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Virulence and genetics at the molecular level of an indigenous strain of *Beauveria bassiana* affected by artificial and mass production media usage for sustainable insect control

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Abstract Molecular biology of indigenous strain of entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillenim (Ascomycota: Hypocreales) (strain Bbs01) based on DNA fingerprinting and enzymatic studies and based on respective RAPD-PCR and acrylamide gel electrophoresis were conducted. The results showed that neither artificial media (PDA, SDA, SDAY, MEA, NA and WA) nor mass production material (cooked rice, paddy, millet and dog feed) effect the fungal genetic stability and protease enzyme activity. Correlation analysis indicated that pathogenicity of Bbs01 to cabbage aphids, *Lipaphis erysimi* (Hemiptera: Aphididae) and flea beetle, *Phyllotreta sinuata* (Coleoptera: Chrysomelidae) were increased with protease activity. Accordingly, the most suitable respective artificial and culture material for *B. bassiana* Bbs01were SDAY and dog food.

Keywords: *Beauveria bassiana*, genetic stability, mass production media, sustainable insect control, virulence

Introduction

Beauveria bassiana (Balsamo) Vuillenim (Acomycota: Hypocreales), a major widespread entomopathogenic fungus has been applied as microbial insecticides for several insect pest species including Order Hemiptera Lepidoptera, Diptera, Hymenoptera and Coleoptera (Tanada and Kaya, 1993). An example is European corn borers (*Oatrinia nubilalis*) (Lewis *et al.*, 1996) in which some strains were utilized for testing microbial insecticides; e.g., Bb147 screening from *O. nubilalis*, commercialized under various trademarks; e.g., Bio-Power, Beauverin, Boverol and Boverosil as well as Naturalis-O and Naturalis-T and Back-off recommended for control of Asiatic corn borer, coffee borer, white

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grubs, bollworm, cutworm, brown planthopper (Copping, 2009). Nevertheless, the microbial insecticide market share is limited and not widespread due to higher cost and shorter self-life compared with chemical insecticide resulting, it's hard to be afforded by low-income farmers. Therefore, in developing countries, the major limitations have been expanded by the benefits of indigenous strains applied with local material cultures (Lopez-Llorca *et al.,* 1999; Posada-Flores, 2008; Sahayaraj and Namadivayam, 2008).

Recently, many efforts have been made with the aim for the effectiveness of native entomopathogenic *B. bassiana* isolate Bbs01 and enrichment culture techniques by using local mass production materials for insect pest control in Thailand as well (Saengyot and Napompeth, 2007). However, several factors; namely, pathogenicity and environmental tolerances, (e.g., light, temperature and moisture) according to the genotypes (Butt *et al.,* 2001) have affected the performance of *B. bassiana* at field condition. Additionally, some reports indicated that an artificial cultural condition influence virulence of *B. bassiana* and may affect its genetic variation (Rodríguez-Gómez *et al.,* 2009). Thence, the types of cultural materials would not only affect the amount of the enzyme production and the molecular genetic, but would also be concerned the crucial factors in the economic investment for the production of a further commercial formula.

The research aimed to point out the suitability of artificial media and culture materials for mass production of Bbs01 involving evaluation on the effects of the different media on pathogenicity and some molecular characteristics including genetic stability and enzymatic activity for resulting in sustainable local resources to maximize the benefits and worthy investment. Hence, this article would lead to crucial conclusion of a suitable artificial medium and materials of mass production of a quantitative and phenotypic molecular quality, which would directly be involved in the effectiveness of the *B. bassiana* isolate Bbs01.

