

**The entomopathogenic nematode *Heterorhabditis megidis*: host
searching behaviour, infectivity and reproduction**

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Propositions

1. Bigger infective juvenile nematodes are not always better. (This thesis)
2. The interaction between plant roots and the natural enemies of the insect pests that attack them is more complex than it appears to be. (This thesis)
3. Plants do not have only smelly bodies but have also smelly feet. (This thesis)
4. The Leonardo da Vinci statement: "We know more about the movement of celestial bodies than about the soil underfoot" is still true.
5. Growing crops in monoculture might be dangerous, but mono-thinking is much more dangerous. (Seshadri, P.)
6. The developing world does not need charity from industrialised countries but cooperation.
7. I write without thinking all what my unconsciousness shouts. Afterwards I think, not only for correcting it, but also to justify what I have written. (Mário de Andrade)
8. It is time to address the needs of the poor in developing countries. It is immoral that they go hungry while food and resources are exported, at very low prices, to developed countries just to pay for expensive technologies and loans.

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Chapter 1

General Introduction

Introduction

The aim of the research presented in this thesis was to investigate the effects of biotic and abiotic factors on the biology and ecology of *Heterorhabditis megidis* (strain NLH-E87.3, Smits *et al.*, 1991) a common, but little studied entomopathogenic nematode in the Netherlands. Particular attention was paid to the searching behaviour of this nematode strain.

Entomopathogenic nematodes are a welcome addition to the natural-enemy pool of insects and can be integrated with various control measures for management of those target pests where individual tactics alone are inadequate. Entomopathogenic nematodes play a role underground reminiscent of that played by insect parasitoids, arthropod predators and microbial pathogens above ground. Like parasitoids or predators, they have chemoreceptors and are motile; like pathogens, they are highly virulent, killing their hosts quickly, and can be cultured easily *in vivo* or *in vitro* (Ehlers, 1996). Entomopathogenic nematodes are among the best studied of a poorly studied group: natural enemies of soil insects. Interest in these beneficial organisms has increased rapidly in recent years and researches are being conducted in many laboratories worldwide (Gaugler *et al.*, 1997). Numerous surveys give evidence of their omnipresence in natural and agricultural soils (Hominick *et al.*, 1996), and new species are described every year with many more isolates waiting for identification and study (Koppenhöfer & Kaya, 1999).

Entomopathogenic nematodes that occur in the families Steinernematidae and Heterorhabditidae possess many qualities that make them excellent biological control agents. They have a broad host range, can easily be mass produced, possess the ability to seek out their host, kill their host rapidly and are environmentally safe. These attributes, combined with others like the easy application by using standard spray equipment, compatibility with many chemical pesticides and no evidence of mammalian pathogenicity (Ehlers & Peters, 1995; Ehlers & Hokkanen, 1996; Boemare *et al.*, 1996) have generated an intense interest in the development of work with these organisms. Nematodes have been used against diverse pests, including those found in the soil, in cryptic habitats, on foliage, in manure and in aquatic habitats (Begley, 1990). However, the soil environment is the one that offers an excellent site for insect-nematode interactions, because more than 90% of insect pests spend part of their life cycle in the soil, and the soil is the natural reservoir of steinernematid and heterorhabditid nematodes (Akhurst, 1986; Gaugler, 1988).

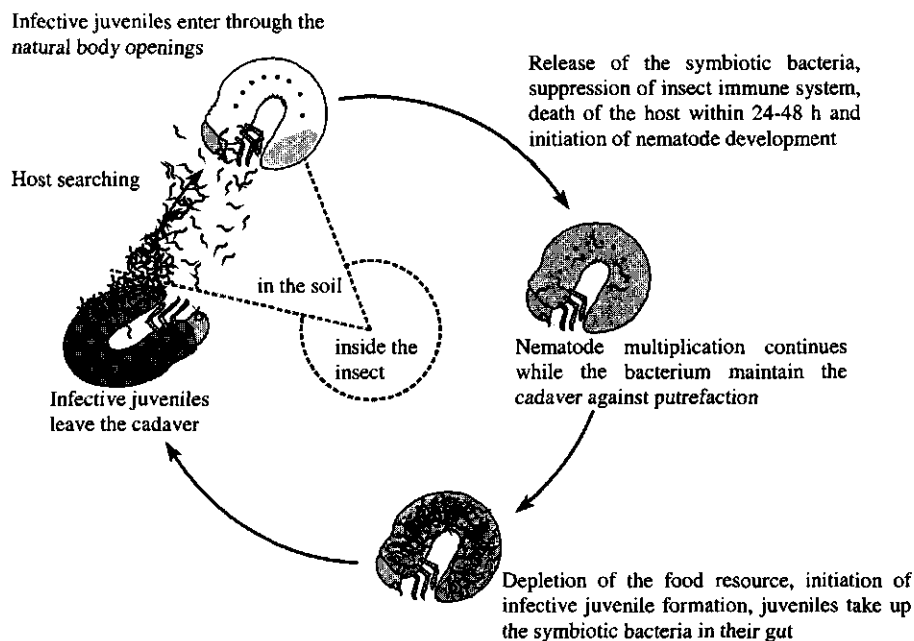


Figure 1. Life cycle of entomopathogenic nematodes (modified from Gerritsen, 1997).

Entomopathogenic nematode biology

Entomopathogenic nematodes belong to the order Rhabditida and comprise two families: Steinernematidae (Chitwood & Chitwood, 1937) and Heterorhabditidae (Poinar, 1975). Each family contains only one genus, *Steinernema* and *Heterorhabditis*, respectively. Both genera contain several species that are mutualistically associated with bacteria of the genera *Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids (Akhurst & Boemare, 1990; Boemare *et al.*, 1993). Individuals of both families have a life cycle that includes eggs, four juvenile stages and an adult stage. Their life cycle consists of a free-living phase of infective juveniles (IJs) occurring in the soil and a propagative phase that occurs inside the insect host (Figure 1). When an insect host has been located, the infective

juvenile enters through the mouth, anus, spiracles, or by direct penetration through the cuticle. If the mode of entry is by mouth or anus, the nematode penetrates the gut wall to reach the haemocoel, and if by spiracles, it penetrates the tracheal wall. Once in the insect body cavity, symbiotic bacteria are released from the nematode gut, which multiply rapidly in the haemolymph and causes rapid insect death (Gotz *et al.*, 1981). The bacteria digest the cadaver and provide food for the exponentially growing nematode population inside, as well as produces antibiotics and other noxious substances that protect the host cadaver from others microbes in the soil (Akhurst, 1982; Thomas & Poinar, 1979).

Species in the genus *Steinernema* are amphimictic (Poinar, 1990). After entering the insect infective juveniles develop into small males or large females. After mating the females lay eggs that hatch as first-stage juveniles that moult successively to second, third and fourth-stage juveniles and then to males and females of the second generation. The adults mate and the eggs produced by these second-generation females hatch as first-stage juveniles that moult to the second stage and may live in the cadaver to become an IJs. Depending on the size of the insect two or three generations can develop inside the dead host.

The life cycle of *Heterorhabditis* species is similar to steinernematids with the difference that the entering infective juveniles develop into self-reproducing hermaphrodites (Poinar, 1990). Strauch *et al.*, (1994) observed that the offspring of the first generation hermaphrodites can either develop into amphimictic adults or into automictic hermaphrodites, and both occur simultaneously (Figure 2). The development into amphimictic adults is induced by favourable nutritional conditions, whereas the development of hermaphrodites is induced by low concentrations of nutrients.

In both genera *Steinernema* and *Heterorhabditis* the late second-stage juveniles cease feeding, incorporate a pellet of bacteria in the bacterial chamber (Smart, 1995), moult to the third stage called infective Juvenile (IJs), leave the deteriorating cadaver and enter the soil to seek new hosts. The cycle from entry of IJs into a host to emergence of new IJs is temperature dependent and varies somewhat for different species and strains. It generally takes about 6-18 days at temperatures ranging from 18-28°C in *Galleria mellonella* (Wouts, 1979; 1980; Poinar, 1990; Nguyen & Smart, 1992).

Host finding

Searching behavior is the means by which animals locate and acquire resources necessary for growth, development, maintenance and reproduction (Lewis *et al.*, 1993). The

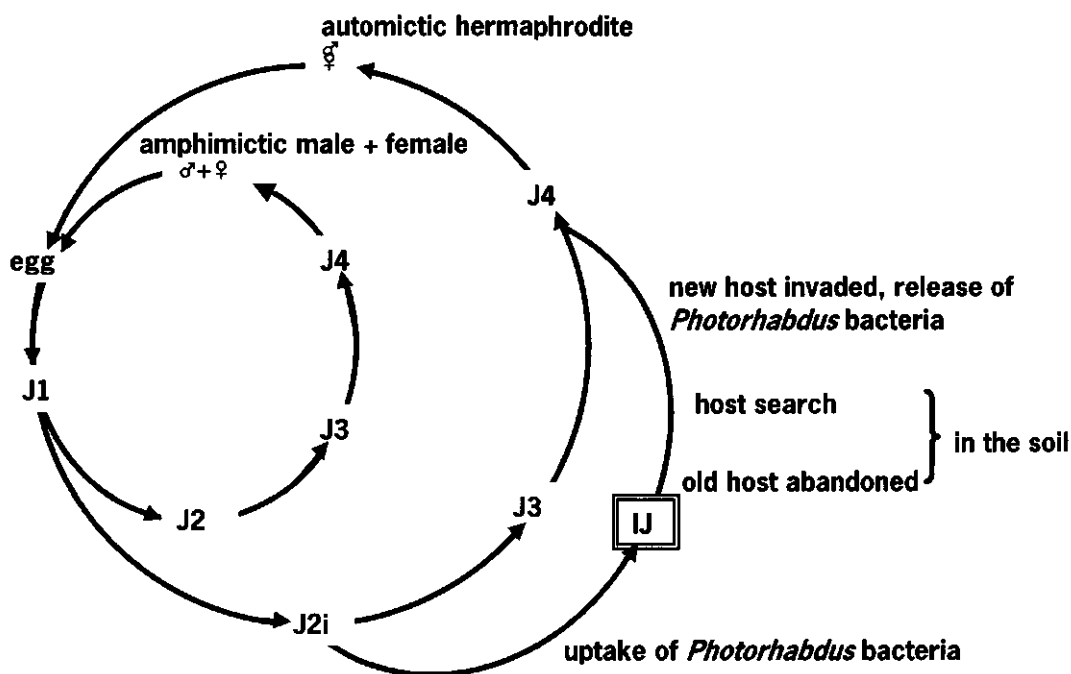


Figure 2. Automatic and amphimictic reproduction of heterorhabditid nematodes (modified from Gerritsen, 1997). J1-J4: juvenile stages; IJ: infective juvenile; J2i: pre-infective juvenile

soil-inhabiting infective juveniles of insect-parasitic nematodes in the families Steinernematidae and Heterorhabditidae locate and parasitize insect hosts in which they develop to adults, mate and reproduce (Poinar, 1990). Lewis *et al.* (1992) reported that host finding behavior of infective juveniles differs from species to species and because of this, two categories of foraging strategies have been recognized: ambushing and cruising (Figure 3A and B).

Ambushers nictate, that is they search by standing on their tail, elevating most of their bodies in a straight position free in the air. Nictation enables the nematode to attach to passing insects near to the soil surface. Ambusher species respond poorly to volatile cues released by hosts and they usually do not react to hosts that are placed only millimeters away. By contrast, cruiser IJs neither nictate nor attach well to passing host but are highly mobile and responsive to long-range host volatiles and are most effective at locating sedentary hosts (Gaugler *et al.*, 1997).

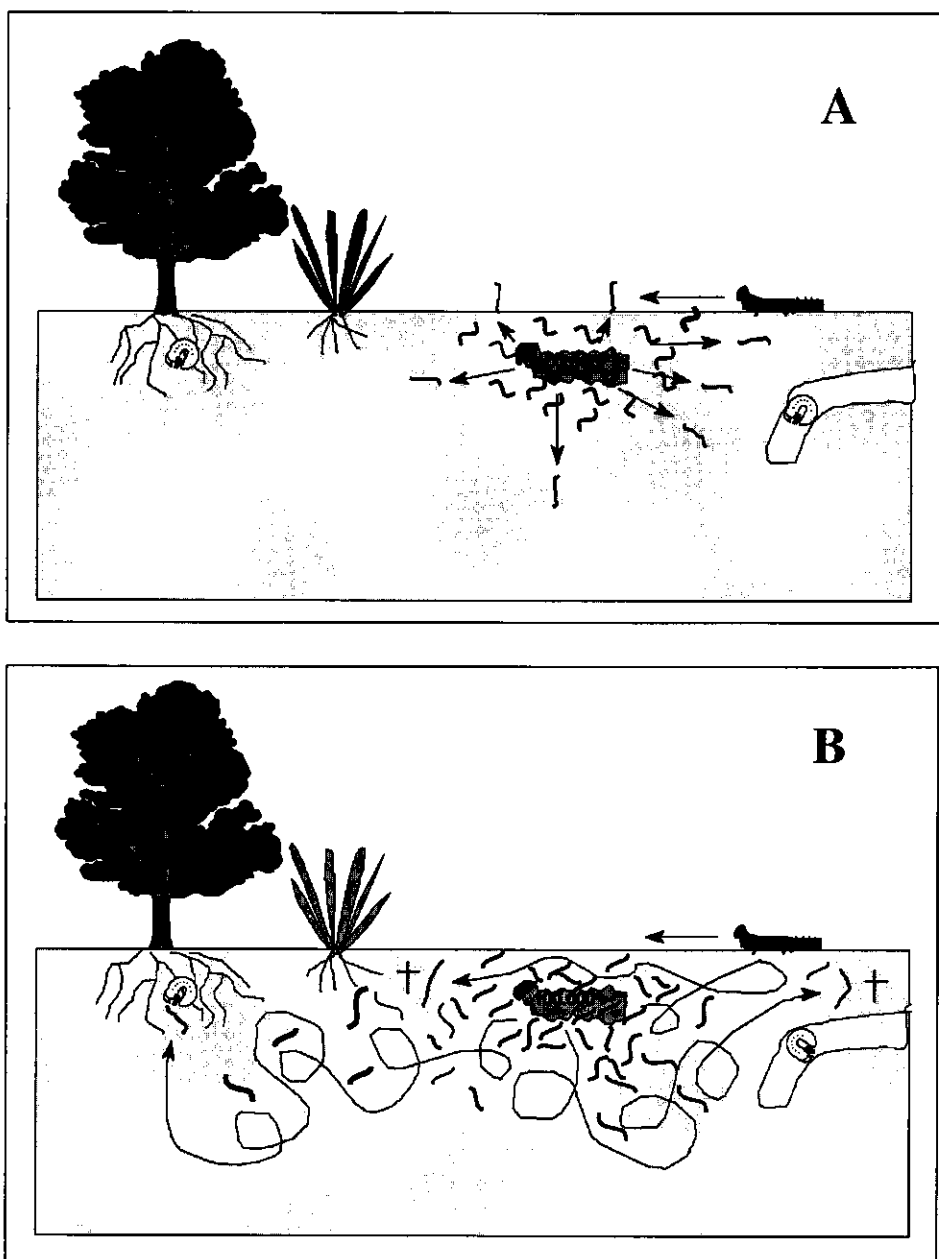


Figure 3. Searching strategies of entomopathogenic nematode species: (A) ambushers and (B) cruisers.

Positive attraction to an insect host is important for effective nematode infection. The cruiser infective stages were observed to locate their insect host by detecting host excretory products (Schmidt & All, 1979, Grewal *et al.*, 1993), sensitivity to carbon dioxide (Gaugler *et al.*, 1980; Lewis *et al.*, 1995a), temperature gradients (Burman & Pye, 1980), moving toward plant roots (Bird & Bird 1986; Choo & Kaya, 1991; Kanagy & Kaya, 1996; Hui & Webster, 2000) and the symbiotic bacteria of the nematodes (Pye & Burman, 1981). However, the understanding of the significance of these responses is incomplete and has to be more extensively studied to find out whether the searching behavior is the result of individual stimuli or of a sequence of host stimuli integrated with other biotic or abiotic factors.

Factors affecting dispersal and host searching behavior of entomopathogenic nematodes

Soil, the natural habitat for entomopathogenic nematodes, varies greatly in chemical composition and physical structure and that, combined with biological factors, makes soil a difficult medium for conducting quantitative studies. Once the infective juveniles leave their host cadavers or are introduced in the soil, they will be subject to various environmental stresses, including biotic and abiotic factors. Both biotic and abiotic influences have been shown to play important roles in persistence, infectivity and movement of these nematodes in their habitats and also in their developmental processes inside the host.

Abiotic factors

In general, soil type (Molyneux & Bedding, 1984; Ishibashi & Kondo, 1986; Kaya, 1990a; Kung *et al.*, 1990a) soil moisture (Kung *et al.*, 1990b, 1991), and soil temperatures (Molyneux, 1985, 1986) are regarded as the three most important abiotic factors which affect entomopathogenic nematode performance. The soil texture affects the ability of nematodes to infect hosts and also affects vertical and horizontal dispersal (Kaya, 1990a). The soil pore space, which in part determines the oxygen concentration, is determined by the arrangement of the soil particles. Nematodes showed to survive better in sandy soils than in clay soils (Kung *et al.*, 1990a). Active movement of IJs is greatest in a water film on a surface. At low soil moistures, for example, nematodes may not be infective because they lack a sufficient water film for effective movement towards their hosts. However, when soils are near their optimum water content, IJs are able to move and initiate a search for hosts causing high rates of infection (Kaya, 1990a).

Temperature is an important environmental factor limiting the success of entomopathogenic nematodes. Infective juveniles are most active between 12 and 32 °C, outside this temperature range penetration and development are very slow. Georgis & Gaugler, (1991) stated that low soil temperature restricts the use of entomopathogenic nematodes in temperate regions of the world. Low temperature is an important factor that limits the pathogenicity of steinernematids and heterorhabditids, either by its influence on the activity of the nematode itself, on the bacterial symbiont, or on both (Griffin & Downes, 1991, Kung *et al.*, 1991). Temperatures higher than 30 °C tend to inhibit nematode development in a host (Milstead, 1981; Gray & Johnson, 1983) and temperatures over 35°C for prolonged periods have adverse effects on infective juveniles (Schmiede, 1962). Knowledge of the region from which the nematodes have originally been isolated appears to be very important in determining the temperature range to which it is best-adapted and an understanding of the effects of soil temperature on nematode survival and infectivity helps to improve the accuracy of field applications (Kaya, 1990b). Besides the influence on nematode performance in the field, temperature plays also an important role for the nematode's thermal requirements during the production and storage process. However, the optimum temperature for persistence in the field may differ from the optimum temperature for laboratory storage. Griffin (1996) reports that the storage temperature and time profoundly affected the performance of two *Heterorhabditis* strains. Selvan *et al.*, (1993b) suggested that entomopathogenic nematodes are adapted to survive prolonged periods of environmental stress and that the nematodes metabolism is temperature dependent. Steinernematids retain a relatively high level of saturated fatty acids that could explain, in part, their better survival during storage as compared to heterorhabditids. Because of distinct physiological and behavioral differences among nematode strains and species a special attention concerning the effects of temperature on their shelf-life and field performance is needed.

Other physical factors have been less studied, but the consensus is that in most target habitats, factors such as pH, photoperiod and salinity are unlikely to be seriously limiting factors (Gaugler, 1981).

Biotic Factors

Populations of entomopathogenic nematodes in the soil are exposed to a variety of natural enemies such as bacteria, fungi, predatory nematodes and arthropods (Smart, 1995). Invertebrate predators including mites and collembolans appear to be especially voracious nematodes-feeders (Epsky *et al.*, 1988; Gilmore & Potter, 1993). Nematophagous fungi are

found in a wide range of soil habitats and are divided in two basic forms: (I) trapping fungi that capture their nematode prey by using specialized hyphae that penetrate into the nematode body cavity (Fowler & Garcia, 1989; Koppenhöfer *et al.*, 1996); (II) endoparasitic fungi that infect their hosts using conidia or zoospores, which attach to the nematode cuticle or are ingested (Timper *et al.*, 1991; Jaffec *et al.*, 1993).

Entomopathogenic viruses, fungi and bacteria can have a negative effect upon nematode development, for instance when IJs penetrate and kill a NPV-infected insect a few days before its death from the virus, the nematodes develop normally until they disintegrate a fragile integument and at this time the development of nematodes ceases (Kaya & Koppenhöfer, 1996). According to Kaya & Burlando (1989), in a host infected with *Bacillus thuringiensis* (*Bt*), nematode development is abnormal. However, when the insect host is exposed to *Bt* and nematodes simultaneously, dual infection occurs and brings about a more prompt death of the insect than an application with the nematode alone (Ishibashi, 1992). When entomopathogenic nematodes and *Beauveria bassiana* are applied simultaneously to a host, the nematodes develop normally (Barbercheck & Kaya, 1990). These authors also observed that if the fungus is applied before the nematode, the antagonistic interaction is temperature dependent and most of the time the fungus simply competes with the nematodes for available resources.

The capability of some steinernematid and heterorhabditid nematode species to search for a host, coupled with the intrinsic capability of these nematodes in the soil, suggests there is considerable selective pressure for soil insects to evolve strategies to avoid parasitism. It is known that many of the adapted soil insects protect themselves from entomopathogenic nematode attack by a series of defensive strategies such as: producing repellent compounds (Thurston *et al.*, 1994); excreting CO₂ discontinuously (Gaugler, 1988); protecting the natural openings with sieve plates and bristles; having a tough epidermis (Eidt & Thurston, 1995); brushing the IJs off with the legs; rubbing the body with an abrasive raster (Gaugler *et al.*, 1994). An infective juvenile can parasitise only a single host, so it must search carefully and assess an insect before committing itself irreversibly. Once a host has been located, recognized and penetrated, the nematode attack still may not succeed if the insect is able to respond with effective physical or physiological immune response. For instance when the penetration route tends to be the gut, the nematode has to be able to pass the peritrophic membrane and get into the haemocoel. In the haemocoel nematodes still have to deal with the insect physiological mechanisms like encapsulation (Peters & Ehlers, 1997).

Despite the fact that most of the entomopathogenic nematode species are able to invade and, in most cases, kill a large number of insect species, especially among the Lepidoptera, Coleoptera and Diptera, significant differences in susceptibility to nematodes were observed (Simões & Rosa, 1996). For instance, larvae of *O. sulcatus* are more difficult to kill by nematodes than other insect species, such as *Galleria mellonella* (Bedding *et al.*, 1983). The developmental stage of the insect also plays an important role in susceptibility to the nematodes. In general early instar larvae are more susceptible than late larval instars, pupae and adults (Simões & Rosa, 1996). The developmental stage of an insect host exerts a strong influence not only on the infectivity of the entomopathogenic nematodes but also on its development and on the number of new IJs produced (Sander & Stanuszek, 1971).

The life cycle of entomopathogenic nematodes is completed in the body of one insect host. When the number of infective juveniles invading a host exceeds an optimal level, exploitative intra-specific competition takes place and the development of nematodes is affected (Sander & Stanuszek, 1971; Zervos *et al.*, 1991; Selvan *et al.*, 1993a; Koppenhöfer & Kaya, 1995). The resulting competition in the host may be antagonistic to the survival of naturally occurring populations or to the establishment of applied nematodes because the infective juveniles will be depleted and little or no progeny production may occur (Kaya & Koppenhöfer, 1996).

Striking differences in efficacy exist not only between steinernematid and heterorhabditid nematodes but also between their various species and strains (Gaugler, 1988). Several studies have demonstrated that the efficacy of various nematode strains differs significantly with the same target insect. According to Glazer *et al.*, (1991) the difference between the nematode-bacteria complex may differ in various aspects that influence the infectivity and virulence of each species or nematode strain. These differences include the invasion rate of the infective juveniles into the host, the time taken for the release of the bacteria and the virulence of the symbiotic bacteria.

Despite a great amount of information available the knowledge of entomopathogenic nematode behavior in the soil environment is still limited. According to Hominick (1990), it is difficult to discover how entomopathogenic nematodes interact with abiotic and biotic factors, because soil is one of the most complex and least known of all habitats. However, for optimization of the control potential of entomopathogenic nematodes against soil insects the understanding of their interactions in the soil environment is necessary.

Objectives and outline of the thesis

Important information is lacking on the fate of nematodes introduced into the soil, on factors regulating their population dynamics, on optimal conditions for the initiation of epizootics, and on the ecological barriers to infection. Our understanding of the host finding behavior of entomopathogenic nematodes is clearly deficient and efforts to integrate behavior with pathogenic function should be encouraged, because such studies will provide fundamental knowledge useful in increasing our ability to use nematodes effectively in biological control programs.

The objective of the research presented in this thesis was to investigate the effects of biotic and abiotic factors on the biology and ecology of *Heterorhabditis megidis* (strain NLH-E87.3) a common entomopathogenic nematode in the Netherlands.

Increasing the population density of nematodes within the host can adversely effect the fitness of the parasite. As the life cycle of entomopathogenic nematodes is completed in the body of a single insect host, density-dependent factors can have an important influence on the population dynamics of nematodes. Although all entomopathogenic nematodes have the same general life history, the response to increasing densities differs from species to species and strain to strain. Hypothesizing that an inverse relationship between inoculum size and infective juvenile performance may occur, experiments testing different doses of IJs of the strain NLH-E87.3 were carried out (**chapter 2**). The intraspecific competition effects on invasion rate, reproduction, IJs length and time to first emergence were tested by using *G. mellonella* larvae.

Temperature is an important factor to consider when examining the use of nematodes as biocontrol agents, because it affects for example survival, mobility, infectivity, reproduction and development of nematodes. Between production and application the nematodes are often stored for a certain period of time. Thus, the thermal tolerance of each nematode species or strain, thought to have potential qualities as a biocontrol agent, may be adjusted in order to improve their shelf life and assure the maintenance of the infective capacity during a storage period. In this respect a series of laboratory experiments were carried out to investigate the effect of several different temperatures on survival, infection and reproduction of a Dutch heterorhabditids strain stored over time (**chapter 3**).

Nematode strains differ in virulence to insect hosts and that the developmental stage of the insect plays an important role in its susceptibility. In most earlier laboratory assays most of the nematodes are cultured mainly in the last larval instar of *G. mellonella*, which is a model of a very susceptible insect with no economic importance as a soil pest. The black vine weevil, *Otiorhynchus sulcatus*, is an important soil pest of a wide range of horticultural crops and ornamental nursery stocks. Entomopathogenic nematodes are one of the alternative control

strategies that have been used to control *O. sulcatus*. Some of the *Heterorhabditis* isolates from NW Europe showed to be effective against *O. sulcatus* larvae and pupae. Working with the hypothesis that *Heterorhabditis megidis* (strain NLH-E87.3), belonging to the NW European group, might have the potential to control *O. sulcatus*, and that the susceptibility for nematodes changes with the larval age with consequences on nematode reproduction was tested. Thus, a series of laboratory experiments were conducted to evaluate the effect of the developmental larval instar of two different insect hosts *G. mellonella* (beehive pest) and *Otiorhynchus sulcatus* (soil pest) (**chapter 4**).

When the infective juveniles obtained from one insect host are being used to control another insect species they must adapt themselves to the new host. I hypothesize that the effectiveness of the reared nematode in relation to another host may differ completely from that known for *G. mellonella*. Therefore, the IJs obtained from the different larval sizes of *G. mellonella* and *Otiorhynchus sulcatus* were tested on their capacity to infect different larval instars of the same or different hosts (**chapter 5**).

Infective juvenile dispersal is an important and necessary factor for host finding. Due to differences in searching behavior, species of entomopathogenic nematodes are divided in two categories: nematode species that search actively for a host are classified as cruisers whereas, the species of nematodes that sit and wait near the soil surface are called ambushers. Some species of *Steinernema* and *Heterorhabditis*, particularly the NW European *Heterorhabditis* nematodes, are known to migrate very actively through the soil in search of a host. Cruiser nematode species use up their limited reserves more quickly than ambushers, and face a series of other stress factors. The effects of aging and density on dispersal behavior of infective juveniles of a NW European *Heterorhabditis* on a 2-dimensional substrate are reported and discussed in **chapter 6**.

Purposing to understand how nematodes search for a host in the soil a series of bioassays in 3-dimensional sand matrices and a Y-tube olfactometer were performed. Approaching the idea that chemical compounds from plant roots, host insects, or compounds resulting from the interaction between plant and insect might affect the host finding process, I hypothesise that the foraging behaviour of infective juveniles changes when they are exposed to chemicals from roots of different species of plants, depending on whether they are given individually, in combination with other plant species or in combination with root feeding insect larvae. The results of this study are reported and discussed in **chapters 7, 8 and 9**.

Finally, in **chapter 10**, the contribution of the results to the existing knowledge is highlighted, and the applicability is discussed in the context of understanding the interactions of entomopathogenic nematodes in the soil environment and optimizing their control potential against soil insects in biological control programs.

Chapter 2

Development of the entomopathogenic nematode *Heterorhabditis megidis* strain NLH-E87.3 in *Galleria mellonella*.

Adapted from: Mari I. C. Boff, Gerrie L. Wiegers, Lonne J.M. Gerritsen & Peter H. Smits. (2000). Development of the Entomopathogenic Nematode *Heterorhabditis megidis* strain NLH-E87.3 in *Galleria mellonella*. *Nematology*, 2: 303-308.

Abstract

Increasing densities of *Heterorhabditis megidis* (strain NLH-E87.3) infective juveniles (IJs) affected establishment, reproduction, length and time to first emergence of the nematodes in larvae of the greater wax moth, *Galleria mellonella*. Although the number of nematodes that established the host increased with increasing dose, percentage of invasion declined. The number of progeny produced per host initially increased with dose. The highest production of IJs per cadaver was reached at a dose of 300 IJs per host, at that dose 62 ± 3.4 IJs were established per cadaver. Production decreased again significantly at higher densities. The smallest infective juveniles were produced at a dose of 1000 IJs per host and the largest at a dose of 300 IJs per host. Time to first emergence of juveniles was generally shorter when the number of IJs inoculated was large (300-3000 IJs/host).

Introduction

Entomopathogenic nematodes in the families Heterorhabditidae and Steinernematidae are lethal parasites of insects. They have one free-living stage with a single function: to infect a host. Once the host is found, infective juveniles penetrate into the hemocoel and release symbiotic bacteria which produce toxins that kill the host in 24-48 h. The bacteria multiply and create the conditions necessary for the nematodes to mature, mate and reproduce (Poinar, 1990). All entomopathogenic nematodes have the same general life history but species differ in host utilization (Dutky, 1956; Reed & Carne, 1967; Selvan & Blackshaw, 1990), searching behaviour (Campbell & Gaugler, 1993 and 1997; Grewal *et al.*, 1994) and reproductive strategies (Poinar, 1990). Intraspecific competition affects progeny production in entomopathogenic nematodes. When the number of infective juveniles penetrating into a host exceeds an optimal level, exploitative intraspecific competition occurs among the developing nematodes, which reduces the total number of progeny emerging from the cadaver (Kaya & Koppenhöfer, 1996). Reduced production of entomopathogenic nematode (Heterorhabditidae and Steinernematidae) progeny from cadavers exposed to high densities of infective juveniles has also been reported by Molyneux *et al.* (1983), Zervos *et al.*, (1991), Selvan *et al.*, (1993a) and Koppenhöfer and Kaya (1995). Increasing population density within the host can also adversely affect the fitness of parasitic nematodes besides reducing progeny. This, for example, has been observed in vertebrate intestinal helminths (Keymer, 1982; Goater, 1992;

Kaitala *et al.*, 1997). Effects of intraspecific competition in insect parasitic nematodes have been noted in mermithid nematodes in mosquitoes and beetles (Tingley & Anderson, 1986; Blackmore, 1992), in pinworms (*Oxyurida*) in the hindgut of cockroaches and in aquatic beetles (Adamson *et al.*, 1992; Adamson & Noble, 1993), and also in Steinernematidae and Heterorhabditidae (Selvan *et al.*, 1993a; Koppenhöfer & Kaya, 1996; Grewal *et al.*, 1997). In this paper we present data on how density dependent factors within *Galleria mellonella* larvae influence the population development of the entomopathogenic nematode *Heterorhabditis megidis* strain NLH-E87.3. We studied the effects of increasing density on nematode establishment reproduction and length.

Material and Methods

Heterorhabditis megidis (NLH-E87.3 strain) (Smits *et al.*, 1991), was cultured in last instar larvae of the greater wax moth, *Galleria mellonella*, at 25°C. Cylindrical plastic containers (diameter 36mm, height 56 mm) with 4 wax moth larvae at the bottom were filled with moist sand (8%, w/w, tap water). Thirty infective juveniles per larva in 1-ml tap water were added to the sand surface. The containers were capped and incubated at 25°C. After 144 hr, the host cadavers were removed to modified White traps (Smits *et al.*, 1991; Lewis & Gaugler, 1994). Small Petri dishes (5 cm), without lid, and holding the cadavers, were floated in water inside a 9 cm Petri dish and stored at 25°C. Infective juveniles emerging from the cadavers crawled over the side of the holding dish into the water where they were trapped. Infective juveniles were collected daily, during 4 days, transferred to tissue culture flasks and stored in tap water at 5°C until testing. All the experiments were performed within 15 days of emergence.

Bioassays were carried out by placing single last-instar larvae of *G. mellonella* in plastic cylinders (50 ml, 36mm diameter) filled with moist (8% w/w) heat sterilised silver sand. Host larvae were individually exposed to nematode doses of 10, 30, 100, 300, 1000 and 3000 infective juveniles that were added to the top of the cylinder in one ml of water. Water without nematodes was added to the controls. The containers were closed with a lid and held at 25°C. Each concentration of infective juveniles was tested on 15 *Galleria* larvae. After 10 hours of exposure the *G. mellonella* larvae were removed from the sand and rinsed with tap water to remove external nematodes. The rinsed larvae were transferred to Petri dishes with moist filter paper and held at 25°C during 144 hours. Of each group of 15 larvae exposed to a

certain dose of infective juveniles, 10 larvae were dissected and digested in a 0.8% pepsin solution and the number of nematodes inside the cadavers, for each dose, was determined. The other 5 larvae were used to study density dependent effects on reproduction, size of nematodes and emergence time. The experiment was performed four times.

Production capacity was assessed by placing each of the five red coloured cadavers into an individual modified White traps. All infective juveniles that emerged from a single host over a period of 5 days after the first appearance of nematodes in the water were harvested and the total nematode suspension was put in a 50-ml tissue culture flask. To assess the total production during the harvest period, the contents of the flask was mixed thoroughly with air bubbles from an aquarium pump and from this suspension 8 samples of 10 μ l were counted under a stereomicroscope using a counting slide.

The length of a hundred individual infective juveniles was measured for each dose. Individuals to be measured were randomly selected from the pooled population. Prior to measurement, infective juveniles were killed by heating them in 5 ml of water in a microwave for 30 seconds.

The time to emergence, *i.e.*, number of days after infection to the moment when infective juveniles started to emerge from the infested host cadaver was determined by checking all the modified White traps daily at 10:00 hours until the first infective juveniles started to come out from the cadaver.

The results were statistically analysed by analysis of variance. Significant F-test ($P \leq 0.05$) are followed by least-significant-difference-test (LSD) ($P \leq 0.05$) for testing pairwise differences between treatment means. Means are reported with standard errors of the mean (\pm SE). Analyses were performed using the statistical program Genstat (Anon., 1993).

Results

At all densities of IJs tested, all wax moth larvae were infected by *Heterorhabditis megidis* (strain NLH-E87.3). No mortality was detected in the control larvae. The number of infective juveniles (IJs) that invaded the host increased with increasing dose. Invasion increased from 4 ± 0.2 at a dose of 10 to 763 ± 28.9 at a dose of 3000 IJs/larva. The number of

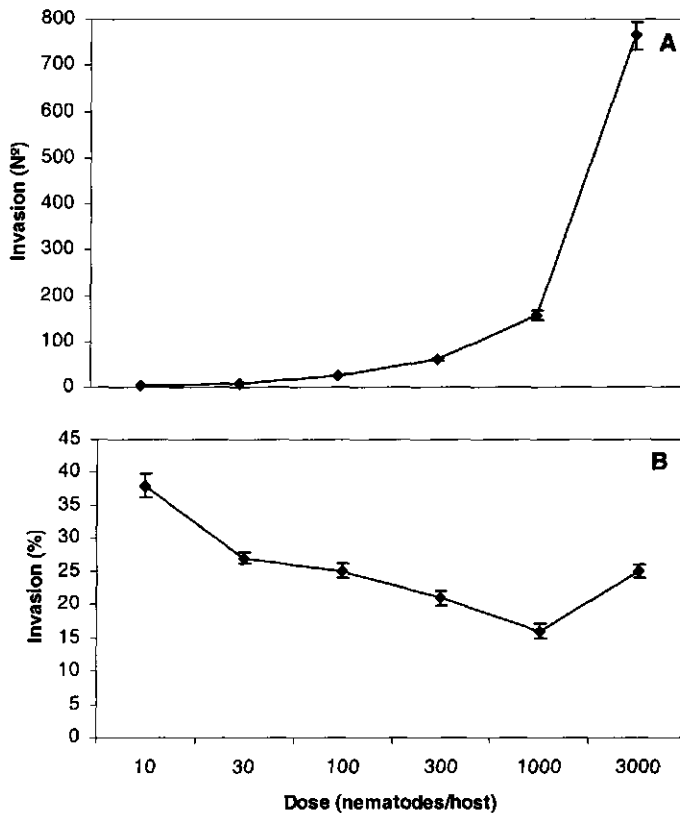


Fig. 1. Mean number (A) and percentage (B) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) present in the haemocoel of *Galleria mellonella* larvae 144 h after exposure. Exposure time was 10 hours. Means \pm SE are shown.

invading nematodes did not increase proportionally to the increase in dose, so there was a decline in the percentage of invading nematodes. Percentage invasion decreased from $38 \pm 1.9\%$ at a dose of 10 to $16 \pm 1.0\%$ at a dose of 1000 and increased again to $25 \pm 1.0\%$ at a dose of 3000 IJs/larva (Fig. 1). Production of infective juveniles initially increased with increasing density up to dose 300 nematodes/host and then declined (Table 1). The largest cumulative production of juveniles occurred when the initial nematode density within the host was 62 ± 3.4 at an exposure dose of 300 IJs/host. No statistical difference in infective juvenile

production was observed among the doses of 10 (4 ± 0.2), 100 (25 ± 1.0) and 1000 (157 ± 10.1) nematodes/host, nor between the doses of 1000 (157 ± 10.1) and 30 (8 ± 0.3) nematodes/host. The strongest effect of nematode density was observed at a dose of 3000 (763 ± 29.0) nematodes/host where each cadaver produced only 10 142 IJs. A clear decline in the number of offspring per invaded infective juvenile was observed with the increase of the dose. At the dose of 3000 IJs/host only 13 new infective juveniles were produced per invaded IJ.

