Limonene for the management of Crape Myrtle Bark Scale (*Acanthococcus lagerstroemiae*)

by

Jordan Michaela Melson-Jordan

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 4, 2024

Keywords: *Acanthococcus lagerstroemiae*, limonene, coccinellids, urban landscape entomology, Herbivore-Induced Plant Volatiles, Integrated Pest Management

Approved by

David W. Held, Chair, Professor: Department of Entomology and Plant Pathology
Arthur Appel, Associate Dean of Research, Professor: Department of Entomology and Plant Pathology
Amanda Strayer-Scherer, Assistant Professor, Extension Specialist: Department of Entomology and Plant Pathology

ABSTRACT

Crape myrtle bark scale (CMBS, *Acanthococcus lagerstroemiae*) is an invasive pest of crape myrtles first introduced to the United States less than 20 years ago. CMBS reduce the aesthetic value of these ornamental trees which presents a risk to the crape myrtle industry through additional management and replacement costs. The current recommendation for management is soil applied systemic neonicotinoids which translocate into the pollen of crape myrtle trees at levels that are toxic to pollinators and other beneficial insects. Limonene is a citrus monoterpene previously studied for its pesticidal activity against numerous arthropod pests, fungi, and bacteria. These studies show that weekly limonene at 1% is effective at controlling CMBS with limited toxicity to lady beetle natural enemies. A LC_{50} for limonene was established at 1.41%. The combination of limonene and yellow panels was not effective for the recruitment of lady beetle natural enemies, but limonene alone (at 1%) was not toxic to lady beetles in the crape myrtle trees. This research provides a new method of chemical control better suited to an environmentally integrated approach for the management of CMBS.

ACKNOWLEDGMENTS

I would like to extend my thanks to my main advisor, Dr. David Held, for his guidance and expertise during my master's experience. I would also like to express my appreciation for my wonderful committee members, Dr. Arthur Appel and Dr. Amanda Strayer-Scherer, for their knowledge and continuous support through the completion of this thesis. I must give a special thanks to Dr. Appel for his in-depth explanations of toxicology and him allowing me to use his laboratory equipment.

Thank you to my fellow lab members for their unending help counting thousands of insects and treating trees. This thesis could not have been completed without their assistance and encouragement. My sincerest thanks go out to Gracie Cotter for her continuous help collecting data and Elijah Carroll for his immense mentorship.

This work could not have been done without the support of the Southern Region IPM Center, Orange Guard Inc., and Florida Chemical Company LLC.

Finally, and most importantly, I will never be able to express my thanks enough for my husband, Carter Jordan, and our Josie and Nacho, as well as my family, for their ceaseless love and support. They are a bigger blessing than they will ever know and provided me with the spark to continue persisting in the best and worst moments. This could not have been done without them.

DEDICATION

To Scotty Wayne Stover, who taught me that life is the most precious thing we could ever dare to learn about.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CMBS	Crape Myrtle Bark Scale
APSA	All Purpose Spray Adjuvant
HIPV	Herbivore-Induced Plant Volatile
IPM	Integrated Pest Management
IGR	Insect Growth Regulator
GLMM	Generalized Linear Mixed Model
DI	Deionized
DBH	Diameter at Breast Height
CL	Confidence Limits
AI	Active Ingredient
LC50	Lethal Concentration expected to kill 50% of the test group

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 CRAPE MYRTLE

Crape myrtles (*Lagerstroemia* spp*.)* are the most popular flowering ornamental tree in the United States with production in 33 states and production almost doubling in the past 20 years (Marwah et al., 2021). Since their introduction over 175 years ago, crape myrtles have become a preferred horticultural plant because of their durability, low maintenance, and susceptibility to relatively few diseases and pests (Chappell et al., 2012). Due to cultivation and breeding techniques, varieties are extremely diverse offering more than 130 cultivars (Wang et al., 2019). The array of cultivars means a large diversity of crape myrtles with different flower colors, bark type, fall leaf color, height, and resistance to cold and pest or disease. This diversity creates a massive market of hardy trees for every environment. However, an accidental pest introduction in 2004 appears to be a growing threat to the crape myrtle industry.

1.2 CRAPE MYRTLE BARK SCALE

1.2.1 Introduction to CMBS

Crape myrtle bark scale (CMBS, *Acanthococcus lagerstroemiae*) was introduced to the United States less than 20 years ago. First reported in a nursery in Richardson, Texas, this scale pest has spread across the southeastern United States (Wang et al., 2019). The largest impact of *A. lagerstroemiae* is its ability to reduce the aesthetic value of the trees. The sessile, white females attach to the bark to lay their eggs. As nymphs and adult females feed, they produce honeydew which encourages the growth of sooty mold. The discoloration of plants covered in sooty mold is typically the first symptom producers or homeowners note (*Figure 1)*. Sooty mold on leaves can decrease photosynthesis, reducing the overall health of the plant. CMBS and subsequent reductions in photosynthesis can cause branch dieback, small sparce flowering, and stunting, and while these issues will not cause severe damage on a mature tree, infestations can result in the death of young plants (*Figure 2)* (Marwah et al., 2021).

1.2.2 Host and range

Despite the name, crape myrtle bark scale can be found on a range of other plants. In several Asian countries, CMBS can infest no less than 13 economically and environmentally important plants (Park et al. 1993, Wang et al., 2016). However, in North America, crape myrtle bark scale have only been reported on crape myrtle and American beautyberry (*Callicarpa americana* L.) (Wang et al., 2016, 2019). Along with crape myrtles, CMBS are considered to major threats to persimmons and pomegranates, although they have not yet moved to those hosts in the United States. Wu et al. (2021) studied the suitability of six different *Lagerstroemia* species, as well as one other native Lythraceae plant. The study showed that suitability differed significantly between species, and although all species were susceptible to infestation, *L. speciosa* could only sustain very low populations (Wu et al., 2020). During my research with trees on the Auburn University campus, I noted a multi-year infestation on a set of crape myrtle trees (cultivar Dynamite). Populations on those trees never reached the level of infestation noted on other varieties (Natchez) on campus.

Figure 6: Shrub under a CMBS-infested crape myrtle that has sooty mold.

Figure 7: CMBS damage on crape myrtle. Infested trees may flower less, have delayed leaf flush or thinned canopies in addition to sooty mold.

This shows potential for the development of new cultivars with low CMBS suitability. While focus in the United States is mainly on CMBS impact to their namesake, understanding the full biological and geographical range of the pest can give insight into better control and management.

Acanthococcus lagerstroemiae originated in East Asia and is now found throughout Asia, expanding from Beijing, China to India and Korea (Park et al. 1993, Wang et al., 2016). As of 2021, crape myrtle bark scale was confirmed in at least 14 states (Alabama, Arkansas, Florida, Georgia, Kansas, Louisiana, Mississippi, New Mexico, North Carolina, Oklahoma, Pennsylvania, Tennessee, Virginia, and Washington D.C.) since it was first reported in 2004 (Marwah et. al., 2021, Skvarla and Schneider, 2022). Wang et al. (2019) determined the potential range of crape myrtle bark scale in the United States ranges from the furthest point south to around the 43rd parallel north from Idaho to Maine. Cold temperatures were much more limiting to CMBS spread than warm temperatures (Wang et al., 2019). The major goal of observing and studying CMBS range and behavior is to prevent the spread of this major ornamental pest, and understanding their potential allows for better preventative strategies.

1.2.3 Biology

Crape myrtle bark scale populations on crape myrtles can grow rapidly due to their high fecundity (Wang et al., 2019). The first instar nymphs, known as crawlers, hatch from eggs and are the mobile dispersing stage. The crawlers disperse along the branches and go through several nymphal stages before becoming sessile; females and males have three and five nymphal stages respectively (Wang et al., 2016). The males enter their pre-pupal and pupal stages, where they are pink and non-feeding while surrounded in white sacs. Adult male CMBS are also pink but are alate and lack functional mouthparts unlike the other developmental stages. Since females are wingless and remain sessile after the third molt, adult males seek out the females for reproduction. Once the female has fertilized eggs, she begins to produce a white ovisac where she lays between 114-320 eggs and decreases in size before dying (Wang et al., 2016). Each stage can be found in *Figure 3*. The ovisac seems to function as a barrier to protect the eggs from natural enemies and humidity loss while they develop. Once the eggs hatch, the cycle starts again, but the crawlers can only disperse over short distances. In field experiments with sticky cards and infested plants, Cornish (2021) determined wind can disperse crape myrtle bark scale crawlers up to 13.7 m from an infested plant, but most crawlers were captured on cards at the short and moderate distances of 0 m and 4.5 m from the infested source plant. Evidence did show potential long-distance dispersal by birds nesting in the trees (Cornish, 2021). However, Cornish (2021) notes that it only takes one crawler that develops into a reproductive female to start an infestation on a new host tree. The movement of infested plants over state or county boundaries through commercial exchanges is one ways CMBS are introduced over long distances, as low-density populations on a host plant are hard to observe and often go undetected at border checkpoints (Cornish, 2021).

Depending on their location, CMBS can have two to four generations per year (Wang et al., 2016) as the development of *A. lagerstroemiae* is highly temperature-dependent.

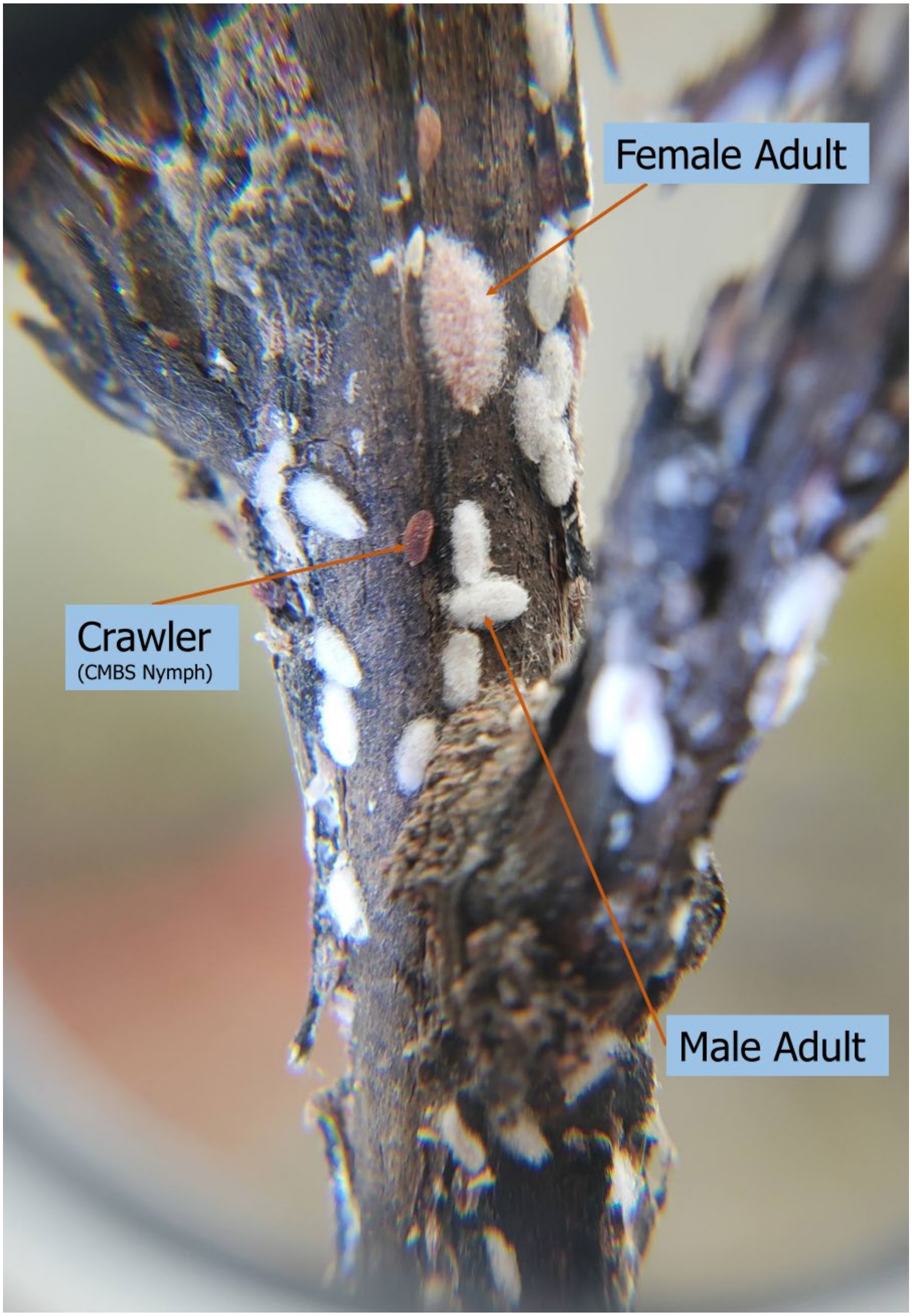

Figure 8: CMBS life stages on crape myrtle branch.

