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THE CONTINUAL FORMING AND CONTRIBUTION OF INFECTIVE JUVENILES PRODUCED VIA *ENDOTOKIA MATRICIDA* OF ENTOMOPATHOGENIC NEMATODES IN THE FAMILY OF STEINERNEMATIDAE AND HETERORHABDITIDAE

Yuliantoro Baliadi[a], E. Kondo[b], and T. Yoshiga[b]

[a]*Indonesian Legume and Tuber Crops Research Institute, Jalan Raya Kendal Payak, PO Box 66 Malang 65101, Indonesia*
Phone (0341) 801468, Fax. (0341) 801496, E-mail: blitkabi@telkom.net
[b]*Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, Japan*

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ABSTRACT

The non-feeding developmentally arrested infective juveniles (IJs) of entomopathogenic nematodes in the family of Steinernematidae and Heterorhabditidae seek out a susceptible insect host and initiate infections. The aim of the research was to examine the continual forming and contribution of IJs produced via *endotokia matricida* (IJs-EM) of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. carpocapsae*. The research was conducted at the Laboratory of Nematology of the Saga University, Japan (April 2001-April 2002) and the Laboratory of Nematology of the Indonesian Legume and Tuber Crops Research Institute (June 2003-October 2004). The nematode progenies were investigated using the greater wax moth, *Galleria mellonella*, pre-inoculated with 50 IJs at 25°C. Results showed that three reproductive adult generations were observed at day 18[th]. There were 135,000, 128,000 and 133,000 IJs per insect cadaver produced in *H. bacteriophora*, *S. glaseri* and *S. carpocapsae*, respectively. *Endotokia matricida* contributed a higher number of IJs than that of a normal mode of IJs production. The ratios are 81%, 28% and 64% for *H. bacteriophora*, *S. glaseri*, and *S. carpocapsae* of the IJs total production, respectively. Among the generations, the highest contribution of IJs was come from the third adult generation bearing *endotokia matricida*, i.e., 63%, 24% and 51% for the three nematode species. Although the IJs-EM were more transparent compared to the normal IJs, they were morphologically similar. The results show that *endotokia matricida* has a pivotal role in a species maintenance and survival strategy of entomopathogenic nematodes in extreme environmental conditions.

[***Keywords***: Entomophilic nematodes, Steinernematidae, Heterorhabditidae, *endotokia matricida*, infective juveniles]

INTRODUCTION

The entomopathogenic nematodes (EPNs) in the families of Steinernematidae and Heterorhabditidae are the only obligate lethal insect-parasitic nematodes possessing an optimal balance of biological control. They have a broad host range, posses higher ability to search for hosts, recycle in the soil environment, and are harmless to birds and mammals (Burnell and Stock 2000). They also have an extremely broad host range and variation in foraging strategies and host associations which potentially offer the ability to control species with diverse life histories, especially against pests in the soil (Poinar 1990; Campbell and Gaugler 1991). The non-feeding developmentally arrested infective juveniles (IJs) seek out a susceptible insect host and initiate infections. The IJs is the life stage of interest for inundative biological control considerations because it is only one formulated and applied.

Both nematode families are unique among the rhabditis in having an intra-uterine birth of IJs causing maternal death, *endotokia matricida* (Wang and Bedding 1996; Baliadi *et al*. 2001). By employing this alternative route of post-embryonic development, EPNs can optimize their chance of encountering a suitable host without unnecessary waste of limited host food resources (Johnigk and Ehlers 1999a; Baliadi *et al*. 2001). Its function in EPNs is to provide optimal conditions for offspring development when the environmental conditions are becoming detrimental (Johnigk and Ehlers 1999b).

Poinar (1990) and Baliadi *et al*. (2003) demonstrated that IJs produced from *endotokia matricida* (IJs-EM) occupy many morphological and physiological similarities to the normal IJs arising from egg laid by hermaphrodite or female adult. This means that IJs development will also be repeated through the similar phenomenon in the next life cycle (Downes 1995). Therefore, we expect large natural populations of EPNs emerged from any insect cadavers contained IJs-EM (Wang and Bedding 1996; Baliadi *et al*. 2001).

