LOW YIELDS IN AVOCADOS (*PERSEA AMERICANA*): IS POOR POLLINATION A CONTRIBUTING FACTOR AND CAN IT BE ENHANCED?

by

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DECLARATION

I hereby certify that this dissertation is my own work, except where duly acknowledged. I also certify that no plagiarism was committed in writing this dissertation.

Signed ______________________

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In his 1624 prose, John Donne penned ‘no man is an island’. As I come closer to fulfilling the needs of my MSc(Agric) in Horticulture, the importance of relying on the help of others is a prominent lesson I will walk away with. It is with this thought that I would like to express my thanks to the following institutions and individuals:

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RESEARCH OUTPUTS

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Du Toit ES, Stanton MS. 2020. Low yields in avocados: is poor pollination a contributing factor and how can it be enhanced? South African Avocado Growers Association Annual Conference, Tzaneen, South Africa. Presented by MS Stanton on behalf of the research team.

ABSTRACT

The avocado (*Persea americana* Mill*.)* is a widely demanded fruit worldwide. While its palatable taste is one of the reasons it is favoured so, the fleshy interior contains a wide range of vitamins, minerals and other health boosting components. Not only this, but the pulp and seed are also valued in the oil-processing, pharmaceutical and cosmetic industries. The demand for avocados is thus extensive, and production of the fruit has increased five-fold over the last 35 years to meet this demand.

Despite centuries of selection and hybridisation, avocado trees are still characterised by a low-yielding nature. The oil-rich flesh, alternate bearing, competition between vegetative and reproductive flushes, and disease proliferation all contribute to low yields in commercial orchards. There is another factor, widely investigated and yet poorly understood, that further reduces yields in avocados: sub-optimal pollination.

When cultivated in its native region of central Mexico, many endemic insect species facilitate pollination between the synchronous, dichogamous avocado flowers. As avocado cultivation expands outside its native region, honey bee hives are installed in orchards to improve pollination. However, more attractive blooms of other angiosperms (citrus, for example) can cause a mass honey bee exodus out of the avocado orchards. This results in reduced pollination and consequently, fruit set and yield are seriously compromised. This dissertation aimed to explore the notion of low pollination rates in avocados. Factors inherent to the avocado's physiology, such as floral morphology and scent were considered in conjunction with external influences, including cold stress and nutrient availability. Furthermore, practical management strategies to combat low pollination in avocado orchards were surveyed.

Simple methods were used to manually count honey bee activity amongst the canopies of a range of cultivars. Honey bees appeared to favour 'B-type' cultivars, such as *P. americana* 'Fuerte' and 'Zutano'. Visitations to flowers open in the female and male phases showed honey bees are more partial to functionally male flowers. This may be due to the dual reward of pollen in addition to nectar, as well as the grander floral display when the flowers are open in the male phase.

Floral volatile exudation also plays an important role in pollinator attraction. Solid-phase microextraction (SPME) and thermal desorption with comprehensive gas chromatography with time-of-flight mass spectrometry (GC x GC-TOFMS) showed that depending on the avocado flowers' cultivar and sexual phase, distinct floral volatiles are released. Flowers sampled in the actively male phase generally released a higher concentration of volatiles, which may contribute to them being preferred by honey bees. In addition to this, linalool (a volatile known for its honey bee attracting properties) was detected in very little amounts in the avocado flowers. This may explain why flowers that exude higher concentrations of linalool (like citrus, for example) are more attractive to honey bees.

With these findings in mind, practical methods to encourage honey bee foraging amongst avocado flowers were investigated. Flowering lavender (*Lavandula stoechas*) and lemongrass (*Cymbopogon citratus*) essential oils were used to attract honey bees to an avocado orchard in bloom. Honey bee activity was then monitored over a period of time during the flowering season. The lemongrass lures proved effective in increasing honey bee activity, while the lavender plants increased fruit set in trees experiencing an 'off' production year. This highlights the importance of promoting pollination in orchards suffering from reduced flowering sites during 'off' years. Therefore, this study pioneered possible solutions that can be introduced into commercial avocado orchards to mitigate the problem of low pollination by honey bee vectors. However, these methods will not be effective if the pollen itself is compromised.

As avocado production expands into sub-tropical and temperate regions the trees experience more cold stress. A novel pollen counting methodology was used in conjunction with light microscopy to evaluate the effects of cold temperature on pollen development at flower anthesis. There were many problems observed with the anthers and pollen, including absent and empty compartments, and malformed pollen grains. *P. americana* 'Fuerte' anthers appeared to be more adept at maintaining pollen health during cold periods. The number of pollen counted in flowers sampled during the actively female and male phases was also different, suggesting the overnight closure between the phases is sensitive to cold temperature. Considering the avocado trees used in this study are regularly exposed to temperatures below the minimum required for successful meiosis in tropical plants, it is reasonable to suggest these trees will suffer from inadequate pollen production and development. These findings highlight the need for further investigation into how pollen development in avocado trees is affected by cold stress, especially when the trees are cultivated in cooler climates.

Lastly, nutrient analysis of floral material (a factor poorly understood in its relation to avocado production) was analysed using nitric acid (HNO_3) digestions and inductively coupled plasma (ICP) analytical methods. The results showed how flowers accumulate varying amounts of potassium, boron, phosphorous, and calcium based on their cultivar, sexual phase, and sampling location. Furthermore, there was a differential accumulation of nutrients between the reproductive and vegetative growth points. These findings show how the critical reproductive sites have a greater ‘pull’ for nutrients. This was the first known attempt to investigate avocado flower nutrition and consider the impact thereof on pollination. While the direct role of many of these nutrients on pollen development in avocados is not well known, other reports claim these nutrients can protect against the effects of cold stress, improve overall pollen health, and even repel honey bees when present in high concentrations.

Ultimately, this dissertation highlighted the importance of applying holistic investigative methods to explore the notion of low pollination in avocados. This study found that many factors, including honey bee preferences for certain flowers based on sexual phase and scent, in addition to external factors such as cold stress and nutrient availability, may play interconnected roles that limit pollination and fruit set in avocados. Furthermore, the possibility of improving pollination in avocado orchards by using attractive measures was highlighted. Therefore, this study was successful in evaluating the causes for sub-optimal pollination in avocados and potential solutions thereof. However, as is the case with scientific investigation, this study also uncovered additional facets that require deeper understanding. Future research should thus focus on long-term studies in commercial orchards to evaluate the roles played by alternate bearing, drought, and insecticide sprays on pollinator activity. In addition, the influence of cold stress and nutrient deficiencies on specific physiological processes such as pollen production must be investigated. This will also require the development of floral nutrient statuses, which can be achieved during long-term studies.

Keywords: cross-pollination, Fuerte, Hass, gas chromatography, volatiles, essential oils, insect lures, light microscopy, cold stress, pollen, nutrient stress, fruit

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LIST OF ABBREVIATIONS

%	percent
º C	degrees Celsius
CV	canonical variates
CVA	canonical variate analysis
dH_20	distilled water
E	east
GA	gluteraldehyde
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GC-O	gas chromatography-olfactometry
GC x TOFMS	comprehensive gas chromatography with time-of-flight mass spectrometry
ha	hectare
HIV	human immunodeficiency virus
ICP	inductively coupled plasma
HS-SPME	headspace-solid phase microextraction
kgs	kilograms
kts	kilotons
lbs	pounds
LDL	low density lipoprotein
LM	light microscopy
M	molar
m	meter
mm	millimeter
Mts	megatons
N	north
n	replication
PAM	primary axis meristem
PAR	photosynthetically active radiation
PVA	polyvinyl alcohol
RH	relative humidity
RPA	relative peak association

S	south
SAM	secondary axis meristem
SPME	solid phase microextraction
TDS	thermal desorber system
USA	United States of America
v	volume
W	west
µm	micrometer

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GENERAL INTRODUCTION

The avocado (*Persea americana* Mill.) is a tropical tree belonging to the botanical family Lauraceae (Kostermans 1957). The avocado's popularity is not only owed to its taste and texture, but the varied health benefits that are associated with consumption of the fruit (Bergh 1992a, Wigg *et al*. 1996, Bouic 2002, Lottenberg 2002, Pahua-Ramos *et al*. 2014). Since its domestication in Mesoamaerica roughly 10 000 years ago (Smith 1966; Galindo-Tovar *et al*. 2008), worldwide avocado production totalled almost 6.5 million metric tonnes in 2018 (Shahbandeh 2020). Cultivation in areas external to the avocados region of origin began in the 18th century in Indonesia and South America (Silva and Ledesma 2014). Currently, Mexico is the largest producer of avocados, followed by the Dominican Republic and Peru (Statista 2019).

Despite centuries of selection and hybridisation, commercial avocado orchards are hindered by a low-yielding nature (Trochoulias and O'Neill 1976, Chen *et al*. 2009). Characteristics of the fruit itself, such as its high lipid content and large seed, reduce the inherent yield capabilities compared to other fruits like apples (USDA 2002, Wolstenholme 1986, Ranade and Thiagarajan 2015). However, many experts believe yields are further reduced by factors such as sub-optimal pollination, alternate bearing, competition between reproductive and vegetative shoots, and diseases such as *Phytophthora cinnamomi* (Whiley *et al*. 1986, Sedgley 1987, Ish-Am *et al*. 1999, Mickelbart *et al*. 2012). This study investigates the first of these factors and aims to use a holistic approach to examine the variables that contribute to low pollination rates. This study will also examine practical solutions to the problem.

Authorities on the subject of pollination in avocados base their argument for cross-pollination on the flowering nature of the avocado (Stout 1923, Clark 1924, Bergh 1968). As is the case with the dichogamous avocado flowers, there is a temporal separation of the active female and male phases. When the avocado flower first opens, the stigmas are pollen-receptive, and the anthers have yet to dehisce. The same flower then closes and reopens for a second time the following day, at which time the stigmas have browned and the anthers release pollen (Nirody 1922). The case for cross-pollination in avocados was strengthened by a 2009 study by Alcaraz and Hormaza in which hand-pollinated flowers set 15 % more fruit compared to open pollinated flowers.

When cultivated in its region of origin many native insects act as pollinators for avocado flowers (Nieto 1985, Ish-Am *et al*. 1999). However, trees located in foreign orchards cannot rely on these insects for pollination. To combat this problem commercial growers often install honey bee hives into their orchards (Bergh 1992b, Roubik 1998, Ish-Am and Lahav 2011). Despite these installations avocado orchards are still plagued by insufficient pollination rates (Ish-Am 2005). This may be due to inherent floral qualities, such as volatile exudation and flower structure, as well as external influences, such as cold and nutrient stress (Cheung and Stephenson 1993, Issarakraisila and Considine 1994, Dell and Huang 1997, Ashman 2000, Afik *et al*. 2006, Choudhurry *et al*. 2013).

This dissertation therefore aims to investigate whether or not pollination can be enhanced in avocado orchards by determining why bees do not favour the avocado trees, and how external factors such as cold stress and floral nutrient status impact pollen development. Furthermore, possible solutions to this problem will also be explored.

Therefore, the main objectives of this study were:

- To test whether or not honey bees exhibit a preference for certain avocado cultivars, thereby allowing commercial growers to potentially select more favourable cultivars.
- If it is found to be the case that there is a preference, to determine the volatile profile of avocado flowers belonging to different cultivars.
- To investigate the possibility of increasing pollinator activity in commercial avocado orchards using attractive measures.
- To determine the effects of cold temperature on pollen development in avocados cultivated in a sub-tropical orchard.
- To determine the nutrient concentration of the flowers of different avocado cultivars to resolve whether or not nutrient limitations reduce pollination.

To visualise the holistic importance of this study, an organogram depicting the interconnectedness is presented in Figure A. As we progress through this dissertation, Chapters 2 through 6 will aim to fill the gaps in the understanding of how low pollination contributes to low yields in avocados, thus fulfilling the aims of this study.

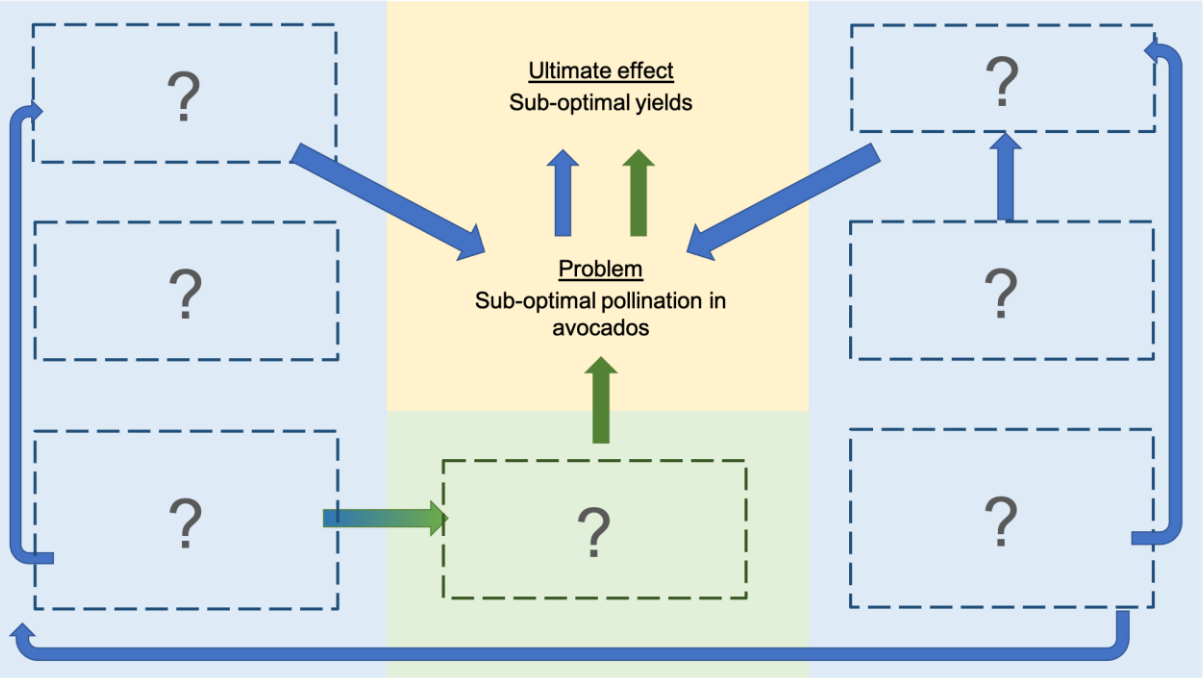

Figure A. Organogram depiction of the holistic approach employed in this study to determine how sub-optimal pollination in avocados contributes to the ultimate effect of sub-optimal yields. Blue arrows represent pathways that undermine pollination in avocados and lead to yield reductions. Green arrows represent pathways that promote pollination in avocados and minimise yield reductions.

A detailed literature review will be discussed in Chapter 1 of this dissertation. Following this, Chapter 2, titled ‘Testing honey bee preferences for avocado (*Persea americana* Mill.) flowers by monitoring activity surrounding different cultivars and flower sexual phases in trees’, will analyse honey bee activity amongst the canopies of a variety of avocado cultivars. These analyses were conducted twice: while the flowers were active in the female and male phases.

Chapter 3 ‘The volatile profile of the avocado (*Persea americana* Mill.) flower’ will use Solid-phase microextraction (SPME) and comprehensive gas chromatography with time-of-flight mass spectrometry (GC x GC-TOFMS) techniques to determine what volatiles are exuded by avocado flowers. After extensively consulting the available literature, we believe this will allow us to describe the volatile profile of seven avocado cultivars for the first time.

After the role of volatile exudation in attracting honey bees will be made clear in Chapter 3, Chapter 4 ‘Improving pollination in avocado (*Persea americana* Mill.) orchards using honey bee attractants’ will analyse the efficacy of using essential oil lures and flowering lavender to enhance pollination in a small avocado orchard.

In Chapter 5 ‘Novel counting methodology and light microscopy reveal pollen abnormalities in cold stressed avocado (*Persea americana* Mill.) flowers cultivated in sub-tropical climates’, the effects of cold stress on pollen production and health late in the flower development process will be explored. We believe this study will be the first of its kind to investigate the role of cold stress on pollen development in avocados cultivated in sub-tropical regions, specifically for flowers at anthesis.

In Chapter 6 - ‘Floral elemental content and its potential influence on pollination in avocados (*Persea americana* Mill.)’ we will expand on the findings of Chapter 5 by determining the influence of floral nutrient status on pollen development and subsequent pollination. This will be the first known attempt to examine floral nutrient status in avocados and explore the effects thereof on pollination. Nitric acid (HNO_3) digestions and inductively coupled plasma (ICP) analysis will show any differences in floral nutrient accumulation of potassium, phosphorous, boron, and calcium based on flower cultivar, sexual phase, a flowers position within the canopy, and sampling location. This chapter will also show how the direct role of these compounds in pollen development in avocados is poorly understood, thus uncovering yet another gaping hole in the multifaceted understanding of pollen development, and pollination as a whole, in avocados.

Lastly, Chapter 7 will conclude this dissertation by summarising the findings of Chapters 2 through 6. This will allow us to evaluate the success of this study by comparing our findings with the aims explained above. Furthermore, practical methods for improving pollination in avocados will be discussed. We will close this dissertation by examining and recommending future research opportunities.

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CHAPTER 1

LITERATURE REVIEW

1.1 *Persea americana* (Mill.)

1.1.1 Economic importance and uses

In the United States alone, avocado (*Persea americana* Mill.) consumption rose from 437 million lbs (198 million kgs) in 1985 to an all-time high of 2 647 million lbs (1 200 million kgs) in 2019 (Shahbandeh 2020a). In the last 20 years, global avocado production has almost tripled from 2.71 million metric tonnes in 2000 to 6.41 million metric tonnes in 2018 in order to meet the ever-increasing demand for the fruit (Shahbandeh 2020b). The latest annual production statistics report Mexico (2.3 Mts) to be by far the largest producer of avocados. They are followed by the Dominican Republic (661.3 kts), and Peru (535.9 kts). Kenya (364.9 kts) is the largest producer in Africa and Israel (138.7 kts) the largest in Eurasia (Statista 2019). Duarte *et al*. (2016) have attributed the increase in avocado production to advancements in post-harvest technology, a more open and accessible trading market, and the health benefits of the fruit.

Containing a myriad of vitamins, healthy fats, fibre, and phytochemicals (Fulgoni *et al*. 2013), the avocado is one of the most nutritionally dense fruits. Regular consumption of the avocado has the potential to reduce low-density lipoprotein (LDL) cholesterol levels (Bergh 1992a, Lottenberg 2002), play a role in cancer (Bouic 2002) and diabetes prevention (Pahua-Ramos *et al*. 2014), and even HIV-infection (Wigg *et al*. 1996), among many others. Not only is the avocado edible in its fresh form, but when processed into pulps and oils, it is also an invaluable resource for the pharmaceutical and cosmetic industries (Teixeira *et al*. 1992, Duarte *et al*. 2016).

1.1.2 Taxonomy, origin, distribution, and horticultural varieties

The avocado belongs to the botanical family Lauraceae (Kostermans 1957), along with other important tree crops and ornamentals including *Cinnamomum camphora*, *Laurus nobilis*, and *Ocotea* sp (Scora and Bergh 1990). Most of the species in the Lauraceae family are either tropical or sub-tropical species (Bergh and Ellstrand 1986). According to Williams (1976), more than 1 000 species within the Lauracea family are shrubs and very large trees, the majority of which are found in the Americas and Asia. Within the Lauraceae family there are approximately 2 850 species belonging to 45 different genera (Christenhusz and Byng 2016). The *Persea* is one such genus, which itself contains roughly 50 species (Bergh and Ellstrand 1986). Within the *Persea* genus, there are two sub-genera, *Eriodaphne* and *Persea* (Bergh 1992b). The avocado, *Persea americana*, belongs to the latter.

The avocado owes its nomenclature to the region in which it is believed to have originated from – central Mexico on the North American continent (Storey *et al*. 1986). Along with many other food crops like beans and rice (Gepts and Bliss 1985, Londo *et al*. 2006), the avocado is a cultigen (Chen *et al*. 2009). This means the avocado was domesticated through human intervention over millennia, making it difficult to precisely determine its origin (Spencer and Cross 2008). Historians have reported instances of avocado consumption as far back as 8 000 BC (Smith 1966). It was during this time Mesoamerican inhabitants began to domesticate the avocado (Galindo-Tovar *et al*. 2008).

As a result of centuries of selection, three distinct horticultural varieties have arisen (Chen *et al*. 2009). Bergh (1992b) describes these botanical varieties as the West Indian (*americana*), Guatemalan (*guatemalensis*) and Mexican (*drymifolia*) races. Bergh (1992b) elaborates further by explaining how the West Indian varieties are better suited to cultivation in tropical climates, while the Mexican races will be better suited to cooler environments.

1.1.3 Cultivation history and advancements

The avocado has since been introduced throughout the world, with Indonesia reporting uses of the fruit in the mid-1700's, South America in the early-1800's and South Africa and Australia almost a century later (Silva and Ledesma 2014). The selection for desired characteristics in seedling propagation began in Florida in the USA during the early 1900's (Schaffer *et al*. 2013).

Commercial avocado orchards are hindered by low-yielding trees in most of the production regions throughout the world (Trochoulias and O'Neill 1976). There are numerous theories proposed by scholars to explain the low yielding nature of the avocado. The avocado is a unique fruit in that it has a remarkably high lipid content between 12-24 %, while carbohydrates total 0.8 - 4.8 % (Araújo *et al*. 2018), especially when compared to other fruits like apples, which contain roughly 15 % carbohydrates and only trace amounts of lipids (USDA 2002). There is a great difference in the amount of energy stored in fats and carbohydrates (Taiz *et al*. 2014) (Table 1.1). As they produce oil-storing fruits, avocado trees have lower production potentials compared to sugar-storing fruits (Wolstenholme 1986, Ranade and Thiagarajan 2015). Wolstenholme (1986) suggests another major reason for the inherent low yielding nature of avocado trees is due to the large seed and the 'concentrated food reserves' it contains.

Table 1.1 Comparison of the amount of energy stored in 1 g of fat and 1 g of carbohydrates (Taiz *et al*. 2014)

Energy Source (1g)	**Amount of energy (kcal)**
Fat	9.3
Carbohydrate	3.8

In spite of the influence of the oil-rich fruit itself in limiting yields in avocados, many experts have proposed additional reasons such as low pollination rates when cultivated outside their native regions (Ish-Am *et al*. 1999), complications brought on by alternate bearing (Mickelbart *et al*. 2012), competition between reproductive and vegetative growing points (Sedgley 1987), and diseases such as root rot caused by *Phytophthora cinnamomi* (Whiley *et al*. 1986). Further investigation into the notion of low pollination in avocados revealed there to be many variables that had the potential to discourage pollination.

1.2 Pollination

1.2.1 Synchronous dichogamy

Before these variables can be discussed in greater detail, the flowering pattern of the avocado must be understood. As is the case with over 100 important food crops, avocados benefit immensely from insect pollinators (Rader *et al*. 2020). The structure of the avocado flower has long been known to act as a barrier to self-pollination (Clark 1922). Avocado flowers are perfect, containing both female and male reproductive structures (Davenport 1986). The dichogamous nature of the flowers was first described by Nirody (1922), the female phase with receptive stigmas opens prior to the male phase which is characterised by anther dehiscence. Based on their flowering pattern, Stout (1933) classified avocado cultivars as ‘A-type’ or ‘B-type’ – the flowers of ‘A-type’ cultivars are functionally female when they first open in the morning, close overnight and reopen the following afternoon in the functional male phase. The flowers of ‘B-type’ cultivars are functionally female in the afternoon, close overnight and reopen the following morning in the male phase. It is this dual opening and separation of the active female and male phases that leads many experts to believe the avocado flower requires cross-pollination to increase fruit set (Stout 1923, Clark 1924, Bergh 1968). The importance of cross-pollination was made even more apparent in a study by Alcaraz and Hormaza (2009a), which showed a 15 % increase in fruit set in hand-pollinated avocado flowers compared to open-pollinated flowers.

1.2.2 Pollinators and low pollination rates

Cross-pollination is therefore vital to ensure adequate fruit set in avocados. When cultivated in its native central Mexico, there are numerous insects that can act as pollinators for the avocado flower, including stingless bees, flies, wasps, and beetles (Nieto 1985, Ish-Am *et al*. 1999). However, as the tree has become widely cultivated outside its native region, the flowers can no longer rely on visitations by these insects to facilitate cross-pollination. Bergh (1992b), Roubik (1998) and Ish-Am and Lahav (2011) highlight the importance of honey bees as they have become the main pollinator for commercially grown avocado trees outside the native region (Figure 1.1).

Figure 1.1. Honey bee pollinating the female phase of the 'Hass' avocado flower in the Politsi Valley, Tzaneen during the 2019 flowering season.

The efficacy of beehive introductions into commercial orchards is contested by many experts. The findings of Ish-Am (2005) suggest that in spite of the copious amount of blooms produced by avocado trees, insufficient pollinator activity within an orchard leaves many flowers unpollinated and unfertilised. This may be due to honey bees showing preference for other floral sources over those of the avocado (Afik *et al*. 2006), as they often leave the avocado orchards in which their hives are placed to forage for more attractive flowers (Afik *et al*. 2008). This behaviour is especially problematic in Mediterranean regions, where the flowering seasons of the avocado and indigenous flora overlap (Monzon *et al*. 2020). Afik *et al*. (2006) suggest there to be compounds present in the nectar of the avocado flower that contributes to its low attractiveness.

To improve cross-pollination in avocado orchards, pollinizing varieties are intercropped with pollinated (fruiting) cultivars (Schnell *et al*. 2009). According to Alcaraz and Hormaza (2009b), a good pollinizer must have an overlapping flowering period, as well as a complementary sexual phase cycle (‘A-type’ cultivars complement ‘B-type’ cultivars), to the pollinated varieties. Some of the popular intercroppings include ‘Hass’ (‘A-type’) and ‘Bacon’ (‘B-type’) (Kobayashi *et al*. 2000) and ‘Simmonds’ (‘A-type’) and ‘Tonnage’ (‘B-type’) (Borrone *et al*. 2008). In South Africa specifically, ‘B-type’ cultivars ‘Bacon’, ‘Ettinger’, and ‘Zutano’ are potential pollinizers for ‘Hass’ and ‘Maluma Hass’ (both ‘A-type’ cultivars) (Bezuidenhout *et al*. 2018). The proximity of pollinated and pollinizing cultivars varies from orchard to orchard. For example, some growers may choose to plant pollinizers in every sixth row in the orchard (Fetscher *et al*. 2000).

1.2.3 Floral volatiles and their perception by the honey bee

Muhlemann *et al*. (2014) describe how plants exude floral volatiles to facilitate many delicate ecological interactions, including pollinator attraction. When perceived by pollinators, the volatiles can communicate details not only about the characteristics of the floral source, but information relating to the specific location of nectar within the flower (Baldwin 2010). Due to specialised volatile detectors, a honey bee is able to select flowers that exude more favourable volatile odours (Galizia and Menzel 2000), which may explain why honey bees favour other floral sources over those of the avocado. The volatile profile of the avocado flower has yet to be described, highlighting the need for investigation into the effect of avocado floral volatiles on honey bees.

Volatile detection can also serve as a practical solution to the problem of low attractiveness of avocado blooms to honey bees. Previous tests on other crops have showed the potential of attracting honey bees using essential oil sprays (Waller 1969, Malerbo-Souza *et al*. 2004) and intercropping with more attractive plants (Moghaddam *et al*. 2020). These represent two interesting methods that may be exploited by avocado farmers to improve honey bee activity within the orchards.

1.3 External factors contributing to low pollination rates

1.3.1 Meiosis in eukaryotes

There may be factors external to the avocado tree itself that also contribute to inadequate pollination by influencing flower development. In plants (like all eukaryotes), meiosis serves the purpose of creating variation from generation to generation through the shuffling of genetic material. Meiosis involves two sequences of cell-division and chromosomal separation, the purpose of which is to halve the number of chromosomes in a cell (Mercier *et al*. 2015). As explained by O’Connor (2008), in order to maintain a consistent chromosome number between generations germ cells must undergo meiosis.

In angiosperms, the flowers are the reproductive structures where meiosis occurs. According to Harrison *et al*. (2010), for a plant to effectively complete its life cycle meiosis must be initiated at a precise time and place. This generally occurs after the plant has undergone enough vegetative growth and transitions into the reproductive phase, characterised by flower induction in angiosperms. Unlike humans, in which the reproductive ‘germ cells’ are already present during embryogenesis, vegetative and germ cells of higher plants are all produced from the shoot meristems which arise during embryogenesis (Gilbert 2000). The vegetative cells can then transition to reproductive cells due to the ‘totipotent’ nature of plant cells (Vasil and Vasil 1972).

1.3.2 Flower induction and the ABC model of flower development

Davenport (1986) highlights the difference between floral induction and initiation: the former describes the initiation of flowering gene transcription, while the latter, which always occurs after induction, refers to the ‘first physiological and histological expression’ of flower organs.

A simplistic explanation given by Gilbert (2000) states that the transition from the vegetative to the reproductive phase begins when a signal transmitted by the leaves travels to the shoot apex, where flowering is then initiated. The details of these flowering signals require further study in order to be understood fully. However, there are some factors both internal and external to the plant that account for the transition between vegetative and reproductive development (Bernier 1981). Some of these triggers include temperature in impatiens (Mattson and Erwin 2003), destruction of apical dominance (aka decapitation) in pistachios (Nikpeyma 2020), and photoperiod in chrysanthemums (de Jong 1989).

Whilst the exact biochemical pathway of the transition between vegetative and reproductive development is yet to be fully described, it is known that genetics and gene expression play a major role (Theißen and Melzer 2013). First proposed by Coen and Meyerowitz (1991), the ABC model of flower development explains how certain genes, and the interaction and mutation thereof, have a major influence on the development of floral structure. The flowers of different angiosperm species have distinct arrangements of the four organs – the sepals, petals, stamens, and carpels – in concentric whorls. The classical ABC model is used to assign functions to certain genes that play a role in organ differentiation, first described for *Arabidopsis thaliana* (Li *et al*. 2003). Making up the outer whorl, the sepals are controlled by A genes. The petals make up the next whorl, development of which is coded for by the interaction of A and B genes. The third whorl, the stamens, develop when both B and C genes are expressed. Lastly, carpels make up the innermost whorl and the development of which is controlled by C genes alone.

For reproduction and species survival, all flowers must contain stamens and carpels, but these structures may be present in different flowers on the same or separate tree. In contrast, the sepals and petals may not be found in some flowers. Litt and Kramer (2010) explain how many basal angiosperms and monocots diverge from the 'standard' flower by possessing only 3 of these organs: the tepals (fused sepals and petals), stamens, and carpels.

Based on the simplistic ABC model of floral differentiation the anthers, being a part of the stamen along with the filament, require the expression of both B and C genes. Meiosis takes place in the anthers and produces four haploid cells from a single diploid parent cell (Dawe 1998). Not only do the anthers provide the developing pollen grains with structural support and protection, but the anther walls also contain a tapetum layer which is a crucial supplier of nutrients and signals (Goldberg *et al*. 1993). After meiosis is complete, pollen grains start to develop from microspores (Ma 2005). In this process, various cellular divisions and differentiations must take place to produce one vegetative cell and two sperm cells (Li and Ma 2002). The four pollen sacs within the anthers contain many microspores, the 'mother cells'. This mother spore is diploid and will undergo meiosis to form a tetrad of four haploid microspores. Each microspore represents the first phase of the male gametophyte generation. Vacuolization then commences and the nucleus, which was originally at the centre of the cell, is displaced outwards. This nucleus undergoes two rounds of mitosis. The first-round results in the production of a large vegetative cell and a small generative cell. This generative cell undergoes a second round of mitosis and results in the production of two sperm cells. While the pollen mature in the anthers, they dehydrate, but rehydrate after pollination. This allows the pollen to germinate on the stigma and a pollen tube then begins to grow. This pollen tube will grow down the style until it reaches the ovule. The two sperm cells travel through this tube and subsequently fertilise the ovule (Li and Ma 2002).

Schroeder (1952) made one of the first attempts to describe how meiosis progresses specifically in avocados. His findings are briefly summarised as follows: a conical meristematic mass in the axis of the bud scales gradually flattens as the perianth becomes visible. The perianth elongates and curves towards the centre. The stamen primordia arise, followed by the pistil, the final organ to develop. Cell division on the funiculus causes the ovule to bend towards the receptacle, resulting in the anatropous ovule structure. Megasporogenesis commences as the ‘hypodermal archesporal cell’ produces both the ‘megaspore mother cell’ and ‘primary parietal cell’. Subsequent divisions of the megaspore mother cell generates an additional four megaspores. Three of these megaspores degenerate while the remaining chalazal megaspore becomes the single-celled embryo sac. A series of gametophytic divisions and nuclei migrations precede nuclei fusion. Subsequent elongation of the embryo sac and nuclei degeneration occurs, followed by ultimate embryo sac maturation. Microsporogenesis occurs when the flower buds are approximately 1 mm long.

1.3.3 Transition from vegetative to reproductive growth in *P. americana* trees

Salazar-García *et al*. (1999) found that the transition from vegetative to reproductive bud development in avocados occurs when the summer shoot expansion concludes. Early findings of Venning and Lincoln (1958) highlighted the role of the primary axis meristem (PAM) in the development of inflorescence bracts and leaf primordia. Beneath the PAM, secondary axis meristems (SAMs) are found and most commonly, but not always, result in the formation of inflorescences (Bernier *et al*. 1981). Once the SAMs have been activated, the PAM shifts from a convex form to a more flattened one. Salazar-García and Lovatt (2002) have concluded that this structural conversion is a critical stage in the changeover to reproductive growth and is known as the ‘transition phase’ and was observed to occur in ‘Hass’ trees from the end of July and into August in California. Buds within the shoots that have developed during the spring and summer growth flushes contain the flower primordia (Bender 2002). Salazar-García *et al*. (1999) showed that flower organs started to develop at what they called developmental Stage 5 of apical inflorescence buds. They also reported that between Stage 7 and Stage 8 (the cauliflower stage, occurring towards the end of February in ‘Hass’ trees in California), meiosis occurs in the anthers and by the time the flowers had reached Stage 10, mitosis had resulted in the formation of two-celled pollen grains.

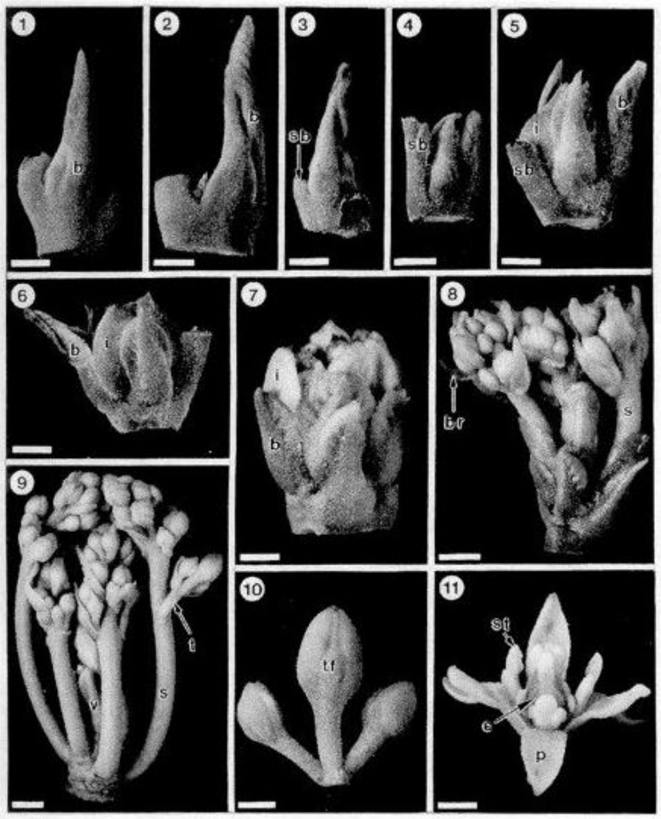

Figure 1.2. Microscopic depiction of avocado inflorescence developmental stages 1 through 11 (Salazar-Garcia *et al*. 1999). Meiosis in avocados occurs between stages 7 and 8.

1.3.4 Influence of cold stress and nutrient availability on avocado flower development

As aforementioned, the avocado flower is described as protogynous, synchronous and dichogamous (Nirody 1922, Davenport 1986). The influence of cold temperatures on the closing and opening of the avocado flowers has been widely reported on. Whilst a period of low temperatures is required for flower induction in some avocado cultivars (Bender 2002), temperatures lower than a night-time minimum and daytime maximum of 21 °C interrupt the regular flowering pattern in avocados (Bergh 1974). Issarakraisila and Considine (1994) report temperatures below 15 ºC to negatively influence pollen development in tropical trees. Given that temperate countries such as New Zealand are fast becoming major avocado producers (Naamani 2007), the influence of cold temperature and elevational effects on avocado pollen development must be investigated.

Pollen development is also influenced by nutrient availability in addition to ambient temperature. Upon exploring the current literature available on the subject, little information was found detailing the exact role of nutrients on pollen development, especially in avocados. However, as Cheung and Stephenson (1993) report, during meiosis the sporophytic generation must have an adequate nutrient supply if pollen is to develop optimally. Furthermore, boron and potassium deficiencies have been shown to affect pollen development and extreme deficiencies thereof may lead to male sterility (Dell and Huang 1997, Choudhurry *et al*. 2013). It is therefore reasoned that a trees nutrient status will influence pollen development, and if deficiencies are present, sub-optimal pollen development will diminish pollination and ovule fertilisation.

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CHAPTER 2

TESTING HONEY BEE PREFERENCES FOR AVOCADO (*PERSEA AMERICANA* MILL.) FLOWERS BY MONITORING ACTIVITY SURROUNDING DIFFERENT CULTIVARS AND FLOWER SEXUAL PHASES IN TREES

2.1 Abstract

Avocado (*Persea americana* Mill.) flowers are protogynous, synchronous and dichogamous. As a result, they require cross-pollination for ovule fertilisation and fruit set. Outside its native region of central Mexico, the primary pollinator for the avocado is the honey bee (*Apis mellifera)*. It is common practice to place beehives in commercial avocado orchards during the flowering season, but the exodus of these honey bees out of the orchards to forage among favourable flower sources has been documented. As the honey bee shows a clear preference for certain flower species, this study aimed to determine if honey bees discriminated between avocado flowers of different sexual phase and cultivar. The differences in honey bee activity between a commercial and non-commercial avocado orchard was also monitored. By using a 1 x 1 m frame to quantify honey bee activity, results suggest that honey bees show a preference for different cultivars. In addition to this, honey bee activity was continuously higher around actively male flowers. This is consistent with the findings of other experts who report higher honey bee activity around male flowers. This may be due to the dual reward of nectar and pollen offered by the actively male avocado flowers, in addition to their larger, more attractive floral display. Furthermore, it can be theorised that there may be a compound, or mix of compounds, released from different avocado flowers that will affect their ability to attract honey bee pollinators. This highlights the importance of placing favourable pollinisers in avocado orchards, especially by mixing compatible ‘A-type’ and ‘B-type’ cultivars. Lastly, the effects of drought on honey bee activity were also observed during the study.

Keywords: pollination, pollinators, attraction, repulsion, sub-tropical, fruit, cultivation, avocados

2.2 Introduction

With high concentrations of beneficial compounds such as healthy fats and numerous vitamins, the avocado (*Persea americana* Mill.) is a widely popular fruit (Duarte *et al*. 2016). In the United States alone, the demand for avocados increased three-fold between 2001 and 2017 (USDA AMS 2020, Cho 2021). With the increased demand, production has expanded. This is clearly observable in Mexico, where an excess of 231 000 ha of avocado trees were planted in 2017, compared to only 168 000 ha in 2013 (Shahbandeh 2020).

Whilst greater acreage will lead to increased harvests, the low fruiting nature of the avocado tree is one of the factors that prevents producers from reaching optimal yields, especially when the tree is cultivated outside of its native region (Bergh 1976, Ish-Am 2005, Garner and Lovatt 2016). Many experts believe inadequate pollination is a factor that contributes to the low production figures (Ish-Am and Eisikowitch 1998).

Whilst the avocado flower is monoecious, the protogynous, synchronous dichogamy of the flower promotes cross-pollination (Ish-Am and Eisikowitch 1993). Avocado varieties are classified as ‘A-type’ or ‘B-type’ cultivars (Stout 1923). ‘A-type’ cultivars, which includes varieties such as ‘Hass’ and ‘Maluma Hass’, have functionally female flowers open in the morning, which close and reopen as functional males the following afternoon (Stout 1923, Peterson 1955, Davenport 1986). ‘B-type’ cultivars, which includes varieties such as ‘Fuerte’ and ‘Zutano’, have functionally female flowers open in the afternoon, which close and reopen as functional males the following morning (Stout 1923, Peterson 1955, Davenport 1986). Not only is cross-pollination necessary for ovule fertilisation and subsequent fruit growth, but cross-pollinated fruit is also ‘stronger’ and more likely to survive until it reaches a harvestable size (Vrecenar-Gadus and Ellstrand 1985). It has therefore been suggested that avocado grower’s select ‘B-type’ cultivars as pollinisers in their orchards to facilitate cross-pollination for ‘A-type’ cultivars (McGregor 1976, Bezuidenhout *et al*. 2017).

2.2.1 Problem statement

Cross-pollination is most commonly facilitated by insect vectors in avocados. When cultivated in its native Mexico, the avocado is pollinated by indigenous species of wasps and bees (Free and Williams 1976, Perez-Balam *et al*. 2012). However, in order to meet the ever-increasing demand for the fruit, avocado production has increased outside its native region. In these areas, the main avocado pollinator is the honey bee. (Stout 1923, Ish-Am and Eisikowitch 1991, Ish-Am and Eisikowitch 1993). Unfortunately, previous studies have shown that honey bees often show a preference for other flowers over those of the avocado (Afik *et al*. 2008). This may be due to a higher ‘reward’ offered by other plants (Ish-Am and Eisikowitch 1998), in addition to the pollen and nectar of the avocado not being suited to the needs of the honey bee (Ish-Am 1994).

