Steinernema carpocapsae

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[Original Article]

Pathogenicity of the axenic entomopathogenic nematode *Steinernema carpocapsae* against *Galleria mellonella* and *Spodoptera litura* larvae

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Pathogenicity of the entomopathogenic nematode *Steinernema carpocapsae* depends on its symbiotic bacterium *Xenorhabdus nematophila*. To understand the pathogenicity of the nematode itself, and its influence on insect tissues, we compared nematode invasion, insect mortality, nematode development in insects, and differences in the effects on the insect tissues between infection by axenic or monoxenic nematodes. Invasion of axenic infective juveniles (IJs) was lower than that of monoxenic IJs. Axenic as well as monoxenic nematodes killed both axenic and xenic *Galleria mellonella* and *Spodoptera litura* larvae, although it took a longer time for axenic nematodes to kill the insects, especially axenic ones. Axenic nematodes grew and reproduced in insects; however, their growth and reproduction were delayed as compared to that of monoxenic nematodes. Tissue destruction of axenic IJ-infected insect larvae was delayed as compared with monoxenic IJ-infected insect larvae, and suppression of insect hemolymph melanization was not observed in the axenic nematodes. These results suggest that *S. carpocapsae* is able to kill insects and partly degrade insect tissues in the absence of any bacteria; however, the pathogenicity of axenic nematodes is weaker than that of monoxenic nematodes. Nematol. Res. 46(2), 39–44 (2016).

Key words: axenic, *Galleria*, *Spodoptera*, *Steinernema*, *Xenorhabdus*

INTRODUCTION

Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* show strong pathogenicity to a wide range of insects. Infective juveniles (IJs) seek insect hosts in soil, and invade them through their natural openings or through the skin. After invasion, IJs release their symbiotic bacteria, which produce insect toxins, into the hemocoel of the host thereby rapidly killing the host (Dowds and Peters, 2002). Thus, the pathogenicity of entomopathogenic nematodes is largely dependent on their symbiotic bacteria. The insecticidal toxins such as toxin complex (TC) and other potential virulence agents produced by the symbiotic bacteria have been studied (Forst and Nealson, 1996; Dowds and Peters, 2002).

In contrast to the symbiotic bacteria, information on the pathogenicity of the nematode itself is limited because of the difficulty of evaluation. Some studies on axenic nematodes have indicated that axenic steinernematids have pathogenicity to insects, but axenic *Heterorhabditis*, at least *H. bacteriophora* Poinar, does not (Boemare *et al.*, 1983; Burman, 1982; Ehlers *et al.*, 1997; Gerritsen and Smits, 1993; Han *et al.*, 1991; Han and Ehlers, 2000). In the case of *S. carpocapsae* (Weiser) Wouts, Mráček, Gerdin & Bedding, Han and Ehlers (2000) demonstrated that the axenic IJs produced by a monoxenic culture with *Xenorhabdus* bacteria isolated from *S. glaseri* (Steiner) Wouts, Mráček, Gerdin & Bedding could kill insects. Nevertheless, only limited information is available on the infectivity of axenic IJs, the growth of axenic nematodes in insects, and the effects of axenic nematodes on insect tissues.

The aims of the present study are to examine the pathogenicity of IJs obtained using an axenic culture developed by us (Fuchi *et al.*, 2016) against insects with different susceptibilities to entomopathogenic nematodes, and to clarify the influences of the symbiotic bacteria on infectivity in IJs and parasitism of axenic nematodes on insect tissues and hemolymph as well as on the development and propagation of axenic nematodes in the insect.

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MATERIALS AND METHODS

Nematodes:

S. carpocapsae (All) were monoxenically maintained on dog food agar medium (DFA) (Hara *et al.*, 1981) and used for the experiments.

Insects:

Galleria mellonella Linnaeus, which is highly susceptible to *S. carpocapsae* (Kondo and Ishibashi, 1988), was maintained using beehives. *Spodoptera litura* Fabricius larvae are less susceptible to the nematode and were maintained on an artificial diet (Okada, 1977).

