Deanship of Graduate Studies
Al-Quds University

Laboratory Investigations on the Tolerance of Entomopathogenic Nematodes *Heterorhabditis* and *Steinernema* Species to Desiccation and Hypoxia

Amani Jubra'al Awad Abu Sa'da

M.Sc. Thesis

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Department of Applied Earth and Environmental Studies
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Thesis Approval

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Jerusalem-Palestine

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Dedication

I dedicate this work to my beloved parents for their unlimited enthusiasm, encouragement and support, to my dearest brothers Issa and Fadi for their continuous help especially in typing this manuscript and to my dearest fiancé Muhannad for his continuous encouragement, patience and support in achieving my ambition.

Signed: …….
Amani Jubra'al Awad Abu Sa'da
Date: ……

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis or any part of the same has not been submitted for a higher degree to any other university or institution.

Signed: …….

Amani Jubra'al Awad Abu Sa'da

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Amani Jubra'al Awad Abu Sa'da

Abstract

This study evaluated the effect of desiccation and hypoxia on the dauer juveniles (DJs) survival rate of eight *Heterorhabditis* and seven *Steinernema* strains collected from diverse habitats.

Tolerance to desiccation was assessed after incubation of DJs in 25% glycerol at 25ºC for 24, 48 and 72 hr. Hypoxia was tested by sealing the DJs in 300 µl tubes at 25ºC for 24, 48 and 72 hr.

Percent survival was determined microscopically by prodding each DJ. The local isolate *S.abbasi*-09 (Gaza isolate) was the most tolerant strain to desiccation stress which showed 93% survival rate after 72 hr of incubation, followed by *S.abbasi* (Oman isolate), however this isolate was the most tolerant to hypoxia stress (survival rate was 86% after 72hr of treatment). Tolerance to desiccation correlated poorly with hypoxia tolerance.

Differences in tolerance to hypoxia within and among isolates cannot be explained since mechanisms of tolerance to hypoxia are poorly understood. One hypothesis to be investigated is that nematode may enter into a quiescent stage where low level of metabolism reduces oxygen consumption. On the other hand, nematodes may have oxygen storage or regeneration capability to overcome the low oxygen concentration

H.tayseerae showed the least level of tolerance to desiccation while *H.tayseerae*-06 was the least tolerant isolate to hypoxic conditions. These two strains are considered to be weak bio-control agents in areas which have low humidity and oxygen respectively. The three *S.abbasi* isolates (*S.abbasi*-08, 09 and the reference isolate *S.abbasi*) and *H.bacteriophora*-05 are recommended for further studies, experimentation, and field test application in the area of bio-control especially in semi-arid and arid climates.

بحث مخبري لمدى تحمل بعض أصناف النيماتودا الممرضة للحشرات من الجنسين *Heterorhabditis* و*Steinernema* لكل من الجفاف ونقص الأكسجين

ملخص

إن دراسة أهمية تأثير العوامل البيئية وغير البيئية خاصة الجفاف ونقص الأكسجين في التربة على فعالية النيماتودات يساعد في تحديد الموسم والمناخ والطبيعة الجغرافية الملائمة لاستخدام هذه الديدان من أجناس النيماتودا.

إن التغييرات الموسمية للعوامل غير الحيوية، خاصة الجفاف ونقص الأكسجين، تنعكس سلبا على فعالية الطور المعدي لحشرات التربة كعامل هام في المكافحة الحيوية.

يهدف البحث لدراسة تأثير الجفاف ونقص الأكسجين على الطور المعدي لثمانية أطياف من النيماتودات التي تنتمي إلى جنس *Heterorhabditis* وسبعة أطياف تنتمي إلى جنس *Steinernema* والتي عُزِلت من مناطق بيئية مختلفة.

ولمعرفة مدى مقاومة النيماتودات للجفاف قمنا بوضع الطور المعدي من النيماتودات في بيئة تحتوي على 25% جليسرول على درجة 25 مئوية لثلاث فترات زمنية 24, 48 و 72 ساعة. أما قياس فعالية النيماتودات لنقص الأكسجين فقد تمت عن طريق وضعها داخل أنبوب محكم الإغلاق بحجم 300 مايكرولتر على درجة حرارة 25 مئوية لثلاث فترات زمنية 24, 48 و 72 ساعة. إن تحديد نسبة مقاومة النيماتودات بعد التعرض للصدمة يتم بواسطة وخز كل نيماتودا باستخدام رأس مدبب أثناء المشاهدة بالميكروسكوب.

سجل الصنف المحلي المعزول من غزة *S. abbasi*-09 أعلى قدرة تحمل للجفاف بعد 72 ساعة وهي 93% يليه صنف *S. abbasi* المعزول من عُمان وقد أظهرت النتائج قدرته العالية على تحمل نقص الأكسجين وسجل 86% بعد 72 ساعة من المعالجة.

أظهرت التحاليل الإحصائية ارتباطاً ضعيفاً بين مقاومة مختلف أطياف النيماتودا للجفاف وبين مقاومتها لنقص الأكسجين.

إن الفروقات بين مقاومة أطياف النيماتودا من كلا الجنسين لنقص الأكسجين غير واضحة وما زالت بحاجة إلى دراسة أعمق ولكن هناك فرضيات قد تفسر هذه الظاهرة وهي أن النيماتودات تدخل في مرحلة سكون تحت تأثير هذا النقص وبالتالي تحتاج إلى كمية أكسجين قليلة لتقوم بعملياتها الحيوية أو ربما باستطاعة النيماتودا خزن كمية معينة من الأكسجين لتقوم باستخدامها تحت الظروف الصعبة خاصة نقص الأكسجين.

سجل الصنف *H. tayseerae* الذي ينتمي إلى جنس *Heterorhabditis* أقل نسبة مقاومة للجفاف بينما سجل الصنف *H. tayseerae*–06 أقل نسبة مقاومة لنقص الأكسجين. يعتبر هذان الصنفان الأقل

فاعلية من حيث إستخدامهما في التربة كوسيلة مكافحة حيوية خاصة في المناطق الجافة أو شبه الجافة أوالتي تعاني من نقص الأكسجين.

صنف *S. abbasi*, والذي يتضمن الأطياف التالية: *S. abbasi*–08، 09 وَ *S. abbasi* والصنف *H. bacteriophora*-05 من الجنس *Heterorhabditis* تعد من الأطياف التي تستحق دراسات مستقبلية خاصة في مجال إستخدامها في الحقل, ويوصى بها للإستخدام كوسيلة للمكافحة الحيوية خاصة في المناطق شبه الجافة والتي تتميز بمناخ ذي درجات حرارة مرتفعة.

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Abbreviation	Description
EPNs	Entomopathogenic Nematodes
et. al.	And others
USA	United States of America
Kg	Kilogram
NA	Not Available
IJs	Infective Juveniles
DJs	Dauer Juveniles
mm	Millimeter
m	Meter
%	Percent
BSL	Below sea level
ASL	Above sea level
ºC	Degree Centigrade (Celsius Scale)
spp.	Species
ATP	Adenosine Tri-Phosphate
HSP	Heat Shock Protein
Desc.	Desiccation
g	Gram
H.	*Heterorhabditis*
S.	*Steinernema*
cm	Centimeter
ml	Milliliter
hr	hour
No.	Number
e.i.	That Is
IPM	Integrated Pest Management

Chapter One

Introduction

1.1. Overview

Chemical pesticides are commonly used for the control of many agricultural pests. However, many problems are associated with using pesticides, one major problem is the failure to control pests, this is due to the appearance of new pest strains that are resistant to applied pesticides. The lack of specificity and the harming of beneficial insects is another major problem (Brent, 1987).

Environmental contamination is yet a third problem related to pesticide application. As the applied pesticide or its breakdown products may persist in soil and water causing groundwater contamination, poisoning and destroying habitats of a large number of life forms (Dempster, 1987).

Therefore, to avoid environmental risks of chemical pesticides, efforts have been directed to the use of alternative methods in controlling pest populations. One of these alternatives is the application of biological control. Before commercializing nematodes as a safe alternative to chemical pesticide, it is important to determine their biological properties, such as heat tolerance, infectivity and tolerance to desiccation and hypoxia. Persistence of EPNs in soil, infectivity, development, maturation and reproduction of Entomopathogenic Nematodes (EPNs) are influenced by many abiotic factors. Therefore to overcome environmental obstacles and achieve successful biocontrol application, research has been conducted to select improved nematodes that withstand environmental stresses (Van Driesche and Bellows, 1996).

1.2. Statement of the study Problem

EPNs mainly from the genera *Heterorhabditis* and *Steinernema* lose fitness and capacity to act as an ideal bio-control agent in infecting and destroying agricultural insect hosts in response to seasonal fluctuations of the environmental conditions, especially desiccation (low humidity) and hypoxia (low soil oxygen).

1.3. Aim of the study

The aim of this study is to answer the following question:
To what extent desiccation and hypoxia will affect the dauer juveniles survival rate of eight *Heterorhabditis* and seven *Steinernema* species?

1.4. Objectives of the study

1. To compare variation in the tolerance to desiccation and hypoxia among local and reference isolates of different EPNs species.
2. To determine the correlation between climatic origin of different EPNs species and their survival rate under environmental stress conditions; especially in response to desiccation and hypoxia.
3. To identify and select the most tolerant nematode species to both desiccation and hypoxia to be used as an ideal bio-control agents.
4. To determine the correlation between the DJs tolerance of different EPNs species to desiccation and hypoxia.

Chapter Two

Literature Review

2.1. Introduction

Insect parasites belonging to the phylum Nematoda have been known since the 17th century. However, it was not before 1930's that serious consideration was given to using nematodes in the control of insects (Smart, 1995).

Nematodes, in general, are microscopic roundworms; the nematodes that are pathogenic to insects are called Entomopathogenic Nematodes (EPNs), only belong to the genera *Heterorhabditis* and *Steinernema* (Van Driesche and Bellows, 1996), which possess the potential to infect and control a wide range of agriculturally important pests. Such insect pests which cause disease to crops, soil and the environment. Thus EPNs are considered safe and ideal bio-control agents (Iraki *et al*., 2000). In this study, biological control is viewed as "the use of environmentally harmless natural enemies to suppress a pest population to a minimal level rendering it less abundant and damaging" (Van Driesche and Bellows, 1996).

Biological control was used for controlling insects, mites, and weeds (Cameron *et al*., 1989). Application of the method then broadened to include other invertebrates, plant pathogens, and even some vertebrates (Stirling, 1991). Over 1200 programs of biological control have been used to control more than 543 species of target insects. Several families of mites; which include: rust mites (Abou-Awad and El-Banhawy, 1986), tarsonemid mites (Huffaker and Kennett 1956) and the spider mites, all have been targets of biological control (Mc Murtry, 1982).

