KKU Res. J. 2012; 17(5):762-768
http : //resjournal.kku.ac.th

Conidial production of entomopathogenic fungi in solid state fermentation

Thet Thet Mar[1] *and Saisamorn Lumyong* [1*]

[1] *Microbiology Division, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand*
* *Correspondent author: scboi009@chiangmai.ac.th*

Abstract

Six naturally collected entomopathogenic fungal isolates were grown on five cereal grains; rice, wheat, rye, corn and sorghum, as solid inoculum in order to measure the linear growth, spore production, and their pathogenicity. Among the grains, corn gave the highest growth rate and rye showed poor mycelium growth for all isolates except *Paecilomyces lilacinus* CMUCDMT02. In all grain substrates, *P. lilacinus* grew significantly faster than the others. *Isaria tenuipes* CMUCDMF02 and *Beauveria bassiana* CMUCDMF03 produced fruiting bodies on corn and sorghum 15 days after inoculation. Spore productivity on sorghum yielded the maximum amount of spores for *P. lilacinus* CMUCDMT02 and *Metarhizium flavoviride* CMUCDCT01 whereas rice yielded the greatest amount of spore for *B. bassiana* CMUCDMF03. Germination at 48 hours was over 80% for all isolates that had been incubated for 60 days. Among all tested isolates, pathogenicity of *P. lilacinus* was highest against *Bactrocera* sp. (1×10^6 spore/ml). The data from this experiment could be employed for the mass production of entomopathogenic fungi for biological control purposes.

Keywords: Entomopathogenic fungi, mycelial growth, mass production, grains, sporulation

1. Introduction

Entomopathogenic fungi (EPFs) can be manipulated in several ways for use in biocontrol, but must be available in large quantities (1). Production processes for fungal biopesticides must be low-cost and a high yield concentration of viable, virulent, and persistent spores (2). There are three types of production system such as submerged (liquid) fermentation, surface cultivation (solid), and diphasic fermentation. Methods for commercial production of conidia are usually done on solid substrates like cereal grains, rice or other starch-based substrate (3). Moreover, several nutritional studies have been undertaken in production and sporulation of filamentous fungi such as *B. bassiana*, *M. anisopliae* and *I. fumosorosea* (4, 5, 6). In Thailand, a great deal of research on entomopathogenic fungi has been extensively carried out during the past 20 years. However, the production of locally isolated entomopathogenic fungi in suitable

media for large scale application has not yet been studied. Therefore our work aimed with the production of entomopathogenic fungi on various cereal grains without addition of any supplements, using simple technologies with low inputs, in order to evaluate linear growth, spore production, substrate moisture content, and quality control parameters, such as germination, purity and their pathogenicity.

2. Materials and Methods

Six isolates such as: 2 isolates of *B. bassiana, M. anisopliae, M. flavoviride, I. tenuipes* and *P. lilacinus* collected from natural habitats of Northern Thailand were used in this study. The solid substrates evaluated were rice, wheat, rye, corn and sorghum. Grains were boiled until they were soft but not cooked. The boiled grains were placed inside an 18 cm long test tube filling 10 cm of the test tube height and plugged with silicon. Test tubes were sterilized by autoclaving at 121˚C for 30 minutes. After cooling, solid media were inoculated with 1 cm diameter mycelial disc at the center and incubated at room temperature (25±2˚C) until the percent moisture of the substrates reached a stable level. Each treatment was replicated 3 times. Linear growth of fungal isolates was recorded five days after inoculation. The moisture content of substrates was calculated by (W-B) - (D-B)/ (W-B)× 100. Where: W= Weight of test tube plus wet spores; D= Weight of test tube plus dry spores; B= Weight of test tube.

Conidial production was determined 60 days after inoculation. Approximately 0.05-0.1 gram of a fresh sample was weighed and added to 10 ml of 0.1 % Tween 80. Once all the spores were suspended, dilution series was carried out (10^{-1}, 10^{-2} and 10^{-3}) and spores were counted using a haemocytometer. Germination and purity of fungal inocula were performed with 100 µl of conidial solution (1×10^{6} conidia/ml) plated on PDA and incubated at 25±2˚C for 48 hours and 3–5 days, respectively. Other quality control parameters such as viability and purity of conidia were measured.

