

Optimization of controlled pollination in avocado (*Persea americana* Mill., Lauraceae)



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ABSTRACT

Avocado has a singular synchronous protogynous dichogamy breeding system that promotes outcrossing. In this work different steps have been optimized to improve controlled pollinations in avocado in order to perform basic studies of reproductive biology and directed crosses in breeding programs. The results show that, in order to achieve successful fruit set, male flowers should be collected when all the anthers have dehisced and the pollen transferred by direct contact of the anthers with the stigma during the female stage using preferentially determinate inflorescences and pollinating at least 11 flowers per inflorescence. To perform crosses between cultivars of complementary floral groups, the flowers should be stored under high relative humidity during the hand pollination process. When the crosses involved cultivars of the same floral group, the male flowers should be collected just before anther dehiscence and could be stored for several days at 4 °C. Pollen application should be performed preferentially on flowers within determinate inflorescences or within indeterminate inflorescences where vegetative growth has been removed. The results obtained are useful to optimize controlled pollinations in avocado and can help to increase fruit set in breeding programs and avoid the incorrect interpretation from pollination experiments in studies of reproductive biology.

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1. Introduction

Pollination is a key step to assure fertilization and seed production and, consequently, is a critical stage in sexual plant reproduction (Darwin, 1876). Although for many plants the amount of pollen grains that arrive on the stigma is far in excess of the amount required to fertilize all the ovules, poor seed set or low yield is often caused by insufficient pollination (Burd, 1994; Larson and Barrett, 2000; Ashman et al., 2004) both in animal-pollinated and wind-pollinated plants (reviewed in Friedman and Barrett, 2009).

Controlled pollinations are widely used in fruit and forest tree breeding programs to combine desirable traits from selected genotypes and to perform different studies of reproductive biology such as pollen limitation, self-incompatibility, length of the effective pollination period or pollen competition. In some particular cases, controlled hand pollination is a powerful tool to improve both yield and fruit quality (Burd, 1994; Gonzalez et al., 1998; Lora et al., 2006). However, the usually high costs and the relatively low fruit production associated with performing controlled crosses

in some woody perennial species results in the use of alternative approaches to produce seeds from controlled crosses. This is the case in avocado (*Persea americana* Mill.) where usually hand pollination has been reported as feasible for producing a few fruits but is not generally used for breeding purposes where a high number of seedlings are usually needed. The most common method is to collect fruits from the desired parents planted closely, and sometimes caged together with honeybees, the most common pollinator in commercial production, and analyze the paternity of the fruits produced with molecular markers to select the progeny of the desired crosses (Lahav and Lavi, 2013). Avocado is an evergreen fruit tree native to tropical and subtropical regions of Mexico, Guatemala and the Pacific Coast of Central America where it was domesticated in ancient times and that is currently cultivated in many different tropical and subtropical regions worldwide.

Although avocado flowers are bisexual having both functional male and female organs, those are separated in time through a synchronous protogynous dichogamous breeding system. Each flower opens twice, the first functionally as a female flower; then the flower closes and the following day the flower reopens functionally as a male flower (Davenport, 1986). Avocado can be considered as heterodichogamous, a special case of synchronized dichogamy with two morphs of genotypes with reciprocal flowering behavior

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(Endress, 2010), since the different avocado cultivars can be classified based upon their floral behavior in two groups: A and B. In the type A cultivars, the flowers open in the morning as functionally females, close at midday and reopen the afternoon of the following day functionally as males; flowers of the type B cultivars open as functionally female flowers in the afternoon, close overnight and reopen the following morning in the male stage (Stout, 1923). For this reason, performing crosses that involve genotypes of the same floral group is difficult. Moreover, avocado is an extreme case of a species with a very low fruit to flower ratio, with less than 1% of the flowers produced able to set fruits due to a massive drop of flowers and developing fruitlets (reviewed in Alcaraz et al., 2013) making the development of progenies with high number of individuals difficult.

Although a number of techniques [such as optimizing the location and management of the trees, avoid excessive shading, performing the controlled crosses in high (on) bearing years, girdling after initial fruit set, selecting highly producing cultivars as female parents, selecting an appropriate male parent or reducing competition between vegetative and reproductive growth] have been proposed to increase fruit set after hand pollination in avocado (reviewed in Lahav and Lavi, 2013), an evaluation of the main factors affecting pollen quality and fruit set in controlled pollinations is still needed. Thus, the purpose of this study was to establish a standardized method to perform controlled pollinations in avocado trees to carry out reproductive biology studies and increase the efficiency in the development of progenies with desirable characteristics in breeding programs. The importance of this information lies in that several inferences derived from the results obtained in these studies directly depend upon the performance of the hand pollination protocols used (reviewed in Stone et al., 1995). For that, we describe different steps that can be optimized to improve pollen management for controlled crosses in avocado: pollen application, length of stigmatic receptivity, pollen conservation and type of inflorescence. Finally, the efficiency of hand pollination on fruit set was compared with fruit set obtained in flowers left to open pollination.

