DEVELOPING A FUNGAL BASED TRAPPING STATION FOR THE CONTROL OF FRUIT FLIES (DIPTERA: TEPHRITIDAE) IN HAWAII

A THESIS SUBMITTED TO THE GRADUATION DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER’S IN SCIENCE

IN

ENTOMOLOGY

MAY 2023

By

Christian Streit

Committee:

Ikkei Shikano, Chairperson

Dara Stockton

Jia-Wei Tay

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ACKNOWLEDGEMENTS

I thank Yahuah my Elohim for the life and ability that He has given me. For without Him I would have nothing. I would like to thank my wife, Alysson, for all of her long hours of understanding and support while I worked late nights. I thank my children for understanding and encouraging me to complete my degree. I thank Dr. Ikkei Shikano for the opportunity to pursue my degree and all of his guidance in developing my work. His insight has helped me become an adept entomologist and researcher. Thank you to my committee, Dr. Jia-Wei Tay and Dr. Dara Stockton for their insight and direction. I want to thank all of my lab mates: Morgan Pugh, Sarah Pennington, April Grumner, Kevin Armstrong, Maya Montoya-Pimolwatana for their camaraderie and shared experiences as we developed our work together.

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CHAPTER 1: Fruit Fly (Diptera: Tephritidae) History and Management

Abstract

Fruit flies (Diptera: Tephritidae) are a cosmopolitan species with many members of this family known to be economically significant pests. Research to understand the lifecycles and unique behaviors of these flies have been important to exploit critical points of development to control their populations. Many management strategies have been proposed, studied, and implemented across the world and have been met with success. But, with the ability of these flies to disperse, not all methods are applicable or successful in all regions. Entomopathogenic fungi are being studied in conjunction with the Tephritidae to determine efficacy against all life stages. Entomopathogenic fungi are capable of inducing high rates of mortality in adult fruit flies. The limiting factor of entomopathogenic fungi is its susceptibility to UV radiation. For this reason, it is widely used as a soil drench. Development of a product that incorporates entomopathogenic fungi to target adult fruit flies would be far more efficacious, adding one more tool to a widening array of management tools.

Multiple stages of testing and development were performed, starting with determining the efficacy of *Beauveria bassiana* against three invasive tephritid fruit flies in Hawaii: *Bactrocera dorsalis*, *Ceratitis capitata*, and *Zeugodacus cucurbitae*. After confirming that *B. bassiana* kills adult fruit flies, we determined the lethal concentrations and developed a formulation that would be effective in a passive bait station, which included incorporation of lures and thickening agents.The effectiveness of each addition or modification to the formulation was assessed by measuring the mortality of exposed flies and the numbers of spores they picked up. The

developmental goal of creating a formulation that could be vectored and dispersed across a population via horizontal transmission was confirmed, at least in lab cage trials. This fungal formulation could potentially be used as a new IPM tool for the control of tephritid fruit flies in both conventional and organic cropping systems.

Introduction

Across the world fruit flies (Diptera: Tephritidae) are known for their diversity and negative impacts on agriculture production of fruits and vegetables [1]. This family Tephritidae is moderately large, with over 4000 documented species [2,3] and are endemic to Africa, Asia, Australia, the Pacific, and Central and South America [4–16]. Tephritidae are primarily distributed throughout temperate, subtropical, and tropical parts of the world [17]. Tephritid flies are also known to be excellent colonizers due to their strong ability to adapt to new regions, climates, and host species [18]. Of the known tephritid flies, there are about 250 species that are economically significant due to their deleterious impacts on production and trade of agricultural commodities [19]. The rise in insecticide resistance in tephritid fruit flies [20] has also driven the need for alternative control methods.

Entomopathogenic fungi (EPF) have been shown to be effective in the control of multiple pest species [21–23]. Recent studies have shown that EPF's like *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metsch.) Sorokin are effective at achieving high rates of mortality in adult fruit flies [24–28]. Commercial EPF products are available but are not able to be applied in a way that would effectively control these fruit flies. Development of a product that incorporates these EPF could become a powerful addition to many integrated pest management (IPM) programs across the State, Nation, and Globe.

Impacts

In the literature on tephritid fruit flies, the bulk of the published work focuses on negative economic trade impacts and the best control methods of a handful of family members. Many lists of the most important agricultural and horticultural pests include members of Tephritidae [15,29–32]. Some of the most prominent and well-studied members are the South American fruit fly, *Anastrepha fracterculus* (Wiedemann), the Mexican fruit fly, *A. ludens* (Loew), West Indian fruit fly, *A. obliqua* (Macquart), Oriental fruit fly, *Bactrocera dorsalis* (Hendel), the olive fruit fly, *B. oleae* (Rossi), Queensland fruit fly, *B. tryoni* (Froggatt), peach fruit fly, *B. zonata* (Saunders), Mediterranean fruit fly(med fly), *Ceratitis capitata* (Wiedemann), apple maggot fly, *Rhagoletis pomonella* (Walsh), and the melon fruit fly, *Zeugodacus cucurbitae* (Coquillett). The ability for these flies to invade new areas is exacerbated by the increase of globe trade over the past few decades. The trade of agricultural commodities has the inherent risk of introducing exotic pests that are costly and difficult to control or eradicate [33].

In California, *C. capitata* reintroduced nearly annually through Los Angeles [34]. As a result, the California Department of Food and Agriculture (CDFA) spends roughly $15 million annually on eradication and monitoring programs to prevent invasive establishment. If *C. capitata* were to establish in California, the estimated losses are over $1 billion annually in crop loss, export sanctions, and treatment costs [35]. In the United States, many states are at risk of having a tephritid pest introduced and potentially establish. Texas and Florida are also agricultural states that have had to deal with tephritid introductions. Spending millions of dollars to eradicate Oriental and Mediterranean fruit flies [36]. Only in Hawaii are these invasive fruit flies established. Resulting in strict quarantines on exporting agricultural commodities.

In Hawaii there are five invasive tephritids. *Zeugodacus cucurbitae* (Coquillett) (Melon fly) was the first to arrive in Hawaii in 1895 [37,38] and then in 1910, *Ceratitis capitata* (Wiedemann) (Mediterranean fly) arrived [39,40] effectively colonizing and damaging the papaya industry. During World War II the heavy traffic of troops and supplies going back and forth from the pacific allowed for the *Bactrocera dorsalis* (Hendel) (Oriental fly) to make its way to Hawaii in 1944 [41,42]. Governments began to become more aware of the dangers of inadvertently introducing new species and began to restrict the free flow of goods through quarantine restrictions in and out of Hawaii. The next tephritid, *Bactrocera latifrons* (Hendel) (Malaysian fruit fly), made its way into the State even with the quarantine restrictions in 1983 [43,44]. The most recent introduction, as of 2019, with all the modern quarantine measures, was the *Bactrocera oleae* (Gmelin) (olive fruit fly) which has become established on Hawaii Island and Maui [45]. The extent of my research focuses on the three older introductions of Melon fly, Med fly, and Oriental fruit fly.

Economic damage and strict quarantine strategies are the result of these introductions into Hawaii [46]. With over 400 different species of fruit and vegetables that these fruit flies are known to infest throughout Hawaii. Some of the more economically important crops to Hawaii are: Citrus, *Citrus* spp.; coffee, *Coffea arabica* L.; eggplant, *Solanum melongena* L.; guava, *Psidium guajava* L.; mango, *Mangifera indica* L.; melons, *Cucumis melon* L.; papaya, *Carica papaya* L.; passion fruit, *Passiflora edulis* Sims.; persimmon, *Diospyros kaki* L.; tomato, *Solanum lycopersicum* L.; cucurbits, *Cucurbita pepo* L.; [47]. Aside from the hurdles of producing these susceptible crops, exporting them from Hawaii requires postharvest treatment, which further exacerbates an already difficult agricultural economy[47]. In the 1970's melon

flies in Hawaii caused approximately $15 million in crop losses [48]. At the turn of the century, an estimate for the economic impact of melon fly, med fly, and oriental fly in Hawaii was about $300 million annually [49]. The presence of these fruit flies has impacted more than the wallets of farmers. These flies are impacting farmer's willingness to plant crops that are known to be susceptible hosts, limiting the potential production of certain crops in Hawaii [50].

Tephritid flies are destructive in many nations and have become exceptionally destructive on island ecosystems without natural predators to control them [51]. Fruit flies possess many attributes that make them exceptionally difficult to control and destructive if left unchecked. Their ability to be transported undetected by human activity makes their introductions into new regions easy. Natural high dispersal capabilities make these new introductions even more devastating if the flies are able to establish on a host or closely related species [52]. Populations can then explode out of control during warm and humid periods [53]. Across each genus in the Tephritidae many characteristics are shared. However, each fruit fly species has its own unique behaviors as well that can be exploited to potentially control them.

Biology & Ecology

The lifecycles of tephritid fruit flies are uniform across most species within the family. Each tephritid goes through four primary phases during its lifetime. Egg, larval, pupal, and adult phases. The first three phases are the immature stages and visual species identification can be difficult. During the adult phase most tephritid's are easily identified by their unique body marking and wing patterns. Proper identification is critical to ensuring the proper management, quarantine, and control programs are implemented [54].

Eggs are elongated, glistening white, rounded at one end, approximately 1mm in length [55]. They are laid into young fruits and deposited in clusters. Some will lay eggs in batches or individually, depositing eggs in as many fruits as possible over the course of the female's lifetime. The incubation time of the eggs is impacted primarily by temperature and substrate. The substrate type impacts the time it takes for eggs to hatch in melon flies. Melon fly eggs laid in cucumbers take between 24 to 38 hours to hatch [56], on watermelon approximately 28 hours [57], and on bottle gourd, *Lagenaria siceraria* (Mol.) Standl. eggs took between 34 and 47 hours to hatch [58]. Temperature impacts how many eggs will hatch and the time it takes for them to hatch [53].

Once eggs hatch, the larvae (maggots), begin to burrow deeper into the fruit. The three instar phases are characteristic of all tephritids [59]. Each successive instar phase takes progressively longer than the one before it. The first instar is tiny and not much larger than the egg and translucent white. Second instars are slightly larger than first instars, becoming creamier in color. Third instars are the most noticeable with yellow-creamy white colored bodies and their dark mandibular hooks [55,60]. In almost all tephritids, third instars exhibit a jumping behavior as they become fully mature [17]. Larvae tense and fold their bodies in half then relax particular muscles launching its body into the air and many inches away from the fruit to an area to pupate in the soil [17,55].

Making their way into the soil, larvae then become sluggish and contract their bodies longitudinally [19,58]. Pupae are small, cylindrical, and barrel-shaped. Their colors vary by species, ranging from creamy tan to reddish-brown to dark brown-grey [55,61]. The pupal period ranges by species, host fruits, and multiple external factors like temperature, moisture content of

the soil, and soil type [62,63]. As flies eclose from their puparium, they make their way to the surface of the soil. Here the teneral adults seek out a safe place to dry their bodies and wings before becoming active, searching for a food source. Each adult fly is going to exhibit unique behaviors, body coloration, and wing patterns that are characteristic of that species.

Mediterranean flies (*C. capitata*) are smaller than other tephritids. With an average body length of 3.5-5 mm and wingspan of 8-10 mm [64]. Their bodies are short and stubby with black, yellow, and brown markings [61,65]. Their thorax is creamy yellow-white with symmetrical black blotches and black bristles scattered across the whole thorax [65]. The scutellum of med flies are inflated and shiny black [61]. The abdomen is yellow-brown in color with two narrow transverse lightly colored bands on the basal half [66]. Wings are typically held in a drooping position, mostly hyaline with black, brown, and yellow markings. There are dark streaks throughout the wing and anterior and anal cells, with a large yellow brown band across the middle of the wing [61,66]. Males are identifiable from females by the pair of bristles with enlarged dark spatulate tips next to their eyes [61].

Melon flies (*Z. cucurbitae*) are smaller than an average house fly with their average body size between 8-10 mm long and a wingspan of 14-17 mm [55,60]. Males are typically smaller than the females and females are identified by their ovipositor [60]. Their bodies are a reddish-brown with three distinct lateral yellow vitta and a yellow scutellum [55,67,68]. The abdomen is reddish-brown with 5 tergites. A “T” pattern is formed by a transverse dark band across the T3 tergite is intersected by a thin medial line connecting the T3-T5 tergite. This “T” marking is sometimes faint [60,68]. Their wings are predominantly hyaline with dark costal

banding along the anterior margin and interior cup with fuscous markings at the margin of the wing tip and at the dm-cu cross vein [60,68,69].

Oriental flies (*B. dorsalis*) are similar in size to the melon fly. The average body size is between 8-10 mm long and a wingspan of 14-16 mm [70]. The thorax is predominantly black with yellow lateral vitta and a yellow scutellum [71]. Abdomens have 5 tergites with a small sixth tergite in females and yellow to orange, brown. The T3-T5 tergites have black medial stripes and a transverse dark line on T3, forming a "T" on the abdomen [14,71]. Oriental flies wings are mostly hyaline with a dark costal banding along the anterior margin of the wings [70,71].

Most tephritids become sexually mature within a few days, with some taking up to two-weeks to mature, after eclosion and finding a food source [72]. Sexually mature flies will aggregate together forming a lek. Leks are formed before sunset and the light intensity is most intense [73,74]. In these leks, each territory within the lek will be occupied by one male who is actively defending his site against other males [75,76]. Males will exhibit aggressive defensive actions like head butting and lunging at encroaching males. During these leks males secrete pheromones to attract females. Mediterranean fly males exhibit pheromone calling [77]; males curl their abdomen upward exposing their rectal epithelium excreting a bubble of pheromone, while vibrating their wings to disperse the pheromone toward nearby females [76,78].

Once a female has found a male, they will copulate for anywhere from a few hours to throughout the night [17]. Gravid females will then leave in search of a place to lay their eggs. Preoviposition period, fecundity, and daily eggs laid are directly impacted by temperature for tephritids found in Hawaii [79]. When a female is attempting to find a suitable location for her

eggs, she will use both olfactory and visual stimuli to determine fruits that are suitable [73,80]. The females will probe the surface of fruits with their labellum and ovipositor, preferring to oviposit in damaged areas and cracks of the fruits [73,74]. Some have reported that gravid females will lay more eggs in an area where other conspecific females are present and laying [81–83]. Conflicting observations of oriental fly females will defend their oviposition sites against any other females of the same or different species [17].

Current Control Methods and Shortcomings

Since tephritid fruit flies were introduced into Hawaii many different control methods have been implemented to reduce the damage they can inflict, including mechanical, cultural, biological, physical, and chemical controls. In combination, these methods have been successful at reducing fruit fly damage on Hawaii farms. However, each has its own inherent pitfalls that make a single solution to keep the fly populations below a level of economic damage challenging.

Mechanical

Most mechanical controls applied today are most effective against insects with limited movement capabilities. Fruit flies pose more of a challenge to growers who wish to control the insects by mechanical methods. Some growers have successfully reduced infestation rates of tephritidae in their field by netting, bagging, or wrapping fruits. Reducing the amount of produce available to gravid females [84,85]. Augmentoriums have been utilized in Hawaii to cover infested plants and fruits to keep emerging adults from dispersing and mating [86]. Mechanical

controls do not require any intensive learning or skill to implement them and there are little to no negative impacts to the environment. The greatest limitation of mechanical controls being successful in most systems is the time and labor required to effectively protect each crop [87]. For small scale producers wrapping can be more effective and lucrative. As production scale increases mechanical controls become a less viable option for crop protection.

Cultural

Reduction in pest prevention and damages can be achieved through different farming practices and techniques. Cultural controls can vary in application from the use of resistant varieties to crop rotation and timing of planting to crop residue removal. Since tephritid fruit flies are highly polyphagous and mobile, most cultural control methods do not sufficiently control or reduce populations. The cultural practice of sanitization by removing and disposing of infested plants and fruits is effective at breaking the reproduction life cycle of the flies. By physically removing potentially infested fruit either by crushing and burying or by solarization in bags, populations can be reduced significantly [19,84]. These cultural practices can be beneficial at hindering pest populations from reaching economically damaging levels. They do however require a lot of intimate knowledge of the pest's biology and understanding the timing of when to implement these controls. If these controls are not implemented at the correct time they will not be as effective at reducing pest pressure [86,88].

Chemical

Starting in the 20th century, many different inorganic insecticides (e.g. Bordeaux [copper(II) sulfate and lime] plus nicotine sulfate, lead arsenate, Paris green [copper(II)

acetoarsenite]) were sprayed to little effect on crops to reduce infestation and emergence [37,78,90]. Further development of pesticides like DDT in the 1940's had some effect on tephritid fruit fly control [91,92]. The most commonly used insecticide since the 1950's is the organophosphate, malathion. As more research was done to understand fruit fly behavior and dietary requirements, combining insecticides with proteinaceous bait sprays became the recommended method of controlling fruit flies for decades [88,93,94]. At the turn of the century GF-120® NF Naturalyte® Fruit Fly Bait (Dow AgroSciences, Indianapolis, IN, USA) containing spinosad, a toxin derived from a soil-dwelling bacterium, was introduced. This environmentally friendlier option was heavily relied upon in Hawaii as the primary method of control for Z. *cucurbitae* [84,88,95]. Targeting areas around the cropping systems and known roosting locations instead of directly spraying the crop [96–98]. Due to the effectiveness of chemical applications for many decades farmers have relied heavily on pesticides for tephritid control. This has led to a high level of resistance in Hawaii tephritids to spinosad and other classes of insecticides when inadequate rotations of pesticides are being used [99–103]. The ability of these pesticide resistant populations to persist in the environment reduces the efficacy of many pesticides for the control of tephritid's [20,102].

Behavioral

Fruit fly behavior controls are focused on disrupting mating of males and females with or without the use of pheromones and semiochemicals. The two most prominent methods are Male Annihilation Technique and Sterile Insect Technique.

Male Annihilation Technique (MAT) strives to decrease the male population of a species to reduce the number of mating interactions that may occur, reducing the overall population

[104]. A key mechanism that makes MAT effective against tephritids is the use of synthesized male-specific chemicals like methyl eugenol (ME; 4-allyl-1, 2-dimethoxybenzene-carboxylate), cuelure (C-L; 4-(*p*-acetoxyphenyl)-2-butanone), and raspberry ketone (RK; 4-(4-Hydroxyphenyl)butan-2-one) [105,106]. Combining these attractants with a toxicant or fumigant forms the basis for MAT. Pestiferous tephritids have been eradicated successfully using MAT on multiple islands throughout the pacific and Indian ocean [107–112]. Fiberboard [107,109,110,112–114], coconut husks [115,116], and cotton wicks [117–120] are soaked in the attractant-toxicant mixture and then distributed across the infested regions. These concentrated lure-toxicant combinations have been effective at reducing pesticide applications and tephritid pest pressure in field and forested regions. MAT can be used to eradicate invasive tephritids, but financial restrictions and geographic distribution of pest species can determine the success of a MAT program. Multiple MAT programs that were initially set out to eradicate a specific tephritid species later became suppression programs [105,110,114]. Sterile Insect Technique (SIT) was first proposed with its principles by E.F. Knipling and Raymond Bushland in the 1950's as a method to suppress or eradicate pest species by the release of sterilized insects [122,123]. Fruit flies have been the subject of many SIT programs across the globe, each with different goals. The earliest SIT programs in the United States occurred in Hawaii to eradicate *C. capitata*, *B. dorsalis*, and *Z. cucurbitae* [107,124,125]. The most successful SIT eradication programs have been accomplished on Islands in the Pacific; *Z. cucurbitae* from Rota [112] and Okinawa [111,126], and *B. dorsalis* from the Mariana Islands [107]. Regional programs across Australia [127–129], Central America [130–133], Asia [111,126,134–137], the Mediterranean [138–143], and Africa [144,145] had varied levels of success achieving eradication and suppression of

tephritids. One of the limiting factors for SIT to be a viable long-term component to IPM programs is the economic viability of producing sterile insects and funding for extended projects [130,131,136,144,145].

Biological

Use of natural enemies to control pest populations have been applied against many different species of plant and insect pests. Natural enemies or control agents include parasitoids, predators, and pathogens. For most Tephritidae, biological control for most of the 20th century consisted of classical and augmentative biological control programs [51,146,147]. Biological control programs began as early as 1914 with the first introductions of Opiine Braconid (Hymenoptera) parasitoids in Hawaii [148]. Subsequent releases followed, with one the largest biological control programs against fruit flies from 1947-52, where 29 parasitoid species were collected from across the world and reared in Hawaii for release [149–152]. Many of the programs began as classical programs [148,153] but had to shift to augmentative control programs due to the inability of parasitoid agents to become established in the wild [51,153–156]. *Psyttalia fletcheri* (Silvestri) was successfully established in Hawaii on *Z. cucurbitae* with parasitism percentages that varied according to the host fruit species [157]. Although a level of control is afforded through established parasitoid wasps, parasitism is not adequate alone to fully control tephritids in many programs [51,153,158,159].

The lifecycle of most tephritid flies leaves them susceptible to predation in their larval and pupal state [160]. Although predation may attribute to some larval and pupal mortality it has not been the primary focus of most research. Observations of ants, beetles, spiders, and even mice have been implicated in preying upon third instar larvae exiting fruit and pupae

[51,157,161]. Garcia et.al. found there were a total of 56 species of predators associated with tephritid fruit flies in the Americas and Hawaii with ants as the most likely to predate on tephritids [157].

Pathogens and biological pesticides that utilize nematodes, bacteria, fungi, and viruses are being brought to the forefront in the biological control of fruit flies. Some pathogens are more effective at controlling the flies while they are in the soil (nematodes, fungi) while others may be able to be applied to adults (fungi, bacteria, viruses). Research into gut symbiosis and compatible bacterial pathogens, like *Wolbachia*, are being tested and applied to tephritids as another option for biological control [162,163]. During the pupal stage many tephritids (*Ceratitis*, *Bactrocera*, *Anastrepha*, *Rhagoletis*) are susceptible to entomopathogenic nematodes (EPN) [164–170]. EPN's could be an effective part of an IPM program to control fruit flies during the soil stage of their lifecycle[171].

Entomopathogenic fungi are another biological control agent that are being examined in more depth for their application toward the control of fruit flies. Due to the nonspecific nature of EPF's they can be applied toward a wide array of pest species [172,173]. Economically significant genera of fruit flies have been studied for their susceptibility to *B*. *bassiana* and *M. anisopliae* [174–179]. Multiple life stages have been shown to be susceptible to EPF infection; larvae [179–183], pupae [26,174,178,179,181–185], adult [25,26,175–177,179,181,183,186–188]. Soil drenching is the only practical application at this time to employ EPF into any cropping system without exposing the fungi to excessive amounts of ultraviolet light [174,178,179,183,185,189]. However, adult tephritids are more susceptible to

fungal infection than the pupal phase [179]. This limits the ability of EPF to be applied toward the longest phase of a fruit flies life cycle.

Opportunity to Develop a New Management Tool

There are a multitude of IPM tools that can be utilized for the control of tephritids globally. All IPM strategies that are implemented to control fruit flies aim to reduce fly populations below economic thresholds. Damage from female fruit flies during oviposition results in unmarketable products and persistence of pest populations [84,85]. Many geographical, environmental, and economic factors influence the effectiveness and availability of some IPM tools to producers and conservationists [190]. Considerations for organic or small scale-producers to reduce reliance on chemical pesticides has led to a shift in research. There is an emphasis on researching and developing control programs that are organic, affordable, environmentally sound, and easy to implement [191].

Multiple studies have tested the efficacy of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metsch.) Sorokin to multiple life stages and genera of tephritids [25,26,174–189]. Both *B. bassiana* [192] and *M. anisopliae* [193] are commercially available. Although many studies have shown the efficacy of EPF and suggest their incorporation into fruit fly IPM programs [174,179,185–188], only a few have considered using EPF to control the adult populations [27,181,194,195].

