CHAPTER 6

Spore Production of EPFs in Solid State Fermentation

6.1 Introduction

Recently, BCAs are taking part in the integrated control program of agricultural pests in crop production. Management of insect pests in the form of biological measure have been encouraged and invested by large multinational agrochemical companies as bio-pesticides since 1980s (Charnley and Collins, 2007). The use of conidia as mycoinsecticides is warranted as they are a naturally-infective propagule. The high number of fungal propagules used in biocontrol approach requires a cost-effective production and stabilization process that delivers viable, infective fungal propagules (Goettel and Roberts, 1992; Wraight *et al*., 2001; Jackson, 2007). Production and formulation are critical to the commercial development of a fungal biocontrol agent. EPFs can be manipulated in several ways for use in biocontrol, but must be available in large quantities (Alves and Lopes, 2008).

Mass production for inundative control (release the large number of biocontrol agent relative to the number of target species) is known as the microbial pesticide or mycoinsecticide approach (Tanada and Kaya, 1993). There are three types of production system such as submerged (liquid) fermentation, surface cultivation, and diphasic fermentation, have been employed. However, Deshpande (1999) demonstrated that solid state fermentation is advantageous because it is easy to carry out and raw material is cheap. Large-scale production has been carried out on

on agricultural, brewing or other wastes though such media can be too variable and of low immediate metabolic availability (Dalla Santa *et al*., 2005). Jackson, 1997 stated that mass production and formulation processes directly influence the cost, shelf life, virulence, and field efficacy of fungal products.

Moreover, several nutritional studies have been undertaken in production and sporulation of filamentous fungi such as *B. bassiana*, *M. anisopliae* and *I. fumosorosea* (Rombach, 1988; Cruz *et al*., 1993; de la Torre and Cardenas-Cota, 1996). In Thailand, a great deal of research on EPFs has been extensively carried out during the past 20 years. However, the production of locally isolated EPFs in suitable media for large scale application has not yet been studied. Therefore our work related with the spore production of traditional fungal pathogens on various cereal grains (surface cultivation) in order to investigate the long-term establishment of fungal isolates in the absence of host.

6.2 Objective

To examine the suitable growth substrate for the large scale conidial production of entomopathogenic fungi**.**

6.3 Methodology

6.3.1 Preparation and inoculating of substrates

In vitro production of EPFs was conducted to test the effect of different solid substrates on the growth and sporulation of fungi. Solid substrates of rice, wheat, rye, corn and sorghum were prepared according to Karanja *et al*. (2010) with some modification. 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. Linear growth of the mycelium, number of spores, germination of spores, moisture content of substrate and purity of spores were analysed in order to determine the most suitable medium of production.

6.3.2 Linear growth

Cooled test tubes containing different solid media were inoculated with 1 cm diameter mycelial disc at the center and incubated at room temperature for 60 days. Moisture percent was checked before harvesting. Each treatment was repeated three times. Linear growth of fungal isolates was recorded five days after inoculation.

6.3.3 Spore production

In order to investigate the shelf-life of tested fungal isolates, fungal strains were incubated for 60 days and inoculum production was examined. Approximately 0.05-0.1 g of a fresh sample was weighed and added to 10 ml of 0.1% Tween 80. The solution was shaken vigorously in order to suspend all the conidia in the water by using a vortex shaker. Once all the spores were suspended, dilution series was carried out (10^-1,-2 and -3) and spores were counted using a haemocytometer (INSECT PATHOLOGY MANUAL, Section VII). In some cases, if all the conidia were not suspended in the solution, the entire solution was diluted in a large volume of Tween 80.