Currently, nematodes are used against soil-inhabiting insects that attack citrus, cranberries, turf, and ornamental plants. EPNs have been registered for commercial application in many countries: USA, Australia, Japan, and Europe (Caroli *et al.*, 1996). As a result, EPNs are considered a superior alternative to hazardous chemical pesticides (Jagdale, 1997).

2.2. The extent of chemical pesticides consumption in Palestine

Application of chemical pesticide is on the rise. The need to feed 11 billion humans and their farm animals, lead to intensive culturing and created a real need to control agricultural pests (Nebel and Wright, 2000). With reference to the survey conducted by the Applied Research Institute of Jerusalem (ARIJ, 1995), the total cultivated area of the West Bank is about two million dunums, one hundred thousand dunums of which are under irrigation while 1.6 million dunums are rain fed, the remaining 300 thousand dunums are fallow lands. In Gaza, nearly half the total area (about 175,000 dunums) is cultivable land (Laeremans and Sourani, 2006).

According to (Samer Farah thesis study 2007) results showed a significant increase in the number of chemical pesticides used especially during the last ten years: insecticides contribute 30.5% of applied pesticide, acaricide 7.9%, fungicides 36.2%, fertilizers 3.4%, herbicides 16.9% and others 5.1%.

As shown in Fig. (2.1), it is estimated that the total quantity of pesticide including methyl bromide used in the West Bank is around 493.83 tons per year (Saleh *et al.,* 1995).

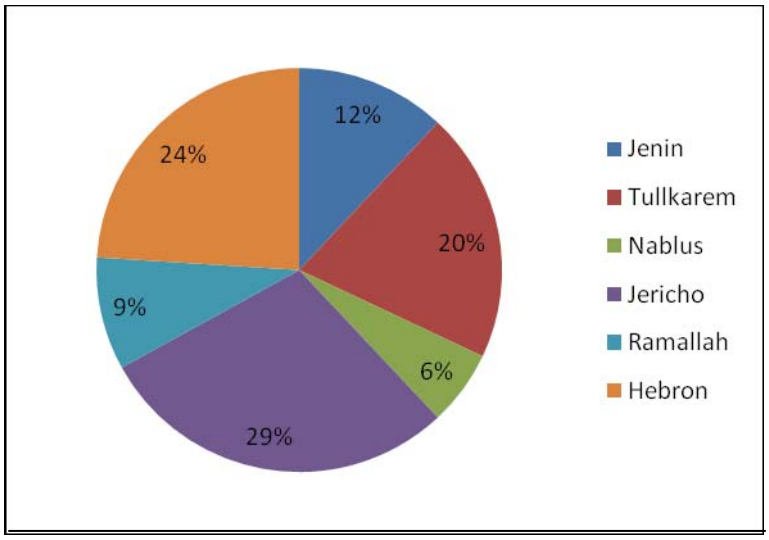

Fig.2.1: Average pesticide consumption in the West Bank according to districts (Saleh *et al.*, 1995).

In Gaza Strip 771 tons of pesticides are used these consist of: 36% as insecticides, 19% fungicides and 20% herbicides according to Palestinian National Information Center (PNIC, 1999). These different chemical pesticides can be grouped into six major chemical categories: chlorinated hydrocarbons, organophosphates, carbamates, thiocyanates, dinitrophenols, and fluoroacetates (Mastsumura, 1985).

Unfortunately, Palestinian farm workers use these pesticides extensively unaware of their risks and hazardous effects on their health and the environment as well. (Saleh *et al*., 1995).

2.2.1. Risks of using chemical pesticides

Agriculture is the backbone of the Palestinian economy, contributing 33% and 24% of the Gross National Products in the West Bank and Gaza strip, respectively. The overuse of chemical pesticides in agriculture in recent years especially in irrigated areas has had many negative side effects (Saleh *et al*,.1995).

Evaluation of pesticide residual effect has shown that many pesticides are not readily degradable and constitute a major source of environmental contamination, especially of underground and surface water. Contaminated water resources lead to the poisoning of life forms including humans (Dempster, 1987). Furthermore, it disturbs the ecological balance as a result of the development of pesticide resistance and, in some cases, the increase in pest population.

Indeed, the number of insects that developed resistance to pesticides has increased dramatically since the 40's of the last century (Brent, 1987). In addition, the nonspecific action of chemical pesticides kills the pest's natural enemies, which are usually more sensitive to pesticides than the pest itself (Croft, 1990). Pollution of the environment and reduction of biodiversity at its current dramatic rate cannot be justified. Some countries have approved legislations to control and regulate pesticide application (Nebel and Wright, 2000).

As a result it would be useful to minimize the use of pesticides and encourage alternative methods in controlling field pest populations. Researchers are working toward finding a non-chemical, non-residual method for controlling field pests. There are two major substitutes to reduce the use of chemicals. Abiotic and biotic alternatives are efficient in controlling field pests and reducing the risks of damage to life forms, water, and environment (Bauer and Kaya, 2001). These alternatives are discussed below.

2.3. Abiotic approaches to pest control

2.3.1. Solarization

Solarization is a nonchemical process for controlling field pests, weeds and diseases, it is a soil solarization technique which will control many soil inhabiting pests and weeds, this technique is based on capturing radiant heat energy from the sun, causing biological, chemical and physical changes in the soil. A polyethylene plastic nylon that allows the sunlight to penetrate is placed on the top of moist soil increasing the temperature of the soil to a level that are lethal to many soil inhabiting organisms such as weeds, plant parasitic nematodes, insects, and soil mites. Solarization leads to an increase in crop yield, plant growth, and crop quality. Although solarization reduces the need for chemical pesticides, it is a nonspecific method that kills all susceptible life forms under solarization (Katan, 1981).

2.3.2. Ultraviolet light

Ultraviolet light is also a technique used to controlling soil inhabiting pests. This technique is based on the exposure of the soil inhabiting organisms to very short pulses of ultraviolet light emitted at a wavelength that can be absorbed by surface coloring chemicals in the insect like integument. The coloring chemicals act as a heat sink to the ultraviolet photons, as a result the harmful undesired organisms are selectively heated without affecting adjacent food objects and causes irreversible fatal damage to targeted organisms. This method is probably expensive, cannot reach below the surface and need special equipment. Therefore, it cannot be applied on a large scale (Bond and Grundy, 2001).

2.4. Biotic approaches to pest control

Biopesticides are pest management tools that are based on beneficial microorganisms like: bacteria, viruses, fungi, and Entomopathogenic Nematodes (EPNs), which are considered as efficient biocontrol agents for controlling soil pests (Van Emden and Service, 2004).

2.4.1. Bacteria

More than 90 species of naturally occurring bacteria, insect specific bacteria have been isolated from insects, plants, and soil. The main interest is given to *Bacillus thuringiensis,* a species that has been developed as a microbial insecticide, for examples the primary hosts of *Bacillus thuringiensis* are caterpillars and larvae of beetles and flies (Van driesche and Belows, 1996). There is a different variety of this bacterium that produce toxic proteins which are fatal to certain insect groups. The two other bacteria used in pest control are *Bacillus popilliae* which attack beetles, especially the Japanese beetle and *Bacillus sphaericus* which is used to attack and kill mosquito larvae (Van Emden and Service, 2004).

2.4.2. Viruses

Sixteen families of viruses have been found to infect insects. Members of the family Baculoviridae are efficient biocontrol agents and can be used commercially, because they are lethal to insects (Van Driesche and Bellows, 1996). The most important viruses used in pest control belong to Nuclear Polyhedrosis Viruses (NPVs) and Granulosis Viruses (GVs) subgroups. For example a major success with viruses is the control of the sawfly *Neodiprion sertifer*, which is found in Canada's forest trees using a nuclear polyhedrosis virus (Van Emden and Service, 2004).

2.4.3. Fungi

The infectious fungal family that causes disease to insects is the Entomophthoraceae within the Zygomycotina or Deuteromycotina subdivisions. Fungal epidemics can cause high levels of mortality in affected arthropod populations. For example, the fungus *Zoophthora radicans* was introduced to Australia to suppress the Lucerne aphid *Therioaphis trifolii* (Van Driesche and Bellows, 1996).

2.4.4. Nematodes

Entomopathogenic nematodes (EPNs) of the families *Heterorhabditidae* and *Steinernematidae* are considered as an ideal method for controlling agricultural pests (Klein and Georgis, 1992). EPNs application is safe for plants and animals; this high degree of safety means that applications in the field don't need safety equipment, unlike hazardous chemical pesticides, or even *Bacillus thuringiensis.* The application re-entry time and dose does not contaminate the surrounding environment and no need to be reintroduced or repeated (Smart, 1995).

However, EPNs are susceptible to chemical pesticides as well as biotic factors. Infective Juveniles (IJs) or dauer juveniles (DJs) are compatible with a wide range of chemical but not all agricultural chemicals under field conditions. Studies have shown that *Heterorhabditis bacteriophora* is the most sensitive species among EPNs to physical stress (Selvan *et al.*, 1996). Moreover, many of the chemicals that were considered to be toxic to nematodes actually have a transient effect only; as nematodes recovered quickly when exposure is terminated (Van Emden and Service, 2004). In Palestine there is only laboratory conducting research on EPNs.

2.5. Research on EPNs in Palestine

At the beginning of the last decade, the laboratory of the UNESCO Biotechnology center at Bethlehem University initiated a large-scale research project on these bio-control agents. About ten strains were isolated from Bethlehem area and Gaza Strip. Two of these strains (Pal-H-01 and Pal-H-02) were identified as *H.indica* by conventional crosses (Iraki *et al* ., 2000), while the other eight by comparison of the restriction digestion profiles of the Internal Transcribed Spacer (ITS) region with the corresponding profiles of the reference strains. The candidate reference strains were selected based on morphological dimension of the DJ stage (Sansour, 2000), (Sansour *et al*., 2001). Also, the genetic relatedness among the identified strains was determined by RAPD-PCR technique. Another field of research on EPNs conducted in this laboratory involved characterization of biological traits including infectivity to larvae of the wax moth *Galleria mellonella* (Sansour, 2000), heat tolerance of the DJs and the effect of heat shock on their infectivity (Sandouka, 2003).

The research on heat tolerance of these nematodes and the effect of heat shock on their infectivity showed that the penetration capacity into *Galleria mellonella* larvae for the two EPNs species "*Heterorhabditis indica* and *Heterorhabditis bacteriophora*", decreased as a result of exposing the DJs to 40°C for one hour. The DJs penetration capacity of the *H.indica* strain was greater than that of *H.bacteriophora*. However, preconditioning the DJs at 35°C before exposure to 40°C improved the penetration capacity of *H.bacteriophora* DJs to about 37 folds compared to only 3 folds improvement in the DJs penetration of *H.indica*. Development of the penetrated DJs to hermaphrodite was also decreased when the DJs were exposed to 40°C for one hour without preconditioning compared to preconditioned DJs. The total reproduction of the DJs inside the Galleria larvae showed a tendency to decrease with the increase in the number of DJs injected inside the larvae at 25°C in both species (Sandouka, 2003).

