BIOCONTROL POTENTIAL OF ENTOMOPATHOGENIC NEMATODES (EPNs) AND ENTOMOPATHOGENIC FUNGI (EPF) AGAINST THE SOIL-DWELLING STAGES OF SPOTTED-WING DROSOPHILA (*DROSOPHILA SUZUKII*)

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ABSTRACT

Spotted wing drosophila (SWD), a highly invasive pest of small fruits such as blueberries and raspberries, causes significant yield losses. Females have serrated ovipositors, allowing them to lay eggs in unripe fruits, making them unsellable. High fecundity and short generation time also contribute to the challenges in managing this fly. Current management heavily relies on using broad-spectrum insecticides, which increases its resistance. Recently, there has been an increasing interest in biological control and other sustainable tactics to reduce reliance on insecticides. This study investigates the potential of using entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) as alternative control methods. Lab experiments in 2022 and 2023 tested commercially available EPNs and EPFs against SWD's soil-dwelling stages. Nine EPN species and three EPF species were assessed at various concentrations against the soil-dwelling stages of SWD. Results showed that three EPN species (*Steinernema feltiae, Steinernema carpocapsae, and Steinernema glaseri*) and one EPF species (*Beauveria bassiana*) reduced SWD mortality by more than 50%. When combined, EPF and EPNs achieved more than 90% reduction. Although these findings suggest that EPNs and EPF may not wholly control SWD populations, they could be beneficial additions to SWD management strategies.

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CHAPTER 1. LITERATURE REVIEW

1.1 Spotted wing drosophila

Spotted-wing drosophila (*Drosophila suzukii*) is one of the most important pests of soft-skinned and small stone fruits, including blueberries (Walsh et al., 2011). The invasive species, originating from southeastern Asia, was initially identified in Hawaii during the 1980s. Subsequently, it was discovered in California in 2008 and spread throughout the West Coast in 2009. In 2010, it was first observed in Florida, Utah, the Carolinas, Wisconsin, and Michigan. (Spotted Wing Drosophila, MSU, 2020). Compared to other *Drosophila species*, *Drosophila suzukii* infests undamaged fruit near harvest time using its serrated ovipositor, leading to physical harm to the fruit and creating opportunities for other pathogens to enter (Walsh et al., 2011).

1.1.1 Pest Biology and Host Range

SWD belongs to the *D. suzukii* subgroup in the *D. melanogaster* species group of the subgenus Sophophora (Diptera: *Drosophilidae).* A distinguishing morphological feature used to distinguish SWD from other drosophilas is the existence of black spots on the leading wing edge of males and a large, serrated ovipositor in females (Kikkawa and Peng, 1938; Walsh et al., 2011; Cini et al., 2012). Female insects can deposit as many as 380 eggs in a span of 2 to 3 weeks, leading to significant reproduction rates (Kanzawa 1939).

Raspberries, cherries, blueberries, and blackberries are the most crucial crop hosts (Bellamy et al., 2013; Burrack et al., 2013). Some fruits, such as apples, tomatoes, and pears, can be infested if they are previously damaged or split (Steffan et al., 2013). Cultivated fruits and numerous wild plants may be potentially significant hosts (Mitsui et al., 2010; Cini et al., 2012; Poyet et al., 2014; Lee et al., 2015).

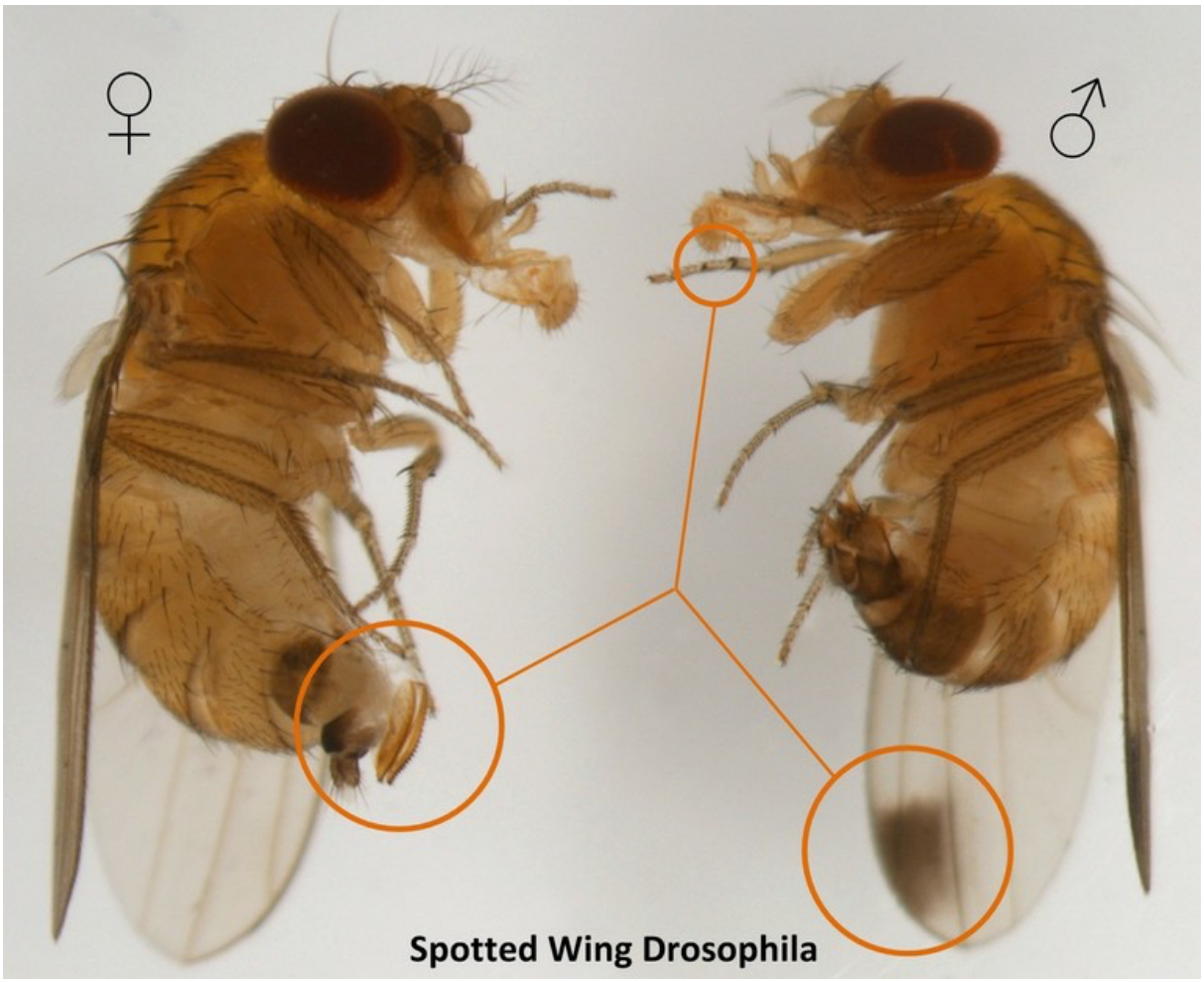

Figure 1: SWD males (right) have dark spots at the tip of each wing. Females (left) lack these spots but have a serrated egg-laying structure. (Photograph courtesy Christelle Guédot, UW-Madison Entomology).

1.1.2 Life Cycle and Damages

Female fruit flies have a unique way of laying eggs. Using their serrated ovipositor, they cut through the surface of fruits, depositing approximately 1-3 eggs per fruit and up to 7-16 eggs per day. Over the course of her life, a female fruit fly can lay several hundred eggs. These eggs have a pair of "breathing tubes" attached to one end, protruding from the hole they were laid in. Once hatched, the maggots feed on the interior of the fruit, causing it to decay, change color, and

eventually collapse. The spoiled fruit becomes susceptible to secondary infections by fungi and bacteria. While pupation can occur both inside and outside the fruit, the larvae are typically found outside (Woltz and Lee 2017). During the summer, adult SWD can survive for 3-9 weeks, while those that overwinter can live for many months. SWD can rapidly reproduce and generate successive generations when suitable hosts and temperatures are present (Spotted Wing Drosophila, WSU, 2021).

1.1.3 Current Economic Status of SWD

SWD damage causes up to $500 million in annual yield losses in the US (Rochi, 2021). Yeh et al., (2020) used a 30% yield reduction value for the wild raspberry variety in Maine and found a revenue loss of over $6.8 million. In a one-year study, Minnesota raspberry farmers experienced a sales decline of around $2.36 million (DiGiacomo et al., 2019). From 2009 to 2014, the raspberry industry in California suffered a revenue loss of about $39.8 million because of spotted wing drosophila (Farnsworth et al., 2016).

1.1.4 Management practices

In order to manage the spread of spotted wing drosophila (SWD), many farmers rely on a combination of cultural practices and the use of chemical insecticides (Rogers et al., 2016). Currently, field sanitation is considered the most effective approach to combat Drosophila suzukii. This involves labor-intensive tasks such as solarizing the soil and removing overripe and fallen fruits. Several studies (Vogt 2012; Haye et al., 2016; Farnsworth et al., 2017) have highlighted the importance of these methods in controlling SWD. Countries around the world have taken steps to prohibit the use of soil treatments, insecticides, disinfectants, and post-harvest treatments due to their harmful effects on the environment. Instead of relying on these chemicals, some have turned to using nets to cover fruit-bearing shrubs and trees as an alternative option. However, the long-

term effectiveness of these nets is still uncertain and requires further study (Stacconi et al., 2015; Englert and Herz, 2016; Haye et al., 2016). It's important to note that chemical control alone is not a sustainable solution for managing SWD, as excessive use of insecticides can lead to resistance and environmental contamination (Van Timmeren and Isaacs, 2013; Asplen et al., 2015). Biological control is a method used to decrease the long-term expenses associated with managing spotted-wing drosophila and to assist farmers in developing more economically and environmentally sustainable farms (Haye et al., 2016, Schetelig et al., 2018).

Drosophila suzukii poses a significant threat to crops, and it is important to find safe and eco-friendly ways to control it. While there are ongoing assessments of native natural enemies, their direct impact on D. suzukii has not yet been studied in the field (Stacconi et al., 2015). Therefore, alternative control methods are needed to manage this pest (Wiman et al., 2016).

1.2 Biological control

Smith introduced the concept of "Biological control" in 1919. Biological control involves utilizing natural enemies like predators, parasitoids, and pathogens to diminish pest populations. It plays a crucial role in integrated pest management and usually requires active human participation (Waage & Greathead, 1998). It is an ecological strategy that leverages knowledge of food-web interactions to proactively regulate pest populations. When properly implemented, biological control offers a reliable and methodical approach to pest management (Hajek 2004; Vincent et al., 2007).

In biological control, various techniques are used to manage pests. One of these techniques, known as classical biological control, involves the introduction of non-native species to control pest populations. A notable example of this method's success occurred in 1900 when the predatory ladybird *Rodolia cardinalis* was introduced to combat the citrus pest *Icerya purchasi*

in Mediterranean Europe. Another instance of success was the release of the parasitoid *Aphelinus mali* in the 1930s to control the woolly apple aphid *Eriosoma lanigerum* in apple orchards across Europe. Additionally, the introduction of the parasitoid *Bathyplectes anurus* to manage the alfalfa weevil *Hypera postica* also yielded positive results (Bryan et al., 1993).

Augmentative biological control involves introducing biological control agents into areas where natural enemies are scarce or absent in order to manage pest populations. This can be accomplished through either inoculative releases or inundative releases (Jeffers & Chong, 2021). Inoculative augmentation entails releasing a beneficial agent onto crops once during the season. This agent then regulates pests for the remainder of the season through their offspring. Inundative releases are planned when pests are only controlled by the released beneficial agents themselves, as future generations typically do not survive to manage potential pest outbreaks. In such cases, more frequent releases are necessary throughout the season (Thomas 2019; Toscan 2017). An example of inoculation involves introducing predatory rove beetles into a greenhouse to establish them with the goal of eliminating the need for future releases. (Bennison et al., 2009; Bennison et al., 2008). An example of inundation-based biological control is the large-scale release of entomopathogenic nematodes 2.5 billion per hectare to target soil-dwelling pests (Barbercheck 2004). While entomopathogenic nematodes are naturally present in the soil, it is believed that their abundance is typically not enough to achieve efficient economic control (Stuart et al., 2006). In both cases, the objective of releasing natural enemies is to enhance the current population in order to attain sufficient biological control. (Hajek 2018).

The approach of conservation biological control focuses on manipulating the environment, such as habitat and plant diversity, as well as modifying agricultural practices and pest

management to enhance the populations and effectiveness of naturally occurring predators and parasites (Jeffers & Chong 2021; Eilenberg et al., 2001).

