An-Najah National University
Faculty of Graduate Studies

Effect of Heat Shock on Performance of Entomopathogenic Nematodes strains *"Heterorhabditis indica* and *Heterorhabditis bacteriophora"* inside *Galleria mellonella* Larvae

By

BASMA ABDULLA YOUSEF SANDOUKA

Under the Supervision

Of

Dr. Yacoub Batta and Dr. Naim Iraki

Submitted in Partial Fulfillment for the Requirements for the Degree of Masters in Environmental Sciences at the Faculty of Graduate Studies at An-Najah National University, Nablus, Palestine
3003

Effect of Heat Shock on Performance of Entomopathogenic Nematodes strains *"Heterorhabditis indica* and *Heterorhabditis bacteriophora"* inside *Galleria mellonella* Larvae.

By

BASMA ABDULLA YOUSEF SANDOUKA

This Thesis was defended successfully on the 26th July, 2003 and approved by

Committee Members		**Signature**
Dr. Yacoub Batta	**(Supervisor)**	
Dr. Naim Iraki	**(Co supervisor)**	
Dr. Issam Rashed	**(Internal examiner)**	
Prof. Dr. Adnan Shquir	**(External examiner)**	

Dedication

TO

**MY PARENTS FOR THEIR SUPPORT, MY SISTERS AND BROTHERS,
MY HUSBAND AND MY BEST FRIENDS
FOR THEIR UNDERSTANDING AND ENCOURAGEMENT**

Acknowledgments

I would like to thank my advisor Dr. Ya'koub Batta for his support and advice. My deep thanks and appreciation is addressed to my advisor Dr. Naim Iraki, director of the UNESCO/BETCEN at Bethlehem University, for his continuous efforts, support, and advise, which were very useful for achieving this Thesis.

Also I would like to thank the administration of Bethlehem University especially the faculty members of the Biology Department for their support. My special thanks are adressed to my friends at the UNESCO/BETCEN at Bethlehem University, Omar Issa, Micheal Sansour, Amani Abu sa'da, Silvana Vuskovic, Arwa Saleh, Rabeha Elyan, Nasser Elayasseh, Christine Qamar, for their help and support. Special thanks to Ghadeer Abu El hawa, Jawan Tawil, and Rami Arafeh, for their continuous encouragement. And to my friend Eman Kassem.

This work was supported by a fellowship from the UNESCO Biotechnology Action Council, through the UNESCO Biotechnology Educational and Training Center (BETCEN) for Palestinian Territory and Arab Countries, and in part by a grant from the DFG/Germany.

List of Contents

List of Tables

List of Figures

LIST OF ABBREVIATIONS

Entomopathogenic Nematodes	EPNs
Heat Shock Proteins	HSPs
Infective Juveniles	IJs
Nutrient Bromothymol Blue Triphenyl tetrazolium chloride Agar	NBTA
Analysis of Variances	
ANOVA	
Lethal Dose	LD
Lethal Time	LT
Kilo Dalton	KDa
Liquid Culture Medium	LCM

Abstract

Temperature is the most important factor affecting survival, infectivity, development, and reproduction of entomopathogenic nematodes, therefore, it is necessary to study the effect of heat shock on their penetration, development, and reproduction capabilities inside a target insect.

The penetration capacity into Galleria mellonella larvae of the infective juveniles of two EPNs species; *Heterorhabditis indica* and *Heterorhabditis bacteriophora*, decreased as a result of exposing the IJs to the temperatures of 35^{o}C and 40^{o}C and relaxed for two days at 25^{o}C compared to the control at 25^{o}C. The decrease in penetration of IJs from both species was more significant at 40oC than that at 35oC. Furthermore, the IJs penetration capacity of the locally isolated strain *H. indica* was greater than that of *H. bacteriophora* strain, which was isolated from temperate climatic region, at both temperatures. However, the penetration capacity of *H. bacteriophora* IJs was improved as a result of preconditioning the IJs at 35^{o}C before exposure to 40^{o}C about 37 folds compared to 3 folds improvement in the penetration of *H. indica* IJs, when the obtained penetration rates were compared to values observed at 40^{o}C.

The development of the penetrated IJs to hermaphrodites was also decreased when the IJs were exposed to 40^{o}C without preconditioning. The recovery to hermaphrodites did not exceed 45% of that in the control in both nematode species. While upon the preconditioning treatment, the percent of *H.indica* IJs developed to hermaphrodites was close to that of the control and the development of *H. bacteriophora* IJs was only 72%. These results indicate

that preconditioning was less efficient in reducing the inhibitory effect of the lethal temperature 40^{o}C on the development of *H. bacteriophora* IJs to hermaphrodites.

The total reproduction of the IJs, expressed per IJ injected inside larva of Galleria mellonella, showed a tendency to decrease with the increase in the number of IJs injected inside the larvae at 25oC in both species. Also this decrease was observed with the temperature increase. The yield of the IJs at the density (26 IJs/ larvae) did not exceed 12 and 15% of that when 3 IJs per larvae were used at 35oC for *H. indica* and *H. bacteriophora* respectively.

Preconditioning *H. bacteriophora* IJs improved their reproductive capacity by 2.6 and 3 folds compared to 1.8 and 1.1 folds in the reproduction improvement of *H. indica* IJs. This result supports our suggestion that preconditioning the IJs at 35^{o}C before exposure at 40^{o}C, gives the opportunity for heat shock proteins synthesis which plays a protective role in reducing the negative effects of stress such as, temperature and crowd ness.

Chapter One
Introduction

Introduction

1.1 Biological control

Chemical pesticides have been successfully used to control many agricultural pests since more than a century now. However, their exessive use in agricultur, posed many undesirable problems. First, pests may become resistant after continuous use of a certain chemical compound. This means that application of that pesticide will not treat the problem (Brent, 1987). Environmental contamination is another problem caused by pesticides. Second, many of these chemicals are not degradable or their degradation is a slow process, such as (DDT). So they persist in the soil and lakes and cause contamination of underground water leading to poisoning of many living organisms and destruction of their natural habitat (Dempester, 1987). Third, most of the chemical pesticides are not selective and act against benefical insects as well leading to a severe impairment of the natural biological balance. Therefore, to avoid environmental risks of chemical pesticides, efforts have been made to use altermative methods to control pests' populations. One of these alternatives is the application of biological control.

Biological control is the use of environmentally harmless natural enemies of pests to reduce the population of the latter to a level below the economically harmful threshold level (Van Driesche and Bellows, 1996). The natural enemies used in biocontrol belong to different taxonomic groups of diverse biological traits.

These biocontrol agents attack their target hosts and kill them in one of three ways. In Predation the natural enemy kills the host and consumes its cadaver. Predation is the most widespread method used by natural enemies from insects' orders, such as the *Thysanoptera* (thrips) and *Coleoptera* (beetles). Another way of killing the host is by parasitism, in this way the enemy parasitizes the host and develops inside it leading to its death, such as in the case of many arthropods of the families *Ichneumonidae* and *Braconidae*. The third way in which natural enemies may kill their hosts is by causing a disease which leads to decomposition of their bodies. Natural enemies that cause disease to insects are called entomopathogens. These pathogens include species of bacteria in the genus *Bacillus* (Becker and Schwinm, 1993), fungi in the family *Entomophthoracae*, viruses in the family *Baculviridae*, and nematodes in the families *Heterorhabditae* and *Steinernematidae* (Van Driesche and Bellows, 1996). In general natural enemies of pests that could be propagated at low costs could be candidates for use as biocontrol agents (Bedding, 1981).

1.2 Entomopathogenic nematodes

Insect-parasitic nematodes are microscopic round-worms that are pathogens to a wide range of insects (Koltal *et al.,* 1994). Out of fourty known families of nematodes associated with insects, only two are of special interest to the biocontrol concept, Steinernematidae and Heterorhabditae, because they are the only nematode families that cause mortality to insects without causing any damage to humans, plants, and livestock (Boemare *et al.,* 1996). Nematodes belonging to these families were proved as the most effective biocontrol agents

against soil-dwelling insects, and insects that live part of their life cycle in the soil, mainly because of their ability to look for the insect and infect it (Ehlers and Peter, 1995).

Of all developmental stages of nematodes (Figure 1.2), only the third stage infective juvenile (IJ) (figure 1.1) can survive in the soil outside the host and search for insects to infect (Kaya and Guagler, 1996). These IJs carry in their intestines symbiotic bacteria from the genera Xenorhabdus (Thomas and Poinar, 1979) and Photorhabdus (Boemare *et al.,* 1993), for Steinernema spp. and Heterorhabditis spp., respectively. These IJs enter the insect haemocoel through natural openings (e.g. spiracles, mouth, anus) or penetrate through the cuticle of certain insects (Bedding and Molyneux, 1982). Once the IJs are inside the haemocoel, they release their bacteria, which proliferate rapidly and kill the insect within few days by septicaemia (Figure 1.2). The penetrated IJs start to feed on the proliferating bacteria and develop to the fourth stage juvenile (Lunau *et al.,* 1993).

Figure 1.1: The infective juvenile of *Heterorhabditis* spp. at 100X.

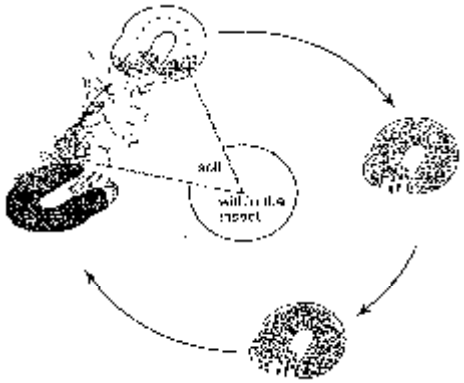

Figure 1.2: Life cycle of Entomopathogenic nematodes (Gerritsen, I. J. M. , 1997).

In the first reproductive cycle of *Heterorhabditis* spp. the feeding J4 develop to hermaphrodities while in subsequent cycles they may develop to females, males, or hermaphrodites (Zioni *et al.,* 1992; Strauch *et al.,* 1994) (Poinar, 1975) (Figure 1.3). In *Steinernema* spp. the J4 always develops to either male or female (Elawad *et al.,* 1999). The males and females mate and then the females, or hermaphrodites lay fertilized eggs, from which the first juvenile stage (J1) hatchs. While feeding on symbiotic bacteria, the J1 develops into the second-stage juvenile (J2), then to the third-stage juvenile (J3) (Figure 1.4). The nematodes continue reproduction until the amount of bacteria becomes limited. At this stage, the reproduction cycle shifts to produce IJs instead of the J3 stage. The IJs will leave the insect cadaver and search in the soil for a new insect to infect. The reproduction cycles of nematodes inside the insect proceeds for 6-18 days depending on the type of the host and nematodes species (Poinar 1990; Zioni *et al.,* 1992).

The relationship between the nematodes and bacteria is symbiotic mutualistic. The nematodes feed on this bacteria and cannot reproduce inside

the insect without the presence of its symbiont (Han and Ehlers, 2000) (knodo and Ishibshi, 1991). On the other hand the bacteria cannot enter the haemocoel and cause infection without the nematodes (Poinar, 1975). The symbiotic bacteria are gram-negative, non-sporeforming and thus, they are of low resistance to the environmental conditions. Because of the latter characteristic it has been found only inside nematodes and their infected hosts (Akhrust and Boemare, 1990; Poinar, 1990).

