weeks since July. To detect a possible relationship between total number of hand-pollinated flowers per inflorescence and fruit set percentage at maturity, Pearson's correlation coefficients at the 0.05 significance level were computed. Chi-square test for 2×2 contingency tables, with Yate's correction for continuity, was used to determine possible survival differences between fruits set at the beginning or the end of the flowering season.

2.6. Characteristics of Fruits Derived from Self- and Cross-Fertilization

To determine whether the embryo genotype had an effect on fruit weight, a 'Hass' orchard with 'Fuerte' as pollinizer was used. In this orchard, 882 'Hass' fruits were collected from 12 trees. The weight, diameter, and length of the fruits and their seeds were measured, and DNA was extracted from the embryo to perform a paternity analysis. Fruit diameter (at the broadest part) and length were measured with a digital Digi-Max slide caliper (Merck, Darmstadt, Germany). DNA extraction and paternity analysis were carried out from each embryo, as described in Section 2.4.

Male parent assignment was made with the program Cervus 3.0 [55]. The weight of fruits derived from self- and cross-pollination was compared using a Student's *t*-test. To establish a possible relationship between embryo genotype and fruit and seed characteristics (diameter, length, and weight), a Pearson correlation was computed. The comparisons of means between self and outcrossed fruit were made with a Student's *t*-test.

3. Results

3.1. Pollen Deposition at the Male and Female Stages during the Flowering Season

At the end of the female stage, 78% of the flowers showed no pollen grains on the stigma (Figure 1), and 14% showed only one to five pollen grains. Thus, only about 8% of the flowers analyzed received more than five pollen grains. Considering only the flowers that received at least one pollen grain, the average number of pollen grains deposited on the stigma of female flowers was 6.77. As expected, the number of pollen grains observed at the end of the male stage (average 9.1) was higher than that on flowers collected at the female stage, although still 45% of the flowers analyzed presented no pollen grains on the stigma at the end of the male stage (Figure 1). Significant differences between male and female stages were found not only for the number of pollen grains adhered (Student's *t*-test; $t = 5.276$; d.f. = 443, $p < 0.01$) but also for pollen germination (Student's *t*-test; $t = 3.617$; d.f. = 443, $p < 0.01$).

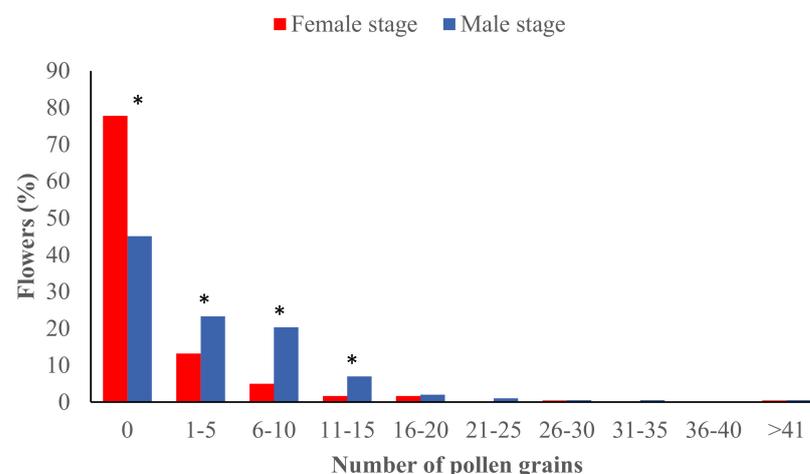


Figure 1. Percentage of flowers at the male and female stages left to open pollination with different pollen load sizes (number of pollen grains) on the stigma during the avocado flowering season. An asterisk into each range of the number of pollen grains indicates significant differences ($p < 0.05$) between percentages of flowers at the female and male stages (Chi-square test).

Moreover, differences in both stages for pollen adhesion and germination were found during the flowering season (Table 1). Thus, in the female stage, differences were observed both on pollen adhesion ($F_{3,239} = 3.262$, $p = 0.022$) and pollen germination ($F_{3,239} = 3.661$, $p = 0.013$) among weeks throughout the flowering season. Pollen germination ($p = 0.024$) and pollen adhesion ($p = 0.041$) were significantly higher in the first than in the last week of the flowering season. Similarly, flowers in the male stage showed differences in pollen adhesion ($F_{3,198} = 7.763$, $p < 0.001$) and pollen germination ($F_{3,198} = 11.417$, $p < 0.001$) throughout the flowering season. Both pollen adhesion and germination were significantly higher during the first week of the flowering season ($p < 0.01$). Differences were also found in the percentage of stigmas of female and male stage flowers with pollen during the different weeks of the flowering season (Figure 2).

