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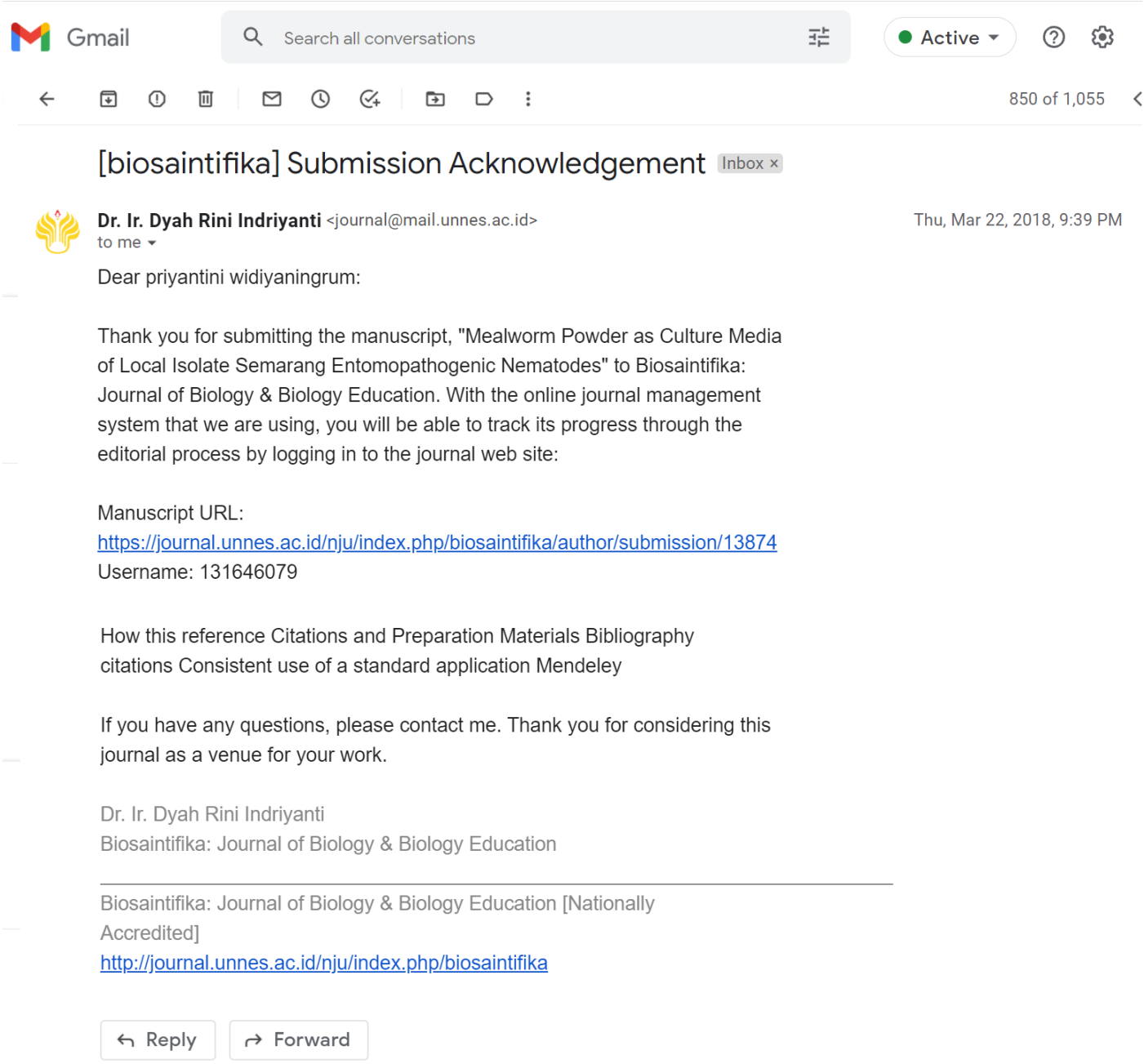

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Mealworm Powder as Culture Media of Local Isolate Semarang Entomopathogenic Nematodes

