

कीट पतंगों के नियंत्रण में प्रयुक्त *स्टीनरनीमा थर्मोफिलम* एवम जैवबिषों के  
अनुप्रयोग प्रद्योगिकी का अध्ययन

**STUDIES ON APPLICATION TECHNOLOGY OF *Steinernema  
thermophilum* AND THE BIOTOXICANTS INVOLVED IN  
MANAGING INSECT PESTS.**

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**STUDIES ON APPLICATION TECHNOLOGY OF *Steinernema thermophilum* AND THE BIOTOXICANTS INVOLVED IN MANAGING INSECT PESTS.**

By  
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**A thesis submitted to the faculty of Post-Graduate School,  
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## **CERTIFICATE**

This is to certify that the thesis entitled “**Studies on application technology of *Steinernema thermophilum* and the biotoxicants involved in managing insect pests**” submitted to the faculty of post graduate school, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Nematology** by **Anes K.M.**, embodies the result of *bonafide* work carried out by him under my supervision and guidance. No part of the thesis has been submitted by him for any other degree or diploma.

I further certify that any help or information received during the work on this has been duly acknowledged.

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## 1. INTRODUCTION

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Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae are soil inhabiting insect pathogens that possess potential as biological control agents (Gaugler and Kaya, 1990; Gaugler, 2002). Most biocontrol agents require days or weeks to kill their hosts, yet nematodes, working with their symbiotic bacteria, kill insects within 24-48 h. The non-feeding infective juvenile seeks out insect hosts, and penetrates into the insect body, usually through natural body openings (mouth, anus, spiracles) or areas of thin cuticle. These nematodes carry symbiotic bacteria (*Xenorhabdus* for steinernematids and *Photorhabdus* for heterorhabditids), which are released in the insect haemocoel where the bacteria multiply and cause septicemia, thus resulting in the mortality of the insect host. The nematodes feed upon the bacteria, liquefies insect and mature into adults. Thus, entomopathogenic nematodes are a nematode-bacterium complex. They are safe for the plant health, human health, soil and the environment and hence are exempted from registration in many developed countries. In USA alone, there are more than hundred private companies, which are producing, formulating and marketing these nematodes. In India, work on steinernematids was first initiated by Rao and Manjunath (1966) who imported DD-136 strain of *S. carpocapsae* for its use against insect pests of rice and sugarcane. There after several researchers worked on exotic strains of *S. glaseri* (NC 34), *S. feltiae* and *Heterorhabditis bacteriophora* and reported their bioefficacy against local insect pests, but results were inconsistent under field conditions.

*S. thermophilum* described by Ganguly and Singh (2000) from India has been found to be heat tolerant and efficacious against several insect pests of national importance (Ganguly and Gavas, 2004a; Ganguly and Gavas, 2004b). Being indigenous, it is well adapted to local environmental conditions. The symbiotic bacterium associated with *S. thermophilum*, has been isolated, characterized and found to be a new species which has been described as *Xenorhabdus indica* (Somvanshi *et al.*, 2006).

Judicious integration of various crop production and crop protection options with minimum possible damage to the environment is inevitable for the sustainability of agricultural system. When we integrate different components, they interact each other with

varying effects, range from antagonism to synergism where in one component complement to the action or the effect of the other. The wise combination of components which complement each other without harming the ecosystem, ultimately leads to better performance of agricultural system. Therefore, the success of any pest management program depends on the judicious integration of various management options in an ecologically sound and economically viable manner. The precise knowledge on the interaction effects of entomopathogenic nematodes with other agricultural practices, which are commonly used for both crop production and crop protection, would facilitate its efficient integration in pest management programs. Entomopathogenic nematodes may be combined with other agricultural chemical and control agents for various purposes. Nematodes and other control agents which are compatible with each other can even be tank-mixed and applied together, to achieve better efficacy besides saving the time and labor. The types of interactions between nematodes and other agents may range from effects on viability and virulence of the bioagent, its recycling ability and susceptibility of the target pest.

On *S. thermophilum*, work was done to find its host range (*in vitro*), ecological requirements and foraging strategies (Ganguly and Gavas, 2004a; Ganguly and Gavas, 2004b). This species being indigenous heat tolerant entomopathogenic nematode with wide host range, there is a need to generate information on its application technology with respect to its compatibility with various chemical pesticides and adjuvant/surfactants to be used in spray mixture. The precise knowledge about its compatibility with other bioagents, organic amendments and its effect on the non-target beneficial organisms would facilitate its use in integrated pest management programs. Besides this, there is a possibility of developing broad-spectrum biopesticidal formulations by combining mutually compatible bioagents with synergistic effects.

In this nematode bacterium insect (ENBI) complex, the insect cadaver does not give any putrid odor, as it does not get infected by any other microorganism. In addition, the bacterial symbiont actually contributes more towards the insect mortality by the production of an array of bio-toxicants. Therefore, there is also a need to identify the bio-toxicants involved in this ENBI complex, especially from the symbiotic bacteria *X. indica* which is associated with *S. thermophilum* as it is a potential source for its exploration for novel bio-toxins. Due to various environmental and health problems caused by the excessive use of

chemical pesticides, the precise knowledge of the toxins, from *S. thermophilum* or its symbiotic bacterium would be of immense use in developing new generation bio-pesticides.

Keeping in view the above mentioned gaps in knowledge, the present investigation is proposed to fulfill the following objectives:

1. To know the compatibility of *Steinernema thermophilum* with different chemical pesticides;
2. To know the compatibility of *Steinernema thermophilum* with different adjuvant/surfactants, for use in spray mixtures;
3. To know the effect of different bioagents and organic amendments on the survival of *S. thermophilum*; and
4. To isolate and study the nature of biotoxins from the symbiotic bacterium associated with *S. thermophilum*.

## 2. BACKGROUND

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This chapter give the general information about the different research areas of the present study. The previous work and related works done are furnished below inorder to emphasis the importance of present investigation.

### 2.1 Compatibility of entomopathogenic nematodes with chemical pesticides

Entomopathogenic nematodes are often applied in conjunction with other biological and chemical pesticides, fertilizers, and soil amendments and it is usually more economical to `tank-mix' nematodes with one or more inputs for application. Infective juveniles (IJs) of entomopathogenic nematodes are tolerant to short exposures (2- 6 h) of most agrochemicals (Rovesti and Deseo, 1990) and thus can be tank-mixed for application. However, some pesticides can reduce nematode viability and virulence (Zimmerman and Cranshaw, 1990; Patel and Wright, 1996; Grewal *et al.*, 1998).

Several studies have been conducted on the effects of agrochemicals including acaricides, fungicides, herbicides, and insecticides on different species of entomopathogenic nematodes including *Steinernema carpocapsae* (Rao *et al.*, 1975; Hara and Kaya, 1982, 1983; Rovesti and Deseo, 1990; Zimmerman and Cranshaw, 1990; Kaya *et al.*, 1995); *S. feltiae* (Rovesti and Deseo, 1990; Zimmerman and Cranshaw, 1990; Grewal *et al.*, 1998; Krishnayya and Grewal, 2002) *S. bicornutum* (Hussaini *et al.*, 2001); *S. glaseri* (Koppenhöfer and Kaya, 1997), and *Heterorhabditis bacteriophora* (Poinar *et al.*, 1990; Rovesti and Deseo, 1991; Kaya *et al.*, 1995; Rovesti *et al.*, 1988; Koppenhöfer and Kaya, 1997). The results from these experiments indicate that nematode species differ in their susceptibility to pesticides. The infective juveniles can tolerate exposure to many agrochemicals and certain pesticides can even stimulate and interact synergistically with the nematodes, but certain chemicals can be detrimental. As nematode species can differ in sensitivity to chemical pesticides (Grewal, 2002), extrapolation of results from one pesticide-nematode species combination to another is difficult and not recommended.

The organophosphate oxamyl increased *S. carpocapsae* efficacy against *Agrotis segetum* synergistically, but only in fumigated soil, probably by enhancing the nematodes

nictation behavior (Ishibashi, 1993). The pyrethroid tefluthrin had a weak synergistic effect on efficacy of *S. carpocapsae* and *H. bacteriophora* against *Diabrotica virgifera* larvae, probably because sub lethal tefluthrin doses caused a paralytic and convulsive response in the insects that may have increased the nematode susceptibility (Nishimatsu and Jackson, 1998). The synergistic interaction of *Heterorhabditis* spp. and *S. glaseri* with the neonicotinoid imidacloprid in scarab larvae is well documented (Koppenhöfer and Kaya, 1998; Koppenhöfer *et al.*, 2003; Anuar and Daniel 2009). Imidacloprid reduces the grub's defensive behavior resulting in increased nematode attachment and penetration (Koppenhöfer *et al.*, 2000b). However, *S. kushidai* and *S. scarabaei*, two rather scarab-specific species, generally do not interact with imidacloprid. Imidacloprid does not compromise nematode recycling in grubs (Koppenhöfer *et al.*, 2003). Another neonicotinoid insecticide thiomethoxam (Meridian 25%) were found to be compatible with *H. megidis*, *S. feltiae*, *S. glaseri* and *S. carpocapsae* whereas non-compatible interactions were also observed for the same formulation with *H. bacteriophora* (Koppenhöfer *et al.*, 2003; Alumai and Grewal, 2004). Alumai and Grewal (2004) also established the compatibility of Spinosad (Conserve SC) with *H. bacteriophora* and *S. carpocapsae*.

There are few studies on the compatibility of herbicides with EPNs (Rovesti *et al.*, 1988; Rovesti and Deseo, 1990, 1991; Forschler and Gardner 1990; Gutierrez *et al.*, 2008). In most cases, these studies showed that the herbicides tested did not have a significant effect on nematode viability and infectivity. Fernando and Morton (2009) found that the two herbicides glyphosate and MCPA individually have a negligible effect on *S. feltiae* and a moderate effect on *H. bacteriophora*. However, the exposure of these EPNs to glyphosate + MCPA caused significantly higher mortality (26.33-57.33%) than glyphosate (0.67-15%) or MCPA (2.33-19%) alone. For all herbicide treatments, concentrations and exposure times, *S. feltiae* was more tolerant to the herbicides than *H. bacteriophora*. These results confirmed the possible synergistic effect of the pesticide combinations on the mortality in entomopathogenic nematodes.

Krishnayya and Grewal (2002) recorded 100 % mortality of *S. feltiae* when exposed to fungicides cinnamaldehyde (Cinnamate) and hydrogen dioxide/peroxyacetic acid mixture (ZeroTol) within 4 hours and 120 hours respectively. While another fungicide azoxystrobin (Abound) caused no nematode mortality.

All these studies clearly indicate that nematode species differ in their compatibility with a specific agrochemical. Even though infective juveniles of entomopathogenic nematodes can tolerate exposure to many agrochemicals and some of them even stimulate or interact synergistically with the nematodes, some can be detrimental. Even some chemicals used as inert ingredients or adjuvants in formulations can be toxic to nematodes (Krishnayya and Grewal, 2002). As nematode species can differ in sensitivity to different agrochemicals and even different formulations of the same chemical, compatibility of each formulation with the specific nematode species should be evaluated before any recommendation for their combined application.

## **2.2 Compatibility of entomopathogenic nematodes with adjuvant/surfactants**

Entomopathogenic nematodes (EPN) can be one alternative to ineffective chemical insecticides (Cherry *et al.*, 2004). Desiccation tolerant EPNs could be well adapted for foliage application against insect pests (Baur *et al.*, 1995). However, the efficacy on foliage of soil-borne nematodes is affected by abiotic factors like UV-radiation (Gaugler *et al.*, 1992; Nickle and Shapiro 1992), extreme temperature or drought (Glazer, 2002). Additionally, efficacy can be enhanced through improved formulations or addition of adjuvants (Wright *et al.*, 2005; Shapiro-Ilan *et al.*, 2006a). To prolong nematode survival on the leaf, several adjuvants have been evaluated (Glazer 1992; Baur *et al.*, 1997; Mason *et al.*, 1998). Schroer *et al.* (2005) screened several adjuvants for toxicity on nematodes, plants or insects and tested different combinations of surfactants and polymers for their potential to improve nematode efficacy. They recorded a two fold increase in DBM mortality at 80% relative humidity (RH) and fivefold increase at 60% RH in leaf disc assays when spraying nematodes in 0.3% xanthan or 0.3% surfactant (Rimulgan). The surfactant–polymer formulation (SPF) reduced the time to kill DBM by 50% ( $LT_{50}$ ) from >40 to <25 h.

Formulations of some pesticides may differ in toxicity to different nematodes due to the use of different surfactants. Compounds from the neem tree, *Azadirachta indica* A. Juss, are widely used as repellents, antifeedents, insect growth regulators and ovicides (Schmutterer, 1990). However, there are conflicting reports on the effects of neem products on entomopathogenic nematodes (Stark, 1996; Rovesti and Deseo, 1989). Neem as pure oil at the field recommended concentrations (5-10 mL L<sup>-1</sup>) had no effect on the viability and

virulence of *S. feltiae* up to 120 h incubation. However, the neem formulation Nimbecidine and neem oil when mixed with a bactericidal soap (commonly used as a surfactant with neem oil) caused 13 - 25% mortality of *S. feltiae* (Krishnayya and Grewal, 2002). This toxic effect was attributed to the soap that alone caused about 24% mortality. Neither neem oil Nimbecidine nor soap had any effect on nematode virulence. It demonstrated that the surfactants that are usually recommended as 'tank-mix' applications can be toxic to the nematodes and should be evaluated for compatibility prior to use.

### **2.3 Compatibility of entomopathogenic nematodes with organic manures/soil amendments.**

The addition of organic and inorganic soil amendments to improve soil fertility and plant growth is among the oldest of agricultural practices. Many of these amendments have nematicidal effects (Muller and Gooch, 1982; Rodriguez-Kabana, 1986). Georgis and Gaugler (1991) recognized the potential of soil amendments to limit the efficacy of entomopathogenic nematodes when they encouraged researchers to record the recent history of fertilizer use in field test plots. Inorganic fertilizers are likely to be compatible with nematodes in tank mixes and should not reduce the effectiveness of nematodes used for short-term control as biological insecticides, but may interfere with attempts to use nematodes as inoculative agents for long-term control. Organic manure used as fertilizer may encourage nematode establishment and recycling (Bednarek and Gaugler, 1997).

Shapiro *et al.* (1996) determined the effect of three fertilizers (fresh cow manure, composted manure, and urea) on the virulence of *S. carpocapsae* (Weiser) against the greater wax moth, *Galleria mellonella* (L.). They observed Urea and fresh manure decreased virulence of *S. carpocapsae* under laboratory conditions. However, in field experiments, only the fresh manure reduced nematode virulence, and the effects being more rapid in a soil with reduced organic matter than in a soil with higher levels of organic matter.

Bednarek and Gaugler (1997) investigated the impact of inorganic and organic fertilizers on the infectivity, reproduction, and population dynamics of entomopathogenic nematodes. Prolonged (10- to 20-day) laboratory exposure to high inorganic fertilizer concentrations inhibited nematode infectivity and reproduction, whereas short (1-day) exposure increased infectivity. *Heterorhabditis bacteriophora* was more sensitive to adverse

effects than *S. feltiae* and *S. anomalae*. In field studies, organic manure resulted in increased densities of a native population of *S. feltiae*, whereas NPK fertilizer suppressed nematode densities regardless of manure applications.

Different fertilizers can reduce the fitness of entomopathogenic nematodes (Bednarek and Gaugler, 1997; Shapiro *et al.*, 1996). These studies did not demonstrate if the reduced nematode fitness affects ability to prevent plant injury under field conditions. Ultimately, it is a reduction of plant injury that defines the success of a pest management strategy.

Pai and Hou (2005) observed a lower persistence of *S. abbasi* in the soil containing non-decomposed organic fertilizers, whereas 32 days of nematode persistence in soil containing 0.5 - 2.0% decomposed organic fertilizer made of cattle, pig, or chicken feces. However, in soil containing chemical fertilizers, nematode persistence was found to be severely hampered.

Mustard (*Brassica* and *Sinapis* spp.) green manures tilled into the soil act as biofumigants that are toxic to plant-parasitic nematodes, providing an alternative to synthetic soil fumigants. Recardo *et al.* (2009) found a trend toward lower rates of EPN infection in fields, where mustard green manures were applied, compared to the control. Mustard-exposure trials were conducted both in laboratory arenas and then in larger microcosms in the greenhouse. In all the trials they used *G. mellonella* larvae as hosts and included multiple EPN species in the genera *Steinernema* (*Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. riobrave*) and *Heterorhabditis* (*Heterorhabditis bacteriophora*, *H. marelatus*, and *H. megidis*). Among these 7 EPN species, only *S. feltiae* was found not affected by exposure to mustard extract or mustard leaf mulch.

#### **2.4 Interaction of entomopathogenic nematodes with other bioagents**

Pest control can be greatly improved by using combinations of bio-control agents; their increased efficacy may be due to synergistic or additive effects. In studies targeting insect pests, synergistic interactions have been observed from certain combinations of entomopathogenic nematodes with other pathogens. For example, synergistic effect on virulence to *Hoplia philanthus* (Coleoptera: Scarabaeidae) was observed in combinations of *H. megidis* or *S. glaseri* with *Metarhizium anisopliae* (Ansari *et al.*, 2004b). Combining EPNs with the bacteria *Paenibacillus popilliae*, formerly known as *Bacillus popilliae* Dutky,

or *Bacillus thuringiensis* Berliner Buibui strain gave significantly higher control of white grubs than either agent alone (Thurston *et al.*, 1994; Koppenhöfer & Kaya, 1997). Nematode-fungus combinations generally result in additive effects on target mortality, but in some cases, mortality caused by the individual agents was too high to allow for significant improvement (Koppenhöfer and Grewal 2005; Ansari *et al.*, 2006). Ansari *et al.* (2006) reported additive or synergistic effects of the combination of *M. anisopliae* with *S. kraussei* against overwintering larvae of the black vine weevil, *Otiorhynchus sulcatus*, in strawberry grow bags. In some instances, the interactions between EPNs and fungi are neither additive nor synergistic, but still result in higher pest mortality than that caused by either agent when used alone (Choo *et al.*, 2002; Acevedo *et al.*, 2007).

A combination of *H. bacteriophora* and *Beauveria bassiana* against *Spodoptera exigua* (Barbercheck and Kaya, 1991) and *S. carpocapsae* with *Beauveria brongniartii* against the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis* (Choo *et al.*, 2002) resulted in higher mortality than that caused by either the nematode or fungus alone. In addition, Glare (1994) observed that the combination of the fungus *M. anisopliae* Sorokin with the bacterium *Serratia entomophila* caused a synergistic effect on the mortality of early instars of grubs but not the later ones of *Costelytra zealandica* (White). Koppenhöfer *et al.* (1999) also demonstrated that combining *H. bacteriophora* and *S. glaseri* with *Bacillus thuringiensis* subspecies *japonensis* Buibui strain caused additive and synergistic effects against masked chafers, *Cyclocephala hirta* and *C. pasadenae*, in the greenhouse and under field conditions. Ansari *et al.* (2004) reported stronger synergistic effects between *M. anisopliae* and *H. megidis* or *S. glaseri* against third-instar *Hoplia philanthus* when the fungus was applied for at least 3 or 4 weeks before the addition of nematodes.

The underlying mechanisms of these synergistic interactions are unclear, but it is postulated that one agent may stress or alter the behavior (e.g., feeding or movement) of the target insect, making it more susceptible to the other agent. For example, *M. anisopliae* infected insects may be less mobile, which gives EPNs more time to penetrate the host (Ansari *et al.*, 2004). In contrast, insects exposed to *B. thuringiensis* often feed less, causing debilitation, which increases their vulnerability to EPNs (Koppenhöfer and Kaya, 1997).

However, interactions between EPN and fungus can also lead to antagonistic effects (Shapiro-Ilan *et al.*, 2004; Ansari *et al.*, 2005). Shapiro-Ilan *et al.* (2004) investigated the interaction effect of *H. indica* or *S. carpocapsae* with the fungus *B. bassiana*, *M. anisopliae*, or *Paecilomyces fumosoroseus*, or the bacterium *Serratia marcescens* on pecan weevil, *Curculio caryae*. They observed antagonism in all pathogen combinations, except *H. indica* combined with *M. anisopliae*, for which additive effects were observed.

Whether the interaction between EPNs and insect pathogenic fungi is additive, antagonistic, or synergistic depends on the EPN species, insect host, application rate, and time of application (Koppenhöfer and Kaya, 1997; Ansari *et al.*, 2004, 2006).

The antimicrobial nature of metabolites produced by the growing bacteria is well documented (Webster *et al.*, 2002). Several of the metabolites are known to have antifungal (Barbercheck and Kaya, 1990; Chen *et al.*, 1994), nematocidal (Han and Ehlers, 1999; Hu *et al.*, 1999) or insecticidal effects (Bowen *et al.*, 1998). However, information about the antimicrobial effects of *Xenorhabdus* and *Photorhabdus* on entomopathogenic fungi is very few. Diverse toxic metabolites have been described in several fungal biological control agents including species of *Beauveria*, *Metarhizium* and *Paecilomyces* (Vey *et al.*, 2001). Some of these metabolites have been found to display antibiotic, fungicidal or insecticidal properties against insect pests and diseases (Kershaw *et al.*, 1999; Vey *et al.*, 2001).

Ansari *et al.* (2005) investigated the mutual effects between the symbiotic bacteria of entomopathogenic nematodes, *Photorhabdus luminescens* and *Xenorhabdus poinarii*, and different entomopathogenic fungi. The results revealed that *P. luminescens* is antagonistic to *Metarhizium anisopliae*, *Beauveria bassiana*, *B. brongniartii* and *Paecilomyces fumosoroseus* by inhibiting their growth and conidial production, whereas the fungal growth was not inhibited by *X. poinarii*. Crude extract from *M. anisopliae* were found to be antibacterial to *P. luminescens* and *X. poinarii* at 1000 µg/ml and inhibited their growth, but had no effect at 100 or 10 µg/ml. They also studied the influence of the crude extract of *M. anisopliae* on the dispersal of infective juveniles (IJs) of *Heterorhabditis megidis* and *Steinernema glaseri* and observed no toxic effects even at highest concentration (1000 µg/ml).

All these studies indicate that, if one wants to combine entomopathogenic nematodes with fungi or bacteria in order to achieve a better control of the insect pest, in an economically viable manner one should be aware of all possible interactions between biocontrol agents.

## **2.5 Interspecific interactions of entomopathogenic nematodes.**

The occurrence of more than one species of entomopathogenic nematodes within the same location is not unusual. Numerous field surveys have shown that steinernematid species often occur sympatrically (Amarasinghe *et al.*, 1994; Stuart and Gaugler, 1994; Campbell *et al.*, 1995; Sturhan, 1999; Mráček *et al.*, 2005a; Puza and Mráček, 2005). Entomopathogenic nematodes do not avoid entering hosts already infected with heterospecifics, both naturally (Bovien, 1937), and experimentally (Lewis *et al.*, 2006). Thus, there is a potential for interspecific interactions between sympatric nematode species. In the field, the interactions are thought to be mediated by differing foraging strategies and host preferences (Alatorre-Rosas and Kaya, 1990; Koppenhöfer and Kaya, 1996). Vertical distribution and patchiness probably contribute to the coexistence (Stuart *et al.*, 2006). While there are advantages of using two or more *Xenorhabdus* strains / species simultaneously, intra-specific / inter-generic competition must be considered. Different EPN/EPB complexes have been shown to invade the same insect. Interspecies competition involves bacteriocins and xenocins (Boemare *et al.*, 1992), phage-derived bacteriocins (Thaler *et al.*, 1995, 1997) and colicin E3 type killer proteins (Singh and Banerjee, 2008). Sicard *et al.* (2006), monitored experimental inter-specific competition between two EPN species, *S. carpocapsae* and *S. scapterisci* and their respective EPB symbionts, *X. nematophila* and *X. innexi*, within an experimental insect-host (*Galleria mellonella*). The authors suggested *Xenorhabdus* not only provides *Steinernema* with a food source, but also gives them new abilities to deal with biotic parameters such as competitors. However, the simultaneous use of more than one *Xenorhabdus* is limited by their tolerance to each other.

So far, a number of laboratory studies have addressed inter-specific interactions between steinernematids. But at present not all aspects of the interspecific interactions in entomopathogenic nematodes are understood. In the laboratory, heterorhabditids and steinernematids cannot coexist in the same host, because neither can develop on the

symbiotic bacteria of the other genus (Koppenhöfer *et al.*, 1995). So far, various effects of the mixed infection on the reproduction of steinernematids have been reported. According to San-Blas *et al.* (2008), some EPN species prefer dead hosts to living ones as in dead hosts the nematodes do not have to overcome the insect's immune system or defense strategies.

Kondo (1989) observed *S. carpocapsae* to be a stronger competitor than *S. glaseri* in mixed infections of *S. litura*. He recorded an 80% reduction in the progeny of *S. glaseri* that emerged from co-infected *S. litura* while *S. carpocapsae* was not affected. Koppenhöfer *et al.* (1995) suggested that *S. litura* is more susceptible to *S. carpocapsae* than to *S. glaseri* and found an opposite situation in *G. mellonella* which authors considered to be equally susceptible to both nematode species. The superiority of *S. glaseri* was ascribed by the authors to its faster development and less specific association with symbiotic bacterium. On the other hand, Neumann and Shields (2006) did not observe any evidence of multiple infection of *G. mellonella* while studying the interactions of *S. carpocapsae*, *S. feltiae* and *H. bacteriophora* in sand columns even though nematodes infected hosts in close proximity. When *S. carpocapsae* and *H. bacteriophora* simultaneously competed for the same insect species in Petri dishes, *S. carpocapsae* infected significantly more *Galleria* hosts than *H. bacteriophora* (Alatorre-Rosas and Kaya, 1991). Lewis *et al.* (2006) concluded that species of *Steinernema* can develop together in one insect host, though one species may suffer the effects of the competition more than the other.

Mráček *et al.* (2005b) studied the interaction effect of Canadian strains of two sympatric entomopathogenic nematodes *S. affine* and *S. kraussei*. When *G. mellonella* was infected with a 1:1 mixture of both species at rates of 50 and 100 IJs per-host, and both species developed into 1st generation adults, whereas second generation adults and IJs were purely *S. affine*. Puza and Mráček (2009) found a similar situation while evaluating the interactions of the same two species in a series of laboratory experiments. The invasion rate of *S. affine* was not affected by the mixed infection whereas the invasion of the latter species was strongly reduced. *S. affine* out-competed *S. kraussei* in all treatments and the progeny production of the latter species occurred only sporadically. Generally, in the presence of the latter species, *S. affine* was able to infect and multiply in a higher number of hosts in comparison to single species infection, especially at a low inoculation rate. Interestingly *S. affine* invaded, multiplied and dominated also in hosts already infected and even killed by *S.*

*kraussei* producing a normal amount of progeny. But they observed better performance of both the species in single infections of *G. mellonella*, and *S. kraussei* was even more effective than *S. affine*, being able to infect more hosts and produce more progeny. In addition, no difference in the duration of development between studied species was observed and both species share the same symbiotic bacterium *Xenorhabdus bovienii* (Saux *et al.*, 1998; Boemare, 2002). But the cause of the dominance of *S. affine* remains unclear. Generally the results suggest that the interactions between steinernematid species can be more complex, including a positive effect of one species on another.

Temperature has profound effects on all aspects of the biology of entomopathogenic nematodes, affecting development, respiration, survival, dispersal, host finding and infectivity (Griffin, 1993). Hilal and Alper (2005) observed negative interaction between *H. bacteriophora* and *S. feltiae* at 3 different temperatures (12, 18 and 25°C) on the last instar larvae of *Tenebrio molitor*. When *S. feltiae* and *H. bacteriophora* were applied separately, both species had approximately the same percentage of mortality, except at 12°C. In the combined application, competition ability of *H. bacteriophora* increased to 64 % at 25°C (efficiency 3, 8 and 12.5 %), whereas competitive ability of *S. feltiae* decreased by 6 % (efficiency 72, 85 and 80 %), but *S. feltiae* dominated in all temperatures. Not only did both species affect each other negatively because of the competition, but also total insect mortality was negatively affected when the 2 species were used together. Notably 2 species were not found together in the same insect larvae.

Differences in nematode infection among the different species are due to either their mutualistic bacteria or their response to environmental conditions such as temperature; or both of them. The speed of releasing of bacteria into the haemolymph from the dominating species may be higher than that of suppressed one and the nematode also may grow and reproduce faster than suppressed one in the insect haemolymph, thus giving the competitive advantage to the former (Alatorre-Rosas and Kaya, 1991). Second approach concerns toxic metabolites of either its symbiotic bacteria or nematode which may slow down the reproduction or development of their competitor. Toxins or bio-products from the nematode or bacteria may prevent the development of another co-inhabitant (interference competition) (Alatorre-Rosas and Kaya, 1991). Besides these, metabolic rate, processing of host tissues by

symbiotic bacteria and the physiological parameters required for growth differ between nematode species (Selvan *et al.*, 1993). Therefore, the steinernematids and heterorhabditids can complete their development in the same host but cannot survive (Alatorre and Kaya, 1990). The temperature also affects the development of the symbiotic bacteria of these nematodes. Temperature is one of the most important factors limiting the success of entomopathogenic nematodes.

## **2.6 Interaction of entomopathogenic nematodes with non-target organisms**

Under Laboratory Conditions entomopathogenic nematodes have been proved to be effective in controlling some foliar pests (Trdan *et al.*, 2009), but they do have some negative properties, e.g., the wide spectrum of their efficacy includes a negative influence on beneficial organisms (Hazir *et al.*, 2003). Variable responses have been reported relating to the susceptibility of beneficial insects and other arthropods to EPNs. Up to now, studies on the nontarget effects of entomopathogenic nematodes have been performed on various species of nontarget organisms, and a large range from complete harmlessness to pronounced harmful effect was established (Bathon 1996, Farag 2002, Powell and Webster 2004). The results of some field trials show a moderate influence of entomopathogenic nematodes on nontarget arthropods or even the absence of such an effect (Georgis *et al.*, 1991). Bathon (1996) reported that mortality can be observed among the nontarget organisms, but the influence of these agents should be temporary and local and so only a part of the population is under attack. Georgis *et al.* (1991) demonstrated a negligible influence of entomopathogenic nematodes on nontarget organisms if they are used only in short term pest control.

Helena *et al.* (2009) concluded the pronounced nontarget effect of EPNs (*Steinernema feltiae*, *S. carpocapsae*, *Heterorhabditis bacteriophora*) on the larvae of Two spotted lady bird beetle (Coleoptera: Coccinellidae) and green lacewing (Neuroptera: Chrysopidae). Farag (2002) reported a high mortality of the larvae of *Coccinella undecimpunctata* L. caused by *H. taysearae* and *S. carpocapsae* strain S2 in a laboratory assay, and therefore did not recommend the use of entomopathogenic nematodes when these predators are present on the plants in high number. Likewise, *H. bacteriophora* and *S. carpocapsae* under laboratory conditions were very harmful to the predators like

*Coleomegilla maculata* (De Geer), *Olla nigrum* (Mulsant), *Harmonia axyridis* (Pallas), and *Coccinella septempunctata* L. In contrast, Shapiro-Ilan and Cottrell (2005) found lady bird beetles to be substantially less susceptible to nematode infection compared with a known susceptible insect, the black cutworm *Agrotis ipsilon*.

Kaya and Hotchkin (1981) and Kaya (1978a, 1978b) reported that EPNs could affect the interactions between braconid and tachinid parasitoids and their hosts. Moreover, Shannag and Capinera (2000) observed that *S. carpocapsae* is directly or indirectly detrimental to the development of *Cardiochiles diaphaniae* Marsh (Hymenoptera: Braconidae), a parasitoid of the melon worm *Diaphania hyalinata* (L.) and the pickleworm *D. nitidalis* Stoll (both Lepidoptera: Pyralidae).

No published records indicate a susceptibility of mites to EPNs. However, mites have been related to population reductions of EPNs. Ishibashi *et al.* (1987) conducted a study on predators of EPNs and observed that collembolans, mites, tardigrades and nematodes (mononchid and dorylaimid) preyed upon *S. carpocapsae* and found no deleterious effects of EPNs on them. Moreover, Epsky *et al.* (1988) demonstrated that the mesostigmatid mite *Gamasellodes vermivorax* Walter (Acarina: Ascidae) reduced *S. carpocapsae* efficacy against *G. mellonella* larvae. In addition, they observed that the endeostigmatid mite *Alycus roseus* Koch (Acarina: Bimichaeliidae) completed its development from late instar nymphs to adults and produced viable eggs when feeding on IJs of *S. carpocapsae*. Inera and Davis (1983) reported that *Hypoaspis* spp. feed on plant parasitic nematodes, and Brodsgaard *et al.* (1996) showed, that *H. miles* predated on IJs of *S. feltiae*. Several species of steinernematids attach onto mites that serve as phoretic hosts (Epsky *et al.*, 1986).

