Pollination: In vitro, in nets and open trees; avocado flower characteristics and bee activity

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ABSTRACT
The first study entailed an in-vitro pollination study and field trials. The field trials included fruit set determination on a) caged trees with bees ('Hass' x 'Hass' and 'Hass' x 'Zutano'), b) open pollinated trees, and on c) caged trees without bees ('Hass' x 'Hass' and 'Hass' x 'Zutano'). Fruit set and harvest counts were taken between October and March for three consecutive seasons. In vitro pollination studies were also done from July to September of the above three seasons.

Fruit drop occurred dramatically from October to December and then declined noticeably up until harvest time. A clear relation between fruit number per 'Hass' tree and fruit size was found – more fruit – smaller fruit. 'Zutano' as a pollinizer for 'Hass', incorporating bees, slightly increased fruit set and yield on 'Hass'. It is still questionable if this small difference justifies interplanting 'Zutano' with 'Hass' to increase fruit set, although flowering of this B-type cultivar's 'Zutano' did not always synchronize with 'Hass' flowering. Therefore, the demand for other or newer cultivar pollinizers is essential.

In vitro results are based on the number of pollen tubes reaching the ovary and entering the ovule. In 2014 more 'Zutano' pollen tubes reached the ovary. In 2015 'Ettinger' pollen tubes were more successful in entering the 'Hass' ovules. In 2016 'Zutano' and 'Bacon' pollen tubes were more successful in reaching the ovary and entering the ovules.

The second study in 2017 and 2018 focused on the facets of volatile exudation and flower characteristics, which may limit bee activity, pollination and subsequent yield. Volatiles contribute to the characteristic scent of the flower. Volatile profiles of a range of avocado cultivars were done. Using thermal desorption with comprehensive gas chromatography time of flight mass spectrometry (GC x GC-TOFMS), it was found that volatiles exuded by a flower differed based on both the sexual phase and the cultivar. Some of the major volatiles detected were Limonene, Pinene and Eucalyptol, among others. Bees did not clearly respond when exposed to specific volatiles in an olfactometer, but more research is required. Mineral element constituents of avocado flowers may influence bee behaviour, however, more research is necessary to confirm results obtained. Inner floral nectaries produce nectar during the female phase, while outer nectaries function during the male phase. Male phase flowers therefore supply both nectar and pollen as rewards for visiting bees and seem to be more attractive to bees than female phase flowers. Extreme environmental factors such as temperature will influence pollen viability.

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 pollinizer. 'Hass' being an A-type cultivar, the pollinizer must be a B-type cultivar.

Clark and Clark (1923, 1926) reported on the benefits of cross-pollination against self-pollination for increasing fruit set and yield. Since then many attempts have been made to "prove" that avocados are out-breeders and that pollinizers are essential for good fruit set.

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 pollinizers 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 pollinizer 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.

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 and avocado flowers also do not open under such conditions.

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 pollinizers for 'Hass' and the role of bees in pollination of avocados under South African conditions.

The first study ran for three seasons (2015-2016; 2016-2017; 2017-2018). The results of the 2015-2016 and 2016-2017 seasons were presented in the 2016 and 2017 SAAGA yearbooks. The 2017 in vitro results were reported in the 2018 SAAGA yearbook and for this report the results presented in this report cover the consecutive seasons combined. The second study is preliminary, but indicates that flower characteristics influence bee activity.

MATERIALS AND METHODS

The first study was done in orchards of ZZ2. It comprised of two parts, namely (A) in vitro trials and (B) field trials. Data trees were subjected to the same standard cultural practices as the remaining orchard trees.