Furthermore, the effect of other insect pathogenic agents (*Beauveria bassiana* and *Serratia merscecense*) on the invasion and recovery of the insect-pathogenic nematode *Heterorhabditis indica* (Pal-H-01) was studied. Infection of *Galleria mellonella* larvae for 24 hr with *S.marcescens* or *B.bassiana* before nematode infection dramatically reduced the invasion of DJs into the insect. Recovery and total production of nematodes was profoundly suppressed by pre infection with *S.marcescens* or *B.bassiana* (Dar-Issa, 2000).

The results of these studies indicate that the survival of different stages of EPN could be affected by various abiotic and biotic environmental conditions.

To understand the nature of these effects it is necessary to know the developmental biology of these worms.

2.6. Biology of EPNs

Entomopathogenic nematodes in the families *Heterorhabditidae* and *Steinernematidae* have the potential to control a wide range of agriculturally important pests that infest crops, soil, and the environment. These are considered to be as best alternatives to chemical pesticides (Klein, 1990). The qualities that make the two families excellent biocontrol agents include: host seeking capability, broad host range, high virulence, and safe to mammals, crops and environment (Gaugler, 1988).

The two genera *Heterorhabditis* and *Steinernema* contain the most important species of EPNs. Currently, there are six species in *Heterorhabditis* and sixteen species in *Steinernema* that can infect and kill field insects (Smart, 1995).

2.6.1. EPNs life cycle

The third stage infective juvenile (IJ) or Dauer Juvenile (DJ) Figure (2.2) is the only free–living nematode stage that can survive outside the host for many months without food, and posses a greater resistance to environmental extremes as compared with the other developmental stages (Grewal and Taylor, 2002). It also moves freely from one insect to another (Iraki *et al.*, 2000).

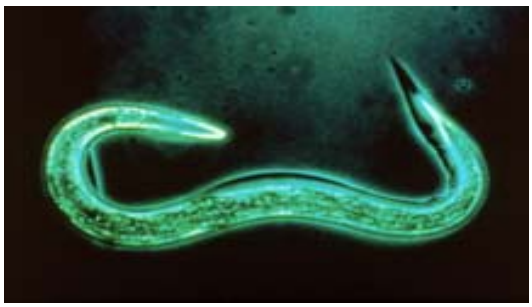

Fig.2.2: The infective juvenile of *Heterorhabditis* spp. (Hazir *et al*., 2003)

Infective Juveniles range from 0.4-1.1 mm in length and can be observed with a compound light microscope, their intestines contain symbiotic bacteria, from the genera *Photorhabdus* (Boemare *et al.*, 1993) and *Xenorhabdus* (Georgis and Manweiler, 1994) for *Heterorhabditis* spp. and *Steinernema* spp. respectively. The bacteria are released into the host after the infective juvenile has gained entry to the insect haemocoel by way of natural openings spiracles, mouth, anus and cuticle (Bedding and Molyneux, 1982). In the haemolymph, bacteria start to multiply rapidly causing septicemia, eventually killing the insect within 24-72 hours, see Figure (2.3) (Selvan *et al.*, 1996).

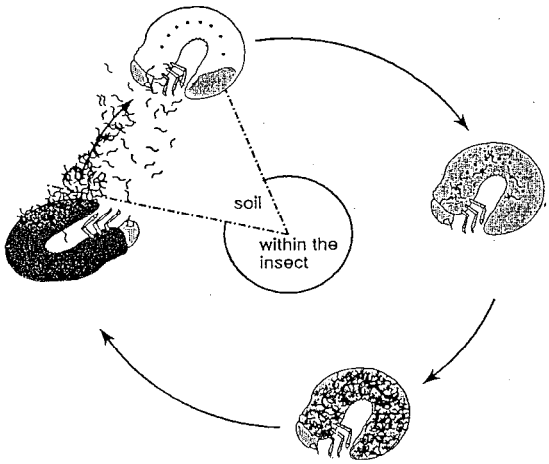

Fig.2.3: The Life cycle of EPNs showing a soil larva (white) infected (grey) then destroyed by the nematode (black) (Gerritsen, 1997).

Inside the insect host, the non-feeding DJ starts to feed on the bacteria and their metabolic byproducts, develops into the fourth stage J4, see Figure (2.4) (Poinar, 1990). Stage J4 of *Heterorhabditis* spp. develops into hermaphrodite, while in *Steinernema* spp. it develops into male and females of the first generation (Georgis and Manweiler, 1994).

In the second generation the fourth juvenile stage J4 develops to male, female, or hermaphrodite in *Heterorhabditis* spp., the adults (males and females) mate and the eggs produced by these second-generation females (or hermaphrodites) hatch as first juvenile stage (J1) that develops to the second juvenile stage (J2) (Smart, 1995). The late second juvenile stage (J2) ceases feeding and develops into the third juvenile stage (J3) which is called infective juvenile (IJ) or dauer juvenile (DJ) and leaves the insect cadaver in search for new hosts (Gouge *et al*., 2000). Generally, the nematodes complete 2-3 generations within the host, after which, free living infective juveniles (IJs) emerge to seek new hosts (Selvan *et al*., 1993).

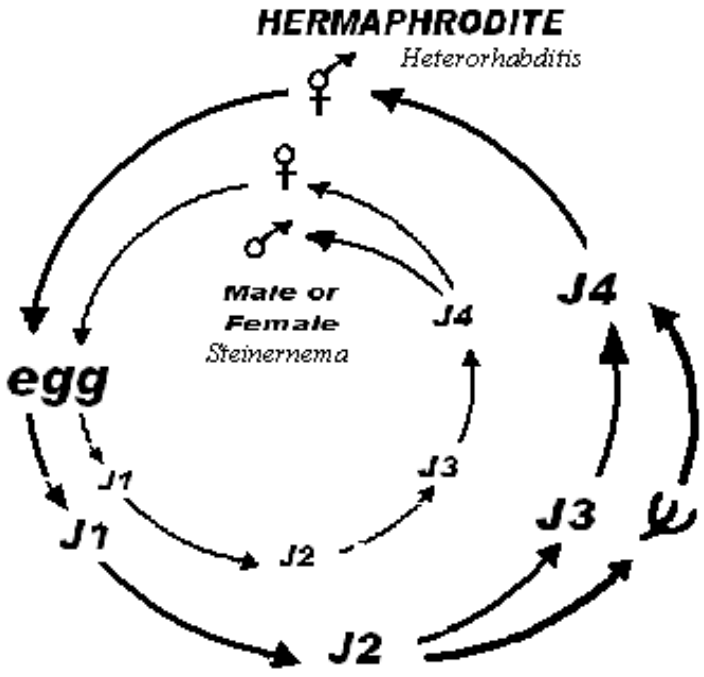

Fig.2.4: The developmental stages of *Heterorhabditis* and *Steinernema* EPNs from egg to adult male and female passing through four Juveniles (J1-J4) stages, completing the cycle by producing more eggs (Gerritsen, 1997)

2.6.2. Symbiotic relationship between nematode and bacteria

Entomopathogenic nematodes from the genera *Heterorhabditis* and *Steinernema* kill insects by the mutualistic bacteria, *Photorhabdus* and *Xenorhabdus* spp., members of the family Enterobacteriaceae which are symbiotically associated with EPNs that belong to the families Heterorhabditidae and Steinernematidae (Burnell and Stock, 2000). Because the nematodes cannot reproduce inside the insect without the bacteria and the bacteria cannot enter the haemocoel without the nematode (Ishibashi and Kondo, 1990), therefore, nematodes act as vectors transporting the symbiotic bacteria into the haemocoel of insect host. The bacteria are gram negative, peritrichous motile and facultative anaerobic rods (Georgis and Manweiler, 1994).

2.6.3. Specificity of EPNs to host

Specific host recognition by EPNs is considered an important mechanism to maintaining host affinities (Selvan *et al.*, 1993). Nematode ability to penetrate into the insect haemocoel occurs by the release of proteolytic enzyme that is a specific factor. Another specific factor is the nematode ability to attack insect defenses through evasion and destruction of the insect antibacterial factors (Simoes and Rosa, 1996). Contact with insect excretory products, cuticle or gut contents are main factors for host recognition by (EPNs). Other behavioral activities help nematodes to locate insect targets include nematode alterations in frequency and duration of search parameters: body waving, backward crawling, forward crawling, head waving, stopping and head thrusting (Grewal *et al*., 1993).

2.7. Interaction between natural enemies

Regardless of the way in which biocontrol is implemented, interaction between pest populations takes one of the following forms: competition, parasitism, predation, or pathogenecity (Van Emden and Service, 2004).

2.7.1. Competition

Two or more organisms may compete for the same limited resources of food, oxygen, and space. Naturally, the stronger of those organisms with superior numbers will thrive (Van Driesche and Bellows, 1996). Competition can become important with respect to weed control and to a smaller extent with beneficial insects; however its application in biological control is limited (Debach, 1991). Previous studies indicated that (EPNs) tend to avoid the attraction of new invaders; upon infection with EPNs the host insect *Galleria mellonella* larvae secretes a substance to prevent further invasion and avoid competition (Caroli *et al.,* 1996).

2.7.2. Parasitism

A parasite lives in, on, or with another organism and obtains its food and/or shelter at host's expense (Van Emden and Service, 2004). Parasitic insects and microbes are important in the biological control of many pests, an insect that is parasitic on other insects during its immature stages, but is free living as an adult, is called a parasitoid. Parasitoids are insects with an immature stage that develops on or in an insect and kills the host (Greathead and Greathead, 1992). Most parasitoids are small flies and are often common in flowering plants such as fruit crops, because parasitoids must be adapted to the life cycle, physiology, and defenses of their hosts; many are limited to one or a few closely related host species. The accurate identification

of the host and its parasitoid species is critical for application of parasitoids as biological control agents (Debach, 1991).

2.7.3. Predation

A predator is an organism that attacks, kills and feeds on one or many other individual organisms during its life time (Van Emden and Service, 2006). Several kinds of predators feed on insects; vertebrate insectivores usually feed on many insect species and rarely focus on a specific prey (Debach, 1991). Insects and related predators are more often used in biological control because they feed on a smaller range of prey species, lady beetles, ground beetles, and flower bugs are good examples of insect predators (Van Driesche and Bellows, 1996). Predators are the best known agents; they capture, kill and consume a large quantity of prey individuals during their development. Good example is birds, frogs, bats, and other larger animals for terrestrial pests. However, with crop pests the great majority of predators are arthropods such as spiders, mites and insects (Van Emden and Service, 2006).

