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DEVELOPMENT AND PATHOGENICITY OF INFECTIVE JUVENILES ORIGINATING VIA *ENDOTOKIA MATRICIDA* IN AXENIC STEINERNEMATID NEMATODES

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ABSTRACT

Development and insecticidal activity of axenic infective juveniles (IJs) originating from endotokia matricida in maternal bodies of ***Steinernema glaseri*** *and* ***S. carpocapsae*** *were investigated with a comparison to IJs developed in monoxenic culture. In comparison with the monoxenic steinernematids, the axenic ones grew slower and produced fewer IJs when they were cultured in a sterile chicken liver extract medium supplement with an autoclaved nematode-infected* ***Galleria mellonella*** *larva. The phenomena of endotokia matricida, an intra-uterine development of hatched juvenile, occurred in an axenic culture as did the monoxenic ones. Although it occurred faster in monoxenic culture, the ratio of females bearing endotokia matricida was more numerous in axenic ones. These axenic females also produced IJs through the endotokia matricida phenomenon. Compared to the normal IJs develop in monoxenic culture, the IJs originated via endotokia matricida of axenic nematodes showed lower insecticidal activity and it could not reproduce in G. mellonella cadaver.*

Key words: *aseptic culture, endotokia matricida, insect mortality, intra-uterine development, monoxenic culture, nematode invasion,* ***Steinernema carpocapsae*****,** ***Steinernema glaseri***

INTRODUCTION

Investigation on *endotokia matricida,* an intra-uterine development of juveniles*,* has attracted recent interesting as an alternatively post-embryonic development strategy of entomopathogenic nematodes (EPNs) in the family Heterorhabditidae and Steinernematidae (Baliadi *et al*., 2001; Johnigk and Ehlers, 1999b). The main function of this intra-uterine birth is to provide optimal conditions for infective juveniles (IJs) development when the environmental conditions are becoming detrimental (Serycynska *et al*., 1974; Baliadi *et al*., 2001). Low food supply, high population densities, non-functional vulva, and nematode ageing (Johnigk and Ehlers, 1999a; Kondo and Ishibashi, 1991; Poinar, 1990) have been found to induce the forming of this phenomenon.

Nematodes grown axenically in defined medium have proved to be a valuable tool for the determination of nutrient requirements of the nematodes, infective juvenile (IJs) recovery and formation and for pathogenicity bioassays in the absence of bacteria cells and their metabolic activities (Han and Ehlers, 2000; Hansen and Cryan, 1966). Axenization on Steinernema nematodes have been conducted so far in order to elucidate the role of their symbiotic bacteria *Xenorhabdus* spp. on nematode reproduction (Despommier and Jackson, 1972; Gerritsen *et al.,* 1992; Poinar and Thomas, 1966) and/or pathogenicity of the nematode-bacterium complexes (Boemare *et al*., 1982; Ehlers *et al.,* 1997; Peter and Ehlers, 1997; Serycynska *et al.,* 1974). However, none of above axenization studies focused on the development of *endotokia matricida*.

The pathogenicity of IJs depends also on their physiological state. As they are non-feeding, IJs quality is rely upon the storage lipids and bacterial symbiont (Gerritsen *et al.,* 1998; Han and Ehlers, 2000; Selvan *et al*., 1993; Baliadi *et al*., 2003). A positive correlation has been found between monoxenic IJs produced via *endotokia matricida* and low of pathogenicity of EPNs (Baliadi *et al*., 2003). A bioassay based on the number of nematode invades *Galleria mellonella* can be used for evaluating the pathogenicity of axenic IJs originated via *endotokia matricida*.

The present study was performed to elucidate the role of symbiotic bacteria in the development of *endotokia matricida* of *Steinernema glaseri*, and *S. carpocapsae* in axenic culture and to measure the pathogenicity of the normal IJs developed in axenic media and of the IJs originated via *endotokia matricida* of the axenic nematodes.