Estimation of IJs-EM population in final yields of IJs progenies is less well studied in EPNs, and reports are sometimes contradictory (Wang and Bedding 1996; Johnigk and Ehlers 1999a; Baliadi *et al*. 2003). The objective of the study was to describe the continual forming of IJs-EM and its contribution to the final yields of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. carpocapsae* using the wax moth larvae, *Galleria mellonella* as a host.

MATERIALS AND METHODS

Three species of EPNs used *were H. bacteriophora* (strain HP88), *S. glaseri* (strain unidentified), and *S. carpocapsae* (strain All). They were maintained at 25°C on larvae of the greater wax moth, *G. mellonella*. The *G. mellonella* were maintained continuously on used bee-comb at 28-30°C. The last instar larvae with an average fresh body weight of 154 ± 45 mg were used.

Nematode inoculation on *G. mellonella* larvae was conducted at 25°C. If not particularly mentioned, *ca*. 50 IJs suspended in 0.4 ml of 0.01% (v/v) formalin solution were deposited onto a filter paper in a plastic Petri dish (5.5 cm in inner diameter) with a *G. mellonella* larvae. To obtain active IJs, all juveniles emerging out of the hosts on a trap were allowed to migrate through a nylon sieve (pore size of 30 µm), then treated with 1% sodium dodecyl sulphate for 20 minutes to eliminate the parasitic forms, and rinsed three times by centrifugation using sterile Ringer's physiological saline solution (NaCl 9.0 g, KCl 0.4 g, $CaCl_2$ 0.4 g, NaH_2CO_3 0.2 g, H_2O 1000 ml) (White 1927).

Dissection of Insects and Examination of Nematode Development and *Endotokia Matricida*

The insect cadavers were gently dissected in Ringer's solution under the microscope. All nematode development stages were identified and counted once outside the parent using serial dilution when necessary. Insect cadavers dissection and nematode counts were carried out at 2-18 days after nematode inoculation on five insect cadavers which had a successful development of nematode populations. Because of the highly viscous nature of cadavers infected with *H. bacteriophora*, dissected cadavers with this species were repeatedly washed with surplus Ringer's solution before counting. Eighteen days after inoculation, each remaining insect cadaver was placed individually on a White trap (White 1927) and the final IJs produced per host cadaver were counted. The experiments were performed three times.

At 5, 8, and 14 days after nematode inoculation, the nematodes were dissected out of the insect cadavers under a dissecting microscope to count the populations of developmental hermaphrodites and adult females with/without *endotokia matricida* of the first, second, and third generations using serial dilution when necessary. Thirty each of adult nematodes bearing *endotokia matricida* were randomly selected from 5-8 host cadavers then transferred individually into Petri dishes (3 cm in inner diameter) containing 2 ml Ringer's solution. The dishes were placed in a polyethylene container (32.5 cm length, 25.0 cm wide and 11.5 cm depth) with a sheet of moist filter paper (Whatmann no. 1) and incubated at 25°C for allowing the IJs-EM emerged out to the solution before counting. The experiments were performed three times.

Physiological Conditions of IJs Produced via *Endotokia Matricida*

To examine the physiological conditions of the resulting IJs-EM, 40 hermaphrodites and adult females of the first, second and third generations collected following the above method were transferred individually into Petri dishes (3 cm inner diameter) containing Ringer's solution. Then, when the first juvenile stage (J1) hatched inside the maternal body (the beginning of *endotokia matricida*), the nematodes were immediately placed in new dishes containing Ringer's solution. The ensheathment (loss of the extracuticle or sheath) ratios of IJs-EM and normally developed outside maternal body (in former Petri dishes) were counted.

For further evaluation of the IJs-EM quality, the recovery of infective juveniles, the moment when the IJs exited from its dauer stage was examined within 48 hours with 2-hour intervals after nematode incubation. A hundred IJs-EM and developed normally outside maternal body were divided into 10 groups then transferred on Petri dishes (5 cm inner diameter) containing nutrient bromothymol agar medium (Woodring and Kaya 1988).

