Finding the best polliniser for 'Hass' avocado and the effect of bees as pollinators

MM Bezuidenhout, ES du Toit and PJ Robbertse

Department of Plant Production and Soil Sciences, University of Pretoria
Private Bag X20, Hatfield 0028, SOUTH-AFRICA
E-mail: elsa.dutoit@up.ac.za

ABSTRACT

The study entailed an in vitro pollination study and field trials. The field trials consisted of encaged 'Hass' trees with bees, encaged 'Hass' x 'Zutano' trees with bees, open pollinated 'Hass' trees and encaged 'Hass' trees with no bees inside. For testing the viability of cross pollination with 'Fuerte' as a polliniser, fruit set on 'Hass' trees at increasing distances from an adjacent 'Fuerte' block was determined. In vitro pollination and volatile tests were done in September 2016. Fruit set counts were taken in October and again in December 2016. Further counts will be made at harvesting during March/April.

Results from 2016 in vitro pollinations on 'Hass' flowers showed that out of the four pollinisers tested, 'Zutano' pollen outperformed those of 'Hass', 'Bacon', 'Fuerte' and 'Ettinger' although 'Bacon' pollen performed second best. 'Hass' trees in nets together with 'Zutano' did have an increased fruit set, although heavy fruit drop still occurred between October and December. Bees increased fruit set dramatically. Volatiles collected from flowers in the male and female stages of 'Hass' and 'Fuerte' differed not only between flowering stages, but also between the two cultivars.

INTRODUCTION

Bender (2002) gave a short description of the origin of 'Hass' and the cultivar characteristics. He found that the 'Hass' cultivar was selected by Rudolph Hass in the 1920s and originated as a chance seedling variety. He also mentioned that 'Hass' has a very long harvest season and is known to be the top quality avocado available, but it is also known to have poor fruit set in some locations.

Growers are continuously looking for higher yields and believe that it should be possible to increase the present yields by finding the best polliniser. 'Hass' being an A-type cultivar, the polliniser must be a B-type cultivar.

The benefits of cross-pollination against self-pollination for increasing fruit set and yield was already reported by Clark and Clark (1923, 1926). Since then many attempts have been made to 'prove' that avocados are out-breeders and that pollinisers are essential for good fruit set. Degani *et al.* (1997) and Degani and Goldring (1989) showed that abscission of avocado fruitlets and fruit characteristics were greatly influenced by the pollen parent.

Garner *et al.* (2008) had contradictory results and found that outcrossing is not the primary factor affecting fruit persistence and ultimately yield. This report highlights the fact that the question about the effectiveness of pollinisers is not yet properly answered and requires more research. This study was therefore conducted to re-investigate the problem under South African conditions.

A good polliniser without pollinators is of no use and Clark (1923) already reported the importance of bees as pollinators. Peterson (1955) concluded that large dipterous and hymenopterous insects are necessary for pollinating avocados. Ish-Am and Eisicowitch (1993 and 1988) wrote several articles that implied the importance of bees as pollinators for avocado, but that avocado flowers are not very attractive to honeybees.

According to Dixon (2004), eight equivalent hives should be used per hectare for trees six to ten meters high. He also mentioned that no less than four hives should be used per hectare. About 20-30 bees need to be working on one tree in order to attain good enough pollination. Weather conditions such as cold (<17°C), wet, windy and very cloudy days will reduce bee activity and cause a reduction in pollination.

Arpaia and Hofshi (2004) discussed different aspects regarding avocado pollination and about pollinisers and cross pollination. They mentioned three points to consider: 1. Synchrony of flowering cycle with 'Hass' flowering cycle, 2. multiple pollinisers

give a better overlap, and 3. Spatial placement of pollinisers. They also emphasised the importance of pollinating insects and the importance of bees. In their report they list 259 references of which most refer to pollination and fruit set.

In his report on avocado pollination, Dixon (2004) wrote that flower opening is highly dependent on temperature and this can affect the overlap of the male polliniser flowers with the female 'Hass' flowers. Such temperatures will have a great influence on cross pollination success. Bender (2002) also pointed out that low temperatures can cause delayed and irregular flowering.