MATERIALS AND METHODS

Nematodes and bacterial cultures: *In-vivo* culture of *S. glaseri* and *S. carpocapsae* was carried out on the last instar of *G. mellonella* (Dutky *et al.,* 1964). The IJs were harvested from the insect cadaver using white traps and stored in sterile distilled water at 25° C (Woodring and

Kaya, 1988) for 12 hr before use. The symbiotic bacteria were isolated by external sterilization of the IJs, crushing, and streaking on agar plates (Akhurst, 1983).

Axenic nematode: Female adults of *S. glaseri* and *S. carpocapsae* obtained from 4 to 6 day-old *G. mellonella* cadavers infected with ca. 250 IJs were surface-sterilized in mixed antibiotic solution of amikasin and streptomycin sulfate (5 mg each/ml) for 24 hr. These surface-sterilized nematodes were then used in further experiments.

To obtain axenic IJs originate via *endotokia matricida*, the female adults developed in axenic media bearing *endotokia matricida* (6–7 days after inoculation) were kept in shallow physiologically balance Ringer's solution to allow the intra-uterine developmental IJs migrated from maternal cadavers. The originating juveniles were treated with 0.1% sodium dodecyl sulphate for 20 min for killing the parasitic juveniles.

Microscopic Observations of Nematode Development and *Endotokia Matricida*

Nematode development: Three each surface-sterilized adult females of *S. glaseri* and *S. carpocapsae* were inoculated into an axenic chicken liver extract medium (soy peptone 4 g, heated chicken liver extract 10 ml, deionized water 90 ml) supplemented with an autoclaved nematode-infected larva of *G. mellonella* (Kondo and Fuchi, 1998) and into a monoxenic medium (a dog-food agar plates pre-inoculated with symbiotic bacteria). In addition, sufficient time was elapse to ensure the female is mated. The axenic and monoxenic cultures were examined for the period of 144 hr for the nematode development and the beginning of *endotokia matricida*.

***Change in the rate of* endotokia matricida**: Following after the experimental noted above, the ratio of adult females bearing *endotokia matricida* was examined at 24 hr intervals for 12 days after nematode inoculation. At each period, nematode culture plates examined for the rate of adult females bearing *endotokia matricida*. For nematode counting, serial dilution was employed if necessary. The progress of *endotokia matricida* was examined at an hour interval for 24 hr after the incubation of 40 females in petri dishes (3 cm in inner diam.) containing 2 ml Ringer's solution. The experiment was repeated four times with four replicates.

Juvenile production: Juvenile production through *endotokia matricida* by the 1st and 2nd generation females of *S. glaseri* and *S. carpocapsae* was investigated. The juveniles were classified into parasitic and infective stage juveniles based on their size and morphological characteristics (Johnigk and Ehlers, 1999a). The experiment was performed four times with four replicates.

Yields of infective juvenile: Total IJs produced by the axenic and monoxenic nematode of *S. glaseri* and *S. carpocapsae* was counted 21 days after nematode inoculation of three surface-sterilized adult females. The experiment was performed four times with four replicates.

Pathogenicity of Infective Juveniles Produced Via *Endotokia Matricida* of Axenic Nematodes

Nematode invasion and insect mortality: The pathogenicity was determined using the methods of Woodring and Kaya (1988) with modification. Briefly, *G. mellonella* larvae were placed in petri dishes (5 cm in inner diam.) padded with filter paper. About ca. 250 IJs produced in axenic and monoxenic media and those originated via *endotokia matricida* of axenic and monoxenic female adults suspended in 0.4 ml of 0.01% formalin solution were applied per petri dish and maintained at 25° C. The pathogenicity was examined by measuring the invasion rate and the lethal effect of invading nematodes towards *G. mellonella* larvae. The invasion rate was assessed for 24 hr at 2 hr interval by dissecting five insect larvae at the point time and for 9 days at 1-day interval after inoculation. The experiments were performed four times with four replicates.

