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In vitro culture of local isolate Semarang entomopathogenic Nematodes on insect powder media

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Abstract. This research aims to analyze the population of EPNs isolate of Semarang cultivated in liquid media containing cricket (*Gryllus bimaculatus*) and mealworm (*Tenebrio molitor*) powder. Each treatment combination (doses of powder 0.5; 1.0; 1.5 and 2.0 g) was replicated five times. Each investing 1000 infective juveniles at the first breeding. The EPNs population was calculated every week for 4 weeks. The data were analyzed by ANOVA, followed by Tukey's test. The results showed EPNs isolate Semarang be able to reproduce in the liquid media with cricket and mealworm powder. The difference between insects had no significant effect on the EPNs population, but the dose of powder influenced significantly ($\alpha < 0.05$). The EPNs population on 2nd week showed no difference between control media, 0.5 g, and 1.0 g media, while doses of 1.5 and 2 g are significant. This study concluded that cricket and mealworm powder are effective as EPNs culture media replacing the in vivo method. The optimum doses in this study were 0.5 g insect powder, and harvesting is suggested to be conducted during the second week. This result can be an alternative to the mass cultivation of EPNs to the development of local bioinsecticides.

1. Introduction

Entomopathogenic nematodes (EPNs) have great potential to use as a biological control agent against insect pests, especially for soil-dwelling insects in an integrated pest management program. Host seeking capability, quick knockdown, exemption from registration, ease of application along with ease of production, high fecundity, and environment safety are attributes that make the EPNs special and exclusive from many other biological control agents [1]. EPNs of the genera Heterorhabditis and Steinernema are commercially used to control pest insects in many countries as bioinsecticide [2]. They are symbiotically associated with bacteria. Steinernema spp. symbiotic with Xenorhabdus bacteria, while *Heterorhabditis* spp. with Photorhabdus bacteria [3]. *Steinernema* sp. dan Heterorhabditis sp. has a wide host spectrum [4].

Bioinsecticide products-based EPNs have the potential as an alternative to chemical insecticides because they do not harm non-targeted insect, safe, and environmentally friendly [5], as well as have a broad range of hosts, i.e., more than 200 species of host insects [6]. For mass production, EPNs can be cultivated through in-vivo and in vitro methods [7,8] and solid or liquid media [9]. Breeding EPNs in vivo is very simple, reliable, and produce high-quality nematode, but it needs a lot of energy and budget. While in vitro culture has lower production costs, by providing artificial medium rich in nutrients and enough oxygen [10]. EPNs that has been widely developed in various countries generally utilize the exploration results of infective juveniles (IJs) from a local isolate. In this phase, IJ can survive outside

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its host media in vitro for several weeks to several months, although the activity will gradually decline over time [11-12].

Local EPNs isolates have been found in many regions with their respective characteristics, including local EPNs isolate from Semarang, Indonesia. EPNs exploration from various locations of land in Semarang concluded that the genera of *Steinernema* sp. dominated the EPN, and the highest population was found in soil samples from the farm area. In the laboratory, local isolate EPNs were cultured in vivo by utilizing mealworm larvae (Tenebrio molitor) as feed [13]. The previous research has formulated some artificial media from various sources of nutrients such as Wouts, Bedding, and Han methods [14]. EPNs cultivation using mixed media formulated [15] showed that EPNs could increase the population from early inoculation with a very varied population and tended to be even lower. In-vitro breeding of the same formula did not necessarily produce the same EPNs population, as many factors influence. Factors affecting EPNs survival in vitro media are including species, environmental factors, and food reserves. The ability of the nematode to survive for a period of time without a host is highly dependent on those factors.

Most of the in vitro media formulas contain nutritional components of animal and plants thus are less similar to the nutritional composition of the insect body. Non-insect dietary components are thought to be poor essential elements for the growth of EPN and its symbiotic bacteria. The productivity of EPNs culture can still be optimized if the formula is modified by adding components similar to the nutritional composition in the hemolymph of insect host, or components that can support the synthesis of essential metabolites for EPN and its bacterial symbiont [16]. In this study, we would study the insect powder formula (cricket and mealworm powder) as an in vitro media. In addition to possessing nutritional characteristics and components similar to host insects, these two insect larvae are available abundantly, sold freely on the market as domestic fodder, and are easy to cultivate. The nymph of cricket are known to contain 74.5% water; ash 1.25%; crude fat 4.43%; crude fiber 1.73%; crude protein 16.91%, and gross energy 6548 cal/ g. The content of mealworm is 70.14%; 1.26%; 8.03%; 3.31%; 15.62% and 6414 cal/g [17]. The media in the powder form was selected to facilitate handling and mixing with other materials. The purpose of this study was to examine the effectiveness of insects powder as in vitro culture media of Semarang isolate EPNs. This study is effective when the highest population in the treatment was not significantly different or better than the population of the EPNs intelligent control.