6.3.4 Percent moisture (INSECT PATHOLOGY MANUAL, Section VII)

The percent moisture content of the substrate was determined by using the formula:

$$\% \text{ moisture content} = \frac{(W\text{-}B) - (D\text{-}B)}{(W\text{-}B)} \times 100$$

Where:

W= Weight of test tube plus wet spores

D= Weight of test tube plus dry spores

B= Weight of test tube

6.3.5 Germination and purity of conidia

For the germination and purity of spores, a small sample was diluted to 10^6 conidia ml^{-1} in 0.1% Tween 80 and spread on PDA media using a sterile glass spreader, and incubated at room temperature (25±2˚C) for 48 hours and 3-5 days, respectively. This was repeated three times for all isolates. The percentage of viable spores was determined by counting spores. Proportions of germinating and non-germinating spores were taken to determine percent viability. The sample size was 100 conidia per plate. For assessing the biological purity of dry spore, colony forming units (CFU) were checked and counted for contaminating microorganisms. Three replicates were used for each isolate.

6.3.6 *In vitro* pathogenicity

Virulence of conidia products on fruit fly (*Bactrocera* spp.) was tested according to Anand *et al*. (2009) with some modifications. Conidial suspensions were

done as mentioned above. Two to three days old pupae, surface sterilized with 1% (v/v) sodium hypochlorite, were dipped in 200 µl of 10^6 conidia ml^{-1} solution for 2 minutes with gentle shaking. For each treatment 10 pupae were used and experiments were repeated three times. Pupae were transferred to 15 ml bioassay glass vials as described in Anand *et al*. (2009) and incubated at 25˚C and 70% RH. Control pupae were treated with sterilized distilled water containing 0.1% Tween 80. Tested pupae were checked daily for 14 days after incubation.

6.3.7 Statistical analysis

The data (mean number of spores) was analyzed using the SPSS program version 16.0 (SPSS Inc., Chicago, IL) to determine variance. The treatment means were compared using Tukey's HSD Post-hoc test at $P<0.05$. Pupal mortality was adjusted for natural mortality in the control one using Abbott's formula (Abbott, 1925).

6.4 Results

6.4.1 Linear growth of entomopathogenic isolates on grains

There were significant differences ($P<0.001$) in the linear growth of different isolates on the different substrates. Among the solid substrates, corn gave the highest and rye the lowest growth rate for all isolates. *Metarhizium flavoviride, P. lilacinus* and *B. bassiana* (MG03) reached maximum growth rate of 10 cm in corn substrate 30 days after incubation. Rice, sorghum and wheat only supported moderate growth. However, the mycelium of *P. lilacinus* MT02 grew very well in all solid substrates (Fig. 6.1).