Biological control is a highly regarded approach used to manage insect populations without the use of chemical insecticides, which can have detrimental effects on the environment and human health. Insects can also develop resistance to these chemicals over time. In the context of greenhouse cultivation, biological control often involves the strategic introduction of natural predators to suppress pest populations. Greenhouses create an environment conducive to rapid insect population growth, with high temperatures and relative humidity fostering ideal conditions for these pests to thrive (Van Lenteren and Woets 1988). These are ideal conditions for augmentative biological control – natural enemies are lacking, and conditions are optimal for prey build-up.

1.2.1 Limitations and Compatibility with Other Management Practices

While biological controls have been effective in managing plant diseases, they do have some limitations. For example, introducing non-native species as a control method can lead to these species becoming invasive and causing negative impacts by spreading beyond their original introduction region (Jennings et al., 2017). Another limitation is that it generally takes longer to suppress pest populations compared to synthetic pesticides. This is because the organism being controlled may take several days to die, and predatory species require some time to establish effective pest suppression (Bale et al., 2007). It's also important to note that biological control doesn't completely eliminate pests, but rather helps to regulate their population (Lenteren J.C 1997b, 1998).

In the world of pest control, using biological methods and pesticides together is a bit of a challenge (Hajek 2004). This is because the active components in pesticides don't just target the

pests, but they can also harm the natural predators and parasites that help keep the pest populations in check (Cloyd and Bethke 2011; Hassan and Veire 2004). Various studies have looked into how different pesticides impact entomopathogenic nematodes – some pesticides are toxic to these helpful organisms, while others are not (Rovesti and Deseo 1991). One possible solution to this issue is to carefully time and plan the use of pesticides to minimize their impact on beneficial organisms involved in biological control (Hassan and Veire 2004).

1.3 Entomopathogenic nematodes

Entomopathogenic nematodes are insect parasites that infect hosts with the help of their mutually associated bacteria symbiont (Shapiro-Ilan et al., 2014). Three families of nematodes are known for their ability to infect and ultimately kill their hosts: *Rhabditidae, Steinernematidae, and Heterorhabditidae.* Among these, the families *Heterorhabditidae and Steinernematida*e have been the focus of extensive research (Kaya and Gaugler 1993; Georgis et al., 2006). With their ability to infect a wide variety of hosts, quick reproduction, and adaptability to both laboratory and natural environments, bacteriophages play a key role in enhancing soil health. They are also known for their environmentally friendly nature and can be effectively used alongside various chemical and biological pesticides (Lacey and Georgis 2012).

1.3.1 Lifecycle

The infective juvenile stage (IJ), also known as the dauer stage, represents the only free-living phase during which the organism roams the soil or waits for passing insects. During this stage, the juvenile enters its host insect through natural openings such as spiracles, mouth, anus, or occasionally by penetrating the insect's cuticle (Shapiro-IIan et al., 2016). In the case of *Heterorhabditis*, the infective juveniles (IJs) expel the symbiotic bacteria by regurgitating them, whereas in *Steinernema*, the IJs eliminate the bacterial cells by excreting them into the body cavity

of the insect. (Ciché et al., 2006). These bacteria rapidly reproduce and fatally infect their host within a span of 24 to 48 hours. Following the host's demise, the infective juvenile stages continue to consume host tissue, undergo molting, and reproduce, resulting in 1-3 subsequent generations. When the food source is exhausted, the infective juvenile stages persist within the host cadaver, actively seeking out new hosts (Kaya and Gaugler 1993).

1.3.2 Rearing

Out of over 90 species of *Steinernematids and Heterorhabditis* that have been identified, only 13 of them are currently being commercially produced (Shapiro-IIan et al., 2014). When it comes to commercial purposes, an in vitro method is commonly used, usually in liquid culture and sometimes in solid fermentation. Conversely, the in vivo method is favored for laboratory research due to its lower cost and simplified technique requirements (Shapiro-Ilan and Gaugler 2002). The final instar caterpillars of the greater wax moth, known as *Galleria mellonella*, are frequently utilized as hosts because they are highly susceptible to a broader variety of nematode species and are readily available (Abd ElAzim et al., 2019). A single larva has the potential to produce anywhere from 1 to 3.5 × 10^5 infective juveniles (Ijs) (Dutky et al., 1964; Milstead and Poinar 1978). Mealworm larvae (*Tenebrio molitor*) are the preferred choice for advanced nematode production because of their strong structural integrity, high susceptibility to nematodes, low cost, and the absence of cocoon production, which could potentially interfere with nematode inoculation (Shapiro-IIan et al., 2016; Blinova and Ivanova 1987).

1.3.2.1 In Vivo method

Numerous researchers have documented various in vivo culture techniques to cultivate entomopathogenic nematodes. These methods all revolve around utilizing a system known as the white trap, with minor variations. This system capitalizes on the natural movement of infective

juveniles as they depart from the host cadaver upon emergence (Shapiro-IIan and Gaugler 2002). Hosts are inoculated with nematodes on a dish or tray lined with filter paper or other substrate conducive to nematode infections. These dishes or trays are then placed in an incubator for 2 to 5 days at a standard temperature of 25°C, which is optimal for most nematodes. After infection, dead cadavers display distinct colors based on the nematode species they are infected with. Cadavers infected with *Steinernema sp*. typically show a gray, brown, or beige color, while those infected with *Heterorhabditis sp*. exhibit a brick red to orange hue (Shapiro-IIan et al., 2016).

To collect the infective juveniles, the infected cadavers are placed in a white trap. This involves placing a smaller petri dish containing the cadaver within a larger petri dish filled with water. As the infective juveniles emerge from the host cadaver, they crawl over the filter paper, up and over the edges of the petri dish, and into the surrounding water. The final step involves collecting and storing the nematodes at the optimal temperature of 25°C and a relative humidity between 75% and 100% (Grewal et al., 1994b).

1.3.2.2 In Vitro method

Initially, attempts to produce EPN in a lab setting using a solid medium were not successful (Glaser 1932). However, a breakthrough occurred when researchers discovered the significance of bacterial symbionts in the reproduction of EPN, leading to the establishment of a method for mass production in vitro (Poinar and Thomas 1966). In vitro production involves a technique where solid or liquid media are inoculated with a pure culture of symbiont bacteria. The process consists of four main steps: preparing the solid media, inoculating it with symbiotic bacteria, introducing axenic nematodes, and finally harvesting the nematodes (Tourtois 2014).

1.3.3 EPN Application

Entomopathogenic nematodes have been utilized as a bio-insecticide in a few specific agricultural settings. These include turfgrass, greenhouse and nursery environments, mushroom cultivation, and a select few field crops and orchards (Grewal et al., 2005). The use of nematodes to control Japanese beetles in turfgrass was one of the earliest applications (Glaser et al., 1935). White grubs, which belong to the Scarabaeidae family of the Coleoptera order, pose a significant threat to turfgrass as they feed on the roots of various grass species (Jackson 1992). Due to their destructive nature, white grubs are considered to be among the most damaging insect pests in turfgrass. Consequently, extensive research has been carried out to explore the efficacy of nematodes in controlling white grub populations.

Entomopathogenic nematodes offer the unique advantage of being compatible with conventional spray equipment, making application convenient and accessible (Grewal et al., 2005). These beneficial nematodes are typically applied at high rates, with up to 2.5 billion infective juveniles per hectare in an aqueous solution (Barbercheck 2004). The combination of high application numbers and compatibility with traditional equipment classifies them as biopesticides, despite not being regulated by the EPA. They can also be directly applied with other biological and chemical pesticides, fertilizers, and soil amendments (Krishnayya et al., 2002).

Entomopathogenic nematodes from the families *Heterorhabditidae* and *Steinernematidae* are widely used to control insect pests (Kaya and Gaugler 1993; Georgis et al., 2006). The nematode-associated beneficial bacteria cause the death of the insect host within 24-48 hours after an infective juvenile has entered the insect. The infective juveniles are the sole independent stage of the nematodes and occur naturally in soil. Consequently, EPNs are most efficient in controlling insect pests that have life stages in the soil (Koppenho¨fer and Fuzy 2007). Entomopathogens can

effectively control fruit flies in all life stages, including adults, pupae in soil, and larvae in fruits (Daniel and Wyss 2009; Oreste et al., 2015; Yousef et al., 2017). In a study by Lee et al. (2019), it was reported that up to that point, 18 research papers had been published on the use of entomopathogenic nematodes to combat spotted-wing drosophila (SWD). The results showed that several species of nematodes had a positive impact in reducing SWD counts or successfully penetrating the insects. Specifically, *Heterorhabditis bacteriophora* was effective in 10 out of 16 trials, *Steinernema carpocapsae* in 13 out of 17 trials*, S. feltiae* in 16 out of 18 trials*, S. kraussei* in 2 out of 3 trials, and an unspecified *Steinernema* species from a hazelnut orchard in 2 out of 2 trials.

1.4 Entomopathogenic Fungi

1.4.1 Introduction

Entomopathogenic fungi are a group of microorganisms that have the ability to infect, parasitize, and ultimately kill arthropod pests. They offer an alternative to traditional chemical pesticides in organic farming, offering a more environmentally friendly pest control method (Bihal et al., 2023). Entomopathogenic fungi (EPF) are present worldwide and have a competitive advantage over other types of entomopathogens due to their ability to invade insect hosts by penetrating the insect integument without the need for ingestion. (Wakil et al., 2022). Shah & Pell, (2003) reported that previous studies indicate that entomopathogenic fungi have minimal impact on non-targets, making them a safer option for use in integrated pest management (IPM) compared to chemical insecticides. Fungi need high humidity for disease initiation which is considered a major disadvantage (Roberts & Hajek 1992, pp 144-159). EPFs typically have a slow killing rate of 2-3 weeks, compared to chemical insecticides, which may take 2-4 hours (Khan et al. 2012).

The research conducted by Bihal et al. in (2023) revealed the presence of a diverse range of entomopathogenic fungi. They identified a total of 12 distinct types of entomopathogenic fungi in the Oomycetes category, 399 types of Microsporidia, 65 types of Chytridiomycota, 474 types of Entomophtoromycota, 283 types of Basidiomycota, and a total of 476 types of Ascomycota. Among these, species from the Ascomycota and Entomophtoromycota phyla are most commonly found in nature. Notably, entomopathogenic fungi belonging to the phylum Entomophtorales such as Conidiobolus, Furia, Erynia, and Entomophaga have shown highly effective pest control properties. However, their practical application is limited by the lack of advanced breeding technologies in laboratory settings, hindering their widespread use in bio-preparation methods. On the other hand, saprophytic fungi from the phylum Ascomycota are frequently utilized. Other biopesticides are derived from entomopathogenic fungi including the genera Beauveria, Paecilomyces, Metarhizium, Lecanicillium, and Isaria. Due to their versatility, these fungi can effectively control a wide spectrum of insect pests. This information is supported by research studies conducted by Khan et al., (2012), Jaihan et al., (2016), Araújo J.P et al., (2016), Castro T et al., (2016), Rı'os-Moreno et al., (2016), and Litwin et al., (2020).

1.4.2 Host infection by EPF

The process of EPF infecting a host can be categorized into three stages: (1) attachment of spores to the cuticle, spore germination, and formation of appressoria that penetrate the cuticle; (2) invasion into the hemocoel, rapid growth, and suppression of the immune response; and (3) formation of fungal reproductive structures (conidial or ascospore in the case of Ascomycetes) (Ramirez-Camejo et al., 2022).

Entomopathogenic fungi invade insects by penetrating the external covering of the insect's body. The process of infection starts when spores adhere to the exoskeleton of the arthropod

because of electrostatic and hydrophobic forces. Following this attachment, lytic enzymes and secondary metabolites are activated. (Skinner et al., 2014). The fungal hyphae start to develop after invading the insect's body cavity. Some EPF release blast conidia or spores that enter the insect's hemolymph and form additional hyphae, which stay within the body and disturb the insect's internal processes. As the infection advances, the insect's initially soft body becomes rigid because of the pathogenic fungus absorbing fluids. (Bihal et al., 2023).

1.4.3 EPF in field condition

Dara et al. reported an outbreak of *Entomophora muscae* in Mississippi, United States, on spotted-wing drosophila in June 2017 during cool and wet weather conditions. Adult spotted-wing drosophila with white/gray powdery fluff, which was sporulating, were found deceased or unable to move on fig plants. In subsequent lab tests, it was observed that approximately 25% of adult spotted-wing drosophila died after being exposed to sporulating house fly cadavers (Becher et al., 2017).

The majority of commercially produced fungi belong to species such as *Beauveria, Metarhizium, Lecanicillium, and Isaria.* These species are chosen because they are relatively easy to mass produce (Roberts & Humber 1981). The family of entomopathogenic fungi typically consists of opportunistic species that can infect various insects and cause their death by producing toxins that trigger defensive reactions in the host (Mondal et al., 2016*). Beauveria bassiana* can parasitize numerous arthropod hosts, such as the pine caterpillar, European corn borer, and green leafhoppers. Therefore, it is typically considered a nonselective pesticide (Islam et al., 2021).

