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Breakthroughs in Honey Bee Health: Continuous-Release Mist Diffusion of Thymol-Based Essential Oils

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Abstract

Honey bee (*Apis mellifera*) pollination is responsible for approximately 80% of all cultivated crops. Unfortunately, reports suggest losses of 30-50% of all bee colonies in the US. The greatest single contributor to the decline of bee health is the *Varroa* mite. Synthetic chemicals are currently used to control *Varroa*, but the mites are developing methods of resistance. Essential oils (EOs) may be a viable alternative. EOs are cheaper, environmentally-friendly, and pose fewer health risks to bees and consumers. Furthermore, *Varroa* have not yet developed resistance to EOs. EOs' shortcoming is the limitation of exposure. Humidity and temperature affect the rate of evaporation and the mites' exposure to the EOs. Thymol-based essential oils dispersed via a battery-operated mist diffuser would provide effective miticide efficacy without causing harm to honey bees due to the natural miticide properties of the EOs and the ability of the mist diffuser to maintain a constant temperature and humidity. Utilizing a gel capsule system to rear larvae/pupae/*Varroa*, thymol-based EOs were found to be effective against *Varroa* without harming bees. Miticide activity was as follows: thyme>oregano>rosemary>spearmint> control (vegetable glycerin). Currently, all commercially available thymol-centered systems are gel-based and work by direct contact with the mite. The use of mist diffusers effectively eliminates fluctuations in temperature and humidity and was more cost-effective than commercially available thymol-based systems (US$3.20 versus US$15-$18 per application). Continuous-release mist diffusion permits the disbursement of EOs throughout the entire hive, perhaps including the brood cell where natural miticides do not reach and where *Varroa* reproduction occurs.

Keywords: Varroa destructor; Apis mellifera; mites; honey bees; essential oils; mist diffusion

1. Introduction

Honey bees, *Apis mellifera,* are crucial for agriculture, responsible for over 80% of all cultivated crops in the United States (Randall, 2020). In fact, bee pollination accounts for approximately US$15 billion in added crop value (Bryant, 2006). Additionally, bees also produce honey, pollen, royal jelly, beeswax, propolis, and venom for nutritional and medicinal uses. In the US, there are approximately 2.5 million commercially farmed honey bee hives and around 500,000 colonies kept by hobbyists and semi-professional bee keepers (Frazier, et al., 2013). Unfortunately, these numbers are declining at a rapid rate. Various reports suggest a loss of 30% to 50% of bee colonies in the US, (Colony Collapse Disorder, 2021) , its lowest point in the past 50 years. The greatest single contributor to

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the decline of bee health is the *Varroa destructor* mite (Rosenkranz, et al., 2010). A single *Varroa* mite can shorten the lifespan of a bee by one-third, and two mites can shorten it by one-half (Bryant, 2006). *Varroa* weakens and ultimately kills colonies by out-reproducing their host.

Varroa is an ectoparasitic mite that exploits the honey bee's life cycle. It feeds on the fat bodies of developing honey bee larvae and adult bees and aggressively reproduces within an infected bee colony. Recent research by Ramsey, Ochoa, Bauchan (2019) brings to light the *Varroa* mite's focus on the fat body tissue, and not the hemolymph as previously believed, which ultimately render the honey bee susceptible to harm from pesticides. *Varroa* mites also facilitate the transmission of multiple viruses and other diseases which have been linked to honey bee mortality (Moore, et al., 2019). Normally, honey bee larvae develop into pupae inside the individual brood cell of the wax comb and emerge as adults.

Female *Varroa* mites attach onto an adult or immature honey bee and enter the bee hive's brood cells to reproduce. The female *Varroa* punctures the underside of the abdomen of the pupae and feeds on the fat body cells before laying the first egg. Like honey bees, the male *Varroa* develops from an unfertilized egg. The female continues to lay about one egg per day. These eggs develop into daughters that mature and mate with the male before the bee emerges from the brood cells. Immature and male *Varroa* can only be found in the capped brood cell. Only the mature *Varroa* female will survive after leaving the brood cells. All immature mites and *Varroa* males die soon after the adult bee emerges. The female mite then repeats the cycle by entering cells of other developing larvae (Hunt, & Given, 2021).

