EFFICACY OF PLANT ESSENTIAL OILS AND DETOXIFICATION MECHANISMS IN *CHORISTONEURA ROSACEANA*, *TRICHOPLUSIA NI*, *DYSAPHIS PLANTAGINEA* AND *MYZUS PERSICAE*

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ABSTRACT

The obliquebanded leafroller, *Choristoneura rosaceana*, and the rosy apple aphid, *Dysaphis plantaginea* are serious pests in apple orchards throughout North America, while the green peach aphid, *Myzus persicae* and the cabbage looper, *Trichoplusia ni* are serious pests in vegetable greenhouses. In an effort to reduce the impact of these pests on their respective crops, growers typically resort to multiple insecticide applications per year for the control of each pest. However, concerns regarding the risk of such pesticides to human and environmental health have led to renewed calls for the development of reduced risk pesticides.

In the following, 17 essential oils were screened against each pest species to identify those which could be used to develop novel essential oil-based insecticides and the most toxic of these were further evaluated to determine their LC_{50} and LD_{50} values. Patchouli oil was found to be among the most toxic to all four species. Thyme oil was also toxic to both *C. rosaceana* larvae and *D. plantaginea* adults, while citronella oil demonstrated high toxicity to *D. plantaginea*. Garlic and lemongrass oils were also identified as potential candidates for *T. ni* control and lavender oil was identified as the second most toxic essential oil to *M. persicae*.

Through this work, it was noted that there appeared to be a role for detoxification enzymes in detoxifying these essential oils. Accordingly, the detoxicative abilities of each insect and the potential role of patchouli oil in inducing these enzymes were assessed. Esterase activity was highest in *M. persicae* while glutathione *S*-transferase activity followed the order of *M. persicae* > *D. plantaginea* > *C. rosaceana* > *T. ni*. Cytochrome P450 activity was only detected in some samples, and consequently, results were less conclusive. The potential for incorporating these essential oils into an essential oil-based insecticide is discussed.

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I would like to dedicate this thesis to my parents, Jack and Adelia Machial, for their assistance over the years. As a young girl, they always encouraged my ideas and taught me to think for myself. I am the woman that I am today thanks to them, and their influence will continue to guide who I am into the future.

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CO-AUTHORSHIP STATEMENT

Chapter 1

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Murray Isman assisted in the development of concepts and provided valuable information on the subject matter. I conducted the literature review and wrote the chapter.

Chapter 2

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Ikkei Shikano performed the labour for the bioassays on *T. ni* under my supervision. Michael Smirle provided the azinphosmethyl resistant line of leafrollers and assisted with the LCR and LDR calculations using software he owned/licensed (PoloPlus). Roderick Bradbury provided access to and instruction on the use of the GC-MS device. He also assisted with confirming the identification of the essential oil constituents. Murray Isman provided insight into the project design and provided valuable information on the subject matter. I designed and conducted all experiments and analyzed the data with the exception of the points specifically outlined above. I also wrote the manuscript.

Chapter 3

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Chapter 4

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1 INTRODUCTION[1]

In the ongoing arms race against insect pests, plants have evolved a variety of natural defences. These defences can range from physical barriers such as trichomes, waxes and pitch, to chemical defences including a variety of toxins (e.g. glucosinolates in *Brassicae* and phenolic glycosides in willow (*Salix*)) and chemical cues which can be used to attract predators and parasitoids.[1-4] Yet, as would be expected with natural evolution, many insects have adapted to overcome these defences and they continue to feed on their host plants.[4-7] Among these resistance strategies are avoidance of plant toxins by adjusting feeding behaviour, suppressing host-plant defences, sequestering or excreting toxins, and detoxifying toxins.[2, 7, 8] The monarch butterfly provides a classic example of an insect which uses several of these methods of resistance to survive on its host plant, milkweed (*Asclepieas* sp). Trenching, or cutting the leaf veins allows the larvae to avoid the plant toxins, cardenolides from the milkweed are sequestered for protection from predators, and various enzymes are used to detoxify other chemicals.[2, 7] However, this arms race can be impacted by a variety of different factors.

In nature, a natural balance has developed as a complex relationship between trophic levels. Plant growth is impacted by the environmental and soil conditions in which it grows, which in turn impacts the suitability of the plant as a host for feeding insects and the development of various plant defences. This affects the control of these herbivorous insects by predators, parasitoids and various microorganisms (e.g. bacteria, viruses and entomopathogenic fungi). Each trophic level is impacted by the

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levels below it with environmental conditions exerting various effects at each level.[1, 2] This interaction is all well in nature, however, as humans, we are rarely satisfied.

In an effort to protect our valuable crops (e.g. food, timber, and other crop plants) from insect pests, we have resorted to the use of pesticides. Pesticide use increased drastically with the introduction of organochlorines, organophosphates and carbamates following the Second World War, however concerns about impacts on the environment and human health have led to the search for safer pesticides.[9, 10] This has led to a renewed interest in the use of botanical pesticides, taking advantage of the natural chemical defences used by plants. The application of natural products for pest control is not a new phenomenon with several botanicals having documented uses for more than 150 years.[10, 11] Among the most well-known botanicals used to date are pyrethrum, rotenone, nicotine and neem oil.[9, 10, 12, 13] Pyrethrum is currently the most commercially used botanical insecticide, while nicotine use has declined significantly due to concerns surrounding human toxicity.[9, 11] These two biopesticides have also spawned two separate classes of reduced-risk chemical pesticides (the pyrethroids and neonicotinoids) by creating pesticides with chemically related structures to their natural variants. However, these chemical pesticides often still have numerous non-target effects and can create a different set of problems.[14-16] In addition, they are not compatible with organic cropping systems, meaning that alternatives are still required.

1.1 Essential Oils

Another group of botanical pesticides which has received increased attention recently are plant derived essential oils. Derived from the steam distillation of the foliage and/or

other plant parts (e.g. flowers and fruit) of particular aromatic plants, plant essential oils have been investigated for their antimicrobial, insecticidal, fungicidal and herbicidal activities.[9, 17, 18] Essential oils have been extracted from members of the Apiaceae (carrot), Lamiaceae (mint), Myrtaceae (myrtle) and Rutaceae (citrus) plant families, while several aromatic Poaceae (grass) species have also been used, including various *Cymbopogon* species. Highly aromatic species from other plant families have also been studied. These oils typically consist of complex mixtures of highly volatile compounds including mono- (C_{10}) and sesquiterpenes (C_{15}), phenols and alcohols. Among the major constituents that make up some of these oils are trans-anethole, caryophyllene, carvacrol, 1,8-cineole, citronella, eugenol, limonene, linalool, menthol, α-pinene, pulegone and thymol.[17, 19-21] They are generally considered to be non-persistent in the environment and typically regarded as being compatible with integrated pest management (IPM) programmes.

Much of the research on essential oils in the past 20 to 30 years has focused on the use of essential oils as repellents and as fumigants against various stored product pests. The essential oils of numerous plants have been tested against the rice weevil (*Sitophilus oryzae*), the maize weevil (*S. zeamais*), the red-flour beetle (*Tribolium castaneum*), the bean weevil (*Acanthoscelides obtectus*), and other stored product pests. Nutmeg oil significantly impacts both *S. zeamais* and *T. castaneum* populations and depending on the concentrations used, has fumigant and/or repellent properties, and can suppress F1 progeny.[22] The essential oils of several *Ocimum* species have also been shown to have fumigant effects against various pests, including *S. zeamais* and *T. castaneum*, and further investigations into the specific components of the oils

found that eugenol and camphor, the major components of *O. suave*, *O. basilicum*, and *O. kilimandsharicum*, are largely responsible for the observed bioactivity.[23-25] Don-Pedro also reported the fumigant effects of citruspeel oil against *Callosobruchus maculatus* F., *S. zeamais* and *Dermestes maculatus*, showing a range of bioactivity.[26-28] In addition, the essential oils of *Mentha viridis*, *Eucalyptus globulus*, *Mentha microphylla*, *Rosmarinus officinalis* and *Lavendula hybrida* caused reductions in the numbers of eggs laid by *Acanthoscelides obtectus* females, through both reduced fecundity and increased egg retention (oviposition deterrence) and they also showed strong toxic effects against *A. obtectus* adults (dependent on sex and essential oil used).[29, 30]

While less research has been conducted on other pest species, there is a growing collection of results. For example, 2% concentrations of lemongrass (*Cymbopogon citratus*) oil effectively inhibited oviposition and protected leaf discs from consumption by the cotton leafworm *Spodoptera littoralis*.[31] Out of 53 essential oils tested as fumigants, the oils of caraway seed (*Carum carvi*), citronella java (*Cymbopogon nardus*), lemon eucalyptus (*Eucalyptus citriodora*), pennyroyal (*Mentha pulegium*) and peppermint (*Mentha piperita*) applied at 14 x 10^{-3} µl/ml air caused >90% mortality in the two-spotted spider mite (*Tetranychus urticae*).[32] Sampson et al. found that essential oils containing (E)-2-tridecenal, (E)-2-tetradecenal or carvacrol (e.g. the essential oils from several *Saturega* and *Thymbra* species) as major constituents have high levels of activity in contact bioassays with the turnip aphid, *Lipaphis psuedobrassicae*.[33] Tansy oil has also been found to impact the growth and fecundity of obliquebanded leafrollers

(*Choristoneura rosaceana*),[34] and various essential oils have been shown to be both repellent and attractant to the codling moth (*Cydia pomonella*).[35]

This is still a growing field, and one facing a number of challenges. To this point, while there have been few instances of commercially available essential oil-based insecticides, there is an opportunity for these products. Indeed, there are several companies which produce essential oil based products commercially, including EcoSMART Technologies in the U.S. which has produced several commercial products. Continuing research will expand the knowledge base, and will assist in the development of additional essential oil-based pesticide alternatives.

1.2 Apple Orchard IPM

Apple producers face a variety of challenges from apple pests. In Pacific Northwest and British Columbia apple orchards, there are numerous insect pests which require management to prevent significant damage to crops. This includes the codling moth (*C. pomonella*), the obliquebanded leafroller (*C. rosaceana*), the rosy apple aphid (*Dysaphis plantaginea*), the apple maggot (*Rhagoletis pomonella*) and the apple clearwing moth (*Synanthedon myopaeformis*), as well as various other pests such as the two-spotted spider mite (*Tetranychus urticae*). In the case of the apple maggot and apple clearwing moth, both have only just recently been introduced into British Columbia and while the apple maggot has not yet reached interior apple orchards, the apple clearwing moth has been found in interior commercial apple orchards and is becoming a serious concern for apple producers.

1.2.1 Obliquebanded leafroller (*Choristoneura rosaceana* Harris)

Of these major apple pests, *C. rosaceana* (see Figure 1.1), has increased in severity from a secondary pest to a primary pest due to changes in pest management techniques for the codling moth. It is native to North America, is widely distributed, and can be a pest on a wide range of rosaceous species.[36, 37] In the past, the use of pesticides to control the codling moth also controlled *C. rosaceana* populations, limiting the ability of the population to reach damaging levels. However, in combination with the development of insecticide resistance, along with the reduced need for pesticide applications for the codling moth following the introduction of mating disruption and the Sterile Insect Release (S.I.R.) program, *C.rosaceana* has emerged as a new major pest of concern.[38-42] Another challenge with the leafroller is that while it is typically a univoltine pest, there is evidence that it can have more than one generation per year in warm areas, including possibly in the southern Okanagan Valley.[43]

Figure1.1. Fifth instar (left) and adult (right) obliquebanded leafroller, *Choristoneura rosaceana.*

Leafrollers typically overwinter as late second to third instar larvae in hibernacula. In the early spring, they emerge and begin feeding on flower buds and then move to the expanding leaves where they roll leaves or web leaves together in order to form a protected feeding space.[42] *C. rosaceana* larvae will typically go through six instars before pupating.[37] After emergence, the adults mate and the females lay their eggs in groups on the apple leaves. Emerging larvae can then rapidly disperse to new locations on silk threads, at which point, they can begin feeding again.[44] Early season damage can occur to the flower buds as well as developing fruitlets, while the summer generation can cause significant damage to developing fruits.

One of the challenges faced by apple growers is the development of insecticide resistance. There are numerous examples of insecticide resistance in leafroller larvae and there are reports of cross resistance to other insecticides (e.g. the insect growth regulators, tebufenozide and methoxyfenozide).[38, 40, 45] While there has been research looking at alternative control strategies (e.g. mating disruption[46] and kaolin clay applications[47]), insecticides remain the most effective strategy for control. However, to reduce the risk of insecticide resistance, additional options are required.

1.2.2 Rosy apple aphid (*Dysaphis plantaginea* Passerini)

Another major apple pest, *D. plantaginea* (see Figure 1.2), affects many apple orchards in North America and Europe and can lead to serious economic losses if not controlled. *D. plantaginea* is a specialized host-alternating species, overwintering as eggs, then hatching in the early spring and feeding on apple leaves. In the summer, it develops into an alate form which then migrates to plantain (*Plantago* spp.) in the understory and

continues to feed and replicate. Changing photoperiods with longer nights in mid-September cause the aphids to develop into gynoparae (winged females) which return to apple to produce the oviparae (the sexual females). Winged males develop on the plantain a few weeks later, then fly to the apple trees and mate with the oviparae, which then deposit eggs under the bark.[48] Feeding damage by developing and adult aphids on apple leaves creates an open gall which causing leaves to curl and thicken and can impact the development of nearby apple fruitlets.[49, 50] Feeding damage can also impact the ability of infested branches to flower in the following year.[48] Other effects of aphid feeding are a reduction in the efficacy of chemical thinning agents as well as reduced natural fruit drop resulting in greater labour costs to manually remove the excess fruit.[50] Affected apples are typically smaller in size, may be deformed and are of lower quality (lower fruit firmness and reduced storage capacity) at harvest (personal observation, Machial and Isman, unpublished).

Figure 1.2. Rosy apple aphid, *Dysaphis plantaginea*, on apple (left) and plantain (right).

Damage can vary from year to year with some years exhibiting very little damage while other years can have 20% or higher yield loss due to aphid damage if no chemical controls are applied.[48] The use of aphicides is common practice for the control of *D. plantaginea*, however, there has been research to assess the potential for using alternate controls including the application of kaolin clay,[51] relying on natural predators and augmenting natural predator populations,[52-54] and considering the use of resistant strains of apple.[55, 56] However, at this point, none of the alternative control methods is at the level where it can provide sufficient control of this pest, meaning that growers will continue to rely on pesticide application.

1.3 Greenhouse IPM

Vegetable and floral greenhouses face a different set of challenges from insect pests than apple orchards, in part because of the different crops and pests, but also because of the different growing system. Because vegetable and floral greenhouses can operate year round, they typically maintain optimal temperatures and lighting conditions that can extend the growing season, which also provides an optimal environment for insect pests to thrive. This allows pests to go through multiple generations a year, whereas they may only go through one or two generations outside of the greenhouse environment. Amongst the major greenhouse pests are the cabbage looper (*Trichoplusia ni*), green peach aphid (*Myzus persicae*), greenhouse whitefly (*Trialeurodes vaporariorum*), western flower thrips (*Frankliniella occidentalis*) and the two-spotted spider mite (*T. urticae*).[57] Greenhouse growers have had to change the focus of their pest management strategies as the increased number of insect generations per year can

also increase the incidence of insecticide resistance. This has led to an increased reliance on IPM strategies including biological control, using biopesticides such as *Bacillus thuringienesis* var. *kurstaki* (BTk), trap cropping and mass trapping.[57]

1.3.1 Cabbage looper (*Trichoplusia ni* Hübner)

T. ni (see Figure 1.3) is a highly polyphagous pest which is a problem on various greenhouse vegetable crops including pepper, cucumber and tomato. Adult moths enter greenhouses from nearby fields and lay their eggs individually on the plants and/or other surfaces. Hatching larvae will feed on suitable hosts and will go through 5-6 instars before pupating.[58] Once the adults emerge from their pupae, they mate and continue the cycle. Given that greenhouses provide near optimal growing conditions throughout the year, they can have multiple overlapping generations per year.[59] Although different crops can influence *T. ni* developmental rates and survivability,[60] the multiple overlapping generations can allow looper populations to increase rapidly inside greenhouses, even in crops which are not ideal host plants.

Figure 1.3. Late instar larvae (left) and adult (right) cabbage looper, *Trichoplusia ni.*
Source: Ontario Ministry of Agriculture Food and Rural Affairs
(http://www.omafra.gov.on.ca/IPM/english/tomatoes/insects/cabbage-looper.html)

As a major defoliator, *T. ni* larvae can cause significant damage to the foliar canopy, which can reduce the photosynthethic capacity of the plants in the greenhouse. This in turn can result in smaller fruits or longer production times, all of which cost the greenhouse grower in the long run. But *T. ni* larvae can also feed directly on developing fruits, damaging the fruit and reducing the marketable yield. Accordingly, greenhouse growers have a relatively low tolerance for *T. ni*. This often leads to the spray application of BTk or spinosyn based products, but the extensive use of these pesticide applications has led to problems with resistance development. For example, there are recent reports of increased tolerance or resistance in *T. ni* to BTk applications.[59] This has led to the renewed search for new control alternatives that are also compatible with the greenhouse production system.

1.3.2 Green peach aphid (*Myzus periscae* Sulzer)

M. persicae (see Figure 1.4), is a major pest on a wide variety of crops in addition to being a serious problem for greenhouse growers. The life cycle of *M. persicae* is similar to that of *D. plantaginea*, except that there can be many secondary hosts for *M. persicae*, and it also has options when it comes to deciding how to overwinter. Although the primary/overwintering hosts for *M. persicae* are *Prunus* spp., this aphid can survive in their absence with little difficulty in temperate areas which has allowed the green peach aphid to become distributed throughout many areas of the world.[61] Factors such as daylength, temperature, nutritional status and population density can influence the production of the various aphid morphs.[61] For example, alate females can develop in response to high population density, which enables the aphids to spread to areas with lower population densities. In addition, as daylength shortens, the aphids appear to have the ability to overwinter both as diapausing eggs, or as hibernating parthenogenetic morphs, giving the aphids greater options for survival.[62]

Figure 1.4. Green peach aphid, *Myzus persicae*, infesting cabbage seedlings.

In the greenhouse environment, feeding aphids produce significant quantities of honey dew, making leaves and fruits sticky and providing a site for the growth of sooty mold. *M. persicae* is also an important vector of over 100 plant viruses including potato virus Y and potato leafroll virus, making it one of the most important aphid pests.[61, 63] This, along with the high reproductive rate of *M. persicae* and its ability to remain anholocyclic on its secondary hosts under favourable conditions (such as a greenhouse), has ensured that this aphid remains a major target for greenhouse pest management.[64] The high reproductive rate has also created another challenge. The green peach aphid can develop resistance to insecticides rapidly, restricting the available aphicide options for growers.[65, 66] Like the rest of the pests listed above, this has led to the continued search for novel pest management strategies.

