Insecticidal Activity and Cuticle Degrading Enzymes of Entomopathogenic Fungi Against *Plutella xylostella* (Lepidoptera: Plutellidae)

Naradorn Chui-Chai[1,2], Patcharin Krutmuang[1,2], Sarunya Nalumpang[1,2], Supamit Mekchay[2,3], Chartchai Khanongnuch[4] and Yaowaluk Chanbang[1*]

[1]*Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand*

[2]*Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand*

[3]*Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand*

[4]*Division of Biotechnology, School of Agro-industry, Faculty of Agro-industry, Chiang Mai University, Chiang Mai 50100, Thailand*

**Corresponding author. E-mail: yaowaluk.c@cmu.ac.th*

ABSTRACT

Fourteen isolates of Beauveria spp. and eleven isolates of Metarhizium spp., entomopathogenic fungi, were selected to screen for entomopathogenic efficiency to control 2nd instar larvae of diamondback moth, Plutella xylostella. The median lethal times (LT_{50}) were estimated at 10^8 conidia ml^{-1} concentration and revealed to be distinguishable into two groups. A first group comprised the 5 higher efficacy isolates (BCC4810, BCC4849, BCC17599, Bb.5335 and Bb.2637) with a LT_{50} ranging from ca. 25 to 35 h, while the second group comprised the 5 lower efficacy isolates (BCC1707, BCC1858, BCC12636, BCC14841 and BCC22353) with a LT_{50} ranging from ca. 73 to 145 h. The highest efficacy of Beauveria sp. and Metarhizium sp. were Bb.5335 and BCC4849 with the 96-h lethal concentration (LC_{50}) of 2.66×10^6 and 3.11×10^5 conidia ml^{-1}, respectively. All isolates were preliminary investigated for chitinase production on 15% (w/v) colloidal chitin agar. Eight of ten isolates were positive and the highest clear zone was detected in BCC17599, while 2 isolates, BCC14841 and Bb.2637, were negative. In addition chitinase production in liquid culture showed that Bb.5335 was the greatest chitinase activity at the 11-day cultivation. Further study revealed that all 8 positive isolates produced protease since 1 day of cultivation and chitinase activity tended to rise up after the highest protease activity. Moreover, chitinase and protease activities were associated with the insecticidal activities. Metarhizium isolates showed higher insecticidal efficacy and hydrolytic enzyme activities than in Beauveria isolates. We suggested that the insecticidal effects of entomopathogenic fungi could be associated with cuticle hydrolytic enzyme activity.

Key words: Entomopathogenic fungi, *Plutella xylostella,* Cuticle degrading enzymes

INTRODUCTION

Diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae), is known well as an important crucifer insect pest. Recently in Thailand, the increased frequency of outbreaks has caused severe cruciferous vegetables loss annually (Shelton, 2004) likely due to global climate changes. The consequence of this phenomenon affects directly agricultural practices, and prevention of pest attacks. Therefore, pesticide treatments have been increased to suppress DBM attacks in recent years (Vanit-Anunchai, 2006; Tirado et al., 2008). In Thailand farmers also raised pesticide treatments to DBM, causing an increased level of insect resistance and environmental effects. In addition the natural enemies would be affected as well (Rushtapakornchai and Vattanatangum, 1986;

Jungbluth, 1996; Pedigo and Rice, 2006). Talekar and Shelton (1993) reported that many alternative approaches can be adopted to control DBM populations. The most prominent among these is the utilization of entomopathogenic fungi which may have several advantages such as no harm to non-target species, wide host range, ability to self-replicate, compatibility with other control approaches and safety (Gullan and Cranston, 2005). Furthermore, most of entomopathogenic fungi produce hydrolytic enzymes during breaching through the insect cuticle, which is composed mainly of chitin and it is hardly degraded. In particular, chitinase and protease are important enzymes involved in the infection process of entomopathogenic fungi (St. Leger et al., 1996). This research aimed to study the infestation process and the infestation efficacy of entomopathogenic fungi to control the larvae of diamondback moth and some mechanisms involved in larval mortality.