Production of progenies: Dead *G. mellonella* larvae were removed from the petri dishes, rinsed with sterile water to eliminate nematodes from insect surfaces, transferred individually onto a filter paper stripe moistened with 0.01% ($^{v}/_{v}$) formalin solution in a test tube. Test tubes with infected cadaver were placed in tightly sealed box, and incubated at 25° C. After 21 days, the IJs emerging on the inner wall of the test tubes and on the surface of filter paper stripe were collected and counted. The experiments were performed four times with four replicates.

Statistical analysis: Significance of mean differences of ratio of females bearing *endotokia matricida* and number of IJs yielded was determined using Studenized *t* test. Significance of mean differences of nematode invasion and insect mortality was subjected to analysis of variance (ANOVA), and multiple comparisons of mean between treatmens were evaluated with the DUNCAN multiple range test (DMRT test).

RESULTS

Microscopic Observation of Nematode Development and *Endotokia Matricida*

Nematode development: Figure 1 shows the eggs laid by the surface-sterilized gravid females of *S. glaseri* and *S. carpocapsae* in axenic medium hatched, moulted into the 2nd, 3rd, and 4th stage juveniles at periods 12 to 48 hr after inoculation. The immature and mature females *S. glaseri* developed faster than *S. carpocapsae*, although they showed the beginning of *endotokia matricida* at the same time (about 144 hr after inoculation). In comparison, the development of monoxenic nematodes included the beginning of the *endotokia matricida* was faster than that of axenic ones.

***Change in rate of* endotokia matricida**: When the females of axenic *S. glaseri* and *S. carpocapsae* had matured, it was full of eggs. While the females continued the egg lying period, about 6–7 days after inoculation, 1st stage juveniles hatched inside the ovaries of few females (Fig. 2). A rapid increased in proportion of females bearing *endotokia matricida* in the axenic population began 8–9 days after inoculation, reaching a level of 18% for *S. glaseri* and 14% for *S. carpocapsae* in which the rate was started higher than that monoxenic females. At 12 days after inoculation, the rate of axenic females showing *endotokia matricida* of *S. glaseri* and *S. carpocapsae* was 6 and 4% higher than those of monoxenic ones. In addition, in monoxenic cultures, the nematode continued their development into the 3rd or even the 4th generation, but development in axenic cultures mostly ceased at the 2nd or the 3rd generation.

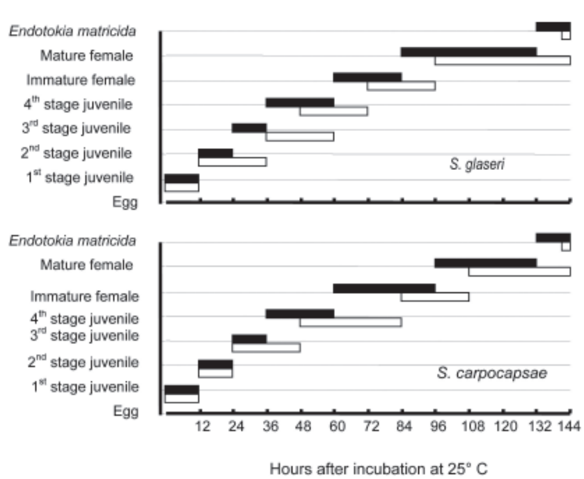

Figure 1. Nematode development and occurrence of endotokia matricida in the 1st generation females of *S. glaseri* and *S. carpocapsae* cultured axenically (■) and monoxenically (□)

The progress of *endotokia matricida* of *Steinernema* spp. grew axenically and monoxenically occurred through a similar manner, although the progress in axenic nematode was faster than in monoxenic ones.

Juvenile production: The number of an axenic intra-uterine juveniles produced by the 1st generation females was significantly higher ($p < 0.05$, *t* test) than that of the 2nd generation ones (Table 1).