1.4 Research Objectives

In the ongoing struggle to control insect pests such as *C. rosaceana*, *D. plantaginea*, *T. ni* and *M. persicae*, we will continue to rely on a variety of different control strategies, including the use of insecticides. However, our extensive use of insecticides in the past has led to serious environmental problems, as well as the development of insecticide resistance. Both of these issues have led to renewed calls to investigate and develop new reduced-risk alternatives which are compatible within an IPM program. As discussed above, one alternative is to take advantage of some of the natural strategies developed by plants and manipulate them for our pest control needs by developing botanical insecticides such as essential oil-based insecticides.

Essential oil-based insecticides have several advantages over current insecticides. They are highly volatile and thus non-persistent in the environment, they are composed of a complex mixture of constituents which is more likely to reduce the risk of resistance development, and they can work via several potential modes of action (e.g. fumigation, contact toxicity, deterrence, etc), which can also reduce the risk of resistance development. Many essential oils are also relatively harmless to humans, meaning there is a reduced risk to workers. However, there is still little known about how essential oils work and why they work the way they do. In addition, while some essential oils or their constituents are toxic to one insect species, they may be entirely ineffective against another insect species.[20] Potential reasons for this idiosyncrasy include the presence or lack of target binding sites, or the detoxicative abilities of the insect, but research on this is still limited.

In the following project, 17 essential oils were screened against *C. rosaceana*, *D. plantaginea*, *T. ni* and *M. persicae* in an effort to find essential oils which could be used to develop future essential oil-based insecticides. The most toxic of these oils were further evaluated in order to determine their LC_{50} and LD_{50} values. The composition of these essential oils was also determined to ensure that valid comparisons can be made in future studies. In addition, through the course of this work, it was noticed that each insect species responded differently to different essential oils and in many cases, the insects were able to recover from their exposure to the essential oils, suggesting a potential role for detoxification enzymes in breaking down the constituents in the oils. Accordingly, the potential influence of essential oils on inducing detoxification enzymes and the possible roles for these detoxification enzymes was also studied.

The main goal of this research was to develop an understanding of how essential oil-based insecticides could be used to manage four important pest species in British Columbia apple orchards and greenhouses, and to develop an understanding of the role that detoxification enzymes may play in the overall toxicity of these oils. As a part of this, one objective was to determine which essential oils are the most toxic to *C. rosaceana*, *D. plantaginea*, *T. ni* and *M. persicae*. Based on these results, the other objective was to determine which detoxification enzymes are active in these insects by looking at esterase, glutathione *S*-transferase and cytochrome P450 activity and to determine the inducibility of these enzymes in response to pre-treatment with an essential oil. The following hypotheses guided the research conducted as a part of this thesis:

1. Essential oils can cause significant toxicity in *C. rosaceana*, *D. plantaginea*, *M. persicae* and *T. ni*, and can be incorporated into essential oil-based insecticides.
2. Detoxification enzyme activity by esterases, glutathione *S*-transferases and cytochrome P450 enzymes can influence the toxicity of essential oils and can be influenced by essential oils.

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2 EVALUATION OF THE TOXICITY OF 17 ESSENTIAL OILS AGAINST *CHORISTONEURA ROSACEANA* (LEPIDOPTERA: TORTICIDAE) AND *TRICHOPLUSIA NI* (LEPIDOPTERA: NOCTUIDAE)[2]

2.1 Introduction

Botanical pesticides have a long history protecting food crops and stored products from insect pests, with documented uses going back 150 years or earlier.[1] Despite this, synthetic chemical pesticides have dominated the commercial pest control market since the 1950s. The insecticide market accounts for a large portion of the World's chemical pesticides, yet as more information becomes available, there is increased concern surrounding the negative ecological and health impacts of these products.[2] These concerns are exacerbated by insect pests becoming resistant to many insecticides, which typically requires higher doses to achieve effective control.[3] Increasing incidence of insecticide resistance has led to a renewed interest in developing insecticides with alternate modes of action, lower environmental impact, greater compatibility with integrated pest management (IPM) programs, and reduced health risk to humans and wildlife. Much of this focus has been on the use of insect growth regulators (IGRs) and microbial insecticides such as *Bacillus thuringiensis* (Bt), although there has also been a

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renewed focus on the development of botanical insecticides, including those based on essential oils.[2]

Essential oils are complex mixtures of highly volatile plant chemicals that are typically derived from the steam distillation of aromatic plant foliage. Commonly used in aromatherapy, many of these oils have been shown to possess medicinal, antibacterial, antifungal and insecticidal activities.[4-6] The fumigant effects of many essential oils are well established with extensive research focusing on the control of stored product pests.[7-9] Many essential oils are also acutely toxic to a variety of insects and can impose more chronic, sublethal effects on growth.[10-13] As a result of their highly volatile nature, essential oils are not persistent in the environment and are less likely to leave residues on food products. These favorable properties of essential oils suggest that products based on them may be viable options as a part of IPM.

The obliquebanded leafroller, *Choristoneura rosaceana* (Harris) and the cabbage looper, *Trichoplusia ni* (Hübner) are two serious orchard and greenhouse pests in the Pacific Northwest and British Columbia, Canada, respectively. *C. rosaceana* larvae can inflict significant apple losses due to their direct feeding on developing fruit. Larvae can also damage other fruit crops such as cherries and raspberries.[14, 15] Development of azinphosmethyl resistance and cross-resistance with several other insecticides (including the IGRs tebufenozide and methoxyfenozide) has led to control failures of *C. rosaceana* larvae in some interior British Columbia orchards.[16] Such a lack of effective control has left growers with fewer control options, primarily *B. thuringiensis* var. *kurstaki* (Btk) and spinosad. Greenhouse crops can be severely defoliated by *T. ni* as a

result of the insect's rapid development and six or more overlapping generations per year. While insecticide resistance is usually less of a concern with *T. ni*, some growers have started noticing increasing tolerance to Btk.[17]

In this study, we screened a selection of 17 commonly available essential oils for toxicity against both *C. rosaceana* and *T. ni* larvae and determined the LC_{50} and LD_{50} values for the most toxic of these oils. The response of *C. rosaceana* larvae to treatment with the essential oils was also monitored over a 24 hour period in order to determine the effects of these oils on the insects. Finally, resistance ratios were ascertained by using an azinphosmethyl resistant strain of *C. rosaceana* in order to determine if such oils could be considered in resistance management programs. I predict that some essential oils will cause significant mortality to both insect species and based on previous reviews of essential oil toxicity,[4] the response is expected to occur rapidly. Furthermore, given that essential oils are composed of complex mixtures of constituents, it is hypothesized that there will be no significant difference in toxicity between the susceptible and azinphosmethyl resistant strain.

2.2 Materials and Methods

2.2.1 Chemicals

Essential oils from seventeen plant species were obtained from two commercial sources (Table 2.1). Polysorbate 80 (Sigma-Aldrich) was used as an emulsifier and a control for some of our bioassays. Acetone (Fisher Scientific) was used as a solvent/emulsifier

and a control in other bioassays. Acetonitrile (Fisher Scientific) was used as a solvent for GC-MS.

Table 2.1. List of essential oils, their species names, plant family and the source of each oil used.

Common name*	Plant species	Plant family
Cedarwood oil[1]	*Juniperus virginiana* L.	Cupressaceae
Cinnamon oil[2]	*Cinnamomum zeylanicum* Blume	Lauraceae
Citronella oil[2]	*Cymbopogon nardus* L.	Poaceae
Clove bud oil[1]	*Eugenia caryophyllata* L.	Myrtaceae
Eucalyptus oil[1]	*Eucalyptus globulus* Labill.	Myrtaceae
Garlic oil[2]	*Allium sativum* L.	Alliaceae
Grapefruit oil[1]	*Citrus paradisi* Macf.	Rutaceae
Lavender oil[1]	*Lavandula angustifolium* Mill.	Lamiaceae
Lemongrass oil[2]	*Cymbopogon citratus* D.C.	Poaceae
Marjoram oil[1]	*Thymus mastichina* L.	Lamiaceae
Niaouli oil[1]	*Melaleuca viridiflora*	Myrtaceae
Patchouli oil[1]	*Pogostemon cablin* Benth.	Lamiaceae
Pennyroyal oil[2]	*Mentha pulegium* L.	Lamiaceae
Peppermint oil[2]	*Mentha piperita* L.	Lamiaceae
Rosemary oil[2]	*Rosmarinus officinalis* L.	Lamiaceae
Tea tree oil[1]	*Melaleuca alternifolia* L.	Myrtaceae
White thyme oil[2]	*Thymus vulgaris* L.	Lamiaceae

*Essential oils were obtained from the following sources: 1=Escents Aromatherapy, 2=EcoSMART Technologies

2.2.2 Insect maintenance

C. rosaceana and *T. ni* larvae were obtained from laboratory colonies that have been maintained at the University of British Columbia (UBC), Vancouver, BC, Canada for >30 and >50 generations, respectively. Both colonies were reared on artificial diet no. 9795 (Bio-Serv Inc., Frenchtown, NJ) supplemented with finely ground alfalfa and vitamins (no. 8045, Bio-Serv Inc). Each species was reared in a separate growth chamber set at 21-24°C and 16:8 h LD photoperiod. The azinphosmethyl resistant strain of *C. rosaceana* was obtained as pupae from the Pacific Agricultural Research Centre (PARC), Summerland, BC, Canada and reared at UBC for one generation as described

above prior to being introduced into the bioassays. Selection protocols in the development of this strain have been described previously.[18]

2.2.3 Comparative toxicity of essential oils

Essential oils were initially screened against first instar *C. rosaceana* and *T. ni* by preparing emulsions of the oils in 0.1% aqueous polysorbate 80. 9.95 ml of the aqueous polysorbate solution and 50 µl of essential oil were placed in a glass vial and vortexed for 10 seconds for a final concentration of 5 µl ml^{-1}. For each treatment, 25 neonate larvae were placed inside 10 cm diameter pyrex Petri dishes lined with filter paper and sprayed with the emulsion using a pump sprayer. Larvae were transferred to five-5 cm diameter Petri dishes which each contained a 1 × 1 × 0.5 cm piece of artificial diet. These smaller Petri dishes were placed in a growth chamber at 22°C with a 16:8 h LD photoperiod, and the larvae were permitted to feed. The aqueous polysorbate 80 solution, minus the oil component, was used as a control. Mortality was assessed for larvae at 24 hours under a dissecting microscope by probing the insects with a fine brush. Larvae were deemed to be alive if they moved in response to the brush's touch and they were considered to have died if they remained motionless. Death was confirmed at 48 hours to ensure that the larvae did not simply recover from temporary paralysis. Each experiment was replicated four times for a total of 100 insects per essential oil.

2.2.4 Determination of LC_{50} and LD_{50} values

Based on the results from the screening assay, the two essential oils most toxic to *C. rosaceana* larvae (thyme and patchouli oils) and the three essential oils most toxic to *T. ni* larvae (patchouli, lemongrass and garlic oils) were selected for determination of LC_{50} and LD_{50} values.

The spray application method described for the screening assay above was used for LC_{50} determination. A selection of 4-5 concentrations for each oil was prepared by mixing with aqueous polysorbate 80. Aqueous polysorbate 80 alone was used as the control. As before, the mixture was sprayed directly on 25 neonate larvae placed inside a 10 cm pyrex Petri dish which was lined with filter paper. Treated larvae were then transferred to five-5 cm diameter Petri dishes containing a 1 × 1 × 0.5 cm piece of artificial diet. The dishes were then placed in a growth chamber and mortality was assessed at 24 hours and confirmed at 48 hours. Each bioassay was replicated three times for *C. rosaceana* and 4 times for *T. ni*.

A topical application method was used on third instar larvae for both species for LD_{50} determination. Four or five doses for each oil were prepared by blending the oil in 1 ml of acetone. Acetone alone was used as the control. A 0.5 µl aliquot of the test solution was applied dorsally to each of 20 third instar larvae using a Hamilton microsyringe with a repeating dispenser. Treated larvae were then transferred to four-5 cm diameter Petri dishes containing a 1 × 1 × 0.5 cm piece of artificial diet and placed in a growth chamber as above. Mortality was assessed at 24 hours and confirmed at 48 hours; each bioassay was replicated 3-5 times.

2.2.5 Response of *C. rosaceana* to patchouli oil and thyme oil

Patchouli oil and thyme oil were applied topically to third instar *C. rosaceana* larvae at a rate of 10 µg insect^{-1} using acetone as a carrier as per the method above. Acetone alone was used as a control. The responses of the larvae were monitored at intervals ranging from 30 seconds post-treatment to 24 hours after treatment. Affected larvae were classified into two categories: those convulsing or unable to control their movements; and those which were paralyzed or moribund (not moving or at or near death). A total of 20 insects were used per treatment and each treatment was replicated 3 times.

2.2.6 Gas chromatography-mass spectrophotometry

The major constituents of garlic, lemongrass, patchouli and thyme oils were identified by gas chromatography/mass spectrometry (GC/MS) using a Varian 3900 GC system with a Saturn 2100T ion trap mass-selective detector (Varian Inc., Walnut Creek, CA). The column used was a FactorFour Capillary column VF-5 ms 30 m x 0.25 mm ID DF=0.25 with a low bleed/MS coating. Injections were performed by a Varian CP-8410 autosampler with an injection volume of 1 µl using pure helium at 1.0 ml min^{-1} as the carrier. The temperature profile used 80°C for 0.5 min, followed by an increase of 10°C min^{-1} for 22.0 min, then held at 300°C for 2.0 min for a total run time of 24.5 min. Constituents were identified by comparing spectra with both Saturn and NIST (National Institute of Standards and Technology) libraries. Relative proportions of the constituents were calculated using peak area by dividing the area under each peak by the total area.

2.2.7 Impact of azinphosmethyl resistance on essential oil toxicity

Using the spray application method described above, the azinphosmethyl-resistant strain of *C. rosaceana* was also tested to determine the LC_{50} values. Groups of 25 neonate larvae were sprayed with four concentrations of an emulsion of thyme oil or patchouli oil mixed with aqueous polysorbate 80. Aqueous polysorbate 80 alone served as an appropriate control. Larvae were immediately transferred to five-5 cm diameter Petri dishes containing a 1 cm × 1 cm × 0.5 cm piece of artificial diet. After larvae incubated in a growth chamber at 22°C with a 16:8 h LD photoperiod for 24 hours, mortality was assessed and then reassessed at 48 hours to confirm death. Each of these bioassays was replicated 3 times.

2.2.8 Data analysis

Mortality data (proportion of insects that died) from the screening assays were analyzed for analysis of variance (ANOVA) and means were compared using Duncan's multiple range test using SPSS 16.0 (SPSS Inc, Chicago, IL). Probit analysis was used to calculate LC_{50} and LD_{50} values and lethal concentration ratios (LCR) and lethal dose ratios (LDR) were calculated using PoloPlus (LeOra Software, Berkeley, CA). LCRs and LDRs were calculated to provide statistical comparisons between one essential oil treatment versus another. Abbott's correction was applied when required.[19]

2.3 Results

2.3.1 Comparative toxicity of the essential oils

Of the 17 essential oils (Table 2.1) tested against *C. rosaceana* larvae, 6 oils demonstrated significant mortality at 5.0 µl ml^{-1} (Table 2.2). Of these, thyme oil (*T. vulgaris*) and patchouli oil (*P. cablin*) were the most toxic, inducing 64.0% and 97.0% mortality, respectively. As the two most toxic essential oils, these were selected for further testing.

Four essential oils were significantly toxic to neonate *T. ni* larvae when screened at 5.0 µl ml^{-1} (Table 2.2). Patchouli, lemongrass (*C. nardus*) and garlic (*A. sativum*) oils caused significantly higher toxicity than the rest of the essential oils tested (48.0%, 53.0% and 74.0%, respectively) and accordingly, these three oils were selected for additional tests.

Table 2.2. Summary of screening results from 17 essential oils applied as a spray emulsion to first instar *C. rosaceana* larvae and first instar *T. ni* larvae at a concentration of 5.0 µl ml^{-1}.

	Mortality (%)	
Essential oil source	*C. rosaceana*	*T. ni*
Control	0.0 ± 0.0[a]	1.0 ± 1.0[a]
A. sativum	22.2 ± 3.7[c-e]	74.0 ± 11.8[d]
C. zeylanicum	17.0 ± 5.3[b-e]	18.0 ± 6.8[ab]
C. paradisi	8.0 ± 5.4[a-c]	1.0 ± 1.0[a]
C. citratus	8.0 ± 4.0[a-c]	53.0 ± 6.6[c]
C. nardus	3.0 ± 1.0[ab]	19.0 ± 8.2[ab]
E. globulus	6.0 ± 3.8[ab]	2.0 ± 2.0[a]
E. caryophyllata	23.2 ± 5.7[de]	27.0 ± 12.6[b]
J. virginiana	29.0 ± 11.5[e]	7.0 ± 1.9[ab]
L. angustifolium	6.0 ± 1.2[ab]	7.0 ± 2.5[ab]
M. alternifolia	6.0 ± 2.0[ab]	5.0 ± 1.9[a]
M. viridiflora	6.0 ± 2.0[ab]	2.0 ± 1.2[a]
M. pulegium	15.1 ± 5.7[a-e]	4.0 ± 1.6[a]
M. piperita	11.2 ± 4.3[a-d]	16.0 ± 5.4[ab]
P. cablin	97.0 ± 1.9[g]	48.0 ± 12.8[c]
R. officinalis	5.0 ± 5.0[ab]	1.0 ± 1.0[a]
T. masicina	7.0 ± 3.0[ab]	3.0 ± 1.9[a]
T. vulgaris	64.0 ± 7.1[f]	10.0 ± 2.6[ab]

*Values are mean (±SE) of n = 4 replicates with 25 first instar larvae per replicate. Means in each column followed by the same letter are not significantly different (Duncan test, $P < 0.05$).