MATERIALS AND METHODS

Entomopathogenic fungi cultures

The fungal isolates (Table 1) were obtained from National Center for Genetic Engineering and Biotechnology (BIOTECH) of Pathum Thani, Thailand for BCC1399, BCC1701, BCC1707, BCC1858, BCC4810, BCC4849 BCC5797, BCC12636, BCC14532, BCC14841, BCC16041, BCC17599, BCC18058, BCC18059, BCC19012, BCC22353, BCC22355 and BCC25950 and from Lampang Agricultural Research and Training Center, Rajamangala University of Technology Lanna Lampang, Thailand for Bb.4591, Bb.5335, Bb.6241, Bb.2637, B.7683, M.6079 and M.7965. All isolates were grown on Potato Dextrose Agar (PDA) and Malt Agar (MA) at appropriate temperature (25 and 30°C).

Table 1. List of entomopathogenic fungi isolated from Thailand.

Isolate	Species	Original host	Sampling location
BCC14532	*Beauveria bassiana*	Coleoptera-adult	Northern Thailand
BCC14841	*Beauveria bassiana*	Unknown	Northern Thailand
BCC16041	*Beauveria bassiana*	Coleoptera-adult	Northern Thailand
BCC17599	*Beauveria* sp.	Homoptera-cicada adult	Phetchaburi
BCC18058	*Beauveria bassiana*	Unknown	Unknown
BCC18059	*Beauveria bassiana*	Unknown	Unknown
BCC19012	*Beauveria bassiana*	Coleoptera	North Eastern Thailand
BCC22355	*Beauveria bassiana*	Homoptera-hopper	Northern Thailand
BCC25950	*Beauveria bassiana*	Insecta-adult	North Eastern Thailand
Bb.4591	*Beauveria bassiana*	Coleoptera-Curculionidae	Chanthaburi
Bb.5335	*Beauveria bassiana*	Hymenoptera-ant	Phetchaburi
Bb.6241	*Beauveria bassiana*	Unknown	Unknown
Bb.2637	*Beauveria bassiana*	Unknown	Unknown
B.7683	*Beauveria* sp.	Unknown	Tak
BCC1399	*Metarhizium flavoviride*	Homoptera-hopper	Southern Thailand
BCC1701	*Metarhizium anisopliae*	Homoptera-hopper	Southern Thailand
BCC1707	*Metarhizium flavoviride*	Hemiptera-nymph	Central Thailand
BCC1858	*Metarhizium anisopliae*	Coleoptera-Lampyridae	Phetchaburi
BCC4810	*Metarhizium anisopliae*	Treehole materials	Thailand
BCC4849	*Metarhizium anisopliae*	Treehole materials	Thailand
BCC5797	*Metarhizium anisopliae*	Unknown	Unknown
BCC12636	*Metarhizium anisopliae*	Coleoptera-adult Elaterdae	Unknown
BCC22353	*Metarhizium anisopliae*	Orthoptera-cricket	Central Thailand
M.6079	*Metarhizium* sp.	Homoptera	Ranong
M.7965	*Metarhizium* sp.	Unknown	Nakhon Ratchasima

Insects rearing

Plutella xylostella was introduced from crucifer farmers' field at Muang, Mae Rim and San Sai districts, Chiang Mai, Thailand. The larvae were reared under laboratory conditions in 19×30×10 cm transparent plastic boxes and provided with fresh Chinese kale leaves every 1-2 days as food for development to adult. Adults were transferred into the new transparent plastic boxes of the same size, where fresh Chinese kale plants were provided for egg laying on leaves and supplied with a small cotton piece soaked in 25% (v/v) honey solution for adults feeding. The eggs laid on Chinese kale leaves were allowed to hatch and larvae were able to develop on leaves. New fresh leaves were served everyday for larval growth until larvae reached to the second instar for bioassays.