Wang et al. (2019) showed the time for CMBS to complete one generation is about four months at 25 °C and three and a half months at 30 °C. However, the same study showed complete mortality of the eggs at 32 °C. Nymphs never reach a reproductive stage at a constant 20 °C (Wang et al., 2019). The daily average temperatures in subtropical areas such as Louisiana and Texas remain above 20 °C from mid-Apr through Oct and then decrease to less than 20 °C until the following mid-April, showing the possible time of crawler emergence and beginning of overwintering (Wang et al., 2019). This estimates that crape myrtle bark scale should go through two generations in these subtropical areas. Areas with more tropical temperatures allow the scales to produce even more generations, allowing populations to increase quickly (Xie et al., 2022). Additionally, Vafaie et al. (2020) estimated up to four overlapping generations in various parts of the United States based on their research in Texas, Arkansas, and Louisiana, and are continuing their work to accurately create a degree-day model for CMBS. While *A. lagerstroemiae* overwinters as all but the adult stage in Asia, in the United States it has only been reported to overwinter as the nymphal stage (Wang et al., 2016) and nymphs can be active throughout the winter (Wright et al, 2023). In Auburn, Alabama, CMBS nymph aggregations were observed on the trunk and base of the trees; however, this gregarious behavior was only observed in the winter months (*Figure 4)*. Wang et al. (2019) also observed significant heat and cold tolerance in the nymphal stage which can vary in the population depending on time of year, allowing for even greater chances of survival.

Figure 9: CMBS Crawlers forming aggregations in the winter months in Alabama.

1.3 IMPACTS ON THE INDUSTRY

As previously mentioned, crape myrtles are an economically valuable ornamental, but these pests are a threat to the industry. A 2018 and 2019 survey with producers in eight southern states showed that 61-72% were of the belief that the sale and use of crape myrtles would decrease and 30-34% believed that the value of crape myrtles would decrease simply because of crape myrtle bark scale (Marwah et al., 2021). Of the 79 respondents, 30-43% agreed that their desire to grow crape myrtles would significantly decrease if the infestations could not be controlled (Marwah et al., 2021). Chong (2022) reported a crape myrtle specialist in South Carolina estimated a $138,000 loss if he was required to cull all heavily infested trees in a single nursery block, not including insecticide and labor costs required to treat lightly or moderately infested trees. In several states, stop-sale restrictions have been placed on nurseries with CMBS infestations for fear of spreading the pest, and this decrease in sales could lead to the exact reduction in value surveyed producers are concerned about (Wang et al., 2016).

1.4 MANAGEMENT AND CONTROL

1.4.1 Natural enemies and biological control

Natural enemies of crape myrtle bark scale include both predators and parasitoids, notedly several species of coccinellids within the genera *Chilocorus* and *Hyperaspis, Harmonia axyridis*, as well as chrysopids (*Figure 5 and Figure 6*) (Wang et al., 2016).

Figure 10: *Hyperaspis bigeminata* mating on CMBS-infested crape myrtle.

Figure 11: Ladybeetle larva next to CMBS females.

A single fourth instar *Chilocorcus cacti* can consume nearly 400 CMBS eggs in 24 hours in the laboratory (Wang et al., 2016). In a Texas study conducted over two years at 26 different crape myrtle sites, a number of coleopteran predators such as *C. cacti, H. axyridis, H. bigeminata,* and *H. lateralis* were found feeding on *A. lagerstroemiae* (Gilder et al., 2021). No parasitoids of CMBS, native or non-native, have been observed in the United States; however, there are several species of parasitoids that affect this pest in their native range (Suh, 2019, Gilder et al., 2021).

Of course, with CMBS having such a large geographic range, different lady beetle species provide differing levels of control depending on the location of the infestation. Each predator species has its own specific optimal conditions determining its range and capabilities as an effective control measure. Research into non-chemical controls like biological control is increasing as more countries are placing stricter regulations on pesticide use (Hulot and Hiller, 2021).

The biology and ecology of a species is extremely important when considering its use as a biological control agent. As generalist predators, lady beetles often are better at tracking prey density than controlling it. Thus, a common issue with the use of lady beetles for classical biological control is their tendency to abscond. The other concern, as usual with classical biological control, is non-target effects (Wang et al., 2016). As many coccinellids are generalist predators, intense research is necessary before their release into a new environment as there can be severe consequences for the native ecosystem (Wang et al., 2016). The mentioned species above and other coccinellids, however, have great potential for augmentative and conservation biological control (Wang et al., 2016). It has been shown that yellow panels in crape myrtle trees can act as a visual lure for several coccinellid species, especially native ones (Ibiyemi, 2020). If we can increase the local populations of natural enemies superficially or raise the local carrying capacity, these predators can help prevent and maintain pest populations with limited chemical applications. Using a combination of biological and chemical control can often provide even greater levels of control; however, many insecticides have disastrous negative effects on beneficial insects.

1.4.2 Management using chemical control

With an invasive pest such as *Acanthococcus lagerstroemiae*, control and management are some of the most pressing issues faced by researchers and growers alike. The most common form of control is soil-drench chemical applications of neonicotinoids such as dinotefuran and imidacloprid (Wang et al., 2016; Vafiae, 2016). With Bayer's introduction of imidacloprid in the mid-1990s, neonicotinoids were widely adopted due to their broad-spectrum uses, limited number of treatments, long residuals, and fewer non-target effects. Neonicotinoids have low mammalian toxicity, which encourages its use; imidacloprid is currently the second most used agrochemical worldwide (Krischik et al., 2015). However, while the mammalian toxicity is low, the translocation of these insecticides into honeydew of sap sucking insects (Quesada et al., 2020; Calvo-Agudo et al., 2019) and in crape myrtle pollen (Thurmond, 2019), both at concentrations that pose risk to beneficial insects, have resulted in exploring alternative, IPM-friendly practices for CMBS (*Figure 7)*. Research conducted on four different species of coccinellids and two species of butterfly larvae showed that feeding on imidacloprid treated flowers significantly reduced survival in three of the four species of beetles as well as both butterfly larvae (Krischik et al., 2015).

Figure 12: Lady beetle larva found accumulated around base of crape myrtle in Auburn, Al one week after imidacloprid treatment. Most were moribund or assumed to be dead.

Alternately, some selective insect growth regulators (IGRs) like pyriproxyfen have been shown to be less lethal to coccinellid natural enemies (Liu and Stansly., 2004). Treating crape myrtle bark scale with neonicotinoids like imidacloprid may handle the pests, but it is incompatible with the core principles of integrated pest management.

An interesting approach to management is the use of biorational terpenes, aromatic plant volatiles found in essential oils, and herbivore-induced plant volatiles (HIPVs) as attractants, deterrents, and insecticides. Limonene, a citrus extract, has been studied for its deterrent and insecticidal properties suggesting its use as a plant-based natural product for chemical pest control (Ibrahim et al., 2001). Many HIPVs like limonene can also attract predators to the plant to feed on the herbivores attacking them, creating a mutually beneficial relationship between plant and predator (Takabayashi and Dicke, 1996). Additional work by Ibiyemi (2020) evaluated the combination of yellow panels and HIPV lures to further recruit ladybeetle natural enemies as a method to increase the biological control provided by the beetles.

1.5 LIMONENE AS A POTENTIAL MANAGEMENT TOOL FOR CMBS

Limonene is an aliphatic monoterpene and is the major component in citrus fruit peel oil constituting more than 95% of the oil composition. The d-isomer (denoted as D-limonene) is its most common naturally occurring form and is recognized as the fragrance of oranges (National Center for Biotechnology Information, 2023). Limonene in its various forms has shown pesticidal activity against numerous arthropod pests like scale insects, mites (Gadelhaq et al. 2022), medically important mosquitoes (Theochari et al., 2020), and even fungi and bacteria (Azeem et al., 2022; Costa et al., 2019). Hollingsworth (2005) was among the first to demonstrate the efficacy of limonene against scale insects. In that study, a 1% limonene mixture controlled from 69-100% of mealybugs (*Rhizoecus* spp.) and green scale (*Coccus viridis* (Green)). Gadelhaq et al. (2022) recently determined the modes of action for limonene in pigeon feather lice (*Columbicola columbae*) are neuromuscular through acetylcholinesterase inhibition and physical through distortion of the cuticle on contact.

The unique quality of limonene is it has also shown some potential as an attractant for lady beetle natural enemies of crape myrtle bark scale. It has been shown that limonene in aphid honeydew secretions can strongly attract *Harmonia axyridis* in research concerning *H. axyridis* as a secondary pest (Leroy et al., 2012). The presence of limonene in a plant also significantly stimulates oviposition behavior of *Harmonia axyridis* females (Alhmedi et al., 2010). An insecticide that targets the pest directly and indirectly through the attraction of natural enemies providing free control is an ideal application of IPM principles.

However, limonene may require specific adjuvants or microencapsulation to resolve its insolubility in aqueous environments, volatility, and instability. Encapsulation of nanoemulsions with surfactants can increase its stability, water dispersibility, and bioavailability, which can greatly affect its efficacy in the field (Choi and McClements, 2020). Hollingsworth (2005) developed a microemulsion using a solution consisting of 1% limonene with two adjuvants, 0.75% APSA-80 and 0.10% Silwet L-77. Limonene in solution with adjuvants at those rates was safe for most plants and has led to further research and development of safer, limonene-based biorational insecticides.

The impact of insecticides on biological control agents can be mitigated through the choice of insecticide, dosage, or timing of application. The current neonicotinoid insecticides for CMBS management are incompatible with beneficial insects and biological control, but the development of new methods and chemical treatments may allow us to decrease environmental effects and implement new and improved strategies for CMBS management. Understanding both the effective chemical treatments for CMBS and the pest's natural enemies can help find a control method more compatible with IPM principles.

1.6 RESEARCH OBJECTIVES

The crape myrtle industry is highly economically and environmentally valuable, but with the introduction of crape myrtle bark scale, the industry is facing a threat that must be handled. CMBS can rapidly establish on a tree or in a nursery, but the current control methods are not sustainable and can harm beneficial insects. The overall goal of this study is to evaluate the potential of limonene as an IPM-compatible, alternative treatment for the management of crape myrtle bark scale. The following objectives were developed to address the use of limonene as a CMBS-reduction method:

1. Compare seasonal treatments of limonene for CMBS control.
2. Determine if limonene can compete with neonicotinoids and other available chemicals recommended for the management of CMBS.

3. Conclude if the combination of limonene and yellow panels will provide control of crape myrtle bark scale and be effective for the recruitment of lady beetle natural enemies of CMBS.
4. Determine the dose response for limonene and other adjuvants against adult lady beetles.

CHAPTER 2: EFFICACY OF LIMONENE AGAINST CRAPE MYRTLE BARK SCALE IN POTTED CRAPE MYRTLES

2.1 INTRODUCTION

Limonene is an aliphatic monoterpene and is the major component in citrus fruit peel oil constituting more than 95% of the oil composition. The d-isomer (denoted as D-limonene) is the most common naturally-occurring form and is recognized as the fragrance of oranges (National Center for Biotechnology Information, 2023). Limonene in its various forms has shown pesticidal activity against numerous arthropod pests like scale insects, mites (Gadelhaq et al. 2022), medically important mosquitoes (Theochari et al., 2020), and even fungi and bacteria (Azeem et al., 2022; Costa et al., 2019). Hollingsworth (2005) was among the first to demonstrate the efficacy of limonene against scale insects. In that study, a 1% limonene mixture controlled from 69-100% of mealybugs (*Rhizoecus* spp.) and green scale (*Coccus viridis* (Green)). Gadelhaq et al. (2022) recently determined the modes of action for limonene in pigeon feather lice (*Columbicola columbae*) are neuromuscular through acetylcholinesterase inhibition and physical through distortion of the cuticle on contact.