2. Materials and methods

The experiments were carried out during two consecutive years (2011–2012) in a 25 years old experimental 'Hass' (floral type A) grafted on 'Topa Topa' seedling rootstocks avocado orchard located at the IHSM la Mayora in Málaga (Spain) at latitude 36°45'N, longitude 4°4'W and altitude 35 m above sea level. Long-term climate data (50 year average) for this location show annual average mean temperatures of 18.5 °C, average maximum temperatures of 28.9 °C in the hottest month (August), average minimum temperatures of 9.8 °C in the coldest month (January) with an average annual rainfall of 536 mm.

2.1. Comparison of hand pollination methods

To determine the best methods of pollen application on the stigma, two techniques were tested: pollination using a paintbrush and pollination by direct contact with the anthers of male stage flowers. For the pollination with a paintbrush, a total of 64 inflorescences distributed in 21 trees were selected and from 4 to 30 flowers were hand pollinated in each inflorescence with a total of 597 'Hass' flowers hand pollinated with pollen from 'Fuerte' (floral type B). Pollen was obtained from male flowers of 'Fuerte' collected when all the anthers had dehisced. The anthers were separated from their filaments and placed in a Petri dish. For the pollination with direct contact of the anthers of 'Fuerte' flowers with the stigmas of 'Hass' flowers, a total of 153 inflorescences distributed

in 30 trees were selected with a total of 645 'Hass' flowers hand pollinated. In both cases, the rest of the non-pollinated flowers in the inflorescences were removed.

The number of flowers and fruits retained by the trees were monitored every two weeks from the end of the flowering season until fruit maturity.

Statistical analyses were performed using SPSS 17.00 statistical software (SPSS Inc., Chicago, USA). Percentage data were normalized by arcsine-square root transformation prior to analysis. A Chi-square test for two way interaction contingency tables with Yate's correction for continuity was used to compare fruit set rate using the two hand pollination methods.

2.2. Length of stigmatic receptivity

Stigmatic receptivity was evaluated during the female stage by measuring the ability of the stigma to support pollen adhesion and pollen germination. Sixty 'Hass' flowers were hand pollinated in the field by direct contact with the anthers of 'Fuerte' male flowers at three different times: at flower opening in the female stage, in the middle of the female stage and just before the female flowers started to close. Hand pollinated flowers at each time were fixed the next day and the fixed pistils were prepared and squashed for microscope observations (see Section 2.9).

Two-way ANOVA was performed to determine the ability of the stigma to support pollen adhesion and germination (stigmatic receptivity) at three different moments of the female stage with pollen adhesion and pollen germination percentage as the dependent variables and time as the explanatory variable. Prior to analysis, data were first subjected to a Kolmogorov–Smirnov normality test and the homogeneity of variance was assessed with Levene's test. Whenever ANOVA results indicated a significant difference, a pairwise comparison of means by least significant difference test (LSD) at $P \leq 0.05$ was carried out.

2.3. Pollen density on the stigmas

With the purpose to determine the minimum number of anthers necessary to perform successful hand pollinations, the relationship between pollen density and pollen germination was analyzed by modifying the number of pollen grains deposited on the stigma using different number of anthers.

For this, female stage 'Hass' flowers were collected at anthesis and maintained in wet florist foam in the laboratory. Hand pollinations were performed by direct contact of the stigma with the anthers of male-stage 'Fuerte' flowers collected when all the anthers had dehisced. Five different density treatments were established based on the number of anthers used to perform hand pollinations (1, 3, 6, 9, 18) and between 17 and 33 flowers were pollinated for each treatment. Flowers were fixed 24 h later in FAA (70% ethanol: glacial acetic acid: formalin [18:1:1; v/v/v]) (Johansen, 1940) and prepared for microscope observations. Pollen adhesion, pollen germination and pollen tube growth were evaluated.

To test whether the pollen behavior differed depending on the number of anthers used for hand pollination, two-way ANOVA was performed with pollen adhesion and pollen germination as the dependent variables and the number of anthers as the independent variable. Differences between groups were determined by the LSD test ($P \leq 0.05$). To establish a possible correlation between the number of anthers used in hand pollination and pollen adhesion and germination on the stigma, Pearson's correlation coefficients at the 0.05 significance level were computed.