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Abstract

Many researchers confirmed that entomopathogenic nematodes (EPNs) of the genera *Heterorhabditis* and *Steinernema* can be cultivated in vitro using artificial media that containing animal nutrition. However, artificial media with insect component hasn't been widely studied. This research aims to discover the population of EPNs isolate of Semarang cultivated in mealworm powder media. Five treatment doses of mealworm powder (*Tenebrio molitor*) were tested in this research, i.e: 0.5; 1.0; 1.5; 2.0 g. Culture media using 1 g of mealworm larvae was used as control. The best treatment was further tested for its pathogenicity on *Macrotermes* sp. at seven levels of invective juvenile (IJs) : 0; 50; 100; 150; 200; 250; 300 IJs/mL. Each treatment was repeated five times. The EPNs population and termites mortality were analyzed using ANAVA, whereas pathogenic value on LD_{50} and LD_{90} was calculated using Probit analysis. The result showed EPNs population were significantly difference (LSD; $\alpha > 0.05$), likewise on termites mortality. The EPNs isolate of Semarang optimally at 1g mealworm powder and pathogenicity against termites based on LD_{50} and LD_{90} values at 220 JI/ml and 410 JI/ml doses, respectively. In conclusion, this result can be an alternative to mass cultivation of EPNs, in effort of development of local bioinsecticides.

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Key words: artificial media, *Macrotermes* sp., Mealworm powder, EPNs isolate of Semarang

INTRODUCTION

Pests insects control using chemical pesticide has caused many negative impacts such as pest resistance, secondary pests explosion, poisoning in humans, and environmental damage (Horowitz and Ishaaya, 2013). Efforts to suppress the use of chemical insecticides have been widely practiced, including an alternative use of mass-produced entomopathogenic nematode (EPNs). the EPN is a parasite for potential pest insects that live underground or above the ground.The EPNs is recently used to control pests in Indonesia (Sucipto, 2008).

The EPNs are a micro-worm, easily found in soil in different parts of the world and has been known as an effective and environmental friendly biocontrol agent (Herbert and Blair, 2007; Nugrohorini, 2010). The results of the EPNs isolate of Semarang (Indriyanti

et al., 2014) revealed that the EPN found was dominated by the genus *Steinernema* sp. and the highest abundance was found on farmland area.

The EPNs can be cultivated using both in-vivo or in-vitro media (Uhan, 2008; (Mulyaningsih, 2010) and solid or liquid media (Malik, 2010). Many researchers confirmed that EPNs of the genera *Heterorhabditis* and *Steinernema* can be cultivated in vitro using artificial media that containing animal nutrition (Chaerani, 2011); (Indriyanti and Muharromah, 2016). However, artificial media with insect component hasn't been widely studied. Mass cultivation in vitro using artificial media without insect substitution still has a weakness because the composition of nutrients has not fully resembled the components of hemolymph of natural host, so the population growth is not optimum. (Chaerani et al. (2012) found that the quality of EPNs grown in an artificial media using mixtures of the chicken intestine, beef fat and egg yolk has shorter and wider morphometrics, also lower pathogenicity than those in vivo cultivation. Artificial media by Indriyanti et al. (2015) using soy powder, chicken liver, and dog food revealed that the highest EPNs population was achieved at the second week then its population decreased. This condition is presumably because artificial media haven't been able to provide the essential nutrients needed for the growth of EPNs and their symbiotic bacteria, such as the nutrients in the insect hemolymph (Chaerani et al, 2012).

Artificial media using mealworm powder is selected because it is cheap and easy in mass cultivation. The mealworm powder easily is stored for a long time, and its composition similar than mealworm larvae. This research aims to discover the population of EPNs isolate of Semarang cultivated in mealworm powder media. These artificial media are potential if the growth of EPNs Semarang isolate is not significantly different or better than the growth of EPNs in vitro as control.

METHODS

Research was conducted at Laboratory of Biology, Universitas Negeri Semarang, started from February - June 2017. The EPNs was isolated from soil sample in area of the poultry farm at Gunungpati, Semarang. The EPNs were multiplied using mealworm larvae (*Tenebrio mollitor*) by white trap method and filter paper 281–324 (Kaya and Stock, 1997). The breeding room is conditioned at room temperature 25° ± 2°C. Infective Juveniles (IJs) harvesting for the test sample is performed in the first week after incubation.

Preparation of Artificial Media

Mealworm larvae was first baked in the oven at 60°C until dried, then grinded into powder. The sample ±10 grams were analyzed proximate, and others are stored for preparation of artificial media test. Control media were determined using 1g of mealworm larvae, while artificial media were made four treatment with the following composition.

P_0 : control media using 1g of mealworm larvae + 30 ml distilled water.

P_1 : medium with 0.5 g of mealworm powder + 0.2 g agar + 30 ml of distilled water.

P_2 : medium with 1 g of mealworm powder + 0.2 g agar + 30 ml of distilled water.