Bahreini, et al., (2020) clarify, in order to control the spread of *Varroa*, bee keepers initially used acaricides, pyrethroids, and organophosphates pesticides. Unfortunately, these chemicals have been found to increase the early replacement of the queen, heighten mortality in adults and brood, reduce body weight in queens, decrease the amount of lipids, carbohydrates and proteins in workers. Furthermore, these synthetic compounds have also been found to accumulate in the beeswax which impacts the development of bee larvae (Bahreini et al., 2020). The frequent use of these synthetic miticides to control *Varroa* infestations has resulted in the development of resistance to many of the chemical components of these miticides (Bahreini et al., 2020; Traynor, et al., 2016). Likewise, the excessive or improper use of synthetic compounds has also resulted in the contamination of hive products which pose a health threat to both hive bees and human consumers (Traynor et al., 2016). For these reasons, the need for alternative *Varroa* control measures is critical.

Essential oils are an alternative to chemical pesticides. They are cheaper, environmental-friendly, and pose fewer risks to the health of bees and consumers. Most importantly, *Varroa* have not developed resistance to essential oils for honey bee mite control (Ghasemi, et al, 2011; Damiani, et al., 2009). Essential oils are concentrated hydrophobic liquids extracted from aromatic plants and capture the "essence" or characteristic odor of the plants. Only plants containing more than 0.1% oil can be called essential oils (Imdorf, et al., 1999). The essential oil composition of each plant is unique. Terpenes (mainly monoterpenes) are the main components of essential oils. A wide variety of essential oils (over 150) have been tested as potential miticides; unfortunately, very few of them have proven successful when tested in field trials (Sabahi, et al., 2017).

A key problem evidenced in trials conducted to evaluate essential oils as miticides in hives is the consistency and significant variability that exists between seasons and localities. Sabahi et al. (2017) concluded that one factor affecting this variability is the pattern of climatic conditions which are dependent upon the ambient temperature and relative humidity which can affect the properties of essential oils such as the rate of evaporation of the oils and, consequently, the mites' exposure to the essential oils.

Currently, numerous essential oil compounds have been evaluated for miticidal activity. One of the proven successful essential oils is thymol. Thymol is a phenolic monoterpene that is present in many plants such as thyme, basil, rosemary, mint, and sage. It works by disorienting the mite and blocking its pores

(Tennessee's Honey Bees, 2021). The efficacy of thymol has previously studied and has been recommended to be effective against *Varroa*. Thymol is the only compound of essential oils widely used in beekeeping with 70%-90% efficacy against *Varroa* (Garrido, 2018). Thymol also has the added advantage of being active against fungus (chalkbrood) and some efficacy with tracheal mites (Davis, & Ward, 2003). The most widely used and popular products with thymol as a main ingredient are Apiguard®, ApiLifeVar® and Thymovar®. These systems only kill mites on the adult bees, but do not reach the reproducing *Varroa* mites in the brood cells (Garrido, 2018).

In light of the current crisis surrounding honey bees and the *Varroa* mite, new solutions are desperately needed. The following essential oils: thyme, oregano, spearmint, *and* rosemary were evaluated for miticide efficacy and safety. The use of a battery-powered mist diffuser to deliver essential oils may allow for the continuous-release of thymol regardless of environmental conditions, perhaps reaching the brood cell where *Varroa* reproduction takes place.

2. Materials and Methods

2.1 Safety Precautions

Appropriate attire and proper safety and handling precautions were taken during the collection and handling of *Varroa* mites, honey bee larvae and pupae. Appropriate attire consisted of a complete beekeeping suit which includes: hat with veil, full body suit, and foot gear. Mentors from Stockton and Rutgers provided guidance and oversight during the handling and collection process.