The current controls for spotted-wing drosophila (SWD) primarily involve using a variety of insecticides such as spinosad, organophosphates, pyrethroids, and neonicotinoids. However, biological control methods could provide a more cost-effective and environmentally friendly

approach to managing SWD (Girod and Turlings, 2019). Nevertheless, these programs might face difficulties because wild fruits can act as a reservoir for this highly adaptable and mobile pest, leading to the reinfestation of managed crops. (Lee et al., 2015). Parasitoids and predators have been used as a biological control for SWD. Parasitoids have been playing an essential role in regulating the population of SWD (Carton et al., 1986; Fleury et al., 2004). In laboratory experiments, *Ganaspis brasiliensis* and *L. japonica*, which are larval parasitoids collected from South Korea, were found to readily develop from SWD, according to a study by Daane et al., 2016. Certain predatory bugs, such as *Orius species* (Anthocoridae), have been observed preying on spotted wing drosophila (SWD) in raspberry crops in the USA (Walsh et al., 2011). These bugs were also found in infested fruit samples in Spain, as noted by Arnó et al. in 2012. The next step in biological control could involve using EPNs and EPF. Based on several studies, further screening for more effective nematodes may be necessary.

My thesis research has three major objectives. 1. Determine the effectiveness of various types of entomopathogenic nematodes on the soil-dwelling stages of SWD. 2. Determine the effectiveness of entomopathogenic fungi on the soil-dwelling stage of SWD. 3. Evaluate the effectiveness of using entomopathogenic nematodes and fungi in soil-dwelling stages of SWD.

CHAPTER 2. ENTOMOPATHOGENIC NEMATODES AND ENTOMOPATHOGENIC FUNGI AGAINST SOIL-DWELLING STAGES OF SWD AND INFESTED BLUEBERRY

2.1 Introduction

Drosophila suzukii, a species of fruit fly, has quickly expanded its presence across the major fruit production regions in the United States, Europe, Canada, and Mexico (Cini et al., 2012; Asplen et al., 2015). Females' prominent serrated ovipositor enables them to infest ripening fruits before harvest, and larvae's internal feeding renders the fruit unmarketable (Hauser et al., 2011; Walsh et al., 2011). Moreover, they have a broad host range, including blackberries, blueberries, cherries, peaches, raspberries, strawberries, and grapes. Current control programs for D. suzukii rely solely on insecticides, leading to resistance (Van Timmerren and Isaacs 2013)

Entomopathogenic nematodes (EPNs) from the families Heterorhabditidae and Steinernematidae are available commercially for controlling various insect pests (Wakil et al.,2021). The use of entomopathogenic nematodes in the biological control of insect pests has long been a common practice in agricultural systems like turfgrass, greenhouses, nurseries, mushrooms, and orchards (Grewal et al., 2005).

In Vivo, the production of nematodes is typically carried out on a small scale and is well-suited for the cottage industry or niche markets (Shapiro-Ilan et al., 2014). The most commonly used hosts for rearing nematodes in vivo are mature larvae of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), and the yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). This involves using insect hosts since entomopathogenic nematodes are obligate parasites of insects (Shapiro-Ilan et al., 2002). The infective juveniles enter insect hosts through natural openings such as the mouth, anus, and spiracles. Once inside the insect host,

the juvenile nematodes release their symbiotic bacteria and kill the host within 24-48 hours. The nematodes then complete their development within the host, going through one to three generations. After depleting the host's resources, thousands of infective juveniles emerge from the cadaver in search of a new host. (Kaya and Gaugler 1993).

Infection of spotted-wing drosophila larvae was more successful than similar trials with spotted-wing drosophila pupae, possibly because nematodes have difficulty penetrating pupae (Garriga et al., 2017, Hübner et al., 2017). The lure and infect strategy of attracting pests to a source of nematodes was tested by Mastore et al., 2021. EPN Combined administration with Bt significantly increased effectiveness compared to treatments with single bioinsecticides.

An EPF is a microorganism that can infect, parasitize, and exterminate arthropod pests. Typically employed as a substitute for traditional chemical pesticides in organic agriculture, they also have origins in biotechnological production and traditional Chinese medicine (Bihal et al., 2023). Entomopathogenic fungi infect insects by directly penetrating their outer integument. Infection initiation involves spores attaching to the arthropod's exoskeleton due to electrostatic and hydrophobic forces, as well as the activation of lytic enzymes and secondary metabolites (Skinner et al., 2014). The insect's soft body stiffens as the infection advances because of the pathogenic fungus absorbing fluids (Donzelli et al., 2010).

In various research studies, it has been observed that *B. bassiana* can reduce both the oviposition of D. suzukii on uninfested berries and the emergence of adult flies from previously infested berries. However, other studies have found conflicting results (Gargani et al., 2013; Cuthbertson et al., 2014; Liburd and Rhodes 2021). Furthermore, Yousef et al., (2017) conducted an experiment using *Metarhizium brunneum*-based devices and reported a 62.2% adult mortality and an 84.7% reduction in fecundity in a lure-and-kill experiment for SWD.

The following experiments were conducted to evaluate the effectiveness of entomopathogenic nematodes and entomopathogenic fungi against soil-dwelling stages of SWD.

2.2 METHOD AND MATERIALS

2.2.1 Spotted-wing drosophila colony

SWD pupae and larvae were obtained from the berry crops entomology lab at Michigan State University. The flies were carefully reared in drosophila vials containing a diet of cornmeal, white table sugar, nutritional yeast, and agar. The diet was prepared following the protocol published by the National Drosophila Species Stock Center in 2019. A dry mix of 62.5g of cornmeal, 100g of white table sugar, 35g of nutritional yeast, and 22.5g of agar is prepared and mixed with 1300 ml of boiled de-ionized water. The mixture was continuously stirred to prevent the formation of clumps for 15 minutes at high temperatures; then, the mixture was removed from the high heat and allowed to cool down. A solution of propionic acid, ethanol, and tegosept is added to the diet to prevent mold growth before the diet is syringed into the vials that contain small coffee paper strips. The vial is set aside to cool at room temperature to prevent condensation from settling in the vial. The vials are closed using a droso plug and stored in the fridge until ready for the infestation.
CO2 gas is used to knock out the flies and move them into new vials. Flies from the same generation are used. 60-100 knocked flies are placed in the new vials, and the vials are placed on their side until the flies have woken up. Vials from the same generation are placed in the tray and the growth chamber with a controlled temperature of 23.5ºC, 60% humidity, and 16:8 day: night cycle, ensuring consistent conditions for the experiment."

Third instar larvae were used for the larval experiment, and no more than three-day-old pupae were used for the pupal experiment.

2.2.2 Entomopathogenic nematode colony

The EPN species were obtained from the USDA, ARS, SEA, SE Fruit, and Tree Nut Research Laboratory and then reared in the laboratory following the protocol published in "In Vivo production of entomopathogenic nematodes" by Shapiro-IIan 2016. Wax worms (*Galleria mellonella*) were used as the insect host. Nematodes were inoculated into a 100mm petri dish lined with filter paper. Ten wax worms were placed in the petri dish, and 1 ml of EPN suspension (250-1000 ijs per ml) was added to the plate. After inoculation, the plates were incubated in a dark place at room temperature of 25 °C for 2-4 days. Nematode-infected cadavers change color depending on the species of EPNs used for the infection. The worm turns reddish when infected with Heterorhabditis, and for Steinernematids, they turn brown or tannish. After five days, the cadaver is transferred to white traps, which use the natural migration of progeny ijs away from the host cadaver upon emergence for harvesting. Emergence can start as early as 7 days post-infection and last 3 weeks. The white trap consists of a dish or tray on which the cadaver rests and is surrounded by water. Nematodes move towards water harvested by collecting the water in tissue culture flasks. The flask was refrigerated between 4 °C and 10 °C until needed for the experiment.

2.2.3 Entomopathogenic fungi colony

USDA, ARS, SEA, SE Fruit, and Tree Nut Research Laboratory provided us with three commercial entomopathogenic fungi required for the experiments. Three different species of entomopathogenic fungi used for the experiment were *Beauveria bassiana strain GHA*, *Met 52* containing beneficial fungus *Metarhizium anisopliae*, and *PFR-97* containing beneficial fungi *Cordyceps fumosorosae*.

2.2.4 Experiment 1a – Entomopathogenic nematodes efficacy against SWD larvae, pupae, and infested fruit

We conducted a study to examine the efficacy of entomopathogenic nematodes (EPNs) on SWD's larval and pupal stages. The experiment followed a completely randomized design (CRD). Initially, we used three different species of commercially available entomopathogenic nematodes (*Steinernema carpocapsae, Steinernema feltiae, and Heterorhabditis bacteriophora*) at the rate of 50 infective juveniles (ijs) per insect for the experiment. We used round test tubes with a plastic cover filled with 10g of play sand with an initial moisture content of 0%. The soil was air-dried at room temperature and then autoclaved at 121 °C for 45 minutes. 1 ml of the EPN solution was added to make the final moisture level 10%. The soil moisture recommended by Peters (2005) was used for conducting insect-based bioassays to control the quality of EPNs. For control, 1 ml of distilled water was used. For larval assay, five third instar larvae were placed on the soil surface, and for pupal assay, five three-day-old pupae were placed about 5mm beneath the soil surface. The test tubes were placed in a growth chamber at 25°C L:D 16:8, 70 - 80% RH. After eight days, the experiment was terminated, and the number of emerged adults was counted. It was then repeated five times and replicated ten times per treatment.

Later, we increased the number of nematode species to nine to provide more options and improved the experiment. Nine species of entomopathogenic nematodes (*Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg))* were used for the experiment. We used nematodes in different concentrations: 25, 50, 75, and 100 infective juvenile stages (ijs) per square centimeter. We also used two-ounce Jello shot cups for the experiment. Each chamber

contained 20g of play sand with an initial moisture content of 0%. The soil was air-dried at room temperature and then autoclaved at 121 °C for 45 minutes. We pipetted 1 ml of EPN solution onto the soil surface and added 1 ml of distilled water to achieve final soil moisture of 10%. The control treatment received 2ml of distilled water. For the larval experiment, ten 3rd instar SWD larvae were placed on the soil surface, and for pupal assay, ten three-day-old pupae were placed about 5mm beneath the soil surface., sourced from a Michigan colony, were placed into each chamber after inoculation. All cups were placed on a plastic tray with a wet paper towel and covered with plastic bags to retain moisture. The cups were then incubated at 25°C L:D 16:8, 70 - 80% RH. The experiment was repeated three times. Mortality was assessed based on adult emergence after seven days. Virulence was assessed by subtracting the number of adults who emerged from the total number of individuals (Hübner et al., 2017).

In the infested fruit experiment, we placed blueberries in a 32-oz plastic deli cup with adult spotted wing drosophila (SWD) for 24 hours. After removing the adults, we selected blueberries containing 10 + 2 eggs for the experiment. We conducted a similar experiment to the first one. The cups were then kept in an incubator at 25°C with a light: dark cycle of 16:8 and at 70-80% relative humidity. We repeated the experiment three times. After 14 days, the experiment was terminated, and we recorded the number of emerged flies.

2.2.5 Experiment 1b – Entomopathogenic fungi against SWD larvae and pupae

We conducted a study to examine the effect of EPF on the larval and pupal stages of SWD and the required concentrations. The experiment followed a completely randomized design (CRD). We used three species of fungi at three different concentrations: 1 X 10^6 conidia mL^{-1}, 1 X 10^7 conidia mL^{-1} and 1 X 10^8 conidia mL^{-1}). We used two-ounce Jello shot cups for the experiment. Each chamber contained 20g of play sand with an initial moisture content of 0%. The soil was air-

dried at room temperature and then autoclaved at 121 °C for 45 minutes. We pipetted one ml of EPF solution onto the soil surface and added one ml of distilled water to achieve a final % soil moisture of 10%. The control treatment received two ml of distilled water. For the larval experiment, ten 3rd instar SWD larvae were placed on the soil surface, and for pupal assay, ten three-day-old pupae were placed about 5mm beneath the soil surface., sourced from a Michigan colony, were placed into each chamber after inoculation. All cups were placed on a plastic tray with a wet paper towel and covered with plastic bags to retain moisture. The cups were then incubated at 25°C. The experiment was repeated three times. Mortality was assessed based on adult emergence after seven days. Virulence was assessed by subtracting the number of adults who emerged from the total number of individuals (Wakil et al., 2022)

2.2.6 Experiment 1c – Entomopathogenic nematodes and fungi combined against SWD larvae and pupae

After completing experiments 1a and 1b, we selected the top three entomopathogenic nematodes and one entomopathogenic fungi for the next phase. *Steinernema feltiae, Steinernema carpocapsae, and Steinernema glaseri* - at two concentrations: 50 ijs per cm^{-2} and 100 ijs per cm^{-2}. Based on the results of experiment 1b, we determined that *Beauveria bassiana* was most effective at a higher dose of 1 X 10^8 conidia mL^{-1}, so we used this dose for further experiments. The laboratory assay was set up like experiments 1a and 1b. We pipetted one ml of EPF solution onto the soil surface and added one ml of EPN solution to achieve a final % soil moisture of 10%. The control treatment received two ml of distilled water. The experiment was repeated three times. Mortality was assessed based on adult emergence after seven days. Virulence was assessed by subtracting the number of adults who emerged from the total number of individuals.