(A) In vitro

Standard practices used for the in vitro tests

A-cultivar flowers, in early anthesis (opening in the female phase), were collected. The flowers were placed in Petri dishes containing a gel made up of 15% agar, 10% sucrose and 0.05% boric acid and allowed to open. B-type cultivar flowers that were open in the female phase were collected the previous afternoon and placed in Petri dishes containing the same medium. The B-type cultivar 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 A-type cultivar flowers. The A-type cultivar flowers were then pollinated with the B-type cultivar pollen. The A-type cultivar flowers were then incubated at 25ºC for 24 hours and then fixed in a Carnoy solution (ethanol, chloroform and acetic acid in the ratio of 60:30:10). Fixation of the flowers from each Petri dish were done in separate glass test tubes and marked according to treatment applied and the date of pollination.

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 ovule.

(B) Field trials

The trial was conducted in Tzaneen on a 'Hass' orchard inter planted with 'Zutano' trees that belong 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. In 2016 four trees per treatment were docketed and ten flowering shoots per tree on four sides of the tree (N, E, S and W) were marked for counting fruit set. In 2014 and 2015 two trees per treatment were tagged and ten shoots per tree. In 2017 two trees per treatment were tagged and fifteen shoots per tree on the four sides of the tree (N, E, S and W). Two nets were set up with no bees inside, one net with 'Hass' x 'Hass' and the other with 'Hass' x 'Zutano'. Fruit set counts were conducted between October to February 2015, 2016, 2017 and 2018. Harvest counts were done in March 2016, 2017 and 2018.

Statistical analysis

The data were analyzed using the statistical programme GenStat® (Payne, 2014).

A generalized linear model (GLM) analysis was applied to the in vitro pollination trial with a logarithmic link function, to test for differences between the treatment effects. 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

(A) In vitro pollination trial

Success rate of pollen tubes reaching and ultimately entering the ovary were the most important criterion for determining effective pollination. The entrance of the pollen tube into the ovule is an important criterion and was also used by Sedgley (1997a) as a measure for effective pollination.

The results for the 2014-2015 season are given in Figure 1. As depicted in Figure 1, 'Zutano' pollen germinated better on the 'Hass' pistil compared to 'Hass' pollen. 'Zutano' pollen tubes were also more successful in moving down the 'Hass' style compared to 'Hass' pollen tubes. However, there was no significant difference between the numbers of 'Hass' or 'Zutano' pollen tubes that reached the 'Hass' ovary.

The results for the 2015-2016 season are given in Figure 2. It is clear in Figure 2 that there was no difference in the number of pollen tubes that reached the 'Hass' ovary whether or not 'Hass' was pollinated with 'Ettinger', 'Zutano' or 'Hass' pollen. However, 'Ettinger' pollen tubes were more successful in entering the 'Hass' ovules. Guil and Gaziet (1992) also found that better yields were obtained in 'Hass' orchards planted next to 'Ettinger'. Degani *et al*. (2004) also regarded 'Ettinger' as a potent pollinizer for 'Hass'.

The results for the 2016-2017 season are given in Figure 3. As shown in Figure 3, 'Zutano' pollen tubes were more successful in reaching the ovary and entering the ovule.

(B) Field trial

The results for the 2015-2016 season are given in Figure 4. According to Figure 4, the initial fruit set count taken in October 2015 showed insignificant difference between 'Hass' trees in nets with or without 'Zutano'. Rows with only 'Hass' trees in open rows had the lowest initial fruit set compared to the other treatments. Trees in nets had slightly higher fruit set than the open trees.

From October 2015 to December 2015, fruit drop occurred quite dramatically. The results for the