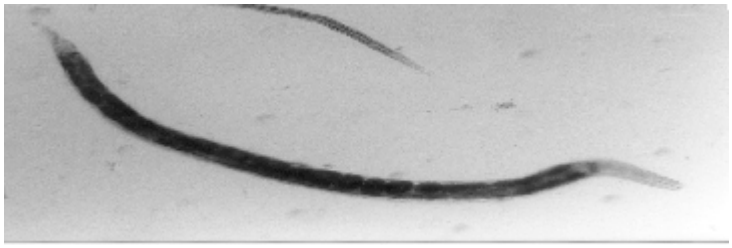

Figure 1.3 The hermaphrodite of *Heterorhabditis* spp. 100X.

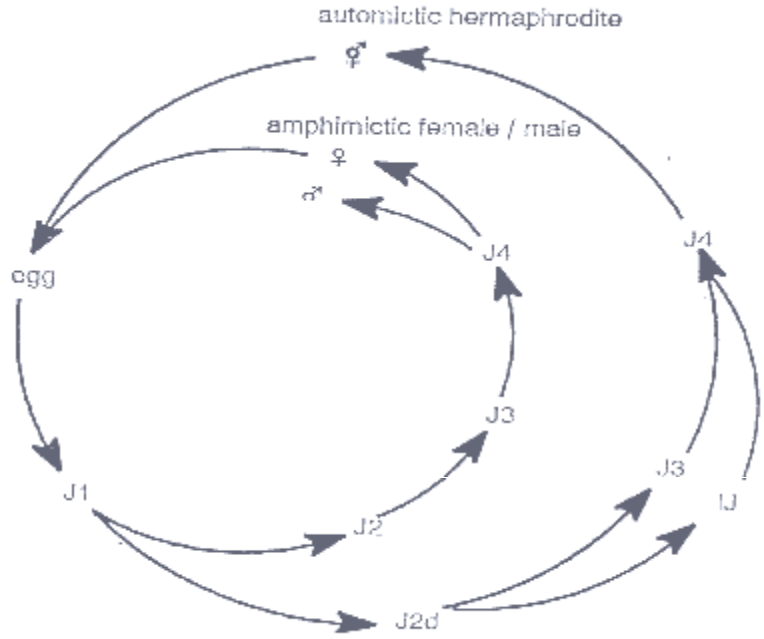

Figure 1.4 The developmental stages of *Steinernema* and *Heterorhabditis* entomopathogenic nematodes (Gerritsen, I. J. M., 1997).

1.3 Factors affecting EPNs efficacy

Although EPNs are known to control a wide range of pest species, some biotic and abiotic factors limit their use as bioinsecticides (Glazer and Gol'berg, 1989). Biotic factors that adversely affect the survival of EPNs and their symbiotic bacteria include; antibiosis, competition, natural enemies and host susceptibility (Kaya and Koppenhofer, 1996; Klien, 1990). On the other hand, abiotic factors include extremes in soil conditions such as temperature, moisture, and texture, as well as ultraviolet light may influence nematode persistence (Kaya, 1990).

1.3.1 Antibiosis

Some insect pests release chemicals upon infection with certain pathogen such as EPNs. These chemicals may adversely affect the reproduction of nematodes (Kaya and Koppenhofer, 1996). Such antibiosis effects are also due to chemicals that are released from some plants through the pests to the soil. Plant chemicals may negatively affect the host finding behavior of the IJs. One of the best-studied plant chemicals affecting nematodes is ∝-terthienyl (Gommers, 1972; Gommers and Bakker, 1988). Survival and infectivity of *S. glaseri* were significantly lowered in the presence of ∝-terthienyl, indicating a toxic effect (Kanagy and Kaya, 1996).

1.3.2 Competition

Competition occur when a huge number of a certain pathogen, or more, infect a host. The competition is mainly on food resources and oxygen.

Intra-specific

Intra-specific competition occurs when the number of IJs penetrating into a host exceeds an optimal level. This kind of competition takes place among the developing nematodes, and causes reduction in the number of progeny emerging from the host cadaver. If the number of establishing IJs exceeds the carrying capacity of the host, null production of progeny may be reached. Such density-dependent effects have been observed with *S. carpocapsae* (Selvan *et al.,* 1993), *S. glaseri* and *Heterorhabditis* spp. (Zervos *et al.,* 1991). However, *H. bacteriophora* appeared to be less susceptable to intra-specific competition than *S. carpocapsae* and *S. glaseri* (Zervos *et al.,* 1991).

Inter-specific

Inter-specific competition occurs when more than one species of EPNs (Choo *et al.,* 1996) (Glazer, 1996) or EPNs and other pathogens compete for survival resources inside the same host. The outcome of such a kind of competition usually causes a local extinction of one of the competing species. For example, Steinernematid spp. usually excludes Heterorhabditis spp. when both infect the same host (Alatorre-rosas and Kaya, 1990; 1991). On the other hand, laboratory studies showed that two Steinermenatid species can temprory co-exist in a host. This is probably because one species can feed on the bacteria of the other species (Koppenhofer *et al.,* 1995a). However, by time one species will domain in the environment (Koppenhofer and Kaya, 1996).

EPNs may compete with other pathogens such as, bacteria, viruses (Kaya and Brayton, 1978), fungi (Barbercheck and Kaya, 1991). Entomopathogenic

viruses and bacteria may cause a negative effect on the development of the competing nematodes. On the other hand, EPNs can out-compete entomopathogenic fungi for the same host individual when both the nematodes and the fungi are applied simultanuosly (Barbercheck and Kaya, 1990). However, if the fungus is applied before the nematodes, the interaction between them will be temperature dependent (Barbercheck and Kaya, 1990).

1.3.3 Natural enemies

Soil may contain organisms that can antagonize EPNs and reduce their persistence (Timper and Kaya, 1989). The best studied natural enemy to EPNs is the nematophagous fungus, Hirsutella rhossliensis. It was found that this fungus can cause higher mortality in S. glaseri comapred with H. bacteriophora. Accordingly, Timper and Kaya (1992) suggested that H. bacteriophora is probably more effective than Steinernema spp. in controling insect pests where nematode-parasitic fungi are abundant. Other predators of EPNs include mites and collembolans (Kaya, 1990).

1.3.4 Host susceptibility

Host susceptibility is one of the most important factors determining the efficiency of EPNs as biocontrol agents. Epsky and Capinera (1994) recommended that determinations of host mortality percentages and nematodes invasion rates should be done simultaneously for assessment of nematode infectivity. Usually the most susceptible hosts are those more easily penetrated by the nematodes (Glazer, 1992; Caroli *et al.,* 1996). Dunphy and Webster

(1986) reported about a variation in the efficacy of two S. carpocapsae strains; the Mexican and DD136 strains, against Galleria Mellonella larvae. They suggested that the difference in efficacy could have been caused by unequal numbers of individuals invaded that host. This was based on the fact that they detected no difference in virulence of either the symbiotic bacteria or the combination of nematodes and bacteria when injected into the insect heamocoel.

1.3.5 Soil moisture

Nematodes survival and movement in soil are highly affected by the soil water content (Gouge *et al.,*2000; Koppenhofer *et al.,*1995b). The greatest survival of Steinernematids tends to be at relatively low soil moistures but only if the rate of water-loss from the soil is slow (Patel *et al.,*1997). Gradual water-loss reduces physical damage caused by desiccation and provides sufficient time for biochemical changes to take place (Simons and Poinar, 1973). Kung *et al.,* (1991) demonstrated that survival of S. carpocapsae and S. glaseri placed in sandy loam was highest at low soil moisture levels of 2% and 4%, respectively, compared with higher levels of 8 and 16%. Therefore, ensuring sufficient moisture in soil after field application of EPNs is an important factor for achieving a greater efficacy with the appropriate nematode species (Molyneux and Bedding, 1984). On the other hand, very high levels of moisture can inhibit nematode infectivity by restricting their movement in the soil. Also, it may decrease the survival of nematodes by decreasing the concentration of oxygen in the soil (Georgis and Manweiler, 1994).

1.3.6 Soil texture

The nematode movement, host finding ability and survival are also affected by the soil texture and its pore size (Kaya and Gaugler, 1993). Horizontal and vertical dispersal of nematodes, as well as their pathogencity and survival decrease as the overall proportion of silt and clay increase (Kung *et al.,* 1990). Sandy loam and sand have large pore sizes and low moisture potentials, compared to clay loam and clay. These properties of sand provides rich aeration environment for nematodes and enough space for free movement (Molyneux and Bedding, 1984). Kung et al. (1990) for example, demonstrated that the survival of S. glaseri and S. carpocapsae was best in sandy loam and sandy soil, respectively compared to other kinds of soil.

1.3.7 Ultraviolet radiation

EPNs are extremely sensitive to ultraviolet (U.V.) radiation (Kaya and Gaugler, 1993). Heterorhabditis nematodes appear to be among the most vulnerable of all entomopathogens to U.V. inactivation. IJs of H. bacteriophora exposed to medium-wave U.V. radiation were inactivated at radiation levels that did not affect S. carpocapsae (Gaugler *et al.,* 1992). Hence, it is recommended to apply such a U.V.-sensitive EPNs species in the late afternoon and to irrigate the soil after their application (Georgis and Manweiler, 1993).

1.3.8 Temperature

Temperature is the most important factor affecting the survival, infectivity, development and reproduction of EPNs (Grewal *et al.,* 1993). Selvan and

coworkers (1996) reported that the survival of IJs of H. bacteriophora decreased with increasing exposure temperature. On the contrary larval mortality of nematodes, reproduction and development were found to be faster at 20^{o}C and 40^{o}C than that at lower temperatures for H. megidis and S. carpocapsae strains, repectively (Saunders and Webster, 1999).

All organisms adapt phenotypically and genetically to changes in thermal environment (Bennett *et al.,* 1990). Thermal adaptation was observed among nematodes species isolated from diverse geographical regions (Grewal *et al.,* 1994). In most cases, species isolated from warm regions tolerate high temperatures more than those isolated from temperate regions (Grewal *et al.,* 1993). Genetic selection was suggested as a mean to enhance tolerance of EPNs to temperature and other stresses (Glazer *et al.,* 1997).

Nematodes propagated at lower temperatures had the capacity to withstand freezing, but they could not tolerate higher temperatures compared to those propagated at elevated temperatures (Jagdale and Gordon, 1997; 1998). Also Grewal *et al.,* (1996) observed that thermal limits for virulence, development, and reproduction of H. bacteriophora and S. anomali, were extended following prolonged sub-culturing at temperatures higher or lower than those of stacking cultures. However, the selection approach depends on the availability of sufficient genetic diversity for a particular trait in the laboratory populations (Glazer *et al.,* 1991a). This problem can be overcome through the discovery of new strains of EPNs with favored biological properties, such as heat tolerance, desiccation tolerance, high infectivity and persistence (Glazer *et al.,* 1996).

Such traits may be the result of chance discovery, laboratory screening efforts, and field surveys (Glazer *et al.,* 1993; Van Driesche and Bellows, 1996). In the case of heat tolerance trait, new strains of EPNs from regions of warm climates, such as Negav desert in Israel (Glazer *et al.,* 1991b) and seashores of Hawaii (Hara *et al.,* 1991) were isolated and found to be heat tolerant.

Once beneficial traits are discovered among natural populations, these traits can be combined through hybridization to create superior strains (kaya and Gaugler, 1993). Hybridization can be a powerful approach for genetic improvement of EPNs. Shapiro *et al.,* (1997) demonstrated that the heat tolerance trait of the IS5 strain was succesfully transferred through conventional crosses to HP88 strain, both of H. bacteriophora.

