CHAPTER 4

Infectivity of Insect Pathogenic Fungal Isolates to Fruit Fly Pupa, *Bactrocera* spp. (Diptera: Tephritidae)

4.1 Introduction

Biological control agents such as (EPFs) can be used as a component of integrated pest management (IPM) of many insect pests. Under natural conditions, these pathogens are a frequent and often cause natural mortalities of insect populations. The main drivers behind the push for myco-insecticides are the need for more specific agents as components of IPM programmes due to concerns over chemical residues on human health and the environment.

Fruit flies belonging to the family Tephritidae are the world's worst pests of fruits causing enormous economic loss every year (White and Elson-Harris, 1992; Aluja *et al*., 1996; Armstrong and Jang, 1997). The total number of species within this family exceeds 4,000. Approximately 10% of them are serious pests distributed around the world in temperate, subtropical and tropical areas (Christenson and Foote, 1960; Weems *et al*., 1999; Singh, 2003). In Thailand and other South-East Asian (SEA) countries, the genus Bactrocera is known for being one of the major pests of tropical fruits and vegetables (Hardy, 1973; Drew and Romig, 1997). These flies attack different fruit including rose apple, mango, banana, etc (Sukhirun *et al*., 2009). The production of fruit and vegetables in Thailand generate important sources of income. These crops represent an important part of the gastronomic culture for Thai people (Sarango, 2009).

The frequent use of insecticides in controlling fruit flies in fruits and vegetable has not resulted in sustainable management of the pest. The use of insecticides as the only way to control pests in fruit and vegetables causes environmental pollution and hygienic problems that represent a risk for people and animals (Gallo, 2007). In Thailand residues of organophosphate and organochloride and other compounds have been detected in soil, water and crops (Thapinta and Hudak, 1998). Resistance problems due to the overuse of such insecticides and high residues in the sprayed vegetables are some of the concerns that necessitate some form of management. This is a big problem for consumer and also export in agricultural business.

Control manners used in the integrated management system are fruit bagging, protein bait spraying, methyl eugenol trap, parasitoid, predator and sterile male release which affect the fruit fly adults (Shelly, 1995; Vargas *et al.*, 2007). On the other hand, the microbial control of fruit flies can be a process that can partially replace other methods of control in integrated management programs for these insects, especially the use of agrochemicals, presenting economic and environmental advantages for tropical fruit (de Oliveira, 2010). Biopesticides based on EPFs show promising for insect pest management. EPFs offer greater opportunity for biological control of adult fruit flies compared to bacteria and viruses, which must be ingested to be effective.

Furthermore, *Cordyceps* is EPFs that parasitize insects and spiders. *Cordyceps militaris* is an entomopathogenic fungus parasitic on the larvae of Lepidoptera, has long been used as a traditional oriental medicine for eternal youth. Several studies have found the infection of this fungus to larvae and pupae of beech caterpillar *Syntipistis punctatella* Motschulsky (Lepidoptera: Notodontidae) (Liebhold *et al.*, 1996; Sato *et al*., 1997; Kamata *et al*., 1997). In order to achieve the stromata production of this fungus, Harada *et al*. (1995) injected ascospore suspension into *Mamestra brassicae*, and Sato and Shimazu (2002) injected hyphal body suspension into lepidopteran and coleopteran pupae.

Not so much research on the use of native fungal isolates and *Cordyceps* in fruit fly control has been conducted in Thailand. Recently, Aemprapa (2007) studied the effectiveness of 7 isolates of *Metarhizium* sp., 12 isolates of *Beauveria* sp., and 1 isolate of *Hirsutella citriformis*, from the culture collection of the National Center for Genetic Engineering and Biotechnology, against fruit fly was performed in Lampang Agricultural Research and Training Centre, Lampang, Thailand. In nature, fruit fly pupation takes place in the soil, and the control strategies of the pupal stage is one of the appropriate method in maintaining the population of fruit fly to some extent.

Therefore, in order to investigate the pathogenesis activity of insect pathogenic fungal isolates in controlling fruit fly, the experiment was laid down with the following objectives.