Insect bioassays

The fourteen isolates of *Beauveria* spp. and the eleven isolates of *Metarhizium* spp. were tested onto larvae of *P. xylostella* with the fungal conidia suspension. Conidia were harvested from the surface of 15 days old fungal cultures by spreading on the surface of the mycelium 3-5 ml of sterile 0.1% (v/v) Tween80 solution. Conidia were collected by gently scraping the surface of the colonies. The conidia suspension was determined by using a Neubauer haemacytometer and adjusted to a concentration of 1×10^8 conidia ml^{-1} for use as inoculation. The treatments composed of 25 fungal isolates and the experimental design was completely randomized design with 3 replications. In each 9 cm Petri dish ten second-instar larvae on sterile filter paper were directly sprayed with 1 ml of conidia suspension using a sprayer. The control was sprayed with 1 ml of sterile 0.1% (v/v) Tween80 solution. After post-inoculation, larvae were fed with a Chinese kale leaf (approximately 6×7 cm) which was initially disinfected with 0.5% (v/v) sodium hypochlorite solution followed washing twice with sterile distilled water and air dried. Every 48 hrs, Chinese kale leaves were changed as needed. Mortality was examined at 24-h intervals after exposure for 7 days. The data was corrected by Abbott's formula (Abbott, 1925) when the mortality of *P. xylostella* larvae occurred in the control and was subjected to probit analysis for calculation of LT_{50} and LC_{50} (Finney, 1971). The mortality data of 25 isolates were analyzed for median lethal times (LT_{50}). Median lethal concentrations (LC_{50}) were calculated for the five isolates associated with the highest LT_{50} and the five lowest LT_{50} considering together both *Beauveria* and *Metarhizium* isolates. LC_{50} were calculated at 96 h and concentrations of 1×10^5 - 1×10^9 conidia ml^{-1}.

Chitinase assay

Preparation of colloidal chitin Ten grams of chitin powder from crab shells (practical grade, Sigma) was dissolved with 100 ml of 85% phosphoric acid and stored in cold place overnight at 4°C. The viscous chitin solution was then washed with tap water several times until a final pH value of 7. Colloidal chitin slurry was collected with filtering through a thin layer of white cloth sheet for using in enzymatic assays.

Isolation and screening The ten isolates which associated with the highest and lowest activity from previous assay were grown on PDA media and incubated at 30 and 25°C for 5 days. Subsequently, fungal colonies were transferred onto chitin agar selective medium comprising of colloidal chitin 150 g l^{-1}, NaCl 0.250 g l^{-1}, KH_2PO_4 0.375 g l^{-1}, $MgSO_4\cdot7H_2O$ 0.125 g l^{-1}, $(NH_4)_2C_6H_6O_7$ 0.625 g l^{-1}, $CaCO_3$ 0.375 g l^{-1}, glycerol 87% 6.5 ml l^{-1} and agar 15 g l^{-1} and incubated at 30°C for 7 days. The experimental design was completely randomized design with 3 replications. The chitinase activity was determined based on ability to hydrolyzed chitin. The diameter of fungal clear zone and colony size were measured in millimeters (Maketon and Orosz-Coghlan, 2008; Sridevi and Mallaiah, 2008).

Colorimetric measurement The chitinase-producing-fungal isolates were grown on PDA media for 7 days and inoculated into 75 ml of enzyme producing medium (EPM), pH 5.0 comprising of colloidal chitin 20 g l^{-1}, NaCl 0.250 g l^{-1}, KH_2PO_4 0.375 g l^{-1}, $MgSO_4\cdot7H_2O$ 0.125 g l^{-1}, $(NH_4)_2C_6H_6O_7$ 0.625 g l^{-1}, $CaCO_3$ 0.375 g l^{-1} and glycerol 87% 6.5 ml. The cultures were incubated on a rotary shaker (200 rpm) at 30°C for 25 days. Samples were collected at 2-d intervals

and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatants were assayed immediately for chitinase activity. A 125 µl of supernatant was mixed with 125 µl of 5% (w/v) colloidal chitin as the substrate enzyme, and 125 µl of supernatant was mixed with 125 µl of 0.1M acetate buffer, pH 5.0 to act as enzyme control and 125 µl of 5% (w/v) colloidal chitin was mixed with 125 µl of 0.1M acetate buffer, pH 5.0 for act as substrate control. The mixtures were incubated at 37°C for 20 minutes after that the reaction was stopped by adding 250 µl of 1% (w/v) dinitrosalicylic acid solution (DNS) followed by boiling for 10 minutes. After cooled down, 5 ml of distilled water was added to the reaction mixture. Then, the color development was measured for the absorbance at the wavelength of 540 nm (Genesys 10UV) (Miller, 1959). One unit (IU) corresponds to the amount of enzyme that hydrolyzes 1 µmole of N-acetylglucosamine per minute at 37°C.