P_3 : medium with 1.5 g of mealworm powder + 0.2 g agar + 30 ml of distilled water

P_4 : medium with 2 g of mealworm powder + 0.2 g agar + 30 ml of distilled water

All components were then mixed continously. Each media was placed in a brown glass bottle (75 ml volume,; 4.5cm in diameter). Inside the bottle, polyurethane sponge (4.5 cm diameter; 2 cm height) was placed to absorb media liquid. Bottles were then closed and sterilised using autoclave for 45 minutes in 121°C. After sterilisation, artificial media was rested for cooling off. Each Media was then inoculated by 1mL suspension which contained $1x10^3$ IJs. Bottle lid was then closed and incubated in room temperature for two weeks. This research repeated five times in each treatment.

Population Density of EPNs

EPNs was observed each week for two weeks. Observation of population density was conducted by taking 50 µl EPN culture from the bottle using a micropipette. The population density was calculated by sampling under binocular microscope (100x magnification). The sample were dripped on an object glass and counted using hand counter. Each sample count was repeated three times to count the average. Only IJs was actively moving, forming the letter S or in position J which was considered alive, while the dead ones (looks straight like a needle, not moving) were not counted.

Population density is calculated by the formula:

$$\text{Population}\left({}^{\text{IJs}}/_{\text{mL}}\right) = \frac{n}{t} \text{ x (Y)}$$

where Y = average number of IJs in 50 µL

n = volume 1 ml (1000 µL)

t = sample volume (50 µL)

Pathogenicity Test on Termites

The pathogenic test was performed by examining the mortality of termites in seven dosage IJs : 50, 100; 150; 200; 250; and 300 IJs/mL. The pathogenic test was also

performed by calculating the LD_{50} and LD_{90} values using Probit analysis. Each doses level was repeated five times. Trials used a plastic cup (10 cm high, 6 cm in diameter), filled with 25 g of sterile sand soil. The soil was made moist by adding 10 ml of distilled water, then put 1mL IJs according to the treatment dosage, as well as the worker termites as much as 50 heads/cup. As a protector, into each cup, are added 1.5 g pieces of cardboard. All cups are incubated in dark space for 48 hours. Termites mortality after 48 hours is calculated using the formula:

$$\text{Mortality}(\%) = \frac{\text{number of dead termites}}{\text{total number of termites}} \text{x } 100\%$$

To determine the pathogenicity of EPNs as the breeding result based on LD_{50} and LD_{90} values, mortality data were converted using the Abbot formulation in advance if mortality was found between 5-20% in the control treatment. Formula Abbott (1925) in WHO (2009) is:

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

X = percentage of mortality on treatment

Y = percentage mortality in control treatment.

Other measured parameter as supporting main data were pH, temperature, air humidity, and light intensity. Average data of EPNs population on 2^{th} weeks were statistically analysed using one way Anova in significancy level of 5% and Post hoc test (LSD test). Data analysis was done by SPSS 18 software. To find out the value of LD_{50} and LD_{90}, this research used Probit analysis with program Minitab 17.

RESULTS AND DISCUSSION

Proximate Analysis of Mealworm Powder

The results of proximate analysis of mealworm powder showed high protein, fat, and energy content that could potentially be a source of nutrients. According to Ravzanaadii *et al.* (2012), larvae of mealworms contain 63.34% protein which is rich in amino acid Isoleucine, leucine, and lysine. According to Lokeshwari and Shantibala, 2010), insects including mealworm larvae have the potential to become a source of protein for humans and animals, because of the abundance of availability as well as more environmentally friendly compared to the source of protein from vertebrates.

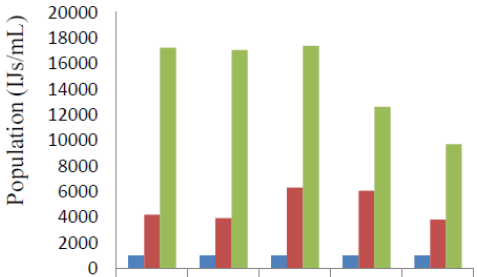
Table 1. Proximate analysis results of mealworm powder

Component	Results of Analysis
Water (%)	32.89
Ash (%)	7.98
Fat (%)	8.27
Crude Fiber (%)	9.89
Crude Protein (%)	71.84
Bruto Energy (Cal/gram)	6414*)

Notes: data analysis of Animal Nutrition Laboratory, UNDIP (2017)
*) data analysis of Center for Biological Resources, Faculty of Agricultural Technology, IPB (Ninasari, 2014).

Population Growth of EPNs

The population growth of EPNs on various mealworm powder is presented in Figure 1, while the results of statistical analysis are presented in Table 2.