Limonene as a formulated product for plant uses may require specific adjuvants or microencapsulation to resolve its insolubility in aqueous environments, volatility, and instability. Encapsulation of nanoemulsions with surfactants can increase its stability, water dispersibility, and bioavailability, which can greatly affect its efficacy in the field (Choi and McClements, 2020). Hollingsworth (2005) developed a microemulsion using a solution consisting of 1% limonene with two adjuvants, 0.75% APSA-80, and 0.1%

Silwet L-77. Limonene in solution with adjuvants at those rates was safe for most plants with limited phytotoxicity and has led to further research and development of safer, limonene-based biorational insecticides.

Crape myrtle bark scale (CMBS, *Acanthococcus lagerstroemiae*) is an exotic scale introduced to the United States through Texas less than 20 years ago. The largest concern surrounding this pest is their ability to decrease the aesthetic value of ornamental crape myrtle trees, as well as their spread to other hosts and geographic areas. In the United States, CMBS have high fecundity and up to four asynchronous generations which can make control difficult. The most common method of management for *A. lagerstroemiae* is soil-applied, systemic neonicotinoids that translocate into the pollen of crape myrtles at levels that present a high contact and dietary exposure for honeybees and other pollinators (Quesada et al., 2020; Calvo-Agudo et al., 2019; Thurmond, 2019). As crape myrtles produce large amounts of pollen and may fill a pollen dearth in urban environments, alternative methods of control are necessary. Some selective insect growth regulators (IGRs) like pyriproxyfen have been shown to be less lethal to coccinellid natural enemies and also require evaluation against CMBS along with limonene (Liu et al., 2004).

Unpublished work (Carroll and Held) has suggested limonene may have a place in the management of CMBS. Field collected crape myrtle cuttings were either left untreated or treated with about 8 mL of limonene (Orange Guard®, 5.8% limonene, Orange Guard Inc., Marina, CA). Cuttings in water tubes with residues of the limonene product that were 14, 10, 7, 3 days old or freshly dried were randomly placed onto potted, infested crape myrtles with active crawlers. Cuttings representing all residue ages and untreated spaced were placed around the same pots with leaves touching the infested plant for 72 hours. Although crawlers were found on all cuttings, the fresh-treated cuttings had the fewest number of crawlers (3.5 ± 0.78) which was about half of the number on cuttings with a 7 day limonene residue and about 25% fewer than non-treated control cuttings (13.3 ± 2.1). These findings provided sufficient evidence for us to continue evaluating limonene for CMBS control in the field.

Based on this previous research, the following objectives were developed: 1) to determine the most efficient short-term limonene application frequency for the management of CMBS, 2) to compare the efficacy of limonene and other chemicals available for the control of CMBS, and 3) to determine the dose response for limonene and other adjuvants against a common adult lady beetle reported to attack CMBS.

2.2 MATERIALS AND METHODS

2.2.1 Plant Materials

Potted crape myrtle trees (Natchez) infested with CMBS were grown in plastic pots growing in a bark-based nursery potting mix (50:50 bark sand mix). Infestations were initiated or augmented by attaching cuttings from infested trees onto non-infested or lightly infested trees using parafilm. For the outdoor experiment, trees were 1.95 m tall on average in 5 gallon (trade) pots. Pots were placed in a 4 x 4 block in a full sun, grassy area with a structure on one side and a gravel road on the other (*Figure 8).* Trees received water daily.

Figure 13: Experimental layout for experiment evaluating the application frequency of limonene on infested potted trees.

For the greenhouse experiment, infested plants (Natchez) in 3 gallon (trade) pots were approximately 1 m tall with very little variation in height. Trees were arranged so only individuals sharing treatments touched and were watered daily as well.

2.2.2 Limonene application frequency

To study the effects of short-term (5 wk) limonene applications on crape myrtle bark scale populations and associated lady beetles, the experiment was arranged in a randomized complete block where a block of four trees were grouped together into a replicate based on similar numbers of CMBS in a pre-treatment count. For pre- and post-treatment samples, CMBS counts were taken on 10 cm portions of three random branches and 10 cm of the trunk with every sample taken on different random sections. Those counts were then used to place each plant into one of four blocks, each block containing trees with similar scale counts. One of four treatments were then randomly assigned to each tree within each block.

The four treatments included a control where no limonene was applied (non-treated control) and limonene (Orange Guard, 5.8% limonene, Orange Guard Inc., Marina, CA) applied either weekly (4 applications), biweekly (2 applications), or after each rain. Plants assigned to the after-rain treatment received 6 total applications. All treatments were applied with a hand pumped backpack sprayer to the entire tree until runoff. The non-treated control was also included to account for changes in CMBS populations due to predation by endemic lady beetle populations or other factors.

Every week after the initial treatment, the number of adults and crawlers were counted in the same way as the pre-treatment counts, 10 cm of the trunk as well as 10 cm of three random branches. Lady beetle surveys were conducted by taking beat samples on four branches of each tree with each branch receiving three beats. The lady beetles were collected into labeled plastic sandwich bags and taken back to the lab in a cooler for immediate identification. After identifying the lady beetles to species, they were released back outdoors.

2.2.3 Comparison of limonene to standard insecticides

A greenhouse experiment was conducted to compare the efficacy of limonene to the commonly recommended insecticides for CMBS control (Vafaie, 2016). These included systemic neonicotinoids, and an insect growth regulator.

Thirty-six CMBS-infested crape myrtle trees were spaced so that plants had no contact with one another benches in the greenhouse. Double-sided tape was placed on the base of each tree to monitor for crawler activity (Vafaie, 2016). The tapes were checked weekly to confirm crawler activity and then removed before the experiment began. Pre-treatment counts were taken using the previous methods and used to assign trees with similar numbers of CMBS into common blocks (replicates) in a randomized complete block design with six replicates of six trees each. Each of the six trees in all replicates were randomly assigned to one of these six treatments: untreated (negative) control, water-treated (positive) control, limonene, dinotefuran, imidacloprid, or pyriproxyfen. The list of chemicals, commercial names, manufacturers, and product rates can be found in *Table 1.* Positive and negative controls were included in each replicate to control for the effects of spraying during the applications.

Table 1: Treatments, manufacturer information, and rates for chemical comparison experiment

Treatment	Commercial Name	% A.I.	Manufacturer Info	Label Rate
Limonene	Orange Guard®	5.8%, diluted with tap water to 1% limonene	Orange Guard Inc., Marina, CA	Spray until runoff
Dinotefuran	Zylam® Liquid Systemic Insecticide	10%	PBI-Gordon Corporation, Kansas City, MO	0.4 fl oz per ft ht
Imidacloprid	Mallet® 2F T&O	21.4%	Nufarm Americas Inc., Alsip, IL	0.2 fl oz per ft ht
Pyriproxyfen	Distance® Insect Growth Regulator	11.23%	Valent U.S.A. Corporation, Walnut Creek, CA	12 fl oz per 100 gal
Untreated	n/a	n/a	n/a	n/a
Water	n/a	n/a	n/a	Spray until runoff

As CMBS are exceedingly small, sprayable treatments can physically remove the pest from the host, regardless of the chemical applied. Applications of the treatments were made based on the chemical label after weekly counts had been taken. Treatments were applied as either a soil drench or foliar spray, and trees requiring spray treatments were moved outside the greenhouse temporarily for chemical application and drying (*Figure 9).* Mallet® (imidacloprid) and Zylam® (dinotefuran) rates were determined based on the average height of trees per the label or 15.91 ml and 35.02 ml per tree, respectively. The amount of product prescribed was mixed in one gallon of water then applied to the potting media.

Figure 9: Treatment using foliar spray with a backpack sprayer outside the greenhouse.

Pyriproxyfen was applied again 14 days after the initial application and Orange Guard®, diluted to 1% limonene, was re-applied weekly. Each week before the re-application of these treatments, *in situ* counts were taken using the same methods previously outlined to evaluate the effects of the treatments on CMBS population numbers.

2.2.4 Ladybeetle toxicity

Due to the concern for pollinators and natural enemies with the use of imidacloprid (Krischik et al., 2015) and the effects of other horticultural oils on insects (Ibrahim et al., 2001), a test of the potential toxicity of limonene and formulation adjuvants (Hollingsworth, 2005), was conducted against the lady beetle natural enemies of CMBS. On trees with or without attractants, Ibiyemi (2020) encountered mostly adult lady beetles on crape myrtles in Auburn, Alabama. Due to Ibiyemi's (2020) work with attractants, we know adult lady beetles can be recruited to an infested tree, increasing the likelihood they may be directly sprayed with limonene.

Adult *Hippodamia convergens* (convergent lady beetle) were sourced from Tip Top Bio Control (White City, OR). Once received by mail, they were stored in a 34.6 x 21 x 12.4 (5.7 L) plastic container with a mesh top in a growth chamber set to 20 ± 1 °C with a 16:8 (L:D) photoperiod. They were supplied with water through both a cotton wick and misted daily with water. The lady beetles were supplied with CMBS on fresh crape myrtle cuttings from infested trees. Before each experiment, beetles were cooled in the refrigerator for an average of 10 min to reduce their activity.

Limonene as undiluted essential oil was obtained from a commercial source (Florida Chemical Company, LLC, Winter Haven, FL) and used to prepare solutions of

0.5%, 1.0%, and 6.0% limonene, also denoted as low, normal, and high rates. Two adjuvants used by Hollingsworth (2005), 0.75% APSA-80 (Amway), 0.1% Silwet L-77 (PhytoTech Labs, Lenexa, KS) were used in each limonene solution. The 0.75% APSA-80 and 0.1% Silwet L-77 used for a 1% (normal) limonene solution were multiplied by 0.5 and 6 for the 0.5% (low) and 6% (high) limonene rates. All limonene concentrations were tested with each adjuvant at each rate. Orange Guard® is a commercially available formulation of limonene but the components that comprise the 94.2% of the formulation are not disclosed. In this experiment, Orange Guard® diluted to a 0.5% and 1% limonene solution were also used for comparison. A water control was also used (*Table 2).* Each treatment was prepared as a 25 ml solution within 10 min of use in experiments.

A preliminary experiment was conducted to assess the mortality of adult *H. convergens* when in contact with limonene residue. Twenty petri dishes were treated with 1 ml of either the 1% limonene formulation (10 dishes) or the 1% limonene diluted Orange Guard® (ten dishes). Immediately after treatment, ten adult lady beetles were each placed in five formulation petri dishes and five Orange Guard® petri dishes. The remaining ten petri dishes were allowed to dry (approximately 15 min) before a lady beetle was placed in each dish. Beetles were observed for 48 hours.

For the topical application experiment, five active lady beetles were placed in a 1 oz (29.6 ml) plastic diet cup with a small hole drilled in the lid. Each treatment was applied as 100 µl in an airbrush sprayer directly to the beetles in the cup. This application rate did not allow for ponding or puddling of the treatment in the cup.

Table 2: Treatments, Concentrations, and Combinations used in the topical bioassay with adult lady beetles. (% by volume)

0% Limonene	0.5% Limonene (Low)	1% Limonene (Normal)	6% Limonene (High)
Water	0.5% Limonene 0.375% APSA-80 0.05% Silwet	1% Limonene 0.75%APSA-80 0.1% Silwet	6% Limonene 4.5% APSA-80 0.6% Silwet
n/a	0.5% Limonene	1% Limonene	6% Limonene
n/a	0.375% APSA-80	0.75% APSA-80	4.5% APSA-80
n/a	0.05% Silwet	0.1% Silwet	0.6% Silwet
n/a	0.5% Limonene 0.375% APSA-80	1% Limonene 0.75% APSA-80	6% Limonene 4.5% APSA-80
n/a	0.5% Limonene 0.05% Silwet	1% Limonene 0.1% Silwet	6% Limonene 0.6% Silwet
n/a	0.375% APSA-80 0.05% Silwet	0.75% APSA-80 0.1% Silwet	4.5% APSA-80 0.6% Silwet
n/a	8% Orange Guard	17% Orange Guard	100% Orange Guard

Each cell represents a different treatment or treatment combination with varying amounts of limonene.

Lady beetle mortality was recorded at 1, 5, 10, 30, 60, and 120 min after application and again at 24 hrs. This experiment was then repeated, with a total of ten beetles treated with each individual treatment.

2.2.5 Statistical Analyses

All data was analyzed and graphed using R statistical software (R Core Team, 2023). With all data, an ANOVA test was run with a Tukey’s post-hoc test. A generalized linear mixed model was then used to analyze both CMBS populations and lady beetle populations on the potted trees. Week and tree were each considered random variables to account for variability over time and location. Both sets of CMBS data were fitted to a negative binomial distribution to account for overdispersion in the data with time and tree considered as random factors. Lady beetle mortality data was fitted to a binomial distribution, which was then used to calculate LC_{50} values. That data was then used to produce a Kaplan-Meier survivorship curve and a Cox proportional-hazards model (Cox, 1972) to analyze differences between the treatments and concentrations. Both concentration effect and time until death were analyzed. Non-statistical significance was considered when $P > 0.05$. “Near significance” was noted at $P > 0.1$ for the consideration of biological significance.

