Tropical Life Sciences Research, 21(1), 11–19, 2010

Spore Density and Viability of Entomopathogenic Fungal Isolates from Indonesia, and Their Virulence against *Aphis gossypii* Glover (Homoptera: Aphididae)

Siti Herlinda*

Department of Plant Pest and Disease, Faculty of Agriculture, Sriwijaya University,
Jl. Raya Palembang-Prabumulih Km. 32, Ogan Ilir 30662, Sumatera Selatan, Indonesia

Abstrak: Tujuan kajian ini adalah untuk mengukur ciri-ciri keafiatan, seperti kepadatan dan keupayaan hidup sendiri spora, menentukan tahap kebisaan pencilan spesies kulat *Beauveria bassiana* dan *Metarhizium anisopliae* terhadap nimfa aphid (*Aphis gossypii*). Pencilan kulat tersebut didapatkan dari pelbagai species serangga, termasuk *Plutella xylostella*, *Hypothenemus hampei*, *Bronstispa longissima*, *A. gossypii*, *Tenebrio molitor*, dan *Leptocorisa acuta*, yang dikumpulkan dari pulau-pulau di Indonesia, iaitu Sumatera, Jawa, dan Sulawesi. Nimfa instar ketiga aphid diinokulasikan secara topikal dengan konidia pencilan kulat entomopatogen dengan kepadatan 10^6 konidia ml^{-1}. Kesemua pencilan-pencilan *B. bassiana* dan *M. anisopliae* dapat menghasilkan konidia dengan kepadatan yang sangat tinggi. Pencilan *M. anisopliae* MaAg, yang didapatkan dari aphid, memiliki kepadatan spora paling tinggi iaitu 6.70×10^8 konidia ml^{-1}. Di antara pencilan *B. bassiana*, keupayaan hidup sendiri konidia paling tinggi dimiliki oleh pencilan CPJW8, yang berasal dari *Chrysodeixis chalcites*, dengan purata keupayaan hidup sendiri sebanyak 39%. Di antara pencilan *M. anisopliae*, keupayaan hidup sendiri paling tinggi dimiliki oleh pencilan-pencilan MaAg and MaLa, yang berasal dari *L. acuta*, dengan purata keupayaan hidup sendiri sebanyak 33% dan 32% masing-masing. Semua pencilan *B. bassiana* dan *M. anisopliae* menjangkiti nimfa aphid, dengan tahap kematian antara 64% hingga 94%. Tiga pencilan yang paling berbisa menjangkiti aphid adalah BBY715 (94%), MPx (92%), dan MaTm (92%), dan yang paling lemah adalah MaLa (64%). BBY715, pencilan yang paling berbisa menjangkiti aphid, memiliki *lethal time median* (LT_{50}) paling singkat terhadap nimfa aphid, iaitu 2.97 jam, dan MaLa memiliki LT_{50} paling lama iaitu 61.81 jam.

Kata kunci: *Beauveria bassiana, Metarhizium anisopliae*, *Aphis gossypii*, Entomopatogenik

Abstract: The focus of this study was on quantifying fitness attributes, such as spore density and viability, and determining the virulence level against aphid (*Aphis gossypii*) nymphs of isolates from the fungal species *Beauveria bassiana* and *Metarhizium anisopliae*. The fungal isolates were obtained from several insects, including *Plutella xylostella*, *Hypothenemus hampei*, *Bronstispa longissima*, *A. gossypii*, *Tenebrio molitor*, and *Leptocorisa acuta*, that were collected from Indonesian islands, such as Sumatera, Java, and Sulawesi. Third instar aphid nymphs were inoculated via topical application of 10^6 conidia ml^{-1} of the entomopathogenic fungal isolates. All of the *B. bassiana* and *M. anisopliae* isolates could produce very dense spores. The *M. anisopliae* isolate MaAg, which was obtained from the aphid, had the highest spore density at 6.70×10^8 conidia ml^{-1}. Among the *B. bassiana* isolates, the highest conidial viability belonged to isolate CPJW8, which was obtained from *Chrysodeixis chalcites*, with a 39% average viability. Among the *M. anisopliae* isolates, the highest viabilities belonged to the isolates MaAg

