WHERE HAVE ALL THE FLOWERS GONE? WHY AVOCADO PRODUCES SO MANY FLOWERS THAT RESULT IN SO FEW FRUITS

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INTRODUCTION

The avocado (*Persea americana* Mill.) is an evergreen subtropical fruit tree that evolved in the humid tropical highlands of Central America, including southern Mexico, Guatemala and Honduras where it was domesticated and cultivated since ancient times (Galindo-Tovar et al., 2008). It is a member of the Lauraceae, a mostly subtropical or tropical family included within the order Laurales in the evolutionary early-divergent angiosperm clade Magnoliid.

The avocado flowering is dichogamous, protogynous and synchronous. The flowers are bisexual having both functional male and female organs although separated in time through a synchronous protogynous dichogamous breeding system that prevents self-pollination and promotes outcrossing. Each avocado flower opens twice during two consecutive days. On the first day the flowers is functionally female with a white receptive stigma; then the flower closes and the following day the flower reopens functionally as a male flower, when the anthers dehisce (Davenport, 1986). Avocado cultivars are classified in two groups (A or B) based upon their flowering behaviour (Nirody, 1922). In type A cultivars, the flowers open in the morning 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 in the female stage, close in the evening and reopen the following morning in the male stage (Stout, 1923). However, this floral cycle is highly sensitive to environmental conditions particularly to cool night temperatures (Stout, 1923; Lesley and Bringhurst, 1951; Bergh, 1974; Sedgley and Annells, 1981 Pattemore et al., 2018).

One of the main limitations in avocado production worldwide is the massive abscission of flowers and developing fruits mainly in the first two months after flowering. As a result, final yield is below 1% of the flowers produced (Cameron et al., 1952; Segdley, 1980; Lahav and Zamet, 1999; Garner et al., 2008; Garner and Lovatt, 2008). Several factors are involved in this massive drop of flowers and developing fruitlets, such as extreme temperatures (Sedgley, 1977; Sedgley, 1987; Sedgley and Annells, 1981), the genotype of the seed (Degani et al., 1989; 1990; 1997), alternate bearing (Hodgson, 1947; Hodgson and Cameron, 1935), or nutrient deficiencies

(Embleton et al., 1959; Lahav and Zamet, 1999; Whiley, 1990). Although all these factors affect yield, additional information to determine the reasons behind the fact that most avocado flowers will drop without becoming fruits is needed in the different avocado producing regions worldwide. In this work, we summarize the results obtained on avocado flowering and fruit set mainly in Spain, under a Mediterranean climate. The observations have been performed at latitude 36º 45' N, with an annual average of the mean temperatures of 19.4ºC, an average high temperature in the warmest month (August) of 29.4ºC, and an average low temperature in the coldest month (January) of 9.3ºC. The average annual rainfall is 435 mm. Temperatures at 'Hass' avocado flowering range from 9ºC to 27ºC with an average of 16ºC with a relative humidity ranging from 45 to 94% with an average of 72%. Consequently, the results obtained represent those under the particular environmental conditions of Southern Spain and, consequently, they might not be directly applicable to other growing conditions with different climates.

Avocado is prone to alternate bearing. Our results show that a similar drop pattern occurred in hand-pollinated flowers in the high (on) and low (off) bearing years suggesting that the main difference in production between both years is due to the higher production of flowers during the high bearing year, corroborating previous results suggesting that the percentage of abscission does not depend on flower intensity (Garner and Lovatt, 2008; Garner et al., 2008).

The need of pollinizer varieties

Due to the especial avocado flowering behavior, a common recommendation is to interplant complementary type A and B cultivars to increase yield. A significant effect of the distance to the pollinizer in final yield has clearly been shown in Israel where a positive effect of interplanting complementary cultivars in close proximity on yield was found (Degani et al., 1989; 1997). Conclusions are not that clear in California where the results have ranged from some correlation (Bergh and Gustafson, 1958, 1966; Bergh and Garber, 1964; Bergh, 1966, 1968; Vrecenas-Gadus and Ellstrand, 1985; Kobayashi et al., 2000), to the absence of correlation (Garner et al., 2008). Similarly, in South Africa, no clear effects of distance to the pollinizer on yield have been obtained (Robbertse et al., 1996; Johannsmeier and Morudu, 1999). Consequently, the relative impact of outcrossing on final yield in 'Hass' is still a subject of controversy, since the studies have been performed in avocado growing areas with different environmental conditions. Moreover, a common observation in different parts of the world is that satisfactory yields can occur in orchards composed of a single variety (usually 'Hass') probably due to close-pollination (pollination during the overlap of opening male and closing female flowers in the same inflorescence or tree) (Davenport, 1986). This overlapping could be considered as a "bet-hedging" strategy of the avocado tree to ensure fertilization when the opportunities for outcrossing are limited and may help to explain fruit production in single cultivar blocks (Davenport, 1989; Davenport et al., 1994). Under the environmental conditions of Southern Spain, it is common to observe flowers in different sexual stages at the same time both among trees of the same genotype and within the same tree during several hours (Alcaraz and Hormaza, 2009). The floral behavior also showed a wide variation during the blooming season; thus, at the beginning of the blooming season, when temperatures are cooler, the floral cycle was longer than the cycle observed at the end of flowering and, in this period, overlapping between the male and female stages could be observed during four hours. The floral behavior was mainly altered in days characterized by lower temperatures. 'Hass' flowers showed a one day delay in the cycle, and the flowers opened in the male stage at the third day of the flower cycle. The type B genotypes showed higher sensitivity to low temperatures as had been reported previously (Lesley and Bringhurst, 1951; Sedgley, 1977; Sedgley and Annell, 1981; Sedgley and Grant, 1983); thus, under those conditions, flowers opened in the female stage in the evening and, some of them, remained open during the morning and reopened the fourth day in the morning as male flowers.

