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PATHOGENICITY, DEVELOPMENT AND REPRODUCTION OF THE ENTOMOPATHOGENIC NEMATODE *Steinernema* sp., IN MEALWORM *Tenebrio molitor*

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ABSTRACT

The pathogenicity, development and reproduction of *Steinernema* sp., isolate Skpr-20/Str, were studied using *Tenebrio molitor*. Results revealed that pathogenicity, development and reproduction were significantly influenced by nematode doses. Although the number of invading IJs increased with increasing dose, percentage penetration declined. The IJs reached adulthood within 3 days. Females laid eggs from day 4-7. All eggs remaining inside uterus develop inside the maternal body. The first female bearing *endotokia matricida* was observed on day 5. In a sand-based assay, nematode was more pathogenic at lower dose instead of higher ones, where optimum dose was 80 nematodes per larva and average number of progeny per female was 5438. Under crowded conditions, development proceeds to IJ stage instead of the J3. The average length and width decreased with increasing of nematode doses. The IJ produced in cadavers infested with 640 nematodes per larva was significantly smaller (492 ± 6.4 µm) than offspring from other doses. The number of days which nematodes first emerged from the cadavers decreased with increasing dose. IJ first emerged at the average of 10-13 days at high IJ densities. It is concluded that the wide experimental characteristic of EPNs is also true for *Steinernema* sp., isolate Skpr-20/Str.

Keywords: development, emergence period, *endotokia matricida*, entomopathogenic nematode, isolation, pathogenicity, *Steinernema* sp., isolate Skpr-20/Str

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INTRODUCTION

Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae possess tremendous potential as biological control agents of insect pests and several advantages over chemical pesticides (Gaugler and Kaya, 1990; Georgies, 2002; Kaya *et al.,* 2006; Shapiro-Ilan *et al.,* 2006). For example, they can actively seek their hosts, recycle in the soil environment, and are environmentally safe (Akhurst and Boemare 1990; Somasekar *et al*., 2002). These insect-killing nematodes are currently considered the second most utilized biocontrol agent against insect pests after *Bacillus thuringiensis* (Glazer, 1998; Bedding 1998; Divya and Sankar, 2009). Advances in mass production, formulation and application technology, and discovery of numerous isolates and strains have culminated in a surge of scientific and commercial interest in these insect-killing nematodes (Gaugler and Kaya, 1990; Burnell and Stock, 2000). EPNs have a mutual association with bacteria of the genus *Xenorhabdus* (Akhurst and Boemare, 1990).

They have one-free living stage with a single function: to infect a host. Once the host is found, infective juvenile (IJ) penetrates into the haemocoel and release symbiotic bacteria which produce toxins that kill the host in 24-48 h. The bacteria multiply and create the optimal conditions necessary for the nematodes reproduction (Poinar Jr., 1990). This rapid mortality permits the nematodes to exploit a range of hosts and spans nearly all insect orders (Poinar Jr., 1979). These nematodes have been isolated from natural population in Indonesia

(Griffin *et al.,* 2000; 2001; Stock *et al.,* 2004; Achadian *et al.,* 2005; Pratiwi *et al.,* 2005; Santoso *et al.,* 2009) and used for control of some important insect pests (Chaerani and Waluyo, 1996; Baliadi 2004; Bakti 2004; Achadian *et al.,* 2005; Pratiwi *et al.,* 2005; Achdiya *et al.,* 2008; Prabowo and Indrayani, 2009; Santoso *et al.,* 2009).

All EPNs have the same general life history but the species differ in host utilization (Selvan and Blackshaw, 1990), searching behavior (Grewall *et al.,* 1994) and reproductive strategies (Poinar Jr., 1990). Intraspecific competition affects progeny production in EPNs. When the number of IJ penetrating into a host exceeds an optimal level, exploitative intraspecific competition occurs among the developing nematodes, which reduce the total number of progeny emerging from the cadaver (Kaya and Koppenhofer, 1999; Selvan *et al.,* 1993). Increasing population density within the host can also adversely affect the fitness of parasitic nematodes besides reducing progeny. Effects of intraspecific competition have been noted in EPNs (Selvan *et al.,* 1993; Grewall *et al.,* 1997). The present study investigates how density dependent factors within *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae influence pathogenicity, development and reproduction of EPN *Steinernema* sp., isolate Skpr-20/Str, originated from Sukapura Probolinggo.

