In vivo and *in vitro* interactions between two entomopathogens: the bacterium *Serratia marcescens* and the nematode *Heterorhabditis indica*

Omar M. Darissa and Naim M. Iraki

UNESCO Biotechnology Educational and Research Center (UNESCO BERCEN), Bethlehem University, P.O. Box 9, Bethlehem, Palestine, e-mail: odarissa@bethlehem.edu

Accepted for publication 16 September 2013

Summary. The influence of interaction between *Serratia marcescens* and *Heterorhabditis indica* on the penetration, development and proliferation of the latter inside *Galleria mellonella* larvae was investigated. The preinfection with *S. marcescens* reduced the penetration of the nematode infective juveniles (IJ) into the infected larvae by 62%. Simultaneous application of the pathogens, however, reduced the IJ penetration only by 21.6%. The development of the juvenile stage into adults was reduced when the pathogens were applied sequentially by 87%, while simultaneous application caused a reduction of 47%. When the IJ were manually injected into larvae preinfected with *S. marcescens*, their development into adults was reduced by 96%. Finally, a total inhibition of the nematode reproduction was observed inside *S. marcescens* preinfected larvae. The above findings indicate that the sustainability of this entomopathogenic nematode, when used as a biocontrol agent, or in indoor experimentation, should be carefully evaluated if the soil is heavily contaminated with *S. marcescens*.

Key words: biological control, *Galleria mellonella*, *Photorhabdus luminescens*, antagonism.

Entomopathogenic nematodes (EPN) are roundworms belonging to the phylum Nematoda. Most of these insect-pathogenic nematodes are found in the families Steinernematidae and Heterorhabditdae (Gaugler & Kaya, 1990; Kaya, 1993). Nematodes of these two families are symbiotically associated with insect pathogenic bacteria. Entomopathogenic nematodes from the family Steinernematidae are associated with bacteria from the genus *Xenorhabdus*, while those from the family Heterorhabditdae are symbiotically linked with bacteria from the genus *Photorhabdus* (Koppenhoefer *et al*., 1995; Chaston *et al*., 2011).

Most of the nematode life cycle occurs inside the host body. The only stage that inhabits the soil is the actively motile, non-feeding infective juvenile (IJ) stage whose individuals are covered by a sheath that probably provides a better protection against environmental conditions (Campbell *et al.*, 1995). In the soil, the IJ penetrates the insect's body through spiracles, mouth, or anus and then discharge the symbiotic bacteria, stored in their gut, into the insect's haemocoel. The bacteria feed on the insect tissue, proliferate and produce toxins that kill the insect (Gaugler & Kaya, 1990; Hazir *et al*., 2003). While feeding on the proliferating symbiotic bacteria, the nematodes develop through several stages into adults (Poinar, 1990; Johnigk & Ehlers, 1999). The cycle continues until the source of food (bacteria) becomes limiting. In this situation, IJ are produced and leave the cadaver to search in the soil for other insects to infect (Johnigk & Ehlers, 1999).

Serratia marcescens is a gram negative, motile rod, facultative anaerobe bacterium belonging to the family Enterobacteriaceae (Hejazi & Falkiner, 1997). Most strains of *S. marcescens* produce typical red pigments known as prodigiosin. Although *S. marcescens* was reported as an opportunistic human pathogen (Kurz *et al*., 2003) it still possesses a great potential as a fungal and insect pathogen, mainly because of its capability to produce chitinases, lipases and proteases (Sikorowski & Lawrence, 1998). These hydrolytic enzymes are necessary for the degradation of the insects' cuticle and internal tissues to allow the penetration of the pathogen (Bogo *et al*., 1998). This bacterium was reported in the literature to have a profound destructive effect on many insects (Bell *et*

al., 1981; Krieg, 1987) such as the adult blowflies, *Lucilia sericata* (O'Callaghan *et al.*, 1996), the apple pest *Phagoletis pomonella* (Lauzon *et al.*, 2003), the tobacco budworm *Heliothis virescens* (Sikorowski *et al.*, 2001) and the fungus *Magnaporthe poae*, which causes the summer patch disease of turfgrass (Kobayashi *et al.*, 1995). When the bacterium enters the haemocoel, it multiplies rapidly and causes death in 1-3 days (Sikorowski, 1985).

Serratia marcescens has been isolated from eggs of insectary-reared *Heliothis zea* (Bell, 1969) and from field-collected egg masses of the European corn borer *Ostrinia nubilalis* (Lynch *et al.*, 1976); it has also frequently been recovered from soils (Kobayashi *et al.*, 1995), and has been isolated from the hemolymph of *G. mellonella* pre-infected with several species of EPN (Gouge & Snyder, 2006). Moreover, a bacterial strain of *S. nematodiphila* that shared high 16S rRNA sequence similarities to *S. marcescens* has been identified as the symbiotic bacterium associated with *Heterorhabditidoides chongmingensis* (Zhang *et al.*, 2009). Insects that are either targets of EPN-mediated biocontrol programmes, or those used for indoor or laboratory research on EPN might be exposed to infection with *S. marcescens* from the soil without an immediate effect on their survival (Sikorowski, 1985). In such a case, the infecting bacterium may interfere with the infectivity and reproduction of the nematodes leading to a decrease in their sustainability. The possible interactions of this bacterium with EPN and its effect on their sustainability have not been studied adequately. This work was conducted to investigate the interactions between *S. marcescens* and the EPN *Heterorhabditis indica* and their effect on the infectivity and reproduction of the latter inside *G. mellonella* larvae and to reveal part of the mechanisms of these interactions by an *in vitro* experimentation.