2.3 RESULTS

2.3.1 Limonene application frequency

There were significant treatment effects (ANOVA, $P < 0.001$) on CMBS populations indicating the importance of application frequency of limonene during this 5 wk experiment. During this experiment, a crawler hatch occurred which increased counts on control trees over time to >1000 per sample at the last collection. Control trees had 5.5 times (1.68 – 18.09; 95% CL) more CMBS than trees with weekly limonene applications ($P = 0.0049$, *Figure 10*). Trees treated after rain had 3.6 times (1.09 – 11.93; 95% CL) fewer CMBS than control trees ($P = 0.035$). Trees treated weekly were not significantly different from those treated after rain ($P = 0.487$), and trees treated biweekly had similar numbers of CMBS as control trees ($P = 0.19$). Results of the GLMM based on comparisons to the control group are provided in *Table 3*. There was no significant treatment effect on lady beetle populations. However, lady beetle populations remained consistently low throughout the experiment which may be the reason for insignificant results.

Table 3: Generalized linear mixed model results for CMBS population data in treatment frequency experiment.

Treatments	**Estimate**	**Std. Error**	**z value**	***P* value**
Control(Intercept)	6.116	0.437	13.996	<2e-16 *
Biweekly	-0.794	0.606	-1.310	0.190
After Rain	-1.284	0.610	-2.106	0.0352 *
Weekly	-1.707	0.607	-2.814	0.0049 *

Statistical significance is denoted by an asterisk (*) in the P value column.

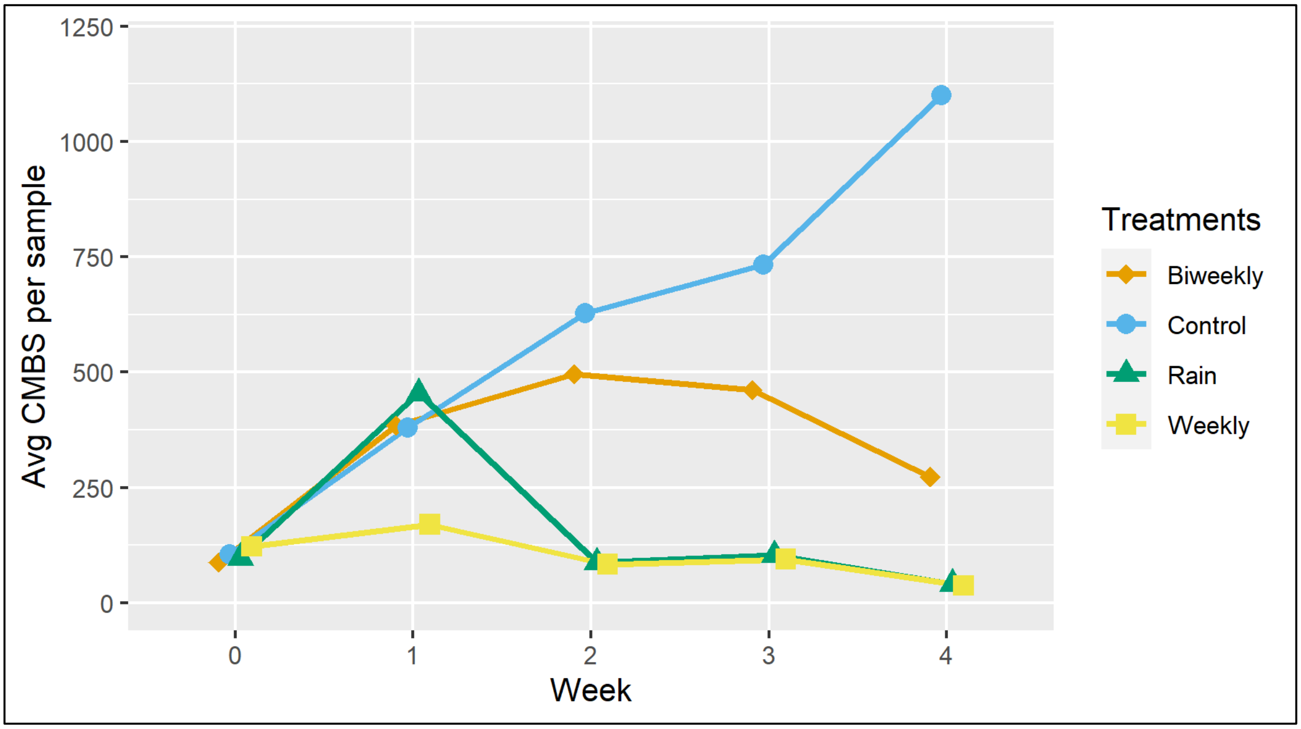

Figure 10: Average population of CMBS on trees treated with limonene (Orange Guard®) at different application frequencies.

2.3.2 Comparison of limonene to standard insecticides

In the 2-month greenhouse experiment comparing limonene to the currently recommended insecticides for CMBS, there were significant differences among treatments (ANOVA, $P < 0.001$). Untreated trees in this experiment averaged approximately 356 CMBS per sample at each time point (*Figure 11*). Trees that received weekly applications of limonene had 81% less CMBS per sample than the untreated trees ($P = 0.043$), and untreated trees had 5.3 times (1.05 – 26.08; 95% CL) more CMBS than limonene treated trees. Only limonene treated trees were significantly different from untreated trees. Populations declined on imidacloprid treated trees but were not significantly different from the untreated trees ($P = 0.760$). Imidacloprid trees also had 4.1 times (0.82 – 20.38; 95% CL) more CMBS than limonene treated trees; however, these results were not statistically significant ($P = 0.0867$, *Table 4*). When all treatments are compared to limonene, only untreated was significantly different ($P = 0.043$) with imidacloprid being “nearly significant” ($P = 0.086$, *Table 5)* showing potential biological significance.

Table 4: Generalized linear mixed model results using untreated as reference for CMBS populations in greenhouse experiment.

Treatments	Estimate	Std. Error	z value	P value
Untreated(Intercept)	5.540	0.589	9.404	<2e-16 *
Dinotefuran	-0.799	0.819	-0.975	0.330
Imidacloprid	-0.255	0.819	-0.311	0.760
Limonene	-1.661	0.821	-2.023	0.043 *
Pyriproxyfen	-0.836	0.820	-1.020	0.308
Water	-0.479	0.819	-0.584	0.559

Statistical significance is denoted by an asterisk (*).

Table 5: Generalized linear mixed model results using limonene as reference for CMBS populations in greenhouse experiment.

Treatments	Estimate	Std. Error	z value	P value
Limonene(Intercept)	3.880	0.590	6.573	4.93e-11 *
Untreated	1.661	0.820	2.023	0.043 *
Dinotefuran	0.862	0.820	1.051	0.293
Imidacloprid	1.406	0.820	1.715	0.086 **
Pyriproxyfen	0.824	0.820	1.006	0.315
Water	1.182	0.820	1.441	0.150

Statistical significance is denoted by an asterisk (*) P<0.1 is denoted by a two asterisks (**)

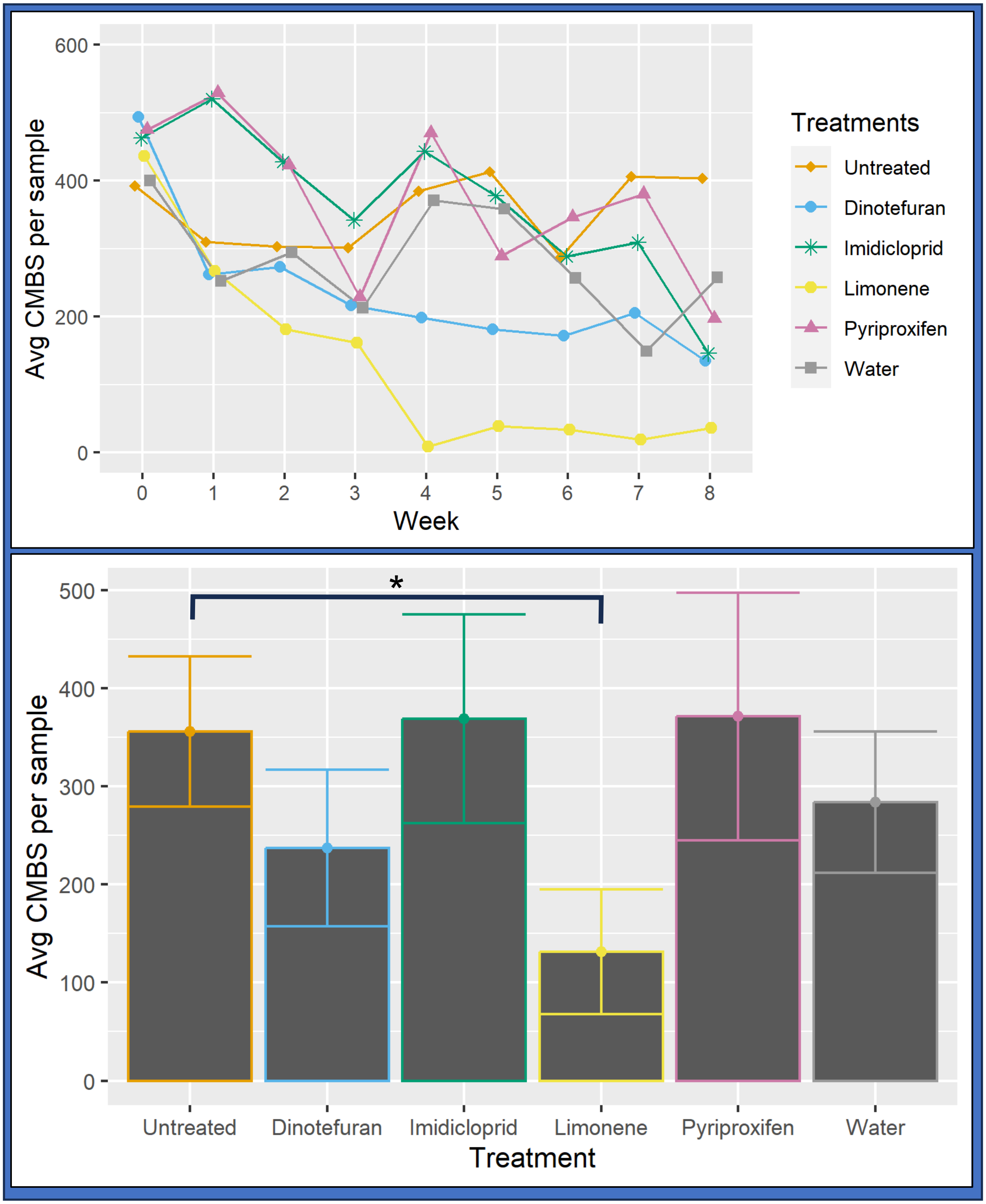

Figure 11: CMBS population by treatment over time (top) and overall treatment effect (bottom) Significance from untreated control ($P < 0.05$) is designated by an asterisk (*)

2.3.3 Ladybeetle Toxicity

There was low to no mortality (0-1%) for adult *H. convergens* lady beetles that contacted fresh or dried residues of the 1% limonene mixture or 1% Orange Guard®.

Survival of lady beetles treated topically with limonene or Orange Guard® was not significantly different from those treated with water (GLMM, $P = 0.571$ and 0.193, respectively) regardless of concentration (*Figure 12*). Orange Guard® and limonene also were not significantly different from each other ($P = 0.360$). Every other mixture including an adjuvant caused mortality greater than water. APSA and any formulation including APSA had higher mortality than the others. While only Orange Guard® and water were indifferent when using limonene as the reference, when Orange Guard® became the reference, Silwet alone was also insignificant from Orange Guard® ($P = 0.209$). Limonene applied alone had an LC_{50} value of 1.41% limonene and the Orange Guard® had an LC_{50} value of 1.16% limonene.

The Kaplan-Meier plots and Cox proportional-hazards model showed statistically significant effects of both treatment and concentration on time until death (both $P <$ 2.2e-16, *Table 6, Figure 13*). Limonene was the closest treatment to water in the Cox proportional-hazards model with a coefficient of 1.66. All treatments (excluding water) showed some toxicity toward the lady beetles. Limonene and Orange Guard® had the longest time until death across all concentration rates, with the shortest time being 60 min at the high rate of Orange Guard®.