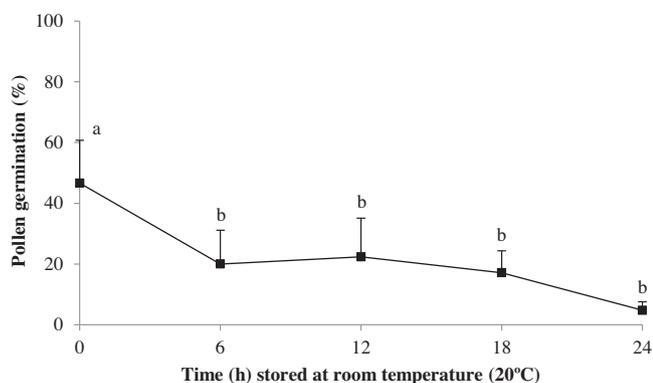


Fig. 1. Percentage of *in vitro* germination (mean \pm SE) of pollen from male stage avocado flowers maintained during several hours at room temperature (average of 20 °C). Bars followed by the same letter are not significantly different ($P > 0.05$) by LSD test.

2.4. Pollen conservation

The two most important abiotic factors that affect pollen viability are relative humidity and temperature. The first step was to study the changes over time in pollen viability under different environmental conditions; thus, ‘Fuerte’ male flowers were collected and maintained at room temperature (average of 20 °C, ranging from a minimum of 17 °C to a maximum of 22 °C). *In vitro* pollen germination was tested as described previously (Alcaraz et al., 2011) at different times: at the moment of collection, 6, 12, 18 and 24 h after collection. Three replicates were made for each time.

Due to the fast decrease observed in pollen viability at room temperature (see Section 3, Fig. 1), a method for short-term pollen storage was developed. Since avocado shows protogynous dichogamy, different pollen conservation mechanisms should be considered based on the floral groups of the parental genotypes. If the crosses are made using genotypes of complementary floral groups, the pollen collection can be made just before performing the hand pollinations although, with the purpose of optimizing the number of crosses during the effective period of pollination, pollen collection can be performed once a day, especially if the genotypes of interest for the crosses are far away from each other. Thus, the establishment of a short-term pollen storage method under orchard conditions without reducing pollen viability will be useful. For this, *in vivo* germination tests were used to compare the viability of pollen from male flowers collected at pollination time was and of pollen from male flowers stored for 3 h in a box with high relative humidity (95%). Male flowers from ‘Fuerte’ were collected (when all the anthers had dehisced) and stored in a semi-open petri dish maintained in a box with high relative humidity under the tree during 3 h. A total of 74 flowers were hand pollinated with fresh pollen and 77 with stored pollen. Pollination with fresh and stored pollen was made at the same time to avoid a possible effect of the stigmatic receptivity on pollen behavior. The next day, these flowers were fixed and prepared for microscope observation (see Section 2.9).

When pollinations are made using genotypes of the same floral group, the pollen has to be stored for a longer time. To check the effect of pollen storage at 4 °C, pollen viability was evaluated daily using both *in vivo* and *in vitro* germination tests during the five days following pollen collection. A total of 250 flowers of ‘Hass’ at the male stage were collected at the time of anther dehiscence and 250 before anther dehiscence and maintained in the fridge (4 °C) for several days. Pollen from 40 flowers stored at 4 °C collected at the moment of anther dehiscence and 40 collected before this stage, was spread daily on two Petri dishes and germinated *in vitro* following Alcaraz et al. (2011). Germination was evaluated after 24 h

of incubation at room temperature. For *in vivo* studies, 10 flowers distributed in different inflorescences were hand pollinated with pollen from flowers (collected before and after anther dehiscence) stored from 1 to 5 days at 4 °C. Hand pollinated flowers were fixed the following day in FAA and the number of pollen grains adhered and germinated was counted (see Section 2.9). *In vitro* and *in vivo* measures were repeated during four consecutive weeks.

To estimate pollen germination variation after storage at room temperature, a Generalized Linear Model was performed. Student’s *t* test at the 0.05 significance level was used to detect differences on pollen adhesion and pollen germination among flowers hand pollinated using fresh pollen and those pollinated with pollen from male flowers maintained under high relative humidity for three 3 h. Data of pollen germination *in vitro* from flowers stored during several days at 4 °C were analyzed with one-way ANOVA and two-way ANOVA was performed to test the pollen viability *in vivo* of flowers stored at 4 °C from 1 to 5 days, with time as the independent variable and pollen adhesion and pollen germination as the dependent variables and, in both cases, when the ANOVA results showed significant differences, comparison of means among groups were made with the LSD test.

2.5. Effect of pollen conservation on fruit set

To estimate the effect of pollen storage on fruit set, 57 inflorescences distributed in six ‘Hass’ trees were labeled. Five flowers were hand pollinated daily using pollen from ‘Fuerte’ collected the day before and stored at 4 °C and other 5 using pollen from ‘Fuerte’ collected at the time of pollination. Each hand pollinated flower was labeled with a paper tag. In every inflorescence, flowers were hand pollinated during all the blooming season and the open non-pollinated flowers were removed daily to avoid competition. A Chi-square test for 2 \times 2 contingency tables, with Yate’s correction for continuity, was used to test whether differences on fruit set exits using pollen stored at 4 °C and fresh pollen.