1.4 Thermotolerance and Heat shock proteins

All tested organisms respond to heat and other physiological stresses, such as anoxia and toxins, by a rapid and intense synthesis of a group of proteins called heat shock proteins (HSPs) (Lindquist and Craig, 1988). HSPs are the most broadly distributed class of known proteins and are highly conserved (Angelidis *et al.,* 1991). They are also synthesized constitutively in living cells and fulfill essential functions under nonstressful conditions. For example, these proteins are believed to assist in the initial folding of the newly synthesized proteins and the transport of proteins into the mitochondria and chloroplasts (Liang and MacRae, 1997; Hartl and Martin, 1992). HSPs are classified into five groups on the basis of their molecular masses; the small HSPs (less than 40

kDa); the HSP60s (60 ± 5 kDa); the HSP70s (73 ± 5 kDa); the HSP90s (approximately 90 kDa); and the HSP100s (105± 5 kDa) (Lindquist and Craig, 1988).

Heat shock treatment at sub-lethal temperatures usually affects the protein conformation by causing it to unfold. Thus, creating many solvent-exposed hydrophobic regions which would normally be buried in the protein native state (Hendrick and Hartl, 1993).

The identification of selectively induced synthesis of HSPs in cells exposed to moderate stresses has led to the conclusion that the role of HSPs is to help these cells to cope with unfolded proteins. This is either by binding to them thereby preventing (premature) folding and aggregation, and marking them for proteolysis, or by mediating correct folding (Hartl and Martin, 1992; Hendrick and Hartl, 1993). Hence, these cells gain a transient resistance to elevated temperatures.

This phenomenon is known as thermotolerance (Gerner and Schneider, 1975; Carretero *et al.,* 1991). Acquired thermotolerance and the associated synthesis of HSPs have been reported in cells of organisms ranging from yeast to mammalian systems. However, each organism has its own threshold of induction temperature for enhanced synthesis of HSPs depending on the temperatures in its natural environment (Dietz, 1994). For example, in the fruit fly Drosophila melanogaster, induction occurs between 33-37^{o}C, the common temperatures on warm summer days (Lindquist, 1980). While, in thermophilic

bacteria growing at 50^{o}C, the proteins are induced when temperatures are raised to 60^{o}C (Trent, 1996).

Induction of HSPs, particularly the HSP70, in Heterorhabditis spp. of EPNs was observed after exposing the IJs to the sub-lethal temperature 35^{o}C and relaxing them at 25^{o}C (Hashmi *et al.,* 1998; Hashmi *et al.,* 1997). The treated IJs tolerated heat shock at 40^{o}C more than the untreated ones (Selvan *et al.,* 1996).

1.5 Objectives of the study

- Studying the effect of heat shock on the penetration, development and production of two strains of EPNs (*H. indica* and *H. bacteriophora*) inside *G. mellonella* larvae.
- Determining the effect of intraspecific competition on the total IJs production of the two strains of EPNs inside *G. mellonella* larvae.

Chapter Two
Materials and Methods

2.1 Rearing of *Galleria mellonella*

Adults of the wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) were placed in glass jars containing nutrient medium and pieces of tissue paper. The jars were covered with metal nets and tissue paper to allow ventilation. During three to four days the moth female lay eggs on the tissue paper.

The paper holding the eggs was collected and disinfected by submerging the pieces of tissue paper in 10% formaldehyde solution for 90 minutes, then washed under running water for one hour. The eggs were left for air dry and then incubated at 33^{o}C in the dark on a nutrient medium. The medium contained; 200g autoclaved Honey, 183g Glycerin, 47g Yeast extract, and 4g Nepagine.

The mixture was homogenized by heating to 50-60^{o}C then mixed with 320g of Oatmeal. The eggs hatched and developed into last instar larvae within 3-4 weeks.

2.2 Source of nematodes

Two strains of EPNs were used in this study. The first strain was *Heterorhabditis indica*; strain Beth 22, which was isolated from Bethlehem area in Palestine (Iraki *et al.*, 2000). The second strain was *Heterorhabditis bacteriophora*; strain HB, a hybrid of two *H. bacteriophora* strains originating from temperate climatic regions. This strain was obtained from R. Gaugler, Rutger University, New Burnswick, NJ, and USA. Both strains were reared in *G. mellonella* larvae as described by Woording, 1988.

2.3 Heat shock treatment

Five ml of Ringer solution containing 5000 of one week-old IJs were transferred into a 100 ml flask containing 15 ml of deionized water at the appropriate temperature. The flasks were covered with cotton to allow gas exchange, and were held in a water bath attached to a shaker to provide continuous orbital shaking at 200 rpm. The IJs were exposed to three heat shock regimes; at 35°C for one hour; at 40°C for one hour and at 35°C for one hour followed by a latency period at 25°C for three hours before exposure to 40°C for one hour. At the end of each heat treatment, the flasks were kept in the dark at 25°C for overnight in one treatment, and for two days in another one. The IJs were then used to infect *G. mellonella* larvae in the sand.

2.4 Determination of IJs penetration into Galleria larvae and development to hermaphrodites

For the determination of penetration and development to hermaphrodites, a sample of fourty larvae of *G. mellonella* (average weight of 0.153g) were trapped in 24 multi-well plates (Costar®, USA). Each well of 1.55 cm diameter and 1cm height (Figure 2.1) was used to trap one larva. Wells were filled with autoclaved sand moistened to 10% with Ringer solution (Woording and Kaya, 1988). The Ringer solution contained (g/l); 9.0 NaCl, 0.42 KCl, 0.48 $CaCl_2.6H_2O$, and 0.20 NaH_2CO_3.

The infection of Galleria larvae with heat-treated infective juveniles was carried out by pouring onto the sand 100 µl suspension in Ringer solution.

Figure 2.1: The multi-well plates used for trapping the *Galleria mellonella* larvae for infection with entomopathogenic nematodes.

The number of IJs in the infecting suspension was adjusted to the value of LD_{50} of each strain; 55 IJs for *H. indica*, and 160 IJs for *H. bacteriophora* (Iraki *et al.*, 2000). The wells were covered with a lid and incubated for 26 hours at 25^{o}C in the dark.

At the end of the 26 hours infection period, the fourty larvae of each sample were randomly divided into two groups of twenty each; the first group was dissected and incubated in pepsin solution for 90 minutes at 37^{o}C with continuous rotary shaking at 120 rpm. The pepsin solution contained 8 g/l pepsin (Sigma), 23 g/l NaCl (Difco), 20 ml of concentrated HCl and 940 ml distilled water. The pH of the solution was adjusted to 2.0 using concentrated HCl. The number of penetrated IJs into the insect was then counted under the inverted microscope. The second group of the larvae was left on wet filter

paper (Wattman no.1) for 72 hours, and then dissected to determine the number of hermaphrodites under the microscope. All experiments were independently repeated twice. Data obtained from the two experiments were pooled and then statistically analyzed.

2.5 Establishment of a monoxenic culture of *H. bacteriophora* (strain HB)

2.5.1 Isolation of the symbiotic bacteria

Photorhabdus luminescens, the symbiotic bacteria of *H. bacteriophora* was isolated from the haemolymph of infected *G. mellonella* larvae. Five larvae were exposed to 100 IJs each, and incubated in the dark at 25^{o}C (see section 2.4). After 30 hours of incubation the larvae were disinfected with 2% sodium hypochlorite solution for 2 minutes followed by a thorough wash with sterile water. Each larva was then transferred to 9 cm Petri-dish containing NBTA medium (Akhrust, 1980). The NBTA medium contained (g/l) 37 Nutrient agar (Difco), 0.25 Bromothymol blue (Sigma), and 10 2,3,5-Triphenyl tetrazolium chloride (Sigma), dissolved in one litre of tap water. The larvae, in the NBTA plates, were carefully pricked with a sterile surgical needle to release the haemolymph without reaching the inner gut (Akrust, 1980).

Using a loop, a sample of the haemolymph was streaked on the NBTA medium. The streaked plates were incubated in the dark at 25^{o}C for 24 or 48 hours till the appearance of bacterial colonies. A well-separated typical colony of *P. luminescens* was sub-cultured on fresh NBTA plates for additional

purification. On colony of the purified culture was transferred into LCM medium, and placed on a shaker at 250 rpm. in the dark at 25°C. The LCM medium contained per liter of tap water; 10g Nutrient broth (Difco), 10 g Tryptic soy broth (Difco), 5 g Yeast extract (Difco), 5 g Peptone (Difco), 5 g NaCl, 0.35 g KCl, 0.21 g $CaCl_2.2H_2O$, and 30 ml Corn oil. After 48 hours of incubation the bacterial culture was either used for experiments or mixed with 20% sterile glycerol and stored at –80°C. To assess the isolation of the correct symbiotic bacteria, a 24-hours bacterial culture was inoculated into Wout's agar plates (Wouts, 1981), and then disinfected IJs of *H. bacteriophora* strain HB were added to the plates. The plates were then incubated at 25°C in the dark for 5-7 days and inspected for development of IJs. The Wout's medium contained per liter tap water; 16 g Bacto nutrient broth, 12 g Bacto agar, and 5 g Corn oil.

2.5.2 Isolation of the eggs

Larvae of *G. mellonella* were infected in sand with IJs of *H. bacteriophora* (strain HB). Four days after infection, the dead larvae were dissected in a petri-dish containing ringer solution (composition described in section 2.4) to release the hermaphrodites. The hermaphrodites were collected and washed for several times with Ringer solution to remove the tissues of the dissected larvae. Hermaphrodites were then placed in a small test tube containing 2 ml of Ringer solution and small pieces of razor blades. The tube was vortexed for 1 minute to rip up the hermaphrodites and release the eggs from them. The solution was then filtered through 55µm mesh. The filtered solution, which contained the eggs, was then transferred to a sterile eppendorf

tubes and washed three times with sterile Ringer solution through centrifugation for 2 minutes at 2000 rpm. A sterilization buffer was then added and the eppendorfs were shacked gently by hand for 4 minutes. The sterilization buffer contained 1.5 ml of 4 M NaOH, 0.5 ml of 12% NaCl, and 10 ml of distilled water. The sterilization buffer was removed and the eggs were washed twice with sterile Ringer solution.

The disinfected eggs were distributed into wells of 24 multi-well plates containing 300 µl/well of LCM medium and incubated for 48 hours in the dark at 25^{o}C till the eggs hatched into the first juvenile stage (J1). The hatched juveniles were transferred from the LCM medium into Wout's agar plates (Wouts, 1981), which were previously inoculated with 24 hours-old culture of *H. bacteriophora* symbiotic bacteria. The plates were then incubated in the dark at 25^{o}C. The first juvenile stage (J1) developed into adults 5-7 days after inoculation, and the IJs appeared after 4 weeks.

2.6 Determination of total production

A known number (1-40) of monoxenic IJs (Luanu *et al.*, 1993), which were aseptically heat treated as described in section 2.3 were placed in a petri-dish in about 20µl drops of Ringer solution. The IJs from each drop were withdrawn under the microscope with a one ml syringe (Samwoo) and then injected under the cuticle of *G. mellonella* larvae. The syringe was then washed three times with 40µl of sterile water. The washing water was collected and examined under the microscope for the presence of IJs. The number of injected IJs was determined by subtracting the number of IJs left in the wash water from

the initial number placed in the drop. Non heat-shocked IJs were used as the control treatment; larvae were injected with 20µl of sterile Ringer solution as a negative control. Larvae were then left on a wet filter paper for 48 hours in the dark at 25^{o}C. After the death of the larvae, each was transferred to a white trap (Woording, 1988) (Figure 2.2), and incubated for three weeks in the dark at 25^{o}C. IJs emerged from each larva were collected and the larva was dissected in a Ringer solution to release the remaining nematodes inside the cadaver. The number of produced IJs inside and outside each larva was determined under the microscope. Each experiment was repeated twice independently. Data obtained were pooled and then statistically analyzed.