MATERIALS AND METHODS

Isolation of *Heterorhabditis indica* and *Serratia marcescens*. The entomopathogenic nematode *H. indica* used in this work was isolated from Bethlehem area, Palestine, and identified together with their symbiotic bacterium *Photorhabdus luminescens* as described by Iraki *et al.* (2000). The IJ used in this work were 2-week-old obtained from a monoxenic culture of the nematode maintained as described by Lunau *et al.* (1993).

Serratia marcescens used in this study was isolated from soil in Jericho district, Palestine. To identify the isolate, a biochemical determination using the Appendix Profile Index (API test) as well as amplification and sequencing of the 16S rRNA gene of the strain were employed according to standard protocols (Zhu *et al.*, 2007).

Rearing of *G. mellonella*. The wax moth *G. mellonella* was cultured on an artificial diet (200 g honey, 183 g glycerin, 47 g yeast extract, 4 g nepagine and 320 g wheat bran). In all experiments the last-instar stage was used.

Isolation of the symbiotic bacteria. The symbiotic bacterium *Photorhabdus luminescens* was isolated from the haemolymph of *G. mellonella* larvae infected with the nematode *H. indica* as described by Ehlers *et al.* (1990) and cultured as reported by Akhurst (1980).

Inoculation of *G. mellonella* larvae with *S. marcescens* and IJ of *H. indica*. The last instar larvae of *G. mellonella* were buried in 10% moistened construction sand in a 1.55 cm diam. × 1cm height wells of a multi-well plate (1 larva per well). The moistening of a volume of sand sufficient to fill a given number of wells was done with Ringer solution (Akhurst, 1980); the control treatment consisted of a suspension of 20 h-old *S. marcescens* culture, or a Ringer solution containing IJ of a concentration that provided 100 IJ well^{-1}. For simultaneous infections, *S. marcescens* suspension was mixed with IJ to maintain 10% moisture and 100 IJ well^{-1}. The mixing of the pathogen suspension with sand was done thoroughly in a beaker to ensure a uniform distribution. Larvae in sand containing either pathogen were incubated for 24 h. At the end of each infection period, the larvae were removed from the sand and washed 3 times with sterile distilled water. The excess water on the surface of larvae was wiped with sterile filter paper. When additional inoculation with nematodes was applied, each washed larva was exposed to 100 IJ in sand for 24 h then washed and maintained in 5 cm diam. Petri dishes lined with wet filter paper. In each treatment, ten larvae were used and the mortality was recorded daily for 4 days. The experiments were repeated three times independently.

Recovery of *S. marcescens* inside dead larvae. The recovery of *S. marcescens* from dead *Galleria* larvae was performed by the surface sterilization of the dead larvae with 70% ethanol, then inoculates from the haemolymph were streaked on nutrient bromothymole triphenyltetrazolium chloride agar (NBTA) medium (Akhurst, 1980) to observe the typical red-pigmented *S. marcescens* colonies.

Determination of growth rates of *P. luminescens* in the presence of *S. marcescens*. A volume of 50 µl of 20 h-old culture of *S. marcescens* or *P. luminescens* was smeared each on

9-cm diam. Petri dish containing solid NTYP medium. The NTYP medium contained (l^{-1} tap water) 10 g nutrient broth (Difco), 10 g tryptic soy broth (Difco), 5 g yeast extract (Sigma), 5 g peptone (MERCK), 5 g NaCl (MERCK), 0.35 g KCl (MERCK), and 0.21 g $CaCl_2 2H_2O$ (MERCK). Filter paper disks (Wattman No. 1), 0.6 cm diam., were soaked in either *S. marcescens* or *P. luminescens* 20 h-old liquid cultures and part of them was mounted on NTYP medium already smeared with *P. luminescens* or *S. marcescens*, respectively. The other part of the disks was mounted on non-inoculated medium (control). Each treatment included four plates, each containing four disks. All of the plates were incubated in the dark at 25°C. The growth of the bacteria was recorded daily for 3 days by measuring the diameter of the culture surrounding the paper disks. The experiments were repeated three times independently.

Determination of IJ and fourth-stage juvenile (J4) survival in *S. marcescens* liquid culture. Fifty thousand (± 20) IJ of *H. indica*, suspended in a volume of 1 ml of Ringer solution, were added to 50 ml of a newly inoculated *S. marcescens* culture. The inoculation of the bacterial culture was performed by adding 0.5 ml of 20 h-old culture to 50 ml NTYP liquid medium in a 250 ml flask. Similar amounts of IJ were added to 50 ml of sterile NTYP solutions as the control treatment. In all treatments, flasks were kept at continuous rotary shaking (200 rpm) at 25°C in the dark. After 24 or 48 h, three samples of 1 ml each were taken from each treatment and poured into 5 cm diam. sterile Petri dishes for recording the number of surviving nematodes under the microscope.