Materials and methods

Pathogenicity of Beauveria isolate 01 (Bbs01) to the target insect pests effected by different artificial media and cultural materials

Cabbage aphids, *Lipaphis erysimi* (Hemiptera: Aphididae), flea beetle, *Phyllotreta sinuata* (Coleoptera: Chrysomelidae) and cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) were collected from the field and mass cultured according to the appropriate methods of each pest species (information not shown). The spore suspension of the Bbs01 was obtained from stock culture

culturing on different artificial media comprising of potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), Sabouraud dextrose agar supplemented with yeast extract (SDAY), malt extract agar (MEA), nutrient agar (NA) and water agar (WA), as well as four types of sterilized mass production media including, cooked rice, paddy, millet and dog feed. Then, the fungus was subjected to pathogenicity test on those three insect pests according to Lacey (1997) and Tanada and Kaya (1993). The experiments were divided into two series: study of the spore suspension obtained from respective six artificial media and the four type of mass production materials as described above compared to an untreated control (0.02% of distilled water mixed with Tween 80). Death and healthy population data were collected and corrected Percent Cumulative Mortality (PCM) were calculated using Abbott's formula (Abbott, 1925) as follows:

$$\text{Corrected (\%)} = 1 - \frac{\text{n in T after treatment}}{\text{n in Co after treatment}} \times 100$$

Where: n = Insect population, T = treated, Co = control

The experimental design was a completely randomized design (CRD) with five replications. Corrected mortality of the target insects of both studies were subject to statistical analysis using Duncan's Multiple Range Test (DMRT).

Genetic study of B. bassiana isolate Bbs01 grow on different artificial media and local cultural materials

The genetic pattern of the *B. bassiana* isolate Bbs01 was obtained by deoxyribonucleic acid (DNA) fingerprinting with a random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). Initially, the Bbs01 was grown in a broth media including PDB, SDB, SDBY, MEB, NB and WB in accordance with Alves *et al.* (2002). Growing materials cooked rice, paddy, millet and dog feed, was prepared by grinding in distilled water at the ratio of 1:1. Then, 100 milliliters of each solution was poured separately into 125 ml flasks and sterilized. Spores from 30-day-old cultures on respective media were collected for the genomic DNA extraction following the modified procedure of Maurer *et al.* (1997). Next, fungal proteins were removed by a lysis buffer containing proteinase K at 65°C for one hour. Then, the genomic DNA was extracted using chloroform: isoamyl alcohol or isopentyl alcohol with a ratio of 24:1 and three replications, centrifuged at 12,000 rpm for 15 minutes, and washed with 70% of ethanol, and the water was removed with a vacuum dryer. The fungal DNA was dissolved in 300-500 µl of a Tris EDTA buffer, and the content of the RAPD markers were increased by PCR with primers; namely, OPA-3 and OPB-10 (Operon Technologies, Alabama, USA). The investigation of the genetic pattern of the

fungus was done in accordance with Castrillo *et al.* (2003) with a PCR. After the initial denaturation at 35 cycles of 95 ℃ for four minutes, denaturation at 94°C for 40 seconds followed by annealing at 37°C for 40 seconds, and an extension at 72°C for 40 seconds with final extensions at 72°C for four minutes were conducted, respectively. The PCR products was checked through with 1.5% of agarose gel electrophoresis and stained gel with ethidium bromide (EtBr). The DNA bands were detected under ultraviolet radiation and compared with the DNA marker bands of 500 and 1,000 bp for estimating the number of DNA molecules.

Enzymatic study of B. bassiana isolate Bbs01 grow on different artificial media and local cultural materials

Protease production of the Bbs01 was studied according to method by Kucera (1971). Bbs01 grown on respective liquid media was incubated at a 150 rpm shaker at 35°C for three days before percolation and centrifugation (10,000 rpm for 15 minutes). The protease molecule was determined by 12.5% of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) that contained 0.3% of the gel without induction at room temperature for an hour in 2.5% of Triton X-100 solution for washing SDS and enzyme denaturation. The gel was incubated in 0.01 M of Tris-HCl buffer pH 8 and 10 mmol of CaCl2 pH 8.0 for five-eight hours. Then, the gel was stained with 0.1% of Coomassie brilliant blue R-250 (CBB G-250) for three hours, and the reaction was stopped by the addition of methanol and acetic acid for one hour. Finally, the protein molecular weight was estimated by comparing the gel bands with a standard marker.