Table 1. Pollen adhesion and percentage of pollen germination (mean \pm standard error) in all the flowers collected at the end of the female and male stages. Means with the same letter within each stage and for each parameter evaluated were not significantly different at $p < 0.05$ (GLM followed by Tukey HSD test). Comparison of pollen adhesion and germination between both stages were determined using a Student's t -test.

Period	Female Stage	Pollen Adhesion	Male Stage	Pollen Adhesion	Comparison between Stages		
	N	Mean \pm SE	N	Mean \pm SE	t	d.f.	p
1st week	72	2.96 \pm 0.87 a	70	7.01 \pm 1.03 a	3.013	140	0.003
2nd week	46	0.23 \pm 0.11 ab	50	1.3 \pm 0.30 b	3.27	94	0.01
3rd week	82	1.36 \pm 0.43 ab	45	2.87 \pm 0.244 b	2.27	125	0.025
4th week	43	0.51 \pm 0.27 b	37	3.02 \pm 0.64 b	3.772	78	0
Period	Female Stage	Germination (%)	Male Stage	Germination (%)	Comparison between Stages		
	N	Mean \pm SE	N	Mean \pm SE	t	d.f.	p
1st week	72	2.33 \pm 0.81 a	70	5.2 \pm 0.86 a	2.43	140	0.016
2nd week	46	0.73 \pm 0.28 b	50	1.04 \pm 0.52 b	3.14	94	0.02
3rd week	82	0.96 \pm 0.38 ab	45	1.02 \pm 0.20 b	0.111	125	0.911
4th week	43	0 b	37	1.56 \pm 0.38 b	4.39	78	0.006

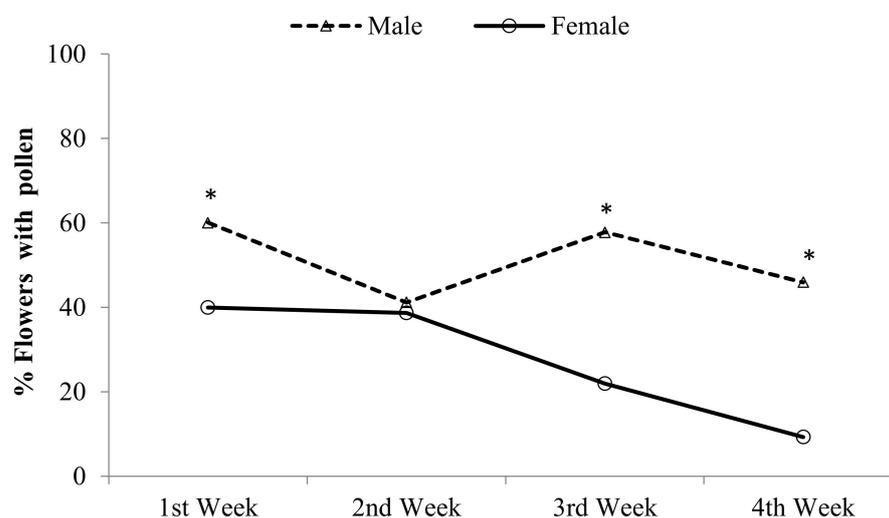


Figure 2. Percentage of avocado flowers at the male and female stages left to open pollination with at least one pollen grain on the stigma during the avocado flowering season. The asterisks indicate significant differences between male and female stages at $p < 0.05$ (Student's t -test).

3.2. Embryo and Endosperm Development in Flowers Pollinated at the Female and Male Stages

At anthesis, the avocado embryo sac is formed by seven cells and eight nuclei characteristic of the Polygonum-type structure, which is the most common embryo sac developmental pattern in angiosperms. Figure 3 shows the changes in the embryo sac several days after hand pollination of avocado flowers at the female and male stages. The mature embryo sac contained starch granules within the egg cell and the synergid cells in the

proximity of nucleolus (Figure 3a,b), and the egg cell shows a large nucleus at the chalazal region such as described by Sedgley [56].