2.7.4. Pathogenicity

Pathogenecity is the ability of a microorganism to cause an infection to a host. (Debach, 1991). Insects like other animals and plants may be infected with bacteria, fungi, protozoans, nematodes, and viruses. This infection reduces the rate of feeding and growth of insect pest, slow or prevents their reproduction or kills them (Van Driesche and Bellows, 1996).

2.8. Effect of environmental factors on persistence of EPNs

Before discussing the most important Abiotic and Biotic factors that affect the survival of nematodes and their symbiotic bacteria, knowledge of the Palestinian climatic zones table (2.2) is necessary to the understanding of the effect of environmental factors on the persistence of EPNs.

Table 2.2: Characteristics of Palestinian climatic zones (rainfall, humidity and temperature) (ARIJ, 1994, 1997, 2003).

Geologic Zone	Altitude(m)	Climate	Rainfall (mm/year)	Mean annual average of relative humidity (%)	Temperature (ºC)
The Jordan Valley region	375 BSL	Semi-tropical (desert)	158-200	50-60	19-38
The eastern slopes regions	200 BSL-800 ASL	Semi-arid to arid	200-400	60-65	13-27
The central highlands region	400-1000 ASL	Mediterranean	400-700	60-70	12-26
The semi-coastal region	100-400 ASL	Mediterranean	200-450	60-80	13-25

2.8.1. Abiotic factors

Environmental stresses including temperature fluctuation, ultraviolet radiation (UV), soil texture, soil moisture and aeration can adversely affect EPNs persistence, survival and reducing its efficacy in controlling soil pests (Bauer and Kaya, 2001).

2.8.1.1. Temperature

Temperature is one of the major determining abiotic factors that affect EPNs persistence, temperatures above 30ºC or below 9ºC negatively affect persistence, but the severity of the impact depends on EPNs species (Kaya, 1990), when water is available different nematode species can better tolerate higher temperatures (Bauer and Kaya, 2001).
Studies have shown a correlation between persistence of different nematode species of *Heterorhabditis* and *Steinernema* at different temperatures and their geographic origin; for example tropical isolates persist better at high temperatures than temperate isolates (Kaya, 1990). Nematode survival is affected by the degree of activity and the amount of their food reserves, nematodes can enter an immobile coiled state associated with enhanced survival (Bauer and Kaya, 2001), as an example EPN isolate (IS-5) from under fruit trees in the arid Negev desert is highly tolerant to temperatures above 30ºC (Glazer *et al.,* 1996).

Also a study conducted at the UNESCO center at Bethlehem University has shown that half the population of EPN isolates (*S.abbasi*-08 and *S.abbasi*-09) from under fruit trees in the Gaza strip could survive for 79 and 101 hr at 40°C, respectively (Iraki *et al.*, 2006).

2.8.1.2. Ultraviolet radiation

Nematodes are extremely sensitive to solar ultraviolet radiation (Gaugler *et al*., 1992). UV radiation in the range of 290-400 nm causes damage to DJs. *Heterorhabditis bacteriophora* DJs are especially sensitive and are inactivated by exposure levels that are tolerated by the DJs of *S.carpocapsae* (Bauer and kaya, 2001).

2.8.1.3. Soil texture

Soil texture and pore size affect the persistence of EPNs through affecting their movement, host finding ability and survival. Studies showed that horizontal and vertical nematode dispersal, pathogenecity and survival decrease as the overall proportion of silt and clay increase (Georgis and Manweiler, 1994). In sterile clay or clay loam, *S.glaseri* and *S.carpocapsae* infective juvenile survival declines by 50-60% in the first 21 days and continues declining at 1 to 5% for the following 90 days. Meanwhile survival in sterile sand or sandy loam is 20% higher (Kaya, 1990).

2.8.1.4. Soil moisture

Soil moisture is the other major determining abiotic factor that affects EPNs survival and movement. Dry conditions (desiccation) adversely affect nematode survival and motility (Patel *et al.*, 1997). Nematodes must have a film of water surrounding their bodies to move freely (Glazer, 1996). Very high moisture levels can also inhibit nematode infectivity and reduce survival of EPNs. Desiccation does not equally affect all nematode species, (Molyneux and Bedding, 1984); *S.carpocapsae* is less susceptible than *Heterorhabditis* species (Solomon *et al*., 1999). Rapid desiccation is quickly fatal to many *Heterorhabditis* and *Steinernema* spp. (Bauer and Kaya, 2001). Some free-living stages of animal and plant-parasitic nematodes can survive exposure to dry conditions for many years (Glazer, 1996) through entering an anhydrobiosis state which is usually reached following a slow rate of water loss (Crowe *et al.*, 1979). In one study, survival of *S.carpocapsae* and *S.glaseri* nematodes is highest at low soil moisture level (Kung *et al.*, 1991). As a result DJs have evolved morphological, physiological, and behavioral adaptations to reduce rates of drying (Bauer and Kaya, 2001).

2.8.1.5. Aeration

Since nematodes are aerobic organisms, low oxygen availability can reduce their survival (Glazer, 1996). Persistence of *S.carpocapsae* or *S.glaseri* infective juveniles decline as oxygen levels decline from 20% to 1% (Kung *et al.*, 1991). Also, very wet soils, or soils with a high

content of clay or organic matter are almost always fatal to EPNs; probably because of oxygen depletion. Excess soil moisture negatively affects infectivity of *S.carpocapsae* and *S.glaseri* DJs (Bauer and Kaya, 2001).

2.8.2. Biotic factors

The biotic components of the soil environment adversely affect the persistence of EPNs by: antibiosis, competition, natural enemies, and host susceptibility (Kaya and Koppenhofer, 1996).

2.8.2.1. Antibiosis

It is an association between two species in which one species is actively harmed by the production of toxins by the other harmful species, antibiosis can take place through the release of plant chemicals from plant roots into the soil, and such chemicals may negatively affect the host finding ability and reproduction ability of EPNs (Kaya and Koppenhofer, 1996). As an example of antibiosis; the black walnut tree *Jugfans nigra* secretes toxic chemical substance called juglone that harms soil inhabiting organisms mainly EPNs (Bauer and Kaya, 2001).

2.8.2.2. Competition

Competition will affect the persistence of EPNs in the soil profile by limiting their access to suitable microsites and hosts. Competition may occur either inter-specifically or intra-specifically among DJs for insect hosts and for nutrients within insect hosts, both types of competition reduce nematode potential (Kaya and Koppenhofer, 1996).

In interspecific competition individuals of different species compete for the same resources in an ecosystem (Bauer and Kaya, 2001). Interspecific competition between two *Steinernema* species shows that both can co-exist in a host, but one species will dominate in the environment and cause local extinction of another nematode species (Kaya and Koppenhofer, 1996). *Steinernema* species usually excludes a *Heterorhabditis* species in the infection (Alatorre-rosas and Kaya, 1990); this is due to production of bacteriocins by the bacterial species *Xenorhabdus* of Steinernematids nematodes. Bacteriocins inhibit the growth of *Photorhabdus* bacterial species of *Heterorhabditis* nematodes, allowing *Steinernema* spp. to out compete *Heterorhabditis* spp. (Baur and Fuxa, 2002).

Intraspecific competition occurs when members of the same species compete for the same resources in an ecosystem (Solomon, 2002). In biological control process this kind of competition occurs when host penetrating infective juveniles exceed the optimal level (Selvan et al., 1993).

2.8.2.3. Natural enemies

Natural enemies like many forms of plants, animals, microbes, and fungi are in competition with or preying on nematodes (Kaya and Koppenhofer, 1996). Natural enemies adversely affect EPNs efficacy and decrease their persistence in the soil (Timper and Kaya, 1992). The natural enemy nematophagous fungus, *Hirsutella rhossiliensis,* causes higher mortality in *Steinernema glaseri* nematodes compared to *Heterorhabditis bacteriophora.* The differential susceptibility to the fungus may be associated with the *H.bacteriophora* cuticle which increases strain tolerant to this kind of natural enemy. Invertebrate predators including mites and collembolans are other example on natural enemies feeding on EPNs (Kaya and Koppenhofer, 1996). Studies showed that *S.feltiae* persistence decline with increased numbers of Mesostigma mites *Gamasellodes vermivorax* (Bauer and Kaya, 2001).

2.8.2.4. Host susceptibility

EPNs efficacy is influenced by the host susceptibility, which is determined by the ability of nematodes to penetrate the host insect. The most susceptible hosts are usually those most easily penetrated by nematodes. In addition, susceptibility of different insects to *Xenorhabdus* bacteria depends upon the ability of the host defense response to limit bacterial reproduction (Georgis and Manweiler, 1994). At the UNESCO lab infectivity test was done for Pal-H-01 and Pal-H-02 results showed that LD_{70} after 26 hr exposure to *G. mellonella* was 25 for Pal-H-01, 55 for Pal-H-02 (Iraki *et al*., 2000).

2.9. Physiological response of EPNs during abiotic stress

The survival of DJs in the soil is affected by a number of environmental factors, particularly desiccation and hypoxia stress (O'leary, 1997), surviving desiccation relies on the ability of the organism to control the rate of water loss; a slow rate of water loss is a prerequisite for EPNs survival. Desiccation and hypoxia stress factors can negatively affect EPNs fitness. EPNs vary in their response to these factors, many organisms including some nematodes; can withstand complete desiccation by entering a condition known as anhydrobiosis "life without water" where their metabolism is fully arrested (Patel and Wright, 1997).

The biochemical and metabolic changes which occur in anhydrobiotic nematodes, typically involve an increase in trehalose and glycerol content and a reduction in lipid and glycogen content. The role of trehalose in anhydrobiosis is to reduce bulk and structural water, and to inhibit oxidative damage (Jagdale and Grewal, 2003). Trehalose is also believed to stabilize cell membranes by direct interaction between hydroxyl groups on the trehalose and the phospholipids resulting in the formation of glass-like structures, this depresses the phase transition temperature in dry phospholipids, effectively protecting membranes during desiccation by replacing the water associated with the phospholipid bilayer with trehalose. These changes maintain the fluidity of the membrane, conserve their structural and functional integrity, and prevent cell leakage during rehydration that may result from membrane damage (Womersley and Smith, 1981).

In addition to trehalose, many proteins are induced during stress conditions like Desc47 (Solomon *et al*.,2000), actin, proteasome, regulatory particle (ATP ase), GroEL chaperonin, GroES co- chaperonin (members of the Hsp 60 family of chaperons) and transposase are induced proteins in response to osmotically desiccated DJs(Chen et al., 2005). For example, as mentioned in (Sandouka, 2003) thesis study it is suggested that the improvement in penetration and development of IJs achieved upon preconditioning at 35°C is related to synthesis of the HSPs.