RESULTS AND DISCUSSION

Dissection of Insects and Examination of Nematode Growth and Development and *Endotokia Matricida*

Table 1 show that the infected insect killed rapidly within 24-48 hour. The invading IJs resumed developing, moulted to the J4 stage and reached adulthood within two (*H. bacteriophora*) or three days (*S. glaseri* and *S. carpocapsae*) after nematode inoculation at

25°C. Nematode reproduction continued over two or three generations until the nutrient status of the insect cadavers deteriorated whereupon adult development was suppressed and thousands IJs accumulated at 18 days after nematode inoculation (Table 1). Hermaphrodites of *H. bacteriophora* developed 4 days after nematode inoculation. They laid eggs from day 5th until 7th. All eggs which remained inside the uterus after the period of egg laying developed inside the maternal body. The first hermaphrodites bearing *endotokia matricida* were observed at day 5th. The first IJs recovered from the insect cadavers were examined at day 8th. We suspected it originated from *endotokia matricida*. The second amphimictic females laid some eggs initially but the third females did not deposit any eggs and all of the juveniles developed inside the maternal body. After 10 days, the number of IJs increased significantly due to the emergence from the hermaphrodites and amphimictic females and that from eggs which were laid inside the insect cadavers. The population increased drastically when all of the IJs developed inside the maternal body of the third generation females emerged. The population dynamics of *S. glaseri* and *S. carpocapsae* were different from those found in *H. bacteriophora* (Table 2 and 3).

Estimation of IJs produced via *endotokia matricida* is based on the number of hermaphrodites or female adults developed, bearing *endotokia matricida* at each nematode generation and the number of IJs developed in the maternal body (Table 4). The invading IJs of *H. bacteriophora* gave three adult generations over 18 days in *G. mellonella* larvae, but the last female generation did not oviposit. Sixteen hermaphrodites that developed from the initial IJs retained 498 ± 72 eggs, 48-56% of which developed into IJs within the mother body, but contributed about 3.2% to the final yields of IJs eventually emerged from the insect cadavers. The second generation females derived from the original hermaphrodites also retained an average of 68 ± 16 eggs within the body which 74-86% later became IJs, contributing 14.8% to the total yields of IJs. The third (last) generation females did not oviposit and all of their eggs (38 ± 12 eggs) hatched and developed into IJs via *endotokia matricida*, contributing 58-63% to the total IJs produced (Table 4). From all three generations, about 81% of the final population of IJs were originated via *endotokia matricida* or only 19% were developed normally in insect cadavers.

Contribution of *Steinernema* spp. to the IJs production was different from that of found in *H. bacteriophora*. Although the first generation females produced a higher number of eggs, the number of eggs retained in the body was fewer than that of *H. bacteriophora*. About 25% and 51% of IJs of *S. glaseri* and *S. carpocapsae*, respectively, came from the last generation females, up to 3.4% and 12% or 0.7% of the IJs came from the second or first generation females. Unlike the third generation of *H. bacteriophora* and *S. glaseri*, in which all the eggs retained developed into IJs, a

Table 1. Population dynamics of *Heterorhabditis bacteriophora* in *Galleria mellonella* cadaver at 2-18 days after inoculation by 50 infective juveniles (IJs) per host.