2.2.2 Aim of the study

Considering the low fruiting nature of the avocado tree and the requirement for adequate cross-pollination to facilitate fruit set, in conjunction with the preference shown to other flowers by the honey bee pollinator, this study aimed to determine if honey bees showed preferences for flowers belonging to different avocado cultivars.

2.2.3 Objectives of the study

To realise the aim of this study, specific objectives were laid out. Bee framing techniques were used to quantify bee activity surrounding different cultivars. Due to the importance of honey bee visitations to both sexual phases of the avocado flower, preference for flower phase was also considered. Finally, to account for the effects of beehive placements in commercial orchards, honey bee activity was monitored in both commercial and non-commercial orchards.

2.2.4 Hypotheses

Due to previous studies reporting on enhanced pollinator activity surrounding male flowers, it is expected that honey bee activity will differ based on the sexual phase of the avocado flower. Furthermore, it can be reasoned that genetic differences between avocado cultivars may contribute to perceivable differences by honey bees which could influence the pollinator’s preferences.

2.2.5 Significance of the study

By determining if honey bee activity is greatly influenced by avocado cultivar, flower phase and location, recommendations can be made to select more favourable cultivars as pollinisers. This will then lead to improved cross-pollination and ‘stronger’ fruit, which will have a greater chance of reaching maturity.

2.3 Materials and Methods

2.3.1 Locality and environmental conditions

Trials were conducted over a two-year period. During the first year, sampling was performed in the commercial avocado orchards of ZZ2 Farms in Tzaneen, Limpopo (23º63’S, 30º04’E). This area experienced minimum temperatures between 3° C and 13° C, and maximum temperatures of 25° C to 30° C during late winter and early spring when sampling took place. Approximately 6.93 mm of rainfall was received at this time. Detailed environmental conditions for the sampling period are reported in Appendix A. Five cultivars were selected based on their importance highlighted by ZZ2 researchers who were responsible for funding the project in that year. The cultivars selected were ‘Hass’, ‘Fuerte’, ‘Maluma Hass’, ‘Galil’ and ‘Zutano’. Two different orchards were used, the first constituted a 36-year-old ‘Hass’ and ‘Fuerte’ mixed planting and the second constituted a 5-year-old ‘Maluma Hass’, ‘Galil’ and ‘Zutano’ mixed planting. ‘Hass’ trees were grafted on a Guatemalan rootstock and were spaced 7 m within and between rows, while ‘Fuerte’ trees were grafted on a ‘Duke 7’ rootstock and were spaced 7 m within and between rows. ‘Maluma Hass’, ‘Galil’ and ‘Zutano’ trees were grafted on ‘Duke 7’ rootstocks and were spaced 4 m within rows and 6 m between rows. The trunks of all trees were treated with polyvinyl alcohol (PVA) paint post-harvest to prevent sunburn. Honey bee activity was monitored in the morning and afternoon to account for the different sexual phases of the avocado flower. Four trees (n = 4) were used as replicates, and the counting was repeated for a second time on a different day. The orchards had beehives in place during the flowering period.

In the second year, this project was no longer being funded by the individuals responsible for the first year's sampling. Therefore, alternative arrangements were made. In this year, sampling was conducted in the commercial avocado orchards of Westfalia in Limpopo, Tzaneen (23º45'S, 30º05'E and 23º44'S, 30º04'E) (Figure 2.1). This area experienced minimum temperatures between 1 °C and 11° C, and maximum temperatures between 22° C and 26° C at the time of sampling. Only 0.1 mm of rainfall was received. Detailed environmental conditions for the sampling period are reported in Appendix A. Due to the change of sampling location, it was only possible to evaluate honey bee activity of 'Hass' and 'Fuerte' trees. 'Hass' sampling was conducted on a 1.5 ha, 7-year-old orchard in the Politsi Valley of Tzaneen. 'Fuerte' sampling was conducted on a 4.43 ha, 46-year-old orchard in the Politsi Valley in Tzaneen. Five trees (n = 5) were used as replicates, and counting was repeated for a second time on a different day. The first round was completed in July, and the second in September. It must be noted that during the trial period, the region was experiencing an extreme drought and the Tzaneen dam only had 5.5 % of its capacity in water (AgriSA 2020). During the September sampling, an extreme cold front had also just passed. The orchards had beehives in place during the flowering period.

During the second sampling year honey bee activity was also monitored on a 12-year-old, non-commercial avocado orchard on the Experimental Farm at the University of Pretoria's Hillcrest Campus (25°45'S 28°16'E) (Figure 2.1). Minimum temperatures ranged between 1° C and 16° C, while maximum temperatures ranged between 18° C and 32° C. Approximately 2.7 mm of rainfall was received between July and September, and trees received supplemental drip irrigation every second day. Detailed environmental conditions for the sampling period are reported in Appendix A The orchard consisted of a mixed planting of 'Hass' and 'Fuerte' cultivars, grafted on 'Dusa' and 'Latas' rootstocks. The 'Fuerte' trees were flowering far less prolifically than the 'Hass' trees, indicating the former were experiencing an 'off-year'. Trees were planted 5 m apart within rows and 6 m apart between rows. To lower the probability of sunburn the trunks of the trees were painted with white PVC paint earlier in the growing season. Five trees (n = 5) were used as replicates, and counting was repeated for a second time on a different day. The avocado orchard is in close proximity to a small number of beehives 100 m away from the trees. Located 200 m in the opposite direction to the hives was a lemon orchard.

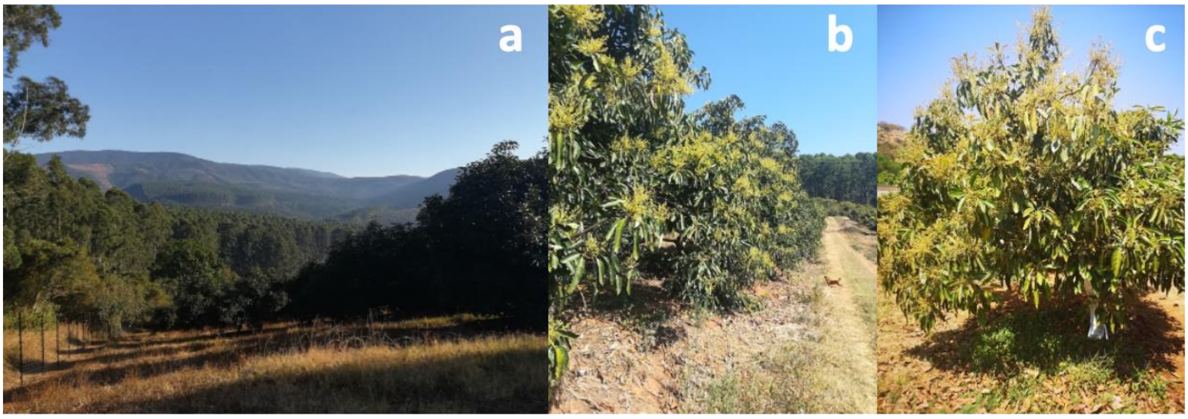


Figure 2.1. (a) Commercial avocado orchard in the Politsi Valley in Tzaneen during the 2019 flowering season. (b) Flowering ‘Hass’ trees in the Politsi Valley in Tzaneen during the 2019 flowering season. (c) Flowering ‘Hass’ tree in the avocado orchard on the Experimental Farm of the University of Pretoria during the 2019 flowering season.

2.3.2 Honey bee monitoring

In order to determine the frequency of honey bee visitations to the different cultivars a 1 x 1 m lightweight frame was held half a meter away from the canopy of the trees. This distance was far enough away to exclude external influences by samplers on bee activity whilst maintaining clear visibility. Measurements were taken for all four cardinal directions of the tree (N, E, W and S) (Figure 2.2). The number of honey bees flying into the frame were counted for a period of 60 seconds. This manual method of counting honey bees is similar to the methodology described by Calzoni and Speranza (1998). Due to the limited personnel available to assist in the counting, the sampling period was reduced from three minutes to 60 seconds. These conditions were made clear to the individuals responsible for counting prior to sampling to promote counting uniformity. The frame was introduced to create a consistent reference area. This counting technique, although simplistic, is a useful method of quantifying insect activity when more sophisticated alternatives are not available, as was the case in this study. Counts were conducted at the same time every day to exclude temporal variations in honey bee activity. Due to the dichogamous nature of the flowers, the avocado trees contained a mix of functional female and male flowers between the hours of 12:00-14:00. This period can be viewed as a ‘transitional phase’. Therefore, the morning samplings took place between 9:00-12:00 and the afternoon samplings took place between 14:00-17:00 in all instances where honey bee activity was monitored. As both the Tzaneen and Pretoria-based orchards experience summer rainfall, all sampling was conducted on clear, sunny days thus excluding the influence of shaded conditions on honey bee activity. In both of these orchards the trees were in full bloom with flower clusters evenly distributed throughout the canopy.

Figure 2.2. Photographic illustration of the bee framing method using 1 x 1 m frames in the commercial orchards of ZZ2 Farms in Tzaneen during the 2018 flowering season.

2.3.3 Data analysis

Due to the different treatment groups analysed in the first (five cultivars) and second year (two cultivars), different analytical procedures were required. For the first year, the general linear model (GLM) analysis was used in conjunction with the Poisson distribution and logarithmic link in all analyses. Fisher’s protected least significant difference (LSD) test was used to discuss differences between treatments. For the second year, a nested factorial analysis was followed by Fisher’s LSD test to determine significant differences. Both sets of analyses were accomplished using the statistical program GenStat® (VSN International 2017).

2.4 Results and Discussion

Differences in honey bee activity based on avocado flower cultivar and flower phase were immediately observable. Counts are presented as ‘bee visits per minute’, which refers to the number of bees flying into the frame over the 60 second counting period. Differences were observed for flower cultivar in both the 2018 and 2019 flowering season (Figure 2.3 and Figure 2.4).

In 2018, the ‘B-type’ cultivars ‘Fuerte’ (8.37 bee visits per minute) and ‘Zutano’ (7.81 bee visits per minute) experienced greater bee activity compared to the ‘A-type’ cultivars ‘Hass’ (3.23 bee visits per minute), ‘Maluma Hass’ (1.88 bee visits per minute) and ‘Galil’ (1.656 bee visits per minute) (Figure 2.3). ‘B-type’ cultivars are functionally female in the afternoon and functionally male in the morning, with the opposite being true for ‘A-type’ cultivars. In the 2019 sampling season the ‘A-type’ cultivar ‘Hass’ located at the Experimental Farm of the University of Pretoria was visited the most (23.13 bee visits per minute), while the ‘Hass’ trees in Tzaneen were visited the least (2.69 bee visits per minute) (Figure 2.4). This stark contrast in bee activity between commercial and non-commercial orchards may be explained by the spray programs implemented in the former (Ish-Am *et al*. 1999). The ‘Fuerte’ trees located at the Pretoria and Tzaneen sampling sites recorded 10.5 and 14.7 bee visits per minute, respectively. The lower honey bee activity surrounding the ‘Fuerte’ trees in Pretoria is likely due to the ‘off’ production year, which is characterised by decreased flower production and pollinator activity (Wolstenholme *et al*. 2010).

Due to the differences in climate, orchard management practices and nature of the trees themselves it would not be prudent to compare the two datasets (2018 and 2019) with one another. Furthermore, if the drought during the 2019 sampling period is considered, it can be reasoned that the 2018 dataset would be a better indicator of true honey bee preferences. Therefore, it can be suggested that ‘B-type’ cultivars are more attractive to honey bees.

Current understanding suggests there to be little to no studies reporting on honey bees’ preferences for certain avocado cultivars. According to Afik *et al*. (2006), there may be a pattern of reduced honey bee activity around avocado flowers due to repellent compounds in their nectar. This fact laid the basis for the investigations that will be discussed in Chapter 3. There are also factors external to the avocado itself which may affect honey bee behaviour.

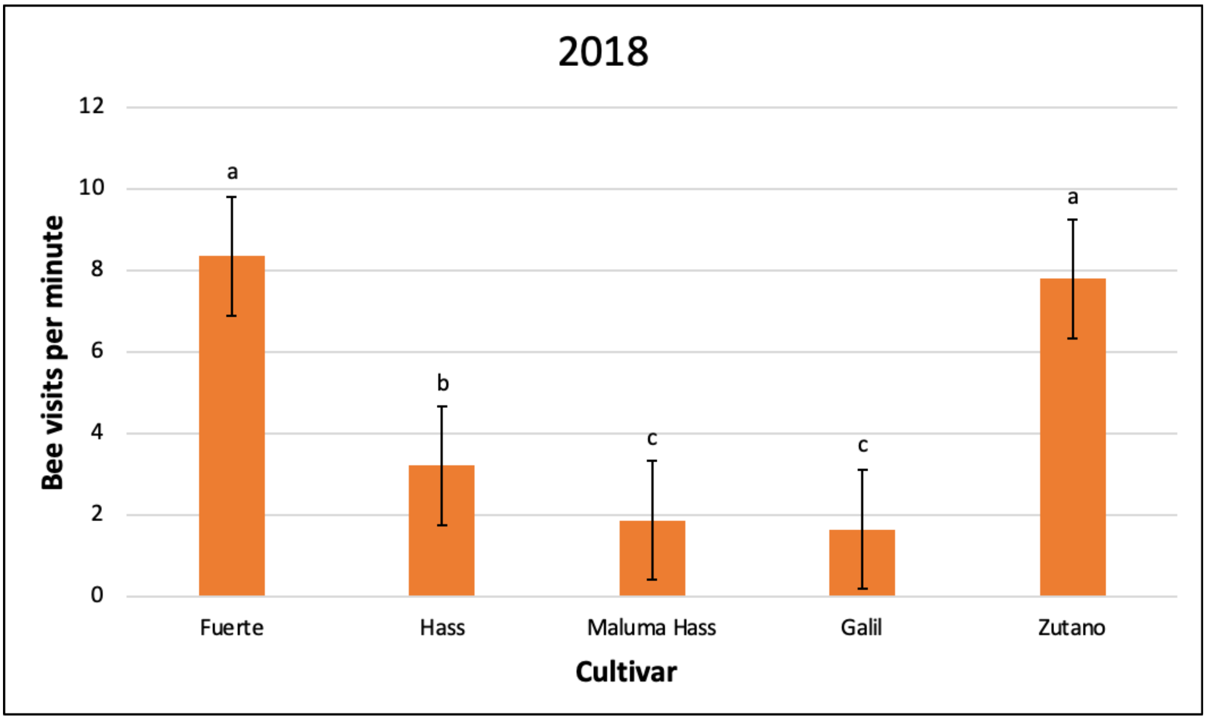


Figure 2.3. Bee visits per minute to different avocado cultivars in Tzaneen during the 2018 flowering season. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

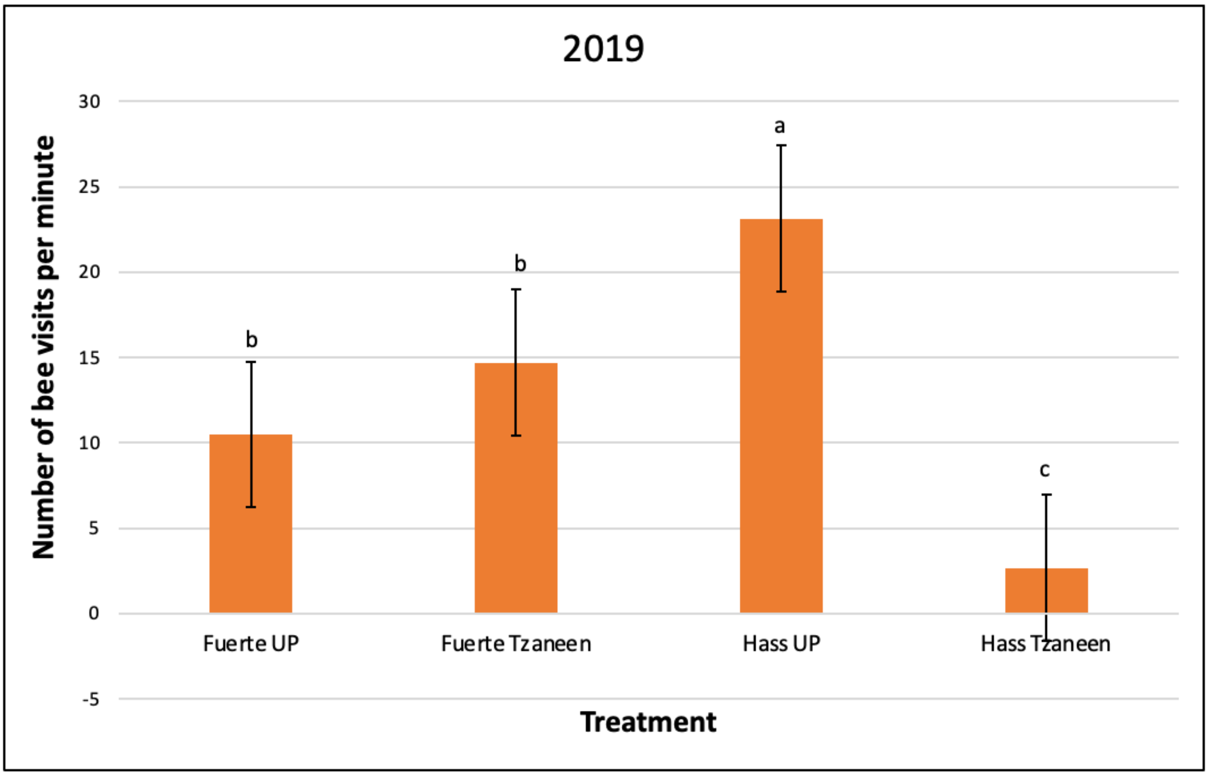
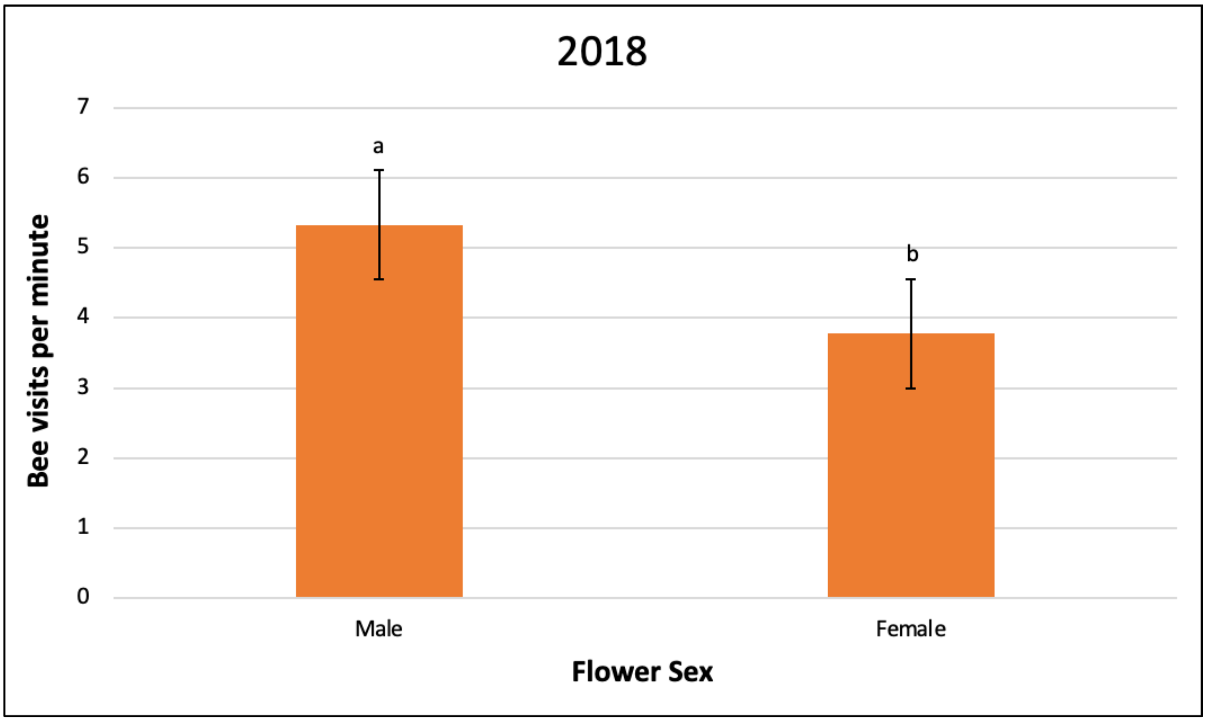


Figure 2.4. Bee visits per minute to different avocado cultivars in Tzaneen and the Experimental Farm of the University of Pretoria (UP) during the 2019 flowering season. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

There were consistent results for honey bee preferences shown towards flower phase (Figure 2.5). In both 2018 and 2019, the male phase (5.33 and 15.52 bee visits per minute, respectively) of the avocado flowers was visited more often than the female phase (3.78 and 11.77 bee visits per minute, respectively). These findings are in accordance with a report by Ashman (2000) who explains how pollinators visit male flowers more often compared to the female flower of the same species.

Differences in floral size may also explain the differential pollinator activity between the male and female phase of avocado flowers. When in the male phase, the avocado flowers appear larger and are more conspicuous compared to those in the female phase. It is this 'reduced floral display' that may also contribute to decreased female flower visitation (Bell 1985, Ashman *et al*. 2005). Experts within the entomological field explain how honey bees show a preference for a certain flower based on nectar quality through direct sampling (Cameron 1981, Gonzalez *et al*. 1995), or 'morphological signs' such as flower size and colour (Geber 1985, Gonzalez *et al*. 1995). It is suggested that worker bees who forage for both nectar and pollen prefer to visit male flowers (Gonzalez *et al*. 1995). However, the findings of Goodwin and Steven (1993) report that honey bees prefer female kiwifruit (*Actinidia deliciosa*) flowers over the males. This suggests there is something further afoot, in addition to the factors mentioned above, that explains why honey bees prefer male avocado flowers. This will be explored in greater detail in Chapter 3.

As a consequence of honey bees preferring actively male flowers, the actively female flowers in mixed orchards of 'A-type' and 'B-type' cultivars may experience a decline in honey bee visitation, which could limit pollination and fruit set (Vrecenar-Gadus and Ellstrand 1985). Chapter 4 details potential solutions to this problem.



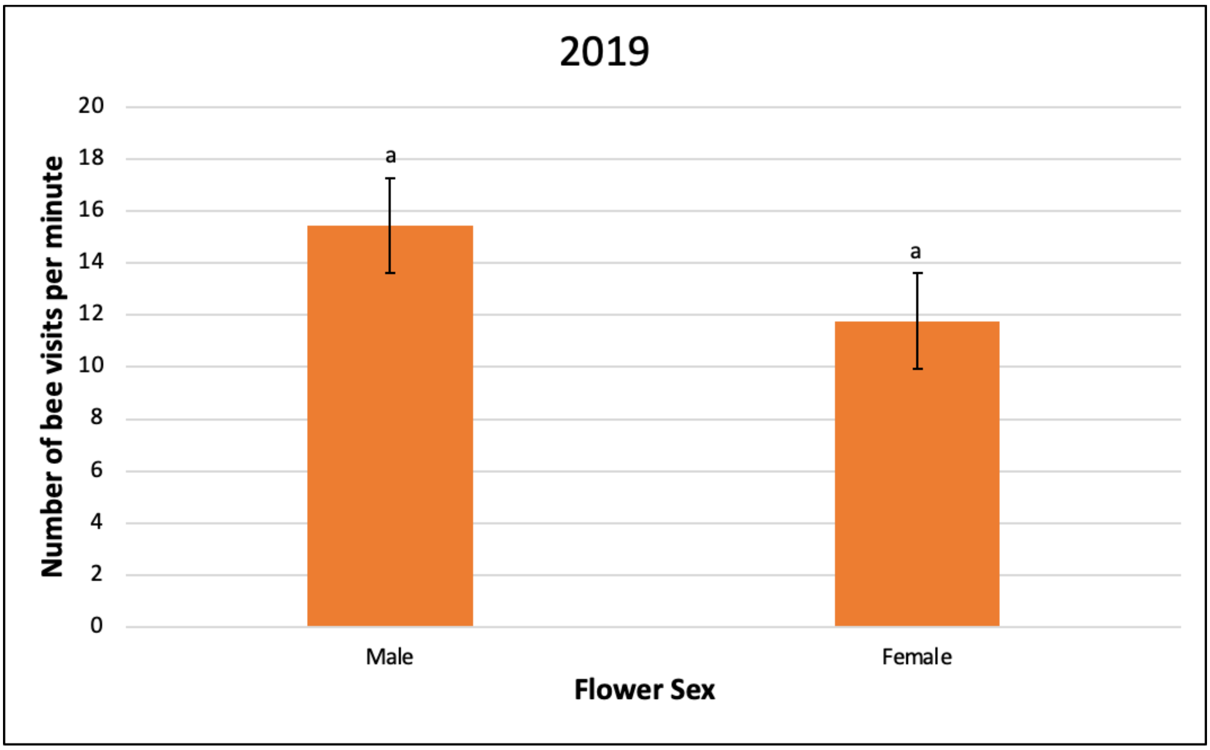


Figure 2.5. Bee visits per minute to avocado trees while flowering in different sexual phases in Tzaneen during the 2018 and 2019 flowering season. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

In 2019, the idea of comparing honey bee activity between commercial and non-commercial avocado orchards yielded interesting results. During the flowering period, it is common practice for commercial farmers to install beehives in their orchards (Stout 1923, Ish-Am and Eisikowitch 1993). It was therefore expected that there would be greater honey bee activity in the commercial orchards in Tzaneen. However, it was observed that the orchard on the Experimental Farm of the University of Pretoria had higher bee activity (Figure 2.6). Significant differences were detected for the interaction effect between the area and flower sexual phase treatments (Figure 2.7). The stark contrast in bee activity between the orchards on the Experimental Farm in Pretoria and in Tzaneen may be due to the drought the latter experienced during the sampling period (AgriSA 2020). The negative impact of water-stress on avocado fruit set has been well documented (Michelakis 1993). However, to our knowledge, the specific influence of water-deficit on flowering physiology has not. Rering *et al*. (2020) reported the effect of drought on floral volatile emission. These floral volatiles serve many purposes, with the most important arguably being to attract pollinators (Schiestl *et al*. 2014).

Therefore, it can be reasoned that due to the drought Tzaneen was experiencing in 2019 (AgriSA 2020), honey bee activity could have been greatly reduced. The influence of drought will therefore also influence pollinator behaviour, besides the differences caused by flower cultivar and sexual phase. The lower honey bee activity in Tzaneen also draws attention to the influence of spraying programs in commercial orchards, and how they undermine pollination by reducing honey bee activity (Ish-Am *et al*. 1999).

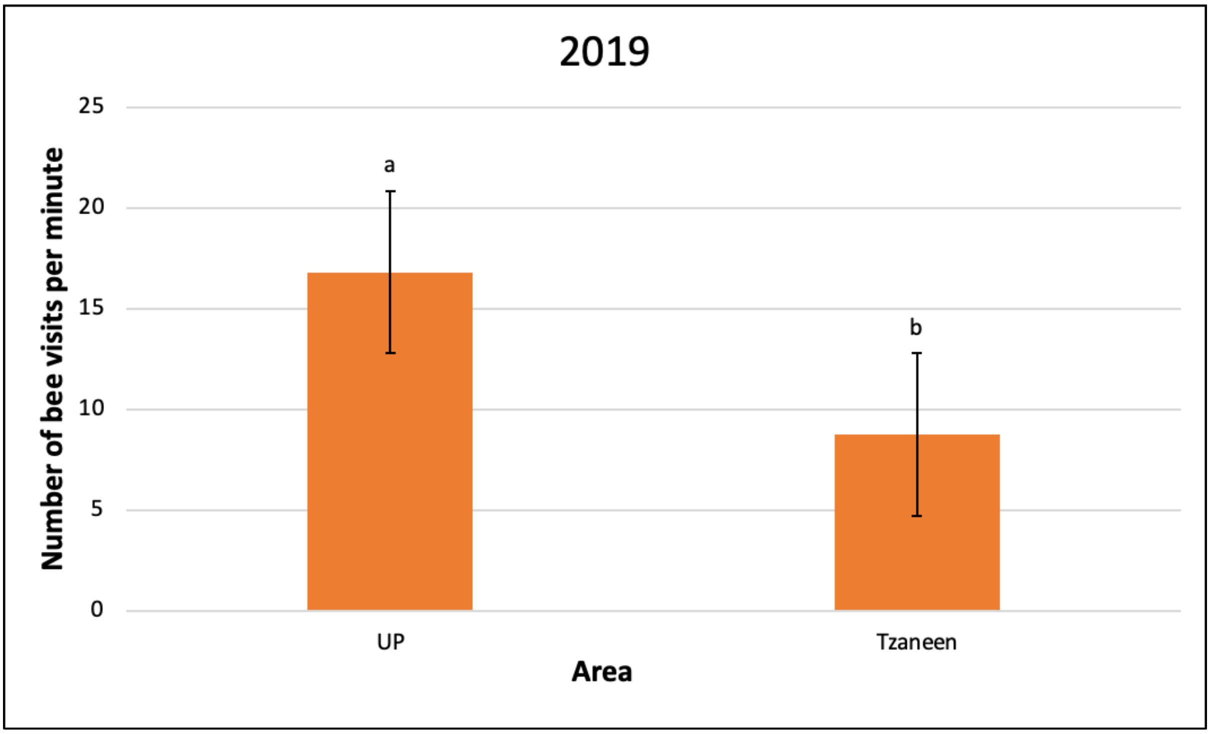


Figure 2.6. Bee visits per minute to avocado trees located at the Experimental Farm of the University of Pretoria and commercial orchards in the Politsi Valley in Tzaneen during the 2019 flowering season. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

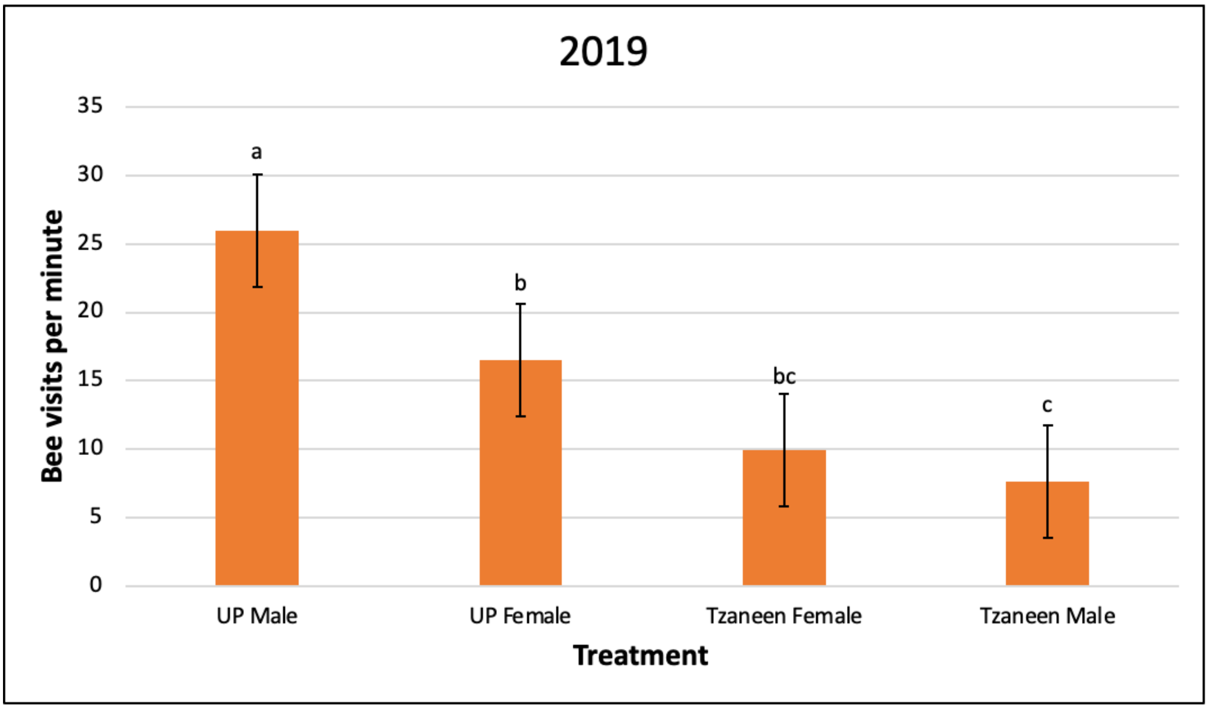


Figure 2.7. Bee visits per minute to avocado trees located at the Experimental Farm of the University of Pretoria and commercial orchards in the Politsi Valley in Tzaneen during the 2019 flowering season. An interaction effect between area and flower phase is observable. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

In a study conducted by Vicens and Bosch (2000), it was reported that wind speed, solar radiation and temperature affected honey bee activity in apple orchards. Due to the fact that the sample trees were in close proximity to other cultivars and the changes in sampling location, long term studies are necessitated to properly understand the effects of these variables on honey bee preferences, especially if factors like successful wind-pollination in avocados are considered (Ying *et al*. 2008). Du Toit and Swart (1993) report pollen-foraging honey bee activity to be greatest during the morning. At this time, ‘B-type’ cultivars will be open in the male phase with pollen available, which could be why pollen-foragers were more prevalent in the morning. However, Du Toit and Swart (1993) did not mention which avocado cultivars were used in their study. This highlights the potential of applying the methods implemented by Du Toit and Swart to different orchards of only ‘A-type’ and ‘B-type’ cultivars. This will allow for a greater understanding of how honey bee foraging is influenced by a flowers sexual phase and time of day.

At the close of this chapter, we can start to fill in the blanks of the organogram presented at the start of this dissertation (Figure 2.8).

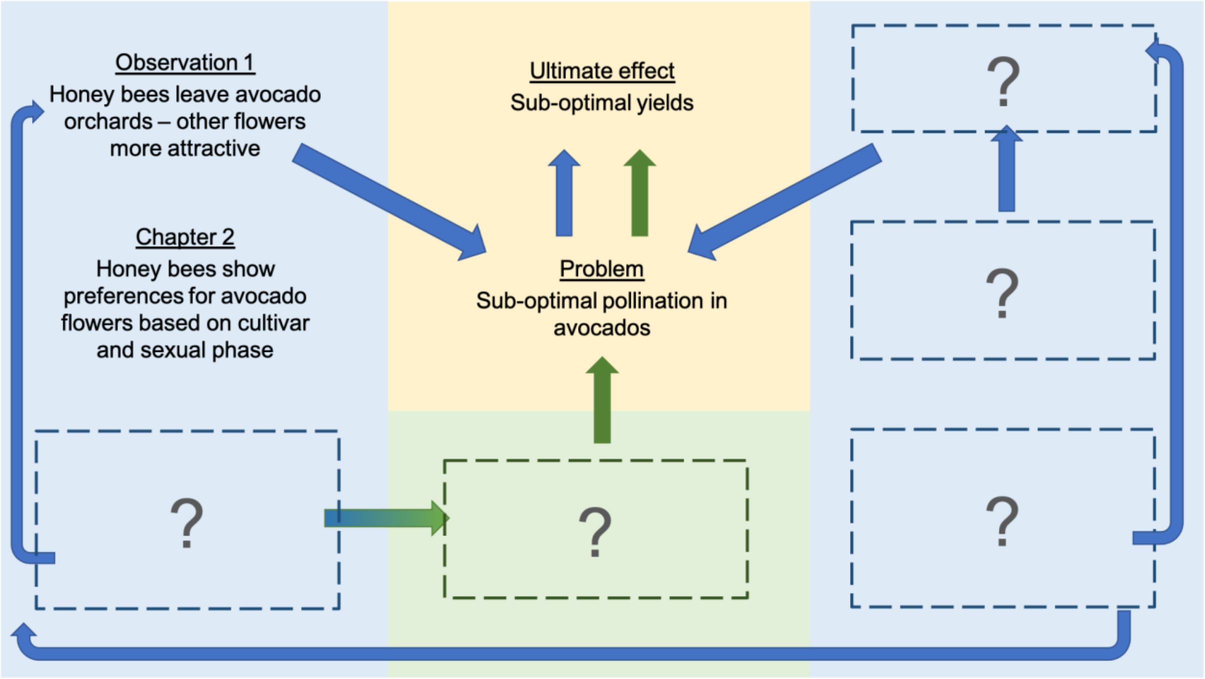


Figure 2.8. Organogram depiction of the holistic approach employed in this study to determine how sub-optimal pollination in avocados contributes to the ultimate effect of sub-optimal yields. The findings of Chapter 2 laid the basis for showing honey bees' preferences for certain avocado cultivars and flower sexual phase. Blue arrows represent pathways that undermine pollination in avocados and lead to yield reductions. Green arrows represent pathways that promote pollination in avocados and minimise yield reductions.

As honey bees have been observed to leave avocado orchards to forage among more attractive plantings, this chapter determined that honey bees also show a preference for avocado flowers on the basis of cultivar and sexual phase. Chapter 3 aims to conclude why this preference is so.

2.5 Conclusion

This study has shown that honey bees do show a preference for different avocados based on cultivar and sexual phase of the flower. Honey bee activity was continuously higher around actively male flowers. This may be due to the greater reward of pollen and nectar, and an enhanced floral display exhibited by flowers in the male phase. A consistent preference for actively male flowers may limit visitations to actively female flowers, reducing pollination and fruit set.

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CHAPTER 3

THE VOLATILE PROFILE OF THE AVOCADO (*PERSEA AMERICANA* MILL.) FLOWER

3.1 Abstract

Plants emit a wide range of volatile compounds for varied reasons. Arguably, one of the most important purposes thereof is in the deployment of mechanisms to facilitate delicate ecological relationships. The most vital of which is the process of pollination. The avocado (*Persea americana* Mill.) tree is no exception to this rule; however, the plant is hindered by the fact that the flowers are not particularly attractive to honey bee pollinators. This fact laid the basis for this investigation, which aimed to analyse the volatile profiles of avocado flowers in detail. Flowers were collected from a commercial orchard. Solid-phase microextraction (SPME) sampled the floral headspace of the flowers. Subsequent analysis was accomplished by thermal desorption with comprehensive gas chromatography with time-of-flight mass spectrometry (GC x GC-TOFMS). The results showed that depending on the sex of the flower and the cultivar of tree to which it belongs, contrasting volatiles will be released. The main class of volatiles to which these compounds belong were the mono- and sesquiterpenes. Honey bees have been shown to exhibit preferences for certain compounds. Therefore, it can be reasoned that the honey bee will show a preference for certain cultivars and as a result, pollinate these trees to a greater extent. By determining which cultivars promote honey bee activity, recommendations can be made to farmers about planting pollinator-attracting trees which will subsequently enhance yields of the avocado fruit.

Keywords: scent, headspace, gas chromatography, solid phase microextraction, time of flight mass spectrometry, pollination, attraction, honey bees, olfactometry

3.2 Introduction

Plants are complex organisms, with each species having a set of mechanisms aimed at maximizing reproduction and survival. One of these mechanisms includes the production and exudation of volatile compounds. According to Klatt *et al*. (2013), the primary function of volatile production is to facilitate cross-pollination by attracting insect facilitators.

3.2.1 Problem statement

Avocado (*Persea americana* Mill.) flowers are characterised as having “synchronous dichogamy” and therefore require pollination by insect vectors to successfully set fruit due to the temporal separation of the sexual phases of the flower (Nirody 1922). Avocados cultivated outside their native region of central Mexico rely on honey bees visiting the flower to facilitate pollination (Davenport 1986) (Figure 3.1). However, honey bees do not like the flowers of the avocado tree and will rather leave the orchards in which their hives are situated to forage on more attractive flowers, like that of macadamia (Gazit and Degani 2002). The notion that bees do not like avocado flowers is supported by Afik *et al*. (2008), who claim that honey bees will leave avocado orchards to forage in more desirable plantings of citrus trees.

Figure 3.1. Photographic illustration of a honey bee (*Apis mellifera*) gathering nectar from an avocado flower in the male phase.

Whilst some scholars may argue that the oil-rich fruit is the main limiting factor influencing the yield potential of avocado trees, many researchers have found that the majority of mature avocado fruits are the result of cross-pollination (Vrecenar-Gadus and Ellstrand 1985). Therefore, it is vital to determine whether or not sub-optimal pollination may further reduce the already low-fruiting nature of the avocado tree.

3.2.2 Aim of the study

According to Gruter and Ratnieks (2011), a species-specific mixture of certain volatile compounds will create a ‘floral constancy’, thereby promoting repeated honey bee visitation to preferred flowers. Determining the exact floral volatile composition of avocado flowers is therefore extremely important. A fully described avocado flower volatile profile will allow for the determination of which compounds are present and in what quantities. A subsequent comparison of the compounds will create a better understanding of which favoured and non-favoured volatiles are exuded from the avocado flowers. This may offer an explanation as to why honey bees show a preference for alternative flower sources.

3.2.3 Objectives of the study

The volatile profiles will be determined by sampling the floral headspace of avocado flowers with Solid-phase microextraction (SPME), with subsequent gas chromatography-olfactometry (GC-O) analysis. SPME was developed in the 1990’s and has since become a valuable tool for chemical compound extraction (Arthur and Pawliszyn 1990). It makes use of a thin, cylindrical column encasing fibres to which floral volatiles adsorb (Pawliszyn 1997, Wilson *et al*. 2018). GC-O then allows for a reliable analysis of these chemicals by desorbing the compounds and yielding a gas-chromatograph. This method will therefore allow for the creation of a detailed volatile profile of the avocado flower (Rusanov *et al*. 2011).