Table 1. Invasion, 5 days yield and reproduction rate of infective juveniles in *Galleria mellonella* larvae exposed to *Heterorhabditis megidis* (strain NLH-E87.3) at different doses.

Data followed by the same letter are not significantly different from each other. ($P \leq 0.05$).

Dose (nematode/host)	Establishment ($n^\circ \pm SE$)	Total Production (IJs / host $\pm SE$)	Reproduction Rate (per invaded IJ)
10	4 ± 0.2	$28\ 107 \pm 2379$ b	7027
30	8 ± 0.3	$25\ 470 \pm 1492$ c	3148
100	25 ± 1.0	$30\ 450 \pm 1015$ b	1216
300	62 ± 3.4	$39\ 272 \pm 882$ a	663
1000	157 ± 10.1	$27\ 243 \pm 1082$ bc	174
3000	763 ± 29.0	$10\ 142 \pm 176$ d	13

The data in figure 2 show that infective juveniles produced in cadavers infested with 1000 nematodes/host were significantly smaller ($767 \pm 6.1 \mu\text{m}$) than offspring from other doses. The maximum infective juvenile length occurred at the dose of 300 IJs/host ($805 \pm 3.96 \mu\text{m}$). No length differences were observed among infective juveniles that came out of larvae infested with 10, 100 and 3000 IJs/host nor between those originated from larvae infested with a dose of 10 and 30 IJs.

The number of days after which nematodes first emerged from the cadavers decreased with increasing dose (Fig. 3). Infective juveniles first emerged on average 13 to 15 days after infestation at high IJs densities, while in larvae infested with 30 and 10 IJs/host the emergence period began 18 and 20 days after infection, respectively.

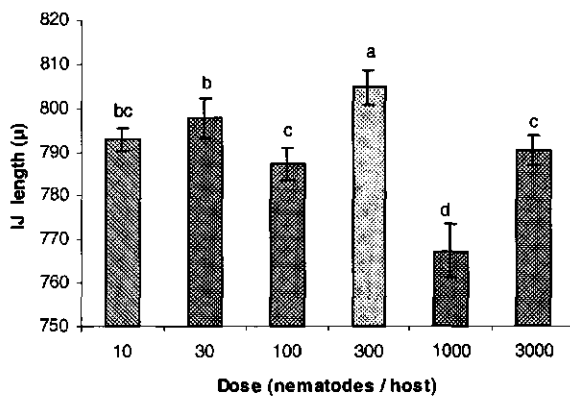


Fig. 2. Length (\pm SE) of infective juveniles of *Heterorhabditis megidis* strain NLH-E87.3 produced in *Galleria mellonella* larvae exposed to various nematode doses. Bars with the same letter are not significantly different ($P = 0.05$).

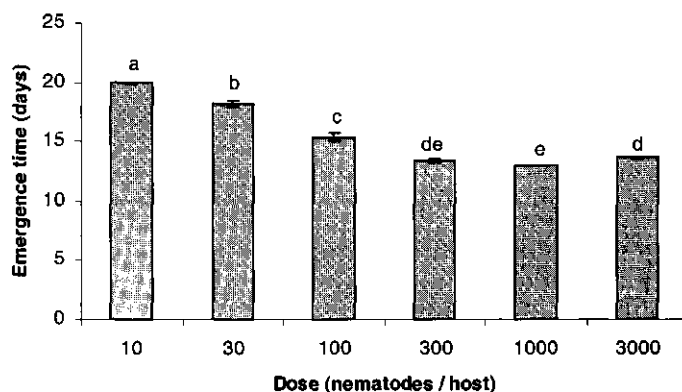


Fig. 3. Effect of inoculum size on the mean duration of time to the first emergence (\pm SE) of infective juveniles of *Heterorhabditis megidis* strain NLH-E87.3 from *Galleria mellonella* larvae. Bars with the same letter are not significantly different ($P = 0.05$).

Discussion

The results on infectivity indicate that, although the number of invading nematodes increased with increasing dose, the percentage of invasion declined. The proportion of IJs infecting *G. mellonella* declined with increasing dose until 1000 nematodes/host, and increased again at the dose of 3000 nematodes/host. Mannion & Jansson (1993) using a sand bioassay arena similar to that used in this study found that the invasion capacity of *Heterorhabditis* sp. continued for several days and that the higher the dose, the higher the proportion of the nematodes that invaded later. Fan & Hominick (1991a) using a series of host exposures and different doses (10-300 nematodes/host) found that percentage infection of *Heterorhabditis* sp. was relatively constant over a range of doses. Selvan *et al.* (1993a) working with 10 different doses (10 - 6 400) also found that the percentage infection of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* declined with increasing dose. With the exposure time of 10 hrs used in our experiment no more than 38.3% of the applied IJs migrated through a 5,5 cm sand column and established in the host. The results seem to support the conclusion of Fan & Hominick (1991a) and Selvan *et al.* (1993a) that only a proportion of the applied nematodes are able or willing to invade a host at a given time. We assessed the IJs establishment 144 hours after exposure. It is possible that large proportions of IJs invaded died soon after invasion and were not found at dissection moment. As soon as the IJs were harvested from a modified White trap they were transferred to a temperature of 5°C for a period of 15 days. According to Griffin (1996) and Fan & Hominick (1991b) this storage condition appears to induce most of the IJs into a state that they lose their ability to parasitize. Griffin (1996) reported that IJs of *Heterorhabditis* sp. stored at a low temperature and tested at a higher temperature show an initial lower peak of infectivity followed by a larger peak after some weeks of storage. A U-shaped curve was observed by Fan & Hominick (1991b) in their studies with *Steinernema* species stored at a temperature of 5°C that also regained the infectivity capacity after a period of storage. These effects may have played a role also in our experiment.

Host utilisation and the metabolic rate of processing host tissues by symbiotic bacteria influences the survival and reproduction of nematodes and differs between nematode species. Selvan *et al.* (1993a) stated that *H. bacteriophora* was able to tolerate high nematode densities within the host whereas *S. carpocapsae* was not able to survive and reproduce above an initial dose of 200 nematodes per host. Molyneux *et al.* (1983) reports that IJs of *Heterorhabditis*

spp. were only able to reproduce in *Lucilia cuprina* larvae subjected to low dosages of IJs, whereas *Steinernema* spp. were not able to reproduce at any dosage. We found that *H. megidis* (strain NLH-E87.3) was able to reproduce on *G. mellonella* larvae at all doses. However, the offspring production, obtained during the determined harvesting period of 5 days, significantly dropped when a large number of infective juveniles invaded the host. The reproduction rate decreased from 7027 IJs per established IJ at a dose of 10 IJs/larva to 13 IJs per established IJ at a dose of 3000 IJs/larva. It is however important to point out that, even if the reproduction rate per invaded IJ decreased proportionally with an increase in dose, the cumulative production of juveniles did not show the same tendency. Zervos *et al.* (1991) found that the cumulative production of *H. heliothidis* decreased with an increase in the IJ inoculum and the largest production occurred at a dose of 25 IJs/host at 25°C. Selvan *et al.* (1993a) observed that the production of IJs of *H. bacteriophora* increased with increasing the initial density up to approximately 100 IJs/host. The highest number of infective juveniles were produced when the initial nematode density within the host was 61.4 ± 1.1 . Our results on the production of infective juveniles agree only with the finding of Selvan *et al.* (1993a). We choose to count the total production of five days after first emergence of IJs in the water. IJs emergence actually continues longer but within the 5 days period the majority of nematodes emerged as was also found by Selvan *et al.* (1993a). The total production per *Galleria* cadaver was not very high. This is probably explained by the relatively high temperature (25°C) used in our experiment. Mason & Hominick (1995) found higher production of *H. megidis* in *Galleria mellonella* at 15°C and 20°C than at 25°C.

Our studies of density-dependent effects on the length of infective juveniles indicate that the longest IJs were produced at densities that produced the largest number of IJs. These results do not agree with the results found by Selvan *et al.* (1993a). These authors concluded that the longest infective juveniles were produced at the lowest densities and not at the densities that produced the largest number of infective juveniles. But it should be considered that the nematode species used are different. The fact that the longest IJs were found at a dose of 300 and the shorter at a dose of 1000 IJs per host, and that the IJs' length did not show clear linear correlation with doses, suggests that at specific establishment rates the developmental conditions and population dynamics of IJs inside the cadavers are influenced by a complexity of interactions.

For the nematode strain we tested, the time to first emergence decreased with increasing dose until a dose of 300 IJs per host. The influence of IJs dose on emergence period may be

direct, by affecting the number of generations in a cadaver before emergence, or indirect, by affecting the growth rate of the bacterial symbiont or the biochemical composition of the cadaver.

Several factors affect the *in vivo* production of entomopathogenic nematodes, and these factors interact in unpredictable ways (Zervos *et al.*, 1991). Selvan *et al.* (1993a) stated that density-dependent factors play an important role in fecundity of entomopathogenic nematodes. They can act directly by affecting the number of IJs produced by infested cadavers, or indirectly by influencing the longevity of IJs. To be successful an entomopathogenic nematode and its associated bacteria must overwhelm the host immune system as a part of the initial infection process. The immune response of a resistant host may affect the establishment in a density-dependent manner (Dunphy & Thurston, 1990). In our experiments, host immunity does not explain the density dependent effects on *H. megidis* (strain NLH-E87.3) establishment and reproduction because *G. mellonella* showed no immune response to the used pathogen species. The effects of density on *H. megidis* (strain NLH-E87.3) infectivity, fecundity and other parameters seem to result from intraspecific competition for nutrients and /or space.

Chapter 3

Effect of storage time and temperature on infectivity, reproduction and development of *Heterorhabditis megidis* in *Galleria mellonella*.

Adapted from: Mari I.C. Boff, Gerrie L. Wieggers & Peter H. Smits. Effect of storage time and temperature on infectivity, reproduction and development of *Heterorhabditis megidis* in *Galleria mellonella*. *Nematology* (in press)

Abstract

The effect of temperature, dose and storage period on the infectivity and development of *Heterorhabditis megidis* (strain NLH-E87.3) infective juveniles (IJs) was studied in the laboratory. Infective juveniles were stored at 5, 10, 15 and 20°C for a period of up to 70 days (10 weeks). Every second week, mortality, infectivity, reproductive capacity, time to emergence and length of infective juveniles were examined. *Galleria mellonella* larvae were infested with a dose of one and 30 IJs, respectively. As the period of storage increased the number of active nematodes, the infectivity and the reproduction of IJs decreased both at low and high temperatures. Time to first emergence and body length of infective juveniles were significantly affected by time, storage temperature and inoculum level. Independent of the dose and storage periods, the highest infectivity and optimal development were observed when IJs were stored at 10 and 15°C.

Introduction

Entomopathogenic nematode species in the families Heterorhabditidae and Steinernematidae are lethal insect parasites which have a mutualistic association with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Boemare *et al.*, 1993; Thomas & Poinar, 1979). All entomopathogenic nematode life-stages of these two families are endoparasitic, except the infective juvenile stage, which emerges into the soil to find and infect new hosts (Campbell *et al.*, 1998; Glazer, 1996). The free-living, infective stage nematode, which does not feed, carries its bacterial symbiont monoxenically within a ventricular portion of the intestine or throughout the intestinal or pharyngeal lumen. These bacteria are released into the insect hemocoel. Inside a hosts the bacteria multiply producing toxins that kill the insect in 24-48 h and create the conditions necessary for the nematodes to mature, mate and reproduce (Poinar, 1990).

Many species of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae are commercially mass-produced and have successfully been introduced to many markets (Ehlers, 1996; Jung, 1996). Although infective juveniles (IJs) of entomopathogenic nematodes have been recovered from soils of a wide variety of climatic

regions in every continent, their development and performance as biological control agents, not only in the field but also in mass reproduction systems are influenced by many factors. Host species, storage time and temperature, moisture conditions, soil type, soil structure, the nematode isolate used, and the quality of the nematode product are some of the abiotic and biotic factors that influence the success of performance and commercialisation of IJs (Georgis & Gaugler, 1991; Fan & Hominick, 1991b; Griffin, 1993; Grewal *et al.*, 1994a; Glazer, 1996; Steiner, 1996; Jagdale & Gordon, 1997 and 1998). Entomopathogenic nematodes of both genera have been cultured commercially for over a decade. The methods used for production vary from *in vivo* for small-scale production used by small farmers or laboratories to *in vitro* using liquid culture fermentors. *Steinernema* species are consistently and effectively produced *in vitro* and steinernematid-based products are less expensive to produce and can be stored longer than heterorhabditid-based products (Georgis & Gaugler, 1998). Although the production and use of heterorhabditid species lags behind steinernematids, there are situations in which they are more efficient as in the case of *Otiiorhynchus sulcatus* that causes economic losses in high and medium cash crops (Klein, 1990). During a storage period there are many factors affecting the IJs performance. Georgis (1990) points out that the metabolism of entomopathogenic nematodes is temperature driven and that it can determine pathogenicity and viability of these organisms in biocontrol programmes. Jung (1996) stated that IJs age with time and that their biological and physiological characteristics during their shelf life influence their field efficacy. Despite a large amount of information and the research advances on entomopathogenic nematodes, shelf life and fitness of heterorhabditid nematodes are still poorly understood. The objective of this study was to investigate the influence of storage time and temperature on infectivity, fecundity and development of *Heterorhabditis megidis* (NLH-E87.3) a Dutch strain, that still not much studied but perhaps it has a great potential as biological control agent against soil-inhabiting insects in Western Europe.

Materials and Methods

Nematodes

Heterorhabditis megidis (strain NLH-E87.3) (Smits *et al.*, 1991) was cultured in last-instar wax moth larvae (*Galleria mellonella*) at a temperature of 20°C. Sixty last instar wax moth

larvae were separated in groups of four. The larvae were put at the bottom of 50 ml plastic cylinders filled with moist sand (8% w/w tap water) and exposed to a dose of 400 infective juveniles (IJs) added in 1-ml of tap water to the sand surface. The containers were closed and incubated in the dark at 20°C. After 96 hours the red-coloured infected wax moth larvae were transferred from the sand to a modified White trap (Smits *et al.*, 1991; Lewis & Gaugler, 1994). Each group of infested larvae was placed on a layer of moist filter paper in a 5 cm Petri dish without lid. The small dishes holding the cadavers were floated in water inside large Petri dishes (15 cm diameter) with lid and stored in the dark at 20°C. IJs that emerged from the cadavers and moved from the small dish to the water layer were collected daily for a period of 3 days. All the collected IJs were mixed to make a standard suspension then distributed and stored in 30 ml of non-sterile tap water in tissue culture flasks at densities of 4 500 IJs per ml. Flasks with the pooled IJs were stored at a temperature of 5°C, 10°C, 15°C or 20°C for periods up to 10 weeks. Before being used in the experiments the IJs were washed with tap water and left at room temperature (18-20°C) for two hours.

Survival

Survival of infective juveniles over time at their respective storage temperatures was estimated by taking twelve 10µl samples of a well-mixed water-nematode suspension and observing their movements and / or shape under a dissecting microscope and counting the total number of living and dead IJs. Five replicate flasks were used for each storage temperature tested. The assays were performed at three different times.

Infectivity assays

The infective ability of IJs stored at different temperatures over time was tested by using two different doses of IJs. Two series of bioassays were carried out.

Dose of 30 IJs - Sixty single last-instar *G. mellonella* larva were placed at the bottom of plastic cylinders (50-cm³, 36 mm diameter). The cylinders were filled to the top with moist (8% w/w) fine-grained silver sand (particle size 0-0.5 mm, heat sterilized, 9 h, 180°C). Four groups of 15 pots were formed with the filled plastic cylinders one for each IJs storage temperature. Each individual larva of each group was exposed to a dose of 30 IJs. Nematodes were added to the top of the cylinder, on the sand surface, in one ml of water. Only water was added to the controls. The containers were closed with a lid and placed in the dark at 20°C for a period of 48 h. In these assays each pot was considered as one repetition and each assay was

performed three times.

Dose of one infective juvenile - Following the methodology described above, four groups (one for each IJs storage temperature) of 30 individual *G. mellonella* larvae were exposed to a dose of only one infective juvenile (IJ). Each IJ was released on the sand surface by using a called "nematode fishing-rod" (eyebrowhair glued to a stick). The containers were closed and transferred to a dark 20°C chamber for 48 h. Here as mentioned above each pot represents one repetition and all bioassays were performed three times.

After two days of exposure and incubation at a temperature of 20°C, the *G. mellonella* larvae of both bioassays were removed from the sand and rinsed with tap water to remove any nematodes from the outer surface. The larvae were transferred to moist filter paper and incubated separately at 20°C for 144 h. Tests were conducted in the 1st, 2nd, 4th, 6th, 8th and 10th week of storage after the harvesting period. Mortality was assessed for both experiments 144 hours after application of the IJs by counting the total number of living and dead larvae. In this study a larva was considered as dead by IJs infection when it presented the characteristic red-brownish colour. To assess the penetration rate of the nematodes the dead larvae exposed to 30 IJs were dissected and digested in a 0.8% pepsin solution (Mauleon *et al.*, 1993) and the number of nematodes inside were counted.

Reproduction

To determine the number of nematodes produced per infested larva, experiments were carried out as described in the infectivity bioassays using the doses of one and 30 IJs per larva. After the exposure of 48 h the infected red-coloured host cadavers from both IJs doses were removed from the sand, rinsed, and individually transferred to individual modified White traps (Smits *et al.*, 1991; Lewis and Gaugler, 1994). The modified White traps were incubated in dark at 20°C. All the infective juveniles emerging from a single host over a period of 5 days were harvested and the total nematode suspension was put in a 50-ml flask. The content of each flask containing the nematode suspension from individual cadavers was mixed thoroughly using air bubbles and from this suspension 8 samples of 10 µl were counted under a stereomicroscope using a counting slide. The harvesting period of 5 days was based on previous personal observations in assays done with the same strain at the same temperature. For the purpose of this study, we defined the start of emergence as the moment when nematodes moved from the small Petri dish holding the cadaver into the water. Three replicate experiments were done, each using five cadavers per treatment.

Infective Juvenile measurements

The effect of storage temperature over time on the body length of IJs was determined by measuring individuals from the original suspension i.e., those used for the bioassays, here called "parents", and individuals originating (offspring) from cadavers initially exposed to a dose of one or 30 IJs parents.

"Parents measurement" - One hundred IJs from each standard suspension stored at different temperatures were measured at week zero and 10 weeks later. Individuals to be measured were randomly selected from the standard suspension. Live IJs were killed by heating them in 2 ml of tap water for 30 s in a microwave oven (700-Watts). The length of the straightened IJs was measured using a microscope with a graticule incorporated into one of the eyepieces.

Offspring measurement - Infective juveniles harvested from each cadaver of each combination of dose (one or 30 IJs), storage temperature and time were separately mixed forming standard IJs suspensions. From these suspensions one hundred IJs, randomly selected, were measured adopting the procedure described above.

Time to emergence

The number of days after infection up to the moment when infective juveniles started to emerge was determined by checking all traps under a dissecting microscope, daily at 14:00 h until the moment that the first infective juveniles started to emerge from the cadaver.

Statistical analysis

The results were analysed by analysis of variance. Significant F-tests ($P \leq 0.05$) were followed by least-significant-difference-tests (LSD) for testing pairwise differences between treatment means. Means are reported with standard errors of the mean (\pm SE). Analyses were performed using the statistical program Genstat 5 version 4.1 (Genstat Committee, 1997)

Results

Survival - Most IJs survived up to 10 weeks at all storage temperatures (Table 1). At the storage temperatures of 10 and 15°C over 90% of IJs were still active after 10 weeks. However, at storage temperatures of 5 and 20°C the number of living IJs changed significantly from the first to the tenth week particularly at 20°C.

Table 1. Percentage of living infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) stored in water over time at different temperatures.

T (°C)	Storage Time (weeks)					
	1	2	4	6	8	10
5	100 aA	97 aA	94 bB	92 bC	87 bD	84 bE
10	100 aA	100 aA	97 aA	95 aAB	94 aB	92 aC
15	100 aA	100 aA	97 aA	97 aA	95 aB	94 aB
20	100 aA	97 aA	92 cB	81 cC	70 cD	53 cE

Lower case letters correspond to comparisons among storage temperatures. Uppercase letters correspond to comparisons among storage times. Data followed by same letters are not significantly different from each other ($P < 0.05$).

Infectivity - The effect of storage temperature and storage time on the infectivity of the nematode strain *H. megidis* (NLH-E87.3) is shown in Figures 1 and 2. *G. mellonella* larvae infested with one infective juvenile (IJ) per larva showed over 30% mortality at all temperatures up to four weeks of storage (Fig. 1). After this, at the 6, 8 and 10th week, the same level of mortality was observed only when IJs were stored at temperatures of 10 and 15°C. Independent of the storage period, the dose of 30 IJs per larva caused a mortality of more than 80% when IJs were stored at temperatures of 5, 10 and 15°C (Fig. 2). No mortality, at either dose, was observed when larvae were exposed to nematodes stored at a temperature of 20°C for 10 weeks. Storage temperature and time also influenced the number of IJs established per larva (Fig. 3). At 10 weeks of storage the penetration capacity of IJs was less affected when they were stored at temperatures of 10 and 15°C than at 5 and 20°C. The maximum penetration capacity of nematodes stored at 5 and 20°C was seen after the first and second week of storage, respectively. After this at both temperatures the numbers of established IJs decreased to very low levels at 5°C and to zero at 20°C.

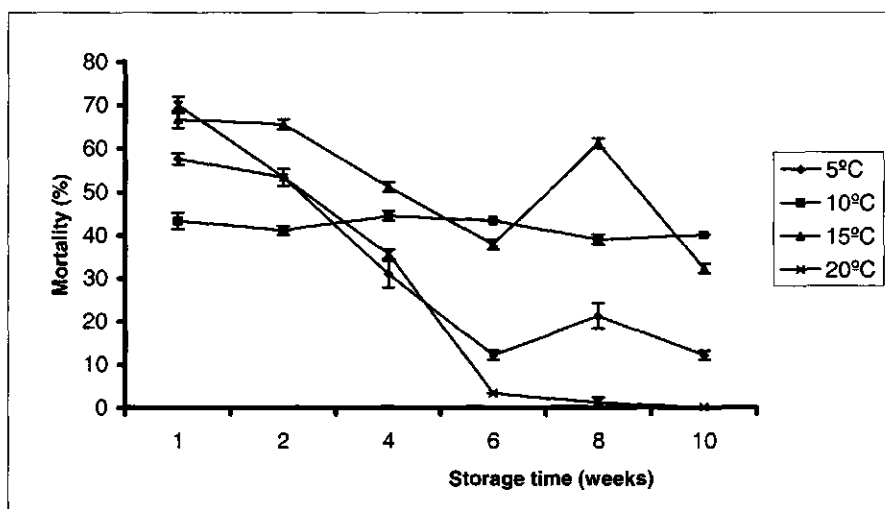


Fig. 1. Mortality of *Galleria mellonella* larvae exposed to a dose of one infective juvenile of *Heterorhabditis megidis* (NLH-E87.3) stored over time at different temperatures.

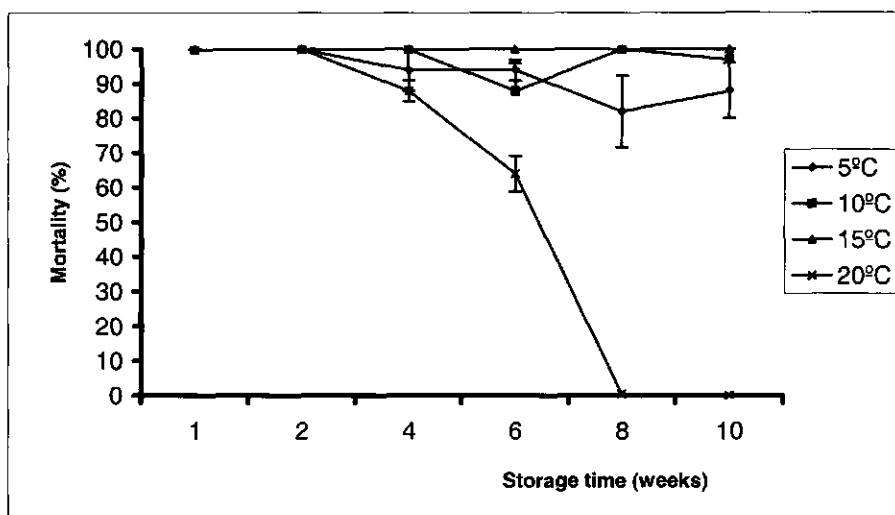


Fig. 2. Mortality of *Galleria mellonella* larvae exposed to a dose of 30 infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) stored over time at different temperatures

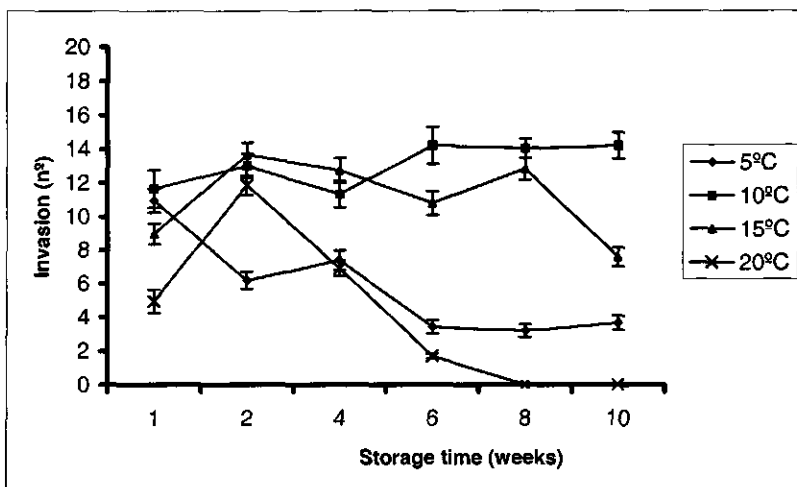


Fig. 3. Number of nematodes invading *Galleria mellonella* larvae exposed to a dose of 30 infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) stored at different temperatures over time.

Reproduction - Results of the production of infective juveniles showed that when host larvae were infested with one IJ, the lowest reproduction occurred at a storage time of one week at all storage temperatures except at 20°C (Fig. 4). IJs stored at 10 and 15°C produced more juveniles than those stored at 5 and 20°C. No infection, and consequently no production, was observed at 20°C after a storage period of 8 and 10 weeks (Fig. 3). Production of IJs from larvae exposed to a dose of 30 IJs (Fig. 5) decreased after two weeks of storage at all temperatures. No great differences in production were observed when larvae were exposed to IJs stored between 4 and 10 weeks at temperatures of 5, 10 and 15°C. Data in Figure 6, show that the production of IJs per established IJ is much higher in larvae infested with one IJ, than in larvae infested with 30 IJs, but the overall average production per cadaver is higher when *G. mellonella* larvae are exposed to the dose of 30 IJs (Fig. 5). The nematodes reproduction seem to improve in time and the process seems also to be influenced by the storage temperature the maximal production per established IJ was obtained after 8 weeks storage at 5°C, 6 weeks storage at 10°C and 4 weeks storage at 15 and 20°C.

Infective juvenile measurement - The length of IJs after 10 weeks of storage was significantly influenced by the storage temperature (Fig. 7). Nematodes stored at

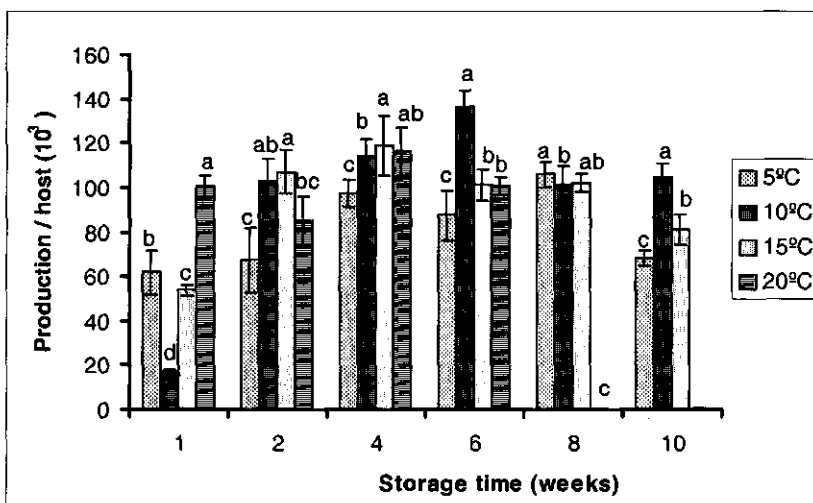


Fig. 4. Average number of infective juveniles that emerged from five individual cadavers of *Galleria mellonella* exposed to one infective juvenile of *Heterorhabditis megidis* (NLH-E87.3) stored over time at different temperatures. Columns with the same letter are not significantly different ($P < 0.05$). Comparisons are between temperatures within the storage periods

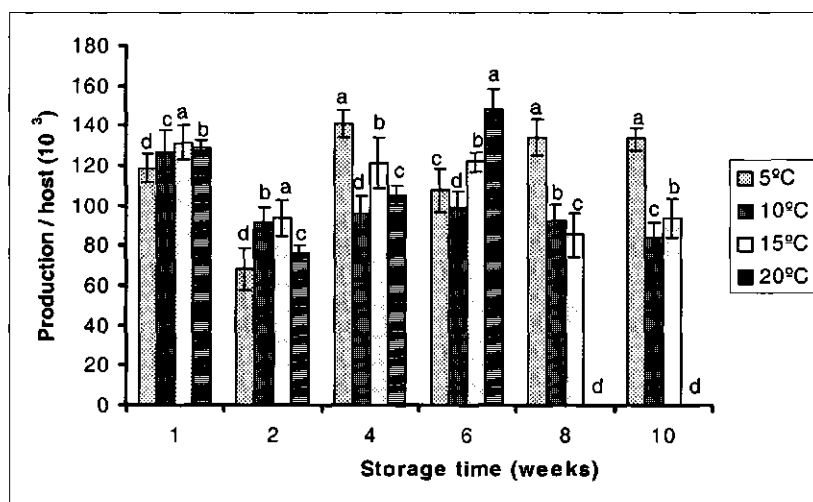


Fig. 5. Average number of infective juveniles emerged from five individual cadavers of *Galleria mellonella* each exposed to a dose of 30 infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) stored over time at different temperatures. Columns with the same letter are not significantly different ($P < 0.05$). Comparisons are between temperatures within the storage periods.

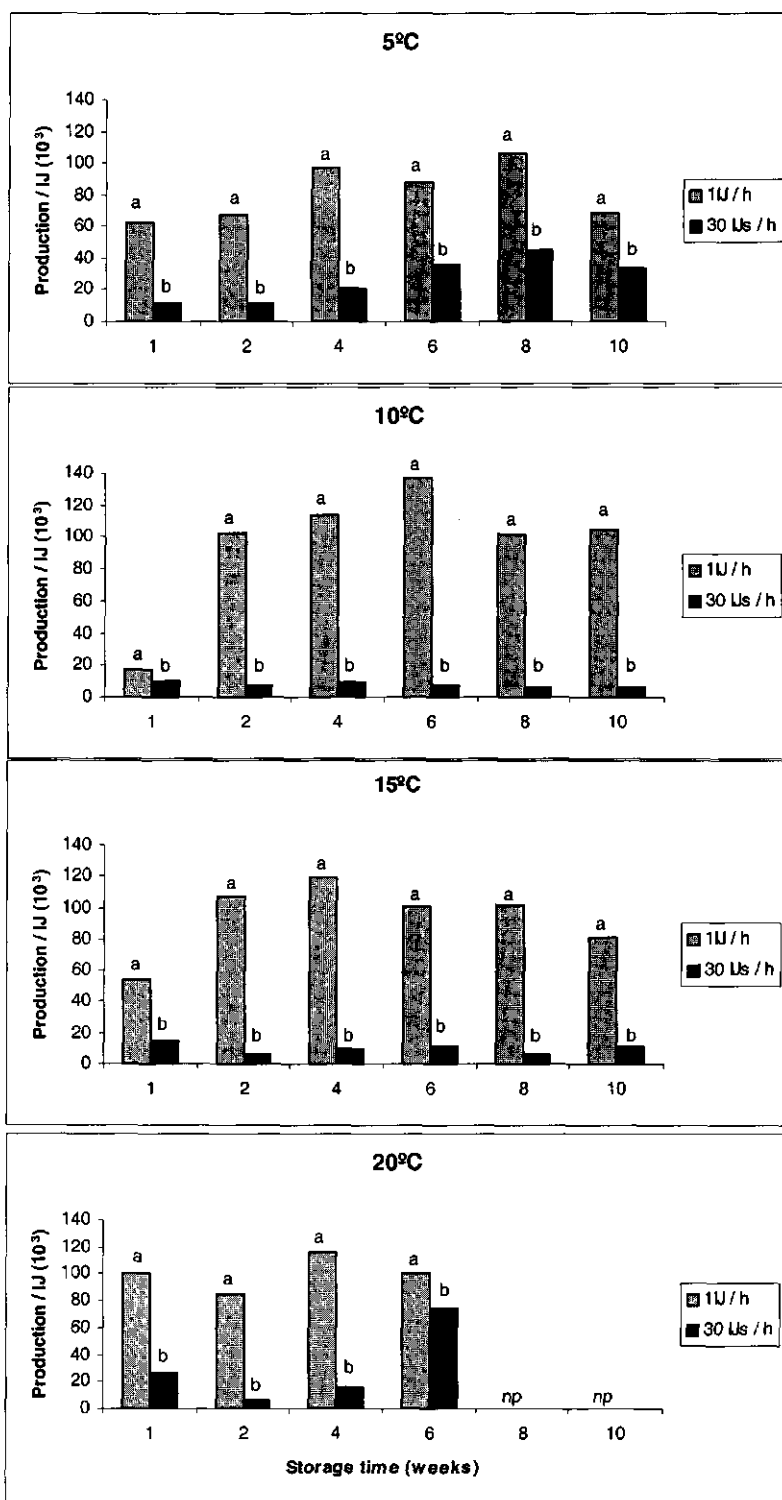


Fig. 6. Production per infective juvenile that established in *Galleria mellonella* larvae exposed to doses of one and 30 infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) stored over time at different temperatures. Columns followed by the same letter are not significantly different ($P < 0.05$).

temperatures of 10 and 15°C did not differ significantly in body length, neither between storage times nor between storage temperatures. These temperatures were more suitable for this nematode species than 5 and 20°C, where a significant body length reduction was found after 10 weeks. At all temperatures the inoculum level did not influence the body length of progeny and the conditions during storage of the IJs did not influence the length of their offspring.

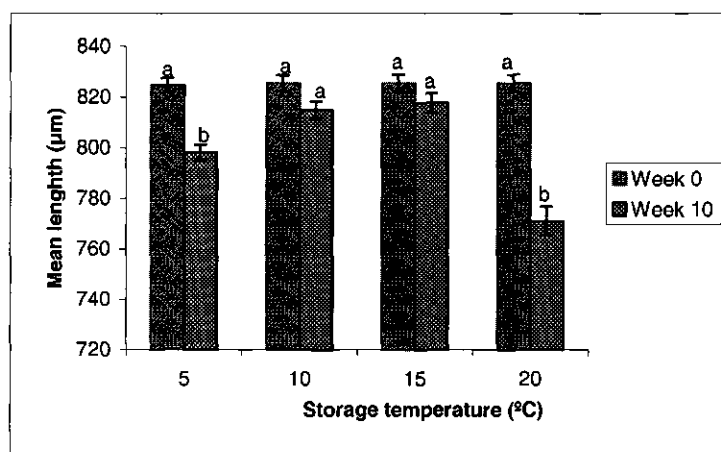


Fig. 7. Mean length ($n = 100$) of *Heterorhabditis megidis* (NLH-E87.3) infective juveniles stored at different temperatures during 10 weeks. Columns with the same letter are not significantly different ($P < 0.05$). Comparisons are between (storage time).

Time to emergence - At both doses no IJs emerged from host larvae exposed to IJs stored at 20°C for 8 and 10 weeks. The time to first emergence at a dose of 1 IJ/host was significantly higher at the first week of storage at all temperatures. There was no significant interaction

between storage temperature and time. Although the differences were small and not always consistent, at most storage periods the time to first emergence was longer when the infection had occurred by IJs stored at 5 and 20°C than at 10 or 15°C (Fig. 8 A). When *G. mellonella* larvae were infested with a dose of 30 IJs, infective juveniles emerged significantly later following storage at 5°C at all storage periods (Fig. 8 B). No significant difference was observed between storage time and temperatures of 10 and 15°C.