2.2 *Varroa* Mite Collection

A. mellifera colonies with bee hives naturally infested by *Varroa destructor* mites were utilized for this study. Live *Varroa* were collected from Stockton University from hives in Galloway, NJ and from Rutgers University from hives in Eastampton Township, NJ. All *Varroa* collection was conducted on October 10, 2020 to coincide with increased *Varroa* populations in the fall/early winter and to minimize interference with honey production during the spring.

For this experiment, live *Varroa* was collected from the powdered sugar roll methodology as described by the Honey bee Health Coalition (2016). However, the roll/shake was performed over a white paper plate and sprayed with a spray bottle containing water to dissolve the powdered sugar. A paintbrush was utilized to collect all live *Varroa*. All collected live *Varroa* were placed into a clean, damp paper towel. The towel was folded up gently with the mites inside and placed into a petri dish for transport.

2.3 Larvae/Pupae Collection

Fourth-instar worker bee larvae and capped brood larvae were collected from established *A. mellifera* colonies at Stockton University from hives in Galloway, NJ on October 10, 2020. Following larvae collection methods as described by Jack, et al., (2020), honeycomb brood frames were placed into an incubator at 94 degrees Fahrenheit (34.4 degrees Celsius) at 65% relative humidity (RH). After 3 hours, larvae crawled out of the cells and paper was placed underneath the combs to catch the larvae. Each larvae was carefully examined for the presence of attached mites. For the capped brood, cells were uncapped and brood was cautiously removed with a pair of fine tipped forceps as described by Honey Bee Health Coalition. Pupae were carefully checked for the presence of mites. All larvae and pupae were examined with a probe, all reacting larvae and pupae were scored as live (Honey Bee Health Coalition, 2016).

2.4 Gelatin Capsule System

As described by Jack et al. (2020), all collected live larvae and pupae were placed into size "0" gelatin capsules (7 mm, Now Foods, Bloomingdale, IL, USA). Using a number 2 insect pin (BioQuip, Rancho Dominguez, CA, USA), small ventilation holes were made in the capsules to simulate the porous capped brood cells. *Varroa* females collected from the powdered sugar roll system were individually inserted into a gelatin capsule containing a larva or a pupa. Each capsule contained a larva/pupa and one or two *Varroa* depending on availability. The capsules were then placed vertically into an empty micro-pipette tip container and maintained in an incubator (FBA Magicfly-MF014, Hong Kong, China) at 94 degrees Fahrenheit (34.4 degrees Celsius) at 75% RH. According to Jack et al. (2020), the gelatin capsule system is reported to have a mite survival rate of >95%. The temperature and humidity were monitored with the Broodminder mobile app (Broodminder, Stoughton, WI, USA).

2.5 Essential Oils and Continuous-Release Battery-Operated Mist Diffuser

For this experiment, four USDA, certified organic, premium food-grade essential oils, thyme, oregano, spearmint, and rosemary, were evaluated along with a control consisting of organic, premium food-grade vegetable glycerin (Plant Guru, Plainfield, NJ, USA).

Continuous-Release Treatments 1,2,3,4 consisted of essential oils (1-thyme, 2-oregano, 3-spearmint, 4-rosemary) for continuous-release via a battery-powered mist diffuser (AirWick® Essential Mist Essential Oil Diffuser, Reckitt Benckiser, Slough, England). Each individual incubator (FBA Magicfly-MF014, Hong Kong, China) contained one of the tested essential oils in a continuous-release battery-powered mist diffuser (or vegetable glycerin only in the control) along with the gelatin capsule system (Jack et al., 2020). This methodology is designed to simulate the brood cell environment within the hive. Sabahi et al. (2017) evaluated oxalic acid, oregano/clove, and oregano alone utilizing a continuous-release electric diffuser. However, in light of the potential fire safety concerns and the lack of accessible electrical outlets in the field, the mist diffuser for this experiment was battery-powered.