3.2.4 Hypotheses

Based on the findings of Chapter 2, it is expected that distinct floral volatiles will be detected for certain cultivars and flower sexual phase, and thus play a role in honey bee attraction. Furthermore, due to the honey bee's preference for other flowers (such as citrus) it can be reasoned that either repellent compounds will be present in high amounts, or attractive compounds will be lacking in the headspace of avocado flowers.

3.2.5 Significance of the study

To our knowledge, the exact volatile composition of avocado flowers has yet to be described. The honey bee perceives the volatiles emitted from a food source through olfactory signals (Menzel 1984). However, the variety and abundance of the volatiles in the outdoor environment run the risk of overwhelming the honey bee's perceptive abilities (Bruce and Pickett 2011). This is avoided by the highly sensitive and specialised volatile detectors, which allows the bee to select more favourable odours (Galizia and Menzel 2000). This explains why honey bees show a preference for certain flowers (Ashman 2000) and may account for the exodus of honey bees out of avocado orchards into more preferable ones. By determining whether or not honey bees show preferences for certain cultivars, commercial growers can select favourable pollinizing varieties. Pollination will thus be increased, consequently improving fruit set and yields.

3.3 Materials and Methods

3.3.1 Locality and environmental conditions

This experiment took place in the orchards at ZZ2 Farms in Tzaneen, located in the province of Limpopo in South Africa (23º63'S, 30º04'E). Flower samples were collected in the late winter, during the middle of August in both years. Two rounds of analysis were completed, the first being completed in 2016 and the next in 2018. This region is characterised as having a sub-tropical climate, with mild, frost free winters and hot, humid summers. In the period of late winter when sampling took place, temperatures ranged between minimums of 2° C and 3° C, and maximums of 29° C and 30° C for 2016 and 2018, respectively. Between July and September of 2016 and 2018, 2.58 mm and 6.93 mm of rainfall was received, respectively. No rain was received on the sampling days. Detailed environmental conditions for the sampling period are reported in Appendix A.

A 36-year-old ‘Hass’ and ‘Fuerte’ mixed planting and a 5-year-old ‘Maluma Hass’, ‘Galil’ and ‘Zutano’ mixed planting were used. ‘Hass’ trees were grafted on Guatemalan rootstocks and were spaced 7m within and between rows, while ‘Fuerte’ trees were grafted on ‘Duke 7’ rootstocks and were spaced 7m within and between rows. ‘Maluma Hass’, ‘Galil’ and ‘Zutano’ trees were grafted on ‘Duke 7’ rootstocks and were spaced 4m within rows and 6m between rows. The trunks of all trees were treated with PVA paint post-harvest to prevent sunburn.

3.3.2 Floral material sampling

The 2016 sampling focused on collecting 50 flowers of two cultivars, ‘Hass’ and ‘Fuerte’, with four replicate sample sets (n = 4). Both the male and female phases of the flowers were collected and kept separate. Sampling was completed in the morning and afternoon to facilitate the collection of both the female and male phases of the flower.

The 2018 sampling included a wider range of cultivars in addition to ‘Hass’ and ‘Fuerte’, namely ‘Maluma Hass’, ‘Galil”, ‘Ettinger’, ‘Zutano’ and an unnamed cultivar labelled as ‘Cultivar 17’. For each cultivar 50 flowers were sampled, which were replicated three times (n = 3).

3.3.3 Chemicals used for flower preservation

In order to maintain the turgidity and hydration of the flowers after collection, the samples were placed into a semi-solidified sucrose-enriched agar medium (Appendix B) in plastic containers, with the peduncles facing downward (Figure 3.2). It is important that no other constituents be added to this medium to limit interference with volatile detection.

3.3.4 Headspace sampling of avocado flowers onto polydimethylsiloxane (PDMS) silicone rubber multichannel traps (MCTs)

The flowers were sampled into multichannel traps (MCTs) using a sorptive technique developed in-house by colleagues at the University of Pretoria (Naudé and Rohwer 2013).

In 2016, 200 ml of floral headspace was sampled at a rate of 3.33 ml/s for 60 seconds. The MCTs containing the floral compounds were desorbed in a thermal desorber system (TDS) connected to a gas chromatogram (GC).

In 2018, 400 ml of floral headspace was sampled using a pump onto MCTs at a rate of 3.33 ml/s for 120 seconds. The MCTs containing the sorbed compounds were desorbed in a TDS connected to a GC.

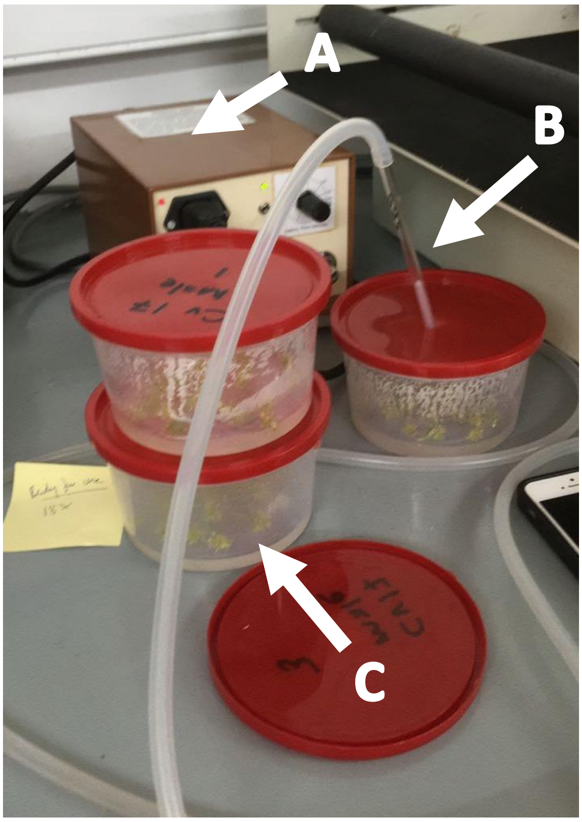

Figure 3.2. Headspace sampling of avocado flowers maintained in an agar medium. A – pump to extract volatiles in the floral headspace, B – polydimethylsiloxane (PDMS) silicone rubber multichannel traps (MCTs), C – flowers embedded in the agar medium in a sealed container.

3.3.5 Volatile analysis using thermal desorption-comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (TD-GCxGC-TOFMS)

For both 2016 and 2018, compound separation was completed using a LECO Pegasus 4D GC x GC-TOFMS with an Agilent 7890 GC (LECO Africa (Pty) Ltd., Kempton Park, South Africa). The system contained a secondary oven and a dual stage modulator. Nitrogen gas was used for the hot jets, and liquid nitrogen was used to cool nitrogen gas for the cold jets. The carrier gas, helium (He), was of ultra-high purity grade (Afrox, Gauteng, South Africa). Conditions of the TD-GC x GC-TOFMS are reported in Appendix C.

Tentative identification of compounds was based on the comparison of the sample mass spectra to that of the National Institute of Standards and Technology (NIST14) library, with compounds exhibiting a ≥ 75 % match being reported.

3.3.6 Data analysis

Due to the sensitivity of the sampling and identification methods, much of the data were unevenly distributed and had heterogenous variances. Kim *et al*. (2011) highlight these as regular problems associated with ‘experimental variations that cause problems with metabolomic analysis and data processing’. Due to this, it was recommended that the sampling length (60 seconds vs 120 seconds) and amount of volume (200 ml vs 400 ml of floral headspace) sampled be doubled in the second sampling year. The dataset was also large and highly correlated, which required a great amount of processing power. Therefore, in order to make valid comparisons, multivariate analyses were used. According to Bartholomew (2010), multivariate analysis allows for the interpretation of “interrelationships between many variables”.

Given this, it was decided that the best analytical method would be canonical variate analysis (CVA), commonly known as linear discriminant analysis. This method is suited to showing differences between treatment groups as opposed to individuals within those groups (Digby and Kempthorne 1987). Upon beginning the CVA, the large number of variables was reduced to smaller sets of variables which accounted for the majority of the variability. This new set of variables, known as canonical variates (CV), become linear combinations of the original dataset and are used as loading vectors for the original data. This was accomplished using the statistical program GenStat® (VSN International 2017).

3.4 Results and Discussion

For the 2016 trial, a total of 185 compounds were detected. A two-dimensional plot accounting for 97.8 % of the total variation of the 20 most prevalent compounds showed the similarity between the four treatment groups (Figure 3.3). In such a plot, treatment points closer to one another are more similar and treatment points further from one another are more dissimilar. When treatment clusters overlap, there are no significant differences at a 5 % confidence level (Krzanowski 1988). The results in Figure 3.3 showed a clear contrast in compounds exuded from the 'Hass' and 'Fuerte' flowers. There were no significant differences ($p \leq 0.05$) between the actively male and female 'Hass' flowers, but differences were significant between the actively male and female 'Fuerte' flowers. The compounds that discriminated the most between treatment groups were eucalyptol and α-pinene.

In 2018 over 1600 compounds were detected, partly due to the increase in the number of treatments and partly due to the increase in sampling length and volume of floral headspace. The results for the 2018 trial yielded a two-dimensional plot accounting for 80.06 % of the total variation of the 16 most prevalent volatile compounds (Figure 3.4). These results showed that the volatile profiles of the 'Ettinger' male, 'Fuerte' male, 'Galil' female, 'Zutano' male and 'Hass' female flowers to be the most unique ($p \leq 0.05$). The compounds that discriminated the most between treatment groups were α-pinene, eucalyptol and germacrene D. The comprehensive datasets are archived at the Department of Plant and Soil Sciences at the University of Pretoria and can be accessed upon request.

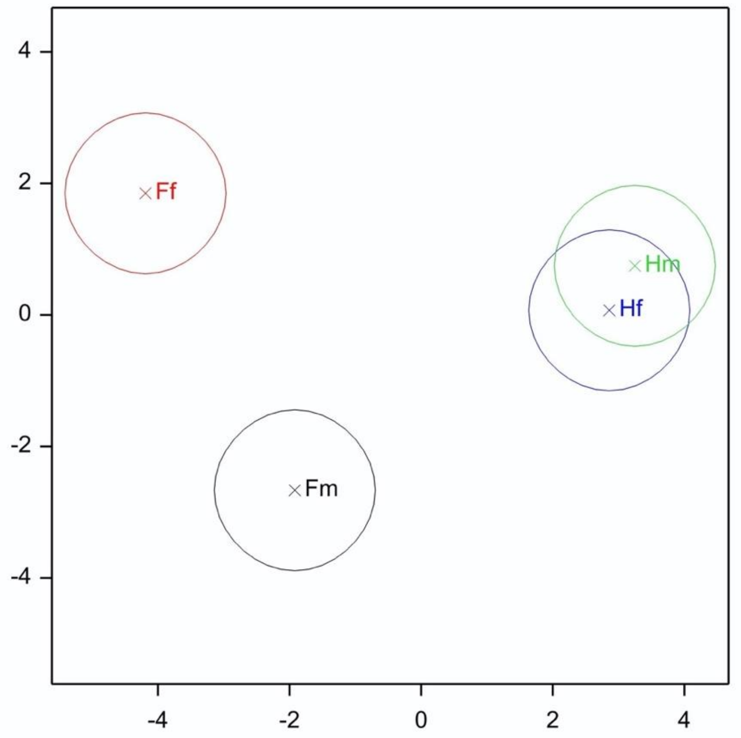

Figure 3.3. Plot of the canonical variate analysis (CVA) of treatment means in the 2016 sampling season. Ff (red) – 'Fuerte' female, Fm (black) – 'Fuerte' male, Hm (green) – 'Hass' male, Hf (blue) – 'Hass' female. Note the overlapping clusters of the 'Hass' treatment groups. Significance is detected when treatment clusters do not overlap (p ≤ 0.05).

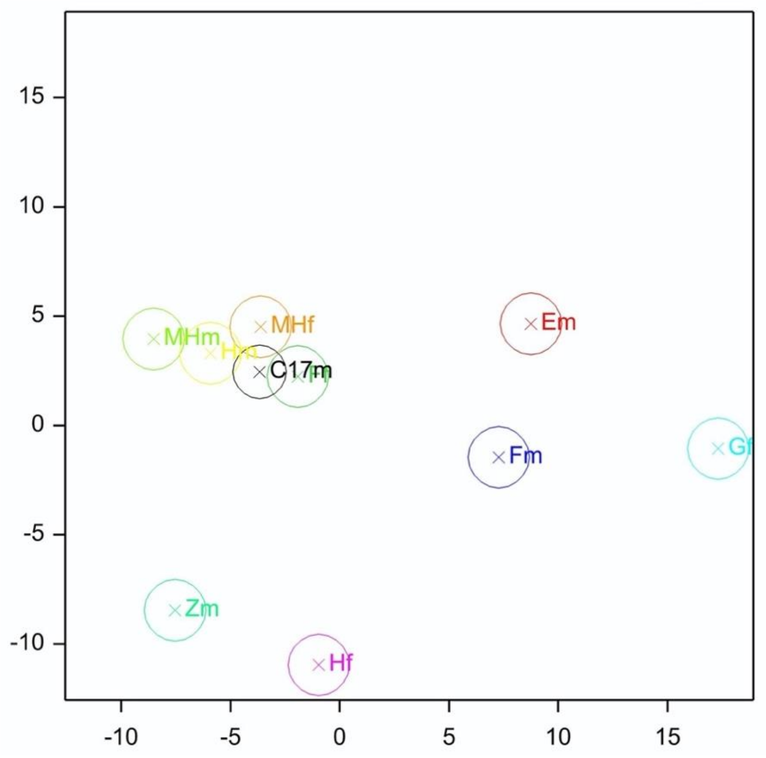

Figure 3.4. Plot of the canonical variate analysis (CVA) of treatment means in the 2018 sampling season. MHm (light green) – 'Maluma Hass' male, Hm (yellow) – 'Hass' male, MHf (orange) – 'Maluma Hass' female, C17 (black) – Cultivar 17, Ff (dark green) – 'Fuerte' female, Em (red) – 'Ettinger' male, Fm (dark blue) – 'Fuerte' male, Gf (light blue) – 'Galil' female, Zm (turquoise) – 'Zutano' male, Hf (pink) – 'Hass' female. Significance is detected when treatment clusters do not overlap (p ≤ 0.05).

After concluding that there were differences in volatile profiles between treatment groups, it would be important to know what quantities were exuded. When considering the 'Hass' and 'Fuerte' cultivars only, it was found that in both 2016 and 2018 the five most prominent volatiles found within the floral headspaces were limonene, α-pinene, β-pinene, α-phellandrene and eucalyptol (Figures 3.5). All of these compounds are terpenoids, classified specifically as monoterpenes. According to Knudsen *et al*. (1993) secondary plant metabolites are mostly terpenoids, many of which are volatile compounds that contribute to floral scent in countless plant species.

Based on the reports of Afik *et al*. (2006), avocado production is hindered by the honey bee's preference for other flowers, like that of citrus crops, compared to those of the avocado. It would therefore be useful to compare the volatile profile of citrus flowers with that of the avocados to determine if there are any differences that may account for this preference. According to the results of a study by Azam *et al*. (2013), headspace-solid phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) analysis of nine citrus varieties detected 110 flower volatiles. The majority of these volatiles were monoterpenes and sesquiterpene hydrocarbons. The headspace of the avocado flowers consisted of mainly monoterpenes and sesquiterpenes, with monoterpenes being found in much higher concentrations. Another interesting aspect that needs to be highlighted from the Azam *et al*. (2013) study is the main volatiles found in the headspace of citrus flowers. The monoterpene linalool was released in abundance from the citrus flowers, ranking highest in the majority of the nine cultivars sampled. Linalool was not found to be present in the 2016 avocado flower samples and was detected in trace amounts in the 2018 samples. It is for this reason that linalool was included in Figure 3.5. Henning *et al*. (1992) have reported on the attractiveness of linalool to honey bees. When exposed to optimised concentrations of linalool in a screened flight chamber, it was observed that it had an attractive influence on honey bees. This result led Henning *et al*. (1992) to suggest that plant breeders, in the case of alfalfa specifically, select varieties with increased linalool concentrations. Therefore, the lack of linalool in the floral headspace of avocado flowers may contribute to their lack of attractiveness. However, it may not be as simple as selecting avocado varieties with higher concentrations of a single volatile compound. For many pollinators, a specific mixture of volatiles is required for the perception of a floral scent (Wright *et al*. 2005, Byers *et al*. 2014). Galen *et al*. (2011)

explain how one volatile can have an attractive and deterrent effect depending on the concentration in which it is released.

Additional inferences can be made from the results depicted in Figure 3.5. A general trend, one especially prevalent in the 2016 dataset, shows that the volatiles exuded from ‘Fuerte’ flowers have a higher relative peak association (% RPA) compared to those of the ‘Hass’ flowers. In Chapter 2, differences in honey bee activity surrounding the different sexual phases belonging to different avocado varieties were described. Honey bees appeared to favour ‘B-type’ cultivars, such as ‘Fuerte’ and ‘Zutano’. This preference may be due to the increased exudation of floral volatiles from these flowers, which plays a vital role in pollinator attraction (Schiestl *et al*. 2014). Chapter 2 also made the honey bees preference for male avocado flowers clear. This chapter discussed the morphological aspects that may lead to this preference, but the impact of volatile emissions has not been explored. In the case of some, but not all, of the five volatiles depicted in Figure 3.5, the male phase of the flowers exuded a higher percentage compared to the female phased flowers of the same cultivar. Klatt *et al*. (2013) explain how strawberry (*Fragaria x ananassa* Duchesne) flowers with enhanced volatile emissions are more detectable and thus more attractive to pollinators. Therefore, it appears that avocado flowers active in the male phase are more attractive to honey bees because they exude a higher volume of volatiles.

The differences in the average % RPA between the 2016 and 2018 sampling years must be mentioned (Figure 3.5). In spite of the longer sampling period (60 seconds vs 120 seconds) in 2018, the % RPA for the 5 most popular compounds was much lower. This is most likely due to the almost ten-fold increase in the total amount of compounds detected in 2018. Thus, the overall contribution of the five most prevalent volatiles will ultimately be less, which explains the lower % RPA.

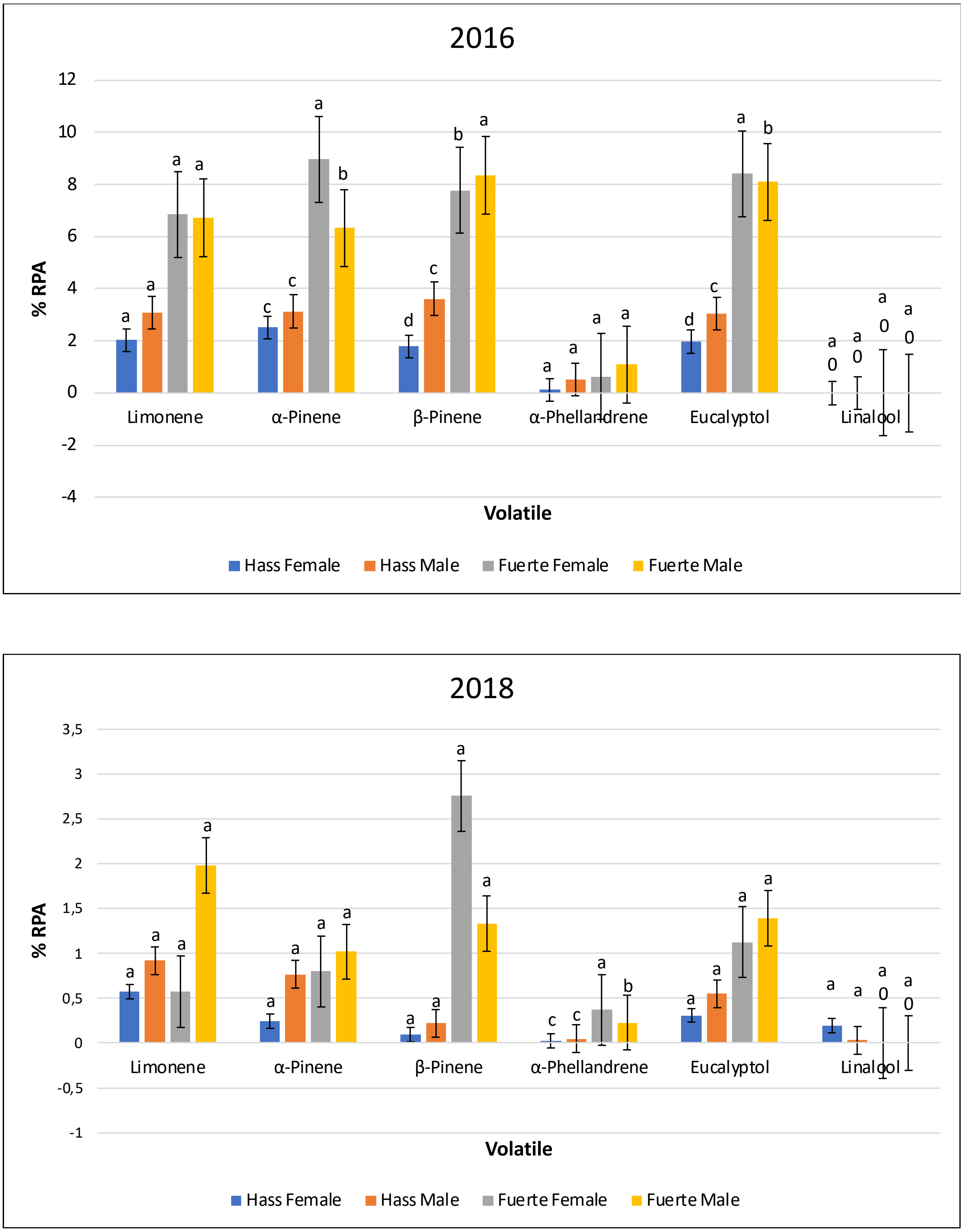

Figure 3.5. Major volatiles found in the headspace of ‘Hass’ and ‘Fuerte’ flowers active in the female and male phases in the 2016 and 2018 sampling season. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

In gender dimorphic plants, male flowers are often visited more than their female counterparts (Ashman 2000, Ashman *et al*. 2005). Studies focusing on pollinator preference for hermaphroditic flowers showed that female flower visitation was increased by 50 % when these flowers were treated with extracts of hermaphroditic scents (Ashman *et al*. 2005). This is interesting to note as a honey bee's preference for citrus flowers has been highlighted above. A citrus flower is 'perfect', containing simultaneously functional male and female organs (Krezdorn 1978). As an avocado flower is synchronously dichogamous, the hermaphroditic nature of citrus flowers may further explain why they are favoured by honey bees.

These findings allow us to add another piece to our low pollination puzzle (Figure 3.6).

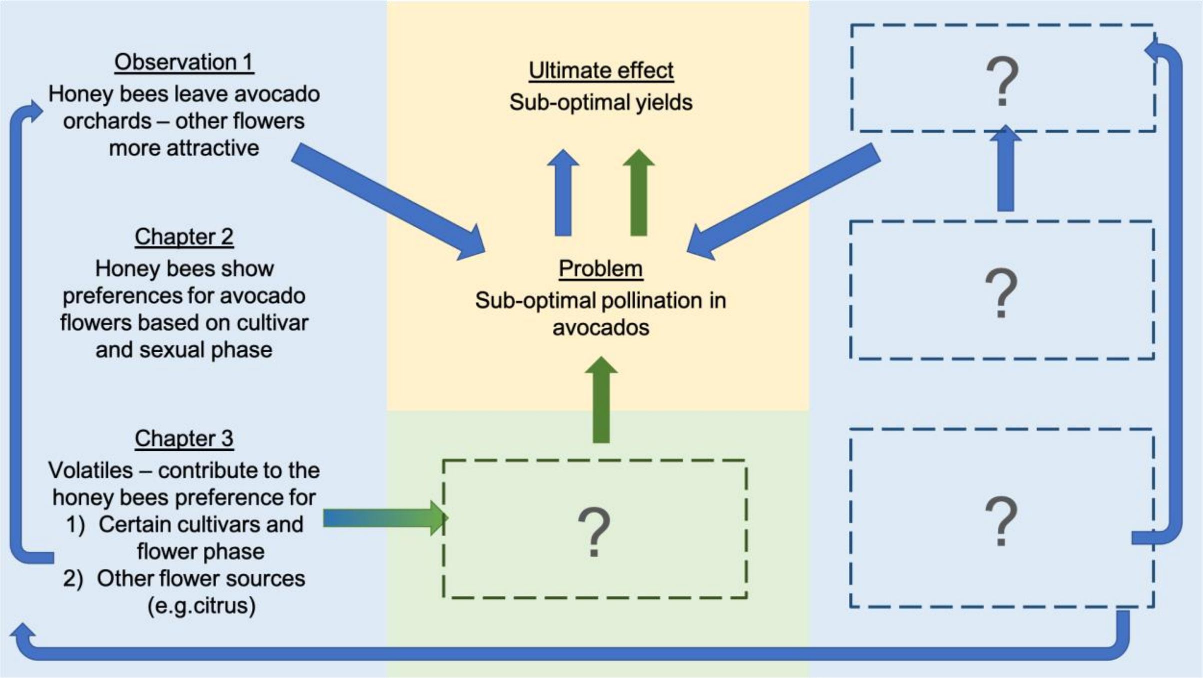

Figure 3.6. Organogram depiction of the holistic approach employed in this study to determine how sub-optimal pollination in avocados contributes to the ultimate effect of sub-optimal yields. Chapters 2 and 3 uncovered the role played by volatile emissions in influencing honey bee preferences for avocado flowers. Blue arrows represent pathways that undermine pollination in avocados and lead to yield reductions. Green arrows represent pathways that promote pollination in avocados and minimise yield reductions.

Chapter 3 has highlighted the influence of floral volatile emission on a honey bees' preference for avocado flowers. This allowed for the determination that 1) the male phase of the flowers release more volatiles compared to those in the female phase, and 2) 'B-type' flowers ('Fuerte' for example') generally release more volatiles compared to 'A-type' flowers. These two factors could explain why honey bees favour those flowers, as Chapter 2 discussed. Lastly, the low concentrations of linalool (a compound prevalent in the headspace of citrus flowers) may explain why honey bees find other flowers more attractive. All these factors contribute to the problem of low pollination in avocados that ultimately leads to yield reductions.

3.5 Conclusion

Through the use of floral headspace sampling and GC-TOFMS techniques, the difference in volatile emission between avocado flower phase, and cultivar to which those flowers belong, was made clear for the first time. For the 'Hass' and 'Fuerte' cultivars, the volatiles limonene, α-pinene, β-pinene, α-phellandrene and eucalyptol were released in the highest quantities. Furthermore, 'Fuerte' flowers released higher quantities of most of the volatiles compared to 'Hass' flowers. Also, actively male avocado flowers generally exude higher concentrations of volatiles compared to their female counterparts.

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CHAPTER 4

IMPROVING POLLINATION IN AVOCADO (*PERSEA AMERICANA* MILL.) ORCHARDS USING HONEY BEE ATTRACTANTS

4.1 Abstract

There are numerous insect pollinators for the avocado (*Persea americana* Mill.) tree in its native central Mexico. However, the honey bee is the primary pollinator in other regions. Many experts believe poor pollination is a yield-limiting factor in avocado production, a reason which may be due to the honey bees' preference for other flower sources. Honey bees have often been observed leaving an avocado orchard to forage amongst more attractive flowers. This study attempted to determine whether or not attractants could be used to limit this honey bee exodus. Two separate treatments were employed by intercropping 'Hass' and 'Fuerte' trees with *Lavandula stoechas* and hanging lemongrass (*Cymbopogon citratus*) essential oil lures in their canopies. The lemongrass lures were shown to have the greatest effect on honey bee activity, prolonging bee visitation even as flowering decreased throughout the season. The lavender plants had little to no effect on bee activity compared to the control groups. However, the lavender did appear to improve fruit set in trees experiencing an 'off' production year where flower production was low, suggesting lavender can be effective in mitigating the effects of low honey bee activity during 'off' years.

Keywords: flowering, attractants, lemongrass, lavender, essential oils, lures, scent, intercropping, yields, alternate bearing

4.2 Introduction

When cultivated in their region of origin, there are a multitude of insect pollinators for the avocado (*Persea americana* Mill.) tree (Wysoki *et al*. 2002). Due to the open nature of the avocado flower, it can be potentially pollinated by a variety of insects (Visscher and Sherman 1998). The avocado flowers are synchronously dichogamous (Nirody 1921, Davenport 1986, Ish-Am and Eisikowitch 1993). There is thus a temporal separation between the periods in which the female and male organs are active. For all cultivars, the avocado flowers open first in the actively female phase. These flowers close and then the same flower reopens the following day in the active male phase. The flowers of ‘A-type’ cultivars, such as ‘Hass’, are actively female in the morning and actively male in the afternoon (Stout 1923). The flowers of ‘B-type’ cultivars, such as ‘Fuerte’ are functionally female in the afternoon and functionally male in the morning (Stout 1923). To facilitate adequate cross-pollination, it is common practice to install a variety of ‘B-type’ cultivars to act as pollinisers for ‘A-type’ cultivars in commercial orchards (McGregor 1976, Bezuidenhout *et al*. 2017). As reported by Nieto (1985), pollinators of avocados in the south of Mexico include honey bees, flies, wasps and beetles. Stingless bees and wasps are believed to be the primary pollinators of the avocados grown in their native environments (Castañeda-Vildózola *et al*. 1999, Ish-Am *et al*. 1999, Perez-Balam *et al*. 2012). However, since the avocado has been cultivated outside its native region since the 1700’s, honey bees have become the primary commercial pollinator in these regions (Bergh 1992, Roubik 1998) (Figure 4.1).

Waller (1969) proved that alfalfa (*Medicago sativa*) fields sprayed with geraniol were effective in attracting honey bees. Malerbo-Souza *et al*. (2004) found similar success with compounds such as eugenol, citral and lemongrass extract applied to sweet orange blooms. According to Toungos (2019), lemongrass is an effective honey bee lure often used in the commercial beekeeping industry.

However, manipulating perceived floral scent may not be the only method of attracting honey bees. Moghaddam *et al*. (2020) conducted a study which showed the positive effects of intercropping onion (*Allium cepa*) with ajwain (*Carum copticum*) and chamomile (*Matricaria chamomilla*) to increase pollination of the onion flowers. Other suitable plants to attract honey bees include *Lavandula stoechas* (Pawelek *et al*. 2009), flowering basil (*Ocimum basilicum*) (Pereira *et al*. 2015), and sunflowers (*Helianthus annuus*) (Jones and Gillett 2005).

Figure 4.1. Honey bee (as indicated by the white arrow) pollinating an actively male 'Fuerte' avocado flower.

4.2.1 Problem statement

Honey bees have been known to leave the orchards in which their hives are placed to forage for more attractive flowers (Afik *et al*. 2008). This is especially problematic for synchronous, dichogamous trees like the avocado which require insect visitations to facilitate cross-pollination (Ish-Am and Eisikowitch 1993). In Israel, it has been reported that as few as five percent of all the flowers produced by an avocado tree receive sufficient pollination (Ish-Am & Eisikowitch 1998). In Chapter 2, a honey bee's preference for avocado flower based on cultivar and flower phase was discussed.

4.2.2 Aim of the study

Chapter 3 reported on the distinct volatiles exuded from the flowers of different avocado cultivars while active in the distinct sexual phases. Therefore, this study aimed to investigate the possibility of luring honey bee pollinators to an avocado orchard using attractants.

4.2.3 Objectives of the study

This study sought to improve pollination and fruit set in an avocado orchard using two honey bee-attractive methods. The first entailed placing lemongrass (*Cymbopogon* *citratus)* essential oil lures in the canopy of the trees, whilst the second analysed the effects of intercropping with flowering lavender (*Lavandula stoechas*).

4.2.4 Hypotheses of the study

It is expected that the lemongrass lures and intercroppings of lavender will be attractive to honey bees. Due to the placement of the lemongrass lures in the canopy, it is predicted that these will be more effective in improving bee activity surrounding the canopy of the avocado trees. Furthermore, if the attractants do increase honey bee activity it is likely that fruit set will be improved.

4.2.5 Significance of the study

Alcaraz and Hormaza (2009) reported a 15 % increase in fruit set in hand-pollinated avocado flowers. These findings highlight the problem of inadequate pollination in avocados, an issue which may be caused in part due to the honey bees' non-preference for the avocado flowers (Afik *et al*. 2008). If previous studies have shown the efficacy of attractants on improving pollination in other species, then it is important to explore the viability of these methods to increase pollination in avocado flowers.

4.3 Materials and Methods

4.3.1 Locality and environmental conditions

Trials were conducted in a 12-year-old *P.americana* orchard on the Hillcrest Campus Experimental Farm of the University of Pretoria (25°45'S 28°16'E) (Figure 4.2). During the time of sampling (August – September 2019) minimum temperatures fluctuated between 5° C and 15° C, and maximum temperatures fluctuated between 18° C and 32° C. At this time 2.7 mm of rainfall was recorded. The trees received supplemental drip irrigation every second day. The orchard experienced sunny, clear days on every sampling day. Detailed environmental conditions for the sampling period are reported in Appendix A.

The orchard is a mixed planting of ‘Hass’ and ‘Fuerte’ trees, grafted on ‘Dusa’ and ‘Latas’ rootstocks, respectively. Trees were planted 5 m apart within rows and 6 m apart between rows. To protect the trees from sunburn, the trunks of the trees were whitewashed with PVC paint earlier in the growing season. The avocado orchard is in close proximity to a small number of beehives 100 m away from the trees. Located 200 m in the opposite direction to the hives is a lemon orchard.

Sampling was completed during the avocado flowering season in September 2019. Two methods of attracting honey bees to the avocado orchards were implemented: lemongrass essential oils (adapted from Waller 1969 and Malerbo-Souza *et al*. 2004) and flowering *L.stoechas* plants (adapted from Pawelek *et al*. 2009 and Moghaddam *et al*. 2020). Treatments were applied to both ‘Hass’ and ‘Fuerte’ trees. Each treatment and a control for each cultivar were replicated three times (n = 3).

It must also be noted that there was a mixture of trees in ‘on’ and ‘off’ years (Figure 4.3). Many ‘Hass’ trees appeared to flower more profusely than the ‘Fuerte’ trees, indicating the former were experiencing an ‘on’ flowering year and the latter an ‘off’ flowering year. Due to limitations imposed by COVID-19 restrictions, this study could not be repeated for a second year in 2020.

Figure 4.2. Google Earth screenshot of the avocado orchard (surrounded by the white square) on the Experimental Farm at the University of Pretoria’s Hillcrest campus.

Figure 4.3. Flowering ‘Hass’ (left) and ‘Fuerte’ (right) avocado trees. Note the profuse flowering on the ‘Hass’ tree, showing the contrast between the ‘on’ and ‘off’ flowering years that the orchard was experiencing.

4.3.2 Lemongrass and lavender treatments

Based on the findings of Balbuena *et al*. (2012) and Pawelek *et al.* (2009), lemongrass essential oils and *Lavandula stoechas* were used as honey bee attractants in this trial. Due to the prolonged flowering season of the avocado, the once-off spraying methodologies as described by Waller (1969) and Malerbo-Souza *et al*. (2004) were modified. Considering the extended flowering season of the avocado trees, the once-off sprays would not have been ideal to attract honey bees for an effective time period. Therefore, lures were hung in the canopies to continuously release attractive compounds. The methodology described by Moghaddam *et al*. (2020) entailed the interplanting of chamomile between rows of alfalfa. As the avocado orchard used in this trial could not be altered in any permanent way, potted lavender plants were placed in a ring next to one another under the canopies instead. The lavender were all flowering profusely during the trial.

To install the lemongrass treatments, the openings of essential oil vials were covered with a fine mesh (Figure 4.4a). The mesh prevented insects from falling into the essential oil whilst at the same time allowed for the volatilization and perception of the scent. These vials were then hung on the tree, one vial was hung on one branch in each of the cardinal directions (N, E, W and S) (Figure 4.4b). For the lavender treatments, 24 flowering lavender plants were placed in a circle at the base of the tree (Figure 4.5). The bases of the pots were sealed with cling wrap to enhance water retention. The plants were manually irrigated once every two days.

Figure 4.4. (a) Photo depicting the Dis-Chem pharmacy branded lemongrass 100 % essential oil in a container with the opening covered in a fine mesh. (b) Photographic illustration of a lemongrass essential oil container hanging on an avocado branch, facing in the southerly direction.

Figure 4.5. (a) Photographic illustration of 24 flowering *Lavandula stoechas* plants arranged in a ring under a ‘Fuerte’ tree. The bases were wrapped in cling film to prevent excess water loss. (b) Photographic illustration of 24 flowering *L.stoechas* plants in 5 litre pots under a ‘Fuerte’ tree.

4.3.3 Honey bee monitoring

In order to determine the frequency of honey bee visitation to the different cultivars a 1 x 1 m lightweight frame was held 0.5 m away from the canopy of the trees (modified from Calzoni and Speranza 1998 as described in Chapter 2) (Figure 4.6). Measurements were taken for all of the four cardinal directions of the tree (N, E, W and S) during the morning and afternoon to record activity for the male and female phase of the flowers. As mentioned in Chapter 2, the hours between 12:00 and 14:00 could be seen as a ‘transition phase’ during which time the actively female flowers started to close and the actively male flowers started to open in the ‘Hass’ trees, and vice versa for the ‘Fuerte’ trees. Therefore, the morning counts were conducted between 9:00-12:00 and the afternoon counts between 14:00-17:00.

Figure 4.6. Demonstration of the method of monitoring honey bee activity using a 1 x 1 m frame held 0.5 m away from the edge of the canopy.

The number of honey bees flying into the frame were counted for a period of 60 seconds. These readings were repeated over the course of five days as the season progressed.

4.3.4 Measuring fruit set

In order to measure the efficacy of the treatments in not only attracting honey bees, but the impact thereof on fruit set too, a branch was tagged on each of the four cardinal directions (N, E, W and S) of the control, lemongrass and lavender trees (Figure 4.7).

Figure 4.7. Photographic demonstration of how the avocado branches were tagged. The number of inflorescences, flowers and fruit set were counted from where the tag was placed to the end of the branch.

The number of inflorescences and flowers were counted prior to the introduction of the treatments. At the end of October in 2019, fruit set and fruit set percentage (%) of all the flowers previously counted was measured using the following formula:

Fruit set percentage (%) = (Total number of fruitlets counted ÷ Total number of flowers counted) x 100

4.3.5 Data analysis

A factorial analysis was followed by Fisher's LSD test to determine significant differences. This was accomplished using the statistical program GenStat® (VSN International 2017).

4.4 Results and Discussion

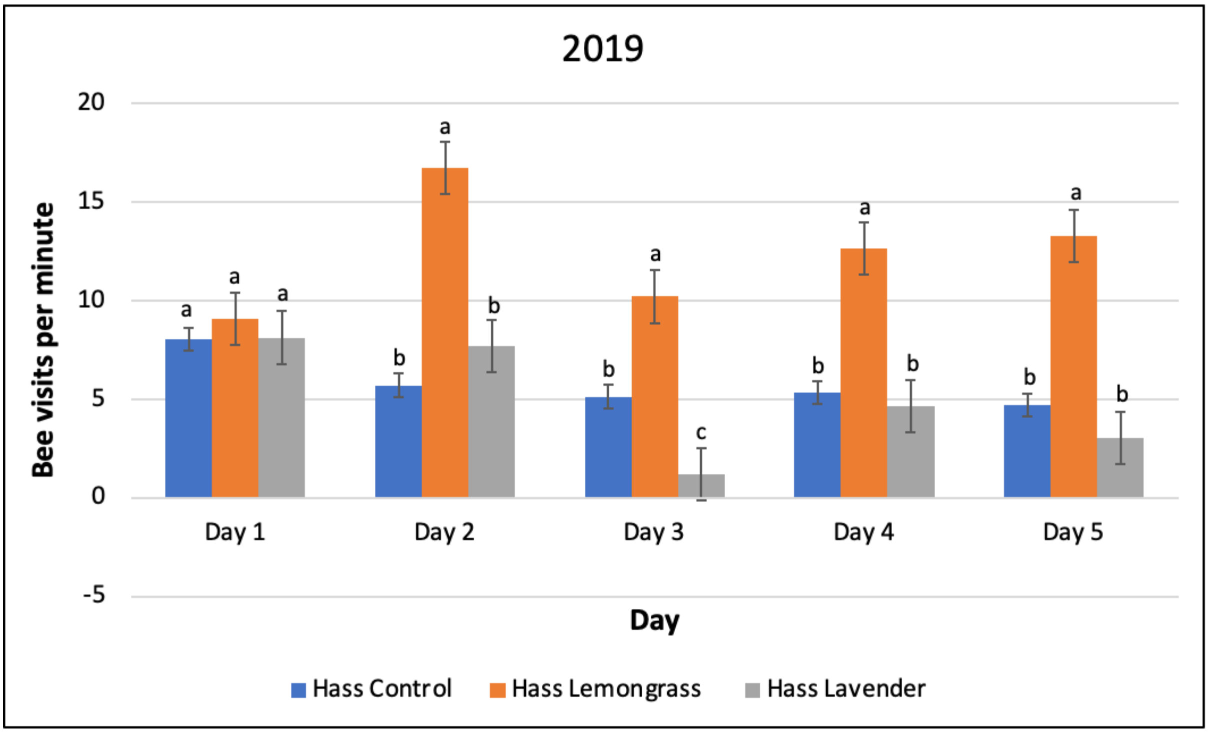
The bee framing results for the four directions of each tree, as well as the morning and afternoon samplings, were combined to get an average for each treatment as there were no patterns of bee activity based on tree direction and time of sampling. The results for the 'Hass' and 'Fuerte' trees are depicted in Figure 4.8. For the 'Hass' trees (Figure 4.8), bee activity surrounding the control group steadily decreased over the course of the five sampling days (8.04 bee visits per minute declined to 4.71 bee visits per minute). The trees that received the lavender treatment also experienced a decline in bee activity, although to a slightly larger extent compared to the control (8.13 bee visits per minute declined to 3.04 bee visits per minute). The trees that received the lemongrass treatment exhibited a steady increase in bee activity (9.08 bee visits per minute increased to 13.04 bee visits per minute).