For determining survival of the J4 stage, about 50 nematodes of this stage were selected from a monoxenic culture under the microscope and incubated in 20 ml NTYP medium inoculated with 0.2 ml of 20 h-old *S. marcescens* culture and then were incubated for 2 days at 25°C in the dark under continuous rotary shaking (200 rpm). As a control treatment, a similar number of J4 was incubated at the same conditions in NTYP medium. The number of nematodes that survived was monitored in the whole culture under aseptic conditions after 24 and 48 h of incubation. Each of the above experiments was repeated twice.

Determination of penetration, development and reproduction of IJ inside *G. mellonella* larvae. The *Galleria* larvae of treatments involving infection with nematodes were divided at the end of the infection period into three groups, each of 10 larvae, for the purpose of determining penetration, development to adults, and reproduction. To determine penetration of IJ into the insect, larvae were washed then each dissected separately in a 5 cm diam. Petri dish and incubated in pepsin solution for 2 h at 37°C with continuous rotary shaking (120 rpm). The pepsin solution contained 8.0 g l^{-1} pepsin (Sigma), 23 g l^{-1} NaCl, and 940 ml distilled water. The pH of the solution was adjusted to 2.0 with HCl (Mauleon *et al.,* 1993). The IJ that penetrated into the insect were detected in the digested tissues and counted under the microscope then calculated as percent of the applied IJ.

Larvae of a second group were washed and left on Ringer-wetted filter paper for 72 h and then each larva was dissected in a 5.5 cm diam. Petri dish to determine the number of adults (hermaphrodites) by direct observation under the microscope.

To determine total production of nematodes, each larva of a third group was washed and left on a White trap (White, 1927) for 2 weeks in the dark at 25°C. IJ were collected from the trap and counted under the microscope together with the adults left in the cadaver. All of the treatments in this experiment were repeated three times independently.

Injection of IJs into *G. mellonella* larvae. IJ, suspended in 20 µl of sterile Ringer solution, were injected with a 1000 µl syringe into the haemolymph of last instar *Galleria* larvae. The number of injected IJ was 30 ± 3 IJ $larva^{-1}$. The development of IJ into adults was recorded 4 days after injection.

Statistical analysis. All the data collected from repeated experiments were combined and statistically analysed by paired samples t-test using the SPSS 9.0 software by comparing two samples each time and doing so for all the possible comparable combinations.

RESULTS

Identification of the entomopathogenic bacterium. The 16SrRNA sequence of the Jericho bacterial isolate (NCBI accession number KC414199) showed 100% identity to *S. marcescens* (data not shown). The API results of the bacterium further identify the isolate as *S. marcescens* (Data not shown).

Pathogenicity of *S. marcescens* and *H. indica* to *G. mellonella* larvae. While *S. marcescens* caused only 10% mortality of *Galleria* larvae 4 days after infection, the IJ of *H. indica* caused 100% mortality two days after infection. When both pathogens were applied simultaneously, each at