There are critical stages in the process that lead to fruit set and fruit development. Bender (2002) gave a short description of the processes that lead to fruit set. First, pollen has to reach the pistil, it then has to germinate and form a pollen tube. The pollen tube has to move successfully down the style to reach the ovary. The pollen tube should then deliver the sperm cells to the egg cell and central cell inside the ovule to accomplish successful fertilisation. The ovary will form the flesh of the avocado fruit and the ovule will form the seed with the embryo. Dixon (2002) stated that competition between pollen tubes in the style leads to stronger seed. The seed provide plant growth regulators that will aid the development of the fruit according to Bender (2002). If one if these stages are interrupted or disturbed, fruit formation and development will be halted.

Garner *et al.* (2008) explained in their report on fruit abscission and alternate bearing that even if fruit set were successful, critical abscission stages will cause a lot of the fruit to drop before maturity is reached. Garner (2008) concluded that fruit drop can be more severe with adverse climatic conditions, poorly applied cultural practices and poor tree health.

According to an article written by Afik (2006), bees have to collect pollen as well as nectar in order for them to visit the male and also the female flowers. If they collect only pollen, they will not visit the female flowering stages and pollination cannot occur. Clearly the nectar component is quite important and it would, most possibly, be the nectar that releases volatiles. Nectar is released by the three inner staminodes and six outer nectaries. The three inner staminodes are active in the female phase but spent in the male phase while the outer nectaries are active in the male phase but still inactive in the female phase (Personal observation). Afik (2006) stated that avocado nectar contains Perseitol and a high amount of Sucrose. Afik then concluded that they still cannot give an explanation why bees are not attracted to avocado flowers as much as other flowers like citrus.

Most of the research mentioned in this introduction was done either in the United States or in European countries and it was regarded necessary to repeat some of the work under South African conditions. The aim of this study was therefore to study the effectiveness of different pollinisers for 'Hass' and the role of bees in pollination of avocados under South African conditions.

The project is running for the second season. The results of the 2015-2016 season were presented in the previous yearbook (SAAGA Yearbook vol 39) and the results presented in this report are from the 2016-2017 season.

MATERIALS AND METHODS

The study was done mostly in orchards of ZZ2 and partly in an orchard on the experimental farm of the University of Pretoria. It comprised of two parts, namely in vitro trials and field trials. Data trees were subjected to the same standard cultural practices as the remaining orchard trees.

For the in vitro pollination study, three sets of six 'Hass' flowers, in early anthesis (opening in the female phase), were collected from 11 different trees. The flowers were placed in Petri dishes containing a gel made up of 5% agar, 10% sucrose and 0.05% boric acid and allowed to open. 'Zutano', 'Ettinger', 'Bacon' and 'Fuerte' flowers that were open in the female phase, were collected the previous afternoon and placed in Petri dishes containing the same medium. The flowers were kept at 25°C during the night and they opened the next morning in the male phase, providing the pollen for in vitro pollination with the female 'Hass' flowers. The 'Hass' flowers were then pollinated with the 'Zutano', 'Ettinger', 'Bacon' and 'Fuerte' pollen and kept at 25°C until the next morning, allowing the pollen to geminate and pollen tubes to move down the style. The flowers were then fixed in a Carnoy solution (ethanol, chloroform and acetic acid in the ratio of 60:30:10). The six flowers from each petri dish were fixed in a separate container.

The third set of opened 'Hass' flowers were placed in a fridge at about 4°C to prevent them from closing until 12:00 when they were returned to ambient temperature in order to warm up before pollination. 'Hass' flowers at early anthesis in the male phase were collected and the anthers allowed to open to provide pollen for self-pollinating the female 'Hass' flowers. The self-pollinated 'Hass' flowers were also kept at 25°C until the next afternoon (24 hours) when they were also fixed in Carnoy solution.

The pollinated flowers fixed in the Carnoy solution were taken to a laboratory at the University of Pretoria where the pistil of each flower was excised and placed in a small container containing 20% alcohol. The excised pistils were then placed in 5M NaOH to soften, followed by rinsing in tap water, cleared in 30% Jik, rinsed again before being placed in Aniline Blue for staining the pollen tubes. Thereafter the samples were kept in the dark until further treatment. Squash preparations were made of each pistil and viewed under a fluorescent microscope. For each pistil the number of pollen grains on the stigma were counted as well as the number of pollen tubes germinated, the number of pollen tubes moving down the style, the number of pollen tubes reaching the ovary and the number of pollen tubes entering the ovary.