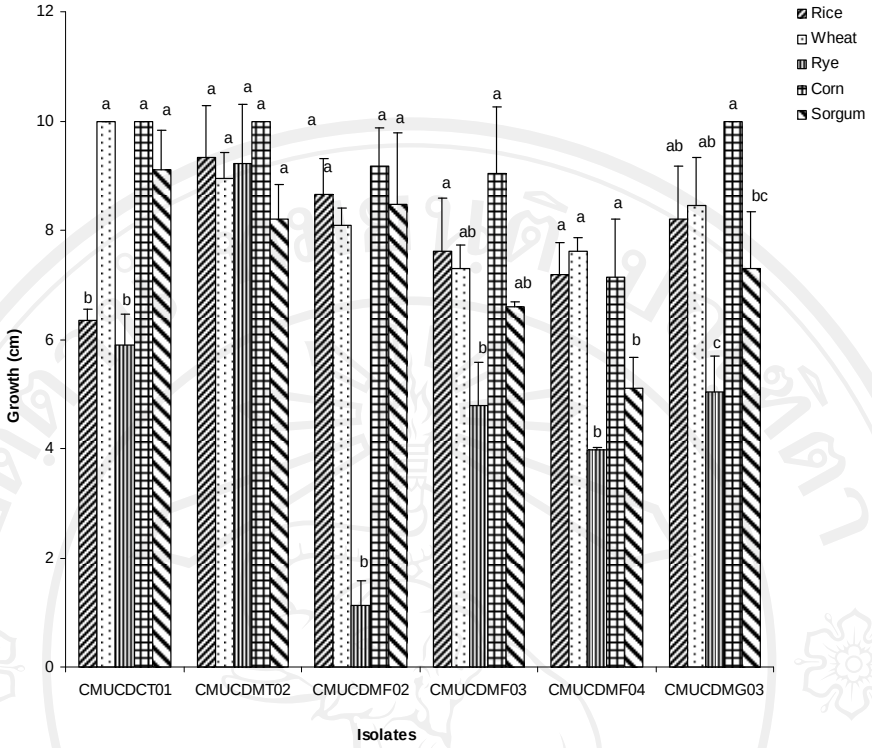

Figure 6.1 Mean linear growth of fungal isolates cultured on five grains at 25 ± 2 ˚C under normal day and night at 30 days after inoculation. The *same letter* above *bars* within a same graph indicates no significant difference according to the Tukey's HSD Post-hoc test at $P < 0.05$. Bars mean standard deviation levels

The daily growth rate of fungal isolates for 30 days incubation were shown in Figure 6.2. All tested fungi grew well in all substrates except *I. tenuipes* MF02 in rye. *Paecilomyces lilacinus* grew well in rice, rye and corn, whereas *M. flavoviride* CT01 grew in wheat, corn and sorghum from the beginning of experiment (Fig. 6.2).