For instance, it was ca. 18.6 and 56.4 for the 1st generation females and ca. 6.8 and 32.4 for the 2nd generation females of *S. glaseri* and *S. carpocapsae*. These value was significantly smaller than that produced by the monoxenic nematodes, particularly on the 1st nematode generations. The ratio of the axenic IJs to the total number of juveniles produced by the 1st generation females was higher than that of monoxenic ones. The ratio was ca. 47.8 and 61.9% for axenic and ca. 40.9 and 37.7% for the monoxenic *S. glaseri* and *S. carpocapsae*, respectively. These greater ratios were more markedly in the 2nd generation adults. These axenic IJs corresponded to IJs produced in monoxenic culture in body size and structure.

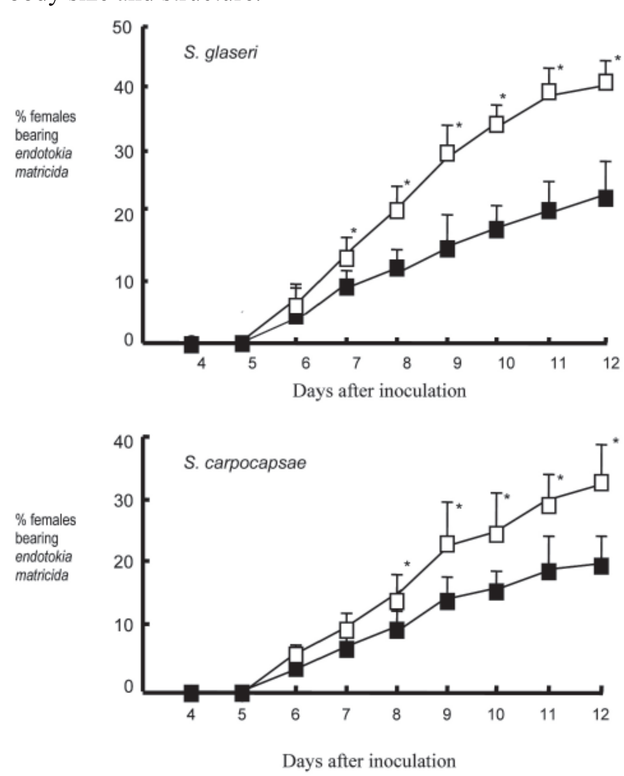

Figure 2. Occurrence of endotokia matricida in female adults of *S. glaseri* and *S. carpocapsae* cultured axenically (□) and monoxenically (■). The ratio of female adults bearing endotokia matricida was examined at 24 hr intervals for 12 days after nematode incubation at 25° C. Data are the mean of four replicates with standard deviation. Asteriks indicate statistical difference between the two culture conditions (*t test*, $p < 0.05$).

Yields of infective juveniles: The nematode of *S. glaseri* and *S. carpocapsae* grew and reproduced in axenic media supplemented with an autoclaved nematode-infected larva of *G. mellonella*. The yield of IJs in axenic media was significantly lower ($p < 0.05$, *t* test) than that of monoxenic dog-food media seeded with *Xenorhabdus* spp. (Figure 3). Fourteen days after nematode inoculation number of axenic *S. glaseri* IJs per petri dish was 38% of monoxenic cultures (ca. 34,000) and it was 37% for *S. carpocapsae*.

