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Original Article

Ecological characterization of local entomopathogenic nematodes *Steinernema* sp. and its pathogenicity test against termites *Macrotermes* sp.

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Abstract

The evaluation of the ecological characteristics of local entomopathogenic nematodes (EPNs) *Steinernema* sp. using one-way classification experimental design was performed in this study. Infective juveniles (IJs) were maintained by *in vivo* culture, and its viability test at six storage temperatures, nine levels of pH and six levels of humidity were determined. The best viability was further tested for its pathogenicity on *Macrotermes* sp. Pathogenicity LC_{50} was calculated using Probit analysis. ANOVA was used to analyse the viability of IJs in all ecological parameters. The statistical analysis showed that viability of IJs was significant in all of the ecological parameters (LSD test; $\alpha < 0.05$). The optimum viability of IJs was obtained at the temperature ranged from 21-27 °C ; pH 7.5-8.0 and moisture level of 80-100 %. Based on the Probit analysis, the pathogenicity LC50 was achieved at concentration 242 IJs/ml in 48 hours.

Keywords: ecological characteristics, local EPNs, pathogenicity, *Steinernema* sp., *Macrotermes* sp.

1. Introduction

The entomopathogenic nematodes (EPNs) have been proven as effective biological agents to control the insect pests. This advantage has been widely tested in many regions in Indonesia by utilizing EPNs local isolates. However, the limitation of EPNs shelf life and their limited ability to tolerate the environmental stresses are still problems in maintaining the EPNs viability and their pathogenicity in comercial product. More than 90 species of EPNs have been commercialized as biological control agents (Shapiro-Ilan *et al*., 2014), while the local isolates of EPNs are likely to have different ecological characters in each region (Chaerani, 2011; Hazir *et al*., 2004; Morton & Garcia-del-Pino, 2009). Specifically, the ecological characteristics affect the activity, toxicity and virulence of EPNs (Lewis *et al*., 2006; Rohde *et al*., 2010), so their viability and pathogenicity can decrease in a different abiotic environment.

The exploration of local EPNs is expected to answer the needs of an area with similar ecological character. *Steinernema* sp. is a local isolate from Semarang that was recently explored (Widiyaningrum & Indriyanti, 2013). The abundance of *Steinernema* sp. has been found in manure soil, i.e. 67.411 IJs/ml, with a clumped distribution pattern (Indriyanti *et al*., 2015). The commercial production of this species is potential. The production of this species is potentially profitable as long as its viability and pathogenicity is maintained. Therefore, this study examined *Steinernema* sp. on their tolerance for temperature, pH and moisture at liquid storages to provide an *in vitro* culture medium with suitable ecological characteristics. Data range of temperature, pH, and humidity from this study results will be applied to set the condition of laboratory for local EPNs bio-control product development, especially biotermiticide.

2. Materials and Methods

The experiment series were conducted at Laboratory of Biology, Department of Biology Faculty of Mathematics

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and Natural Sciences Universitas Negeri Semarang, Indonesia during January - May 2016.

2.1 Soil sampling, trapping and identification of EPNs

Soil samples were collected from the poultry farm in Gunungpati district, Semarang, Indonesia. Each of 0.5 kg soil samples were collected from eight sampling sites. The samples were taken from 15-20 cm depth. The eight soil samples were then combined, and added with water to moisten the soil. The samples were placed into plastic containers with a lid and returned to the laboratory.

The EPNs were baited with late instar *Tenebrio molitor* larvae (Coleoptera: Tenebrionidae). To prevent the larvae from escaping, they were placed in a few small steel mesh cages (diameter 3.5 cm; height 2.5 cm). Ten larvae were put into each cages. The baited samples were then incubated in the dark at room temperature (27 °C). The samples were checked for any dead larvae after 3–5 days. The cadavers of potential were washed in sterile water then placed on white traps. In this study, using modified white traps followed procedures described by Kaya and Stock (1997). White traps were then cultivated in the laboratory at room temperature 27 °C until the emergence of infective-stage juveniles (IJs) occurred. IJs were harvested and stored in water at 27 °C.

Isolated EPNs were identified by visual observation and morphological characterization. Visual observation was done by appearance of *T. molitor* cadavers. Cadavers with a dark brown or ochre coloration were usually parasitized by steinernematids, whereas brick red to dark purple cadavers were parasitized by heterorhabditids (Orozco *et al*., 2014). For morphological characterization, this was followed by morphological assessments of adult males and IJs. Assessments were based on important diagnostic characters as suggested by Nguyen (2007) and Stock *et al*. (2004). Morphological observation was conducted using a digital binocular microscope Olympus CX41 equipped with a computer screen and camera.