The majority of current methods are passive in their control of females, while many IPM strategies successfully target the males. Repeatedly, MAT has proven to be successful in

controlling male fruit flies. Exploiting the attractiveness male lures to target females via horizontal transmission of insecticides has been shown effective at reducing females in the field [196,197]. Horizontal transmission to control entire populations by exploiting males, using them as vectors, to deliver insecticides can be an effective way to reduce broadcast spraying of pesticides [27]. EPF could be employed in a similar fashion to these attract and kill stations[198]. Horizontal transmission of *B. bassiana* and *M. anisopliae* in multiple tephritid species has been confirmed to induce significant reductions in female numbers after exposure to infected males [27,179,194,195].

To effectively utilize EPF in an attract-and-kill fashion to control tephritid fruit flies, multiple requirements must be met. First, the development of a carrier agent that prolongs the viability of EPF spores by protecting them from water exposure and ultraviolet radiation [199]. Second, the development of an auto-dissemination station that can disperse the EPF product to the target members of a population. Some researchers have suggested that such stations could significantly increase the efficacy of EPF as a major tool for control of fruit flies while reducing the risk toward non-target species [27,194,195,199]. Third, an effective lure that is compatible with the EPF spores [194]. All three components must be considered in depth through lab and field experiments before determining the applicability of EPF in controlling adult tephritid fruit flies.

Research Goals

The goal of this thesis is to develop a formulation of entomopathogenic fungi (*B. bassiana*) that can be incorporated into an auto-dissemination device (to be developed later).

The primary objectives are:

1. To develop a novel formulation using *B*. *bassiana* that can passively disperse lethal numbers of fungal spores to adult fruit flies.
2. To disperse fungal spores to sexually mature female flies by using male flies as the vector.
3. To refine the formulation to enhance attractiveness to the male flies, enhance spore pick-up, and maximize persistence in the field.

CHAPTER 2: Developing a Formula and Fungal Based Trapping Station for the Control of Tephritid Flies in Hawaii

1. Introduction

Tephritidae (Diptera) is a globally distributed fruit fly family, containing many species that are considered to be major economical pests [1,19,200–204]. Tephritid flies cause severe damage through the oviposition activities of females [73,112,113]. Females puncture the fruits which become scarred and have discoloration at the puncture site as eggs hatch and larvae (maggots) feed and bore into the fruits[17,73,74,152]. This life cycle can cause up to 100% loss of fruit crops and remaining produce is subject to strict quarantine regulation being imposed on exporting countries [19,85,99,152,174,205,206]. Tephritid fruit flies first invaded Hawaii in the late 1800's. Currently, there are five introduced tephritid species, all of which harm Hawaii's agricultural sector and limit their ability to export produce to the continental United States. The first tephritid was *Zeugodacus cucurbitae* (Coquillett) (melon fly) 1895 [37,38]; followed by *Ceratitis capitata* (Wiedemann) (Mediterranean fruit fly) 1910 [39,40]; *Bactrocera dorsalis* (Hendel) (Oriental fruit fly) 1944 [41,42]; *Bactrocera latifrons* (Hendel) (Malaysian fruit fly) 1983 [43,44]; *Bactrocera oleae* (Gmelin) (olive fruit fly) 2019 [45].

Management of these invasive fruit flies have shifted from broadcast sprays of crops with chemical insecticides to methods that target specific life stages and behaviors. These include field sanitization to kill eggs and larvae, insecticidal soil drenches to kill late-stage larvae and pupae, releases of biological control agents (i.e., parasitoids) that target larvae, sterile insect technique (SIT), and insecticide-laced bait sprays and attract-and-kill bait stations [20,99,100,190,207,208]. Bait stations contain an insecticide with either male lures to target

mainly male flies or a protein source to mainly target reproductively immature females, who need protein to develop their ovaries. Notably, none of the available eradication tools, including bait sprays and bait stations, target reproductively mature females. This is important because mature females are significantly less attracted to protein baits than immature females [209,210]. Moreover, two established fruit fly species in the U.S. (olive fly, *Bactrocera oleae*, in California [211] and melon fly, *Zeugodacus cucurbitae*, in Hawaii [212]) have exhibited resistance to Spinosad, which is the active ingredient in the most widely used protein bait GF-120. Additionally, *B. dorsalis* has shown a propensity to develop resistance to Spinosad in selection experiments in the lab [101] and resistance alleles have been found in field-populations of Mediterranean fruit fly, *Ceratitis capitata*, in Spain [213]. Thus, an over-reliance on GF-120 to target female fruit flies may be an unsustainable strategy.

Many tephritids exhibit unique mating behaviors where males will aggregate together to form leks [206,214]. This behavior gives an opportunity for the formulation to be dispersed amongst both sexes of the flies through mating. *Z. cucurbitae* are known to gather at dusk on non-host plants, which surround crop fields. They release pheromones to attract females while defending small territories from competing males [215]. These pheromones have been synthesized into parapheromones, which play a large role in current trapping and monitoring programs globally [73,216,217]. One of the more recent IPM tools incorporated into *Z. cucurbitae* management in Hawaii is a bait station that contains fipronil and a male lure (Amulet C-L; 0.34% active ingredient fipronil, 9.39% C-L, 90.27% inert ingredients; BASF, Research Triangle Park, NC, USA) [196]. The male flies are attracted to the lure (Cue-lure), which they consume, and in doing so, contact and ingest the insecticide fipronil. Fipronil is relatively slow-acting and provides the males with enough time to horizontally transmit the fipronil to

reproductively mature females via contact during courtship or through their regurgitant during food-sharing [196]. Field experiments in Hawaii demonstrated that the Amulet C-L bait stations significantly reduced the numbers of female *Z. cucurbitae* [196].

Here, we developed a formulation of the fungal entomopathogen *Beauveria bassiana* that would kill three species of fruit flies through direct contact with the spore formulation and through horizontal transfer of spores. Fungal biopesticides have been shown to be effective against tephritid fruit flies but the optimal method of application has not been determined [27,180,183,194,205,219,220]. So far, fungal pathogens have been tested as soil drenches to target late-stage larvae and pupae [28,185], mixed into protein baits to target adults [181], and directly applied to adults [24,26,27,186,187,205]. Our research aimed to exploit the same behavioral mechanism as Amulet C-L to vector *B. bassiana* spores throughout a population. Fungal pathogens take at least several days to kill their hosts. Thus, there is ample time for spore-contaminated adult males to transfer spores to sexually mature females during courtship and mating. Horizontal transmission of *B. bassiana* (Bals.) Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Claviciptaceae) have been demonstrated in several Tephritidae species [26,27,194,195,221,222].

Our goal was to develop a *B. bassiana* formulation that could be used in a bait station. The desired characteristics of the formulation was one that maximizes spore longevity and pickup of spores by male flies. We assessed whether those males would horizontally transfer the spores to female flies. We used a commercially available fungal biopesticide, BotaniGard, and mixed it in a carrier oil, then added abrasives to increase fly mortality and thickening agents and male lures to increase spore pickup. At various developmental stages, our formulation was tested on *Z. cucurbitae*, *B. dorsalis*, and *C. capitata*.

2. Materials & Methods

2.1 Colony Maintenance & Rearing Methods

Three species of fruit flies were maintained in a laboratory at The University of Hawaii at Manoa. All stages of the flies were maintained in temperature-controlled rooms at 25°C, approximately 70% RH and natural lighting from windows. Adult flies were housed in mixed-sex groups of 500 flies in 30 × 30 × 30 cm plastic and mesh cages (BugDorm, Mega View Science Co., Ltd., Taiwan). They were provided with water-soaked cotton balls and sucrose and yeast hydrolysate (MP Biomedicals LLC, Solon, OH) for sugar and protein sources, respectively. Mated fruit flies (at least 10 days old) were given access to fruits for oviposition for 24 hours. *Z. cucurbitae* were provided with zucchini, *B. dorsalis* with ripe papayas, and *C. capitata* with clementine oranges with the rind peeled back. After 24 hours, fruits were removed from the cages and placed into 1 L plastic cups. The cups were lined with a coffee filter and had holes at the bottom to allow liquid from the decomposing fruit to drain. These cups were placed into another 1 L cup, which collected the liquid. The stacked cups containing fruits were placed in a secondary container (28 L clear storage container), which was covered with a mesh cloth. Fresh fruits were added to the cups daily to ensure larvae had enough food to fully develop before pupation. The final instar larvae wriggled or jumped out of the fruits and onto the bottom of the secondary container where they pupated. Pupae were collected from the bottom of the secondary containers.

The *Z. cucurbitae* colony was originally collected from infested zucchini fruits from a commercial farm inEwa, HI. Experiments on this population began at generation F6. *B. dorsalis* and *C. capitata* colonies were obtained from the USDA Agricultural Research Service Pacific Basin Branch (Hilo, HI). All flies were used after they became sexually mature, which was

approximately 14 d after eclosion for *Z. cucurbitae* and *B. dorsalis* and 10 d for *C. capitata* fruit flies.

2.2 Lethal Concentrations Against Three Sp.

We used a dip method to determine the lethal concentrations of *B. bassiana* to *Z. cucurbitae, B. dorsalis,* and *C. capitata* (LC50 and 90). An aqueous stock suspension at the highest label rate of BotaniGard® ES (1×10^9 conidia per ml) was prepared. The stock suspension was serially diluted to produce six concentrations (2×10^8, 4×10^7, 8×10^6, 1.6×10^6, 3.2×10^5, 6.4×10^4 conidia/ml) and a control of distilled water.

Three groups of 15 flies (45 flies total) were aspirated into 30 cm × 5 cm (L × D) clear plastic tubes with mesh covering each end of the tube. These tubes were then dipped into each concentration of *B. bassiana*, while the suspension was being continuously stirred to ensure that the spores were evenly dispersed. As soon as a tube was fully submerged, it was immediately removed and placed on a paper towel to drip dry. Once the excess solution was off the tube, the flies were released into a cage and provided with water, sugar, and yeast hydrolysate. Mortality was monitored daily for 14 d. Each day, dead flies were removed from the cages and placed in a high humidity chamber (i.e., small cups with a damp paper towel) to stimulate sporulation of the cadavers and confirm that the flies were infected by the fungi when they died. Flies that died within 24 h were assumed to have died from handling and were removed from analyses. Nine cages of fifteen flies (a total of 135 flies) were assessed at each concentration per species with three replicates. The bioassay was repeated with ten concentrations for *Z. cucurbitae* and *B. dorsalis* using the same methods ($1x\ 10^9$, $1x\ 10^8$, $5x\ 10^7$, $1x\ 10^7$, $5x\ 10^6$, $1x\ 10^6$, $5x\ 10^5$, $1x\ 10^5$,

1x 10^4, 1x 10^3 conidia/ml). Nine cages of twenty flies (180 flies total) were tested at each concentration per species with three replicates. The bioassay was not repeated for *C. capitata.*.

2.3 Formulation Development

2.3.1 Testing Existing Products on Different Substrates

Two commercial products containing *B. bassiana* were tested for efficacy against *Z. cucurbitae*. Aprehend® (ConidioTec, Centre Hall, PA, USA) is a ready-to-use ultra-low volume formulation spray designed to manage bed bugs (*Cimex lectularius* L.) (Hemiptera: Cimicidae). Aprehend is sprayed on along bed frames, baseboards, walls, etc. to produce a barrier that bed bugs will walk across while searching for a blood meal and pick up fungal conidia [222]. BotaniGard® ES (BioWorks, Inc., Victor, NY, USA) is a product formulated to be diluted and sprayed in greenhouses, nurseries, and fields to control a wide variety of soft-bodied insects. Both Aprehend and BotaniGard® ES contain *B. bassiana* strain GHA at different concentrations and different inert ingredients. BotaniGard® ES was diluted in Heavy Mineral Oil (Fisher Scientific Co., Fair Lawn, NJ) to 2.0 x 10^{10} conidia per ml, while Aprehend is a ready-to-use product and 2.2 x 10^9 conidia per ml.

Each product was applied to three different materials: filter paper, fabric (97% cotton; 3% spandex), and PIG® Oil-Only Absorbent Mat (Polypropylene) (New Pig Corp., Tipton, PA, USA). BotaniGard in mineral oil ("BGM formulation") and Aprehend were applied to each material and left to aerate for 24 h in the dark at 25°C. The lid of a 9 cm petri dish lid was lined with each treated material. Petri dishes had a hole on the side for flies to be aspirated in. Twenty *Z. cucurbitae* flies were aspirated into each treated petri dish and left for 15 min to ensure flies had sufficient time to walk around in the dish. The flies were then released into 30 × 30 × 30 cm

mesh cages with water, sugar, and yeast hydrolysate. Mortality was monitored daily for 14 d. Each day, dead flies were removed from the cages and placed in humidity chambers to confirm infection status as described above.. Two replicates of each treated material were tested for each formulation, with reciprocal control cages for each material.

2.3.2 Addition of an Abrasive Material (Diatomaceous Earth)

Once we determined that the BotaniGard formulation was more effective than Aprehend, albeit at a higher concentration of spores, we further customized the formulation to be more effective for fruit flies. The first modification to the formulation of BotaniGard in mineral oil (2.0×10^{10} conidia per ml) was the addition of an abrasive material, diatomaceous earth (DE), at three concentrations (2.5%, 5%, 10%). DE is commonly used in gardens to control arthropod pests. In addition to absorbing insects' cuticular lipids, DE has sharp edges that scratch the surface of the arthropod's exoskeleton and causes it to desiccate. Many studies have shown that the addition of DE can enhance the efficacy of fungal entomopathogens[223–225]. They hypothesize that the scratches made by DE to the insect's exoskeleton improve the adherence and penetration of fungal hyphae. The formulation containing each concentration of DE was applied to 10 × 10 cm strips of white cotton fabric and then left to aerate for 24 hours in the dark at 25°C. A 50 ml conical bottom centrifuge tube (Thermo Fisher Scientific Inc., Waltham, MA) with a 1 cm diameter hole at the bottom of the tube, which served as an exit hole for the flies, was lined with the treated fabric. A group of twenty mixed-sex *Z. cucurbitae* were then placed into the top end of the tube and the lid was closed. The small opening on the bottom of the tube was inserted into a 30 × 30 × 30 cm mesh cage and the flies were allowed to exit the tube on their own (via walking) and enter the cage. It was not possible to control the amount of time spent in the tube. We could not force the flies to exit as flies that attempted to fly in the tube became stuck on the

oily treated fabric. The flies spent anywhere from two seconds to two minutes in the tube. Each treated fabric was used two times for a total of forty flies and three replicate cages were set up for each treatment. Fabric treated with only mineral oil was used as a control. Each cage contained water, sugar, and yeast hydrolysate, and mortality was monitored daily for fourteen days. Each day, dead flies were removed from the cages and placed in humidity chambers to confirm infection status as described above in section 2.3.1.

2.3.3 Test of DE-Incorporated Formulation on Three Fruit Fly Species

Upon determining that 10% DE significantly improved the efficacy of the BotaniGard formulation diluted in mineral oil ("BMD formulation" [BotaniGard + mineral oil + 10% DE] on fabric), we tested it on *C. capitata*, *B. dorsalis*, and *Z. cucurbitae*. Each species was exposed to the modified formulation or a control (BMD formulation without BotaniGard) using the fabric-lined tube method described above in section 2.3.2. Individual treated fabric strips were used for each DE percentage. Each treatment had three replicate cages with 40 flies per cage. Each day, dead flies were removed from the cages and placed in humidity chambers to confirm infection status as described above in section 2.3.1.

2.4 Horizontal Transmission of Formulation

2.4.1 Horizontal Transmission – Forced Male Contact

In this experiment, males were forced to contact the fungal spores (naturally by walking over the spores) to determine if spore-contaminated males were capable of transferring the spores onto naïve females during courtship and mating. Pupae of *C. capitata*, *B. dorsalis*, and *Z. cucurbitae* were placed in individual 10 ml plastic cups. The sex of the newly emerged flies were

visually determined, and they were placed in their respective male or female cages to prevent mating. The flies were provided with water, sugar, and yeast hydrolysate and maintained for 14 days for *Z. cucurbitae* and *B. dorsalis* and 10 days for *C. capitata* until they were sexually mature. Groups of twenty unmated sexually mature females were transferred to 30 × 30 × 30 cm mesh cages. Twenty males were forced to contact the BMD formulation by using the method described above in section 2.3.2. The males were allowed to walk out of the treatment tube directly into the cage containing untreated females. Two treated and two control cages were set up for each species. Each day, dead male and female flies were counted and removed from the cages and placed in humidity chambers to confirm infection status as described above in section 2.3.1.

2.4.2 Horizontal Transmission – Passive Male Contact

In this experiment, we examined whether males will voluntarily acquire a lethal quantity of spores and transfer enough spores to kill sexually mature females. We set up six cages. In three of the cages, we hung an inverted yellow plastic cup (12 cm height x 9 cm diameter opening; Universal Distribution Center LLC, Edison, NJ) lined with BMD formulation-treated fabric. The other three cages received control cups lined with untreated fabric. Each cup was aerated for 24 h prior to placing in the cages. A species-specific male lure plug cuelure (C-L; 4-(*p*-acetoxyphenyl)-2-butanone; Scentry Biologicals Inc., Billings, MT) for *Z. cucurbitae*, methyl eugenol (ME; 4-allyl-1, 2-dimethoxybenzene-carboxylate; Scentry Biologicals Inc., Billings, MT) for *B. dorsalis,* and trimedlure (TML; t Butyl-4(or5)-chloro-2-methyl cyclohexane carboxylate; Scentry Biologicals Inc., Billings, MT) for *C. capitata* was hung inside their respective cups to encourage males to enter. We then released 20 male and 20 female unmated

sexually mature flies (14±2 days old) into each cage. Each cage contained water, sugar, and yeast hydrolysate, and mortality was monitored daily for fourteen days, including the sex of the dead fly. Dead flies were removed from the cages and placed in humidity chambers to confirm infection status as described above in section 2.3.1.The spore-treated cups remained in the cage throughout the duration of the experiment.

2.5 Germination & Formulation Longevity

Next, we determined the longevity of the BMD formulation under simulated field conditions. In each location, six inverted plastic yellow cups (Universal Distribution Center LLC, Edison, NJ) were hung side by side. Five cups were lined with fabric saturated with the BMD formulation. One of the treated fabric liners was used to determine spore germination rates and the other four for *Z. cucurbitae* mortality bioassays. One cup without a treated liner contained a HOBO® MX2300 Series Data Logger (Onset Computer Co., Cape Cod, MA) to monitor temperature and relative humidity. On the roof of Gilmore Hall at the University of Hawaii at Manoa, we selected one location that was exposed to direct sunlight for most of the day and another location that was shaded for most of the day. We also hung cups in the laboratory as a control (25±1º C; 70±5% RH). Treated cups were tested for 12-week period during the months of May to August.

Viability of the spores were assessed at 0, 1, 2, 3, 4, 6, 8, and 12 weeks post environmental exposure by measuring the germination rate of the spores. We followed the protocol in Shikano et al. 2019 with some modifications [226]. A one cm^2 piece of fabric was cut from the treated fabric and placed in a glass vial containing 5ml of odorless kerosene (Klean Heat Kerosene Alternative, Klean Strip, Memphis, TN). The vial was vortexed for one minute to release the spores from the fabric. The spore suspension was then plated on Sabouraud Dextrose

Agar (SDA) (10 cm petri dish) by pipetting three 10μl drops on each plate and gently tilting in a circular motion to spread the droplets without the droplets touching each other. Plates were then incubated at 25°C for 18 hours. After incubation, spores were counted under a phase-contrast microscope at 400x zoom. Spores were considered germinated when the germ tube was longer than the diameter of the conidia. The first 300 conidia were counted per drop and the average of all three drops was used to estimate the germination rate for each plate. There were three SDA plates used per location. The initial (week 0) germination rate was measured the day the BMD formulation was made, which was one day before the treated cups were hung at their respective locations.

The efficacy of freshly treated fabric (week 0) was tested following the tube method described above in section 2.3.2. At week 2, 4, 6, and 8, we collected the weathered formulation-soaked fabric strips from each location and lined the inside of a 50 ml centrifuge tubes and tested for fly mortality as described previously (method described in 2.3.2). Each treatment had three fabric strips; each strip had two groups of 15 flies (30 per treated strip) pass through per replicate. Three replicate cages per treatment were run simultaneously, with each cage containing 30 flies (90 flies total). Each cage contained water, sugar, and yeast hydrolysate, and mortality was monitored daily for fourteen days. Dead flies were removed from the cages and placed into small cups with a damp paper towel to determine if the flies died from fungal infection.

2.6 Formulation Optimization

2.6.1 Changing the Carrier Oil

The mineral oil used in our BMD formulation may be a potential deterrent to the flies. Therefore, we compared the attraction of *Z. cucurbitae* to alternative oils, which included soybean oil, canola oil, peanut oil, (J.M. Smucker Co., Orrville, OH) and castor bean oil (NOW foods Inc., Bloomingdale, IL). Pieces of fabric (7.5 × 7.5 cm) were soaked with each oil and let drip dry and aerate for 24 hours prior to testing. One oil-soaked fabric piece was placed in a 10 cm petri dish and a cotton wick (3.75 cm) soaked in a 9:1 water-yeast hydrolysate solution was placed in the center of the fabric piece. Four fabric pieces, each treated with a different oil (canola, soybean, peanut, and castor oils), with protein wicks were then placed in separate corners of a 40 × 40 × 60 cm cage containing 250 ± twenty mixed-sex flies. The petri dish lids were removed at the same time and a timelapse recording for each petri dish was taken for one hour. Each time lapse video was analyzed to determine the number of visitations to each oil sheet. Three cages of flies were run simultaneously, with the position of the oil-treated fabrics randomized in each cage to account for positional bias. Flies were starved of protein for one day prior to testing. The most preferred oil (i.e., canola oil) was then tested against mineral oil (Heavy Mineral Oil; Fisher Scientific Co., Fair Lawn, NJ) using the same methods, except with only two petri dishes per cage.

2.6.2 Adding a Phagostimulant

Incorporation of a phagostimulant, such as sugar, could increase the spore pick-up rate by encouraging the flies to probe at the formulation. Two sugar sources were tested, table sugar (C&H Sugar Co., Crockett, CA) and Grandma's Original unsulphured molasses (B&G Foods Inc., Parsippany, NJ). Both sugar sources were incorporated into the BotaniGard + canola oil + 10% DE formulation at a rate of 1 part sugar source to 10 parts formulation. Pieces of fabric (7.5 × 7.5 cm) were soaked with the formulation with or without the sugar source and left to aerate

for 24 h. They were then placed in individual 10 cm petri dishes. One fabric piece treated with sugar-added formulation was placed in a 40 × 40 × 60 cm cage with a piece of fabric treated with the formulation without sugar for a two-choice test. Each cage contained 250 ± 20 flies and three cages were set up for each sugar source. Time lapse videos were recorded and analyzed as described above (section 2.6.1). The flies were sugar-starved for one day prior to testing.

2.6.3 Thickening the Formulation

We added thickening agents to the formulation to reduce settling of spores in the oil and to increase adherence of the formulation on the flies. Three thickening agents were separately incorporated into the formulation with the preferred carrier oil (BotaniGard + canola oil + 10% DE). The thickening agents were glyceride flakes (MDF) (Mono and Diglyceride flakes, Modernist Pantry LLC., Eliot, ME), Dermofeel Viscolid MB (DV) (Evonik Co., Hopewell, VA), and cornstarch (ACH Food Companies Inc., Chicago, IL). The MDF and DV thickening agents were added to the formulation at a concentration of 10% (or 1g per 10 ml of oil) to achieve a Vaseline-like consistency. The cornstarch was directly mixed into the formulation at a rate of 1.4g/ml of oil. To dissolve the MDF and DV, canola oil was heated to 60° C (140° F).The oil containing MDF or DV was cooled to room temperature before mixing in the DE and BotaniGard. All of the thickeners produced a formulation with a consistency similar to petroleum jelly.