*Corresponding author: sherlinda_hpt_fp@unsri.ac.id

and MaLa, which were obtained from *L. acuta*, with a 33% and 32% average viabilities, respectively. All of the *B. bassiana* and *M. anisopliae* isolates were virulent against aphid nymphs, with mortality rates ranging from 64% to 94%. The three most virulent isolates were BBY715 (94%), MPx (92%), and MaTm (92%), and the least virulent isolate was MaLa (64%). BBY715, the most virulent isolate, had the shortest lethal time median (LT_{50}) against aphid nymphs at 2.97 hours, and MaLa had the longest LT_{50} at 61.81 hours.

Keywords: *Beauveria bassiana, Metarhizium anisopliae*, *Aphis gossypii*, Entomopathogenic

INTRODUCTION

The aphid (*Aphis gossypii* Glover) is one of the most serious pests of the chilli plant in Indonesia. This pest, which can infest a wide variety of vegetables, has a cosmopolitan distribution. On its own, the aphid can cause enough damage to reduce the productivity of chilli crops by up to 25% (Miles 1987). In addition, *A. gossypii* also serves as a vector for many different viruses and can transmit 76 viral diseases (Satar *et al.* 1999). Taken together, these characteristics provide the aphid with the capability to destroy entire crops.

The use of synthetic insecticides to control aphids on chilli plants has, unfortunately, resulted in serious problems, such as the killing of beneficial insects and wildlife, insecticide resistance, and insect pest resurgence (McKenzie & Cartwright 1994). With an increasing demand for environmentally sound pest control measures, alternative methods to control the pest have been proposed and developed, including the use of biological control agents such as entomopathogenic fungi (Herlinda *et al*. 2008).

Entomopathogenic fungi, such as *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sor., are common, soil-borne fungi that occur worldwide (Bidochka *et al.* 2000). *B. bassiana* is responsible for white muscardine disease, in which infected insects become covered with a layer of white mould (Alves *et al.* 2002; Klinger *et al.* 2006), and *M. anisopliae* is responsible for green muscardine disease (Santiago *et al.* 2001). The fungi attack the immature and adult stages of several insect orders, such as Lepidoptera (Thalib *et al*. 2005), Hemiptera (Liu *et al*. 2002), and Diptera (Quesada-Moraga *et al*. 2006).

There are many different strains of these fungi that exhibit considerable variation in their virulence levels and host ranges (Soetopo 2004). Spore viability might be one factor that influences virulence. The loss rate of spore viability of *B. bassiana* varies among the isolates (Aregger 1992). In fact, the decline in conidial viability of this fungus has correlated with the decline in the rate of host mortality after infection. Although spore viability, which is likely not the only influencing factor, does affect virulence, there is also an innate virulence that is specific to each isolate. This innate virulence appears to be the most important factor in determining the pathogenicity of the isolate (Soetopo 2004). In Indonesia, *Entomophthora* sp. are known to naturally infect the aphid, but further investigations of aphid infection by *B. bassiana* and *M. anisopliae* are needed, especially with respect to their usefulness as an alternative method of

pest control. This research was performed in order to quantify the fitness attributes, such as spore density and viability, and to determine the virulence level against aphid (*A. gossypii*) nymphs of isolates from the fungal species *B. bassiana* and *M. anisopliae*.

MATERIALS AND METHODS

Rearing of Aphids

Aphid nymphs were reared in the Entomological Laboratory (Department of Plant and Disease, Faculty of Agriculture, Sriwijaya University) under ambient environmental conditions, including a temperature of 25°C–27°C and a humidity level of 70%–85%. The nymphs were collected from a chilli field in South Sumatera and reared in plastic cylindrical containers 8 cm in diameter and 34 cm in length. The nymph diet consisted of chilli leaves, which were replaced daily. New progeny were collected daily. Two-day-old nymphs were used for the virulence tests.