For the 'Fuerte' trees (Figure 4.8), both the control (from 4.75 bee visits per minute to 1.54 bee visits per minute) and lavender (from 3.92 bee visits per minute to 0.67 bee visits per minute) groups showed a decline in bee activity over the course of the five sampling days. Observations for the 'Fuerte' lemongrass treatments were similar to that for the 'Hass' flowers, which appeared to increase bee activity over the course of the sampling period (from 2.45 bee visits per minute to 3.71 bee visits per minute).

The control group was important not only in terms of comparing the effects of the lavender and lemongrass treatments, but it also gave an indication of the gradual decrease in bee activity as the flowering season progressed (Figure 4.8). Throughout the sampling period it was noted that flowering steadily decreased, and the flowers began to set fruit.

For both 'Hass' and 'Fuerte', the control and lavender treatments decreased at a similar rate. This indicates that the lavender appeared to have little to no influence on increasing honey bee activity surrounding the canopy of the avocado trees. Based on the findings of Afik *et al*. (2008) honey bees will prefer to forage for more attractive flowers than remain in avocado orchards. The nectar found in lavender flowers is rich in sugar, which in addition to the colourful bracts atop the inflorescences, aid in pollinator attraction (Herrera 1997). It appears that in spite of this, the lavender flowers did not attract honey bees to the vicinity of the avocado canopy, nor did they lure honey bees away from the canopy. Therefore, in spite of Afik *et al*. (2008) claiming larger orchards of more attractive flowering plants being able to influence honey bee activity it appears that is not the rule for all flowers, at least from what these results suggest. It is widely reported that the health of a honey bee colony is dependent on a variety of floral resources (Naug 2009), therefore, it would not be prudent to advise farmers to remove all sources of floral competition, even during the avocado flowering season.

Once again, the lemongrass treatment affected the ‘Hass’ and ‘Fuerte’ trees similarly (Figure 4.8); honey bee activity appeared to increase slightly over the course of the treatment period (9.08 bee visits per minute increased to 13.29 bee visits per minute for ‘Hass’ and 2.45 bee visits per minute increased to 3.71 bee visits per minute for ‘Fuerte’). Lemongrass is proposed to be as effective in attracting honey bees as the lure pheromone produced by the bee’s Nasonov gland (Toungos 2019), which our findings corroborate. Balbuena *et al*. (2012) also report of the efficacy of lemongrass essential oil in attracting honey bees. It therefore appears that lemongrass lures could be effective in improving honey bee activity in an avocado orchard. This could be especially useful to prolong honey bee activity towards the end of the flowering season when flower numbers are low. To confirm these findings, further studies should include different attractants of the same nature. For example, the attracting effects of live lavender and lemongrass plants (or essential oil concentrates of both plants) should be compared with one another to determine that it is the attractive scent influencing honey bee activity, and not concentration of that scent.



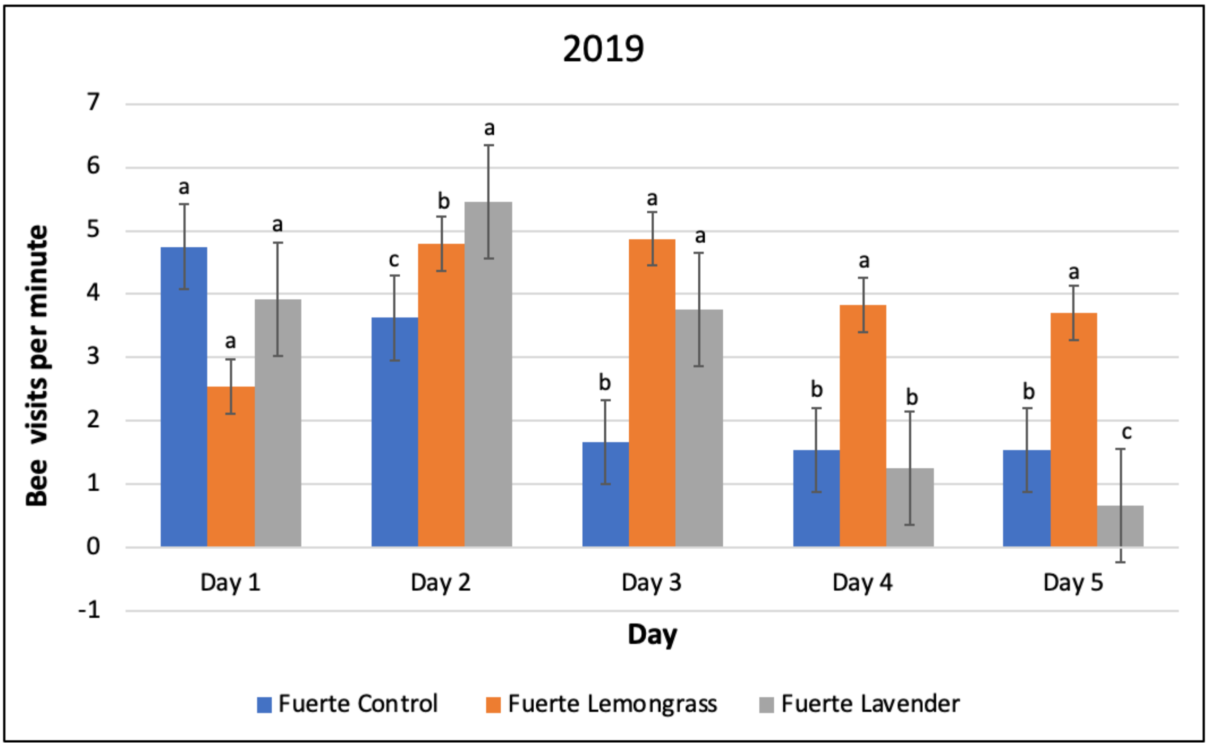


Figure 4.8. Honey bee visits to the treatments applied to ‘Hass’ and ‘Fuerte’ trees over the course of the five sampling days in 2019. Blue bars – Control, Orange bars – Lemongrass treatment, Grey bars – Lavender treatment. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

It was immediately clear that the difference in honey bee activity between the ‘Hass’ and ‘Fuerte’ trees was drastic. As Figure 4.9 and Figure 4.10 show, the ‘Hass’ trees had on average more inflorescences, and more flowers overall, compared to the ‘Fuerte’ trees. According to Akter *et al*. (2017), pollinators prefer plants that have a greater number of flowers. The reason for this preference may be due to many factors, such as a perceived higher reward offered by a larger number of flowers in addition to the flowers being easily detected from a farther distance (Giurfa *et al*. 1996). This pattern of behaviour may also explain the increase in honey bee activity surrounding the ‘Hass’ trees that received the lemongrass treatment. As reported in Figure 4.10, it was recorded that these ‘Hass’ trees had over double the number of flowers (1 012 flowers) compared to the next highest treatment group (426 flowers). This highlights the importance of flower production in avocados and contrasts the differences between trees in ‘on’ and ‘off’ production years. This study draws attention to the problems associated with compromised flower production, as emphasised by Akter *et al*. (2017) and Giurfa *et al*. (1996). Compromised flower production compounds the complications brought about by alternate bearing, as not only will the number of potential fruiting sites be reduced, but the flowers that are present will likely not receive sufficient pollination.

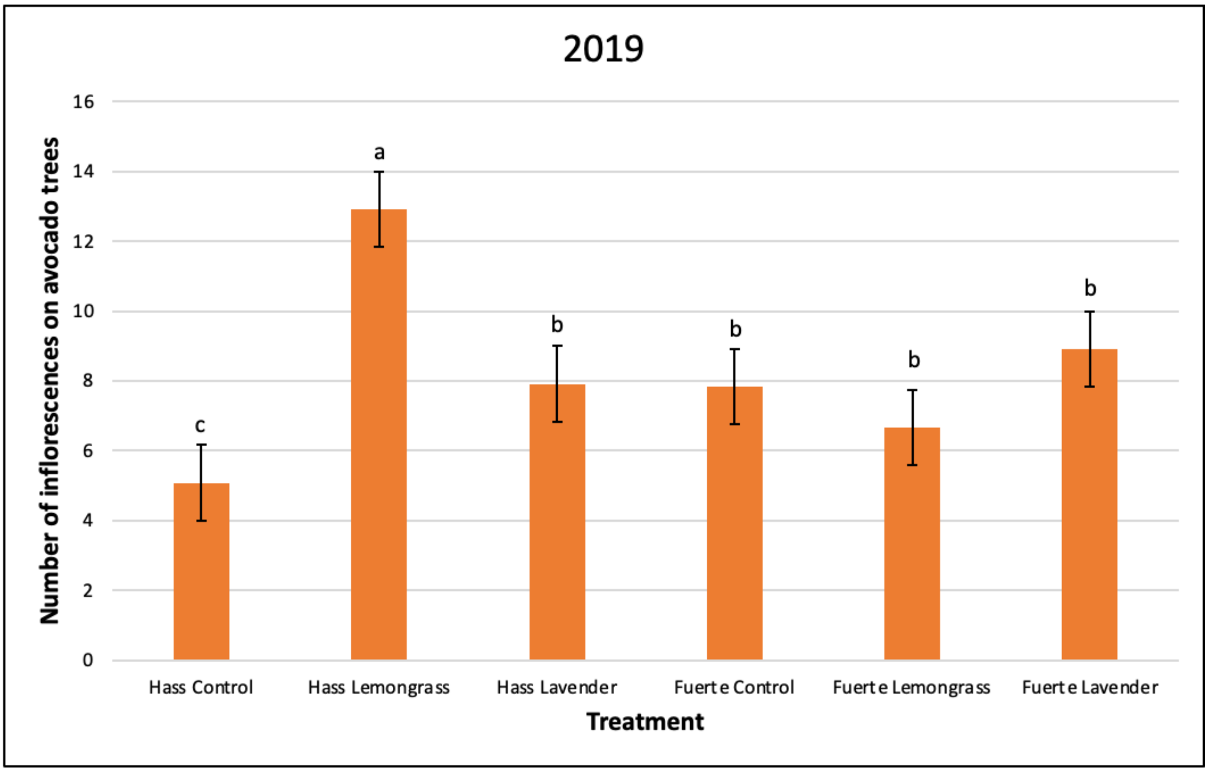


Figure 4.9. Number of inflorescences counted on the tagged branches of the ‘Hass’ and ‘Fuerte’ trees. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences ($p \leq 0.05$).

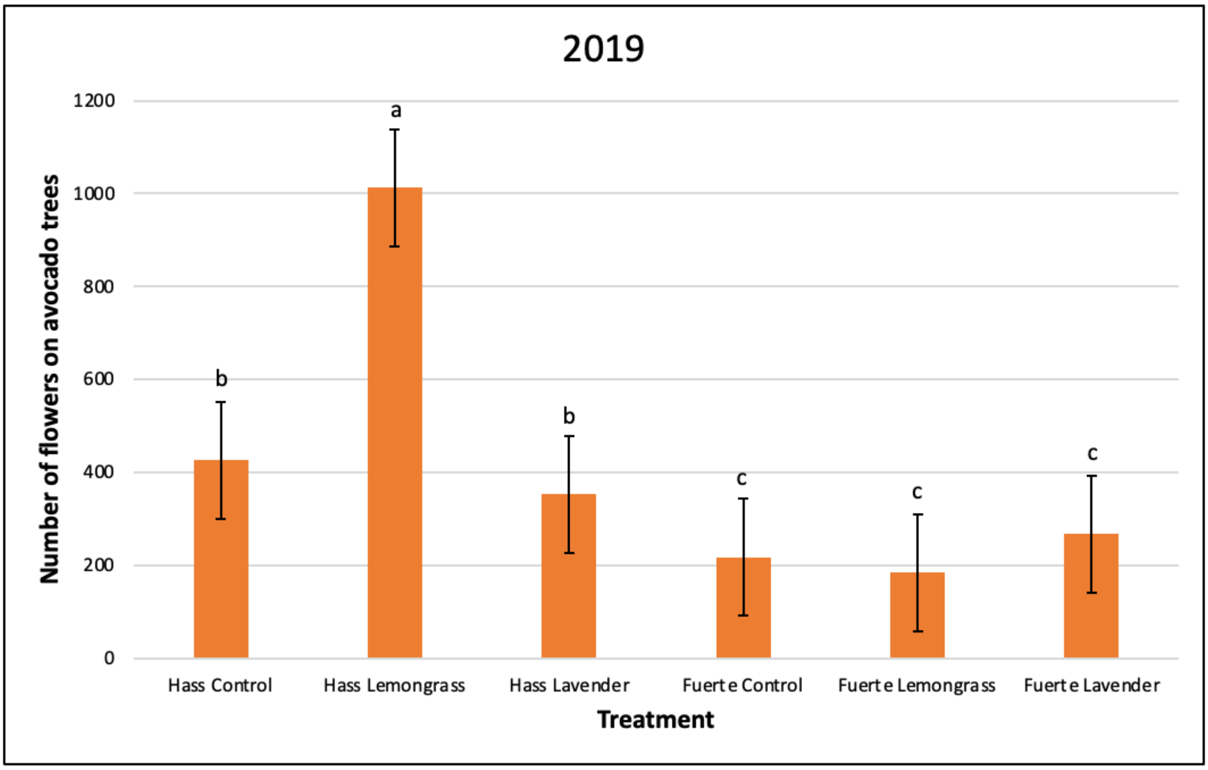


Figure 4.10. Number of flowers counted on the inflorescences of the tagged branches of the ‘Hass’ and ‘Fuerte’ trees. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences ($p \leq 0.05$).

Fruit set (Figure 4.11) and fruit set percentage (Figure 4.12) of the tagged branches were also recorded. For the ‘Hass’ trees, fruit set followed a similar pattern based on the number of flowers recorded. The general pattern showed that if a tree produced more flowers, more fruitlets would be set. Whilst the ‘Hass’ lemongrass treatment did exhibit the greatest number of set fruit, this cannot be credited to the attractant alone (Figure 4.12). These trees had a far greater proportion of flowers, which likely explains the higher fruit set. However, when looking at the fruit set percentages, the lemongrass treatment increased the fruit set percentage (1.52 %) for the ‘Hass’ trees compared to the control (1.32 %) and lavender treatments (0.88 %) (Figure 4.12). The ‘Hass’ lavender treatment group did exhibit the lowest fruit set percentage overall (0.88%), indicating that lavender may have had a negative effect on pollination that was otherwise unobservable when honey bee activity alone was considered. Due to the fact that no previous research has been conducted into the effects of intercropping avocado orchards with attractants, it is difficult to conclude whether attractants will always reduce fruit set. As our findings contradict the reports of other investigations using different attractants in plantings of different species (Jones and Gillett 2005, Pawelek *et al.* 2009, Pereira *et al*. 2015, Moghaddam *et al*. 2020), it is important that this study be repeated on a larger scale using a variety of known attractants.

For the ‘Fuerte’ trees (Figure 4.12), the lemongrass treatment experienced the highest fruit set and fruit set percentage (3.01 %), followed by the lavender treatment group (1.83 %). The trees in the control groups recorded the lowest fruit set (Figure 4.11) and fruit set percentage (Figure 4.12) (1.27 %). The low fruit set recorded for all the ‘Fuerte’ trees is most likely due to the low number of flowers produced overall. According to Salazar-Garcia *et al*. (1999), avocado trees will produce fewer flowers during an ‘off’ year. As Figure 4.12 shows, the ‘Fuerte’ trees in this trial were indeed experiencing such. The ‘Fuerte’ lemongrass treatment exhibited the highest fruit set percentage (3.01 %) of all treatments, followed by the ‘Fuerte’ lavender treatment group (1.83 %). Whilst commercial farmers will be more focused on overall fruit set, small-scale farmers or those experiencing an ‘off’ year may be able to make the most of the limited number of flowers produced by using attractants such as lemongrass, lavender plantings or variations thereof.

Yields for ‘Fuerte’ trees can be as low as 0.015 % in commercial avocado orchard, however, the average fruit set is reported to be 1.5 % (Lahav and Zamet 1999). As Figure 4.14 clearly shows, the fruit set observed in this trial was well below and above this number, ranging from 0.88 % - 3.01 %. The lemongrass and lavender treatments applied to the ‘Fuerte’ trees may have played a role in improving the fruit set that was observed.

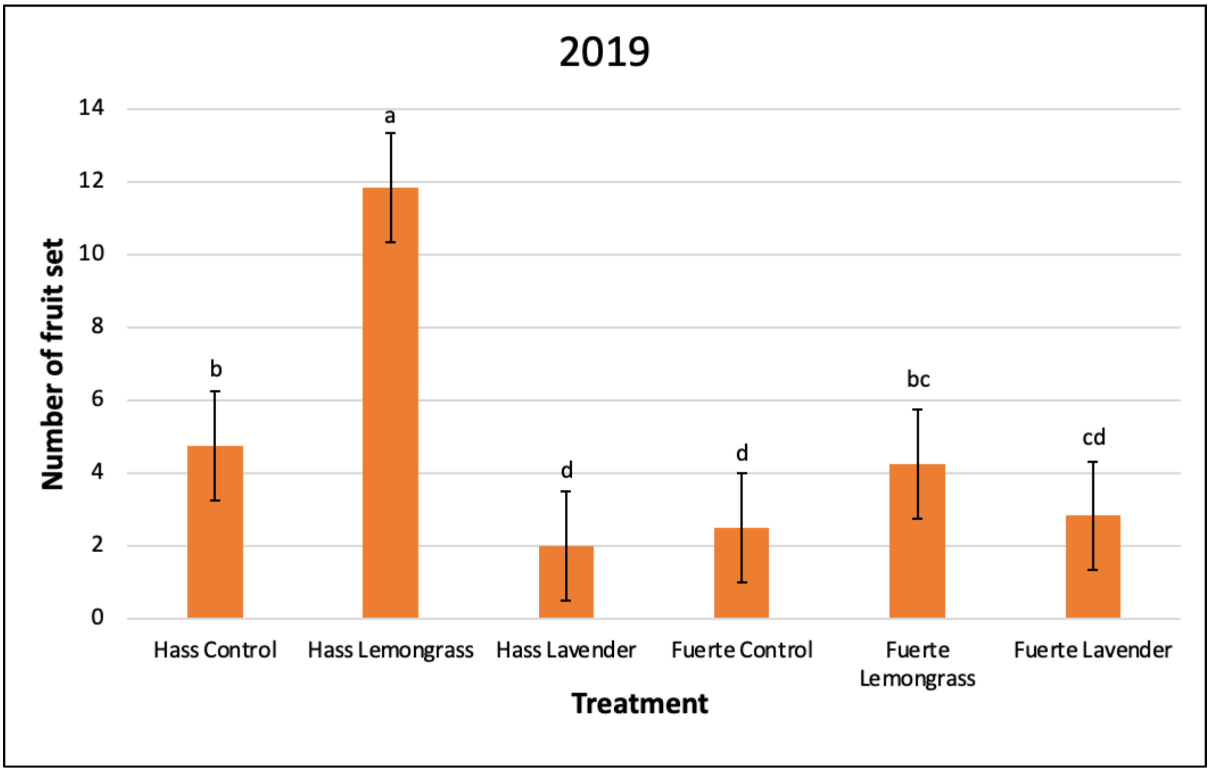


Figure 4.11. Fruit set on the flowers of the tagged branches of the ‘Hass’ and ‘Fuerte’ trees. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

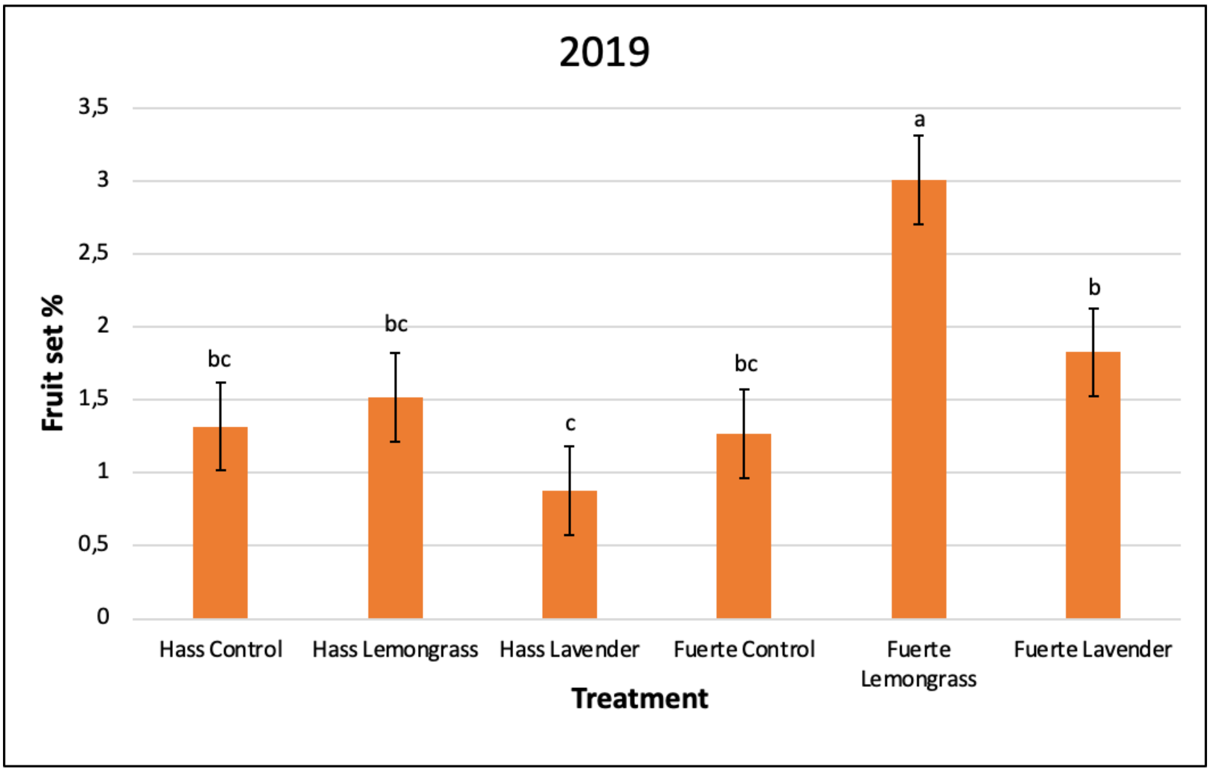


Figure 4.12. Percentage of flowers that set fruit of the tagged branches of the ‘Hass’ and ‘Fuerte’ trees. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences (p ≤ 0.05).

Throughout the sampling period in the orchard on the Hillcrest Experimental Farm, a number of other insects were recorded on and around the avocado flowers. While the exact numbers of these insects were not recorded, flies, ants, and lady bugs were among them (Figure 4.13). As reported by Ish-Am and Eisikowitch (1998), honey bees are not ideal pollinators for avocado flowers and as a result contribute to the low pollination rates. Although many insects have been identified as potential pollinators for avocados, including flies, wasps, beetles and thrips (Vithanage 1990), whether or not any of these could be as effective as honey bees has been poorly reported on. Therefore, the influence of these pollinators on avocado pollination should be made clear before any amendments are made to commercial orchards. This is especially important for attractants like lemongrass which can act as a deterrent to some insect species (Arias *et al*. 1992) and as a result may reduce pollination. This cannot be ignored as Garabaldi *et al*. (2014) highlighted the fact that crop yields often increase when they are visited by a range of different pollinator species.

Figure 4.13. Lady bug (*Coccinella* sp.) on an avocado flower active in the male phase.

Gazit and Degani (2002), Afik *et al*. (2006) and Afik *et al*. (2008) all make note of how citrus orchards located near to avocado plantings will lure honey bees away from the avocados. As aforementioned, there was a lemon orchard 200 m away from the avocado trees used in this study. Therefore, if this trial is to be expanded upon in the future a commercial avocado orchard located no less than two kilometres away from a citrus planting should be used. Alternatively, considering this study supports the possibility of using attractants to improve pollination in avocados, investigations into close plants of citrus trees should also be investigated as an alternative.

Ultimately, intercropping orchards that experience low flowering with pollinator-attractive plants could be useful in maximizing an already compromised yield potential. Both the lemongrass and lavender treatments, or variations thereof, could be useful in improving honey bee activity. This suggests that honey bees can be influenced by different scents and flower morphologies within an avocado orchard. However, further studies in commercial orchards on a larger scale are required, and the off-target effects of lures on deterring other beneficial insects must be understood before concrete conclusions can be drawn.

This chapter represents an attempt to extrapolate the findings of Chapters 2 and 3 and exploit our knowledge to improve pollination in avocados, thereby filling another gap in our understanding of low pollination in avocados (Figure 4.14).

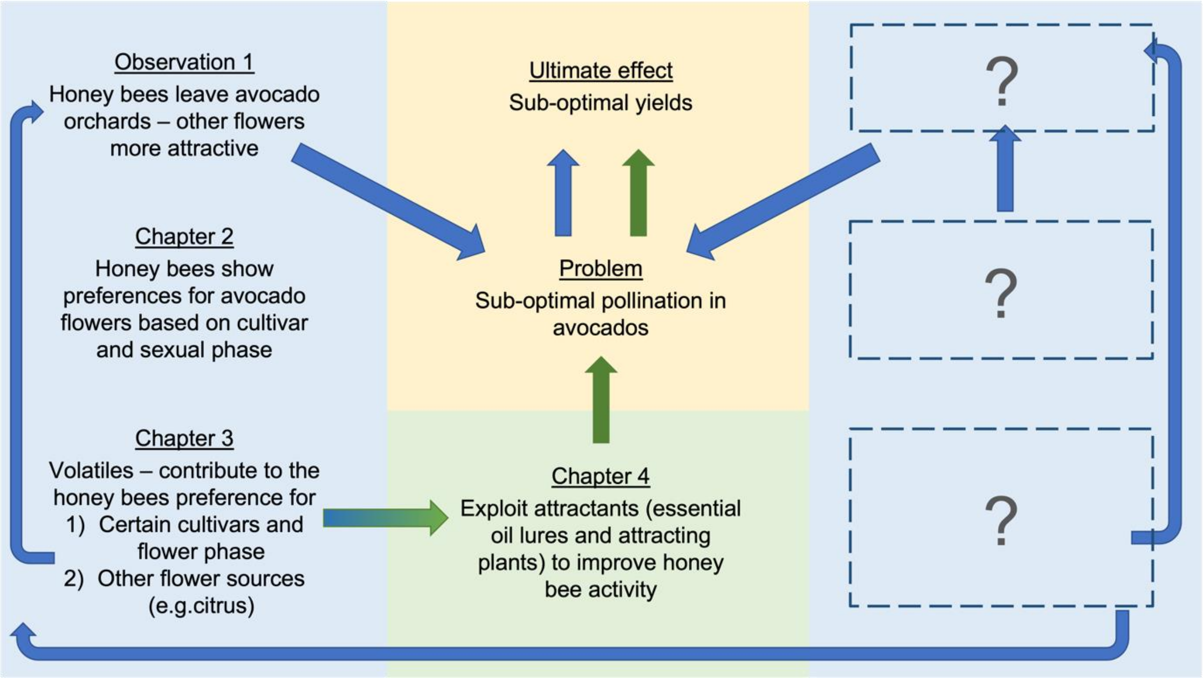


Figure 4.14. Organogram depiction of the holistic approach employed in this study to determine how sub-optimal pollination in avocados contributes to the ultimate effect of sub-optimal yields. Chapter 4 showed how attractants can be implemented to improve honey bee activity in avocado orchards. Blue arrows represent pathways that undermine pollination in avocados and lead to yield reductions. Green arrows represent pathways that promote pollination in avocados and minimise yield reductions.

The viability of using attractants to improve pollination in avocados was made clear for the first time in this pilot study. This represents a potential solution to offset the problem of honey bees showing preference for more attractive flowers in competition with avocado blooms.

4.5 Conclusion

The lemongrass essential oil lures showed promise in attracting honey bees towards to canopy of the avocado trees. Not only did this treatment increase bee activity, but it was effective in increasing overall fruit set and fruit set percentage of all the flowers recorded, too. It can therefore be reasoned that lemongrass essential oils could be effective in not only increasing honey bee activity among avocado flowers but they are also useful towards the end of the flowering season when flower numbers start to decline. The lavender plants had little to no effect on honey bee activity within the canopy of the avocados. However, this treatment did appear to improve fruit set in the 'Fuerte' trees, which were experiencing an 'off' production year at the time of this trial.

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CHAPTER 5

NOVEL COUNTING METHODOLOGY AND LIGHT MICROSCOPY REVEAL POLLEN ABNORMALITIES IN COLD STRESSED AVOCADO (*PERSEA AMERICANA* MILL.) FLOWERS CULTIVATED IN SUB-TROPICAL CLIMATES

This chapter is currently under consideration for publication in the South African Journal of Botany.

5.1 Abstract

Unsynchronised flowering in avocados (*Persea americana* Mill.) as a result of cold stress is well studied. However, the influence of cold temperatures on pollen development late in the flower development process in avocados lacks investigation. To our knowledge, this study represents the first investigation into pollen development in avocados cultivated in sub-tropical regions experiencing cold temperatures during late flower development. A novel pollen counting methodology, believed to be the first of its kind, was used to counteract the problem of inconsistent cutting depths encountered with semithin sectioning preparations for light microscopy. Anthers were prepared for light microscopy using a series of ethanol dehydrations and subsequent resin embedding. Problems with anthers themselves were perceived as absent and/or empty compartments. While many pollen grains appeared to be healthy, some were malformed, and a few were observed as empty shells. *P. americana* 'Fuerte' anthers appeared to be more adept at maintaining pollen health during cold periods while *P. americana* 'Hass' performed better during warm periods; and the influence of a warm-front just prior to anther collection in one of the sampling years may have improved overall pollen health. This suggests pollen health is dependent on sufficiently warm temperatures late in the flower development process if the pollen is to mature properly. A slight difference in the amount of pollen was also detected in the anthers of flowers sampled in the male and female phases, suggesting the overnight closure of these dichogamous flowers is physiologically important and potentially sensitive to cold temperatures. Furthermore, analysis of the temperature trends for the sampling area showed that the avocado trees are regularly exposed to sub-15 ^{o}C temperatures during the period of meiosis, a critical level in tropical plants. These findings suggest avocados grown in sub-tropical and temperate areas which experience low temperatures during and after the period of meiosis may result in less pollen and a higher degree of abnormalities being produced. This will further compound the problem of low pollination in commercial avocado orchards and yields will be compromised.

Keywords: meiosis, counting, abnormalities, temperature, stress, flowering, pollination, semithin sectioning, starch

5.2 Introduction

The avocado, *Persea americana* Mill., is believed to have originated from Mexico and has adapted to grow in the tropical climate of that area (Storey *et al*. 1986). As the avocado trees have become widely cultivated outside their native region, they become exposed to new environmental stressors for which they have limited adaptive mechanisms. One such stressor is cold temperature.

The effect of cold stress on avocado flowering is widely reported on, with visible effects such as unsynchronised flowering and low yields being the result thereof (Sedgley and Annells 1981). Unsynchronised flowering is especially problematic in species like the avocado that exhibit synchronous dichogamy (Robinson *et al*. 1926). As is the case with over 100 important food crops, avocados benefit immensely from insect pollinators (Rader *et al*. 2020). The structure of the avocado flower has long been known to act as a barrier to self-pollination (Clark 1922). Avocado flowers are perfect, containing both female and male reproductive structures (Davenport 1986). The dichogamous nature of the flowers was first described by Nirody (1922), where the female phase with receptive stigmas opens prior to the male phase during which time the anthers dehisce. Based on their flowering pattern, Stout (1933) classified avocado cultivars as ‘A-type’ or ‘B-type’. The flowers of ‘t-Type’ cultivars are functionally female during their first opening in the morning, close overnight, and reopen the following afternoon in the functional male phase. On the other hand, flowers of ‘B-type’ cultivars are functionally female in the afternoon, close overnight and reopen the following morning in the male phase (Stout 1933). It is this dual opening and separation of the active female and male phases that leads many experts to believe that the avocado flower requires cross-pollination to increase fruit set (Stout 1923, Clark 1924, Bergh 1968). The importance of cross-pollination was made even more apparent in a study by Alcaraz and Hormaza (2009), which showed a 15 % increase in fruit set in hand-pollinated avocado flowers compared to open-pollinated flowers.

5.2.1 Problem statement

In a 2018 study on avocado pollination, *P. americana* ‘Fuerte’ anther samples were taken from a commercial orchard in Tzaneen, South Africa, after a cold front had passed (Du Toit 2018). Light microscopy analysis showed what appeared to be deformed pollen grains (Figure 5.1) in the anthers of these flowers. This led to the theory that cold temperatures may not only result in the observable effect of unsynchronised flowering, but also have detrimental effects on pollen maturation late in the flower’s development.

Abnormal pollen development in rice as a response to cold stress can be diagnosed by deformed anthers, sterile pollen grains and low levels of ovule fertilisation (Oda *et al*. 2010). The disastrous influence of cold temperatures on anther development is supported by Sharma and Nayyar (2016), who explain that cold stress increases abscisic acid (ABA) metabolism and decreases ABA catabolism in susceptible plants. The resulting higher ABA concentration reduces expression of ‘tapetum cell wall bound invertase and monosaccharide transport genes’, which diminishes the amount of carbohydrates available for healthy anther development. Furthermore, cold stress supresses the production of gibberellins which are important for protein synthesis in developing anthers and ultimately, overall anther health (Plackett *et al*. 2011, Kwon *et al*. 2015, Sharma and Nayyar 2016).

5.2.2 Aim of the study

Given that avocado production is increasing outside the tree’s native region, especially in temperate regions like New Zealand, the influence of cold temperatures on pollen health and development must be explored. This study will analyse the effect of cold stress on pollen health late in the flower development process.

5.2.3 Objectives of the study

Anther samples were collected during a cold and warm period, thus allowing for the observation of pollen abnormality differences between the two periods. Preparation prior to microscopic observations required the use of microtome semithin sectioning. Michler (2008) describes how this technique is suited to the sectioning of flat surfaces. However, due to the curved nature of the anthers, in addition to uneven pollen dispersal throughout the pollen chambers, it was difficult to cut the samples at consistently even depths. Therefore, a novel pollen counting technique was developed to observe the effects of cold stress on the health and development of avocado pollen.

5.2.4 Hypotheses

The trees used in this study are cultivated in an area that experiences frosty winters. At this time, the trees begin to flower. As these avocados originate from tropical climates (Storey *et al*. 1986) they will likely experience physiological stress in response to cold stress. Furthermore, different cultivars are better suited to endure cold stress (Bergh 1992). When these facts are considered in conjunction with the observations in Figure 5.1, it is expected that pollen health will be influenced by cold stress and cultivar.

5.2.5 Significance of the study

Considering the already-known effects of cold temperature on pollen development in other species, in conjunction with the low pollination rates in avocados, it is imperative that the pollen that is available for pollination be healthy and viable. This highlights the importance of this study, believed to be the first of its kind to investigate the influence of cold stress late in the flower's development on pollen health of two *P. americana* cultivars using light microscopy.

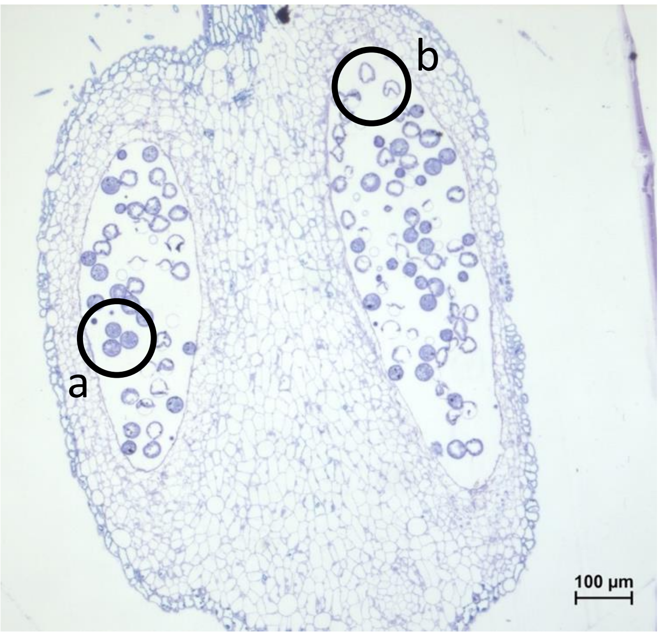


Figure 5.1. 'Deformed' pollen grains inside 'Fuerte' anther compartments sampled from a commercial avocado orchard in Tzaneen, Limpopo (Used with permission from Du Toit 2018). Cluster 'a' – 'healthy' pollen, cluster 'b' – 'deformed' pollen.

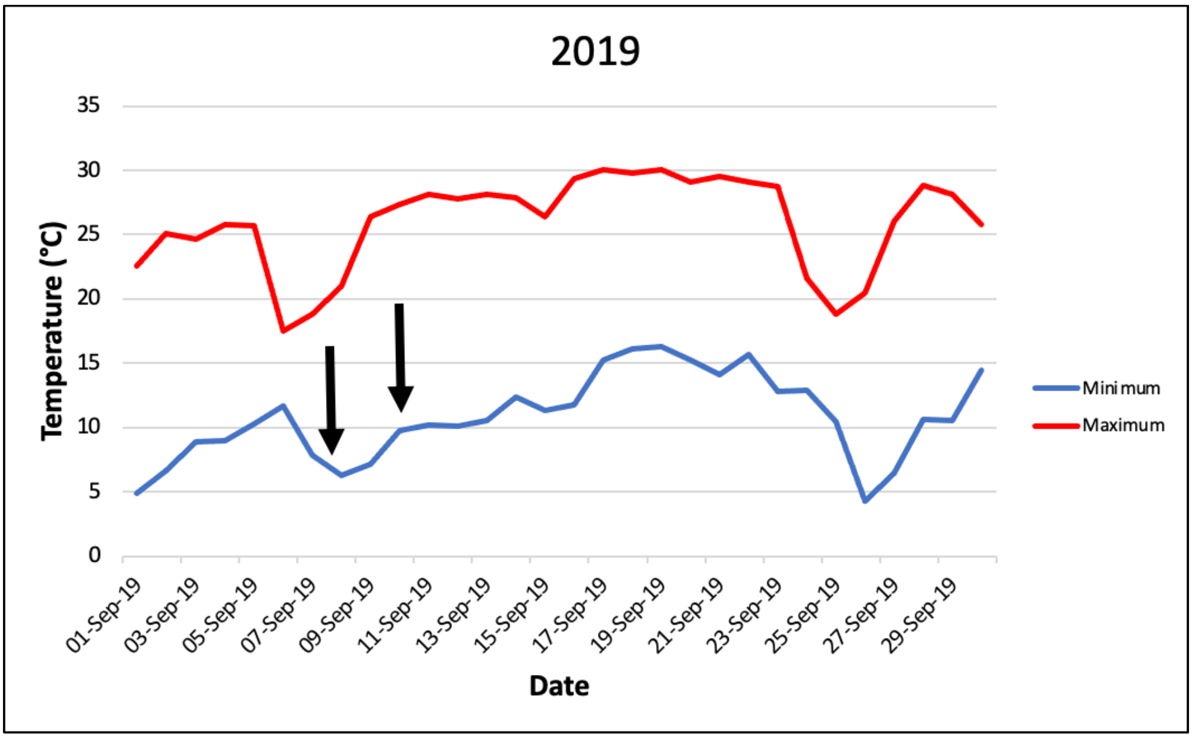
5.3 Materials and Methods

5.3.1 Locality and environmental conditions

Trials were conducted over a period of two years on 12-year-old avocado trees at the Experimental Farm of the University of Pretoria (25°45'S 28°16'E), located 1380 m above sea level. Annual temperature figures for this site are presented in Figure 5.2. The orchard is a mixed planting of *P. americana* 'Hass' and *P. americana* 'Fuerte' trees, grafted on *P. americana* 'Dusa' and 'Latas' rootstocks, respectively. Flowers were taken from both cultivars. Both the female and male phases of the flowers were sampled even though the anthers are not 'active' during the former. Due to the dichogamous nature of the avocado flowers, the importance of cold temperatures during flower closure between the male and female phases could thus be analysed.

This process was completed first in September 2019 followed by a second round in August 2020 (Figure 5.2). In order to determine the effect of cold temperatures on anther and pollen development, samples were taken on a 'cold day' and a 'warm day'. A smart phone weather application (HUAWEI Weather 11.0.1.520 for Android) was used to initially identify a cooler and warmer day. After the sampling periods, the weather records were collected from the University of Pretoria’s weather station (station number 0513435A4 located at 25°45’S 28°16’E).

As mentioned in Chapter 4, the orchard on the Experimental Farm was plagued by alternate bearing, with the *P. americana* ‘Hass’ trees appearing to be experiencing an ‘on’ year and the *P. americana* ‘Fuerte’ trees experiencing an ‘off’ year in the first sampling season (2019).



