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Interaction between the biocontrol agents; Entomopathogenic nematodes, *Serratia marcescens*, and *Beauveria bassiana* isolated from Palestine

By

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TO
MY FATHER AND MOTHER FOR THEIR
CONTINUOUS SUPPORT,
TO MY BROTHERS AND SISTERS, AND TO
MY DEAREST FRIEND WITH MY LOVE
AND RESPECT

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Entomopathogenic Nematodes	EPNs
Entomopathogenic Bacteria	EPB
Entomopathogenic Fungi	EPF
Infective Juveniles	IJs
Juveniles 4	J4
16 Subunit Ribosomal RNA	16SRNA
N-Bromothymole Triphenyl tetrazoum chloride Agar	NBTA
Potato Dextrose Agar	PDA
Yeast Salts	YS
Paired t-Test	t-Test
AnalySIS Profile Index	API

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Abstract

Biological control agents, such as nematodes, bacteria, and fungi have been successfully used as alternatives of chemical pesticides. Their use involves in some cases a combination of two or more agents. The combined agents may interact with one another during their application either outside or inside the target insect leading to either increased or decreased efficiency of the biological control process. The nature of such interactions and their effect on the efficacy of each biocontrol agent has not been given an adequate attention yet. In this work, we isolated, identified, and characterized a new isolate of entomopathogenic bacterium *S. marcescens*. We studied the effect of the new isolate and another entomopathogenic fungal isolate *Beauveria bassiana* on the invasion and recovery of the insect-pathogenic nematode *Heterorhabditis indica*, strain Bethlehem 11. Both of the fungal isolates and nematode strain were isolated at the UNESCO Biotechnology Center at Bethlehem University. The insect used in this study was last instar larvae of the wax moth *Galleria mellonella*.

Experiments aiming at studying in-vitro interactions showed that the survival of the nematode Infective Juveniles (IJs) stage is not affected by incubation in *S. marcescens* or *B. bassiana* cultures for 48 hours at continuous shaking. However, the survival of the Juvenile 4 stage (J4) was substantially reduce by both pathogens. Infection of *G. mellonella* larvae for 24 hours with *S. marcescens*, or *B. bassiana before* nematode infection slightly decreased insect mortality, and dramatically reduced the invasion of nematode IJs into the insect. That is; when the insect larvae were infected with nematode IJs alone (control), the penetration reached

7.37%. However, when the *Galleria* larvae were preinfected with *S. marcescens* or *B. bassiana* for 24 hours the penetration was restricted to 1.16% and 1.72% respectively.

Similar effect of preinfecting the insect was obtained on the recovery of nematode infective Juveniles into adults. The recovery of IJs in larvae preinfected with either *S. marcescens* or *B. bassiana* was 0.61% and 1.2% respectively, compared to 7.0% recovery in the control. Also, the total production of nematodes was suppressed by preinfection for 24 hours with *S. marcescens* or *B. bassiana.* While preinfection with the first pathogen totally hindered nematode production, preinfection with the second pathogen restricted nematode production to 2.1×10^3 individuals/larvae, compared to $106X10^3$ individuals/ larvae in control. Moreover, preinfecting larvae with the fungal pathogen for periods of 48, 72, and 96 hours totally inhibited nematode production. These findings should be taken in consideration when we evaluate the efficacy and sustainability of EPNs as biocontrol agent when applied simultaneously with *S. marcescens* or *B. bassiana* or in fields previously treated with these agents.

Chapter I
General Introduction

Chapter I

General Introduction

1.1 Biological control as an alternative to synthetic chemical pesticides.

1.1.a Synthetic chemical pesticides.

During the last few decades it became evident that biological control could be one of the alternatives for replacing the use of hazardous chemical pesticides. Synthetic chemical pesticides are those chemicals that are manufactured and used for controlling pests in agriculture and human environment. A large number of synthetic chemical pesticides of different chemical composition were developed and used during the last century. These different chemicals can be grouped into six major groups; chloronated hydrocarbons (e.g. DDT), organophosphates (e.g. Parathion), carbamates (e.g. Zectran), thiocyanates (e.g. Lethane), dinitrophenols (e.g. Dinex), and fluoroacetates (e.g. Nissol). Large part of these pesticides inhibits the enzyme choline estarase in the insect's nerve system (Matsumura, 1985). These Chemicals have been successfully used since long time ago to control a wide range of pests in agriculture, and they contributed to the substantial increase in agricultural productivity during the last century.

However, only in the last decades of the twentieth Century, when sophisticated equipment for detection of chemicals became available, together with the intensive research on their toxicology, it became clear that there are many risks in their use. First, many pesticides are not readily degradable and constitute a major source of environmental contamination including contamination of underground water (Dempster, 1987). Second, chemical pesticides are not effective for long periods due to development of resistance in the target insect. This resistance will be transmitted to subsequent generations and generate a new resistant pest population. Indeed, the number of insects that developed resistance to pesticides has increased dramatically since the 1940s (Brent, 1987). Consequently, there is a continuous need for developing new pesticides to replace the ones against which resistance has developed. Third, chemical pesticides may kill the pest's natural enemies, which are usually more sensitive to the pesticide than the pest itself (Croft, 1990). Certainly, such a consequence may lead to impairing the biological balance and to the appearance of new pests. Fourth, pesticides may harm the human health in different ways. For example, chemical pesticides residues in crops and their continuous accumulation in soil, water, etc, have caused human poisoning (Dempster, 1987; Newton, 1988; Metcalf, 1980).

The accumulation of public awareness regarding the risks of continued use of chemical pesticides has led scientists to seek for alternative methods to control pest populations. Biological control, which involves the use of pest natural enemies, might be one of the potential substitutes of chemical pesticides.

1.1b Biological control

Biological control is defined as the use of natural enemies to suppress the pest population to a level below the harmful one. Naturally, under certain circumstances the size of one pest population may increase much above its normal level. Due to the existence of natural enemies this will lead to an eventual increase of the natural enemy population, which in turn will act to suppress the first population and bring it back to the original level. Hence, the process of biological control is at the population level, where the population of one species is lowered by the population of another one (Driesche and Bellows, 1996). Biological control could be implemented in three ways. First, the classical way, which is the importation of natural enemies from an area where the target pest originated to the area where it should be controlled. Second, the conservation of naturally existing enemies, which includes avoiding the factors that can negatively affect them together with providing them with the needed resources that help them to proliferate. Third, biological

control could be implemented by augmentation. There are two types of augmentation. The first type involves release of small amounts of a natural enemy at early stages of the crop cycle, where they can reproduce and their offspring will also contribute to the control of the pest. This kind of augmentation is known as inoculative release (Driesche and Bellows, 1996; King *et al.*, 1985). The second type of augmentation, known as mass release, involves release of large amounts of the natural enemy for a direct control of the pest population (Driesche and Bellows, 1996). Regardless of the way in which biocontrol is implemented, interaction between pest populations occur in one of the following types; competition, parasitism, predation, or pathogenecity.

Competition:-

Two or more species may compete with one another for shared resources such as food, oxygen, and space. The result of such an interaction may lead to the elimination of one of the competing species. This type of competition is called competitive exclusion. For example, Akhurst (1982) showed that the symbiotic bacteria of a nematode species inhibited the bacterial symbionts of another nematode species by producing antibiotics. Also, a species may be completely replaced from a certain area by other similar species. This kind of competition is called the competitive replacement (diamond and Case, 1986). Alatorre-Rosas and Kaya (1991) demonstrated that *H. bacteriophora* was unable to co-

exist with *Steinernema carpocapsae*, and they hypothesized that *S. carpocapsae* may release its symbiotic bacteria in a shorter time than *H. bacteriophora*. Moreover, Boemare *et al.*, (1993) proposed that the bacteriocins produced by *Xenorhabdus* bacteria, the symbionts of *S. carpocapsae*, which are more potent than antibiotics (Davis *et al.*, 1968) do kill the symbiotic bacteria of *Heterorhabditis* species *Photorhabdus luminescens*. Two competing species may coexist if the interspecific competition is much less caustic than the intraspecific one. This means that the results of crowding on the shared resource are not more severe than on other resources. Moreover, the coexisting species may exploit the resource in different ways. For example, one species may eat in the daylight while the other eats at night. Such subdivision of the food resource is called Resource Partitioning (Dorit *et al.*, 1991). In nature, more than one natural enemy of the same insect may exist (Kaya and Koppenhofer, 1996). For example, both the mite *Alycus rosens* and a collembolan, *Hypogastura scotti* are predators of the IJs of the EPN *S. carpocapsae* (Epsky *et al.*, 1988). These natural enemies may compete with one another leading to a reduced ability of the nematode to kill the insect. Another example, Barbercheck and Kaya (1991) concluded that the fungus *Beauveria bassiana* and the nematodes *H. bacteriophora* or *S. carpocapsae* competitively interact inside the soil-borne larvae of *Spodoptera exigua* resulting in higher total mortality of the insect.

Moreover, the competitions between different species of EPNs in the same host resources were investigated by several workers (Alatorre-Rosas and Kaya, 1990; Koppenhofer and Kaya, 1996). They showed that the outcome of such interactions depend on many factors including the time needed for the EPN to develop inside the host, the inoculum size, and on the host species. In general, reports in the literature indicate that EPNs tend to avoid attacking already infected host (L. Caroli *et al.*, 1996). Hominick and Reid (1990) proposed that *G. mellonella* secret a substance upon infection with EPNs to prevent furthers invasion and hence avoid competition between EPNs.

Paratism:-

A parasite can be defined as an organism that obtains its food from another organism (host) and lives inside it or on its surface. In the first case the parasite is called endoparasite (e.g. *Encarsia strenua*), and in the second case it is known as ectoparasite (e.g. ticks). Insects that parasite on the egg, larvae, or pupa of another insect are called parasitoids. A parasitoid eats the tissues of its host and leaves it dying. For example, the braconid moth is a parasitoid of the tomato worm where it spends its larval stage within the worm and pupates on its back. Parasites are known to have high reproductive rate to compensate for the fact that they can live only in highly limited and stable environment.

Predation:-

A predator is an organism that kills and eats its prey. Predators can be divided into two categories. First, ambush, where the predator sits and waits for its prey. A typical example of this category are spiders. Ambush predators depend on the active movement of their prey and protect themselves from exposure to large number of enemies by the sitting and waiting strategy. The second type of predators includes those that actively seek for their prey. For example, the dragonflies those search for mosquitoes in the airspace.

Pathogenecity:-

The ability of a microorganism to infect a host is called pathogenecity. If the infection produces symptoms, then it is considered as a disease. Infectious microorganism should be able to produce virulence factors that help them to establish the disease. Such virulence factors include proteases, chitinases, toxins, adhesins, capsules, etc. For example, entomopathogenic bacteria, which are symbiotically associated with nematodes, produce lipases and proteases that kill the host insect. Also, the proteases and chitinases produced by certain microorganisms such as *Serratia marcescens* are virulence factors that act against various host insects such as the banana weevil cosmopolites.

1.1.c Targets of Biological Control

Biological control has been used to control insects, weeds, mites, plant pathogens, and other pests (Julien, 1992; Stirling, 1991; Madsen, 1990). Over 1200 programs of biological control have been used to control more than 543 species of insect pests of different orders (Greathead and Greathead, 1992). Also, more than 116 species of weed plants in different families have become targets for biological control by using plant pathogens or invertebrate herbivores (Julien, 1992). Different species of mites including rust mites, (Gruys 1982) tarsonemid mites, (Huffaker and Kennett, 1956) and the spider mites (McMurtry, 1982) have become targets for biological control. The control of mites involved mainly conservation of mite predators, and in other cases augmentative release of mite predators (McMurtry, 1982).

1.2 Evaluation of Biological Control

Biological control seems to be a feasible way to reduce the use of the hazardous chemical pesticides. In some cases, where the pesticides fail to control a disease, a biocontrol agent could have a good privilege. For example, many soil-borne diseases, like those caused by some *Fusarium spp.* in peanut are difficult to control by pesticides. However, the use of specific biocontrol agents like *Bacillus subtillis* in

the *Fusarium* case, seems to be the only solution till now (Whipps, 1992). Also, in most cases the development of a biocontrol agent is shorter than that of a pesticide. For example, it takes eight years to introduce a pesticide into market, while a period of five years is usually enough for a biocontrol agent to be ready for sale (Becker and Schwinn, 1993). However, this statement is not always true, EPNs for example, were discovered as biocontrol agents in 1927, but only about 15 years ago they started to be used as a biocontrol agents. This was mainly because of the lack of techniques for mass production of EPN in low costs. Becker and Schwinn (1993), compared the costs of developing a chemical pesticide to those of a biocontrol agent. They found that in the United States, for example, it costs US $ 0.5 million to register a natural, non-modified biocontrol agent, while it costs US $ 20 million for a chemical pesticide. However, these data doesn't mean that biocontrol is cheaper than chemicals. Indeed, a chemical pesticide produced in USA for example may be useful all over the world. In contrast, a biocontrol agent (e.g. EPNs) might be efficient for a given insect in it is origin, however it may not be useful in other areas with different climates.

Biocontrol agents are highly accepted at the public level in the countries were they are used (Becker and Schwinn, 1993). This might be mainly due to their safe use in the field since their action is against

specific targets unlike the broad-spectrum chemical pesticides. Indeed, the success of biological control in the field depends largely on the increased specificity of a biocontrol agent to its target pest and also on the availability of the suitable environmental conditions such as humidity, temperature, etc. However, there is a chance for any biocontrol agent to loose its desired activity. Such a change is most likely to happen when the biocontrol agent is cultured on artificial media (Becker and Schwinn, 1993). Continuous subculturing of a biocontrol agent might affect its genetic stability in a way that it is no more effective for biological control applications.

1.3 Biocontrol Agents

There are many organisms belonging to bacteria, fungi, viruses, nematodes, and protozoa that are pathogenic to insects (Tanada and Kaya, 1993; Burge, 1988; Moore *et al.*, 1987). These agents have a diverse biological properties, such as the infectivity rate, heat tolerance, foraging styles, and specificity to host, which help scientists to use them in successful biocontrol programs Driesche and Bellows, 1996). Parasitoids for example, have been used for the biocontrol of insects such as aphids (Greathead, 1986a). Also, the caccinellid beetles, which are predator agents, were used to control the spider mites which have no parasitoids (Driesche and Bellows 1996). Many of the biocontrol agents are commercially available as insecticides (Falcon, 1985). As a classical

example of commercially marketed biocontrol agent we may mention the bacterium *Bacillus thuringiensis* which controls many insects including the larvae of Lepidopetra and Diptera (Lambert and Peferoen, 1992). In the last two decades, this bacterium was used in many Integrated Pest Management (IPM) programs to control insect pests attacking vegetable crops (Trumble *et al.*, 1994 and 1997), some soil living larvae such as *Agrotis segetum* (Lene Thomsen, *et al.*, 1998), and for protecting forests from insect pests infestation (Jacek Hilszczanski, 1998). Toxins of *B. thuringiensis* were applied into field as powder or in water suspension (Jacek Hilszczanski, 1998). Also, the genes coding for *B. thuringiensis* toxins were transferred into plants such as tobacco and cotton to control insects such as *Spodoptera exigua* (Reitz and Trumble, 1998; Robison *et al.*, 1994). Another example are members of the Baculoviridae virus family which are known to cause disease only to insects (Payne, 1986). Among fungi and nematodes used as biocontrol agents we may mention the fungi *Zoophthora radicans* for controlling *Therioaphis trifolii* (Monell) *f. maculata* (Milner *et al.*, 1982), *Beuavaria bassiana* for controlling the Colorado potato beetle (Fargues *et al.*, 1980) the nematodes *Steinernema scapterisci* for controlling *Scapteriscus spp.* (Parkman *et al.*, 1993) and *Heterorahbditis bacteriophora* for controlling the larvae of *Sitona lineatus* (Jaworska and Kopek, 1998).

Additional information about the fungus *Beauveria bassiana*, the

entomopathogenic nematodes *Heterorahbditis spp.* and the bacterium *Serratia marcescens*, is provided in the remaining sections of this chapter.

1.3.1 The bacterium *Serratia marcescens*

Serratia is a genus of the family Enterobacteriaceae, which is part of the group *Klebsielleae*. *Serratia* contains approximately 13 described species, of which the most commonly known is *Serratia marcescens.* This is a gram negative, motile rods, and facultative anaerobe bacterium. Most strains of *S. marcescens* produce typical red pigments known as Prodiginines. The bacterium produces hydrolytic enzymes including; chitinase, proteases, DNAase, Esterase, and Lipase. It is a human pathogen responsible for a large percentage of nosocomial infections including urinary and respiratory tract infections, cystic fibrosis, and in burn-associated infections (ref).

S. marcescens posses the properties of facultative pathogens. It has mechanism for infecting susceptible body tissues and damaging it by growing in the gut. Also, the bacterium is normally present in the environment (Bucher, 1963, Bell *et al.*, 1981). In the literature, many workers describe the ravaging effect of *S. marcescens* on insects (Bell *et al.*, 1981, Krieg, 1987). However, *S. marcescens* could be present in small numbers of many healthy insects without causing pathogenicity unless the larval vigor is greatly reduced (Sikorowski, 1985). On the other hand, if the bacterium enters the hemocoel, it multiplies rapidly and

causes death in one to three days (Sikorowski, 1985). In many works, *S. marcescens* has been recovered frequently from healthy, diseased, and dead insects such as *Dacus dorsalis* and *Carpocapsa pomonella* (Krieg, 1987, Thomas and Poinar, 1973). Also, the bacterium was isolated from eggs of insectary reared *Heliothis zea* (Bell, 1969), and from field-collected egg masses of the European corn borer *O. nubilalis* (Lynch *et al.*, 1976).

S. marcescens was reported as fungal and insect pathogen mainly because of its capability to produce chitinases and proteases (Sikorowski and Lawrence, 1998). These chitinases and proteases play a role in the penetration of the insect cuticle by degrading the cuticle and internal tissues (M. R. Bogo *et al.*, 1998). Also the chitinase enzyme acts by inhibiting spore germination and hyphal elongation of certain fungal pathogens. For example, the chitinase gene from the fungus *Trichoderma harzianum* was transferred to the grapevines *Vitis Vinifera L.* cultivars Merlot and Chardonnay for the control of fungal pathogens (J. R. Kikkert *et al.*, 1998). Chitinolytic strains of *S. marcescens* were used to suppress diseases caused by *Sclerotium rolfsii* (Ordentlich and Chet, 1987), and *Fusarium ozysporum* f.sp. *pisi*.. Also, *S. marcescens* caused significant mortality to the adult blowflies *lucilia sericata* (M. O'Calllagham *et al.*, 1996). Moreover, there is a strain of *S. marcescens* that can kill the corn earworm in two to three days with less than 10 bacteria per insect.