Preparation of Entomopathogenic Fungal Isolates

The *B. bassiana* and *M. anisopliae* isolates used in this study, which include PD2, KBC, CPJW8, BBY715, PD1, MPx, MaAg, MaCc, MaTm, and MaLa, were all specific to this laboratory (Table 1). These isolates were originally gathered from various sites within Indonesia from different insect species, including *Plutella xylostella*, *Hypothenemus hampei*, *Bronstispa longissima*, *A. gossypii*, *Tenebrio molitor* and *Leptocorisa acuta*. The isolates were purified on glucose yeast agar (GYA). After purification, the isolates were grown on slants of GYA medium supplemented with chitin from the small mole cricket, kept in 1.5 x 13 cm glass test tubes, and incubated for 10 days.

Table 1: Original hosts and places of origin for each of the *B. bassiana* and *M. anisopliae* isolates.

Fungus	Code of isolate	Insect host origin	Places of origin
B. bassiana	PD2	*Plutella xylostella*	Pagaralam, South Sumatera
	KBC	*Chrysodeixis chalcites*	Curup, Bengkulu
	CPJW8	*Chrysodeixis chalcites*	Cipanas, West Java
	BBY715	*Hypothenemus hampei*	Jember, East Java
	PD1	*Plutella xylostella*	Pagaralam, South Sumatera
M. anisopliae	MPx	*Bronstispa longissima*	Manado, North Sulawesi
	MaAg	*Aphis gossypii*	Pagaralam, South Sumatera
	MaCc	*Corcyra cephalonica*	Inderalaya, South Sumatera
	MaTm	*Tenebrio molitor*	Palembang, South Sumatera
	MaLa	*Leptocorisa acuta*	Pemulutan, South Sumatera

Density and Viability of the Fungal Spores

The fungal spores were harvested from the slant culture, and each isolate was separately suspended in 250 ml water. Spore density was calculated using the method of Singleton and Sainsbury (1981), after analysis of a 1 ml sample of the suspension in a haemocytometer.

Spore viability was determined by measuring the rate of spore germination. A suspension containing 10^6 propagules in 100 µl was spread onto GYA medium, which was then incubated at room temperature for 24 hours. The viable propagules per unit volume were determined by multiplying the total count, which was estimated with a haemocytometer, by the percentage of germination.

Fungal Virulence

The conidia from the slant culture of each isolate were separately suspended to obtain a density of 10^6 conidia ml^{-1}. Ten aphid nymphs were inoculated by topical application of 10 µl of the conidia inoculum, with 5 replications. The nymphs were observed every 12 hours, and dead insects were removed, followed by confirmation of fungal infection. Only those nymphs that contained fungi that had undergone sporulation were considered for the determination of fungal virulence. The resulting data was used to calculate the rate of mortality and the lethal/time median (LT_{50}). The LT_{50} was determined by probit analysis based on the method by Finney (1971). The calculation employed the program SAS-STAT in SAS 6.12 (SAS Institute, USA).

RESULTS AND DISCUSSION

Spores Density and Viability

All *B. bassiana* and *M. anisopliae* isolates could produce very dense spores (Table 2). The *B. bassiana* isolates with the highest spore densities were CPJW8 and PD1, and the *M. anisopliae* isolates with the highest spore densities were MaAg and MaCc. The MaAg isolate had the overall highest spore density at 6.70×10^8 conidia ml^{-1}.