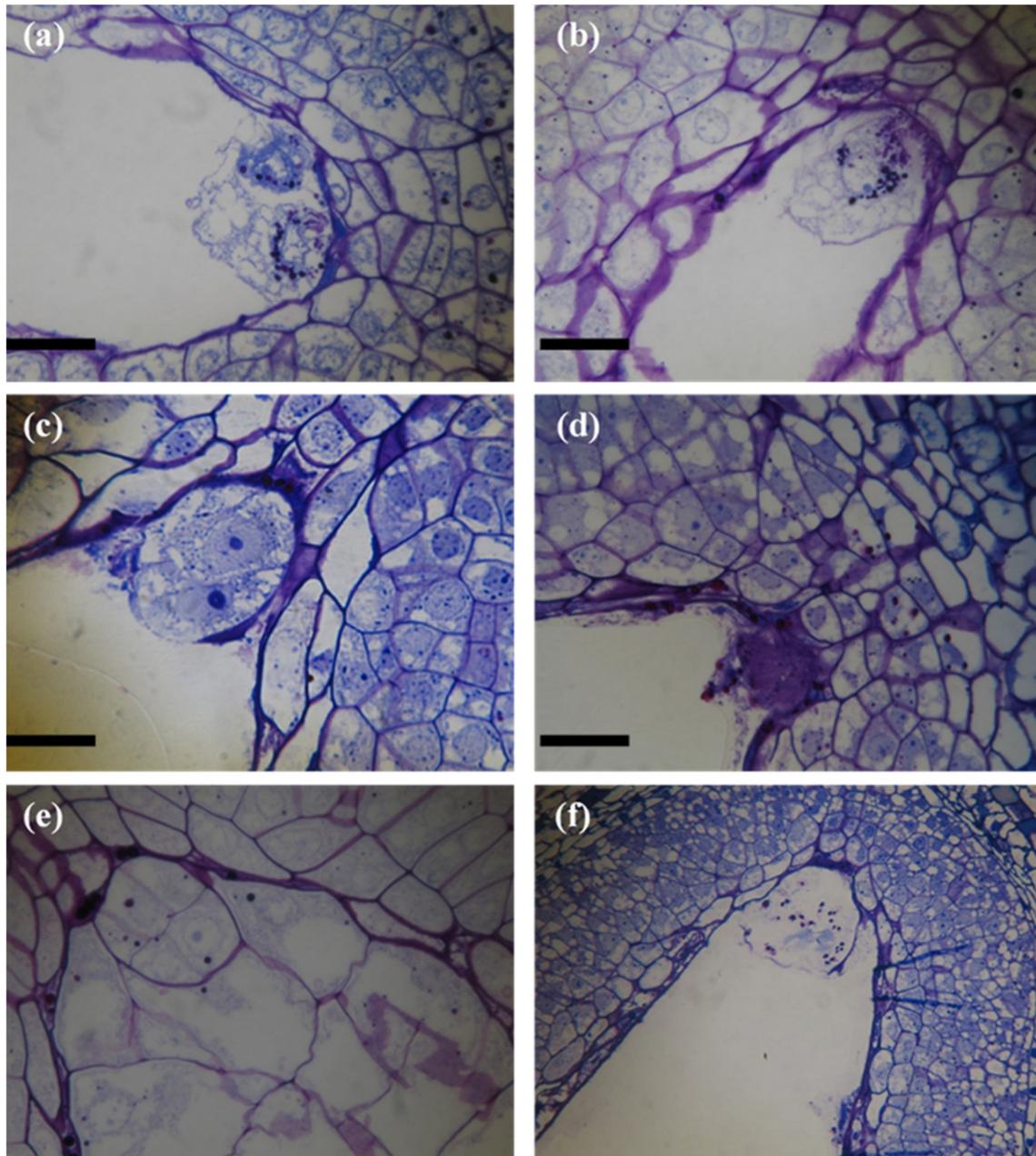


Figure 3. Embryo and endosperm development after pollen deposition on the stigma of avocado flowers at the female and male stages. (a) Embryo sac with two synergid cells, with nuclei at the micropilar end, 1 day after pollination at the female stage. (b) Synergid cell with the nucleus surrounded by starch grains 1 day after pollination at the male stage. (c) Zygote division 10 days after pollination at the female stage. (d) No signs of fertilization 10 days after pollination at the male stage. (e) Multicellular pro-embryo and multicellular endosperm 16 days after pollination at the female stage. (f) Degenerated embryo sac 16 days after pollination at the male stage. Bar =20 μ m. Stain: Pas and toluidine blue.

Two days after hand pollination in the female floral stage, large starch granules were observed in the micropilar region of the embryo sac. Later, the cellular endosperm started its division, and several days later, the embryo was formed by a few cells showing a spherical structure (Figure 3c,e). Following pollination, the synergids elongated, and the cytoplasm of one of them became darker and more granular. However, in non-pollinated

flowers, the starch granules accumulated in the egg cell, and no degeneration of the two synergid cells was observed for several days. A similar development was observed in those flowers pollinated during the male stage where no evidences of embryo and endosperm development were observed (Figure 3d,f) in seventy-five analyzed samples under the different relative humidities, suggesting that fertilization does not take place when the flowers are pollinated at the male stage. Moreover, the retention of flowers pollinated in the male stage on the trees decreased drastically three weeks after anthesis.

