THE USE OF PALM KERNEL CAKE IN THE PRODUCTION OF CONIDIA AND BLASTOSPORES OF *Metarhizium anisopliae* var. *major* FOR CONTROL OF *Oryctes rhinoceros*

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ABSTRACT

The yield of conidia and blastospores of Metarhizium anisopliae *var.* major *produced on maize supplemented with palm kernel cake (PKC) was estimated. In maize supplemented with 5 g and 10 g of PKC, the fungus produced 4.3 g and 5.6 g of conidia respectively. This is significantly higher than the yield of conidia produced on maize alone, which was 2.03 g. The fungus produced submerged spores termed as blastospores, in a simple liquid medium consisting of glucose and PKC. The formation of blastospores during the fermentation process was monitored. The results showed that the young blastospores are round to ovoid, with 5.0-5.5 µm in diameter and matured blastospores are commonly ellipsoid with dimensions ranging from 5.0-5.5 µm x 12.5-15.5 µm. The highest yield of the blastospores was 3.26 x* 10^6 *blastospores* ml^{-1}*, produced at seven days after fermentation. The conidia and blastospores were equally effective in controlling the third instar larvae of the* Oryctes rhinoceros*. Sixteen days after treatment, both inocula killed 100% larvae, with infection of between 94.3% and 97.1%. The* LC_{50} *values for both inocula were almost similar, at 9.1 days for conidia and 9.5 days for blastospores. This study showed that PKC can be used as a supplement to produce conidia and blastospores of the fungus,* M. anisopliae *var.* major*, and that the blastospores are potential for the biocontrol of the rhinoceros beetle.*

Keywords: *Metarhizium anisopliae* var. *major*, *Oryctes rhinoceros*, conidia, blastospores, palm kernel cake.

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INTRODUCTION

Most of the entomopathogenic fungi including *Metarhizium anisopliae* var. *major* commonly produce two types of infectious conidia, depending on the types of media used during the growing stage. On solid substrates, the fungi produce conidia and in liquid media, they produce submerged spores or also well-known as blastospores. The conidia of *M. anisopliae* var. *major* was proven effective in killing the rhinoceros beetle *Oryctes rhinoceros*, therefore it has been used in the management programme of the pest (Ramle *et al.*, 2007). Studies on the use of blastospores of *M. anisopliae* var. *major* as biocontrol agent of rhinoceros beetle are still lacking. On the other hand, blastospores for other fungi such as *Beauveria bassiana*, *Paecilomyces farinosus* and *M. anisopliae* var. *acridum* have been successfully used to control various insect pests (Chelico and Khachatourians, 2003; Kassa *et al.*, 2004).

Palm kernel cake (PKC) is one of the by-products from the oil palm mill. The high fat content in PKC makes it a popular alternative source of protein and energy for animal feeds (Hishamudin, 2001; Wan Zahari and Alimon, 2004). In the fermentation technology involving microbes, PKC can be used

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as a carbon source to replace the sugar-based commercial carbon source (Abd-Aziz *et al.*, 2008), which is normally expensive. Many successful production studies of entomopathogenic fungi have been reported, but mostly grains were used as a sole nutrient without any additive agents to improve fungal sporulation (Aregger, 1992; Mendonca, 1992; Sahayaraj and Namasivayam, 2008). Works on improvement procedures using different carbon sources or supplementary growth enhancer were mainly on the propagation of fungal mycelia or blastospores in a liquid media (Campbell *et al.*, 1978; Samsinokova *et al.*, 1981; Humphrey *et al.*, 1990).

In this study, the production of conidia and blastospores of *M. anisopliae* var. *major* on media supplemented with PKC was evaluated. In addition, the effectiveness of both types of conidia in killing the larvae of the rhinoceros beetle was also determined.