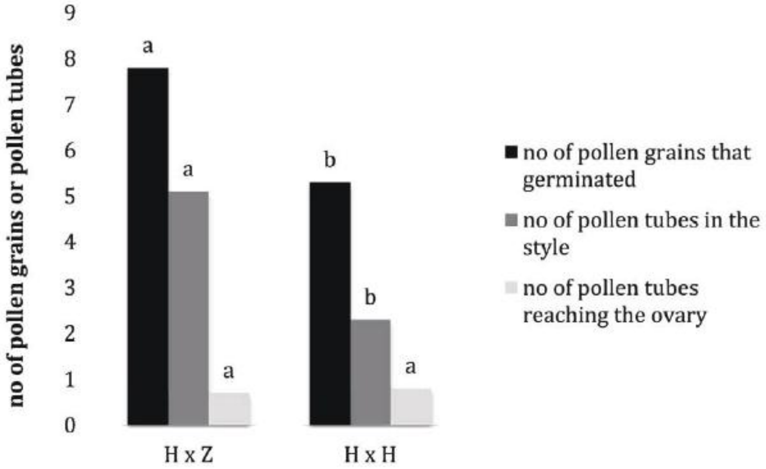

Figure 1. Pollen performance for in vitro pollinated 'Hass' x 'Zutano' and 'Hass' x 'Hass' flowers during the 2014-2015 season.

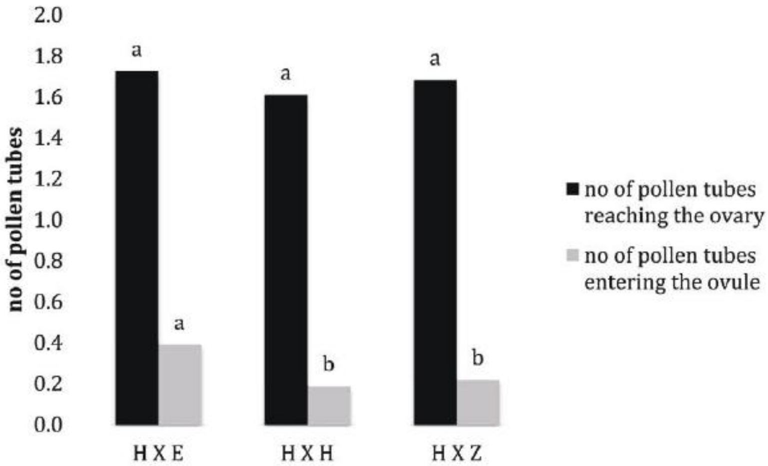

Figure 2. Pollen performance for in vitro pollinated ('Hass' x 'Zutano'), ('Hass' x 'Ettinger') and ('Hass' x 'Hass') flowers during the 2015-2016 season.

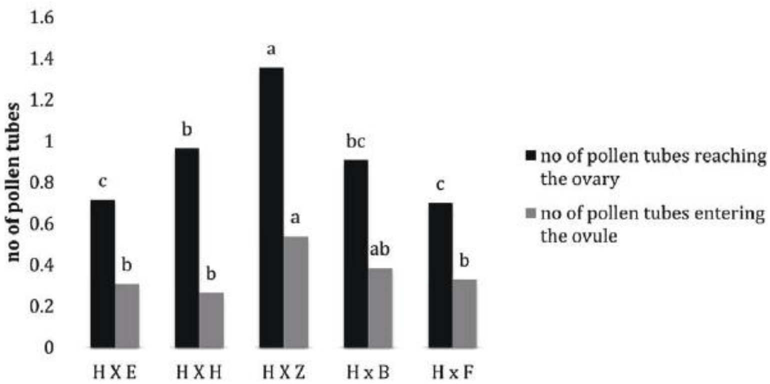

Figure 3. Pollen performance for in vitro pollinated ('Hass' x 'Zutano'), ('Hass' x 'Fuerte'), ('Hass' x 'Bacon'), ('Hass' x 'Ettinger') and ('Hass' x 'Hass') flowers during the 2016-2017 season.

December count showed no significant difference between 'Hass' trees in nets and open 'Hass' trees. The only slight difference was between open 'Hass' trees interplanted with 'Zutano' as a pollinizer and open trees consisting of pure 'Hass' stands. Open 'Hass' trees planted with 'Zutano' had a slightly higher fruit set compared to pure 'Hass' stands. In the nets however, 'Hass' trees inter-planted with 'Zutano' gave the same fruit set as 'Hass' only nets.

During the last count in February 2016, there was insignificant difference between open trees interplanted with 'Zutano' and open trees planted in pure 'Hass' rows. There were, however, a difference between 'Hass' and 'Zutano' in nets and only 'Hass' trees in nets. From December 2015 to February 2016 fruit drop still occurred, but to a lesser extent.

