

ENTOMOPATHOGENIC NEMATODES: THEIR INTERACTIONS WITH PLANT  
PATHOGENS AND INSECTICIDES IN THE SOIL

by

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Patricia D. Navarro entitled "Entomopathogenic nematodes: Their interactions with plant pathogens and insecticides in the soil." and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

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## **DEDICATION**

For my parents, husband and daughter

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## ABSTRACT

Entomopathogenic nematodes (EPNs) in the families Heterorhabditidae and Steinernematidae, and their bacterial symbionts, have been studied intensively because of their role as natural mortality factor for soil-dwelling arthropods, and their potential as biological control agents for belowground insect pests. Moreover, EPN are recognized as key players in regulating soil food webs and triggering trophic cascades. However, most studies of interactions with EPN have been conducted under laboratory setting and simplified conditions, without consider the dynamic of the EPN and their interactions with other soil components in a wider context. In this respect, knowledge of the effect that other soil organisms or human induced factor may have on EPN dynamic and life cycle in the soil may contribute to improve tactics for their implementation and success as natural regulators of herbivores.

The present investigation focused on the interactions of EPN with a selection of insecticides, and biotic (saprobic fungus and plant parasitic nematodes) elements that may be present in the soil, and may potentially interact with EPN. Specifically, I investigated how these factors may affect the life cycle (host search behavior, virulence and reproduction) of EPN. Appendix A shows the effect that a group of selected synthetic and biological insecticides have on EPN virulence and reproduction. The results obtained from this study revealed that most combinations of EPN and insecticides under study increased the mortality of the insect host. However, it was also found that some of these combinations reduced the nematode progeny production and emergence of IJs from the insect cadaver. In contrast in Appendix B, when examining the effect of the saprobic fungus *Fusarium oxysporum* in the life cycle of the EPN *Heterorhabditis sonorensis*, it was found that this fungus negatively affected the virulence and reproduction of



the EPN in the insect host. In the third study of this dissertation (Appendix C) the interactions studied considered the effect of two EPN on an organism of a different trophic guild, the plant parasitic nematode *Tylenchulus semipenetrans*. This plant parasitic nematode causes serious diseases in citrus plants by infecting their roots and defoliating their branches. Previous studies have shown that some EPN species may negatively affect the life cycle of plant parasitic nematodes by reducing the damage produced by this plant parasite. Results from this study confirm the antagonistic effect between the selected EPN and the citrus nematode. Specifically, it was found that the presence in the soil of both EPN reduced the survival of infective juveniles of the citrus nematode and their penetration to the root. Moreover, the presence of EPN had an antagonistic effect in the production of eggs of *T. semipenetrans* females.

## INTRODUCTION

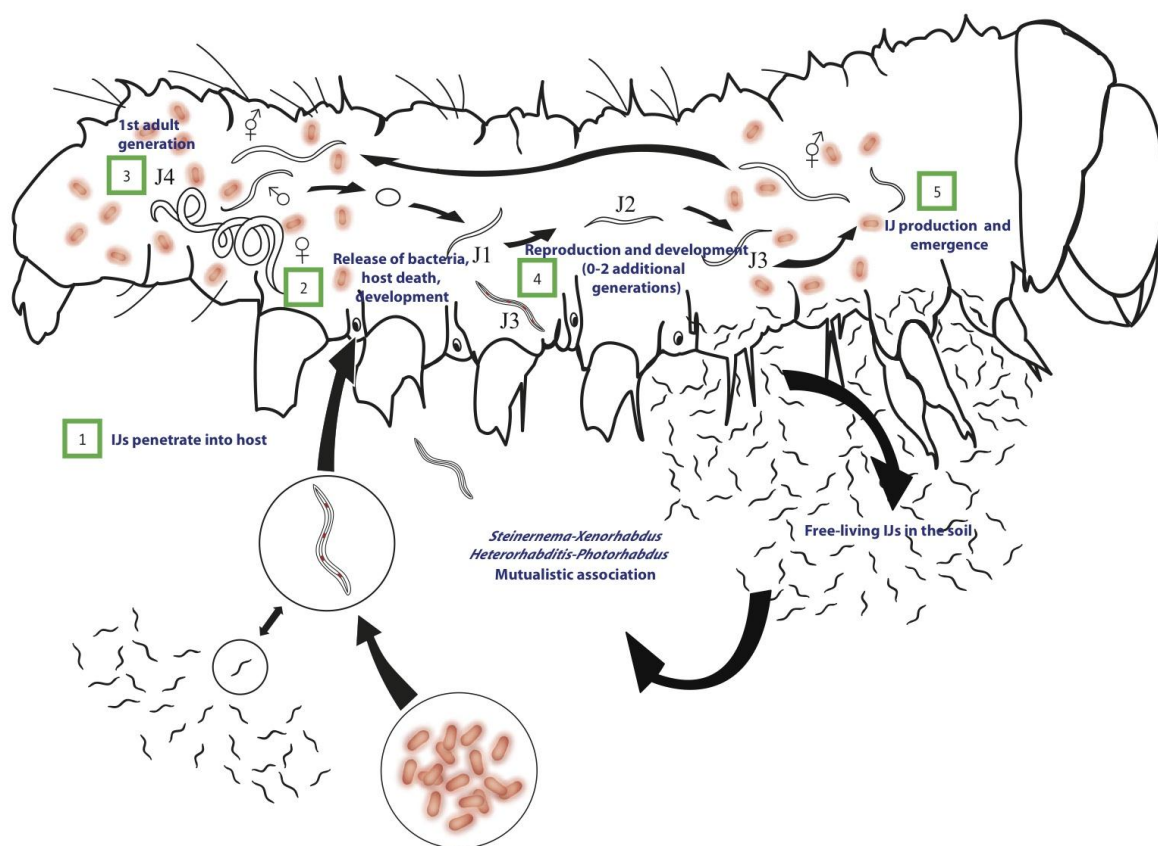
### 1. Literature review

#### 1.1 Entomopathogenic nematodes: their biology and perspectives as model systems

Nematodes are a highly diverse and ubiquitous Phylum. More than 28,000 species have been described, and the global species richness has been estimated between 500,000 to 1 million species (Hugot, et. al. 2001; Gaugler and Bilgrami, 2004). Nematodes can be found in terrestrial, fresh water and marine environments. Their life styles range from free-living to parasites of plants and animals including humans. The most studied nematode species is *Caenorhabditis elegans* (Rhabditidae), which is currently considered a eukaryote model organism for research in biology and biomedical fields. However, many other nematode species are emerging at present as powerful model organisms for studying diverse disciplines including ecology, physiology, developmental biology, and evolution.

Within this group are the so-called entomopathogenic or insect pathogenic nematodes (hereafter referred as EPN). Two nematode families fall into this category: Steinernematidae and Heterorhabditidae. These two nematode families have many common characteristics, for example, their mutualistic association with enteric bacteria (Gamma-Proteobacteria, Enterobacteriaceae) carried in their intestine. Moreover, they also share their need to parasitize an insect host. Briefly, the life cycle of these nematodes is as follows. The only free-living stage, also known as the infective juvenile (IJ) vectors the symbiotic bacteria from one insect to another (Gaugler and Kaya, 1990). Once the IJs encounter a suitable insect in the soil, it penetrates it

through the mouth, anus or spiracles making its way to the insect haemocoel (Kaya, 1993). *Heterorhabditis* nematodes have a buccal cuticular tooth that helps them penetrate the insect cuticle (Bedding and Molyneux, 1982). When the IJs reach the insect haemocoel, the bacteria are released and kill the host by a massive septicemia in 24 to 48h. One to three generations of nematodes may develop inside the insect cadaver, which feed on the symbiotic bacteria and degraded insect tissues. Thus, these new generations massively colonized the insect carcass. Once food is depleted, the new generations of IJs, coming from the last adult generation, abandon the cadaver to seek a new host (Poinar, 1990) (Figure 1).



**Figure 1.** Generalized life cycle of an entomopathogenic nematode (modified from S. P. Stock).

EPN and their symbiotic bacteria are pathogenic to a wide range of insects and have successfully been used in biological control and integrated pest management programs worldwide (Duncan and McCoy, 1996; Gaugler et al., 1997; Lacey and Unruh, 1998; Shapiro and McCoy, 2000). Many important insect pests, such as the citrus weevil, *Diaprepes abbreviatus* (McCoy, 1999), *Cydia pomonella* (Lacey et al. 2006) in apples orchards, *Amyelois transitella* in almonds (Rice et al. 1978), fungus gnats (Jagdale et al., 2004) flowers thrips (Ebbsa et al. 2001), crickets (Parkman et al. 1996), and scarab beetles (Koppenhöfer et al. 1997, 1999) have successfully been controlled by these entomopathogens.

Different isolates and species show substantial variation in behavior, host range, infectivity, reproduction, and environmental tolerances. This biological variation has stimulated interest in more fully characterizing the genetic diversity of these nematodes, because new strains and species may have different biological and/or ecological traits that could be more useful than those currently considered as biological control agents against agriculturally important pests (El-Borai et al. 2007, Oestergaard et al. 2006, Shapiro-Ilan et al. 2006, Vinciguerra and Clausi, 2006). In this respect, realization of the practical use of these nematodes has spurred developments across a far broader scientific front. Certainly, an intensive worldwide search for new EPN has resulted in an exponential growth of the number of new species and the discovery of thousands of new isolates worldwide (Stock and Goodrich-Blair, 2008).

EPN and their bacterial symbionts offer many advantages as model organisms. Their ability to be reared under laboratory conditions (*in vivo* and *in vitro*), their short span (ranging

from 7 to 21 days), long-term storage aptitude, and genetic tractability, are some of these advantages. These traits have set EPN and their symbiotic bacteria as a tractable model system that can be used for advancing research in other disciplines, such as soil ecology, symbiosis and evolutionary biology (Stock and Goodrich-Blair, 2008; Campos-Herrera et al. 2012).

## **1.2. EPN and the soil environment**

### **1.2.1. Effect of biotic and abiotic factors on EPN biology in the soil**

As mentioned above, EPN and their symbiotic bacteria are effective agents of biological control against numerous insect and arthropod pests (Duncan and McCoy, 1996; Gaugler, 1997; Lacey and Unruh, 1998; Shapiro and McCoy, 2000). However, many biotic and abiotic factors are known to play key roles in the persistence, dispersal and reproduction of EPN in the soil. Among abiotic factors, physical and chemical soil characteristics (e.g. moisture, temperature, pH, texture, structure and bulk density) have been shown to impact EPN occurrence, movement, and persistence (Georgis and Gaugler, 1991; Kung et al. 1990; Kaya and Gaugler, 1993; Glazer, 2002; Shapiro–Ilan et al. 2002). Other abiotic factors such as temperature (Kaya, 1990; Grewal et al. 1994), UV radiation (Gaugler and Boush, 1978), and pH (Jaworska, 1993) can affect EPN dispersal and survival in the soil.

There are also numerous biotic factors that influence predator–prey, host–parasite, and other food web interactions (Rosenheim et al. 1995). Similar to other soil fauna, the diversity of EPNs and their interactions can also be altered by the presence of other organisms. A few studies

have examined the interaction of EPN with soil microorganisms including bacteria (Boemare et al. 1993), protozoa (Poinar and Hess, 1988), predators and scavengers (Small, 1988), beetles and ants (Baur et al. 1998; Akhurst and Boemare, 1990), and nematophagous fungi (Gray, 1987; El-Borai, 2011). The interactions among these organisms and EPN are varied, and range from beneficial to detrimental for nematode survival and overall fitness (Koppenhöfer et al. 1997; Ansari et al 2005; Acevedo et al. 2007; El-Borai et al. 2011).

Additionally, EPN-infected insect cadavers also represent a resource with which soil organisms can interact. The EPN-infected cadavers have been shown to repel scavengers such as ants, and thereby provide some measure of protection for the developing EPN inside the insect host (Baur et al. 1998; Zhou et al. 2002). Studies have attributed the “ant-repellent” activity to the production of secondary metabolites by the EPN symbiotic bacteria (Zhou et al. 2002). Moreover, research conducted to detect the effect of the symbiotic bacteria *Xenorhabdus bovienii* and *Photorhabdus luminescens* to suppress the pecan fungus *Fusicladosporium effusum* revealed the fungicidal effect of both symbiotic bacteria toward this plant pathogen. Results from this study showed that the metabolic activity of these bacteria had a similar effect than both synthetic fungicides tested (dodine and fenbuconazole) (Shapiro et al. 2009).

In spite of these observations, the effects of biotic factors on EPN still remain as an unexplored topic for research, probably due to methodological limitations. Moreover, most studies have considered agricultural settings as opposed to natural ecosystems. In this context, Strong et al., (1996, 1999) established the baseline for consideration of EPN as promoters of trophic cascades in the soil, using a natural ecosystem at Bodega bay (California) as model

system. The authors documented interactions between the EPN *Heterorhabditis marelatus* (Nematoda: Heterorhabditidae), its natural host the ghost moth (*Hepialis californicus*), which feeds on bush lupine (*Lupinus arboreous*) in sand dune habitats. In this study, Strong and collaborators (1996, 1999) demonstrated how abiotic factors such as soil moisture could promote EPN survival, thus increasing mortality on root borers, which in turn released bush lupines from herbivore. This study was the first one to demonstrate the direct and indirect roles of EPN in trophic cascades.

Subsequent studies considered the role of EPN in the context of multitrophic interactions (Rasman et al. 2005; Bezemer et al. 2005), demonstrating that plants may manipulate EPN against herbivore to their own benefit. These authors stressed the importance of EPN in above- and belowground interactions and used them as good examples to explain the “bodyguard” hypothesis (Elliot et al., 2000), which postulates that plants can recruit natural enemies of their herbivores. This perspective has also been extrapolated into pest management approaches. For example, Rostás et al. (2003) stated that insect pest management should have a multitrophic-level approach for a more realistic and critical perspective in understanding herbivores, and their interactions with natural enemies.

While tripartite interactions among plants, plant pathogens and insect herbivores have been well documented (Agrawal et al. 1999; Paul et al. 1981; Rostás et al. 2003), the possible impact of plant pathogen-herbivore interactions on other organisms of the same and/or different trophic guild, such as entomopathogens, has been mostly neglected. Plant pathogens are known to alter plant chemistry, and therefore to affect the plant’s susceptibility to herbivores. However, knowledge on the interactions between plant pathogens and insect pathogens and their effect on herbivores remains largely unknown and should be further investigated.

### 1.2.2. EPN interactions with management practices

As discussed above, success of EPN to control a targeted pest can be impacted by numerous abiotic and biotic parameters. Moreover, these parameters can also be altered by management practices and decisions, including: EPN species chosen to control a given pest, the targeted crop (including planting density, variety selection), tillage regime, fertility inputs, and pesticide use (reviewed in Shapiro–Ilan et al. 2002; Barbercheck and Hoy, 2005; Stuart et al. 2006).

For example, in managed systems, lack of physical disturbance or adequate soil moisture, and aeration has favored the inundative application of EPN (Shapiro–Ilan et al. 2002). Moreover, reduced tillage practices have been shown to favor nematode abundance and recovery in the soil (Brust, 1991; Shapiro et al. 1999; Hummel et al. 2002; Millar and Barbercheck, 2002; Campos-Herrera et al. 2008).

Although, there is a good amount of information available about how certain insecticides interact with EPN for control of important insect pests, there is also a dearth of information about the effect that synthetic pesticides have on EPN life cycle and survival in the soil. In this respect, the presence of IJs in the soil is a key factor for the success of EPN as regulators of herbivores in the short and long-term effect. If IJ populations are reduced, low levels of insect control may be expected (Kaya, 2002). Persistence of EPN in the soil is also influenced by rates of virulence (i.e., the nematodes capability to infect and penetrate an insect larva), and progeny production (Kaya, 1985). Many studies have demonstrated that certain EPN and insecticide combinations reduce the nematode performance (virulence and reproduction). In this respect, not only their persistence and recycling in the soil is affected, but also their role as regulators of insect pests.



This background summary clearly points out that it is critical to understand the dynamics of EPN in the soil in relation to other organisms of the same and different trophic guilds. With this knowledge, EPN would be properly applied in the field, and the success of the application could be better predicted. To address some of these caveats in the study of EPN, the present research has focused on a selection of biotic and human-induced elements that may interact with EPN in soil ecosystems, impacting their survival, performance and dispersal. Knowledge on the interactions that EPN have with other soil components should be taken into consideration, not only for enhancing their efficacy in integrated pest management programs, but also to better understand their role in food webs and trophic cascades belowground.

### **1.3. Dissertation format**

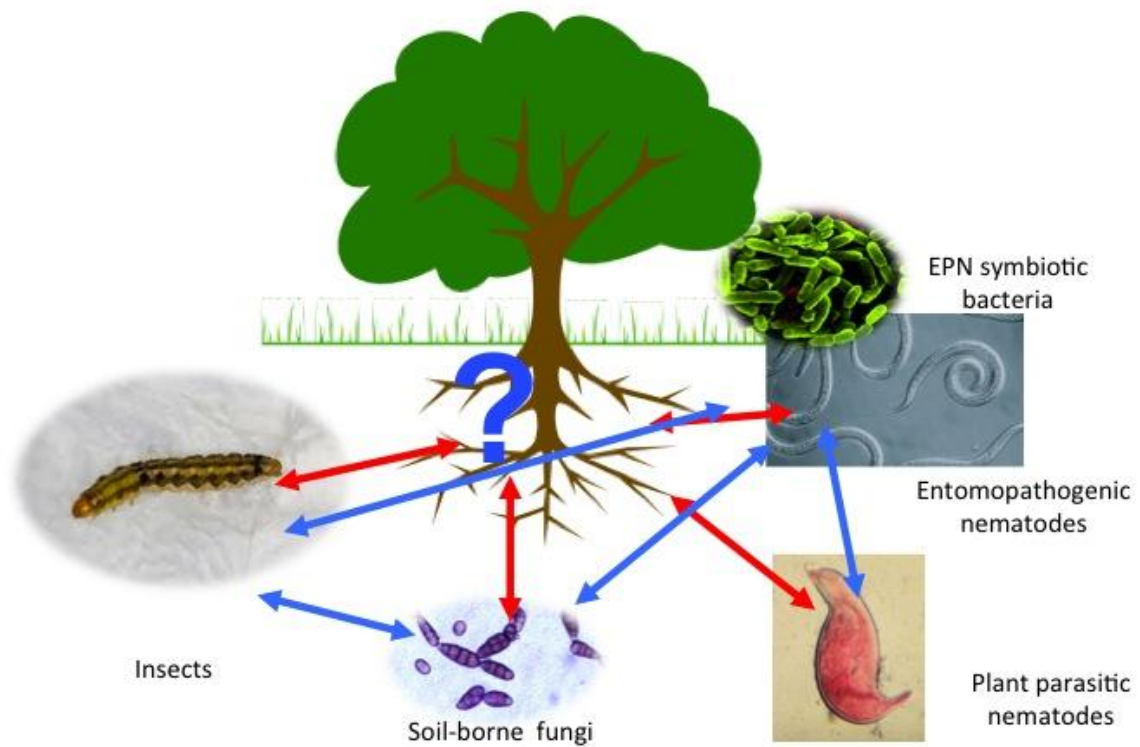
The goal of this investigation is to study the effect of a selected group of insecticides and biotic soil components on EPN life cycle during their interaction with the insect host. The introduction and the appended sections provide background and a selection of cases and examples that justify the present study. Detailed methodology and results are included as three appendices, each of which is intended for publication as an independent paper in a peer-reviewed journal.