4.2 Objectives

4.2.1 To determine the virulence strains of pathogenic fungi to fruit fly (*Bactrocera* spp.).

4.2.2 To compare the entomopathogenic activity between telomorph and anamorph of tested EPFs.

4.2.3 To evaluate the pathogenicity of the effective strains against fruit fly pupa in pot experiments.

4.3 Methodology

4.3.1 Fruit fly collection and handling

The infested fruits with ovipositional scars or marks of larval infestation of tephritid fruit fly were collected from rose apple and star fruit trees. Last instar larvae of average size 7-11 mm in length were collected from the infested fruits and preserved in the plastic bottle containing a layer of dry sand at the bottom (Htar Htar Naing *et al.*, 2008) in order to pupate in the soil (Fig. 4.1). When pupae turn brown, at least 2 days after pupation, were sterilized with 0.5% (v/v) sterilized sodium hypochlorite solution prior to bioassay.

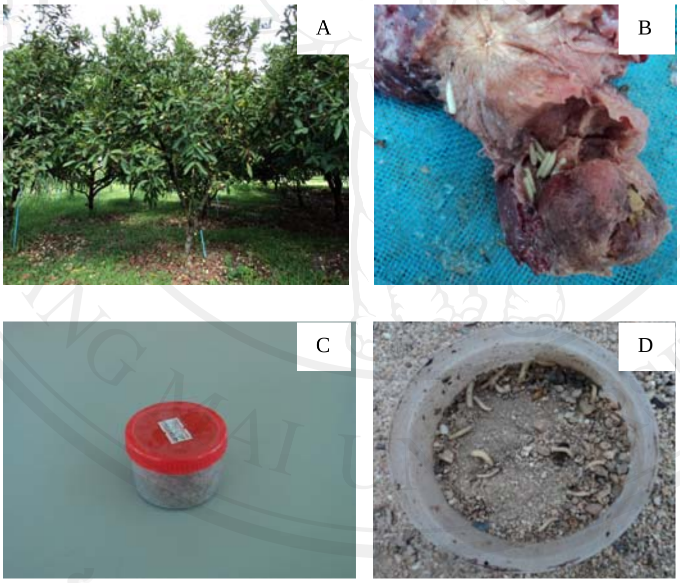

Figure 4.1 Fruit fly collection and rearing. A. rose apple plantation; B. fruit fly larvae infecting rose apply fruit; C. plastic container for collecting last instar larvae; D. last instra larvae in container

4.3.2 Fungal isolates and preparation of conidial suspensions

Totally eight entomopathogenic fungal isolates were used to investigate their pathogenicity against fruit fly pupa. Among them six were collected isolates from naturally infested insects, and *Cordyceps militaris* BCC 17791 and *C. pesudomilitaris* BCC19370 obtained from BIOTEC, Thailand. Fungal strains were cultured on PDA medium and incubated at 25±2˚C.

Conidia were harvested by scraping the surface of 14-days old culture. Spores were suspended in 0.1% Tween-80 solution in glass vials. Conidial suspensions were vortexed to become a homogeneous suspension. Conidia were then quantified with a haemocytometer following serial dilutions (10^1, 2, 3 etc.) in sterile distilled water containing 0.1% Tween-80.

Fungal suspension for two species of *Cordyceps* was cultured in 250-ml Erlenmeyer flasks containing 50 ml potato dextrose broth. Each flask was inoculated with the mycelia discs of fungal isolates and incubated at room temperature (25 ± 2˚C) for 7 days on a reciprocal shaker (125 rpm). The cultured media were filtered through sterilized muslin cloth to remove entangled hyphae. The liquid filtrate was diluted with sterile distilled water containing 0.1% Tween-80 (modified Sato and Shimazu, 2002). The viability of conidia was determined by spread-platting 1ml of 1×10^6 conidia ml^{-1} suspension on PDA (Goettel and Inglis, 1997). Plates were incubated at room temperature (25 ± 2˚C) and germinated spores were counted 100 spores by placing sterilized microscopic cover slip on each plate and replicated three times.

