



Murdoch
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Biological responses and control of California red scale *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae)

by

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Submitted to Murdoch University in fulfilment of the requirements for the

degree of

Doctor of Philosophy

College of Science, Health, Engineering and Education

Murdoch University

Perth, Western Australia

March 2020

Declaration

The work described in this thesis was undertaken while I was an enrolled student for the degree of Doctor of Philosophy at Murdoch University, Western Australia. I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution. To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged.

Khalid O. Mohammed

Date: March 10, 2020

Acknowledgements

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا (٨٥).

The research for this thesis was undertaken in the School of Veterinary and Life Science, Murdoch University. I would like to express my heartfelt gratitude to my supervisors Professor Yonglin Ren and Dr Manjree Agarwal “Postharvest Biosecurity and Food Safety Laboratory Murdoch” for their support with enthusiasm, constructive editing, and patience throughout the years of this wonderful project. I deeply appreciate their encouragement, assistance and for being so willing to take me on as a student.

I would like to express my sincere gratitude to all those who helped me in completing this thesis. I thank all the members of the Postharvest Biosecurity and Food Safety Laboratory staff members in the past and present especially Mr James Newman and Mr Xin Bob Du for their helpfulness and supporting use of the experimental equipment and facilities.

My sincere gratitude also goes to Professor Giles Hardy and Professor Ismail Karaka. Their names were not officially listed in my supervisors, but indeed they are both playing an indispensable role in directing me to complete my thesis. They gave me great support and help in the statistical analysis of raw data, in conceiving questions addressed and setting assumptions in models, and in checking and revising papers.

I thank the Manager of Murdoch University farm in Whitby, Western Australia Bob Fawcett for allowing us doing some experiments and collecting citrus fruit specimens for this study.

I would like to thank my family; my wife Saba Al-qassab and my kids for their love, support and endurance. Special thanks to my mother, brothers and sisters who share happiness and good wishes. Thank you for trusting me! Thanks to all my friends, especially Brian Smith and Penny Kirkland-Smith, for encouragement and good wishes.

I was nominated to study a PhD by Mosul University, and this PhD project was funded by Iraqi Ministry of High Education and Scientific Research. I am very grateful indeed for this support.

Abstract

In many citrus areas around the world and within citrus-producing regions of Australia, the California red scale (CRS), *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), is considered the most important pests of citrus. The main biological control agents of *Ao. aurantii* in this zone are the parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae). In order to improve the biological control of *Ao. aurantii* several biotic and abiotic factors were studied, that may affect the efficiency of *A. melinus* in the laboratory and the field.

More concretely, reproductive potential and age-specific fecundity schedules of *Ao. aurantii* were studied in the laboratory at constant temperatures (20, 23 and 27°C), while the biological parameters of its parasitoid *A. melinus* were conducted at 27°C. Results revealed that the net reproduction rate (R_0) was considerably higher for *Ao. aurantii* than *A. melinus*, which reached 28.14 at 27°C, indicating its high reproductive capacity. Moreover, the net reproduction rate obtained for *A. melinus* indicates a low substitution potential for each female having *Ao. aurantii* as a host under laboratory conditions. The intrinsic rate of increase (r_m) of *A. melinus* (0.188 ♀/♀/day) was significantly greater than that of *Ao. aurantii* (0.080) at 27°C.

Plants produce volatile organic compounds (VOCs) in response to herbivore attack, and these VOCs can be exploited by parasitoids of the herbivore as host location cues. The VOCs from non-infested and *Ao. aurantii*-infested citrus fruit were investigated using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS). The data showed that more than 52 different compounds were identified, and different emissions associated attributed to herbivore activity were found for all fruit species (lemon, orange, mandarin and Tahitian lime). However, a single compound was exclusively produced by infested lemon fruit, while two compounds were significantly increased, and two compounds were only present in non-infested lemon. Five compounds were significantly increased in infested mandarins. For orange, five compounds were increased, and five compounds were exclusively presented in infested fruit. For lime fruit, eighteen of these compounds were increased, one was decreased, whereas five compounds were produced exclusively from infested lime fruit. Two putative herbivores-induced plant volatiles, d-limonene and β -ocimene, were significantly increased by *Ao. aurantii* infestation in all infested fruit, regardless of the citrus species.

Subsequently, the preferences of female parasitoid on infested or healthy fruit in olfactometer bioassays were evaluated. Then in order to understand the magnitude of volatile attractiveness,

the innate attractiveness of VOCs to *A. melinus* females in varying densities were tested in the laboratory. The results of the olfactometer assays that tested the behaviour of *A. melinus* to the different compounds emitted from infested and non-infested citrus fruit showed no such preference when compared between non-infested and infested oranges, mandarins and lime fruit; whilst, there were significant preferences for lemon fruit infested with *Ao. aurantii* over non-infested ones. For assessment, the attraction of synthetic Herbivore induced plant volatiles (HIPVs), four different concentrations (5,10, 15 and 20 $\mu\text{l/ml}$) of d-limonene and β -ocimene were investigated. However, mated *A. melinus* females preferred the reward-associated VOC more than hexane control in the case of d-limonene at the tested dosages of 15 and 20 $\mu\text{l/ml}$, β -ocimene at tested dosages of 10, 15 and 20 $\mu\text{l/ml}$.

Finally, this study evaluated the dispersal ability of released *A. melinus* adults and their effect on the parasitism percentage, using d-limonene and β -ocimene with yellow sticky traps and scoring percentage parasitism on infested fruit. Under field conditions, the natural enemies' effectiveness in controlling pests is largely correlated with their capability to spread towards infested crops. In this study, d-limonene and β -ocimene were examined for their attractiveness to California red scale parasitoid *A. melinus* in the field after augmentative releases. Field experiments demonstrated that lures baited with isolates of d-limonene and/or β -ocimene, which significantly attracted some species of natural enemies but had no significant impact on others. The number of *A. melinus* captured during the whole trial was greater in the traps treated with volatiles than the control. Finally, the overall parasitism rates were not increased by synthetic HIPV lures, but there was evidence that lures may increase parasitism of California red scale when there is a decrease in the amount of volatile organic compounds due to lack of healthy and infested fruit. In conclusion, HIPVs can potentially play important roles in attracting and exploiting natural enemies to reduce pest infestations.

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Publications

Journal Publications

- Mohammed, K., Karaca, I., Agarwal, M., Newman, J. and Ren, Y., 2020. Age-specific life tables of *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) and its parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae). *Turkish Journal of Agriculture and Forestry*, p.44. [doi:10.3906/tar-1905-36](https://doi.org/10.3906/tar-1905-36) (Chapter 2).
- Mohammed, K., Agarwal, M., Newman, J. and Ren, Y., 2017. Optimisation of headspace solid-phase microextraction conditions for the identification of volatiles compounds from the whole fruit of lemon, lime, mandarin and orange. *Journal of Biosciences and Medicines*, 5(03), pp.176-186. <http://dx.doi.org/10.4236/jbm.2017.53019> (Chapter 3).
- Mohammed, K., Agarwal, M., Du, X.B., Newman, J. and Ren, Y., 2019. Behavioural responses of the parasitoid *Aphytis melinus* to volatiles organic compounds (VOCs) from *Aonidiella aurantii* on its host fruit Tahitian lime fruit *Citrus latifolia*. *Biological Control*, 133, pp.103-109. <https://doi.org/10.1016/j.biocontrol.2019.03.015> (Chapter 4).
- Mohammed, K., Agarwal, M., Li, B., Newman, J., Liu, T. and Ren, Y., 2020. Evaluation of d-limonene and β -ocimene as Attractants of *Aphytis melinus* (Hymenoptera: Aphelinidae), a Parasitoid of *Aonidiella aurantii* (Hemiptera: Diaspididae) on Citrus spp. *Insects*, 11(1), p.44. <https://doi.org/10.3390/insects11010044> (Chapter 5).
- Mohammed, K., Agarwal, M., Newman, J. and Ren, Y., 2020. D-limonene and β -ocimene Attract *Aphytis melinus* to Increase Parasitism of California Red Scale *Aonidiella aurantii* (Hemiptera: Aphididae) on Citrus. Submitted to the Journal of Insects.

Conferences (Presentations and posters)

- Mohammed, K., M. Agarwal, J. Mewman, and Y. Ren. The possibility of increasing attraction of *Aphytis melinus* onto California red scale. Citrus technical forum and field day in Mildura, South Australia, Australia on 1st-2nd March 2017.
- Mohammed, K., M. Agarwal, J. Mewman, and Y. Ren. Optimization and Validation for Determination of VOCs from Lime Fruit *Citrus aurantifolia* (Christm.) with and without California Red Scale *Aonidiella aurantii* (Maskell) Infested by Using HS-SPME-GC-FID/MS. WASET – 19th International Conference on Entomology (ICE) in Paris, France on 19th-20th October 2017.

Mohammed, K., M. Agarwal, J. Newman, and Y. Ren. The possibility of increasing attraction of *Aphytis melinus* onto California red scale. 1st Murdoch University annual research symposium in Western Australia, Australia on the 8th of November 2017.

Mohammed, K., M. Agarwal, J. Mewman, and Y. Ren. Age-specific life tables of the California red scale *Aonidiella aurantii* (Mask.) (Hemiptera: Diaspididae) and its Parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae). Citrus technical forum in Adelaide, South Australia, Australia on 6th-7th March 2019.

Mohammed, K., M. Agarwal, J. Mewman, and Y. Ren. Behavioural response of the parasitoid *Aphytis melinus* DeBach to *Aonidiella aurantii*-induced volatile organic compounds (VOCs) from Tahitian lime fruit *Citrus latifolia* (Rutaceae). Citrus technical forum in Adelaide, South Australia, Australia on 6th-7th March 2019.

List of abbreviations

Σ	Sum	HPLC	High-Performance Liquid Chromatography
\times	Finite rate of increase	HS	Head Space
$^{\circ}\text{C}$	The degree celsius	HS-SPME	Headspace Solid-Phase Microextraction
μl	Microliter	Ix	Survival Rate
μm	Micrometre	LSD	Least Significant Difference
V_m	Plasma transmembrane potential	m/z	Mass-to-Charge Ratio
AHS	Headspace autosampler	ml	Millilitre
ANOVA	Analysis of variance	mm	Millimetre
Ca^{2+}	Cytosolic calcium ions	MS	Mass Spectrometric
CAR/DVB	CarbowaxDivinylbenzene	MS	Mass Spectrometry
Cm	Centimetre	mx	Female Fecundity
CO_2	Carbon dioxide	NIST	National Institute of Standards and Technology
DH	Dynamic headspace	OBPs	Odorant Binding Proteins
ECD	Electron capture	ORNs	Olfactory Receptor Neurons
E-nose	Electronic nose	PEG	polyethylene glycol
EPP	Estimated Percentage Parasitism	RH	Relative Humidity
FAO	The Food and Agriculture Organization	RI	Rendition Index
FID	The Flame Ionisation Detector	r_m	Intrinsic Rate of Natural Increase
FPD	Flame Photometric	R_o	Net Reproductive Rate
GC	Gas-Chromatography	RT	Retention Time
GC-MS	Gas-Chromatography Mass Spectrometry	SHS	Static Headspace
GRR	Gross Reproduction Rate	SPME	Solid-Phase Microextraction
He	Helium	Spp	Species Pluralism, the Latin for multiple species
HIPPOs	Herbivore-Induced Plant Protection Odors	SPSS	Statistical Package for the Social Sciences
HIPVs	Herbivore-Induced Plant Volatiles	TCD	Thermal Conductivity
HI-VOCs	Herbivore-Induced Volatile Organic Compounds	T_o	Mean generation time
H_2O_2	Hydrogen peroxide	VOCs	Volatile Organic Compounds

Chapter One

General Introduction and Literature Review

1.1. General Introduction

Scales are among the most abundant and varied group of citrus pests (Bennett and Alam, 1985; Rose, 1990). They have a tremendous host range and can settle on citrus leaves, twigs, branches and fruits. The families, Coccidae (soft scales) and Diaspididae (armoured scales) are the most important. The species of armoured scales in the genera of *Aonidiella* are believed to be native to the same Asian regions as citrus (Rose, 1990). Perhaps this is substantiated by the fact that many effective parasitoids used against these scales are from those areas (DeBach, 1976). California red scale *Aonidiella aurantii* (Maskell), considered the most important armoured scale species attacking citrus (Ebeling, 1959).

Aonidiella aurantii is a key pest in many subtropical and temperate parts of the world, e.g. South Africa, North Africa, Australia, California, Texas, Mexico and parts of South America (Bennett et al., 1976; Anonymous, 1984; Gill, 1988; Smith et al., 1997; Bedford et al., 1998). *Ao. aurantii* gives birth to 100– 150 mobile young, called crawlers, at a rate of two or three a day over a 6 – 8 week period. The crawlers emerge from under their mother's scale cover and search for a suitable feeding site on leaves, shoots or fruit. Crawlers wandering on the tree canopy can be blown by the wind into neighbouring trees or orchards. Once a crawler settles, it inserts its mouthparts into the plant and starts feeding. It secretes a white waxy covering, and at this stage is called a 'whitecap'. *Aonidiella aurantii* skin is attached to the scale cover, giving the cover its typical red colour. The development stage and sex of red scale can be determined by the shape and size of the scale cover. After the second stage, scales can be identified as male or female. The scale cover of males is elongated, while the scale cover of females is circular.

Aonidiella aurantii adult males develop through a prepupal and pupal stage under a scale cover, before emerging as delicate, winged insects. Males are attracted to the female by a pheromone and die after mating. In first-stage and second-stage females, and third-stage (i.e. adult) unmated females, the scale cover is not attached to the body of the scale. When the first stage and second-stage females are moulting, and when third-stage females have the scale cover, it is attached to the body of the scale. In the tropics, there are six or seven generations per year in comparison with two to four generations in higher latitudes.

Aonidiella aurantii attacks all the above-ground parts of the tree, fruits, leaves and wood sucking the sap on the plant tissue with their long, piercing-sucking mouthparts (Beardsley Jr and Gonzalez 1975). It feeds by inserting its mouthparts into plant tissues and sucking the sap.

Stylet track paths are intracellular with most paths terminating in parenchyma cells (palisade and spongy mesophyll of leaves, cortex of twigs, and flavedo of fruit), suggesting that the insect prefers to feed on this tissue (Washington and Walker 1990). The damage caused by *Ao. aurantii* may be a direct a consequence of the feeding activity of the pest or indirect associated with the presence of scales on fresh fruit (University of California Integrated Pest Management, 1984; Smith et al., 1997; Bedford, 1997).

The pesticide resistance and secondary pest outbreaks are the reason behind the temporary use of new insecticides as a solution to *Ao. aurantii* problem. Greater reliance on biological control will be necessary for long-term control of *Ao. aurantii*. *Aonidiella aurantii* can be successfully managed by the augmentative release of natural enemies (Haney, 1992). Scales are often well controlled by beneficial predators and parasitoids, except when these natural enemies are disrupted by ants, dust, or application of persistent, broad-spectrum insecticides. *Ao. aurantii* has numerous predators and parasitoids (Rosen and DeBach, 1978; Hely et al., 1982; Forster et al., 1995), the range and number of which vary with location, in relation to prevailing climate conditions and host stage requirements (Forster et al. 1995).

The most important primary parasitoids of *Ao. aurantii* in Australia are *Aphytis chrysomphali*, *Aphytis lingnanensis*, *Aphytis melinus*, *Encarsia citrina*, *Encarsia perniciosi* [Hymenoptera: Aphelinidae] and the red scale strain of *Comperiella bifasciata* [Hymenoptera: Encyrtidae] (Wilson, 1960; Hely et al. 1982; Furness et al. 1983; Smith et al. 1997). Ectoparasitoids that belong to the genus *Aphytis* Howard considered more effective than the endoparasitoids and the predators (Rosen and DeBach, 1976; 1979; 1990; Rosen, 1994).

Species of *Aphytis* are ectoparasitoids and females lay their eggs externally on the body of their host. As natural enemies, the species of *Aphytis* are generally superior in effectiveness to the endoparasitoids and predators of armoured scale insect and for this reason, several species have been successfully employed in biological control projects (Rosen, 1994). Naturally occurring species of *Aphytis* are responsible for keeping the populations of many potential pests at subeconomic levels, and they are the most effective natural enemies controlling *Ao. aurantii* (Rosen and DeBach, 1979).

Aphytis melinus is commercially reared for release in citrus orchards to control *Ao. aurantii* (Grafton-Cardwell et al., 2011). Such augmentative biological control through *A. melinus* releases is used in many citrus-growing regions (Mazih, 2008; Zappalà, 2010; Grafton-Cardwell et al., 2011; Olivas et al., 2011; Zappalá et al., 2012). *A. melinus* females are more

important to produce because they are responsible for attacking the host (via host feeding and oviposition) and, by extension, actually controlling pests.

Plants normally release small quantities of volatile chemicals, but when herbivorous insects damage a plant, much more volatiles are released in addition to increasing the amount of the same VOCs emitted from non-infested plants. VOCs identity varies with the plant species and the herbivorous insect species. Volatile terpenoids and other compounds emitted from plants in response to insect damage allow insect parasitoids to distinguish between infested and non-infested plants and thus aid in locating hosts or prey. *A. melinus* uses learned, volatile cues from host plants as long-range attractants to potential habitats of *Ao. aurantii* (Morgan and Hare, 1998). Once on the plant, then the wasp forages for hosts by walking.

In order to determine the influences and the roles of VOCs in attracting the parasitoid *Aphytis melinus* to the citrus fruit infested with *Aonidiella aurantii* and to find out their role in the regulation of *Ao. aurantii* populations in the laboratory and citrus orchards, I undertook a comprehensive study of the role of VOCs to enhance biological control of California red scale in the laboratory and orchards in Western Australia. Specifically, I aimed to:

- Study the biological parameters of *Ao. aurantii* and its parasitoid *A. melinus* under laboratory conditions at different temperatures.
- Study the feasibility of the SPME technique for identification VOCs and their potential as a routine method for physiological studies on citrus fruit.
- Identify the chemicals emitted from non-infested and *Ao. aurantii*-infested citrus fruit.
- Investigate the response of *A. melinus* to VOCs from citrus fruit infested with *Ao. aurantii* and their response to d-limonene and β -ocimene through two-choice olfactometer bioassay.
- Examine d-limonene and β -ocimene for their attractiveness to *Ao. aurantii* parasitoid *A. melinus* in the field after augmentative releases.

1.2. Literature Review

1.2.1 Citrus

Citrus ranks first in fruit production in the world, and it is the most commonly grown fruit crops (FAOSTAT, 2012). Citrus fruit belong to the family of Rutaceae, where the leaves typically have transparent oil glands, and the flowers contain an annular disk (Kale and Adsule, 1995). Citrus was previously considered to originate from south-eastern Asia, China and the east of Indian Archipelago from at least 2000 BC (Swingle, 1943; Webber et al., 1967; Gmitter and Hu, 1990); however, the most recent research indicates the Himalayas as the origin (Wu et al., 2018). It is still unclear as to where wild citrus originated. The suggestion is that the seed was spread vast distances by human activity, water bodies and animals (Spiegel-Roy et al., 1996). Around 1000AD citrus fruit was introduced to the new world via the trade routes of Africa to the eastern Mediterranean basin by the Arab traders while the Crusaders brought the fruit to Italy, Spain and Portugal (Scora, 1975). Citrus cultivation now occurs in over 137 countries over six continents in the subtropical and tropical regions, between 40° north and south latitude (Ismail and Zhang, 2004). The Mediterranean region produces around 20% of citrus production. Southern China, the Mediterranean Basin (including southern Spain), South Africa, Australia, the southern United States, Mexico and parts of South America are the major commercial citrus growing areas (Liu et al., 2012; Youseif et al., 2014). Today, within Australia, around 1,900 growers plant over 28,000 hectares of citrus. The major production regions are in the Riverland, South Australia; Murray Valley, Victoria and New South Wales; Riverina, New South Wales and the Central Burnett region in Queensland. There are also additional plantings throughout Western Australia, inland and coastal New South Wales, regions in Queensland, as well as smaller plantings in the Northern Territory (Citrus Australia, n.d). Western Australia currently produces about 15,000 tonnes of citrus, of which most are sold on the local market, with an increasing export market. The industry includes navels and Valencia oranges, mandarins, lemons, limes, tangelos and native citrus. The breakdown of the main citrus types grown in WA based on area planted is presented below (Figure 1.1.) (Department of Primary Industries and Regional Development, 2016).

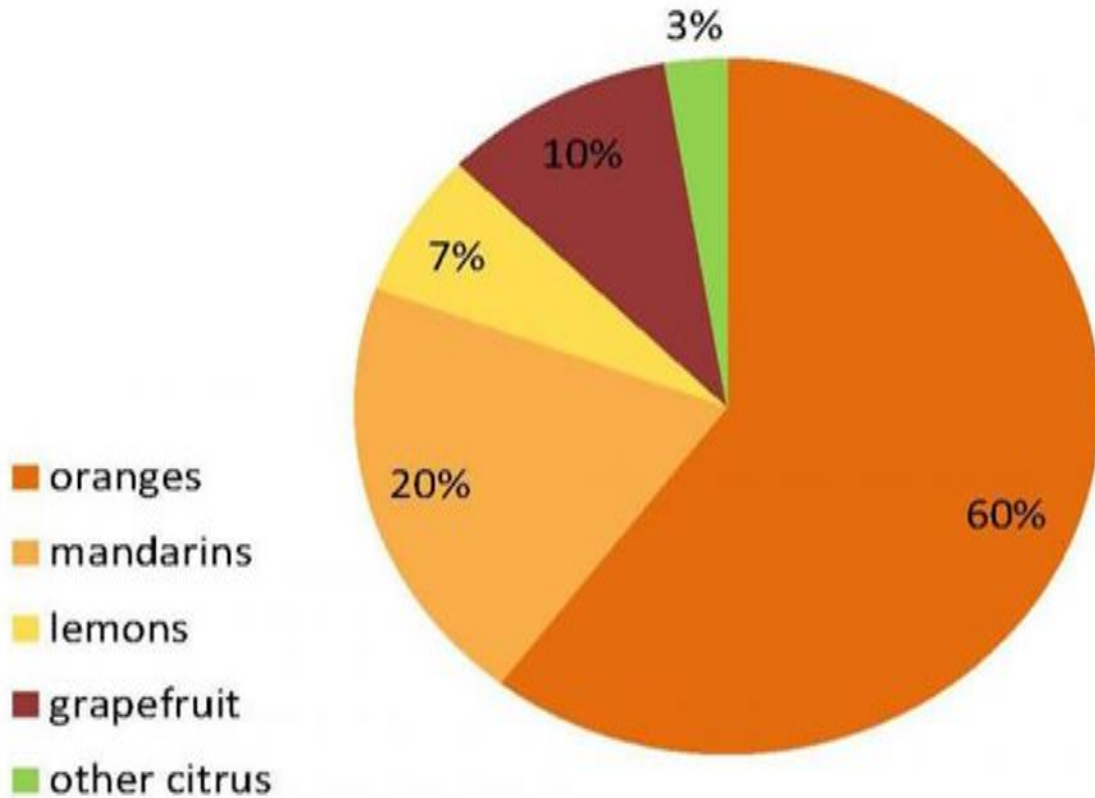


Figure 1.1. The main citrus fruit types grown in Western Australia (DPIRD)

Citrus fruits are largely produced for their juices and essential oils as a by-product. Citrus fruits contain a variety of minerals, vitamins, fibre, and phytochemicals such as carotenoids, flavonoids, and limonoids, which appear to have biological activities and health benefits. Evidence shows that citrus fruit have antioxidant and antimutagenic properties that promote positive effects on bone, cardiovascular, and immune system health (Codoñer-Franch and Valls-Bellés, 2010). Citrus oils, obtained mainly from the peel and cuticles of the fruit are utilised by the food industries as flavourings (Colombo et al., 2002). Also, for its fragrance in perfume and aromatherapy (Koroch et al., 2007).

As with other fruits, citrus is attacked by several pre- and postharvest pests that affect fruit quality. Scale insects are one of the main pests of citrus. California red scale *Aonidiella aurantii* (Maskell) is a cosmopolitan and polyphagous insect which is potentially a severe pest of citrus in California, Australia, New Zealand, Mexico, Chile, Argentina, Brazil, Israel, the eastern Mediterranean islands (Ebeling, 1959), and South Africa (Bedford et al., 1998).

1.2.2 The California red scale, *Aonidiella aurantii* (Maskell)

1.2.2.1. Systematic classification of *Aonidiella aurantii*

In 1878 William Maskell described California Red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), *Aspidiotus* (Maskell). The description was based on scale observed on orange and lemon fruit imported to Auckland, New Zealand, from Sydney, Australia (Compere, 1961; Charles and Henderson, 2002). It was transferred to the genus *Aonidiella* by Howard Lester McKenzie in 1937 (McKenzie, 1937).

Aonidiella aurantii belongs to the order Hemiptera, superfamily Coccoidea, family Diaspididae, which are classified as armoured scale insects. This is a large family of highly evolved specialised plant pests. The family's taxonomy was based almost exclusively on the adult female's characteristics. The Diaspididae are ranked at the tip of evolutionary lineage as the most advanced species of coccid (Ben-Dov, 1990).

California red scale, *Aonidiella aurantii* is classified as follows:

Kingdom Animalia

Subkingdom Eumetazoa

Phylum Arthropoda

Subphylum Hexapoda

Class Insecta

Order Hemiptera

Suborder Sternorrhyncha

Superfamily Coccoidea

Family Diaspididae

Tribe Aspidiotini

Genus *Aonidiella*

Species *aurantii* (Maskell)

1.2.2.2 Origin and Distribution

The establishment of *Ao.aurantii* in Australia and New Zealand was before 1840 as the result of introduction (Compere, 1961). The genus *Aonidiella* is native to south-eastern Asia, an area between India and south-eastern China (Bodenheimer, 1951; Hely et al., 1982; Bedford et al., 1998; Longo et al., 1995). *Aonidiella aurantii* is widely distributed throughout the world, in all tropical and subtropical regions where citrus is grown (Ebeling, 1959; Clausen, 1978; Rosen and DeBach, 1978). The most damaging pest of citrus worldwide is *Ao. aurantii* (Ebeling, 1959; Compere, 1961; Hely et al., 1982; Smith et al., 1997; Bedford et al., 1998; OEPP/EPPO, 2004). The pest, *Ao. aurantii* is distributed throughout the Mediterranean Basin, South Africa, the tropical and subtropical zone of South America, Australia, New Zealand, Pacific Islands, Indian peninsula, Philippines, China, Middle East and Japan (Ebeling, 1959; Rosen and DeBach, 1978; Bedford et al., 1998; Pekas et al., 2010). In Australia, *Ao. aurantii* was introduced before 1840 (Compere, 1961). According to Maelzer (1979), *Ao. aurantii* occurs in both irrigated and non-irrigated areas of Australia. The two climatic zones affecting these areas are inland semi-arid and humid coastal areas. The citrus industry along the River Murray in South Australia, Victoria and NSW, and the Murrumbidgee River in NSW and the non irrigated areas of NSW and south-eastern Queensland is affected. It also occurs as a pest in the Northern Territory and Western Australia (Ebeling, 1959; Rose, 1990).

1.2.2.3. Host plants

Aonidiella aurantii is a polyphagous pest species, attacking a wide variety of plants belonging to at least 77 plant families (Borkhsenius, 1966). Major host plants of *Ao. aurantii* include all species and varieties of citrus [Sapindales: Rutaceae: Aurantioideae: Aurantieae], apple trees (*Malus domestica* Borkh), pear trees (*Pyrus communis* L.), olive trees (*Olea europaea* L.), pomegranate trees (*Punica granatum* L.), carob trees (*Ceratonia siliqua* L.), walnut trees (*Juglans regia* L.), avocado (*Persea americana* Mill. [Laurales: Lauraceae]), mulberry trees (*Morus alba* L.), quince trees (*Cydonia oblonga* L.), laurel trees (*Laurus nobilis* L.), fig (*Ficus* spp. [Rosales: Moraceae]), passionfruit (*Passiflora* spp. [Malpighiales: Passifloraceae]) palm trees, and many ornamentals such as the majority of the species belonging to the family Rosaceae, and various *Solanum* species etc. (Ebeling, 1959; Beardsley and González, 1975; Hely et al., 1982; Miller and Davidson, 1990; Smith et al., 1997).

1.2.2.4. Injurious effects of *Aonidiella aurantii*

One of the most important citrus pests is *Ao. aurantii*. This global and polyphagous pest attacks all above-ground parts of citrus trees. It prefers feeding on fruit, leaves and twigs and branches. It will also go after older corky branches and trunks, but not readily (Ebeling, 1959; Hare et al., 1990). The sap is sucked from the plant tissue by the long, filamentous mouthparts causing the weakened of the plant's organs while the action of the toxic saliva caused deformations (Beardsley and Gonzalez, 1975; Washington and Walker, 1990). The feeding activity of the *Ao. aurantii* may cause direct damage. Fruit and branches, when heavily infested, can be completely encrusted with all life cycle stages of the pest. Leaf drop, defoliation and dieback of twigs and branches are the result of severe infestations. Leaving the pest unchecked can result in large branches being killed and if severe enough whole trees. Tree damage is most likely to occur in late summer and early autumn when scale populations are highest, and water stress on the tree is greatest (Ebeling, 1959; Hely et al., 1982; Smith et al., 1997; Bedford et al., 1998). Branches suffer the most injurious infestations, followed by leaves, and fruit (Quayle, 1911a). This result could be caused by the destruction of cortex cells exposing the vascular cambium to pathogens and dehydration. (Washington and Walker, 1990). This may be due to high scale populations being related to rates of parasitism lower than those that occur on leaves and fruit (Walde et al., 1989; Murdoch et al. 1995). Undoubtedly, the most serious damage caused by *Ao. aurantii* is indirect, which is the appearance of scales on fresh fruit. (Crouzel et al., 1973; Smith et al., 1997; Bedford et al., 1998). The main economic impact of *Ao. aurantii* is due to the overseas export markets and local markets not accepting fruit with evidence of scale, as opposed to its effects of the loss of yield and deforming fruit (McLaren and Buchanan, 1973). Because it is easily transported on fruit and plant material, *Ao. aurantii* is included in several countries quarantine lists. (Burger and Ulenberg, 1990). For instance, in South Africa fruit may be downgraded if more than six scale insects > 1 mm diameter are found on oranges marketed as export grade. For lemons and soft citrus, the thresholds are five and four scale insects, respectively (Bedford et al., 1998). Even though scale can be removed from the fruit during washing, brushing and water-jetting in the packing shed, the fruit may still not be acceptable for sale as fresh fruit because of scale damage.

Aonidiella aurantii was cited as the most important pest in the Mediterranean basin (Tena and Garcia-Marí, 2010). It was the most serious pest in California until the mid-1980s. Infestations were particularly serious in the interior San Joaquin Valley. However, by using an integrated pest management strategy which reduced overall insecticide treatments and utilised more

selected formulas, natural and augmented populations of *Ao. aurantii* predators grew in the area. Thus, pest populations were maintained below economic damage levels (Haney et al., 1992; Luck et al., 1992, 1996). In Australia, *Ao. aurantii* is viewed as a key pest, and its control determines the entire pest management approach (Smith et al., 1997; Papacek, 2006).

1.2.2.5. Life cycle stages of *Aonidiella aurantii*

1.2.2.5.1. Morphology and developmental stages of *Aonidiella aurantii*

Extensive studies of the morphology of *Ao. aurantii* have been investigated and reviewed (Dickson, 1951; Ebeling, 1959; Perez, 1972; Beardsley and Gonzalez, 1975; Bedford and Georgala, 1978; Hely et al., 1982; Foldi, 1990; Takagi, 1990; Forster et al., 1995; Smith et al., 1997; Bedford et al., 1998). In diaspidids, the adults present a marked sexual dimorphism. Adult females retain the appearance of larval stages, have no wings or legs and remain sessile. The body structure of the adult female is morphologically a nymph. Therefore, it is considered neotenic. The segmentation of the adult female body is obscured since parts of the head and thorax are fused into a constricted area that is called pygidium. The pygidium bears the anus dorsally and the vagina ventrally. On the pygidium are also present wax pores and tubes that lead to wax glands that open on the dorsal and ventral surfaces of its posterior tip. On the other hand, the adult diaspidid male develops wings and fly to find a mate. They have well-developed antennae, front wings and legs and are mobile.

The developmental stages of *Ao. aurantii* differ between the two sexes. Females pass through three instars and males through five. The first instar passes through three stages: the mobile crawler, the sessile whitecap and nipple stage. It is the only stage in the life cycle when the scale cover has no orange pigmentation. The second instar rotates inside the scale cover and produces wax material to form the circular armour surrounding the orange ring formed by the combination of first instar cast skin and scale cover. The third instar virgin female body becomes separated from its scale cover, and a new band of wax appears as a grey ring around reddish bands of wax produced by the earlier stages. Prepupal, pupal and adult males do not become attached to the scale cover. Prepupa is orange-yellow with the dark red or brown eyes. The light-yellow adults have a dark band on their dorsal thorax. They remain under their scale covers for a few days until their single pair of wings expand and dry. The adult emerges from under its scale cover by pushing its way backward under the cover. Each instar is separated from the next one by a moulting stage. During the instar stage, the body has a yellow colouration and can be easily separated from the cover (in the case of adult females until

insemination takes place), while during the moult stage, the body becomes orange and cannot be separated from the cover. Great differences may be found in the average size of the scale cover depending on the instar of *Ao. aurantii*, plant substrate upon which the insect feeds, geographic location, time of the year and probably nutritional status of the host plant (Ebeling, 1959; Carroll and Luck, 1984; Walde et al., 1989; Hare et al., 1990; Hare and Luck, 1991, 1994; Forster et al., 1995; Hare and Morgan, 2000).

For all stages of *Ao. aurantii* as for the rest of the armoured scales (Figure 1.2.), except for the crawler and male adult, the soft scale body is covered by the presence dorsally of an “armour” or “scale” cover formed from filaments of proteinaceous material and wax, both produced by integumentary glands essentially located on the pygidium, and exuviae (cast larval skins) combined and cemented together by an anal liquid and its purpose to protect the insect body from physical aggressions and adverse climatic conditions. The scale cover is not a part of the insect. Therefore, it can be removed without disturbing the insect’s life cycle; however, without the scale cover, the insect will die from desiccation. The filaments come out through ducts that open on the dorsal surface at the end of the pygidium. The pygidium is lifted or arched to rub the under the surface of the scale cover with its smooth dorsal surface that contains duct openings. The pygidium is moved slowly so that the covering material can adhere to the scale cover. The cover consists of wax secreted by glands of the pygidium and exuviae that are incorporated during the moult. There is also a ventral cover that is elaborated of secretions of ventral wax glands plus incorporated ventral exuvial residues. This cover is very thin and serves to separate the insect from the host plant (Foldi, 1990). During the first and second instar male and female stages, and the third instar virgin female stage, the scale body is covered by the scale cover, but not attached to it. In contrast, the bodies of first moult males and females, second moult females, and third instar mated females are attached to their scale covers.

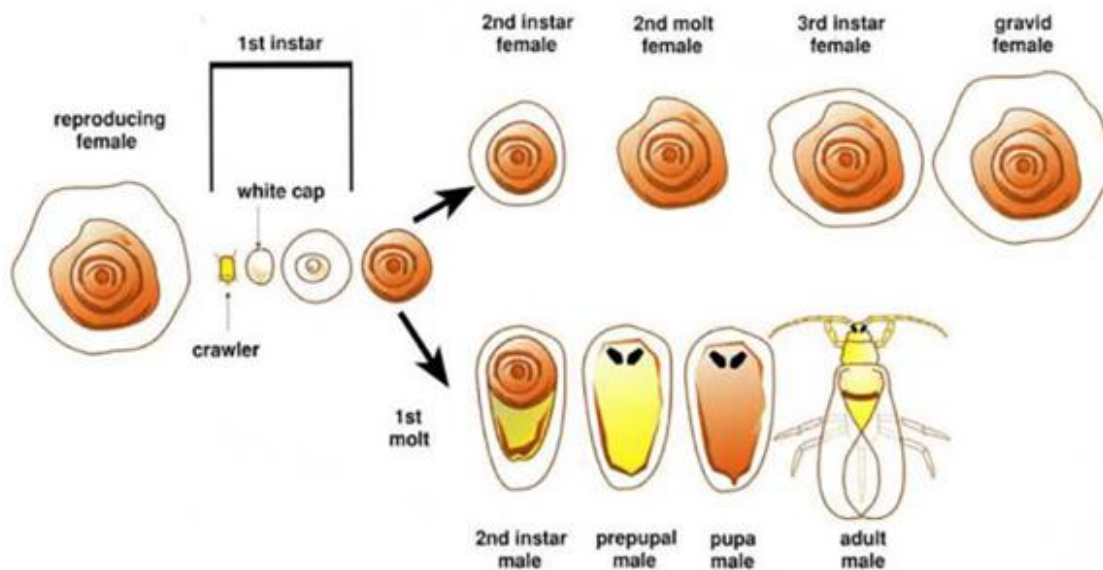


Figure 1.2. Cover forms of the California red scale male and female development stages (Graphic by G. Conville).

In *Ao. aurantii* the female cover is almost circular whereas it is elongate in males. The cover is of similar reddish-orange colour in females and males. Although the cover of *Ao. aurantii* is thin and semitransparent, its physical properties, namely its hardness and impermeability, constitute an effective barrier against chemical insecticides, preventing them from reaching the body underneath.

1.2.2.5.2. Sex ratio

Quayle (1911) suggested that *Ao. aurantii* males outnumber females from January to July (mid-winter to mid-summer). Males are more common than females in spring than in other seasons; in southern California, the sex ratio (male: female) was 1:0.6 from January to July (mid-winter to mid-summer) in contrast to 1:1 from mid-summer to mid-winter. For populations of the scale reared on orange seedlings, Nel (1933) reported ratios of 1:0.4 for the first half of 1930 and 1:1.6 for the second half. Ebeling (1959) reported ratios of 1:2.6 on mature Valencia and 1:2.57 on mature Eureka lemon.

1.2.2.5.3. Ecology and Life cycle of *Aonidiella aurantii*

1.2.2.5.3.1. Influence of abiotic factors

A change of climatic conditions influences the development rate of *Ao. aurantii*. The key conditions appear to be temperature, the amount of rainfall and humidity and these conditions influence the number of generations of insects per year. The generations can vary from two to seven as a function of conditions between the emergence of the mobile nymphs and reproduction and death of the adults. (Beardsley and González, 1975; Kennett and Hoffmann, 1985; Grout et al., 1989; Murdoch et al., 1989; Smith et al. 1997; Asplanato, 2000). Generally, a higher number of generations is observed in zones with low humidity and relatively high temperatures (Bodenheimer, 1951). Extreme temperatures have an impact on *Ao. aurantii* development (Nel, 1933; Willard, 1973; Abdelrahman, 1974a; Zhao, 1990), and these impacts differ with the life-cycle stage. First, second and third instars are the most resistant to low temperatures, whereas females with crawlers are of intermediate resistance. On the other hand, moults and male pupae and prepupae are highly susceptible to low temperatures (Abdelrahman, 1974a). According to the same author, low temperatures are the most determinant factor for the abundance and distribution of the scale. Various authors have noted the impact of temperature on life cycles. The temperature has the least impact on the first instar, is greater on the second and has maximum impact on the combined third instar (Bliss et al., 1931). Strofberg (1937) noted the same differences in the impact on each instar. Nel (1933) estimated the mean numbers of days spent by females were 13.0, 3.4, 8.6, 3.6 and 60.3 days for the first instar, first molt, second instar, second molt and third instar, respectively.

Stofberg (1937) determined that on leaves outdoors in South Africa, the *Ao. aurantii* female period varies between 61 days (mid-summer) to 138 days (winter) to move from a crawler to crawler. In laboratory conditions, at a controlled temperature of 27°C degrees, *Ao. aurantii* female takes approximately 47 days to develop from a crawler to a mature crawler producing female. (Luck, 1995). Willard (1972) found that females and males complete their development in 44.3 and 25.2 days, respectively at 29°C; whereas it takes 209 days for females and 149 for males at 15°C. Interestingly, Yu and Luck (1988) found that developmental time was the same under constant and fluctuating temperatures in the field. Moreover, high summer temperatures cause a significant decrease in the body size of *Ao. aurantii* with serious implications for its biological control (Yu, 1986; Yu and Luck, 1988; Hare et al., 1990).

Various studies have examined the developmental threshold and the thermal constant of *Ao. aurantii*. The developmental threshold for female *Ao. aurantii* reared on leaf disks floating on the water was 11.6°C (Bimboni, 1970; Willard, 1972). Yu and Luck (1988) on lemon fruits found a similar threshold of 11.6°C for females and 12.6°C for males; whereas the thermal

constant to be 639.8 and 331 degree-days (DD) for females and males, respectively. Under variable temperatures in the field, Kennett and Hoffmann (1985) established the average period required for *Ao. aurantii* to complete the whole cycle in about 615 DD (growing degree days) using 11.7°C as threshold development for the scale. Similarly, Rodrigo (1993) in orange orchards, calculated a thermal constant 753 DD using 11.6°C as threshold development. The average degree days for California red scale cycle estimated in 650 DD by Forster et al. (1995), 652 DD by Murdoch et al., (1995), While Asplanato and Garcia-Marí (2001) estimated the average degree days in 667 DD.

The population densities of *Ao. aurantii* depend on the temperature and relative humidity. According to Bodenheimer (1951), optimum conditions for the development of *Ao. aurantii* are temperatures between 23 and 27.5°C and 70–80% R.H. Nevertheless, Smith et al., (1997) reported that in Australia, *Ao. aurantii* develops under high temperatures 30-38°C and even low relative humidity. Moreover, temperature affects fecundity of female *Ao. aurantii*. Willard (1972) obtained a maximum average of 267 of nymphs per female at 30°C and a minimum of 46 nymphs at 15°C.

1.2.2.5.3.2. Influence of biotic factors

Aonidiella aurantii attacks all plant canopy substrates, wood, leaves and fruits. However, key factors such as survival, fecundity and ultimate scale size are functions of the substrate on which the scale grows. Bliss et al., (1931) reported that for all instars, development was more rapid upon fruit than upon the stems and leaves of potted seedlings, and leaves seemed to be somewhat more favourable than stems. Carroll and Luck (1984) reported that fruits were the best substrate for *Ao. aurantii* development, followed by leaves while wood was the least favourable substrate. Bliss et al. (1931) and Carroll (1980) reported more rapid development occurring on fruit, followed by leaves, and stems. Smith (1957) observed that the length of the red scale life cycle on six host plants varied, and that it was shorter on yucca (*Yucca elata* Engelm. [Asparagales: Agavaceae]) and agave (*Agave decipiens* Baker [Asparagales: Agavaceae]) than on lemon, sago palm (*Cycas revoluta* Thunb. [Cycadales: Cycadaceae]), orange, and grapefruit, respectively. Grout et al. (1989) also reported more rapid development on lemon trees than on orange and grapefruit trees. Furthermore, Perez (1972) concluded that development was more rapid on grapefruit than on oranges. Moreover, scales grown on fruits presented higher survival and fecundity than those grown on leaves or twigs (Bodenheimer, 1951; Willard, 1972; Atkinson, 1977; Carroll and Luck, 1984; Hare et al., 1990; Hare and Luck, 1991).

