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Research Article

Pathogenicity of Four Entomopathogenic Nematodes Species to *G. mellonella* Larvae

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Abstract

Entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema* are obligate and lethal insect parasites. In last decade they are widely used as biological control agents for pest insects of commercial crops, therefore research in this area is directly linked to agriculture. In this study, the pathogenicity of four nematode species (*Heterorhabditis bacteriophora*, *Steinernema glaseri*, *Steinernema scarabaei* and *Steinernema feltiae*) was tested against *Galleria mellonella* larvae. Infective stage of EPNs together with their symbiotic bacteria kills insect host within 48 hours. The results show that mortality of insect host correlates with number of invaded infective juveniles (IJs). The invasion process is very fast, IJs enters insect host within a few hours. The importance of digestive tract as entering site was clearly demonstrated, larvae with empty gut are much more sensitive to nematode infection. Nemato-bacterial complex is very effective system overcoming insect immune defences. Encapsulation as the only one cellular reaction is activated, but in very low rate and was detected only during infection of *H. bacteriophora*.

Keywords: *Galleria mellonella*, *Steinernema*, *Heterorhabditis*, entomopathogenic nematodes, encapsulation

1. Introduction

Insects are the most successful group of animals and exist in environment where the potential for infection by viruses, bacteria, fungi, protozoans as well as various metazoan parasites and parasitoids is great (Narayanan 2004). In last decade, molecular mechanisms that regulate the insect immune response become fully appreciated. Insect have an effective and rapid immune system against microbial infections, protozoans and parasites attacks, which is similar to the innate immune system of vertebrates. Insects have a well developed innate response, but are generally thought to lack an acquired immune system. At the first level of insect defence the pathogens are rebuffed by physical barriers, including the cuticle and peritrophic membrane of the gut. Upon breaching these barriers pathogens meet with an arsenal of robust and efficacious immune defence mechanisms. The innate immune system of insects is subdivided into humoral and cellular defences (for a review see Stanley and Miller 2006). In insect cellular immune responses, haemocytes (blood cells) are involved in phagocytosis of microbes, microaggregation, nodule formation and encapsulation of large pathogens (Gupta 2002), while humoral immune responses include synthesis of antimicrobial peptides such as cecropins and lysozyme, haemolymph clotting system and activation of the prophenoloxidase cascade leading to melanisation (Gupta 2001). Entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema* are obligate and lethal insect parasites. In last decade they are widely used as biological control agents for pest insects of commercial crops including many important lepidopteran, dipteran and coleopteran species (Burnell and Stock 2000, Ehlers 2001). The third stage of nematodes called infective juveniles (IJs) is capable of seeking out hosts and occurs free living in the soil. *Steinernema* gains entry into the host

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through natural openings (mouth, anus and spiracles), *Heterorhabditis* also gains entry by abrading the intersegmental membranes of the insect using a dorsal tooth (Burnell and Stock 2000). Both *Heterorhabditis* and *Steinernema* are symbiotically associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively. The bacterial symbiont is required to kill the insect host (usually within 24-48 hours) and to digest the host tissues. The bacterial symbionts contribute to the symbiotic relationship by establishing and maintaining suitable conditions for nematode reproduction (Boemare et al. 1997), providing nutrients and antimicrobial substances that inhibit the growth of a wide range of microorganisms (Akhurst 1982).

The exit from the developmentally arrested infective stage of nematodes (recovery) is a response to a yet undescribed food signal produced by symbiotic bacteria in living insect, infective juveniles recovery is approximately 95% within 1 day (Strauch and Ehlers 1998).

On the other hand insects as host organisms defend themselves against nematode-bacterial infection by encapsulation of nematodes and producing antibacterial components against gram negative symbiotic bacteria.

The aim of this study is to compare the pathogenicity of four different EPN species in *G. mellonella* and bring new details about nematode infection.

2. Material and Methods

2.1 Entomopathogenic Nematodes and Insect

Freshly propagated IJs of four EPNs species were used for the experiments: *Heterorhabditis bacteriophora* (mix of American cultures, e-nema®, Germany), *Steinernema glaseri* (American culture, e-nema®, Germany), *Steinernema scarabaei* (isolated from USA) and *Steinernema feltiae* (isolated from Czech Republic, locality Prosenice). IJs were checked microscopically for viability and diluted in tap water to defined suspension (25 IJs/50µl) that applied on last instar *Galleria mellonella* larvae. Wax moth larvae (*Galleria mellonella* L., Lepidoptera, Pyralidae) reared in the dark at 30°C on artificial diet according to Haydak (1936) was used as host for nematode infection because of its ideal susceptibility.