Figure 2.2: Mini-white trap used for determination of total nematodes production; inside one Galleria larva.

2.7 Statistical analysis

The collected data, from the experiments of the effect of heat shock on the penetration and recovery of the nematodes *H. indica* and *H. bacteriophora*

inside *Galleria mellonella* (Table 1, and 2) obtained in this study were pooled (2 independent experiments) and then statistically analyzed by one-way ANOVA test. Also the data on the effect of EPNs density and heat treatments on the total reproduction of the IJs of both strains were analyzed using the same test. Means were tested for significant differences at P-value ≤ 0.05 using least significant difference test (LSD). The one-way ANOVA test was calculated using the SPSS 9.0 software obtained from Bethlehem University Computer Center.

Chapter Three
Results and Discussion

3.1 Effect of heat shock on the IJs penetration

Exposing the IJs of the two entomopathogenic nematode species (*H. indica* and *H. bacteriophora*) to the sublethal temperature of 35^{o}C for 1 hour followed by an overnight latency period, resulted in a significant decrease in their penetration into Galleria larvae compared to the control (25^{o}C). While exposing the IJs of both species to the lethal temperature of 40^{o}C for 1 hour caused a total inhibition in their penetration capacity. However, the penetration capacity of *H. indica* IJs was significantly greater than that of the *H. bacteriophora* at 35^{o}C (Table 3.1). When the results are expressed as percent of control, the penetration capacities of *H. indica* and *H. bacteriophora* were 49.74% and 42.42%, respectively.

Apparently the preconditioning of IJs of both species for one hour at the sublethal temperature, decreased the inhibitory effect of the lethal temperature 40^{o}C on the penetration of IJs of both nematodes species, where the penetration of IJs of *H. indica* and *H. bacteriophora* were 6.47% and 8.48% that of the control, respectively (Table 3.1). However, this improvement effect was not statistically different.

Table 3.1 Effect of three heat shock regimes on the penetration and development capacities of *Heterorhabditis indica* and *H. bacteriophora* IJs inside *Galleria mellonella* larvae.

Heat-shock regime	Penetration (%)		Development to hermaphrodites (%)	
	H. indica	***H. bacteriophora***	***H. indica***	***H. bacteriophora***
a-Control (25°C)	100.00(19.30[a])	1100.00 (16.50[b])	100.00 (77.72[h])	100.00 (87.27[l])
b- 35°C	49.74 (9.60c)	42.42 (7.00d)	80.41 (62.50j)	91.66 (80.00k)
c- 40°C	00.00 (0.00e,f)	00.00 (00.00[e,g])	00.00 (00.00l,m)	00.00 (00.00l,n)
d- 35°C + 40°C	6.47 (1.25f)	8.48 (1.40g)	64.84 (50.40m)	94.12 (82.14n)

- Figures in parentheses represent the actual percent values (the average number of the penetrated IJs into the larvae divided by the number of the applied IJs).
- The results are means of two independent experiments.
- Means in parentheses with the same lowercase letters are not significantly different ($P > 0.05$).

Like its effect on penetration, the heat treatment at 40°C caused a total inhibition of the development of the penetrated IJs to hermaphrodites in both nematode species (Table 3.1). Furthermore, the preconditioning at 35°C failed to induce a statistically significant reduction in the inhibitory effect of the treatment at 40°C. Apparently, the development of the IJs to hermaphrodites seemed to be more than 50% of the control in both strains. However, the applied statistical analysis showed that these differences are insignificant.

Synthesis of heat-shock proteins as a response to heat stress is a well-known phenomenon documented in many organisms, including entomo-pathogenic nematodes (Hendrick and Hartl, 1993; Hashmi *et al*., 1997). These proteins are believed to protect the organisms' functional proteins from heat-

induced unfolding and formation of aggregates (Liang and McRae, 1997). Preconditioning of the organism at sublethal temperatures was proven to enhance survival of IJs at the lethal temperature of 40^{o}C (Selvan *et al*., 1996). The improved survival was suggested to be attributed to the *de-novo* synthesis of 70kDa heat-shock proteins by the entomopathogenic nematode *H. bacteriophora* (Selvan *et al*., 1996).

In this work, preconditioning of IJs at 35^{o}C failed to significantly decrease the inhibitory effect of the heat shock treatment. It should be remembered that the latency period at 25^{o}C which we applied following the heat shock treatment was for overnight. We hypothesized that this latency period have been too short for allowing the occurrence of all biochemical processes aided by heat shock proteins to repair the damage caused to the penetration and development mechanisms by the heat treatment at 40^{o}C. To test these hypothesis we repeated the previously described experiments but with a latency period of 48 hours instead of overnight.

When the latency period following the heat-shock treatment of the IJs was extended from overnight to 48 hours before application to nematodes, the performance of the treated IJs in term of penetration and development was improved under all heat treatments (compare data in Table 3.2 to that in Table 3.1). For example, the heat treatment at 35^{o}C caused only about 20% and 37% inhibition on the penetration of *H. indica* and *H. bacteriophora* IJs, respectively, when the latency period was 48 hours compared to inhibition rates of 50% and 58% by the same temperature at a latency period of overnight. Also the

development of IJs of both strains to hermaphrodites was improved at all heat shock treatments when the latency period was extended to 48h. the most apparent effect of extending the latency period was the capacity of the IJs to penetrate and develop, although at low rates, after treating them at 40^{o}C. This capacity was totally absent when the latency period was restricted to overnight (Tables 3.1 and 3.2).

In general, the observed improved performance of IJs of both strains upon prolongation of the latency period supports our assumption regarding the time period required for biochemical processes to repair the damage caused by elevated temperatures. The repair processes may involve de-novo synthesis of functional proteins necessary for rebuilding of the mechanisms involved in penetration and development of the infective juveniles.

There were differences between the strains in their response to heat treatments at a latency period of 48 hours. The penetration capacity of the *H. indica* IJs was significantly greater than that of the *H. bacteriophora* IJs at both the sublethal and lethal temperatures. When results were expressed as percent of control, the penetration capacities of *H. indica* IJs were 81.38% and 6.11% at 35^{o}C and 40^{o}C, respectively.

The corresponding capacities of *H. bacteriophora* IJs were 63.09% and 0.59 % at 35^{o}C and 40^{o}C, respectively (Table 3.2). When the IJs of both nematode species were preconditioned at the sublethal temperature (35^{o}C) before exposure to 40^{o}C, the inhibitory effect of the lethal temperature on the penetration of IJs of both nematode species was decreased significantly. IJs

penetration rates of both species were about 20% that of the control. However, when these rates were compared to

Table 3.2 Effect of three heat shock regimes on the penetration and development capacities of *Heterorhabditis indica* and *H. bacteriophora* IJs inside *Galleria mellonella* larvae. The latency period after the application of the heat shock treatment was 48 hours.

Heat-shock regime	Penetration (%)		Development to hermaphrodites (%)	
	H. indica	*H. bacteriophora*	*H. indica*	*H. bacteriophora*
a-Control (25°C)	100.00 (18.80[a])	100.00 (16.80[b])	100.00 (98.40[i])	100.00 (94.04[p])
b- 35°C	81.38 (15.30c)	63.09 (10.60d)	95.02 (93.50k)	102.29 (96.20[l])
c- 40°C	6.11 (1.15e)	0.59 (0.10f)	7.92 (7.80m)	44.72 (42.06[m])
d- 35°C+ 40°C	19.14 (3.60g)	22.02 (3.70h)	98.78 (97.20n)	71.88 (67.60[o])

- Figures in parentheses represent the actual percent values (the average number of the penetrated IJs into the larvae divided by the number of the applied IJs).
- The results are means of two independent experiments.
- Means in parentheses with the same lowercase letters are not significantly different ($P > 0.05$).

The corresponding values obtained at 40°C, the penetration of *H. bacteriophora* IJs was improved by 37 folds compared to only three folds improvement in the penetration of the *H. indica* IJs. The thirty seven-folds improvement in the penetration of the *H. bacteriophora* IJs upon preconditioning at 35°C (Table 3.2) might be attributed to an induced synthesis of heat-shock proteins that protected certain functional proteins involved in the penetration process. In the absence of preconditioning, the greater values of *H. indica* IJs penetration at 40°C compared to those of *H. bacteriophora* indicate that the former IJs are probably protected by a constitutive synthesis of heat-shock

proteins. The basis for this assumption must be proved by documenting a *de-novo* and constitutive synthesis of heat-shock proteins by the two nematode species.

3.2 Effect of Heat Shock on the Development to Hermaphrodites

The development of the penetrated IJs to hermaphrodites was reduced dramatically in both nematode species when the IJs were exposed to 40^{o}C without preconditioning. Although the difference in recovery to hermaphrodites between the two species was not statistically significant, the greater value (*H. bacteriophora*) did not exceed 45% that of the control (Table 3.2). However, upon preconditioning the IJs at 35^{o}C before exposure to the lethal temperature, IJs of the two species showed significant difference in development to hermaphrodites. While the percent of *H. indica* IJs developed to hermaphrodites was close to that of the control, the development of *H. bacteriophora* IJs was only 72% (Table 3.2).

These results indicate that the preconditioning treatment was less efficient in reducing the inhibitory effect of the lethal temperature on the development of *H. bacteriophora* IJs to hermaphrodites.

Since the survival at 40^{o}C of the latter IJs was improved upon preconditioning at 35^{o}C (Selvan *et al*., 1996), and since their penetration capacity was enhanced under the same conditions (Table 3.2), we suggest that the biochemical processes involved in the development of *H. bacteriophora* IJs to hermaphrodites are not protected by the same heat-shock proteins protecting the biochemical mechanisms of IJs penetration into the insect.

3.3 Effect of the heat shock on the IJs reproduction

The infective juveniles reproduction capability expressed per IJ injected inside *G. mellonella* larvae at 25^{o}C showed a tendency to decrease with the increase in the number of injected IJs of both species. The highest IJs yield was obtained when four IJs were injected per larva, while the lowest production occurred at the highest IJs density (36 IJs/larva) (Figure 3.1). However, this reduction in reproduction was not any more significant at higher IJs densities (20-36 IJs/larva) for both *H. indica* and *H. bacteriophora* species. In H. *indica*, total IJs yield, expressed per host, did not differ significantly at all IJs densities except that at 36 IJs/ larvae (Figure 3.2). *H. bacteriophora* showed the same results except that at the IJs densities 8, 15, and 36 IJs/ larva (Figure 3.3). In addition, *H. indica* produced greater number of IJs than *H. bacteriophora* at all IJs densities and temperature treatments.

