

Morphological study, production and cryopreservation of blastospores of entomopathogenic fungi

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ABSTRACT: Entomopathogenic fungi have been known as potential candidates for insecticides and metabolite producers. The aims of this study were to investigate characteristic and development patterns of blastospores and to determine optimal culture conditions for blastospore production of entomopathogenic fungi in the genera *Akanthomyces*, *Cordyceps*, *Hirsutella*, *Metarhizium*, and *Torribiella*. Production of blastospores was induced using Grace's insect cell medium (GICM) supplemented with foetal bovine serum (FBS) except the genus *Torribiella*. Out of 39 fungal isolates, 18 isolates could produce blastospores, most of them belonged to the genus *Cordyceps*. Observation on characteristics and formation of blastospores revealed that blastospores produced from all genera shared common characters and formation. However, some distinctive characters were observed in the genus *Akanthomyces*. Effects of culture media, types of inoculum and cultivating condition were examined for the production of highest numbers of blastospores (up to 10^7 – 10^8 blastospores/ml), revealing different requirements in each fungal isolate for the production of blastospores. Cryopreservation of blastospores revealed that freeze-drying method could be used to preserved blastospores of *C. brongniartii*, *Hirsutella* sp. 02 and *Metarhizium* sp. 02 except *A. pistillariiformis*. This study is the first comprehensive investigation on development patterns of blastospores and factors underpinning effective culture conditions for blastospores production of the selected entomopathogenic fungi.

KEYWORDS: entomopathogenic fungi, blastospores, cryopreservation

INTRODUCTION

Entomopathogenic fungi are important natural regulators of insect populations as they can generally infect a broad range of insects and cause mortality of the insect hosts. Since they are considered natural mortality agents and environmental safe, the fungi have been proposed as a group of potential candidates for microbial insecticides to control agricultural pests [1–4]. For example, *Beauveria bassiana* 36 was investigated for its virulence and pathogenicity on the infected larvae of *Helicoverpa armigera*, the most serious pest of several plant species, in the recent report [5]. Entomopathogenic fungi have also been signified as a valuable source of secondary bioactive metabolites. Several bioactive compounds have been isolated from *Cordyceps* spp., for instance, antimalarial cordypyridones A-D from *C. nipponica* [6, 7], bioanthracenes from *C. pseudomilitaris* [7], antimalarial red naphthoquinones from *C. unilateralis* [8], and beauvericin-like compound from *C. militaris* [9].

The entomopathogenic fungi consist of various taxa and do not form a monophyletic group. Most

of common and important entomopathogenic fungi are grouped in the order Hypocreales which belongs to the phylum Ascomycota. These include the anamorphic phases (*Beauveria* spp., *Hirsutella* spp., *Metarhizium* spp., *Nomuraea* spp., and *Paezilomyces* spp.) and the teleomorphic phase (*Cordyceps* spp.). Meanwhile, other entomopathogenic fungi (*Entomophthora* spp., *Entomophaga* spp., *Pandora* spp., and *Zoopthora* spp.) belong to the order Entomophthorales of the phylum Zygomycota. Mode of production of asexual and sexual propagules has been proposed as a key basis for identification of these fungi. Besides the vegetative propagules, other structures, such as rhizoids, resting spores, hyphal bodies (including protoplasts and blastospores), and sclerotia are also important for the classification of a fungal species or a genus [10]. Unlike other fungal groups, the entomopathogenic fungi can produce secondary spore-type which is commonly referred to blastospore [11, 12].

Formulation of microbial insecticides from entomopathogenic fungi often centers on mass-production of aerial conidia [1–3]. Interestingly, it has been evidenced that blastospores demonstrated

stronger infection ability than conidia in terms of faster germination rate on the insect cuticle and higher level of bioactivity and insecticidal efficacy against various insect pests [13–15], which thus make blastospores the propagules of choice for the production of a biological insecticide. However, *in vitro* cultivation of blastospores is still very fastidious in some fungal genera because traditional culture conditions for fungal growth are unfavourable for the production of blastospores thus different parameters are required [16]. In addition, information on blastospore development and characteristics of individual entomopathogenic fungal genus is still sparse, which hinder the use of blastospores as microbial pesticides.

Since entomopathogenic fungi are a rich source of natural bioactive compounds and demonstrate certain advantages over the use of chemical insecticides, production of the microbial insecticides, particularly exploitation of the blastospore as an active ingredient, is challenging. In addition, understanding the production and development patterns of blastospores of the fungi is also crucial for developing blastospores as microbial insecticides. In response to those challenges, culture conditions for the production of blastospores, including blastospore development patterns, should be defined. The aim of the present study was to investigate characteristics and development patterns of blastospores of the selected entomopathogenic fungal genera. In addition, culture conditions to induce the production of blastospores were also determined. Besides difficulties in producing blastospores for morphological study and mass-production for industrial use, storage of fungal cultures is also very important that these cultures should be preserved in a physiologically and genetically stable state in order to maintain their valuable properties. Therefore, long-term preservation method via freeze-drying of the blastospores were also investigated.

MATERIALS AND METHODS

Fungal isolates and maintenance

Fungal isolates of the entomopathogenic fungi used in the present study were from the genera *Akanthomyces*, *Cordyceps*, *Hirsutella*, *Metarhizium*, and *Torrubiella*. All fungal isolates were maintained on Potato Dextrose Agar (PDA) (HiMedia®) and incubated at 25 °C for 10–15 days.