Earthworms improve soil conditions (aeration, drainage and organic matter content) and they are able to change soil structure, move large amount of soil and affect micro floral and faunal diversity (Brown, 1995; Doube and Brown, 1998). Many associations (phoretic, paratenic intermediate or sole host) between nematodes and earthworms had been reported (Gunnardson and Rundgren, 1986; Poinar, 1978; Timper and Davies, 2004), however there is no report on any adverse effect of earthworms by entomopathogenic nematodes. Several authors have observed the non-susceptibility of earthworms to steinernematids (Capinera *et al.*, 1982; Nguyen and Smart, 1991; Shapiro *et al.*, 1993) and to the slug-parasitic nematode

*Phasmarhabditis hermafrodita* Schneider (Nematoda: Rhabditidae) (Grewal and Grewal, 2003). Some authors think that earthworms could be used as vectors to introduce and/or disperse beneficial organisms including EPNs (Eng *et al.*, 2005; Shapiro *et al.*, 1993, 1995). Raquel *et al.* (2006) studied the interaction of *S. feltiae* with earthworm species *Eisenia foetida* and reported about 20 – 90 % of the earthworms as transmitting nematodes with reduced mobility through their gut without any pathogenic effect on earthworms.

Ten day post infected hosts, especially of steinernematids, contain dauers (Kaya and Stock, 1997) and were scavenged by ants; therefore the dauers could attach to the ants. Scavenging ants have a significant impact on steinernematid nematodes because they consume dead insects containing the parasitic life stages of entomopathogenic nematodes (Baur *et al.*, 1998). Even though the ants do not consume the entire contents of the cadaver, the remaining nematodes within the cadaver die due to desiccation. Baur *et al.* (1998) observed rapid desiccation of cadavers once one or two openings made through the host cuticle. In addition to consuming the cadavers, the larger species of ants (i.e., *Veromessor andrei* and *Formica pacifica*) also carry the cadavers to their nests. These ants removed 4 of the 10 cadavers containing *S. feltiae* by dragging them into the openings of the colony where as the cadavers containing *H. bacteriophora* were not disturbed. This result suggests that ants could play a role in dispersing the dauer stage of steinernematids but not of heterorhabditids.

Under laboratory conditions *H. bacteriophora* can kill spiders, woodlice and Diplopoda (Bathon, 1996). However, in many cases the natural habitat of these organisms would prohibit nematode infection. In field experiments with *H. bacteriophora* no or only transient effects on soil borne non-target species could be observed (Bathon 1996). Under laboratory conditions effects against the semi-aquatic snail *Oncomelania hupensis* could be observed. But, due to the low density of entomopathogenic nematodes in the upper soil layers, a severe impact to snails is unlikely (Bathon 1996). Under natural conditions *H. bacteriophora* will not affect vertebrates (Bathon 1996). Hazardous effects were recorded for tadpoles (*Bufo marinus*, *Hyla regilla*, *Xenopus laevis*) and for the lizard *Anolis marmoratus* (Bathon 1996). However, these effects could only be observed under artificial conditions (Petri dish with high numbers of nematodes), which did not reflect the natural environment of the vertebrates and the application method for the nematodes. It was demonstrated that

under realistic natural conditions no hazard for tadpoles (e.g. aquarium with water) were recorded (Bathon, 1996).

Entomopathogenic nematodes do not affect higher vertebrates such as rats, rabbits or monkeys (Poinar *et al.*, 1982; Bathon, 1996). The bacterium *X. bovienii* has been proven harmless for chicks and mice (Poinar *et al.*, 1982).

## **2.7 Biotoxins produced by symbiotic bacteria associated with entomopathogenic nematodes**

Species of the genus *Xenorhabdus* are gram-negative entomopathogenic bacteria, which live symbiotically with nematodes of the genus *Steinernema*. The nematode bacterium association is highly toxic to many insect species and in most cases the bacteria alone are highly virulent when they enter the insect haemocoel (Forst and Neilson, 1996). The metabolites produced by the symbiotic bacteria help to evade the insect immune system, enzymes such as proteases, lipases and phospholipases to maintain a food supply during reproduction (Thaler *et al.*, 1998) and antifungal and anti-bacterial agents to prevent degradation or colonization of the insect carcass, while bacteria and nematode reproduce (Akhurst and Dunphy, 1993). The entomopathogenic bacterium *Photorhabdus luminescens*, the symbiont of *H. bacteriophora* has been shown to express complex toxin (tc) genes, which is lethal to insects (Bowen and Ensign, 1998). French-Constant and Bowen (1999) have indicated that *Xenorhabdus* spp. also secrete toxins similar to those found in *P. luminescens*. The details of the modes of action of the various toxins of *Xenorhabdus* and *Photorhabdus* are still elusive. Injection of the Tca complex of *P. luminescens* strain W14 or ingestion by *Manduca sexta* larvae damaged the midgut cells, resulting in shedding of the midgut epithelium (Blackburn *et al.*, 1998). Injection of *A24tox* into lepidopteran larvae caused the larvae to cease feeding almost immediately. The histological studies indicated that the main site of action for the toxin was insect midgut (Brown *et al.*, 2004).

Insects killed by *Steinernema* spp. do not putrefy and bacteriological tests have shown that the bacterial symbionts are the predominant microorganism in the cadaver, at least during the early stages of nematode development (Forst and Neilson, 1996; Boemare *et al.*, 1997). This implies the production of antimicrobial compounds by these bacterial symbionts. The lack of putrefaction could be due also to the fact that the bacterial symbionts

reproduce so rapidly that they out-compete decomposers such as fungi and other bacterial species in the parasitized insect (Jarosz, 1996a, 1996b). It has been speculated that insect gut microflora and other invading microorganisms cannot establish in the insect body cavity because of the presence of the large, symbiont population and of its antimicrobial compounds (Boemare *et al.*, 1997). Indeed, the antimicrobial secondary metabolite production is an important characteristic of *Xenorhabdus* spp. (Paul *et al.*, 1981; Akhurst, 1982). A number of antibiotic compounds produced by these bacteria have been identified. The first groups of antibiotics discovered from these bacteria were stilbenes and indole derivatives (Paul *et al.*, 1981). Since then numerous studies have resulted in the identification of several novel, antimicrobial agents from different species of these bacteria. These compounds are active against a broad spectrum of plant and animal pathogenic fungi under laboratory conditions (Chen *et al.*, 1994; Li *et al.*, 1995; Chen, 1996), and, for example inhibit economically important plant pathogenic fungi including *Botrytis cinerea*, *Ceratocystis ulmi*, *Mucor pitifomis*, *Pythium coloratum*, *P. unimum*, *Phytophthora infestans* and *Rhizoctonia solani* in Petri plate tests.

Insect-nematode-bacterium tripartite associations such as those involving *Xenorhabdus* species provide attractive systems for both discoveries of new natural products, identification of novel compounds involved in inter kingdom signaling and antibiotics (Park *et al.*, 2009). The evolution of the entomopathogenic *Xenorhabdus* bacteria has resulted in a broad, inter-specific, diversity of compounds with antimicrobial activity. These antagonize related, or non-related, competitors in the insect cadaver in the soil (Sicard *et al.*, 2006). To compete successfully with invaders, EPB species produce several water-soluble and non-polar compounds with antibiotic activity (Paul *et al.*, 1981; McInerney *et al.*, 1991a; Sztaricskai *et al.*, 1992; Sundar and Chang, 1993; Webster *et al.*, 1996, 2002). The water-soluble peptide antimicrobial compounds xenocoumacin 1 (Xcn1) and 2 (Xcn2), both of which are hybrids of amino and carboxylic acid moieties, are the major antibiotics produced in broth culture by *X. nematophila* strain all (McInerney *et al.*, 1991a). Both Xcn1 and Xcn2 were also shown to be produced in the haemocoel of *Xenorhabdus*-infected insect cadavers (Maxwell *et al.*, 1994). Xcn1 is active against gram-positive and gram-negative bacteria and several fungal species, while Xcn2 is less active against the bacteria and inactive against the fungal species examined (McInerney *et al.*, 1991b). Molecular genetic analysis has identified

a 14 gene complex involved in the biosynthesis of Xcn1 and conversion to Xcn2 (Park *et al.*, 2009).

The antibioticly active, non-purified, cell-free liquid cultures, of *Xenorhabdus* strains are effective against a large spectrum of invaders, from bacteria through fungi to protozoa. Böszörményi *et al.* (2009) showed that secondary metabolite(s) produced by *Xenorhabdus budapestensis* effectively reduced fire blight indexes on apple trees greenhouse conditions and also exerted strong toxicity on both zoospores and cystospores of *Phytophthora nicotianae*.

### 3. MATERIALS AND METHODS

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The following materials and methods were used for achieving the various objectives of the present investigation.

#### 3.1 Nematode culture

Pure culture of Infective juveniles (IJs) of *Steinernema thermophilum*, *S. glaseri* and *Heterorhabditis indica* were obtained from the EPN Genomics laboratory, Division of Nematology, Indian Agricultural Research Institute, New Delhi. The cultures of these entomopathogenic nematodes were maintained on 4<sup>th</sup> instar larvae of greater wax moth, *Galleria mellonella* (L.) (Kaya and Stock, 1997). The emerging IJs from the insect cadavers were harvested in Sterile distilled water using a modified White's trap, and stored at 15° C in BOD incubator. The IJs for subsequent studies were used within 2-4 weeks of storage.

**3.2 Greater wax moth (*G. mellonella*):** The initial culture of *G. mellonella* (greater wax moth) was obtained from the EPN Genomics laboratory, Division of Nematology, IARI, New Delhi. It was subsequently reared on a semi-synthetic diet.

Table 3.1: Ingredients of artificial diet for *G. mellonella* (for one set of 10,000 to 12,000 eggs, distributed in 4 jars of 2 litre capacity to yield approximately 3000 larvae per jar):

Ingredients	Quantity
Corn meal	400 g
Wheat flour	200 g
Wheat bran	200g
Milk powder	200g
Yeast	100g
Honey	350ml
Glycerine	350ml

All the dry contents of the diet were mixed thoroughly in a clean flat container and then honey and glycerine were added slowly while mixing the contents. The mixture was kneaded thoroughly to obtain semisolid golden yellow colored dough.

**3.2.1 Planting of eggs in the medium and after care:** The prepared medium was transferred to the jar so that it occupied only one fourth volume in the jar. It should be a wide mouthed jar for better air circulation and easy handling. Egg masses (each containing 500 eggs) collected from previous cultures were placed on the medium in the jar. Normally 2-3 egg masses after hatching will give rise to 600 to 800 larvae. The jars were incubated at 28° C. The eggs hatched within a week and hatched larvae become visible in 3 to 4 days. They were voracious feeders and attained a length of 2 – 2.5 cm within a fortnight. If there was a need to arrest the growth of the larvae, they were transferred to beaker containing wood scrapings and placed at 15°C.

The pupation of larvae took place in the jar and adults emerged from pupal case within 10-12 days. Adults were fed on 20-30 % honey solution through a cotton wick. After mating, they laid eggs in clusters around the rim or on the tissue paper used for covering the jars. These eggs were further processed for next generation.

### **3.3 Study on compatibility of *Steinernema thermophilum* with chemical pesticides, surfactants and commercial formulations of bioagents**

Compatibility of *S. thermophilum* was evaluated with different chemical pesticides, surfactants/adjuvants and commercial formulations of bioagents.

#### **3.3.1 Pesticidal formulations and surfactants/adjuvants used**

##### **3.3.1.1 Chemical pesticides**

Twelve insecticides (Tagride, Talstar, Marshal, Dursban, Superkiller, Decis, Thiodan, Confidor, Karate, Tagtara, Tracer and Hostathion), six herbicides (Fenoxone, Atrataf, C lencher, Isogourd Stomp and Validamycin) and three fungicides (Bavistine, mancozeb. Hexaconazole) were selected for the compatibility study. These chemicals were procured from FMC India Pvt. Ltd., Dowe Agrosiences India Ltd., Dhamka Agritech Ltd., Bayer Crop Science Ltd., Biostadt India Ltd., Tropical Agrosystem India pvt Ltd., Jai Chemicals India Ltd., Gharda Chemicals Ltd. and Cyanamid Ltd.

### **3.3.1.2 Commercial formulations of bioagents**

Formulated products of various bioagents namely Baba, Metarhizium, Mycomite, Niyrantran, Nisarga, Varsha and Sparsha were procured from Multiplex Biotech Pvt. Ltd. and used for the compatibility study.

### **3.3.1.3 Surfactants/adjuvants**

Compatibility study were carried out with a total of eight surfactants/adjuvants viz. Apsa –80, CMC, Glycerol, Liquid paraffin, Starch, Triton X 100, Tween 20 and Tween 80. These surfactants/adjuvants were procured from Himedia Laboratories, SRL Pvt. Ltd., Qualigens fine chemicals and Amway distributors.

**3.3.2 Viability study:** Two concentrations of surfactants (1 % and 3 %) and four different concentrations of chemical pesticides and commercial formulations of bioagents viz. double the maximum recommended dose (DMD), maximum recommended dose (MD), half of the maximum recommended dose (HMD) and lowest recommended doses (LD) were evaluated for their compatibility with *S. thermophilum*. Only former three doses were used in case of those pesticides for which HMD is lower than LD. The required concentrations were prepared using sterile distilled water and 20 µl of nematode suspension in sterile distilled water with about 50 infective juveniles of *S. thermophilum* were added into 2 ml of each concentrations of insecticidal solution in each well of the 6 well plates. The bottoms of the wells were marked into square sections to facilitate counting. Viability of infective juveniles exposed to different concentrations of pesticides was evaluated after 24, 48 and 72 h up to 7 days of exposure, under a stereomicroscope. The observations on the viability of IJs treated with surfactants/adjuvants were observed in 10, 20 and 30 days after treatment. Nematodes that did not move even after prodding were considered dead.

**3.3.3 Infectivity study:** Stock solutions of the pesticides for filter paper bioassay were prepared in sterile distilled water at double strength of the required concentration. About 2000 infective juveniles of *S. thermophilum* in 1 ml of sterile distilled water were mixed with 1 ml of each stock solution in each well of the 6 well plates. Infective juveniles in sterile distilled water were used as control and all the treatments were held at 25±1°C. The infectivity of infective juveniles after 48 hours of exposure to the highest recommended

doses of each insecticide and one month exposure to 3 % of surfactants were evaluated on 4<sup>th</sup> instar larvae of *G. mellonella* by filter paper bioassay in 12 well plates. The IJs treated with pesticides were washed with sterile distilled water. Fifty infective juveniles in 50µl suspension from each treatment were removed and added to each well containing double layer of Whatman No. 1 filter paper and wells were diluted by adding equal amount of double distilled water to each well. One larva of *G. mellonella* was added to each well. Each treatment was replicated four times and the plates were incubated at 25±1°C. The mortality of *G. mellonella* larvae was recorded after every 24 h. The cadavers of *G. mellonella* larvae were transferred to Petri plates of 10 cm diameter size containing double layers of Whatman No. 1 filter paper and observed for the emergence of infective juveniles.

### **3.4 Study on compatibility of *Steinernema thermophilum* with other bioagents**

#### **3.4.1 Bioagents used in the study**

Two bacterial (*Bacillus thuringiensis* and *Pseudomonas fluorescens*) and six fungal bio agents (*Trichoderma viride*, *T. harzianum*, *Paecilomyces lilacinus*, *Pochonia chlamidosporium*, *Metarhizium anisopliae* and *Beauveria bassiana*) were used in the present investigation. All the fungal bioagents were obtained from Indian Type culture collection (ITCC), Division of Mycology and Plant Pathology, IARI, New Delhi. The bacterial bioagents were procured from National Centre for Integrated Pest Management (NCIPM), New Delhi. The bacterial bioagents were maintained in nutrient broth (NB) medium and the fungal bioagents were maintained in Sabouraud's dextrose agar (SDA) medium and potato dextrose agar medium (PDA).

#### **3.4.2 Culturing of fungal bioagents**

Seed cultures of all the fungal bioagents used were obtained from ITCC, Division of Plant Pathology, IARI, New Delhi. Sabouraud's dextrose agar with 0.2% yeast extract (SDA+Y), was used as medium for culturing both *B. bassiana* and *M. anisopliae*. Potato dextrose agar medium was used for culturing all other fungal bioagents.

### 3.4.3 Preparation of medium

Table 3.2: Ingredients used for Sabouraud's dextrose agar medium (Thomas, 1974)

<b>Ingredients</b>	<b>Quantity</b>
Mycological Peptone	10 g
Dextrose	40 g
Agar	15 g
Distilled water	1 Litre
Yeast extract	0.2%

NB: Sabouraud's dextrose broth is without agar

Table 3.3: Ingredients used for potato dextrose agar medium (Readymade @ 39 g/l)

<b>Ingredients</b>	<b>Quantity</b>
Potatoes infusion from	200 g
Dextrose	20 g
Agar	15 g
Distilled water	1 Litre

NB: Potato dextrose broth (PDB) is without agar

Table 3.4: Ingredients used for Nutrient agar medium (Readymade @ 28 g/l)

<b>Ingredients</b>	<b>Quantity</b>
Peptic digest of animal tissue	5 g
Beef extract	1.5 g
NaCl	5 g
Agar	15 g
Distilled water	1 Litre

NB: Nutrient Broth (NB) is without agar

Table 3.5: Ingredients used for Lipid agar medium

<b>Ingredients</b>	<b>Quantity</b>
Nutrient agar medium	28 g
Olive oil	5 ml
Distilled water	1 Litre

The media supplied by M/S Himedia were used for PDA, PDB and NB. For the preparation of media where agar is present, all the ingredients were mixed in a small amount of lukewarm water and stirred to dissolve. After the ingredients were dissolved, the volume was made up to one litre. The solution was boiled and after boiling, the medium was autoclaved for 30 minutes at 15 psi and allowed to cool for 15 minutes. The cooled medium was poured into Petri plates (10mm x15mm) under laminar airflow chamber. After pouring, a pinch of streptomycin was mixed with the medium to avoid bacterial contamination. It was then allowed to solidify for one day. The Petri plates were sealed with paraffin wax to avoid contamination. For all the media where agar was not used, all the ingredients or specified quantity of readymade medium was mixed with distilled water and made the volume to 1 litre. It was then transferred to conical flasks or test tubes and the flasks/test tubes were plugged with surgical grade cotton. The medium was sterilized in an autoclave at 15 psi pressure for 30 minutes.

**3.4.4 Viability study:** Seventy two hours old cultures of bacterial bioagents (*B. thuringiensis* and *P. fluorescens*) in nutrient broth medium and 14 days old cultures of fungal bioagents in PDB medium (*Trichoderma viride*, *T. harzianum*, *Paecilomyces lilacinus* and *Pochonia chlamydosporium*) and SDB medium (for *Metarhizium anisopliae* and *Beauveria bassiana*) were used for the viability study. The bacterial growth of 72 h in nutrient broth medium was considered as standard stock (S). Whereas the standard stock (S) of fungal bioagents were prepared by grinding 14 days old fungal cultures in their respective medium using a churner and filtered through muslin cloth. The four different concentrations of bacterial bioagents (S, S/2, S/4 and S/8) and Standard (S) concentration of fungal bioagents were evaluated for their compatibility with *S. thermophilum*. The required concentrations were prepared using sterile distilled water from the standard stock solution (S) and 20 µl of nematode suspension in sterile distilled water with about 50 infective juveniles of *S. thermophilum* were added into 2 ml of each concentration of bioagents in each well of the 6 well plates. The bottoms of the wells were marked into square sections to facilitate counting. Viability of infective juveniles exposed to different concentrations of bioagents was evaluated after 24 h, 48 h, 72 h and 7 days of exposure, under a stereomicroscope. Nematodes that did not move even after prodding were considered dead.

**3.4.5 Infectivity study:** Infectivity study was carried out with Standard stock solutions (S) of the fungal bioagents. About 2000 infective juveniles of *S. thermophilum* in 1 ml of sterile distilled water were mixed with 1 ml of each standard stock solution (S) in each well of the 6 well plates. Infective juveniles in sterile distilled water were used as control, and all the treatments were held at  $25\pm 1^{\circ}\text{C}$ . The infectivity of infective juveniles after 48 h of exposure to each fungal bioagents was evaluated on 4<sup>th</sup> instar larvae of *G. mellonella* by filter paper bioassay in 12 well plates. The IJs exposed to fungal bioagents for 48 h, were washed thrice in sterile distilled water. From each treatment, 50 $\mu\text{l}$  suspension containing about 50 infective juveniles were added to each well lined with Whatman No.1 filter paper and then equal amount of double distilled water was added. One *G. mellonella* larva was added to each well, each treatment was replicated four times and the plates were incubated at  $25\pm 1^{\circ}\text{C}$ . The mortality of *G. mellonella* larvae was recorded at 24 h interval. The cadavers of *G. mellonella* larvae were transferred to Petri plates of 10 cm diameter size containing two layers of Whatman No. 1 filter paper and observed for the emergence of infective juveniles.

**3.4.6 Interaction study:** The mutual interaction of *S. thermophilum* and its symbiotic bacterium *X. indica* with *M. anisopliae* and *B. bassiana* were evaluated in 3 different culture media namely Lipid agar medium (LA), Nutrient agar medium (NA) and Sabouraud's dextrose agar (SDA) medium. The symbiotic bacteria *X. indica* was isolated from *S. thermophilum*. The interaction study was carried out by simultaneously inoculating symbiotic bacteria alone and nematode alone with *M. anisopliae* and *B. bassiana*. All the inoculations were carried out in front of the laminar airflow chamber. Nematodes were inoculated after surface sterilization in 0.1 % Hyamine solution. The surface sterilized IJs of *S. thermophilum* @ 100 IJs/plate in 50  $\mu\text{l}$  of sterile distilled water and mycelia of 10 days old growth of respective fungal bioagents were used for inoculation.

#### **3.4.6.1 Surface sterilization of Infective juveniles (IJs)**

The IJs of *S. thermophilum* produced *in vivo* using *G. mellonella* were surface sterilized by Hyamine 10X (Methylbenzethonium chloride). About 10,000 to 15,000 IJs were placed into 0.1% Hyamine 10X for 15 to 30 min. Subsequently, three washings were done in sterilized water to remove traces of Hyamine.

#### **3.4.6.2 Study on interaction of *X. indica* with *M. anisopliae* and *B. bassiana*.**

The study was carried out on three different growth media (LA, NA and SDA) Both bacteria and respective fungal bioagents were inoculated simultaneously on both sides of the media in the Petri plates. Each treatment was replicated 3 times and incubated at  $25\pm 1$  °C. The observations were taken for 1 month at 4 days interval.

#### **3.4.6.3 Study on interaction of *S. thermophilum* with *M. anisopliae* and *B. bassiana*.**

The study was carried out on three different growth media (LA, NA and SDA) by simultaneous inoculation of nematode and fungi as well as by delayed inoculation of nematodes after 10 days of fungal inoculation. The nematode and fungal bioagents were inoculated on both sides of respective media in the Petri plates. Each treatment was replicated 3 times and kept at  $25\pm 1$  °C. The observations were taken up to 1 month at 4 days interval.

### **3.5 Study on compatibility of *Steinernema thermophilum* with Organic amendments:**

Organic amendments used were neem cake, bone meal, farm yard manure (FYM) and vermicompost.

#### **3.5.1 Viability study**

The compatibility of *S. thermophilum* with bone meal, neem cake, FYM and vermicompost were evaluated. The respective organic amendments mixed with sterile distilled water in the ratio 1:2 and strained through muslin cloth were considered as double strength of standard stock solution (DS). The viability study was carried out with four different concentrations (S, S/2, S/4 and S/8) of these organic amendments. About 2000 infective juveniles of *S. thermophilum* in 1 ml of sterile distilled water were mixed with 1 ml of each double strength of required concentration in each well of the 6 well plates to get 2 ml of required concentration with 2000 IJs in each well. Infective juveniles in sterile distilled water were used as control and all the treatments were replicated five times and held at  $25\pm 1$ °C. Viability of infective juveniles exposed to different concentrations of organic amendments was evaluated after 5 days and 10 days of treatment by removing 50 µl from each treatment, under a stereomicroscope. Nematodes that did not move even after prodding were considered dead.

### **3.5.2 Infectivity study**

Infectivity study was carried out with Standard stock solutions (S) of the organic amendments. About 2000 infective juveniles of *S. thermophilum* in 1 ml of sterile distilled water were mixed with 1 ml of each double strength of standard stock solution (DS) in each well of the 6 well plates. Infective juveniles in sterile distilled water were used as control and all the treatments were held at  $25\pm 1^{\circ}\text{C}$ . The infectivity of infective juveniles after 10 days of exposure to each standard stock solution (S) of organic amendments was evaluated on 4<sup>th</sup> instar larvae of *G. mellonella* by filter paper bioassay in 12 well plates. From each treatment 50 $\mu\text{l}$  suspension containing about 50 infective juveniles was added to each well containing 2 layers of filter paper and equal amount of double distilled water was added to each well. One *G. mellonella* larva was added to each well, each treatment was replicated four and the plates were incubated at  $25\pm 1^{\circ}\text{C}$ . The mortality of *G. mellonella* larvae was recorded at 24 h interval. The cadavers of *G. mellonella* larvae were transferred to Petri plates of 10 cm diameter size containing double layers of Whatman No. 1 filter paper and observed for the emergence of infective juveniles.

## **3.6 Study on interspecific interaction of entomopathogenic nematodes**

### **3.6.1 Nematodes**

The interspecific interaction of different entomopathogenic nematodes (EPNs) *Steinernema thermophilum*, *S. glaseri* and *Heterorhabditis indica* were carried out. Pure cultures of Infective juveniles (IJs) of these EPNs were obtained from the EPN Genomics laboratory, Division of Nematology, Indian Agricultural Research Institute, New Delhi. The cultures of these nematodes were maintained on 4<sup>th</sup> instar larvae of greater wax moth, *Galleria mellonella* (L.) (Kaya and Stock, 1997). The emerging IJs from the insect cadavers were harvested in Sterile distilled water using a modified White's trap, and stored at  $15^{\circ}\text{C}$  in BOD incubator. The IJs for the interaction study were used within 2-4 weeks of storage.

### **3.6.2 Interaction study on Greater wax moth larvae**

The interspecific interactions among *Steinernema thermophilum* (St), *S. glaseri* (Sg) and *Heterorhabditis indica* (Hi) on 4<sup>th</sup> instar larvae of greater wax moth (*G. mellonella*) were carried out. The experiment was done in 12 well plates with one insect larva in each well of the plate. Each nematode species alone and different combinations of these EPNs (St+Sg,

St+Hi, Sg+Hi) were inoculated @ of 100 IJs per insect larva. In the treatments where combinations of EPNs used, 50 IJs of each species were applied to achieve a total of 100 IJs/larva. All the treatments were replicated 5 times and the experiment was carried out at 27 and 35 °C. The observations on time taken for mortality of larvae in each treatment were recorded and cadavers were transferred to modified White's trap. The date on which IJs emerged from each cadaver was recorded and the emerging IJs from each cadaver were harvested separately. The total number of IJs of each species emerged from each cadavers killed by different EPNs and EPN combinations were also counted separately.

### **3.6.2 Interaction study on Lipid agar medium**

The interaction of *S. thermophilum* with *X. poinarii* and *Photorhabdus luminescens* was studied on Lipid agar medium in Petri plates at 27 and 35 °C. Both *S. thermophilum* and above mentioned bacteria were inoculated simultaneously on both the sides of Lipid agar medium in the Petri plates. The observations on development of nematode and bacteria were recorded at 2 days interval.

## **3.7. Effect of entomopathogenic nematodes on non-target beneficial organisms.**

### **3.7.1 Beneficial organisms used**

The impact of Entomopathogenic nematodes, *S. thermophilum*, *S. glaseri* and *H. indica* were evaluated on one species of ants (*Messor himalayanus*), one species of spider (*Neoscona theisi*) and one species of earthworm (*Eisinea foetida*). The earthworm species was procured from the Division of Entomology, IARI, New Delhi, where as the spiders and ants were collected directly from IARI research farm.

### **3.7.2 Effect of entomopathogenic nematodes on earthworms**

The experiment was carried out in 100 ml capacity plastic bottles containing 20 grams of vermicompost. The EPN species were added @ 100 IJs/g compost and 500 IJs/g compost in each bottles and the earthworms were released @ 4 earthworms/bottle. All the treatments were replicated five times and held at 25±1 °C. The observations on survival of earthworms were recorded on 7<sup>th</sup> and 14<sup>th</sup> day of the treatment. Fourth instar larvae of *G. mellonella* @ 4 larvae/bottle were added on 10<sup>th</sup> day of treatment to confirm the presence and

survival of IJs in the vermicompost. The dead larvae from each bottle were collected at 24 h intervals.

### **3.7.3 Effect of entomopathogenic nematodes on spider**

The experiment was carried out in 12-well plates lined with two layers of Whatman No. 1 filter paper. Two doses (100 IJs/well and 500 IJs/well) of the three species of entomopathogenic nematodes in 100 µl of sterile distilled water were applied in the each well of twelve well plates. After the release of IJs in the wells, the spiders were released as one spider/well of the twelve well plates. Ten spiders were included in each treatment and experiment was held at 25±1 °C. The observations on mortality of spiders were recorded at every 24 h after treatment. The cadavers were observed for the emergence of next generation Infective juveniles.

### **3.7.3 Effect of entomopathogenic nematodes on ants**

The experiment was carried out in transparent plastic plates of 10 mm diameter lined with 2 layers of Whatman No. 1 filter paper. Each plate was provided with 50 µl of honey solution in small containers to feed the ants. The infective juveniles of entomopathogenic nematodes were released @ 1000 IJs/plate in 300 µl of sterile distilled water. Ten ants were released in each plate to yield a dosage of 100 IJs/ant. All the treatments were replicated five times and the experiment was held at 25±1 °C. The observations on mortality of ants were taken at every 24 hours after the treatment.

### **3.8. Pot experiment to evaluate the efficacy of *S. thermophilum* alone and in combination with pesticide and bioagents.**

The bioagents *M. anisopliae*, *B. bassiana* and bio-pesticide spinosad were used along with *S. thermophilum* for the pot experiment. The experiment was carried out on 4<sup>th</sup> instar larvae of *Helicoverpa armigera* infecting tomato cv. Pusa Ruby under glass house conditions (Temperature: 5 - 20°C; Relative Humidity- 55 - 75 %). The following treatments were used in the experiment.

	<b>Treatments</b>
T1	<i>S. thermophilum</i> @ 2000 IJs/ml

T2	<i>S. thermophilum</i> @ 1000 IJs/ml
T3	<i>M. anisopliae</i> @ 2*10 <sup>7</sup> spores/ml
T4	<i>B. bassiana</i> @ 2*10 <sup>7</sup> spores/ml
T5	Tracer @ 200 ppm
T6	Tracer @ 100 ppm
T7	<i>S. thermophilum</i> (1000 IJs/ml) & <i>M. anisopliae</i> (2*10 <sup>7</sup> spores/ml)
T8	<i>S. thermophilum</i> (1000 IJs/ml) & <i>B. bassiana</i> (2*10 <sup>7</sup> spores/ml)
T9	<i>S. thermophilum</i> (2000 IJs/ml) & Tracer (200 ppm)
T10	<i>S. thermophilum</i> (1000 IJs/ml) & Tracer (100 ppm)
C	Control (water)

The surfactant APSA-80 @ 0.03 % was added in all the treatments and control.

### 3.8.1 *Helicoverpa armigera*

The larvae for the initial cultures were collected from natural infestations on chickpea (*Cicer arietinum*) and redgram (*Cajanus cajan*) fields in the IARI farm. It was subsequently maintained in controlled environment room (Insectary) in the Division of Nematology, where temperature, photoperiod and humidity were controlled. The environmental conditions were: day: 25±2°C, night: 20±2°C, synchronized with a 14:10 hour light: dark photoperiod and Humidified to 70 – 85 % relative humidity.

Table 3.6: Ingredients of diet for culturing *H. armigera*

Ingredients	Quantity
Chickpea seed, finely ground	84 g
L-Ascorbic acid	3 g
Casein	5 g
Formaldehyde	1 ml

Multivitamin	1 ml
Sorbic acid	1 g
Streptomycin sulphate	0.2 g
Methyl 1-4-hydroxybenzoate	2 g
Cholesterol	0.2 g
Distilled water	400 ml
Yeast (dried, bakers)	11 g
Agar (powder)	11 g

**Method:** Agar was boiled in 400 ml of water in a sauce pan for about 15 minutes. There after yeast was added while stirring. It was removed from heat and all other ingredients were mixed using mixer/grinder at high speed until an even consistency was obtained. The mixed contents were poured into shallow trays in front of a laminar flow cabinet to cool and set for 30-60 minutes. The trays with diet were stored in clean plastic bags and kept in a fridge. If not required immediately the diet can be stored satisfactorily in a freezer in sealed bags.

Table 3.7: Ingredients of sucrose solution for *H. armigera* adult.

Ingredients	Quantity
Honey/Sucrose	20 g
Distilled water	200 ml
Multivitamin	10 Dops
Vitamin E capsule	2/3
Ascorbic acid	1 – 2 g
Methyl 1-4-hydroxybenzoate	0.1 g

### **3.8.2 Preparation of fungal spore suspension**

#### **3.8.2.3 Inoculation of fungi**

Ten days old cultures of *M. anisopliae* and *B. bassiana* cultured on Sabarauds Dextrose agar medium in the Petri plates was used. Using a sterilized needle, a small amount of mycelia was inoculated to the media. Then the Petri plate was sealed with paraffin wax and kept in BOD incubator at 27<sup>0</sup>C for 10 days. When the plate was fully covered with fungal spores, they were harvested for preparing spore suspension.