To determine protease activity, a virulent factor of the *B. bassiana* isolate Bbs01. The fungus was cultured on respective artificial media and local cultural materials. Briefly, adjusted $1x10^6$ spores/ml spore suspension (dissolved in distilled water) of Bbs01 obtained from 15 days old was prepared. Then one milliliter of spore suspension was added to 250 ml Erlenmeyer flask each contained 100 ml of respective artificial medium, PDB, SDB, SDBY, MEB, NB, and WB and the flask were on a shaker at 28 ±1°C at 180 rpm for 24 hours. The pH of each artificial medium was adjusted to 8.0 and incubated at 28°C with 180 rpm for 56 hours. The hypha was corrected with filtering cloth, washed with sterilized water two times and separated by centrifugation at 4,000 rpm for five minutes. The supernatant obtained from each flask was evaluated enzymatic activity according the method of Kunitz (1947). The substrate of the protease mechanisms was prepared by mixing 2 g of casein (Sigma) in 0.01 M of Tris-HCl pH 8.0 and 10 mmol of CaCl2 pH 8.0 was adjusted to 100 ml by

the distilled water. Then, 400 µl of the substrate solution was added to the supernatant that dissolved in 200 µl of the mixture of 0.01 mol of Tris-HCl pH 8.0 and 10 mmol of 10 mM $CaCl_2$, and incubated at 35°C for 10 minutes. Then, 1.2 mol of trichloroacetic acid (TCA) was added, centrifuged at 4,000 rpm for 5 minutes, which measured the absorbance of the fungal samples by a spectrophotometer at 280 nm. The protease activity was calculated by the rate of producing tyrosine per minute. Enzymatic activity by mean of unit per minus were recorded from 5 replications in CRD before statistically analyzed using Duncan's Multiple Range Test (DMRT) for means comparing.

Protease activity assay in different local cultural materials, cooked rice, paddy, millet and dog feed

The paddy and millet were prepared by washing with water and soaked in distilled water for 24 hours. Then, the materials were 50% steamed cooked for 15 minutes and dried on a bamboo tray for 30 minutes. The adult dog food brand of 'Plemo' with a liver flavour was soaked in water for 1.5 minutes and dried. Then, each material was ground with a grinder adding some water while grinding before 100 g of each was tranfered into 250 ml flasks. The fungal spores were scratched into the distilled water and adjusted to $1x10^6$ spores/ml. The suspensions were incubated on a shaker at 150 rpm at 35℃ for 72 hours, and the fungal hyphae were filtered through a filter cloth. Then, collected hyphae were rinsed with distilled water two times and centrifuged at 10,000 rpm for 15 minutes. This was followed by the determination of the Bbs01 protease activity by acrylamide gel electrophoresis. Enzymatic activity by mean of unit per minus were recorded from 5 replications in CRD before statistically analyzed using Duncan's Multiple Range Test (DMRT) for means comparing.

Correlation analysis of pathogenicity and protease activity for B. bassiana isolate Bb01 associated with different artificial media and local cultural materials

The study was carried out through the series of 1) the relationship of different artificial mediums and 2) growing materials on protease reaction. The correlation of the protease activity and pathogenicity of the Bb01 culture on PDA, SDA, SDAY, MEA, NA and WA as well as of the difference type of mass production materials: cooked rice, paddy, millet and dog feed were analyzed for the restriction on *Lipaphis erysimi* (Kal.), *Phyllotreta sinuata*, and *Spodoptera litura* using linear regression according to Le Clerg *et al.* (1966) Snedecor and Cochran (1967) Wadley (1967).