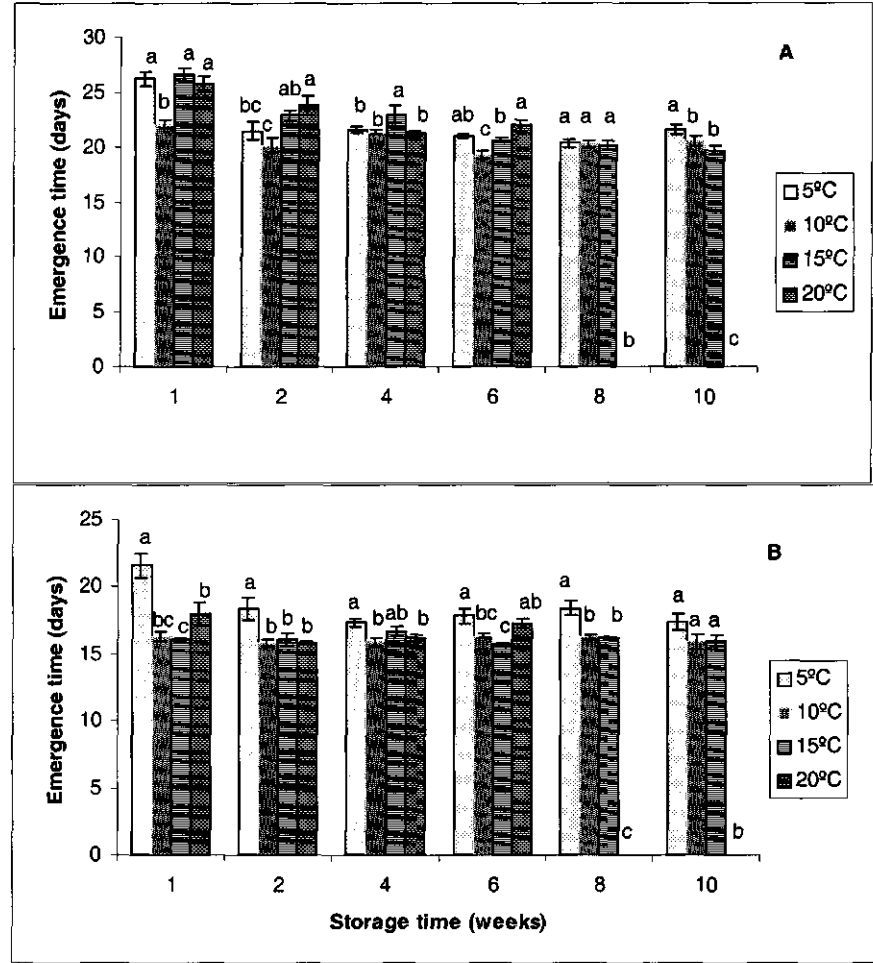


Fig. 8. Effect of storage temperature and time on the mean number of days to first emergence of infective juveniles from *Galleria mellonella* larvae ($n = 5$) exposed to a dose of one (A) and 30 (B) infective juveniles of *Heterorhabditis megidis* (NLH-E87.3). Columns with the same letter, for each group of data per storage time are not significantly different. ($P < 0.05$).

Discussion

A comparison between the different storage temperatures over time reveals that IJs of *H. megidis* (strain NLH-E87.3) survive and perform better when stored at 10 or 15°C, than at 5 or 20°C. After 4 weeks of storage the number of living IJs started to decrease significantly at a temperature of 5°C and dropped dramatically to about 50% at 20°C. Similar results were also observed by Fitters & Griffin (1996) and Griffin *et al.*, (1994) who worked with isolates of *Heterorhabditis* stored over periods of time at 20°C. Shapiro *et al.*, (1997), working with *H. bacteriophora* strains, found that IJs survived significantly longer at 25°C than at 10°C. Selvan *et al.*, (1993b) stated that in general in free-living non-feeding stages of animal parasitic nematodes lipids form the major energy reserve and that high lipid content suggests that organisms are adapted to survive prolonged periods of environmental stress. Lewis *et al.*, (1995b) observed that *H. bacteriophora* IJs stored at a temperature of 25°C survived for a maximum period of 7 weeks and when they compared metabolic rates (J/μg/day) of Steinernematids and Heterorhabditids they noticed that it increased with age for both groups. Heterorhabditids initial metabolic rate was higher than the Steinernematids and increased week by week. Selvan *et al.*, (1993c) proposed that the higher percentage of unsaturated fatty acids in *Heterorhabditis* sp. was the reason for their poorer survival over storage time. They also stated that species of entomopathogenic nematodes that are sit-and-wait foragers live longer than those that are active foragers. Gaugler & Campbell (1991), observed that the active movements of infective juveniles of *H. bacteriophora* resulted in more energy spent when compared with infective juveniles of *Steinernema carpocapsae*. Our results suggest that infective juveniles of *H. megidis* (strain NLH-E87.3) are physiologically better adapted to storage at temperatures of 10 and 15°C than to temperatures of 5 and 20°C, were their survival, infectivity and reproduction were reduced to critical levels.

Fan & Hominick (1991b) and Griffin (1996) found that the number of nematodes infecting a host decreases after storage at low temperatures but is followed by an unexpected increase with passage of time, indicating that cold temperatures induce in most of the nematodes a state in which they lose their ability to parasitise a host and also that an obligatory period of cooling is required before most nematodes regain their infectivity. From our results there are indications of a similar phenomenon only during a period of 6 weeks with a clear difference in production but not in mortality or invasion rates. After 6 to 10 weeks a decrease of infectivity occurred and infective juveniles became less infective.

Considering the many other biotic and abiotic factors involved in a field the poor response to low storage temperatures of this strain may also have negative implications for its use as an

inundative biological control agent and may explain, in part, field-trial data obtained by Smits (1992) in which a huge numbers of nematodes were required to obtain 50% mortality levels of grubs. Jung (1996), observed that the infectivity of *Heterorhabditids* HUK (NW European group) and HK 122 (Irish group) stored at 20°C increased with the increasing of storage time. Contrary to the results of Jung (1996), a clear and progressive decline in infectivity of IJs stored at 20°C was observed at both doses which may be due to the fact that nematodes' metabolism is temperature dependent. High temperatures increase the rates of lipid reserve used and decrease the time that the IJs remain viable and pathogenic. Selvan *et al.* (1993b) demonstrated that *Steinernematids* retain a relatively high level of saturated fatty acids, which could explain, in part, the higher ability of invasion and establishment as compared to *Heterorhabditids*. The decline of bacterial colony-forming units (CFUs) could be another factor decreasing the infectivity. Lewis *et al.*, (1995b) showed that IJs of *H. bacteriophora* stored at 25°C retained an average of 624 *Photorhabdus luminescens* CFUs in the first week. By the second week only 50% of this number remained viable and by the 6th week only 1.8% of the initial number of bacterial CFUs remained.

The best individual reproduction per IJ of *H. megidis* (NLH-E87.3) was obtained from larvae treated with a dose of one IJ, but the best yield per cadaver was obtained at a dose of 30 IJs per larva. Host larvae infested with IJs stored over time at temperatures of 10 or 15°C were those that showed the highest production. A mean maximum of $136\,492 \pm 7\,320$ IJs per *G. mellonella* larva infested by one IJ stored for a period of 6 weeks at a temperature of 10°C was produced within a period of 3 weeks. Our results on reproduction are in concordance with those of Mason & Hominick (1995). Studying the reproduction ability of *Heterorhabditis megidis* (UK and Dutch strain) that were stored for one week at 15°C and tested at 20°C, also found that production following the injection of only one IJ per larva resulted in markedly higher individual yields than those presented by each juvenile in larvae exposed to a dose of 10 IJs. Despite the difference between storage temperature, time and methodology our juveniles production levels also agree with those found by Wang & Bedding (1996) who started with an inoculum of only one IJ of *H. bacteriophora* per larva of *G. mellonella* and obtained a population of 150 000 IJs. Zervos *et al.*, (1991) stated that several factors affect the *in vivo* production of IJs and that these factors interact in unpredictable ways affecting the number of generations passed through before emergence of IJs. These factors include the growth rate of the bacterial symbiont, the biochemical composition of the cadavers and consequently the amount of available food sufficient for continuous reproduction and increase of the population. The lowest production in our experiments occurred when a higher number of IJs invaded the host. This may reflect

crowding effects that are illustrated by the rate of invasion per larva (Fig. 3) and, by fact that in larvae infected with 30 IJs, each established IJ produced less offspring than when larvae were infested with only one IJ (Fig.6). At storage temperatures of 10 and 15°C, IJs showed high levels of infectivity and caused high mortality and consequently lower reproduction per individual. These results agree with the findings of Selvan *et al.*, (1993a) and Boff *et al.*, (2000) who reported that density of nematodes inside one larva has an important influence on the nematodes' fecundity by affecting the number of IJs that were produced by a cadaver due to an intraspecific competition for nutrients or space. In general it can be concluded that nematode production varies with temperature and storage time of infective juveniles and that the optimal production was most likely to occur when a small, rather than a large, number of infective juveniles was inoculated at a optimum temperature.

Infective juveniles from cadavers exposed to a dose of 30 IJs started to emerge earlier than those from cadavers infested with the dose of only one IJ. The influence of IJ doses on emergence period may be direct, by affecting the number of generation in a cadaver before emergence, or indirect, by affecting the growth rate of bacterial symbiont or the biochemical composition of the cadaver (Zervos *et al.*, 1991; Selvan *et al.*, 1993a; Boff *et al.*, 2000). At a dose of 30 IJs per larva an increasing time to first emergence was observed for those nematodes previously stored at 5°C. These results seem to be more related to the influence of storage temperature than to the number of IJs that invaded the host because even when a few IJs invaded the host the time to first emergence was longer than that of other storage temperatures. At a dose of only one IJ, storage time instead of storage temperatures was the factor that prolonged the time to first emergence.

Fitters *et al.*, (1998) reported that energy reserves depletion caused decreasing rate of activity and size of Heterorhabditids nematodes during storage. A similar phenomenon was also observed in our study. Storage temperatures of 5 and 20°C significantly affected body length of IJs during 10 weeks of storage. We believe that the measurement differences observed during a storage period could be related to physiological and metabolic processes that may be altered by the low and high temperature of storage and could explain in part, also why IJs stored at the "extreme" temperatures survived less and were also less infective.

In laboratory studies we could observe that storage conditions, particularly temperatures outside the optimum have a great effect on the performance of the nematodes. Clearly much effort is still needed to understand all the effects of storage temperature and time on infectivity, with a view to manipulation of storage conditions for optimal conditioning of the nematodes, leading to better results in field applications.

Chapter 4

Influences of host size and host species on the infectivity and development of *Heterorhabditis megidis* (strain NLH-E87.3)

Adapted from: Mari I. C. Boff, Gerrie L. Wiegers & Peter H. Smits. Influences of host size and host species on the infectivity and development of *Heterorhabditis megidis* (strain NLH-E87.3). *BioControl* (in press)

Abstract

The infectivity, time to first emergence of infective juveniles (IJs), total number of IJs per insect and IJs body length of the entomopathogenic nematode *Heterorhabditis megidis* (strain NLH-E87.3) after development in larvae of two insect hosts, *Galleria mellonella* (greater wax moth) and *Otiorhynchus sulcatus* (vine weevil) was studied. At a dose of 30 IJs, larvae of *G. mellonella* show to be significantly more susceptible than *O. sulcatus* larvae. At a dose of one IJ, vine weevil larvae were more susceptible. The number of invading infective juveniles (IJs) increased with host size while the host mortality at a dose of one IJ decreased with the increase of host size. Time to first emergence was longer at a dose of one IJ per larva and increased with the increase of host size in both insect species. Reproduction of IJs differed between host species, host sizes and doses of nematodes. Generally, the IJs body size increased with an increasing host size. The longest infective juveniles were produced at the lowest IJ doses. Results are discussed in relation to the influence of different host species and their different sizes on the performance of *H. megidis* (strain NLH-E87.3) as a biological control agent.

Introduction

Insect parasitic nematodes of the genus *Heterorhabditis* are considered effective biological control agents of soil dwelling insect pests. These entomopathogenic nematodes (EPNs) play a role underground comparable to that played by parasitoids above ground, killing and reproducing inside the immature stages of their host species (Kaya, 1990a). The free-living infective juveniles (IJs) seek hosts in the soil but neither feed nor reproduce there. After being invaded by IJs, host insects are killed within one or two days by the symbiotic bacteria (*Photorhabdus luminescens*) released from the gut of the IJs. Soon after, the IJs begin to feed on the bacteria and on the digested host tissues and develop to reproductive stages. The symbiotic bacteria produce antibiotics and other chemicals that protect the host cadaver from decay caused by microorganisms in the soil (Akhurst and Boemare, 1990). Nematodes in the genus *Heterorhabditis* are hermaphroditic, and a single IJ can give rise to hundreds to

thousands of offspring per host within a month after invading a host insect. Thus, a single IJ of *Heterorhabditis* sp. has the potential to colonise a new area or conserve a local population. When resources inside the host cadaver become exhausted, reproduction shifts to the production of IJs, which can disperse in search of new hosts (Kaya, 1990a). The developmental stage of the insect plays an important role in its susceptibility to pathogenic agents. The effect of host age and size on parasitoids has been studied extensively both in the laboratory and the field (Murdoch et al., 1997; Morgan and Hare, 1997). Concerning EPNs, far less has been done to study the effect of the developmental stage of host insect species on their infective ability and reproductive capacity. Some studies (Geden et al., 1985 and 1986; Kaya, 1985; Fuxa et al., 1988; Smits et al., 1994; Jansson, 1996; Shapiro et al., 1999) show that the infectivity, reproduction and development of EPNs vary among nematode species or strains and between target insects and their developmental stages. The purpose of the current study was to determine whether different host species, host sizes and nematode doses have an influence on the infective capacity and development of *Heterorhabditis megidis* (strain NLH-E87.3) regarding their size and production of new IJs.

Material and Methods

Nematodes

Heterorhabditis megidis (strain NLH-E87.3) (Smits et al., 1991) used in the experiments was cultured in last-instar wax moth larvae (*Galleria mellonella*) at a temperature of 20°C. Groups of four wax moth larvae were placed at the bottom of 50 ml plastic cylinders filled with moist sand (8% w/w tap water). One hundred and twenty infective juveniles (IJs) per container in 1-ml of tap water were added to the sand surface. The containers were closed and incubated in the dark at 20°C. After 96 hours the red-coloured infected wax moth larvae were transferred from the sand to a modified White trap (Lewis and Gaugler, 1994). Each group of infested larvae was placed on a layer of moist filter paper in a 5 cm Petri dish without lid. The small dishes holding the cadavers were floated in water in a large Petri dish (15 cm diameter) with lid and stored in the dark at 20°C. All IJs that emerged from the cadavers and moved to the water layer were collected daily for a period of 4 days. Emerging IJs were mixed and stored in 30 ml non-sterile tap water at 10°C for a period of at least 10 days and no longer than two weeks. To prevent the presence of dead IJs before being used in the experiments, the

IJs were rinsed with tap water and allowed to pass through a sieve lined with a cotton-wool filter into tap water and left at room temperature (18-20°C) for half an hour.

Insects

Galleria mellonella larvae were reared on a mixture of 900 g of liquid honey, 900 g of glycerin, 200 g of bees wax, 400 g of yeast flakes and 1300 g of whole meal at 25°C. The larvae were separated by size into groups of small (67 ± 0.9 mg), medium (119 ± 1.9 mg) and large (300 ± 3.2 mg) larvae. *Otiorhynchus sulcatus* (vine weevil) larvae were reared on roots of *Astilbe chinensis* (Saxifragaceae) plants in a greenhouse at 20°C. The larvae collected from the *Astilbe* pots were sized into groups of small (14 ± 0.4 mg), medium (34 ± 0.6 mg) and large (67 ± 0.4 mg), transferred to the laboratory and used immediately.

Infectivity and Establishment

To study the invasion efficiency of *H. megidis* (strain NLH-E87.3) on small (S), medium (M), and large (L) larvae of *G. mellonella* and *O. sulcatus*, single larvae of each size were placed at the bottom of plastic cylinders (50-ml, 36-mm diameter). The cylinders were filled with moist, heat-sterilized silver sand (8% w/w). For each combination of IJs doses and host species sizes, groups of 30 individual host larvae *G. mellonella* or *O. sulcatus* were exposed to doses of a single IJ released on the top of the sand filled plastic cylinders by using a so-called "nematode fishing-rod" (eyebrowhair glued to a small wooden stick) or 30 IJs applied in one ml of tap water with a pipette. The containers were closed with a lid and placed in the dark and incubated at 20°C. One ml of water was added to the controls. All the experiments were done two times. After 48 hours of exposure the *G. mellonella* and *O. sulcatus* larvae were removed from the sand and rinsed with tap water to remove possible IJs adhering to the body surface. The larvae were dried on tissue paper and transferred individually to small plastic Petri dishes lined with moist filter paper and held at a temperature of 20°C during a period of 144 hours. After this period, mortality of both insect species at each dose and size was assessed by counting the number of dead and living larvae. Only those larvae that became red were considered killed by nematodes. Seven days after inoculation the establishment rate of IJs in larvae exposed to the dose of 30 IJs was assessed. Fifteen larvae were dissected and digested in a 0.8% pepsin solution (Mauleon et al., 1993) and the nematodes were counted. At a dose of one IJ establishment was considered equal to death of host.

Reproduction

For each nematode dose and host size ten red-coloured infected larvae of *O. sulcatus* and eight of *G. mellonella* were transferred individually to modified White traps (Lewis and Gaugler, 1994). All the IJs emerging from a single cadaver and moving from the small dish to the water layer were collected daily over a period of 5 days, mixed and stored in 50 ml of tap water in a 200 ml tissue culture flask. At the end of the 5 days collecting period the number of IJs in each flask was assessed. The content of the flasks was mixed thoroughly with air bubbles and from this suspension 8 samples of 10 μ l were counted under a stereomicroscope using a counting slide. The average of 8 aliquots was taken as the estimate of the number of IJs released from each cadaver. As the larvae were weighed before being exposed to the nematodes, it was possible to determine the number of IJs produced per mg of larval body. The progeny production per established IJ at a dose of 30 IJs was calculated by dividing the total IJ production per cadaver by the average number of IJs established per larva (data obtained from the dissected larvae in the infectivity and establishment experiment).

Body length

Length of IJs was measured to see if there were size differences among infective juveniles originating from different host species and hosts sizes. Individuals to be measured were randomly selected from the pooled population. Prior to the measurement, IJs were heat killed on a glass slide in a microwave oven (700-Watts) during 30 seconds. The measurement was made on the body length of the IJs, excluding the second stage cuticle. For each host species and each host size group 100 IJs were measured.

Time to first emergence

The number of days it took IJs to emerge from the infested cadaver was determined by examining each modified White trap daily at a fixed time, for the presence of the first nematodes on the cadaver.

Statistic analyses

All data were analysed by ANOVA using the statistical program Genstat 5 version 4.1 (Genstat Committee, 1997). The minimal level of significance was taken as $P \leq 0.05$.

Results

Infectivity and Establishment

The dose of 30 IJs per larva killed 100% of all sizes of wax moth larvae whereas the dose of one IJ gave mortality levels of 65 % for small larvae and around 30% for medium and large larvae (Fig. 1 *Galleria*). When exposed to a dose of 30 IJs all size groups of vine weevil larvae showed mortality levels of 70 to 85%. The dose of one IJ gave 60-80% mortality of small and medium size vine weevil larvae. Only 10% of large larvae were killed by a dose of one IJ (Fig. 1 *Otiorhynchus*). At a dose of 30 IJs per larva the proportion of the infective juveniles established in larvae of *O. sulcatus* increased with larval size (Fig. 2). The same tendency can be observed with *G. mellonella* larvae but there were no significant differences between the establishment rate for small and medium size larvae. Significantly more IJs established in *G. mellonella* than in *O. sulcatus* larvae, even when we compare small (70 mg) wax moth and large (70 mg) vine weevil larvae.

Reproduction

The production of IJs from infected wax moth and vine weevil larvae increased with larval size at both doses (Table 1). The accumulated production of IJs in wax moth larvae exposed to a dose of 30 IJs was for all size classes significantly higher than the production obtained from larvae infected with only one IJ. Medium size wax moth larvae exposed to a dose of 30 IJs produced the highest number of IJs per milligram of insect body. No production was obtained from small vine weevil larvae exposed to one IJ per larva. Progeny production per established IJ increased with the increase of the host size for both insect species and was significantly higher when larvae were exposed to a dose of one IJ. Comparing the total production per milligram of body weight between small *G. mellonella* (70mg) and large *O. sulcatus* (70mg) it is clear that *G. mellonella* larvae generally produced significantly more infective juveniles than *O. sulcatus* larvae.

Body length

Inoculation doses and host sizes were shown to have an influence on body length of progeny. In general, larvae exposed to a dose of one IJ (Fig. 3) produced the longest progeny. Independent of the insect species and the used dose of IJs, large cadavers produced the longest IJs whereas shortest IJs were produced from small cadavers.

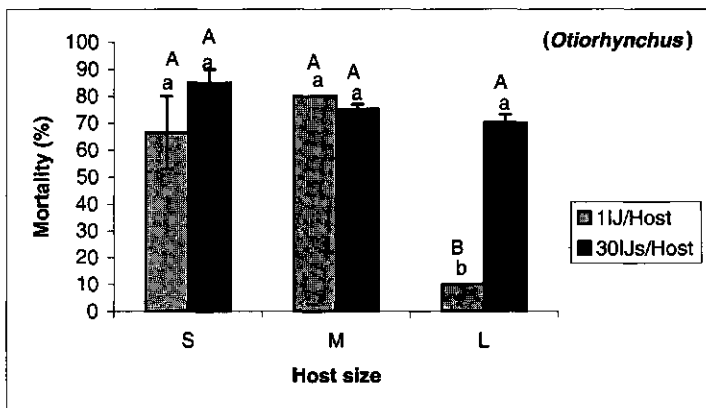
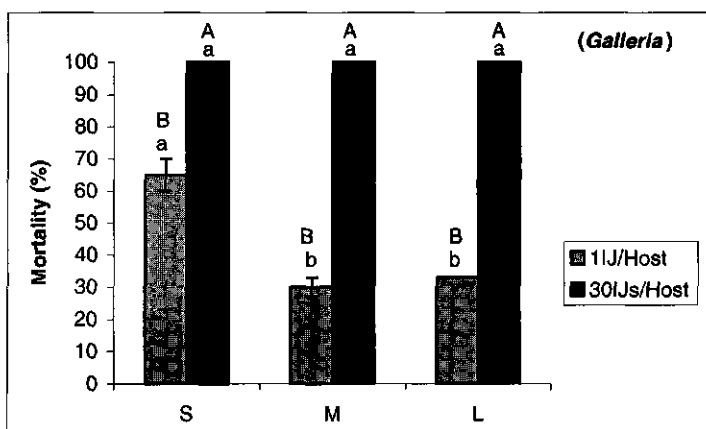


Figure 1. Larval mortality (\pm SEM) of *G. mellonella* and *O. sulcatus* exposed to different doses of infective juveniles (IJs) of *H. megidis* (strain NLH-E87.3). Capital letters correspond to the 6 pairwise comparisons of low and high doses of IJs for the three-host sizes of both insects. Lower-case letters correspond to comparison of mortality when the three different host sizes were exposed to same dose. Columns indicated by different letters are significantly different ($P \leq 0.05$).

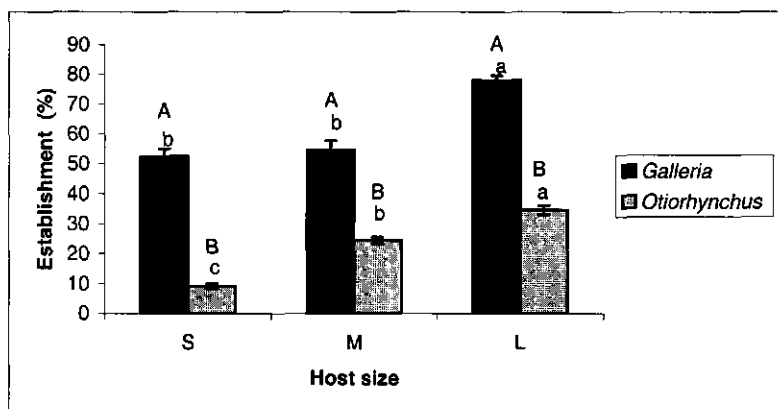


Figure 2. Percentage (\pm SEM) of established infective juveniles (IJs) in small (S), medium (M) and large (L) larvae of *G. mellonella* and *O. sulcatus* exposed to 30 IJs of *H. megidis* (strain NLH-E87.3). Capital letters correspond to comparison between host species. Lower-case letters correspond to comparison between different larval sizes of the same host species. Columns indicated by different letters are significantly different ($P \leq 0.05$).

Table 1. Production of infective juveniles from different host sizes and host species exposed to different doses of infective juveniles of *H. megidis* (strain NLH-E87.3).

Dose	Host	Host Size	Number of Infective Juveniles (IJs)*		
			IJs per host	IJs/ IJ established	IJs/mg of host
1 IJ / Host	<i>G. mellonella</i>	Small	16 042 \pm 440 c	16 042 \pm 440 c	229 \pm 6 b
		Medium	35 110 \pm 4 618 b	35 110 \pm 4 618 b	293 \pm 39 b
		Large	113 392 \pm 6 713 a	113 392 \pm 6 713 a	378 \pm 22 a
	<i>O. sulcatus</i>	Small	NP	NP	NP
		Medium	2 751 \pm 233 b	2 751 \pm 233 b	92 \pm 8 a
		Large	7 777 \pm 564 a	7 777 \pm 564 a	111 \pm 8 a
30 IJs/host	<i>G. mellonella</i>	Small	20 924 \pm 1 452 c	1 308 \pm 91 c	299 \pm 21 c
		Medium	63 364 \pm 2 590 b	3 960 \pm 162 b	528 \pm 22 a
		Large	145 014 \pm 5 431 a	6 305 \pm 236 a	483 \pm 18 b
	<i>O. sulcatus</i>	Small	723 \pm 46 c	241 \pm 15 c	52 \pm 5 c
		Medium	3 386 \pm 326 b	484 \pm 47 b	113 \pm 11 b
		Large	11 918 \pm 1 209 a	1 192 \pm 121 a	170 \pm 17 a

* Number of Infective Juveniles = mean \pm standard error of the mean. NP = No production. For each column values followed by the same letter are not statistically significantly different at $P \leq 0.05$. Comparisons are between each host size group.

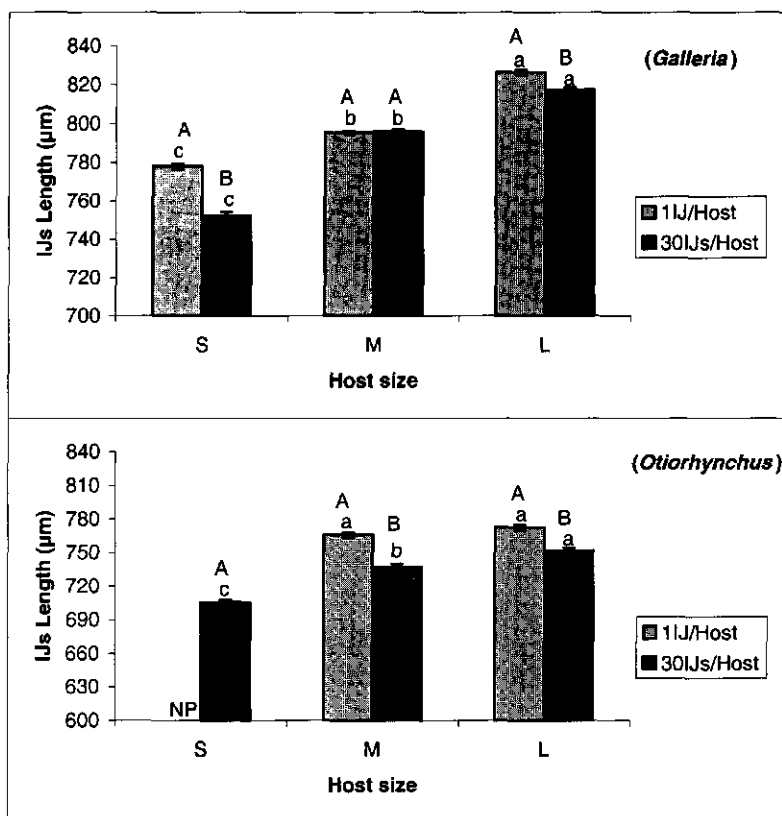


Figure 3. Body length of infective juveniles (IJs) produced in small (S), medium (M) and large (L) larvae of *G. mellonella* and *O. sulcatus* exposed to different doses of *H. megidis* (strain NLH-E87.3). Means (\pm SEM) of 100 measurements. Capital letters correspond to pairwise comparison between doses of IJs. Lower-case letters correspond to comparisons between host sizes. Columns indicated by different letters are significantly different ($P \leq 0.05$). NP = no production.

Time to first emergence

No IJs emerged from small larvae of *O. sulcatus* exposed to a dose of one IJ per larva. For both insects tested, time to first emergence differed significantly between doses and IJs emerged significantly later from larvae exposed to a dose of one IJ (Fig. 4). Independent of the dose, time to first emergence increased with increasing size of *G. mellonella*. However, in larvae of *O. sulcatus* exposed to 30 IJs, a significant decrease in day of first emergence with increasing size was observed.

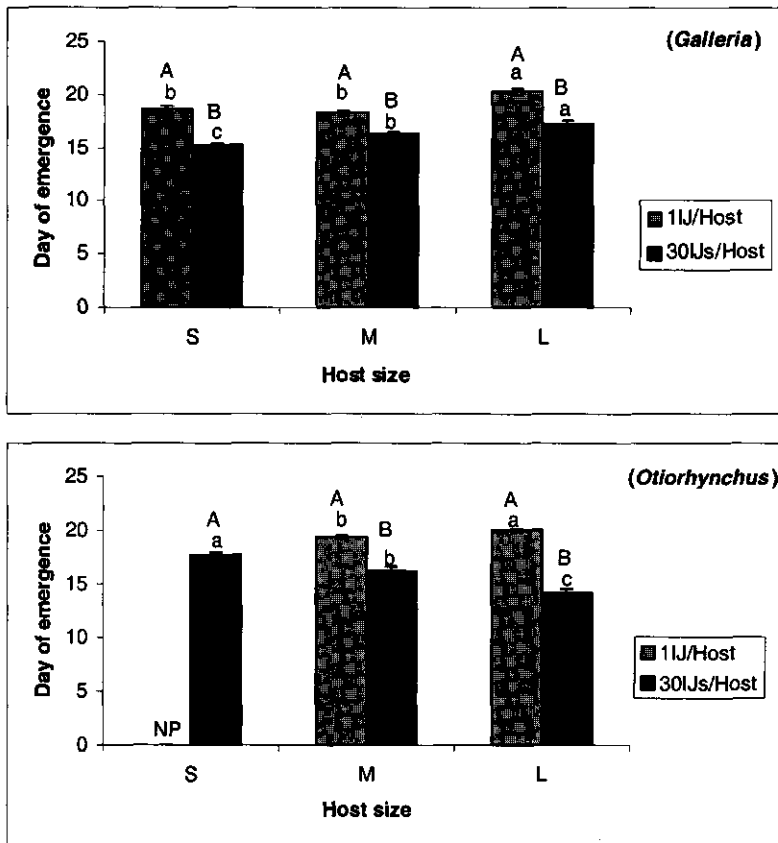


Figure 4. Time to first emergence of infective juveniles (IJs) from small (S), medium (M) and large (L) larvae of *G. mellonella* and *O. sulcatus* exposed to different doses of *H. megidis* (strain NLH-E87.3). Capital letters indicate comparisons between IJs doses and lower-case letters indicate comparisons between host sizes. Columns indicated by different letters are significantly different ($P \leq 0.05$). NP = no production.

Discussion

Our results indicate that there are no differences in the susceptibility between small, medium and large larvae of *G. mellonella* when exposed to a dose of 30 IJs. However, with a dose of one IJ small larvae of *G. mellonella* were more susceptible (65% mortality) than medium and large larvae that showed only 30% mortality. The results further indicate that

small and medium size *O. sulcatus* larvae are suitable hosts and that a dose of one IJ is sufficient to kill more than 65% of the small and medium larvae. However, the same dose (one IJ) proved to be lethal for only 10% of the large larvae. These results are in agreement with the findings of Shapiro et al., (1999) and Kaya and Hara (1981) who reported that the infectivity of IJs differed among developmental stages of a host insect. Kaya and Hara (1981) also reported that generally, soil-inhabiting insects are more resistant than those living above ground. In our study we observed that *O. sulcatus* larvae are in general less susceptible, larvae exposed to a dose of 30 IJs showed lower establishment rates than *G. mellonella* larvae. Jansson (1996) reported that the infectious capacity of one IJ of *H. bacteriophora* HP88 in two insect hosts, *Cylas formicarius* (third instar) and *G. mellonella* (late instar) did not differ between hosts. In our case it differed not only between host species but also between the different larval size of the same hosts. In the first step of infection, IJs have to move toward the insect host and invade. Under natural conditions the larval stages of *O. sulcatus* are located near or between the roots of plants and as they are feeding they are not very mobile creating in this way a favourable situation for infection. During the experiment no food was offered. Larvae were not immobilised and were just put at the bottom of a plastic cylinder filled with sand. Large vine weevil larvae could actively move to various parts of the cylinder and possibly escape or avoid encountering IJs. Another reason that can partly explain why the nematode establishment decreased in larger larvae exposed to a single IJ is that the larger and older the larvae the thicker and harder the body integument will be. On the other hand higher attractivity of large larvae would be expected by the increase of host-associated cues e.g. CO₂, host faeces or cuticle excretory products (Lewis et al., 1993, Schmidt and All, 1978). These differences in susceptibility between *G. mellonella* and *O. sulcatus* larvae could also be due to the fact that *G. mellonella* larvae were reared on an artificial diet and *O. sulcatus* on plants. Barbercheck et al., (1995) and Barbercheck and Wang (1996), reported that a secondary plant metabolite, cucurbitacin D affected the susceptibility of *Diabrotica undecimpunctata howardi* to entomopathogenic nematodes and the *in vitro* growth of *Xenorhabdus* and *Photorhabdus* (the bacterial symbionts of EPN. Until now there are no references reporting on the effects of *Astilbe chinensis* chemical compounds (secondary metabolites) on the biology of insect pests in terms of transferring protection against their natural enemies.

The total amount of IJs produced per larva, per mg of host body and per IJ established in both insect hosts was highly influenced by the larval body size and the insect species. At a dose of 30 IJs per larva the production of IJs was related to an increase in the number of established IJs but in the case of one IJ per host, the yield was influenced by the host size.

This phenomenon seems to be related with the mechanism of the pathogenicity of the nematode species with its symbiotic bacteria. The rate at which bacteria act on the insect's organism producing food for the nematodes depends on its multiplication in the insect body. The bacterial multiplication, in our study, seems to be dependent on a differentiation in the defensive system of the insect species, the host stage, and the initial dose of invasive IJs which increased the chance of mass injection of bacteria into the host's haemolymph. Thus low levels of bacteria or no multiplication could be one of the causes of non-production observed in small vine weevil larvae exposed to a dose of one IJ. Dutky et al. (1964) observed that the yield of IJs depends on the body dimensions of the host larvae. Similar to our results they got an increase in IJs production with increasing host size.

We observed that besides the host species and body dimensions, the initial dose also had an important influence on the development of IJs. The number of invading IJs increased with the increasing host size but the production of IJs per established IJ did not increase in proportion as compared to the situation when the larvae were infested by only one IJ. Boff et al. (2000) also observed that the larger the inoculum the smaller the number of IJs produced per cadaver. The progeny production obtained from large *G. mellonella* larvae infested with a dose of one IJ per larva was lower than the production obtained by Wang and Bedding (1996) but higher than that obtained by Mason and Hominick (1995) from larvae infested with a single *H. megidis* (UK and Dutch strain). Our five-day cumulative IJ production from medium and large *G. mellonella* larvae exposed to a dose of 30 IJs is also higher than the total production obtained by O'Leary et al. (1998) from *Tenebrio molitor* larvae exposed to a dose of approximately 25 IJs of *H. megidis* (strain UK211).

In general, for both host species, the longest IJs were produced at the lowest dose (1 IJ/larva). At this dose it was also observed that the time to first emergence was longer and the progeny production was lower than in larvae exposed to 30 IJs. For *O. sulcatus* the size of nematodes increased with the increase of host size only when larvae were exposed to 30 IJs. Our results agree with Selvan et al. (1993a) who observed that the longest IJs were produced at lowest densities and with Craig and Webster (1982) who reported that density and host size directly influence the size of the nematode *Mermis nigrescens*. In this study we observed that the larger the host and the lower the initial dose the longer the nematodes remain inside the cadavers, and consequently the larger their size.

Periods to first emergence differed between the three larval sizes for both insect hosts. Infective juveniles from large *G. mellonella* larvae required more time to emerge from the cadavers. In *O. sulcatus* IJs took a significantly longer period to emerge from small larvae

exposed to an initial dose of 30 IJs than from medium and large larvae. However, at a dose of one IJ the period to first emergence was significantly longer in large than in medium size larvae. It is well known that incubation time or time to first emergence of IJs is related to depletion of food reserves and intraspecific competition within the host cadaver (Wang and Bedding, 1996). Results on the development of *H. megidis* strain NLH-E87.3 using large larvae of *G. mellonella* obtained by Boff et al. (2000) indicated that the larger the number of invading IJs the shorter the period to first emergence. However, the results obtained by testing different size larvae of *G. mellonella* showed that the period of incubation was influenced much more by food than by the number of invading IJs. In *O. sulcatus* the effects of overcrowding was clear, large larvae attracted more IJs and consequently the incubation period was shorter than when the larvae were invaded only by one IJ. Jansson (1996) observed that the time to first emergence of three different species of *Heterorhabditis* was considerably shorter in the weevil *C. formicarius* than in *G. mellonella*. The author concluded that this happened due to the smaller size of the weevil compared with *G. mellonella*. However, when we compare times to first emergence between the two tested hosts with the same size and treated with a dose of 30 IJs, a significant difference ($P < 0.05$) is observed. In large *O. sulcatus* IJs required less time to emerge from the cadavers than from small *G. mellonella* larvae. In this case host nutrients available to the nematodes may trigger the emergence.

The present study demonstrated that the infectivity, reproduction and length of progeny of *H. megidis* strain NLH-E87.3 differ between insect host species, between the different developmental stages of an insect species and between different inoculum sizes. In order to check the effects of the three above mentioned factors on the quality of the progeny of this nematode strain more experiments have been carried out (Boff et al., (a) in press).

Chapter 5

Host influences on the pathogenicity of *Heterorhabditis megidis*.