However, *S. marcescens* is not safe for agricultural use without an appropriate engineering towards rendering it harmless to humans and other mammals. This could be achieved by transferring the genes that code for the enzymes, that kill the insects, to harmless bacterial (e.g. *E. coli*) strains that can be used safely for agricultural applications. Indeed, chitinase genes from *S. marcescens* were cloned into an *E. coli* strain which was then used to control *S. rolfsii* in bean plants (Shapria *et al.*, 1989). Furthermore, *E. coli* bacteria transformed for the chitinase gene were used for controlling plant pathogenic fungi by adding it to irrigation water (Schickler *et al.*, 1993).

1.3.2 The fungus *Beauveria bassiana*

The entomopathogenic fungus *Beauveria bassiana* from the class Hyphomycetes is known to have a broad host range of insects (Goettel *et al.*, 1990). Many pests of the orders Coleoptera, Lepidoptera, and Hemiptera are susceptible to different strains of *B. bassiana* (Tanada and Kaya, 1993; Humber and Soper, 1986). It could be used to control aphids, (Dorschner *et al.*, 1991; Feng *et al.*, 1990) but it is pathogenic to non target aphid predators (Magalhaes *et al.*, 1988). Another use of *B. bassiana* involved field applications to control the coffee berry borer as well as the larvae of the Colorado potato beetle (Fargues *et al.*, 1994).

Products of *B. bassiana* were registered for field applications in the United States for the control of the fire ant *Solenopsis invicta* (David *et al.*, 1994).

Formulation of Entomopathogenic Fungi (EPF) for field application includes mainly a mixture of the spores, a wetting agent, and a carrier (Samson *et al.*, 1988). The carriers used for this purpose may include inert particles which carry the spores to the target site (Inglis, 1996). Oil is the most used carrier for such applications because it stimulate spore germination, protect the fungus from UV radiation and high temperatures, and help the spores to attach to the hydrophobic surfaces of the insect cuticle (Inglis, 1996).

1.3.2.1 Mode of action

The mechanism by which *B. bassiana* penetrates into the insect and kills it involves several steps. First, the spores are attached chemically and physically to the insect's external cuticle (Atuahene and Dopperlreiter, 1982), or respiratory tracheal wall (Clark *et al.*, 1968), or to the wall of the alimentary tract (Broome *et al.*, 1976; Yanagita, 1987). Second, the spores germinate and produce an apical swelling of germ tubes which is called the appressorial cell. The appressorial cell helps the fungus to attach to the insect surface. This step requires suitable conditions including optimal temperature, carbon and nitrogen sources.

Third, the fungus penetrates the insect's cuticle mechanically using the germ tubes and appressorrium, and enzymatically by producing lipases, chitinases and proteases, which digest the tissues of the insect. Fourth, the fungus overcomes the host defense systems, kills it and reproduces inside the cadaver. The fungus escape from the insect's defense systems by forming fungal protoplasts, which can't be recognized by the insect immune system. The protoplasts develop hyphae, which produce toxins such as beauvericine. The hyphae feed on the nutrients resulting from the digestion of tissues. All of these events lead to insect's death. Finally, the fungus repentrate the insect's cuticle from inside to outside and produces conidiophores with high quantity of spores, which spread in the environment and infect other insects (Hajek and Leger, 1994; Broome J. R *et al.*, 1976).

1.3.3 Entomopathogenic nematodes (EPN's)

Nematodes in general are roundworms belonging to the phylum Nematoda. Nematodes that are pathogenic to insects are called Entomopathogenic Nematodes (EPNs). They have no risks to livestock, plants, and human health. Therefore, EPNs were registered commercially for field application in many countries including USA, China and in Europe (Poinar, 1986). Most of the nematodes being used in biological control are found in the families *Steinernematidae* and *Heterorhabditdae*

(Gaugler and Kaya, 1990; Kaya, 1993). Nematodes of these two families are associated with symbiotic bacteria, which kill the insect through septicemea (Kaya, 1985). EPNs from the family *Steinernematidae* are associated with bacteria from the genus *Xenorhabdus* while EPNs from the family *Heterorhabditdae* are found in association with bacteria from the genus *Photorhabdus* (Thomas and Poinar, 1979; Koppenhofer *et al.*, 1995).

1.3.3.1 The life cycle of EPN's

The infective stage of the nematodes, which is found in the soil, penetrates the insect's body through spiracula, mouth, and anus. Upon their penetration they release the symbiotic bacteria stored in their gut into the insect's haemocoel (Figure 1.1). The bacteria proliferate and produce exotoxins (e.g. lecithinase), endotoxins (e.g. polysaccharides), and antibiotics (e.g. indole derivatives) that kill the insect. The infective Juvenile recovers into Juvenile 4 and then it develops into hermaphrodite in *Heterorhabditdae* while in *Steinernematidae* it develops into males and females (Bowen *et al.*, 1988; Paul *et al.*, 1981; Gaugler and Kaya, 1990). Most of the nematode life cycle takes place inside the host body while the only stage that inhabits the soil is the infective juvenile stage. In subsequent rounds of *Heterorhabditdae* life cycle, males and females develop from J4 (Luanu *et al.*, 1993). Females and hermaphrodites do lay fertilized eggs from which the J1 stage hatches. It feeds on the symbiotic

bacteria and develops into J2, J3, J4, and adults. The cycle continues till the source of food (bacteria) becomes limited, then the J1 stage will develop through J2 stage into the Infective Juveniles stage (IJs) (Poinar, 1990). Infective Juveniles carry in their gut small amounts of symbiotic bacteria. They are motile, non-feeding nematodes, which will move out of the insect's cadaver and search in the soil for other insects to infect (Campbell and Guagler, 1995).

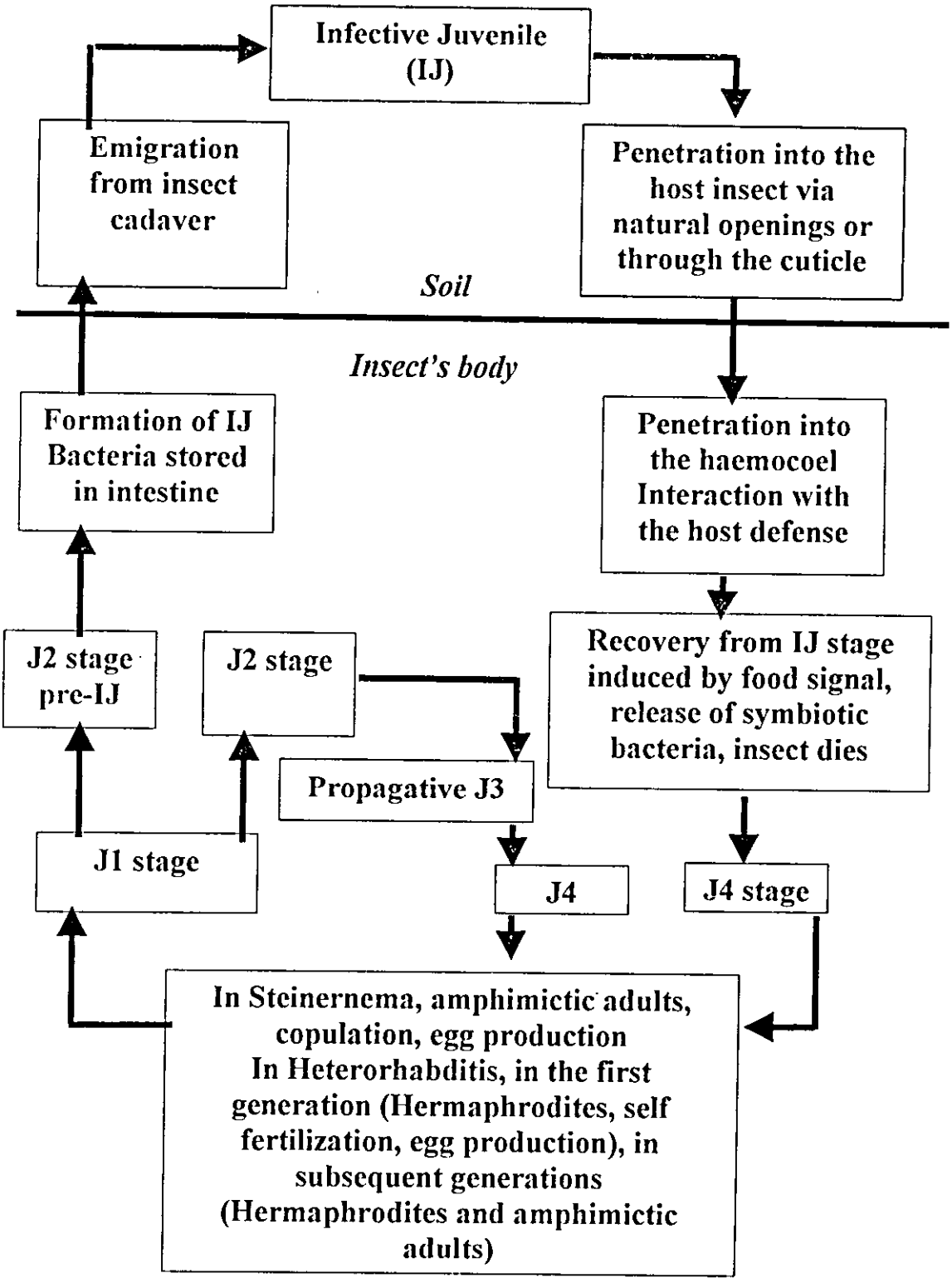


Figure 1.1: Life cycle of entomopathogenic nematodes from the *Steinernema* and *Heterorhabditis* genera.

1.4 Interaction between biocontrol agents

The interactions resulting from combining different biocontrol agents against a given target insect haven't been studied sufficiently (Barbercheck and Kaya, 1991). Choo *et al.* (1996) used a combination of two EPNs species to control the larvae of western spotted cucumber beetle, *Diabrotica undecimpunetata*. They found that no advantage is gained by using such a combination for controlling the mentioned insect. That is, the mortality of the insect resulting from combining the two nematode species was not higher than of any of them alone. On the other hand, the same combination (*S. carpocapsae* and *H. bacteriophora)* showed higher mortality percentage against *Galleria mellonella* than any of each agent alone (Alatorre-Rosas and Kaya, 1991). Kamionek *et al.* (1974a, b) found that a combination of two agents would result in higher mortality than using any of the agents alone if the insect host is very susceptible to both pathogens alone. In contrast, if the host is not very susceptible to one or both pathogens when applied alone, the combination wouldn't result in an synergistic effect on the insect mortality (Kamionek *et al.*, 1974 a,b, and Choo *et al.*, 1996). In the above two examples, we may explain the results as follows: *Galleria mellonella* is known for its sensitivity for entomopathogenic nematodes and hence the combination resulted in higher mortality than using any agent alone, however,

Diabrotica undecimpunctata might not be a very susceptibility for one or both of the nematodes used.

Combining biocontrol agents with different foraging strategies should increase the efficacy of biological control (Kaya *et al.*, 1993). This is because of the possibility that agents with different foraging strategies could encounter larger range of insects (including the mobile and resting) than an agent alone. The nematode *S. carpocapsae*, for example, are good enemies for the soil-surface *S. lineatus* adults since they do sit and wait foraging. On the other hand, *S. lineatus* larvae, which fed on root and root nodules, were successfully controlled by the active searching forager, *H. bacteriophora* (Jaworska and Kopek, 1998).

Fungi such as *B. bassiana* , *Melashizium anisoptiae, Paealomyces farinosus*, and *P. fumoso-roseus* where successfully combined with the EPN *H. bacteriophora* and *S. carpocapsae* to increase the mortality of the pea weevil *Sitona lineatus* (Jaworska and Ropek, 1998). Furthermore, the release of *H. bacteriophora* into soil infested with *B. bassiana* resulted in higher total mortality of *Sodoptera exigua* than any of the agents alone (Barbercheck and Kaya, 1991). Also, a combination of EPN and the bacterium *Bacillus thuringiensis* subspecies *japonensis* (Btj) increased field and greenhouse efficacy against Scarab Grubs (Koppenhofer *et al.*, 1999). On the other hand, the nematode *Steinernema fltiae* showed

greater control of the artichoke plume moth than combining it with Bt var. *Kurstaki* (Bari and Kaya, 1984).

1.5 Importance of studying the interaction

In most studies, the use of a single biocontrol agent showed better biocontrol efficacy than having multiple biocontrol agents together (Myers *et al.*, 1989). A successful biocontrol process shouldn't be evaluated only by higher moralities of the target insect as measured in the laboratory but also on the sustainability of the biocontrol agent in the field. In the soil there are organisms that may reduce the efficiency of EPNs as biocontrol agents (Patricia Timper and Kaya H.K., 1989). For example, both *B. bassiana* and EPNs that occur in soil were not able to reproduce inside a single host (Barbercheck and Kaya, 1991). Also, it was found that EPNs prefer to infect a healthy insect rather than a one that is preinfected with *B. bassiana* (Barbercheck and Kaya, 1991). The Entomopathogenic bacteria (EPB) *S. marcescens*, were isolated from soil samples (Kobayashi *et al.*, 1995) so it may occur in insects where EPNs are to be applied. Hence, it is important to check the effect of such bacteria on the sustainability of EPNs. Such effect should be investigated for the ability of EPNs to; penetrate preinfected host, recover inside it, and produce normal offspring over time.

1.6 Objectives of this study:

The study aims at:

1. Characterization of an entomopathogenic bacterial strain newly isolated from Jericho, Jordan Valley.
2. Determining the infectivity of the newly isolated bacterial strain to *Galleria mellonella.*
3. Studying the effect of the newly isolated bacterial strain and *B. bassiana* on the survival of Infective Juveniles, and J4 stages of *H. indica*, Bethlehem 11 strain.
4. Studying the in-vitro interactions between the symbiotic bacterium of the *H. indica* strain Bethlehem 11; *Photorhabdus luminescence* and the Jericho 11bacterial strain or the fungus *B. bassiana.*
5. Studying the effect of infecting *Galleria mellonella* larvae with the newly isolated bacterial strain or the fungus *Beauveria bassiana* on the invasion and the subsequent proliferation of the entomopathogenic nematode *Heterorhabditis indica*, strain Bethlehem 11, inside the larvae.

Chapter II
Materials and Methods

Chapter Two
Materials and Methods

2.1 Rearing of *Galleria mellonella.*

Adults of the wax moth *G. mellonella* (Lepidoptera: Pyralidae) were placed in glass jar, which contained nutrient medium and strips of tissue paper. The female adults, developed from caterpillars, laid eggs on the tissue paper strips. The eggs were disinfected with 10% formaldehyde for 90 minutes, followed by intensive wash with running water for 1hour. The disinfected eggs were placed in sterile glass jar with larvae-feeding medium. The medium contained, gram per liter: 200 autoclaved honey, 183 Glycerol, 47 Yeast Extract, and 4 antifungal Nipagine, all ingredients were mixed and stirred at 50-60°C. When softened, 320.0 g of oatmeal were added to the mixture. The glass jar containing the eggs and the media were incubated at 32°C in the dark. The eggs hatched to larvae after 2-3 weeks of incubation, and developed to the last instar larvae, which were used in the experiments.

2.2.1 Isolation of *Serratia marcescens*

The bacterial strain was isolated from the haemocel of *G. mellonella* larvae which was used as a bait for nematodes in a soil sample

collected from Jercho (Sansour M. MSc Thesis, 2000). This infected dead larvae was removed from the soil, washed and disinfected with 1% sodium hypochlorite for 2 minutes, then washed three times with sterile distilled water then it was wiped using sterilized filter paper. The haemolymph of the disinfected larvae was then streaked on NBTA medium in 9cm petridishes and incubated for 24 hours in the dark. The NBTA medium contained in gram per liter, 37.0 Nutrient agar (Difco), 0.25 Bromothymol blue (Sigma), and 1% 2,3,5-Triphenyl tetrazolium chloride (Sigma), and solubilzed in tap water. A well-separated colony was cultured in liquid BSA medium for 24 hours in the dark at continuous rotary shaking at 200 r.p.m. A stock of the bacterial culture was brought into 15% glycerol and stored at -20°C. Part of this culture was used to infect a new Galleria larvae followed by isolation on NBTA of another inoculum from the haemolymph and culturing it on BSA medium. This cycle was repeated 8 times. The BSA medium contained, in gram per liter, 10.0 Nutrient broth (Difco), 10.0 Tryptic soy broth (Difco), 5.0 Yeast extract (Difco), 5.0 Peptone (Difco), 5.0 NaCl, 0.35 KCl, .021 $CaCl_2.2H_2O$ and solublized in tap water.

2.2.2 Determining the infectivity of the isolated bacterial strain against *G. mellonella.*

Aliquots of 250µl of either the bacterial strain suspension or its Millipore filtered medium were mixed with one gram of Galleria food in

a 5.5cm-diameter petridish. Five larvae of *G. mellonella* (0.173g weight in average) were housed in each dish and incubated in the dark at 25°C. Non-contaminated food was used as control. For each treatment five replicates were used. Mortality of larvae was recorded everyday. Infectivity of the bacterial strain was determined also by injecting 50µl of either bacterial suspension or Millipore filtered medium into the haemolymph of Galleria larvae and incubated as described above. For each treatment 10 replicates were used. All experiments were repeated twice independently.

2.2.3 Identification of the bacterial isolate

2.2.3.1 General characterization

The bacterial strain was characterized morphologically under the microscope. Also a biochemical determination using the Appendix Profile Index (API test) and gram staining were used to identify the isolate. In an attempt to study the effect of medium composition on pigment production by the bacteria, the isolate was cultured on the following media; NBTA, solid and liquid BSA and YS. The YS medium contained, in gram per liter 0.5 $NH_4H_2PO_4$, 0.5 K_2HPO_4, 0.2 $MgSO_4$, 5.0 NaCl, and 5.0 yeast extract (Difco). The solid and liquid cultures were incubated at 25°C in the dark. The liquid cultures were put on a rotary shaker at 200 r.p.m.

2.2.3.2 Molecular identification

The bacterial isolate was sent to the Deutscue Sammlung Von Mikroorganismen und Zelliulturen GmbH (DSM2) in Germany for identification. The 16S rRNA gene of the strain was extracted, amplified by the PCR, and sequenced as described by Rainey F. A. *et al* 1996 and Maldak B. L. *et al* 1996. The sequenced gene was compared to the sequences of the same gene in other bacteria.