The field trials consisted of three parts: (1) A trial done on 'Hass' trees with 'Zutano' trees as the polliniser, (2) a trial with varying distances between pol-

liniser orchard and 'Hass' trees and (3) analysis from volatiles collected from 'Hass' and 'Fuerte flowers'. The first trial was done in Tzaneen on a six-year old 'Hass' orchard interplanted with 'Zutano' trees belonging to ZZ2. Four cages containing both 'Hass' and 'Zutano' trees with bees and four cages containing only 'Hass' trees with bees were used. For open pollination, trees in rows containing 'Hass' and 'Zutano' were used as well as rows with only 'Hass' trees. Four trees per treatment were used and ten flowering shoots per tree on four sides of the tree (N, E, S and W) were marked for counting fruit set. In the 'Hass' x 'Zutano' orchard at ZZ2, two nets with 'Hass' trees were set up with no bees inside. A pollen trap was put up on one of the bee hives in the 'Hass' x 'Zutano' orchard in Tzaneen to monitor pollen collection by bees.

The second trial was also done in Tzaneen on a 28-year old ZZ2 orchard containing 'Hass' trees on the one side and 'Fuerte' trees on the other side. Fruit set counts were made on 'Hass' trees 7 meters, 21 meters, 35 meters and 49 meters away from the 'Fuerte' block.

Fruit set counts were made in October 2016 and again in December 2016. A third count will be made in April/May when the harvest data will also be collected.

Volatile tests on 'Hass' and 'Fuerte' flowers were performed as a joint project with the Chemistry department of the University of Pretoria. Four replicates each of male and female stage flowers of 'Hass' and 'Fuerte' were collected in Petri dishes containing the same medium as for the in vitro tests.

Replicate sample sets (n=4) were sampled at midday (n=2) and at late afternoon (n=2). The headspace of avocado flowers placed in agar was sampled, using an inhouse developed sorptive method [1]. Two hundred millilitre of floral headspace was sampled onto MCTs at a rate of 3.33 ml/s for 60 s. The MCT containing the sorbed compounds was desorbed in a Thermal Desorber System (TDS) connected to a GC x GC-TOFMS.

Headspace sampling was performed by Egmont Rohwer, Yvette Naudé and Tyra Horngren. Analyses by thermal desorption with comprehensive gas chromatography time of flight mass spectrometry (TDS-GC x GC-TOFMS), data processing, interpretation and statistical analyses were performed by Dr. Yvette Naudé. Laboratory resources were provided by Prof. Egmont Rohwer (Department of Chemistry, University of Pretoria).

Statistical analysis

The data were analysed using the statistical program GenStat® (Payne, 2014).

A generalised linear model (GLM) analysis was applied to the in vitro pollination trial with a logarithmic link function, to test for differences between the three treatment effects. These treatment effects include HxH, HxZ and HxE. Means were compared with Fisher's protected least significant test at the 5% level.

REML, or linear mixed model, analysis was applied to the total number of fruit set. A pseudo split-plot analysis was used with treatments as whole plots and sides of a tree as split-plots. Means were compared with Fisher's protected least significant test at the 1% level as residuals after analysis were Normal, but with heterogeneous treatment variances.

RESULTS AND DISCUSSION

In vitro pollination trial

The results for the 2016-2017 season are presented in Figure 1 to 6.

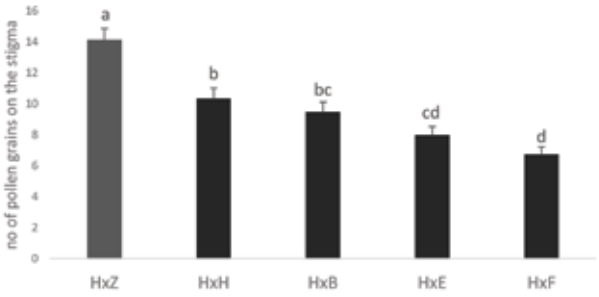

Figure 1. Average number of pollen grains on the stigma for in vitro pollinated 'Hass' x 'Zutano', 'Hass' x 'Bacon', 'Hass' x 'Ettinger', 'Hass' x 'Fuerte' and 'Hass' x 'Hass' flowers.

