



## Research Article

### *In Vitro* Cultivation of *Aphelenchoides besseyi* Christie on Fungal Cultures

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#### ABSTRACT

White tip disease on rice caused by *Aphelenchoides besseyi* is categorized as emerging infectious disease in Indonesia because the disease incidence and its geographical distribution are increasing rapidly. Until presently, there is no information related with mass culture technique of *Ap. besseyi* in Indonesia. Thus, studies to obtain pure population of *Ap. besseyi* are necessary to be conducted to support various research aspects in the future. This research is aimed to study *Ap. besseyi* pure mass culture technique by rearing the nematode on three fungal species. Three different fungal cultures on PDA medium, i.e. *Alternaria padwickii*, *Fusarium semitectum*, and *Botrytis cinerea* were tested as *Ap. besseyi* cultivation media. To obtain the optimum condition for nematode cultivation, the fungal cultures were incubated in three different temperature. The nematodes were surface sterilized with 0.1% streptomycin sulfate followed by washing with sterilized water before infesting the nematode into fungal culture. Subsequently, 25 sterilized nematodes were infested into 7 days old of fungal culture and incubated at 20°C, 25°C and 30 °C. After 21 days, nematodes were harvested and counted the final population. Among fungal species tested as rearing medium, the best culture for *Ap. besseyi* reproduction was on *Al. padwickii* culture at 25°C with average final population up to 9,115 per petri dish and reproduction factor 364.6. At a 30°C, *Ap. besseyi* was failed to develop its population in all the fungal cultures tested. It indicated that the optimum temperature for reproduction of *Ap. besseyi* on those of fungal species was 20–25°C.

Keywords: *Aphelenchoides besseyi*; *in vitro* culture nematode; reproduction factor; temperature

#### INTRODUCTION

*Aphelenchoides* is a genus that is consisted of parasitic nematodes on high-level plants, associated with insects, and most species are microphagous nematodes. Some *Aphelenchoides* species recognized as major pests include *Aphelenchoides besseyi* Christie, *Ap. ritzemabosi* (Schwartz) Steiner and Buhner, *Ap. fragariae* (Ritzema Bos) Christie and *Ap. arachidis* Bos. (De Waele, 2002). *Ap. besseyi* are recognized to cause white tips on rice and are distributed in rice fields around the globe (EPPO, 2017).

White tips were first found in Indonesia in 2014 and caused a distinctive symptom of the ends of rice leaves to turn white (Wiyono *et al.*, 2017). In Java, *Ap. besseyi* have been reported at Banten (Lebak), West Java (Bogor, Sukabumi, Subang, and Indramayu), Central Java (Klaten, Sragen, Sukoharjo, Boyolali, Pati, and Pemalang), Special Region of Yogyakarta (Yogyakarta and Sleman), East Java (Magetan, Blitar, Tuban, Gresik, Nganjuk, and Banyuwangi) (Diana, 2018).

*Ap. besseyi* is an ectoparasite and seedborne nematode. Nematodes have been reported to be found on seed glumes. Dormant nematodes will re-establish when preferred humidity levels are reached. Nematodes move through water on plant surfaces and migrate to leaves and stem growing points to obtain nutrients. Optimum temperature for the development of this nematode is approximately 21–25°C and nematodes have been reported to survive in dry condition for 2–3 years (Hockland, 2004).

Besides infecting high-level plants, this nematode also feed on fungi. *Ap. besseyi* mass rearing on fungal species may serve as an efficient method to obtain high populations of pure nematodes, which will support further research of this species. Fungal species known to support growth for *Ap. besseyi in vitro* rearing include *Fusarium solani* (Huang *et al.*, 1972), *Aerobasidium pullulans* (Huang *et al.*, 1979), *Alternaria tenuis* (Todd & Atkins, 1958), and *Botrytis cinerea*. In addition, fungal cultures known as the best fungi for *Ap. besseyi* include *Alternaria alternata*, *Fusarium moniliforme*, *Phoma medicaginis*, *F. solani*,

*F. equiseti* (Gokte & Mathur, 1989), *Bipolaris oryzae* and *Magnaporthe salvinii* (Jamali *et al.*, 2008).