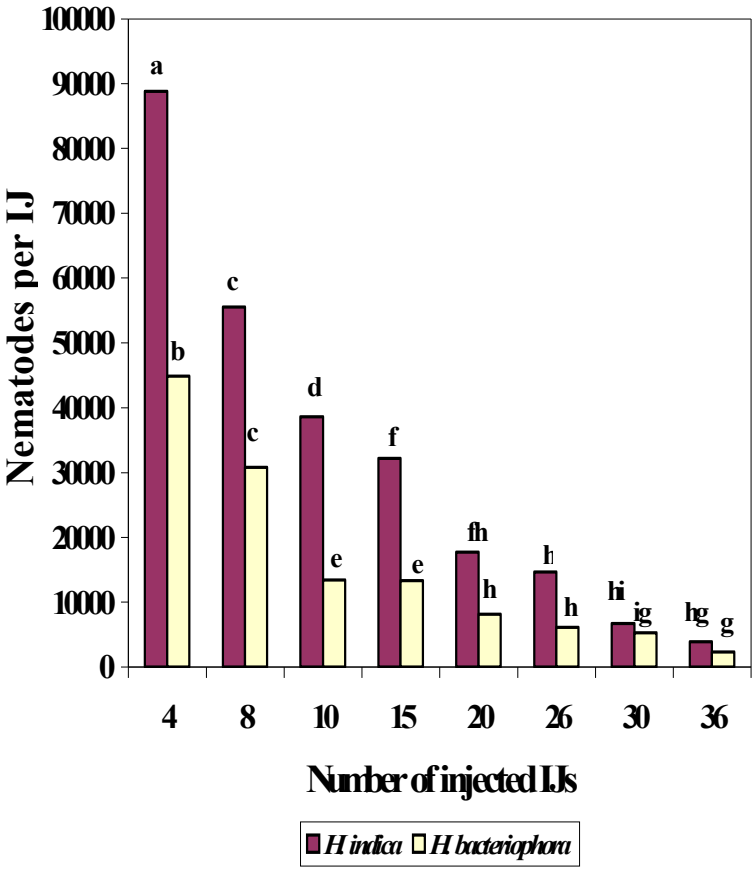

Figure 3.1 : Total nematodes production of *Heterorhabditis indica* and *H. bacteriophora* IJs inside the larvae of *Galleria mellonella* at 25°C (Results are average of two independent experiments; similar letters indicate non-significantly different values using one -way ANOVA test).

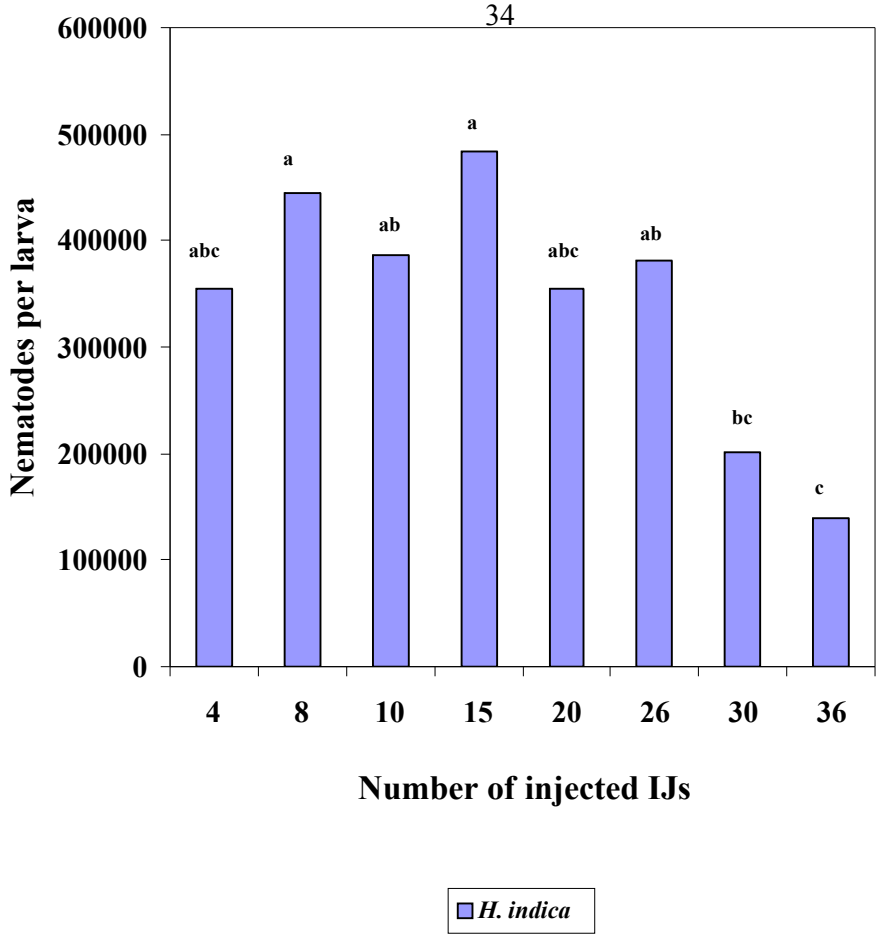

Figure 3.2 : Total nematodes production of *Heterorhabditis indica* per larva of *Galleria mellonella* at 25°C. Results are average of two independent experimements (similar letters indicate non-significantly different values $(P > 0.05)$.

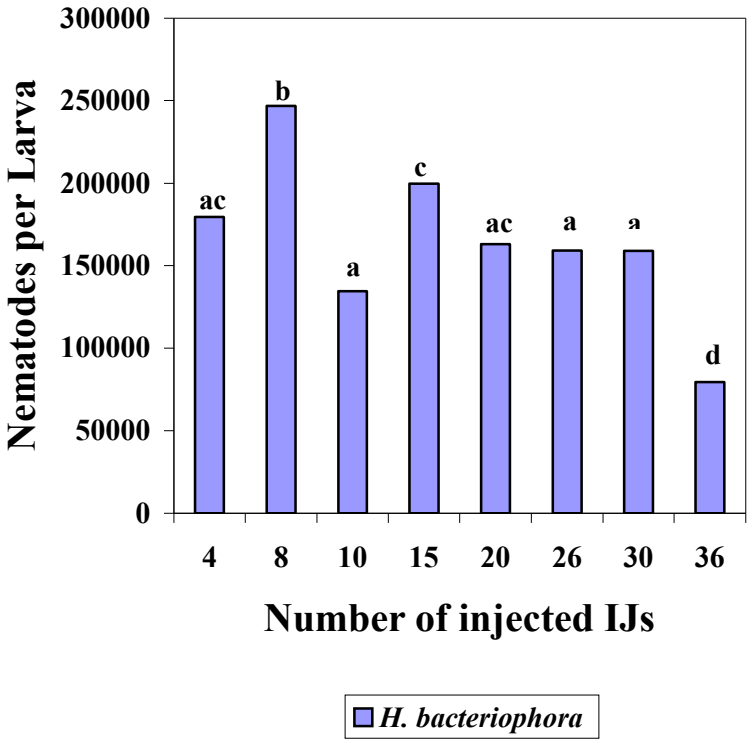

Figure 3.3 : Total nematodes production of *Heterorhabditis bacteriophora* expressed per larva of *Galleria mellonella* at 25°C. Results are average of two independent experiments; similar letters indicate non -significantly different values ($P > 0.05$).

Reproduction capability of the IJs of both species treated at 35^{o}C for one hour, did not show any significant change in response to the raise in the treatment temperature from 25 to 35^{o}C. On the other hand, the reductive effect of increasing the IJs densities on reproduction was significantly observed within the same species at the same temperature, also difference in reproduction between the two species at same IJs densities and temperature (Figure 3.4). The IJs yield at a density of 26 IJs/larva did not exceed 12% and 15% of that when 3 IJs per host were used at the same temperature for *H. indica* and *H. bacteriophora* respectively.

IJs of *H. indica* resisted the effect of heat shock at the lethal temperature 40^{o}C for one hour and produced 60% of the IJs produced at 25^{o}C, when 3 IJs/larva were used (Figure 3.5). While *H. bacteriophora* IJs reproduction capability was almost totally inhibited when treated at the same heat shock temperature and used to inject the Galleria larvae at the same density.

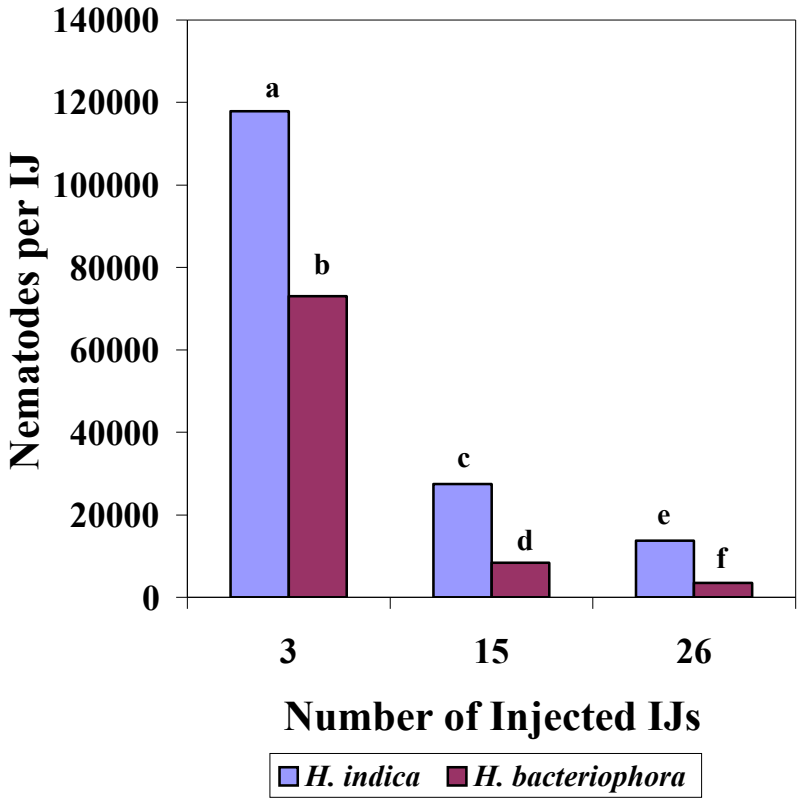

Figure 3.4 : Effect of the heat treatment at 35°C on the proliferation of the infective juveniles of *Heterorhabditis indica* and *H. Hacteriophora* inside the larvae of *Galleria mellonella.* Results are average of two independent experiments.

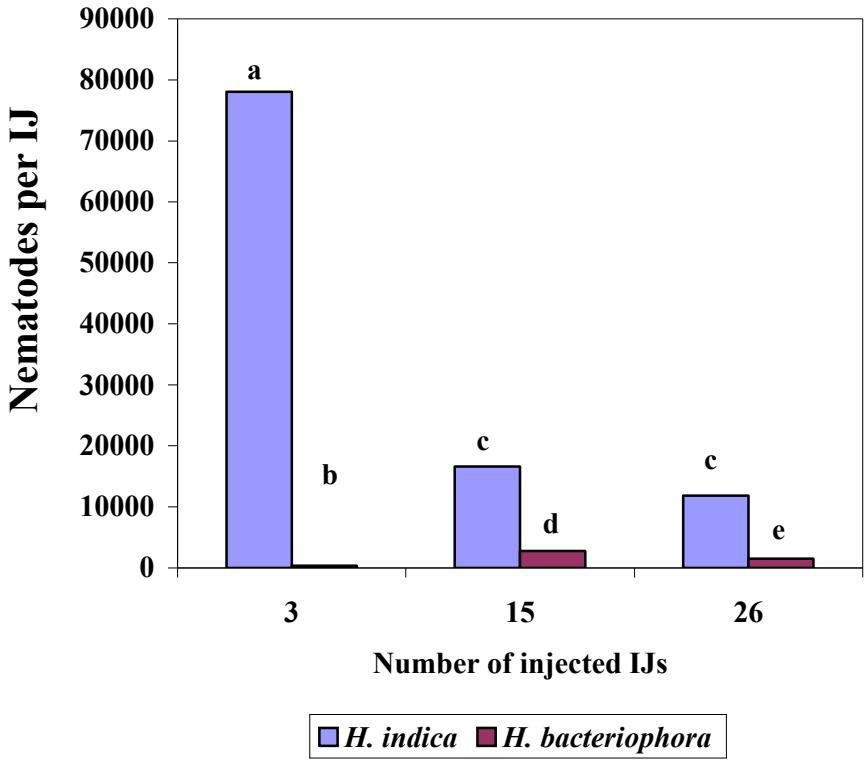

Figure 3.5 : Effect of the heat treatment at 40°C on the proliferation of the infective juveniles of *Heterorhabditis indica* and *H. bacteriophora* inside the larvae of *Galleria mellonella* . Results are average of two independent experiments; similar letters indicate non - significantly different values ($P > 0.05$) .