In appendix A, I described the interactions between two EPN species and three synthetic and one biological insecticide, and the effect that their combined application and application timings have on EPN fitness.

Appendix B focuses on the interactions between a plant pathogenic/saprobic fungus, *Fusarium oxysporum* f. sp. *asparagi*, on the EPN's life cycle in relation to the insect host.

In appendix C, I evaluated the effect of two EPN species on the plant parasitic nematode, *Tylenchulus semipenetrans* (Tylenchida: Tylenchulidae). Specifically, I assessed the effect of EPN on *T. semipenetrans* root infection and reproduction.

## Nematode Interactions in the Rhizosphere



**Figure 2.** Soil pathogens and their interactions with entomopathogenic nematodes in the soil (from S.P. Stock).

## CURRENT STUDY

The methods, results, and conclusions from this research are presented in the appended manuscripts. The following is a summary of the most important findings of each study.

### **2.1 Interactions of Two Arizona-Native Entomopathogenic Nematodes with Synthetic and Biological Insecticides**

In appendix A, I assessed the interactions of two Arizona-native EPN species: *Heterorhabditis sonorensis* (Caborca strain) and *Steinernema riobrave* (SR-5) in combination with three synthetic (imidacloprid, dinotefuran, indoxacarb), and one biological (*Bacillus thuringiensis* subsp. *kurstaki*) insecticides. Specifically, I evaluated the following EPN parameters: a) virulence (measured as insect mortality); b) establishment of infective juveniles in the cadaver (measured as the number of nematode that penetrated the larva); and c) reproductive fitness (measured as progeny production or the number of nematodes that emerged from the larva), considering the effect of EPN application time. The nature of the interactions between EPN and insecticides (synergistic, additive, or antagonistic) was also determined. Fourth instar *Helicoverpa zea* (Lepidoptera: Noctuidae) was used as the insect host.

Results from this study showed that most of the combinations of EPN and insecticides were more effective in killing *H. zea* larvae than either agent used alone. Synergistic interactions were observed between *H. sonorensis* with *Btk* and *S. riobrave* with indoxacarb. Although certain combinations of EPN and insecticides increased insect mortality, a reduction in progeny

production was observed. Reduction in EPN progeny production may have detrimental effects for EPN persistence in the soil.

## **2.2 Interactions between the entomopathogenic nematode *Heterorhabditis sonorensis* (Nematoda: Heterorhabditidae) and the saprobic fungus *Fusarium oxysporum***

In Appendix B, I assessed interactions between the soil-borne fungus *F. oxysporum* f. sp. *asparagi* and the entomopathogenic nematode *Heterorhabditis sonorensis* (Caborca strain). In this study, I hypothesize that the presence of this saprobic fungus may interfere with the nematode's ability to infect and reproduce in an insect host, and therefore, negatively affect the nematode life cycle. For this purpose, the following nematode parameters were evaluated: a) host search behavior, b) virulence c) establishment, and d) reproduction. Additionally, the effect of fungal extracts on the nematode's symbiotic bacteria was assessed *in vitro*. Results from this study indicated that *F. oxysporum* did not affect the ability of *H. sonorensis* to search a host, nor interfered with the nematode's ability to penetrate the insect. However, nematode virulence and progeny production were negatively affected by the presence of *F. oxysporum*. The fungal crude extracts inhibited the growth of the symbiotic bacteria at a concentration of 10 mg/ml. These results support the hypothesis that saprobic fungi negatively affect key stages of the *H. sonorensis*' life cycle during their interaction with the insect host.

### **2.3 Effect of two entomopathogenic nematode species on the citrus nematode *Tylenchulus semipenetrans* (Tylenchida: Tylenchulidae)**

In Appendix C, I studied the effect of two EPN species, *Steinernema riobrave* (TX strain) and *Heterorhabditis bacteriophora* (NC1) on the citrus nematode (*Tylenchulus semipenetrans*) life cycle. The citrus nematode is an important pest that infects citrus plants by penetrating the root and reproducing inside of it. Successful infection and reproduction of the citrus nematode inside the root increases the root weight and produces a slow defoliation of the aerial part of the plant. Previous studies have demonstrated that the presence of EPN may negatively affect the life cycle of plant parasitic nematodes by reducing their population of infective juveniles in the soil. Therefore, the goal of this investigation was to determine if the presence of *S. riobrave* and *H. bacteriophora* might negatively affect the infection and reproduction stages of this plant parasitic nematode on the citrus plant. To achieve this goal I assessed two parameters of the citrus nematodes biology: a) root penetration (measured as fresh weight and number of J2 in the soil) and b) egg production by adult parasitic females. Additionally, I considered two EPN delivery methods (EPN-infected cadavers and IJ aqueous suspension), and two application timings (simultaneous and subsequent). Results of this investigation showed that both EPN *S. riobrave* and *H. bacteriophora* negatively affect root penetration and egg production of *T. semipenetrans*. Specifically, I found that the presence of EPN reduces the number of citrus nematode infective juveniles (J2) in the soil. This observation was positively correlated with the reduction of citrus root fresh weight. Moreover, parasitic females of the citrus nematode were also negatively impacted by the presence of either EPN species. This was translated in a reduction of the number of eggs that they produced when compared to the controls. Therefore, both species of

entomopathogenic nematodes, *S. riobrave* and *H. bacteriophora*, showed negative effects on the life cycle of *T. semipenetrans*, which directly benefit the health of the plant by reducing the damage of their roots. These observations may have future consequences in the use of EPN, for control of this plant parasite.

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**APPENDIX A**

INTERACTIONS OF TWO ARIZONA-NATIVE ENTOMOPATOGENIC NEMATODES  
WITH SYNTHETIC AND BIOLOGICAL INSECTICIDES

Submitted to Biological Control

## Interactions of Two Arizona-Native Entomopathogenic Nematodes with Synthetic and Biological Insecticides

P. D. Navarro, J. G. McMullen II and S. P. Stock

### Abstract

In Arizona as in other parts of USA, alternatives for chemical control of insect pests are needed, and entomopathogens such as nematodes (EPN) are a promising alternative for pest management. EPN have been shown to control many insect pests, either applied alone or in combination with other microbial or chemical agents. In this study, we assessed the interactions of two Arizona-native EPN species: *Heterorhabditis sonorensis* (Caborca strain) and *Steinernema riobrave* (SR-5 strain) in combination with three synthetic (imidacloprid, dinotefuran, indoxacarb) and one biological (*Bacillus thuringiensis* subsp. Kurstaki) insecticide. Fourth instar *Helicoverpa zea* (Lepidoptera: Noctuidae) was used as the insect host. EPN virulence (measured as the number of nematodes that penetrated the larva) and reproductive fitness (measured as progeny production or the number of nematodes that emerged from the larva) were evaluated considering the effect of EPN application time. The nature of the interactions (synergistic, additive, or antagonistic) was also determined. Our data showed that most of the combinations of EPN and insecticides were more effective in killing *H. zea* larvae than either agent used alone. Moreover synergistic interactions were observed between *H. sonorensis* with *Bacillus thuringiensis* subsp. kurstaki and *S. riobrave* with indoxacarb. However, while these combinations increased insect mortality, they also reduced significantly progeny

production of the nematodes. This aspect may have a future impact when considering recycling of these nematodes in the soil, affecting the long-term effect of EPN application.

**Keyword:** *S. riobrave*, *H. sonorensis*, *Helicoverpa zea*, insecticides, interactions.

## 1. Introduction

Arizona and Southern California are characterized by a diversity of irrigated desert vegetable crops such as cotton, alfalfa, citrus, melons, lettuce and small grains, which are produced at various times through the year (Anonymous 1987). This crop diversity together with favorable climatic conditions (high temperatures, dry conditions and other abiotic factors) provides an ideal habitat for a number of insect pests (Anonymous 1987). Among these pests, lepidopterans such as beet armyworms, loopers, heliothines, cutworms, and other caterpillars are considered major pest problems in the Southwest of United States (Kerns and Palumbo, 2009). At present, management of these lepidopteran pests typically involves a combination of approaches including cultural practices, insect monitoring, and synthetic or biological insecticides (*Bt*). However, synthetic insecticides still remain the most widely used method for insect control in vegetables (Kerns and Palumbo, 2009). The prevalent usage of chemical insecticides has generated several problems, such as insecticide resistance, outbreaks of secondary pests and decrease of biodiversity (Lacey et al. 2001). For this reason, the search for environmentally friendly strategies for pest management is imperative.

One alternative approach is the use of biological control agents, such as entomopathogenic nematodes (a.k.a. EPN) (Gaugler, 1999; Grewal et al. 2005). These nematodes are obligate and lethal pathogens of a wide range of insect pests, including lepidopterans, coleopterans and dipterans. The third-stage juvenile of EPN nematode, also known as the infective juvenile (IJ), is the only free-living stage, and it is responsible for vectoring pathogenic bacteria (Gamma-Proteobacteria, Enterobacteriaceae) from one insect to

another (Gaugler and Kaya, 1990). These bacteria kill the insect host in a short period of time (24-48h) by massive septicemia (Walsh and Webster, 2003).

Several studies have shown the efficacy of EPN to control different insect pests, when used alone or combined with other pathogens or synthetic pesticides. Thus, many insecticides, nematicides, fungicides and acaricides have been tested to determine their compatibility with EPN (Rovesti et al. 1988; Barbosa et al. 1996; Zimmerman and Cranshaw, 1990). Results from these studies are variable, depending on the type of chemical and nematode species studied (Koppenhöfer and Grewal, 2005). For example, the insecticide carbaryl (1-naphthyl methylcarbamate) showed a positive compatibility with *Steinernema carpocapsae* and *Steinernema feltiae* (Das and Divakumar, 1987), which indicates that these EPN species can tolerate the exposure to carbaryl. In contrast, the same insecticide showed a negative compatibility with the EPN *Heterorhabditis bacteriophora* (Zimmerman and Cranshaw, 1990).

A number of studies have demonstrated the synergistic effect of combine low risk insecticides with EPN (Koppenhöfer and Kaya, 1997,1998; Koppenhöfer et al. 2000). A synergistic effect is defined as the supplemental action of two agents of insect control, in this case EPN and insecticide, which result in a greater effect than the sum of their effects alone (Koppenhöfer and Grewal, 2004). For example, the insecticide imidacloprid (1-(6-chloro-3-pyridilmethyl)-N-nitroimidazolin-2-ylideneamine) had a synergistic effect in combination with *H. bacteriophora* (Koppenhöfer and Kaya, 1998; Koppenhöfer et al. 2000, 2002) or *S. carpocapsae* (Alumai and Grewal, 2005), increasing mortality of *Cyclocephala hirta* LeConte

and *C. pasadenae* Casey (Coleoptera: Scarabaeidae), when compared with the application of *H. bacteriophora* or *S. carpocapsae* alone.

Until now, most of the studies considering combinations of EPN and insecticides have been focused in the use of commercially available EPN species, and how effective these combinations are for control insect pests. In this study, we assessed the effect of a selection of synthetic and biological reduced risk insecticides in the fitness (virulence and reproduction) and recycling of IJs in the soil. For this purpose, we chose two Arizona-native EPN *Heterorhabditis sonorensis* (Caborca strain) and *Steinernema riobrave* (SR-5 strain). These EPN species were selected because they are endemic to the southwest and could be considered in pest management programs in this region. Results obtained from this investigation should contribute to enhancing management strategies for insect control.

## 2. Materials and Methods

**2.1. Insects, Nematodes, and Insecticides.** The corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae) was the insect host selected for these experiments. This insect host resulted less susceptible to EPN than the greater wax, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Navarro, unpublished data), therefore more suitable for assessing interactions between EPN and insecticides. Eggs of *H. zea* were obtained from Benzon Research<sup>®</sup> and reared under laboratory conditions at 28°C, 80% RH following procedures described by Waldbauer et al. (1984). Fourth instar larvae were used in all assays. During experiments, larvae were fed with 5g of corn earworm artificial diet (Southland Products Inc.). Both AZ-native EPN were

propagated *in vivo* using the fifth instar of *G. mellonella* following procedures described by Stock and Goodrich-Blair (2012). Infective juveniles less than two weeks old (i.e., time after initial emergence from insect cadavers) were used in each assay and stored at 13° C. The chemical and biological insecticides considered were: imidacloprid [Merit<sup>®</sup> 75 WP] (Bayer, NC); dinotefuran [Scorpion<sup>™</sup> 35SL] (Gowan, Yuma, AZ); indoxcarb [Avaunt<sup>®</sup>] (Dupont, Wilmington, DE); and *Bacillus thuringiensis* subsp. *kurstaki* [*Btk*] (Green Light<sup>®</sup>, San Antonio, TX). These insecticides were chosen because are commonly used for control of important lepidopteran and coleopteran pests and are considered of reduced risk toxicity.

**2.2. Nematode and Insecticide Concentrations.** Before initiation of the experiments, the lethal concentration 50 (LC<sub>50</sub>) of EPN and insecticide were determined for proper assessment of interactions. The following LC<sub>50</sub> were measured: 1) EPN against *H. zea*, 2) insecticide against *H. zea*, and 3) insecticide against EPNs.

All assays to determine the LC<sub>50</sub> (except the ones to determine the LC<sub>50</sub> of insecticides toward EPN) were conducted in SOLO<sup>®</sup> plastic cups (1 oz.) containing 4g of sterile sand, where one *H. zea* larva was exposed to different EPN or insecticides concentrations. EPN and insecticide concentrations considered are shown in Table 1 and 2, respectively. One milliliter of inoculum (nematode or insecticide) was applied directly to each larva at the start of the experiment. For treatments considering *Btk*, the insecticide was applied to diet, based on the rate of insecticide shown in Table 2. Each larva was fed once with 5 g of artificial diet at the start of the experiment. Ten replicates were conducted for each treatment (concentration). Treatments

were organized in blocks and each block was repeated three times. Larval mortality was recorded after 10 days.

Assays to determine the effect of the insecticides on each EPN species were conducted in a 12-well plate arena, where each EPN species was evaluated separately. One milliliter of each insecticide concentration tested in combination with 100 IJs was added to each well of the plate. Each experiment was repeated three times. From each replicate (plate), six wells were randomly chosen, and the number of dead nematodes was recorded after 10 days. The immobile nematodes were probed with a needle to determine if they were dead or alive. Mortality data were subjected to probit analyses (Finney, 1964) using the statistical software SPSS (SPSS 20.0, 2012).

**2.3. Interactions between EPN and Insecticides.** In these experiments we evaluated nematode virulence (assessed as insect mortality and IJ establishment in the insect host) and progeny production. IJ's establishment was measured as the number of nematodes that penetrated the larva. Progeny production was measured as the number of nematodes that emerge from the larva. The effect of EPN application time in relation to the application of the insecticides was considered. Each nematode species was evaluated separately.

2.3.1. *EPN virulence*: Treatments consisted of four insecticides (imidacloprid, dinotefuran, indoxacarb and *Btk*), and three EPN application timings: 1) EPN applied first, insecticide applied 24h later, 2) insecticide applied first, EPN applied 24h later, and 3) simultaneous application of EPN and insecticide. Nematode and insecticide inocula concentrations were based on results from experiments in section 2.2. The assays were conducted in SOLO<sup>®</sup> cups filled with 4 g of



sterile sand, where a single larva was added per cup. Each larva received 1 ml of inoculum (nematode and/or insecticide), which was applied at the different times explained above. Positive controls consisted of 1 ml of nematode or insecticide inoculum. Negative controls consisted of the application of 1 ml of distilled water per cup. Ten larvae (1 larva = 1 replicate) were evaluated for each treatment (concentration) and controls. The experiment was arranged in a completely randomized block design. Each block was conducted three times. Cups were incubated at  $25 \pm 1$  °C and 80% RH. Larval mortality was recorded after 10 days post-inoculation. Once data of insect mortality were recorded from all cups, half of the insect cadavers obtained from each treatment were used to record EPN establishment. The other half of the cadavers was used to record progeny production (see section 2.3.2). To evaluate nematode establishment (i.e., number of IJs inside each insect cadaver), we used the enzymatic digestion method described by Mauleón et al. (1993).

*2.3.2. EPN reproductive fitness:* Cadavers were thoroughly rinsed in distilled water, and individually placed in modified White traps (Kaya and Stock, 1997). Daily observations were made to record the first day of nematode progeny emergence. Emerging IJs were collected from each White trap 10 d after the first day of emergence, as described by Koppenhöfer and Kaya (1999), and stored in tissue culture flasks at 4° C until counted. Insect cadavers that did not produce progeny were not considered.