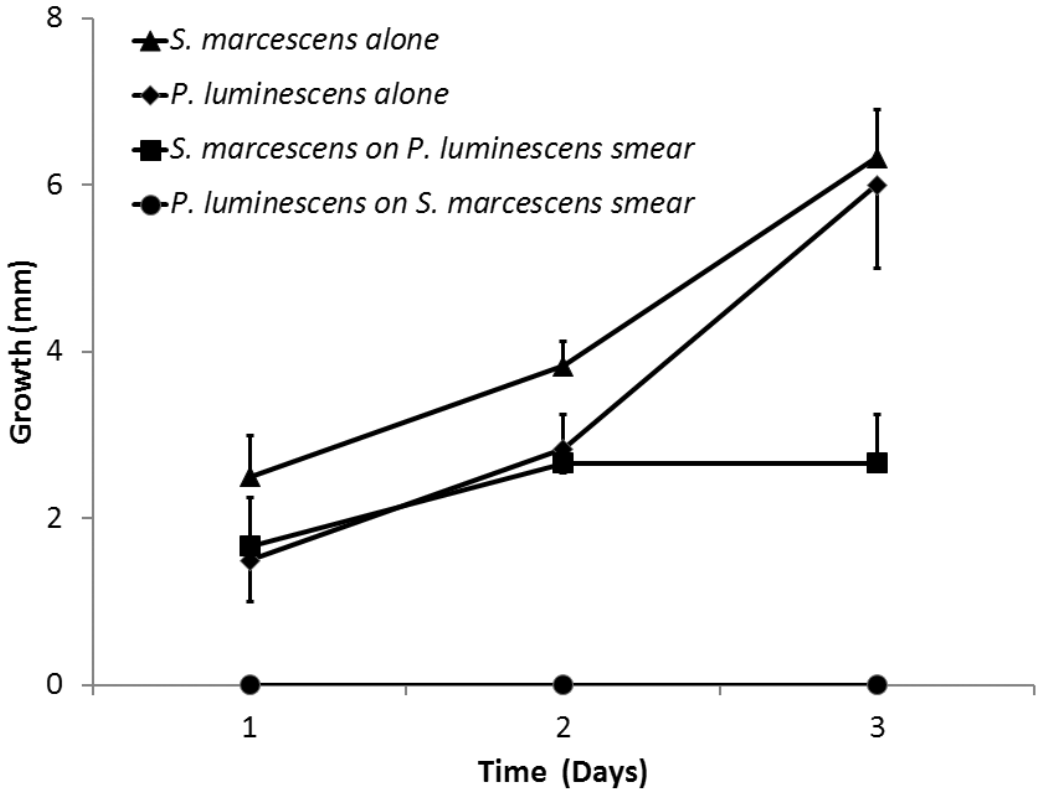

Fig. 1. Growth rates on BSA medium, measured as an increase in culture diameter (mm), of *Photorhabdus luminescens* on a smear of *Serratia marcescens* and of *S. marcescens* on a smear of *P. luminescens*. Error bars (shown either as a minus or a plus to avoid overlapping) represent the standard deviation of three independent experiments.

the same concentration as when applied alone, the mortality was similar to that obtained from application of nematodes alone (Table 1). These results indicate that the combined application of *S. marcescens* with nematodes has no additive effect on mortality of *Galleria* larvae.

However, when the nematodes were applied following a 24 h infection with *S. marcescens*, the mortality was 86% 2 days after the application of nematodes (3 days after the application of *S. marcescens*) compared with 100% mortality obtained when nematodes were applied either simultaneously with *S. marcescens* or alone (Table 1).

Effect of *S. marcescens* on the growth of *P. luminescens*. The growth of *P. luminescens* on a smear of *S. marcescens* was totally inhibited (Fig. 1). On the other hand, the growth of *S. marcescens* on a smear of *P. luminescens* was inhibited by 42%, as calculated after 3 days of co-incubation, compared to the control treatment where *S. marcescens* disks were mounted on non-inoculated medium (Fig. 1).

Survival of IJ and J4 of *H. indica* in the presence of *S. marcescens*. Although the survival of the IJ was not affected after 48 h of incubation in the *S. marcescens* culture, the survival of the J4 stage was dramatically reduced to 1.9% (Table 2).

Table 1. Percentage mortality of *Galleria* larvae infected with *Serratia marcescens* or infective juveniles (IJ) of *Heterorhabditis indica* for 24 h at different infection orders. Values are means of results from three independent experiments. Values with different superscripts are significantly different.

Pathogen mode of applicatio	Percentage mortality of larvae			
Days after 1st infection	1	2	3	4
S. marcescens alone	0	0	0	10 [d]
Infective juveniles alone	0	100 [a]	100 [a]	100 [a]
Simultaneous: *S. marcescens* with IJ	0	100 [a]	100 [a]	100 [a]
Sequential: *S. marcescens* then IJ	0	25 [b]	86 [c]	100 [a]

Table 2. Percentage survival of infective juveniles (IJ), and fourth-stage juveniles (J4) of *Heterhabditis indica* in liquid culture of *Serratia marcescens*. Values are means of results from two independent experiments. Values in columns representing the same nematode stage and have different superscripts that are significantly different.

Time (h)	In *S. marcescens*		Control (NTYP medium)	
	IJ	J4	IJ	J4
24	98.1 [a]	65.4 [b]	99.2 [a]	97.7 [a]
48	98.7 [a]	1.9 [c]	99.1 [a]	95.9 [a]

Effect of *S. marcescens* on the penetration and development to adults of IJ inside *G. mellonella* larvae. To determine the penetration of nematodes into the insect, the nematode-infected larvae were washed and digested in pepsin to recover the penetrated IJ. The number of detected IJ in each larva was calculated as a percentage of the total number of the nematodes that were applied. Preinfecting the *Galleria* larvae for 24 h with *S. marcescens* significantly reduced the penetration of IJ from 7.4% in the control to 2.8% (Table 3). A non-significant effect on penetration was observed when the microbial pathogen was applied simultaneously with the IJ (Table 3).

Table 3. Effect of infection with *Serratia marcescens* on the penetration of infective juveniles (IJ) of *Heterorhabditis indica* into *Galleria* larvae, on their development into adults, and on their reproduction inside the insect. Values are means of results from three independent experiments. Values with different superscripts are significantly different in the same column.

Pathogen mode of application	Mean % penetration of IJs	Mean % development to adults	Calculated % development of penetrated IJs	Reproduction: nematodes × 10^3/ larva	Presence of *S. marcescens* in haemolymph
Sequential: *S. marcescens* then IJ	2.8 [a]	0.9 [a]	32.0	0 [a]	+
Simultaneous: *S. marcescens* with IJ	5.8 [b]	3.7 [b]	63.7	5 [b]	+
IJ alone	7.4 [b]	7.0 [c]	94.9	106 [c]	–