Chapter Three

Materials and methods

3.1. Rearing the nematode's insect host (*Galleria mellonella*)

The adults of the wax moth *Galleria mellonella,* the host insect of EPNs, belongs to the family Pyralidae, order Lepidoptera. To propagate the moth, adult moths were trapped in a glass jar containing nutrient medium: 200g autoclaved honey, 183g glycerol, 47g yeast extract and 5g of Nipagine antibiotic, all the contents were mixed and homogenized by heating at 50ºC- 60ºC, then 320 g of autoclaved oatmeal were added to the homogenate, the medium was dispensed in sterile jars (Chandler *et al*., 1997) and covered with strips of tissue paper. After one week, eggs laid on the strips of tissue paper by the adult trapped moth were collected; the eggs were disinfected by immersing in 10% formaldehyde for 90 minutes followed by washing with running distilled water for 60 minutes. After drying, eggs were covered with nutrient medium which was prepared as above. The inoculated jars were incubated at 30ºC, eggs hatch and develop to the last instar larvae during two weeks of incubation, larvae were collected and used in desiccation and hypoxia experiments.

3.2. In vivo propagation of nematodes

In this study, nine different isolates of EPNs were collected from Gaza Strip and Bethlehem area (Table 3.1) by the laboratory of the UNESCO Biotechnology Center at Bethlehem University. Another six isolates (reference strains) (Table 3.1) were obtained from the laboratories of Dr. Shamseldeen, Egypt (*H.tayseerae* and *S.abbasi*) and Dr. Ralf-Udo Ehler, Germany (LN2, SF, HB and Russ.). All strains were maintained by rearing on larvae of *Galleria mellonella* at 25ºC. DJs were collected by white trap Figure (3.1) (Woodring and Kaya, 1988). Identification and characterization of some biological traits of the nine local strains were performed as described in chapter 2 section 5.

Table 3.1: Identity of EPNs isolates, local isolates and imported isolates (shaded rows)

Serial Number	Identity Genus/species	Population	Code Number	Year of isolation	Original locality
1	*Heterorhabditis indica*	B11	Pal-H-01	1997	Battir/Bethlehem
2	*H.indica*	B22	Pal-H-02	1997	Artas/Bethlehem
3	*Steinernema. feltiae*	B33	Pal-S-03	1998	Wad Fukin/ Bethlehem
4	*S.palestini*	G11	Pal-S-10	1998	Al Shati'/Gaza
5	*H.bacteriophora*	G22	Pal-H-05	1998	Al Shati'/Gaza
6	*S.abbasi*	Fig1	Pal-S-09	1999	Bet Lahia/Gaza
7	*H.tayseerae*	S.orange	Pal-H-07	1999	Bet Hanoon/Gaza
8	*H.tayseerae*	GF1A	Pal-H-06	1999	Bet Hanoon/Gaza
9	*S.abbasi*	GF1B	Pal-S-08	1999	Bet Hanoon/Gaza
10	*H.indica*	LN2	reference	NA	Europe
11	*S. feltiae*	SF	reference	NA	Europe
12	*H.tayseerae*	H.tayseerae	reference	NA	Egypt
13	*S.abbasi*	S.abbasi	reference	NA	Oman
14	*H.bacteriophora*	HB	reference	NA	Europe

15	*S.arenarium*	Russ.	reference	NA	Russia

Fig. 3.1: White trap used in the propagation of EPNs

3.3. Determination of tolerance of EPNs to desiccation

Tolerance of EPNs populations to desiccation was evaluated by dehydrating the DJs in glycerol solution as described by Glazer and Salame 2000. Five thousand DJs in 0.5ml deionized water were mixed with 0.5ml of 50% glycerol solution to obtain a final glycerol concentration of 25%. The nematodes mixed in glycerol are placed in 24 multi-well plates, six wells per nematode strain. Meanwhile, additional six wells containing nematodes in water at the same nematode concentration were used as a control. Each nematode population was incubated at 25ºC for 72 hour. Nematodes were sampled every 24 hr, rehydrated in 9ml of water at 25ºC. Nematode survival was determined microscopically every 24 hr intervals post-rehydration by prodding each juvenile with a metallic needle. The inactive nematodes were considered dead if they did not respond to prodding. The experiment was independently repeated two times.

3.4. Determination of tolerance of EPNs to hypoxia

Tolerance of EPNs populations to hypoxia was evaluated by storing the DJs in 0.3ml of water in eppendorf tubes as described by (Grewal and Taylor, 2002). Six thousand DJs were held in each tube with the lids tightly closed. Three tubes were prepared for each population; incubation was done at 25ºC for 72 hours. Samples were collected every 24 hr and the nematodes were transferred into 5 cm diameter petridishes containing 5.5 ml of water and incubated at 25ºC for 24 hr to recover from hypoxia before prodding. Three 5 cm diameter dishes containing nematodes at the same concentration were used as control and were held without hypoxia at 25ºC. Nematode survival was determined after the nematodes were allowed to recover from hypoxia, using light microscope and prodding each DJ with a metallic needle. The inactive nematodes were considered dead if they did not respond to prodding. The experiment was independently repeated two times.

3.5. Statistical analysis

The obtained data of the two independent experiments conducted in triplets. The data for the tolerance of fifteen *Heterorhabditis* and *Steinernema* spp. to desiccation or hypoxia were pooled and tested for normality using Shapiro – Wilk test. General Linear Model (GLM) procedure

wereare used to test the interactions between variables. Tukey's studentized range test at $P \leq 0.05$ was employed to determine statistical differences among and within nematode strains. Correlation between both treatments (desiccation and hypoxia) were calculated using Microsoft Ooffice Eexcel software (Microsoft office excel, 2006)

Chapter Four

Results and discussion

4.1. Determination of tolerance of EPNs to desiccation:

4.1.1. Determination of tolerance of ~~eight~~ *Heterorhabditis* spp. to desiccation:

Desiccation was separately imposed on DJs ~~The affect of desiccation imposed on DJs stage~~ of eight ~~strains of~~ *Heterorhabditis* strains~~isolates~~ Table (3.1) by incubation in 25 % glycerol. Data was pooled from two independent experiments conducted in triplicates (Appendix 1). Percent survival was determined microscopically and pooled data is shown in Figure (4.1 A). Percent survival was scored longitudinally at three time intervals; 24, 48 and 72 hr. A~~a~~fter 24 hr of desiccation, survival of the eight strains ranged between ~~ranged between~~ 30-91 ~~among the eight DJs strains~~. No significant difference was observed within species except for ~~With one exception;~~ *H.indica* which differed ~~differed~~ from the other two locally isolated *H.indica* strains Figure (4.1A). ~~, for the remaining strains, no significant intraspecies difference was observed after 24 hr of desiccation.~~ However, the survival pattern~~is picture~~ changed when desiccation was scored after 48 and 72 hr. Significant differences in the survival pattern of DJs were observed as shown in Figure (4.1B and C). ~~survival was scored after 48 and 72 hr. Significant differences in survival pattern were observed as shown in Figure (3.1B and C).~~ The DJs of the locally isolated strain *H.bacteriophora-05* ~~DJs~~, showed the highest level of survival and tolerance to desiccation (53% after 72 hr incubation in 25% glycerol), in contrast to its weak tolerance to heat (LT_{50} was 3 hr at 40°C (Iraki *et al*., 2006). On the other extreme, *H.tayseerae* including the local isolate *H.tayseerae-07* showed the lowest level of survival (11 and 16% respectively after 72 hr incubation), in comparison to heat tolerance, *H.tayseerae* strains DJs survived better than desiccation and maintained LT_{50} of 6 hr at 40°C for *H.tayseerae*-07 (Iraki *et al*., 2006). Desiccation ~~The~~ ~~s~~survival rate~~al of the remained isolates~~ ranged from 26-85 % after 48 hr and 16-53% after 72 hr. The control groups which were not exposed to desiccation, maintained 100% survival rate at all three time periods for all ~~(no desiccation)~~ ~~of the~~ eight *Heterorhabditis* isolates ~~, maintained 100% survival at all three time periods~~ (not shown).

4.1.2. Determination of tolerance of ~~seven~~ *Steinernema* spp. to desiccation:

Similar data was~~ere~~ obtained for the effect of desiccation imposed by incubation in 25% glycerol on DJs of seven strains of *Steinernema* spp. Table (3.1). Data was pooled from two independent experiments conducted in triplicates (Appendix 1). The results ~~DJs of seven strains of *Steinernema* spp. listed in Table (3.1)~~ showed little variation in survival pattern between isolates scored after 24 or 48 hr, where survival rate ranged from 57-98% Figure (4.2 A and B). However, survival rate scored after 72 hr of desiccation ranged~~varied~~ between 28-93% among the seven tested *Steinernema* strains. One locally isolated strain, *S.abbasi*-09, showed the highest (93%) survival average even after 72 hr of desiccation. Other *S.abbasi* isolates were tolerant to desiccation as well ranking just below *S.abbasi*-09 but higher than the remaining isolates. The results suggest that *S.abbasi* especially *S.abbasi*-09 to be the most tolerant to desiccation over the test period Figure (4.2C). Superior tolerance to heat stress was also obviously pronounced for *S.abbasi*, *S.abbasi*-08 and *S.abbasi*-09 with LT_{50} of 101, 79 and 83 hr at 40°C, respectively (Iraki *et al*., 2006). ~~survival pattern showed no significant difference with *S.abbasi* and *S.feltiae* strain the results are illustrated in Figure (4.2 C).~~ The control~~s~~ group~~s~~ (no desiccation) of the seven *Steinernema* isolates maintained 100% survival rate at all three time periods (not shown).