To provide continuous-release, two alternating mist diffusers were used in each incubator to provide 24/7 release as each mist diffuser automatically shuts down after eight hours of continuous use. The AirWick Mobile App was utilized to ensure that all diffusers were operating properly. To calculate the rate of evaporation of the essential oil, the mist diffuser container was weighed while empty and then weighed again when filled with the essential oil at the beginning (the net weight was obtained by subtracting the tare weight from the gross weight) and at the end of the trial. Control Treatment 5 contained no essential oil, only vegetable glycerin. Cumulative mite and bee survival was recorded at 24, 48, 72, 96, 120, 144, 168, 192, and 216 hours.

In total, 88 live larvae/pupae and 91 live mites were collected from the apiaries at Stockton University and Rutgers University. After 12 hours, all collected mites and larvae/pupae were assessed for movement as a measurement of lividity using fine tipped forceps. In total, 56 live larvae/pupae and 63 live mites were recovered to commence the experiment. Each live larvae/pupae was separated into its own gel capsule and 1 (or 2 mites) was allocated to each larvae/pupae.

3. Results and Discussion

The recorded mite and bee mortality from the diffusion of tested essential oils in 24-hour increments are provided in Table 1.

Table 1: Number of fallen *Varroa* and fallen larvae/pupae by essential oil tested

E.O. Tested	Time (hours)	# of Fallen Varroa	# of Fallen Larvae/Pupae
Thyme	24	5	0
	48	13	0
	72	13	0
	96	13	0
	120	13	0
	144	13	5
	168	13	9
	192	13	11
	216	13	11
	240	13	11
Oregano	24	3	0
	48	13	0
	72	13	0
	96	13	0
	120	13	0
	144	13	4
	168	13	8
	192	13	11
	216	13	11
	240	13	11

Spearmint	24	0	0
	48	0	0
	72	1	0
	96	5	0
	120	8	0
	144	9	3
	168	12	5
	192	12	8
	216	12	11
	240	12	11
Rosemary	24	0	0
	48	0	0
	72	3	0
	96	9	0
	120	13	0
	144	13	2
	168	13	7
	192	13	10
	216	13	10
	240	13	11
Control	24	0	0
	48	0	0
	72	0	0
	96	1	0
	120	2	0
	144	3	2
	168	6	4
	192	10	7
	216	13	11
	240	13	12
(yellow)	Time to last fallen Varroa		
(green)	Time to last fallen larvae/pupae		

Across all essential oils, an increase in the hours of exposure led to an increase in *Varroa* mortality as depicted in Figure 1. After 48 hours, all *Varroa* mites in both the thyme and oregano had completely fallen. After 120 hours, all mites exposed to the rosemary had succumbed. After 144 hours, all mites exposed to spearmint had fallen. For the control, containing only vegetable glycerin, after 240 hours, all mites had succumbed. The overall miticide activity from the first recorded mite fall to last recorded mite fall was as follows in terms of essential oil efficacy: thyme > oregano > rosemary > spearmint > control. Statistical significance was determined using regression analysis. All p-values were statistically significant (p-value $\leq$ 0.05) across all mite falls (thyme (p=.00000651); oregano (p=.0000451); spearmint (p=.00391); rosemary (p=.000294)).