Thickening the formulation eliminated the need for a fabric lining in the cups.Thus, approximately 3g of each thickened formulation was applied evenly to the inside of 454 ml yellow plastic cups. Four cups treated with each thickened formulation were hung in a direct sun-exposed area and shaded area on the roof of Gilmore Hall and in the laboratory as described previously (section 2.5) to assess the impacts of the thickeners on spore longevity. Germination

rates were assessed every two weeks (0, 2, 4, 6, and 8 weeks) using the methods described in section 2.5, except instead of cutting a 1 cm^2 piece of treated fabric, a 1 cm^2 area inside the cup was swabbed. Additionally, we hung up cups containing fabric liners treated with non-thickened formulation for comparison.

In addition to spore viability, we tested the effectiveness of the weathered thickened formulations on fly mortality. Mortality tests were conducted with the same thickened formulations described above, except that we did not test the DV-thickened formulation. The cornstarch and glyceride flake-thickened formulations were applied to modified 50 ml centrifuge tubes (described in section 2.3.2). The treated tubes were then hung inside an inverted yellow plastic cup and hung in a direct sun-exposed area and a shaded area on the roof of Gilmore Hall and in the laboratory. This experiment was conducted at the same time as the spore viability tests of the weathered thickened formulations. Mortality tests were conducted every two weeks (0, 2, and 4 weeks) as described in section 2.3.2. Briefly, the treated tubes were removed from the yellow cups and a mixed-sex group of 25 *Z. cucurbitae* were passed through each tube to allow all flies to walk over the formulation. Two replicates per thickening agent and location were used, with 25 flies per replicate (50 flies total per treatment and location). Controls for each thickened formulation without BotaniGard were also tested. Flies exited the tubes into a 30 × 30 × 30 cm mesh cage and were provided water, sugar, and yeast hydrolysate. Mortality was monitored daily for 14 days, and sporulation of cadavers confirmed. Next, we tested whether thickening the formulations would reduce runoff of the formulation in the inverted cups at various temperatures. Runoff tests were performed in incubation chambers set to one of four constant temperatures (40, 35, 30, and 25°C). Inverted yellow plastic cups (Universal

Distribution Center LLC, Edison, NJ) were weighed to the nearest 0.01 g and re-weighed after each thickened formulation was applied. The treated cups were then hung in the incubation chambers and weighed weekly for two weeks to determine the amount of formulation that dripped out of each cup. Three formulations were tested with a fabric substrate and without a fabric substrate for a total of six treatments: 1) non-thickened canola oil on fabric, 2) cornstarch-thickened canola oil on fabric, 3) glyceride flake-thickened canola oil on fabric, 4) non-thickened canola oil directly on cup, 5) cornstarch-thickened canola oil directly on cup, 6) glyceride flake-thickened canola oil directly on cup. Four replicate cups were set up for each treatment and temperature.

2.6.4 Incorporating Liquid Lure

Plugs of male parapheromones to lure male fruit flies are standard practice in monitoring populations and for the male annihilation technique. For our formulation, we hypothesized that incorporating a liquid form of the lures directly into the formulation would increase visitation and potentially oral probing. Therefore, C-L was added to the non-thickened canola oil formulation (BotaniGard + canola oil + 10% DE) for *Z. cucurbitae* and ME for *B. dorsalis*. Lures were added to the formulation at 0.1, 1, and 10%. The lure-incorporated formulations were then assessed for effects on spore viability and fly attraction. Three pieces of fabric (7.5 x 7.5 cm) were saturated with each of the lure-added formulations and hung in the lab out of direct light. Pieces of fabric saturated with the formulation without lure served as the control. Spore viability over time was assessed by cutting a one cm^2 piece of the treated fabric each week for three weeks, and germination rates were determined following the methods described in section 2.5.

For testing male attraction, we treated the fabric with the same lure-incorporated formulation as described above. Two-choice tests with controls that did not have any lure

incorporated (as in section 2.6.2.) were tested. Each cage contained 250 ± 20 flies with three replicate cages per treatment. Time lapse videos were recorded and analyzed as described above (section 2.6.1.). *Z. cucurbitae* (14 ± 2 days old), *B. dorsalis* (14 ± 2 days old), and *C. capitata* (10 ± 2 days old) flies were used to ensure males were sexually mature and at the age when they would be most attracted to parapheromones [227,228].

2.7 Final Comparison (Testing Improvements & Spore Pick-up)

2.7.1 Spore Pickup Rates

We assessed the impacts of combining the most attractive carrier oil with thickening the formulation and incorporating a liquid lure on the numbers of spores picked up by male flies. All treatment formulations contained BotaniGard and 10% DE with the following remaining ingredients: (1) canola oil, (2) canola oil + cornstarch, (3) canola oil + lure, (4) canola oil + cornstarch + lure. We also included the(5) BMD formulation (BotaniGard, mineral oil, and 10%DE). All non-thickened formulations were applied to fabric while thickened formulations were directly applied to the inside of a modified 50 ml centrifuge tube. Each tube was aerated for 24 h prior to exposing flies. Five male *Z. cucurbitae* were then passed through the treated tubes into a 30 × 30 × 30 cm cage where they were then recaptured and placed into a 5 ml glass vial containing 1 ml of odorless kerosene (i.e., five flies in 1 ml of odorless kerosene). The vial was vortexed for 1 min to release the spores from the flies and the spore suspension was loaded onto a hemocytometer. Spore counts were performed at 400x magnification under a phase contrast microscope and the estimated numbers of spores per ml (i.e., number of spores per five flies) was calculated. Six replicate vials were counted per formulation, with five flies per vial and three 10 µl drops of spores were counted per replicate.

2.7.2 Horizontal Transmission – Passive Contact

We conducted a passive horizontal transmission experiment to determine how the final formulation, which contains a thickening agent and male lure (C-L), compares to the non-thickened mineral oil and canola oil formulations. All treatment formulations contained BotaniGard and 10% DE with the following remaining ingredients: (1) canola oil, (2) canola oil + cornstarch, (3) canola oil + lure, (4) canola oil + cornstarch + lure, and (5) BMD (BotaniGard, mineral oil, and 10%DE). We followed the methods described in section 2.4.2 with some modifications. All non-thickened formulations were applied to fabric lining the inside of yellow plastic cups while thickened formulations were directly applied to the inside wall of the cups. Each treated cup was aerated for 24 hours. Twenty male and twenty female sexually mature virgin *Z. cucurbitae* were placed into each cage. A treatment cup with a C-L plug was then hung in each cage. Daily mortality was recorded for 20 d, and sporulation of cadavers confirmed. Treatment cups remained in the cage throughout the duration of the experiment. Four replicate cages were set up for each treatment.

2.7.3 Horizontal Transmission – Passive Contact – Final Formulation vs. Mineral Oil Formulation

Lastly, we tested our final formulation (canola oil + cornstarch + lure + DE) applied directly to the inside of the yellow cup against our initial formulation (mineral oil + DE), which was applied to fabric lining the inside of the cup. This passive horizontal transmission experiment was conducted as described in section 2.4.2 and 2.7.3 using *Z. cucurbitae* (with C-L plugs) and *B. dorsalis* (with ME plugs). Three replicate cages were set up for each treatment.

2.8 Statistical Analyses

Lethal concentrations were calculated using a generalized linear model using a binomial distribution and probit link. Probit analysis assumed that the percent response (fly deaths) is related to the log concentration (concentration of spores) as the cumulative normal distribution [229]. Lethal concentrations with 100% and 0% mortality were excluded from the data analysis. The germination of *B. bassiana* spores were analyzed by generalized linear model (GLM) using a binomial distribution. The survival times, mean, and median survival times were obtained by Kaplan-Meier survival estimator [230,231]. In all mortality trials, flies that survived beyond 14 days were censored from the data set. The mean survival time of the Kaplan-Meier estimation becomes biased when more than 30% of the data is censored while the median survival time is minimally biased [232]. Survival differences between the entire distributions of survival curves were compared using nonparametric log-rank tests weighing each death with the Kaplan-Meier estimate of survival as a log-rank (rho = 0) [233,234]. Spore germination percentages and thickening agent run-off tests were analyzed using three-way repeated measures ANOVA with multiple pairwise comparisons to determine the group mean differences with Bonferroni adjustment. Choice tests for different oils, lure concentrations, and phagostimulants were analyzed using GLM using a Poisson distribution with least-squares pairwise comparison. All analyses were performed on R version 4.1.0.

3. Results

3.1 Lethal Concentrations

Pairwise comparisons using a log rank test of the lethal concentration (LC) curves with a bonferroni adjustment indicated that there was a statistical significance between each species in trial one and two. With the closest curve in trial one being that of *Z. cucurbitae* and *B. dorsalis* ($p = 0.034$) and the curves between *B. dorsalis* and *C. capitata* ($p = <0.0001$), and *Z. cucurbitae* and *C. capitata* ($p = <0.0001$). There was a greater significance between the LC curves of *Z. cucurbitae* and *B. dorsalis* ($p = <0.0001$) in trial 2 (Figure 1.;Figure 2.; Table 1.). In the second trial, we increased the number of spore concentrations and obtained better lethal concentration curves. Along with the pairwise comparison, the probit analysis LC_{50} or LC_{90} showed that *Z. cucurbitae* had a longer mean and median survival time at most concentrations (Table 2). *Z. cucurbitae* required a higher concentration of spore during both iterations of the trial. This is why we used *Z.cucurbitae* as the primary testing fly going forward.

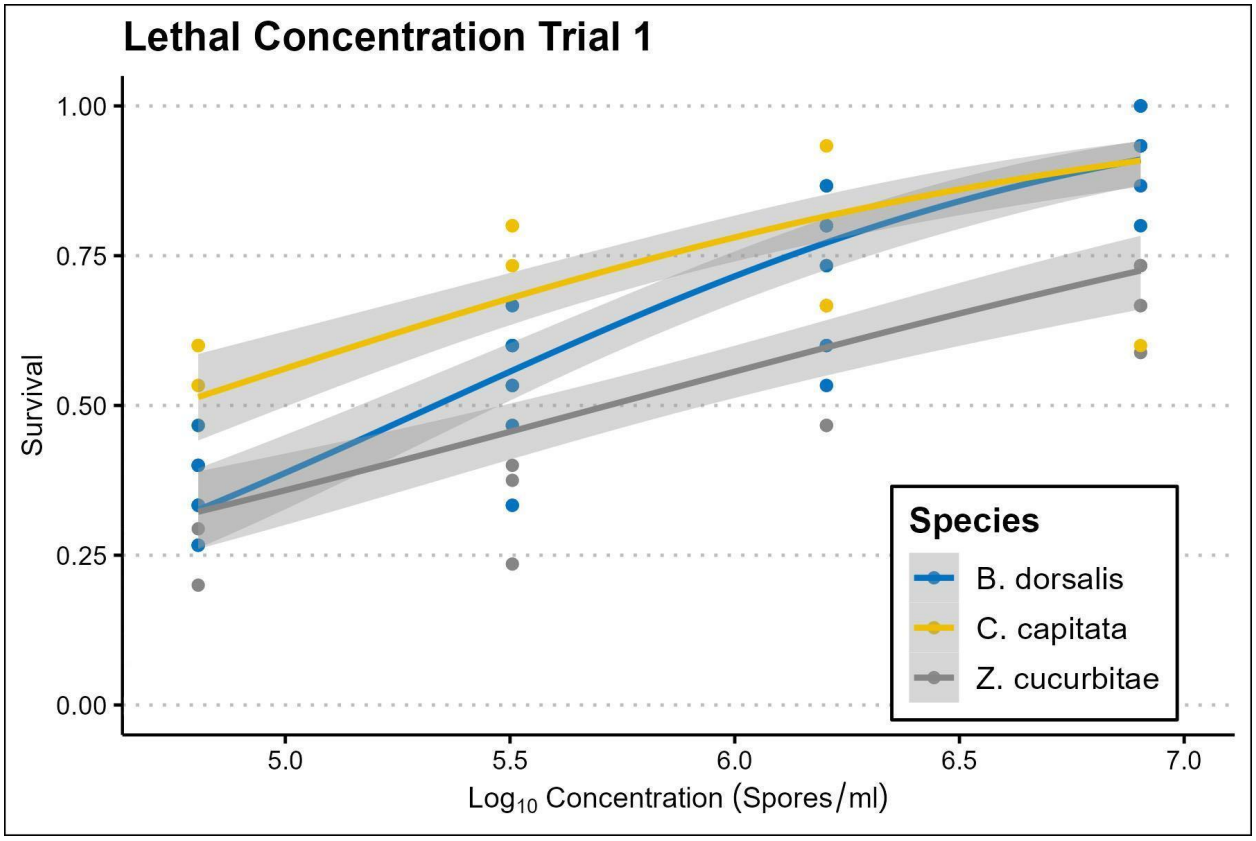

Figure 1. Lethal concentration survivorship curves from probit analysis of Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae exposed to multiple concentrations of Botanigard® ES in aqueous suspension. The data is from Trial 1. Gray shading indicates 95% confidence intervals. Data from the control and highest concentration (2.0e+08 spores per/ml), which mainly produced 0 and 100% mortality, were excluded from the analysis.

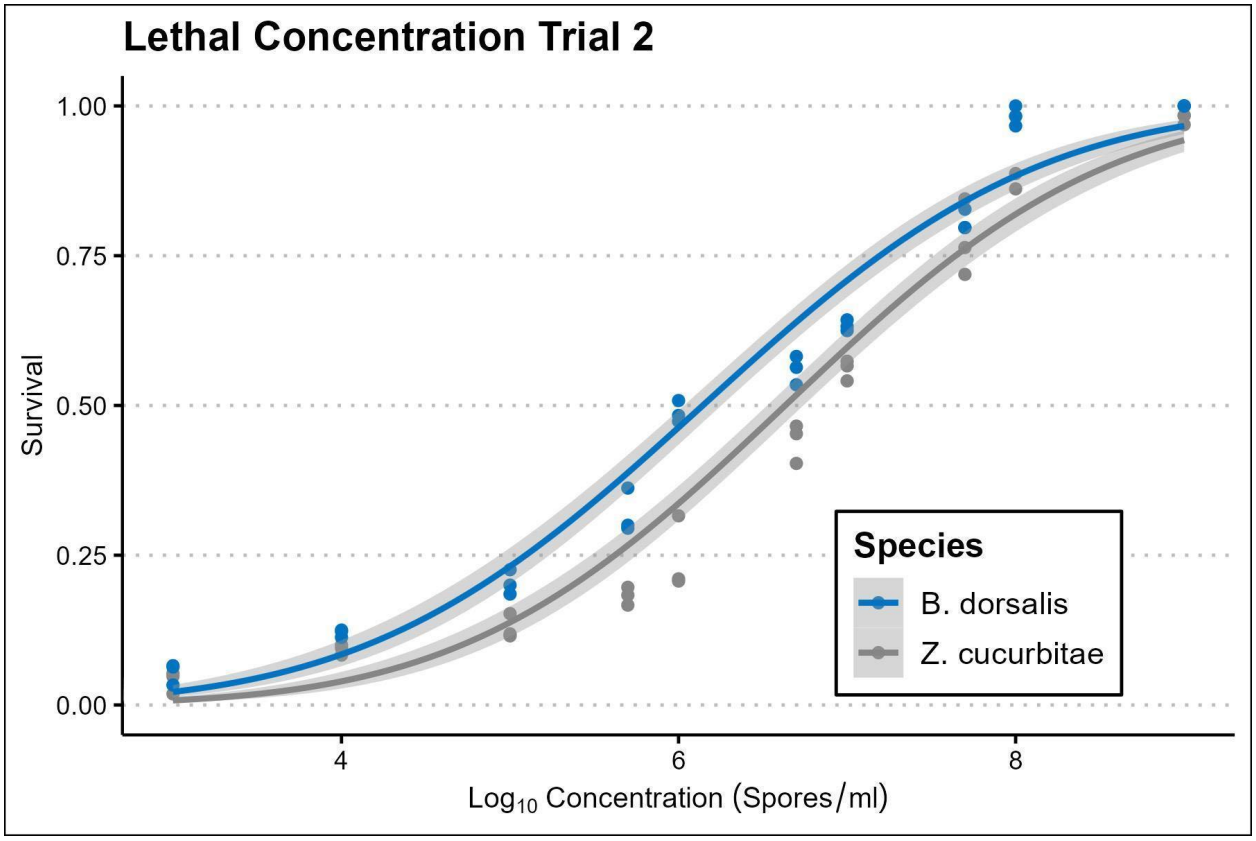

Figure 2. Lethal concentration survivorship curves from probit analysis of Bactrocera dorsalis, and Zeugodacus cucurbitae exposed to multiple concentrations of Botanigard® ES in aqueous suspension. The data is from Trial 2. Gray shading indicates 95% confidence intervals. Data from the control which produced 0% mortality, were excluded from the analysis.

Table 1. Probit analysis results, consisting of lethal concentrations (LC50 and LC90) for Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae, exposed to multiple concentrations of Botanigard® ES in aqueous suspension. Trial 1 used six serially diluted concentrations of Botanigard® ES and Trial 2 used 10 serial dilutions.

		Trial 1					
Species	LC level	LC (spores/ml)	Lower 95% CL	Upper 95% CL	$\chi 2$	df	Slope
B. dorsalis	50	2.18E+05	1.52E+05	2.98E+05	3.83	3	0.91
B. dorsalis	90	5.57E+06	3.73E+06	9.27E+06	3.83	3	0.91
C. capitata	50	5.62E+04	2.30E+04	1.02E+05	0.04	2	0.62
C. capitata	90	6.61E+06	3.37E+06	1.88E+07	0.04	2	0.62
Z. cucurbitae	50	4.61E+05	1.07E+05	1.24E+06	17.92	4	0.71
Z. cucurbitae	90	2.95E+07	8.59E+06	3.53E+08	17.92	4	0.71

Trial 2							
Species	LC level	LC (spores/ml)	Lower 95% CL	Upper 95% CL	$\chi 2$	df	Slope
B. dorsalis	50	1.17E+06	4.98E+05	2.31E+06	42.44	15	0.66
B. dorsalis	90	1.00E+08	4.76E+07	2.73E+08			
Z. cucurbitae	50	3.44E+06	1.92E+06	5.64E+06	42.98	20	0.68
Z. cucurbitae	90	2.66E+08	1.54E+08	5.20E+08			

Table 2. Kaplan-Meier survival times for Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae, exposed to multiple doses of Botanigard® ES in aqueous suspension. Mean and median survival times and total dose mortality show the variability in each cohort's susceptibility to fungal infection after exposure to B. bassiana.

Lethal Concentration Survival Times per Dose Exposure

Trial 1

		Control	$6.4x10^4$	$3.2x10^5$	$1.6x10^6$	$8.0x10^6$	$4.0x10^7$	$2.0x10^8$
C. capitata	Mean† [a]	9.74	9.03	7.79	6.35	4.61	1.24	1.00
	(± SE)	(0.450)	(0.474)	(0.426)	(0.402)	(0.361)	(0.045)	(0.000)
	Median† [a]	>14	13	7	6	4	1	1
	0.95 LCL	-	8	5	5	2	1	1
	0.95 UCL	-	-	9	7	4	1	1
	Mortality	45.19%	51.85%	67.41%	81.48%	91.11%	100.0%	100.0%
B. dorsalis	Mean† [a]	-	-	9.70	7.91	4.78	2.26	1.10
	(± SE)			(0.378)	(0.372)	(0.304)	(0.171)	(0.038)
	Median† [a]	>14	>14	10	6	5	1	1
	0.95 LCL	-	-	8	6	4	1	1
	0.95 UCL	-	-	-	8	5	2	1
	Mortality	6.67%	34.07%	55.56%	73.33%	93.33%	99.26%	100%
Z. cucurbitae	Mean† [a]	-	-	-	8.70	7.74	4.14	1.77
	(± SE)				(0.451)	(0.424)	(0.260)	(0.189)
	Median† [a]	>14	>14	>14	9	7	4	1
	0.95 LCL	-	-	-	7	5	3	1
	0.95 UCL	-	-	-	13	9	5	1
	Mortality	20.00%	35.04%	41.30%	60.00%	73.72%	97.04%	98.52%

Trial 2

		Control	$1.0x10^3$	$1.0x10^4$	$5.0x10^4$	$1.0x10^5$	$5.0x10^5$	$1.0x10^6$	$5.0x10^6$	$1.0x10^7$	$1.0x10^8$	$1.0x10^9$
B. dorsalis	Mean† [a]	-	-	-	-	-	9.93	9.33	8.24	6.44	4.01	2.21
	(± SE)						(0.341)	(0.347)	(0.378)	(0.312)	(0.228)	(0.106)
	Median† [a]	>14	>14	>14	>14	>14	>14	8	6	6	3	2
	0.95 LCL	-	-	-	-	-	8	7	6	5	3	1
	0.95 UCL	-	-	-	-	-	-	-	8	6	4	2
	Mortality	2.20%	5.43%	12.00%	20.45%	31.84%	48.88%	55.95%	63.31%	84.75%	98.31%	100.0%
		Control	$1.0x10^3$	$1.0x10^4$	$1.0x10^5$	$5.0x10^5$	$1.0x10^6$	$5.0x10^6$	$1.0x10^7$	$5.0x10^7$	$1.0x10^8$	$1.0x10^9$
Z. cucurbitae	Mean† [a]	-	-	-	-	-	-	-	9.57	7.03	5.28	2.95
	(± SE)								(0.343)	(0.338)	(0.283)	(0.217)
	Median† [a]	>14	>14	>14	>14	>14	>14	>14	10	6	5	1
	0.95 LCL	-	-	-	-	-	-	-	8	5	5	1
	0.95 UCL	-	-	-	-	-	-	-	-	6	5	2
	Mortality	2.15%	4.02%	9.29%	12.94%	18.24%	24.42%	43.93%	56.00%	77.40%	90.71%	97.35%

† Survival times derived from Kaplan-Meier analysis. [a] Dashes denote that estimation could not be performed because of low mortality.

3.2 Formulation Development

3.2.1 Testing Existing Products on Different Substrates

When *Z. cucurbitae* were forced to contact a dilution of BotaniGard in mineral oil (BGM) and the bed bug biopesticide, Aprehend, 69% of flies exposed to Aprehend were still alive 14 d post exposure compared to only 35% of flies alive after exposure to BGM ($\chi^2 = 57.9$, DF = 1, P= 3e-14; Figure 3). Survival differences showed the BGM formulation was more effective than Aprehend regardless of the substrate on which each formulation was applied (Figure 3): fabric ($\chi^2 = 43.3$, DF = 1, $P < 0.0001$), filter paper ($\chi^2 = 7.5$, DF = 1, $P = 0.006$), PIG® Oil-Only Absorbent Mat ($\chi^2 = 16.2$, DF = 1, $P < 0.0001$). BGM formulation was more effective on fabric than filter paper ($\chi^2 = 21.7$, DF = 1, $P < 0.0001$) and PIG® Oil-Only Absorbent Mat ($\chi^2 = 19.6$, DF = 1, $P < 0.0001$).