2.2.4 Establishing a growth and standard curves of the newly bacterial isolate.

A volume of 0.5 ml of the bacterial stock was precultured in 50 ml BSA medium in a 250ml flask. After 24 hours of incubation at 25°C at continuous shaking at 200 r.p.m in the dark, 0.5 ml of the culture were inoculated in another 250 ml flask containing 50 ml BSA medium (composition described in section 2.2.1). Samples from the culture were taken for determining turbidity every two hours up to 18 hours, and one additional sample after 24 hours. The turbidity of the culture was determined by measuring optical density (O.D) at 560 nm using the spectrophotometer (Milton Roy Spectronic 401). Another set of samples were taken at the same time intervals and diluted under aseptic conditions $X10^{-2}$ - X10-^{10} in sterile saline solution. One microliter from each dilution was cultured on solid BSA medium. The number of Colony Forming Units (CFU) was counted after 48 hours of incubation at 25°C.

2.3 Harvesting *B. bassiana* spores.

The fungus *B. bassiana* used in this study was isolated by Mahassneh Mohammad (MSc Thesis 1999) and grown on Potato Dextrose Agar (PDA) medium in 9cm petridish at 25°C and 14 hours photoperiod for 5 days. The PDA medium contained, in g per liter 4.0 potato extract, 20.0 dextrose, and 15.0 agar. Twenty milliliters of autoclaved distilled water were added to each petridish. The spores were released from the conidiophores by gentle hand shaking of the petridish. The liquid containing the released spores was then passed through a mira cloth filter to separate the spores from the mycelium. The number of spores per ml was determined using the hemacytometer. The spores were stored at 4°C till use.

2.4 Effect of *S. marcescens* and *B. bassiana* on the survival of infective juveniles, juveniles 4 (J4), and hermaphrodite stages of *H. indica* strain Bethlehem 11.

The entomopathogenic nematode *H. indica*, strain Bethlehem 11 was isolated by Sansour Michael (MSc Thesis 2000). One hundred IJs/ml were added to a newly inoculated *S. marcescens* culture; 0.5 ml of 18 hours old culture inoculated in 50 ml BSA medium in a 250 ml flask. Similar amounts of IJ's were added to 50 ml of sterile Ringer or BSA solutions not containing *S. marcescens*. The Ringer solution contained, in gram per liter 9.0 NaCl, 0.42 KCl, 0.48 $CaCl_2.6H_2O$, and 0.20 $NaHCo_3$

dissolved in H_2O. In all treatments flasks were kept at continuous rotary shaking (200 r.p.m.) at 25C° in the dark. After 12, 24, and 48 hours 3 samples of 1 ml each were taken from each treatment and poured into 5.5cm sterile petridish for recording IJ survival under light microscope on a laminar flow bench.

For determining survival of the J4 stage, about 50 nematodes were incubated in 20 ml BSA medium containing 0.2 ml of either 18-20-hours old *S. marcescens* culture, or a suspension of 48 hours old germinated spores of *B. bassiana* (2.0×10^7 spores/ ml). Samples were incubated for 2 days at 25°C in the dark under continuous rotary shaking at 200 r.p.m. As a control similar numbers of J4 were incubated at the same condition in either BSA medium or sterile deionized water. The number of survived nematodes was counted under the inverted microscope each 24 hours for 48 hours.

2.5 Sequential infections on filter paper of *Galleria* Larvae with nematodes and either the new isolate of *S. marcescens* or *B. bassiana.*

The interaction between the biocontrol agents; *H. indica* Bethlehem 11 strain and either *B. bassiana* or the new isolate of *S. marcescens*, Jericho 11strain were studied inside larvae of the insect *Galleria mellonella.* In each treatment, twenty last instar larvae (average weight of 0.17g) were placed each in one well of a multi-well plate

(Corning, England, each well of 1.55cm in diameter and 1 cm height) lined with Wattman #1filter paper. Dual infections using 50 IJs of *H. indica* Bethlehem 11 strain (collected from bedding culture as described by Bedding R. A., 1981) and 25 µl of either *S. marcescens* Jericho 11 strain (20 hours old culture) or *B. bassiana* (2.5×10^6 spores/ml) were performed in the following order: *H. indica* Bethlehem 11 IJs were applied one day after the application of *S. marcescens*, Jericho 11strain or two days after the application of *B. bassiana.* Another treatment involved simultaneous application of *H. indica* Bethlehem 11 strain and either *S. marcescens* Jericho 11, or *B. bassiana.* The application of the pathogens was done as follows: A volume of 25µl of suspension from each pathogen was used per each *G. mellonella* larvae. The agents were poured directly on the insect's body inside the well. When the larvae were infected by only one pathogen, 25µl of Ringer solution were used to reach a 50µl total volume.

Mortality of *G. mellonella* was recorded everyday. The number of recovered hermaphrodites was counted after four days of application of the Infective Juveniles under the microscope. Each experiment was repeated three times independently.

2.6 Effect of the *S. marcescens* new isolate and *B. bassiana* on the growth of the symbiotic bacteria *Photorahpdus luminescens.*

A volume of 50µl of *S. marcescens* (20 hours old culture), *B. bassiana* (2 X10^7 spores/ml) and *P. luminescens* (24 hours old culture) were smeared separately on 9-cm petridish containing solid BSA medium. Several paper disks (Wattman # 1 filter paper) of 0.6-cm diameter were soaked in the *S. marcescens*, *B. bassiana,* or *P. luminescens* cultures. Part of the disks soaked with either *S. marcescens* or *B. bassiana* were placed in plates smeared with *P. luminescens*, While the other part were placed on plates containing the medium only (control). Moreover, disks soaked with *P. luminescens* were placed in the plates smeared with either *S. marcescens* or *B. bassiana.* Furthermore, *P. luminescens* disks were placed on plates containing the medium only (control). Each treatment included four plates each containing four disks. All of the plates were incubated in the dark at 25°C. The growth of the organisms was recorded daily by measuring the diameter of the culture circle surrounding the paper disks. The experiments were repeated three times independently.

2.7 Establishing a monoxenic culture of *H. indica* Bethlehem 11 strain.

Infective Juveniles of *H. indica*, strain Bethlehem11, which were collected from a white trap, figure 2.3, (white, 1927) were used for

infecting Galleria larvae. After 4 days of infection the dead Galleria larvae were dissected in a petridish containing Ringer solution. Hermaphrodites were collected and washed for several times to remove the tissues of the dissected larvae. The excess solution was filtered through a 55µm mesh. Hermaphrodites were collected from the filter and placed in a small test tube containing 2 ml of Ringer solution together with small pieces of razor blades. The tube was vortexed for about 1 minute to rupture the hermaphrodites and release the eggs. The contents of the tube were then filtered through 55µm mesh. The filtrate, which contained the eggs, was transferred to eppendorf tubes and washed 3 times with Ringer solution through centrifugation for 2 minutes at 2000 rpm. The washed eggs were transferred to a sterile eppendorf tube and washed twice with sterile Ringer solution. The sterilization buffer was added and the mixture was shaken for 4 minutes by hand. The sterilization buffer contained; 1.5 ml of 4M NaOH, 0.5 ml of 12% NaOCl, and 10 ml of distilled water. The buffer was removed by spinning the egg suspension and washing it twice with Ringer solution. At the end of the second centrifugation the supernatant was discarded and 300µl of sterile BSA medium were added. The disinfected eggs, suspended in BSA medium were transferred into wells of a multi-well plate containing 200µl of BSA medium (1-2 suspension droplets/well) and incubated for 48 hours till the hatching of Juvenile 1 stage. The newly hatched Juvenile 1 stage were transferred from the egg-incubation medium

into Wout's agar plates (Wouts, 1981) (3-4 droplets/plate). Before transferring the Juvenile 1 suspension, each Wout's plate was inoculated with 3-4 droplets of 24 hours-old culture of the nematode symbiotic bacteria *photorhabdus luminescens*. This bacterium was isolated from Galleria larvae infected with *H. indica* as described by Sansour Michael (MSc Thesis 2000). The Wout's plates containing the Juvenile 1 and symbiotic bacteria were incubated in the dark at 25°C. Adults were observed 5 to 7 days after inoculation and IJs developed after 4 weeks. The Wout's medium contained 16.0 g/L Bacto nutrient broth, 12.0 g/L Bacto agar, 5.0 g/L corn oil and solublized in deionized water.

2.8 Interaction between *H. indica* Bethlehem 11 strain, and either *S. marcescens*, or *B. bassiana*, inside *Galleria mellonella* larvae.

The last instar larvae of *G. mellonella* were placed in multi-well plates (1 larvae per well see figure 2.1) containing 10% moistened sand. The moistening was done with appropriate volumes of Ringer solution (control), suspension of 20 hours - old *S. marcescens* culture, or 2X 10^7 spores/ml of *B. bassiana* spore suspension. The incubation period with *S. marcescens* was 24 hours, while 0, 24, 48, 72, and 96 hours incubation periods were applied for *B. bassiana*. At the end of the infection period, the larvae were washed 3 times with sterile distilled water and excessive

water was removed with sterile filter paper. The washed larvae were exposed for 24 hours to 100 IJs obtained from a monoxenic culture and mixed in 10%-wet sand. The larvae were divided into three groups. The first group of larvae was dissected and incubated in pepsin solution for 2 hours at 37C° at continuous rotary shaking (120 rpm). The pepsin solution contains 8.0g/l pepsin (Sigma), 23g/l NaCl (Difco), and 940ml distilled water. The pH of the solution was adjusted to 2.0 with HCl. The number of IJs penetrated into the insect was then counted under the microscope. The second group of the larvae was left on wet filter paper for 72 hours, and then dissected to determine the number of adults (hermaphrodites) under the microscope. The third group was left on a White trap (figure 2.2) in the dark at 25°C for 2 weeks to determine the total production of nematodes. Insect mortality for each treatment was recorded daily after the infection with the entomopathogen. Inoculates from the haemolymph of dead *G. mellonella* larvae from all treatments were streaked on either NBTA medium for detecting *S. marcescens* and *P. luminescens* or PDA medium for detecting *B. bassiana.* The control treatment involved infection of *Galleria mellonella* larvae with each of the entomopathogens alone. Ten larvae were used for each treatment. All of the experiments were repeated three times independently except for the one involved studying the effect of preinfecting *Galleria* larvae with *B. bassiana* for 0,

24, 48, 72, and 96 hours on the penetration, recovery, and total production of the nematode *H. indica* where it was repeated only twice.

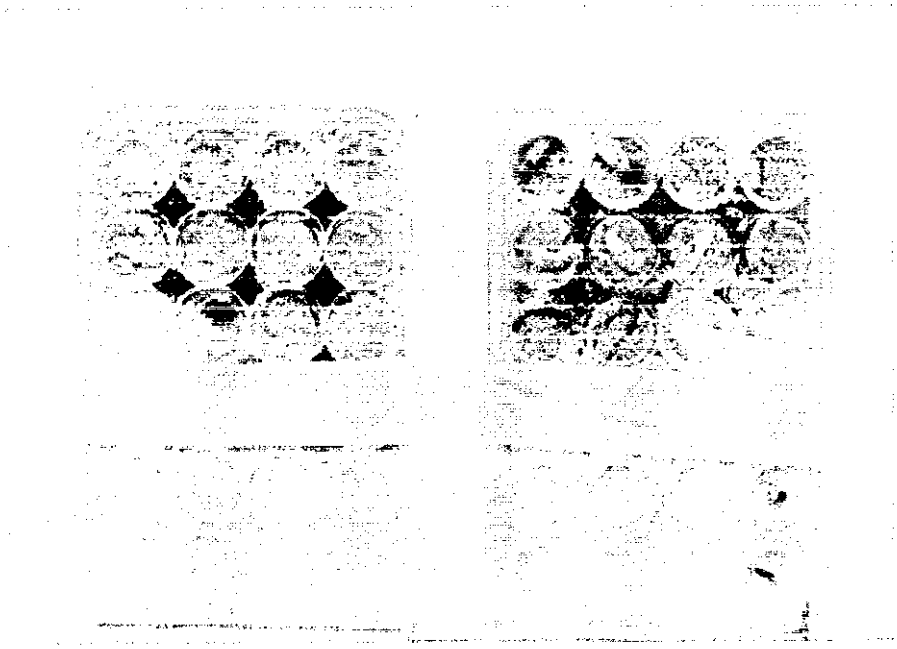


Figure 2.1 The multi-well plates used for infecting *Galleria mellonella* larvae

2.9 Statistical analysis

The collected data, from the interaction experiments (Tables 3.8, and 3.10), observed in this study including the effect of either *S. marcescens* and *B. bassiana* on the penetration, recovery, and proliferation of the nematode *H. indica*, Bethlehem 11 strain were statistically analyzed by paired samples t-test. The data in table 3.9 concerning the effect of *S. marcescens* and *B. bassiana* on the recovery of the injected IJs into adults were also analyzed by the same test. Samples were said to be significantly different if the significance value (2-tailed) was less than 0.05. The paired samples t-test values were calculate using the SPSS 9.0 software obtained from the Bethlehem University Computer Center.

Figure 2.2 The mini-White trap used for studying the total production of nematodes.

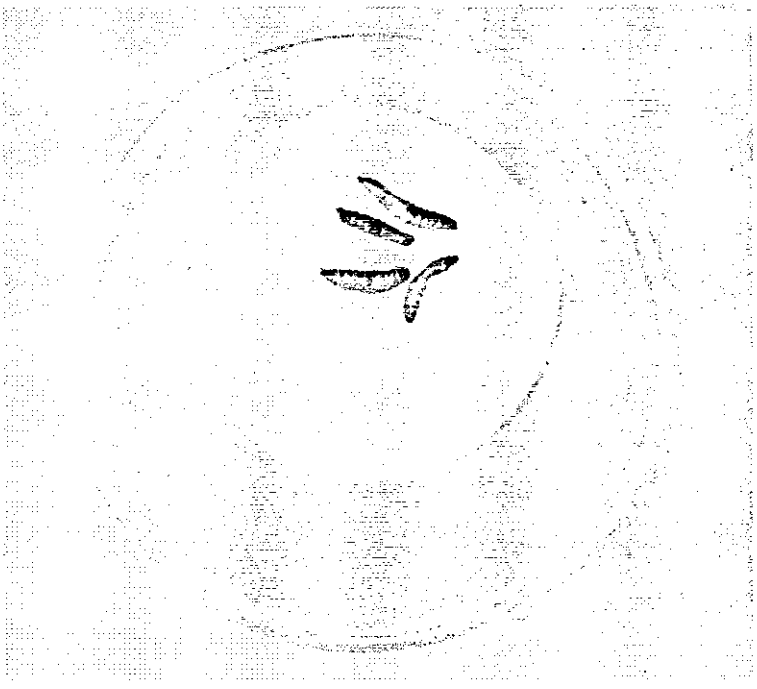


Figure 2.3 White trap method for collecting the IJs of *H. indica* Bethlehem 11 strain.

Chapter III
Results and Discussion

Part One
Characterization of Jericho 11bacterial strain

Chapter 3

Results and Discussion

3.1 Isolation and identification of *Serratia marcescens*

The Galleria larva that was infected and killed inside the soil sample, was disinfected and placed onto White trap. This was done originally for the purpose of collecting infective juveniles of entomopathogenic nematodes*. After two weeks, the suspension containing the infective juveniles was collected and filtered through Whattman #1 filter. The nematode-free filtered liquid was capable of infecting Galleria larvae when it was poured on larvae housed in a petridish lined with filter paper. The larvae died after 4days and they turned red postmortem. The cadaver was disinfected and the haemolymph was streaked on NBTA medium. The streak produced red colonies (Fig. 3.1).

*In Summer 1997, at the early stages of research work on entomopathogenic nematodes at the UNESCO Biotechnology Center at Bethlehem University, Michael Sansour, Dr. Nida Salah, and Dr. Naim Iraki collected soil samples from Al-Auja in the Jordan Valley for the purpose of isolating new strains of these nematodes. The isolation process employed the baiting technique, in which one Galleria larva was trapped inside a net and covered with soil. After two weeks, the infected dead larva was washed, disinfected and transferred onto White trap for collecting the "Infective juvenile", stage in Ringer solution. The "Infective Juveniles", suspended in Ringer solution were used to infect other Galleria larva. One of the infected dead larva was dissected to isolate a single adult female, which was used to initiate a pure line of nematodes. The "Infective Juveniles" of the pure line were able to "kill" Galleria larvae, proliferate inside the cadaver and produce another generation of "Infective Juveniles". The cycle was repeated several times successfully. Meanwhile, the pure line of nematodes was sent for identification to the laboratory of Dr. Ralf-Udo Ehlers,

The colonies were used for infecting additional larvae, which in turn showed the same color postmorten, as well as the same color of colonies obtained from streaking the haemolymph on NBTA medium. This cycle was repeated 8 times, each time giving the same effect on larvae and the same colony color. Gram staining and microscopic (X1000) examination of cells taken from the colonies showed that they are gram negative and coccobacilli in shape.

The above preliminary characterizations of the isolate indicate that it is an insect-pathogenic gram-negative bacterium. The red color of the colonies is typical to some strains of the insect-pathogenic bacterium *Serratia marcescens* (Stienhaus 1959). The red pigment is water insoluble substance known as prodigiosin (Brock and Madigan 1991). However, prodigiosin, and prodigiosin-like substances are produced by other species of bacteria, such as *Vibrio* spp., *Pseudomonas* spp., and *Alteromonas* spp. (Kreig 1987, Lindberg 1976). Hence, the red pigmentation of our new isolate can't be used exclusively as an indicator

at Kiel University, in Germany. Surprisingly, the nematodes were identifies as saprophytic nematodes rather than entomopathogenic. Saprophytic nematodes cannot infect and kill insects. They live on dead cadavers. The ultimate question that was asked is how the Galleria larvae were killed? Attempting to isolate a killing factor other than the nematodes, the nematodes suspension collected from White trap was filtered under aseptic conditions through Wattman # 1 filter. The filtered liquid was used to infect Galleria larvae. The larvae died and then they were disinfected with sodium hypochlorite, and the haemolymph was streaked on NBTA medium. The streak developed colonies of red – color (figure 3.1). When these colonies were smeared on Galleria larvae which were incubated on wet filter paper, they caused death of the insect after 3 days (Figure 3.2). In a later stage of this work, these colonies were identifies as *Serratia marcescens.*

for its identity as *Serratia marcescens*. The coccibacilli shape of cells of our isolate is different from the rod shape that was most frequently reported for bacteria from the genus *Serratia* (Davis *et al.*, 1973).