The viability of EPNs was observed at 100× magnification. The IJs population counting was performed using the sampling method by counting dish and hand counter. Only lived nematodes were counted. Dead nematodes did not move and rigid like needles, while that lived nematodes actively move and swerve (Laznik & Trdan, 2014; Yadav & Lalramliana, 2012). The suspension of IJs with an average density 1000 IJs/ml was employed to the tolerance test of temperature, pH and humidity. In a previous study, IJs of *Steinernema* sp. can survive in a suspension of up to two week at room temperature (27 °C), then diminishing in viability (Widiyaningrum & Indriyanti, 2013).

2.2 Temperature tolerance test

The tolerance test was performed using one-way classification experimental at six storage temperatures (30, 27, 24, 21, 18 and 15 °C). Each of 5 ml suspension with density of 1,000 IJs/ml was added to the petri dish, and each treatment was repeated five times. After 24 hours incubation, the viability of IJs in each petri dish was calculated using the formula:

Viability (%) = (Population the EPNs after 24 hours) / (Initial population) x 100%

The room temperature on the optimum viability was applied to the tolerance test of IJs to acidity.

2.3 Acidity tolerance test

The tolerance test was performed using one-way classification experimental design at nine pH levels: 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Each treatment was repeated five times. Some IJs suspension with known population was added to the beaker glass. By using a pH meter, the pH value was adjusted according to the experimental design by adding the $Ca(OH)_2$. After the required pH had been reached, the density was recalculated to determine the initial viability. After 24 hours, the viability of IJs in each petri dish was calculated. The acidity on the optimum viability was applied to humidity tolerance test.

2.4 Humidity tolerance test

The tolerance test to humidity was conducted using one-way classification experimental at six levels of moisture: 100%, 90%, 80%, 70%, 60% and 50% in a sponge medium. The sponge medium was prepared by placing the sterile polyurethane sponges (thickness: 1 mm, diameter: 9 cm) into the petri dish (10 cm in diameter). In this procedure, the sponge in full saturated water is assumed as 100% humidity. The distilled water (15 ml) was added to saturate the sponge. In order to make 100% humidity, 10 ml distilled water was added to 5 ml IJs suspension (200 IJs/ml) in each petri dish. The decrease in 10% humidity was obtained by reducing 10% volume of distilled water from the full suspension. Thus, distilled water and the IJs suspension were added to the petri dish as follows, respectively: (A) 5 ml IJs + 10 ml of distilled water; (B) 5 ml IJs + 8.5 ml of distilled water; (C) 5 ml IJs + 7 ml of distilled water; (D) 5 ml IJs + 5.5 ml of distilled water; (E) 5 ml IJs + 4 ml of distilled water; and (D) 5 ml IJs + 2.5 ml of distilled water. After 24 hour incubation, all suspensions in sponge were squeezed to get out the suspension. The suspension volume and density were recalculated (IJs/ml).

2.5 Pathogenicity test against *Macrotermes* sp.

The pathogenicity test was designed using one-way classification experimental at six density levels of IJs *Steinernema* sp.: 0, 50, 250, 500, 750 and 1,000 IJs/ml. This treatment used IJs taken from *in vivo* breeding which has been conditioned at the optimum temperature of 24 °C, pH 8.0 and 100% humidity for one week. The effect of the EPNs on termite mortality was observed every 24 hour, i.e. 24, 48 and 72 hours after IJs application; while pathogenicity LC_{50} was calculated by Probit analysis (Finney, 1971). Trials were conducted in the polyethylene terephthalate (PET) plastic cup filled with 25 g of sterilized sandy soil, and added with 5 ml of distilled water to moist the soil. One ml IJs of EPNs was predetermined on its density; next, it was sprayed on the soil, and then, 50 worker termites were poured into the cup. A few pieces of cardboard (2 g) were added into each cup for hiding during incubation in a dark room. Data of mortality was observed after 24, 48 and 72 hours. Termite cadavers were examined under a stereomicroscope connected with a computer screen. If mortality in the controls is between 5% and 20%, results with the treated samples are corrected using Abbott's

formula by Abbott (1925) cited in World Health Organization (WHO, 2009):

$$\text{Mortality } (\%) = [\frac{X - Y}{100 - Y}] \times 100$$

where X = percentage mortality in the treated sample and Y = percentage mortality in the control.