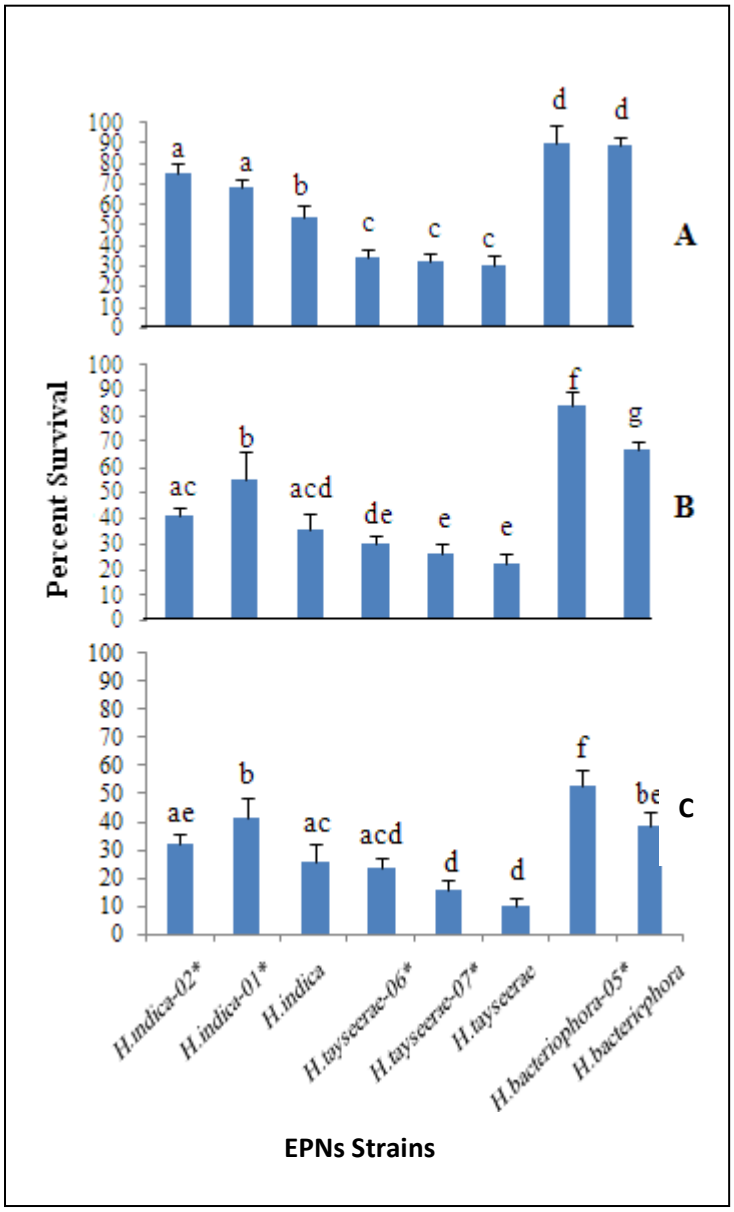

Fig. 4.1: Mean survival rate of DJs of *Heterorhabditis* ~~spp~~strains~~.~~ after A: 24 hr, B: 48 hr and C: 72 hr of desiccation in 25 % glycerol. Bars with the same letter(s) do not differ significantly at P ≤ 0.05, the figure illustrates the means of six experiments, standard deviation is indicated. Local isolates are identified *~~. (*) indicates local isolates~~.

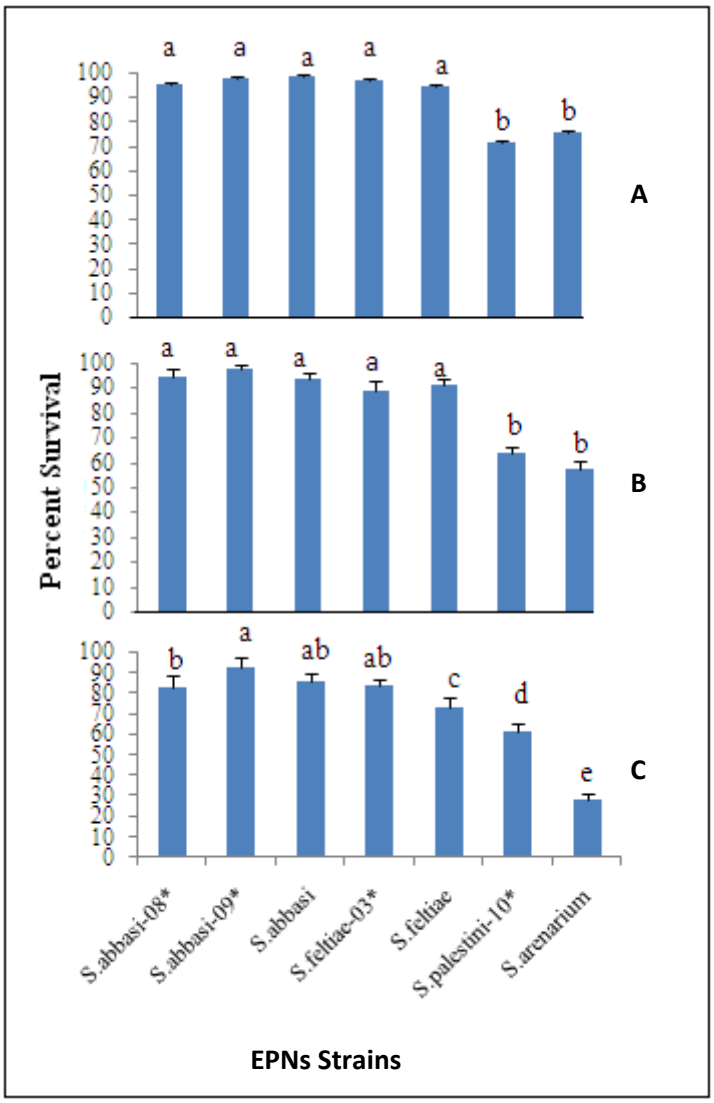

Fig. 4.2: Mean survival rate of DJs of *Steinernema* strains~~spp.~~ after A: 24 hr, B: 48 hr and C: 72 hr of desiccation in 25 % glycerol. Bars with the same letter(s) do not differ significantly at P ≤ 0.05, the figure illustrates the means of six experiments, standard deviation is indicated. Local isolates are identified *. ~~(*) indicates local isolates.~~

4.1.3. Effect of desiccation on ~~Comparison on the effect of desiccation on~~ *Heterorhabditis* in relation to ~~and~~ *Steinernema*:~~spp.~~

Tolerance to desiccation within genera, species and among all tested strains is summarized in Figure (4.3). The results indicate that DJs of *Steinernema* spp. are generally more tolerant than *Heterorhabditis* spp. (except for the Russian strain, *S.arenarium*~~ a Russian strain~~). The most tolerant strain was the local isolate *S.abbasi*-09 followed by ~~an~~other *S.abbasi* (imported). DJs of the eight *Heterorhabditis* spp. may~~can~~ be viewed to have poor to moderate levels ~~tolerance~~of tolerance to desiccation. Based on the presented data, it appears that *Steinernema* spp. especially *Steinernema abbasi*-09 is more likely to survive in fields with low humidity level than would~~will~~ most *Heterorhabditis* spp. isolates, ~~especially at elevated temperatures (> 25ºC)~~. The observed high sensitivity, or poor tolerance, to desiccation ~~observed~~ in *H.tayseerae* isolate, may be tied to its ~~the result of~~ adaptation to its original habitat; moist soils of Egypt ~~(Egyptian strain) conditions of its native habitat~~ (Shapiro-Ilan *et al.*, 2005). Similarly, the superior ability of *S.abbasi-09* (Gaza strain) to withstand desiccation could be due to selection pressure brought about by its native warm and dry environment. This view is further supported by the second ranking tolerant strain *S.abbasi* which was isolated in Oman. Nematode cuticle plays an important role in controlling water loss as described by Wright and Newall 1980, another factor

is the accumulation. ~~Metabolic changes such as induction of high concentrations~~ of both trehalose and glycerol which serves as a general stress protectant (Jagdale and Grewal, 2003). There are substantial biological differences between *Heterorhabditis* and ~~*in*~~*Steinernema*; while induction of high concentrations of trehalose and glycerol take place in *Steinernema* spp. in response to desiccation stress or cryptobiosis (Charwat *et al.*, 2002). Accumulation of trehalose and glycerol is not observed in *Heterorhabditis* spp. this difference in biological response to desiccation between the two genera, offers an attractive explanation to differences in survival patterns of the two genera (O'leary and Burnell, 1997).

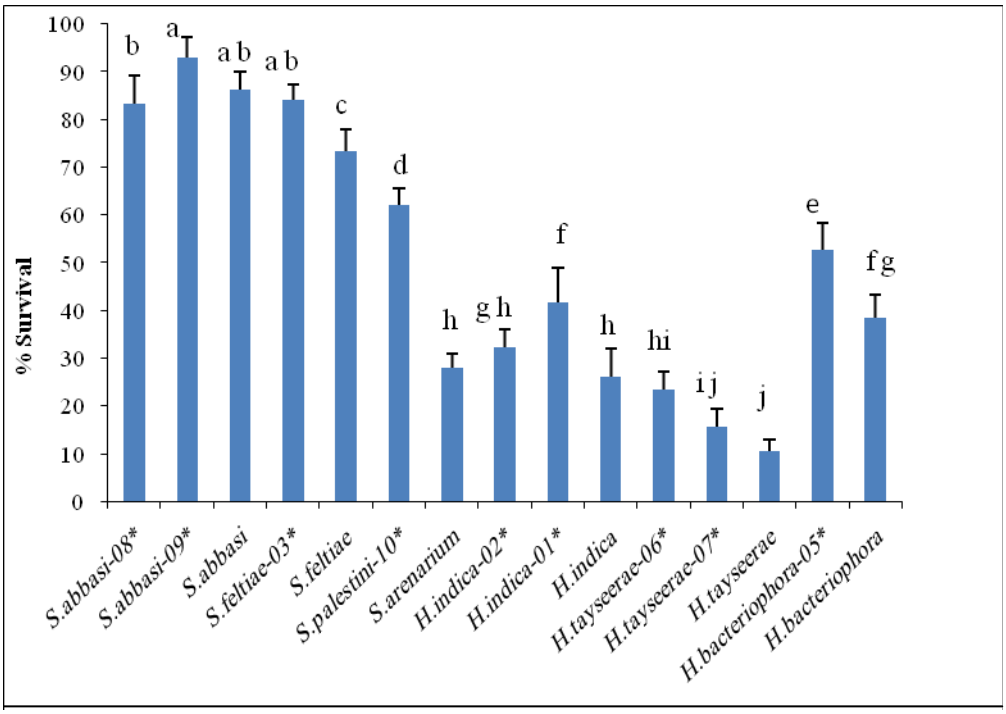

Fig. 4.3: Mean survival rate of DJs of both *Heterorhabditis* and *Steinernema* strains after 72 hr of desiccation in 25 % glycerol. Bars with the same letter(s) do not differ significantly at $P \leq 0.05$, the figure illustrates the means of six experiments, standard deviation is indicated. Local isolates are identified *

4.2. Determination of tolerance of EPNs to hypoxia:

4.2.1. Determination of tolerance of *Heterorhabtidis* spp. to hypoxia:

The effect of another environmental factor known as hypoxia (reduced available oxygen) was tested on DJs stage of EPNs. Determination of the survival rate, therefore tolerance, of eight *Heterorhabditis* strains to hypoxic conditions was carried in a similar longitudinal study as in desiccation experiments, i.e. after 24, 48 and 72 hr of incubation in a closed system. Data was pooled from two independent experiments conducted in triplicates (Appendix 2). The pooled results expressed as survival rate are presented in Figure (4.4). The results Figure (4.4A) showed no variation among all isolates during the first 24 hr, this result is to be expected since oxygen depletion does not occur instantaneously and the effect of hypoxia will not be observed during the first period. No significant decrease in survival rate of all strains which maintained a survival level above 96%. After 48 hr of incubation, a decrease in survival rate of some strains was more pronounced than others, it varied between 73 and 96% Figure (4.4B). The lowest observed survival level was noticed with DJs of the local isolate *H.tayseerae*-06 (73%) while the DJs of another local isolate, *H.bacteriophora*-05, showed the highest survival level (96%) with low to moderate tolerance to heat stress (LT_{50} of 3 hr at 40°C) (Iraki *et al.*, 2006). After 72 hr of incubation, survival ranged from 61 to 84%, Figure (4.4C). Although, the percent survival rate was shifted down at 72 hr relative to that at 48 hr, while the survival profile did not change. On

the other hand the *H.tayseerae*-06 isolate and *H.bacteriophora*-05 maintained their lowest and highest ranks respectively. The controls of the eight *Heterorhabditis* isolates maintained 100% survival rate during the three time points over the period of experiment.