Adapted from: Mari I. C. Boff, Gerrie L. Wieggers & Peter H. Smits. Host influences on the pathogenicity of *Heterorhabditis megidis*. *BioControl* (in press)

Abstract

The infectivity of infective juveniles (IJs) of *Heterorhabditis megidis* (strain NLH-E87.3) produced on small, medium and large larvae of *Galleria mellonella*, and on medium and large larvae of *Otiorynchus sulcatus* was tested under laboratory conditions against *G. mellonella* and *O. sulcatus* larvae. Infective juveniles originating from small *G. mellonella* exposed to an initial dose of one IJ were more infectious than those from small cadavers exposed to a dose of 30 IJs. Independent of the initial inoculum size, IJs from small cadavers of *G. mellonella* were more infectious than those from medium and large cadavers. At a dose of one IJ per larva, IJs originating from medium size *O. sulcatus* cadavers were more infective against *G. mellonella* than against *O. sulcatus* larvae. Large *G. mellonella* larvae were less susceptible to all IJ batches than medium and small sized larvae.

Introduction

Entomopathogenic nematodes (EPNs) of the family Heterorhabditidae are obligate pathogens in nature. In cooperation with specific symbiotic bacteria, *Photorhabdus luminescens*, they are able to infest and kill a large number of insect species, especially in the orders Lepidoptera, Coleoptera and Diptera (Simões & Rosa, 1996). The nematodes develop and reproduce inside the insect cadaver from which infective juveniles (IJs) are released into the soil, where they persist without feeding and seek out new hosts. Intrinsic factors (e.g. genetic, physiological, and behavioral characteristics) and external factors affect the IJs' performance (Curran, 1993). Abiotic factors, such as temperature, soil texture, soil moisture and ultraviolet light affect the nematode persistence (Kaya, 1990a; Smits, 1996). Biotic factors like antibiosis, competition and natural enemies influence the survival of entomopathogenic nematodes and their symbiotic bacteria (Kaya, 1990a; Kaya and Thurston, 1993). The close relationship between nematodes and their insect hosts suggests a particular susceptibility of the insect. The susceptibility of different developmental stages of insect hosts shows great variation and also there is large intrinsic variation in virulence of the nematode species or strains (Geden et al., 1985; Fuxa et al., 1988; Glazer, et al., 1991; Smits et al., 1994; Simões and Rosa, 1996; Jansson, 1996; Doucet et al., 1998). The influence of host age or size on parasitoids and predator biology has been extensively studied and reviewed. However, the role of the developmental stage of the insect host in which the nematodes

develop and susceptibility to EPNs as well as progeny quality has not been studied much. Despite studies by Kakouli-Duarte and Hague (1999) and Gouge and Hague (1995) the influence of host size on the biology and performance of IJs needs further investigation. The more so because ecologically, EPNs are obligate pathogens (Kaya, 1990a) and in order to persist in the soil environment they need to reproduce inside a host. There are also strong suggestions that EPNs play a substantial role in the soil, protecting both natural vegetation and agricultural plants from root-feeding insects (Hominick & Reid, 1990). However, the success of a long-term biological control program is strictly related to the population dynamics and intrinsic characteristics of both insect hosts and EPN species. The purpose of this work was to study the pathogenicity of infective juveniles originating from different sized larvae of two insect host species, *Galleria mellonella* and *Otiorhynchus sulcatus*, exposed to different doses of parental nematodes.

Material and Methods

Nematodes

Heterorhabditis megidis (strain NLH-E87.3) (Smits et al., 1991) was cultured in small (67 ± 0.9 mg), medium (119 ± 1.9 mg) and large (300 ± 3.2 mg) larvae of the greater wax moth, *Galleria mellonella*, as well as in medium (34 ± 0.6 mg) and large (67 ± 0.4 mg) larvae of the vine weevil, *Otiorhynchus sulcatus*, at 20°C. Twenty larvae of each size group of each host species were used. Using a sand column assay (plastic cylinders of 50 ml, 36 mm diam) each larva was exposed separately to a dose of one or 30 IJs. After 96 hours of exposure, infected larvae were transferred to a modified White trap (Lewis & Gaugler, 1994). Each infested larva was placed on a moist filter paper in a 5 cm Petri dish without lid. The dishes holding the cadavers were floating in water inside a large Petri dish (15 cm diam) with lid and stored in the dark at 20°C. Infective juveniles were collected daily for 3 days. Harvested IJs were mixed and stored in 30 ml of non-sterile tap water at 10°C for a period no longer than two weeks. Before being used the IJs were rinsed with tap water and left at room temperature (18-20°C) for half an hour.

Insects

The greater wax moth was reared at 25°C on a diet of liquid honey (900 g), glycerine (900 g), bees wax (200 g), yeast flakes (400 g) and wheat meal (1300 g). Vine weevil larvae

were reared on *Astilbe chinensis* plants in a greenhouse at approximately 20 -25°C. Larvae of both species were collected from their rearing place, weighed, and used immediately.

Infectivity tests

The effect of host species and developmental stage on the infective capacity of *H. megidis* was compared using infective juveniles emerging from different sized cadavers of the two above mentioned insect pests, exposed to two different inoculum doses of IJs. An outline of the experiments carried out in this study is detailed in Table 1. Single small, medium and large larvae of *G. mellonella* and large larvae of *O. sulcatus* (sized as mentioned before) were placed at the bottom of plastic cylinders (50 ml, 36mm diam) filled with moist heat sterilised silver sand (8% w/w). For each host species and size two groups of 30 individual host larvae were exposed to a dose of either one or 30 IJs. Single IJs were added to the top of the sand using a "nematode fishing rod" (eyebrowhair glued to a small wooden stick). Doses of 30 IJs were applied in one ml of water with a pipette. One ml of tap water was added to the controls. The plastic cylinders were closed with a lid and placed at 20°C in a dark climate room. Forty eight hours later *G. mellonella* and *O. sulcatus* larvae were removed from the sand, rinsed with tap water to wash off the nematodes from the body surface, individually transferred to Petri dishes lined with moist filter paper and held at 20°C. Mortality was assessed after 7 days.

To assess the establishment rate of IJs, the larvae exposed to a dose of 30 IJs were dissected after digestion in a 0.8% pepsin solution (Mauleon et al., 1993) and the established nematodes were counted. Using different culture batches all the treatments were performed twice. No control mortality was observed for *O. sulcatus* or for *G. mellonella* larvae.

Statistic analysis

All data were analysed by analysis of variance (ANOVA) using the statistical program Genstat 5 version 4.1 (Genstat Committee, 1997). The minimal level of significance was taken as $P < 0.05$.

Table 1. Sets of experiments carried out in the present study. The infective capacity of infective juveniles (IJs) originating from different sized larvae of *Galleria mellonella* and *Otiorhynchus sulcatus* exposed to a dose of one and 30 nematodes was tested against different sizes of *G. mellonella* and *O. sulcatus* larvae at a dose of 1 or 30 IJs.

Nematodes origin	Tested Insect Host							
	<i>Galleria mellonella</i>						<i>Otiorhynchus sulcatus</i>	
	Tested dose						Tested dose	
	1 IJ / larva			30 IJs / larva			1 IJ/L	30IJs/L
	S*	M	L	S	M	L	L	L
Dose 1 IJ/Larva								
Small <i>G. mellonella</i> (SG1)	X	X	X	X	X	X		
Medium <i>G. mellonella</i> (MG1)	X	X	X	X	X	X		
Large <i>G. mellonella</i> (LG1)	X	X	X	X	X	X		
Dose 30 IJs/Larva								
Small <i>G. mellonella</i> (SG30)	X	X	X	X	X	X	X	X
Medium <i>G. mellonella</i> (MG30)	X	X	X	X	X	X	X	X
Large <i>G. mellonella</i> (LG30)	X	X	X	X	X	X	X	X
Medium <i>O. sulcatus</i> (Mvw30)	X	X	X	X	X	X	X	X
Large <i>O. sulcatus</i> (Lvw30)	X	X	X	X	X	X	X	X

* Host size: small (S), medium (M) and large (L) larvae.

Results

Infectivity against G. mellonella of IJs, originating from G. mellonella exposed to 1 IJ

Single infective juveniles originating from small cadavers of *G. mellonella* were more infective than those originating from medium or large cadavers (Fig. 1A). Infective juveniles from medium sized cadavers killed fewer medium sized hosts. Infective juveniles from large cadavers caused mortality levels around 40 % among all host sizes. No differences in mortality (always 100%) were observed when different sized hosts were exposed to a dose of 30 IJs. Data presented in Fig. 1B show that IJs from small cadavers were also much more invasive than those originating from medium and large ones. When tested at a dose of 30 IJs/larva significantly fewer IJs from medium and large cadavers were able to establish in large hosts. Mortality never occurred in the untreated larvae (control) neither in this nor in the following experiments.

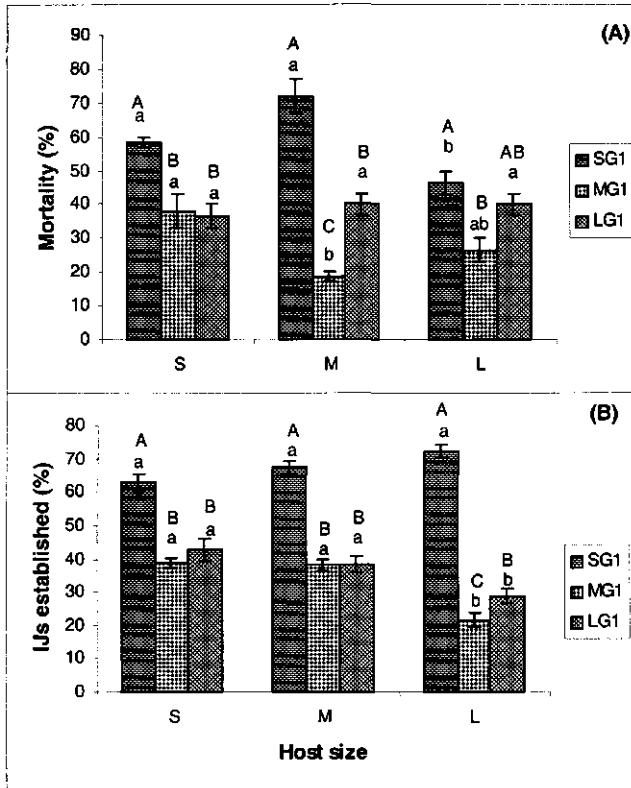


Figure 1. Infective capacity of *Heterorhabditis megidis* (NLH-E87.3) reared in small (SG1), medium (MG1) and large (LG1) larvae of *Galleria mellonella* and tested at a dose of one IJ/larva (A) and 30 IJs/larva (B) against small (S), medium (M) and large (L) *G. mellonella* larvae. Capital letters represent comparisons between IJs of different origin to the same host size group. Lower case letters represent comparisons of IJs with the same origin between host sizes. Columns marked with the same letter are not statistically different at $P \leq 0.05$.

Infectivity against G. mellonella of IJs, originating from G. mellonella exposed to 30 IJs

Irrespective of inoculum and host size the highest mortality levels were caused by IJs originating from small cadavers (Fig. 2 A and B). Infective juveniles originating from medium and large cadavers obtained the lowest level of mortality against small and medium sized hosts, respectively. Independent of the target host size the highest establishment rate was recorded for IJs originating from small cadavers (Fig. 2 C). The establishment capacity of IJs decreased with increasing size of the cadaver from which they had been recovered.

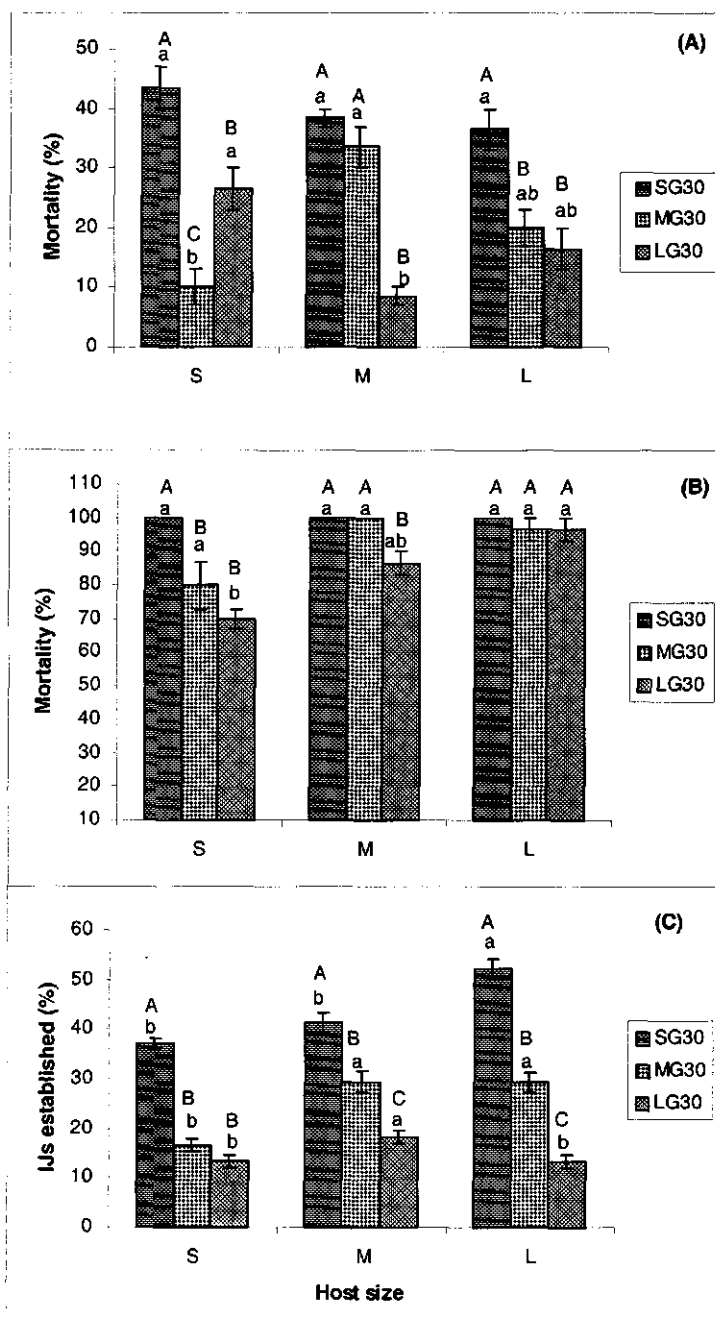


Figure 2. Infective capacity of *Heterorhabditis megidis* (NLH-E87.3) reared in small (SG30), medium (MG30) and large (LG30) larvae of *Galleria mellonella* tested at a dose of one IJ/larva (A) and 30 IJs/larva (B and C) against small (S), medium (M) and large (L) *G. mellonella* larvae. Capital letters represent comparisons between IJs of different origin to the same host size group. Lower case letters represent comparisons of IJs with the same origin between host sizes. Columns marked with the same letter are not statistically different at $P \leq 0.05$.

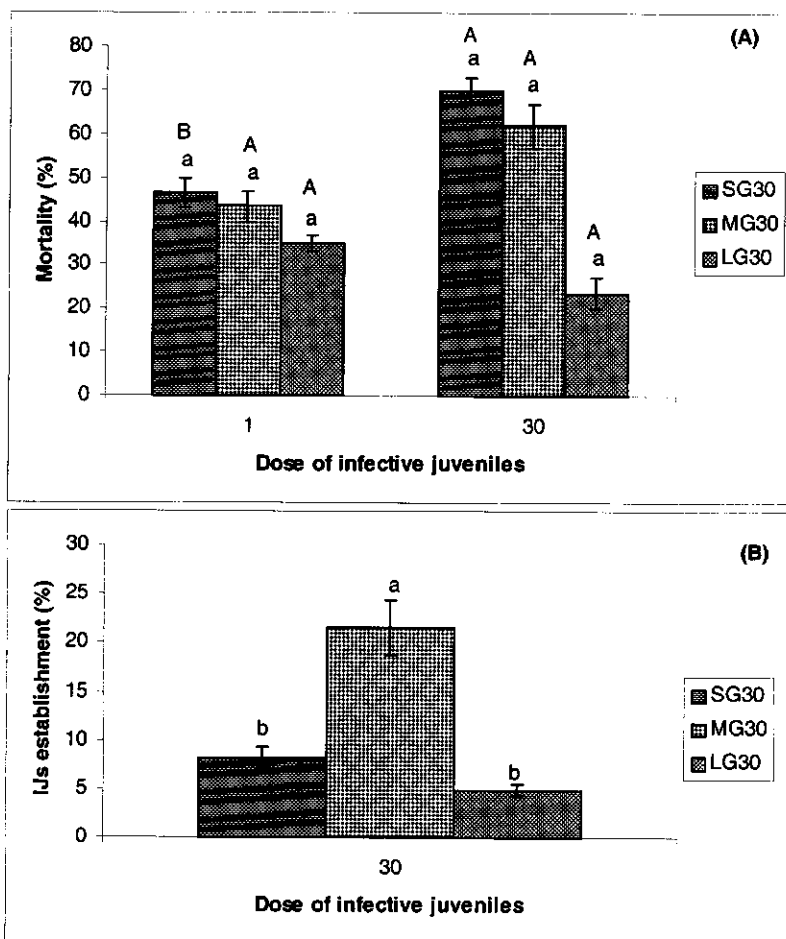


Figure 3. (A): Mortality of large larvae of *Otiorynchus sulcatus* exposed to a dose of one and 30 infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) originated from small (SG30), medium (MG30) and large (LG30) *Galleria mellonella* larvae. (B): Establishment rates in larvae of *O. sulcatus* exposed to 30 infective juveniles. Capital letters represent comparisons of different doses of IJs with the same origin. Lower case letters represent comparisons of the same dose of IJs with different origin. Columns marked with the same letter are not statistically different at $P \leq 0.05$.

Infectivity against O. sulcatus of IJs, originating from G. mellonella exposed to 30

Figure 3 A shows that IJs originating from small and medium sized cadavers used at a dose of 30 IJs per host were more infective to vine weevil larvae than those from large cadavers. Independent of IJ origin, the mortality of *O. sulcatus* larvae exposed to a single IJ was 35-45%. This level of mortality is always higher than that of *G. mellonella* larvae

exposed to a single IJ originating from medium and large hosts exposed to one or 30 IJs. A higher percentage of IJs from medium sized *G. mellonella* cadavers established in *O. sulcatus* larvae than IJs from small and large sized cadavers. Establishment capacity did not differ between IJs from small and large cadavers (Fig. 3B).

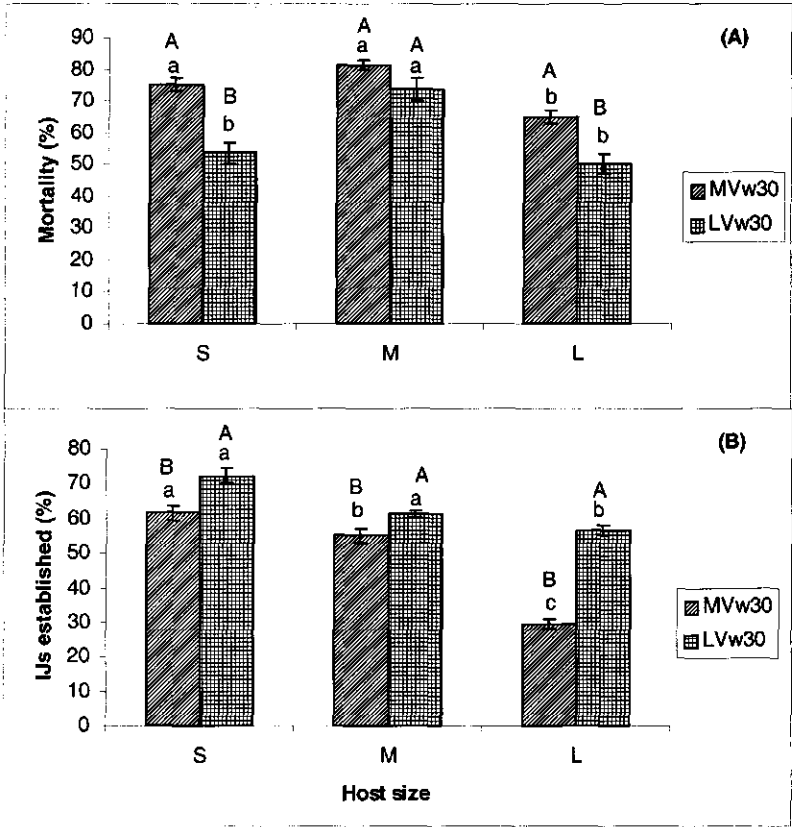


Figure 4. Infective capacity of *Heterorhabditis megidis* (NLH-E87.3) reared in medium (MVw30) and large (LVw30) larvae of *Otorhynchus sulcatus* tested at a dose of one IJ/larva (A) and 30 IJs/larva (B) against small (S), medium (M) and large (L) *Galleria mellonella* larvae. Capital letters represent comparisons between IJs of different origin to the same host size group. Lower case letters represent comparisons of IJs with the same origin between host sizes. Columns marked with the same letter are not statistically different at $P \leq 0.05$.

Infectivity against G. mellonella of IJs, originating from O. sulcatus exposed to 30 IJs

Single IJs originating from medium sized cadavers killed significantly more small and large hosts than single IJs from large cadavers. Infective juveniles originating from large cadavers showed to be more effective against medium sized hosts (Fig. 4A). No differences of host mortality (always 100%) were observed when IJs from medium and large larvae were used at a dose of 30 IJs per host. The percentage of IJs establishing in *G. mellonella* decreased with the increase of host size and IJs from large cadavers were significantly more invasive than those from medium sized cadavers (Fig. 4B).

Infectivity against O. sulcatus of IJs, originating from O. sulcatus exposed to 30 IJs

A dose of 30 IJs originating from medium sized cadavers killed significantly more vine weevil larvae than a dose of one IJ (Fig 5A). No statistical differences in the mortality were observed when vine weevil larvae were exposed to IJs of different origin used at the same dose. The establishment rate of IJs was low at only 20% and was not influenced by the origin of the IJs (Fig. 5B).

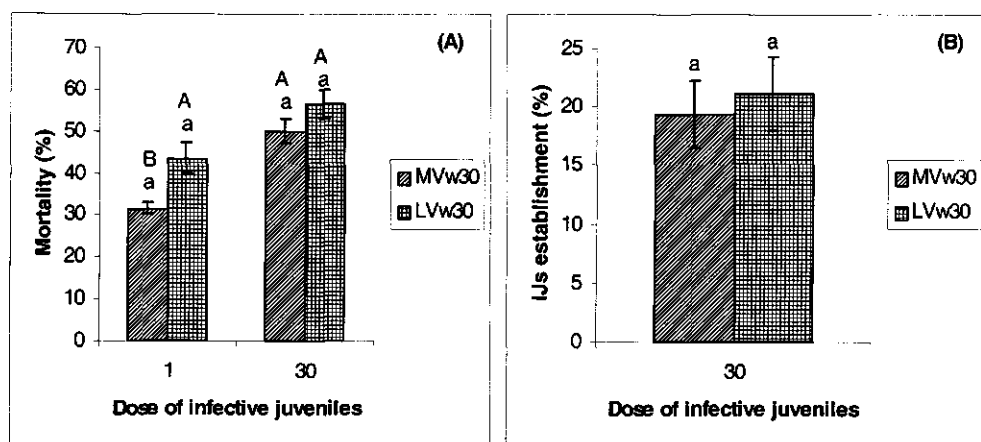


Figure 5. (A) Infective activity of *Heterorhabditis megidis* (NLH-E87.3) originated from medium (MVw30) and large (LVw30) larvae of *Otiiorhynchus sulcatus* against large larvae of *O. sulcatus*. (B) Establishment rates in larvae exposed to 30 infective juveniles. Capital letters represent comparisons of IJs dose within IJs origin. Lower case letters represent comparisons of IJs dose between IJs origin. Columns marked with the same letter are not statistically different at $P \leq 0.05$.

Discussion

Infective juveniles originating from small *G. mellonella* larvae showed to be more invasive and caused the highest level of mortality at both tested doses against all different sizes of *G. mellonella* larvae. Similar results were observed when they were tested against large larvae of *O. sulcatus*. This trend has also been observed for the infective ability of *Steinernema feltiae* originated from *Bradysia paupera* and other small hosts (Gouge and Hague, 1995). The IJs originating from medium sized vine weevil cadavers tested at a dose of one IJ/larva against different sized *G. mellonella* larvae caused higher mortality but were less invasive than IJs from large cadavers. The results found with IJs from *O. sulcatus* were the opposite of those found for IJs originating from *G. mellonella* cadavers. Although the data are not always statistically different, we observe that IJs from larger *O. sulcatus* cadavers were more pathogenic than those from smaller cadavers. Kakouli-Duarte and Hague (1999) observed that two different sizes of IJs were produced in large *O. sulcatus* larvae. Both sizes were able to re-infest small, medium and large larvae as well as being pathogenic to large *G. mellonella* larvae and the smallest IJs were more pathogenic for small vine weevil larvae.

Searching for a spontaneous mutation in entomopathogenic nematodes, Tomalak (1994) suggested that small IJs of *Steinernema feltiae* are better able to invade smaller insect hosts, but because smaller IJs are likely to have a lower energy content no advantage was found when they were compared with normal size IJs. In our study, however, we observed that smaller IJs that have been produced by smaller larvae of *G. mellonella* and *O. sulcatus* (Boff et al., (b) in press) were all the time more aggressive to all host sizes than those classified as medium and larger IJs.

O'Leary et al. (1998), investigating the effect of the day of emergence of *H. megidis* strain UK211 from insect cadavers, observed that IJs emerging early display a better initial host-finding ability when compared with the IJs that emerged later. The IJs that showed the highest infectivity in our experiments originated from small hosts. As a result they were small in size and had a shorter incubation period in the host than infective juveniles developing in medium and larger hosts. Although the experiments are not completely comparable, our results show similarity to those of O'Leary et al. (1998) and suggest that a common physiological mechanism may exist that triggers the host-finding and infective ability in early emerged IJs.

Murdoch et al., (1997) stated that large parasitoids might be able to search faster and hence have higher attack rates and that older or larger hosts receive larger numbers of parasitoids. We did not compare the dynamics of infection between the IJs of different origin but we observed that most of the time the better performance was achieved by IJs originating from smaller instead of those from medium or large insect hosts. The establishment rate of IJs in different sized *G. mellonella* larvae was higher when larvae were exposed to IJs originating from small *G. mellonella*. In general, a significant difference in invasive and pathogenic capacity of IJs was found related with the initial parental dose. For example, *G. mellonella* larvae initially infested with a dose of one IJ produced a more aggressive offspring against *G. mellonella* than those exposed to a parental dose of 30 IJs. We were, unfortunately, not able to observe this effect in *O. sulcatus* because of the lack of nematode production from small cadavers and lack of sufficient small and medium size vine weevil larvae.

Kaya and Hara (1981) reported that the infectivity of IJs differs between the different developmental stages of a host insect and generally soil-inhabiting insects are more resistant than those living above ground. Effects of the developmental stage of a host insect, in which IJs developed, on the infective capacity of IJs can clearly be observed in the present work in the experiment in which a dose of one IJ originating from different sized cadavers was used against *G. mellonella* larvae. Related to the last statement of Kaya and Hara (1981) we also observed that independent of the IJs' origin, *G. mellonella* larvae, a non soil pest, were more susceptible than larvae of *O. sulcatus*, a soil pest, but only when larvae were exposed to a dose of 30 IJs. However, the same tendency can not always be observed when larvae of both insect hosts were exposed to a single IJ. Bedding and Molyneux (1982) observed that *Heterorhabditis* IJs invaded *O. sulcatus* and *G. mellonella* larvae mainly via the cuticle or the spiracles, respectively. One possible explanation for the decrease in susceptibility in large *O. sulcatus* larvae is that the integument becomes hard in old larvae and acts as a barrier for IJs invasion (Eidt and Thurston, 1995).

We conclude that significant differences in infectivity were observed between IJs originating from different host species. The IJs originating from small cadavers of *G. mellonella* showed the highest infectivity against all sizes of both host species tested. Also the initial dose used to obtain IJs, and thereby the conditions during nematode reproduction played an important role in the infectivity of the nematodes. The results clearly show that the conditions in which IJs are grown have a severe impact on their later infectivity and performance.

Chapter 6

Effects of density, age and host cues on the dispersion of *Heterorhabditis megidis* (strain NLH-E87.3)

Adapted from: Mari I. C. Boff & Peter H. Smits. Effects of density, age and host cues on the dispersion of *Heterorhabditis megidis* (strain NLH-E87.3). *Biocontrol Science and Technology*, (accepted)

Abstract

Agar plate assays were used to assess the effect of density, incubation time and age of nematodes and the presence of insect hosts on the dispersal of infective juveniles (IJs) of *Heterorhabditis megidis* (strain NLH-E87.3). Infective juveniles dispersed faster and further at high densities than at low densities. Dispersal was also influenced by the age of the IJs. Individuals stored for a period of 1.5 and 4.5 weeks showed to be more active than those stored for 2.5 and 3.5 weeks. The presence of a host insect enhanced the dispersion of nematodes. After 90 minutes IJs responded positively to host cues from *Galleria mellonella* but poorly to cues from *Otiorynchus sulcatus* larvae.

Introduction

Foraging is the process whereby organisms search their habitat for suitable resources necessary for development, maintenance and reproduction. In the biological control context predators and parasitoids respond to physical and chemical stimuli that lead them to potential prey. In general search orientation consists of two phases, ranging, a relatively linear locomotion in which limited resource information is available, and localised search, a more convoluted and restricted search that occurs after information has been gained about an available resource (Miller & Strickler, 1984).

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae have only one free-living stage, the infective juvenile (IJ) (Poinar, 1990). The infective juveniles (IJs) are responsible for finding and penetrating a suitable host. Once the insect hemocoel is reached, IJs release their associated bacteria, which rapidly multiply and kill the host producing a suitable environment for nematode development and production of new IJ generations. The strategy that IJs adopt to find hosts varies between species. Some entomopathogenic nematode species (e.g. *Steinernema carpocapsae*, *S. scapterisci*) wait for passing hosts at or near the soil surface, whereas other species such as *Heterorhabditis bacteriophora*, *H. megidis*, *S. glaseri* and *S. anomali* continuously move through the soil in search of hosts (Grewal *et al.*, 1994b).

Dispersal is a behavioural mechanism used by entomopathogenic nematodes to locate habitats for survival and infection. Dispersal movement of entomopathogenic nematodes can be active or passive (Kaya, 1990a). Infected insects, phoretic hosts, water films, incrustated soil

in farm machinery, and nursery plants are listed as some agents that play a role in the passive dispersal of entomopathogenic nematodes whereas active dispersal depends on the nematodes themselves. Active dispersal includes nematode movement away from the host cadaver to sheltered microenvironments or to preferred soil depths. Active dispersal movement has advantages for IJs because it increases the chances for encountering a susceptible host as well as for survival (Kaya & Gaugler, 1993; Ishibashi & Kondo, 1990).

Interest in the use of entomopathogenic nematodes for biological control has stimulated research. Studies have emphasised the infection behaviour of the free-living infective stage. Behavioural aspects of infective juveniles not linked directly with infection, such as dispersal, have however been little less studied (Kaya & Gaugler, 1993).

Grewal *et al.*, (1994b) concluded that IJs of *H. megidis* (HO1 strain) cruise to find hosts. However, it is not known if the searching behaviour presented by this nematode strain can be the same in another *Heterorhabditis megidis* strain isolated from NW Europe. Thus, the objective of this work was to study the effects of nematode density, nematode age, time of exposition and host cues on the dispersal behaviour of infective juveniles of *Heterorhabditis megidis* (strain NLH- 87.3) on a 2-dimensional smooth agar substrate.

Materials and Methods

Nematodes and Insects

The entomopathogenic nematode *Heterorhabditis megidis* (strain NLH-E87.3) (Smits *et al.*, 1991) was cultured at 15°C in *Galleria mellonella* larvae. Forty *G. mellonella* larvae were separated in groups of five and each group was placed at the bottom of a cylindrical plastic container (diameter 36 mm, height 56 mm) filled with heat-sterilised silver sand (8%, w/w, tap water). One hundred and fifty infective juveniles (IJs) in one ml of tap water were added to the top sand surface. The containers were capped and incubated at 15°C for 5-7 days. Parasitized larvae were then placed in modified White traps (Lewis and Gaugler, 1994), and again incubated at 15°C. The emerging IJs were collected daily during a period of 6 days. The total of the harvested IJs was mixed and stored in 35 ml tap water in an aerated tissue culture flask at 15°C. The age of IJs was calculated from the time the first IJs were observed in the water layer of the modified White trap. To prevent the presence of dead or less active IJs in the assay plate, the IJs were rinsed with 15°C tap water and allowed to pass through a sieve lined with a coarse paper filter (Ederol n° 261) into tap water before being tested.

The tested insect larvae were last-instar *G. mellonella* and *Otiornychus sulcatus* (black vine weevil). *G. mellonella* larvae were reared according to standard procedures on artificial medium that consisted of a mixture of 900 g liquid honey; 900 g glycerin; 200 g bees wax; 400 g yeast flakes and 1300 g wheat meal at a temperature of 25°C. *Otiornychus sulcatus* larvae were reared on *Astilbe chinensis* plants in a greenhouse at a temperature of 20°C.

Assay arenas

A modification of the bioassay system described by Grewal et al., (1994b) was used to test the effects of density, time, age and presence of hosts on dispersal of IJs. A 14 cm diameter plastic Petri dish was divided into 4 equal quarters and a grid of 1 cm wide concentric rings around the center point (0.1 cm) was drawn on the bottom. Seventy five ml of 4% water agar were poured into each dish and allowed to cool for 1 h. The dishes with agar were covered, sealed with parafilm and transferred to a 15°C chamber 10 hr before being used. The center of the lid contained an access port (1cm in diameter) which could be sealed with a rubber stopper, allowing nematodes to be pipetted on the agar surface at the center point, without removing the lid.

Bioassays

Density effects

Doses of 10, 100, 500 and 1000 of 2.5 weeks old IJs were used to test the effect of nematode density on dispersal. In each dose the pooled IJs were concentrated in 100 µl and transferred to the centre point (0.1cm diameter) of the assay dish through the access port in the lid using a micropipette. The excess water at the release point was removed by touching the water droplet with a long paintbrush hair connected to a piece of filter paper (by capillary forces the water was absorbed by a piece of filter paper and the IJs were soon able to move on the agar surface). The test dishes were incubated in the dark at 15°C. After 30 min of incubation the agar of all pre-marked concentric rings of each quarter were cut out with a scalpel and rinsed separately with tap water. All IJs rinsed from each section of each quadrant were counted in a counting dish under a dissecting microscope. Five replicates were done for each IJ concentration.

Age effects

Following the methodology described above, IJs which were 1.5, 2.5, 3.5 and 4.5 weeks old were tested at a dose of 100 per plate. Thirty minutes after incubation the agar of each quarter was divided as explained above and the recovered nematodes from 5 replicates were counted.

Time effects

To test the dispersion of IJs over time the described methodology for the density effect bioassay was followed. Fifteen 14 cm-diameter plastic Petri dish arenas were prepared and 100 cleaned and active 2.5 weeks old IJs were applied on the surface of the agar at the release point. The dishes were divided into three groups of five and incubated at 15°C in the dark for 30, 60 and 90 min, respectively. Subsequent to each incubation period, the distribution of IJs was determined by counting the nematodes located in each part of the grid squared dish. No nematodes were found to reach the edge of the dishes.

Host effects

The dispersion of 2.5 weeks old IJs was tested using the assay arenas described above but now provided with live larvae of *G. mellonella* or *O. sulcatus*. One medium sized larva (120mg) of *G. mellonella* was placed into a small plastic gauze sack. The sack with the larva was placed on the agar surface and positioned exactly in the middle of one quadrant at a distance of 5-cm from the centre. In the opposite quadrant an empty sack was used as a control. In the tests using *O. sulcatus* larvae, two larvae (60 mg each) were put in the sack and it was also located 5 cm from the centre. Once the test dishes were prepared they were sealed with parafilm and immediately a dose of 100 IJs was pipetted on the centre point through the access port. The assay arenas were incubated at 15°C in the dark for 30, 60 and 90 min. All experiments were done 5 times. The relative position of the insect host quarter was different in each of the replicates and the position of the plates in the incubator was randomised. The distribution patterns were checked 30, 60 and 90 min after inoculation, respectively, and the IJs located in each section of each quadrant were counted under a dissecting microscope. The larvae were removed from the dishes and held at 25°C for 7 days. No mortality was observed in any of the exposed host larvae.

Statistical Analyses

Data on nematode dispersal and response to host cues were analysed using Generalized Linear Model (GLM) with Poisson distribution and a logarithmic link. Data are reported as mean \pm standard error of the mean. Analyses were performed using the Genstat (Genstat 5 Committee, 1997) statistical program.

Results

Density effects

Data presented in figure 1 show that the initial density of IJs influenced the dispersal on agar surfaces. At the lowest dose all IJs moved out of the centre but not further than 2-3 cm. At the concentration of 100 most IJs were found at 0-1 cm from the centre after 30 min but some moved 4-5 cm. At densities of 500 and 1000 nematodes most were found at a distance of between 0-1 and 4-5 cm from the centre but some even moved 6-7 cm. No significant difference in the distribution pattern was observed between the two highest doses. Infective juveniles did not show aggregation behaviour, they moved throughout the entire assay arena. At higher densities nematodes moved further from the release point than at lower densities.

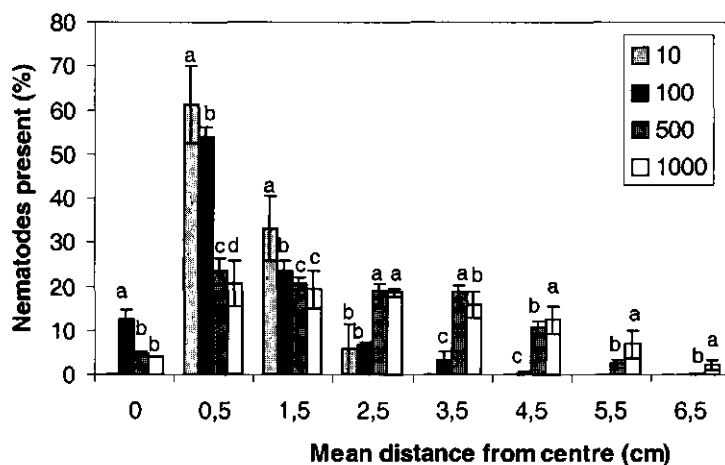


Figure 1. Mean dispersal activity \pm SE ($n = 5$) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) on agar surface. Nematodes were tested at a dose of 10, 100, 500 and 1000 and applied at the centre of each agar arena. Their position on the dishes was recorded after 30 min. For each plate ring, means followed by the same letter are not significantly different ($P \leq 0.05$).