Biochemical tests, using the API 20E system were conducted on the new isolate. The overall results of these tests showed 89% similarity to the bacterium *Serratia marcescens* (Table 3.1 and Appendix I). The 11% dissimilarity on the other hand might be attributed to the possibility of being our isolate a new strain or perhaps a new species of the genus *Serratia*. Because of this uncertainty a more sophisticated method of identification was employed.

Comparison of the 16S rRNA gene sequence is a commonly used method for identification of bacteria (Maidak *et al.*, 1996)). The 16S rRNA gene sequence of our new isolate was determined and compared to the corresponding sequences of other bacteria (Figure 3.2). The highest homology (99.3%) was found between our isolate and *Serratia marcescens* (Table 3.2). Based on this result, and on the API 20E tests, we concluded, at high level of confidence, that our new isolate is *Serratia marcescens*. Following this conclusion the strain was named as Jericho 11. The question whether the new isolate is a distinct strain of the species *marcescens* could be answered after conducting further molecular- based identification tests, such as DNA-DNA hybridization.

Figure 3.1 A smear of the *S. marcescens*, Jericho 11 strain on NBTA showing the red colonies of the new bacterial isolate.

Table 3.1: Biochemical characteristics of the newly isolated bacterial strain using the API 20E method. Results are percent positivist of the test.

Test	Result
Beta-Galactosidase	94%
Arginine dihydrolase	0
Lysine Decarboxylase	95
Ornithine decarboxylase	95
Citrate utilization	96
H2S production	0
Urease	28
Tryptophan deaminase	0
Indole production	0
Voges Proskauer	99
Gellatine hydrolyses	1
Acid production from:	
Glucose	100
Mannitol	100
Inositol	0
Sorbitol	0
Rhammonse	5
Sucrose	0
Melibiose	0
Amygdalin	90
Arabinose	1
Cytochrome oxidase	0
Nitrite production	100

494 bp RNA
Base count: 124 A, 115 C, 163 G, 92 T, 0 others.

1CAGATTGAAC GCTGGCGGCA GGTTAACAC ATGCAAGTCG
AGCGGCAGGGGAGCT 61 TGCTCCCTGG GTGACGAGCG
GCGGACGGGT GAGTAATGTC TGGGACCTGATGGAG121
GGGGATAACT ACTGGAAACG GTAGCTAATA CCGCATAACG
TCGCAAAAGACCCCC 181 ACCTTCGGGC CTCTTGCCAT
CAGATGTGCC CAGATTAGCTAGTAG GTGGGGTAAT
241GGCTCACCTA GGCGACGATC CCTAGCTGAGAGGAT
GACCAGCCAC ACTGGACTG 301 AGACACGGTC
CAGACCGGGAGGCAG CAGTGGGGAA TATTGCACAA
TGGGCGCAAG 361 CCTGCCATGCCGCG TGTGTGAAGA
AGGCCTTCGG GTTGTAAAGC ACTTTCAGAGGAGGAAGG
TGGTGAGCTT AATACGCTCA TCAATGACG
TTACTCGCAAGAAGCACC 481 GGCTAACTCC GTGC

Figure 3.3: The partial sequence of the 16SrRNA of the newly isolated *S. marcescens* bacterial strain.

Table 3.2: Percent homology between the partial 16S rRNA gene sequence of the newly isolated strain and that of other bacteria.

Bacteria	Percent of homology with the unknown strain
Serratia marcescens	99.3%
Erwinia carotorora	96.9%
Citrobacter freundii	96.9%
Klebsiella planticola	96.3%
K. terrigena	96.1%
Entterobacter	95.8%
Serratia faecaria	<95%
S. odosicera	<95%
S. grinesii	<95%
S. fonticula	<95%
S. plymutica	<95%
S. proteamaculaus	<95%

3.2 Characterization of the newly isolated *S. marcescens* culture

3.2.1 Culture pigmentation

When a liquid BSA medium was inoculated with bacterial cells taken from red colonies of *S. marcescens* developed on NBTA medium, the culture color was light brown, but not pink (Table 3.3). In order to test the possibility that the medium composition has an effect on the appearance of red color in the culture, our isolate was grown on two other media in addition to NBTA. Each of these media was used in the liquid state, with or without oil, and in the solid form (using 0.8% agar) in the presence or absence of oil (Table 3.3). In the liquid medium the pink-red color appeared only if oil was added, regardless of the medium type. In contrast, the oil had no effect on color appearance if the medium was solidified with agar. These results could be explained as follows: It is known from the literature that the red pigment prodigiosin secreted by *Serratia marcescens* is water insoluble. The oil droplets suspended in the liquid medium served as a hydrophobic surface for the adherence of the pigment, which in turn caused an increased concentration of the substance leading to appearance of the color. Another possible role of the oil in the appearance of the red pigment is its function as a metabolite, necessary for the synthesis of the colored substance. However, this possibility becomes unlikely when we inspect the results of color appearance on the

solid medium (Table 3.3). In these types of media the pink-red color did appear in the presence and absence of oil. This observation suggests that the medium solidifying agent, that is, the agar, acted as an adsorbent and a concentrator of the pigment molecules. This assumption was tested by adding autoclaved powdery agar to already autoclaved liquid BSA medium. After 24 hours of culture the insoluble agar particles became pink while the liquid medium remained not pigmented (data not shown). The color of the bacterial culture in liquid BSA medium, which did not contain oil, was light brown (Table 3.3). When this suspension was spun at 1000g, the color of the pellet was pink-red indicating that the red pigment was present in the culture but at very low concentration (Figure 3.4)

Table 3.3: Pigmentation of *S. marcescens*, Jericho 11 strain cultures at different types of growth media.

Growth medium	**Liquid**		**Agar solidified**	
	+ oil	**- oil**	**+ oil**	**- oil**
NBTA	Not tested	Not tested	Not tested	Dark red
YS	Pink-red	Gray	Pink-red	Pink-red
BSA	Pink-red	Light brown	Pink-red	Pink-red

Figure 3.4: The colored material resulted from spinning the liquid culture of *S. marcescens*, Jericho 11 strain at 1000g

3.2.2 Growth and standard curve

As a new isolate, it was necessary to determine the typical growth rate of *S. marcescens* on the BSA medium, which was used for the growth of other organisms in this work (e.g. the symbiotic bacteria of the nematodes). Knowledge about the rate of accumulation of alive cells in a bacterial culture is important for determining the culture age at which these cells are at maximal density. This knowledge is particularly important for the experiments involved determination of the isolate infectivity to Galleria and its interaction with other pathogens. These two parameters are highly affected by the number of alive cells in a given culture volume and the amount of toxins, antibiotics and other pathogenicity factors secreted by the bacterial cells into the growth medium.

Because of the above considerations, we determined the rate of accumulation of total cells in the culture spectrophotometrically, through measurement of the changes in turbidity with time. The results of this measurement indicated that the culture enters the stationary phase 24 hours after inoculation (Figure 3.5). Culture turbidity is not attributed solely to alive cells, but also to dead cells as well as to extracellular material secreted into the growth medium. Hence, we determined the number of alive cells, capable of dividing and forming colonies at each

growth stage. The results of this determination showed that the number of colony forming units (CFU) is maximal (4.5×10^{11}/ml) when the culture is 24 hours old (Figure 3.6).

Based on the data presented in these two figures we decided to use a 20 hours old culture in all infectivity experiments of this work. At this age the culture is almost at maximum density of C.F.Us and turbidity.

3.2.3 Preliminary tests of infectivity of *Serratia marcescens*, Jericho 11strain against *G. mellonella* larvae.

The infectivity of the newly isolated strain was determined by infecting the Galleria larvae through the digestive tract and by direct injection into the haemocoel. In both cases, suspension culture of bacteria was used, as well as a Millipore - filtered medium of the same culture. Four days after the infection through the food, the mortality reached 40% regardless whether the infecting factor was suspension cells or filtered medium (Table 3.4). However, on the sixth day after infection, the filtered medium caused mortality two folds greater than that caused by the suspension. This difference is probably due to the presence of a repellent substance secreted by the alive bacterial cells and prevented the larvae from approaching the contaminated food. Similar effect of other bacteria-contaminated food was reported on other insects, such as the

adult tsetse, *Glossina morsitans* (Kaaya GP and Darji N 1989), and adult *Heliothis virescens* (Sikorowski and Lawrence 1998).

The mortality effect of *Serratia marcescens* is mediated through hydrolytic activity of chitinases and proteases (Kless, H *et al.*, 1989). These enzymes are necessary for the penetration of the bacterium into the haemolymph through the midgut tissues. Inside the haemolymph the bacteria proliferate rapidly, kill the insect and continue to digest its tissues.

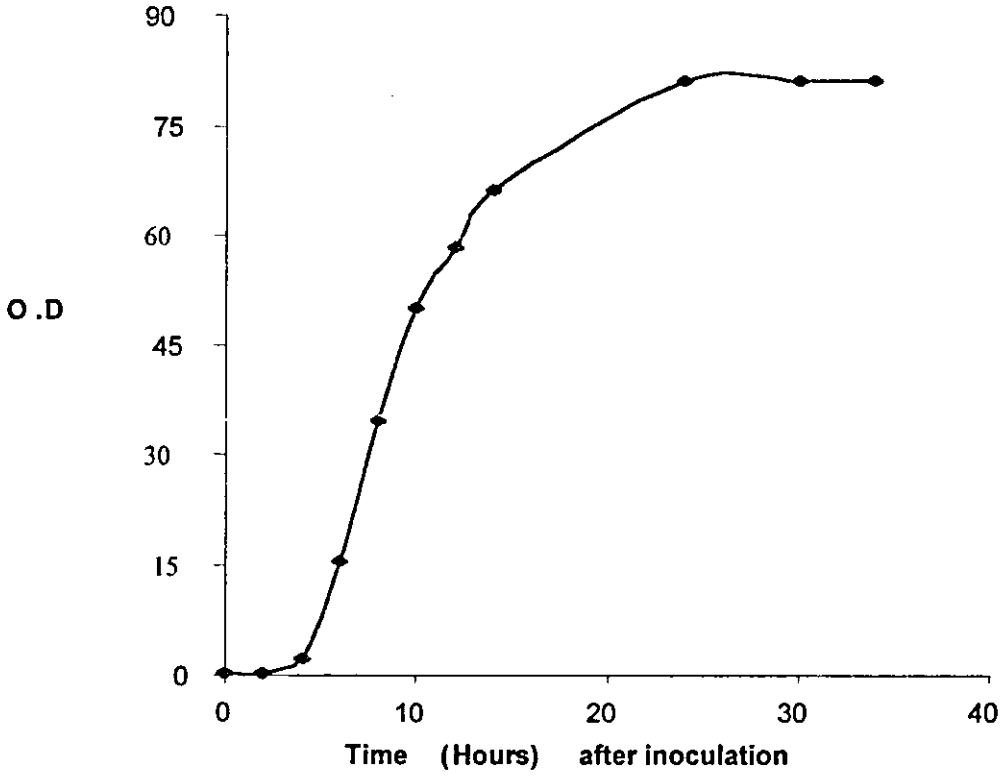


Figure 3.5: Growth curve of *Serratia marcescens* Jericho 11strain on BSA medium. Culture O.D was measured at 560nm.

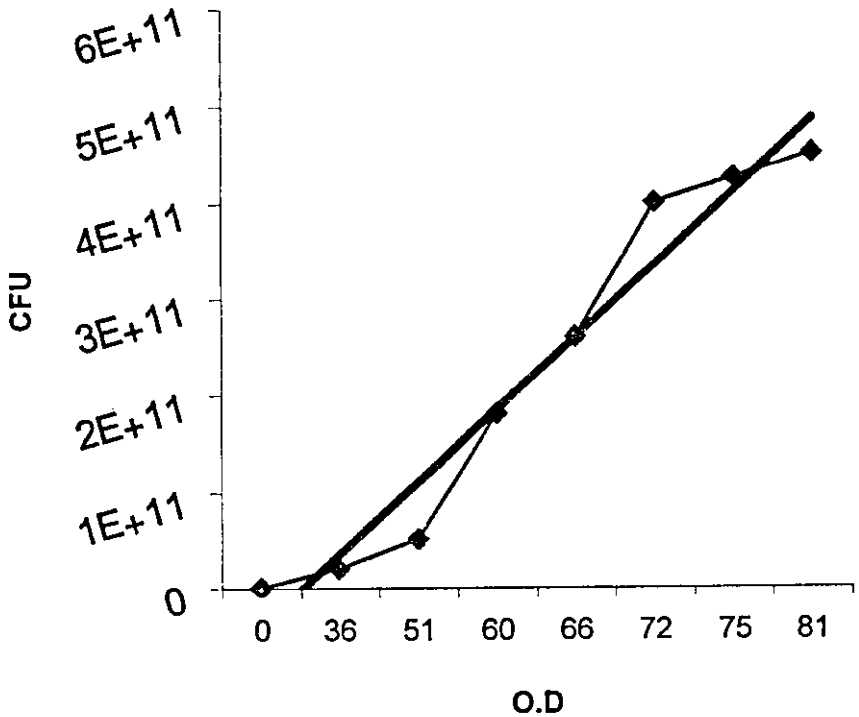


Figure 3.6: Standard curve of *Serratia marcescens*, Jericho 11 strain on BSA medium. Culture O.D. was measured at 560nm.

Table 3.4: Infectivity of *Serratia marcescens,* Jericho 11 strain to larvae of *Galleria mellonella.* The larvae were fed with food mixed with bacterial suspension or with Millipore-filtered medium from the same culture.

	Percent of mortality		
Time (days)	Control	Bacterial suspension	Filtered medium
1	0	0	0
2	0	0	0
3	0	0	0
4	0	40	40
5	0	40	60
6	0	40	80
7	0	60	80

Part Two

A study of the interaction between *H. indica*, *S. marcescens* and *B. bassiana*

3.3 Interaction between *S. marcescens*, *B. bassiana*, and *H. indica* Bethlehem 11strain against *G. mellonella* larvae on filter paper.

S. marcescens may inhabit soil and may infect soil-dwelling insects. Among these insects some are agricultural pests against which biocontrol programs might be applied. Entomopathgenic nematodes are being used successfully as biocontrol agents against soil pests (Barbercheck and kaya, 1991). Hence, it is important to study the effect of *S. marcescens* on the efficiency of entomopathogenic nematodes in controlling insects. For this purpose, Galleria larvae were infected with the nematodeInfective Juveniles (IJs), on a wet filter paper in wells of multi-well plate. Together with, or before this infection the larvae were infected with *S. marcescens*. The effect of *S. marcescens* on the mortality of Galleria and on the recovery of IJs into hermaphrodites were recorded 2 and 4 days after the nematode application, respectively. The results in Table 3.5 show that adding *S. marcescens* cells to the IJs inoculum did not cause a significant change in nematode infectivity to Galleria larvae (95% mortality caused by the combined application of pathogens compared to 100% caused by nematodes alone). However, the ability of IJs to recover into adult hermaphrodite was decreased by more than two folds when they were applied together with *S. marcescens* (42% recovery compared to 17%). This trend was

increased when the larvae were infected with *S. marcescens* 24 and 48 hours before application of IJs (7% and 4%, respectively). Interestingly, when the infection with the bacterial pathogen preceded the application of nematodes, the mortality of the treated larvae decreased by 10%. The sharp decrease in recovery of IJs might be a result of one or several effects of the bacterial cells. First, the *S. marcescens* inhibits the penetration of IJs into the insect. Second, the bacteria secretes, inside the insect, toxins, or antibiotics, that inhibit the proliferation of the nematode symbiotic bacteria leading to inhibition of nematode feeding and recovery. Third, the *S. marcescens* cells secrete digestive enzymes that act directly on IJs or adults, leading to a decreased percent of recovery into hermaphrodites. Finally, it is possible that one or more of these effects act together to reduce nematode recovery.

The slight decrease in mortality of Galleria larvae upon adding another infection with *S. marcescens* before application of nematodes might be attributed to interactions between the Serratia strain and the symbiotic bacteria of the nematodes. The nematodes alone killed 100% of the larvae after 2 days from infection. Whereas, *S. marcescens* cells killed only 10% of the insect population at the end of the same period of infection (Table 3.5). These results imply that at these experimental conditions, the nematodes are more virulent to Galleria larvae than

S.marcescens. At normal conditions, the nematodes kill the insect by means of their symbiotic bacteria, which proliferates and secretes toxins and hydrolytic enzymes. It is possible that the Serratia cells secrete antibiotics and other antibiosis agents that suppress the proliferation of symbiotic bacteria, leading to a slight delay in the lethal effect of the nematode symbiont. Also, it might be equally possible that preinfection with *S. marcescens* decreases the penetration of IJs into the insect. Such an effect would decrease the number of the more virulent symbiotic bacteria as well as the number of adults recovered from IJs.

The fungus *Beauveria bassiana* is a commercial biocontrol agent used to control wide range of agricultural pests (Brian and Joseph, 1996). In some cases it was used in combination with other biocontrol agents, such as entomopathogenic nematodes. Many research works focused on the effect of combining *B. bassiana* with other agents on the mortality of the target insect. However, the effect of *B. bassiana* on the recovery and proliferation of entomopathogenic nematodes was not studied sufficiently (Barbercheck and Kaya, 1989). We conducted preliminary experiments attempting to determine the effect of *B. bassiana* on the recovery of Infective Juveniles inside Galleria larvae. The results indicate that *B. bassiana* causes a substantial inhibition of IJs recovery inside the larvae (Table 3.6).

Table 3.5: Effect of *Serratia marcescens* on the infectivity (measured as percent mortality) of IJs of *H. indica,* strain Bethlehem 11 against *Galleria mellonella* larvae. Mortality of larvae was recorded at the end of the exposure period. When two pathogens were applied sequentially, the mortality was recorded at the end of the exposure period of the second pathogen. The infection of larvae with all pathogens was conducted on filter paper. The percent recovery of IJs was calculated from the number of hermaphrodites recorded inside the dead Galleria in relation to the number of IJs poured on the insect (50IJs).