#### **3.8.2.4 Preparation of spore suspension**

The spores from 10 days old cultures of *B. bassiana* and *M. anisopliae* were harvested by flooding the plates with sterile distilled water and carefully stirred to break the conidial chains and to suspend the spores. The concentrated spore suspensions from one plate of each species were mixed with 10 ml sterile distilled water, 5 g of glass beads and Apsa 80 (0.03 %) and stirred for 30 minutes on a magnetic stirrer. After serial dilution, two different concentrations ( $4 \times 10^7$  and  $2 \times 10^7$  spores/ml) of spore suspension were prepared by using haemocytometer after serial dilutions (Muerrle *et al.*, 2006).

**3.8.3 The experimental design:** 25 days old seedlings of tomato cv. Pusa Ruby procured from Centre for Protected Cultivation and Training (CPCT), IARI, New Delhi, were transplanted in plastic pots of 6" diameter filled with potting mixture. The seedlings were maintained in the glass house of the Division of Nematology, IARI. At 90 days after transplantation when the plants started flowering, the fourth instar larvae of *H. armigera* starved for one day were released on the plants @ 10 larvae/pot and covered with thin polythene bags of 50 µm thickness to prevent larval escape. The larvae were released in the morning and allowed to establish on the plants for 36 hours and the treatments were applied on the next day evening. The observations on the mortality of larvae were recorded at every 24 hour interval up to 5 days and the last observation on 10<sup>th</sup> day of treatment. The cadavers were collected at each interval and kept in moist filter paper in Petri plates and observed for the emergence of IJs.

### **3.9 The extraction and evaluation of bio-toxins from the symbiotic bacterium *X. indica* associated with *S. thermophilum***

#### **3.9.1 Isolation of the bacteria**

The bacteria *X. indica* which is symbiotically associated with *S. thermophilum* was isolated by following the procedure described by Akhurst (1980).

1. The IJs of *S. thermophilum* were surface sterilized by Hyamine 10X (Methylbenzethonium chloride). About 10,000 to 15,000 IJs were placed into 0.1% Hyamine 10X for 15 to 30 min. Subsequently, three washings were done in sterilized water to remove traces of Hyamine.
2. The surface sterilized IJs were suspended in yeast salts (LB) or nutrient broth media in the small 1.5 ml Eppendorf's tube.
3. The IJs were crushed manually by a sterile tissue grinder/crusher.
4. One loop full of this crushed suspension was then streaked on the indicator NBTA (In 1 litre water: Peptone 5.0g, Beef extract 3.0g, agar 15g, Bromothymol blue 0.025g and 2,3,5-Triphenyl Tetrazolium Chloride 0.04g) media plates. Five replications were made for each media. The plates were incubated at 28 °C for 2-3 days.

#### **3.9.2 Chemicals and reagents**

Chemicals and reagents required for the study were purchased from M/S Merck India limited, Acros Organics, Sd fine chemicals and Qualigens Fine Chemicals. Various fungal and bacterial media were procured from Hi Media Laboratories. Solvents used in extraction, separation, crystallization and column chromatography were of laboratory grade. Solvents required for mass spectroscopy were HPLC grade.

#### **3.9.3 Extraction of the biotoxins**

**3.9.3.1 Test culture:** Cells of *X. indica* were isolated from the entomopathogenic nematode *S. thermophilum* and cultured.

**3.9.3.2 Preparation of media:** 30 g of readymade Soybean Casein Digest medium (Tryptic Soy Broth) was suspended in 1000 ml of distilled water and stirred well to dissolve it completely. It was then transferred to conical flasks and the flasks were plugged with surgical grade cotton. The medium was sterilized in an autoclave at 15 psi pressure for 30 minutes. Glass Petri plates and other required glass wares were sterilized in a hot air oven at 80 °C for 2 hours. The *Xenorhabdus indica* isolated from *S. thermophilum* in pure form were cultured in the Soybean Casein Digest medium (Tryptic Soy Broth). The medium after the inoculation of bacteria were incubated at 27±1°C and 150 rpm in an incubator shaker. Then 72 hours old culture broths were used for the isolation of biotoxin.

**3.9.3.3 Isolation of the biotoxins:** The biotoxins were sequentially isolated using three solvents namely, Hexane, Ethyl acetate and Butanol in a separatory funnel to partitioned thrice successively to give three corresponding solvent fractions. The culture broths were mixed with equal volume of the respective solvents in a separatory funnel with gentle shaking for 5 minutes. The solvent fractions were passed through anhydrous sodium sulphate (20 g) to remove the traces of water if any. The solvents were removed under reduce pressure (100 bar) below 40 °C in rotary evaporator to obtain corresponding residues. The extraction process was repeated five times with 4 L. batch of bacterial broth to obtain sufficient quantity of the corresponding extract.

**3.9.3.4 Preparation of the standard stock:** 100 mg of residues of Hexane, Ethyl acetate and Butanol fractions of bio-toxins of *S. thermophilum* were dissolved in 2 ml of acetone to yield a standard stock of 50000 ppm stock solution. Required concentrations for toxicity studies against nematodes, insects and fungi were prepared by diluting the standard stock in emulsified water (0.5% Tween 80).

#### **3.9.4 Toxicity evaluation of various solvent fractions on J<sub>2</sub> of root knot nematode *Meloidogyne incognita***

Three different concentrations (500 ppm, 1000 ppm and 2000 ppm) of solvent extracts of *S. thermophilum* were tested for its toxicity against second stage juveniles of *M. incognita* by immersion bioassay in twelve well plates. One ml of nematode suspension with about 50 J<sub>2</sub> in sterile distilled water was added in to 1 ml of double the required

concentrations of solvent extracts in each well of 12 well plates. All the treatments were replicated five times and held at  $25 \pm 1$  °C. The observations on nematode mortality/survival were recorded in every 24 h interval.

### **3.9.5 Toxicity evaluation of various solvent fractions on insect larvae**

The toxicity of different solvent extracts of *S. thermophilum* against 4<sup>th</sup> instar larvae of *G. mellonella* and *Spodoptera litura* was evaluated both by oral feeding and micro injection. Three different concentrations viz. 1000 ppm, 5000 ppm and 10000 ppm of solvent extracts were tested. Oral toxicity was studied by putting 50 µl of above mentioned concentrations of solvent extracts in 0.5 cm<sup>2</sup> of the diet in each well of the 6 well plates with one larvae in each well. The micro injection study was carried out by injecting 20 µl of these three concentrations of all the solvent extracts into the haemocoel of the larvae through the prolegs by using micro injector. All the treatments were replicated five times and observed of larval mortality in every 24 hours.

### **3.9.6 Toxicity evaluation of various solvent fractions on fungal pathogens**

The fungicidal activities of different solvent fractions were evaluated by poisoned food technique against *S. rolfisii* at six different concentrations of 1000, 500, 250, 125, 62.5, and 31.25 ppm. A 5 mm thick disk of fungus was placed in different Petri plates. Plates treated with acetone were taken as control. Both treated and control plates were kept in BOD incubator at  $28 \pm 1$  °C till the fungus growth in the control plate was completed (4-6 days) after which the readings were taken.

### **3.9.7 Electron Spray Ionization- Mass Spectroscopy analysis of biotoxins:**

Mass spectroscopy was carried out using Thermo Electron LC-MS-MS. Spectrometer (Thermo Electron Corporation, USA) equipped with P-2000 quaternary pumps. Detection of mass was done by electron spray ionization (ESI) source in positive mode with Finnigan LCQ tune plus program fitted with MAX-detector. Xcalibur software was used for the purpose of identification, quantification and fragmentation pattern. The following MS parameters were optimized in direct infusion mode: Spray voltage (5 kV), Sheath gas flow rate (21 mL min<sup>-1</sup>), Auxiliary gas flow rate (4.5 mL min<sup>-1</sup>), Spray current (0.91 µA), Capillary temperature (210°C-230°C), Capillary voltage (7.5V) and Tube lens offset (40).

## 4.1 RESEARCH PAPER I

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### **Compatibility of Entomopathogenic nematode *Steinernema thermophilum* (Nematoda: Rhabditida) with pesticidal formulations**

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**Abstract:** The compatibility of entomopathogenic nematode, *Steinernema thermophilum* with four different doses of the twenty six pesticidal formulations, namely Tagride, Talstar, Marshal, Superkiller, Decis, Confidor, Karate, Tracer, Hostathion, Tagtara, Dursban, Thiodan (Insecticides), Stomp, Atrataf, Isoguard, Fernoxone, Clincher, Validamycin (Herbicides), Zinthane, Contaf, Bavistine (Fungicides), Baba, Metarhizium, Mycomite, Niyrantran, Sparsha, Nisarga and Varsha (Formulations of bioagents) was tested. More than 98 % survival of the infective juveniles of *S. thermophilum* was recorded even after 7 days of treatment in all the tested doses of formulations except Hostathion, Dursban, Thiodan and double the highest recommended dose of Fernoxone. The virulence of the infective juveniles exposed to the highest recommended doses of various formulations was evaluated in laboratory filter paper bioassay by treating them on the 4<sup>th</sup> instar larvae of *Galleria mellonella* after an exposure period of 48 h. The bioassay study confirmed the retention of virulence by the infective juveniles after their exposure to the above-mentioned formulations except Hostathion, Dursban and Thiodan. Our study confirms that these formulations except Hostathion, Dursban and Thiodan in their recommended doses are absolutely compatible with *S. thermophilum* and could be tank mixed in spray mixtures of respective pesticidal formulations.

**Key words:** Insecticides, Herbicides, Fungicides, Commercial formulations of bioagents, *S. thermophilum*, Compatibility.

#### **4.1.1 Introduction**

Nematodes in the families Steinernematidae and Heterorhabditidae are soil inhabiting lethal insect pathogens that possess potential as biological control agents (Gaugler and Kaya, 1990; Gaugler, 2002). Most biocontrol agents require days or weeks to kill their hosts, yet nematodes, working with their symbiotic bacteria, kill insects in 24-48 hours.

These groups of nematodes are found to be lethal to a broad range of insect pests. However, they have very good bio-safety records with respect to the plant health, human health, soil and the environment.

*S. thermophilum* described by Ganguly and Singh (2000) from India has been found to be heat tolerant and efficacious against several insect pests of national importance (Ganguly *et al.*, 2004; 2004b). Being an indigenous, it is well adapted to local environmental conditions. Precise knowledge about its compatibility with other management options, which are commonly used against various pests and diseases, would facilitate its use in integrated pest management programs.

Over the years, several workers have evaluated the effects of various agrochemicals including acaricides, fungicides, herbicides and insecticides on different species of entomopathogenic nematodes (Rao *et al.*, 1975; Hara and Kaya, 1982, 1983; Kaya *et al.*, 1995; Grewal *et al.*, 1998; Hussaini *et al.*, 2001; Krishnayya and Grewal, 2002; De Nardo *et al.*, 2003; Aldomario *et al.*, 2010). The results of all these experiments had indicated that nematode species differ in their compatibility with different agrochemicals. Infective juveniles of entomopathogenic nematodes can tolerate exposure to many agrochemicals and some of them stimulate or interact synergistically with the nematodes, some can be detrimental. Even some chemicals used as inert ingredients or adjuvants in formulations can be toxic to nematodes (Krishnayya and Grewal, 2002). Therefore, compatibility of each formulation with the specific nematode species should be evaluated before any recommendation for their combined application.

In this study, we have evaluated 26 pesticidal formulations including 12 insecticides, 6 herbicides, 3 fungicides and 7 commercial formulations of bioagents for their compatibility with entomopathogenic nematode *S. thermophilum*.

## **4.1.2 Materials and methods**

### **4.1.2.1 Entomopathogenic nematodes**

Infective juveniles of entomopathogenic nematode, *Steinernema thermophilum* already available in our laboratory were cultured *in vivo* using 4<sup>th</sup> instar larvae of greater wax moth (*Galleria mellonella*) as per the method of Kaya and Stock (1999) and harvested using

modified White's trap. The aqueous suspensions of infective juveniles (IJs) were stored at 15 °C till use. The nematode stocks with 100% survival were used within one-week after the harvest. For maintaining the virulence potential the cultures were renewed at an interval of 4 weeks.

#### **4.1.2.2 Pesticidal formulations**

Altogether 26 commercially available pesticidal formulations, including 12 insecticides, 6 herbicides, 3 fungicides and 7 biopesticides were used for the compatibility study. The insecticidal formulations were comprised of neonicotinoids, synthetic pyrethroids organocarbamate, organophosphate and biopesticides, which are detailed in Table 4.1.1. All the herbicidal and fungicidal formulations used in the study were also of diverse chemical nature, and their details are provided in Table 4.1.2 & 4.1.3, respectively. The biopesticidal formulations used are listed in Table 4.4.4, providing the details of their respective trade name, bioagent, target pest, type of formulation and source.

#### **4.1.2.3 Pesticide dosage**

Four doses viz, double the maximum recommended dose (DMD), maximum recommended dose (MD), half of the maximum recommended dose (HMD) and the lowest recommended doses (LD) of pesticidal formulations which are commonly used for the pest management in various agricultural crops in India were tested. For those formulations for which HMRD is lower than LD, we eliminated the later. The recommended doses of chemical pesticides were calculated based on the recommendations of Central Insecticides Board and Registration Committee, Faridabad, Haryana, India (<http://www.cibrc.nic.in/mupi.pdf>). However for the commercial formulations of bioagents, we followed the manufacturer's recommendations. Complete details about all the tested pesticidal formulations are given in Table 4.1.1 to 4.1.4

#### **4.1.2.4 Compatibility tests**

All the pesticides were tested at three/four different concentrations viz. double the maximum recommended dose, maximum recommended dose, half of the maximum recommended dose and the lowest recommended doses (if applicable). The required concentrations of the insecticides were prepared using sterile distilled water. Then 20 µl of

Table 4.1.1: List of insecticidal formulations used in the study

Sl. No.	Trade name	Technical name	Group	% a.i.	HRD (PPM)	Source
1.	Tagride	Acetamiprid	NN	20 SP	200	Tropical Agrosystem India Pvt. Ltd.
2.	Talstar	Bifenthrin	SP	10 EC	1600	FMC India Pvt. Ltd.
3.	Marshal	Carbosulfan	OC	25 EC	2000	FMC India Pvt. Ltd.
4.	Dursban	Chlorpyrifos	OP	20 EC	10000	Dowe Agrosience India Ltd.
5.	Superkiller	Cypermethrin	SP	10 EC	7000	Dhamka Agritech Ltd.
6.	Decis	Deltamethrin	SP	2.8 EC	1350	Bayer Crop Science Ltd.
7.	Thiodan	Endosulfan		35 EC	2675	Bayer Crop Science Ltd.
8.	Confidor	Imidachloprid	NN	17.8 SL	560	Bayer Crop Science Ltd.
9.	Karate	Lambdacyhalothrin	SP	5 EC	1675	Biostadt India Ltd.
10.	Tracer	Spinosad	BP	45 SC	200	Bayer Crop Science Ltd.
11.	Tagtara	Thiomethoxam	NN	25 WG	500	Tropical Agrosystem India Pvt. Ltd.
12.	Hostathion	Triazophos	OP	40 EC	4000	Bayer Crop Science Ltd.

*NN – Neonicotinoid, SP – Synthetic pyrethroid, OC – Organocarbamate, OP – Organophosphate, BP - Biopesticide*

Table 4.1.2: List of Herbicidal formulations used in the study

Sl. No.	Trade name	Technical name	Group	% a.i.	HRD (PPM)	Source
1.	Fernoxone	2,4 –D Sodium salt	UC	80 WSP	5000	Jai Chemicals India Ltd.
2.	Atrataf	Atrazine	TH	50 WP	9000	Jai Chemicals India Ltd.
3.	Clincher	Cyhalofop-butyl	PH	10 EC	1600	Jai Chemicals India Ltd.
4.	Isoguard	Isoproturon	UH	50 WP	2700	Gharda Chemicals
5.	Stomp	Pendimethalin	DA	30 EC	10000	Cynamid

*UC – Unclassified herbicide, TH – Triazine herbicide, PH – Phenoxy herbicide, UH – Urea herbicide, DA – Dinitroaniline herbicide*

Table 4.1.3: List of Fungicidal formulations used in the study

Sl. No.	Trade name	Technical name	Group	% a.i.	HRD (PPM)	Source
1.	Contaf	Hexaconazole	TF	5 EC	2000	Rallis India Ltd.
2.	Bavistin	Carbendazim	BF	50 WP	666	Bayer Crop Science Ltd.
3.	Zinthane	Mancozeb	ZF	75 WP	4000	Tropical Agrosystem India Pvt. Ltd.
4.	Validamycin	Validamycin	AF	3 L	2666	Dhamka Agritech Ltd.

TF – Triazole fungicide, BF – Benzimidazole fungicide, ZF – Zinc fungicide, AF – Antibiotic fungicide

Table 4.1.4: List of commercial formulations of bioagents used in the study

Sl. No.	Trade name	Bio-agent	Target	Formulation	HRD (g/L)	Source
1.	Baba	<i>Beuveria bassiana</i>	Insect	WP	3	Multiplex Bio-tech Pvt. Ltd.
2.	Metarhizium	<i>Metarhizium anisopliae</i>	Insect	WP	3	Multiplex Bio-tech Pvt. Ltd.
3.	Mycomite	<i>Paecilomyces fumosoroseus</i>	Mite	WP	3	Multiplex Bio-tech Pvt. Ltd.
4.	Niyantran	<i>P. lilacinus</i>	Nematode	WP	1	Multiplex Bio-tech Pvt. Ltd.
5.	Sparsha	<i>Pseudomonas fluorescens</i>	Fungus/virus/Bacteria	WP	3	Multiplex Bio-tech Pvt. Ltd.
6.	Nisarga	<i>Trichoderma viride</i>	Fungus	WP	4	Multiplex Bio-tech Pvt. Ltd.
7.	Varsha	<i>Verticillium lecani</i>	Nematode	WP	4	Multiplex Bio-tech Pvt. Ltd.

nematode suspension with about 50 IJs of *S. thermophilum* in sterile distilled water was mixed with 2 ml of each concentrations of insecticidal solution in each well of the 6 well plates. Each treatment was replicated 5 times and all experiment was conducted in two trials. Stock solutions of the pesticides for filter paper bioassay were prepared in sterile distilled water at double strength of the required concentration. About 2000 infective juveniles of *S. thermophilum* in 1 ml of autoclaved double distilled water were mixed with 1 ml of each stock solution in each well of the 6 well plates. Each treatment was replicated four times and infective juveniles in sterile distilled water were used as control.

The experimental design used was Completely Randomized Design (CRD) and all the experiments were conducted at  $25\pm 1^{\circ}\text{C}$ .

**4.1.2.5 Viability study:** Observations on nematode survival were recorded after 24, 48 and 72 h up to 7 days of exposure, under a stereomicroscope. Nematodes that did not move even after prodding were considered dead.

**4.1.2.6 Infectivity study:** The infectivity of IJs after 48 hours of exposure to the highest recommended dose of each formulation was evaluated on 4<sup>th</sup> instar larvae of *G. mellonella* by filter paper bioassay in 12 well plates. All four replications of a single treatment collected together in 50 ml beaker and diluted by adding equal volume of sterile distilled water and stirred well. After allowed to rest for 30 minutes, supernatant liquid was withdrawn and the rinsing processes were repeated three times. Then 50  $\mu\text{l}$  of nematode suspension with about 50 IJs from each treatment were removed and added to each well of 12 well plates containing 2 layers of Whatman No. 1 filter paper and each well was diluted with adding 50  $\mu\text{l}$  of sterile distilled water. Four *G. mellonella* larvae were added in four different wells as one larva in each well for each replication. Each treatment was replicated four and the plates were incubated at  $25\pm 1^{\circ}\text{C}$ . The mortality of *G. mellonella* larvae was recorded in every 24 h and the cadavers of *G. mellonella* larvae were transferred to Petri plates of 4 cm diameter containing double layers of Whatman No. 1 filter paper and observed for the emergence of IJs.

**4.1.2.7 Statistical analysis:** All the data were subjected to statistical analysis and differences between treatment means were estimated by LSD test at  $P < 0.05$  probability.

### 4.1.3 Results

In all the concentrations of nine insecticidal formulations namely, Tagride, Talstar, Marshal, Superkiller, Decis, Confidor, Karate, Tracer and Tagtara, 100 % IJs were found viable after their exposure to all the four doses of these formulations up to 72 h (Table 4.1.5). There was no record of mortality of IJs in any of the treatments of Tagride and Tracer even after 7 days of exposure. The IJs exposed to double the highest recommended dose of Tagtara induced only 1.4 % mortality of IJs after 7 days of treatment which was the maximum recorded mortality when compare to different doses of above mentioned insecticidal formulations. These mortality data were found statistically non-significant.

Among the different herbicidal and fungicidal formulations evaluated, double the highest recommended dose of Fernoxone recorded significant level of mortality from 48 hours of treatment and a maximum of 13.4 % mortality was recorded in 7 days of its exposure. However any other treatments of the Fernoxone or all the doses of other herbicidal and fungicidal formulations did not yield significant level of nematode mortality up to 7 days of treatment (Table 4.1.6).

The IJs exposed to the highest recommended doses of all the above mentioned pesticidal formulations for a period of 48 hours, when treated on the 4<sup>th</sup> instar larvae of *G. mellonella*, could induce 100 % mortality within 72 hours (4.1.8). As 48 hours of treatment is a standard time interval for the insect bioassay studies with EPN, the data on mortality of *G. mellonella* induced by IJs after the same time interval were represented separately in Figs. 4.1.1, to 4.1.3

A marginal reduction in percentage mortality of *G. mellonella* in 48 hours of treatment was observed in the treatments of IJs exposed with Tagride, Talstar and Decis as compared to control. However the IJs exposed to pesticidal formulations Superkiller, Confidor, Karate, Tracer and Tagtara induced a higher rate of mortality than control in 48 hours of treatment and in addition Karate induced 100 % larval mortality. The rate of mortality caused by IJs exposed to Marshal was on par with the control. But all these differences in the rate of mortality were statistically non-significant (CD – 20.9) except Karate treated IJs which induced significantly higher rate of insect mortality (Table 4.1.8). The emergences of IJs from all these cadavers were observed within 6 – 8 days of their

mortality. This result showed that some of pesticidal formulations like Superkiller, Confidor, Karate, Tracer and Tagtara could activate the IJs to become more virulent to cause faster rate of infection and pathogenicity. It indicated that these insecticidal formulations in their recommended doses are safe for *S. thermophilum*.

Among the herbicidal and fungicidal formulations, infective juveniles treated with Atrataf, Clincher and Bavistine showed a marginal improvement in its virulence (75 % larval mortality in 48 hours) towards 4<sup>th</sup> instar larvae of *G. mellonella* when compared with control. The IJs treated with Fernoxone, Validamycin, Contaf and Zinthane induced larval mortality of 62.5 % in 48 hours, which was on par with control. While Isoguard and Stomp reduced the virulence of IJs marginally (56.3 % larval mortality) when compared with control. But none of these treatments exhibited statistically significant differences (Table 4.1.9).

Significant reduction in the viability of IJs was recorded in all the doses of Hostathion and 3 higher doses of Dursban and Thiodan in all the time intervals (Table 4.1.5). The double the highest recommended dose of Hostathion (800 ppm) induced 48.6 % nematode mortality in 7 days and 9, 11.3 and 18 % mortality in 24, 48 and 72 hours of treatment respectively. Other 3 doses, 4000, 2000 and 625 ppm of Hostathion caused nematode mortality of 38.1, 33.2 and 16.1 % respectively in 7 days of exposure. The surviving IJs in all the treatments, even in the lowest recommended doses of Hostathion were found to be less active as compared to that in any other formulations or control evaluated. An increase in the level of mortality of IJs was observed as the duration of exposure increased in all the four doses of Hostathion. The data on mortality of IJs in the four doses of Hostathion in all the time intervals were found significant as compared to control. There were no significant differences in the mortality among the 2 highest doses of Hostathion in first 2 time intervals and between highest recommended dose and half of the recommended dose in 72 hours and 7 days of treatment (Table 4.1.5). The Lowest recommended dose in all the time intervals showed significantly lower nematode mortality when compared with other doses of Hostathion. But we could observe a marked reduction in activity of IJs in all the treatments of Hostathion. Even though double the highest recommended dose of Hostathion induced a maximum of 48.6 % mortality of IJs after 7 days of treatment, all the surviving IJs in the same treatment were found to be highly inactive ie,

Table 4.1.5: Effect of different doses of insecticidal formulations on Infective Juveniles (IJs) of *S. thermophilum*

Pesticidal formulation	Treatments (Dose in ppm)	% mortality of IJs			
		24 Hour	48 Hour	72 Hour	7 Days
<b>Tagride</b>	<b>T1 (400)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (200)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (100)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Talstar</b>	<b>T1 (3200)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (1600)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.6</b> (4.6)
	<b>T3 (800)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>1.1</b> (5.9)
<b>Marshal</b>	<b>T1 (2000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (1000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.6</b> 4.3
	<b>T3 (500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Dursban</b>	<b>T1 (20000)</b>	<b>2.9</b> (9.8)	<b>6.2</b> (14.4)	<b>29.3</b> (32.8)	<b>66.5</b> (54.7)
	<b>T2 (10000)</b>	<b>0.5</b> (4.2)	<b>4.9</b> (12.7)	<b>14.1</b> (22.0)	<b>47.6</b> (43.6)
	<b>T3 (5000)</b>	<b>0.9</b> (5.3)	<b>3.4</b> (10.6)	<b>12.3</b> (20.6)	<b>24.7</b> (29.8)
	<b>T4 (500)</b>	<b>0.0</b> (0.4)	<b>1.7</b> (7.6)	<b>4.0</b> (11.6)	<b>6.9</b> (15.3)
<b>Superkiller</b>	<b>T1 (14000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.7</b> (4.9)
	<b>T2 (7000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (3500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T4 (500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Decis</b>	<b>T1 (2700)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>1.3</b> (6.5)
	<b>T2 (1350)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (675)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T4 (150)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (4.2)
<b>Thiodan</b>	<b>T1 (5250)</b>	<b>7.6</b> (16.0)	<b>11.0</b> (19.4)	<b>17.5</b> (24.7)	<b>49.4</b> (44.7)
	<b>T2 (2675)</b>	<b>1.8</b> (7.7)	<b>6.3</b> (14.6)	<b>10.0</b> (18.4)	<b>36.2</b> (37.0)
	<b>T3 (1338)</b>	<b>0.7</b> (4.9)	<b>1.8</b> (7.8)	<b>3.7</b> (11.1)	<b>8.5</b> (16.9)
	<b>T4 (400)</b>	<b>0.4</b> (3.8)	<b>0.4</b> (3.8)	<b>0.9</b> (5.4)	<b>1.3</b> (6.6)

<b>Confidor</b>	<b>T1 (1120)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (560)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (4.2)
	<b>T3 (260)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Karate</b>	<b>T1 (3350)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.6</b> (4.3)
	<b>T2 (1675)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (838)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>1.2</b> (6.4)
	<b>T4 (400)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Tracer</b>	<b>T1 (400)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (200)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (100)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Tagthara</b>	<b>T1 (1000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>1.4</b> (6.8)
	<b>T2 (500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (4.2)
	<b>T3 (250)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>1.3</b> (6.6)
	<b>T4 (50)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Hostathion</b>	<b>T1 (8000)</b>	<b>9.0</b> (17.5)	<b>11.3</b> (19.6)	<b>18.0</b> (25.1)	<b>48.6</b> (44.2)
	<b>T2 (4000)</b>	<b>7.5</b> (15.9)	<b>9.1</b> (17.6)	<b>12.3</b> (20.5)	<b>38.1</b> (38.1)
	<b>T3 (2000)</b>	<b>4.8</b> (12.7)	<b>6.1</b> (14.3)	<b>11.4</b> (19.7)	<b>33.2</b> (35.2)
	<b>T4 (625)</b>	<b>1.5</b> (7.0)	<b>2.9</b> (9.9)	<b>4.9</b> (12.8)	<b>16.1</b> (23.7)
<b>Control</b>	<b>C1</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.6</b> (4.4)
	<b>C2</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>SEM +</b>		<b>1.3</b>	<b>1.5</b>	<b>1.3</b>	<b>2.4</b>
<b>CD (P= 0.05)</b>		<b>2.5</b>	<b>3.0</b>	<b>2.6</b>	<b>4.9</b>

(T1 – Double the Highest Recommended Doze; T2 – Highest Recommended Doze (HRD); T3 – Half of the HRD; T4 – Lowest Recommended Doze)

\* Data in parenthesis are arcsine transformed values

Table 4.1.6: Effect of different doses of herbicidal and fungicidal formulations on Infective Juveniles (IJs) of *S. thermophilum*

Pesticidal formulation	Treatments (Dose in ppm)	% mortality			
		24 Hour	48 Hour	72 Hour	7 Days
<b>Fernoxone</b>	<b>T1 (10000)</b>	<b>0.9</b> 5.3	<b>2.2</b> 8.5	<b>4.3</b> 12.0	<b>13.4</b> 21.5
	<b>T2 (5000)</b>	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.9</b> 5.6
	<b>T3 (2500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> 4.0
	<b>T4 (1250)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> 4.1
<b>Atrataf</b>	<b>T1 (18000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (9000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (4500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> 3.8
	<b>T4 (1425)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Clincher</b>	<b>T1 (3200)</b>	<b>0.5</b> 4.0	<b>0.5</b> 4.0	<b>0.5</b> 4.0	<b>0.5</b> 4.0
	<b>T2 (1600)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> 3.9
	<b>T3 (500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Isoguard</b>	<b>T1 (5400)</b>	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.5</b> 3.9
	<b>T2 (2700)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (1350)</b>	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.5</b> 3.9
<b>Stomp</b>	<b>T1 (20000)</b>	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.5</b> 3.9	<b>0.5</b> 3.9
	<b>T2 (10000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.9</b> 5.3
	<b>T3 (5000)</b>	<b>0.5</b> 4.1	<b>0.5</b> 4.1	<b>0.5</b> 4.1	<b>0.5</b> 4.1
	<b>T4 (3550)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Validamycin</b>	<b>T1 (21400)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (10700)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> 3.9
	<b>T3 (5350)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Bavistin</b>	<b>T1 (5400)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> 3.7
	<b>T2 (2700)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (1350)</b>	<b>0.4</b> (3.7)	<b>0.4</b> (3.7)	<b>0.4</b> (3.7)	<b>0.4</b> (3.7)
<b>Contaf</b>	<b>T1 (2000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> 3.9

	<b>T2 (1000)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (500)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Zinthane</b>	<b>T1 (21400)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (10700)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> 3.9
	<b>T3 (5350)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Control</b>	<b>C1</b>	<b>0.5</b> 4.0	<b>0.5</b> 4.0	<b>0.5</b> 4.0	<b>0.5</b> 4.0
	<b>C2</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>SEM +</b>		<b>1.4</b>	<b>1.4</b>	<b>1.4</b>	<b>2.0</b>
<b>CD (P= 0.05)</b>		<b>2.9</b>	<b>2.9</b>	<b>2.7</b>	<b>4.1</b>

(T1 – Double the Highest Recommended Doze; T2 – Highest Recommended Doze (HRD); T3 – Half of the HRD; T4 – Lowest Recommended Doze)

\* Data in parenthesis are arcsine transformed values

Table 4.1.7: Effect of different doses of commercial formulations of bio-agents on Infective Juveniles (IJs) of *S. thermophilum*

Pesticidal formulation	Treatments (Dose in g/L)	% mortality			
		24 Hour	48 Hour	72 Hour	7 Days
<b>Baba</b>	<b>T1 (6)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (3)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.9</b> (5.4)
	<b>T3 (1.5)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> (3.6)
<b>Metarhizium</b>	<b>T1 (6)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (3)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.9</b> (5.5)
	<b>T3 (1.5)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Mycomite</b>	<b>T1 (6)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (4.1)
	<b>T2 (3)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> (3.6)
	<b>T3 (1.5)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> (3.6)
<b>Nyantran</b>	<b>T1 (2)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (1)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (0.5)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.9</b> (5.5)
<b>Sparsha</b>	<b>T1 (6)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)

	<b>T2 (3)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>1.4</b> (6.8)
	<b>T3 (1.5)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<b>Nisarga</b>	<b>T1 (8)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (4)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T3 (2)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (3.9)
<b>Varsha</b>	<b>T1 (8)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>T2 (4)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> (3.7)
	<b>T3 (2)</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> 3.8)
<b>Control</b>	<b>C1</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
	<b>C2</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> (3.7)
	<b>C3</b>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (3.9)
<b>SEM ±</b>		<b>0.0</b>	<b>0.0</b>	<b>0.0</b>	<b>1.9</b>
<b>CD (P= 0.05)</b>		<b>0.0</b>	<b>0.0</b>	<b>0.0</b>	<b>4.0</b>

(T1 – Recommended Doze (RD); T2 – Half of the RD; T3 – 1/4<sup>th</sup> of RD)

\* Data in parenthesis are arcsine transformed values

Table 4.1.8: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* infected with IJs of *S. thermophilum* exposed to the highest recommended dozes of insecticidal formulations for 48 hours.