Infective juveniles preconditioned at 35^{o}C for one hour before exposure to 40^{o}C showed an improved IJs production than that when the IJs were directly treated at 40^{o}C alone. This improvement in reproduction capacity was not signifcantly different from that at 35^{o}C in both species at the densities used, except for *H. bacteriophora* at 15 IJs/larva (Figure 3.6). In addition, upon preconditioning *H. bacteriophora* IJs the reproduction was not significantly different from that of *H. indica* at the IJs densities 15 and 26 IJs/larva, indicating that the improvement in the reproduction capacity of *H. bacteriophora* was greater than that in *H. indica*. Improvement in the *H. bacteriophora* IJs total yield was 2.6 and 3 folds, compared to the 1.8 and 1.1 folds improvement in *H. indica* IJs total yield at densities of 15 and 26 IJs per larva, respectively (Figure 3.6).

The total reproduction of *H. bacteriophora* per injected IJ decreased as the number of the injected IJs increased, as a result of intraspecific competition which is one of the most important density-dependent factors affecting a parasite population within a host. At high nematodes densities, rapid host utilization by both the nematodes and the bacteria results in an inadequate nutrition. Therefore, the detrimental effects of density on entomopathogenic nematodes reproduction inside the Galleria larvae appear to result from intraspecific competition for nutrients and space.

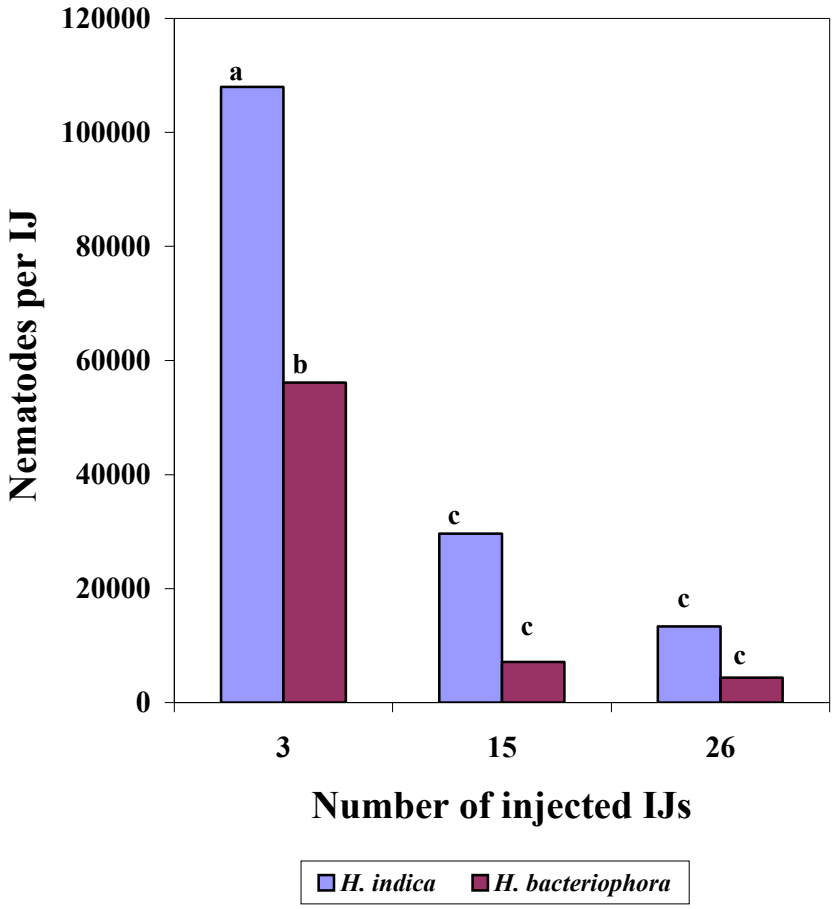

Figure 3.6 :Eeffect of the heat treatment at 35+40°C on the proliferation of the infected juveniles of *Heterorhabditis indica* and *H. bacteriophora* inside the larvae of *Galleria mellonella* . Results are average of two independent experiments; similar letters indicate non - significantly different values ($P > 0.05$).

In this work, crowdness negatively affected the number of infective juveniles produced inside the Galleria larvae in both species (Figure 3.1). This agrees with the results of (Selvan *et al.*,1993) who showed that the IJs production increased with increasing the initial IJs density up to a certain limit, depending on the nematode species used, then declined. Also he found that the longest infective juveniles of *S. carpocapsae* and *H. bacteriophora* were produced at lower densities and not at higher densities that produced larger number of IJs. Since longer IJs store more nutrients they can be expected to survive for a longer periods than shorter IJs, thus their opportunity to locate a new host may increase (Selvan *et al.*, 1993).

Injecting wax moth larvae of nearly the same size eliminated the effect of space on the productivity of the IJs. Therefore, when the total production was experssed per injected larvae no significant change was observed at most of the IJs densities of *H. indica* and *H. bacteriophora*, except unexplainable difference appeared at a particular IJs densities in both species (Figure 3.2).

A significant interaction between the heat-shock temperature and the density of the injected IJs of *H. indica* was observed. The highest IJs yield was at 25^{o}C and at the lowest IJs density 4 IJs/larva (Figure 3.1), while the lowest IJs production was obtained at 40^{o}C at the highest IJs density (26 IJs/larva) (Figure 3.5). The influence of temperature on the total reproduction of the IJs inside the larvae may be either by affecting the number of generations passed through before emergence or by affecting the growth rate of the symbiotic bacteria inside the larvae (Zervos *et al.*, 1991).

Variation among the IJs of the same species appeared clearly in the response of *H. bacteriophora* IJs to the heat treatment at 40^{o}C. The IJs at low density could not tolerate the effect of the lethal temperature on their reproduction, while the IJs of the same species showed a significant tolerance to 40^{o}C at higher densities 15 and 26 IJ/larva (Figure 3.5), at which there is an increased opportunity to have a temperature tolerant IJs than that at the density 3IJs/larva.

Conclusion

1. 35^{o}C has a moderate effect on the penetration, development and reproduction capacities of the IJs of *H. indica and H. bacteriophora*. Treatment for one hour at 40^{o}C has a sever effect on the penetration, development and reproduction capacities of the IJs of *H. indica and H. bacteriophora.*

2. Preconditioning the IJs at 35^{o}C before exposure to the lethal temperature 40^{o}C significantly improves their penetration, development and reproduction capacities if they are allowed a 48 hours latency period after the heat shock treatment.

3. It is suggested that the improvement in penetration and development of IJs achieved upon preconditioning at 35^{o}C is related to synthesis of HSPs and to biochemical repair processes that require a period of time longer than overnight.

4. *H. indica* IJs showed higher rates of penetration into the insect, development, and reproduction capacities than *H. bacteriophora* IJs. Therefore, it is advised to use H. indica EPNs in regions of warm climates.

5. The reproduction capacity of the IJs of both species decreases as the density of the IJs injected inside the Galleria mellonella larvae increase. This inhibitory effect is attributed to intra-specific competition.

References

Akhurst, R. J. (1980). Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *J. Gen. Microbiol. 121*: 303-309.

Akhurst, R. J. and Boemare, M. E. (1990). Biology and Taxonomy of *Xenorhabdus*. In: Gaugler, R. & Kaya, H. K. (Eds.). Entomopathogenic Nematodes in Biological Control. Boca Raton, Fl., CRC Press. pp. 75-87.

Alatorre-Rosas, R. and Kaya, H.K. (1990) Inter-specific competition between entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* for an insect host in sand. *J. Invertebr. Pathol*. *55*: 179-188.

Alatorre-Rosas, R. and Kaya, H. K. (1991). Interactions between two entomopathogenic nematode species in the same host. *J. Invertebr. Pathol.* 57: 1-6.

Angelidis, C. E., Lazaridis, I. and Pagoulatos, G. N. (1991). Constitutive expression of heat-shock protein 70 in mammalian cells confers thermotolerance. *Eur. J. Biochem*. *199*: 25-39.

Barbercheck, M. E. and Kaya, H. K. (1990). Interactions between *Beauveria bassiana* and the Entomogenous Nematodes, *Steinernema feltiae* and *Heterrhabditis heliothidis*. *J. Invertebr. Pathol. 55*: 225-234.

Barbercheck, M. E. and Kaya, H. K. (1991) Competitive interactions between entomopathogenic nematodes and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in soil borne larvae of *Spodoptera exigua* (lepidoptera: Noctuidae). *Environ. Entomol*. *20 (2):* 707-712.

Becker, J.O. and Schwinn, F.J., (1993). Control of Soil-borne Pathogenes with living bacteria and fungi: status and outlook. *Pestic. Sci. 37*: 355-363.

Bedding, R. A. (1981). Low cost in vitro mass production of *Neoaplectana* and *Heterorhabditis* species (Nematoda) for field control of insect pests. *Nematologica* 27: 109-114.

Bedding, R. A., and Molyneux, A. S. (1982). Penetration of insect cuticle by infective juveniles of *Heterorhabditis* spp.. *Nematologica.* 28: 354-59.

Bennett, A. F., Dao, K. M. and Lenski, R. E. (1990). Rapid evolution in response to high-temperature selection. *Nature* 346: 79-81.

Boemare, N. E., Akhurst, R. J. and Mourant, R. G. (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *Int. J. Syst. Bacteriol.* 43:249-255.

Boemare, N., Laumond, C. and Mauleon, H. (1996). The entomopathogenic nematodes-bacterium complex: Biology, life cycle and vertebrate safety. *Biocontrol Sci. Technol.* 6: 333-345.

Brent, K.. J. (1987). Fungicide resistance in crops – its practical significance and management. In: Brent, K. J. and R. k. Atkin (Eds.). Rational Pesticide Use, Proceedings of the Ninth Long Ashton Symposium. Cambridge University Press. Cambridge. UK. pp 137-151.

Caroli, L., Glazer, I. and Gaugler, R., (1996). Entomo-pathogenic

infectivity assay: comparison of penetration rate into hosts. *Bioc. Sci. Technol.* *6*: 227-233.

Carrettero, M. T., Carmona, M. J. and Dietz, J. L. (1991). Thermotolerance and heat-shock proteins in chironomus. *J. Insect Parasitol.* 37: 239-246.

Choo, H. Y., Koppenhoffer, A. M., and Kaya, H. K. (1996). Combination of two entomopathogenic nematode species for suppression of an insect pest. *J. Econ. Entomol.* 89(1): 97-103.

Crowther, J. R. (1995). ELISA: Theory and Practice. In: Walker, J. M. (Ed.). *Methods in molecular biology, 42*. Humana Press, Totowa, New Jersey.

Dempster, J. P. (1987). Effects of pesticides on wildlife and priorities in future studies. In: Brent, K.J. and Atkin R.k. (eds). *Rational Pesticide Use, Proceedings of the Ninth Long Ashton Symposium*. Cambridge University Press. Cambridge. UK. pp.17-25

Dietz, T. J. (1994). Acclimation of the threshold induction temperatures for 70-kDa and 90-kDa heat shock proteins in the fish *Gillichthys mirabilis. J. Exp.Biol.* 188: 33-338.

Dunphy, G. B. and Webster, M. (1986). Temperature effects on the growth and virulence of *Steinernema feltiae* strains and *Heterorhabditis heliothidis*. *J. Nematol.* 18 (2): 270-272.