**2.4. Statistical Analysis.** All experiments were analyzed using ANOVA, and the differences among means were determined using Tukey's test. For experiments where the insect mortality was determined, the number of dead larvae was recorded and the percentage mortality was

corrected using Abbott's formula (Abbott, 1925). Before to analysis, in order to meet the assumption of normality, data were arcsine (insect mortality) and log10 (establishment and progeny production) transformed. Mortality (%), nematode establishment, and nematode progeny production were considered as response variables. Timings of application were considered as explanatory variables. All statistical analyses were conducted using JMP<sup>®</sup> 8.0.2 (SAS Institute, 2008). The nature of the interactions (additive, antagonistic, or synergistic) between EPN and insecticides was determined based on the analysis used by Nishimatsu and Jackson (1998). The expected mortality of larvae was calculated based on the formula  $P_E = P_o + (1-P_o) (P_1) + (1-P_o) (1-P_1) (P_2)$ , where  $P_E$  is the expected mortality on combination of EPN and insecticide,  $P_o$  is the mortality in the control,  $P_1$  is the mortality after treatment with the insecticide alone, and  $P_2$  is the mortality after treatment with the nematode alone. The determination of  $X^2$  was calculated through the formula  $X^2 = (L_0 - L_E)^2 / L_E + (D_0 - D_E)^2 / D_E$ , where  $L_0$  is the number of living larvae observed,  $L_E$  is the number of living larvae expected,  $D_0$  is the number of dead larvae observed, and  $D_E$  is the number of dead larvae expected. The parameter  $X^2$  was used to test the hypothesis for independence of variables (df=1 and  $P=0.05$ ). Combinations of nematode and insecticide where  $X^2 < 3.84$  were defined as additive. Synergism was denoted by  $X^2 > 3.84$  and  $P_C > P_E$ ; antagonism was defined as  $X^2 > 3.84$  and  $P_C < P_E$ , where  $P_C$  is the observed mortality of the insecticide and nematode combination.

### 3. Results

**3.1. Nematode and Insecticide Concentrations.**  $LC_{50}$  obtained for *H. sonorensis* and *S. riobrave* against *H. zea* was 7 and 3 IJs/larva, respectively (Table 1). The  $LC_{50}$  obtained for imidacloprid, dinotefuran, indoxacarb and *Btk* against *H. zea* was of 1.4, 1.0, 33.0 and 1.5 mg/L

respectively (Table 2). When different insecticides concentrations were evaluated on both nematode species, not significant effect on nematode mortality was observed compared with the control (average nematode mortality = 30%). Therefore, the selected insecticides were considered compatible to both EPN species. The  $LC_{50}$  previously calculated against *H. zea* (Table 2) were used to assess all further interactions.

### **3.2. Interactions between EPN and Insecticides.**

3.2.1. *EPN virulence*: Not significant effect of blocks was observed in all ANOVAs performed in this investigation. The virulence of *H. sonorensis* and *S. riobrave* (measured as insect mortality) was significantly affected, when these nematodes species were combined with the selected insecticides at different application times (Fig. 1 and Fig. 2). Moreover, the nature of their interaction varied depending on the insecticide and time of application (Table 3). Most of the combinations evaluated resulted in additive effects on mortality of *H. zea*; however some synergistic and antagonistic effects were also observed.

The simultaneous application of *H. sonorensis* and *Btk* resulted synergistic (Table 3) achieving 100% of insect mortality (Fig. 1A). This combination was considered the best for control of *H. zea*. Other combinations considering the EPN species *H. sonorensis* also achieved high percentage of insect mortality, for example: one alternated combination of *H. sonorensis* and dinotefuran (Fig. 1B), and the simultaneous application of *H. sonorensis* with dinotefuran (Fig. 1B) or imidacloprid (Fig. 1D); however those interactions were additive (Table 3). Insect mortality from both alternated application of *H. sonorensis* and *Btk* resulted statistically similar to the application of *H. sonorensis* alone (Fig. 1A). All combinations (simultaneous and

alternated) of *H. sonorensis* with dinotefuran resulted in higher insect mortality (90-100%), when compared with the application of either the insecticide or the nematode alone (Fig. 1B). For all combinations of *H. sonorensis* and indoxacarb not significant differences were observed with the application of nematode alone (Fig. 1C). For combinations of *H. sonorensis* with indoxacarb the interactions ranged from additive (simultaneous and the alternated: nematode first, insecticide 24 h after) to antagonistic (alternated: insecticide first, nematode 24 h after). All interactions of *H. sonorensis* with imidacloprid were additive. The simultaneous and one of the alternated applications (insecticide first, nematode 24 h after) of *H. sonorensis* and imidacloprid caused more than 90% of insect mortality, and were statistically different to the application of *H. sonorensis* alone. The other alternated application (nematode first, insecticide after) was not significantly different to the application of *H. sonorensis* alone (Fig 1D).

With respect to combinations of *S. riobrave* with Btk, insect mortality for all application timings evaluated were not statistically different than the observed with the application of *S. riobrave* alone (50%) (Fig.2A). All interactions of *S. riobrave* with Btk were considered additive. High larval mortality was also observed in all combined applications of *S. riobrave* with dinotefuran (Fig. 2B). The nature of the interactions between this insecticide and *S. riobrave* were additive. The simultaneous application of *S. riobrave* and indoxacarb, and one alternated application (nematode first, insecticide 24 h after) resulted synergistic, with larval mortality of 97% in both cases (Fig. 2C). The other alternated application (indoxacarb first, *S. riobrave* 24 h after) was additive.

Interactions between *S. riobrave* and imidacloprid were all considered antagonistic. Indeed, *H. zea* mortality challenged by both, insecticide and nematode, did not differ significantly from that observed with the application of the nematode alone (Fig. 2 D).

With respect to IJ establishment, none of the pesticides and application timings considered in this study affected the ability of either *H. sonorensis* or *S. riobrave* to penetrate the insect host (Table 4). For both EPN species, the average number of penetrating IJ was 1.5 IJs/larva, which was similar to the average number of IJs that penetrated each larva in the controls.

*3.2.2. EPN reproductive fitness:* Results for progeny production varied significantly among the different insecticides and application timings considered (Fig. 3 and Fig. 4). For *H. sonorensis*, all application timings with Btk caused a significant reduction in the number of emerging IJ ( $F_{3,32}=15.6$ ;  $p<.0001$ ). Indeed, progeny production was reduced four times when compared to that observed in the control (Fig 3A). In contrast, IJ progeny was not affected by any of the application timings for either dinotefuran or imidacloprid, (Figs. 3B and 3C, respectively). Similarly for indoxacarb, the number of emerging IJs did not differ significantly when compared to the control for both alternated applications (Fig. 3D). But a reduced IJ progeny was observed when *H. sonorensis* and indoxacarb were applied simultaneously ( $F_{3,32}=2.3$ ;  $p=0.008$ ) (Fig. 3D).

Progeny production was significantly reduced for combinations of *S. riobrave* with indoxacarb and Btk (Fig. 4A and 4B, respectively) when compared with the application of *S.*

*riobrave* alone. In contrast, all combination timings of *S. riobrave* with dinotefuran caused an increase in the number of emerging IJs (Fig. 4C). Simultaneous applications of *S. riobrave* with imidacloprid also resulted in a significant increase of emerging IJs when compared with the application of *S. riobrave* alone. However, progeny production was not affected in either of the alternate application timings of *S. riobrave* and imidacloprid (Fig. 4D).

#### 4. Discussion

In this study we evaluated the interaction of two Arizona-native EPN species with a selection of synthetic and biological insecticides. Specifically, we assessed the effect and timing of application of imidacloprid, dinotefuran, indoxacarb and *Btk* when combined with *H. sonorensis* and *S. riobrave*. All these insecticides resulted compatible in their combination with EPN, not affecting the survival of the nematode. Throughout most of the combinations evaluated in this study, the addition of the selected insecticides improved larval mortality of *H. zea* compared with the application of EPN alone.

For most of tested combinations of EPN and insecticides, the interactions were considered additive. Synergistic interactions were found for the simultaneous application of *Btk* and indoxacarb with *H. sonorensis* and *S. riobrave*, respectively. Moreover, the application of indoxacarb 24 h after the application of *S. riobrave* also resulted synergistic. For all these cases, the achieved mortality of *H. zea* increased from 50% to 97% or more. These synergistic results agree with previous finding reported by Koppenhöfer and Kaya, 1997,1998; Koppenhöfer et al. 2000), who found that the mixture of EPN with reduced risk insecticides (imidacloprid, *Bacillus*

*thuringensis* sub. sp. *japonensis* [*Btj*], and neem) act synergistically for control of insect pest. However, in our study, the effect of the application timings on the nature of the interactions was different when compared with the results obtained by Koppenhöfer and Kaya (1997). These authors found that the simultaneous application of *Heterorhabditis bacteriophora* and *Btj* resulted antagonistic for the control of scarab beetles. In contrast, our results showed that this combination had a synergistic effect on mortality of *H. zea*. We speculate that the discrepancy of results may be related to the different affinity that the midgut receptors have to the *Bt* toxin in each insect host species (*Cyclocephala hirta* versus *H. zea*). Additionally, Koppenhöfer and Kaya (1997) also reported that to achieve a synergistic interaction between EPN and *Btj* on scarab beetle mortality, the insects had to be exposed to *Btj* at least 7 days prior to EPN application. Our results indicated that previous exposure of *H. zea* larvae to *Btk* was not necessary to obtain synergistic effects with *H. sonorensis*.

With respect to interactions between EPN and indoxacarb, to our knowledge, no previous studies have considered this chemical in combination with EPN. In the present study, this insecticide was found to interact synergistically when applied simultaneously with *S. riobrave* or when applied 24h after the EPN. Indoxacarb is a non-systemic insecticide that targets the nervous system of the insect by blocking sodium channels and causing paralysis of the larva (Environmental Protection Agency, 2000). Similarly to *Btk*, this insecticide causes feeding cessation, which affects the behavioral, morphological and physiological strategies of the larva to avoid natural enemies in the soil (Gaugler et al. 1994), and turns the larva more susceptible to EPN infection. In fact, our experiments revealed that larvae treated with the simultaneous

application of EPN and Btk or indoxacarb died earlier (24-48 hours after inoculation) when compared with imidacloprid and dinotefuran (average: 5-7 days).

In contrast, the simultaneous applications of EPN with either Btk or indoxacarb caused a drastic reduction in nematode progeny production. Larva from these treatments not only died faster than those treated with imidacloprid and dinotefuran, but also rapidly dried when placed in White traps for further collection of nematode progeny. We speculate that the desiccation effect caused by these insecticides (*Btk* and indoxacarb) negatively interfered with nematode reproduction and emergence of IJs. In this respect, Poinar et al. (1990), found that the developing stages of EPN inside the insect host resulted reduced in size or even not reproduced at all when *Btk* toxin was previously inoculated to the insect host. He attributed the reduced development and reproduction of IJs to competition between the nematodes' symbiotic bacteria and Bt, therefore limiting food resources for IJs.

With respect to antagonistic interactions, we found that all combinations of *S. riobrave* with imidacloprid and one alternated combination of *H. sonorensis* with indoxacarb resulted antagonistic. Based in previous studies reported by Koppenhöfer and Kaya (1998), Koppenhöfer et al. (2000, 2002), and Alumai and Grewal (2004), we expected to found synergistic interactions in combinations of EPN and imidacloprid; however the opposite results were observed. According to these authors, the nature of the interactions not only varied with the nematode species, but also with the rate of insecticide considered and the insect host. For example, Koppenhöfer and Kaya (1998) found that imidacloprid in combination with *Steinernema glaseri* or *Heterorhabditis bacteriophora* resulted synergistic, but additive with *Steinernema kushidae*.



In our study, we not only consider different nematodes species, but also a different insect host. The effect of imidacloprid on larval mortality of *H. zea* was not observed until four to five days after inoculation. In fact, most larvae treated with imidacloprid were quite active during the first five days post-inoculation. Therefore, we speculate that during these days *H. zea* larvae were still able to avoid nematodes infection, which can also be explained by the highly aggressive behavior of this insect species (Capinera, 2008). In relation to this, Koppenhöfer et al. (2000) reported that the mechanism of synergy attributed to imidacloprid on mortality of grubs is the reduction of the defensive behavior showed by the insect, which result in a higher nematode attachment and penetration to the host. Therefore, this insecticide also affects grooming and evasive behavior of the insect host against nematodes.

With respect to the effect of these insecticides on nematode's penetration and establishment, the results of this investigation showed that none of the tested insecticides, in any of their combinations with both EPN species, interfered with the normal process of nematode penetration and establishment.

Simultaneous combinations of *H. sonorensis* with *Btk* and *S. riobrave* with indoxacarb are feasible alternatives to improve management of *H. zea* in the field. While in this study we found that progeny production was reduced with these insecticides, we expect that the increased number of hosts succumbing to nematode infection may improve nematode recycling in the soil. In this respect, further greenhouse and field trials are warranted to further investigate their short and long-term persistence in the soil.

**Acknowledgments**

This study constituted partial fulfillment for P. Navarro's Ph.D. degree. We acknowledge the USDA Sustainable Agriculture Research and Education (W-SARE) Graduate Grant Program for partially funding this research. We acknowledge MGE@MSA/Project 1000 (Arizona State University) for partially funding to P. Navarro. We are also thankful to M. Lamoreaux for technical assistance. We extend our gratitude to I. Molnar, Y. Carrière and P. Ellsworth (dissertation committee members) for their insightful comments and suggestions during this investigation and preparation of this manuscript.

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Table 1. Lethal concentration (LC<sub>50</sub>) calculated for *Heterorhabditis sonorensis* and *Steinernema riobrave* against *Helicoverpa zea*.

EPN	Concentrations Tested (IJ/ml)	<i>n</i>	Slope ±SE	LC <sub>50</sub> (IJ/larva)	95% CL	$X^2$ <sup>a</sup>	$p > X^2$
<i>H. sonorensis</i>	0, 1, 5, 10, 100, 200	30	2.1 ± 0.8	7	2.3 – 9.1	1.3	0.48
<i>S. riobrave</i>	0, 1, 5, 10, 100, 200	30	1.6 ± 0.4	3	0.9 – 4.2	1.6	0.40

<sup>a</sup> Pearson chi-square statistics



Table 2. Lethal concentration (LC<sub>50</sub>) calculated for all insecticides toward *Helicoverpa zea*, and the EPN *H. sonorensis* and *S. riobrave*.

Insecticide <sup>a</sup>	Concentrations Tested (mg/L)	Species <sup>b</sup>	<i>n</i>	Slope ±SE <sup>c</sup> (mg/L)	LC <sub>50</sub>	95% CL	X <sup>2d</sup>	P>X <sup>2</sup>
Imida	1, 1.95, 3.9, 7.8, 11.7	<i>H. zea</i>	30	1.88 ± 0.5	1.4	0.4 - 2.4	2.1	0.59
	1, 1.95, 3.9, 7.8, 11.7	<i>H. sono</i>	9	(-)				
	1, 1.95, 3.9, 7.8, 11.7	<i>S. rio</i>	9	(-)				
Dinote	1, 2, 4, 8, 12	<i>H. zea</i>	30	3 ± 0.4	1.0	0.3 - 1.2	1.7	0.83
	1, 2, 4, 8, 12	<i>H. sono</i>	9	(-)				
	1, 2, 4, 8, 12	<i>S. rio</i>	9	(-)				
Indoxa	1, 2.5, 25, 250, 2500	<i>H. zea</i>	30	2.1 ± 0.9	33.0	22 - 56	1.1	0.73
	1, 2.5, 25, 250, 2500	<i>H. sono</i>	9	(-)				
	1, 2.5, 25, 250, 2500	<i>S. rio</i>	9	(-)				
<i>Btk</i>	1.1, 2.1, 4.1, 8.6, 12.8	<i>H. zea</i>	30	1.1 ± 0.1	1.5	1.1 - 2.2	1.6	0.14
	1.1, 2.1, 4.1, 8.6, 12.8	<i>H. sono</i>	9	(-)				
	1.1, 2.1, 4.1, 8.6, 12.8	<i>S. rio</i>	9	(-)				

<sup>a</sup> Imida: Imidacloprid; Dinote: Dinotefuran; Indoxa: Indoxacarb; *Btk*: *Bacillus thuringensis* sub specie *kurstaki*.

<sup>b</sup> *H. sono*: *Heterorhabditis sonorensis*; *S. rio*: *Steinernema riobrave*.

<sup>c</sup> (-): Mortality not significantly different from the control; LC50s could not be calculated.

<sup>d</sup> : Pearson chi-square statistics

Table 3. Nature of the interactions of EPN-insecticide application timings on larval mortality of *Helicoverpa zea*.

Insecticide	Nematode	Treatment <sup>a</sup>	Mortality of <i>H. zea</i>			
			Observed	Expected	$X^2$ <sup>b</sup>	Interaction <sup>c</sup>
<i>Btk</i>	<i>H. sonorensis</i>	N/I	70	75	0.32	Additive
<i>Btk</i>	<i>H. sonorensis</i>	I/N	67	75	0.83	Additive
<i>Btk</i>	<i>H. sonorensis</i>	N+I	100	75	8.39	Synergistic
Dinotefuran	<i>H. sonorensis</i>	N/I	97	84	2.01	Additive
Dinotefuran	<i>H. sonorensis</i>	I/N	90	84	0.42	Additive
Dinotefuran	<i>H. sonorensis</i>	N+I	97	84	2.01	Additive
Indoxacarb	<i>H. sonorensis</i>	N/I	73	72	0.01	Additive
Indoxacarb	<i>H. sonorensis</i>	I/N	47	72	8.68	Antagonistic
Indoxacarb	<i>H. sonorensis</i>	N+I	87	72	3.12	Additive
Imidacloprid	<i>H. sonorensis</i>	N/I	80	85	0.29	Additive
Imidacloprid	<i>H. sonorensis</i>	I/N	93	85	0.75	Additive
Imidacloprid	<i>H. sonorensis</i>	N+I	97	85	1.60	Additive

Table 3 cont'd

Insecticide	Nematode	Treatment <sup>a</sup>	Mortality of <i>H. zea</i>			Interaction <sup>c</sup>
			Observed	Expected	$X^2$ <sup>b</sup>	
<i>Btk</i>	<i>S. riobrave</i>	N/I	63	70	0.70	Additive
<i>Btk</i>	<i>S. riobrave</i>	I/N	57	70	2.40	Additive
<i>Btk</i>	<i>S. riobrave</i>	N+I	57	70	2.41	Additive
Dinotefuran	<i>S. riobrave</i>	N/I	97	90	0.48	Additive
Dinotefuran	<i>S. riobrave</i>	I/N	93	90	0.07	Additive
Dinotefuran	<i>S. riobrave</i>	N+I	97	90	0.48	Additive
Indoxacarb	<i>S. riobrave</i>	N/I	97	74	7.43	Synergistic
Indoxacarb	<i>S. riobrave</i>	I/N	87	74	2.43	Additive
Indoxacarb	<i>S. riobrave</i>	N+I	97	74	7.43	Synergistic
Imidacloprid	<i>S. riobrave</i>	N/I	63	88	7.30	Antagonistic
Imidacloprid	<i>S. riobrave</i>	I/N	50	88	16.81	Antagonistic
Imidacloprid	<i>S. riobrave</i>	N+I	63	88	7.30	Antagonistic

<sup>a</sup> I/N: insecticide applied first, nematode applied 24h later; N/I: nematode applied first, insecticide applied 24h later; N+I: nematode and insecticide applied at the same time

<sup>b</sup> Interaction based on  $X^2$  mortality ratio (expected : observed).