The rate of spore germination was used as a measure of viability (Soetopo 2004). A spore was considered to be viable if its germtube length was twice the diameter of the propagule or if conspicuous swelling of the spore was visible. The isolate CPJW8, which was isolated from *C. chalcites*, had highest conidial viability among the *B. bassiana* isolates, with a 39% average viability. The isolates MaAg, which was isolated from *A. gossypii*, and MaLa, which was isolated from *L. acuta*, had the highest viabilities among the *M. anisopliae* isolates, with 33% and 32% average viabilities, respectively (Table 2).

Table 2: Density and spore viability of *B. bassiana* and *M. anisopliae* isolates.

Code of isolate	Conidial density (x 10^8 conidia ml^{-1})	Conidial viability (%)
PD2	1.59	33.00
KBC	1.49	36.00
CPJW8	4.83	39.00
BBY715	1.81	31.00
PD1	5.15	28.00
MPx	2.84	31.00
MaAg	6.70	33.00
MaCc	6.69	29.00
MaTm	1.19	28.00
MaLa	3.10	32.00

Fungal Virulence

All of the *B. bassiana* and *M. anisopliae* isolates were pathogenic to the aphid nymphs, with mortality rate between 64% and 94% (Fig. 1 and Table 3). The three most virulent isolates were the *B. bassiana* isolate BBY715 (94%) and the *M*. *anisopliae* isolates MPx (92%) and MaTm (92%). The least virulent isolate was the *M*. *anisopliae* isolate MaLa (64%). The variation in the mortality rates could possibly be explained by differences in the innate virulence and conidial viability between each isolate.

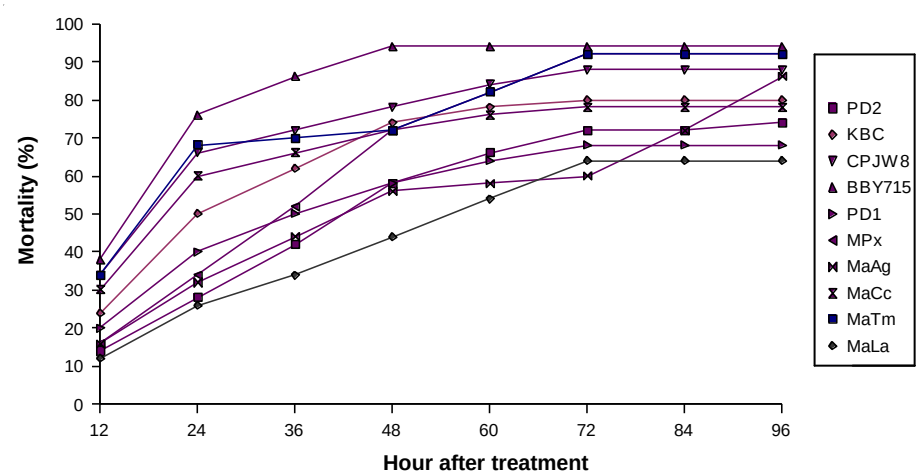

Figure 1: Cumulative mortality of aphid nymphs that were treated with different isolates of *B. bassiana* and *M. anisopliae* at a concentration of 10^6 conidia ml^{-1}.

Table 3: Lethal time median (LT_{50}) of *B. bassiana* and *M. anisopliae* isolates against aphid nymphs at a concentration of 10^6 conidia ml^{-1}.

Code of isolate	Mortality (%)	LT_{50} (hours)	95% Confidence limit	
			Lower	Upper
PD2	74.00	49.61	42.55	56.13
KBC	80.00	27.46	–5.37	42.29
CPJW8	88.00	12.51	–25.23	28.39
BBY715	94.00	2.97	–85.69	23.85
PD1	68.00	45.50	34.39	54.57
MPx	92.00	35.76	30.28	40.46
MaAg	86.00	49.77	42.87	56.22
MaCc	78.00	17.93	–44.36	37.08
MaTm	92.00	16.31	3.06	25.06
MaLa	64.00	61.81	54.49	70.13