*Ap. besseyi* are able to reproduce parthenogenically or amphimictically. Parthenogenesis is an asexual reproduction where female nematodes produce eggs cells without fertilization. This behavior causes nematodes to be able to produce many offspring. However, competition to obtain nutrient also increases. Amphimictic is a sexual reproduction where sperm and egg cells from different individuals meet (cross fertilization). Nematodes are able to mate freely and produce fertile offspring. Population of *Ap. besseyi* from Russia were able to parthenogenesis on *Fusarium solani* cultures (Sudakova & Stoyakov, 1967), while *Ap. besseyi* populations from Taiwan were able to reproduce amphimictically on *Aerobasidium pullulans* cultures (Huang *et al.*, 1979). To our knowledge, until today there has not been scientific information on mass rearing of *Ap. besseyi* populations from Indonesia on fungal culture mediums. Mass rearing techniques for *Ap. besseyi* is essential for further research. Therefore, this study aims to investigate fungal species medium and temperature combinations for optimum *Ap. besseyi* rearing.

## MATERIAL AND METHODS

### *Ap. besseyi* and Fungal Culture

*Ap. besseyi* used in the study were collected from rice seeds, variety Pak Tiwi-1, and obtained from Balai Besar Padi Sukamandi Subang. Nematode extraction was done using the Baermann funnel method based on procedures from International Seed Testing Association (ISTA) (2018), by cutting 5 g of seed hilum. After cutting hilum, seeds were placed on cloths and immersed in water. Incubation was done for 24 hours at 25°C. Incubation results were extracted using a 500 mesh. *Ap. besseyi* surfaces were sterilized using streptomycin sulphate (0.1%) for 10 minutes followed by rinsing with sterilized water for 3 times. Fungal species used as rearing medium of *Ap. besseyi* were *Alternaria padwickii*, *Fusarium semitectum*, and *Botrytis cinerea*. Cultures were obtained from the Mycology Laboratory, Faculty of Agriculture, Bogor Agriculture Institute. Fungal colonies were grown on Potato Dextrose Agar (PDA) medium on 9-cm petri dishes. Fungal cultures were incubated at 25°C and grown until

entire surfaces of medium were covered with mycelium.

### *Ap. besseyi* rearing on Fungal Cultures

Twenty five nematodes were infested to each petri dish when mycelium of tested fungal species has entirely covered the medium. Each petri dish was stored in a black plastic bags and incubated in dark conditions at temperatures of 20°C, 25°C, and 30°C. Nematodes were harvested 21 days after infestation, followed by population counts. Mediums were sliced and extracted using a modified Baermann funnel method. Numbers of juvenile and mature nematodes were calculated using the formula from Coyne *et al.* (2014):

$$N = \frac{V}{v} \times n$$

N = nematode population

V = suspension volume in bottle

v = suspension volume in syracuse dish

n = average number counted under microscope

Total number of juvenile and mature nematodes were used as final population densities. Reproduction factor (FR) was calculated accordance to the formula used by Jamali *et al.* (2008):

$$FR = \frac{Pf}{Pi}$$

Pf = final nematode population

Pi = beginning nematode population

### Experimental Design and Data Analysis

This study was designed as a Factorial Complete Randomized Design. The first factor were the three fungal species used as medium and the second factors were the three incubation temperatures Treatment combination tested were nine and each treatment combination consisted of nine replications. Data obtained were processed using *Excel 2013* and analyzed using *Minitab 16 Statistical Software* with a Tukey post-hoc test at 95% confidence level.