The ultimate (cover and body) size *Ao. aurantii* attains also varies substantially among citrus species and substrates within species. *Ao. aurantii* are largest when they grow on fruits, smallest when they grow on wood and of intermediate size when they grow on leaves (Carroll and Luck, 1984; Luck and Podoler, 1985; Hare et al., 1990; Hare and Luck, 1991). Similarly, *Ao. aurantii* is largest when reared on lemon and grapefruit compared to orange or mandarin (Hare et al., 1990; Hare and Luck, 1991, 1994). The presence of size variability is a key factor in the effectiveness of biological control of scale. The main natural enemy of *Ao. aurantii* is the adult *Aphytis* parasitoids (Hymenoptera: Aphelinidae). Their effectiveness depends on the size of their hosts.

1.2.3. Biological control of *Aonidiella aurantii*

Biological control is founded on the premise that the natural enemies of the pest are utilized (Opoku-Debrah et al., 2013). Therefore, it is necessary to practice the rearing and release of their natural enemies such as parasitoids, predators and pathogens (Moore, 2002; Opoku-Debrah et al., 2013) and these natural enemies help preserve the ecological balance by controlling the population of their hosts or prey (Balmer et al., 2014). Altering this ecological balance results in certain insect species becoming pests (Desneux et al., 2007). Therefore, biological control seeks to correct the ecological balance in one of the following ways:

- **Classical Biological Control:** Involves the amalgamation of natural enemies with pests that have relocated to new geographical areas (Zeddies et al., 2001; Kipkoech et al., 2006). The most positive successful biological control projects repeatedly demonstrate that the introduction of *Ao. aurantii* parasitoids and predator enemies are successful in the elimination of *Ao. aurantii*. For instance, importation of *Ao. aurantii* parasitoids and predators has been demonstrated to reduce the scale population in citrus-producing areas of California, Australia and South Africa (DeBach, 1969; Smith, 1978; Furness et al., 1983; Bedford, 1997).
- **Augmentation Biological Control:** This approach organises activities targetted at increasing the population of natural enemies. Such techniques as mass culture and periodic inoculative release have been shown to result in a suppression of pest populations (Opoku-Debrah et al., 2013).
- **Conservation Biological Control:** This approach features the enhancement of biodiversity in an agro-ecosystem to protect and optimise the survival and effectiveness of natural enemies (Straub et al., 2008). The environment is manipulated to favour natural enemies

species by eliminating or mitigating adverse features. Examples of this approach are the elimination of ants or providing lacking requisites. For example, facilitating nectar sources and honeydew providing plants has been successful. (Rosen and DeBach, 1978).

In nature, it is rare for any pest not to have one or more parasitoids or predators (Rosen and DeBach, 1991). The most biocontrol reliable system has a reciprocal density-dependent relationship between the predator or parasitoid and its prey or host. In other words, the host is regulated by its enemy and the enemy is limited by the number of hosts (Huffaker and Messenger, 1964). The key premise of biological control is that many noxious species are amenable to control and regulation by their natural enemies. Empirical evidence supported by theoretical considerations appears to favour host-specific parasitoids over predators. Parasitoids are more closely adapted and synchronised in interrelationship because their lower food requirements allow them to maintain a balance with their host at a lower host density and the young stages do not have to search for their food (Doutt and DeBach, 1964).

The scientific approaches leading up to the biological control of *Ao. aurantii* are recognised as the most thorough in biological controls methods. During 1889-1947 varieties of exotic natural enemies were introduced into California for *Ao. aurantii* control. The earliest of these approaches introduced coccinellid predators, of which more than 40 species were imported to the US (Compere, 1961). After most of the predators had failed to establish consideration was then given to parasitoids. Since 1940, even with inadequate information, a huge emphasis was placed in finding parasitoids in the pests' origin area (Flanders, 1943; Smith, 1942). Finally, after unsuccessful attempts of the introduction of many natural enemies, DeBach et al. (1955) outlined the basis of the biological control of *Ao. aurantii*. Namely, the biological control of the pest was possible under certain circumstances through a combination of different parasitoids, and this is also considered as the longest campaign in the history of biological control (Kennett et al., 1999).

The chemical control of *Ao. aurantii* is difficult because of its frequent, recurrent infestations, due to the elimination of natural enemies and resistance to products used for *Ao. aurantii* control. Populations of *Ao. aurantii* developed resistance to organophosphate and carbamate insecticides in California, South Africa, Israel and Australia (Forster et al., 1995; Bedford, 1997; Levitin and Cohen, 1998; Collins et al., 2007). Experiments have demonstrated that *Ao. aurantii* has developed resistance to chlorpyrifos and methidathion (Grafton-Cardwell et al., 2001; Vehrs and Grafton-Cardwell, 1994; Martínez et al., 2005).

On the other hand, their unique lifestyle appears to make armoured scale insects more amenable to biological control by natural enemies than by other groups of injurious organisms. Hence, it is essential to encourage the activity of parasitoids and predators to *Ao. aurantii* by the study of the existing species and the periods of the year they are most abundant in order to look for suitable species for release. The negative effects of some chemicals, like pyridaben, methidathion or carbaryl, on parasitoid populations has been associated with the increase of *Ao. aurantii* densities up to 15 times that of the untreated controls (Amalin et al., 2003; Grafton-Cardwell and Reagan, 2005). An alternative approach to chemical control is through enhancing the activity of natural enemies. This strategy attempts to minimise the use of pesticides and focuses on preserving existing and introducing missing natural enemies that will biologically manage citrus pests. *Ao. aurantii* has numerous predators and parasitoids (Hely et al., 1982; Forster et al., 1995), the range and number of which vary with location, prevailing climate conditions and host stage requirements (Forster et al., 1995). Parasitoids are considered to be more effective than predators (DeBach et al., 1953; Ebeling, 1959; Hely et al., 1982; Murdoch et al., 1995; Sorribas and Garcia-Marí, 2010). In some regions, *Ao. aurantii* is also parasitised by one or more entomopathogens (Searle, 1964; Bedford et al., 1998; Hely et al., 1982; Smith et al., 1997; Xie et al., 2012; Dao et al., 2016).

1.2.3.1. Classification of parasitoids

Adult female parasitoids lay their eggs in or on other insects, resulting in the death of the host. The parasitoid larvae develop by feeding on the host bodies (Wajnberg et al., 2008). There are two types of parasitoids based on their egg allocation and the developing of the larval system: Ectoparasitoids and endoparasitoids. Ectoparasitoids are idiobionts: they permanently paralyse their hosts and prevent further development of the host after initial parasitisation; have long adult lifespans and low fecundity; typically involves a host life stage that is immobile (e.g., an egg or pupa), and almost without exception, they live outside the host; and have ‘fast’ larvae (relatively rapidly developing) and slow (relatively long-lived) adults. In contrast, endoparasitoids are nobionts with slow larvae and fast adults; they allow the host to continue its development and often do not kill or consume the hosts until the host is about to either pupate or become an adult. Therefore, it typically involves living within an active, mobile host. Koinobionts can be further subdivided into endoparasitoids, which develop inside of the host, and ectoparasitoids, which develop outside the host body, though they are frequently attached or embedded in the host's tissues. It is not uncommon for a parasitoid itself to serve as the host

for another parasitoid's offspring. The latter is termed as a secondary parasitoid, or hyperparasitoid; most of such species known are in the insect order Hymenoptera (Askew and Shaw, 1986; Jervis and Kidd, 1986; Godfray, 1994; Thompson, 1999; Traynor and Mayhew, 2005).

Both ectoparasitoids and endoparasitoids can be either solitary (when only one larva develops per single host) or gregarious (when more than one larva develops upon an individual host). Parasitoids may have one generation to one generation of the host or two or more generations to one of the host. When females emerge with a reduced number of ovarian eggs and more eggs are produced along with its life it is named as synovigenic, whereas proovigenic females complete oogenesis before eclosion and generally lay their eggs soon after emergence. Proovigenic parasitoids most often develop as koinobiotic endoparasites, developing in hosts that survive and continue to feed and grow following parasitism (Godfray, 1994; Quickie, 1997).

Synovigenic parasitoids (females emerge with at most only a fraction of their total egg complement) offer an ideal opportunity to study how insects manage their larval and adult resources. These parasitoids lay mature eggs during their entire adult life. Females lay their eggs in or on the bodies of their hosts and feed off them as they develop. The host is also the main source of nutrients for the foraging female (Jervis and Kidd, 1986; Heimpel and Collier, 1996; Jervis et al., 2001).

The most important primary parasitoids of *Ao. aurantii* in Australia are *Aphytis chrysomphali*, *Aphytis lingnanensis*, *Aphytis melinus*, *Encarsia citrina*, *Encarsia perniciosi* [Hymenoptera: Aphelinidae] and the red scale strain of *Comperiella bifasciata* [Hymenoptera: Encyrtidae] (Wilson, 1960; Furness et al., 1983; Hely et al., 1982; Smith et al., 1997). Species of *Aphytis* are ectoparasitoids and females lay their eggs externally on the body of their host. The two species of *Encarsia* and *Comperiella bifasciata* are endoparasitoids and females lay their eggs within the body of their host.

1.2.3.2. Ectoparasitoids of *Ao. aurantii*: *Aphytis* Howard

Ectoparasitoids play important roles in regulating *Ao. aurantii* populations in many countries (Rosen and DeBach, 1979; Smith et al., 1997; Bedford et al., 1998). Their life cycle stages include the egg, three larval instars, the pupa and the adult. Adults are very small, yellow or greyish parasitoids with mostly hyaline wings that usually do not exceed one millimetre in length, and develop exclusively as primary ectoparasitoids of diaspidid scales. Their antennae

is comprised of six segments, with the basal segments being much smaller than the distal segment. They usually emerge by pushing their way out through a hole that has been chewed in the covering scale or merely they push their way out under the edge (Rosen and DeBach, 1979).

Identification and separation of *Aphytis* species are extremely difficult. According to Rosen and DeBach (1979), this is due to their minute size, the lack of reliable taxonomic characters, the common occurrence of sibling species and the fact that in many species males are rare and thus, hybridisation tests are impossible.

The genus *Aphytis* is classified as follows:

Kingdom: Animalia
Subkingdom: Eumetazoa
Phylum: Arthropoda
Subphylum: Hexapoda
Class: Insecta
Order: Hymenoptera
Suborder: Apocrita
Superfamily: Chalcidoidea
Family: Aphelinidae

About 105 species of *Aphytis* have been described (Rosen, 1994). Most species of *Aphytis* are biparental. However, a relatively high proportion is uniparental. The biparental species exhibit arrhenotokous reproduction, unfertilised eggs developing into males and fertilised eggs into females. Unmated females produce male progeny, whereas mated female produces both sexes. The uniparental species of *Aphytis* exhibit thelytokous parthenogenesis, female producing female progeny without fertilisation by males. However, males are produced regularly in these species at a very low rate (Doutt, 1959; Rosen and DeBach, 1979).

Adult females use their ovipositor to pierce the scale cover of their hosts laying one or more eggs under the cover, outside of the scale body. Therefore, they only parasitise host stages in which the scale cover is not attached to the scale body (Rosen and DeBach, 1978, 1979). Eggs are teardrop in shape translucent, whitish, and adhere to the surface of the scale insect (Rosen and DeBach, 1978; Forster et al., 1995). Larvae feed by sucking the body fluids of their hosts,

resulting in their eventual death. Fully grown larvae excrete characteristic meconial pellets and pupate under the empty scale. The species of *Aphytis* are ectoparasitoids and are more effective than endoparasitoids and predators as enemies of armoured scale insects, and in this way, several species have been successfully employed in biological control projects (Rose, 1994). Subeconomic levels of many potential pests have been achieved by effectively using species of *Aphytis* (Rosen and DeBach, 1979).

Adult *Aphytis* were not observed parasitising the first instar of *Ao. aurantii*, and rarely deposit eggs on scales less than 0.15 mm² (i.e., smaller second instar) (Yu, 1986; Opp and Luck, 1986). *Aphytis* females prefer to oviposit on the third instar of virgin *Ao. aurantii* because they are a larger food source for their progeny to develop on than second instars, and more offspring can be produced per host (Forster et al., 1995). Based on the biology of *A. melinus*, *Aphytis* attacking *Ao. aurantii* lay 40% of their eggs on second instar (males and females) and prepupal male scales and 60% of eggs on virgin female scales (Richardson, 1978). Moulting stages are not accepted as hosts because their body becomes attached to the cover and also because their tegument becomes heavily sclerotised and is too hard to be pierced by the ovipositor. Similarly, mature, mated females of California red scale are not accepted as hosts by *Aphytis* (DeBach, 1969; Baker, 1976; Forster et al., 1995). However, the size of the grey skirt of second and third instar virgin females also influences oviposition behaviour by *Aphytis* species. The longer second instar and third instar, virgin females feed, the wider their grey skirt becomes. Therefore, *Aphytis* may prefer a large, second instar scale with a wide grey skirt, over a young third instar with a narrow grey skirt. This behavioural by *Aphytis* may be more important than host size in the host selection process (Forster et al., 1995). Abdelrahman (1974b), stated that not only host size but also host quality influences host preference by *A. melinus*.

1.2.3.2.1. Life history and morphology of *Aphytis*

Aphytis are holometabolous. Their development includes the following stages: egg, three larval instars and toward the end of the larval period, the third-instar larva enters a short prepupal stage, then pupae and adult.

Aphytis pre-ovipositional behaviour is characterised by five phases and these are drumming, turning, drilling, probing and ovipositing. When the adult female has located the host, the female walks forward to drum the scale cover with her antennae until she encounters the margin. Then she walks backwards, rotating left to the right. She uses her ovipositor to drill through the scale cover into the host body. She then lays the eggs underneath the scale cover

of the host where they adhere to the surface through a small adhesive pad. *Aphytis* females lay their eggs on either the dorsal or the ventral side of the host body (Abdelrahman, 1974b). *A. melinus* lay most of its female eggs on the dorsum of a scale-insect beneath its cover, and most of its male eggs under the scale insect's body (Luck et al., 1982). The probing of the host's body prior to oviposition serves not only to provide accessibility for the eggs but also to paralyse the host preventing it from becoming unsuitable for the development of her progeny (Rosen and DeBach, 1979). *Aphytis* eggs hatching time is a function of temperature. At 20°C, it takes almost five days whereas at 26.7°C eggs hatch in two days (Yu, 1986; Yu and Luck, 1988). *A. melinus* passes around 18% of the total developmental time as egg (Yu and Luck, 1988). Host size and host stages affect *Aphytis* behaviour, size, fecundity and sex ratio (Opp and Luck, 1986; Reeve, 1987). Luck et al. (1982) reported that numbers of eggs laid per host by *A. melinus* and *A. lingnanensis*, facultatively gregarious species, depends on host size. Small hosts, such as the second instar, produced small *Aphytis* with a male-biased sex ratio. The third instar, a larger host, produced much larger *Aphytis* and a more balanced sex ratio (Reeve, 1987).

After *Aphytis* hatch, the larvae develop through three instars. As larvae begin to feed externally on the scale body, they grow in size and the host, depleted of body fluids, gradually shrinks. Larval instars can be distinguished by their size and shape. The first instar larva is ovoid, segmentation is usually not clearly visible, but in some specimens, the head and 12 body segments are evident. The second instar larva segmentation is much more visible than in the first instar. The third instar is considerably larger than the second, elongate, rounded anteriorly and somewhat narrower posteriorly. All feeding ceases, and the larva excretes faecal material in the form of several brown or black meconial pellets. These pellets remain under the scale cover after the adult emerges. *Aphytis* pass around 36% of their developmental time as larvae, i.e. almost 11 days at 17°C, and four days at 26.7°C or 30°C (Rosen and DeBach, 1979; Yu and Luck, 1988; Forster et al., 1995). The prepupal stage is similar to the larval stages, and it is usually milky white but with no colouration in the gut. It then enters a resting period, during which a rapid metamorphosis takes place. Approximately 8% of *Aphytis* development time is in the prepupae stage. Differentiation of pupal structures becomes evident with the appearance of pupal mouthpart, legs and wing cases. *Aphytis* pupa is exarate, flattened dorso-ventrally and wider than thick. The antennal cases are present on each side of the head. The sexes are recognisable in this stage (Rosen and DeBach, 1978). Initially, eyes during the pupal stage, are white. They then move to red, dark red, blackish and eventually green (Forster et al., 1995). The green-eyed pupal stage lasts only a day before the adult emerges (Abdelrahman, 1974b). The

pigmentation of the thorax and abdominal tergites can in this stage be used to distinguish species of *Aphytis* (Rosen and DeBach, 1978). In some cases, the pupae colouration may be an important character that helps distinguish among *Aphytis* (Rodrigo, 1993; Sorribas et al., 2008).

Adult *Aphytis* emerge by either pushing underneath the scale cover or by chewing on it at an exit hole. The entire process takes about thirty minutes at room temperature. Both males and females are minute, yellowish and difficult to distinguish without augmentation. The cephalic and thoracic exuvium remain recognisable after emergence, whereas the abdominal exuvium is often fragmented. After emergence from the host, adult *Aphytis* rest for a while then start preening. Adults move mainly by running, and long-distance dispersal occurs by flying. Low air movement likely aids the adults flying (Rosen and DeBach, 1979). The unmistakable signs attesting the fact that a dead armoured scale insect had earlier been parasitised by *Aphytis* is the distinctive meconia and characteristic exuvia, as well as the oval exit hole when present.

1.2.3.2.2. Biology and ecology of *Aphytis*

Aphytis species have shorter development periods than their hosts. Periods will vary between species, but all are dependent on climatic conditions, mainly temperature and humidity (Yu and Luck, 1988). At 26.7°C, *A. melinus* takes almost two weeks to complete its entire development whereas, at 17°C, one month is required. Most species are multivoltine i.e. develop continuously throughout the year. *A. melinus* have been found to have two to three generations to one of its host *Ao. aurantii* (Yu and Luck, 1988; Murdoch et al., 1995).

Most species of *Aphytis* are biparental and reproduce sexually. However, a relatively high proportion is uniparental. At oviposition females control the sex of their offspring. Fertilised eggs produce females, whereas unfertilised ones produce males (Flanders, 1953; Rosen and DeBach, 1979). Female *Aphytis* are essentially monogamous. They mate only once, and the sperm is stored in the spermatheca for egg fertilisation. On the other hand, males are polygamous, capable of mating with several females (Rosen and DeBach, 1979).

The female paralyses the host by inserting venom through her ovipositor before oviposition (Rosen and DeBach, 1979; van Lenteren, 1994). Paralysed scales stop rotating (Fischer, 1952). The wasp injects an unknown substance into the scale body. As the host is paralysed, it stops growing and its size at the moment of oviposition represents the food available for the parasitoid offspring. The number of parasitoids per host as they are facultative gregarious is correlated with its size and also may be influenced by host and parasitoid density (Rosen and

DeBach, 1979; Luck et al., 1982). Emerging *Aphytis* females have no or few eggs, and these develop and mature in the ovaries continuously throughout the female's life. Thus, it is considered a synovigenic species (Rosen and DeBach, 1979; Opp and Luck, 1986; Collier, 1995). Casas et al. (2000) showed that under field conditions, *A. melinus* produces about six mature eggs per day. *Aphytis* pre-oviposition examination of the host serves to prevent parasitising of an already parasitised host (superparasitism). Avoidance of superparasitism is essential for host selection by *Aphytis*. According to Abdelrahman (1974c), females of *A. melinus* recognise a recently parasitised host by the odour left by the first wasp. Rejection is based on an external examination, and the wasp needs only two to three seconds to discriminate if a host is parasitised (van Lenteren, 1994). Even if the external odour wears off, internal probing with the ovipositor can still determine if the host is parasitised (Rosen and DeBach, 1979). According to Van Lenteren and DeBach (1980), *Aphytis* parasitoids are also able to discriminate between unparasitised hosts and hosts parasitised by conspecifics.

Murdoch et al. (1989) noted a significantly higher level of *A. melinus* parasitism of *Ao. aurantii* in the exterior parts of tree canopies as compared to the interior wood trees. Walde et al. (1989) found while the population of *Ao. aurantii* was high, parasitism by *A. melinus* was low, on trunks and woody branches compared to external branches and leaves. They attributed the low level of parasitism to the small size of *Ao. aurantii* on wood relative to other substrates. However, by calculating expected levels of parasitism on wood, Walde et al. (1989) pointed out that the difference of scale size could only account for around 10% of the observed difference and was not the main explanation for the presence of the refuge. Subsequently, Hare and Morgan (2000) showed that parasitism was related to lower levels of o-caffeoyltyrosine in scale covers of the small scales on wood, than in large scales on fruit and leaves. They considered the lower levels of O-caffeoyltyrosine were probably a consequence of the reduced nutritional quality of bark as a substrate for scale survival and growth (Hare and Morgan, 2000). Outcomes of the relationships between parasitism by *Aphytis* species and *Ao. aurantii* densities vary among studies. Atkinson (1977) reported that the relationship between *Aphytis* species and *Ao. aurantii* were density-dependent. However, many other studies report that there were no density-dependent or aggregation relationships between *A. melinus* and *Ao. aurantii* (Reeve and Murdoch, 1985; Smith and Maelzer, 1986; Reeve, 1987; Murdoch et al., 1995).

Aphytis oviposit only in hosts whose body is not attached to the cover, i.e. they avoid parasitising moult stages. Mature females similarly are not accepted as host as they are heavily sclerotised (DeBach and White, 1960; Forster et al., 1995). As mentioned earlier, first instars

and small-sized hosts usually are not parasitised; they are used for host-feeding instead. In biparental species, male offspring result from unfertilised and females from fertilised eggs. Most *Aphytis* female come from larger instar hosts (Luck and Podoler, 1985; Opp and Luck, 1986). Females usually allocate female eggs to large hosts and male eggs to small hosts as sex ratio is also affected by host size (Charnov et al., 1981; Luck et al., 1982; Luck and Podoler, 1985; Yu, 1986). Females can control the sex of their offspring at oviposition by producing unfertilised eggs to generate males or fertilised eggs to generate females (Flanders, 1953; Rosen and DeBach, 1979).

1.2.3.2.3. Factors affecting *Aphytis* efficiency

Aphytis efficiency as biological control agents is a function of both biotic and abiotic factors. These factors limit distribution and parasitoid abundance. Temperature, humidity and light are the most important abiotic factors, and the biotic factors that affect *Aphytis* efficiency are host scale, host plant, the availability of food for adults and ant activity (Rosen and DeBach, 1979).

1.2.3.2.3.1. Abiotic factors

Temperature has a strong effect on *Aphytis* development and thus influences their ability to regulate the target pest population. The main mortality factor of *Aphytis* in the field is extreme temperature. Low winter temperatures in inland California caused nearly a 100% mortality of *Aphytis chrysomphali* and *Aphytis lingnanensis* pupae ((Rosen and DeBach, 1979). Furthermore, substantial rates of mortality were observed during the hot and dry months of July, August and September (Rosen and DeBach, 1979). In the laboratory, experiments on the combination of high temperatures with low humidity negatively affected adult *Aphytis* survival (Kfir and Luck, 1984). In these experiments, the life span of the adult *Aphytis*, provided with honey as a food, ranged from one up to 14 days. Abdelrahman (1974a) reported that all *A. chrysomphali* stages are susceptible to extreme heat (all eggs die at 32°C), but less susceptible to extreme cold temperature than the respective stages of *A. melinus*.

The development threshold for *A. melinus* was determined to be 11°C while that for *A. chrysomphali* was found to be 8.5°C (Abdelrahman 1974a). Different thresholds are reported by Kfir and Luck (1984) which for *A. melinus* were 6.77°C and *A. chrysomphali* 5.91°C. A study by Yu (1986) determined that a lower temperature threshold for *A. melinus* was 9.65°C. Seasonal variations also occur between them as *A. melinus* predominate in the summer months and *A. chrysomphali* in winter (Sorribas et al., 2010). For biparental species, the sex ratio is also impacted by temperature. Lower temperatures produce a biased male population (Rosen

and DeBach, 1979; Kfir and Luck, 1979). A temperature of 15.6°C for a day resulted in 74% of the progeny of *A. lingnanensis* being male. However, at a temperature of 26.7°C, approximately 33% of the progeny were male. (Rosen and DeBach, 1979). When female *A. lingnanensis* mated at 26.7°C, were then exposed to -1.1°C for six hours and finally were allowed to oviposit at an optimal temperature, all their progeny were males. According to Rosen and DeBach (1979), this result suggests that low temperature killed the sperm stored in the female spermatheca. Similar male-biased sex-ratios under the influence of low temperatures were found for *A. melinus* (Kfir and Luck, 1984). The optimum temperature for reproduction for *A. chrysomphali* is 27°C (Rosen and DeBach 1979). Numbers of eggs/female were 25.1 at 25°C, compared to 21.3 at 20°C, and 6.6 at 30°C (Abdelrahman 1974a). DeBach and Sisojevic (1960) who mentioned that reproduction for *A. chrysomphali* was best at low temperatures and poorest at high temperatures, whereas it was vice-versa for *A. lingnanensis*. DeBach and Argyriou (1967) noted that at 15.6°C, *A. melinus* and *A. lingnanensis* are quite sluggish and inactive, and that average fecundity between 15.6 to 20°C ranged from 11 to 17 progenies per female, compared to 28.2 at 26.7°C.

Variations in humidity and light have been seen to be less important than temperature. Survival rates are significantly impacted by high temperatures. However, high temperatures, when combined with low humidity, negatively affect adult *Aphytis* survival. At 32°C and 10% relative humidity, all *Aphytis* fail to survive beyond 24 hours. Increasing relative humidity to 40% resulted in a three-day period before death (Kfir and Luck, 1984). The impact of low humidity is greater on the adult stage of *A. lingnanensis* as the immature stages are normally protected. Adult longevity at 20% relative humidity was less than 1/3 or that at 80% RH (DeBach et al., 1955). When high temperature is accompanied by low relative humidity extreme impacts are observed. At 32°C and 20%RH, the population was one-twentieth of that at 21°C and 80% RH (DeBach et al., 1955). Light is thought to affect flight initiation and searching (Rosen and DeBach, 1979). *Aphytis melinus* is sensitive to the wavelength of light exposed to them. For example, yellow and green sticky traps captured more individuals than white, blue, fluorescent yellow, black or red ones (Moreno et al., 1984).

1.2.3.2.3.2. Biotic factors

Scale insects affect *Aphytis* abundance and efficiency in various ways. As already mentioned, not all developmental stages of the host scale are suitable for *Aphytis*, i.e. moults and gravid females are not accepted for parasitism because of their hard integument. Besides, the developmental stages which potentially can serve as hosts are not of the same quality. There is

substantial variation in host size depending on the developmental stage (Yu, 1986; Reeve, 1987; Hare et al., 1990). Moreover, before oviposition, the female paralyses the host by inserting venom through her ovipositor (Van Lenteren, 1994). Thus, host size represents the resources available for the developing parasitoid and is probably the most reliable cue of host quality for *Aphytis* (Hare and Luck, 1991).

The efficiency of *Aphytis* as a biological control agent is likely indirectly influenced by the host plant. Field studies show that the body size of *Ao. aurantii* varied as a function of plant substrates, locality, time of year and likely the nutritional stage of the host plant (Pekas, 2011b). Larger scales are preferred by *A. melinus* (Yu, 1986) and will parasitize the scale on fruit in preference to those elsewhere. *A. melinus* reared on *Ao. aurantii*-infested lemon leaves (*Citrus limon*) produced nearly twice the number of female progeny compared with parasitoids reared on *Ao. aurantii*-infested leaves of grapefruit (*Citrus paradisi*), orange (*Citrus cinensis*) or Satsuma mandarin (*Citrus unshiu*) (Hare and Luck, 1991). Furthermore, parasitoids raised on lemon leaves had higher initial egg loads, followed by those reared from grapefruit, mandarin and orange.

Host size is known to have a strong influence on various fitness components of adult *Aphytis*. For *Ao. aurantii*, Richardson (1980), based on the biology of *A. melinus*, concluded that *Aphytis* attacking *Ao. aurantii* lay 40% of their eggs on second instar (males and females) and prepupal male scales and 60% of eggs on virgin female scales. *Aphytis* females prefer to oviposit on the third instar and virgin *Ao. aurantii* because they are a larger food source for their progeny to develop on than second instars, and more offspring can be produced per host (Forster et al., 1995). However, the size of the grey margins of second instar and third instar virgin females also influences oviposition behaviour by *Aphytis* species. Reeve (1987) mentioned that, in the field, second instar and prepupal were attacked at a rate equal to or greater than that for third instars, whereas Abdelrahman (1974c), based on a laboratory experiment, noted that the order of preferred hosts for *A. melinus* was third instar, second instar and prepupa. Abdelrahman (1974c) also stated that not only host size but also host quality influences host preference by *A. melinus*. Luck et al. (1982) reported that numbers of eggs laid per host by *A. melinus* and *A. lingnanensis*, facultatively gregarious species, depends on host size.

Aphytis behaviour, size, fecundity and sex ratio are a function of host size and stage. Host size positively correlates with the size of adult males and females of both *A. lingnanensis* and *A. melinus* (Opp and Luck, 1986; Yu, 1988; Reeve, 1987; Rosenheim and Rosen, 1992). In 2006,

Pina noted that *Ao. aurantii*, show a similar positive relationship between the size of 2nd instar males and 3rd instars to *A. chrysomphali* size when the scale was reared on lemons. Size is a key variable as the majority of parasitoids are Hymenoptera and adult size is believed to influence offspring fitness by effecting longevity, fecundity (females) or searching capacity (Godfray, 1994). Adult *A. chrysomphali* that emerged from large hosts (third instar females of *Ao. aurantii*) were significantly larger and lived almost twice compared with parasitoids that emerged from smaller hosts (second instar males) (Pina, 2006). A similar positive relationship exists between the number of mature eggs and adult size of *A. lingnanensis* and *A. melinus* (Opp and Luck, 1988) and *A. chrysomphali* (Pina, 2006). Host size also affects *Aphytis* fecundity; larger hosts yield more fecund *Aphytis*.

In biparental *Aphytis* species, sex ratio is also affected by host size. Small hosts, such as the second instar, produced small *Aphytis* with a male-biased sex ratio. The third instar, a larger host, produced much larger *Aphytis* and a more balanced sex ratio (Reeve, 1987). As already pointed out, the available hosts for *Aphytis* vary greatly in size due to developmental stage, plant substrate or time of the year (Reeve, 1987; Yu, 1988; Walde et al., 1989; Hare et al., 1990; Hare and Luck, 1991, 1994; Hare and Morgan, 2000; Pekas, 2011b). As a result, larger hosts are of higher quality because they are expected to give place to more “fit” parasitoids. Under laboratory conditions, *A. melinus* allocated female eggs mostly to hosts larger than 0.39 mm² (in the body area of *Ao. aurantii*) and *A. lingnanensis* to hosts larger than 0.55 mm² (Luck et al., 1982; Luck and Podoler, 1985). In a posterior field study, Yu (1986) confirmed that *A. melinus* laid female eggs mostly to hosts larger than 0.39 mm². Luck et al. (1982) reported that numbers of eggs laid per host by *A. melinus* and *A. lingnanensis*, facultatively gregarious species, depends on host size. For instance, on *Ao. aurantii* 0.46 to 0.60 mm² in size, 33% of the hosts received two *A. melinus* eggs, while the remainder received one. The same size class of host received only one *A. lingnanensis* egg. For scales 0.61 to 0.75 mm², *A. lingnanensis* began to express gregariousness as 33% of the hosts in this size range received two eggs, and the remainder received one.

To have an effective biological control program, a high female-biased sex ratio is desired because females attack the pest by ovipositing and host feeding. Also, females augment parasitoid populations, thus the fewer females, the poorer rate of population growth. The key element for a successful classical and augmentative biological control program is the size of the available hosts (Ode and Hardy, 2008). A biological control program for *Ao. aurantii* with *A. melinus* was implemented in the San Joaquin Valley of California. The importance of host

size was confirmed when scarcity of suitable female producing host for *A. melinus* (larger than 0.39 mm²) resulted in a small population, and the consequence was poor biological control, especially in the summer (Luck et al., 1996; Luck et al., 1999). As a solution, augmentative releases of commercially produced *A. melinus* are used to suppress *Ao. aurantii* populations in that area (Moreno and Luck, 1992).

Adults' food availability is a key factor in the efficiency of parasitoids as biological control agents. The activity and abundance of pests are suppressing by the carnivorous parasitoid larvae. However, adults require non-host food, primarily carbohydrates to cover their energetic requirements. Adult *Aphytis* parasitoids derive their carbohydrate nutrition in nature, mainly from nectar and honeydew (Avidov et al., 1970; Heimpel and Rosenheim, 1995). Food provided by plants can have a striking impact on various life-history traits of natural enemies. It has been demonstrated that in the absence of an adequate plant food source longevity (Heimpel et al., 1997; Wäckers, 2001; Lee et al., 2004) and reproduction of natural enemies (Winkler et al., 2006) is seriously compromised. Therefore, the availability of adequate food sources is expected to affect the efficacy of natural enemies and consequently, the outcome of biological control. For *Aphytis*, availability of an adequate sugar source is crucial for adults. Already since the first mass rearing efforts, it was noticed that most adults die within 24 hours unless honey, sugar-water or a similar carbohydrate source was provided (DeBach and White, 1960). Moreover, host-feeding alone cannot enhance longevity; it can do so only when the wasps have in addition, access to a sugar source and the longevity of adult *A. melinus* that had no access to a sugar source did not exceed three days (Heimpel et al., 1997). Similarly, fecundity was also seriously compromised. Presumably, nectar from citrus and other floral species, as well as hemipteran honeydew, are the main carbohydrate sources (Rosen and DeBach, 1979). However, like other synovigenic insects, the females require proteinaceous nutrition for continuous ovigenesis. Proteinaceous nutrition is obtained by predatory host-feeding by which the parasitoid feeds on the body fluids of the host (Forster et al., 1995). After drilling through the scale cover, the female thrusts the ovipositor into the body of the host. When the ovipositor is at last carefully withdrawn, a tiny straw-like feeding tube remains (Rosen and DeBach, 1979). The host-fed scale develops large brown necrotic spots and dies within several hours to a few days. However, mortality by host-feeding is rather difficult to quantify because of host die and dry up, and thus they cannot be distinguished from hosts that have died due to other abiotic factors. Certain host stages that are not acceptable for oviposition by *Aphytis*, such as first-instar and first moult stages are readily utilized for host-feeding

(Abdelrahman, 1974b; Heimpel and Rosenheim, 1995). Host-feeding is a typical ability of many synovigenic species (Jervis and Kidd, 1986).

1.2.3.2.4 *Aphytis melinus* DeBach

The most successful and widely spread biological control agent is *Aphytis melinus* of the *Aphytis* parasitoids genus (Murdoch et al., 1989; DeBach and Rosen, 1991; Forster et al., 1995). *A. melinus* is native to northern India and Pakistan. Since 1956, it has been used to control *Ao. aurantii* and *Aspidiotus nerii* Bouché (Rosen and DeBach, 1978). *Aphytis melinus* has been either accidentally introduced or mass-reared and released in almost all the citrus areas where *Ao. aurantii* is found. *A. melinus* was first described in 1959 by Paul DeBach from specimens from *Ao. aurantii* collected on rose in New Delhi and Gurgaon in India and Lahore in Pakistan in 1956 (DeBach, 1959). It was introduced into Australia in 1961 from the University of California, Riverside (Furness et al. 1983; Malipatil et al. 2000). The establishment was achieved at Boundary Bend in Victoria in 1963 (Furness et al., 1983). Biological Services Inc., was Australia's first commercial insectary and It was established in Loxton in 1971 for mass rearing of *A. melinus* (Furness et al., 1983; George, 1984). *Aphytis melinus* releases are now widely used in the citrus districts Victoria, South Australia, Western Australia and inland New South Wales citrus districts, and in Alice Springs in the Northern Territory (Smith et al., 1997).

Aphytis melinus can be described as a gregarious, biparential, arrhenotokous species (DeBach, 1959; Rosen and Debach, 1979). While females are produced by fertilised eggs unfertilised ones result in males (Rosen and DeBach, 1979). Abdelrahman (1974b) noted that fertilised females produce both males and females., this indicates that only some of their eggs are fertilised. Also noted was that older *A. melinus* females only lay male eggs towards the end of their life and these females were only seen to have mated once. Host size and quality determine the degree of gregariousness. (Abdelrahman, 1974b, Luck et al., 1982). Also noted was that larger hosts were more likely to have had several eggs deposited in them. (Luck et al., 1982).

Aphytis melinus is considered a superior competitor in the field, very often displacing other previously existing *Aphytis* species, because it is better adapted to dry and hot climates (Rosen and DeBach, 1979). The biology of *A. melinus* has been widely studied. The life cycle of *A. melinus* on oleander scale on lemon fruit takes 12–13 day at 26.7°C and 50% RH. Each oviposition requires about 12 minutes, and females produce an average of 24 progeny, laying an average of 2.8 eggs per scale (DeBach and Sundby, 1963; Rosen and DeBach, 1978, 1979). The development time for different parasitoid instars, in degree-days, was calculated as an

average of several temperatures (Murdoch et al., 1995) as follows: 42.7°C days from egg to larva, 88.1°C days from larva to prepupa, 19.3°C days from prepupa to pupa and 93.4°C days from pupa to adult.

Host identification in *Aphytis melinus* is mediated by a non-volatile kairomone, O-caffeoyltyrosine, in scale covers. Variation in concentrations of this kairomone in scale covers is thought to contribute to differences in levels of parasitism that occur on substrates such as fruit, leaf and bark (Hare and Morgan, 2000). Variation in scale size on different substrates within a citrus tree (the largest scales occur on fruits, smallest on wood, and intermediate-sized scales on leaves) may also contribute to the differences in parasitism rate (Luck and Podoler, 1985). Murdoch et al. (1989) observed in grapefruit trees in a grove that the fraction parasitised by *A. melinus* was significantly greater on the exterior substrates than on wood. This pattern was consistent in all three vulnerable stages of scale (second instar females, virgin females, and second instar males). The parasitism rate in the exterior (twigs) exceeded that in the interior by 6-fold (in virgin females) to 27-fold (in the second instar). Low parasitism rates in the interior may have been caused by the parasitoid's response to the bark substrate.

1.2.4. Age-specific fecundity tables

Reproduction is a key life-history process affecting the evolution and success of a genotype. (Williams, 1966). To measure reproduction rates, several approaches to life table analysis have been developed. These highlights the impact of population growth of various sources of intrinsic and extrinsic mortality. Methods include age-specific life tables for both laboratory and field studies. Observations forms of key mortality factors analyse, and time are varied over time in the life table. Life tables prepared for cohorts or populations are schedules of births and deaths grouped by relevant factors. Under laboratory conditions factors such as food quality, temperature, humidity and photoperiod can be varied, and thus the intrinsic population birth and death rates observed (van den Bosch et al., 1982).

The composition of the population is dependent on demographic processes such as births, deaths, immigration, and emigration. The timing of these processes also plays a critical role; a population with high juvenile mortality will have a very different structure from a population with high mortality in the post-reproductive years. A life table records matters of life and death for a population. It summarises the likelihood that organisms in a population will live, die, and/or reproduce at different stages of their lives. A standard method is to collect data on a cohort, or group of individuals all born at the same time. Life tables constructed this way are

called cohort life tables. They can then be used to determine age- or stage-specific fecundity and mortality rates, survivorship, and basic reproductive rates, which in turn can be compared from cohort to cohort enabling analysis of their annual variation.

Population parameters are determined by two basic factors which are the likelihood of an individual surviving and then the likelihood of it breeding successfully. Furthermore, age becomes of paramount importance as the very young and old do not breed. It has been shown that young individuals often experience higher rates of mortality. Combining these basic parameters in a life table format can reflect age-specific survivorship and age-specific fecundity. These data allow the derivation of information to measure the rate of population growth and also project future population size.

The intrinsic rate of increase is a basic parameter in which an ecologist may wish to establish an insect population. It is defined as the rate of increase per head under specified physical conditions, in an unlimited environment where the effects of increasing density do not need to be considered (Birch, 1948). Chapman (1931) referred to biotic potential, which includes fertility rates, sex ratio, and survival rate. Stanley (1946) described the concept of the environmental index, which gives a measure of the relative desirability of different conditions, but It does not give an internal rate of increase in the population of the insect. Stanley (1946) added that the evidence of the rate of increase possible under different natural conditions at the same time gives a measure of the relative suitability of different environments. Birch (1948) noted that a fixed value is used to determine the population increase in unlimited environments and a method of calculating the internal rate of increase was based on the survival and productivity rates of females because they are responsible for the increase in population size. The following basic requirements for calculating the internal rate of increase were determined:

- Survivorship from birth to age-class x is denoted l_x . (l for live)

$$l_x = N_x/N_0 \text{ (N for number)}$$

This is the likelihood of living to a given age. l_x decreases continually through age classes, but this does not mean that old animals are more likely to die than young animals.

- Fecundity Schedule.

$$m_x = 1/2 \text{ number of offspring born to a parent of age } x.$$

For each offspring produced, male and female parents are each credited with 1/2 of an offspring produced. To see the logic of this, remember that in the sexual organism, each individual must leave two offspring for an exact replacement. In practice, m_x is usually measured as female offspring per female of age x (m for maternity). This is because paternity is usually unknown, so numbers of offspring per male cannot be measured. In some cases, male reproduction is known, and m_x is measured as 1/2 of total offspring for each parent.

These requirements are placed in tables called fecundity tables. The total number of individuals resulting in the time unit (m_x) represents the gross reproducible rate (GRR), ($GRR = \sum m_x$), which is the total lifetime reproduction in the absence of mortality. This is the average lifetime reproduction of an individual that lives to senescence and is useful in considering potential population growth if all ecological limits (predation, competitors, disease, starvation) were removed for a population. GRR is rarely if ever attained in nature, but it is useful to consider how far below this a population is held by ecological limits.