<sup>c</sup> Additive:  $X^2 < 3.84$ ; Synergistic:  $X^2 > 3.84$  and  $P_C > P_E$ ; Antagonistic:  $X^2 > 3.84$  and  $P_C < P_E$ , where  $P_C$  is the observed mortality of the insecticide and nematode combination and  $P_E$  is the expected mortality of the combination.

Table 4. One-way ANOVA. Effect of nematode/insecticide application timings on nematode establishment

EPN	Insecticide	DF Model	DF Error	F value	<i>p</i> -value
<i>H. sonorensis</i>	Imidacloprid	3	32	0.13	0.93
	Dinotefuran	3	32	1	0.40
	Indoxacarb	3	32	0.44	0.72
	<i>Btk</i>	3	32	0.20	0.89
<i>S. riobrave</i>	Imidacloprid	3	32	1.96	0.13
	Dinotefuran	3	32	0.23	0.87
	Indoxacarb	3	32	0.77	0.51
	<i>Btk</i>	3	32	0.34	0.79

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- Figure 1 Corrected mortality ( $\pm$  SE) for *H. zea* exposed to combinations of *H. sonorensis* with different insecticides A) *Btk*, B) dinotefuran, C) indoxacarb, D) imidacloprid. Different letters above bars indicate statistical differences (based on Tukey's test,  $p=0.05$ ). Data plotted are untransformed. References: C = control (distilled water), I = insecticide, N = nematode, I/N = insecticide first, nematode 24h later, N/I = nematode first, insecticide 24h later, N+I = nematode and insecticide applied simultaneously.
- Figure 2 Corrected mortality ( $\pm$  SE) for *H. zea* exposed to combinations of *S. riobrave* with different insecticides A) *Btk*, B) dinotefuran, C) indoxacarb, D) imidacloprid. Different letters above bars indicate statistical differences (based on Tukey test,  $p=0.05$ ). Data plotted are untransformed. References C = control (distilled water), I = insecticide, N = nematode, I/N = insecticide first, nematode 24h later, N/I = nematode first, insecticide 24h later, N+I = nematode and insecticide applied simultaneously.
- Figure 3 *Heterorhabditis sonorensis* progeny production ( $\pm$  SE) when applied with A) *Btk*, B) dinotefuran, C) imidacloprid, and D) indoxacarb in alternating sequence [insecticide before nematode (N/I) or nematode before insecticide (I/N)] or in mixture (N+I). Different letters above bars indicate statistical differences (based on Tukey's test,  $p=0.05$ ). Data plotted are untransformed. References C = control (distilled water), I = insecticide, N = nematode, I/N = insecticide first, nematode 24h later, N/I = nematode first, insecticide 24h later, N+I = nematode and insecticide applied simultaneously.

Figure 4 *Steinernema riobrave* progeny production ( $\pm$  SE) when applied with A) *Btk*, B) indoxacarb, C) dinotefuran, and D) imidacloprid in alternating sequence [insecticide before nematode (N/I) or nematode before insecticide (I/N)] or in mixture (N+I). Different letters above bars indicate statistical differences (based on Tukey's test,  $p=0.05$ ). Data plotted are untransformed. References C = control (distilled water), I = insecticide, N = nematode, I/N = insecticide first, nematode 24h later, N/I = nematode first, insecticide 24h later, N+I = nematode and insecticide applied simultaneously.



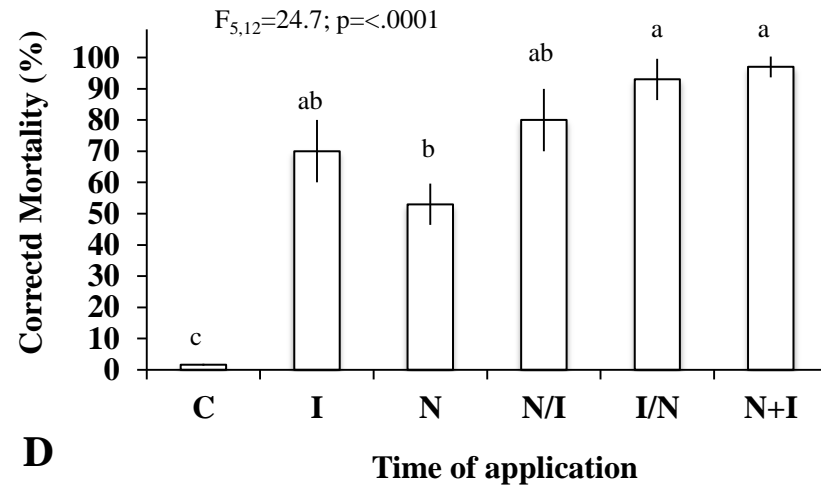
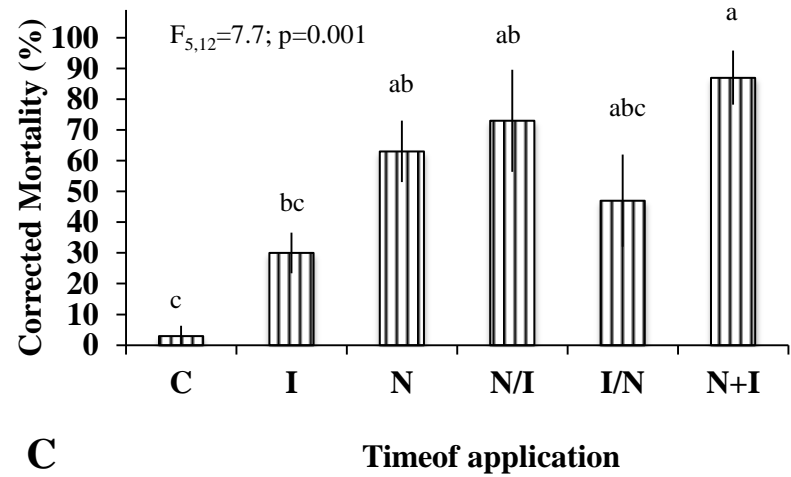
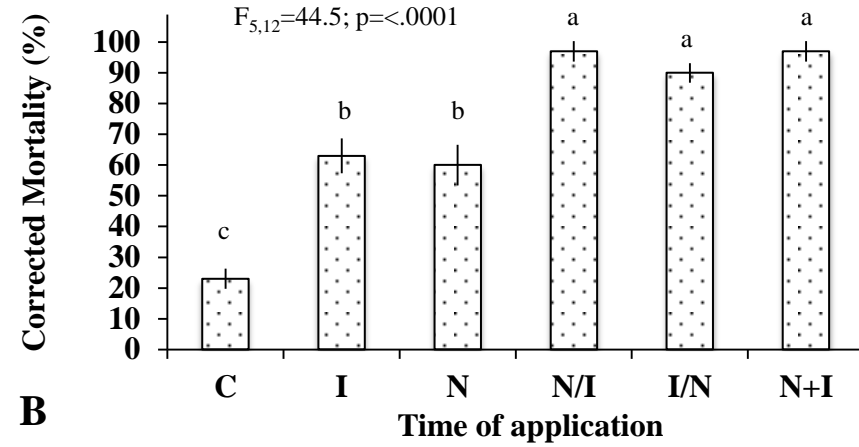
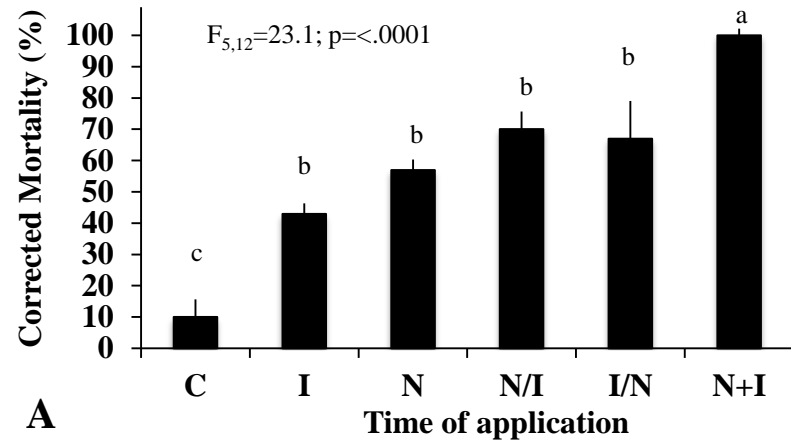


Fig. 1

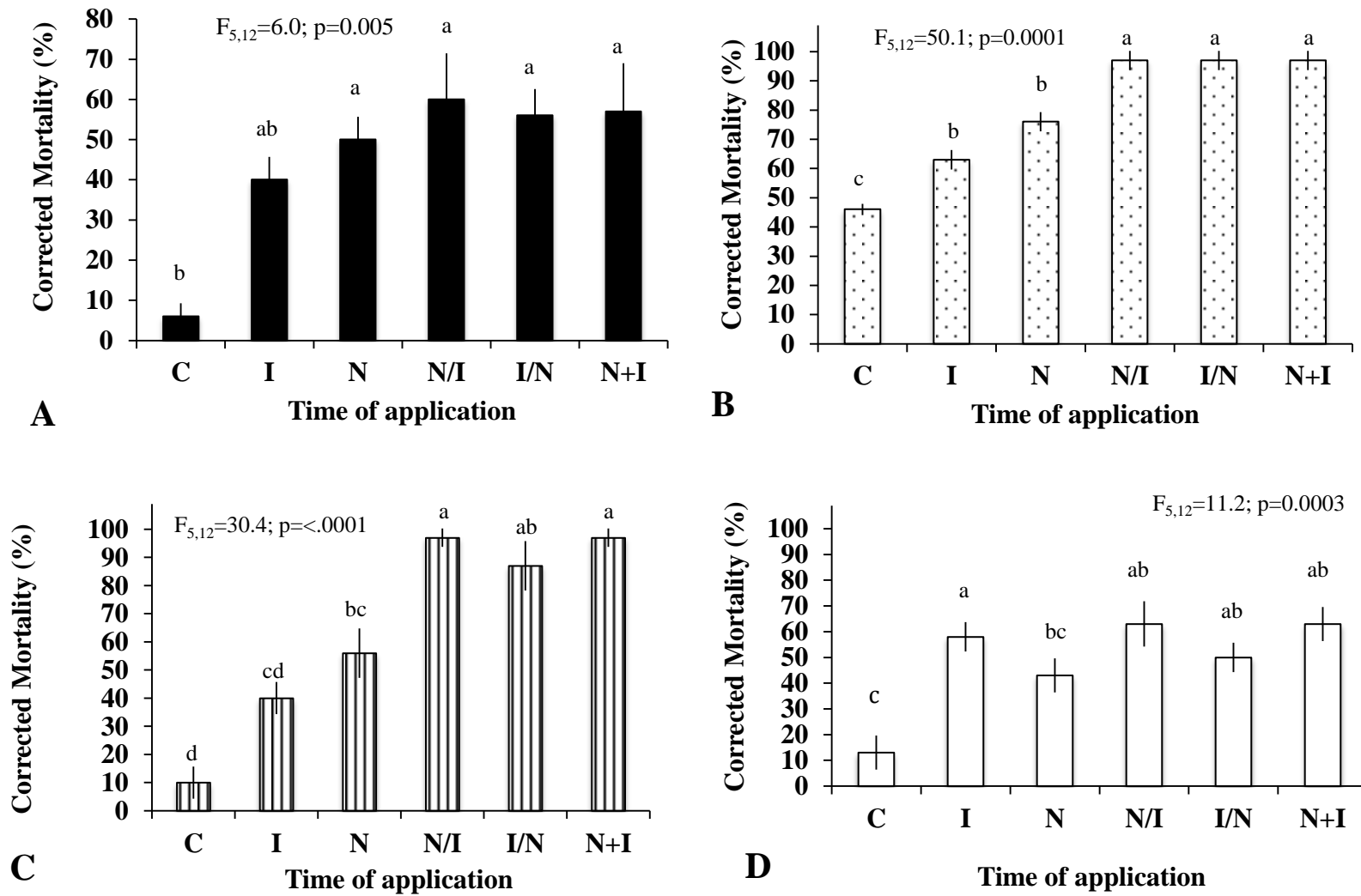


Fig. 2

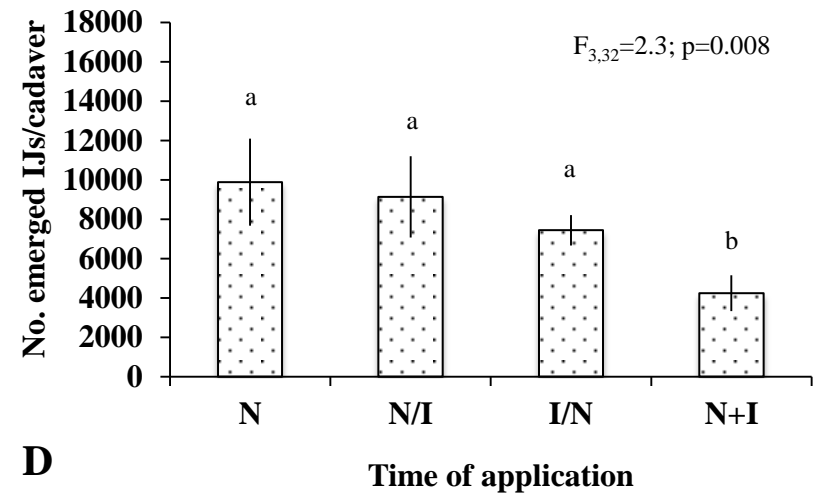
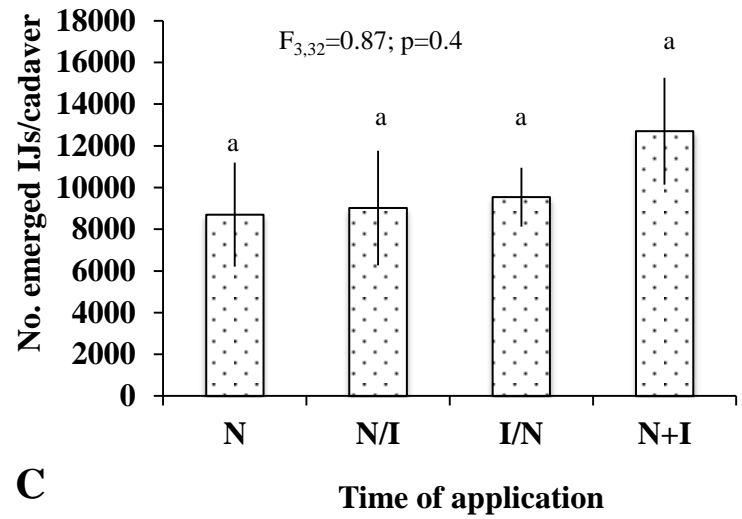
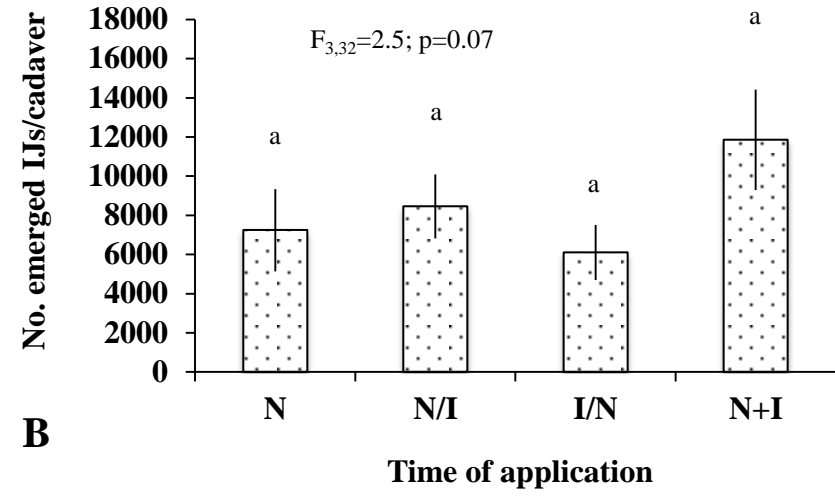
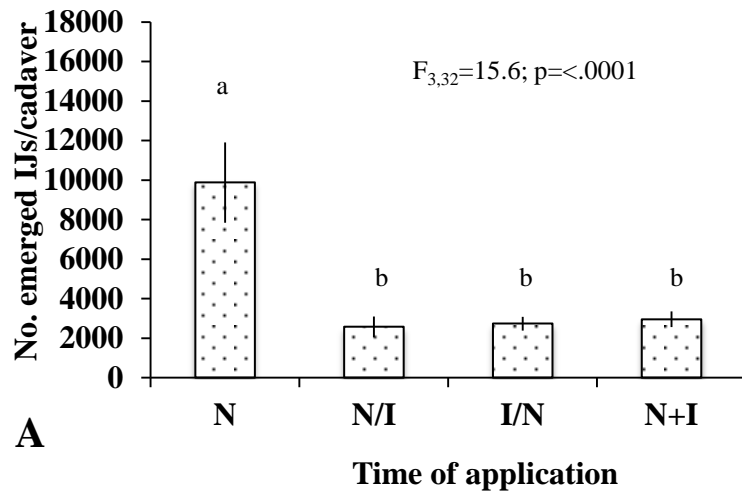