4.3.3 *In vitro* screening of virulence isolates against fruit fly pupa

Laboratory bioassay of collected EPFs was conducted in order to screen out the effective strains against fruit fly, *Bactrocera* spp. *In vitro* screening was done according to Anand *et al.* (2009) with some modification. Two-three days old pupae were surface sterilized with 0.5% (v/v) sodium hypochlorite and dipped for 2 min in conidial suspensions. Four conidial suspensions (1×10^5 conidia ml^{-1} to 1×10^8 conidia ml^{-1}) of six isolates of collected fungal pathogens and fungal mycelial filtrates of two species of *Cordyceps* were used in screening bioassay. For each dilution, 10 pupa ml^{-1} were used. The treated pupae were transferred to 15ml sterilized glass vials containing wetted cotton wool and incubated in incubation chamber (25 ± 2˚C and 70 ± 2% RH). Control pupae were treated with sterile distilled water containing 0.1% Tween-80. Treated pupae were examined for their emergence and mycosis daily for 10 days after inoculation. Mycosis was confirmed by microscopic examination. Each experiment was replicated three times.

4.3.4 Recovery test of fungi from infected pupae

Infected pupae with external fungal growth were transferred to PDA plates for the confirmation of infecting species. The pure cultures were examined under microscope to identify their morphological feature of mycelia and conidial structures.

4.3.5 Soil bioassays

Pathogenic activity of selected virulence strains in controlling *Bactrocrea* pupa was done in pot experiments during February, 2011 to April, 2011. Three selected strains: *M. flavoviride, P. lilacinus* and *B. bassiana* MF03 were used in the soil bioassay experiments. Soil bioassay was done using pot (11×7×7½ cm), and conidial suspensions were applied in two ways as drenching and pre-mixing with soil in controlling fruit fly pupae (Anand *et al*., 2009). Autoclaved soil was used and sterilized pupae were placed below the soil surface. Conidial suspension was prepared to the final volume of 10^8 conidia g^{-1} in both experiments. Pots were kept under the shade net and the temperature during the experimental period was between 26.1±2˚C to 29.1±2˚C. The RH of soil was maintained about 80% throughout the bioassay. Dead pupae were examined under microscope after washing with sterilized distilled water. The experiments were carried out three replicates.

4.3.6 Statistical analysis

Pupal mortality was adjusted for natural mortality in the control using Abbott's formula (Abbott, 1925). Lethal concentration and lethal times were calculated by using probit analysis. The treatment means were compared using Tukey's HSD Post-hoc test at 5% probability using the SPSS program version 16.0 (SPSS Inc., Chicago, IL).

4.4. Results

4.4.1 Virulence of fungal isolates

All tested fungal isolates were pathogenic to fruit fly pupa. Mortality of pupae was assessed by analyzing dead pupae and emergence as well as mortality rates for emerged adult flies. Significant differences in pupal mortality were observed between different isolates with different concentrations. In screening bioassay, *M. flavoviride*, *P. lilacinus* and *B. bassiana* isolate MF03 showed the highest efficacy against *Bactrocera* sp. pupa. Mortality of these isolates caused 81.44% (F= 25.49; d.f. =9, 29; P<0.0001), 85.22% (F= 25.93; d.f. =9, 29; P<0.0001), and 85.22% (F= 17.39; d.f. =9, 29; P<0.0001) at 10^7 conidia ml^{-1} and 100% in each at 10^8 conidia ml^{-1}, respectively.

The control mortality was only 10%. No significant differences in infectivity were found among these three isolates (Table 4.1).

Table 4.1 Percent mortality of dead *Bactrocera* pupae after 10 days exposure of six fungal isolates with various conidial concentrations

Species	Percent mortality of fruit fly pupa 10 days after treatment			
	1×10^5 (conidia ml^{-1})	1×10^6 (conidia ml^{-1})	1×10^7 (conidia ml^{-1})	1×10^8 (conidia ml^{-1})
M. flavoviride (CT01)	25.89c	29.67c	81.44b	100.00a
P. lilacinus (MT02)	22.22b	33.33b	85.22a	100.00a
I. tenuipes (MF02)	18.56c	22.22c	40.78b	59.22a
B. bassiana(MF03)	29.67d	48.11c	85.22b	100.00a
M. anisopliae (MF04)	18.56b	22.22b	44.44ab	66.67a
B. bassiana (MG03)	14.78b	25.89b	40.78ab	63.00a

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

Nevertheless, fruit fly mortality was significantly affected by conidial concentrations ($P<0.01$). The higher the concentration level, the greater the number of dead fruit flies. On the other hand, the virulence of *I. tenuipe* MF02, *M. anisopliae* MF04 and one strain of *B. bassiana* MG03 were found to be the moderate infectivity towards pupae, even at the highest dose of conidia. When two species of *Cordyceps* were investigated for pathogenicity against *Bactrocera* sp., both *C. militaris* (BCC91) and *C. pseudomilitaris* (BCC70) were less effective against fruit fly pupae (Table 4.2).