Ehlers, R.U. and Peters, A., (1995). Entomopathogenic nematodes in biological control: Feasibility, perspectives and possible risks. In: Hokkanen, H.M.T. and Lynch, J.M. (Eds*). Biological Control: benefits and risks.* Cambridge University Press, U.K. pp.119-136.

Elawad, S. A., Gower, S. R. and Hague, N. G. M. (1999). The life cycle of *Steinernema abassi* and *S. riobrave* in *Galleria mellonella*. *Nematol. 1*:762-764.

Epsky, N. D. and Capinera, L. (1994). Invasion rate as a measure of efficacy of the entomogenous nematode *Steinernema carpocapsae* (Rhabditidae: Steinernematidae). *J. Econ. Entomol. 87(2):* 366-370.

Gaugler, R., Bednarek, A. and Campbell, J. F. (1992). Ultraviolet interaction of Heterorhabditid and Steinernematid nematodes. *J. Invertebr. Pathol.*59: 155-160.

Gerner, E. W. and Schneider, M. J. (1975). Induced thermal resistance in Hela cells. *Nature 256*: 500-502.

Gerritsen, L. J. M. (1997). Symbiotic interaction between the bacterium *Photorhabdus luminescens* and the entomopathogenic nematode *Hetero-rhabditis*. Research institute for plant protection. pp. 1-19.

Glazer, I. (1992). Invasion rate as a measure of infectivity of Steinernematid and Heterorahbditid nematodes to insects. *J. Invertebr. Pathol. 59*:90-94.

Glazer, I. (1996). Survival mechanism of entomopathogenic nematdes. *Biocontrol Sci. Technol. 6*: 373-378.

Glazer, I. and Gol'berg, A. (1993) Field efficacy of entomopathogenic nematodes against the beetle *Maladera matrida* (Coleoptera, Scarabaeidae). *Biocontrol Sci. Technol. 3*: 367-376.

Glazer, I., Gaugler, R. and Segal, D. (1991a). Genetics of the nematode *Heterorhabditis bacteriophora* strain HP88; The diversity of beneficial traits. *J.*

Nematol. 23: 324-333.

Glazer, I. and Gol'berg, A. (1989). Laboratory evaluation of steinernematid and heterorhabditid nematodes for control of the beetle *Maladera matrida*. *Phytoparasitica 17*: 3-11.

Glazer, I., Kozodoi, E., Hashmi, G. and Gaugler, R. (1996). Biological characteristics of the entomopathogenic nematode *Heterorhabditis* sp. IS-5: A heat tolerant isolate from Israel. *Nematologica 42*: 481-492.

Glazer, I., Liran, N. and Steinberger, Y., (1991b). A survey of entomopathogenic nematodes (Rhabditida) in the Negev desert. *Phytoparasitica 19*: 291-300.

Glazer, I., Liran,. N., Poinar, G.O.Jr. and Smits, P.H. (1993). Identification and biological activity of newly isolated heterorhabditid populations from Israel. *Fund. Appl. Nematol.16*: 467-472.

Glazer, I., Salame, I. and Segal, D. (1997). Genetic enhancement of nematicide resistance in entomopathogenic nematodes. *Biocontrol Sci. Technol.* 7: 499-512.

Gommers, F. J. (1972). Increase of the nematicidal activity of α- terthienyl and related compounds by light. *Nematologica 18*: 458-462.

Gommers, F. J. and Bakker, J. (1988). Physiological diseases induced by plant responses or products. In: Poinar, G. O., Jr. and Jansson, H.B. (Eds.) *Diseases of Nematodes*. CRC Press, Boca Raton. Fl. 2: 22-33.

Gouge, D. H., Smith, K. A., Lee, L. L. and Henneberry, T. J., (2000). Effect of soil moisture on the vertical distribution of *Steinernema riobrave* (Nematoda:

Steinernematidae). *J. Nematol. 32 (2)*: 222-228.

Grewal, P. S., Gaugler, R. and Shupe, C. (1996). Rapid changes in thermal sensitivity of entomopathogenic nematodes in response to selection at temperature extremes. *J. Invertebr. Pathol. 68*: 65-73.

Grewal, P. S., Gaugler, R., Kaya, H. K. and Wusaty, M. (1993). Infectivity of the entomopathogenic nematodes *Steinernema scapterisci* (Nematoda: Steinernematidae). *J. Invertebr. Pathol. 62*: 22-28.

Grewal, P. S., Selvan, S. and Gaugler, R. (1994). Thermal adaptation of entomopathogenic nematodes: Niche breadth for infection, establishment, and reproduction. *J. Therm. Biol. 19*: 245-253.

Han, R. and Ehlers, R.-U. (2000). Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic *in vivo* conditions. *J. Invertebr. Pathol. 75*: 55-58.

Hara, A. H., Gaugler, R., Kaya, H. K. and LeBeck, L. (1991). Natural populations of entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae) from the Hawaiin Islands. *Environ. Entomol. 20*: 211-216.

Hartl, F.U. and Martin, J., (1992). Protein folding in the cell: The role of molecular chaperones Hsp70 and Hsp60. *Annu. Rev. Biophys. Biomol. Struct. 21*: 293-322.

Hashmi, G., Hashmi S., Selvan, S., Grewal, P. and Gaugler, R., (1997). Polymorphism in heat shock protein gene (Hsp70) in entomopathogenic nematodes (Rhabditida). *J. Therm. Biol.22 (2)*: 143-149.

Hashmi, S., Hashmi, G., Glazer, I. and Gaugler, R., (1998). Thermal

response of the *Caenorhabditis elegans* Hsp70 encoding gene. *J. Experim. Zool. 281*: 164-170.

Hendrick, J. P. and Hartl, F. U., (1993). Molecular chaperone functions of heat-shock proteins. *Ann.. Rev. Biochem*. 62: 349-384.

Iraki, N., Salah, N., Sansour, M. A., Segal, D., Glazer, I., Johnigk, S. –A., Hussein, M. A. and Ehlers, R.U. (2000). Isolation and characterization of two entomopathogenic nematode strains, *Heterorhabditis indica* (Nematoda; Rhabditida), from West Bank, Palestinian territories. *J. Appl. Entomol.123*: 1-7.

Jagdale, G.B. and Jordon, R. (1998). Effect of propagation temperatures on temperature tolerances of entomopathogenic nematodes. *Fundam. Appl. Nematol. 21(2):* 177-183.

Jagdale, G.B. and Jordon, R. (1997). Effect of recycling temperatures on the infectivity of entomopathogenic nematodes. *Can. J. Zool. 75*: 2137-2141.

Kanagy, J. M. N. and Kaya, H. K. (1996). The possible role of marigold root and α-terthienyl in mediating host finding by Steinernematid nematodes. *Nematologica* (in press).

Kaya, H.K., Burlando, T.M. and Thurston, G.S., (1993). Two entomopathogenic nematode species with different search strategies for insect suppression. *Environ. Entomol. 22*: 859-864.

Kaya, H. K. (1990). Soil ecology. In: Gaugler, R. and Kaya, H. K. (Eds.). *Entomopathogenic Nematodes in Biological Control*. CRC Press. Boca Raton, Florida.

Kaya, H. K. and Brayton, M. A. (1978). Interaction between *Neoplectana*

carpocapsae and agranulosis virus of the armyworm *Pseudaletia unipuncta. J. Nematol.*10: 350-354.

Kaya, H. K. and Gaugler, R., (1993). Entomopathogenic nematodes: a developing biological control technology. In: Evans, K., (Ed.). *Agricultural Zoology Reviews Intercept Andover 6*: 63-94.

Kaya, H. K. and Koppenhofer, A.M., (1996). Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. *Biocontrol Sci. Technol. 6*: 357-371.

Klein, M. G. (1990). Efficacy against soil-inhabiting insect pests. In: R. Gaugler and H. K. Kaya (Eds.).*Entomopathogenic Nematodes in Biological Control.* CRC Press. Boca Raton, Florida.

Knodo, E. and Ishibashi, N. (1991). Dependency of three steinernematid nematodes on their symbiotic bacteria for growth and propagation. *Japanese J. Nematol. 21*: 11-17.

Kolatl, H., Glazer, I. and Segal, D., (1995). Reproduction of the entomopathogenic nematode *Heterorhabditis bacteriophora* Poinar, 1976: hermaphroditism *vs* amphimixis. *Fundam. Appl. Nematol. 18(1)*: 55-61.

Koppenhofer, A. M. and Kaya, H. K. (1996). Coexistence of two Steinernematid nematode species (Rhabditidae: Steinernematidae) in the presence of two host species. *Appl. Soil Ecol. 4*: 221-230.

Koppenhoffer, A. M., Kaya, H., Shanmugam, S. and Wood, G. L. (1995a). Interspecific competition between Steinernematid nematodes within an insect host. *J. Invertebr. Pathology 66*: 99-103.

Koppenhoffer, A. M., Kaya, H., Shanmugam, S. and Taormino, S. P. (1995b). Infectivity of entomopathogenic nematodes (Rhabditida: Steinernematidae) at different soil depths and moistures. *J. Invertebr. Pathol. 65(2)*: 193-199.

Kung, S., Gaugler, R. and Kaya, H.K., (1990). Soil type and entomopathogenic nematodes persistence. *J. Invertebr. Pathol. 55*: 401-406.

Kung, S., Gaugler, R. and Kaya, H. K. (1991). Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematodes persistence. *J. Invertebr. Pathol. 57*: 242-249.

Liang, P. and MacRae, T. H. (1997). Molecular chaperones and the cytoskeleton. *J. Cell Sci. 110*: 1431-1440.

Lindquist, S. (1980). Varying patterns of protein synthesis during heat shock: Implications for Regulation. *Dev. Biol. 77*: 463-479.

Lindquist, S. and Craig, E. A. (1988). The heat-shock proteins. *Ann. Rev. Genet. 22*: 631-677.

Lunau, S., Stoessel, S., Schmidt-Peisker, A. J. and Ehler, R.U., (1993). Establishment of monoxenic inocula for scaling up in vitro cultures of the entomopathogenic *Steinernema* spp. And *Heterorhabditis* spp.. *Nematologica 39*: 385-399.

Molyneux, A. S. (1986). *Heterorhabditis* spp. and *Steinernema Neoaplectana* spp.: Temperature, and aspects of behavior and infectivity. *Exp. Parasitol. 62*: 169-180.

Molyneux, A. S. and Bedding, R. A. (1984). Influence of soil texture and

moisture on the infectivity of *Heterorhabditis* sp. D1 and *Steinernema glaseri* for larvae of the sheep blowfly, *Lucilia cuprina*. *Nematologica 30*: 358-365.

Patel, M. N., Perry, R. N. and Wright, D. J. (1997). Desiccation survival and water contents of entomopathogenic nematodes, *Steinernema* spp. (Rhabditida: Steinernematidae). *Internat. J. Parasitol. 27*: 61-70.

Poinar, G. O., Jr. (1975). Description and biology of a new parasitic rhabditoid *Heterorhabditis bacteriophora* nov. gen., nov. sp. *Nematologica 21*:463-470.

Poinar, G. O., Jr. (1990), Biology and taxonomy of Steinernematids and Heterorhabditidae. In: Gaugler, R. & Kaya, H. K. (Eds.). *Entomopathogenic nematodes in Biological control.* Boca Raton, Fl., CRC Press. 23-61.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual (3). Cold spring harbor laboratory press (2 Ed).