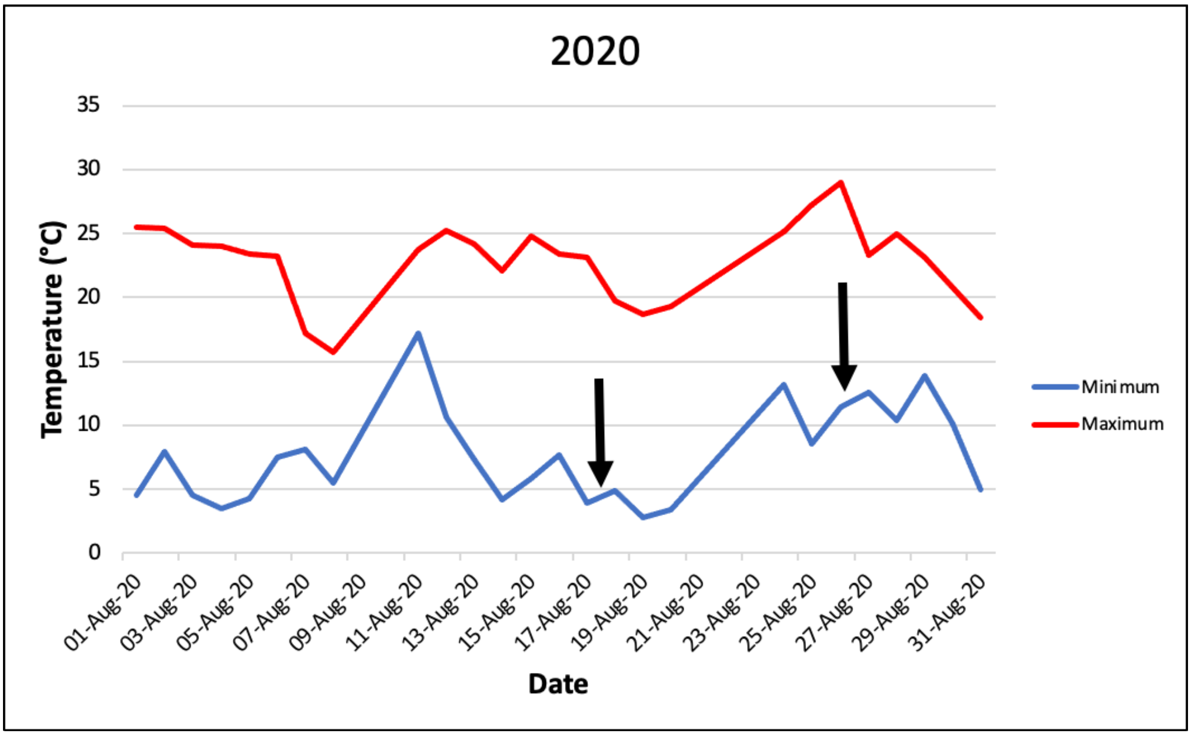


Figure 5.2. Daily minimum (blue line) and maximum (red line) temperatures (°C) for the Experimental Farm at the Hillcrest Campus of the University of Pretoria for September 2019 and August 2020. Black arrows indicate the days on which anther samples were collected.

5.3.2 Anther fixation

Flowers were sampled from the four cardinal directions (N, E, W, S) of the canopy (n = 3) at inflorescence developmental Stage 11 (Salazar-García *et al*. 1999) (Figure 5.3). When active male flowers were collected, care was taken to harvest flowers shortly after they had opened to prevent pollen loss as the anthers start to dehisce. After the flowers had been collected, the anthers were removed using a scalpel on a dissection microscope. The anthers were placed in a 2.5 % glutaraldehyde (GA) fixative solution [25 % GA: 0.075 M phosphate buffer: 100 % distilled water (dH20), in proportions 1:5:4 v/v/v] until they were prepared for analysis using light microscopy.

Figure 5.3. Sampling of the female phase of a 'Hass' avocado flower using tweezers to remove the flower. Note how the anthers lie flat against the sepals and petals. Arrow indicates a closed flower bud.

5.3.3 Microscope preparations

Following the protocol outlined by Fannon *et al*. (1993) the anthers were removed from the GA fixative and washed with a 0.075 M phosphate buffer three times. Between each wash, the samples were centrifuged for 15 minutes in the phosphate buffer. This was followed by a series of dehydrations using a series of ethanol concentrations (30 %, 50 %, 70 %, 90 % and 100 %). In each ethanol concentration the samples were centrifuged for 15 minutes. The series of 100 % ethanol was repeated two times and the anthers were left in the final round of 100 % ethanol for 30 minutes. After the final ethanol treatment, the samples were treated with a stabilised epoxy resin (9.9 g benzoyl peroxide / 500 g resin) and ethanol mix [100 % medium grade LR White resin (London Resin Company Ltd), 100 % ethanol 1:1 v/v] and were centrifuged for one hour. This mixture was removed and replaced with 100 % LR White resin and centrifuged for four hours. Finally, the anthers were placed into sample moulds and fresh resin was added, and sample numbers were assigned (Figure 5.4a). The samples were then placed in the oven at 50 °C for 36 hours of polymerisation (Figure 5.4b).

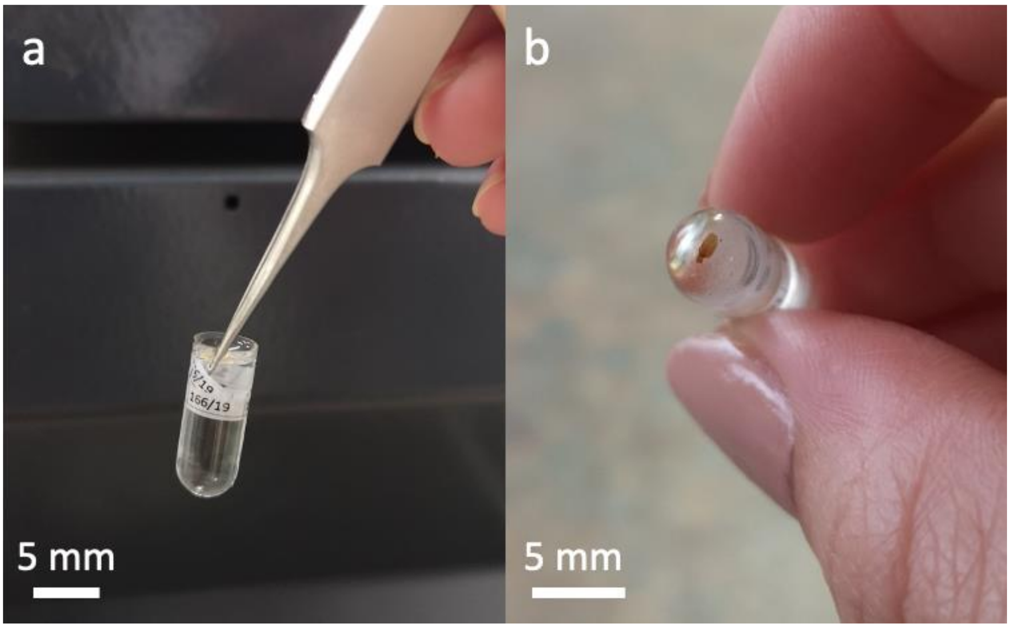


Figure 5.4. (a) Sample moulds with assigned numbers before polymerisation. (b) Polymerised anther sample ready to be sectioned along the vertical plane.

5.3.4 Light microscopy and counting

Due to the curvature of the anthers and uneven pollen distribution throughout the chambers, a new method for pollen counting in avocados needed to be developed as it was not possible to consistently cut anther samples at identical depths for light microscopy investigation. Samples were subjected to block trimming and semithin sectioning (1 µm thick) using a microtome along the vertical axis of the anther. Five sections were made per anther to account for pollen distribution variability that resulted from the inconsistent sectioning depths. These were mounted and stained using Toluidine blue. After this, the samples were analysed using a light microscope and the pollen was counted. To further counteract the inconsistent sampling depths, counting was presented as the number of pollen per compartment size (µm^2 converted to mm^2), as opposed to overall pollen number. Compartment size was calculated by multiplying the length and width of the compartment using the scale as a guide (Figure 5.5).

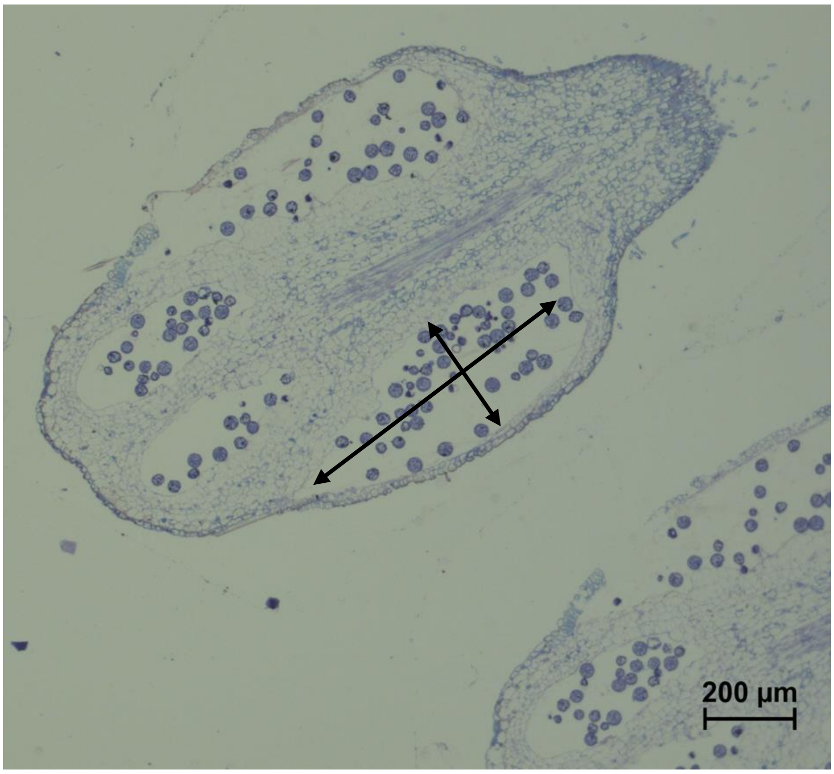


Figure 5.5. Calculating compartment size by multiplying the length and width of the compartment, using the scale (bottom right) as a guide.

5.3.5 Data analysis

Data required the use of non-parametric tests, and the Mann-Whitney U (Wilcoxon rank-sum) test was used to detect significant differences ($p \leq 0.05$). This was accomplished using the statistical program GenStat® (VSN International 2017).

5.4 Results and Discussion

When viewing the anthers using the light microscope, many anthers appeared to have a fine structure (Figure 5.6a), but problems with the structure of other anthers was observed: compartments had no or very little pollen (Figure 5.6b) and/or compartments were absent entirely (Figure 5.6c). However, upon investigating the anomaly of missing compartments, it was found that a healthy avocado anther can have two or four chambers (Schroeder 1943).

Furthermore, it was determined that pollen was viewable in three conditions: in an observably healthy, full form (labelled as ‘healthy’) (Figure 5.7a); as irregular shapes (labelled as ‘deformed’) (Figure 5.7b); and as empty shells (labelled as ‘empty’) (Figure 5.7c). These findings are in accordance with the observations made by Tsvetova and Elkonin (2013) who identified pollen abnormalities in sorghum. The methodology of these researchers was thus employed in this study based on their reports on the following three pollen conditions: ‘fertile’, ‘irregularly shaped’ and ‘incompletely filled with starch’.

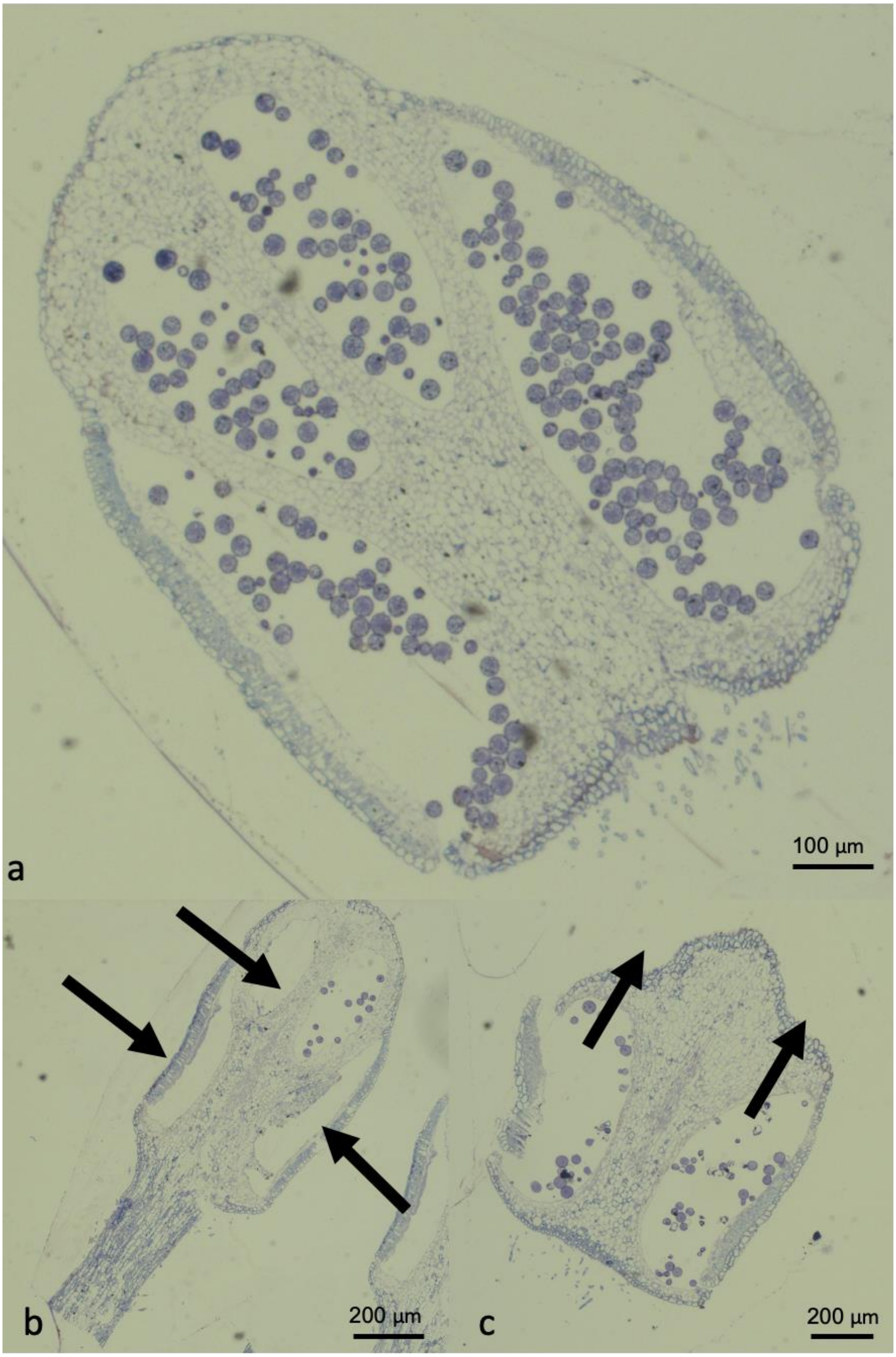


Figure 5.6. Light microscope view of *Persea americana* anthers stained with Toluidine blue. (a) 'Healthy' pollen spread throughout the four chambers. (b) Empty first, third and fourth chambers indicated by the arrows. (c) Anther with only two chambers, arrows indicate missing chambers.

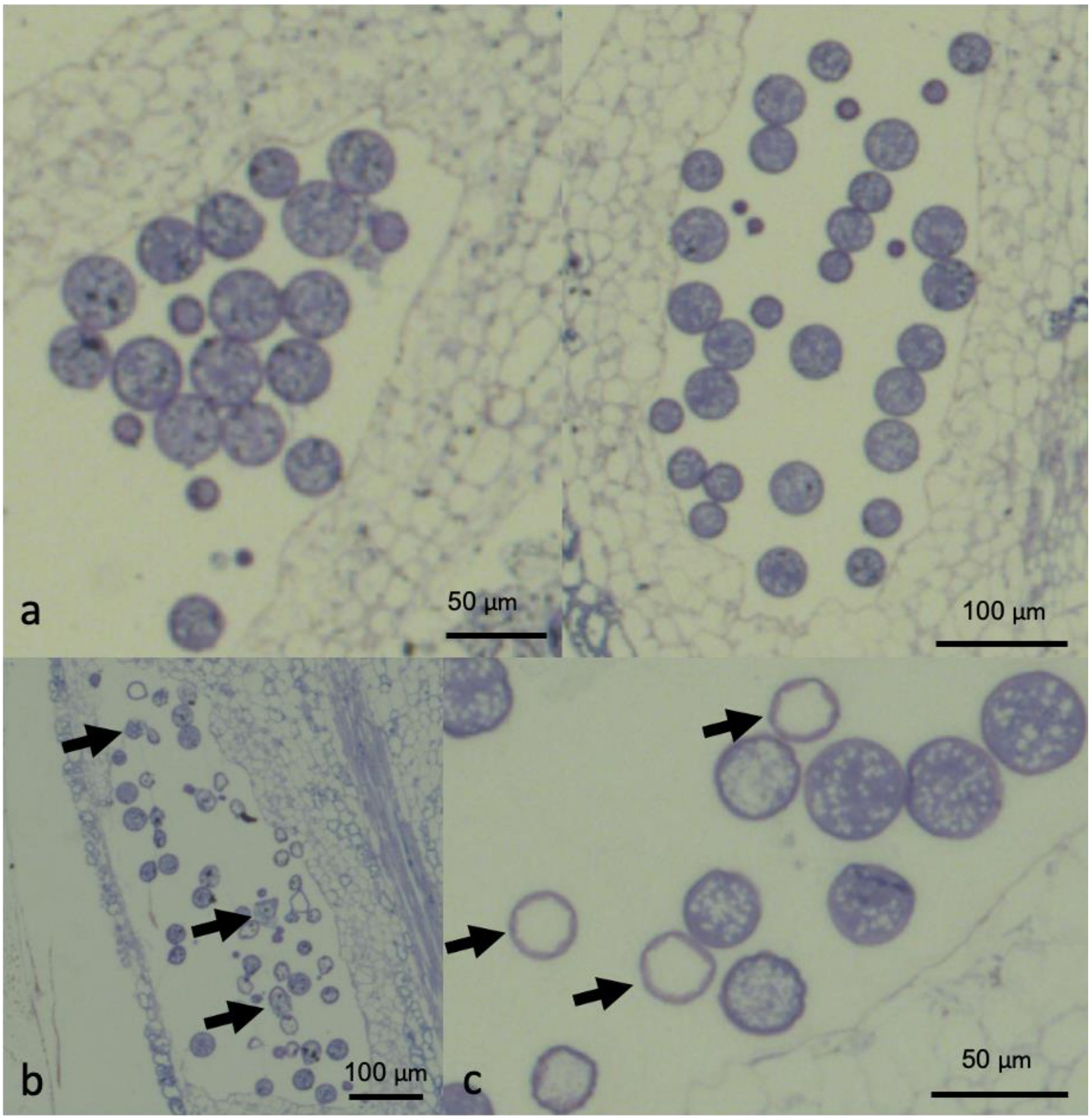


Figure 5.7. Light microscope view of *Persea americana* pollen stained with Toluidine blue. (a) ‘Healthy’ viable pollen. (b) ‘Deformed’ pollen as indicated by the arrows. (c) ‘Empty’ pollen grains as indicated by the arrows.

To keep the discussion of results as easy to follow as possible, the total pollen count will be reviewed first. Within this discussion, the differences observed for the cooler and warmer sampling days, flower cultivar, and flower sexual phase will each be discussed in their own paragraphs. Secondly, the percentages of healthy, deformed and empty pollen will be discussed. Again, the differences observed for the cooler and warmer sampling days, flower cultivar, and flower sexual phase will be allocated individual paragraphs.

Figure 5.8 depicts the differences in total pollen counted between the cooler and warmer sampling days. In 2019, the majority of the anthers sampled on the warmer day had a higher total pollen count (147.5 pollen grains per mm^2) compared to those on the cooler day (83.0 pollen grains per mm^2). Conversely, the majority of the anthers sampled on the cooler day had a higher total pollen count (195.6 pollen grains per mm^2) compared to those on the warmer day (161.4 pollen grains per mm^2) in 2020. When considering these patterns in conjunction with the temperatures recorded in Figure 5.2 it is clear that there was a very warm period prior to the cooler sampling date in 2020. Furthermore, in spite of attempting to sample anthers on warm and cool days using a smartphone weather forecast in 2019, temperature records showed that the two sampling days did not differ greatly in minimum temperature.

In 2019, it also appeared that the *P. americana* ‘Fuerte’ anthers contained more pollen (98.3 pollen grains per mm^2) compared to the *P. americana* ‘Hass’ flowers (67.7 pollen grains per mm^2) when samples were collected on the cooler day, but the *P. americana* ‘Hass’ flowers had more pollen (153.2 pollen grains per mm^2) compared to the *P.americana* ‘Fuerte’ flowers (141.9 pollen grains per mm^2) on the warmer day (Figure 5.8). A similar trend was observed in 2020. According to Bergh (1992), Mexican avocado cultivars are more tolerant of cold temperatures compared to Guatemalan or West Indian cultivars. Both *P. americana* ‘Fuerte’ and *P. americana* ‘Hass’ are hybrids of Mexican and Guatemalan varieties, with *P. americana* ‘Hass’ containing a mostly Guatemalan genome (Wood 1984). Previous studies have shown *P. americana* ‘Fuerte’ to be less susceptible to cold injury in terms of branch and foliar damage (Cooper *et al*. 1957), however, the specific role of cold temperatures on pollen development in avocados is not at all well studied. Schroeder (1955) makes brief comments about *P. americana* ‘Fuerte’ anthers producing more viable pollen grains in unfavourable climates compared to low-yielding cultivars such as *P. americana* 'Weisel Fuerte'. Considering the *P. americana* 'Fuerte' anthers (98.3 pollen grains per mm^2) sampled in 2019 (Figure 5.8) appeared to have more pollen in the anthers than *P. americana* 'Hass' (67.7 pollen grains per mm^2) during cooler periods sampled during cooler periods it is reasonable to suggest cold temperatures will influence pollen development differently for certain cultivars.

In 2020, anthers sampled from the *P. americana* 'Fuerte' (208.7 pollen grains per mm^2) and *P. americana* 'Hass' (208.2 pollen grains per mm^2) female flower phases had slightly more pollen than their male counterparts (195.4 and 170.1 pollen grains per mm^2, respectively) during cooler periods, but anthers from the male flower phases (179.8 pollen grains per mm^2) had a higher pollen count than those from the female phase (143.2 pollen grains per mm^2) during the warmer periods (Figure 5.8). This trend was only observed with the *P. americana* 'Fuerte' samples in 2019. As aforementioned, the flowers in this study were collected during inflorescence developmental Stage 11. Therefore, even though meiosis had occurred months before in these anthers, these results suggest cold temperatures may still influence pollen development just prior to flower anthesis. Low temperatures during flower anthesis has been shown to decrease the yield of an avocado orchard (Coit 1927). Robbertse *et al*. (1998) reported that this yield reduction is due to retarded pollen tube growth post-pollination. Furthermore, day and night temperatures below 21 °C disrupt the avocado's flowering cycle which may contribute to a reduction in pollination (Ish-Am and Eisikowitch 1989).

Considering the role played by temperature on the flowering pattern of avocado flowers, Cuevas and Cabezas (2007) highlighted the importance of endogenous factors by showing that selective removal of floral organs influenced the opening and closing of the flower. Sedgley (1977) reports stigmas collected in the male phase contain more callose, which plays a role in retarding pollen tube growth to promote cross-pollination. In addition to this, Chapter 6 will discuss how actively male flowers appear to accumulate more nutrients, such as boron, compared to the actively female flowers. Therefore, the findings of this study highlight the importance of determining how overnight cold temperatures may affect processes within the flower that ultimately determine healthy pollen development. However, current understanding suggests little investigation has been conducted into determining the physiological processes that occur within the avocado flower during the period of closure between the female and male phase, and how this influences pollen development. Therefore, this study highlights the importance of determining how cold temperatures influence the number of pollen within the anthers at flower anthesis and during the overnight closure between the flower sexual phases.

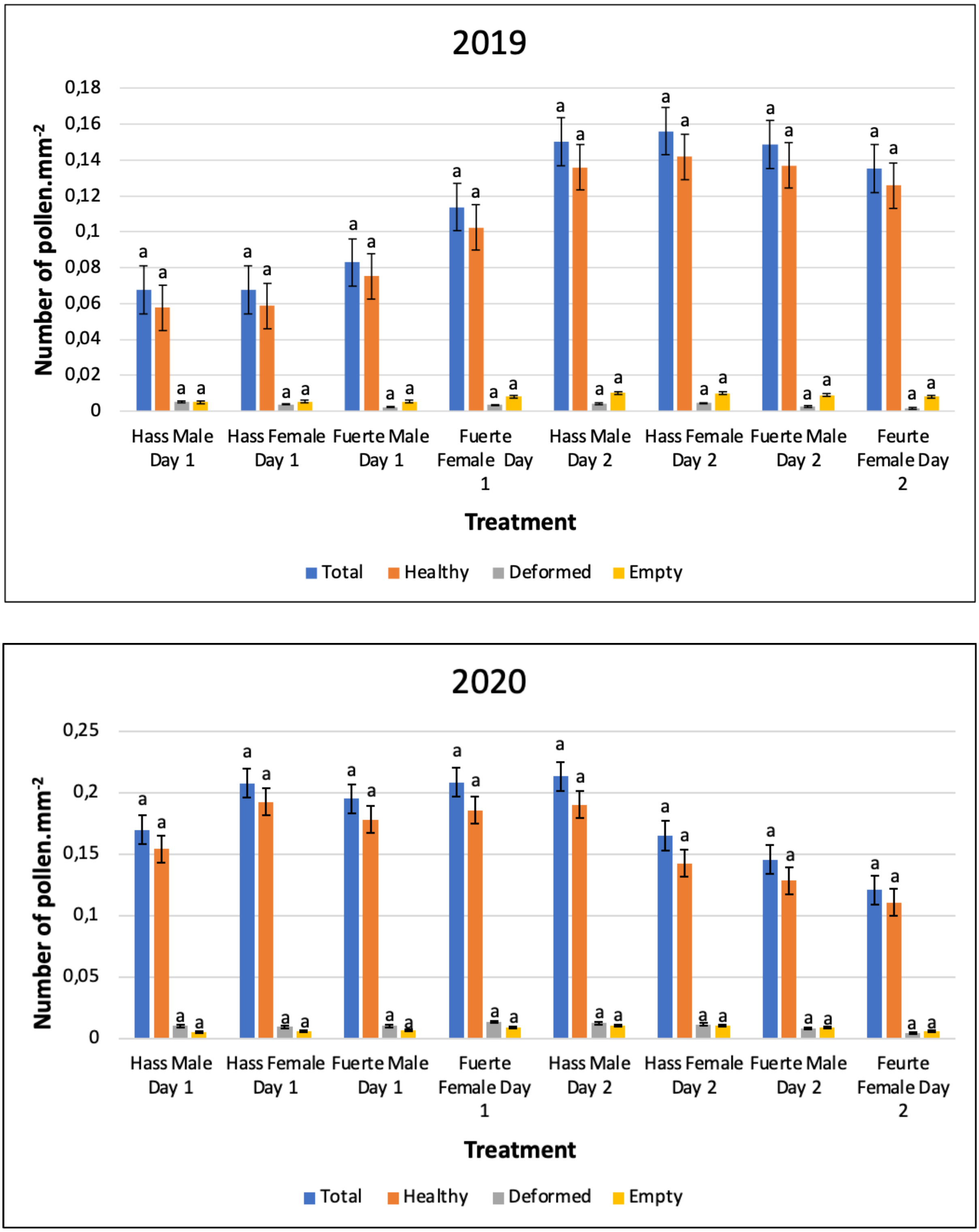


Figure 5.8. Average number of total, ‘healthy’, ‘deformed’, and ‘empty’ pollen counted in the compartments of *P. americana* anthers (pollen.mm^{-2}) in the 2019 and 2020 sampling seasons. Day 1 refers a cool sampling day; Day 2 refers to a warm sampling day for both seasons.

While total pollen counts were helpful in uncovering the effects of cold temperature on overall pollen counts, the number of deformed and empty pollen that was observed also warranted further study. In the first sampling season of 2019, far more problematic pollen grains were observed (Figure 5.9). Furthermore, there was a higher proportion of empty pollen grains detected during the cooler sampling period. These results are contrasted with the results from the 2020 sampling season where the overall healthy pollen count was higher and very little differences in healthy, deformed and empty pollen between the cooler and warmer sampling days was observed (Figure 5.9). Considering all of the problematic pollen observed, the majority thereof were classified as ‘empty’ in 2019 while a higher proportion were ‘deformed’ in 2020.

There was a more apparent difference in ‘healthy’, ‘deformed’ and ‘empty’ pollen grains for the different flower cultivars in 2019 (Figure 5.9). Generally, *P. americana* ‘Fuerte’ had a higher proportion of empty pollen grains (24.5 %) compared to *P.americana* ‘Hass’ (19.1 %), while *P. americana* ‘Hass’ had more deformed pollen (4.1 %) compared to *P.americana* ‘Fuerte’ (1.4 %), especially on the cooler sampling day. In 2020, slightly healthier pollen (89.9 %) was observed on the warmer sampling day compared to the cooler day (88.7 %) (Figure 5.9). There were no observable patterns for differences in ‘healthy’, ‘deformed’ or ‘empty’ pollen between flowers sampled in the male or female phases for both 2019 and 2020 (Figure 5.9).

These differences in pollen structure (‘empty’ versus ‘deformed’) may be key in determining the effects of cold temperature both in the early and late stages of pollen development in avocados. Ohnishi *et al*. (2010) found that cold stress during the critical stages of flower development in soybeans (*Glycine max*) results in abnormally shaped pollen being produced. Furthermore, Datta *et al*. (2002) explained how starch accumulation is an important ‘indicator for pollen maturation’. Chung *et al*. (2014) showed how genes involved in sucrose transportation in rice (*Oryza sativa*), which is essential for starch synthesis in sink tissues, are downregulated at low temperatures between 15 and 20 °C. Thus, if these findings are applied to the pollen abnormalities observed in this study it can be reasoned that low temperatures early in the avocado floral development stage may result in ‘deformed’ pollen being produced. In addition to this, cold stress later on in the flower’s development may lead to decreased starch accumulation. Therefore, the ‘empty’ pollen observed in this study may be caused by a reduced expression of sucrose transporting genes. The resulting pollen would therefore have not been sufficiently mature had the anthers dehisced. This could also explain why fewer ‘empty’ pollen were detected in 2020, as there was a period of warm weather just prior to sampling which may have improved sucrose translocation and subsequent starch accumulation in the pollen.

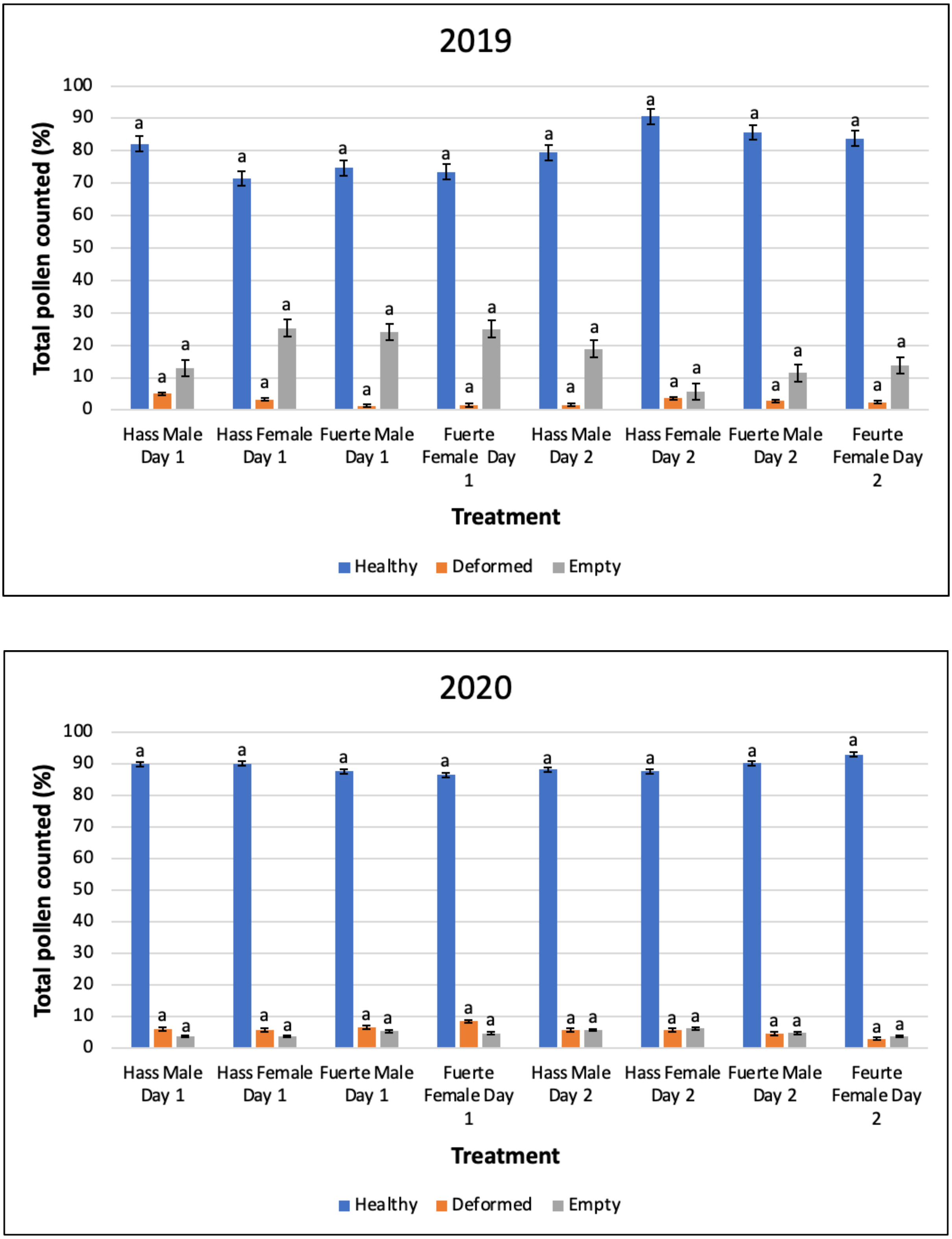
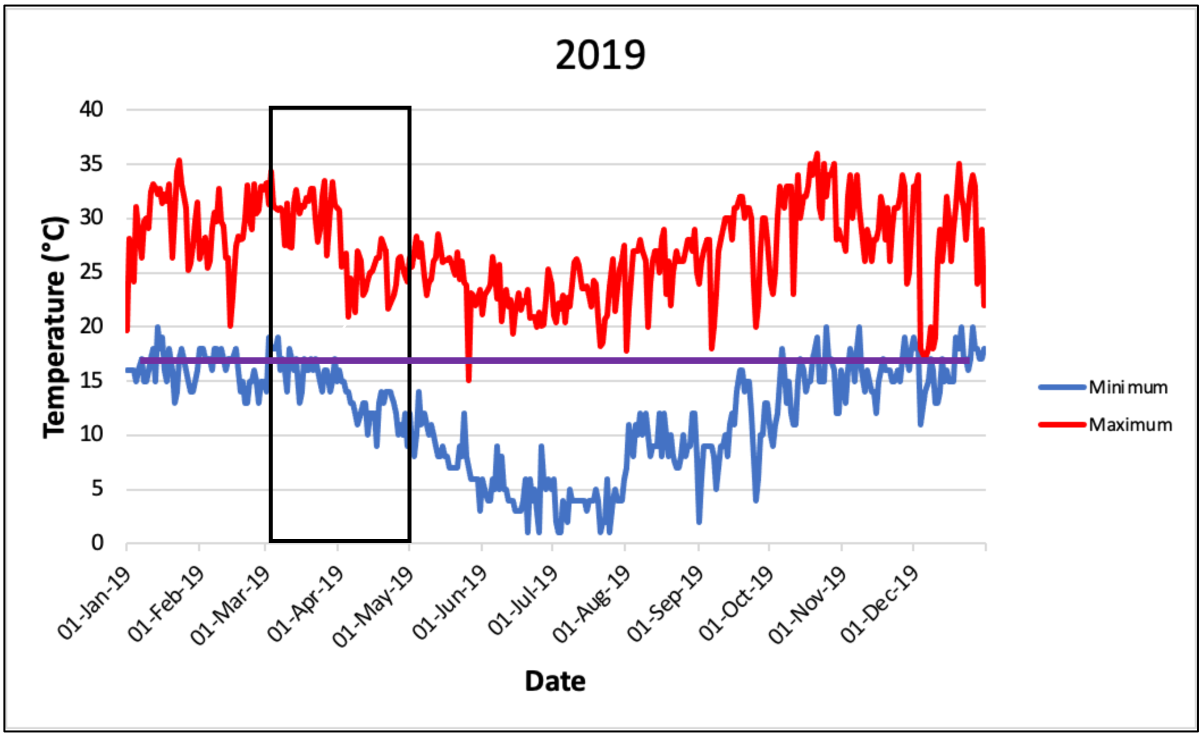


Figure 5.9. ‘Healthy’, ‘deformed’ and ‘empty’ pollen as percentages of total pollen counted in the compartments of *P.americana* anthers during the 2019 and 2020 sampling seasons. Vertical bars (±) indicate standard errors of the mean (SEM), different letters represent significant differences ($p \leq 0.05$).

While this study focused on the influence of cold temperatures during flower anthesis on pollen development and health, cold stress during the meiotic period cannot be ignored. According to Issarakraisila and Considine (1994), cold temperatures can be especially damaging to tropical trees ‘between the meiosis and pre-vacuolate stages’ of pollen development, with temperatures below 15 ºC being especially damaging. Based on the findings of Salazar-García *et al*. (1998), meiosis occurs between developmental Stages 7 and 8 in avocados, and the process is complete once the flower buds have reached the cauliflower stage (Stage 8). Based on the findings of Kotze (1979), this process may occur anywhere between March and May in South Africa, depending on the climate. Given that the anthers for this study were sampled at flower anthesis, it would be useful to know what temperatures the avocado trees were exposed to during the meiotic periods in 2019 and 2020. Therefore, temperature data were extracted from weather station 0513435A4 (located at 25°45’S 28°16’E) for 2019 and 2020 (Figure 5.10). These records show that the orchard from which the pollen in this study were collected regularly experiences minimum temperatures of less than 15 ºC during the meiotic period.

Batygina and Vasilyeva (2003) emphasised the importance of adequate temperatures both during and after meiosis if pollen is to develop correctly. Oda *et al*. (2010) explained how low temperatures can cause the tapetum, which nourishes the developing pollen, to degrade in rice anthers. Furthermore, the findings of Sharma and Nayyar (2016) suggested that plants susceptible to cold stress accumulate ABA within the anthers, which supresses the activity of the genes involved in the generation of the tapetum. When these observations are considered, in conjunction with the fact that the trees used in this study were regularly exposed to temperatures below the critical level of 15 ºC during the meiotic period, it is reasonable to suggest that cold temperatures during and after meiosis may play an additional role in observed pollen abnormalities.

Despite tree nutritional status not being the focus of this chapter, the role of boron on pollen viability cannot be ignored. As aforementioned, the trees used in this study were experiencing alternate bearing. According to Harkness (1959) and Thorp (2011), the foliar boron content of avocado trees in an ‘on’ year is greater than that in an ‘off’ year. Boron plays an important role in pollen viability in avocados (Whiley *et al*. 1996), and deficiencies thereof have been shown to reduce the fertility of floral male organs of other plant species (Dell and Huang 1997), possibly by limiting ‘nucleic acid exchange’ during meiosis (Batygina and Vasilyeva 2003). Considering the anthers of *P. americana* ‘Fuerte’ (experiencing an ‘off’ year in 2019) contained more pollen compared to the anthers of *P.americana* ‘Hass’ (experiencing an ‘on’ year in 2019) that were sampled on a cool day, it is reasonable to suggest that cold temperatures have a far more devastating effect on pollen viability in avocados compared to boron deficiencies. This finding reiterates the importance of this study and supports the need for further investigation into the role of cold temperatures on pollen development in avocados cultivated in sub-tropical and temperate climates.



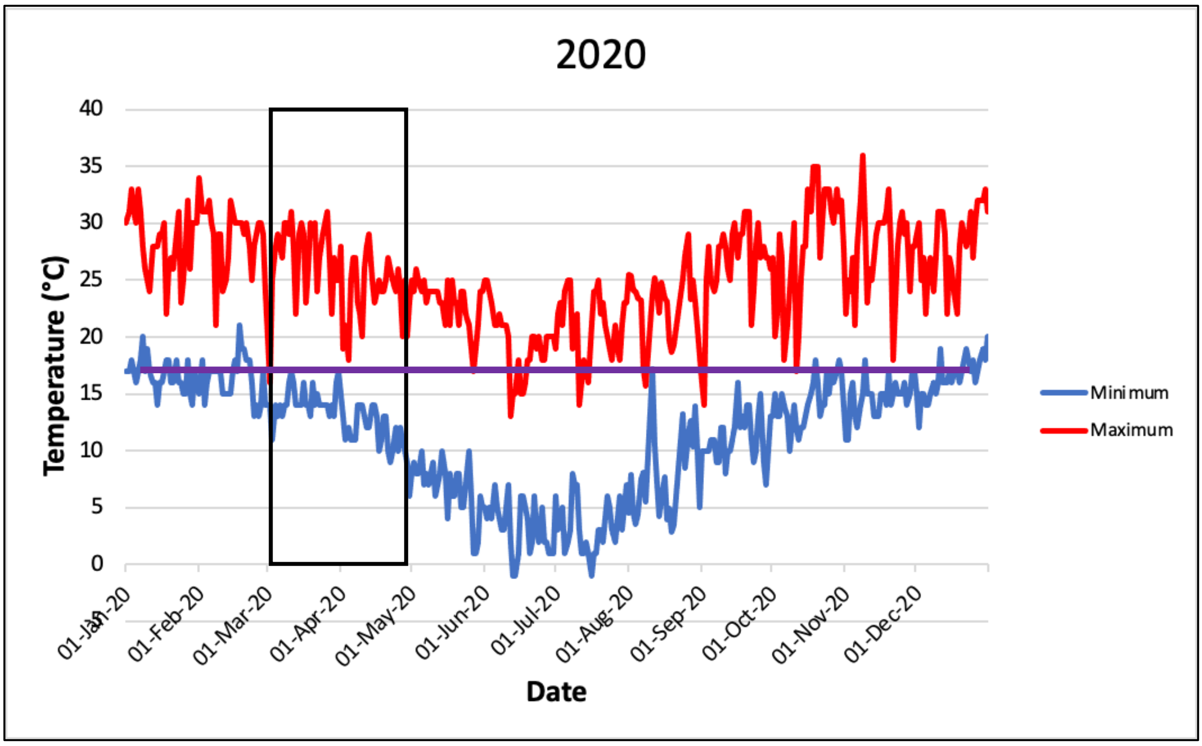


Figure 5.10. Minimum (blue line) and maximum (red line) temperatures (°C) for the Experimental Farm at the Hillcrest Campus of the University of Pretoria for 2019 and 2020. Data was collected from the weather station numbered 0513435A4. The black rectangle indicates the period of floral differentiation and meiosis for *Persea americana* in South Africa (Kotze 1979). The purple line indicates the critical temperature of 15 ºC for pollen development in tropical tree species (Issarakraisila and Considine 1994).