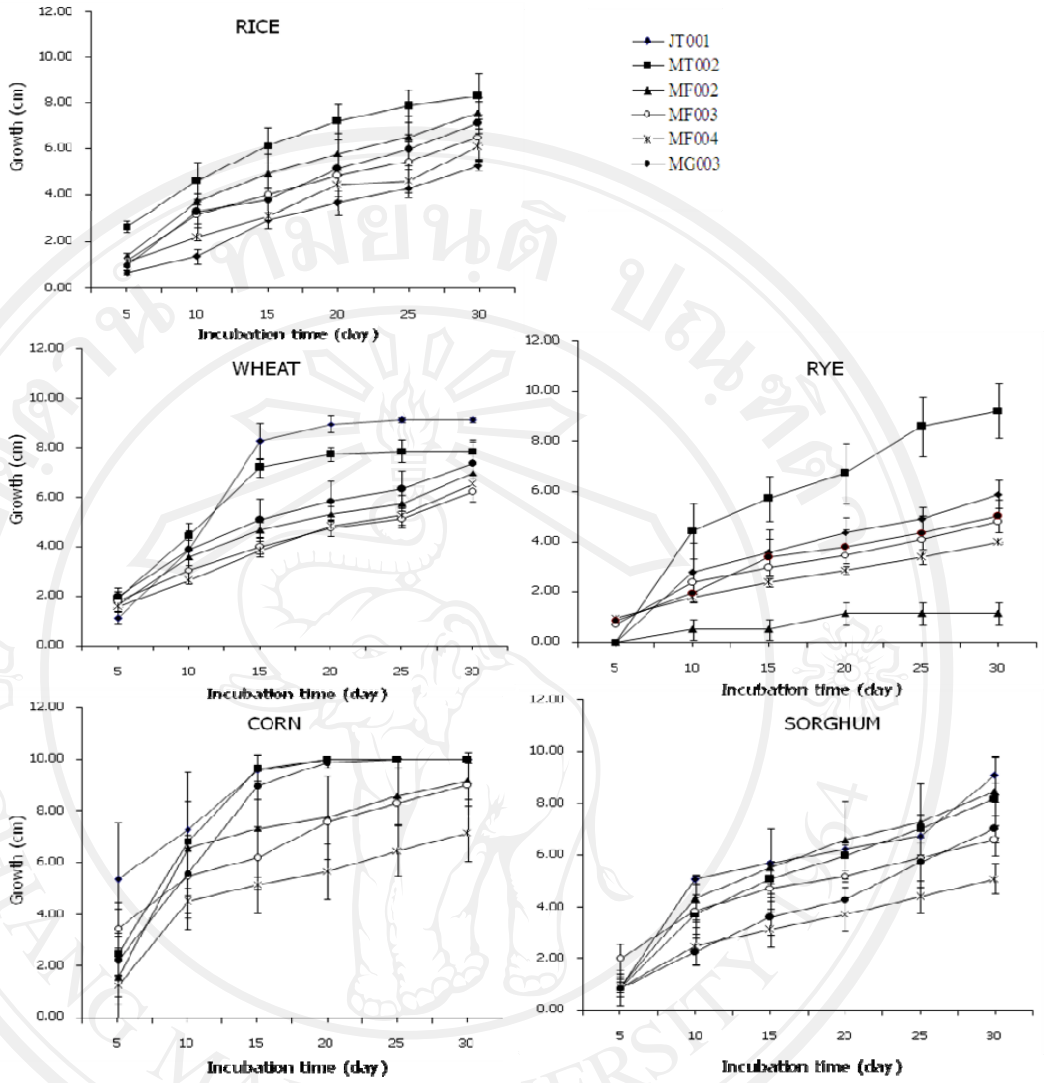

Figure 6.2 Mean daily growth rate (cm) of entomopathogenic fungi on different solid substrates at room temperature (25±2˚C) for 30 days under normal day light periods. Bars represent the standard deviation of three replicated means

6.4.2 Spore production and moisture content of fungal inocula

The number of spores as well as the rate of viability varied between isolates. Spores were counted 60 days after inoculation, when the moisture percent of substrates was 7-10%. The highest number of spores from the grain substrates were

observed when *P. lilacinus* MT02 (530.6±31.6) and *M. flavoviride* CT01 (102.8±11.4) were grown on in sorghum medium whereas the rice substrate yielded the greatest amount of spores for *B. bassiana* (21.8±13.0) MF03 (Table 6.1). The number of spores in the rye substrate was the lowest especially for *I. tenuipes* MF02 (0.5±0.06).

Table 6.1 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]					
	M. flavoviride CT01	*P.lilacinus* MT02	*I. tenuipes* MF02	*B. bassiana* MF03	*M. anisopliae* MF04	*B. bassiana* MG03
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 show the means and standard deviations of three biological replicates. Data with different letters in the same column 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

6.4.3 Germination and purity of fungal inocula

There were no significant differences ($P<0.05$) in the germination of spores of fungal strains (Table 6.2). Germination at 48 hours was over 80% for all isolates and there were no other contaminated microorganisms that had been incubated for 60 days.

Table 6.2 Percent germination of harvested dry spores on PDA, and the virulence activity of spores against fruit fly pupa (*Bactrocera* spp.).

Strains	% germination of fungal isolates on PDA					Virulence of harvested inocula against *Bactrocera* spp. pupa		
	Rice	Wheat	Rye	Corn	Sorghum	% Mortality	LT 50 (days)	LT90 (days)
M. flavoviride (CT01)	85	86	89	94	93	68.81 b	9	14
P. lilacinus (MT02)	87	93	85	94	93	100 a	8	10
I. tenuipes (MF02)	89	91	83	92	91	74.33 ab	11	>14
B. bassiana (MF03)	93	83	85	93	95	95.54 ab	9	11
M. anisopliae (MF04)	82	81	86	91	93	74.33 ab	11	>14
B. bassiana (MG03)	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 test within the same treatment. LT: lethal time.

6.4.4 Virulence of fungal inocula against fruit fly pupa

Percent mortality and lethal times of tested fungi are shown in Table 6.2. In pathogenicity test, all fungal isolates were pathogenic to *Bactrocera* spp., between 68.81 to 100%. The mortality of *Bactrocera* spp. was 100% in *P. lilacinus* MT02 when spores were harvested 60 days after inoculation.