Price (1997) states that population growth depends on the number of females and their production of offspring. This is expressed in the expected productivity, and that the expected productivity during all stages represents the net reproductive rate, which is R_0 (Net reproductive rate = $\sum l_x m_x$) which is the average number of offspring produced by an individual in its lifetime, taking normal mortality into account. l_x is the odds of living to age x , m_x is the average of offspring produced at that age, so the product $l_x m_x$ is the average number of offspring produced by individuals of age x . Summed across all ages, this is average lifetime reproduction. R_0 is also called the replacement rate:

$R_0 < 1$ individuals not fully replacing themselves, population shrinking

$R_0 = 1$ individual exactly replacing themselves, population size stable

$R_0 > 1$ individuals more than replacing themselves, population growing

Krebs (1999) suggests that the age-specific fecundity tables can be used to calculate the mean generation time (T_0) and to calculate the internal growth rate (r_m), which is the basic function of the expression of insect populations because it illustrates the relationship between productivity, generation duration and survival rate.

Semelparous species = big bang breeders, which breed only once in life (e.g. salmon, many insects) T = egg to egg time, or newborn to newborn time (obviously).

$$T_0 = \frac{\sum x l_x m_x}{\sum l_x m_x}$$

To understand this, we have to know about the numerator. $l_x m_x$ is the average number of offspring born to female at age x , as discussed above. If we weight each offspring by the age of the mother, x , and then sum across all ages, we have the mother's age when each offspring was born, summed across all offspring born in her life. The denominator ($\sum l_x m_x$) is equal to the total number of offspring born. Dividing the numerator by the denominator gives the mean age of a female when each of her offspring was born. In other words, if most offspring are produced when mothers are old, generation time will be longer than if most offspring are produced when mothers are young.

R_0 measures reproduction based on individual lifetimes (offspring produced per individual per lifetime), and most models of population growth measure growth based on births - deaths per unit time, where time does not have to be a generation - often years are the units. The most common measure of population growth is the intrinsic rate of increase, r_m .

$$r_m = \ln R_0 / T$$

When: $R_0 = 1$, $r_m = 0$, stable population

$R_0 < 1$, $r_m < 0$, shrinking

$R_0 > 1$, $r_m > 0$, growing

1.2.5. Volatile organic compounds

Volatile organic compounds (VOCs) are compounds of organic chemicals. Their characteristics permit them to evaporate under normal indoor atmospheric conditions of temperature and pressure (Orecchio et al., 2017). Thus, compounds can be transported under suitable conditions areas far from their source of emission. VOCs play a major role in our environment (Dicke and Loreto, 2010). Almost any kind of tissues (Peñuelas and Llusà, 2001; Dudareva et al., 2006), and types of vegetation (trees, shrubs grass, etc.) release green leaf volatiles containing both nitrogen-containing compounds and aromatic compounds (Holopainen et al., 2010; Holopainen and Gershenzon, 2010), or in response to a variety of stimuli. Plant emissions of VOCs play a role in a wide range of ecological functions, and they

are involved in both biotic and abiotic functions (Spinelli et al., 2011; Vivaldo et al., 2017). Plants have developed the ability to use VOCs for many tasks, as outlined below;

- as an indirect defence against insects (Mumm et al., 2003).
- to attract pollinators (Dudareva and Pichersky, 2000).
- for a plant to plant communication (Baldwin et al., 2006; Heil and Karban, 2010).
- for thermotolerance and environmental stress adaptation (Holopainen and Gershenzon, 2010).
- for defence from predators (War et al., 2012).

Animal life requires access to green plants to exist and wherever plants grow, insects are also found. The interactions between plants and insects can be both antagonistic and mutualistic (Schoonhoven et al., 2005). Studies have focused on the antagonistic relationship of insects feeding on plants as well the mutualistic relationship of plant pollination by insects. Plants have evolved several different types of defences against herbivores, and herbivores have evolved different traits to overcome these defences. Plant chemistry is probably the most important factor regulating insect feeding and distribution (Schultz, 1988). The ability of plants to produce toxins that act as a defence against herbivorous insects and the ability of insects to detoxify plant toxins has been described as an evolutionary arms race (Ehrlich and Raven, 1964; Harborne, 2014). As elsewhere in this world, the enemy of an enemy is my friend. Plants have been shown to interact with the enemies of herbivores i.e. the third trophic level. VOCs were noted to be an important factor in interactions with parasitoids of the third trophic level (Price et al., 1980). This area has been extensively studied, and very specific interactions between plants and the natural enemies of herbivores have been shown to be mediated by plant volatiles (Dicke et al., 2003; Turlings and Wäckers, 2004; Wajnberg and Colazza, 2013).

1.2.5.1. Volatile compounds released from the plants

Hundreds of different compounds can be found in the volatile blends released from any plant (Raguso, 2004). Blends can be unique to a plant family, but many are found in different and unrelated plants (Knudsen et al., 1993). Volatile compounds are released by metabolic activities taking place within the plant parts such as fruit, leaves, shoots or flowers. Infested plant parts produce significantly different compound profiles as a result of the physiological condition of the plant as well as the species of the cultivar. Metabolic changes in plants influence the profile of volatile compound released by them. Alterations in the environment,

plant age, stage of development, stress effects and the presence of disease or herbivores have been shown to alter the volatile compound profile. Volatile plant metabolites can be used as an indicator of the disease, stress or herbivore presence. Natural variations within species, however, challenge the use of volatile compound profiles as a true measure of these factors. Thus, it is vital to determine which volatile compounds can act as biomarkers for a plant or herbivore which will be different from compounds produced as a result of environmental or nutrient stress (Sankaran et al., 2010).

The scientific community has shown great interest in using the emission of trace gases by plants for the purposes of scientific research. Suitable understanding of this activity has major implications for plant defences and the response of insects to chemical signals (Laothawornkitkul et al., 2009). Over time many herbivores have developed means of neutralising or evading some of these defences. The laws of natural selection favour plants that developed effective defensive traits to deal with herbivore created damage (Agrawal and Rutter, 1998; Heil et al., 2000; Ness, 2003). Chemical defence has been shown to be one of the most effective strategies used by plants (Mortenson, 2013), and this strategy is useful both at short and long ranges. Secondary plant metabolites such as VOCs are not seen as important to essential metabolic processes. Abundant evidence exists showing that plant emission of VOCs can serve as a plant defence by facilitating multitrophic interactions (Berenbaum and Zangerl, 1996; Rasmann and Agrawal 2009). Defences can be direct (e.g., oviposition deterrence) or indirect (e.g., recruitment of natural enemies) (Kessler and Baldwin, 2001; D'Alessandro and Turlings, 2006; Heil, 2008). When a direct defence is in place, the plant emits chemicals that are targeted at the herbivore and result in retarded growth or death (Onzo et al., 2012). An indirect response results in the production of volatile chemicals (semiochemicals) by the plant in response to the presence of the herbivore (Pare and Tumlinson, 1999). Natural enemies of the attacking herbivores are attracted by the plant's release of volatile chemicals (Fatouros et al., 2005). The three-way relationship between the plant-herbivore and the third trophic level is known as a tritrophic relationship (Hare, 2011). These volatile phytochemicals attract natural enemies of the damaging herbivores and thus increase their mortality (Yu et al., 2008).

1.2.5.2. Volatiles released by citrus fruit

The VOCs detected around wounded, and infested citrus species include the monoterpenes, which include limonene, pinene, sabinene and myrcene (Droby et al., 2008). Monoterpene hydrocarbons are normally the most abundant volatile compounds in all volatiles regardless of the source conditions (Droby et al., 2008). The most abundant volatile compound in citrus,

limonene, was shown to increase from 34% to 95% of total emissions as the fruit ripened (Flamini and Cioni, 2010; Al-Khshemawee et al., 2017). While the volatile profile of citrus fruit varies between species, but this is not necessarily the same between cultivars within a species (Birla et al., 2005). The cited concentration range of the 17 most predominant volatiles associated with *Citrus sinensis* fruit from both Navel and Valencia cultivars are acetaldehyde, ethanol, ethyl acetate, hexanal, ethyl butanoate, α -pinene, sabinene, β -myrcene, limonene, β -ocimene, 1-octanol, λ -terpinene, linalool, 1- α -terpineol, decanal, dodecanal and valencene (Birla et al., 2005; Flamini et al., 2007; Droby et al., 2008; Al-Khshemawee et al., 2017). The emission of volatile compounds from healthy ripe grapefruit (*Citrus paradisi* L.) was shown to consist almost exclusively of monoterpene hydrocarbons (Flamini and Cioni, 2010).

1.2.5.3. Herbivore-Induced Plant Volatiles

Plants will usually release small amounts of volatile chemicals which accumulated in storage sites in the leaves and other plant parts (Pare and Tumlinson, 1999). When herbivores damage a plant, it increases its production of volatile compounds (Pare and Tumlinson 1999; Kendra et al., 2011). The chemicals which increased by herbivore feeding is called herbivore-induced plant volatiles (HIPVs). Upon herbivore infestation, the combination of tissue damage and oral elicitors produced by insects, induce a defence signalling cascade in plants. Within seconds, depolarisation of plasma transmembrane potential (V_m) occurs, followed by an increase in the level of cytosolic calcium ions (Ca^{2+}) within minutes (Zebelo and Maffei, 2014). Hydrogen peroxide (H_2O_2) is produced within minutes to hours due to an oxidative burst caused by herbivory (Fürstenberg-Hägg et al. 2013). This is followed by kinases and production of the phytohormones, jasmonic acid and salicylic acid within hours (Fürstenberg-Hägg et al., 2013; Zebelo and Maffei, 2014). Jasmonic acid is usually associated with damage by chewing insects while salicylic acid is usually associated with damage by sucking insects or pathogens, with reports of crosstalk between the pathways of both phytohormones (Fürstenberg-Hägg et al. 2013). Activation of defence genes within hours follow phytohormone production, and metabolic changes, including volatile production, occurs within hours to days (Fürstenberg-Hägg et al., 2013; Zebelo and Maffei, 2014). The composition of plant VOCs can vary depending on several factors including plant species, herbivore species, type and duration of damage, and abiotic stresses (Hilker and Meiners, 2002; Dicke et al., 2009; Morawo and Fadamiro, 2014a,b; Ngumbi and Kloepper, 2016). Most VOCs involved in plant defence are products of the lipoxygenase, shikimic acid and terpenoid pathways (Pichersky and Gershenzon, 2002). Plants constitutively release small amounts of certain compounds which

may attract foraging insects (Morgan and Hare, 1998; Wäckers, 2004). For instance, a few monoterpenes such as d-limonene and β -ocimene, are emitted by an undamaged citrus plant (Lin et al., 2016). The combination of tissue damage caused by feeding and action of elicitors from oral insect secretions induces increased emission of constitutive and newly synthesised volatiles (War et al., 2012; Al-Khshemawee et al., 2017).

1.2.5.4. synthetic herbivore-induced plant volatiles

Many articles revealed the role of herbivore-induced plant volatiles (HIPVs) on natural enemies attraction. However, the role of these compounds in effecting arthropod herbivore population suppression at the field scale is still to be demonstrated. Shimoda et al. (1997) recorded more predatory thrips on sticky cards near spider mite-infested bean plants than on traps near non-infested plants. Bernasconi et al. (2001) trapped more natural enemies near plants damaged and treated with caterpillar regurgitant, than near undamaged, untreated plants. Despite the accumulation of a substantial volume of literature on the biology and chemistry of HIPVs during the past 10–15 years (Pare et al., 1999; Kessler and Baldwin, 2001; Scutareanu et al., 2003; War et al., 2011; Peñaflores and Bento, 2013; Tamiru and Khan, 2017; Gebreziher, 2018), field demonstration of the potential of synthetic HIPVs as beneficial insect attractants did not occur until recently (James, 2003a). Synthetic volatiles have been deployed as lures within crops (Rodriguez-Saona et al., 2011), and although some success has been demonstrated concerning the attraction of arthropod predators and parasitoids (James, 2003b; James and Grasswitz, 2005), other studies show that HIPVs can have repellent effects on natural enemies (Braasch et al., 2012). As a result, various synthetic HIPVs have been tested to manipulate natural enemy behaviour in agroecosystems (Zhu and Park, 2005; Jones et al., 2011; Woods et al., 2011). However, the use of these HIPVs for this purpose has remained controversial because there is the risk of disrupting biological control by confusing the natural enemies instead of helping them during prey or host location, as well as the possibility of increasing ecological risks by unintentionally attracting the herbivores themselves (Kaplan, 2012a). A way to improve the negative effects of HIPVs on biological control is by combining different tactics to conserve natural enemies such as HIPVs and companion plants, in an approach known as ‘attract-and-reward’. Under this scenario, a synthetic HIPV is used to attract natural enemies while a floral resource is used to provide food and thus conserve their populations (Simpson et al., 2011a, 2011b; Gordon et al., 2013). The use of synthetic HIPVs as ‘Herbivore-Induced Plant Protection Odors’ (HIPPOs) has the potential to provide a novel yet practical strategy for improving the efficacy and reliability of conservation biological control in a variety of

agricultural ecosystems (James et al., 2005). The first direct evidence for the potential of synthetic HIPV as field attractants for beneficial insects came from this research group (James 2003a, b) which demonstrated attraction of some insect species and families to methyl salicylate (MeSA) and (Z)-3-hexenyl acetate (HA) in Washington hop yards. Several studies have shown that natural enemies are attracted to HIPVs in agro-ecosystems (e.g., James and Grasswitz, 2005; Lee, 2010; Jones et al., 2011; Rodriguez-Saona et al., 2011; Woods et al., 2011). For example, James (2003b) reported that beneficial insects from the families *Syrphidae*, *Geocoridae*, *Anthocoridae*, and *Miridae* are attracted to MeSA and (Z)-3 hexenyl acetate in hops. Less evidence currently exists in support of the role of natural enemy attraction to HIPVs will lead to reduced pest populations in agricultural systems. For example, in soybean, MeSA increases the attraction of predatory insects, such as members families *Syrphidae* and *Chrysopidae*, and reduces soybean aphid, *Aphis glycines* Matsumura, populations (Mallinger et al., 2011). in cotton, α -farnesene and (Z)-3 hexenyl acetate attracted the parasitoid *Anaphes iole* Girault, and this attraction increased parasitism rate of *Lygus lineolaris* Palisot de Beauvois eggs (Williams et al., 2008).

1.2.5.4.1. D-limonene

D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a monocyclic monoterpene (Figure 1.3.) with a lemon-like odour and is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). D-limonene is the primary bioactive food components (BAFC) of citrus peel oil comprising 75% of lemon peel oil, 95% of orange peel oil, and 87% of mandarin peel oil (Sun, 2007).

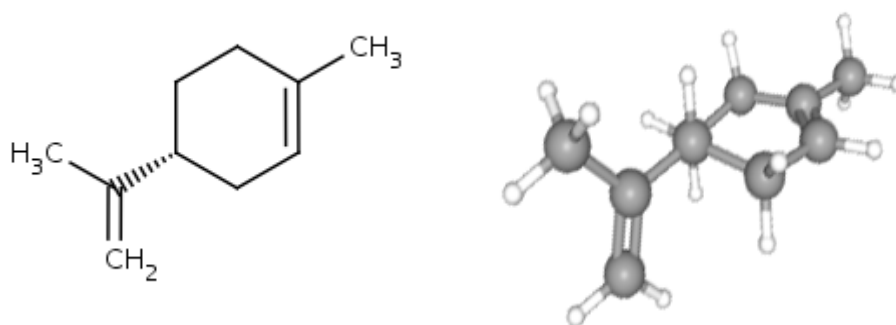


Figure 1.3. D-limonene

Because of its pleasant citrus fragrance, d-limonene is considered as a GRAS (Generally Recognized as Safe) material by the U.S. Food and Drug Administration and plays an important role in flavours and fragrances as well as acts as a cleaning/degreasing agent in industrial and in household applications (Mira et al., 1999; Aissou et al., 2017). It is also used in the manufacture of resins, as a wetting and dispersing agent and in insect control (International Agency for Research on Cancer, 1993; Budavari, 1996). D-limonene is found in healthy fruit but is quantitatively elevated by insect herbivory or mechanical injury of the fruit peel (Kendra et al., 2011; Chamberlain et al., 2012; van der Walt, 2012; Lin et al., 2016). D-limonene is one of most significant emitted volatiles of citrus and was previously identified to attract natural enemies, and its production is elevated due to the feeding injury on citrus fruit by pests, which consequently attracts predators and parasitoids to their prey or host on fruit enemies (Wei et al., 2013; Dias et al., 2014; Song et al., 2017). Therefore, it could further be suggested that production of d-limonene in citrus is part of the indirect defence strategy against pests infestation by attracting its natural enemies. Thus d-limonene plays a key role as long-range attractants natural enemies to infested fruit. d-limonene is only a long-range attractant for parasitoids, and those female parasitoids require an extra cue(s) to finally locate their hosts on fruit (Zimba, 2014).

1.2.5.4.2. β -ocimene

β -ocimene (3,7-dimethyl-1,3,6-octatriene) is a monoterpene and it has two stereoisomers, cis- and trans- β -ocimene (or (Z)- and (E)- β -ocimene, respectively), which are the cis and trans forms of the central double bond (Figure 1.4.). β -ocimene is a very common plant VOC released in large amounts from the leaves, fruit and flowers of many plant species (Farré-Armengol et al., 2017; Hosni et al., 2010; Chamberlain et al., 2012). This acyclic monoterpene can play several biological functions in plants, by potentially affecting floral visitors and also by mediating defensive responses to herbivory (Farré-Armengol et al., 2017). β -ocimene has a medium strength, tropical, green and woody odour with vegetable nuances.

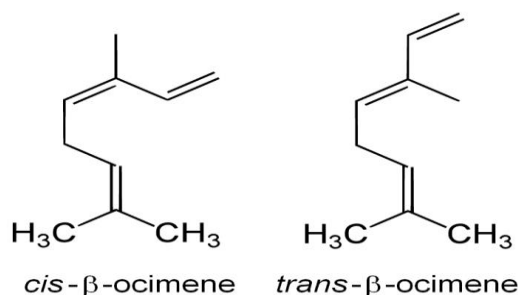


Figure 1.4. β-ocimene

β-ocimene is not only abundantly emitted by plant reproductive structures but is also a common VOC emitted from plant tissues (Hansen and Seufert, 1999; Wang et al., 2007). Phytophagous insects can identify the VOC blends that are constitutively emitted by the plants in the community, including β-ocimene, and use them as chemical cues to identify their host plants (Røsteliën et al., 2000; Kariyat et al., 2013). β-ocimene also plays important defensive roles in plant tissues by mediating tritrophic interactions with parasitoids and predators of herbivores (Farré-Armengol et al., 2017). Herbivore-infested plants induce increased emissions of VOCs such as β-ocimene from damaged and undamaged tissues in a systemic defensive response (Miresmailli et al., 2010; Miresmailli et al., 2012; Copolovici et al., 2014; Kendra et al. 2011; Lin et al., 2016). Parasitoids and predators of herbivores are attracted to the VOCs emitted from infested plants, which in most cases include important proportions of β-ocimene, that indirectly help plants to cope with herbivorous attacks in a tritrophic interaction (De Moraes et al., 1998; De Moraes et al., 2001). The stronger production and emission of β-ocimene from herbivore-attacked citrus fruit compared to undamaged fruit suggests that this compound plays an active role in indirect insect defences. Therefore, it could further be suggested that production β-ocimene in citrus is part of the indirect defence strategy against pests infestation by attracting its natural enemies. Thus β-ocimene play a key role as long-range attractants of natural enemies to infested fruit.

1.2.6. Host search in parasitoids

A crucial factor of female parasitoids in host interactions is to choose host individuals having different qualities that qualify them to be desirable individuals in the process of parasitism. The parasitoids reproductive success is dependent on the ability of the female to locate its host by exploiting a variety of physical and chemical cues associated with their hosts (Hare, 2011). It is well known that host plant and herbivore cues can guide female parasitoids to their hosts (Paré and Tumlinson, 1999; Canale and Benelli, 2011; Goubert et al., 2013). Olfactory cues

play a major role in parasitoid behavioural ecology (Dicke and Loon, 2000). They are used as both pre-alighting cues, involved in host habitat location and the selection of suitable host plants; and as post-alighting cues, involved in searching for and parasitising the host insect (Eben et al., 2000; Rousse et al., 2007; Segura et al., 2012). The use of olfactory cues by foraging parasitoids can thus be thought of as influencing behaviour in four sequential steps: finding the habitat, host location within the habitat, host acceptance, and assessing host suitability for offspring survival (Vinson, 1976; Steidle and van Loon, 2003; Chesnais et al., 2015). Herbivores can acquire plant chemicals in their diet (Despres et al., 2007). When herbivores emit certain plant-associated compounds, they can serve as kairomones used by parasitoids to locate their hosts. These kairomones may originate from the body, frass or even trails left by the host (Alborn et al., 1995; Chuche et al., 2006; de Rijk et al., 2016). Arguably, plants have larger biomass and produce more abundant volatiles than herbivorous insects (Turlings et al., 1995). Thus, due to the high levels of biomass in the plant as compared to herbivorous insects, the plant produces far higher amounts of volatiles that are believed to play a key role as long-range cues in the orientation of parasitoids during host searching. Conversely, host cues, i.e. odour of oral secretions and excreta, including feeding vibrations or sounds are believed to act as close-range cues in host finding (Paré and Tumlinson, 1999). However, once parasitoids find host plant patch and make an appropriate landing on a plant, herbivore host-specific odours become critical short-range cues in the later phase of host location process (Afsheen et al., 2008; de Rijk et al., 2013; Colazza et al., 2014).

The parasitoid's foraging environment is described as highly complex (Lewis et al., 1998), due to a myriad of other volatiles released by the many herbivorous insects, host plant and dead organic matter (Beyaert et al., 2010). Parasitoids in tritrophic systems often use a combination of odour cues from both lower trophic levels for host location. Many species are attracted to green leaf volatiles released due to feeding activity of their host herbivore but do not show the same response to volatiles of mechanically damaged plants (Dicke et al., 1990; Takabayashi et al., 1998; Du et al., 1998). In parasitoids, host foraging begins with an active search of host habitat and the host, a selective process that is mostly mediated by odour cues from plants. However, interferences from another olfactory, visual and acoustic stimulus exist in natural environments. For effective host location, parasitoids must develop strategies to use the most reliable cues available during the time of the day that they are most active (Turlings et al., 2005). Once in the micro-habitat of the host, parasitoids rely on other host-specific chemicals and visual cues for recognition and acceptance of hosts. Parasitoids have been shown to

overcome the difficulties of complex foraging environments by learning cues associated with their hosts (Paré and Tumlinson, 1999; Yu et al., 2008). The parasitoids host location is assisted by odour specificity usually provided by recognising the specific blending ratios of volatile chemicals released by each plant. (Paré and Tumlinson, 1999). In addition, herbivores produced volatiles provide information about host quality and age i.e. whether the parasitoid is parasitised or not (Fatouros et al., 2005; Chu et al., 2014).

An important attribute is their ability to display learning to associate VOCs with their desired host. Parasitoids can cope with a variable foraging environment (Van Alphen et al., 2003). Most parasitoids have demonstrated the ability to learn a variety of cues; however, others can be only able to learn a few specific signals (Mumm and Hilker, 2005). Thus, parasitoids are broadly divided as specialists (utilising one or relatively few host species) or generalists (utilising several host species). Generalist parasitoids show the ability to adapt to a higher variation of cues from their polyphagous hosts. They are thus believed to have higher abilities to learn the odours associated with their various hosts. The level is seen as using their innate abilities (Vet and Dicke, 1992; Ngumbi et al., 2012). In contrast, specialist parasitoids show lower learning abilities and depend on innate abilities to perceive their host-related cues (Geervliet et al., 1998). Variation in cue perception and learning abilities in parasitoids is believed to be among other factors influenced by phenotypic and genotypic factors (Vet and Dicke, 1992). Gu and Dorn (2000) demonstrated that the ability of parasitoids to perceive cues related to their hosts is heritable. Further, cue perception in parasitoids is also influenced by biotic factors, i.e. age, hunger, reproductive state (Greiner et al., 2002), which affect the physiological state of the insect (Gadenne and Anton, 2000).

Several studies have tested parasitoid attraction to single VOCs to identify the specific compounds responsible for the recruitment of parasitoids (James and Price, 2004; James and Grasswitz, 2005; Wei et al., 2007; Ngumbi et al. 2012). However, there is still an ongoing debate on whether specific single components or the entire natural suite of plant odours elicit complete behavioural responses in parasitoids (van Wijk et al., 2011).

1.2.7. Insect olfaction and olfactory learning in parasitoids

Parasitoids use two organs primarily to detect odours. These are the antennae and specialised mouthparts called the maxillary palps (Li and Liberles, 2015). Inside these olfactory organs, are neurons called olfactory receptor neurons which, as the name implies, house receptors for scent molecules in their cell membrane. Most of the olfactory receptor neurons typically reside

in the antenna. Olfactory receptor neurons (ORNs) are housed within small sensilla. Apart from the ORNs, the olfactory sensilla consist of a number of auxiliary (or enveloping) cells that have supportive functions and are involved in the development of the sensilla during ontogeny. In most insects, trichoid sensillar type (trichoid sensilla are the dominating sensillum type on the antennae of many insect species) is innervated by 2-3 neurons, but in Hymenoptera sensilla trichodea often contain around ten neurons (Hallberg and Hansson, 1999). Another character typical for Hymenoptera is a high abundance of sensilla placodea (Hallberg and Hansson, 1999; Bleeker et al., 2004). Odours access the sensilla through pores in their walls. The odour molecules are transported through the sensillum lymph by water-soluble odourant binding proteins (OBPs). The selectivity of insect odour detection is thought to depend partly on selectivity in these odour-OBP bindings, but mainly on the specificity of receptor sites of the odour receptor neuron (Stengl et al., 1999). Briefly, the odour-OBP complex interacts with receptor proteins in the dendritic membrane of the odour receptor neuron. These interactions give rise to a change in receptor potential, and if the potential is above a threshold, action potentials are triggered in the neuron (Todd and Baker, 1999). After receptor activation, the odour-OBP complex is deactivated and dissolved, followed by degradation of the odour molecule by enzymes in the lymph (Stengl et al., 1999). The action potentials of different odour receptor neurons have different amplitudes and waveforms, which often allow discrimination between recorded neurons within one sensillum (Todd and Baker, 1999). However, it is the frequency of action potentials rather than their size and shape that reflects the strength of the response and affects the behaviour. These action potentials are transmitted in the olfactory axons of the antennal nerves ending in the antennal lobe, where the olfactory signal is processed before being transferred to higher integrative centres of the brain (Todd and Baker, 1999). Insects are capable of smelling and differentiating between thousands of volatile compounds both sensitively and selectively (Carragher et al., 2015; Syed, 2015). Sensitively is how to attune the insect is to very small amounts of an odorant or small changes in the concentration of an odorant. Selectivity refers to the insects' ability to tell one odorant apart from another.

Olfactory learning in parasitoid foraging behaviour has now been demonstrated for many wasps (Meiners et al., 2003; Olson et al., 2003; Schurmann et al., 2009; Ngumbi et al., 2012; Luo et al. 2013; Canale et al., 2014; Frederickx et al., 2014; Wilson and Woods, 2016). Parasitoids have innate preferences for environmental odours (e.g. host-derived stimuli) (Segura et al., 2016; Wilson and Woods, 2016), but their behaviour can be modified by learning from various experiences and storing the learned information (memory) for subsequent use,

and they can use this learned information for faster host finding (Blande et al., 2007; Canale et al., 2014; Giurfa, 2015). Studies have shown that parasitoids have been able to overcome the challenges posed by the complex foraging environment by developing abilities to learn the cues associated with their hosts (Paré and Tumlinson, 1999; Yu et al., 2008).

1.2.8. Volatile isolation techniques

The analysis of volatile compounds by static headspace analysis is widely used (Miller and Stuart, 1999). However, in many analyses, the gas-sampled static headspace method using an airtight syringe lacks the sensitivity required (Miller and Stuart, 1999). Many techniques are used for the qualitative and quantitative analysis of volatile compounds, but the combination of gas chromatography and mass spectrometry is the most efficacious. Techniques that allow enrichment of headspace volatile compounds for analysis can be achieved using solid-phase microextraction (SPME), static headspace (SHS), dynamic headspace (DH), electronic nose (E-nose) and headspace autosampler (AHS). Volatile isolation techniques generally involve two steps: collection the odour from the sample matrix, separation and identification of the volatile compounds (Teranishi, 1998; Wampler, 2001).

1.2.8.1. Solid-phase microextraction (SPME)

The twin disciplines of analytical chemistry are evolving, and the rapid pace of evolution has fostered a demand for new methods to keep up with laboratories' mounting efficiency and sensitivity requirements. One such method that has gained interest in recent years is solid-phase microextraction (SPME), an off-column pre-concentration technique that is adaptable to gas-chromatography mass spectrometry (GC-MS) analysis. In 1990, SPME was developed and used by Arthur and Pawliszyn for quantitative trace analysis of volatile and semi-volatile compounds (Pawliszyn, 1999). The SPME device generally looks like a normal syringe (Figure 1.5). It consists of a plunger, a needle and other components which allow a device to penetrate through the sample chamber and GC septum. Fused silica fibre is a principal part of this tool because the chosen polymer coated on the fibre will work as an adsorbent for the volatile compounds. The thickness of the coating can range from 5 to 100 μm depending on the chosen coating. This still enables the fibre to fit into a needle attached to the SPME device (Martos and Pawliszyn, 1998; Reineccius, 2002; Turner, 2006). This technique utilises a fibre to adsorb analyte molecules from its environs, gathering them and accumulating them on the fibre surface before transferring them to the injection port of the GC. SPME technique allows for good selectivity, as different adsorption fibres can be chosen depending on the target compound to be analysed (Wang, 1997). The thickness and chemistry of the fibre can be tailored to the specific needs of the analyst; SPME fibre coatings have different chemical properties (Table 1.1). With non-polar fibre coatings such as polydimethylsiloxane (PDMS) proving adept at collecting non-polar analytes, and polar coatings such as polyethylene glycol (PEG) showing preference to polar compounds. The most popular SPME fibres are made of a polydimethylsiloxane stationary phase (Wang, 1997). By choosing the correct fibre for the analyte to be collected, the sensitivity of the analysis can be improved (Wang, 1997).

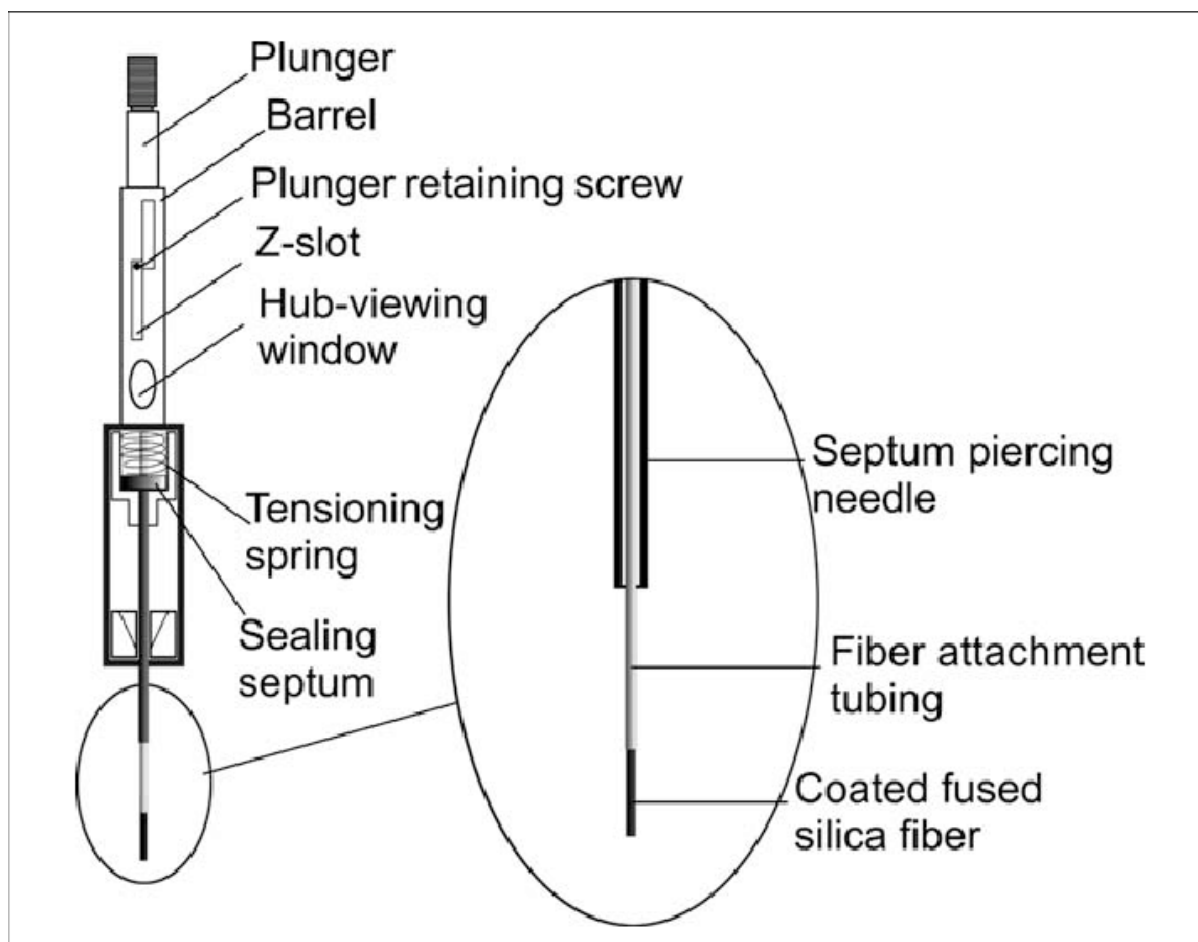


Figure 1.5. Schematic diagram of an SPME device.

Table 1.1. Summary of common commercially-available SPME fibres.

Fibre types	Polarity	Max. Operating Temp (°C)	Target Compounds
Polydimethylsiloxane (PDMS)	Non-Polar	280	Volatiles and nonpolar semivolatiles
PolydimethylsiloxaneDivinylbenzene (PDMS/DVB)	Bipolar	270	Polar volatiles
PDMS-Carboxen (PDMS/CAR)	Bipolar	320	Gases and volatiles
PolydimethylsiloxaneDivinylbenzene Carboxen (PDMS/DVB/CAR)	Bipolar	270	Odours and flavours
CarbowaxDivinylbenzene (CAR/DVB)	Polar	265	Polar analytes (alcohols)

Polyethylene Glycol (PEG)	Polar	250	Polar analytes (alcohols)
Polyacrylate (PA)	Polar	320	Polar semivolatiles (phenols)

The SPME extraction principle is described as an equilibrium process in which the volatile analytes are partitioned between the stationary phase of the fibre and headspace (Wang, 1997). SPME allows solventless extraction by using fused silica or stainless-steel fibre coated with a thin polymer. This acts as a solvent and allows the compounds to be extracted (Pawliszyn, 1999). The analytes absorb to the polymer coating on the SPME fibre, which is mounted onto a syringe like a device (Wang, 1997). SPME is a relatively new technique which is simple and appropriate for heat-sensitive materials (Richter and Schellenberg, 2007). SPME has been described as an isolation method which “extracts, concentrates and provides a means to directly deliver the concentrated and extracted analytes into the interface of GC or HPLC” (Martos and Pawliszyn, 1998). This is because SPME involves the insertion of a coated silica fibre into or above the sample; hence extraction of volatile components by the organic solvent is unnecessary (Richter and Schellenberg, 2007). Moreover, if the fibre is applied to the headspace above the sample, concentrated volatiles can be readily obtained without interferences from food matrices and other non-volatile compounds. SPME can combine extraction, concentration and introduction of volatile analytes in one step which reduces preparation time and, in some cases, this fact increases sensitivity over other extraction techniques (Pawliszyn, 1999). Therefore, SPME is claimed as a rapid method compared to distillation since a few steps can be omitted. It follows that the main advantage of SPME over other techniques is that the commercially available SPME devices are convenient and user-friendly. Among the myriad applications that have been published on to date, the technique has found use for the assay of samples as diverse as foods (de Fatima Alpendurada, 2000; Xu et al., 2016), drugs (Kataoka, 2003; Lord and Bojko, 2012; Pragst and Balikova, 2006), environmental pollutants (de Fatima Alpendurada, 2000; Shoeib and Harner, 2002; Stiles et al., 2008), and many other samples.

SPME sampling can be performed in three basic modes: direct extraction, headspace extraction and extraction with membrane protection (Jinno et al., 2007). Direct extraction is a technique

in which the fibre is directly applied to the sample, and the analytes are then transferred straight from the sample matrix to the coating. In contrast, headspace techniques involve adsorption of the analytes in the headspace above the sample. This is to prevent damage of fibre from high molecular-weight and other non-volatile interferences present in the sample. For the membrane protection approach, membrane serves as the barrier to prevent the fibre from damage and some contaminants, but it still allows the analytes to be adsorbed directly onto the fibre (Figure 1.6.).

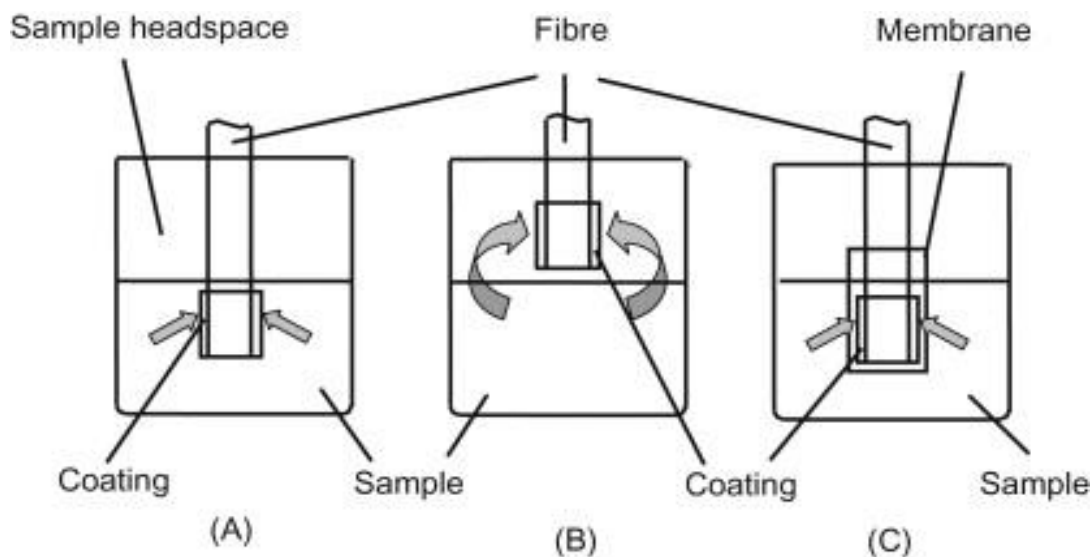


Figure 1.6. Mode of SPME operation A) Direct extraction, B) Headspace configuration, C) Membrane protection approach (Jinno et al., 2007)

Among these three modes, the headspace technique is probably the most widely applied in many studies since sample degradation can be avoided and concentrated analytes can be collected faster. This is because the non-volatile and semi-volatile compounds cannot interfere with the extraction. This eliminates the need for liquid-liquid extraction, solid-phase extraction, and many of the other arduous, time-consuming sample preparation procedures that are endemic to traditional analysis methods. As a result, many types of samples can be run immediately, with sensitivity equal to or greater than what liquid injection would confer, and—due to SPME’s ability to bypass the sources of error that tend to crop up in multi-step workups—often with superior accuracy (Pawliszyn, 1997; Vas and Vekey, 2004). In contrast, membrane protection is a time-consuming process as the analytes have to travel through the membrane before reaching the fibre. However, the membrane can be modified to improve selectivity and also it can be used to extract the low molecular-weight volatile components (Pawliszyn, 1997).

SPME makes use of a very small volume of sorptive phase. Absorption of volatiles onto the fibre is allowed to proceed until the rate of adsorption is equal to the rate of release. Heat can change this equilibrium to allow the concentrated volatiles to be released; therefore thermal desorption of the enriched material is required (Burger et al., 2006). The aroma isolation

performed by SPME headspace technique can be started by placing the coated fibre in the space above the food sample and allowing to adsorb the volatile compounds for a certain time. Subsequently, the SPME needle is removed from the headspace, and then it is directly injected to the GC. Once the fibre is placed in the GC inlet, heating causes the volatile compounds adsorbed by the fibre to be released into the GC column. Finally, they will be further separated and characterised by GC or GC-MS (Martos and Pawliszyn, 1998; Reineccius, 2002; Turner, 2006). The principle of the SPME method is simply described by the schematic diagram shown in Figure 1.7. SPME does not require cryofocusing of volatiles onto the column, and thermal desorption occurs almost immediately after injection of the sample into the chromatography system, which eliminates the requirement for a thermal desorption system (Burger et al., 2011).

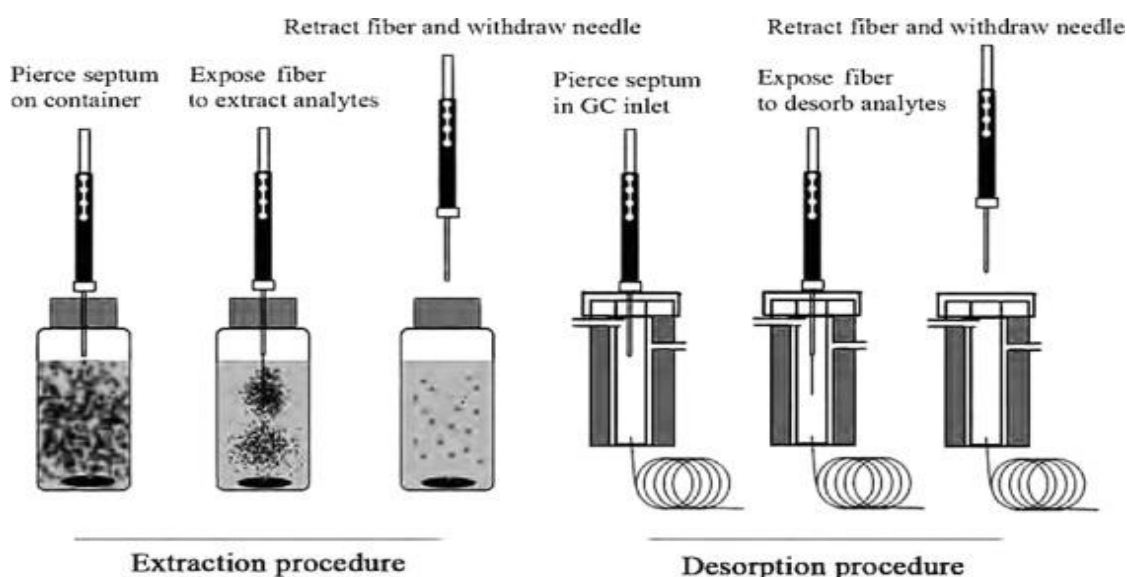


Figure 1.7. Principle of SPME A) Extraction procedure, B) Desorption procedure (Reineccius, 2002)

1.2.8.2. Identification of volatile mixtures

The identification of volatile compounds is performed after they have been isolated from food matrices. Gas chromatography (GC) is a conventional method and most widely used instrument in the aroma studies, particularly those which employ mass spectrometry (MS) as the detector.