2.6. Fruit set and type of inflorescence

Avocado inflorescences are determinate thyrses that result in functionally determinate or indeterminate inflorescences depending on the abortion of the terminal vegetative bud (Chanderbali et al., 2013). In this work one hundred functionally determinate inflorescences, 100 functionally indeterminate inflorescences and 70 functionally indeterminate inflorescences where the terminal vegetative shoots were removed were hand pollinated using different type B pollen donor cultivars: ‘Bacon’, ‘BL516’ (‘Marvel’), ‘Fuerte’ and a pollen mix of the three pollinizers. Approximately 25 functionally determinate and indeterminate inflorescences and between 15 and 20 functionally indeterminate inflorescences without vegetative growth were hand pollinated using each of the four treatments. The pattern of flower/fruit drop was estimated weekly during the month following the flowering season and monthly since August. Fruit set was compared among the three types of inflorescences and among the four treatments for each type of inflorescence.

A Chi square test was performed to compare fruit set in the three groups of inflorescences established and among the different treatments for each type of inflorescence.

2.7. Number of flowers used per inflorescence to increase fruit set

Due to the differences observed on fruit set among the different types of inflorescences studied (see Section 3), the effect of the number of hand pollinated flowers on percentage of fruit set and in final fruit number per inflorescence was analyzed separately. To estimate the optimal number of flowers that must be

pollinated in an inflorescence to assure fruit set, 92 functionally indeterminate, 83 functionally determinate and 61 functionally indeterminate removing the vegetative growth were used to perform hand pollinations. Four different groups were established based on the number of hand pollinated flowers per inflorescence: 1–5, 6–10, 11–15 and 16–20 flowers. The rest of the non-pollinated flowers in the inflorescence were removed. A total of 1424 flowers were hand pollinated in functionally determinate inflorescences, 1170 in functionally indeterminate inflorescences and 749 in functionally indeterminate inflorescences with the vegetative shoot removed. The pattern of flower/fruit drop was estimated weekly during the months following the flowering season (from May to July) and monthly since August. Percentage of fruit set among the groups established was calculated for each type of inflorescence.

GLM was computed to compare the percentage of fruit set and the number of fruits per inflorescence among the four groups based on the number of hand pollinated flowers per inflorescence and, when significant differences were found, the pairs of means were compared using least significant differences (LSD) at the $P \leq 0.05$ level of significance.

2.8. Fruit set in controlled and open pollinations

After the establishment of the best method for hand pollination (see Section 3), fruit set was tested in two populations of flowers, one hand pollinated and the other left to open pollination. To estimate the fruit set in the population of flowers left to open pollination, 400 inflorescences were labeled and the initial number of flowers was counted (12,903 flowers). The fruit set in the population of hand pollinated flowers was estimated in the experiment of the effect of pollen storage on fruit set described above. After the end of the flowering season, the number of fruits remaining in these inflorescences was determined weekly and after August twice a month. A Chi-square test was used to compare proportion of final fruit set obtained in hand pollinated flowers and those left to open pollination.

2.9. Microscope preparations

Pistils fixed in FAA from the different experiments were washed three times with distilled water, 1 h each wash, and left overnight in 5% sodium sulfite. On the following day, the pistils were autoclaved at 1 kg/cm² for 10 min in 5% (w/v) sodium sulfite to soften the tissues and stained with 0.1% (v/v) aniline blue in 0.1 N K₃PO₄ for callose (Linskens and Esser, 1957). Preparations were observed under a Leica DM LB2 microscope with UV epifluorescence using a BP 515–560 exciter filter and an LP 590 barrier filter.

3. Results and discussion

Hand pollination methods should be described providing information not only about pollen management (time of collection, viability, storage in the orchard, application, etc.) during the process but also about the capacity of the stigma to support pollen adhesion and pollen germination at the moment of pollen application; this is especially important in species such as avocado with protogynous dichogamy where often crosses involving genotypes of different flower type are performed.

3.1. Pollen application techniques

Fruit set of hand pollinated flowers by direct contact of the stigma with the anthers (2%) was higher than that obtained when the pollen was applied on the stigma using a paintbrush (0.9%) although the differences between both methods of pollen application were not statistically significant ($\chi^2 = 3.024$, $df = 1$, $P = 0.082$),

probably due to the very low number of fruits relative to the number of flowers in avocado. The additional manipulation of the pollen when the paintbrush is used could account for these differences. If hand pollination is performed by direct contact with the dehiscent anthers of male flowers, pollen application must be done carefully to avoid damage to the stigma. Moreover, when the paintbrush is used for pollen application, an additional step in which the anthers are separated from the flowers and placed in a Petri dish is required making the overall process more slow. Due to these results, the method of direct application of pollen from the anthers was used for the rest of the experiments.