In vitro virulence of conidia products was tested on fruit fly (*Bactrocera* sp.). Two to three days old pupae, surface sterilized with 1% (v/v) sodium hypochlorite, were dipped in 200 µl of 10^{6} conidia/ml solution for 2 minutes with gentle shaking (modification of Anand *et al., (*7)). Treated pupae were transferred to 15 ml bioassay glass vials and incubated at 25˚C and 70% RH. Tested pupae were checked daily for 14 days after incubation. The data (mean weight of spores) was analyzed using the SPSS program version 16.0 (SPSS Inc., Chicago, IL) to determine variance.

3. Results and Discussion

From this study it was clear that all the tested fungi were able to grow on a wide variety of cereal grains. Humber (8) stated that the growth characteristic of the vast majority of EPFs is clearly affected by the supply of nutrients. Our finding showed that almost all isolates grew on all grain substrates for 60 days, even though no nutrient supplements were added. Among the solid substrates, corn gave the highest growth rate and rye showed the lowest for all isolates. Rice, sorghum and wheat recorded moderate fungal growth. However, the mycelium of *P. lilacinus* CMUCDMT02 grew very well in all solid substrates (Figure 1). The daily growth rate of all tested entomopathogenic fungi grew well in all substrates except *I. tenuipes* CMUCDMF02 in rye from the beginning of experiment (Fig. 2).

The number of spores as well as the rate of viability varied between isolates. High spore numbers is one of the main criteria for choosing a fungal pathogen for biological control of pests in the field (9). The

highest number of spores from the grain substrates were observed when *P. lilacinus* CMUCDMT02 (530.6±31.6) and *M. flavoviride* CMUCDCT01 (102.8±11.4) were grown in sorghum medium whereas the rice substrate yielded the greatest amount of spores for *B. bassiana* (21.8±13.0) CMUCDMF03 (Table 1). Ibrahim and Low (10) and Sharma *et al.* (11) reported that rice was a suitable media for the mass culture of *B. bassiana* and the cereal was also suitable for the mass production of other deuteromycete fungi. For all isolates, rice substrate produced large number of spores in tested fungi except for *M. flavoviride* CMUCDCT01 and *P. lilacinus* CMUCDMT02. Moreover, with the agreement of Samson (12), *Beauveria* sporulates well on sterilized rice; we found that rice showed maximum spore production for *B. bassiana* strains CMUCDMF03 and CMUCDMG03. Gopalakrishnan *et al.* (13) reported that sorghum was the ideal cereal for the mass production of *P. farinosus*.