Modern gas chromatography (GC) was invented by Martin and James in 1952 and has become one of the most important and widely applied analytical techniques in modern chemistry (James and Martin, 1952; Teranishi, 1998). In general, chromatography is used to separate mixtures

of chemicals into individual components (Reineccius, 2002). Principle of GC-MS combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyse complex organic and biochemical mixtures (Skoog et al., 2007). The GC-MS instrument consists of two main components. The gas chromatography portion separates different compounds in the sample into pulses of pure chemicals based on their volatility (Oregon State University, 2012) by flowing an inert gas (mobile phase), which carries the sample, through a stationary phase fixed in the column (Skoog et al., 2007). Spectra of compounds are collected as they exit a chromatographic column by the mass spectrometer, which identifies and quantifies the chemicals according to their mass-to-charge ratio (m/z). These spectra can then be stored on the computer and analysed (Oregon State University, 2012).

The process performed by GC-MS begins by injecting the mixture into the carrier gas of the GC part. Then, the carrier gas and the sample are introduced continuously to the column. The compounds that are less likely to be adsorbed by column will pass down first while the remainder will be retained and eluted later (Gudzinowicz et al., 1976). After separation by GC, the identification is carried out in the MS part. The main principle of MS is to measure mass to charge ratio of gas-phase ions. When the sample molecules enter into the MS ion source, they are all transformed into ionised fragments. Then, they are allowed to travel through magnetic or electrical fields in a low-pressure environment. After that, the interaction between the field and ions causes separation of the charged particles. It is expressed as ion signals and captured by the detector. The mass spectrum is the result generated from this measurement, demonstrating the relative abundance of the molecular ions and its fragment ions against the mass to charge ratio. From the mass spectrum, the different compounds generally demonstrate ion signals differently, and therefore this property is used for quantitation and identification purposes (Reiner and Clement, 1990; Kitson et al., 1996). With the integration of GC and MS, the identification can be carried out by automatically matching the mass spectra of a reference library to the mass spectra of the sample components (Babushok et al., 2007). Then, the retention time and its identity of the compounds are readily available. Figure 1.8. shows the basic components of GC-MS. GC comprises of injector port, oven column and interface which is used to remove the carrier gas from the sample, so the flow rate is in a range that mass spectrometer can tolerate. For MS, there are three major components; ion source, mass analyser and detector. GC-MS different parts and their functions are discussed below (Bartle and Myers, 2002) (Figure 1.8.).

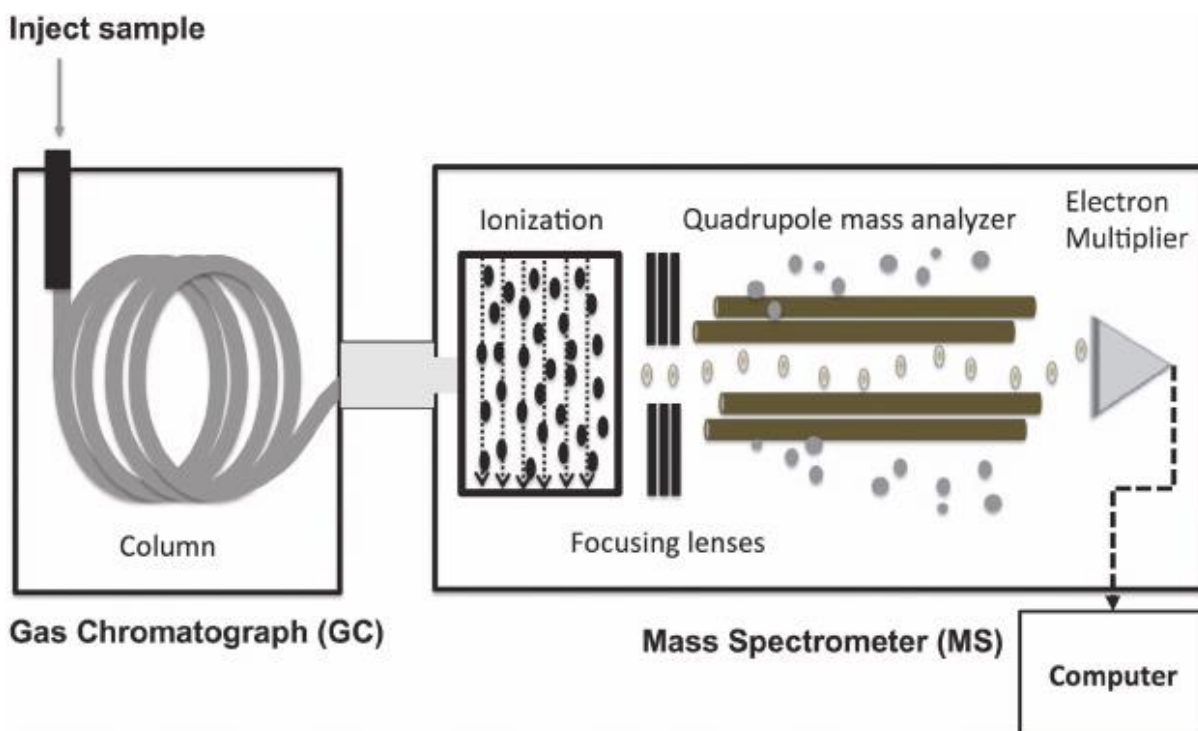


Figure 1.8. Schematic diagram of GC-MS ([Il-Young Kim, 2016](#)).

1.2.8.2.1. Gas supply

The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependent upon the type of detector which is used. The carrier gas nowadays is helium or hydrogen, passes from a cylinder through a pressure- or flow-rate-controlling device to the sample injector at the column inlet. It is usual to purify the gases to ensure high gas purity and gas supply pressure (Gas Supply and Pressure Control from theory and Instrumentation of GC-GC Channel). The carrier gas system also contains a molecular sieve to remove water and other impurities.

1.2.8.2.2. Injector

Here the sample is volatilised, and the resulting gas entrained into the carrier stream entering the GC column (Sampling Techniques and Sample Introduction from Theory and Instrumentation of GC-GC Channel).

1.2.8.2.3. Column

The column is at the centre of the analytical gas chromatography; the quality of the separation achieved by the whole system can be that of the column only. Gas Chromatography uses a gaseous mobile phase to transport sample components through columns either packed with coated silica particles or hollow capillary columns containing, the stationary phase coated onto

the inner wall. Capillary GC columns are usually several meters long (10-120 m is typical) with an internal diameter of 0.10-0.50 mm, while packed GC columns tend to be 1-5 meters in length with either 2 or 4 mm internal diameter (GC columns from Theory and Instrumentation of GC) (Hussain and Maqbool, 2014). Capillary column independently realised in 1957 by Golay (Golay, 1958). In a capillary column, the stationary phase is coated on the inner wall, either as a thin film (wall-coated open tubular) or impregnated into a porous layer on the inner (porous layer or support coated open tubular), and a single channel replaces the different paths taken by solute molecules as they pass through the (inevitably) non-uniform packing (i.e. a bundle of capillaries). Capillary columns work at a lower temperature and give much better separation in equal times, or the same separation in shorter time Compared to other column types (Table 1.2.).

Table 1.2. Comparison of a wall-coated capillary, support-coated open tubular, and packed columns.

Features	Wall-coated capillary	Support-coated open tubular	Packed
Length (m)	10–100	10–50	1–5
Internal diameter (mm)	0.1–0.8	0.5–0.8	2–4
Liquid film thickness (µm)	0.1–1	0.8–2	10
Capacity per peak (ng)	<100	50–300	10,000
Resolution	High	Moderate	Low

Several column materials were employed in the early history of capillary GC: copper, nickel, stainless steel, and even nylon tubing, but the relative inertness and transparency of glass columns were quickly recognised as advantageous by their fragility and activity towards highly polar analytes. These problems were, to a large extent, solved by the invention of fused-silica columns in 1979 by Dandeneau and Zerenner (1979). Such columns, manufactured by a process originally based on fibre-optic technology, are highly flexible, durable and chemically inert. Externally coated with a protective layer of polyimide and with an immobilised film of one of a wide variety of stationary phases, fused-silica columns provide the means of separation of almost all mixtures to be analysed; the few exceptions, such as permanent gases and low molecular-weight compounds, are separated on porous layer open-tubular columns with, for example, alumina-based stationary phases (Ettre and Purcell, 1974).

With the advent of capillary columns, greater precision was required in the pneumatic control systems. Control of the gases required to run a GC has been through a combination of on-off valves, forward and back-pressure regulators, needle valves and mass-flow-control regulators. These have evolved together with the instrumentation, such that today we see total feedback controls to maintain constant flow rates of the carrier gases by monitoring the gas pressures and flow rates that, in turn, control electronic regulators. Much of the flow regime in capillary instrumentation is closely associated with the requirements of sample injection, and this is now possible through the automatic computer control of the pneumatics. As the instruments have evolved, we have seen a trend towards the use of keypads, either on the instrument or on a separate keypad, to set the conditions of the instrument. With the advent of the modern PC, control tended to move to control and data-acquisition programs on the PC. Now, it is possible with the automatic flow-control modules to function under mass control or pressure control of the carrier gas. This allows a choice of constant pressure, constant flow or even pressure programming.

Gas chromatography has ovens that are temperature programmable, the temperature of the gas chromatographic ovens typically ranges from 5°C to 400°C but can go as low as -25°C with cryogenic cooling (GC Temperature Programming from The Theory and Instrumentation of GC) (Hussain and Maqbool, 2014). For precise work, column temperature must be controlled to within tenths of a degree. The optimum column temperature is dependent upon the boiling point of the sample. As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes. Minimal temperatures give good resolution but increase elution times. If a sample has a wide boiling range, then temperature programming can be useful. The column temperature is increased (either continuously or in steps) as separation proceeds.

1.2.8.2.4. Detection in Gas Chromatography

There are many detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A non-selective detector responds to all compounds except the carrier gas, a selective detector responds to a range of compounds with a common physical or chemical property, and a specific detector responds to a single chemical compound. Detectors can also be grouped into concentration-dependent detectors and mass flow-dependent detectors. The signal from a concentration-dependent detector is related to the concentration of solute in the detector and does not usually destroy the sample. Dilution of with make-up gas will lower the response of the detector. Mass flow-dependent detectors usually

destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependant detector is unaffected by make-up gas. Table 1.3. shows the tabular summary of common GC detectors. An automated titration system generated the first gas chromatograms. But, in 1954, Ray used the temperature (and hence electrical resistance) change of a filament of a thermal conductivity-measuring device, a katharometer, as a means of detection (Ray, 1954). The katharometer remained popular for packed-column work because of its response to most analytes, but the requirement of trace analysis and the development of the capillary column quickly resulted in a new emphasis. Rather than use bulk properties (based, e.g. on gas density, flow-impedance, and gravimetry - a sensitive version of the latter was proposed by Martin (Bartle and Myers, 2002) in 1962 as potentially the “ideal detector for GC”), more sensitive ionisation-based detectors were investigated.

Table 1.3. Common GC detectors

Detector	Type	Support gases	Selectivity	Detectability	Dynamic range
Flame ionisation (FID)	Mass flow	Hydrogen and air	Most organic cpds.	100 pg	10 ⁷
Thermal conductivity (TCD)	Concentration	Reference	Universal	1 ng	10 ⁷
Electron capture (ECD)	Concentration	Make-up	Halides, nitrates, nitriles, peroxides, anhydrides, organometallics	50 fg	10 ⁵
Nitrogen-phosphorus	Mass flow	Hydrogen and air	Nitrogen, phosphorus	10 pg	10 ⁶
Flame photometric (FPD)	Mass flow	Hydrogen and air possibly oxygen	Sulphur, phosphorus, tin, boron, arsenic, germanium, selenium, chromium	100 pg	10 ³
Photo-ionization (PID)	Concentration	Make-up	Aliphatics, aromatics, ketones, esters, aldehydes, amines,	2 pg	10 ⁷

			heterocyclics, organosulphurs, some organometallics
Hall electrolytic conductivity	Mass flow	Hydrogen, oxygen	Halide, nitrogen, nitrosamine, sulphur

1.2.8.2.4.1. The flame ionisation detector (FID)

The flame ionisation detector passes sample and carrier gas from the column through a hydrogen-air flame. The hydrogen-air flame alone creates few ions, but when an organic compound is burned, there is an increase in ions produced. In a flame ionisation detector (FID), electrodes are placed adjacent to a flame fueled by hydrogen/air near the exit of the column, and when carbon-containing compounds exit the column, they are pyrolysed by the flame (Harris, 1999; Higson, 2004). This detector works only for organic / hydrocarbon containing compounds due to the ability of the carbons to form cations and electrons upon pyrolysis which generates a current between the electrodes (Harris, 1999; Higson, 2004). The increase in current is translated and appears as a peak in a chromatogram. FIDs have low detection limits (a few picograms per second), but they are unable to generate ions from carbonyl-containing carbons (Harris, 1999). FID compatible carrier gasses include helium, hydrogen, nitrogen, and argon (Harris, 1999; Higson, 2004). The FID rapidly became, and has remained, extremely popular, overtaking a number of other ionisation detectors proposed at the same time (Ettre and Zlatkis, 1967). Its low cost, unsurpassed linearity, linear dynamic range and sensitivity for carbon-containing compounds still make it the universal detector of choice.

1.2.8.2.4.2. Mass Spectroscopy (MS)

Mass Spectroscopy (MS), also called GC-MS. Mass Spectroscopy (MS) is highly effective and sensitive, even in a small quantity of sample. This detector can be used to identify the analytes in chromatograms by their mass spectrum (Skoog et al., 2013). It was realised early that the structural information and selectivity available from mass spectrometry (MS) made the combination of MS with GC the most effective technique for the analysis of complex mixtures. The separation of the phase ions is achieved within the mass spectrometer using electrical and magnetic fields to differentiate ions. In the ion source, the products are ionised before analysis in the mass spectrometer. There are several very popular types of mass analyser associated with routine GC-MS analysis, and all differ in the fundamental way in which they separate species

on a mass-to-charge basis. Mass analysers require high levels of vacuum to operate predictably and efficiently. The ion beam that emerges from the mass analyser has to be detected and transformed into a usable signal. The detector is an important element of the mass spectrometer that generates a signal from incident ions by either generating secondary electrons, which are further amplified, or by inducing a current (generated by moving charges). Modern instruments will also allow controlling MS parameters from a computer by using specially designed software. The mobile-phase called as carrier gas must be chemically inert. The helium gas is most commonly used; however, argon, nitrogen, and hydrogen are also used.

As the individual compounds elute from the GC column, they enter the electron ionisation (mass spectrometry) detector. There, they are bombarded with a stream of electrons, causing them to break apart into fragments. These fragments can be large or small pieces of the original molecules. The fragments are charged ions with a certain mass. The mass of the fragment divided by the charge is called molecular weight (or mass-to-charge, m/z , ratio). Since most fragments have a charge of +1, the M/Z usually represents the molecular weight of the fragment. A group of 4 electromagnets (called a quadrupole, focuses each of the fragments through a slit and into the detector. The quadrupoles are programmed by the computer to direct only certain M/Z fragments through the slit, while the rest bounce away.

The computer has the quadrupoles cycle through different M/Z 's one at a time until a range of M/Z 's is covered. This occurs many times per second. Each cycle of ranges is referred to as a scan. The computer records a graph for each scan. The x-axis represents the M/Z ratios. The y-axis represents the signal intensity (abundance) for each of the fragments detected during the scan. This graph is referred to as a mass spectrum. The data is then sent to a computer to be displayed and analysed. The computer linked to the GCMS has a library of samples to help in analysing this data (Agilent Technologies, 2012). Data for the GC-MS is displayed in several ways. One is a total-ion chromatogram, which sums the total ion abundances in each spectrum and plots them as a function of time. Another is the mass spectrum at a particular time in the chromatogram to identify the particular component that was eluted at that time. A mass spectrum of selected ions with a specific mass to charge ratio, called a mass chromatogram, can also be used. The great advantages of time-of-flight MS lie in the possibilities for accurate mass measurement (in contrast to the unit m/z resolution of “bench-top” MS), which allow the molecular formulae of ions to be determined, and rapid rates of accumulation of spectra (up to 500 Hz), which allow GC peaks with widths as narrow as 12 ms to be identified (Van Deursen et al., 2000).

1.2.9. Olfactometer apparatuses

Resource searching in parasitoids involves the exploitation of a wide range of stimuli such as visual, chemical and vibratory cues (Benelli et al., 2013). Olfactory cues play a major role in parasitoid behavioural ecology (Dicke and Loon, 2000). Parasitoids possess a relatively efficient olfactory mechanism and have been considered good models for insect olfaction studies (Meiners et al., 2002; Rains et al., 2004; Harris et al. 2012; Ngumbi et al., 2012). Olfaction is, however, the main modality used by parasitoids in the host location (Thompson, 1999). Many studies have demonstrated that plant feeding by herbivorous insects induces production of volatiles that consequently attracts parasitoids (Hare, 2011; Kaplan, 2012b; Benelli et al., 2013). VOCs emitted here are a combination of cues from the host plant and the herbivore and its products (frass, regurgitate or silk) (Hare, 2011; Kessler et al., 2001). Furthermore, many plants are known to have evolved induced resistance in which herbivore feeding activates the synthesis of HIPVs that aid parasitoids to locate their specific hosts (Gols et al., 2011; Hare, 2011). Olfactometer experiments are commonly used in chemical ecology research to study how arthropods locate their hosts (Bruce et al., 2005; Heil, 2004; Natale et al., 2003). In particular, the host searching behaviour of parasitoid wasps, which lay their eggs in or on insect herbivores, has been studied in great detail (Vet et al., 1995). It has been shown that such parasitoids use herbivore-induced volatile organic compounds (HI-VOCs) emitted by plants under herbivore attack to localise hosts for oviposition (Mumm and Dicke, 2010). To overcome the problem of unspecific cues often provided by HI-VOCs, as an example, many parasitoids are capable of olfactory learning, which enables them to link host presence to specific odours and thereby increase the chances to distinguish reliable plant signals from unreliable ones (Vet et al., 1995; Meiners et al., 2003). Therefore, parasitoids can read the information that is essential to them. For herbivorous insects, in particular, information regarding the quality of the plant as a food source or oviposition site is critical (Clark et al., 2011; Johnson et al., 2006).

Studying the response of insects to phytochemicals necessitates the use of appropriate tools to quantify insect behaviour (Stelinski and Tiwari, 2013). Olfactometers are widely used to study relative attraction or repellence of phytochemicals to insects, but the data collected are generally restricted to noncontact of the insect with the source of the odour (Isman, 2002). Many scientists use the term "olfactometer" to refer to a device used to study insect behaviour in the presence of an olfactory stimulus. It consists of a tube with a bifurcation (with "T" or "Y" shape) where an insect walks and decides between two choices, usually clean air versus

air carrying an odour. This is why this device is also called dual choice olfactometer (Beavers et al., 1982; Otálora-Luna et al., 2009). The Y- or T-tube olfactometer bioassays are among the techniques that have been in use for decades (Rotheray, 1981; Wei and Kang, 2006; Ngumbi et al., 2012). They typically allow for comparing the response of parasitoids to a test odour from one arm and control from the other arm. However, testing the preference of parasitoids between two or more treatment odours require the use of multi-choice olfactometers. Four and six-choice olfactometer (Patterson, 1970; Turlings et al., 2004) are commonly used in preference tests. The four or six arms olfactometer provides a neutral central zone which is surrounded by four or six very distinct odour boundaries which the test insects can enter, sample the odour and then either stay or leave and move into another area of the apparatus, like the four or six odours can be offered to test insects at the same time (Patterson, 1970; Turlings et al., 2004). To avoid mixing up of odours, the air is sucked out of the system at a flow rate equal to or greater than the sum of inlet flows. Discrete choices made by parasitoids are often recorded as counts or proportions.

In a Y- or T-tube olfactometer, the probability that an insect ends up in one of the arms by chance is 50 percent. The probability of this potential error is reduced to 25 percent in a four-choice olfactometer (Vet et al., 1983). Davison and Ricard (2011) recently reviewed various models for analysing data generated from olfactometer bioassays. Notwithstanding, the choice of olfactometer type to be used should depend on the objectives of the study. There is a continued need to develop laboratory monitoring assays to evaluate insect behaviour in a manner that is efficient, reliable, and ecologically relevant. Allowing insect test subjects to have physical contact with the plant material to feed and oviposit affords the concurrent collection of data on additional parameters related to behaviour and may increase the ecological relevance of the study. This technique also combines the benefits of a controlled laboratory setting with the increased ecological relevance afforded by allowing the insect to have physical contact with the leaf sample to feed and freely oviposit (Coffey et al., 2016).

1.2.10. Research Objectives

The ultimate objective of this study was to investigate the potential of using synthetic volatiles d-limonene and β -ocimene in attracting the parasitoid *A. melinus*. The process involved the assessment of the female parasitoid innate or conditioned behavioural responses in the presence of *Ao. aurantii*-infested citrus fruit. However, several objectives were to be met in order to realise the ultimate objective.

Firstly, to better manage *Aonidiella aurantii*, it is crucial to understand its occurrence and population dynamics under a range of temperatures. The development of an age-specific life table for an *Ao. aurantii* and the parasitoid *Aphytis melinus* were investigated on butternut squash at three different temperatures so that the rate of development of the insect, age-specific mortality, survival of the original population with time, and age-specific fecundity could be determined (Chapter 2).

The second objective was to determine the optimal method for accurate, rapid and cost-effective extraction of VOCs from citrus fruit infested with *Aonidiella aurantii* using HS-SPME fibre coupled with gas chromatography-mass spectrometry (GC-MS) (Chapter 3).

The third objective involved olfactometer bioassays with female *A. melinus* parasitoids using fruit and volatile compounds identified in *Ao. aurantii* citrus infested fruit. This investigation enabled the determination of specific compounds in infested fruit that were attractive to *A. melinus* female parasitoids and to determine whether there were distinct and interpretable behavioural responses from female *A. melinus* female parasitoids that could be associated with *Ao. aurantii* infested fruit. This enabled confirmation of specific behavioural responses in *A. melinus* that were exclusively elicited in the presence of infested fruit. Within the context of this investigation, the host searching behavioural sequence in *A. melinus* female parasitoid was elucidated (Chapter 4 and 5).

The final objective was to shed light on the effects of the application of d-limonene and β -ocimene on the attraction of parasitoids *A. melinus* and other natural enemies to citrus infested with *Ao. aurantii* and their role as well on the increasing the parasitism percentages on the pest *Ao. aurantii* (Chapter 6).


Chapter Two

**Age-specific life tables of *Aonidiella aurantii*
(Maskell) (Hemiptera: Diaspididae) and its
parasitoid *Aphytis melinus* DeBach
(Hymenoptera: Aphelinidae)**

Statement of Contribution

Title of Paper	Age-specific life tables of <i>Aonidiella aurantii</i> (Maskell) (Hemiptera: Diaspididae) and its parasitoid <i>Aphytis melinus</i> DeBach (Hymenoptera: Aphelinidae)
Publication Status	Published
Publication Details	Turkish journal of Agriculture and Forestry, 44. pp, 77-86


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Contribution to the Paper	Methodology, funding acquisition, formal analysis, investigation, data curation and writing—original draft preparation
Overall Percentage (%)	55%
Signature	 Date:17/March/2020


Co-Author Contributions

By signing the statement of contribution, each author certifies that:


- The candidate's stated contribution to the publication is accurate (as detailed above).
- Permission is granted for the candidate to include the publication in the thesis
- The sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Overall Percentage (100%)	10%
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2.1. Abstract

Biological parameters of the California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) were determined under laboratory conditions at three different temperatures (20, 23 and 27°C) on butternut squash (*Cucurbita moschata* Duchesne ex Lamarck) (Cucurbitaceae), while the biological parameters of its parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) were conducted just at 27°C. The percentage survival of *Ao. aurantii* ranged between 80.0% and 88.3%. The highest mortality was recorded during the adult stage with mortalities ranging between 12% and 20%. On *C. moschata* the total development time was 93.1 ± 9.73 , 81.8 ± 7.13 and 65.7 ± 6.37 days, while the adult longevity was 54.65 ± 0.71 , 47.05 ± 0.97 and 39.35 ± 1.07 days at 20, 23 and 27°C, respectively. Oviposition period of *Ao. aurantii* was 44.3 ± 0.51 , 40.65 ± 0.41 and 34.5 ± 0.45 days at 20, 23 and 27°C, respectively. Average fecundity was 73.25 ± 1.827 , 109.7 ± 3.569 and 129.35 ± 4.564 individuals at 20, 23 and 27°C, respectively. For *A. melinus*, adult longevity was 19.24 ± 0.73 days, and average fecundity 62.7 ± 2.81 eggs at 27°C. The pre-oviposition period was 0.82 ± 0.05 days, oviposition period 15.7 ± 0.52 days and post-oviposition period 2.21 ± 0.09 days. The intrinsic rate of increase (r_m) of *A. melinus* (0.188 female/female/day) was significantly greater than that of *Ao. aurantii* (0.080) at 27°C. These laboratory results demonstrated that *A. melinus* is an effective parasitoid for decreasing *Ao. aurantii* populations. Fecundity of *Ao. aurantii* and *A. melinus* was determined with Enkegaard equation. The best-fit parameters of fecundity were calculated as $a = 0.410$, $b = 0.099$; $a = 0.624$, $b = 0.098$; $a = 0.661$, $b = 0.091$ and $a = 1.190$, $b = 0.179$ for *Ao. aurantii* at 20, 23 and 27°C, and *A. melinus* at 27°C, respectively.

2.2. Introduction

Australia has an important role in citrus (*Citrus* spp., Rutaceae) production in the world, and this production has increased day by day (FAO, 2017). Many pest species such as *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), *Aonidiella aurantii*, *Sasseita oleae* (Gomez-Menor Ortega) (Hemiptera: Coccidae), *Toxoptera citricada* (Kirkaldy) (Hemiptera: Aphididae) and *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillaridae) have been observed in WA (Western Australia) citrus orchards (Sonia, 2006). Growers have followed various methods to avoid damage caused by these pests. Biological control is one of these methods (FAO, 2017; Pekas, 2011a; Uygun and Satar, 2008). There are 32 species in the genus *Aonidiella* which is a genus of scale insects in the family of Diaspididae (Hemiptera), the armoured scale insects (Ben-Dov, 2006). California red scale *Aonidiella aurantii* (Maskell), occurs on numerous host plants throughout the world. They attack different crops, such as fruit trees and ornamental

plants all over the world and cause heavy damage to the plants. Individual species infest leaves, fruits, branches, main stems, trunks and roots. They are distributed throughout the world except in the cold extremes of the Arctic and Antarctic regions (Miller, 2005). California red scale, *Ao. aurantii* is one of the most important pests infesting citrus trees in different parts of the world (Garcerá et al., 2008; Badary and Abd-Rabou, 2010; Hill, 2008; Karaca and Uygun, 1992; Uygun et al., 1995). California red scale feeds on various parts of their host plants, such as twigs, leaves or fruit (Beardsley and Gonzalez, 1975), harming them by inserting its mouthparts deep into plant tissue and sucking sap from parenchyma cells and injecting toxic saliva into the plants during the feeding process. Severe infestations of the scale cause leaf drop, defoliation, and dieback of twigs and branches. Young trees may die in the absence of effective controls (Hely et al., 1982; Smith et al., 1997).

The control of *Ao. aurantii* has encountered many difficulties, which has raised interest in alternative control methods (Vacas et al., 2010). *Ao. aurantii* has numerous predators and parasitoids (Hely et al., 1982; Forster et. al., 1995). Among the parasitoids, *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) is known to be relatively important on *Ao. aurantii* (Erlor and Tunç, 2001). It was introduced into Australia in 1961 from the University of California, Riverside (Furness et al., 1983; Malipatil et al., 2000). It is now widely distributed in the citrus orchards of Victoria, South Australia, Western Australia and inland New South Wales, and in Alice Springs in the Northern Territory (Smith et al., 1997). *A. melinus* is commercially reared for release in citrus orchards to control *Ao. aurantii* (Grafton-Cardwell et al., 2008). Regarding parasitoids, females are more important to produce, because they are responsible for parasitising the host (via oviposition and host feeding) and, thus, basically keeping pests population under an economic threshold. A plethora of experiments have been conducted to determine the environmental conditions contributing to the increase in female production (Ode and Hardy, 2008).

For the success of a crop protection program, it is essential to know some detailed biological information about the pest of interest. This information can be obtained by constructing the life-table providing different mortality factors acting in sequence on the successive developmental stages. The intrinsic rate of increase (r_m) is of value, as it is the means of describing the potential growth of a population under given climatic and food conditions; it is an important parameter in inductive strategic and management models of the pest populations (Abou Hatab, 1999; Bayoumy et al., 2009). Few studies have been conducted on the biological

parameters of the California red scale and its parasitoid *A. melinus* (Heimpel, et al., 1997; Badary and Abd-Rabou, 2010). Therefore, the aims of the present work were to study the life history and behaviour of *Ao. aurantii* and its parasitoid *A. melinus* at three constant temperatures (20, 23 and 27°C) under laboratory conditions simultaneously.

2.3. Materials and methods

2.3.1. Rearing of *Aonidiella aurantii* and *Aphytis melinus*

Specimens of *Ao. aurantii* were initially collected in the field in 2016 from a population of *Ao. aurantii* in a citrus orchard located in WA (32.30°S 116.01°E 69m AMSL), Australia. Stock cultures of *Ao. aurantii* were kept on butternut squash (*Cucurbita moschata* Duchesne ex Lamarck), in the climate chamber laboratory conditions [27°C, 65 ± 5% RH, L16: D8 photoperiod] in the Murdoch University insect culture room. The adult wasps of *A. melinus* used to start the colony were provided by Biological Services (Adelaide, Australia) a commercial insectary. *Aphytis melinus* adults used in the experiments were reared following the method developed by (Opp and Luck, 1986), for rearing *A. melinus* in the climate chamber at (26°C, 40-60% RH, L16: D8 photoperiod). This method is commonly used to produce these insects in commercial insectaries. The production method is based on rearing the pest *Ao. aurantii* on butternut squash. When the host reaches the third instar, which is the preferred age for the parasitoid to laid eggs and maximise progeny production, the infested squash with third instar scales were exposed to a 2-day old adult parasitoid (male and female) in a ventilated cage (30 cm W × 30 cm H × cm D) with honey distributed on plastic trays. Adult parasitoids emerged about 11-14 days later. Parasitoids produced have participated in the experiments within one day after emergence.

2.3.2. Survival, Longevity and Fecundity of *Aonidiella aurantii*

Uniform groups of *Ao. aurantii* were obtained by place the cut up butternut squash and left undisturbed for 24 h on the *Ao. aurantii* colony maintained in the insect culture room at Murdoch University. After which, the butternut squashes were removed, and ten randomly selected settled crawlers (those with the stylet inserted into the fruit and already forming the waxy cover) were enclosed by a microplastic cage (small plastic thimble, 3 cm diameter with fine holes on its top) affixed with the help of modelling clay to the surface of butternut squash. Butternut squash and nymphs were labelled to allow them throughout the experiment. An age-specific life table of *Ao. aurantii* was constructed at three different constant temperatures (20, 23 and 27°C). The infested butternuts were kept in a ventilated polystyrene box (30 cm × 30

cm× 30 cm). Each treatment consists of three thimbles for each squash fruit and each of these thimbles placed over ten of *Ao. aurantii* nymphs. There were two pieces of fruit per box (six replicates) were kept on a plastic tray in identical climatically controlled cabinets (HWS Ningbu southeast equipment Co. Lt D. China) maintained at (20, 23 and 27°C, 65 ± 5% RH and L16: D8 photoperiod). The development of *Ao. aurantii* individuals were observed daily using a stereomicroscope (×20) until the death of the adult females.

2.3.3. Survival, Longevity and Fecundity of *Aphytis melinus*

Newly emerged adult parasitoids were shifted to an Agilent glass vial (1.5 ml). The vial was then plugged with a cotton bung. A drop of honey was supplied to each vial. After 24 hours, a female parasitoid was confined under a microplastic cage (small plastic thimble, 3 cm diameter with fine holes on its top) affixed with the help of modelling clay to the surface of butternut squash containing ten individual females of 3rd instar stage of *Ao. aurantii*. A virgin female and male of *A. melinus* were placed into each of these cages as the parasitoid prefers to oviposit on the initial stages of this host (Heimpel et al., 1997). A stereoscopic magnifying glass (×2.5) was used to observe the mating. The parasitoids were transferred every 24 hours to a new cage and observations continued until the female parasitoid died (Qiu et al., 2007). Determining the number of eggs laid without removing the armoured scale of the nymphs was not possible, as this process leads to the death of the nymph (Watson, 1990). A parallel experiment, identical to that used in the longevity and oviposition experiment, was therefore undertaken. Non-parasitized individuals were inverted to check for egg remains or un-hatched parasitoid eggs, indicating unsuccessful parasitism or infertile eggs, respectively (Urbaneja et al., 2007). Observations were recorded to get the information on pre-oviposition, oviposition, and post-oviposition periods and the number of eggs laid each day. Parasitised nymphs were kept determining when the adult parasitoid emerged in order to estimate developmental time, survivorship (a number that emerged divided by total number parasitised). The experimental unit was a group of ten female parasitoids. Each experimental unit was replicated three times.

2.3.4. Design and statistical analysis

A completely randomised design was used in all the experiments. An analysis of variance (ANOVA) was subsequently performed, and means of survivorship, developmental times, longevity and fecundity were compared using Tukey's multiple comparison tests ($P < 0.05$). Subsequently, all data were analysed using Levene's test to ensure homogeneity of variance.

2.3.5. Life table parameters

The main purpose of age-specific fecundity table studies is to predict the rate of population growth of *Ao. aurantii* and *A. melinus*. A fertility life table was constructed by making a list of the data collected on the developmental and reproductive biology of the species as per formulae provided by (Birch, 1948). Daily adult numbers observation from the first day of female exclusion until complete female death, daily survival rate (l_x) and female fecundity (m_x) were calculated according to the equation of life tables to predict the growth rate of the population under laboratory conditions at 27°C. From the fertility and survival rate, several population growth parameters including the net reproductive rate (R_0), mean generation time (T_0), and intrinsic rate of natural increase (r_m) was calculated using the formulas suggested by (Carey, 1993), where x is the age of individuals in days, l_x is the age-specific survival, and m_x is the age-specific number of female offspring. Age-specific life table parameters of insects were calculated according to the Euler-Lotka equation (Birch, 1948). These parameters are:

Net reproductive rate	$R_0 = \sum l_x m_x$
Intrinsic rate of natural increase	$1 = \sum l_x m_x e^{-r_m \cdot x} \quad (r_m)$
Mean generation time,	$T_0 = \ln R_0 / r_m$
Gross reproduction rate,	$GRR = \sum m_x$
Finite rate of increase,	$\lambda = e^{r_m}$

Age-specific eggs laid by a female were described by Enkegaard equation: $F(x) = a \cdot x \cdot e^{-b \cdot x}$ (Enkegaard, 1993; Hansen et al., 1999). Where $F(x)$ is the daily age-specific fecundity rate (eggs/female/day), x is the female's age in days, a and b are constants. Day 1 is the first day of oviposition period. Analyses were done by using JMP (ver. 5), MS Excel (ver. 2003), SPSS (ver. 24.0) and CurveExpert Pro (ver. 1.6.7) software.

2.4. Results

2.4.1. Survival, Development and Oviposition of *Aonidiella aurantii*

The survival rate of the immature state of *Ao. aurantii* at different temperatures (20, 23 and 27°C) were similar but varied between 80.0 and 88.0% at these three temperatures (Table 2.1). The average mortality recorded at the three different temperatures was 0.16%. The highest mortality occurred during the preadult stage, with values ranging between 0.12% and 0.20% (depending on the temperature). Based on these results, we can assume that there were no

significant differences in the survival of *Ao. aurantii* reared on *C. moschata* under these different temperatures.

Table 2.1. Effect of temperature on the survival of immature stage (1st to 3 instars) *Aonidiella aurantii* reared on *Cucurbita moschata* in the laboratory at 20, 23 and 27°C, 65.0±5.0% RH and 16: 8D photoperiod.

Temperature	<i>n</i>	1 st instar (%)	2 nd instar (%)	3 rd instar female (%)	Gravid female (%)	Survival (%)
20°C	60	98.33	95.00	95.00	83.33	83.3 a
23°C	60	100	98.33	98.33	88.33	88.3 a
27°C	60	100	98.33	98.33	80.00	80.0 a

The duration of each developmental stage was affected by temperature (Table 2.2). The results indicated that 27°C was an adequate test temperature for the *Ao. aurantii* life cycle studies and resulted in the highest oviposition (129.35±4.56 eggs/female), the shortest incubation period (4.45±0.14 days) and adult longevity (39.35±1.07 days).

Table 2.2. Duration of the California red scale *Aonidiella aurantii* stages at three constant temperatures (20, 23 and 27°C, 65.0±5.0% RH and 16: 8D photoperiod) reared on fruits of *Cucurbita moschata*.

Developmental Stages	Duration ¹ (days) Mean ± SE		
	20°C	23°C	27°C
1 st instar	12.15±0.38 a	10.85±0.32 b	8.65±0.27 c
2 nd instar	12.65±0.26 a	10.95±0.16 b	8.45±0.24 c
3 rd instar	13.05±0.22 a	12.3±0.20 a	9.11±0.44 b
Pre-oviposition period	8.85±0.26 a	6.15±0.19 b	4.45±0.14 c
Oviposition period	44.35±0.51 a	40.65±0.41 b	34.5±0.45 c
Total eggs·female ⁻¹	73.25±1.86 c	109.7±3.57 b	129.35±4.56 a
Adult longevity	54.65±0.71 a	47.05±0.97 b	39.35±1.07 c
Adult's lifespan	93.1±9.73 a	81.8±7.13 b	65.7±6.37c

¹Means within each row followed by different letters differ significantly ($p < 0.05$) based on Tukey's test. Data are means ± standard errors.

2.4.2. Survival, Development and Oviposition of *Aphytis melinus*

The *A. melinus* mortality was greatest in older individuals. The adult *A. melinus* individuals started to die from day ten until day 18 and then showed a sharp drop on days 13 and 16 (Figure 2.1).

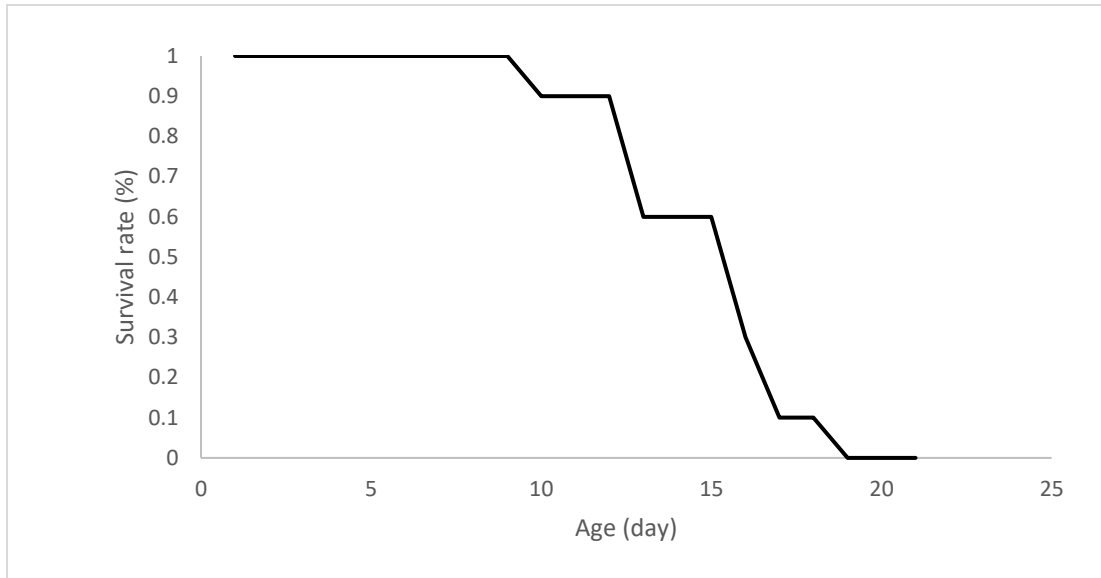


Figure 2.1. Survival rate (%) of female *Aphytis melinus* in days at 27°C, 65 ± 5% RH and a photoperiod of 16: 8 h (L: D).

The oviposition period of *A. melinus* lasted an average 15.7±0.52 (Table 2.3). The average oviposition rate was 3.95±0.22 eggs female⁻¹ day⁻¹. Each female laid an average total of 62.7±2.81 eggs. The pre-oviposition period was 0.82±0.05 day; adult longevity was an average of 19.24±0.73 days, while the egg-pupal stage was 11.25±0.12 day.

Table 2.3. Oviposition periods, longevity, fecundity and oviposition rates of *Aphytis melinus* at 27°C, 65.0 ± 5.0% RH and a photoperiod of 16 L: 8 D

Developmental Stages	Duration of the stages, days ¹
	Mean ± SE
Egg-Pupal stage	11.25 ± 0.12
Preoviposition	0.82 ± 0.05
Oviposition	15.7 ± 0.52
Postoviposition	2.21 ± 0.09
Adult Longevity	19.24 ± 0.73
Oviposition	Number of eggs
Total eggs·female ⁻¹	62.7 ± 2.81
Oviposition rate (Eggs·female ⁻¹ ·day ⁻¹)	3.95 ± 0.22

¹ Data are reported as the mean ± standard error.

2.4.3. Life table parameters

Aonidiella aurantii had a higher net reproduction rate (R_o) and mean generation time (T_o), and conversely, intrinsic rate of increase rate (r_m) than *A. melinus* (Table 2.4).