2.2 Experimental Design

Conditions for natural infection were established in 24-well microtitre plate filled partly with moistured sand (10% humidity). Each insect larva was in individual well in contact with 25 IJs. In following experiments 10 IJs were injected in *G. mellonella* larvae according to Peters and Ehlers (1997). After 24 hours of incubation at laboratory temperature was one half of larvae dissected and recovered or encapsulated IJs were searched microscopically in the haemocoel. Remaining larvae were used for determination of natural mortality after 48 hours and counting of invaded IJs in cadavers.

To determine time-dependence of the invasion *G. mellonella* larvae were in contact with *S. feltiae* or *H. bacteriophora* IJs for 1, 2, 3 or 4 hours, then washed and incubated without nematodes at the conditions used before. 24 hours starving larvae and normally feeding larvae were used to compare their susceptibility to nematode-bacterial infection. Each experimental group contained at least 10 *G. mellonella* larvae.

3. Results

All four nematode species invaded *G. mellonella.* larvae, but with some differences in rate and effectivity (Fig. 1).

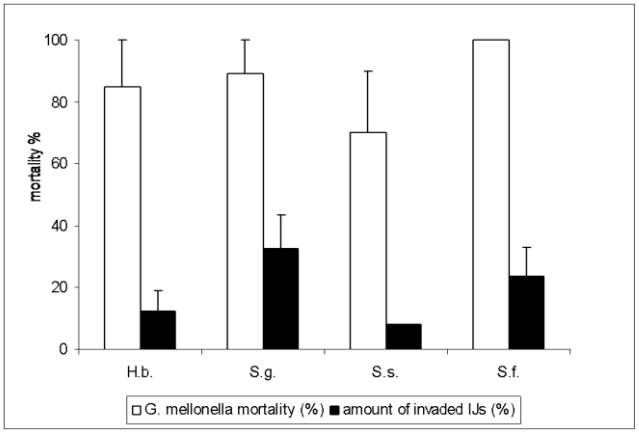

Figure 1. Mortality of *G. mellonella* larvae after natural invasion of *Heterorhabditis bacteriophora* (H.b.), *Steinernema glaseri* (S.g.), *Steinernema scarabaei* (S.s.) and *Steinernema feltiae* (S.f.). Black columns show the amount of invaded IJs (as percentage from total IJs in the assay), average values ± S.D. are included.

S. feltiae killed *G. mellonella* in 100%, opposite *S. scarabaei* only in 70 ± 20%, remaining two species caused approx. 85-90% mortality. The pathogenicity of EPNs correlates with the number of invaded IJs as shown on Fig. 1; the lowest amount of IJs shows *S. scarabaei*. Recovery of invaded IJs was in all EPN species 90-100%. Haemocytes were found attached to the nematode cuticle after natural invasion only in *G. mellonella* invaded by *H. bacteriophora* where 9% of IJs was partly encapsulated.

Injection of all nematode species caused 100% mortality and recovery of IJs was 90-100% except *S. glaseri*, where only 20% recovery was detected (most of IJs were non-recovered and 30% dead). Encapsulation was detected only in *G. mellonella* infected by *H. bacteriophora* in the 10% frequency. The encapsulation response was observed as disappearing cellular capsules without melanisation that varied considerably among individual insects and was initiated mainly at the tail region of the IJs.

Nematode invasion is very fast; within 3-4 hours 50% of host insect can be infected as shown on *S. feltiae* or *H. bacteriophora* (Fig. 2). Under the same condition as with normally feeding larvae (Fig. 2), starving larvae were more susceptible to nematode infection and after 4 hours reached approx. 90% mortality (Fig. 3).

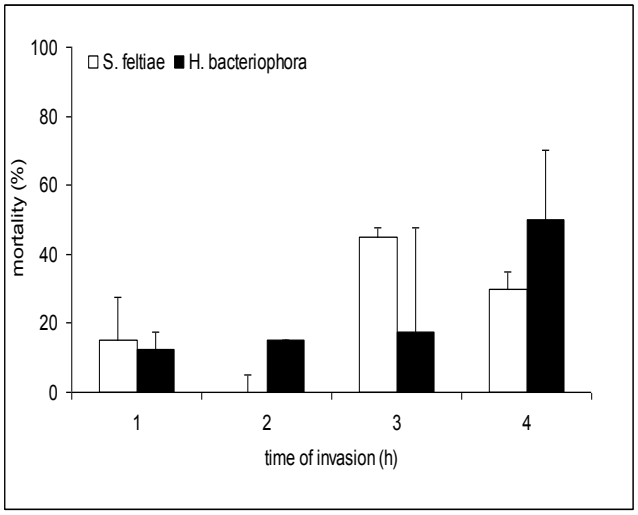

Figure 2. Mortality of *G. mellonella* larvae after short-time infection (1 - 4 hours) of *Steinernema feltiae* or *Heterorhabditis bacteriophora*, average values ± S.D. are included.