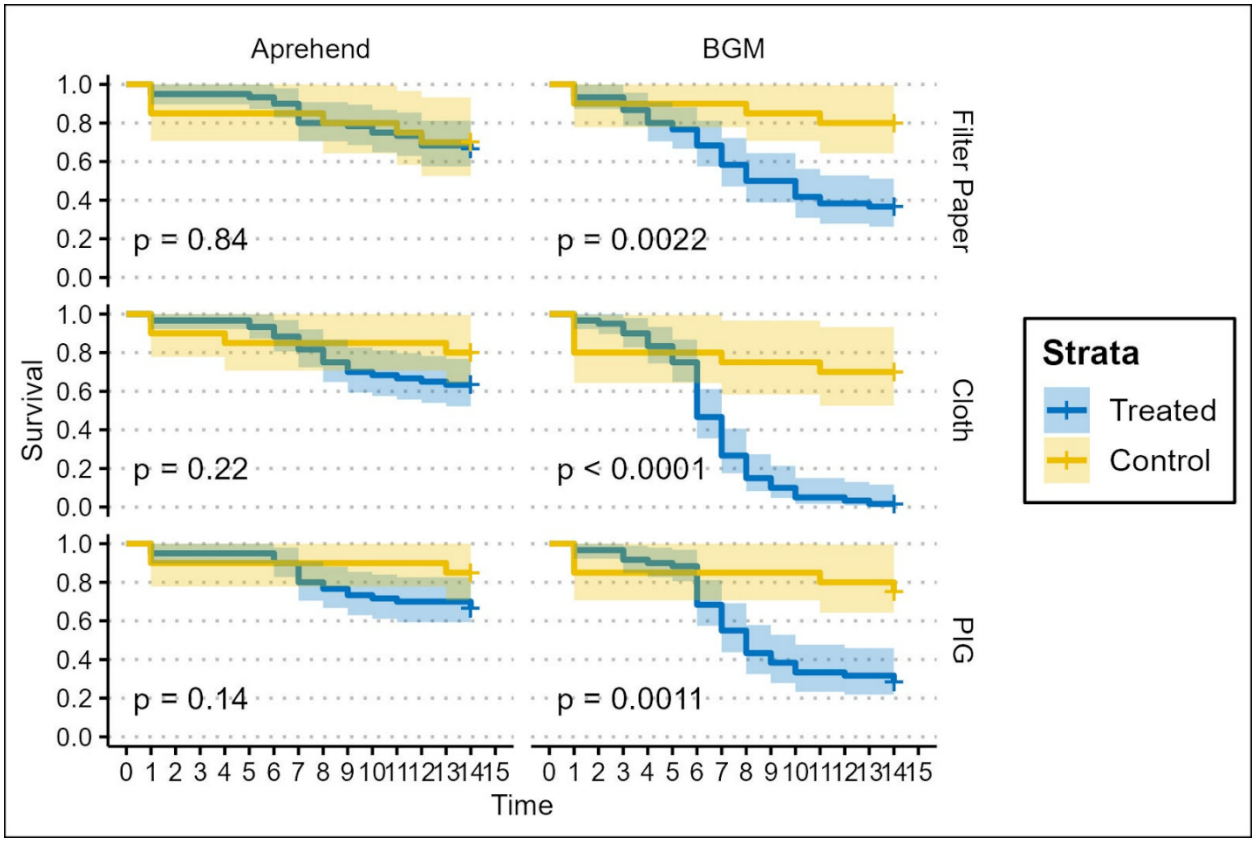

Figure 3. Kaplan-Meier survival curves of Aprehend® and the BotaniGard in mineral oil formulation (BGM) on three substrates: filter paper, cotton cloth fabric, and PIG® Oil-Only Absorbent Mat. Survival curves are shaded by 95% confidence intervals with individual p-values between each control and treatment. Zeugodacus cucurbitae flies were exposed to each treatment for five minute periods before release and monitoring for mortality. After 14 days exposure, all escaped and surviving flies were censored from the analysis. The BGM formulation treatment was statistically more effective than Aprehend® treatment on each treated substrate: filter paper ($p = 0.00041$), cloth ($p = <0.0001$), and PIG® Oil-Only Absorbent Mat ($p = <0.0001$).

3.2.2. Abrasive Trials Results

The incorporation of the diatomaceous earth (DE) to the mineral oil formulation killed *Z. cucurbitae* more quickly and increased overall mortality (Figure 4). Mortality was greatest when 10% DE was incorporated into the formulation as opposed to no DE (0% DE; $\chi^2 = 106$, DF = 1, $P < 0.0001$), 2.5% DE ($\chi^2 = 75.7$, DF = 1, $P < 0.0001$) and 5% DE ($\chi^2 = 66.6$, DF = 1, $P < 0.0001$).

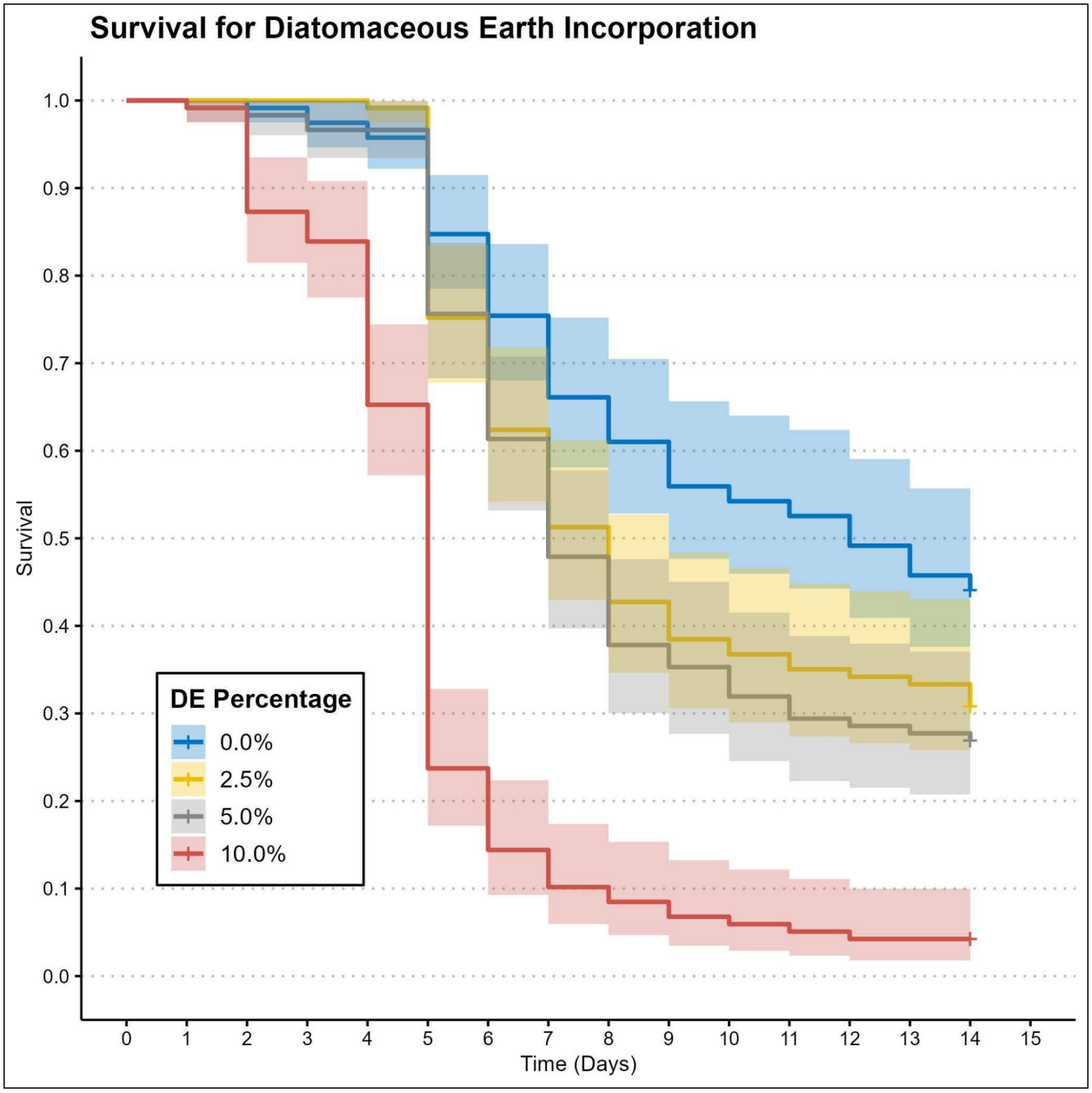

Figure 4. Kaplan-Meier survival curves of the BotaniGard in mineral oil formulation (BGM) with diatomaceous earth incorporated at 2.5, 5.0, and 10.0%. Survival curves of Zeugodacus cucurbitae are shaded by 95% confidence intervals. Flies were passed through 4-inch treated tubes and monitored for mortality for 14 days after exposure, all escaped and surviving flies were censored from the analysis.

3.2.3. Mortality Trials Results

Z. cucurbitae, B. dorsalis, and *C. capitata* all exhibited high mortality when they were forced to walk over the BMD (BotaniGard + mineral oil + 10% DE) formulation (Figure 5). *Z. cucurbitae* took longer to die than *C. capitata* ($\chi^2 = 38$, DF = 1, $P < 0.0001$) and *B. dorsalis* ($\chi^2 = 50.2$, DF = 1, $P < 0.0001$) (Table 3). Since *Z. cucurbitae* had the highest median survival time, it appeared to be the most resilient to *B. bassiana* infection. Therefore, it was used as the target fly for testing for most of my thesis research.

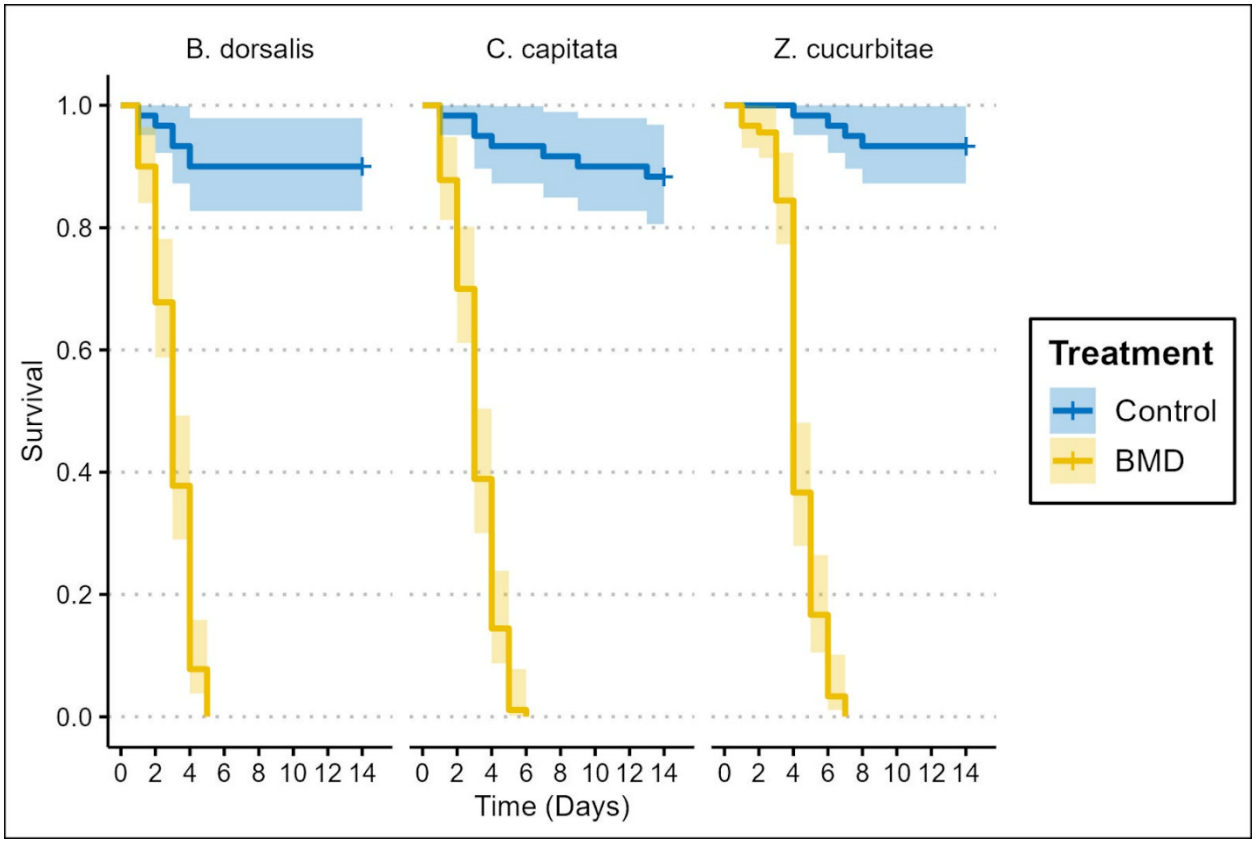

Figure 5. Kaplan-Meier survival curves of the BotaniGard in mineral oil formulation with diatomaceous earth incorporated at a concentration of 10.0% (BMD). Survival curves of Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae are shaded by 95% confidence intervals.

Table 3. Kaplan-Meier survival times for Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae, exposed to Botanigard® ES in mineral oil formulation containing 10% diatomaceous earth (BMD). Mean and median survival times, mortality and fungal infection percentage show the efficacy of the BMD formulation against each species.

Mortality following forced contact with the BMD formulation and fungal infection percentages							
Species	Treatment	% Mortality	Mean Survival Time (± SE) [a]	Median Survival Time	% Fungal Infection[b]	$\chi 2$	P
B. dorsalis	Treated	100%	3.03±0.12	3	93.33%	117	<0.0001
	Control	10%	-	>14	0.00%		
C. capitata	Treated	100%	3.12±0.13	3	96.67%	127	<0.0001
	Control	12%	-	>14	0.00%		
Z. cucurbitae	Treated	100%	4.33±0.12	4	95.56%	144	<0.0001
	Control	7%	-	>14	0.00%		

[a] Dashes denote that estimation could not be performed because of low mortality. [b] Confirmation of dead flies by fungal infection percentages were calculated from the mortality percentages.

3.3 Horizontal Transmission of Formulation

3.3.1. Forced Horizontal Transmission Trials Results

When males were forced to contact the formulation and then were released into cages containing sexually mature unmated females, significant female mortality occurred (Table 4). Female mortality was lower and lagged male mortality (Figure 6 A, C, E), which is indicative of females acquiring a low dose of spores from the males. Even with lower mortality in male *Z. cucurbitae* compared to the other species, female mortality was observed. Significant differences between control and treated sexes survival curves also showed there was a difference in the mortality times (Table 5).

Table 4. Kaplan-Meier survival times for Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae, which were forced to contact the BMD formulation. All three species showed female mortality and survival times followed the males at a slight lag.

Forced Horizontal Transmission Mortality following male contact to BMD formulation subsequently exposed to unexposed females

Species	Sex	Treatment	% Mortality	Mean Survival Time (± SE) [a]	Median Survival Time	% Fungal Infection [b]
B. dorsalis	Males	Control	5.0%	-	>14	0.00 %
	Females	Control	20.0%	-	>14	0.00 %
	Males	Treated	100%	4.12±0.12	4	95.12%
	Females	Treated	87.5%	8.25±0.55	7	94.29 %
C. capitata	Males	Control	60.0%	-	9.5	0.00 %
	Females	Control	25.0%	-	>14	0.00 %
	Males	Treated	100%	6.80±0.58	3	97.50%
	Females	Treated	82.5%	4.08±0.52	5	90.91%
Z. cucurbitae	Males	Control	0.00%	-	>14	0.00 %
	Females	Control	0.00%	-	>14	0.00 %
	Males	Treated	57.5%	10.1±0.58	9	91.30%
	Females	Treated	37.5%	12.1±0.40	14	85.71%

[a] Dashes denote that estimation could not be performed because of low mortality. [b] Confirmation of dead flies by fungal infection percentages were calculated from the mortality percentages.

Table 5. Kaplan-Meier log rank comparison of the BMD formulation between treatment and sex for Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae, with males forced into contact with the BMD formulation and subsequently exposed to sexually mature, unmated adult female flies.

Forced Horizontal Transmission Survival Differences				
Species	Log Rank Comparison	$\chi 2$	df	p
B. dorsalis	Control Male – Control Female	3.7	1	0.05
	Control Male – Treated Male	81.5	1	<0.0001
	Control Female – Treated Female	30.7	1	<0.0001
	Treated Male – Treated Female	56.2	1	<0.0001
C. capitata	Control Male – Treated Male	22.3	1	<0.0001
	Control Female – Treated Female	28.6	1	<0.0001
	Treated Male – Treated Female	11	1	<0.0001
Z. cucurbitae	Control Male – Treated Male	31.4	1	<0.0001
	Control Female – Treated Female	14	1	<0.0001
	Treated Male – Treated Female	7.3	1	0.007

3.3.2. Passive Horizontal Transmission Trials Results

When male and female flies were presented with an inverted yellow cup containing a male lure plug and the cup lined with the mineral oil formulation, mortality of both sexes was higher than their respective controls (Table 7). For *Z. cucurbitae* and *C. capitata,* female mortality lagged behind male mortality, indicating that males contacted more spores and/or contacted the spores earlier than females (Figure 6. D, F; Table 6). Since *Z. cucurbitae* and *C. capitata* females are not attracted to their respective male lures, the results suggest that the males entered the cup, contacted the spores, and transferred them to the females. *B. dorsalis* males and females died at a similar rate. This was likely because females are attracted to males who have fed upon ME, and we observed female *B. dorsalis* entering the cups and probing at the ME plug alongside the males (Figure 6 B). Further testing is needed to determine the affinity of *B. dorsalis* females to ME plugs without male contact. *C. capitata* had high rates of control mortality. This might be attributed to lower colony health or higher activity of the flies; *C. capitata* males were observed engaging in more intense/aggressive behaviors towards one another in the cage than the other two species.

Table 6. Kaplan-Meier survival times for Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae, which were passively exposed to the BMD formulation.

Passive Horizontal Transmission Mortality following unexposed male and females with access to a BMD formulation treated trap						
Species	Sex	Treatment	% Mortality	Mean Survival Time (± SE) [a]	Median Survival Time	% Fungal Infection [b]
B. dorsalis	Males	Control	8.33%	-	>18	0.00%
	Females	Control	6.67%	-	>18	0.00%
	Males	Treated	100.0%	5.19±0.19	5	90.00%
	Females	Treated	100.0%	6.49±0.21	6	84.44%
C. capitata	Males	Control	46.67%	-	>18	0.00%
	Females	Control	28.33%	-	>18	0.00%
	Males	Treated	100.0%	5.41±0.21	6	93.33%
	Females	Treated	100.0%	6.87±0.29	7	97.78%

Z. cucurbitae	Males	Control	8.33%	-	>18	0.00%
	Females	Control	11.67%	-	>18	0.00%
	Males	Treated	94.44%	7.9±0.39	7	95.29%
	Females	Treated	90.00%	11.1±0.46	10	93.83%

[a] Dashes denote that estimation could not be performed because of low mortality. [b]Confirmation of dead flies by fungal infection percentages were calculated from the mortality percentages.

Table 7. Kaplan-Meier log rank comparison of the BMD formulation between treatment and sex for Bactrocera dorsalis, Ceratitis capitata, and Zeugodacus cucurbitae, with sexually mature unmated adult males and females given access to a trap treated with BMD formulation.

Passive Horizontal Transmission Survival Differences				
Species	Log-Rank Comparison	χ2	Df	p
B. dorsalis	Control Male – Treated Male	69	1	<0.0001
	Control Female – Treated Female	96.9	1	<0.0001
	Treated Male – Treated Female	5.9	1	0.01
C. capitata	Control Male – Treated Male	39.8	1	<0.0001
	Control Female – Treated Female	70.6	1	<0.0001
	Treated Male – Treated Female	23.3	1	<0.0001
Z. cucurbitae	Control Male – Treated Male	102	1	<0.0001
	Control Female – Treated Female	63.8	1	<0.0001
	Treated Male – Treated Female	25.9	1	<0.0001

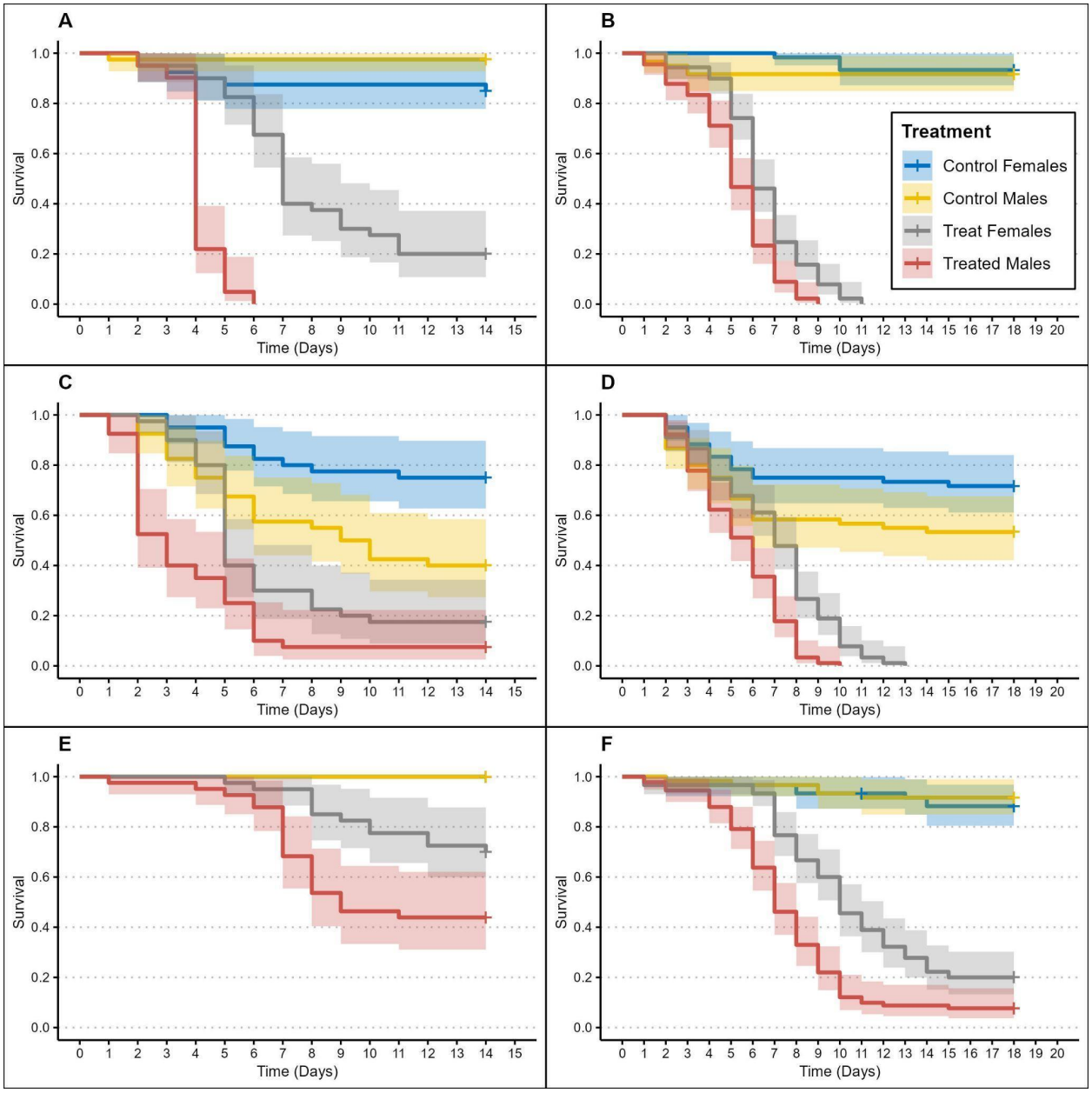

Figure 6. Kaplan-Meier survival curves of the BotaniGard in mineral oil formulation with diatomaceous earth incorporated at 10.0% (BMD) with 95% confidence intervals. Horizontal transmission trials for B. dorsalis ([A] forced; [B] passive), C. capitata ([C] forced; [D] passive), Z. cucurbitae ([E] forced; [F] passive). Flies were censored from the data if they escaped out of cages and after 14 days for forced trials and 18 days for passive trials.

3.4. Germination & Formulation Longevity

3.4.1. Germination Testing Results

Germination rates declined steadily over a 12 week period in all conditions (Figure 7). Least-squares pairwise comparison showed that germination rates of the BMD formulation declined significantly faster under full sun than in the lab ($p = 0.0015$), while there were no significant differences between lab and shade ($p = 0.36$), or shade and sun ($p = 0.07$).