Table 2.4. Life table parameters and statistics of *Aonidiella aurantii* fed on butternut squash (*Cucurbita moschata*) and their parasitism by *Aphytis melinus* under laboratory conditions

Parameter ¹	<i>Aonidiella aurantii</i>			<i>Aphytis melinus</i>
	20°C	23°C	27°C	27°C
Net reproductive rate (R_o)	30.79 a	48.78 b	61.02 c	28.14 a
Mean Generation time (T_o)	62.31 a	55.53 b	51.39 c	19.06 d
Intrinsic rate of increase (r_m)	0.055 a	0.070 b	0.080 c	0.188 d
Finite rate of increase (λ)	1.056 a	1.072 b	1.083 c	1.207 d
Gross reproduction rate (GRR)	37.09 a	55.43 b	65.16 c	32.15 d

Means within each row followed by different letters differ significantly based on Tukey's test ($p < 0.05$).

Parameters show that the *Ao. aurantii* population grows 61.02 times at 27°C in one generation. The net reproduction rate of females for each female of a generation, was considerably higher for *Ao. aurantii* than *A. melinus* which reached 28.14 at 27°C (Table 2.4), which indicates the high reproductive capacity of *Ao. aurantii* when fed butternut squash. Moreover, the net reproduction rate obtained for *A. melinus* indicates a low substitution potential for each female having *Ao. aurantii* as a host under laboratory conditions. The intrinsic rate of increase (r_m), defined as a population's capacity to multiply, was higher for *A. melinus* (0.188) than *Ao. aurantii* which was 0.080 at 27°C (Table 2.4).

The parameters (a, b and R^2) of the Enkegaard regression model applied on the age specific fecundity rate (m_x) of *Ao. aurantii* and *A. melinus* are given Figure 2.3. The relationship between days and fecundity was higher at all temperatures.

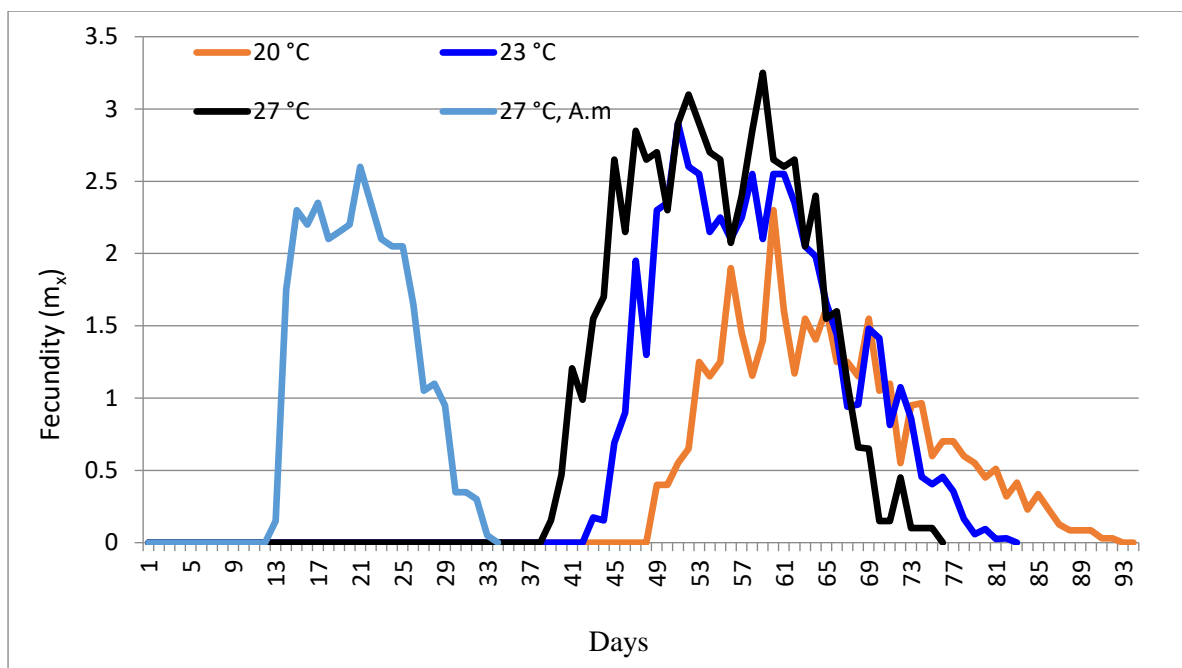


Figure 2.2. Age-specific fecundity (m_x) of *Aonidiella aurantii* reared on fruits of *Cucurbita moschata* at three constant temperatures (20, 23 and 27°C) and *Aphytis melinus* (A.m) at 27°C.

2.5. Discussion

The use of a constant temperature provides a useful reference point for the performance of *Ao. aurantii*. The survival values of *Ao. aurantii* varied between 0.88% and 0.80%. These values are essentially similar to those obtained by (Karaca, 1990) at 26°C on lemon, orange and grapefruit, and higher than the survival of *Ao. aurantii* on mandarin. Karaca (1990) indicates in a study on the development, survival and longevity of *Ao. aurantii* on different citrus species, in which survival was only 0.90, 0.81, 0.85 and 0.61 on lemon, orange, grapefruit and mandarin respectively. These differences could be due to the varieties of the fruit species studied and laboratory conditions being different from those used in the present study. The average mortality of *Ao. aurantii* is approximately similar to that obtained by (Vanaclocha et al., 2012) on a lemon fruit at 26°C when the mortality percentage was 15%, and to those obtained by (Karaca, 1990) on lemon, orange, grapefruit and higher than those in mandarin where the highest mortality (39%) was recorded for adults reared on mandarin fruits.

The life cycle and development rates of *Ao. aurantii* females were significantly different depending on the temperature used except for the duration of the third instar which did not differ significantly between 20°C and 23°C. In addition, the pre-oviposition period, oviposition period, longevity of adults and the mean number of eggs per *Ao. aurantii* female were significantly different depending on the temperature. In general, the duration of each developmental stage recorded in this study were consistent with those determined for *Ao. aurantii* by Badary and Abd-Rabou, (2010), and less than those determined by Karaca (1990). In other species of *Aonidiella*, the average developmental time ranged between 94 days and 45 days depending on the temperature used for *Aonidiella orientalis* (Flaih, 2007), 65 days for *Aonidiella citrina* in California, and the reproductive period lasts 60 days under a constant temperature of 27.8°C (Nel, 1933). Temperature is the most important abiotic factor affecting growth, development rate, oviposition and survival of *Ao. aurantii*. With high temperatures within optimal limits, all processes occur significantly faster, which results in rapid ageing of the females with a significant increase in the total number of eggs laid and reduced longevity of the adult female. Likewise, the number of eggs laid per day increases with increasing temperature (Pekas, 2011b). This last parameter, together with the increased rate of oviposition and nymphal development, increases the potential pest status of *Ao. aurantii* in warm citrus-growing areas.

Aphytis melinus females showed gradual mortality over time, which can be attributed to death by natural ageing of the individuals. The developmental period of *Aphytis spp.* is usually short and depends on climatic conditions, principally temperature and humidity (Yu and Luck, 1988). For example, at 26.7°C, *A. melinus* completes its development in almost a fortnight, whereas it takes one month at 17°C. Nutrition also contributes to longevity in the ectoparasitoid species *A. melinus*. For example, greater longevity was observed in females that were fed than those not allowed to host feed (Heimpel et al., 1997). As a result, greater availability of females could occur over time when the temperature is appropriate, and there is food, but this is not necessarily associated with an increase in the parasitism rate. *Aphytis melinus* has been found to have two to three generations to one of its host *Ao. aurantii* (Yu and Luck, 1988). The duration of the longevity period of *A. melinus* is approximately consistent with that obtained by (Vanaclocha et al., 2012) who determined that at 26.7±1.5°C the duration of the longevity period of *A. melinus* was 20.5 days, while the total number of eggs per female was about 11.7 eggs less than that produced by *A. melinus* at 26.7±1.5°C. The diet of adult parasitoids influences lifetime reproductive success (Flaih, 2007). Adult females of many species obtain

materials required for egg maturation by feeding upon host insects (host feeding), and materials necessary for adult maintenance and survival are acquired by host feeding and by feeding upon any of a number of sugar sources (Jervis, 1996; Heimpel and Collier, 1996).

These results of life table parameters confirm the potential of *A. melinus* to control *Ao. aurantii*. However, this characteristic must be compared with that of other species such as other *Aphytis* spp., that can use the same field substrate. Studies carried out by Orphanides, (1984) point out that interspecific competition and competitive displacement occurs between *Aphytis* spp. which are *Ao. aurantii* parasitoids. So other species or subspecies could also affect biological control success in the field. By comparing the life table parameters of *Ao. aurantii* and *A. melinus*, it can be seen that there are significant differences in all of the five parameters studied (Table 2.4). The net reproductive rate (number of females for each female of a generation) is significantly higher for *Ao. aurantii* (61.02) than those for *A. melinus* (28.14). Generation time, the meantime between two successive generations is significantly longer in *Ao. aurantii* (51.39 days) than *A. melinus* (19.06 days), and this is interpreted as favourable for increasing the numbers and efficiency of the parasitoid (La Rossa et al., 2002). The intrinsic rate of increase (r_m), which indicates the ability of a population to increase in abundance from generation to generation is an essential indicator of the potential of a parasitoid to control its host (MERcaDo et al., 2014; Persad and Khan, 2002). The r_m of *A. melinus* (0.188) is significantly greater than that of *Ao. aurantii* (0.080) at 27°C, (Table 2.4). The relationship between days and fecundity was well described using the Enkegaard regression model; at 20, 23 and 27°C for *Ao. aurantii*; $R^2= 0.833$, $a= 0.410$, $b= 0.099$; $R^2= 0.799$, $a= 0.624$, $b= 0.098$; $R^2= 0.715$, $a= 0.661$, $b= 0.091$ respectively and at 27°C for *A. melinus*; $R^2= 0.841$, $a= 1.190$, $b= 0.179$. Most of the eggs were laid within the first half of the oviposition period (Figure 2.3). The influence of increased temperature was clearly seen for *Ao. aurantii* by the increased fecundity peak at an earlier age. Thus, the fecundity curve turning to the left at 27°C earlier than other temperatures. It is most clearly seen for *A. melinus* as well. Understanding the variations of *Ao. aurantii* population response to different temperatures could have useful implications for its management. In addition, determination of life table parameters and their variation allows us to estimate maximal responses to estimate their biotic potential under specific conditions. these data could be coupled with *A. melinus* population dynamics models to develop *Ao. aurantii* management strategies involving biological control programs in the field.

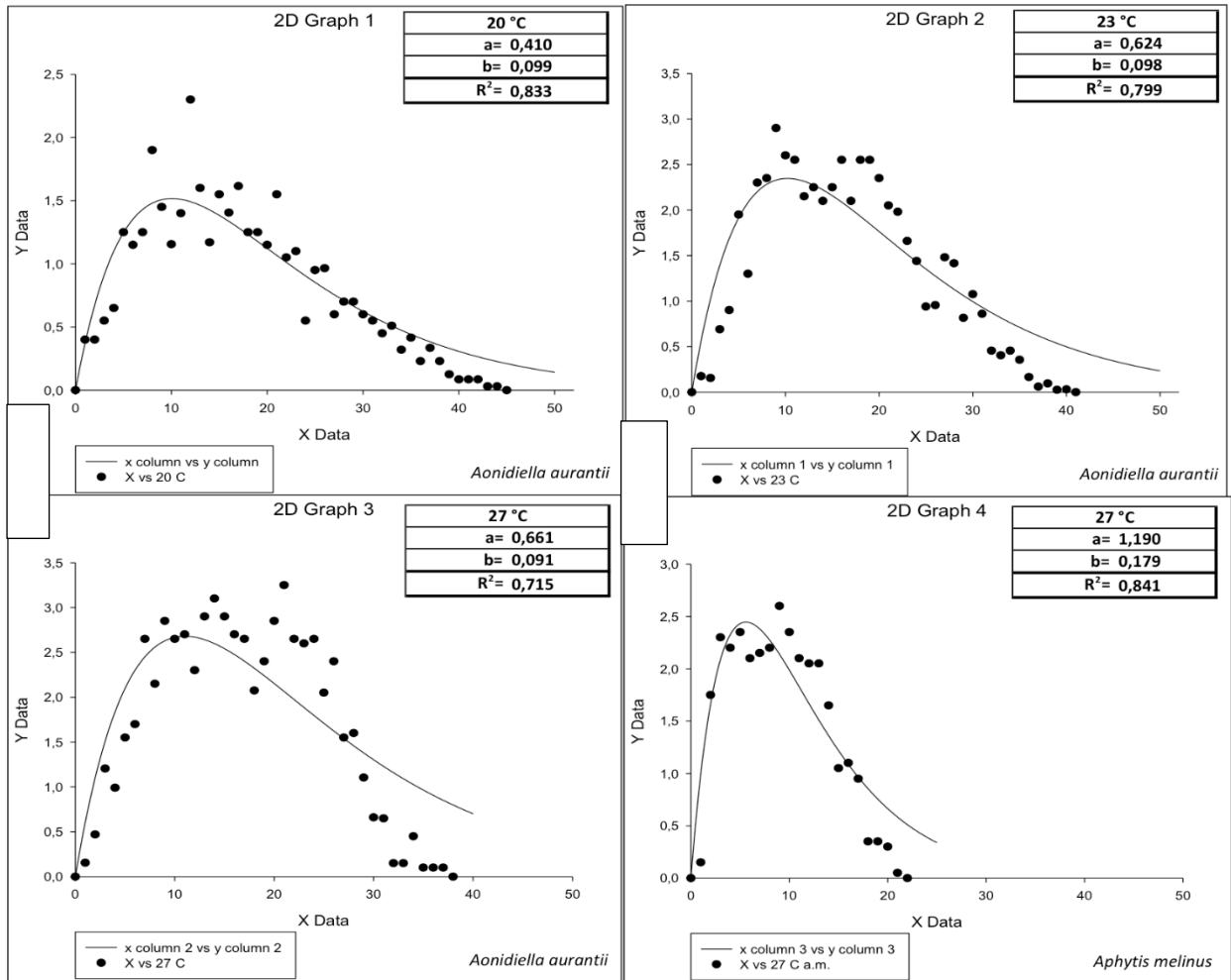


Figure 2.3. Enkegaard distribution of *Aonidiella aurantii* and *Aphytis melinus*.


Chapter Three

Optimization of Headspace Solid-Phase Microextraction Conditions for the Identification of Volatiles Compounds from the Whole Fruit of Lemon, Lime, Mandarin and Orange

Statement of Contribution

Title of Paper	Optimization of Headspace Solid-Phase Microextraction Conditions for the Identification of Volatiles Compounds from the Whole Fruit of Lemon, Lime, Mandarin and Orange
Publication Status	Published
Publication Details	Journal of Biosciences and Medicines, 5(03). pp, 176-186.

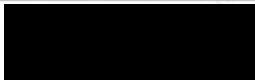
Principal Author


Name of Principal Author (Candidate)	Khalid Mohammed
Contribution to the Paper	Methodology, funding acquisition, formal analysis, investigation, data curation and writing—original draft preparation
Overall Percentage (%)	70%
Signature	 Date: 17/March/2020

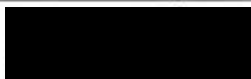
Co-Author Contributions

By signing the statement of contribution, each author certifies that:

- The candidate's stated contribution to the publication is accurate (as detailed above).
- Permission is granted for the candidate to include the publication in the thesis
- The sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Overall Percentage (100%)	10%
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3.1. Abstract

Plants emission of volatile organic compounds (VOCs) is playing an important role in plants interactions with biotic and abiotic factors. An optimum method has been developed for extracting volatile organic compounds (VOCs) which contribute to the aroma of different species of citrus fruit (orange, lemon, lime, and mandarin). Headspace solid phase microextraction (HS-SPME) combined with gas chromatography (GC) coupled with flame ionization detection (FID) is used as a very simple, efficient and non-destructive extraction method. A three phase 50/30 μ m PDV/DVB/CAR fibre was used for the extraction process. The optimal sealing time for volatiles reaching equilibrium from whole fruit in the headspace of the chamber was 20, 16, 8 and 16 hours for lemon, lime, mandarin, and orange respectively. Optimum fibre exposure times for whole fruit were 2, 4, 2 and 2 hours for lemon, lime, mandarin, and orange respectively. Three chamber volumes (500, 1000 and 2000 ml) were evaluated for the collection of VOCs with the 500 ml chamber being selected. The 500 ml chamber produced the highest quality peak areas and quantity of extracted volatiles. As a result of fruit respiration, the percentage of oxygen (O₂) of all citrus fruit species in 500 ml chamber decreased from 21.8% to 18.8% in the 20 hours sealing time, while carbon dioxide (CO₂) contents increased to 2.9% also in the 20 hours sealing time. The results of this study showed the feasibility of this technique for identifying VOCs from four of the citrus fruit species and its potential as a routine method for physiological studies on citrus fruit or on other fruit species.

3.2. Introduction

Citrus is a genus of flowering trees and shrubs in the family of Rutaceae and is considered among the most important horticultural industries in the world. Citrus fruit have the highest international trade value of all food (Liu et al., 2012). The contribution of the citrus industry to the world economy is estimated at more than \$10 billion USD annually (Ladanyia, 2010). The Australian citrus industry considers as one of the largest fresh fruit industries in Australia, and definitely the largest fresh fruit exporter with an annual average export volume of 170,000 tons and a value of \$190 million AUD (Authority, 2010). Citrus fruit are used not only for foods and drinks but also in perfumes, cosmetic, soaps, and many other aromatic products. They are widely grown in the world's tropical and subtropical regions and are native to parts of China, India, New Caledonia and northern Australia (Manner et al., 2006). Taxonomic identification is confounding because there are many species, but citrus can generally be classified into the following categories: sweet oranges (*Citrus sinensis*), mandarins (*C. unshi*), tangerines (*C. tangerina*, and *reticulata*), and (*C. clementine*)), sour/ bitter oranges (*C. aurantium*), lemons

(*C. lemon*), limes (*C. aurantifolia* and *latifolia*), grapefruit (*C. paradisi*) and pummelos (*C. grandis*), hybrids (e.g., tangelos, tangors, and limequats), and citrons (*C. medica*, which have a rind that is used primarily for confectionary and is only commercially grown in limited areas). All of which are exported to various markets around the world (Ortiz, 2002).

Citrus species produce essential oils in their fruits which are used in soap, food, perfumes, repellents and others (Tan et al., 2011). Citrus consumption depends closely on their aroma and flavour. Chemically, the aroma and flavour are given by the presence of volatile compounds that impress the olfactory receptors. Detailed analysis of the aroma components of citrus fruits is important for citrus industries to ensure the production of quality foods and free of pests and pathogens. Citrus have been considered as emitters of volatile organic compounds (VOCs) in chamber studies under controlled environmental conditions (Fares et al., 2012). These VOCs can be extracted by various techniques, such as microwave-assisted hydro distillation extraction, solvents and recently, solid phase microextraction (SPME) which is used to profile and quantify these compounds. Headspace solid phase microextraction (SPME) nowadays, is considered the method of choice for most of the volatile extraction in flavour chemistry/food (Cavalli et al., 2003; González-Mas et al., 2009) and particularly in Citrus (Jia et al., 1998; Choi and Min, 2004). There are many methods for obtaining citrus volatiles, such as citrus oil (essential oil) and these methods have been extensively reviewed (Atti-Santos et al., 2005; Sikdar et al., 2016). Unfortunately, the aroma of extracted oils rarely represents the delicate natural aroma of citrus, because of uncontrolled temperature during the steam distillation process (Automatik and Minyak, 2013). Likewise, other extraction methods using ground samples and solvents also frequently fail to capture the natural aromas (Sparinska and Rostoks, 2015). To identify VOCs produced by citrus or other fruit, it is necessary to develop easy to operate, repeatable, sensitive, rapid and cost-effective method. Until now, there are no studies about the use of headspace solid phase microextraction (HS-SPME) technique for the whole fruit of citrus species. The SPME method is excessively used for the analysis of volatile compounds. The HS-SPME technique is a new, simple, rapid, eco-friendly and solvent-free sample preparation technique for the extraction of volatile compounds (Najafian and Rowshan, 2012; Bicchi et al., 2000). The HS-SPME technique gives simultaneously tens or hundreds of possible volatile compounds and also provides interesting results when gas chromatography (GC) is combined with either Flame Ionization Detector (FID) or mass spectrometric detection (MS), but it must be optimized for the volatiles being targeted (Dorea et al., 2008; Jeleń et al., 2012). Many factors can affect the optimization of extraction conditions, such as the correct

fibre and an appropriate chamber for capturing the VOCs, the temperature used during extraction and the extraction time from the headspace (Nongonierma et al., 2006). So far, there has been no systematic work on optimizing extraction conditions for whole fresh citrus fruit. Therefore, this study will determine the optimal conditions of sealing time, extraction time and chamber size for citrus fruit volatile isolation by the headspace solid phase microextraction (HS-SPME) technique with gas chromatography coupled with Flame Ionization Detector GC-FID.

3.3. Materials and Methods

3.3.1. Reagents

An n-hexane 95% was purchased from Sigma-Aldrich Australia, catalogue number 270504-2L. Ethanol was purchased from MERK (Germany) (high-performance liquid chromatography HPLC grade), and the n-Alkane standard (C7- C30) was purchased from Sigma-Aldrich Australia, catalogue number 49451-U.

3.3.2. Apparatus and Equipment

An Agilent Technologies gas chromatograph 7829A (serial number CN14272038) fitted with an HP-5MS column (30 m x 0.25 mm, film thickness 0.25 µm, RESTEK, catalogue number 13423) non-polar, with a flame ionization detector (FID) was used. The SPME extractions were carried out in three-phase divinylbenzene/carboxen/polydimethylsiloxane DVB/CAR/PDMS fibre, 50/30 µm (Sigma-Aldrich Australia, catalog number 57347-U), which was designed for analytes with a broad range of polarities (suitable for C2-C20 range) (Chen and Lin, 2004; Bourgou et al., 2012) (Sigma-Aldrich Australia, catalog number 57347-U), attached to a manual SPME holder (Supelco Inc.). The fibres were conditioned as recommended by the manufacturer and the supplier specifications before analyses. A 500, 1000 and 2000 ml Pyrex (Silverlock Packaging; JG2701 FL, JG2879 FL and JG2901 FL respectively) glass jar with a 5 mm port drilled into one side, into which septa (20633 Thermogreen® LB-2 Septa, plug) was placed and was used for collection of citrus fruits VOCs. Aluminium foil 150 m × 44 cm (Vital Packaging Company) was used to cover the glass jar opening and extract volatile organic compounds (VOCs) emitted from fruits. Witt OXYBABY® 6.0 (WIT-Gasetechnik GmbH and Co KG T, Germany) was used for monitoring head space fruit concentration of carbon dioxide, oxygen, and nitrogen from respiring fruits was by inserting the needle through jars septa.

3.3.3. Samples

Fresh samples of orange, lemon, lime and mandarin fruit were purchased from different vendors at local shopping centres. The orange, lemon and mandarin were weighed at approximately 150 g and the lime at approximately 100 g. The fruit was checked and washed well with warm water to get rid of the wax, and then conditioned at room temperature ($25\pm 1^\circ\text{C}$) for 24 hours before the experiment was conducted.

3.3.4. Gas Chromatogram Condition

The GC-FID run time was 45 minutes; the oven column temperature ranged from $50\text{-}250^\circ\text{C}$, programmed at $5^\circ\text{C}/\text{min}$, with a final hold time of 5 min. Helium (He) was used as the carrier gas at 1.1 ml/min constant flow, and detector (FID) temperatures of 290°C , injection port temperature 250°C , and the GC-FID instrument was operated under the splitless mode.

3.3.5. Optimization of Solid-Phase Microextraction

For optimization of the HS-SPME, the variables chosen were sealing time, extraction time and different volumes of chambers for extraction; the fibre extraction temperature of $25\pm 1^\circ\text{C}$; the weight of samples were kept constant. In order to optimize the sealing time, extraction times and chambers volumes and all factors influencing the equilibrium between the analyses and the fibre were taken into consideration. Different sealing times, different extraction times and different extraction chambers volumes were used for the different species of fresh citrus fruit. The fibres were cleaned between each extraction by placing them into the GC injection port for 15 min at 250°C to ensure the absence of carry-over peaks and contaminants in blanks and next injections to have excellent repeatability between the injections. All three fibres were calibrated using standard n-alkene C7-C30 after dilution in the ratio of 1/10 ml in n-hexane, and then desorbed for one hour at room temperature, and this procedure was repeated twice with three replications before analysis. The results are presented as mean values.

3.3.5.1. Optimization of Sealing Time

To determine the best sealing time; the citrus fruits species were individually sealed in 1000 ml glass jars for (2, 4, 8, 12, 16 and 20 hours). The extraction efficiency of the six different sealing time was determined by comparing the peak area of the eight compounds from all citrus fruit species under the same extraction time, SPME fibre, desorption time, and GC conditions. The fibres were exposed to the headspace (HS) of the glass jars for 2 hours. After exposure,

the fibres were retrieved and injected into the heated injection port (250°C) of a GC-FID and desorbed for 10 minutes. Each sample was replicated three times.

3.3.5.2. Optimization of Extraction Time

Each fibre was exposed to the HS of the 1000 ml glass jar containing individual citrus fruit for different time periods (1, 2 and 4 hours). After exposure, the fibre was retrieved and injected into the heated (250°C) injection port of a GC-FID and desorbed for 10 minutes. Each sample was replicated thrice.

3.3.5.3. Optimization of Chambers Volume

To determine the most efficient extraction method of the VOCs emitted by citrus fruits, a comparison was made between different volumes of glass jars (500, 1000 and 2000 ml). The results showed that 500 ml glass jar volume achieved higher efficiency for VOCs extraction from all citrus fruit samples, so the 500 ml jar was chosen because it was efficient for capturing the VOCs emitted. Individually, citrus fruits were placed into the glass jar and the opening covered with aluminium foil and incubated at 25±1°C for 8 hours sealing time and 2 hours extraction time. Each sample was conducted in triplicate.

3.3.6. Gas Composition inside the Glass Jars

An Oxybaby gas analyser was used for monitoring headspace composition of the respiration of the citrus fruit during the sealing time in 500 ml glass jar. Three replicates were used to determine the gas composition.

3.3.7. Data Analysis

The GC data, including retention time and peak area, were collected and integrated into the chromatography software Agilent Chem-station, and then exported to Microsoft Excel for further analysis. The repeatability of replicates from the same sample was verified by checking the chromatogram pattern features such as detected peak retention times and peak areas. To compare volatile emissions between fruit species, the variance between peak areas was analysed. Differences in volatile emissions between non-infested and infested fruits were analysed using ANOVAs, followed by LCD tests.

3. 4. Results

3.4.1. O₂ and CO₂ Headspace Concentration

As a result of fruit respiration, the O₂ concentration decreased during the first two hours in varying proportions reaching 19.7% and 20.6% in orange fruit and lime, respectively. As for the lemon and mandarin fruit, oxygen consumption rate was the same as the control (20.2%) (Figure 3.1(a)). After 20 hours of sealing time, the oxygen level dropped to 18.8% and 20% for oranges and lime, respectively. Carbon dioxide had a lower accumulation in the first two hours of sealing time. After four hours of sealing time, CO₂ levels built up to 0.5% in lime and 1.1% in orange (Figure 3.1(b)). Carbon dioxide gas production continued to increase in all citrus fruit species and reached a level of 2.9%, 1.8%, 1.7% and 1.1% after 20 hours for orange, lemon, mandarin, and lime, respectively.

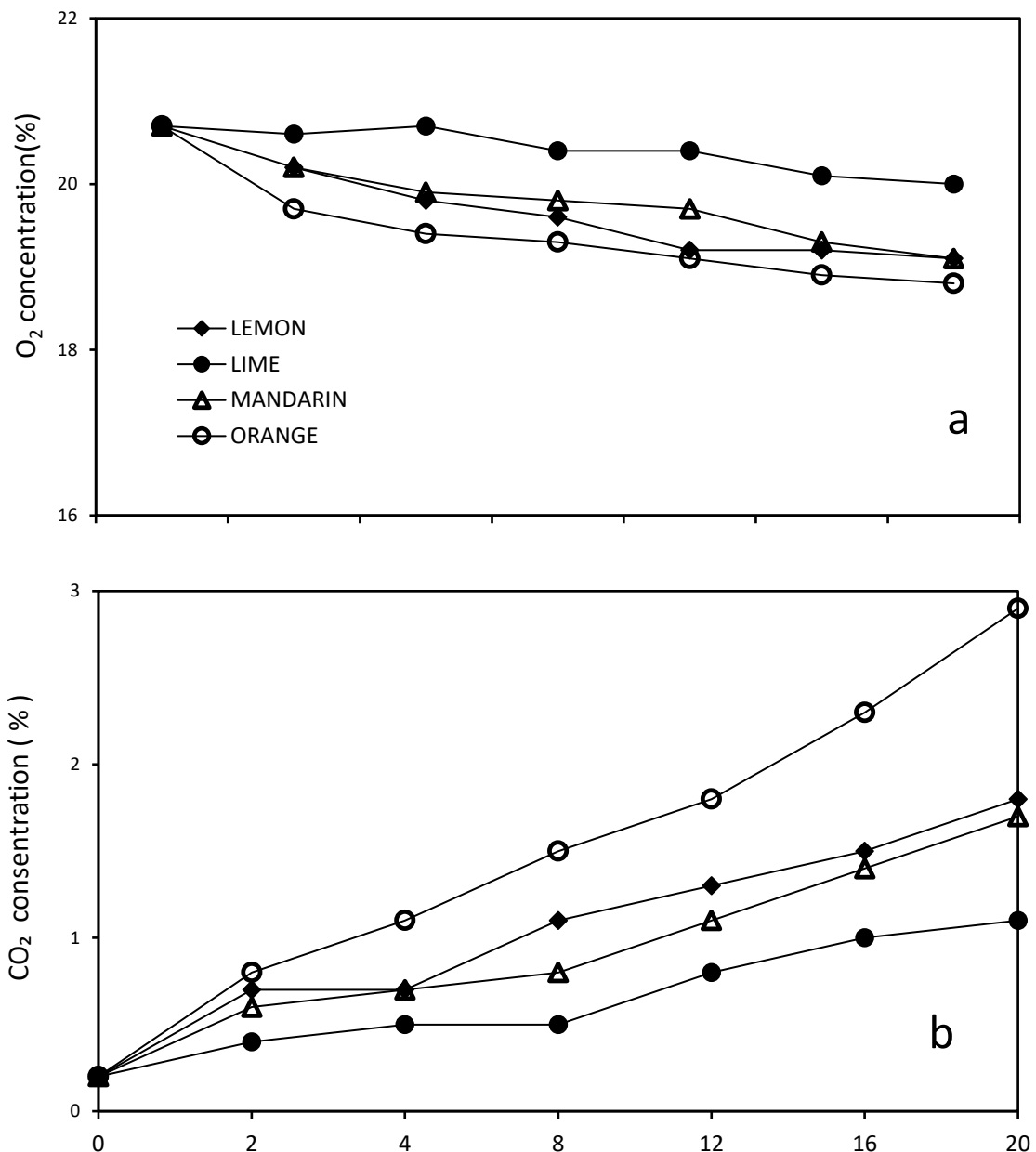


Figure 3.1. Effect of different sealing time on headspace gas composition O₂ (a) and CO₂ (b) of different citrus species.

3.4.2. Analysis of Volatile Organic Compounds from the Fruit of Different Citrus Species at Different Sealing Times

Total peak areas from the different samples sealed for 2, 4, 8, 12, 16 and 20 hours are compared in Figure 3.2. The levels of volatile compounds were significantly different between those collected at different sealing times. This result showed that 20, 16, 8 and 16 hours sealing period achieved higher efficiency for VOCs extraction from lemon, lime, mandarin and orange samples respectively. Therefore, the 20, 16, 8 and 16 hours sealing time for lemon, lime, mandarin, and orange respectively were selected for subsequent studies.

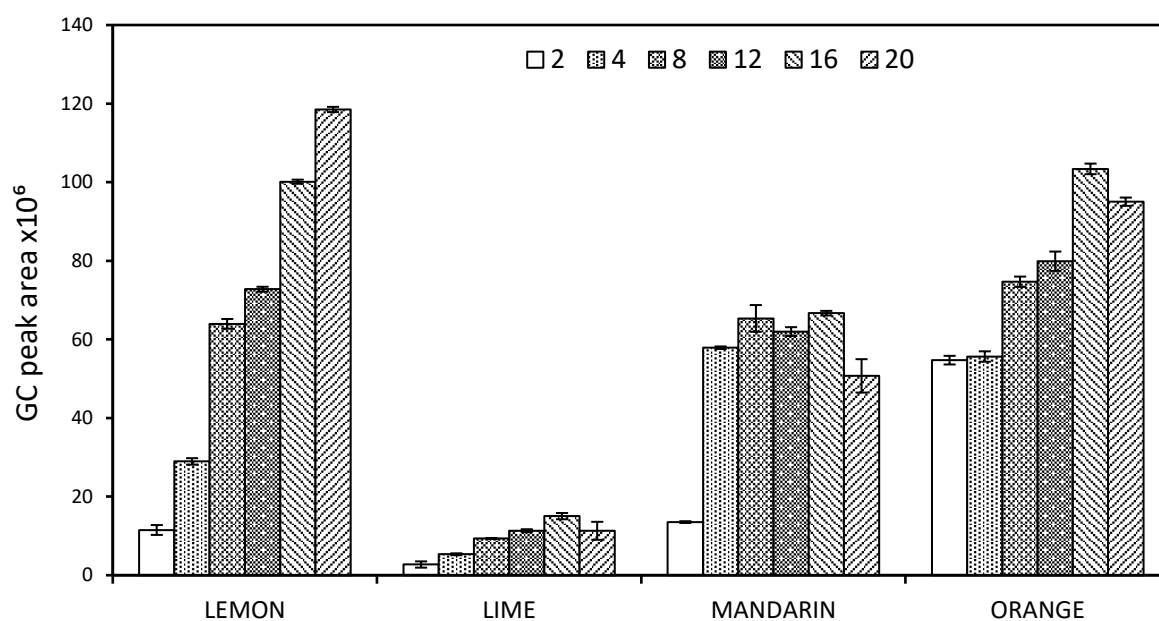


Figure 3.2. Peaks of volatile organic compounds (units) produced by different citrus fruit species with 2, 4, 8, 12, 16 and 20 hours sealing time in sample preparation. Error bars are LSD at 5% (n = 3).

3.4.3. Analysis of Volatile Organic Compounds from the Fruit of Different Citrus Species at Different Fibre Extraction Times

The amount of the volatile compounds did not differ significantly between those collected at two and four hours from lemon and mandarin, while there were significant differences between them and those collected at one hour (Figure 3.3). Therefore, two hours was selected for

subsequent studies for both lemon and mandarin. In contrast, there were significant differences in the amounts of VOCs produced at the different extraction times from lime and orange fruit (Figure 3.3). Therefore, two and four hours were selected for best extraction time to absorb the VOCs emitted from the lime and orange, respectively.

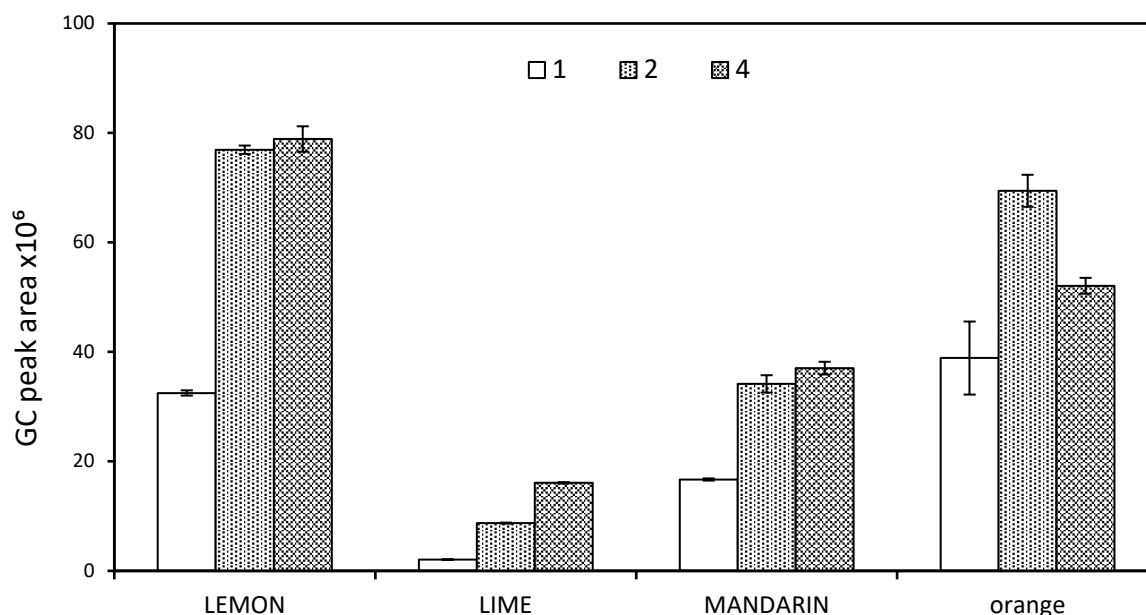


Figure 3.3. Effects of extraction time with citrus fruit species on the peak area of volatile organic compounds at 1, 2 and 4 hours. Error bars were LSD at 5% (n = 3).

3.4.4. Selection of Chamber Volume

The extraction efficiency of the three different chambers volumes (500, 1000 and 2000 ml glass jar) was evaluated by comparing the peak area of the eight compounds from all citrus fruit species under the same extraction time, SPME fibre, desorption time, and GC conditions. There were significant differences between the three different chambers size, so the 500 ml jar was chosen because it was optimum for capturing the released VOCs (Figure 3.4).

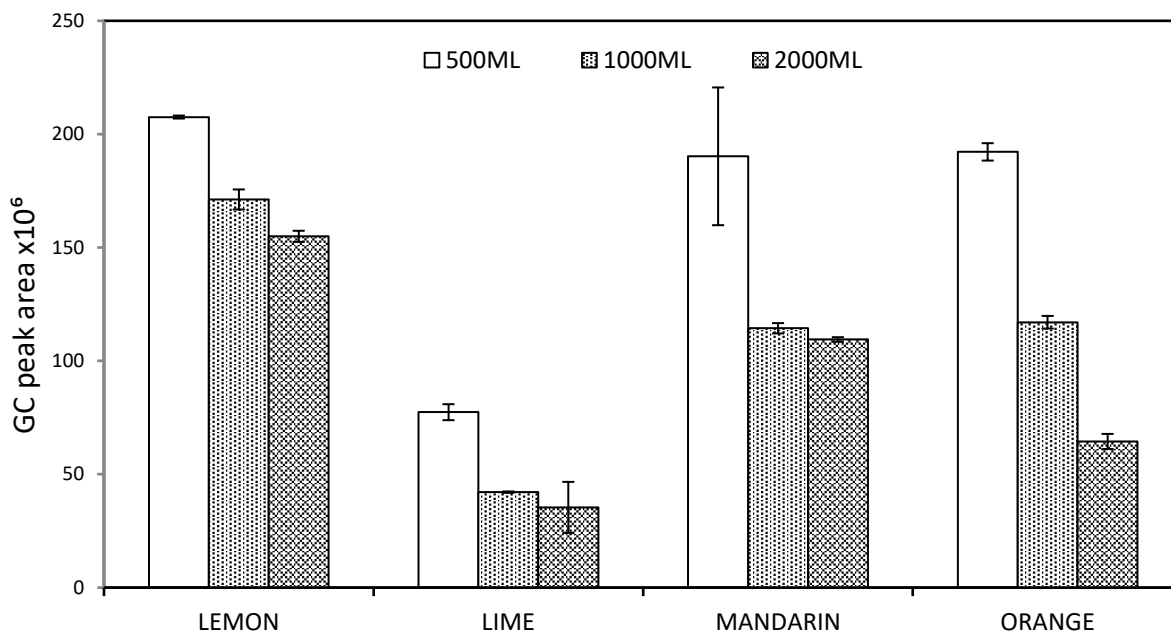


Figure 3.4. Peaks of volatile organic compounds (units) produced by different citrus fruit species extracted by three sizes of chambers (500, 1000 and 2000 ml). Error bars are LSD at 5% (n = 3).

3.5. Discussion

The concentration of the CO₂ and O₂ into glass jars is the main factor effect on the production of some volatile organic compounds related to the fruit's aroma (Both et al., 2014). Our results support previous findings in the literature which showed that O₂ consumption was directly related to CO₂ production (Almenar et al., 2007). Extremely low O₂ levels (0.5 kPa) decreased the emission of straight-chain esters related to the aroma of 'Royal Gala' apples (Both et al., 2014), while Barker (1928) found that carbon dioxide injury on oranges took the form of an unpleasant bitter flavour.

Most of our knowledge about citrus volatiles has been obtained from studies of processed juices and the peel essential oils, essence oils, and aqueous essences used to flavour juice products (Jia et al., 1998; Shaw, 1991). In contrast, optimization and extraction studies on aroma volatiles in fresh citrus fruit have rarely been reported. Optimization of isolation conditions was carried out using a one litter glass jar with two factors: time needed to reach equilibrium in the headspace and the fibre exposure time. Samples were analysed by GC-FID. The criteria were a higher number of peaks and greater total area of the chromatogram. The determination of the optimum time of sealing is essential to obtain maximum efficiency of the SPME fibres

for particular VOCs. The equilibrium between the citrus fruit species and its volatiles within the glass jar had an impact on the final volatile extraction by the SPME fibre. Normally if there is no significant difference between the sealing time, less sealing time is preferred, which agrees with the study by Facundo et al., (2013) who isolated a number of high-quality volatiles from the headspace of whole banana using 140 minutes sealing time compared with 15 minutes for banana pulp. In the present study a significant difference was observed between different sealing times, so a long sealing time of 20, 16, 8 and 16 hours for lemon, lime, mandarin, and orange, respectively was selected to isolate high-quality volatiles.

Extraction temperature and time are significant parameters in HS-SPME, since both influence the equilibrium during extraction of volatile compounds (Zhang et al., 2007). In this study, the optimal extraction time from the fibre for all citrus fruit species was two hours, except for lime at four hours which is longer than the time used by Nardini et al., (2013) who reported 40 minutes as the optimum extraction time for volatile compounds produced by some species of citrus fruit juice. The best conditions for isolating volatiles from the headspace of whole banana fruit was 120 minutes fibre exposure, while for the banana pulp the best conditions was a 60 minutes exposure time (Facundo et al., 2013). This difference is most likely due to the difference in the fruit extraction methods, the head space volume and extraction temperature, since the extraction time depends on the chemical nature of the compounds present, the distribution constant, the fibre polymeric phase, and to the size of the molecular mass (e.g., polyunsaturated fatty acids and other compounds are expected to require longer extraction times depending on their lower partitioning and diffusion coefficient). In the present study, the results indicated that there were differences between all citrus fruit species by total peak area. Apparently, more volatiles were emitted in two hours extraction time from orange fruit, and there were no significant differences between two and four hours extraction time from lemon and mandarin, while total peak area from lime fruit reached high levels after four hours extraction time (Figure 3.3), and this is because different species have different types and amounts of compounds. In general, all plants have the ability to emit VOCs, and the content and composition of these VOCs will depend on the plant species and plant organ.

