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Mass Cultivation of Entomopathogenic Nematode in Artificial Media

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Abstract

Entomopathogenic nematodes Entomopathogenic nematodes (EPN) of the genera *Heterorhabditis* and *Steinernema* are commercially used to control pest insect. EPN is widely cultivated through in-vivo and in vitro methods. This research aims to discover the abundance of EPN cultivated in various artificial media. Seven types of media composition were tested in this research: media A (yeast + soybean powder), media B (yeast + chicken liver), media C (yeast + dog food), media D (yolk + soybean powder), media E (yolk + chicken liver), media F (yolk + dog food), and media G (yeast + yolk + dog food). Each media was inoculated in 1.2x103JI/mL. The growth of EPN was observed weekly in 4 weeks. Results showed that EPN could be cultivated using various media; media D, E, F, and G. Highest abundance of EPN is found in the second week of media D for 28164 JI/ml.Cell harvesting is suggested to be conducted during the first and second week to obtain maximum abundance of EPN.

How to Cite

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INTRODUCTION

Pesticide usage frequency to control pests is increasing nowadays (Rahim, 2010). Continous usage of synthetic pesticide results in secondary pests explosion, elimination of non target insects, and environmental damage, including animals that compose the ecosystem structure (Djunaedy, 2009).

Pests insects control using highly pathogenic natural pesticides or agents needs to be increasing supported. Some of them are entomopathogenic nematode, bacteria, virus, and entomopathogenic fungi. Entomopathogenic nematode (EPN) is recently used to control pests in Indonesia (Sucipto, 2008). EPN is a parasite for potential pest insects that live underground or above the ground.

Entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* are commercially used to control pest insect. They are symbiotically assosiated with bacteria (Ehler, 2001). *Steinernema* sp. dan *Heterorhabditis* sp. have wide hosts spectrum (Rahim, 2010; Afifah et al, 2013). EPN kill Lepidopteran, Coleopteran, Dipteran, and Hymenopteran rapidly, around 24-48 hours (Griffin et al, 2005; Nugrohorini, 2010). EPN invade the insect host through natural opening (mouth, anus, tracheae or directly through the cuticle). In the haemolymph the nematodes encounter optimal condition for reproduction (Ehler, 2001).

EPN can be cultivated in bulk using both in-vivo or in-vitro media (Uhan, 2008) and solid or liquid media (Malik, 2010). In-vitro cultivation of EPN uses various insects, such as Hongkong caterpillar (*Tenebrio molitor*) (Safitri et al, 2013). In-vitro breeding of EPN is conducted using artificial media (Somwong & Petcharat, 2012; Yoo et al, 2000). EPN cultivation in high protein-dog food media was reported by Wagiman (2003), meanwhile the usage of cow kidney and duck egg for breeding is reported by Malik (2010).

EPN supply is mostly restricted, even in agricultural area. In the other hand, EPN supply is required to control pest insect in such area.

EPN is now produced in form of natural pesticides. However, this EPN is not durable, easily damaged, and quite expensive. Farmers require appropriate and low cost technology to breed EPN. One of the components of cultivation technology is EPN cultivation media. EPN cultivation is urgently required to meet the demand of natural control agents in agricultural area. Therefore, modification of media formula is needed to meet the demand of accesible and affordable. The appropriate technology for EPN cultivation needs modification in media formula to meet the demand of accesible and affordable EPN cultivation technology for farmers.

This research aims to present informations about EPN cultivation using mixed media that contain yeast, dog food, soybean powder, chicken liver, and yolk. The result is expected to encourage farmers to cultivate EPN independently in agricultural area.

METHODS

Research was conducted at the Laboratory of Biology, Semarang State University started from February to August 2014.

EPN multiplication

EPN population were originated from biopesticides obtained in Jember, East Java. EPN were multiplied using Hongkong caterpillar (*Tenebrio mollitor*) as media in white trap method. White trap consists of a jar (6 cm in height and 13 cm in diameter. White trap process was conducted for 7 days. After the process, EPN were harvested. Water that contains EPN was then used for EPN cultivation in several artificial media.

Production of EPN Cultivation Media

EPN cultivation media was made in seven formulas. Each formulas was coded as A, B, C, D, E, F, and G. Compositions of each media are listed below in Table 1.

All media were then made into powder except yolk. Soybean was grinded into powder using grinder. Chicken liver was first baked using oven in 70°C until dried, then grinded into powder. Dog food was grinded into powder, meanwhile yeast had already been in powder. Yolk was obtained from chicken egg in liquid.