Protease assay

Preparation of casein substrate Two grams of casein powder from bovine milk (Sigma) was dissolved with 100 ml of 0.1 M acetate buffer, pH 5.0 and then put in microwave at 600 watts for 3-4 times. After the casein powder formed gum, the mixtures were then stirred and heated for 30 minutes. Only the liquid part of mixtures was used as substrate.

Colorimetric measurement Similar samples as in the previous enzyme assay which collected from EPM were used as the crude enzyme sources and determined as follows slightly modified caseinolytic method (Sundararajan et al., 2010). A 100 µl of supernatant was mixed with 400 µl of 2% (w/v) casein to act as substrate enzyme, 100 µl of supernatant was mixed with 400 µl of 0.1 M acetate buffer, pH 5.0 to act as enzyme control and 400 µl of 2% (w/v) casein was mixed with 100 µl of 0.1 M acetate buffer, pH 5.0 to act as substrate control. The mixtures consisting of 500 µl were incubated at 37°C for 30 minutes and stopped reaction with 10% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 12,000 rpm for 10 minutes. A 700 µl of supernatant mixture was collected and transferred to new tube in which 1 ml of 0.4 M $NaCO_3$ was added. The mixtures consisting of 1.7 ml were reacted with 100 µl of 0.5 N Folin Ciocalteu's phenol reagent and incubated at 37°C for 30 minutes. The color development was measured for the absorbance at the wavelength of 660 nm (Genesys 10UV). One unit (IU) corresponds to the amount of enzyme that hydrolyzes 1 µmole of tyrosine per minute at 37°C.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 for windows (SPSS Inc., 2007). The variable of insect bioassays and enzyme activities from separated flasks were analyzed using one-way ANOVA. Means of 3 replicates in bioassay tests were compared by Duncan's Multiple Range Test and preliminary chitinase activity means displayed with standard error were compared using Tukey HSD. The correlation studies were showed as R value from subjecting to linear regression.

RESULTS

Insect bioassays

Second instar larvae of *P. xylostella* were exposed to twenty five isolates of entomopathogenic fungi, *Metarhizium* spp., and *Beauveria* spp., and the results showed that the median lethal times (LT_{50}) of *Beauveria* spp. ranged from 32.29 to 73.26 h while *Metarhizium* spp. ranged from 26.28 to 144.74 h (Table 2). Moreover, the 25 isolates of entomopathogenic fungi were distinguished into 2 groups by statistic of LT_{50} values as five higher efficacy isolates (H) including BCC4810, BCC4849, BCC17599, Bb.5335 and Bb.2637 with LT_{50} ranged from 26.28 to 34.98 h, while five lower efficacy isolates (L) were BCC1707, BCC1858, BCC12636, BCC14841 and BCC22353 ranged from 73.26 to 144.74 h (Table 2). The greatest efficacy of *B. bassiana* (Bb.5335) and *M. anisopliae* (BCC4849) were confirmed with the LC_{50} value at 96-h and showed at 2.66×10^6 conidia ml^{-1} with 95% confidence limits of 8.15×10^5-7.2×10^6 and 3.11×10^5 conidia ml^{-1} with 95% confidence limits of 9.97×10^4-7×10^5, respectively. The lowest efficacy of each fungal genus (BCC14841 and BCC22353)

also were confirmed as 9.44×10^6 conidia ml^{-1} with 95% confidence limits of 3.26×10^6-2.71×10^7 and 2.88×10^8 conidia ml^{-1} with 95% confidence limits of 5.1×10^7-1×10^{10} respectively. The LT_{50} values were associated with the LC_{50} values of *Beauveria* and *Metarhizium* isolates which performed high efficacy of insecticidal activity in the isolates with low LT_{50} values.

Table 2. Effects of entomopathogenic fungi against the 2nd instar larvae of diamondback moth under laboratory conditions at 25.2±2.4°C and room humidity (60-70 %RH).