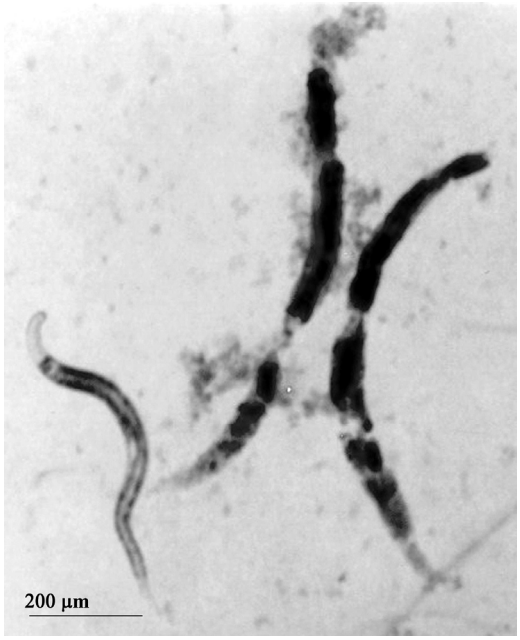

Fig. 2. Photograph of the fourth-stage juvenile (J4) of *Heterorhabditis indica* after incubation in liquid medium for 48 h. The two J4 on the right side were incubated in medium inoculated with *S. marcescens* for 48 h, while the small one on the left side was incubated in *S. marcescens*-free medium as a control.

The development of IJ into adults was reduced as a result of infecting the larvae with *S. marcescens*. When this bacterial pathogen was applied together with, or before, the application of IJ, the development of the latter into adults was lower than their penetration rates (Table 3). When development to adults values were calculated as a percentage of the penetrated IJ, rather than a percentage of the applied IJ, the percentage development to adults of IJ inside the larvae infected by simultaneous application of *Serratia* and IJ was 64%, *i.e.*, an inhibition of 31% compared with the control. However, when *S. marcescens* cells were applied 24 h before application of nematodes, the development to adults of IJ was only 32%, which is lower than the control by 63%. This level of inhibition is greater by two-fold than that observed upon simultaneous application of both pathogens. Increasing the number of IJ inside the *Serratia*-preinfected larvae may improve the rate of development to adults. To test this assumption we injected IJ, in relatively large numbers (27-31), into *Galleria* larvae preinfected with *S. marcescens*. The development to adults of IJ inside S. *marcescens*-preinfected *Galleria* was only 2.6% compared with 71.9% in control, indicating an inhibition of 69%

(Table 4). This level of inhibition is close to the one observed when IJ naturally penetrated into the larva pre-inoculated with *S. marcescens* (Table 3). This result implies that 31 IJ and their symbiotic bacteria inside preinfected *Galleria* are still insufficient to overcome the antibiosis of *S marcescens*. The data presented in Table 4 indicate that 2 days after injecting 31 IJ into *Serratia*- preinfecting larva, the mortality was 100%, which is the same level as in control. Hence, increasing the number of IJ infecting each *Galleria* from 2.8 to 31.0 has increased mortality of larvae from 86% to 100%. It should be pointed out that when IJ were injected into a non-preinfected *Galleria*, the percentage development to adults was lower than that obtained when IJ were applied by normal penetration; 71.9% (Table 4) compared with 94.9% (Table 3).

Effect of *S. marcescens* on the proliferation of *H. indica* inside *G. mellonella* larvae. The results presented in Table 3 indicate that in the absence of *S. marcescens,* one single *Galleria* larvae might accommodate the production of 106 $\times 10^3$ nematodes (mainly IJ). If the larva is exposed to IJ together with *S. marcescens*, however, the total production of nematodes per larva drops to 5×10^3, a reduction of more than 21-fold. Furthermore, when larvae were exposed to *S. marcescens* for 24 h before the application of IJ, the nematode could not reproduce at all (Table 3). We have shown that under the latter infection regime, some IJ do penetrate (2.8%) and recover (32%) inside the *S. marcescens*-infected larvae. It should be pointed out that *S. marcescens* cells were discovered in haemolymph of larvae preinfected with the bacterium as well as in larvae infected simultaneously with the bacterium and IJ (Table 3).

Table 4. Development of infective juveniles (IJ) to adults of *Heterorhabditis indica* upon injection into *Galleria mellonella* larvae following preinfection with *Serratia marcescens* for 24 h. The results were observed 4 days after IJ injection. Values are means of results from three independent experiments. Values with different superscripts are significantly different.

Treatment	Average number of injected IJ larva^{-1}	Mean % development to adults	% Mortality 2 days after injection
S. marcescens preinfection	31	2.6^{b}	100
No preinfection	27	71.9^{a}	100

DISCUSSION

Serratia marcescens is a pathogen to many insects and its pathogenicity was attributed to the secretion of chitinases and proteases (Bell *et al*., 1981; Krieg, 1987; Bogo *et al*., 1998; Sikorowski & Lawrence, 1998). Although the penetration of *S. marcescens* into the insect's haemolymph through the cuticle has not been demonstrated, there are some reports in the literature referring to evidence for penetration through the digestive tract (Kurz *et al*., 2003). In the soil, the bacterium might be attached to soil particles and when these particles are ingested by the insect the bacterium may penetrate into the insect's haemolymph with the aid of secreted chitinases, proteases, and lipases.