Mixed media in each treatment was then added with 0,2 gram gelatin and 30 mL aquadest. All components were then mixed continously. Each media was placed in a glass bottle (12 cm in height and 7 cm in diameter). Inside the bottle, a white sponge (6 cm in height and 1 cm in width) was placed to absorb media liquid. Bottles were then closed and sterilised using autoclave for 30 minutes in 121°C. After sterilisation, media was rested for cooling off. Each Media was then inoculated by $1.2x10^3$ JI/mL EPN suspension (JI = Juvenil infectiv ; ml = mili liter) Bottle lid was then closed and inoculated in room temperature (27-31°C; 65-87%). This research required seven types of treatment and repeated three times in each treatment.

Table 1. Composition of EPN cultivation media

Media composition	*Yeast* (g)	Yolk (g)	Soybean powder (g)	Chicken liver (g)	*Dog food* (g)	Gelatin (g)	Aquadest (mL)
Yeast + soybean powder (A)	1	-	1	-	-	0.2	30
Yeast + Chicken liver (B)	1	-	-	1	-	0.2	30
Yeast + dog food (C)	1	-	-	-	1	0.2	30
Yolk + soybean powder (D)	-	1	1	-	-	0.2	30
Yolk + chicken liver (E)	-	1	-	1	-	0.2	30
Yolk + dog food (F)	-	1	-	-	1	0.2	30
Yeast+ yolk + dog food (G)	0.5	0.5	-	-	1	0.2	30

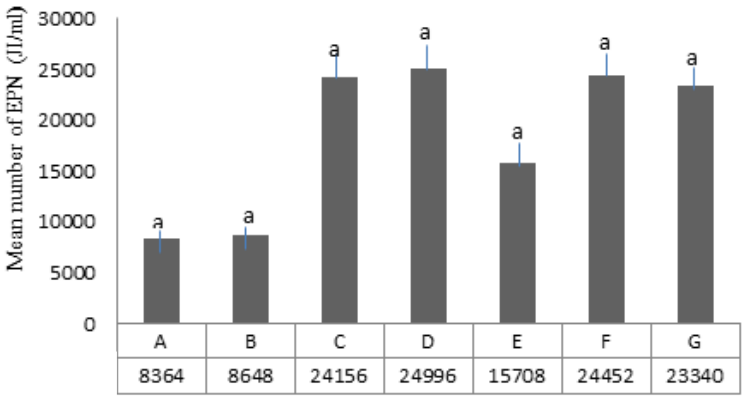

Figure 1. Mean number of EPN in various media in 1st week

Inf.: media A (yeast +soybean powder), media B (yeast +chicken liver), media C (yeast + dog food), media D (yolk+soybean powder), media E (yolk+chicken liver), media F (yolk+ dog food), and media G (yeast +yolk+ dog food)

Observation of EPN

EPN was observed each week for four weeks. Observation of EPN abundance was conducted by taking 0.05 ml EPN culture from the bottle. EPN culture was then dropped in glass slide and observed using binocular microscope (400x magnification scale). EPN was counted using handcounter. Each sample count was repeated three times to count the average. EPN population was counted using this equation:

EPN Population JI/mL = x JI EPN amount

Other measured parameter were pH, temperature, air humidity, and light intensity. Average data of EPN abundance from 1st to 4th week were statistically analysed 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.

RESULT AND DISCUSSION

In vitro EPN cultivation in several media showed various result in each treatment. In the 1st week, all media had the population increased from early inoculation ($1.2x10^3$ JI/mL). Results of EPN abundance in each week is shown below in Figure 1 – 4.

ANOVA test of mean number of EPN (Figure 1) showed that there was no any significant difference between various media in the st week. ($F = 1.566$; $df = 6$; $P = 0.194$; $P>0.05$). However, media C (yeast + dog food), D (yolk + soybean powder), and G (yeast + yolk + dog food) were prone to produce higher EPN population compared to media A (yeast + soybean powder) and B (yeast + chicken liver). EPN could grow in all media, but media C, D, F, and G gave better result than media A and B. In the 1st week, there was an increase in number of EPN in all media. This

was caused by the remaining nutrients in EPN cultivation media.