2. Methods

Research was conducted at the Biology Laboratory, Faculty of Mathematics and Natural Science, Universitas Negeri Semarang, Indonesia. In this research, EPNs cultivation treatment was performed in a room at a temperature of 25 ^{o} ± 2 ^{o}C, following the optimum temperature for culturing Semarang isolate EPNs [18]. EPNs of Semarang isolate was obtained from the collection of previous research.

2.1. EPNs multiplication

EPNs were multiplied using mealworm larvae (*Tenebrio mollitor*) as media in the white trap method [19]. White trap is modified using jar (height 6 cm; diameter 10 cm). The pots used had a convex surface at the bottom as well as a canal around the edges. A filter paper of diameter 10 cm was put on the convex side and then added with 30 mL of aquadest. The water will wet the filter paper, and the rest flows to fill the moat. The white trap process was conducted for 7 days. After incubation, EPNs in the water were harvested. Water that contains EPNs was then used for EPNs cultivation in treatment.

2.2. Insect powder preparation

The nymph of crickets and mealworm larvae were collected as needed. Crickets were selected that lack of wings (nymphs 3), while mealworms with an average length of 3 cm. Each insect was then dried in temperature of 60 ^{o}C for 5 hours, mashed with a blender, and stocked in jars with silica gel (to absorb moisture). Before used, each insect powder was sampled for proximate analysis.

2.3. EPNs Cultivation Media

EPNs cultivation media was made in two kinds of insect powder, that is the culture media using crickets powder (CP), and the culture media using mealworm powder (MP). Each insect powder was made in 4 formulas. Each formula was coded as C1, C2, C3, C4, M1, M2, M3 and M4. The control treatment used nymph of crickets (C0) and mealworm larvae (M0), respectively, as much as 1 g (8 nymph) and 1 g of mealworm. (15 larvae). Compositions of each media are listed in Table 1.

Table 1. Composition of EPNs Cultivation Media

No	Code	Composition
1.	C0	1.0 g cricket nymph on white trap method
2.	C1	0.5 g CP + 0.2 g gelatin + 30 mL aquadest
3.	C2	1.0 g CP + 0.2 g gelatin + 30 mL aquadest
4.	C3	1.5 g CP + 0.2 g gelatin + 30 mL aquadest
5.	C4	2.0 g CP + 0.2 g gelatin + 30 mL aquadest
6.	M0	1.0 g mealworm on white trap method
7.	M1	0.5 g MP + 0.2 g gelatin + 30 mL aquadest
8.	M2	1.0 g MP + 0.2 g gelatin + 30 mL aquadest
9.	M3	1.5 g MP + 0.2 g gelatin + 30 mL aquadest
10.	M4	2.0 g MP + 0.2 g gelatin + 30 mL aquadest

All treatments were done in 5 replications. Beside of control, all components in treatment were then mixed continuously. Each media was placed in a glass bottle (volume 75 mL; height 12 cm; diameter 4.5 cm). Inside the bottle, a white sponge polyurethane (diameter: 4.5 cm, thickness: 2 cm) was placed to absorb media liquid. Bottles were then closed and sterilized using autoclave for 60 minutes in 121ºC. After sterilisation, media was rested for cooling off. Each Media was then inoculated by 103 IJs/mL. Bottle lid was then closed and incubated at room temperature of 25 º± 2 ºC. The control treatment was done by the white trap method and filter paper. White trap was modified using jars. As much as 1 g of mealworm larvae and 1 g of cricket nymphs were put on the surface of the filter paper, then it was infected by 103 IJs using sprayer until flushed. In a dead state, the cadaver is arranged circularly on filter paper. The concave side around the bottom of the jar was added by 30 mL aquadest. Water will wet the filter paper, and the rest flows to fill the moat. Then the jars were closed and incubated indoors with other treatment bottles.

Observation was carried out by taking a sample of 50 µL of suspension from each treatment bottle using a micropipette and observed under a binocular microscope (100x magnification), equipped with a camera and connected to a computer screen. EPNs were counted using hand counter. Each sample count was repeated 3 times to compute the average, including the control group. EPNs population was calculated every week. Other measured parameters were initial and final pH of all suspension, change the color and aroma (odor) that arise, pH, temperature, and air humidity. Average data of EPNs abundance from 1st to 4th week were statistically analyzed using one way F test (ANOVA) in significancy level of 5% and Post hoc test (Tukey test). Data analysis was done by SPSS 18 software.