Dai	Number of nematodes of various stages per host							
	Eggs	J1	J2	IJs	J3/J4	Male	H/F	Total
2	0	0	0	18 ± 5	0	0	0	18
3	0	0	0	7 ± 4	8 ± 4	0	3 ± 2	18
4	56 ± 14	34 ± 6	0	4 ± 2	4 ± 3	0	7 ± 3	105
5	244 ± 64	68 ± 23	32 ± 12	0	5 ± 2	0	14 ± 4	363
6	1268 ± 168	1408 ± 152	985 ± 78	0	28 ± 8	0	16 ± 6	3705
7	1096 ± 249	966 ± 89	1544 ± 122	0	142 ± 24	18 ± 4	46 ± 10	3812
8	0	1858 ± 104	1620 ± 146	54 ± 14	294 ± 36	105 ± 24	188 ± 22	4119
9	4136 ± 296	2480 ± 144	2854 ± 243	462 ± 78	560 ± 44	168 ± 28	294 ± 28	11,054
10	3882 ± 124	2906 ± 240	3688 ± 264	3858	2480 ± 104	470 ± 36	982 ± 34	18,266
11	426 ± 34	2636 ± 245	3864 ± 206	11,876	2858 ± 138	922 ± 45	1620 ± 54	24,022
12	0	240 ± 34	2256 ± 152	28,000	3618 ± 180	1226 ± 68	2038 ± 154	37,378
13	0	0	1280 ± 98	38,240	1418 ± 102	1460 ± 83	2206 ± 123	44,604
14	0	0	0	60,060	644 ± 42	852 ± 64	1988 ± 98	71,544
15	0	0	0	121,400	0	446 ± 38	1592 ± 86	123,438
16	0	0	0	134,182	0	246 ± 52	1340 ± 62	135,764
17	0	0	0	141,000	0	0	424 ± 36	141,424
18	0	0	0	135,062	0	0	0	135,062

Data are the mean of four insect cadavers with standard deviation. Dai is days after nematode inoculation. The experiments were performed three times using 10 insect cadavers. H/F is hermaphrodite/female.

Table 2. Population dynamics of *Steinernema glaseri* in *Galleria mellonella* cadaver at 2-18 days after inoculation by 50 infective juveniles (IJs) per host.

Dai	Number of nematodes of various stages per host							
	Eggs	J1	J2	IJs	J3/J4	Male	H/F	Total
2	0	0	0	17 ± 4	0	0	0	17
3	0	0	0	4 ± 2	9 ± 4	2 ± 2	2 ± 1	17
4	358 ± 42	204 ± 18	0	2 ± 4	5 ± 2	5 ± 2	9 ± 2	583
5	438 ± 42	314 ± 36	240 ± 52	2 ± 0	44 ± 10	7 ± 2	12 ± 3	1057
6	3166 ± 204	1208 ± 184	392 ± 34	0	122 ± 24	5 ± 1	13 ± 4	4906
7	4564 ± 186	2988 ± 206	1874 ± 178	0	864 ± 60	43 ± 2	59 ± 2	10,392
8	2614 ± 65	5280 ± 264	2652 ± 240	461	1254 ± 42	84 ± 1	142 ± 2	12,487
9	4124 ± 278	3864 ± 24	2803 ± 167	2021	1472 ± 108	246 ± 1	294 ± 11	14,644
10	7820 ± 234	6436 ± 145	5946 ± 259	2148	2560 ± 134	822 ± 1	1598 ± 14	27,330
11	4104 ± 218	4872 ± 132	4483 ± 184	7944	3284 ± 274	1683 ± 1	2730 ± 45	29,100
12	908 ± 56	2868 ± 82	2853 ± 166	18,041	2475 ± 158	2892 ± 1	1432 ± 45	31,469
13	0	154 ± 12	2374 ± 129	29,415	1962 ± 146	984 ± 1	1032 ± 45	35,921
14	0	28 ± 8	2198 ± 152	51,810	1861 ± 153	583 ± 26	874 ± 11	57,364
15	0	0	1851 ± 68	92,603	1438 ± 174	124 ± 31	698 ± 14	96,714
16	0	0	1560 ± 40	108,000	1059 ± 158	0	332 ± 45	120,951
17	0	0	0	123,094	962 ± 146	0	152 ± 45	124,208
18	0	0	0	128,600	638 ± 74	0	132 ± 45	129,370

Data are the mean of four insect cadavers with standard deviation. Dai is hours after nematode incubation. The experiments were performed three times using 10 insect cadavers. H/F is hermaphrodite/female.

Table 3. Population dynamics of *Steinernema carpocapsae* in *Galleria mellonella* cadaver at 2-18 days after inoculation with 50 infective juveniles (IJs) per host.