MATERIALS AND METHODS

The experiments was conducted at Laboratory of Nematology of Indonesian Legume and Tuber Crops Research Institute, Malang in the period of July to October 2010.

Soil Sampling and Nematode Extraction

Soil sample has been collected from rhizosphere of several crops at village Batu and Sukapura, East Jawa. Three sample points have been determined randomly in 2 to 4 m^2 area. Using shovel, subsample of 100 cm^2 surface x 20 cm depth was taken to the laboratory. The shovel was cleaned and disinfected by ethanol 70% before use. About 150 g of soil were placed in the plastic container, and 5 – 10 larvae of *T. mollitor* were introduced on the soil surface, and covered with lid. The container was then inversely placed and incubated at room temperature. After 3 to 4 days, the cadavers were rinsed with sterile water and the nematode was extracted based on White trap method (Kaya and Stock, 1997). The suspension of nematode obtained from this trap was kept in the refrigerator at 10 °C temperature for further study. To obtain the true EPN, *ca* 100 nematodes consisting of male and female were suspended in 10 ml water and poured into the Petri dish with sterile filter paper at the bottom. Five larvae of *T. mollitor* were then released into the dish and allowed in contact with nematode for 3 – 4 days. The dead larvae were subject to White trap, and the nematodes freed from the host cadaver were then considered pathogenic. For identification, the nematode were put in the Ringer solution on the object glass and covered with ring glass. Adams and Nguyen (2002) methodologies were used for nematode identification. For further study, mass propagation of the nematode was done using *T. mollitor* as insect host and followed by White trap method as described above.

Preparation of Nematodes

We have chosen the nematode *Steinernema* sp., isolate Skpr-20/Str, as an isolate for development studies because it caused the highest mortality to the larvae of *T. molitor*. The nematode was cultured in last instar larvae of *T. molitor*, at room temperature. Plastic containers Pot R. 100 (diam. 5.5 cm, height 5.0 cm) with five *T. molitor* larvae at the bottom were filled with moist soil (8%, w/w, tap water). Fifty IJs per larva in 1-ml tap water were added to the sand surface. The containers were capped and incubated at room temperature. After 144 h, the host cadavers were removed to modified White traps (Lewis and Gaugler 1994). Petri dishes (5 cm), without lids and holding the cadavers, were floated in water inside a 9 cm Petri dish and stored at room temperature. IJ emerging from the cadavers crawling over the side of the holding dish into the water were trapped. IJ were collected daily, for 4 days, transferred to tissue culture flask and stored in tap water at 5 °C to testing. All the experiments were performed within 15 days of emergence.

Nematode growth and development

The *T. molitor* cadavers were gently dissected in Ringer's solution under the microscope. All nematode development stages were identified and counted once outside the

cadaver using serial dilution when necessary. Dissection and nematode counts were carried out for 2 to 8 days after nematode inoculation on ten insect cadavers which had a successful development of nematode populations. The experiments were performed three times using 10 insect cadavers.

Pathogenicity of nematode

Bioassays were carried out by placing single last-instar larvae of *T. molitor* in plastic container Pot R. 30 (diam. 4.0 cm, height 3.0 cm) filled with moist (8% w/w) heat sterilized soil. Host larvae were individually exposed to nematode doses of 10, 20, 40, 80, 160, 320, and 640 IJ that were added to the top of the container in 1 ml of water. Water without nematodes was added to the controls. The containers were closed with a lid and held at room temperature. Each dose of IJ was tested on 20 *T. molitor* larvae. After 10 h exposure, the *Tenebrio* larvae were removed from the soil and rinsed with tap water to remove external nematodes. The rinsed larvae were transferred to Petri dishes with moist filter paper and held at room temperature for 144 h. Of each group of 20 larvae exposed to a certain dose of IJ, ten larvae were dissected and digested in a 0,8% pepsin solution and the number of nematodes inside the cadavers for each dose was determined. The other 10 larvae were used to study density dependent effects on reproduction, size of nematodes and emergence time. The experiment was performed four times.