The results for the 2016-2017 season are given in Figure 5. According to Figure 5, fruit set on 'Hass' trees in the nets was higher than on the open trees even after fruit abscission took place from October to December. There were no significant difference between 'Hass' trees in nets with 'Zutano' as a pollinizer and nets without 'Zutano' as a pollinizer. No significant difference between open rows with only 'Hass' trees and open rows with 'Zutano' planted between the 'Hass' trees were found. Trees that initially set more fruit tend to have more fruit that dropped, as can be seen in the 'Hass' x 'Zutano' nets (Fig. 5). Zutano did not have an effect on fruit set in 'Hass' according to fruit set counts taken in the 2016-2017 season.

The results for the 2017-2018 season are given in Figure 6. According to Figure 6, there was no significant difference between 'Hass' trees in nets and open 'Hass' trees. Robbertse (1998) also found that there was no difference between caged 'Hass' x 'Hass' and between open 'Hass' x 'Hass' trees. However, 'Hass' trees in the 'Hass' x 'Zutano' nets performed slightly better than 'Hass' trees in the 'Hass' x 'Hass' nets (Fig. 6).

As seen in Figure 6, 'Hass' trees in open 'Hass' x 'Zutano' rows had

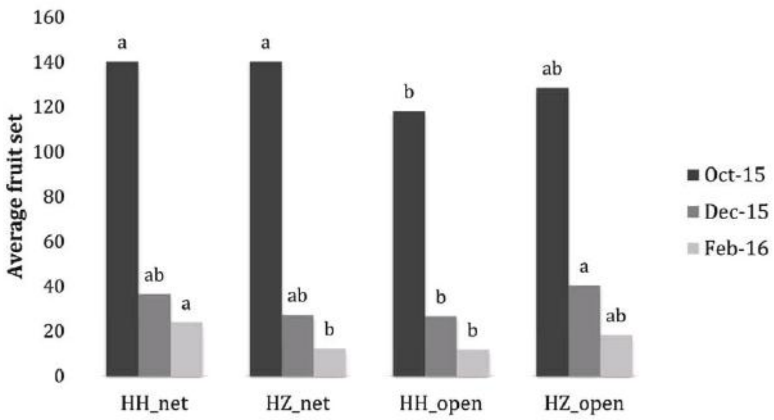

Figure 4. Fruit set counts on 'Hass' trees taken in October 2015, December 2015 and February 2016 for four different treatments with bees i.e. 'Hass' x 'Hass' in nets, 'Hass' x 'Zutano' in nets, 'Hass' x 'Hass' in open rows and 'Hass' x 'Zutano' in open rows.

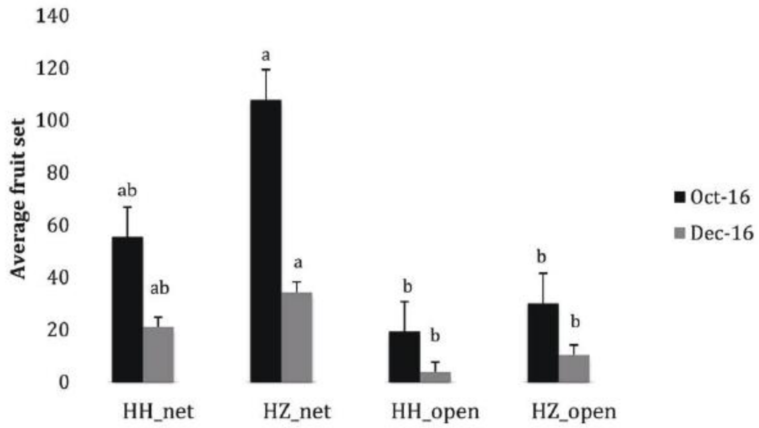

Figure 5. Fruit set counts on 'Hass' trees taken in October 2016 and December 2016 for four different treatments i.e. 'Hass' x 'Hass' in nets, 'Hass' x 'Zutano' in nets, 'Hass' x 'Hass' in open rows and 'Hass' x 'Zutano' in open rows.