Figure 1 shows that 'Zutano' pollen outperformed the other polliniser cultivars with regards to pollen availability. When there is more pollen available, there is a better chance for a bee transferring the pollen to a female pistil. There is also a better chance for fertilisation to occur when there is a higher rate of pollination that could lead to better fruit set.

According to Figure 3, 'Zutano' had the highest number of pollen tubes moving down the 'Hass' styles. This could indicate better receptiveness of the 'Hass' stigma for 'Zutano' pollen. A higher number of pollen tubes in the style, could lead to higher rate of competition between the pollen tubes. With higher competition, only the "strongest" pollen tubes will reach the ovary and possibly enter and fertilise the ovule. This could mean that fertilisation of a 'Hass'

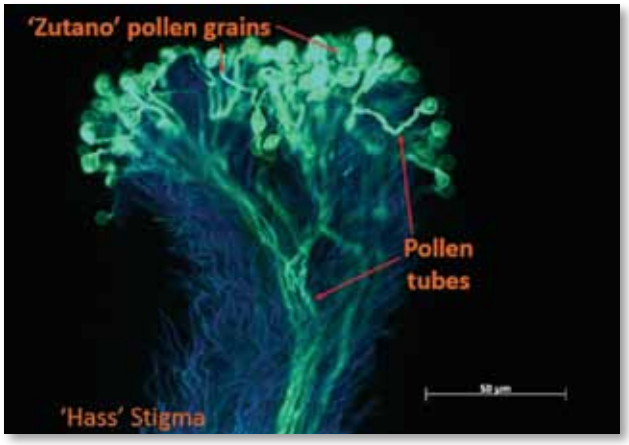

Figure 2. Illustration of the number of 'Zutano' pollen grains germinating on a 'Hass' stigma.

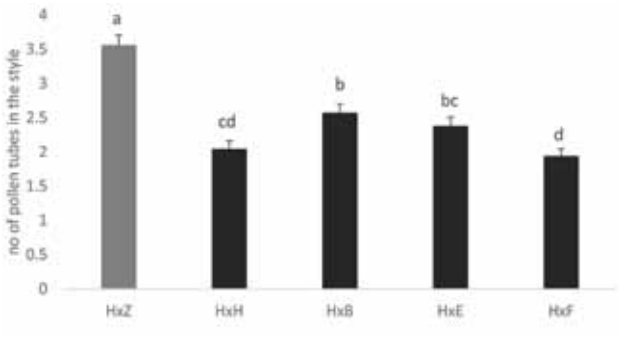

Figure 3. Average number of pollen tubes in the style for in vitro pollinated 'Hass' x 'Zutano', 'Hass' x 'Bacon', 'Hass' x 'Ettinger', 'Hass' x 'Fuerte' and 'Hass' x 'Hass' flowers.

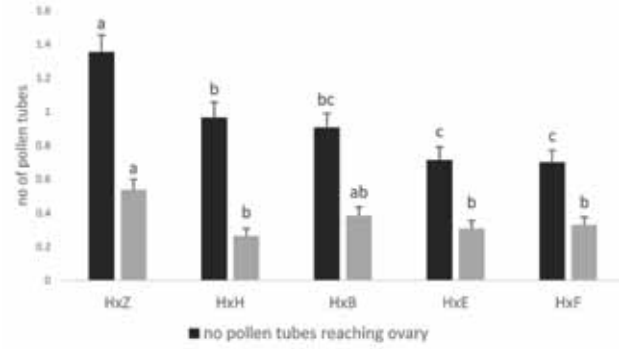

Figure 5. Average number of pollen tubes reaching the ovary and number of pollen tubes entering the ovule after in vitro pollination of 'Hass' x 'Zutano', 'Hass' x 'Bacon', 'Hass' x 'Ettinger', 'Hass' x 'Fuerte' and 'Hass' x 'Hass' flowers.