ANOVA test of mean number of EPN in the 2nd week (Figure 2) presented the result of (F =3.172; df= 6; P<0.05). This showed that there was a difference in average abundance of EPN cultivated in various media. Tukey test showed different mean number of EPN which is shown below in Figure 2.

During the 2nd week, EPN population, cultivated in various media, was fluctuative. EPN population in media D, E, and A was increasing from the 1st week to 2nd week. Media D (yolk and soybean powder) increased from 24996 JI/mL to 28164 JI/mL. Media E (yolk and chicken liver) increased from 15708 JI/mL to 21720 JI/mL. Media A (yeast and soybean powder) increased from 8364 JI/mL to 14.652 JI/mL. Media G, F, C, and B decreased consecutively. Media G (yeast, yolk, and dog food) decreased from 23340 JI/mL to 16212 JI/mL. Media F (yolk and dog food) decreased from 24452 JI/mL to 15888 JI/mL. Media C (yeast and dog food) decreased from 24156 JI/mL to 9948 JI/mL. Media B (yeast and chicken liver) decreased from 8648 JI/mL to 7092 JI/mL.

EPN abundance during the 2nd week increased significantly. This was caused by abundance nutrient in the media. However, EPN activity started to produce waste.

In the 3rd week, there was a difference of cultivated EPN abundance in various media (F=4.776; df=6; P= 0.002; P< 0.05). Post Hoc test using Tukey resulted different EPN abundance shown in Figure 3.

EPN population in all cultivation media was decreased during the 3rd week, consecutively started from media F (yolk + dog food) with highest population (10596 JI/mL), meanwhile the lowest was media C (948 JI/mL). EPN population cultivated in media C and B was even lower than the initial number before EPN inoculation.

The decrease was mainly caused by the lack of nutrient and increasing waste inside the media. This lack of nutrient was caused by increasing usage of the population during the 2nd week, which has the highest number of EPN population. This high EPN population resulted in high waste production. Increasing waste and decreasing nutrients in the media caused decreasing EPN population.

EPN cultivation required optimum nutrients, such as carbohydrate, fat, and protein. According to Johnigk *et al* (2004), carbohydrate and oxygen depletion in the media leads to declining of EPN population. Oxygen depletion induces the rising of carbondioxyde level in the media. It will cause media becomes more alkaline, proven by the rising of pH level from 5 to 8.

Anova test of Mean number of EPN in the 4th week showed result of (F= 16.504; df= 6; P=0.000; P<0.05) (Figure 4). There was a difference in average abundance of EPN population cultivated in various media. Post Hoc test using Tukey resulted different EPN abundance, as shown in Figure 4.

In the 4th week, EPN population cultivated in various in vitro media underwent depletion in

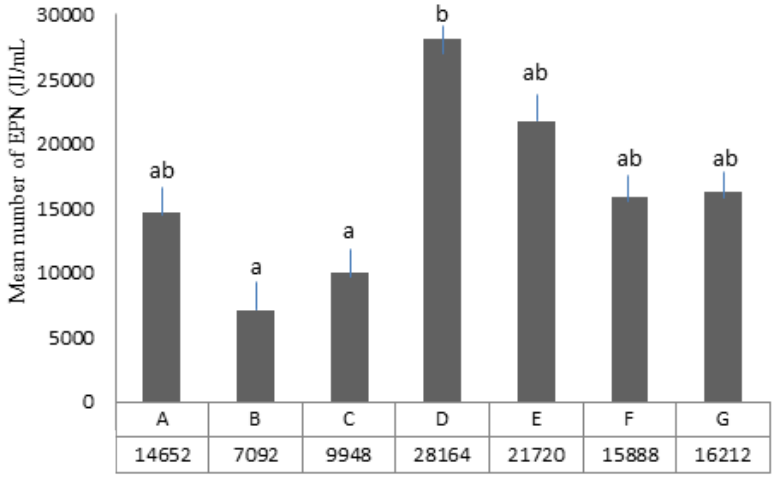

Figure 2. Mean number of EPN in various media during the 2nd week.