The decision on whether to plant and in which number pollinizer varieties is highly relevant in economic terms because the price of the fruits produced by the pollinizers is usually significantly lower than those derived from 'Hass'. Therefore, the cost of using a space of the orchard to interplant pollinizer trees could be higher than the higher input derived from the possible increasing yields obtained by outcrossing (Schnell et al., 2009). An interesting alternative could be the use of pollinizer varieties as wind breakers planted at short distances, especially in avocado orchards of small size. Moreover, the selection of the pollinizer genotypes is highly dependent on the environmental conditions of each producing region because a good pollen donor must present a good overlapping in the flowering season with the pollinated cultivar.

Under our growing conditions, using molecular markers to check paternity (Alcaraz and Hormaza, 2011), we found a decrease in the percentage of outcrossing with increasing distance from the pollinizers (Bacon and Fuerte) similar to previously described results (Vrecenas Gadus and Ellstrand, 1985; Degani et al., 1989; 1997; Kobayashi et al., 2000; Garner et al., 2008). However, no significant correlation between outcrossing rate and yield was observed, except in off years (Alcaraz y Hormaza 2011). This could mean that the use of pollinizers could be required in situations with a strong alternate bearing or if stresses to the tree at flowering (extremely high or low temperatures) are common. In fact, the effect of the distance between cultivars on yield has been mainly explained in terms of a selective drop of fruits resulting from self-fertilization (Degani et al., 1989; 1990; 1997). Thus, in spite of the high percentage of self-pollination reported under different environmental conditions (Davenport, 1989; Davenport et al., 1994), most of the final fruits are the result of outcrossing (Vrecenar-Gadus and Ellstrand, 1985; Degani et al., 1986, 1989, 1997, 2003; Goldring et al., 1987; Robbertse et al., 1997; Chen et al., 2007; Borrone et al., 2008). In order to study this topic under our environmental conditions, after the flowering season, several fruits were tagged based on the week of fertilization (at the end, mid and start of the 'Hass' flowering season). A polyethylene net was placed on the ground under each 'Hass' tree to collect all the falling fruitlets from June to commercial harvest in March the following year. Our results show a high drop of fruits derived from self-fertilization from June to August. However, if we take into account the time of fertilization, most of those fruits were fertilized at the end of the 'Hass' flowering season, suggesting that this selective fruit drop could be mainly due to resource competition rather than to genetic selection among developing embryos. In addition, under our growing conditions, at the end of the 'Hass' flowering season no 'Fuerte' pollen is available and, thus, the fruits fertilized at the end of the 'Hass' flowering season are the result of self-pollination. These fruits have to compete for nutritive resources with those derived from flowers pollinated at the beginning of the 'Hass' flowering season when 'Fuerte' pollen was available. Consequently, the results obtained indicate that outcrossing is probably not the main limiting factor in 'Hass' yield under the growing conditions of Southern Spain and that competition by available resources could be the main reason behind fruit drop during the months following the flowering season. This competition occurs not only among developing fruits but also with the new vegetative flush that starts at the end of the flowering period. However, additional studies in other avocado producing area should be performed to compare with these observations.

The diversity of insect pollinators as a yield limiting factor

The percentage of fruit set obtained in hand-pollinated flowers can be fifteen times higher than in flowers left to open-pollination, suggesting that pollination is one of the limiting factors in avocado commercial production and that a higher fruit set could be obtained by increasing the supply of pollen to the flower during the female phase. Studies performed in Southern Spain indicate that approximately 90% of the flowers do not receive pollen during the female stage, although this percentage is lower at the end of the flowering period, when temperatures are higher and, consequently, more adequate both for avocado fertilization and for insect activity.