MATERIALS AND METHODS

Sources of *M. anisopliae* var. *major*

The *M. anisopliae* var. *major* isolate MaST was maintained on a 90 mm petri plate of potato dextrose agar (PDA) and incubated at 28°C for two weeks. Spore solutions were prepared by adding 15 ml of 0.02% Tween 80 solution into the plate cultures. The conidia were separated from the media by scraping using an 'L' shaped inoculation needle. The spore solution was transferred into a 50 ml screw-capped bottle and the bottle was vigorously shaken. The spore solutions were then filtered via glass wool into a new bottle and the concentration was determined using a haemocytometer. The spore suspensions were diluted to final concentration of 2.0×10^7 conidia ml^{-1}.

Production of Conidia on Solid Media Supplement with Palm Kernel Cake

The production of conidia was conducted using two fermentation techniques following Ramle *et al.* (2007). Mycelia of the fungus was produced in a 500 ml conical flask with medium prepared by mixing 1.25 g yeast extract, 2.5 g peptone, 5.0 g dextrose, 0.13 g chloramphenicol and 250 ml distilled water. All ingredients were homogenously mixed and dissolved by heating in a microwave oven for several minutes. The media was sterilised by autoclaving at 121°C for 20 min and cooled at room temperature. The flask was then inoculated by adding 3 ml spore suspensions as previously prepared. The flask was transferred into a refrigerated orbital shaker and at 166 rpm, 28°C for four consecutive days.

Broken maize (grade B) was used in sporulation stage of the fungus. The debris was removed by washing the maize in a sieve with 2.0 mm mesh. The floating debris or husks were removed and the maize was kept in water for about 40 min. The sieve was then lifted to air-dry the maize for 5 min. The maize was then transferred into 90 pieces of autoclavable plastic bags, each with 250 g maize. In Treatment 1 (T1), 30 bags were added with maize supplemented with 5 g PKC in each bag. In Treatment 2 (T2) another 30 bags were added with 10 g PKC in each bag. Prior to utilising as supplement, the PKC was sieved via a 0.5 mm mesh sieve to remove the debris. For control, 30 bags contained only maize without PKC. The bags were vigorously shaken, sealed and sterilised by autoclaving at 121°C for 20 min. Inoculation was made by adding 3 ml of mycelia solution into each bag and then incubated in darkroom at 28 ± 3°C for 30 days.

The yield of conidia produced in a bag was estimated by washing and filtration methods following Ramle *et al.* (2007). In each treatment, the yield of conidia was estimated from three bags at 30 days after fermentation. Harvesting was done by adding 100 ml of 0.02% tween 80 into the bag, and finally shaking the bag vigorously to separate the conidia from the maize. The mixtures of spore solutions were filtered via filter paper (Whatman 1) under vacuum for collection of conidia. Drying was done by placing the wet conidia into a refrigerator at 5°C for 10 hr and then transferred into a biosafety cabinet for 1 hr. The weight of the dried conidia was then recorded.

Production of Blastospores in Liquid Media Supplemented with Palm Kernel Cake

The blastospores were produced in a 1 litre conical flask with a liquid media prepared by mixing 5 g sucrose, 0.25 g chloramphenicol, 2.5 g PKC and 500 ml distilled water. The mixture was homogenously mixed and dissolved by heating in a microwave oven for 3-5 min and sterilised by autoclaving at 121°C for 20 min. The flasks were inoculated with 3 ml of conidial solutions at concentration of 2.0×10^7 conidia ml^{-1}. Then, the flasks with the inoculated broth were fermented in a refrigerated orbital shaker, programmed at 166 rpm, 28°C for six days. Data of production of blastospores was then recorded at four, five, six and seven days after fermentation (DAF).

At each DAF, the numbers of blastospores were estimated from three flasks. Prior to harvesting, the morphological characteristics of the blastospores were recorded. Harvesting was done by homogenising the liquid media using a homogeniser at 4500 rpm for 30-45 s. The mixture was then sieved using a 180 mesh sieve to separate the blastospores from the mycelium. The volume of blastospore solution was recorded and the concentration was estimated using a haemocytometer technique. The viability of blastospores was estimated by plating

100 μl blastospore solution onto the PDA plates. The germinated blastospores were counted under a microscope at 10X magnifications after 24 hr incubation at 28°C.