3. Results and Discussion

3.1. EPNs Population on Insect Powder Media

The result of EPNs cultivated in various insect powder media during 4 weeks is shown in Figure 1. In the 1st week, all media had the population increased from early infestation (103 IJs/mL). The EPNs population has increased by 4-5 times, in all treatment both of cricket and mealworm powder.

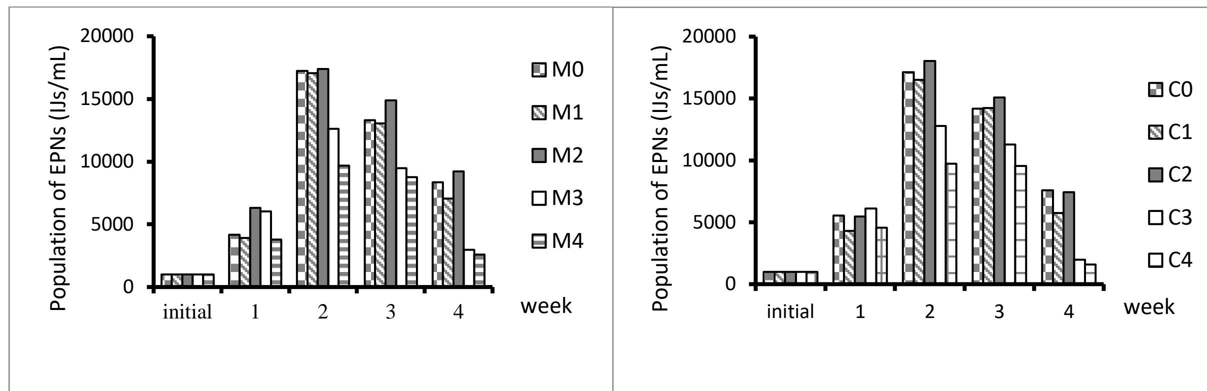

Figure 1. EPNs population in the cricket powder media (left) and mealworm powder media (right) during 4 weeks of observation.

This study indicates that the EPNs were able to live and reproduce on the insect powder media. The remaining nutrients caused this in EPNs cultivation media. Environmental factors and food reserves significantly affect the viability of the EPNs. In this research, EPNs cultivation treatment was conducted in a room at a temperature of 25 ± 2 ºC following the optimum temperature for culturing Semarang isolate EPNs [19]. Under sufficient nutritional conditions, Steinernematidae will reproduce one to three generations within the same host and produce a new generation within 7-10 days [20].

Mass cultivation of EPNs in artificial media using houseflies extract (*Musca domestica*) has been observed. The results prove that *Steinernema innovationi* was able to reproduce better than the culture media in vivo. That research concludes that the use of insect-based artificial media may be a competitive alternative technology in the EPNs mass production without the need for significant investment of medium and liquid fermentation equipment. If the availability of the nutrients is abundant, the life cycle of the EPNs could be faster. Still, on the contrary, if there is not enough nutrition, the nematode life cycle may be longer [21].

Population increases were observed in the second and third weeks, then decreased sharply during the fourth week. An increase in the number of EPNs in all treatments indicated that Semarang isolate EPNs could grow and reproduce on insect powders. All treatments peaked in the second week, highest in P2 media, followed by P0 and P1. In the 3rd and 4th weeks, all treatments decreased. In this study, the higher dose of insect powder (1.5 g and 2 g) did not cause a higher EPNs population. EPNs' Population on 2nd week, 3rd, and 4th for treatment of P3 and P4 were lower than the other three treatments (P0, P1, and P2). The small population in P3 and P4 was thought to be related to changes in the culture media, especially odor, pH, and color of cultivation media that observed after the 4th week (Table 2).

The result of pH measurement and qualitative observation (color of solution and odor) at the beginning of treatment showed that initial pH ranged from 7.30 - 8.0. The pH range following the optimum requirement for the survival of Semarang isolate EPNs [21], while the final pH observations at the 4th week fell in the field of 5.70 - 7.78. The pH range of the 4 - 6 medium decreased the growth of symbiotic bacteria and IJ survival [22]. In medium containing higher protein (1.5 gram and 1.0 gram), protein degradation into ammonia is possible, so that the higher protein content and the more ammonia formed in the bottle. This condition caused a more turbid coloration of the media and gave a stronger ammonia odor, thereby affecting the survival of the EPN. On the contrary, in the treatment of P0, P1, and P2, the pH decreases slightly. Despite the presence of ammonia gas, it is suspected to be within tolerable limits of EPN reproduction continuity.