Fig. 3

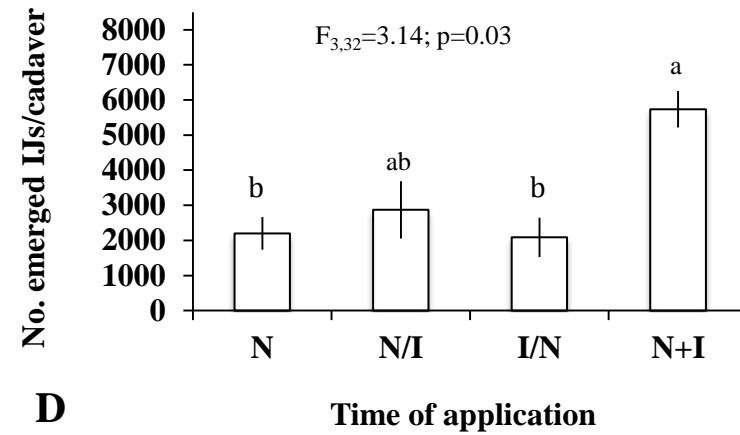
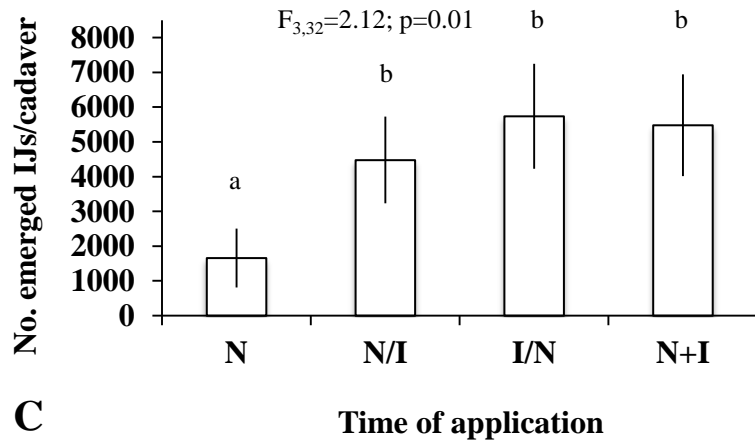
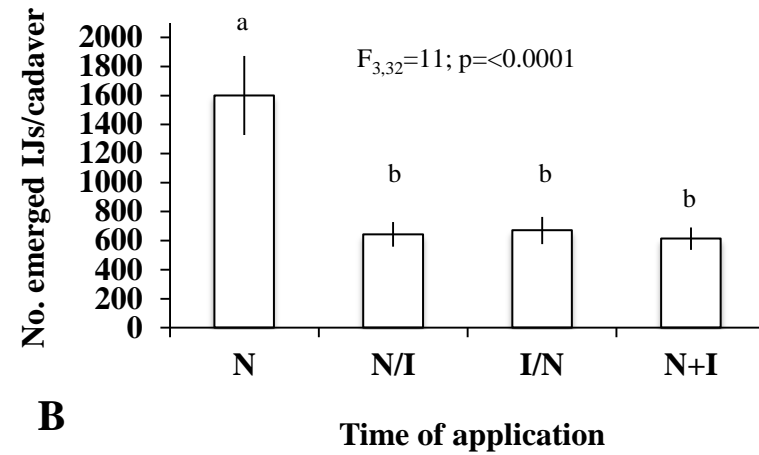
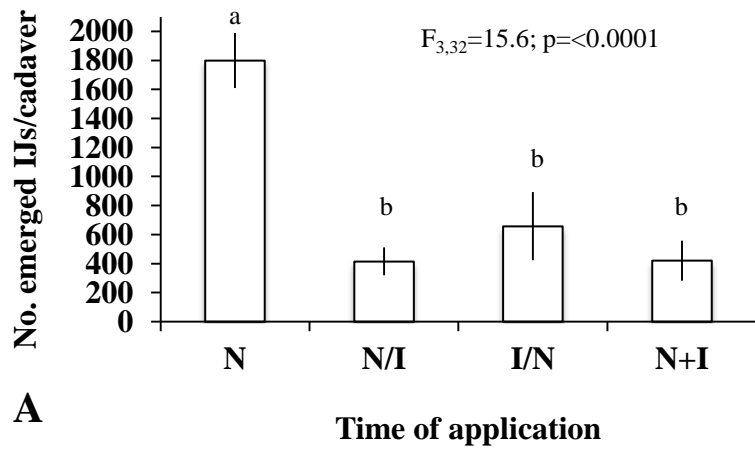


Fig.

**APPENDIX B**

INTERACTIONS BETWEEN THE ENTOMOPATHOGENIC NEMATODE  
*HETERORHABDITIS SONORENSIS* (NEMATODA: HETERORHABDITIDAE) AND THE  
SAPROBIC FUNGUS *FUSARIUM OXYSPORUM*

To be submitted to the Journal Biological Control

**Interactions between the entomopathogenic nematode *Heterorhabditis sonorensis*  
(Nematoda: Heterorhabditidae) and the saprobic fungus *Fusarium oxysporum***

Patricia D. Navarro and S. Patricia Stock

**Abstract**

The soil-borne fungus *F. oxysporum* f. sp. *asparagi* can potentially interact with the soil-inhabiting entomopathogenic nematode *Heterorhabditis sonorensis*. We assessed the impact of *F. oxysporum* f. sp. *asparagi* on *H. sonorensis* (Caborca strain) life cycle, considering the following parameters: a) ability to search for a host, b) virulence (measured as insect mortality), c) establishment (measured as the number of nematodes that penetrated the larva), and d) reproduction (measured as the number of infective juveniles or IJs that emerged from the insect cadaver). Specifically, presence of fungal mycelium in the soil was also used to evaluate the physical effect of this saprobic fungus on *H. sonorensis* IJs' host search process. Fungal spores were used to evaluate the effect of the fungus on nematode virulence, establishment and reproduction. Additionally, the effect of *F. oxysporum* fungal extracts on the nematodes' symbiotic bacteria, *Photorhabdus l. sonorensis*, was assessed *in vitro*. Our results indicate that fungal mycelia did not significantly affect the nematodes' ability to search and establish on an insect host. However, interaction of the *H. sonorensis* with the fungal spores in the soil reduced the ability of the nematodes to kill an insect host and also reduced their progeny production. Additionally, *in vitro* assays showed that growth of the symbiotic bacteria was inhibited at the highest concentration of fungal extract evaluated (10 mg/ml).

These results demonstrate that presence of *F. oxysporum* in the soil may affect *H. sonorensis* life cycle in an insect host.

**Keywords:** entomopathogenic nematode, *H. sonorensis*, life cycle, fungus, interactions

## 1. Introduction

Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) have an indirect role in protecting plants, by parasitizing a wide range of insect in natural and agricultural systems (Duncan and McCoy, 1996; Lacey and Unruh, 1998; Shapiro and McCoy, 2000). The third-stage juvenile (a.k.a. infective juvenile or IJ) is the only free-living stage that is responsible for vectoring pathogenic bacteria (Gamma-Proteobacteria, Enterobacteriaceae) from one insect to another (Gaugler and Kaya, 1990). These bacteria are responsible for killing the insect host, which usually happens in a short period of time (24-48h) by massive septicemia (Walsh and Webster, 2003). In the search for a suitable host, the IJs interact with other soil-inhabiting organisms such as predatory nematodes, arthropods, parasitoids and a wide range of soil bacteria and fungi (Kaya and Koppenhöfer, 1996; Sher et al. 2000; Stuart et al. 2006). The nature of these interactions is varied and ranges from beneficial to detrimental for nematode survival and overall fitness (Koppenhöfer et al. 1997; Ansari et al. 2005; Acevedo et al. 2007; El-Borai et al. 2011).

Many studies have focused on the interactions between entomopathogenic nematodes (hereafter referred as EPN) and soil fungi, in particular entomopathogenic fungi (EPF) and nematophagous fungi (NF) (Koppenhöfer et al. 1997; El-Borai, 2011) reporting varied results. For example, Ansari et al. (2005) observed positive interactions (additive and synergistic) between an EPF and two EPN species, *Heterorhabditis megidis* and *Steinernema glaseri*, in relation to nematode virulence in laboratory studies. In contrast, Acevedo et al. (2007) found that the EPF *Metarhizium anisopliae* (isolate LPP45) had a negative effect on persistence of *Heterorhabditis bacteriophora* in the field. Strong et al. (1996, 1999) found that nematode



trapping fungi suppressed populations of *Heterorhabditis marelatus*, in coastal sandy soils of California. In this study, these authors concluded that the fungi had an impact on the dynamics of plants, insect, and entomopathogenic nematode populations in this ecosystem. Stock et al. (2009) also observed that 70% of the insects infected with the nematode *Heterorhabditis sonorensis* (Caborca strain) in an asparagus field (Sonora, Mexico) were also infected by the saprobic fungus *Fusarium oxysporum* f. sp. *asparagi* (Ascomycota: Hypocreales). In this study, the authors speculated that the presence of *F. oxysporum* could interfere with the dynamics of the EPN populations in this crop. This fungus is considered a pathogen of asparagus crops in North America (Meyer et al. 1971; La Mondia and Elmer, 1989) but also has a saprobic role colonizes dead organic matter remained in the soil. Different strains and *formae speciales* of this fungus vary widely in their virulence and host range (Langton, 1969; Smith and Graca, 1988). Moreover, certain *formae speciales* have been reported to produce secondary metabolites such as beauvericin, which has insecticidal effects (Logrieco et al. 2002).

There is a dearth of information with respect to the nature of the interaction between saprobic fungi and EPN, either in the soil or within the insect host. It is not known the role that saprobic fungi have on the EPN life cycle, how this interaction may directly affect the survival and fitness of these nematodes, and how insect population dynamic may be indirectly affected in the soil. We hypothesize that the presence of *F. oxysporum* f. sp. *asparagi* may interferes with the life cycle of nematode-bacteria complex *H. sonorensis/Photorhabdus luminescens sonorensis*, not only during the host search process, as a free living nematode, but also within the insect host in the establishment and reproduction. To test this hypothesis, we

conducted a series of experiments under laboratory conditions. We evaluated the effect of this saprobic fungus on the following nematode parameters: a) ability to search for the host, b) virulence, c) establishment, d) progeny production. Additionally, the effect of *F. oxysporum* fungal extracts on the growth of the symbiotic bacteria was also assessed. Information obtained from this study not only contribute to better understand the direct effect of saprobic fungus on EPN fitness and the indirect effect on insect population dynamic in the soil, but also will contribute to improve tactics of insect pest management for control of important lepidopterans pest as *H. zea*.

## 2. Material and methods

### 2.1. Rearing of insects, nematode and fungus.

Fourth instar larvae of *Helicoverpa zea* (Lepidoptera: Noctuidae) was the insect host for these experiments. Rationale for this insect choice was based on preliminary studies, which showed that *Galleria mellonella* (L) (Lepidoptera: Pyralidae), a commonly used host for EPN bioassays, is hyper-susceptible to EPN when compared with *H. zea* (Navarro, unpublished data).

Larvae were reared and maintained in the laboratory at 28°C and 80% RH following the procedures described by Waldbauer et al. (1984). During experiments, larvae were fed individually with 5 g of artificial diet (Southland Products Inc.).

The EPN *Heterorhabditis sonorensis* (Caborca strain) was propagated *in vivo* in *G. mellonella* fifth instars, following procedures described by Stock and Goodrich-Blair (2012). Infective juveniles less than two weeks old (storage time after initial emergence from insect cadavers) were used for all assays.

The fungus *F. oxysporum* f. sp. *asparagi* was originally isolated from infected insect cadavers found in an asparagus fields in Caborca, Sonora state, Mexico. Its taxonomic identity was confirmed by morphological and molecular (ITS rDNA sequence analysis) methods. Sequence records, frozen tissues, and lyophilized stocks (accession #BMP 1421) are hosted in Dr. B. Pryor's laboratory at the School of Plant Sciences, University of Arizona. The fungus

was propagated on Potato Dextrose Agar (PDA). Spore concentration was adjusted according to procedures described by Goettel and Inglis (1997).

**2.2. Effect of *F. oxysporum* on IJ's ability to search for a host.** In this study we seek to determine the physical effect (i.e. presence in the soil) that fungal mycelia may have on the ability of IJs to locate a suitable host in the soil. To assess this goal, different ecological state of the fungus (mycelia ages 7, 10 and 15-day old) were evaluated and the following parameters were measured: a) nematode virulence and b) IJ establishment (i.e., the number of IJs that successfully penetrate the larva).

The fungal mycelium was grown on PDA, following methods developed by Weiss (1957). The sand column assay described by Thurston et al. (1994) was used for this assay (Fig. 1). Briefly, two PVC tube sections (8 cm height, 5 cm diam.) were attached one on top of the other. The top and bottom of the column were capped with 5 cm petri dishes. Six *H. zea* larvae were added to the bottom of the lower section, which was filled with 300g of autoclaved sand adjusted to 15% moisture (8 ml of distilled water/100cm<sup>3</sup> of sand). A PDA disc (5cm diam. x 0.5 cm thick) with fungal mycelium was added on the top of the bottom column. The top column was connected to the bottom column and filled with sand. The columns were attached to each other and wrapped with parafilm (Fig. 1). One milliliter of *H. sonorensis* suspension (100 IJs/ml) was inoculated onto the sand surface of the top column. The top section of the column was covered with a 5 cm Petri dish lid. The columns were incubated in the dark at 25°C for 5 days. Two controls were used for this assay; one consisted of a PDA disc without mycelium and the other without mycelium or PDA disc. The control

with the PDA disc was used to discard the possibility that IJs were stuck in it. Treatments were arranged in blocks, each treatments consisted of ten columns with six larvae/column (1 column=1 replicate). Blocks were repeated three times.

*2.2.1. Nematode virulence (insect mortality):* Insect mortality was evaluated by counting the number of dead larvae per column. Cadavers showing the typical red EPN-infection symptoms were considered as killed by *H. sonorensis*. Insect mortality was recorded from each block five days after initial inoculation.

*2.2.2. Nematode establishment (penetration efficiency):* Half of the insect cadavers obtained from the previous experiment were used to measure IJ establishment, which was assessed by counting the number of IJs that managed to successfully infected each larva. For this purpose, larvae were enzymatically digested following procedures described by Mauleón et al. (1993), and the number of IJs inside the larva was recorded from each block five days after initial inoculation.

**2.3. Effect of fungal spores on nematode fitness.** Before the initiation of these experiments, a preliminary assay was performed to determine the effect of *F. oxysporum* spores on survival of *H. sonorensis* (see Appendix I). Subsequently, the effect of fungal spores on nematode fitness was determined considering the following parameters: a) virulence b) IJ establishment in the insect host, and c) progeny production. Three application timings (treatments) were considered: 1) alternate I: EPN applied first, fungus applied 24h later, 2) alternate II: fungus

applied first, EPN applied 24h later), and 3) simultaneous: EPN and fungus applied at the same time.

Nematode and fungus inocula considered in this study were based on preliminary LC<sub>50</sub> experiments (see Appendix II). Inocula concentrations of 7 IJ/ml and 10<sup>6</sup> spores/ml for *H. sonorensis* and *F. oxysporum*, respectively, were considered suitable for assessing interactions between these two organisms in the insect host.

2.3.1. *Nematode virulence*. Assays were conducted in SOLO<sup>®</sup> plastic cups (1oz.) containing 4g of sterile sand. *H. zea* larvae were placed individually in each cup, where 1 ml of inoculum (fungal spores and/or IJ suspension) was applied directly to the sand according to each treatment (application timing). Treatments were arranged in blocks, each treatment consisted of ten cups (1 cup=1larva). Blocks were repeated three times. Cups were incubated at 25 ±1°C and 80% RH. To assess insect mortality, the number of dead larvae was recorded from each treatment. To evaluate the type of interactions between the two pathogens (synergistic, additive or antagonistic) we followed the methodology proposed by Nishimatsu and Jackson (1998).

2.3.2. *Nematode establishment*: Half of the insect cadavers obtained from each block of the previous experiment (2.3.1) were used to assess IJ establishment in the insect host. IJ establishment (or penetration efficiency) was assessed by enzymatic digestion (Mauleón et al.1993) of 5 days old cadavers (see section 2.2.2). Digested cadavers were opened and the

number of IJs found inside was determined from each block. Control consisted of nematode applied alone.

*2.3.3. Nematode progeny production:* The second half of the cadavers obtained from each block of the virulence assays (2.3.1) were used to assess progeny production, or the number of IJ that emerged from the insect cadaver. For this purpose, each insect cadaver was individually placed in a modified White traps following procedures described by Koppenhöfer and Kaya (1999). Emerging IJs were collected from each block during 10 days after the first day of nematodes emergence. Controls consisted of nematode applied alone.

**2.4. Effect of fungal crude extracts on symbiotic bacteria.** In this assay we evaluated the bactericidal effect of *F. oxysporum* crude extracts on the nematodes' symbiotic bacterium, *Photorhabdus luminescens sonorensis*. Crude extracts of *F. oxysporum* were obtained following procedures described by Ansari et al. (2004) and Bandani et al. (2000). Briefly, bacteria were grown overnight on Luria broth (LB). A two hours subculture was made, and 100 µl were plated onto LB pyruvate (1%) agar. Four fungal crude extract concentrations were evaluated: 0.01, 0.1, 1, 10 mg crude extract/ml of MetOH. Methanol and distilled water were considered as controls. Three diffusion papers discs (0.5 cm diameter; Sensi-Disc™) were placed on each plate where a single concentration of crude extract was inoculated on each disc at 0 hr. Treatments were arranged in blocks, each treatment consisted of five plates with three discs each (1 plate= 3 replicates) for a total of fifteen observations per treatment. Blocks were repeated three times. Plates were incubated in the dark at 25°C. The presence/absence of a zone of inhibition in the bacterial lawn was recorded for all treatments

at 12, 24 and 48h after inoculation. Presence of a zone of inhibition was considered as an indicator for an antagonistic effect of the fungal crude extracts against bacterial growth. Additionally, the size of the zone of inhibition was calculated based on the average of two diameters measured in two perpendicular directions (Shapiro-Ilan et al. 2009).

## 2.5. Statistical analysis.

2.5.1. *Effect of F. oxysporum on nematodes ability to search the host*: For this experiment, insect mortality and EPN establishment were analyzed using one-way ANOVA. Differences among averages were determined using Tukey's range test ( $p = 0.05$ ).