3.3. Effect of Pollen Load Size on Fruit Set

From the 4570 hand-pollinated flowers, only 127 (2.78%) developed into fruits. A high variation in the number of pollen grains deposited on the stigma ($n = 2200$) was found ranging from 0 to 95, and the number of pollen tubes showed a reduction from the upper part of the pistil (an average of 4.06) to the base of the style (an average of 2.95).

Comparisons were made among three groups of flowers: group 1 was formed by flowers that dropped early after hand pollination, group 2 by those that initiated fruit development but dropped, and group 3 by flowers whose fruits were maintained on the tree until harvest time. Within the inflorescences, each flower was treated as an event with two possible outcomes 'able to set fruit' or 'not able to set fruit'.

The probability of a flower to develop into fruit was significantly affected by the number of pollen grains adhered (Wald $\chi^2 = 44.51$, d.f. = 1, $p < 0.0001$) and germinated (Wald $\chi^2 = 55.15$, d.f. = 1, $p < 0.0001$) on its stigma. Multivariate GLM results showed significant differences for all dependent variables (number of pollen grains adhered, germinated, and percentage of pollen germination) among the different groups established according to the date of abscission (Lambda de Wilks = 0.186, $F_{3,22} = 1919.23$, $p < 0.001$). Significant differences were observed on germinated pollen grains ($F_{2,22} = 31.83$, $p < 0.001$), pollen adhesion ($F_{2,22} = 32.13$, $p < 0.001$), number of pollen tubes at the top of the style ($F_{2,22} = 56.55$, $p < 0.001$), at the base of the style ($F_{2,22} = 57.28$, $p < 0.001$) and percentage of pollen germination ($F_{2,22} = 59.60$, $p < 0.001$). The significant differences among groups established based on flower fate for each of the dependent variables are shown in Figures 4 and 5. All the dependent variables showed a significant correlation ($n = 2200$) (Table 2). In the style of flowers that set fruit, the number of pollen grains adhered, germinated, and the number of growing pollen tubes was higher than in those that dropped.

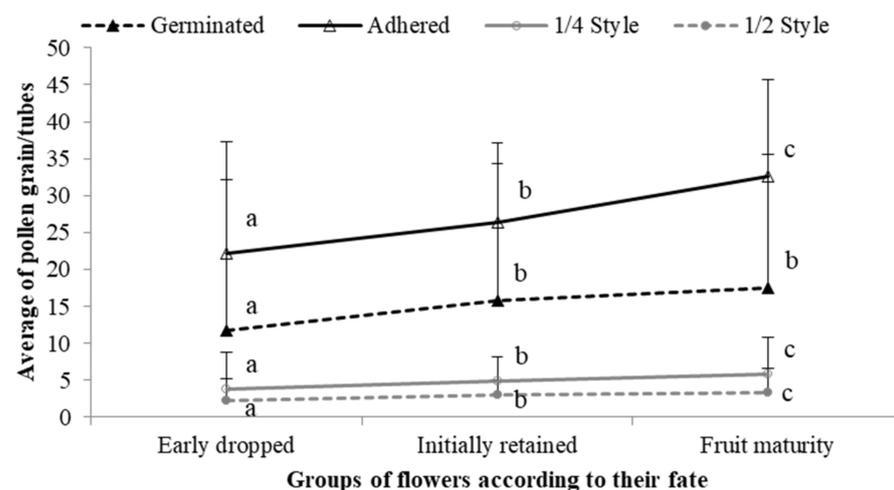


Figure 4. Pollen grains adhered and germinated and pollen tube number at the upper part of the style and at the middle of the style (where the styles were sliced) in avocado flowers. The values are presented as mean, and error bars are standard deviations. In the same dependent variable, the same letter indicates no significant differences ($p > 0.05$, Tukey HSD test).