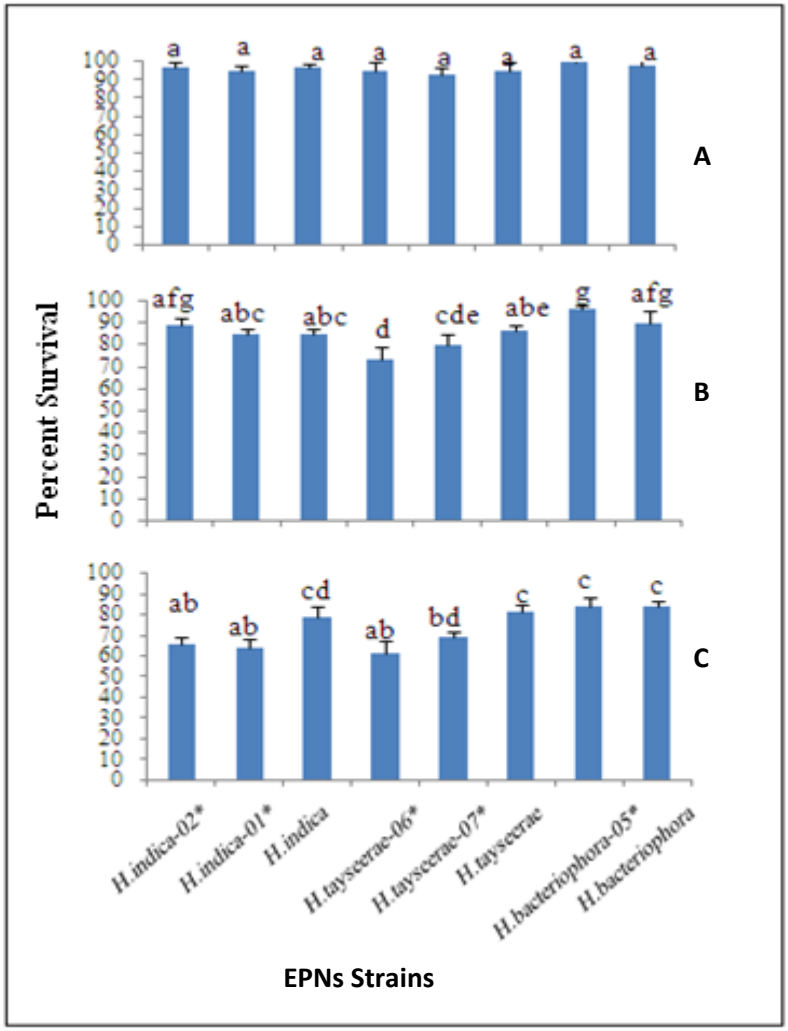

Fig. 4.4: Mean survival rate of DJs of *Heterorhabditis* strains after A: 24 hr, B: 48 hr and C: 72 hr of hypoxia. Bars with the same letter(s) do not differ significantly at $P \leq 0.05$, the figure illustrates the means of six experiments, standard deviation is indicated. Local isolates are identified *

4.2.2. Determination of tolerance of *Steinernema* spp. to hypoxia

The seven *Steinernema* isolates Table (3.1) that were subjected to desiccation, were also tested for their tolerance to hypoxia. Strains were individually placed in sealed tubes, to prevent replenishing of oxygen. Data was pooled from two independent experiments conducted in triplicates (Appendix 2). Survival scored after 24 hr showed *S.abbasi* isolates to maintain viability near 100%, Figure (4.5A). Other isolates showed significant decrease in survival rates; the lowest was observed for *S.arenarium* 81% and 83% for *S.palestini*-10. The hypoxia survival rate profiles after 48 and 72 hr exposure showed minor differences among strains. A significant drop in the survival rates of the local strains *S.abbasi*-08 and *S.abbasi*-09 relative to the reference *S.abbasi* strain isolated in Oman was observed Figure (4.5B and C). The optimal and minimal survival rate for 48 hr time point were different from those obtained after 72 hr of incubation. *S.abbasi* has scored the highest survival rate (94%) after 48 hr and (86%) after 72 hr of incubation. On the survival scale, *S.arenarium* continued to occupy the lowest rank, its survival average was 73% after 48 hr incubation then dropped to 62% at the end of incubation after 72 hr

of hypoxic treatment. The control DJs of the seven *Steinernema* isolates maintained 100% survival rate at the three time period (not shown).

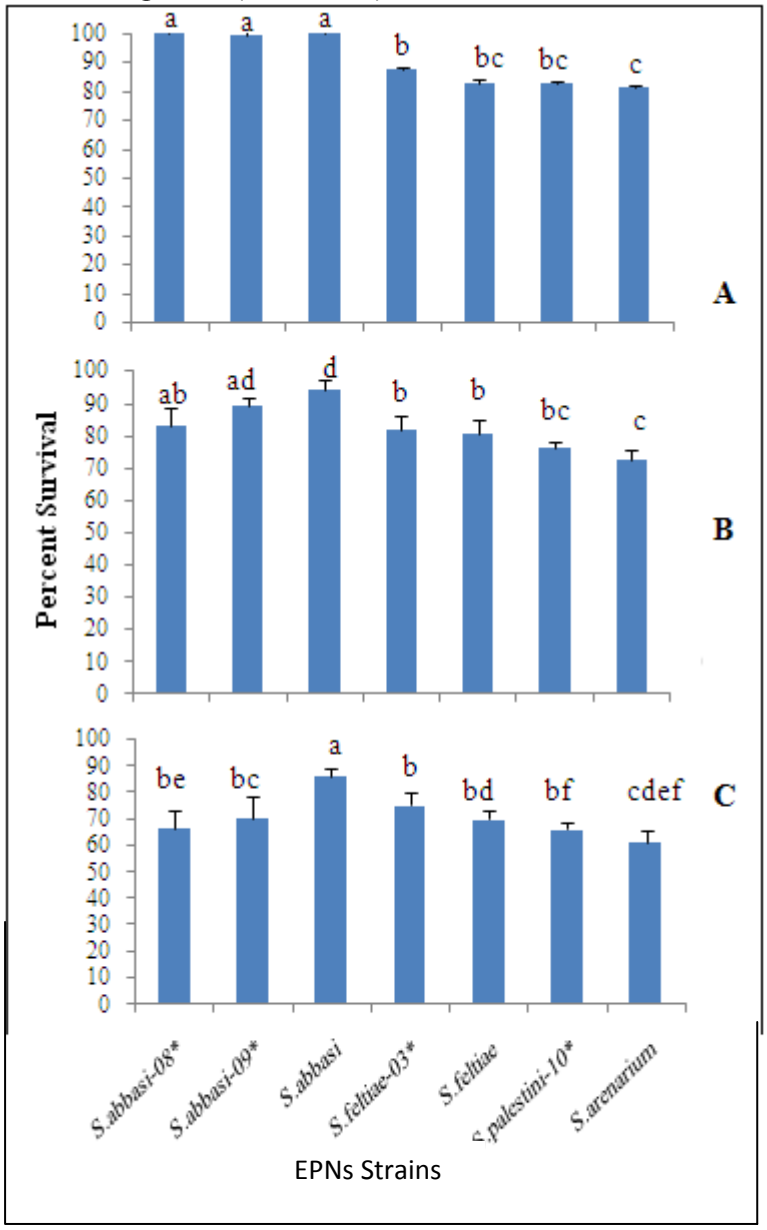

Fig. 4.5: Mean survival rate of DJs of *Steinernema* strains after A: 24 hr, B: 48 hr and C: 72 hr of hypoxia. Bars with the same letter(s) do not differ significantly at $P \leq 0.05$, the figure illustrates the means of six experiments, standard deviation is indicated. Local isolates are identified *

4.2.3. Effect of hypoxia on ~~Comparison on the effect of desiccation on~~ *Heterorhabditis* in relation to ~~and~~ *Steinernema*:~~spp.~~

In order to observe and compare the DJs ability of the two genera, *Heterorhabditis* and *Steinernema*, to tolerate hypoxia, survival data obtained after 72 hr of exposure to hypoxic conditions were pooled, statistically analyzed, and summarized Figure (4.6). First observation, is that the range of survival rates under hypoxic conditions is narrow, falling between 61% and 86%, unlike that of desiccation which spreads over the survival range of 11-93%. Second observation is that the profile of survival under hypoxia is different from that of desiccation or even heat tolerance (Iraki *et al*., 2006); tolerance to hypoxia did not produce any discernable pattern that is similar, opposite or related to that observed with desiccation. In other words, no dividing border line can be drawn between the two genera in reference to their tolerance and susceptibility to hypoxia. The mechanisms and means by which nematodes can withstand hypoxia are poorly understood and are still under investigation. Trehalose and glycerol may be

suspected to play a role in the process. However, this view is weakened by the inability of *Heterorhabditis* spp. to accumulate trehalose and glycerol leaving the observed high level of tolerance to hypoxia seen in *Heterorhabditis bacteriophora*-05 with no explanation. Another hypothesis to be investigated is that nematode may enter into a quiescent stage where low level of metabolism reduces oxygen consumption. On the other hand, nematodes may have oxygen storage or regeneration capability to overcome the low oxygen concentration. Myoglobin found in the muscle cells of diving birds and mammals is an established fact on oxygen storage protein (Meyer, 2004).