Results

Effect of artificial media and local cultural materials on DNA fingerprinting of the B. bassiana isolate Bbs01

The DNA fingerprints of the *B. bassiana* isolate Bbs01 used RAPD-PCR, OPA-3, OPB-10 for growing in PDA, SDA, PDA, MEA, and NA in which there were differences in the transmitted pattern. In this case, five DNA bands of 500-1,000 bp were shown (Figure 1) while the molecular genetics of the Bbs01 growing in different materials (cooked rice, paddy, millet and dog food) showed the same genetic pattern (Figure 2).

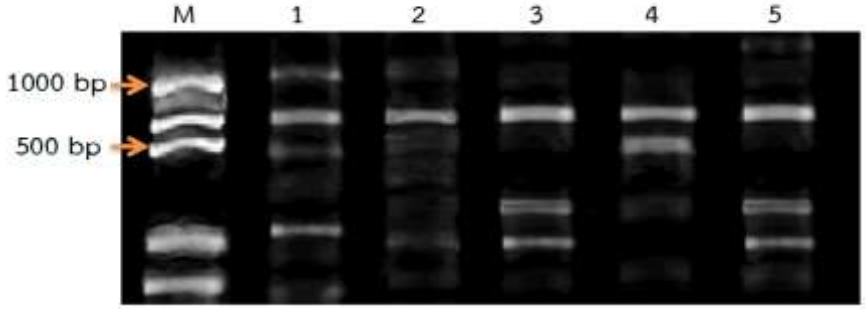


Figure 1. RAPD-PCR product using primer OPA-3 of fungi *B. bassiana* (M) and the gene of the *B. bassiana* Group 1 that was the same gene from fungi growing in PDA (1), SDA (2), PDA (3), MEA (4), and NA (5), respectively

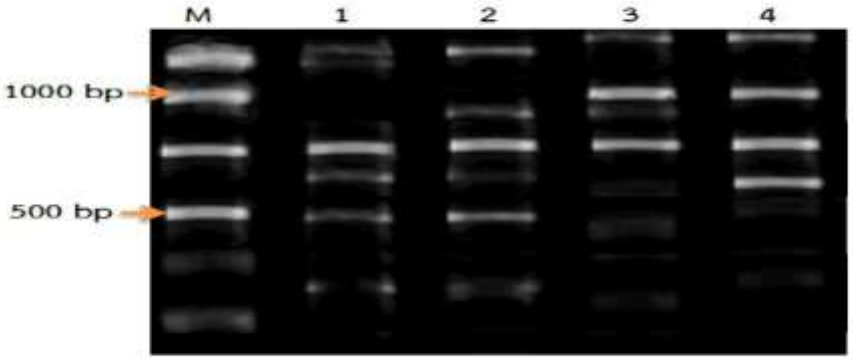


Figure 2. RAPD-PCR product using primer OPA-3 of fungi *B. bassiana* (M) and the gene of the *B. bassiana* group 1 that had the same genetic pattern growing in cooked rice (1), paddy (2), millet (3), and dog feed (4), respectively.
***** M meant a marker with a molecular size of 500-1,000 bp

Confirmation of the protease enzyme in culture media

The protease activity was indicated as the virulence of the Bbs01. After growing the Bbs01 under different artificial media; namely, PDA, SDA, SDAY, MEA and NA, the protease content was detected using 12.5% of SDS-PAGE. The Bbs01 recorded the same band of the protease molecular weight of 66 kDa fragment determined by SD-PAGE as shown in Figure 3 and 4.

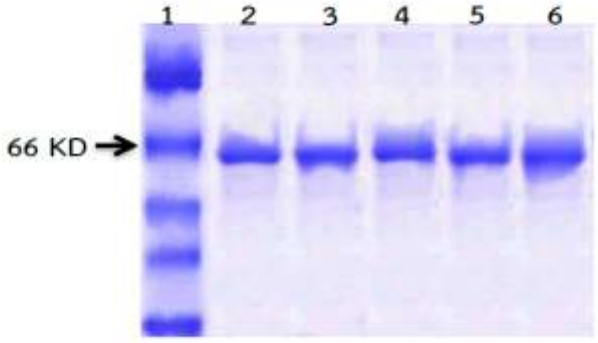


Figure 3. Protease production of the *B. bassiana* isolate Bbs01 using 12.5% of SDS-PAGE and stained with Coomassie brilliant blue R-250 for three hours. The 1st band was a protein marker while the other bands were protein extracted from various fungal inoculated artificial media including PDA, SDA, SDAY, MEA, and NA, respectively