Production Capacity

Production capacity was assessed by placing each of the 10 cadavers into individual modified White traps. All IJ that emerged from a single host over a period of 5 days after the first appearance of nematodes in the water were harvested and the total nematode suspension was put in a 50-ml tissue culture flask. To assess the total production during the harvest period, the contents of the flask were mixed thoroughly with air bubbles from an aquarium pump and from this suspension eight samples of 10 µl were counted under a stereomicroscope using a counting slide.

Size of Nematode

The length and width of a hundred individual IJ were measured for each dose. Individuals to be measured were randomly selected from the pooled population. Prior to measurement, IJs were killed by hating them in 5 ml of water in a microwave for 30 s.

Time of Nematode Emergence

The time to emergence, i.e. number of days after infection to the moment when IJ started to emerge from the infested host cadaver, was determined by checking all the modified White traps daily at 09:00 am until the first IJ started to come out from the cadaver.

Data Analysis

The results were statistically analyzed by analysis of variance. Significant *F*-tests ($P \leq 0.05$) were followed by least significant different tests (LSD) ($P \leq 0.05$) for testing differences among treatments. Means were reported with standard errors of the mean (±SE). Analysis was performed using the statistical program MSTAT.

RESULTS AND DISCUSSION

Soil Sampling and Nematode Extraction

A total of 50 soil samples were collected in Batu and Sukapura. By using the larvae of *T. molitor* bait, *Steinernema* sp. was present in eight samples collected from Batu and Sukapura (Table 1). Isolate Skpr-20/Str induced the highest mortality (76 ± 26 %) of *T. molitor* larvae at 74 h after baiting, followed by isolate Skpr-23/Str (64 ± 18 %). The lowest one was isolate Batu-6/Str (16 ± 8%). Identification study, based on the occurrence of adult male and female at first generation, morphological characters of IJ and bioluminescen, showed that all nematodes felt only into family of Steinernematidae (Table 2). Each sample consisted of soil to a depth of 10-15 cm, and Wouts (1991) suggests that Steinernematid tend to distribute near the soil surface, unlike Heterorhabditid that can move deeper in the soil. Most of the Heterorhabditids found in Indonesia are isolated from coastal area (Griffin *et al.,* 2000). Due to the certain limitations, *Steinernema* sp. identification until species level was not undertaken in this study. EPNs reported that they had a worldwide distribution as they had been isolated from every inhabited continent and many islands (Hominick *et al.* 1996), from different soil types and from natural habitats to disturbed agroecosystems (Kaya and Koppenhofer 1999). Achadian *et al.,*

(2005) used *T. molitor* bait isolated *Steinernema* sp. from Jolondoro and Semboro sugarcane plantation, which is also located at East Java agroecosystem. So far, *Steinernema hermaphroditum* n. sp (Stock *et al.,* 2004) and *Heterorhabditis indica* (Griffin *et al.,* 2000) were found as recorded species of EPNs in Indonesia; however, this is only a preliminary conclusion since the survey is still in progress.

Nematode Growth and Development

As shown in Table 3, the infected insects were killed rapidly within 24-48 hrs. The invading IJs resumed development, moulted to the J4 stage and reached adulthood within three days after nematode inoculation. In more detail, immature females appeared likewise within two days after inoculation and adult female developed three days after inoculation. The females laid eggs from day 4 to 7. All eggs which remained inside the uterus after the period of egg-laying developed inside the maternal body. The first female bearing *endotokia matricida* was observed at day 5. Then, the first IJs recovered from the insect cadavers were examined on day 8, and we suspected it was originated from *endotokia matricida*.

Table 1. Eight isolates of the entomopathogenic nematode originated from rhyzosphere of carrot, cabbage, sweet potato, banana and tomato of village Batu and Sukapura.