2.5.2. *Effect of fungal spores on EPN fitness*: Insect mortality was estimated using logistic regression for binary response, with insect mortality and application time as response and explanatory variable, respectively. Dummy-coded variables were used. The general linear model platform was considered for this purpose (JMP, 2010). Averages of treatments were compared with the applications of nematode alone using linear contrasts. To determine the nature of the interaction (additive, antagonistic, or synergistic) between *H. sonorensis* and *F. oxysporum*, the expected mortality and  $X^2$  were determined. The expected mortality of larvae was calculated based on the formula  $P_E = P_o + (1-P_o) (P_1) + (1-P_o) (1-P_1) (P_2)$ , where  $P_E$  is the expected mortality on combination of *H. sonorensis* and *Fusarium*,  $P_o$  is the mortality in the control,  $P_1$  is the mortality after treatment with *F. oxysporum* alone, and  $P_2$  is the mortality after treatment with the nematode alone. The determination of  $X^2$  was calculated through the formula  $X^2 = (L_o - L_E)^2 / L_E + (D_o - D_E)^2 / D_E$ , where  $L_o$  is the number of living larvae observed,  $L_E$  is the number of living larvae expected,  $D_o$  is the number of dead larvae



observed, and  $D_E$  is the number of dead larvae expected. The parameter  $X^2$  was used to test the hypothesis of independence ( $df=1$  and  $P=0.05$ ). When combinations of nematode and *Fusarium* resulted in a  $X^2 < 3.84$ , the interaction was considered additive. Synergism was determined when  $X^2 > 3.84$  and  $P_C > P_E$ ; antagonism was considered when  $X^2 > 3.84$  and  $P_C < P_E$ , where  $P_C$  is the observed mortality by *Fusarium* and nematode combination and  $P_E$  is the expected mortality of the combination.

EPN establishment and progeny production were analyzed using one-way ANOVA, with number of IJs that penetrated the larva, and number of IJs that emerged from the cadaver as response variables, respectively. Time of application was considered as the explanatory variable in both cases. Differences among averages were determined using Tukey's studentized range test ( $P = 0.05$ ).

*2.5.3. Effect of fungus crude extracts on symbiotic bacteria:* The size of the inhibition zone for different fungus extract concentrations at different times was analyzed using repeated measures (MANOVA). Following ANOVA analysis, a paired t-test was used to compare averages over time. All statistical analyses of this investigation were conducted using JMP<sup>®</sup> 8.0.2 (SAS Institute, 2008), except for those analysis where other software was mentioned.

### **3. Results**

**3.1. Effect of *F. oxysporum* on nematodes' ability to search for the host:** In these experiments we evaluated the effect of presence of *F. oxysporum* mycelia in the soil on the ability of *H. sonorensis* to find and successfully infect a host. For this, different mycelia ages

were tested. No significant effect of blocks was observed for all ANOVAs performed in this investigation. Results from this study indicated that for all mycelia ages tested, 50% of the insects exposed in each column were parasitized by *H. sonorensis*. The average number of nematodes that penetrated each larva was 1.5 IJs. These results were statistically similar to those observed in the controls. Therefore, presence of *F. oxysporum* mycelia (regardless of its age) in the soil did not significantly affect the ability of *H. sonorensis* to search and kill an insect host ( $F = 0.9$ ;  $df = 4, 45$ ;  $P = 0.42$ ) and penetrate it ( $F = 1.8$ ;  $df = 4, 45$ ;  $P = 0.14$ ).

### **3.2. Effect of fungal spores on EPN fitness:**

3.2.1. *Nematode virulence*: In this assay we assessed the effect that fungal spores have on *H. sonorensis* ability to kill the insect host and establish inside of it. Results from these experiments revealed that the tested application timings had a significant effect on insect mortality when compared with the mortality in the control (nematode alone) (logistic regression for binary response ( $X^2 = 31.8$ ,  $df = 5, 174$  p-value =  $<0.0001$ ). A linear contrast analysis of each application time versus the application of nematode alone is shown in Table 1. Data indicates that *F. oxysporum*'s spores (at any application timing) when applied with *H. sonorensis*, significantly reduced nematode virulence when compared with the application of *H. sonorensis* alone. Two out of three application times evaluated (simultaneous and one alternated: nematode first and fungus applied 24h later) resulted in an antagonistic interaction (Table 2). Therefore, the presence of *F. oxysporum* spores in the soil has a negatively effect on *H. sonorensis* virulence.

3.2.2. *Nematode establishment*: Presence of *F. oxysporum* spores did not have an impact on the number of IJs that penetrated a larva (data not shown) when compared with the control (EPN only) (one-way ANOVA;  $F = 0.2$ ;  $df = 3,40$ ;  $P = 0.8$ ). On average, 1.3 IJs managed to penetrate each larva in the presence of fungal spores.

3.2.3. *Nematode progeny production*: Progeny production was significantly reduced when *Fusarium* spores and *H. sonorensis* were applied simultaneously (one-way ANOVA;  $F = 4.3$ ;  $df = 3,40$ ;  $P = 0.008$ ) (Fig. 2). In this treatment, the total number of emerging IJs was about half the number of IJs observed in the treatment with *H. sonorensis* alone (14500 IJs/cadaver). For both alternated applications, progeny production was similar to that observed in the control.

**3.3. Effect of fungal crude extracts on symbiotic bacteria**: *In vitro* growth of *Photorhabdus luminescens* was only inhibited by the highest tested concentration of fungal crude extracts (10 mg/ml) (multivariate repeated measures analysis  $F = 26.5$ ;  $df = 5,85$ ;  $P < .0001$ ) (Fig. 3). The formation of a zone of inhibition (halo) was observed at 12h after inoculation when a crude extract concentration of 10 mg/ml was applied. The largest diameter of the inhibition zone was observed at 24 h post-inoculation (Pair sample t-test,  $t = 5.14$ ;  $df = 14$ ;  $p = < .0001$ ) with a diameter of 15 mm.

#### 4. Discussion

In this study we demonstrated that presence of mycelia and spores of *F. oxysporum* in the soil had different effects in the life cycle of the EPN *H. sonorensis*. Specifically, the presence of mycelia did not affect the nematode's ability to search a host; neither affected the nematodes' virulence nor the IJ establishment in the insect host. In contrast, the presence of fungal spores in the soil negatively affected nematode virulence and reproduction in the host.

We predicted that fungal mycelia would be a physical barrier that would interfere with the ability of the IJs to access an insect host. Specifically, we speculated that IJs would get trapped on the mycelium pads that were placed in the sand column assays. However, nematodes entanglement or entrapment was not observed. On average, a similar amount of IJs on the bottom portion of the column were found in both the treatment columns (containing the mycelium pads) and in the controls (without mycelium pads) (data not shown). This clearly indicates that the presence of mycelia did not interfere with the movement of IJs in the sand column to locate their host.

With respect to the effect of *F. oxysporum* spores on *H. sonorensis* fitness, our data indicated that either, the simultaneous or alternated (*Fusarium* first, EPN 24 h later) applications reduced the nematode's virulence. Certainly, when these application timings were evaluated, insect mortality decreased about 30% when compared with the application of nematodes alone. Nematodes virulence measures the overall pathogenic effect caused by the nematode-symbiotic bacteria complex in killing an insect, host (Dillman et al. 2012). Therefore, *F. oxysporum* may not only have an effect on the nematodes, but may also interfere

with their symbiotic bacteria. In relation to this, our data showed that *F. oxysporum* crude extracts at the highest concentration tested (10 mg/ml) inhibited *Photorhabdus luminescens* growth. These findings points out that some chemical interaction must exist between the fungus and the nematodes' symbiotic bacterium inside the insect host. In this respect, it is known that *F. oxysporum* produces a wide range of secondary metabolites, and many of them are known to antagonize several microorganisms in the soil (Teeter-Barsch and Roberts, 1983; Hallmann and Sikora, 1996; Majumdar et al. 2008). To our knowledge not previous studies have been conducted to determine the interaction between *F. oxysporum* and the EPN's symbiotic bacteria. However, Ansari et al. (2005) found that crude extracts of the entomopathogenic fungus *Metarhizium anisopliae* inhibited growth of *Photorhabdus luminescens* and *Xenorhabdus poinarii* (Vey et al. 2001).

We hypothesize that the spores of the soil-borne fungus *F. oxysporum* may passively gain access to an insect by attaching to the IJ's body. Once inside the insect host, the fungus may interfere with the nematodes' symbiotic bacteria antagonizing it or reducing its growth. A reduction of symbiotic bacteria population in the insect cadaver, may then impact nematode fitness, in particular reproduction in the insect host. In relation to this, our data showed a significant reduction in IJ progeny for all treatments when compared with the control (nematodes alone).

Based on these findings, we conclude that *F. oxysporum* f. sp. *asparagi* has an antagonistic effect on the *H. sonorensis*-*P. luminescens* complex, in particular in relation to those stages of the life cycle that take part in an insect host. Successful establishment,

development and reproduction of the EPN-bacteria complex in an insect host are key factors for their survival and dissemination in the soil. EPN IJs being the dispersive and resistant stage may encounter many antagonistic microbes in the soil, including saprobic fungi. The effects these antagonists may have on EPN, may not only directly affect their life cycle but may also indirectly interfere their role as natural regulators of insect populations in natural and agricultural ecosystems. Results obtained from this investigation set the start point for further investigations on EPN and soil-microbe interactions which should be taken into account when implementing and assessing success of EPN in pest management programs.

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Table 1. Linear contrasts for insect mortality caused by *Fusarium* fungal spores comparing different EPN application timings versus nematode alone.

Contrast <sup>b</sup>	Estimate	SE	$X^2$	$p$	Confidence Interval (95%)	
					Lower	Upper
L <sub>1</sub> <sup>a</sup> : N/F vs. N	-0.34	0.26	1.69	< .0001	-0.86	0.17
L <sub>2</sub> : F/N vs. N	-0.61	0.27	5.49	0.0191	-1.16	-0.09
L <sub>3</sub> : N+F vs. N	-0.77	0.28	8.35	0.0039	-1.35	-0.24

<sup>a</sup> L<sub>1</sub>: Contrast 1; L<sub>2</sub>: Contrast 2; L<sub>3</sub>: Contrast 3.

<sup>b</sup> N: Nematode alone; N/F: Nematode 1<sup>st</sup> / *Fusarium* 24 h later; F/N: *Fusarium* 1<sup>st</sup> / Nematode 24h later; N+F: Nematode and *Fusarium* applied at the same time.

Table 2. Effects of different combined applications of *H. sonorensis* and *F. oxysporum* f. sp asparagi on *H. zea* larval mortality.

Treatment <sup>a</sup>	Observed	Expected	X <sup>2</sup>	Interaction
N/F	34	68	17	Antagonistic
F/N	45	55	1.8	Additive
N+F	40	64	9	Antagonistic

<sup>a</sup> N/F: nematode applied first, *Fusarium* applied 24h later, F /N: *Fusarium* applied first, nematode applied 24h later, F+N: *Fusarium* and nematode applied simultaneously.



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- Figure 1 Sand column assay. A) Column set-up: (1) upper section covered by a 5 cm Petri dish lid (2) lower section containing *H. zea* larvae; B) mycelia disc, C) PVC columns filled with sand
- Figure 2 Fungal spores assay: Number of IJs emerged ( $\pm$  SE) for treatments considering different times of application
- Different letters above bars indicate statistical differences (based on Tukey test,  $P=0.05$ ). Data plotted are untransformed data. References: N = nematode applied alone; N/F = nematode applied first, *Fusarium* applied 24h later; F/N = *Fusarium* applied first, nematode applied 24 h later; N+F = nematode and *Fusarium* applied simultaneously.
- Figure 3 Effect of *Fusarium* crude extracts at 10 mg/ml on *Photorhabdus* symbiotic bacteria at 24h post-inoculation.