Pathogenicity Test of Conidia and Blastospores against Larvae

Conidial solutions were prepared by adding 30-day old culture bags with 100 ml sterilised distilled water with 0.02% Tween 80. The bag was vigorously shaken to separate the conidia from the maize and the conidial mixtures were then filtered via glass wool into a new sterilised bottle. The concentration of conidia was estimated by a haemocytometer and the solution was diluted to 2 x 10^6 conidia ml^{-1}. The blastospore solutions were prepared from a 6-day old culture flask containing 500 ml liquid media supplement with PKC. Prior harvesting, the media were homogenised to separate the blastospores from the mycelia. The collection of blastospores was done by filtering the mixture via a 180 μm mesh sieve. The concentration of blastospores was estimated by a haemocytometer and diluted to 2 x 10^6 blastospores ml^{-1}.

A pathogenicity study was done against the third instar larvae (L3) of *O. rhinoceros* larvae. Bioassay was conducted in a plastic box with a dimension of 20 cm length x 15 cm width x 15 cm height, half-filled with sterilised medium of a mixture of oil palm rotting tissues with soil. In T1, the medium was mixed with 10 ml conidial solutions and T2 with 10 ml blastospore solutions. For control, the medium was only mixed with 10 ml of sterilised distilled water. The L3 larvae of *O. rhinoceros* was then added into each container and placed in a laboratory at 28 ± 3°C for 16 days. The test was conducted using a randomised complete block design with seven replications and five L3 larvae per replicate. Data on larval mortality and infection was recorded every alternate day for a period of 16-day after treatment (DAT). Fungal infection on larval cadavers was confirmed by placing a group of four to five cadavers in a wet container for two to four days for mycoses to develop.

Data Analysis

The dry weight of conidia, numbers of blastosopres, percentage germination of conidia and blastospore were analysed using one-way analysis of variance (ANOVA). The effects of conidia and blastospores on mortality and infection of L3 larvae were analysed using two way ANOVA. To stabilise variance of binomial, data of percentages of germination, mortality and infection were transformed using arcsine transformation before performing the analyses of ANOVA and means were compared by Duncan's Multiple Range Test (DMRT) at P=0.05 (SAS, 1997). The lethal time 50% (LT_{50}) values of conidia and blastospores for killing the L3 larvae was estimated by Probit analysis (Finley, 1971; Wigley and Kalmakoff, 1977).

RESULTS AND DISCUSSIONS

In this study, supplement of PKC into a solid substrate had improved the production of conidia of *M. anisoplaie* var. *major* (*Figure 1*). The yield of conidia in bags with 10 g PKC was 5.61 g, and was significantly higher (P<0.01) than the yield of