Table 6: Cox proportional hazards model on lady beetle survival using water as a reference.

Treatments	**Coef**	**Std. Error**	**z value**	**P value**
APSA	3.325	0.582	6.055	1.40e-09 *
APSA+Limonene	3.500	0.582	6.010	1.86e-09 *
APSA+Silwet	3.526	0.582	6.055	1.40e-09 *
Limonene	1.666	0.598	2.788	0.005 *
Orange Guard®	2.356	0.588	4.005	6.21e-05 *
Silwet+Limonene+APSA	3.536	0.582	6.073	1.25e-09 *
Silwet	2.981	0.585	5.096	3.47e-07 *
Silwet+Limonene	3.182	0.583	5.455	4.91e-08 *

Statistical significance from the water control **is denoted by an asterisk (*).**

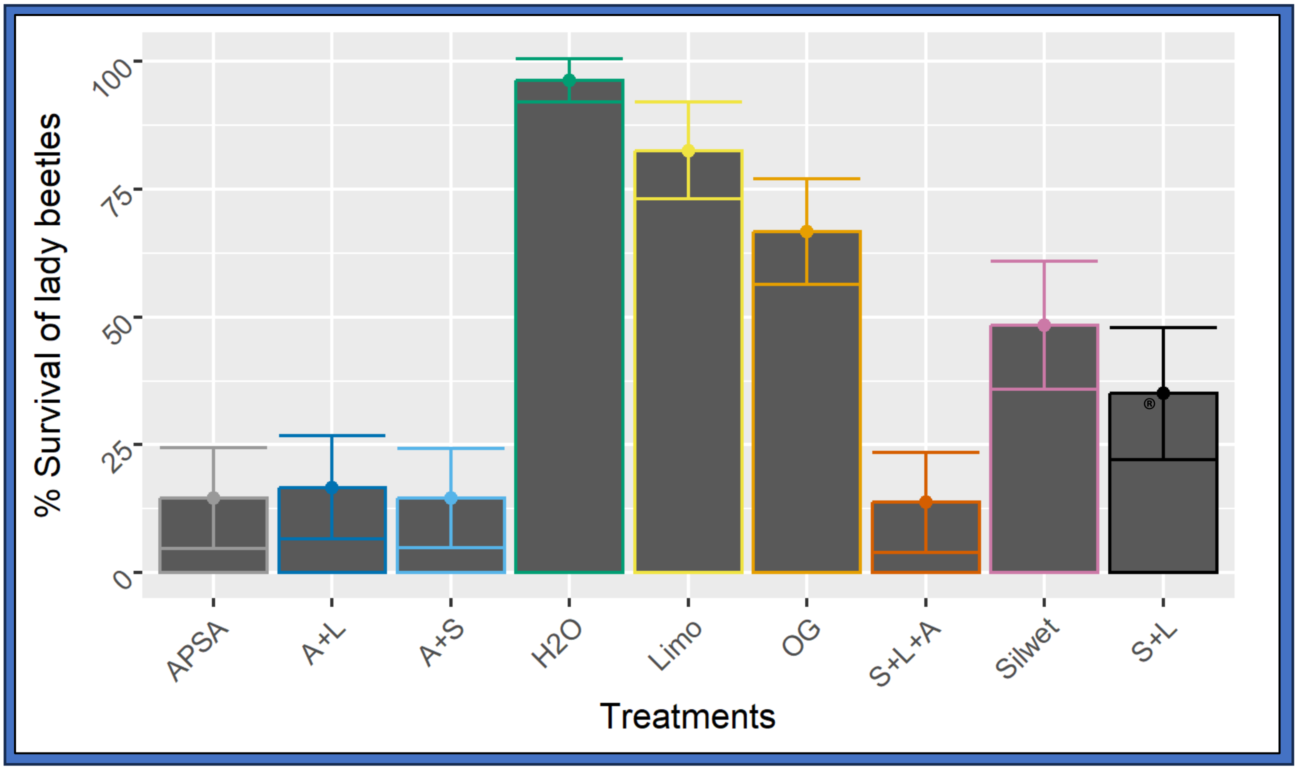

Figure 12: Overall treatment effect on lady beetle percent survival over 24 hours after treatment including confidence limits.

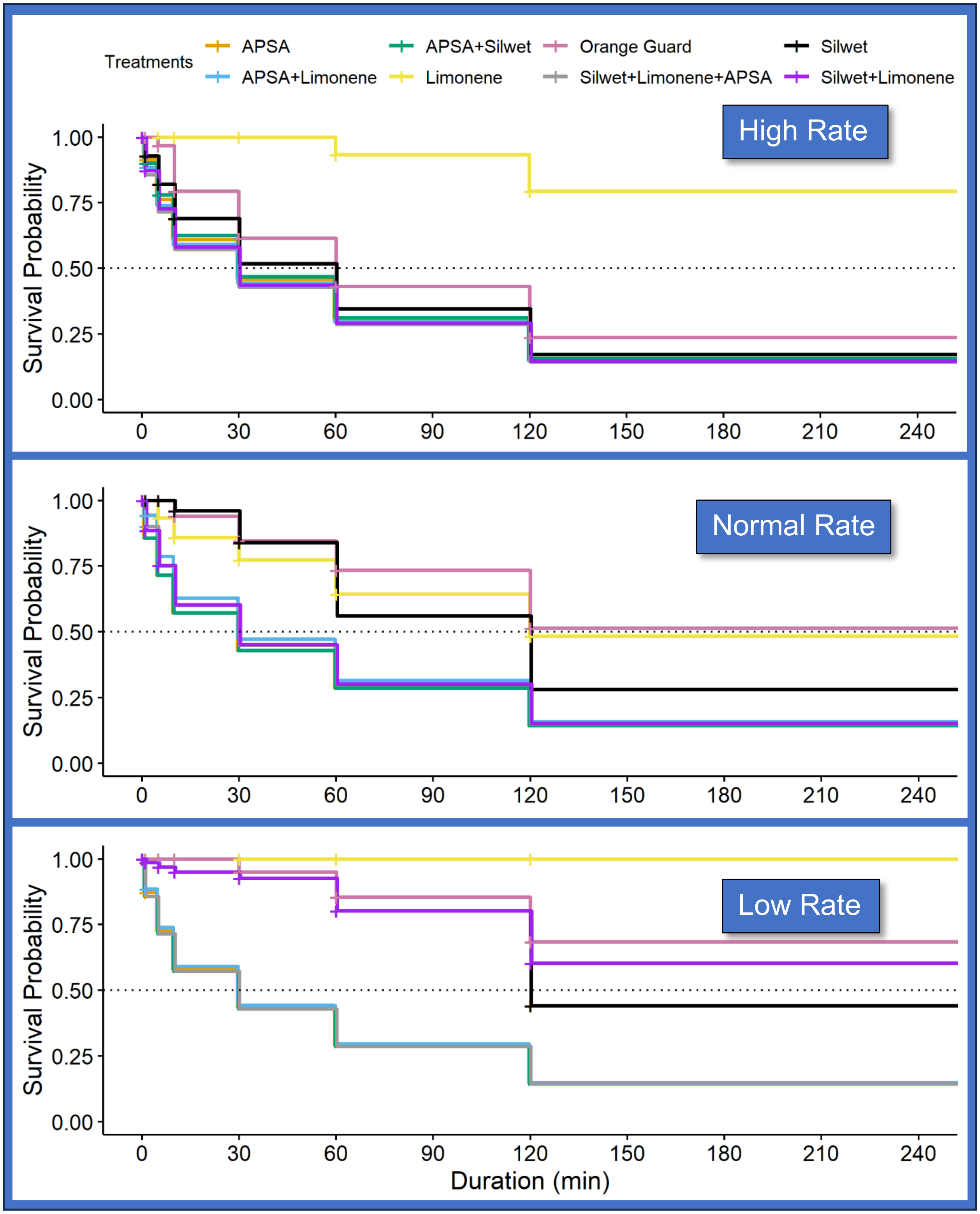

Figure 13: Kaplan-Meier survival curves (survival probability over time) from lady beetle toxicity assays, separated by concentration with 50% survival denoted with a dotted line. Rate were 0.5%, 1%, and 6% for low, normal and high respectively. 24 hrs is not shown for higher clarity of early event activity.

2.4 DISCUSSION

Since being introduced to North America, the management of CMBS has been dependent on insecticides, primarily systemic neonicotinoids. While being effective against the pest, the insecticides can negatively impact non-target, beneficial insects such as honeybees (Thurmond, 2019) or natural enemies that are present on crape myrtle trees (Ibiyemi, 2020; Gilder et al., 2021). In this study, we present evidence that limonene, an essential oil can be used to manage CMBS with reduced risk to lady beetles, one of the common predators of CMBS.

This study shows massive insight to the potential of limonene as a biorational alternative to systemic neonicotinoids for the management of CMBS. Surprisingly, the most commonly recommended neonicotinoid for CMBS control, imidacloprid, was not significantly different from the control population. The IGR pyriproxyfen did not significantly manage the CMBS. The other neonicotinoid tested, dinotefuran, did generally work better than imidacloprid but was statistically similar. Not only do we know these neonicotinoids are harmful for pollinators and natural enemies (Quesada et al., 2020; Calvo-Agudo et al., 2019), they also did not provide sufficient control of the pests in our greenhouse experiment.

The limonene application frequency experiment was the first evaluation of the efficacy against CMBS in the field and allowed us to develop an effective treatment plan for control of CMBS. Although both after-rain treatments and weekly treatments equally controlled the CMBS populations, more treatments and consequently more product was needed suggesting that weekly treatments were more efficient. Control populations greatly increased in both potted tree experiments, with the treatment frequency control group soaring to over 900% the initial population. CMBS is not a pest that can be ignored. It requires treatment at the first signs of infestation as problems quickly become noticeable as the populations grow.

While the lady beetle population data collected from the outdoor potted trees was scarce, we were able to identify several key species in Auburn, Alabama feeding on CMBS, namely *Hyperaspis bigeminata* and *Harmonia axyridis. Hyperaspis bigeminata* is native to the United States, and several species of the genus are scale specialist (Wang et al., 2016). Further research into the potential of *H. bigeminata* for biological control may prove useful. We also noted that most lady beetles collected from the infested trees were adults.

Due to this finding, we chose adults for our lady beetle toxicity experiments. Adult lady beetles are the most likely to be affected flying into and out of a treated crape myrtle. Also, since adult lady beetles are known to abscond, they have the potential to leave the treated area when disturbed by sprays; thus, it was imperative to the acute toxicity of the chemicals. Due to inaccessibility, immature stages were not used and need further research; however, results from the adult toxicity study were promising. Although the LC_{50} is lower than any easily accessible formulated product like Orange Guard®, we used only a 1% solution in all experiments. Orange Guard® diluted to 1% limonene is lower than the LC_{50} calculated and may be regarded as safe for the adult lady beetles. Also as noted, the LC_{50} of limonene was higher than the 1% formulation used in the potted plant experiments, and the 1% solution offered high control of CMBS.

Limonene also had a longer time until death regardless of concentration, suggesting that toxicity in the field may be less with their ability to escape treatment areas.

This experiment raised concerns that the adjuvants used were indeed toxic to the adult lady beetles both on their own and in combination when sprayed directly on the beetles. While limonene alone may not be harmful to lady beetles (*Figure 12)*, the other components were. APSA showed significant toxicity to lady beetles and cause mortality quickly. Fortunately, no toxicity was recorded from contact with a treated surface, limiting its toxicity to when actively sprayed. Limonene formulations are extremely important because of the compound's volatility and insolubility (Choi and McClements, 2020). Further investigation into safer adjuvants and emulsifiers should be completed to increase the IPM-compatibility of limonene.

Overall, limonene is effective for the control of crape myrtle bark scale when applied weekly and has the potential to replace systemic neonicotinoids in the control of CMBS. CMBS can very quickly colonize a tree or nursery, but the current control methods are not sustainable and are harming beneficial insects. Limonene can help mitigate those ecological issues and is a safer alternative for pollinators and other beneficials. The research toward and adoption of more IPM-friendly methods of control is critical to the success of the current ornamental industry. Further research on the use of limonene at landscape scale may provide more insight into its efficacy, natural enemy interactions, and potential in IPM.