There were significant differences between the three volumes of glass jars used to capture VOCs. The 500 ml glass jar proved to be the best size in capturing the optimum VOCs emitted from different citrus species, which agrees with the study by (Lu et al., 2014) who used a 2000 ml glass jar to extract a number of high-quality VOCs from 1500 g of peach and pear fruit.

3.6. Conclusion

This study concluded that headspace solid-phase microextraction combined with gas chromatography and flame ionization detection could be used to detect VOC/s from whole citrus fruit species without the need for cutting, or extracting the juice and essential oils and the optimum condition for sealing and extraction time, were 20, 16, 8 and 16 hours headspace equilibrium and 2, 4, 2 and 2 hours fibre exposure time for lemon, lime, mandarin, and orange, respectively. At the same time, the 500 ml jar was chosen because it was optimum for capturing the released VOCs.


Chapter Four

Behavioural responses of the parasitoid *Aphytis melinus* to volatiles organic compounds (VOCs) from *Aonidiella aurantii* on its host fruit Tahitian lime fruit *Citrus latifolia*

Statement of Contribution

Title of Paper	Behavioural responses of the parasitoid <i>Aphytis melinus</i> to volatiles organic compounds (VOCs) from <i>Aonidiella aurantii</i> on its host fruit Tahitian lime fruit <i>Citrus latifolia</i>
Publication Status	Published
Publication Details	Biological Control, 133. pp, 103-109.


Principal Author

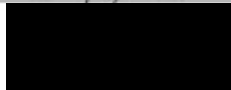
Name of Principal Author (Candidate)	Khalid Mohammed
Contribution to the Paper	Methodology, funding acquisition, formal analysis, investigation, data curation and writing—original draft preparation
Overall Percentage (%)	55%
Signature	 Date: 17/March/2020


Co-Author Contributions

By signing the statement of contribution, each author certifies that:


- The candidate's stated contribution to the publication is accurate (as detailed above).
- Permission is granted for the candidate to include the publication in the thesis
- The sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Overall Percentage (100%)	5%
Signature	 Date 17/March/2020

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Contribution to the Paper	Methodology, supervision, data curation and writing—review and editing
Overall Percentage (100%)	10%
Signature	 Date 17/March/2020

4.1 Abstract

Aphytis melinus DeBach (Hymenoptera: Aphelinidae) is a parasitoid of *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae). However, the cues used by *A. melinus* for host location have not been extensively investigated. Many studies have shown that mating parasitoids are strongly attracted by specific volatiles from infested plants. This paper investigated the response of *A. melinus* to volatiles from Tahitian lime fruit infested with *Ao. aurantii* through two-choice olfactometer bioassays. First, we identified the chemicals emitting from non-infested and *Ao. aurantii*-infested lime fruit via gas chromatography-mass spectrometry analyses and solid phase microextraction and identified 26 volatile organic compounds (VOCs). Eighteen of these were increased by *Ao. aurantii* infestation, and one was decreased, whereas five compounds were produced exclusively from infested fruit. Innate positive chemotaxis of mating *A. melinus* females toward lime fruit and their VOCs was then tested in olfactometer assays. Compared to the control without fruit, female *A. melinus* showed significantly greater attraction to healthy and *Ao. aurantii*-infested lime fruit, while there were no significant differences in the attraction of the parasitoid between healthy fruit or fruit infested with the second and third instar of *Ao. aurantii*. Among the two synthetic compounds tested, d-limonene and β -ocimene elicited a strong attraction for parasitoids at tested dosages (15 μ l/ml), indicating an intrinsic response to this compound as a short-range attractant. Results from this study suggest that *A. melinus* parasitoids mainly rely on olfactory cues in host habitat location and that d-limonene and β -ocimene are the major attractants in infested fruit volatiles. Practically, field applications of this volatile may play a significant role in attracting more *A. melinus* to enhance the efficiency of biological control of *Ao. aurantii* in the future.

4.2. Introduction

Limes are an attractive fruit sought after in many countries due to their unique flavour and acidity, and they also serve as a source of industrial and add-value food products (Bosquez-Molina et al., 2002). Australia is interested in growing many different varieties of lime with unique flavours for different uses and has recently developed commercial production of the Tahitian lime. Citrus genetic analysis indicates the origin of the Tahitian lime is southeast Asia, specifically the east and north-east of India, north Myanmar, southwest China and throughout the Malay Archipelago (Moore, 2001). These days, Tahitian limes are grown throughout tropical and subtropical areas of the world. Mexico is the top commercial producer (Crane and Osborne, 2013) followed by Brazil, Israel, and Australia (Roy et al., 1996).

California red scale, *Aonidiella aurantii* (Hemiptera: Diaspididae), is one of the most significant pests of citrus worldwide (Pekas, 2011b). In Australia, California red scale is considered a key pest and its control is part of an entire pest management program (Papacek, 2006). The use of pesticides to control the scale is difficult, as treatment is often followed by short periods of infestations, highlighting the pest's ability for resistance to various chemicals and the impact chemical control has on the natural enemies of the scale in the field (Pekas, 2011a). Efforts are currently focussing on enhancing biological control programs using synthetic VOCs and finding ways these compounds can be used commercially, with many of them now contributing to established practices in Integrated Pest Management (IPM) (Witzgall et al., 2010). The ectoparasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) is a key natural enemy of *Ao. aurantii* (Sorribas and Garcia-Mari, 2010). It was introduced into Australia in 1961 from the University of California, Riverside by CJR Johnston (Malipatil et al., 2000) and has been used ever since to control California red scale. Currently, several commercial insectaries produce *A. melinus* to meet the demands of citrus growers wishing to use biological control for *Ao. aurantii* (Grafton-Cardwell et al., 2013).

Plants are continuously under a wide array of biotic and abiotic stresses, of which biotic stress due to herbivorous insects is a significant constraint in crop production (Sharma et al., 2009). When plants are infested upon by insects, there is an herbivore-induced release of volatiles from the plants which subsequently provides reliable host location cues for the natural predators and parasitoids of these pests, a phenomenon known as an indirect defence of plants (Zimba et al., 2015, Lin et al., 2016, Morawo, 2017). Plant volatiles are detectable as long-range cues for foraging parasitoids to locate host plant patches (Steidle and Van Loon, 2003) whereas these herbivore-specific odours are considered as reliable short-range indicators of host presence. Parasitoids likely resolve the detectability-reliability problem by using plant volatiles as long-range cues and herbivore odours as short-range cues (Obonyo et al., 2010, Colazza et al., 2014). Natural selection should favour the development of highly adaptive host location strategies. Consequently, parasitoids use olfactory cues to locate their hosts and which could potentially be applied to the biological control of target pest species (Uefune et al., 2012, Zimba et al., 2015, Giunti et al., 2016a). There have been a plethora of studies looking at how parasitoid foraging is enhanced through the use of cues derived from plants infested with pests, but there is a lack of specific research into the attraction of *A. melinus* due to VOCs emitted from citrus infested with *Ao. aurantii* (Mohammed et al., 2017a).

To test the hypothesis that *A. melinus* uses host-associated olfactory cues for host location, this study first evaluated the use of the HS-SPME method coupled with the GC-MS technique as potential technologies for identifying the effect of California red scale herbivory on VOCs emitted from the fruit of the Tahitian lime. Secondly, we evaluated the preferences of female *A. melinus* for healthy or infested fruit in a two-choice bioassay; then, we tested the innate attractiveness to these females of d-limonene and β -ocimene in varying concentrations. This study highlights the potential practical application of the development of attractants for *A. melinus* to enhance its efficiency as a bio-control agent against *Ao. aurantii*.

4. 3. Materials and methods

4.3.1. Plant material

Fruit from ten-year-old Tahitian lime trees were collected in September 2017, from Western Australia (31.95°S 116.03°E 70 m AMSL). Trees used sprinkler irrigation systems, and no insecticides were applied. Infested and non-infested fruits were collected manually, stored in jars (diameter 10 cm, length 20 cm) and transferred to laboratory conditions within one hour. Although there was no evidence of *A. melinus* orientation toward California red scale sex pheromone (Morgan and Hare, 1998, Pekas et al., 2015), there were no virgin females among scales on the infested lime fruit. Among infested fruits, we selected those attacked the most by second and early third instar nymphs. Healthy fruits were chosen by avoiding crushed and naturally damaged specimens. Before being tested, fruits were stored in laboratory conditions for 5–7 days.

4.3.2. Insect colonies

The California red scale *Ao. aurantii* culture was established by rearing from an infested citrus orchard located in Western Australia (32.30°S 116.01°E 69 m AMSL) in 2017. Laboratory cultures of California red scale were reared on butternut squash (*Cucurbita moschata* Duchesne ex Lamarck) in the Murdoch University insect culture room. Fresh pupa of *A. melinus* was purchased from the Biological Services commercial insectary (Adelaide, Australia), and maintained under laboratory conditions (26°C, 40–60% RH, L16: D8 photoperiod) until adult emergence. *A. melinus* is an ectoparasitoid, so pupae could be easily isolated from the host without disrupting their development. From adult emergence until their use in bioassays, parasitoids were held in chambers (1 cm diameter, 4 cm length) and were supplied honey for nutrition. All parasitoids used in bioassays were naïve females without previous contact with

suitable hosts, i.e. infested or non-infested fruits. After placing parasitoids in the conditioning vial for a day, parasitoids were removed and placed in an empty chamber. Female parasitoids, on the day of bioassay, were isolated and held individually in a glass chamber with a drop of honey until tested.

4.3.3. Headspace volatile entrainments

Samples of Tahitian lime fruit, either infested or not, were placed separately into a 500 ml glass jar and left to equilibrate gases for 16 hours. Volatiles were collected by a solid-phase microextraction (SPME) fibre with 50/30 μm Carboxen/DVB/PDMS (2 cm) (Sigma-Aldrich, Bellefonte, USA) coating. VOCs were collected and trapped by inserting the SPME into the glass jar and exposing it to the headspace. After two hours at room temperature, the fibre was withdrawn into the needle and inserted into the GC–MS injection port system and released directly onto the column by thermal desorption as described by Mohammed et al. (2017b). The desorption time of the SPME fibre was ten minutes in the injection port (Mohammed et al., 2017a). Six replicates were conducted for each treatment. Tested fruit were of uniform weight and similar to those used in the behavioural assays in terms of freshness and maturity, with each sample consisting of one fruit weighing about 150 g. Infested citrus were selected with 300–400 second and early third instars of *Ao. aurantii*. Only intact fruit were selected, crushed or otherwise damaged fruits were discarded. Empty glass jars were used as control and was treated to the same conditions as used for healthy and infested fruit.

4.3.4. Coupled gas chromatography-mass spectrometry

Volatile organic compounds were thermally desorbed and analysed with gas chromatography Agilent GCMS 7820A coupled to a mass spectrometer detector 5977E (Agilent Technologies, USA) and a DB 35 ms capillary column (30 m \times 250 μm \times 0.25 μm) (Santa Clara, CA 95051, USA). The carrier gas was 99.99% helium supplied by (BOC Gas, Sydney, Australia). The GC-MS operation conditions were programmed as follows: The temperature of the injector port was 250°C. The oven temperature programmed at 60°C and increased to 270°C by (5°C/min). The flow rate of the column was 1:1 ml/min and the splitless injection was 20 ml/min at 1.5 min. The total GC-MS run time was 45.40 min. Compound peaks were deconvoluted by AMDIS version 2.72 and identified by searching the NIST 2014 MS database (the US National Institute of Standards and Technology) with retention index confirmation. The experiment was repeated two times to confirm the chemicals.

4.3.5. Bioassays

A glass Y-tube olfactometer was used to record behavioural responses of *A. melinus* to pairs of different volatile sources. To assess if the synthetic HIPVs or infested Tahitian lime fruits were attractive for *A. melinus*, a parasitoid was considered to choose a cue option when it remained in the same chamber for at least 20 seconds searching actively for a host, and the replicate was deemed to be complete when the parasitoid left the chamber. Parasitoids that had not made a choice two minutes later were not considered. Two synthetic HIPVs (d-limonene purity 97%, 98% EE (GLC) purchased from Sigma-Aldrich and β -ocimene purity $\geq 90\%$ purchased from Toronto Research Chemicals), were selected to evaluate their role in attracting *A. melinus* towards their host. The concentrations were adopted to simulate the natural airborne concentrations of these compounds. Tested dose of chemicals was selected by using β -ocimene standard. The peak area of β -ocimene and d-limonene in mass chromatogram was calibrated with serial dilution (1 μ l to 15 μ l) of β -ocimene standard and injecting each of them into gas chromatography and extrapolating the sample concentration from the standard curve. To assess the attraction of synthetic HIPVs, two different concentrations of d-limonene and β -ocimene (5 and 15 μ l/ml) were dissolved in hexane and offered to mating females in Y-tube bioassays. In each trial, 5 ml of solution was applied to Whatman 90 mm filter paper and placed in a 1-litre desiccator. After solvent evaporation (ca. 20 s), the solution was connected to an arm of the Y-tube; the other arm received 5 ml of pure hexane additive to a Whatman 90 mm filter paper as a control. In the first bioassay, parasitoids were given a choice between healthy Tahitian lime fruit (i.e., fruit with no mechanical or herbivore damage) versus infested Tahitian lime fruit (i.e., lime fruit infested with *Ao. aurantii* second and third instars). In the second bioassay, parasitoids were given a choice between healthy or infested Tahitian lime fruit versus a blank. In the third bioassay, parasitoids were given a choice between 5 and 15 μ l/ml of d-limonene and β -ocimene, respectively, and the additive on Whatman 90 mm filter paper versus just the hexane additive on the filter paper. The set-up of the Y-tube olfactometer was comparable to that previously described by Morgan and Hare (1998) with slight modifications. Briefly, the Y-tube olfactometer had an internal diameter of 2.4 cm, a 15 cm stem and 7 cm lateral arms at a 60° angle with ground glass fittings through which humidified air was passed (0.5 ml/s through each arm, controlled by up-stream flow meters). An extending glass tube was connected to each arm (2.4 cm diameter 15 cm long). The extending glass tube prevented the escape of insects. The air was pumped through Teflon tubing, purified by the passage through activated charcoal, then passed through a chamber into which fruit and insect material could

be introduced. The air then passed through the olfactometer and once assembled, the apparatus was left to stabilise for 15 minutes prior to use.

All tests were performed in a constant-temperature room at $24 \pm 1^\circ\text{C}$. The Y-tube was lit from above by fluorescent lamps, and the surrounding areas (around and below) were shrouded by placing a white paper around the Y-tube to block out any visual cues. For each bioassay, a single female parasitoid was introduced into the central arm tube of the olfactometer. Once the parasitoid reached the arm divide where she had to make a choice, a timer was started. If the parasitoid remained stationary for more than two minutes, it was considered to be generally unresponsive, and was excluded from the assays and replaced with a new specimen.

For each replicate, seven parasitoids were assayed. After each replicate, and after removing the odour sources, the apparatus was cleaned with water and ethanol, then dried and heated in an oven at 60°C for 30 min between replicates, and overnight between treatments. Then, new fresh sources were introduced into the aeration chambers, and another replicate of seven parasitoids were assayed. Fresh and ripe Tahitian lime fruit were tested in bioassays to reduce the variation in volatile emission rates between replicates. The parasitoids efficiency recordings were used to assess the overall activity of wasps and determine the preference for each choice. Activity was assessed by summing the number of entries into both arms by each parasitoid.

4.3.6. Statistical analysis

To compare volatile emissions between infested and non-infested fruit, the variance between peak areas was analysed for each compound. Differences in volatile emissions between non-infested and infested fruits were analysed using ANOVAs, followed by Tukey's post hoc tests. Principal Component Analysis (PCA) was achieved on normalised values of each VOC to derive different variables (principal components) by summarising the original data. PCA was performed using Metaboanalyst 3.0 (a comprehensive online tool suite for metabolomics data analysis). To compare the parasitoid proportion that chose a specific cue, a likelihood chi-square test with Yates correction (with $\alpha=0.05$) was used for each choice-test.

4.4. Results

4.4.1. Identification of volatiles

Tahitian lime fruit infested with *Ao. aurantii* emitted higher levels of volatile compounds compared with non-infested lime fruit. A total of 26 compounds were identified emanating

from non-infested and infested fruit. Compounds commonly associated with earth's atmosphere (e.g., toluene and benzene), as well as compounds associated with the analytical system (e.g., siloxanes or phthalates), were excluded from the list. These compounds were also found in control samples. The complete list of identified chemicals is presented in (Table 4.1). The PCA (Fig. 4.1) showed that the number of volatile compounds from infested fruit was higher than those from non-infested fruit. The separation of some replicates from the rest is due to varied performance with the fibres. It can be emphasized that two oval zones presented data in 95% statistical confidential intervals, which means it looks far from others, but there was statistical significant difference between it and the rest. Differential VOCs emissions attributable to pest activity were found. Eighteen compounds significantly increased in infested lime fruit, and these compounds were: oxime-, methoxy-phenyl- (F = 8.50; $P = 0.016$), β -terpinene (F = 11.86; $P = 0.001$), β -myrcene (F = 5.41; $P = 0.001$), isoterpinolene (F = 2.07; $P \leq 0.001$), d-limonene (F = 6.94; $P \leq 0.002$), β -cis-ocimene (F = 2.41; $P = 0.001$), β -ocimene (F = 0.03; $P \leq 0.001$), γ -terpinene (F = 2.43; $P \leq 0.001$), terpinolene (F = 0.851; $P \leq 0.001$), (E)-4,8-dimethylnona-1,3,7-triene (F = 0.92; $P \leq 0.001$), nonanal (F = 1.48; $P \leq 0.001$), cosmene (F = 2.81; $P \leq 0.001$), methyl salicylate (F = 0.82; $P \leq 0.001$), undecane, 4,8-dimethyl- (F = 10.16; $P = 0.001$), caryophyllene (F = 10.39; $P = 0.006$), δ -selinene (F = 0.14; $P = 0.004$), (Z,E)- α -farnesene (F = 10.52; $P \leq 0.001$), 7-epi- α -Selinene (F = 0.50; $P \leq 0.001$). In contrast, just linalool (F = 1.37; $P \leq 0.042$) decreased during infestation and were characteristic of uninfested lime fruits. Five volatiles were exclusively produced by infested fruits and these volatiles were β Thujene (F = 10.40; $P \leq 0.001$), trans-d-limonene oxide (F = 16.00; $P \leq 0.001$), 1-isopropenyl-3-propenylcyclopentane (F = 16.00; $P \leq 0.001$), terpinen-4-ol (F = 16.00; $P \leq 0.001$) and trans- γ -bisabolene (F = 10.26; $P \leq 0.001$).

Table 4.1. Mean (mean \pm SE) peak area values of VOCs (n = 6) emitted by non-infested and *Aonidiella aurantii*-infested Tahitian lime fruits

RT	RI	COMPOUNDS	F	P Value	Infested fruit Mean \pm SE (s)	Healthy fruit Mean \pm SE (s)
4.355	1301	oxime-, methoxy-phenyl-	8.50	0.016	98.52 \pm 11.85 A	50.57 \pm 1.54 B
4.921	966	β -thujene	10.40	<0.001	62.95 \pm 2.68 A	0 \pm 0 B
5.828	1028	β -terpinene	11.68	<0.001	30.01 \pm 0.28 A	2.63 \pm 0 B
6.131	991	β -myrcene	5.41	0.001	154.11 \pm 13.15 A	19.34 \pm 4.29 B
6.686	1086	isoterpinolene	2.07	<0.001	82.43 \pm 2.09 A	8.91 \pm 1.11 B
6.850	1119	1,3,8-p-menthatriene	1.08	0.889	35.88 \pm 6.80 A	37.91 \pm 11.78 A
6.951	1018	d-limonene	6.94	0.002	988.50 \pm 23.05 A	270.84 \pm 94.39 B
7.100	1038	β -cis-ocimene	2.41	0.001	242.77 \pm 21.90 A	27.85 \pm 5.29 B
7.329	1037	β -ocimene	0.03	<0.001	169.39 \pm 2.56 A	118.32 \pm 2.44 B
7.566	1060	γ -terpinene	2.43	<0.001	457.59 \pm 17.81 A	26.82 \pm 6.32 B
8.216	1088	terpinolene	0.851	<0.001	115.75 \pm 2.13 A	17.98 \pm 4.56 B
8.334	1116	(E)-4,8-dimethylnona-1,3,7-triene	0.92	<0.001	22.91 \pm 0.31 A	9.79 \pm 0.69 B
8.422	1099	linalool	1.37	0.042	24.52 \pm 5.51 B	54.33 \pm 8.51 A
8.498	1104	nonanal	1.48	<0.001	60.43 \pm 1.61 A	17.77 \pm 2.77 B
8.881	1131	cosmene	2.81	<0.001	311.92 \pm 3.69 A	5.92 \pm 1.80 B
9.263	1139	trans-d--limonene oxide	16.00	<0.001	5.63 \pm 0.01 A	0 \pm 0 B
10.001	1095	1-isopropenyl-3-propenylcyclopentane	16.00	<0.001	6.48 \pm 0.15 A	0 \pm 0 B
10.084	1177	terpinen-4-ol	16.00	<0.001	11.15 \pm 0.28 A	0 \pm 0 B
10.462	1192	methyl salicylate	0.82	<0.001	10.31 \pm 0.25 A	2.60 \pm 0.34 B
12.450	1212	undecane, 4,8-dimethyl-	10.16	0.001	6.19 \pm 0.06 A	2.99 \pm 0.33 B
14.834	1419	caryophyllene	10.39	0.006	28.76 \pm 4.53 A	4.73 \pm 0.81 B
15.364	1523	7-epi-cis-sesquisabinene hydrate	7.21	0.177	5.73 \pm 2.87 A	0.84 \pm 0.84 A
15.974	1493	δ -Selinene	0.14	0.004	147.85 \pm 7.26 A	91.01 \pm 5.94 B
16.304	1491	(Z,E)- α -farnesene	10.52	<0.001	259.07 \pm 15.82 A	13.70 \pm 2.24 B
16.584	1517	7-epi- α -selinene	0.50	<0.001	19.81 \pm 0.68 A	7.77 \pm 0.45 B
18.221	1533	trans- γ -bisabolene	10.26	<0.001	12.54 \pm 0.98 A	0 \pm 0 B

Values in the same row followed by different letters are significantly different. A and B indicates significant at $P < 0.05$; RT is retention times; RI is retention index; F is F statistic; SE is the standard error.

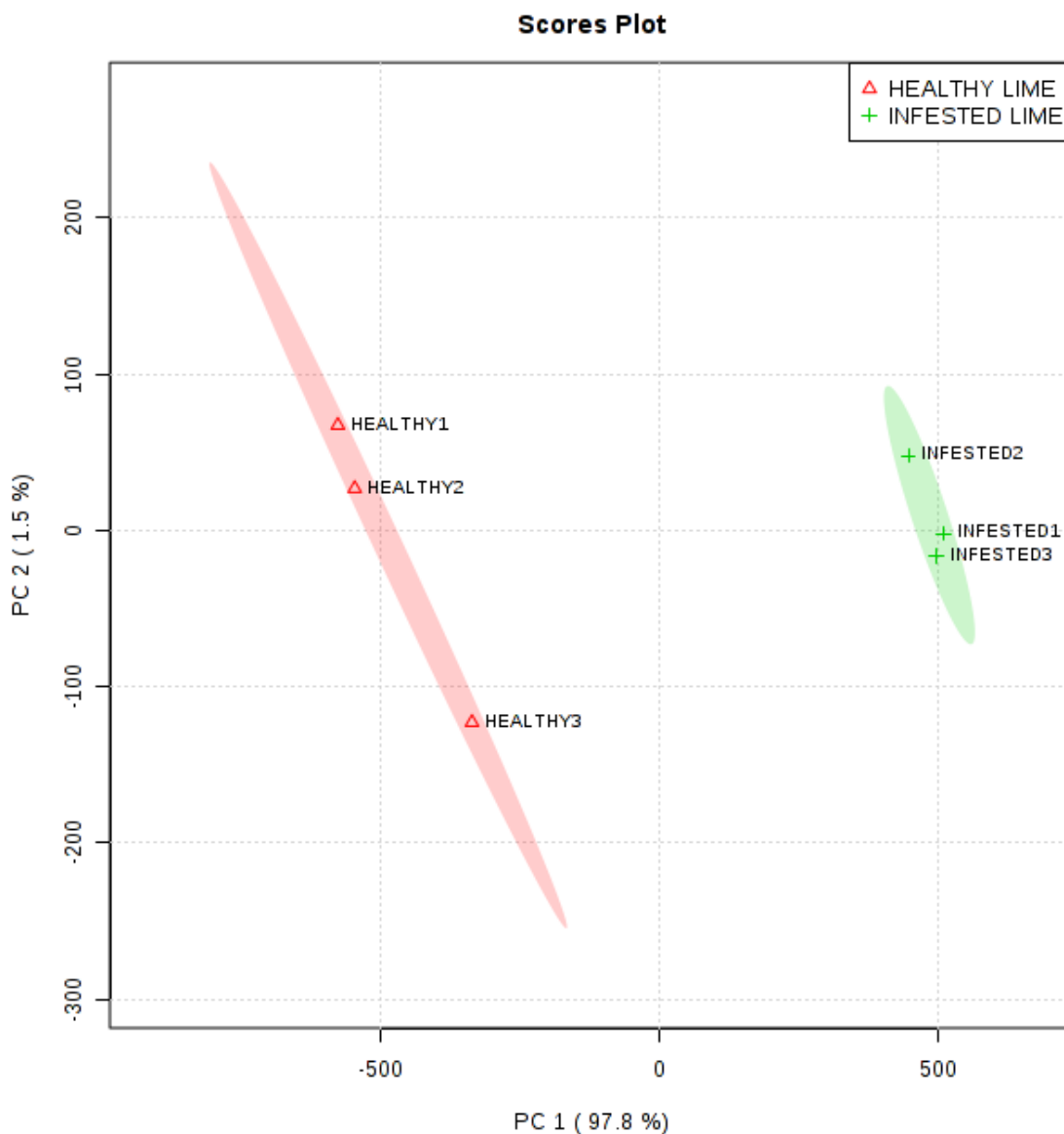


Figure 4.1. Score plot shows the separation pattern of metabolic data obtained from three biological samples of non-infested and *Ao. aurantii*-infested Tahitian lime fruits

All chemical classes were increased in infested lime fruit. Monoterpenes ($F = 14.475$; $P \leq 0.001$), represented 35.37% of total volatile emissions, sesquiterpenes ($F = 23.482$; $P \leq 0.001$), represented 15.17%, and hydrocarbons ($F = 7021.591$; $P \leq 0.001$), represented 30.55%, while other compounds present 18.9% (Table 4.2).

Table 4.2. Mean (mean± SE) peak areas (n = 6) of various VOC chemical classes emitted from non-infested and *Aonidiella aurantii*-infested Tahitian lime fruits.

Chemical class	Infested lime(±SE)	Uninfested lime(±SE)	F	P Value
Hydrocarbons	117.67± 48.72A	15.61± 6.57B	7021.591	≤ 0.001
Aldehydes	79.48 ± 10.07 A	34.17 ± 7.47 A	0.228	0.644
Alcohols	70.13± 12.73A	15.50 ± 4.73B	16.835	≤ 0.002
Esters	3.44±0.08 A	0.86±0.12 A	0.878	0.420
Monoterpene	184.10± 42.41A	41.73± 13.29B	14.475	≤ 0.001
Sesquiterpene	78.96± 22.93A	19.68± 7.87B	23.482	≤ 0.001

Values in the same row followed by different letters are significantly different. A and B indicates significant at $P < 0.05$; F is; F statistic; SE is the standard error.

4.4.2. Olfactometer assays of *Aphytis melinus*

The results of the olfactometer assays that tested the behaviour of *A. melinus* to the different compounds emitted from infested and non-infested lime fruit are shown in (Figure 4.2). For mated female *A. melinus* parasitoids, no significant differences were recorded in the parasitoids' choice for infested fruits over healthy ones ($\chi^2 = 0.429$, $df = 1$, $P = 0.513$) (Figure 4.2). Of the 21 wasps, nine preferred healthy fruit and 12 chose *Ao. aurantii* infested fruit (Figure 4.2). Female wasps were significantly more attracted to volatiles emitted by healthy limes than to the control ($\chi^2 = 8.048$, $df = 1$, $P = 0.005$), and preferred volatiles emitted by infested over those emitted by the control ($\chi^2 = 10.714$, $df = 1$, $P = 0.001$) (Figure 4.2).

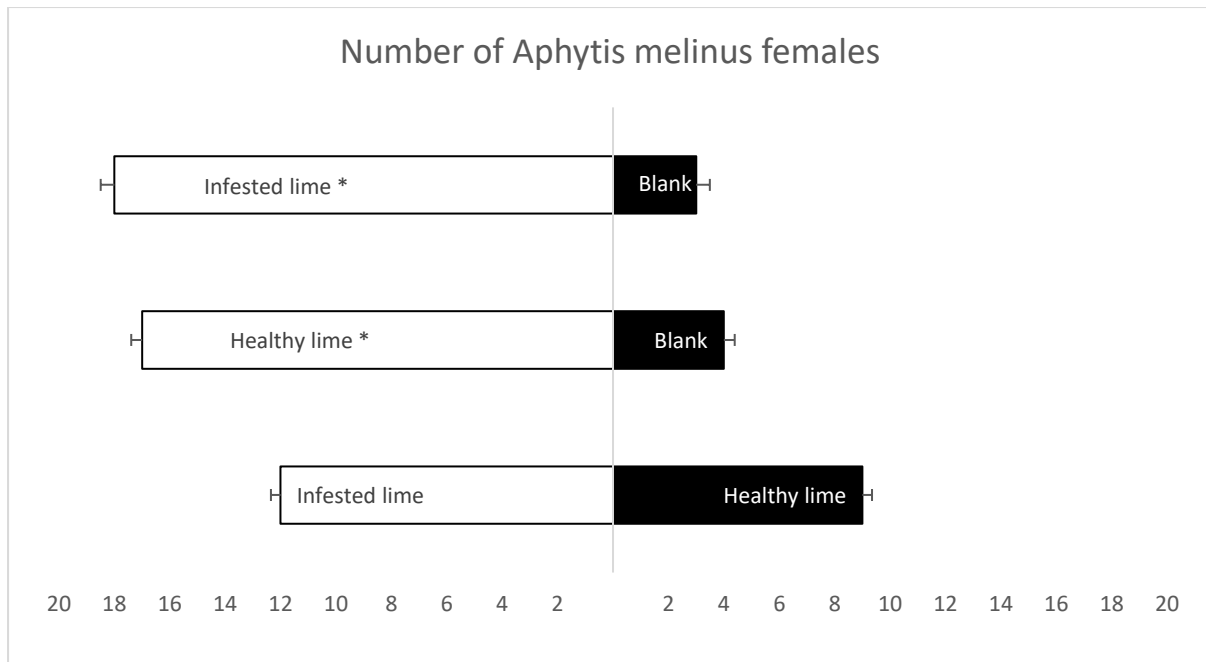


Figure 4.2. The attractiveness of *Aonidiella aurantii*-infested fruits towards *Aphytis melinus* females. Two-choice bioassays were conducted with different combinations of blank and lime fruit, infested or not by California red scale. Seven wasps were tested in each replicate; three replicates were done. For each test, asterisks indicate significant differences in the number of wasps landing on each cue (χ^2 test with Yates correction, $P < 0.05$).

Mated *A. melinus* females, with no previous oviposition experience, were not significantly attracted by d-limonene at tested dosages (5 $\mu\text{l/ml}$) ($\chi^2 = 0.429$, $\text{df} = 1$, $P = 0.513$), whereas significant differences in preferences between d-limonene at tested dosages (15 $\mu\text{l/ml}$) ($\chi^2 = 3.857$, $\text{df} = 1$, $P = 0.050$) and the control were observed for mated parasitoids. Similarly, β -ocimene was less attractive to *A. melinus* at tested dosages (5 $\mu\text{l/ml}$) ($\chi^2 = 2.333$, $\text{df} = 1$, $P = 0.127$) than β -ocimene at tested dosages (15 $\mu\text{l/ml}$) ($\chi^2 = 8.048$, $\text{df} = 1$, $P = 0.005$) (Figure 4.3). Only four parasitoids were attracted to the control while 17 preferred β -ocimene at tested dosages (15 $\mu\text{l/ml}$).

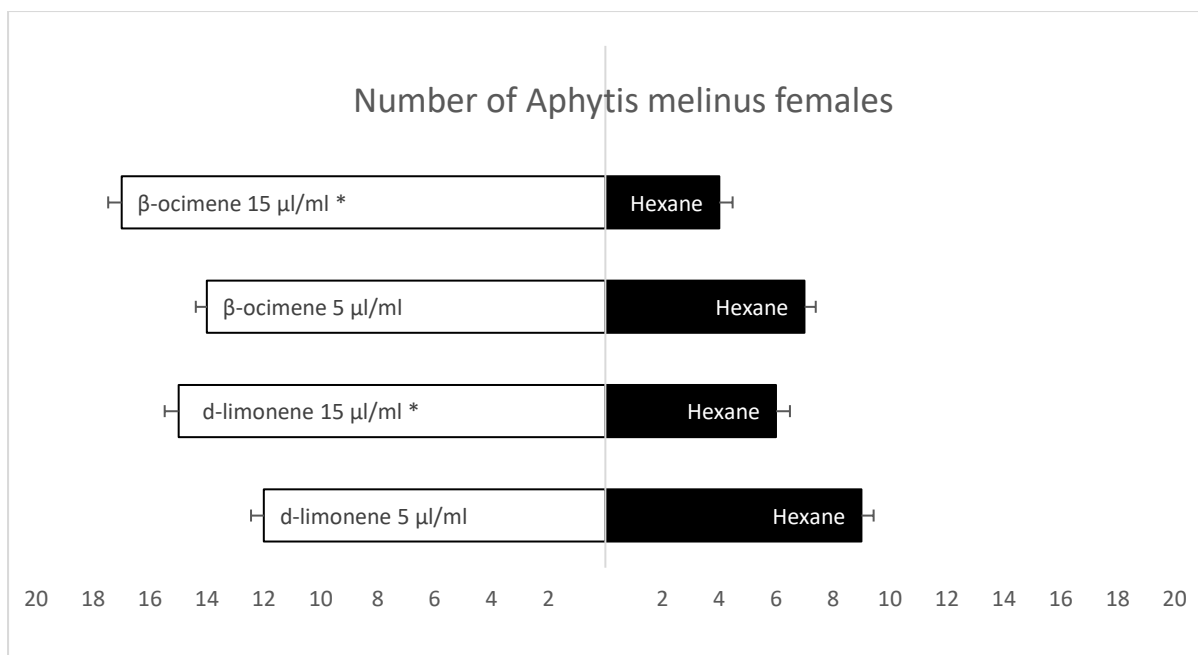


Figure 4.3. Behavioural responses of *Aphytis melinus* females in two-choice bioassays conducted with synthetic HIPV chemicals versus hexane. Twenty-one wasps were tested in each experiment. For each experiment, asterisks indicate significant differences in the number of females landing on the two given cues (χ^2 test with Yates correction, $P < 0.05$).

4.5. Discussion

There have been several studies on volatile emissions of non-infested lime fruit (Mohammed et al., 2017a, b). Our chemical analysis of the headspace of non-infested and infested Tahitian lime fruit provided similar results to these previous studies. However, we also detected volatile chemicals that had not been previously identified in the headspace of lime fruits. To determine if the change in volatile emissions could explain parasitoid behaviour, *Ao. aurantii*-infested and healthy lime fruits were analysed. Among 26 VOCs identified by SPME and GC-MS techniques, 18 volatiles were found to increase in infested lime fruit. Most of these are already known as constituents of lime fruit odours (Atti-Santos et al., 2005, Mohammed et al., 2017a), although we noticed quantitative changes in VOCs emissions among infested and non-infested limes which is not uncommon even in similar tritrophic systems (Hern and Dorn, 2002). Indeed, several plants react to pest infestation by producing blends of metabolites with changes in their proportions (Giunti et al., 2016a).

Olfactory stimuli from infested fruit are known to be essential during host location behaviour for many parasitoids (Zimba et al., 2015, Giunti et al., 2016a). In this paper, we investigated the capability of *A. melinus* to respond to plant chemical cues associated with the damage induced by a phytophagous pest. Host selection conventionally consists of three steps: host habitat and host location, host recognition and acceptance and host suitability/regulation (Chesnais et al., 2015). Plants under herbivore attack emit abundant and volatile odours that are cues, highly detectable over long distances by natural enemies, mainly applicable to the host habitat/community and host location step. Biological assays conducted in the olfactometer showed that *A. melinus* somewhat, but not significantly, orientated preferentially towards the source of HIPVs (i.e. a lime fruit infested by *Ao. aurantii*) compared to non-infested fruit, suggesting that the presence of *Ao. aurantii* on the fruit is important for host location. Simultaneously, we observed that *A. melinus* orientated significantly towards non-infested fruit compared to the control (empty chamber), and slightly more to the infested fruit. VOCs cues attract female *A. melinus* toward both infested and non-infested host plant. Parasitoids learned these cues by associating odours from the host plant with host presence. They had no natural preferences for scale insect or host plant volatile stimuli. Contrary to previous studies, we found no evidence of orientation toward VOCs cues from the attacked host stage (Morgan and Hare, 1998). Despite significant differences in the amount of VOCs emitted from infested and non-infested fruit, in these biological assays, a somewhat higher number of individuals moved to odour sources emitting from infested fruit, probably because the amounts of VOCs released in these systems were still high compared to infested fruit. This result further suggests that healthy fruit can produce volatiles that may act as attractants of *A. melinus*, but these volatiles may be down-regulated by *Ao. aurantii* through volatile profile modification, which plays a key role in the host location behaviour of *A. melinus* parasitoids. The presence of chemicals produced exclusively, or in a higher amount, by infested lime fruit, and the evoking of behavioural responses of mating *A. melinus* females by VOCs from infested fruit, supports our hypothesis that VOCs could, over a short-range, increase the percentage of attraction, and play a pivotal role during host-seeking. This finding suggests the presence of short distance cues from infested fruit elicit a searching activity in parasitoids. The relative importance of long-range and short-range cues to the parasitoid host location process is well known (Wang et al., 2010, Morawo, 2017). Plants under herbivore attack emit abundant and volatile odours containing cues that are highly detectable over long distances by natural enemies and are mainly related to the host habitat/ community and host location step. Over a short-range, in terms of parasitoid host recognition and acceptance, natural enemies might search for more parasitoid host-specific

chemicals. Odours derived from parasitoid host are highly reliable as they precisely indicate the host species, although they are less volatile and abundant. Thus, parasitoid host odours commonly function as cues for natural enemies, but only over short distances (Colazza et al., 2014). For example, it has been reported that cues in hosts were more important in short-range host recognition for the parasitoid *Cotesia sesamiae* (Cameron) while host plant stimuli served as important long-range indicators of the presence of its host, *Busseola fusca* (Fuller) (Obonyo et al., 2010).

Although most VOCs are present in healthy fruit, the emission rate for some is elevated by herbivory of *Ao. aurantii*. Among these volatiles, d-limonene and β -ocimene seemed to be elevated and thus are considered as important compounds in the process of attracting natural enemies towards the pests preying on the host plants (Zimba et al., 2015, Lin et al., 2016,). The two compounds are among the major terpenes found in citrus fruit rind (Hosni et al., 2010; Chamberlain et al., 2012), and have been described previously as a constituent of lime fruit (Atti-Santos et al., 2005, Mohammed et al., 2017a). However, since β -ocimene and d-limonene have been demonstrated to attract many species of parasitoids (Kang et al., 2018, Zimba et al., 2015, Lin et al., 2016,), these two compounds can be considered as putative kairomones for *A. melinus*. Several studies have shown that increased emission of d-limonene and ocimene constitutes an indirect defense system in plants, as they attract natural enemies of herbivorous insects (Zimba et al., 2015). Both these synthetic volatile compounds associated with *Ao. aurantii* infested fruit elicited almost similar responses to *A. melinus* parasitoids in the Y-tube bioassays. d-limonene and β -ocimene at tested dosages (15 μ l/ml) were significantly attractive to female parasitoids compared with solvent stimulus, suggesting their role as attractants in infested fruit volatiles. Apart from this, these are found not only in lime fruit, but in other citrus species (Zhang et al., 2017, Sarrou et al., 2013). Some compounds; for example, methyl salicylate which are significantly higher in infested limes has not been selected as there are already many studies on its roles in attraction of natural enemies (Gadino et al., 2012, Mallinger et al., 2011), and also it has not been detected in the most of the healthy citrus fruit species (Lin et al., 2016,). These two synthetic compounds d-limonene and β -ocimene were therefore found to have a significant impact on *A. melinus* behaviour, particularly in the search for a host on infested lime plants. Overall, this study demonstrated that *Ao. aurantii* induced fruit volatiles, particularly d-limonene and β -ocimene, play a key role in aiding *A. melinus* female parasitoids to locate their host habitat (fruit).

Our results support the hypothesis that chemical cues produced by healthy or infested lime fruit with *Ao. aurantii* attack route the host location behaviour of *A. melinus* females, acting as long range kairomones. SPME and GC-MS analysis have also supported the presence of volatiles attributable to herbivore activity. In detail, we found 23 volatiles increasing or exclusively emitted by infested lime fruit. Interestingly, our preliminary studies showed two common VOCs produced naturally in most of citrus fruit species and increased as response of *Ao. aurantii* infestation: d-limonene and β -ocimene. The findings of this study suggest that d-limonene and β -ocimene could play important roles in improving biological control programs (Uefune et al., 2012). However, due to the general occurrence of these compounds in most plants, chemical analysis of *Ao. aurantii* and its kairomonal activity should also be examined, which could most likely provide a more specific short-range cue for *Ao. aurantii* infested fruit. Indeed, synthetic kairomones for parasitoids have been successfully tested in field conditions (Mallinger et al., 2011, Uefune et al., 2012). Nevertheless, a deeper knowledge of the mechanisms behind HIPVs on the foraging behaviour of beneficial arthropods in the field is still needed before any possible commercial application. Further research is warranted to assess the role of the identified compounds on *A. melinus* females in field conditions, in order to evaluate their potential use in improving California red scale biological control programs.


Chapter Five

Evaluation of d-limonene and β -ocimene as attractants of *Aphytis melinus* (Hymenoptera: Aphelinidae), a parasitoid of *Aonidiella aurantii* (Hemiptera: Diaspididae) on Citrus spp.