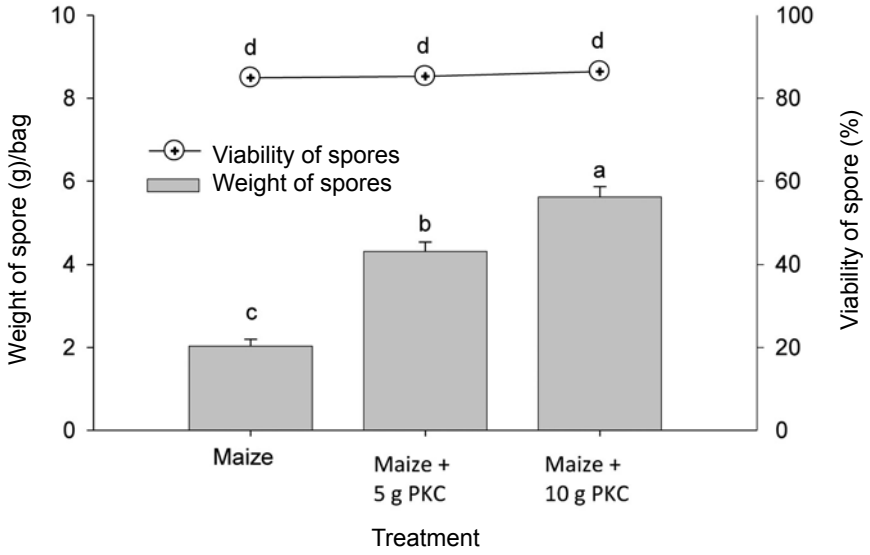

Figure 1. Yield of conidia of Metarhizium anisopliae *var.* major *grown on maize alone and maize supplement with 5 g and 10 g palm kernel cake in each bag. Bars or line graphs with the same letters are not significantly different by Duncan Multiple Range Test at P=0.05.*

conidia in bags with 5 g PKC, which was 4.30 g. More importantly, the yield of conidia produced on media with both rates of PKC were two times higher as compared to yield of conidia produced on broken maize alone (2.03 g per bag). The viability of conidia produced in all treatments was more than 85%, and not significant (P>0.05) between treatments. A higher fat content in PKC could have possibly provided the essential nutrients for the fungus to induce sporulation. Findings of this study supported the earlier study of using oil as a fat source to improve spore production (Ramle *et al.*, 2005). An addition of 2 ml palm oil in 250 g maize had significantly increased the spore yield from 3.8 g to 5.8 g per bag.

Most reports showed that the entomopathogenic fungi such as *Beauveria* sp., *Paecilomyces* sp. and *Metarhizium* sp. produced blastospores when grown in a liquid medium (Humphreys *et al.*, 1990; Jenkins and Prior, 1993; Kleespies and Zimmermann, 1998; Lozano-Contreras *et al.*, 2007). The production of blastospores of *M. anisopliae* var. *major* in liquid media with PKC is reported in this study. The formation of blastospores was monitored. Based on morphological structures, three distinct shapes of blastospores of *M. anisopliae* var. *major* were identified. The new blastospores were formed at the tip of the fungal mycelium (*Figures 2a* and *2b*) until it matured (*Figures 2c, 2d* and *2e*). Young detached blastospores are ovoid to ellipsoid in shape with a dimension of 2.5-3.0 μm x 2.0-3.0 μm (*Figure 3b*). The immature blastospores are swollen ellipsoidal to cylindrical with rounded ends, with a dimension 7.5-10.0 μm x 2.5-3.0 μm (*Figure 3b*). The matured blastospores are commonly elongated ellipsoidal to cylindrical in shape, with dimensions 2.5-3.0 μm x 12.5-15.0 μm (*Figure 3c*).

The production of blastospores had significantly increased (P<0.01) over time from 2.0 x 10^6 blastospores ml^{-1} at four DAF to a peak at 3.3 x 10^6 blastospores ml^{-1} at seven DAF (*Figure 4*). The viability of blastospores was maintained high, more than 90% (*Figure 4*). The yield of blastospores depends on the liquid medium compositions. For examples *M. flavoviridae* produced more than 1.5 x 10^9 blastospores ml^{-1} in media with 20 g sucrose and 20 g yeast (Jenkins and Prior, 1993). The *M. anisopliae* var. *anisopliae* produced up to 1.9 x 10^8 blastospores ml^{-1} in a medium enriched with 5% lecithin as compared to only 1.9 x 10^7 blastospores ml^{-1} in the standard medium (Kleespies and Zimmermann, 1998). For the fungus *P. fumosoroseus*, medium containing peptone and yeast extract produced 10^{10}