Insecticidal Formulations	% Mortality		
	24 Hour	48 Hour	72 Hour
<b>Tagride</b>	<b>43.8</b> (41.5)	<b>68.8</b> (56.1)	<b>100.0</b> (90.0)
<b>Talstar</b>	<b>68.8</b> (56.1)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
<b>Marshal</b>	<b>68.8</b> (56.1)	<b>81.3</b> (64.4)	<b>100.0</b> (90.0)
<b>Dursban</b>	<b>6.3</b> (14.5)	<b>12.5</b> (20.7)	<b>18.8</b> (25.7)
<b>Superkiller</b>	<b>93.8</b> (75.6)	<b>93.8</b> (75.6)	<b>100.0</b> (90.0)
<b>Decis</b>	<b>56.3</b> (48.6)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
<b>Thiodan</b>	<b>12.5</b> (20.7)	<b>31.3</b> (34.0)	<b>37.5</b> (37.8)
<b>Confidor</b>	<b>75.0</b> (60.0)	<b>93.8</b> (75.6)	<b>100.0</b> (90.0)
<b>Karate</b>	<b>93.8</b> (75.6)	<b>100.0</b> (90.0)	<b>100.0</b> (90.0)

<b>Tracer</b>	<b>68.8</b> (56.1)	<b>87.5</b> (69.3)	<b>100.0</b> (90.0)
<b>Tagthara</b>	<b>56.3</b> (48.6)	<b>87.5</b> (69.3)	<b>100.0</b> (90.0)
<b>Hostathion</b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)
<b>Control</b>	<b>56.3</b> (48.6)	<b>81.3</b> (64.4)	<b>100.0</b> (90.0)
<b>SEM</b>	<b>13.3</b>	<b>9.7</b>	<b>3.3</b>
<b>CD (P= 0.05)</b>	<b>28.7</b>	<b>20.9</b>	<b>7.1</b>

\* Data in parenthesis are arcsine transformed values

Table 4.1.9: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* when treated with IJs of *S. thermophilum* exposed to highest recommended dozes of Herbicidal and fungicidal formulations for 48 hours.

<b>Pesticidal Formulations</b>	<b>% Mortality</b>		
	<b>24 Hour</b>	<b>48 Hour</b>	<b>72 Hour</b>
<b>Fernoxone</b>	<b>12.5</b> (20.7)	<b>62.5</b> (52.3)	<b>100.0</b> (90.0)
<b>Atrataf</b>	<b>18.8</b> (25.7)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
<b>C lencher</b>	<b>18.8</b> (25.7)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
<b>Isoguard</b>	<b>18.8</b> (25.7)	<b>56.3</b> (48.6)	<b>100.0</b> (90.0)
<b>Stomp</b>	<b>18.8</b> (25.7)	<b>56.3</b> (48.6)	<b>100.0</b> (90.0)
<b>Validamycin</b>	<b>12.5</b> (20.7)	<b>68.8</b> (52.3)	<b>100.0</b> (90.0)
<b>Bavistin</b>	<b>12.5</b> (20.7)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
<b>Hexaconazole</b>	<b>18.8</b> (25.7)	<b>62.5</b> (52.3)	<b>100.0</b> (90.0)
<b>Mancozeb</b>	<b>18.8</b> (25.7)	<b>62.5</b> (52.3)	<b>100.0</b> (90.0)
<b>Control</b>	<b>18.8</b> (25.7)	<b>62.5</b> (52.3)	<b>100.0</b> (90.0)
<b>SEM</b>	<b>12.4</b>	<b>10.3</b>	<b>0.0</b>
<b>CD (P= 0.05)</b>	<b>27.6</b>	<b>22.9</b>	<b>0.0</b>

\* Data in parenthesis are arcsine transformed values

Table 4.1.10: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* inoculated with IJs of *S. thermophilum* exposed to recommended dozes of commercial formulations of bioagents for 48 hours.

Formulations	% Mortality		
	24 Hour	48 Hour	72 Hour
<b>Baba</b>	<b>6.3</b> (14.5)	<b>56.3</b> (48.6)	<b>100.0</b> (90.0)
<b>Metarhizium</b>	<b>12.5</b> (20.7)	<b>62.5</b> (52.3)	<b>100.0</b> (90.0)
<b>Mycomite</b>	<b>12.5</b> (20.7)	<b>68.8</b> (56.0)	<b>100.0</b> (90.0)
<b>Niyrantran</b>	<b>18.8</b> (25.7)	<b>56.3</b> (48.6)	<b>100.0</b> (90.0)
<b>Sparsha</b>	<b>12.5</b> (20.7)	<b>68.8</b> (56.0)	<b>100.0</b> (90.0)
<b>Nisarga</b>	<b>18.8</b> (25.7)	<b>62.5</b> (52.3)	<b>100.0</b> (90.0)
<b>Varsha</b>	<b>12.5</b> (20.7)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
<b>Control</b>	<b>18.8</b> (25.7)	<b>68.8</b> (56.0)	<b>100.0</b> (90.0)
<b>SEM</b>	<b>12.2</b>	<b>9.9</b>	<b>0.0</b>
<b>CD (P= 0.05)</b>	<b>28.2</b>	<b>22.9</b>	<b>0.0</b>

\* Data in parenthesis are arcsine transformed values

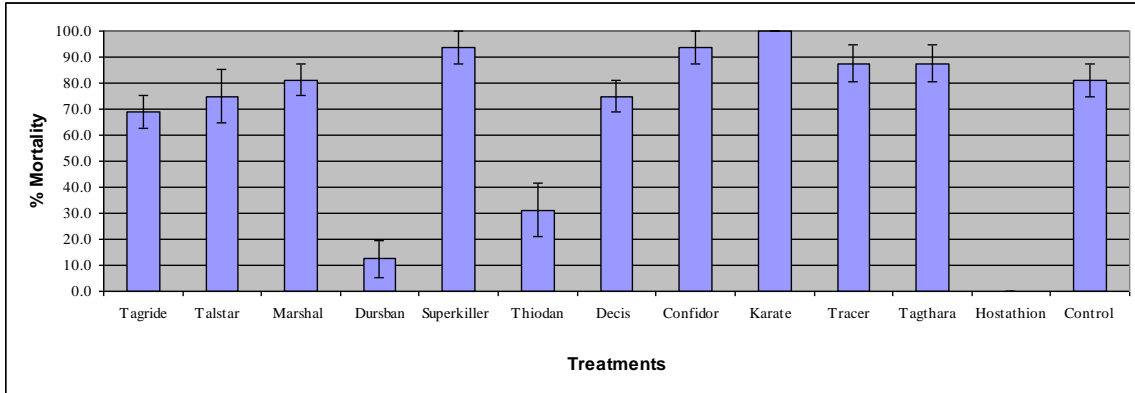


Figure 4.1.1: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* after 48 hours when infected with IJs of *S. thermophilum* exposed to highest recommended doses of insecticidal formulations for 48 hours

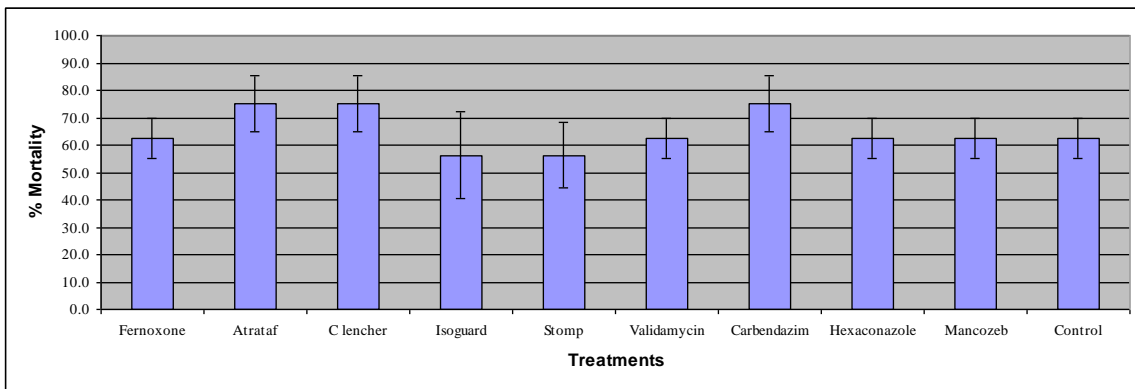


Figure 4.1.2: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* after 48 hours when infected with IJs of *S. thermophilum* exposed to highest recommended doses of herbicidal and fungicidal formulations for 48 hours

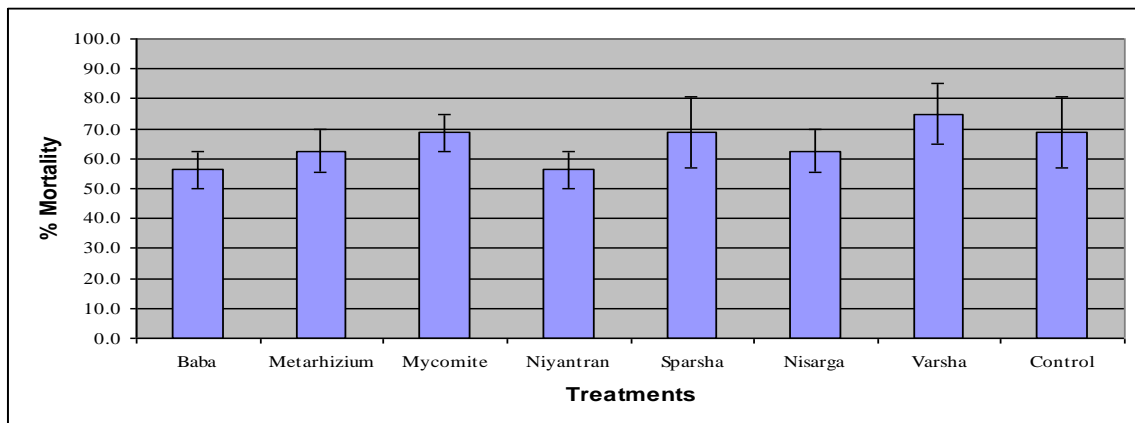


Figure 4.1.3: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* after 48 hours when infected with IJs of *S. thermophilum* exposed to highest recommended doses of commercial formulations of bio-agents for 48 hours

they were almost dead. The loss of virulence of IJs was evident as no mortality of *G. mellonella* larvae was observed when IJs exposed to highest recommended dose of Hostathion for 48 hours treated on it (Table 4.1.8). This study indicated that Hostathion is not compatible with *S. thermophilum* and it is not advisable to mix them together.

The significant levels of mortality of IJs were observed in all the doses of Dursban and three higher doses of Thiodan after 7 days of treatment (Table 4.1.5). The rate of mortality increased in all the treatments of both the formulations from 24 hours to 7 days. A maximum of 66.5 % and 49.4 % mortality was observed in double the highest recommended doses of Dursban and Thiodan respectively after 7 days of treatment and the former was the maximum recorded mortality among all the treatments of pesticides. All the treatments of Thiodan except lowest 2 doses in 24 h, were found to be significantly different in inducing mortality of infective juveniles. There were no significant differences among the nearest doses of Dursban in 48 hours of treatment and between 2<sup>nd</sup> and 3<sup>rd</sup> doses in 24 and 72 h of treatment. Even though the lowest recommended dose of Dursban caused only 6.6 % of nematode mortality, it was statistically significant. The reduction in the virulence of infective juveniles treated with highest recommended doses of Dursban and Thiodan were observed as it induced larval mortality of only 18.8 and 37.5 % respectively after 72 h of treatment (Table 4.1.8). It indicates that some of the Infective juveniles retained their virulence even after their exposure to these two insecticides. But only 50 % of cadavers killed by Dursban treated infective juveniles showed emergence of next generation infective juveniles, whereas there was no emergence of infective juveniles from any of the cadavers killed by Thiodan treated nematodes.

There was no record of mortality of infective juveniles in all the tested doses of commercial formulations of bioagents up to 72 hours of treatment (Table 4.1.7). Even though up to 1.4 % mortality was recorded after 7 days of exposure in half of the recommended dose of Sparsha, it was non-significant as compared to the control. In all the other treatments the mortality ranged from 0 to 0.9 % after 7 days of treatment and none of these mortality data were significant when compared to the control. The infective juveniles exposed to recommended doses of these commercial formulations of bioagents induced 100 % mortality of 4<sup>th</sup> instar larvae of *G. mellonella* in 72 hours and the mortality levels were non-significant in any of the time intervals (Table 4.1.10).

#### 4.1.4 Discussions

Studies on compatibility of any bioagent with other management options including agrochemicals are essential for their integration in the integrated pest management programs. There are studies, addressing the compatibility of different entomopathogenic nematodes with several pesticidal formulations. Most of the nematode pesticide combinations were found compatible where as few of them were incompatible (Grewal *et al.*, 2005). The compatibility of Imidacloprid (Merit 75 WP) with Entomopathogenic nematodes *Heterorhabditis bacteriophora* and *S. carpocapsae* were reported in several studies (Koppenhöfer *et al.*, 2003; Alumai and Grewal, 2004). Anuar and Daniel (2009) observed the synergistic interaction between *H. bacteriophora* and Imidacloprid against white grubs. Another neonicotinoid insecticide Thiomethoxam (Meridian 25%) were found to be compatible with *H. megidis*, *S. feltiae*, *S. glaseri* and *S. carpocapsae* where as non-compatible interaction were also observed for the same formulation with *H. bacteriophora* (Koppenhöfer *et al.*, 2003; Alumai and Grewal, 2004). Alumai and Grewal (2004) also established the compatibility of Spinosad (Conserve SC) with *H. bacteriophora* and *S. carpocapsae*. Previous studies on compatibility of different entomopathogenic nematodes and agrochemicals indicated that the compatibility varies in different EPN - pesticide combinations. Even some inert ingredients used in the pesticide formulations can be detrimental to the survival of entomopathogenic nematodes (Krishnayya and Grewal, 2002). As nematode species can differ in sensitivity to different agrochemicals and even different formulations of the same chemical, compatibility of each formulation with the specific nematode species should be evaluated for their integration. The compatibility of indigenous entomopathogenic nematode *S. thermophilum* with different pesticidal formulations was evaluated for the first time. In the present study we established the compatibility of *S. thermophilum* with 23 pesticidal formulations, and these formulations in their recommended doses could be tank mixed and applied together. As compatibility of entomopathogenic nematodes and pesticides varies with different species and formulations, the compatibility of each pesticide - EPN combination should be established before any recommendations for their combined application.

#### 4.1.5 Conclusion

Our study has demonstrated that, the pesticidal formulations namely, Tagride (Acetamiprid), Talstar (Bifenthrin), Marshal (Carbosulfan), Superkiller (Cypermethrin), Decis (Deltamethrin), Confidor (Imidachloprid), Karate (Lambdacyhalothrin), Tracer (spinosad), Tagtara (Thiomethoxam), Atrataf (Atrazine), 2,4-D Sodium Salt (Fenoxone), Cyhalofop-butyl (Clincher), Isoguard (Isoproturon), Stomp (Pendimethalin), Validamycin (Validamycin), Zinthane (Mancozeb), Bavistin (Carbendazim), Contaf (Hexaconazole), Baba (*B. bassiana*), Metarhizium (*M. anisopliae*), Mycomite (*P. fumosoroseus*), Niyrantran (*P. lilacinus*), Sparsha (*P. fluorescens*), Nisarga (*T. viride*) and Varsha (*V. lecani*) are compatible with entomopathogenic nematode *S. thermophilum* and these pesticidal formulations in their recommended doses could be tank mixed with the same nematode. However Hostathion, Dursban and Thiodan were found to be non-compatible with *S. thermophilum*.

## 4.2 RESEARCH PAPER II

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### **Compatibility of entomopathogenic nematode, *Steinernema thermophilum* (Nematoda: Rhabditida) with different surfactants/adjuvants commonly used in spray mixtures**

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**Abstract:** The compatibility of indigenous heat tolerant entomopathogenic nematode, *Steinernema thermophilum* with different surfactants/adjuvants commonly used in the spray mixtures was evaluated. The infective juveniles (IJs) of *S. thermophilum* were exposed to 2 different doses (1% and 2%) of 8 surfactants/adjuvants (Apsa 80, CMC, glycerol, Liquid Paraffin, Starch, Triton X 100, Tween 20 and Tween 80) for a period of one month. All these surfactants/ adjuvants found to be compatible with *S. thermophilum* as they did not affect either viability or virulence of the nematode even after their exposure to the same nematode for a period of one month.

**Key words:** Compatibility, *S. thermophilum*, surfactants/adjuvants.

#### **4.2.1 Introduction**

The environmental and health concerns caused by indiscriminate use of chemical pesticides have lead to an increasing interest for the use of entomopathogenic nematodes (EPNs) (Nematoda: Rhabditida) for the control of various agricultural pests. The steinernematids are obligatorily associated with symbiotic bacteria *Xenorhabdus* spp., which contribute to the rapid killing of their host insects (Han and Ehlers, 2000). They can be easily mass cultured *in vitro* (Ehlers, 2001), have good safety records on non-target organisms especially regarding their effects on predators and pollinators. In addition, no evidence exists for mammalian pathogenicity (Ehlers, 2003). Successful use of entomopathogenic nematodes for the pest management depends on various biotic and abiotic factors. A number of these factors can be controlled and optimized to improve efficacy (Shapiro-Ilan *et al.*, 2006a). For example, a biotic factor that can enhance efficacy is the choice of nematode species or strain in relation to the target host (Shapiro-Ilan *et al.*, 2002, 2006a). The major obstacle for successful pest control with EPNs are low persistence in the foliage especially due to low

relative humidity (RH) resulting in quiescence of the nematodes and then lethal desiccation of the infective dauer juveniles (DJs) before they have penetrated the host. Hence application under favorable soil moisture levels is critical (Georgis and Gaugler, 1991; Kaya and Gaugler, 1993). Webster (1973) proposed manipulation of habitats to favor DJ survival and infectivity before application. Manipulation of macro environment (weather) is impossible. Maintaining the micro climate (substrate humidity) may give success in the control of insect pests using EPNs (Navaneethan *et al.*, 2010). Thus, application technology and possibly improved formulation can help to overcome the problems with low RH. Additionally, efficacy can be enhanced through improved formulation or addition of adjuvants (Wright *et al.*, 2005; Shapiro-Ilan *et al.*, 2006a). Surfactants are one group of adjuvants that can enhance nematode efficacy (Wright *et al.*, 2005). Schroeder and Sieburth (1997) observed increased nematode suppression of the Diaprepes root weevil, *Diaprepes abbreviatus* (L.), with the addition of surfactants.

The indigenous entomopathogenic nematode *S. thermophilum* described by Ganguly and Singh (2000) were found to be heat tolerant and efficacious against a number of insect pests (Ganguly *et al.*, 2004). A clear understanding on the compatibility of *S. thermophilum* with different adjuvants/surfactants will be useful in mixing the compatible adjuvants/surfactants along with the suspension of this nematode to enhance its field efficacy. Besides, it will be useful in making novel formulations of this entomopathogenic nematode. So the present study was carried out to evaluate the compatibility of *S. thermophilum* with a total of 8 adjuvants/surfactants.

#### **4.2.2 Materials and method**

The entomopathogenic nematode used in the investigation was cultured *in vivo* on 4<sup>th</sup> instar larvae of *Galleria mellonella*. The infective juveniles (IJs) emerged from the cadavers were harvested in White's trap, stored at 15 °C and used within 1 week of storage. A total of 8 surfactants/adjuvants namely APSA 80, Carboxy Methyl Cellulose (CMC), glycerol, Liquid Paraffin, Starch, Triton X 100, Tween 20 and Tween 80 were used for the present study. These surfactants/adjuvants procured from Himedia Laboratories, SRL Pvt. Ltd., Qualigens fine chemicals and Amway distributors. Two different concentrations of these surfactants/adjuvants (1 % and 3 %) were prepared in sterile distilled water. The experiment

was carried out in 6 well plates where 2 ml of required concentrations of each surfactant was taken in each well of the plates and 50 infective juveniles of *S. thermophilum* in 20 µl of sterile distilled water was added to each well. All the treatments were replicated five times and infective juveniles in the sterile distilled water were used as control. The bottoms of the wells were marked in to square sections to facilitate counting and the experiment was conducted at  $25 \pm 1$  °C. The observations on the survival of infective juveniles in the respective treatments were taken at an interval of 10 days up to 30 days. The virulence of infective juveniles of *S. thermophilum* exposed to 3 % of the surfactants for a period of 30 days were evaluated on 4<sup>th</sup> instar larvae of *G. mellonella*. Double the required concentrations (6 %) of the surfactants were prepared in sterile distilled water. One ml of the double strength of surfactants were taken in each well of the 6 well plates and 1 ml of the infective juveniles of *S. thermophilum* in sterile distilled water was added to the each well. All the treatments were replicated 5 times and these were then incubated at  $25 \pm 1$  °C. All the replications of the same treatment were pooled together and 50 IJs in 50 µl suspension were added to each well of the 12 well plates lined with double layer of Whatman No. 1 filter paper. Each well was diluted with 50 µl of sterile distilled water and 4<sup>th</sup> instar larvae of *G. mellonella* were placed in each well. There were four replications and each comprised of four larvae in separate wells. The nematode treated larvae in the 12 well plates were kept at  $25 \pm 1$  °C and observed for larval mortality after every 24 hours. The cadavers collected from different treatments were transferred to White's trap and observed for the emergence of infective juveniles.

The experimental design used was Completely Randomized Design (CRD) and all the data were subjected to statistical analysis and differences between treatment means were estimated by LSD test at  $P < 0.05$  probability.

### **4.2.3 Results**

A maximum of only 4.1 % mortality of infective juveniles were observed in 3 % concentration of Tween 80 followed by 3.7 and 3.6 % nematode mortality in 3 % concentrations of Tween 20 and Apsa 80 respectively after 30 days of treatment. The lowest mortality of infective juveniles were recorded in 1 % concentration of Glycerol and Starch (0.5 % mortality) after 30 days of treatment. The 3 % concentration of Tween 20 recorded a mortality level of 2.6 % after 20 days of exposure, which was the highest mortality recorded

among all the treatments for the same time duration. In control also, the mortality of infective juveniles was recorded, which was 0.4, 1.2 and 1.6 % after 10, 20 and 30 days. So, none of the observations in any of the treatments were statistically significant (Table 4.2.1).

The infective juveniles exposed to 3 % concentrations of all these surfactants/adjuvants for a period of 30 days when treated on 4<sup>th</sup> instar larvae of *G. mellonella* induced 100 % larval mortality within 72 hours of treatment and next generation infective juveniles emerged from all the cadavers. The level of mortality in 24 hours and 48 hours of treatment showed some differences among the treatments. Among the surfactants Apsa 80, Triton X 100 (87.5 %) Starch and Tween 20 (93.8 %) observed to improve the level of insect mortality in 48 hours. The IJs treated with surfactants namely CMC, Liquid paraffin and Tween 80 recorded the lowest larval mortality of 62.5 % after 48 hours of treatment and these levels of mortality were found to be significantly different from that of starch and Tween 20 (93.8 % larval mortality). However any of these mortality data were statistically significant when compare with the control (Table 4.2.2 & Fig. 4.2.1).

#### **4.2.4 Discussion**

As different surfactants act differently with nematodes in their compatibility/toxicity and each nematode species may also differ in sensitivity to different adjuvants (Grewal *et al.*, 2005) the compatibility of each adjuvant with specific nematode species should be evaluated for better result. Here we observed the mortality of IJs of *S. thermophilum* ranging from 0.5 to 4.1 % in different treatments of adjuvants after 30 days of exposure. The reason for the mortality of infective juveniles in all the treatments could be natural rather than due to treatment effects, as we could also observe 1.6 % of nematode mortality in the control in 30 days of treatment and the mortality data in different treatments are statistically non significant when compared with control. The IJs exposed to 3 % concentrations of different adjuvants for a period of 30 days when treated on 4<sup>th</sup> instar larvae of *G. mellonella* recorded a variation in larval mortality in different treatments ranging from 62.5 to 93.8 % in 48 hours, but all the treatments induced 100 % larval mortality in 72 hours. This observation confirmed the retention of virulence in IJs exposed to 3 % concentrations of all the surfactants for a period of 30 days. So our study found that these adjuvants/surfactants are compatible with *S. thermophilum* without any harmful effect on either viability or the virulence of this

Table 4.2.1: Effect of different surfactants/adjuvants on Infective Juveniles (IJs) of *S. thermophilum*

Treatments	Concentration	% Mortality of IJs		
		10 DAT	20 dat	30 DAT
<b>Apsa 80</b>	3 %	<b>0.4</b> (3.6)	<b>0.8</b> (5.2)	<b>3.6</b> (11.0)
	1 %	<b>0.0</b> (0.4)	<b>0.5</b> (4.1)	<b>1.0</b> (5.9)
<b>CMC</b>	3 %	<b>0.0</b> (0.4)	<b>0.5</b> (4.0)	<b>2.0</b> (8.1)
	1 %	<b>0.0</b> (0.4)	<b>0.5</b> (4.2)	<b>2.1</b> (8.4)
<b>Glycerol</b>	3 %	<b>0.0</b> (0.4)	<b>0.4</b> (3.6)	<b>0.8</b> (5.2)
	1 %	<b>0.0</b> (0.4)	<b>0.5</b> (4.1)	<b>0.5</b> (4.1)
<b>Liquid Paraffin</b>	3 %	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>1.2</b> (6.3)
	1 %	<b>0.0</b> (0.4)	<b>0.5</b> (4.1)	<b>1.0</b> (5.8)
<b>Starch</b>	3 %	<b>0.0</b> (0.4)	<b>0.8</b> (5.1)	<b>1.6</b> (7.2)
	1 %	<b>0.0</b> (0.4)	<b>0.5</b> (3.9)	<b>0.5</b> (3.9)
<b>Triton X 100</b>	3 %	<b>0.3</b> (3.3)	<b>0.3</b> (3.3)	<b>1.0</b> (5.8)
	1 %	<b>0.0</b> (0.4)	<b>0.9</b> (5.5)	<b>1.4</b> (6.8)
<b>Tween 20</b>	3 %	<b>1.1</b> (5.9)	<b>2.6</b> (9.4)	<b>3.7</b> (11.1)
	1 %	<b>0.5</b> (3.9)	<b>0.9</b> (5.5)	<b>1.8</b> (7.8)
<b>Tween 80</b>	3 %	<b>1.0</b> (5.8)	<b>1.5</b> (7.1)	<b>4.1</b> (11.7)
	1 %	<b>1.0</b> (5.6)	<b>1.4</b> (6.9)	<b>2.9</b> (9.8)
<b>Control</b>		<b>0.4</b> (3.6)	<b>1.2</b> (6.2)	<b>1.6</b> (7.2)
<b>SEM</b>		<b>1.8</b>	<b>2.8</b>	<b>3.2</b>
<b>CD (P= 0.05)</b>		<b>3.7</b>	<b>5.9</b>	<b>6.8</b>

Table 4.2.2: Virulence of Infective juveniles of *S. thermophilum* on 4<sup>th</sup> instar larvae of *G. mellonella* after one month exposure to different surfactants/adjuvants

Treatments	% Mortality of insect larvae		
	24 Hour	48 Hour	72 Hour
Apsa 80	43.8 (41.4)	87.5 (69.3)	100.0 (90.0)
CMC	18.8 (25.7)	62.5 (52.3)	100.0 (90.0)
Glycerol	37.5 (37.8)	75.0 (60.0)	100.0 (90.0)
Liquid Paraffin	37.5 (37.8)	62.5 (52.3)	100.0 (90.0)
Starch	62.5 (52.3)	93.8 (75.6)	100.0 (90.0)
Triton X 100	31.3 (34.0)	87.5 (69.3)	100.0 (90.0)
Tween 20	37.5 (37.8)	93.8 (75.6)	100.0 (90.0)
Tween 80	37.5 (37.8)	62.5 (52.3)	100.0 (90.0)
Control	37.5 (37.8)	81.3 (64.4)	100.0 (90.0)
SEM	6.6	10.2	0.0
CD (P= 0.05)	15.0	23.0	0.0

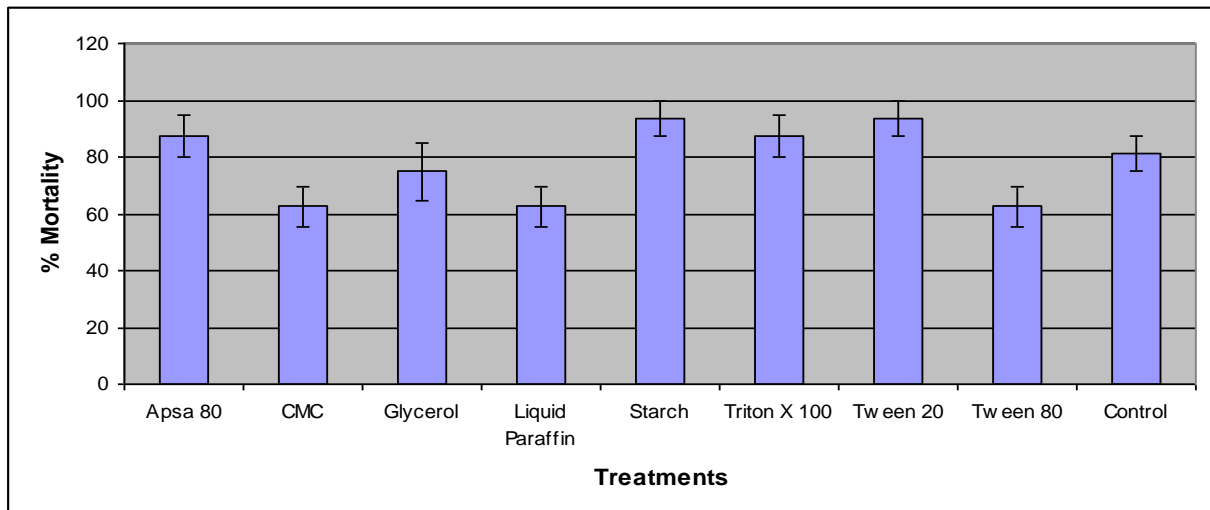


Figure 4.2.1: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* after 48 hours of treatment with IJs of *S. thermophilum* exposed to surfactants/adjuvants for a period of 1 month

entomopathogenic nematode. The recommended concentration of most of the adjuvants for the field application of pesticides or biopesticides is only 0.05 to 0.5 %. As we established the compatibility of these adjuvants up to a concentration of 3 %, it will be absolutely safe to mix these adjuvants with the suspension of *S. thermophilum* in the recommended concentrations to enhance the efficacy of this entomopathogenic nematode. As the field efficacy of entomopathogenic nematodes can be enhanced through improved formulation or addition of adjuvants (Wright *et al.*, 2005; Shapiro-Ilan *et al.*, 2006a) this information could be useful in the better use of *S. thermophilum* against various insect pests.

#### **4.2.5 Conclusion**

Conclusively the surfactant/adjuvants namely Apsa 80, CMC, glycerol, Liquid Paraffin, Starch, Triton X 100, Tween 20 and Tween 80 at 3 % concentrations have no adverse effect on the survival or virulence of infective juveniles of *S. thermophilum* up to a period of 1 month. So our result suggests that these surfactants/adjuvants are not harmful for the same nematode and it could be tank mixed at recommended concentrations to enhance the field efficacy of *S. thermophilum*.

## 4.3 RESEARCH PAPER III

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### **Compatibility of entomopathogenic nematode *Steinernema thermophilum* (Nematoda: Rhabditida) with other bio agents and organic amendments**

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**Abstract:** The compatibility of entomopathogenic nematode *Steinernema thermophilum* was evaluated with different bioagents namely *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces lilacinus*, *Pochonia chlamydosporium*, *Trichoderma viride*, *T. harzianum* (fungal bioagents) *Bacillus thuringiensis* and *Pseudomonas fluorescens* (bacterial bioagents) as well as with organic amendments like bone meal, farm yard manure (FYM), neem cake and vermicompost. All the fungal bioagents and organic amendments were found to have no harmful effect on the infective juveniles of *S. thermophilum*, where as both the bacterial bioagents induced significant mortality of infective juveniles, in the immersion bio-assay study. The retention of virulence of infective juveniles after exposing to the fungal bioagents and organic amendments was evaluated on 4<sup>th</sup> instar larvae of *Galleria mellonella*. However, *S. thermophilum* and its symbiotic bacteria were observed to suppress the growth of fungal bioagents namely *B. bassiana* and *M. anisopliae* in the solid growth media without any adverse effect on the growth and development of the nematode. None of the entomopathogenic fungi or bacteria evaluated was found compatible with *S. thermophilum* and it is not advisable to combine both for the same target. However, *S. thermophilum* could be combined with fungal bioagents like *P. lilacinus*, *P. chlamydosporium*, *T. viride* and *T. harzianum* which target other pathogenic organisms of agricultural crops. The organic amendments like bone meal, FYM, Neem cake and vermicompost can also be applied in combination with *S. thermophilum*.