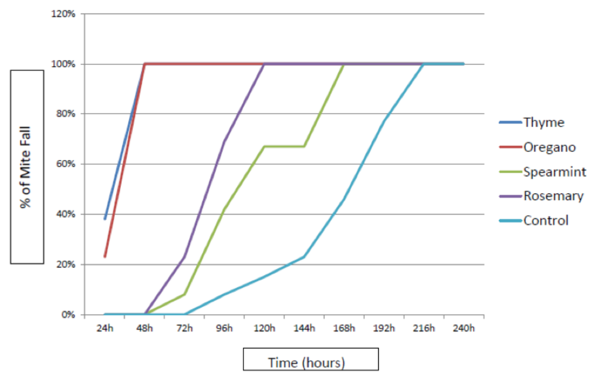

Figure 1. Percentage of *Varroa* mites fallen by essential oil per 24-hour periods

Across all essential oils, increasing the hours of exposure did not lead to an increase in honey bee mortality as depicted in Figure 2. Honey bee mortality was first noted at 144 hours for thyme, oregano, rosemary and spearmint. These values are comparable to the placebo which also noted honey bee larvae/pupae mortality at 144 hours. Overall bee mortality from first fall to last fall was as follows: thyme = oregano > rosemary > spearmint = placebo. All p-values were not statistically significant (p-value $\leq$ 0.05) across all bee larvae/pupae fall (thyme (p=.176); oregano (p=.594); spearmint (p=.255); rosemary (p=.452)).

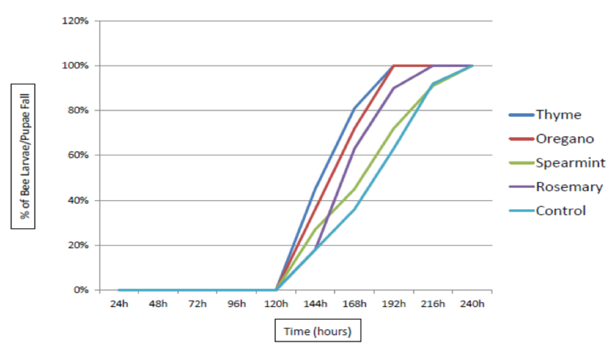

Figure 2. Percentage of bee larvae/pupae fallen by essential oil per 24-hour periods

4. Discussion

This investigation of various essential oils demonstrates the effectiveness of thymol-based essential oils and a battery-operated continuous-release mist diffusion delivery system as an effective miticide. All tested essential oils were found to be effective. The mist diffuser system was successful in its dispersal of the essential oils independent of temperature and humidity variables.

A key advantage of using essential oils in hives is that it is generally recognized as safe and environmentally-friendly. In fact, all the essential oils utilized for this experiment were premium food-grade. Due to the sustained lividity of the larvae/pupae from this investigation, the essential oil concentration tested is concluded to be safe for honey bees.

Furthermore, a brief analysis of the cost-effectiveness of the battery-operated continuous-release mist diffuser technology in concert with essential oils was found to be more economical than the annual cost of commercially available thymol products. The average cost per application of the continuous-release diffuser technology and essential oil is approximately US$3.20 per hive per application (assuming the use of 2 diffusers) in comparison to currently available thymol-based commercial products at US$15-US$18 per hive per application.

5. Conclusion

The goal of this in vitro study was to demonstrate that continuous-release mist diffusion delivery of thymol-based essential oils can serve as an effective miticide while also being safe for bees. The results of this in vitro investigation warrant further examination of the continuous-release mist diffusion delivery system and the essential oils of thyme, oregano, and rosemary in a field investigation. Because the effectiveness of essential oils is dependent on multiple factors, including temperature, humidity, and time of year (Sabahi, et al., 2017), the use of a battery-operated continuous-release mist diffuser system minimized these variables and, perhaps, may allow for miticide exposure inside the capped brood cells. Currently, available thymol-centered systems are gel-based and work only by contact with the mite (Honey Bee Health Coalition, 2016). As technology evolves, other continuous-release devices (solar powered diffusers) may also be investigated. Currently, no natural miticides penetrate into brood cells where *Varroa* reproduction takes place (Garrido, 2018). The use of thymol-based essential oils with a continuous-release mist diffuser delivery system may one day be incorporated with other control measures to effectively manage *Varroa* mites and other pests in order to enhance the chance of colony survival and residue-free hive products.

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