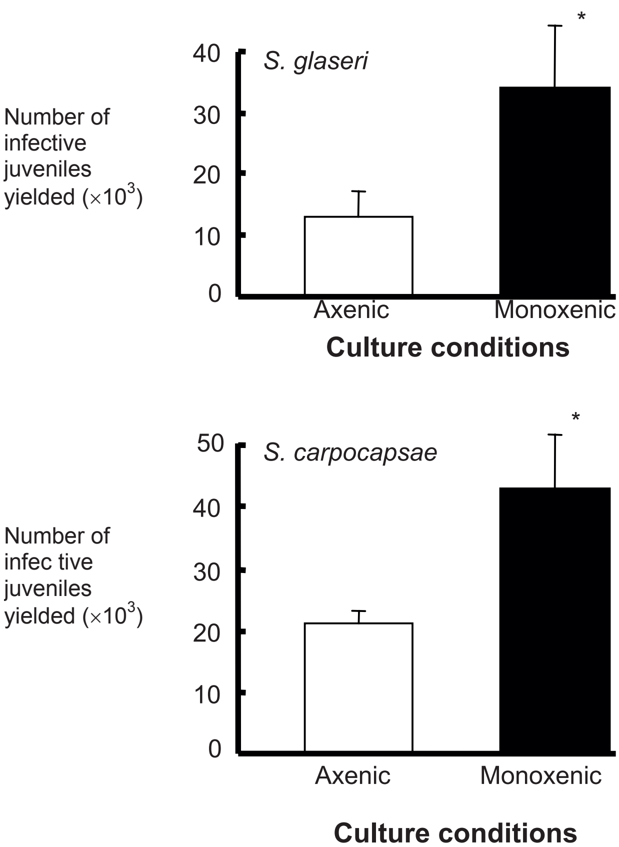

Figure 3. Yields of infective juveniles of S. glaseri and S. carpocapsae cultured axenically and monoxenically for 21 days after nematode incubation at 25° C. Data are the mean of four replicates with standard deviation. Asterisks indicate statistical difference between the two culture conditions (*t test*, $p < 0.05$)

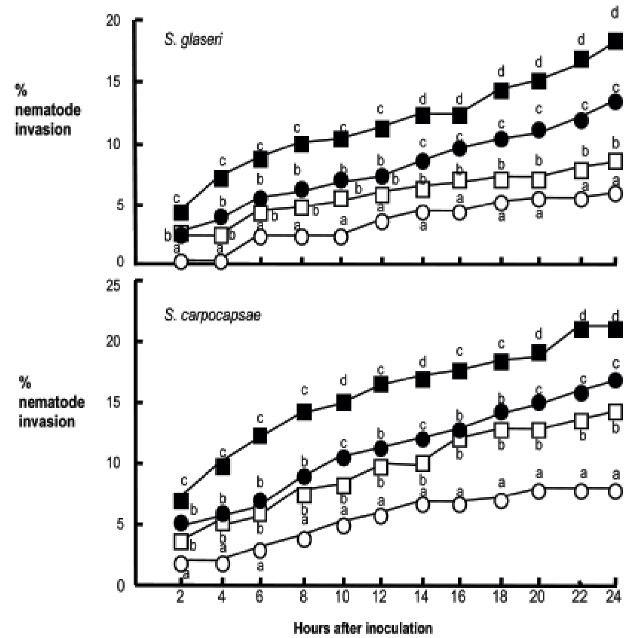

Figure 4. Nematode invasion to *G. mellonella* larvae inoculated with infective juveniles (IJs) originated via and not via *endotokia matricida*. Values followed by the same letters in each observation period re not significantly different (DMRT test, $p < 0.05$)

○ Axenic IJs originated via *endotokia matricida*
● Monoxenic IJs originated via *endotokia matricida*
□ Axenic IJs originated not via *endotokia matricida*
■ Monoxenic IJs originated not via *endotokia matricida*

Nematode invasion and insect mortality: The number of nematodes invades increased with the increase of post-inoculation time. The significantly lowest ($p < 0.05$, DMRT test) percentage of nematode invasion was for IJs that originated via *endotokia matricida*, followed by IJs developed on axenic media, originated via *endotokia matricida* of monoxenic female adults, and that developed on monoxenic dog-food agar media.

The values at 24 hr after nematode inoculation was 5; 6; 8; 13; 2; and 18% for *S. glaseri* and it was 8; 14; 16; and 21% for *S. carpocapsae* (Figure 4).

Table 1. Intra-uterine development of parasitic juveniles (PJs) and infective ones (IJs) in the 1st and 2nd generation females of *S. glaseri* and *S. carpocapsae* cultured axenically and monoxenically.