Induction and observation of blastospore development patterns and morphology

Optimum concentrations of the foetal bovine serum (FBS; GIBCO®, USA) used for the production of blastospores were determined. Ten entomopathogenic fungal isolates belonged to five fungal genera (*Akanthomyces pistillariiformis*, *Akanthomyces* sp. 01, *Cordyceps brongniartii*, *Cordyceps* sp. 01, *Cordyceps* sp. 02, *Hirsutella* sp. 01, *Hirsutella* sp. 02, *Metarhizium* sp. 01, *Metarhizium* sp. 02, *Torrubiella* sp. 01, and *Torrubiella* sp. 02) were selected for the study. A mycelial plug (0.5 mm in diameter) of each entomopathogenic fungal strain was transferred into 1 ml of Grace's insect cell medium (GICM; GIBCO®, USA) containing 0.60 g/l L-glutamine, 3.33 g/l lactalbumin hydrolysate, 3.33 g/l yeastolate, and 3% (w/v) glucose and supplemented with different concentrations of FBS (0, 1, 5 and 10% (v/v)) in a 24-well microtitre plate. The cultures were incubated at 28 °C under static condition and the formation of blastospores was investigated daily for 14 days using inverted microscope. Formation, characteristics and morphological development of blastospores were observed daily using an inverted microscope.

Large scale cultivation of blastospores

To monitor rate of blastospore production, either conidia suspensions or mycelial plugs were used. Conidia suspensions (10^3 conidia/ml) were prepared using sterile distilled water containing 1% (v/v) of tween 80. Conidia suspensions (200 μ l) or 5 mycelial plugs, were transferred into 5 ml of yeast extract-peptone-glucose (YPG) broth or GICM in a 6-well plate and incubated at 28 °C under static condition and shaking condition at 100 rpm. The numbers of blastospores were counted using haemocytometer (Boeco, Germany). The experiment was conducted in triplicate. The conditions giving blastospore concentration of more than 10^8 blastospores/ml were selected for the production of blastospores to be used in the cryopreservation process.

Cryopreservation of blastospores of entomopathogenic fungi

Blastospores were filtered through double layers of sterile Miracloth and adjusted to desired concentration of 10^7 – 10^8 blastospores/ml. Each blastospore suspension was centrifuged at 6 600 rpm for 5 min to remove the culture media and the pellet washed twice with sterile distilled water. The resulting blas-

Table 1 Determination of FBS concentration for blastospore production (blastospores/ml).

Fungal isolate	FBS concentration (%)			
	0	1	5	10
<i>A. pistillariiformis</i>	10 ²	10 ⁵	10 ⁸	>10 ⁸
<i>Akanthomyces</i> sp. 01	–	–	–	–
<i>C. brongniartii</i>	10 ⁵	10 ⁷	10 ⁸	>10 ⁸
<i>Cordyceps</i> sp. 01	10 ⁴	10 ⁶	10 ⁷	10 ⁷
<i>Hirsutella</i> sp. 01	–	–	10	10 ²
<i>Hirsutella</i> sp. 02	–	–	10 ²	10 ⁴
<i>Metarhizium</i> sp. 01	–	–	10 ²	10 ⁴
<i>Metarhizium</i> sp. 02	–	10	10 ⁴	10 ⁶
<i>Torrubiella</i> sp. 01	–	–	–	–
<i>Torrubiella</i> sp. 02	–	–	–	–

blastospore pellets were used within 2 h after washing. Protective agent (5% (w/v) trehalose) was prepared in 0.1 M potassium phosphate buffer (pH 7.0) and sterilized at 115 °C for 15 min. Blastospore pellet was resuspended in 0.1 ml of the protective agent and filled in an ampoule. Primary vacuum drying was performed for the minimum of 3 h, followed by secondary drying at 0.090 mbar at –60 °C for 20 h. Ampoules were sealed and stored at 5 °C in the dark.

Blastospore viability was evaluated before drying, immediately after drying and after one-year storage. Freeze-dried blastospores were dispersed in 0.1 ml rehydration fluid (peptone 5 g, yeast extract 3 g, MgSO₄ 1 g, and distilled water to 1 l, pH 7.0). Rehydrated blastospores were transferred into microtitre plate wells containing 1.5 ml of GICM. After 8–15 h of incubation, one drop of 2 N HCl was added to halt the germination process. Germination of blastospores was assessed microscopically; and counting was done in duplicate from two ampoules. Revived cultures were also observed for morphological and genetic stability, such as changes in pigmentation or colony morphology.

RESULTS

FBS concentration for the production of blastospores

Ten entomopathogenic fungal isolates belonged to five fungal genera (*Akanthomyces*, *Cordyceps*, *Hirsutella*, *Metarhizium*, and *Torrubiella*) were selected for the study. Mycelial plugs of each isolate were grown in GICM supplemented with 0, 1, 5 and 10% (v/v) FBS. The results revealed that blastospore production was observed from the genus *Cordyceps* and *A. pistillariiformis* without addition of FBS (Table 1). With addition of FBS, the production

Table 2 Screening of blastospore production from 39 entomopathogenic fungal isolates.

Genera	No. of isolates tested/ No. of blastospore producing isolates	Frequency of blastospore production (%)
<i>Akanthomyces</i>	9/3	33
<i>Cordyceps</i>	16/10	63
<i>Hirsutella</i>	5/3	60
<i>Metarhizium</i>	5/2	40
<i>Torrubiella</i>	4/0	0

of blastospores was significantly enhanced. FBS supplementation was necessary in the genera *Hirsutella* and *Metarhizium* as blastospore production was induced at high concentration of FBS (5 and 10% (v/v)). Meanwhile, none of the *Torrubiella* and *Akanthomyces* sp. 01 isolates produced blastospores. This might infer specific preference of each fungal strain for blastospore production *in vitro*. Although 5% (v/v) FBS was sufficient for most isolates, 10% (v/v) FBS was chosen for the entire study based on satisfactory blastospore yield.