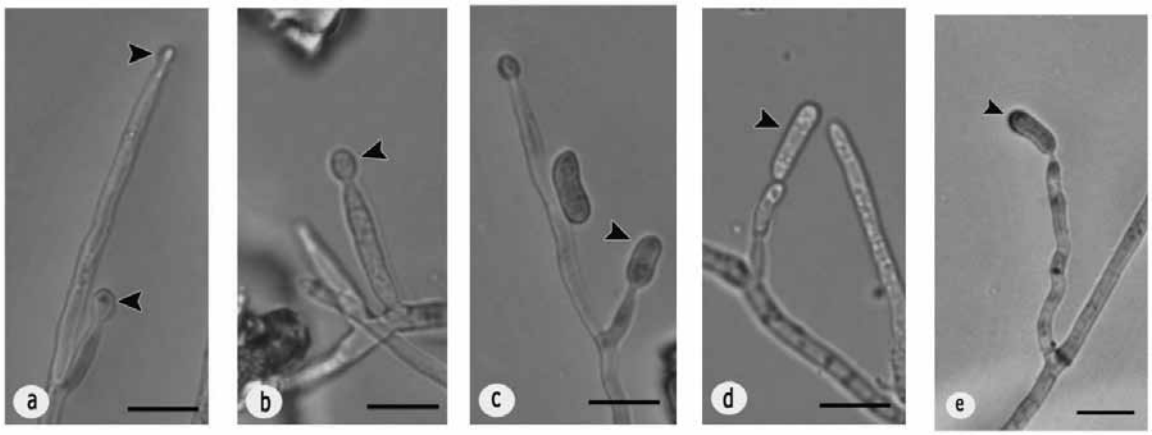

Figure 2. The developmental stages of Metarhizium anisopliae *var.* major. *Arrows showing the formation of young (a, b and c) and matured (d and e) blastospores in liquid medium containing palm kernel cake as a carbon source. Bar = 10 µm.*

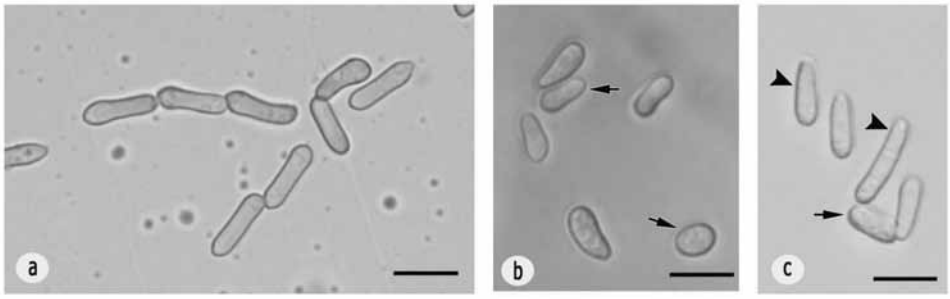

Figure 3. The morphological characteristics of matured conidia (a). Arrows showing detached young (b) and matured blastospores (c) of Metarhizium anisopliae *var.* major. *Bar = 10 µm.*

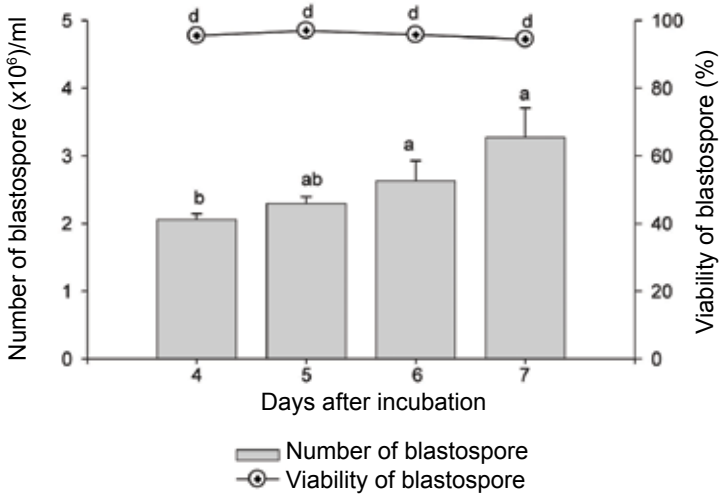

Figure 4. Production of blastospores of Metarhizium anisopliae *var.* major *in liquid media supplemented with palm kernel cake over time. Bars or line graphs with the same letters are not significantly different by Duncan Multiple Range Test at P=0.05.*

blastospores ml^{-1}, significantly higher as compared to the medium with amino acids that only produced 2.4 x 10^{8} blastospores ml^{-1} (Lazano-Contreras *et al.*, 2007).