Results of previous studies [14], which used soy flour, chicken livers, and dog food as a culture media, show that the pH of the media at the 4th week increased compared to the initial pH. In this case, differences in the source of nutrients used allegedly helped determine changes in the pH of the media during the incubation period.

Table 2. Color, odor, and pH alteration in various cultivation media.

doses (g)	pH Media Cricket's Powder		pH Media Melworm's Powder		Color of solution (th^4 week)	Odor of Ammonia
	initial	end	initial	end		
In vivo	8.04	7.78	7.98	7.56	Brownish	Low
0.5	7.30	7.24	7.95	7.45	Clear, brownish	Low
1.0	7.42	7.24	7.67	7.39	Clear, brownish	Low
1.5	7.63	5.92	7.48	6.56	Dark, dark brown	High
2.0	7.48	5.70	7.76	6.06	Dark, dark brown	High
In vivo	7.87	6.77	8:00	7.42	Clear, brownish	Low
0.5	7.68	7.07	7.57	7.44	Clear, brownish	Low
1.0	7.57	7.43	7.68	7.08	Clear, brownish	Low
1.5	7.63	6.06	7.52	6.75	Dark, dark brown	High
2.0	7.54	5.86	7.68	6.55	Dark, dark brown	High

3.2. Analysis of EPNs Population on 2th-week cultivation

Based on population data for 4 weeks' observation (Figure 1), the highest population was found at 2nd week. Therefore, analysis of variance on population data of the second week was performed to know the optimum powder dose. The study showed that the different kinds of insect powders did not significantly affect the EPN population, but the powder does treatments had a significant effect. It revealed that there was no interaction influence between the two factors (Figure 2). Furthermore, different test results on powder dose variation showed that P0, P1, and P2 were not significantly, while P3 and P4 media were substantially different from the other 3 dose treatments (Tukey's test; $\alpha < 0.05$). The insignificant differences between EPN populations of cultured in vivo (P0) within vitro cultured EPN populations (media P1 and P2) indicated that the availability of nutrients, composition, and environmental conditions of the three treatments were relatively similar. The water content analysis of cricket and worm samples at the beginning of the study was 63% and 55%, respectively. That is, every 1 gram of fresh insects used in vivo medium can be assumed to have a dry weight of 0.37 g and 0.45 g, respectively. Thus, the treatment of P1 and P2 (0.5 g and 1 g of insect powder) are assumed to contain nutrients similar to the in vivo culture.

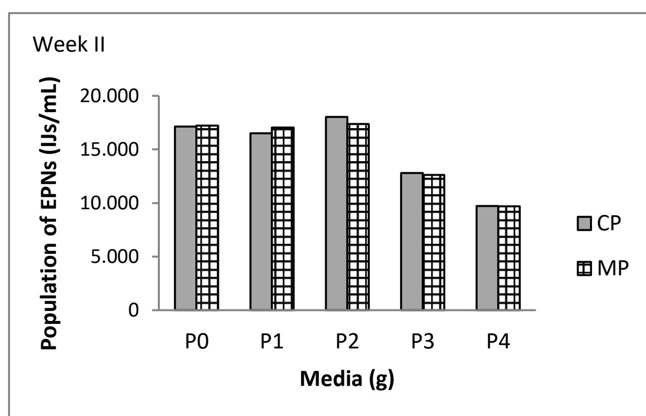

Figure 2. The Average of EPN population in artificial powder media of cricket and mealworm in the 2nd week. Bars followed by the same letters show no significant difference (Tukey's test; $\alpha > 0.05$)

The drying process of insects in powder-making does not seem to affect the nutritional composition of insects significantly. This is due to the drying process under sunlight or at a maximum temperature of 60 °C will not damage the material composition, but only reduce the water content. By reducing the

moisture content, the concentration of constituents such as proteins, carbohydrates, fats, and minerals will increase, while vitamins and colorant generally diminished or destroyed [23].

4. Conclusion

This study concluded that crickets and mealworm powder are active as EPNs culture medium replacing the in vivo method. The optimum doses in this study were 0.5 g insect powder, and harvesting is suggested to be conducted during the second week to obtain maximum population of EPNs. The findings of this study also inform farmers that EPNs can be easily cultivated using simple and available materials.

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