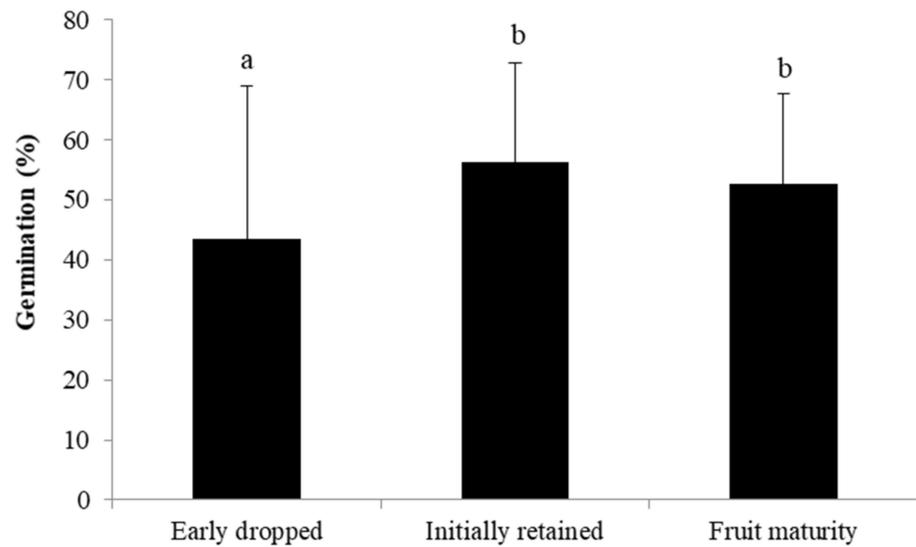


Figure 5. Percentage of avocado pollen germination on the stigma of different flower groups established based on fruit abscission data. Error bars are \pm standard deviations. The same letter indicates no statistically significant differences at $p < 0.05$ (GLM analyses followed by Tukey HSD test).

Table 2. Pearson correlation among the different avocado pollination parameters analyzed. ** Correlation is highly significant at $p < 0.01$.

Pollen	Germinated	Adhered	% Germination	Tubes 1/4 Style	Tubes 1/2 Style
Germinated	1.00	0.886 **	0.593 **	0.788 **	0.708 **
Adhered	0.886 **	1.00	0.437 **	0.739 **	0.660 **
% Germination	0.593 **	0.437 **	1.00	0.643 **	0.646 **
Tubes 1/4 Style	0.788 **	0.739 **	0.643 **	1.00	0.907 **
Tubes 1/2 Style	0.708 **	0.660 **	0.646 **	0.907 **	1.00

Likewise, pollen behavior was analyzed after the presence of different pollen loads on the stigma. The percentage of flowers with pollen tubes at the different levels of the style was significantly lower when the number of pollen grains deposited on stigma ranged from 1 to 5 pollen grains than when pollen loads were higher than 5. When the number of pollen grains deposited on the stigma was higher than 20, the percentage of flowers with germinated pollen grains and pollen tubes in the style was significantly higher than those observed with fewer than 20 pollen grains (Figure 6).

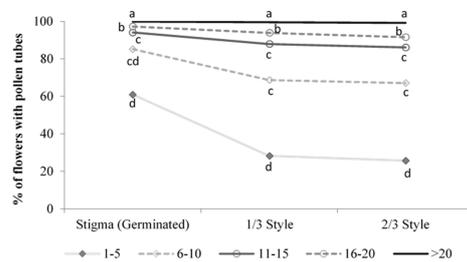


Figure 6. Percentage of flowers with pollen tubes at the different levels of the style in relation to the pollen load size on the avocado stigma. Different letters indicate significant differences between groups at $p < 0.05$ (GLM analysis followed by Tukey HSD test).

However, higher stigmatic pollen loads may not always translate directly into a higher probability of fruit set. In this work, flowers with different pollen load sizes were found in the different groups established based on abscission date (Figure 7).

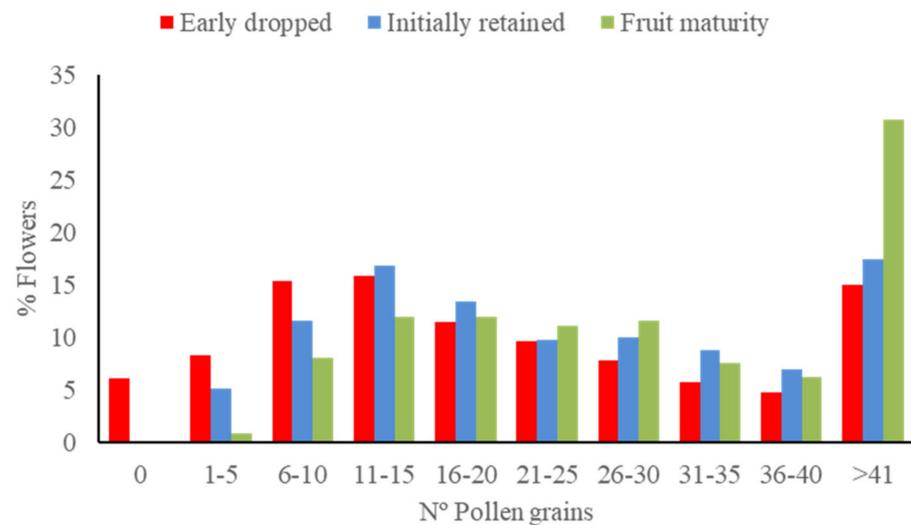


Figure 7. Histogram showing the percentage of avocado flowers into each range of pollen load size on the stigma for each group established based on the fate of the flower/fruit (early dropped, initially retained, and fruit maturity).