2.3.2 Essential oil composition

The major constituents of garlic, lemongrass, patchouli and thyme oil were identified using GC-MS analysis and are listed in Table 2.3. Five sulfur-containing constituents and one unknown chemical (6.7%) were identified from the garlic oil sample. Six constituents were identified from lemongrass oil with citral and trans-verbenol accounting for 79.2% of the constituents identified. Patchouli alcohol was the most abundant compound identified from patchouli oil, followed by several isomers of guaiene (31.2% combined) and patchoulene (11.2% combined). Five additional minor

constituents were identified from patchouli oil in addition to caryophyllene, cedran-diol and γ-gurjunene. Thyme oil contained seven constituents including most importantly, thymol (57.8%) and ρ-cymene (28.6%). Additional minor compounds were detected but not identified as their concentrations were too low to be analyzed with mass spectroscopy.

Table 2.3. Major constituents identified from the four most insect toxic essential oils identified with GC-MS and their relative proportions in the pure oil.

	Essential oil source			
	A. sativum	*C. citratus*	*P. cablin*	*T. vulgaris*
Major constituents	% v/v			
Borneol	---	---	---	0.9
Camphene	---	10.7	---	1.6
Carvacrol	---	---	---	4.8
Caryophyllene	---	3.9	2.2	---
Caryophyllene oxide	---	2.9	---	2.1
Cedran-diol	---	---	0.8	---
Citral	---	47.1	---	---
ρ-Cymene	---	---	---	28.6
Diallyl disulfide	35.2	---	---	---
Geraniol	---	3.2	---	---
α-Guaiene	---	---	13.4	---
δ-Guaiene	---	---	15.4	---
Guaiene isomer	---	---	2.4	---
γ-Gurjunene	---	---	2.2	---
Linalool	---	---	---	4.1
Methyl 2-propenyl trisulfide	6.6	---	---	---
Methyl 1-propenyl disulfide	4.5	---	---	---
α-Patchoulene	---	---	9.2	---
β-Patchoulene	---	---	2.0	---
γ-Patchoulene	---	---	5.5	---
Patchouli alcohol	---	---	40.1	---
di-2-Propenyl trisulfide	26.2	---	---	---
Thymol	---	---	---	57.8
Trans-verbenol	---	32.1	---	---
3,3' Thiobis-1-propene	20.7	---	---	---

2.3.3 LC_{50} and LD_{50} values

In *C. rosaceana* neonates, the emulsion of patchouli oil had a LC_{50} of 2.8 µl ml^{-1} (Table 2.4), and was twice as toxic as the thyme oil emulsion (LCR = 0.51 (0.43-0.61), $P < 0.05$). Topical application of patchouli oil in acetone was also significantly more effective than thyme oil, however the difference was less pronounced (LDR = 0.70 (0.59-0.83), $P < 0.05$). No control mortality was observed.

T. ni larvae were considerably more tolerant to topical application of essential oils than *C. rosaceana* larvae, and somewhat more tolerant to spray emulsions (Table 2.4). Contrary to preliminary screening results, patchouli oil was nearly as toxic as garlic oil (LD_{50} = 25.7 µg $insect^{-1}$ vs. 22.7 µg $insect^{-1}$), however the difference was still significant (LDR = 0.886 (0.797-0.984), LCR = 0.71 (0.61-0.83), $P < 0.05$ for both). Lemongrass oil was significantly less toxic than both oils, with topical treatments of lemongrass oil being less than one half as toxic as either garlic or patchouli oils (LDR = 0.38 (0.33-0.43), $P < 0.05$, and 0.42 (0.36-0.50), $P < 0.05$, respectively). Control mortality was negligible.

Table 2.4. LC_{50} and LD_{50} values of garlic, lemongrass, patchouli and white thyme essential oils against first and third instars of *C. rosaceana* and *T. ni*. LC_{50} and LD_{50} values are followed by 95% confidence intervals.

Insect species	Essential oil source	LC_{50} (µl ml^{-1})	Slope*	LD_{50} (µg^{-1} insect)	Slope*
C. rosaceana	*P. cablin*	2.8 (2.6-3.3)	4.34 (0.41)	7.8 (6.6-9.5)	2.57 (0.28)
	T. vulgaris	5.6 (4.9-6.3)	4.21 (0.68)	11.2 (9.5-13.3)	4.92 (0.50)
Azinphosmethyl resistant *C. rosaceana*	*P. cablin*	4.3 (3.6-5.3)	4.28 (0.45)	---	---
	T. vulgaris	11.0 (6.9-22.1)	1.95 (0.20)	---	---
T. ni	*A. sativum*	3.3 (2.6-4.4)	4.64 (0.43)	22.7 (21.0-25.1)	8.42 (0.84)
	C. citratus	7.2 (5.9-9.0)	2.37 (0.18)	60.5 (51.9-71.1)	3.55 (0.42)
	P. cablin	4.7 (4.0-5.5)	3.62 (0.35)	25.7 (22.8-30.2)	4.23 (0.56)

*Slope of the probit line followed by SE

2.3.4 Response of *C. rosaceana* larvae to essential oils

Third instar *C. rosaceana* larvae responded rapidly to treatment with both patchouli and thyme oils at a rate of 10 µg $insect^{-1}$ (see Figures 2.1 and 2.2). Over 70% of larvae began convulsing or showing uncontrolled movements within the first 30 seconds after treatment. Within the first 5 minutes, all insects had responded to both the patchouli and thyme oil treatments with 86.7% and 91.7% of insects, respectively, showing clear symptoms of paralysis (unable to move when touched with a fine brush). However, 30 minutes after treatment, some of those paralyzed insects began to show signs of recovery and, after 3 hours, only 48.3% of larvae treated with thyme oil were still paralyzed. Insects treated with patchouli oil took slightly longer to recover but the trend was similar. Within the final 12 hour period though, the number of insects that were either paralyzed or moribund increased again to 70.0% and 73.3% for thyme and patchouli oils respectively, with many of the insects showing clear signs of death (e.g.

no response to touch or oxidized hemolymph on the outside of the body). None of the control insects showed any symptoms in response to the application of acetone alone.

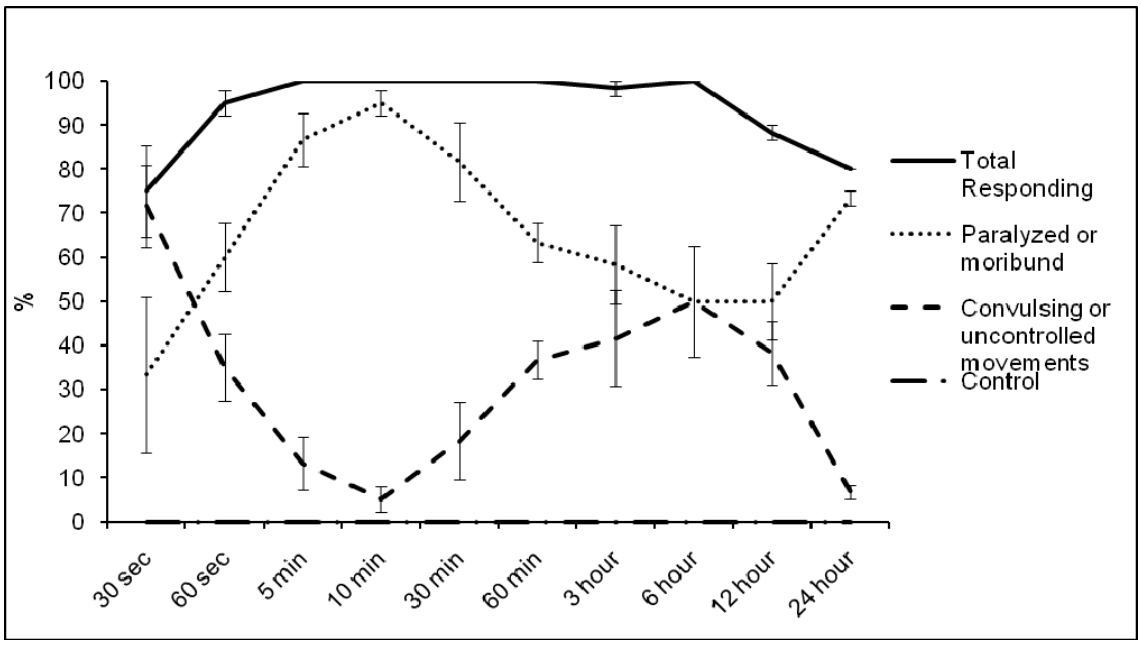

Figure 2.1. Responses of third instar *C. rosaceana* larvae treated topically with patchouli oil (10 µl insect^{-1}) over a 24 hour period. (Mean±SE).

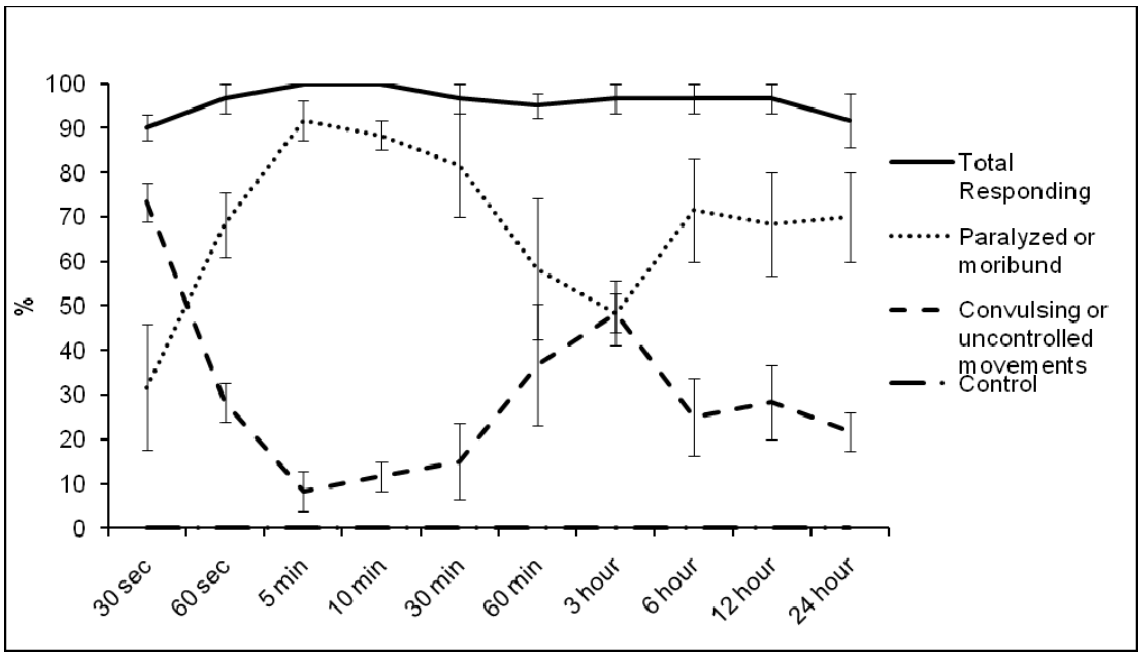

Figure 2.2. Responses of third instar *C. rosaceana* larvae treated topically with thyme oil (10 µl insect^{-1}) over a 24 hour period. (Mean±SE).

2.3.5 Impact of resistance on essential oil toxicity

First instar azinphosmethyl-resistant *C. rosaceana* were significantly more tolerant to both patchouli and thyme oils than the susceptible strain with LC_{50} = 4.3 µl ml^{-1} for patchouli oil and LC_{50} = 11.0 µl ml^{-1} for thyme oil (Table 2.4). The resistant strain demonstrated a 1.5-fold tolerance for patchouli oil (LCR = 0.43 (0.36-0.53), $P < 0.05$) and a 2.0-fold tolerance for thyme oil (LCR = 0.51 (0.40-0.65), $P < 0.05$).

2.4 Discussion

Preliminary screening demonstrated a wide range of toxicity among the 17 essential oils tested, and a majority of oils demonstrated little to no toxicity at the screening concentration of 5.0 µl ml^{-1}. Patchouli oil was identified as one of the most toxic essential oils against both lepidopteran species. However similar patterns were not observed with the other oils. While thyme oil was the second most toxic oil to *C. rosaceana* larvae in screening assays, it was one of the least toxic oils to *T. ni* larvae. Garlic and lemongrass oils were among the least toxic oils to *C. rosaceana* larvae, although they were the most toxic to *T. ni* larvae. The results corroborate earlier observations that toxicity of specific essential oils among insect species is highly variable.[4] Furthermore, even individual essential oil constituents such as limonene and linalool can differ in toxicity depending on the strain of the insect species used.[20]

In comparing the toxicity of patchouli oil and thyme oil to *C. rosaceana*, we found that the application method can affect the toxicity of thyme oil. Patchouli oil was two times more effective than thyme oil when sprayed as an emulsion, but only 1.4 times as

effective via topical application. The use of acetone as the carrier may have facilitated the penetration of thyme oil through the cuticle, resulting in a lower LD_{50}. Patchouli oil has been previously observed to cause tissue destruction,[21] and in observations with topically-treated *C. rosaceana* larvae, there was evidence of oxidized hemolymph (based on its brown colouration) on the surface of the larvae 24 hours after treatment. Patchouli oil toxicity has also been reported for the housefly, *Musca domestica*,[22] and it is both toxic and repellant to the termite *Coptotermes formosanus.*[21] Thyme oil is amongst the most effective oils against a variety of insect pests, including the mosquito *Culex quinquefasciatus*[11] and the pine processionary moth, *Thaumetopoea pityocampa*.[23] In a separate study with a related species of the pine processionary moth, *T. wilkinsoni*, thymol was shown to cause 50% mortality at 5 µl ml^{-1}, while carvacrol caused 65% mortality at the same concentration.[24] Another constituent of thyme oil, camphene, caused decreased growth rate and lower pupal weight in the western spruce budworm, *C. occidentalis*, demonstrating the potential sublethal effects on growth.[25]

Following the topical application of patchouli and thyme oils at a rate of 10 µl $insect^{-1}$ (~LD_{80} level), third instar *C. rosaceana* larvae also exhibited a variety of symptoms including convulsions (spasms resulting in flailing of the body), followed by paralysis within the first 5 minutes. This indicates a likely neurotoxic mode of action. Indeed, work conducted by Enan found that exposure to various essential oil constituents resulted in changes to octopamine and tyramine receptor binding activity, cAMP levels and [CA^{2+}] levels, in both *Drosophila melanogaster* and *Periplaneta americana*, supporting this hypothesis.[26, 27] Interestingly though, after approximately 30 minutes,

some recovery was observed, suggesting a potential role for metabolic enzymes in the detoxification of these oils. However, due to the relatively high dose of essential oils used, while some recovery was observed at 24 hours, most insect subjects did not survive. In previous work with *Spodoptera litura* and *T. ni*, thymol, the major constituent of thyme oil, was found to be metabolized to its 3-*O*-*β*-glucoside and excreted, providing further evidence to suggest that detoxification enzymes may play a role in the observed temporary recovery.[28]

While the toxicity of patchouli oil was similar for both *C. rosaceana* and *T. ni* neonate larvae, 3rd instar *T. ni* larvae were more than 3 times more tolerant to patchouli oil than 3rd instar *C. rosaceana* larvae. Given that LD_{50} values for both garlic oil and lemongrass oil were comparable, it is expected that the increase in LD_{50}s is due to increased tolerance by *T. ni* larvae, which could be due to differences in larval size or more efficient detoxification of plant toxins. The potential role for the activity of the insects' detoxification enzymes are presented in Chapter 4 and these results may provide insight into the developmental cause for insect tolerance.

The specific composition of essential oils can also profoundly impact toxicity as synergistic effects among constituents in the oil can influence toxicity.[10, 29, 30] However, individual constituents can be highly toxic themselves. In experiments with *Sitophilus zeamais* and *Tribolium castaneum*, two serious stored product pests, two major constituents of garlic oil were screened for toxicity. Diallyl trisulfide and methyl allyl disulfide were both highly toxic to these pests depending on the life stage tested. These tests indicate that diallyl trisulfide and methyl allyl disulfide impart much of the toxicity to

garlic oil.[31] Citral, the major constituent of lemongrass oil, has been shown to be both toxic and phototoxic against developing *T. ni* larvae.[32] Given that our sample of lemongrass oil contained 47.1% citral, it is expected that much of the toxicity of lemongrass oil was linked to the presence of citral.

The comparison of the azinphosmethyl-resistant and susceptible strains of *C. rosaceana* indicated a 1.5 and 2.0-fold tolerance for both patchouli and thyme oil, respectively. In comparison, the azinphosmethyl resistant strain used was previously described to be ~38 times as resistant to azinphosmethyl versus the susceptible laboratory strain.[18] This indicates that the cross-resistance to the essential oils conferred by azinphosmethyl resistance is likely to be negligible and may be due to interspecific differences between the two strains rather than a specific resistance mechanism. Accordingly, essential oil-based insecticides could be used for resistance management of azinphosmethyl resistant leafrollers or any other product with a different mode of action than patchouli or thyme oils.

Our results indicate that patchouli and thyme oils, properly formulated, may be useful for the control of *C. rosaceana* larvae and possibly used for managing resistance. *T. ni* larvae may be effectively controlled with patchouli oil as well as garlic and lemongrass oils. Further work will be required to establish the mode(s) of action of these oils and to develop more effective formulations. It will also be important to determine the impact of these oils on other insects to safeguard beneficial species such as bees, predatory insects and parasitoids. As phytotoxicity is a concern for some essential oils,[33] consideration must be made to protect the crops from any phytotoxic effects. To our

knowledge, there are no studies that have tested the phytotoxicity of patchouli, thyme, garlic and lemongrass oils. Determination of these factors will ensure that insecticides developed from these essential oils are fully compatible with an IPM program.

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3 INSECTICIDAL ACTIVITY OF 17 ESSENTIAL OILS AGAINST THE ROSY APPLE APHID, *DYSAPHIS PLANTAGINEA* PASSERINI AND THE GREEN PEACH APHID, *MYZUS PERSICAE* SULZER[3]

3.1 Introduction

Development of novel aphicides is an ongoing task as aphid pests continue to develop resistance to currently available products. Two pests that continue to receive attention in British Columbia agriculture are the rosy apple aphid, *Dysaphis plantaginea* Passerini (Homoptera: Aphididae), and the green peach aphid, *Myzus persicae* Sulzer (Homoptera: Aphididae). *D. plantaginea* is the most serious aphid pest on apples and is considered one of the most serious apple pests, both in North American and European apple orchards.[1, 2] This species reproduces on apple trees (*Malus domestica* Borkhausen) during the spring before moving to its secondary host plant, *Plantago* spp. in the summer.[2] Feeding damage in the spring results in the development of leaf galls and causes leaves to curl, protecting feeding aphids from pesticide applications.[1, 3] Apple fruitlet development is impacted even at low aphid numbers, resulting in small, deformed fruit near infested leaves, possibly due to toxins in the aphid saliva.[4, 5] This damage has led to a very low tolerance for this pest and accordingly, the use of aphicides is often recommended.