Table 4.2 Mortality of *Bactrocera* pupae infected by the fungal suspension of two species of *Cordyceps* after 10 days exposure

Species	% mortality	LC_{50} (95%CI) * log(conidia ml^{-1})	LC_{90} (95%CI) log(conidia ml^{-1})	LT_{50} (95%CI) (days)	LT_{90} (95%CI) (days)
C. pseudomilitaris	22.22	13.24	26.89	11.92	19.14
BCC70		(9.13-201.18)	(16.34-552.0)	(10.18-15.70)	(15.45-27.69)
C. militaris	18.56	15.51	31.24	11.77	17.81
BCC91		(0.00-0.00)	(0.00-0.00)	(10.23-14.98)	(14.70-24.75)

Note: * means 95% Confident interval

The rate of pupae mortality increased with increasing dosage up to 10^8 conidia ml^{-1}. *Paecilomyces lilacinus* achieved the shortest time to attain 50 % mortality (LT_{50}=3.54 days), whereas the time for isolate MF03 was 3.71 days and 4.15 days for *M. flavoviride.* In case of *Cordyceps* sp., the lethal dose and time to kill 50% of fruit fly might need higher concentration and longer time (Table 4.2).

Fruit fly pupae were significantly sensitive to all collected isolates after 3 days at a concentration of 10^8 conidia ml^{-1}. The LD_{50} value of isolate MF03 was lower than the others but this was not significantly different from MT02 and CT01 (Table 4.3). The time taken for the isolates to kill 50% of fruit fly pupae is shown in Table 4.3.

The entomopathogenic activity of tested isolates was confirmed by the presence of fungal hyphae on the bodies of dead flies (Fig. 4.2). Percent mycosis in dead flies was significant among isolates. *Paecilomyces lilacinus*, *M. flavoviride* and *B. bassiana* MF03 showed the highest mycosis in dead pupa after 5 days. The appearance of mycosis on dead pupae was shown first in isolate MT02 followed by CT01 and MF03. Though isolate MG03 showed less virulent infectivity to fruit fly pupa, the colonization of fungal mycosis reached the highest in 5 days treated. The least amount of mycosis was observed in *M. anisopliae* even at 10^8 conidia ml^{-1}, and the appearance was delayed when compared with others. Emerged adults from pupae of treated isolates showed mycosis on the surface in all treatments.

Table 4.3 Lethal concentrations and lethal times of each tested isolates infected fruit fly, *Bactrocera* spp. for 10 days at a concentration 10^8 conidia ml^{-1}

Isolates	LC_{50} (95%CI)* log(conidia ml^{-1})	LC_{90} (95%CI) log(conidia ml^{-1})	LT_{50} (95%CI) (days)	LT_{90} (95%CI) (days)
M. flavoviride (CT01)	5.15(3.94-6.08)	8.78(7.55-11.49)	4.15(3.83-4.46)	5.87(5.46-6.44)
P. lilacinus (MT02)	4.96(3.74-5.88)	8.54(7.36-11.03)	3.54(3.22-3.84)	5.15(4.76-5.71)
I. tenuipes (MF02)	7.47(6.35-9.56)	13.95(11.17-21.55)	7.60(7.08-8.21)	11.41(10.44-12.85)
B. bassiana(MF03)	4.72(3.90-5.37)	8.21(7.37-9.58)	3.71(3.37-4.03)	5.53(5.11-6.11)
M. anisopliae (MF04)	7.12(6.13-8.70)	12.88(10.59-18.55)	7.14(6.65-7.68)	10.76(9.91-11.99)
B. bassiana (MG03)	7.28(6.24-9.09)	13.39(10.87-19.92)	7.04(6.46-7.72)	11.64(10.50-13.37)

Note: * means 95% Confident interval

However in two isolates of *Cordyceps* sp., though these two isolates infected the fruit fly (*Bactrocera* spp.), fungal mycosis was not observed on the surface of dead cadavers. Daily mortality rate is shown in Fig. 4.3.