Dai	Number of nematodes of various stages per host							
	Eggs	J1	J2	IJs	J3/J4	Male	H/F	Total
2	0	0	0	26 ±5	0	0	0	26
3	0	0	0	8 ±4	15 ± 4	2 ± 1	2 ± 0	27
4	820 ± 52	520 ± 52	0	4 ±0	6 ± 2	4 ± 2	7 ± 3	1361
5	2184 ± 131	1846 ± 122	496 ± 78	2 ±1	296 ± 2	6 ± 2	14 ± 4	4844
6	3948 ± 260	2220 ± 251	2143 ± 141	0	1094 ± 108	108 ± 22	124 ± 202	9637
7	8254 ± 488	9741 ± 260	8322 ± 232	0	2206 ± 182	940 ± 41	1455 ± 82	30,918
8	9253 ± 226	10,406 ± 218	14,063 ± 204	5108	3042 ± 174	2405 ± 120	2604 ± 102	46,881
9	2883 ± 134	3743 ± 194	18,714 ± 190	8420	3265 ± 192	2941 ± 134	2780 ± 92	42,746
10	482 ± 62	2962 ± 186	22,024 ± 188	16,892	4049 ± 260	4023 ± 188	4204 ± 144	54,636
11	44 ± 10	1864 ± 122	36,396 ± 278	44,452	2094 ± 108	2068 ± 104	3768 ± 125	90,668
12	0	981 ± 45	17,143 ± 141	94,860	1806 ± 182	1284 ± 82	1542 ± 88	117,616
13	0	280 ± 26	9140 ± 232	110,782	1442 ± 174	482 ± 26	804 ± 82	120,230
14	0	0	0	122,082	1036 ± 98	63 ± 12	372 ± 40	123,553
15	0	0	0	124,730	844 ± 62	0	46 ± 10	125,620
16	0	0	0	128,286	360 ± 38	0	0	128,646
17	0	0	0	136,664	202 ± 24	0	0	136,866
18	0	0	0	134,000	0	0	0	134,000

Data are the mean of four insect cadavers with standard deviation. Dai is hours after nematode incubation. The experiments were performed three times using 10 insect cadavers. H/F is hermaphrodite/female.

part of juveniles of *S. carpocapsae* which originally retained within their mother developed into parasitic juvenile stages. From three generations, 29% and 64% of the final populations of *S. glaseri* and *S. carpocapsae* IJs were originated via *endotokia matricida* (Table 4).

Physiological Conditions of Infective Juveniles Produced via *Endotokia Matricida*

The loss of extracuticle or sheath was higher on IJs-EM compared to the normal IJs (Fig. 1). In *H. bacteriophora*, the IJs-EM developed inside the first,

Table 4. Estimation of *endotokia matricida* (EM) contribution to the final yields of infective juveniles of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. carpocapsae* in the larvae of *Galleria mellonella* after inoculation with *ca*. 50 IJs per host.

Nematode generation	*H. bacteriophora*	*S. glaseri*	*S. carpocapsae*
First generation			
Total hermaphrodite/female developed	16 ± 5	12 ± 6	10 ± 4
Ratio of hermaphrodite to female bearing EM (%)	54-58	48	52
Ratio of IJs developed per hermaphrodite to female (%)	48-54	42	40
Number of IJs per hermaphrodite/female	268 ± 56	72 ± 20	98 ± 30
Total IJs in all hermaphrodite/female	4288 ± 644	864 ± 62	980 ± 48
Contribution (%)	3.2	0.7	0.7
Second generation			
Total female developed	294 ± 64	363 ± 52	438 ± 60
Ratio of female bearing EM (%)	68-86	58-78	56-76
Ratio of IJs developed per female (%)	84-96	70-86	64-82
Number of IJs per female	68-88	12 ± 4	38 ± 12
Total IJs in all females	19,992 ± 3405	4356 ± 984	16,644 ± 2865
Contribution (%)	14.8	3.4 ± 0.5	12.5
Third generation			
Total female developed	2038 ± 856	3944 ± 580	3768 ± 602
Ratio of female bearing EM (%)	94-100	82-94	78-88
Ratio of IJs developed per female (%)	100	100	90-94
Number of IJs per female	42 ± 14	8 ± 2	18 ± 4
Total IJs in all females	85,344 ± 6088	31,522 ± 2244	95,232 ± 7206
Contribution (%)	63.2	24.7	50.9
Final yields of IJs per *G. mellonella*	135,000 ± 14,000	128,000 ± 10,000	133,000 ± 13,000
Total contribution of EM (%)	81.2	28.8	64.1