3.2. Length of stigmatic receptivity

The stigma supports pollen hydration, germination and initial pollen tube growth and the length of stigmatic receptivity plays an important role in the effective pollination period and subsequent fruit set (Nepi and Pacini, 1993; Tangmitcharoen and Owens, 1997; Sornsathapornkul and Owens, 1998; Aleemullah et al., 2000).

After pollen application by direct contact with the anthers of male flowers collected at the time of anther dehiscence, significant differences were observed on pollen adhesion ($F_{2, 106} = 4.33$, $P = 0.016$) and pollen germination ($F_{2, 106} = 4.52$, $P = 0.013$) among the different times analyzed during the female stage. Maximum pollen adhesion was observed in the middle of the female stage whereas the maximum germination took place at flower opening and just before flower closing.

Taken together, these results suggest that the avocado stigma maintains its capacity to support pollen adhesion and pollen germination during all the length of the female phase and, thus, that the pollen could be applied on the stigma along the whole female stage although the stigma seems to provide higher adhesion during the middle part of this period.

3.3. Number of pollen grains on the stigma

In avocado, although only one pollen grain penetrating the ovary is needed for fertilization of the single ovule present in the flower, the percentage of flowers with a pollen tube reaching the ovary is affected by the number of pollen grains deposited on the stigma (Shoval, 1987). This phenomenon is called population effect and it is common to a wide range of angiosperms species (reviewed in Cruden, 2000).

In this work, Pearson correlation analysis showed a significant correlation between the number of anthers used in hand pollination and the number of pollen grains adhered on the stigma (Pearson = 0.255, $P = 0.004$) as well as between pollen adhesion and germination (Pearson = 0.577, $P < 0.0001$).

The relationship between pollen density and pollen behavior was analyzed modifying the number of pollen grains deposited on the stigma using different number of anthers. Statistical differences were observed on pollen adhesion based on the number of anthers used to perform hand pollinations ($F_{5, 122} = 5.232$, $P = 0.001$). Thus, the maximum number of pollen grains adhered was obtained using consecutively 6 anthers for pollination, although no significant differences were observed when 3, 9 and 18 anthers were used in hand pollination. The minimum number of pollen grains adhered to the stigma was 5 using just one anther and the maximum number was 52 using 18 anthers. Previous reports in avocado (Shoval, 1987) suggested that at least 20 pollen grains must be deposited on the stigma to assure fertilization. The use of 6 anthers to perform hand pollination guarantees the deposition of enough pollen grains to assure ovule fertilization (an average of 22 pollen grains) although the results obtained in this work show that fertilization can take place with a lower number of pollen grains on the stigma. In fact, when less than 20 pollen grains are deposited on the stigma,

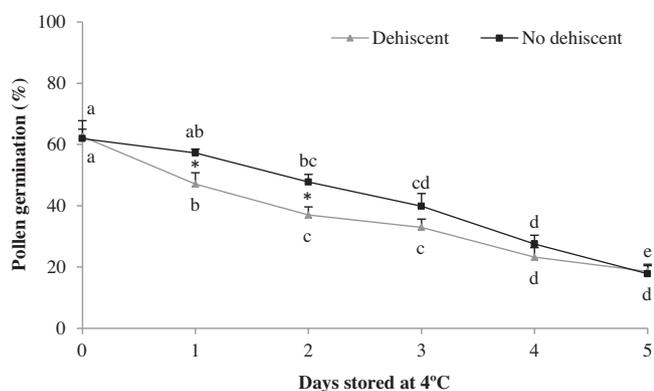


Fig. 2. *In vitro* germination of pollen stored at 4°C during several days in male stage avocado flowers collected before and after anther dehiscence. Data are means and the bars indicate the SE. The same letters within each group indicate no significant differences on pollen germination at different times of storage ($P > 0.05$) by LSD *post-hoc* test. Asterisk indicates significant differences on pollen germination between male stage flowers stored at the dehiscent and non dehiscent anther stage ($P < 0.05$) by Student's test.

a pollen tube can be seen near the ovule in all the pistils visualized under microscope.

3.4. Pollen conservation

A fast decrease in viability of pollen stored at room temperature has been observed during the hours following pollen collection (Fig. 1) ($F_{4,10} = 7.935$, $P = 0.004$). This rapid decrease in the germination capability of pollen suggests that the normal temperature conditions during flowering in most avocado growing areas (under our growing conditions during the years of study the average mean temperature at flowering was 17.2°C, ranging from an average minimum of 11.5°C to an average maximum of 21.5°C) are not suitable for long term pollen storage and, therefore, storage at lower temperature will be generally required.