Inf.: media A (yeast +soybean powder), media B (yeast + chicken liver), media C (yeast + dog food), media D (yolk + soybean powder), media E (yolk + chicken liver), media F (yolk + dog food), and media G (yeast + yolk + dog food)

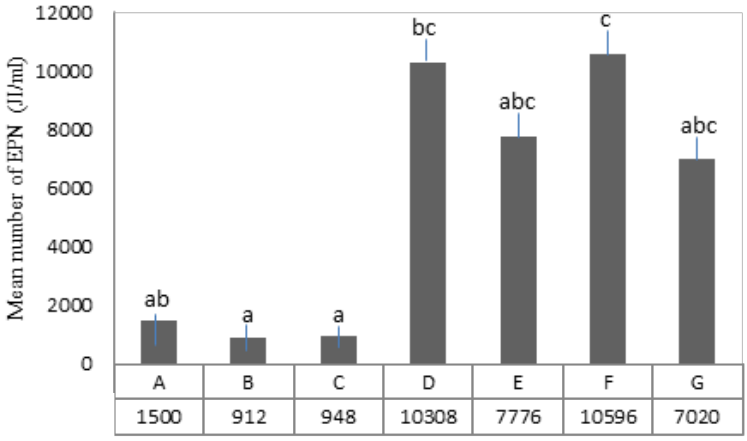

Figure 3. Mean number of EPN in various media during the 3rd week.

Inf.: media A (yeast +soybean powder), media B (yeast + chicken liver), media C (yeast + dog food), media D (yolk + soybean powder), media E (yolk + chicken liver), media F (yolk + dog food), and media G (yeast + yolk + dog food)

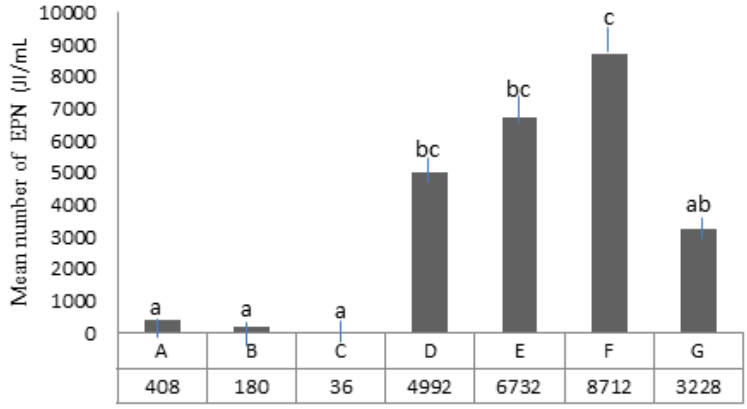

Figure 4. Mean number of EPN in various media during the 4th week.

Inf.: media A (yeast +soybean powder), media B (yeast + chicken liver), media C (yeast + dog food), media D (yolk + soybean powder), media E (yolk + chicken liver), media F (yolk + dog food), and media G (yeast + yolk + dog food)

number of population, same as the 3rd week. The depletion occured in all media, starting from media F (yolk + dog food; 8712 JI/mL) to media C (yeast + dog food; 36 JI/mL). EPN population in media A, B, C, were even lower than the initial number (1.2 x 10^3 JI/mL).

Result of EPN abundance cultivated in various in vitro media during 4 weeks is shown in Figure 5.

According to given data in Figure5, it is concluded that the abundance of EPN reached its peak during the 2nd week in media D, while the least abundance of EPN is shown in Figure 5 is media B.

The best media to cultivate EPN during 4 weeks was media D since it produced the highest abundance of EPN. Media D, composed of yolk and soybean powder. It has the biggest escalation of EPN population number during the 1st and 2nd week. According to Rahayu (2003), yolk contains 29,8% of fat, 15,22% of protein, and 1,92% of carbohydrate. High fat contents in the yolk is used as main source of nutrient for EPN, since EPN has restricted ability to synthesize fat (Gil et al, 2002). Soybean powder has high protein content that is important to build new tissue in EPN population that were cultivated in various in vitro media (Cao et al, 2013). Soybean powder contained 40,5% of protein, 20,5% of fat, and 22,2% of carbohydrate.

EPN is required in high amount for pest control system. This research showed that harves-

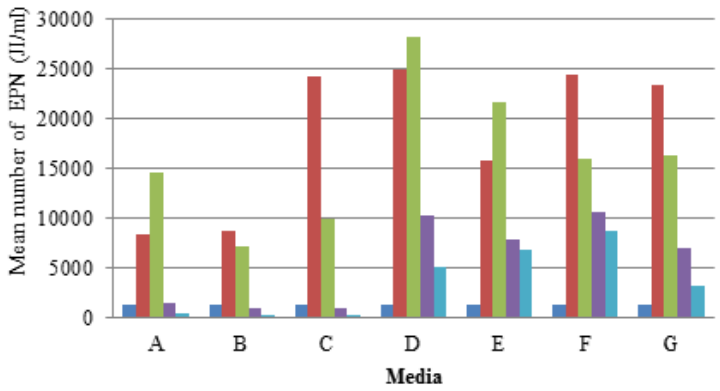

Figure 5. Mean number of EPN during 4 weeks.