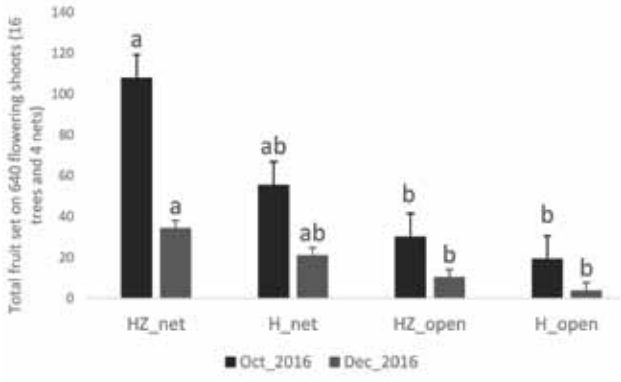

Figure 7. Average fruit set count taken in October 2016 and December 2016 for 640 flowering shoots in four different treatments.

ovule with 'Zutano' pollen could lead to a fruit that is less prone to abscission due to its vigour.

As shown in Figure 5, 'Zutano' pollen tubes were more successful in reaching the ovary. This emphasises our hypothesis that the stronger the competition in the style, the larger the chance of fertilisation due to the vigour of the pollen tube. This could mean that 'Zutano' might be considered as a polliniser for 'Hass'. 'Bacon' can also be considered while 'Fuerte' and 'Ettinger' pollen did not perform better than 'Hass' pollen based on pollen tube performance.

Figure 4. Illustration of pollen tubes moving down the 'Hass' style.

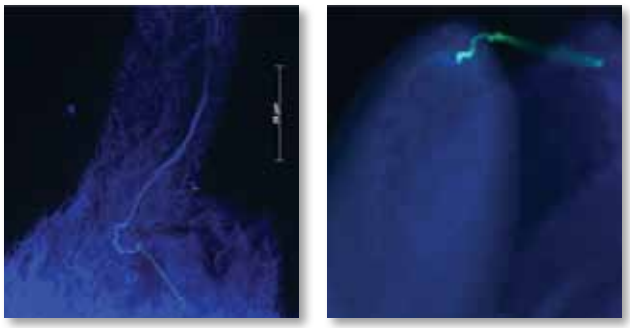

Figure 6. Illustration of a pollen tube that reached the ovary and a pollen tube entering the ovule.

Field trial

The results for the 2016-2017 season are given in Figure 7 to 13.

According to Figure 7, fruit set on 'Hass' trees in the nets was higher than on the open trees. This is a clear indication of the importance of bees for pollination and fruit set. This can also be seen in Figure 8 where trees in nets with bees had more fruit than the nets without bees. 'Zutano' as a polliniser gave a slightly higher fruit set, but there were almost no significant difference between the 'Hass' x 'Zutano' and the 'Hass' x 'Hass' treatment.

After the first count it was found that fruit set in the 'Hass' row closest to the 'Fuerte' block was high, where many small fruit were set per flowering shoot. The fruit set count dropped as the distance increased further away from the 'Fuerte' block. After the second count, however, most of the fruit dropped and there were no significant differences in the number of counted fruit at the different distances away from the 'Fuerte' block. The most fruit per flowering shoot were two to four, irrespective of the distance from the polliniser. Fruit drop between October and December was again very high.

Pollen traps

Pollen kernels found in the pollen trap are shown in Figure 10. About 60% of the kernels consisted purely

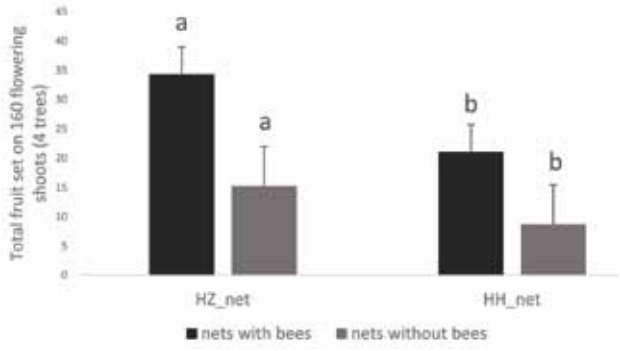

Figure 8. Average fruit set count for 160 flowering shoots in nets with bees and nets without bees.