Axenic *G. mellonella* were cultured on an autoclaved artificial diet (10 ml honey, 10 ml glycerol, 10 ml water, 0.5 g/L ascorbic acid, and 120 g cereal) modified from Poinar (1978). Eggs were washed with 70% ethanol for 3 min, then washed with sterilized deionized water three times, and finally transferred onto a dry autoclaved filter paper to remove excess water. These eggs were later transferred to 150 g of the artificial diet medium in a 600 ml glass bottle with an autoclavable plastic lid, and the lid was loosely closed for aeration. The *G. mellonella* larvae that were non-aseptically cultured on the artificial diet were defined as xenic larvae.

Axenic *S. litura* larvae were cultured on a modified artificial diet in which sorbic acid, p-hydroxybenzoic acid, formalin, and L-ascorbic acid were omitted from the original recipe (Okada, 1977). This autoclaved artificial diet was mixed well with 5 ml of 0.6% L-ascorbic acid solution (0.22 µm-filter-sterilized) before solidification. Eggs were surface sterilized by washing with 70% ethanol for 6 min, followed by soaking in a solution comprising 70% ethanol and 0.1% mercuric chloride for 3 min (Kamano, 1973). Surface-sterilized eggs were washed with autoclaved distilled water three times, and were transferred onto a piece of sterile filter paper, which was then placed on the axenic artificial diet (200 g) in a 600 ml glass bottle with an autoclavable plastic lid, and the lid was loosely closed for aeration. Xenic *S. litura* was non-aseptically cultured on the original artificial diet as described by Okada (1977).

Axenicity of the axenic insect culture was confirmed by streaking a portion of the artificial diet and feces from the insect culture onto a yeast extract-peptone medium plate.

Production of axenic IJs:

An axenic liquid static culture of *S. carpocapsae* was achieved as described by Fuchi *et al.* (2016). Briefly, first generation gravid females obtained 5 days after IJs were inoculated to DFA were axenized by soaking in a solution containing 5 mg of amikacin sulfate (Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan) and penicillin G sodium salt (Sigma-Aldrich Co. LLC., Tokyo, Japan) for 24 h, and then transferred to a base medium modified from Cryan *et al.* (1963) (4% soy peptone, 3% yeast extract, and 10% heated liver extract) for hatching. Approximately 200 juveniles from gravid females were transferred into a 6 cm Petri dish containing 2 ml of the base medium and an autoclaved *G. mellonella* larval cadaver (approximately 100 mg) that was infected with *S. carpocapsae*. Nematodes were then statically cultured in the dark at 25℃. A monoxenic culture was established by transferring approximately 200 hatched juveniles to DFA seeded with their symbiotic bacteria for comparison.

Invasion assay:

Invasion was evaluated by using the filter paper method (Ishibashi and Takii, 1993). Approximately 100 of the axenic or monoxenic IJs suspended in 0.4 ml of 1/2 insect saline (0.35 g Na₂HPO₄, 0.15 g KH₂PO₄, 0.2 g NaCl, 100 ml deionized water) were inoculated into a Petri dish (inner diam. 5.3 cm) containing a piece of filter paper (No.1, diam. 5.5 cm) (Advantec Toyo Kaisha Ltd., Tokyo, Japan) placed at the bottom of the dish. Then, the final instar of a xenic insect larva was released into the dish, and the dish was incubated in the dark at 25℃. The number of invading nematodes was counted every 3 h for 24 h. Before counting the invaded nematodes in the insects, the body surface of each insect was well washed with deionized water to remove IJs attached onto the surface. Then, the insect was dissected from the posterior to the anterior end by using a pair of scissors, and was floated in 1/2 insect saline with the dissected side facing down. The number of nematodes was counted after 24 h. Ten replicates were used and the experiments were repeated twice.

Mortality assay:

Mortality was evaluated using 50 IJs as an inoculum and axenic or xenic insects as described above. Three hours after releasing a single axenic or xenic insect larva into a Petri dish containing 50 axenic or monoxenic IJs, the larva was washed with 0.01% formalin solution, transferred to a new Petri dish without nematodes, and incubated at 25℃. Mortality was recorded every 24 h for 8 days. Ten replicates were used and the experiments were repeated three times.

Development and propagation of nematodes in insects:

Nematode development and propagation in insects were observed under a stereomicroscope after the dissection of inoculated insects. Fifty IJs were inoculated per plate by the filter paper method as described above. The insects were dissected every 2 days, and the nematodes in an insect were recovered as described above. Axenic *G. mellonella* or *S. litura* larvae were inoculated with axenic IJs, and xenic insects were inoculated with monoxenic IJs.