	P0	P1	P2	P3	P4
Early inoculation	1000	1000	1000	1000	1000
1st Week	4183	3927	6307	6039	3800
2nd Week	17231	17048	17376	12620	9692

Figure 1. Mean population of EPNs in various media
Notes: P0 (*In vivo* media); P1 (0.5 g of mealworm powder); P2 (1 g of mealworm powder); P3 (1.5 g of mealworm powder); P4 (2 g of mealworm powder).

Figure 1. explains that in the 1st week and 2nd week there has been increased from early inoculation. This indicates that EPNs isolate of Semarang can grow in artificial media with nutritional source of mealworm powder. According Ramakuwela *et al.*, 2016) artificial media using fruit fly pulp could be a competitive technological alternative in mass cultivation of EPNs. This is potentially because without having to invest heavily in artificial media and fermentation equipment. Population growth that has begun to be seen

in the 1st week and higher in the 2nd week is due to the life cycle of genera *Steinernema* sp. takes approximately 14 days for development of the eggs until a new generation IJs (Wagiman et al., 2003). In the sufficient nutritional conditions, Steinernematidae will reproduce one to three generations within the same host and produce a new generation within 7-10 days (Wouts, 1991). In this case, the environmental and nutrient reserve factors are very influential on the survival of EPNs (Rahoo *et al.*, 2016). In this study, the laboratory environment is conditioned at 25 ±1°C as the optimum temperature for the EPNs isolation of Semarang under in vitro ((Widiyaningrum et al., 2016).

The high EPN population at 2nd week is thought to be the peak of population growth. Physiologically, 2nd week period or 14 days after inoculation was the period in which IJs phase 3. It is said to have the best resistance because it is still inside the cuticle that serves as a protector (Sulistyanto, 2000). Multiplication of local EPNs by (Indriyanti et al., 2015) using some artificial media without insect (mixed from soy bean powder, chicken liver, dog food) found that the highest population was also achieved at 2nd week.

Table 2. Population of EPN isolate of Semarang in 1st and 2nd week

Treatment	Population in 1st week (IJs/mL)	Population in Week 2 (IJs/mL)
P_0	4.183[a]	17.231[a]
P_1	3.927[a]	17.148[a]
P_2	6.307[b]	17.376[a]
P_3	6.039[b]	12.620[b]
P_4	3.800[c]	9.692[c]

Inf: different letters in the same column for each treatment indicate significant differences (LSD test; $\alpha < 0.05$).

Based on the statistic analysis of population at the 1st week (Table 2), it showed that the dosage of mealworm powder have significantly influenced on EPNs population ($\alpha > 0.05$). The EPNs population on the treatment of 0.5 g mealworm powder (P1) was not significantly different than those *in vivo* media (control), while the other three treatments showed the difference. The highest population was seen in the the treatment of 1 g mealworm powder (P2) and was significantly different from control. In line to the mean population of the 1st week, the highest EPNs population at 2nd week was achieved in treatment P2, and the lowest population at P4 on both observations. However based on the LSD test, P2, P1 and control were not significantly different. Thus, using 0.5 g of mealworm powder on artificial media of EPNs are the best.

High protein in the artificial media didn't cause the EPNs population to increase, instead it decreased. According Indriyanti and Muharromah (2016) waste product of EPNs metabolism and micro contaminant inside the artificial media obstruct the EPNs growth, resulted in declining EPNs population. Supporting data showed that Inital pH of each media were ranged from 7.30 to 8.0. This pH range corresponds to the optimum requirement for survival of EPNs of Semarang isolate (Widiyaningrum et al., 2017). After the 2th week of treatment, the pH level was relatively constant, but on qualitative observation (color and odor) of cultivation media changed from light brown to dark brown. Over time, in media containing higher protein (1.5 g and 1 g), the possibility of degradation of the protein compounds into ammonia gas is also higher. Therefore, the higher the protein content, the more ammonia is formed in the bottle. This condition causes smoother media color changes and emits stronger ammonia odors. Thus it affected EPNs survival. Solid media causes protein degradation which disrupted the activity of EPNs and its symbion bacteria and eventually it causes the death of juvenile infective nematodes. In contrast to the conditions of treatment P0, P1, and P2, it is presumed that the availability of nutrients is still within tolerable limits for continuity of EPNs reproduction until 2nd week.