Pathogen	Exposure period (hours)	%Mortality of Galleria	% Recovery of IJs
IJs	48	100	42
S. marcescens together with IJs.	48	95	17
S. marcescens then IJs	24 48	90	7
S. marcescens then IJs	48 48	90	4
S. marcescens	48	10	-

While the simultaneous application of the fungus and nematodes had no effect mortality, it did decrease the recovery of IJs by more than 50%. Furthermore, when larvae were infected with the fungus for periods of 24, and 48 hours before the application of nematodes, the recovery of IJs into hermaphrodites dropped to 31% and 22%, respectively, compared to 42% in the control. Also, the mortality of Galleria larvae dropped to 85% upon 48 hours preinfection with the fungus, compared to 100% in control. Infecting the larvae for a period of 48 hours with the fungal pathogen alone failed to cause any death of the infected insect. This indicates that at the pathogen concentrations used in this work, the nematodes are much more virulent than *B. bassiana* (Table 3.6). I propose that like the case of *S. marcescens*, the fungus *B. bassiana* acts on the symbiotic bacteria, or directly on the nematodes causing either decrease in IJs penetration or a suppression of the proliferation of the symbiont.

Table 3.6: Effect of *Beauveria bassiana* on the infectivity (measured as percent mortality) of IJs from *H. indica,* strain Bethlehem 11 against *Galleria mellonella* larvae. Mortality of larvae was recorded at the end of the exposure period. When two pathogens were applied sequentially, the mortality was recorded at the end of the exposure period of the second pathogen. The infection of larvae with all pathogens was conducted on filter paper. The percent recovery of IJs was calculated from the number of hermaphrodites recorded inside the dead Galleria in relation to the number of IJs poured on the insect (50IJs).

Pathogen	Exposure period (hours)	%Mortality of Galleria	% Recovery of IJs
IJs	48	100	42
B. bassiana together with IJs.	48	100	19
B. bassiana.	24		
then IJs	48	95	31
B. bassiana	48		
then IJs	48	85	22
B. bassiana	48	0	-

3.4 In-Vitro interaction between the nematode *H. indica* and either *S. marcescens* or *B. bassiana.*

3.4.1 Survival of IJs, and J4 stages of the nematode *H. indica* inside *S. marcescens*, and *B. bassiana* cultures.

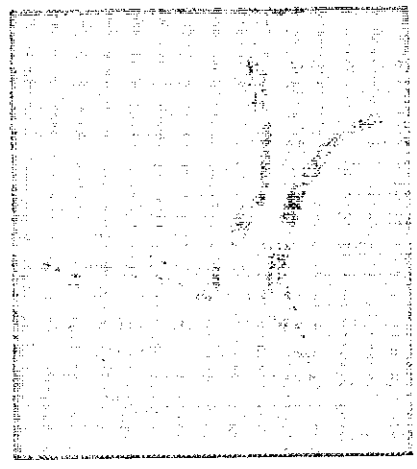
In the previous section it was mentioned that *S. marcescens* and *B. bassiana* may have a direct effect on the survival of the nematode inside the infected larvae. In order to test that, we incubated IJs, and Juvenile 4stages, of the nematode inside cultures of *S. marcescens* and *B. bassiana*. The mixed organisms were kept under continuous shaking for a period of 48 hours. After 24 and 48 hours of incubation, the survival of the nematode Juveniles was determined under the microscope. The results in Table 3.7 show that the survival of the IJs was not affected after 48 hours of incubation neither in the *S. marcescens* nor in the *B. bassiana* cultures. In contrast, the survival of the J4 stage was dramatically reduced to 2% after 48 hours of incubation in the bacterial culture and to 65% in the fungal culture. Certainly we cannot conclude about a difference in the virulence of these two cultures against the J4 stage because the concentration of organisms in each culture is different from the other. On the other hand, we may conclude, at high level of confidence that the J4 stage is much more sensitive to *S. marcescens* culture than the IJ. Similar observation, but less dramatic, could be related to the sensitivity of the J4 to *B. bassiana* culture (Table 3.7). The difference in sensitivity between

these two stages might be related to the fact that IJs are coated with double cuticle that may provide them with a better protection from the hydrolytic enzymes secreted by the bacterial culture. This assumption is supported by the destructive effect of Serratia culture on the J4 tissues (Figure 3.7). A more feasible reason could be the fact that IJs are non-feeding stage and when they recover into the subsequent J4 stage they start feeding immediately. The feeding process employed by the J4 stage may involve ingestion of symbiotic bacteria, or perhaps *S. marcescens* cells and toxins secreted by them.

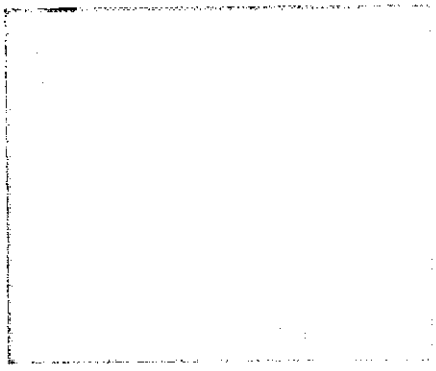
The above findings may provide a strong basis for interpreting the reduced recovery of IJs in Galleria larvae preinfected with *S. marcescens* or *B. bassiana*, which was mentioned in the previous section. Preinfecting Galleria larvae with either of the microbial pathogens may establish, inside the larva, a population of microorganisms that buffers the proliferation of the subsequently introduced nematodes. The toxins and enzymes secreted by these microorganisms may act directly on the feeding J4 stage, which is coated by a single cuticle.

Table 3.7: Percent survival of Infective Juveniles (IJs), Juvenile 4 (J4), of *H. indica,* strain Bethlehem 11 in the presence of either *Serratia marcescens* or *Beauveria bassiana.* known amounts of nematodes were incubated in BSA medium or sterile deionized water and inoculated with 18-20 hours old *S. marcescens* culture or suspension of *B. bassiana* germinated spores. Treatments and control were incubated for 48 hours at continuous shaking (180rpm) in the dark at 25°C.

Time (hours)	Incubation with *S. marcescens*		Control J4 in BSA medium		Incubation with *B. bassiana*		Control J4 in water	
	IJs	J4	IJs	J4	IJs	J4	IJs	J4
24	98.1	65.4	99.2	97.7	97.3	80.8	99.2	94.0
48	98.7	1.9	99.1	95.9	97.1	65.4	98.6	97.7



a



b

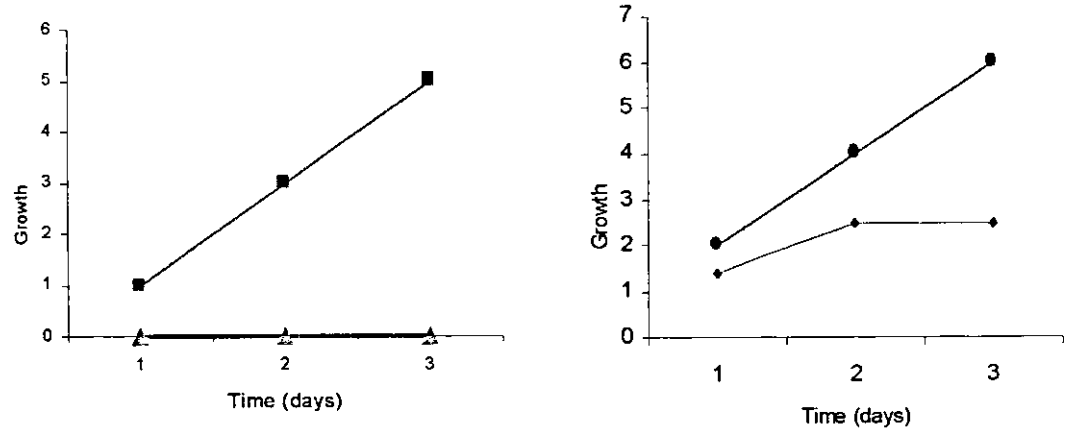
Figue 3.7: The Juvenile 4 stage of the nematode *H. indica* treated with *S. marcescens*:
a: The two large J4 were incubated in *S. marcescens* culture for 48 hours, the small J4 was not treated (control). (10x)
b: A J4 with the red color due to infection with *S. marcescens*. Photographed after 48 hours incubation with *S. marcescens* culture. (40x)

3.4.2 In-Vitro interaction between symbiotic bacteria of the *H. indica* nematode and *S. marcescens*.

In the previous section we hypothesized that the microbial pathogens may suppress the proliferation of the nematode symbiotic bacteria through various antibiosis processes. The consequence of such an inhibition would ultimately lead to a rapid depletion of the food resources available for the developing nematode stages, which in turn would result in a reduced recovery of hermaphrodites. Attempting to find factual basis for the hypothesized in-vivo interactions between the pathogens, we measured the growth rates of the *H. indica* symbiotic bacteria *Photorhabdus luminescens* in the presence of *S. marcescens*. The growth of *P. luminescens* on a smear of *S. marcescens* was totally inhibited (Figure 3.8 a, and 3.9 E), indicating that the latter produces some antibiosis factors, for example prodigiosin and *B*-lactams (Tomohiko sato *et al.*, 1998), that inhibit the growth of the nematode symbiotic bacterium. When a filter paper disc saturated with 20 hours-old *S. marcescens* culture was placed on a smear of *P. luminescens* the growth of the Serratia culture was inhibited by a bout 60% after 3 days of culture compared to the control (Figure 3.8.b, and 3.9 A). These results indicate that there are mutual growth-inhibiting effects between the two pathogens and that the *S. marcescens* is a stronger inhibitor.

These two insect-pathogenic bacteria may have similar interactions inside the Galleria larva. The *S. marcescens* reaches the larval haemolymph through the digestive tract and it is ingested together with contaminated food, soil particles, or filter paper as the case in our experiment (Sikorowski *et al.*, 1992). Since the bacteria penetrates the wall of the digestive midgut by means of proteinases and chitinases, the rate of penetration depends on the amount of cells ingested by the insect. Once the bacterium reaches the haemolymph it proliferates rapidly and kills the insect within 1-3 days (Sikorowski *et al.*, 1992). The symbiotic bacteria reach the haemolymph while carried by their infective juveniles. IJs enter the haemolymph, through midgut or other routes, such as spiracula. When IJs are applied to an insect that has already ingested small amount of *S. marcescens* cells, the physical penetration of the IJs offers an opening through which the bacterial cells may enter and reach the haemolymph. When both kinds of bacteria are present in the haemolymph, they become exposed to mutual inhibitory effects, such as those documented in figures 3.8 a, b, and 3.9 A, and B. The consequence of such effects would be a decrease in the pathological effect of the symbiotic bacterium on the infected insect, which in turn may lead to a delay in mortality. The results presented in the previous section (Table 3.5) show that the mortality of larvae caused by Infective Juveniles is 90% if the Juveniles are applied after preinfection with *S. marcescens*

compared to a 100% mortality when the nematodes were applied without preinfection with the bacterium. We suggest that this reduced mortality is attributed to the inhibitory effect of *S. marce*scens on the proliferation of the virulent symbiotic bacterium.



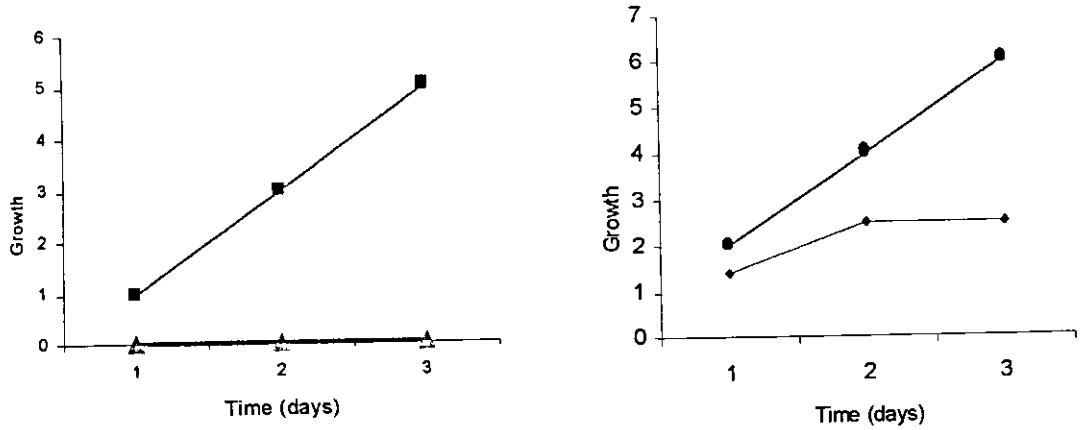
a b

Figure 3.8: Growth rates on BSA medium, measured as an increase in culture diameter (mm), of *P. luminescens* on a smear of *S. marcescens* (a), and of *S. marcescens* on a smear of *P. luminescens* (b).

a:
- *P. luminescens* alone ■
- *P. luminescens* on *S. marcescens* smear ▲

b:
- *S. marcescens* alone ●
- *S. marcescens* on *P. luminescens* smear ◊

bacterium. As for the *Serratia marcescens* we also propose here that these in-vitro interactions could be a basis for explaining the delayed mortality of Galleria larvae when *B. bassiana* spores were applied 48 hours before IJ application (Table 3.6). If the fungal mycelium inhibited the growth of *P. luminescens* in-vitro, then it is likely that same effect may take place inside the Galleria larvae. Some antibiosis agents could be secreted by the fungus inside the larva where they suppress the proliferation of the insect pathogenic symbiotic bacteria leading to a slower death of the insect. The recovery of IJs into J4 and then into adults depends to a large extent on the amount of symbiotic bacteria on which these stages feed. Hence, if symbiotic bacteria become scarce, the recovery of IJs into adults will be reduced.



a b

Figure 3.10: Growth rates on BSA medium, measured as an increase in culture diameter (mm), of *P. luminescens* on a smear of germinated *B. bassiana* spores (a), and of germinated *B.bassiana* spores on a smear of *P. luminescens* (b).

a:

- *P. luminescens* alone ■
- *P. luminescens* on smear of germinated *B. bassiana* spores ▲

b:

- *B. bassiana* alone ●
- *B. bassiana* on *P. luminescens* smear ◊

3.5 Effect of *S. marcescens*, and *B. bassiana*, on the penetration, recovery, and proliferation of *H. indica*, Bethlehem 11 strain inside *G. mellonella* larvae.

In section 3.3 we discussed the work aimed at investigating the effect of the two microbial insect-pathogens *S. marcescens* and *B. bassiana* on the infectivity and recovery of IJs. The recovery, measured in these experiments, was the total number of advanced J4 stage and hermaphrodites detected in each larva and expressed as percent of total number of IJs that were applied to the insect during infection. In section 3.4 we discussed results of in-vitro interactions between the symbiotic bacteria and the other two microbial pathogens. These results provided us with a basis for explaining the observed reduced recovery and pathogenicity of the insect-pathogenic nematode. However, reduced recovery (when recovery is calculated as described above) may result not only from certain inhibitory effects of the pathogens on the symbiotic bacteria or of some hydrolytic enzymes that hydrolyze the tissues of the developing nematode stage, but also merely from reduced penetration of the IJs into the larva. Therefore, it was necessary to determine the effect of infecting Galleria larvae with *S. marcescens* and *B.bassiana* on the capability of the IJs to penetrate into the insect.

3.5.1 Effect of *S. marcescens* and *B. bassiana* on the penetration and recovery of H. *indica* Infective Juveniles inside *G. mellonella* larvae.

In order to unify the system for pathogen application, an appropriate volume of pathogen suspension of known concentration was mixed with sand to from a 10% moist medium. The Galleria larvae were trapped in the inoculated sand and incubated for 24 hours. When the infectious sand contained IJs alone or a mixture of IJs and *S. marcessens*, all of the larvae in the sample died as early as two days from the start of the infection (Table 3.8). This result implies that adding *S.marcessens* to IJs does not increase mortality. Interestingly, if cells of *S.marcescens* were introduced to larvae 24 hours before application of IJs, the mortality of larvae was reduced to 86% compared to 100% in control (Table 3.8). Similar trend was observed with *B.bassiana* –preinfected larvae. Sequential infection of larvae with *B.bassiana* for 24 hours followed by infection with IJs for the same period of time resulted in 55% mortality, 3 days after the first infection, which is 2 days after the application of nematodes (Table 3.8). In summary, both *S. marcescens* and *B. bassiana* when applied 24 hours before the IJs, they reduce, the mortality caused by nematodes. This observation is similar to the one observed when the infection was carried out on filter paper (section 3.3). Reduced mortality of target insects as a result of dual application of two kinds of entomopathogenic nematodes

was reported in the literature recently (Choo *et al.*, 1996). Authors of these reports suggested that when the competition between the two applied pathogens involves mutual antibiosis, it will lead to a reduced mortality of the host. Under the conditions used in our study, the nematodes exhibited more virulence to Galleria larvae than *S. marcescens* or *B. bassiana* (Tables 3.5 and 3.6). If each of the latter two pathogens suppresses the growth and reproduction of the symbiotic bacteria, as was shown in sections 3.4.2 and 3.4.3, then the ultimate consequence of such a suppression would be reduced fatality of the host.

However, we should not neglect the possibility that the reduction in percent mortality could be a result of decreased penetration of IJs into the larvae upon preinfection with *S. marcescens* or *B. bassiana*. For determining the penetration of nematodes into the insect, the nematode-infected larvae were washed and digested in pepsin to uncover the penetrated IJs. These IJs are resistant to pepsin digestion due to their thick cuticle. The number of detected IJs in each larva was calculated as percent of the total number of the nematodes that were applied during the infection. Preinfecting the Galleria larvae for 24 hours with either *S. marcescens* or *B.bassiana* significantly reduced the penetration of IJs from 7.37% in the control to 2.8% and 1.72% respectively (Table 3.8). A non siginficant effect on penetration was observed when each of the microbial pathogens was applied simultaneously with the IJs (5.80 and 6.0%). A

decreased penetration of IJs into a host that has already been infected with other nematode was reported by several workers (Hominick 1990). They proposed that the infected larvae secrete certain substance that is sensed by IJs and causes them to avoid penetration into the infected insect. This kind of behavior has an obvious biological importance in that it prevents overpopulation of the host, which may lead to a detrimental competition on food resources. To the best of our knowledge, there are no reports in the literature documenting similar behavior of nematodes when the insect is infected by bacterial pathogens. We propose that the low penetration into Galleria larvae preinfected with *S. marcescens* or *B.bassiana* is due to secretion of certain substance either by the infected larvae, or the infecting pathogen. The hypothesized substance, in turn, repells the Infective Juveniles, or neutralizes the chemical attraction existing between nematodes and insects. The symbiotic bacteria inoculum, released into the haemolymph of Serratia or Beauveria preinfected larva, will be in small quantity due to a decreased number of penetrating IJs. The presence of a limited inoculum of the symbiotic bacteria will be reflected in a lower level of virulence against the host and in a weaker antibiosis reaction against the pathogen that had already colonized the insect. As a result, the growth of the symbiotic bacteria remains inferior to that of the reinfecting pathogen, which continues to proliferate and to exert its inhibitory effects on nematode development.