Screening for fungal isolates capable for blastospore production

Production of blastospores was screened from 39 entomopathogenic fungi using GICM supplemented with 10% (v/v) FBS. Out of 39 fungal isolates, 18 isolates were able to produce blastospores (Table 2). Most of them belonged to the genera *Cordyceps* and *Hirsutella* (10/16 and 3/5, respectively) while blastospore production was detected in few isolates of *Akanthomyces* and *Metarhizium*. In addition, none of *Torrubiella* isolates produced blastospores in GICM supplemented with 10% (v/v) FBS.

General characteristics and formation patterns of blastospores

Observation on characteristics and formation patterns of blastospores revealed that blastospores produced from all genera shared common characters and formation patterns (Table S1). However, some distinctive characters could be observed in some fungal isolates. In the *Akanthomyces* spp., blastospore formation was generally observed after 1–2 days of incubation. The spores were hyaline, small, round or oval shapes with 4–10 μm average in length. They were usually produced from hyphal tips and formed clusters. After released into the medium, blastospores immediately produced a new budding cell. In addition, it can be observed that

Akanthomyces spp. demonstrated unique characteristics of blastospores that differed from other genera in terms of shape, size and budding position on the hypha. In the *Cordyceps* spp., blastospore formation was generally observed after 2–3 days of incubation. The spores were hyaline, ellipsoidal to cylindrical shapes which connected to the mycelium by a short neck. Average length of blastospore was 10–25 μm . In addition, the spores usually produced from hyphal tips and sometimes from sides. In the *Hirsutella* spp., blastospore formation was generally observed after 2–5 days after inoculation depending on fungal strains. The spores were mainly hyaline, small, narrow with cylindrical to club-shapes. However, some blastospores were ellipsoidal, cylindrical to long rod shapes. The length of blastospores was between 15–20 μm . Blastospores of *Hirsutella* were found to be generated very rapidly by budding both from the mother cells and from the hyphal sides. In the *Metarhizium* spp., blastospore formation was generally observed after 2–3 days of incubation. The spores were hyaline, ellipsoidal to cylindrical shapes and usually produced from hyphal sides with an attachment through a short neck. Average length of blastospore was 10–20 μm .

Detailed morphological development patterns of blastospores

Representative isolates of each fungal genus (*C. brongniartii*, *Hirsutella* sp. 02, *Metarhizium* sp. 02, and *A. pistillariiformis*) were chosen as model organisms, based on their ability to produce large numbers of blastospores and their industrial importance, to study the morphological development of blastospores.

C. brongniartii is one of the most important species among *Cordyceps* spp. This fungus can produce cuticle-degrading peptidases, which can potentially be used in agricultural and medical applications. Colony appearance of *C. brongniartii* grown on PDA at 28 °C for one week were woolly to powdery with approximately 19.3 \pm 1.6 mm in diameter (Fig. 1a). Colony color was white to cream and yellowish-brown to dark brown on the reverse side resemble to its anamorph. After three weeks of incubation, the colony changed to brown and slender stalks of stromata were produced on the colony (Fig. 1b). Conidia were abundant, elliptical, with thick smooth walls and borne along the sides and ends of repeatedly branched hyphae to form large clusters. Size of conidia was 10.3 \pm 1.4 μm \times 4.7 \pm 0.5 μm (Fig. 1c). Blastospore formation of *C. brongniartii* was observed daily

in GICM supplemented with 10% (v/v) FBS and the developmental process was initially examined from the stage of conidia development. The onset of conidia germination was marked by gradual swelling of conidia until it was approximately 30–100% larger than the original size after 8 h of incubation (Fig. 1d). After that the emergence of unipolar germ tubes were detected at 12 h. Further elongation of the germ tubes and septation of hypha were apparent after 16 h of incubation (Fig. 1e). During this stage, bipolar or multipolar growth of young mycelia from the conidium occurred. After elongation of mycelia, a blastospore appeared laterally or sometimes terminally from mycelia after 24 h of incubation. Blastospores were connected to mycelium by a short neck. These spores propagated by production through a pore and bulged out near the septa. Release of free blastospores occurred after 36 h of incubation (Fig. 1f). At this stage, blastospores were hyaline, single-celled with thin smooth-wall resemble protoplast-like cells. Detached blastospores were mostly elliptical shape. The average sizes of blastospores were 10.9–23.4 μm \times 3.1–3.2 μm . Then, the spores' elongation proceeded and a cross-walled was made to separate part of the stretched blastospores after 48 h of incubation (Fig. 1g). These short mycelia derived from blastospores could reproduce new blastospores on the third day of cultivation (Fig. 1(h,i,j)). New generation of blastospores could be obtained from reproduction of newly grown mycelia.

Colony appearance of *Hirsutella* sp. 02 grown on PDA at 28 °C for 7 days was flat with short aerial mycelia and moist colony surface with approximately 14.24 \pm 1.13 mm in diameter (Fig. 2a). Colony color was cream to golden brown and dark brown on the reverse side. Blastospores were hyaline, single-celled with smooth-wall and appeared laterally or sometimes terminally from mycelia after 2 days of incubation (Fig. 2b). The release of free blastospores was observed after 2.5 days of incubation. Detached blastospores were mostly elliptical to rod shape. The average size of detached blastospores was 11.1–22.6 μm \times 3.6–4.0 μm (Fig. 2c). After release of blastospores, the spores were elongated and a cross wall was made to separate part of the stretched blastospores after 3.5 days of incubation (Fig. 2d). However, these short mycelia derived from blastospores could not reproduce new blastospores as observed in *C. brongniartii*. In this case, new blastospores were generated from budding of the mature blastospores only.