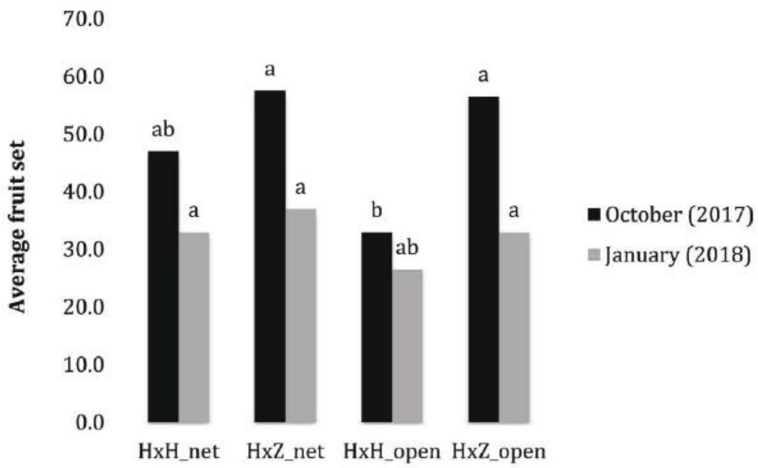

Figure 6. Fruit set counts on 'Hass' trees taken in October 2017 and January 2018 for four different treatments i.e. 'Hass' x 'Hass' in nets, 'Hass' x 'Zutano' in nets, 'Hass' x 'Hass' in open rows and 'Hass' x 'Zutano' in open rows.

significantly higher fruit set compared to 'Hass' trees in open 'Hass' x 'Hass' rows in October 2017. After fruit abscission, 'Hass' trees in open 'Hass' x 'Zutano' rows had only a marginal higher fruit set. It is important to take note that pollinizer 'Zutano' did slightly increase fruit set on 'Hass' trees, in spite of the fact that 'Zutano' flowering did not always synchronize with 'Hass' flowering which could have influenced outcrossing.

Results for the harvest count taken in March 2017 and March 2018 are given in Figure 7. In March 2017 during harvesting, fruit counts inside the nets were much higher compared to fruit counts on the open trees. According to Figure 7A, there were slight differences between the number of fruit on 'Hass' trees planted alone and between 'Hass' trees interplanted with 'Zutano' as a pollinizer. The open 'Hass' trees interplanted with 'Zutano' gave, on average, 56 (about 19%) more fruit than the open 'Hass' trees planted alone (Fig. 7A). In the nets, the 'Hass' trees planted with 'Zutano' gave 21 more fruit on average compared to nets with only 'Hass' trees inside. Therefore, incorporating 'Zutano' with 'Hass' did increase fruit set in 'Hass' to a small extent.

In March 2018, the open 'Hass' trees interplanted with 'Zutano' gave, on average, 106 (about 27%) more fruit than the open 'Hass' trees planted alone (Fig. 7B). This was quite a big difference and did indicate that 'Zutano' did increase fruit set in 'Hass' in this season. In the nets, the 'Hass' trees planted with 'Zutano' gave 14 more fruit on average compared to nets with only 'Hass' trees inside (Fig. 7B). The difference was not that major when comparing fruit set inside the nets (Fig. 7B). Thus, when bees were concentrated in the nets, they worked with what they had available. On the open trees where more bees and 'Zutano' pollen was available, 'Hass' trees interplanted with 'Zutano' gave a 27% increase in yield. Schaffer (2013) mentioned that substantial yields could still be attained with self-pollination, however, today yield expectations are increasing, which makes the