No. sample	Isolates	Originated	Vegetation	Mortality of *Tenebrio molitor* larva 72 h after baiting (%)	EPNs detection (+/-)
6	Batu-6/Str	Batu	Carrot	16 ± 8	+
12	Batu-12/Str	Batu	Cabbage	58 ± 18	+
15	Batu-15/Str	Batu	Sweet potato	40 ± 16	+
25	Skpr-2/Str	Sukapura	Cabbage	52 ± 18	+
30	Skpr-5/Str	Sukapura	Carrot	46 ± 18	+
35	Skpr-10/Str	Sukapura	Banana	28 ± 10	+
45	Skpr-20/Str	Sukapura	Cabbage	76 ± 26	+
48	Skpr-23/Str	Sukapura	Tomato	64 ± 18	+

Table. 2. Occurrence of male and female and first generation, bioluminescens, length and width of infective juvenile base for identifying the genus of eight nematodes isolated from Batu and Sukapura

Isolates	Occurrence of ♀ and O at 1st generation	bioluminescens	Length and Width of IJ (µm)				Nematode genera
			Length		Width		
			×	range	×	range	
Batu-6/Str	+	-	568	498-638	24	22-28	*Steinernema*
Batu-12/Str	+	-	570	490-613	24	22-30	*Steinernema*
Batu-15/Str	+	-	572	494-615	26	20-27	*Steinernema*
Skpr-2/Str	+	-	566	502-620	24	20-29	*Steinernema*
Skpr-5/Str	+	-	569	501-622	26	23-29	*Steinernema*
Skpr-10/Str	+	-	568	496-594	24	24-30	*Steinernema*
Skpr-20/Str	+	-	568	490-602	24	22-30	*Steinernema*
Skpr-23/Str	+	-	566	504-640	26	21-30	*Steinernema*

Remarks= +/- indicates present and not present

Studies revealed that the females did not lay all of their eggs and they remained hatched and developed inside maternal body (Wang and Bedding, 1996; Baliadi *et al.,* 2001), eventually emerging as IJs (Johnigk and Ehlers, 1999). These IJs must be direct result of a feedback mechanism (probably a combination of reduction of quantity and/or quality of food) (Wang and Bedding 1996). Our present results is in accordance with Poinar Jr. (1990) who observed that all *Steinernema* females deposit some eggs in the initial stages but become ovoviviparous later in their development.

Pathogenicity of Nematode

At all densities of IJ tested, all *Tenebrio* larvae were infected by *Steinernema* sp., isolate Skpr-20/Str. Morphological characteristics of this isolate were described in Table 4. No mortality was detected in the control larvae. Although the number of IJ that invaded the host increased with increasing dose, the percentage of penetration declined. Obviously, living *T. molitor* larvae seem to release substances which result in various degree of invasion. Invasion increased from 3 ± 0.2 at dose of 10 to 33 ± 1.6 at a dose of 640 IJ/larva. The number of invading nematodes did not increase as proportionally as the increase in dose ($Y=-0.0333x + 23.297$; $R^2 = 0.766$), so there was a decline in the percentage of invading nematodes. The nematode penetration efficiency decreased from 32% at a dose of 10 to 5.16% at a dose of 640 IJ/larva (Figure 1). The same phenomenon was observed on the number of nematodes attached to the insect cuticle. These results showed that part of IJs that found their host did not penetrate or invaded the *T. molitor* larvae, and most of the inoculated IJs still stayed in the Petri dish lid (Table 5).

Table 3. Population dynamics of the *Steinernema* sp., isolate Skpr-20/Str in *Tenebrio molitor* cadaver 2 – 8 days after inoculation by 50 infective juveniles per host.

	Number of nematodes of various stages per host							
dai	Eggs	J1	J2	IJs	J3/J4	Male	F	Total
2	0	0	0	10±0.8	0	0	0	10
3	0	0	0	2±0.1	4±0.2	2±0.4	2±0	10
4	320±22	120±14	0	0	1±0.01	4±0.2	5±0.4	450
5	884±28	682±40	196±26	0	96±8	6±0.6	14±4$^{(EM)}$	1878
6	1260±58	1640±68	844±36	0	894±56	68±8	84±14	4790
7	4068±220	6644±180	4202±164	0	1802±120	240±20	268±42	17224
8	6178±246	6894±188	8056±180	806	2248±89	890±38	1264±56	26336

Remarks = Data are the mean of four insect cadavers with standard deviation. dai is hours after nematode incubation. IJs is infective juveniles. The experiments were performed three times using 10 insect cadavers. F is Female. (EM) is the first occurrence of female bearing *endotokia matricida*

Table 4. Morphological characteristics of infective juveniles of *Steinernem*a sp.,isolate Skpr-20/Str, originated from Sukapura

Characters	Value (µm) *	
	×	Range
Length	568	490-602
Width	24	22-30
Length between head and *excretory pore*	42	38-52
Length between head and nervous rings	86	80-92
Length between head and esophagous base	114	105-184
Tail length	49	46-58
Tail width	13	10-15

Remarks= an average value from 40 IJs.