Autopsy examination:

Changes in the internal appearance of nematode-infected insects were compared with uninfected ones. Nematode-infected or uninfected insects were dissected along a ventral mid-body line every 24 h for 7 days after the inoculation of axenic or monoxenic IJs for nematode-infected insects, and 1/2 saline without nematodes was used for uninfected controls as described above. Changes in the digestive organ and fat body were recorded every 24 h. Hemolymph was collected by cutting one of the forelegs, and its melanization was examined by observing the color of bled hemolymph on a glass slide.

RESULTS

Infectivity and insect mortality:

The invasion rate of IJs obtained by using the axenic culture showed a tendency to be lower than those using the monoxenic culture in both the xenic insect larvae (Fig. 1).

Insect mortality reached 100% in both axenic and xenic insects inoculated using axenic and monoxenic IJs; however, the mortality fluctuation differed between the combinations (Fig. 2). Mortalities of the xenic *G. mellonella* and *S. litura* larvae caused by monoxenic IJs reached 100% at days 2 and 3, respectively; however, in both xenic insects, 100% mortality caused by axenic IJs was delayed (Figs. 2A, 2B). Similarly, mortalities of the axenic *G. mellonella* and *S. litura* larvae caused by monoxenic IJs reached 100%; whereas in both axenic insects 100% mortality caused by axenic IJs was also delayed (Figs. 2C, 2D).

Development and propagation of nematodes in insects:

Axenic nematodes grew in axenic insects; however, their growth was delayed as compared to that of
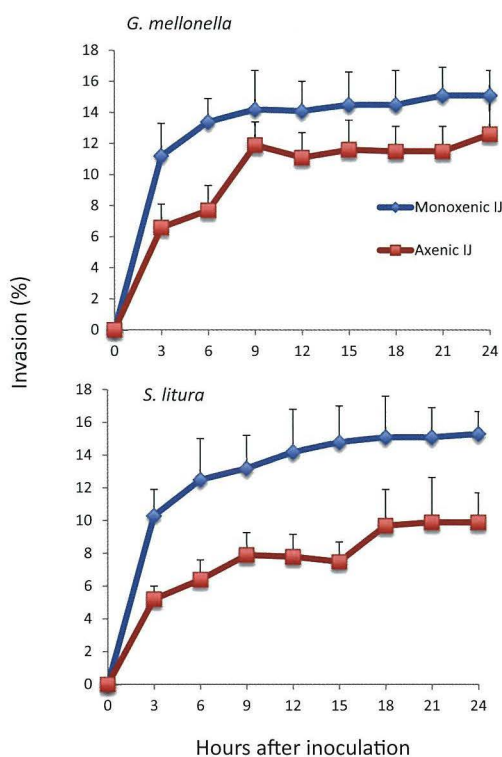

Fig. 1. Invasion rate of xenic and axenic infective juveniles (IJs) of *Steinernema carpocapsae* in the last instar larvae of xenic *Galleria mellonella* or *Spodoptera litura*. Each value is the mean of 20 replications with standard deviation (vertical line).
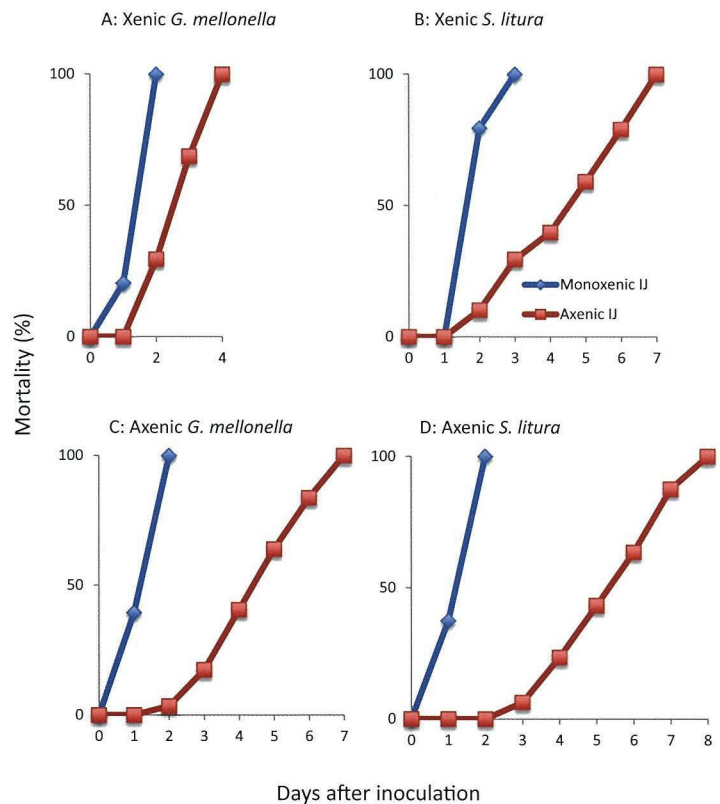
Fig. 2. Mortality of the last instar larvae of xenic and axenic *Galleria mellonella* or *Spodoptera litura* after inoculation with infective juveniles (IJs) of *Steinernema carpocapsae*. Each value is the mean of 30 replications with standard deviation (vertical line).