6.5 Discussion

In this study, almost all fungal isolates grew well on all cereal grains incubated for 60 days. Aregger (1992) stated that the culture method of fungi on cereals allows a

simple and fast method for mass production of conidia and sporulating mycelium. Several authors have reported an intermediate carbon/ nitrogen source achieves the best yield in terms of growth of the selected fungi. Humber (2008) stated that the growth characteristics of the vast majority of EPFs are clearly affected by the supply of nutrients. However, in this experiment, no attempts were made to add of any nutrient sources in order to obtain maximum growth and sporulation, but almost all tested entomopathogenic fungi produced large amount of spores.

High spore numbers is one of the main criteria for choosing a fungal pathogen for biological control of pests in the field (Robl *et al*., 2009). El Damir (2006) and Pandey and Kanaujia (2008) found that conidial production of EPFs is affected by the type of growing medium. Silva and Loch (1987) opined that the organism could easily be multiplied on polished rice grains. Boiling the rice grains before sterilization resulted in higher spore yields. We found that sporulation was highest in rice for tested fungal isolates with the exception of *P. lilacinus* and *M. flavoviride.* Moreover, the similar result reported by Ibrahim and Low (1993) that rice produced significant number of spores for the mass culture of *B. bassiana*.

On the other hand, sorghum was the ideal cereal for the mass production of *P. farinosus* (Gopalakrishnan *et al*., 1999). Sporulation was highest when *Nomuraea rileyi* was cultured on crushed sorghum enriched with N- supplement (Vimaladevi, 1994 and Kulkarni, 1999). In our findings, sorghum showed the most effective media for the mass production of *P. lilacinus* and *M. flavoviride* with the significant number of spores.

Sporulation likely occurs upon nitrogen depletion in the presence of carbohydrate (Robl *et al*., 2009). 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. During the present study, because of the relatively high carbohydrate content, sorghum grain produced a significantly higher amount of inoculum than other substrates. On the other hand, the possible reason of reducing the inoculum number in corn substrate assumes the composition and structure of the grains. The same results pointed out by Shah *et al*. (2005) and Mustafa and Kaur (2009) that an increase in radial growth did not result in a simultaneous increase in conidial yield.

The reduction in mycelial growth and inoculum of *I. tenuipes* in rye may be due to the differences between the characteristic of the fungi. Lacey and Neven (2006) reported that substantial growth of *Mascoda albus* on the rye seeds was observed after a 72 h exposure period, and dehydrated rye culture of *M. albus* was adequate for controlling blue mold (*Penicillium expansum)* of apples, when used as a fumigant at 21 ˚C for 24h (Jimenez and Mercier, 2005).

The rate of germination is an important factor in the infection process, with faster germinating conidia generally considered to have a greater chance of causing infection (Charnley, 1989). Likewise, maintenance of a high viability during storage is essential for effectiveness and thus market acceptance of fungus-based biopesticides (Hedgecock *et al*., 1995; Hong *et al*., 2001). It was found that all tested entomopathogenic fungi were capable of maintaining their viability above 80% and longer storage time for 60 days incubation period.

Maximum spore stability of the conidia of commonly employed entomopathogenic fungi requires drying to low moisture content 4-5% (Hedgecock *et*

al., 1995: Hong *et al*., 2001). In our experiment, the substrates were incubated until the lowest percent moisture without additional drying of fungal inocula was achieved. The moisture content of the inocula was 5-7% at the time of spore extraction. On the other hand, all tested entomopathogenic fungi were capable of maintaining viability above 80% after a 60 day incubation period.

The longer the solid substrate is exposed without fungal colonization, the higher the chance of contamination. Thus, a medium which promotes a high concentration of propagules in a shorter period of time must be chosen for inoculation of the solid medium (Mascarin *et al.*, 2010). Fortunately, no traces of contaminating microorganisms were found in our finding when the inocula were stored for 60 days in solid grains. There were no reports for the pathogenic activity of the aerial conidia against fruit fly (*Bactrocera* spp.). Nevertheless, the highest efficacy to cause disease was found in *P. lilacinus* when fruit fly pupae were dipped in spore suspensions at the concentration of 1×10^6 conidia ml^{-1}.