Time effects

The spontaneous dispersal rate of 100 IJs on agar in different periods of incubation and in absence of host cues is presented in figure 2. At all incubation periods most of the IJs moved away from the release point into the first and second ring. After 30 min, 14% of the IJs remained in the centre, 62% had moved an average distance of 0-1 cm, 18% moved 1-2 cm and 6% moved at least up to 2-3 cm from the release point. No aggregation behaviour was observed.

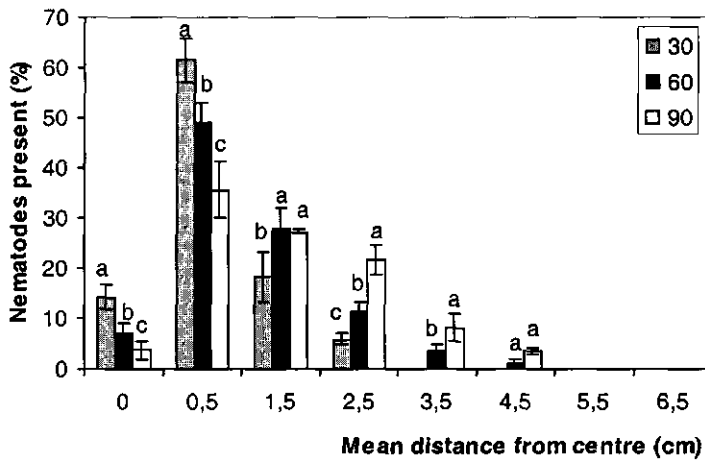


Figure 2. Dispersal activity of *Heterorhabditis megidis* (strain NLH-E87.3) IJs after 30, 60 and 90 minutes of incubation on agar plates in absence of host cues. Means (\pm SEM) of 5 replications, bars indicated by different letters are significantly different ($P \leq 0.05$). Comparisons are between time of incubation.

Age effects

Figure 3 shows that despite the major number of nematodes moved at least 2 cm far from the delivering point. The youngest (1.5 weeks old) and the oldest (4.5 weeks old) IJs were those that moved covering the total area of the assay arena and those 2.5 and 3.5 weeks old stayed more near the release point.

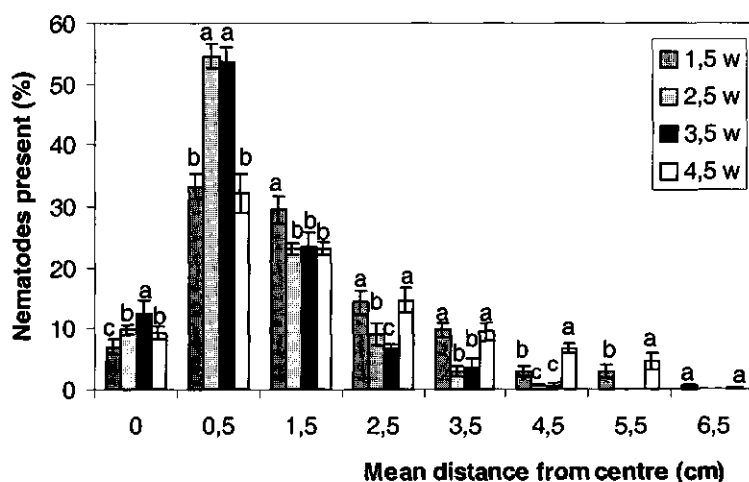


Figure 3. Effects of age on dispersal activity of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3). Means (\pm SEM) of 5 replications, bars indicated by different letters are significantly different ($P \leq 0.05$). Comparisons are within age at the same plate sector.

Host effects

The distribution of infective juveniles in response to the presence of a host insect on agar is shown in Figure 4. A significant response of IJs toward the quarter with the live *G. mellonella* larva was observed, but only after 90 min of incubation (Figure 4A). In contrast, IJs responded poorly to the presence of live larvae of *O. sulcatus* (Figure 4B). After 30 or 60 min IJs did not show any directional preference, they were distributed randomly over the four quarters. However, after 90 min significantly more IJs were found located in the quarter left of the exposed attractive source. Figure 5 shows that the presence of host cues stimulated the dispersal movement of IJs. This effect could already be seen after 30 min and increased with time.

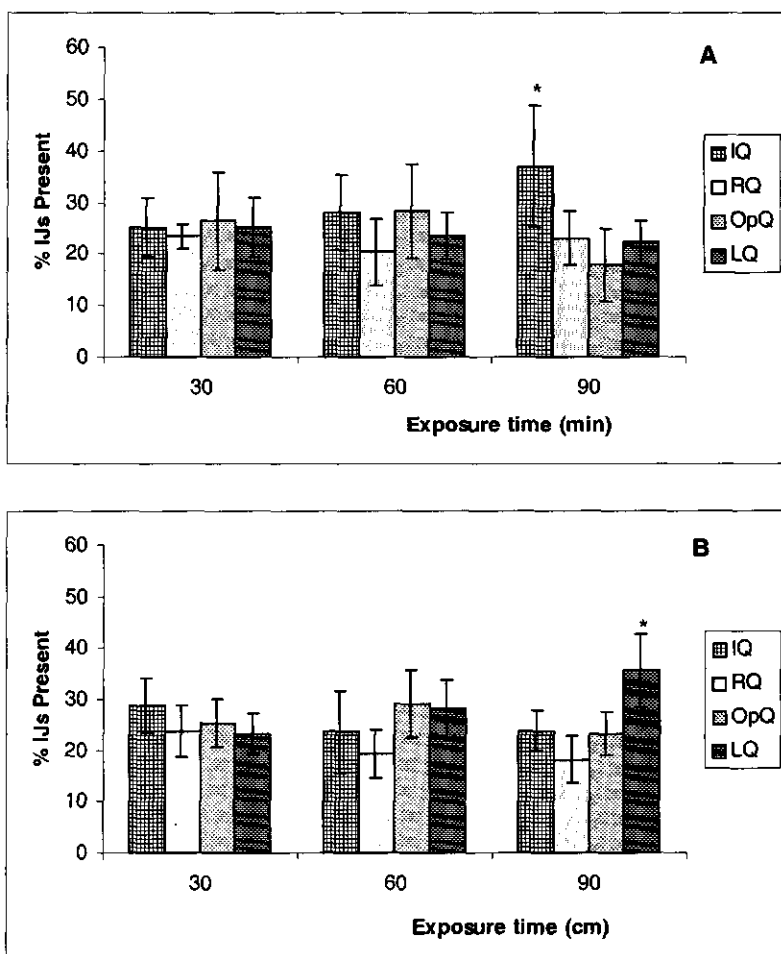


Figure 4. Response of *Heterorhabditis megidis* (NLH-E87.3) IJs to *Galleria mellonella* larvae (A) and *Otiiorhynchus sulcatus* larvae (B) in different time of exposure on agar plates. Means (\pm SEM) of 5 replications. The legend items are IQ = insect quarter; RQ = right quarter; OpQ = opposite quarter and LQ = left quarter in clockwise with the starting point at insect quarter.* Significantly different at $P \leq 0.05$.

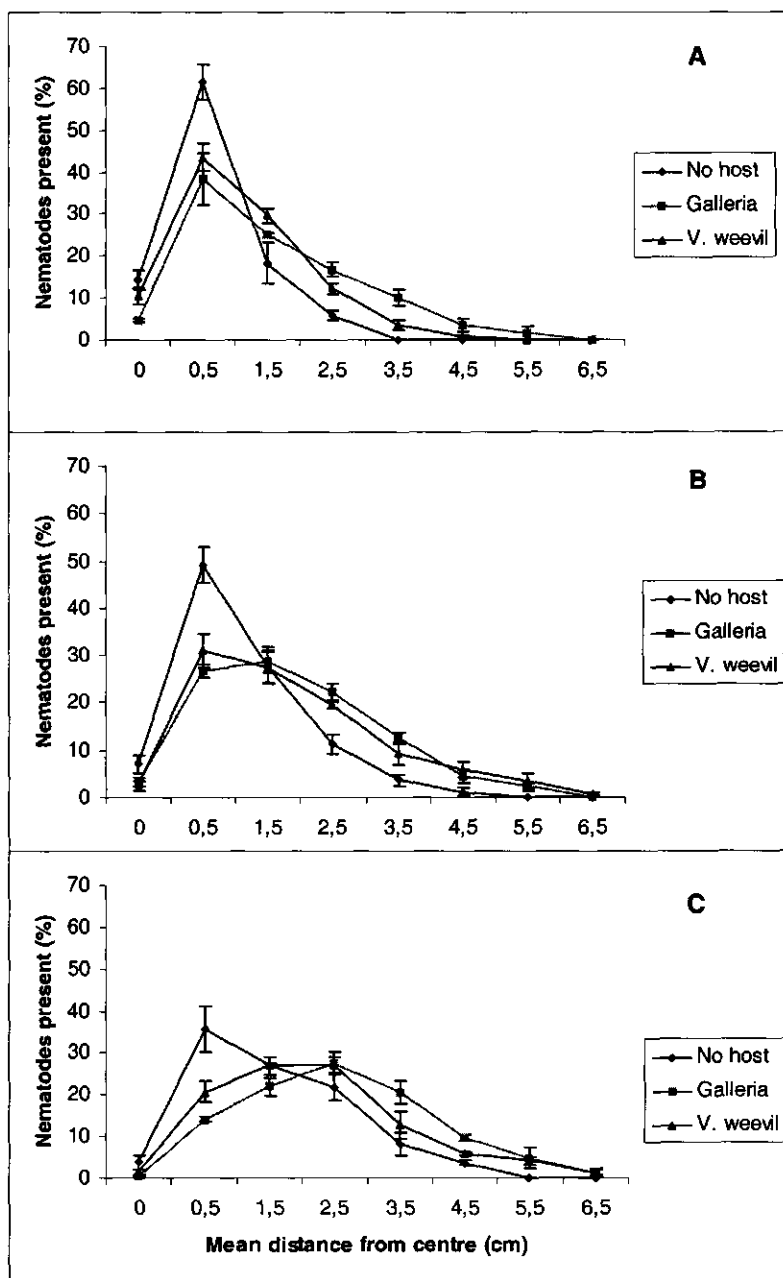


Figure 5. Effect of presence or absence of insect host on dispersal of *Heterorhabditis megidis* (NLH-E87.3) on agar at different exposition periods 30 min (A); 60 min (B) and 90 min (C). Means (\pm SEM) of 5 replications.

Discussion

Under natural conditions in the soil, IJs active dispersal and host finding is constricted by soil particle size, pore diameter, water content, temperature (Kaya, 1990a), and the relative activity of the nematodes categorised as cruiser or ambusher species (Grewal *et al.*, 1994b). In cruising search the IJs move continuously through the environment, searching for prey. Grewal *et al.* (1994b) concluded that *Heterorhabditis bacteriophora* and *H. megidis* are species that cruise to find hosts.

In all of our agar assays either in the presence or absence of hosts no nematodes show ambush behaviour (infective juveniles standing on their tails awaiting attachment to a passing host) on a smooth surface. Similar trends in behaviour were also observed when IJs of the same strain were tested on agar plates covered with sand grains (unpublished data). Our results are supported by Grewal *et al.* (1994b) who observed that IJs of *H. megidis* (HO1 strain) did not nictated neither on smooth or rough substrate. All IJs moved away from the release point by sinuous movements and in permanent contact with the agar surface. We also observed that the IJs movement became faster and IJs went further when other IJ touched them. This kind of "mutual dispersal help" or "stimulation by contact" could clearly be observed in the experiments in which different doses of IJs were tested (Figure 1). At the highest doses, nematodes dispersed faster and significantly more IJs were found in the ring 6-7cm from the release point.

Lewis *et al.* (1997) observed that IJs of *Steinernema carpocapsae* became more mobile with age. Age influence on mobility of IJs was also observed in our experiments. Infective juveniles of *H. megidis* stored during a period of 2.5 and 3.5 weeks showed less activity than those stored for 1.5 and 4.5 weeks. Fan and Hominick (1991b) demonstrated that the infectivity patterns of *Steinernema* spp. displayed a U-shaped curve. The infection levels were initially high, then decreased, but increased back to the original level after several weeks. Our results on dispersion seem to show a similar pattern and the dispersion ability might be related with the infective capacity. Carrying out host finding experiments on agar plates, O'Leary *et al.* (1998) observed that the first IJs of *H. megidis* emerging from host cadavers showed poor dispersal both in the presence or absence of a host whereas the last group of IJs to emerge dispersed well and their host-finding ability improved with increased duration of storage. The results presented by O'Leary *et al.* (1998) indicate that there are differences between IJs from different days of emergence. We worked with pooled samples from the total number of IJs that emerged from the cadaver during a period of 6 days so it was not possible to observe the

dispersal differences related with emergence period. Nonetheless our results agree with the findings that the dispersal ability improves with increased duration of storage.

In both experiments on incubation time and host finding ability, IJs tested in absence of a host moved randomly. However, when an insect host was added to the assay plates a difference in dispersal patterns could be observed (Figure 5). Although the IJs did not show an immediate directional response, the presence of an insect host stimulated their dispersion. Nematodes moved randomly over the agar surface for more than one hour before a response to host cues could be observed. After 90 min nearly 40 % of the IJs tested aggregated in the *G. mellonella* quarter and most of them near the insect. At the same incubation period most of the IJs exposed to *O. sulcatus* cues aggregated in the left quarter. We do not know the reason for this aggregation but the poor response to *O. sulcatus* larvae observed in this study on agar plates was also observed in a 3-dimensional choice experiment with sand (Boff *et al.*, submitted). Host search by infective juveniles is believed to include direct orientation toward host-released stimuli such as CO₂ (Gaugler *et al.*, 1991), excretory products (Schmidt & All, 1978, 1979; Grewal *et al.*, 1993) and temperature gradients (Burman & Pye, 1980; Byers & Poinar, 1982). In our study we did not measure the CO₂ production but the different response of NLH-E87.3 IJs to the two insect species is apparently in concordance with the findings of Gaugler *et al.* (1991) who found that a last instar *G. mellonella* larva produced more CO₂ per hour than last instar larvae of Coleopteran species as *Leptinotarsa decemlineata* and *Popillia japonica* and consequently attracted more IJs. The less attractivity of IJs to *O. sulcatus* larvae could also be caused by production of intrinsic excretory products that repel the IJs. Thurston *et al.* (1994) observed that IJs of *Steinernema carpocapsae* were attracted to CO₂ and faeces produced by *G. mellonella* larvae but were repelled by *L. decemlineata* faeces. Apparently, there are no cues from *O. sulcatus* larvae that arrest at or attract IJs of the tested strain.

Chapter 7

Influence of insect larvae and plant roots on the host-finding behaviour of *Heterorhabditis megidis*.

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(Submitted to *Biocontrol Science and Technology*)

Abstract

We studied the host-finding and dispersion behaviour of *Heterorhabditis megidis* (strain NLH-E87.3) in the presence of *Galleria mellonella* or *Otiorhynchus sulcatus* larvae and strawberry roots. In large Petri dishes (19 cm diameter) filled with moist sand (8 % w/w) and incubated at 15°C over 24 hours infective juveniles responded positively to the presence of *G. mellonella*, to roots of a single strawberry plant and to *O. sulcatus* larvae in direct contact with roots of a single strawberry plant. A neutral or negative response was observed when infective juveniles were presented with only *O. sulcatus* larvae or a combination of several strawberry plants with *O. sulcatus* larvae, either in contact or not in contact with the roots. Infective juveniles responded strongly to the combination of plant roots and feeding larvae indicating that the tritrophic interaction formed by infective juveniles - *O. sulcatus* larvae - strawberry plants may be an infochemical-mediated interaction.

Introduction

Dispersal by free-living infective juveniles is a behavioural mechanism that entomopathogenic nematodes use to locate new habitats for survival and infection. Entomopathogenic nematodes belonging to the family Heterorhabditidae are highly mobile and responsive to long-range host volatiles (Lewis *et al.*, 1992). Chemoreception is the main sensory mode used by entomopathogenic nematodes in orientating to their hosts (Kaya & Gaugler, 1993). Infective juveniles move through the substrate searching for a host and respond strongly to host-derived compounds (Schmidt & All, 1979; Gaugler *et al.*, 1980; Grewal *et al.*, 1993 and 1994b; Lewis *et al.*, 1993; Thurston *et al.*, 1994; Hui & Webster, 2000; Boff *et al.*, (c) in press). Infective juveniles of entomopathogenic nematodes have also been demonstrated to move toward plant roots and germinating seeds (Bird & Bird, 1986; Lei *et al.*, 1992; Kanagy & Kaya, 1996; Hui & Webster, 2000). The possibility of the use of plant

roots as a host habitat finding mechanism has been suggested by Kaya *et al.* (1987) and Choo *et al.* (1989). They observed that the ability of *Heterorhabditis heliothidis* to find a host was impaired by high root density but not by the presence of sparse corn, tomato, or marigold roots. Studies have also shown however, that the roots of certain plant species release substances that arrest, or are repulsive to, entomopathogenic nematodes (Lei *et al.*, 1992; Kanagy & Kaya, 1996; Boff *et al.*, (c) in press). Since most soil-inhabiting insects feed on plant roots, chemical cues from damaged or undamaged plant roots may also be used by entomopathogenic nematodes for host habitat finding. Cues from intact and wounded grass roots showed to have influence on the host finding ability of infective juveniles of *S. glaseri* and *H. bacteriophora* (Wang & Gaugler, 1998). Despite the above-mentioned results, Downes and Griffin (1996) pointed out that most of these studies might not be relevant for the natural behaviour of infective juveniles because the laboratory bioassays to select and test the efficacy of entomopathogenic nematodes are generally performed on agar or in sand columns with *G. mellonella* larvae as a host. In order to understand searching strategies it is required that studies are carried out under conditions that allow the expression of host-finding responses in a more natural context. Only few researchers have performed experiments with a soil pest such as *Otiorynchus sulcatus*. Using vertical sand and soil column arenas Westerman and Godthelp (1991) and Van Tol and Schepman (1999) found that some *Heterorhabditis* strains were not attracted by last instar larvae of *O. sulcatus*. Van Tol *et al.* (1998) and Van Tol and Schepman (1999) observed higher mortality of *O. sulcatus* larvae in the presence of plant roots. Working with an Y-tube sand olfactometer Boff *et al.* (c) in press) observed that infective juveniles of *H. megidis* were poorly attracted by *O. sulcatus* larvae but the combination of strawberry roots and *O. sulcatus* larvae showed strong attraction. The present study was undertaken to determine whether *H. megidis* (strain NLH-E87.3) nematodes are attracted to host insects and plant roots in a 3-dimensional sand matrix: a large Petri dish (19 cm Ø) filled with moist fine sterilised sand.

Materials and Methods

Nematodes

Heterorhabditis megidis (strain NLH-E87.3) (Smits *et al.*, 1991) was reared in last instar of wax moth larvae (*Galleria mellonella*) at a temperature of 15°C. Groups of four wax moth

larvae were placed on the bottom of 50-ml plastic cylinders filled with moist sand (8% w/w tap water). One hundred and twenty infective juveniles (IJs) in 1-ml of tap water were added to the sand surface of each cylinder. The containers were closed and incubated in the dark at 15°C. After 120 hours of incubation the red-coloured infected wax moth larvae were removed from the sand to a modified White trap (Lewis & Gaugler, 1994). The White traps were stored in the dark at 15°C. All the IJs that emerged from the cadavers and moved to the water layer were collected daily for a period of 6 days. Emerging IJs were mixed and stored in 30 ml of tap water at a temperature of 15°C for a period of two weeks. To prevent dead nematodes being used in the experiments, IJs were rinsed with tap water and allowed to pass through a sieve lined with a coarse paper filter (Ederol n°. 261) into tap water and were left at room temperature for half an hour before the experiments.

Insects

Galleria mellonella larvae were reared on a mixture of 900 g of liquid honey, 900 g of glycerin, 200 g of bees wax, 400 g of yeast flakes and 1300 g of wheat meal at a temperature of 25°C. Larvae of *Otiorhynchus sulcatus* (vine weevil) were reared on *Astilbe chinensis* plants in a greenhouse at a temperature of approximately 20 - 25°C. The larvae collected from the *Astilbe* pots were transferred to the laboratory and used immediately.

Plants

Young organically grown strawberry plants (Var. Elsanta, 3.5 - 4.0 g green weight) were collected from the field, transplanted into trays filled with potting soil and transferred to a climate chamber (20°C; L:D = 16:8). Plants were grown for 20 days to let them recover from the transplanting and form new roots. At the time of the experiments each plant was carefully removed from the soil, rinsed with tap water to remove all soil debris and immediately transferred to the experimental environment. Complete plants, i. e. with roots and aerial parts were used for all assays.

Assay arenas

Large glass Petri dishes (19 cm Ø) divided in 4 equal quarters filled with a leveled layer of 3 cm (1065 g) of heat sterilized sand (grain size 0 - 0.5 mm) containing 8% (w/w) tap water were used to test the nematode's response to host and root cues (Figure 1).

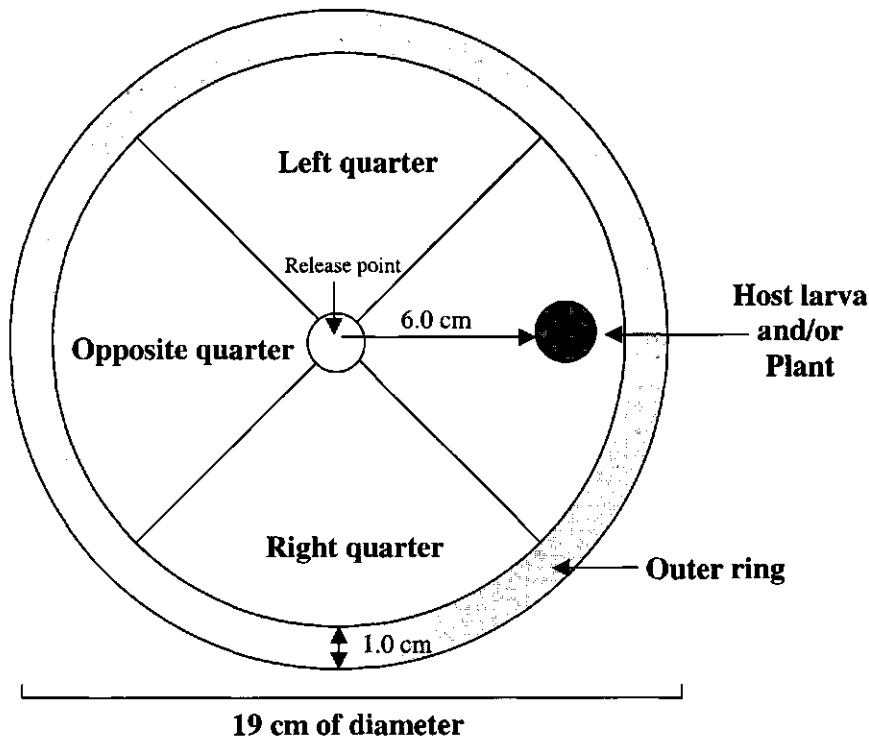


Figure 1. Schematic presentation of the experimental arena used to test the distribution of entomopathogenic nematodes in sand.

Nematode Recovery

Nematodes were recovered from the sand using a modified Cobb's decanting and sieving method (Klein Beekman *et al.*, 1994). In short, the amount of sand of each sampled dish region was put in a glass cylinder (1 l) and 600 ml of tap water was poured on it in one go. After 10 seconds the supernatant was poured into a 2-liter beaker. Two times more the remaining sand was mixed with 600 ml of tap water but now the supernatant was collected and added to the beaker after 15 seconds. The total volume of water with nematodes was poured through a sieve (0.025 mm) and the nematodes were washed from the sieve onto a coarse paper filter (Ederol n°. 261) in an extraction sieve placed in a shallow tray filled with tap water. Next day the sieve was carefully removed and the water suspension with the

nematodes was poured into a 50-ml plastic tube. Nematodes were left to settle and the total suspension was reduced to 10 ml. The number of nematodes from each tube was assessed by counting the total 10-ml suspension in counting grid dishes under a dissecting microscope.

Assays

Effects of insect larvae and plant roots on nematode dispersion were tested by carrying out the following series of assays: (i) *Galleria mellonella* larvae. (ii) *Otiiorhynchus sulcatus* larvae. (iii) A single strawberry plant. (iv) A single strawberry plant with *O. sulcatus* larvae. (v) Four strawberry plants and *O. sulcatus* larvae in direct contact with the roots of one of the plants. (vi) Four strawberry plants and *O. sulcatus* larvae not in contact with the roots of one of the plants.

General assay description

Insect assays

Single larvae of *G. mellonella* (± 200 mg) or 3 *O. sulcatus* larvae (± 60 mg each) were placed in a muslin bag (5 x 7.5 cm) filled with the same sand mixture (8% w/w) as used in the Petri dishes. The filled muslin bag was tied with a cotton string and inserted into the sand layer exactly in the center of one of the quarters, 6 cm from the center of the dish (Figure 1).

Plant and insect assays

Intact roots of whole single strawberry plants were placed in muslin bags (5 x 7.5 cm) filled with the same sand mixture (8% w/w) as used in the Petri dishes. To some root bags three larvae of *O. sulcatus* were added and left in direct contact with the roots. To other root bags three larvae of *O. sulcatus* were added, but the contact between larvae and roots was prevented by enclosing the larvae themselves in a small muslin bag filled with 0.78 g of moist sand and inserting this bag into the bag with the plant roots. To avoid a possible escape of the larvae the top part of the bag was carefully tied around the plant stem.

In the assays with plants or a combination of plant roots and larvae the bags were inserted in the center of one dish quarter as shown in Figure 1. In the assays with four plants, three without larvae and one with larvae, the bags were inserted in the center of each dish quarter.

Once the assay plates were prepared they were covered with glass lids and incubated at a temperature of 15°C under a light regime of 16:8 h to build up a chemical gradient. Ninety-six

hours later about 1000 infective juveniles were pipetted on the center of each plate into a hole made with a cork borer (1.5 cm diameter and 2 cm deep). After releasing the IJs the hole was refilled with the same sand, dishes were covered again and incubated at 15°C for 24-h more. After the incubation period of 24- h all the sand of each dish was sampled for the presence of nematodes. Using a cork borer of 2.2 cm of diameter, samples of 24 g of sand were taken around the nematode release point in the center of the dishes. Using a cork borer of 4.0 cm of diameter, samples of 48 g of sand were taken around the position of the muslin bag with larvae or plants. The whole outer ring of the sand (1 cm wide, ± 148 g of sand) was sampled separately using an aluminum ring. The rest of the sand was divided in 4 quarters (± 221 g of sand each) by using a cross-shaped cutting instrument. Each assay consisted of five dishes with larvae, plant or a combination of both and one, the control dish with a muslin bag filled with sand only. All the assays were performed 3 times with different batches of nematodes.

Statistical Analyses

Data on nematode dispersal and response to host and plant roots cues were analysed using Generalized Linear Model (GLM) with Poisson distribution and a logarithmic link. Data are reported as mean \pm standard error of the mean. Analyses were performed using the Genstat (Genstat 5 Committee, 1997) statistical program.

Results

Figure 2 shows the average distribution of *H. megidis* juveniles over the total of 18 untreated control dishes filled only with sand. It is clear that 98% of the nematodes move away from the release point, showing their basic searching activity (cruisers) and their vitality. Only 2% were found in the outer ring showing that the nematodes do not accumulate there to escape from the dish. The other 96% of the nematodes were found to be evenly distributed over the four quarters of the dishes, showing there was no unidirectional attraction present in the experiments. The recovery rate of the 1000 nematodes released in each dish was around 90% in all of the dishes indicating good conditions for the nematodes and a high efficiency of the modified Cobb's recovery method.

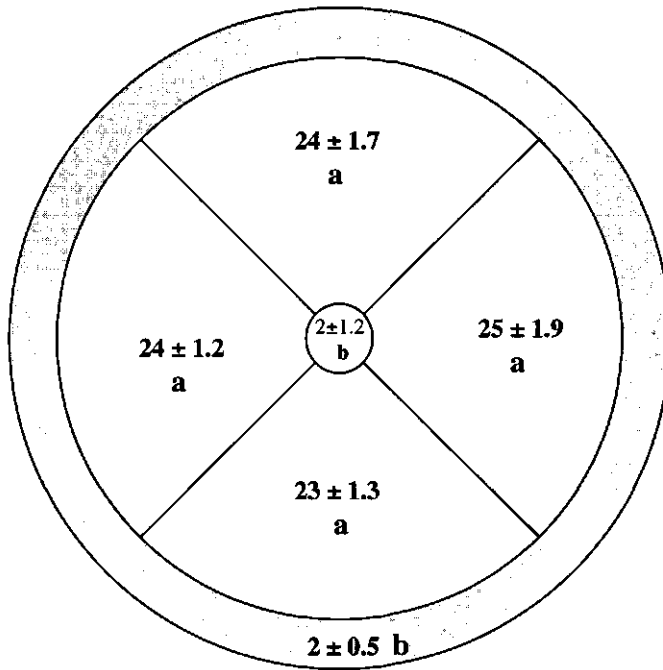


Figure 2. Distribution (mean percentage \pm SEM) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) in a large Petri dish (19 cm \varnothing) filled with only moist sand (control treatment). Dishes were incubated at 15°C for 96 h to build up a chemical gradient and 24 h for nematode distribution. A dose of 1000 infective juveniles was used. The nematode recovery rate was 89 %. Means followed by the same letter are not significantly different ($P \leq 0.05$).

Galleria mellonella

Data presented in figure 3 show that the nematodes respond strongly to the presence of *G. mellonella* larvae. The proportion of infective juveniles recovered from the quarter with the insect was significantly higher than from other quarters. From the total number of applied nematodes, 13% were located in the area directly around the insect (larva cage). Though less than 1 % of IJs were found established in the larvae they caused 100% mortality. Few nematodes were found in the outer region and significantly less IJs were recovered from the opposite than from the right and left quarters.

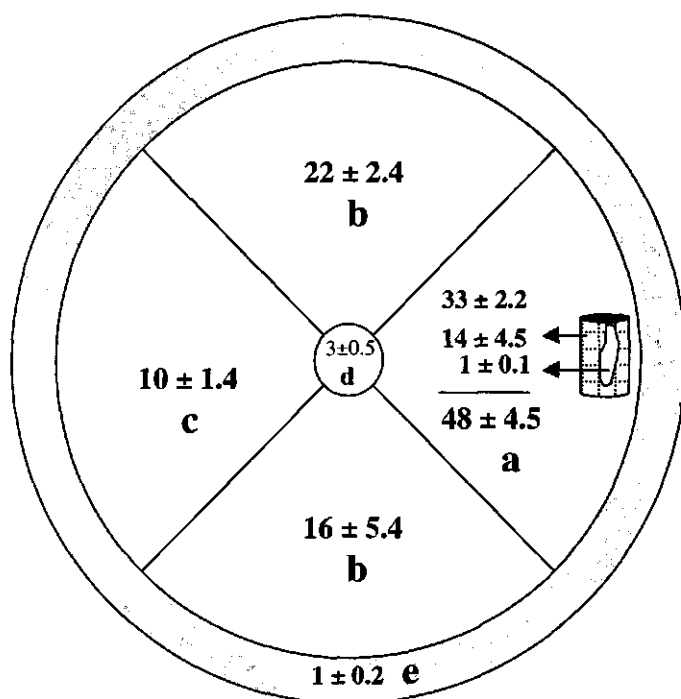


Figure 3. Distribution (mean percentage \pm SEM) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) in large Petri dishes (19 cm \varnothing) filled with moist sand and with a *Galleria mellonella* larvae present in one of the quarters. Dishes were incubated at 15°C for 96 h to build up a chemical gradient and 24 h for nematode distribution. A dose of 1000 infective juveniles was used. The nematode recovery rate was 92 %. Means followed by the same letter are not significantly different ($P \leq 0.05$).

Otiiorhynchus sulcatus

Infective juveniles responded poorly to the presence of *O. sulcatus* larvae (Figure 4). No significant difference was observed between the number of IJs recovered from the quarter with the larvae, the right and the opposite quarter. Despite the fact that 7% of the applied nematodes were recovered from the insect cage, no larval mortality was observed. The proportion of IJs that remained in the inoculation point was less than 5%, as was the percentage found in the outer region.

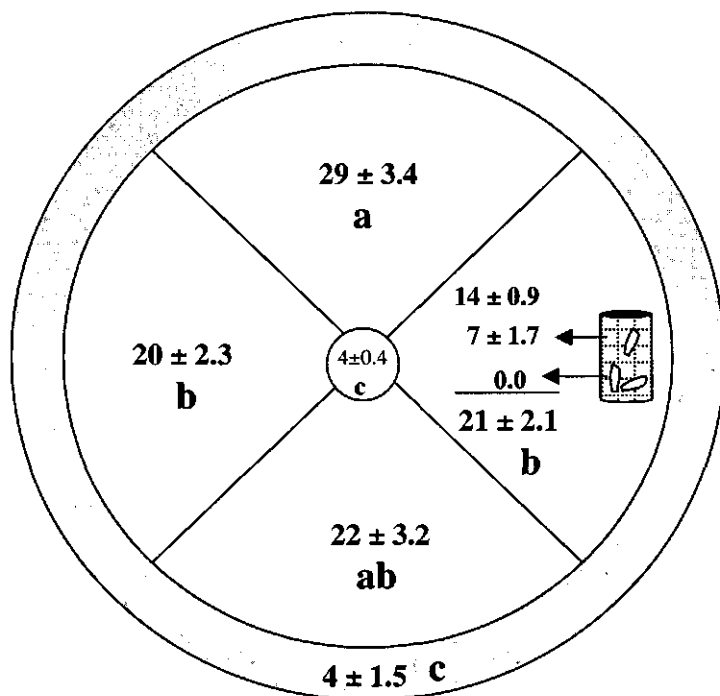


Figure 4. Distribution (mean percentage \pm SEM) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) in large Petri dishes (19 cm \varnothing) filled with moist sand and with *Otiiorhynchus sulcatus* larvae present in one of the quarters. Dishes were incubated at 15°C for 96 h to build up a chemical gradient and 24 h for nematode distribution. A dose of 1000 infective juveniles was used. The nematode recovery rate was 93 %. Means followed by the same letter are not significantly different ($P \leq 0.05$).

Single strawberry plant

There was a strong positive response of the IJs to strawberry plants (Figure 5). At the recovery time more than 60 % of the applied nematodes were found to be located in the plant quarter. Out of these 60 %, a total of 16% of IJs were aggregated near the roots. There was no difference between the number of IJs recovered from the right, left and the outer plate region. Less than 1 and 1.5% of IJs were recovered from the center and opposite regions, respectively.

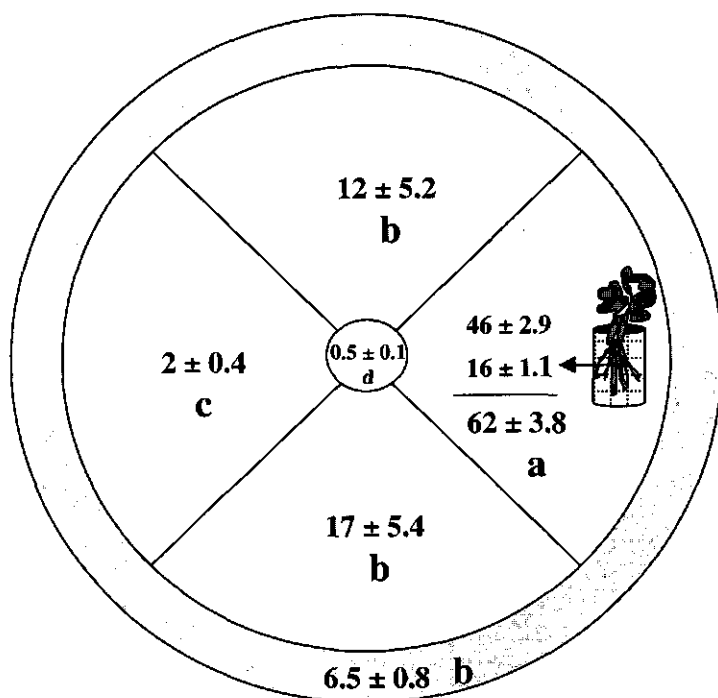


Figure 5. Distribution (mean percentage \pm SEM) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) in large Petri dishes (19 cm \varnothing) filled with moist sand and with a single strawberry plant present in one of the quarters. Dishes were incubated at 15°C for 96 h to build up a chemical gradient and 24 h for nematode distribution. A dose of 1000 infective juveniles was used. The nematode recovery rate was 88 %. Means followed by the same letter are not significantly different ($P \leq 0.05$).

Single strawberry plant with *O. sulcatus* larvae

The combination of strawberry roots and *O. sulcatus* larvae demonstrated to have a strong attraction on the nematodes, but not stronger than that of strawberry plants alone. Figure 6 shows that 26% of the initially released IJs were found aggregated near the roots plus larvae region. Another 25 % were recovered from the insect-plant quarter. Despite the high number of IJs recovered from the insect-plant quarter no larval mortality was observed. There was no significant difference in the proportion of nematodes found between the right, opposite, left and outer regions. The proportion of IJs staying in the inoculation point was less than 1%.