monoxenic nematodes. In both xenic insect species, the first generation adults were observed 2 days after inoculation of monoxenic IJs, whereas those of axenic nematodes were observed in both axenic insects 4 days after inoculation. Offspring were observed in all xenic insects infected with monoxenic nematodes at day 4, whereas in axenic *G. mellonella* and *S. litura* larvae infected with axenic nematodes, offspring were observed at days 10 and 12, respectively. IJs produced in xenic insects infected with monoxenic nematodes were observed at days 8 and 10 for *G. mellonella* and *S. litura*, respectively, while those in axenic insects infected with

axenic nematodes were observed at days 14 and 16 for *G. mellonella* and *S. litura*, respectively.

Autopsy examination:

The insect cadavers that were infected with monoxenic IJs displayed a yellowish-brown color (Figs. 3Ab, 3Cb), whereas the body color of axenic insect larvae infected with axenic IJs did not change in both insect hosts (Figs. 3Aa, 3Ca). Tissue destruction of the fat body was observed in *G. mellonella* by 2 days and in *S. litura* between 2 and 3 days after inoculation into the cadaver infected with monoxenic IJs, whereas the onset of tissue
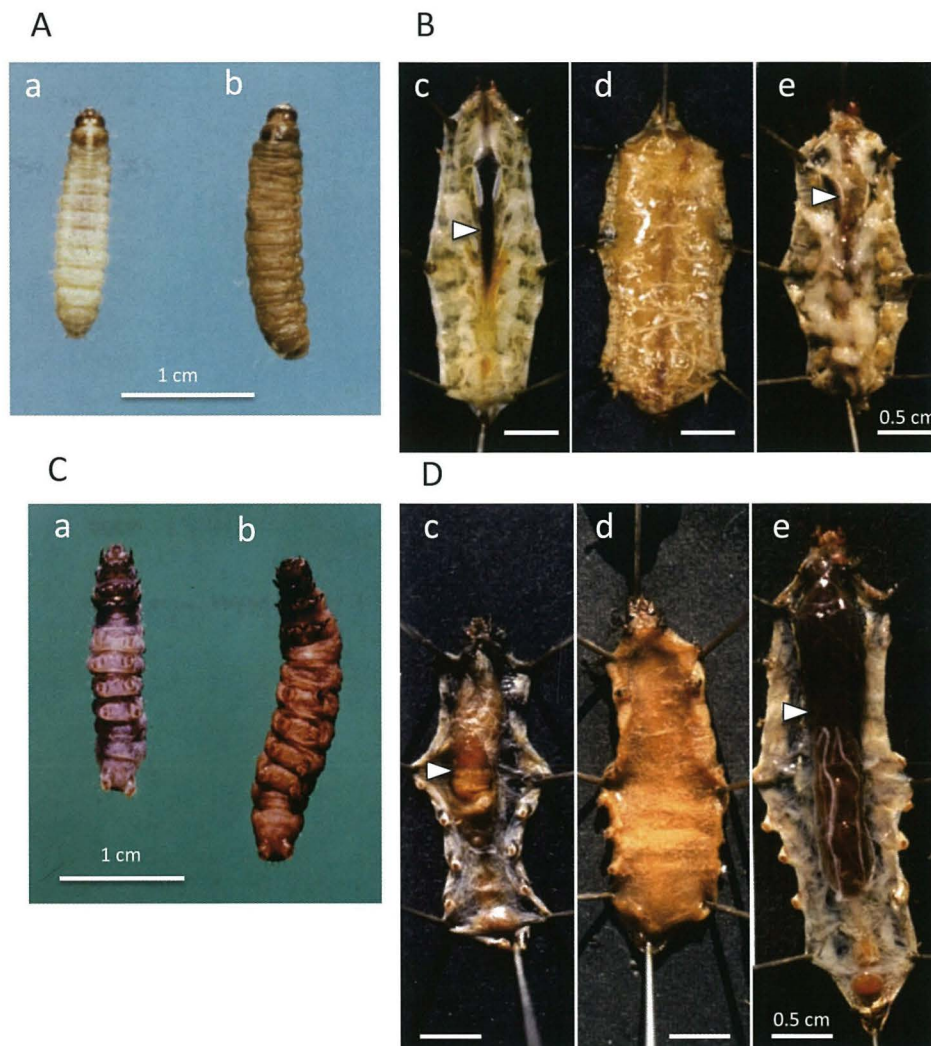