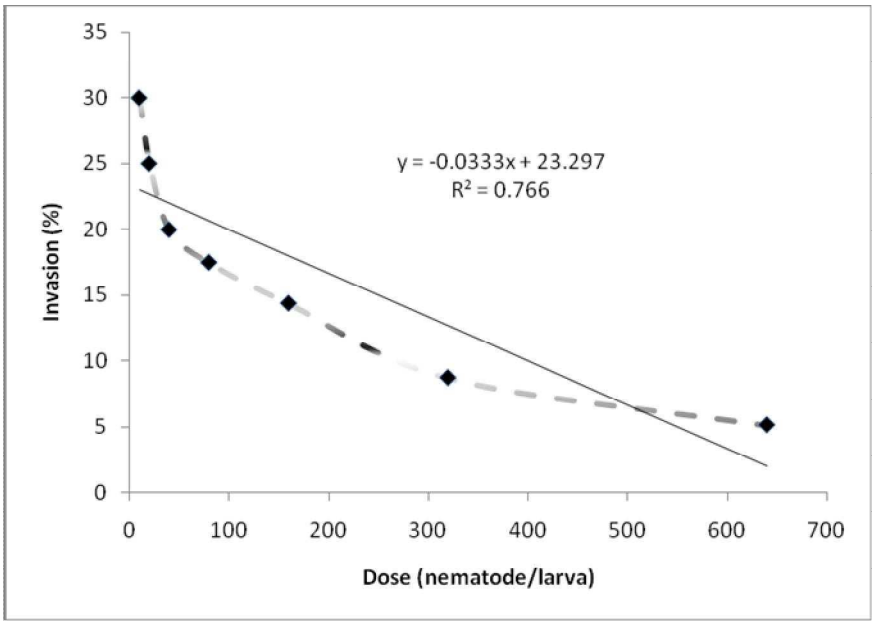

Figure 1. Mean percentage of infective juveniles of *Steinernema* sp., isolate Skpr-20/Str, present in the hemocoel of *Tenebrio molitor* larvae 144 h after exposure

Table 5. Number of nematodes recovered in the hemocoel, cuticle of *Tenebrio molitor* larvae and in petri dish lid 144 h after exposure.

Dose (nematode/larva)	Number of nematodes		
	Insect hemocoel	Insect cuticle	Petridish lid
10	3.2 ± 0.2 (32%)	2.4 ± 0.1 (24%)	4.4 (44%)
20	5.4 ± 0.4 (27%)	4.2 ± 0.6 (21%)	10.4 (52%)
40	8.4 ± 0.5 (21%)	8.0 ± 2.2 (20%)	23.6 (59.0%)
80	14.0 ± 0.8 (17.5%)	18.4 ± 4.4 (23%)	47.6 (59.5%)
160	23.6 ± 1.2 (14.75%)	36.4 ± 6.2 (22.75%)	100.0 (62.5%)
320	28.2 ± 1.4 (8.81%)	58.2 ± 6.8 (18.19%)	233.6 (73%)
640	33.0 ± 1.6 (5.16%)	62.6 ± 8.2 (9.78%)	544.4 (85.06%)

Remarks= value in the percentage of insect hemocoel is the nematode penetration efficiency calculated by the following formula: No nematodes penetrated into insect larva/No added nematodes x 100%.

Selvan *et al.* (1993) working with ten different doses (10-6400) also found that the infection percentage of *Steinernema carpocapsae* and *H. bacteriophora* declined with increasing dose. With the exposure time of 10 h used in our experiment, no more than 30% of the applied IJ migrated through a 5.5 cm soil column and established in the host. The results seem to support the conclusion of Fan and Hominick (1991) and Selvan *et al.* (1993) where only a proportion of the applied nematodes are able or willing to invade a host at a given time. We assessed IJ invasion 144 h after exposure, and it is possible that large proportions of invaded IJ died soon after invasion and were not found by dissection.