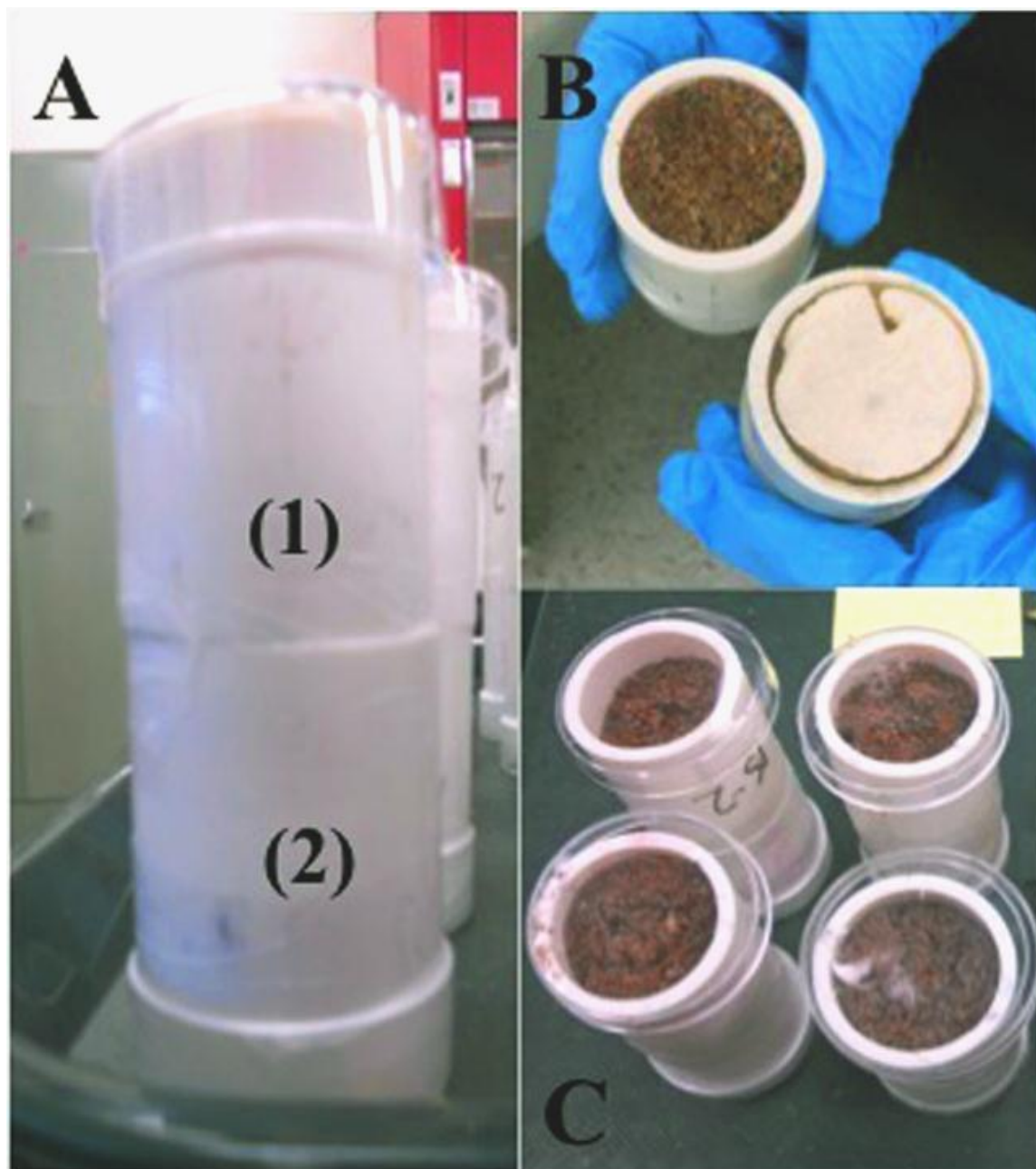


Fig. 1

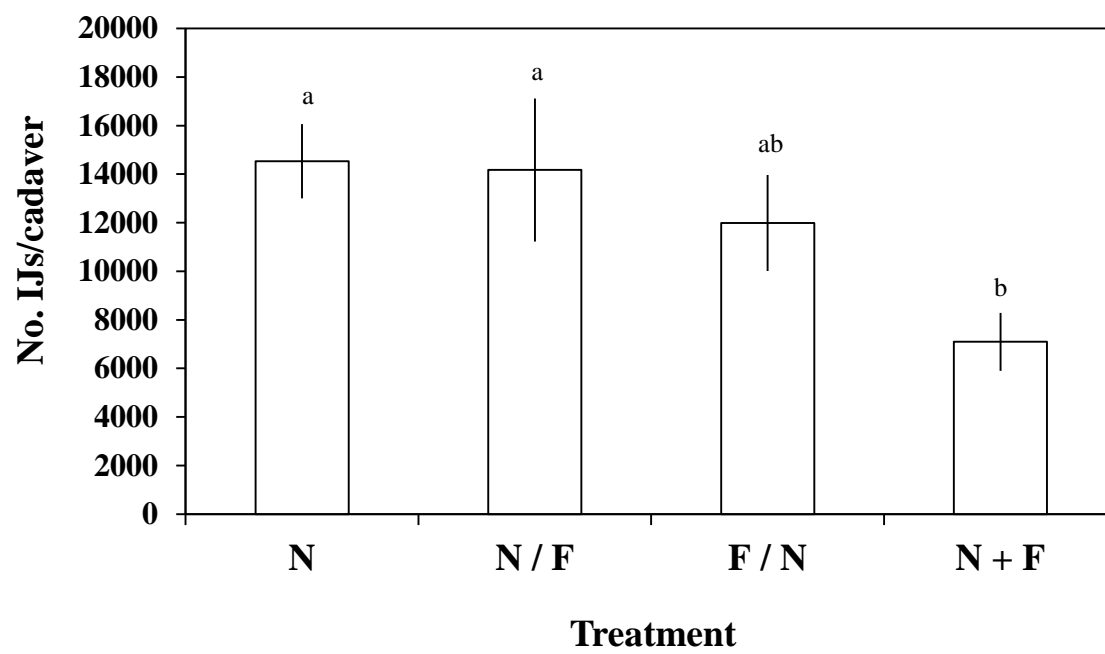


Fig. 2

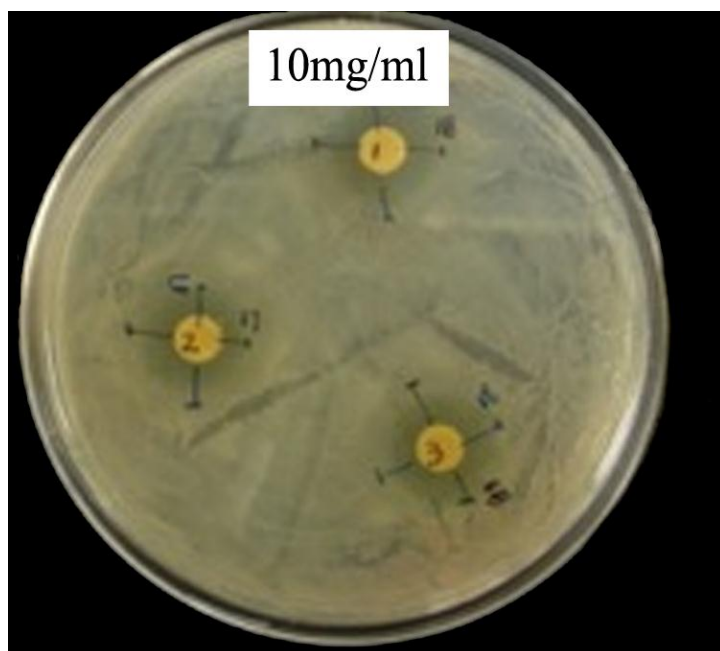
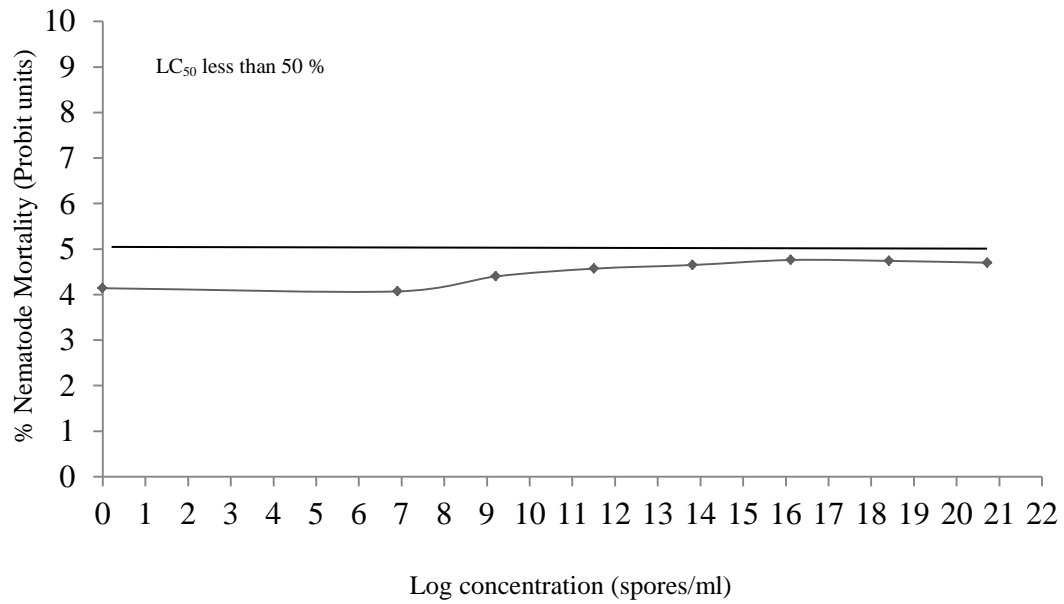


Fig. 3

**Appendix I**

Forty-eight hours log dose-response curve (on probit scale) of the effect of spores of *Fusarium oxysporum* f. sp. *asparagi* on survival of *Heterorhabditis sonorensis* (Caborca).



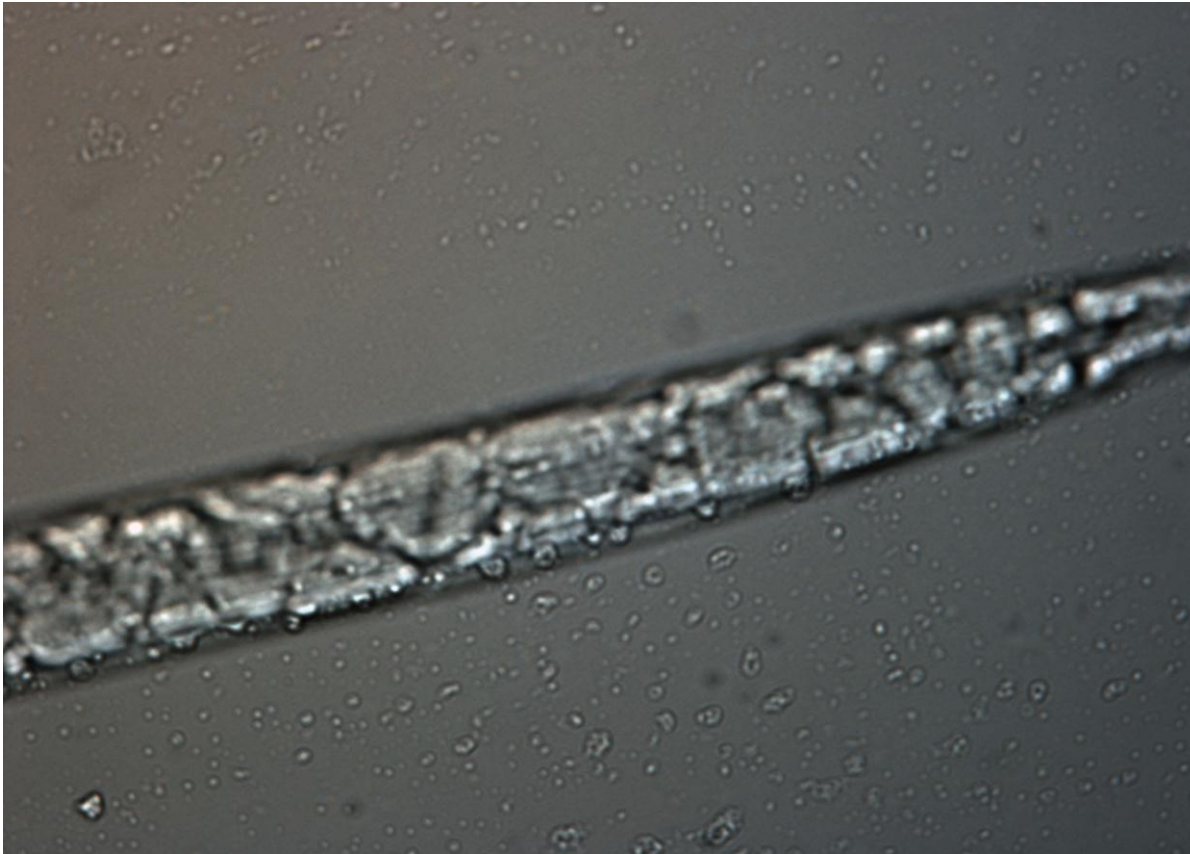
## Appendix II

Lethal Concentration (LC<sub>50</sub>) calculated for *Heterorhabditis sonorensis* (Caborca strain) and *Fusarium oxysporum* f. sp. asparagi toward *Helicoverpa zea*

Organism	Concentrations	<i>n</i>	Slope ±SE	LC <sub>50</sub>	95% CL	<i>X</i> <sup>2</sup>	P> <i>X</i> <sup>2</sup>
Tested							
<i>H. sonorensis</i>	0, 1, 5, 10, 50, 100 IJ/ml	30	0.73 ± 0.2	6.53	4.2 - 8.3	1.8	0.39
<i>F. oxysporum</i>	10 <sup>3</sup> , 10 <sup>4</sup> , 10 <sup>5</sup> , 10 <sup>6</sup> , 10 <sup>7</sup> 10 <sup>8</sup> , 10 <sup>9</sup>	30	0.96 ± 0.6	10 <sup>6</sup>	10 <sup>5</sup> - 10 <sup>7</sup>	2.1	0.76

**Appendix III**

Microscopy image of *Fusarium* spores attached to IJ's of *H. sonorensis* (Caborca) surface.



**APPENDIX C**

EFFECT OF TWO ENTOMOPATHOGENIC NEMATODES SPECIES ON THE  
CITRUS NEMATODE *TYLENCHULUS SEMIPENETRANS* (TYLENCHIDA:  
TYLENCHULIDAE)

**To be submitted to the Journal of Invertebrate Pathology**



## Effect of two entomopathogenic nematode species on the citrus nematode

### *Tylenchulus semipenetrans* (Tylenchida: Tylenchulidae)

Patricia D. Navarro and S. Patricia Stock

#### Abstract

The citrus nematode, *T. semipenetrans*, is one of the most debilitating citrus pests worldwide (Duncan and Cohn, 1990; Verdejo-Lucas and Kaplan, 2002). This nematode is an obligate parasite that reproduces only on roots of host plants. Nematode females become semi-endoparasites and sedentary by burrowing its anterior end deep inside the root cortex while the posterior end remains outside in the soil. They establish feeding sites within the root cortex composed of nurse cells that surround the female nematode head. The posterior portion of the adult female protrudes from the root and is surrounded by a gelatinous matrix in which eggs are deposited (Cohn, 1965).

At present very few alternatives are available for control of this nematode. One of the choices for substitution of chemical nematicides is the consideration of biological control agents such as entomopathogenic nematodes (hereafter referred as to EPN). Previous studies have demonstrated that presence of entomopathogenic nematodes (EPN) may negatively affect the life cycle of plant parasitic nematodes (PPN) by reducing their population of infective juveniles (J2) in the soil. In this study, two EPN species, *Steinernema riobrave* (TX strain) and *Heterorhabditis bacteriophora* (NC1) were tested to assess their effect on *T. semipenetrans*. The following parameters of this

PPN were evaluated: a) root penetration (measured as fresh weight and number of J2 in the soil) and b) egg production. Two EPN delivery methods (EPN-infected cadavers and IJ aqueous suspension) and two application timings of EPN (simultaneous and sequential to the PPN) were examined. Results of this investigation showed that both EPN species reduced the number of citrus nematode J2 in the soil. This observation was positively related with the reduction on citrus root fresh weight. Moreover, parasitic females of the citrus nematode were also negatively impacted by the presence of either EPN species by reducing the number of eggs when compared with the control. Results obtained from this investigations demonstrated that the presence of EPN in the soil directly benefit the health of the plant by reducing the damage produced by *T. semipenetrans* in the roots. These observations may have future consequences in the use of EPN for control of this plant parasite.

**Key words:** *citrus nematode*, interactions, *S. riobrave*, *H. bacteriophora*

The citrus nematode, *Tylenchulus semipenetrans* (Tylenchida: Tylenchulidae) is considered one of the most debilitating citrus pests worldwide (Duncan and Cohn, 1990; Verdejo-Lucas and Kaplan, 2002). This nematode is an obligate parasite that reproduces only on living roots of plants. The second stage juveniles (J2) penetrate the roots of the host plant and mature to females. Females are semi-endoparasites; they burrow their head deep inside the root cortex, while their posterior end remains outside the root. Gravid females lay eggs outside the root, which are surrounded by a gelatinous matrix (Cohn, 1965).

Although *T. semipenetrans* does not kill citrus trees, it significantly reduces tree vigor, growth and productivity (Duncan and Cohn, 1990). Environmentally friendly alternatives for control of this nematode are needed to reduce the impact of highly toxic chemical nematicides, which have detrimental effects to humans, wildlife and ground water (McClure and Schmitt, 1996; Anonymous, 2002). One alternative to chemical nematicides is biological control agents such as entomopathogenic nematodes (hereafter referred to as EPN). These nematodes have been shown to exert control of a wide range of insect pests and plant pathogens including plant parasitic nematodes (Vega and Kaya, 2012).

Many studies have shown the effect of EPN in suppressing plant parasitic nematodes in both, field and greenhouse settings (Ishibashi and Kondo 1986; Lewis et al. 2001; Jagdale et al. 2002; Pérez and Lewis, 2004; Shapiro-Ilan et al. 2006, Shahina and Tabassum, 2009). For example, several *Steinernema* spp. have been reported to suppress

root-knot nematodes, *Meloidogyne* spp. (Bird and Bird, 1986; Grewal et al. 1997). Similarly, *Heterorhabditis bacteriophora* was shown to suppress the stubby nematode, *Pratylenchus pratensis* in turf grass (Smitley et al. 1992). Suppression has been attributed to physical (i.e., crowding of EPN IJs near the roots of host plants) and chemical (i.e., by allelochemicals produced by the EPN's symbiotic bacteria) factors (Bird and Bird, 1986; Grewal et al. 1999; Hu et al. 1999).

In this study we assessed interactions between two EPN species, *Steinernema riobrave* (TX strain) and *Heterorhabditis bacteriophora* (NCI strain) and the citrus nematode, *T. semipenetrans*. Specifically, we evaluated the effect of these EPN species on root penetration by citrus nematode J2 and on the production of eggs by females of *T. semipenetrans*. Two different EPN delivery methods and two inoculation timings were examined. Assessment of these parameters will contribute to our understanding of the interactions between EPN and this plant-parasitic nematode. Results from this study will also assist in making predictions on the feasibility of EPN as biocontrol agents of the citrus nematode.

## 1. Material and Methods

**1.1. Nematode species and rearing methods.** The citrus nematode, *T. semipenetrans*, was isolated from an infested lemon orchard at the Yuma Agricultural Center (YAC), University of Arizona (Yuma, AZ). Second stage juveniles (J2), who are the free-living stage that invades the roots, were extracted from soil samples using the mist chamber technique described by Viglierichio and Schmitt (1983). Collected J2s were propagated and maintained in two-month old rough lemon seedlings (*Citrus wolkameriana*). Seedlings were grown in cone-tainers<sup>®</sup> (Stuewe & Sons, Inc., Oregon, USA) that were filled with 200 g of pasteurized sandy soil at  $25^{\circ}\text{C} \pm 2$  and 30% relative humidity.

The EPN *S. riobrave* (TX strain) and *H. bacteriophora* (NC1 strain) were propagated *in vivo* in fifth instar wax moth larvae, *Galleria mellonella* (Lepidoptera: Pyralidae), following procedures described by Stock and Goodrich-Blair (2012). Emerging infective juveniles (IJs) from cadavers were collected from modified White traps, (Kaya and Stock, 1997) during 10 days after the first day of nematodes emergence and stored at  $15^{\circ}\text{C}$  before being used for the assays. IJs were then allowed to acclimatize at room temperature for 1 h.

**1.2. Bioassays.** Two-months old rough lemon seedlings grown in cone-tainers<sup>®</sup> were used in all bioassays. Seedlings were individually inoculated with 12,000 *T. semipenetrans* J2s. Two EPN delivery treatments were examined: i) aqueous suspension (1,000 IJ/seedling, applied in 1.5 ml volume) and ii) EPN-infected *G. mellonella* cadavers. For each EPN species, larvae of *G. mellonella* (average weight:  $300 \pm 50$  mg)

were infected on filter paper in Petri dishes (90 mm) with an inoculum of 100 IJs per insect. Three days old cadavers were removed from the Petri dishes and used in experiments.

For each delivery treatment, two EPN application times were considered: i) Simultaneous: EPN and citrus nematode applied at the same time, and ii) Sequential: EPN applied 8 weeks after citrus nematode inoculation. This time frame allowed *T. semipenetrans* J2 to penetrate the root system and mature into adult females. Controls were rough lemon seedlings infested with *T. semipenetrans* only. There were a total of 12 seedlings per treatment (EPN delivery method and application time). Experiments were run separately for each EPN species. Experiments were arranged in a completely randomized design block and repeated three times. The following parameters were evaluated: i) J2 penetration into the root, and ii) egg production (number of eggs/g of root).

1.2.1. *Effect of EPN on citrus nematode J2 penetration into the root*: Penetration of *T. semipenetrans* J2 was measured in two ways: i) fresh root weight and ii) number of J2 remaining in the soil. Measurement of root fresh weight is a parameter commonly used to indirectly account for nematode penetration in the root. To account for J2 present in the soil, the soil of each container was removed and placed in Baermann funnels in a mist chamber for 24 h. The total number of J2 present in each sample was counted using serial dilutions (Hooper, 1970).

1.2.2. *Effect of EPN on citrus nematode egg production.* *T. semipenetrans* gelatinous egg masses present in each seedling's roots were extracted to account for the number of eggs/g of root. Egg extraction was performed following the H<sub>2</sub>O<sub>2</sub> method described by Tarjan (1972). Briefly, roots were gently rinsed with 20% bleach solution, placed on a rubber stopper and shaken for 90 sec. The runoff was poured through 500 and 80 mesh sieves. The content was rinsed 3 times with distilled water. Once eggs were collected they were poured into a 20 ml beaker and rinsed again before counting. The number of eggs found per root was recorded and the number of eggs/gram of root calculated.

**1.3. Statistical Analysis.** Data of fresh root weight, J2 penetration and egg production were log<sub>10</sub> transformed, analyzed using two-way ANOVA. Application method (aqueous suspension vs. EPN-infected cadaver) and timing (simultaneous vs. sequential) were considered as explanatory variables. All possible interactions between these variables were tested. Means were compared using Tukey test ( $\alpha=0.05$ ). All analyzes were conducted using JMP<sup>®</sup> 8.0.2 (SAS Institute, 2008).

## 2. Results

### 2.1. *Effect of EPN on citrus nematode (J2) penetration*

2.1.1. *Fresh root weight:* For *S. riobrave*, the delivery method had a significant effect on fresh root weight when compared with the control (*T. semipenetrans* only) (Table 1). Indeed, root weight was reduced significantly with either EPN delivery method. However, no difference was observed between the two delivery methods (Fig. 1A).

For *H. bacteriophora*, a significant interaction effect on root fresh weight was observed between the EPN delivery method and application timing evaluated (Table 1). A reduction in fresh root weight was observed in all treatments; however, differences were not statistically different when compared with the controls ( $p < 0.05$ ) (Fig. 1B)

2.1.2. *Number of J2 present in the soil*: For both EPN species, the interaction delivery method x application timing had a significant effect on the number of J2 in the soil (Table 1). The simultaneous application of *S. riobrave*, for either delivery method, caused a high reduction of the number of J2 in the soil (Fig. 2A). However, no significant reduction in the number of J2 was found for the sequential application (of either delivery method) when compared to those observed in the control. In contrast, a significant reduction of the number of J2 in the soil was found for *H. bacteriophora* for all delivery methods and application timings (Fig. 2B).