For the confirmation and identification of infected isolates, fungal mycosis on the surface of dead pupae was transferred to agar plates (PDA). Pure cultures of each isolate showed typical characteristics of mycelia and conidia of tested fungi used in the bioassay.

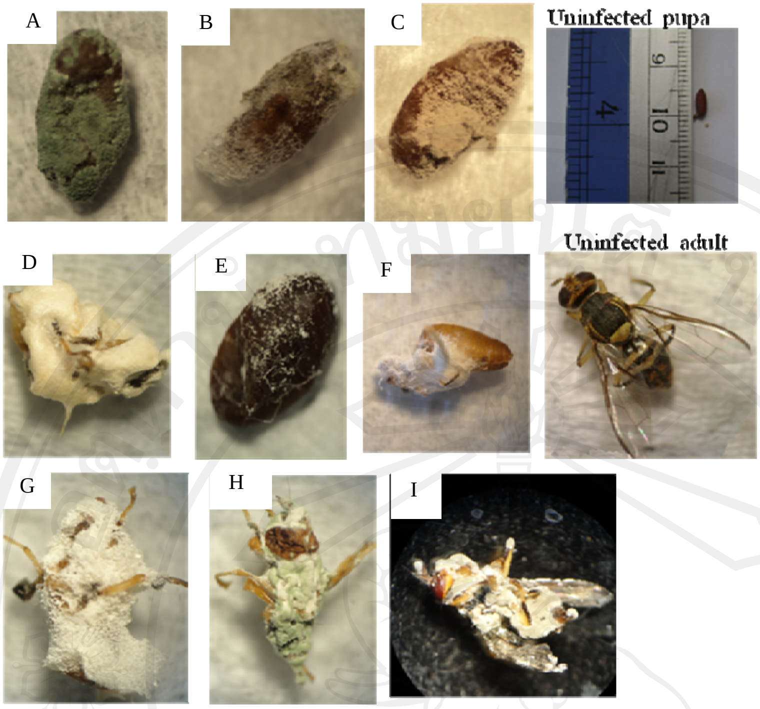

Figure 4.2 Fungal mycosis of tested entomopathogenic isolates on infected fruit fly (*Bactrocera* spp.) pupae and adult. A. *Metarhizium flavoviride*; B. *M. anisopliae*; C. *Paecililacinus lilacinus*; D. *Beauveria bassiana* MF03; E. *B. bassiana* MG03; F. *Isaria tenuipe*; G. *B. bassiana* MF03 (adult); H. *M. flavoviride* (adult); I: *B. bassiana* MG03 (adult)

4.4.2 Pathogenic activity of fungal isolates in soil bioassay

The entomopathogenic activity of selected three isolates showed no significant difference in both drenching and premixing of conidial suspension with soil. However, pupal mortality was observed first in the premixing with soil treatment (Figure 4.4). In addition, external formation of mycelium was observed on *B. bassiana,* MF03, when pupae were treated with the premixed conidial solution with soil.