2.3 Statistical analysis

Emerged adults were counted, and Virulence was assessed by subtracting the number of adults who emerged from the total number of individuals. The data underwent variance analysis and were compared using Fisher's LSD ($p < 0.05$). These analyses were conducted using the R statistical program.

2.4 Results

2.4.1 Experiment 1a – Entomopathogenic nematodes against SWD larvae and pupae

A) SWD larvae, pupae, and infested fruit experiment with three species of entomopathogenic nematode

All three species of EPN infected SWD larvae and pupae. Dissection of dead larvae and pupae revealed the presence of adult and juvenile nematodes. In the case of SWD larvae ($F= 64.34$, $df = 3$, $p < 0.05$), treatments with *Heterorhabditis bacteriophora (Hb)* resulted in a lower mortality of 22%. *Steinernema feltiae* showed higher mortality followed by *Steinernema carpocapsae*. Treatments with *Steinernema feltiae* have 78% and *Steinernema carpocapsae* have 66% host infections, respectively (Figure 2.1). All EPN species tested against pupae were less effective. In SWD pupae assay ($F = 25.31$, $df = 3$, $p < 0.05$), *Heterorhabditis bacteriophora (Hb)* resulted in 15% mortality, and *Steinernema feltiae (Sf) and Steinernema carpocapsae (Sc)* resulted in 65% and 55% mortality respectively (Figure 2.2). There was no significant difference between *Sc* and *Sf.* When the infested fruit experiment was carried out ($F = 0.291$, $df = 3$, p-value = 0.804). There was a significant difference between treatments and control, but there was no significant difference between the three treatments. So, no further experiment was carried out with infested fruit.

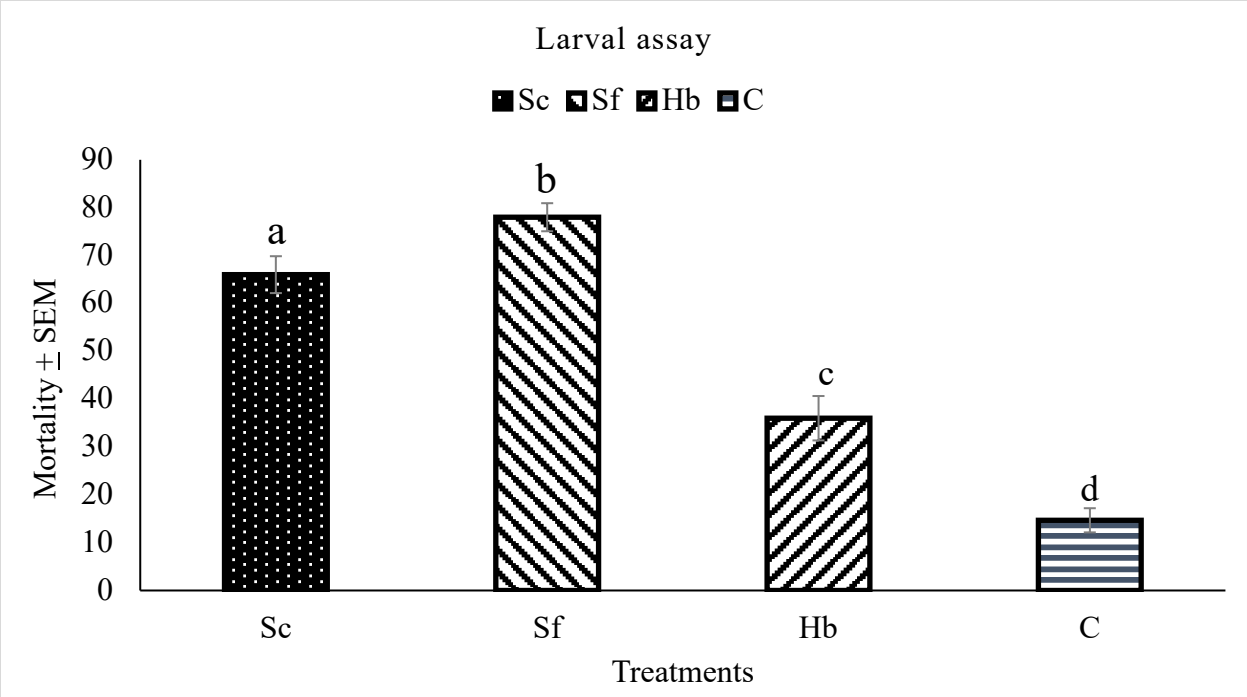

Figure 2.1: Mortality of SWD larvae (Mortality ± SEM) after infection with *Steinernema carpocapsae* (Sc), *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb), Control (C) at a rate of 50 IJs per host at 25°C in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

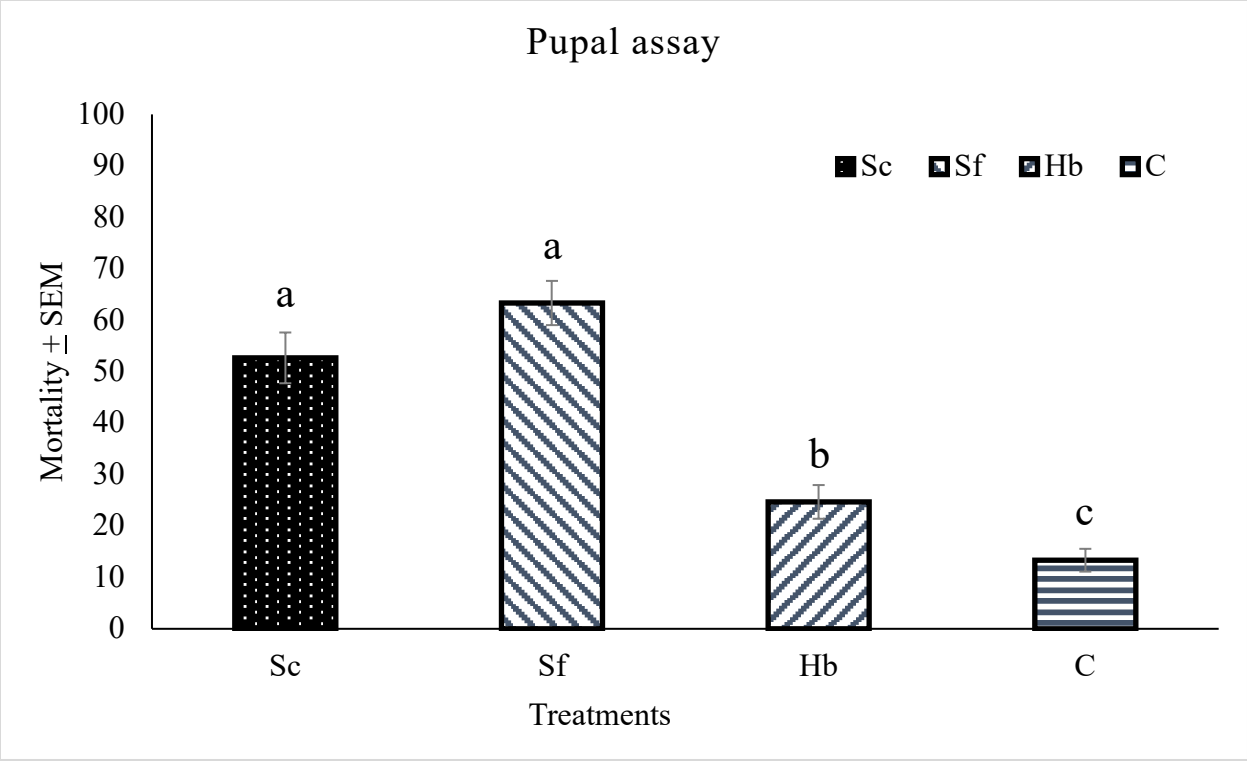

Figure 2.2: Mortality of SWD pupae when infected with *Steinernema carpocapsae* (Sc), *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb), and Control(C). Infective juveniles were applied at 50 ijs per host at 25 °C. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

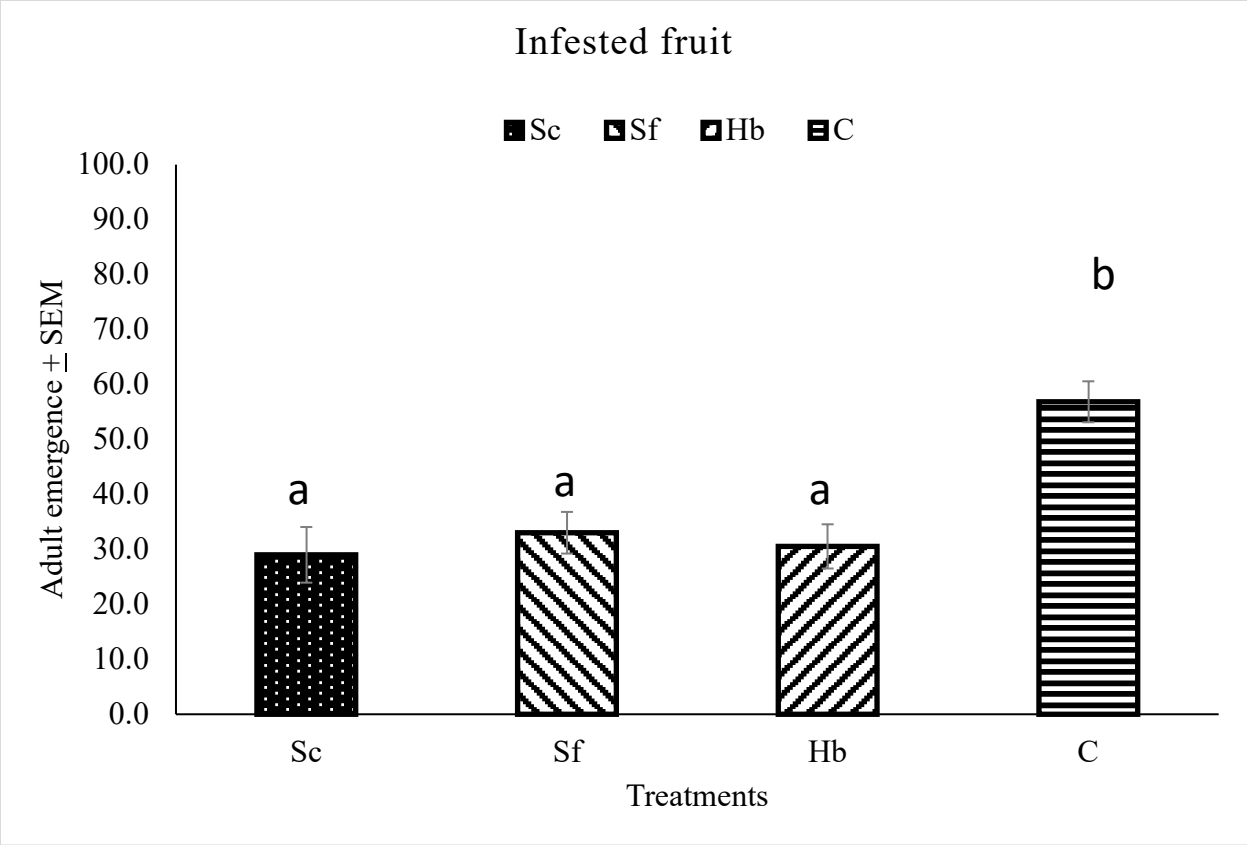

Figure 2.3: Adult Emergence ± SEM of infested fruit when infected with *Steinernema carpocapsae* (Sc), *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb), and Control(C). Infective juveniles were applied at 50 ijs per host at 25 °C. Different letters denote significant differences and NS = No significant difference.

B) SWD larvae and pupae experiment with nine species of entomopathogenic nematodes

In this study, we aimed to examine the impact of nine different species of entomopathogenic nematodes on the population of SWD larvae and pupae. We used four different rates to compare the efficacy of these entomopathogenic nematodes (EPNs). We can see significant difference between treatments. When Steinernema feltiae were applied at different concentrations (25 ijs cm-2, 50 ijs cm-2, 75 ijs cm-2, 100 ijs cm-2), it resulted in 62%, 65%, 70%, and 80% larval mortality, respectively. These results were significantly different from other nematodes at similar

concentrations. Similarly, when Steinernema carpocapsae and Steinernema glaseri were applied at 100 ijs cm-2, they caused 72% and 62% larval mortality, respectively. The remaining six EPNs caused the least mortality, even at higher concentrations as illustrated in Figures 2.4, 2.5, 2.6, and 2.7.