Metarhizium spp. is one of the most potential

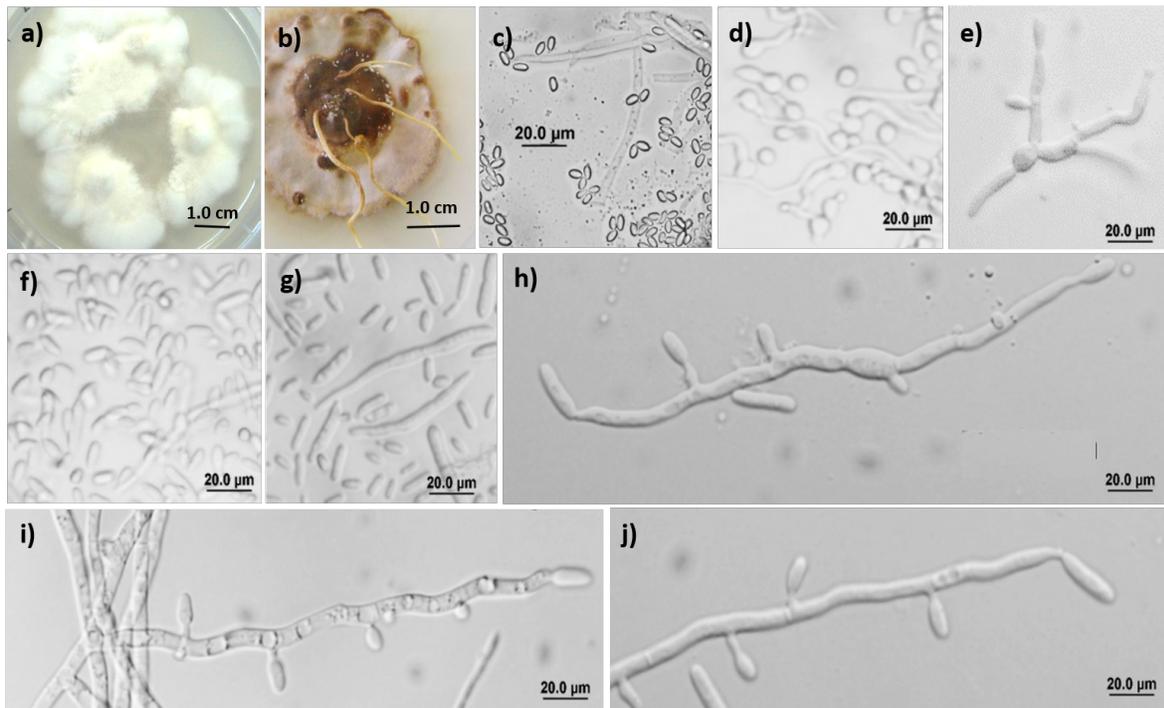


Fig. 1 Characteristics and morphological development patterns of blastospores of *C. brongniartii*. The liquid culture was grown in GICM supplemented with 10% FBS and incubated at 28 °C under static condition. (a) Colony of *C. brongniartii* on PDA incubated at 28 °C for 7 days; (b) colony after incubation for 3 weeks; (c) conidia; (d) gradual swelling of conidia and emergence of a unipolar germ tube at 8–12 h; (e) blastospore formation from germinating conidia at 16–24 h; (f) released blastospores at 36 h; (g) germination and elongation of blastospores at 48 h; (h,i,j) generation of blastospores from a mature mycelium at 54 h.

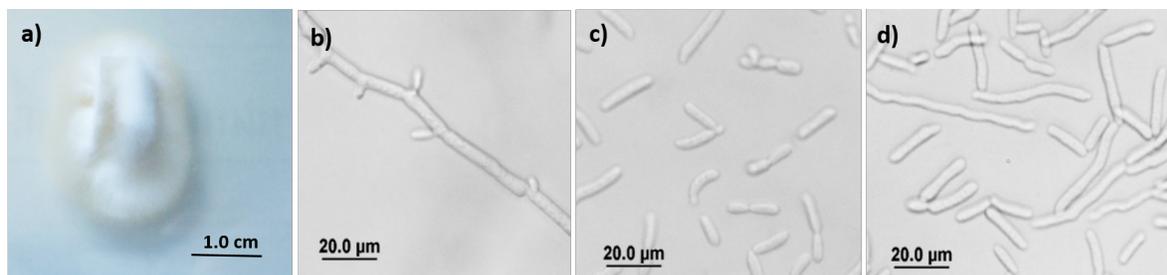


Fig. 2 Characteristics and morphological development patterns of blastospores of *Hirsutella* sp. 02. The liquid culture was grown in GICM supplemented with 10% FBS and incubated at 28 °C under static condition. (a) Colony of *Hirsutella* sp. 02 on PDA incubated at 28 °C for 7 days; (b) blastospore formation after 2 days of incubation; (c) released blastospores after 2.5 days of incubation; (d) germination and elongation of blastospores after 3.5 days of incubation.

entomopathogenic fungi which is used as biological insecticide to control a number of pests, such as grasshoppers, termites, thrips, etc. Colony appearance of *Metarhizium* sp. 02 grown on PDA at 28 °C for one week was flat and velvety with approximately 22.61 ± 2.15 mm in diameter (Fig. 3a). Colony color was white to creamy and yellow to orange on the reverse side. Conidia were single-celled,

cylindrical or ovoid, forming chains (Fig. 3b). Blastospores were observed after 2 days of cultivation and appeared laterally or sometimes terminally from mycelia. They were hyaline, single-celled with smooth-wall (Fig. 3cd). Detached blastospores were mostly elliptical to rod shape with the average size of $15.0\text{--}21.3 \mu\text{m} \times 3.6\text{--}4.7 \mu\text{m}$. After released into the medium, enlargement of blastospores oc-