Sulistyanto and Ehlers (1998) stated that pathogenicity of EPNs and their bacterial symbionts were a symphony of several sequences played on well tuned instruments. The pathogenesis starts with host recognition and host finding, proceeding during conquest of the

host's defenses, and the activation of the general pathogenic metabolism culminates in the death of the host. Important pre-invasive steps with influence on the antagonistic potential of bacto-helminthic complexes are host finding and penetration activity. Our results indicated that many IJs reached the *T. molitor* larvae; however, they failed to invade the potential host. Peters and Ehlers (1994) demonstrated that the dose-mortality relation of *S. feltiae* was correlated with its penetration activity. Some *Steinernema* sp. seemed to produce dauer juveniles that were either infective or non-infective, roughly in a ratio of three infective individuals to seven non-infective ones. It can be assumed that non-infective dauer juveniles will become infective over time to avoid over-exploitation of available hosts. The actual insecticidal efficacy has been comprised of a small proportion of active IJs and remaining large proportion of quiescent or inactive to infection (Baliadi 2008).

Production Capacity

Results of the experiment revealed that production of IJ initially increased with increasing density up to 80 nematodes per larva and then declined (Table 6). The largest cumulative production of juvenile occurred when the initial nematode density within the host was 14 ± 0.8 at an exposure dose of 80 IJ/host. No statistical difference in IJ production was observed among the dose of 40 (8.4 ± 0.5 invaded) and 160 (23.6 ± 1.2 invaded); and 10 (3.2 ± 02 invaded) and 20 (5.4 ± 0.4 invaded).

Nematode reproduced in cadaver of *T. molitor*. The mean number of IJ produced per cadaver did differ significantly. The strongest effect of nematode density was observed at a dose of 640 (33 ± 1.6) nematodes/host where each cadaver produced only 12 860 ± 568 IJ. A clear decline in the number of offspring per invaded IJ was observed with the increase of the dose. Because it is impossible to discriminate between male and female IJs, our results were all obtained after the IJs reached adulthood in the *T. molitor* larvae. The IJs of *Steinernema* sp., isolate Skpr-20/Str, developed into either males or females. As the nutritional quality within the cadaver deteriorates, the nematodes develop into IJs sequestering the mutualistic bacteria in their intestine. Assuming that sex ratio of male and female of invaded IJ is 1:1, at dose of 10 IJ/host, 15 251.25 new IJs were produced per invaded IJ and at dose of 640 IJ/host, only 779.39 new IJs were produced per invaded IJ.

The reproduction strategies employed by EPNs can also give some vital clues about the behavior of these nematodes in natural soil. Once a host is located and invaded, the fecundity of each invading nematode is dependent on the number of invading individuals and the size of the host; a large host invaded by few nematodes will result in the highest fecundity (Leij, 1995). Host utilization and the metabolic rate of processing host tissues by symbiotic bacteria influence the survival and reproduction of nematodes and differ among nematode species. Selvan *et al.* (1993) stated that *H. bacteriophora* was able to tolerate high nematode densities within the host, whereas *S. carpocapsae* was not able to survive and reproduce above an initial dose of 200 nematodes per host. Molyneux *et al.* (1983) reported that IJ of *Heterorhabditis* spp. was only able to reproduce in *Lucilia cuprina* larvae subjected to low dosages of IJ, whereas *Steinernema* spp. was not able to reproduce at any dosage. However, the offspring production, obtained during the determined harvesting period of 5 days, significantly dropped when a large number of IJ invaded the host. The reproduction rate decreased from 15 251.25 IJ per invaded IJ at a dose of 10 IJ/larva to 779.39 IJ per invaded IJ at a dose of 640 IJ/larva. It is however important to point out that even if the reproduction rate per invaded IJ decreased proportionally with an increase in dose, the cumulative production of juveniles did not show the same tendency. Zervos *et al.,* (1991) found that cumulative production of *H. heliothidis* decreased with an increase in the IJ inoculums and the largest production occurred at a dose of 25 IJ/host at 25 °C. Selvan *et al.,* (1993) observed that the proportion of IJ of *H. bacteriophora* increased with the increase of the initial density up to approximately 100 IJ/host. The highest number of IJs was produced when the initial nematode density within the host was 14 ± 0.8. We chose to count the total production over 5 days after first emergence of IJ in the water. IJ emergence actually continues longer but within the 5-day period the majority of nematodes emerged, as was also found by Selvan *et al.* (1993). The total production per *Tenebrio* cadaver was not very high. This is probably explained by the relatively high temperature used in our experiment. Mason and Hominick (1995) found higher production of