Inf.: media A (yeast +soybean powder), media B (yeast + chicken liver), media C (yeast + dog food), media D (yolk + soybean powder), media E (yolk + chicken liver), media F (yolk + dog food), and media G (yeast + yolk + dog food)

Table 2. Color, aroma, and shape alteration in various cultivation media.

Media	Color		Aroma		Shape	
	Initial	End	Initial	End	Initial	End
A	Light brown	Dark brown	Fresh	Odorless	Semi solid	Semi solid
B	Light brown	Dark brown	Fresh	Rather smelly	Semi solid	Semi solid
C	Brownish yellow	Brown	Fresh	Rather smelly	Semi solid	Semi solid
D	Brownish yellow	Black brown	Fresh	Rather stingy	Semi solid	Liquid
E	Brownish yellow	Dark brown	Fresh	Stingy	Semi solid	Liquid
F	Light brown	Black brown	Fresh	Odorless	Semi solid	Liquid
G	Light brown	Dark brown	Fresh	Rather smelly	Semi solid	Semi solid

Inf.: media A (yeast +soybean powder), media B (yeast + chicken liver), media C (yeast + dog food), media D (yolk + soybean powder), media E (yolk + chicken liver), media F (yolk + dog food), and media G (yeast + yolk + dog food)

ting EPN population was best conducted during the 2nd week. The cultivation media still has adequate amount of carbohydrate, protein, and fat for EPN growth.

During the 3rd and 4th week, nutrients in the media was degraded into another compound that obstruct the EPN growth and induce the depletion of EPN population. This was proven by increasing pH level from 5 to 8.

Waste product of EPN metabolism and micro contaminant inside the media obstruct the EPN growth, resulted in declining EPN population. The color of cultivation media also changed from brown to dark brown. Aroma alteration also occured in the media, from fresh to stingy aroma. The media also underwent shape alteration from solid to liquid. Color, aroma, and shape alteration is affected by composition of each media and waste product of EPN metabolism from the cultivated population in closed condition.

End product of EPN metabolism also affect the pH level of EPN cultivation media. Inital pH of each media were ranged from 5 to 5,5. After the 4th week of treatment, the pH level increased to 8 to 9 (Table 3). pH level that ranged from 5 to 9 is still tolerable for EPN cultivation (Djunaedy, 2009). This pH level alteration is mainly affected by EPN activity in the media itself. pH level alteration in the media is also caused by degradation of other nitrogenic compound (Prihantini et al, 2005). Ammonia in the media will be toxic for the EPN and threat the EPN cultivation. High ammonia level in the media obstruct EPN cultivation (Fadilah et al, 2009).

Table 3. pH measurement in various EPN cultivation media

Media	pH	
	Initial	End
A	5,5	9
B	5	8
C	5	8
D	5,5	8
E	5,5	8
F	5	8
G	5	8

Media A, which were composed of yeast and soybean, underwent the most significant pH change to 9. Yeast and soybean powder have high protein contain, respectively 50% and 40,5%. High protein contain in the media would be degraded into ammonia. High ammonia contain in the media induced the pH change into alkaline condition.

Light is an important factor that affects EPN cultivation. Direct exposure of light toward EPN could inhibit the EPN growth (Ehlers, 2001). In this research, EPN cultivation treatment was conducted in a room, so the EPN was not directly exposed to sunlight. Measured light intensity was 3,3 – 27,2 Lux.

This research findings inform farmers that EPN can be easily cultivated using media D (yolk + soybean powder), media E (yolk + chicken liver), media F (yolk + dog food), and media G (yeast + yolk + dog food). EPN population is best harvested in the 1st and 2nd week after inoculation.

CONCLUSION

EPN cultivation in various media for four weeks showed its different population of EPN in each week. In the 1st week, EPN abundance was increased. In the 2nd week, EPN population was increased and then decreased. In the 3rd and 4th week, the EPN population was gradually decreased in all cultivation media. EPN cultivation media with the the best result was media D composed of yolk and soybean powder, producing highest EPN population of 28164 JI/mL in the 2nd week.

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