Data are the mean of 10 *G. mellonella* cadavers with standard deviation. Total females developed at each generation were examined at 5, 8, and 14 days after nematode inoculation for counting the number of first, second, and third adult generations. The number of IJs originated via *endotokia matricida* was examined in Petri dishes containing Ringer's solution in which females bearing *endotokia matricida* incubated. Final yields of IJs were counted at 18 days after nematode inoculation.

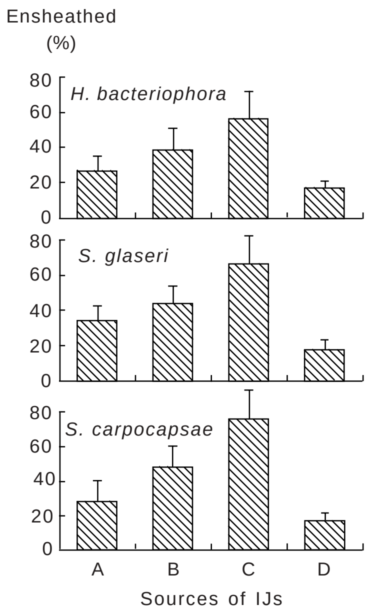

Fig. 1. The loss of extracuticle or ensheathed on infective juveniles-*endotokia matricida* developed inside the first (A), second (B), and third (C) adult generations of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. carpocapsae* compared to the normal IJs (D).

second, and third adult generation bodies lost the extracuticle or ensheathed. Ensheathed IJs-EM was more profoundly found on *S. glaseri* and *S. carpocapsae* after leaving the maternal body. In both nematode genera, the highest ratio of ensheathed IJs was observed on the first hermaphrodite or female adults bearing *endotokia matricida*, followed by the second and third female adult generations, respectively. These ensheathed IJs-EM completed the stages after they had emerged out from the maternal cadavers.

In vivo cultures IJs-EM frequently resume development upon emergence from their maternal cadavers (recovered or exited from "dauer" stage), whereas normal IJs that developed inside the insect cadavers appeared not to be resume development. The highest ratio of IJs-EM recovered from dauer stage was observed on *S. carpocapsae*, followed by *S. glaseri* and *H. bacteriophora* (Fig. 2). The IJs of *Steinernema* spp. were recovered faster than that of *H. bacteriophora* IJs. In *H. bacteriophora*, the IJs started to exit dauer stage at 6 hours after nematode incubation. In contrast, it was 2 hours for *S. glaseri* and *S.*