Although *Prunus* spp. are the primary hosts for *M. persicae*, this aphid has a vast range of secondary plant hosts and is a problem worldwide.[6] *M. persicae* can vector over 100

[3] A version of this chapter will be submitted for publication. Machial, CM, Bradbury, R and Isman, MB. (2010) Toxicity of 17 essential oils to the rosy apple aphid, *Dysaphis plantaginea*, and the green peach aphid, *Myzus persicae*.

plant viruses, including most notably the potato leaf roll virus and potato virus y,[6, 7] and honeydew secretion by the aphids can lead to the development of sooty mold caused by *Ascomycete* sp. Owing to these factors, there is often also a very low tolerance for this aphid and in some cases, multiple applications of aphicides are used.

One of the biggest challenges with both *M. persicae* and *D. plantaginea* is that because of their rapid development and short generation times, both species can develop resistance to aphicides rapidly.[6-10] This has led to extensive efforts to find alternative solutions including augmentation of biological controls,[11, 12] use of conservation biological control,[13] adjustments in the timing of pesticide applications,[14] and alternative control options. Kaolin clay has been studied as an alternative control for both aphid species with somewhat positive results,[8, 15, 16] while neem products derived from the Indian neem tree (*Azadirachta indica*) have shown promise for the control of *M. persicae*.[17] Various essential oils have also been tested against *M. persicae* with demonstrated toxic and repellent effects.[18-22] To date, there are no known studies that have considered essential oils for the control of *D. plantaginea*, although Cross tested garlic extracts for autumn control of this pest.[14]

The goal of the present research was to screen a selection of essential oils in an effort to identify those that warrant further consideration and development as aphicides. A selection of 17 commonly available essential oils were screened against both *D. plantaginea* and *M. persicae* adults and based on the results of this screening, additional experiments were conducted to further assess the toxicity of the essential oils with the highest mortality in the screening assays. In addition, it is expected that if the

essential oils target similar tissues and have similar modes of action in both aphid species, then both species will have comparable responses to the essential oils.

3.2 Materials and Methods

3.2.1 Chemicals

A selection of 17 commonly available essential oils was obtained from two sources (see Table 2.1). Acetone and acetonitrile were purchased from Fisher Scientific and polysorbate 80 was purchased from Sigma-Aldrich. Soil Moist polymer (polyacrylamide gel granules) was obtained from a local garden supply store.

3.2.2 Insect culture

Dysaphis plantaginea was collected from plantain plants located within a 5 m radius of apple trees on the University of British Columbia (UBC) campus. No pesticides had been applied to these trees and surrounding plant material for at least five years. *Myzus persicae* was obtained from cabbage plants in the horticulture greenhouse at UBC. The greenhouse has had limited pesticide application for the previous 3 years with biocontrol used for the control of aphids, western flower thrips (*Frankliniella occidentalis*) and the greenhouse white fly (*Trialeurodes vaporariorum*).

D. plantaginea was reared inside 1 L clear plastic containers (height 15.2 cm) containing five leaves cut from *Plantago lanceolata* in 250 ml of fully expanded Soil Moist polymer in water. Contact between the aphids and the Soil Moist polymer was restricted by placing the leaves through a layer of paraffin wax placed on top of the Soil Moist. These aphid cages were sealed with a plastic lid with the center cut out and replaced by shear fabric to allow for airflow. *M. persicae* was also reared as above

except that two *Brassica rapa* var. *chinensis* (pak choi) leaves were used in each cage along with one *Brassica oleracea* var. *capacitate* 'Stonehead' (cabbage) leaf as an alternate food source in the event that the pak choi leaves wilted before leaves were replaced. The aphid cages were kept in a growth chamber (22°C, 16:8 h LD photoperiod). Leaves were replaced weekly and the Soil Moist was cleaned or replaced weekly or as required.

3.2.3 Preliminary assessment of the toxicity of 17 essential oils

The 17 essential oils listed in Table 2.1 were initially screened against both *D. plantaginea* and *M. persicae*. Emulsions of essential oil were prepared inside glass vials by vortexing 50 µl of essential oil in 9.95 ml of 0.1% aqueous polysorbate 80 to give a final concentration of 5.0 µl ml^{-1} essential oil in solution. This concentration was selected as preliminary experiments indicated that a 5.0 µl ml^{-1} solution would provide an appropriate range of mortality. Groups of 10-adult apterous aphids were placed on glass 10 cm diameter Petri dishes lined with a 10 cm diameter filter paper and sprayed using a pump sprayer until the filter paper was wet and all aphids had been exposed. Aqueous polysorbate 80 solution was used as a control. Aphids were transferred in groups of 5 onto a 2 cm diameter leaf disc that was placed inside a 5 cm diameter Petri dish that also contained a piece of cotton moistened with 300 µl of distilled H_2O. Leaf discs were cut using a #13 cork borer and the moistened cotton was used to maintain turgidity of the leaf discs and to maintain humidity in the Petri dish. Leaf discs were cut from *P. lanceolata* for the bioassays with *D. plantaginea* and from *B. rapa* var. c*hinensis* for the bioassays with *M. persicae*. The aphids were then transferred to a growth chamber (22°C, 16:8 h LD photoperiod). Mortality was assessed at 24 hours by viewing

aphids under a dissecting microscope. Adult aphids were scored as dead if they did not respond to touch using a fine brush. Each experiment was repeated 3 times and the two essential oils demonstrating the highest mortality against each aphid species were selected for further research. Thyme oil was also selected for further study with *D. plantaginea* as it was one of the most effective essential oils against another apple pest, the obliquebanded leafroller, *Choristoneura rosaceana* (see Chapter 2), and could be impacted by field applications of thyme oil meant to control emerging overwintering *C. rosaceana* larvae.

3.2.4 Gas chromatography-mass spectrophotometry

The major constituents of citronella, lavender, patchouli and thyme oils were determined and quantified via gas chromatography-mass spectrophotometry (GC-MS) using a Varian 3900 GC system with a Saturn 2100T ion trap mass selective detector (Varian Inc., Walnut Creek, CA). Data was collected and analyzed on a PC using Varian GC/MS Worskstation software. A FactorFour capillary column VF-5ms 30m x 0.25mm 1D DF=0.25 with a low bleed/MS coating was used. One µl samples were injected into the column by a Varian CP-8410 autosampler using a split ratio of 100:1 in the injection port. 99.999% UHP helium with a column flow rate of 1.0 ml min^{-1} was used as a carrier gas. The temperature profile started at 80°C for 0.5 min and was followed by an increase of 10°C min^{-1} for 22.0 min, then held at 300°C for 2.0 min for a total run time of 24.5 min. The MS ion trap temperature was 220°C with a manifold temperature of 80°C and transfer line temperature of 300°C. Scan time for the MS detector (used in electron ionization mode) was 0.58 seconds per scan with a mass scan range of 40-650 m/z and maximum ionization time in the ion trap was 25,000 µs. Individual constituents were

identified by comparison with both Saturn and NIST (National Institute of Standards and Technology) libraries.

3.2.5 Determination of LC_{50} and LD_{50} values for selected essential oils

Along with thyme oil, patchouli and citronella oils were selected for further study with *D. plantaginea* and patchouli oil and lavender oil were selected for further study with *M. persicae*. Four to five concentrations of the essential oils ranging from 0.5-25.0 µl ml^{-1} in 0.1% aqueous polysorbate 80 were prepared and groups of 20-25 adult aphids were sprayed using the method described for the screening assay above. The aqueous polysorbate 80 solution was applied for control treatments. Aphids were transferred in groups of 4-5 to five-5 cm diameter Petri dishes containing a piece of moistened cotton and either a 2.0 cm diameter *P. lanceolata* or *B. rapa* var. *chinensis* leaf disc (for bioassays with *D. plantaginea* or *M. persicae*, respectively). The Petri dishes containing the aphids were then placed in a growth chamber (22°C, 16:8 h LD photoperiod) and mortality was assessed at 24 hours as above. LC_{50} values were calculated for each essential oil tested.

LD_{50} values were determined using topical bioassays with essential oils in acetone. Four to five doses of essential oils ranging from 5-50 mg ml^{-1} were prepared by dissolving the oils in acetone. Acetone alone was used as the control. Using a Hamilton microsyringe with a repeating dispenser, 0.2 µl aliquots of each solution were applied to groups of 20 adult aphids. Treated aphids were then transferred in groups of 4-5 to 5 cm diameter Petri dishes containing moistened cotton and a leaf disc as above. Aphids were then placed in a growth chamber and mortality was assessed at 24 hours.

All bioassays with *D. plantaginea* were repeated 3-6 times while all bioassays with *M. persicae* were repeated 3-4 times.

3.2.6 Data analysis

Analysis of variance (ANOVA) was used to analyze mortality data from the screening assays. Means were compared using Duncan's test. Probit analysis was used to determine LC_{50} and LD_{50} values and corresponding 95% confidence intervals. All analyses were completed using SPSS 16.0 (SPSS Inc, Chicago, IL) and Abbott's correction was applied where required (Abbott 1925).

3.3 Results

3.3.1 Assessment of toxicity of the essential oils

In preliminary screening bioassays with the 17 essential oils tested, 10 essential oils demonstrated significant toxicity to adult *D. plantaginea* with 50% or greater mortality at the screening concentration of 5.0 µl ml^{-1} (Table 3.1). However, considerable variability was observed for many of the oils. For example, tea tree oil caused an average of 60.0% mortality, however individual replicate mortality ranged from 20-90%, while the variability was even higher for pennyroyal oil with individual replicates ranging from 10-90% mortality. This was not the case for all essential oils and the most toxic oils (patchouli, citronella, and peppermint oils) produced more consistent results. Of the oils tested, the two most toxic essential oils - patchouli oil (90% mortality) and citronella oil (80%) - were selected for further testing. Thyme oil was also selected for further testing against *D. plantaginea* even though it was the third least effective essential oil tested

(33.3% mortality) as it was the second most effective oil for another apple pest species, *Choristoneura rosaceana* (see Chapter 2).

The essential oils were much less toxic to *M. persicae* adults. Only patchouli, lavender and peppermint oils caused significant mortality at 5.0 µl ml^{-1} (50%, 46.7% and 30%, respectively), while all other essential oils caused less than 15% mortality. Unlike *D. plantaginea*, low variability was observed between replicate treatments. As the two most toxic essential oils to this species, patchouli and lavender oils were selected for further study.

Table 3.1. Results from preliminary screening bioassays with 17 essential oils applied at a concentration of 5.0 µl ml^{-1} as a spray emulsion to adult *D. plantaginea* and *M. persicae*.

	Mortality (%)	
Essential oil source	*D. plantaginea*	*M. persicae*
Control	5.0 ± 3.4^{a}	0.0 ± 0.0^{a}
A. sativum	36.7 ± 16.7^{a-d}	0.0 ± 0.0^{a}
C. zeylanicum	16.7 ± 3.3^{ab}	3.3 ± 3.3^{a}
C. paradisi	66.7 ± 6.7^{c-e}	0.0 ± 0.0^{a}
C. citratus	70.0 ± 10.0^{c-e}	13.3 ± 6.7^{a}
C. nardus	80.0 ± 11.5^{de}	13.3 ± 8.8^{a}
E. globulus	40.0 ± 15.3^{a-d}	3.3 ± 3.3^{a}
E. caryophyllata	60.0 ± 20.8^{b-e}	3.3 ± 3.3^{a}
J. virginiana	70.0 ± 10.0^{c-e}	6.7 ± 3.3^{a}
L. angustifolium	56.7 ± 3.3^{b-e}	46.7 ± 3.3^{c}
M. alternifolia	60.0 ± 20.8^{b-e}	3.3 ± 3.3^{a}
M. viridiflora	30.0 ± 20.8^{a-c}	3.3 ± 3.3^{a}
M. piperita	73.3 ± 8.8^{c-e}	30.0 ± 5.8^{b}
M. pulegium	50.0 ± 23.1^{b-e}	13.3 ± 13.3
P. cablin	90.0 ± 5.8^{e}	50.0 ± 5.8^{c}
R. officinalis	36.7 ± 16.7^{a-d}	0.0 ± 0.0^{a}
T. mastichina	46.7 ± 12.0^{a-e}	6.7 ± 3.3^{a}
T. vulgaris	33.3 ± 6.7^{a-c}	13.3 ± 3.3^{a}

*Values are mean (± SE) of n = 3 replicates with 10 adult aphids per replicate. Means in each column followed by the same letter are not significantly different (Duncan test, $P < 0.05$)

3.3.2 Essential oil composition (GC-MS)

Table 3.2 lists the major constituents of citronella and lavender oils as identified with GC-MS analysis along with their relative proportions (calculated according to peak area). Major constituents for patchouli and thyme oils are listed in Table 2.3. Chemical profiles differed markedly between the oils with very few compounds in common among them. A total of 11 compounds were isolated from citronella oil including one unknown chemical. Of the identified compounds, nearly half of the oil was composed of citronellal (48.7%) while geraniol and a geraniol isomer made up an extra 18.8%. Lavender oil was composed primarly of linalool and linalyl acetate (89.3% combined), two constituents commonly found in lavender oil. An additional four constituents were identified including 1,8-cineole, camphor and terpinen-4-ol. Patchouli oil had the most diverse chemical profile with a total of 13 isolated constituents, including one unknown compound and several isomers of patchoulene and guaiene. Patchouli alcohol was the most abundant constituent of patchouli oil at 40.1%. Thymol (57.8%) and ρ-cymene (28.6%) were the major components in thyme oil, while an additional five constituents were identified with quantities ranging from 0.9% to 4.8%.

Table 3.2. Identified major constituents and their relative proportions as isolated from citronella and lavender oils using GC-MS.

	Essential oil source	
	C. nardus	*L. angustifolium*
Major constituents	% v/v	
1,8-Cineole	---	3.9
Camphene	4.5	---
Camphor	---	3.2
Caryophyllene	---	1.9
Citronellal	48.7	---
α-Cedrene	6.5	---
β -Elemene	5.2	---
Elemol	6.0	---
Geraniol	15.1	---
Geraniol isomer	3.7	---
Isopulegal	3.3	---
Linalool	---	47.6
Linalyl acetate	---	41.7
Menthyl acetate	5.3	---
Terpinen-4-ol	---	1.6

3.3.3 LC_{50} and LD_{50} determinations

Patchouli oil was twice as toxic as citronella oil and more than three times as toxic as thyme oil when applied as a spray emulsion to *D. plantaginea* adults. However, when applied topically in acetone, there was a surprising reversal (see Table 3.3). Thyme oil was the most toxic oil when applied topically with a LD_{50} of 2.5 µg insect^{-1} compared to 2.9 µg insect^{-1} for citronella oil and 3.1 µg insect^{-1} for patchouli oil. Given that the 95% confidence intervals overlap considerably for all three oils, there is no significant difference between the toxicity of the topically applied oils.

Table 3.3. LC_{50} and LD_{50} values for adult *D. plantaginea* and *M. persicae* treated with citronella, lavender, patchouli, and thyme oils applied as a spray emulsion or topical application, respectively. Values are followed by 95% confidence intervals.

Insect species	Essential oil source	LC_{50} (µl ml^{-1})	Slope*	LD_{50} (µg $insect^{-1}$)	Slope*
D. plantaginea	*C. nardus*	4.8 (3.4-7.2)	1.69 (0.15)	2.9 (2.5-3.3)	2.93 (0.29)
	P. cablin	2.4 (1.6-3.4)	1.45 (0.13)	3.1 (2.5-3.9)	3.49 (0.34)
	T. vulgaris	7.4 (6.3-8.9)	2.54 (0.27)	2.5 (2.2-2.9)	2.87 (0.30)
M. persicae	*L. angustifolium*	20.0 (11.7-49.6)	2.02 (0.21)	5.5 (4.9-6.1)	3.20 (0.32)
	P. cablin	8.2 (7.3-9.1)	5.33 (0.70)	3.4 (3.0-3.8)	3.38 (0.33)

*Slope of the probit line followed by (SE)
**Mortality assessed at 24 hours post-treatment

Application of higher concentrations of all three essential oils caused noticeable responses in the treated rosy apple aphids and the response was typically rapid. Topical treatment with thyme oil often caused fluid to be exuded via the cauda at a high rate (see Figure 3.1b), followed by paralysis of the aphid. Patchouli oil caused disruptions in the cuticle with both spray emulsion and topical applications (see mottling in Figure 3.1c), although these were not always fatal (especially at lower concentrations) and some aphids survived for more than 48 hours and continued to give birth to offspring. Many aphids also exuded fluid through the cauda following treatment with patchouli oil and while paralysis did occur, it took longer than for aphids topically treated with thyme oil. Citronella oil caused cuticle disruptions at higher concentrations although responses typically took longer to develop.

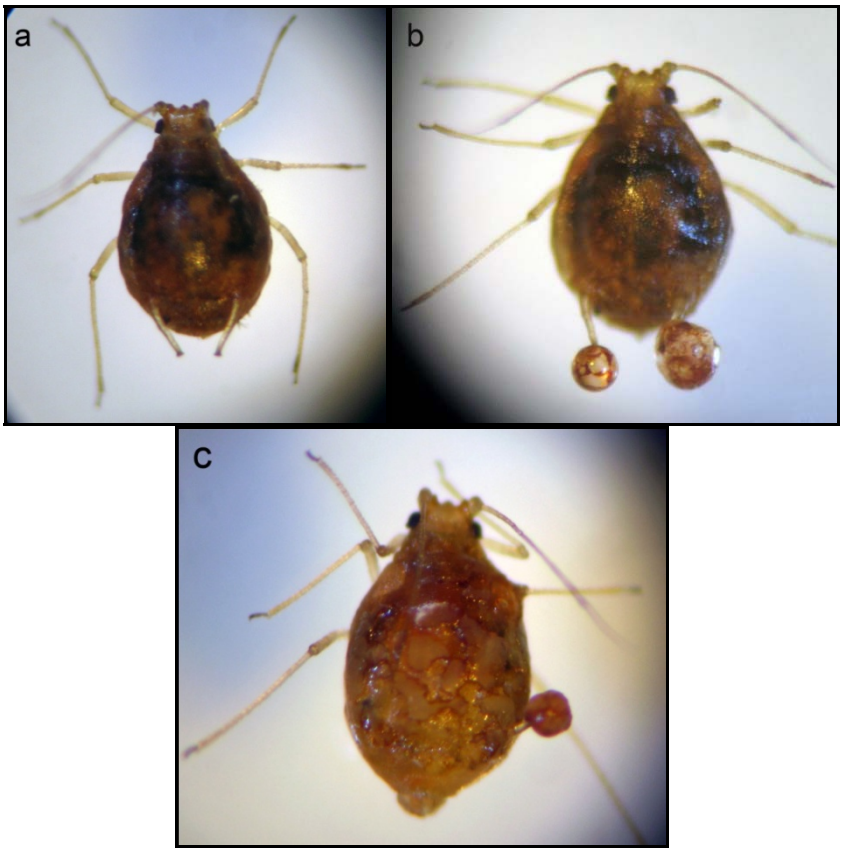

Figure 3.1. Untreated *D. plantaginea* (a) and *D. plantaginea* treated with thyme oil (b) or patchouli oil (c).