Table 3.9: Recovery of Infective Juveniles (IJs) of the nematode *H. indica* injected into *Galleria mellonella* larvae after preinfection with *Serratia marcescens* and *Beauveria bassiana.*

Preinfecting pathogen	Exposure period (hours)	Average number of injected IJs/larva	Mean % recovery	% Mortality 2 days after injection
Control		27.1	71.90	100
S. marcescens	24	31.1	02.65	100
B. bassiana	24	33.7	78.80	100
B. bassiana	48	34.3	58.30	Not determined
B. bassiana	72	46.0	47.60	Not determined
B. bassiana	96	36.0	0	Not determined

this level of inhibition is close to the one observed when IJs naturally penetrated into the preinfected larva (Table 3.8). This result implies that 31 IJs and their symbiotic bacteria inside preinfected Galleria are still insufficient to overcome the antibiosis of *S marcescens*. Injection of IJs into larvae preinfected for 24 hours with *B. bassiana*, showed the same level of recovery as in control (Table 3.9). However, at natural penetration of IJs, the recovery was 30% less than that of the control (Table 3.8). This level of inhibition is probably related to the small number of natuarlly penetrated IJs, which released a small amount of symbiotic bacteria. The fact that we could eliminate the inhibitory effect of a 24 hours-preinfection by increasing the number of IJs, implies that a larger inoculum of symbiotic bacteria could overcome the antibiotic effects of a not well - established infection of *B. bassiana*. On the other hand, under preinfection periods of 48, and 72 hours the recovery dropped below the control level to 58 and about 48% respectively. These drops in recovery reflect the suggested harmful antibiosis action of *B. bassiana* against the nematodes. Extending the period of preinfection allows establishment of a more developed mycelium inside the infected larva. The well-developed mycelium would secrete larger amounts of antibiosis substances that will act on the nematode symbiotic bacteria or directly on the nematodes. This trend continued till we reached a null recovery when the preinfection period lasted 96 hours. In summary, under conditions used in our

experiments, increasing the number of IJs inside the 24 hours- preinfected larvae did improve the recovery level when the preinfecting pathogen was *B. bassiana*, but it had no effect when the preinfectant was *S. marcescens.*

In previous work we observed that when larvae were preinfected for 24 hours with *S. marcescens* and *B. bassiana* before IJs application, the mortality caused by nematodes infection dropped to 86% and 55% respectively. We suggested that the observed drop is due to antibiotic action of the preinfecting pathogen against the virulent symbiotic bacteria, or to a limited inoculum of symbiotic bacteria resulting from inhibited penetration of IJs (2.8 and 1.7 IJs per larva- Table 3.8). The data presented in table 3.9 indicate that two days after injecting 31 and 34 IJs into Serratia- or Beauveria preinfecting larva, respectively, the mortality was 100%, which is the same level as in control. Hence, increasing the number of IJs infecting each Galleria from 2.8 and 1.7 to 31 and 34 has increased larvae mortality from 86% and 55% to 100%. This observation strongly supports our previous assumption that the reduced mortality, caused by IJs in larvae that had been infected with the Serratia and Beauveria pathogens, is a consequence of inhibited IJ penetration and limited inoculum of symbiotic bacteria.

It should be pointed out that when IJs were injected into a non preinfected Galleria, the percent recovery was lower than that obtained when IJs were applied by normal penetration (71.9% -Table 3.9, compared

to 95% -Table 3.8). We propose that the observed difference is related to natural variation among individuals of the same population of IJs. Such a variation was reported by Hominick *et al* (1990). They found that not all individuals of a given population of IJs are capable of penetrating into the insect. Furthermore, not all of those that do penetrate can recover into adults. We injected into the larvae a population of IJs that had not passed through selection for penetration potency. The recovery potential of these non-selected individuals is probably lower than that of individuals capable of penetrating into the insect by themselves. In other words, there might be a certain level of correlation between penetration capability and recovery of IJs into adults.

recommendation concerning the use of *S. marcescens*, *B. bassiana*, and insect-pathogenic nematodes in a biocontral program, it was necessary to study the effect of these bacterial and fungal pathogens on the reproductive capability of the nematode inside the infected insect. For this purpose, Galleria larvae that were infected with microbial pathogens under various infection regimes were exposed to the nematode IJs for 24 hours and left to die in a petridish. The dead larvae were then transferred to mini White traps and left for two weeks. Part of the larvae sample were used for testing the presence of the microbial pathogens by streaking on NBTA medium before transfer to White trap.

The results presented in table 3.10 indicate that in the absence of *S. marcescens* and *B. bassiana*, one single Galleria larvae might accommodate the production of 106 $x10^3$ nematodes (mainly IJs). If the larva is exposed to IJs together with *S. marcescens*, however, the total production of nematodes per larva drops to $5 x10^3$, a reduction of more than 21 fold. Furthermore, when larvae were exposed *to S. marcescens* for 24 hours before the application of IJs, the nematode could not reproduce at all (Table 3.10). We have shown that under the latter infection regime, some IJs do penetrate (2.8%) and recover (32%) inside *the S. marcescens*-infected larvae. Our failure to detect any nematode production neither in White trap nor in insect cadaver, after two weeks of incubation (Table 3.10, and Figure 3.14), strongly indicates a direct action of *S. marcescens*

on the small number of adults that recovered from the penetrated IJs. Based on our observation concerning the survival of IJs and hermaphrodites in *S. marcescens* culture (section 3.4.1), we propose that *S. marcescens* cells inside the infected larva have secreted hydrolytic enzymes, which were responsible for the digestion of either J4 or hermaphrodites, which recovered from the small number of IJs that had penetrated into the insect. Moreover, this effect could be achieved only if the *S. marcescens* inoculum is given sufficient time for proliferation and secretion of an adequate amount of hydrolytic enzymes before application of nematodes. When the exposure time to *S. marcescens* is parallel to that of nematodes (simultaneous application), the nematode Infective Juveniles reach the haemolymph and release their symbiotic bacteria in a shorter period of time than that required for *S. marcescens*. As a result, the symbiotic bacteria inoculum proliferate and allow recovery of IJs into adults before the *S. marcescens* cells succeed to multiply and spread in the whole insect's body. Furthermore, the antibiotic effect of the symbiotic bacterium against S. *marcescens* may reduce the hydrolytic effect of the latter against the developing nematode stages. This effect would ultimately lead to a partial nematode proliferation (Table 3.10). It should be pointed out that *S. marcescens* cells were discovered in haemolymph of larvae preinfected with the bacterium as well as in larvae infected simultaneously with the bacterium and IJs (Table 3.10). This finding indicates that under

the conditions used inour eperiments, entomopathogenic nematodes can coexist and reproduce together with *S. marcescens,* only if both pathogens are applied simultaneously.

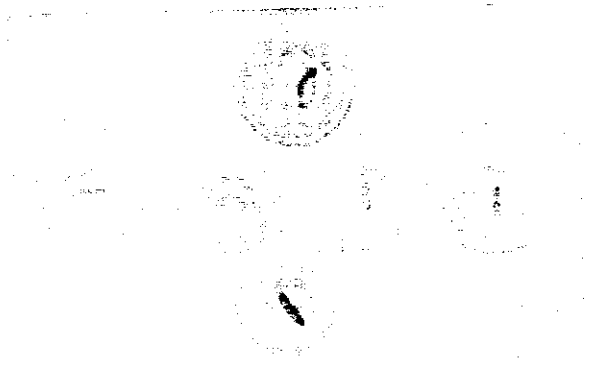


Figure 3.14: White trap used to collect Infective Juveniles from infected Galleria larvae for determination of total nematode production under various infection conditions with the microbial pathogens *S. marcescens* and *B. bassiana*:
Top: Control, Galleria infected with *H. indica* IJs.
Bottom: red Galleria cadaver of larva infected with *S. marcescens* before IJ infection.
Middle: cadavers of larvae that were infected with *B. bassiana* for (from right to left) 24, 48, 72,and 96 hours before the nematode infection.

To investigate the effect of *B. bassiana* on the reproduction of the nematode *H. indica* we conducted five types of infection regimes; simultaneous application of the fungus with the nematode, application at 24,48,72, and 96 hours before nematode infection. The results (Table 3.10) show that under all of these infection regimes, the total production of nematodes was substantially inhibited, but at different levels of inhibition. Simultaneous application of *B. Bassiana* and IJs allowed production of 35 $x10^3$ nematodes per larva compared to 106 $x10^3$ in the control. This means an inhibition of about 3 folds. A more severe inhibition of nematode production was observed when the larvae were infected with the fungus for 24 hours before nematode application. This relatively short period of preinfection was sufficient to cause about 50-fold inhibition of nematode production (2.6 $x10^3$ compared to 106 $x10^3$ in the control). Preinfecting the larvae with the fungus for periods of 48 hours and longer caused a total inhibition of nematode reproduction. These results indicate that the more developed mycelium in the infected larva, the stronger the effect that it exerts either directly on the developing stages of nematodes or on the proliferation of their symbiotic bacteria. The moderate inhibition caused by the simultaneous application of the fungus and nematodes is probably due to the relatively longer time period required for the fungus to penetrate into the insect. The symbiotic bacteria is carried into the insect haemolymph by means of the actively moving infective juveniles, a process which is most

likely faster than the germination of the fungal spores and their penetration of the cuticle. However, if the symbiotic bacteria reach the haemolymph faster, the results indicate that they cannot totally inhibit the growth of the mycelium nor could they eliminate its effect. This assumption is based on the results presented in table 3.10 where, the simultaneously infected and the 24 hours-preinfected larvae showed development of mycelium in their haemolymph. Moreover, the fact that this mycelium could not penetrate to the external surface of the dead insect and reach the sporulation stage implies that the fungus was affected by antibiosis exerted by the nematode symbiotic bacteria. Indirect support to this explanation comes from the observation that mycelial penetration to the outer surface of the cadaver and sporulation took place only in treatments that inhibited nematode reproduction (preinfection for 48 hours and longer). In general we may conclude that the fungus *B. bassiana* and the nematode *H. indica* cannot co reproduce in the same insect. Similar observation was reported by Barbercheck and Kaya (1990). The above conclusion has a great practical importance when planning a biocontrol program involving combined application of *B. bassiana* and insect-pathogenic nematodes.

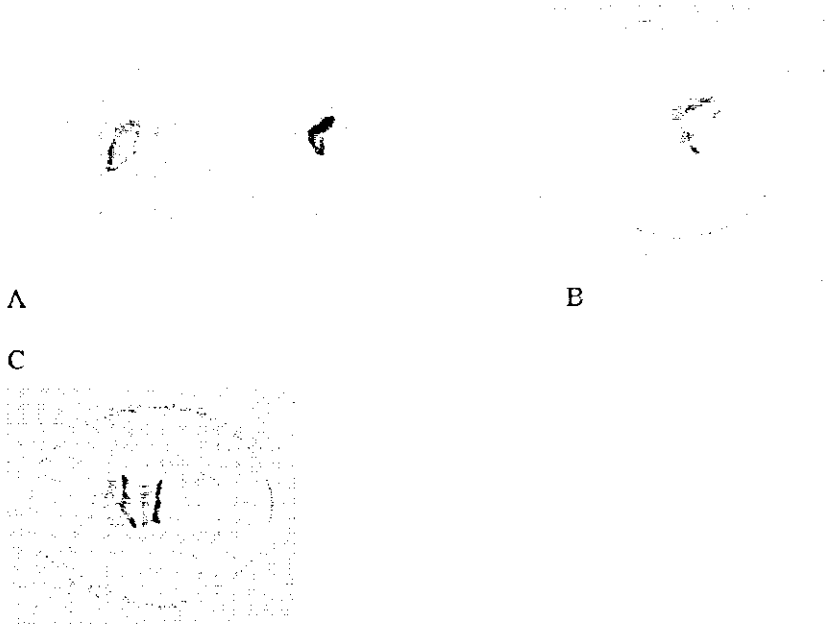


Figure 3.15: Typical colors of Galleria cadavers resulting from infection with different pathogens A; The red color due to infection with *S. marcescens*, B; White color (mycelium) due to infection with *B. bassiana* , C; Orange color due to infection with *H. indica,* Bethlehem 11 strain.

RECOMMENDATIONS:

Results of our work showed that entomopathogenic nematodes are sensitive to antibiotic effects that might be imposed by the *S. marcescens* new isolate. These effects were reflected in inhibiting the nematode penetration into the insect and its proliferation inside the cadaver. Based on these findings we recommend the following:

1- Avoid application of EPNs for pest control in *S. marcescens*-infested soils.

2- Apply severe precaution steps to prevent contamination of EPN mass production plants with this bacterium.

3- Develop a new research direction aiming at investigating the effect of this bacterium on the survival and development of plant parasitic nematodes.

The fungus *B. bassiana* is an insect-pathogen, commercially used biocontrol agent. Our work showed that it couldn't co-reproduce together with entomopathogenic nematodes inside the same insect. The capability to reproduce inside the target insect is one of the main required characteristics of a successful biocontrol agent. Hence, a very important recommendation stemming from our work is as follows:

4- Avoid using a combination of *B. bassiana* and the entomopathogenic nematode *H. indica* in the same biocontrol program. Also, we

recommend not using this entomopathogenic nematode in a field that has been treated with *B. bassiana*.

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Appendix I: statistical analysis

Paired Samples t - test for the data presented in this work.

Table I: Penetration of IJs into Galleria larvae (data from table 3.8). S: *S. marcescens*, N: nematode, B: *B. bassiana*, A: simultaneous application, C: control, P: penetration. 1,2,3,4: number of days of preinfection before nematode application.

Pair	Paired samples statistics				df	t	Sig. (2tailed)
	Mean	SD	95% Confidence Interval of the Difference				
			Lower	Upper			
S1NP – NCP	-6.20	4.21	-7.83	-4.56	27	-7.783	.0001
SANP - NCP	-1.55	4.33	-3.23	.125	27	-1.899	.068
B1NP – NCP	-5.65	3.66	-7.07	-4.23	27	-8.174	.0001
B2NP – NCP	-5.92	4.23	-7.56	-4.28	27	-7.411	.0001
B3NP – NCP	-5.67	4.04	-7.24	-4.11	27	-7.435	.0001
B4NP – NCP	-7.37	4.65	-9.17	-5.56	27	-8.385	.0001
BANP – NCP	-1.37	4.60	-3.16	.405	27	-1.586	.124

Table II: Recovery of IJs into adults (data from table 3.8). S: *S. marcescens*, N: nematode, B: *B. bassiana*, A: simultaneous application, C: control, R: recovery. 1,2,3,4: number of days of preinfection before nematode application.

Pair	Paired samples statistics				df	t	Sig. (2tailed)
	Mean	SD	95% Confidence Interval of the Difference				
			Lower	Upper			
S1NR – NCR	-6.42	2.22	-8.45	-4.40	27	-6.512	.0001
SANR – NCR	-3.28	3.85	-4.77	-1.78	27	-4.503	.0001
B1NR – NCR	-5.85	4.41	-7.56	-4.14	27	-7.026	.0001
B2NR – NCR	-7.03	4.83	-8.91	-5.16	27	-7.701	.0001
B3NR – NCR	-7.03	4.83	-8.91	-5.16	27	-7.701	.0001
B4NR – NCR	-7.03	4.83	-8.91	-5.16	27	-7.701	.0001
BANR – NCR	-3.92	4.08	-5.51	-2.34	27	-5.093	.0001

Table III: Recovery of injected IJs into adults (data from table 3.9). S: *S. marcescens*, N: nematode, B: *B. bassiana*, A: simultaneous application, C: control, R: recovery. I: injection 1,2,3,4: number of days of preinfection before nematode application.

Pair	Paired samples statistics				df	t	Sig. (2tailed)
	Mean	SD	95% Confidence Interval of the Difference				
			Lower	Upper			
S1NRI – NCRI	-69.3	19.4	-76.78	-61.71	27	-18.85	.0001
B1NRI – NCRI	-6.92	23.7	-2.268	-16.12	27	-16.12	.134
B2NRI – NCRI	-13.6	29.1	-24.92	-2.335	27	-2.335	.020
B3NRI – NCRI	-24.2	48.4	-43.01	-5.470	27	-5.470	.013
B4NRI – NCRI	-71.9	18.7	-79.16	-64.65	27	-64.65	.0001

Table IV: Reproduction of nematodes (data from table 3.10). S: *S. marcescens*, N: nematode, B: *B. bassiana*, A: simultaneous application, C: control, R: Reproduction. 1,2,3,4: number of days of preinfection before nematode application.

Pair	Paired samples statistics				df	t	Sig. (2tailed)
	Mean	SD	95% Confidence Interval of the Difference				
			Lower	Upper			
S1NR– NCR	-106000	1784.7	-106692	-105308	27	-314.2	.0001
SANR – NCR	-101000	1774.5	-101688	-100312	27	-301.1	.0001
B1NR – NCR	-103840	1885.4	-104601	-103078	25	-280.8	.0001
B2NR – NCR	-106000	1784.7	-106692	-105308	27	-314.2	.0001
B3NR – NCR	-106000	1784.7	-106692	-105308	27	-314.2	.0001
B4NR – NCR	-106000	1784.7	-106692	-105308	27	-314.2	.0001
BANR – NCR	-70800	1785.7	-71492.4	-70107.5	27	-209.7	.0001

api 20 E

Ref. 20 100

ENTIFICATION SYSTEM FOR
ITEROBACTERIACEAE AND OTHER
RAM-NEGATIVE RODS

struction Manual
rsion C

I 20 E is a standardized identification system for Ente-
bacteriaceae and other non-fastidious Gram-negative
ds which uses 23 miniaturized biochemical tests and a
ta base. The complete list of those organisms that it is
ssible to identify with this system is given in the identi-
ation table (p. 22 of this instruction manual).

Inciple

e API 20 E strip consists of 20 microtubes containing
hydrated substrates. These tests are inoculated with a
cterial suspension which reconstitutes the media.
ring incubation, metabolism produces colour changes
at are either spontaneous or revealed by the addition
reagents.

e reactions are read according to the **Interpretation**
ble and the identification is obtained by referring to
e **Identification Table**, the **Analytical Profile Index** or the
ntification software.

The API 20 E kit allows the performance of 25 identifications. It consists of :

- 25 API 20 E strips
- 25 incubation boxes
- 25 report sheets
- 1 clip seal
- 1 instruction manual.