3.4. Fate of Fruits Resulting from Selfing vs. Crossing

A total of 11 flowers became fruits from 1314 self-hand pollinated flowers (0.84% fruit set), whereas 40 of 1314 ‘Hass’ cross-hand pollinated with pollen from ‘Fuerte’ set fruit (3.04% fruit set). Thus, those fruits derived from cross-pollination showed a higher probability of reaching the maturity stage than those self-pollinated ($\chi^2 = 15.677$, $p < 0.001$).

3.5. Fruit Set According to the Date of Anthesis

No correlation was found between the total number of hand-pollinated flowers in the inflorescence and the number of those that became fruits (Pearson’s correlation = 0.131, $p = 0.315$).

Figure 8 shows the percentage of fruit set obtained according to the percentage of hand-pollinated flowers in the inflorescence. From the 2284 flowers initially hand-pollinated in the inflorescence, 77 became fruits, whereas from 2284 hand-pollinated during the second half of the flowering season, 44 produced fruits. The test of contingency showed that the time of fertilization could confer the same advantages to compete for resources and, consequently, earlier flowers in the blooming season would have higher chances to become fruits ($\chi^2 = 9.245$; d.f. = 1; $p = 0.002$).

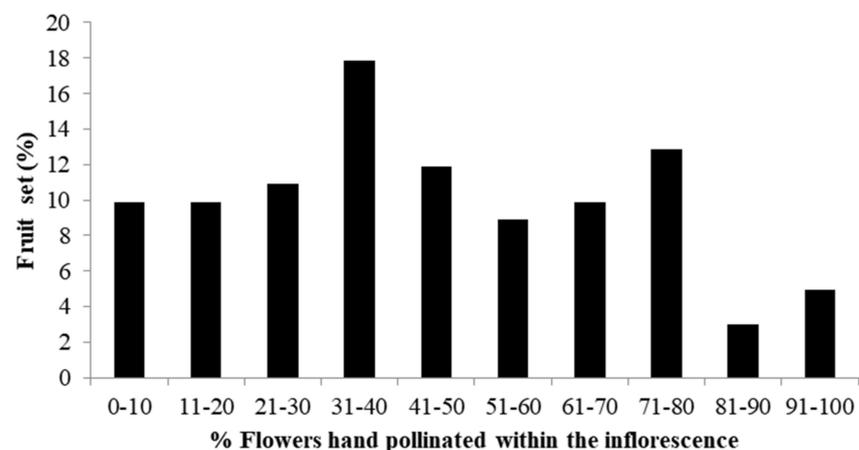


Figure 8. Percentage of fruit set obtained based on the percentage of hand-pollinated flowers in the inflorescence.

3.6. Effect of the Genotype of the Male Parent on Fruit Size

From 882 fruits collected in the 'Hass' orchard that used 'Fuerte' as pollinizer, the paternity test showed that 539 derived from self-pollination and 343 from outcrossing (39% of allogamy).

Significant differences were observed in fruit characteristics between selfed and outcrossed fruits (Figure 9) for fruit weight ($t = -4.003$, f.d. = 677.29, $p < 0.001$) and fruit diameter ($t = -3.923$, f.d. = 880, $p < 0.001$), whereas no significant differences were observed for fruit length ($t = 0.191$, f.d. = 880, $p = 0.848$). Moreover, differences were observed on seed weight ($t = -2.056$, f.d. = 880, $p = 0.04$), although neither on seed diameter ($t = 1.697$, f.d. = 882, $p = 0.09$) nor on seed length ($t = 0.721$, f.d. = 880, $p = 0.471$).