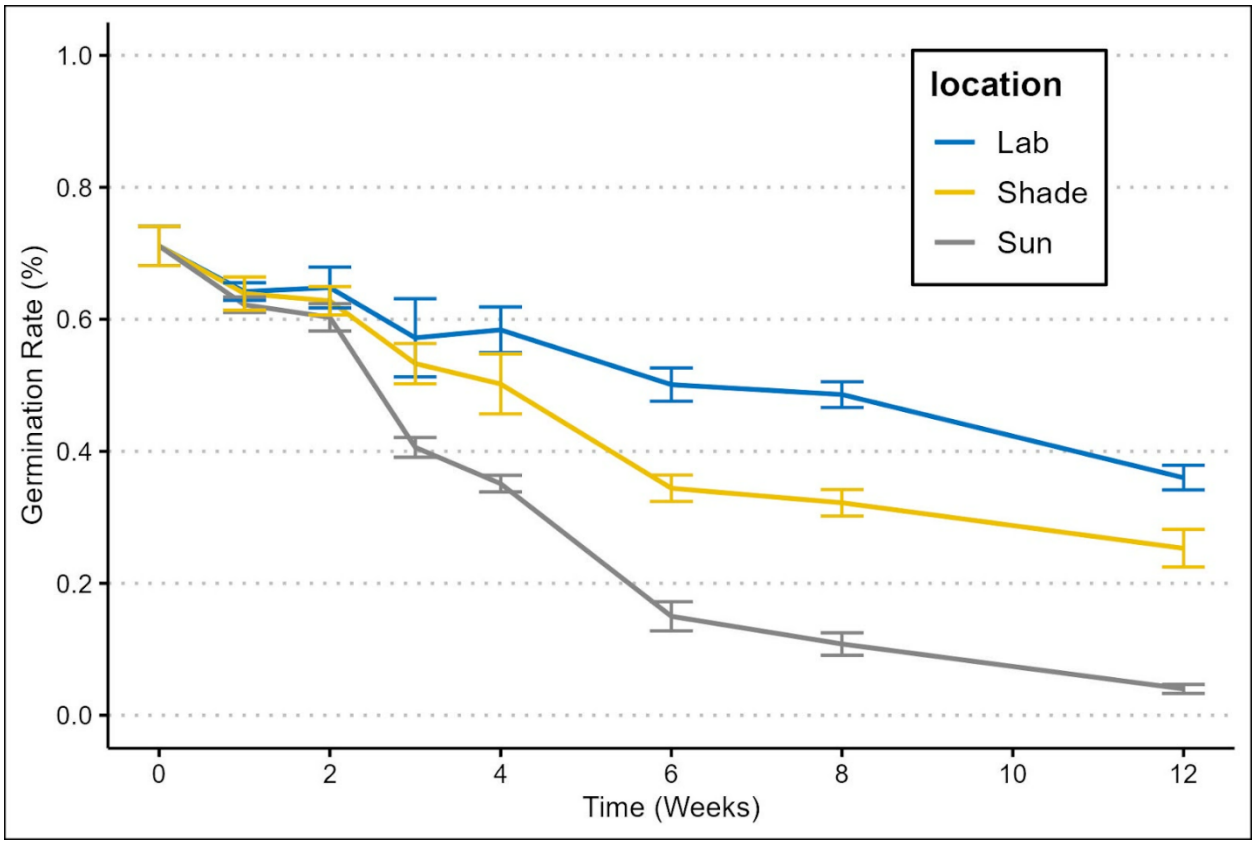

Figure 7. Average germination rates (± SE) of the BotaniGard in mineral oil formulation, with 10% diatomaceous earth incorporated (BMD), during 12 weeks of exposure in control (lab), direct sun (sun), and indirect sun (shade) conditions.

3.4.2. Weathered Formulation Mortality Trials Results

Fabric treated with the BMD formulation were hung inside inverted yellow cups under simulated field conditions on the roof of Gilmore Hall. Initial testing to confirm our expected mortality gave similar results as before at week 0 ($\chi 2 = 197$, df = 1, p = <0.0001). Compared to treated fabric stored in the laboratory, exposure of *Z cucurbitae* to the weathered treated fabrics in the shade and the sun resulted in significantly lower mortality (Figure 8; Table 8). Effectiveness of the treated fabrics in the lab decreased over time. By week four, the sun and shade treatments were rendered ineffective.

Table 8. Kaplan-Meier log rank comparison of the BMD formulation between treatment and location for Zeugodacus cucurbitae, with sexually mature unmated adults forced into contact with weathered strips of fabric soaked in the BMD formulation.

Weathered Formulation Survival Differences				
Week	Log-Rank Comparison	$\chi 2$	DF	p
0	Control–Lab	42	1	<0.0001
2	Control–Lab	39.8	1	<0.0001
	Control–Sun	1.3	1	0.3
	Control–Shade	6.7	1	0.009
	Lab–Sun	29.5	1	<0.0001
	Lab–Shade	16	1	<0.0001
	Sun–Shade	2.2	1	0.1
4	Control–Lab	25.4	1	<0.0001
	Control–Sun	0	1	1
	Control–Shade	0.2	1	0.7
	Lab–Sun	24.5	1	<0.0001
	Lab–Shade	21.1	1	<0.0001
	Sun–Shade	0.1	1	0.7

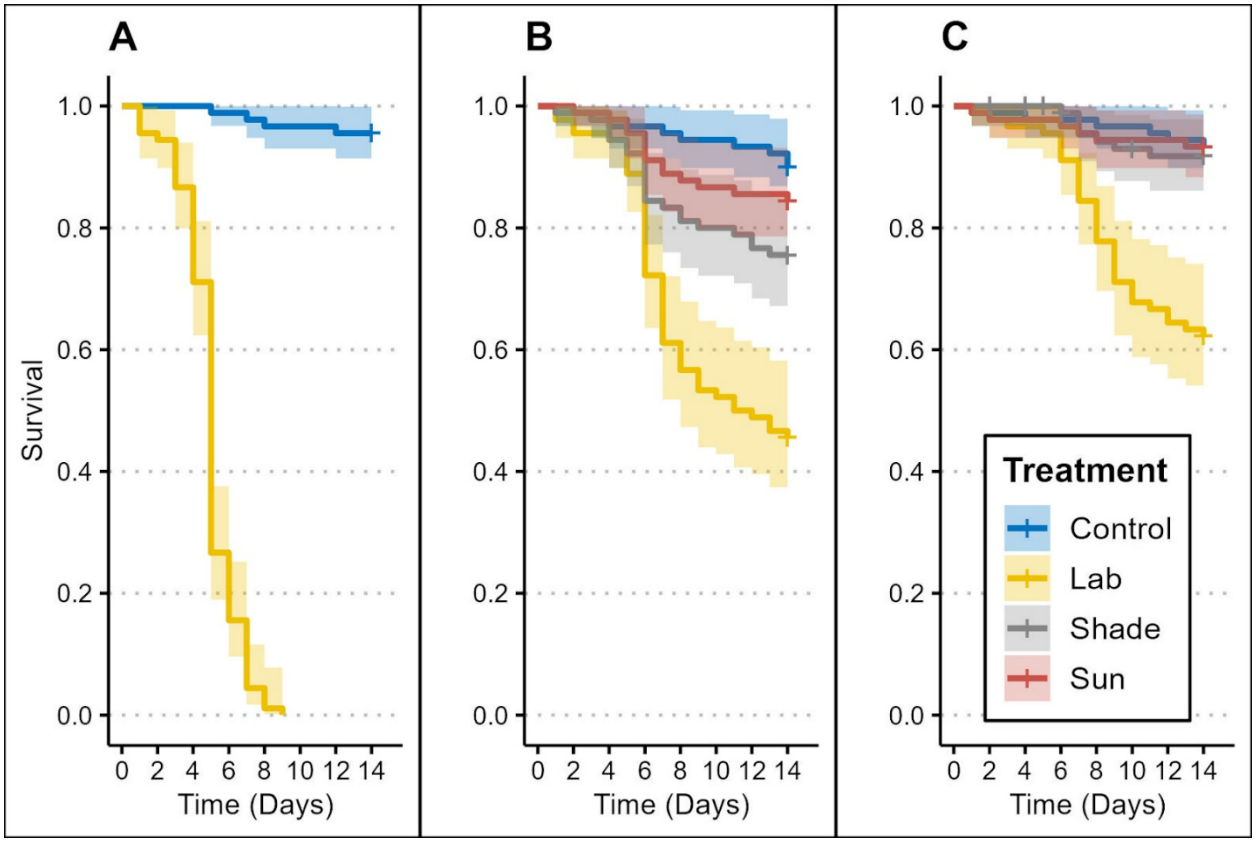

Figure 8. Kaplan-Meier survival curves with 95% confidence intervals of Z. cucurbitae that were forced to contact the weathered BMD formulation. BMD treated fabrics were exposed to three climatic conditions; control (lab), direct sun (sun), and indirect sun (shade). Mortality tests at two week intervals, A) week 0, B) week 2, C) week 4, were done to determine longevity and efficacy of the BMD formulation after initial exposure. Flies were censored from the data if they escaped out of cages and after 14 days.

3.5 Formulation Improvement

3.5.1. Carrier Oil Testing Results

The carrier oil significantly influenced the visitation of *Z. cucurbitae* to a yeast hydrolysate-treated cotton wick ($F = 7.923$, df = 4, $p = 0.004$). More *Z. cucurbitae* visited the protein wick on canola oil-soaked fabric than castor ($p = <0.0001$), peanut ($p = <0.0001$), or soybean oils ($p = <0.0001$) (Figure 9 A). The average time spent on the oil-treated fabric per visit

was not statistically significant between the oil types. I then compared canola oil and mineral oil in a choice test and found that canola oil was greatly preferred ($p > 0.0001$) or was far less repellent than mineral oil (Figure 9 B).

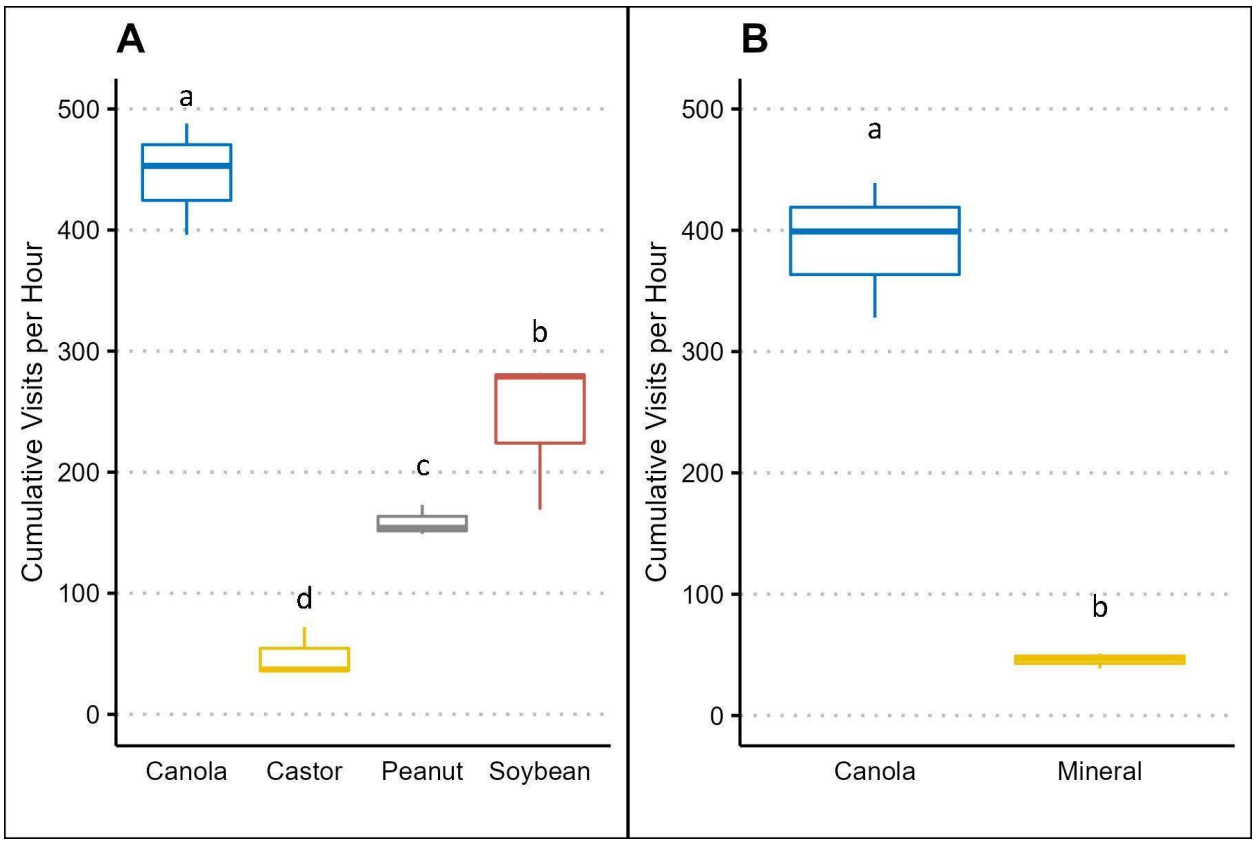

Figure 9. A) Boxplot of the number of visits by Z. cucurbitae to potential carrier oils. B) The preferred oil (canola) was then compared in a two choice test against mineral oil Significant differences (Tukey HSD) are indicated by different lower case letters.

3.5.2. Phagostimulant Testing Results

Incorporation of a sugar or molasses to the formulation (BotaniGard + canola oil + DE) did not increase fly attraction and probing relative to the formulation without these phagostimulants (Figure 10). Fly visitations did not significantly change with the addition of table sugar ($F = 0.38$, df = 1, $p = 0.56$) or molasses ($F = 0.153$, df = 1, $p = 0.71$). The amount of

time flies spent on the sugar-incorporated ($p = 0.71$) or molasses-incorporated ($p = 0.75$) formulations also did not change.

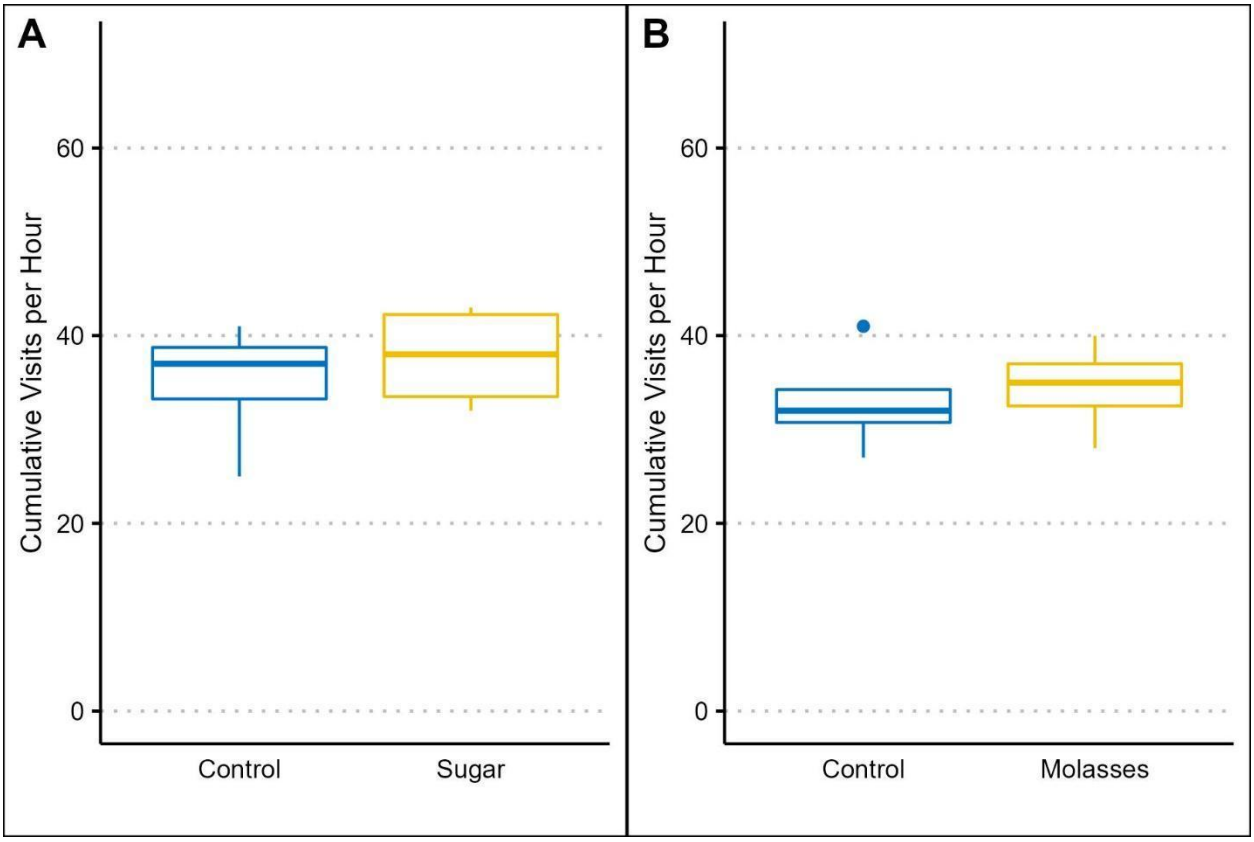

Figure 10. Boxplot of the number of visits by Z. cucurbitae flies to canola oil with or without sugar A) and molasses B). Least-squares means showed no significant difference between oil with and without a carbohydrate source incorporated.

3.5.3. Thickening Agent Results

We incorporated three thickening agents to the formulation (BotaniGard + canola oil + DE) and assessed spore viability over an eight-week period under simulated field conditions and in the lab. Our results showed that both cornstarch and glyceride flakes had no negative impact on germination rates relative to the BMD formulation while Dermofeel Viscolid caused significant decreases in spore germination (Figure 11). Three-way repeated measures ANOVA

showed that there are significant differences in germination rates between thickening agent, location, and the time (week) (Table 9). There are statistical differences ($p = <0.0001$) when a one-way ANOVA between the location and week with thickening agent type as the effect were run including cornstarch, glyceride flakes, Dermofeel Viscolid, and the BMD formulations (Appendix A: Table 1.). One-way ANOVA between the location and week with formulation as the effects were run again, censoring the Dermofeel Viscolid formulation from the analysis. This resulted in no significant differences in germination rates between formulations except at week 6 in the sun location ($p = 0.0005$) and week 8 in the lab location ($p = 0.0008$) cornstarch thickened formulations had higher germination rates (Appendix A: Table 2.). Further pairwise t-test comparisons with Bonferroni adjustment (Appendix A: Table 3.) indicated that the cornstarch thickened formulation had better germination at week 6 than the glyceride flakes ($p = 0.01$) and the BMD formulation ($p = 0.016$) in the sun. Also at week 8 cornstarch thickened formulation had better germination than glyceride flakes ($p = 0.003$) and the BMD formulation ($p = 0.041$) in the lab. Difference in germination rates between locations matched closely to what was seen in earlier germination trials (Figure 11). Pairwise t-test comparisons with Bonferroni adjustments of formulation type and week by the location were run. This showed a trend of the lab kept formulations having a higher germination rate with the sun kept formulations having the lowest germination rates, and the shade kept formulations germination rates falling in between the lab and sun (Appendix A: Table 4.).

Table 9. Three-way repeated measure ANOVA of formulation type, location, and time (week). Each BMD formulation thickened with cornstarch (CORN), Dermofeel® Viscolid (DMV), and mono- and di-glyceride flakes (GF).

Thickened Formulation Germination Rate Three-way ANOVA Comparing the Effect of Formulation Type [a], Location, and Time (Week)						
Effect	DFn	DFd	F	p	p<0.05	ges

Formulation [a]	3	6	1149.555	<0.0001	*	0.976
Location	2	4	596.231	<0.0001	*	0.893
Week	4	8	4393.777	<0.0001	*	0.988
Formulation [a]: Location	6	12	1.295	0.33		0.043
Formulation [a]: Week	12	24	83.559	<0.0001	*	0.913
Location: Week	8	16	37.579	<0.0001	*	0.737
Formulation [a]: Location: Week	24	48	20.824	<0.0001	*	0.784

[a] Formulation type is the comparison of the formulations with different thickening agents incorporated.

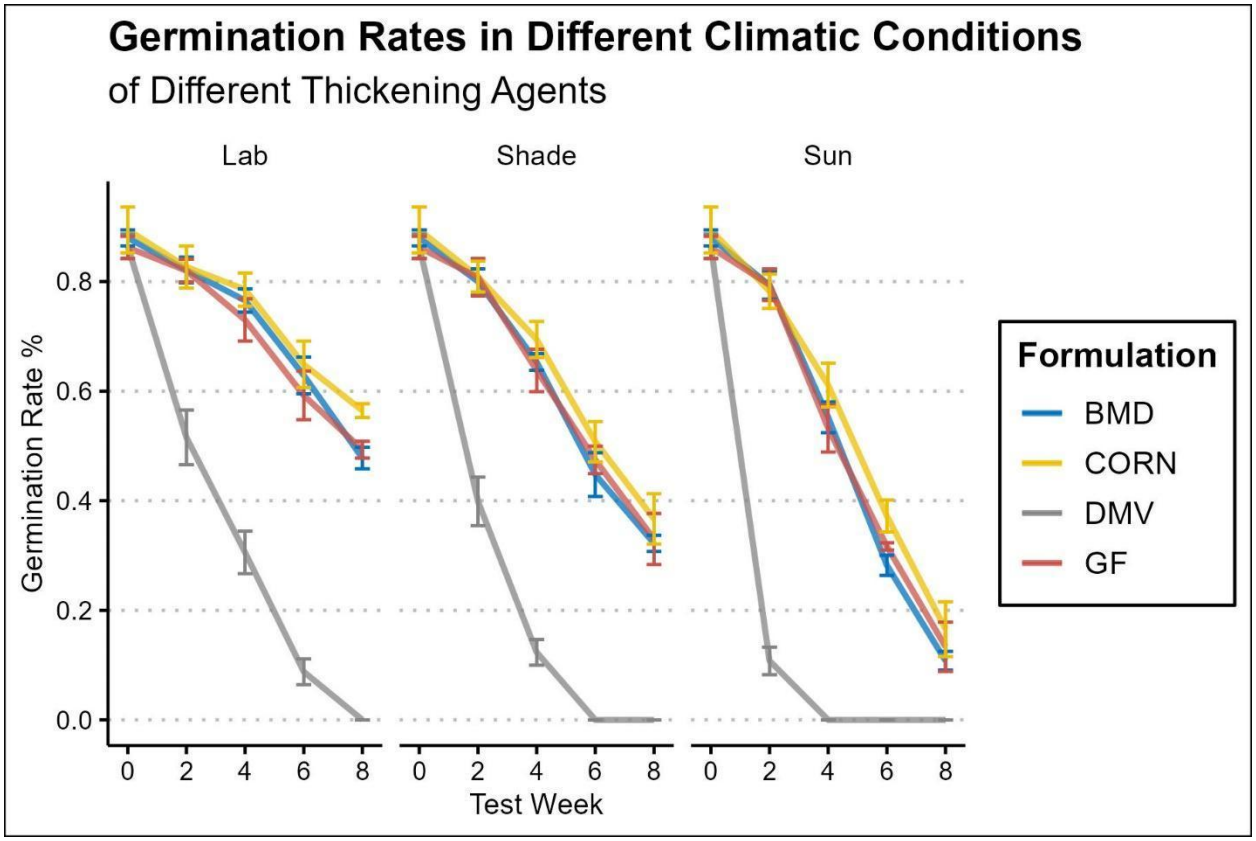

Figure 11. Average germination rates (± SE) BMD formulation thickened with cornstarch (CORN), Dermofeel® Viscolid (DMV), and mono- and di-glyceride flakes (GF) during 8 weeks of exposure in control (lab), direct sun (sun), and indirect sun (shade) conditions.