To use API 20 E, the following are necessary :

- Suspension Medium, 5 ml (ref. 20 110)
- Reagent kit (ref. 20 120) or the individual reagents (ref. 70 400 to 70 460 and 70 540)
- Zn reagent (ref. 70 380)
- Mineral oil (ref. 70 100)
- Pipettes or PSIpettes (ref. 70 250)
- API 20 E Analytical Profile Index (ref. 20 190) or identification software
- Ampoule rack (ref. 70 200)

The following additional products when necessary :

- API OF Medium (ref. 50 110). Test for the determination of fermentative or oxidative metabolism.
- API M Medium (ref. 50 120). Test for motility of facultative anaerobic bacteria.

Plus the following general laboratory equipment :

- incubator (35-37°C), refrigerator, Bunsen burner, marker pen.

Storage

API 20 E strips are in an aluminium pouch. Once opened the pouch should be re-sealed using the clip seal (supplied with the kit) to preserve the remaining strips : place the open end of the pouch along the seal and carefully clamp between the two parts. The strips may then be kept for up to 10 months after the pouch has been opened, at 2-8°C (or until the expiry date indicated on the packaging, if this comes before).
Most of the reagents should also be stored at 2-8°C and may be kept for up to 1 month after the ampoules have been opened.
The **OX** reagent is very sensitive to light : wrap the bottles in aluminium foil.

Composition of media and reagents

- *Suspension Medium* (ref. 20 110) : demineralized water
- **TDA** reagent (ref. 70 400) for the detection of tryptophane deaminase:

ferric chloride	3.4 g
demineralized water	100 ml

- Reagents for the detection of indole

JAMES Reagent (ref. 70 540)

Compound J 2183	0.5 g
HCl N qsp	100 ml

or

IND reagent

paradimethylaminobenzaldehyde	5 g
isoamyl alcohol	75 ml
HCl 37 %	25 ml

- Voges Proskauer reagents for the detection of acetoin :

VP 1 (ref. 70 420)

potassium hydroxide	40 g
demineralized water	100 ml

VP 2 (ref. 70 430)

alpha naphthol	6 g
ethanol	100 ml

- Griess reagent for the detection of nitrites :

NIT 1 (ref. 70 440)

sulfanilic acid	0.8 g
acetic acid 5N	100 ml

NIT 2 (ref. 70 450)

N-N-dimethyl-1-naphthylamine	0.6 g
acetic acid 5N	100 ml

- **OX** (ref. 70 460) for the detection of oxidase :

tetramethyl-p-phenylenediamine	1 g
isoamyl alcohol	100 ml

1. Preparation of the strip

- prepare an incubation box, tray and lid, and distribute about 5 ml of water into the honeycombed wells of the tray to create a humid chamber.
- record the strain reference on the elongated tab of the tray.
- place the strip in the tray.
- perform the oxidase test on an identical colony as follows :
  - place a piece of filter paper on a glass slide.
  - moisten the paper with 1 drop of water.
  - take the chosen colony with a wooden or glass applicator and rub it onto the moistened filter paper.
  - add 1 drop of **OX** reagent
  - a deep VIOLET coloration which appears within 1 or 2 minutes indicates a POSITIVE REACTION.

NOTE : API 20 E should only be used with non-fastidious Gram-negative rods. Fastidious organisms having demanding nutritional requirements and requiring appropriate handling precautions (i.e. *Brucella* and *Francisella*) are not included in the **API 20 E** database. Alternative procedures must be used to exclude or confirm their presence.

2. Preparation of the inoculum

- open an ampoule of **Suspension Medium** (ref. 20 110) (or sterile distilled water without additives.)
- with the aid of a pipette, remove a single well-isolated colony from an isolation plate.
- carefully emulsify to achieve a homogeneous bacterial suspension.

3. Inoculation of the strip

- with the same pipette, fill both the tube and cupule of tests CIT, VP, GEL with the bacterial suspension.
- fill only the tubes (and not the cupules) of the other tests.
- create anaerobiosis in the tests ADH, LDC, ODC, URE and H_2S by overlaying with **mineral oil.**
- close the incubation box and incubate at 35-37°C for 18-24 hours.

4. Reading of the strip

- after 18-24 hours at 35-37°C, read the strip by referring to the **Interpretation Table.**
- record all spontaneous reactions on the **report sheet.**
- **If the glucose is positive** and/ or 3 tests or more are positive : reveal the tests which require the addition of reagents.
  - **VP Test** : add 1 drop of **VP 1** and **VP 2** reagents. Wait at least 10 minutes. A BRIGHT PINK or RED colour indicates a POSITIVE reaction to be recorded on the **report sheet.**
  - **TDA Test** : add 1 drop of **TDR** reagent. A DARK BROWN colour indicates a POSITIVE reaction to be recorded on the **report sheet.**
  - **IND Test :** add 1 drop of **JAMES** reagent. The reaction takes place immediately : a PINK colour developed in the whole cupule indicates a POSITIVE REACTION to be recorded on the **report sheet.**
    or
    add 1 drop of **IND** reagent. Wait 2 minutes. A RED RING indicates a POSITIVE reaction to be recorded on the **report sheet.**
  - **NO_2 Test :** add 1 drop of each of **NIT 1** and **NIT 2** reagents to the GLU tube. Wait 2 to 3 minutes. A RED colour indicates a POSITIVE reaction. A negative reaction (yellow) may be due to the reduction to nitrogen (as sometimes evidenced by gas bubbles) : add 2 to 3 mg of **Zn** to the GLU tube. After 5 minutes, if the tube remains YELLOW this indicates that (N2) is POSITIVE and is to be recorded on the **report sheet.** If the test turns PINK-RED, this is a NEGATIVE reaction ; the nitrates still present in the tube have been reduced by the Zinc.
- **If the glucose is negative** and the number of positive tests is less than or equal to 2, do not add reagents.
  - inoculate 2 **API OF Medium** to confirm the metabolism of glucose.
  - streak a MacConkey agar plate.
  - check for motility by inoculating 1 **API M Medium** for fermentative organisms or by microscopic observation.
  - reincubate for 24 hours.
  - add the reagents as described above.
  - record the strip and supplementary test results on the **report sheet** by referring to the **Interpretation Table.**

5. Identification

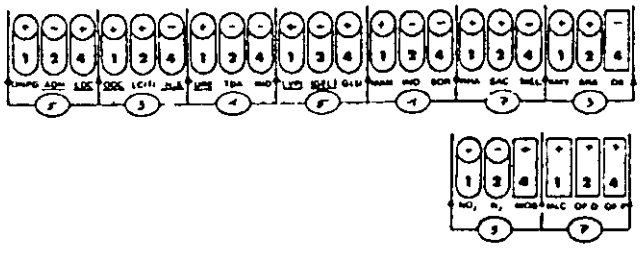
- using the **IDENTIFICATION TABLE** : compare the results recorded on the **report sheet** with those given in the table.
- with the **Analytical Profile Index** or the identification software : the pattern of the reactions obtained must be coded into a NUMERICAL PROFILE.

On the **report sheet,** the tests are separated into groups of 3 and a number 1, 2, or 4 is indicated for each. By adding the numbers corresponding to POSITIVE reactions within each group, a 7-digit profile number is obtained for the 20 tests of the **API 20 E** strip. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

In some cases, the 7-digit profile is not discriminatory enough and supplementary tests should be carried out :

- reduction of nitrates to nitrites (NO_2)
- reduction of nitrates to N_2 gas (N_2)
- motility (MOB)
- growth on MacConkey agar medium (McC)
- oxidation of glucose (OF-O)
- fermentation of glucose (OF-F)

Example :



5 315 173-57 : Enterobacter gergoviae

the **Technical Assistance Service** : allows the bacteriologist to consult our laboratory for any unlisted profiles. In all cases, features of interest such as source of the specimen, colonial and microscopic morphology, patient history, serology etc., must be taken into consideration for the identification obtained.

Disposal

ter use, all ampoules, strips and incubation boxes must
autoclaved, incinerated, or immersed in a disinfectant
ior to disposal.

Quality Control

The media, strips and reagents are systematically quality controlled at various stages of their manufacture. For those who wish to perform their own quality control tests, it is recommended that the following stock cultures be used, to obtain the results below.

1. *Klebsiella pneumoniae pneumoniae* NCTC 8172
2. *Enterobacter cloacae* ATCC 13047
3. *Proteus vulgaris* ATCC 13315
4. *Pseudomonas aeruginosa* ATCC 10145

	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
1.	+	−	+	−	+	−	+	−	−	+	−	+	+	+	+	+	+	+	+	+	−
2.	+	+	−	+	+	−	−	−	−	+	−	+	+	−	+	+	+	+	+	+	−
3.	−	−	−	−	−	+	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−
4.	−	+	−	−	+	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	+

Limitations

The API 20 E systems is intented for the identification of those non-fastidious, Gram-negative rods included in the database (see identification table on p. 22) and exclusively for them. It cannot be used to identify any another organisms or to exclude their presence.

Interpretation of the test results should be made by a competent microbiologist who should also take into consideration the patient history, the source of the specimen, colonial and microscopic morphology and, if necessary, the results of any other tests performed, particularly the antimicrobial susceptibility patterns.

api 20 E

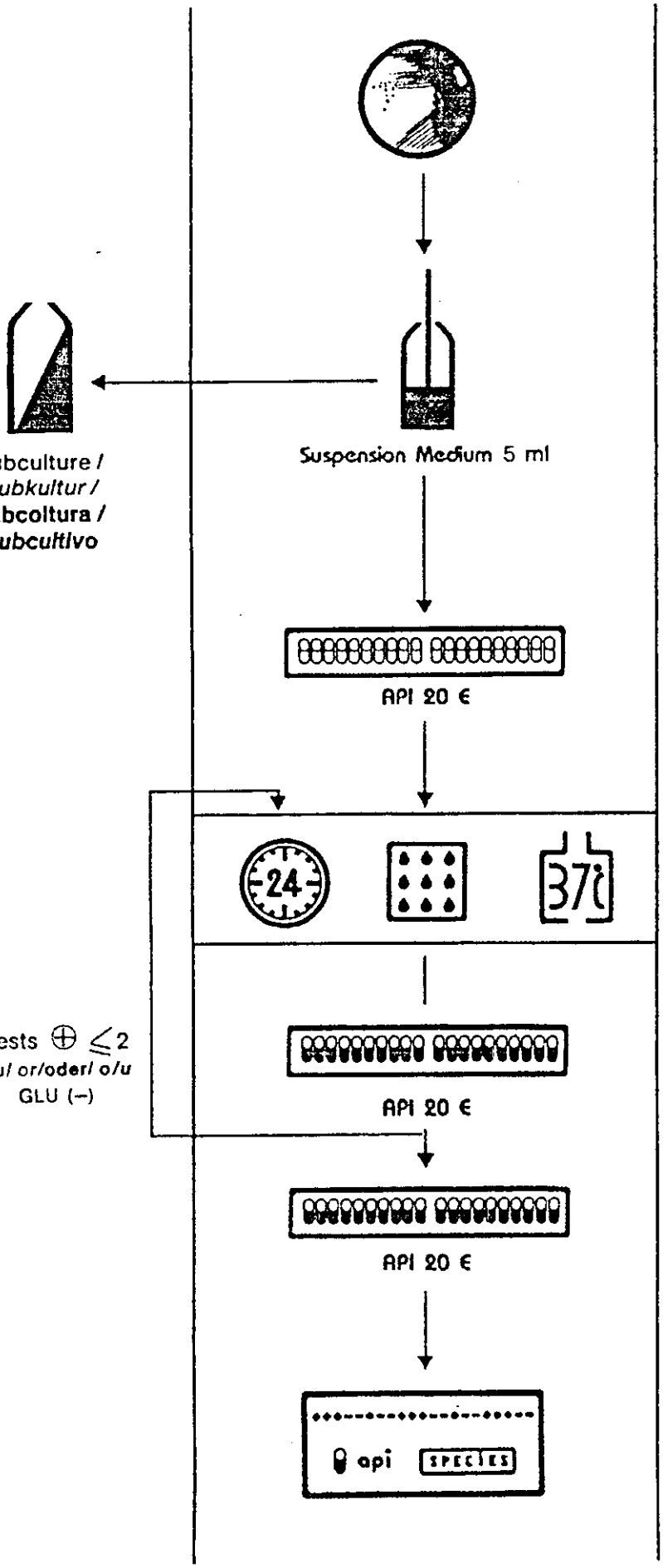
INTERPRETATION TABLE

TESTS	SUBSTRATES	REACTIONS/ENZYMES	RESULTS	
			NEGATIVE	POSITIVE
ONPG	ortho-nitro-phenyl-galactoside	beta-galactosidase	colourless	yellow (1)
ADH	arginine	arginine dihydrolase	yellow	red/ orange (2)
LDC	lysine	lysine decarboxylase	yellow	orange
ODC	ornithine	ornithine decarboxylase	yellow	red/ orange (2)
CIT	sodium citrate	citrate utilization	pale green/ yellow	blue-green / blue (3)
H_2S	sodium thiosulphate	H_2S production	colourless/ greyish	black deposit/ thin line
URE	urea	urease	yellow	red/ orange
TDA	tryptophane	tryptophane desaminase	TDA/ immediate	
			yellow	dark brown
IND	tryptophane	indole production	JAMES Reagent/ immediate or IND 2 mn	
			JAMES colourless pale green-yellow IND yellow ring	JAMES pink IND red ring
VP	sodium pyruvate	acetoin production	VP 1 + VP 2 / 10 mn	
			colourless	pink/ red
GEL	Kohn's gelatin	gelatinase	no diffusion of black pigment	diffusion of black pigment
GLU	glucose	fermentation/ oxidation (4)	blue/ blue-green	yellow
MAN	mannitol	fermentation/ oxidation (4)	blue/ blue-green	yellow
INO	inositol	fermentation/ oxidation (4)	blue/ blue-green	yellow
SOR	sorbitol	fermentation/ oxidation (4)	blue/ blue-green	yellow
RHA	rhamnose	fermentation/ oxidation (4)	blue/ blue-green	yellow
SAC	sucrose	fermentation/ oxidation (4)	blue/ blue-green	yellow
MEL	melibiose	fermentation/ oxidation (4)	blue/ blue-green	yellow
AMY	amygdalin	fermentation/ oxidation (4)	blue/ blue-green	yellow
ARA	arabinose	fermentation/ oxidation (4)	blue/ blue-green	yellow
OX	on filter paper	cytochrome-oxidase	OX / 1-2 mn	
			colourless	violet
NO_3-NO_2	GLU tube	NO_2 production	NIT 1 + NIT 2 / 2-3 mn	
			yellow	red
		reduction to N_2 gas	Zn	
			red	yellow
MOB	API M or microscopic	motility	non motile	motile
MAC	MacConkey medium	growth	absence	presence
OF	glucose (API OF)	fermentation : closed oxidation : open	green green	yellow yellow

a very pale yellow should also be considered positive
an orange colour after 24 hours of incubation must be considered negative
reading made in the cupule (aerobic)
fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.

api 20 E

MÉTHODOLOGIE/PROCEDURE/METHODIK
PROCEDIMENTO/TECNICA



SELECTION/AUSWAHL
SELEZIONE/SELECCION

INOCULUM/INOKULUM
SOSPENSIONE/INOCULO

1 colonie
colony
Kolonie
colonia

INOCULATION/BEIMPFEN
INOCULO/INOCULACION

- CIT
- VP
- GEL
- ADH ➡ ODC
- H_2S - URE

INCUBATION/INKUBIEREN
INCUBAZIONE/INCUBACION

LECTURE/READING/ABLESEN
LETTURA/LECTURA

- TDA : **TDA**
- IND : **JAMES**
  ou/or/**oder**/**o**/*u* **IND**
- VP : **VP1 + VP2**
- NO_2 : **NIT 1 + NIT 2**
  in GLU
- OX : **OX**

INTERPRETATION/INTERPRETAZIONE
INTERPRETACION

- Tab. Identif.
- Cat. Analy.
- Software

api 20 E (V3.2)