The honeybee (*Apis mellifera*) is the most common pollinator in avocado orchards worldwide. However, the honeybee was introduced in America by the Spaniards (Roubik, 1998) and, consequently, the avocado has evolved in its area of origin in the presence of other insects native to the Americas. In Central America, more than 100 different species of Hymenoptera, Diptera, Coleoptera or Heteroptera have been observed visiting avocado flowers (Free, 1993; Can-Alonzo et al., 2005). The most common pollinators are honeybees, stingless bees (Apidae, *Meliponinae*), wasps, flies or beetles (Angel, 1984; Crane, 1992; Free and Williams, 1976; Papademetriou, 1976, Ish -Am et al., 1999). In Southern Mexico, the most common insects visiting avocado flowers are diptera, followed by honey bees (Pérez-Balam et al., 2012). In South Africa, about 50 different species have been observed visiting avocado flowers being the honey bee the most common followed by different species of diptera (Eardley and Mansell, 1996). Similar observations have been made in New Zealand, where moths have also been described visiting avocado flowers at night (Pattemore et al. 2018). In Spain, we have observed mainly species of himenoptera, diptera and, to a lesser extend, lepidoptera and coleoptera. Among the himenoptera, besides honeybees and bumblebees, several species of solitary bees have been found, which have already been described as efficient pollinators of several fruit tree crops (Garibaldi et al., 2011; Klein et al., 2012). Among diptera, syrphids are particularly common.

We have performed different trials with the objective of increasing the number of flowers that receive pollen during the female phase by increasing the number of honey beehives in commercial avocado orchards. Although the percentage of flowers that receive pollen increased significantly, still the number of flowers receiving pollen is low suggesting that additional insects could play a role to increase productivity (Garibaldi et al., 2013). Preliminary work with bumblebees and syrphids, suggest that honeybees, bumblebees and flies are capable of transporting avocado pollen grains in their bodies. The honeybee is capable of transporting a higher number of pollen grains than the other insects but most of them are transported in the corbiculae and, consequently, are not viable for fertilization. Observations in closed nethouses indicate that the yield in trees enclosed with syrphids and those left at open pollination was very similar and higher than yield in trees enclosed with bumblebees. This suggests that different species of syrphids can be good candidates as additional pollinating insects in commercial avocado orchards.

Additional studies in the different avocado producing areas are required to evaluate the efficiency of the different insects visiting avocado flowers in each regions in order to optimize management of avocado pollinators.

A low proportion of flowers receive pollen during the female phase, and of those, an important percentage receive few pollen grains. Although the probability of a flower becoming into fruit is affected by the number of pollen grains deposited on the stigma, some flowers with a low stigmatic load could be able to set fruits and reached the maturity stage. All these evidence indicate that the lack of pollination could be one of the limiting factors in commercial avocado production. However, although pollination is a requirement for avocado fruit set, we also found an early abscission pattern in both hand- and open-pollinated flowers, suggesting that other factors besides lack of pollination contribute to the massive drop of avocado flowers and fruitlets.

Effect of environmental conditions on pollen germination and pollen tube growth

Environmental conditions not only affect flowering behavior but also the progamic phase, from pollination to fertilization. The effective pollination period (EPP) was defined by Williams (1970) as the period during which a flower maintains its capacity to be transformed into fruit. This period is conditioned by three events: stigmatic receptivity, pollen tube kinetics and ovule longevity (review in Sanzol and Herrero, 2001).

In spite of the importance of the length of stigmatic receptivity on the period of effective pollination, little is known about the effect of environmental conditions on EPP in avocado. Several works have reported that stigmas are no longer receptive in flowers opening in the male stage although sometimes pollen grains can germinate but the pollen tubes do not reach the ovule (Sedgley, 1977). Our studies under orchard conditions indicate that a high percentage of flowers receive pollen during the male stage, but no evidence of embryo and endosperm development were observed, suggesting that fertilization did not take place in spite of pollen germination and tube growth. However, other studies have reported that fertilization can occur in this situation (Davenport, 1989, 1991; Davenport et al., 1994).

We have studied the effect of different temperatures and relative humidities on stigmatic receptivity and pollen tube growth through the style. Thus, the effect of four temperatures (15, 20, 25 and 30ºC) and three relative humidities (50, 75 and 95%), that are representative of the variation observed under our growing conditions during the 'Hass' flowering season, on stigmatic receptivity were analyzed in approximately 60 flowers per treatment maintained in wet florist's foam and also under field conditions. Differences were observed on the adequate conditions to obtain the maximum pollen adhesion and germination in both male and female flower stages. The maximum pollen adhesion and germination was observed at 20 and 25ºC and high RH in both male and female stages. These results were similar to those described in different avocado cultivars (Sahar and Spiegel-Roy, 1984; Loupassaki et al., 1997) and to the conditions required for good fruit set (Sedgley, 1987; Sedgley and Annells, 1981). Moreover, we found that at temperatures higher than 25ºC, pollen germination decreased mainly in the male stage probably due to rapid stigma degeneration.