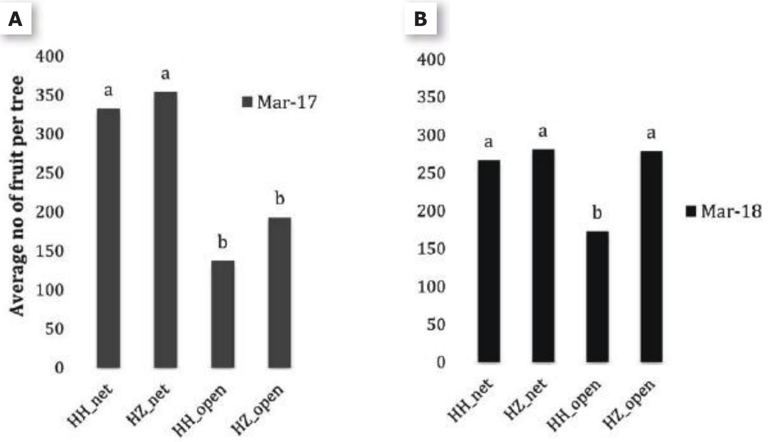

Figure 7 (A and B). Harvest count on 'Hass' trees (average number of fruit per tree) taken in March 2017 and in March 2018 for four different treatments i.e. 'Hass' x 'Hass' in nets, 'Hass' x 'Zutano' in nets, 'Hass' x 'Hass' in open rows and 'Hass' x 'Zutano' in open rows.

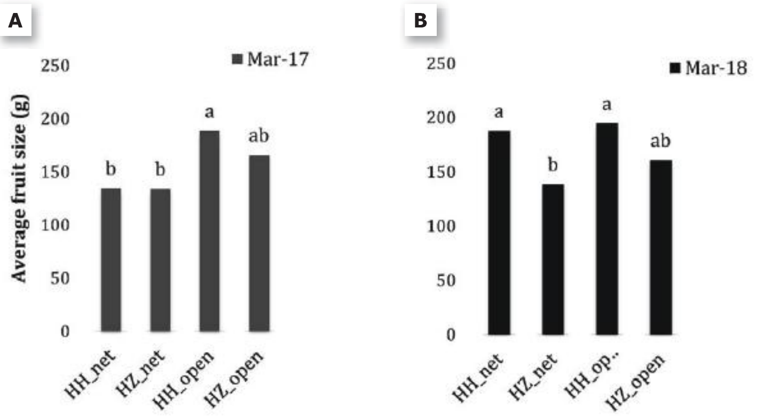

Figure 8 (A and B). Average fruit size on 'Hass' trees taken after harvesting in March 2017 and in March 2018 for four different treatments i.e. 'Hass' x 'Hass' in nets, 'Hass' x 'Zutano' in nets, 'Hass' x 'Hass' in open rows and 'Hass' x 'Zutano' in open rows.

demand for an efficient pollinizer essential. Garner *et al.* (2008) found that outcrossing is not the primary factor affecting fruit persistence and ultimate yield. In our case, we found that 'Zutano' flowering period was not well synchronized with 'Hass' flowering period, which made outcrossing for bees difficult and in spite of that, fruit yield on 'Hass' trees still increased where 'Zutano' pollen was available.

Robbertse (1998) also found that nets without bees had very low yields. Bender (2014) mentioned that it was noticed by commercial farmers that 'Hass' trees planted near 'Zutano' had a higher yield and that the effect is greater when the 'Hass' tree is only one tree away from the 'Zutano'.

Comparing Figures 7 and 8, it can be seen thet there is a clear relationship between fruit number on a tree and fruit size. Where there are more fruit on the tree, the fruit are smaller, and with fewer fruit on the tree, the fruit are bigger.

Depending on the demand, it seems possible that fruit size can be manipulated by controlling the number of fruit per tree.

CONCLUSION

According to in vitro results, 'Ettinger' and/or 'Zutano' could be recommended as pollinizers for 'Hass'. Field trials showed that 'Zutano' has a significant impact on 'Hass' at the initial fruit set, but after fruit abscission the differences become very small. Fruit abscission is high from October to December and decline from December to harvest time in March. Trees that develop many fruit tend to have smaller fruit and trees that develop less fruit tend to have larger fruit. By changing the fertiliser and irrigation programme, trees might hold/keep their fruit yield and size until harvesting. The final assumption is that 'Zutano' could increase fruit set in Hass, only when sufficient numbers of bees are available and synchronized flowering between the cultivars during the season.