Fig. 3. Intact and dissected last instar larvae of *Galleria mellonella* larvae (A and B) and *Spodoptera litura* (C and D) after inoculation with infective juveniles (IJs) of *Steinernema carpocapsae*. A: Dorsal views of an axenic *G. mellonella* larva inoculated with axenic IJs (a) and xenic one with monoxenic IJs (b). B: Comparison of internal body organs of axenic *G. mellonella* inoculated with axenic IJs (c) and xenic one with monoxenic IJs (d), and non-inoculated xenic control (e). C: Ventral views of an axenic *S. litura* larva inoculated with axenic IJs (a) and a xenic larva with monoxenic IJs (b). D: Comparison of internal body organs of axenic *S. litura* inoculated with axenic IJs (c) and xenic one with monoxenic IJs (d), and non-inoculated control (e). Photographs B and D were taken 7 days and 8 days after inoculation, respectively. The arrowhead indicates intestine.

destruction was observed 2 days after the inoculation of axenic IJs for axenic *G. mellonella* and after the death of axenic *S. litura*. The midgut and Malpighian tubules of larvae infected with monoxenic IJs were destroyed by the propagation of nematodes in the dead larvae (Figs. 3Bd, 3Dd), and the degraded tissues and hemolymph were mixed, whereas axenic insects infected with axenic IJs were not severely damaged, even after 7 days of nematode inoculation. The damage of Malpighian tubules was not observed in *G. mellonella*, but those were partly damaged in *S. litura*. The midgut of *G. mellonella* appeared blackened (Fig. 3Bc) as compared with that of the uninfected midgut (Fig. 3Be), whereas the midgut of *S. litura* appeared faded (Fig. 3Dc) as compared with that of the uninfected midgut (Fig. 3De).

Melanization was not observed in the hemolymph infected with monoxenic IJs; similar observations were made in *S. litura* until 2 days after inoculation. In the axenic larvae infected with axenic IJs, melanization was observed until 4 days after inoculation for *G. mellonella* and 6 days for *S. litura*. Melanization was not observed in the dead larvae in both cases.

DISCUSSION

We examined the pathogenicity of axenic IJs produced using the axenic medium supplemented with a nematode-infected insect larva. The axenic *S. carpocapsae* IJs showed pathogenicity to *G. mellonella* larvae, which is consistent with the results by Han and Ehlers (2000), and other reports additionally suggest the potential of axenic steinernematid IJs to kill insects (Burman, 1982; Ehlers *et al.*, 1997). To confirm the pathogenicity of axenic IJs, we also used *S. litura*, which is less susceptible to *S. carpocapsae* in comparison with the highly susceptible *G. mellonella* (Kondo and Ishibashi, 1988), and demonstrated that axenic IJs showed pathogenicity to *S. litura*, as well. Interestingly, in the present study, we demonstrated that there was a difference in invasion and mortality rates between the monoxenic and axenic IJs in both insects: the invasion rate showed a tendency to be higher and the time taken to kill the insects was faster in monoxenic IJs. These results suggest that the presence of symbiotic bacteria in the intestine of IJs influences the behavior of IJs, resulting in a higher penetration rate and stronger pathogenicity of monoxenic IJs. Even though there was a small possibility that the difference in culture conditions used to produce IJs affected the penetration and insect mortality rates, there was no difference between the growth of adults in the axenic and monoxenic cultures, and a large number

of IJs were obtained by using the axenic culture (Fuchi *et al.*, personal observation).