Isolate	LT_{50} (hours)±SE	95% confidence limits
BCC1399	41.03±11.02^{ab1}	19.65-60.30
BCC1701	52.05±4.02^{ab}	28.42-71.15
BCC1707	112.63±25.62^{cde}	87.65-176.87
BCC1858	86.69±3.97^{bcd}	70.70-101.05
BCC4810	31.04±0.87^{a}	19.65-41.43
BCC4849	26.28±2.28^{a}	7.09-40.25
BCC5797	62.80±16.36^{abc}	37.93-94.21
BCC12636	117.33±14.18^{de}	89.11-185.28
BCC14532	57.31±14.66^{ab}	37.89-73.83
BCC14841	73.26±14.72^{abcd}	54.98-91.32
BCC16041	56.95±2.57^{ab}	42.07-69.82
BCC17599	34.98±2.31^{ab}	17.80-48.60
BCC18058	41.20±8.47^{ab}	13.01-63.55
BCC18059	49.50±4.41^{ab}	32.91-63.51
BCC19012	49.94±18.91^{ab}	36.12-61.71
BCC22353	144.74±9.33^{abc}	55.66-240.70
BCC22355	62.46±8.31^{e}	42.50-79.76
BCC25950	38.21±3.32^{ab}	26.27-49.51
Bb.2637	33.18±8.63^{ab}	11.73-49.58
Bb.4591	64.15±23.42^{abc}	27.62-56.03
Bb.5335	32.29±6.76^{ab}	16.43-45.02
Bb.6241	38.24±6.34^{ab}	24.47-50.19
M.6079	71.00±1.57^{abcd}	55.48-84.66
B.7683	47.59±19.62^{ab}	31.15-60.61
M.7965	47.79±7.09^{ab}	33.31-60.74

[1]Means within columns followed by same letter were not significantly different (P<0.01)

Enzyme assays

Preliminary investigation of chitinase activity on 15% (w/v) colloidal chitin agar revealed that these isolates could degrade chitin substrate. BCC1707, BCC1858, BCC4810, BCC4849, BCC12636, BCC17599, BCC22353 and Bb.5335 showed positive results in forming clear zones, while BCC14841 and Bb.2637 were negative. BCC17599 revealed the highest ratio (diameter of fungal clear zone / colony size) but not significantly greater than BCC1858, BCC4849 and BCC4810. As we observed, chitin activities were found at the first day from the isolates which associated with high insecticidal efficacy. However, those chitin activities did not perform consistently (Table 3).

Table 3. Measuring diameters of clear zone and colony size on 15% colloidal chitin agar for 7 days at 30°C.

Day / Isolate	Ratio (mean±SE)[1]						
	1	2	3	4	5	6	7
BCC1707	0±0.00a[2]	1.30±0.02[b]	1.22±0.06b[c]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]
BCC1858	1.30±0.10[bc]	1.22±0.04[b]	1.23±0.01[c]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]
BCC22353	0±0.00[a]	1.44±0.10[b]	1.11±0.00[b]	0±0.00[a]	1.26±0.03[c]	1.08±0.01[b]	1.00±0.00[b]
BCC4849	1.36±0.12[bc]	1.14±0.00[b]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]
BCC12636	0±0.00[a]	0±0.00[a]	0±0.00[a]	1.11±0.06[b]	0±0.00[a]	0±0.00[a]	0±0.00[a]
BCC4810	1.48±0.03[c]	1.15±0.03[b]	1.34±0.01[c]	1.28±0.01[c]	1.05±0.00[b]	1.07±0.03[b]	1.02±0.02[b]
BCC14841	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]
Bb.2637	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]
Bb.5335	0.4 ±0.47[ab]	1.39±0.08[b]	1.25±0.24[c]	1.24±0.03[c]	1.26±0.06[c]	1.13±0.03[b]	1.23±0.03[c]
BCC17599	1.63±0.03[c]	1.47±0.13[b]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]	0±0.00[a]

[1]Ratio between fungal clear zone and colony size

[2]Means within columns followed by same letter were not significantly different (P<0.01)

Determining the enzyme activities in liquid culture showed that chitinase activity of H group ranged from 6.58 to 181.05 mUnit ml^{-1} and Bb.5335, which produced the highest chitinase activity, reached to 181.05 mUnit ml^{-1} at day 11. While L group ranged from 4.39 to 68.58 mUnit ml^{-1} and BCC1858, which produced the highest chitinase activity, was 68.58 mUnit ml^{-1} at day 25 (Figure 1a). The H and L groups, based on protease activity, showed the highest protease activity ranged from 9-d to 10-d The ranging of H group was 50.24 to 55.24 mUnit ml^{-1} and ranging of L group was 54.41 to 57.93 mUnit ml^{-1}.