Formulation runoff testing showed that cornstarch and glyceride flakes significantly decreased the amount of formulation that melted off of the traps (Figure 12 A) while reducing the need for a fabric substrate. BMD formulation with and without a fabric substrate had significantly higher rates of run-off when compared to either thickened formulation (p =

<0.0001). Cornstarch and glyceride flake thickened formulations held equally well (p = 0.537) and fabric substrate had no significant benefit to reduce runoff. The cornstarch thickened formulation without the fabric held better than the glyceride flakes without fabric at each temperature 25 (p = 0.004), 30 (p = 0.02), 35 (p = 0.001), and 40° C (p = 0.029) (Figure 12 B)

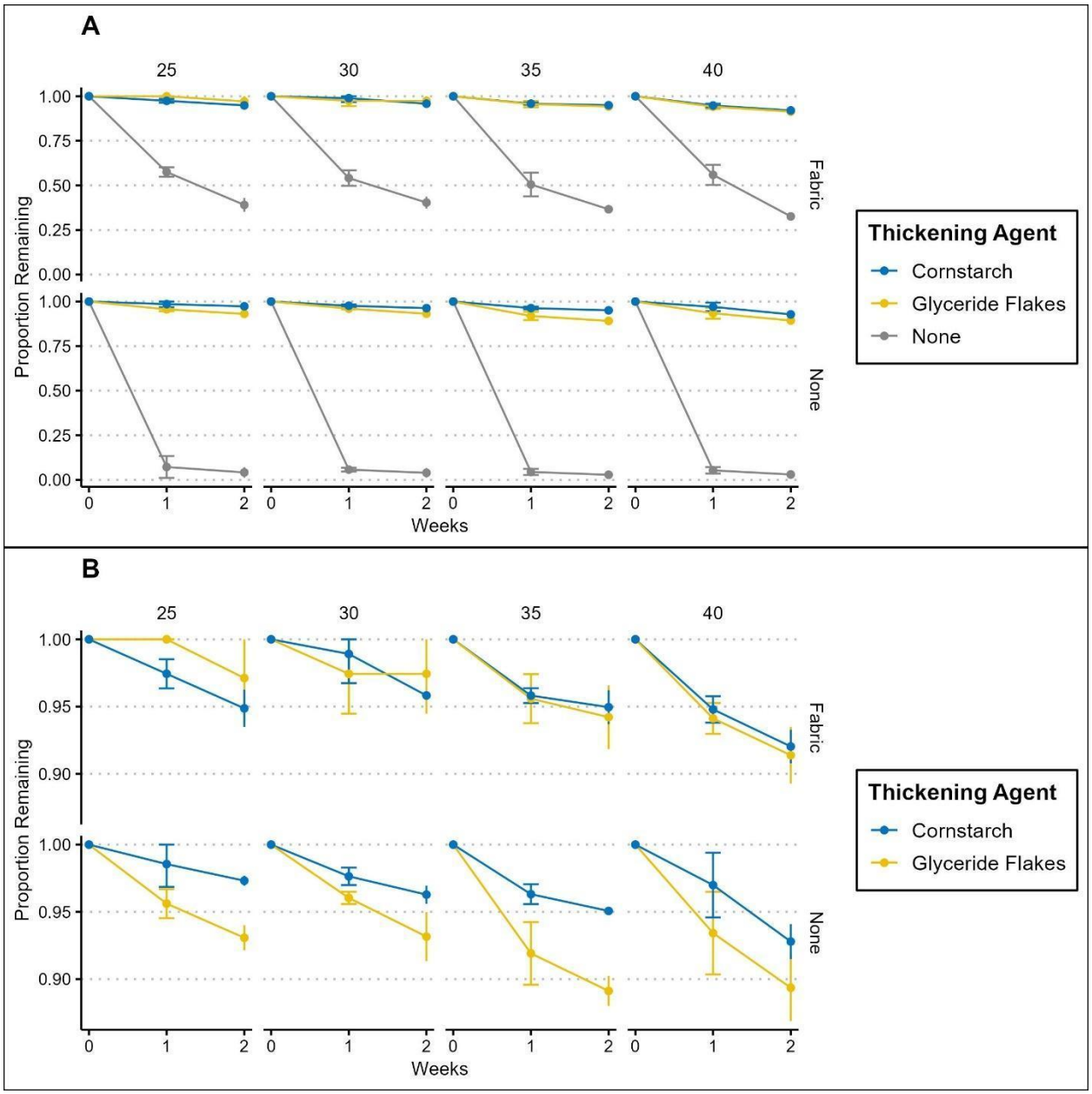

Figure 12. A) The mean amount (± SE) of cornstarch, glyceride flake and non-thickened BMD formulations that dripped out of an inverted plastic cup when exposed to constant temperatures of 25, 30, 35, and 40℃. The formulations were applied on a cloth fabric lining (fabric) or directly to the plastic surface (none). B) Close up of figure 12A (reduced y-axis values), to compare only the cornstarch and glyceride flake-thickened formulations.

Modified centrifuge tubes lined with BMD formulation on fabric, or treated on the inside wall with cornstarch and glyceride flake-thickened formulations were hung inside inverted yellow cups and weathered at three locations (as described in 2.5). These tubes were used for testing the formulations' effectiveness against flies at biweekly intervals for four weeks during the spring (March 18, 2021 – April 15, 2021) (Figure 13). Tubes treated with cornstarch and glyceride flake-thickened formulations killed significantly more flies than the BMD formulation at each location and week (Table 10) There were no significant differences between the cornstarch and glyceride thickened formulations except at week 4 in the shade location, where the cornstarch-thickened formulation had higher efficacy than the glyceride flake-thickened formulation ($\chi 2 = 16.9$, df = 1, p = <0.0001). (Table 11).

A second trial was conducted from July 29, 2021 – August 26, 2021 comparing only the cornstarch and glyceride flake formulations. Formulations maintained at each location were effective in killing melon flies relative to their controls until at least week 2 (Table 12 and 13; Figure 14). Germination rates of the spores declined dramatically in the sun and shade locations in Week 4, which coincided with much higher average outside temperatures than the first two week (Table 14). Multiple days between week 2 and 4 had high temperatures of 38.4ºC± 0.94. *B. bassiana* has a maximum thermal threshold for growth of 35ºC (95ºF) [235,236].

Table 10. Kaplan-Meier log rank comparison of the survival curves of Z. cucurbitae adult flies exposed to BMD formulation thickened by cornstarch and glyceride flakes, which had been

exposed to three climatic conditions; control (lab), direct sun (sun), and indirect sun (shade) for 0, 2 and 4 weeks.

Weathered Thickened Formulation Fly Survival Differences					
Week	Location	Log-Rank Comparison	$\chi 2$	DF	p
0	Lab	BMD – Cornstarch	8.8	1	0.003
		BMD – Glyceride Flakes	8.0	1	0.005
2	Lab	BMD – Cornstarch	42.5	1	<0.0001
		BMD – Glyceride Flakes	37.1	1	<0.0001
	Sun	BMD – Cornstarch	56.7	1	<0.0001
		BMD – Glyceride Flakes	28.3	1	<0.0001
	Shade	BMD – Cornstarch	51.1	1	<0.0001
		BMD – Glyceride Flakes	47.5	1	<0.0001
4	Lab	BMD – Cornstarch	22.7	1	<0.0001
		BMD – Glyceride Flakes	21.7	1	<0.0001
	Sun	BMD – Cornstarch	27.1	1	<0.0001
		BMD – Glyceride Flakes	22.1	1	<0.0001
	Shade	BMD – Cornstarch	63.5	1	<0.0001
		BMD – Glyceride Flakes	28.2	1	<0.0001

Survival differences were calculated using the G^{ρ} ($\rho = 0$; log rank) family of tests to compare survival curves.

Table 11. Kaplan-Meier mean and median survival times for Zeugodacus cucurbitae exposed to BMD formulation thickened by cornstarch and glyceride flakes, which had been exposed to three climatic conditions; control (lab), direct sun (sun), and indirect sun (shade) for 0, 2 and 4 weeks.

Weathered Formulation Mortality Survival Time Table							
Week	Location	Thickening Agent	Treatment	% Mortality	Mean Survival Time (± SE)[a]	Median Survival Time	% Fungal Infection[b]
0	Lab	Cornstarch	Control	12.00%	-	>14	0.00%
			Treated	100.0%	4.35±0.19	4	85.33%
		Glyceride Flake	Control	16.00%	-	>14	0.00%
			Treated	100.0%	4.35±0.24	4	88.00%
		None	Control	5.33%	-	>14	0.00%
			Treated	90.67%	5.64±0.43	5	94.12%
2	Lab	Cornstarch	Control	2.67%	-	>14	0.00%
			Treated	100.0%	4.11±0.19	4	96.00%

		Glyceride Flake	Control	9.33%	-	>14	0.00%
			Treated	98.67%	4.25±0.40	4	91.89%
		None	Control	1.33%	-	>14	0.00%
			Treated	54.67%	8.65±0.58	6	90.24%
	Sun	Cornstarch	Control	1.33%	-	>14	0.00%
			Treated	96.00%	4.96±0.29	4	93.06%
		Glyceride Flake	Control	1.33%	-	>14	0.00%
			Treated	78.67%	6.23±0.50	4	96.61%
		None	Control	5.33%	-	>14	0.00%
			Treated	33.33%	10.6±0.58	4	92.00%
	Shade	Cornstarch	Control	6.67%	-	>14	0.00%
			Treated	94.67%	4.81±0.36	4	97.18%
		Glyceride Flake	Control	8.00%	-	>14	0.00%
			Treated	93.33%	5.08±0.39	4	92.86%
		None	Control	5.33%	-	>14	0.00%
			Treated	42.67%	10.11±0.56	4	93.75%
4	Lab	Cornstarch	Control	0.00%	-	>14	0.00%
			Treated	78.67%	8.35±0.48	8	89.93%
		Glyceride Flake	Control	0.00%	-	>14	0.00%
			Treated	74.67%	8.31±050	8	91.07%
		None	Control	0.00%	-	>14	0.00%
			Treated	33.33%	-	>14	84.00%
	Sun	Cornstarch	Control	0.00%	-	>14	0.00%
			Treated	54.67%	9.91 ±0.49	10	90.24%
		Glyceride Flake	Control	0.00%	-	>14	0.00%
			Treated	50.67%	10.57±0.46	12	89.47%
		None	Control	0.00%	-	>14	0.00%
			Treated	13.33%	-	>14	60.00%
	Shade	Cornstarch	Control	0.00%	-	>14	0.00%
			Treated	81.33%	6.84 ±0.46	6	95.08%
		Glyceride Flake	Control	0.00%	-	>14	0.00%
			Treated	65.33%	9.96 ±0.44	10	85.71%
		None	Control	0.00%	-	>14	0.00%
			Treated	21.33%	-	>14	87.50%

[a] Dashes denote that estimation could not be performed because of low mortality. [b] Confirmation of dead flies by fungal infection percentages were calculated from the mortality percentages.

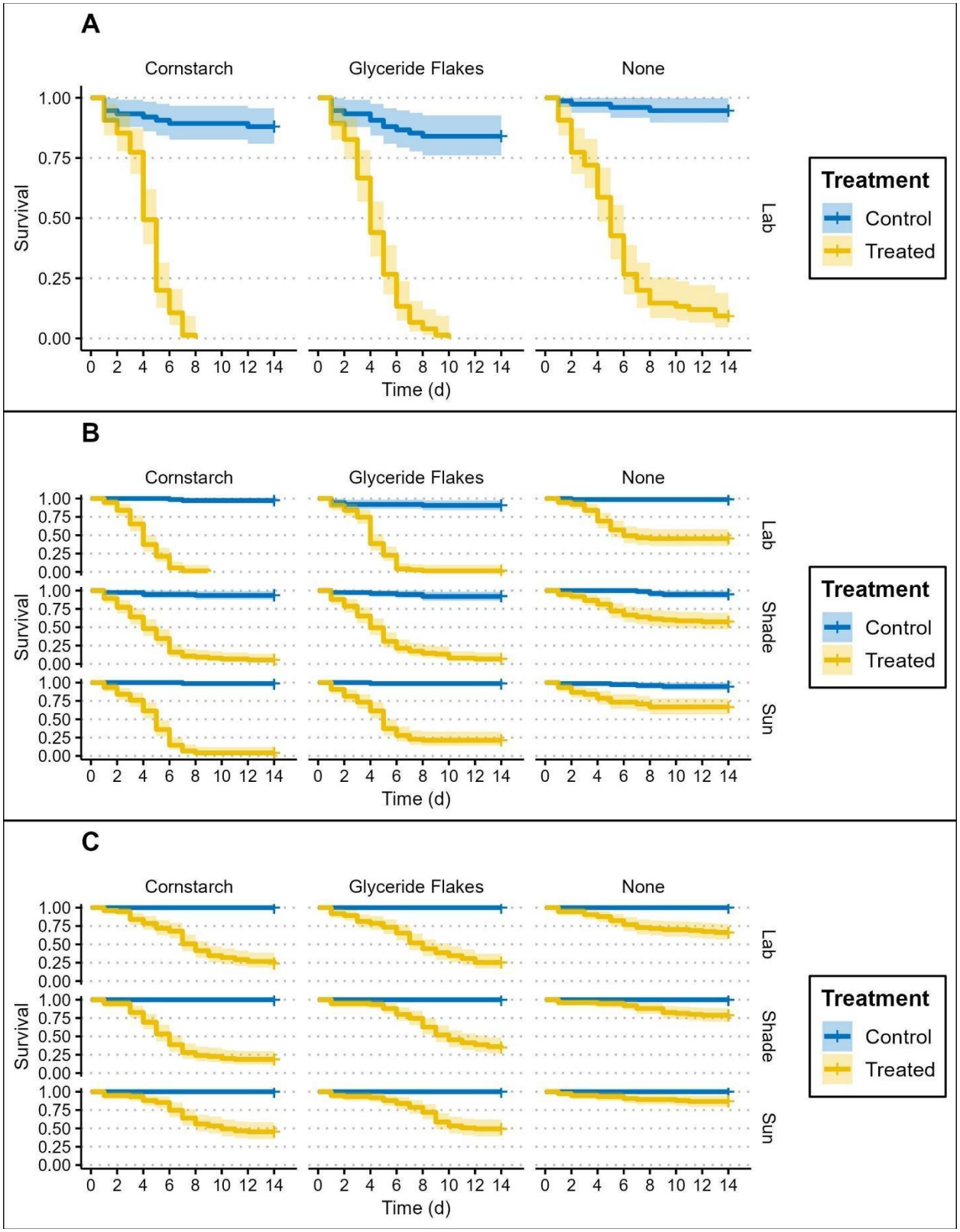
A
Cornstarch
Glyceride Flakes
None
Survival
1.00
0.75
0.50
0.25
0.00
0 2 4 6 8 10 12 14
Time (d)
Lab
Treatment
Control
Treated
B
Cornstarch
Glyceride Flakes
None
Survival
Lab
Shade
Sun
Time (d)
Treatment
Control
Treated
C
Cornstarch
Glyceride Flakes
None
Survival
Lab
Shade
Sun
Time (d)
Treatment
Control
Treated

Figure 13. Kaplan-Meier survival curves with 95% confidence intervals of Z. cucurbitae adults exposed to weathered thickened BMD formulation. Cornstarch and glyceride flake-thickened BMD formulations were exposed to control (lab), direct sun (sun), and indirect sun (shade) over a four-week period (March 18, 2021 – April 15, 2021). Testing intervals were at A) week 0, B) week 2, and C) week 4. Thickened formulations maintained significant efficacy over the four-week period compared to the non-thickened BMD formulation during the same period. Flies were censored from the data if they escaped out of cages and after 14 days.

Table 12. Kaplan-Meier log rank comparison of the survival curves of Z. cucurbitae adult flies exposed to BMD thickened formulations exposed to control (lab), direct sun (sun), and indirect sun (shade) for 0, 2, and 4 weeks. Sexually mature unmated adult flies were forced to contact the weathered treated fabric strips.

Weathered Thickening Agent Mortality Results					
Week	Thickening Agent	Log-Rank Comparison	$\chi 2$	DF	p
0	Cornstarch	Control–Lab	129	1	<0.0001
	Glyceride Flakes	Control–Lab	138	1	<0.0001
2	Cornstarch	Control–Lab	103	1	<0.0001
		Control–Sun	106	1	<0.0001
		Control–Shade	96.5	1	<0.0001
	Glyceride Flakes	Control–Lab	103	1	<0.0001
		Control–Sun	89.4	1	<0.0001
		Control–Shade	95.6	1	<0.0001
4	Cornstarch	Control–Lab	111	1	<0.0001
		Control–Sun	0.1	1	0.7
		Control–Shade	0.6	1	0.5
	Glyceride Flakes	Control–Lab	110	1	<0.0001
		Control–Sun	0.2	1	0.6
		Control–Shade	0.7	1	0.4

Table 13. Kaplan-Meier survival times for Zeugodacus cucurbitae, forced in contact with thickened BMD formulations exposed to control (lab), direct sun (sun), and indirect sun (shade) for 0, 2, and 4 weeks. High temperatures (up to 40°C (104 °F)) between weeks 2 and 4 significantly reduced spore viability.

Weathered Formulation Mortality Survival Time Table							
Week	Location	Thickening Agent	Treatment	% Mortality	Mean Survival Time	Median Survival Time	% Fungal Infection[b]

					(± SE) [a]		
0	Lab	Cornstarch	Control	12.00%	-	>14	0.00%
			Treated	100.0%	3.91±0.14	4	97.33%
		Glyceride Flake	Control	9.33%	-	>14	0.00%
			Treated	100.0%	3.84±0.15	4	94.66%
2	Lab	Cornstarch	Control	10.0%	-	>14	0.00%
			Treated	100.0%	4.46±0.28	4	
		Glyceride Flake	Control	10.0%	-	>14	0.00%
			Treated	100.0%	3.88±0.26	4	98.0
	Sun	Cornstarch	Control	6.00%	-	>14	0.00%
			Treated	98.00%	4.80±0.28	4.5	97.96%
		Glyceride Flake	Control	6.00%	-	>14	0.00%
			Treated	96.00%	5.50±0.23	4	97.92%
	Shade	Cornstarch	Control	6.00%	-	>14	0.00%
			Treated	100.0%	5.50±0.25	5	100.0%
		Glyceride Flake	Control	8.00%	-	>14	0.00%
			Treated	96.00%	4.56±0.25	4	94.00%
4	Lab	Cornstarch	Control	8.00%	-	>14	0.00%
			Treated	100.0%	3.04±0.20	3	98.00%
		Glyceride Flake	Control	10.00%	-	>14	0.00%
			Treated	100.0%	4.22±0.27	3	96.00%
	Sun	Cornstarch	Control	8.00%	-	>14	0.00%
			Treated	10.0%	-	>14	60.00%
		Glyceride Flake	Control	6.00%	-	>14	0.00%
			Treated	4.00%	-	>14	50.00%
	Shade	Cornstarch	Control	6.00%	-	>14	0.00%
			Treated	10.00%	-	>14	60.00%
		Glyceride Flake	Control	8.00%	-	>14	0.00%
			Treated	4.00%	-	>14	100.0%

[a] Dashes denote that estimation could not be performed because of low mortality. [b] Confirmation of dead flies by fungal infection percentages were calculated from the mortality percentages.

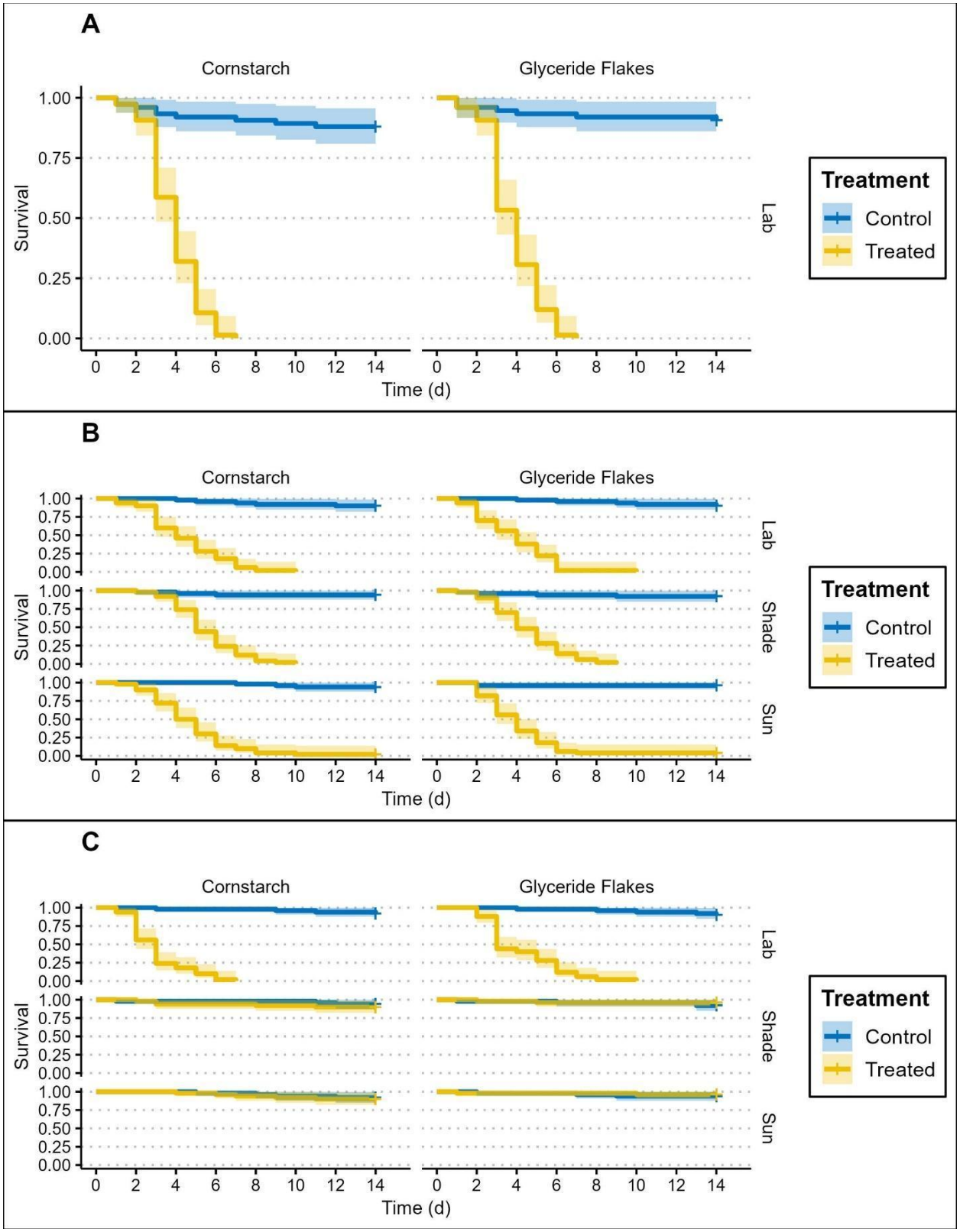
A
Cornstarch
Glyceride Flakes
Survival
1.00
0.75
0.50
0.25
0.00
0 2 4 6 8 10 12 14
Time (d)
Lab
Treatment
Control
Treated
B
Cornstarch
Glyceride Flakes
Survival
Lab
Shade
Sun
Time (d)
Treatment
Control
Treated
C
Cornstarch
Glyceride Flakes
Survival
Lab
Shade
Sun
Time (d)
Treatment
Control
Treated

Figure 14. Kaplan-Meier survival curves with 95% confidence intervals of Z. cucurbitae adults exposed to weathered thickened BMD formulation. Cornstarch and glyceride flake-thickened BMD formulations were exposed to control (lab), direct sun (sun), and indirect sun (shade) over a four-week period (July 29-August 26, 2021). Testing intervals were at A) week 0, B) week 2, and C) week 4. The upper temperatures reached 40˚C (104 ˚F) during a heat wave between weeks 2 and 4. Flies were censored from the data if they escaped out of cages and after 14 days.

Table 14. Germination rates of cornstarch and glyceride flake-thickened and non-thickened BMD formulations (±SE) over a four-week period in Trials 1 and 2. Mean temperature and relative humidity (±SE) differed in each location and may have impacted the spore viability and longevity of each formulation.