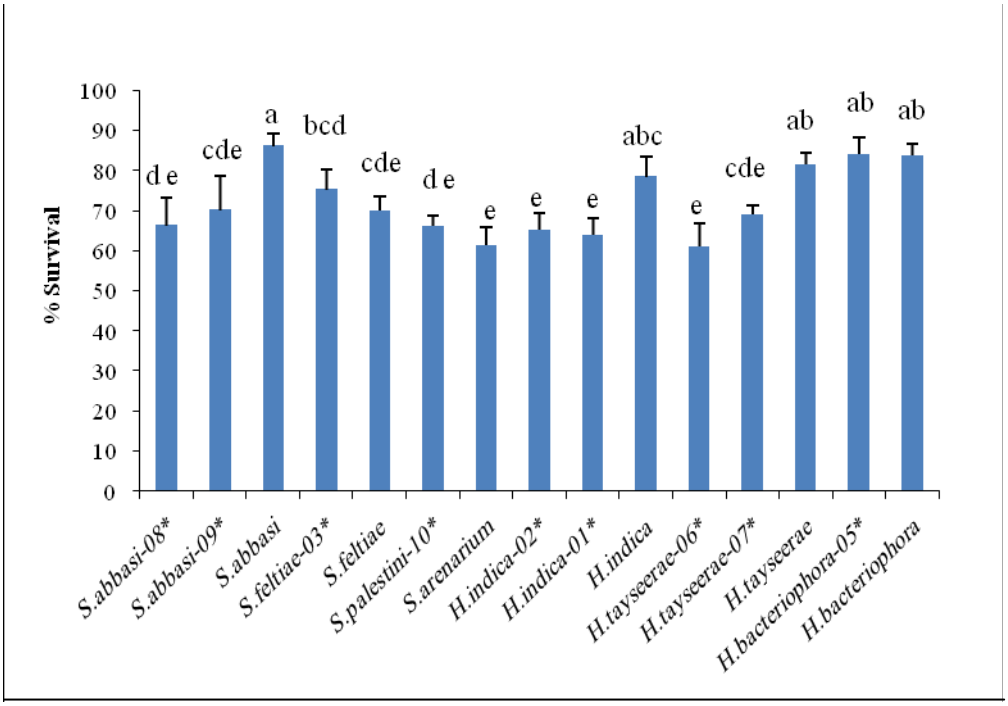

Fig. 4.6: Mean survival rate of DJs of both *Heterorhabditis* and *Steinernema* strains after 72 hr of hypoxia. Bars with the same letter(s) do not differ significantly at $P \leq 0.05$, the figure illustrates the means of six experiments, standard deviation is indicated. Local isolates are identified *

4.3. Correlation analysis between desiccation and hypoxia:

Correlation analysis using Microsoft Office Excel software, correlation between desiccation and hypoxia was examined ($r = 0.12$) which indicates a poor correlation between the two variable. In other words, survival mechanisms of DJs to desiccation is different than that of hypoxia and each one has its own survival mechanism.

Chapter Five

Conclusions and recommendations

5.1. Conclusion

- The observed variation in hypoxia and desiccation tolerance among the tested isolates is consistent with the assumptions made in the statement of the problem and the objectives of this study.

- *Steinernema* isolates especially *Steinernema abbasi*-09 were found to have a relatively superior ability to tolerate high level of desiccation and low soil oxygen concentration.

- *H.bacteriophora*-05 isolate showed the highest level of desiccation and hypoxia tolerance compared to the eight *Heterorhabditis* spp.

- *H.tayeseerae* and *H.tayseerae*-06 isolates showed the lowest levels of tolerance compared to desiccation and hypoxia, respectively.

- No significant variation was observed in relation to tolerance to hypoxia among all stains.

- Extreme desiccation conditions (72 hr incubation in 25% glycerol) caused a significant decrease in survival rate among all strains.

- It is believed that the mechanism of desiccation tolerance in EPNs is different than that of hypoxia tolerance.

5.2. Recommendations

- *Steinernema abbasi* isolates should be the focus of field experiments addressed to a possible successful implementation of biological control in semi-arid to arid regions.

5.3. Future studies

- Field experiments should be addressed to determine a possible interaction between temperature, hypoxia and humidity and its effect on efficacy of EPNs as bio-control agents.

- Future studies must concentrate on the survival mechanisms of EPNs in hypoxia conditions.

- Hybridization through cross breeding to yield a superior EPNs hybrid with superior traits.

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المراجع العربية:

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Appendices

Appendix 1: Desiccation treatment raw data:

Time	24hour		24hour		48hour		48hour		72hour		72hour	
Strain	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
S.abbasi												
S.abbasi-08	91	9	99	1	90	10	98	2	81	19	88	12
	93	7	98	2	92	8	98	2	79	21	89	11
	93	7	98	2	93	7	95	5	74	26	88	12
S.abbasi-09	98	2	96	4	96	4	99	1	89	11	97	3
	98	2	98	2	95	5	99	1	91	9	95	5
	99	1	98	2	96	4	99	1	87	13	98	2
S.abbasi	100	0	98	2	95	5	90	10	86	14	88	12
	100	0	95	5	97	3	95	5	81	19	83	17
	100	0	97	3	93	7	92	8	90	10	90	10
H.indica												
H.indica-02	81	19	80	20	45	55	43	57	34	66	30	70
	77	23	75	25	40	60	36	64	37	63	35	65
	71	29	70	30	42	58	40	60	31	69	27	73
H.indica-01	65	35	72	28	46	54	65	35	39	61	53	47
	66	34	72	28	44	56	64	36	35	65	45	55
	62	38	73	27	45	55	66	34	34	66	44	56
H.indica	50	50	61	39	30	70	32	68	30	70	19	81
	46	54	57	43	40	60	34	66	30	70	18	82
	56	44	54	46	46	54	32	68	31	69	28	72
S.feltiae												
S.feltiae-03	98	2	95	5	90	10	90	10	86	14	82	18
	97	3	97	3	95	15	82	18	87	13	88	12
	98	2	98	2	89	11	85	15	82	18	80	20

Time	24hour		24hour		48hour		48hour		72hour		72hour	
Strain	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
S.feltiae	98	2	90	10	93	7	89	11	76	24	80	20
	98	2	93	7	94	6	90	10	70	30	75	25
	98	2	89	11	93	7	88	12	67	33	72	28
S.palestini												
S.palestini-10	74	26	70	30	65	35	67	33	62	38	57	43
	67	33	75	25	63	37	60	40	60	40	61	39
	78	22	65	35	66	34	62	38	65	35	67	33
S.arenarium	79	21	80	20	55	45	60	40	27	73	25	75
	76	24	75	25	53	47	55	45	25	75	30	70
	73	27	72	28	58	42	62	38	29	71	32	68
H.tayseerae												
H.tayseerae-06	30	70	35	65	33	67	29	71	25	75	22	78
	28	72	38	62	28	72	30	70	29	71	25	75
	33	67	40	60	35	65	28	72	21	79	19	81
H.tayseerae-07	27	73	35	65	25	75	20	80	18	82	10	90
	35	65	28	72	28	72	25	75	20	80	13	87
	38	62	30	70	32	68	28	72	15	85	18	82
H.tayseera	22	78	30	70	22	78	25	75	11	89	15	85
	33	67	33	67	18	82	28	72	9	91	10	90
	28	72	35	65	19	81	20	80	9	91	9	91
H.bacter												
H.bacter-05	88	12	100	0	82	18	93	7	52	48	56	44
	85	15	96	4	85	15	86	14	55	45	49	51
	79	21	95	5	78	22	83	17	60	40	44	56
H.bacterio	95	5	86	14	70	30	64	36	45	55	39	61
	87	13	92	8	65	35	67	33	39	61	33	67
	90	10	87	13	67	33	71	29	42	58	33	67

Appendix 2: Hypoxia treatment raw data

Time	24hour		24hour		48hour		48hour		72hour		72hour	
Strain	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
S.abbasi												
S.abbasi-08	100	0	100	0	84	16	88	12	60	40	73	27
	100	0	99	1	80	20	84	16	63	37	69	31
	100	0	100	0	75	25	89	11	58	42	75	25
S.abbasi-09	100	0	100	0	92	8	92	8	63	37	75	25
	99	1	100	0	86	14	90	10	64	36	78	22
	99	1	100	0	88	12	88	12	61	39	80	20
S.abbasi	100	0	100	0	93	7	90	10	88	12	89	11
	100	0	100	0	97	3	95	5	84	16	82	18
	100	0	100	0	98	2	93	7	90	10	84	16
H.indica												
H.indica-02	100	0	95	5	92	8	88	12	65	35	60	40
	98	2	93	7	85	15	86	14	70	30	63	37
	99	1	97	3	92	8	90	10	64	36	70	30
H.indica-01	98	2	95	5	83	17	85	15	58	42	67	33
	97	3	93	7	86	14	88	12	69	31	66	34
	96	4	92	8	83	17	82	18	60	40	63	37
H.indica	98	2	95	5	86	14	83	17	76	24	75	25
	100	0	93	7	86	14	87	13	82	18	72	28
	97	3	97	3	84	16	81	19	86	14	80	20
S.feltiae												
S.feltiae-03	88	12	90	10	80	20	85	15	70	30	79	21
	85	15	87	13	77	23	82	18	68	32	80	20
	87	13	88	12	79	21	88	12	78	22	77	23

Time	24hour		24hour		48hour		48hour		72hour		72hour	
Strain	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
S.feltiae	82	18	85	15	78	22	79	21	65	35	68	32
	85	15	80	20	80	20	83	17	73	27	75	25
	79	21	87	13	75	25	88	12	69	31	70	30
S.palestini												
S.palestini-10	85	15	80	20	78	22	76	24	68	32	70	30
	82	18	85	15	77	23	74	26	64	36	65	35
	81	19	83	17	74	26	78	22	67	33	63	37
S.arenarium	80	20	79	21	75	25	70	30	64	36	65	35
	85	15	80	20	77	23	69	31	59	41	55	45
	82	18	80	20	72	28	73	27	67	33	59	41
H.tayseerae												
H.tayseerae-06	98	2	95	5	77	23	75	25	62	38	69	31
	98	2	90	10	73	27	72	28	60	40	65	35
	100	0	89	11	64	36	79	21	52	48	59	41
H.tayseerae-07	90	10	89	11	80	20	83	17	69	31	69	31
	98	2	95	5	79	21	87	13	70	30	65	35
	96	4	90	10	77	23	73	27	72	28	70	30
H.tayseera	99	1	91	9	84	16	86	14	80	20	85	15
	99	1	93	7	90	10	84	16	83	17	84	16
	100	0	88	12	88	12	87	13	78	22	79	21
H.bacter												
H.bacterio-05	100	0	98	2	97	3	95	5	80	20	88	12
	100	0	99	1	98	2	94	6	79	21	85	15
	100	0	99	1	95	5	97	3	82	18	90	10
H.bacterio	100	0	95	5	95	5	88	12	85	15	79	21
	100	0	97	3	97	3	85	15	82	18	83	17
	100	0	92	8	90	10	84	16	88	12	85	15