The highest protease activity of H and L groups were 55.24 mUnit ml^{-1} from isolate BCC12636 and 57.930 mUnit ml^{-1} from isolate BCC17599 respectively (Figure 1b). The chitinase activity was initial rise up after the protease activity was the highest peak at 9-d to 10-d and also found that the highest chitinase activity was found on the late day.

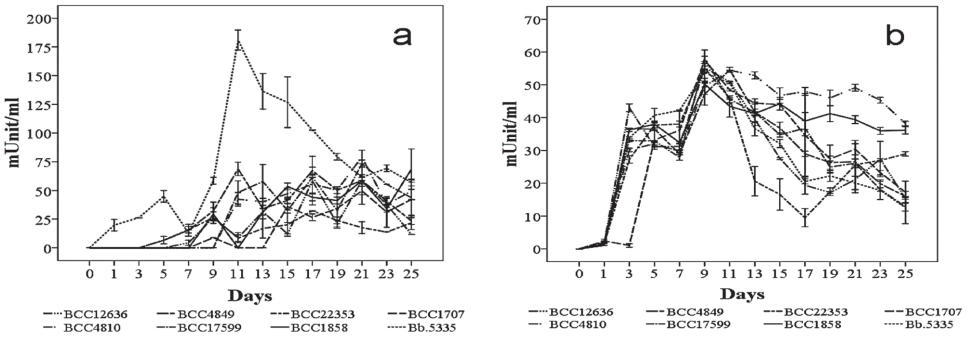

Figure 1. The amount of chitinase activity (a) and protease activity (b) in liquid culture after 25 days incubation at 30°C.

Correlation studies

The correlation coefficient of 0.579 (P=0.08) at day 1 showed that increase of ratio between fungal clear zone of all ten pathogenic fungi and colony size is negative correlated LT_{50}. While the correlation coefficient of *Metarhizium* spp. (P=0.017, r=0.891) is significant higher than those of *Beauveria* spp. (P=0.587, r=0.413). After day 1, the correlation coefficient tends inconsistency

in overall fungi and in every single genus (Table 4).

Table 4. Correlation coefficient (r) between LT_{50} value of 10 isolates, *Metarhizium* and *Beauveria* onto 2nd instar larvae of diamondback moth testing and ratio between fungal clear zone and colony size.

Parameter		Day / Fungus	1	2	3	4	5	6	7
LT_{50} (hour)	Ratio[1] (mm)	Overall[2]	0.579	0.033	0.236	0.178	0.037	0.005	0.043
		Metarhizium spp.	0.891*	0.111	0.147	0.244	0.105	0.027	0.014
		Beauveria spp.	0.413	0.565	0.373	0.373	0.373	0.373	0.373

[1]Ratio between fungal clear zone and colony size
[2]*Beauveria* spp., n=4; *Metarhizium* spp., n=6
*= significant at $P<0.05$

Chitinase activity of all 8 isolates (with high and low efficacy observed by the LT_{50} from both *Beauveria* spp. and *Metarhizium* spp.) showed significant correlation with LT_{50} on day 17 through day 23 while the protease activity showed significant correlation with LT_{50} on day 7 through day 9.

Between *Metarhizium* LT_{50} and enzyme activities the results showed that there was significant correlation of *Metarhizium* and chitinase activity on day11 (P=0.035, r=0.844), 17 (P=0.009, r=0.83), 19 (P=0.001, r=978), and protease activity on day 7 (P=0.032, r=0.850), 9 (P=0.024, r=0.871) respectively (Table 5).

Table 5. Correlation coefficient (r) between LT50 values of 8 isolates, *Metarhizium* and *Beauveria* onto 2nd instar larvae of diamondback moth testing and chitinase and protease activities in liquid culture.