FUTURE RESEARCH ON FLOWER CHARCTERISTIC AND BEE ACTIVITY

Preliminary observations on bee activity amongst different avocado cultivars showed that flower visits were generally highest when the flowers on the trees were in the functional male phase. Additionally, more bees appeared to visit the flowers of the B-type cultivars 'Fuerte' and 'Zutano'. It is therefore reasonable to suggest that bees have a preference for specific flower phase and cultivar. With regards to why bees prefer male flowers in particular, may be due to the higher "energetic reward" that these flowers offer compared to the female flowers. When in the male phase, flowers offer not only a source of sugar and amino acid-rich nectar, but protein-rich pollen as well, the latter of which is not available in the female stage flowers (Carter *et al*., 2006).

After determining that honeybees do prefer the flowers of certain cultivars, the reason for this needed to be answered. According to Afik *et al*. (2006), the volatiles from the flowers may influence bee activity, which our findings above support. Therefore we can reason that the differences in bee activity amongst the different cultivars and flower phases may be explained by the different volatiles exuded by the flowers. It is the volatile emitted by the flower that acts as the primary attractant for the honeybees.

In order to determine which volatiles were exuded from the flowers, headspace sampling was conducted at the orchards of ZZ2 farms. Subsequently, the samples were analyzed by thermal desorption with comprehensive gas chromatography time of flight mass spectrometry (TDS-GC x GC-TOFMS) with the help of the Department of Chemistry at the University of Pretoria (Naudé and Rohwer, 2013). This process was conducted first in 2017 and repeated in 2018, with the later study including a wider variety of cultivars.

The results showed that the volatile profiles differed on the basis of both sex and cultivar of the flower. The volatiles that were ubiquitously expressed amongst all the flowers were Limonene, Eucalyptol and Pinene. Limonene was expressed to a greater extent within the male flowers. Limonene is also found in high quantities in citrus flowers. Afik *et al*. (2006, 2007) found that honeybees favour the nectar of citrus flowers above that of the avocado flowers. Therefore, the volatile Limonene may aid in attracting honeybees, and flowers high in this compound may be preferred.

The volatiles that are unique to certain flower phase and cultivar is also of interest because these compounds may be the reason for higher preference being shown to particular flowers. Linalool is relatively unique to the female 'Hass' flowers, and α-Cubebene is expressed in 'Fuerte' and 'Ettinger' only. Limited studies on the effect of these volatiles on bee behaviour have been done. This study therefore needed to conduct preliminary experiments to determine which volatiles were favoured by honeybees. This was accomplished with the use of an olfactometer, which tests a bee's preference for a particular scent. Results showed that the volatiles Citral and Pinene were attractive to honeybees. However, these findings need to be repeated in order to gain a better understanding of what volatiles honeybees prefer.

The final aspect relating to volatile emission was determining whether or not factors such as genetics or environmental conditions can influence volatile exudation.

'Hass' and 'Fuerte' flowers from the orchards at the ZZ2 farms and the University of Pretoria were analyzed for their elemental composition. Potassium was found to be higher within the 'Fuerte' flowers (11647 mg/kg) compared to the 'Hass' flowers (10984 mg/kg). In terms of Phosphorous, 'Hass' flowers (3160 mg/kg) had higher concentrations than 'Fuerte' flowers (2961 mg/kg). According to Afik *et al*. (2014), the mineral constituents of the avocado flowers, in particular Potassium and Phosphorous, are repellant to flower visitors. Environmental factors such as temperature, and cultural practices such as fertiliser applications and irrigation schedules can influence the elemental composition of the flowers. Further studies are required to determine whether or not the aforementioned concentrations will ultimately deter honeybees and how this can be mitigated.

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