	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO2	N2	MOB	MLC	OF-O	OF-F
Buttiauxella agrestis	100	0	3	85	66	0	0	0	0	0	0	100	100	0	9	100	0	82	100	100	0	100	0	100	100	100	100
Budvicia aquatica	100	0	0	0	0	0	60	0	0	0	0	100	100	0	0	100	0	0	0	100	0	100	0	0	100	100	100
Cedecea davisae	100	77	0	100	77	0	0	0	0	85	0	100	100	15	0	0	100	0	100	0	0	99	0	87	100	100	100
Cedecea lapagei	90	100	0	0	90	0	0	0	0	100	0	100	90	0	0	0	0	10	100	10	0	99	0	87	100	100	100
Cedecea neteri	100	80	0	0	100	0	0	0	0	80	0	100	100	20	100	0	100	0	100	20	0	99	0	87	100	100	100
Citrobacter diversus/amalonaticus	97	50	0	93	82	0	2	0	92	0	0	100	99	4	92	94	27	17	96	95	0	100	0	95	100	100	100
Citrobacter freundii	83	32	0	32	73	69	3	0	7	0	0	100	98	11	95	98	35	66	61	99	0	98	0	95	100	100	100
Edwardsiella hoshinae	0	0	100	99	0	94	0	0	100	0	0	100	100	0	0	0	100	0	0	75	0	100	0	100	100	100	100
Edwardsiella tarda	0	0	100	99	1	94	0	0	100	0	0	100	0	0	0	0	0	0	0	1	0	100	0	98	100	100	100
Enterobacter cloacae	98	75	1	88	85	0	1	0	0	85	0	100	98	12	80	79	96	83	99	99	0	100	0	95	100	100	100
Enterobacter aerogenes	98	0	88	98	60	0	2	0	0	73	0	99	99	93	99	93	99	99	99	99	0	100	0	97	100	100	100
Enterobacter agglomerans 1	99	0	0	1	16	0	3	2	1	60	8	99	99	20	13	66	77	26	57	84	0	85	0	85	100	100	100
Enterobacter agglomerans 2	100	0	0	0	92	0	0	3	97	51	23	100	87	46	67	86	98	83	100	100	0	85	0	85	100	100	100
Enterobacter agglomerans 3	100	2	1	2	88	0	0	1	30	80	24	98	97	11	19	94	99	91	100	97	0	85	0	85	100	100	100
Enterobacter agglomerans 4	96	0	0	3	62	0	1	1	1	93	1	99	99	90	51	96	62	84	66	96	0	85	0	85	100	100	100
Enterobacter agglomerans 5	99	0	0	13	44	0	13	13	56	1	1	99	91	31	13	99	1	88	99	99	0	85	0	85	100	100	100
Enterobacter amnigenus 1	96	26	1	100	62	0	0	0	0	78	0	100	99	0	1	100	99	98	99	99	0	100	0	92	100	100	100
Enterobacter amnigenus 2	98	79	1	100	90	0	0	0	0	40	0	100	99	0	99	100	5	100	99	99	0	100	0	100	100	100	100
Enterobacter intermedius	99	1	2	100	1	0	0	0	0	11	0	100	95	0	68	100	32	100	99	99	0	100	0	92	100	100	100
Enterobacter gergoviae	100	0	35	100	70	0	95	0	0	91	0	100	99	25	0	100	99	100	99	100	0	100	0	90	100	100	100
Enterobacter sakazakii	100	93	0	89	94	0	1	0	41	81	37	100	100	85	44	99	99	99	99	99	0	100	0	96	100	100	100
Enterobacter taylorae	100	68	0	99	98	0	0	0	0	89	0	100	100	0	0	100	1	0	100	100	0	100	0	99	100	100	100
Erwinia spp	13	3	1	0	5	0	0	0	0	88	17	97	77	3	26	1	91	19	42	41	0	85	0	85	100	100	100
Erwinia nigrifluens	11	0	0	11	0	0	0	0	0	67	56	100	100	76	99	100	100	100	100	100	0	85	0	85	100	100	100
Escherichia coli 1	88	5	74	70	0	3	2	0	99	0	0	99	97	3	90	82	41	67	20	82	0	100	0	95	100	100	100
Escherichia coli 2	47	10	45	30	0	2	1	0	77	0	0	97	84	2	42	35	4	34	12	90	0	98	0	5	100	100	100
Escherichia fergusonii	96	1	99	100	1	0	0	0	99	3	0	100	99	0	0	87	0	3	99	97	0	100	0	93	100	100	100
Escherichia hermannii	100	0	6	100	5	0	0	0	100	0	0	100	100	0	0	97	44	0	97	99	0	100	0	99	100	100	100
Escherichia vulneris	100	64	42	0	0	0	1	0	0	0	0	100	100	0	1	95	7	78	85	100	0	100	0	100	100	100	100
Ewingella americana	90	0	0	0	96	0	0	0	0	99	1	100	100	0	0	5	0	0	90	1	0	100	0	60	100	100	100
Hafnia alvei 1	62	1	98	95	57	0	15	0	0	56	0	99	99	0	1	97	1	0	26	98	0	100	0	85	100	100	100
Hafnia alvei 2	52	1	97	98	5	0	1	0	0	15	0	95	92	0	1	1	1	0	5	18	0	100	0	0	100	100	100
Klebsiella ornithinolytica	100	0	92	99	92	0	75	0	100	50	0	100	100	100	100	100	100	100	100	100	0	100	0	0	100	100	100
Klebsiella oxytoca	89	0	80	0	89	0	78	0	99	44	0	100	100	98	100	94	100	100	100	100	0	100	0	0	100	100	100
Klebsiella pneumoniae pneumoniae	100	0	73	0	79	0	78	0	0	75	0	100	90	73	99	95	100	99	99	99	0	100	0	0	100	100	100
Klebsiella pneumoniae ozaenae	94	23	30	0	20	0	1	0	0	1	0	98	94	49	57	55	8	79	90	83	0	92	0	0	100	100	100
Klebsiella pneumoniae rhinoscleromatis	25	0	2	0	1	0	1	0	0	1	0	92	100	71	71	42	87	1	92	25	0	100	0	0	100	100	100
Kluyvera spp	89	0	30	99	56	0	0	1	76	0	0	100	99	0	37	83	69	99	95	99	0	95	0	94	100	100	100
Koserella trabulsii	100	0	100	100	100	0	0	0	0	0	0	100	100	0	0	100	0	100	100	100	0	100	0	0	100	100	100
Leclercia adecarboxylata	100	0	0	0	0	0	15	0	86	0	30	100	100	0	4	100	55	100	99	100	0	100	0	100	100	100	100
Moellerella wisconsensis	86	0	0	0	29	0	0	0	21	7	0	100	0	0	0	0	100	100	0	0	0	90	0	0	100	100	100
Morganella morganii	1	1	5	98	2	1	99	91	97	0	0	97	1	0	0	0	1	0	0	1	0	88	0	95	100	100	100
Proteus mirabilis	1	1	1	98	57	83	99	98	2	4	63	96	1	0	0	0	1	1	1	1	0	93	0	95	100	100	100
Proteus penneri	0	0	0	0	3	17	100	99	0	0	17	100	0	0	0	0	100	0	1	0	0	99	0	85	100	100	100
Proteus vulgaris	0	0	0	0	31	83	98	98	92	0	52	97	1	1	0	4	89	0	65	1	0	100	0	94	100	100	100
Providencia alcalifaciens	0	0	0	0	94	0	0	98	99	0	0	99	2	2	0	0	8	0	0	2	0	100	0	96	100	100	100
Providencia rettgeri	1	1	0	0	70	0	98	99	90	0	0	98	62	78	2	42	34	0	11	1	0	98	0	94	100	100	100
Providencia stuartii	1	0	0	0	89	0	30	90	97	0	0	98	3	84	0	0	16	0	0	3	0	100	0	85	100	100	100
Rahnella aquatilis	100	0	0	0	66	0	0	7	0	100	0	100	100	0	98	98	100	85	98	95	0	100	0	6	100	100	100
Salmonella arizonae	98	46	96	98	50	97	0	0	0	0	0	100	99	0	99	96	1	64	0	99	0	100	0	99	100	100	100
Salmonella choleraesuis	0	22	97	97	4	65	0	0	0	0	0	100	99	0	85	95	0	20	0	0	0	100	0	95	100	100	100
Salmonella paratyphi A	0	1	0	99	0	5	0	0	0	0	0	100	99	0	99	99	0	96	0	89	0	100	0	95	100	100	100
Salmonella spp	3	69	96	95	75	85	0	0	0	0	0	100	97	33	93	93	2	78	1	94	0	100	0	94	100	100	100
Salmonella typhi	0	2	99	0	0	8	0	0	0	0	0	100	99	0	99	0	0	94	0	0	0	100	0	87	100	100	100

	CAPR	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO2	N2	MOB	NAC	OF-O	OF-F
Serratia ficaria	100	0	0	0	100	0	0	0	0	50	81	100	100	50	85	92	100	75	95	96	0	92	0	100	100	100	100
Serratia fonticola	100	0	85	99	60	0	0	0	0	0	0	100	100	88	100	96	18	99	100	95	0	99	0	91	100	100	100
Serratia liquefaciens	96	0	81	99	77	0	6	0	0	52	60	100	99	75	98	10	99	70	97	97	0	100	0	95	100	100	100
Serratia marcescens	94	0	95	95	96	0	28	0	1	60	85	100	99	71	91	1	98	68	97	19	0	95	0	97	100	100	100
Serratia odorifera 1	95	0	97	84	87	0	0	0	99	40	90	100	99	99	99	99	100	99	99	85	0	99	0	100	100	100	100
Serratia odorifera 2	95	0	96	2	87	0	0	0	99	65	90	100	99	99	99	99	0	89	99	95	0	89	0	100	100	100	100
Serratia plymuthica	99	0	0	0	65	0	0	0	0	65	50	100	90	55	58	8	100	85	95	98	0	99	0	50	100	100	100
Serratia rubidaea	99	0	73	0	83	0	4	0	0	69	76	98	98	58	1	3	84	82	94	99	0	100	0	85	100	100	100
Shigella spp	7	0	0	4	0	0	0	0	35	0	0	96	63	0	15	7	7	22	0	52	0	100	0	0	100	100	100
Shigella sonnei	96	0	0	97	0	0	0	0	0	0	0	100	99	0	1	75	2	1	0	97	0	100	0	0	100	100	100
Tatumella ptyseos	0	0	0	0	10	0	0	72	0	5	0	99	0	0	0	0	80	27	9	1	0	90	0	0	100	100	100
Yersinia aldovae	25	0	25	50	0	0	100	0	0	0	0	95	100	90	100	0	6	0	5	50	0	100	0	0	100	100	100
Yersinia enterocolitica	81	0	0	90	0	0	93	0	66	8	0	99	99	25	98	2	100	4	88	69	0	98	0	2	100	100	100
Yersinia frederiksenii	95	0	0	99	0	0	98	0	99	1	0	100	99	11	95	100	100	0	97	57	0	98	0	5	100	100	100
Yersinia intermedia	95	0	0	100	0	0	97	0	97	2	0	100	99	63	95	99	99	97	98	52	0	98	0	5	100	100	100
Yersinia kristensenii	87	0	0	87	0	0	100	0	99	0	0	99	99	62	99	0	0	0	99	87	0	98	0	5	100	100	100
Yersinia pestis	68	0	0	0	0	0	0	0	0	8	0	99	97	0	71	0	0	0	18	23	0	47	0	0	99	100	100
Yersinia pseudotuberculosis	77	0	0	0	13	0	96	0	0	0	0	98	97	0	0	77	0	70	22	29	0	95	0	0	100	100	100
Acinetobacter spp	0	0	0	0	48	0	1	1	0	10	10	60	0	0	0	2	1	60	2	56	0	3	0	0	90	98	0
Moraxella spp	0	0	0	0	1	0	1	0	0	4	2	0	0	0	0	0	0	0	0	0	100	9	0	0	35	0	0
Pasteurella aerogenes	70	0	0	95	0	0	95	0	0	0	0	99	0	90	0	10	99	0	0	80	85	100	0	0	100	100	100
Pasteurella multocida	4	0	0	25	0	0	0	0	88	0	0	29	74	0	68	0	77	0	0	0	79	52	0	0	2	23	23
Pasteurella spp	60	0	1	10	0	0	25	0	15	7	3	35	10	10	10	1	35	1	2	1	80	59	0	0	9	33	33
Pseudomonas aeruginosa	0	87	0	0	92	0	35	0	0	2	74	97	0	0	0	0	1	11	0	18	97	12	56	97	100	98	0
Pseudomonas cepacia	58	0	32	18	72	0	1	0	0	4	48	65	3	1	1	0	14	0	24	18	90	40	0	99	88	97	0
Pseudomonas fluorescens/putida	0	74	0	0	68	0	1	0	0	22	27	41	0	0	0	3	0	25	1	20	97	26	0	100	96	93	0
Pseudomonas pseudomallei	0	72	0	0	18	0	0	0	0	1	81	95	79	75	79	0	72	0	64	18	100	0	92	100	100	100	0
Pseudomonas spp	3	10	0	0	54	0	3	0	0	25	10	10	0	0	0	2	1	6	1	9	93	48	35	99	85	49	0
Xanthomonas maltophilia	60	0	48	0	75	0	0	0	0	0	89	2	0	0	0	0	0	0	0	0	4	26	1	100	91	49	0
Aeromonas hydrophila/caviae	98	90	6	0	18	0	0	0	85	20	85	100	100	1	3	10	97	1	75	84	100	97	0	95	99	99	99
Aeromonas salmonicida	5	60	0	0	2	0	0	0	15	1	75	46	54	0	0	1	1	0	5	0	96	98	0	95	99	99	99
Aeromonas sobria	99	97	80	1	80	0	0	0	85	80	95	97	99	1	1	1	80	1	75	20	99	98	0	95	99	99	99
Listonella damsela	0	100	51	0	0	0	94	0	0	10	6	50	0	0	0	0	6	0	0	0	100	100	0	25	99	99	99
Plesiomonas shigelloides	95	95	100	100	0	0	0	0	100	0	0	99	0	99	0	0	0	0	0	0	100	99	0	95	99	99	99
Vibrio alginolyticus	0	0	97	62	60	0	2	0	100	10	55	100	100	0	0	0	100	0	5	2	100	47	0	100	99	94	94
Vibrio cholerae	94	1	94	96	62	0	0	0	100	40	87	98	97	0	0	0	94	0	5	0	100	96	0	100	96	99	99
Vibrio hollisae	6	0	0	0	0	0	0	0	94	0	0	20	0	0	0	0	0	0	0	5	100	100	0	0	99	99	99
Vibrio metschnikovii	20	85	88	0	30	0	0	0	50	63	100	100	100	30	30	5	100	0	0	0	0	0	0	80	99	99	99
Vibrio mimicus	99	1	85	90	65	0	0	0	90	3	95	99	95	0	0	0	0	0	1	0	100	95	0	100	95	99	99
Vibrio parahaemolyticus	0	0	100	89	63	0	8	0	100	5	63	100	96	0	0	3	1	0	12	36	100	63	0	100	98	99	99
Vibrio vulnificus	100	0	68	90	81	0	0	0	99	18	99	100	36	0	0	0	0	0	90	1	99	54	0	100	99	99	99
Achromobacter spp	5	1	0	0	85	0	45	1	0	10	1	1	1	1	1	5	1	1	4	1	100	42	60	99	99	47	0
Bordetella/Alcaligenes spp	0	0	0	0	60	0	59	2	0	28	4	0	0	0	0	0	0	0	0	0	95	62	1	99	90	0	0
Chromobacterium violaceum	0	98	0	0	59	0	0	0	19	0	89	98	0	0	0	0	11	0	0	0	92	75	0	89	99	99	99
Chryseomonas luteola	86	59	0	0	94	0	0	0	0	43	13	84	0	13	0	16	4	16	2	78	1	30	0	100	91	94	0
Flavobacterium indologenes	20	0	0	0	12	0	92	0	85	0	84	0	0	0	0	0	0	0	0	0	99	20	0	0	57	90	10
Flavobacterium meningosepticum	70	0	0	0	18	0	1	0	81	1	91	0	0	0	0	0	0	0	0	0	99	6	0	0	48	93	6
Flavobacterium odoratum	0	0	0	0	56	0	85	0	0	7	71	0	0	0	0	0	0	0	0	0	100	0	0	0	84	2	2
Flavimonas oryzihabitans	0	0	0	0	95	0	0	0	0	60	6	40	0	15	1	2	0	11	0	60	0	7	0	100	99	99	0
Shewanella putrefaciens	0	0	0	80	83	90	0	0	0	6	93	6	0	0	0	0	9	0	0	2	100	96	0	100	96	9	0
Sphingobacterium multivorum	96	0	0	0	30	0	92	0	0	75	10	46	0	0	0	0	25	2	7	17	96	0	0	0	84	96	0
Sphingomonas paucimobilis	73	0	0	0	35	0	6	0	0	31	7	10	0	0	0	2	23	0	3	12	50	0	0	92	0	85	0
Weeksella virosa	0	0	0	0	8	0	0	0	85	0	88	0	0	0	0	0	0	0	0	0	99	0	0	0	3	0	0
Weeksella zoohelcum	0	0	0	0	2	0	95	0	25	0	15	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0

% de réactions positives après 24-48 h à 35/37°C / *% of positive reactions after 24-48 h at 35-37°C*

% der positiven Reaktionen nach 24-48 St. bei 35-37°C

% de las reacciones positivas después de 24-48 H a 35-37°C / % di reazioni positive dopo 24-48 ore a 35-37°C

دراسة التفاعل بين عوامل المكافحة الحيوية: النيماتودات الممرضة للحشرات، *Serratia marcescens* و *Beauveria bassiana* المعزولة من فلسطين

ملخص

تستخدم عوامل المكافحة الحيوية مثل النيماتودات الممرضة للحشرات، والبكتريا والفطر في مكافحة الآفات الزراعية لتقليل استخدام المبيدات الحشرية. في بعض الحالات يتضمن استخدامها الدمج بين أكثر من عامل. في مثل هذه الحالات قد يحدث تفاعل بين العوامل أثناء تطبيقها. هذا التفاعل قد يحصل خارج أو داخل الحشرة مما يؤدي إلى زيادة أو نقصان في فعالية العامل الحيوي المستخدم. ان طبيعة هذه التفاعلات وأثرها على فعالية العامل الحيوي لم تعطى حقها من الاهتمام والدراسة. في هذا البحث، عزلنا وصنفنا عزلة جديدة من البكتريا الممرضة للحشرات. وكذلك درسنا تأثير معاملة الحشرة أولا بالبكتيريا *Serratia marcescens* أو بالفطر *Beauveria bassiana* على نسبة هجوم وتطور النيماتودا الممرضة للحشرات *Heterorhabditis indica* من النوع Bethlehem II.

استخدمنا فى هذه الدراسة يرقة حشرة الشمع (*Galleria mellonella*) الحساسة لعوامل المكافحة الحيوية. أظهرت النتائج الأولية أن بقاء الطور المعدي (Infective Juveniles) من العزلة Bethlehem II لم يتأثر عند حضنه بـ *Serratia marcescens* أو بـ *Beauveria bassiana* لمدة ٤٨ ساعة في حين أن الطور الرابع (J4) تأثر بكلا العاملين. مع أن معاملة الحشرة لمدة ٤٨ ساعة بـ *Serratia marcescens* أو *Beauveria bassiana* لم يحدث تأثير ملموس على سرعة موت الحشرة، ألا أنه أثر على قدرة النيماتودات على مهاجمة الحشرة وتطورها في داخلها. بمعنى اخر، اصابة الحشرة بالنيماتودات

لوحدها (الضابط) كانت ٧,٣٧% بينما معاملة الحشرة بالبكتيريا أو بالفطر أو لا لمدة ٢٤ ساعة قلص نسبة دخول الطور المعدي للنيماتودات اليها ل ١,١٦% و ١,٧٢% بالتعاقب.

تأثير مشابه على قدرة تطور الطور المعدي للنيماتودات الى حشرات يافعة عند معاملة حشرة الشمع بالبكتيريا أو الفطر قبل النيماتودات (٠,٦١ % و ١,٢% بالتعاقب بالمقارنة مع ٧,٠% للضابط). كذلك أثرت هذه المعاملة على الإنتاج الكلي للنيماتودات (٤٠٢٠ نيماتود /للحشرة الواحدة المعاملة بالبكتيريا، و ٢١٦١ نيماتودا للحشرة الواحدة المعاملة بالفطر بالمقارنة مع ١٠٧١٠٦ للحشرة الواحدة غير المعاملة.

أظهر هذا العمل أن معاملة حشرة الشمع بالبكتيريا لمدة ٢٤ ساعة أو لغاية ٩٦ ساعة بالفطر قلل نسب دخول وتطور وانتاج النيماتودا *H. indica* من النوع II Bethlehem. يجب أخذ هذه الحقائق بالحسبان لتقييم فعالية واستمرار البقاء للنيماتودات عند استخدامها في مثل هذه الحالات.