The innate virulence of each isolate appears to be the most important factor that determines its level of pathogenicity. BBY715, MPx, and MaTm were the three most virulent isolates, and they were originally isolated from *H. hampei*, *B. longissima*, and *T. molitor*, respectively. These three insect hosts are all members of the order Coleoptera. Insects belonging to the Coleoptera order tend to have thicker and harder integument than insects from other orders. When the host integument is hard and thick, the fungal germ tube will produce an appressorium that can form a penetration plate, penetration tube, and hyphal body for improved invasion (Tanada & Kaya 1993). Thus, as the BBY715, MPx, and MaTm isolates adapted and developed the ability to infect the hosts with thicker and harder integument, they consequently became more virulent. The fungal characteristic of insect host origin, therefore, might have an important role in determining the innate virulence of the isolate.

The level of nymph mortality varied among the isolates. These variations could potentially be a result of the differences in conidial density and viability of the isolates. The viability of conidia does influence insect mortality, since host mortality increases as spore viability increases. Both the conidial viability and the rate of mortality for the isolate CPJW8 was higher than those of KBC, PD1, and PD2 (Table 2). A decrease in the conidial viability of *B. bassiana* has been correlated with the decrease in the rate of mortality for the insect host, *Helicoverpa armigera,* due to *B. bassiana* infection (Soetopo 2004). Our data indicated that the spore viability might not be the only factor that affects virulence. There might be a relationship between the rate of germination and the rate of mortality. In the case of BBY715, this isolate had a lower germination percentage (31%) but a higher pathogenicity (94%) compared to that of both KBC (80%) and PD2 (94%). As previously mentioned, an important factor that influences the pathogenicity is the innate virulence of the isolate.

All isolates were able to infect aphid nymphs 12 hours after treatment (Fig. 1), and the rate of mortality for each isolate continuously increased until 48 hours after treatment. The isolate BBY715 took the shortest time to kill the aphid

nymphs, requiring less than 48 hours to kill more than 50% of the aphid nymphs whereas the isolate MaLa took the longest time. The time period to the level of lethal infection was very short, occurring in less than 12 hours, which indicated that all these isolates had a high rate of pathogenicity against aphid nymphs.

The LT_{50} also varied among the isolates (Table 3). BBY715 had the shortest LT_{50} against the aphid nymphs at 2.97 hours, while MaLa had the longest LT_{50} at 61.81 hours. Lower values of LT_{50} have been demonstrated to lead to higher rates of infection, which indicates that the isolate has a higher virulence level (Facundo *et al*. 2001). A short period of lethal infection indicates a high level of pathogenicity or virulence of a pathogen and, in contrast, a long period of lethal infection indicates a low level of pathogenicity (Tanada & Kaya 1993; Fuxa & Richter 2004).

The majority of the infected aphid nymphs died. In addition, infected nymphs that were still alive displayed a lack of appetite and decreased mobility. Dead nymphs became hardened and stiff, with mycelia and conidia appearing on the nymphs. Dead insect hosts infected by *M. anisopliae* showed the same symptoms as those infected by *B. bassiana*, except for the colour of the hyphae, which was greenish white. All *B. bassiana* and *M. anisopliae* isolates in this study were able to infect the aphid nymphs. In general, infections by fungi can be categorised as latent, chronic, or acute (Tanada & Kaya 1993). All the *B. bassiana* and *M. anisopliae* isolates in this study caused acute infection, given the reduced appetite and activity of the infected nymphs. The characteristics of the fungal isolates in this study with respect to pathogenicity and virulence indicates that they could potentially be good candidates for an alternative method to control the aphid and protect the chilli plant from damage caused by this pest.

ACKNOWLEDGEMENT

Financial support for this research was provided by the Project of Competent Research Fund (Directorate General of Higher Education, Department of National Education, Indonesia, Budget Year 2008 with Contract Number: 004/HIKOM/DP2M/2008, 25th June 2008). Special thanks to Simona Vauka for her assistance with the laboratory work.

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