Regarding pupae, the EPNs were found to be less effective. Steinernema feltiae and Steinernema carpocapsae, when applied at 100 ijs cm-2, resulted in 65% mortality, and Steinernema glaseri caused 62% mortality at the same concentration. All other EPNs, regardless of concentration, caused less than 50% mortality.

As a result of these experiments, three EPN species - *Steinernema feltiae, Steinernema carpocapsae,* and *Heterorhabditis bacteriophora* - were chosen for further experiments.

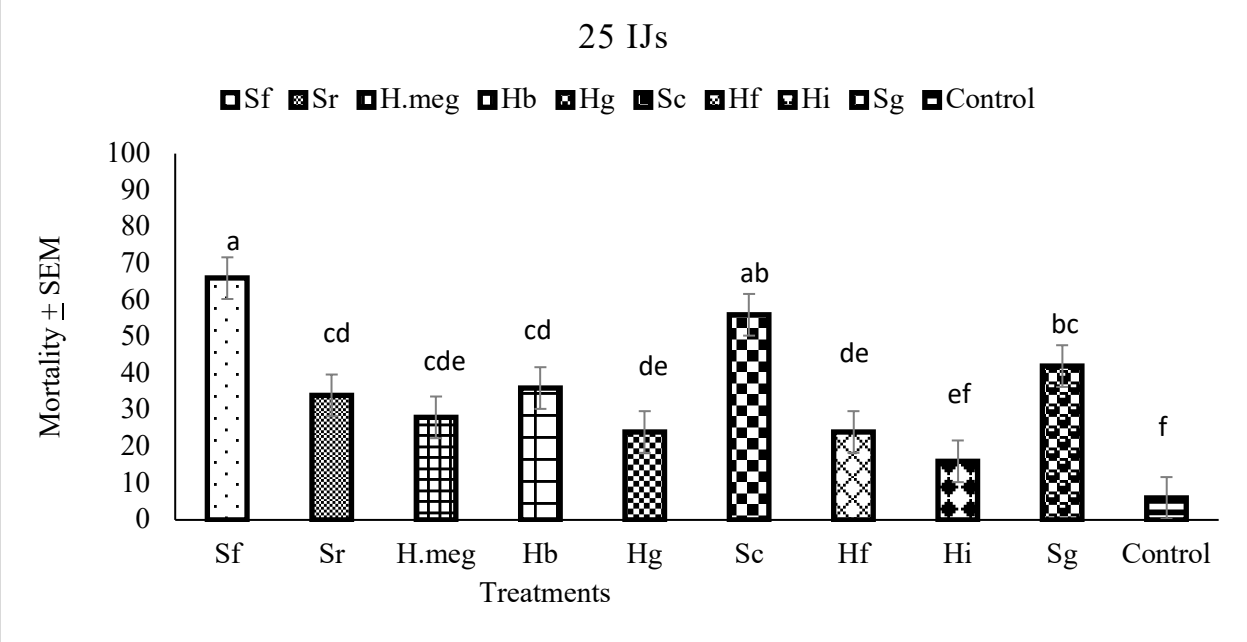

Figure 2.4: Mortality percentage of (Mortality ± SEM) of SWD larvae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg*)(25IJs

Figure 2.4 (cont'd)

cm^{-2} , and Control(C) in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

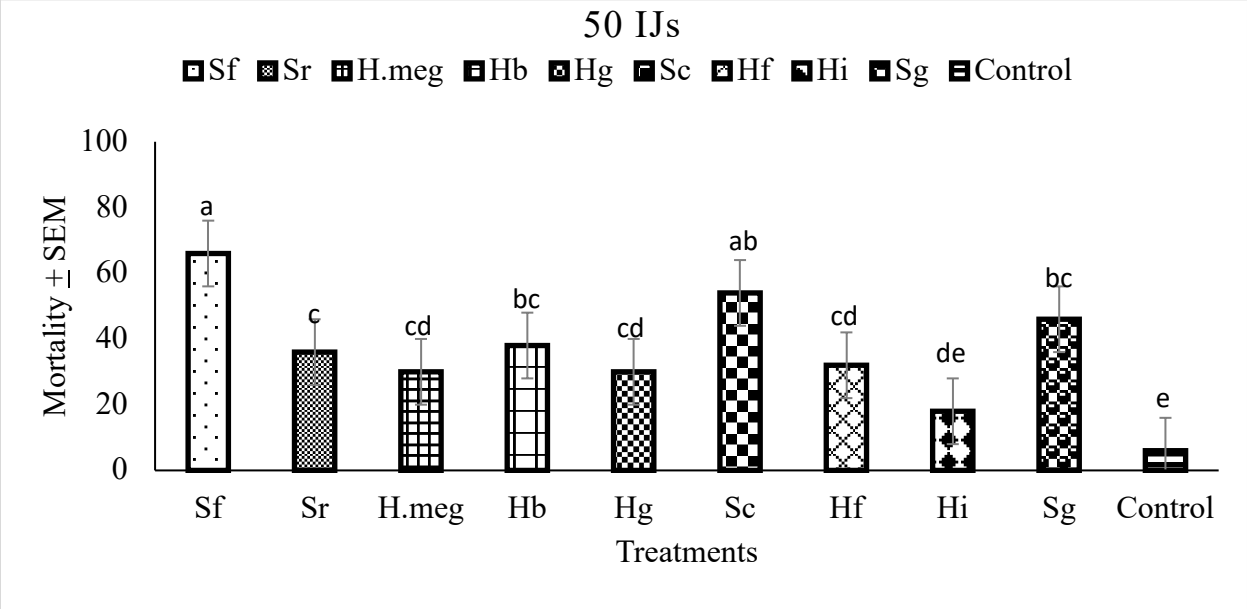

Figure 2.5: Mortality percentage of (Mortality ± SEM) of SWD larvae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg)* and Control(C) at 50 IJs cm^{-2} in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

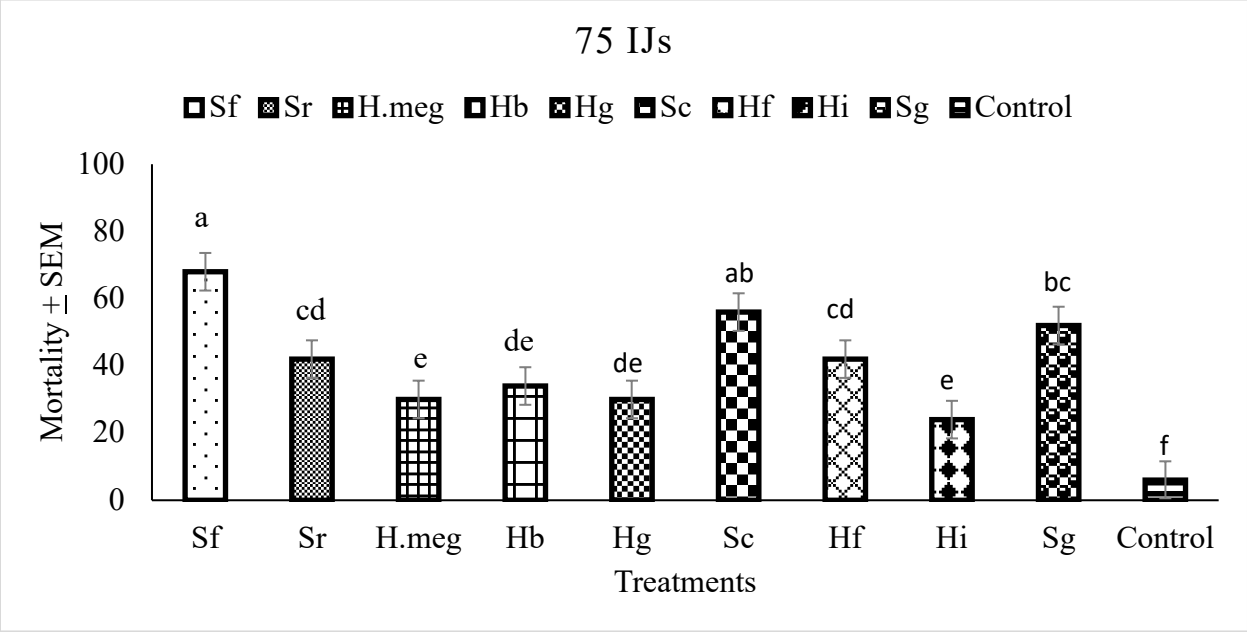

Figure 2.6: Mortality percentage of (Mortality ± SEM) of SWD larvae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg)* at 75 IJs cm^{-2} and Control(C) in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

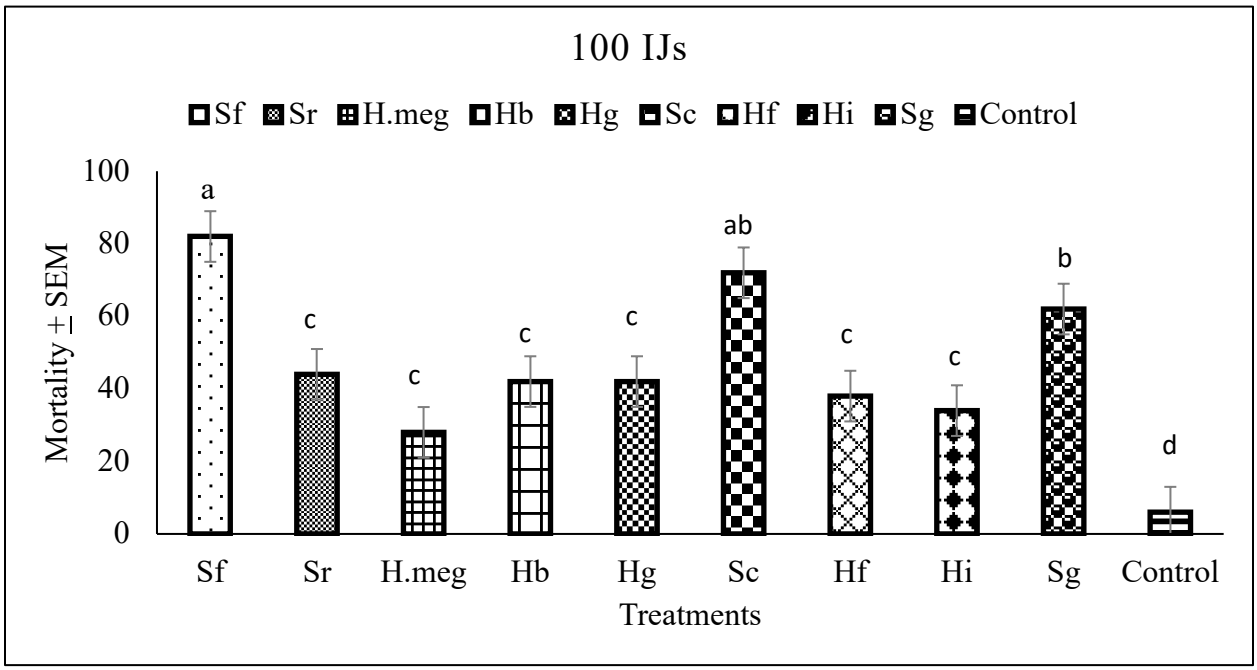

Figure 2.7: Mortality percentage of (Mortality ± SEM) of SWD larvae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg)* at 100 IJs cm^{-2} and Control(C) in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