M. persicae adults were significantly more tolerant to the patchouli oil emulsion than were *D. plantaginea*, as indicated by the lack of overlapping confidence intervals (Table 3.3). However, when applied topically in acetone, *M. persicae* was not significantly more tolerant than *D. plantaginea* (3.4 µg insect^{-1} versus 3.1 µg insect^{-1}, respectively) demonstrating different responses depending on the carrier solution used. In addition, while patchouli oil was significantly more toxic than lavender oil to *M. persicae*, the difference with the topical application was not as strong as with the spray emulsion application (the spray emulsion was 2.4 times as toxic in comparison to the topical application which was 1.6 times as toxic). The effects of the essential oils were not as visible on *M. persicae* in comparison to *D. plantaginea*. Patchouli oil did cause rapid

paralysis (within less than 2 minutes after application) at high concentrations and in several aphids, the cuticle had lost structural strength and internal components of the aphids leaked out at 24 hours. The effects of lavender oil took considerably longer to occur, and were often only apparent 20 minutes or longer after treatment.

3.4 Discussion

Identifying essential oils which may be suitable for use as pesticides is often difficult due to the high variability in responses between both target species and the composition of the oils.[23] What works against one insect species may not work against another, and such interspecific differences in susceptibility of insects to essential oil toxicity are notoriously idiosyncratic.[23] Accordingly, it can become necessary to screen large numbers of oils in order to find one or two which demonstrate levels of toxicity which warrant further development. In preliminary toxicity screening bioassays, patchouli oil was found to be the most effective essential oil against both *D. plantaginea* and *M. persicae*. However, it was nearly twice as toxic to *D. plantaginea* as *M. persicae*, and in both cases, the next most toxic essential oil to each species was one of the less toxic oils to the other (although lavender oil did still cause significant mortality to *D. plantaginea*). Overall, *D. plantaginea* adults were more susceptible to the essential oils with 10 essential oils causing significant mortality in comparison to only 3 essential oils for *M. persicae*.

There are several potential reasons that both aphid species responded so differently to these oils. *D. plantaginea* is a specialist aphid, feeding on only apple and plantain leaves and tissues, and accordingly, is likely to have a more restricted and specialized

set of detoxification enzymes. As a generalist aphid species with a host range encompassing over 30 plant families,[6] *M. persicae* would be expected to have considerably higher detoxicative abilities which would protect it from an increased set of potential toxins. Full size adult *D. plantaginea* developing on *Plantago* spp. also appeared to be slightly smaller in size than full size adult *M. persicae*, which may also explain why *D. plantaginea* adults were more susceptible to the essential oils applied at the same concentration. An additional reason for the differences may be due to differences in the number or type of specific neuroreceptors. If one species lacks a specific neuroreceptor, then a chemical that would trigger a response in one species may not trigger the same response in the other species. Unfortunately, little is yet known about the types of neuroreceptors in aphids and what they respond to, so additional work would be required to validate this hypothesis.

Oddly, only *D. plantaginea* demonstrated highly variable results with some of the essential oils (this variability continued in additional assays with several essential oils, data not shown). It is unlikely that this was due to problems with the essential oil emulsions, coverage or insect condition as all aphids were treated using identical techniques and not all essential oils caused variable differences in mortality. The presence of different clonal lines should not be an issue either as the colony was started from 5-10 aphids which were obtained from within a 50 meter radius. It is also unlikely that different aphid morphs are a cause as all aphids had been reared on plantain for more than 10 generations. In addition, a preliminary assay with *D. plantaginea* collected from apple trees did not demonstrate any significant differences in mortality between aphid morphs as the LC_{50} for patchouli oil on these aphids was 2.2 µl ml^{-1}

(Machial CM, unpublished), suggesting that toxicity should be similar regardless of the development stage of the aphid.

In further tests with both aphid species, patchouli oil had the lowest LC_{50} values, further supporting the initial screening results. There are several potential modes of action for patchouli oil applied to both aphid species. Some of the constituents may have neurotoxic effects on the aphids, resulting in the observed paralysis and excess fluid exudation from the cauda. The octopaminergic system and the tyramine receptor cascade have both been identified as potential targets of several essential oil constituents, controlling cAMP production, coupling to chloride channels, and ultimately causing the excitation or depression of various nervous responses.[24-27] Octopamine has been implicated in the excitation of the dorsal unpaired medican (DUM) neuron in several insects, controlling the contractions of the abdomen in both locusts,[28] and the American cockroach, *Periplaneta Americana*.[29] It is possible that the rapid excretion of fluid via the cauda could be mediated by this system. Tissue disruption is also hypothesized to play an integral role as many adult *D. plantaginea* developed clear dorsal lesions in response to the application of patchouli oil (see Figure 3.1c). Although previous studies with aphids have not looked at the effects of patchouli oil, Zhu et al.[30] assessed the toxic and repellent effects of patchouli oil and the main constituent, patchouli alcohol, against termites. They also noticed neurotoxic effects and tissue destruction inside the exoskeleton, suggesting that patchouli oil and patchouli alcohol may “dissolve” the exoskeleton and disrupt internal membranes.

The inclusion of thyme oil in the bioassays with *D. plantaginea* to determine the LC_{50} and LD_{50} values produced an interesting set of results. Thyme oil was originally

included for further testing even though it was one of the least toxic essential oils in the screening process as it had been found to be the second most toxic essential oil to *C. rosaceana* larvae (see Chapter 2). Given the timing of emergence for both pests in the spring, there is the potential to control both species simultaneously. When applied as a spray emulsion in aqueous polysorbate 80, thyme oil was 3 times less toxic to adult *D. plantaginea* than patchouli oil. However, when applied topically using acetone as a carrier, it was more toxic than patchouli oil. It is possible that this increase in relative toxicity was due to the acetone acting as a better carrier, allowing the thyme oil to penetrate the exoskeleton and come into direct contact with the internal membranes and the nervous system. This could explain the rapid neurotoxic response manifested as rapid paralysis and fluid exudation via the cauda following topical application. In screening assays with a selection of essential oils dissolved in dimethyl sulfoxide (DMSO) and mixed in water, Sampson[31] also found that essential oils that were high in carvacrol and thymol demonstrated amongst the greatest toxicity to the adult turnip aphid, *Lipaphis pseudobrassicae*. Accordingly, it is evident that the carrier used can play a very important role in toxicity and this should warrant greater attention in the screening and development of essential oil based insecticides.

As with thyme oil, the toxicity of citronella and lavender oils to *D. plantaginea* and *M. persicae* was greatly increased (relative to patchouli oil) by the use of acetone as a carrier. This suggests that the use of solvents or adjuvants which aid in emulsification, increase spreading or facilitate penetration of the insect cuticle and tissues would be beneficial in the development of aqueous emulsions of essential oils as aphicides. Much of the previous research with citronella oil has been directed towards its use as a

repellent, especially for mosquitos;[32, 33] however, previous research with citronellal, the major constituent of citronella oil has also demonstrated direct toxic effects to other insect pests, including the wireworm, *Agriotes obscures* (LD_{50} = 404.9 µg insect^{-1})[34] and the tobacco cutworm, *Spodoptera litura* (LD_{50} = 111.2 µg insect^{-1}).[35] In *S. litura*, treatment with citronellal caused high levels of hyperactivity followed by paralysis and death, while paralysis was also observed in *A. obscures*, indicating a likely neurotoxic mode of action. Other essential oils containing linalool and linalyl acetate (the major constituents of lavender oil) have also been demonstrated to possess moderate toxicity against *L. pseudobrassicae*.[31]

Beyond the toxic effects of these essential oils, there may be other effects which could increase the potential usefulness of these essential oils in the field. For example, both thyme and rosemary oils have been found to be repellent to *M. persicae*, and the essential oil constituents linalool, camphor and α-terpineol demonstrated repellency at higher concentrations.[18] Pulegone (specifically the R-(+)-pulegone enantiomer) was also able to deter *M. persicae* and to change probing and feeding patterns for the full 24 hour duration of the test.[36] Even if the essential oils are unable to reach all aphids on the plant and cause 100% mortality, the potential for repellent or deterrent effects may cause the aphids to fall off the plants and expose them to additional predators or unfavourable environmental conditions and may reduce reinfestation of the crops.

While this research has identified several essential oils that warrant further investigation in the development of essential oil-based insecticides for the control of *D. plantaginea* and *M. persicae*, additional research is still required to determine the exact modes of action and penetration of these oils. This information can be useful in determining

which emulsifiers to use, which, as the results shown here demonstrate, can have a significant impact on toxicity. Determining the role of detoxification enzymes in these aphids can also assist in identifying potential synergists which can be used to increase toxicity and reduce the amount of active ingredient required. Although detoxification of aphicides is typically linked to esterases,[7, 9, 10] there may also be other mechanisms involved in the detoxification of these chemically diverse compounds. Finally, in the case of *D. plantaginea*, there were other essential oils that demonstrated significant toxicity in screening assays and it may be worthwhile to investigate those essential oils further, especially given that some of those oils had highly variable results. Different emulsifiers or carriers may provide more consistent and useful results and at the same time, identify additional essential oils of value.

3.5 References

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4 EFFECT OF PATCHOULI OIL ON THE DETOXIFICATION ENZYMES IN *DYSAPHIS PLANTAGINEA*, *MYZUS PERSICAE*, *CHORISTONEURA ROSACEANA* AND *TRICHOPLUSIA NI*[4]

4.1 Introduction

Insects are exposed to a variety of toxins in their environment and in the plants they eat, and accordingly, they possess a variety of different strategies for avoiding the effects of these toxins. Among the most important mechanisms that insects have evolved in developing resistance to these toxins are various detoxification enzymes. Of these, esterases, glutathione *S*-transferases (GST) and cytochrome P450 enzymes are the most well-known and studied.[1-6] In nature, these enzymes metabolize plant toxins, typically increasing their solubility and enhancing their elimination from the insect. These enzymes are also involved in breaking down insecticides, and in many cases, they have been found to be inducible, both by the insecticide and by the plant toxins the insects feed on.[7-9] This can lead to the over production of these enzymes or enhanced detoxicative abilities, which in turn can result in insecticide resistance, requiring the development of new pest management products or tools when resistance does occur.

The induction of detoxification enzymes and the ability of insects to effectively detoxify chemicals can depend on various factors. Enzymes in different species and even strains of insects can be inducible to differing degrees, while the age of insects can also influence the inducibility of detoxification enzymes. The specific toxin can influence

[4] A version of this chapter will be submitted for publication. Machial, CM and Isman, MB. (2010) Effect of patchouli oil on the detoxification enzymes in *Dysaphis plantaginea*, *Myzus persicae*, *Choristoneura rosaceana*, and *Trichoplusia ni.*

inducibility as well. For example, Yu and Hsu found that aldrin epoxidase and GST enzymes were much more inducible in sixth instar fall armyworm (*Spodoptera frugiperda*) larvae than second instar larvae, and while *S. frugiperda* enzyme activity was highly inducible, that of the diamondback moth (*Plutella xylostella*) was not.[9] Similarly, Sintim et al. found that feeding on sesame leaves versus a control diet led to a 6-fold increase in the activity of GST enzymes in *Spodoptera litura*, and first and second instar larvae had lesser capacity to detoxify toxins than older larvae.[10]

Apart from the inducibility of detoxicative enzymes, another factor that can play an important role in the detoxicative abilities of insects is simply which specific enzymes are responsible for the detoxification of toxins in insects. Cytochrome P450 enzymes (P450s) oxidize a wide range of substrates and are regularly implicated in resistance to plant toxins and insecticides, but most research thus far has focused on dipteran, lepidopteran, blattarian and orthopteran species.[11-15] GSTs have been implicated in resistance to all major insecticide classes, however specific GST enzymes have been difficult to identify.[16] Most work with GSTs has been performed on various lepidopteran species, as well as mosquitoes and a variety of other insects.[8, 16] Esterases can hydrolyze and sequester insecticide esters and are often implicated in organophosphate resistance, especially in smaller insects such as aphids where insecticide resistance is often mediated by the amplification of esterase genes resulting in the overproduction of esterases.[17-19]

In Chapters 2 and 3, plant-derived essential oils were assessed for their insecticidal activity, however, during the scope of this research, I frequently observed that *Choristoneura rosaceana* and *Trichoplusia ni* larvae, as well as *Dysaphis plantaginea*

and *Myzus persicae* adults would appear to be affected by the essential oils, only to recover at a later point in time. In addition, each species responded differently to the essential oils with some essential oils being toxic to one species, but not the others. This observation suggested a role for detoxification enzymes in the metabolism of essential oils. Indeed, other work conducted with essential oil constituents confirms that detoxification enzymes can be used to metabolize these chemicals. Exposure to thymol in *S. litura* and *T. ni* larvae resulted in the conversion and excretion of its 3-O-β-glucoside while *trans*-anethole was hydroxylated on the side chain methyl group.[20] *S. litura* has also been shown to transform limonene to uroterpenol and perillic acid,[21] and α-terpineol to 7-hydroxy- α-terpineol and oleuropeic acid.[22] In line with previous work with other plant toxins, this suggests that detoxification enzymes play an important role in the toxicity of essential oils, and indirectly, whether essential oils can be successfully utilized as bioinsecticides.

Accordingly, the overall purpose of this study was to determine the detoxicative abilities of *C. rosaceana*, *T. ni*, *D. plantaginea*, and *M. persicae* and to determine the influence of patchouli oil in inducing esterase, glutathione *S*-transferase, and cytochrome P450 detoxification enzymes. More specifically, I predicted that the two lepidopteran species, *C. rosacaeana* and *T. ni* would show higher levels of cytochrome P450 content than the two aphid species, whereas the two aphid species, *M. persicae* and *D. plantaginea*, would be expected to have higher general esterase activity. In addition, given that both *T. ni* and *M. persicae* demonstrated greater tolerance to patchouli oil, as well as many other essential oils, compared to *C. rosaceana* and *D. plantaginea* (see Chapters 2 and

3), I expected that *T. ni* and *M. persicae* would demonstrate greater enzyme activity compared to *C. rosaceana* and *D. plantaginea*, respectively.

4.2 Materials and Methods

4.2.1 Chemicals

Patchouli oil was purchased from Escents Aromatherapy. All chemicals used in the enzyme preparations and assays were purchased from Sigma Aldrich.

4.2.2 Insects

All insects used were obtained from laboratory colonies reared at the University of British Columbia. *C. rosaceana* and *T. ni* were reared on an artificial diet (No. 9795, Bio-Serv Inc., Frenchtown, NJ) supplemented with finely ground alfalfa and vitamins (No. 8045, Bio-Serv Inc) as described in Chapter 2. *D. plantaginea* was reared on *Plantago lanceolata* leaves and *M. persicae* was reared on *Brassica rapa* var. *chinensis* (pak choi) and *Brassica oleracea* var. *capacitate* 'Stonehead' (cabbage) leaves as described in Chapter 3.

Third instar *C. rosaceana* and *T. ni* larvae (1-2 days old) and adult apterous *D. plantaginea* and *M. persicae* were used in enzyme experiments. To assess the effects of patchouli oil on the activity of general esterase, GST and P450 enzymes, a total of 70 third instar larvae from each lepidopteran species and 380 adult aphids from each aphid species were topically treated with LD_{20} levels of patchouli oil as determined from data presented in Chapters 2 and 3. Exact doses used per insect are listed in Table 4.1. Treated larvae were place in 2 ml Solo cups containing artificial diet while adult aphids

were placed in 5 cm diameter Petri dishes containing two leaf discs (*P. lanceolata* for *D. plantaginea* and *B. rapa* var. *chinensis* for *M. persicae*) and a piece of moistened cotton to maintain humidity. The treated insects were then placed in a growth chamber (22°C, 16:8 h LD) for 8 hours before they were collected for whole body enzyme extractions. Control groups were not treated with patchouli oil.

Table 4.1. Dose of patchouli oil applied per insect as a pre-treatment prior to whole body enzyme extractions. All doses correspond to LD_{20} levels.

Insect Species	Dose per insect (µg)
C. rosaceana	5.57
T. ni	20.71
D. plantaginea	1.39
M. persicae	1.91

4.2.3 Enzyme preparation

Whole body enzyme extractions were prepared from pre-treated and untreated insects. Fifty third instar *C. rosaceana* and 50 *T. ni* larvae from each treatment group were used, as were 300 adult *D. plantaginea* and *M. persicae*. Only live insects from the groups pre-treated with patchouli oil were selected for this experiment. Each set of insects was homogenized in 1 ml of homogenizing buffer (1 mM EDTA, 0.1 mM DTT, 0.5 mM PMSF and 10% glycerol in 0.1 M sodium phosphate buffer, pH 7.5) in a glass-Teflon homogenizer with 10 vertical passes. Four ml of homogenizing buffer was added to the homogenate and the solution was then centrifuged at 8,000 *g* (the maximum speed of the machine) for 15 minutes in a Sorval RC-5B centrifuge. Two hundred µl was removed from the supernatant for each treatment, placed in labelled microcentrifuge tubes, and then stored in a -80°C freezer for later use in esterase assays. One hundred and fifty µl was stored for esterase assays while a 50 µl sample was stored separately for protein content determination. The remaining supernatant was centrifuged for 1 hour

at 100,000 *g* in a Beckman L8-80 ultracentrifuge with a Ti70 rotor. From this, the supernatant was collected for GST assays while the microsomal pellet was resuspended in 2.2 ml of homogenizing buffer for use in P450 experiments. The prepared enzyme solutions were placed in labelled microcentrifuge tubes and stored at -80°C. Fifty µl of each homogenate was placed in separate tubes and stored for protein content determination. All enzyme preparations were prepared on ice at 0-5°C and each insect treatment was replicated three times.