Although a large number of pollen tubes is recorded at the stigma, the number of growing pollen tubes decreases along the style (Tomer and Gottreich, 1975; Sedgley, 1979) and, finally, only one reaches the ovary and fertilizes the ovule. As in other species (reviewed in Hedhly et al., 2009), avocado pollen tube growth through the style is highly dependent on temperature (Sedgley, 1977; Sedgley and Annells, 1981; Sedgley and Grant, 1983). Thus, pollen tubes grow faster at higher temperatures. In flowers maintained at 30ºC the pollen tubes reach the ovary 4 hours after pollination and 8 hours in those maintained at 20ºC although in both conditions the pollen tubes were observed near the ovule 24 hours after pollination. However, at lower temperatures (10ºC), two days after pollination no pollen tubes were observed penetrating the ovule. This observation agrees with the low fruit set obtained under cold conditions in avocado (Sedgley, 1977; Sedgley and Annells, 1981; Sedgley and Grant, 1983).

Thus, besides adequate pollination, appropriate environmental conditions are also needed for an adequate fertilization. Nevertheless, while pollination and fertilization is a requirement for fruit set, those two factors do not appear to be sufficient for a flower to develop into a fruit.

Nutritive reserves of the flowers

No morphological differences are apparent between avocado flowers that drop and those that will produce fruits (Sedgley, 1980). In avocado, reproductive and vegetative growth occur simultaneously and, consequently, both developmental stages compete for resources provided by either reserve mobilization or photosynthesis (Scholefield et al., 1985; Wardlaw, 1990). Early fruit abscission has been attributed to low carbohydrate reserves in the trees (Davie et al., 1995) and fruit drop has been shown to be accompanied by the reduction of carbon export from the leaves (Finazzo et al., 1994). The presence of starch in the ovary of avocado flowers has been reported at anthesis (Segdley, 1979; Alcaraz et al., 2010). We have analyzed the role of nutritive reserves of the flower on fruit set with an image analyzing system attached to the microscope that allows detecting and measuring differences among flowers (Alcaraz et al., 2013). The results show that, whereas no external differences are apparent among avocado flowers at anthesis, a wide variability exits in starch content at flower opening. Differences among flowers could be related to the probability of producing a fruit, since a very small proportion of the flowers present high starch content in the ovary. In order to establish the implications of nutritive reserves accumulated in the flowers on reproductive success we designed an experiment in which 40 'Hass' flowers in the female stage were hand-pollinated daily using pollen from 'Fuerte'. After pollination, each flower was labelled and the style was cut off the following day once the pollen tubes had grown along the style. The ovaries were, thus, maintained in the tree and fruit set could be examined for each labelled flower. The results obtained demonstrated a significant correlation between starch content in the flowers at anthesis and fruit set suggesting that the capacity of a flower to become a fruit could be preconditioned by the nutritive status of the flower (Alcaraz et al., 2013). High starch content at anthesis would thus be a necessary for fruit set. Additional similar experiments were performed in New Zealand and Spain, under different edaphoclimatic conditions measuring additional compounds in the cut styles, such as different soluble sugars (manoheptulose, fructose, sacarose, perseitol) or boron. The results were similar to those reported previously with starch. The flowers that eventually produce fruits show a higher flower quality (expressed in terms of higher sugar and boron content) than those that will finally drop both in Spain and New Zealand (Boldingh et al., 2016).

In short, flowering is a critical step in avocado production. Both intrinsic and extrinsic factors are involved in reproductive success and a good knowledge of the different aspects that play a role in this phase in different edaphoclimatic conditions is necessary to optimize avocado production. This is especially urgent in tropical regions since most of the studies performed so far in this topic have been carried out in temperate regions. The combination of all these experiments indicates that multiple factors are responsible for the low fruit set in avocado and that a holistic approach should be followed in order to optimize avocado fruit set. On the one hand, the opportunities for pollination should be increased by optimizing the diversity of pollinating insects. Moreover, taking into account that the floral behaviour is very sensitive to environmental conditions (temperature and relative humidity) it could be interesting to design mixed plantation systems involving two or more pollinizer genotypes that would have the advantage of decreasing the possible negative effect of a lack of synchrony depending on the environmental conditions. Moreover, although avocado has been described as a species with a marked protogynous dichogamy, it is common to observe flowers in different sexual stages at the same time among trees of the same genotype and even within the same tree allowing self-pollination. The influence of the starch reserves of the flower at anthesis on fruit set suggests that the nutritive status of the tree and the allocation of resources to the developing inflorescences and flowers could play an important role on fruit set, being a necessary although not sufficient condition for fruit setting.

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