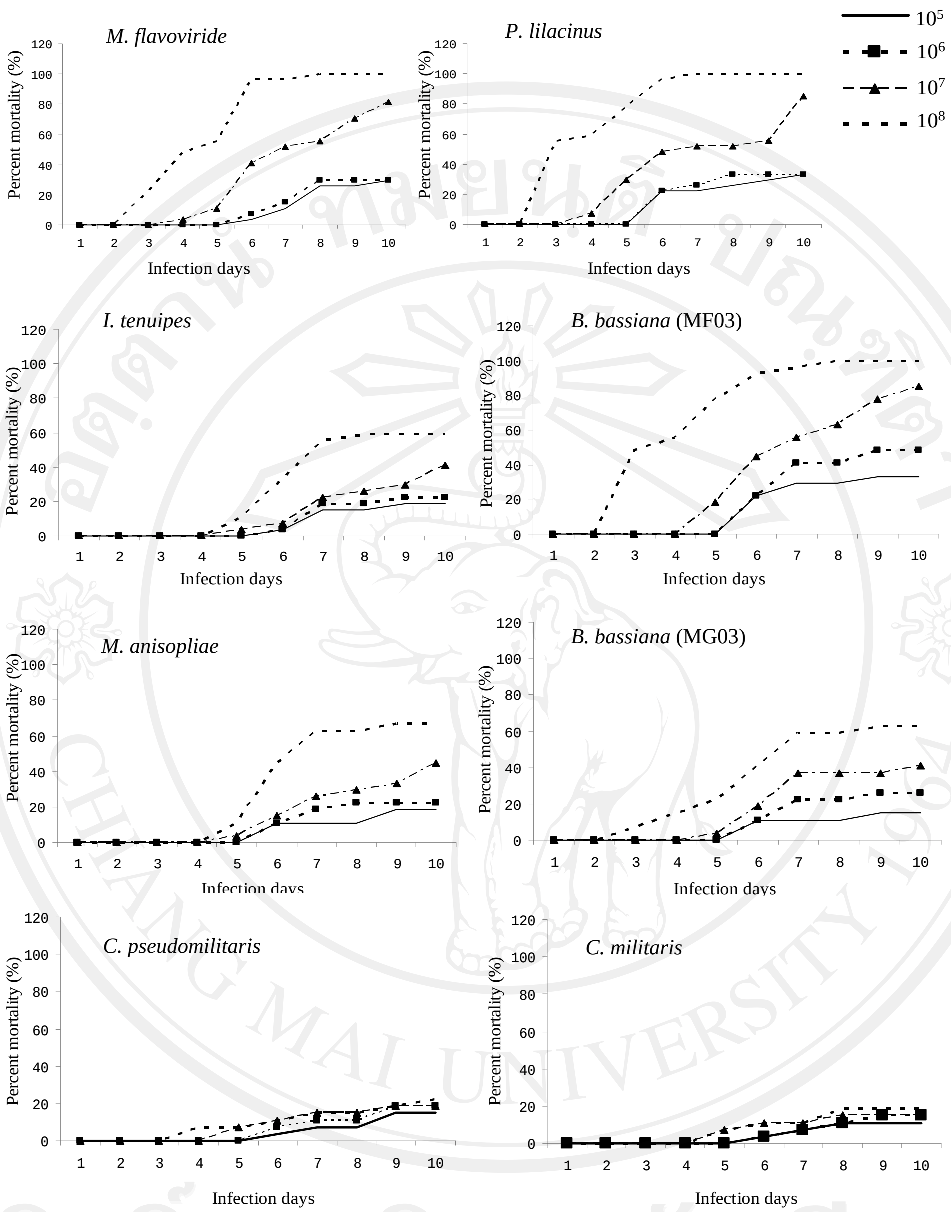

Figure 4.3 Daily mortality percentage of fruit fly (*Bactrocera* spp.) infecting different fungal isolates with different conidial concentration incubated for 10 days at 25˚C

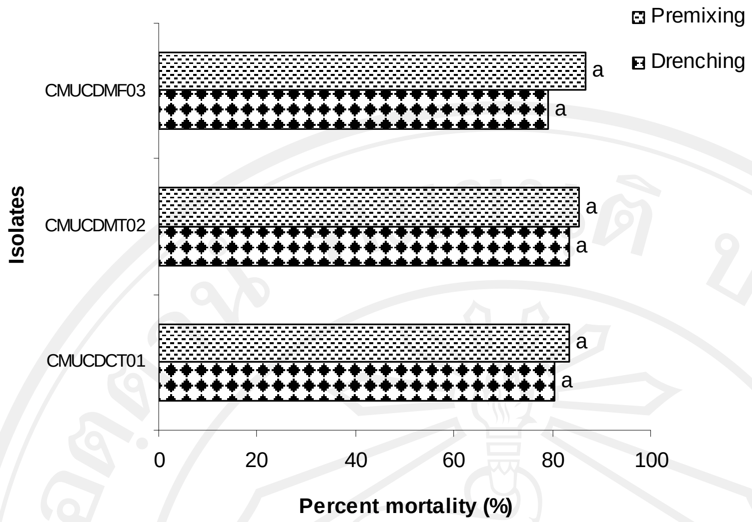

Figure 4.4 Mean percent mortality of fruit fly in different soil bioassay at a concentration of 10^8 conidia ml^{-1} after 14 days treated. Data with same letters within a same graph indicates no significant difference according to Tukey's HSD Post-hoc test at $P < 0.05$