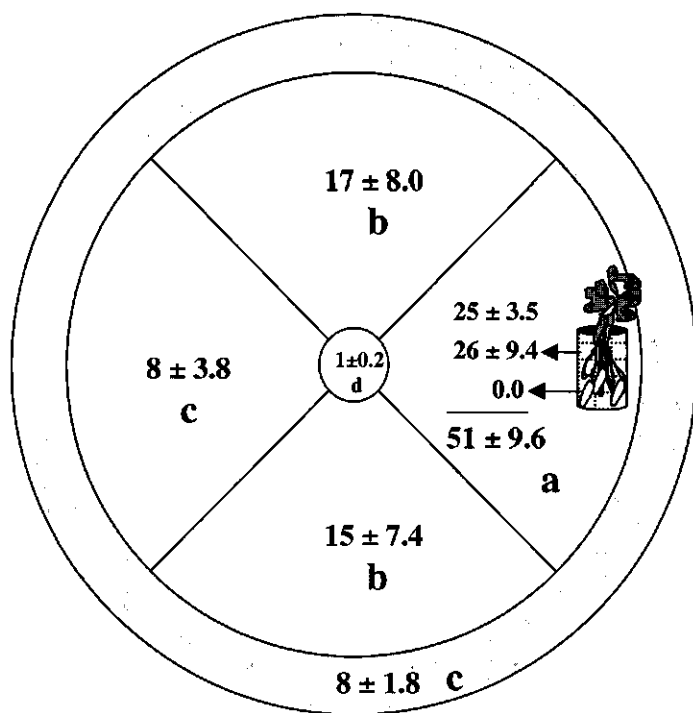


Figure 6. Distribution (mean percentage \pm SEM) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) in large Petri dishes (19 cm \varnothing) filled with moist sand and with a combination of roots of a single strawberry plant and three larvae of *Otiiorhynchus sulcatus* present in one of the quarters. Dishes were incubated at 15°C for 96 h to build up a chemical gradient and 24 h for nematode distribution. A dose of 1000 infective juveniles was used. The nematode recovery rate was 91 %. Means followed by the same letter are not significantly different ($P \leq 0.05$).

Four strawberry plants and *O. sulcatus* larvae

Infective juveniles confronted with the choice between three strawberry plants without larvae and one plant with *O. sulcatus* larvae either allowed or not allowed contact with the roots, surprisingly moved away from the stimuli (Figure 7 A and 7 B). In both assays, more than 40% of the IJs moved from the inoculation point passing through the sand with roots

and/or larvae to the outer region. Considering the proportion of nematodes recovered from each quarter, the presence of larvae led to an increase of the number of nematodes recovered but only in the case when larvae were in contact with the roots and able to feed on and damage the roots. More than 23 % of the IJs were recovered from the section with larvae in contact with roots (Figure 7A). However, in the case where larvae were not able to contact the roots less than 10 % of IJs were recovered (Figure 7B).

Discussion

Orientation of infective juveniles of *H. megidis* (strain NLH-E87.3) towards and away from stimuli varied, depending on the source and nature of these stimuli. Infective juveniles responded positively to *G. mellonella* larvae, to a single strawberry plant, and to the combination of a single strawberry plant and *O. sulcatus* larvae but negatively or neutral to *O. sulcatus* larvae and to stimuli from 4 strawberry plants. There was a significant difference between the response of the infective juveniles to *G. mellonella* and *O. sulcatus*. The present data support those found by Boff *et al.* (c) in press). Using a sand olfactometer these authors observed that after 24 h of incubation significantly more infective juveniles were recovered from the side where *G. mellonella* larvae were placed than from the *O. sulcatus* compartments. Westerman and Godthelp (1991) and Van Tol and Schepman (1999) also observed that some NW European *Heterorhabditis* spp. were not attracted to larvae of *O. sulcatus*.

Looking at the proportion of IJs that moved towards and reached the *O. sulcatus* bag and taking into account that one infective juvenile can be enough to cause infection, one would expect some level of infection. However, as it was observed by Van Tol and Schepman (1999) not one larva was found to be infected. Larvae of *O. sulcatus* live and pupate in the soil, where they will naturally come into contact with nematodes. It is likely that this insect has developed some resistance to, or avoidance of, penetration and that it will be less susceptible than *G. mellonella*, which does not naturally come into contact with nematodes.

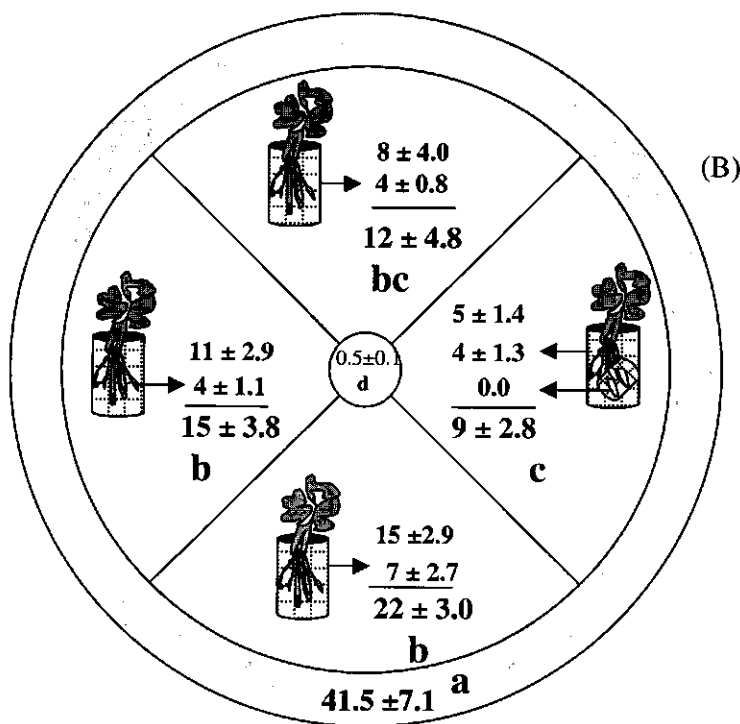
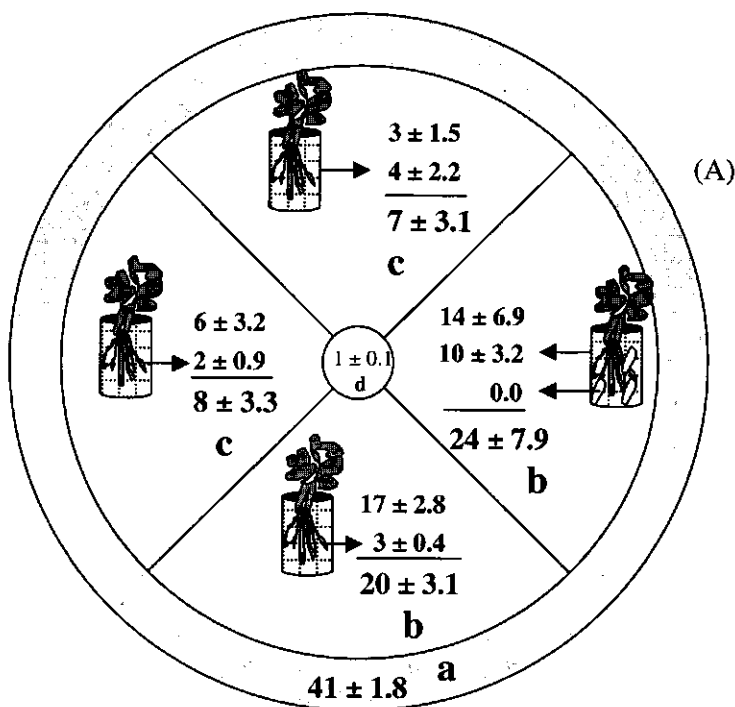


Figure 7. Distribution (mean percentage \pm SEM) of infective juveniles of *Heterorhabditis megidis* (strain NLH-E87.3) in large Petri dishes (19 cm \varnothing) filled with moist sand and with four single strawberry plants. One of the plants was provided with three *Otiiorhynchus sulcatus* larvae in contact (A) or not in contact (B) with the roots. Dishes were incubated at 15°C for 96 h to build up a chemical gradient and 24 h for nematode distribution. A dose of 1000 nematodes was used. The nematode recovery rate was 82 % for (A) and 88 % for (B). Means followed by the same letter are not significantly different ($P \leq 0.05$).

The distribution patterns of the nematodes and the "attraction" to host larvae and/or plant roots can be caused and explained by two behavioural mechanisms. Nematodes can be attracted over a distance of many centimetres to a source of odour or chemical gradient and home in on the source of the stimulant, thereby accumulating near the plant roots or insect larvae. The other mechanism is a combination of random movement combined with a short-range arrestment response. It is difficult to conclude from these experiments which mechanism lies at the base of the distribution patterns found in our experiments. It is clear from the low numbers of nematodes remaining at the release point and the very even distribution found in the control dishes without stimulants, that *H. megidis* nematodes move actively throughout the dish also without apparent stimulants being present. The results of some of the experiments with high numbers of nematodes present very close to the stimuli and with much lower numbers on the opposite side suggest there may be a long-range attraction, otherwise the lower numbers would be more evenly spread over the dish. The results with the plants and insect larvae where a relatively large proportion of the nematodes were always found in the compartment directly around the roots or larvae indicates that there certainly is a short range arrestment response of plant roots and insect larvae, even of vine weevil larvae.

Infective juveniles responded positively to strawberry roots and to the combination of strawberry roots and *O. sulcatus* larvae. The finding that intact strawberry roots attracted this nematode strain contrasts with the results obtained by Boff *et al.* (c) in press). Using an Y-tube olfactometer filled with sand these authors found that strawberry roots had a repulsive effect. Similar trends of repellence were also observed when IJs of NLH-E87.3 were confronted with cues from an intact strawberry plant on agar (unpublished results). These

contrasting results indicate that beside the effects of root-produced compounds on nematode responses there is also the influence of the used experimental system. Bird and Bird (1986), Lei *et al.* (1992), Wang and Gaugler (1998) and Hui and Webster (2000) also observed positive effects of intact plant roots on the response of entomopathogenic nematodes.

There is evidence that entomopathogenic nematodes respond positively to the presence of natural or unnatural sources of CO₂ (Gaugler *et al.*, 1980, Lewis *et al.*, 1993). However, as intact plant roots and the associated microbial components produce a series of compounds that lead to an accumulation of chemicals forming concentration gradients in the rhizosphere (Croll, 1970), CO₂ can not be considered the only compound influencing the behaviour of entomopathogenic nematodes.

Measuring the root respiration of strawberry plants, Blanke (1995) observed that strawberry roots contribute little to CO₂ emission in the soil when compared with the microbial soil respiration, which is about 50 times higher. In our study we simulate a more natural system with sand, plant roots and insect larvae. The strawberry roots used in our assays were rinsed with tap water before transplantation to the sterile sand, still, some part of the micro-flora and fauna on the roots might have been present and contributed to produce attractive compounds when a single plant was offered. However, when nematodes were confronted with cues from more than one strawberry plant, the CO₂ level and its diffusion in combination with other root exudates might have impaired the directional response. Kingler (1965) also reports that high concentrations of CO₂ inhibited the directional movements of some species of plant parasitic nematodes.

Damage caused by insects on the aerial part of the plants induce the production of secondary metabolites favoring plant defense (Dicke, 1999; McCall *et al.*, 1993). If these phenomena happen above ground it is not unlikely that this may also occur in the soil were the plant roots are attacked by many insect species. In our study infective juveniles responded positively to the combination of strawberry roots and damage or feeding activity of *O. sulcatus* larvae. The proportion of nematodes recovered from the "insect-plant cage" was nearly two-folds compared with roots offered separately and about three times more than for *O. sulcatus* larvae alone.

Damage to roots is likely to cause elevated root temperatures and CO₂ production, as well as the production of other chemical compounds that play a role in the defense action of plants (Rhodes & Wooldorton, 1978; Uritani & Ōba, 1978). A clear example that insect damaged roots produce and release secondary metabolites that attract nematodes can be seen in the assays carried out with roots of four strawberry plants. The proportion of infective juveniles

found to be aggregated around the plant cage with larvae in contact with the roots was higher than the proportion found around the plant cage in which larvae were not in contact with the roots. Parallel or aggregated to the production of allelochemicals, the vibration (noise) caused by root feeding activity might be another means by which infective juveniles locate their host species.

There are not many examples referring to the behaviour of *Heterorhabditis* species in the presence of plant roots with a root-feeding insect. However, our results are strongly supported by the results obtained by Boff *et al.* ((c) in press) and Van Tol and Schepman (1999). The first authors observed that in a Y-tube olfactometer infective juveniles of *Heterorhabditis megidis* responded positively to the combination of strawberry roots and *O. sulcatus* larvae. The second authors reported that plant roots of *Waldsteinia ternata* had an positive influence on the mortality and infection of *O. sulcatus* larvae by infective juveniles of *Heterorhabditis* sp..

To summarize, the present study indicates that nematodes moved more directly and positively toward *G. mellonella* larval cues than toward *O. sulcatus* larval cues. Intact strawberry roots exude compounds that positively attract infective juveniles but when more plants are present the behavioural pattern of the infective juveniles changes to avoid the plants and to move to the outer ring of the dish. The combination of plants and feeding larvae is very attractive, suggesting that the tritrophic interaction "strawberry plant - *O. sulcatus* - infective juveniles" may be an infochemical mediated interaction.

Chapter 8

Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer.

Adapted from: Mari I. C. Boff, Frans C. Zoon & Peter H. Smits. Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. *Entomologia Experimentalis et Applicata* (in press)

Abstract

The host-searching behaviour of *Heterorhabditis megidis* strain NLH-E87.3 in the presence of insect hosts and plant roots, offered individually and in combination, was studied using a newly developed Y-tube olfactometer filled with sand. Within a period of 24 hours infective juveniles (IJs) were significantly attracted to living *G. mellonella* larvae and caused 100% larval mortality. *Otiorynchus sulcatus* larvae, however, did not elicit host-oriented movement of IJs and no larval mortality was observed. Roots of strawberry plants induced a negative response in IJs. The combination of strawberry roots and *O. sulcatus* larvae, however, strongly attracted IJs leading to 37% host mortality. It was shown that this type of Y-tube choice arena is a useful tool in studying the searching behaviour of entomopathogenic nematodes in a semi-natural habitat.

Introduction

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have the potential to control a wide range of insect species that occur in soil and cryptic habitats. They have proven to have certain advantages over chemicals, as these bio-control agents are non-polluting, environmentally safe and acceptable in most countries over the world (Smart, 1995).

Only the third stage (infective juveniles) can survive outside an insect host and move from one insect to another. The infective juvenile carries symbiotic bacteria in its intestine (*Xenorhabdus* spp. for steinernematids or *Photorhabdus* sp. for heterorhabditids). When the infective juvenile reaches the hemocoel of a host it releases the bacterial cells which multiply rapidly in the hemolymph and provide essential food for the nematodes' development and reproduction. When the newly produced nematodes become infective juveniles they leave the cadaver and search for a new host. The infective juveniles of both genera utilize different strategies to locate hosts, acting either as "ambushers" that wait for passing insect hosts or as "cruisers" that search actively for a host by moving through the soil (Grewal et al., 1994b; Lewis et al., 1993).

Although it is not understood exactly how the infective juveniles find hosts in soil, it is believed that entomopathogenic nematodes of both genera somehow utilize chemoreception (Downes & Griffin, 1996). Most of the species respond positively to host-associated cues such as CO₂ and or faeces (Schmidt & All, 1978, 1979; Gaugler et al., 1980; Lewis et al., 1993; Grewal et al., 1993).

Since most soil-inhabiting insects feed on plant roots, cues from damaged or undamaged plant roots may also be used by entomopathogenic nematodes for host habitat finding. The possibility of using plant roots as a host habitat finding mechanism was first demonstrated by Kaya et al. (1987) and Choo et al. (1989). Entomopathogenic nematodes have also been found to respond to germinated seeds (Hui & Webster, 1998), to the meristematic region of tomato seedlings (Bird & Bird, 1986), to marigold and tomato roots (Kanagy & Kaya, 1996), and to cues from intact and wounded grass roots (Wang & Gaugler, 1998). Choo & Kaya (1991) showed that the presence of corn roots increased the proportion of nematodes entering *G. mellonella* larvae. Downes & Griffin (1996), however, pointed out that this is not an adequate investigation of the natural behaviour of infective juveniles, because *G. mellonella* does not normally feed on plant roots.

In order to understand searching strategies of entomopathogenic nematodes it is necessary to confirm observations made under less natural conditions with studies carried out under conditions that allow the expression of host-finding responses in a more natural context. Most of the laboratory bioassays to select and test the efficacy of entomopathogenic nematodes are performed on agar or in sand columns with *G. mellonella* larvae as a host. Few researchers have performed experiments with a soil pest such as *Otiorynchus sulcatus* in soil columns. Using vertical soil column arenas Westerman & Godthelp (1991) and van Tol & Schepman (1999) found that some *Heterorhabditis* strains were not attracted by last instar larvae of *O. sulcatus*. van Tol et al. (1998) and Van Tol & Schepman (1999) observed a difference in mortality of *O. sulcatus* larvae in the presence or absence of plant roots. The present study was undertaken to determine whether infective juveniles of *Heterorhabditis megidis* (strain NLH 87.3) are attracted to host insect and root-produced chemostimulants in a 3-dimensional sand matrix: a horizontal Y-tube choice apparatus filled with moist sand.

Material and Methods

Nematode, insect culture and Plants.

Infective juveniles (IJs) of *Heterorhabditis megidis* (Poinar et al., 1987), (strain NLH-E87.3 Smits et al., 1991)) were reared in last instar larvae of wax moth (*Galleria mellonella* (L)) at 15 °C. Infective juveniles were harvested from modified White traps (Smits et al., 1991), stored at 15 °C in aerated tissue culture flasks with 35 ml of tap water. All experiments were performed with IJs harvested three to six days after initial emergence in the water layer and tested within 3 weeks of production. Before being used in the experiments, the IJs were rinsed with tap water and left at room temperature (18-20 °C) for 15 minutes. The test insect larvae were last-instar *G. mellonella* and *Otiorynchus sulcatus* (Faber). Larvae of *G. mellonella* were reared according to standard procedures on artificial medium that consisted of a mixture of 900 g of liquid honey; 900 g of glycerin; 200 g of bees wax; 400 g of yeast flakes and 1300 g of wheat whole meal at a temperature of 25 °C. *Otiorynchus sulcatus* larvae were reared on *Astilbe chinensis* plants in a greenhouse at a temperature of 20 to 25 °C. The larvae collected from the *Astilbe* pots were transferred to the laboratory and used immediately.

Young organically grown strawberry plants (Var. Elsanta, 3.5 – 4.0 g fresh weight) were collected from the field, transplanted into trays filled with potting soil and transferred to a 20 °C greenhouse. Plants were held for 15 days to let them recover from the transplanting and form new roots. At the time of the experiments each plant was carefully removed from the soil, rinsed with tap water to remove all soil debris and immediately transferred to the experimental environment.

Assay arena.

The Y-tube choice apparatus (Figure 1) provides an arena consisting of a basal Y connector divided in two parts: the "opposite compartment" and the "centre compartment" and two pairs of pipe pieces to form the Y-tube arms. Each Y-tube arm was composed by connecting two pipe pieces (68 mm long) denominated compartment "At and Bt" for the treatment arm; compartment "An and Bn" for the arm without treatment ("neutral"). The material used to build the assay arenas was dark grey hard PVC tubing for sewage discharge. All the parts of the Y-tube olfactometer were successively filled with fine sterilized silver sand (8 %w/w) and connected to form a closed system.

Experiments.

Behavioural responses of IJs to the presence of larvae of *G. mellonella*, *O. sulcatus*, strawberry plants, strawberry plants plus *O. sulcatus* larvae and to only

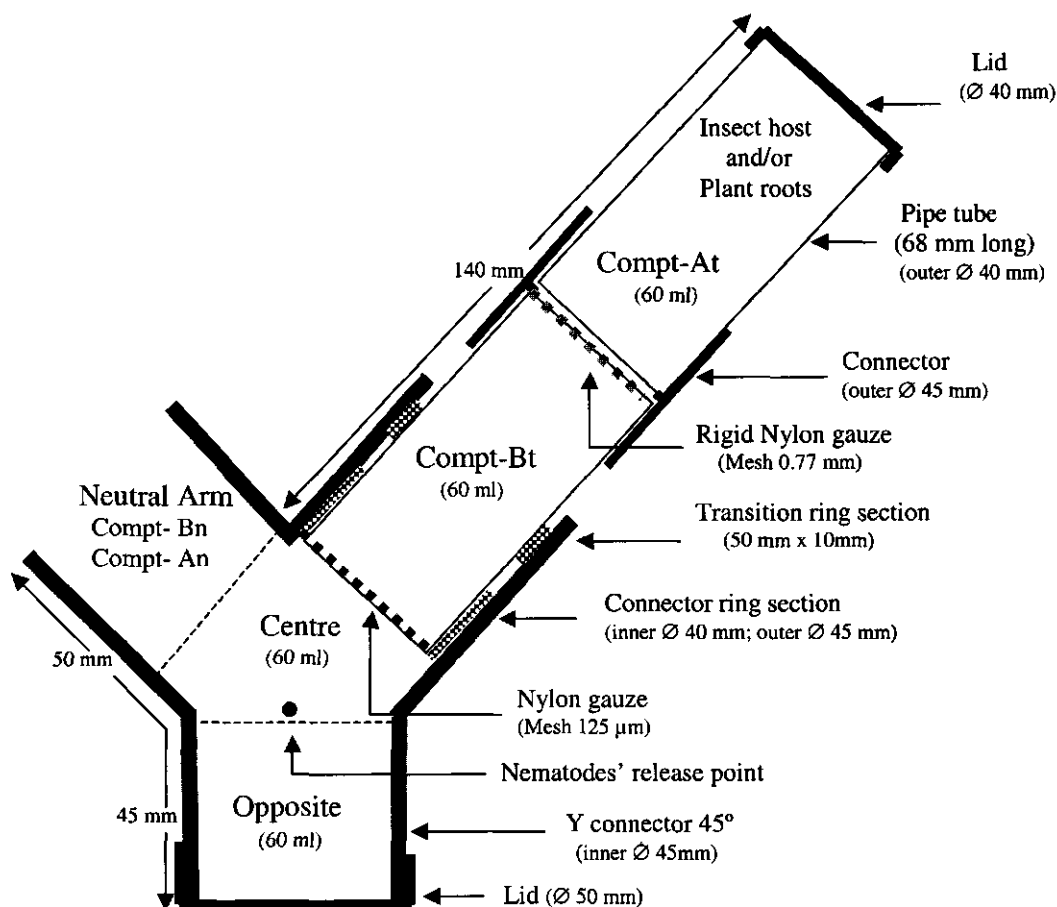


Figure 1. Schematic drawing of a Y-tube choice arena for recording preferential responses of entomopathogenic nematodes to insect and roots stimuli in soil.

sand were tested by using the above described assay arenas. The experiment was performed in 12 runs with fresh batches of nematodes. Each run was composed of four replicates of a Y-tube set-up (treatment) and one control. Thus, runs of each set-up were done three times.

G. mellonella set-up. One last instar *G. mellonella* larva (0.35 g) was placed in the compartment At of the Y-tube choice apparatus (Figure 1) and confined to the bottom part by adding sand up to the top. The assay units were incubated in a horizontal position in the dark at 15 °C for 24 h to form a chemical gradient. Twenty-four hours later one ml of tap water with 1000 ($\pm 10\%$) IJs was pipetted in the centre through a hole of 15 mm made in the sand with a cork borer. After

nematode application, the sand was gently replaced and the Y-tube was again closed with a lid and incubated horizontally again for 24 h at 15 °C. Subsequently the fraction of IJs in each compartment was assessed. Nematodes were recovered from the sand using a modified Cobb's decanting and sieving method (Klein Beekman et al., 1994). The amount of sand of each compartment was put in a 1-liter glass cylinder and 600 ml of tap water was poured over it. After 10 seconds the supernatant was poured into a 2 liters beaker. Two times more the remaining sand was mixed with 600 ml of tap water, but now the supernatant was collected and added to the beaker suspension after 15 seconds. The total beaker suspension with the nematodes was poured through a sieve (mesh size 0.025 mm). The nematodes were washed from the sieve on to a coarse paper filter (Ederol n°. 261) in an extraction sieve placed in a shallow tray with tap water. Twenty-four hours later the sieve was carefully removed and the suspension was collected into a 50 ml plastic cylinder. Nematodes were allowed to settle and the total suspension was reduced to 10 ml. The active nematodes from the soil of each compartment of the Y-tubes were counted in a counting dish under a dissecting microscope. The insect larvae that were used as an attractive source were incubated in a 20 °C chamber for 5-7 days and dissected to assess the number of infective juveniles established.

O. sulcatus set-up. Two L 4 *O. sulcatus* larvae (0.06 g each) were placed together in compartment At of the Y-tube arena. The assay was conducted further following the above-described methodology.

Strawberry plants set-up. Individual whole plants were placed in compartment At of the Y-tube choice apparatus. The space between the tube walls and the roots was filled with sand (8% w/w). The surface around the stems at the top end of the tube was sealed with aluminium foil and parafilm. After filling the assay units, they were incubated at a temperature of 15 °C under a light-dark regime of L16:D8 to build up a chemical gradient. Forty-eight hours later a dose of 1000 (\pm 10%) IJs was applied as described above. After 24 h of horizontal incubation the IJs from each compartment were recovered as described above.

Strawberry plants plus O. sulcatus larvae set-up. Compartment At was filled with two L 4 larvae of *O. sulcatus* and an individual strawberry plant. The larvae were put in contact with the strawberry roots and allowed to feed on them for 48 h. The free space between roots and larvae was filled with sand (8% w/w). Further methodology was as described above for the strawberry plants set-up.

Statistical analysis. For the comparisons between Y-tube compartments within treatments a generalised linear model (GLM) was used with distribution Poisson and a logarithmic link. As batch effects were not found ($P \leq 0.05$) treatment effects on whole-tube variables were tested

in ANOVA based on a completely randomised design with five treatments (including control) each with 12 replicates. Analyses were performed using the Genstat (Genstat 5 Committee, 1997) statistical program.

Results

Figure 2 shows the average distribution of infective juveniles over the 12 control Y-tube units. It is clear that 90% of the nematodes did not move away from the centre compartment. The other 10% of the nematodes were found evenly distributed between the two Y-arms and the opposite compartment of the olfactometer, showing that there was no attraction.

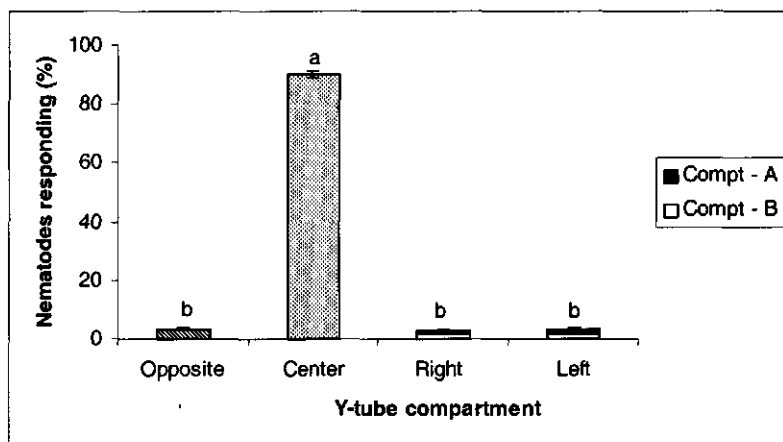


Figure 2. Distribution (mean percentage \pm s.e.) of infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) in a Y-tube olfactometer filled with only moist sand (control treatment) and incubated at 15 °C. Compt-A and Compt-B correspond to the pipe pieces that compose either the right or left Y-tube arms. Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

Galleria mellonella - Twenty-four hours after inoculation a high proportion of infective juveniles showed a positive response to cues produced by *G. mellonella* larvae (Figure 3). During the exposure time more than 50% of the inoculated IJs moved at least 10 cm in the direction of the insect compartment. Only 2% of the IJs were found established in the larvae but this was enough to cause 100% of larval mortality.

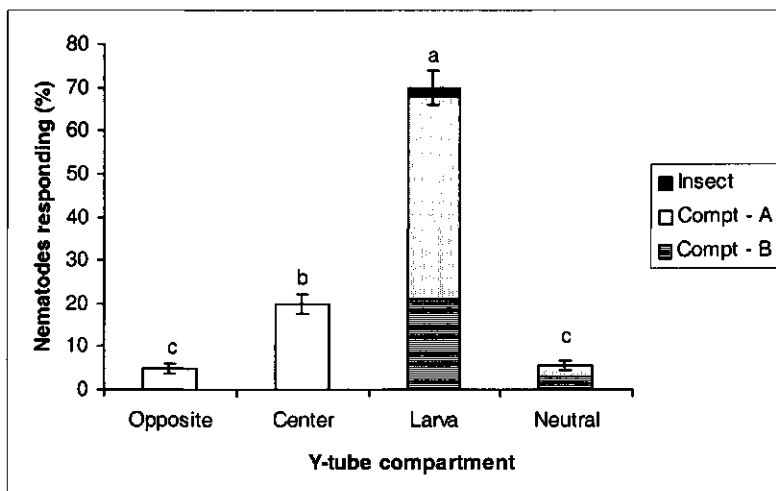


Figure 3. Distribution (mean percentage \pm s.e.) of infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) in response to cues from *Galleria mellonella* larvae in a Y- tube olfactometer filled with moist sand and incubated at 15 °C. Host larvae were placed in the top compartment (Compt- At) of one arm of the olfactometer referred to as "larva" arm. The other olfactometer arm with only sand is called "neutral" arm. The terms Compt-A and Compt-B correspond to the pipe pieces that compose either the "larva" or "neutral" Y-tube arms (see Figure 1). Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

Otiiorhynchus sulcatus - A reaction of IJs to the presence of *O. sulcatus* larvae was observed (Figure 4). The percentage of IJs that moved towards the larvae arm was lower than that observed in the *G. mellonella* treatment (Figure 3), but much higher than that observed in the untreated tubes (Figure 2). A significant percentage of IJs (42%) remained in the Y-tube centre compartment. The active IJs did not move preferentially to the arm with the *O. sulcatus* larvae as they did in the assays with *G. mellonella* larvae. No IJs were found established in the larvae and consequently all the exposed larvae remained alive.

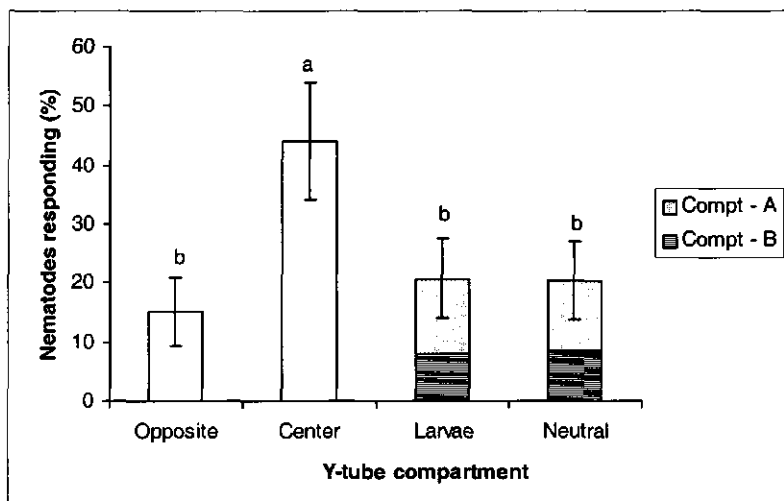


Figure 4. Distribution (mean percentage \pm s.e.) of infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) in response to cues from *Otiorynchus sulcatus* larvae in a Y- tube olfactometer filled with moist sand and incubated at 15 °C. Host larvae were placed in the top compartment (Compt- At) of one arm of the olfactometer referred to as "larva" arm. The other olfactometer arm with only sand is called "neutral" arm. The terms Compt-A and Compt-B correspond to the pipe pieces that compose either the "larva" or "neutral" Y-tube arms (see Figure 1). Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

Strawberry plants - Data in Figure 5 show that IJs had a strong, negative chemotaxis toward strawberry roots. More than 50% of the applied IJs moved to the "opposite compartment". Within the group of IJs that responded "positively", however, significantly more IJs were recovered from the treatment arm with plant roots than from the neutral arm.

Strawberry plants plus O. sulcatus larvae - Fifty-one percent of the inoculated IJs showed a positive directional response to cues from the combination of roots and larvae (Figure 6). From the proportion of IJs, which had a positive response, 34 % were recovered from soil in the "plant + larvae" compartment and only 0.2 % had established in the larvae causing a mortality level of 37 %.

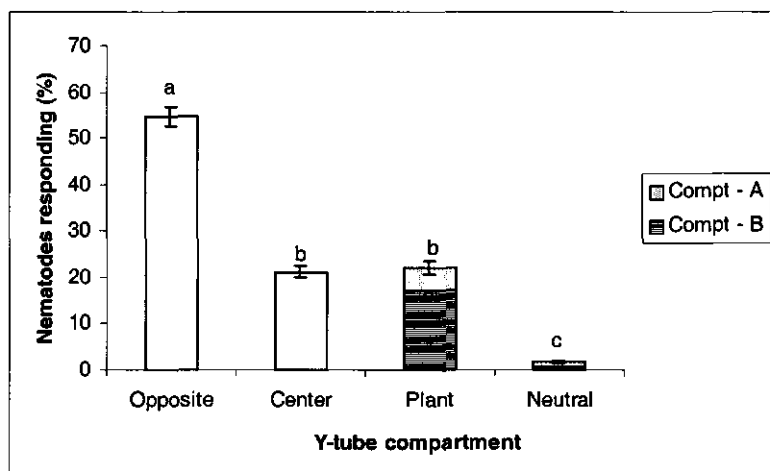


Figure 5. Distribution (mean percentage \pm SE) of infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) in response to cues from intact roots of strawberry plants in a Y- tube olfactometer filled with moist sand and incubated at 15 °C. Plants were placed in the top compartment (Compt- At) of one arm of the olfactometer referred to as "plant" arm. The other olfactometer arm with only sand is called "neutral" arm. The terms Compt-A and Compt-B correspond to the pipe pieces that compose either the "plant" or "neutral" Y-tube arms (see Figure 1). Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

The recovery rate of the nematodes released in different set-ups was always over 94% (Table 1) indicating good conditions for the nematodes and a high efficiency of the modified Cobb's recovery method. The whole-tube variables like percentage of active nematodes i.e. nematodes that moved out of the centre compartment and preference are also summarized in Table 1.

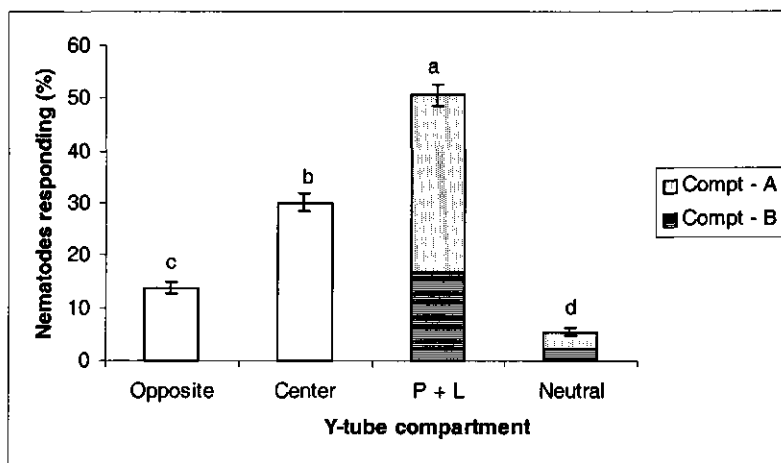


Figure 6. Distribution (mean percentage \pm SE) of infective juveniles of *Heterorhabditis megidis* (NLH-E87.3) in response to cues from roots of strawberry plants and *Otiorhynchus sulcatus* larvae in a Y-tube olfactometer filled with moist sand and incubated at 15 °C. Plant and the larvae were placed together, in the top compartment (Compt- At) of one arm of the olfactometer referred to as "P+L" arm. The other olfactometer arm with only sand is called "neutral" arm. The terms Compt-A and Compt-B correspond to the pipe pieces that compose either the "plant + larvae" or "neutral" Y-tube arms (see Figure 1). Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

Table 1. Comparisons of treatment effect on whole-tube variables. Means followed by different letters are significantly different from each other (LSD, $P \leq 0.05$).

Treatments	Recovery (%) ^a	IJs Active (%) ^b	Preference of active nematodes (%) ^c		
			Treated Arm	Neutral Arm	Opposite Comp.
Control	94 ns	10 d	30 b	36 a	34 b
<i>G. mellonella</i>	95	80 a	87 a	7 c	6 d
<i>O. sulcatus</i>	95	56 c	40 b	29 b	32 bc
Strawberry plants	97	78 a	29 b	2 d	69 a
Strawb. + <i>O. sulc.</i>	96	70 b	73 a	8 c	20 c

^a Calculated assuming an inoculum of 1000 infective juveniles.

^b Percentage of total recovered nematodes in "opposite" + At + Bt + An + Bn compartments of the Y-tube.

^c Percentage of active nematodes present in the treated arm (At + Bt), neutral arm (An + Bn) or "opposite compartment", respectively (see Figure 1).

Discussion

The assay arena used in this study for measuring responses of *H. megidis* (NLH-E87.3) to cues produced by live host larvae or intact plants appeared to provide consistent results. In the tests with *G. mellonella* larvae, IJs proved to have the ability to move and respond positively to insect host cues although *G. mellonella* is not a soil insect. Within an exposure period of 24 hours a significant percentage of IJs made a choice and moved over a horizontal distance of at least 10-cm through moist sand and reached the insect compartment causing 100% of host mortality. It was observed before that the presence of *G. mellonella* larvae influenced the vertical distribution of IJs of strain NLH-E87.3 (Westerman, 1995). It is not clear whether the stimuli involved are volatile, water soluble or both, although Grewal et al. (1993) found that IJs of *H. megidis* and *H. bacteriophora* on agar responded directionally to volatile host cues. Cruiser species like *H. megidis* tend to search continuously through the environment, and therefore rely heavily on chemical cues for locating their hosts (Grewal et al., 1994 b).

In our study a different behaviour of IJs towards *G. mellonella* compared to *O. sulcatus* larvae was observed. Nematodes responded strongly to the presence of *G. mellonella* larvae, more than 70% moved into the arms, most of which accumulated in the insect arm close to the insect (Table 1, Figure 3). In presence of *O. sulcatus* larvae, about 40% of the IJs moved into the arms, but they did not accumulate close to the insect (Table 1, Figure 4). The fact that the overall response of the nematodes is lower for *O. sulcatus* larvae than for *Galleria* larvae could be due to the size of the larvae or the quantity and composition of stimuli produced by either species. For instance, it is documented that *G. mellonella* produces relatively large quantities of CO₂ (Gaugler et al., 1991). More interesting than the quantitative difference in response is the difference in preference for the arm in which the host is present. It seems that *G. mellonella* larvae produced stimuli that both activated and attracted (or arrested) the IJs resulting in an apparent positive directional response. In contrast, *O. sulcatus* stimuli seemed only to activate the nematodes resulting in a random movement without aggregation or arrestment near the host. Our results are similar to those of Westerman & Godthelp (1991) and van Tol & Schepman (1999) who also observed that some NW European *Heterorhabditis* spp. were not attracted to larvae of *O. sulcatus*.