If this project is to be repeated in the future, samples should be taken from orchards located in different climatic zones. This should yield a more reliable indication of how temperature affects pollen development.

This chapter posited cold stress in avocado trees cultivated in sub-tropical climates as another factor contributing to sub-optimal pollination in avocados (Figure 5.11).

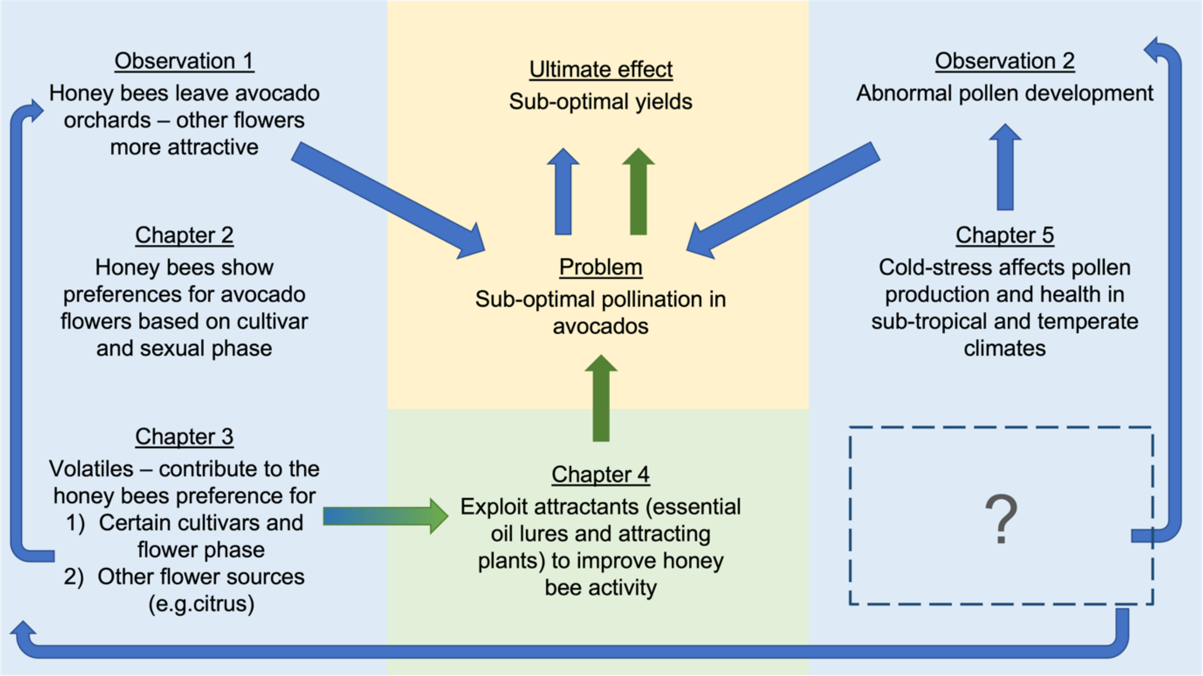


Figure 5.11. Organogram depiction of the holistic approach employed in this study to determine how sub-optimal pollination in avocados contributes to the ultimate effect of sub-optimal yields. Chapter 5 proposed cold stress as a contributing factor to sub-optimal pollination by reducing healthy pollen production. Blue arrows represent pathways that undermine pollination in avocados and lead to yield reductions. Green arrows represent pathways that promote pollination in avocados and minimise yield reductions.

By observing abnormal pollen in the anthers of avocados cultivated in sub-tropical climates, Chapter 5 made use of novel-pollen counting techniques to facilitate the first investigation into the effects of cold stress on pollen development in avocados cultivated outside their native tropical regions. The observations in this chapter laid the foundations for the final investigation explored in Chapter 6.

5.5 Conclusion

With the use of novel pollen counting methodologies and light microscopy, the observation of abnormal pollen in the anthers of the sampled flowers suggest cold temperatures do play a role in pollen development in avocados. *P. americana* 'Fuerte' flowers appeared to be better accustomed to producing more pollen in cooler periods, while *P. americana* 'Hass' proved superior in warmer periods. The different amount of pollen observed in anthers sampled during the male and female phases suggest pollen development remains ongoing during the overnight closure between the flower sexual phases. Lastly, if 15 ºC is taken as a critical level for meiosis in tropical plants, avocados grown in sub-tropical and temperate regions may experience sub-optimal pollination due to abnormal pollen development as a result of low temperatures during meiosis.

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CHAPTER 6

FLORAL ELEMENTAL CONTENT AND ITS POTENTIAL INFLUENCE ON POLLINATION IN AVOCADOS (*PERSEA AMERICANA* MILL.)

6.1 Abstract

In avocados (*Persea americana* Mill.), very little research has been invested into determining how nutrient deficiencies specifically affect pollen development. Expanding on the findings of Chapter 5, the nutrient concentrations of avocado flowers were investigated with the aim of determining the influence thereof on pollen development and pollination. Samples from different cultivars, flower sexual phases, position within the canopy (top or bottom) and sampling location (a commercial orchard or non-commercial orchard) were processed using nitric acid (HNO_3) digestions followed by inductively coupled plasma (ICP) analytical methods. There were a range of significant differences detected for the various elements, including potassium, boron, phosphorous, and calcium. Flowers of different cultivars, sexual phase and sampling location accumulated these nutrients in distinct ways. Furthermore, this study revealed the limited research that has been conducted into the role that these and other elements play in the development of avocado pollen. Finally, by comparing the elemental content of the leaves and flowers we showed that elements tend to accumulate in higher concentrations in the latter, suggesting there is a different 'pull' for elements between the reproductive and vegetative organs during the flowering period. The differences in nutrient accumulation between the flowers and leaves allowed us to develop what is believed to be the first recommendations for floral nutrient statuses for potassium, boron, phosphorous and calcium.

Keywords: nutrients, deficiencies, pollen, health, yields, potassium, phosphorous, boron, calcium, standards

6.2 Introduction

As depicted in Chapter 5, a 2018 sampling of 'Fuerte' pollen grains taken from a commercial avocado (*Persea americana* Mill.) orchard showed what appeared to be malformed pollen grains when the samples were observed under a light microscope (Figure 5.1). Chapter 5 has already discussed the influence of cold temperature on pollen development. This chapter is going to focus on the role of nutrient status within the flowers and their possible influence on pollen development.

Based on the reports of Cheung and Stephenson (1993), adequate pollen development is dependent on the nutritional status of the sporophytic generation as it is from these sporophytes that the pollen draws its nutrients. Research focusing on the role of nutrients in other plant species has shown that boron deficiencies contribute to sterility in the male floral organs (Dell and Huang 1997); potassium levels influence pollen development and germination (Choudhurry *et al*. 2013); phosphorous influences the amount of pollen produced in cucurbits (Lau and Stephenson 1994); and calcium is an important constituent of pollen walls of *Torenia fournieri* (Wang and Dobritsa 2018).

6.2.1 Problem statement

A multitude of studies focusing on the influence of nutrient availability on fruit set in avocados has been conducted (Coetser *et al*. 1993, Bard and Wolstenholme 1997, Selladurai and Awachare 2019). However, little is known about how these nutrients affect pollen development directly, let alone specifically for the avocado. Some nutrients can repel honey bee pollinators when present in excessive amounts (Afik *et al*. 2006), and their accumulation in the flowers of avocados could thus contribute to the honey bee's non-preference therefor.

6.2.2 Aim of the study

Nutrient diagnostics in avocados primarily uses leaves as sampling material (Du Plessis and Koen 1992). Nutrient standards for avocado flowers have, to our knowledge, not been adequately investigated. The aim of this study was thus to focus on how flowers of different avocado cultivars, sexual phase (when the flowers are actively male or female on the tree) and sampling location accumulate a range of nutrients. The influence thereof on pollen development, and pollination as a whole, can thus be better understood.

6.2.3 Objectives of the study

Flower sampling and subsequent analysis using inductively coupled plasma (ICP) will highlight the differences in nutrient accumulation in the different treatment groups. Potassium (K), boron (B), phosphorous (P), and calcium (Ca) concentrations will be quantified. In spite of the lack of literature explaining how these nutrients affect pollen development in avocados directly, by examining their influence in other plants their role in avocados can be better understood. The potential influence of floral nutrient status on pollen development and the translocation of these nutrients throughout the canopy will determine their influence on successful pollination in avocados. A secondary influence of floral nutrient status is that of pollinator repulsion, as Afik *et al*. (2006) reported on the repelling effects of some nutrients. Therefore, the objectives of this study are to analyse the presence of these elements in the flowers of avocados. Foliar samples will also be gathered to compare nutrient accumulation between the leaves and flowers of avocados, thus examining the different ‘pull’ for nutrients when the trees are in flower. By comparing the nutrient accumulation between floral and foliar material, the first recommendations for floral nutrient status will be developed. This will be achieved by calculating ratios between the amount of potassium, boron, phosphorous, and calcium detected in the flower and leaf samples. Following this, these ratios can be used to convert the foliar standards of Selladurai and Awachare (2019) and Dixon (2008) to recommendations for floral nutrient status.

This study will not explore the role of inputs such as fertilisers or irrigation, nor will it examine the physiological pathways affected by nutrient deficiencies. However, it will highlight the need for individual, detailed studies such as these.

6.2.4 Hypotheses

Throughout this dissertation, disparities between avocados of certain cultivar, sexual phase, and locality have highlighted differences in honey bee activity (Chapter 2) and volatile exudation (Chapter 3). It is thus expected that floral elemental content will differ based on the same patterns observed in these chapters. Due to the pollen abnormalities observed in Chapter 5, it is reasonable to suggest element deficiencies may be a contributing factor.

6.2.5 Significance of the study

To our knowledge, this study will be the first of its kind to analyse nutrient status in the flowers of avocados and attempt to relate them to pollen development. Furthermore, this study will investigate how integral floral nutrient status is to not only pollen development, but honey bee repulsion in addition. The development of the first floral nutrient recommendations will assist farmers in detecting elemental deficiencies or surpluses that could negatively influence pollination. Thus, a proper understanding of floral nutrient status is exceptionally important for commercial farmers as it will affect two aspects of pollination: healthy pollen development and successful pollination of that pollen.

6.3 Materials and Methods

6.3.1 Locality and environmental conditions

Trials were conducted in two different regions over a two-year period. Flower samples were collected from commercial orchards of Westfalia in Limpopo, Tzaneen. Two orchards were used to collect ‘Hass’ and ‘Fuerte’ flowers. ‘Hass’ flowers were collected from a 1.5 hectare, 7-year-old orchard in the Politsi Valley (23º45’S, 30º05’E). ‘Fuerte’ flowers were collected from a 4.43 hectare, 46-year-old orchard, also in the Politsi Valley (23º44’S, 30º04’E). All trees were grafted onto ‘Duke 7’ rootstocks. The trees in both locations were planted in loamy, well-drained soil. The spraying and fertilisation programs for both the commercial orchards were similar. During the sampling period (July to September), this region experienced minimum temperatures between 1 °C and 11 °C in 2019, but 0 °C and 6 °C in 2020. Maximum temperatures ranged between 22 °C and 26 °C in 2019, and 26 °C and 35 °C in 2020. The non-commercial orchard was located on the Experimental Farm at the University of Pretoria's Hillcrest Campus (25°45'S 28°16'E). Within the orchard there were 'Hass' and 'Fuerte' trees grafted on 'Dusa' and 'Latas' rootstocks. The trees in the non-commercial orchard received no pesticide or fertiliser applications during the two sampling years. During the sampling period, this region experienced minimum temperatures between 1 °C and 16 °C in 2019, and -2 °C and 16 °C in 2020. Maximum temperatures ranged between 18 °C and 32 °C in 2019, and 14 °C and 31 °C in 2020. Detailed environmental conditions for the sampling period are reported in Appendix A.

6.3.2 Flower and leaf sampling

Two cultivars were focused on in this study, namely 'Fuerte' and 'Hass'. Flower samples were collected from two different regions to compare the differences in elemental content between a commercial orchard and a smaller, non-commercial orchard. In order to determine the differences in elemental translocation throughout the canopy of the trees, samples were collected from the top and the bottom of the canopy. In order to reach the top of the canopy, a small ladder was used (Figure 6.1). When sampling from the bottom of the canopy, the lowermost flowers were selected. Three trees (n = 3) were used for each area and cultivar. For each treatment between 50 and 150 flowers were collected and placed in a plastic petri dish with the lid removed thereby allowing the flowers to dry.

Figure 6.1. Demonstration of flower collection from the top of a ‘Fuerte’ tree using a small step ladder.

In the second sampling year leaves were also collected. Leaves were collected from ‘Hass’ and ‘Fuerte’ trees, from the top and the bottom of the canopies, and from both the commercial and non-commercial orchards. Four trees (n = 4) were used for each treatment group. From each tree 20 leaves were sampled over a period of two days. Leaves were collected at the same time as the flowers during the late winter to early spring.

6.3.3 Processing of floral and foliar material for nutrient analysis

The flowers and leaves were left to air dry. To fully dehydrate the samples, they were placed in an oven at 40 ºC for 24 hours. Samples were then placed in a grinder to process the dried flowers and leaves into a coarse texture (Figure 6.2) and 0.3 g of the samples were decanted into microwaveable digestion tubes. Into these tubes,10 ml of nitric acid (HNO_3) was added, and the samples were placed in an Anton Paar Multiwave 3000 microwave for 45 minutes. Following digestion, samples were decanted into a falcon tube and deionised water was added to fill the tubes to the 25 ml mark. The tubes were sealed, shaken and analysed using inductively coupled plasma (ICP) techniques at the Soil Science division of the Plant and Soil Sciences Department at the University of Pretoria in Pretoria, Gauteng.

Figure 6.2. Photographic illustration of dried avocado flowers ground to a coarse powder in plastic petri dishes sealed with masking tape.

The results from the ICP analysis were given in mg/l and needed to be converted to mg/kg. For this purpose, the following two formulas were used for sequential conversion of the results of the ICP given in mg/l to mg/kg. ‘C’ - concentration, ‘DF’ - dilution factor.

$$1.\ C\ (\text{mg/l}) = [\text{Soil ICP concentration (mg/l)} \times \text{DF}] - \text{blank ICP concentration}$$

$$2.\ C\ (\text{mg/kg}) = \frac{C\ (\text{mg/l}) \times 0.025\ \text{l}}{0.0003\ \text{kg}}$$

6.3.4 Data analysis

Data were analysed using a nested factorial. Significant differences between the treatment groups were then determined using Fisher’s protected least significant differences (LSD) test. This was accomplished using the statistical program GenStat® (VSN International 2017).

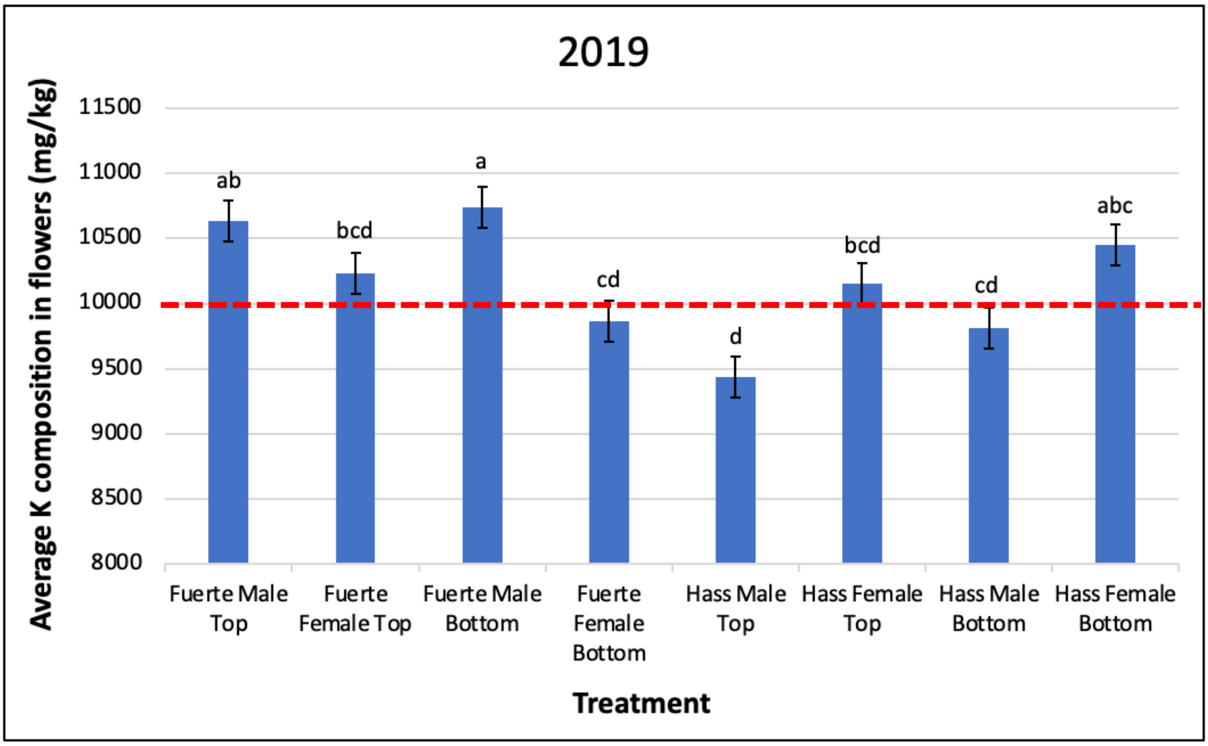
6.4 Results and Discussion

In order to simplify the discussion of results, the individual elements are first presented in dedicated graphs. As aforementioned, to our knowledge there are no nutritional floral standards for avocados. Therefore, foliar standards will be used instead. The foliar standards used were gathered from reports by Selladurai and Awachare (2019) for potassium, boron, and phosphorous, and Dixon (2008) for calcium.

Figure 6.3 highlights the differences in floral potassium content in 2019 and 2020. For both years, 'Fuerte' flowers appeared to accumulate more potassium compared to the 'Hass' flowers. In 2019, the actively male 'Fuerte' flowers contained more potassium (10 369 mg/kg to 10 738 mg/kg) compared to the active females (9866 mg/kg to 10 234 mg/kg) of the same cultivar, while the opposite was true for the 'Hass' flowers. However, this trend was not consistently observed in both sampling years (Figure 6.3). There was also no observable pattern for differences in potassium accumulation at the top and bottom of the canopy. Selladurai and Awachare (2019) suggest that the optimum foliar potassium concentration lie between 1-2 % (10 000 - 20 000 mg/kg). As Figure 6.3 shows, the floral samples are all on the lower end of this spectrum (9 436 mg/kg to 12 936 mg/kg), with some treatments in 2019 even falling below the standards of Selladurai and Awachare (2019).

Potassium plays a vital role in flowering (Fan *et al*. 2001) and when available in the form of potassium nitrate (KNO_3), influences pollen germination and development (Choudhurry *et al*. 2013). Oosthuyse and Berrios (2015) claim KNO_3 sprays have the potential to improve yields in avocados, especially when growth retardants such as paclobutrazol are used to limit excessive vegetative growth. Whilst little information relating directly to the role of potassium in pollen development is available, White and Karley (2010) explained the importance of the element in the 'reproductive organs' of angiosperms and the role it plays in cell metabolism, growth and development. It is reported that potassium has the potential to offset the negative effects of cold stress in susceptible plants (Wang *et al*. 2013), which includes 'Hass' avocados especially (Zaro *et al*. 2019). It is interesting to relate the increased potassium content of the 'Fuerte' flowers to the higher pollen count in the anthers of these flowers sampled during the cooler periods in Chapter 5. As the literature discussed above states, potassium can improve the cold tolerance of a plant. Therefore, the increased accumulation of potassium in the ‘Fuerte’ flowers may be a reason for the higher pollen count in the anthers of these flowers.

Furthermore, Choudhurry *et al*. (2013) highlight the importance of potassium for *in vitro* pollen germination, suggesting the element does play a role in pollen health and viability. Therefore, while the direct effects of potassium on pollen development cannot be made clear from this study, it is reasonable to suggest potassium levels have the potential to directly influence pollen health, thereby supporting the need for future research into the topic.



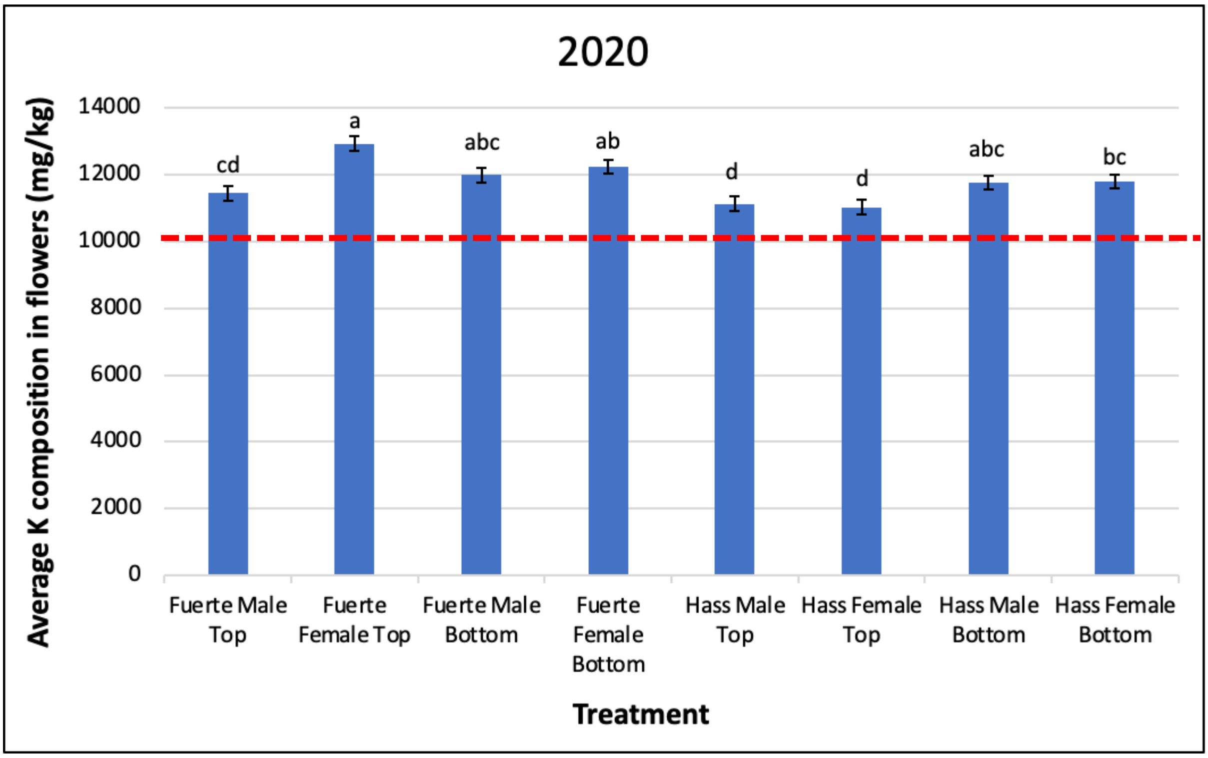
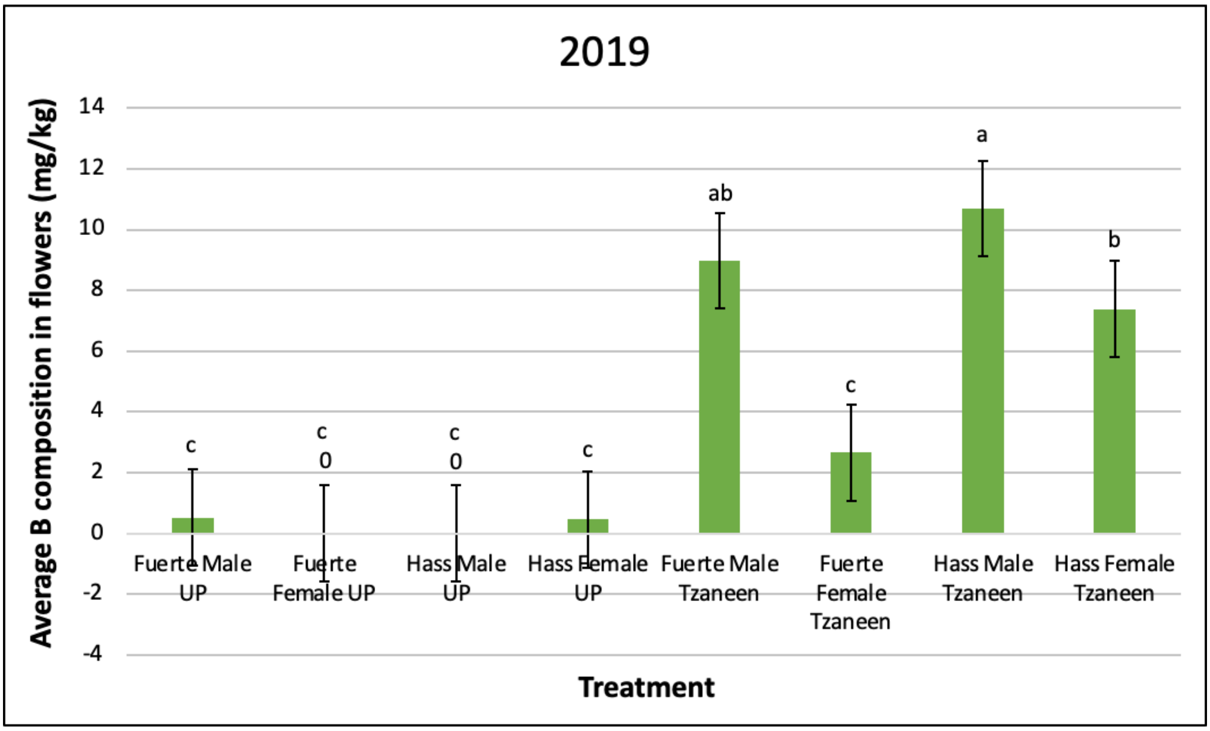


Figure 6.3. Average potassium (K) composition in avocado flowers (mg/kg) sampled during the flowering seasons in 2019 and 2020. Letters indicate significant differences ($p \leq 0.05$) between the treatments sampled from the same orchard. Vertical bars (±) indicate standard error (SE) of the mean. *The red line indicates the minimum recommended potassium threshold in avocado leaves (Selladurai and Awachare 2019).*

The results of the floral boron content are depicted in Figure 6.4 and Figure 6.5. What is immediately clear is that the 2019 samples were all severely deficient in boron when compared to the standards of Selladurai and Awachare (2019) that suggests the leaves contain more than 20 ppm (20 mg/kg) of the element. Similar to the potassium results, there were inconsistent trends associated with average boron content in the different cultivars and flower sexual phase (Figure 6.4) when both sampling years were considered. However, what these results may suggest is that when boron availability is limited, it is translocated to the organs in which there is a dire need therefor. This can be observed in Figure 6.4 where boron appeared to accumulate more in the male phase of the flowers sampled in Tzaneen.

Another interesting finding presented in Figure 6.4 is the difference in floral boron content between 2019 and 2020. In 2020, considerably more boron was detected in the flowers in spite of no fertiliser applications in the non-commercial orchard. In 2020, foliar samples were also collected in addition to floral samples. While this will be discussed in detail later in this chapter, it must be briefly mentioned now as it may explain the differences in floral boron content observed between 2019 and 2020. In 2020, the average floral boron content ranged from 3.41 to 38.65 mg/kg compared to 0 to 0.53 mg/kg in 2019. In 2020, the average foliar boron content ranged from 0 to 2.48 mg/kg. Therefore, the increased levels of boron observed in 2020 may be due to the translocation of boron from the leaves to the flowers, as illustrated in Figure 6.4. According to Brown and Hu (1996) boron is generally considered to be phloem immobile. However, our theory is in fact supported and perhaps confirmed by Minchen *et al.* (2011). These researchers proved that boron forms a phloem-mobile complex with perseitol, thus allowing the translocation of boron from mature leaves to developing inflorescences. The increased boron content in the ‘Fuerte’ samples in 2020 can also be due to alternate bearing. Boron accumulation increases in the flowers of trees experiencing ‘on’ years (Harkness 1959, Thorp 2011). As Chapter 4 made it clear, the ‘Fuerte’ trees in the orchard on the Experimental Farm at the University of Pretoria were experiencing an ‘off’ production year in 2019. This may explain why boron concentrations were lower that year (Figure 6.4).

Coetser *et al*. (1993) highlighted the importance of boron in pollen germination, fruit set and overall fruit growth. According to Cheng and Rerkasem (1993), boron deficiencies result in poorly developed pollen and anthers in wheat. This results in reduced male fertility because inadequate boron levels negatively impact microsporogenesis during meiosis in the anthers (Dell and Huang 1997). According to Batygina and Vasiliyeva (2003), boron deficiencies limit 'nucleic acid exchange', which in turn interrupts meiosis and ultimately leads to abnormal pollen formation. Therefore, it can be reasoned that when boron stores are limited, it will be preferentially allocated to the flowers in the male phase to perhaps maximise the chances of the tree producing viable pollen. Therefore, further studies focusing on this anomaly of preferential boron translocation in times of deficiency is required.



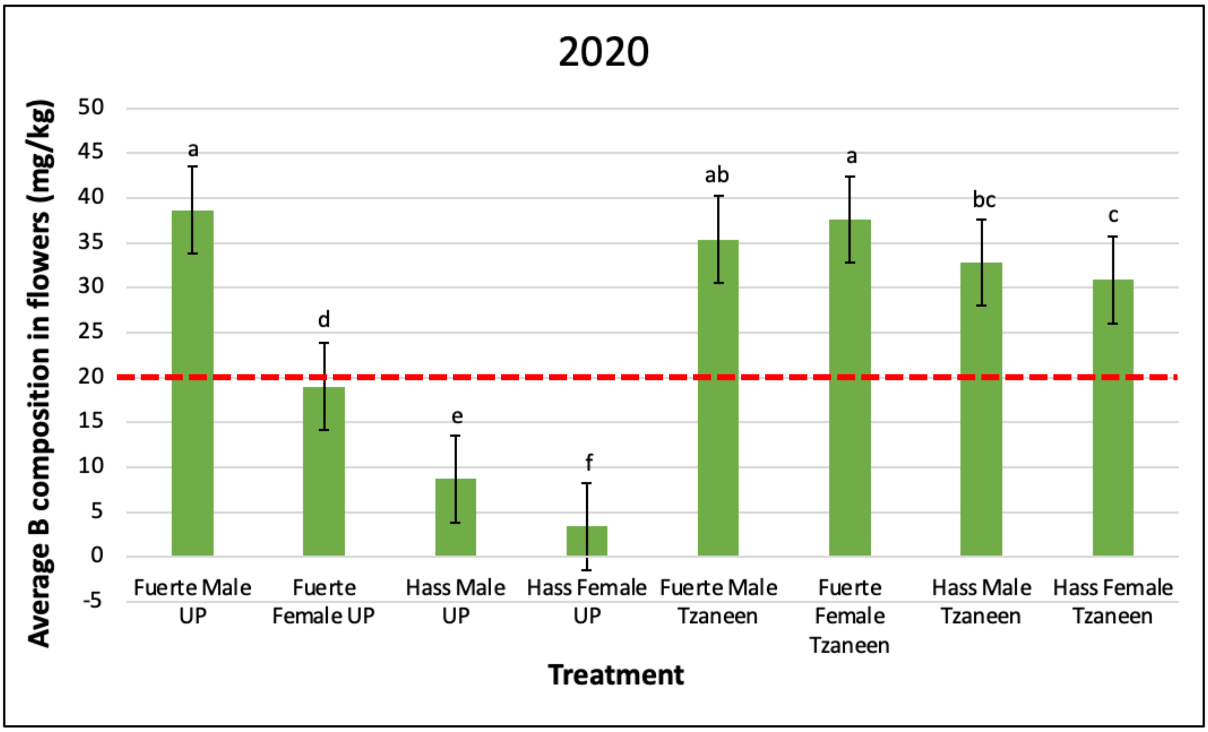


Figure 6.4. Average boron (B) composition in avocado flowers (mg/kg) sampled during the flowering seasons in 2019 and 2020 for the interaction effect between flower cultivar, flower phase and sampling location. Letters indicate significant differences ($p \leq 0.05$), and vertical bars (±) indicate standard error (SE) of the mean. *The red line indicates the minimum recommended boron threshold in avocado leaves (Selladurai and Awachare 2019).*

The differences in boron content between the top and bottom of the avocado canopy, as well as that for the samples taken from the Experimental Farm at the University of Pretoria and a commercial orchard in Tzaneen, are given in Figure 6.5. While differences in boron accumulation for the top and bottom of the canopy were observed, they were inconsistent throughout the two years. However, the floral samples taken from the commercial orchard did have a consistently higher boron concentration. In a commercial orchard, such as the one used for the purposes of this study, foliar boron sprays are commonly used to address the problem of boron deficiencies in South African orchards (Abercrombie 2011). Therefore, the higher floral boron levels of the samples taken from the commercial orchard is to be expected. What is interesting to note is that one of the only studies conducted into floral boron contents suggests pollen tube growth is maximised when the flowers contain between 50 mg/kg – 75 mg/kg of boron (Robbertse *et al*. 1998). None of the flowers sampled, including those in the commercial orchard, came close to reaching these levels of boron. Therefore, it can be reasoned that the current boron spray programs are not supplying the flowers with sufficient levels of the nutrient, and this may be contributing to poor pollen health and as a result may be limiting pollination, fruit set and yield. Therefore, it is suggested that commercial farmers consider increasing the amount of boron applied to their trees, especially during the flowering season. Furthermore, the development of boron-efficient rootstocks could supplement foliar boron sprays of phloem-mobile forms of boron to improve uptake and translocation of the nutrient (Wang *et al.* 2016). It is recommended that this aspect be focused on in future studies.

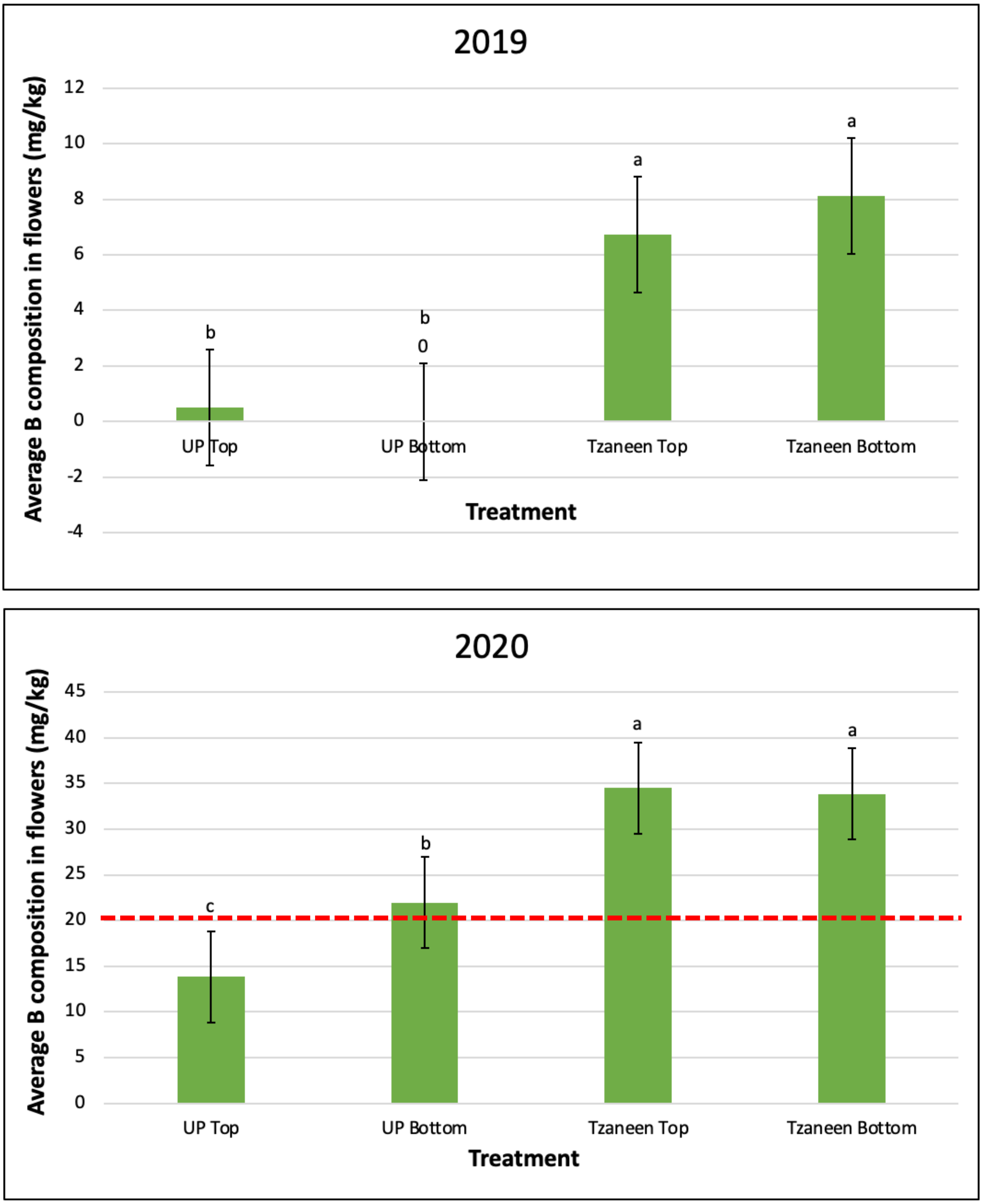


Figure 6.5. Average boron (B) composition in avocado flowers (mg/kg) sampled during the flowering seasons in 2019 and 2020. Letters indicate significant differences ($p \leq 0.05$), and vertical bars (±) indicate standard error (SE) of the mean. *The red line indicates the minimum recommended boron threshold in avocado leaves (Selladurai and Awachare 2019).*

The results for the floral phosphorous contents are given in Figure 6.6 and Figure 6.7. There was an interaction effect for flower phase and sampling location (Figure 6.6). Overall, the female phase of the flowers appeared to accumulate more phosphorous compared to the male phase, a trend which is clear in Figure 6.6. Similar to the results for boron, the flowers sampled from the commercial orchard had a higher phosphorous concentration. Based on the recommendations given by Selladurai and Awachare (2019) which suggested a foliar phosphorous concentration between 0.1 % - 0.25 % (1000 mg/kg – 2500 mg/kg), almost all the samples were found to contain more than this amount.

Adequate phosphorous fertilisation, either using foliar sprays or trunk injection, is essential in avocado production to protect the root system from *Phytophthora* root rot (Lovatt 1990). Adequate phosphorous levels also help avocado trees maintain a healthy canopy (Lahav and Kadman 1980). However, the direct influence of phosphorous on pollen development in avocados is poorly understood. Lau and Stephenson (1994) highlighted the importance of phosphorous on the amount of pollen produced per flower, and size thereof, in *Cucurbita pepo*. The authors refer to the findings of Stanley and Liskens (1974) and Mengel and Kirkby (1982), who explain the importance of phosphorous metabolization during pollen germination and the detrimental effect of phosphorous deficiencies on reproduction. However, due to the high concentration of phosphorous in the flower samples, it can be reasoned that even if its direct role in avocado pollen development is poorly understood, it is unlikely to be a limiting factor in this case.

What must be mentioned is the potential repellent effects of excess phosphorous on honey bees. Afik *et al*. (2006) claimed that both potassium and phosphorous repel honey bees. The discussion pertaining to the potassium content of the flowers above showed the compound to be present within the recommended foliar range. The same cannot be said for the phosphorous content within the flowers. This is important to consider when looking at the interaction effect of cultivar and flower phase (Figure 6.7) and how it could relate to the honey bee preferences discussed in Chapter 2. The phosphorous content tended to be higher in the active female flowers, which may be an additional reason why active male flowers were continuously preferred by honey bees.