## RESULTS AND DISCUSSION

Fungal species used as rearing medium of *Ap. besseyi* are all considered as plant pathogens. *Al. padwickii* and *F. semitectum* are pathogen that infect rice seeds in the fields and storages, while *B. cinerea* is a pathogen on strawberries which causes grey mold. Colony of *Al. padwickii* on PDA medium were gray with smooth mycelium. Colony located

on the underside of the PDA medium was black. Conidiophores were dark colored, elongated, and conidia contained 7–8 septas that were oval shaped. Macromorphological characteristics of *F. semitectum* on PDA mediums contained dense mycelium and was white to brownish depending on its age. Abundant microconidia were oval shaped, in general contained three septas, hypha microscopically were transparent and possessed septas. Colonies of *B. cinerea* on PDA medium were greyish brown with white cotton-like mycelium on the edges. Conidiophores were long, slim, contained hyaline, irregularly branched on top, and apical cells were larger and round (Figure 1).

Rearing of *Ap. besseyi* resulted in various growth stages of nematodes, including juvenile to mature nematodes (Figure 2). Female nematodes were elongated, with lengths of 0.66–0.75 mm and slender. Mouths were round, did not contained striations and in general were wider than their neck or parallel with their body width. Heads were flat and round on its anterior with a strong and sclerotized supporting structure, which forms the head structure and function to point their thin and slightly offset stylets. Cuticles were smooth annulation. Sizes and shapes of male nematodes were similar to

females, except for the characteristics of their reproduction structure.

Results of *Ap. besseyi* rearing on three culture species showed that nematode population varied between culture species and incubation temperature. Population of *Ap. besseyi* reached the highest number, 9,115 individuals, when reared on *Al. padwickii* at 25°C, while the lowest population were obtained from nematodes reared on *B. cinerea* at 30°C (Table 1). Optimum temperature for *Ap. besseyi* development is 21–25°C, their life cycles last for about 10 days at 21°C, eight days at 23°C, and nematode did not grow at 13°C (Bridge *et al.*, 2005).

On fungal mediums, *Ap. besseyi* have been reported to be able to finish their life cycle at 20°C (Rajan *et al.*, 1990) and between 23–30 °C (Huang *et al.*, 1972). Research have shown that temperature affect *Ap. besseyi* development. At incubation temperatures of 20°C and 25°C, *Ap. besseyi* populations inoculated on *Al. padwickii* and *F. semitectum* showed higher populations compared with nematodes incubated at 30°C. Temperatures between 20 to 25°C were the most suitable conditions for *Ap. besseyi* development. Although oviposition, molting, and egg hatching still occurred at 30°C, *Ap. besseyi* were not able to development well. Nurjanah *et al.* (2016) stated that