The experiments with strawberry plants show that nematode movement was stimulated by the presence of intact plant roots. A large proportion of IJs moved away from the roots to the opposite compartment, but also some were found in the arm with the roots (Table 1,

Figure 5). It seems that some nematodes were attracted by stimuli from the roots but most were repelled. In recent experiments with the same Y-tube system and *Thuja occidentalis* plants also attraction of entomopathogenic nematodes to roots was found (Boff, unpubl.). The situation with strawberry plants may reflect a different balance between attractive and repellent root exudates. It can also be a case of density of roots and the level of stimuli. Kaya et al. (1987) and Choo et al. (1989) observed root density effects on infective ability of nematodes. Both authors found a negative correlation between root density and infection rate of *G. mellonella* in sandy soil. In addition, Choo & Kaya (1991) proved that in humic soil the presence of sparse roots of sweet corn increased the proportion of IJs entering *G. mellonella* larvae. Lei et al. (1992) observed that the chemical and temperature gradients around the root system of ball cabbage and radish attracted IJs of *H. zealandica*. These authors suggested that the attraction of entomopathogenic nematode species to roots might aid them in locating their insect hosts. Many other authors working with in vitro arenas also reported on the attraction of entomopathogenic nematodes to plant roots (Bird & Bird, 1986; Kanagy & Kaya, 1996; Wang & Gaugler, 1998; Hui & Webster, 1998; van Tol et al., 1998).

In the experiment with strawberry plants combined with *O. sulcatus* larvae the IJs were strongly attracted to the combination, at least much stronger than to either strawberry plants or *O. sulcatus* larvae alone and this result resembles that of *G. mellonella* (Table 1). The large difference between the separate and combined situation is the presence of larval feeding and damage to the roots. It is therefore likely that either exudates from damaged roots, stimuli from insect faeces or saliva or possibly the vibration associated with feeding activity elicit attraction or arrestment by the nematodes. It is also surprising that in the combination of *O. sulcatus* larvae and plants 37% host mortality was found. In assays with only *O. sulcatus* larvae no mortality was observed despite the presence of sufficient numbers of nematodes in the larvae compartment. It seems that feeding activity is a necessity for the nematodes to find and parasitize *O. sulcatus* larvae and that they mainly enter the larvae together with the food through the mouth. Similarly Wang & Gaugler (1998) reported that IJs of *S. glaseri* and *H. bacteriophora* responded positively to detached grass roots, but when wounded roots were presented as alternative to intact roots IJs were attracted more by wounded roots than by intact grass roots.

Dicke (1999) stated that in tritrophic systems herbivory results in cell damage and a subsequent emission of volatile compounds that are highly attractive to natural enemies of the herbivores. Damage to roots is likely to cause elevated root temperatures and CO₂ production, as well as the production of other chemical compounds that play a role in the defence action

of plants (Rhodes & Wooldorton, 1978; Uritani & Ōba, 1978). This phenomenon appears to be an additional cue that may help IJs to locate their host species.

Obviously, *H. megidis* (strain NLH-E87.3) is attracted particularly towards the *O. sulcatus* larvae in feeding activity. Remarkably, feeding activity appears to be also the key process in the penetration and establishment of this nematode strain in this host species. This might explain the success of this nematode strain as a biocontrol agent of *O. sulcatus* larvae in pot plants and nursery stock and throws a new light on host finding behaviour of entomopathogenic nematodes. The Y-tube arena used to conduct the present study provides a meaningful tool for studying the performance of entomopathogenic nematodes in their natural environment.

Chapter 9

Behavioural response of *Heterorhabditis megidis* (strain NLH-E87.3) towards plant roots and insect larvae.

Adapted from: Mari I. C. Boff, Rob H. W. M. van Tol & Peter H. Smits. Behavioural response of *Heterorhabditis megidis* (strain NLH-E87.3) towards plant roots and insect larvae. *BioControl*, (in press).

Abstract

The behavioural response of infective juveniles (IJs) of *Heterorhabditis megidis* (strain NLH-E87.3) to cues from roots of strawberry (*Fragaria x ananassa* Duch.), thuja (*Thuja occidentalis* L.) and black vine weevil, *Otiorynchus sulcatus*, larvae was studied. Choice assays were conducted in an Y-tube olfactometer filled with moist sand. Infective juveniles were activated by the presence of intact roots of both strawberry and thuja plants. Some nematodes aggregated in the compartments with roots but most moved away from the roots to the opposite side. Given a choice, IJs showed a preference for strawberry roots above *O. sulcatus* larvae. No difference in preference was observed between thuja roots and *O. sulcatus* larvae. The combination of strawberry roots with vine weevil larvae was preferred above roots alone. In the assays with thuja roots and larvae versus thuja roots alone, however, IJs were stimulated to move but showed preference for the opposite compartment away from the arms with roots and larvae. Nematodes responded differently to mechanically damaged roots as opposed to roots damaged by vine weevil larvae. In assays with damaged thuja roots, IJs were most attracted by the roots damaged by larvae, whereas in the strawberry assays IJs showed a clear preference for the mechanically damaged roots. When challenged with a choice between strawberry and thuja roots, IJs moved preferentially to strawberry than to thuja roots. A preference for the combination of strawberry roots plus larvae over the thuja roots plus larvae was also observed.

Introduction

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* are effective biological control agents of soil pests. They possess attributes of insect parasitoids, predators and microbial pathogens (Kaya and Gaugler, 1993) and today are considered the only really promising biological insecticides in soil (Downes and Griffin, 1996). The host finding behaviour of infective juveniles differs from species to species. Some species depend on a sit and wait "ambush" strategy to find their hosts, others such as most *Heterorhabditis* species actively search for hosts and are described as "hunters or cruisers" (Grewal et al., 1994).

Predators and parasitoids often integrate visual, auditory, tactile, and chemical cues in locating hosts. Entomopathogenic nematodes seem to rely mainly on chemical cues (Kaya and Gaugler, 1993). The role of chemical perception in host finding has been studied less

extensively for entomopathogenic nematodes than for plant parasitic nematodes that respond strongly to chemical concentration gradients (Croll, 1970). It was demonstrated that entomopathogenic nematodes react to host-released substances (Schmidt and All, 1979; Gaugler et al., 1980; Grewal et al., 1993 and 1994; Lewis et al., 1993; Thurston et al., 1994; Hui and Webster, 2000; Boff et al., in press) and also to plant roots and germinating seeds (Bird and Bird, 1986; Lei et al., 1992; Kanagy and Kaya, 1996; Hui and Webster, 2000; Boff et al., in press). Studies have also shown, however, that roots of certain plant species release substances that arrest or repel entomopathogenic nematodes (Lei et al., 1992; Kanagy and Kaya, 1996; Boff et al., in press).

Since most soil-inhabiting insects live nearby or feed on plant roots, chemical cues from undamaged or damaged plant roots may also be used by entomopathogenic nematodes for host habitat location. Wang and Gaugler (1998) reported that infective juveniles of *Steinernema glaseri* and *Heterorhabditis bacteriophora* showed a distinct host finding ability when presented with intact or wounded grass roots. Infective juveniles of *H. megidis* also were attracted to the combination of plant roots and larvae of *O. sulcatus* (van Tol and Schepman, 1999; Boff et al., in press).

Although the use of entomopathogenic nematodes for controlling root-feeding insects is at a stage of rapid development, the impact of integrating insect, plants and habitat signals on the host-finding process has not been much studied. The objective of this study was to examine the effect of combinations of plant roots and host insects on the searching behaviour of *H. megidis* (strain NLH-E87.3) in a 3-dimensional assay arena.

Materials and Methods

Nematode

Heterorhabditis megidis (strain NLH-E87.3, Smits et al., 1991) was reared in larvae of *Galleria mellonella* at 15 °C. All infective juveniles (IJs) that emerged from the cadavers and moved to the water layer of the modified White traps (Lewis and Gaugler, 1994) were collected daily for 6 days. Harvested IJs were mixed and stored at 15 °C in aerated tissue culture flask with 35 ml of non-sterile tap water at densities of 5 000 IJs/ml. Experiments were performed with 2-week-old IJs. Before being used in the experiments, the IJs were rinsed with tap water and allowed to pass through a sieve lined with a coarse paper filter (Ederol n°. 261) into tap water and left at room temperature for 30 min.

Insects

Otiorhynchus sulcatus larvae were reared on *Astilbe chinensis* plants in a greenhouse at a temperature of 20 - 25°C. Larvae collected from the *Astilbe* pots were transferred to the laboratory and used immediately. For all assays, larvae weighting 67 ± 0.4 mg were used.

Plants

Young organically grown strawberry plants (*Fragaria x ananassa* cv. 'Elsanta') (3.5 - 4.0 g fresh weight) were collected from the field, transplanted into trays with potting soil and placed in a climate chamber (20°C; L:D = 16:8). Plants were grown during 20 days to let them recover from the transplanting and form new roots. At the time of the experiments, each plant was carefully removed from the soil, rinsed with tap water to remove all soil debris and immediately transferred to the Y-tube compartment.

Rooted cuttings (10 - 15 cm high) of thuja (*Thuja occidentalis* cv. 'Brabant') were transplanted into trays with potting soil and placed in a climate chamber (20°C; L:D = 16:8). The thuja plants were grown for more than 20 days. Before the plants were used in an assay, the roots were cleaned with tap water to remove all soil debris and immediately transferred to the Y-tube compartment. The two plant species, strawberry and thuja, were selected for the experiments because the roots and crown of both plants are susceptible to attacks of *O. sulcatus* larvae.

Assay arena

A slight modification of a Y-tube olfactometer described in chapter 8 was used (Figure 1). Each olfactometer consisted of a basal Y-connector with a central part (60 ml) and two pairs of pipe pieces (68 mm long; 60 ml each) to form the Y-tube arms. At the bottom end of the Y-tube a single pipe piece (68 mm long; 60 ml) was connected. Between the centre compartment and the three arms, nylon gauze (mesh 125 μ m) was placed to facilitate separation of the sand in each compartment. The two compartments in which larvae and roots were placed were separated from by rigid nylon gauze (mesh 0.77 mm) to prevent the larvae and/or the roots from moving into the next compartment. The whole olfactometer was filled with moist (8% w/w) fine-textured silver sand (particle size 0-0.5mm). The open ends of the olfactometer without plants were closed with PVC lids, the ends of those with plants were sealed with aluminium foil and parafilm put tightly around the plant stems.

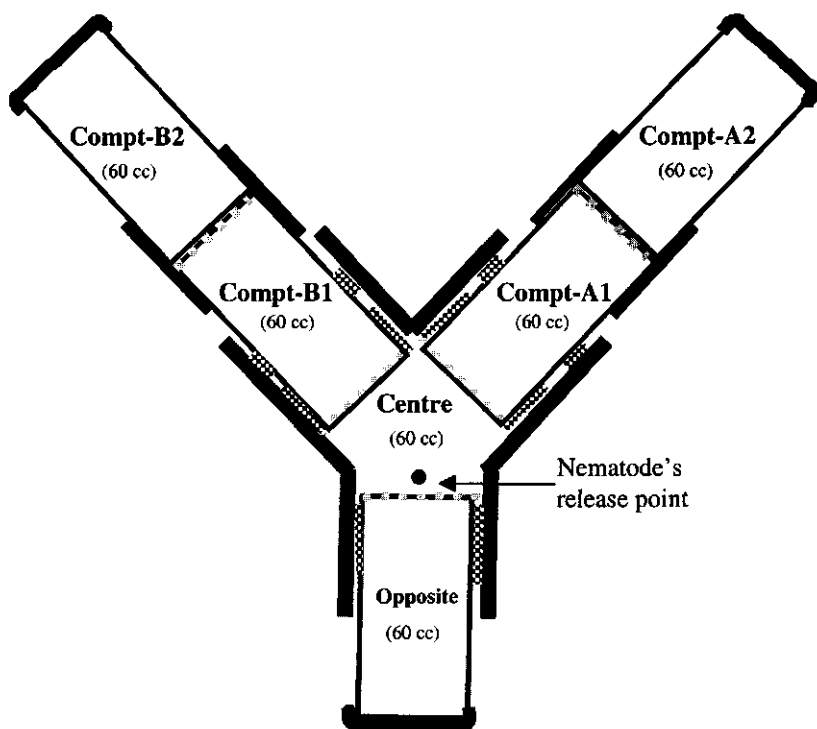


Figure 1. Schematic drawing of Y-tube olfactometer for evaluating effects of plant roots and insect host produced substances on the behavior of entomopathogenic nematodes. (modified from Boff et al., in press).

Bioassays

The behavioural response of IJs to the presence of intact or damaged strawberry and thuja roots either alone or in combination with *O. sulcatus* larvae, was tested by carrying out the following series of choice bioassays.

strawberry plants assays: strawberry plants with intact roots versus only sand (neutral); strawberry plants with intact roots versus *O. sulcatus* larvae; strawberry plants with roots plus *O. sulcatus* larvae versus strawberry plants with intact roots; strawberry plants with roots damaged by *O. sulcatus* larvae versus strawberry plants with mechanically damaged roots.

thuja plants assays: thuja plants with intact roots versus only sand (neutral); thuja plants with intact roots versus *O. sulcatus* larvae; thuja plants with roots plus *O. sulcatus* larvae versus thuja plants with intact roots; thuja plants with roots damaged by *O. sulcatus* larvae versus thuja plants with mechanically damaged roots.

strawberry plants versus thuja plant assays: strawberry plants with intact roots versus thuja plants with intact roots; strawberry plants with roots plus *O. sulcatus* larvae versus thuja plants with roots plus *O. sulcatus* larvae.

Using different batches of nematodes, each bioassay was performed two times. Each assay was composed of four separate but identical Y-tube arenas with the treatment and one control Y-tube arena with both arms filled with moist sand only.

Bioassay procedure

Individual strawberry or thuja plants and/or six larvae of *O. sulcatus* were placed in the arm compartments A2 and/or B2 (Figure 1). The space between the tube walls and the roots and/or larvae was filled with sand (8% w/w). After being completely filled, the assay units were horizontally incubated in a climate chamber (15°C; L:D = 16:8 h) to build up a chemical gradient. Forty-eight hours later, by removing the opposite compartment, a concentration of 1000 (\pm 5%) IJs was applied at the release point (Figure 1). After the nematodes had been released, the opposite compartment was reconnected and the Y-tubes were again incubated under the same conditions for another 24 h. Nematodes from each Y-tube compartment were recovered by using a modified Cobb's decanting and sieving method (Klein Beekmann et al., 1994; Boff et al., in press). The active nematodes from the soil of each compartment of the Y-tubes were counted in a counting dish under a dissecting microscope. The insect larvae used in the assays were transferred to Petri dishes lined with moist filter paper and incubated separately at 20°C for 120 h. After this period of incubation the larval mortality levels was assessed by counting the total number of living and dead (red-brownish colour) larvae. The establishment rate of nematodes was assessed by dissecting the dead larvae and counting the nematodes established.

Making damaged roots.

Insect-feeding damaged roots were obtained by adding six *O. sulcatus* larvae to a compartment with an individual strawberry or thuja plant. Mechanically damaged roots were obtained by removing randomly about 10% of the roots with a pair of scissors before placing the plants in the compartment tube. Both the compartments with plants plus larvae and the compartments with mechanically damaged roots were incubated in a climate chamber (15 °C; L:D = 16:8 h). After 96 hours of incubation, both larvae and plants were removed. The insect-damaged and the mechanically-damaged roots were washed with tap water and placed into new compartment tubes with new moist sand and immediately connected to the Y-tube unit.

From this moment the assays were conducted further following the above-described methodology.

Statistical analyses

For the comparisons between Y-tube compartments within treatments a generalized linear model (GLM) was used with Poisson distribution and a logarithmic link. Analyses were performed using the Genstat (Genstat 5 Committee, 1997) statistical program.

Results

Figure 2 shows the average distribution of infective juveniles over the 20 control Y-tube arenas. A high proportion of nematodes (85%) did not move away from the centre compartment and the other 15% of the nematodes were found evenly distributed in the two Y-tube arms and the opposite compartment, showing that there was no attraction to any of the Y-tube extremities.

The recovery rate of the released nematodes was always over 95% in all of the assays indicating good conditions for the nematodes and a high efficiency of the recovery method utilised.

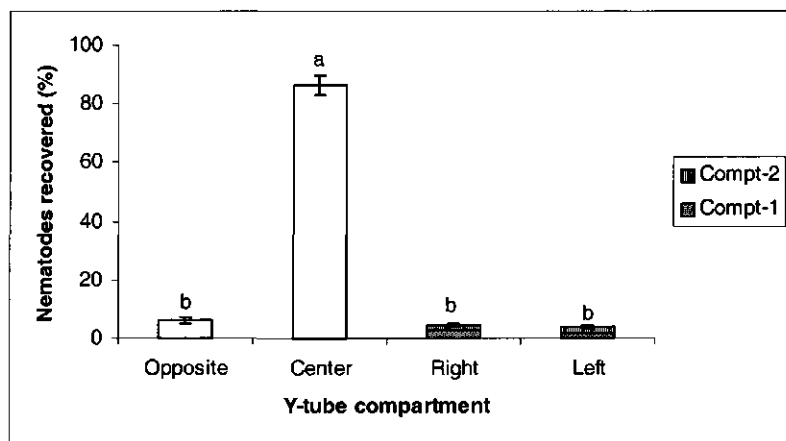


Figure 2. Distribution (mean percentage \pm s.e.) of infective juveniles of *Heterorhabditis megidis* (NLH-E 87.3) in a Y-tube olfactometer filled with only moist sand (control treatment) and incubated at 15 °C. Compt-1 and Compt-2 correspond to the pipe pieces that compose either the right (A) or left (B) Y-tube arms. Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

Strawberry assays

Roots versus only sand

Figure 3A shows that IJs were stimulated to move by the presence of strawberry roots. Only 20% of the nematodes remained in the centre compartment as opposed to 85% in the control Y-tubes (Figure 2). Most of the nematodes (55%) were found in the opposite compartment and apparently repelled by the compounds produced by the roots of strawberry plants. Within the group that responded "positively" in direction to the upper part of the Y-tube, however, significantly more IJs (25%) were recovered from the plant roots arm than from the "neutral" arm filled only with sand. In the arm with roots, most of the IJs were not found in the A2 compartment where the roots were present but in the A1 compartment somewhat removed from the roots.

*Roots versus *O. sulcatus* larvae*

Figure 3B shows that when IJs were confronted with intact strawberry roots versus *O. sulcatus* larvae, they were highly stimulated to move out of the centre compartment. Significantly more nematodes (41%) moved towards the arm with the plant roots than towards the host larvae arm or towards the opposite compartment. As compared with the previous experiment, it is striking that much more nematodes aggregated near the strawberry roots and also less were found in the opposite compartment. Despite the fact that 10 % of the IJs were recovered from the host larvae compartment, no larval mortality was observed.

*Roots plus *O. sulcatus* larvae versus roots*

Figure 3C shows that again most (65%) of the IJs were stimulated to move out of the centre compartment by the presence of roots and larvae. The active IJs clearly preferred the arm with the combination of strawberry roots and *O. sulcatus* larvae to the arm with only roots. As compared to both previous assays, the very low percentage of nematodes found in the opposite compartment is striking. It seems that the combination of larvae and roots and probably feeding activity and damaged to the roots is highly attractive to the nematodes. Surprisingly again no larval mortality was observed despite the large numbers of nematodes present in the vicinity of the vine weevil larvae.

Larvae damaged roots versus mechanically damaged roots

Figure 3D shows that nematodes reacted better to mechanically damaged strawberry than to strawberry roots previously damaged by vine weevil larvae. It shows that the compounds released by mechanically damaged roots are also attractants of entomopathogenic nematodes.

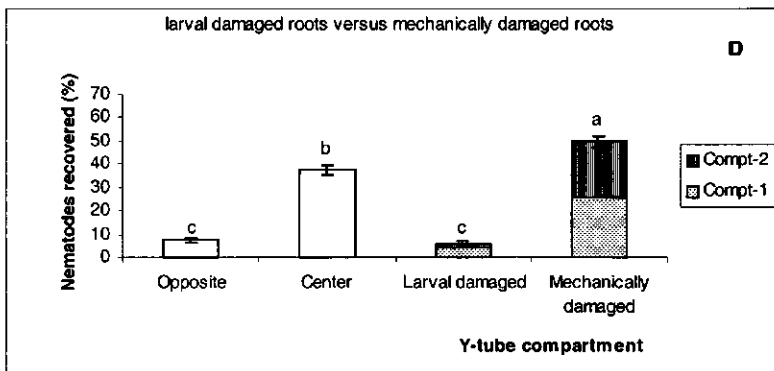
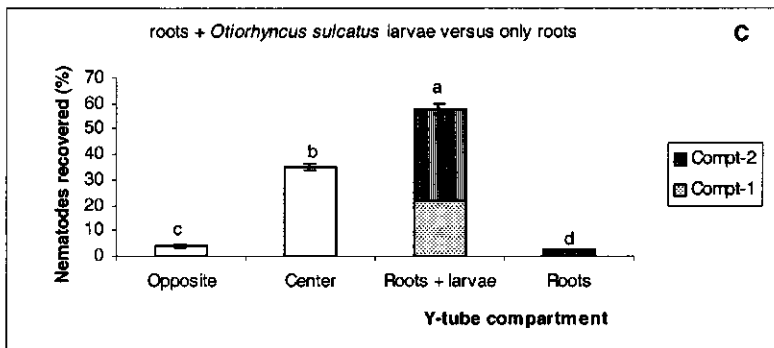
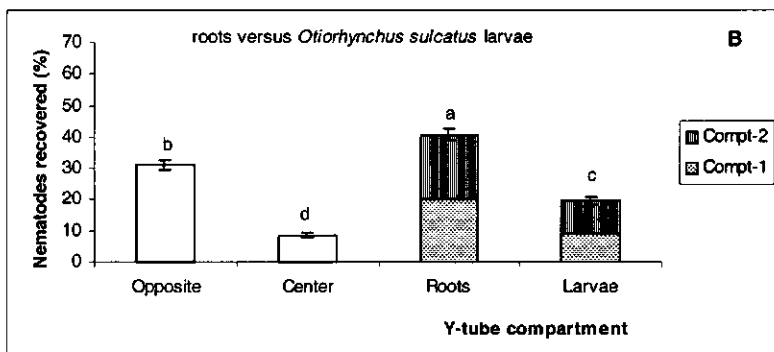
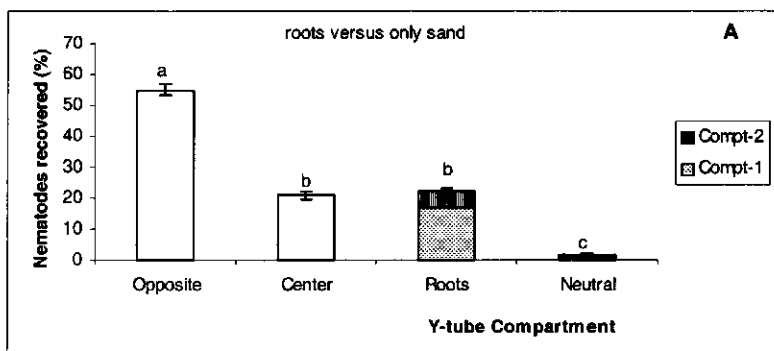


Figure 3. Distribution (mean percentage \pm s.e.) of infective juveniles of *Heterorhabditis megidis* (NLH-E 87.3) in response to cues from strawberry (*Fragaria x ananassa*) roots and/or *Otiorynchus sulcatus* larvae placed in the top compartment (Compt-A2 or B2) of each arm of a Y-tube olfactometer filled with moist sand and incubated horizontally at 15 °C. The terms Compt-1 and Compt-2 correspond to the pipe pieces that compose either the right (A) or left (B) Y-tube arms (see Figure 1). Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

Thuja assays

Roots versus only sand

Figure 4A shows that IJs were stimulated to move by the presence of thuja roots. As was the case in the comparable assay with strawberry plants (Figure 3A), IJs were partly attracted to the roots arm, but most of them moved to the opposite compartment.

Roots versus O. sulcatus larvae

Figure 4B shows that if vine weevil larvae are added to the system, as compared to the previous assay series, the "repellent" effect of the thuja roots disappears. Vine weevil larvae and thuja roots are about equally attractive. But still more than 50% of the inoculated IJs remained or returned to the centre compartment. No mortality of vine weevil larvae was found.

Roots plus O. sulcatus larvae versus roots

Figure 4C shows that if the combination of roots and vine weevil larvae is tested against thuja roots alone, more nematodes are found in the roots and larvae compartment, as was the case with strawberries (Figure 3C). About 80% of the IJs were stimulated to move out of the centre compartment but surprisingly almost 50% moved to the opposite compartment. This is quite different from the results in the comparable strawberry experiment and difficult to explain. Again no larval mortality was observed.

Larvae damaged roots versus mechanically damaged roots

Figure 4D shows that in contrast to the strawberry assays, the IJs were more strongly attracted to the insect-damaged roots than to the mechanically damaged roots. Furthermore most nematodes were found aggregated in the compartment 2 of the arm very close to the damaged roots.

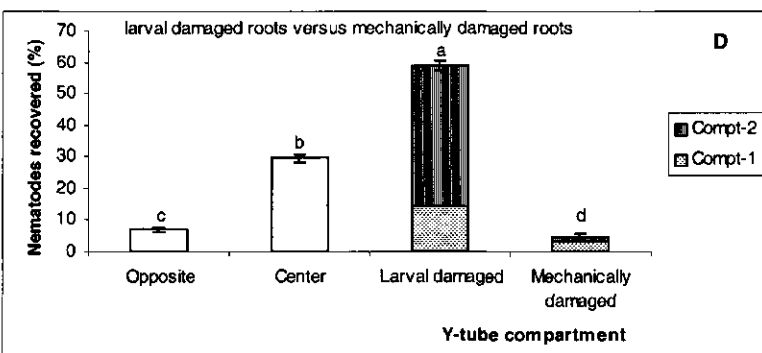
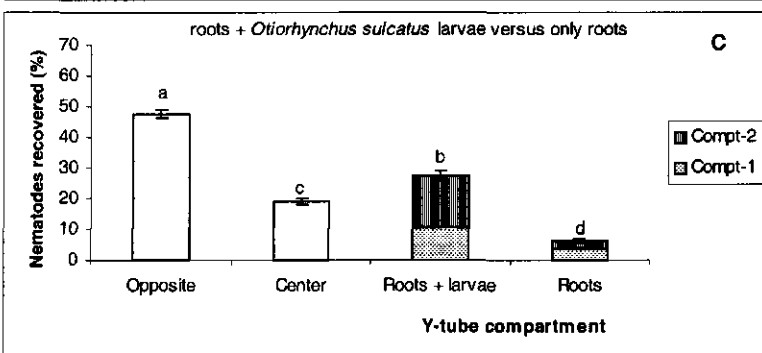
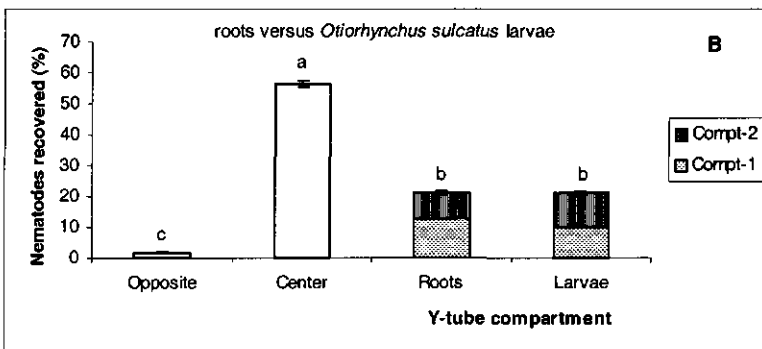
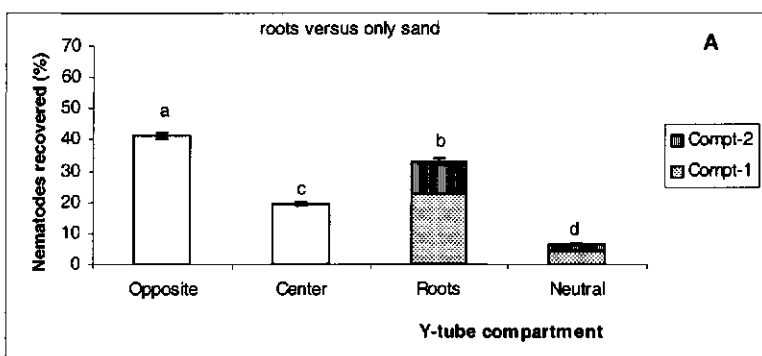


Figure 4. Distribution (mean percentage \pm s.e.) of infective juveniles of *Heterorhabditis megidis* (NLH-E 87.3) in response to cues from thuja (*Thuja occidentalis*) roots and/or *Otiorynchus sulcatus* larvae placed in the top compartment (Compt-A2 or B2) of each arm of a Y- tube olfactometer filled with moist sand and incubated horizontally at 15 °C. The terms Compt-1 and Compt-2 correspond to the pipe pieces that compose either the right (A) or left (B) Y-tube arms (see Figure 1). Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

Strawberry plants versus thuja plants

Strawberry roots versus thuja roots

The IJ distribution pattern presented in Figure 5A shows that more than 50% of the IJs were found in the centre compartment and very few in the opposite compartment, despite the presence of both strawberry and thuja roots. In the assays with the roots of each plant separately, 40–60% of the nematodes were apparently repelled by the roots and found in the opposite compartment (Figure 3A and Figure 4A). It is also surprising that the nematodes show strong preference to the strawberry roots above thuja roots.

Strawberry roots plus O. sulcatus larvae versus thuja roots plus O. sulcatus larvae

Figure 5B shows that nematodes are stimulated to move by the presence of substances produced by the combination of plant roots and larvae. The combination of strawberry roots and *O. sulcatus* larvae attracted more nematodes than the combination of thuja and *O. sulcatus* larvae. In the combination of strawberry and larvae most of the IJs were aggregated in the compartment with the roots and larvae, whereas in the combination of thuja and larvae IJs were evenly distributed. No larval mortality was observed either in the larvae presented with strawberry roots or thuja roots.

Discussion

This study shows that IJs are stimulated to move, probably by the presence of chemical cues from either roots or larvae or combinations of both. Without those stimuli (control treatment, Figure 2), more than 85% of the nematodes remained in the centre compartment. With stimuli present generally only 20–40% of the IJs were found in the centre compartment

24 h after their release. Nematodes of *H. megidis* are always described as a typical cruiser species that move randomly through soil in search for hosts (Grewal et al., 1994). The results of our experiments, however, show a different pattern. It seems that IJs of the tested strain

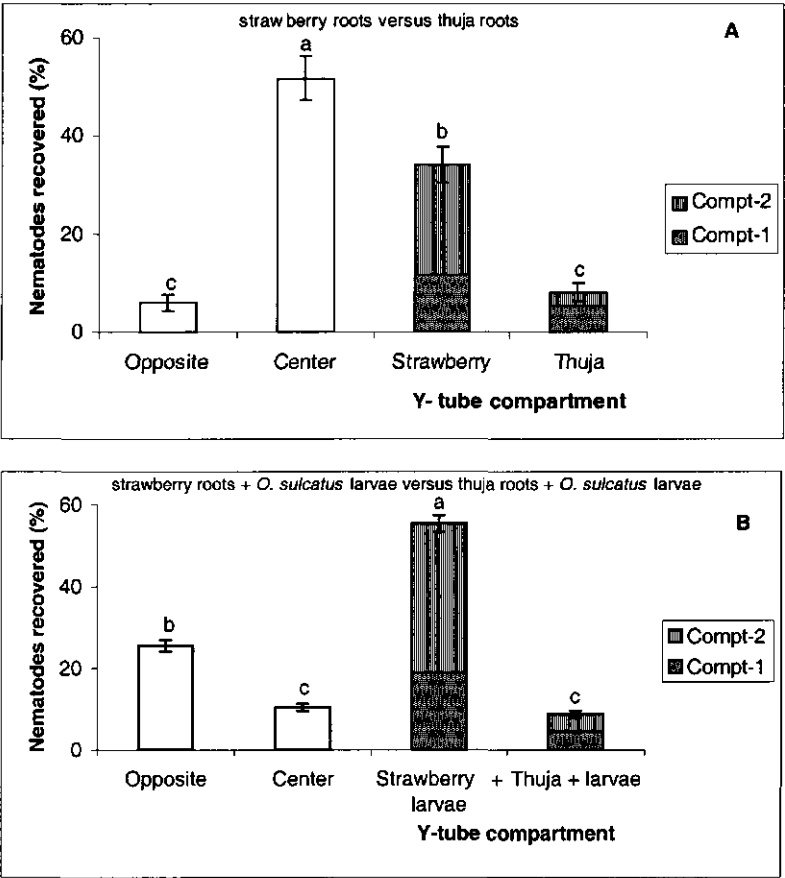


Figure 5. Distribution (mean percentage \pm s.e.) of infective juveniles of *Heterorhabditis megidis* (NLH-E 87.3) in response to cues from intact roots of strawberry or thuja plants (A) or the combination of both strawberry and thuja plants with *Otiiorhynchus sulcatus* larvae (B). The different treatments were placed in the top compartment (Compt-A2 or B2) of each arm of a Y- tube olfactometer filled with moist sand and incubated horizontally at 15 °C. The terms Compt-1 and Compt-2 correspond to the pipe pieces that compose either the right (A) or left (B) Y-tube arms (see Figure 1). Columns followed by the same letter are not significantly different ($P \leq 0.05$). The error bars refer to the whole column.

may sit and wait when no stimuli are present and only become activated when stimuli are detected. Of course if *H. megidis* react to stimuli from a broad range of hosts and plants, as may be the case, in a natural situation there may always be stimuli present and the behaviour may be that of a cruiser species.

Infective juveniles showed a mixed response to intact plant roots. Some IJs were found aggregated near the strawberry or thuja roots, but most of them were found in the opposite compartment, suggesting that compounds produced by the intact plant roots had a repellent effect on IJs. The finding that IJs react to stimuli from intact plant roots by moving to the opposite direction of the roots compartment agree with earlier results in Y-tube tests with strawberry roots (Boff et al., in press). However, in experiments with the same nematode strain and strawberry plants in large Petri dishes filled with sand (Boff et al., submitted), contrasting results were found. In large Petri dishes IJs were positively attracted to the roots of a single strawberry plant but were repelled by the presence of four strawberry plants, suggesting there may be a variable response to differences in stimulus concentrations created by a group of plants as well as influences of the experimental set-up. The negative response to intact plant roots displayed by the tested nematode strain is might be induced by the secretion of some specific root compounds as observed by Castro et al. (1989) and Diez and Dusenbery (1989) for plant parasitic nematodes.

When challenged with both *O. sulcatus* larvae and intact roots of strawberry, IJs were more activated to move than when challenged with intact roots of thuja plants and *O. sulcatus* larvae. In the assays with strawberry roots versus larvae the IJs responded by moving preferentially to the plant root arm whereas, in the assays with thuja roots versus larvae, the nematodes that moved out of the centre did not show a defined preference. An earlier study had already shown that vine weevil larvae alone did stimulate nematodes to move but that no preferential aggregation near the larvae occurred (Boff et al., in press). Compared with the response displayed in the control treatments it is clear that nematodes react to the presence of vine weevil larvae, but in a choice experiment they are not able to locate the larvae or discriminate them from plant roots.

Roots damaged either by feeding activities of vine weevil larvae or by a pair of scissors are highly attractive for *H. megidis* IJs. Results from assays with thuja plants suggested that roots damaged by larval feeding are more attractive than mechanically damaged roots and possibly plant signalling by insect damaged roots plays a role in attraction (van Tol et al., submitted). Our series of experiments with strawberry plants (Figure 3D), however, did not show the same results as with thuja plants. The fact that mechanically damaged roots of strawberry plants were more attractive suggests that general wound-related molecules might be responsible for the attraction of

nematodes. The different results between the strawberry and thuja might be related to differences in quantitative and qualitative release of stimuli. The results of van Tol et al. (submitted) though strongly suggest the involvement of synomone production by thuja in relation to larval damage, which is obviously not produced by strawberry when pre-damaged by larval feeding. It is possible that strawberry did not yet developed such a mechanism of SOS signal production in relation to *O. sulcatus* attacks simply because it never was a primary host plant for this flightless insect in history, but recently introduced by mankind in strawberry fields. This hypothesis is supported by several reports showing that strawberry is not a suitable food plant for the vine weevil. Smith (1932) reports no preference for strawberry and lower oviposition rates. Further, high mortality of larvae and adult weevils feeding on strawberry (Smith, 1932; Evenhuis, 1978; Van Tol, unpublished) and absence of olfactory attraction to strawberry leaves (Van Tol et al., 2000) are in strong contrast with the oviposition, survival and olfactory attraction to several other host plants tested. This may in part explain the observed differences between the tested plant species. However, more biological assays combined with chemical analysis are needed to elucidate the nature of chemical compounds produced by the different plant species and their insect pests that are involved in the mechanism of attraction of entomopathogenic nematodes.

We did not yet analyse the nature of the stimuli produced by roots, larvae or mechanically damaged roots. They are likely to be volatile molecules rather than non-volatile molecules as is the case for stimuli that plant-parasitic nematodes use for host plant location (Bargmann and Morri, 1997). A likely candidate is of course CO₂, a general kairomone produced as an end product of metabolism by plants, microorganisms, and other soil animals. CO₂ has been shown to be involved in the long-distance attraction of plant-parasitic nematodes (Klinger, 1965; Robinson, 1995) and it also attracts entomopathogenic nematodes (Gaugler et al., 1980; Lewis et al., 1993; Robinson, 1995). In addition to CO₂ several others gradients exist around physiologically active roots, such as various ions, sugars, amino acids, other organic acids, phenolic compounds and chelating agents (Perry and Aumann, 1998). Damage to roots may also cause elevated root temperatures as well as the production of CO₂ and chemical compounds that play a role in the defense mechanism of plants (Rhodes and Woollorton, 1978; Uritani and Ōba, 1978).