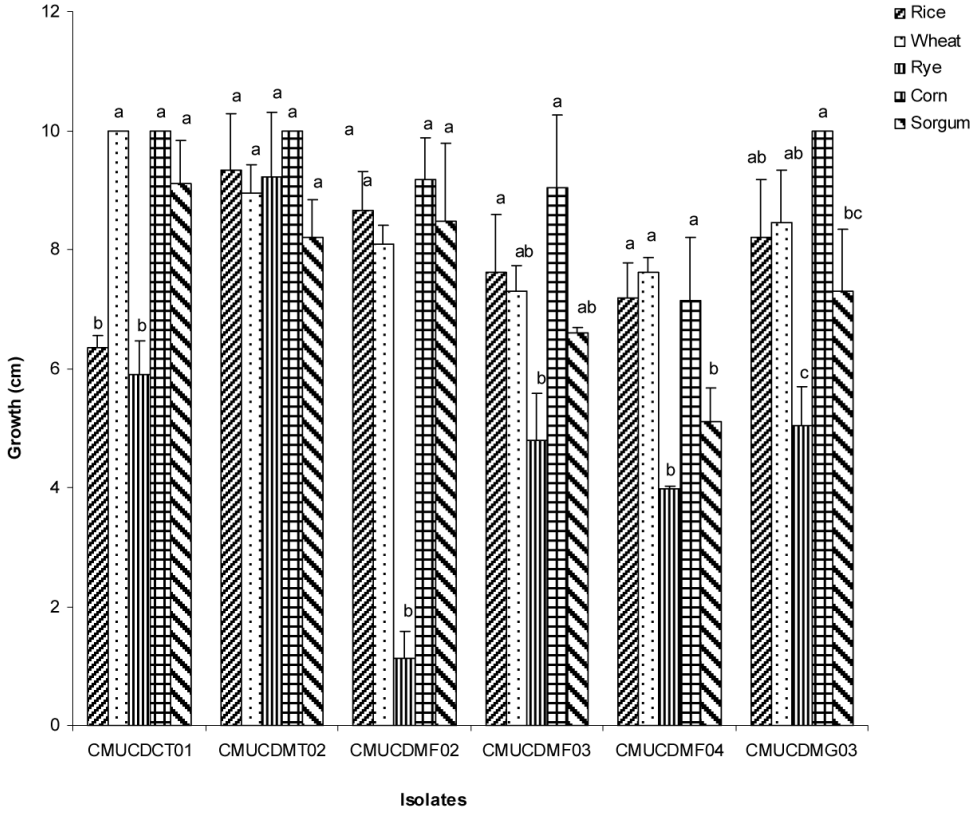

Figure 1. Mean linear growth of fungal isolates cultured on five grains at 30 days after inoculation. The *same letter* above *bars* within a graph indicates no significant difference according to the Tukey's HSD Post-hoc test at $P < 0.05$

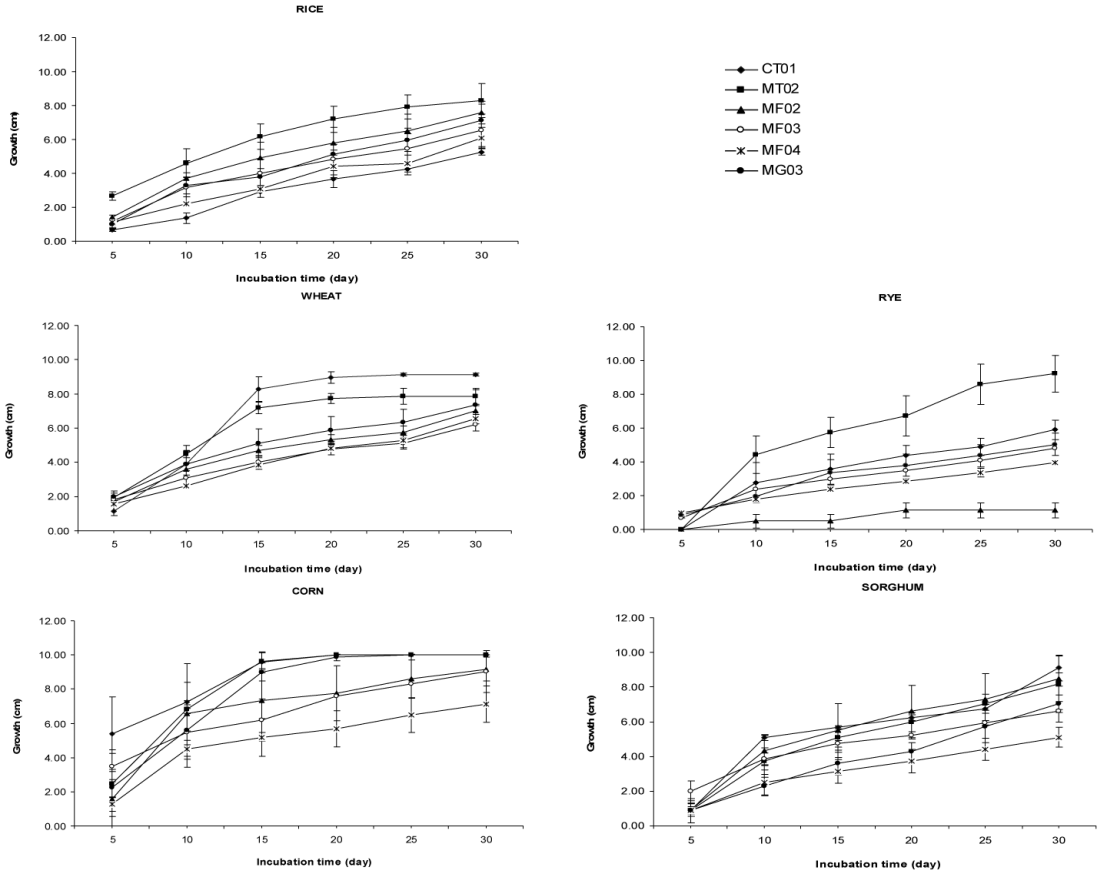

Figure 2. Mean daily growth rate of entomopathogenic fungi on different solid substrates at room temperature (25±2˚C) for 30 days