The pathogenicity of conidia and blastospores of *M. anisopliae* var. *major* against the L3 larvae of *O. rhinoceros* is shown in *Table 1*. The treatment of conidial solutions caused 5.7% mortality at 8 DAT and then rapidly increased to 91.4% at 10 DAT. Blastospores caused mortality as early as two DAT, and slowly increased until it reached 65.7% at 10 DAT. Subsequently, the L3 larval mortality in both treatments increased and reached 100% at 16 DAT. The larval mortality for both treatments at every DAT was not significantly different (P>0.05). The number of dead larvae infected by *M. ansiopliae* in both treatments was more than 91%. Based on the time required to kill 50% larvae, both treatments had about the same killing speed (*Table 2*). The LT_{50} values for conidia and blastospores were 9.1 days and 9.5 days, respectively.

The percentage of mortality and infection, and the LT_{50} values in this study were about the same as reported by earlier workers. Latch (1976) found that three isolates *M. anisoliae* var. *major* killed 100% larvae of *O. rhinoceros* between 7 and 16 DAT. A previous study by Ramle *et al.* (1999) using two isolates found that the LT_{50} values for both isolates were between 8.9 and 9.1 days. In a more recent study (Ramle *et al.*, 2006), all four isolates caused 100% mortality to L3 larvae *O. rhinoceros* at 12 and 14 DAT and the LT_{50} values for all isolates were less than 10 days .

CONCLUSION

In this study, the supplement of the PKC in the substrate had improved the production of conidia of *M. anisopliae* var. *major* by more than two times. As the supply of PKC is abundant and cheap in Malaysia, the use of the material in the mass production plant for the production of conidia is recommended. In the long run, it will reduce cost, increase productivity and the capacity of the plant. The blastospores are proven as good as conidia in killing the larvae of *O. rhinoceros*. At 16 DAT, conidia caused 97.1% infection whilst, blastospores caused 91.4% infection. Therefore, both types of

TABLE 1. PERCENTAGE MORTALITY AND INFECTION OF LARVAE OF *Oryctes rhinoceros* AFTER BEING TREATED WITH CONIDIA AND BLASTOSPORES OF *Metarhizium anisopliae* var. *major*

Treatment	Percentage of cumulative mortality of larvae over time (days after treatment)								% Infected larvae*
	2	4	6	8	10	12	14	16	
Control	0.0a	0.0a	2.9a	2.9a	5.7a	5.7a	5.7a	5.7a	5.7a
Conidia	0.0b	2.9a	2.9a	5.7a	91.4b	94.3b	100b	100b	97.1b
Blastospores	5.7b	8.6a	8.6a	11.4a	65.7c	88.6b	94.3b	100b	91.4b

Note: Data in column with the same letters are not significantly different by Duncan Multiple Range Test at P=0.05. *Percentage of infected larvae was recorded at 16 days after treatment.

TABLE 2. THE LT_{50} VALUES FOR CONIDIA AND BLASTOSPORES OF *Metarhizium anisopliae* var. *major* ON LARVAE OF *Oryctes rhinoceros*

Types of spores	LT_{50} (days)	Fiducial limit 95% (days)	Regression	Chi-square Test	SE slopes
Conidia	9.12	8.77 – 9.58	Y = 13.99x - 8.46	7.51	0.135
Blastospores	9.47	8.95 – 10.02	Y = 9.54x - 4.31	8.50	0.116

Note: Y - log of days, x - empirical probit of mortality, SE - standard error.

conidia are equally effective. Although blastospores have a short life span in the field, the production is relatively easy, especially in the process of scaling up as well as to control key parameters during the fermentation. Further research is needed to fully use the potential of blastospores as another biocontrol agent of *O. rhinoceros*.

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