The ratio of nutrient availability, the extent of breeding space is thought to affect the availability of oxygen, thus affecting survival. According to Indriyanti and Muharromah (2016), it is necessary to consider the number of nutrients and capacity of artificial media space to ensure the need for carbohydrates, proteins, and fats for the growth of EPNs within a certain time. According to Grewal et al. (2005) factors influencing the success of the mass production of EPNs with liquid media such as oxygen supply, media composition, temperature, bacterial biology, and population-nematodes. Similar to this research, the success of EPNs production using mealworm flour media is not only influenced by above factors but also is influenced by the pH and humidity of the room. (Dwijayanto et al., 2016) stated that the nutritional and fat content contained in mealworm is considered good as a food source that supports the colony breeding. Therefore, the content of fat and protein in mealworm flour in a certain amount can meet the needs of life in adapting to breed and in metabolic processes.

Similarly, the high composition makes the protein in the medium is also high compared to fat, it is beyond the needs of nematodes that require only 40% protein and 60% fat. The increased protein will lead to an increase in ammonia concentration in the medium because ammonia is the result of hydrolysis of the protein (Suherman et al.,

2013). The formation of ammonia will be toxic to the nematodes and will make the symbiotic bacteria decrease. The decrease of symbiotic bacteria in the breeding medium resulted in fat synthesis process which is not running optimally. Without the source of fat in the cultivation media, the EPNs can not reproduce normally, so the population declines. (Sahabuddin et al., 2014) stated that the higher the CO_2 concentration, the lower the pH. According to previous research (Widiyaningrum et al., 2017) it is stated that the best pH for breeding *Steinernema* sp. ranged from 7.5 and 8.

Pathogenicity test of EPNs against *Macrotermes* sp.

The pathogenicity of EPNs cultivation was tested using seven dosage and determining LD_{50} and LD_{90} values. The average mortality of termites on each treatment within 48 hours of observation is presented in Table 3.

Table 3. Mortality of *Macrotermes* sp on Various Dosage of EPNs within 48 hours.

Dosage	Replication					Average (%)
0	12	8	4	2	16	8.4[a]
50	8	20	14	16	8	13.2[a]
100	22	20	8	20	30	20[a]
150	30	18	36	30	26	28[b]
200	28	42	58	52	46	45.2[c]
150	50	40	64	60	78	58.4[d]
300	60	70	64	80	86	72[e]

Note: different letter notation indicate significant differences (LSD test; $\alpha < 0.05$).

Table 3 indicates that the higher dosage of EPNs, the higher termites mortality rate. Statistically, mortality at 50 and 100 IJs/mL was not significantly different from control. However, the other four treatments were significantly different ($\alpha > 0.05$). The highest mortality was found in a treatment dose of 300 IJs/mL with 72% mortality. This suggests that a dose of 300 IJs/mL is the best. This data is in line with previous research (Widiyaningrum et al., 2016) which obtained data of 250 IJs/mL to achieve mortality of more than 50%. Probit analysis of converted mortality data is presented in Table 4.

Table 4. LD_{50} and LD_{90}-48 hours values on pathogenicity test of EPNs on termites.

Lethal Dose	The population of EPNs (IJs/mL)
50	220
90	410

Based on Table 4, the EPNs isolate Semarang succeeded in killing up to 50% of the test animals at a dose of 220 IJs/mL, while the killing ability of dose 410 IJs/mL is up to 90%. According to (Chapuis et al., 2012), the more IJs to enter into the insect host body, it will cause septicemia and die more quickly. To prove that termite mortality was caused by EPNs infection, dead termites were observed under microscope by 10x10 magnification. The observed samples showed EPNs in the abdomen of termites (Figure 2). In addition, ground termites infected by EPNs are characterized by changing body colors like brown caramels and dark brown. The texture of the body became soft and discharged fluid and over time would be destroyed. This is due to the character of the Symbionese bacterium *Steinernema* sp., ie, xenorhabdus produced pigments causing discoloration of the cuticle of its host (Safitri et al., 2012).

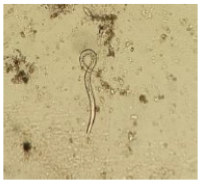


Figure 2. Observation of abdominal termites infected with EPN

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In the previous study, (Widiyaningrum et al, 2017) found that LD50 value the 24 hours pathogenicity test had not reached 50% mortality, but within 48 h termites mortality reached over 50%. According to (Widiyaningrum et al., 2016), when the number of IJs entering the host's body exceeded the optimal limit, competition appeared and occurred between IJs and affected in reducing the mean number of migrating larvae from an insect.

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CONCLUSION

The mealworm powder was proved potential to be used as a cultivation media of EPNs isolate Semarang, optimally at dose of 0.5 g. The pathogenicity against termites

based on LD_{50} and LD_{90} values was achieved at consecutive doses of 220 JI/mL and 410 JI/mL. This research findings inform the people that EPNs can be easily cultivated using artificial media.

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