3.4.1. Crosses between complementary flowering type genotypes

With the objective to optimize the number of crosses during the time when male flowers with dehiscent anthers of type B cultivars ('Fuerte') coexist with the female flowers of type A cultivars ('Hass'), that under our environmental conditions lasts approximately 3 h, the pollen collection could be made once a day. Pollen moisture content may be further altered during storage depending on the relative humidity maintained in the storage container and pollen hydration influences pollen viability and germination (Nepi et al., 2001; Franchi et al., 2002; Pacini et al., 2006).

No significant differences were observed neither in pollen adhesion ($t = 0.187$, $df = 142.22$, $P = 0.852$) nor in pollen germination ($t = 0.138$, $df = 143.43$, $P = 0.890$) in hand pollinated flowers using fresh pollen and pollen stored under conditions of high relative humidity for 3 h. Thereby, male flowers must be collected when all the anthers become dehiscent to perform crosses among genotypes of complementary groups in an open Petri dish and the male flowers (pollen source) should be stored in a high relative humidity environment (95%) allowing the pollen to maintain its viability.

3.4.2. Crosses between non-complementary flowering type genotypes

In vitro studies showed that pollen germination of pollen collected at anther dehiscence was significantly reduced with increasing time of pollen storage ($F_{5,40} = 24.218$, $P < 0.001$) (Fig. 2). Similar results were obtained when the male flowers were collected before anther dehiscence ($F_{5,34} = 29.272$, $P < 0.001$) (Fig. 3). Moreover, after one ($t = -2.606$, $df = 8.7$; $P = 0.029$) and two days

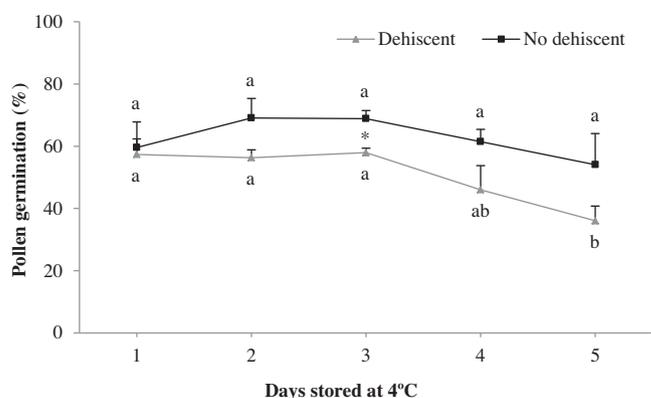


Fig. 3. *In vivo* germination of pollen stored at 4°C during several days of male stage avocado flowers collected before and after anther dehiscence. Data are means and bars indicate the SE. Different letters in the same group indicate significant differences in pollen germination ($P < 0.05$) using LSD *post-hoc* test. Asterisk indicates significant differences ($P < 0.05$) between pollen from dehiscent and non dehiscent anthers.

($t = -2.934$, $df = 13$; $P = 0.012$) of pollen storage, significant differences were observed in the capacity of pollen to germinate based on the developmental stage of the anthers collected from male flowers.

Regarding *in vivo* studies, the number of days of pollen storage at 4°C had a significant effect on the percentage of pollen germination (Fig. 3) of male flowers stored at anther dehiscence ($F_{4,17} = 4.284$, $P = 0.022$), although no significant effect was observed for pollen adhesion ($F_{4,17} = 2.336$, $P = 0.115$). The pollen from male flowers stored before anther dehiscence showed no variation neither in the capacity of pollen adhesion ($F_{4,17} = 0.113$, $P = 0.976$) nor in percentage of germination ($F_{4,17} = 0.918$, $P = 0.483$) (Fig. 4). When the pollen behavior *in vivo* was compared daily between male flowers stored before and at anther dehiscence, significant differences were obtained only after three days of storage ($t = -3.649$, $df = 4.627$, $P = 0.017$) (Fig. 3).

Previous studies in avocado showed that under orchard conditions the pollen grains maintain its capacity to germinate after six days (Papademetriou, 1975) although its capacity to fertilize the ovule was not analyzed; however, our results show that the capacity of pollen to germinate decreases significantly after 6 h of storage. Likewise, studies performed with the cv. Fuerte showed a similar low viability of stored pollen since pollen stored for one month at 4°C at different relative humidities was capable of germination and penetrating the ovule whereas germination of pollen stored at 25°C was greatly reduced and that pollen was not able to penetrate the ovule (Sedgley, 1981).