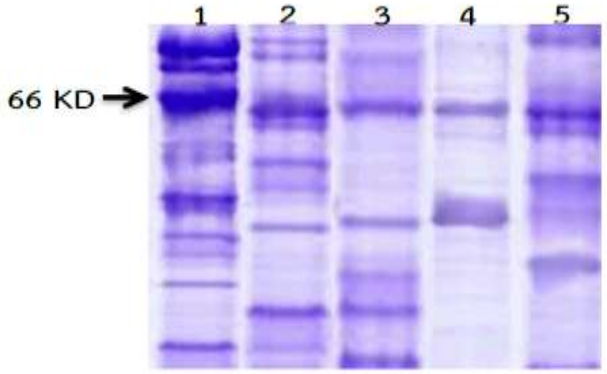


Figure 4. Protease production of the *B. bassiana* isolate Bbs01 using 12.5% of SDS-PAGE and stained with Coomassie brilliant blue R-250 for three hours. The. 1st band was a protein marker while the other bands were protein extracted from various fungal inoculated material including cooked rice, paddy, millet, and dog feed, respectively

Protease activity of the Bbs01 grown on difference artificial media and local cultural materials

The proteolytic enzyme produced by Bbs01 was detected by using a casein substrate. In laboratory scale, both the artificial media and growing materials had a high significance on the protease content (p=0.01). This enzyme remained within the range of 0.85±0.16 - 1.99±0.09 U/ml. The most production of protease was found in SDAY similar to those of MEA and SDA whereas PDA had the lowest protease activity (Table 1). For the growing materials, the highest protease reaction was found in the dog food (1.86+0.08 U/ml) similar to millet (1.79±1.16 U/ml). However, the activity of the protease ranged from 1.17 ± 0.10 U/ml (growing in cooked rice) to 1.86±0.08 U/ml (growing in dog feed) (Table 2).

Table 1. Protease activity produced from the B. bassiana isolate Bbs01 growing in different artificial media

Media	**Protease Activity (unit/ml ± SD)**
Potato Dextrose Agar (PDA)	0.85 ± 0.16c
Sabouraud Dextrose Agar (SDA)	1.43 ± 0.08a
SDA with Yeast Extract (SDAY)	1.99 ± 0.09b
Malt Extract Agar (MEA)	1.91 ± 0.06a
Nutrient Agar (NA)	1.32 ± 0.08b
Water Agar (WA)	inability to cultivate the sufficient amounts for testing