**Key words:** Compatibility, *S. thermophilum*, Fungal bioagents, Bacterial bioagents.

#### **4.3.1 Introduction**

Entomopathogenic nematodes belonging to the families Steinernematidae and Heterorhabditidae are obligate parasites. They kill the insect host with the aid of a mutualistic bacterium, which is carried in their intestine (*Xenorhabdus* spp. and *Photorhabdus* spp. are

associated with *Steinernema* spp. and *Heterorhabditis* spp., respectively). The nematodes complete 2–3 generations within the host, after which free living infective juveniles emerge to seek new hosts (Poinar, 1990). Pest control can be greatly improved by combination of compatible bio-control agents. In several studies targeting insect pests, synergistic (Barbercheck and Kaya, 1991; Choo *et al.*, 2002; Ansari *et al.*, 2004) as well as antagonistic (Shapiro-Ilan *et al.*, 2004; Ansari *et al.*, 2005) interactions have been observed with certain combinations of entomopathogenic nematodes with other bioagents. The interactions between EPNs and insect pathogenic fungi can be additive, antagonistic, or synergistic depending on the EPN species, insect host, application rate, and time of application (Koppenhöfer & Kaya, 1997; Ansari *et al.*, 2004, 2006).

In the present investigation, we have evaluated six fungal bioagents (*Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces lilacinus*, *Pochonia chlamydosporium*, *Trichoderma viride* and *T. harzianum*) 2 bacterial bioagents (*Bacillus thuringiensis*, *Pseudomonas fluorescens*) and 4 organic amendments (Bone meal, neem cake, FYM and vermicompost) for their compatibility with indigenous entomopathogenic nematode *Steinernema thermophilum*.

### **4.3.2 Materials and Methods**

The entomopathogenic nematode *Steinernema thermophilum* already available in our laboratory was cultured *in vivo* on 4<sup>th</sup> instar larvae of *Galleria mellonella* (Kaya and Stock, 1997) for use in the present study. The infective juveniles were harvested by modified White's trap, stored at 15 °C and used for the study within 1 week of storage. All the fungal bioagents used in the study were procured from Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology, IARI, New Delhi. The bacterial bioagents were procured from National Centre for Integrated Pest Management (NCIPM), New Delhi. The organic amendments use for the study was collected from the Division of Agronomy, IARI, New Delhi.

**4.3.1.1 Viability study:** Fifteen days old cultures of fungal bioagents namely *B. bassiana* and *M. anisopliae* cultured in Sabouraud's Dextrose broth medium and other bioagents grown in Potato Dextrose Broth medium were used for the immersion bio-assay study. The

bacterial bioagents of 72 hours growth in Nutrient broth medium were used for the present investigation. The fungal and bacterial bioagents grown in their respective media for above mentioned time period were considered as standard stock solution (S). The organic amendments mixed with water in the ratio 1:1 (Organic amendments: water) and strained through muslin cloth was considered as double strength of standard stock (2S). Four different concentrations of bacterial cultures and extracts of organic amendments (S, S/2, S/4 and S/8) and Standard stock (S) of the fungal bioagents were evaluated for their effect on the survival of infective juveniles of *S. thermophilum*. All the required concentrations of the bacterial bioagents and organic amendments were prepared by dilution of the Standard stock with sterile distilled water. The immersion bio-assay study was carried out in 6 well plates marked with square sections in the bottom of the wells to facilitate nematode count. The infective juveniles @ 50 specimens in 20 µl of sterile distilled water were added to each well of the 6 well plates with 2 ml of required concentrations of respective bioagents. All the treatments were replicated 5 times and the infective juveniles in sterile distilled water as well as in respective growth media were used as control. The observations on nematode survival/mortality were taken at 24 h, 48 h, 72 h and 7 days after the treatment. The virulence potential of IJs in different dilutions of organic amendments was also evaluated in 6 well plates, where 1ml of double strength of required concentrations in each well was mixed with 1 ml of nematode suspension containing about 2000 infective juveniles of *S. thermophilum*. All the treatments were replicated 5 times and the infective juveniles in sterile distilled water were used as control. The observations on nematode survival were recorded on 5<sup>th</sup> and 10<sup>th</sup> day of treatment by removing about 25 µl of the suspension from each well. All the treatments were held at 25±1 °C

#### **4.3.1.2 Infectivity study**

The infective juveniles exposed to S/2 concentration of fungal bioagents and S concentration of organic amendments for a period of 48 h was evaluated for their infectivity on 4<sup>th</sup> instar larvae of *G. mellonella*. The infective juveniles were washed 3 times with sterile distilled water and 50 IJs from each treatment were drawn and added on double layers of Whatman No. 1 filter paper in each well of the 12 well plates. Four *G. mellonella* larvae were added in four different wells with one larva in each well. Each treatment was replicated four times and the plates were incubated at 25±1°C. The mortality of *G. mellonella* larvae

was recorded at 24 h interval and the cadavers were shifted to Petri plates of 4 cm diameter containing double layers of Whatman No. 1 filter paper and observed for the emergence of IJs.

#### **4.3.1.3 Interaction study on solid culture media**

The mutual interaction of entomopathogenic fungi *B. bassiana* and *M. anisopliae* with *S. thermophilum* and its symbiotic bacterium, *Xenorhabdus indica* was evaluated on 3 different culture media namely, Lipid agar (LA) medium, Nutrient agar (NA) medium and Sabouraud's dextrose agar (SDA) medium in 10 cm diameter Petri plates. The infective juveniles of *S. thermophilum* surface sterilized with 0.1 % hyamine solution was used for the interaction study. The study was carried out by the simultaneous inoculation of *S. thermophilum* or *X. indica* along with *B. bassiana* or *M. anisopliae* on 2 sides of the same Petri plate as well as by delayed inoculation of *S. thermophilum* on the plates where fungal bioagents were already established. All the treatments were replicated 3 times and held at  $25 \pm 1$  °C. The observations on the growth and development of bioagents were recorded at 24 h interval.

#### **4.3.3 Results**

There was no record of nematode mortality by any of the fungal bioagents up to 72 h of treatment. Even after 7 days of exposure, a maximum of only 0.9 % mortality was recorded in *Paecilomyces lilacinus* and none of the treatments yielded any significant mortality (Table 4.3.1). The infective juveniles exposed to S/2 concentration of the fungal bioagents induced 100 % larval mortality of 4<sup>th</sup> instar larvae of *G. mellonella* in 72 hours and next generation infective juveniles were emerged from all the cadavers (Table 4.3.2). It showed that the exposure to fungal bioagents did not affect the virulence potential of nematodes. In contrast, both the bacterial bioagents induced higher rate of mortality of infective juveniles in all the concentrations evaluated (Table 4.3.3). The level of mortality of infective juveniles was observed to increase as the concentration and time of exposure increased. The standard stock of the *B. thuringiensis* and *P. fluorescens* induced a nematode mortality of 91.4 and 80.1 % respectively in 7 days of treatment. Among the different treatments of *B. thuringiensis*, highest concentration (S) showed significant difference in mortality only with lowest concentration (S/8) in 24 hours and there were no significant

Table 4.3.1: Effect of standard stock<sup>a</sup> (S) concentration of different fungal bio-agents on Infective juveniles (IJs) of *S. thermophilum*

Treatments	% Mortality of IJs at different exposure period			
	24 Hour	48 Hour	72 Hour	7 Days
<i>B. bassiana</i>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> (3.8)
<i>M. anisopliae</i>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
<i>P. lilacinus</i>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.9</b> (5.5)
<i>P. chlamydosporium</i>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.9</b> (5.5)
<i>T. viride</i>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (4.1)
<i>T. harzianum</i>	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
Control-1 (PDB)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.4</b> (3.8)
Control-2 (SDA)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.5</b> (3.9)
Control-3 (Water)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)	<b>0.0</b> (0.4)
SEM	0.0	0.0	0.0	2.0
CD (P= 0.05)	0.0	0.0	0.0	4.6

\* Data in parenthesis are arcsine transformed values; a-72 h old culture broth

Table 4.3.2: Virulence of *S. thermophilum* against 4<sup>th</sup> instar larvae of *Galleria mellonella* after the exposure of infective juveniles to S/2 concentration of different fungal bioagents for a period of 48 hours

Treatments	% Mortality of insect larvae		
	24 Hour	48 Hour	72 Hour
<i>B. bassiana</i>	31.3 (34.0)	68.8 (56.0)	100.0 (90.0)
<i>M. anisopliae</i>	25.0 (30.0)	68.8 (56.0)	100.0 (90.0)
<i>P. lilacinus</i>	31.3 (34.0)	62.5 (52.3)	100.0 (90.0)
<i>P. chlamydosporium</i>	25.0 (30.0)	68.8 (56.0)	100.0 (90.0)
<i>T. viride</i>	37.5 (37.8)	68.8 (56.0)	100.0 (90.0)
<i>T. harzianum</i>	31.3 (34.0)	62.5 (52.3)	100.0 (90.0)
Control-1 (PDB)	25.0 (30.0)	75.0 (60.0)	100.0 (90.0)
Control-2 (SDA)	31.3 (34.0)	62.5 (52.3)	100.0 (90.0)
Control-3 (Water)	25.0 (30.0)	75.0 (60.0)	100.0 (90.0)
SEM	7.5	13.4	0.0
CD (P= 0.05)	17.0	30.2	0.0

\* Data in parenthesis are arcsine transformed values

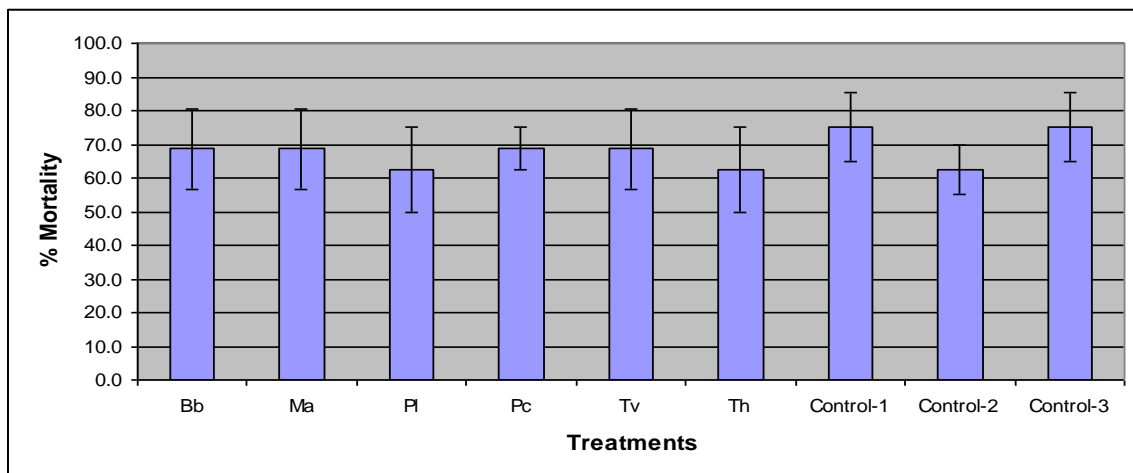


Figure 4.3.1: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* in 48 hours when treated with IJs of *S. thermophilum* exposed to S/2 concentration of fungal bioagents for a period of 48 hours

Table 4.3.3: Effect of different concentrations of Bacterial bio-agents on Infective juveniles of *S. thermophilum*

Treatments		% Mortality of IJs at different exposure period			
		24 Hour	48 Hour	72 Hour	7 Days
<i>B. thuringiensis</i>	T1 (S)	<b>7.9</b> (16.3)	<b>23.0</b> (28.7)	<b>40.1</b> (39.3)	<b>91.4</b> (73.0)
	T2 (S/2)	<b>3.2</b> (10.3)	<b>6.4</b> (14.7)	<b>16.0</b> (23.6)	<b>33.3</b> (35.3)
	T3 (S/4)	<b>1.2</b> (6.4)	<b>3.7</b> (11.1)	<b>5.6</b> (13.6)	<b>9.9</b> (18.3)
	T4 (S/8)	<b>0.6</b> (4.5)	<b>1.2</b> (6.3)	<b>1.2</b> (6.3)	<b>4.8</b> (12.7)
<i>P. fluorescens</i>	T1 (S)	<b>45.6</b> (42.5)	<b>67.6</b> (55.3)	<b>76.2</b> (60.8)	<b>80.1</b> (63.5)
	T2 (S/2)	<b>28.5</b> (32.3)	<b>42.1</b> (40.4)	<b>49.5</b> (44.8)	<b>66.4</b> (54.6)
	T3 (S/4)	<b>6.0</b> (14.2)	<b>8.4</b> (16.8)	<b>13.5</b> (21.6)	<b>20.9</b> (27.2)
	T4 (S/8)	<b>0.9</b> (5.4)	<b>2.7</b> (9.4)	<b>4.4</b> (12.2)	<b>8.0</b> (16.4)
Control (Media)	C1	<b>0.8</b> (5.0)	<b>0.8</b> (5.0)	<b>1.5</b> (7.1)	<b>3.4</b> (10.6)
Control (water)	C2	<b>0.4</b> (3.4)	<b>0.7</b> (4.8)	<b>0.7</b> (4.8)	<b>1.1</b> (5.9)
SEM		3.2	3.4	3.3	3.7
CD (P= 0.05)		7.2	7.6	7.4	8.2

\* Data in parenthesis are arcsine transformed values

difference among other treatments in the same time duration. However after 48 hours of exposure, S/2 concentration also showed significant difference with S/8 concentration in terms of nematode mortality. At 72 h and 7 days interval all the treatments of *B. thuringiensis* except lower two doses showed significant differences in nematode mortality. Among all the treatments of *B. thuringiensis*, only the higher two doses induced significantly higher level of mortality of infective juveniles (9.4% and 33.3 %) when compared with control (1.1 % and 3.4 % mortality). Higher 3 concentrations (S, S/2 and S/4) of *P. fluorescens* induced significant level of nematode mortality in all the time intervals when compared with control. The significant difference in inducing the mortality of infective juveniles was recorded among different treatments of *P. fluorescens* in all the time intervals except between lower two doses at 48 h.

Even though *M. anisopliae* and *B. bassiana* were found to be safe for the infective juveniles of *S. thermophilum*, it was essential to evaluate the effect of *S. thermophilum* and its symbiotic bacteria *X. indica* on these bioagents as both are entomopathogenic and there is a possibility of their direct interaction in the host species. So the mutual interaction of these bioagents with *S. thermophilum* as well as with its symbiotic bacterium *X. indica* was evaluated on 3 different media namely Lipid agar (LA) medium, Nutrient agar (NA) medium and Sabouraud's dextrose agar (SDA) medium in Petri plates (Plates 4.3.1 and 4.3.2). The symbiotic bacterium *X. indica* suppressed the growth of both the bioagents on LA medium as well as in the NA medium, but not in the SDA medium. However the growth of bacteria was not affected by the presence of these fungal bioagents in LA or NA media. But *X. indica* could not grow well in SA medium even in the presence or absence of the fungal bio agents and it indicated that SA is not a suitable medium for bacterial growth. When the fungal bioagents and *S. thermophilum* inoculated simultaneously on the growth media the nematode did not allow the fungus to grow from its point of inoculation (Plate 4.3.3). The nematodes could grow and multiply on the fungal mat of *B. bassiana* and interestingly more number of nematodes was found to aggregate in the area where fungal mat was present, and the nematodes dissolved the fungal mat completely probably with the help of its symbiotic bacteria (Plate 4.3.4). However, the fungal inoculums of *M. anisopliae* were not affected by the nematodes for initial few days, but later on it invaded and dissolved the same (Plate 4.3.3). When *S. thermophilum* was inoculated after the establishment of fungal bio-agent on

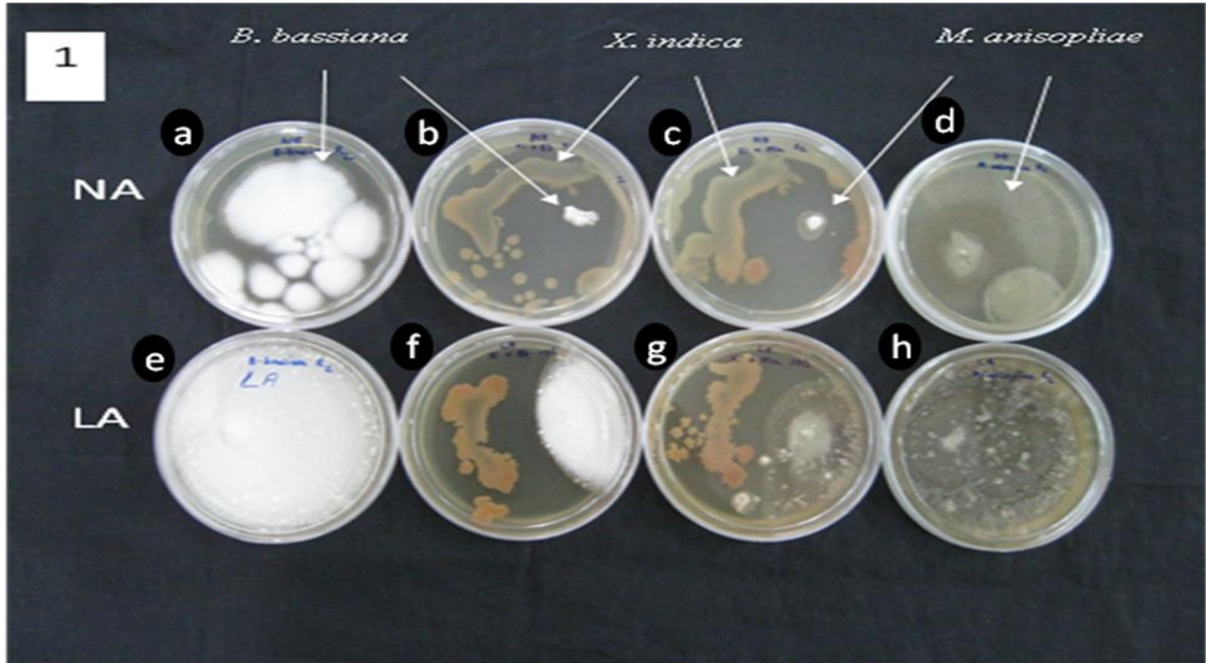


Plate 4.3.1: Interaction of *Xenorhabdus indica* with entomopathogenic fungi on different growth media: NA – Nutrient agar medium (a – d); LA – Lipid agar medium (e – h); a & e – *B. bassiana* alone; b & f – *X. indica* and *B. bassiana*; c & g – *X. indica* and *M. anisopliae*; d & h – *M. anisopliae* alone.

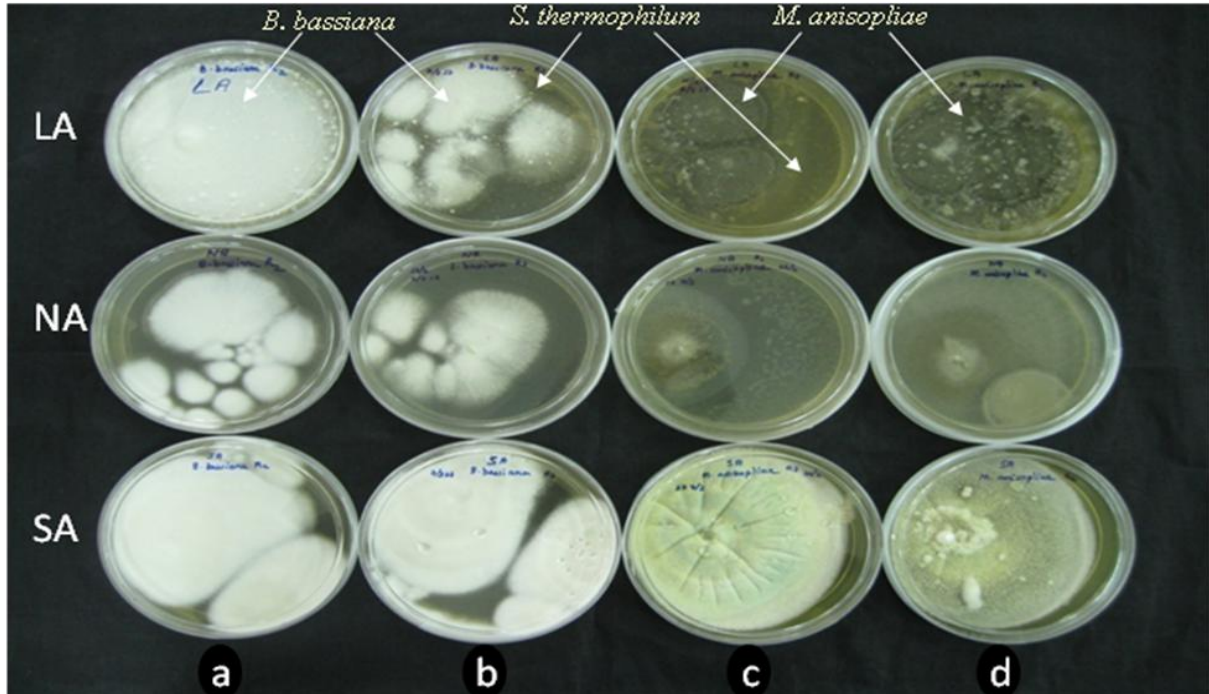


Plate 4.3.2: Interaction of *S. thermophilum* with entomopathogenic fungi on different growth media: LA – Lipid agar medium (e – h); NA – Nutrient agar medium (a – d); SA – Sabouraud's dextrose agar medium; a – *B. bassiana* alone; b – *S. thermophilum* and *B. bassiana*; c – *S. thermophilum* and *M. anisopliae*; d – *M. anisopliae* alone.

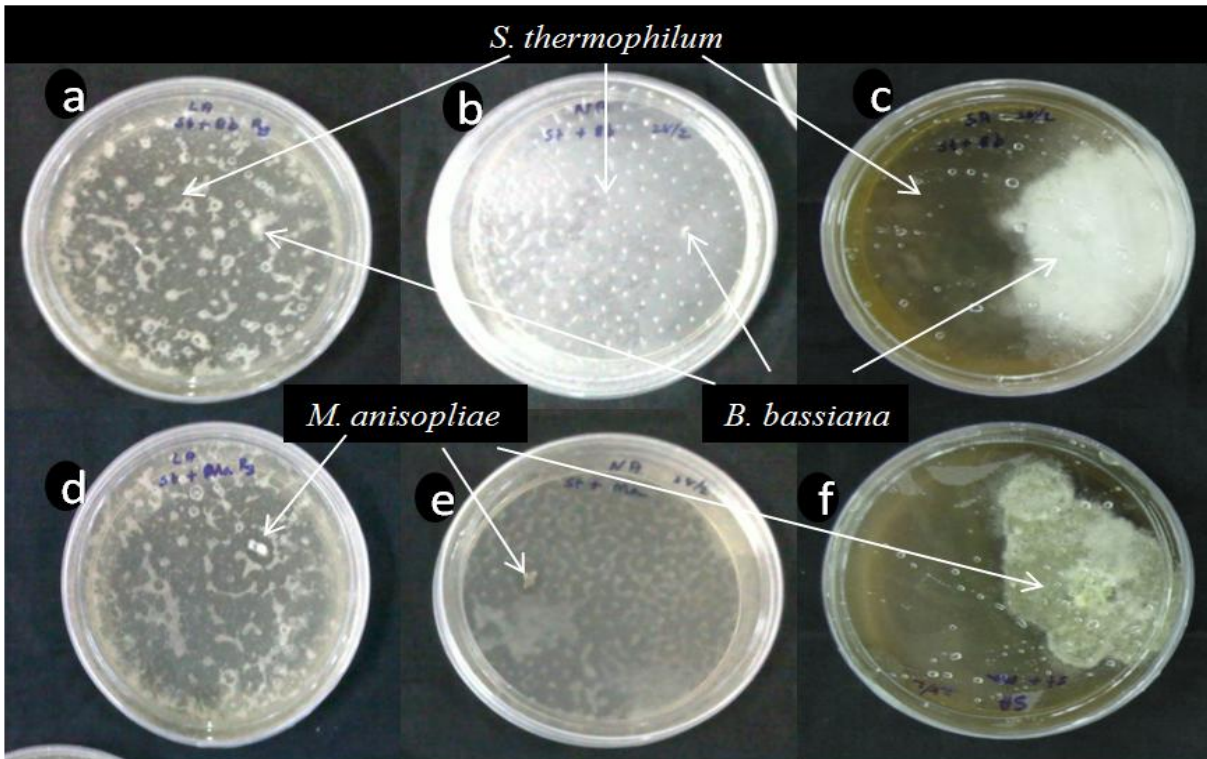


Plate 4.3.3: Interaction of *S. thermophilum* with entomopathogenic fungi when both applied together on different growth media: a & d – Lipid agar medium; b & e – Nutrient agar medium; c & f – Sabouraud's dextrose agar medium.

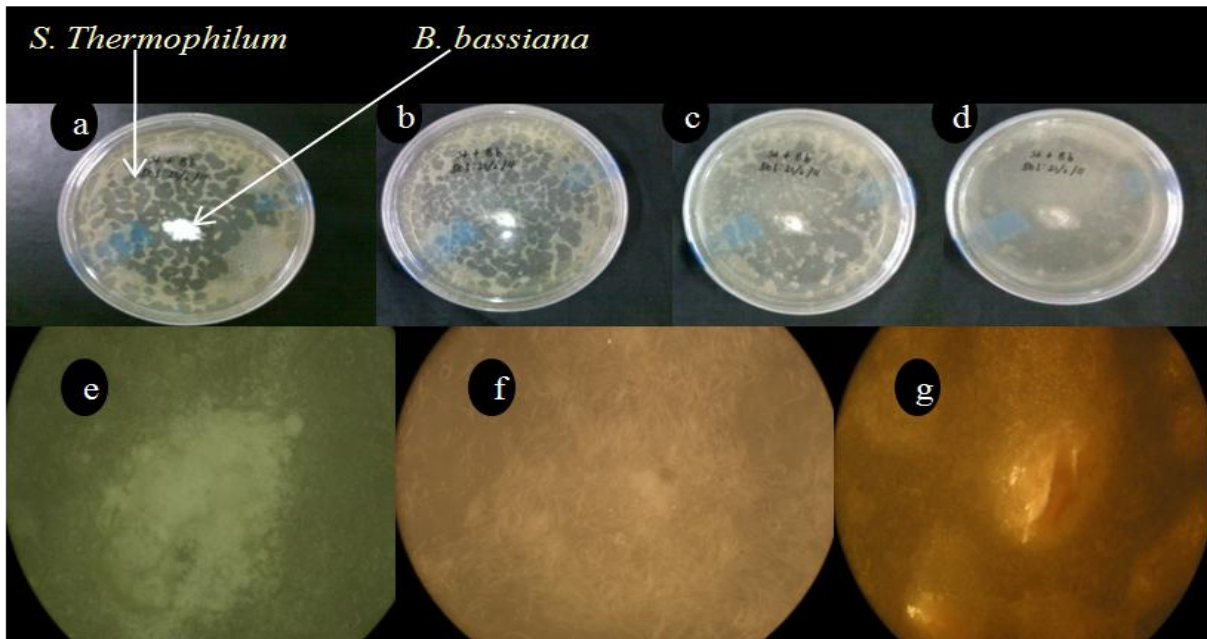


Plate 4.3.4: Interaction of *S. thermophilum* with *Beauveria bassiana* when both applied together on lipid agar medium: a – 2 days after inoculation; b – 4 days after inoculation; c – 8 days after inoculation; d – 16 days after inoculation; e, f, g – *S. thermophilum* developing on the fungal mat of *B. bassiana*

the media, the zone of inhibition were developed by both the fungal bioagents initially. But the zone of inhibition developed by *B. bassiana* disappeared in 2 days, whereas that of *M. anisopliae* remained for about 1 week. In both the cases, *S. thermophilum* could invade and multiply in the area where fungal growth was present and gradually dissolved the fungal mat, even though *M. anisopliae* being more resistant towards the nematode as it remained protected from the nematode attack for considerably long time (Plate 4.3.2).

Among the different organic amendments evaluated, higher concentration of neem cake (S) induced a maximum of 2.4 % nematode mortality in 5 days and it increased to 3.3 % after 10 days of treatment. The level of mortality recorded in the higher concentrations of bone meal, FYM and vermicompost after 10 days of treatment were 2.1, 1.9 and 1.4 % respectively. None of these treatments induced significant level of mortality of infective juveniles (Table 4.3.4). The infective juveniles exposed to standard stock (S) concentrations of these organic amendments for a period of 48 h when treated on 4<sup>th</sup> instar larvae of *G. mellonella* caused 100 % larval mortality within 72 h of treatment and thus confirmed the retention of their virulence. Even though a marginal reduction in the larval mortality was observed in 24 and 48 h in all the treatments when compared with control, the differences were statistically non-significant (Table 4.3.5).

#### 4.3.4 Discussion

There are several reports on the synergistic, additive or antagonistic interaction between entomopathogenic nematodes and other bioagents. Ansari *et al.*, 2004 reported stronger synergistic effects between *M. anisopliae* and *Heterorhabditis megidis* or *Steinernema glaseri* against third-instar *Hoplia philanthus* when the fungus applied for at least 3 or 4 weeks before the addition of nematodes. Koppenhöfer *et al.* (1999) also demonstrated that combining *H. bacteriophora* and *S. glaseri* with *Bacillus thuringiensis* subspecies *japonensis* Buibui strain caused additive and synergistic effects against masked chafers, *Cyclocephala hirta* and *C. pasadenae*, in the greenhouse and under field conditions. Shapiro-Ilan *et al.* (2005b) investigated the interaction effect of the nematodes *Heterorhabditis indica* or *Steinernema carpocapsae* with the fungus *Beauveria bassiana*, *Metarhizium anisopliae*, or *Paecilomyces fumosoroseus*, or the bacterium *Serratia marcescens* on pecan weevil, *Curculio caryae*. They observed antagonism in all pathogen

Table 4.3.4: Effect of different concentrations of organic amendments on Infective Juveniles (IJs) of *S. thermophilum*

Organic amendment	Treatments	% Mortality of IJs	
		5 DAT	10 DAT
Bone meal	T1 (S)	<b>1.6</b> (7.3)	<b>2.1</b> (8.2)
	T2 (S/2)	<b>1.5</b> (7.1)	<b>2.2</b> (8.4)
FYM	T1 (S)	<b>1.0</b> (5.8)	<b>1.9</b> (7.9)
	T2 (S/2)	<b>0.5</b> (4.0)	<b>1.4</b> (6.9)
Neem Cake	T1 (S)	<b>2.4</b> (9.0)	<b>3.3</b> (10.5)
	T2 (S/2)	<b>0.9</b> (5.3)	<b>2.7</b> (9.5)
Vermicompost	T1 (S)	<b>1.4</b> (6.8)	<b>1.4</b> (6.7)
	T2 (S/2)	<b>0.4</b> (3.8)	<b>0.9</b> (5.4)
Control	C	<b>0.5</b> (4.0)	<b>0.9</b> (5.4)
SEM		2.9	2.7
CD (P= 0.05)		6.6	6.2

DAT: Days after treatment

Table 4.3.5: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* when treated with IJs of *S. thermophilum* exposed to different organic amendments

Organic amendments	% Mortality of insect larvae		
	24 Hour	48 Hour	72 Hour
Bone meal	<b>18.8</b> (25.7)	<b>68.8</b> (56.0)	<b>100.0</b> (90.0)
FYM	<b>12.5</b> (20.7)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
Neem Cake	<b>18.8</b> (25.7)	<b>56.3</b> (48.6)	<b>100.0</b> (90.0)
Vermicompost	<b>18.8</b> (25.7)	<b>62.5</b> (52.3)	<b>100.0</b> (90.0)
Control	<b>25.0</b> (30.0)	<b>75.0</b> (60.0)	<b>100.0</b> (90.0)
SEM	<b>12.7</b>	<b>11.4</b>	<b>0.0</b>
CD (P= 0.05)	<b>32.7</b>	<b>29.2</b>	<b>0.0</b>

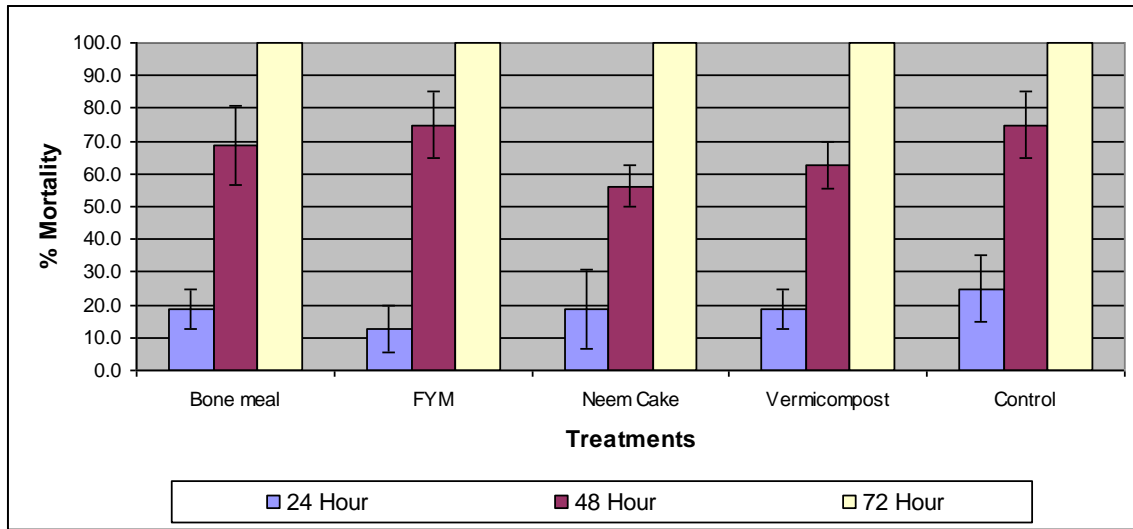


Figure 4.3.2: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* when treated with IJs of *S. thermophilum* exposed to S concentration of organic amendments for a period of 48 hours

combinations, except *H. indica* combined with *M. anisopliae*, for which additive effects were observed. All these studies clearly indicate that the interaction effect varies with different species of entomopathogenic nematode and bioagent combinations.