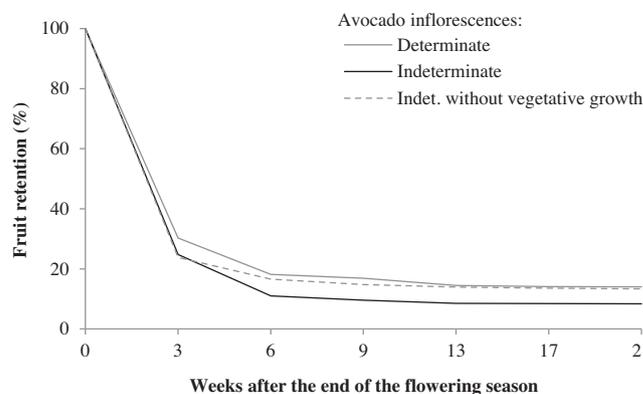


Fig. 4. Fruit set pattern in the different types of hand pollinated avocado inflorescences. The same letter indicates no statistically significant differences by Chi square test ($P > 0.05$).

3.5. Effect of pollen storage on fruit set

The ultimate purpose of pollen storage is successful fertilization and seed production. However, often the studies are limited to the effect of storage on pollen germination (Schroeder, 1942; Papademetriou, 1975) and tube growth and, in some cases, on the capacity to fertilize the ovule (Sedgley, 1981) but fewer studies address the effects on final fruit set. In order to check this point in avocado, a total of 4570 'Hass' flowers were hand pollinated: 2635 with fresh pollen and 1935 with pollen stored at 4 °C overnight. A total of 77 fruits (2.92%) and 50 fruits (2.58%), respectively, reached their maturity stage. No significant differences were found ($N = 4570$, $\chi^2 = 0$, $df = 1$, $P = 0.988$) between flowers pollinated using fresh pollen and those pollinated with pollen stored at 4 °C on final fruit set. Thus, although the percentage of pollen adhesion and pollen germination is reduced after storage for one day, stored pollen is still able to produce fruit set, probably because since only one pollen tube is needed to produce fertilization in avocado, the reduction observed in the number of pollen tubes is not sufficient to impede fertilization.

3.6. Fruit set and type of inflorescence

The type of inflorescences used to perform hand pollinations had an important effect on final fruit set ($\chi^2 = 21.54$, $df = 2$, $P < 0.0001$) (Fig. 4). Thus, functionally indeterminate inflorescences presented lower fruit set than functionally determinate ($\chi^2 = 20.099$, $df = 1$, $P < 0.000$) and than functionally indeterminate in which vegetative growth was eliminated ($\chi^2 = 12.821$, $df = 1$, $P < 0.001$). However, no significant differences were observed between functionally determinate inflorescences and indeterminate without the vegetative shoot. A lower fruit set in functionally indeterminate inflorescences compared to functionally determinate and functionally indeterminate where vegetative growth was removed has been reported in different experiments (Blumenfeld et al., 1983; Cutting and Bower, 1990; Salazar-García and Lovatt, 1998; Salazar-García et al., 2013) and explained in terms of reduced competition within functionally determinate floral shoots (Salazar-García et al., 2013). Usually, most floral shoots produce functionally indeterminate inflorescences although the ratio between functionally determinate and indeterminate inflorescences is dependent on cultivar, edaphoclimatic conditions and shoot age (Salazar-García et al., 2013).

A similar pattern was observed using different pollen donors except when a mix of pollen from three different pollinizers was placed on the stigma; in this case, fruit set showed no statistically significant differences among the type of inflorescence. Fruit set was similar using the different pollinizer genotypes ($\chi^2 = 6.48$, $df = 3$, $P = 0.090$) in each kind of inflorescence when the rest of non-pollinated flowers were removed.

Thus, in order to increase fruit set rate in breeding programs, it seems appropriate to select determinate inflorescences. In years or trees where a high proportion of indeterminate inflorescences is present, removal of the terminal vegetative shoot after hand pollination would also significantly increase fruit set.

3.7. Number of hand pollinated flowers necessary to assure fruit set based on the type of inflorescence

The number of open flowers per inflorescence showed a high variability according to the size of inflorescence, period of the flowering season, the genotype or the year. With the purpose to select the appropriate inflorescences to perform hand pollinations, the knowledge of the effect of the number of pollinated flowers on final fruit set is of interest since non-pollinated and closed flowers must be discarded. Significant differences were observed among two of the three types of inflorescences studied on the number of