CHAPTER 3: CONSERVATION BIOLOGICAL CONTROL IN THE LANDSCAPE

3.1 INTRODUCTION

Crape myrtles (*Lagerstroemia* spp.) are the dominant tree in urban landscapes in the Southeast. Their canopies have a diversity of natural enemies such as spiders, lady beetles, lacewings, and predatory hemipterans (Gilder et al., 2021). Due to their versatility as a pioneer species, they are planted into urban landscapes under a variety of conditions. However, an accidental pest introduction in 2004 appears to be a growing threat to crape myrtles.

Crape myrtle bark scale (CMBS, *Acanthococcus lagerstroemiae*) is an invasive pest of crape myrtle introduced to the United States less than 20 years ago and is now pervasive across the southeastern United States (Wang et al., 2019). The highly mobile, wingless crawlers (nymphs) can cling to other organisms or be transferred through human interaction to infest new trees surprisingly quickly. The largest impact of *A. lagerstroemiae* is its ability to reduce the aesthetic value of the trees. Sooty mold on honeydew secretions can decrease photosynthesis, reducing the overall health of the plant. CMBS and subsequent reductions in photosynthesis can cause branch dieback, small sparce flowering, and stunting, and while these issues will not cause lasting severe damage on a mature tree, infestations can result in the death of young plants (Marwah et al., 2021).

Acanthococcus lagerstroemiae also exhibit highly temperature-dependent development. Depending on their location, CMBS can have two to four generations per year (Wang et al., 2016). In the southeastern United States, CMBS have two peak crawler activity periods in the spring (April-May) and fall (September-October) (Vafaie et al., 2020). While crawler populations increase during peaks, they are active all year long (Wright et al., 2023). CMBS is also highly tolerant of warm temperature and thrive in the urban city centers (Wang et al., 2019).

Because urbanization acts as a biological filter reducing the diversity of coccinelids in the community available for predation in highly urbanized areas (Grez et al., 2019), outbreaks in urban landscapes may improve with conservation or augmentative biological control. As generalist predators, lady beetles often are better at tracking prey density than controlling it. Thus, a common issue with the use of ladybeetles for classical biological control is their tendency to abscond. Coccinellids, however, have great potential for conservation biological control using species already in the system (Wang et al., 2016). Yellow is an attractive color to lady beetles (Alhmedi et al., 2010) and yellow panels in crape myrtle trees can act as a visual lure for several coccinellid species, especially native ones (Ibiyemi, 2020). If we can increase the local populations of natural enemies superficially or raise the local carrying capacity, these predators can help prevent and maintain pest populations with limited chemical applications. Using a combination of biological and chemical control can often provide even greater levels of control; however, many insecticides have disastrous effects on beneficial insects (Krischik et al., 2015).

The most common form of chemical control of CMBS is soil-drench applications of neonicotinoids such as dinotefuran and imidacloprid (Wang et al., 2016; Vafiae, 2016). However, while the mammalian toxicity is low, the translocation of these insecticides into honeydew of sap sucking insects (Quesada et al., 2020; Calvo-Agudo et al., 2019) and in crape myrtle pollen (Thurmond, 2019), both at concentrations that pose risk to beneficial insects, have resulted in exploring alternative, IPM-friendly practices for CMBS.

An interesting approach to bio-rationale management is the use of terpenes, aromatic plant volatiles found in essential oils, and herbivore-induced plant volatiles (HIPVs) as attractants, deterrents, and insecticides (Ibrahim et al., 2001). Limonene, a citrus extract, has been studied for its deterrent and insecticidal properties suggesting its use as a plant-based natural product for chemical pest control (Ibrahim et al., 2001). Limonene in its various forms has shown pesticidal activity against numerous arthropod pests like scale insects, mites (Gadelhaq et al. 2022), medically important mosquitoes (Theochari et al., 2020), and even fungi and bacteria (Azeem et al., 2022; Costa et al., 2019). Previous research (Chapter 2) has shown limonene to be 81% effective against CMBS when applied weekly, while untreated control populations were heightened to over 900%. This, however, did require weekly applications over the entire two-month experiment, compared to a single yearly treatment of soil-drench neonicotinoids. Limiting labor inputs may increase the appeal of limonene as an alternative treatment.

Many HIPVs like limonene have also been used in conservation biological control to attract predators to the plant to feed on the herbivores attacking them, creating a mutually beneficial relationship between plant and predator (Takabayashi et al., 1996). The unique quality of limonene is that it has also shown some potential as an attractant for lady beetle natural enemies of crape myrtle bark scale. Previous research has shown that limonene in aphid honeydew secretions can strongly attract *Harmonia* *axyridis* in research concerning *H. axyridis* as a secondary pest (Leroy et al., 2012). The presence of limonene and a yellow trap is attractive to *Harmonia axyridis* females and can stimulate oviposition behavior (Alhmedi et al., 2010). An insecticide, such as limonene, that targets the pest directly and indirectly through the attraction of natural enemies providing free control is an ideal application of IPM principles. Additional work by Ibiyemi (2020) evaluated the combination of the yellow panels and HIPV lures to further recruit ladybeetle natural enemies as a method to increase the biological control provided by the beetles. However, research studying the effectiveness of limonene for control of CMBS and recruitment of lady beetles in the landscape is limited.

Based on this previous research, the following objectives were developed: 1) to evaluate seasonal treatment plans for infested trees in urban landscapes and 2) to compare conservation biological control approaches (limonene, yellow panels, or both) with insecticide for seasonal impacts on CMBS in an urban landscape.

3.2 MATERIALS AND METHODS

3.2.1 Seasonal Treatments

This experiment was developed to evaluate season-long treatment plans with limonene for the management of CMBS. The treatment plans were developed to coordinate with the two peak crawler hatch periods in the spring and fall and maintained for an entire growing season. Twenty large landscape crape myrtle trees (Natchez) were selected on two sites, ten trees per site, on the Auburn University campus (Auburn, AL USA, *Figure 14*).

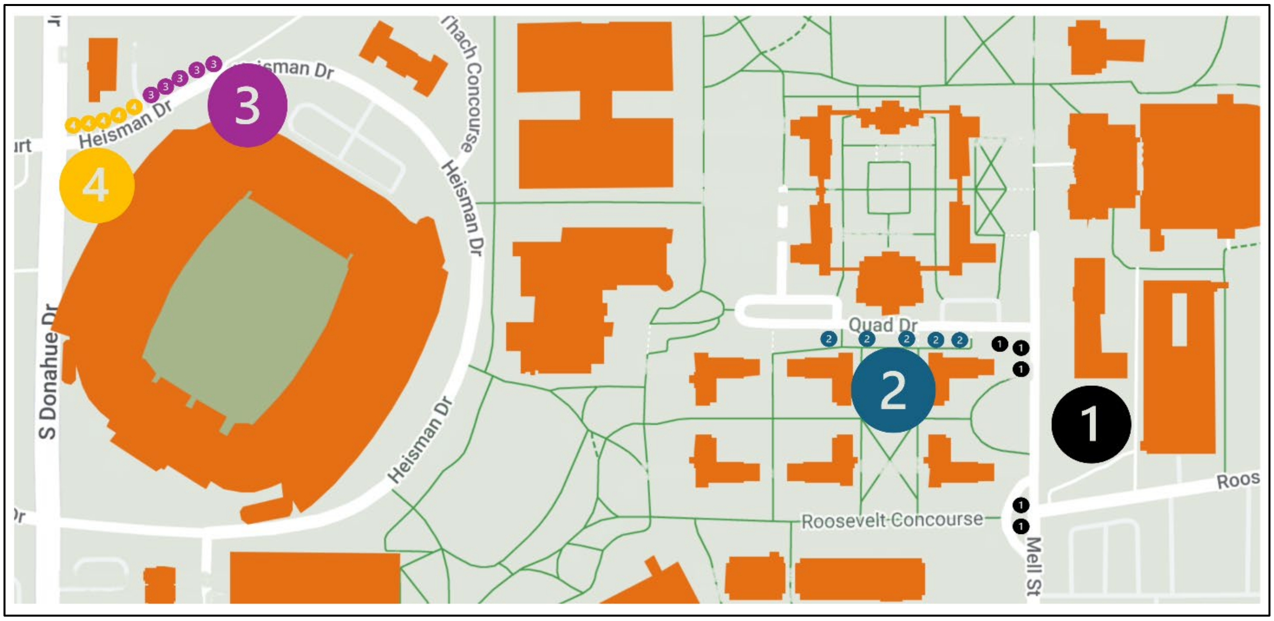

Figure 14: Map of experimental trees on Auburn University campus. Each tree is denoted by a small dot with each replicate sharing the same number and color. Large numbers are used as a signifier of the general area where those replicates are located.

Trees were located in the center of campus along streets, pedestrian walkways, or in parking lots with impervious surface on one or more sides. On each site, trees were at least 3 m apart and there was 640 m between the two groups.

The experimental design was a randomized complete block design using pre-treatment variation in CMBS numbers per tree as a blocking factor. During March 2023, counts of CMBS adults and crawlers were taken by surveying 10 cm portions of five random branches and 10 cm the trunk. Based on these counts, trees were grouped into five blocks (replicates) based on similar numbers of CMBS. Each of the five trees within a block received one of five treatments, with four replications.

The five treatments included the non-treated control and weekly applications of a 1% limonene mixture that included limonene (Florida Chemical Company, LLC, Winter Haven, FL), 0.75% APSA-80 (Amway), and 0.1% Silwet L-77 (PhytoTech Labs, Lenexa, KS). The limonene applications were made either all season (weekly all year), spring only, fall only, or spring and fall. Controls were included to evaluate the true treatment effects of the limonene on CMBS populations rather than changes that were due to other factors. All treatments were applied at 1 liter with a hand pumped backpack sprayer to the trunk and lower branches (ground level to approximately 10 ft) until runoff.

A six-week treatment plan based on the crawler hatch began with spring treatments on 19 May 2023 when high crawler activity was noted. The Spring treatments were made weekly for 6 wks. The week following the last spring treatment, double-sided sticky tape (Vafaie, 2016) was placed around three branches and the trunk, for 72 hr then collected. Crawlers on the tapes were counted under a microscope with the length of tapes measured and all three branches counted together then divided by three to attempt to standardize the tape length. *In situ* counts were performed using the same methods as pre-treatment counts at one month after the last spring treatment and monthly until fall treatments started. Fall treatments began 13 October 2023 and continued with weekly applications for 6 wks. As in spring, following last application, sticky tapes were placed on three branches for 72 hr and number of crawlers counted.

3.2.2 Chemical and conservation biological control

This experiment evaluated the combination of conservation biological control tactic (limonene applications and yellow panels) for control of crape myrtle bark scale and the recruitment of lady beetle natural enemies of CMBS. Three urban sites with large, infested landscape crape myrtle trees around were selected in Auburn, AL with each site having five trees. When the sites were selected, the trees were dormant, and the cultivars could not be confirmed by bloom. All but one tree was the cultivar Natchez. The other tree was an unknown pink-flowering cultivar that was as equally infested as the Natchez trees on the same site.

The experiment was a randomized complete block design and blocked based on location. Pre-treatment counts consisting of 10 cm portions of five random branches and the trunk were taken June 2022. Each tree in a block/site received one of five treatments randomly assigned within the block. The five treatments included an untreated control, weekly applications of a limonene solution, yellow panels alone, limonene in combination with yellow panels, and a soil application of imidacloprid. The rectangular panels were black and white corrugated tree protectors (A.M. Leonard Horticultural Tool and Supply Co., Piqua, OH) cut into rectangular sizes of 30 x 60 cm painted with two layers of yellow primer (3006-1B, Dandelion Chain, Insl-X® Aqua Lock® Plus Primer, Benjamin Moore & Co., Montvale, NJ) and three layers of yellow paint (3006-1B, Dandelion Chain, Benjamin Moore Regal Select, Exterior Soft Gloss, Benjamin Moore & Co., Montvale, NJ) based on the design of Ibiyemi (2020) (*Figure 15)*.

Three panels were hung in the lowest boughs of each tree (approximately 3 m above the ground and 3 m apart) using black cable ties (Utilitech®) (*Figure 16)*. Limonene was prepared as a 1% limonene (Florida Chemical Company, LLC, Winter Haven, FL), 0.75% APSA-80 (Amway),and 0.1% Silwet L-77 (PhytoTech Labs, Lenexa, KS) solution. The limonene solution was mixed within a few hours of application and applied using a backpack sprayer to the trunk and branches up to 10 feet at 2 L of solution per tree. Imidacloprid (Mallet® 2F T&O, 21.4% imidacloprid, Nufarm Americas Inc., Alsip, IL) was applied as a soil drench at 70 mL product per liter of water according to the label rate with 3.8 liters of solution applied to the base of each tree.