Statement of Contribution

Title of Paper	Evaluation of d-Limonene and β -ocimene as Attractants of <i>Aphytis melinus</i> (Hymenoptera: Aphelinidae), a Parasitoid of <i>Aonidiella aurantii</i> (Hemiptera: Diaspididae) on Citrus spp.
Publication Status	Published
Publication Details	Insects, 11 (1). P, 44.


Principal Author


Name of Principal Author (Candidate)	Khalid Mohammed
Contribution to the Paper	Methodology, funding acquisition, formal analysis, investigation, data curation and writing—original draft preparation
Overall Percentage (%)	65%
Signature	 Date: 17/March/2020

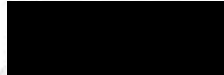
Co-Author Contributions

By signing the statement of contribution, each author certifies that:

- The candidate's stated contribution to the publication is accurate (as detailed above).
- Permission is granted for the candidate to include the publication in the thesis
- The sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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	Date 17/March/2020
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Overall Percentage (100%)	10%
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5.1. Abstract

The volatile organic compounds (VOCs) released from herbivore-infested plants can be used as chemical signals by parasitoids during host location. In this research, we investigated the VOC chemical signals for the parasitoid *Aphytis melinus* to discriminate between *Aonidiella aurantii* (California red scale)-infested fruit and non-infested fruit on three different citrus species. First, we identified the chemical stimuli emanating from non-infested and *Ao. aurantii*-infested citrus fruits via solid phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) analyses and identified 34 volatile organic compounds (VOCs). The GC-MS analysis showed qualitative and quantitative differences between VOCs emitted from non-infested and infested citrus fruit. Two VOCs, d-limonene and β -ocimene, were significantly increased in all infested fruit, regardless of the fruit species. The response of the female adult *A. melinus* to olfactory cues associated with *Ao. aurantii* infested fruit was evaluated using a Y-tube olfactometer. In two-choice behavioural assays, *A. melinus* females preferred infested citrus cues over non-infested fruit. Females showed positive chemotaxis toward these VOCs in all tested combinations involving two dosages of synthetic compounds, d-limonene and β -ocimene, except for d-limonene at a dosage of 10 μ l/ml. The application of these VOCs may help to enhance the effectiveness of bio-control programs and parasitoid mass-rearing techniques.

5.2. Introduction

The citrus industry is considered one of the largest fresh fruit industries in Australia, as well as the largest fresh fruit exporter, with an annual average export volume of 170,000 tonnes and a value of AUS\$190 million (Authority, 2010). Citrus crop spread has determined by the most devastating insect pest, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), causing a heavy loss in production (El-Otmani et al., 2011). *Ao. aurantii* prefers to attack citrus, and it is one of the most important citrus pests worldwide (Tena and Garcia-Marí, 2011). *Ao. aurantii* attacks all citrus species, but there are varying levels of susceptibility, such as in descending order of vulnerability is lemon trees (*Citrus limon* (L.)), grapefruit (*C. paradisi* Macf.), orange (*C. sinensis* (L.) Osbeck) and mandarin (*C. reticulata* Blanco and *C. unshiu* Markovitch) (Habib et al., 1972). California red scale attacks all aerial parts of the citrus tree including fruits, leaves, branches and twigs (Beardsley and Gonzalez, 1975). However, the fruit is the preferred plant substrate by *Ao. aurantii*, followed by leaves, while branches and twigs are the least preferred substrate (Hare et al., 1990). This is economically important as the presence of this scale on fruit considerably reduces their market value, causing huge economic losses (Vacas et

al., 2010). Chemical control of *Ao. aurantii* is difficult and is frequently followed by reinfestation within a short period, resistance to different products used for its control, and the risk of eliminating natural enemies present in the field (Mellado, 2012).

Plant volatiles can serve as semiochemicals to protect plants from insect and pathogen attack, attract beneficial animals, and as communication signals within and among plants (Baldwin, 2010). Terpenoids and other volatiles emitted from plants in response to insect attack allow parasitoids and predators to distinguish between infested and non-infested plants and thus aid in locating hosts or prey (Paré and Farag, 2005). In addition, the herbivorous-induced plant volatiles vary quantitatively and qualitatively related to various biotic and abiotic factors (e.g. number of infesting pests, period and duration of infestation and previous infestation) and they are specific to each plant–pest association (Benelli et al., 2013). It is well-known that insect feeding activity leads to biochemical changes in plants. Plants respond to the presence of pests by activating their defense system (González-Aguilar et al., 2004), but they can also trigger indirect defenses, such as the emission of herbivorous-induced plant volatiles (HIPVs) (Hare, 2011). The role of kairomones on host location by natural enemies has been widely investigated (Dicke and Baldwin, 2010; Kaplan, 2012a). Many parasitoids of phytophagous insects orient to HIPVs, using them as kairomones to guide their search for hosts (Benelli et al., 2013; Hare, 2011) and differential emissions have been reported for *Ao. aurantii*-infested and healthy fruits (Mohammed et al., 2017a).

The aphelinid *A. melinus* is a primary ectoparasitoid of armored scale insects. It is the most commonly used biocontrol agent for California red scale *Ao. aurantii* across the world, through augmentative releases (Rizqi et al., 2006; Tena and Urbaneja, 2015). *A. melinus* females rely on stimuli to successfully locate their host. *Aphytis* use learned volatile cues from host plants as long-range attractants to potential habitats of their hosts (Morgan and Hare, 1998; Mohammed et al., 2019). Previous work with *A. melinus* and California red scale showed that wasps were attracted to the odors of scale-infested lemon fruit (Sternlicht, 1973).

In this study, we investigated the olfactory cues used by *A. melinus* females to locate their host microhabitat. We hypothesize that the HIPVs from *Ao. aurantii* infested citrus fruit may play a pivotal role in affecting *A. melinus* host location. Three different citrus fruit, lemon fruit *Citrus limon* (L.), orange fruit *C. sinensis* (L.) and mandarin fruit *C. reticulata* Blanco, were tested to determine parasitoid attractiveness and VOC emissions. First, the volatiles emitted

from healthy and infested citrus fruits were extracted with SPME fibre and analyzed by gas chromatography-mass spectrometry to determine whether that VOCs are attributable to herbivores' activity and determine possible HIPVs. Subsequently, we evaluated female preferences for infested or healthy fruit in olfactometer bioassays. Finally, to understand the magnitude of volatile attractiveness, the innate attractiveness of VOCs to *A. melinus* females in varying concentrations was tested in the laboratory.

5. 3. Materials and methods

5.3.1. Insect colonies

Parasitoid pupae of *A. melinus*, purchased from Biological Services Commercial Insectary (Adelaide, Australia), were maintained under laboratory conditions until adult emergence. Adults of *A. melinus* were held in plastic cages (30 × 30 × 30 cm) covered with a fine mesh cloth. The wasp colony was reared on *Ao. aurantii*; on butternut squash (*Cucurbita moschata* Duchesne ex Lamarck) (26°C, 40–60% relative humidity and L16: D8 h photoperiod) during their entire life. The original *Ao. aurantii* colony was established in 2016 from infested citrus fruit harvested in Western Australia (32.30° S, 116.01° E; 69 m AMSL). Fresh squash infested with scales (>1500 per squash) were placed in a cage with *A. melinus* adults which were released twice a week to maintain the *A. melinus* colony.

5.3.2. Plant material

Three different citrus species (lemon, orange and mandarin) were used for behavioural assay and GC-MS analysis. Citrus fruit was collected from 25-year-old trees (spaced 4.5 × 5 m apart) on 15 September 2016, from Murdoch University citrus orchard located in Western Australia, same location as mentioned above. Non-infested or infested fruit from each citrus species was collected manually, stored in glass jars (10 cm diameter, 20 cm high) and transferred to laboratory within 1 h. The infested fruit samples were selected which had the most second and early third instar nymphs. Non-infested fruit samples were chosen to be free of any naturally physical damages as much as possible. Prior to test, the fruit were stored in the laboratory at 25 ± 2°C and 45–55% RH for 5–7 days. The tested fruit samples were uniformed in weight, each sample consisting of one piece of fruit weighing about 150 g. The infested citrus fruit were selected with 300–400 s and early third instars of *Ao. aurantii*.

5.3.3. Extraction of VOCs and GC-MS analysis

Random pieces of citrus fruit, either non-infested or infested, were placed individually into a 500 ml glass jar and sealed to equilibrate gases for 8, 16 and 20 h for mandarin, orange and lemon, respectively. The differences in the equilibration time for extracting VOCs for each fruit could be attributed to species characteristics and many other factors such as growing area, weather conditions and non-homogeneity of the fruit ripening stage. After this, VOCs were extracted by inserting and exposing a solid-phase microextraction (SPME) fibre with 50/30 μm Carboxen/DVB/PDMS (2 cm) (Sigma-Aldrich, Bellefonte, PA, USA) to the headspace of the glass jar over the fruit samples at room temperature at $25 \pm 2^\circ\text{C}$ and 45–55% RH for 2 h. The fibre was then withdrawn into the needle and transferred to the injection port of the GC–MS system as described by (Mohammed et al., 2017b). The desorption time of the SPME fibre at GC-MS injector was 10 min (Mohammed et al., 2017a). Six replicates were conducted for each treatment. The VOCs were analysed by GC-MS using an Agilent 7820A GC (Mulgrave, Victoria, Australia), equipped with a DB-35ms (30 m \times 0.25 mm \times 0.25 μm) fused-silica capillary column (Agilent Technologies, Santa Clara, CA, USA), with a mass spectrometer detector 5977E (Agilent Technologies, USA) under splitless mode. The carrier gas was 99.99% helium supplied by BOC (Sydney, Australia). The operation conditions of GC-MS were 250°C injector temperature; the oven temperature was initially programmed at 60°C and increased to 270°C (by 5°C/min); the column flow rate and splitless were 20 ml/min at 1.5 min and 1:1; the total GC-MS run time was 45.4 min. Compound peaks were deconvoluted by AMDIS version 2.72 and identified by searching the NIST 2014 MS database (US National Institute of Standards and Technology, Gaithersburg, MD, USA) with retention index confirmation. The experiment was repeated twice.

5.3.4. Bioassay Procedures

The olfactory response of *A. melinus* to host-associated cues was tested using a glass Y-tube olfactometer purchased from Volatile Collection Systems Co LLC (Gainesville, FL, USA). The Y-tube olfactometer design was similar to that described in (Morgan and Hare, 1998) with slight modifications for the purpose of our research. Briefly, the Y-tube olfactometer consisted of a central tube (2.5 cm diameter, 15 cm long) and two lateral arms (2.5 cm diameter, 11 cm arm's length) with ground glass fittings through which humidified air passed (0.5 ml/sec through each arm, controlled by up-stream flow meters). Each arm was connected to an extended glass tube (2 cm diameter, 6 cm long). The extended glass tube with the mesh barrier prevented the insects from escaping. The air was filtered with activated carbon traps, then

passed through a chamber into each fruit, then VOCs and insects could be introduced. The air passed through the olfactometer and then the system was left to stabilise for 15 min prior to use.

Olfactometer studies were carried out at $25 \pm 1^\circ\text{C}$ (45–55% RH) in a room illuminated with overhead daylight fluorescent tubes. A light was placed over the Y-tube olfactometer, and the surrounding area (around and below) was shrouded with white paper to block out any visual cues. *A. melinus* is an ectoparasitoid, so pupae could be isolated from host and host plant material without disrupting their development. From adult emergence until their use for bioassays, parasitoids were held in tubes (1cm diameter, 4 cm length) and droplets of honey were provided throughout their lives. All wasps used for the bioassays were naive females without any previous contact with infested or non-infested host fruit. After placing parasitoids in the conditioning vial for a day, wasps were removed and placed in an empty container. On the day of bioassay, female wasps were isolated and held individually in glass vials with a drop of honey until tested. A total of 40 replicates (individual *A. melinus*) were conducted for each of the three citrus fruit species, and the four tested dosages of the synthetic VOCs. For each bioassay, a single female wasp was introduced into the central arm tube of the olfactometer. Once the wasp had reached the point where the arm divides (i.e., when the wasp reached a position to make a choice), a timer was started. If the parasitoid remained stationary for two minutes, it was considered as unresponsive. It was then excluded from the study and substituted with another one.

The olfactometer was reversed after half of the wasps in each replicate had been tested. After all, ten parasitoids were assayed, the odour sources were removed, and the apparatus was cleaned with water and acetone, then dried and heated in an oven at 80°C for at least half an hour between replicates. Overnight between treatments, new sources were introduced into the aeration chambers, and a further replicate of ten parasitoids was tested. For bioassays involving fruit, fresh and ripe fruit samples were tested to reduce the variation in volatile emissions between replicates.

To understand whether infested citrus fruit is attractive to *A. melinus*, at the first stage, mated females were allowed to choose between non-infested or infested fruit samples for each citrus species. A wasp was considered to have chosen a cue when it remained in the same chamber for at least 20 s, actively searching for a host. The bioassay replicate was considered complete

when the wasp left the chamber. However, if wasps are unresponsive after two minutes in the system, they were eliminated from the study.

For assessment of the attraction of synthetic VOCs, two different concentrations (10 and 20 $\mu\text{l/ml}$) of d-limonene and β -ocimene (d-limonene purity 97%, 98% EE (GLC) Sigma-Aldrich, and β -ocimene purity $\geq 90\%$ Toronto Research Chemicals, North York, ON, Canada) were dissolved in hexane. In each trial, 5 μl of solution was applied onto a filter paper (1.5 cm \times 1.5 cm Whatman no. 1) and placed in an Erlenmeyer flask (250 ml) stoppered with a sealed adaptor. After solvent evaporation (20 sec), Erlenmeyer flask was connected to an arm of the Y-tube, and another arm received filter paper treated with 5 μl of pure hexane as a solvent control. Forty mated females were tested for each treatment.

For non-infested and infested citrus fruit bioassays, the female's first choice, and search duration (time spent actively searching inside the arm) were recorded. For the bioassay of synthetic VOCs, only the female's first choice was recorded. The activity recordings of parasitoids were used to assess the overall activity and the preference for each choice. The activity was measured by summing the number of entries into both arms by each wasp.

5.3.5. Statistical Analysis

To compare VOCs emissions between infested and non-infested fruit, the variance between peak areas was analysed for each compound and chemical class. Differences in volatile emissions between non-infested and infested fruit were analysed using non-parametric Mann-Whitney U test. Principal Component Analysis (PCA) was achieved on normalised values of each VOC to derive different variables (principal components) that summarise the original data. PCA analysis was performed using Metaboanalyst 3.0 (a comprehensive online tool suite for metabolomics data analysis). A likelihood chi-square test using Yates correction (with $p = 0.05$) for each choice-test was used to compare the proportion of parasitoids choosing a given cue. Time spent in the non-infested plant area and the infested plant area was compared using ANOVA, followed by Tukey's post hoc tests. (SPSS version 24.0, SPSS, Inc., Chicago, IL, USA).

5.4. Results

5.4.1. Identification of volatiles

The data from GC-MS analysis showed that more than 34 different volatile compounds were separated and identified from the infested and non-infested citrus fruit and differential associated emissions attributed to herbivore activity were found for all fruit species (Table 5.1). The two naturally presented compounds in non-infested lemon, orange and mandarin, d-limonene and β -ocimene were significantly increased in the infested fruit (Table 5.1). However, n-hexadecanoic acid was exclusively produced by infested lemon fruit, and 5,4-di-epi-aristolochene was decreased after infestation, while 3,7-dimethyl-(E)-2,6-octadienal and α -bulnesene were only present in non-infested lemon and were not detected in infested lemon. There were no significant differences between the non-infested and infested mandarin fruits, but the compounds of γ -terpinene, alloocimene and alloaromadendrene were significantly increased in infested mandarins (Table 5.1). For orange, compounds were changed only in infested fruit, e.g., three more compounds, alloaromadendrene, 4,8-dimethyl-(3E)-1,3,7-nonatriene, and hexyl caproate were increased, and five compounds, acetic acid hexyl ester, alloocimene, α -tyerpineol, nerolidol and (3E,7E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene were exclusively present in infested fruit (Table 5.1).

The PCA showed that the volatile compound profiles varied with infestation and citrus fruit species (Figure 5.1). D-limonene and β -ocimene were the major VOCs released from all tested fruits. D-limonene emitted 26.42%, 35.92% and 8.50% and β -ocimene emitted 32.83%, 31.51% and 17.46% of total volatile emissions from lemon, mandarin and orange fruit, respectively. Moreover, there were some major VOCs released from infested orange fruit such as alloaromadendrene, which accounted for 24.79% of total volatile emissions, and 19.65% and 23.48% of eudesma-4(14),7(11)-diene from lemon and orange fruit, respectively.

Table 5.1. Quantities of major volatile compounds released by non-infested and *Aonidiella aurantii* -infested citrus fruits through headspace sampling by SPME

Feature ID	RI	Chemical compounds	GC response (Peak areas±SE) ×10 ⁶					
			Lemon		Mandarin		Orange	
			Uninfested	Infested	Uninfested	Infested	Uninfested	Infested
4.464-151.1	949	Oxime-, methoxy-phenyl-	4.235±1.382	4.749±0.370	5.568±1.504	4.512±1.337	12.081±2.677	9.811±0.433
4.924-136.1	966	β-Thujene	n.d	n.d	0.731±0.050	2.686±1.127	n.d	n.d
5.921-136.1	979	β-Pinene	4.464±0.064	5.269±2.531	n.d	n.d	n.d	n.d
6.171-136.1	991	β-Myrcene	3.359±2.009	6.115±2.429	1.211±0.405	6.752±3.215	0.984±0.571	1.636±0.918
6.594-144.1	1011	Acetic acid, hexyl ester	n.d	n.d	n.d	n.d	n.d	2.754±0.4046*
7.043-136.1	1018	d-limonene	30.852±27.394	136.594±18.874*	31.585±6.825	137.129±30.916*	24.716±1.878	40.918±2.773*
7.429-136.1	1037	β-ocimene	67.916±6.775	169.708±13.934*	45.039±18.482	120.268±10.677*	20.812±3.123	83.942±15.674*
7.625-136.1	1047	γ-Terpinene	3.686±2.522	12.214±7.216	7.117±2.606	41.078±11.193*	0.573±0.498	1.633±0.929
8.258-136.1	1088	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	2.449±1.443	1.988±1.018	1.431±0.251	5.572±2.170	n.d	n.d
8.808-150.1	1116	(E)-4,8-Dimethylnona-1,3,7-triene	35.130±15.029	18.611±1.789	17.214±7.774	31.584±16.498	3.774±1.832	32.767±10.184*
9.092-134.1	1131	2,6-Dimethyl-1,3,5,7-octatetraene,E,E-	4.025±0.683	6.200±0.870	0.635±0.319	1.238±0.622	1.561±0.586	3.577±1.367
9.338-136.1	1144	2,4,6-Octatriene,2,6-dimethyl-,(E,E)-	3.653±2.236	5.718±2.126	1.108±0.198	6.906±0.021*	n.d	2.409±0.641*
9.867-128.2	1175	2-Octen-1-ol,(E)-	n.d	n.d	0.191±0.191	0.218±0.218	n.d	n.d
10.086-154.1	1182	L-Terpinen-4-ol	n.d	n.d	0.284±0.146	0.896±0.658	n.d	n.d
10.309-154.1	1189	α-Terpineol	1.081±0.567	1.815±1.334	0.525±0.264	2.975±2.476	n.d	3.004±1.176
10.488-170.2	1200	Dodecane	n.d	n.d	0.785±0.119	1.126±0.195	n.d	n.d
10.614-156.1	1206	Decanal	n.d	n.d	1.272±0.642	1.312±0.667	1.416±0.302	1.438±0.209
11.943-152.1	1270	2,6-Octadienal,3,7-dimethyl-,(E)-	0.753±0.386	n.d	n.d	n.d	n.d	n.d
12.478-212.2	1275	Dodecane,2,6,11-trimethyl-	1.369±0.819	0.745±0.373	1.196±0.051	1.648±0.335	1.371±0.108	1.766±0.213
14.111-200.3	1384	Hexanoic acid, hexil ester	n.d	n.d	n.d	n.d	4.375±1.713	18.305±6.018
14.206-204.2	1398	Cyclohexane,1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	7.433±2.337	5.899±0.894	n.d	n.d	10.546±4.774	20.594±5.217

14.922-204.2	1461	Alloaromadendrene	17.374±5.366	13.587±0.729	3.454±0.358	7.713±0.894*	39.032±7.499	119.291±13.459*
15.236-204.2	1490	α -Bulnesene	1.309±0.805	n.d	n.d	n.d	n.d	n.d
15.384-204.2	1440	Aromandendrene	n.d	n.d	n.d	n.d	4.920±1.749	9.635±2.296
15.784-204.2	1469	5,4-di-epi-aristolochene	2.391±0.345	1.435±0.103	n.d	n.d	3.789±1.474	7.687±0.861
15.941-204.2	1527	Panasinsen	16.519±6.276	10.936±2.061	n.d	n.d	11.113±4.743	20.255±2.813
16.283-204.2	1544	(4aR,8aS)-ra-Methyl-1-methylene-7-(propan-2-ylidene)decahydronaphthalene	51.037±45.977	101.571±76.637	n.d	n.d	100.882±27.236	113.043±38.383
17.039-204.2	1556	Guaia-3,9-diene	1.705±0.296	2.091±0.595	n.d	n.d	n.d	n.d
17.323-222.2	1564	1,6,10-Dodecatrien-3-ol,3,7,11-trimethyl-	3.129±2.278	2.652±1.351	n.d	n.d	n.d	1.247±0.644
17.532-218.2	1581	(3E,7E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene	n.d	n.d	1.941±0.766	2.587±1.083	n.d	1.582±0.821
17.849-220.2	1586	trans-Z-a-Bisaboleneepoxide	1.355±0.319	1.322±0.359	0.142±0.1416	0.155±0.155	n.d	n.d
18.945-222.2	1660	Neointermedeol	3.218±0.332	4.672±0.699	0.348±0.182	0.566±0.288	1.275±0.332	2.305±.389
23.368-256.2	1968	n-Hexadecanoic acid	n.d	0.920±0.920	3.098±1.076	3.336±0.928	n.d	n.d
44.331-722.6	4932	Trimyristin	2.843±2.201	2.088±0.529	1.343±0.458	1.416±0.266	n.d	n.d

Asterisks indicate significant differences between uninfected and infested fruit at $P < 0.05$; Feature ID includes retention time (min) and mass to charge ratio m/z ; RT is retention times; RI is retention index; SE is the standard error; n.d means not detected. Each value represents the peak area (mean±SE) of (n=6).

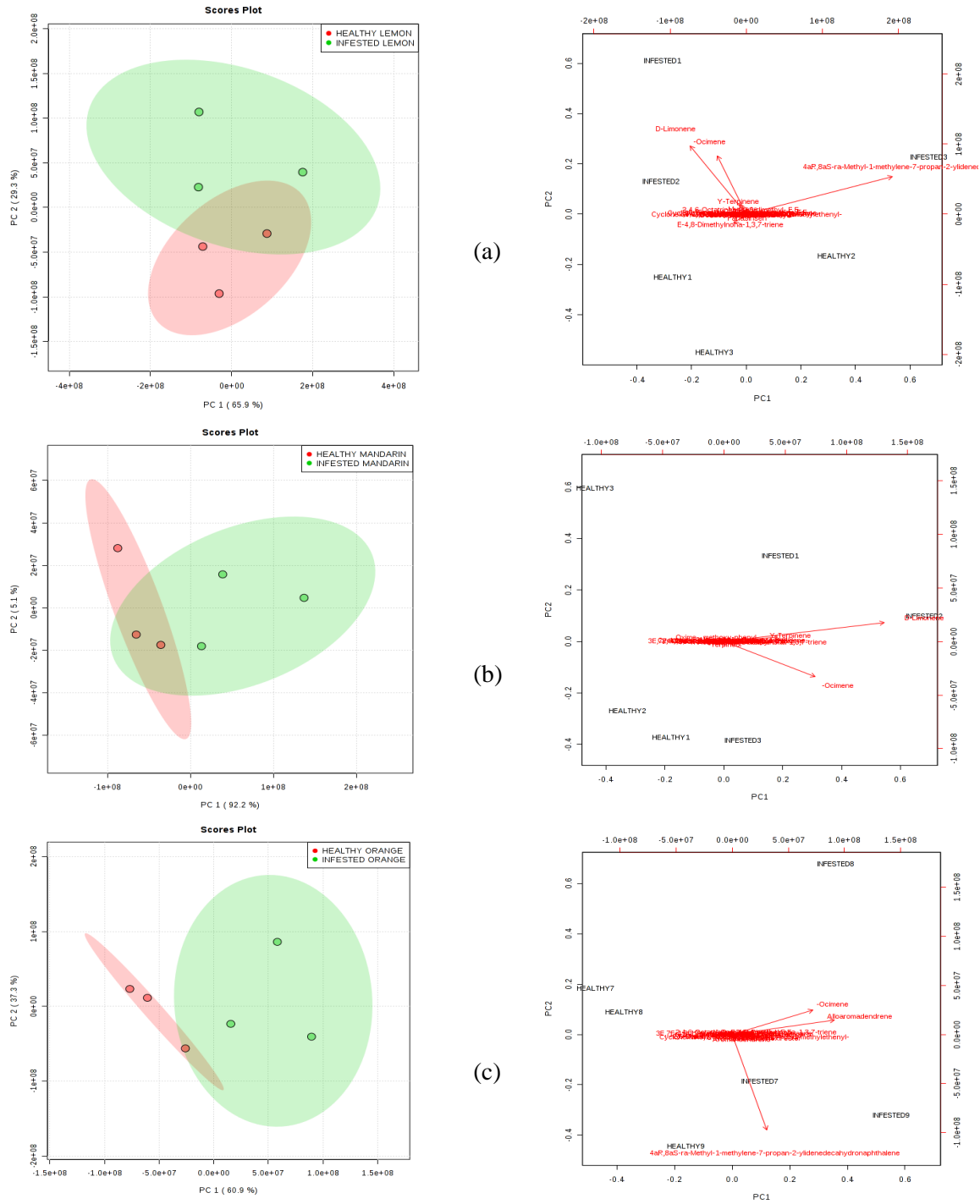


Figure 5.1. Principal Component Analysis (PCA) of volatile profiles from infested and healthy fruit of three different citrus species. PCA scores plot and biplot of lemon (a), mandarin (b) and orange fruit (c), showing volatile correlations with the first and second principal component; PCA score plot, highlighting cluster of volatiles attributable to species or infestation status; PCA biplot highlighting changes in chemicals attribute to species or infestation status.

5.4.2. Behavioural tests

The results of the Y-tube olfactory bioassays are presented in Figure 5.2. When olfactory cues were provided, *A. melinus* mated females showed significant preferences for lemon fruit infested with *Ao. aurantii* over healthy ones, but no such preference was observed when compared between healthy or uninfested and infested oranges or mandarins. (lemon: $\chi^2 = 4.900$, $df = 1$, $P = 0.027$; orange: $\chi^2 = 2.500$, $df = 1$, $P = 0.114$; mandarin: $\chi^2 = 1.600$, $df = 1$, $P = 0.206$).

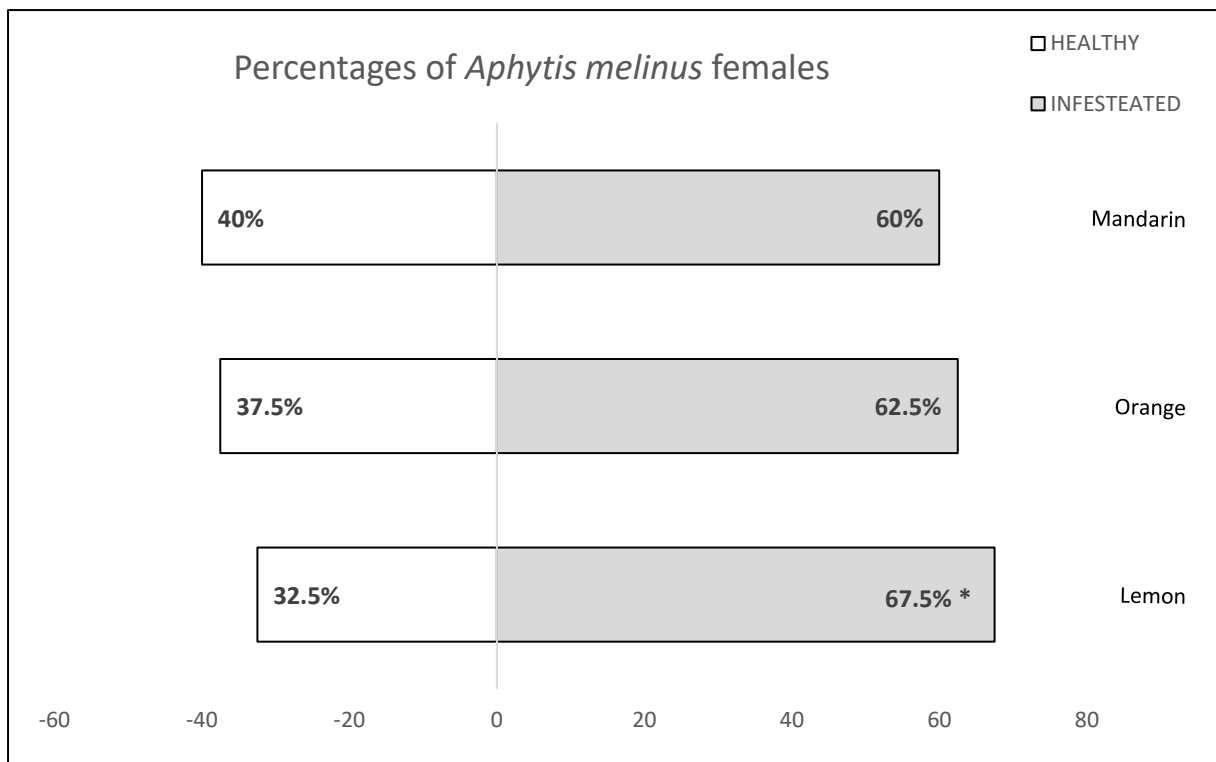


Figure 5.2. Attractiveness of *Aonidiella aurantii*-infested citrus fruit towards *Aphytis melinus* mated females. Two choice bioassays were conducted in a still air arena with citrus fruit, infested or not by California red scale, providing olfactory cues. Forty wasps were tested in each bioassay. For each test, asterisks indicate significant differences in the number of wasps choosing different cue (χ^2 test with Yates correction, $P < 0.05$).

There was no significant time differences of *A. melinus* to choose the chamber connected to *Ao. aurantii* infested citrus fruit. Indeed, *A. melinus* females slightly preferred and taken the initiative to the chambers containing infested fruit rather than health fruit (lemon: $\chi^2 = 26.000$,

df = 21, $P = 0.206$; orange: $\chi^2 = 28.000$, df = 21, $P = 0.140$; mandarin: $\chi^2 = 26.333$, df = 21, $P = 0.194$) (Table 5.2).

Table 5.2. Choice time spent by *Aphytis melinus* females during searching behaviour on healthy and *Aonidiella aurantii* -infested citrus fruits in Y-tube Olfactometry.

Spices	Infested citrus fruit		Healthy citrus fruit		F	P value
	Choice time (Sec)	Replicates	Choice time (Sec)	Replicates		
	Mean±SE		Mean±SE			
<i>Lemon</i>	148.15±13.482	27	133.31±14.604	13	0.558	0.206 ^{ns}
<i>Orange</i>	153.40±10.523	25	119.13±12.084	15	4.573	0.140 ^{ns}
<i>Mandarin</i>	139.53±12.802	23	144.06±15.412	17	0.051	0.194 ^{ns}

Asterisks indicate significant differences in choice time spent by *Aphytis melinus* between uninfested and infested fruit at $P < 0.05$; ns: not significant.4.

Mated *A. melinus* females, with no previous oviposition experience, were significantly attracted to the synthetic VOCs in all tests, with the exception of d-limonene at tested dosage 10 $\mu\text{l/ml}$ ($\chi^2 = 2500$; df = 1; $P = 0.114$). However, mated *A. melinus* females preferred the reward of associated VOC more than hexane control in the case of d-limonene at the tested dosage of 20 $\mu\text{l/ml}$ ($\chi^2 = 7.410$; df = 1; $P = 0.006$), β -ocimene at the tested dosage 10 $\mu\text{l/ml}$ ($\chi^2 = 4.900$, df = 1, $P = 0.027$) and β -ocimene at the tested dosage 20 $\mu\text{l/ml}$ ($\chi^2 = 12.100$; df = 1; $P = 0.001$) (Figure 5.3).

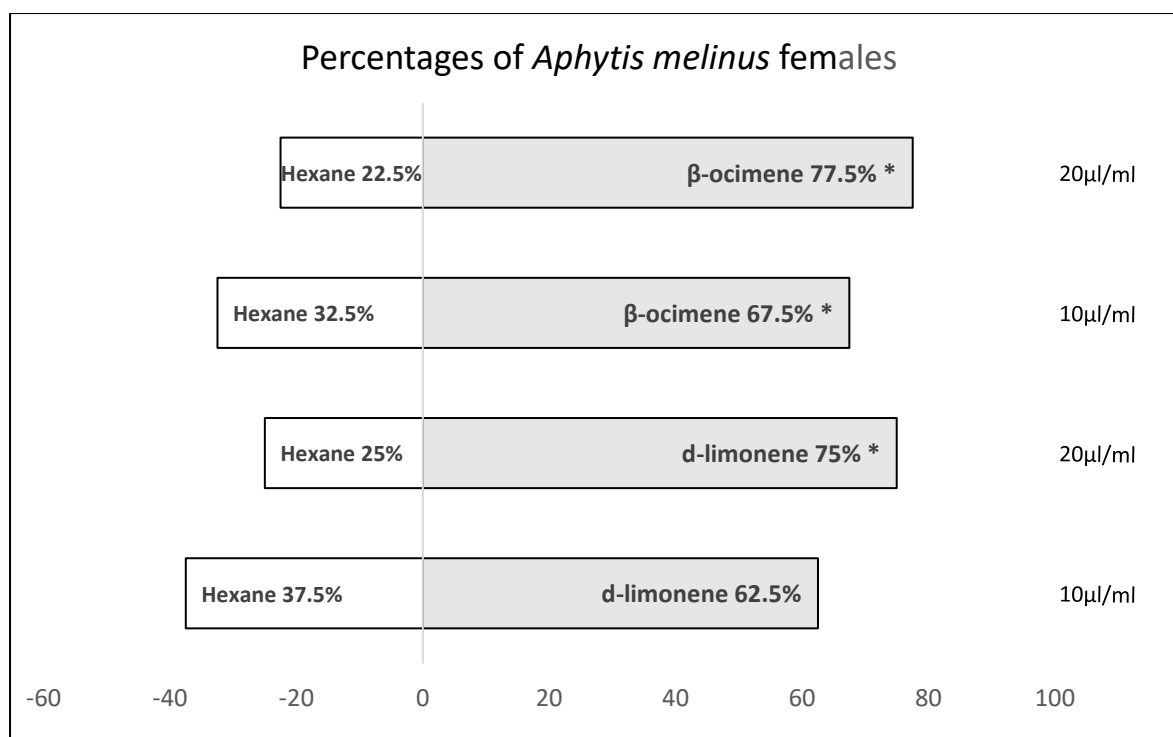


Figure 5.3. Attraction of female *Aphytis melinus* to VOCs differentially emitted by *A. aurantii*-infested citrus fruits. Choice bioassays were conducted in a Y-tube olfactometer presenting one of the two dosage levels (10 and 20 μl/ml) of citrus fruits VOCs vs. hexane. Asterisks indicate significant differences between numbers of VOC and control choices (χ^2 test with Yates' correction, $P < 0.05$).

5.5. Discussion

This report investigated the capability of an aphelinid parasitoid *A. melinus* to respond to citrus fruit chemical cues associated with damage induced by a phytophagous pest. The results indicated that *A. melinus* was able to discriminate between citrus fruit infested with *Ao. aurantii* and non-infested fruit by using olfactory cues.

Among 34 VOCs extracted and identified by SPME and GC-MS techniques, only two volatiles d-limonene, and β -ocimene were increased in all infested three citrus species, which were already known as a constituent of the odours of citrus fruit and citrus oils (Mohammed et al., 2017a; Mohammed et al., 2019; Auta et al., 2018). However, since d-limonene and β -ocimene are attractive to many other parasitoid species (Morgan and Hare, 1998; Mohammed et al., 2019; Kang et al., 2018), these two compounds can be considered as putative kairomones for *A. melinus*.

PCA analysis has highlighted that VOC emissions are particular for each species and chemical. Fruits differentially emitted the VOCs after insect infestation and they change depending on the citrus species. Indeed, several plants react to herbivore damage by producing blends of metabolites that change in number or proportion (Carrasco et al., 2005). The increasing in VOCs follow the infestation could be explained for all species, but we also identified some particular compounds emitted under the condition of *Ao. aurantii* infestation. Besides d-limonene and β -ocimene, mandarin infested fruits increased the emission of another three compounds (Y-Terpinene, 2,4,6-Octatrinene,2,6-dimethyl- and Alloaromadendrene). Infested lemon fruits increased the emission of just d-limonene and β -ocimene and produced n-Hexadecanoic acid. When attacked by *Ao. aurantii*, orange fruits similar to other species increased the production of d-limonene, β -ocimene, (E)-4,8-Dimethylnona-1,3,7-triene and Alloaromadendrene, and exclusively produced five compounds (Acetic acid, hexyl ester, 2,4,6-Octatrinene,2,6-dimethyl-,(E,E)-, α -Terpineol, 1,6,10-Dodecatrien-3-ol,3,7,11-trimethyl- and (3E,7E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene). Most of these compounds are known as common floral compounds, but interestingly some of them are also recognised as pheromones for several hymenopteran species (Pinto-Zevallos et al., 2013).

Since the variance in VOCs depends mainly on the fruit species, we cannot exclude the possibility that other VOCs produced specifically by one species or increased in all species may act as an attractant toward *A. melinus*. Indeed, healthy fruit also produce volatiles that are attractive to parasitoids (Carrasco et al., 2005), and aphelinid parasitoids, such as *A. melinus* could be able to perceive cues from non-infested plants to locate a host (Morgan and Hare, 1998; Mohammed et al., 2019). Therefore, short-range volatiles produced by the plants or as feeding larvae secretion could be useful for successful localisation (Canale, 2003). Overall, since HIPVs are known to act as attractive chemicals for several parasitoids (Carrasco et al., 2005; Zimba et al., 2015; Giunti et al., 2016b), further research is needed to assess the activity of these compounds on parasitoid behaviour. Indeed, understanding tritrophic system communications has potential implications for biological control programs (Uefune et al., 2012).

Olfactory cues from host-infested fruit are known to be essential for parasitoids during foraging for hosts, food and mates in complex environments (Hare, 2011; Clavijo McCormick et al.,

2014). For *A. melinus*, the source of volatile cues that the wasp uses to locate the host species were determined when those hosts occurred on more than one host plant species (Morgan and Hare, 1998). The evidence that olfactory cues from infested fruit evoke behavioural responses from mated *A. melinus* females, and the presence of chemicals that were produced exclusively or in higher amounts by infested citrus fruit, supported our hypothesis that VOCs could act as short-range attractants and played a key role during host-seeking. Biological assays conducted in the olfactometer indicated that *A. melinus* preferentially orientated towards the source of HIPVs, that is, the presence of *Ao. aurantii* on the fruit is crucial for host location.

The VOCs d-limonene and β -ocimene have been reported as bouquet constituents of several citrus species (Mohammed et al., 2017a; Mohammed et al., 2019). d-limonene and β -ocimene were identified as constituents of different infested fruit species that attracted other parasitoids such as *Agathis bishopi* and *Aphidius gifuensis* (Kang et al., 2018; Zimba, 2014). Many experiments have shown that, in the species examined, successful source location requires the presence of an attractive odour (Cardé and Willis, 2008).

It is clear that *A. melinus* was not attracted to the pest when fruit material was absent, suggesting the importance of plant HIPVs in attracting parasitoids, this result is consistent with that host insects do not always produce good cues (Heil, 2008). This study confirmed that the aphelinid parasitoid *A. melinus* uses common herbivore-induced plant volatiles to locate prey. The volatile profile analysis provides complementary information about the composition of the volatile chemicals potentially involved in the behaviours with 34 compounds varying during infestation by *Ao. aurantii*. Further electrophysiology and behavioural assays are expected to provide more accurate identification of the HIPVs that contribute towards attracting *A. melinus* to infested host citrus fruit. Such information may prove useful for further studies aiming at developing semiochemical strategies that could be incorporated into an integrated management approach for enhancing existing pest control techniques for California red scale.

5.6. Conclusions

The aphelinid parasitoid *A. melinus* responded to citrus fruit chemical cues associated with damage induced by California red scale. The analytical data showed that more than 34 different volatile compounds were separated and identified from the infested and healthy citrus species

fruit. Interestingly, the three-species showed two common VOCs increased as a response of *Ao. aurantii* infestation: d-limonene and β -ocimene.

Olfactory cues seem to have a key role in host-seeking behaviour for the three citrus species. Synthetic kairomones may be useful to improve biological control programs especially after HIPVs are recognized as kairomones of a number of parasitic wasps, but still, further studies are necessary to ensure the behavioural activity of this investigated volatiles toward *A. melinus* parasitoids under field condition.