The time taken for insect mortality due to axenic IJs differed between xenic and axenic insect larvae; xenic insects were killed faster than axenic insects, particularly in *G. mellonella* larvae. This phenomenon implies the possibility that insect mortality by IJs is affected by the microorganisms naturally associated with insects, although there is no information on the effects of naturally associated microorganisms on insect host mortality in nematode pathogenicity. In the Gram-positive bacterium *Bacillus thuringiensis* Berliner, which is an opportunistic insect pathogen, *B. thuringiensis*-induced mortality of larvae of the gypsy moth is reported to depend on enteric bacteria (Broderick *et al.*, 2006). IJs of *S. carpocapsae* infect through natural openings such as the mouth and anus, and enter into the insect hemocoel by penetration through the gut of host insects where there are many enteric bacteria. Although *S. carpocapsae* itself has pathogenicity, the enteric bacteria could additionally be introduced by the IJs during the penetration, or the opportunistic bacteria in insects could infect the insects weakened by nematode infection, which resulted in the difference in the time taken for mortality.

Han and Ehlers (2000) reported the liquefaction of hemolymph in the *G. mellonella* larvae treated with axenic *S. carpocapsae*, which is probably a phenomenon similar to the degradation of the *G. mellonella* fat body observed in the current study. On the other hand, midgut and Malpighian tubules of *G. mellonella* and the Malpighian tubules and the fat body of *S. litura* were not severely damaged by the axenic nematodes before the death of insects, which suggests that the degradation of insect tissues infected by the entomopathogenic nematodes with symbiotic bacteria largely depends on their symbiotic bacteria, and the nematode's ability to degrade is low. The partial degradation of the fat body in *G. mellonella* and the Malpighian tubules in *S. litura* as well as the color changes of the midgut in both insects by axenic nematodes suggests that *S. carpocapsae* is able to partly digest those tissues without their symbiotic bacteria and/or these changes were induced by the insect itself in the presence of the nematode. Hemolymph melanization was observed in the insects infected with axenic nematodes until the death of the insect, whereas that was not the case in those infected with monoxenic nematodes. This result suggests that the phenoloxidase cascade, which is an important insect defense system (Eleftherianos and Revenis, 2011), is not suppressed by the nematode itself.

ACKNOWLEDGEMENTS

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英文報文（原著論文）の和文摘要

**無菌の昆虫病原性線虫 *Steinernema carpocapsae* のハチノ
スツヅリガおよびハスモンヨトウ幼虫に対する病原性**

渕　通則・小野雅弥・近藤栄造・吉賀豊司　‥‥‥‥‥‥ 39

　昆虫病原性線虫 *Steinernema carpocapsae* の病原性は共生
細菌 *Xenorhabdus nematophila* に大きく依存する。線虫自
体の病原性や昆虫組織への影響を理解するため、線虫の侵
入、昆虫の死亡率、昆虫体内での線虫の発育、および昆虫
組織への線虫感染の影響について、無菌および保菌線虫の
間で比較した。無菌感染態幼虫の侵入率は、保菌線虫より
も低かった。無菌および保菌線虫は、無菌および無菌化し
ていないハチノスツヅリガおよびハスモンヨトウ幼虫の両
方を殺した。しかし、無菌線虫は、保菌線虫に比べて殺虫
に時間がかかり、無菌昆虫の殺虫により時間がかかった。
無菌線虫は昆虫体内で発育し、増殖したが、保菌線虫に比
べて発育や増殖は遅れた。無菌線虫が感染した昆虫組織の
崩壊は、保菌線虫が感染した幼虫に比べて遅く、昆虫体液
のメラニン化の抑制は無菌の線虫では認められなかった。
以上、*S. carpocapsae* は細菌の非存在下でも殺虫力および
昆虫組織の分解力を有するが、保菌線虫に比べて無菌線虫
は病原性が低いことが示唆された。