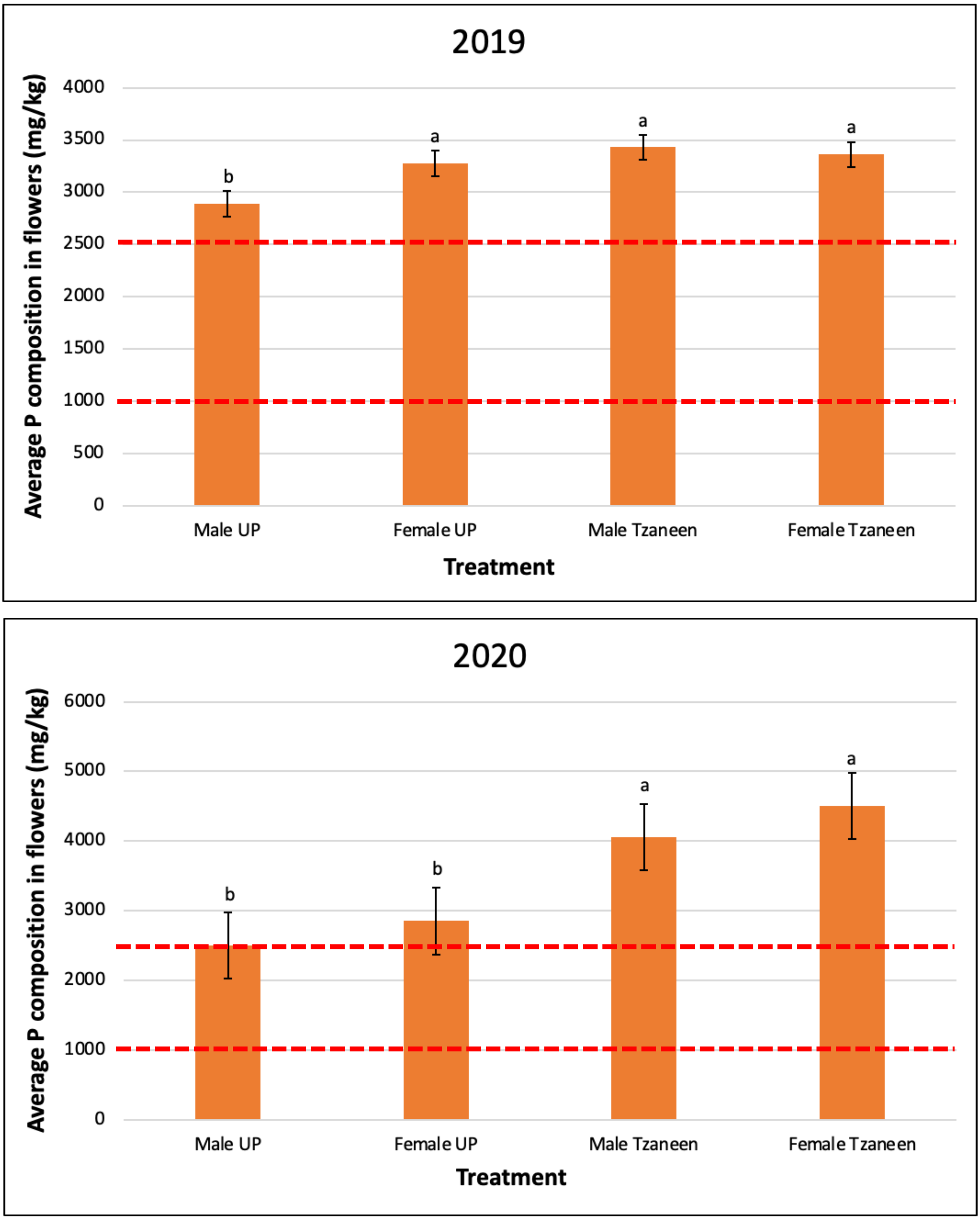


Figure 6.6. Average phosphorous (P) composition in avocado flowers (mg/kg) sampled during the flowering seasons in 2019 and 2020 for the interaction effect between flower phase and sampling location. Letters indicate significant differences (p ≤ 0.05), and vertical bars (±) indicate standard error (SE) of the mean. *The red lines indicate the minimum and maximum recommended phosphorous thresholds in avocado leaves (Selladurai and Awachare 2019).*

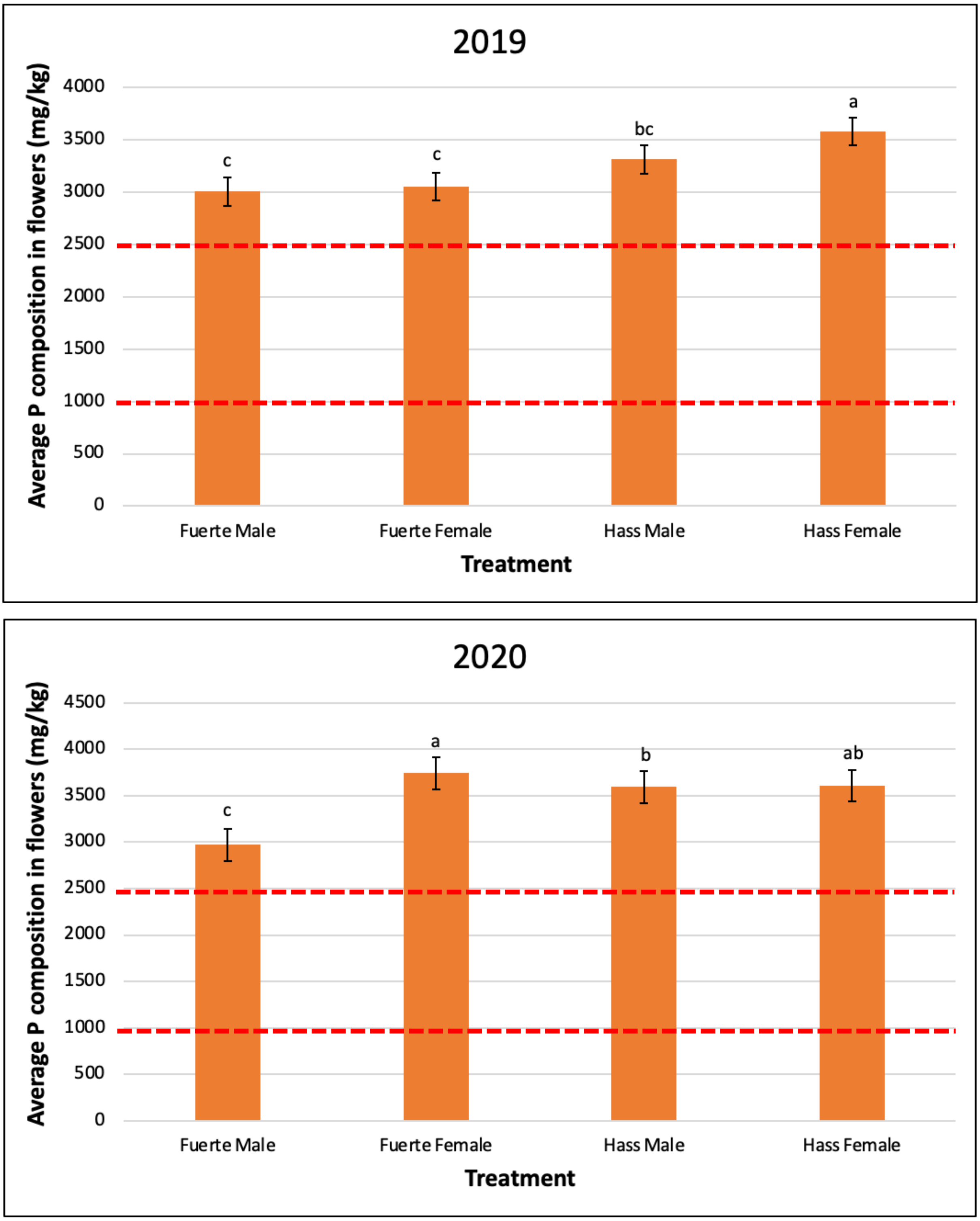
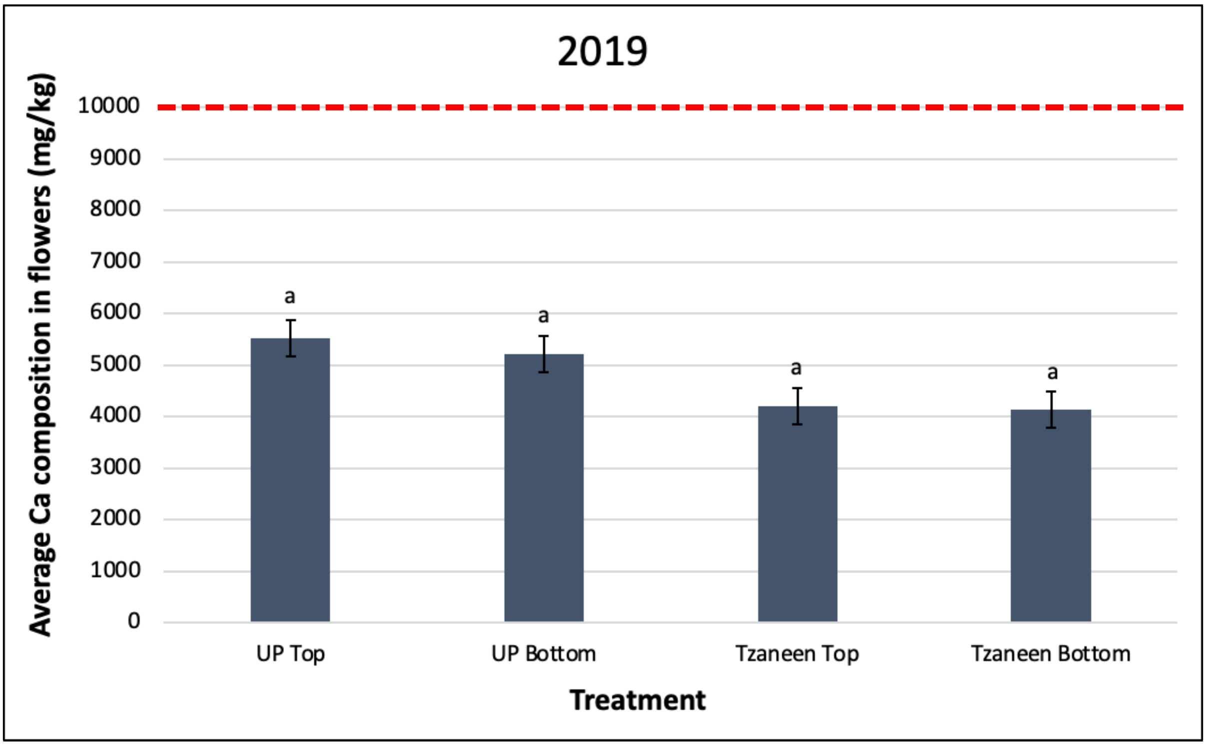


Figure 6.7. Average phosphorous (P) composition in avocado flowers (mg/kg) sampled during the flowering seasons in 2019 and 2020 for the interaction effect between flower cultivar and flower phase. Letters indicate significant differences ($p \leq 0.05$), and vertical bars (±) indicate standard error (SE) of the mean. *The red lines indicate the minimum and maximum recommended phosphorous thresholds in avocado leaves (Selladurai and Awachare 2019).*

The final floral element that is going to be discussed is calcium. Figure 6.8 depicts the results for the sampling location and canopy height interaction effect that was observed. A report by Dixon (2008) suggested that the average foliar calcium concentration be between 1 % – 3 % (10 000 mg/kg – 30 000 mg/kg). What is immediately noticeable is that the samples from both orchards appear to be deficient in calcium. The non-commercial orchard on the Experimental Farm at the University of Pretoria had a higher concentration of calcium compared to the commercial orchard in Tzaneen. This did not follow the pattern observed with the other elements. As Penter and Stassen (2000) explain, when it comes to calcium the absolute soil concentration is far less important compared to its balance with other elements. Therefore, the commercial orchard may be managed in such a way that focuses on balancing soil calcium content as opposed to merely increasing it. Acidic soil conditions will also influence the uptake of the nutrient. Fertiliser selection and ample summer rainfall in the commercial orchard in Tzaneen could acidify the soil, thus minimizing the amount of plant available calcium. Bender and Faber (1999) recommend growers use calcium nitrate, $Ca(NO_3)_2$, as a supplemental calcium source in acidic soils. This fertiliser also offers the additional benefit of preventing *Phytophthora cinnamomi* infection.

Chen *et al*. (2008) conducted one of the pioneer studies that focused on the role of calcium in pollen development. They found that calcium was prevalent in the walls of *Torenia fournieri* pollen grains, which aid in protecting the pollen grains from environmental stressors (Wang and Dobritsa 2018). Keeping in mind that as is the case with most of the elements discussed in this study, the role they play directly in pollen development in avocados is poorly understood. Therefore, considering the supposed deficiencies that Figure 6.8 depicts, studies that aim to determine the effects of increased calcium fertilisation rates on avocado pollen development are warranted.



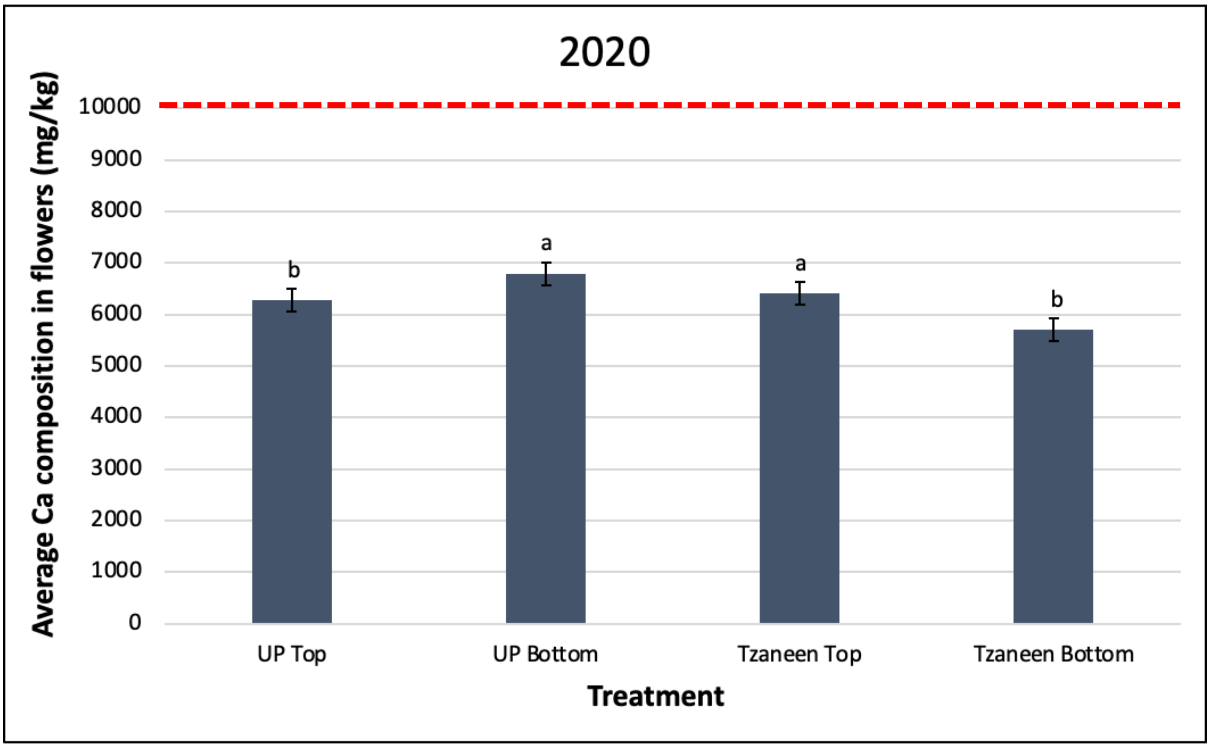


Figure 6.8. Average calcium (Ca) composition in avocado flowers (mg/kg) sampled during the flowering seasons in 2019 and 2020 for the interaction effect between flower sampling location and position within the canopy. Letters indicate significant differences ($p \leq 0.05$), and vertical bars (±) indicate standard error (SE) of the mean. *The red lines indicate the minimum calcium threshold in avocado leaves (Dixon 2008).*

Potassium, boron, phosphorous and calcium were detected in differing amounts based on the sexual phase in which the flowers were sampled. As depicted in Figure 6.9, the following patterns were observed: 1) Higher amounts of boron were detected in the flowers sampled during the active male phase (5.03 mg/kg and 27.57 mg/kg in 2019 and 2020, respectively) compared to those sampled in the active female phase (2.63 mg/kg and 24.34 mg/kg in 2019 and 2020, respectively); and 2) flowers active in the female phase tended to accumulate slightly more phosphorous (3 317 mg/kg and 3607 mg/kg in 2019 and 2020, respectively) compared to flowers active in the male phase (3 159 mg/kg and 3 283 mg/kg in 2019 and 2020, respectively). These findings support the claims made earlier in this chapter. Boron appears to be preferentially allocated to flowers active in the male phase to ensure optimum pollen health at the time of anthesis (Cheng and Rerkasem 1993, Dell and Huang 1997, Batygina and Vasiliyeva 2003). Pollen development and health thereof is dependent on sufficient boron availability, therefore the flowers active in the male phase seem to have a greater ‘pull’ for boron (Eaton 1944, Coetser *et al*. 1993). Furthermore, these findings may also explain the slight differences in the amount of pollen detected in the compartments of anthers sampled in the different sexual phases in Chapter 5, thus reiterating the claim that the overnight closure between the sexual phases are physiologically important. In addition to this, the increased levels of phosphorous [a known honey bee deterrent (Afik *et al.* 2006)] detected in flowers sampled in the female phase support the findings of Chapter 2 in which we showed honey bees consistently preferring avocado flowers active in the male phase. The overall excess levels of phosphorous detected in both the active male and female flowers (Figure 6.9) may also explain why other floral sources (such as citrus) are preferred by honey bees.

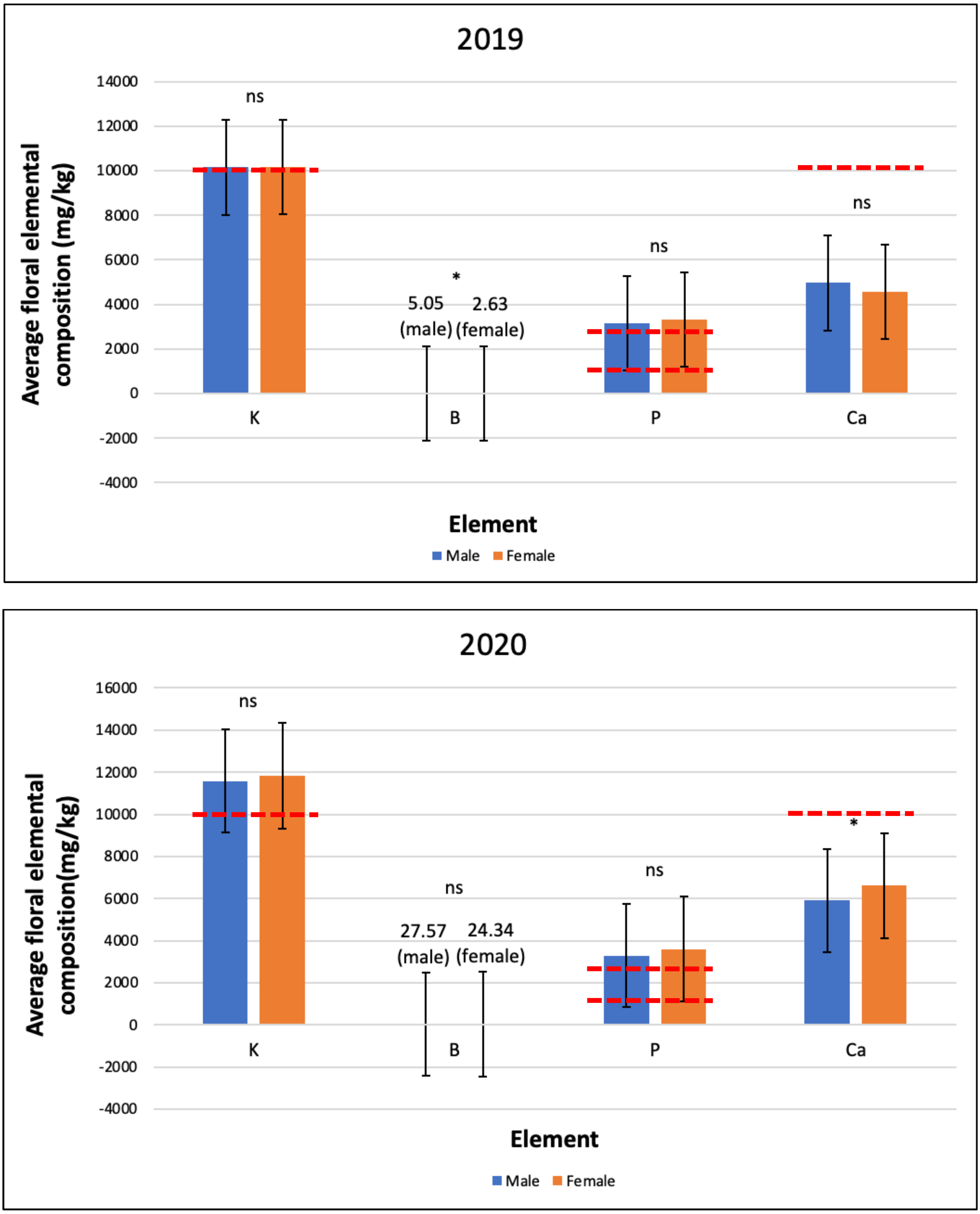
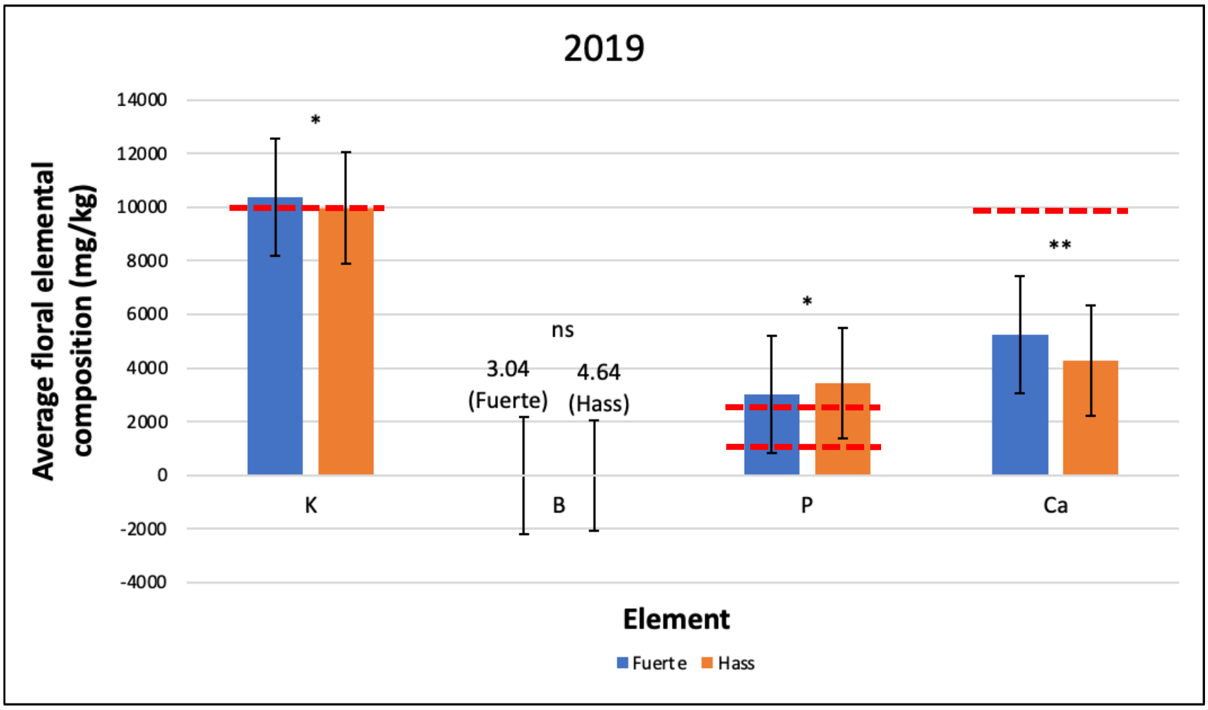


Figure 6.9. Average potassium (K), boron (B), phosphorous (P), and calcium (Ca) composition of the two sexual phases of the avocado flowers (mg/kg) sampled during the flowering seasons in 2019 and 2020. The amounts depict the averages for flowers sampled from both the commercial orchard in Tzaneen and the non-commercial orchard in Pretoria. Blue bars – functionally male flowers, orange bars – functionally female flowers. NS – no significant differences detected, * - significant differences detected ($p \leq 0.05$). Vertical bars (±) indicate standard error (SE) of the mean. *Red lines indicate the minimum and maximum recommended thresholds in avocado leaves, recommended minimum boron levels are 20 mg/kg (Dixon 2008, Selladurai and Awachare 2019).*

Potassium, boron, phosphorous and calcium were also detected in differing amounts based on the cultivar from which the flowers were sampled (Figure 6.10). The following patterns were observed: 1) Potassium accumulated more in ‘Fuerte’ flowers (10 369 mg/kg and 11 991 mg/kg in 2019 and 2020, respectively) compared to ‘Hass’ flowers (9 962 mg/kg and 11 428 mg/kg in 2019 and 2020, respectively); 2) phosphorous accumulated more in ‘Hass’ flowers (3 447 mg/kg and 3 600 mg/kg in 2019 and 2020, respectively) compared to ‘Fuerte’ flowers (3 030 mg/kg and 3 290 mg/kg in 2019 and 2020, respectively), and 3) calcium accumulated more in ‘Fuerte’ flowers (5 261 mg/kg and 6 817 mg/kg in 2019 and 2020, respectively) compared to ‘Hass’ flowers (4 276 mg/kg and 5 718 mg/kg in 2019 and 2020, respectively). Again, these results support the claims made by the findings presented earlier in this chapter. The higher levels of potassium may increase the cold-tolerance of ‘Fuerte’ trees (Wang *et al*. 2013, Zaro *et al*. 2019) and could be a reason for the higher levels of pollen produced in those anthers, as described in Chapter 5. The higher levels of honey bee deterring phosphorous detected in the ‘Hass’ flowers may contribute to the honey bee’s preference for ‘Fuerte’ flowers, as observed in Chapter 2 (Afik *et al*. 2006). In spite of the limited understanding as to how calcium influences pollen development in avocados, it is clear that certain cultivars have differing abilities to accumulate the nutrient in the flowers. As calcium has the potential to protect certain plants from abiotic stress, the higher levels observed in the ‘Fuerte’ flowers may contribute to the cold-tolerating properties of that cultivar (Wang and Dobritsa 2018).



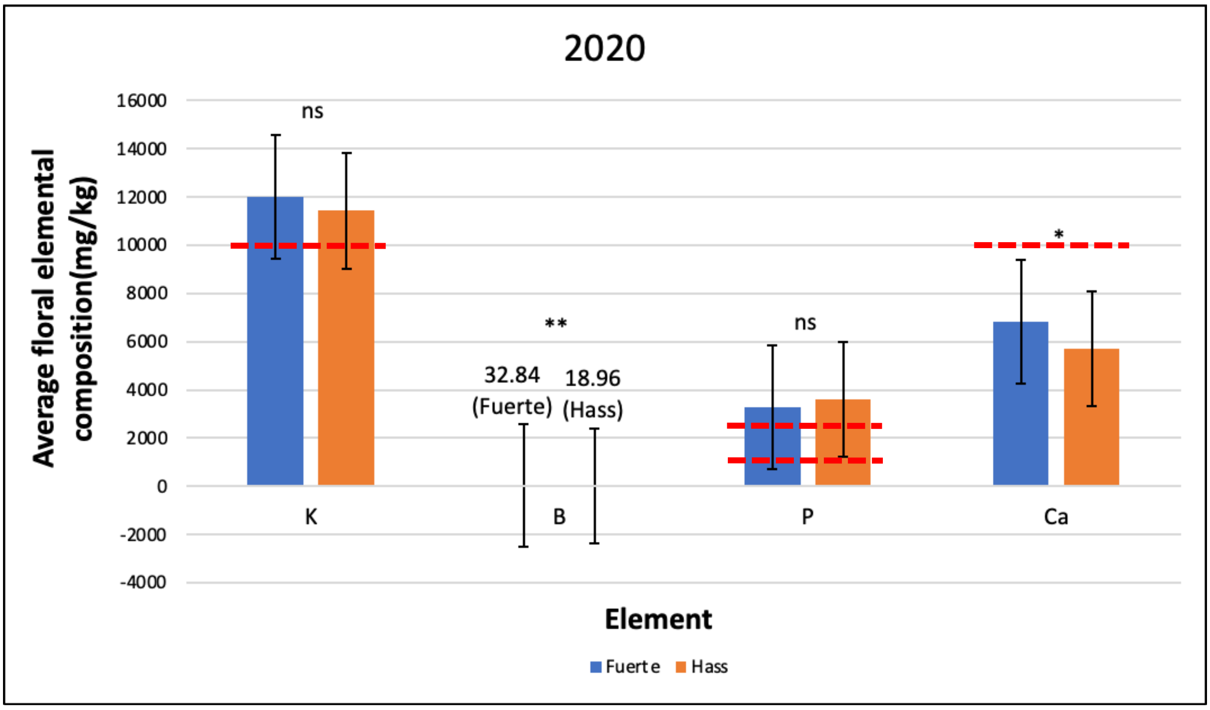


Figure 6.10. Average potassium (K), boron (B), phosphorous (P), and calcium (Ca) composition of the avocado flowers (mg/kg) belonging to the cultivars 'Fuerte' and 'Hass' sampled during the flowering seasons in 2019 and 2020. The amounts depict the averages for flowers sampled from both the commercial orchard in Tzaneen and the non-commercial orchard in Pretoria. Blue bars – 'Fuerte', orange bars – 'Hass'. NS – no significant differences detected, * - significant differences detected, ** - highly significant differences detected ($p \leq 0.05$). Vertical bars (±) indicate standard error (SE) of the mean. *Red lines indicate the minimum and maximum recommended thresholds in avocado leaves, recommended minimum boron levels are 20 mg/kg (Dixon 2008, Selladurai and Awachare 2019).*

In 2020, foliar samples were collected in addition to flower samples. The three main macroelements for the foliar samples are depicted in Figure 6.11. These results show a stark difference in elemental content between the flower and leaf samples. The leaves contained a lower concentration of potassium, phosphorous and calcium (Figure 6.11), and almost all of the samples were deficient in potassium and calcium. Throughout a season, the organs of an avocado tree will exhibit a fluctuating source/sink relationship (Whiley 1994). It appears that the flowers act as a stronger sink for these compounds compared to the leaves during the flowering season. Blumenfeld *et al*. (1989) explain how during periods of anthesis, there is competition for photoassimilates (carbohydrates) between the reproductive and vegetative growth points. However, Finazzo *et al*. (1994) suggest that the accumulation of these photoassimilates was not dependant on organ type, claiming carbohydrates are not a limiting factor affecting fruit set and fruitlet abscission. However, the marked difference in elemental accumulation between the flowers and leaves does suggest the flowers may act as a stronger sink for nutrients. Further research into this viewpoint is required to determine its effects on pollen development and fruit set in avocado trees.

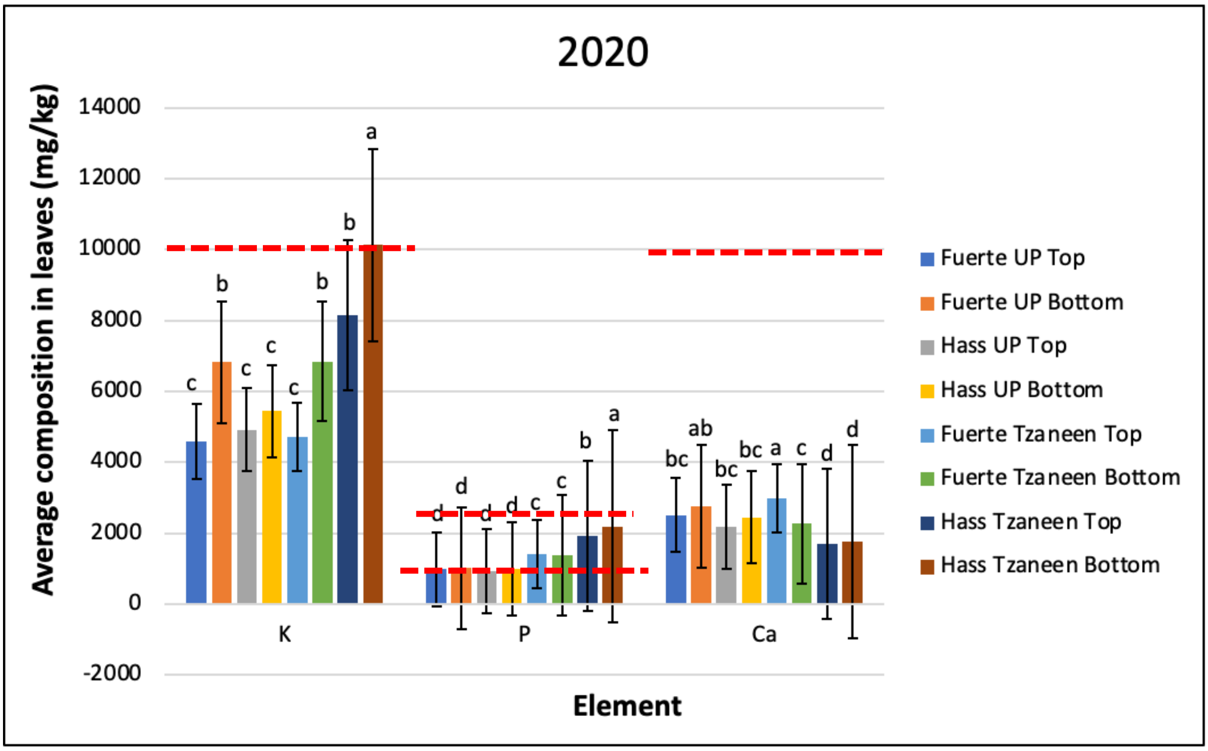


Figure 6.11. Average potassium (K), phosphorous (P), and calcium (Ca) composition (mg/kg) in the leaves of avocados sampled in 2020. Letters indicate significant differences ($p \leq 0.05$), and vertical bars (±) indicate standard error (SE) of the mean. *The red lines indicate the minimum and/or and maximum recommended thresholds in avocado leaves (Dixon 2008, Selladurai and Awachare 2019).*

When it came to foliar boron concentrations significant differences were detected for the cultivar, canopy height and location interaction effects (Figure 6.12). Apart from the 'Fuerte' leaves sampled in Tzaneen, all of the other samples were either completely lacking or exceedingly deficient of boron. Apart from the flowers sampled from the top of the canopy of the 'Fuerte' trees in Tzaneen, all of the flower samples had higher boron concentrations. This strengthens the claim theorised above which suggests flowers act as stronger sinks compared to the leaves. According to Eaton (1944), boron will continue to be remobilised throughout a plant until the requirements in every cell are satisfied. Coetser *et al*. (1993) relied on this explanation when they encountered similar results to this study that detected higher boron content in avocado flowers compared to that of the leaves. These findings thus validate the results of this study which shows flowers tend to act as a stronger sink for boron, in addition to potassium, phosphorous, and calcium, compared to the leaves.

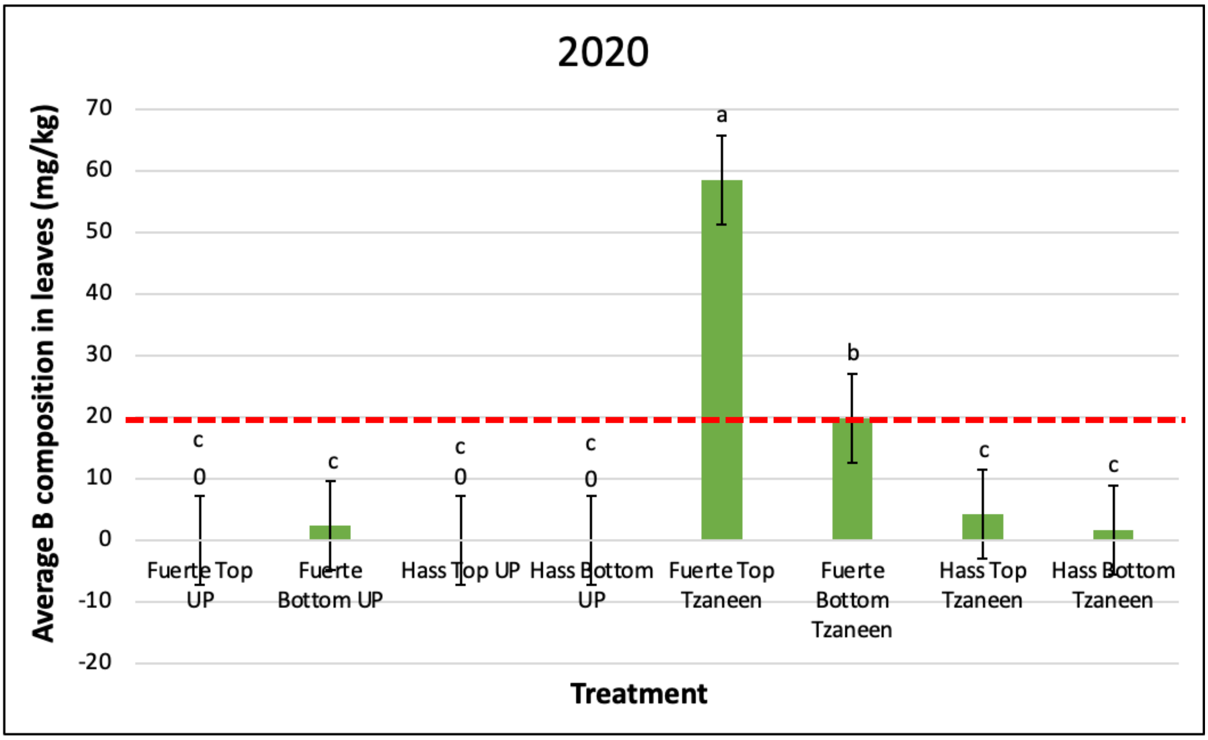


Figure 6.12. Average potassium boron (B) composition (mg/kg) in the leaves of avocado flowers sampled in 2020 for the interaction effect for flower cultivar, position in the canopy and sampling location. Letters indicate significant differences ($p \leq 0.05$), and vertical bars (±) indicate standard error (SE) of the mean. *The red lines indicate the minimum recommended threshold for boron in avocado leaves (Selladurai and Awachare 2019).*

Similar to the findings depicted in Figure 6.10, the leaves of ‘Fuerte’ and ‘Hass’ differentially accumulated nutrients in their leaves. Figure 6.13 shows how ‘Fuerte’ leaves accumulated significantly more boron (17.38 mg/kg) and calcium (26 261 mg/kg) compared to ‘Hass’ leaves (0.68 mg/kg and 20 229 mg/kg for boron and calcium, respectively). These findings are an observable effect of how rootstocks influence nutrient uptake in avocados. Salazar-Garcia *et al.* (2016) claim that certain rootstocks can positively or negatively influence nutrient uptake in ‘Hass’. ‘Duke’ has also been reported to increase foliar phosphorous concentration (Labanauskas *et al*. 1978). However, it cannot be concluded that the trees in the commercial orchards accumulated more phosphorous due to their ‘Duke 7’ rootstocks as these trees received more fertiliser compared to those in the non-commercial orchard that are grafted onto ‘Dusa’ and ‘Latas’ rootstocks (Figure 6.14).