H. megidis in *G. mellonella* at 15 and 20 than at 25°C.

Size of Nematode

The average length and width of IJs showed significant difference. The data (Figure 2) show that the average length ($Y = 2.8095x^2 – 39.39x + 635.71$; $R^2 = 0.9192$) and width ($Y = -1.1857x + 30.857$; $R^2 = 0.967$) decreased with the increase of nematode doses. The IJ produced in cadavers infested with 640 nematodes per larva was significantly smaller (492 ± 6.4 µm) than offspring from other doses. The maximum IJ length occurred at the dose of 10 IJ/larva (598 ± 7.8 µm). No length differences were observed among IJs that came out of larvae exposed to 10, 20, and 40 IJ/larva nor between those originated from larvae with a dose of 80, 160 and 320 IJ. Figure 2 revealed that the maximum width of IJ occurred at dose of 10 IJ/larva (29 ± 1.4 µm) and the minimum one was at dose of 640 IJ/larva (22 ± 0.8 µm) respectively.

Table 6. Invasion, five-day yield and reproduction rate of infective juveniles in *Tenebrio molitor* larvae exposed to *Steinernema* sp., isolate Skpr-20/Str, among different nematode doses.

Dose (IJ/larva)	Invasion (N ± SE)	Total production (IJ/larva ± SE)	Reproduction rate (per invaded IJ) Sex ratio 1:1
10	3.2 ± 0.2	24 402 ± 1024 cd	15 251.25
20	5.4 ± 0.4	25 564 ± 1882 c	9 468.15
40	8.4 ± 0.5	34 256 ± 1640 b	8 156.19
80	14.0 ± 0.8	38 066 ± 1684 a	5 438.00
160	23.6 ± 1.2	32 420 ± 986 b	2 747.46
320	28.2 ± 1.4	23 644 ± 1002 d	1 676.88
640	33.0 ± 1.6	12 860 ± 568 e	779.39

Remarks= Data followed by the same letter are not significantly different from each other ($P \leq 0.05$)

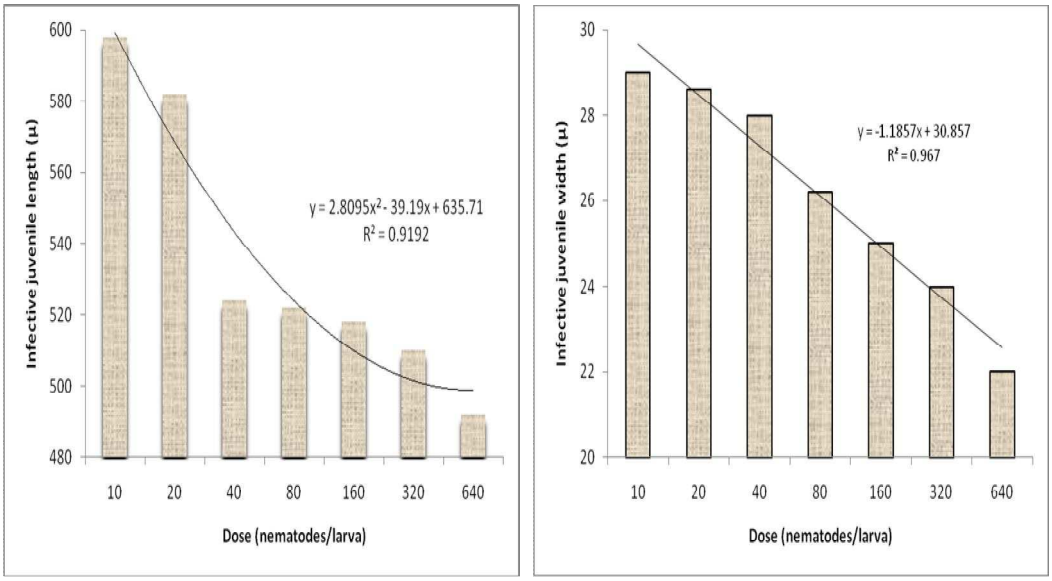

Figure 2. Length of infective juveniles of *Steinernema* sp., isolate Skpr-20/Str, produced in *Tenebrio molitor* larvae exposed to various nematode doses