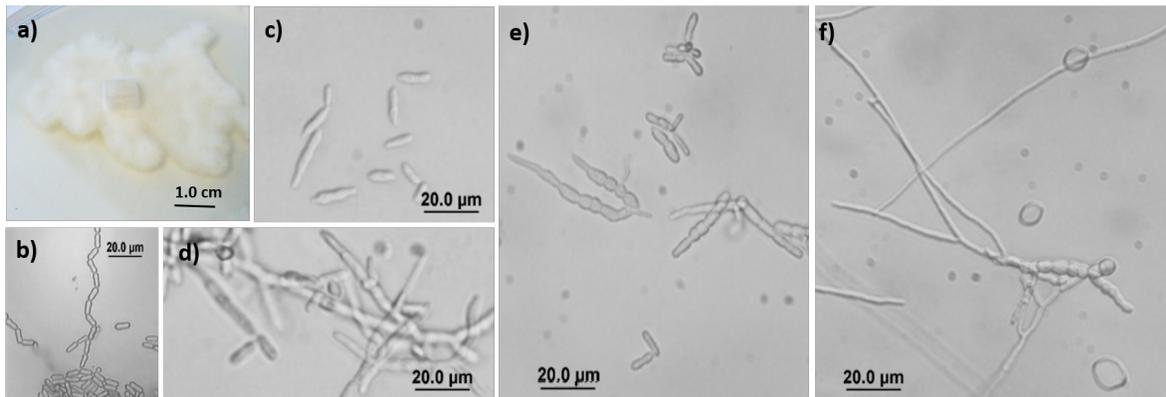


Fig. 3 Characteristics and morphological development patterns of blastospores of *Metarhizium* sp. 02. The liquid culture was grown in GICM supplemented with 10% FBS and incubated at 28 °C under static condition. (a) Colony of *Metarhizium* sp. 02 on PDA incubated at 28 °C for 7 days; (b) conidia; (c,d) blastospore formation after 2 days of incubation; (e,f) germination and elongation of blastospores after 3 days of incubation.

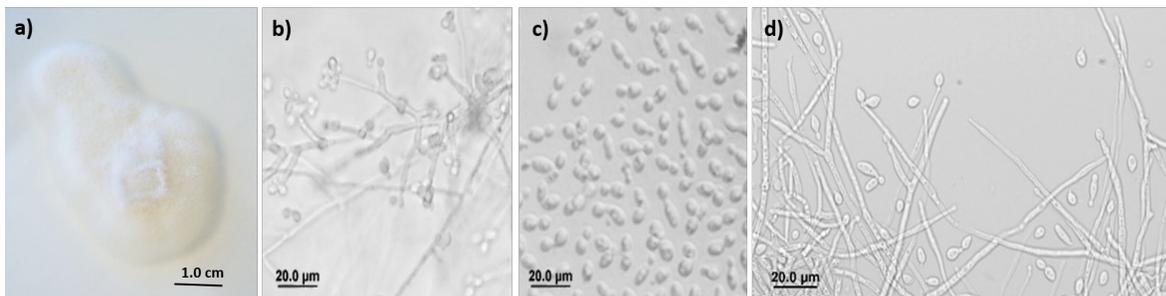


Fig. 4 Characteristics and morphological development patterns of blastospores of *A. pistillariiformis*. The liquid culture was grown in GICM supplemented with 10% FBS and incubated at 28 °C under static condition. (a) Colony of *A. pistillariiformis* on PDA incubated at 28 °C for 7 days; (b) blastospore formation after 2 days of incubation; (c,d) released blastospores.

curred, followed by a production of germ tubes after 3 days of incubation. A cross wall was subsequently made to separate part of the stretched blastospores. During this stage, shape of blastospores were transformed to tamarind-like shapes and size was increased simultaneously (Fig. 3e). After 5 days of cultivation, germinated blastospores became short mycelia and continued to grow slowly in the GICM. However, formation of new blastospores was not detected in this study (Fig. 3f).

Members in the genus *Akanthomyces* can produce valuable toxins and metabolites. Colony appearance of *A. pistillariiformis* grown on PDA at 28 °C for one week was flat and wet on the surface with approximately 12.78 ± 2.05 mm in diameter (Fig. 4a). Colony color was cream to white and yellow on the reverse side. Mycelia grew very slowly in the liquid medium. Blastospores were observed after 2 days of cultivation, which appeared laterally or

sometimes terminally from mycelia (Fig. 4b). They were hyaline, single-celled with smooth-wall with varying shapes from round to oval (Fig. 4c). The average size of blastospores was $4.3\text{--}8.1 \mu\text{m} \times 3.9\text{--}4.3 \mu\text{m}$ and usually produced from hyphal tips and formed clusters. After released into the medium, blastospores began to bud immediately (Fig. 4c). However, new budding cells did not detach from the mother cells which were not the case in *C. brongniartii* and *Hirsutella* sp. 02. No germination of blastospores was observed within 14-day period of observation (Fig. 4d).

According to the observation of developmental process of blastospores, common characteristics of blastospores among the isolates can be summarized. Generally, blastospores are single cells, hyaline, and non-motile with smooth thin-walled resembling protoplast-like cells. They propagated by bursting through a pore on a hyphal fragment and connected

to the mycelium through a short neck. Once ramification of mycelium has taken place under suitable environmental conditions, formation of blastospore was induced. After disconnected, blastospores enlarged in size and shape, followed by initiation of germination to form new mycelia. However, blastospores of some genera underwent through a process of budding similar to that of yeasts.

Effects of media, types of inoculum and cultivating conditions on the production of blastospores

Optimal condition for the production of blastospores of *C. brongniartii*, *Hirsutella* sp. 02, *Metarhizium* sp. 02, and *A. pistillariiformis* were determined. Effects of media, types of inoculum and cultivating conditions were examined for the maximum production of blastospores. Two different media (GICM and YPG) were tested. The results showed that blastospore production of *C. brongniartii* was induced by cultivation in both GICM and YPG liquid media (Fig. 5a). The GICM gave a higher yield of blastospores compared to that of YPG regardless of types of inoculum and cultivating conditions. Meanwhile blastospore production of *Hirsutella* sp. 02, *Metarhizium* sp. 02 and *A. pistillariiformis* could only be induced by cultivation in GICM. Therefore, the GICM was used as culture medium in all subsequent experiments in this study.