The present study on the compatibility of *S. thermophilum* with different bioagents and organic amendments was carried out for the first time. Any of the fungal bioagents evaluated did not induce any significant level of mortality in IJs of *S. thermophilum*. As bacterial symbionts are also associated with entomopathogenic nematode the compatibility evaluation of each bioagents with respective bacterial symbiont is also essential, especially when both targeting the same host. As the target of *S. thermophilum* and bioagents, namely *P. lilacinus*, *P. chlamydosporium*, *T. harzianum* or *T. viride* are different, we could establish the compatibility of these fungal bioagents with the same nematode from the viability study. The fungal bioagents namely *M. anisopliae* and *B. bassiana* were also found to cause no harmful effect on the infective juveniles of *S. thermophilum*. But the mutual interaction of *S. thermophilum* and its symbiotic bacteria *X. indica* with these bioagents were also studied on different growth media as both are entomopathogenic and there is a possibility of their direct interaction in the host species. In the mutual interaction study we observed the suppression of both *B. bassiana* and *M. anisopliae* by *S. thermophilum* and its symbiotic bacteria *X. indica*. The fungicidal metabolites produced by the symbiotic bacteria must be the reason for the suppression of these fungal bioagents by the nematode-bacterial complex. The antimicrobial nature of metabolites produced by symbiotic bacteria is well documented (Webster *et al.*, 2002) and several of the metabolites are known to have fungicidal action (Barbercheck and Kaya, 1990; Chen *et al.*, 1994). Ansari *et al.* (2005) investigated the mutual effects between the symbiotic bacteria of entomopathogenic nematodes, *Photorhabdus luminescens* and *Xenorhabdus poinarii*, and different entomopathogenic fungi. The results revealed that *P. luminescens* is antagonistic to *Metarhizium anisopliae*, *Beauveria bassiana*, *B. brongniartii* and *Paecilomyces fumosoroseus* by inhibiting their growth and conidial production, whereas the fungal growth was not inhibited by *X. poinarii*. So our observation clearly indicated the incompatibility of *S. thermophilum* with *B. bassiana* or *M. anisopliae* targeting the same host.

### 4.3.5 Conclusion

The bacterial bioagents namely *B. thuringiensis* and *P. fluorescens* were found to be harmful to infective juveniles of *S. thermophilum*. Even though fungal bioagents like *M. anisopliae* and *B. bassiana* observed to cause no harmful effect on the survival and infectivity of Infective juveniles of *S. thermophilum* they were found to affect adversely by the nematode *S. thermophilum*. As there is a chance of suppression of these fungal bioagents by *S. thermophilum* when both infect the same host, the combination of *S. thermophilum* with these fungal bioagents are not desirable. So we concluded that combining any of these fungal or bacterial bioagents for the management of same insect host should not be recommended as it reduces the overall efficacy of the bioagents in long run. However it is advisable to combine *S. thermophilum* with fungal bioagents like *P. lilacinus*, *P. chlamydosporium*, *T. harzianum* or *T. viride* as it cause no harmful effect on the survival and infectivity of *S. thermophilum* and there is no chance for their mutual interaction as they target different pathogenic organisms. The organic amendments like Bone meal, FYM, neem cake and vermicompost were observed to have no harmful effect on the survival and infectivity of the infective juveniles of *S. thermophilum*.

## 4.4 RESEARCH PAPER IV

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### **Interspecific interactions of entomopathogenic nematodes and their effect on non-target organisms**

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**Abstract:** The interspecific interactions among the entomopathogenic nematodes *Steinernema thermophilum*, *S. glaseri* and *Heterorhabditis indica* were evaluated on 4<sup>th</sup> instar larvae of greater wax moth *Galleria mellonella* at two different temperatures (27 and 35°C). At 27°C *S. glaseri* was found to dominate over other 2 species and *S. thermophilum* dominated over *H. indica*. At 35°C only *S. thermophilum* could develop and multiply. Even though *S. thermophilum* was observed to be suppressed in the presence of *S. glaseri* at 27 °C, the emergence pattern did indicate that both the species could develop in the same host. However, there was no record of multiple infections of any *Steinernema* species with *Heterorhabditis indica*. The dominance of *S. thermophilum* at higher temperature was further confirmed by its development on Lipid agar medium in the presence of *S. glaseri* and *Photorhabdus luminescens* (the symbiotic bacteria of *H. indica*). The non-target effect of these nematodes on beneficial organisms like earthworm (*Eiseinea foetida*), Spider (*Neoscona theisi*) and ants (*Messor himalayanus*) were also evaluated. These nematodes were found to be safe towards the earthworm species *E. foetida* as it did not cause any earthworm mortality up to 3 weeks of treatment when applied @ 500 IJs/g compost. However, *S. thermophilum*, *S. glaseri* and *H. indica* induced mortality of 20, 10 and 30 %, respectively on Spider species *Neoscona theisi* when applied @ 500 IJs/spider, but no mortality was recorded @ 100 IJs/spider. The *S. thermophilum* and *H. indica* when applied on ant species *M. himalayanus* @ 100 IJs/ant could induce 100 % mortality whereas *S. glaseri* caused 72 % ant mortality after 5 days.

**Key words:** *S. thermophilum*, *S. glaseri*, *H. indica*, interaction, earthworm, ant, spider, beneficial organisms.

#### 4.4.1 Introduction

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae are naturally occurring pathogens of insects (Kaya and Gaugler, 1993; Hominick *et al.*, 1996) and are environmental friendly alternative for insect pest control (Kaya, 1990; Kaya and Gaugler, 1993). Entomopathogenic nematodes do not avoid entering hosts already infected with heterospecifics, both naturally (Bovien, 1937), and experimentally (Lewis *et al.*, 2006). Thus, there is a potential for interspecific interactions between sympatric nematode species. In the field, the interactions are thought to be mediated by differing foraging strategies and host preferences (Alatorre-Rosas and Kaya, 1990; Koppenhöfer and Kaya, 1996). Vertical distribution and patchiness probably contribute to the coexistence of different entomopathogenic species (Stuart *et al.*, 2006). So far, a number of laboratory studies have addressed inter-specific interactions between steinernematids. Kondo (1989) observed *Spodoptera litura* co-infected with *Steinernema glaseri* and *S. feltiae* producing a mixed progeny, while in *Galleria mellonella* co-infected with *S. glaseri* and *S. carpocapsae* the reproduction of the latter species was suppressed (Koppenhöfer *et al.*, 1995). On the other hand, Neumann and Shields (2006) did not observe any evidence of multiple infection of *G. mellonella* while studying the interactions of *S. carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora* in sand columns even though nematodes infected hosts in close proximity. Lewis *et al.* (2006) concluded that species of *Steinernema* can develop together in one insect host, though one species may suffer the effects of the competition more than the other. At present not all aspects of the interspecific interactions in entomopathogenic nematodes are understood. This study examined the interactions between three entomopathogenic nematodes namely *S. thermophilum*, *S. glaseri* and *Heterorhabditis indica* in the experimental host *G. mellonella* under two temperature conditions.

Studies on the nontarget effects of entomopathogenic nematodes on various species of nontarget organisms showed a large range from complete harmlessness to pronounce harmful effect was established (Bathon 1996, Farag 2002, Powell and Webster 2004). The results of some field trials showed a moderate influence of these nematodes on nontarget arthropods or even the absence of such an effect (Georgis *et al.*, 1991). Bathon (1996) reported that mortality can be observed among the nontarget organisms, but the influence of these agents should be temporary and local and so only a part of the population is under

attack. Georgis *et al.* (1991) demonstrated a negligible influence of entomopathogenic nematodes on nontarget organisms when used only in short-term pest control. Earthworms improve soil conditions (aeration, drainage and organic matter content) and they are able to change soil structure, move large amounts of soil and affect micro floral and faunal diversity (Brown, 1995; Doube and Brown, 1998). Many associations (phoretic, paratenic intermediate or sole host) between nematodes and earthworms had been reported (Gunnardson and Rundgren, 1986; Poinar, 1978; Timper and Davies, 2004), and some authors think that earthworms could be used as vectors to introduce and/or disperse beneficial organisms (Eng *et al.*, 2005; Shapiro *et al.*, 1993, 1995). The aim of this study was to evaluate the effect of entomopathogenic nematodes *Steinernema thermophilum*, *S. glaseri* and *H. indica* against epigeic earthworm (*Eisenia foetida*), spider (*Neoscona theisi*) and ant species (*Messor himalayanus*).

#### **4.4.2 Materials and methods**

**4.4.2.1 Nematodes:** The interspecific interaction of different entomopathogenic nematodes (EPNs) namely *Steinernema thermophilum*, *S. glaseri* and *Heterorhabditis indica* were evaluated. The pure cultures of Infective juveniles (IJs) of these EPNs already available in our laboratory were maintained on 4<sup>th</sup> instar larvae of greater wax moth, *Galleria mellonella* (L.) (Kaya and Stock, 1997). The emerging IJs from the insect cadavers were harvested in sterile distilled water using a modified White's trap, and stored at 15° C in BOD incubator. The IJs for the interaction study were used within 2-4 weeks of storage.

**4.4.2.2 Interaction study:** The interspecific interaction of *Steinernema thermophilum* (St), *S. glaseri* (Sg) and *Heterorhabditis indica* (Hi) on 4<sup>th</sup> instar larvae of greater wax moth (*G. mellonella*) were carried out. The experiment was done in 12 well plates with one insect larva in each well of the plate. These EPNs alone and different combinations of these EPNs (St+Sg, St+Hi, Sg+Hi) were treated @ of 100 IJs per insect larva. In the treatments where combinations of EPNs used, 50 IJs of each species were applied to become a total of 100 IJs/larva. There were 6 treatments replicated 5 times and the experiment was carried out at 27 °C and 35 °C. The observations on date of mortality of larvae in each treatment were recorded and cadavers were transferred to modified White's trap. The date on which IJs emerged from the cadaver was recorded and the emerging IJs from each cadaver were

harvested separately. The total number of IJs of each species emerged from each cadaver killed by different EPNs were also counted separately.

**4.2.2.3 Interaction study on Lipid agar medium:** The interaction of *S. thermophilum* with *X. poinarii* and *Photorhabdus luminescens* were carried out on Lipid agar medium in Petri plates at 27 °C and 35 °C. Both *S. thermophilum* and above mentioned bacteria were inoculated simultaneously on both the sides of Lipid agar medium in the Petri plates. The observations on development of nematode and bacteria were recorded in every 2 days interval.

**4.2.2.4 Beneficial organisms used:** The impact of entomopathogenic nematodes, *S. thermophilum*, *S. glaseri* and *H. indica* was evaluated on two species of ants (*Messor himalayanus*), one species of spider (*Neoscona theisi*; Family: Araenidae) and one species of earthworm (*Eisinea foetida*). The earthworm species was procured from the Division of Entomology, IARI, New Delhi, whereas the spiders and ants were collected directly from IARI Research Farm.

**4.2.2.5 Effect of entomopathogenic nematodes on earthworms:** The experiment was carried out in 100 ml capacity plastic bottles containing 20 g of vermicompost. The EPN species were added @ 100 IJs/g compost and 500 IJs/g compost in each bottles and the earthworms were released @ 4 earthworms/bottle. All the treatments were replicated five times and held at 25±1 °C. The observations on survival of earthworms were recorded on 7<sup>th</sup> and 14<sup>th</sup> day of the treatment. Fourth instar larvae of *G. mellonella* @ 4 larvae/bottle were added on 10<sup>th</sup> day of treatment to confirm the presence and survival of IJs in the vermicompost. The dead larvae from each bottle were collected in 24 hour intervals.

**4.2.2.6 Effect of entomopathogenic nematodes on spider:** The experiment was carried out in 12-well plates lined with two layers of Whatman No. 1 filter paper. Two doses (100 IJs/well and 500 IJs/well) of the three species of entomopathogenic nematodes in 100 µl of sterile distilled water were applied in the each well of twelve well plates. After the release of IJs in the wells, the spiders were released as one spider/well of the 12-well plates. Ten spiders were included in each treatment and experiment was held at 25±1 °C. The observations on mortality of spiders were recorded at 24 h after intervals. The cadavers were observed for the emergence of nematode progenies.

**4.2.2.7 Effect of entomopathogenic nematodes on ants:** The experiment was carried out in transparent plastic plates of 10 mm diameter lined with 2 layers of Whatman No. filter paper. Each plate was provided with 50 µl of honey solution in small containers to feed the ants. The infective juveniles of entomopathogenic nematodes were released @ 1000 IJs/plate in 300 µl of sterile distilled water. Ten ants were released in each plate to yield a dosage of 100 IJs/ant. All the treatments were replicated five times and the experiment was held at 25±1 °C. The observations on mortality of ants were taken at every 24 h interval.

### 4.4.3 Results

The three species of entomopathogenic nematodes namely *S. thermophilum*, *S. glaseri* and *H. indica* when treated alone or in different combinations on 4<sup>th</sup> instar larvae of *G. mellonella* @ 100 IJs/larva induced 100 % larval mortality after 72 h of treatment at 27 as well as at 35°C. All the treatments except *H. indica* kept at 35 °C could induce 100 % larval mortality in 48 h of treatment. The majority of the treatments namely *S. thermophilum* at 27 and 35 °C, *S. glaseri* at 27 °C, St+Sg at 27 and 35 °C, St+Hi at 35 °C and Sg+Hi at 27 °C recorded 100 % larval mortality after 24 hours of treatment (Table 4.4.1).

The emergence of next generation infective juveniles from cadavers of *G. mellonella* of both the species of *Steinernema* started from 6<sup>th</sup> day of mortality and it continued up to 8<sup>th</sup> day where as the emergence of *Heterorhabditis* species were observed from 14<sup>th</sup> to 16<sup>th</sup> day. There were no emergences of infective juveniles from the cadavers killed by *S. glaseri* and *H. indica* as well as their combined application at 35 °C. The data on the date of emergence of infective juveniles from different treatments are given in Table 4.4.2.

Among all the treatments, the maximum number of progeny production (emergence of IJs) was recorded from the cadavers killed by *H. indica* kept at 27 °C followed by *S. thermophilum* at 27 °C. About 60 % reduction in the total progeny production was observed from the cadavers killed by *S. thermophilum* at 35 °C when compared with the progeny production at 27 °C. There was no record of IJs emergence from the cadavers killed by *S. glaseri* or *H. indica* at 35 °C, where as a maximum of only about 15000 infective juveniles of *S. glaseri* was emerged from the cadavers killed by the same nematode at 27 °C. Even though the emergence of both *S. thermophilum* and *S. glaseri* was observed from the cadavers co-infected with these two species at 27 °C, there was a marked reduction in the number of IJs

Table 4.4.1: Mortality of 4<sup>th</sup> instar larvae of *G. mellonella* when treated with different entomopathogenic nematodes and their combinations

Treatment	Temperature	% Mortality of insect larvae		
		24 Hours	48 Hours	72 Hours
St	27 °C	100	100	100
St	35 °C	100	100	100
Sg	27 °C	100	100	100
Sg	35 °C	80	100	100
Hi	27 °C	80	100	100
Hi	35 °C	20	60	100
St+Sg	27 °C	100	100	100
St+Sg	35 °C	100	100	100
St+Hi	27 °C	40	100	100
St+Hi	35 °C	100	100	100
Sg+Hi	27 °C	100	100	100
Sg+Hi	35 °C	80	100	100

Table 4.4.2: Date of emergence of Infective juveniles of different entomopathogenic nematodes when treated on 4<sup>th</sup> instar larvae of *G. mellonella*

Treatment	Temperature	% of cadavers with nematode emergence					
		6 <sup>th</sup> Day	8 <sup>th</sup> Day	10 <sup>th</sup> Day	12 <sup>th</sup> Day	14 <sup>th</sup> Day	16 <sup>th</sup> Day
St	27 °C	20	100	100	100	100	100
St	35 °C	40	100	100	100	100	100
Sg	27 °C	100	100	100	100	100	100
Sg	35 °C	0	0	0	0	0	0
Hi	27 °C	0	0	0	0	20	100
Hi	35 °C	0	0	0	0	0	0
St+Sg	27 °C	100	100	100	100	100	100
St+Sg	35 °C	20	100	100	100	100	100
St+Hi	27 °C	0	20	20	60	60	100

St+Hi	35 °C	0	80	100	100	100	100
Sg+Hi	27 °C	80	100	100	100	100	100
Sg+Hi	35 °C	0	0	0	0	0	0

Table 4.4.3: Progeny production of different entomopathogenic nematodes in combined application on 4<sup>th</sup> instar larvae of *G. mellonella* at two temperature conditions

Treatment	Temperature	Total no. of IJs emerged		
		<i>S. thermophilum</i>	<i>S. glaseri</i>	<i>H. indica</i>
St	27 °C	<b>102806</b> (5.01)	-	-
St	35 °C	<b>61820</b> (4.77)	-	-
Sg	27 °C	-	<b>15486</b> (4.17)	-
Sg	35 °C	-	<b>0</b>	-
Hi	27 °C	-	-	<b>168558</b> (5.22)
Hi	35 °C	-	-	<b>0</b>
St+Sg	27 °C	<b>1321</b> (2.90)	<b>13782</b> (4.08)	-
St+Sg	35 °C	<b>51374</b> (4.70)	<b>0</b>	-
St+Hi	27 °C	<b>39624</b> (2.88)	-	<b>35000</b> (1.97)
St+Hi	35 °C	<b>37226</b> (4.54)	-	<b>0</b>
Sg+Hi	27 °C	-	<b>14625</b> (4.15)	<b>0</b>
Sg+Hi	35 °C	-	<b>0</b>	<b>0</b>
SEM		<b>0.70</b>	<b>0.08</b>	<b>0.70</b>
CD (P= 0.05)		<b>1.52</b>	<b>0.17</b>	<b>1.52</b>

St – *S. thermophilum*; Sg – *S. glaseri*; Hi – *H. indica*

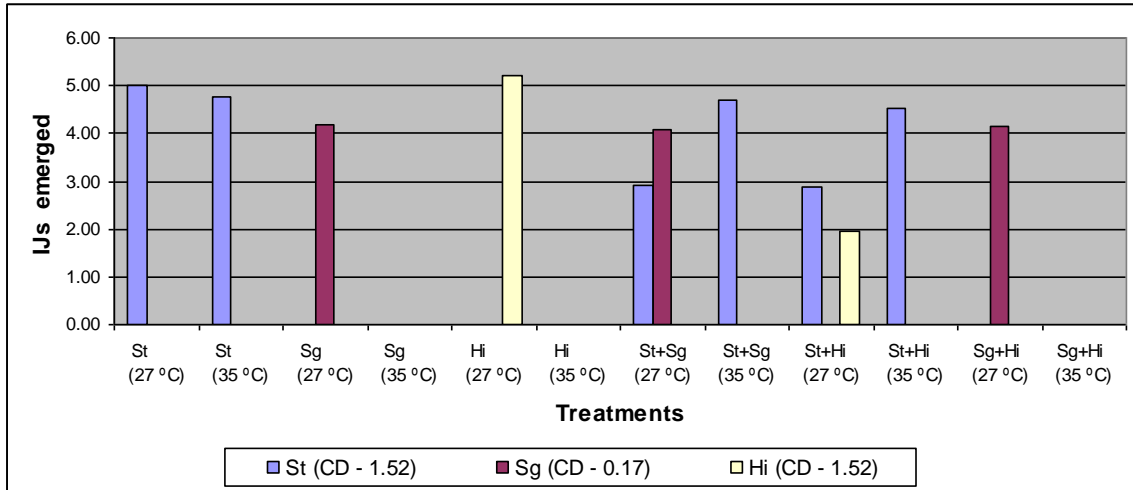


Figure 4.4.1: Progeny production of different entomopathogenic nematode species and their combinations

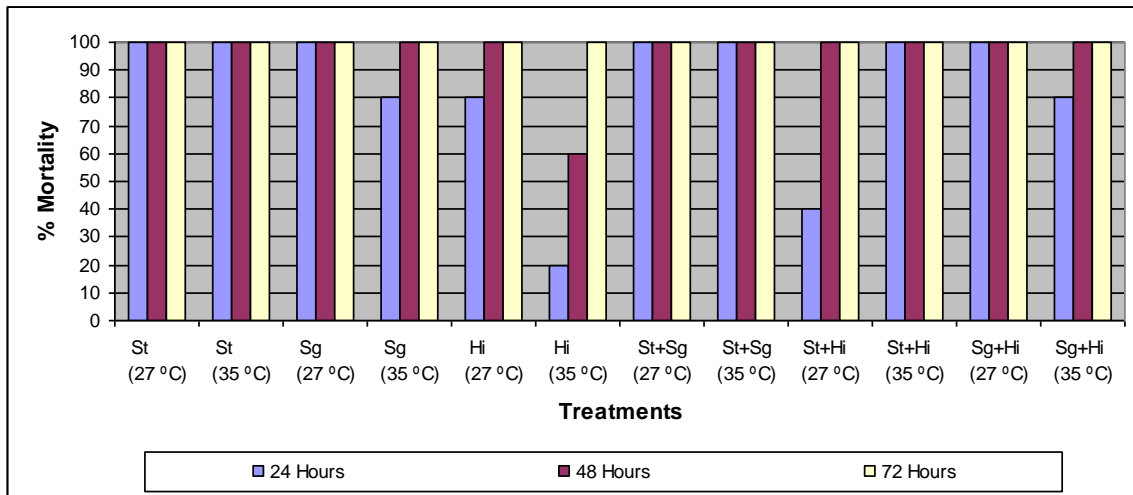


Figure 4.4.2: Mortality of *Galleria mellonella* when treated with entomopathogenic nematode species and their combinations

of *S. thermophilum*. The emergence of only *S. thermophilum* was recorded from the cadavers co-infected with both steinernematids at 35 °C. There was no record on the emergence of any one *Steinernema* species and *H. indica* from the same cadaver. Among the treatments where both *S. thermophilum* and *H. indica* combined together at 27 °C, 40 % of the cadavers recorded the emergence of *H. indica* and remaining 60 % recorded the emergence of *S. thermophilum* with a reduction in the total number of progenies when compared with their individual application. The combination of these two species at 35 °C yielded the emergence of only *S. thermophilum* with about 60 % reduction in the total number of IJs when compared with its individual infection at the same temperature. When *S. glaseri* and *H. indica* applied together at 27 °C, only *S. glaseri* emerged from the cadavers without any significant reduction in total number of infective juveniles. The details on the number of progeny production from different treatments are given in Table 4.4.3 and Figure 4.4.1.

When *S. thermophilum* and *X. poinarii* (Symbiotic bacterium of *S. glaseri*) were inoculated together on the lipid agar medium, *S. thermophilum* developed normally at both the temperature conditions (27 and 35 °C). However, in the treatments where *S. thermophilum* and *P. luminescens* (Symbiotic bacterium of *H. indica*) inoculated together, the zone of inhibition was developed between them only at 27 °C but not at 35 °C. But, *S. thermophilum* was gradually invaded the area of *P. luminescens* and the zone of inhibition developed at 27 °C was disappeared in 10 days of inoculation (Plate 4.4.1).

The entomopathogenic nematodes, *S. thermophilum*, *S. glaseri* and *H. indica* were treated @ 100 IJs/ant on the adults of ant species *Messor himalayanus*. Among these three species, *S. thermophilum* observed to be more pathogenic followed by *H. indica* and least by *S. glaseri*, in all the time durations evaluated (Table 4.4.4). The *S. thermophilum* induced 68 % mortality after 24 hours and 100 % mortality in 120 hours after treatment. The mortality induced by *H. indica* in 24 h was 40 % and up to 100 % in 120 h. Among these three EPNs, *S. glaseri* was found to be least effective against *M. himalayanus* as it induced only 24 % mortality after 24 hours and a maximum of only 72 % in 120 h. The level of mortality induced by *S. glaseri* was found to be statistically significant from that by *S. thermophilum* in all the four time intervals evaluated. The data on mortality caused by *S. glaseri* after 120 h of treatment indicated significant difference from the treatments of both *S. thermophilum* and *H. indica* in the same time duration.

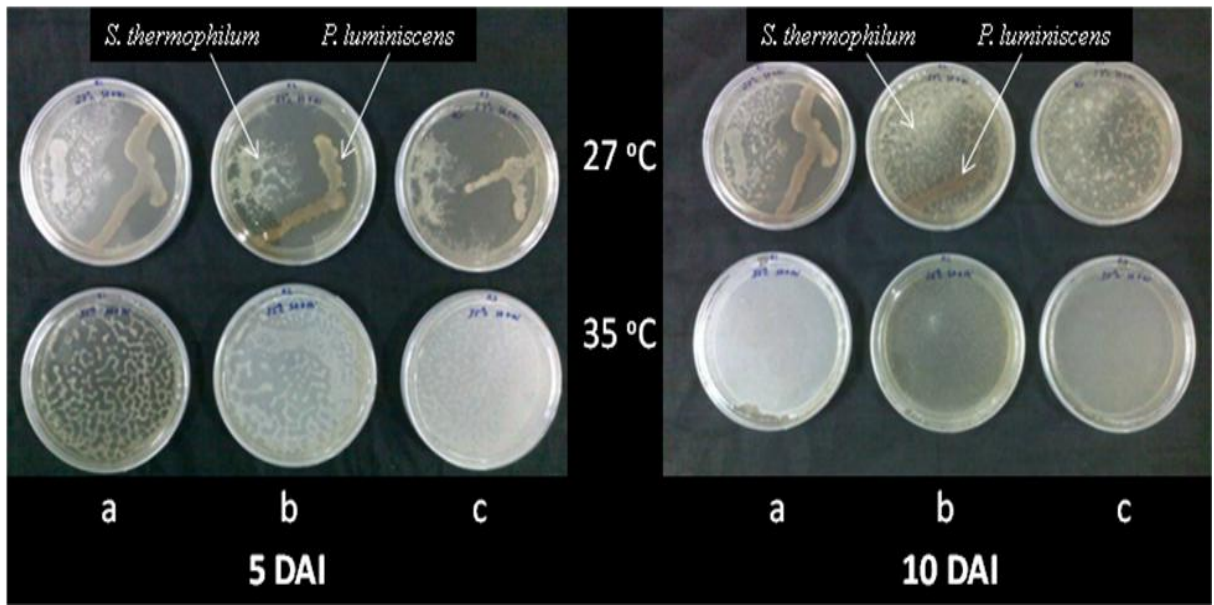


Plate 4.4.1: Interaction of *Steineria thermophilum* and *Photorhabdus luminiscens* on lipid agar media: 5 DAI – 5 days after inoculation; 10 DAI – 10 days after inoculation; 1<sup>st</sup> row – plates kept at 27 °C; 2<sup>nd</sup> row – plates kept at 35 °C; a,b,c – 3 replications of same treatments.

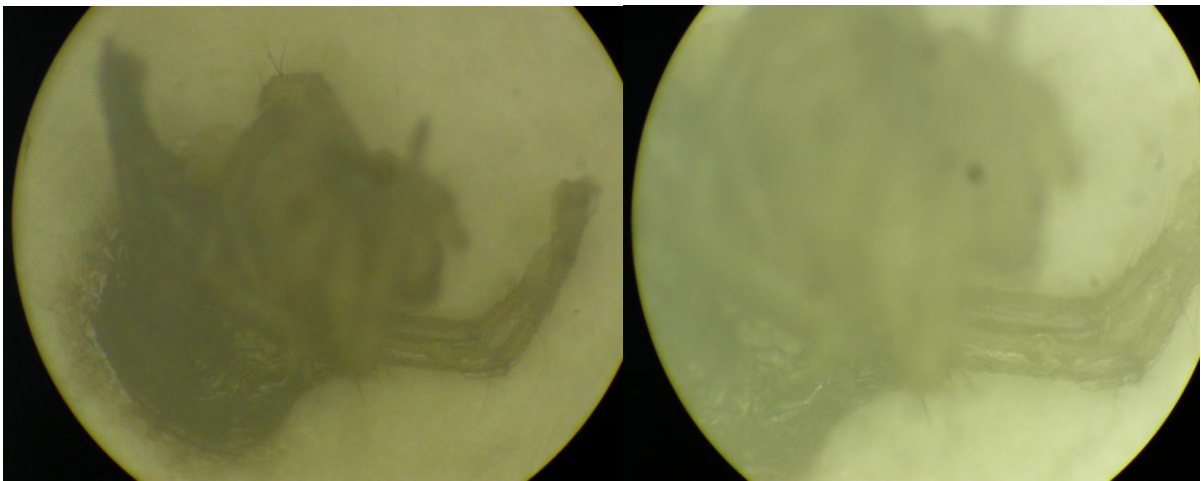


Plate 4.4.2: Infective juveniles of *Steineria thermophilu* emerging from the cadavars of Spider (*Neoscona theisi*)

Table 4.4.4: Mortality of ant species, *Messor himalayanus* by entomopathogenic nematodes when applied @ 100 IJs/ant

Treatments	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
<i>S. thermophilum</i>	68.0 (55.6)	86.0 (68.1)	92.0 (73.6)	98.0 (81.9)	100.0 (90.0)
<i>S. glaseri</i>	24. (29.3)	32.0 (34.5)	40.0 (39.3)	46.0 (42.7)	72.0 (58.1)
<i>H. indica</i>	40.0 (39.3)	48.0 (43.9)	64.0 (53.2)	78.0 (62.1)	100.0 (90.0)
Control	0.0 (0.4)	0.0 (0.4)	0.0 (0.4)	0.0 (0.4)	0.0 (0.4)
SEM	6.1	4.5	5.4	3.9	2.8
CD (P= 0.05)	17.0	12.5	15.1	10.8	7.8

Table 4.4.5: Mortality of Spider species, *Neoscona theisi* by entomopathogenic nematodes

Treatment	% Mortality				
	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
St @ 50 Ijs	0	0	0	0	0
St @ 500 Ijs	10	20	20	20	20
Sg @ 50 Ijs	0	0	0	0	0
Sg @ 500 Ijs	0	10	10	10	10
Hi @ 50 Ijs	0	0	0	0	0
Hi @ 500 Ijs	0	20	30	30	30

Table 4.4.6: Mortality/survival of earthworm species, *Eisenia foetida* and 4<sup>th</sup> instar larvae of *G. mellonella* by entomopathogenic nematodes

Treatment	% Mortality					
	Earthworm			<i>G. mellonella</i>		
	7 Days	14 Days	21 Days	24 Hours	48 Hours	72 Hours
St @ 250 Ijs/g	0	0	0	5	100	100
St @ 100 Ijs/g	0	0	0	0	100	100
Sg @ 250 Ijs/g	0	0	0	0	100	100
Sg @ 100 Ijs/g	0	0	0	0	100	100
Hi @ 250 Ijs/g	0	0	0	0	100	100
Hi @ 100 Ijs/g	0	0	0	0	100	100
Control	0	0	0	0	0	0

On the adult stages of the spider, *Neoscona theisi* a maximum of only 30, 20 and 10 % mortality was recorded by *H. indica*, *S. thermophilum* and *S. glaseri* respectively when applied @ 500 IJs/spider in 120 h after treatment. But there was no record of mortality of spiders up to 120 h in any of the treatments when these EPNs applied @ 50 IJs/spider. The detailed data on the mortality of spiders in different treatments are given in Table 4.4.5. The emergence of infective juveniles of *S. thermophilum* from the cadavers of spider confirmed the infection (Plate 4.4.2).

The entomopathogenic nematodes, *S. thermophilum*, *S. glaseri* and *H. indica* were applied on the earthworms, *Eisania foetida* in the vermicompost medium @ 100 IJs/g and 250 IJs/g compost and observed for the mortality/survival of earthworms up to 21 days. There was no record of mortality of earthworms in any of the treatments evaluated. The survival of infective juveniles of these nematodes was evaluated by putting 4<sup>th</sup> instar larvae of *G. mellonella* in each treatments after 10 days of starting the experiment. As 100 % of larval mortality was recorded in all the treatments within 48 h of exposure, this study confirmed the survival of infective juveniles of these EPNs in the vermicompost medium without causing any harmful effect on the earthworms. The data on the study is presented in Table 4.4.6.

#### **4.4.4 Discussion**

The combined application of different entomopathogenic nematode in all the combinations yielded negative interactions. It indicated that there is no advantage in combining different species of entomopathogenic nematodes to target same insect host. The interspecific interactions of different entomopathogenic nematodes were studied by several workers. Almost all the studies reported dominance of one species over the other with reduction in development of both the species (Kondo, 1989; Koppenhöfer *et al.*, 1995; Lewis *et al.*, 2006). Differences in nematode infection among the different species are due to either their mutualistic bacteria or their response to environmental conditions such as temperature; or both of them. The speed of releasing of the symbiotic bacteria into the haemolymph of dominating species may be higher than that of suppressed one and the nematode also may grow and reproduce faster than suppressed one in the insect haemolymph, thus giving the competitive advantage to the former (Alatorre-Rosas and Kaya, 1991). So far, there is no record on the survival of one *Steinernema* sp. and *Heterorhabditis* sp. in the same host. Our

results indicated the variation in dominance of one species over the other at different temperatures as *S. glaseri* dominated at lower temperature and *S. thermophilum* at higher temperature. So this type of studies will be helpful in finding out the suitable nematode species in a given set of conditions. The survival and development of *S. thermophilum* at higher temperature regimes was reported by Ganguly and Gavas (2004).