2.6 Statistical analysis

The viability of IJs was statistically analyzed and was represented as the mean ± standard error of the average, while termite mortality was corrected using Abbott's formula. The significance of the difference was determined by one-way analysis of variance (ANOVA) and LSD test. P values <0.05 were accepted as statistically significant. Pathogenicity test against termites was calculated using Probit analysis by Minitab software version 17 single user license (Finney, 1971), in order to obtain the lethal concentration (LC_{50}).

3. Results and Discussion

Based on the color changes and important diagnostic characters as suggested by Nguyen (2007) and Stock *et al.* (2004), EPNs were identified as genus *Steinernema* sp. Identified EPNs in this study show the same characteristics as the previous results of the identification (Widiyaningrum & Indriyanti, 2013).

Each 3 g of larvae *T. molitor* produced an average of 2.157 IJs/ml after eight days of incubation. Furthermore, the IJs were cultured in the laboratory as needed for the material under test tolerance of temperature, pH, humidity and the pathogenicity test on termites.

3.1 IJs tolerance to different temperature

The viability IJs of EPNs local isolates of Semarang at six storage temperatures are presented in Table 1. During the experiment, the room temperature as a control in the laboratory was at 27 °C. Based on the statistical analysis, it shows that temperatures have significantly affected on the viability IJs (α<0.05). The lowest viability was found at 30 °C, and the highest was at 24 °C. However, based on the LSD test, the temperature of 21 °C, 24 °C and 27 °C were not different. Thus, the optimum temperature range for storage of IJs on tolerance tests was varied from 21 °C up to 27 °C. When compared with the temperature in the soil sampling sites (range of 26-26.5 °C), then to survive, the IJs tends to live in the temperature range similar to their habitat and less suited to the higher temperatures. The climatic differences between regions and between countries are very influential in this regard. This phenomenon strengthens the evidence that *Steinernema* sp. tolerates the varying temperature in the different area among countries, depending on the ecological characteristics of each region.

The research of the local isolate of *Steinernema* sp. in some countries, such as India (Lalramliana & Yadav, 2015; Lalramnghaki *et al.*, 2016), South Africa (Ramakuwela *et al.*, 2015), and Colombia (Andalo *et al.*, 2011; Torres & Sáenz, 2013) indicated that stability and infectivity of EPNs in the optimum temperature were in the range between 15 °C–30 °C. In Indonesia, the optimum temperature for *in vivo* culture of *Steinernema* sp. isolates from Jember (Yasin, 2005), Tulungagung (Nugrohorini, 2007), Madura (Djunaedy, 2009), Banyumas (Purwiyatno, 2012) is different, within the range of 24 °C-30 °C.

3.2 IJs tolerance to different acidity levels

The optimum temperature (24 °C) was then applied at the room temperature for *Steinernema* sp. tolerance test to various acidity levels. The highest pH treatment in this trial was 9.0, and the lowest was 5.0. The viability of IJs after 24 hours in incubation are presented in Table 2.

The difference acidity of the suspension was proved to have a significant effect on the viability of IJs of EPNs after 24 hours. The highest value was found in the suspension with pH 8.0, and the lowest treatment was found at pH 5.0. The results showed that the viability at pH 8.0 and 7.5 were not significantly different (LSD test; α <0.05); whereas at pH 8.5 and 9.0, the viability were likely decreased significantly. Similarly, the viability of pH 7.0 to pH 5.0 declined considerably in line with declining levels of acidity. In conclusion, the optimum pH for maintaining the viability of IJs ranged from 7.5-8.0.

This finding concurs with a study reported by Somsook and Somsook (2005) which concluded that the survival of EPNs *Steinernema carpocapsae* (Weiser) in liquid culture was hampered by high acidity levels such as pH 3.0 and 4.0, with mortality reached 88%. They said at pH 5.0 and 6.0 EPNs were growing more slowly, while at pH 7.0, 8.0 and 9.0 EPNs were developed steadily. Similarly, pH tolerance test on

Table 1. Viability of IJs after 24 hours at different temperatures.

Temp (°C)	Replication					Averages (%)
	1	2	3	4	5	
15	63.81±0.408	63.00±0.246	57.37±0.880	64.34±0.514	60.32±0.290	61.77±0.648 [a]
18	64.42±0.519	61.86±0.007	61.50±0.065	53.83±1.599	67.52±1.139	61.82±0.666 [a]
21	76.49±0.671	71.08±0.411	68.65±0.897	78.38±1.049	71.08±0.411	73.14±0.688 [b]
24	81.31±0.665	78.44±0.091	76.59±0.279	77.82±0.033	75.77±0.443	77.99±0.151 [b]
27	82.48±0.942	75.73±0.408	75.73±0.408	84.12±1.270	70.80±1.394	77.77±0.884 [b]
30	48.38±0.292	52.70±0.572	48.65±0.238	55.14±1.060	44.32±1.104	49.85±0.653 [c]

Different letters in the same column for each temperature levels indicate significant differences by LSD test (α < 0.05).