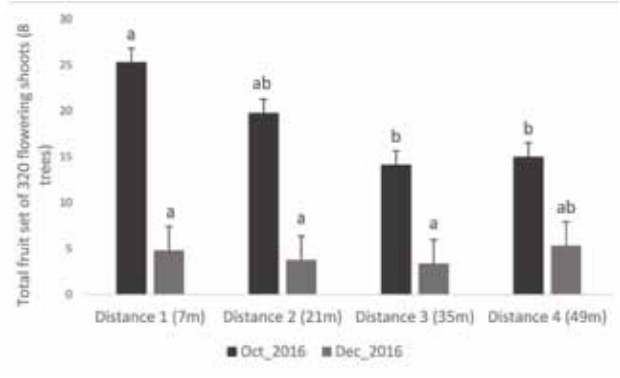

Figure 9. Average fruit set counts on 'Hass' trees: 40 flowering shoots per tree for eight trees per distance for the distances 7 m, 21 m, 35 m and 49 m from pollinizer ('Fuerte') block.

of macadamia pollen while only 40% were avocado pollen. These specific macadamia trees were three kilometres away from the pollen trap, which tells us that the bees travel quite far to collect the pollen that they prefer.

Volatiles

The result of the volatile analysis is illustrated in Figure 13. 'Hass' and 'Fuerte' had very different chemical profiles. There was also a significant difference between the chemical profile of the 'Hass' female and 'Fuerte' male flowers. 'Fuerte' flowers contained higher amounts of Monoterpenes which have herbal, spicy, camphor, peppery and pine scents. It was also

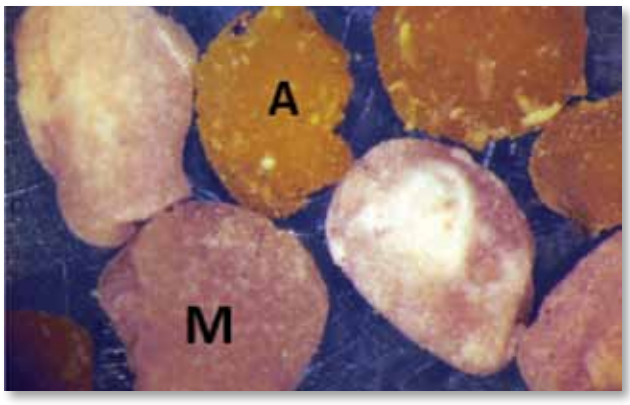

Figure 10. Pollen found in pollen trap: M – macadamia pollen (white), A – avocado pollen (yellow).

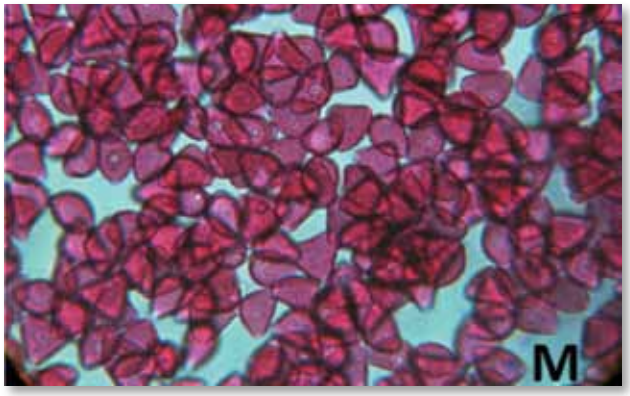

Figure 11. Macadamia pollen from kernel M in Figure 10.

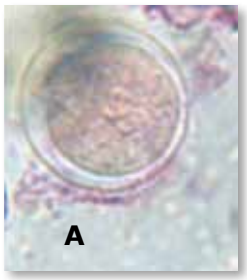

Figure 12. Avocado pollen from kernel A in Figure 10.

high in Estragole with scents of anise and liquorice. 'Hass' flowers contained high amount of Longifolene, Cis, trans-α-Farnesene and ester compounds with sweet, floral, fruity and rose scents. There is a clear contrast between the pungent spicy scents of 'Fuerte' and the sweet scents of 'Hass'.

DISCUSSION

Due to un-synchronised flowering between 'Hass' and 'Zutano' during the 2015-2016 season, causing a lack of 'Zutano' pollen during the flowering of the 'Hass'trees, the results for both the in vitro and field experiments were not very clear. According to 2016-2017 in vitro test results, however, 'Zutano' pollen outperformed 'Bacon', 'Fuerte', 'Ettinger' and 'Hass' pollen with regards to pollen availability, pollen tube movement down the style and pollen tube reaching and entering the ovary. 'Zutano' and 'Bacon' pollen tubes seem to have a better chance of entering the ovule followed by fertilisation. 'Zutano' pollen might produce more competitive pollen tubes and sperm cells that would result in stronger fruit. This agrees with the findings of Degani *et al.* (1998) that fruit derived from cross-pollination were less subjected to abscission. 'Bacon' pollen performed slightly better than 'Fuerte', 'Hass' and 'Ettinger' pollen.