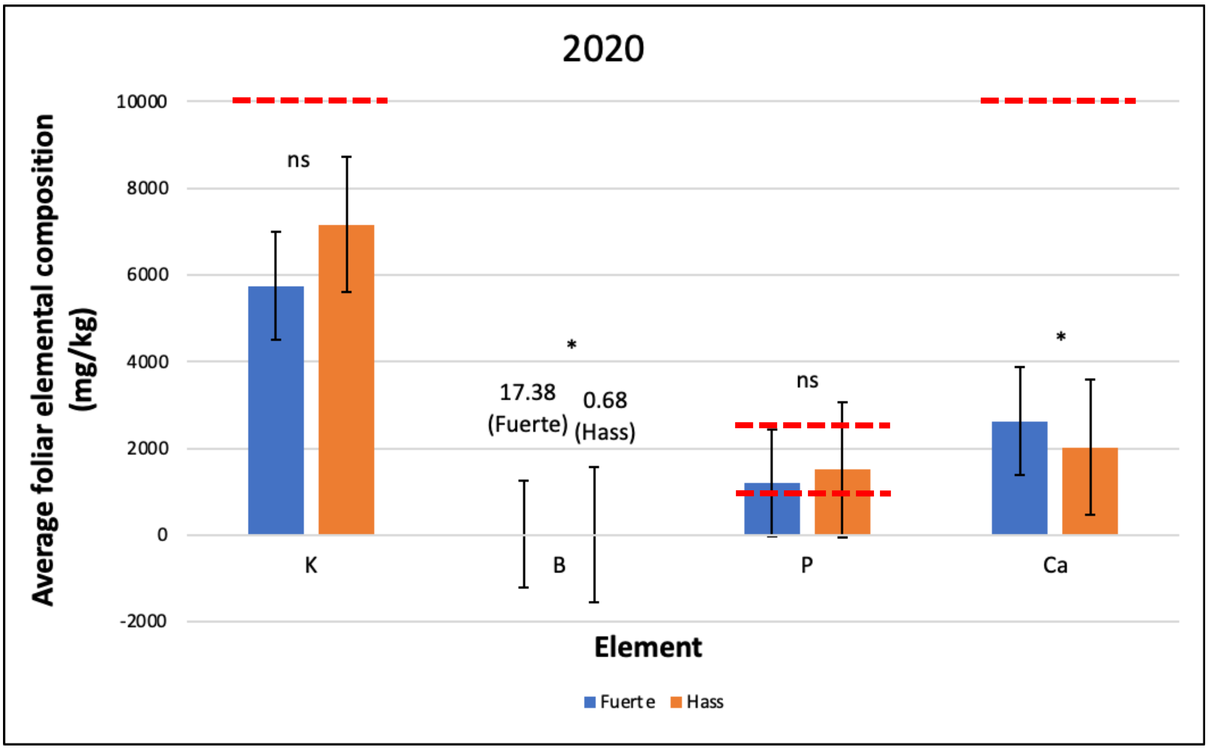


Figure 6.13. Average potassium (K), boron (B), phosphorous (P), and calcium (Ca) composition of the avocado leaves (mg/kg) belonging to the cultivars ‘Fuerte’ and ‘Hass’ sampled during the flowering seasons in 2019 and 2020. The amounts depict the averages for flowers sampled from both the commercial orchard in Tzaneen and the non-commercial orchard in Pretoria. Blue bars – ‘Fuerte’, orange bars – ‘Hass’. NS – no significant differences detected, * - significant differences detected ($p \leq 0.05$). Vertical bars (±) indicate standard error (SE) of the mean. *Red lines indicate the minimum and maximum recommended thresholds in avocado leaves, recommended minimum boron levels are 20 mg/kg (Dixon 2008, Selladurai and Awachare 2019).*

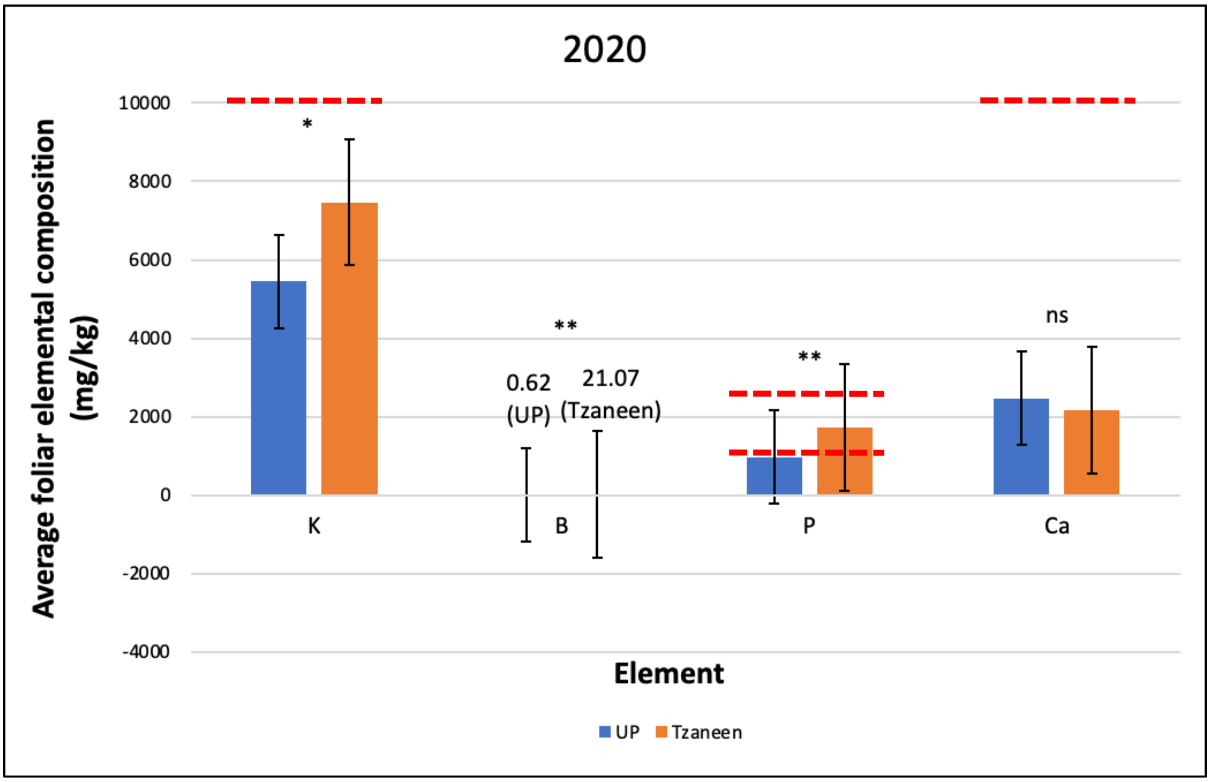


Figure 6.14. Average potassium (K), boron (B), phosphorous (P), and calcium (Ca) composition of the avocado leaves (mg/kg) sampled from the commercial orchard in Tzaneen (orange bars) and the non-commercial orchard at the University of Pretoria (UP) (blue bars). NS – no significant differences detected, * - significant differences detected, ** - highly significant differences detected ($p \leq 0.05$). Vertical bars (±) indicate standard error (SE) of the mean. *Red lines indicate the minimum and maximum recommended thresholds in avocado leaves, recommended minimum boron levels are 20 mg/kg (Dixon 2008, Selladurai and Awachare 2019).*

When the nutrient concentrations of the floral samples are compared to the foliar samples, it is clear that the flowers exert a stronger ‘pull’ for all of the nutrients studied in this chapter. Therefore, the current practice of using foliar samples as an indicator of floral nutrient status is not optimised. When this is considered in conjunction with the fact that flowers require distinct amounts of nutrients compared to the leaves, the need for the development of floral standards becomes apparent. This is a lengthy process and it will take several years to compile thorough standards. Therefore, by comparing the ratios of nutrient allocation between the leaves and the flowers, we can develop a simple set of floral standards in the meantime. Table 6.1 shows the stepwise calculations (from left to right) involved in developing a recommendation for floral nutrient concentrations, the first known attempt to do so.

Table 6.1. Stepwise calculation of developing a simple recommendation for floral nutrient concentrations (mg/kg) of potassium (K), boron (B), phosphorous (P) and calcium (Ca) in avocado flowers.

Element	**Floral averages (mg/kg)**	**Foliar averages (mg/kg)**	**Floral: Foliar**	**Recommended Foliar Concentrations (mg/kg)**	**Amended Floral Concentrations (mg/kg)**
K	10 937.80	6 452.48	1.70	10 000 – 20 000[c]	16 951 – 33 902
B	14.87	9.03	1.65	> 20[c]	> 33 or 50 – 75[a]
P	3 342.21	1 351.33	2.43	1 000 – 2 500[c]	2 473 – 6 183
Ca	5 518.65	2 324.54	2.37	10 000 – 30 000[b]	23 740 – 71 222

Recommended floral concentrations according to Robbertse *et al.* (1998)[a], and amended floral concentrations using the recommended foliar concentrations of Dixon (2008)[b] and Selladurai and Awachare (2019)[c]. In terms of boron (B), it is suggested the recommendations of Robbertse *et al.* (1998) be used to ensure optimum pollen germination.

The amended floral concentrations included in Table 6.1 could be useful to commercial farmers who want to analyse floral nutrient status in their orchards. However, as this chapter has already highlighted, the high concentrations of phosphorous found within the avocado flowers could potentially repel honey bees. Therefore, investigations need to be conducted to determine which phosphorous concentrations deter honey bees from avocado flowers. Furthermore, further studies are required to determine the precise role of these nutrients in avocado flowering physiology which could thus call for higher or lower concentrations than what is reported in Table 6.1.

This chapter thus represents the final cog in the cyclic machine of low pollination in avocados (Figure 6.15).

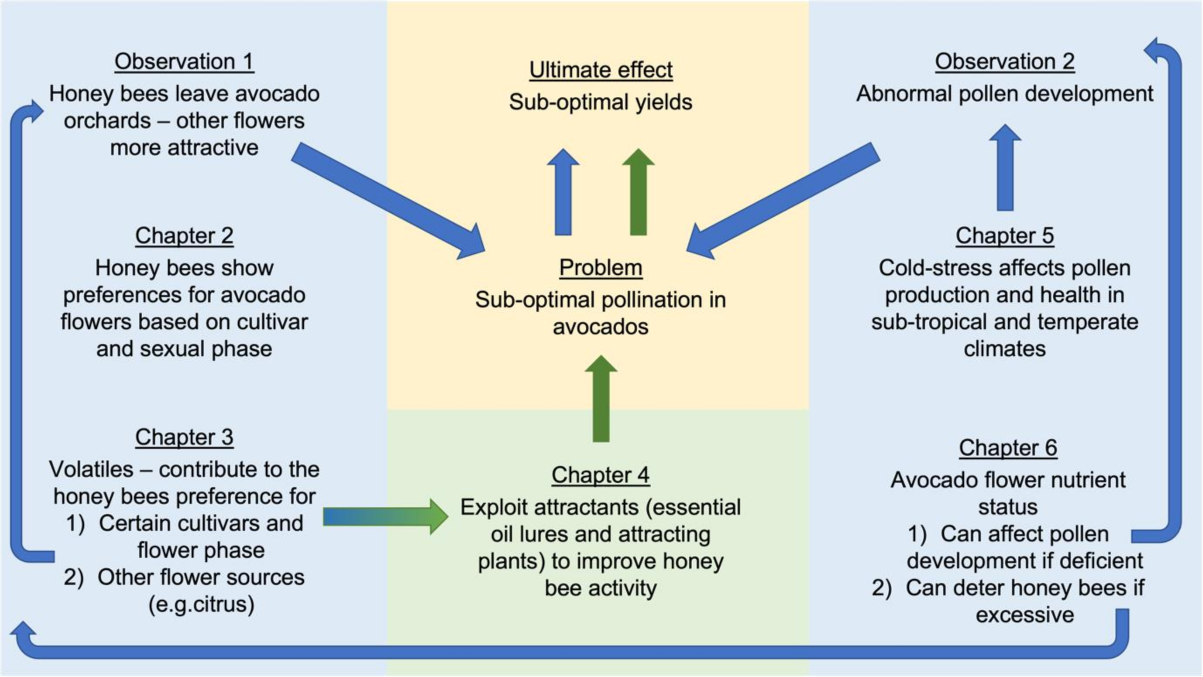


Figure 6.15. Organogram depiction of the holistic approach employed in this study to determine how sub-optimal pollination in avocados contributes to the ultimate effect of sub-optimal yields. Chapter 6 concluded the investigation and determined floral nutrient status can affect both pollen production and honey bee activity. Blue arrows represent pathways that undermine pollination in avocados and lead to yield reductions. Green arrows represent pathways that promote pollination in avocados and minimise yield reductions.

Floral nutrient status was the first variable discussed in this study that had the potential to affect not one, but two facets of low pollination in avocados. Not only can nutrient deficiencies contribute directly towards sub-optimal pollination by compromising pollen development, but nutrient excesses can indirectly influence pollination through their deterring effect on honey bees. These findings make clear the need for the urgent development of floral nutrient statuses, considering the direct role of these nutrients on both pollen development and pollinator activity.

6.5 Conclusion

This study highlighted the differential nutrient allocation to the flowers of certain avocado cultivars, sexual phase (when sampled in the active male or female phases), position within the canopy and sampling location. The preferential allocation of boron, a nutrient essential for pollen development, to actively male flowering sites in deficiency cases was interesting to note. Furthermore, phosphorous, a known honey bee repellent, was present in excessive amounts, especially in the female phase of the flowers. Calcium was observed to be present in very low concentrations in all treatments. Lastly, flower samples had higher elemental concentrations compared to the leaf samples, suggesting there is a differential allocation of elements between the reproductive and vegetative organs during the blooming season.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Over the course of the last two decades avocado production has increased three-fold to meet the increasing demand for the fruit (Shahbandeh 2020a, Shahbandeh 2020b). This has been possible due to the improvements in post-harvest technology, open trading markets and the health aspects of the avocado (Duarte *et al*. 2016).

The benefits associated with avocado consumption are numerous. Containing many vitamins, healthy fats, fibre and phytochemicals, when included in a diet the avocado has the potential to improve and maintain an individual’s health (Bergh 1992a, Wigg *et al*. 1996, Bouic 2002, Lottenberg 2002, Fulgoni *et al*. 2013, Pahua-Ramos *et al*. 2014). The avocado is also used in the manufacturing of non-foodstuff commodities (Teixeira *et al*. 1992, Duarte *et al*. 2016).

The majority of the species belonging to the botanical family Lauraceae are tropical or subtropical trees – the avocado is no exception (Kostermans 1957, Bergh and Ellstrand 1986). Due to its status as a cultigen, scientists have experienced difficulty in determining the avocado’s precise region of origin (Spencer and Cross 2008, Chen *et al*. 2009). In spite of this, it is generally accepted that the avocado originates from the area in and around central Mexico (Storey *et al*. 1986). Three horticultural races have resulted after centuries of selection (the West Indian, Guatemalan, and Mexican) and are differently suited to certain climates (Bergh 1992b).

Over the last three centuries, the avocado has been introduced and commercialised throughout the world (Schaffer *et al*. 2013, Silva and Ledesma 2014). In spite of this, avocado trees are hindered by inherent low yields (Garner and Lovatt 2008, Mickelbart *et al*. 2012). The avocado is an oil-rich fruit with a large seed, development of which demands a substantial energy investment from the tree (Wolstenholme 1986, Ranade and Thiagarajan 2015).

Apart from the role of the fruit itself contributing to the low yielding nature of the avocado trees, other factors such as low pollination, alternate bearing, competition between vegetative and reproductive shoots, and disease infestations also have deleterious effects (Whiley *et al*. 1986, Sedgley 1987, Ish-Am *et al*. 1999, Mickelbart *et al*. 2012).

The avocado flower is characterised as being monoecious and protogynous with a synchronous, dichogamous flowering pattern (Nirody 1922, Davenport 1986, Ish-Am and Eisikowitch 1993). It is due to this separation of the functional female and male phases that leads many experts to believe that optimal fruit set is achieved through cross-pollination (Stout 1923, Clark 1924, Bergh 1968). Alcaraz and Hormaza (2009) highlighted the importance of adequate cross-pollination when their study into the effects of hand-pollination in avocados increased fruit set by 15 %.

Stout (1923) classified avocado cultivars as ‘A-type’ or ‘B-type’, depending on the time of the day the female and male organs are active. The flowers of ‘t-Type’ cultivars open first in the female phase in the morning, close and reopen the following afternoon in the male phase, while the flowers of ‘B-type’ cultivars open in the female phase in the afternoon, close and reopen the following morning in the male phase (Stout 1923, Peterson 1955, Davenport 1986). This leads many professionals in the field of pollination to recommended interplanting ‘A-type’ avocado orchards with ‘B-type’ pollinisers (McGregor 1976, Bezuidenhout *et al*. 2017).

When cultivated in their native region, many insects including stingless bees, flies, wasps, and beetles pollinate the avocado flower (Nieto 1985, Ish-Am *et al*. 1999). However, when cultivated outside this region, the honey bee is often the primary pollinator (Stout 1923, Ish-Am and Eisikowitch 1991, Ish-Am and Eisikowitch 1993). Unfortunately, there are studies discussing the non-preference shown by honey bees towards avocado flowers (Afik *et al*. 2008).

Further investigation into the influence of low pollination in avocado production uncovered a myriad of issues, both internal and external to the avocado tree, that had the potential to limit pollination. This laid the basis for this study, the main aim of which was to determine if low pollination is a contributing factor to low yields in avocados and if it is an issue, to seek possible solutions for the problem.

Considering the necessity for honey bees to facilitate cross-pollination between ‘A-type’ and ‘B-type’ cultivars, it was useful to determine if honey bees exhibited a preference for avocado flowers belonging to different cultivars. By monitoring honey bee activity surrounding avocado trees of different cultivars while the flowers were open in the male and female phases, Chapter 2 of this study found that honey bees do exhibit a differential preference for certain cultivars. While a consistent trend was not clear outright, it appeared that honey bees prefer ‘B-type’ cultivars like ‘Fuerte’ and ‘Zuatno’. Due to the fact that honey bee activity is affected by other factors, such as wind speed and ambient temperature (Vicens and Bosch 2000), these variables may have played a role in why cultivar preferences were not always clear. However, there was a consistent preference shown towards the male phase of the flowers. Many factors may contribute towards this preference, including the increased floral display (Bell 1985, Ashman *et al*. 2005) and the added reward of pollen in addition to nectar that the functional male flowers offer (Gonzalez *et al*. 1995). Furthermore, there are reports of avocado nectar containing repellent compounds (Afik *et al*. 2006), which may be differentially perceived in the different cultivars and flower phases. The preference shown towards the male phase of the avocado flowers may limit visitation to the female phase of the flowers, resulting in reduced pollination and fruit set. Lastly, comparing honey bee activity between commercial and non-commercial orchards highlighted the dramatic effects of drought on honey bee activity. This suggests that no matter how favourable the flowers, their ability to attract honey bees will not outweigh the negative effects of drought.

Floral scent is perceived by honey bees through specialised volatile detectors which allows them to select those that emit more favourable odours (Galizia and Menzel 2000). Honey bees have been observed to leave avocado orchards in which their hives are placed to forage among more attractive flowers (Gazit and Degani 2002, Afik *et al*. 2008). As avocado flowers require cross-pollination to set fruit, this exodus of honey bees may have a negative effect on yield (Vrecenar-Gadus and Ellstrand 1985, Davenport 1986). To our knowledge, the volatile profile of the avocado flower has yet to be fully described. Chapter 3 of this study aimed to solve this gap in knowledge, and in so doing allowed for an improved understanding of what compounds are present and in what quantities, and the effects thereof on attracting or deterring honey bees. Through the use of solid-phase microextraction (SPME) followed by gas chromatography-olfactometry (GC-O) analysis, it was determined that the floral volatile profile was distinct based on flower cultivar and sexual phase. The five most prominent volatiles detected in ‘Hass’ and ‘Fuerte’ flowers were limonene, α-pinene, β-pinene, α-phellandrene and eucalyptol. These compounds are terpenoids, specifically monoterpenes, which contribute to floral scent in many angiosperms (Knudsen *et al*. 1993).

We believe this dissertation is thus the first to fully describe the volatile profile of a variety of avocado cultivars. Afik *et al*. (2006) reported on the honey bee’s preference for citrus flowers over those of the avocado. Through the use of HS-SPME and GC-MS analysis, Azam *et al*. (2013) found the volatile linalool to be released in abundance from citrus flowers. Linalool is known to be attractive to honey bees (Henning *et al*. 1992). This compound was detected in only trace amounts in the headspace of the avocado flowers. Furthermore, previous studies have shown honey bees to prefer the scent of hermaphroditic flowers. Our study showed that there are distinct volatiles released from many of the actively male and female flowers. Therefore, despite the avocado being ‘perfect’, the temporal separation of the male and female phases may cause the flower to be less attractive compared to citrus flowers, which possess simultaneously functional male and female reproductive organs.

In Chapters 2 and 3 we have so far highlighted the potential effects of avocado flower morphology and volatile exudation on honey bees and how they might limit pollination. In Chapter 4 we explored the possibilities of using attractants to improve honey bee activity in a small avocado orchard. There are numerous studies detailing successful methods of manipulating floral scent to attract honey bees, including the use of geraniol sprays in alfalfa fields (Waller 1969) and eugenol, citral and lemongrass extracts in sweet orange plantings (Malerbo-Souza *et al*. 2004). However, none related specifically to avocados. Furthermore, intercropping with pollinator attracting plants has also been an effective method to improve pollination in onions (Moghaddam *et al*. (2020). Lavender, basil and sunflowers are some examples of honey bee attracting plants (Jones and Gillett 2005, Pawelek *et al*. 2009, Pereira *et al*. 2015).

In Chapter 4 we therefore explored the potential of using lemongrass (*Cymbopogon citratus*) essential oil lures and flowering lavender (*Lavandula stoechas*) to attract honey bees to avocado orchards, as we were unable to find any literature investigating these methods. In terms of honey bee activity, interplanting lavender appeared to have little effect (positive or negative) on bee activity directly. This is in spite of the findings of Herrera (1997), who claimed that honey bees are attracted to lavender as a result of the high sugar-content in the nectar of the flowers and the flamboyant display of the inflorescence. The lemongrass lures proved to be more effective in not only improving honey bee activity in the canopy but prolonging this activity towards the end of the flowering season. Lemongrass is reported to be as attractive as the lure pheromones produced by the honey bee's Nasonov gland (Toungos 2019). Based on the differences in flowering intensity between the 'Hass' and 'Fuerte' trees used in this study, the effects of 'on' and 'off' years on honey bee activity were highlighted. Akter *et al*. (2017) claimed that pollinators prefer plants with a greater flowering intensity, and avocado trees that experience 'off' years exhibit reduced flowering (Salazar-Garcia *et al*.1998). This was clearly observed in this study.

Fruit set and fruit set percentages were also recorded in Chapter 4. Generally, trees that flowered more profusely set more fruit. The lemongrass lures and lavender appeared to improve fruit set for trees experiencing 'off' years. Therefore, small-scale farmers or those managing trees in 'off' production years may benefit the most from attractants. During this study, other insects such as flies, ants, and lady bugs were also noticed on the avocado flowers. Whilst many other insects have been reported to be potential pollinators for the avocado, their efficacy compared to honey bees is poorly understood. Arias *et al*. (1992) claim that lemongrass may act as a deterrent on other insects. Therefore, before any attractants are installed into orchards, their potential deterring effect on off-target species must be understood.

The internal aspects of flower morphology, volatile emission and the practical solutions to these problems have been discussed in the first three chapters. However, there are problems external to the flower itself which have the potential to influence pollination. During an unrelated study into pollination in avocados in Tzaneen, South Africa, in 2018 (Du Toit 2018), light microscopy samples showed what appeared to be ‘deformed’ pollen grains. This observation led to the hypothesis that there may be factors external to the flower itself that have the potential to reduce pollination by negatively influencing pollen development. Chapter 5 thus focused on analysing the influence of cold temperature on pollen development. Flowers were sampled in the active female and male phases from ‘Fuerte’ and ‘Hass’ trees during cooler and warmer periods. There was a noticeable difference in the state of the pollen observed: the ‘healthy’, ‘deformed’ and ‘empty’ pollen grains correlated with the findings of Tsvetova and Elkonin (2013) and their studies on sorghum.

Chapter 5 also detailed a novel pollen counting method to mitigate the problems encountered with uneven cutting depths during semithin sectioning. As a result, we were able to report the differences in the amount of pollen produced, and the state of that pollen. The total pollen counts between the cooler and warmer sampling periods differed between the two sampling years. However, there was a degree of consistency with the differences observed between the two cultivars: ‘Fuerte’ anthers sampled during the cooler periods appeared to contain more pollen, while ‘Hass’ anthers totalled more during the warmer periods. ‘Fuerte’ is known to be less susceptible to chilling injury (Cooper *et al*. 1957), and while the direct effects of temperature on meiosis and pollen development in avocados is not well-known, this resistance to chilling injury may extend to improving pollen development during cold periods. This theory is strengthened by the findings of Schroeder (1955), who briefly described how ‘Fuerte’ has the ability to produce more pollen compared to low-yielding cultivars.

Chapter 5 also highlighted the differences in pollen observed between the anthers sampled in the female and male phases. Therefore, in spite of pollen development beginning months before the sampling period, it can be reasoned that pollen development is still ongoing at flower anthesis, and during the overnight closure between the sexual phases. Very little investigation has been dedicated to the importance of this overnight closure, but this study represented the first step in remedying that problem. Sedgley (1977) reported stigmas collected from avocado flowers in the male phase contain more callose, supporting the theory that reproductive organ development is still in progress during the overnight flower closure. The differences in the proportion of healthy, deformed and empty pollen grains was more noticeable during the first sampling season. ‘Fuerte’ appeared to contain more empty pollen, while ‘Hass’ had a higher proportion of deformed pollen. Upon investigating the yearly temperature data for the sampling region, it was shown that temperatures regularly fell below 15 ºC during the periods of meiosis and pre-vacuolate stages of pollen development, a critical level in tropical trees (Issarakraisila and Considine 1994). Low temperatures can cause tapetum degradation in the anthers of rice which negatively influences pollen development (Oda *et al*. 2010). Cold stress also increases abscisic acid accumulation and the subsequent downregulation of tapetum-expressing genes (Sharma and Nayyar 2016), which may explain the pollen abnormalities observed in this study.

In addition to cold temperatures, nutrient availability is also a limiting factor influencing pollen development. Chapter 6 focused on the role of nutrient availability on pollen development and as a result, pollination. This was accomplished through the use of nitric acid (HNO_3) digestions and inductively coupled plasma (ICP) analysis. Our testing aimed to determine the differences in nutrient content between flower cultivar, sexual phase (actively male or female), location within a canopy and sampling region (a commercial and a non-commercial orchard). Two facts were immediately clear: firstly, there are no recommended flower standards for avocado trees (therefore foliar standards were used as a baseline); and secondly, there are limited studies focusing on the direct role of nutrients on pollen development in avocados. In spite of this, we ventured forth with our investigation and we discussed some noteworthy results. In terms of potassium, ‘Fuerte’ flowers were shown to accumulate a higher degree thereof compared to ‘Hass’ flowers. Potassium is believed to offset the effects of cold injury in susceptible plants (Wang *et al*. 2013). When this is considered in conjunction with the higher pollen counts for ‘Fuerte’ anthers discussed in Chapter 5, potassium may be an important factor in ensuring optimal pollen development. Almost all of the flowers sampled in this study were shown to be deficient in boron. Boron deficiencies have been shown to negatively affect the processes of microsporogenesis and nucleic acid exchange during meiosis, which are critical for anther development (Dell and Huang 1997, Batygina and Vasiliyeva 2003). The deficiencies detected in the flowers may explain the abnormalities observed in Chapter 5. Furthermore, when boron was deficient, flowers sampled in the male phase had higher concentrations thereof compared to the flowers sampled in the female phase. This suggests that in times of deficiency, boron is preferentially allocated to the functionally male flowers, potentially to ensure the viability of the pollen. The direct role of phosphorous on pollen development in avocados is not well-described, however, it has been shown to improve pollen count and overall grain size in cucumbers (Lau and Stephenson 1994). However, considering it was present in excessive amounts, it was unlikely to affect pollen development, at least in this study. Functionally female flowers had higher concentrations of phosphorous, a known honey bee repellent (Afik *et al*. 2006). When this is related to the bee preferences discussed in Chapter 2, a higher phosphorous accumulation in the female phase of the flowers may explain why they are less favoured. Lastly, the flowers sampled from a commercial and non-commercial orchard were deficient in calcium. Calcium has been shown to be prevalent in the walls of *Torenia fournieri* pollen grains, which as a result confers a degree of resistance to environmental stress (Wang and Dobritsa 2018).

Chapter 6 also compared foliar and floral samples, and the latter had consistently higher nutrient concentrations. Coetser *et al.* (1993) also noticed the same pattern during their study into the investigation of the role of boron in avocados. During anthesis, the vegetative and reproductive growth points of an avocado tree compete for photoassimilates (Blumenfeld et al. 1989). This is even more apparent in the case of boron, which is continuously remobilised throughout a plant until the requirements in every cell are satisfied (Eaton 1944). This suggests that boron is differentially allocated to reproductive and vegetative shoots in avocado trees.

To our knowledge, the investigation described in Chapter 6 was the first of its kind to analyse nutrient status in the flowers of avocados and relate them to pollen development. This study showed how adequate floral nutrient status is important for not only adequate pollen development, but pollinator attraction too. This highlights the importance of understanding floral nutrient status and how it can affect both the production of healthy pollen, and successful pollination of that pollen. By developing floral nutrient recommendations, we determined avocado flowers should contain between 16 951 mg/kg to 33 902 mg/kg of potassium, 2 473 to 6 183 mg/kg phosphorous, and 23 740 mg/kg to 71 222 mg/kg of calcium. Table 6.1 calls for at least 33 mg/kg of boron to be present in flower samples, based on the amended floral concentration that was calculated using the accumulation ratio between the flowers and leaves. However, to ensure optimum pollen health and germination post-pollination, it is suggested that the recommendation of Robbertse *et al.* (1998), which calls for floral boron concentrations to lie between 50 and 75 mg/kg, be used instead.

At the end of this study, the importance of employing a holistic approach to explore factors potentially limiting to pollination in avocados was made clear. All factors discussed, both internal physiological processes such as volatile exudation and external factors including cold temperatures and nutrient availability, seem to have the potential to limit pollination in avocados. Examples of this interconnectedness include: the role played by phosphorous in pollen development, in addition to its ability to repel honey bees; and the potential of using essential oil lures to attract honey bees, while risking deterring other potential pollinators.

Based on the original aims as described in the beginning of this dissertation, this study achieved the following:

- The honey bee's preference for certain avocado flowers was better understood. Honey bees appear to prefer avocado flowers based on cultivar and sexual phase. Honey bees tend to favour 'B-type' cultivars such as 'Fuerte' and 'Zutano', but further investigation is required to conclude this outright. In addition to this, the male phase of the avocado flowers was consistently preferred.
- For the first time, a floral headspace of *P.americana* 'Hass', 'Fuerte', 'Maluma Hass', 'Galil', 'Ettinger' and 'Zutano' were described. Limonene, α-pinene, β-pinene, α-phellandrene and eucalyptol were the most prominent volatiles in all the flowers. The fact that linalool was detected in only trace amounts may explain why honey bees prefer other flower sources that exude higher concentrations of that volatile. Furthermore, the distinct scents exuded from the male and female flower phases, in conjunction with the fact that honey bees prefer the scent of hermaphroditic flowers (like citrus), could be an additional reason why the avocado flowers are not favoured.
- We discovered that it is possible to improve pollination and subsequent fruit set in avocado orchards using honey bee attractants. While a larger study is required before concrete conclusions can be drawn, it appears that lemongrass

essential oil lures and flowering lavender are especially effective at improving fruit set when the flowering of trees is compromised.

- We developed a novel pollen counting technique to mitigate the obstacles encountered with uneven cutting depths when anthers embedded in resin samples are subjected to semithin sectioning. By cutting multiple sections of an anther, in addition to reporting pollen counts in relation to compartment size, we could evaluate the influence of cold stress on pollen development in avocados. The process of meiosis in tropical fruit, such as the avocado, is compromised when temperatures fall below 15 °C. This may result in pollen abnormalities such as deformed shapes and empty grains. Our results alluded that ‘Fuerte’ anthers are capable of producing more pollen during periods of cold stress.
- The precise role of nutrients on pollen development in avocados is poorly researched. Despite this, our study noted the apparent importance of potassium, phosphorous, boron, and calcium in avocado pollen development. Potassium may play a role in conferring a degree of cold resistance, which may explain why ‘Fuerte’ flowers contained more pollen during cooler periods; and the female phase of the avocado flowers accumulate more phosphorous, which has the potential to repel honey bees and may explain why the male phase of the flowers is preferred. Also, the preferential allocation of boron to the male phase of the flowers, and to flowers in general compared to vegetative shoots, further highlights the important role of boron in avocado production. By comparing the ratio of nutrients accumulated between the leaves and flowers, we developed primary standards for floral potassium, boron, phosphorous, and calcium concentrations.

Throughout this dissertation, new insights were sequentially added to our understanding of how low pollination affects yields in avocados. Figure 7.1 shows how the holistic approach used in this study deepens our understanding of the numerous factors that contribute to low pollination in these trees. By comparing the original organogram presented at the beginning of this dissertation with the complete story presented in Figure 7.1, the success of this dissertation is clear as we have answered the question posed by this dissertation ‘Low yields in avocado’s: is poor pollination a contributing factor and can it be enhanced?’

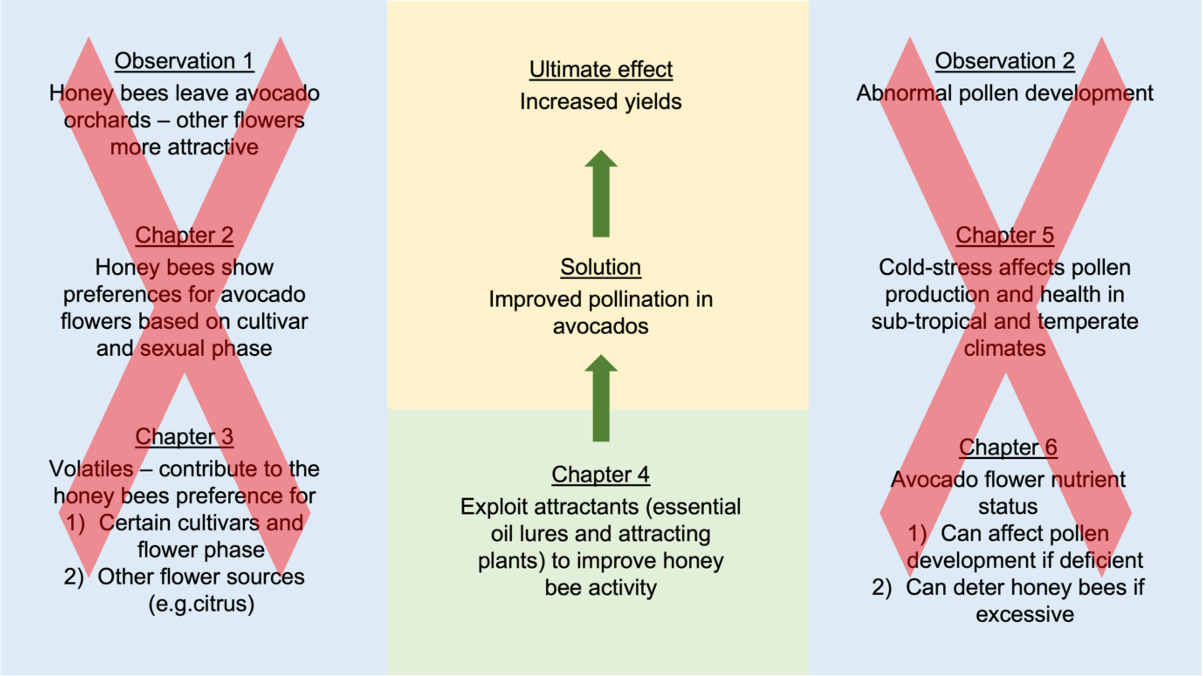

Figure 7.1. Organogram depicting the relationship between the many variables affecting pollination in avocados. If the practical solutions presented in Chapter 4 are thus implemented (green arrows) and the problems presented in Chapters 2, 3, 5 and 6 eliminated (red crosses), pollination will be improved, and avocado yields will increase.

We can therefore conclude that poor pollination is a contributing factor to low yields in avocados and it can be enhanced. Chapter 4 posited practical solutions to the issues described in Chapters 2 and 3. Chapters 5 and 6 highlighted the need for further studies focussing on the direct physiological effects of cold and nutrient stress on pollen development. If the contributing factors discussed in Chapters 2, 3, 5, and 6 are eliminated, honey bee activity and subsequent pollination will increase, and the pollen transferred in those pollination events will be of optimum health. This can be achieved in part using the attracting measures described in Chapter 4. However, further studies are required to develop additional solutions, specifically for the production of optimum amounts of healthy pollen. The remainder of this dissertation will discuss recommendations for how future research can achieve this goal.

Future Recommendations

As the research presented in this dissertation was a part of a master's study, only two sampling years were required. This allowed for a fair representation of honey bee preferences, volatile exudation, pollen development, and floral nutrient status. However, due to the vast number of factors that influence avocado physiology, such as alternate bearing, irrigation, fertilisation, and climate, the methodologies described in this study will yield more results when implemented as part of long-term investigations.

The bee framing techniques described in Chapter 2 should be repeated if we are to conclude undoubtedly that honey bees favour the flowers of 'B-type' cultivars. To achieve this, honey bee activity should be monitored in single cultivar orchards under a range of environmental conditions. If funding is allocated therefor, technological equipment can be used to improve counting precision. One such example of sophisticated methods is described by Ngo *et al*. (2019), whose use of 'real-time imaging' and algorithms allowed the researchers to monitor honey bee activity. By concluding which cultivars are favoured, commercial farmers can select more attractive cultivars and thereby encourage honey bee visitations.

The lack of the volatile linalool in the avocado flowers sampled in Chapter 3 could represent an opportunity for breeders of new avocado cultivars. Noman *et al*. (2017) explore the possibilities of improving flower characteristics in plants, including floral scent. This can be exploited in avocados by developing new cultivars with flowers exuding a greater amount of linalool through the 'manipulation of scent-genes' (Noman *et al*. 2017).

The investigation into the possibility of improving pollination in avocados using attracting measures described in Chapter 4 yielded some interesting results. However, many factors, such as nearby lemon orchards, alternate bearing, and COVID-19 restrictions mean that further studies are required before any concrete recommendations can be made to commercial farmers. Through casual discussions with commercial growers, many already intercrop their orchards with a species of perennial basil. Given the opportunity, it would be useful to investigate the differences in pollinator activity between these orchards and those that instead have clean rows between trees.

Chapter 5 detailed the influence of cold stress on pollen development late in the flowering process. If this pollen analysis is to be repeated in the future, the following is recommended to improve the likelihood of detecting significant differences: instead of trying to predict temperature fluctuations using smartphone applications, sampling anthers from orchards in different locations (ideally located in a temperate and tropical region that experience different temperatures throughout a season) should yield significant differences in pollen count. With this being said, researchers at the South African Avocado Growers Association have noticed the need for determining the effects of cold temperature on pollen development and have shown an interest in expanding the findings of this study. Therefore, more research should be performed to compare pollen development in orchards located in completely different climatic zones to better understand the consequences of cold stress on pollen development, pollination and fruit yield. Furthermore, flowers can be sampled during the meiotic period, thus allowing for detailed investigations into cold stress and pollen development in avocados.

Chapter 6 highlighted the lack of understanding relating nutrient deficiencies directly to their influence on pollen development in avocados. The next step in this process should aim to describe which physiological processes are specifically compromised during deficiency periods. Importantly, avocado producers and researchers alike should endeavour to develop floral nutrient statuses, especially if the findings of Chapter 6 suggest many important nutrients are present in either excessive or insufficient amounts even in commercial orchards. For farmers wanting to analyse their flowers, the amended floral nutrient statuses reported in Table 6.1 can be referred to. Failing this, the accumulation ratios included in Table 6.1 could be used in conjunction with foliar analyses to predict floral nutrient status. As Chapter 6 briefly mentions, the chosen rootstock will play a role in nutrient uptake and translocation throughout the tree (Labanauskas *et al*. 1978). As boron and calcium were both detected in deficient amounts in many treatments, nutrient efficient rootstocks can be used to help prevent deficiency symptoms in poor soils. Furthermore, the differences in nutrient accumulation between ‘Fuerte’ and ‘Hass’ flowers suggests fertiliser programs should be tailored according to the variety of the avocado. Lastly, calcium was extremely deficient in all the flowers sampled in this study. Merely increasing soil calcium applications could influence the uptake of other nutrients and cause further problems (Penter and Stassen 2000). Therefore, foliar calcium sprays and efficient rootstock varieties should be investigated as potential remedies.

In conclusion, this study successfully investigated factors internal and external to the avocado tree that have the potential to limit pollination and contribute to the low yields often observed. Practical solutions to this problem were also evaluated. Not only did this study achieve its original aims, but it also uncovered some glaring gaps in avocado-based research. This includes, but is not limited to, improving our understanding of how other insects contribute to avocado pollination compared to the honey bee outside its native region, determining the exact effects of cold temperature on meiosis and pollen development in avocados when they are cultivated in more temperate climates, and highlighting the exact role nutrients such as potassium, phosphorous, and calcium play in pollen development in avocados.

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APPENDIX A

Environmental Conditions

Table A1. Minimum temperatures (°C), maximum temperatures (°C), rainfall (mm) and relative humidity (%) averages during the sampling periods for the respective orchards used in this study.

	Location					
	ZZ2 Farms, Tzaneen (23º63’S, 30º04’E)		Westfalia, Tzaneen (23º45’S, 30º05’E and 23º44’S, 30º04’E)		Experimental Farm, University of Pretoria (25°45’S 28°16’E)	
Period	July – September 2016	July – September 2018	July – September 2019	July – September 2020	July – September 2019	July – September 2020
Minimum temperature (°C)	2 – 9	3 – 13	1 – 11	0 – 6	1 – 16	-2 – 16
Maximum temperature (°C)	26 – 29	25 – 30	22 – 26	26 – 35	18 – 32	14 – 31
Rainfall (mm)	2.58	6.93	0.10	6.06	2.7	7.77
Relative humidity (%)	41 – 53	38 – 57	41 – 48	50 – 57	30 – 48.5	47 – 50

APPENDIX B

Agar medium

Table B1. Constituents and conditions of the sucrose-enriched agar medium dissolved in one litre of distilled water.

Compound	**Amount (g)**
Sucrose	30
Agar	30
Qualities	
pH	5.6

APPENDIX C

TD-GC x GC-TOFMS conditions

Table C1. Thermal desorption-comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (TC-GC x GC-TOFMS) conditions for volatile detection.

GC	Agilent 7890A
Detector	LECO Pegasus 4D Time-of-Flight Mass Spectrometer
Acquisition rate	100 spectra/s
Mass range	40 to 450 Da
Source temperature	230 °C, Ionisation mode EI+
Detector voltage	1815 Volts
Thermal desorber system (Gerstel)	30 °C (3min) at 60 °C/min to 250 °C (5 min) Transfer line temperature 350 °C Desorption flow rate 100 ml/min
Cryo-cooled inlet system	-100 °C (0.1 min) at 10 °C /s to 250 °C
Splitless time	90 s
Carrier gas	Helium 1.4 ml/min, constant flow mode
Column 1	Rxi-5Sil MS, 30 m x 0.25 mm ID x 0.25 µm film thickness
Column 2	Rxi-17Sil MS, 0.97 m x 0.25 mm ID x 0.25 µm film thickness
Column 1 oven	40 °C for 1.5 min to 280 °C at 10 °C/min, hold 5 min
Column 2 oven offset	5 °C (relative to primary oven)
Modulator offset	15 °C (relative to 2nd oven)
Modulation period	3 s (hot pulse time 0.8 s)
MS line transfer temperature	280 °C