The EPN species studied were found to be safe towards earthworms. Several authors have observed the non-susceptibility of earthworms to different steinernematids (Capinera *et al.*, 1982; Nguyen and Smart, 1991; Shapiro *et al.*, 1993) and to the slug-parasitic nematode *Phasmorhabditis hermafrodita* Schneider (Nematoda: Rhabditidae) (Grewal and Grewal, 2003). Some authors think that earthworms could be used as vectors to introduce and/or disperse beneficial organisms including EPNs (Eng *et al.*, 2005; Shapiro *et al.*, 1993, 1995). Raquel *et al.* (2006) studied the interaction of *S. feltiae* with earthworm species *Eisenia foetida* and reported about 20 – 90 % of the earthworms as transmitting nematodes through their gut without any pathogenic effect on earthworms. However they observed a reduction in the mobility of infective juveniles of *S. feltiae* after passage through the *E. foetida* gut.

All the three species of EPNs evaluated were observed to be pathogenic to spiders and ants at higher doses. Earlier Bathon (1996) reported the pathogenicity of entomopathogenic nematode, *H. bacteriophora* on spiders under laboratory conditions. But he could not observe any effect under natural conditions. This could be due to prohibition of natural habitat of these organisms from nematode infection. We could observe only a maximum of 30 % spider mortality even at a higher dose of 500 IJs/spider, a dose which is unlikely to encounter in the natural habitat of spiders. So there is more possibility of safety of EPNs against spiders under field conditions. But it indicated the need for precautions before the field application of EPNs, as we observed the mortality of spiders, even though at a lower level in very high dose. It will be better to go for field application at a time when spiders are least active.

#### **4.4.5 Conclusion**

We observed no pathogenic effect of *S. thermophilum*, *S. glaseri* or *H. indica* on earthworm species, *E. foetida*. However these EPNs were found to be lethal to spider species,

*N. theisi* and ant species *M. himalayanus* at higher doses. So these EPN species should be applied at the time when beneficial ants or spiders are least active.

### **Evaluation of compatibility of *Steinernema thermophilum* with fungal bioagents and bio-pesticidal formulation spinosad on *Helicoverpa armigera* infecting tomato**

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**Abstract:** A study was carried out to evaluate the compatibility of *Steinernema thermophilum* with biopesticidal formulation, Tracer (spinosad) and fungal bioagents *Metarhizium anisopliae* and *Beuvaria bassiana* on 4<sup>th</sup> instar larvae of *Helicoverpa armigera* infecting tomato under glass house conditions. A maximum of 100 % larval mortality was observed within 96 hours of treatment when Spinosad (@ 200 ppm) and *S. thermophilum* (@2000IJs/ml) combined together whereas Spinosad (@ 200 ppm) and *S. thermophilum* (@ 2000IJs/ml) when treated alone yielded a mortality rate of 100 and 72 % respectively after 10 days of treatment. The larval mortality induced by *M. anisopliae* and *B. bassiana* alone were not statistically significant even though it induced a mortality level of 41.7 and 47.1 % respectively. Our result revealed a synergistic interaction of *S. thermophilum* and Spinosad when both combined and applied together. However *S. thermophilum* interacted additively with *M. anisopliae* and slight antagonistic interaction were observed with *B. bassiana*.

**Key words:** *S. thermophilum*, *M. anisopliae*, *B. bassiana*, Spinosad (Tracer), Synergism, antagonism.

#### **4.5.1 Introduction**

The gram pod borer *Helicoverpa armigera* is a major polyphagous insect pest of national importance causing considerable damage to an array of agricultural crops including tomato. Due to environmental and health concerns caused by indiscriminate use of synthetic chemical pesticides, effective alternative means of pest management is an urgent need. A non-chemical control method utilising the entomopathogenic nematode, as a foliar treatment against larval instars (Williams and Macdonald, 1995; Williams and Walters, 1994, 2000) and their integration with other bioagents and safer pesticide regimes have been investigated. Before developing an IPM technique for the control of any pest, the compatibility of

different management options routinely used needs to be established (Lomer *et al.*, 1999). Several studies have addressed the consequences of direct exposure to solutions of insecticides on the behavior and infectivity of selected entomopathogenic nematode species (Hara and Kaya, 1982, 1983; Rovesti *et al.*, 1988; Zhang *et al.*, 1994; Gordon *et al.*, 1996) and detrimental effects reducing infectivity and motility have been recorded for several compounds (Rovesti and Deseo, 1990; Gaugler and Campbell, 1991). However it has also been suggested that exposure to certain chemicals may stimulate nematode movement and enhance host finding behavior and penetration of the host (Ishibashi and Takii, 1993). The indigenous entomopathogenic nematode *S. thermophilum* described by Ganguly and Singh (2000) from India has been found to be heat tolerant and efficacious against several insect pests of national importance including *H. armigera* (Ganguly *et al.*, 2004). Spinosad is a chemical pesticide of biological origin produced by *Sacharopolyspora spinosin*. Among the different toxic compounds produced by the *S. spinosa* two of them namely spinosin A and spinosin D are found to be more efficacious against insect pest. So these two toxin fractions formulated and named as spinosad (SpinosAD). Entomopathogenic fungi like *M. anisopliae* and *B. bassiana* are also potential biocontrol agents for their use against several insect pests. Pest control can be greatly improved by using combinations of biocontrol agents and their increased efficacy may be due to synergistic or additive effects.

So our study was aimed to evaluate the efficacy of *S. thermophilum*, *M. anisopliae*, *B. bassiana* and Spinosad (Tracer) alone and in different combinations to manage *H. armigera* infesting tomato under glass house conditions.

#### **4.5.2 Materials and methods**

Experiment was carried out on tomato (*Solanum lycopersicum*) var. Pusa Ruby, grown on plastic pots of 6” diameter under glass house conditions. The 4<sup>th</sup> instar larvae of *H. armigera* @ 10 larvae/plant were released on tomato plants after 90 days of transplanting when it started flowering and fruit set. The larvae were starved for 24 hours before their release. All the pots were covered with thin polythene sheets to prevent the escape of larvae and the following treatments were applied after 36 hours of larval release.

All the treatments were applied in hand sprayer after mixing with 0.03 % Apsa 80. Each treatment was replicated 5 times and distilled water with 0.03 % Apsa 80 was served as

control. The observations on larval mortality were recorded in every 24 hours up to 5 days and the last observation was taken on 10<sup>th</sup> day of treatment. All the cadavers collected were kept in White's trap for the observation of the emergence of Infective juveniles. The experiment was carried out from 31<sup>st</sup> January, 2011 to 10<sup>th</sup> February 2011 in the glass house of the Division of Nematology, IARI, New Delhi. The temperature and humidity recorded during the period were Tmin - 06 °C, Tmax – 22 °C, RHmin – 55 % and RHmax – 75 %.

### 4.5.3 Results

The result of the experiment on larval mortality is presented in Table 4.5.1. The level of larval mortality was increased as time advances in all the treatments. The treatments namely spinosad (@ 200 ppm), St + Sp (FD) and St + Sp (HD) recorded significant level of larval mortality in all the time intervals. However St (@ 2000 IJs/ml), St + Bb (HD) and spinosad in half dose (@100 ppm) yielded significant larval mortality in 96 hours of treatment, whereas the treatments St (@1000 IJs/ml) and St + Ma (HD) resulted in significant larval mortality after 120 hours of treatment. Even though Ma and Bb in their separate application caused 41.7 % and 47.1 % of larval mortality after 10 days of treatment, these were statistically not significant at 0.05 % probability. When *S. thermophilum* applied alone in 2 different doses yielded 72 % and 55.6 % larval mortality after 10 days of application. Among all the treatments better result was observed by the combined application of *S. thermophilum* (@ 2000 IJs/ml) and spinosad (@ 200 ppm) as it recorded 100 % larval mortality on 4<sup>th</sup> day (96 hours) of treatment. The emergence of Infective juveniles was recorded from insect cadavers killed by *S. thermophilum* alone and in combination with other components and thus confirmed the role of *S. thermophilum* in the larval mortality (Plate 4.5.2). The treatment effects of spinosad and *S. thermophilum* alone and their combinations are shown in figure 4.5.3.

### 4.5.4 Discussion

The interaction of *S. thermophilum* and spinosad in their different doses was rated as synergistic as it enhanced the level and speed of larval mortality than their individual application. The half dose of spinosad (@ 100 ppm) when combined with half dose of *S. thermophilum* (@1000 IJs/ml) resulted in 97.4 % larval mortality in 5 days of treatment and which is higher than the mortality recorded from even full dose (@ 200 ppm) of spinosad in

Table 4.5.1: Effects of different management options on the mortality of *Helicoverpa armigera* infecting tomato.

Treatments	% Mortality					
	24 Hour	48 Hour	72 Hour	96 Hour	120 Hour	10 Day
<b>St @ 2000 IJs/ml (FD)</b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>18.8</b> (25.7)	<b>51.7*</b> (46.0)	<b>64.0*</b> (53.2)	<b>72.0*</b> (58.1)
<b>St @ 1000 IJs/ml (HD)</b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>10.3</b> (18.8)	<b>18.5</b> (25.5)	<b>38.9*</b> (38.6)	<b>55.6</b> (48.2)
<b>Ma @ 4 * 10<sup>7</sup></b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>8.7</b> (17.2)	<b>16.7</b> (24.1)	<b>41.7</b> (40.2)
<b>Bb @ 4 * 10<sup>7</sup></b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>6.1</b> (14.3)	<b>9.4</b> (17.8)	<b>17.6</b> (24.9)	<b>47.1</b> (43.3)
<b>Sp @ 200 ppm</b>	<b>13.9*</b> (21.9)	<b>36.1*</b> 37.0	<b>55.6*</b> (48.2)	<b>77.1*</b> (61.5)	<b>80.0*</b> (63.5)	<b>100.0*</b> (90.0)
<b>Sp @ 100 ppm</b>	<b>0.0</b> (0.0)	<b>3.0*</b> (10.0)	<b>17.6</b> (24.9)	<b>48.4*</b> (44.1)	<b>67.7*</b> (55.4)	<b>74.2*</b> (59.5)
<b>St + Ma (HD)</b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>3.2</b> (10.4)	<b>9.1</b> (17.6)	<b>38.5*</b> (38.3)	<b>69.2*</b> (56.3)
<b>St + Bb (HD)</b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>9.7</b> (18.1)	<b>29.2*</b> (32.7)	<b>41.2*</b> (39.9)	<b>52.9</b> (46.7)
<b>St + Sp (FD)</b>	<b>11.4*</b> (19.8)	<b>38.9*</b> (38.6)	<b>97.1*</b> (80.3)	<b>100.0*</b> (90.0)	<b>100.0*</b> (90.0)	<b>100.0*</b> (90.0)
<b>St + Sp (HD)</b>	<b>7.1*</b> (15.5)	<b>11.9*</b> (20.2)	<b>74.4*</b> (59.6)	<b>97.4*</b> (80.8)	<b>97.4*</b> (80.8)	<b>100.0*</b> (90.0)
<b>Control</b>	<b>0.0</b> (0.0)	<b>0.0</b> (0.0)	<b>3.4</b> (10.7)	<b>3.4</b> (10.7)	<b>7.1</b> (15.5)	<b>14.3</b> (22.2)
<b>SEM<sub>±</sub></b>	<b>4.0</b>	<b>4.5</b>	<b>7.5</b>	<b>7.9</b>	<b>9.7</b>	<b>12.4</b>
<b>CD (P= 0.05)</b>	<b>8.8</b>	<b>9.9</b>	<b>16.6</b>	<b>17.5</b>	<b>21.4</b>	<b>27.2</b>

St – *S. thermophilum*;

Ma – *M. anisopliae*;

Bb – *B. bassiana*;

Sp – Spinosad

FD – Full doze; HD – Half of the doze

# Data in paranthesis are Arcsine transformed values

\* Significantly different from control

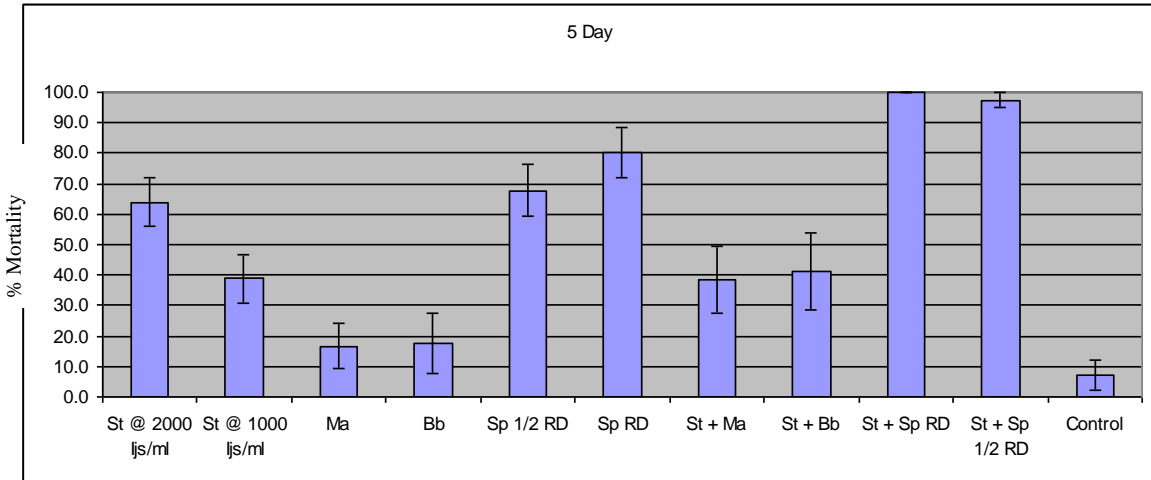


Figure 4.5.1: Comparison on mortality of *H. armigera* infecting tomato after 5 days of treatment with different management options

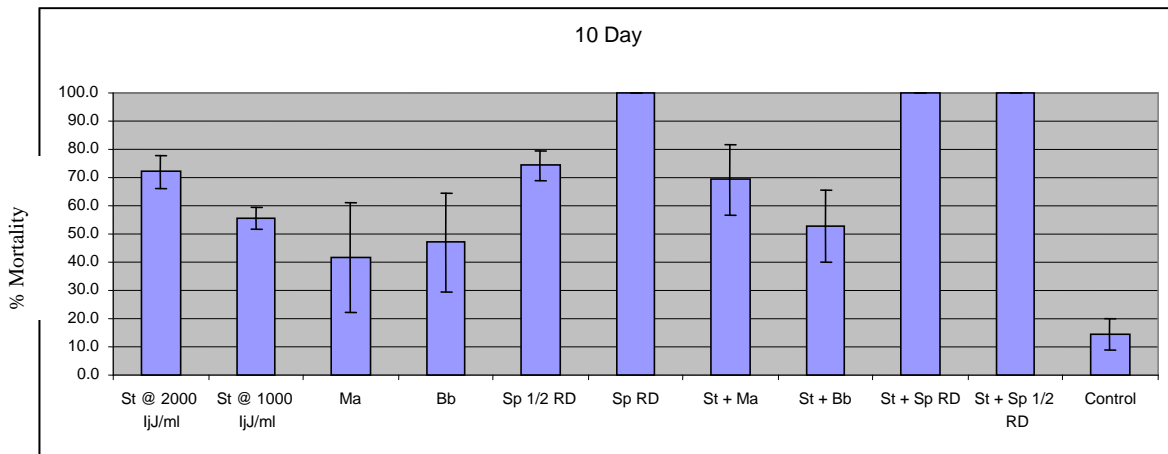


Figure 4.5.2: Comparison on mortality of *H. armigera* infecting tomato after 10 days of treatment with different management options

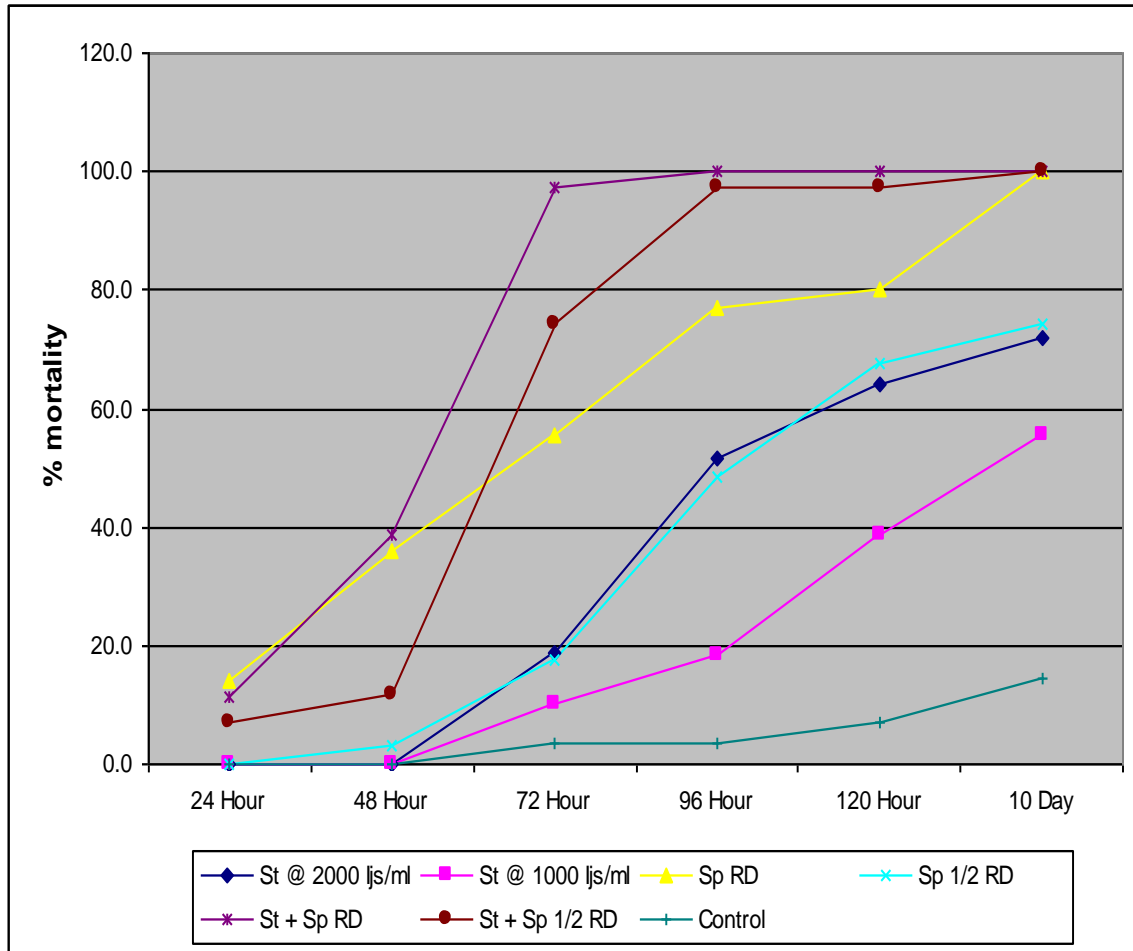


Figure 4.5.3: Comparison on mortality of *H. armigera* on tomato induced by Spinosad, *S. thermophilum* and their combinations

the same time duration. The role of nematode on larval mortality in the combined application with spinosad was obvious as it recorded a sharp increase in the level of mortality from 48 hours to 72 hours (Figure 4.5.3) and which was further confirmed by the emergence of Infective juveniles from the cadavers. In the earlier studies we observed no harmful effect of spinosad (Tracer) on the viability and virulence of the Infective juveniles of *S. thermophilum* and even slight improvement in the virulence of IJs after exposure to double the highest recommended dose (400 ppm) of the same for a period of 48 hours. Further we confirmed the retention of virulence by the IJs of *S. thermophilum* even after its exposure to spinosad in its recommended dose (200 ppm) for a period of one month. There could be several reasons for the synergism between chemical pesticides and entomopathogenic nematodes. When the larvae exposed to the chemical pesticides, the insect reduce its mobility and become weak, thus nematodes get more chance for infection and have to spend little energy to counteract the immune system of its host. Besides this the IJs may get activated when they exposed to pesticidal compounds, thus become more virulent and all these factors ultimately lead to better performance. Furthermore this strategy i.e., combining lower doses of safer pesticides in combination with entomopathogenic nematodes could be a potential alternative to reduce the pesticidal load to the environment as well as to counteract the development of pesticide resistant strains of insect pests.

Both *B. bassiana* and *M. anisopliae* in the dose we applied were found to be not effective as it induced only 41.7 and 47.1 % larval mortality after 10 days of treatment which was statistically non significant. However when *S. thermophilum* when combined with *M. anisopliae* was yielded 69.2 % larval mortality in 10 days of treatment which was statistically significant and rated as additive interaction. When *S. thermophilum* combined with *B. bassiana* recorded only 52.9 % larval mortality after 10 days, which was lower than the rate of mortality caused by *S. thermophilum* alone (55.6 %) and higher than that of *B. bassiana* alone (47.1). So the interaction of *S. thermophilum* and *B. bassiana* were considered as slightly antagonistic. In the earlier studies we observed no reduction in the viability or virulence of IJs of *S. thermophilum* when exposed to the cultures of above two fungal bioagents. But later studies of their interaction on culture media was revealed the suppression of both the fungal bioagents by *S. thermophilum* and its symbiotic bacteria *Xenorhabdus indica*. Among these two fungal bioagents *M. anisopliae* were found to be more competent as



Plate 4.5.1: Pot experiment to evaluate the efficacy of *S. thermophilum* alone and in combination with various bio-agents.



Plate 4.5.2: Emergence of infective juveniles of *S. thermophilum* from the cadavers of *H. armigera* collected from different treatments

compared to *B. bassiana* in the presence of *S. thermophilum*. So, all these result clearly indicate that there is no advantage in combining these fungal bioagents with *S. thermophilum* for the management of insect pests in long run.

#### **4.5.5 Conclusion**

The present investigation revealed the synergistic interaction of *S. thermophilum* and bio-pesticidal formulation spinosad for the management of *H. armigera* infesting tomato. However *S. thermophilum* interacted additively with *M. anisopliae* and slight antagonistically with *B. bassiana*. Eventhough *M. anisopliae* interacted additively with *S. thermophilum*, this effect could be temporary as we observed antagonism between both the bio agents on culture media. So we concluded that the combination of *S. thermophilum* and these fungal bioagents is not advisable for the management of same insect pest. However entomopathogenic nematodes like *S. thermophilum* in combination with lower doses of safer pesticidal formulations like spinosad could be a possible alternative to reduce the pesticide load to the environment as well as to combat the threat of pest resurgence or secondary pest outbreak.

## 4.6 RESEARCH PAPER VI

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### **Extraction and evaluation of biotoxins produced by *Xenorhabdus indica* (Enterobacteriaceae), the symbiotic bacterium associated with entomopathogenic nematode, *Steinernema thermophilum*.**

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**Abstract:** The symbiotic bacterium *Xenorhabdus indica* was isolated from its host *Steinernema thermophilum* and cultured in nutrient broth medium. The metabolites produced by the bacteria were isolated by solvent extraction method by using hexane, ethyl acetate and butanol as solvents. Different concentrations of all the three solvent fractions were treated on 4<sup>th</sup> instar larvae of *Galleria mellonella* and *Spodoptera litura* for evaluating its insecticidal property. We could not detect any insecticidal property up to a concentration of 10000 ppm. When different concentrations of all the solvent fractions treated on J<sub>2</sub> of *Meloidogyne incognita* did not show any nematocidal property up to a concentration of 5000 ppm. However, all the three fractions when studied by poisoned food technique exhibited fungicidal action against *Sclerotium rolfii*. Ethyl acetate fraction exhibited maximum antifungal activity which recorded a maximum of 90.8 % reduction in the fungal growth when treated at 0.1 % concentration. At the same concentration, hexane and butanol fraction showed 52.14 and 78.1 % fungal growth inhibition. The spectroscopic studies to find out the chemical constituents of ethyl acetate fraction revealed the presence of xenocoumacins, nematophins and xenorhabdins.

**Key words:** *Xenorhabdus indica*, solvent extraction, antifungal activity, chemical constituents.

#### **4.6.1 Introduction**

Species of the genus *Xenorhabdus* are gram-negative entomopathogenic bacteria, which live symbiotically with nematodes of the genus *Steinernema*. The combined actions of the nematode and bacterium kill the insect usually within 48 h.

*Xenorhabdus* and *Photorhabdus* are motile Gram-negative bacteria that are highly pathogenic to insects (Burnell and Stock, 2000). The bacteria live in symbiosis with

rhabditoid nematodes belonging to the genera *Steinernema* and *Heterorhabditis* (Vivas and Goodrich-Blair, 2001; Forst and Clarke, 2002). *Xenorhabdus* occurs naturally in a special intestinal vesicle of *Steinernema* (Bird and Akhurst, 1983) while *Photorhabdus* are mainly located in the anterior part of *Heterorhabditis* infective juveniles (Boemare *et al.*, 1996). Upon invasion of the insect, the IJ enters the haemocoel and releases *Xenorhabdus* or *Photorhabdus* into the hemolymph. *Xenorhabdus* spp. and *Photorhabdus* spp. secrete proteinaceous toxin complexes (Boven *et al.*, 1998). Together the bacteria and nematode contribute to the pathogenic process involved in killing the insect host. The combined actions of the nematode and bacterium kill the insect usually within 48 h. The bacteria grow to high cell density and cause septicemia, which break down the host. In the insect cadaver, these bacteria secrete a diverse array of antimicrobial products which are believed to protect the insect cadaver from invasion by other soil organisms. Many strains of *Xenorhabdus* and *Photorhabdus* bacteria produce a variety of metabolites. These metabolites often have overlapping functions, a strategy that is likely to contribute to the success of the nematode–bacteria association against a variety of insect hosts. Also *Xenorhabdus* spp. produce exoenzymes that degrade insect tissues and macromolecules which contribute to the nutrient base that supports bacterial and nematode reproduction. The metabolites produced help to evade the insect immune system, enzymes such as proteases, lipases and phospholipases to maintain a food supply during reproduction (Thaler *et al.*, 1998) and antifungal and antibacterial agents to prevent degradation or colonization of the insect carcass, while bacteria and nematode reproduce (Akhurst & Dunphy, 1993).

The symbiotic bacterium associated with indigenous heat tolerant entomopathogenic nematode *S. thermophilum*, has been isolated, characterized and which has been described as *Xenorhabdus indica* (Somvanshi *et al.*, 2006). There is a need to identify the bio-toxicants involved in this complex, especially from the symbiotic bacteria *X. indica* as it is a potential source for its exploration for novel bio-toxins. So the present study was carried out to isolate the biotoxins produced by the bacteria and to evaluate its insecticidal, nematicidal and fungicidal properties.

## 4.6.2 Materials and Methods

**4.6.2.1 Chemicals and reagents:** Chemicals and reagents required for the study were purchased from Merck India limited and Qualigens Fine Chemicals. Potato-Dextrose-Agar (PDA) used as a medium for antifungal activity was procured from Hi Media Laboratories. Solvents used in extraction were laboratory grade. Solvents required for mass spectroscopy were HPLC grade.

**4.6.2.2 Mass Spectroscopy:** Mass spectroscopy was carried out using Thermo Electron LC-MS-MS. Spectrometer (Thermo Electron Corporation, USA) equipped with P-2000 quaternary pumps. Detection of mass was done by electron spray ionization (ESI) source in positive mode with Finnigan LCQ tune plus program fitted with MAX-detector. Xcalibur software was used for the purpose of identification, quantification and fragmentation pattern. The following MS parameters were optimized in direct infusion mode: Spray voltage (5 kV), Sheath gas flow rate (21 mL min<sup>-1</sup>), Auxiliary gas flow rate (4.5 mL min<sup>-1</sup>), Spray current (0.91 μA), Capillary temperature (210°C-230°C), Capillary voltage (7.5V) and Tube lens offset (40).

**4.6.2.3 Mass culturing of bacteria:** The symbiotic bacteria *X. indica* was isolated from the infective juveniles of *S. thermophilum* which was already available in our laboratory by following the methodology given by Akhurst (1980). Pure culture of *X. indica* was inoculated in Tryptic soy broth medium in 500 ml conical flasks and incubated at 27±1 °C and in 180 rpm in an incubator shaker. Then 72 hours old bacterial cultures were used for the extraction of metabolites by solvent extraction method.

**4.6.2.4 Extraction:** Secondary metabolites were sequentially extracted and partitioned using three solvents namely, hexane, ethyl acetate and butanol in a separatory funnel to give three corresponding solvent fractions. The culture broths were mixed with equal volume of the respective solvents in a separatory funnel with gentle shaking for 5 minutes. The solvent fractions were passed through anhydrous sodium sulphate (20 g) to remove the traces of water if any. The solvents were removed under reduced pressure (100 bar) below 40 °C in rotary evaporator to obtain corresponding residues. The extraction process was repeated five times with 4 L. batch of bacterial broth to obtain sufficient quantity of the corresponding extract.

#### **4.6.2.5 Bioassay**

**4.6.2.5.1 Test concentrations:** 100 mg of residues of hexane, ethyl acetate and butanol fractions (100 mg) of *X. indica* were dissolved in 2 mL of acetone to yield a standard stock of 5 % stock solution. Required test concentrations for bioassay studies against nematodes, insects and fungi were prepared by serial dilution of standard stock solution with emulsified water (0.5% Tween 80).

**4.6.2.5.2 Evaluation of nematicidal activity:** Nematicidal activity of all the three solvent fractions were evaluated by treating second stage juveniles (J<sub>2</sub>) of *Meloidogyne incognita* in three different concentrations (0.05%, 0.1 % and 0.2 %) by water screening method.

**4.6.2.5.3 Evaluation of insecticidal activity:** Insecticidal activity of different solvent fractions was studied on 4<sup>th</sup> instar larvae of *Galleria mellonella* and *Spodoptera litura*. Three different doses (1000, 5000 and 10000 ppm) of solvent fractions were treated on these two species of insect larvae by direct injection into the haemocoel as well as by feeding method.

**4.6.2.5.4 Evaluation of fungicidal activity:** Fungicidal activity of different solvent fractions was evaluated by poisoned food technique against *Sclerotium rolfsii* at six different concentrations of 1000, 500, 250, 125, 62.5, and 31.25 ppm. A 5 mm thick disk of fungus was placed in different Petri plates. Plates treated with acetone were taken as control. Both treated and control plates were kept in BOD incubator at 28±1°C till the fungus growth in the control plate was completed (4-6 days) after which the readings were taken.

#### **4.6.3 Results**

**4.6.3.1 Extraction of biotoxins:** Culture broth of *X. indica* was extracted and partitioned with hexane, ethyl acetate, and butanol to obtain 5 g, 4 g and 7 g of residue, respectively.

**4.6.3.2 Nematicidal property:** There was no mortality of J<sub>2</sub> of *M. incognita* in any of the concentrations of biotoxins evaluated up to 72 hours of treatment. It indicated the absence of any nematicidal toxins in the solvent fractions isolated.

**4.6.3.3 Insecticidal property:** No mortality of insect larvae was recorded in any of the treatments up to 5 days of treatment. It showed the absence of any insecticidal toxins in the solvent fractions of *X. indica* culture isolated.

**4.6.3.4 Fungicidal property:** All three fractions in all the concentrations evaluated showed fungicidal activity against *Sclerotium rolfsii*. Maximum activity was shown by ethyl acetate fraction followed by butanol and least by hexane extract. Ethyl acetate fraction in 0.1% yielded 90 % reduction in the growth of the fungus whereas butanol and hexane fractions in the same concentration recorded 78 and 52 % reduction respectively. Ethyl acetate extract at lowest concentration (31.25 ppm) exhibited highest fungal growth inhibition (45%) followed by butanol (29 %) and hexane extract (26%). The EC<sub>50</sub> value for ethyl acetate, butanol and hexane fractions calculated to be 45, 87 and 51 ppm respectively. As ethyl acetate fraction showed highest fungicidal activity, this fraction was further studied for its chemical composition.