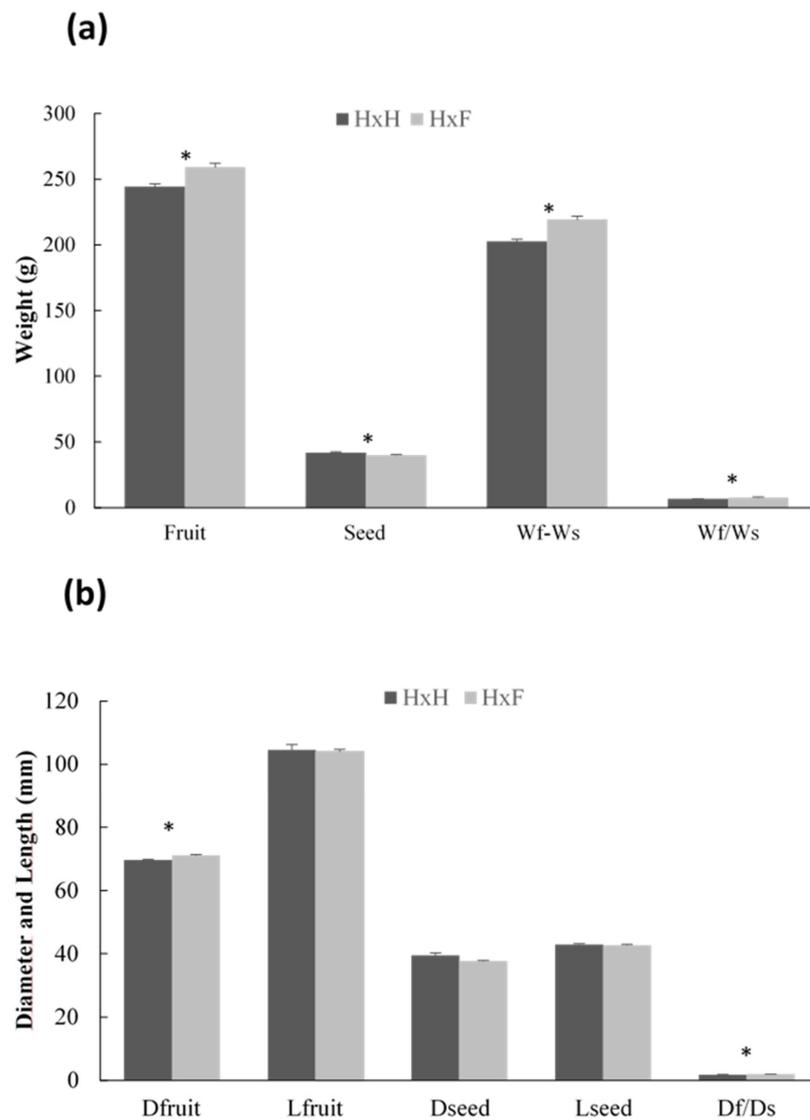


Figure 9. Fruit and seed characteristics in selfed and outcrossed 'Hass' avocado fruits. (a) Mean of fruit and seed weight (g), pericarp weight (g) defined as the difference in fruit and seed weight (Wf-Ws) and fruit: seed weight ratio (Wf/Ws). (b) Mean of diameter and length of fruits (mm) and seed (mm) as well as the ratio between fruit diameter and seed diameter (Df/Dd). The asterisks show significant differences in these parameters between selfed (Hass \times Hass) and outcrossed (Hass \times Fuerte) fruits at $p < 0.05$ (Student's t -test).

4. Discussion

Avocado trees produce a large number of flowers, but only a small fraction (less than 1%) set fruits [11]. This observation is common in a high number of plant species, and several non-exclusive hypotheses have been proposed to explain the reasons why plants have evolved to produce more flowers than fruits: pollinator limitation, resource limitation, or sexual selection. Successful fruit set in plants is dependent on both pollination and subsequent fertilization and several species invest resources in flowers that are not going to produce fruits with the objective to increase pollinator attraction or pollen production.

4.1. The Effect of Pollen Load on Fruit Set

The functional relation between pollen load size and seed or fruit set has been studied in both animal [57–61] and wind [28,62] pollinated plants. Results in different species show that a higher number of pollen grains on the stigma can usually improve the likelihood of fruit set [63–71]. Although avocado flowers have one single ovule, the deposition of at least 20 pollen grains to ensure fertilization has long been considered as a limiting factor in this species for an adequate fruit set [30]. Our results showed that even if there is a positive correlation between pollen load and the likelihood to set fruits, not all the flowers with generous stigmatic loads are able to set fruit and, on the other hand, an important percentage of flowers receiving fewer than 20 pollen grains are able to become fruits. Nevertheless, when five or fewer pollen grains are deposited on the stigma, the capacity to set fruit decreases significantly. As discussed by Alcaraz et al. [12] and Boldingh et al. [9], although pollination is a requirement for fruit set, the capacity of an avocado flower to become fruit could be predetermined by its nutritive status at anthesis.

4.2. Pollination during the Dichogamous Cycle of the Avocado Flowers

Under our environmental conditions, even in conditions that promote high insect activity, most of the avocado flowers showed no pollen grains on the stigma at the end of the female phase. In addition, most of the flowers with pollen grains on the stigma received fewer than five pollen grains during that period. When the flowers open again functionally as male, additional pollen deposition takes place, although the percentage of flowers receiving no pollen was still very high.

Similar results were reported in southern California and Florida by Davenport [31,72–74] and Davenport et al. [75], although the percentage of flowers with pollen at the end of the female stage was even lower than in our case and, consequently, the main pollen deposition occurred during the male phase. Those observations were made under different climatic conditions and using different varieties, and the results suggested that self-pollination in avocado during the male flower stage could be important to assure reproduction in the humid tropics. In our work, the flowers used to determine the pollen load in both stages were collected on optimal days for insect activity (sunny and windless days with temperatures above 20 °C at midday) when an overlap of at least two hours was observed between both sexual stages. It would be of interest to check this situation in other avocado-producing countries since inadequate pollination could be one of the main factors behind the low fruit set in this species. Optimizing the availability of honeybees and other insects known to pollinate avocado flowers could be one priority for avocado growers worldwide.