Two different types of inoculum (mycelial plug and conidia suspension) were also tested. The results showed that conidia suspension gave a slightly higher yield of blastospores than that of mycelial plug when cultivated in the GICM for all tested fungal isolates (Fig. 5a–d). Numbers of blastospores produced from both types of inoculum were not significantly different according to Duncan's multiple range test ($p < 0.05$). Therefore, mycelial plugs were used as a starting inoculum for the production of blastospores due to cost and ease of preparation. Different cultivating conditions, shaking and static were subsequently evaluated. Regardless of inoculum types and culture media, production rate of *C. brongniartii* blastospores under static condition increased slowly; and the numbers of blastospores obtained were lower when compared to those under shaking condition. However, cultivating conditions were species-specific as no blastospore was observed under shaking condition from *Hirsutella* sp. 02, *Metarhizium* sp. 02 and *A. pistillariiformis* cultures.

Time course for the production of blastospores was observed in all entomopathogenic fungal isolates. In the *C. brongniartii*, blastospore formation

initiated at 12–18 hours post inoculation (hpi) and yield of blastospores reached the maximum level at 36–72 hpi. After 78 hpi of cultivation, most of blastospores started to germinate. Regarding cultivating conditions, the highest yield of blastospores was 2.43×10^6 blastospores/ml under static condition when either conidia or mycelial plugs were used as inoculum in GICM. On the other hand, the highest yield of blastospores under shaking condition was $3.28\text{--}4.35 \times 10^8$ blastospores/ml when either conidia or mycelial plugs were used as inoculum in GICM. It can be thus concluded that the best conditions for the production of blastospore of *C. brongniartii* was using GICM as the induction medium under shaking condition. The suitable time course for harvesting fresh blastospores, to be used as specimens for cryopreservation, was approximately at 42–72 hpi after incubation.

In *Hirsutella* sp. 02, blastospore formation was initiated at 36 hpi and numbers of blastospores were exponentially increased within a day. A sudden decrease in spore production rate was subsequently detected, followed by second period of exponential increase in numbers of blastospores. This interesting observation indicated a unique pattern of blastospore development of *Hirsutella* sp. 02 in the GICM. The sudden change in the numbers of blastospores at 84 hpi could arise from the fact that majority of budding blastospores were detached from one another, resulting in an increased numbers of blastospores. Blastospore production reached the maximum level at 96–132 hpi, yielding 4.8×10^6 blastospores/ml. A decline in blastospore numbers occurred immediately due to rapid germination of most blastospores. According to the results, the best conditions for the production of blastospore of *Hirsutella* sp. 02 were cultivating in GICM under static condition. The suitable time course for harvesting fresh blastospores was approximately at 84–144 hpi.

In *Metarhizium* sp. 02, blastospore formation was initiated at 48 hpi. The exponential increase in blastospore numbers was observed within 72 hpi as budding blastospores were detached from one another. The highest numbers of blastospores was observed at 108 hpi, yielding 4.4×10^6 blastospores/ml. A sharp decline in blastospore numbers was observed after 4 days of incubation resulting from rapid formation of emerging young mycelia. It can be thus concluded that the best conditions for the production of blastospores of *Metarhizium* sp. 02 were using GICM as the induction medium under static condition. The suitable time course for harvesting fresh blastospores