4.2.4 Enzyme assays

Enzymes assays were conducted using the methods described in Feng & Isman.[4] α-Naphthyl acetate was used as a substrate to determine general esterase activity using a method adapted from van Asperen[23] by preparing a 3 ml reaction medium containing 0.9 µmol of substrate dissolved in 2.95 ml of 0.1 M phosphate buffer, pH 7.5, and 50 µl of 10x diluted 8,000 *g* supernatant. The reaction was allowed to run for 20 minutes at 25°C and then stopped by adding 0.5 ml of diazo blue solution (prepared by dissolving a solution of fast blue salts in a sodium lauryl sulphate solution at a ratio of 2 parts fast blue salts to 5 parts sodium lauryl sulphate solution). The reaction product was read at 600 nm on a Pharmacia Biotech Ultraspec 3000 spectrophotometer. A standard curve using α-naphthol was prepared for quantifying the final concentrations.

3,4-Dichloronitrobenzene (DCNB) was used as a substrate for determining GST activity. The 1.0 ml reaction mixture contained 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.5, 7.5 µmol of reduced glutathione, and 0.5 ml of the 100,000 *g* supernatant. Samples were preincubated for 3 minutes before the addition of 10 µl of 0.15 M DCNB solution in

ethanol which was used to start the reaction. Ten µl of ethanol was used as the control. The reaction was recorded every 30 seconds for 5 minutes at 344 nm using a Pharmacia Biotech Ultraspec 3000 spectrophotometer. A millimolar extinction coefficient of 10 cm^{-1} for the reaction product *S*-(2-chloro-4-nitrophenyl) glutathione was used for quantitation according to the described method.[4, 24]

The 100,000 *g* microsomal fraction was used for the determination of the abundance of intact cytochrome P450 according to the method of Omura and Sato.[25] In brief, 50 µl of the microsomal fraction was added to 950 µl of homogenizing buffer, mixed and added to a cuvette. One to two mg of sodium dithionite was used to reduce the P450 and CO was bubbled into the mixture. Absorbance values at 450 nm and 490 nm were measured using a Shimadzu UV-2450 UV-Vis spectrophotometer and a millimolar extinction coefficient of 91 cm^{-1} was used to calculate the level of P450 in each sample.[26]

Protein content determination was conducted using a method adapted from Bradford.[27] In brief, 5 µl of 10x diluted protein samples were added to separate wells in a 96 well plate and 250 µl of Bradford Reagent was subsequently added. Samples were incubated at room temperature for approximately 10 minutes and then the absorbance was read at 595 nm. Protein concentration was determined by comparing absorbance against a standard curve.

4.2.5 Data analysis

Enzyme activity levels were analyzed using multivariate analysis of variance (MANOVA) and means were compared using Tukey's test. All analyses were completed using SPSS 16.0 (SPSS Inc, Chicago, IL).

4.3 Results

4.3.1 Esterase activity

Comparisons of the general esterase activity per mg protein revealed no significant interaction between insect species and treatment (i.e., control versus pre-treatment with patchouli oil) ($F_{3,16}$ = 0.43, p = 0.732) (Table 4.2). The main effect of treatment was also not significant ($F_{1,16}$ = 0.10, p = 0.755), however, insect species was significant ($F_{3,16}$ = 25.37, p < 0.0001). Tukey HSD post hoc tests demonstrated that general esterase activity was significantly higher in *M. persicae* adults compared to *D. plantaginea* adults and *C. rosaceana and T. ni* larvae (Figure 4.1). The test also indicated that pre-treatment with patchouli oil did not significantly increase esterase activity.

In terms of general esterase activity per insect, comparisons of activity again revealed no significant interaction between insect species and treatment ($F_{3,16}$ = 0.92, p = 0.455) and no significant effect of pre-treatment with patchouli oil ($F_{1,16}$ = 0.87, p = 0.366) (Table 4.2). There was a significant effect of insect species on general esterase activity ($F_{3,16}$ = 81.35, p < 0.0001). Tukey HSD post hoc tests show that the lepidopteran pests (*C. rosaceana* and *T. ni*) had significantly higher esterase activity per insect than both

aphid species, owing to their larger size (Figure 4.2) (see Table 4.3 for the average weights of insects).

Table 4.2. Esterase activity in adult *D. plantaginea* and *M. persicae*, and third instar *C. rosaceana* and *T. ni* larvae, as well as esterase activity following pre-treatment with patchouli oil.

		Enzyme Activity	
Insect Species	Treatment	nmol/min/mg protein	nmol/min/insect
D. plantaginea	Control	12.30 ± 1.39	5.71 ± 0.45
	Pre-treated	11.92 ± 1.67	5.75 ± 0.57
M. persicae	Control	22.35 ± 1.96	8.76 ± 0.33
	Pre-treated	24.95 ± 3.65	10.18 ± 1.29
C. rosaceana	Control	12.20 ± 2.05	45.34 ± 8.12
	Pre-treated	12.12 ± 0.98	43.86 ± 4.26
T. ni	Control	12.27 ± 0.76	51.80 ± 2.47
	Pre-treated	11.58 ± 1.54	42.67 ± 2.16
Insect Species		$F(3,16) = 25.37$ $p < 0.0001$*	$F(3,16) = 81.35$ $p < 0.0001$*
Treatment		$F(1,16) = 0.10$ $p = 0.755$	$F(1,16) = 0.87$ $p = 0.366$
Insect Species*Treatment		$F(3,16) = 0.43$ $p = 0.732$	$F(3,16) = 0.92$ $p = 0.455$

*Values are mean (± SE) of n = 3 replicates.

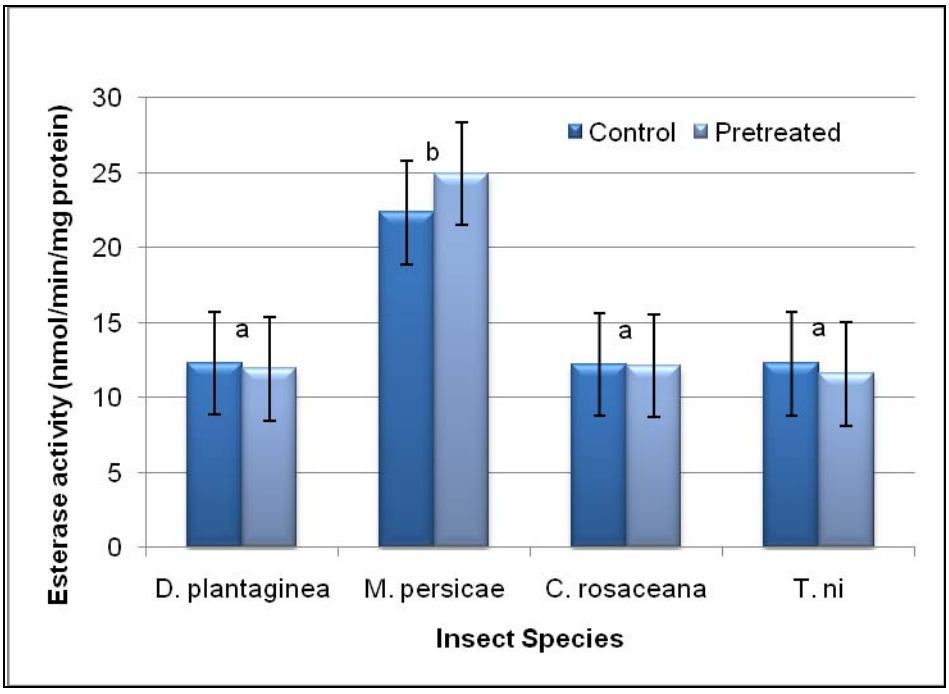

Figure 4.1. Esterase activity (nmol/min/mg protein) in adult *D. plantaginea* and *M. persicae*, and third instar *C. rosaceana* and *T. ni* larvae pre-treated or not treated with patchouli oil. Means are presented with 95% confidence intervals (CI) as determined by the MANOVA. Bars with the same letter above indicate no statistically significant differences between species (Tukey HSD test, $p < 0.05$).

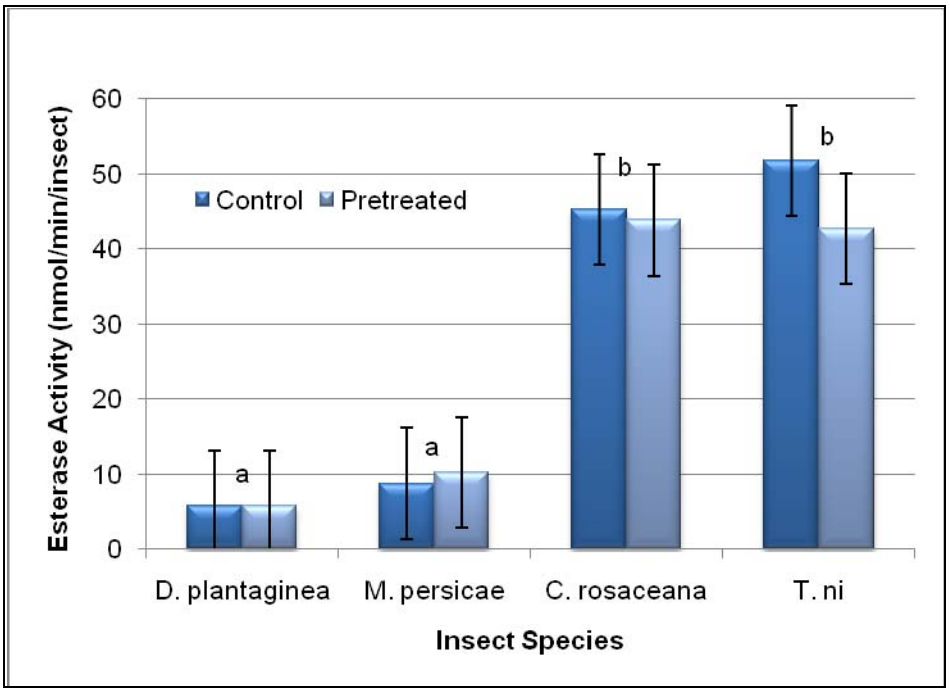

Figure 4.2. Esterase activity (nmol/min/insect) in adult *D. plantaginea* and *M. persicae*, and third instar *C. rosaceana* and *T. ni* larvae pre-treated or not treated with patchouli oil. Means are presented with 95% CI as determined by the MANOVA. Bars with the same letter above indicate no statistically significant differences between species (Tukey HSD test, $p < 0.05$).

Table 4.3. Average weight per insect from control and pre-treated groups.

	Insect Weight (mg/insect)	
Insect Species	Control	Pre-treated
D. plantaginea	0.47 ± 0.047	0.49 ± 0.047
M. persicae	0.40 ± 0.048	0.41 ± 0.026
C. rosaceana	3.72 ± 0.18	3.61 ± 0.068
T. ni	4.27 ± 0.41	3.85 ± 0.64

*Values are mean (± SE) of n = 3 replicates.

4.3.2 Glutathione *S*-transferase activity

Comparisons of GST activity revealed no significant interaction effects between insect species and the pre-treatment or lack of pre-treatment of insects with patchouli oil ($F_{3,16}$ = 1.63, p = 0.221) and no significant effect of treatment ($F_{1,16}$ = 1.89, p = 0.188) (Table 4.4). Insect species did have a significant effect on GST activity ($F_{3,16}$ = 14.93, p <0.0001). As with general esterase activity, *M. persicae* showed significantly higher GST activity per mg protein versus the other insect species, although there was no significant difference between pre-treated and untreated groups (Figure 4.3).

Comparisons of GST activity per insect did not reveal a significant interaction effect between species and treatment ($F_{3,16}$ = 0.97, p = 0.424), nor treatment alone ($F_{1,16}$ = 2.47, p = 0.135) (Table 4.4). Insect species, on the other hand, was again a significant predictor of the effect on GST activity ($F_{3,16}$ = 13.4, p < 0.0001) with Tukey HSD post-hoc tests showing a significant difference in activity between *C. rosaceana* larvae and the two adult aphid species. *T. ni* also showed significantly higher activity than *D. plantaginea* adults (Figure 4.4).

Table 4.4. Glutathione *S*-transferase activity of *D. plantaginea* and *M. persicae* adults, and third instar *C. rosaceana* and *T. ni* larvae, as well as glutathione *S*-transferase activity following pre-treatment with patchouli oil.

		Enzyme Activity	
Insect Species	Treatment	nmol/min/mg protein	nmol/min/insect
D. plantaginea	Control	19.39 ± 4.24	8.98 ± 1.98
	Pre-treated	9.93 ± 1.57	4.82 ± 0.53
M. persicae	Control	25.99 ± 3.86	10.52 ± 2.44
	Pre-treated	29.46 ± 4.24	12.20 ± 2.47
C. rosaceana	Control	15.84 ± 1.75	59.42 ± 8.81
	Pre-treated	10.26 ± 4.21	37.02 ± 15.49
T. ni	Control	7.83 ± 1.33	34.18 ± 8.21
	Pre-treated	7.22 ± 1.58	26.57 ± 5.05
Insect Species		$F(3,16) = 14.93$ $p < 0.0001$*	$F(3,16) = 13.4$ $p < 0.0001$*
Treatment		$F(1,16) = 1.89$ $p = 0.188$	$F(1,16) = 2.47$ $p = 0.135$
Insect Species*Treatment		$F(3,16) = 1.63$ $p = 0.221$	$F(3,16) = 0.97$ $p = 0.424$

*Values are mean (± SE) of n = 3 replicates.

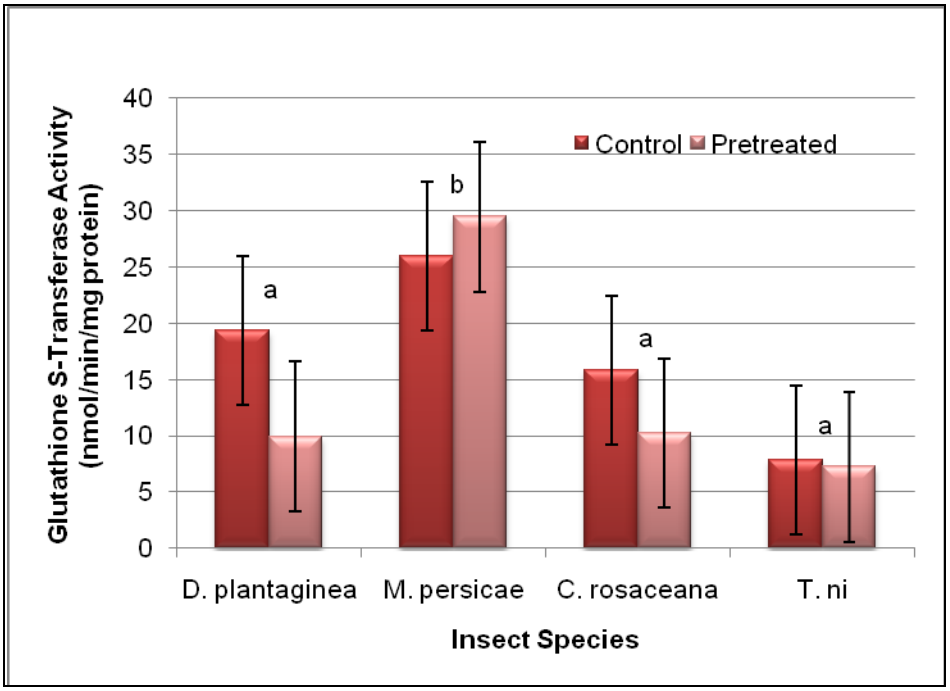

Figure 4.3. Glutathione *S*-transferase activity (nmol/min/mg protein) in adult *D. plantaginea* and *M. persicae*, and third instar *C. rosaceana* and *T. ni* larvae pre-treated or not pre-treated with patchouli oil. Means are presented with 95% CI as determined by the MANOVA. Bars with the same letter above indicate no statistically significant differences between species (Tukey HSD test, $p < 0.05$).

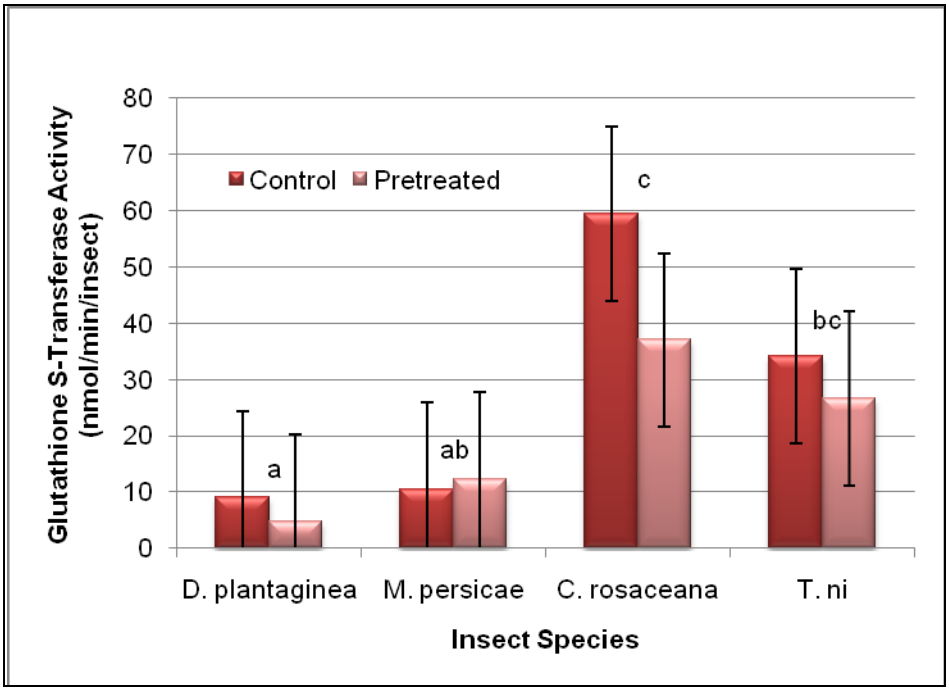

Figure 4.4. Glutathione *S*-transferase activity (nmol/min/insect) in adult *D. plantaginea* and *M. persicae*, and third instar *C. rosaceana* and *T. ni* larvae pre-treated or not pre-treated with patchouli oil. Means are presented with 95% CI as determined by the MANOVA. Bars with the same letter above indicate no statistically significant differences between species (Tukey HSD test, $p < 0.05$).

4.3.3 Cytochrome P450 abundance

I was only able to determine cytochrome P450 content for 11 out of 24 samples, so results are limited. Table 4.5 shows the mean from the samples which had detectable P450 levels, along with how many replicates were used to calculate the mean. From this, third instar *T. ni* larvae had the highest number of samples with detectable P450 content and it appears that pre-treatment with patchouli oil may lead to an increase in the abundance of the active form of cytochrome P450. This is due to the presence of the highest number of replicates with detectable levels as well as the higher average in pre-treated larvae. Similarly, pre-treated *C. rosaceana* larvae also had an extra

replicate with detectable P450 levels and the levels were higher than the single control observation.