Time of Nematode Emergence

The number of days on which nematodes first emerged from the cadavers decreased with increasing dose (Y = - 1.2286x + 18.943; R^2 = 0.9904) (Figure 3). IJ first emerged on average of 10-13 days after infestation at high IJ densities, while in larvae infested with 10-40 IJ/larva, the emergence period began 15-17 days after infection, respectively.

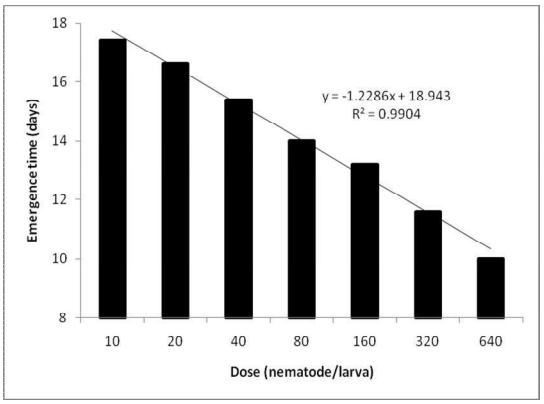

Figure 3. Effect of inoculum size on the mean duration of time to the first emergence of infective juveniles of *Steinernema* sp., isolate Skpr-20/Str, from *Tenebrio molitor* larva.

For the nematode isolate we tested, the time to first emergence decreased with increasing dose until a dose of 300 nematodes per larva. The influence of IJ dose on emergence period may be direct, by affecting the number of generations in a cadaver before emergence, or indirect, by affecting the growth rate of the bacterial symbiont or the biochemical composition of the cadaver.

Several factors affect the *in vivo* production of EPNs, and these factors interact in unpredictable ways (Zervos *et al.,* 1991). Selvan *et al.* (1993) stated that density-dependent factors played an important role in pathogenicity and fecundity of EPNs. They can act directly by affecting the number of IJ produced by infested cadavers, or indirectly by influencing the longevity of IJ. Penetration into the host insect should be an important step in the life history of EPNs as premise for reproduction. The reduction in subsequent invasion would be one of their survival strategies; to evade overcrowding in the host body which will bring the downsized fecundity of adult females leading to less production of IJ (Selvan *et al.,* 1993; Baliadi 2008). To be successful, an EPN and its associated bacteria must overwhelm the host immune response of a resistant host, which may affect invasion in a density-dependent manner (Dunphy and Thurston, 1990). In our experiments, host immunity does not explain the density-dependent effects on *Steinernema* sp., isolate Skpr-20/Str, pathogenicity, development and reproduction because *T. molitor* showed no immune response to the pathogen species used. The effects of density on *Steinernema* sp., isolate Skpr-20/Str, infectivity, fecundity and other parameters seem to result from intraspecific competition for nutrients and/or space.

CONCLUSIONS

The pathogenicity, development and reproduction are influenced by the nematode doses particularly due to the penetration rate of IJ into *T. molitor* larvae. The number of invading nematodes increased with increasing dose, but the percentage of penetration declined. The invading IJs resumed development, moulted to the J4 stage and reached adulthood within three days after inoculation. The females laid eggs from day 4 to 7. All eggs which remained inside the uterus after the period of egg-laying developed inside the maternal body at day 5 (*endotokia matricida*). The optimum dose for nematode penetration and establishment in larvae was 80 nematodes per larvae, while the average number of progeny per female is 5438. The IJ produced in cadavers infested with 640 nematodes per larvae were significantly smaller (492 ± 6.4 µm) than offspring from other doses. The IJ first emerged in the average of 10-13 days after inoculation at high IJ densities. It is concluded that the wide experimental characteristic of EPNs also holds true for the Indonesian nematode *Steinernema* sp., isolate Skpr-20/Str.

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