Chapter Six

D-limonene and β -ocimene Attract *Aphytis melinus* and Increase Parasitism of California Red Scale *Aonidiella aurantii* (Hemiptera: Aphididae) in Citrus Orchards

Statement of Contribution

Title of Paper	D-limonene and β -ocimene Attract <i>Aphytis melinus</i> to Increase Parasitism of California Red Scale <i>Aonidiella aurantii</i> (Hemiptera: Aphididae) on Citrus spp.
Publication Status	Submitted
Publication Details	Insects


Principal Author


Name of Principal Author (Candidate)	Khalid Mohammed
Contribution to the Paper	Methodology, funding acquisition, formal analysis, investigation, data curation and writing—original draft preparation
Overall Percentage (%)	70%
Signature	 Date: 17/March/2020

Co-Author Contributions

By signing the statement of contribution, each author certifies that:

- The candidate's stated contribution to the publication is accurate (as detailed above).
- Permission is granted for the candidate to include the publication in the thesis
- The sum of all co-author contributions is equal to 100% less the candidate's stated contribution

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Methodology, supervision, data curation and writing—review and editing
Overall Percentage (100%)	15%
Signature	 Date 17/March/2020

Name of Co-Author	Yonglin Ren
Contribution to the Paper	Methodology, supervision, data curation and writing—review and editing
Overall Percentage (100%)	15%
Signature	 Date 17/March/2020

6.1. Abstract

Under field conditions, the natural enemies' effectiveness in controlling pests is largely correlated with their capability to spread towards infested crops. We previously reported that *Aphytis melinus* (Hymenoptera: Aphelinidae), a parasitoid of California red scale *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), was attracted to volatiles from citrus fruit infested with *Ao. aurantii* and that d-limonene and β -ocimene *Ao. aurantii*-induced plant volatiles were responsible for this attraction. In this study, d-limonene and β -ocimene were examined for their attractiveness to *Ao. aurantii* parasitoid *Aphytis melinus* in the field after augmentative releases. Both were mixed with paraffin oil for slow release in field experiments to control the population density of *Ao. aurantii* by enhancing their natural enemies. The experiment was conducted in 2018 at Murdoch citrus orchard. A total of 10,000 *A. melinus* adults were released in different spots of the citrus orchard. The spread of the parasitoid was evaluated, for three months after the release, using yellow sticky traps activated with both of d-limonene and β -ocimene and by monitoring the percentage parasitism of the scale on citrus fruit. Field experiments demonstrated that lures baited with isolates of d-limonene and/or β -ocimene, which significantly attracted some species of natural enemies but had no significant impact on other recruitments. The number of *A. melinus* captured during the whole trial was greater in the traps treated with volatiles than the control. Finally, we determined that overall parasitism rates were not increased by synthetic HIPV lures but found evidence that lures may increase parasitism of *Ao. aurantii* when there is a decrease in the amount of volatile organic compounds due to lack of healthy and infested fruit.

6.2. Introduction

California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), is one of the most important pests of citrus worldwide (Pekas, 2011b). Within Australia, *Ao. aurantii* is a major pest of citrus in the citrus producing regions of Queensland, Western Australia, South Australia, Victoria, and inland New South Wales (Smith et al., 1997). *Aphytis melinus* is an important agent in controlling *Ao. aurantii* (Sorribas and Garcia-Marí, 2010) utilising augmentative releases (Moreno and Luck, 1992). When the aim of mass release is a quick effect of natural enemies rather than their establishment, natural enemies must move from the release points and spread throughout the infested area (Corbett and Rosenheim, 1996). The capacity of natural enemies to spread within infested crops is mostly the most important factor in controlling pests in the field conditions (Zappalá et al., 2012). Therefore, the dispersal and host-

location behaviour of wasps, especially released ones, are factors that clearly influence the efficiency of biological control (Suverkropp et al., 2009; Tabone et al., 2012).

Herbivore-induced-plant-volatiles (HIPVs) constitute one particular group of allelochemicals, and these volatiles are composed of many organic compounds that are released when plants become infested by herbivorous insects (Schmelz et al., 2009; Mohammed et al., 2017a; Mohammed et al., 2019; Mohammed et al., 2020). HIPVs are involved in plant communication with natural enemies of the insect herbivores through the attraction of natural enemies and the repulsion of herbivores (Goggin, 2007; Arimura et al., 2009). It has long been expected that manipulation of HIPVs in synthetic or natural form can be used to attract and increase populations of natural enemies within their hosts or preys population or repel pests from crop plants (Kaplan, 2012a,b; Uefune et al., 2012).

To reduce reliance on pesticide use as an urgent need for sustainable agricultural methods, more studies are focusing on the ecological effect of Volatile organic compounds (VOCs) released by plants on herbivores (Shimoda et al., 1997; Bernasconi Ockroyet al., 2001). Direct evidence for the potential of synthetic plant volatiles as field attractants for beneficial insects was investigated (Alhmedi et al., 2010; Lee, 2010). Essential oils, as a natural emission from plants, do not pose the toxicity problems of pesticides to animals and the environment (Prinsloo et al., 2007; Mohan et al., 2011). Plant volatiles can be considered as potential reliable green chemicals for repelling pests and attracting natural enemies of these pests. Their long-distance effects and easy production and manipulation make these molecules excellent prospects for use with crops by spraying or mixing with a slow-releasing carrier to repel insect feeding or ovipositing from host plants and guide them to non-hosts (Bernasconi et al., 1998; Dudareva et al., 2006).

D-limonene and β -ocimene are monoterpenes found within a variety of plants and fruits. d-limonene and β -ocimene are the main terpene compounds found in citrus fruit peel (Chamberlain et al., 2012; Mohammed et al., 2019; Mohammed et al., 2020). The two compounds are both found in healthy fruit but are quantitatively elevated by insect herbivory, or mechanical injury of the fruit peel (Chamberlain et al., 2012; Mohammed et al., 2019; Mohammed et al., 2020). The elevated concentrations of the above two compounds in *Ao. aurantii* infested fruit (Mohammed et al., 2017a; Mohammed et al., 2019; Mohammed et al., 2020) could be due to the feeding injury on the fruit rind caused by *Ao. aurantii*, which consequently attracts *A. melinus* females to their host. d-limonene is a major constituent in

citrus oils (lemon, orange, mandarin, lime, and grapefruit) (Sun, 2007). Despite the use of d-limonene as a repellent for many pests, especially in high concentration (Du et al., 2016; Song et al., 2017), it is also considered as an attractant for many species of parasitoids (Zimba, 2014; Zimba et al., 2015; Song et al., 2017; Mohammed et al., 2019; Mohammed et al., 2020). β -ocimene is considered one of the major compounds in citrus (Sarrou et al., 2013; Mohammed et al., 2017b; Mohammed et al., 2019; Mohammed et al., 2020). β -ocimene, also emitted from plants, can serve as a chemical cue for the attraction of parasitoids or predators of plant herbivores (Zimba, 2014; Kang et al., 2018; Mohammed et al., 2019; Mohammed et al., 2020). Therefore, it could be further suggested that the production of d-limonene and β -ocimene in citrus is part of the indirect defence strategy against *Ao. aurantii* infestation by attracting its natural enemies. Thus, d-limonene and β -ocimene play a key role as long range attractants of *A. melinus* females to *Ao. aurantii* infested citrus fruit. This study aimed to evaluate the dispersal ability of released *A. melinus* adults and their effect on the parasitism percentage, using d-limonene and β -ocimene with yellow sticky traps and scoring percentage parasitism on infested fruit. These methods have the advantage of providing both qualitative and quantitative data on the parasitoids' presence and distribution in space.

6.3. Materials and Methods

6.3.1. Insect releases

Parasitoid pupae used in the experiment, which were purchased from Biological Services Commercial Insectary (Adelaide, Australia), were maintained under laboratory conditions until adults emerged. Ten thousand parasitoid adults were released at many release points not exceeding five meters from the locations of the chemicals dispensers and equal to the number of them because low mobility of the wasps can reduce the spread, resulting in high levels of control close to the release point and decreasing effectiveness with distance, at least in the first few generations after the release (Zappalá et al., 2012). This number of *A. melinus* adults is recommended by Biological Services (commercial insectary). The trial started on 6th September – in order to keep background parasitism by naturally occurring *A. melinus* low – when the parasitoid is scarce in the field (Pekas, 2011b) to synchronise with the presence of virgin adult female *Ao. aurantii*, which is the preferred instar of the parasitoid (Moreno and Luck, 1992). The adult parasitoids released were less than 48 hours old, collected by using an insect aspirator vacuum and the number quantified based on estimation. They were then segmented, and groups of around 500 adults were placed in 20 ml vials. These were then carried

to the field in a refrigerated box and attached to the mid of the trees 150–200 cm above the ground.

6.3.2. Attractants

Yellow sticky traps attract *A. melinus* adults (Moreno et al., 1984; Sorribas and Garcia Mari, 2010). Besides, researchers indicate that *A. melinus* females are attracted to airborne cues from hosts, i.e. *Ao. aurantii* virgin females and host-infested fruit (Bernal and Luck, 2007; Mohammed et al., 2019; Mohammed et al., 2020). Lures were developed for experiments to test the attraction of d-limonene and β -ocimene to arthropods in the field (d-limonene purity is 98% EE (GLC), which has been purchased from Sigma-Aldrich, and β -ocimene purity is \geq 90%, which has been purchased from Toronto Research Chemicals) because of their large increase in herbivore-induced citrus trees volatiles compared to the healthy trees, which are commercial availability and low cost. d-limonene and β -ocimene lures were tested at the dosage of 20 μ l (Mohammed et al., 2020). Yellow sticky traps (101 mm x 173 mm) that are often used to monitor insects in fields (Laubertie et al., 2006) were attached to orange trees and placed in the middle of the tree 150–200 cm above the ground. Every seven days, 20 μ l of d-limonene and β -ocimene solution formulated in paraffin oil (for slow release of the infochemical), as well paraffin oil as the control, were deposited on a 1-cm-diameter rubber septum dispensers that were placed on the top of the yellow sticky traps. The slow dispensers were first placed in the citrus orchard on 6th September. There were 13 weekly trapping periods, the first just before the release and the others over the following 84 days. Sticky traps and lures were replaced weekly between 13th September to 22nd November. Once collected, the old traps were placed inside transparent plastic bags and taken to the Murdoch University laboratory, where the numbers of *Aphytis spp.* adults and other natural enemies were counted under a stereomicroscope, and the abundance of each species was recorded (Table 1).

Aphytis spp. were ascribed to *A. melinus* because this is by far the most abundant species suited to temperate conditions (Sonia, 2006) and because of the great numbers that were released. Four fruits per tree were randomly collected 150–200 cm above the ground every week on the same trees on which the traps were hung on in order to assess parasitism by *A. melinus* both in the trees treated by d-limonene and β -ocimene dispensers and in the trees treated by paraffin oil as a control. In the laboratory, the number of live and parasitised scales in these samples were scored.

6.3.3. Experimental design of citrus orchard

This was conducted in an unsprayed experimental citrus orchard of Murdoch University located in WA (32.30°S 116.01°E 69m AMSL), Australia, during spring and early summer (September–November) 2018. The trees in the experimental field were 25-year-old orange trees (*C. sinensis* (L.)), which were planted in a 5 x 5 m grid. The trial was conducted in two 1-acre plots, about 500 m apart from each other. The trial consisted of three treatments in the citrus orchards: (1) only paraffin oil as the control; (2) β -ocimene release; and (3) d-limonene release, and the experiment was repeated twice to confirm the results. Single yellow trap sticks with the 1 cm diameter rubber septum were placed 20 m apart in a Latin square design with three replicates per treatment (12 dispensers and 12 traps total). No herbicides or insecticides were used in the entire experimental area. The temperature was obtained through the climate Data Online by the bureau of meteorology.

6.3.4. Statistical analyses

The estimated percentage of parasitism (EPP) was measured using the following formula:

$$\text{EPP} = 100 \times (\text{Np} / \text{NI} + \text{Np})$$

Here, Np is the number of scale instars bearing *A. melinus*, and NI is the number of live *Ao. aurantii* instars that are suitable hosts for this parasitoid. Population densities of the EPP, as well as the *A. melinus* captured, recorded in infested fruit and traps in trees treated with d-limonene and β -ocimene dispensers and trees treated with only paraffin oil dispensers as a control, were compared among the infochemical releaser tests using a one-way analysis of variance (ANOVA). Raw data that did not pass the Kolmogorov Smirnov test for normality and the Levene test for equality of variances was subjected to square-root transformation before being analysed followed by Tukey's honestly significant difference (HSD) test. Correlation analysis was used to assess the relationship between the numbers of *A. melinus* captured and EPP during the whole trial (SPSS version 24.0, Chicago, IL, USA).

6.4. Results

6.4.1. The abundance of Natural Enemy Adults

The main natural enemies of *Ao. aurantii* found in the trials in order of abundance were *Aphytis* spp., *Comperiella bifasciata*, *Rhizobius lophanthae* and *Mallada* spp. Based on the total number of natural enemy species attracted, d-limonene and β -ocimene attracted more than the control paraffin oil did (Table 6.1). Analyses conducted for three months (September, October, and November) revealed that significantly greater numbers of *Aphytis* spp. were trapped in the yellow sticky traps treated with d-limonene and β -ocimene dispensers than the traps treated

with paraffin oil in October and November, while there was no significant difference observed in September. The number of *Mallada spp.* attracted to traps were significantly higher in October but not in September and November. There were no significant differences in the percentage of *Comperiella bifasciata* attraction towards traps despite the increase in the number of wasps oriented towards the traps treated with VOCs compared to those treated with just paraffin oil. *Rhizobius lophanthae* was significantly more numerous in the β -ocimene traps during September and October. Most of these species showed a trend of increasing abundance not only in the d-limonene and β -ocimene but also in the control area as the season progressed. *Ao. aurantii* natural enemies and their diversity are presented in Table 6.1.

Table 6.1. Season (September–November) (Mean \pm SE) of CRS’s natural enemies found in yellow sticky traps with controlled-release d-limonene, β -ocimene and paraffin oil dispensers in citrus orchard during 2018.

Beneficial insect	Time	Treatments			P value	F
		d-limonene	β -ocimene	Paraffin oil		
<i>Aphytis melinus</i>	September	1 \pm 0.389 a	1 \pm 0.348a	0.583 \pm 0.229a	0.591	0.534
	October	5.0833 \pm 1.062a	5.667 \pm 1.281a	0.917 \pm 0.229b	0.003	7.134
	November	6.267 \pm 1.089a	6.375 \pm 1.080a	1.5 \pm 0.389b	0.001	8.473
<i>Comperiella bifasciata</i>	September	2.333 \pm 1.047a	1.75 \pm 0.708a	0.583 \pm 0.193a	0.248	1.457
	October	3.667 \pm 1.170a	3.667 \pm 1.227a	1.583 \pm 0.553a	0.26	1.403
	November	4.933 \pm 1.244a	4.6 \pm 1.249a	1.933 \pm 0.511a	0.102	2.408
<i>Mallada spp.</i>	September	0.4167 \pm 0.193	0.583 \pm 0.229	0.167 \pm 0.112	0.289	1.29
	October	2.833 \pm 0.534a	2.417 \pm 0.417a	0.917 \pm 0.260b	0.007	5.79
	November	2.667 \pm 0.832a	2.467 \pm 0.723a	0.667 \pm 0.187a	0.065	2.913
<i>Rhizobius lophanthae</i>	September	2.5 \pm 0.584 ab	2.917 \pm 0.712a	0.667 \pm 0.225b	0.015	4.785
	October	3.167 \pm 0.878ab	3.5 \pm 0.925a	0.667 \pm 0.142b	0.021	4.37
	November	4.867 \pm 0.920a	4.933 \pm 0.983a	2.267 \pm 0.511a	0.054	3.345

6.4.2. *Aphytis melinus* captures

The total number of *A. melinus* captured by the yellow sticky traps positioned under rubber septums that contain d-limonene or β -ocimene was greater than the numbers captured by the traps under the rubber septums filled with only paraffin oil as a control (Figure 6.1). Significant differences in the weekly captures of *A. melinus* between d-limonene and β -ocimene on the one hand and the paraffin oil, on the other hand, were recorded in the fifth week after release (R + 35d) and seventh (R + 49d), eighth (R+56d), 10th (R + 70d) and 12th week (R + 84) after

the parasitoids were released. In the pre-releases, first (R + 7d), second (R + 14d), third (R+ 21d), fourth (R + 28d), sixth (R + 42d), ninth (R + 63d), and the 11th week (R+ 77d) after the release, only very few parasitoids were trapped, and there were no significant differences in the numbers of wasps trapped (Figure 6.1).

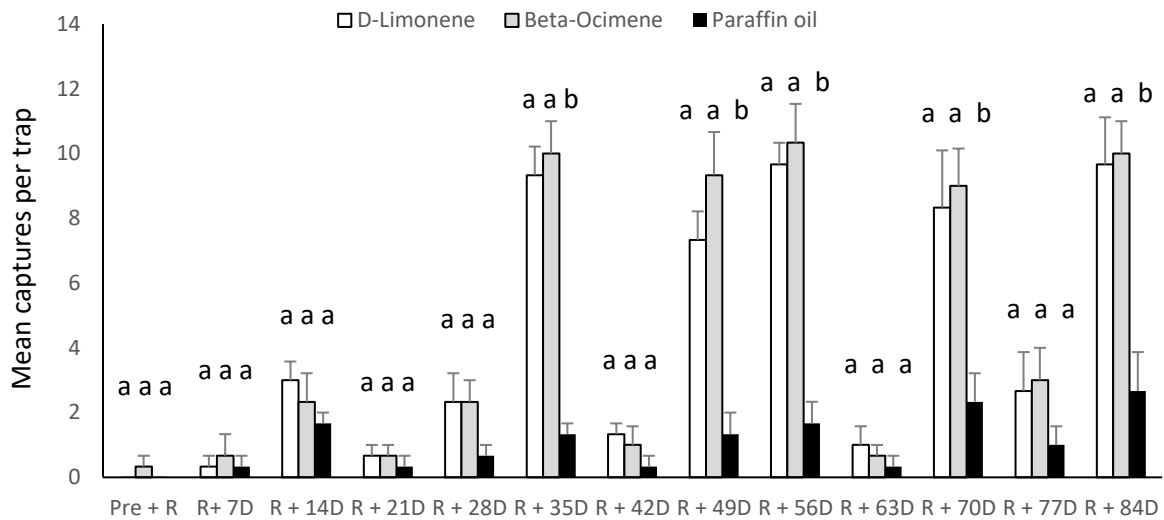


Figure 6.1. Mean number of adult *Aphytis melinus* captured (\pm SE) in yellow sticky traps located in citrus orchards baited with controlled-release d-limonene, β -ocimene and paraffin oil dispensers during 2018 growing season in each of the thirteen weeks of the study. Different letters in the same time interval are significantly different (ANOVA $P < 0.05$).

6.4.3. Estimated percentage parasitism (EPP)

The EPP by *A. melinus* did not differ significantly between trees' content, VOCs dispensers and paraffin oil dispensers, before and after the release. Except 35 days after the release, parasitism percentage was significantly greater in trees containing β -ocimene dispensers than in the other trees with d-limonene or just paraffin oil dispensers (Figure 6.2).

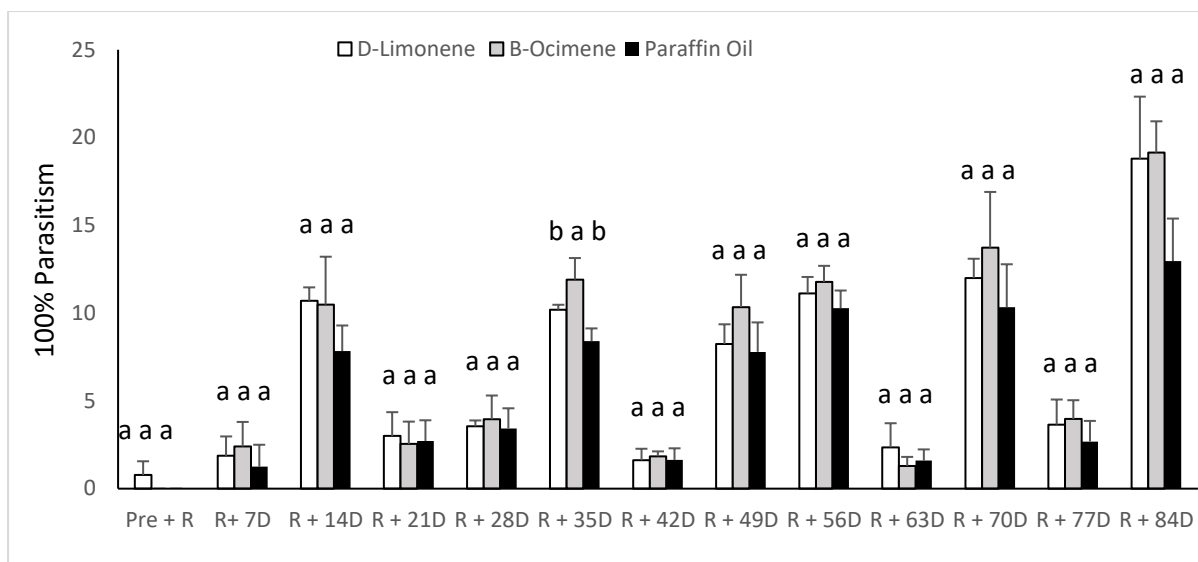


Figure 6.2. Mean number of adult *Aphytis melinus* captured (\pm SE) in citrus orchards baited with controlled-release d-limonene, β -ocimene and paraffin oil dispensers during 2018 growing season in each of the thirteen weeks of the study. Different letters in the same time interval are significantly different (ANOVA $P < 0.05$).

6.4.4. Pattern of parasitism

The cumulative number of *A. melinus* captured in the sticky traps under the d-limonene ($P = 0.912$; $N = 13$; $P \geq 0.000$), β -ocimene ($P = 0.902$; $N = 13$; $P \geq 0.000$), and paraffin oil dispensers ($P = 0.961$; $N = 13$; $P \geq 0.000$) were significantly correlated with EPP. The regression analysis showed that the number of wasps captured in traps under d-limonene ($R^2 = 0.316$; $F = 5.089$; $P = 0.045$) and β -ocimene ($R^2 = 0.264$; $F = 3.939$; $P = 0.073$) dispensers was also dependent on the available host density but not for the number of wasps captured in traps under paraffin oil dispensers ($R^2 = 0.068$; $F = 0.808$; $P = 0.388$). Furthermore, the EPP in trees with d-limonene ($R^2 = 0.052$; $F = 0.599$; $P = 0.455$), β -ocimene ($R^2 = 0.103$; $F = 1.267$; $P = 0.284$), and paraffin oil ($R^2 = 0.129$; $F = 1.634$; $P = 0.227$) was not correlated significantly to the density of vulnerable hosts. The mean minimum temperatures during the three months of the experiment (September, October and November) were (7.3, 12.5 and 12.1°C), while the mean maximum temperature was (20.1, 23.2 and 24.8°C) respectively.

6.5. Discussion

The potential of synthetic herbivore-induced plant volatiles (d-limonene and β -ocimene) in a crop dispensed via controlled-release sachets for enhancing the recruitment and residency of some beneficial insects is further supported by the field data presented here. The citrus orchard experiment demonstrated positive responses by certain insect species to d-limonene and β -

ocimene-baited trees. Further, our results suggested that biological control of *Ao. aurantii* was improved by d-limonene and β -ocimene in the citrus experiment.

Previous studies have indicated that wasps often use volatile cues for host location, which makes them ideal targets for biological control programs (De Moraes et al., 1998; Quilici and Rouse, 2012; Mohammed et al., 2019; Mohammed et al., 2020). Some of these wasps, which were shown previously, significantly responded to d-limonene and/or β -ocimene (e.g., *Aphytis melinus*, *Agathis bishopi* and *Aphidius gifuensis*) (Zimba, 2014; Kang et al., 2018; Mohammed et al., 2019; Mohammed et al., 2020). *A. melinus* showed a significant response to d-limonene and β -ocimene in October and November. In the current study, both synthetic HIPVs also attracted wasps in the family Encyrtidae and predators in two families (Chrysopidae and Coccinellidae). Encyrtids and *Comperiella bifasciata* are beneficial insects with the former being important parasitoids of *Ao. aurantii* but has not responded significantly to both synthetic HIPVs. Chrysopidae (*Mallada spp.*) has significantly responded to d-limonene and β -ocimene in this experiments in October, while Coccinellidae (*Rhizobius lophanthae*) responded significantly to just β -ocimene in September and October for the first time. *Mallada spp.* are an excellent general predator in the larval stage, while ladybird *Rhizobius lophanthae* adult and larva feed on scale insects at all stages. Both of these predators are predatory of many pests such as Aphids, Greenhouse whitefly, Scales, Mealybugs and Moth eggs and small caterpillars. The attraction of these families to d-limonene and β -ocimene has not previously been recorded.

The success of many augmentation biological control programs depends on the dispersal ability of the natural enemy released (Kölliker-Ott et al., 2004; Lavandero et al., 2010), and on the other hand, the dispersal ability of these natural enemies towards their hosts or preys depends on many factors. These factors can affect the physiological perception and behavioural response by arthropods to volatiles under field conditions. VOCs (e.g. composition and quantity of blends, their emission, and degradation), distances at which they are bioactive, and the physiological state of arthropods that are the putative volatile receivers are all important biotic factors and can interact with abiotic factors, such as wind speed and direction, to affect arthropod response (Williams et al., 2017). It is important to have many rather than a few or only one release point in augmentative programs (Zappalá et al., 2012). Insects behaviour which drove by olfactory, among another sensory system parts, influences mate finding, food searching, avoidance of enemies, and competition. (Lima and Dill, 1990; Schoonhoven et al., 2005). Olfactory stimuli from infested fruit are known to be essential during host location behaviour for many parasitoids (Zimba et al., 2015; Giunti et al., 2016). In this study, *A.*

melinus dispersed progressively from the release points to other citrus trees in the orchard over a period of 12 weeks. Our results show that releases of *A. melinus* during spring (September–November), when virgin female scales are most abundant relative to the other stages, reduces the percentage of scale-infested oranges in the release orchards. As abiotic conditions were variable in the field, the number of *A. melinus* caught in the traps was quite low in the first month (September) when compared with that in October and November. The compatibility in the number of parasitoids captured in the two treatments (control vs VOCs) during the first month indicates that this was mainly due to low temperature during the release of wasps as the temperature affects the duration of development of *Aphytis* which, in turn, influences their ability to regulate the pest's populations (Rosen and DeBach, 1979). The results (Figure 1) revealed that the significant higher attraction of *A. Melinus*, which was captured on the sticky traps hanged on citrus trees with d-limonene and/or β -ocimene dispensers than that on trees with just paraffin oil dispensers in October and November, was likely due to the relative increase in the attractiveness of these trees owing to the presence of the synthetic HIPVs.

Regarding the incidence of parasitism, we focussed on the incidence of parasitism and found that the presence of the HIPV dispensers increased the incidence of parasitism slightly by *A. melinus* under the field conditions compared to parasitism percentage on the absence of HIPV dispensers (Figure 2). In particular, the similarity in the incidence of parasitism in the two treatments (control vs synthetic HIPVs) during the whole trail indicates that this was possibly due to the presence of many fruits infested with *Ao. aurantii*, both on the trees carrying synthetic HIPVs dispensers and those carrying paraffin oil dispensers, especially if we know that the amount of the d-limonene or β -ocimene in the dispensers is approximately equal to the amount of this volatiles emitted from infested fruit (Mohammed et al., 2020). After 7, 21, 42 and 63 days, the numbers captured was almost zero, mainly because of the life cycle of the released parasitoids, i.e., on these dates, the parasitoid may have been in the larval stages of their life cycle. Regarding the parasitism percentage, the parasitism recorded 35 days after the release was significantly higher in trees hanging β -ocimene dispensers compared other treatments. During other weeks, the EPP was more uniform in all the release trees, with no significant differences between the treatments.

From the viewpoint of plant defence strategies, the emission of the HIPVs that attract the carnivorous natural enemies of herbivores is considered to constitute an indirect defence against herbivores (Arimura et al., 2009; Allmann and Baldwin, 2010). Importantly, by combining the blend of synthetic volatiles from *Ao. aurantii*-infested trees with those released

by infested citrus trees in the field, we were able to increase the incidence of parasitism of *Ao. aurantii* slightly by the parasitoid *A. melinus* present in the surrounding environment.

The analysis of the potential association between cumulative parasitoid captures and total EPP, and between these two parameters and the density of susceptible hosts, highlighted that d-limonene and β -ocimene influenced parasitoid dispersal (in terms of captures) towards their host. By contrast, the non-significant association on sticky traps under paraffin oil dispensers suggest that, under the infestation conditions recorded in our trial, VOCs is more important in determining parasitoid dispersal towards their host. Habitat location over longer distances may rather be accomplished by using cues, which are associated with the habitat in general than with the host itself (Kost, 2008), and that leads to the fact that the lack of a significant association between EPP and host density under the infestation conditions recorded in our trial, which d-limonene and β -ocimene have no influence in the increase of parasitism percentage, maybe due to the equal quantities of synthetic d-limonene and β -ocimene released from septum dispensers and infested fruit from one side and other side; it may be because of the parasitoids strategies that predict that oviposition preference of parasitoids should correlate with host suitability for offspring development (Mills, 2009). To increase the EPP rate, we think that it needs to experiment with higher concentrations of these synthetic VOCs used in comparison with their percentage emitted from infested fruit.

D-limonene and β -ocimene are a common plant volatile, which can be found in the rind of citrus fruits, such as lemons, limes, mandarin, and oranges (Mohammed et al., 2017b; Mohammed et al., 2019; Mohammed et al., 2020). However, it is also an induced volatile in citrus trees damaged by *Ao. aurantii* (Mohammed et al., 2017a; Mohammed et al., 2019; Mohammed et al., 2020). The attractiveness of d-limonene and β -ocimene to *A. melinus* was demonstrated in olfactometer tests (Mohammed et al., 2019; Mohammed et al., 2020). In the current field study, d-limonene and β -ocimene were attractive to *A. melinus*. Both compounds also attracted beneficial insects in the current field study.

Chapter Seven

General Discussion

7.1. Introduction

This study has made a significant research contribution in three specific areas: a) identified the quantitative and qualitative changes of volatile organic compounds from citrus fruit after infestation with *Aonidiella aurantii*, b) examined responses of the parasitoid *Aphytis melinus* towards *Ao. aurantii*-infested and non-infested citrus fruit and to synthetic volatile compounds, and c) demonstrated that lures baited with the synthetic volatiles d-limonene and β -ocimene play a significant role in attracting *A. melinus* in the field to *Ao. aurantii*.

The main aim of this study was to examine the possibility of the synthetic volatiles d-limonene and β -ocimene to attract *A. melinus* to infested fruit and to increase the percentage parasitism of *Ao. aurantii* by *A. melinus*. In order to achieve this aim, five experimental chapters were conducted. These experiments progressed from understanding (1) how to quantify age-specific birth and death rates of *Ao. Aurantii* and its parasitoid *A. melinus*, which enabled predictions to be made on the growth or decline of *Ao. aurantii* and its parasitoid *A. melinus* populations (Chapter 2); (2) identification of volatile compounds emitted from healthy and *Ao. aurantii*-infested citrus fruit (Chapter 4 and 5); (3) identification of which volatile compounds produced by *Ao. aurantii* infested fruit are attractive to female *A. melinus* parasitoids (Chapter 4 and 5); (4) determination of whether there are distinct observable and interpretable behavioural responses from *A. melinus* that can be associated with *Ao. aurantii* infested fruit (Chapter 4 and 5); and (5) the determination of whether attraction by parasitoids to synthetic herbivore-induced plant volatiles (HIPVs) is present in the field, and if synthetic volatiles can be practically applied to attract *A. melinus* (Chapter 6).

The major findings from these experiments are discussed in more detail below:

The use of natural enemies to control pests should be based on the availability of a comprehensive life table of the pest and its natural enemies. Life tables are powerful and necessary tools for analysing and understanding external factors such as various temperatures have on the growth, survival, reproduction, and intrinsic rate growth of insect populations (Chi and Su, 2006). Understanding the effect of the main abiotic factor, temperature, on the development and fecundity of and its parasitoid *A. melinus* is a useful way of predicting the activity and population dynamics of both the pest and the parasitoid in the field. Consequently, this will allow the release of the parasitoid at the most appropriate time to most effectively

control the *Ao. aurantii*. The aim of my first experimental chapter (Chapter 2), was to develop an age-specific life table based on population growth of *Ao. aurantii* and *A. melinus* using a range of biological and ecological parameters under different temperature conditions. This was successfully achieved, and consequently it is now possible to calculate the life expectancy of *Ao. aurantii* and *A. melinus*. This information can be used for biological control by predicting which is the most suitable temperature in terms of the longevity and the number of off springs for both *Ao. aurantii* and its parasitoid *A. melinus*, thus we can predict the particular instars which will be subjected to maximum mortality. On this basis, we can plan to manage *Ao. aurantii* during its most susceptible life stage to its parasitoid *A. melinus*. In Chapter 2, 27°C was found to be the best temperature to provide the highest net reproduction rate (R_0) and mean generation time (T_0), and conversely, intrinsic rate of increase (r_m) for *Ao. aurantii* in comparison to *A. melinus*. The intrinsic rate of increase (r_m) was higher for *A. melinus* (0.188) than *Ao. aurantii* (0.080) at 27°C. These life table parameters confirmed the potential of *A. melinus* to control *Ao. aurantii*. Consequently, these life table parameters will be of value to researchers in the future.

The emission of volatile organic compounds (VOCs) from plants is involved in a wide class of ecological functions, as VOCs play a crucial role in plant interactions with biotic and abiotic factors (Vivaldo et al., 2017). The aim of the second experimental chapter (Chapter 3), was to determine the best conditions for extracting VOCs from some non-infested and *Ao. aurantii* infested citrus fruit species. The SPME technique coupled to the GC FID/MS was found to be a robust, rapid and reliable method to analyse VOCs. To my knowledge, this is the first study to successfully use this methodology on VOCs from fresh fruit of different citrus species. Specifically, a range of different parameters (chamber volume, sealing and extraction times) were optimised for the analysis of emitted VOCs to ensure the maximum release of VOCs from the fibre without compromising the composition of VOCs released. In brief, a chamber volume of 500 ml, a sealing time of 20, 16, 8 and 16 hours for lemon, lime, mandarin, and orange respectively, and an extraction time of 2, 4, 2 and 2 hours for lemon, lime, mandarin, and orange respectively were found to provide the optimal parameters for determining VOCs from infested and non-infested fruit.

Plants produce VOCs in response to herbivore attack, and these VOCs can be exploited by parasitoids of the herbivore as host location cues. The aim of the third experimental chapter

(Chapter 4), was to separate and identify the VOCs emitted from non-infested and infested Tahitian lime fruit with *Ao. aurantii* and to examine the ability of some VOCs in attracting the parasitoid *A. melinus* towards its host *Ao. aurantii*. 18 VOCs were shown to significantly increase in *Ao. aurantii*-infested lime fruit, these were: oxime-, methoxy-phenyl-, β -terpinene, β -myrcene, isoterpinolene, d-limonene, β -cis-ocimene, β -ocimene, γ -terpinene, terpinolene, 1,3,7-nonatriene, 4,8-dimethyl-, (3E)-, nonanal, cosmene, methyl salicylate, undecane, 4,8-dimethyl-, caryophyllene, δ -selinene, (Z,E)- α -farnesene, 7-epi- α -selinene. In contrast, just linalool decreased during infestation and were characteristic of non-infested lime fruits. Five volatiles were exclusively produced by infested fruits and these volatiles were β -thujene, trans-d-limonene oxide, 1-isopropenyl-3-propenylcyclopentane, terpinen-4-ol and trans- γ -bisabolene.

D-limonene and β -ocimene were chosen to show their role in increasing the attraction of *A. melinus* towards their hosts *Ao. aurantii* based on a literature review of the previous studies (Zimba, 2014; Zimba et al., 2015; Song et al., 2017; Kang et al., 2018), and on the increasing in the amount of these two VOCs after infestation with *Ao. aurantii*. The innate positive chemotaxis of mating *A. melinus* females toward lime fruit and their VOCs were tested in olfactometer assays. Compared to the control without fruit, female *A. melinus* showed a significantly greater attraction to healthy and *Ao. aurantii*-infested lime fruit. In addition, in these biological assays, a higher but not significantly different number of individuals moved to odour sources emitted from infested fruit, probably because the amounts of VOCs released from non-infested fruit in these systems were still high compared to infested fruit. Plant odours can be recognised from a longer range and are considered to be more detectable for foraging parasitoids to locate host plants (Vet and Dicke, 1992; Röse et al., 1997; Steidle and Van Loon, 2003; Morawo, 2017), while herbivore-specific odours are short-range cues considered to be more reliable indicators of host presence. These studies further suggest that healthy fruit can produce volatiles that may act as attractants of *A. melinus*, but these volatiles may be down-regulated by *Ao. aurantii* through volatile profile modification, which plays a key role in the host location behaviour of *A. melinus* parasitoids. The synthetic compounds, d-limonene and β -ocimene both elicited a strong attraction for the parasitoid at 15 μ l/ml, indicating an intrinsic response to these compounds as a short-range attractant. These results highlight the important roles played by d-limonene and β -ocimene in mediating the attraction of *A. melinus* to *Ao. aurantii*. In general, these results suggest that herbivore-related experiences such as oviposition

play an important role in host discrimination by specialist parasitoids. Nevertheless, the results also showed that the plant-related experience plays an important role in shaping the foraging decisions made by parasitoids. This is besides serving as highly detectable long-range cues for locating host habitat (Vet and Dicke 1992).

Based on the results from Chapters 4, the aim of the fourth experimental chapter (Chapter 5), was to repeat this work but using other citrus fruit species in order to confirm the results. In total, more than 34 compounds were detected from non-infested and *Ao. aurantii*-infested lemon, orange and mandarin fruit. However, n-hexadecanoic acid was exclusively produced by infested lemon fruit, with decreases in 5,4-di-epi-aristolochene and increases in d-limonene and β -ocimene after infestation. Whilst, 2, 6-octadienal, 3,7-dimethyl-(E)- and α -bulnesene were only detected from non-infested lemon. For mandarin fruits, γ -terpinene, alloocimene, alloaromadendrene, d-limonene and β -ocimene were significantly increased in infested mandarins. For orange, compounds changed only in infested fruit, e.g., five compounds, alloaromadendrene, 1,3,7-nonatriene, 4,8-dimethyl-, (3E)-, hexyl caproate, d-limonene and β -ocimene increased, and five compounds, acetic acid, hexyl ester, alloocimene, α -terpineol, nerolidol, 1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl- and (3E,7E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene were only present in infested fruit. Interestingly, two HIPVs, d-limonene and β -ocimene, were significantly increased by *Ao. aurantii* infestation in all infested fruit, regardless of the citrus species. Thus, these results supported the findings in Chapter 4 where these two VOCs attracted *A. melinus* towards their host *Ao. aurantii*. In two-choice behavioural assays, *A. melinus* females non-significantly preferred infested citrus cues over non-infested fruit with the exception of infested lemon fruit, which attracted significantly more female *A. melinus*. In addition, these results are compatible with the first step of the parasitism strategies which indicates that host selection consists of three steps: host plant habitat and host insect location, host recognition and acceptance and host suitability/regulation (Chesnais et al., 2015). Female *Ap. melinus* showed positive chemotaxis toward d-limonene and β -ocimene in all dosages combinations of d-limonene and β -ocimene, except for d-limonene at 10 μ l/ml. These results support the findings in Chapter 4 and agree with other studies where both plant- and herbivore-related experiences play important roles that affect foraging decisions in parasitoids, especially at short-range (Röse et al. 1997).

The results obtained from the laboratory studies (Chapters 4 and 5) encouraged the application of the highest dosages of both d-limonene and β -ocimene in the field to determine their roles

in attracting natural enemies towards *Ao. aurantii*-infested citrus and increasing the parasitism percentage of *Ao. aurantii* (Chapter 6). In brief, d-limonene and β -ocimene were mixed with paraffin oil for slow release and examined for their attractiveness to *A. melinus* in the field after augmentative releases. A total of 10,000 *A. melinus* adults were released in different spots of a citrus orchard covering two acres. The spread of the parasitoid was evaluated, for three months after release, using yellow sticky traps and by monitoring the percentage parasitism of the *Ao. aurantii* on citrus fruit. The lures baited with d-limonene and β -ocimene, in the field significantly attracted some species of natural enemies but had no significant impact on others. The main natural enemies of *Ao. aurantii* found in the field in order of abundance were *A. melinus*, *Comperiella bifasciata*, *Rhizobius lophanthae* and *Mallada* spp. Analyses conducted over three months (September, October, and November) revealed significantly greater numbers of *A. melinus* were trapped in the yellow sticky traps treated with d-limonene and β -ocimene dispensers than the traps treated with paraffin oil in October and November, while there were no significant differences observed in September. The estimated percentage of parasitism (EPP) by *A. melinus* did not differ significantly between trees contain VOCs dispensers and trees contain paraffin oil dispensers, before and after the release of the parasitoids. Except 35 days after the release, parasitism percentage was significantly greater in trees contain β -ocimene dispensers than in the other trees contain d-limonene or just paraffin oil dispensers. Finally, in this experiment the overall parasitism rates were not increased by synthetic VOCs lures but there was evidence that lures may increase parasitism of *Ao. aurantii* when there is a decrease in the levels of VOCs due to lack of non-infested and infested fruit. Therefore, it can be concluded that the production of d-limonene and β -ocimene in citrus is potentially part of the indirect defence strategy against *Ao. aurantii* infestation by attracting its natural enemies. Thus, d-limonene and β -ocimene play a key role as long range attractants of *A. melinus* females to *Ao. aurantii* infested citrus fruit.

7.2. Future research

This study has found potential avenues for future research, these include:

- Examining d-limonene and β -ocimene at doses higher than 20 μ l/ml individually and in combination. This might lead to improved attraction of *A. melinus* towards *Ao. aurantii*-infested fruit than observed in the current study.
- Evaluating the role of synthetic VOCs d-limonene and β -ocimene in attracting other natural enemies to *Ao. aurantii* in citrus orchards.

- Assessing the effectiveness of other types of VOCs and their mixtures in attracting *A. melinus* and other natural enemies to *Ao. aurantii*-infested citrus.
- Assessing the impact of abiotic factors to better understand the behaviour of *A. melinus* by exploiting host–plant interactions for improved pest management is encouraged.
- Studying non-volatile organic compounds extracted from the *Ao. aurantii* armour and bodies and whether these will increase parasitism of *Ao. aurantii* by *A. melinus*.

7.3. Conclusion

Through this study, a significant stride has been made towards the role of the synthetic volatiles, d-limonene and β -ocimene, in increasing the attractiveness of the parasitoid *A. melinus* towards *Ao. aurantii*-infested citrus fruit. In light of the observations made in this study, a better understanding of the biology of *Ao. aurantii* is essential for improving the mass production of *A. melinus* for their subsequent release into the field. It was evident in this study that the infestation of citrus fruit by *Ao. aurantii* increased the emission of some VOCs, while others were exclusively emitted after the infestation. These VOCs have been classified as herbivore-induced plant volatiles (HIPVs), which are considered highly-detectable signals by parasitoids to help them locate the host habitat. Therefore, it is suggested that chemical analysis of *Ao. aurantii* -infested citrus fruit, be examined to ascertain the chemical compound/s that are responsible for eliciting attraction behaviour in *A. melinus* females. Given the poor innate oriented movement of female *A. melinus* towards *Ao. aurantii* -infested fruit in the field, it is advisable to consider conditioning *A. melinus* female parasitoids to compounds in infested fruit in order to enhance a stronger oriented movement of parasitoids towards *Ao. aurantii* -infested fruit. This will allow the more effective control of *Ao. aurantii* in commercial citrus orchards.

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