Table 1 Mean spore number per gram of fungal isolates incubated on various solid substrates at room temperature

Grains	Number of spores per one gram of substrate (x 10^9)[a]					
	CMUCDCT01	CMUCDMT02	CMUCDMF02	CMUCDMF03	CMUCDMF04	CMUCDMG03
Rice	86.2±12.0ab	399.3±6.7b	22.5±9.9a	141.0±48.6a	21.8±13.0a	57.6±35.3a
Wheat	6.8± 1.6d	4.2 ±0.9c	1.7±0.2ab	21.7±4.9b	11.1±2.6a	29.8±7.7a
Rye	10.7±1.9cd	9.1 ±6.3c	0.5±0.06b	35.5 ±3.8ab	1.8 ±0.8a	47.4±11.8a
Corn	53.4±13.8bc	59.2±22.9c	3.3±0.8ab	11.1 ±3.1b	4.4 ±0.7a	11.1 ±4.1a
Sorghum	102.8±11.4a	530.6±31.6a	10.7±1.2ab	69.3±22.9ab	15.4 ±3.0a	53.2 ±16.9a

Note: The results are mean and standard deviation of three replicates. Data with different letters indicates a significant difference at $P<0.001$ according to Tukey's HSD Post-hoc test within the same treatment.

[a] Average ± standard deviation error from triplicate samples

The result also concurred that the maximum spore productivity of *P. lilacinus* CMUCDMT02 yielded on sorghum. The number of spores in rye substrate was the least especially in *I. tenuipes* CMUCDMF02 (0.5 ±0.06). Robl *et al.* (9) demonstrated that sporulation likely occurs upon nitrogen depletion in the presence of carbohydrate. For optimum sporulation a medium is required where extensive mycelial growth is followed by spore production. A nutrient rich medium would not stimulate sporulation while a nutrient poor medium would not offer extensive mycelial growth. However, in this experiment, no attempts were made to add of any nutrient sources in order to obtain maximum growth and sporulation, and almost all tested entomopathogenic fungi produced spore numbers. There were no significant differences (P<0.05) in the germination of spores of fungal strains (Table 2).

Maintenance of a high viability during storage is essential for effectiveness and thus market acceptance of fungus-based biopesticides (Hedgecock *et al.* (14) Hong *et al.* (15). It was found that all tested entomopathogenic fungi were capable of maintaining a percent germination above 80%, 60 days after incubation. According to Hedgecock *et al.* (14) and Hong *et al.* (15) maximum spore stability of the conidia of commonly employed entomopathogenic fungi requires drying to low moisture content (4-5%). In this experiment, moisture content at the time of spore counting was 5-7%.

Table 2 Percent germination of spores, percent mortality and lethal time of isolates at 25±2˚C

Strains	% germination on solid substrates					Pathogenicity		
	Rice	Wheat	Rye	Corn	Sorghum	% Mortality	LT 50 (days)	LT90 (days)
CMUCDCT01	85	86	89	94	93	68.81 b	9	14
CMUCDMT02	87	93	85	94	93	100 a	8	10
CMUCDMF02	89	91	83	92	91	74.33 ab	11	>14
CMUCDMF03	93	83	85	93	95	95.54 ab	9	11
CMUCDMF04	82	81	86	91	93	74.33 ab	11	>14
CMUCDMG03	85	95	87	95	94	78.25 ab	11	>14

Note: The results are mean and standard deviation of three replicates. Data with different letters indicates a significant difference at P<0.001 according to Tukey's HSD Post-hoc test within the same treatment. LT: lethal time

There were no reports for the pathogenicity of mass producing conidia against fruit fly (*Bactrocera* sp.). Nevertheless, Posada-Flórez (16) demonstrated that pathogenicity of *B. bassiana* was over 92.5% against *H. hampei*, when spores were harvested 15 days after inoculation. In all pathogenicity tests, all fungal isolates were pathogenic to *Bactrocera* sp., between 68.81 to 100% with the spore concentration *ca.* 1×10^6 spore/ml. The mortality of *Bactrocera* sp. was 100% in *P. lilacinus* CMUCDMT02 when spores were harvested 60 days after inoculation (Table 2).