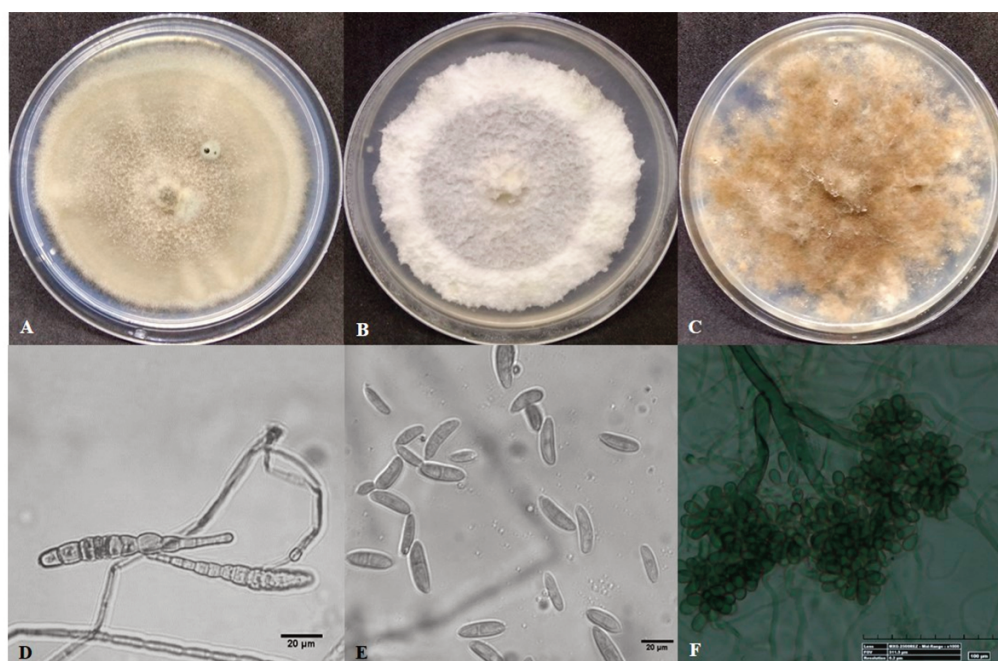


Figure 1. Fungal cultures and microscopic appearances of species used as rearing mediums of *Aphelenchoides besseyi*: *Alternaria padwickii* (A, D), *Fusarium semitectum* (B, E), *Botrytis cinerea* (C, F)

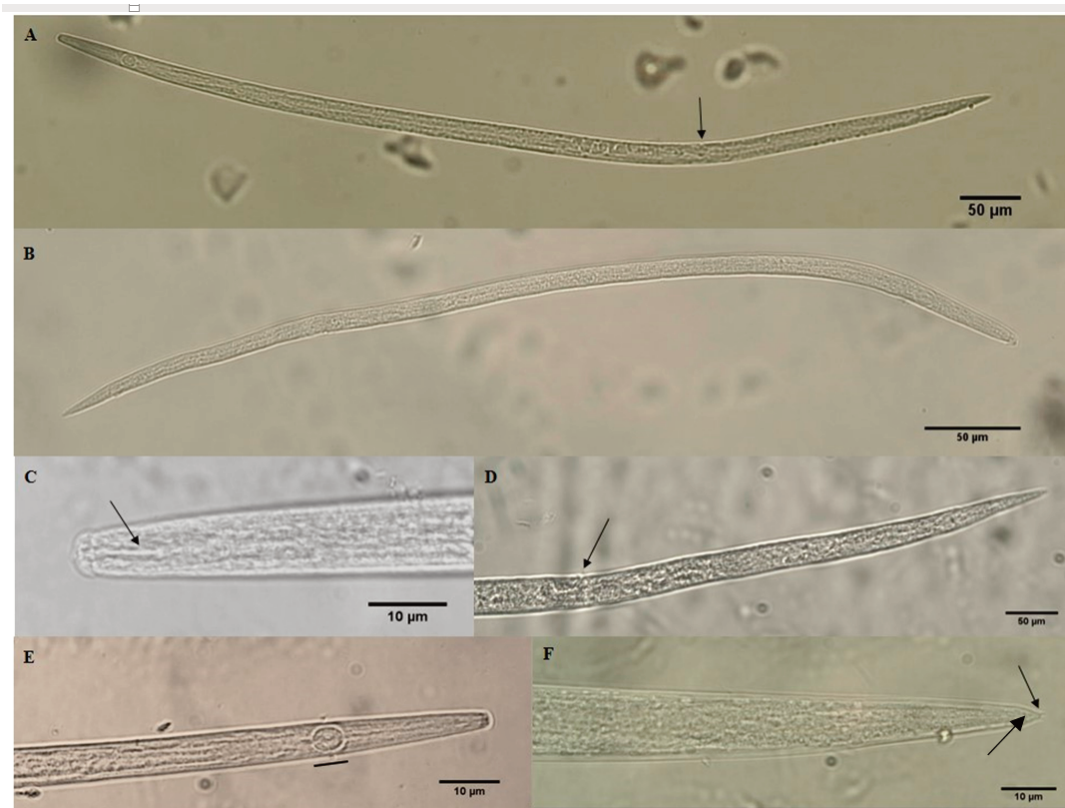


Figure 2. *Aphelenchoides besseyi* morphology from nematodes reared on fungal cultures: (A) mature nematode, (B) juvenile nematode, (C) stylet, (D) vulva, (E) metacarpus, (F) mucron