Day	Correlation coefficient (r)			
	LT_{50} (hour)			
	Chitinase activity		Protease activity	
	Overall[1]	*M.*[2]	Overall	*M.*
0	0.000	0.000	0.000	0.000
1	0.347	0.000	0.045	0.034
3	0.347	0.000	0.299	0.256
5	0.324	0.015	0.094	0.090
7	0.030	0.069	0.323	0.850*
9	0.019	0.207	0.458	0.871*
11	0.656	0.844*	0.480	0.704
13	0.590	0.655	0.554	0.722
15	0.605	0.671	0.483	0.640
17	0.805*	0.923**	0.531	0.696
19	0.830*	0.978**	0.400	0.639
21	0.783*	0.769	0.198	0.544
23	0.750*	0.738	0.082	0.298
25	0.391	0.272	0.002	0.366

[1]*Beauveria* spp., n=2; *Metarhizium* spp., n=6
[2]*Metarhizium* spp., n=6
*= significant at $P<0.05$, **= significant at $P<0.01$

DISCUSSION

The results of insect bioassays were found to be similar with the ones found by Silva et al. (2003) where LT_{50} was calculated for *B. bassiana, M. anisopliae, Beauveria* spp., *Metarhizium* spp., with values ranging from 1.1 to 4.3 days, from 0.7 to 5.8 days, from 1.3 to 3.1 days and from 1.1 to 6.0 days, respectively. Masuda (2000) reported that *B. bassiana* treated on diamondback moth larvae showed the conidia invasion into host approximately 7 h after inoculation and complete mortality within 15 h. In other words, the 100% mortality was found after 15 h, and the approximate 50% mortality of the 4th instar larvae was found after 11 h. In our test the LT_{50} of 2nd instar larvae of DBM at 25°C was 1.1 days equal to 26 h in BCC4849 (*M. anisopliae*). It showed that there are many factors involved in larval mortality such as time of conidia invasion, insect stages, and incubation temperatures. Loc and Chi (2007) indicated that the diamondback moth larvae were more susceptible to *Metarhizium* spp. than to *Beauveria* spp. In our test, the LT_{50} of *M. anisopliae* (BCC4849) was the most effective isolate. In contrast, Godonou et al. (2009) reported that *B. bassiana* efficiency was greater than *M. anisopliae* efficiency. A major of insect exoskeleton component is chitin structure (chitin microfibrils) embedded with protein matrix (Chapman, 1998; Klowden, 2002; Gullan and Cranston, 2005). When entomopathogenic fungi attacked insect host, the enzyme chitinase is used in order to breaching through the insect cuticle (St. Leger et al., 1996). In our study it was found that Bb.5335 isolate produced the greatest chitinase activity at the 11-day cultivation, and all 8 positive isolates produced protease since the first day of cultivation and chitinase activity tended to rise up after the highest protease activity. Moreover, chitinase and protease activities were associated with the insecticidal activities. In integumental system, insect protein comprises more than half the dry weight of insect cuticle (Klowden, 2002; Nation, 2008). The other minor components composed of chitin, phenol and lipids (Nation, 2008). Thus protease in both *Beauveria* spp. and *Metarhizium* spp. would involve significantly in protein degradation. Both protease and chitinase were hydrolytic enzyme which play a role as cuticle degradation when entomopathogenic fungi penetrated through insect cuticle (St. Leger et al., 1986). *Metarhizium* isolates in our experiments showed higher insecticidal efficacy and hydrolytic enzyme activities than in *Beauveria* isolates. Therefore, we suggested that the insecticidal effects of entomopathogenic fungi could be directly linked with the activity of cuticle hydrolytic enzymes.

CONCLUSION

The entomopathogenic fungi, BCC4849 and Bb.5335, can be promoted to farmer for using in *P. xylostella* control. Enzyme activity profiles showed that protease activity was found before chitinase activity. Moreover, both protease and chitinase activities showed the great correlation to the efficacy of *P. xylostella* larvae control and also correlated with duration of infection. Particular correlation of *Metarhizium* spp. showed greater than *Beauveria* spp.

ACKNOWLEDGEMENTS

This research is partially supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education. (AG-BIO/PERDO-CHE)

REFERENCES

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18: 265-267.

Chapman, R. F. 1998. The insects: Structure and function. 4th ed. Cambridge University Press, Cambridge.

Finney, D. J. 1971. Probit analysis. 3rd ed. Cambridge University Press, Cambridge.