Treatments were applied 16 June 2022 and were continued through October. One month after the first treatment and each consecutive month, counts of CMBS crawlers and adults were taken using the methods from the pre-treatment counts. Lady beetle surveys were also conducted by taking beat samples of four branches of each tree with each branch receiving three beats. Lady beetles were collected in plastic sandwich bags, taken back to the lab, and placed in the freezer until identification.

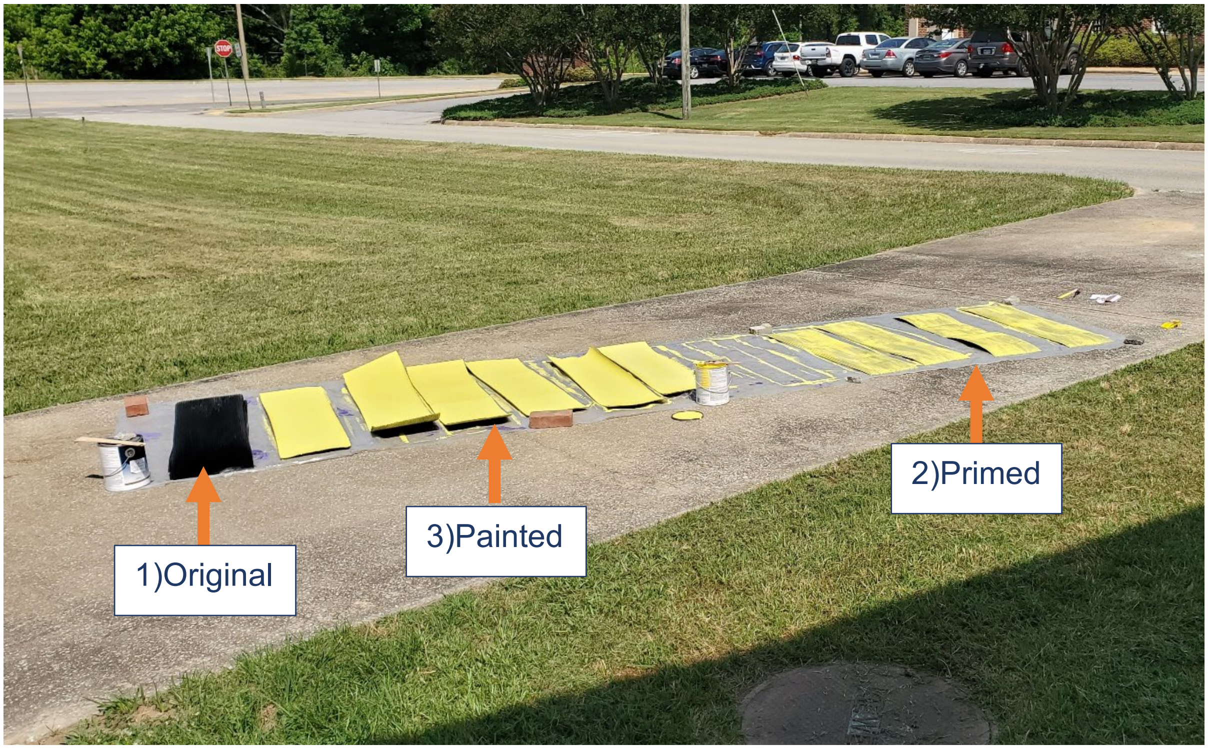

Figure 15: Yellow panels in different stages of preparation for use in the experiment.

Figure 16: Yellow panels hanging in crape myrtle trees to attract lady beetle natural enemies of CMBS. This treatment was evaluated alone or in combination with weekly applications of limonene.

3.2.3 Statistical Analyses

All data was analyzed and graphed using R statistical software (R Core Team (2023)). We used generalized linear models to analyze both CMBS and lady beetle populations. Both sets of CMBS data (tapes and *in situ* counts) were fitted to a negative binomial distribution to account for overdispersion in the data with time and tree considered random factors. Non-statistical significance was considered when $P > 0.05$. “Near significance” was noted at $P > 0.1$ for the consideration of biological significance.

3.3 RESULTS

3.3.1 Seasonal Treatments

This experiment compared different application timings of limonene across a growing season. Only weekly treatments of limonene significantly differed from the control. Control trees had 5.96 times (1.89 – 18.84; 95% CL) more CMBS than trees that received weekly applications of limonene ($P = 0.002$, *Table 7*). However, fall treatments and spring + fall treatments were noted to be “nearly significant” ($P = 0.099$ and 0.077, respectively) relative to control. While not statistically significant, this may be biologically significant with spring + fall treatments offering some control of CMBS. When compared to weekly applications of limonene, spring trees are also “nearly significant” ($P = 0.060$). There was no significant treatment effect on tape crawler counts ($P = 0.464$) with either the ANOVA or GLMM. Nevertheless, the average number of crawlers on control tapes (8.625) was higher than the averages for all other treatments (*Table 8).*

Table 7: Results from generalized linear mixed model on CMBS population data from seasonal treatment experiment.

Treatments	Estimate	Std. Error	z value	P value
Control(Intercept)	3.044	0.604	5.037	4.72e-07 *
Fall	-0.955	0.579	-1.648	0.099 **
Spring + Fall	-1.027	0.581	-1.769	0.077 **
Spring	-0.673	0.576	-1.169	0.243
Weekly	-1.785	0.587	-3.040	0.002 *

Statistical significance is denoted by an asterisk (*) in the P value column and P<0.1 is denoted by two asterisks (**).

Table 8: Crawlers captured on sticky tapes for 72 hrs 1 wk after the last application of limonene

Treatments	Mean number of crawlers
Control	8.625
Fall	3.750
Spring + Fall	3.500
Spring	5.000
Weekly	3.875

3.3.2 Chemical and conservation biological control

Limonene, limonene + panels, and imidacloprid were significantly different from the control. Control trees had 24.8 times (4.48 – 137.32; 95% CL) more CMBS than limonene treated trees (*P* < 0.001, *Table 9*) and 8.41 times (1.48 – 47.73; 95% CL) more CMBS than limonene + panel trees (*P* = 0.016, *Figure 17*). Control trees also had 11.4 times (1.95 – 66.28; 95% CL) more CMBS than imidacloprid treated trees (*P* = 0.002). There was no significant treatment effect with panels alone compared to control (*P* = 0.154). Also, there was no significant difference between limonene, limonene + panels, and imidacloprid.

Table 9: Results from generalized linear mixed model on CMBS population data from limonene and panels experiment.				
Treatments	**Estimate**	**Std. Error**	**z value**	**P value**
Control(Intercept)	5.819	0.688	8.457	< 2e-16 *
Limonene	-3.212	0.873	-3.679	< 0.001 *
Limonene+Panels	-2.129	0.886	-2.403	0.016 *
Imidacloprid	-2.432	0.899	-2.707	0.007 *
Panels	-1.200	0.842	-1.426	0.154
Statistical significance is denoted by an asterisk (*).				

When comparing treatments to limonene, there is a significant difference between limonene and panels (*P* = 0.020), but when the reference becomes imidacloprid, there is not a difference between imidacloprid and panels (*P* = 0.165). Treatments did not significantly affect lady beetle population. However, the average number of lady beetles collected per sample was 2.64, so low counts may have affected these results. It should be noted that most adults and larvae (by assumption) collected were *Hyperaspis bigeminata.*

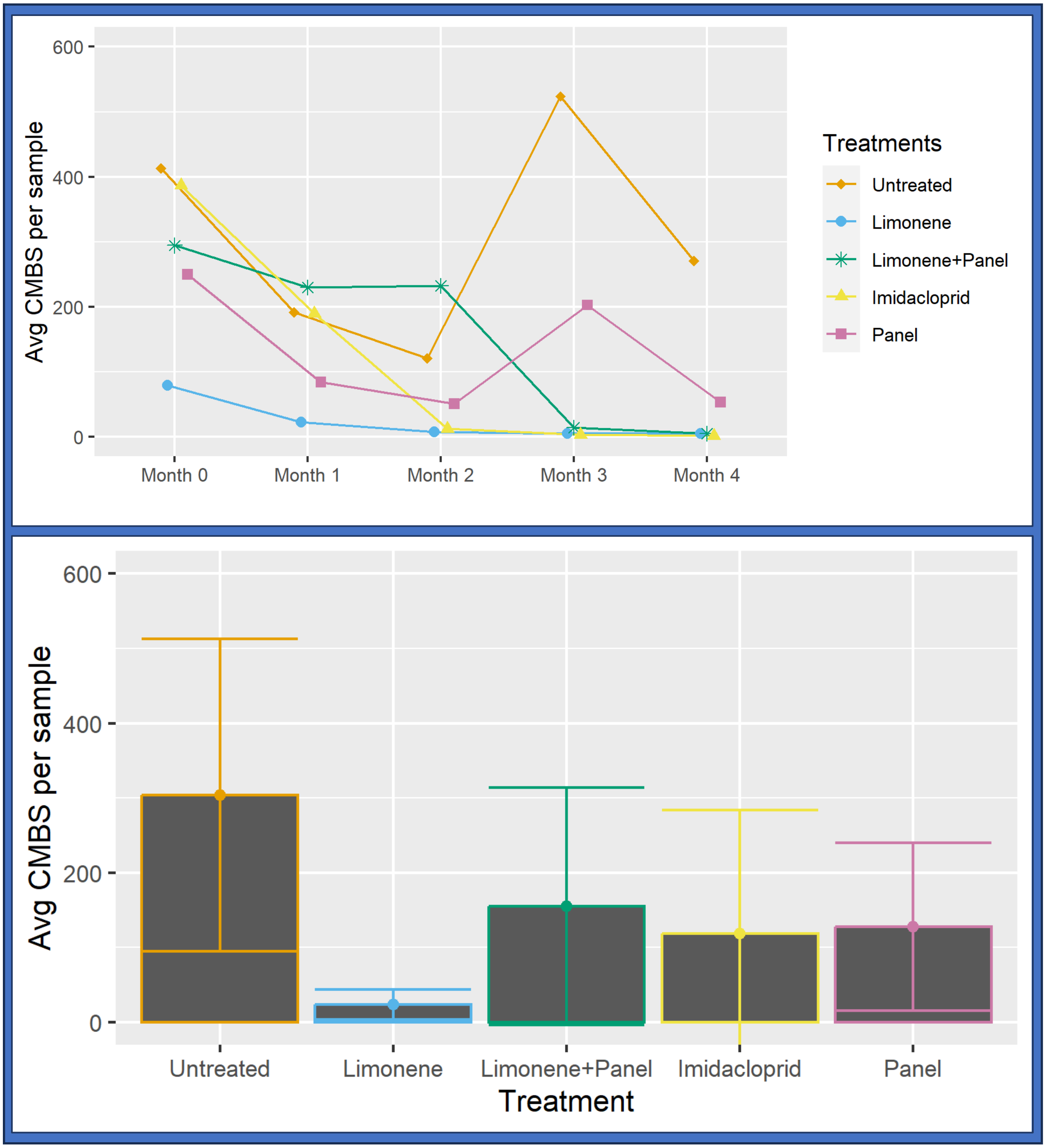

Figure 17: Average CMBS population by treatment over time (top) and overall treatment effect (bottom) in limonene and panels experiment

3.4 DISCUSSION

Since being introduced, management of CMBS has been largely dependent on the use of systemic insecticides. The translocation of active ingredients or bioactive metabolites into the pollen of crape myrtle flowers has implications for flower visiting beneficial insects (Thurmond, 2019). Using insecticides that are toxic to those natural enemies and other beneficial insects only creates further dependence on chemical control. We cannot continue to focus only on managing the pest while we create a lethal environment for other organisms. Past research has shown the utility of limonene or visual attractant for conservation biological control of CMBS, but those greenhouse or small assays had not been applied to a spatial or seasonal scale applicable for adoption by landscape managers or arborists. This was the rationale for the present study.

In Chapter 2, potted plant experiments in the greenhouse and outdoors suggested that a weekly application of a 1% limonene solution could significantly reduce CMBS, and those reductions were equal to or better than reductions achieved by most conventional insecticides used for CMBS control. When these tactics were applied to infested landscape trees over the course of a growing season, only weekly treatments of 1% limonene were effective at controlling crape myrtle bark scale at levels comparable to soil applied imidacloprid. In the seasonal treatment experiment, fall, spring and fall, and weekly crawler tape averages were lower than control or spring only tapes (*Table 7)*. This could be due to short-term crawler decreases following treatment, but the evidence was inconclusive. Further research is required in order to develop a limited labor treatment plan. While weekly applications require more labor and costs for landscape service, it is possible for homeowners or gardeners with only a few crape myrtles to apply these results to management CMBS without systemic insecticides.