Weathered thickened formulation germination percentage, temperature, and relative humidity					
Trial 1 (March 18, 2021 – April 15, 2021)					
Week	**Location**	**Temperature (˚C) (± SE)**	**RH (± SE)**	**Thickening Agent**	**Germ % (± SE)**
0	Lab	23.49 (± 0.16)	68.36 (± 1.97)	Cornstarch	91.56 (± 0.99)
				Glyceride Flakes	91.70 (± 1.67)
				None [a]	90.44 (± 2.73)
2	Lab	23.53 (± 0.07)	72.16 (± 0.99)	Cornstarch	86.66 (± 2.59)
				Glyceride Flakes	86.09 (± 3.18)
				None [a]	87.12 (± 3.02)
	Sun	27.15 (± 4.19)	63.53 (± 8.718)	Cornstarch	85.55 (± 4.76)
				Glyceride Flakes	83.87 (± 2.01)
				None [a]	84.98 (± 2.78)
	Shade	25.37 (± 2.21)	64.76 (± 8.06)	Cornstarch	85.92 (± 4.17)
				Glyceride Flakes	85.80 (± 5.01)
				None [a]	85.79 (± 4.86)
4	Lab	23.46 (± 0.07)	71.82 (± 1.94)	Cornstarch	83.73 (± 3.02)
				Glyceride Flakes	82.49 (± 3.93)
				None [a]	82.60 (± 3.50)
	Sun	26.81 (± 3.76)	65.72 (± 14.63)	Cornstarch	79.67 (± 5.57)
				Glyceride Flakes	77.71 (± 6.94)
				None [a]	78.91 (± 2.83)
	Shade	25.59 (± 2.27)	67.73 (± 8.72)	Cornstarch	81.22 (± 5.80)
				Glyceride Flakes	80.35 (± 6.17)
				None [a]	81.44 (± 2.83)
Trial 2 (July 29, 2021 – August 26, 2021)					
Week	**Location**	**Temperature (˚C) (± SE)**	**RH (± SE)**	**Thickening Agent**	**Germ % (± SE)**
0	Lab	23.21 (± 0.12)	66.76 (± 0.93)	Cornstarch	94.71 (± 4.07)

				Glyceride Flakes	95.02 (± 2.80)
2	Lab	23.73 (± 0.08)	69.30 (± 1.12)	Cornstarch	90.93 (± 1.26)
				Glyceride Flakes	91.32 (± 2.96)
	Sun	27.36 (± 3.86)	67.22 (± 8.52)	Cornstarch	87.99 (± 2.73)
				Glyceride Flakes	87.10 (± 3.84)
	Shade	26.09 (± 2.47)	68.01 (± 8.75)	Cornstarch	88.67 (± 1.67)
				Glyceride Flakes	89.08 (± 4.41)
4	Lab	23.39 (± 0.12)	70.71 (± 0.99)	Cornstarch	84.47 (± 1.33)
				Glyceride Flakes	82.59 (± 2.59)
	Sun	32.67 (± 3.31)	76.99 (± 11.72)	Cornstarch	42.52 (± 3.84)
				Glyceride Flakes	41.54 (± 2.87)
	Shade	31.85 (± 3.48)	74.69 (± 7.96)	Cornstarch	47.33 (± 3.86)
				Glyceride Flakes	42.30 (± 7.07)

Temperatures and relative humidity are based on two-week averages from HOBOs MX2300 Series in situ. [a] None is the non-thickened BMD formulation.

3.5.4. Liquid Lure Incorporation

Incorporating species-specific liquid lure at the 10% concentration significantly increased the number of visits of *B. dorsalis* ($p = <0.0001$) and *Z. cucurbitae* ($p = <0.0001$) when compared to controls without any lure. Lower concentrations elicited no significant increase in visitation (Figure 15 A, B). Incorporation of liquid C-L and ME lures to the formulation did not negatively impact spore germination rates over the three-week period (Figure 15 C; Appendix B: Table 1.). One-way ANOVA and TukeyHSD test showed that incorporation of liquid lure at a 10% concentration increased the numbers of spores the flies picked up. C-L at 10% significantly increased spore pick-up compared to 1.0% ($p = 0.033$), 0.1% ($p = 0.0004$), and 0% ($p = 0.0003$). ME at 10% lure increased spore pick-up compared to 1.0% ($p = <0.0001$), 0.1% ($p = <0.0001$), and 0% ($p = <0.0001$).

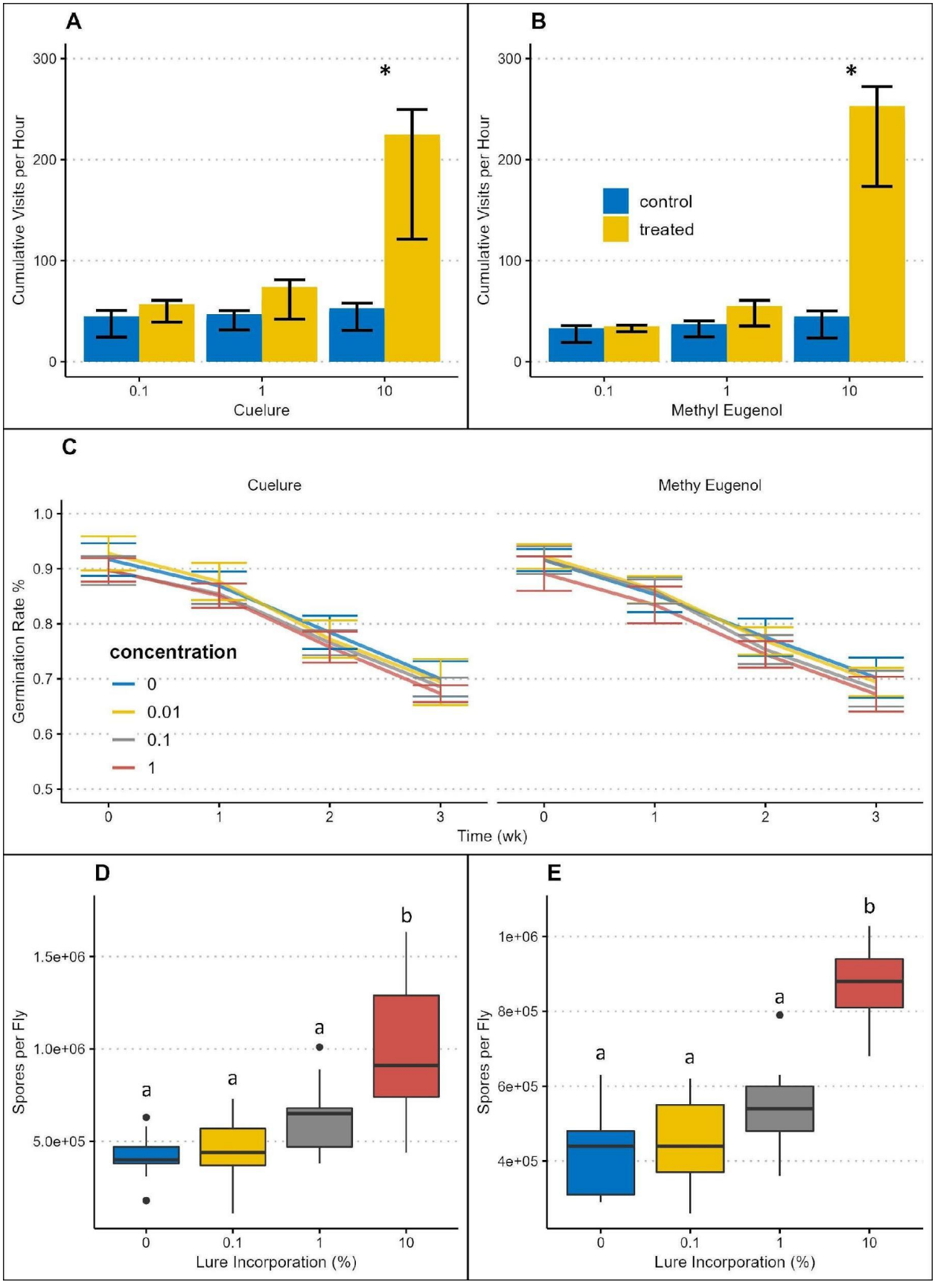
A
Cumulative Visits per Hour
300
200
100
0
*
0.1
1
10
Cuelure
B
Cumulative Visits per Hour
300
200
100
0
*
control
treated
0.1
1
10
Methyl Eugenol
C
Cuelure
Methy Eugenol
Germination Rate %
1.0
0.9
0.8
0.7
0.6
0.5
concentration
0
0.01
0.1
1
0
1
2
3
Time (wk)
D
Spores per Fly
1.5e+06
1.0e+06
5.0e+05
a
a
a
b
0
0.1
1
10
Lure Incorporation (%)
E
Spores per Fly
1e+06
8e+05
6e+05
4e+05
a
a
a
b
0
0.1
1
10
Lure Incorporation (%)

Figure 15. Boxplot of the numbers of visits by A) Z. cucurbitae and B) B. dorsalis flies in two-choice tests to the canola oil formulation containing three liquid lure concentrations at 0.1, 1.0, and 10.0% (± SE). Cuelure was used for Z. cucurbitae and methyl eugenol for B. dorsalis. Least-squares means showed a significant difference between formulations with 10.0% lure incorporated for both species. C) Average germination rates (± SE) of the canola oil formulation containing three concentrations of liquid cuelure and methyl eugenol showed that the lures had no negative effect on spore germination. Boxplot of average spore pick-up rates by D) Z. cucurbitae from cuelure-incorporated formulation and E) B. dorsalis from methyl eugenol-incorporated formulation showed that formulations with 10.0% lure increased fly spore pick up rates significantly.

3.5.5. Effects of Formulation Improvements on Spore Pick-Up Rates

For both *Z. cucurbitae* and *B. dorsalis*, adding one of cornstarch or 10% lure to canola oil did not significantly increase spore pickup compared to canola oil alone (Figure 16). However, adding both cornstarch and lure significantly increased spore pickup compared to canola oil alone as well as combinations of canola oil with lure and canola oil with cornstarch (Table 14).

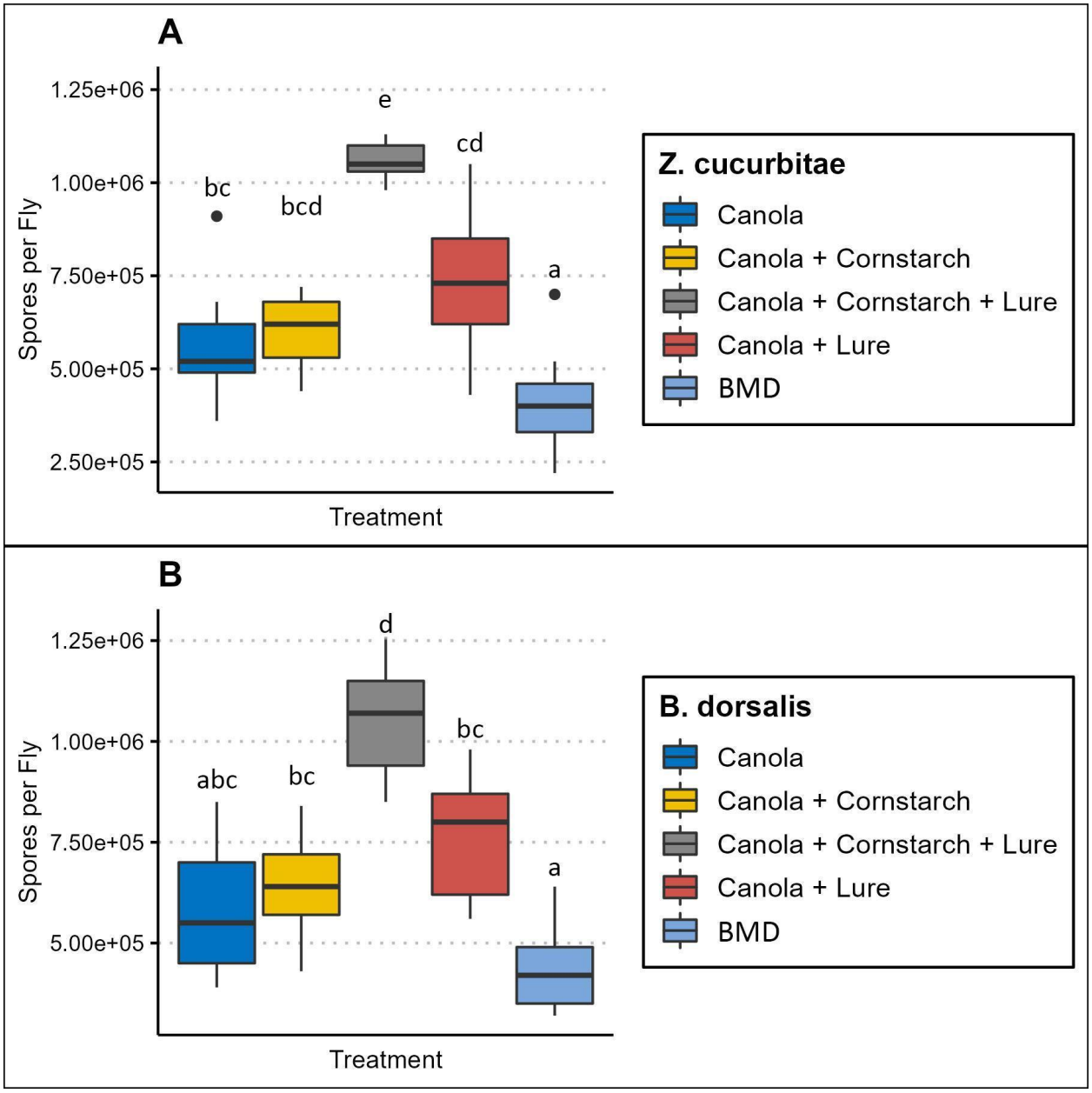

Figure 16. Boxplot of average spore pick-up rates of A) Z. cucurbitae and B) B. dorsalis with each improvement; canola oil, thickening agent, and liquid lure. Tukey HSD test with normal distribution showed that formulations with all the additional components significantly increased the number of spores picked up by the flies.

Table 15. Log-rank pairwise comparison of spore pick-up rates of B. dorsalis and Z. cucurbitae adult flies exposed to BMD formulation and at various stages of modification (carrier oil, thickening agent, and liquid lure).

Species	Pairwise comparison	P
Z. cucurbitae	BMD – Canola	0.15
	BMD – Canola + Cornstarch	0.06
	BMD – Canola + Lure	0.0003
	BMD – Canola + Cornstarch + Lure	<0.0001
	Canola – Canola + Cornstarch	0.99
	Canola – Canola + Lure	0.15
	Canola – Canola + Cornstarch + Lure	<0.0001
	Canola + Cornstarch – Canola + Lure	0.32
	Canola + Cornstarch – Canola + Cornstarch + Lure	<0.0001
	Canola + Lure – Canola + Cornstarch + Lure	0.0002
B. dorsalis	BMD – Canola	0.18
	BMD – Canola + Cornstarch	0.015
	BMD – Canola + Lure	<0.0001
	BMD – Canola + Cornstarch + Lure	<0.0001
	Canola – Canola + Cornstarch	0.81
	Canola – Canola + Lure	0.043
	Canola – Canola + Cornstarch + Lure	<0.0001
	Canola + Cornstarch – Canola + Lure	0.37
	Canola + Cornstarch – Canola + Cornstarch + Lure	<0.0001
	Canola + Lure – Canola + Cornstarch + Lure	0.001

Table 15. Pairwise comparisons of spore pick up rates.

3.6. Final Comparison (Testing Improvements)

3.6.1. Final Formulation Horizontal Transmission Testing Results

When I compared the initial BMD formulation (BotaniGard + mineral oil + DE on a fabric liner) to the optimized final formulation (BotaniGard + canola oil + cornstarch + lure + DE with no liner), for both *B. dorsalis* and *Z. cucurbitae*, the optimized formulation induced significantly higher and faster mortality in both males and females (Table 15 and 16). Overall, all the combined incorporated improvements decreased the time to mortality while achieving remarkably high mortality within 11 days (Figure 17).

Table 16. Kaplan-Meier survival times for Bactrocera dorsalis and Zeugodacus cucurbitae that were passively exposed to the BMD formulation and to the final formulation (Botanigard with diatomaceous earth, canola oil, cornstarch, and liquid lure).

Final Passive Horizontal Transmission Survival Times						
Species	Sex	Treatment	% Mortality	Mean Survival Time (± SE) [a]	Median Survival Time	% Fungal Infection [b]
B. dorsalis	Males	BMD	100.0%	4.21±0.29	4	88.57%
		Final	100.0%	3.43±0.18	4	93.49%
		Control	13.33%	-	>20	0.00%
	Females	BMD	100.0%	5.52±0.27	6	91.90%
		Final	100.0%	4.13±0.22	4	96.67%
		Control	11.67%	-	>20	0.00%
Z. cucurbitae	Males	BMD	100.0%	7.98±0.31	8	88.33%
		Final	100.0%	5.24±0.33	5	93.49%
		Control	5.00%	-	>20	0.00%
	Females	BMD	100.0%	9.53±0.35	10	86.67%
		Final	100.0%	6.75±0.34	7	95.00%
		Control	3.33%	-	>20	0.00%

[a] Dashes denote that estimation could not be performed because of low mortality. [b] Confirmation of dead flies by fungal infection percentages were calculated from the mortality percentages.

Table 17. Kaplan-Meier log-rank comparisons for Bactrocera dorsalis and Zeugodacus cucurbitae that were passively exposed to the BMD formulation and to the final formulation (Botanigard with diatomaceous earth, canola oil, cornstarch, and liquid lure).

Survival Differences Between the BMD and Final Formulation				
Species	Log-Rank Comparison	$\chi 2$	DF	p
Z. cucurbitae	BMD M – BMD F	21.8	1	<0.001
	BMD M – Final M	35.2	1	<0.001
	BMD F – Final F	43.8	1	<0.001
	Final M – Final F	11.2	1	<0.001
B. dorsalis	BMD M – BMD F	8.2	1	0.004
	BMD M – Final M	9.6	1	0.002
	BMD F – Final F	22	1	<0.001
	Final M – Final F	8	1	0.005

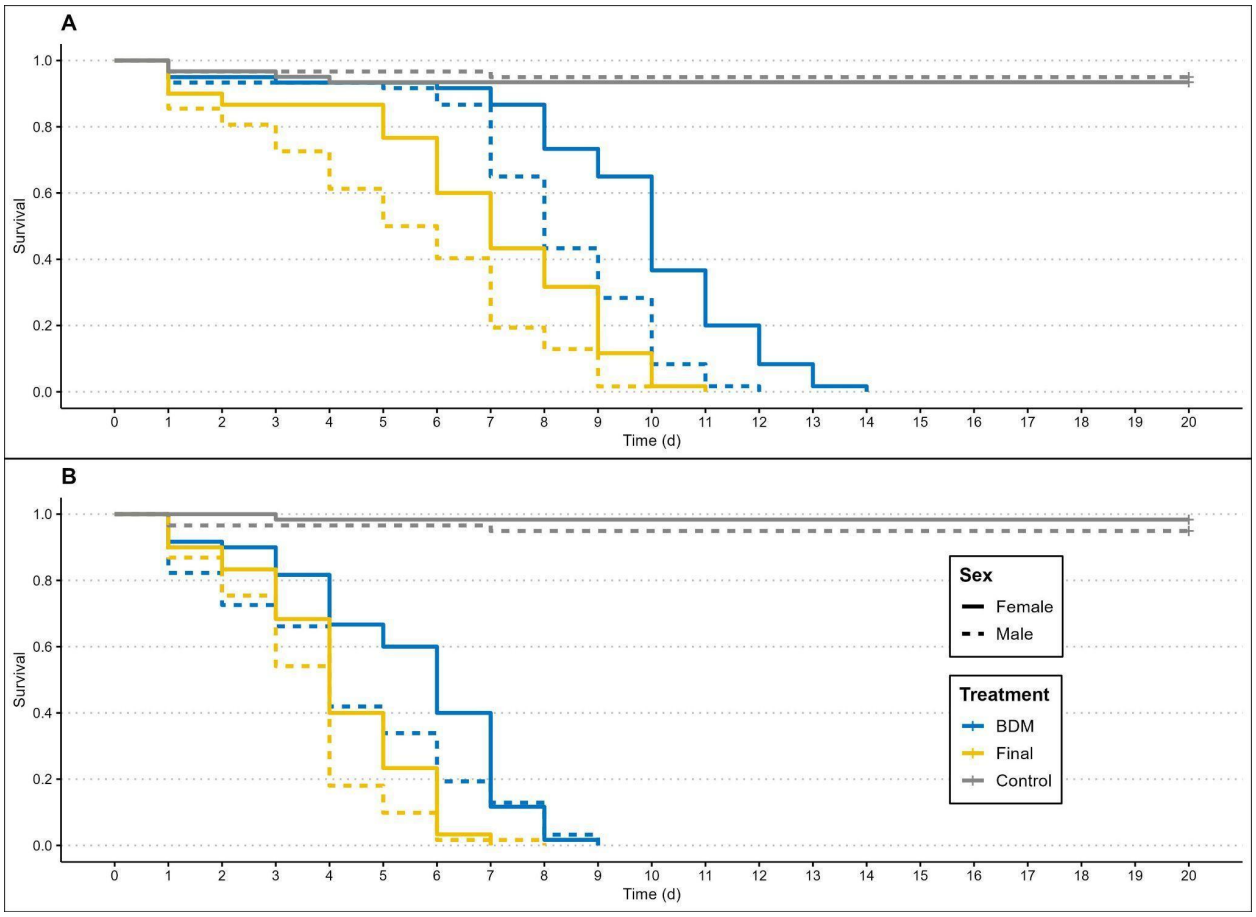

Figure 17. Kaplan-Meier survival times for A) Bactrocera dorsalis and B) Zeugodacus cucurbitae that were passively exposed to the BMD formulation and to the final formulation (Botanigard with diatomaceous earth, canola oil, cornstarch, and liquid lure). Accompanying log-rank tests confirmed that the additions to the formula decreased the time to death in both sexes and species significantly. Flies that survived beyond 20 days were censored out of the analysis.

4. Discussion

First, we demonstrated that BotaniGard ES (*B. bassiana* strain GHA at $2.0x10^{10}$ spores per ml of water) was effective in killing *B. dorsalis*, *C. capitata*, and *Z. cucurbitae*, with *Z. cucurbitae* being the least susceptible of the three species. Since our goal was to use the *B. bassiana* in a bait station, we used mineral oil as the carrier solution rather than water to

maximize longevity of the spores. Various oils have been shown to keep fungal spores from germinating to maintain long-term viability in storage or after application until the spores contact their target insect hosts [237–239]. For example, the fungal biopesticide Aprehend used for bed bug control uses a petroleum distillate-based carrier and putatively lasts up to three months after application (US EPA, Pesticide Product Label, Aprehend, 27 Mar. 2017). After mixing the BotaniGard in mineral oil, we made step-by-step improvements to the "formulation" to maximize fly mortality and spore pickup.

We found that adding diatomaceous earth (DE) to the mineral oil formulation increased total fruit fly mortality and decreased time to death. DE is an abrasive absorbent material that scratches the insect cuticle and absorbs cuticular lipids, which disrupts the insects' ability to prevent water loss [224,240]. These cuticular disruptions also increase the ability of conidial germ tubes to penetrate through the insects' cuticle. Combining *B. bassiana* spores and DE have previously been demonstrated to have synergistic effects that facilitate fungal penetration into insects bodies[223,224,241]. DE is naturally derived from the fossilized remains of phytoplankton and has low environmental impact and is easy to obtain globally [242–244]. It has been utilized to control pests for many stored products [242,245–247] and has been added as an adjuvant in many commercial crop protection products [248–251]. Since DE's mechanism of action is physiological, resistance is unlikely but tolerance may be seen in different insect populations [252–254].