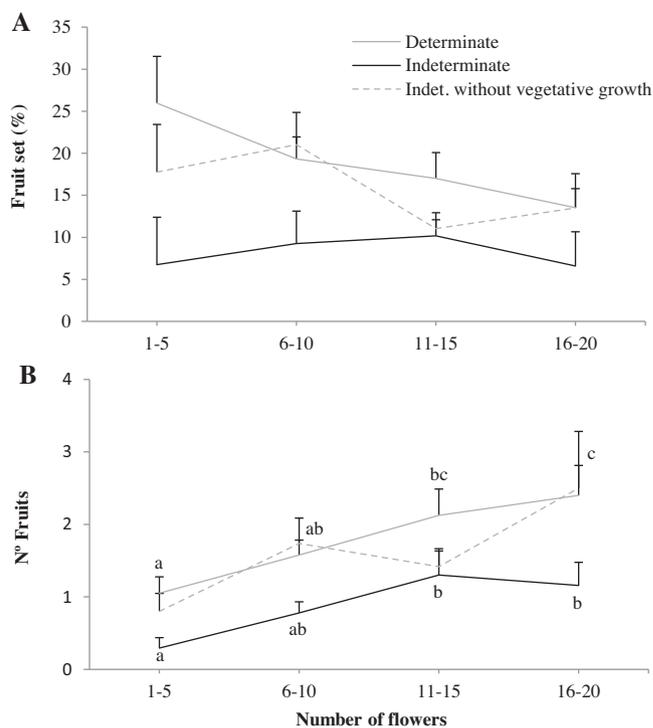


Fig. 5. Fruit set (A) and number of fruits per inflorescence (B) among groups established based on the number of flowers hand pollinated per inflorescence in determinate, indeterminate and indeterminate without vegetative shoot inflorescences in 'Hass' avocado. Data are means \pm SE. The same letter in the same group indicates no significant differences by LSD test ($P > 0.05$).

fruits based on the number of hand pollinated flowers per inflorescence (Fig. 5) (Determinate: $F_{3, 82} = 3.778$, $P = 0.014$; Indeterminate: $F_{3, 91} = 2.923$, $P = 0.038$; Indeterminate without vegetative growth: $F_{3, 60} = 2.923$, $P = 0.071$).

Similar fruit set percentage was observed in the different groups (Indeterminate: $F_{3, 91} = 0.853$, $P = 0.469$; Indeterminate without VG: $F_{3, 60} = 1.032$, $P = 0.385$; determinate inflorescences: $F_{3, 82} = 0.427$, $P = 0.734$). The fruit set in indeterminate inflorescences were lower than in determinate and indeterminate without vegetative growth. Fig. 5 shows fruit set and number of fruits per inflorescence in each group. A significant correlation was found between the number of hand pollinated flowers and final fruit number (Determinate: Pearson = 0.258, $P = 0.008$; Indeterminate: Pearson = 0.385, $P < 0.001$; Indeterminate without vegetative shoot: Pearson = 0.553, $P < 0.001$). The highest fruit set was obtained when from 1 to 5 flowers were hand pollinated per inflorescence in the determinate and indeterminate inflorescences and when 6 to 10 flowers in indeterminate without vegetative growth although, no significant differences were found among the different groups.

Although a relationship between the numbers of hand pollinated flowers per inflorescence and number of final fruits has been observed in this work, the average of fruits per inflorescence at harvest time is lower than two. Since no significant differences were observed neither in the number of fruits per inflorescence nor in fruit set rate in functionally indeterminate and determinate inflorescences between the two last groups (11–15 and 16–20 flowers hand pollinated, respectively), from 11 to 20 flowers could be hand pollinated per inflorescence. In functionally indeterminate inflorescences without vegetative growth, no differences neither in fruit set nor in number of fruit per inflorescence were observed, and, thus, a similar number of hand pollinated flowers per inflorescence to assure fruit set can be recommended.

The fact that similar results have been obtained in this work using different pollinizer genotypes indicates that the hand pollination method described here could probably be extrapolated to different pollen donors.

3.8. Hand pollination vs. open pollination

Fruit set obtained when all the flowers of each inflorescence are hand pollinated using the method described here (2.53%) is significantly higher ($\chi^2 = 102.8$, $df = 1$, $P < 0.001$) than that obtained in flower populations left to open pollination (0.15%). This fifteen fold increase suggests that natural pollination is still a limiting factor in avocado production but also that, in spite of pollinating all the flowers of the inflorescence, still a low percentage of them are able to develop into fruits probably due to differences in flower quality at the time of anthesis (Alcaraz et al., 2010, 2013).

4. Conclusions

The results obtained in this work allow us to improve the approaches used to perform experiments involving hand pollination in avocado. Thus, to achieve successful fruit set in crosses between different floral groups, the male flowers should be collected at anther dehiscence and the pollen transferred by direct contact of anthers with the stigma of functionally female flowers. During the process, the flowers could be stored in a box with high relative humidity in the orchard. When the crosses involved cultivars of the same floral group, the male flowers should be collected just before anther dehiscence and could be stored for several days at 4 °C. In both cases, the pollen application can be done during all the female stage using preferentially determinate inflorescences and pollinating at least 11 flowers per inflorescence.

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