## 2.2. *Effect of EPN on citrus nematode egg production*

The interaction between delivery method and application timing was significant in both EPN species *S. riobrave* and *H. bacteriophora* (Table 1). For *S. riobrave*, a significant reduction in egg production was observed in all possible combinations between delivery method and application timing (Fig. 3A).

In all *H. bacteriophora* experiments, egg production by the citrus nematode females, in both the controls and the treatments, was significantly lower than that observed for *S. riobrave*. Additionally a significant effect of block was observed in the



number of eggs for the species *H. bacteriophora* ( $P < 0.05$ ). In spite of these observations, egg production of *H. bacteriophora* was significantly reduced when EPN were applied simultaneously for either delivery method. Although a reduction in the number of eggs was also observed in the sequential application, the difference was not statistically different compared to that observed in the control.

### 3. Discussion

In this study we evaluated two EPN spp., *S. riobrave* and *H. bacteriophora*, against the citrus nematode *T. semipenetrans*. These EPN have different host foraging strategies, *H. bacteriophora* is a cruiser and *S. riobrave* is an intermediate between cruiser and ambusher. Specifically, we assessed the effect of these EPN spp. on *T. semipenetrans* infection process and reproduction of rough lemon seedlings. Our results indicate that *H. bacteriophora* interfered with *T. semipenetrans* J2 more than *S. riobrave*. Indeed, a higher reduction of *T. semipenetrans* J2 was observed when *H. bacteriophora* was concomitantly present in the soil. We hypothesize that crowding of *H. bacteriophora* IJs around the lemon seedlings roots may have disrupted and interfered with *T. semipenetrans* J2. Therefore, less J2 were able to penetrate and establish in the roots. In this respect, a reduction of root weight was also observed in most of the treatments where both *H. bacteriophora* and *S. riobrave* were applied.

Our results agree with previous studies (Grewal et al. 1999; Perez and Lewis, 2004; Molina et al. 2008), who also reported a temporary suppression of root penetration by plant-parasitic nematodes. Moreover, Grewal et al. (1999) also noticed a reduction in

root penetration of the root-knot nematode, *M. incognita* when heat-killed EPN IJs were applied in the soil. These authors attributed these results to the effect of the EPN symbiotic bacteria (or their byproducts), which may have caused death or suppression of *M. incognita* J2. Although in our study, the effect of EPN symbiotic bacteria was not evaluated, we do not discard this possibility, in particular for the EPN-infected cadaver applications.

With respect to the effect of EPN on *T. semipenetrans*' reproduction, both of the tested species caused a reduction on the number of eggs produced by *T. semipenetrans*. Specifically, a three-fold reduction in the number of eggs was observed with *S. riobrave* (in all treatments) and two-fold reduction was observed with *H. bacteriophora* (simultaneous application of either delivery method) when compared with the controls. These observations agree with those reported by Pérez and Lewis (2002, 2004), who evaluated the same EPN spp. on the root-knot nematode *M. incognita*. These authors found that the simultaneous application of either *S. riobrave* or *H. bacteriophora* provided a greater reduction in the production of eggs by the rot-knot nematode. Although the mechanisms by which this reduction occurs are not known, it has also been suggested that the EPN symbiotic bacteria or their byproducts may be the ones responsible for these results (Grewal et al. 1999; Hu et al. 1999; Molina et al. 2008; Pérez and Lewis, et al. 2002).

Previous studies have considered that the application of EPN-infected cadaver is a more efficient method than the application of the IJ aqueous suspension. It has been reported that the infected-cadaver delivery method improves nematode's dispersal (Shapiro and Glazer, 1996), and enhances IJ survival in the soil (Pérez et al. 2003). In our

study, both delivery methods showed similar results in all parameters evaluated. However, differences were observed when comparing the two application timings, with the simultaneous application being more effective in reducing *T. semipenetrans* J2 in the soil, penetration and egg production than sequential applications.

Finally, our results suggest that both of the tested EPN spp., *S. riobrave* and *H. bacteriophora* can antagonize the citrus nematode at different stages of its life cycle, and therefore, could be considered an alternative for control strategies of this plant pathogen. These results represent interactions under controlled conditions and therefore further studies under natural settings are warranted to confirm these findings.

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Table 1. Two-way ANOVAs showing effects on fresh root weight, number of J2 in the soil, and number of eggs/g root of *Tylenchulus semipenetrans* treated with *Steinernema riobrave* or *Heterorhabditis bacteriophora*.

EPN species	Parameter	Effect	Df	F-value	P-value
<i>S. riobrave</i>	Fresh root weight	Delivery method	2	8.56	0.0003
		Time of application	1	0.73	0.39
		Delivery X Time	2	2.50	0.08
		Error	210	-	-
<i>H. bacteriophora</i>	Fresh root weight	Delivery method	2	6.28	0.002
		Time of application	1	2.74	0.09
		Delivery X Time	2	3.44	0.03
		Error	210	-	-
<i>S. riobrave</i>	No. J2 in the soil	Delivery method	2	5.98	0.003
		Time of application	1	94.55	<.0001
		Delivery X Time	2	1.86	0.1
		Error	210	-	-

**Table 1 cont'd**

EPN species	Parameter	Effect	Df	F-value	P-value
<i>H. bacteriophora</i>	No. J2 in the soil	Delivery	2	46.37	<. 0001
		Time	1	21.82	<.0001
		Delivery X Time	2	40.35	<.0001
		Error	210	-	-
<i>S. riobrave</i>	No. eggs/g root	Delivery	2	13.58	<.0001
		Time	1	3.94	0.04
		Delivery X Time	2	6.08	0.002
		Error	210	-	-
<i>H. bacteriophora</i>		Delivery	2	6.11	0.002
		Time	1	21.16	<.0001
		Delivery X Time	2	3.50	0.03
		Error	210	-	-

## Index of Figures

Figure 1 Effect of (A) *Steinernema riobrave* (B) and *Heterorhabditis bacteriophora* on fresh root weight ( $\pm$  SE)

Different letters above bars indicate statistical differences (based on Tukey test,  $P=0.05$ ). Data plotted are untransformed data. References: DW = distilled water, Cadaver = EPN-infected cadaver;

IJ suspension = IJ aqueous suspension; sequential = application of J2 first and EPN eight weeks later

Figure 2 Effect of (A) *Steinernema riobrave* (B) and *Heterorhabditis bacteriophora* on the number of *T. semipenetrans* J2 found in the soil/cone-tainer ( $\pm$  SE)

Different letters above bars indicate statistical differences (based on Tukey test,  $P=0.05$ ). Data plotted are untransformed data. References: DW = distilled water, Cadaver = EPN-infected cadaver;

IJ suspension = IJ aqueous suspension; sequential = application of J2 first and EPN eight weeks later

Figure 3 Effect of (A) *Steinernema riobrave* (B) *Heterorhabditis bacteriophora* on the number of *T. semipenetrans* eggs per gram of root ( $\pm$  SE)

Different letters above bars indicate statistical differences (based on Tukey test,  $P=0.05$ ). Data plotted are untransformed data. References: DW = distilled water, Cadaver = EPN-infected cadaver;

IJ suspension = IJ aqueous suspension; sequential = application of J2 first and EPN eight weeks later

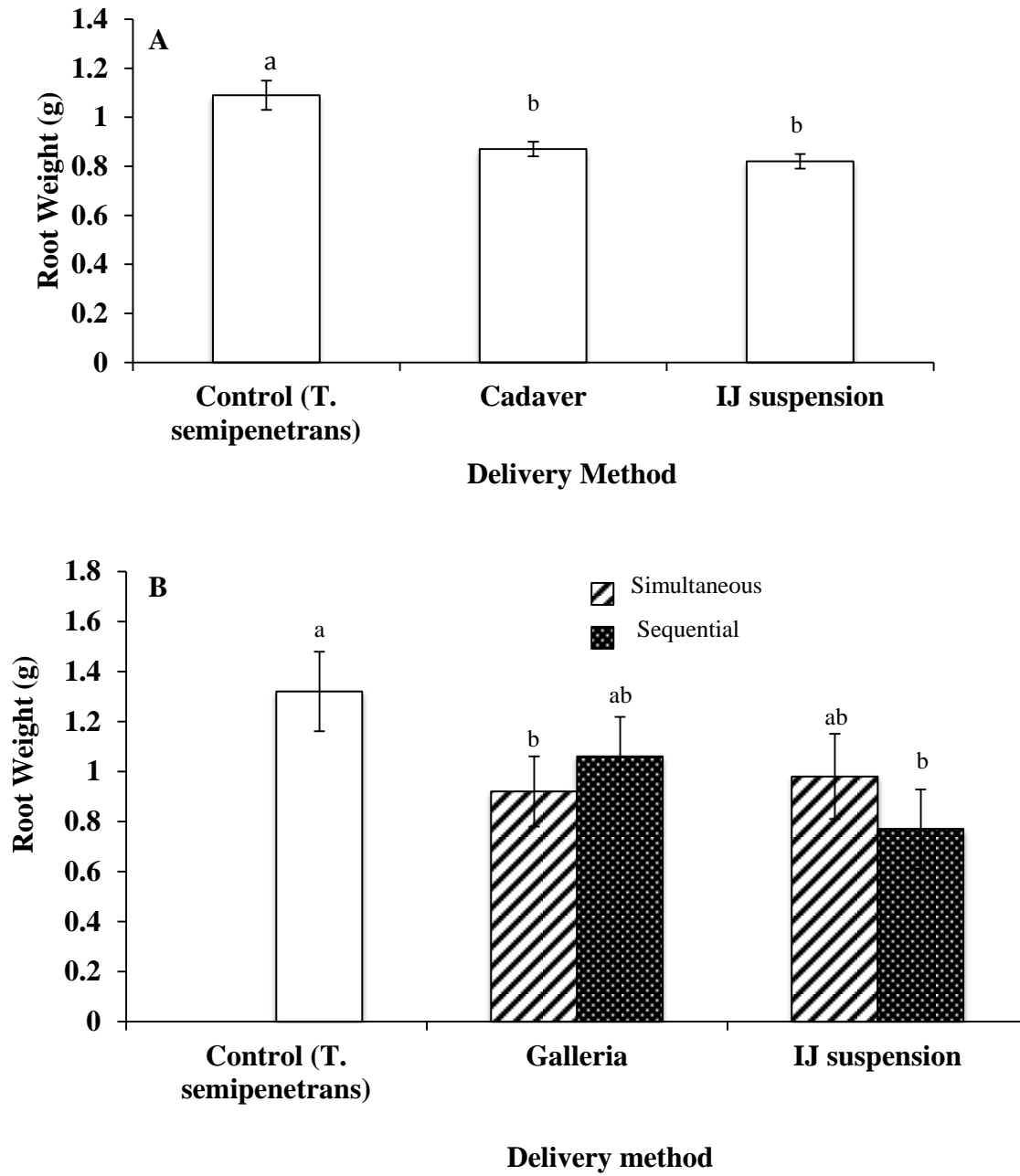


Fig. 1

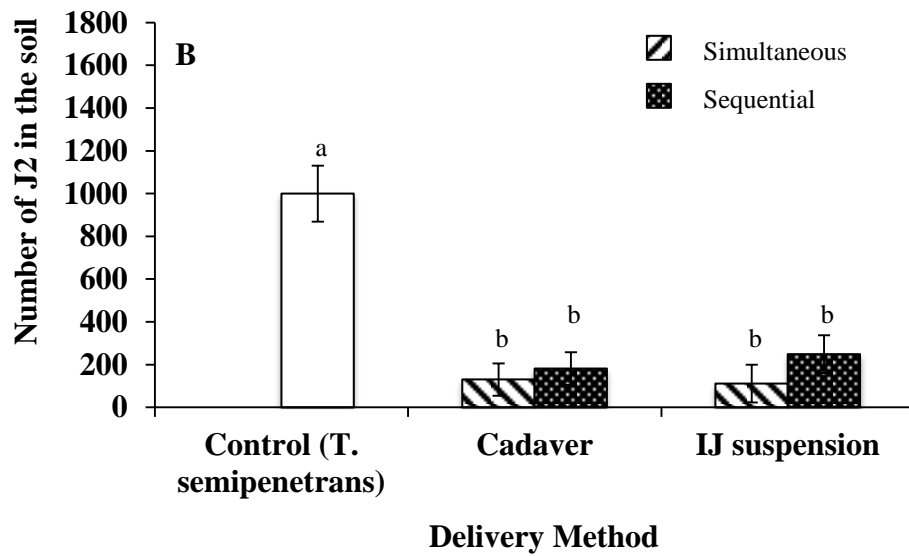
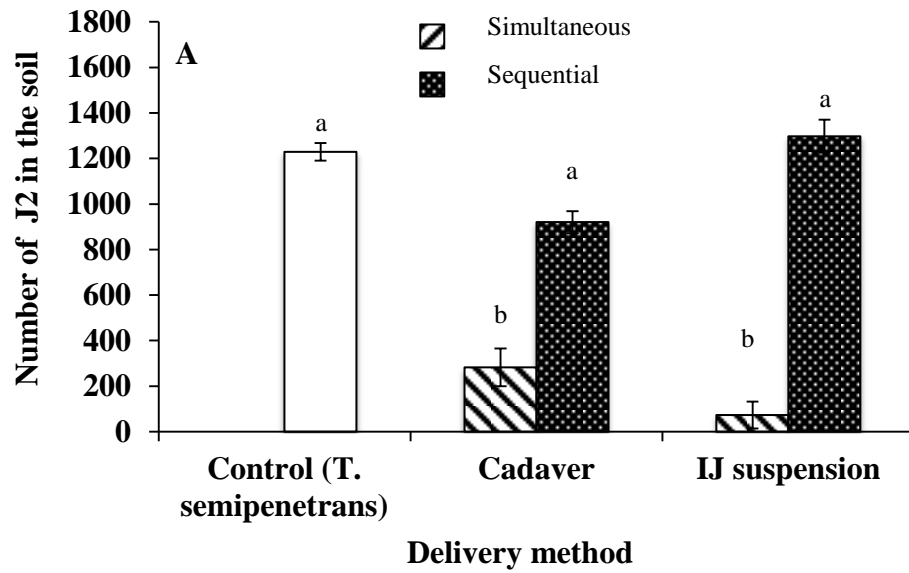


Fig. 2

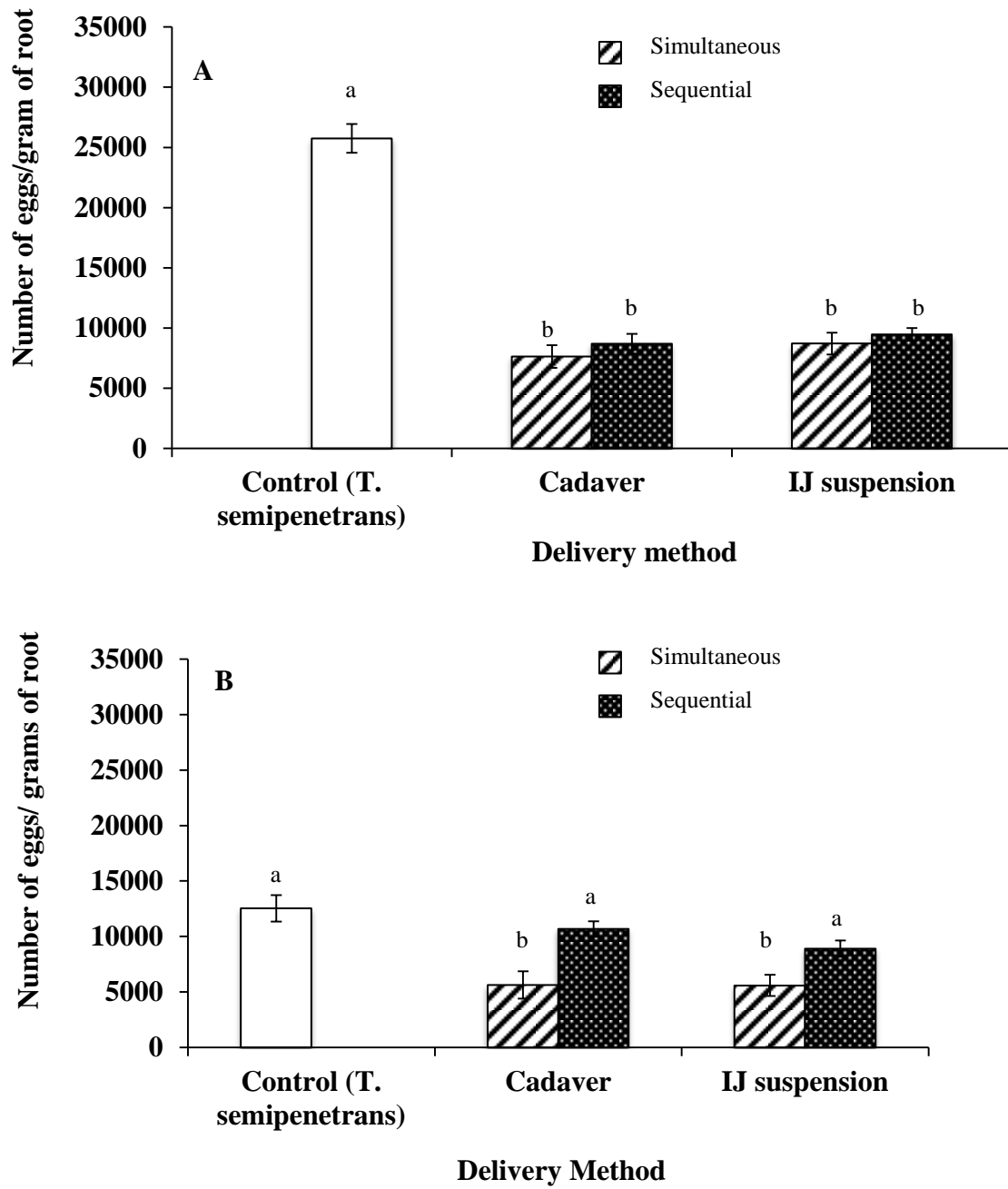


Fig. 3