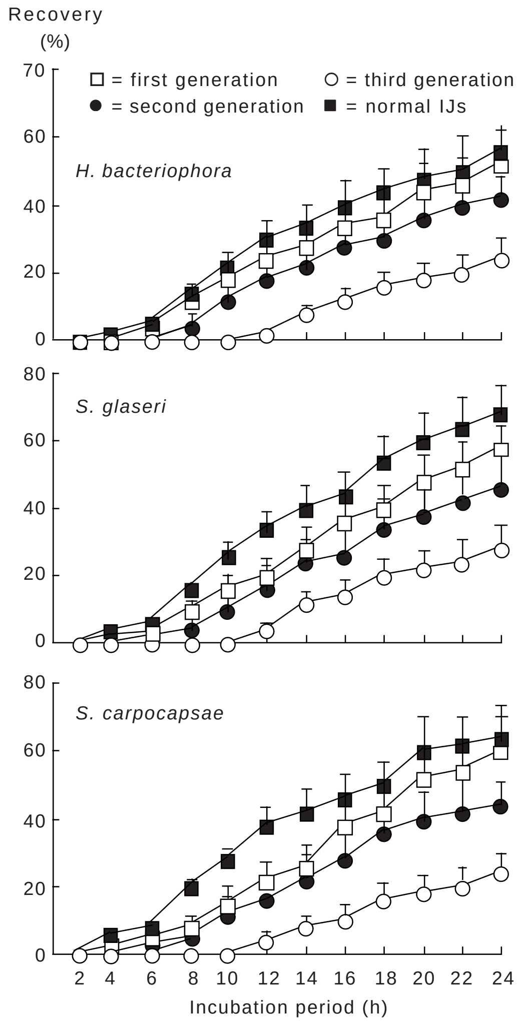

Fig. 2. The recovery of infective juveniles-*endotokia matricida* developed inside the first, second, and third adult generations of *Heterorhabditis bacteriophora, Steinernema glaseri*, and *S. carpocapsae* compared to the normal IJs at 2-24 hours after incubation on nutrient bromothymol agar medium.

carpocapsae. As comparison, the normal IJs of the three nematode species were recovered from dauer stage at about 12, 10, and 8 hours after nematode incubation, respectively.

The study revealed that the formation of both IJs-EM was lower than the yields of *H. bacteriophora* or *S. carpocapsae*. Wang and Bedding (1996) reported that the average of IJs per *G. mellonella* pre-inoculated with one or two IJs was 150,000. The estimation methods of IJ-EM used in the study showed that *endotokia matricida* contributed to the final yields of IJs per host in greater number than that of normal development in insect cadavers. Thus, this is the first case of estimation of the originality of the IJs among EPNs. The ratio of contribution of *H. bacteriophora* and *S. carpocapsae* in the first and second nematode generations to the final yields of IJs were similar to the results of Wang and Bedding (1996), but the contribution of the third nematode generation has not been estimated. IJ-EM plays an important form in the EPN life cycle at least as a survival stage for species maintenance as did normal IJs in drastically extreme environmental change and for further development (Johnigk and Ehlers 1999b). Wojick (1986a; 1986b), Wouts (1979) and Poinar (1990) also identified IJ-EM in the maternal body of *Steinernema* spp. and *Heterorhabditis* spp.

IJs-EM developed intra-uterinary in maternal body of EPNs has been found to have an exceptionally less dense appearance than that of normal IJs (Baliadi *et al*. 2001). It was reported that IJs-EM in the genera of *Steinernema* and *Heterorhabditis* had few storage granules (Han and Ehlers 2000), limited food supply (Wouts 1979) and symbiotic bacteria (Baliadi *et al*. 2001). It is well known that the nematode quality is also related to sheath retention. The results showed that IJs-EM physiologically was weaker than that of normal IJs, particularly in *Steinernema* which provided more ensheathed IJs. Losing the sheath increases the susceptibility to detrimental factors and reduces motility, although it does not affect the nematode pathogenicity (Campbell and Gaugler 1991; 1992). Seemingly, the sheath plugs the anal and oral openings of IJs (Campbell and Gaugler 1992). They also examined that the fitness of IJs-EM incubated on artificial medium was weaker than that of normal IJs.

By comparing the recovery speed and ratio of both IJs types to the exited of dauer stage, following standardization of the assay to nematode culture on nutrient bromothymol agar, we were able to quantify the physiological status of IJs-EM. The present study is in agreement with the results of Burnell (1998) who reported that IJs-EM were more frequently resume development upon emerge from the maternal cadaver than that of normal IJs developed inside insect cadaver. Most of IJs harvested from liquid culture were IJs-EM that developed in limited food supply provided by maternal body (Johnigk and Ehlers 1999a). Thus, our present findings could explain the inferior quality of IJs of *H. bacteriophora* produced *in vitro* liquid culture both in efficacy and soil persistence (Gaugler and Georgis 1991). It also suggests that animal protein, especially insect tissue in our case, is an important ingredient for obtaining high quality of EPN IJs.