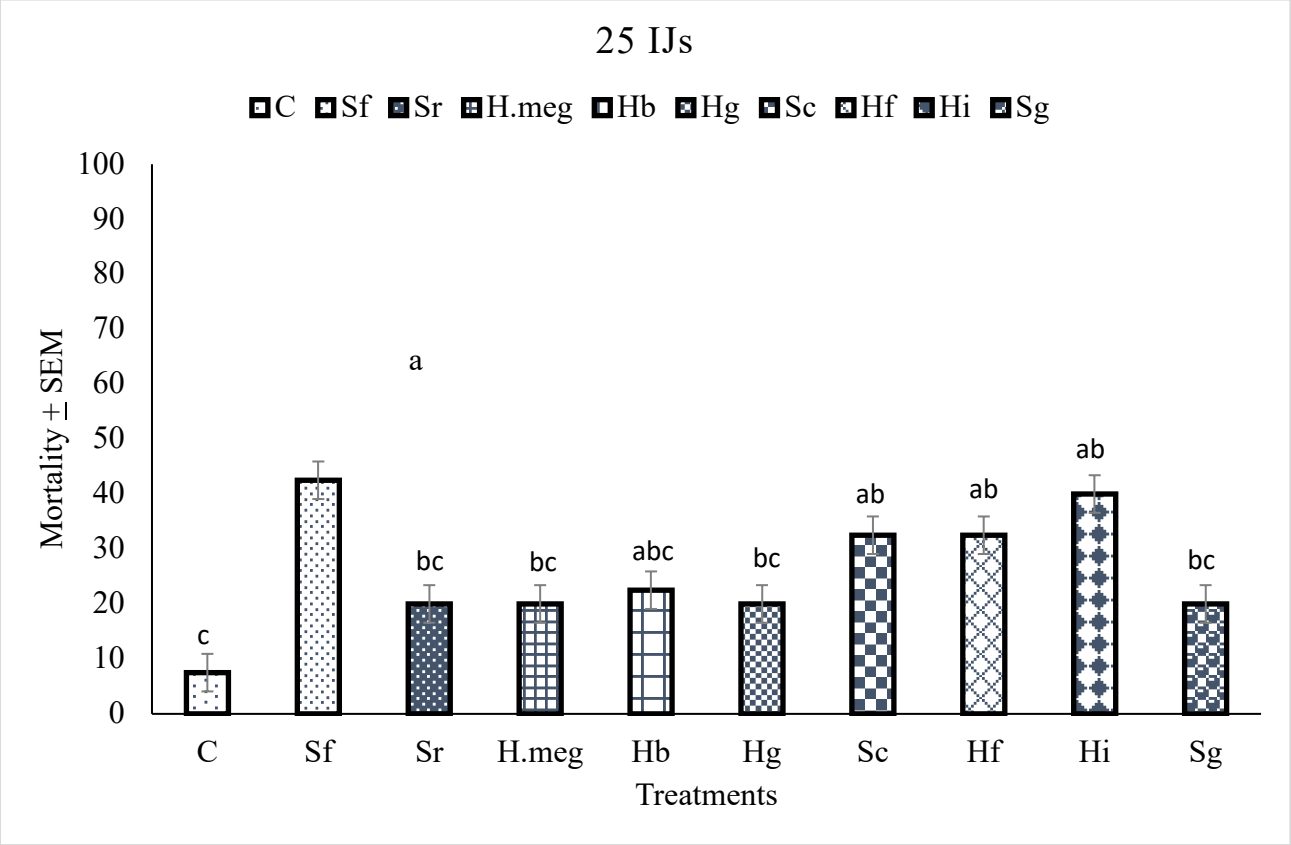

Figure 2.8: Mortality (%) of SWD pupae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg)* at 25IJs cm^{-2} and Control(C) in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

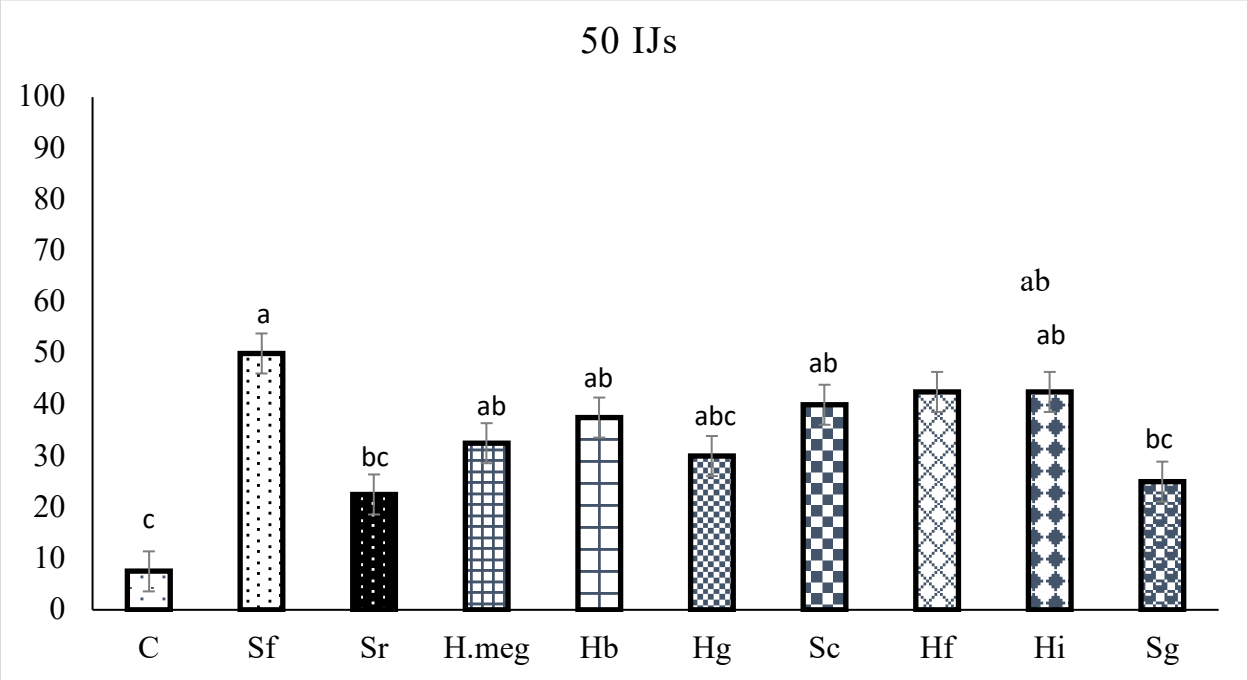

Figure 2.9: Mortality (%) of SWD pupae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg)* at 50Ijs cm^{-2} and Control(C) in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

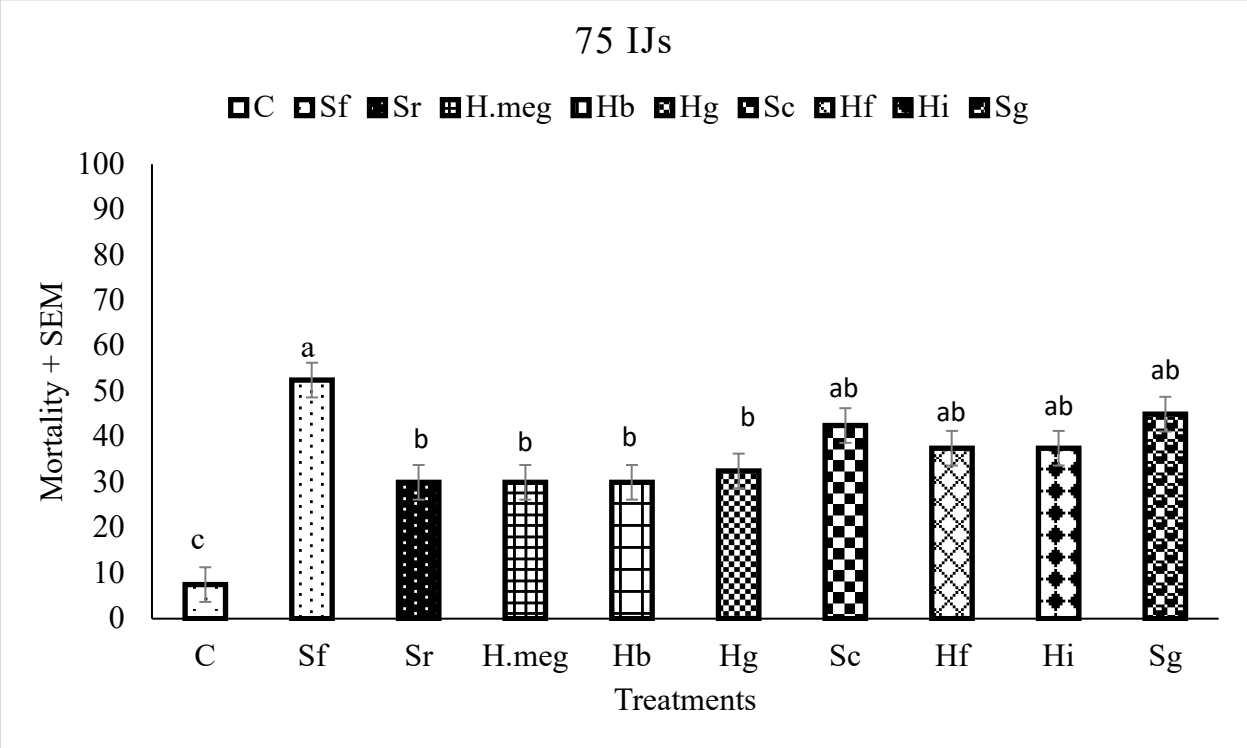

Figure 2.10: Mortality (%) of SWD pupae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg)* at 75 IJs cm^{-2} and Control(C) in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

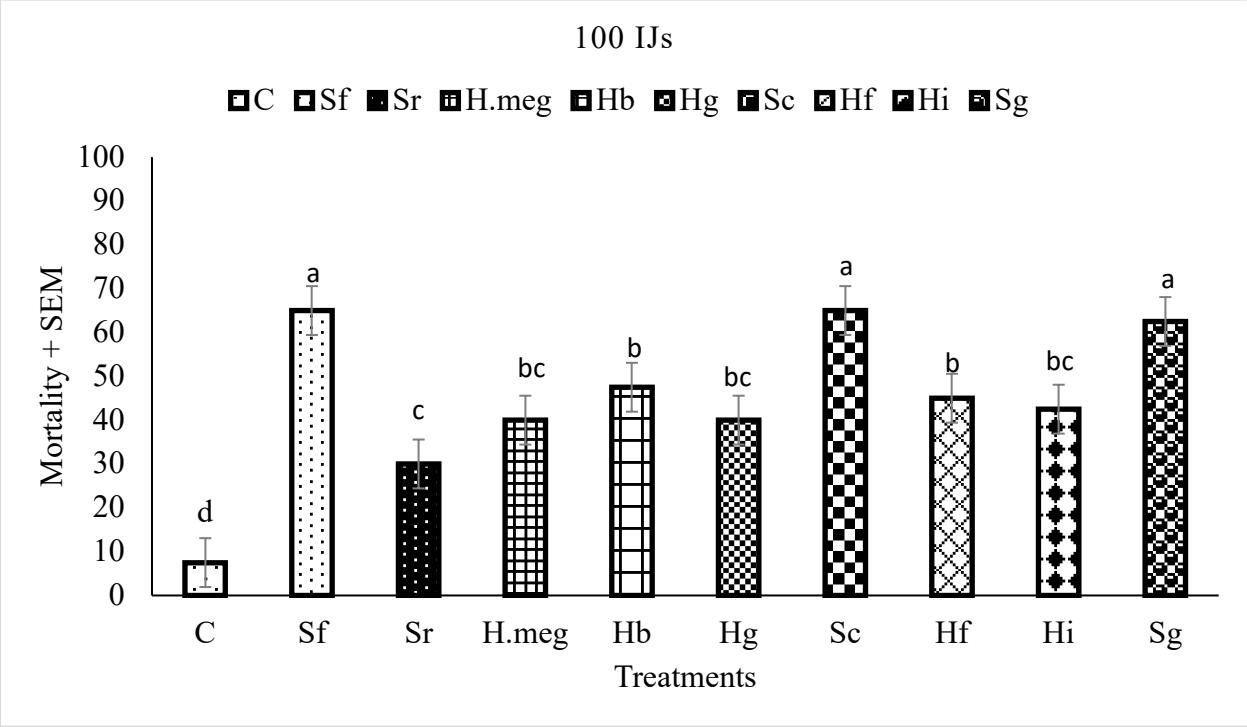

Figure 2.11: Mortality (%) of SWD pupae when treated with *Steinernema feltiae (Sf), Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Steinernema carpocapsae (Sc), Heterorhabditis floridensis (Hf), Heterorhabditis indica (Hi), Steinernema glaseri (Sg)* at 100 IJs cm^{-2} and Control(C) in laboratory conditions. Different letters denote significant differences at $p < 0.05$ (Fisher's LSD).

2.4.2 Experiment 1b – Entomopathogenic fungi against SWD larvae and pupae

Pupae mortality of SWD ($F_{(10,55)} = 38.639$, $p < .05$) when exposed to *Beauveria bassiana (Bb)* at higher concentrations of 1 X 10^8 conidia mL^{-1}, is around 70 %, followed by 54% and 67 % when exposed to 1 X 10^6 conidia mL^{-1}, 1 X 10^7 conidia $mL^{-1)}$. *Cordyceps fumosorosae (Pfr-97)* and *Metarhizium anisopliae (Met-52)* at all concentrations (1 X 10^6 conidia mL^{-1}, 1 X 10^7 conidia mL^{-1} and 1 X 10^8 conidia mL^{-1}) had mortality less than 50%. Figure (2.12).

Larvae mortality ($F_{(10,49)} = 13.17$, $p < 0.05$) when exposed to *Beauveria bassiana (Bb*) at concentrations of 1 X 10^7 conidia mL^{-1} and 1 X 10^8 conidia mL^{-1} was 64% and 52%, respectively. In comparison, at a concentration of 1 X 10^6 conidia mL^{-1} it was around 47%. The other two fungi, *Cordyceps fumosorosae (Pfr-97) and Metarhizium anisopliae (Met-52),* at all concentrations, have mortality lower than 50% Figure (2.13).

At the end of the experiment, *Beauveria bassiana (Bb)* at a concentration of 1 X 10^8 conidia mL^{-1} was used for further experimentation.