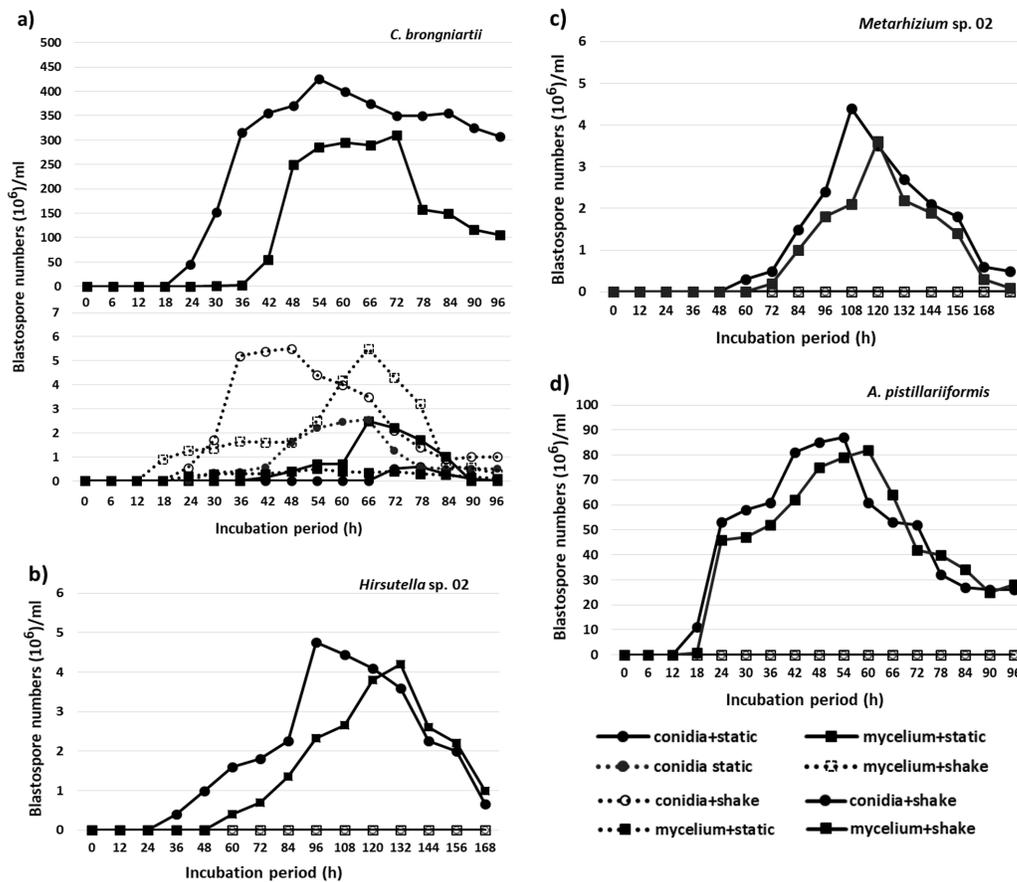


Fig. 5 Production of blastospores of selected entomopathogenic fungi incubated at 28 °C under shaking and static conditions. Mycelial plugs and conidia were used as static inocula. (a) Production of blastospores of *C. brongniartii* in YPG (dotted line) and GICM (solid line); (b) production of blastospores of *Hirsutella sp. 02* in GICM; (c) production of blastospores of *Metarhizium sp. 02* in GICM; (d) production of blastospores of *A. pistillariiformis* in GICM. The results are presented as the mean \pm SD of three independent experiments.

was approximately at 84–144 hpi. In *A. pistillariiformis*, blastospore formation was initiated at 12 hpi and numbers of blastospores were exponentially increased up to 48 hpi, yielding 8.8×10^7 blastospores/ml. Therefore, the best conditions for blastospore production of *A. pistillariiformis* were cultivating in GICM under static condition. The suitable time course for harvesting fresh blastospores was approximately at 24–72 hpi after incubation.

Cryopreservation of blastospores of entomopathogenic fungi

Long-term preservation of blastospores of the entomopathogenic fungi was studied. Freeze-drying was used as a preservation method and 5% (w/v) trehalose was used as a protective agent. Viability

Table 3 Viability of four entomopathogenic fungi using freeze-drying method.

Storage	Viability (%)	
	Preservation	One-year storage
<i>C. brongniartii</i>	31.8 \pm 3.4	21.8 \pm 1.8 ^a
<i>Hirsutella sp. 02</i>	41.8 \pm 2.2	35.6 \pm 0.9 ^b
<i>Metarhizium sp. 02</i>	31.8 \pm 2.2	22.0 \pm 1.7 ^c
<i>A. pistillariiformis</i>	0.00 \pm 0.0	0.00 \pm 0.0

The results are presented as the mean value \pm SD of three independent experiments. Different letters indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

of blastospores was assessed by determination of germ tube formation assay immediately after preser-

vation and after one-year storage at 5°C in the dark. The preservation results revealed that freeze-drying method could be used to preserve fungal blastospores of *C. brongniartii*, *Hirsutella* sp. 02 and *Metarhizium* sp. 02 because satisfactory percentage of viability was obtained after one-year storage, as shown in Table 3. However, the method was not suitable for preserving blastospores of *A. pistillariiformis* since no viability was observed. Observation of revived cells in terms of morphological and genetic stability demonstrated no changes in pigmentation, colony morphology including ability to produce blastospores and reproductive structures.

DISCUSSION

Entomopathogenic fungi in the genera *Akanthomyces*, *Cordyceps*, *Hirsutella*, *Metarhizium*, and *Torrubiella* have been recognized as potential candidates for the production of microbial insecticides to control several agricultural pests and have been identified as valuable sources of bioactive metabolite production. They are considered agriculturally and medically important. Therefore, understanding the morphological development of entomopathogenic fungi is one of the key parameters prior to using blastospores in industrial production. In addition, preservation methods which maintain genetic and physical stabilities of entomopathogenic fungi, were required to avoid alterations during storage.

In the present study, production of entomopathogenic fungal blastospores could be induced by using GICM as a culture medium supplemented with FBS. However, induction of blastospores *in vitro* depends on fungal genera since the findings revealed that none of the isolates in the genus *Torrubiella* could produce blastospores. Similarly, production of blastospores also depends on fungal isolates since blastospore formation could be detected in some isolates of the genera *Akanthomyces*, *Cordyceps*, *Hirsutella* and *Metarhizium* with different efficiency. As blastospore formation has been reported as an indispensable phase in the fungal reproducing cycle [17], failure in the production of blastospores in some fungal isolates could be attributed to inappropriate conditions of cultivation used.

Out of 39 entomopathogenic fungal isolates, 18 isolates were able to produce blastospores. Most of them belonged to the genera *Cordyceps* and *Hirsutella*. Most isolates of *Cordyceps* spp. were capable to produce blastospores (63%) which suggested that *Cordyceps* spp. is non-fastidious and does not

require complex nutrients for growth and development. Common characteristics of blastospores among those four genera were single-cell, hyaline, non-motile with smooth thin-walled, and varied in size and shape. Development patterns of blastospore formation also depends on fungal genera in term of day of spore initiation, spore emerging position, spore budding, and spore germination. It can be noted that blastospore morphology and development of *Akanthomyces* sp. is unique among other genera. This characteristic could be used to distinguish *Akanthomyces* sp. from other fungal genera. This corresponds to a statement from Samson et al [9] who stated that blastospores may be used to classify a genus of entomopathogenic fungi in the same manner as other vegetative propagules or other structures.