Godonou, I., B. James, C. Atcha-Ahowé, S. Vodouhé, C. Kooyman, A. Ahanchéhé, and S. Korie. 2009. Potential of *Beauveria bassiana* and *Metarhizium anisopliae* isolates from Benin to control *Plutella xylostella* L. (Lepidoptera: Plutellidae). Crop Protection 28: 220-224.

Gullan, P. J., and P. S. Cranston. 2005. The insects: An outline of entomology. 3rd ed. Blackwell Publishing, Inc., Malden.

Jungbluth, F. 1996. Crop protection policy in Thailand: economic and political factors influencing. Pesticide policy project, GTZ/University of Hannover, Hannover.

Klowden, M. J. 2002. Physiological systems in insects. Academic Press, San Diego, California.

Loc, N. T., and V. T. B. Chi. 2007. Biocontrol potential of *Metarhizium anisopliae* and *Beauveria bassiana* against diamondback moth, *Plutella xylostella*. Omonrice 15: 86- 93.

Maketon, M., and P. Orosz-Coghlan. 2008. Evaluation of *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) for control of broad mite *Polyphagotarsonemus latus* (Acari: Tarsonemidae) in mulberry. Experimental and Applied Acarology 46: 157-167.

Masuda, T. 2000. Microbial control of the diamondback moth, *Plutella xylostella,* by an entomo pathogenic fungus, *Beauveria bassiana.* II. Effects of temperature on mycoses and conidial invasion time. Japanese Journal of Applied Entomology and Zoology 44: 177-182.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426-428.

Nation, J. L. 2008. Insect physiology and biochemistry. 2nd ed. CRC Press, Taylor and Francis Group, Boca Raton.

Pedigo, L. P., and M. E. Rice. 2006. Entomology and pest management. 5th ed. Pearson Education, Inc., Upper Saddle River.

Rushtapakornchai, W., and A. Vattanatangum. 1986. Present status of insecticidal control of diamondback moth in Thailand. p. 307-312. In N. S. Talekar and T. D. Griggs (eds) Proceedings of the First International Workshop on Diamondback Moth Management. Shanhua, Taiwan.

Shelton, A. M. 2004. Management of the diamondback moth: déjà vu all over again. p. 3-10. In N. M. Endersby and P. M. Ridland (eds) Proceedings of the Fourth International Workshop on the Management of Diamondback moth and Other Crucifer Pests. Melbourne, Australia.

Silva, V. C. A., R. Barros, E. J. Marques, and J. B. Torres. 2003. Susceptibility of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) to the fungi *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorok. Neotropical Entomology 32: 653-658.

SPSS Inc. 2007. SPSS 16.0 Command Syntax Reference SPSS Inc., Chicago.

Sridevi, M., and K. V. Mallaiah. 2008. Factors effecting chitinase activity of *Rhizobium* sp. from *Sesbania sesban*. Biologia 63: 307-312.

St. Leger, R. J., R. M. Cooper, and A. K. Charnley. 1986. Cuticle-degrading enzymes of entomopathogenic fungi: Cuticle degradation *in vitro* by enzymes from entomopathogens. Journal of Invertebrate Pathology 47: 167-177.

St. Leger, R. J., L. Joshi, M. J. Bidochka, N. W. Rizzo, and D. W. Roberts. 1996. Characterization and ultrastructural localization of chitinase from *Metarhizium anisopliae*, *M. flavoviride* and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Applied and Environmental Microbiology 62: 907-912.

Sundararajan, S., C. N. Kannan, and S. Chittibabu. 2010. Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent. Journal of Bioscience and Bioengineering 111: 128-133.

Talekar, N. S., and A. M. Shelton. 1993. Biology, ecology and management of the diamondback moth. Annual Review of Entomology 38: 275-301.

Tirado, R., A. J. Englande, L. Promakasikorn, and V. Novotny. 2008. Use of agrochemicals in Thailand and its consequences for the environment. Greenpeace Research Laboratories, University of Exeter, Exeter.

Vanit-Anunchai, C. 2006. Possibilities and constraints of marketing environmentally friendly produced vegetables in Thailand. Doctoral Dissertation. Hannover University, Hannovor.

none