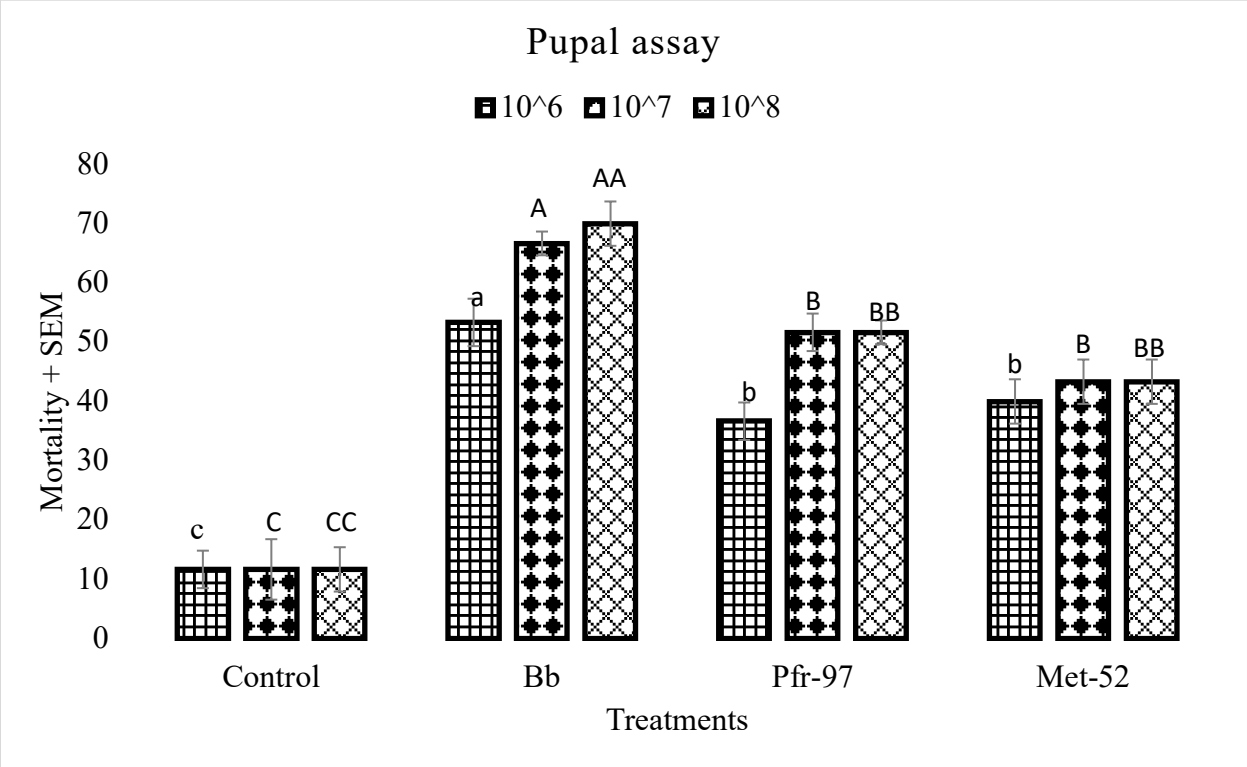

Figure 2.12: Mortality (± SEM) of SWD larvae exposed to *B. bassiana (Bb)*, *Cordyceps fumosorosae (Pfr-97), and Metarhizium anisopliae (Met-52)* at three different concentrations (1 X 10^6 conidia mL^{-1}, 1 X 10^7 conidia mL^{-1} and 1 X 10^8 conidia mL^{-1}) and Control(C) under laboratory

Figure 2.12 (cont'd)

conditions. The letter above the bar denotes a significant difference at $p < 0.05$ (Fisher's LSD).

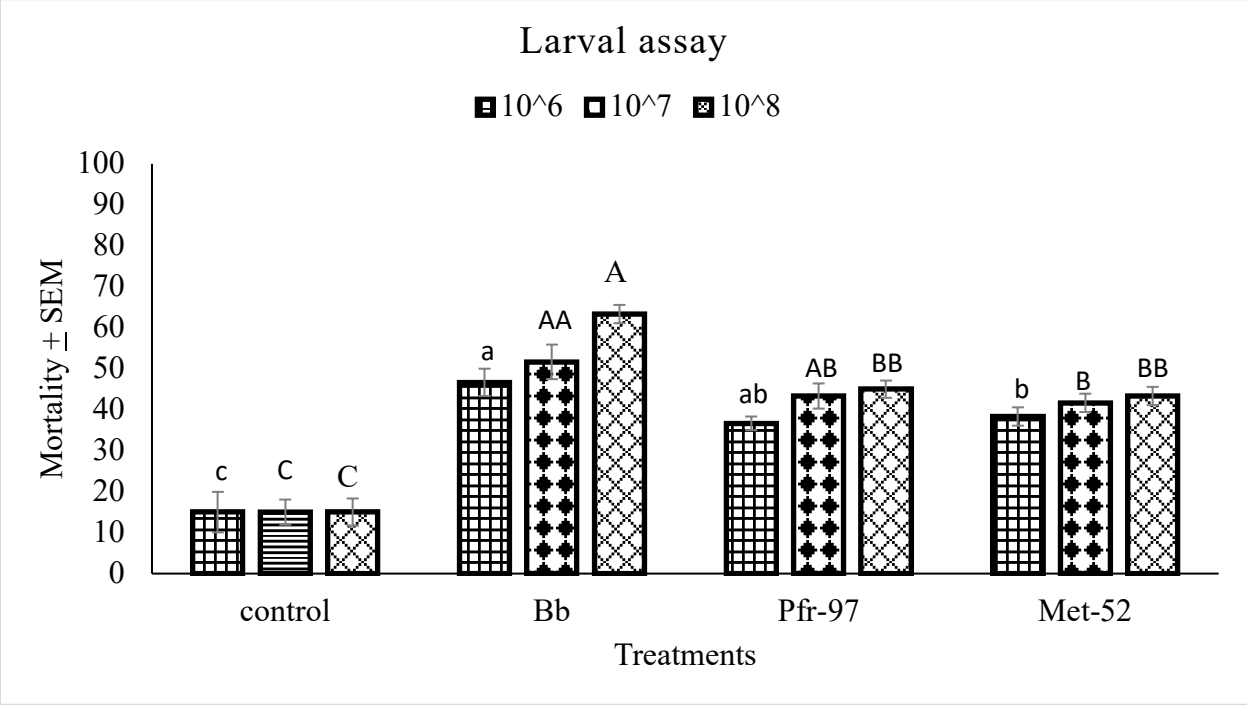

Figure 2.13: Mortality (± SEM) of SWD pupae exposed to *B. bassiana (Bb)*, *Cordyceps fumosorosae (Pfr-97), and Metarhizium anisopliae (Met-52)* at three different concentrations (1 X 10^6 conidia mL^{-1}, 1 X 10^7 conidia mL^{-1} and 1 X 10^8 conidia mL^{-1}) and Control(C) under laboratory conditions. The letters above the bar denote significant differences (Fisher's LSD, $p < 0.05$).

2.4.3 Experiment 1c – Effect of entomopathogenic nematodes and fungi combined against SWD pupae

In a combination experiment, entomopathogenic nematodes *(Steinernema carpocapsae (Sc), Steinernema feltiae (Sf), and Steinernema glaseri (Sg))* at two different concentrations (50ijs cm^{-2}, 100 ijs cm^{-2}) and fungi *Beauveria bassiana (Bb)* at 1 X 10^8 conidia mL^{-1} was used for the concurrent experiment. 98% mortality was observed when *Steinernema carpocapsae* at 100 Ijs cm^{-2} was applied to SWD larvae ($F_{(7,48)} = 13.77$, $p < 0.05$). The lowest mortality of 57 % was observed when *Steinernema glaseri* at the rate of 50 Ijs cm^{-2} was used. *Steinernema feltiae* at 100 ijs cm^{-2} caused 80% mortality. Figure (2.14).

In the pupal experiment ($F_{(7,48)} = 11.60$, $p< 0.05$), 71% mortality was observed when *Steinernema carpocapsae* was applied at the 100ijs cm^{-2} and 61% when *Steinernema carpocapsae* was applied at the same concentration. The lowest mortality was observed with *Steinernema feltiae* at 50IJscm^{-2}.

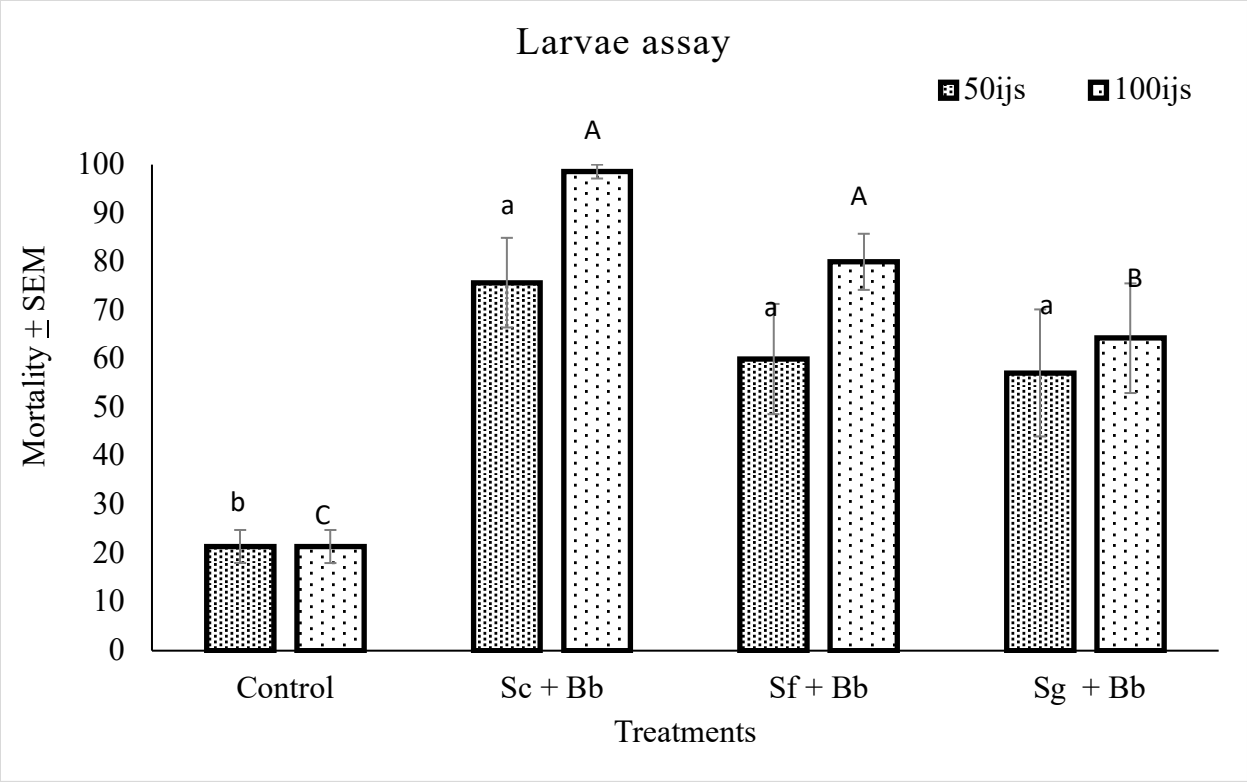

Figure 2.14: Mortality (± SEM) of SWD larvae exposed to combination of entomopathogenic nematodes (*Steinernema carpocapsae (Sc), Steinernema feltiae (Sf), and Steinernema glaseri (Sg*)) at two different concentrations (50ijs cm^{-2}, 100ijs cm^{-2}) and fungi *Beauveria bassiana (Bb)* at 1 X 10^8 conidia mL^{-1} and Control(C) in laboratory conditions. Bars with different letters are significantly different, ANOVA, Fisher's LSD ($p < 0.05$).