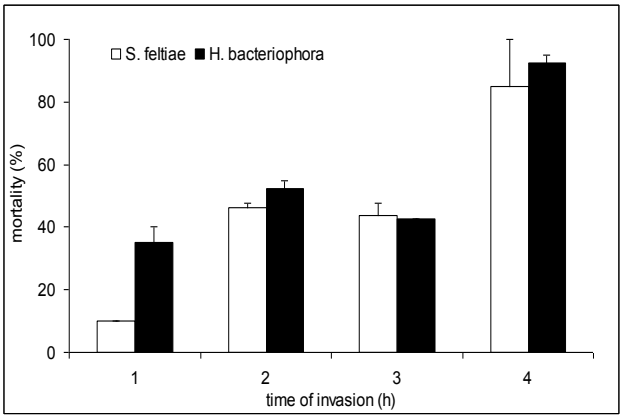

Figure 3. Mortality of starving *G. mellonella* larvae after short-time infection (1 - 4 hours) of *Steinernema feltiae* or *Heterorhabditis bacteriophora*, average values ± S.D. are included

4. Discussion

Species and isolates of entomopathogenic nematodes exhibit differences in host range, infectivity and environmental tolerances. *H. bacteriophora* and *S. feltiae* were used in all experiments as they are common European species widely used in biological control. *S. glaseri* is a tropical-origin nematode (Kaya 1990) and *Steinernema scarabaei* is recently described species from the USA (Koppenhöfer and Fuzy 2003) with a low effectivity against *G. mellonella* - an insect highly susceptible to most known EPNs, but sucessfully used e.g. for control of the European chafer (Cappaert and Koppenhöfer 2003). It was confirmed in this study when *S. scarabaei* caused the lowest mortality from all tested EPN species and the lowest amounts of IJs entered in *G. mellonella* larvae.

All species showed high recovery from IJs stage after entering the host both during natural invasion and after injection except injected *S. glaseri* what can be explained by higher temperature optimum. Cellular defence reaction – encapsulation was detected only against *H. bacteriophora* after natural invasion or injection. This confirms that in the pathogenicity of *H. bacteriophora* towards *G. mellonella* is not important the reaction of host against IJs old J2-cuticle which nematodes loose after entering the host (=exsheathment, Campbell and Gaugler 1992) in contrast e.g. to *Tipula oleracea* where J2-cuticle has an important function in avoiding encapsulation (Peters and Ehlers 1997). In all cases only partly developed and disappearing capsules (not stabilised or

melanised) were detected; neither completely encapsulated IJ was observed, also Li et al. (2007) describes escaption of *H. bacteriophora* from encapsulation in *G. mellonella*. Symbiotic bacteria responsible for pathogenicity can supposedly suppress this cellular immune response since they adhere to and kill the haemocytes before completion of the capsule (Dunphy and Webster 1988, Gerritsen et al. 1998). Other tested species of nematodes did not induce any encapsulation as they are not probably recognised as non-self material (Dowds and Peters 2002). *S. glaseri* was also non-encapsulated in *Popilia japonica* where two surface coat proteins were shown to reduce hemocyte numbers and one of them markedly reduced melanisation and the ability of haemocytes to phagocyte (Wang and Gaugler 1999). Similarly *S. feltiae* in *G. mellonella* was not recognised because of lipoid components on the epicuticle (Dunphy and Webster 1987).

The invasion process is very fast. Natural openings as mouth, anus or spiracles are known as main entry sites for nematode (*H. bacteriophora* can also penetrate cuticle of host, Wang and Gaugler 1998), thus starving larvae with empty gut were used to confirm the importance of host digestive tract at the beginning of nematode infection. IJs enters insect host within a few hours and the importance of digestive tract as entering site was clearly demonstrated; larvae with empty gut are much more sensitive to nematode infection and show better time-dependence mortality. The mortality of insect host also correlates with number of invaded IJs. To summarise nemato-bacterial complex is very effective system using the digestive tract as preferred entering site and overcoming insect immune defences. Encapsulation is activated as the only one cellular reaction although in very low rate and seems to be not effective when more nematodes entered the host or if they already released symbiotic bacteria.

5. Acknowledgements

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