#### **4.6.3.5 Electron Spray Ionization- Mass Spectroscopy analysis of biotoxins:**

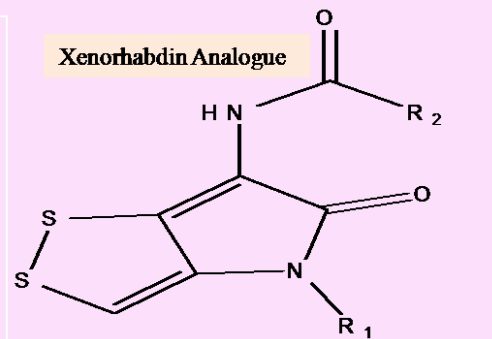
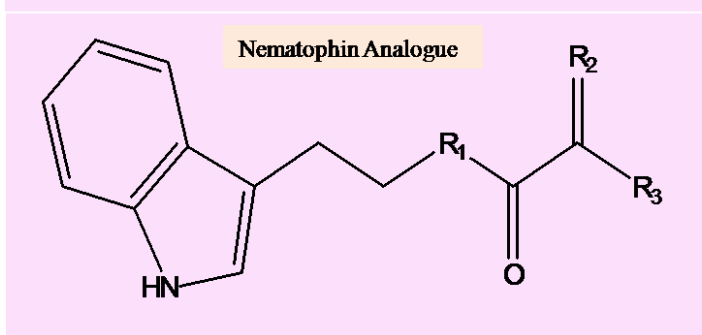
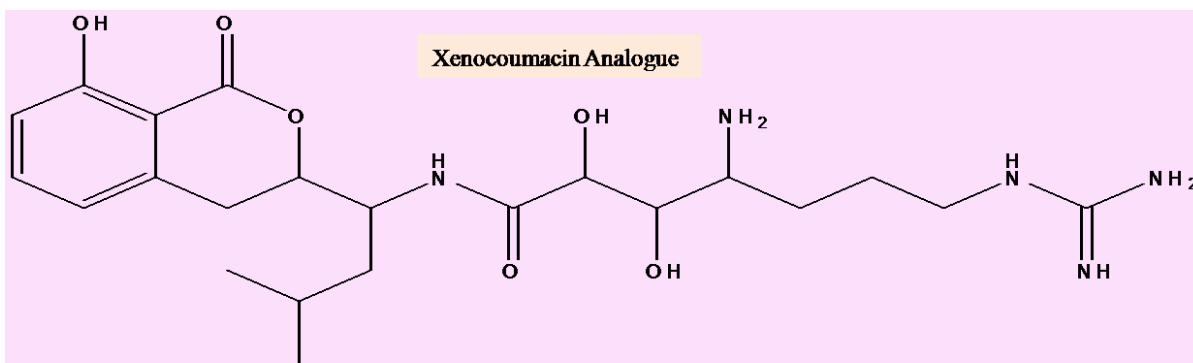
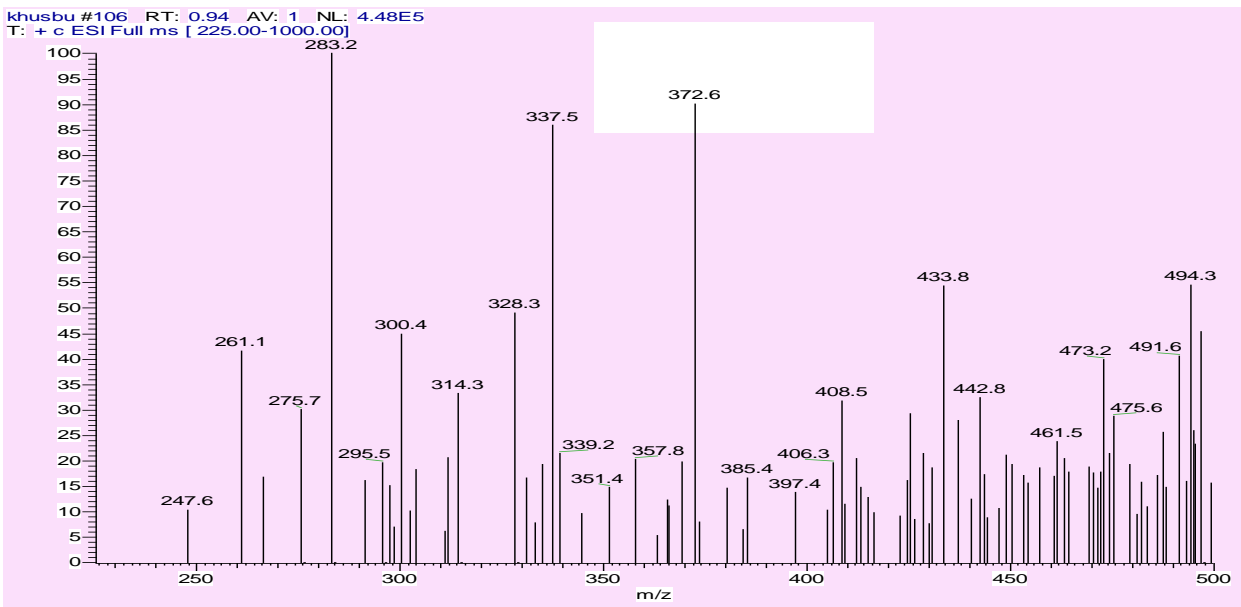
The chemical constituents of most active ethyl acetate extract was analyzed by ESI-MS in direct infusion mode. The total ion chromatogram (TIC) exhibited a number of major peaks, indicating xenocoumacin, nematophin and xenorhabdin analogues (Fig 4.6.1).

#### **4.6.4 Discussion**

During the complex life cycle, the bacteria not only have to kill the insect host using several protein toxins but also have to defeat several other microbes that are direct food competitors. Saprophytic microbes from the soil as well as bacteria adhering to the insect gut or cuticle of the nematode represent other potential sources of competitors that can grow within the insect cadaver. These competitors are eliminated by the symbiotic bacteria, as it becomes the dominant microbial species in the hemolymph. To successfully compete for nutrient resources of the insect *Xenorhabdus* and *Photorhabdus* produce phage-derived bacteriocins (Thaler *et al.*, 1995), colicin E3-type killer proteins (Singh and Banerjee, 2008) and insect toxin complexes (Ffrench-Constant and Waterfield, 2006) as well as several secondary metabolites. The three solvent fractions, we isolated did not show any insecticidal or nematocidal properties, but exhibited fungicidal action. The insecticidal or nematocidal compounds produced by *Xenorhabdus* or *Photorhabdus* are mainly enzymes or biologically active proteins. These enzymes or proteins are unlikely to be isolated by the solvent extraction method as it yields mainly the secondary metabolites. The toxins in the secondary metabolites are more likely to be antibiotic compounds targeting secondary invaders. In the

Table 4.6.1: Antifungal activity of different solvent fractions of *Xenorhabdus indica* biotoxins against *Sclerotium rolfsii*

Concentration (ppm)	% Growth inhibition		
	Hexane extract	Ethyl acetate extract	Butanol extract
1000	52.14	90.8	78.1
500	50.71	72.6	65.5
250	40.0	68.9	59.83
125	35.71	62.7	42.6
62.5	28.57	57.6	38.91
31.25	26.43	45.2	29.4
EC <sub>50</sub>	696.96	48.62	153.09
$\chi^2$	1.01	6.19	1.52
Y	3.56+0.51X	3.65+0.80X	3.11+0.87X



- 1)  $R_1 = \text{NH}$   $R_2 = \text{OH} + \text{H}$   $R_3 = \text{CH}_2\text{CH}_3$
- 2)  $R_1 = \text{NH}$   $R_2 = \text{OH} + \text{H}$   $R_3 = \text{CH}(\text{CH}_3)_2$
- 3a)  $R_1 = \text{NH}$   $R_2 = \text{OH} + \text{H}$   $R_3 = \text{CH}_2\text{CH}(\text{CH}_3)_2$
- 3b)  $R_1 = \text{NH}$   $R_2 = \text{OH} + \text{H}$   $R_3 = \text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$

- 1)  $R_1 = \text{CH}_3$   $R_2 = \text{C}_5\text{H}_{11}$
- 2)  $R_1 = \text{CH}_3$   $R_2 = (\text{CH}_2)_3\text{CH}(\text{CH}_3)_2$
- 3)  $R_1 = \text{CH}_3$   $R_2 = \text{CH}_2\text{CH}(\text{CH}_3)_2$
- 4)  $R_1 = \text{CH}_3$   $R_2 = (\text{CH}_2)_6\text{CH}_3$
- 5)  $R_1 = \text{CH}_3$   $R_2 = (\text{CH}_2)_7\text{CH}_3$

Figure 4.6.1: Total Ion Chromatogram (TIC) of ethyl acetate extract of *Xenorhabdus indica*

present study, all the three solvent fractions exhibited fungicidal property against *S. rolfsii* with more activity by ethyl acetate fraction. Major biotoxins of ethyl acetate extract of *X. indica* were identified as xenocoumacin, nematophin and xenorhabdin analogues. Xenocoumacin analogues include xenocoumacin I and one cyclic derivative. Four different types of nematophin analogues and five substituted derivatives of xenorhabdin were identified in ethyl acetate extract. Such type of tryptamine derivatives was also reported in *X. nematophilus* (McInerney *et al.*, 1991; Li *et al.*, 1998). Compounds described in the literature represent mostly small molecules like benzylideneacetone (Ji *et al.*, 2004), iodine (Fodor *et al.*, 2008), phenethylamides and indole derivatives (Li *et al.*, 1995; McInerney *et al.*, 1991) but also more complex compounds like the xenorhabdins and xenorxides (Li *et al.*, 1998) and xenocoumacins (McInerney *et al.*, 1991) are known. Despite their simple structure, different biological activities have been described for these compounds and their biosynthesis genes have not been identified yet except for the xenocoumacins.

#### **i. Conclusion**

The secondary metabolites produced by *X. indica* was isolated by solvent extraction method with three different solvents namely, hexane, ethyl acetate and butanol. All the three solvent fractions showed fungicidal properties against *S. rolfsii* with maximum activity by ethyl acetate extract. Further studies on the chemical nature of ethyl acetate fraction revealed the presence of antimicrobial compounds like xenocoumacin, nematophin and xenorhabdin.

## 6. DISCUSSION

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The present investigation was carried out to evaluate the compatibility of *Steinernema thermophilum* with different management options, to know the nontarget effect of different entomopathogenic nematode species as well as to isolate and characterize biotoxins produced by its symbiotic bacterium *Xenorhabdus indica*.

Out of the total 26 pesticidal formulations evaluated all of them except Hostathion, Dursban and Thiodan were found to be compatible with *S. thermophilum* in their recommended doses. Earlier studies had shown the differences in compatibility of the pesticidal formulations to different entomopathogenic nematodes (Rovesti *et al.*, 1988; Rovesti and Deseo, 1990). The compatibility of Imidacloprid (Merit 75 WP) with entomopathogenic nematodes *Heterorhabditis bacteriophora* and *S. carpocapsae* were reported in several studies (Koppenhöfer *et al.*, 2003; Alumai and Grewal, 2004). Anuar and Daniel (2009) reported the synergistic interaction between *H. bacteriophora* and this pesticidal formulation against white grubs. Another neonicotinoid insecticide Thiomethoxam (Meridian 25%) was found to be compatible with *H. megidis*, *S. feltiae*, *S. glaseri* and *S. carpocapsae* where as non-compatible interactions were also observed for the same formulation with *H. bacteriophora* (Koppenhöfer *et al.*, 2003; Alumai and Grewal, 2004). Alumai and Grewal (2004) also established the compatibility of Spinosad (Conserve SC) with *H. bacteriophora* and *S. carpocapsae*. As compatibility of entomopathogenic nematodes and pesticides varies with different species and formulations, the compatibility of each entomopathogenic nematode species should be evaluated with each pesticidal formulation, before any recommendations for their combined application. Compatibility of *S. thermophilum* with different pesticidal formulations was evaluated for the first time and we established the compatibility of *S. thermophilum* with 23 pesticidal formulations. This information will be useful to integrate these compatible pesticidal formulations with *S. thermophilum*. Even some chemicals used as inert ingredients or adjuvants in formulations can be toxic to nematodes (Krishnayya and Grewal, 2002), compatibility of different adjuvants with the specific nematode species should be evaluated. All the adjuvants evaluated in the present study were found to be compatible with *S. thermophilum* as it did not cause any significant level of nematode mortality up to one month. These adjuvants could be

useful in improving the field performance of *S. thermophilum* by mixing them while applying in the field or by developing the formulations.

Bacterial bioagents namely *Bacillus thuringiensis* and *Pseudomonas fluorescens* were found to be incompatible with *S. thermophilum* as these bioagents induced significant level of mortality of its infective juveniles. The antimicrobial compounds or enzymes produced by these bioagents could be the reason for nematode mortality. Therefore, these bacterial bioagents should not be combined with *S. thermophilum*. None of the fungal bioagents evaluated were found to cause mortality of *S. thermophilum*. This type of information is not sufficient to ensure the compatibility of two entomopathogenic organisms as there can be possibility of their mutual interaction as well as with symbiotic bacteria of entomopathogenic nematodes inside the target pest. Keeping this in view, we carried out the mutual interaction study of entomopathogenic fungi like *Metarhizium anisopliae* and *Beauveria bassiana* with *S. thermophilum* and its symbiotic bacterium, *Xenorhabdus indica* on artificial media. This study revealed the lethal effect of *S. thermophilum* and its symbiotic bacteria on these fungal bioagents. But we did not see any harmful effect of these fungal bioagents on *S. thermophilum* or *X. Indica* and it indicated the better competence of *S. thermophilum* over these bioagents. The antimicrobial natures of metabolites produced by symbiotic bacteria of entomopathogenic nematodes had already been reported (Webster *et al.*, 2002) and several of these metabolites are known to have fungicidal action (Barbercheck and Kaya, 1990; Chen *et. al.*, 1994). These metabolites must be the reason for the suppression of the bioagents by *S. thermophilum* and *X. Indica*.

Even though organic amendments have been used successfully to create phytopathogen suppressive soils, almost no documentation exists on the effects of these amendments on populations of entomopathogenic nematodes. In field experiments, organic manure used as fertilizer had been reported to have positive (Bednarek and Gaugler, 1997) as well as negative (Hsiao and All, 1997; Shapiro *et al.*, 1999a) effects on nematode establishment and recycling. In our experiment we studied the direct effect of different organic amendments on *S. thermophilum*. We did not find any harmful effect of organic amendments like bone meal, farm yard manure, vermicompost and neem cake on the survival and infectivity of *S. thermophilum*.

Studies on interspecific interaction among entomopathogenic nematode species revealed that, both heterorhabditid and steinernematid could not develop on the same host with combined application. In the laboratory, heterorhabditids and steinernematids cannot coexist in the same host, because neither can develop on the symbiotic bacteria of the other genus (Koppenhöfer *et al.*, 1995). However, various effects of the mixed infection on the reproduction of steinernematids have been reported. Our study showed the development of *S. thermophilum* and *S. glaseri* together in the same host with dominance of *S. glaseri* at lower temperature and *S. thermophilum* at higher temperature. It showed the better competence of *S. glaseri* at lower temperature and *S. thermophilum* at higher temperature. The survival and development of *S. thermophilum* at higher temperature was reported by Ganguly and Gavas (2004). As both of them could develop together in the same host, even though by the suppression of one species, the mutual antagonism between the respective *Xenorhabdus* species of both of these steinernematids must be weak. The change in the dominance of different entomopathogenic nematodes at different temperature ranges showed the importance of the selection of suitable EPN species at a particular climatic condition.

Studies on the nontarget effects of entomopathogenic nematodes have been performed by various workers on different species of nontarget organisms, and a large range from complete harmlessness to pronounced harmful effect was established (Bathon 1996, Farag 2002, Powell and Webster 2004). The results of some earlier field trials showed a moderate influence of entomopathogenic nematodes on nontarget arthropods or even the absence of such an effect (Georgis *et al.* 1991). We have evaluated the effect of *S. thermophilum*, *S. glaseri* and *H. indica* on earthworms, spiders and ants. In our study, earthworms were found to be safe from these entomopathogenic nematodes. Even though many associations between nematodes and earthworms had been reported (Gunnardson and Rundgren, 1986; Poinar, 1978; Timper and Davies, 2004), there is no report on any adverse effect of earthworms by entomopathogenic nematodes. Some authors reported that earthworms could be used as vectors to introduce and/or disperse beneficial organisms including EPNs (Eng *et al.*, 2005; Shapiro *et al.*, 1993, 1995). Spiders and ants were found to be affected by these entomopathogenic nematodes at higher doses. Bathon (1996) reported that mortality can be observed among the nontarget organisms, but the influence of these agents should be temporary and local and so only a part of the population is under attack.

Georgis *et al.* (1991) demonstrated a negligible influence of entomopathogenic nematodes on nontarget organisms if they are used only in short term pest control.

In pot experiment, *M. anisopliae* interacted additively and *B. bassiana* antagonistically with *S. thermophilum* against *Helicoverpa armigera* infecting tomato. There are several reports on the synergistic, additive or antagonistic interactions between entomopathogenic nematodes and other bioagents. Ansari *et al.* (2004) reported stronger synergistic effects between *M. anisopliae* and *H. megidis* or *S. glaseri* against third-instar of *Hoplia philanthus*. Koppenhöfer *et al.* (1999) also demonstrated that combining *H. bacteriophora* and *S. glaseri* with *B. thuringiensis* subspecies *japonensis* Buibui strain caused additive and synergistic effects against masked chafers, *Cyclocephala hirta* and *C. pasadenae*, in the greenhouse and under field conditions. Shapiro-Ilan *et al.* (2005b) investigated the interaction effect of the nematodes *H. indica* or *S. carpocapsae* with the fungi *B. bassiana*, *M. anisopliae*, or *Paecilomyces fumosoroseus*, or the bacterium *Serratia marcescens* on pecan weevil, *Curculio caryae*. They observed antagonism in all pathogen combinations, except *H. indica* combined with *M. anisopliae*, for which additive effects were observed. All these studies indicated that the interaction effect varies with different species of entomopathogenic nematode and bioagent combinations. In the present study Interaction of *S. thermophilum* and *B. bassiana* were considered as slightly antagonistic. In the laboratory experiment we observed no reduction in the viability or virulence of IJs of *S. thermophilum* when exposed to the cultures of above two fungal bioagents. But later studies of their interaction on culture media revealed the suppression of both the fungal bioagents by *S. thermophilum* and its symbiotic bacterium *Xenorhabdus indica*. Among the two fungal bioagents *M. anisopliae* was found to be more competent as compared to *B. bassiana* in the presence of *S. thermophilum*. Hence, all these results indicate that there is no advantage in combining these fungal bioagents with *S. thermophilum* for the management of insect pests in long run.

Compatibility of Spinosad with *S. thermophilum* was confirmed by viability and virulence evaluations in the laboratory. Further studies on compatibility of *S. thermophilum* with biopesticidal formulation Spinosad (Tracer) revealed their synergistic interaction in inducing the mortality of *H. armigera* infecting tomato when both were applied even in minimum dosage. In contrast, *S. thermophilum* or spinosad when applied alone, even their

high doses were less effective. Earlier Alumai and Grewal (2004) reported the compatibility of Spinosad (Conserve SC) with *H. bacteriophora* and *S. carpocapsae*. The synergistic interaction between an EPN and compatible pesticidal formulations can be attributed to several factors. Pesticidal formulations may stimulate the infective juveniles, thus increasing their mobility to find their host insect. Inactivation of insect larvae by the pesticides may increase the chance of host finding by the nematodes or weakening of the host defense mechanism by the pesticides may help nematodes to establish faster. This result indicated the possibility of combining lower doses of safer pesticides in combination with entomopathogenic nematodes as a potential alternative to reduce the pesticidal load on the fragile environment as well as to counteract the development of pesticide resistant strains of insect pests.

In the entomopathogenic nematode-bacteria-insect complex, the insect cadaver does not give any putrid odor, because the bacterium does not allow the growth of any other microorganism. Our studies on bacterial metabolites showed the presence of high fungicidal activity in ethyl acetate fraction when tested against *Sclerotium rolfsii*. The spectroscopic analysis revealed the chemical nature of these fungicidal compounds to resemble with xenocoumacin, nematophin and xenorahbdins. Such type of tryptamine derivatives was also reported in *X. nematophilus* (McInerney *et al.*, 1991; Li *et al.*, 1998).

## 6. SUMMARY AND CONCLUSION

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The present investigation was carried out to evaluate the compatibility of indigenous heat tolerant entomopathogenic nematode *Steinernema thermophilum* with different agrochemicals, bioagents, organic amendments, nontarget organisms and to know the nature of biotoxins produced by its symbiotic bacterium *Xenorhabdus indica*.

The compatibility of different doses of 26 pesticidal formulations including 12 insecticides, 6 herbicides, 3 fungicides and 7 biopesticides was studied. The effect of four different doses namely double the maximum recommended dose, maximum recommended dose, half of the maximum recommended dose and the lowest recommended doses of pesticidal formulations on survival and virulence of infective juveniles of *S. thermophilum* was tested. The pesticidal formulations namely Tagride, Talstar, Marshal, Superkiller, Decis, Confidor, Karate, Tracer, Tagtara, (Insecticides), Stomp, Atrataf, Isogourd, Fernoxone, Clincher, Validamycin (Herbicides), Zinthane, Contaf, Bavistine (Fungicides), Baba, Metarhizium, Mycomite, Nyantran, Sparsha, Nisarga and Varsha (biopesticides) were found compatible with *S. thermophilum* in their recommended doses as it did not affect either viability or virulence of infective juveniles. However, insecticides like Hostathion, Dursban and Thiodan were observed to be incompatible. Double the highest recommended dose of fernoxone was found to be harmful to the survival of infective juveniles, but no harmful effect was recorded for its recommended dose. Conclusively these pesticidal formulations which were found compatible could be tank mixed and applied safely along with *S. thermophilum*.

A total of eight surfactants/adjuvants namely Apsa 80, CMC, glycerol, Liquid Paraffin, Starch, Triton X 100, Tween 20 and Tween 80 in two different doses (1% and 3 %) were evaluated for their compatibility with *S. thermophilum*. All the surfactants/adjuvants tested were found compatible as it did not affect either viability or virulence of infective juveniles for a period of 30 days. So these surfactants/adjuvants can be mixed with *S. thermophilum* for enhancing the field efficacy of this entomopathogenic nematode. Additionally this information will be useful in developing novel liquid formulations of this entomopathogenic nematode species.

The fungal bioagents like *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces lilacinus*, *Pochonia chlamydosporium*, *Trichoderma viride*, *T. harzianum* and bacterial bioagents like *Bacillus thuringiensis* and *Pseudomonas fluorescens* were evaluated for their compatibility with *S. thermophilum*. Significant level of nematode mortality was observed in all the treatments when the infective juveniles of *S. thermophilum* exposed to four different doses of three days old cultures of bacterial bioagents in nutrient broth medium. It showed that these bacterial bioagents are incompatible with *S. thermophilum* and cannot be combined. Fifteen days old cultures of fungal bioagents in their respective culture medium were tested on infective juveniles of *S. thermophilum* and evaluated for their survival up to 7 days and virulence was tested after 48 hours of exposure. None of these fungal bioagents recorded any harmful effect on infective juveniles of *S. thermophilum*. As *M. anisopliae* and *B. bassiana* are entomopathogenic fungi and there is a possibility of their direct interaction with *S. thermophilum* and with its symbiotic bacterium *X. Indica* inside the host, we studied their mutual interaction *in vitro* on the culture media. The mutual interaction study revealed the harmful effect of both *S. thermophilum* and *X. Indica* on the growth and development of both the fungal bioagents. So we concluded that, both the entomopathogenic fungi are incompatible with *S. thermophilum* and should not be recommended for their combined application against the same target pest.

Organic amendments like bone meal, farm yard manure, neem cake and vermicompost were found to be compatible with *S. thermophilum* up to an exposure period of 10 days.

The interspecific interactions among entomopathogenic nematodes *S. thermophilum*, *S. glaseri* and *Heterorhabditis indica* were studied at 27 °C and 35 °C. The infective juveniles of these nematode species separately and in different combinations were tested on 4<sup>th</sup> instar larvae of *G. mellonella* @ 100 IJs/larva. The observations on time taken for mortality, emergence of nematode progenies as well as number of infective juveniles from each cadaver, were recorded. The dominance of *S. glaseri* over other two species and *S. thermophilum* over *H. Indica* was observed at 27 °C. However, only *S. thermophilum* developed normally and dominated over other two species at 35 °C. All the combined applications in any combination resulted in drastic reduction in the number of progeny production as compared with their individual infections. It indicated that there is no

advantage in combining different entomopathogenic nematode species against same insect host rather selection of suitable entomopathogenic nematode species against a particular insect pest under a given environmental condition is critical in the success of EPN application.

The nontarget effect of different entomopathogenic nematodes *S. thermophilum*, *S. glaseri* and *H. indica* on earthworms, spiders and ants was evaluated. These nematode species were found harmless to earthworm species *Eiseinea foetida* up to an exposure period of 21 days. The survival of EPNs in vermicompost in the presence of earthworms was confirmed by putting 4<sup>th</sup> instar larvae of *G. mellonella* in different treatments after 10 days of exposure as it recorded 100 % larval mortality in 48 hours. But, these EPNs were found harmful to spider species *Neoscona theisi* and ant species *Messor himalayanus* @ 500 IJs/spider and 100 IJs/ant respectively.

Compatibility of *Steinernema thermophilum* with biopesticidal formulation, Tracer (spinosad) and fungal bioagents *Metarhizium anisopliae* and *Beauveria bassiana* on 4<sup>th</sup> instar larvae of *Helicoverpa armigera* infecting tomato was studied under glass house conditions. A maximum of 100 % larval mortality was observed within 96 hours of treatment when Spinosad (@ 200 ppm) and *S. thermophilum* (@2000IJs/ml) were combined together where as Spinosad (@ 200 ppm) and *S. thermophilum* (@ 2000IJs/ml) when treated alone yielded a mortality rate of 100 and 72 % respectively after 10 days of treatment. The larval mortality induced by *M. anisopliae* and *B. bassiana* alone were not statistically significant even though it induced a mortality level of 41.7 and 47.1 % respectively. Our result revealed a synergistic interaction of *S. thermophilum* and Spinosad when both were combined and applied together. However *S. thermophilum* interacted additively with *M. anisopliae* and slight antagonistic interaction were observed with *B. bassiana*. This result suggests that EPNs like *S. thermophilum* in combination with lower doses of safer pesticides like spinosad could be an alternative to reduce pesticidal load to the environment with improved efficacy.

The symbiotic bacterium *X. indica* was isolated from *S. thermophilum* and cultured in the nutrient broth medium. Biotoxins produced by *X. Indica* in 72 hours of its growth in nutrient broth medium were isolated by solvent extraction method with different solvents like hexane, ethyl acetate and butanol. The solvent fractions were evaluated for insecticidal,

nematicidal and fungicidal properties. All the three solvent fractions showed fungicidal action against *Sclerotium rolfsii*. As ethyl acetate fraction found to be more effective, further study was carried out to find out the chemical nature of biotoxin. It revealed the presence of xenocoumacins, nematophins and xenorhabdins in the ethyl acetate extract of *X. indica*.

**शीर्षक:** कीट पतंगों के नियंत्रण में प्रयुक्त *स्टीनरनीमा थर्मोफिलम* एवम जैवबिषों के अनुप्रयोग प्रद्योगिकी का अध्ययन

कीटरोगजनक सूत्रकृमि (ई पी एन) *स्टीनरनीमा थर्मोफिलम* की विभिन्न कृषि रसायनों जैसे कि 12 कीटनाशक, 6 शाकनाशक, 3 कवकनाशक, 7 जैवकीटनाशक, 8 सहायक, विभिन्न जैवनाशी व कार्बनिक संशोधनों के साथ अनुरूपता (योग्यता) का परीक्षण किया गया। कृषि रसायनों में से होस्टेथिआन, डर्सबान एवम थायोडान के अतिरिक्त सभी अपनी संस्तुति खुराक में अनुरूप (योग्य) पाये गये। *बैसिलस थूरिन्जिएनसिस* एवम *स्यूडोमोनास फ्लोरिसेंस* नामक जीवाणु जैवनाशी संक्रमित तरुणों ( जुवैनाइल्स ) के उत्तरजीविता में हानिकारक जबकि अध्ययन किये गये सभी कवक जैवनाशी सुरक्षित पाए गये। इसके विपरीत *स्टीनरनीमा थर्मोफिलम* व इसके सहभोजी जीवाणु जीनोरैडस इण्डिका, मेटाराइजियम एनिसोप्ली व ब्यूवैरिया बैसियाना नामक कवक जैवनाशकों की वृद्धि को रोकने में सक्षम रहे। इससे स्पष्ट है कि *स्टीनरनीमा थर्मोफिलम*, मेटाराइजियम एनिसोप्ली व ब्यूवैरिया बैसियाना के साथ विशेषतया तभी अयोग्य पायी जाती है जब दोनों एक ही परपोषी पर लक्ष्य या निशाना बनाते हैं। कार्बनिक संशोधन जैसे कि वर्मिकम्पोस्ट, गोबर की खाद, हडडी का चूरा व नीम केक इत्यादि *स्टीनरनीमा थर्मोफिलम* के अनुरूप पाये गये। ई पी एन की विभिन्न प्रजातियों स्टी0 *थर्मोफिलम*, स्टी0 ग्लैसराइ एवम हेटेरोरैड्डाइटिस इण्डिका के अन्तरजातीय परस्पर प्रभाव के प्रयोग में स्टी0 ग्लैसराइ 27 डिग्री सेल्सियस व स्टी0 *थर्मोफिलम* 35 डिग्री सेल्सियस पर अधिक प्रभावी पाये गये। लेकिन सभी संमिश्रणों के मिश्रित संक्रमणों में एकल संक्रमण की तुलना में कम संतानोपत्ति देखी गयी। ई पी एन की इन तीन प्रजातियों की गैर लक्ष्य प्रभाव के अध्ययन में देखा गया कि ये प्रजातियाँ केंचुओं पर हानिकारक प्रभाव नहीं पहुँचाते हैं जबकि इनकी उच्च खुराक से चींटियाँ व मकड़ियाँ प्रभावित हो जाती हैं। *हेलिकोवर्पा आर्मिजेरा* से ग्रसित टमाटर के पौधों पर स्टी0 *थर्मोफिलम* व जैवनाशक निरुपन (फार्मुलेशन) ट्रेसर (स्पिनोसैड) के पारस्परिक प्रभावों को गमलों में प्रयोग करके देखा गया। इस परिणाम से स्पष्ट हुआ कि स्टी0 *थर्मोफिलम* व स्पिनोसैड कम से कम मात्रा में भी समिश्रित उपयोग से एकल उपयोग के उच्च मात्रा की तुलना में अत्यधिक प्रभावी पाया गया। जो कि पर्यावरण में कीटनाशकों के दबाव को कम करने हेतु एक विकल्प संभव हो सकता है। सहभोजी जीवाणु एक्स0 इण्डिका जनित बिषैले उत्सर्जी पदार्थों को विभिन्न जैसे हेक्सेन, ईथाइल ऐसीटेट एवम ब्यूटेनॉल आदि बिलायकों के साथ पृथक किया गया। चूंकि ईथाइल ऐसीटेट प्रभाज का स्कलीरोसियम रोलपसाइ के प्रति अधिक कवकनाशी प्रभाव देखा गया इसीलिए इसके जैवबिष की रसायनिक प्रकृति जानने हेतु इसका फिर से अध्ययन किया गया। अध्ययन में देखा गया कि एक्स0 इण्डिका के ईथाइल ऐसीटेट निरस्यन्द में जीनोकौमासिन्स निमेटोफिन्स एवम जीनोरैडिन्स आदि उपस्थित होते हैं।

**Title: Studies on application technology of *Steinernema thermophilum* and the biotoxicants involved in managing insect pests**

The compatibility of entomopathogenic nematode (EPN), *Steinernema thermophilum* with different agrochemicals including 12 insecticides, 6 herbicides, 3 fungicides, 7 biopesticides and 8 surfactants/adjuvants, different bioagents and organic amendments were evaluated. Among the agrochemicals, all except Hostathion, Dursban and Thiodan in their recommended doses were found compatible. The bacterial bioagents namely *Bacillus thuringiensis* and *Pseudomonas fluorescens* were found to be harmful to the survival of infective juveniles, where as all the fungal bioagents studied were found to be safe. In contrast, *S. thermophilum* and its symbiotic bacterium *Xenorhabdus indica* could suppress the growth of fungal bioagents namely *Metarhizium anisopliae* and *Beauveria bassiana*. It indicated the incompatibility of *S. thermophilum* with *M. anisopliae* and *B. bassiana* especially when both target the same host. The organic amendments like vermicompost, farm yard manure, bone meal and neem cake were compatible with *S. thermophilum*. The experiment on interspecific interaction of different EPN species namely *S. thermophilum*, *S. glaseri* and *Heterorhabditis indica* showed dominance of *S. glaseri* at 27 °C and *S. thermophilum* at 35 °C. But combined infections in all the combinations resulted in reduced progeny production as compared to single infections. The studies on nontarget effects of these three species of EPNs showed no harmful effect on earthworms, where as ants and spiders were observed to be affected at higher doses. The synergistic interaction of *S. thermophilum* and biopesticidal formulation Tracer (spinosad) was observed in the pot experiment on *Helicoverpa armigera* infecting tomato. This result indicated that the EPNs like *S. thermophilum* in combination with lower doses of safer pesticidal formulations like Spinosad could be a possible alternative to reduce the pesticide load on the environment. The toxic metabolites produced by the symbiotic bacterium *X. indica* were isolated with different solvents like hexane, ethyl acetate and butanol. As ethyl acetate fraction showed more fungicidal action against *Sclerotium rolfsii*, it was further studied for the chemical nature of biotoxin. The studies revealed the presence of xenocoumacins, nematophins and xenorhabdins in the ethyl acetate extract of *X. indica*.

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