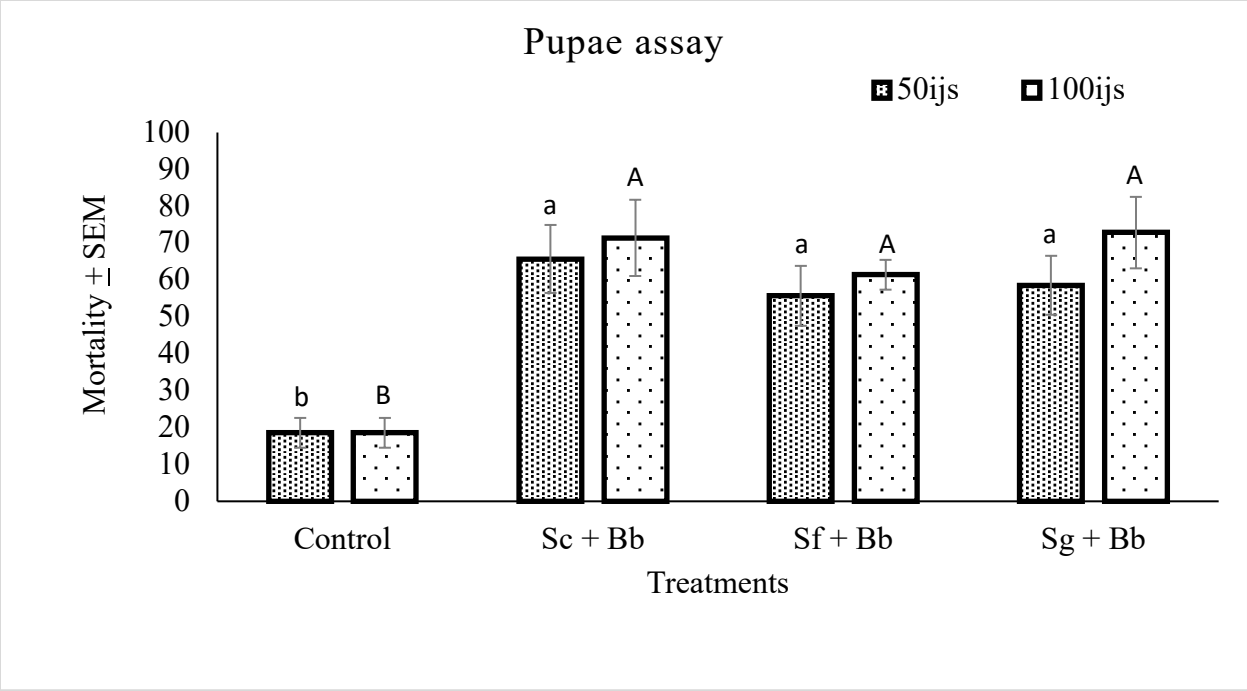

Figure 2.15: Mortality (± SEM) of SWD pupae exposed to combination of entomopathogenic nematodes (*Steinernema carpocapsae (Sc), Steinernema feltiae (Sf), and Steinernema glaseri (Sg*)) at two different concentrations (50ijs cm^{-2}, 100 ijs cm^{-2}) and fungi *Beauveria bassiana (Bb)* at 1 X 10^8 conidia mL^{-1} and Control(C) in laboratory conditions. Bars with different letters are significantly different, ANOVA, Fisher's LSD ($p < 0.05$).

2.5 Discussion

The main objective of this research project was to evaluate the potential of using entomopathogenic nematodes and entomopathogenic fungi to control the soil-dwelling stages of spotted-wing drosophila. The results confirm that spotted-wing drosophila larvae were more susceptible to entomopathogenic nematodes than pupae. This finding is consistent with previous studies on similar flies (Beaver and Calkins 1984; Kopper et al., 2004; Kamali et al., 2013). This difference in susceptibility between larvae and pupae may be attributed to the hardened outer layer of the pupa, which hinders the entry of entomopathogenic nematodes into the host. Yee and Lacey (2003) also suggested that the higher activity and CO2 output of host larvae could be contributing factors. Huber (2017) suggested that the differences in the effectiveness of nematodes could be attributed to their diverse foraging strategies and the behavior of SWD third-instar larvae. Cuthbertson and Audslay (2016) presented contradictory results, indicating that Heterorhabditis bacteriophora displayed a greater mortality rate among SWD pupae.

In our research, we discovered that the larvae of the spotted wing drosophila (SWD) were vulnerable to three different types of entomopathogenic nematodes: *Steinernema feltiae, Steinernema carpocapsae, and Steinernema glaseri*, as opposed to other nematode species such as *Steinernema riobrave (Sr), Heterorhabditis megidis (H.meg), Heterorhabditis bacteriophora (Hb), Heterorhabditis glaseri (Hg), Heterorhabditis floridensis (Hf), and Heterorhabditis indica (Hi).* Specifically, when exposed to a concentration of 100 infective juveniles (ijs) per cm^{-2}, *Steinernema feltiae* caused 72% mortality in SWD larvae, while *Steinernema carpocapsae* and *Steinernema glaseri* resulted in mortalities of 68% and 53%, respectively. Furthermore, we noted that increasing the concentration of infective juveniles led to higher mortality rates among the SWD larvae.

Entomopathogenic fungi, such as *B. bassiana and M. anisopliae*, have shown highly effective outcomes in combating the Mediterranean fruit fly. These fungi can infect adults, larvae, or pupae through various methods (Castillo et al., 2000; Ekesi et al., 2002). For instance, when dealing with lanternflies, *Beauveria bassiana* mycoinsecticides caused 90-93% mortality in nymphs and 82-99% in adults (Clifton & Hajek 2022). Most studies on Entomopathogenic fungi have focused on adult SWD, involving direct spraying and placement in small, treated vials. In certain investigations, *B. bassiana* has been found to reduce *D. suzukii* oviposition on uninfested berries and adult emergence from previously infested berries. However, contradictory outcomes have also been reported in other studies (Gargani et al., 2013; Cuthbertson et al., 2014; Liburd and Rhodes 2021). Yousef et al. (2017) conducted a lure-and-kill experiment using devices based on *Metarhizium brunneum* for SWD, resulting in a 62.2% adult mortality and an 84.7% reduction in fecundity.

Pupae are more susceptible to EPF treatments than larvae and adults (Ibouh et al., 2019). Our results also showed that applying *Beauveria bassiana* at a higher dose of $1X10^{8}$ conidia mL^{-1} caused around 50% larval mortality and 70% pupal mortality.

In recent years, experiments have been conducted combining biological insecticides that are environmentally friendly to increase effectiveness. Some strains of *Bacillus thuringiensis* bacteria and species of entomopathogenic nematodes have been shown to cause SWD mortality in the laboratory. A study by Mastore et al., (2021) found that the combined administration of *S. carpocapsae* with *Bt* significantly increased effectiveness compared to treatments with single bioinsecticides. This increase was observed in terms of larval mortality rate and reduction in treatment time. Muhammad et al., 2020 reported an additive interaction when EPNs *S. riobrave* and *S. carpocapse* combined with *M. brunneum* and *I. Javanica* against apple maggot. Applying

EPF and EPNs resulted in increased mortality of larvae, pupae, and pharate adults of *B. zonata* and *B. dorsalis* in laboratory, glasshouse, and field cage settings when compared to separate treatments. This combined approach has significant potential for fruit fly control (Wakil et al., 2022).

In the context of SWD, the combination of EPNs and EPF has not been previously explored. Our study aimed to assess whether this combination could increase SWD mortality. 80% mortality was observed when *Steinernema carpocapsae* at 100 ijs cm^{-2} and the fungus *Beauveria bassiana (Bb)* at a rate of 1 X 10^8 conidia mL^{-1} were applied to SWD larvae under laboratory conditions. Furthermore, a 77% mortality rate was observed in SWD pupae when *Steinernema feltiae* and the fungus *Beauveria bassiana (Bb)* at a concentration of 1 X 10^8 conidia mL^{-1} were applied, and a 71% mortality rate was observed when *Steinernema carpocapsae* was applied at the same concentration to SWD pupae.

CHAPTER 3. CONCLUSION AND FUTURE RESEARCH DIRECTIONS

My thesis research has three major objectives. 1. Determine the effectiveness of various types of entomopathogenic nematodes on the soil-dwelling stages of SWD. 2. Determine the effectiveness of entomopathogenic fungi on the soil-dwelling stage of SWD. 3. Evaluate the effectiveness of using entomopathogenic nematodes and fungi in soil-dwelling stages of SWD.

My research indicates that EPNs (Entomopathogenic Nematodes) and EPF (Entomopathogenic Fungi) have the potential to effectively control pests. I observed a 72% mortality rate when SWD larvae were exposed to *Steinernema feltiae* and a roughly 70% mortality rate when SWD pupae were infected by *Beauveria bassiana* at higher doses. When used together, they increased the mortality rate. While these findings suggest that EPNs and EPFs will not completely eliminate SWD populations, they could still be valuable additions to SWD management strategies.

Given that all experiments involving entomopathogenic nematodes were carried out in controlled laboratory settings, it is crucial to conduct additional field experiments to gain a more comprehensive understanding of their behavior and dynamics in natural environments. Furthermore, exploring potential combinations or integration of these nematodes with insecticides warrants further investigation through additional studies. In the future, it would be beneficial to explore the development of lure-and-infect strategies, which involve attracting pests and requiring less product to effectively control their population.

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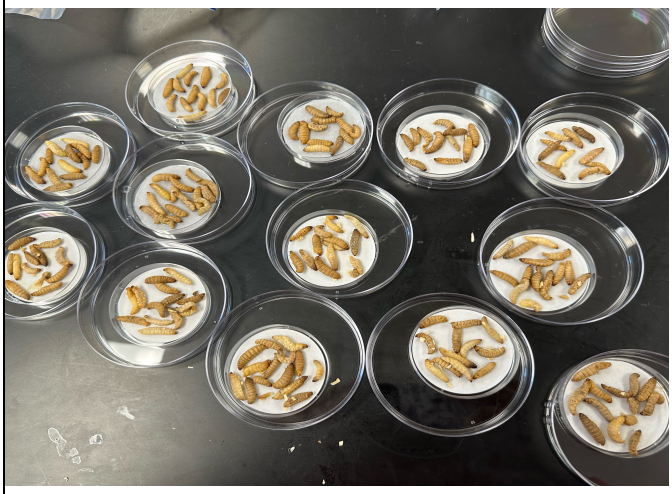
APPENDIX

Experiment setup for larval assay

White trap setup for *Steinernema feltiae*



White trap setup for *Heterorhabditis bacteriophora*

Figure 3: Laboratory experiment setup.

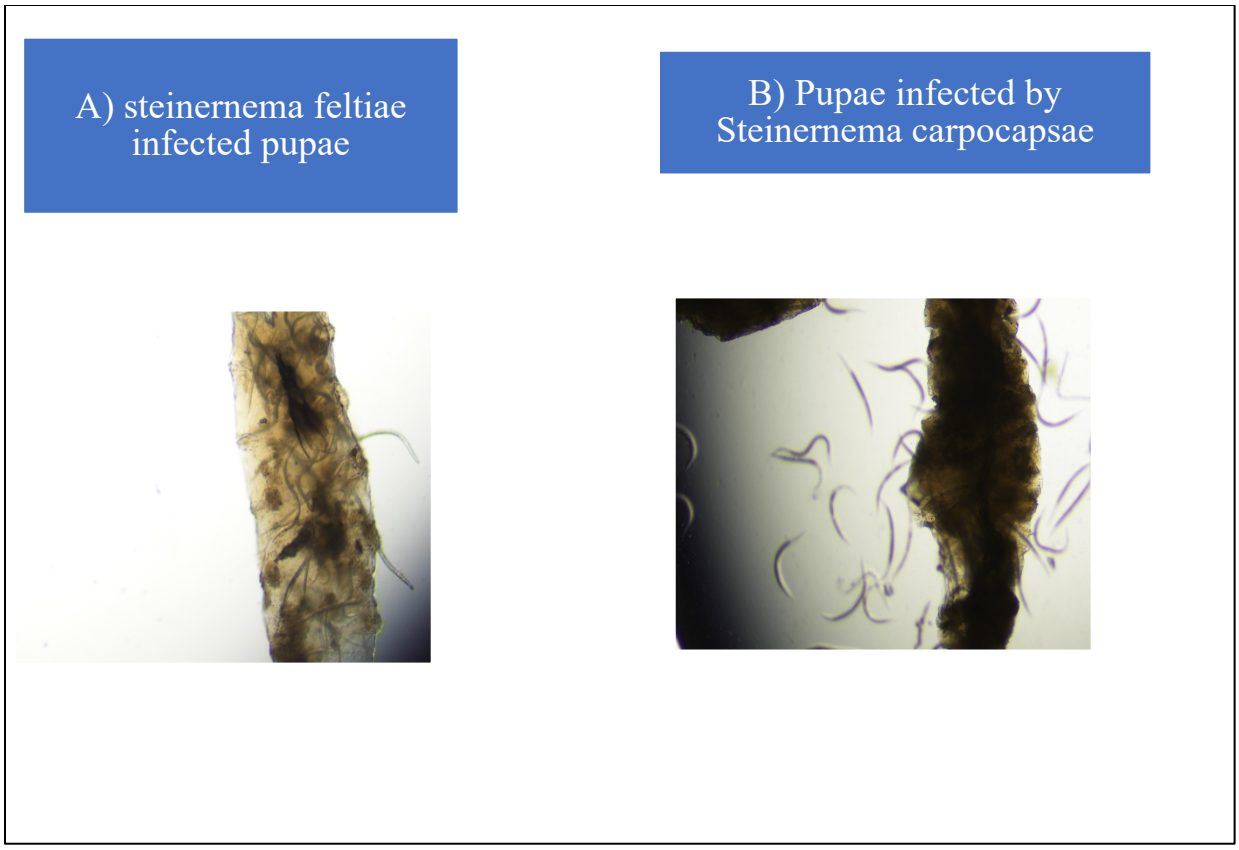


Figure 4: A) SWD pupae after successful host infection in the larval stage. B) SWD pupae after successful host infection in the pupal stage.