In this study we have shown that *S. marcescens* reduced the capacity of *H. indica* to penetrate, develop and proliferate inside *Galleria* larvae and dramatically inhibit the survival of J4 nematodes but not the IJ (Table 2). The difference in sensitivity between IJ and J4 to *S. marcescens* might be related to the presence of the double cuticle in the IJ that may protect them from the hydrolytic enzymes secreted by the bacterial culture. This assumption is supported by the destructive effect of the bacterial culture on the J4 tissues (Fig. 2). Furthermore, since IJ is a non-feeding stage, this may reduce any probability of ingestion of *S. marcescens* cells or toxins as might occur in the J4 stage. The above findings may explain the reduced development to adults and proliferation of nematodes in *Galleria* larvae preinfected with *S. marcescens*.

The results also showed mutual growth-inhibiting effects between *S. marcescens* and *P. luminescens* and that the former is a stronger inhibitor. *Serratia marcescens* is known for the production of antibiosis factors such as prodigiosin and beta-lactam (Tomohiko *et al.*, 1998). These two insect-pathogenic bacteria may have similar interactions inside the *Galleria* larva. The *S. marcescens* reaches the larval haemolymph through the digestive tract and it is ingested together with contaminated food or soil particles (Sikorowski *et al.*, 1992). Since the bacteria penetrate the wall of the digestive midgut by means of proteinases and chitinases, the rate of penetration depends on the amount of cells ingested by the insect. Once the bacterium reaches the haemolymph it proliferates rapidly and kills the insect within 1-3 days (Sikorowski *et al.*, 1992). The symbiotic bacteria reach the haemolymph while carried by their infective juveniles. IJ enter the haemolymph, through midgut or other routes, such as the spiracles. When IJ are applied to an insect that has

already ingested small amount of *S. marcescens* cells, the physical penetration of the IJ offers an opening through which the bacterial cells may enter and reach the haemolymph. When both kinds of bacteria are present in the haemolymph, they become exposed to mutual inhibitory effects, such as those documented in Figure 1. The consequence of such effects might be a decrease in the pathological effect of the symbiotic bacterium on the infected insect, which in turn may lead to a delay in mortality. Reduced mortality of target insects as a result of dual application of two kinds of entomopathogenic nematodes was reported by Choo *et al.* (1996). They suggested that when the competition between the two applied pathogens involves mutual antibiosis, it will lead to a reduced mortality of the host. The results shown in Figure 1 are in agreement with the suggestion of Choo *et al*. (1996). However, we should not neglect the possibility that the reduction in percentage mortality could be a result of decreased penetration of IJ into the larvae upon preinfection with *S. marcescens.* When the number of IJ inside the larvae was increased by injection, the insect mortality becomes comparable to that in the control treatment (Table 4). This strongly supports our previous assumption that the reduced mortality, caused by IJ in larvae that had been infected with *S. marcescens*, is a consequence of inhibited IJ penetration and limited inoculum of symbiotic bacteria.

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

The percentage development of IJ into adults in a *Serratia*-preinfected larva was dramatically reduced to 32% in comparison with 94.9% in the control treatment (Table 3). The reason for this may stem from the 24 h preinfection period, which might be long enough for the *Serratia* cells to colonise the larva haemolymph, proliferate and excrete adequate levels of antibiotics and hydrolytic enzymes. These substances would suppress the proliferation of the nematode symbiotic bacteria, or hydrolyze the developing nematode stages (Figs 1 and 2). Both of these effects lead to decreasing the number of nematode adults that may develop from IJ. Furthermore, the relatively longer period of infection with *S. marcescens* before nematode application causes a substantial decrease in IJ penetration, which means introducing a smaller number of symbiotic bacteria that might not be able to cope with and antagonise the well-established *S. marcescens* inoculum. The lower percentage of development to adults of injected IJ (71.9%, Table 4) compared to the naturally penetrating ones (94.9%, Table 3) is related to natural variation among individuals of the same population of IJs. Such a variation was reported by Hominick & Reid (1990). They found that not all individuals of a given population of IJ are capable of penetrating into the insect. Furthermore, not all of those that do penetrate can develop into adults. We injected into the larvae a population of IJ that had not passed through selection for penetration potency. The potential of development to adults of these non-selected individuals is probably lower than that of individuals capable of penetrating into the insect by themselves. In other words, there might be a certain level of correlation between penetration capability and development of IJ into adults.

In the *Serratia*-preinfection treatment, our failure to detect any nematode production either in White trap or in insect cadaver after 2 weeks of incubation (Table 3) strongly indicates a direct action of *S. marcescens* on the small number of adults that developed from the penetrated IJ. In the case of simultaneously application of both organisms, the IJ are able to reach the insect haemolymph and release their symbiotic bacteria in a shorter period of time than that required for *S. marcescens.* As a result, the symbiotic bacteria inoculum proliferates and allows

development of IJ into adults before the *S. marcescens* cells succeed in multiplying and spreading in the whole insect's body. Furthermore, the antibiotic effect of the symbiotic bacterium against S. *marcescens* may reduce the hydrolytic effect of the latter against the developing nematode stages. This effect would ultimately lead to a partial nematode proliferation (Table 3). These findings indicate that the application of entomopathogenic nematodes as biocontrol agents in soils infested with *S. marcescens* can affect the nematodes sustainability.