4.3. Pollination during the Flowering Season

Pollen deposition varied during the flowering season, being higher in male stage flowers during the first and the third weeks. The maximum pollen adhesion in the female stage occurred during the first week, maybe due to a higher period of coexistence between male and female flowers on the same tree or on trees of the same variety as a consequence of low temperatures that frequently occur at the beginning of the flowering season.

In species, such as avocado, pollinated by insects, pollination is highly dependent on pollinator abundance and pollinator activity that shows variation during the flowering season [76]. In this sense, the number of flowers open simultaneously within the inflores-

cence and within the tree could affect the number of pollinators attracted to a plant [77] and, therefore, the number of flowers visited as reviewed in [78]. At the beginning of the avocado flowering season, the number of honeybees visiting female flowers was lower, probably due to lower temperatures that affect bee activity. A similar observation occurred at the end of the flowering season when, although the temperature conditions were more favorable for bee activity, we found a decrease in the number of open flowers within the inflorescence, probably resulting in a decrease in the attraction of the inflorescence for insects and possibly corresponding with the beginning of the flowering period of other plant species more attractive for bees. On the other hand, the samples were collected during warm and sunny days, and, consequently, our results could have overestimated the overall proportion of flowers receiving pollen because of the role of temperature on flower opening and bee activity. Under our environmental conditions, adverse weather conditions (low temperatures, rainy and windy days) were more frequent at the beginning of the flowering season. Those years where those conditions occur, the behavior of the flowers can be altered, resulting in a lower number of fertilized flowers at the beginning of the flowering season and, consequently, the last flowers opening in the inflorescence will have more chances to set fruit and, therefore, determine the final production. Additional studies are necessary to evaluate the proportion of flowers with pollen at the end of the female stage during the flowering season and how this is affected by the weather conditions.

Once the pollen grains are deposited on the stigma, the time when fertilization took place with respect to the rest of the flowers within the inflorescence could determine the final fate of the embryo. The time of fertilization could be especially important in avocado due to its extended flowering period. Thus, intensive competition between developing fruits and between fruits and vegetative growth takes place within the avocado inflorescences (being more intense at the end of the blooming season). Fertilization time could play an important role conferring some advantages to compete for resources and, therefore, early blooming flowers set proportionately more fruits than late blooming ones. These results are in concordance with the higher fruit set found in determinate than in indeterminate inflorescences since in indeterminate inflorescences, developing fruits compete for resources with the new vegetative growth that takes place at the tip of the inflorescence [11].

4.4. Embryo Development in Flowers Pollinated during the Male Phase

In several studies [31,72–75] carried out under humid tropical conditions in Florida, an extension of stigmatic receptivity and pollen deposition during the male phase has been proposed as the main mechanism to ensure fruit production in avocado. However, our results showed that, under our environmental conditions, although a high percentage of flowers received pollen during the male stage and that pollen was able to germinate on the stigma, neither fertilization nor embryo and endosperm development took place in the 75 flowers examined pollinated in the male phase. This suggests that, under our environmental conditions, the period of effective pollination in avocado is mainly concentrated in the female stage. These unfertilized flowers can remain on the trees for some weeks and finally drop.

4.5. Fruits Resulting from Self- and Cross-Fertilization

When fruits resulting from self- and cross-fertilization compete in the same inflorescence, fruits resulting from cross-fertilization seem to have more chances of reaching the maturity stage. This higher retention of crossed versus selfed fruits has been reported previously in avocado orchards with interplanted A and B cultivars [79–86]. In some of these works, this observation could be a result of higher pollen availability of the complementary cultivars than of pollen from the same variety due to low overlapping between sexual stages in the same tree or among trees of the same variety. In our work, both pollen genotypes were applied at the same time, and the final fruit retention depended only on the genotype of the embryo. Differences in retention among fruits derived from self-pollination

and cross-pollination have also been described in other (sub)tropical fruit crops, such as litchi [87], mango [88,89], or macadamia [90], among others.

4.6. Pollen Parent Effect on Fruit and Seed Characteristics

Fruits derived from cross-pollination not only presented a higher survival rate but also showed differences in weight and seed characteristics compared to fruits derived from self-pollination [81]. Some reports [86] also showed differences in seed size depending on the male genotype. Fruits resulting from cross-fertilization showed desirable traits from a commercial point of view, such as the highest pericarp weight, lower seed weight, and a higher relation between fruit and seed weight than ‘Hass’ fruits derived from selfing.

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