***** The mean values in each column followed by the same letter are not significantly different by DMRT at $p \leq .01$

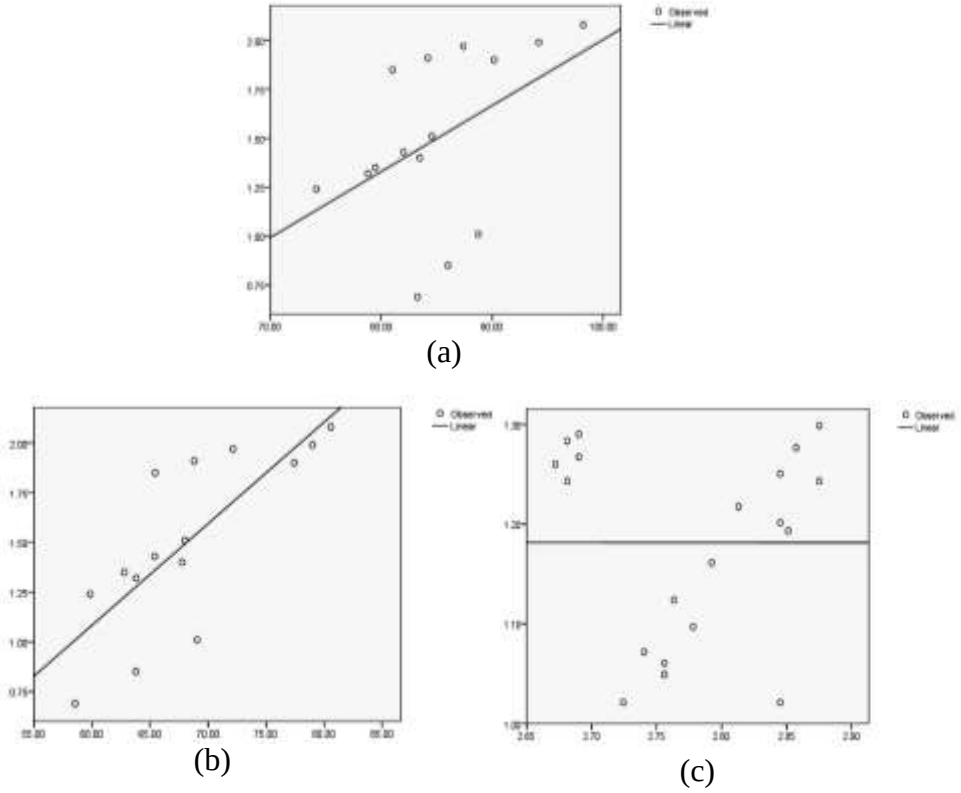
Table 2. Protease activity produced from the B. bassiana isolate Bbs01 growing in growing in different cultural material

Growing Media	Protease Activity (unit/ml ± SD)
cooked rice	1.40 ± 0.25b
paddy	1.17 ± 0.10c
millet	1.79 ± 0.16a
dog feed	1.86 ± 0.08a

***** The mean values in each column followed by the same letter are not significantly different by DMRT at $p \leq .01$

Correlation of pathogenicity and protease activity of the Bb01 grown on difference artificial media and local cultural materials

This research established the greater association of the increasing protease activity and of the virulence of the Bb01 to *L. erysimi* and *P. sinuata*, respectively. The regression coefficients for *L. erysimi* and *P. sinuata* were 0.420 and 0.438, while the lowest regression was found on *S. litura* (0.01) (Figure 5). The correlation analysis indicated that local cultural materials, cooked rice, paddy, millet, and dog food was positively associated with both protease content or pathogenicity of the Bb01 Infested *L. erysimi*, and *P. sinuata* with respective coefficients of 0.447 and 0.772. However, *S. litura* differed from the other two target insects (0.027) (Figure 6).



(a)

(b)

(c)

Figure 5. Linear regression showed the relationship between the protease production by the *B. bassiana* isolate Bbs01 growing in different artificial culture media and the Percent Cumulative Mortality (PCM) of (a) aphid (*Lipaphis erysimi*), (b) flea beetle (*Phyllotreta sinuata*), and (c) cutworm(*Spodoptera litura*)