Studies revealed that the last (third) generation females of *H. bacteriophora* did not oviposit and all of their eggs hatched and developed inside the maternal body (Wang and Bedding 1996), eventually emerging as IJs (Johnigk and Ehlers 1999b; Baliadi *et al*. 2001). This suggests that *endotokia matricida* can act as the sole IJs reproduction methods of the third generation of *H. bacteriophora*. Unlike *H. bacteriophora*, the juvenile stages resulting via *endotokia matricida* in *S. carpocapsae* and *S. glaseri* did not develop into IJs until they had emerged from the maternal body. Our present results is in accordance with Poinar (1990) who observed that the amphimictic females of *H. bacteriophora* appeared completely ovoviviparous and after mating the vulva is nonfunctional, whereas all *Steinernema* females deposit some eggs in the initial stages but become ovoviviparous later in their development. In addition, the first and second generations of *S. carpocapsae* were found to lay a larger proportion of their eggs than did *H. bacteriophora*. The increasing population of females bearing *endotokia matricida* in the second and third nematode generations is considered as a factor of a higher number of IJs originated via *endotokia matricida* (Wang and Bedding 1996).

The functional significance of these alternatively IJs mode of reproduction in EPNs is unclear. For instance, Johnigk and Ehlers (1999a) noted that most of IJs harvested from liquid culture originated from juveniles that developed inside hermaphrodites without any further explanation. However, previous studies showed that through *endotokia matricida*, the nematodes provide optimal conditions for offspring development when the environmental conditions are becoming detrimental (Johnigk and Ehlers 1999b). Thus, in such conditions the nematode is suppressed to develop IJs, an arrested stage best adapted for long-term survival. Possibly when the nematode decides to enter the intra-uterine juvenile development, the food resource might not be sufficient to induce the development of further stage and, consequently, the development to the IJs is induced. These data clearly showed the importance of *endotokia matricida* in survival mechanisms of *Steinernema* and *Heterorhabditis* species, because the IJs are morphologically and physiologically adapted to persist in the environment and search for a new food source (Glazer 1996). Further study of IJs-EM *in vivo* nematode reproduction is deemed necessary for a clear understanding of the regulatory of IJs-EM in life cycle and survival strategies of EPNs.

IJs developed inside or outside the maternal body are equipped with two layers of external membrane: the cuticle of the second molt remains attached to provide additional protection (Campbell and Gaugler 1991), particularly observed in *Steinernema*. Observation under light microscope revealed that the natural openings (mouth and anus) of the IJs-EM are also closed as in normal IJs. It is also found that the final yields of IJs of both nematodes from individual insects were closely similar.

The varied contributions of each nematode generation via *endotokia matricida* to the final yields of IJs are affected by several factors such as different number, body size, and egg retain of the hermaphrodites and female adults, food resources as well as nematode density (Wang and Bedding 1996; Baliadi *et al*. 2001). The population regulation as a result of IJs formation via *endotokia matricida* is also achieved in different manners (Wojick 1986a; 1986b), possibly, a combination of reduction of quantity and/or quality of food. Interestingly, *H. bacteriophora* showed the mechanism for safely sequestering IJs in the second and/or third female generation soon after these females have matured by developing its progeny via *endotokia matricida* in non-functional vulva (Wouts 1979; Poinar 1990). Perhaps characterization of nutrition status inside the maternal body of EPNs will provide answers to this puzzle. A better understanding of the real importance of *endotokia matricida* in the life cycle of EPNs and the recognition of the regulation factors inducing IJs is hoped to facilitate the manipulation and rearing of this species *in vivo* or *in vitro* media, a prerequisite for inclusion of this species in a biological control program of insect pest.

CONCLUSION

Endotokia matricida has a pivotal role in a maintenance and survival of entomopathogenic nematodes. Number of IJs produced via endotokia matricida was higher than that of a normal reproduction, particularly on *S. carpocapsae* and *H. bacteriophora*. The ratios were about 81%, 28%, and 64% of the final yields of IJs for *H. bacteriophora*, *S. glaseri*, and *S. carpocapsae*, respectively. Among the nematode adult generations, the highest contribution of IJs was come from the third adult generation bearing *endotokia matricida*. Although the IJs originated via *endotokia matricida* were more transparent compared to the normal IJs, they were morphologically similar.

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