4.5 Discussion

In this experiment, fruit fly pupae (*Bactrocera* spp.) were susceptible to all tested fungal isolates. This result confirms the previous findings of the entomopathogenic activity of fungal isolates on fruit fly species (Garcia *et al*. 1984; Castillo *et al*. 2000; Lezama-Cutierrez *et al*. 2000; de la Rosa *et al*. 2002; Ekesi *et al*. 2002; Dimbi *et al*. 2003; Uziel *et al*. 2003; Mochi *et al*. 2006; Quesada-Moraga *et al*. 2006; Sookar *et al*. 2008). However, pathogenic activity of isolates differed significantly between each other. In screening bioassays, the mortality of fruit fly pupae varied considerably between isolates with different conidial concentrations. These findings of pupal mortality verify a dose- dependent pattern. This concurs with Angel-Sahagun *et al*. (2005), that the infection of *H. irritans* by three *M. anisopliae, I.*

fumusorosea isolates and one *B. bassiana* isolate was found at the dose of 10^8 conidia ml^{-1}.

In the present study, the impact of increasing conidial concentrations on mortality and emergence of adults was clear in all measured isolates. These results are consistent with those of Bernardine *et al*. (2006) who reported that the adult emergence of *M. domestica* larvae reduced as concentration increased. On the other hand, the pathogenic activity of seven isolates of *M. anisopliae,* five isolates of *B. bassiana* and two isolates of *P. fumosoroseus* against adult *B. zonata* and *B. cucurbitae* was demonstrated at a conidial concentration of 1×10^6 conidia ml^{-1}(Sookar *et al*., 2008).

Among eight tested entomopathogenic fungi, the most virulent strains observed were *M. flavoviride*, *P. lilacinus* and *B. bassiana* (MF03) in laboratory bioassay. These three strains infected fruit fly pupa to kill 50% after 4.15, 3.54 and 3.71 days of exposure to conidia, respectively. Geden *et al.* (1995) reported that *B. bassiana* killed adult house flies within five days, while Watson *et al*. (1995) mentioned the mortality of *Musca domestica* occurred within seven days. However, Mwamburi *et al*. (2010) stated that adult house fly mortality was observed within six days for *B. bassiana* isolates. In their studies, none of the isolates of *P. lilacinus* were pathogenic to these adult house flies, in contrast to our results with *Bactrocera* spp. in Thailand. According to the minimum lethal concentration (LD_{50}), *B. bassiana* (MF03) was found to be highly pathogenic to fruit fly pupa followed by *P. lilacinus* and *M. flavoviride*. This is in agreement with the findings of Aemprapa (2007) who reported that *Beauveria* isolate 6241 killed 50% of *B. dorsalis* pupa.

The least virulent isolates were *C. militaris* (BCC91) and *C. pseudomilitaris* (BCC70). The delay in mycosis, or lack, for cadavers of several insect species inoculated with pathogenic fungi has been reported, despite having been killed by the fungus (Shimazu, 1994; Shimazu *et al*., 2002). In the finding of Sato *et al*. (1997), when larva and pupa of *Quadricalcarifera punctatella* (Motschulsky) were infested by *C. militaris* in three beech forest of northern Japan, the number of fruiting body was highest in site A whereas no fruiting body was observed in site B. In our finding, there was no fungal mycosis present in dead fruit fly when treated with *C. militaris* and *C. pseudomilitaris*. There reason for no mycosis found in dead pupae infected by two species of *Cordyceps* may be due to the infection of other organisms, such as bacteria. Moreover, for the appearance of stromata from dead pupae of *M. brassicae,* it took 80 days after dipping in *C. militaris* ascospores (Harada *et al*., 1995). Therefore, the application method may influence the infection and colonization of fungal pathogens on hosts. In the dipping method, the ascospores require the following stages for infection: germination, penetration to the cuticle and production of hyphal bodies in the haemocoel (Sato and Shimazu, 2002). In case of Ha *et al*. (2005) using ascospore of *C. militaris* to silkworm larvae was quite poor in the incidence of infection.

Virulent isolates were fast-growing with compact and dense mycelium, producing a high yield of conidia on the surface of the culture. These characteristics lead to the highest infective activity against hosts.