Table 2. Viability of IJs after 24 hours at different pH.

pH	Replication					Averages (%)
	1	2	3	4	5	
5.0	6.43±0.155	6.11±0.219	4.82±0.103	4.50±0.167	4.82±0.103	5.34±0.149 [a]
5.5	9.66±0.205	5.68±0.137	6.53±0.137	7.67±0.091	5.97±0.023	7.10±0,119 [a]
6.0	37.75±0.112	34.65±0.620	29.01±0.508	28.73±0.564	27.61±0.338	31.55±0.428 [b]
6.5	75.50±0.658	63.09±0.188	55.70±0.054	69.13±0.282	52.35±0.618	63.15±0.360 [c]
7.0	77.13±0.195	84.76±0.501	77.74±0.903	82.32±0.013	89.33±0.195	82.26±0.361 [d]
7.5	86.25±0.652	90.83±0.264	90.54±0.206	89.11±0.080	90.83±0.264	89.51±0.293 [e]
8.0	96.41±0.327	90.20±0.261	88.24±0.001	89.54±0.393	93.14±0.327	91.50±0.262 [e]
8.5	76.69±0.074	79.45±0.626	75.77±0.110	69.33±1.398	80.37±0.810	76.32±0.604 [f]
9.0	66.29±1.469	70.45±0.637	83.33±1.939	73.86±0.045	74.24±0.121	73.64±0.842 [f]

Different letters in the same column for each pH levels indicate significant differences by LSD test ($\alpha < 0.05$).

local isolates of Madura Heterorhabditis by Djunaedy (2009) also concluded that the optimum medium for EPNs culture obtained at the alkaline condition (pH 8.0). By these findings, the optimum pH was applied to test the tolerance of IJs to moisture. The experimental condition was set at the alkaline suspension (pH 8.0) and storage temperature at 24 °C.

3.3 IJs tolerance to different moisture levels

In this study, the assay of IJs *of Steinernema* sp. tolerance to moisture used a liquid culture as the medium. Therefore, the moisture test was conducted by using petri dish (diameter 10 cm) which was coated with polyurethane sponge (thickness 1 mm; diameter 9.8 cm). The saturation level of water absorbed by the sponge was assumed to be 100% moisture. The viability of IJs at various moisture levels after 24 hours are presented in Table 3.

The viability of IJs on different moistures showed the significant differences and based on LSD test (α <0.05) it was known that viability of IJs at 100%, 90% and 80% moisture were not different. Moreover, the humidity at 70%, 60% and 50% were significantly different each other. The viability of IJs tends to decline as humidity decreases. The lowest viability on this study was found at 50% moisture treatment. The low water content in the sponge is suspected to cause the limited mobility of IJs. Additionally, the IJs were potentially trapped and died in the sponge.

According to Sulistyanto and Muhibuddin (2001), the movement of IJs is very dependent on moisture media. In liquid culture, EPNs need a moist environment up to 100% to be able to move around freely and discover their host. In soil, a low humidity causes a difficult movement on IJs. Moreover, they cannot survive, because of the characteristics of their body that are not tolerant to dry conditions (Radová & Trnková, 2010). If the humidity is only 6%, the mortality can reach 100% (Yadav & Lalramliana, 2012). In this study, IJs *of Steinernema* sp. was survived well in the humidity between 80% and 100%. Furthermore, IJs of *Steinernema* was prepared in the in vivo culture at 24 °C temperature and pH 8.0 with 100% humidity for the pathogenicity test to *Macrotermes* sp.

3.4 Pathogenicity test of IJs of *Steinernema* sp. isolates of Semarang against *Macrotermes* sp.

Pathogenicity test was conducted by giving treatments in several IJs concentration levels against termites, i.e. 0, 50, 250, 500, 750 and 1,000 IJs/ml. Termites mortality was observed after 24, 48, and 72 hours of application. During the observation, the death occurrence of the control group was used as the basic on treatment observation data correction using Abbott formula. The corrected mortality data is presented in Table 4, while the pathogenicity test at LC_{50} was done by Minitab program. In the observation after 24 hours infection, all treatments showed no result in termite mortality by 50%. The highest of termite mortality was found at the concentration of 750 IJs/ml. However, based on LSD test (α> 0.05), it shows that there were no difference in the concentration of 500, 750 and 1,000 IJs/ml. While at the observation at 48 hours after application, the concentration of 250, 500, 750 and 1,000 IJs/ml achieved over 50% mortality and each other was not significantly different (LSD test; α <0.05). Similarly, in the 72-hours observation, all treatments

Table 3. Viability of IJs after 24 hours at different moisture.