Table 4.5. Average abundance of intact cytochrome P450 from samples with detectable levels of P450 in adult *D. plantaginea* and *M. persicae*, and third instar *C. rosaceana* and *T. ni* larvae, as well as following pre-treatment with patchouli oil.

		Enzyme Content	
Insect Species	Treatment	nmol/min/mg protein	# of replicates
D. plantaginea	Control	0.34	1
	Pre-treated	ND	0
M. persicae	Control	0.33	1
	Pre-treated	0.32	1
C. rosaceana	Control	0.029	1
	Pre-treated	0.12	2
T. ni	Control	0.13	2
	Pre-treated	0.22	3

*Means are calculated based on the number of replicates with detectable levels. Values with only one replicate are the value of that sole replicate. ND = Not determined.

4.4 Discussion

In the previous two chapters of this thesis, I document screening a selection of 17 essential oils for toxicity against four insect pests aimed at identifying one or more essential oils worth considering for development as a botanical insecticide. Of the oils tested, patchouli oil, was among the most toxic to all four pests. However, I noted in testing that many treated insects, even at low concentrations, would respond to the treatment, only to recover later, suggesting a potential role for detoxification enzymes. The development of botanical insecticides can be confounded by plant toxins inducing detoxification enzymes, which can reduce the efficacy of botanical insecticides in the field. Accordingly, an essential oil which does not significantly induce detoxification enzyme activity could have greater utility as a part of a botanically-based insecticide.

However, before speculating further, it is first necessary to understand the detoxicative abilities of these pests.

Bearing this in mind, esterase activity in adult *M. persicae* was approximately two times higher per mg protein than for the other insect species, partially supporting the hypothesis that the aphid species would demonstrate the highest esterase activity. Meanwhile, esterase activity was nearly identical for adult *D. plantaginea*, and third instar *C. rosaceana* and *T. ni* larvae. Pre-treatment with patchouli oil led to a small increase in the general esterase activity in *M. persicae*; however this increase was not significant and was not observed in the other insect species. In terms of esterase activity per insect, third instar control *T. ni* and *C. rosaceana* larvae demonstrated the highest general esterase activity, and while the results were not significantly different from pre-treated larvae, there was a trend towards lower overall esterase activity per insect in the pre-treated larvae. This can be explained by the lower weights in pre-treated insects, which may be due to various effects from the patchouli oil pre-treatment (including nervous responses restricting directed movement and feeding, as well as the loss of hemolymph via regurgitation or tissue disruption). Of the two aphid species, the higher activity level per mg protein in *M. persicae* also resulted in higher overall activity per insect, however due to the smaller size of the *M. persicae* adults, the results were not significant.

At 22.35 nmol/min/mg protein, esterase activity levels in *M. persicae* were comparable to previously reported levels ranging between 3.5-118.8 nmol/min/mg protein in a selection of six strains of *M. persicae*.[18] Although the esterase levels were significantly lower in *D. plantaginea*, they were still considerably higher than two other known apple

aphid pests. *Aphis pomi* and *A. spiraecola* converted α-naphthyl acetate at average rates of 1.54 and 2.08 nmol/min/mg protein, respectively,[28] which is approximately 6-8 times lower than the rates found for *D. plantaginea*. It had also been expected that esterase levels would have been somewhat higher in the lepidopteran species. For example, in post-diapause codling moth, *Cydia pomonella*, another serious lepidopteran apple pest, esterase levels in a susceptible strain were 208 nmol β-naphthol/min/mg protein.[29] Levels were over 2000 times higher in *S. litura* as esterase activity ranged from 23.68-121.19 µmol α-naphthol/min/mg protein in 2nd to 3rd instar larvae.[10] Additional work would be required to determine if esterase activity increased in older *C. rosaceana* and *T. ni* larvae.

M. persicae adults also had the highest GST activity at 25.99 and 29.46 nmol/min/mg protein for control and pre-treated aphids, respectively, followed by *D. plantaginea* adults and third instar *C. rosaceana* larvae. Previous work with *M. persicae* has shown that secondary metabolites from *Brassica* plant species can induce GST activity,[30] thus potentially explaining the higher GST activity. Third instar *T. ni* had the lowest GST activity at 7.83 and 7.22 nmol/min/mg protein (in control and pre-treated larvae, respectively). These rates are also comparable with results reported for other insects. Sixth instar *S. frugiperda* larval midguts from insects fed on an artificial diet had higher GST activity at 23.3-36.5 nmol/min/mg protein and 27.5 nmol/min/mg protein for 2nd instar larvae, versus what was observed for the whole body homogenates from *C. rosaceana* and *T. ni*.[7, 9] In comparison, 2nd-3rd instar *S. litura* larvae had lower activity levels (2.92 nmol/min/mg protein) (from whole body homogenates).[10]

As with esterase activity, patchouli oil appeared to induce GST activity in *M. persicae*, however, it may have inhibited activity for all other insect species (not statistically significant). While the differences following pre-treatment with patchouli oil were not significant, the decreases observed between control and pre-treated *D. plantaginea* and *C. rosaceana* were substantial (pre-treated insects showed 2.0 and 1.5 times decreased activity respectively). Accordingly, while the results are unable to confirm this, there does appear to be a trend suggesting that patchouli oil does have an impact on the induction or inhibition of GST activity (either by increasing or decreasing enzyme activity).

Unfortunately the cytochrome P450 assays were unable to show much beyond possible trends due to a lack of usable data and accordingly, there was insufficient evidence to support or refute the hypothesis that cytochrome P450 content would be higher in the lepidopteran pests. There are several potential reasons for this, the most likely being that P450 levels are simply too low in whole body enzyme extracts using the extraction methods used. In the case of the aphids, this is not unexpected as aphids are not typically associated with high cytochrome P450 levels, and indeed, little research is available assessing the cytochrome P450 content or activity in aphids. Based on the available results, it appears that P450 levels were highest in the two lepidopteran species, and there does appear to be a trend suggesting that patchouli oil may induce P450 enzymes. This would be consistent with other research showing that plant compounds can induce P450 enzymes. For example, indole-3-carbinol induced P450 enzymes by a factor of 3.8 in the corn earworm, *Helicoverpa zea*.[9] Caryophyllene, a constituent of patchouli oil (see Table 2.3, Chapter 2), was also shown to induce

cytochrome P450 activity in 5th instar tobacco budworm (*Heliothis virescens*) and adult boll weevils (*Anthonomus grandis grandis*).[31] Indeed, several other investigators have demonstrated the ability of plant toxins to induce cytochrome P450 enzymes.[13, 14, 32]

In summary, my study has demonstrated that esterase and GST activity is higher in *M. persicae* than in the other insects tested, and although the evidence is not conclusive, there is a trend suggesting that patchouli oil may act as both an inducer and an inhibitor of the various detoxification enzymes depending on the species. This supports the hypothesis that *M. persicae* would demonstrate a greater detoxicative capacity than *D. plantaginea*. Indeed, as a generalist species, *M. persicae* would be expected to have a higher detoxicative capacity versus a specialist aphid species such as *D. plantaginea*.[33] However, the hypothesis that *T. ni* would have greater detoxicative abilities than *C. rosaceana* was not supported by the observed results. It is possible that *T. ni* has a higher cytochrome P450 content, however, I was unable to conclusively demonstrate this. The work here provides a starting point for future research, however, it is evident that more research will be required to confirm the trends observed and to ascertain what role essential oils can have on the induction of the detoxification enzymes in these insects, and conversely, what role the detoxification enzymes have in breaking down the essential oil components.

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5 SUMMARY AND DISCUSSION

The push for alternative, reduced-risk pesticides is growing as concerns mount surrounding the human and environmental safety of many current pesticides. Governments are increasing their scrutiny of pesticides with many older and higher risk pesticides undergoing reassessment, and the development of reduced-risk pesticides is now considered a priority. To date, essential oils have not been extensively used as pesticides despite their known insecticidal, herbicidal, fungicidal, and antimicrobial activities,[1-3] in part because of the cost of registering new pesticides, but also because they tend to be less efficacious than conventional products.[4] However the current regulatory environment in places like Canada, the USA and Europe may provide the opportunity for change and their relative safety can counterbalance their lesser efficacy in situations where a premium is placed on human and environmental safety.

With this in mind, it seemed to be an opportune time to look into the use of essential oils as insecticides against four serious agricultural pests found in British Columbia apple orchards and greenhouses. Problems with resistance have reduced the number of pesticides available or increased the doses required for the control of the obliquebanded leafroller (*Choristoneura rosaceana* Harris), the rosy apple aphid (*Dysaphis plantaginea* Passerini), the cabbage looper (*Trichoplusia ni* Hübner) and the green peach aphid (*Myzus persicae* Sulzer). In the case of *D. plantaginea* there is also a lack of available control options for organic growers. This provided the impetus to search for essential oils that could be incorporated into an essential oil-based insecticide. Accordingly, the primary goals of this project were to identify one or more

essential oils that would warrant further development into an essential oil-based insecticide, and to determine the level of toxicity of those essential oils to the target insects. In addition, in the course of this work, I noticed that many insects that initially appeared affected by these essential oils would recover, especially at lower doses, suggesting a role for detoxification enzymes. This led to the second set of goals for the project: to determine the enzymatic detoxicative abilities of these insects; and to determine if exposure to essential oils could induce these enzymes.

Simply identifying essential oils which are toxic to insect pests is not novel, and indeed, some work has been conducted on *M. persicae*,[5-7] *T. ni*,[8-11] and *C. rosaceana.*[12] However, most prior research on these pests focused on the behavioural effects of essential oils.[3, 13, 14] It is noteworthy that much of the research currently available on the toxicity of essential oils to these pests only became available since the start of this project and little research has been reported on the effects of essential oils on the detoxicative abilities of these insects. Accordingly, this research will continue to expand the boundaries of the current knowledge base.

Screening results from Chapters 2 and 3 identified several essential oils worthy of further investigation. Patchouli, thyme, lavender, garlic, lemongrass and citronella oils all elicited rapid responses at higher doses as well as significant toxicity to *C. rosaceana*, *D. plantaginea*, *T. ni* and/or *M. persicae.* Of particular interest, patchouli oil was among the most toxic of the essential oils tested against all four insect species, suggesting that it may act as a broad spectrum insecticide and work against a variety of insect pests. While this essential oil is often regarded as having insect repellent properties, little actual research has been conducted on the toxicity of patchouli oil and

its major constituent, patchouli alcohol. Indeed, a search of the literature reveals a single study of the direct effects of patchouli oil and patchouli alcohol against the Formosan subterranean termite, *Coptotermes formosanus*.[15] Based on the observations from this study and Chapters 2 and 3, it also appears that patchouli oil may have multiple modes of action, including neurotoxicity, as well as tissue destruction (see Figure 3.1). Accordingly, it is conceivable that insects would have difficulty developing resistance to patchouli oil.

Another noteworthy finding is that thyme oil showed substantially higher toxicity in *D. plantaginea* relative to the toxicity of patchouli oil when applied topically with acetone as a carrier versus a spray emulsion in water and polysorbate 80. These results were unexpected based on the results from the screening assay and indeed, thyme oil was only included for further testing against *D. plantaginea* because this species had the potential to be managed at the same time as *C. rosaceana*, if applied to the spring generation of leafrollers. I hypothesized that the acetone assisted the thyme oil in penetrating the cuticle of the aphids. This suggests that the selection of emulsifiers may be just as important in developing essential oil-based insecticides as selecting the correct essential oil active ingredient, especially when dealing with essential oils that may not fully penetrate the insect cuticle on their own.

How essential oils work against insects is still poorly understood. Attractant and deterrent properties are likely mediated by specific chemosensory receptor interactions and often, insect pests can habituate to these effects.[16-18] Toxic effects may be mediated via the insect nervous system, cellular membrane disruption, or a combination of effects. Molecular research has implicated the octopamine and tyramine receptors,[19-

[21] although this has been debated by other researchers.[22] At this point, the specific mode(s) of action of essential oils remain largely unknown and further research may indicate other targets and additional modes of action.

Chapter 2 also addressed the potential for cross-resistance between essential oils and other insecticides. The development of resistance is a major concern for growers, but cross-resistance with other insecticides can also pose a serious problem. In some South Okanagan apple orchards, increased tolerance or resistance to azinphosmethyl in *C. rosaceana* larvae was highly correlated to resistance to the insect growth regulators tebufenozide and methoxyfenozide, providing evidence of cross resistance.[23] To investigate if this could be a concern with an essential oil-based insecticide, an azinphosmethyl-resistant strain of *C. rosaceana* larvae was tested with patchouli and thyme oils. Although LC_{50} values were approximately double and significantly higher than those for the susceptible strain, it is likely that these differences are due to intraspecific differences between the two strains, rather than any substantial sign of cross resistance. This provides evidence to support the idea that essential oil-based insecticides could be used as a part of a resistance management plan for azinphosmethyl-resistant *C. rosaceana* larvae, and quite possibly for other situations.

Of course, the possibility remains that these insects could develop resistance to an essential oil-based insecticide. Selection for resistance to lavender oil vapours was demonstrated in *Acanthoscelides obtectus* with resistance ratios of 8.6 and 4.7 for female and male beetles, respectively.[24] However, to date, there has been little research presented on this subject. In the field, it is unlikely that resistance would be a concern as an essential oil-based insecticide would likely be composed of more than

one essential oil or other component, meaning that insects would have to develop resistance to multiple active ingredients. This would likely require multiple genetic mutations to overcome which in turn could have negative impacts on insect growth and development, causing reduced fecundity and/or reduced survival of eggs and larvae. In addition, responsible use of such products in the field would ensure they are used within a rotation with other pest management products, resulting in reduced selection pressures.

The detoxicative abilities of insects can also influence the efficacy of an essential oil-based insecticide. In Chapter 4, I attempted to determine the activity levels of 3 important detoxification enzymes in these insects: the esterases; the glutathione *S*-transferases; and the cytochrome P450 enzymes. Esterase activity was highest in *M. persicae* and was nearly identical for *D. plantaginea*, *C. rosaceana* and *T. ni*. Results with glutathione *S*-transferases indicated that the relative activity per mg protein followed the order *M. persicae* > *D. plantaginea* > *C. rosaceana* > *T. ni*. Results from cytochrome P450 enzymes were less conclusive. It appears that *M. persicae* has the highest detoxicative abilities, and indeed, in comparing the toxicity data from Chapter 3, it would appear that *M. persicae* adults have a greater capacity to resist essential oils than *D. plantaginea*. This suggests that in aphids, the overall esterase and glutathione *S*-transferase activity may play an important role in determining the efficacy of essential oils to insects. I hypothesized that in the two lepidopteran species, cytochrome P450 enzymes play an important role, with *T. ni* larvae likely having a greater detoxicative capacity given that the essential oils were generally less toxic to *T. ni* larvae (see Chapter 2), however my data was unable to test this hypothesis. Other tests I

conducted with *C. rosaceana* using the cytochrome p450 inhibitor piperonyl butoxide (PBO) does support the role for the cytochrome P450 enzymes in the detoxification of several essential oils including thyme and patchouli oils, providing some support for this hypothesis.[25]

The ability to induce the detoxification enzymes present in insects is another concern with essential oil-based insecticides. Previous research has shown that secondary plant chemicals can induce the activity of detoxification enzymes.[26, 27] For example, *T. ni* fed on a diet including peppermint leaves showed a 4-fold increase in the activity of aldrin epoxidase.[28] Based on the results from Chapter 4, patchouli oil may induce esterase and glutathione *S*-transferase activity in *M. persicae* (although results are not statistically significant), and while the results are inconclusive for cytochrome P450, there appears to be a trend which suggests that patchouli oil may induce cytochrome P450 in both lepidoteran species as well. One concern is that sublethal doses of these essential oils could result in increased enzyme activity that could affect the toxicity of other insecticides. Though unlikely, there is a possibility that this could in turn result in an increased chance of the development of resistance. At this point though, the observed levels of induction (less than1.5 fold) are unlikely to pose any significant risk. Conversely, the relative decreases observed in glutathione *S*-transferase activity in *D. plantaginea* and *C. rosaceana* may actually assist in improving the efficacy of other insecticides which are typically detoxified by glutathione *S*-transferases in these insects.

Another challenge in the development of essential oil-based insecticides is that insect responses can be highly variable depending on the type and composition of essential oils. For example, third instar *T. ni* larvae treated using a combination of blends of the

major constituents of the essential oil of *Litsea pungens* showed that the presence of 1,8-cineole accounted for much of the observed toxicity of *L. pungens* oil.[11] However, the composition of the essential oils can be highly variable and often depends on the plant part it is extracted from, as well as seasonal variability, location, climate and soil. For example, 1,8-cineole isolated from samples of rosemary oil that had been collected at two different locations in Italy ranged in concentration from a low of 7.28% to 55.3%.[29] Accordingly, it becomes necessary to know the composition of the essential oils to be used in essential oil-based insecticides in order to be confident that results can be consistently replicated. When sources do not have the correct concentrations of constituents, it may be necessary to blend essential oils from different sources to create a blend with the correct levels.

So, what does this ultimately mean for the development of essential oil-based insecticides in field applications? Are they a viable alternative to conventional or more established pesticides? The preceding research identified several potential essential oils for further research and development, including the oils of patchouli, thyme, lavender, lemongrass, citronella and garlic. But how likely are any of these essential oils to be incorporated into an essential oil-based insecticide, and what are the challenges related to incorporating them? In order to answer these questions, it can help to address a more specific set of questions. These include:

1. What is the ideal application technology and method?

2. What are the costs and availability of the essential oils and how can costs be reduced?

3. What are the regulations and registration requirements for essential oil-based insecticides?

4. What other factors need to be considered when developing an essential oil-based insecticide?

5. Who would be the most likely to benefit from essential oil-based insecticides?

What is the ideal application technology and method?

When developing novel insecticides, two basic steps that need to be considered when deciding how to formulate the product are how the insecticide will be applied and what dilution rate is required. In order for a novel insecticide to be accepted by growers, it should be amendable to current pesticide application technologies. In the case of apple pest management, the most commonly used technology for applying insecticides is the airblast sprayer which is typically pulled and powered using a tractor. Greenhouses have a variety of different spray equipment options which can be used. However, portable/backpack sprayers and powered boom sprayers are the most likely options that can work with an essential oil-based insecticide. In both cases, this suggests that an essential oil-based application would be most easily applied as an emulsion in water. Accordingly, an emulsifiable concentrate (EC) would likely be the most suitable formulation.