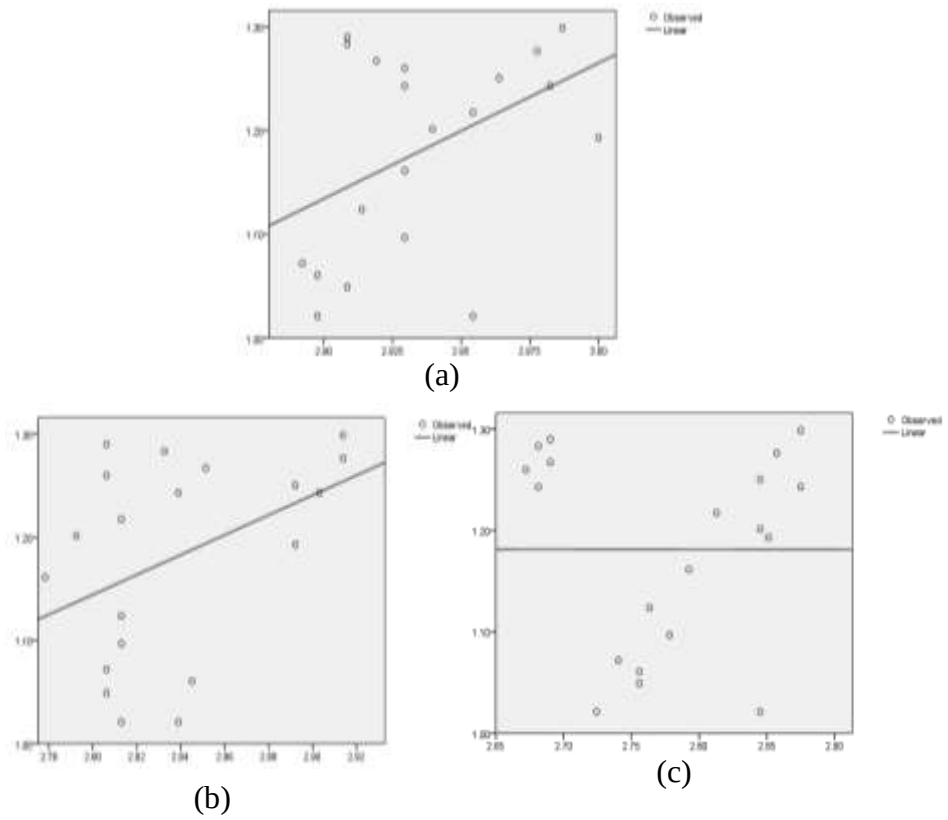


Figure 6. Linear regression showed the relationship between the protease production by the *B. bassiana* isolate Bbs01 growing in different cultural material and the Percent Cumulative Mortality (PCM) of (a) aphid (*Lipaphis erysimi*) (b) flea beetle (*Phyllotreta sinuata*) (c) cutworm (*Spodoptera litura*)

Discussion

Although some reports showed that fungal pathogenicity could be controlled by molecular genetics (Zhang *et al.*, 2009), for this study, the researchers investigated the DNA fingerprinting with RAPD-PCR of the Bbs01 that did not have any significant effect from the difference artificial media and local cultural materials. There was evidence that showed various protease activities from *B. bassiana* were caused by many factors; for instance, strain, geographic origin, and culture medium (Kucera, 1971). Therefore, this research focused on some other substantial factors, which were found to have been caused by the medium and growing materials as well. Accordingly, the artificial medium and growing material may involve the protease activity of the

B. bassiana isolate Bbs01, and fungal pathogenesis. Protease was an enzyme affecting virulence (Inglis *et al.*, 2001). From an experiment conducted in 2012, it was found that the Bbs01 could produce a proteolytic enzyme in all artificial media (PDA, SDA, SDAY, NA, and MEA) and growing materials (cooked rice, paddy, un-millet rice, and dog food) by using 12% of SDS-PAGE and a 66-KD protein marker (Figures 1 and 2). Additionally, the present observation confirmed that the protease activities had a significant difference depending on the artificial medium and growing materials. In this study, the protease activities of SDAY (1.99 ± 0.99 U) were similar to that of MEA (1.91 ± 0.06 U) while that grown in dog food (1.86 ± 0.08 U) did not differ from that of millet (1.79 ± 0.16. U). However, some studies confirmed that the culture medium type was the crucial cause of the virulence of *B. bassiana* (Fan *et al.*, 2007). Thus, there is a positive relationship of the protease activity with the pathogenesis of the *B. bassiana* isolate Bbs01. The results showed the same trend that was the artificial culture media and growing materials were positively related to the virulence to aphid, flea beetle, and cutworm. This meant that the *B. bassiana* isolate Bbs01 could be virulent when protease was being produced.

Acknowledgement

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