Although we used male lures to attract male flies to the inverted yellow cups treated with the mineral oil formulation, we observed that the males were not readily entering the cup and suspected that the mineral oil may be acting as a deterrent. Mineral oil has been shown to deter

oviposition by Queensland fruit flies (*Bactrocera tryoni*) when fruits were dipped in a 0.5% vol/vol aqueous solution versus only water [255]. Mineral oil is a neutral oil and shows little attraction by itself when compared to mineral oil that has attractive secondary compounds incorporated to control *C. capitata* [256]. Due to the viscous nature of mineral oil, trapped flies died but did not decay as quickly, reducing the release of carrion volatiles [257]. Next, we tested the repellency of fruit flies to canola, soybean, peanut, and castor bean oils. We selected canola, soybean, peanut, and castor bean oils for several reasons, but primarily because they are edible oils. Canola and soybean oils have been used as alternatives to wheat germ oil in larval liquid diets for *B. dorsalis*, *C. capitata*, and *Z. cucurbitae* [258,259]and adult *Z. cucurbitae* in the field are commonly found roosting on plants from the Fabaceae (*Phaseolus vulgaris*, *Crotalaria juncea*, *Cajanus cajan*) and Euphorbiaceae (*Ricinus communis*, *Euphorbia geniculata*) plant families [206]. Canola, soybean and peanut oils have also been shown to be effective in maintaining the long-term viability of fungal spores and have been demonstrated to protect spores under high temperatures [260]. In the control of other insects, canola oil has been used as a phagostimulant to increase probing and feeding to increase acquisition of *B. bassiana* spores in grasshoppers [261,262]. Hydrogenated canola oil has been used with *B. bassiana* and aggregation pheromones to produce pellets for the control of the larger grain borer, *Prostephanus truncatus* (Horn) [263]. Our results showed that out of the four oils, the flies were least repelled by canola oil. We then compared the repellency of canola oil-treated fabric and mineral oil-treated fabric in a two-choice test and found that the flies were more likely to land and walk on the canola oil. With studies supporting the repellency of fruit flies to mineral oil [249]and a

higher level of attraction of *Z. cucurbitae* toward canola in our study, we decided to change the carrier oil to canola oil.

Many tephritids have unique mating rituals and behaviors [264,265] with male pheromones playing an important role in attracting females [73,76,266–270]. The need for males to produce pheromones increases their likelihood to engage in pharmacophagy, as imbibing parapheromones increases the likelihood of successful mating [17,271,272]. Exploitation of these parapheromone lures is an effective fruit fly management strategy [36,273]. These lures have mostly been incorporated into solid polymeric matrix plugs, which are then placed in traps for male annihilation. The Amulet bait stations use molded paper fiber soaked in C-L or ME and fipronil to control *Z. cucurbitae* and *B. dorsalis*, respectively [197,274]. Similar attract-and-kill stations have been utilized for many different classes of insecticides to control a variety of pests [263,268,272,275–282], including the facilitation of horizontal transfer of fungal spores between sexes [280,283]. We used a similar approach by incorporating C-L and ME in the canola oil formulation to increase male interaction with the formulation and increase spore pickup and mortality. We hypothesized that the addition of the lure elicited probing behavior in the males because when we placed fungus-killed males in humidity chambers, the growth of white *B. bassiana* spores started on the mouthparts of the male flies, indicating that the mouthpart may have been the site of initial infection. We also tried eliciting probing behavior by adding sugar and molasses to the formulation (without lures), but these phagostimulants did not increase fly attraction to the formulation. Incorporating C-L and ME to the formulation increased horizontal transfer of spores from males to females, which was indicated by higher female mortality, regardless of whether we used forced or passive exposure of male flies to the fungal spores.

Next, we thickened our formulation to prevent separation of the spores and diatomaceous earth and to limit dripping of the formulation from the bait station (Figure 9). Dripping and separation was particularly an issue at elevated temperatures, which is likely to be the environment when the formulation is applied inside a plastic bait station exposed to sunlight in the field. Additionally, thickening the formulation has the added benefit of being easier to apply to the bait station. We found that the thickening agent used for cosmetic products, Dermofeel, was toxic to the fungal spores. However, glyceride flakes and cornstarch were both effective thickeners and had no negative effect on spore viability. Glyceride flakes (monoglyceride and diglycerides from fatty acids) is commonly used in the food, pharmaceutical, and cosmetic industries as an emulsifying agent and potentially has many other industrial applications [284,285]. Cornstarch is a widely used thickening agent in the food industry for its ability to gel liquids. Cornstarch is readily available and inexpensive and is a renewable raw material [286]. The thickened formulation, regardless of whether we used cornstarch or glyceride flakes, killed 100% of *Z. cucurbitae* that were exposed to it. After the thickened formulations were hung outside in the shade and under direct sunlight, both formulations were still highly effective after 2 weeks. While both had significant decreases in efficacy beyond 4 weeks, this may have been due to a heat wave, where daily temperatures peaked at about 39.8° C (103.6° F) during a 6 day period. Due to the ease of incorporating cornstarch into the formulation compared to glyceride flakes, which need to be heated and melted, we used cornstarch for the remainder of the experiments.

The combination of the stickiness of the thickened formulation, the low repellency of the canola oil, and the phagostimulating effect of the liquid lure clearly improved the effectiveness

of the *B. bassiana.* In a passive exposure experiment consisting of a treated bait station in a cage, the final formulation, which contained BotaniGard, canola oil, diatomaceous earth, cornstarch, and liquid lure, was the most effective in killing male and female *Z. cucurbitae* compared to the formulation lacking certain components. Using forced contact experiments by making male flies walk through a treated tube, we found that the flies picked up the most spores when they were exposed to the final formulation. Fungal pathogens have been successfully dispersed among populations of *Cosmopolites sordidus* (banana weevil) via horizontal transmission and pheromone lure exploitation [287]. Ovitraps for disseminating *B. bassiana* to control the spread of the mosquito-borne Dengue fever and Malaria have been successful at reducing adult survival and dispersing fungal spores to uncontaminated mosquitoes [288,289]. Research on the use of fungal pathogens like *B. bassiana* and *Metarhizium anisopliae* (Metsch.) Sorokin for the control of tephritid fruit flies has been growing in recent years. A majority of the research shows that direct contact with the fungal spores in the adult, pupal, and larval stages results in very high mortality rates in the *Ceratitis*, *Bactrocera*, and *Anastrepha* genera [24–26,180,183,205,290]. Horizontal transmission during copulation or contamination during mating (leks) aggregations has also been a focus of a handful of studies [25,27,194,195]. However, development of a successful method of exploiting their behaviors to disseminate fungal spores has been the missing key. We have developed a formulation of *B. bassiana* that effectively transfers spores within a population of fruit flies in a laboratory setting. Field trials are the next step to determine the efficacy of the formulation against wild populations. An auto-disseminating bait station eliminates the need to locate specific roosting areas for the flies and can be used in combination with other IPM strategies.

Multiple types of traps (Jackson traps, McPhail Traps) are used to control many species of tephritid fruit flies [105,106,291]. For conducting field trials, we plan to design a bait station device that will protect the fungal spores from sunlight (ultraviolet radiation) and water infiltration. Ultraviolet radiation from the sun degrades spore viability resulting in low germination rates [292–294] and spores in dry or oil formulations can germinate prematurely without contacting an insect when they come in contact with water, including rain, irrigation or even high humidity [295,296]. Designing a bait station that addresses these pitfalls will be key to successful implementation of this new method.

The development of this formulation creates another potential product that can be utilized to control fruit fly populations. One of the most readily available products that growers can purchase for the control of fruit flies with the similar mechanism of attract-and-kill or attract-and-dispense is Amulet CL (BASF Corp., Research Triangle Park, NC). Unlike other dissemination stations and devices in use today, the formulation we developed is an alternative to chemical control. The use of biopesticides to contain a pest are emphasized as safer alternatives for the environment, people, and non-target pests [297,298]. Moreover, recent detections of pesticide-resistant tephritids emphasize the importance of developing an effective biopesticide with a very different mode of action [99,100,102,103,213]. Lastly, this formulation and method of use can be implemented in both conventional and organic cropping systems, making its application more universal.

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Appendix A

Full post-hoc comparison tables from three-way ANOVA of thickened germination percentages testing. The three effects that were tested were: formulation type (form), location, and time (week). The form types tested were the BMD (canola oil + non-thickened formulation on fabric), cornstarch, BMD + canola oil + cornstarch without fabric (CORN), glyceride flakes, BMD + canola oil + glyceride flakes without fabric (GF), and Dermofeel Viscolid, BMD + canola oil + Dermofeel Viscolid without fabric (DMV). Locations tested were the laboratory as a control (25±1º C; 70±5% RH) (Lab), a shady spot (Shade), and a sunny spots (Sun). Germination was evaluated over time (week) every 14 days for 8 weeks during the fall of 2021 (Sept. 6 – Nov. 1).

Table 1.

Table 1. Simple simple main effect table : One-way Anova (**location*week**) and how formulation type (**form**) effects germination percentages. DMV REMOVED removes significant p's

location	week	Effect	DFn	DFd	F	p	p<0.05	ges
Lab	0	form	2	4	2.326	0.214		0.248
Shade	0	form	2	4	2.326	0.214		0.248
Sun	0	form	2	4	2.326	0.214		0.248
Lab	2	form	2	4	0.071	0.933		0.015
Shade	2	form	2	4	0.058	0.945		0.027
Sun	2	form	2	4	0.114	0.895		0.053
Lab	4	form	2	4	2.018	0.248		0.454
Shade	4	form	2	4	5.609	0.069		0.712
Sun	4	form	2	4	2.796	0.174		0.542
Lab	6	form	2	4	1.475	0.331		0.337
Shade	6	form	1	2	5.111	0.152		0.585

Sun	6	form	2	4	80.138	0.000593	*	0.722
Lab	8	form	2	4	68.013	0.000816	*	0.892
Shade	8	form	2	4	6.187	0.06		0.569
Sun	8	form	2	4	5.523	0.071		0.56

Table 2.

Table 2. Simple simple main effect table: One-way Anova (location*week) and how formulation type (form) effects germination percentages. DMV INCLUDED creates the significant p								
location	week	Effect	DFn	DFd	F	p	p<0.05	ges
Lab	0	form	3	6	0.942	4.77E-01		0.262
Shade	0	form	3	6	0.942	4.77E-01		0.262
Sun	0	form	3	6	0.942	4.77E-01		0.262
Lab	2	form	3	6	83.52	2.77E-05	*	0.955
Shade	2	form	3	6	89.548	2.26E-05	*	0.977
Sun	2	form	3	6	380.067	3.13E-07	*	0.994
Lab	4	form	3	6	118.785	9.87E-06	*	0.982
Shade	4	form	3	6	227.939	1.43E-06	*	0.99
Sun	4	form	3	6	193.19	2.34E-06	*	0.988
Lab	6	form	3	6	138	6.34E-06	*	0.984
Shade	6	form	3	6	222.22	1.55E-06	*	0.987
Sun	6	form	3	6	347.125	4.10E-07	*	0.981
Lab	8	form	3	6	1799.139	2.99E-09	*	0.997
Shade	8	form	3	6	109.254	1.26E-05	*	0.969
Sun	8	form	3	6	20.925	1.00E-03	*	0.862

Table 3.

Table 3. Simple simple pairwise comparison with Bonferroni adjustment :: **Aragorn**: Pairwise t-test (**location*week**) with pairwise comparisons of formulation (**form**) type effects germination percentages. DMV REMOVED removes significant p's

BMD (non-thickened formulation); CORN (cornstarch thickened formulation); GF (glyceride flake thickened formulation)

location	week	.y.	group1	group2	n1	n2	p	p.adj	p.adj.signif
Lab	0	percentage	BMD	CORN	3	3	0.481	1	ns
Lab	0	percentage	BMD	GF	3	3	0.085	0.254	ns
Lab	0	percentage	CORN	GF	3	3	0.231	0.693	ns
Shade	0	percentage	BMD	CORN	3	3	0.481	1	ns
Shade	0	percentage	BMD	GF	3	3	0.085	0.254	ns
Shade	0	percentage	CORN	GF	3	3	0.231	0.693	ns
Sun	0	percentage	BMD	CORN	3	3	0.481	1	ns
Sun	0	percentage	BMD	GF	3	3	0.085	0.254	ns
Sun	0	percentage	CORN	GF	3	3	0.231	0.693	ns
Lab	2	percentage	BMD	CORN	3	3	0.766	1	ns
Lab	2	percentage	BMD	GF	3	3	0.964	1	ns
Lab	2	percentage	CORN	GF	3	3	0.767	1	ns
Shade	2	percentage	BMD	CORN	3	3	0.634	1	ns
Shade	2	percentage	BMD	GF	3	3	0.833	1	ns
Shade	2	percentage	CORN	GF	3	3	0.977	1	ns
Sun	2	percentage	BMD	CORN	3	3	0.756	1	ns
Sun	2	percentage	BMD	GF	3	3	0.968	1	ns

Sun	2	percentage	CORN	GF	3	3	0.713	1	ns
Lab	4	percentage	BMD	CORN	3	3	0.559	1	ns
Lab	4	percentage	BMD	GF	3	3	0.069	0.207	ns
Lab	4	percentage	CORN	GF	3	3	0.28	0.84	ns
Shade	4	percentage	BMD	CORN	3	3	0.108	0.324	ns
Shade	4	percentage	BMD	GF	3	3	0.367	1	ns
Shade	4	percentage	CORN	GF	3	3	0.152	0.456	ns
Sun	4	percentage	BMD	CORN	3	3	0.033	0.098	ns
Sun	4	percentage	BMD	GF	3	3	0.668	1	ns
Sun	4	percentage	CORN	GF	3	3	0.21	0.63	ns
Lab	6	percentage	BMD	CORN	3	3	0.562	1	ns
Lab	6	percentage	BMD	GF	3	3	0.209	0.627	ns
Lab	6	percentage	CORN	GF	3	3	0.343	1	ns
Shade	6	percentage	BMD	CORN	3	3	0.15	0.45	ns
Shade	6	percentage	BMD	GF	3	3	0.399	1	ns
Shade	6	percentage	CORN	GF	3	3	0.036	0.107	ns
Sun	6	percentage	BMD	CORN	3	3	0.005	0.016	*
Sun	6	percentage	BMD	GF	3	3	0.079	0.237	ns
Sun	6	percentage	CORN	GF	3	3	0.003	0.01	*
Lab	8	percentage	BMD	CORN	3	3	0.014	0.041	*
Lab	8	percentage	BMD	GF	3	3	0.222	0.666	ns
Lab	8	percentage	CORN	GF	3	3	0.000986	0.003	**
Shade	8	percentage	BMD	CORN	3	3	0.117	0.351	ns
Shade	8	percentage	BMD	GF	3	3	0.771	1	ns
Shade	8	percentage	CORN	GF	3	3	0.079	0.238	ns
Sun	8	percentage	BMD	CORN	3	3	0.089	0.266	ns
Sun	8	percentage	BMD	GF	3	3	0.251	0.753	ns
Sun	8	percentage	CORN	GF	3	3	0.211	0.633	ns

Table 4.

Table 4. Pairwise T-test grouping (form*week) with pairwise comparisons of formulation (location) type effects germination percentages. DMV REMOVED removes significant p's

BMD (non-thickened formulation); CORN (cornstarch thickened formulation); GF (glyceride flake thickened formulation)

week	form	.y.	group1	group2	n1	n2	p	p.adj	p.adj.signif
0	BMD	percentage	Lab	Shade	3	3	NaN	NaN	
0	BMD	percentage	Lab	Sun	3	3	NaN	NaN	
0	BMD	percentage	Shade	Sun	3	3	NaN	NaN	
2	BMD	percentage	Lab	Shade	3	3	3.36E-01	1.00E+00	ns
2	BMD	percentage	Lab	Sun	3	3	1.10E-02	3.30E-02	*
2	BMD	percentage	Shade	Sun	3	3	7.65E-01	1.00E+00	ns
4	BMD	percentage	Lab	Shade	3	3	1.20E-02	3.70E-02	*
4	BMD	percentage	Lab	Sun	3	3	1.80E-02	5.30E-02	ns
4	BMD	percentage	Shade	Sun	3	3	4.10E-02	1.24E-01	ns
6	BMD	percentage	Lab	Shade	3	3	5.10E-02	1.52E-01	ns
6	BMD	percentage	Lab	Sun	3	3	2.00E-03	7.00E-03	**
6	BMD	percentage	Shade	Sun	3	3	3.10E-02	9.30E-02	ns
8	BMD	percentage	Lab	Shade	3	3	1.60E-02	4.70E-02	*
8	BMD	percentage	Lab	Sun	3	3	2.65E-05	7.95E-05	****
8	BMD	percentage	Shade	Sun	3	3	7.00E-03	2.20E-02	*
0	CORN	percentage	Lab	Shade	3	3	NaN	NaN	
0	CORN	percentage	Lab	Sun	3	3	NaN	NaN	
0	CORN	percentage	Shade	Sun	3	3	NaN	NaN	
2	CORN	percentage	Lab	Shade	3	3	6.88E-01	1.00E+00	ns
2	CORN	percentage	Lab	Sun	3	3	3.86E-01	1.00E+00	ns
2	CORN	percentage	Shade	Sun	3	3	1.50E-02	4.60E-02	*
4	CORN	percentage	Lab	Shade	3	3	2.30E-01	6.90E-01	ns
4	CORN	percentage	Lab	Sun	3	3	1.30E-02	4.00E-02	*
4	CORN	percentage	Shade	Sun	3	3	4.90E-02	1.48E-01	ns
6	CORN	percentage	Lab	Shade	3	3	1.18E-01	3.54E-01	ns

6	CORN	percentage	Lab	Sun	3	3	1.00E-03	3.00E-03	**
6	CORN	percentage	Shade	Sun	3	3	5.10E-02	1.52E-01	ns
8	CORN	percentage	Lab	Shade	3	3	4.30E-02	1.28E-01	ns
8	CORN	percentage	Lab	Sun	3	3	9.00E-03	2.70E-02	*
8	CORN	percentage	Shade	Sun	3	3	5.33E-04	2.00E-03	**
0	GF	percentage	Lab	Shade	3	3	NaN	NaN	
0	GF	percentage	Lab	Sun	3	3	NaN	NaN	
0	GF	percentage	Shade	Sun	3	3	NaN	NaN	
2	GF	percentage	Lab	Shade	3	3	3.92E-01	1.00E+00	ns
2	GF	percentage	Lab	Sun	3	3	3.40E-02	1.02E-01	ns
2	GF	percentage	Shade	Sun	3	3	3.82E-01	1.00E+00	ns
4	GF	percentage	Lab	Shade	3	3	6.90E-02	2.08E-01	ns
4	GF	percentage	Lab	Sun	3	3	4.00E-03	1.10E-02	*
4	GF	percentage	Shade	Sun	3	3	9.80E-02	2.94E-01	ns
6	GF	percentage	Lab	Shade	3	3	2.70E-02	8.10E-02	ns
6	GF	percentage	Lab	Sun	3	3	2.10E-02	6.20E-02	ns
6	GF	percentage	Shade	Sun	3	3	4.40E-02	1.32E-01	ns
8	GF	percentage	Lab	Shade	3	3	4.40E-02	1.34E-01	ns
8	GF	percentage	Lab	Sun	3	3	4.00E-03	1.10E-02	*

Appendix B

Full post-hoc comparison tables from three-way ANOVA of liquid lure incorporated formulation germination percentages testing. The three effects that were tested were: liquid lure type (lure), concentration (%), and time (week). The liquid lure types tested were the C-L (BMD + canola oil + non-thickened formulation on fabric + C-L concentration %) and ME (BMD + canola oil + non-thickened formulation on fabric + ME concentration %). Concentrations were tested at 10.0, 1.0, 0.1, and 0.0%. All tested sheets were hung in the laboratory out of direct sunlight and kept at (25±1º C; 70±5% RH).

Table 1.

Table 1. Pairwise T-test grouping (lure*week) with pairwise comparisons of formulation (concentration) type effects germination percentages.

week	form	.y.	Group 1 (%)	Group 2 (%)	n1	n2	p	p.adj	p.adj.signif
0	Cuelure	percentage	0.00%	0.10%	3	3	0.658	1	ns
0	Cuelure	percentage	0.00%	1.00%	3	3	0.593	1	ns
0	Cuelure	percentage	0.00%	10.00%	3	3	0.598	1	ns
0	Cuelure	percentage	0.10%	1.00%	3	3	0.802	1	ns
0	Cuelure	percentage	0.10%	10.00%	3	3	0.817	1	ns
0	Cuelure	percentage	1.00%	10.00%	3	3	0.742	1	ns
1	Cuelure	percentage	0.00%	0.10%	3	3	0.811	1	ns

1	Cuelure	percentage	0.00%	1.00%	3	3	0.607	1	ns
1	Cuelure	percentage	0.00%	10.00%	3	3	0.015	0.092	ns
1	Cuelure	percentage	0.10%	1.00%	3	3	0.794	1	ns
1	Cuelure	percentage	0.10%	10.00%	3	3	0.801	1	ns
1	Cuelure	percentage	1.00%	10.00%	3	3	0.893	1	ns
2	Cuelure	percentage	0.00%	0.10%	3	3	0.758	1	ns
2	Cuelure	percentage	0.00%	1.00%	3	3	0.555	1	ns
2	Cuelure	percentage	0.00%	10.00%	3	3	0.396	1	ns
2	Cuelure	percentage	0.10%	1.00%	3	3	0.816	1	ns
2	Cuelure	percentage	0.10%	10.00%	3	3	0.587	1	ns
2	Cuelure	percentage	1.00%	10.00%	3	3	0.876	1	ns
3	Cuelure	percentage	0.00%	0.10%	3	3	0.847	1	ns
3	Cuelure	percentage	0.00%	1.00%	3	3	0.657	1	ns
3	Cuelure	percentage	0.00%	10.00%	3	3	0.533	1	ns
3	Cuelure	percentage	0.10%	1.00%	3	3	0.785	1	ns
3	Cuelure	percentage	0.10%	10.00%	3	3	0.746	1	ns
3	Cuelure	percentage	1.00%	10.00%	3	3	0.748	1	ns
0	Methyl Eugenol	percentage	0.00%	0.10%	3	3	0.831	1	ns
0	Methyl Eugenol	percentage	0.00%	1.00%	3	3	0.741	1	ns
0	Methyl Eugenol	percentage	0.00%	10.00%	3	3	0.422	1	ns
0	Methyl Eugenol	percentage	0.10%	1.00%	3	3	0.788	1	ns
0	Methyl Eugenol	percentage	0.10%	10.00%	3	3	0.591	1	ns
0	Methyl Eugenol	percentage	1.00%	10.00%	3	3	0.556	1	ns
1	Methyl Eugenol	percentage	0.00%	0.10%	3	3	0.873	1	ns
1	Methyl Eugenol	percentage	0.00%	1.00%	3	3	0.793	1	ns
1	Methyl Eugenol	percentage	0.00%	10.00%	3	3	0.631	1	ns
1	Methyl Eugenol	percentage	0.10%	1.00%	3	3	0.931	1	ns
1	Methyl Eugenol	percentage	0.10%	10.00%	3	3	0.057	0.343	ns
1	Methyl Eugenol	percentage	1.00%	10.00%	3	3	0.69	1	ns
2	Methyl Eugenol	percentage	0.00%	0.10%	3	3	0.824	1	ns
2	Methyl Eugenol	percentage	0.00%	1.00%	3	3	0.497	1	ns
2	Methyl Eugenol	percentage	0.00%	10.00%	3	3	0.034	0.203	ns

2	Methyl Eugenol	percentage	0.10%	1.00%	3	3	0.647	1	ns
2	Methyl Eugenol	percentage	0.10%	10.00%	3	3	0.367	1	ns
2	Methyl Eugenol	percentage	1.00%	10.00%	3	3	0.74	1	ns
3	Methyl Eugenol	percentage	0.00%	0.10%	3	3	0.849	1	ns
3	Methyl Eugenol	percentage	0.00%	1.00%	3	3	0.189	1	ns
3	Methyl Eugenol	percentage	0.00%	10.00%	3	3	0.501	1	ns
3	Methyl Eugenol	percentage	0.10%	1.00%	3	3	0.744	1	ns
3	Methyl Eugenol	percentage	0.10%	10.00%	3	3	0.245	1	ns
3	Methyl Eugenol	percentage	1.00%	10.00%	3	3	0.772	1	ns