The 2016-2017 field trial study showed that at the first count, better fruit set was obtained in the nets containing both 'Hass' and 'Zutano' trees and bees compared to trees in nets containing only 'Hass' trees with bees. Arpaia and Hofshi (2004) also emphasised the importance of bees as pollinators for avocado. At the second count, however, and due to heavy fruit drop, there was not a significant difference in fruit retention between the two treatments. Fruit drop is a major concern for trying to increase fruit production in 'Hass'. It could be argued that instead of incorporating a polliniser to increase fruit set in 'Hass', a polliniser needs

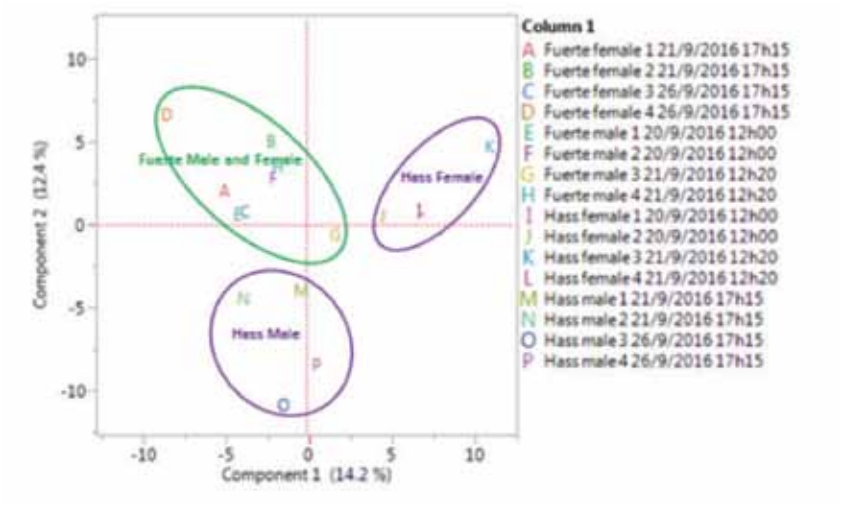

Figure 13. Score plot of a principle component analysis of the chemical compounds detected in the headspace of 'Fuerte' and 'Hass' male and female flowers.

to be included that will reduce the number of fruit drop of the tree. Cross pollinated fruit should, theoretically, be less prone to abscise (Degani *et al.,* 1998). The question still remains, which polliniser to use and whether it will actually improve yield.

Bees do play a major role in pollination and fruit set in avocado. Based on the present results it could be stated that instead of focusing on polliniser trees to increase fruit set, it is more important to introduce hives into the orchard and to make sure that the bees in the hives are healthy and active. Garner *et al.* (2008) found that outcrossing is not the primary factor affecting fruit persistence and ultimate yield. Avocado flowers are not as attractive to bees, although bees still do visit avocado flowers to collect pollen and nectar thus promoting pollination. Bees will travel far to collect pollen that they prefer as was found in the study with pollen traps.

Afik *et al.* (2006) studied the nectar properties of avocado flowers and their perception by bees. They found that avocado nectar and honey were rich in a wide range of minerals including phosphorus and potassium, both of which are repellent to bees. They have not looked at the volatiles in the flowers. In this study, we found that avocado flowers produce a large number of different volatiles which differed between the male and female phases of the flowers and also between the two cultivars that were studied.

CONCLUSION

Although it was found in this study that 'Zutano' pollen outperformed the pollen from other studied cultivars and that that better fruit set was obtained in the nets containing 'Zutano' trees as pollinisers for 'Hass', the results are still unclear. It is therefore necessary to repeat all the experiments for another season to get more clarity on the problem. When choosing a polliniser to be interplanted in a 'Hass' orchard, it is important to make sure that their flowering times coincide with 'Hass' flowering to obtain optimal cross-pollination.

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