Nematode species	Culture conditions	1st generation females		2nd generation females	
		PJs	IJs	PJs	IJs
S. glaseri	Axenic	9.7 ± 3.4 a	8.9 ± 4.0 a	2.4 ± 1.8 a	4.4 ± 2.5 a
	Monoxenic	37.9 ± 8.2 c	26.3 ± 6.6 b	5.7 ± 2.4 a	6.7 ± 3.2 a
S. carpocapsae	Axenic	21.5 ± 6.0 b	34.9 ± 9.3 c	10.2 ± 3.6 b	22.2 ± 7.2 b
	Monoxenic	80.2 ± 18.4 d	48.6 ± 10.2 d	22.2 ± 5.8 c	30.4 ± 8.4 c

Data are the mean of 40 females with standard deviation. Values in the same column followed by the same letter are not significantly different ($p > 0.05$) according to a DMRT-test.

In *G. mellonella* ca. 250 axenic nematodes of *S. carpocapsae* caused significantly lowest ($p < 0.05$, ANOVA) mortality (40%) at 4 days after nematode inoculation (Figure 5) where as it was 18, 75 and 100% for IJs originated via *endotokia matricida* of axenic females, IJs originated via *endotokia matricida* of monoxenic females and normal IJs developed on monoxenic media, respectively. In contrast, no mortality was counted on *G. mellonella* infested either of normal IJs developed in axenic media or of IJs originated via *endotokia matricida* of axenic females.

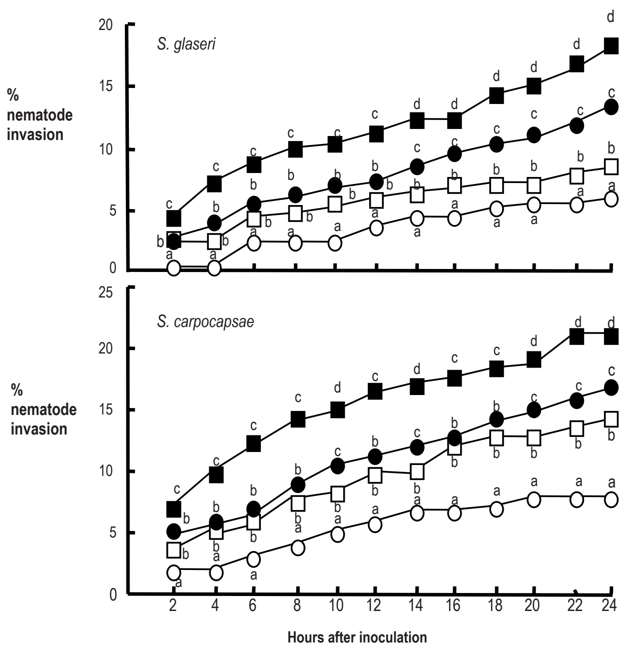

Figure 5. Mortality of G. mellonella larvae inoculated with infective juveniles (IJs) originated via and not via endotokia matricida. For the explanation of symbols, see Figure 4

Production of progenies in G. mellonella larvae: Once inside the body of *G. mellonella* larvae, axenic nematodes followed a similar growth pattern with monoxenic ones. However the former grew significantly more slowly than the latter. Interestingly at 21 days after nematode inoculation, no progenies were obtained from the axenic *S. glaseri* or *S. carpocapsae* infested-*G. mellonella* cadavers.

DISCUSSION

S. carpocapsae and *S. glaseri* developed axenically in media containing a sterile nematode-infected *G. mellonella* larva exhibited many similar growth patterns as on monoxenic media. Some axenic females produced offspring while other had only intra-uterine hatched juveniles or unfertile females, indicating a complete life cycle with progeny can be obtained for *Steinernema* spp. without any digestive contribution of symbionts (Han and Ehlers, 2000; Kondo and Fuchi, 1998). *Steinernema* spp. was not produced on axenic media in a yield comparable to the monoxenic conditions. However they are viable during several generations. These results confirm although the symbiont is clearly not essential for the *Steinernema* spp. reproduction (Akhurst, 1983), in axenic conditions the most suitable menu which usually prepared by the symbionts for their reproduction was not existing. In the present study, some females could not well develop due to the lack of food or mated with the male. In contrast to steinernematids, *H. bacteriophora* females were unable to produce offspring in the same axenic media even though some female adults developed (unpublished data). The symbiotic interaction between *P. luminescens* and *H. bacteriophora* seems to be more specific. Attempts to establish axenic cultures of *Heterorhabditis* spp. have so far been unsuccessful (Lunau *et al.,* 1993), because a substitute diet has not been discovered yet for *Heterorhabditis* spp. (Boemare *et al*., 1997).