From this study it was clear that the tested fungal isolates have grown very well on all substrates used. Our finding showed that *P. lilacinus* CMUCDMT02 and

M. flavoviride CMUCDCT01 grew significantly better than other strains on all substrates. Even though, fungal growth was highest in corn, isolates CMUCDMT02 and CMUCDCT01 had maximum number of conidia on sorghum, while CMUCDMF03 produced significantly higher amount of spore in rice. Germination of all tested EPFs had a higher viability would be practical for the field works and ability to maintain longer shelf-life at room temperature. The method used in this experiment is a promising strategy for the large scale production of conidia as mycoinsecticide with least cost. To our knowledge this is the first report of the conidial production of the fungal isolates collected from naturally infected hosts by using solid-state fermentation in Thailand. Moreover, further work is necessary to find out their virulence against insect host.

4. References

(1) Alves SB, Lopes RB. Controle Microbiano de Pragas na América Latina: Avanços e Desafios. FEALQ, Piracicaba, São Paulo. 2008.

(2) Jackson MA. Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. J Ind Microbiol Biotechnol 1997; 19: 180-7

(3) Goettel MS, Roberts DW. Mass production, formulation and field application of entomopathogenic fungi. in: Lomer, C.J., Prior, C. (Eds.), Biological Control of Locusts and Grasshopper. CAB International, Wallingford, Oxon. 1992. P.230-38.

(4) Rombach MC. Production of *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) sympoduloconidia in submerged culture. Entomophag. 1988; 34: 45-52.

(5) Cruz BPB, Abreu OC, Oliveira AD, Chiba S. Crescimento de *Metarhizium anisopliae* (Metsch.) Sorokin em meios de cultura naturais líquidos. O Biológico. 1993; 49: 111-116

(6) Torre M de la, Cardenas-Cota HM. Production of *Paecilomyces fumosoroseus* conidia in submerged culture. Entomophaga. 1996; 41: 443-453

(7) Anand R, Prasad B, Tiwary BN. Relative susceptibility of *Spodoptera litura* pupae to selected entomopathogenic fungi. BioControl. 2009; 54: 85-92

(8) Humber RA. Evolution of entomopathogenicity in fungi. J Invertebr Pathol. 2008; 98(3): 262-266

(9) Robl D, Sung LB, Novakovich JH, Marangoni PRD, Zawadneak MAC, Dalzoto PR, Gabardo J, Pimentel IC. Spore production in *Paecilomyces lilacinus* (Thom.) Samson strains on agro-industrial residues. Braz J Microbiol. 2009; 40: 296-300

(10) Ibrahim YB, Low W. Potential of mass production and field efficacy of isolates of the entomopathoghenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* on *Plutella xylostella.* J Invertebr Pathol. 1993; 39: 222-232

(11) Sharma S, Gupta RBL, Yadava CPS. Selection of a suitable medium for mass multiplication of entomofungal pathogens. Indian J Entomol. 2002; 64(3): 254-261

(12) Samson RA. Identification: entomopathogenic deuteromycetes, *In*: Burges, H.D. (Ed), Microbial Control of Pests and Plant Diseases. Academic Press, London. 1981.P.93-106

(13) Gopalakrishnan C, Anusuya D, Narayanan K. *In vitro* production of conidia of entomopathogenic fungus *Paecilomyces farinosus.* Entomology. 1999; 24: 389-392

(14) Hedgecock S, Moore D, Higgins PM, Prior C. Influence of moisture content on temperature tolerance and storage of *Metarhizium flavoviride* in an oil formulation. Biocontrol Sci Technol. 1995; 5: 371-377

(15) Hong TD, Gunn J, Ellis RH, Jenkins NE, Moore D. The effect of storage environment on the longevity of conidia of *Beauveria bassiana.* Mycol Res. 2001; 105: 597-602

(16) Posada-Flórez FJ. Production of *Beauveria bassiana* fungal spores on rice to control the coffee berry borer, *Hypothenemus hampei*, in Colombia. J Insect Sci. 2008; 8 (4): 13pp