Previous work suggested that yellow panels in the canopy of infested trees could reduce CMBS populations on infested landscape trees over 2 months (Ibiyemi, 2020). Panels are a less labor-intensive approach to CMBS management. If effective, they could be deployed once for the recruitment of lady beetles during the entire growing season. In the present study, the application of panels alone to infested trees was not effective to reduce the seasonal long abundance of CMBS in the landscape. The reduction in CMBS with the treatment panels and limonene is attributed mainly to limonene. The success of panels is dependent on the recruitment of lady beetles. Ibieymi (2020) reported conflicting results in their experiment with yellow panels. For example, panels in the canopy had the lowest lady beetle counts relative to placement of yellow attractants on the trunk. However, trees with panels in the canopy had significantly lower counts of CMBS. Urbanization can filter species or reduce the abundance of lady beetles (Grez et al., 2019). Using non-baited yellow sticky cards, Grez et al. (2019) surveyed lady beetles in urban greenspaces that varied in levels of urbanization (impervious surfaces and built infrastructure). Lady beetle abundance and richness especially native species were negatively correlated with the proportion of urbanization in the landscape (Grez et al., 2019). In the present study, there were fewer lady beetles to be recruited to trees (evident in counts) and this was likely due to the high level of urbanization on campus around those trees. The effectiveness of panels may therefore only be an effective tactic for infested crape myrtles in suburban or agricultural landscapes (production nurseries) where habitat crop provides a reservoir for lady beetles. A future study along an urban-rural gradient could confirm if an interaction exists between the effectiveness of canopy panels and level of urbanization on CMBS predation and lady beetle recruitment.

In conclusion, the results with limonene applications on landscape trees coupled with the results from Chapter 2 provide strong support for limonene as an effective, conservation biological control tactic for CMBS. Based on our literature review, this is the first biological control approach available for CMBS and one that can reduce our dependence on insecticide inputs for CMBS, especially systemic insecticides. These findings may prove useful when moving toward a more environmentally inclusive approach in the management of landscape ornamentals.

REFERENCES

Alhmedi, A., Haubruge, E., & Francis, F. (2010). Identification of limonene as a potential kairomone of the harlequin ladybird *Harmonia axyridis* (Coleoptera: Coccinellidae). *Eur. J. Entomol.*, *107*(4), 541-548. doi: 10.14411/eje.2010.062

Azeem, M., Zaman, T., Abbasi, A. M., Abid, M., Mozūratis, R., Alwahibi, M. S., & Elshikh, M. S. (2022). Pesticidal potential of some wild plant essential oils against grain pests *Tribolium castaneum* (Herbst, 1797) and *Aspergillus flavus* (link, 1809). *Arabian Journal of Chemistry*, *15*(1), 103482. https://doi.org/10.1016/j.arabjc.2021.103482

Calvo-Agudo, M., González-Cabrera, J., Picó, Y., Calatayud-Vernich, P., Urbaneja, A., Dicke, M., & Tena, A. (2019). Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects. *Proceedings of the National Academy of Sciences*, 116(34), 16817-16822.

Chappell, M., Braman, S., Williams-Woodward, J., & Knox, G. (2012). Optimizing plant health and pest management of *Lagerstroemia* spp. in commercial production and landscape situations in the southeastern United States: A review. *Journal of Environmental Horticulture, 30*, 161-172.

Choi S.J. & McClements D.J. (2020). Nanoemulsions as delivery systems for lipophilic nutraceuticals: strategies for improving their formulation, stability, functionality and bioavailability. *Food Sci Biotechnol*, 29(2), 149–168. https://doi.org/10.1007/s10068-019-00731-4

Chong, J. (2022). Managing crapemyrtle bark scale in nurseries. GrowerTalks. https://www.growertalks.com/Article/?articleid=25873

Cornish, A. (2021). Seasonality, distribution, and biological control of crapemyrtle bark scale, A new invasive threat in Tennessee. Master’s Thesis, University of Tennessee, Knoxville. 2021.

Costa, M. D., Rocha, J. E., Campina, F. F., Silva, A. R. P., Da Cruz, R. P., Pereira, R. L. S., Quintans-Júnior, L. J., De Menezes, I. R. A., De S. Araújo, A. A., De Freitas,

T. S., Teixeira, A. M. R., & Coutinho, H. D. M. (2019). Comparative analysis of the antibacterial and drug-modulatory effect of D-limonene alone and complexed with β-cyclodextrin. *European Journal of Pharmaceutical Sciences*, 128, 158–161. https://doi.org/10.1016/j.ejps.2018.11.036

Cox, D.R. (1972). Regression Models and Life-Tables. *Journal of the Royal Statistical Society Series B-Methodological*, 34, 187-220.

Gadelhaq, S.M., Aboelhadid, S.M., Abdel-Baki, A.-A.S., Hassan, K.M., Arafa, W.M., Ibrahium, S.M. et al. (2022). D-limonene nanoemulsion: lousicidal activity, stability, and effect on the cuticle of *Columbicola columbae*. *Medical and Veterinary Entomology*, 37(1), 63–75. https://doi.org/10.1111/mve.12607

Gilder, K., Gu, M., Merchant, M., & Heinz, K. (2021). Inventory and food web of arthropod fauna associated with *Lagerstroemia* spp. in Texas. Southwestern Entomologist, 46(2), 413-438. doi: 10.3958/059.046.0212

Grez, A. A., Zaviezo, T., Gardiner, M. M., & Alaniz, A. J. (2019). Urbanization filters coccinellids composition and functional trait distributions in greenspaces across Greater Santiago, Chile. *Urban Forestry & Urban Greening*, 38, 337–345. https://doi.org/10.1016/j.ufug.2019.01.002

Hollingsworth, R. (2005). Limonene, a citrus extract, for control of mealybugs and scale insects. *Journal of Economic Entomology*, *98*(3), 772-779. doi: 10.1603/0022-0493-98.3.772

Hulot, J.F. and Hiller, N. (2021). Exploring the benefits of biocontrol for sustainable agriculture – A literature review on biocontrol in light of the European Green Deal', Institute for European Environmental Policy. https://ieep.uk/publications/exploring-the-benefits-of-biocontrol-for-sustainable-agriculture/

Ibiyemi, O.D. (2020). Recruiting lady beetles using olfactory and visual cues for the biocontrol of *Acanthococcus lagerstroemiae* (Hemiptera: Eriococcidae). Masters Thesis, Auburn University. 2020.

Ibrahim, M., Kainulainen, P., & Aflatuni, A. (2001). Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: With special reference to limonene and its suitability for control of insect pests. *Agricultural and Food Science*, 10(3), 243-259. doi:10.23986/afsci.5697

Krischik, V., Rogers, M., Gupta, G., & Varshney, A. (2015). Soil-applied imidacloprid translocates to ornamental flowers and reduces survival of adult *Coleomegilla maculata, Harmonia axyridis*, and *Hippodamia convergens* lady beetles, and larval *Danaus plexippus* and *Vanessa cardui* butterflies. *PLOS ONE*, 10(3), e0119133. doi: 10.1371/journal.pone.0119133

Leroy, P.D., Heuskin, S., Sabri, A., Verheggen, F.J., Farmakidis, J., Lognay, G., Thonart, P., Wathelet, J.-P., Brostaux, Y. and Haubruge, E. (2012). Honeydew volatile emission acts as a kairomonal message for the Asian lady beetle *Harmonia axyridis* (Coleoptera: Coccinellidae). *Insect Science*, 19: 498-506. https://doi.org/10.1111/j.1744-7917.2011.01467.x

Liu, T., & Stansly, P.A. (2004). Lethal and sublethal effects of two insect growth regulators on adult Delphastus catalinae (Coleoptera: Coccinellidae), a predator of whiteflies (Homoptera: Aleyrodidae). *Biological Control, 30*, 298-305.

Marwah, P., Zhang, Y. Y., & Gu, M. (2021). Investigating producers' preferences for crapemyrtle and their perceptions regarding crapemyrtle bark scale. *Horticulturae*, 7(6), 146.

National Center for Biotechnology Information (2023). PubChem compound summary for CID 22311, Limonene, (+/-)-. Retrieved September 29, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Limonene.

Park, J. D., Kim, Y. H., Kim, S. S., Park, I. S., & Kim, K. C. (1993). Seasonal occurrence, host preference and hatching behavior of *Eriococcus lagerstroemiae*. *Korean J. Appl. Entomol*, 32, 83-89.

Quesada, C.R., Scharf, M.E., & Sadof, C.S. (2020). Excretion of non-metabolized insecticides in honeydew of striped pine scale. *Chemosphere, 249*, 126167.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Skvarla, M.J., & Schneider, S.A. (2022). First Record of Crapemyrtle Bark Scale (Hemiptera: Eriococcidae: Acanthococcus lagerstroemiae) from Pennsylvania. *Proceedings of the Entomological Society of Washington, 123*, 862 - 868.

Suh, S.-J. (2019). Notes on some parasitoids (Hymenoptera: Chalcidoidea) associated with *Acanthococcus lagerstroemiae* (Kuwana) (Hemiptera: Eriococcidae) in the Republic of Korea*. Insecta Mundi*, 690, 1–5. https://doi.org/10.5281/zenodo.3670468

Takabayashi, J., & Dicke, M. (1996). Plant—carnivore mutualism through herbivore-induced carnivore attractants. *Trends In Plant Science*, *1*(4), 109-113. doi: 10.1016/s1360-1385(96)90004-7

Theochari I, Giatropoulos A, Papadimitriou V, Karras V, Balatsos G, Papachristos D, Michaelakis A. (2020). Physicochemical characteristics of four limonene-based nanoemulsions and their larvicidal properties against two mosquito species, *Aedes albopictus* and *Culex pipiens molestus*. *Insects*; 11(11):740. https://doi.org/10.3390/insects11110740

Thurmond, A. (2019). Defining and mitigating the impacts of *Acanthococcus lagerstroemiae* (Hemiptera: Eriococcidae) management on pollinators. Masters Thesis, Auburn University. 2019

Vafaie, E., Merchant, M., Xiaoya, C., Hopkins, J. D., Robbins, J. A., Chen, Y., & Gu, M. (2020). Seasonal population patterns of a new scale pest, *Acanthococcus lagerstroemiae* Kuwana (Hemiptera: Sternorrhynca: Eriococcidae), of crapemyrtles in Texas, Louisiana, and Arkansas. *Journal of Environmental Horticulture*, 38(1), 8–14. https://doi.org/10.24266/0738-2898-38.1.8

Vafiae, E., (2016). Bark and systemic insecticidal control of *Acanthococcus (=Eriococcus) lagerstroemiae* (Crapemyrtle Bark Scale) on landscape crapemyrtles, 2016. *Arthropod Management Tests*, Volume 42, Issue 1, January 2017, https://doi.org/10.1093/amt/tsx130

Wang, Z., Chen, Y., & Diaz, R. (2019). Temperature-dependent development and host range of crapemyrtle bark scale, *Acanthococcus lagerstroemiae* (Kuwana) (Hemiptera: Eriococcidae). *Florida Entomologist*, *102*(1), 181. doi: 10.1653/024.102.0129

Wang, Z., Chen, Y., Gu, M., Vafaie, E., Merchant, M., & Diaz, R. (2016). Crapemyrtle Bark Scale: A New Threat for Crapemyrtles, a Popular Landscape Plant in the U.S. *Insects*, *7*(4), 78. https://doi.org/10.3390/insects7040078

Wright, E. R., Chase, K. D., Littlejohn, C., Stiller, A., & Ward, S. F. (2023). Winter activity for Crapemyrtle bark scale, an urban landscape pest. *HortScience*, 58(10), 1237–1241. https://doi.org/10.21273/hortsci17341-23

Wu, B., Xie, R., Knox, G. W., Qin, H., & Gu, M. (2020). Host suitability for crapemyrtle bark scale (*Acanthococcus lagerstroemiae*) differed significantly among crapemyrtle species. *Insects,* 12(1), 6. https://doi.org/10.3390/insects12010006

Xie, R., Wu, B., Gu, M., & Qin, H. (2022). Life table construction for crapemyrtle bark scale (*Acanthococcus lagerstroemiae*): the effect of different plant nutrient conditions on insect performance. *Scientific Reports*, *12*(1), 11472.