As in monoxenic nematodes, females of axenic nematodes lay eggs initially, but egg lying ceases faster and unlaid eggs develop and hatch inside the ovaries and *endotokia matricida* started. Interestingly, the development of *endotokia matricida* phenomenon is not dependent on the presence of symbiotic bacteria, since it was developed in axenically grown females of *S. carpocapsae* and *S. glaseri*. The precise mechanism of the phenomenon is not yet clearly understood. Anyway, many animal parasitic nematodes, which hatch from eggs as 1st, 2nd, or 3rd stage juveniles, require a specific stimulus to induce them to hatch. Moulting and hatching of free-living nematodes are controlled and coordinated by some internal mechanism in the animal. In monoxenic culture these mechanisms partly depend on the presence of symbiotic bacteria as main constituent of food supply (Johnigk and Ehlers, 1999a). In addition, although there was no evidence for this, it would not be surprising if internal secretion of axenic females may regulate the time of production of substances, which cause *endotokia matricida*. Similar to the monoxenic nematodes, the axenic mother's cadaver also supplied the necessary stimulus to continue development, and some of the juveniles passed out the female intestine, which feed upon the storage materials. The ultimate step of the *endotokia matricida*, emergence period, follows a similar pattern to monoxenic nematodes; i.e., the infective juveniles leave the mother's cadaver together with the 1st and 2nd parasitic or infective stage juveniles through natural openings or transcuticular.

Moreover, due to the restricted food supply, the majority of the intra-uterine birth developed into 2nd stage infective and parasitic juveniles. The mechanism of nutrition for these juveniles has not been studied yet, but it is probable that metabolites within the mother's cadaver are the main food source. In the present study, all the maternal tissues are destroyed and consumed by the intra-uterine developing juveniles. These slightly contrast to the monoxenic conditions where not all of the maternal body tissues consumed during the progress of *endotokia matricida*.

It has been widely accepted that axenic *S. carpocapsae* can kill insect by its insecticidal activity (Han and Ehlers, 2000; Poinar and Thomas, 1966), but no entomopathogenic action is recorded with axenic *S. glaseri*. In the present study, axenic *S. carpocapsae* include those produced via *endotokia matricida* have demonstrated a rather active role of the nematode in the pathogenic process. These results supported the previous results (Han and Ehlers, 2000) in which axenic *S. carpocapsae* could kill the *G. mellonella* larvae. The toxic released by the axenic IJs possibly played in the pathogenic process, at the initial stage of parasitism (Burman, 1982) and Palha *et al*. (1998) found that the maximum toxic activity was obtained with 48 hr after incubation (Palha *et al.,* 1998), the necessary time for the nematode to reach the final development in the 3rd juvenile stage. In contrast, axenic *S. glaseri* could not kill *G. mellonella* larvae because they do not produce such kind of toxin (Akhurst, 1986).

In the present study, although the axenic IJs of *S. carpocapsae* produced in axenic media or originated via *endotokia matricida* kill the *G. mellonella* larvae, developed into adults but did not reproduce as many previous reports (Kondo and Fuchi, 1998; Poinar and Thomas, 1966). Its means, although their toxin alone effectively lethal to insect (Burman, 1982), they could not complete reproduction without the intervention of symbiotic bacteria. Apparently axenic nematodes were unable to utilize the host tissues and fluids as sources without the bacterial bioconservation, as they do on artificial diet (Boemare *et al.,* 1997).

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