Moisture (%)	Replication					Averages (%)
	1	2	3	4	5	
50	3.46±0.061	3.08±0.015	3.46±0.061	3.08±0.015	2.69±0.093	3.15±0.049[d]
60	8.85±0.072	9.23±0.054	8.80±0.178	8.85±0.062	9.60±0.112	9.06±0.096[c]
70	76.92±0.076	76.54±0.000	77.69±0.230	77.31±0.154	74.23±0.462	76.54±0.184[b]
80	97.31±0.523	92.69±0.401	96.54±0.369	97.31±0.523	87.31±1.015	94.69±0.566[a]
90	98.96±0.383	98.19±0.229	96.11±0.187	98.70±0.331	93.26±0.757	97.04±0.332[a]
100	98.70±0.425	95.34±0.321	93.52±0.011	96.63±0.145	97.93±0.611	97.10±0.303[a]

Different letters in the same column for each moisture levels indicate significant differences by LSD test ($\alpha < 0.05$).

Table 4. Mortality *Macrotermes* sp. at various concentrations IJs in 24, 48 and 72 hours after application.

Concentration (IJs/ml)	Termite Mortality (%)		
	24 h	48 h	72 h
0	0[a]	0[a]	0[a]
50	20.25 [b]	35.84 [b]	93.90 [b]
250	28.27 [bc]	81.86 [c]	94.51 [b]
500	38.82 [c]	82.30 [c]	100.0 [b]
750	40.51 [c]	83.63 [c]	100.0 [b]
1,000	38.82 [c]	84.96 [c]	100.0 [b]

Different letters in the same column for each time indicate the significant differences by LSD test ($\alpha < 0.05$).

achieved maximum mortality except for controls, and each other there was no differences. The effectiveness of the concentration and duration of IJs *Steinernema* sp. isolates of Semarang on termites killing can be observed from the LC_{50} value using Probit analysis. Based on observation data at 24 and 48 hours (Table 4), LC_{50} value was achieved at the concentration of 1048 IJs/ml and 242 IJs/ml, respectively. This fact indicates that the higher levels of infected IJs, the lower their mortality rate. The concentration of population (IJs density) seems to cause them experiencing competition between individuals regarding space and food (Sucipto, 2008; Baliadi *et al.*, 2012). When the number of IJs penetrating into a host exceeds an optimal level, exploitative intraspecific competetion occurs among the IJs, which reduce the total number of progeny emerging from the cadaver. For these reasons, within 24 hours after application, there was no treatment which resulted in the death of termites reached 50%. Factors that contribute to penetration process of the IJs are the soil moisture, the environmental temperature, and the characteristics of IJs *Steinernema* sp. that are ambushing in finding and attacking the host (Li *et al.*, 2015; Nixon, 2005). Moreover, another factor is the characteristics of termite active movements and their habit of hiding in the dark (Nandika *et al.*, 2003). According to Kaya and Gaugler (1993), the pathogenicity of EPNs also depends on the development of mutualistic bacteria which produce the intracellular and extracellular toxins usually within 24-48 hours after entering the body of the insect. According to Lewis and Clarke (2012), after entering the hemocoel host, EPNs release the symbiont bacteria to produce toxins responsible for killing the host within 24 to 48 hours and provide nutrients for EPNs. In the hemocoel host, EPNs can complete at least three generations, and after that, IJs spread out of the cadaver to find a new host.

4. Conclusions

The optimum viability of IJs *Steinernema* sp. Isolates of Semarang was obtained at the temperature ranged from 21 °C-27 °C; pH 7.5 - 8.0 and moisture of 80% - 100%. Based on the Probit analysis, the pathogenicity LC_{50} on *Macrotermes* sp. 24 hours after the application was achieved at concentration 1048 IJs/ml, while LC_{50} at 48 hours was at 242 IJs/ml. Further research related to *in vitro* culture of IJs *Steinernema* sp. isolates of Semarang in the commercial production is necessary to be conducted. The findings obtained from this study can be used for local EPNs mass production based bioinsecticides product development plan.

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