It is known that damage caused by insects on the aerial part of the plants can induce the production of secondary metabolites that attract enemies of insect pests (McCall et al., 1993;

Dicke, 1999). If these phenomena happen above ground it is not unlikely that this may also occur in the soil where the plant roots are attacked by many insect species. Our results and those of van Tol et al. (submitted) show that insect damaged plant parts in the soil might also emit infochemicals that attract enemies of the insect pests. It would make sense for plants to attract entomopathogenic nematodes to help them in their defense against insects. Long-range orientation to plant roots where insects are feeding, followed by close range orientation to insect cues increase the chance for nematodes to find their host insects in the soil. In addition a directional response to signals emitted by pre-damaged roots and by feeding larvae would be advantageous for the nematodes because by being near to the roots they can easily be ingested. Getting in with the food, IJs have only to penetrate the relatively soft and thin gut wall to get into the haemocoel instead of to encountering other entry barriers.

Many of the soil insects protect themselves from entomopathogenic nematode attack by excreting CO₂ discontinuously (Gaugler, 1988); protecting the natural openings with sieve plates and bristles; having a tough epidermis (Eidt and Thurston, 1995); brushing the IJs with the legs; rubbing the body with the abrasive raster (Gaugler et al., 1994) or by physiological mechanisms like encapsulation (Peters and Ehlers, 1997). We do not know exactly if the lack of infection of the host insects observed in this study was caused by a kind of protection or avoidance strategy or if it was a matter of exposure time. Infective juveniles were submitted to a choice between an array of odors and they might get confused and have spent time in moving around remaining not much time for the infection process.

From our studies and those of others a general behavioral model for the entomopathogenic nematode *H. megidis* starts to become visible. The demonstration that: IJs are highly stimulated by the presence of roots; IJs are strongly attracted to the combination of roots and insect larvae (in feeding activity); IJs are able to make a distinction between insect or mechanically damaged roots; and that IJs are able to make a distinction between root of different plants species, imply that a sophisticated tritrophic interaction among plant roots, insect pests and entomopathogenic nematodes does occur also in the soil systems. This interaction might involve a series of specific semiochemicals, which might include volatiles or diffusible substances. In order to elucidate the supposed compounds involved in this interaction and their exact effect on the host-finding ability of entomopathogenic nematodes, further specific studies are therefore necessary.

Chapter 10

General Discussion

General discussion

The studies presented in this thesis were conducted to elucidate ecological factors that affect the performance of a Dutch *Heterorhabditis megidis* strain (NLH-E87.3). This nematode strain was recovered in 1987 from larvae of *Phyllopertha horticola*, damaging grass in sports fields in the Eindhoven region. Very little information on its biology and ecology are available but this nematode strain may have potential for biological control of many insect pests and may perform better than some well-studied nematode species.

Biological control is applied to reduce populations of pest organisms by the action of parasites, predators, pathogens, antagonists or competitive organisms. Today it stands as a cornerstone of integrated pest management (IPM) and is the foremost alternative to the use of chemical pesticides (Gaugler *et al.*, 1997). Biological control offers a tremendous opportunity to supply agriculture with effective tools for the development of production techniques which minimise impacts on human health and the environment (Ehlers, 1996).

Although the use of entomopathogenic nematodes as biocontrol agents has expanded over the past decades, it is still mainly restricted to high value crops that cover a small proportion of the world agriculture. Besides economic factors the restricted expansion of the use of entomopathogenic nematodes as biocontrol agents is due to various intrinsic biotic and abiotic related factors. As the interest in the use of indigenous or non-indigenous nematodes is increasing, knowledge on the ecology and the specific environmental requirements of each specific species or strain of nematodes that we want to use as a biocontrol agent becomes crucial. Though much research has been done and much progress has been made, especially in the last decade, the ecology and behaviour of infective juveniles (IJs) of *Heterorhabditis megidis* in its natural environment remains obscure.

Working with the hypothesis that increasing the population density of infective juveniles within an insect host would adversely effect the fitness of nematodes, bioassays were conducted to test different doses of nematodes against *G. mellonella* larvae. The results show that, as with many other species and strains of entomopathogenic nematodes, the infectivity and reproduction of the strain NLH-E87.3 is highly influenced by the inoculum size. Although the number of invading IJs increased with the inoculum size, the proportional percentage of invasion declined. Contrary to the results of Koppenhöfer & Kaya (1995) and Zervos *et al.* (1991) we observed that the infective juveniles were able to establish and reproduce at all inoculum sizes. The tested nematode strain showed to have optimal reproduction at a dose of 300 IJs per host, whereas a dose

of 3000 IJs per host showed the lowest reproduction. Looking at the results on progeny production per IJ we observed that the lower the establishment of IJ's, the higher the reproduction per individual IJ is. The observation that progeny production is lower at high infection densities is in concordance with studies of Sandner & Stanuzek (1971); Zervos *et al.* (1991) Selvan *et al.*, (1993a) and Koppenhöfer & Kaya (1995). The longest nematodes were produced at densities that produced most progeny i.e., when larvae were exposed to a dose of 300 IJs. These results contrast with those of Selvan *et al.*, (1993a) who found that the nematode size increased with decreasing nematode density. The unclear correlation observed between inoculum size and length of progeny suggests that a complexity of interactions take place inside the host cadaver. Competition for food inside the host cadaver is pointed out as one of the most important factors that strongly influences IJs size, time to emergence, and the progeny production (Craig & Webster 1982). The earlier emergence of IJs from cadavers exposed to high inoculum densities shows that a competition for nutrients inside the host occurs also in the strain NLH-E87.3. In general we can conclude that optimal nematode reproduction varies among genera, species and nematode strains and optimal reproduction is most likely to occur when an insect host is invaded by a small rather than a large number of IJs.

Temperature is an important environmental factor to consider when examining the use of entomopathogenic nematodes as biocontrol agents because it affects their mobility, survival, development and reproduction. Besides the influence on nematode performance in the field, temperature also plays an important role for the nematode during mass production and storage. The performance of a nematode in a test at a defined temperature will almost certainly depend on its thermal history prior to the test. Moreover, variation between species and strains is to be expected. Even closely related species of nematodes from similar habitats may show considerable variation in response to temperature (Griffin, 1993; Griffin 1996). Studies on the effect of storage temperature over time showed that IJs of *H. megidis* strain NLH-E87.3 performed better when stored at a temperature of 10 or 15 °C, than when stored at 5 or 20°C over a period of 10 weeks. Infective juveniles stored at 5 and 20°C showed significantly decrease in size over time. According to Fitters & Griffin (1996) the decrease in size of *Heterorhabditis* species during the storage period is explained by the rapid utilisation of energy reserves. The survival of IJs stored at 5 and 20 °C showed a typical S-curve. After 4 weeks of storage the numbers of IJs alive started to decrease and after 10 weeks near 50% of the IJs stored at 20 °C were dead. The differences in persistence of IJs of strain NLH-E87.3 at various temperatures reflect, in part, a preference for the temperature range in its original habitat.

When stored at a temperature of 10 or 15 °C and tested at 20 °C, IJs were able to invade and kill the insect host over the entire storage period causing 35 to 100% of host mortality. However,

in nematodes stored at 5 or 20 °C the infectivity ability and host mortality dropped to lower and even to zero levels. Contrary to the results of Jung (1996) who reports that the infectivity of some *Heterorhabditis* species stored at a high temperature (20 °C) increased with the storage time, we observed that IJs stored at 20°C lose their infectivity capacity in few weeks. Normally at low storage temperature the lipid utilisation and other ageing process proceed more slowly, whereas at higher temperatures, the metabolic rate increases and a more rapid utilisation of stored food reserves contributes to a reduction in energy (Jung, 1991; Selvan *et al.*, 1993c; Lewis *et al.*, 1995b).

The best yield of nematodes per insect cadaver was obtained at a dose of 30 IJs/host, whereas the best yield per IJs established was obtained from cadavers exposed to a dose of one IJ. Mason & Hominick (1995) also noted a similar trend in reproduction for both *H. megidis* strains from the UK and the Netherlands. Independent of the storage conditions the reproduction per IJ established was low and the period to first emergence was shorter when larvae were exposed to a dose of 30 IJ. This is a clear example that nematode reproduction is affected by a competition for nutrients or space inside a host cadaver rather than by the temperature and/or storage time.

The effectiveness and development of *H. megidis*, strain NLH-E87.3 changed not only in function of the host species and its larval size but also with the inoculum size. When exposed to a dose of 30 IJs each larval size of *G. mellonella* resulted in maximum mortality, whereas when exposed to one IJ small larvae were more susceptible than medium and large sized larvae. Large *O. sulcatus* larvae also showed to be far more resistant than small and medium larvae, at a dose of one IJ. Our results are supported by those of Kaya and Hara (1981), LeBeck *et al.*, (1993) and Shapiro *et al.*, (1999) who reported that the infectivity of IJs differed among developmental stages of a host insect. One of the reasons that can partly explain why the nematode establishment decreased in larger larvae exposed to a single IJ is that larger and older larvae have a thicker and harder body integument. On the other hand, a higher attractivity of large larvae could be expected because of a higher production host-associated cues e.g. CO₂, host faeces or cuticle excretory products (Lewis *et al.*, 1993, Schmidt & All, 1979).

Besides all differences caused by larval size and IJs dose, it was observed that larvae of *O. sulcatus* were more resistant to the tested nematode strain than *G. mellonella* larvae. There are many reasons that can explain the difference in susceptibility between the two host species used. One is that in general soil-inhabiting insects are more resistant than those living above ground (Kaya & Hara 1981). Under natural conditions larvae of *O. sulcatus* are found feeding near or between the roots of plants creating in this way a favorable situation for nematodes infection. In the bioassays no food was offered to the larvae, reducing in this way the possibility of IJs being ingested by the feeding activity. Insect larvae could also actively move to various parts of the

experimental set-up and possibly "escape or avoid" encountering IJs. Differences in susceptibility could also be due to the fact that *G. mellonella* larvae were reared on an artificial diet and *O. sulcatus* on *Astilbe chinensis* plants. Barbercheck, 1993, Barbercheck *et al.*, 1995 and Jaworska & Ropek, 1994 reported the effect of host plant on nematode-induced mortality and nematode progeny production in *Diabrotica undecimpunctata howardi* and *Sitona lineatus* respectively. However, until now there are no references reporting possible effects of *A. chinensis* chemical compounds (secondary metabolites) on the biology of insect pests in terms of conferring protection against their natural enemies.

Nematodes reproduced in all sizes of *G. mellonella* and *O. sulcatus* except in case of small larvae of *O. sulcatus* exposed to a dose of one IJ. The total amount of IJs produced per larva, per mg of host body and per established IJ in both insect hosts was highly influenced by the larval body size and the insect species.

For both host insects, it was observed that the larger the host and the lower the initial dose, the longer the nematodes remain inside the cadaver and consequently the larger their size. Our results are again supported by Selvan *et al.* (1993a) and Craig and Webster (1982) who reported that density and host size directly influence the body size of the nematode progeny.

An overcrowding effect was clearly observed for *O. sulcatus*. Large larvae attracted more IJs and consequently the time to first emergence was shorter than when the larvae were invaded only by one IJ. However, in *G. mellonella* the period of incubation was influenced much more by the larval size than by the number of invading IJs. Despite the differences between host insects and their physiology we observed that time to first emergence of IJs is strongly influenced by depletion of food reserves and/or by intraspecific competition within the host cadaver.

The differences in effectiveness of IJs originating from different sized hosts of two different species answers the question whether the host size has influence on the performance of IJs. Infective juveniles originating from small *G. mellonella* larvae showed to be more invasive and caused the highest level of mortality at both tested doses against all different sizes of *G. mellonella* and *O. sulcatus* larvae. In *O. sulcatus* the effect of cadaver size was also observed. Infective juveniles from medium sized larvae showed to be more pathogenic against all sizes of *G. mellonella*, whereas IJs originating from large *O. sulcatus* larvae were more invasive and pathogenic against *O. sulcatus* than those originating from medium larvae. The fact that the most infective IJs were originating from small cadavers, and consequently were small in size and had a shorter incubation period than IJs developing in medium and larger cadavers suggests a response to nutrient stress. Under low nutrition conditions the life cycle is shorter and nematodes go to the IJs stage earlier (Strauch *et al.*, 1994). One plausible explanation for the differences in infectivity might be related to the energy contents and physiological mechanisms that may trigger the host-

finding and infectivity ability in shorter and early emerging IJs. Small and fast developed IJs are likely to have less energy reserves and may search faster for a host, whereas those of medium or large size are likely to be more fat and may persist and wait longer before infecting a host. The possibility that the size of the hosts generates a diversification on infective strategies of IJs is very important for the dynamics of host infection process in the soil.

Dispersion of entomopathogenic nematodes can be active or passive (Kaya, 1990a). Active dispersal depends on the nematodes themselves and includes movement away from the host cadaver to sheltered microenvironments or to preferred soil depths, increasing the chances for encountering a susceptible host as well as for survival (Kaya & Gaugler, 1993; Ishibashi & Kondo, 1990). Some nematode species search actively to find their host and are classified as cruisers, whereas others tend to remain stationary adopting an ambush strategy to attack hosts. Assays on a 2-dimensional substrate showed that IJs of the strain NLH-E87.3 adopt a cruiser foraging strategy. All IJs moved away from the release point by sinuous movements in permanent contact with the agar surface. With the increase of IJs density a kind of "mutual dispersal help" or "stimulation by contact" could clearly be observed. The IJs movement became faster and IJs went further when others IJs touched them.

Lewis *et al.* (1997) observed that IJs of *Steinernema carpocapsae* lowered down their nictation rate and became more mobile with age. In our study no change in the searching strategy was observed with the increase of IJs age, but the mobility patterns displayed a U-shaped curve, being high first, then decrease, and show an increase at old age. Infective juveniles stored during a period of 2.5 and 3.5 weeks showed less activity than those stored for 1.5 and 4.5 weeks. Similarly to our results O'Leary *et al.* (1998) observed that IJs of *H. megidis* (strain UKH 211) dispersed well and their host-finding ability improved with increased duration of storage. The results on IJs dispersion show a similar pattern to the infectivity patterns.

In absence of a host IJs moved randomly. However, when an insect host was offered the dispersal patterns changed and a response to host cues was observed. Nematodes showed a positive directional response to *G. mellonella* but not *O. sulcatus* larvae. According to Gaugler *et al.* (1991) a last instar *G. mellonella* larva produces more CO₂ per hour than last instar larvae of Coleoptera species as *Leptinotarsa decemlineata* and *Popillia japonica* and consequently attracts more IJs. Thurston *et al.* (1994) observed that IJs of *Steinernema carpocapsae* were attracted to CO₂ and faeces produced by *G. mellonella* larvae but were repelled by *L. decemlineata* faeces. Probably, besides the differences in CO₂ production, other excretory products released by *G. mellonella* and *O. sulcatus* also have influence on the behaviour of IJs.

The majority of studies on the foraging behaviour of entomopathogenic nematodes has been conducted in non-soil systems, and often these studies have investigated only one variable was

studied at a time. Consequently, such studies do not reflect the nematodes true behaviour in nature, in which they are exposed to a myriad of conflicting chemical signals. The two 3-dimensional matrix, a large Petri dish and a Y-tube olfactometer filled with moist sand, used to conduct assays in the present study, provide a meaningful tool for studying the performance of entomopathogenic nematodes in a more natural environment. In both arenas IJs showed to move randomly without cues, but directional responses were always observed when cue sources were presented.

Plant roots liberate various substances, such as carbon dioxide and amino acids, which form chemical gradients around the root system. These substances in turn modify the quantitative and qualitative microbial composition of the rhizosphere (Croll, 1970). However, the complexity of the constitution of various attractants that are required to initiate a behavioural response in entomopathogenic nematodes such as the cruiser species hinders our understanding of the role that these chemicals play in biological processes. It seems improbable that only one stimulus is predominant, but host finding by IJs is likely the result of a sequence of host stimuli that influence and steer searching behaviour. Furthermore, there is a persistent belief that the attraction of infective juveniles of entomopathogenic nematodes to plant roots may aid the nematodes to locate their insect host, which also tends to accumulate around the roots upon which they feed (Kanagy & Kaya, 1996).

When a foraging nematode is confronted with an array of signals its response may depend on the strength and exposure time as well as on the nature of the stimuli (Hui & Webster, 2000). Besides the factors former mentioned we observed that the experimental set-up also influenced the IJs searching behaviour. In large Petri dishes IJs were attracted to the roots of one strawberry plant but when four plants were presented IJs moved away from the roots or the combination of roots and host larvae to the edges of the arena. In the Y-tube arenas the IJs were observed to be stimulated to move by the presence of roots of a single strawberry and thuja plant but most of them moved away to the opposite arm of the olfactometer. Overall these results suggests that signals emitted from plant roots strongly influenced nematode searching behaviour and that an infective juvenile may either be attracted or repelled by the gradients emanating from the roots exudates. Furthermore, the chemoresponse of foraging nematodes depends on the threshold of the response to host and non-host sources of stimuli, and any modification of these sources modifies their host searching and infection success.

Damage caused by pest insects on the above ground parts of plants induce production of secondary metabolites favoring plant defense mechanisms like attraction predators or parasites of insect pests (Dicke, 1999; McCall *et al.*, 1993). The same phenomenon may also occur in the soil were the plant roots are attacked by many insect species. The hypothesis that plant roots, which

are under insect attack, would be able to produce compounds that attract the infective juveniles to their host was supported by my results. In Y-tube experiments IJs moved preferentially to the arm with roots under attack, not only when it was offered against only sand, but also when nematodes could choose between roots under attack and undamaged roots.

The demonstration that IJs are able to distinguish between plant roots that have been damaged by a natural insect hosts and roots that have been mechanically damaged, is evidence that an intricate tritrophic chemical interaction among plant roots, insect pests and entomopathogenic nematodes occurs in the soil systems. This interaction is complex and might involve a series of specific allelochemicals, which include volatiles or diffusable substances. A logical next step in the research on the behavior of entomopathogenic nematodes is to elucidate the semiochemicals involved in this interaction and their exact effect on the host-finding ability of entomopathogenic nematodes.

The present study has answered important questions about factors that affect the persistence, infectivity and reproduction of *H. megidis*. Also it provided essential insight in the host finding behaviour of entomopathogenic nematodes. Work described in this thesis further has practical implications for biocontrol with entomopathogenic nematodes in general and *H. megidis* in particular. We know now that the quality of IJs of the strain NLH-E87.3 depends on the host size, host species and nematode densities inside a host insect and that some times small nematodes from small hosts are more infective then larger ones. The optimum storage temperature for the tested nematode strain is between 10 and 15°C, and that is higher than the storage temperature of 5 °C that is generally recommended. Studies on host searching behaviour showed differences in reaction to specific signals from plant species that open possibilities for nematode strain selection. Nematodes react positively to the combination of insect larvae and damaged roots and mostly negatively to intact roots which suggests that curative treatments may be more effective than preventive applications.

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Summary

Entomopathogenic nematodes in the families Heterorhabditidae and Steinernematidae have considerable potential as biological control agents of soil-inhabiting insect pests. Attributes making these nematodes ideal biological control agents include their broad host range, high virulence, safety to non target organisms, ability to search for hosts, high efficacy in favourable habitats, high reproductive potential, ease of mass production, ease of application, and compatibility with other control strategies. In **chapter 1**, I give a short review on biology and some of the important biotic and abiotic factors that affect the infectivity and dispersal ability of entomopathogenic nematodes, state the research aims and present the outline of the thesis.

Density-dependent factors within a host can have an important influence on the population dynamics of entomopathogenic nematodes. In **chapter 2** the effects of increasing *Heterorhabditis megidis* (strain NLH-E87.3) density in *Galleria mellonella* larvae were compared. Although the number of nematodes that established in the host increased with increasing dose, the percentage of invasion decreased. The number of progeny produced per host initially increased with dose, but the highest production of infective juveniles (IJs) per cadaver was reached at a dose of 300 IJs per host, when about 62 IJs were established per cadaver. The smallest infective juveniles were produced at a dose of 1000 IJs per host and the largest at a dose of 300 IJs per host. Time to first emergence of juveniles was generally shorter when the number of IJs inoculated was large. Effects of high density appear to result from competition for limited nutrients within the host. For the success of entomopathogenic nematodes in the field, the knowledge on density effects of each nematode species or strain should be taken into account.

Between production and application, the infective juveniles are exposed to environmental stress, especially during storage and shipment. Among the factors hampering the performance of entomopathogenic nematodes, as biocontrol agents are time and the temperature conditions of storage. In **chapter 3** the effect of storage conditions, temperature and time, on the survival, infectivity and development of IJs of *H. megidis* was investigated. Infective juveniles were stored at 5, 10, 15 and 20°C for a period of up to 70 days (10 weeks). Infectivity and reproduction after each storage time and temperature were measured in bioassays with *G. mellonella* larvae exposed to a dose of one or 30 nematodes. The results show that independent of the time of storage, IJs performed best when stored at a temperature of 10 or 15°C. An increase of the storage time caused a decrease of "quality" of the

nematodes stored at 5 and 20°C. The low storage temperature induced most of the nematodes into a state in which they lose their ability to parasitise a host and the temperature of 20°C directly affected the nematode survival. Time to first emergence was affected more by the inoculum size than by the storage conditions. Infective juveniles emerged earlier from cadavers exposed to a dose of 30 nematodes than from those infected by only one nematode. This result shows that the reproduction time inside a host is more tightly regulated by density-dependent constraints than by the storage conditions of the infective juveniles.

Entomopathogenic nematodes are able to invade and, in most the cases, to kill a large number of insect species. However, a close relationship between the nematode and the host is generally observed and this suggests a particular susceptibility of the insect, variable also within its different developmental stages, and an intrinsic virulence of the nematode species or strain. In **chapter 4** I report the results of investigations on the ability of IJs of *H. megidis* to cause infection and reproduce in differently sized larvae of *G. mellonella* and *Otiiorhynchus sulcatus*. Larvae of both insect hosts were weighed, divided in groups of small, medium and large, and exposed to a dose of one or 30 IJs. The number of invading IJs increased with host size while the host mortality at a dose of one IJ decreased with the increase of host size. However, IJs showed to be able to invade and kill each size group of larvae of both insect hosts tested. At a dose of 30 IJs, larvae of *G. mellonella* show to be significantly more susceptible than *O. sulcatus* larvae, whereas at a dose of one IJ, *O. sulcatus* larvae were more susceptible. In general, time to first emergence was longer at the lowest IJs inoculum and increased with the increase of host size in both insect species. The production of progeny differed between host species, host sizes and doses of nematodes. *G. mellonella* larvae produced more nematodes than *O. sulcatus* when the production from larvae of the same size was compared. The total progeny production per larva increased with the increasing larval size but no progeny production was observed in small larvae of *O. sulcatus* exposed to a dose of one IJ. Generally, the IJs body size increased with an increasing host size and the longest infective juveniles were produced at the lowest IJ doses.

Using IJs of *H. megidis* originating from small, medium or large larvae of *G. mellonella*, and from medium or large larvae of *O. sulcatus*, previously exposed to a different IJs dose, I attempted to answer the question as to whether infective juveniles obtained from the different larval size of an insect host are capable of invading and killing larvae of the same host where they originate from and/or a new host. A series of infectivity tests were done (**chapter 5**). Independent of the size of the larvae from which IJs were originating they were capable of infecting larvae of all sizes of its own host and also larvae of a new host. In general IJs

originating from small cadavers of both host insects showed to be more infective than those originating from the medium and large cadavers. When tested at a dose of one IJ per larva, IJs originating from medium *O. sulcatus* cadavers were more infective against *G. mellonella* than against *O. sulcatus* larvae. Large *G. mellonella* larvae were in general less susceptible to all IJ batches than medium and small larvae.

In **chapter 6** an agar-based assay was used to assess the effect of nematode density, nematode age, incubation time and the presence of insect hosts on the dispersal behaviour of IJs of *H. megidis*. Infective juveniles dispersed faster and further at high densities than at low densities. Dispersal was also influenced by the age of the IJs. Nematodes stored for a period of 1.5 and 4.5 weeks showed to be more active than those stored for 2.5 and 3.5 weeks. The presence of a host insect enhanced the dispersion of nematodes. After 90 minutes IJs had responded positively to cues from *G. mellonella* but poorly to cues from *O. sulcatus* larvae.

In **chapter 7** the host-finding and dispersion behaviour of *H. megidis* in the presence of *G. mellonella* or *O. sulcatus* larvae and strawberry (*Fragaria x ananassa* Duch.) roots alone, in the presence of, or under attack by *O. sulcatus* larvae was studied. Bioassays were conducted in Petri dishes (19 cm diameter) filled with moist sand and incubated at 15°C over 24 hours. Infective juveniles responded positively to the presence of *G. mellonella* larvae, to roots of a single strawberry plant, and to *O. sulcatus* larvae in direct contact with roots of a single strawberry plant. A neutral or negative response was observed when infective juveniles were presented with only *O. sulcatus* larvae or a combination of several strawberry plants with *O. sulcatus* larvae, either in contact or not in contact with the roots. Infective juveniles responded strongly to the combination of plant roots and feeding larvae indicating that the tritrophic interaction formed by infective juveniles, *O. sulcatus* larvae, and strawberry plants may be an infochemical-mediated interaction.

In **chapter 8 and 9** a newly developed Y-tube olfactometer filled with sand and incubated at 15 °C was used to test the host-searching behaviour of *H. megidis*. Within an incubation period of 24 hours, IJs were significantly attracted to living *G. mellonella* larvae and caused 100% larval mortality. *O. sulcatus* larvae, however, did not elicit host-oriented movement of IJs and no larval mortality was observed. Roots of strawberry plants induced IJs movement but caused IJs to move away from the plant roots. The combination of strawberry roots and *O. sulcatus* larvae, however, strongly attracted IJs leading to 37% host mortality (**chapter 8**). The results in **chapter 9** showed that IJs were activated by the presence of intact roots of both strawberry (*Fragaria x ananassa* Duch.) and thuja (*Thuja occidentalis* L.). Some nematodes aggregated in the compartments with roots but most moved away from the roots to the

opposite side. Given a choice, IJs showed preference for strawberry roots alone above *O. sulcatus* larvae. No difference in preference was observed between thuja roots and *O. sulcatus* larvae. The combination of strawberry roots with *O. sulcatus* larvae was preferred above strawberry roots alone. In the assays with the combination of thuja roots plus *O. sulcatus* larvae versus thuja roots alone, IJs were stimulated to move but away from both roots plus larvae or only roots arm. When challenged with insect damaged roots and mechanically damaged roots IJs were most attracted by thuja roots damaged by larvae, whereas in the case of strawberry IJs showed a clear preference for the mechanically damaged roots above insect damaged roots. A preference for strawberry roots, alone or in combination with *O. sulcatus* larvae, over thuja roots, in the same condition was always observed. It was also shown that the Y-tube choice arena used to perform the assays is a useful tool in studying the searching behaviour of entomopathogenic nematodes in a semi-natural habitat.

In **chapter 10** the most important research findings and the contribution of the results to the existing knowledge and the supposed applicability of the findings are discussed.

Samenvatting

Insectenparasitaire nematoden in de families Heterorhabditidae en Steinernematidae zijn mogelijk goed te gebruiken als biologisch bestrijdingsmiddel tegen bodeminsecten. Eigenschappen die hen erg geschikt maken zijn onder andere brede waardinsect reeks, hoge virulentie, veiligheid ten opzichte van niet-doelwit organismen, heb vermogen om insecten in de bodem actief op te zoeken, hoge efficiëntie in een goede leefomgeving, hun hoge reproductiesnelheid, makkelijk te vermeerderen in massaproductie, makkelijk toe te passen en compatibiliteit met andere bestrijdingsstrategieën. In **hoofdstuk 1**, geef ik een kort overzicht van de biologie en enkele van de belangrijke biotische en abiotische factoren die een rol spelen bij de infectiviteit en verspreiding van insectenparasitaire nematoden, ik beschrijf het doel van het onderzoek en presenteer de hoofdlijnen van het proefschrift.

Dichtheidafhankelijke factoren in een gastheer insect kunnen een belangrijke invloed hebben op de populatiedynamica van insectenparasitaire nematoden. In **hoofdstuk 2** wordt het effect van een toenemende dichtheid van *Heterorhabditis megidis* (stam NLH-E87.3) in *Galleria mellonella* larven onderzocht. Hoewel het aantal nematoden dat zich in een insect vestigt toeneemt met een toenemende dosis, neemt het percentage nematoden dat binnendringt af. Het aantal nakomelingen per insect neemt in het begin toe met de dosis, maar de hoogste productie per infectieve nematodelarve (infectieve juvenielen = IJs) per kadaver werd bereikt bij een dosis van 300 IJs per insect, wanneer ongeveer 62 IJs zich in het insect vestigen. De kleinste nakomelingen worden geproduceerd bij een dosis van 1000 IJs per insect en de grootste bij een dosis van 300 IJs per insect. De tijd totdat de eerste nakomelingen uit het insect komen was in het algemeen korter wanneer er meer nematoden geïnoculeerd werden. Effecten van hoge dichtheid blijken te ontstaan door voedselcompetitie in het insect. Voor het succes van insecteparasitaire nematoden in het veld moet rekening worden gehouden met dichtheidseffecten bij iedere nematode soort of stam.

Tussen productie en toepassing worden de nematoden blootgesteld aan stress, speciaal tijdens opslag en verzending. De prestatie van de nematode als biologisch bestrijdingsmiddel wordt onder andere bepaald door factoren als opslagtijd en temperatuur tijdens opslag. In **hoofdstuk 3** wordt het effect van opslagcondities, temperatuur en tijd, op het overleven, de infectiviteit en de ontwikkeling van IJs van *H. megidis* onderzocht. De nematoden werden opgeslagen bij 5, 10, 15, en 20°C gedurende een periode van 70 dagen (10 weken). De infectiviteit en reproductie van de nematoden na opslag bij verschillende temperatuur en tijd werd gemeten in biotoetsen waarbij *G. mellonella* larven werden blootgesteld aan een dosis

van 1 of 30 nematoden per larve. De resultaten laten zien dat, onafhankelijk van de opslagtijd, de IJs het best presteerden na opslag bij een temperatuur van 10 of 15°C. Een toename van de opslagtijd zorgde voor een afname van de kwaliteit van de nematoden na opslag bij 5 of 20°C. Na opslag bij lage temperatuur verloren de nematoden hun vermogen tot parasiteren van een insect, terwijl opslag bij 20°C een direct effect had op het overleven van de nematoden. De tijd totdat de eerste nakomelingen uit het insect komen werd meer beïnvloed door de inoculum grootte dan door de opslagcondities.

Insectenparasitaire nematoden zijn in staat om een groot aantal insectensoorten binnen te dringen en, meestal, te doden. Echter, vaak is er een directe relatie tussen de nematode en het insect waar te nemen, wat suggereert dat er een specifieke gevoeligheid van het insect is die varieert tussen ontwikkelingsstadia, en een intrinsieke virulentie van de nematodensoort of -stam. In **hoofdstuk 4** vermeld ik de resultaten van onderzoek naar het vermogen van IJs van *H. megidis* om larven van verschillende grootte van *G. mellonella* en *Otiiorhynchus sulcatus* te infecteren en er in te reproduceren. De larven van beide insectensoorten werden gewogen, verdeeld in groepen van klein, middel en groot, en blootgesteld aan een dosis van 1 of 30 IJs. Het aantal binnendringende IJs neemt toe met de grootte van de insectenlarve terwijl de mortaliteit van de insecten, bij een dosis van één IJ, afneemt bij toename van de grootte van de insectenlarve. Echter, alle grootte groepen van beide insectensoorten konden worden gedood door de nematoden. Bij een dosis van 30 IJs bleken larven van *G. mellonella* significant gevoeliger dan *O. sulcatus* larven, terwijl bij een dosis van één IJ *O. sulcatus* gevoeliger is. In het algemeen is de tijd totdat de eerste nakomelingen uit het insect komen langer bij de laagste inoculum dosis en neemt toe met het toenemen van de grootte van de insectenlarven, in beide insectensoorten. De productie van nakomelingen verschilt tussen insectensoorten, larvegrootte en dosis van nematoden. *G. mellonella* larven produceerden meer nematoden dan *O. sulcatus* larven wanneer larven van dezelfde grootte werden vergeleken. De totale nakomelingenproductie per larve nam toe met de toename van larvegrootte. Bij kleine larven van *O. sulcatus* en een dosis van één IJ werden geen nakomelingen geproduceerd. De lichaamslengte van IJs neemt in het algemeen toe met een toename van de grootte van de larve, en de langste IJs werden geproduceerd bij de laagste IJ dosis.

Door gebruik te maken van IJs van *H. megidis* opgekweekt uit kleine, middelgrote of grote *G. mellonella* larven en gemiddelde of grote *O. sulcatus* larven, heb ik geprobeerd een antwoord te vinden op de vraag of IJs verkregen uit insectenlarven van verschillende grootte in staat zijn om insectenlarven van dezelfde of een nieuwe insectensoort te infecteren en

doden. Er is een serie infectiviteitstoetsen uitgevoerd (**hoofdstuk 5**). De IJs konden larven van alle groottes, zowel van het insect waarin ze waren opgekweekt als een ander insect, infecteren, onafhankelijk van de grootte van de larven waaruit de IJs waren opgekweekt. IJs uit kleine kadavers en van beide insectensoorten, vertoonden een hogere infectiviteit dan IJs uit middelgrote of grote kadavers. Bij een dosis van één IJ per larve waren IJs uit middelgrote *O. sulcatus* kadavers beter in staat *G. mellonella* te infecteren dan *O. sulcatus*. Grote *G. mellonella* larven zijn minder gevoelig voor alle IJ batches dan middelgrote en kleine *G. mellonella* larven.

In **hoofdstuk 6** wordt een agar-biotoetsen gebruikt om het effect te testen van nematodendichtheid, leeftijd, incubatietijd en de aanwezigheid van een insect op het verspreidingsgedrag van IJs van *H. megidis*. De IJs verspreiden zich sneller en verder bij een hoge nematodendichtheid dan bij een lage. De verspreiding werd ook beïnvloed door de leeftijd van de IJs. Nematoden die 1,5 en 4,5 weken werden opgeslagen waren actiever dan nematoden die 2,5 en 3,5 weken waren opgeslagen. De aanwezigheid van een insect versterkte de verspreiding van de nematoden. Na 90 minuten reageerden de IJs positief op de aanwezigheid van *G. mellonella* maar nauwelijks op *O. sulcatus* larven.

In **hoofdstuk 7** wordt het zoek- en verspreidingsgedrag van *H. megidis* getoetst, in aanwezigheid van *G. mellonella* en *O. sulcatus* larven en aardbeiwortels (*Fragaria x ananassa* Duch.) alleen, in aanwezigheid van, of aangevreten door *O. sulcatus* larven. De biotoetsen werden gedaan in Petrischalen (19 cm diameter) gevuld met vochtig zand en geïncubeerd bij 15°C gedurende 24 uur. IJs reageerden positief op de aanwezigheid van *G. mellonella* larven, op wortels van één aardbeienplant, en op *O. sulcatus* larven in direct contact met de wortels van één aardbeienplant. In aanwezigheid van *O. sulcatus* larven alleen of een combinatie van verschillende aardbeienplanten met *O. sulcatus* larven, zowel in contact met de wortels als niet in contact met de wortels, gaf een neutrale of negatieve respons van de IJs. IJs reageerden sterk op de combinatie van plantenwortels en etende larven, wat suggereert dat de tritrofische interactie tussen IJs, *O. sulcatus* larven en aardbeienplanten een door signaalstoffen aangestuurde interactie is.

In **hoofdstuk 8 en 9** wordt gebruik gemaakt van een nieuw ontwikkelde Y-buis olfactometer, gevuld met zand en geïncubeerd bij 15°C, om het zoekgedrag van *H. megidis* te toetsen. Na 24 uur incubatie werden de IJs significant aangetrokken door de levende *G. mellonella* larven en veroorzaakten 100% mortaliteit. *O. sulcatus* larven daarentegen, waren niet aantrekkelijk voor de IJs en er was geen mortaliteit van de larven. Wortels van aardbeienplanten waren afstotend. Echter, een combinatie van aardbeienplanten en *O.*

sulcatus larven was erg aantrekkelijk voor de IJs en 37% van de larven werd gedood (**hoofdstuk 8**). De resultaten van **hoofdstuk 9** laten zien dat IJs worden geactiveerd door de aanwezigheid van intacte wortels van zowel aardbei als thuja (*Thuja occidentalis* L.). Enkele nematoden aggregeerden in de compartimenten met wortels, maar de meeste gingen naar het compartiment waar geen wortels waren. Wanneer ze de keuze hadden tussen aardbeiwortels en *O. sulcatus* larven, hadden IJs voorkeur voor aardbeiwortels. De IJs hadden geen specifieke voorkeur als ze konden kiezen tussen thujawortels of *O. sulcatus* larven. De combinatie van aardbeiwortels met *O. sulcatus* larven had de voorkeur boven aardbeiwortels alleen. In toetsen met een combinatie van thujawortels met *O. sulcatus* larven versus thujawortels alleen werden de IJs wel geactiveerd maar ze bewogen weg van zowel de wortels met larven als van de wortels alleen. In een toets met mechanisch beschadigde wortels versus door insecten aangevreten wortels, werden de IJs bij thuja aangetrokken door aangevreten thujawortels maar bij aardbei aangetrokken door mechanisch beschadigde wortels. Er was altijd een voorkeur voor aardbeiwortels, zowel alleen als in combinatie met *O. sulcatus* larven, boven thujawortels in dezelfde condities. Een Y-buis keuze-toets bied goede mogelijkheden om het zoekgedrag van insectenparasitaire nematoden te bestuderen in een semi-natuurlijke omgeving.

In **hoofdstuk 10** worden de meest belangrijke onderzoeksresultaten, de bijdrage van dit onderzoek aan de bestaande kennis en de mogelijke toepassingen van dit onderzoek bediscussieerd.

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