ACKNOWLEDGEMENT

We would like to thank Prof. Ralf-Udo Ehlers from Christian-Albrechts University, Kiel, Germany for his advice and support.

REFERENCES

AKHURST, R.J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* 121: 303-309.

BELL, J. 1969. *Serratia marcescens* found in eggs of *Heliothis zea*: tests against *Trichoplusia ni*. *Journal of Invertebrate Pathology* 13: 151-152.

BELL, J., KING, E. & HAMMALLE, J. 1981. Some microbial contaminants and control agents in a diet and larvae of *Heliothis* spp. *Journal of Invertebrate Pathology* 37: 243-248.

BOGO, M., ROTA, C., PINTO, H., OCAMPOS, M., CORREA, C., VAINSTEIN, M. & SCHRANK A. 1998. A chitinase encoding gene (*chit1* gene) from the entomopathogen *Metarhizium anisopliae*: Isolation and characterization of genomic and full-length cDNA. *Current Microbiology* 37: 221-225.

CAMPBELL, J., LEWIS, E., YODER, F. & GAUGLER, R. 1995. Entomopathogenic nematodes (Heterorhabditdae and Steinernematidae) seasonal population dynamics and impact on insect populations in turfgrass. *Biological Control* 5: 598-606.

CHASTON, J.M., SUEN, G., TUCKER S.L., ANDERSEN, A.W., BHASIN, A., BODE, E., BODE, H.B., BRACHMANN, A.O., COWLES, C.E., COWLES, K.N., DARBY, C., DE LEON, L., DRACE, K., ZIJIN, D., GIVAUDAN, A., TRAN, E.H., JEWELL, K.A., KNACK, J.J., KRASOMIL-OSTERFELD, K.C., KUKOR, R. 2011. The entomopathogenic bacterial endosymbionts *Xenorhabdus* and *Photorhabdus*: Convergent Lifestyles from Divergent Genomes. *PLoS ONE* 6: e27909. doi:10.1371/journal.pone.0027909.

CHOO, H., KOPPENHOEFER, A. & KAYA, H. 1996. Combination of two entomopathogenic nematode species for suppression of an insect pest. *Economic Entomology* 89: 97-103.

EHLERS, R.-U., STOESSEL, S. & WYSS, U. 1990. The influence of phase variants of *Xenorhabdus* spp. and *Escherichia coli* (Enterobacteriaceae) on the propagation of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*. *Review of Nematology* 13: 417-424.

GAUGLER, R. & KAYA, H. 1990. *Entomopathogenic Nematodes in biological Control*. USA, Boca Raton, CRC Press. 365 pp.

GOUGE, D.H. & SNYDER, J.L. 2006. Temporal association of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) and bacteria. *Journal of Invertebrate Pathology* 91: 147-157.

HAZIR, S., KAYA, H.K., STOCK, S.P. & KESKÜN, N. 2003. Entomopathogenic Nematodes (Steinernematidae and Heterorhabditidae) for Biological Control of Soil Pests. *Turkish Journal of Biology* 27: 181-202.

HEJAZI, A. & FALKINER, F.R. 1997. *Serratia marcescens*. *Journal of Medical Microbiology* 46: 903-912.

HOMINICK, W. & REID, A. 1990. Perspectives on entomopathogenic nematology. In: *Entomopathogenic Nematodes in Biological Control* (R. Gaugler & H.K. Kaya Eds). pp. 327-343. USA, Boca Raton, CRC Press.

IRAKI, N., SALAH, N., SANSOUR, M., SEGAL, D., GLAZER, I., JOHNIGK, S., HUSSEIN, M. & EHLERS, R.U. 2000. Isolation and characterization of two entomopathogenic nematode strains, *Heterorhabditis indica* (Nematoda, Rhabditida), from the West Bank, Palestinian Territories. *Journal of Applied Entomology* 124: 375-380.

JOHNIGK, S.A. & EHLERS, R.U. 1999. Juvenile development and life cycle of *Heterorhabditis bacteriophora* and *H. indica* (Nematoda: Heterorhabditidae). *Nematology* 1: 251-260.

KAYA, H. 1993. Entomogenous and entomopathogenic nematodes in biological control. In: *Plant Parasitic Nematodes in Temperate Agriculture* (K. Evans, D.L. Trudgill & J.M. Webster Eds). pp. 565-591. U.K, Cambridge, Cambridge University Press.

KOBAYASHI, D., GUGLIELMONI, M. & CLARKE, B. 1995. Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass. *Soil Biology and Biochemistry* 27: 1479-1487.

KOPPENHOEFER, A., KAYA, H., SHANMUGAM, S. & WOOD, G. 1995. Interspecific competition between steinerneamtid nematodes within an insect host. *Journal of Invertebrate Pathology* 66: 99-103.

KRIEG, A. 1987. Diseases caused by bacteria and other prokaryotes. In: *Epizootiology of insect diseases* (J.R. Fuxa & Y. Tanada Eds). pp. 323-355. USA, New York, John Wiley and Sons.

KURZ, C., CHAUVET, S., ANDRÈS, E., AUROUZE, M., VALLET, I., MICHEL, G., UH, M., CELLI, J., FILLOUX, A., BENTZMANN, S., STEINMETZ, I., HOFFMANN, J., FINLAY, B., GORVEL, J., FERRANDON, D. & EWBANK, J. 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. *The EMBO Journal* 22: 1451-1460.