Such an essential oil-based insecticide should also be applied using a high volume or dilute application to ensure the highest probability that insects will be contacted with the insecticide. While there may be a fumigant effect of the essential oils that could control

the insects which were not directly contacted, this is not guaranteed and owing to the highly volatile nature of the essential oils, especially in an entirely open system such as an apple orchard, such an effect would be unlikely to have any considerable impact on toxicity. A dilute application (e.g. ensuring foliage is thoroughly wet) applied under cooler conditions in the evening or early morning would ensure appropriate coverage and reasonable drying times. Similar strategies have been previously developed for other essential oil-based pesticides such as EcoTrol™ and Sporan™, produced by EcoSMART Technologies.[30] Now marketed as Ecotec® and Sporatec®, respectively, by Brandt Consolidated, Inc., these formulations can be mixed in water at the prescribed rates and applied using conventional sprayer technologies for each target crop.

What are the costs and availability of the essential oils and how can costs be reduced?

The next step involves deciding which components to include in an essential oil-based insecticide. Based on the results from Chapters 2 and 3, patchouli oil appears to have the most promise as a broad spectrum insecticide as it was the only essential oil tested that was toxic to all 4 species. However, factors such as cost and availability can play a significant role in making the final decision. Given that the essential oils I identified for further development are typically mass produced for use in the fragrance and flavourings industries, availability is unlikely to be an issue. However, the cost is a potential barrier as the price of the formulated insecticide must be comparable to other currently available insecticides if growers are to adopt an essential oil-based insecticide.

Table 5.1 shows prices from various sources for patchouli, white thyme, lavender, lemongrass, citronella and garlic oils, as well as rosemary oil as a reference value.

Rosemary oil is included as it is the main active essential oil ingredient used in both Ecotec® and Sporatec®. As can be seen, only lemongrass and citronella oils are generally less expensive than rosemary oil, possibly due to their extensive use as mosquito repellents and according high availability. While lavender oil is not dramatically more expensive, the prices for patchouli, white thyme and garlic oils are typically considerably higher and could pose a barrier for their use. However, it is evident that prices do vary considerably between sources (e.g. patchouli oil from Sigma Aldrich costs 6.6 times more than from Wholesale Aromatherapy ltd, while white thyme oil is 3.4 times less expensive). This suggests that it may be possible to work with different suppliers to obtain the required essential oils at reasonable prices. And given that rosemary oil is already being used within an affordable commercially available essential oil insecticide, it is expected that if it is possible to source these oils at prices comparable to rosemary oil, it will be possible to develop essential oil-based insecticides that are affordable for growers.

However, as I discussed previously, insect responses can be highly variable depending on the type and composition of essential oils. For example, in the case of patchouli oil, any wholesale source would likely need to have a high concentration of patchouli alcohol. Bulk thyme oil sources should have high thymol concentrations, while citronellal should be a key constituent of citronella oil, and so on. This can be further complicated as different suppliers can have different materials that they call the same thing. For example, the oils of *Cymbopogon citratus* and *C. flexuosus* are often both called lemongrass oil, and indeed, the latter two suppliers listed in Table 5.1 use the

essential oil from *C. flexuosus* for their versions of lemongrass oil while the first two use *C. citratus*.

Table 5.1. Prices of essential oils from various sources and the average price of the essential oils. Prices were obtained from company websites on June 4, 2010.*

	Source				
Essential oil	Sigma Aldrich	Voyageur Soap & Candle Company	Wholesale Aromatherapy ltd.	Essential7[a]	Average Prices[b]
	$/kg	$/L	$/kg	$/L	$/L
Rosemary	94.00	57.40	47.79	134.71	83.47
Citronella	81.00	41.40	53.94	70.66	61.75
Garlic	120.00	n/a	384.10	175.93	226.68
Lavender	193.50	79.90	77.48	124.39	118.82
Lemongrass	102.00	63.90	49.15	63.15	69.55
Patchouli	509.00	190.24	77.03	229.69	251.49
White Thyme	120.00	120.80[c]	412.77	158.01	202.90

*Note: this information is based on products that may differ in quality and have substantially different compositions.
[a]Prices from Essential7 were calculated from an original price per 950 ml. Prices are in USD
[b]Average prices are approximate as essential oils were calculated using the assumptions that 1 kg = 1 L and 1 CAD=1 USD
[c]Actual price of thyme oil was $30.20/250 ml

I presented the composition of the essential oils I tested in Tables 2.3 and 3.3 to present a guideline for the selection of essential oils. However, there may be opportunities to enhance the insecticidal activity of these essential oils while decreasing costs by testing additional types of the same essential oil or by further assessing the efficacy of individual constituents. The fragrance industry typically requires high quality sources of essential oils with a wide range of constituents to ensure that the appropriate scent/flavour is obtained. However, this may not be as much of a concern for essential oils used for insecticidal activities. For example, it was previously shown that 1,8-cineole and α-pinene are the primary constituents which are responsible for the toxicity of rosemary oil to the two-spotted spider mite, *Tetranychus urticae*.[31] Accordingly, ideal

sources of rosemary oil for the control of *T. urticae* should have high concentrations of 1,8-cineole and α-pinene, but it would not matter if other major constituents such as camphor were not present. To use olive oil as an analogy, the first press of olive oil produces extra virgin olive oil with the most flavour, but also the highest cost. Successive presses produce a lower quality and less expensive product. As with essential oils, so long as those presses include the desired concentrations of the necessary constituents, they can be sufficient for the required needs and at a considerably lower price.

Accordingly, while lemongrass, citronella and rosemary oils are all less expensive than patchouli oil, if it can be sourced at lower prices with appropriate concentrations of constituents such as patchouli alcohol, patchouli oil may still be able to make up a critical component of an essential oil-based insecticide. Similarly, while the cost of garlic oil and thyme oil seems prohibitive, there may still be opportunities for these oils, even as minor components of an essential oil-based insecticide. Indeed, there are various other commercially available insecticides either based on or including essential oils, including garlic oil, suggesting that while costs might be a barrier, these barriers can be overcome and such insecticides can be sold.[32]

What are the regulations and registration requirements for essential oil-based insecticides?

The registration requirements for a new pesticide are often considerable and require extensive efficacy data as well as research on the toxicology to non-target organisms as a basis for estimating safety to humans and environmental risks to wildlife. Concerns

related to environmental pollution and ground water contamination must also be addressed. In many cases, the associated costs and time make it unfeasible to proceed with development, unless that pesticide can sell sufficient quantities to surpass those initial costs.[33] These costs typically make it particularly difficult to develop highly specific classes of insecticides which target only one or a few species. This is further complicated as each country has their own pesticide regulations meaning that something that is registered for use in one country still requires registration for use in another country. For example, while Canada and the US are major trading nations, they do not share similar pesticide registration laws, and accordingly, pesticides must undergo separate registration in both Canada and the US. In Canada, pesticide regulations fall under the Pest Control Products Act (PCPA) which is managed by the Pest Management Regulatory Agency (PMRA), while the Environmental Protection Agency (EPA) is responsible for overseeing the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in the US. Because of the different laws, there are significant differences in how products such as essential oil-based insecticides can be introduced to the market.

One advantage in the US is that several essential oils are listed as minimum risk pesticides and accordingly have been exempted from registration requirements. The FIFRA 25(b) exemption list includes citronella oil, garlic oil, lemongrass oil, rosemary oil and thyme oil, among others as active ingredients which do not require registration (US EPA (http://www.epa.gov/opp00001/biopesticides/regtools/25b_list.htm)). The essential oils (active ingredients) from the FIFRA 25(b) list can be combined with the inert ingredients found in List 4A “Inert ingredients of minimal concern”. This list includes a

wide variety of compounds including various emulsifiers, some chemicals with synergistic action, and other chemicals such as wintergreen oil, which can improve the emulsification of essential oils in water (US EPA (http://www.epa.gov/opprd001/inerts/section25b_inerts.pdf)). As a result of this exemption, it is possible to develop formulations and take them to market quickly and with considerably lower cost. This strategy has been used by companies such as EcoSMART Technologies, in developing their lines of essential oil-based pesticides. Accordingly, in the US, it would be easiest to develop essential oil-based pesticides for *C. rosaceana*, *D. plantaginea*, *M. persicae* and *T. ni* based on citronella, garlic, lemongrass and thyme oils. Pesticides developed from patchouli or lavender oils would require registration and, accordingly, would take considerable more time and cost to introduce to the market.

In Canada, there are no such exemptions, meaning that an essential oil-based insecticide would be required to go through registration procedures regardless of the exemption status in the US. Indeed, EcoSMART Technologies approached the PMRA about registration requirements for their exempt essential oil-based pesticides. However, given that there is no exemption language in the PCPA, the PMRA acknowledged that they are unsure of how to handle these products (Isman MB, 2010, pers. comm.). However, there are options for registered products from OECD countries to be introduced to Canadian markets under the User Requested Minor Use Registration (URMUR) program. This allows pesticides with a relatively small market to be introduced at the request of grower groups with little additional cost or effort. Accordingly, if a patchouli oil or lavender oil based insecticide was developed and

registered in the US or any other OECD country, they would likely be able to enter the Canadian market via the URMUR program.

What other factors need to be considered when developing an essential oil-based insecticide?

Ultimately, when developing an essential oil-based pesticide, the actual formulation is critical. As was discussed in Chapters 2 and 3, the carrier and emulsifier used can have a dramatic impact on the toxicity of an essential oil. Accordingly, for an EC formulation, it will be particularly important to find emulsifiers that can enhance toxicity. This is especially important if developing essential oils including thyme, citronella or lavender oils, as the carrier agent used had the greatest impact on the toxicity of these three essential oils. The inclusion of synergists could also increase toxicity while reducing the quantity of essential oil required, thus reducing costs. Including adjuvants such as spreaders would enhance toxicity as well as ensure better coverage of the insecticide on the crop. The stability of essential oil formulations can also be influenced by the use of specific agents known to maintain essential oil stability or by using techniques such as microencapsulation during the manufacturing process. For example, microencapsulation of various essential oils in gelatin, water, Na_2SO_4, glutaraldehyde and NaOH significantly increased their stability, with up to 20% of active principles still available after 30 days.[34] Similarly, nanoencapsulation of garlic oil using polyethylene glycol coated nanoparticles dramatically improved the stability of garlic oil in the control of adult *Tribolium castaneum*. The nanoencapuslated garlic oil was 80% as toxic at 5 months as the first day, while non-encapusalted garlic oil caused only 11% toxicity. In

addition, the chemical composition of the nanoencapsulated garlic oil was still similar to the original composition, even after 5 months had passed.[35]

It is also important to note that an insecticide developed with essential oils does not need to be restricted to just one essential oil or even essential oils only. For example, it may be possible to combine patchouli and thyme oils with lower cost essential oils which showed moderate toxicity, such as peppermint or clove oils. Alternately, essential oils could be added to other insecticides such as pyrethrum based insecticides to enhance their toxicity.

In developing an essential oil-based pesticide, it is also important to understand how such a product could be used within an IPM program. In theory, pesticides should only be applied once pest populations reach densities that are likely to cause economic damage to the crop (e.g. by understanding the economic injury levels (EILs) for a pest). In practice, this strategy is difficult to use as determining the economic thresholds (ETs), i.e. the point at which pest controls should be applied, is often a largely subjective process.[36] This also typically depends on having pest control products that have a rapid response and are efficacious and can successfully reduce pest populations below the ET. However in the case of products that may have a lower efficacy, such as an essential oil-based insecticide, it could be better to use them in combination with other pest management options, rather than to simply rely on the use of EILs and ETs. For example, the use of biological control agents is often a crucial component of greenhouse IPM, however biological controls are often insufficient to reduce pest levels to below economic thresholds if pest populations have been allowed to build up. Accordingly, an essential oil-based insecticide could be used to decrease the pest

population to a level which could then be effectively managed by biological control agents. One recommended strategy may be to apply the essential oil-based insecticide, wait 2-5 days to ensure that the insecticide has had an opportunity to work and has volatilized, leaving no residues on the crop, and then introduce biological controls such as parasitoids and predators. It is important to note here that unlike traditional pesticides, an essential oil-based pesticide does not need to eradicate the pest. Rather, it could be incorporated into an IPM program as a method of managing pest levels.

While the above suggests that an essential oil-based insecticide could be used to reduce insect pest populations to levels that can be managed by other pest management strategies, it could also be used to keep growing pest populations from developing early in the season. For example, in apple orchards, an early season application of an essential oil-based pesticide targeting both *C. rosaceana* larvae and developing *D. plantaginea*, could decrease initial populations to a level where they are unlikely to develop sufficient populations to cause damage later in the season. This could be a particularly effective strategy for *D. plantaginea* as these aphids are only a problem on apple trees for a short period of time. And given that many essential oils volatilize rapidly leaving no residue on crops, most such products could be used at any time during the growing season, including just before or during harvest.

One other factor which needs to be considered is whether the essential oil-based insecticide could leave oily residues on crops. During the course of my research with both patchouli and garlic oils, I noticed that both oils left a sticky, oily residue, particularly at higher concentrations, which did not easily dissipate. This could create

problems if sprayed on a crop which was harvested shortly after as fruits or leaves could still have residues. Accordingly, if an essential oil-based insecticide were to be developed using these essential oils as significant constituents within the insecticide, it may need to be restricted to an early season application before the crop developed. This is particularly important for patchouli oil as it is not considered a food product. Additional testing would be required with complete formulations in order to determine how much residue remained, if any, following application of the insecticide, and to determine how long it would take for that residue to fully dissipate. Testing would also be needed to ensure that these residues did not pose a risk of damage to application equipment such as sprayers.

Finally, the positive and negative effects of an essential oil-based insecticide need to be considered. While most essential oils are generally regarded as safe in the public eye, this doesn't guarantee safety. For example, some essential oil constituents such as *d*-limonene have been implicated in causing contact dermatitis in humans and pets, as has tea tree oil (*Melaleuca alterniflora*).[37] And given that essential oil-based insecticides are insecticides, there is the possibility that beneficial insect populations will be more strongly impacted than the pest species. However this is not always the case. In tests with the two-spotted spider mite, *Tetranychus urticae* and the predatory mite, *Phytoseiulus persimilis*, both rosemary oil and the commercial product, EcoTrol, were more toxic to the spider mite than the predatory mite.[38] In addition, some essential oils may have toxic effects to other organisms. For example, clove oil has been studied as a fish anaesthetic for rainbow trout[39] and many other fish, suggesting that clove oil should not be used close to waterways. Additional research is required to assess the

safety of these essential oils and to reduce the risk of negative effects on non-target insects and the environment. However, with such research, it will be possible to ensure that appropriate safeguards are implemented.

Who would be the most likely to benefit from essential oil-based insecticides?

The most likely group to benefit from essential oil-based insecticides are the organic producers. For example, there are currently few available organic control options for *D. plantaginea*, and accordingly, an essential oil-based insecticide targeting *D. plantaginea* would greatly assist organic apple producers in managing this serious pest. But organic producers are not the only group. Essential oil-based insecticides can also work well in conventional orchards and greenhouses, especially as a resistance management tool, both to help control resistant insect pests as well as to help prevent resistance development by incorporating such insecticides in a pest management rotation with other insecticides. And as stated above, it can be used with other IPM strategies including with biological control agents, which would benefit all producers.

Essential oil-based pesticides can also be used by the home owner, although this would require modifications of the formulations. While EC formulations are useful for commercial applications, ready to use (RTU) formulations would be more suitable for home owners. In this case, the product can simply be used out of the bottle and sprayed on gardens or backyard trees. However, while commercial growers are unlikely to care considerably about the smell of the pesticide, the situation may be different with home owners. Accordingly, it may be necessary to develop odourless formulations or formulations with a lower odour for home use, or with pleasant odours.

For example, garlic and patchouli oils can smell quite strong and could be overwhelming for some home owners. I did notice in separate tests that it was quite easy to mask the smell of patchouli oil with various compounds including methyl salicylate, the main constituent of wintergreen oil, suggesting that it would not be difficult to adjust the odours of the formulations.

Ultimately, what does all of this mean for developing an essential oil-based insecticide from any of the six essential oils identified within this thesis? While I initially suggested patchouli oil as a likely option for inclusion in such an insecticide due to its broad spectrum activity, the above comments demonstrate a patchouli oil-based insecticide faces a considerable number of challenges. However, given the broad spectrum activity of patchouli oil, it is predicted that such an insecticide could be toxic to a variety of other insect pests, increasing the potential market for such an insecticide to a point where it becomes worthwhile to overcome these challenges. If registration were obtained in the US or another OECD country, an URMUR could be started within Canada which would provide Canadian producers access to such an insecticide. This would give the manufacturer an opportunity to proceed with full registration in Canada as well, if desired. It may also be possible to obtain lower quality quantities of patchouli oil that are still highly insecticidal and less expensive, or it may be possible to reduce the amount of patchouli oil required by adjusting the composition of other constituents within the insecticide. Combined, this would allow the production of a product which is still affordable to growers, while remaining profitable for the manufacturer. Additional research could also determine if residues would be a concern in a fully formulated product, and if so, how to reduce the persistence of the residues.

The development of lemongrass or citronella oil based insecticides for *T. ni* and *M. persicae*, respectively, also remains a viable option. Both oils are typically among the least expensive essential oils, and given that both essential oils could be incorporated into a FIFRA 25(b) exempt insecticide quite easily, a product could be rapidly introduced to the market. However, this would mean that unless Canadian pesticide laws change, it is unlikely that such insecticides would be seen in Canadian orchards or greenhouses. The biggest challenge to the development of a thyme or garlic oil based insecticide remains cost. From Table 5.1, these two essential oils were typically among the most expensive, meaning that additional efforts would likely be required to reduce the necessary amount of active ingredient. It's also possible that these oils could be incorporated into other insecticides to enhance the toxicity of those insecticides. Based on the cost of lavender oil as well as the requirement for registration and the relatively limited market, it is unlikely that lavender oil will be developed into a lavender oil-based insecticide for field application, although it could be added as a minor component to other registered essential oil-based insecticides.

While the market for current essential oil-based pesticides is small, there are growing opportunities. Increasing awareness in the general public of the negative environmental and health effects of synthetic pesticides and an improved regulatory environment has meant that the time may be right for the potential growth of this field. There is also very strong interest in using such reduced risk pesticides in Europe, China, Korea, Southeast Asia, and Latin America, providing further opportunities for growth (Isman MB, 2010, pers. comm.). I hope that the research presented in this thesis constitutes a useful

contribution to our knowledge of these pest management materials and will stimulate further research leading to commercial development.

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