Saunders, J. E. and Webster, J. M. (1999). Temperature effects on *Heterorhabditis megidis* and *Steinernema carpocapsae* infectivity to *Galleria mellonella*. *J. Nematol. 31(3)*: 299-304.

Selvan S., Grewal P. S., Leustek T. and Gaugler R. (1996). Heat shock enhances thermotolerance of infective juvenile insect-parasitic nematode *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae). *Experimentia 52*:727-730

Selvan, S., Campbell, J. F. and Gaugler, R. (1993). Density-dependent effects on entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) within an insect host. *J. Invertebr. Pathol. 62*: 278-284.

Shapiro, D.I., Glazer, I. and Segal, D. (1997). Genetic improvement of heat tolerance in *Heterorhabditis bacteriophora* through hybridization. *Biol. Control 8*:153-159.

Simons, W. R. and Poinar, G. O., Jr. (1973). The ability of *Neoaplectana carpocapsae* (Steinernematidae: Nematodea) to survive extended periods of desiccation. *J. Invertebr. Pathol. 22*: 228-230.

Strauch, O., Stoessel, S. and Ehler, R.-U. (1994). Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis. Fundam. Appl. Nematol*. *17*:575-582.

Thomas, G. M. and Poinar, G. O. (1979). *Xenorhabdus* gen. nov., a genus of entomopathogenic nematophilic bacteria of the family Enterobacteriacae. *Internat. J. System. Bacteriol. 29*:352-360.

Timper, P. and kaya, H. K. (1992). Impact of a nematode-parasitic fungus on the effectiveness of entomopathogenic nematodes. *J. Nematol. 24(1)*: 1-8.

Timper, P., and Kaya, H.K. (1989). Role of the second-stage cuticle of entomogenous nematodes in preventing infection by nematophagous fungi. *J. Invertebr. Pathol. 54*: 314-321.

Trent, J. D. (1996). A review of acquired thermotolerance, heat-shock proteins, and molecular chaperons in archaea. *FEMS Micro. Rev. 18*: 249-258.

Van Driesche, R.G. and Bellows, T.S. (1996). Biological Control. Chapman and Hall, New York, USA.Woodring, J.L. and Kaya, H.K. (1988). *Steinernematid and heterorhabditid nematodes: a handbook of techniques*. Southern Cooperative Series Bulletin. 331. Arkansas Agricultural Experiments

Station, Gayetterville, AK. 30pp.

Wouts, W.M. (1981). Mass production of the entomopathogenic nematode *Heterorhabditis heliothides* (Nematoda: Heterorhabditidae) on artificial media. *J. Nematol. 13*:467-469

Zervos, S., Johnson, S. C. and Webster, J. M. (1991). Effects of temperature and inoculum size on reproduction and development of *Heterorabditis heliothidis* and *Steinernema glaseri* (Nematoda: Rhabditoidae) in *Galleria mellonella*. *Canadian J. Zool. 69*: 1261-1264.

Zioni, S., Glazer, I., and Segal, D. (1992). Life cycle and reproductive potential of the nematode *Heterorhabditis bacteriophora* strain HP88. *J. Nematol*. *24*: 352-358.

تأثير الصدمة الحرارية على فعالية نوعين من النيماتودات الممرضة للحشرات؛ *Heterorhabditis indica* و *Heterorhabditis bacteriophora* داخل يرقة عثة الشمع *Galleria mellonella*ا.

الخلاصة:

تعتبر النيماتودات الممرضة للحشرات أحد أهم وسائل المكافحة الحيوية التي تستخدم للسيطرة على الآفات التي تعيش في التربة. إلا أن حساسية هذه النيماتودات لعدد من العوامل البيئية و غير البيئية كارتفاع درجة الحرارة والتنافس مع كائنات أخرى يحد من استخدامها في ظل هذه الظروف. و من هنا تأتي أهمية دراسة تأثير هذه العوامل على فعالية النيماتودات كوسيلة مكافحة وعلى استمراريتها في التربة.

إن تعريض نيماتودات من النوعين *H. indica* **و** *H. bacteriophora* لصدمة حرارية على درجتي الحرارة ٣٥ °م و ٤٥ °م أدى إلى انخفاض قدرة هذه النيماتودات على اختراق الحشرة مقارنة مع قدرتها على الاختراق على درجة ٢٥°م. هذا الانخفاض كان أكبر على درجة الحرارة ٤٠°م منها على ٣٥°م ، و عند نيماتودات النوع *H. bacteriophora* التي تم عزلها من مناطق مناخية باردة ، منها عند نيماتودات النوع *H. indica* التي تم عزلها من فلسطين.

إلا أن النيماتودات أظهرت تحسنا كبيرا في قدرتها على اختراق الحشرة عند تعريضها لصدمة حرارية على درجة ٣٥°م قبل تعريضها للصدمة الحرارية على ٤٠°م. حيث كان التحسن عند نيماتودات النوع *H. bacteriophora* حوالي ٣٧ مرة و عند نيماتودات النوع *H. indica* حوالي ٣ مرات من قدرتها على اختراق الحشرة على درجة الحرارة ٤٠°م.

إن نسبة النيماتودات من كلا النوعين التي اخترقت الحشرة وتطورت داخلها لاكمال دورة حياتها لم تتجاوز ٤٥% من تلك التي تطورت على ٢٥°م نتيجة تعريضها للصدمة الحرارية على ٤٠°م. أما عند تهيئة النيماتودات للصدمة الحرارية على ٤٠°م بتعريضها لدرجة الحرارة ٣٥°م فقد ارتفعت هذه النسبة إلى ١٠٠% في نيماتودات *H. indica* ، و لكنها لم تتجاوز ٧٢% في نيماتودات *H. bacteriophora* بالنسبة للنيماتودات التي تطورت على ٢٥°م.

أما قدرة النيماتودات على إنتاج أجيال جديدة داخل الحشرة فقد أظهرت تناقصا مع زيادة عدد النيماتودات التي تم حقنها داخلها على درجة حرارة ٢٥°م في نوعي النيماتودات، و كذلك مع ارتفاع درجة الحرارة. حيث لم يتجاوز إنتاج النيماتودات الكلي ٢٦ نيماتودة لكل حشرة ١٥% و ١٢% من تلك التي أنتجت عند استعمال ٣ نيماتودات لكل حشرة عند تعريضها لدرجة حرارة ٣٥°م لكل من *H. indica* و *H. bacteriophora* على التوالي.

أما عند تهيئة النيماتودات للصدمة الحرارية، فقد أظهرت نيماتودات *H. bacteriophora* تحسنا في قدرتها على إنتاج أجيال جديدة بمقدار ٢.٦ و ٣ مرات مقارنة مع تحسن مقداره ١.٨ و ١.١ مرة في إنتاج نيماتودات النوع *H. indica* مقارنة مع انتاجها على درجة ٤٠°م ، عند استخدام ١٥ و ٢٦ نيماتودة لكل حشرة على التوالي. هذه النتيجة تدعم الفرضية أن معاملة النيماتودات على درجة الحرارة ٣٥°م قبل تعريضها للصدمة الحرارية على ٤٠°م يعطيها الفرصة لانتاج بروتين يمكن أن يتحمل تأثير الصدمة الحرارية و يلعب دورا هاما في حمايتها عند تعرضها لمثل هذه الظروف الصعبة.

جامعة النجاح الوطنية
كلية الدراسات العليا

تأثير الصدمة الحرارية على فعالية نوعين من النيماتودات الممرضة للحشرات

Heterorhabditis indica و *Heterorhabditis bacteriophora*

داخل يرقة عثة الشمع *Galleria mellonella*

اعداد

بسمة عبدالله يوسف صندوقة

باشراف

د. يعقوب بطه و د. نعيم عراقي

قدمت هذه الاطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم البيئية
بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين
٢٠٠٣

ملخص الدراسة

تعتبر النيماتودات الممرضة للحشرات أحد أهم وسائل المكافحة الحيوية التي تستخدم للسيطرة على الآفات التي تعيش في التربة. إلا أن حساسية هذه النيماتودات لعدد من العوامل البيئية و غير البيئية كارتفاع درجة الحرارة والتنافس مع كائنات أخرى يحد من استخدامها في ظل هذه الظروف. و من هنا تأتي أهمية دراسة تأثير هذه العوامل على فعالية النيماتودات كوسيلة مكافحة وعلى استمراريتها في التربة.

إن تعريض نيماتودات من النوعين *H. indica* و *H. bacteriophora* لصدمة حرارية على درجتي الحرارة ٣٥ °م و ٤٥ °م أدى إلى انخفاض قدرة هذه النيماتودات على اختراق الحشرة مقارنة مع قدرتها على الاختراق على درجة ٢٥°م. هذا الانخفاض كان أكبر على درجة الحرارة ٤٠°م منها على ٣٥°م ، و عند نيماتودات النوع *H. bacteriophora* التي تم عزلها من مناطق مناخية باردة ، منها عند نيماتودات النوع *H. indica* التي تم عزلها من فلسطين.

إلا أن النيماتودات أظهرت تحسنا كبيرا في قدرتها على اختراق الحشرة عند تعريضها لصدمة حرارية على درجة ٣٥°م قبل تعريضها للصدمة الحرارية على ٤٠°م. حيث كان التحسن عند نيماتودات النوع *H. bacteriophora* حوالي ٣٧ مرة و عند نيماتودات النوع *H. indica* حوالي ٣ مرات من قدرتها على اختراق الحشرة على درجة الحرارة ٤٠°م.

إن نسبة النيماتودات من كلا النوعين التي اخترقت الحشرة وتطورت داخلها لاكمال دورة حياتها لم تتجاوز ٤٥% من تلك التي تطورت على ٢٥°م نتيجة تعريضها للصدمة الحرارية على ٤٠°م. أما عند تهيئة النيماتودات للصدمة الحرارية على ٤٠°م بتعريضها لدرجة الحرارة ٣٥°م فقد ارتفعت هذه النسبة إلى ١٠٠% في نيماتودات *H. indica*، و لكنها لم تتجاوز ٧٢% في نيماتودات *H. bacteriophora* بالنسبة للنيماتودات التي تطورت على ٢٥°م. أما قدرة النيماتودات على إنتاج أجيال جديدة داخل الحشرة فقد أظهرت تناقصا مع زيادة عدد النيماتودات التي تم حقنها داخلها على

درجة حرارة ٢٥°م في نوعي النيماتودات، و كذلك مع ارتفاع درجة الحرارة. حيث لم يتجاوز إنتاج النيماتودات الكلي ٢٦ نيماتودة لكل حشرة ١٥% و ١٢% من تلك التي أنتجت عند استعمال ٣ نيماتودات لكل حشرة عند تعريضها لدرجة حرارة ٣٥°م لكل من *H. indica* و *H. bacteriophora* على التوالي.

أما عند تهيئة النيماتودات للصدمة الحرارية، فقد أظهرت نيماتودات *H. bacteriophora* تحسنا في قدرتها على إنتاج أجيال جديدة بمقدار ٢.٦ و ٣ مرات مقارنة مع تحسن مقداره ١.٨ و ١.١ مرة في إنتاج نيماتودات النوع *H. indica* مقارنة مع انتاجها على درجة ٤٠°م ، عند استخدام ١٥ و ٢٦ نيماتودة لكل حشرة على التوالي. هذه النتيجة تدعم الفرضية أن معاملة النيماتودات على درجة الحرارة ٣٥°م قبل تعريضها للصدمة الحرارية على ٤٠°م يعطيها الفرصة لانتاج بروتين يمكن أن يتحمل تأثير الصدمة الحرارية و يلعب دورا هاما في حمايتها عند تعرضها لمثل هذه الظروف الصعبة.