LAUZON, C.R., BUSSERT, T.G., SJOGREN, R.E. & PROKOPY, R.J. 2003. *Serratia marcescens* as a bacterial pathogen of *Rhagoletis pomonella* flies (Diptera: Tephritidae). *European Journal of Entomology* 100: 87-92.

LUNAU, S., STOESSEL, S., SCHMIDT-PEISKER, A. & EHLERS, R.U. 1993. Establishment of monoxenic inocula for scaling up *in vitro* cultures of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis* spp. *Nematologica* 39: 385-399.

LYNCH, R., LEWIS, L. & BRINDLEY, T. 1976. Bacteria associated with eggs and first instar larvae of the European corn borer: Identification and frequency of occurrence. *Journal of Invertebrate Pathology* 27: 229-237.

MAULEON, H., BRIAND, S., LAUMOND, C. & BONIFASSI, E. 1993. Utilisation d'enzyme digestives pour l'e ´tude du parasitisme des Steinernematidae et Heterorhabditidae envers les larves d'insectes. *Fundamental and Applied Nematology* 16: 185-186.

O'CALLAGHAN, M., GARNHAM, T., NELSON, B. & JACKSON, T. 1996. The pathogenecity of *Serratia* strains to *Lucilia sericata* (Diptera: Calliphoridae). *Journal of Invertebrate Pathology* 68: 22-27.

POINAR, G. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditdae. In: *Entomopathogenic Nematodes in Biological Control* (R. Gaugler & H.K. Kaya. Eds). pp. 23-61. Boca Raton, USA. CRC Press.

SIKOROWSKI, P. 1985. Pecan weevil pathology. In: *Pecan weevil: research perspective* (W.W. Neel. Ed.). pp. 87-101. Jackson, MS. Quail Ridge Press.

SIKOROWSKI, P. & LAWRENCE, A. 1998. Transmission of *Serratia marcescens* (Enterobacteriaceae) in Adult *Heliothis virescens* (Lepidoptera: Noctuidae) laboratory colonies. *Biological Control* 12: 50-55.

SIKOROWSKI, P.P., LAWRENCE, A.M. & INGLIS, G.D. 2001. Effect of *Serratia marcescens* on rearing of the tobacco budworm (Lepidoptera: Noctuidae). *American Entomologist* 47: 51-60.

SIKOROWSKI, P., POWELL J. & LAWRENCE, A. 1992. Effects of bacterial contamination on development of *Microplitis croceipes* [Hym: Braconidae]. *Entomophaga* 37: 475-481.

TOMOHIKO, S., HIROKI, K., YASUFUMI, T., TAKAO, K., KAZUO, N., WASSERMAN, H. & SHOJI, O. 1998. Prodigiosins as a new group of H+/Cl symporters that uncouple proton translocators. *The Journal of Biological Chemistry* 273: 21455-21462.

WHITE, G. 1927. A method for obtaining infective nematode larvae from cultures. *Science* 66: 302-303.

ZHANG, C.X., YANG, S.Y., XU, M.X., SUN, J., LIU, H., LIU, J.R., KAN, F., SUN, J., LAI, R. & ZHANG, K.Y. 2009. *Serratia nematodiphila* sp. nov., associated symbiotically with the entomopathogenic nematode *Heterorhabditidoides chongmingensis* (Rhabditida: Rhabditidae). *International Journal of Systematic and Evolutionary Microbiology* 59: 1603-1608.

ZHU, H., ZHOU, W.Y., XU, M., SHEN, Y.L. & WEI, D.Z. 2007. Molecular characterization of *Serratia marcescens* strains by RFLP and sequencing of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *Letters in Applied Microbiology* 45: 174-178.

Darissa, O.M. and Iraki, N.M. Взаимодействие двух энтомопатогенов – бактерии *Serratia marcescens* и нематоды *Heterorhabditis indica* – при изучении *in vivo* и *in vitro*.

Резюме. Было изучено влияние взаимодействия между *Serratia marcescens* и *Heterorhabditis indica* и их проникновение, развитие и распространение в личинках *Galleria mellonella*. Предварительное заражение *S. marcescens* снижало проникновение инвазионных личинок (ИЛ) нематод в зараженные личинки насекомых примерно на 62%. Однако одновременное применение патогенов снижало проникновение инвазионных личинок (ИЛ) только на 21,6%. Развитие личиночной стадии нематод во взрослую снижалось на 87% в случае последовательного применения патогенов, тогда как одновременное применение вызывало снижение на 47%. Когда предварительно зараженные *S. marcescens* насекомые вручную были заражены ИЛ нематод, их развитие во взрослых особей сокращалось на 96%. В итоге, общее подавление репродукции нематод было обнаружено в предварительно зараженных *S. marcescens* личинках моли. Обнаруженные находки означают, что устойчивость этих энтомопатогенных нематод при их использовании в качестве агента биоконтроля или лабораторных экспериментах должна оцениваться с учетом загрязнения почвы *Serratia marcescens*.