Culture media, types of inoculum and cultivating conditions exhibited different impacts on the production of blastospores. The GICM supplemented with FBS could be used to induce the production of blastospores in all fungal genera tested, however, YPG medium could only be used to induce the production of blastospores in the genus *Cordyceps* (*C. brongniartii*). FBS acts to mimic natural condition of haemolymph and contains essential components, such as hormones, vitamins, minerals, trace elements, and growth factors [18], which contributed to the induction of blastospore formation of entomopathogenic fungi *in vitro*. An ability of *C. brongniartii* for production blastospores in YPG medium might be attributed from non-fastidious character of the fungus including strong virulence resembling to its anamorph, *B. brongniartii*. This ability could thus be one of the factors contributing to adaptation ability to diverse environmental conditions. YPG medium was also used to promote the production of blastospores in *B. bassiana* and *C. brongniartii* under shaking condition, yielding 8.5×10^7 blastospores/ml and 5.7×10^6 blastospores/ml, respectively, after 2 days of incubation [19].

In addition to culture media, either mycelial plugs or conidia suspensions could be used as starting inocula since the numbers of blastospores produced from both were not significantly different. Cultivating conditions also showed different impacts on blastospore production because cultivation under static condition was only suitable for *Hirsutella* sp. 02, *Metarhizium* sp. 02 and *A. pistillariiformis*, while *C. brongniartii* produced blastospores in both static and shaking conditions. Production of blastospores under shaking condition has been

described as a suitable condition for the production of blastospores in several entomopathogenic fungi, for example *M. flavoviride*, *B. bassiana*, *P. fumosoroseus*, and *C. unilateralis* [20–23]. However, in the present study, production of blastospores of *Hirsutella* sp. 02, *Metarhizium* sp. 02 and *A. pistillariiformis* was only successful under static condition. Furthermore, those parameters tested also had significant impacts on time course of blastospore formation and determination of cultivation period that generated the highest numbers of blastospores. The observation revealed that initiation of blastospore formation of *C. brongniartii* occurred faster than other isolates. This might be a result from the fact that this fungus is fast-growing when grown on PDA and in GICM while the others are slow-growing.

The findings provided not only characteristics and development patterns of blastospores of entomopathogenic fungi, but also demonstrated the requirements for specific nutrients and conditions for growth and development of individual isolate. Some fungal species might need different types of sugars or nitrogen sources in medium composition, which could affect the numbers of blastospore production, growth and morphology. Formation of blastospores might be affected by age of the culture and loss of virulence due to routine subculturing. As blastospores serve as a virulent determinant in entomopathogenic fungi, loss of virulence due to routine subculturing may result in loss of ability to produce blastospores accordingly.

Long-term preservation of blastospores using freeze-drying method was successful in *C. brongniartii*, *Hirsutella* sp. 02 and *Metarhizium* sp. 02. However, the method was not suitable to preserve blastospores of *A. pistillariiformis* because no viability was observed. This finding might suggest different compositions and properties of blastospores of *Akanthomyces* spp., which make the fungus more sensitive to freeze-drying. This, consequently, leads to a problem in preservation of this fungus. Therefore, further investigation to identify an appropriate technique, conditions and protective agents must be conducted for this genus. In addition, several reports described that selection of optimal growth phase for cryopreservation is also essential for survival of dried spores. For example, the stationary phase cells of *Lactobacillus rhamnosus* gave the highest recovery, after drying, of 31–50%, whereas early log phase cells exhibited 14% survival and lag phase cells showed the highest susceptibility, with only 2% cell survival [24]. Similar results were

reported by Mary et al [25], where significantly higher cell viability was achieved from stationary phase *Rhizobia* cells compared to exponential phase cells. In contrast, higher survival rates of *Sinorhizobium* and *Bradyrhizobium* were produced when sampled in the lag phase of growth [26]. According to the finding from Cliquet and Jackson [20], a defined medium containing basal salts, glutamate, glucose, and zinc can be used to induce optimal concentrations of desiccation-tolerant blastospores of *P. fumosoroseus*. However, in this study the growth phase of entomopathogenic fungi was not determined but only the time course for maximum production of blastospores.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at <http://dx.doi.org/10.2306/scienceasia1513-1874.2020.078>.

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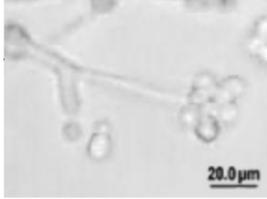
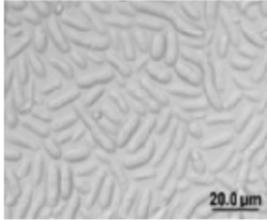
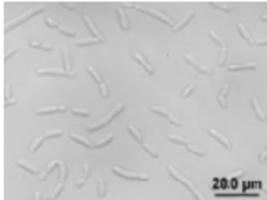
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Appendix A. Supplementary data

Table S1 General characteristics and blastospore formation patterns of the entomopathogenic fungi.

Genera/species	Blastospore morphology	Size (μm)	Days of formation	Budding position	Blastospores (X400 magnification)
<i>A. pistillariiformis</i> <i>Akanthomyces</i> sp. 01 <i>Akanthomyces</i> sp. 02	hyaline, small, round or oval shapes	4–10	1–2	hyphal tips (clusters)	
<i>C. brongniartii</i> <i>C. pseudomilitaris</i> <i>Cordydeps</i> sp. 01 <i>Cordydeps</i> sp. 02 <i>Cordydeps</i> sp. 03 <i>Cordydeps</i> sp. 04 <i>Cordydeps</i> sp. 05 <i>Cordydeps</i> sp. 06 <i>Cordydeps</i> sp. 07 <i>Cordydeps</i> sp. 08	hyaline, ellipsoidal to cylindrical shapes	10–25	2–3	hyphal sides	
<i>Hirsutella</i> sp. 01 <i>Hirsutella</i> sp. 02 <i>Hirsutella</i> sp. 03	hyaline, small, narrow with cylindrical to club-shapes	15–20	2–5	hyphal sides	
<i>Metarhizium</i> sp. 01 <i>Metarhizium</i> sp. 02	hyaline, ellipsoidal to cylindrical shapes	10–20	2–3	hyphal sides	