Table 1. Population and reproduction factor of *Aphelenchoides besseyi* on different fungal cultures and temperatures

Fungal Species	Incubation Temperature (°C)	Individuals			
		<sup>a</sup> Juvenile	<sup>a</sup> Mature	<sup>a</sup> Total	<sup>a</sup> RF
AP	20	2,236 ± 0.38a	3,887 ± 0.34a	6,123 ± 0,32a	244.9 ± 0.32a
FS		1,016 ± 0.29a	1,817 ± 0.29a	2,833 ± 0,28a	113.3 ± 0.27a
BC		99 ± 0.8b	159 ± 0.86b	259 ± 0,89b	10.3 ± 0.53b
AP	25	2,206 ± 0.95a	6,909 ± 0.25a	9,115 ± 0,3a	364.6 ± 0.3a
FS		1,588 ± 0.18a	4,747 ± 0.35a	6,335 ± 0,29a	253.4 ± 0.29a
BC		37 ± 0.89bc	100 ± 1.15b	137 ± 1,13bc	5.5 ± 0.55bc
AP	30	7 ± 0.62c	59 ± 0.4b	65 ± 0,42bc	2.6 ± 0.25bc
FS		24 ± 0.87bc	134 ± 0.65b	158 ± 0,69bc	6.3 ± 0.49bc
BC		4 ± 0.53c	17 ± 0.71b	21 ± 0,75c	0.8 ± 0.21c

Information: AP = *Alternaria padwickii*, FS = *Fusarium semitectum*, BC = *Botrytis cinerea*, RF = Reproduction Factor;

<sup>a</sup>Numbers followed by different letters in the same column are not significantly different (Tukey post-hoc test  $\alpha=0.05$ )

the effects of temperature on embryo development is one the main factor that affect nematode ecology and distribution.

Reproduction factor of *Ap. besseyi* was the highest on *Al. padwickii* and *F. semitectum* incubated at 25°C and *Al. padwickii* at 20°C. Reproduction factor of *Ap. besseyi* reared on *Al. padwickii* incubated at 25°C reached 364.6 times implying that *Ap. besseyi* were able to complete their life cycle and reproduce.

However, when reared on *B. cinerea* at 25°C, reproduction factor only reached 5.5 times. Reproduction factor of nematodes reared on *B. cinerea* at 25°C was lower compared to nematodes reared on *F. semitectum* incubated at 30°C, specifically 6.3 times. This implies that *Ap. besseyi* were still able to survive and reproduce on *F. semitectum* at 30°C even though conditions were not optimum (Table 1).

Food source is an important factor that determines the increase of nematode populations. Food preferences of *Ap. besseyi* can be evaluated based on the population of mature nematodes from different fungi cultures. Several fungal species have essential function and can be considered to increase *Ap. besseyi* reproduction *in vitro* (Rajan *et al.*, 1990; Jamali *et al.*, 2008). The highest *Ap. besseyi* population was obtained from *Al. padwickii* grown on PDA mediums incubated at 25°C. PDA was the best substrate for growing fungi used for *Ap. besseyi* rearing. Nematodes prefer mycelium rather than conidia as food source. Therefore, nematodes are not able to reproduce and survive on medium that induct fungi sporulation (Jamali *et al.*, 2008).

At 25°C, reproduction factor of nematodes reared on *Al. padwickii* was 364.6 times, while *F. semitectum* was 253.4 times. Therefore, *Al. padwickii* grown on PDA and incubated at 25°C may be an alternative to rear *Ap. besseyi* in laboratories for ecological, epidemiological, and molecular testing. However, *Al. padwickii* grows slower than *F. semitectum* causing longer time for preparation before being able to be inoculated with *Ap. besseyi*.

## CONCLUSION

All three fungal species were suitable as mass rearing media for *Ap. besseyi* with population density depending on the combination of fungal species and temperature. Between all fungal species and temperature combinations, *Al. padwickii* inoculated at 25°C was the best combination for *Ap. besseyi* mass rearing.

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